Amphibian survival, growth and development in response to mineral nitrogen exposure and predator cues in the field: an experimental approach

Kerry L. Griffis-Kyle · Mark E. Ritchie

Abstract Mineral nitrogen (N) has been suggested as a potential factor causing declines in amphibian populations, especially in agricultural landscapes; however, there is a question as to whether it remains in the water column long enough to be toxic. We explored the hypothesis that mineral N can cause both lethal and sublethal toxic effects in amphibian embryos and larvae in a manipulative field experiment. We sampled 12 ponds, fertilizing half with ammonium nitrate fertilizer early in the spring, and measured hatching, survival, development, growth, and the incidence of deformities in native populations of wood frog (*Rana sylvatica*) and eastern tiger salamander (*Ambystoma tigrinum tigrinum*) embryos and larvae held in *in situ* enclosures. We found that higher ammonium concentrations negatively affect *R. sylvatica* more strongly than *A. tigrinum*. *R. sylvatica* tended to have lower survival as embryos and young tadpoles, slowed embryonic development, and an increased proportion of hatchlings with deformities at experimentally elevated ammonium. *A. tigrinum* did not experience significantly reduced survival, but their larval development was slowed in response to elevated ammonium and the abundance of large invertebrate predators. Variable species susceptibility, such as that shown by *R sylvatica* and *A. tigrinum*, could have large indirect effects on aquatic community structure through modification of competitive or predator-prey relationships. Ammonium and nitrate + nitrite concentrations were not correlated with other measures that might have affected amphibians, such as pH, pond area, depth, or vegetation. Our results highlight the potential importance of elevated ammonium on the growth, development and survival of amphibians, especially those that breed in surface waters receiving anthropogenic N inputs.

Keywords Amphibian declines · Agriculture · *Rana sylvatica* · *Ambystoma tigrinum tigrinum* · Field experiment

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Introduction

Increasing nitrogen (N) enrichment has become one of the largest and most pervasive threats to our environment (Galloway et al. 2002; Holland et al. 2005). Mineral N in aquatic environments can cause declines in species diversity and changes in community composition, through both direct and indirect effects on individuals and populations via increased productivity, acidification, and potentially toxicity (Camargo and Alonso 2006).

Amphibians are an important group of aquatic organisms that may be susceptible to N enrichment. Amphibians are undergoing widespread declines; some

have been caused by habitat loss and over-exploitation, but many declines are as yet unexplained (Stuart et al. 2004). Uncovering interactions between pollutants such as nitrogen and amphibian survival may be critical to the long-term management and recovery of amphibian populations. Laboratory evidence is growing on the toxic effects of mineral N on amphibians (e.g., Hecnar 1995; Marco et al. 1999; Johansson et al. 2001; Griffis-Kyle 2005; Smith et al. 2006); however, these laboratory studies do not include the interacting factors that might either mitigate or exacerbate relationships in the field. For example, in the field there may be positive effects of enrichment through trophic interactions if N is assimilated and productivity is increased. Additionally, if plants take up the mineral N quickly, organisms may then be exposed to the toxic forms of N for a much shorter period of time.

In buffered systems unlikely to experience acidification, mineral N entering surface waters can have two very different consequences for amphibians and other biota. Mineral N in ponds is assimilated into living tissue at a rate that depends on phosphorus and micronutrient availability (Wetzel 2001). If this process is fast enough, little mineral N will remain in the water column. This assimilation stimulates primary production leading to increased food for herbivores; this potentially reduces competition, thereby increasing survival, growth and development. However, if plants and microbes do not take up the mineral N or only take it up slowly, then the N ions will remain in the water column, potentially causing toxic effects.

Mineral N tends to be highest in surface waters in temperate climates during snowmelt and early spring rains (Meybeck et al. 1989; Brooks and Williams 1999), possibly because plants and microbes that biologically assimilate mineral N are dormant or have greatly reduced activity during winter, allowing N to accumulate in soils. During spring thaw, this N is flushed into surface waters when many species of amphibians are breeding and laying eggs (Wright and Wright 1949; Petranka 1998). This temporal concurrence between amphibian breeding and high N concentration may be critical, because embryos and young larvae are more susceptible to toxins than older larvae and adults (Schuytema and Nebeker 1999a, 1999b), and embryonic exposure to mineral N may increase mortality later in development (Griffis-Kyle 2005). However, assimilation of N in surface waters could potentially benefit amphibians by increasing primary production and hence food supply, and thereby mitigating earlier toxic effects.

We examined the effects of pond fertilization and mineral N concentration (nitrogen from ammonium and nitrogen from nitrite plus nitrate) on amphibian survival and development in 12 ponds in east-central Minnesota. We evaluated two main alternative research hypotheses: (1) early exposure to mineral N negatively affects amphibians through declines in survival and/or slowed development and growth, and (2) elevated early mineral N concentrations have positive effects on amphibian embryo and larval survival, development, and growth through increased plant production and subsequent trophic interactions. Additionally, we explored the hypothesis that high mineral N concentrations early in development increase the incidence of amphibian deformities. We assessed these hypotheses in a field experiment by introducing a pulsed application of ammonium nitrate (NH_4NO_3) fertilizer to ponds and evaluating the effects of the resulting mineral N concentrations on *Rana sylvatica* (wood frog) and *Ambystoma tigrinum tigrinum* (eastern tiger salamander) from the embryo phases into the tadpole and larval stages, using a combination of enclosures and whole pond sampling.

Materials and methods

In 2004, we selected ponds in east central Minnesota from aerial photographs. We randomly selected 12 of a total possible 25 ponds, excluding ponds that had sphagnum mats, clay soils, surface area greater than 1 ha, or adjoined to large wetlands, so as to minimize variation in amphibian communities. We also did not sample ponds adjacent to animal pastures. All ponds were located in an agricultural region within 50 m of cropland, and local amphibian populations were likely to have been exposed to N over time, possibly leading to populations more tolerant of mineral N than populations in non-agricultural areas. All ponds were surrounded by reed canary grass (*Phalaris arundinacea)* and all but two ponds, one treated and one not treated, had broad-leaved cattails (*Typha latifolia*).

We randomly selected six ponds to receive N additions, and six ponds as controls. We sampled mineral nitrogen concentrations on 12 April, and then calculated the approximate pond volume (surface area × depth), to estimate the amount of fertilizer we needed to add to bring whole-pond mineral N concentrations up to 10 mg l^{-1} N. This concentration was slightly higher than concentrations in mesocosm experiments shown to cause 35% mortality in *R. temporaria*, but no effect on *Bufo bufo* (de Wijer et al. 2003) and well below the 7-day LC_{50} calculated for *B. bufo* tadpoles (260 mg l^{-1} NH_4–N, Xu and Oldham 1997) and the 10-day LC_{50} for *Pseudacris regilla* tad-

poles (55.2 mg l^{-1} NH$_4$–N) and *Xenopus laevis* tadpoles (52.9 mg l^{-1} NH$_4$–N) (Schuytema and Nebeker 1999b).

During the period 12–15 April, we established 129 l *in situ* enclosures, five per pond in 12 ponds where the water was approximately 1 m deep. Enclosures were made out of plastic 34 gallon trash cans, in which we drilled 64 holes of 2 cm radius below the water line. Holes were covered with 700 μm mesh to allow water and small invertebrates access to enclosure interiors while excluding potential predators. We did not assess food resources in enclosures but did note the presence of algae and zooplankton. We removed the centers of the lids and replaced the plastic with mosquito netting supported by hardware cloth to exclude invertebrates laying eggs or any other organisms from reaching the amphibians. We placed 30 *R. sylvatica* embryos (Gosner 1960, stage 11) into each of three enclosures and 20 *A. tigrinum* embryos (Shi and Boucaut 1995, stage 16) into each of two enclosures per pond, each enclosure containing eggs from a different egg mass collected from that pond. We placed eggs in a bag made out of a 1 m^2 mesh (700 μm) with the edges gathered and held by a rubber-band that hung in the water in the enclosure so that we could easily find and assess embryos and hatchlings. During the period 15–19 April, we added dissolved ammonium nitrate fertilizer to the ponds. The fertilizer was agitated and dissolved into pond water in a 2 l container and then distributed into the pond while we walked through the water to ensure adequate mixing. We did not add dissolved fertilizer directly next to enclosures, to ensure that eggs were not subjected to short-term high concentrations because of our application procedures. Our calculations overestimated the amount of N needed in the fertilized ponds and additional runoff from a large rain event (18–19 April) and snowmelt created a gradient in all ponds of concentrations 3 days after fertilization ranging from 0.8 to 45 mg l^{-1} total mineral N, averaging 18 mg l^{-1} N (Fig. 1). We cleaned the enclosure screens once a week throughout the experiment to facilitate adequate water exchange between the enclosure and the pond. We measured, staged, and checked all larvae for deformities within 3 days of hatching, then releasing the hatchlings from the mesh bags into the larger enclosures. We repeated these measurements and weighed larvae using a pesola scale at three weeks post-hatch, after which we released all larvae from the enclosures into the ponds. We handled amphibians using small hand-held dipnets and measured them with calipers in small v-shaped troughs full of water so as to minimize stress.
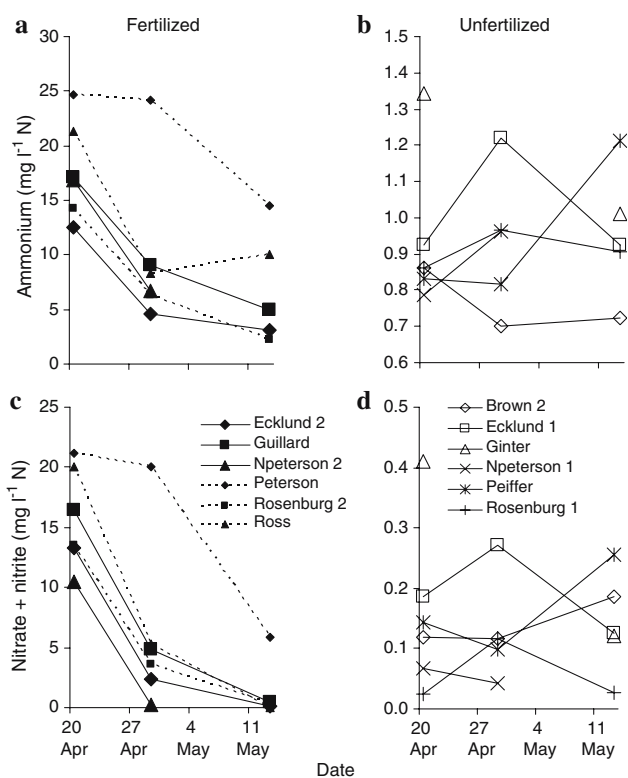

Fig. 1 Change of N concentrations as ammonium and nitrate plus nitrite in six (**a**, **c**) fertilized and six (**b**, **d**) unfertilized experimental ponds from 20 April to 11 May 2004 located in east central Minnesota. Fertilized ponds averaged 18.8 mg l^{-1} NH$_4$–N and 16.3 mg l^{-1} NO$_3$ + NO$_2$–N, with 53% of the mineral N from ammonium. Unfertilized ponds averaged 0.79 mg l^{-1} NH$_4$–N and 0.16 mg l^{-1} NO$_3$ + NO$_2$–N

Site conditions

We sampled water chemistry bi-weekly from March into May 2004. We measured dissolved oxygen (YSI probe, Yellow Springs, OH, USA), pH, conductivity, and temperature (Hannah probe, Woonsocket, RI, USA) with portable ion probes at four randomly located points around the pond 1 m from the edge of emergent vegetation. Water chemistry values were averaged for the pond. We took 20-ml water samples along 1-m transects, 1 m from the edge of emergent vegetation, midway between the surface and substrate at three points around the pond, for a total of 60 ml per pond (Wetzel and Likens 1991). Using a syringe, water samples were filtered through combusted Whatman 25-mm glass fiber filters (GF/F) (Clifton, NJ, USA) and immediately placed on ice. We then froze the samples at the lab and analyzed them within 25 days for mineral N concentration (ammonium, and nitrate plus nitrite) (United States Environmental Protection Agency 1983). All 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).

We tested water from all ponds for the presence of pesticides shortly after amphibians had hatched (15 May) to assess ambient pesticide residue during vulnerable stages. To determine which pesticides were likely to be present, we surveyed farmers for pesticides they had used either in the previous or current year. Then we collected unfiltered water samples from 6 to 10 locations per pond in 1-l acid-washed Nalgene bottles, one combined sample per pond. Samples were immediately put on ice, frozen within 1 hour and shipped within 2 days to Columbia Food Laboratories, Inc. (Corbett, OR, USA) for testing. We did not account for exposure to pesticides applied after May 15.

We sampled the invertebrate community using dip-nets from the sediment surface up to the water surface along a transect between 1 and 3 m from the pond edge, sampling every 3 m. We later used minnow traps in the same locations to sample for fish and larger invertebrates that may have been missed by dip-netting. We calculated an index of predator abundance, including fish, Coleoptera, Hemiptera, and Odonata greater than 3 cm, by calculating the number of individuals caught per trap night per area of the pond. These large predators were caught only infrequently in dip nets. We estimated the longest pond diameter and shortest pond diameter during spring high water to calculate an index of pond surface area. We conducted visual surveys of the percent floating and emergent vegetation coverage. We estimated canopy cover as the proportion of water surface directly under tree canopy. We used a self-tripping benthic dredge sampler to collect benthic sediments and roots.

We searched all ponds for egg masses 1 April through 15 May weekly, using timed visual searches noting location, approximate developmental stage and species of each egg mass. After the first search we only counted new egg masses, determined by developmental stage and location in pond.

Analysis

We calculated the average hatching, survival, and length per pond per species to compensate for clutch effects. Because we were unable to tightly control N concentrations and because concentrations varied over an order of magnitude across ponds, we evaluated N effects using both regression and analysis of variance (ANOVA) ($\alpha = 0.05$). We considered using a probit analysis to analyze amphibian survival data; however, in all cases a probit function did not fit our data (in all cases $\chi^2 > 120.0$, $P < 0.0005$), and therefore we used linear and multiple

regression to examine all amphibian responses to N from ammonium (NH_4–N) and the sum of N from nitrate and nitrite (NO_3 and NO_2–N) (Neter et al. 1996). Ammonium and nitrate plus nitrite were tightly correlated on all sampling dates (Pearson's $r > 0.97$, $P < 0.0005$), so we only report results for ammonium in the text because ammonium is much more toxic than nitrate (Jensen 1995). See Appendix 1 for all regressions of mineral N and predator abundance on *R. sylvatica* and *A. tigrinum* survival, growth, development, and incidence of deformities. Note that by week 3, two ponds had desiccated, one pond was not sampled on April 29 for mineral N and one pond was not sampled for predators because of a large tadpole die-off noted when we arrived at the pond to sample (tadpoles infected with ranavirus, confirmed by United States Geological Service, National Wildlife Health Center, Madison, WI, USA, unpublished report 19100). We used ANOVA to examine differences in physical and chemical parameters between treatments and multivariate analysis of variance to examine differences in chemical parameters between sampling dates (Neter et al. 1996). All dependent variables tested met assumptions for normality (Kolmogorov–Smirnov test) and homogeneity of variance (Levene test of the homogeneity of variance) (SPSS 1997). Average pH was calculated from the logarithm of the reciprocals (Wetzel 2001)

Results

Environmental effects

Before fertilization, ponds on average had no detectable nitrate plus nitrite, but averaged 0.08 mg l^{-1} NH_4–N (ammonium values include both ammonia and ammonium) (standard error SE = 0.01). Just after fertilization (15 through 19 April) and after a large rain event (18 and 19 April), all ponds, including unfertilized, had increased concentrations of ammonium and nitrate plus nitrite. Concentrations and proportions of nitrate, nitrite and ammonium can fluctuate with the addition of 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) (Lewis and Morris 1986; Jensen 1995). Hence, we are unable to calculate the proportion of nitrite versus nitrate for our data, so the effects of the two are inextricably coupled in our analysis.

As time progressed, the amount of available N declined dramatically from levels just after fertilization in almost all ponds (Fig. 1). Later runoff increased

ammonium concentrations in several unfertilized ponds, and maintained concentrations in one fertilized pond. Two ponds were not included in this analysis because they had desiccated by 10 May. Unfertilized ponds all had a greater proportion of N present as ammonium than as nitrate, likely due to nighttime hypoxia which leads to ammonium accumulation (Wetzel 2001). Ammonium is much more toxic than nitrate to aquatic organisms (Jensen 1995). Unfertilized ponds had a significantly greater proportion of mineral N from ammonium than from nitrate or nitrite than fertilized ponds shortly after fertilization ($F_{1,10} = 96.3$, $P < 0.0005$). Later in the year (11 May) the proportion of N from ammonium did not differ between fertilized and unfertilized ponds ($F_{1,8} = 0.1$, $P = 0.8$), suggesting an equilibrium had been reached in conversions between the two forms of N in the fertilized ponds.

Ammonium, and nitrate plus nitrite were correlated with conductivity on all dates (Pearson's $|r| > 0.89$, $P < 0.0005$ in all cases). pH was not related to ammonium or nitrite plus nitrate (Pearson's $|r| < 0.17$, $P > 0.5$ in all cases) or conductivity (Pearson's $|r| < 0.35$, $P > 0.3$ in all cases) on any sampling date.

Based on the farmers' responses regarding pesticide use, we tested for acetochlor (Harness) in one pond, atrazine in all 12 ponds, glyphosate (Roundup, Accord) in all 12 ponds, and 2,4-D in one pond. The minimum detection levels were: acetechlor 1 part per billion (ppb), atrazine 1 ppb, glyphosate 50 ppb, and 2,4-D 1 ppb. One pond had concentrations of atrazine at 1.26 ppb and less than 1 ppb of 2,4-D, and another pond had less than 1 ppb of atrazine present. By chance, both ponds with detectable pesticide concentrations were experimentally fertilized.

There was no significant difference in the number of *R. sylvatica* or *A. tigrinum* egg masses per pond between treatments ($F_{1,10} < 0.8$, $P > 0.3$) (*R. sylvatica*: untreated ponds averaged 27 egg masses, SE 19; fertilized ponds averaged 10 egg masses, SE 4) (*A. tigrinum*: untreated ponds averaged 8 egg masses, SE 3; fertilized ponds averaged 12 egg masses, SE 5).

Ponds did not significantly differ in pH, temperature, dissolved oxygen, pond area, depth, or canopy cover by treatment ($F_{1,10} < 3.6$, $P > 0.1$ in all cases; Table 1). Additionally, ammonium and nitrate plus nitrite on all sampling dates did not vary by pond area (in all cases $r^2 < 0.15$, $F < 1.5$, $P > 0.2$) or by pond depth (in all cases $r^2 < 0.1$, $F < 0.3$, $P > 0.3$).

Amphibian enclosures

Rana sylvatica hatched 11 days after placing the eggs in enclosures, an average of 8 days post-N fertilization.

Table 1 Average chemical and physical parameters in six fertilized and six unfertilized experimental ponds in east central Minnesota after fertilization (samples collected April and May 2004)

	Fertilized	Unfertilized
Chemical parameters		
Mineral nitrogen	18 mg/l N (SE 4.3)	1 mg/l N (SE 0.1)
Conductivity	240.7 (SE 44.9)	103.2 (SE 13.5)
PH	7.4 (SE 0.2)	7.4 (SE 0.1)
Dissolved O_2	11.7% (SE 0.6)	11.8% (SE 0.5)
Temperature	19.8 (SE 0.3)	20.1 (SE 0.9)
Physical parameters		
Area	1,053 m^2 (SE 288)	1,157 m^2 (SE 378)
Depth	1.37 m (SE 0.1)	1.55 m (SE 0.2)
Canopy cover	0.2% (SE 0.2)	1% (SE 0.6)

There was a nonsignificant trend for proportionately fewer *R. sylvatica* hatching in fertilized ponds ($P = 0.1$; Table 2) (untreated ponds averaged 70% hatched, SE 10; fertilized ponds averaged 38% hatched, SE 16), and hatching appeared to decline with increasing ammonium concentration ($r^2 = 0.30$, $F_{1,10} = 4.3$, $P = 0.06$) (Fig. 2a).

The average *R. sylvatica* length within 48 hours of hatching was 7.0 mm (SE = 0.4) and was shorter in fertilized ponds ($P = 0.03$; Table 2). Frog length declined as ammonium increased ($r^2 = 0.60$, $F_{1,8} = 11.8$, $P = 0.009$) (Fig. 2c).

Table 2 Results from ANOVA of pond fertilization treatment on survival, growth, development and deformities of *Rana sylvatica* and *Ambystoma tigrinum tigrinum* at hatching and 3 weeks after hatching in enclosures. Note that by week 3, one fertilized and one unfertilized pond had desiccated, four fertilized ponds no longer had any living *R. sylvatica*, and three ponds, both fertilized and unfertilized, no longer had any living *A. tigrinum*. Gross deformities were not present at week 3

|	Number of ponds (n)		ANOVA	
	Fertilized	Unfertilized	F	P
R. sylvatica				
Hatching				
Percentage of hatched	6	6	2.79	0.1
Length	5	5	6.27	0.03
Development	5	5	12.50	0.008
Deformities	5	5	1.60	0.2
Three weeks				
Percentage of survived	5	5	4.14	0.07
Length	2	5	0.09	0.7
Development	2	5	0.72	0.4
A. tigrinum				
Hatching				
Percentage of hatched	6	6	0.07	0.8
Length	6	6	0.87	0.3
Development	6	6	2.87	0.1
Deformities	6	6	0.50	0.5
Three weeks				
Percentage of survived	5	5	0.24	0.6
Length	3	4	0.12	0.7
Development	3	4	1.21	0.3
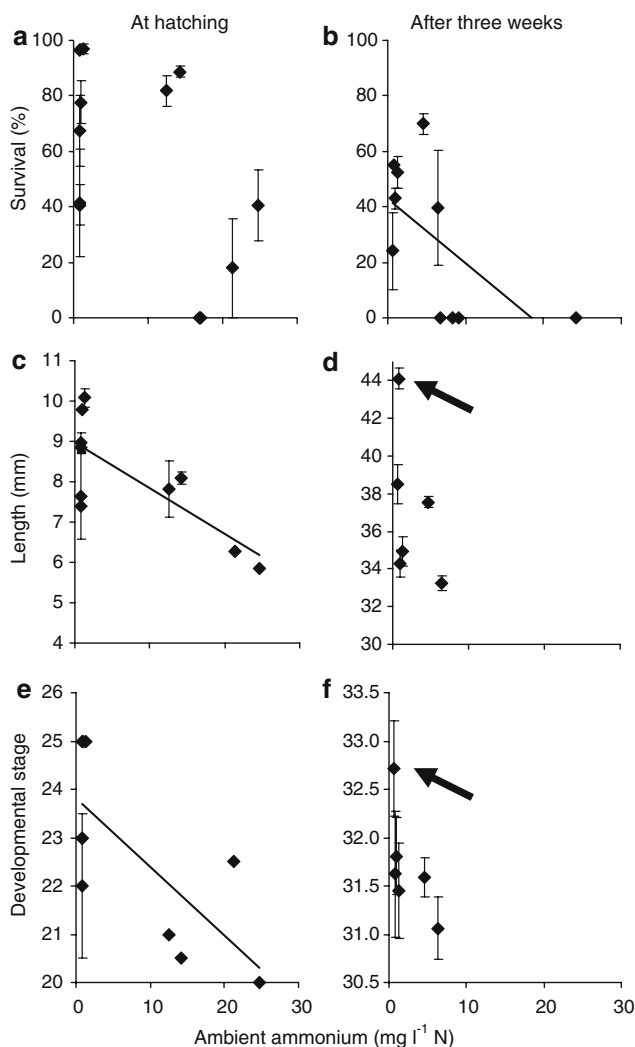
Fig. 2 **a**, **b** Survival, **c**, **d** growth (length), and **e**, **f** Gosner (1960) developmental stage (means ± SE) of *Rana sylvatica* in response to ambient ammonium levels at hatching and after 3 weeks in enclosures in a whole-pond fertilization experiment. Significant linear regressions are noted with trendlines. Note there are no individuals surviving after 3 weeks in ponds with > 6.5 mg l^{-1} NH$_4$–N. The *arrows* in (**e**) and (**f**) indicate the pond with the highest abundance of predators (fish, Coleoptera, Hemiptera and Odonata > 3 cm). *Error bars* are absent for ponds where only one enclosure of several had surviving individuals, and for ponds where individuals hatched at the same stage. We were unable to explain a greater amount of variation by fitting curves to the data

On average, *R. sylvatica* had reached Gosner (1960) stage 21 within 48 hours of hatching. Frogs in treated ponds were less developed at hatching than those in unfertilized ponds (*P* = 0.008; Table 2), and their developmental stage significantly declined in relation to the concentration of ammonium (*r*^{2} = 0.53, *F*$_{1,8}$ = 8.9, *P* = 0.02) (Fig. 2e).

We found deformities in 3.6% of the *R. sylvatica* hatchlings in the pond with the highest concentrations of ammonium and of atrazine (see Fig. 1a, Peterson).

Hence, we were unable to statistically tell the difference between the two predictors. However, we looked to the literature to determine the compound that was most likely to cause the deformities we documented, which included asymmetries in development, edemas, and skin irregularities. Similar deformities were documented for ammonium (Hecnar 1995, Xu and Oldham 1997). At the atrazine concentration present in the pond, we might expect to find abnormalities in sexual development (Hayes et al. 2002), deformities we did not examine. Hence we present regression results for ammonium only. The incidence of deformities at hatching increased at high ammonium concentrations during the hatching period (*r*^{2} = 0.86, *F*$_{1,8}$ = 44. 4, *P* < 0.0005). The incidence of deformities was not significantly related to treatment (Table 2).

Rana sylvatica 3-week survival declined significantly with increasing ambient ammonium (*r*^{2} = 0.51, *F*$_{1,9}$ = 9.4, *P* = 0.01) (Fig. 2b), and tended to be lower in fertilized ponds (untreated ponds averaged 49% survived, SE 7; fertilized ponds averaged 18% survived, SE 12) (statistically not significant) (*P* = 0.07; Table 2). The two fertilized ponds that had surviving *R. sylvatica* tadpoles also had the lowest ammonium concentrations of the fertilized ponds on all dates (see Fig. 1a, Ecklund 2 and Rosenberg 2). No *R. sylvatica* survived in the enclosures in the three ponds with concentrations greater than 6.5 mg l^{-1} NH$_4$–N (*n* = 4) (Fig. 2b).

After 3 weeks *R. sylvatica* exposed to higher ammonium (*β* = −0.1, standardized partial regression coefficient), and exposed to more large predators (large dragon fly larvae, predaceous diving bugs and beetles and fish) (*β* = 168.1, standardized partial regression coefficient) through development, were less developed than those individuals not exposed (*R*^{2} = 0.97, *F*$_{2,2}$ = 28.8, *P* = 0.03) (Figs. 2f, 4c); conversely, these factors did not explain tadpole length (*R*^{2} = 0.66, *F*$_{2,2}$ = 1.9, *P* = 0.3) (Figs. 2d, 4a). Length and development were not significantly associated with treatment (*P* = 0.4; Table 2); however, only two treated ponds had any surviving *R. sylvatica*, so our power to detect differences based on treatment was reduced for this analysis.

Ambystoma tigrinum hatched an average of 24 days after placing the eggs in the enclosures and 22 days post N-fertilization. *A. tigrinum* hatching was not significantly related to treatment (*P* = 0.8; Table 2), to mineral N concentration, or to any other water chemistry parameter (in all other cases Pearson's |*r*| < 0.5, *n* = 12, *P* > 0.08) (Fig. 3b).

Ambystoma tigrinum averaged 14 mm (SE = 0.3) at hatching, but their length was not statistically related to treatment (*P* = 0.3; Table 2), mineral N or other
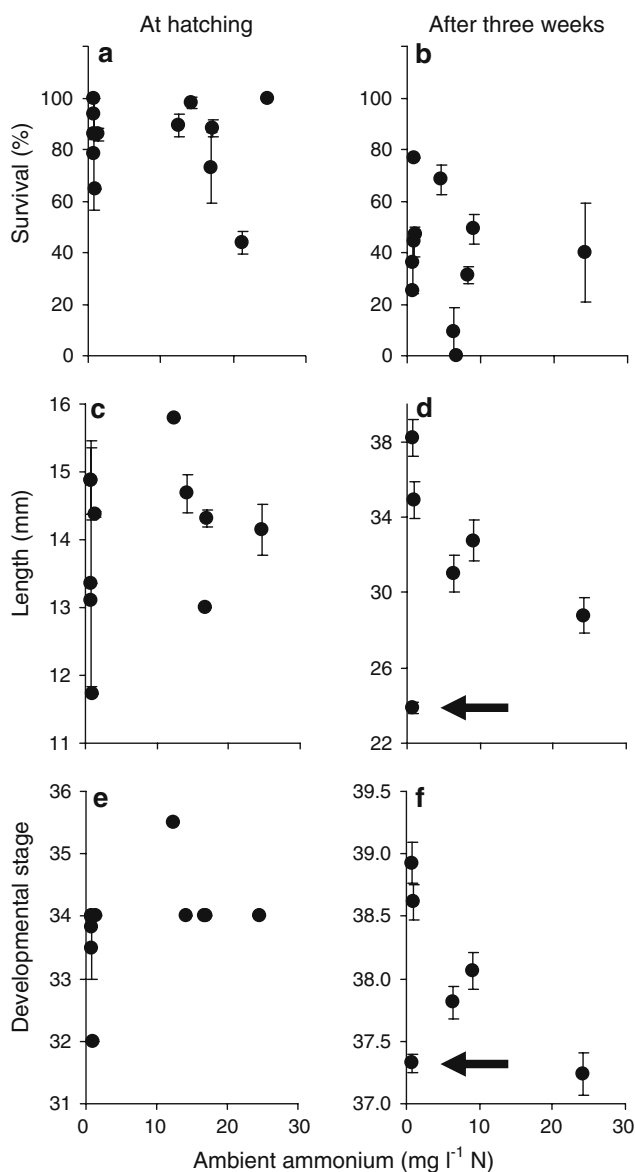
Fig. 3 **a**, **b** Survival, **c**, **d** growth (length), and **e**, **f** Shi and Boucaut (1995) developmental stage (means ± SE) of *Ambystoma tigrinum tigrinum* in response to ambient ammonium levels at hatching and after 3 weeks in enclosures in a whole-pond fertilization experiment. Significant linear regressions are noted with trendlines. Note that *arrows* indicate the pond with the largest abundance of predators such as fish, Coleoptera, Hemiptera and Odonata > 3 cm. *Error bars* are absent for ponds where only one enclosure of several had surviving individuals, and for ponds where individuals hatched at the same stage

water quality parameters (in all cases Pearson's $|r| < 0.4$, $n = 12$, $P > 0.2$) (Fig. 3c).

The developmental stage of *A. tigrinum* at hatching was approximately 34 (SE 0.2) (Shi and Boucaut 1995). Development was not related to treatment ($P = 0.1$; Table 2) or correlated with mineral N or other water chemistry parameter (in all cases Pearson's $|r| < 0.4$, $n = 12$, $P > 0.2$) (Fig. 3e).

Deformities in *A. tigrinum* at hatching ranged from 0 to 7% of hatchlings, and included asymmetries in development, edemas, and skin irregularities. We found salamander deformities in both fertilized and unfertilized ponds. Deformities in *A. tigrinum* were not significantly affected by treatment ($P = 0.5$; Table 2), mineral N or other water chemistry parameter (in all cases Pearson's $|r| < 0.4$, $n = 12$, $P > 0.2$).

Ambystoma tigrinum survival after three weeks in enclosures was not affected by treatment ($P = 0.5$; Table 2), mineral N, or any other water chemistry parameter (in all cases Pearson's $|r| < 0.53$, $n = 11$, $P > 0.1$) (Fig. 3b).

Ambystoma tigrinum exposed to higher ammonium concentrations at hatching and exposed to more large predators (large dragon fly larvae, predaceous diving bugs and beetles and fish) through development, were smaller (NH₄, $\beta = -0.1$; predators, $\beta = -249.7$) and less developed (NH₄, $\beta = -0.3$; predators, $\beta = -2200.0$) than those individuals not exposed ($R^2 > 0.96$, $F_{2,3} > 36.2$, $P < 0.008$) (Figs. 3d, f, 4b, d) (β values are standardized partial regression coefficients). Salamander size and development at 3 weeks were not significantly explained by treatment ($P = 0.3$; Table 2).

Discussion

Our study is one of the first field experiments to demonstrate that ammonium can remain at high enough concentrations in the water column to negatively effect amphibians, especially those amphibians that breed early in the year before plants begin to grow. These results suggest that the timing of reproduction as well as the N dynamics in local water bodies should be considered when managing for amphibian populations. Our results are particularly important in light of the concerns regarding amphibian conservation and chronic declines in some populations (Stuart et al. 2004) and the increasing amount of N entering our environment (Holland et al. 2005; Prud'homme 2005).

It is key to note that in temperate regions the embryonic period for early breeding amphibians coincides with periods of elevated ambient ammonium and nitrate concentrations, which we have shown can have toxic effects on at least two species of amphibians. Hence, this coincidence in timing could have serious implications for amphibian populations in areas that receive large N additions, potentially leading to chronic declines and extirpations. High concentrations early in the season in temperate regions are caused because the concentration of nitrogen in snow, which can be much higher than the concentration in rain, can
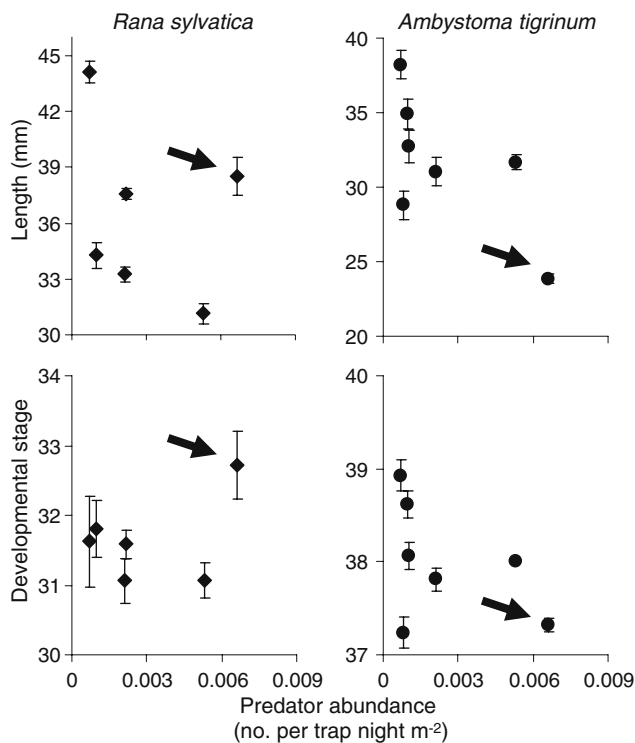
Fig. 4 Influence of invertebrate predator abundance on **a**, **b** length and **c**, **d** developmental stage of *Rana sylvatica* and *A. tigrinum tigrinum* after three weeks in enclosures (means ± SE). Developmental stages of *R. sylvatica* are from Gosner (1960) and development stages of *A. tigrinum* are from Shi and Boucaut (1995). Invertebrate predator abundance is the number of large predators, such as fish, Coleoptera, Hemiptera and Odonata > 3 cm, per trap night, per pond surface area. *Arrows* indicate the pond with the lowest ammonium concentration

supply a significant portion of the nitrogen inputs to a system (Wetzel 2001). This N then enters aquatic systems when the snow melts, before significant biological uptake of N occurs (Oldham 1999). Background levels of mineral N during summer months tend to be less than 1 mg l^{-1} NO$_3$–N, 0.1 mg l^{-1} NH$_4$–N, and 0.02 μg/l NO$_2$–N in temperate systems in the United States (Eddy and Williams 1994; Mueller et al. 1995). However, observational studies have reported concentrations of mineral N in freshwater up to 70 mg l^{-1} NO$_3$–N (McCoy 1972), above 15 mg l^{-1} NH$_4$–N (Mueller et al. 1995), and 18 mg l^{-1} NO$_2$–N (McCoy 1972). These high concentrations occur in systems contaminated by large amounts of biomass decomposition, domestic sewage, and/or fertilizer runoff. Because N pollution is a growing problem, it is important to examine these scenarios of high N concentrations that may provide a greater risk to the amphibians than occur in ponds in a more natural setting.

A chronic reduction in the number of amphibian offspring reaching adulthood can have large implica-

tions for long-term population and metapopulation persistence (Skelly 2001). We have shown that survival in *R. sylvatica* tadpoles declines with increasing ammonium concentrations. Susceptible species, such as *R. sylvatica*, that breed early in the year may be more ecologically vulnerable to mineral N toxicity than other later breeding amphibians, because their breeding is coincident with annual high ammonium concentrations in the water. More resistant species, like the *A. tigrinum*, may only show slowed growth and development in response to ammonium, which could indirectly lead to higher mortality in ephemeral environments through increased time vulnerable to predation and increased risk of pond desiccation as described below.

Delayed embryonic and larval growth and development and delayed metamorphosis can increase the time an individual is vulnerable to gape-limited aquatic predators (Caldwell et al. 1980; Wellborn et al. 1996) and increase the risk of complete mortality of a cohort due to pond desiccation (Griffiths 1997). After 3 weeks, *A. tigrinum* and *R. sylvatica* were shorter in response to both ammonium concentration and an index of predator abundance. Our results in part support laboratory findings that show ammonium and nitrate can slow growth and development, and at concentrations as low as 5 mg l^{-1} NO$_3$–N over the larval period (Johansson et al. 2001) and 6.9 mg l^{-1} NH$_4$–N over 5 days and 10 days depending on the amphibian species (Schuytema and Nebeker 1999b). Additionally, researchers have shown that several forms of mineral N can postpone metamorphosis (Marco and Blaustein 1999; de Wijer et al. 2003; Griffis-Kyle 2007).

The presence of predators, a biotic stressor, has an effect on amphibian size several orders of magnitude greater than ammonium, an environmental stressor. There is a wealth of literature examining the tradeoffs between growth and mortality in response to both biotic and abiotic stresses. For example, some species reduce movements in the presence of predators to become less apparent; however, these individuals tend to grow more slowly (Altwegg 2002; Werner and Peacor 2006), as evidenced in our study by *A. tigrinum*. Other species that may experience additional risks, such as competition, may respond to predators by increasing foraging and becoming more apparent to predators, but developing faster so that they can metamorphose out of the risky environment faster (Relyea 2004; Werner and Peacor 2006), as evidenced in our study by *R. sylvatica*. It is important to note that our experimental individuals were only exposed to predator cues and not to actual predation pressure while in enclosures.

Deformities can have a large impact on amphibian fitness and survival. Many deformed individuals either

cannot metamorphose, or are especially vulnerable to predators during their first winter (Helgen et al. 2000). Mineral N may play a role in increasing deformities in *R. sylvatica* at high ammonium concentrations; however, we only detected deformities (asymmetries in development, edemas, and skin irregularities) in one pond (the pond with the highest ammonium concentration, see Fig. 1a), so our results should be interpreted with caution. Other researchers have found similar deformities in amphibians exposed in the laboratory to ammonium at concentrations as low as 2.5 mg l^{-1} NH$_4$–N (Hecnar 1995, proportion deformed not reported; Xu and Oldham 1997, 7.8% deformed pulsed exposure). Researchers have suggested that more N entering ponds and increasing productivity may increase amphibian deformities by supporting a greater abundance of snails, an intermediate host for the trematode *Ribeiroia* known to cause deformities (Johnson and Lunde 2005). This was probably not the case in the ponds we sampled. *Ribeiroia* tend to cause supernumery limbs, which we did not observe, even in anurans with emergent limbs trapped later in the summer (K. L. Griffis-Kyle, unpublished data).

Species, populations, and individuals have been shown to differ in their susceptibility to mineral N (Hecnar 1995; Marco et al. 1999; Schuytema and Nebeker 1999a, b; Johansson et al. 2001; Ortiz et al. 2004). Variable species susceptibility could have large indirect effects on aquatic community structure through modification of competitive or predator–prey relationships. For instance, *R. sylvatica* exposed to elevated ammonium may experience more mortality from predation as young tadpoles because of initially slowed growth; however, this trend may be changed later in tadpole development as tadpoles respond to predator cues by speeding up development. Conversely, *A. tigrinum* do not appear as susceptible to mineral N as embryos and young larvae of the *R. sylvatica*, but may decline in predatory efficiency as they grow, since ammonium and predator cues slow their growth and development later in ontogeny. Because of these species-specific differences between trophic levels, we could see changes in both competitive and consumptive relationships in nitrogen-enriched environments. Further study is essential to understand how mineral N affects behavioral interactions and community structure.

Industrial fixation of mineral N will continue to increase across the globe (Jeffries and Maron 1997; Prud'homme 2005), and as fixation increases so will the amount of mineral N lost to surface waters. Amphibians in areas that receive these N additions, especially if the amphibians breed coincident to high mineral N concentrations, may be at risk for population declines. We have shown that some early breeding amphibians may be vulnerable to elevated mineral N concentrations early in development, showing increased mortality, delays in growth and development and increased incidence of deformities. Our study is an important first step in understanding how increased concentrations of mineral N effect amphibians under field conditions, and our results are relevant as the amount of available nitrogen added to our environment continues to increase.

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