Herpetological Conservation and Biology 2(1):29-34
Submitted: 5 March 2007; Accepted: 30 March 2007

IMPACT OF AMMONIUM NITRATE ON WOOD FROG (*RANA SYLVATICA*) TADPOLES: EFFECTS ON SURVIVORSHIP AND BEHAVIOR

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***Abstract*.—Nitrogenous based fertilizers, such as ammonium nitrate, are commonly used in agriculture, entering aquatic ecosystems through runoff. Ammonium nitrate has been shown to affect the survivorship and behavior of anurans. We conducted an experiment to examine the potential toxic effects of ammonium nitrate on Wood Frog (*Rana sylvatica*) tadpoles. We also examined whether ammonium nitrate might interact with predator cues to affect tadpole behavior. Ammonium nitrate decreased survivorship of Wood Frog tadpoles ($\geq$ 50 mg L^{-1} NH_4NO_3). Activity level of Wood Frog tadpoles decreased when exposed to ammonium nitrate, as well as in the presence of predator (Mosquitofish, *Gambusia affinis*) chemical cues. Our results suggest that ammonium nitrate can have significant effects on anuran larvae, both through direct toxicological effects on survivorship, but also through behavioral effects.**

Key Words.—**activity; ammonium nitrate; behavior; *Gambusia affinis*; Mosquitofish; *Rana sylvatica*; toxicology; Wood Frog**

INTRODUCTION

Nitrogenous based fertilizers are commonly used in agriculture, entering ponds and other bodies of water through runoff associated with agriculture, industrial and human wastes, livestock waste, lawn fertilizers (Fenn et al. 2003; Holland et al. 2005). The amount of these fertilizers entering freshwater ecosystems is likely to remain the same, or even increase in the future (e.g., Tilman et al. 2001).

Several studies have examined the direct effects of nitrogen fertilizers on larval amphibians. Nitrogenous compounds are known to be lethal to some species of amphibian larvae at or near ecologically relevant concentrations (e.g., Marco et al. 1999; Ortiz et al. 2004; Smith et al. 2005; see also an earlier review by Rouse et al. 1999), but not always (e.g., Allran and Karasov 2000; Hatch and Blaustein 2000; Vaala et al. 2004).

Fewer studies have examined the sublethal effects of nitrogenous compounds on larval amphibians. Such sublethal effects can include decreased growth (e.g., Allran and Karasov 2000; Johansson et al. 2001; Smith et al. 2005), changes in the timing of metamorphosis (e.g., Edwards et al. 2006), and changes in behavior (e.g., Marco and Blaustein 1999; Marco et al. 1999; Hatch and Blaustein 2000). Sublethal effects have the potential to affect interactions between larval amphibians and their competitors and predators. In many instances, sublethal effects of a variety of pollutants have been shown to affect the community structure of ponds by altering the antipredator response of amphibian larvae (e.g., Bridges 1999; Lefcort et al. 1999; Rohr and Crumrine 2005) but they may also affect competition (e.g., Smith et al. 2006). Thus, pollutants have the potential to alter the interactions between amphibians and their predators.

We studied the effects of ammonium nitrate on the survivorship and behavior (activity level in the presence and absence of cues from a potential predator, the Mosquitofish *Gambusia affinis*) of Wood Frog (*Rana sylvatica*) tadpoles. Nitrogenous fertilizer run-off into surface waters frequently peaks with early spring rains or snow melt (see Rouse et al. 1999; see also discussions in Hecnar 1995; Ortiz-Santaliestra et al. 2006); thus, early spring breeders, such as Wood Frogs, might be particularly affected by nitrogen-based fertilizers, such as ammonium nitrate. Previous studies have shown that Wood Frog tadpoles are affected, either lethally or sublethally, by chronic exposure to ecologically relevant concentrations of nitrite (Griffis-Kyle 2005, 2007), but not to acute exposure to $\leq$ 20 mg L^{-1} of nitrate or $\leq$ 6 mg L^{-1} of nitrite (Smith, in press). We also examined the influence of tadpole age/size on the effects of ammonium nitrate.

MATERIALS AND METHODS

Several (> 4) wood frog egg masses were collected from a pond on the Denison University Biological Reserve, Licking Co., Ohio and brought into the laboratory to hatch and develop for approximately one week prior to our first experiment. Plastic containers, approximately 19 x 14 x 5 cm, containing 700 ml of solution, were used for this experiment. The tadpoles were exposed to one of four nominal ammonium nitrate treatments (replicated 5 times): control = 0 mg l^{-1}, low = 50 mg l^{-1} (= 11.2 mg l^{-1} NH_4^+, = 38.8 mg l^{-1} NO_3^-), medium = 100 mg l^{-1} (= 22.5 mg l^{-1} NH_4^+, = 77.5 mg l^{-1} NO_3^-), and high = 200 mg l^{-1} (= 33.8 mg l^{-1} NH_4^+, = 116.3 mg l^{-1} NO_3^-) made using ammonium nitrate crystals (CAS#61184-52-2; Fisher Scientific, Rochester, NY) and aged tapwater (water chemistry measured on aged tapwater used: Salinity = 0.3 ppt; pH = 8.5; dissolved oxygen = 9.40 mg l^{-1}; Nitrate-N < 1 ppm; Ammonia-N $\leq$ 0.1 ppm; Phosphate-P $\leq$ 1 ppm; Hardness = 176 ppm). These concentrations are within observed concentrations of nitrate or ammonium nitrate found in nature (10-250 mg l^{-1}; Xu and Oldham 1997; Rouse et al. 1999; Ortiz et al. 2004) and tested on other species of anuran tadpoles (see Table 1). Six tadpoles were placed in each replicate container creating a density of 8.6 tadpoles l^{-1}, which is at

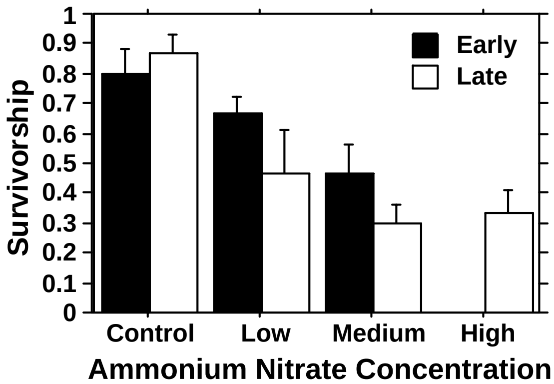

FIGURE 1. Effect of ammonium nitrate concentrations and tadpole age (early vs. late – see text) on the survivorship of Wood Frog (*Rana sylvatica*) tadpoles for one week. Means are given ± 1 SE N = 5 replicates for all means.

the lower end of the range of densities used in other experiments on ammonium nitrate (see Table 1).

Tadpoles were exposed to solutions for one week. All tadpoles were fed the same amount of ground Purina Rabbit Chow on the first day of exposure and on the fourth day of exposure. Excess food and feces were removed before the food was added on the fourth day. After the week of exposure, survivorship was determined using the number of tadpoles still alive in each container. Behavioral observations began after the survivorship data were collected. We recorded the number of movements of two focal tadpoles per container for ten minutes. Containers were divided into four quadrants, and we considered a tadpole to have moved each time it crossed a line into a different quadrant. After these initial observations were made, we added 50 ml of water from a holding tank (water volume = 5 l) occupied by 20 *G. affinis* for at least 1 week. Tadpoles were then given ten minutes to re-acclimate, and then observations on activity level were repeated for two focal tadpoles in each container as outlined above. We thus had pre- and post-cue activity level observations for each container (see Kruuk and Gilchrist 1997; Marquis et al. 2004 for similar methods).

A second set of experiments and behavioral observations ("late experiment") were conducted two weeks after the first set of experiments to allow for growth and development of the tadpoles. The two sets of experiments used the same methods. Tadpoles used in the second experiment were not exposed to ammonium nitrate until the start of the second experiment. Mean mass of 10 haphazardly selected tadpoles at the start of the early experiment was 0.080 ± 0.0005 g. Mean mass of 10 haphazardly selected tadpoles at the start of the late experiment was 0.094 ± 0.009 g. In both experiments tadpoles were Gosner stage 26 (Gosner 1960).

To examine the effects of tadpole age (early vs. late) and the nominal concentration of ammonium nitrate (control, low, medium, or high) on tadpole survivorship, we used a two-way ANOVA (α = 0.05) on the arcsin-square root transformed survivorships (i.e., proportion of tadpoles surviving for each replicate). We used repeated-measure ANOVAs (α = 0.05) to examine the effects of predator cue (as a repeated measure) and ammonium nitrate on the activity levels of the tadpoles. Preliminary analyses of activity using the number of surviving tadpoles as a covariate found no significant effects of the covariate and no significant interactions of the covariate and ammonium nitrate concentration. Due to the complete mortality of tadpoles at the high concentration in the early experiment, we used separate analyses of the activity levels for the two experiments.

RESULTS

Survivorship.—Survivorship of tadpoles significantly decreased as the concentration of ammonium nitrate increased (Fig. 1; $F_{3,32}$ = 23.7, P < 0.0001; all pairwise Fisher's PLSD tests among ammonium nitrate groups were significant at $P \leq 0.003$, except for the low vs. medium concentration comparison, which was not significant). Tadpole age had no effect on survivorship (Fig. 1; $F_{1,32}$ = 0.56, P = 0.46). However, the interaction of treatment and age did show a significant effect on tadpole survivorship. Mortality at low and medium concentrations of ammonium nitrate was slightly higher in the late experiment than in the early experiment; whereas, at the high concentration, mortality was complete in the early experiment, but not in the late experiment (Fig. 1, $F_{3,32}$ = 5.99, P = 0.0023).

Activity Level.—For the early experiment, activity was, in general, lower at low and medium concentrations of ammonium nitrate than in the control (Fig. 2A; $F_{2,12}$ = 8.98, P = 0.0041; Fisher's PLSD: control vs. low, P = 0.0012; control vs medium, P = 0.026). Activity was also significantly lower in the presence of the potential predator cue (Fig. 2A; $F_{1,12}$ = 7.46, P = 0.018). There was no significant interaction between ammonium nitrate concentration and the presence of the potential predator cue ($F_{2,12}$ = 1.96, P = 0.18).

For the late experiment, activity was not significantly affected by ammonium nitrate concentration, although activity levels did tend to be lower in the presence of ammonium nitrate (Figure 2B; $F_{3,14}$ = 1.43, P = 0.28). Activity tended to be lower in the presence of the potential predator cue, but the difference was not

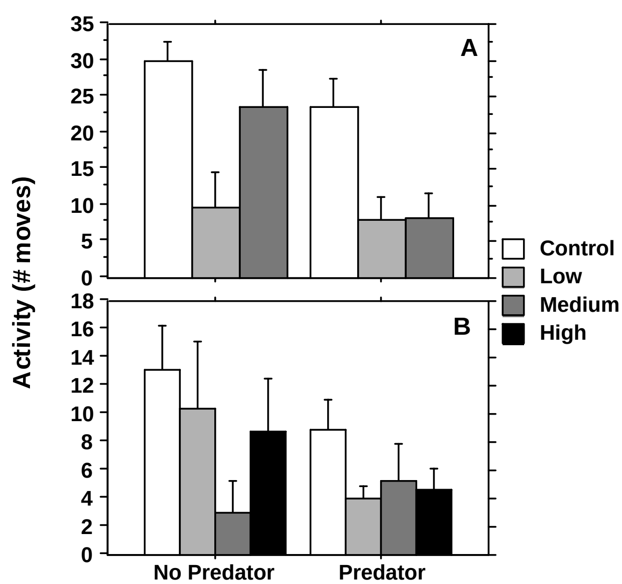

FIGURE 2. Effects of ammonium nitrate concentrations and the presence of a predator (*Gambusia affinis*) cue on the activity of Wood Frog (*Rana sylvatica*) tadpoles in the (A) early experiment (total mortality at the high concentration precluded measuring of activity), and (B) late experiment. Means are given ± 1 SE.

TABLE 1.— A review of the susceptibility of anuran tadpoles for exposure to ammonium nitrate. Values given are mg L^{-1} of ammonium nitrate unless otherwise noted.

Species	Duration	Lethal Effects (> 50% mortality) [mg l^{-1} NH_4NO_3]	Not lethal (< 25% mortality for all treatments) [mg l^{-1} NH_4NO_3]	Tadpole Density [tadpolesl^{-1}]	Source
Bufo americanus	96 h	80-228		10	Hecnar (1995)
B. bufo	15 d	1143		12-20	Ortiz et al. (2004)
B. bufo	96 h-7 d	≈ 2193		12.5	Xu and Oldham (1997)
B. calamita	8-12 d	648		?	Ortiz-Santaliestra et al. (2006)
B. calamita	15 d		Up to 1143	12-20	Ortiz et al. (2004)
Crinia signifera	21 d		Up to 19.4	19.8	Hamer et al. (2004)
Discoglossus galganoi	15 d	259		?	Ortiz-Santaliestra et al. (2006)
D. galganoi	15 d	1143		12-20	Ortiz et al. (2004)
Hyla arborea	15 d	286		12-20	Ortiz et al. (2004)
Limnodynastes peronii	91 d		Up to 19.4	39.6	Hamer et al. (2004)
Litoria aurea	150 d	12.9		9.9	Hamer et al. (2004)
Osteopilus septentrioinalis	13 d	228.6		2	Punzo and Law (2006)
Pelobates cultripes	8-15 d	259		?	Ortiz-Santaliestra et al. (2006)
P. cultripes	15 d		Up to 1143	12-20	Ortiz et al. (2004)
Pleurodeles waltl	15 d		Up to 1143	12-20	Ortiz et al. (2004)
Pseudacris regilla	10 d	281-570		4 – 6	Schuytema and Nebeker (1999)
P. triseriata	96 h	143		10	Hecnar (1995)
Rana clamitans	96 h	> 286		10	Hecnar (1995)
R. pipiens	96 h	143		10	Hecnar (1995)
R. sylvatica	7 d	50-100		8.6	This Study
Xenopus laevis	10 d	281-570		15	Schuytema and Nebeker (1999)

statistically significant (Fig. 2B; $F_{1,14} = 3.69$, $P = 0.075$). There was no significant interaction between ammonium nitrate concentration and the predator cue ($F_{3,14} = 1.41$, $P = 0.28$).

DISCUSSION

Our study suggests that ammonium nitrate can have a significant negative effect on the survivorship of Wood Frog tadpoles, at least at the range of concentrations used in this experiment. Our findings place the susceptibility of Wood Frog tadpoles to ammonium nitrate within the range of susceptibilities to ammonium nitrate found for a wide range of other species of anurans (Table 1). Thus, while Wood Frogs may not be physiologically more prone to ecological effects of ammonium nitrate exposure, they may be at more risk than other anurans if, as previous studies have suggested (see Hecnar 1995; Rouse et al 1999; Ortiz-Santaliestra et al. 2006), fertilizer runoff and contamination of surface waters peaks in the late winter or early spring when Wood Frogs breed.

Our results suggest that the negative effects of exposure to ammonium nitrate may change with age in Wood Frog tadpoles (e.g., total mortality at high concentrations in the early experiment, but 33% survivorship during the late experiment). The age and size differences in our study were relatively small, and all the tadpoles were the same developmental stage when tested. However, Ortiz-Santaliestra et al. (2006) found that a difference in only four days was enough to change the susceptibility of the hatchlings of *Pelobates cultripes* to ammonium nitrate, suggesting that small changes in age or size may be able to affect susceptibility of anuran tadpoles to ammonium nitrate. Other studies have also found tolerance for exposure to toxic substances can change over the development or growth of tadpoles (e.g., Harris et al. 2000; Smith 2001).

Our results show that Wood Frog tadpoles tend to decrease activity when exposed to ammonium nitrate at both early and late exposure times (albeit only significantly in the early experiment). Exposure to ammonium nitrate has been shown to reduce activity or feeding rate in a variety of other amphibian larvae (e.g., Hecnar 1995; Watt and Oldham 1995; Xu and Oldham 1997). The decrease in activity level of tadpoles or larvae in the presence of ammonium nitrate may actually decrease predation risk since activity in tadpoles frequently increases predation (see Lefcort 1996; Anholt et al. 2005). However, reduced activity could have negative effects since foraging, and consequently growth, are often functions of activity levels (Skelly and Werner 1990; Relyea and Werner 1999). Reduced activity may also affect the competitive abilities of tadpoles because activity level has sometimes been correlated with competitive ability in tadpoles (e.g., Morin and Johnson 1988; Werner 1992; Semlitsch 1993; Dayton and Fitzgerald 2001), but not always (e.g., Smith et al. 2004). Thus the consequences of these sublethal, behavioral effects of ammonium nitrate on Wood Frog tadpoles may depend on the ecological context of the exposure (e.g., whether predators or competitors are present or not).

Wood Frog tadpoles have been shown to decrease activity in the presence of fish (*Umbra limi*, Relyea 2000; *Lepomis* gibbosus, Richardson 2001; *L. macrochirus*, A.R. Awan and G.R. Smith In Press). In general, Wood Frog tadpoles in our experiment showed a decrease in activity level when exposed to water containing chemical cues from a potential predator, *G. affinis*, but this reduction in activity was only statistically significant in the early experiment. Size does not appear to affect the ability of Mosquitofish to prey upon tadpoles (Webb and Joss 1997); therefore, all tadpoles in this experiment would likely be susceptible to predation by the Mosquitofish. Lawler et al. (1994) found that younger tadpoles (Gosner Stage 26) of *Rana aurora draytoni* reduced activity levels in the presence of Mosquitofish, but older tadpoles (Gosner Stage 33-36) showed no reduction in activity. Other species of tadpoles show no response to the presence of Mosquitofish cues (Hamer et al. 2002; G.R.

Smith et al. unpubl. data). There was no significant interaction between predator cue presence and exposure to ammonium nitrate. Thus, there was no evidence that exposure to ammonium nitrate modified the response of tadpoles to the cues of Mosquitofish.

Acknowledgments.—Egg masses were collected under a permit from the Ohio Department of Natural Resources, and the experiments conducted with approval by the Denison University IACUC (#05-005; #06-007).

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