EFFECTS OF ATRAZINE AND NITRATE ON NORTHERN LEOPARD FROG (RANA PIPiens) LARVAE EXPOSED IN THE LABORATORY FROM POSTHATCH THROUGH METAMORPHOSIS

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Abstract—A recent ecological risk assessment of the herbicide atrazine found that the ecosystems at greatest risk within North America are the streams, rivers, and reservoirs of the midwestern corn-growing regions. Habitats adjacent to application areas could be exposed to high levels of atrazine during periods of amphibian activity such as breeding and migration. Because fertilizer application coincides both spatially and temporally with atrazine application in agricultural areas, we tested the effects of atrazine and nitrate on northern leopard frog (Rana pipiens) larval in the laboratory. Larvae were exposed to atrazine (0, 20, and 200 μg/L) and nitrate (0, 5, and 30 mg NO₃-N/L) from first-feeding stage through metamorphosis in a factorial design. Atrazine concentrations in metamorphosing juveniles were approximately six times the concentration in the water, indicating bioconcentration of atrazine by larvae. Atrazine, nitrate, and their interaction had no significant effect on development rate, percent metamorphosis, time to metamorphosis, percent survival, mass at metamorphosis, or hematocrit. However, nitrate slowed growth of larvae. Though this growth inhibition is statistically significant, it is probably not biologically important when compared with natural variation in the environment. Thus, concentrations of atrazine and nitrate commonly found in the environment do not appear to pose a significant threat to R. pipiens larvae through direct toxicity.

Keywords—Atrazine Nitrate Amphibian decline Rana piperis

INTRODUCTION

Numerous species of amphibians are declining in many parts of the world [1–3]. In the western United States alone, more than a dozen species of anurans have disappeared from much of their historic ranges [4]. Because many of these species have disappeared from seemingly pristine sites [3], a search began for global causes of amphibian declines, such as climate changes, ultraviolet radiation, acid precipitation, and disease [2,5–7]. However, the cause for the decline can often be linked to local anthropogenic influences such as habitat destruction and fragmentation, introduction of nonnative predators or competitors, or the presence of environmental contaminants [8–10]. In fact, there is often a lower species richness and abundance of amphibians at agricultural sites where pesticides and nitrate fertilizers are applied than at adjacent non-agricultural habitats [11]. However, because the presence of contaminants at agricultural sites is often confounded with less suitable habitat [11], laboratory studies on the effects of pesticides and fertilizers on amphibians become necessary.

Atrazine is the most heavily used agricultural pesticide in North America, with more than 50 million kilograms applied to 25 million hectares annually in the United States [12]. A recent ecological risk assessment of atrazine found that the ecosystems at greatest risk within North America are the streams, rivers, and reservoirs of the Midwestern corn-growing regions [13]. A survey of 76 Midwestern reservoirs revealed 92% to be contaminated with atrazine [13]. Though levels in rivers and streams rarely exceed 20 μg/L, concentrations at the field edge can reach 250 μg/L [13], and levels as high as 740 μg/L have been detected in run-off waters from treated cornfields following spring application [12]. Furthermore, the half-life of atrazine in water can range from 3 to 90 d, depending on water salinity and pH [13]. Considering the timing of application and the persistence of atrazine, habitats adjacent to application areas could be chronically exposed to consistently high levels of atrazine that could negatively impact amphibians during periods of migration and breeding. In addition, atrazine decreases the number of red blood cells in numerous taxa, including mice [14], dogs [15], and fish [16,17]. Though several studies have determined the acute effects of atrazine on survival and deformity [18–20], there is a lack of information on the chronic effects of ecologically relevant concentrations of atrazine on survival, growth, and metamorphosis of amphibian larvae.

Nitrate from fertilizer application is correlated both temporally and spatially with atrazine application [12]. In the last 50 years, nitrogen fertilizer use in the United States has increased from less than 0.45 million metric tons/year to more than 9.98 million metric tons/year [21]. Surface waters normally contain nitrate at levels up to 1 mg/L; however, concentrations above 5 mg/L usually indicate anthropogenic contamination [22]. Nitrate levels from lakes in Illinois, USA, from 1973 to 1991 averaged 1.06 mg NO₃-N/L, though some concentrations were as high as 39 mg NO₃-N/L [10]. Nitrate-nitrogen in subsurface drainage from Indiana corn crops fertilized with 285 kg N/ha from 1984 to 1988 was seldom less than 10 mg NO₃-N/L and was generally 20 to 30 mg NO₃-N/L [21]. Adult amphibians are likely to be exposed to nitrate as they cross treated agricultural fields to reach breeding sites, and embryos and larvae are at risk from runoff into surface waters [23]. Baker and Waights found sodium nitrate (40, 100
Toxicity of atrazine to anuran amphibians

mg NO₃/L (L) to cause retarded growth and increased mortality of common toad (Bufo bufo) [24] and Australian tree frog (Litoria caerulea) [25] larvae. Other researchers found that, when larvae of several species of amphibians are exposed to ammonium nitrate, they suffer disequilibrium, developmental abnormalities, and increased mortality in acute and chronic studies at levels comparable to those found in the environment [22,23]. Furthermore, nitrate can be converted to nitrite through bacterial reduction in the gut of the amphibians consuming plant material containing nitrate. Nitrite is reported to induce methemoglobinemia in larvae of Rana catesbeiana by oxidizing the Fe²⁺ to Fe³⁺, thus preventing oxygen transport [26].

Atrazine is capable of interacting synergistically with other agricultural chemicals to decrease survival of amphibian larvae [18], and it is possible that an interaction between atrazine and nitrate could impair survival, growth, or metamorphosis of larvae. One possible mechanism of interaction involves the oxygen-carrying capacity of larval blood. Because nitrate (from reduced nitrite) can cause methemoglobinemia and atrazine is known to reduce circulating erythrocytes, we propose that this combined effect may increase mortality and decrease growth and metamorphosis in larvae exposed to both atrazine and nitrate. Considering this possibility and the fact that atrazine and nitrate co-occur ecologically, we studied the effects of atrazine and nitrate separately and in combination on three larval frog (Rana pipiens) larvae from posthatch through metamorphosis.

MATERIALS AND METHODS

Study organisms

Four clutches of R. pipiens eggs were purchased from Nasco (Fort Atkinson, WI, USA). The supplier artificially induced four gravid female R. pipiens to spawn, and each egg clutch was fertilized with the sperm from a different male. The clutchs were transported to the laboratory in Madison (Wisconsin, USA) the same morning they were fertilized. Each clutch was placed in a separate 2.5-gallon aquarium with 7 L of dechlorinated water and placed in an incubator at 22°C on a 14:10 light-dark cycle until the embryos hatched into larvae, approx. 6 d later. Partial water changes were done every 12 h during this period by decanting approximately 80% of the water in each aquarium and replacing it with clean dechlorinated water.

Experimental design

Laboratory experiments were conducted on the University of Wisconsin-Madison campus (Madison, WI, USA) at the Center for Limnology aquarium facilities located in the Olin Science and Engineering building. Larvae were exposed from Gosner [27] stage 25 (both gills covered with opercula; feeding begins) through metamorphosis to three concentrations of atrazine (0, 20, 200 µg/L) and three concentrations of nitrate (0, 5, 30 mg NO₃-N/L) in a factorial design for a total of nine treatments. These nine treatments were replicated for each of the four clutches, resulting in a total of 36 experimental units (2.5-gallon aquarium). Twenty larvae were placed in each aquarium containing 7 L of treatment solution. The concentrations for each chemical were chosen to bracket the environmentally relevant ranges, with the low concentration representing a more typical exposure from contaminated surface waters and the higher concentration representing levels from a runoff event or in areas adjacent to the site of application [12,13,21,22].

Each experimental unit was randomly assigned to a position in the array of basins that provided a water bath with a constant temperature of 22°C. Treatment solutions in each aquarium were changed out completely and renewed with new solution every 48 h in a static renewal design. The pH of each aquarium was measured twice every 48 h, once immediately after solution renewal and again immediately prior to renewal of treatment solution. During each renewal, any dead larvae were removed and mortality recorded. Following each water change, a water sample was taken from the tanks with nitrate treatments and nitrate-nitrogen concentrations were measured using a Hach pocket colorimeter (Loveland, CO, USA). Then larvae were fed a mixture of 75% (w/w) chlorella, 25% of romaine lettuce, 18.75% (w/w) rabbit chow (8360 rabbit chow, Harlan Teklad, WI, USA), and 6.25% (w/w) TetraMin® fish flakes (TetraSales, Blacksburg, VA, USA) as 15% (w/w) of the average body mass per tadpole times the number of tadpoles per tank [28]. Feeding at this rate is considered nonlimiting [28], and we noticed food remaining at each 48-h renewal. Separate water samples were collected once a week for determination of atrazine concentration and were refrigerated at 4°C in borosilicate amber glass vials (03-3771, Fisher Scientific, Fairlawn, NJ, USA) with TetraCap®-lined caps (03-3771-1A, Fisher Scientific) until analysis by enzyme-linked immunosorbent assay (ELISA). Aquaria were aerated with air stones throughout the course of the experiment. Every 7 d, length (to the nearest mm) and stage (27) were measured for 10 randomly selected larvae from each tank. Individuals were collected at metamorphosis (Gosner stage 42 [27]) and were transferred into a second series of 2.5-gallon aquaria with 1,000 ml treatment solution, and the aquaria were placed on a slant to provide a bank for developing juveniles to climb out of the water. Metamorphs were not fed during this period. Upon tail resorption (approximately 5–7 d after front-limb emergence), the juveniles were anesthetized with 0.5 g/L MS-222 (Sigma Chemical, St. Louis, MO, USA). Mass of each juvenile was measured after blotting excess water from the body. Blood was taken by cutting the systemic arch of the heart and collecting it in a heparinized capillary tube. Hemocrit was determined according to standard methods. The juvenile was then euthanized via decapitation while still under anesthesia. The experiment was terminated after 138 d when more than 90% of the tadpoles completed metamorphosis. Only one or two larvae remained in each aquarium at experiment termination (60 of the initial 720 larvae in the experiment).

Treatment solutions

Solutions were prepared with dechlorinated charcoal-filtered water, pH 8 (approximately), hardness 324 mg/L as CaCO₃, and 11.5 mg/L of dissolved oxygen from Line 2 in the Water Science and Engineering Lab at the University of Wisconsin-Madison. Four liters of a 20 mg/L stock solution was prepared on a weekly basis from 99% pure compound atrazine (ChemService, West Chester, PA, USA). Seven milliliters of stock were diluted into the 7 L of water to reach the 20 µg/L concentration, and 70 ml of stock were diluted into the 7 L of water to reach the 200 µg/L concentration. Sodium nitrate (NaNO₃; Sigma) was the source of nitrate in the experiment, and nitrate stock and treatment solutions were prepared in a similar manner. We selected sodium nitrate as the source of nitrate instead of ammonium nitrate because we wanted to
isolate the toxicity of the nitrate ion. Baker and Wrights attributed the toxicity of sodium nitrate (40, 100 mg NO₃⁻/L) in Litoria caerulea larvae to the nitrate ion and not the sodium ion because they saw no statistically significant effects on growth or survival of larvae in the sodium chloride controls [25].

The pH of treatment solutions during the course of the experiment ranged from 8.0 ± 0.1 (n = 1.078) immediately after solution renewal to 8.4 ± 0.1 (n = 1.078) immediately prior to renewal. Measured atrazine concentrations differed significantly from target values for the 20 μg/L treatments at 19.19 ± 0.27 (t = -2.959, n = 52, p = 0.005). However, concentrations did not differ from nominal for the 200 μg/L treatments at 192.18 ± 4.16 (t = -1.877, n = 52, p = 0.066). Similarly, measured nitrate concentrations differed significantly from target values, i.e., 5.4 ± 0.1 for the 5 mg NO₃⁻/L treatments (t = 6.24, n = 204, p < 0.001) and 29.2 ± 0.2 for the 30 mg/L treatments (t = -3.83, n = 193, p < 0.001). However, these measured concentrations were within the range of acceptability (±10% of the target concentrations [18]) and were used to verify that no gross errors were made during dosing of atrazine and nitrate.

Residue analysis

The ELISA (Strategic Diagnostics, Newark, DE, USA) was used to determine residue levels of atrazine in tissues of metamorphosed juveniles [29]. Four juveniles per experimental unit were euthanized as above, placed separately in a glass scintillation vial, and frozen at -80°C for residue analysis for atrazine. These frogs did not have blood taken. Atrazine was extracted from individual juvenile frogs according to procedures outlined by Allgrod et al. [29]. Two hundred microliters of extract were diluted with atrazine-diluent 1:50 (v/v) for frogs from 200 μg/L nominal concentration treatment solutions or 1:5 (v/v) for frogs from 20 μg/L nominal concentration treatment solutions to achieve the range for analysis by ELISA. These diluted samples were stored at 4°C in Teflon-capped amber borosilicate glass vials until analysis.

Statistical analyses

Means from all individuals in each aquarium were used in analyses of all parameters with the exception of growth and development. Growth and development data were repeated measures of a randomly selected subsample of 10 of the 20 larvae per tank. Mortality was calculated at the end of the experiment by dividing the number of larvae that died in each aquarium during the experiment by 20 (the initial number of larvae in the aquarium). Time to 50% metamorphosis was calculated as the number of days for 50% (10 of 20) of larvae to reach metamorphosis. Percent mortality, metamorphosis, and hematocrit data were transformed by taking the arcsine of the square-root of the proportion to normalize the data for analyses. Normality and equal variance assumptions were verified by examination of residual plots for random distribution. Raw data complied with these assumptions and were used for analyses of all other parameters.

Effects of atrazine, nitrate, and the interaction of atrazine and nitrate were determined by multivariate analysis of variance in SAS®, Version 6.12 [30], using a mixed model with Satterthwaite degrees of freedom. Clutch was included in the random statement to account for variability among clutches. Significant differences among clutches were determined by a likelihood ratio test in which we took the difference between the -2 residual log likelihood of the model without clutch in the random statement and the -2 residual log likelihood of the model with clutch in the random statement. This difference was compared on a χ² distribution with 1 df. When clutches were found to be significantly different for a given parameter, the Satterthwaite option uses the treatment-by-clutch interaction (if it is significant) as the error term instead of the mean square error. Larval length and stage were analyzed by repeated-measures analyses, and only the first eight weeks of data were used in these analyses because larvae began metamorphosing on the ninth week. We considered p-values < 0.05 to indicate significant differences.

RESULTS

Atrazine, nitrate, and their interaction had no significant effect on percent metamorphosis, time to metamorphosis, percent survival, mass at metamorphosis, or hematocrit (Table 1). However, nitrate significantly decreased larval length (F₁,213 = 4.04, p = 0.0190), though these differences in growth were never more than a couple of millimeters (Fig. 1A). For example, in week 4, larva exposed to 30 mg/L NO₃⁻/N were 29.5 ± 0.3 mm, while control larvae measured 31.3 ± 0.3 mm. Though there were no consistent effects of atrazine on growth (Fig. 1B), we found a significant atrazine-by-time effect (F₁,213 = 2.48, p = 0.0001) and atrazine-by-nitrate-by-
Fig. 1. Growth of Rana pipiens larvae exposed to (A) sodium nitrate as NO$_3$-N and (B) atrazine. Larvae from four separate egg masses were exposed to three concentrations of atrazine and three concentrations of nitrate in a factorial design for a total of 36 aquariums. Replicates (each a mean value from 10 tadpoles per aquarium) are pooled within each chemical and exposure concentration.

Fig. 2. Development of Rana pipiens larvae exposed to (A) sodium nitrate as NO$_3$-N and (B) atrazine. Larvae from four separate egg masses were exposed to three concentrations of atrazine and three concentrations of nitrate in a factorial design for a total of 36 aquariums. Replicates (each a mean value from 10 tadpoles per aquarium) are pooled within each chemical and exposure concentration. Tadpoles were staged according to Gooner [27].

time effect ($F_{2,21} = 1.80, p = 0.0107$). Likewise, there were no consistent effects of either atrazine or nitrate on development (Fig. 2), although we did find a significant atrazine-by-nitrate-by-time effect. Clutches differed significantly only for developmental stage ($\chi^2 = 7.081, p = 0.0082$).

Residue concentrations from juveniles exposed as larvae to 20 µg/L atrazine were found to contain 128.48 ± 16.93 µg/L atrazine (n = 12 aquariums), while juveniles exposed to 200 µg/L atrazine as larvae contained 1,147.08 ± 133.73 µg/L atrazine (n = 12 aquariums). Thus, atrazine concentrations in metamorphosed juveniles were approximately six times the concentration in the water, indicating bioconcentration of atrazine by larvae.

**DISCUSSION**

Nitrate slowed growth of R. pipiens larvae. Such a decrease in growth as a larva can have a significant detrimental impact later in the life of a frog by decreasing survival [31], size as an adult [31], rate of sexual maturation [32], mate selection [33], and locomotion ability for predator evasion [34]. The growth inhibition of larvae exposed to nitrate in our study was significant statistically; however, this finding does not necessarily imply ecological significance. There are many other natural environmental variables, both biotic and abiotic, that can affect growth of anuran larvae to a greater degree than that caused by nitrate exposure. Growth and development can be affected by food availability [35], temperature [35], density of larvae [36], reduction in water volume [37], and presence of predators or competitors [38].

We were able to detect small differences with high significance because we were able to control many of the environmental variables that affect growth and development. In our experiment, temperature was controlled for all aquaria and food was adjusted on a per capita basis. Density was not a factor in our experiment because the same number of larvae were distributed among all aquaria at the beginning of the experiment. Furthermore, density did not substantially change among treatments during the course of the experiment because there were no significant differences among treatments in percent metamorphosis, percent mortality, or time to metamorphosis.

Larson et al. [39] found dose-dependent effects of atrazine on the growth and development of tiger salamander (Ambystoma tigrinum) larvae. Ambystoma tigrinum larvae exposed to 250 µg/L atrazine progressed to metamorphic climax at the same time but at lower mass than controls. In contrast, larvae exposed to 75 µg/L atrazine reached metamorphic climax later.
but at similar size and mass as controls. Furthermore, they found that both concentrations of atrazine raised the levels of thyroxine in the larvae, while only the lower concentration of atrazine increased corticosterone. They argued that, while thyroxine tends to speed up metamorphosis, corticosterone prevents the conversion of triiodothyronine (T3) to thyroxine (T4), thus slowing metamorphosis and allowing greater growth between developmental stages. The fact that we did not see altered growth or development of R. piperi larvae exposed to atrazine may be due to differences in species’ sensitivity or differences in formulation of atrazine between the studies.

We are aware of only one study that examined bioconcentration of atrazine in amphibians, and it found no bioconcentration of atrazine in R. catesbeiana tadpoles [40]. However, the bioconcentration factor of sex (approximately) that we found for metamorphosed juveniles is within the range reported in the literature for fish (<0.27-12) [13].

Atrazine significantly decreased (p < 0.01) hemoglobin in Nile fish (Oreochromis niloticus and Cichlidae auratus) exposed to 6 mg/L atrazine for 14 d (55.7 and 51.1%, respectively) [16]. Hemoglobin levels were 16.4% lower (p < 0.05) in Tilapia mossambica exposed to 1.1 mg/L atrazine for 15 d [17]. However, the concentrations of atrazine that decrease hemoglobin levels in fish are much higher than the range in which we tested. This fact may explain why we found no decrease in hemocrit in R. piperi juveniles exposed to atrazine as larvae. According to the endpoints we examined in the laboratory, concentrations of atrazine and nitrate commonly found in the environment do not appear to pose a significant threat to R. piperi larvae through direct toxicity.

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