Adaptation to osmotic stress provides protection against ammonium nitrate in Pelophylax perezi embryos

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Natural resistance to salinity minimizes the impact of chemical fertilizers on amphibian embryos.

Keywords: Amphibian decline Intraspecific variation Local adaptation Multiple stressors Salinity

a b s t r a c t

The negative effects of pollution on amphibians are especially high when animals are additionally stressed by other environmental factors such as water salinity. However, the stress provoked by salinity may vary among populations because of adaptation processes. We tested the combined effect of a common fertilizer, ammonium nitrate (0–90.3 mg $N–NO_3NH_4$/L), and water salinity (0–2&) on embryos of two Pelophylax perezi populations from ponds with different salinity concentrations. Embryos exposed to the fertilizer were up to 17% smaller than controls. Survival rates of embryos exposed to a single stressor were always below 10%. The exposure to both stressors concurrently increased mortality rate (>95%) of embryos from freshwater. Since the fertilizer was lethal only when individuals were stressed by the salinity, it did not cause lethal effects on embryos naturally adapted to saline environments. Our results underscore the importance of testing multiple stressors when analyzing amphibian sensitivity to environmental pollution.

1. Introduction

Because of the permeability of their skin, amphibians are very sensitive to osmotic stress; therefore, water salinity constitutes a critical factor in habitat suitability and survival probabilities of these animals (Beebee, 1985). Salinity levels above concentrations typical of freshwaters (0–0.5&) may produce diverse physiological malfunctions, such as an alteration of respiratory function (Mahajan et al., 1979) or abnormalities in the circulatory system (Parsons et al., 1990). Nevertheless, some amphibian populations are quite capable of living in brackish waters (0.5–32& salinity) and, exceptionally, in saline waters (32–37.5&). Some coastal populations of Bufo calamita in Sweden breed in waters with a 4& salinity (Andrén and Nilson, 1985), while other populations of this species can breed close to the Baltic Sea, in water bodies with a 28& salinity (Gislen and Kauri, 1959). Uchiyama et al. (1990) found individuals of Fejervarya cancrivora, the amphibian with the highest salinity tolerance found so far, in ponds with the same salt concentration as that of seawater (33&). However, these amphibians that appear in brackish or saline waters are specific populations that are exceptionally tolerant of moderate or even high salinity levels, while other populations of these same species are unable to live in osmotically stressful environments (Gomez-Mestre and Tejedo, 2003). There are no known cases of an amphibian species whose populations are all adapted to osmotic stress.

Water pollution is another factor that may affect different populations of the same amphibian species in a distinct way. For example, Johansson et al. (2001) found that Rana temporaria tadpoles from an area with a high nitrate load, as a consequence of the use of fertilizers, were more resistant to the chemical than were tadpoles from a nitrate-free area. The fluctuations in environmental levels of many pollutants in the water might act as selection pressures for the organisms inhabiting these waters (Holloway et al., 1990). Therefore, the tolerance of certain amphibian populations to some agricultural chemicals to which they are usually exposed could be the consequence of genetic adaptation. For example, Egea-Serrano et al. (2009) suggested that a local adaptation to nitrogen contamination had happened among Pelophylax perezi populations inhabiting areas exposed to agricultural pollution over the last three decades. However, while the existence of genetic adaptation has been demonstrated for some of the observed salinity-resistant populations (e.g., Gomez-Mestre and Tejedo, 2003), there is as yet no evidence, to our knowledge, of

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genetic adaptation of amphibians to environmental pollution. As suggested by some authors, the pace of the environmental change in the case of pollutants would be too rapid to allow the genetic adaptation of animals to chemical stress (Bürger and Lynch, 1997). Consequently, geographic variations in sensitivity to pollutants would therefore be due to environmentally induced sources such as phenotypic variation (Falfushinska et al., 2008) or maternal effects (Räsänen and Kruuk, 2007).

While intraspecific differences in amphibian sensitivity to a specific stressor have been occasionally elucidated, little is known about how different populations of the same species respond to the combined effects of various factors. Because previous research has demonstrated that the presence of two or more factors can cause sublethal chemical stressors to become lethal when conditions are altered (Hatch and Blaustein, 2003; Relyea, 2003), examining the roles of multiple stressors is essential if we are to understand how to protect community processes. Testing chemicals individually can underestimate the risk posed to wildlife (Sih et al., 2004). A number of papers published during the last decade emphasize that combinations of stressors may be enhancing the negative effects of each stressor alone, often having interactive effects not predicted from single-factor studies (e.g., Blaustein et al., 2003; Boone and James, 2003; Boone et al., 2005, 2007; Johnson et al., 2006; Relyea and Mills, 2001). With regards to nitrogenous compounds, deleterious consequences have been noted in response to their combination with other environmental factors such as pesticides (Boone et al., 2005), UV–B radiation (Hatch and Blaustein, 2000, 2003; Macías et al., 2007), or low pH (Hatch and Blaustein, 2000). In addition, Egea-Serrano et al. (2009) demonstrated that the exposure of amphibian larvae to combinations of different nitrogenous compounds affected larval survival and food consumption more severely than did exposure to single compounds.

The combined effects of nitrogen pollution and high salinity levels could be especially relevant in water bodies impacted by agricultural activities. Chemical fertilizers applied to crop fields constitute by far the main source of inorganic nitrogen pollution in freshwater ecosystems (Vitousek et al., 1997). In addition, agricultural activities indirectly increase water salinity, because of the substitution of natural perennial vegetation by crops of herbaceous plants with small root systems that do not penetrate deeply into the soil (Hart et al., 2003). Shallow rooting may reduce the soil retention of nutrients and minerals, which are then easily transported by runoff to the nearby water bodies. Loman and Lardner (2006) found, in Southern Sweden, that ponds located in agricultural lands showed higher conductivity and nitrogenous compound concentrations than did ponds outside the agricultural areas.

We hypothesize that the combined exposure to a common fertilizer, ammonium nitrate, and increased water salinity may have synergistic toxic effects on amphibian embryos, and that these effects may vary as a function of the local adaptation to any of these stressors. Previous research has demonstrated that embryos are generally not as sensitive as young larvae when exposed to chemical stress (Meredith and Whiteman, 2008; Ortiz-Santaliestra et al., 2006), which is commonly attributed to the protective role of the gelatinous envelope (Edginton et al., 2007; Marquis et al., 2006). However, the embryonic jelly coat is permeable to some toxic chemicals such as the pyrethroid insecticide a-cypermethrin (Greulich and Pflugmacher, 2003).

Inorganic nitrogen, including ammonium nitrate, may inhibit the eclosion (Ortiz-Santaliestra et al., 2007) or decrease embryonic growth and development, which ultimately reduces survival probabilities during the larval stage (Griffis-Kyle, 2005). Increased levels of nitrate (25 mg $N–NO_3^-$/L) and nitrite (>3.5 mg $N–NO_2^-$/L) inhibit hatching success and cause mortalities in hatched amphibian larvae of several amphibian species (Marco et al., 1999). Ponds with higher levels of nitrate have been reported to have lower densities of egg masses and poorer hatching success for three amphibian species (Laposata and Dunson, 2000). An increased level of ammonia (up to 1.27 mg/L) caused a decrease in egg hatch in Rana aurora and Ambystoma gracile (De Solla et al., 2002). Concentrations over 0.6 mg/L of unionized ammonia decreased survival, increased the prevalence of abnormalities and slowed growth and development in Lithobates clamitans embryos. Similar effects were reported for Lithobates pipiens embryos with concentrations in excess of 1.5 mg NH_3/L (Jofre and Karasov, 1999).

Regarding the impact of salinity on amphibian aquatic stages, contrary to what happens with inorganic nitrogen, embryos would be, in most of cases, more sensitive than are larvae to osmotic stress, especially during the gastrulation and neurulation stages (Viertel, 1999). A review of the available literature indicates that embryos of non-adapted amphibian populations can tolerate moderate salinity concentrations. For example, Viertel (1999) estimated a 'no observed effect concentration' (NOEC) of 2.2& salinity for embryonic R. temporaria. Similarly, Microhyla ornata embryos were not affected by a 2& salinity concentration (Padhye and Ghate, 1992), while survival rates over 50% were observed in Rhinella marina at 3.5& salinity (Ely, 1944), in R. temporaria at 3.7& (Viertel, 1999), and in B. calamita at 4& salinity concentration (Beebee, 1985). On the other hand, higher concentrations may impair embryonic survival. For example, Beebee (1985) reported 100% mortality in B. calamita embryos in waters with a salinity concentration of 7&.

The aim of the present study was to analyze the intraspecific differences in the embryonic sensitivity of two Iberian green frog (P. perezi) populations to a combined exposure to ammonium nitrate and osmotic conditions corresponding to fresh or brackish waters. For this purpose, we used embryos from two localities with similar nitrogen load and different salinity levels.

P. perezi occurs throughout the Iberian Peninsula, where it is one of the most abundant amphibians, and also can be found in southern France. It occupies almost all kinds of water bodies in all types of habitats and is known to tolerate brackish water (Kadel and Hemmer, 1980) and moderate concentrations of water pollution, as deduced from its presence in highly eutrophic agricultural habitats (Llorente et al., 2002). In the region where we collected the eggs for the present study, embryonic development lasts for an average of 8–12 days, depending essentially on the temperature (Diego-Rasilla and Ortiz-Santaliestra, 2009). The duration of larval development is highly variable and depends on the environmental conditions, with metamorphs usually emerging between 8 and 12 weeks after laying. Recent research has revealed that larval survival is affected by exposure to environmentally relevant levels of ammonium, but not by nitrate or nitrite (Egea-Serrano et al., 2009). We are unaware of the existence of experimental data that quantify the resistance of P. perezi to osmotically stressful environments.

2. Materials and methods

We collected P. perezi eggs from two locations in West Central Spain in April 2005. The eggs from the first population (ZA) were collected in the Lagunas de Villafáfila Natural Reserve, in Zamora province (41° 50′ 08″ N, 5° 35′ 24″ W). The eggs from the other population (SA) were collected in the Prado de la Hermida lake, in Salamanca province (41° 05′ 36″ N, 5° 36′ 48″ W). Both places have similar climatic conditions and are located in agricultural regions dominated by cereal crops. We selected these places because we had previous data on their salinity and nitrogen concentrations. According to those previous measurements, we expected differences between localities in terms of salinity but not in inorganic nitrogen concentrations. At the moment of egg collection, we measured water salinity (conductivity-meter Hanna HI 9835; Hanna Instruments®, Eibar, Spain), pH (pH-meter Hanna HI 8314; Hanna Instruments®, Eibar, Spain), dissolved oxygen (oximeter Handylab OXI/Set; Schott-Geräte®, Hofheim, Germany), total hardness (test Visocolor ECO 931029; Macherey–Nagel®, Düren, Germany), and nitrate, nitrite, and total ammonia concentrations (tests Visocolor ECO 931041, 931 044 and 931 008, respectively; Macherey–Nagel®, Düren, Germany). The obtained

measurements are shown in Table 1. As expected, salinity levels were substantially different between localities. While levels at SA were within the normal range for freshwater systems (0–0.5&), waters from ZA could be considered as moderately brackish. Therefore, our comparison was of individuals from two populations with a different history in their environmental osmotic conditions.

Eggs at Gosner stages 10–11 (<24 h; Gosner, 1960) from five different clutches were collected from both locations on the same day. The eggs were carried to the laboratory immediately after collection, where they were incubated at an air temperature of 18 °C and with a natural photoperiod. We used 54 containers with 300 mL of tap water filtered with activated carbon to remove chlorine and ammonia. Concentrations of nitrate, nitrite and total ammonia after filtering were below detection limits. Conductivity of the filtered water was 5.5 ms/cm. Each container was assigned to a population, a nominal ammonium nitrate level (0, 22.6, 90.3 mg $N–NO_3NH_4$/L) and an initial salinity level (0&, 0.4&, 2&) in such way that each combination of treatments was replicated three times in a block design. The two experimental salinity levels were similar to those measured at the original ponds (see Table 1). The lowest ammonium nitrate level was selected from the nitrate legal maximum allowed for drinking waters (European Council, 1998); the highest fertilizer level was consistent with that expected in the amphibian breeding ponds located in agricultural environments just after the drainage of fertilizers by rains (Ortiz-Santaliestra et al., 2006; Scholefield et al., 1996). We did not consider the concentrations of nitrate, nitrite or ammonia measured at the ponds where eggs were collected because they were too low to be considered as potentially stressful. Moreover, some analyses made in these ponds during the amphibian reproductive season in subsequent years showed maximum levels above 100 mg $N–NO_3^-$/L and 10 mg $N–NH_4^+$/L (unpublished data). To obtain the experimental levels, we added commercial sodium chloride and/or ammonium nitrate salt with 99% purity (Merck®, Darmstaad, Germany) to the water. Water in all containers was changed every 6 days in a typical static-renewal design (Stephen, 1975); during that period, ammonium nitrate levels were not readjusted, so we expected a decrease in concentration of nitrate and ammonium over time, as happens in the field after fertilizer runoff (Bogardi et al., 1991). Inside each container we placed 20 eggs from the corresponding population (four eggs from each of the five clutches). The jelly coat of the eggs was left intact in order to retain the natural conditions of the egg environment as much as possible. The experiment began the day after egg collection, when embryos were at Gosner stage 12, and lasted for 12 days. We chose this 12-day period from the available data on embryonic development duration of the tested populations. All of the surviving embryos hatched between days 10 and 11 of the experiment, but none of them was yet able to swim when the experiment ended.

We recorded hourly temperatures during the experiment with a data-logger Hobo Water Temp Pro v2 (Onset Computer®, Bourne, MA, USA) submerged in an additional container with the same characteristics as those used in the experiment. Water pH and conductivity were checked every 4 days, and concentrations of nitrate and total ammonia were measured at days 6 and 12. All levels were readjusted at day 6, after water renewal.

Mortality rates in each container were calculated every 3 days. To analyze the effects of population, salinity and ammonium nitrate, as well as their interactions, on embryonic survival, we used a three-way repeated measures analysis of variance with the increase of mortality rates (arcsine of square root transformed) over time as the dependent variable. At the end of the experiment, we recorded the total length (mouth to tail tip) of survivals with a digital caliper ACHA 17-264 (d ¼ 0.01 mm; ACHA®, Eibar, Spain). We did a logarithmic transformation of larval lengths and analyzed them with a three-way analysis of variance (ANOVA). When the full three-way factorial models provided significant results, we conducted ANOVAs for each factor or combination of factors that produced significant effects, and the p-values were adjusted with a Bonferroni correction for multiple testing. Statistical analyses were run with the software SPSS 11.5 for Windows (SPSS Inc.®, Chicago, IL, USA).

3. Results

No mortality occurred in controls. Both ammonium nitrate and salinity, as well as their interaction, significantly increased overall embryonic mortality over time. In addition, sensitivity to both stressors, either alone or in combination, was different between populations (Table 2). Embryonic survival in the ZA population was not affected by any of the stressors (over 90% in all treatments), while a significant increase in lethality was observed for SA embryos exposed to either fertilizer or salinity. In this population, mortality was significantly higher only at the highest ammonium nitrate and salinity levels when compared to controls; at 90.3 mg $N–NO_3NH_4$/L, the overall embryonic mortality was 24%, while less than 2% of the individuals exposed to 22.6 mg $N–NO_3NH_4$/L died. With regards to salinity, the overall mortality of SA embryos was 32% at the higher level and 12% at the lower one. Nevertheless, this significant lethality suffered by SA embryos was exclusively provoked by the synergistic effect of the two stressors; while the mortality rates caused by a single stressor were always lower than 2% (Bonferroni test: $p > 0.05$), the increase of salinity augmented the lethality caused by ammonium nitrate in such way that more than 95% of embryos exposed simultaneously to 90.3 mg $N–NO_3NH_4$/L and 2& salinity had died by the end of the experiment (Fig. 1).

The total length of survivors did not vary significantly between populations, with mean values of 7.71 mm in SA, and 7.49 mm in ZA. According to the full three-way factorial model, both ammonium nitrate and salinity affected the length of hatchlings, although we did not detect any statistical interaction of the population of origin with the effects of any of the stressors or their interaction (Table 3). Ammonium nitrate reduced the growth rate of embryos in both populations. Hatchlings from the SA population were on average 8–16% smaller than controls, depending on the fertilizer level, while ZA individuals exposed to the highest ammonium nitrate level showed a total length that was 17% lower than that of the controls. However, in the case of salinity, the Bonferroni adjustment for multiple testing did not reveal any significant effect of this specific stressor on length in any of the populations (Fig. 2).

Mean water temperature was 17.2 °C, with an average daily variation of 2.2 °C. Mean water pH was 7.27 (range 7.18–7.41). Neither the ammonium nitrate concentration nor the salinity level affected water pH ($p > 0.05$). Mean salinity levels per treatment, calculated as the average values between the initial salinity concentrations and those measured before water change (day 6) and at the end of the experiment (day 12), were slightly higher than the initial values (0.22&, 0.57& and 2.48&). The concentration of ammonium nitrate did not affect significantly water salinity ($F_{2,15}$ ¼ 0.211; p ¼ 0.812). Average fertilizer concentrations per treatment were 0, 17.6 and 76.6 mg $N–NO_3NH_4$/L; water salinity did not affect these concentrations ($F_{2,15}$ ¼ 0.001; p ¼ 0.999).

Table 1
Water parameters measured in the ponds of origin of the embryos at the moment of collection.

Parameter	SA population	ZA population
Conductivity (ms/cm)	626	3180
Salinity (&)	0.40	2.04
pH	7.40	7.42
Dissolved oxygen (mg/L)	12.43	13.91
Total hardness (mg $CaCO_3$/L)	250.6	250.6
Nitrate (mg N/L)	2	1
Nitrite (mg N/L)	0.01	BDL[a]
Total ammonium (mg N/L)	0.15	0.6

[a] BDL: below detection limits.

4. Discussion

We observed a clear intraspecific difference in the sensitivity of P. perezi embryos to ammonium nitrate and osmotic stress. The individuals from the ZA population, native to a more saline area, were more tolerant than were those of the SA population. This was evident not only for moderate salinity levels but also for fertilizer exposure. The ammonium nitrate concentration that did not affect individuals from the ZA population under the high salinity treatment was highly lethal to SA embryos.

In the P. perezi population from non-saline waters (SA), increased salinity levels acted as a stress source. The embryos of this population appear to be very sensitive in comparison with other cases of salinity-exposed amphibian embryos. For salinity concentrations close to our highest treatment (around 2&), no lethal effects were detected in M. ornata (Padhye and Ghate, 1992) or R. temporaria (Viertel, 1999) embryos. Survival rates over 50% were also found in embryos of other species exposed to salinity

Table 2
Effects of ammonium nitrate (NO_3NH_4) and salinity on the evolution of embryonic mortality over time in two P. perezi populations. Data from the three-way full factorial model comparing both populations, as well as those from the separate two-ways analyses for each population are shown.

	Source of variation	Sum of squares	df	Mean squares	F	p
Full model	Time	0.725	1	0.725	70.717	<0.000001
	Time × population	0.240	1	0.240	23.412	0.000025
	Time × salinity	0.295	2	0.147	14.372	0.000026
	Time × NO_3NH_4	0.826	2	0.413	40.281	<0.000001
	Time × population × salinity	0.156	2	0.078	7.615	0.001746
	Time × population × NO_3NH_4	0.379	2	0.190	18.502	0.000003
	Time × salinity × NO_3NH_4	0.522	4	0.131	12.740	0.000001
	Time × population × salinity × NO_3NH_4	0.419	4	0.105	10.213	0.000012
	Error	0.369	36	0.010		
ZA population	Time	0.065	1	0.065	8.637	0.008776
	Time × salinity	0.024	2	0.012	1.579	0.233503
	Time × NO_3NH_4	0.043	2	0.022	2.845	0.084433
	Time × salinity × NO_3NH_4	0.049	4	0.012	1.614	0.214095
	Error	0.136	18	0.008		
SA population	Time	0.899	1	0.899	69.543	<0.000001
	Time × salinity	0.427	2	0.213	16.500	0.000085
	Time × NO_3NH_4	1.162	2	0.581	44.921	<0.000001
	Time × salinity × NO_3NH_4	0.892	4	0.223	17.246	0.000006
	Error	0.233	18	0.013		

concentrations up to 4& (Beebee, 1985; Ely, 1944). Other studies have reported a high embryonic sensitivity of certain amphibian species exposed to 250–945 mg chloride/L (approximately 0.4–1.5& salinity, Karraker and Ruthig, 2009; Turtle, 2000); however, these studies analyzed the effects of road de-icing salt which, in addition to sodium chloride, contains other chemicals such as sodium ferrocyanide (Paschka et al., 1999) or different heavy metals (Oberts, 1986) that may have had separate toxicity.

Apart from these effects on embryos, amphibians exposed to osmotic stress may show reduced larval growth and metamorphosis rates, and increased larval and adult mortality. For example, Sanzo and Hecnar (2006) calculated a 96 h median lethal concentration of 2.6& salinity in Lithobates sylvaticus. Christy and Dickman (2002) observed that sublethal salinity levels reduced the developmental rate of Litoria aurea tadpoles. This reduction of growth or developmental rate enhances the negative effect of salinity by delaying metamorphosis and thus prolonging larval exposure to the stressor.

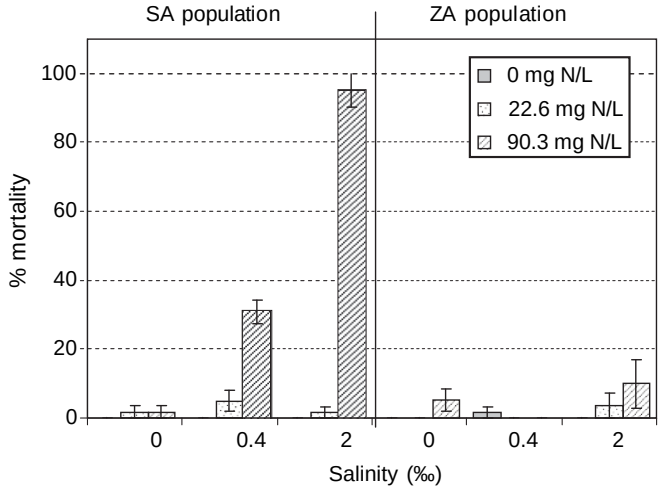

Fig. 1. Mortality rates (±SE) per treatment of P. perezi embryos from two populations after 12 days of exposure to different ammonium nitrate and salinity levels.

In contrast to these deleterious effects of increased salinity on amphibians, the adaptation of some populations to brackish or even saline environments has also been reported. For example, Gomez-Mestre and Tejedo (2003) demonstrated that the B. calamita populations from Southern Spain native to brackish water had a higher salinity tolerance than did freshwater populations, suggesting local adaptation. Furthermore, individuals from brackish waters that were transplanted to freshwater environments showed the same survival probabilities as did those native to freshwaters, indicating that an increased tolerance to osmotic stress does not imply a loss of performance in freshwater. However, Turtle (2000) also conducted transplant experiments with Ambystoma maculatum from roadside pools with high salinity levels and from woodland pools with low salinity concentrations. This author found that embryos from roadside pools had higher survival rates when transferred to woodland pools, possibly indicating that they were not adapted to the conditions of their native pool. Nevertheless, Turtle (2000) studied vernal pools affected by highway runoff, and additional stressors other than salinity could be acting on individuals growing in these pools. These transplant experiments conducted both by Gomez-Mestre and Tejedo (2003) and Turtle (2000) are useful for resolving the question of whether the resistance to salinity of some populations lies in a genetic adaptation or is simply because of a phenotypic acclimation with no evolutionary implications. B. calamita populations studied by Gomez-Mestre and Tejedo (2003), native from a naturally brackish environment, would be genetically adapted and therefore its resistance to salinity would be an adaptive, inheritable character. On the other hand, the A. maculatum populations analyzed by Turtle (2000) inhabiting roadside pools would be able to resist high salinity concentrations as a consequence of an environmentally induced phenotypic acclimation, and no genetic adaption to brackish or saline environments would exist in these populations. Considering that ZA embryos showed the same survival rates regardless of whether salinity concentration was similar to that of their native habitat or lower, we could conclude that resistance to salinity in this population would be the consequence of a genetic adaptation.

With regards to the effects of ammonium nitrate, the concentrations used in the present study, in absence of any salinity effect, caused only a reduction in embryonic growth rate. Negative effects of environmentally relevant concentrations of ammonium nitrate on growth rates of amphibian aquatic stages have been reported in

Table 3
Effects of ammonium nitrate (NO_3NH_4) and salinity on the total length of hatchlings of two P. perezi populations after 12 days of exposure. Data comparing both populations and those from each population separately are shown.

Source of variation	Sum of squares	df	Mean squares	F	p
Population	0.001	1	0.001	0.715	0.403672
Salinity	0.009	2	0.004	4.629	0.016674
NO_3NH_4	0.052	2	0.026	28.070	<0.000001
Population × salinity	0.002	2	0.001	0.827	0.445855
Population × NO_3NH_4	0.000	2	0.000	0.152	0.859232
Salinity × NO_3NH_4	0.006	4	0.002	1.723	0.167657
Population × salinity × NO_3NH_4	0.002	4	0.001	0.603	0.663044
Error	0.032	34	0.001		

a number of previous studies (Ortiz et al., 2004; Ortiz-Santaliestra et al., 2006; Schuytema and Nebeker, 1999; Watt and Oldham, 1995; Xu and Oldham, 1997). The disadvantages of a reduced size in amphibian aquatic stages are well known. Individuals that reach metamorphosis with a smaller size have lower survival probabilities during the juvenile period than do larger metamorphs (Berven, 1990; Semlitsch et al., 1988; Smith, 1987). Nevertheless, our experiment was not prolonged during the larval stage and thus we cannot confirm that the observed reduction in growth rate during the embryonic period would have been reflected in a lower size at metamorphosis.

A review of the available literature on the effects of ammonium nitrate on amphibians affirms the consideration of P. perezi as a tolerant species. In previous experiments, we have detected lethal effects of the same ammonium nitrate levels as those used in this experiment on embryos and larvae of Hyla arborea, Bufo bufo and Discoglossus galganoi (Ortiz et al., 2004). The mortality rate of Pseudacris regilla embryos after 10 days of exposure to 101.8 mg N–NO_3NH_4/L was 80%, and the mortality rate of Xenopus laevis embryos after 5 days of exposure to the same concentration was 53.5% (Schuytema and Nebeker, 1999). As in P. perezi, embryos of other species have been found to be more resistant to ammonium nitrate. For example, identical levels to what were used in the present study did not reduced survival of Pelobates cultripes or Triturus pygmaeus embryos (Ortiz-Santaliestra et al., 2006, 2007).

There are some cases where amphibian populations are known to tolerate moderate levels of contamination (e.g., Semlitsch et al., 2000). With regards to nitrate, Johansson et al. (2001) reported that R. temporaria individuals from a population naturally exposed to a high nitrate load were less sensitive to the negative effects of this chemical than were the individuals from a non-exposed population. Egea-Serrano et al. (2009) also found that P. perezi tadpoles from a nitrogen polluted area showed lower mortality rates when exposed in the laboratory to high concentrations of ammonium, either alone or in combination with nitrate and nitrite. These authors suggested that populations breeding in habitats exposed to high levels of toxicant nitrogenous compounds may have evolved rapidly in response to environmental nitrification in a pronounced process of selection. Bridges and Semlitsch (2000), who observed differences in sensitivity to the pesticide carbaryl among Lithobates sphenocephala populations, also suggested a process of local adaptation to the chemical to explain the reported differences. These hypothetical phenomena of rapid local adaptation to anthropogenic pollution seem compatible with the idea that environmental stress, especially stress of contemporary anthropogenic origin, is a strong force generating local adaptations and rapid evolution (Carroll et al., 2007; Hoffmann and Hercus, 2000). However, contrary to what happens with salinity in those populations that historically inhabit brackish environments, it is not clear whether the population-dependent tolerance to anthropogenic contamination reported by Johansson et al. (2001), Egea-Serrano et al. (2009) or Bridges and Semlitsch (2000) is actually because of a genetic adaptation, and further research would be needed to clarify this question. Nonetheless, in the present study we did not detect differences between populations in sensitivity to ammonium nitrate alone, as we would expect from the fact that the levels of nitrogenous compounds were similar in both ponds.

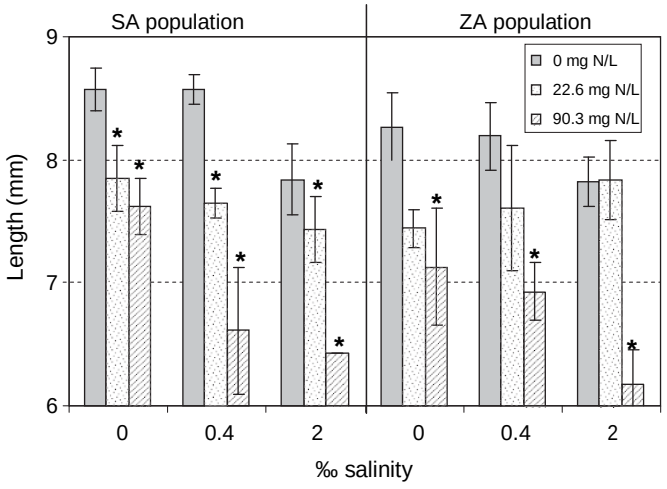

Fig. 2. Larval length per treatment in two P. perezi populations after 12 days of exposure to different ammonium nitrate and salinity levels. Asterisks indicate the treatments that were significantly different from the controls ($p < 0.05$) according to the Bonferroni tests for multiple comparison adjustment.

In spite of the absence of differences between populations in sensitivity to ammonium nitrate alone, we found a high toxicity in SA embryos when the fertilizer was combined with high salinity levels, while no similar effects were observed in ZA individuals. Since lethality is not caused by a single factor, but is a consequence of synergistic effects between the two stressors, the natural adaptation of ZA individuals to salinity possibly prevents these animals from suffering any ill effects of the ammonium nitrate at the levels tested. Analyzing the combined effect of nitrite and ambient UV radiation, Macías et al. (2007) found a synergism of different magnitude in two P. perezi populations, but did not describe any phenomenon of adaptation to any stressor that could have reduced the overall effect of the combination.

Some of the mechanisms related to the exposure to nitrogenous fertilizers or osmotically stressful environments may contribute to the explanation of why the population adapted to salinity was also more tolerant to ammonium nitrate toxicity. Gosner and Black (1957) confirmed that high salinity levels prevented water diffusion into the egg because of the alteration of osmotic equilibrium; therefore, the volume of the space inside the egg did not increase and embryos suffered malformations as they were growing. An excess of ammonium or nitrate in the water could have the same effect on water exchange throughout the egg envelope by altering the osmotic equilibrium. Embryos adapted to salt should have a mechanism to avoid the reduced uptake of water, and this strategy

could be also beneficial when dealing with high levels of inorganic nitrogenous compounds. Wright et al. (2004) demonstrated that one of the physiological mechanisms involved in the high resistance of F. cancrivora to osmotically stressful environments is an active regulation of the urea–ornithine cycle. Under highly saline conditions, juvenile frogs increase the rate of urea synthesis to accumulate osmotically active metabolites thus equilibrating the osmotic potential inside and outside the organism and avoiding an excessive dehydration. Furthermore, urea synthesis and excretion is the main route of ammonia detoxification for amphibian aquatic stages (Schmuck et al., 1994). Therefore, we could expect that those species with a higher efficiency in the activation of the urea–ornithine cycle will be able not only to resist high salinity, but also to efficiently detoxify ammonia. However, urea–ornithine cycle happens in the liver, and since the liver in anuran amphibians does not develop until early larval stages (Duellman and Trueb, 1994), this hypothesis may not be appropriate to explain the observed effects on P. perezi embryos. In general, organ development in amphibian embryos is minimal, so the physiological mechanisms to avoid the negative effects of high salinity and/or inorganic nitrogen should be related to active transport of water and ions across the skin and egg membrane. Unfortunately, nothing is known about how these organisms respond to osmotic stress at the physiological level.

Finally, the mechanism involved in the tolerance to osmotic stress and inorganic nitrogen could be related to the characteristics of the gelatinous envelope. Water diffusion across the jelly coat depends not only on the osmotic conditions inside and outside the egg, but also on the thickness and structure of the envelope. The embryonic jelly coat is made of glycoproteins, mucopreoteins and carbohydrates (e.g., Freeman, 1968), and it has a high diffusion capacity for hydrophilic substances such as sodium, chlorine, nitrate or ammonium (Salthe, 1963). However the thickness and structure of the jelly coat, and therefore its permeability to these substances, may vary (Carroll et al., 1991; Seymour, 1995). Adaptation to osmotic stress could involve a gelatinous envelope that would be less permeable to the uptake of ions or to the loss of water, which indirectly would benefit the organisms when dealing with inorganic nitrogenous chemicals.

5. Conclusions

We have demonstrated that the genetic adaptation of P. perezi embryos to moderately saline environments helps them to cope with an excess of inorganic nitrogen in the water. As the lethality produced by these two factors is a consequence of their combined action, the fact that one of the factors does not act as a true stressor in one of the populations minimizes the overall risk for that population. To our knowledge, the case reported in the present study is the first in amphibians that demonstrates how the adaptation to a natural stressor may indirectly generate a resistance against the toxicity of a chemical. Our results emphasize the necessity of evaluating multiple stressors, arising from both natural factors and anthropogenic impacts, as the potential causes for currently observed declines in amphibian populations (Linder et al., 2003). In addition, the high intraspecific variability in the susceptibility to one or all of these stressors needs to be evaluated when planning conservation strategies at a local scale.

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