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Source: BIOS, 79(4) : 139-149

Published By: Beta Beta Beta Biological Society

URL: https://doi.org/10.1893/0005-3155-79.4.139

Research Article

Effects of atrazine on embryonic development of fathead minnows (*Pimephales promelas*) and *Xenopus laevis*

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Abstract. Atrazine is currently the most widely used herbicide in the U.S. Its effects on embryonic development of *Xenopus* and fathead minnows was analyzed in this study. Embryos were exposed to one of five different exposures: atrazine (0, 20, or 250 μg/L) or ethynyl estradiol (EE2) (1 or 100 μg/L), all containing 0.025% acetone:methanol. Fish and frogs were exposed immediately after fertilization until no later than 180h in development. At all concentrations, atrazine did not significantly affect timing of development, muscle contractions, embryonic length, yolk diameter, or mortality. Atrazine-exposed frogs and fish, however, did exhibit altered morphology. EE2 did not affect timing of development or muscle contractions of frogs and fish. EE2-exposed fish did have larger yolk diameters than the others exposed to atrazine, possibly indicating slower uptake of nutrients from the yolk sac. However, EE2-exposed frogs had no significantly altered timing of development, muscle contractions, embryonic length, mortality, or morphology. There was also no effect of atrazine or EE2 on cartilage development of frogs. Though few effects were seen this early in development, other studies do demonstrate the effects of atrazine later in development.

Introduction

With a projected future extinction rate is 4% per decade, the percent of species in North American temperate freshwater ecosystems is being depleted as rapidly as tropical forests (Lydeard and Mayden, 1995; Naiman and Turner, 1999; Ricciardi and Rasmussen, 1999). Approximately 20% of the world's freshwater fish species are already extinct or in serious decline (Moyle and Leidy, 1992). Freshwater dependent organisms, such as amphibians, have been facing global declines for several decades, regardless of geographical and temporal variability (Houlahan et al., 2000).

Many factors contribute to freshwater species declines, including habitat loss and destruction, species invasions, overharvesting, secondary extinctions, chemical and organic pollution, and global climate change (Allan and Flecker, 1993). Chemical and organic pollutants play a role in 38% of North American extinctions (Miller et al., 1989; Allen and Flecker, 1993). Richeter et al. (1997) found that fish populations in the eastern U.S. suffer from nonpoint source pollution.

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Bios 79(4) 139–149, 2008

Environmental toxins

Many recent studies point toward the prevalence and hazards of potentially toxic synthetic compounds in wastewater and sewage sludge (Birkett and Lester, 2003; Kanda et al., 2003). Endocrine disrupting compounds (EDCs) are often a significant component of wastewater effluent. While many EDCs are found in wastewater as a result of human activities, natural (E1, estriol [E3]) and synthetic (EE2, mestranol) hormones are generally the greatest contributors to the estrogenic activities in sewage effluents and the receiving water (Rodgers-Gray et al., 2000; Aerni et al., 2004). Anthropogenically-derived EDCs, such as those used as surfactants in domestic and industrial products, have been detected in air, soil, sediments, and biota (Ying et al., 2002), and they ultimately end up in groundwater, rivers, and lakes (Esperanza et al., 2004).

Atrazine, an EDC and a member of the s-triazine family of herbicides, is one of the most significant pollutants in water (Phyu, 2005). It is the most widely used herbicide in the United States and it was applied to 84% of corn acres at a rate of 1.01 lb/acre in 2000 (Bringolf, 2004). Between 1992 and 1998, atrazine was discovered in 85% of surface-water samples from urban and agricultural areas, and the concentration of atrazine exceeded 0.1 μg/L in 34% of agricultural streams (Bringolf, 2004). Although atrazine concentrations rarely exceed 20 μg/L in streams and lakes (Solomon, 1995; lran and Karasov, 2000), it has been found as high as 200 μg/L in static bodies of water that receive runoff from atrazine-treated farms (Kadoum, 1978).

More research is focusing on the impacts of atrazine and other agricultural chemicals on freshwater fauna (i.e. Richter et al., 1996; Diana et al., 2000; Allran and Karaov, 2000). The acute and chronic effects of atrazine on freshwater fauna have been extensively studied. Two model organisms for this research are *Xenopus laevis* frogs (i.e. Carr et al., 2002; Sullivan and Spence, 2002; Tavera-Mendoza et al., 2002a; Freeman and Rayburn, 2005) and Pimephales promelas (fathead minnows; i.e. Detenbeck et al., 1995; Bringolf et al., 2003). In *X. laevis*, a high concentration (800 μg/L) of atrazine has been found to alter timing of metamorphosis (Freeman and Rayburn, 2005), while environmentally relevant concentrations (<25 μg/L) seem to have little or no effects on metamorphosis (Carr et al., 2002). Atrazine causes a significant increase in the frequency of secondary oogonia in *Xenopus* tadpoles during sexual differentiation. This reduces the total number of germ cells in the ovary by 20% (Tavera-Mendoza et al., 2002 a). In another study by Tavera-Mendoza et al. (2002 b), atrazine-exposed tadpoles had a 57% reduction in testicular volume, spermatogonial cell nests were reduced by 70%, and nursing cells were reduced by 74%. Since cell nests represent the primordial germ cells for the reproductive life of these organisms, the reduction in spermatogonial cell nests and nursing cells suggests that environmentally relevant concentrations (21 μg/L) of atrazine during sexual differentiation might significantly reduce reproduction during the organism's life.

Although atrazine is known to have an effect upon *Xenopus* frog embryos, its effects on fathead minnow embryos is not as well documented. Detenbeck et al. (1995) did not see any significant effects of atrazine on growth of fathead minnow larvae at 25 to 75 μg/L, and Bringolf et al. (2003) found that atrazine did not have a significant effect on reproduction in adult fathead minnows at 5 and 50 μg/L. While the aforementioned studies on fatheads found no effects on larvae or adult reproduction, these studies have not considered effects on early developmental stages. Our study will compare the effects of atrazine on *Xenopus* frog and fathead minnow embryos in order to better understand the effects of atrazine on developmental stages of freshwater fauna.

Materials and Methods

Experimental design

Adult fathead minnows (age seven months) were obtained from Chesapeake Cultures (Hayes, VA, USA). The adult fish had no known chemical exposure either prior to or during the experiment. Fish were kept in 17-L glass aquariums containing dechlorinated, aerated water, as well as one PVC habitat for laying eggs. Each

aquarium (four in total) contained two females and one male. Fish were fed twice daily: goldfish flakes (Tetrafin) in the morning, and frozen brine shrimp (San Francisco Bay Brand) in the evening. Water temperature was maintained at 25–30°C, photoperiod at 16:8h light:dark, and pH range 7.3–8.2, following the methods of Bringolf (2003).

When at least 50 eggs were obtained at one time, the PVC habitats with eggs were removed and placed in a beaker with dechlorinated water. The exposure chemicals were made using an acetone:methanol solution as the carrier solvent. The treatments were prepared and placed into petri dishes (two for each exposure) and the eggs evenly distributed to all five treatments in duplicate. Eggs were maintained at room temperature (22°C ± 1°C) for one to two hours to observe developmental stages and morphology.

Adult *Xenopus* frogs were obtained from a colony maintained at Ithaca College. The frogs were separated by sex and kept in large white bins half full with dechlorinated water. Frogs were fed salmon bits three times per week; temperature was maintained at room temperature (22°C ± 1°C), light at 12:12h light:dark. The frogs were induced to fertilize in the following manner: two males and two females were removed from bins and placed in four separate, large bowls, where they were left for 24 hours. They were injected with human chorionic gonadotropin (Sigma, St. Louis, MO, USA), 1 unit/μL water. Males and females received 75 and 600 units respectively. Immediately after injection, one male and one female were placed in one container, the other two in another, and were left overnight to mate. The next morning, the eggs were collected, placed into a petri dish, de-jellied with 2% cysteine, and then rinsed three times with water. The fertilized eggs were then separated and placed into a petri dish. The chemicals were then prepared in 0.1 MMR buffered solution (pH 7.4) and the eggs were divided into the various exposures.

Chemical exposures

All stock solutions were diluted into dechlorinated water (for the fish) or 0.1xMMR solution (for the *Xenopus*) immediately after fertilization:

1. The negative control contained an acetone: methanol solution (50:50 v/v, 2.5 mL) in distilled water (for fish) or 0.1 MMR buffered solution (for frogs) to 100 mL.
2. The two positive controls contained ethynyl estradiol (EE2) (Sigma, St. Louis, MO, USA), 1 μg/L and 100 μg/L. The first stock solution of EE2 (Stock A) contained 100 mg EE2 in 100 mL of acetone:methanol was made (1000 μg/mL). Stock A (1mL) was diluted in acetone:methanol to 100 mL, creating Stock B (10 μg/mL). Stock B (1mL) was then diluted in acetone: methanol to 100mL, creating Stock C (100 μg/L). All stocks were stored in glass bottles, refrigerated, and diluted into water as needed.
   a. For the high exposure (100 μg/L), Stock B (1 mL) was diluted to 100 mL in dechlorinated water or 0.1 MMR solution.
   b. For the low exposure (1 μg/L), Stock C (1 mL) was added to 100 mL distilled water or 0.1 MMR buffered solution.

   *EE2 has a half-life of 7 days in water (Jurgens, 2002), so these solutions were refreshed daily to ensure a constant concentration.
3. The experimental samples contained atrazine (Chem Service, Inc. West Chester, PA, USA). Two concentrations, 20 μg/L and 250 μg/L, were tested. The first stock solution, Stock A, contained 100 mg atrazine in 100 mL acetone: methanol (1000 μg/mL). Stock A (1 mL) was then diluted in acetone: methanol to 100 mL, creating Stock B (10 μg/mL). Both stock solutions were stored in brown bottles to prevent photolysis and were kept in a freezer at −20°C (Phyu, 2004).
   a. For the high exposure (250 μg/L), Stock A (2.5 mL) was diluted in distilled water or 0.1 MMR buffered solution to 100 mL.
   b. For the low exposure (20 μg/L), Stock B (0.2 mL) was diluted in distilled water or 0.1 MMR buffered solution to 100 mL.

   *Atrazine has a half-life of 24 days in water (Moore 1999), but in order to be consistent with the EE2 samples, the atrazine solutions were refreshed daily.

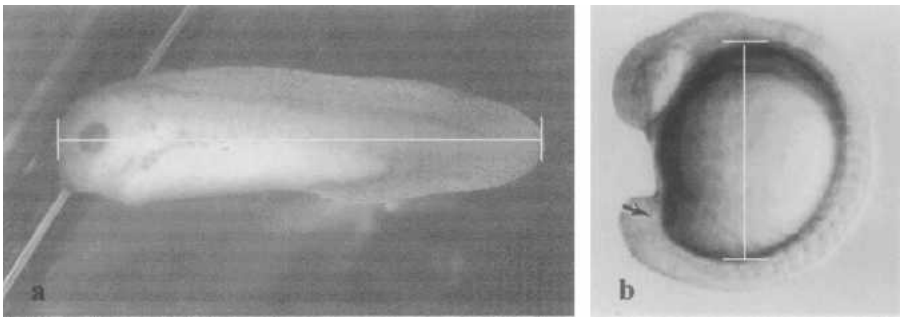

Figure 1. (a) Frog and fish embryonic length measured head to tail. (b) Fish yolk measured at the longest diameter present (white lines indicate areas of measurement).

Embryological endpoints

Frog and fish embryonic stages, timing of development/hatching time, number of muscle contractions per 20 seconds, embryonic length (frogs, fish after hatching; Figure 1a), yolk diameter (fish only; Figure 1b), mortality, and morphology (skeletal deformities, pigmentation, presence of eye pigmentation, eye size, awkward swimming) were monitored and recorded every six hours for one week during the exposure period—intervals of monitoring was dependent on the stage of development (more frequently during early development, less frequently later in development). Pictures from Nieuwkoop's Normal Table of *Xenopus* (1994) were used as a reference. Digital images were taken with a digital camera mounted on a Nikon SMZ1500 stereoscope, and images were processed with Adobe Photoshop.

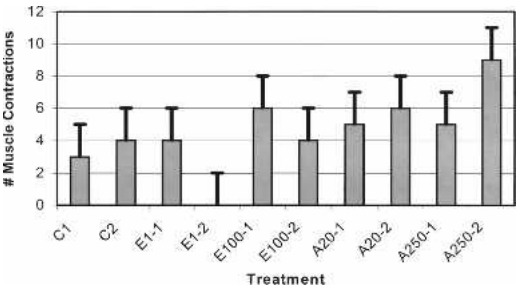

Figure 2. Fathead Minnow embryo muscle contractions, stage 26. Each bar represents total contractions observed in five embryos over 20 seconds.

Clearing and staining

Using the methods of Potthoff (1984), embryos were cleared and stained to observe devel-

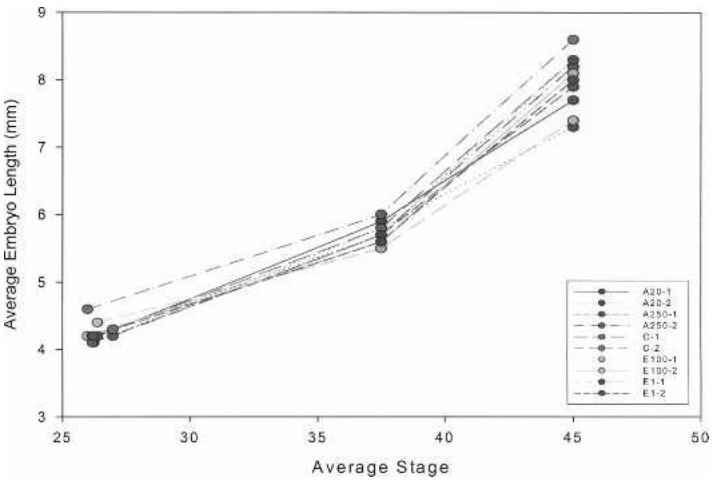

Figure 3. Xenopus average embryo length of control, EE2 (100 µg/L), atrazine (20 µg/L), and atrazine (250 µg/L) at each measured stage.

opment of cartilage and calcification of bones. Due to a lack of fish embryos obtained, the procedure was only done with *Xenopus*. All steps were done in 5 mL vials with four embryos in each and were mildly agitated on a nutator. Solutions were replaced using a 3 mL plastic pipette. Embryos were fixed in 4% paraformaldehyde in PBS for 48 hours at 4°C, with one intermediate solution change. Embryos were then dehydrated at room temperature: they were washed twice for five minutes each in PBS; next they were dehydrated in 50% ethanol for 24 hours; finally they were dehydrated in 100% ethanol for 48 hours with one intermediate solution change. Cartilage was then stained with a solution that contained 100% ethanol (70 mL), acetic acid (30 mL), and Alician blue (20 mg) for a final volume of 100 mL. Specimens were incubated in the staining solution at room temperature for 24 hours. Embryos were then washed in saturated sodium borate solution for 9–12 hours. They were then bleached with a solution containing 3% hydrogen peroxide (15 mL), and 1% potassium hydroxide (85 mL), for 20 minutes. Specimens were rinsed three times for five minutes each in PBS. Next, the trypsin digestion so-

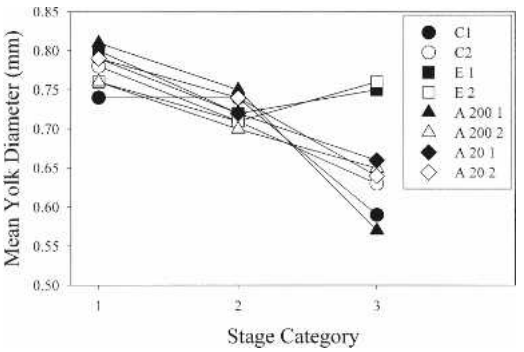

Figure 4. Mean fish yolk diameters for each treatment at each stage category (1: stages 15–20, 2: 21–25, 3: 26–30). Stage and treatments have significant effects on yolk diameter ($p < 0.05$ for stage category 3.) Data obtained from pilot study in 2005.

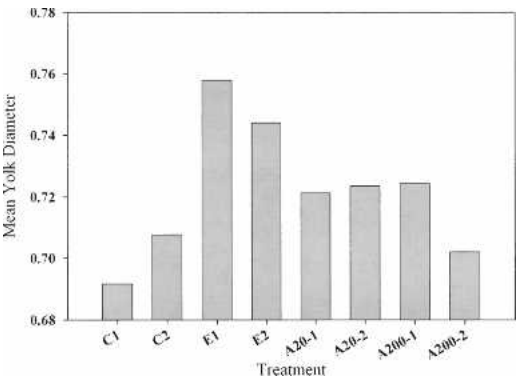

Figure 5. Mean fish yolk diameter in stages 26–30 for each treatment. Yolk diameters of embryos in E1 significantly different from those in all other treatments ($p < 0.05$). C1 and C2: Control, E1 and E2: EE2 (100 µg/L), A20–1 and A20–2: atrazine (20µg/L), A200–1 and A200–2: atrazine (200 µg/L). Data obtained from pilot study in 2005.

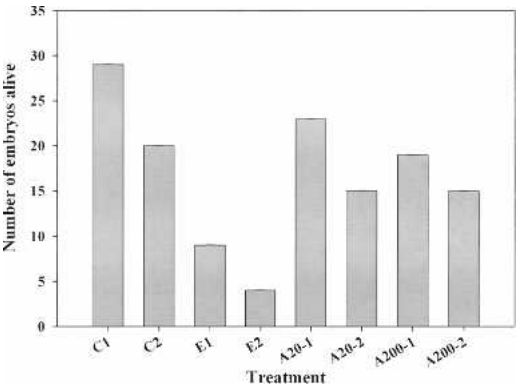

Figure 6. Average number of fish embryos alive in each treatment for stages 26–30. C1 and C2: Control, E1 and E2: EE2 (100 µg/L), A20–1 and A20–2: atrazine (20µg/L), A200–1 and A200–2: atrazine (200 µg/L). Data obtained from pilot study in 2005.

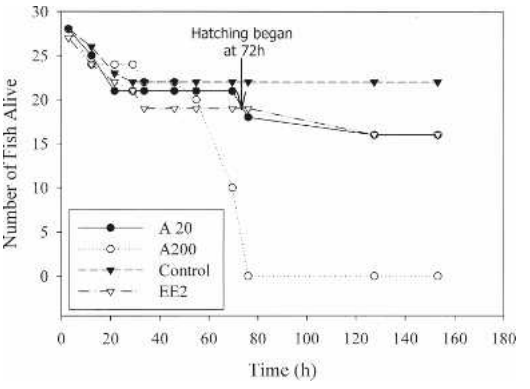

Figure 7. Average number of fathead minnow embryos alive in each treatment over the course of the entire experiment. Embryos hatched at approximately 72h, when half of the fish embryos exposed to EE2 were dead. By the next reading at 80h, which corresponded with stage 29, all embryos were dead. Samples from each treatment were averaged; data obtained from pilot study in 2005.

lution was prepared (saturated sodium borate (35 mL), deionized water (65 mL), and trypsin (1g)); specimens were incubated in the trypsin digestion solution at room temperature for an hour. Bone was then stained with a solution of 1% potassium hydroxide with 0.1g/100 mL Alizarin red, at room temperature for 24 hours. Next, specimens were incubated in trypsin digestion solution for 40–48 hours to destain the embryos. Finally, the embryos were preserved first in glycerol: 1% potassium hydroxide (30%:70%) for 2–3 days at room temperature, second in glycerol: 1% potassium hydroxide (60%:40%) for 2–3 days at room temperature, and lastly in 100% glycerol. Digital images were taken in the same process described above.

Results

Results are based on examination of fathead minnow and *Xenopus* embryos (10 per exposure,

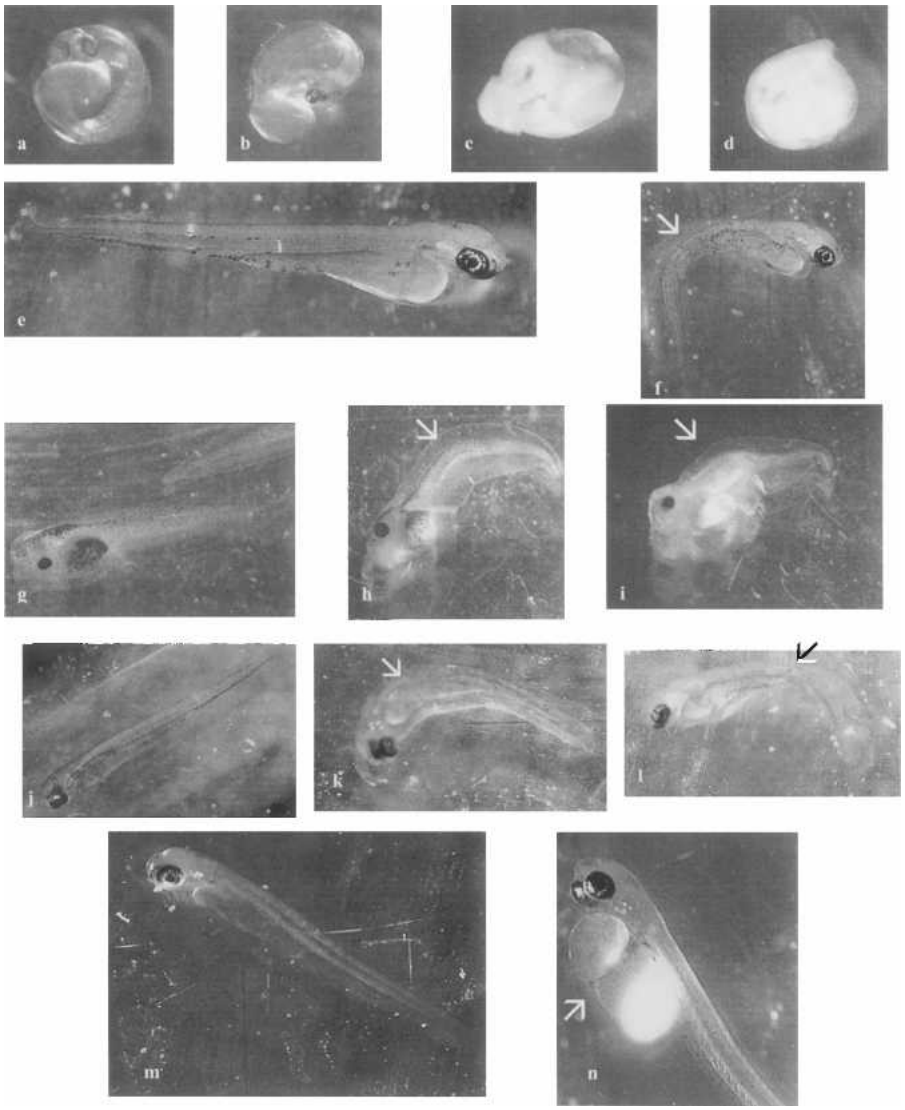

Figure 8. Fathead minnow embryos: (a, b) Exposed to control, 72h, stage 29; (c, d) Exposed to EE2 [100 µg/L], 72h, stage 29; (e, f) Exposed to EE2 [100 µg/L], 59h, stage 27; (g, h, i) Exposed to atrazine [20 µg/L], 150h, stage 40; (j, k, l) Exposed to [20 µg/L], 59h, stage 28; (m, n) Exposed to atrazine [250 µg/L], 59h, stage 26.

respectively), exposed to atrazine (20 µg/L and 250 µg/L), positive control of EE2 (1 and 100 µg/L), and a negative control of acetone/ methanol solution in water (0.025%).

Staging and muscle contractions

There were no effects of treatments on the timing of development (based on stages), or on number of muscle contractions per 20 seconds observing period of either the fathead minnow or *Xenopus* (Figure 2).

Embryonic length (*Xenopus* and fathead minnows) and yolk diameter (fathead minnows)

While the treatments did not affect the frog or fathead minnow embryonic length (Figure 3), yolk diameter was affected by EE2 (100 µg/L) in stages 26–30 (Figures 4 and 5).

Mortality

Mortality was measured as number of fish and frogs alive in each stage for each treatment. Treatments had no effects on the number of *Xenopus* and fish alive, but during the pilot, in stages 26–30, the number of fish alive in EE2 was, though not significantly, much less than the rest of the treatments (p-value = 0.062; Figure 6). By the time they hatched (around 72h), all the fish were dead (Figure 7). In the next trials, embryos exposed to EEs (100 µg/L) did survive past hatching.

Fish morphology

In the pilot study, all fish embryos exposed to EE2, 100 µg/L, developed normally until they hatched, at which point all the embryos died (Figure 8a-d). All the fish embryos exposed to atrazine at 20 µg/L survived, but some developed abnormal spine curvatures, causing them to swim in short, sporadic movements. One of the embryos, shown in Figure 8i, had an abnormal eye that was loosely attached to its head and would flutter separately from the rest of the body. There was also a constriction in the yolk observed in one embryo exposed to the high concentration of atrazine (Figure 8n). This may indicate problems in the uptake of nutrients from the yolk which may further affect development.

The frequency of abnormalities is higher in the fathead minnows exposed to atrazine (20 µg/L) than any other treatment (see Figure 9).

***Xenopus* morphology**

Xenopus developed abnormally in both concentrations of atrazine. Some embryos exposed to atrazine (20 µg/L) were seen to lack pigmentation (Figure 10d). Others at the same concentration were seen to have curvatures in their spine, most often curved laterally (Figure 10f, 10g). An embryo exposed to atrazine (200 µg/L) exhibited much less pigmentation and smaller eyes at 82h (Figure 10i). An embryo exposed to atrazine (250 µg/L) also showed lacking pigmentation (Figure 10k).

***Xenopus* stained cartilage and bone**

Since bone has not ossified this early in development, bone development could not be observed. However, cartilage was visible, but there were no observed effects of treatments on its development (Figure 11a-d).

Discussion

Concentrations used in this study were based on those occurring naturally and those that would produce clean results. The fathead minnow and *Xenopus* embryos were exposed to EE2 at 1 µg/L, an environmentally relevant concen-

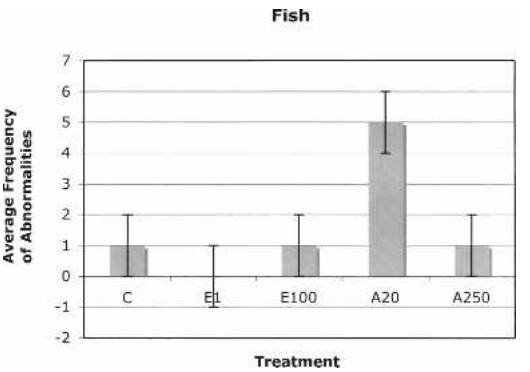

Figure 9. Frequency of abnormalities in fathead minnow embryos.

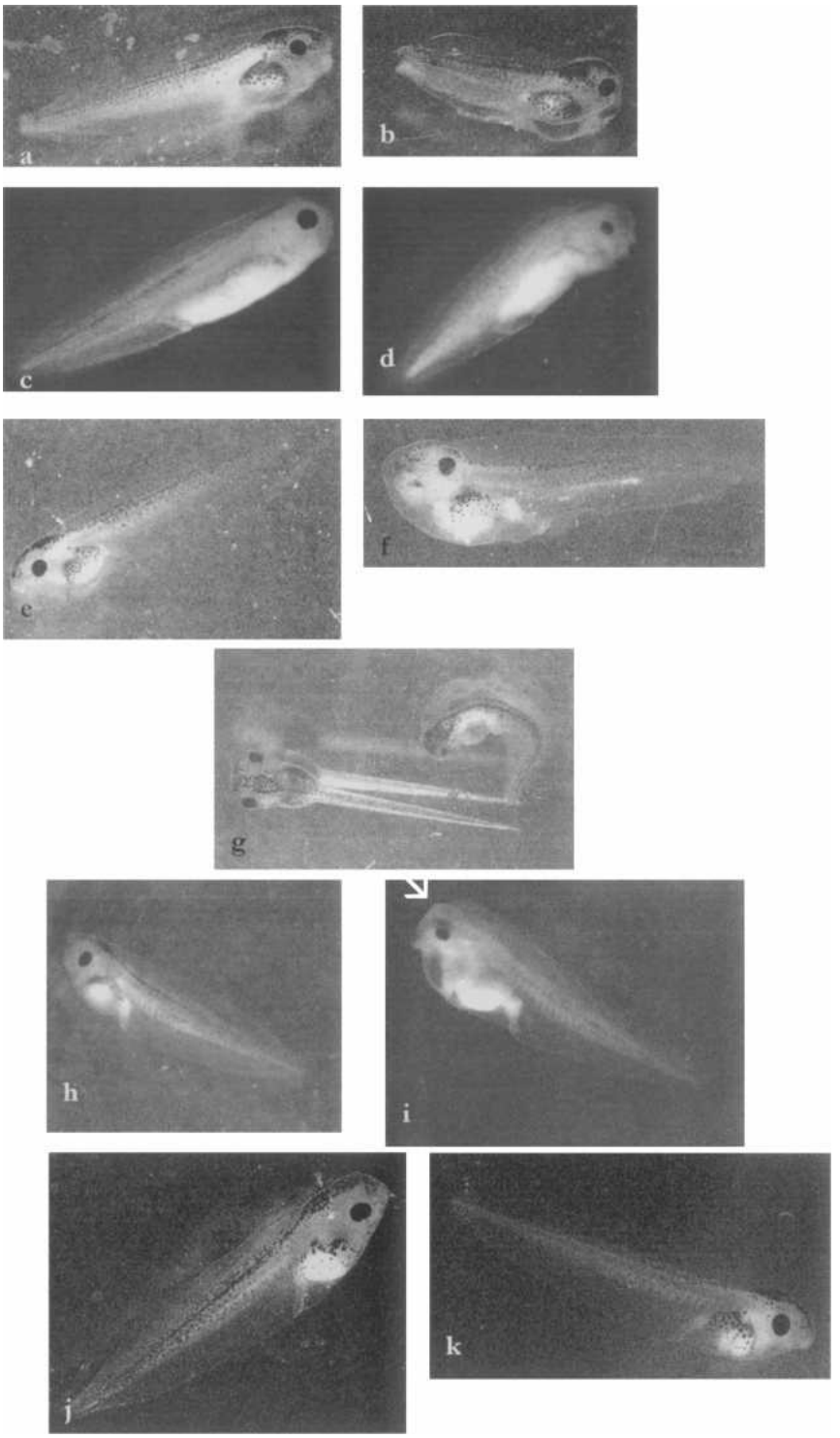

Figure 10. *Xenopus* embryos: (a,b) Exposed to EE2 [100 µg/L], 79h, stage 37; (c, d) Exposed to atrazine [20 µg/L], 51h, stage 27; (e, f) Exposed to atrazine [20 µg/L], 79h, stage 37; (g) Exposed to atrazine [20 µg/L], 79h, stage 37; (h, i) Exposed to atrazine [200 µg/L], 82h, stage 42; (j, k) atrazine [250 µg/L], 79h, stage 37.

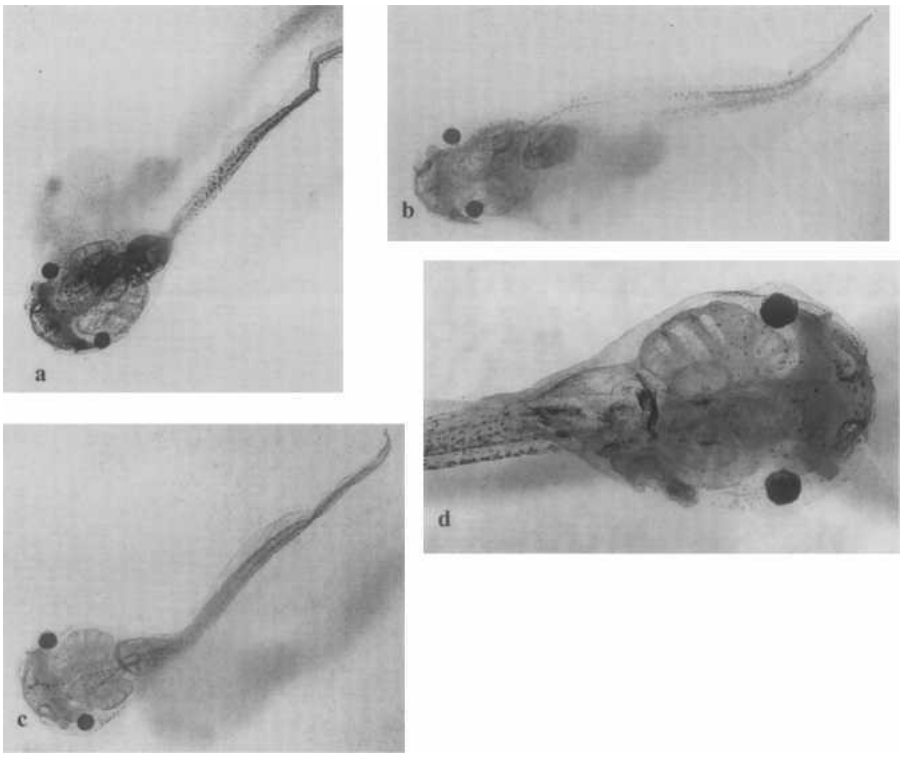

Figure 11. *Xenopus* embryos with stained cartilage: (a,b) exposed to EE2 (100 µg/L), 288h, stage 49; (c) exposed to atrazine (250 µg/L), 288h, stage 49; (d) exposed to atrazine (20 µg/L), 288h, stage 49.

tration, and 100 µg/L, an acute concentration that would produce positive results. Embryos were exposed to atrazine at both 20 µg/L and 250 µg/L to see effects of a naturally occurring concentration (20 µg/L), and an acute concentration (250 µg/L) to produce results.

Although atrazine had no significant effects on the timing of development and muscle contractions of the fathead minnows and *Xenopus* frog embryos, Alvarez (2005) found that the growth of red drum fish larvae was significantly affected. Atrazine, at the "environmentally realistic" doses used (0, 40, 80, and 500 µg/L), "significantly reduced red drum larval growth rate by 7.9–9.8%, thereby, increasing the duration of the highly vulnerable larval period" (Alvarez, 2005). There may not have been significant effects on the timing of development of fathead minnows or *Xenopus* in this study, but with more testing and a larger sample size, differences may be seen.

The embryonic length of *Xenopus* was not significantly altered when exposed to atrazine at either concentration. This is consistent with the results of other studies, which have found that "chronic exposures to atrazine at concentrations between 0.1 and 25 µg/L did not affect *Xenopus* age, length, or weight" (Coady et al., 2005). However, the yolk diameter of the fathead minnows was significantly altered as a result of exposure to EE2 ($p < 0.05$), an observation that has not been seen in other studies. The yolk diameter decreased at a much lower rate than the negative control group and the atrazine exposed groups, showing that it took up the nutrients much more slowly. No other studies considered the effects of atrazine on yolk diameter in fish.

The observed effects of atrazine on mortality, or lack thereof, were also consistent with other studies. Atrazine did not significantly alter the mortality of either the fathead minnows or *Xenopus* frogs. Ward (1985) found that at exposures less than thirty days, there was no significant effect on mortality rates of estuarine fauna, which

included fathead minnows. This is consistent with our results since our study lasted no more than seven days for either species, and there was difference in mortality of either the frogs or fish exposed to atrazine. As for the EE2, however, the concentration used in this study, although used to produce positive results, may have been too high since 100% mortality was observed in all the fathead minnows. Future experiments should use a lower concentration that will still produce positive results just short of mortality.

The morphological abnormalities observed in both the fathead minnows and *Xenopus* frogs may be the most useful endpoint. The skeletal system is an important indicator of water pollution, as cited by Bengtsson (1979). It is also noted that spinal deformities, which we saw in the fathead minnows exposed to atrazine, may occur at various stages in development, but most damage occurs at the egg stage or immediately afterwards (Bengtsson, 1979). In the *Xenopus* frogs exposed to atrazine, in addition to abnormal development of the eyes and insufficient pigmentation, there may also be other morphological abnormalities in areas that are not visible from the outside of the fish and frogs, including formation of the organs such as the brain, liver, pancreas, and reproductive organs.

Atrazine has been known to have effects on the secondary sex characteristics of male *Xenopus* frogs. For instance, Coady et al. (2005) found mixed gonads from a 10 µg/L atrazine-exposed frog as well as gonad size irregularity. This, however, was found post-metamorphosis, not during embryological development. Our study has determined that atrazine has minimal effects on timing of development, embryonic length, yolk diameter, and mortality, while there were effects on overall morphology. Atrazine may have its greatest effects on wildlife later in their life, but its effects during early development may not be as obvious.

Acknowledgements. Thanks to the Ithaca College Biology Department for funding and use of facilities, and the Beta Beta Beta Research Scholarship Foundation Fund for funding. Thanks also to Susan Allen-Gil and Marc Servetnick for help with the project.

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Received 19 July 2007; accepted 21 December 2007.