

Sublethal effects of nitrite on eastern tiger salamander (*Ambystoma tigrinum tigrinum*) and wood frog (*Rana sylvatica*) embryos and larvae: implications for field populations

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Abstract Ephemeral pools, which can have high animal biomass and low dissolved oxygen, may be prone to nitrite accumulation. As such, it is important to understand how exposure to nitrite might affect development and growth of amphibians that breed in these ephemeral pools. Wood frog (*Rana sylvatica*) and eastern tiger salamander (*Ambystoma tigrinum tigrinum*) embryos and tadpoles and young larvae were exposed to elevated concentrations of nitrite derived from sodium nitrite: 0, 0.3, 0.6, 1.2, 2.1, 4.6, and 6.1 mg l⁻¹ NO₂-N. Increasing nitrite exposure slowed embryonic and larval development in both the eastern tiger salamander and the wood frog, reduced growth in tiger salamander embryos and larvae, and delayed metamorphosis in the wood frog. At concentrations less than 2 mg l⁻¹ NO₂-N nitrite delayed hatching, and at concentrations above 2 mg l⁻¹ time to hatching decreased causing more individuals to hatch at less developed stages. Nitrite also increased asynchrony in tiger salamander hatching. The sublethal effects of nitrite on

amphibian development, growth and hatching could have serious repercussions on amphibian fitness in ephemeral environments. Potential increases in mortality on field populations caused by sublethal effects of nitrite are discussed.

Keywords Metamorphosis · Growth · Development · Ephemeral ponds · Nitrogen · Amphibian declines

Introduction

Over the past several decades amphibian populations worldwide have been declining (Stuart et al. 2004). Researchers have linked these declines to various causes including habitat fragmentation and destruction, exotic species invasions, harvesting, pollution, and natural fluctuations (Berger 1989; Phillips 1990; Griffiths and Beebee 1992; Blaustein et al. 1994; Pounds and Crump 1994; Reaser 1996; Carey et al. 1999; Kiesecker et al. 2001). Some of these declines may be related to the increasing amounts of mineral nitrogen (N) (nitrite, nitrate, ammonium) entering our surface waters (Rouse et al. 1999). Over the last century, increased fossil fuel consumption, fixation of previously unavailable gaseous N₂ for fertilizer, as well as other anthropogenic activities have doubled the amount of available N in the environment (Vitousek et al. 1997). This mineral N can have direct toxic effects on aquatic organisms.

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High concentrations of nitrate and ammonium can be harmful to organisms through the conversion of nitrate to nitrite and direct toxicity of ammonium (Jensen 1995). Nitrite may enter organisms with food, diffuse through dermal layers as HNO_2 , or enter by active diffusion through the gills of aquatic organisms through the chloride uptake mechanism. Additionally, nitrate and ammonium are transformed into nitrite in the animal's digestive tract (Jensen 1995; Lewis and Morris 1986). Nitrite then accumulates in the aquatic organism's tissues extracellularly at concentrations much higher than in the environment (Eddy et al. 1983).

Pollution, high animal biomass, or any factor, such as pH, sodium, or dissolved oxygen that differentially affects *Nitrobacter* spp. (bacteria that transform nitrite to nitrate) and *Nitrosomonas* spp. (bacteria that transform ammonium to nitrite) can cause an accumulation of nitrite in the water column (Lewis and Morris 1986; Jensen 1995; Neuman et al. 2001; Sampaio et al. 2002). Ephemeral ponds often experience high animal biomass and low dissolved oxygen, so they may be susceptible to the accumulation of nitrite, particularly if they receive additional N inputs from agriculture or other sources.

Nitrite can cause direct lethal effects in amphibians (Huey and Beitinger 1980a; Marco et al. 1999; Smith et al. 2004; Griffis-Kyle 2005), but the sublethal effects, especially on amphibians that breed in ephemeral ponds in agricultural regions, need to be explored. Hatching variability and growth and development of embryos and larvae of the eastern tiger salamander (*Ambystoma tigrinum tigrinum*) and the wood frog (*Rana sylvatica*) collected from ephemeral ponds in an agricultural area in Minnesota were examined to accomplish this. These effects were examined into the larval period for tiger salamanders and through metamorphosis for wood frogs.

Materials and methods

Study organisms

Wood frogs (*Rana sylvatica*) are common in the northern United States and Canada and breed in

shallow ponds. Eastern tiger salamanders (*Ambystoma tigrinum tigrinum*) are common in eastern North America and into Canada. In Minnesota, adults of both species arrive at ponds of varying hydroperiods as the ice melts and lay their eggs over several weeks. Three wood frog egg masses were collected from three ponds and 18 eastern tiger salamander egg masses were collected from six ponds in 2004. Egg masses (wood frog Gosner (1960) stage 10; tiger salamander Shi and Boucaut (1995) stage 16) were returned to the laboratory and separated into smaller clusters for hatching experiments.

Well water used in the experiment was from Cedar Creek Natural History Area, Bethel Minnesota and was filtered to remove rust, sediments, chlorine, lead, and bacteria using an Omnicfilter system (filters RS-55 and CB3) (Delavan, WI, USA). The filtered water had pH 8.2, 304.5 $\mu\text{S}/\text{cm}$ conductivity, 121 mg l^{-1} total alkalinity, <2 mg l^{-1} total hardness, 0.05 mg l^{-1} $\text{NO}_3\text{-N}$, 0 mg l^{-1} $\text{NO}_2\text{-N}$, and 0 mg l^{-1} $\text{NH}_3\text{-N}$. Lighting approximated the natural photoperiod in this area of Minnesota during May, 15 h of light and 9 h dark, and provided the full spectrum of light radiation. Containers were cleaned for all experiments twice weekly, keeping the same nitrite treatment concentration (sodium nitrite 99.60% pure, A.C.S. Fisher Scientific, Fairlawn, NJ, USA).

At three times during the experiment, testing water was sampled for mineral N just before the containers were cleaned. Fifty milliliters of fluid were collected from each of five pseudo-randomly chosen containers per species (Microsoft Excel pseudo-random number generator). The samples were filtered through combusted Whatman 25 mm (GF/F) glass fiber filters (Clifton, NJ, USA) and immediately (<2 min) frozen (U.S. Environmental Protection Agency 1983). These samples were analyzed for ammonium, nitrate and nitrite colorimetrically with an Alpkem auto-analyzer (College Station, TX, USA) at Cedar Creek Natural History Area (modified from Environmental Sciences Section 1991). Dissolved oxygen (YSI probe, Yellow Springs, OH, USA), pH and temperature (Hannah probe, Woonsocket, RI, USA) were measured in all containers weekly.

Embryo experiments

Egg masses were split randomly into groups of 10 eggs so that each group had eggs from at least two different egg masses. Eggs that were discolored were excluded from the test. Each group was placed into a clear 3 l Sterilite container, with five replicate containers per treatment per species, and seven treatment levels (0, 0.3, 0.6, 1.2, 2.1, 4.6, and 6.1 mg l⁻¹ NO₂-N derived from sodium nitrite) (Griffis-Kyle 2005). Containers were randomly assigned locations on two shelving units. Eggs were checked daily for hatchlings. Experiments ran for 10 days, until all eggs either hatched or became discolored and began to break down. All individuals were staged (Gosner 1960; Shi and Boucaut 1995) and digitally photographed with a digital dissecting scope (Motic DMW143, B.C. Canada). Images of tadpoles and larvae were later measured (Motic Images 2000, Version 1.3, B.C. Canada). Developmental stage and length were averaged within the replicates and the replicates were analyzed for treatment effects.

Larvae and tadpole experiments

For the tadpole and larval experiment, 30 randomly chosen wood frogs and 30 randomly chosen tiger salamanders that hatched during the embryo experiments were used per treatment level of nitrite for a total of 210 wood frogs and 210 tiger salamanders. Individuals were removed from the hatching containers within 24 h of hatching. If fewer than 30 individuals hatched for a given species in a given treatment, the number available was used. Early tadpole and larval survival were tested at nitrite concentrations of 0, 0.3, 0.6, 1.2, 2.1, 4.6, and 6.1 mg l⁻¹ NO₂-N. All tadpoles and larvae were housed individually in opaque Solo cups containing 0.4 l of water. After 3 weeks, wood frog tadpoles were moved into individual containers holding 2.0 l of water. Daily, tiger salamander larvae were fed brine shrimp larvae, and tadpoles were fed algae. Tadpoles were also supplied with boiled lettuce and fish food ad libitum. Mortality was tallied daily.

Tiger salamanders were examined for 26 days of exposure because of the large amount of food

they require. After 26 days tiger salamanders were staged (Shi and Boucaut 1995), weighed and measured with calipers. Wood frogs were examined through metamorphosis. Time to metamorphosis, and mass and size at metamorphosis were recorded for the wood frogs.

Treatment conditions

Actual treatment concentrations are reported below as ranges, but these ranges were not significantly different between species (analysis of variance: $F = 1.4$, $df = 1$, $P = 0.3$); therefore, treatment ranges are reported for both species combined. For embryos, nitrite increased between cleanings, and actual treatment concentrations were as follows: 0–0.0, 0.3–0.5, 0.6–0.7, 1.2–1.5, 2.1–2.3, 4.6–4.9, and 6.1–7.7 mg l⁻¹ NO₂-N. For tadpoles and larvae the actual treatments concentrations were 0–0.27, 0.3–0.59, 0.6–0.9, 1.2–1.4, 2.1–2.2, 4.6–5.0, and 6.1–7.6 mg l⁻¹ NO₂-N. Ammonium averaged 0.3 mg l⁻¹ N (standard error (SE) = 0.01) in the embryo treatments and 0.5 mg l⁻¹ N (SE = 0.01) in the tadpole and larvae treatments. Nitrate averaged 0.18 mg l⁻¹ N (SE = 0.02) over all treatments and life stages. pH averaged 8.2 (SE = 0.003), and temperature averaged 17.8 C (SE = 0.03).

Analyses

Size and development are bivariate data and correlated, and as such were examined using conditional bivariate normal correlation models (Neter et al. 1996). These models are used to make inferences about one predictor variable on a response variable conditional on the value of a second predictor variable. In this study, I examined the effect of nitrite on amphibian size controlling for development, and the effects of nitrite on development controlling for salamander size. If univariate data showed a nonlinear response to nitrite (Neter et al. 1996), responses were examined by fitting curves to the data (SPSS 1997). All other relationships were examined using linear regression unless scatterplots indicated nonlinear relationships (Neter et al. 1996; SPSS 1997). All residual plots were examined for deviations of the data from the model tested (Neter et al. 1996).

Results

Eastern tiger salamander

Nitrite exposure significantly affected hatching time for the eastern tiger salamander (fitted cubic curve: $r^2 = 0.30$, $df = 32$, $P < 0.01$), at first increasing time to hatching for concentrations up to $2.1 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$ and then decreasing time to hatching for higher concentrations (Fig. 1A). The variation (standard error, SE) in hatching day increased with increasing nitrite concentration (linear regression: $r^2 = 0.43$, $df = 33$,

$P < 0.0005$) (Fig. 1B) increasing asynchrony in hatching.

For the eastern tiger salamander, nitrite has a significant negative effect on developmental stage at hatching controlling for the size of the hatchling (bivariate normal correlation model: $R^2 = 0.58$, $F = 22.2$, $P < 0.0005$; nitrite $B = -0.09$, $P < 0.0005$) (Fig. 2 A, C, D). Nitrite also had a significant negative effect on salamander hatchling size controlling for development (bivariate normal correlation model: $R^2 = 0.45$, $F = 12.9$, $P < 0.0005$; nitrite $B = -0.15$, $P = 0.05$) (Fig. 2 B, C, D).

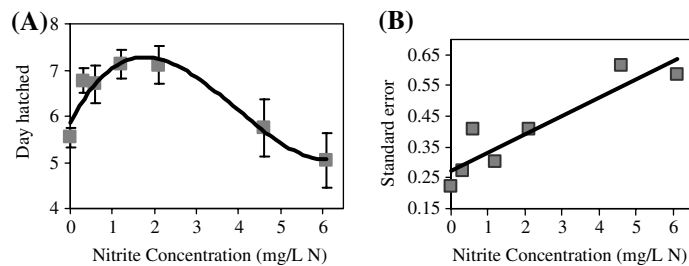


Fig. 1 (A) Average day hatched (fitted cubic curve: $r^2 = 0.30$, $P = 0.01$) and (B) variation in hatching day (linear regression: $r^2 = 0.43$, $P < 0.0005$) for eastern tiger

salamander replicates are significantly affected by nitrite concentration. Error bars in (A) represent standard errors

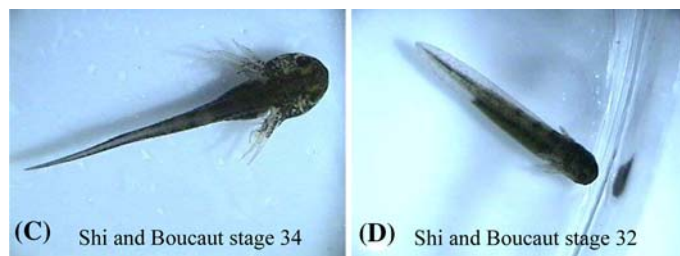
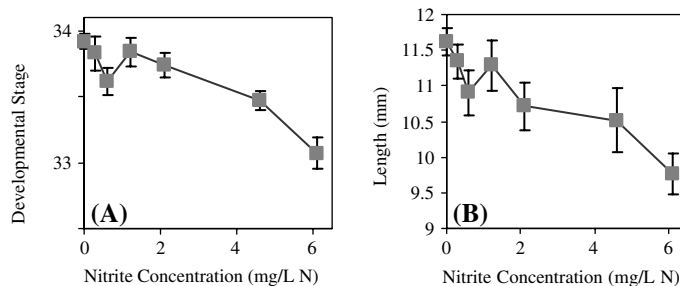


Fig. 2 Nitrite concentration is inversely related to eastern tiger salamander (A) development (Shi and Boucaut (1995) stages) (bivariate normal correlation model: $R^2 = 0.58$, $P < 0.0005$) and (B) length (bivariate normal correlation model: $R^2 = 0.45$, $P < 0.0005$) at hatching.

Boxes represent means of replicates, and error bars represent standard errors. Photographs of eastern tiger salamanders taken within 24 h of hatching show differences in development and size for individuals exposed to (C) $0 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$ and (D) $6.1 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$

After 3 weeks of exposure, salamander development was negatively effected by nitrite especially at low concentrations (fitted cubic curve: $r^2 = 0.37$, $df = 161$, $P < 0.0005$) (Fig. 3A). Salamander size was also negatively associated with increasing nitrite concentration, controlling for development, after 3 weeks of exposure (bivariate normal correlation model: $R^2 = 0.73$, $F = 217.6$, $P < 0.0005$; nitrite $B = -0.30$, $P = 0.04$) (Fig. 3B).

Wood frogs

Nitrite exposure had no significant effect on the average day a replicate of wood frogs hatched or the SE associated with that hatching (linear regression in both cases: $R^2 < 0.02$, $df = 30$, $P > 0.4$) (Fig. 4).

Nitrite exposure slowed wood frog development controlling for the individual's size at hatching (bivariate normal correlation model: $R^2 = 0.52$, $F = 16.3$, $P < 0.0005$; nitrite $B = 0.09$, $P = 0.01$) (Fig. 5 A, C, D), but did not affect the size of wood frog embryos controlling for the individual's development (bivariate normal correlation model: $R^2 = 0.43$, $F = 11.2$, $P < 0.0005$; nitrite $B = 0.06$, $P = 0.3$) (Fig. 5B).

Wood frogs took longer to reach metamorphosis as nitrite concentration increased (linear regression: $r^2 = 0.19$, $df = 47$, $P = 0.002$) (Fig. 6A), and wood frog mass was positively related to nitrite concentration (linear regression: $r^2 = 0.12$, $df = 47$, $P = 0.02$) (Fig. 6B). However time to metamorphosis (exposure time) was a much stronger predictor of mass at metamorphosis for wood frogs (linear regression: $r^2 = 0.35$, $df = 47$, $P < 0.0005$). In fact, adding nitrite concentration as a predictor variable to the regression of wood frog mass related to exposure days only accounted for an increase in the R^2 of 0.02, and in a stepwise procedure nitrite concentration was excluded from the model. Note that all wood frogs at the two highest concentrations, 4.6 and 6.1 mg l⁻¹ NO₂-N, died before metamorphosis (Griffis-Kyle 2005).

Discussion

My results show that nitrite negatively affects both eastern tiger salamander and wood frog embryos and larvae by causing reductions in growth and delays in development, and for the tiger salamander increasing variability in hatching

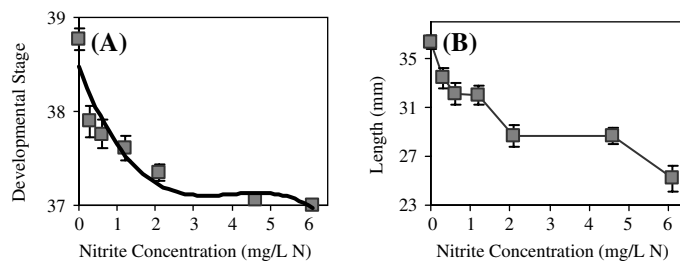


Fig. 3 Averages and standard error for eastern tiger salamander (A) development (Shi and Boucaut (1995) stages) (fitted cubic curve: $R^2 = 0.37$, $P < 0.0005$) and (B)

length (bivariate normal correlation model: $R^2 = 0.73$, $P < 0.0005$) after 26 days of exposure showing a negative relationship with nitrite concentration

Fig. 4 (A) Average day hatched and (B) variation in hatching day for wood frog replicates are not significantly related to nitrite concentration (linear regression in both cases: $r^2 < 0.02$, $P > 0.4$)

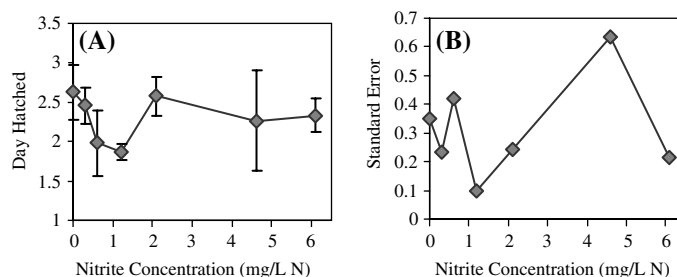
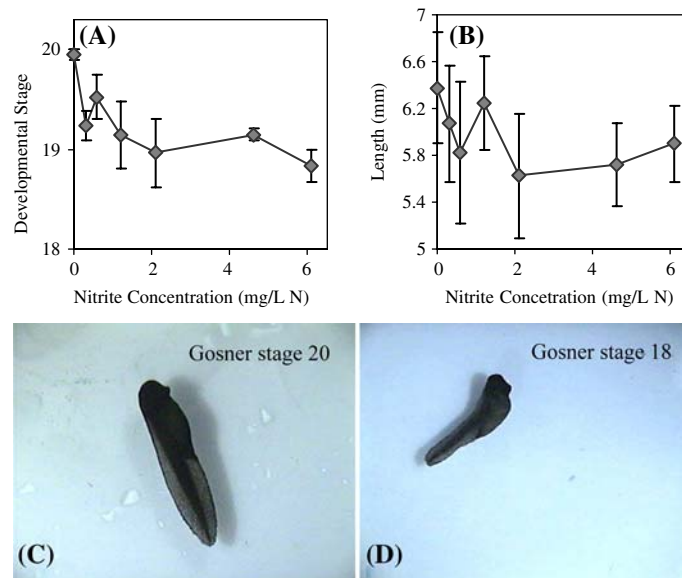


Fig. 5 Nitrite concentration is inversely related to wood frog (A) development (Gosner (1960) stages) (bivariate normal correlation model: $R^2 = 0.52$, $P < 0.0005$) but not to (B) length (bivariate normal correlation model) at hatching. Diamonds represent means of replicates, and error bars represent standard errors. Photographs of wood frog tadpoles taken within 24 h of hatching show differences in development for individuals exposed to (C) $0 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$ and (D) $6.1 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$



time. For the wood frog, these delays in development translated into an expanded larval period, delaying metamorphosis as compared to individuals not exposed to nitrite. These sublethal effects may influence amphibian survival especially in ephemeral environments.

Increasing nitrite exposure first increased the amount of time to hatching, then at concentrations above approximately $2 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$ hatching occurred more quickly. Those individuals that hatched at the higher concentrations also tended to be less developed, suggesting that nitrite causes embryos to hatch before they might otherwise in the absence of nitrite. Additionally, as nitrite concentration increased, the variability associated with hatching date increased, leading to asynchronous hatching within replicates.

Nitrite tends to delay development and reduce size in amphibians at hatching. Embryos exposed to nitrite tended to hatch before their respiratory systems were fully developed. A greater proportion of wood frog embryos exposed to higher concentrations of nitrite hatched at Gosner (1960) stages 18 and 19, before circulation begins in the gills (Duellman and Trueb 1986). A greater proportion of tiger salamander embryos exposed to higher concentrations of nitrite hatched at Shi and Boucaut (1995) stage 32, before their gills branched. Branching of the gills provides a greater surface area per volume ratio for oxygen

diffusion, resulting in more efficient oxygen uptake. Thus, it appears that nitrite reduces oxygen consumption efficiency at hatching for both wood frogs and eastern tiger salamanders.

Tiger salamander larvae exposed to higher nitrite concentrations tend to be smaller than those exposed to less nitrite after 3 weeks of exposure, whereas wood frogs exposed to higher nitrite tend to metamorphose at a larger mass. This is not as counterintuitive as it may seem. All tiger salamanders were measured on the same day without regard to development, whereas wood frogs were measured at a specific developmental stage (Gosner stage 45). As nitrite concentration increases, it takes longer for wood frogs to reach a given developmental stage (metamorphosis), giving individuals more time to accumulate mass before completing metamorphosis into the terrestrial juvenile form.

Marco and Blaustein (1999) also found that nitrite slows growth and development and delays metamorphosis in Cascades frog (*Rana cascadae*) tadpoles. They examined tadpoles shortly before metamorphosis, from Gosner (1960) stage 39 through stage 45, and suggested the slowed growth and delayed metamorphosis were due to high oxygen consumption throughout metamorphosis (Feder 1982) and reduced oxygen uptake by the blood due to nitrite exposure (Huey and Beitinger 1980b). Whereas, Marco and Blaustein

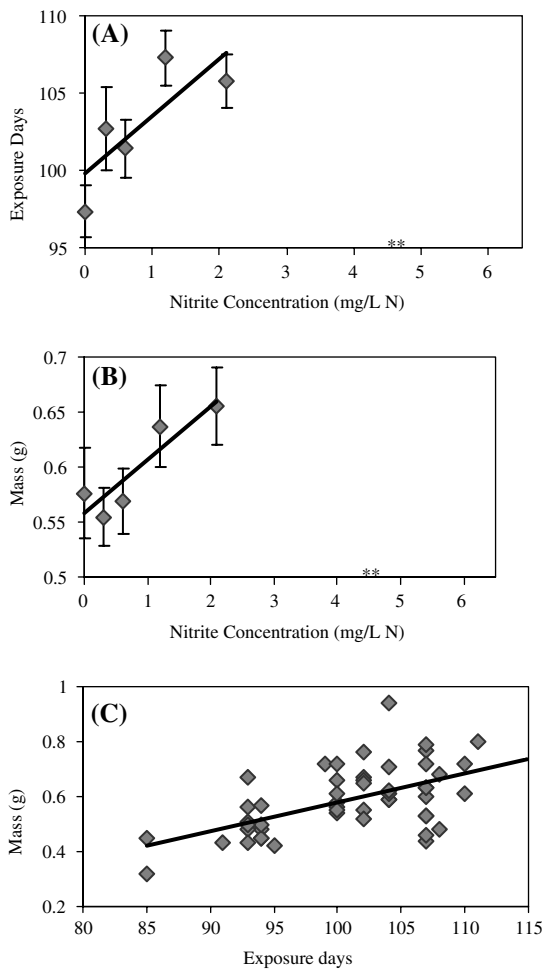


Fig. 6 (A) Time to metamorphosis is positively related to nitrite concentration (linear regression: $r^2 = 0.19$, $P = 0.002$). (B) Mass of wood frogs at metamorphosis (Gosner stage 45) is positively related to nitrite (linear regression: $r^2 = 0.12$, $P = 0.02$). (C) Mass of wood frogs at metamorphosis is related to exposure time (linear regression: $r^2 = 0.35$, $P < 0.0005$), a relationship much stronger than the relationship between mass and nitrite. Error bars represent standard errors and * indicate treatments in which all individuals died

(1999) only examined tadpoles late in development, my data demonstrates that development is slowed throughout both the embryonic and larval periods.

Implications for field populations

Very few studies have measured nitrite in the field because it is only expected to accumulate

when there are asymmetries in denitrification and nitrification; however, researchers have found elevated concentrations greater than $0.1 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$ (Mackerness and Keevil 1996; Rice and Jung 2004), and even up to $18 \text{ mg l}^{-1} \text{ NO}_2\text{-N}$ (McCoy 1972). This research examined concentrations from 0 to 6.1 mg l^{-1} , within the range of nitrite concentrations documented in the field. Ephemeral ponds can experience high animal biomass, anoxic conditions and in many areas receive mineral N runoff, all potential causes of asymmetries in the reactions that may lead to an accumulation of nitrite. Accumulation of nitrite may indirectly increase mortality in field populations of amphibians through consequences of the sublethal effects presented here.

Amphibian embryos and larvae in ephemeral environments are faced with a number of causes of mortality including pool desiccation and predation. Nitrite can potentially amplify the proportion of the population taken by these factors. For example, ephemeral pools dry and can cause full mortality in the cohort of amphibians within the pool if they have not completed metamorphosis (Berven 1990). This research shows nitrite can delay metamorphosis, which could lead to an increased frequency of zero reproductive output for any given pond, significantly decreasing individual fitness and potentially reducing population growth. Second, most invertebrate predators in ephemeral pools are size-limited in the prey they can take based on the predator's body size and method of capture and consumption (Wellborn et al. 1996). Any stressor, such as nitrite, that slows growth should make amphibians susceptible to predation for a longer period of time; thus, leading to a greater proportion of the cohort that is lost to aquatic predators. Third, nitrite causes both wood frogs and tiger salamanders to hatch before their respiratory structures are fully developed, likely reducing oxygen uptake efficiency. This reduction in efficiency is especially important after hatching because individuals are faced with predator avoidance and other activities that require more oxygen than required by the movements the embryos make within the eggs. Lastly, nitrite tends to increase the variation associated with hatching date in the tiger salamander. Increasing variation in hatching time,

called asynchronous hatching, can increase predation risk. In synchronous hatching, where all individuals tend to hatch over a short period of time, the predator population becomes satiated on hatchlings and some individuals escape. However, if individuals hatch over a greater range of time, predators can consume a larger proportion of the hatchlings because they do not necessarily become satiated (Drickamer and Vessey 1986); suggesting nitrite exposure could indirectly increase population mortality by increasing the asynchrony in hatching.

These results suggest that nitrite could indirectly increase amphibian mortality from predators and the environment through sublethal effects on hatching, growth, and development. As the amount of nitrogen entering aquatic ecosystems continues to increase (Bumb and Baanante 1996; National Atmospheric Deposition Program 2005), more ephemeral pools may develop increased nitrite concentrations, potentially causing or increasing population declines in amphibians through both lethal (Huey and Beitinger 1980a; Marco et al. 1999; Smith et al. 2004; Griffis-Kyle 2005) and sublethal pathways.

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