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Effects of Ammonium Nitrate on Larval Survival and Growth of Four Iberian Amphibians

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Abstract The purpose of this study was to understand the differences in tolerance to ammonium nitrate in four Iberian amphibians. Results showed a negative effect on amphibian survival and larvae total length. Three different tolerance groups on amphibian survival were established. The two first groups showed a higher reduction, around 90% and between 70 and 80% after 96 h exposition at the maximum concentration tested (180 mg NH_4NO_3/L), while the third group showed no mortality in any concentration tested. In addition, a reduction in larvae total length was also detected for all four species, with *Bufo bufo* as the most sensitive species.

Keywords Fertilizers · Land use · Interspecific tolerance · Sensitivity

Some of the more ubiquitous contaminants in agricultural landscapes are associated with the use of fertilizers, which in recent years have been receiving a great deal of attention (Mann et al. 2009). It is well documented that nitrogen fertilizers are changing many aspects of abiotic and biotic environments (AMAP 1998). Elevated concentrations of nitrate and/or ammonia in surface water of agricultural landscapes may be hazardous to many wildlife species (Bogardi et al. 1991). Subsequent runoff after rainfall results in elevated concentrations of inorganic nitrogenous compounds in surface and ground waters. The major sources of nitrate contamination in water are the agricultural application of nitrogen-based mineral fertilizers, manure (Bogardi et al. 1991), and atmospheric deposition of nitric compounds (Jenkins 1999). Ammonium (NH_4^+), nitrite (NO_2^-) and nitrate (NO_3^-) are the most common ionic (reactive) forms of dissolved inorganic nitrogen in aquatic ecosystems (Rabalais 2002). Many amphibians breed opportunistically in aquatic habitats embedded in agricultural landscapes. These species may be sensitive to pollutants not only during the terrestrial adult phase, but also during aquatic embryonic and larval stages (García-Muñoz et al. 2009, 2010a, in press). Amphibian species suffer increased mortality, especially during their aquatic stages, due to habitat acidification or water pollution (e.g. Gibbs et al. 2005). Therefore, the contribution of nitrate may be one potential agent contributing to global decline of amphibian populations (Blaustein and Wake 1995).

In the southern Iberian Peninsula, different toxic substances are used in intensive agriculture (García-Muñoz et al. 2009), these include ammonium nitrate which is one of the most commonly chemical fertilizers used in the world. Fertilization with ammonium nitrate mainly occurs during late winter in temperate regions. During early spring rains, nitrate drainage occurs into water bodies used by amphibians for reproduction (Ortiz-Santaliestra et al. 2006). Since spring runoff may coincide with amphibian reproduction, nitrogen compounds are potential hazards to amphibian populations. A strong tolerance of some amphibians to environmental concentrations of these

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compounds has been documented (Sullivan and Spence 2003). However, different species have different tolerances to contaminants. The purpose of this study was to investigate the sensitivity to ammonium nitrate for four Iberian amphibians, *Bufo bufo*, *Bufo calamita (=Epidalea calamita)*, *Pelodytes ibericus* and *Pelophylax perezi*.

Materials and Methods

Culture: Four anuran species were used in the present study: *Bufo bufo* (Bb), *Bufo calamita* (Bc), *Pelodytes ibericus* (Pi) and *Pelophylax perezi* (Pp). Amphibians were collected from different wetlands situated in the Alto Guadalquivir region (southern Spain), and in an area with no evidence of pollution (UTM: *B. bufo*: 30S 436040, 4169244; *B. calamita* and *P. ibericus*: 30S 448100, 4220986; *P. perezi*: 30S 426744, 4175154). At least six different clutches (to ensure natural genetic variability) from each species were collected in early development stages (Goner stage 10–12; Gosner 1960).

Egg masses were brought into the laboratory immediately after collection and placed in aquariums (20 L with 100 eggs of the same species per aquarium with water from a highland spring without contamination) (pH: 7.2–7.8; alkalinity: 170–250 mg/L; hardness: 129 mg/L; $NO_3^- < 0.1$ mg/L). Water was renewed every 48 h. Eggs were maintained at 20°C, on a 12 h light: 12 h dark cycle in a temperature-controlled chamber. Individuals were allowed to develop to Gosner stage 25 before acute toxicity testing. The different species reached to this stage at different times, so experiments were conducted at different times using the same conditions. This Gosner stage was selected for different reasons: (1) tadpoles are free swimming; (2) they begin to feed; and (3) the larvae remains in this life-stage for a longer time, while their weight and length increases (García-Muñoz et al. 2009).

All toxicity tests were static, and were conducted in 1 L. Ammonium nitrate (purity: 99%; Merck KgaA®, Darmstadt, Germany) was used to prepare concentrations to be used in acute toxicity tests. Exposure conditions were confirmed using a photometer (Filterphotometer PF11; Gesant-Stickstoff; N-total, TN_b 22 y TN_b 220) following a colorimetric technique (detection limit: TN_b 22 = 0.5 mg/L; TN_b 220 = 5 mg/L). No statistical differences were detected among ammonium nitrate concentration replicates or between the different experiments for all species. The same highland spring water used in culture was used to prepare the different concentrations. Tests consisted of 10 individuals per each container with five replicate containers per exposure concentration including the control. Tests were maintained in a temperature-controlled chamber at 20°C with a 12 h light: 12 h dark cycle. Larvae were not fed during the experimental period. Tests were conducted for 96 h. Every 48 h the water was renewal (80%) in each aquarium maintaining the same initial concentration.

Tests were conducted using five different nominal concentrations of ammonium nitrate (0, 22, 45, 90 and 180 mg NH_4NO_3/L). The selected concentration of ammonium nitrate was consistent with peak concentrations that are expected to appear in water bodies after runoff of fertilizer applied in agricultural landscapes (Scholefield et al. 1996). Individuals were visually examined once every 24 h, larval mortality was monitored and dead larvae were removed. Water temperature and pH were checked daily (pH varied between 7.20 and 7.80). The temperature controlled chamber keep water temperature at 20°C (±0.5°C). No statistical differences in pH or water temperature were detected among treatments within the same experiment and between the different experiments with all species. The experiments were concluded after 96 h of exposure and at the end of these experimental periods, individuals were placed in Petri dishes with 1 cm depth of water. Individuals were photographed against a grid paper using a CANON S40 camera. Measurements of total lengths (from snout to tail tip; before and after exposure) were done with the help of an image program (Image J).

Statistical analysis: A repeated measure ANOVA was performed with cumulative mortality rates (arcsine of square root transformed) at four times (24, 48, 72 and 96 h in exposure) as dependent variable, and ammonium nitrate concentration (22, 45, 90 and 180 mg NH_4NO_3/L) and species (Bb, Bc, Pi and Pp) as categorical variables. A Tukey post hoc test for pairwise comparisons was used to detect significant differences. A Kolmogorov-Smirnoff Z test was conducted before repeated measures ANOVA to confirm normality of transformed mortality rates. In all cases, the Z test resulted in p values greater than 0.05.

As different species showed a different growth rate, a multiple regression analysis was used for all species, with larval length (log transformed) as dependent variable and ammonium nitrate concentration as categorical variable. Software Statistica 7 for Windows was used for the statistical analysis.

Results and Discussion

Mortality rates after 24, 48, 72 and 96 h of exposure for all species are shown in Fig. 1. No mortality occurred in any control vessels during the experimental period, for all species. No mortality occurred in any treatment group with *P. perezi* tadpoles. The results of repeated measures ANOVA are shown in Table 1 and Fig. 1. Three different tolerance groups were detected, the first one was composed by the most sensitive species (*P. ibericus*), showing

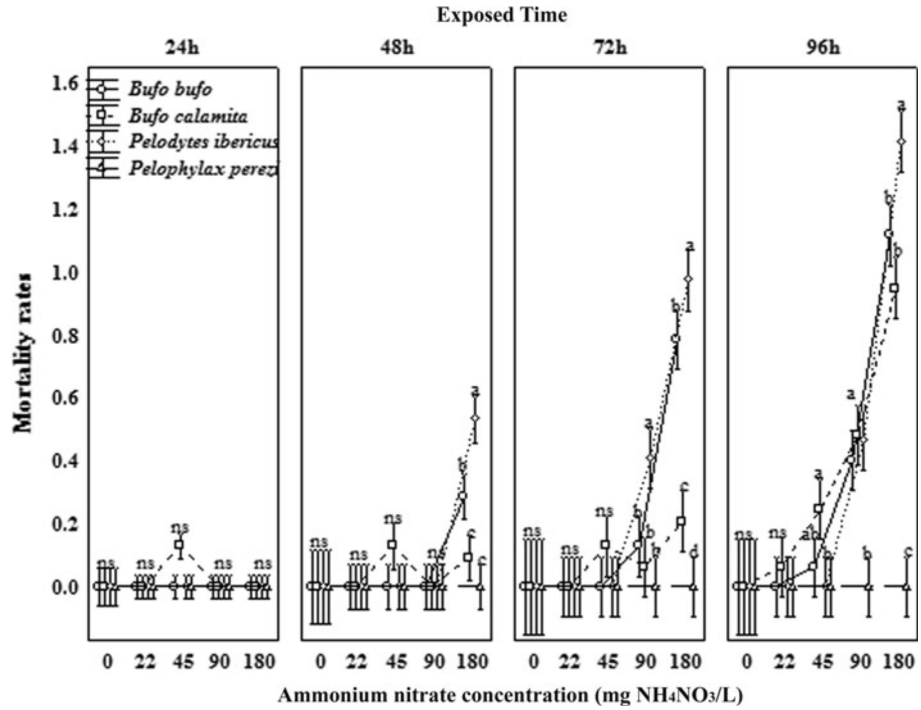

Fig. 1 Larval mortality after 24, 48, 72 and 96 h of exposure at different ammonium nitrate concentrations, in *B. bufo*, *B. calamita*, *P. ibericus*, *P. perezi*, (*vertical bars* denote CL = 95%). Different letters denote different groups at $p < 0.01$ (Tukey post-hoc test)

Table 1 Statistical results of repeated measures ANOVA

	SS	*df*	MS	*F*	*p*
Intercept	3.952	1	3.952	305.627	0.000
Species	1.495	3	0.498	38.549	0.000
N	8.132	4	2.033	157.244	0.000
Species*N	4.592	12	0.383	29.598	0.000
Error	0.879	68	0.013		
Time	2.861	3	0.954	153.338	0.000
Time*species	1.227	9	0.136	21.918	0.000
Time*N	5.725	12	0.477	76.712	0.000
Time*species*N	2.674	36	0.074	11.942	0.000
Error	1.269	204	0.006		

Repeated measures ANOVA analyzes the effects of ammonium nitrate concentration (N), species, and time on larval mortality (arcsine of square root transformed)

mortality around 90% after 96 h exposition, at the maximum concentration tested (180 mg NH_4NO_3/L). The second group was composed by the species with intermediate sensitivity (*B. bufo* and *B. calamita*) that showed mortalities around 70–80% after 96 h exposition at the maximum concentration tested. Finally, the third group was composed by the most tolerant species (*P. perezi*) that showed no mortality at any concentration tested.

Ammonium nitrate exposure also caused a reduction in larval total length at the end of the experimental time in all species, when compared with control groups [initial total length (mm) ± standard deviation: Bb = 6.65 ± 0.36; Bc = 6.5 ± 0.43; Pi = 6.4 ± 0.23; Pp = 6.35 ± 0.21]. The results of multiple regressions analysis confirmed the negative effect of ammonium nitrate exposition on larval total length (Table 2). *B. bufo* showed the highest slope meaning that exposure to these concentrations had a higher effect on growth compared with the other species (*P. perezi*, *B. calamita* and *P. ibericus*) that showed similar slopes (Fig. 2, Table 2).

The obtained results reported that ammonium nitrate can be seriously hazardous for amphibian survival, as has been suggested previously (Hecnar 1995). Although a high variability in amphibians' sensitivity to ammonium nitrate has been reported in the literature, there is considerable controversy in relation with the effect of ammonium nitrate on amphibians' length (Mann et al. 2009). On this study, exposure to ammonium nitrate not only had a lethal effect, showed by the increased mortality in exposed larvae, but also had a sub-lethal effect on total length. On the one hand, the negative effect of ammonium nitrate on growth rate has been observed in amphibians' species (e.g. Egea-Serrano et al. 2009; Sullivan and Spence 2003). On the other hand, some other studies observed no significant effect of fertilizers on larval final size (Hecnar 1995). Our study showed that ammonium nitrate had a negative effect on larval size, in all species, after 96 h of exposition to a concentration of 45 mg NH_4NO_3/L and to higher concentrations (90 and 180 mg NH_4NO_3/L). Reduced total length is correlated with longer developmental time and smaller size at metamorphosis (Breden and Kelly 1982). In addition, an increase in developmental time can affect the total number of individuals which can complete their metamorphosis successfully, since some of these species

Table 2 Multiple regression analysis results for all studied species

Species	Source of variation	Beta	SE of beta	B	SE of B	n	*t*	*p* level
Bb	Concentration	−0.883	0.061	−0.044	0.003	60	−14.555	0.000
Bc	Concentration	−0.539	0.106	−0.026	0.005	63	−5.081	0.000
Pi	Concentration	−0.672	0.133	−0.025	0.005	31	−5.051	0.000
Pp	Concentration	−0.884	0.056	−0.029	0.002	71	−15.909	0.000

Studied species: Bb, *B. bufo*; Bc, *B. calamita*; Pi, *P. ibericus*; Pp, *P. perezi*. Total length (log transformed) was the dependent variable and ammonium nitrate concentration was the independent variable (0, 22, 45, 90, 180 mg NH_4NO_3/L)

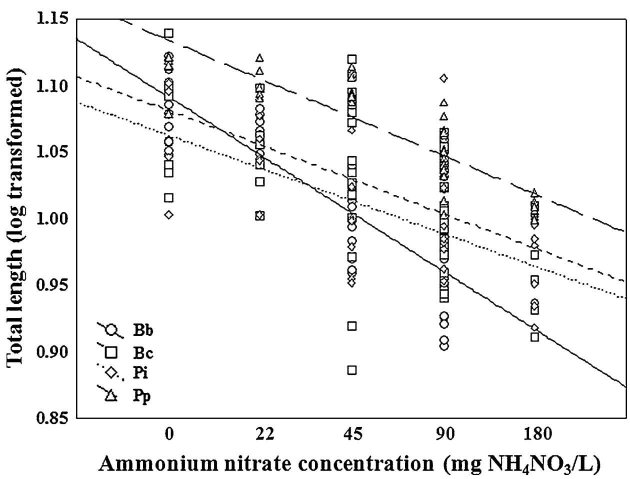

Fig. 2 Result of multiple regression analysis, for all species [Bp, *B. bufo*; Bc, *B. calamita*; Pi, *P. ibericus*; Pp, *P. perezi* (Pp)] for total length reached (log transformed) at the end of the experimental period (96 h). Ammonium nitrate concentration expressed in mg NH_4NO_3/L. The different lines represent the slopes of multiple regressions for each species

(*B. bufo, B. calamita* and *P. ibericus*) use small and temporary wetlands that suffer rapid desiccation processes (Sinsch 1998), and fitness later in life is directly related to size at metamorphosis (Semlitsch et al. 1988). The effect of length reduction may have been caused by increased in energetic costs involved in detoxification pathways (Wright and Wright 1996), so further physiological studies are needed to identify the mechanisms involved.

Our results showed that exposition to 180 mg NH_4NO_3/L caused significant mortality in *B. bufo*, *B. calamita* and *P. ibericus*. No mortality was observed in *P. perezi* at the highest concentration tested. Significant mortality has been also described in *Rana sculenta* complex after 96 h of exposure to 20 mg NH_4NO_3/L (Berger 1989). By contrast, mortality did not occur until exposure to 387.5 mg NO_3^--NH_4NO_3/L (175 mg NH_4NO_3/L) in *Triturus helveticus* (Watt and Jarvis 1997). Ortiz-Santaliestra et al. (2006) found a negative effect in *B. calamita* larval length, but nevertheless lethal effects were observed under four days of exposure to the highest ammonium nitrate level (225.8 mg NH_4NO_3/L). Some mechanisms for ammonium nitrate toxicity have been proposed. Hecnar (1995) indicated that nitrate may be reduced to nitrite in the gut, thus resulting in nitrite toxicity. Nitrite induces methahemoglobinemia as a secondary effect of exposure to nitrate, which may provide an explanation for toxicity at relatively low levels of nitrate. Egea-Serrano et al. (2009) defend that different tolerance responses between different populations of the same species can be explained by a hypothetical rapid evolution, presumably caused by the intense farming activities developed in the polluted areas during the past three decades.

The responses to ammonium nitrate exposition differed between species, and confirm *P. perezi* as the most tolerant species tested. *P. perezi* occurs throughout the Iberian Peninsula and it‘s one of the most abundant species of amphibians. It occupies almost all kinds of water bodies in all types of habitats and is known to tolerate brackish, polluted and highly eutrophic waters (García-Muñoz et al. 2010a). It can be interesting to merge the obtained results with amphibian’s data on spatial distribution in agricultural landscapes (García-Muñoz et al. 2010b). The mentioned study relates the presence of the most sensitive species, *B. bufo* and *P. ibericus*, with wetlands classified by medium or high conservation status. By contrast, the wetlands with higher agriculture intensification degree in their watersheds, which is correlated with a high nutrient enrichment (nitrogen and phosphorus), present only the most tolerant species founded in this study, *P. perezi*.

Like the autecology that allows us to generate hypotheses on the distribution of organisms in relation to the variation of environmental factors, ecotoxicology could generate hypotheses about the current amphibian’s distribution in relation to the presence, concentration and persistence of pollutants.

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