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Effects of Nitrate on Embryos of Three Amphibian Species

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Abstract Embryos of three aquatic breeding amphibian species, *Ambystoma mexicanum, Hyla chrysoscelis*, and *Rana clamitans*, were exposed to increasing levels (0, 5, 10, 30, 60, 100, 300, and 500 mg/L) of nitrate-N (NO_3^-) in laboratory, static-renewal experiments. Lethal effects were recorded from Gosner stage 2 (*H. chrysoscelis* and *R. clamitans*) or Harrison stage 2 (*A. mexicanum*) to time of hatching. Date of hatching and length at hatching were also compared between treatments for *A. mexicanum*. No significant differences in mortality between treatments were found between the three species. *A. mexicanum* in the 300 and 500 mg/L treatments hatched significantly earlier than individuals in the other treatment groups and, consequently, were significantly shorter in length at hatching. However, no effect on length was detected when days until hatching was considered as a covariate in the analysis. This study supports other recent research showing little to no effect of NO_3^- on amphibian embryos. The lack of effect at such high nitrate concentrations raises questions about the specific mechanisms responsible for protecting amphibians from NO_3^- during embryonic development, especially when compared to other chemicals that have shown more deleterious effects.

Keywords Nitrate · Agriculture · Amphibians · Embryos · *Hyla chrysoscelis* · *Rana clamitans* · Axolotl

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Degradation of aquatic habitats poses a severe threat to amphibian populations. The embryos, early hatchlings, and larvae of aquatic breeding species may be the most susceptible to decreases in water quality caused by contaminants in agricultural runoff. Embryos of these species commonly breed in agricultural ponds and have permeable membranes that absorb environmental chemicals from the water during development. Newly-hatched larvae may remain exposed to these chemicals for weeks to months (Duellman and Trueb 1986; Laposata and Dunson 1998; Kiesecker et al. 2001).

Nitrate (NO_3^-), the most stable and abundant of the nitrogen ions present in cropland and manure run-off, may pose a threat to young stages of amphibians when it is converted to NO_2^- in the gut. The ion NO_2^- changes hemoglobin in the blood to methehemoglobin and reduces the ability of the blood to carry oxygen (Hall 1991). Although both sublethal and lethal effects of nitrate (NO_3^-) on the survival of aquatic amphibian larvae have been well documented (Marco et al. 1999; Schuytema and Nebeker 1999; Allran and Karasov 2000), effects on embryos are less researched. One known study by Laposata and Dunson (1998) found no effect of NO_3^- on hatching success, percent deformed larvae, or survival of wood frog (*Rana sylvatica*), Jefferson salamander (*Ambystoma jeffersonium*), spotted salamander (*Ambystoma maculatum*), and American toad (*Bufo americanus*) embryos when exposed to concentrations of up to 40 mg/L from an early embryonic stage until time of hatching (Laposata and Dunson 1998). This suggests that amphibian embryos are highly tolerant to NO_3^-, because they do not show negative effects at high concentrations.

This is in contrast to NO_2^-, which has been shown to contribute to significant declines in hatching success and larval mortality of amphibians at low concentrations

(Greulich and Pflugmacher 2003) and to a number of other ions shown to have sublethal and lethal effects on amphibian embryos at concentrations common in agricultural environments. Such ions include a number of metals, hydrogen (pH), and boron (Freda et al. 1990; Whiteman et al. 1995; Laposata and Dunson 1998; Greulich and Pflugmacher 2003; Rohr et al. 2003; Griffis-Kyle 2005).

In this paper, we present research findings showing effects of NO_3^- on three additional aquatic breeding species. To our knowledge, no other studies estimating NO_3^- toxicity to amphibian embryos have been published since Laposata and Dunson (1998). We specifically chose concentrations greatly exceeding those of previous studies with the goal of identifying the limits of embryo tolerance to the NO_3^- ion.

Materials and Methods

Hyla chrysoscelis and *R. clamitans* egg masses were obtained from forested ponds in western Kentucky. *Ambystoma mexicanum* embryo masses were purchased from The Indiana University Axolotl colony. At the onset of the experiments, the anuran embryos were at the Gosner (1960) stage 2 stage of development, and the *A. mexicanum* embryos were at Harrison (1969) stage 2.

Buffer solutions reflected pH and alkalinity of the species' natural habitat, based on field measurements during collection and information taken from The Indiana University Axolotl Colony website (http://www.indiana.edu/~axlotl/). For experiments on *A. mexicanum*, a medium-hard buffer solution was prepared; for those on *H. chrysoscelis* and *R. clamitans*, a soft water buffer solution was prepared (Standard Methods 1998). Incubator temperatures were set according to temperatures at which each of the species was expected to thrive in a natural environment, based on a review of similar species in other studies and reference literature (Duellman and Trueb 1986; Baker and Waights 1994, 1998; Allran and Karasov 2000).

Stock solutions of 1,000 mg/L NO_3^- were prepared weekly by adding 6.07 g of $NaNO_3$ to 1 L of buffer solution in a volumetric flask. New treatment solutions were prepared prior to each addition by adding an appropriate amount of stock to a 2 L volumetric flask and diluting with the buffer solution. Treatment solutions were prepared by adding the appropriate amount of $NaNO_3$ to buffer solution to obtain the following target concentrations of NO_3^-–N: 0 mg/L (control), 5, 10, 30, 60, 100, 300, and 500 mg/L. Fingerbowls of 200 mL volume were filled to within 1 cm of the top with treatment solution and placed in an incubator set at either 16°C (*A. mexicanum*) or 25°C (*R. clamitans*, *H. chrysoscelis*) and a 12:12 light/dark cycle. The experimental design included four replicates per treatment, and 10 individuals per replicate.

For the *H. chrysoscelis* and *R. clamitans* experiments, NO_3^- dissolved oxygen (DO), alkalinity, and temperature concentrations of test water were measured at the beginning of experiments and within a select container of each concentration at the end of experiments (64 h for *H. chrysoscelis* and 96 h for *R. clamitans*; n = 2). Because of the longer length of the embryo experiments for *A. mexicanum*, water was replenished approximately every 72 h and test water was measured before each replenishment and in select containers following the 72 h period (n = 14). The previous solutions were then partially removed through decanting in all bowls and new solutions were added.

The pH of test water was measured by probe (ORION Instruments, Waltham, MA, USA), and alkalinity was measured by titrating to pH 4.8 using a HACH digital titrator (HACH Instruments, Boulder, CO, USA). All NO_3^- samples collected during the experiments were analyzed using a LACHAT 8000 Quickchem Flow Injection Analyzer with dilutor (LACHAT Instruments, Milwaukee, WI, USA). All DO samples collected during the experiments were measured by probe (YSI Instruments, Loveland, OH, USA) to ensure that concentrations were within a range of 7–10 mg/L.

Every 24 h, the number of live embryos remaining was recorded and dead embryos were removed. The experiment was conducted until all embryos had either died or hatched. For *A. mexicanum*, total larval length at hatching was determined by placing the larvae onto a piece of paper and measuring the distance between the snout to the end of the tail using a digital caliper (0.01 precision).

Statistical tests for significant differences were conducted using Statview software (SAS Institute, Cary, NC, USA). Significant differences in survival and hatching date (*A. mexicanum*) were determined using multiple comparisons ANOVA. A linear regression was used to investigate the effect of hatching date on larval length at hatching. An ANCOVA model, with time to hatching as the covariate, was used to detect differences in size at hatching (*A. mexicanum)*. The Bonferroni adjusted significance level of 0.00625 was used to detect significant differences between treatments for all ANOVA and ANCOVA analyses. Arcsine transformations were performed on all percentage data prior to analysis.

Results and Discussion

Table 1 shows general environmental conditions measured during the experiments. Table 2 shows measured NO_3^- concentrations. All of the NO_3^- concentrations were within 10% of target values (0, 5, 10, 30, 60, 100, 300, and 500 mg/L NO_3^-N), except the 30 mg/L treatment for *R. clamitan*, which was within 20% of the target value.

Table 1 General environmental conditions for each test species, ±SE in parenthesis

	pH	Temperature (°C)	Alkalinity (mEq/L)
A. mexicanum	7.91 (0.04)	19.2 (0.1)	0.789 (0.016)
H. chrysoscelis	7.37 (0.12)	24.0 (0.0)	0.345 (0.044)
R. clamitans	7.52 (0.08)	24.0 (0.0)	0.256 (0.026)

All *A. mexicanum* embryos hatched by day 22, all *R. clamitans* embryos hatched by 96 h, and all *H. chrysoscelis* embryos hatched by 64 h. Mortality did not vary significantly with NO_3^- concentration (Table 3) for *A. mexicanum* ($F_{7,24} = 0.506$, $p = 0.218$), *R. clamitans* ($F_{7,24} = 1.910$; $p = 0.112$), or *H. chrysoscelis* ($F_{7,24} = 0.936$; $p = 0.498$).

For the *A. mexicanum* experiment, significant differences were found in time to hatching. ($F_{7,24} = 7.670$; $p < 0.0001$). A post hoc Bonferroni test revealed that the 0, 5, 30, 60, and 100 mg/L treatments hatched significantly earlier than the 300 mg/L treatment ($p < 0.00625$) and the 0, 5, 10, 30, 60, and 100 mg/L treatments hatched significantly earlier than the 500 mg/L treatment ($p < 0.00625$; Fig. 1). Date of hatching was significantly related to length at hatching according to a linear regression ($F_{1,30}$; $p = 0.0103$; $r^2 = 0.200$). However, no significant differences were found in length at hatching when date of hatching was considered as a covariate ($F_{7,16} = 0.804$; $p = 0.596$; Fig. 2).

Even low levels of NO_3^- had no effect on survival of embryos of all three species, and few sublethal effects for *A. mexicanum*. These findings are consistent with a previous study showing no effect on embryos of four other amphibian species exposed to up to 40 mg/L NO_3^- (Laposata and Dunson 1998). However, pH, various pesticides, dissolved metals, boron, elevated levels of dissolved organic carbon, and NO_2^- have all been found to have significant effects on mortality and/or hatching success in other studies (Freda et al. 1990; Freda 1991; Whiteman et al. 1995; Laposata and Dunson 1998; Greulich and Pflugmacher 2003; Griffis-Kyle 2005). This raises questions about mechanisms responsible for protecting embryos from some pollutants and not others.

Several potential explanations exist for this invulnerability. The nature of NO_3^- toxicity may be such that NO_3^- ions have to be ingested or absorbed before they can become toxic. Since embryos lack means of uptaking the ions, they may be essentially immune during development. Another possibility is that embryos have not yet developed the enzymes necessary to convert NO_3^- to harmful NO_2, which has been found to have lethal effects on embryos (Griffis-Kyle 2005).

Yet another mechanism may be the role of the perivitelline membrane in protecting the developing embryo. Immediately after hatching, the perivitelline membrane surrounding the embryo fills with water from its

Table 2 Actual measured concentrations of NO_3^-N, in mg/L, during experiments (±SE)

	Treatment							
	0	5	10	30	60	100	300	500
A. mexicanum	0.002 (0.96)	5.22 (0.23)	10.57 (0.46)	32.29 (1.66)	61.69 (2.76)	104.96 (3.18)	312.19 (13.06)	520.21 (14.44)
H. chrysoscelis	0.001 (0.00)	5.41 (0.23)	10.17 (0.25)	29.28 (0.46)	56.54 (0.69)	95.19 (4.34)	295.14 (5.84)	499.24 (10.21)
R. clamitans	0.030 (0.03)	6.00 (1.78)	10.55 (0.89)	36.58 (0.88)	69.355 (9.18)	110.72 (7.76)	312.83 (24.69)	511.44 (3.64)

Table 3 Comparison of proportion of embryos surviving (±SE in parentheses) for each treatment category. No significant differences were detected for *A. mexicanum* ($F_{7,24} = 0.506$, $p = 0.218$), *H. chrysoscelis* ($F_{7,24} = 0.936$, $p = 0.498$), and *R. clamitans* ($F_{7,24} = 1.910$; $p = 0.112$) using ANOVA

	Treatment							
	0 mg/L	5 mg/L	10 mg/L	30 mg/L	60 mg/L	100 mg/L	300 mg/L	500 mg/L
A. mexicanum	0.950 (0.035)	0.925 (0.042)	0.925 (0.042)	0.850 (0.057)	0.925 (0.042)	0.900 (0.048)	0.875 (0.033)	0.925 (0.042)
H. chrysoscelis	0.625 (0.165)	0.650 (0.029)	0.725 (0.111)	0.625 (0.125)	0.650 (0.104)	0.650 (0.029)	0.900 (0.041)	0.600 (0.122)
R. clamitans	0.925 (0.048)	0.650 (0.155)	0.675 (0.075)	0.975 (0.025)	0.900 (0.071)	0.850 (0.096)	0.775 (0.193)	0.975 (0.025)

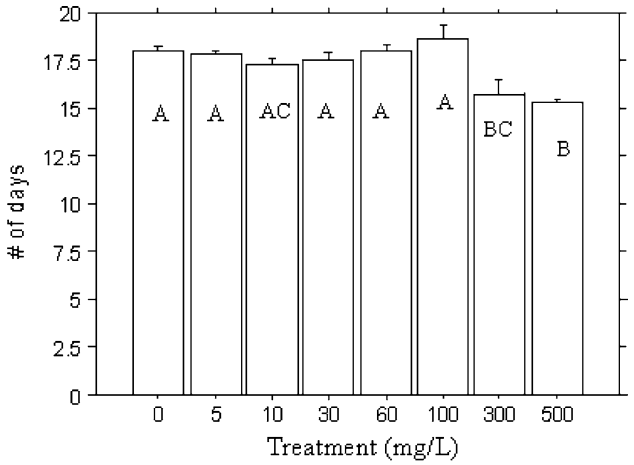

Fig. 1 Number of days until hatching (±SE) with exposure to increasing NO_3^-–N concentration (±SE) for *A. mexicanum*. ANOVA showed significant differences between treatments ($F_{7,24} = 7.670$; $p < 0.0001$) with different letters

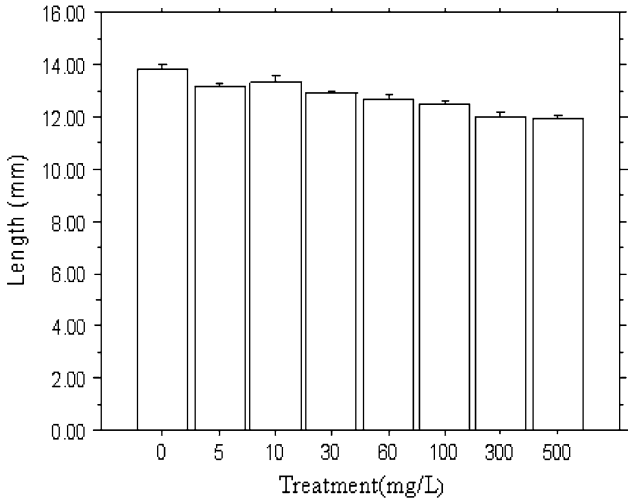

Fig. 2 Length at hatching for *A. mexicanum* (±SE) with exposure to increasing NO_3^-–N concentration. ANCOVA, with days until hatching as covariate, showed no significant difference in length at hatching between treatments ($F_{7,16} = 0.804$; $p = 0.596$)

surroundings, which provides a protective barrier (Duellman and Trueb 1986). After hatching, the osmotic pressure of fluid in the embryo potentially slows the movement of negatively charged ions like NO_3^- into the membrane and, consequently, may have a dilution effect. This is a similar process to how ammonia toxicity is prevented in embryos when osmotic pressure causes water to diffuse from adjacent egg capsules to the perivitelline membrane (Duellman and Trueb 1986). In this case, only ions that are able to penetrate this membrane would have deleterious effects on embryos. As result, the water quality to which embryos are initially exposed may be critical. Our study assumed that the concentrations of NO_3^- into which the embryos hatched was low (forested ponds in the case of *H. chrysoscelis* and *R. clamitans* and a controlled laboratory environment in the case of *A. mexicanum*). However, the quality of water into which the eggs hatched was not tested, and further research is needed. Future experiments should work to simulate the deposition of embryos into an already polluted environment in order to test these varying hypotheses.

Although statistically significant, only a small difference was detected in days until hatching for *A. mexicanum* in the highest concentrations. Early hatching has been documented in response to pH, and has been suggested as an adaptive way of gaining mobility and avoiding stressful environmental conditions (Whiteman et al. 1995). Because individuals that hatched earlier were also shorter, early hatching could have negative ramifications for fitness and predation vulnerability. However, this effect was only found in extremely high concentrations (300 and 500 mg/L NO_3^-N), which exceed those documented for agricultural run-off (Peterjohn and Correll 1984). No studies could be found showing the range of concentrations present in agricultural ponds, where embryos of aquatic breeding species are often deposited. More research is needed to understand why amphibian embryos are not affected by high concentrations of NO_3^- and the implications of this finding for amphibian conservation.

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