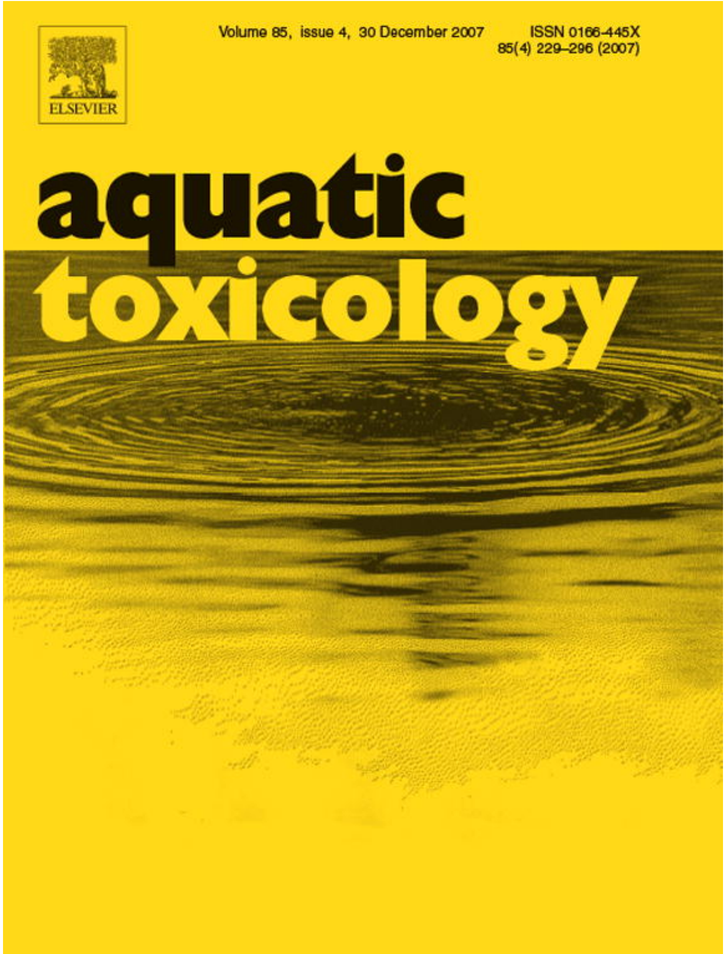
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Effects of ammonium nitrate exposure and water acidification on the dwarf newt: The protective effect of oviposition behaviour on embryonic survival

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Abstract

Embryonic mortality in many aquatic animals, including most amphibian species, is usually very high. In addition to mechanical and chemical defences, some species have developed behavioural patterns that can increase egg survival. For example, females of some newt species protect their eggs by wrapping them in leaves of aquatic plants. We have studied the effects of ammonium nitrate (nominal concentration of 90.3 mg $N–NO_3NH_4$/L) and water acidification (pH 4–5) on egg wrapping behaviour of the dwarf newt, *Triturus pygmaeus*, and on whether this specific behaviour may protect embryos from contamination. Although either ammonium nitrate or low pH did not inhibit oviposition, the mean percentage of eggs that were wrapped by the females was significantly lower at low pH than in controls. In order to assess the potential effects of oviposition behaviour on embryonic survival, we exposed simultaneously wrapped and unwrapped eggs to ammonium nitrate and acid pH during their development. After 25 days of exposure, ammonium nitrate reduced length and developmental stage at eclosion of the exposed individuals, regardless of whether they were wrapped or unwrapped. The fertilizer caused a significantly higher mortality in unwrapped than wrapped eggs. The potential impact of water pollution on amphibians in the field may include not only direct effects on embryonic and larval survival but also alteration of breeding behaviours, which may reduce reproductive success and ultimately affect population's condition.

Keywords: Amphibian decline; Behavioural toxicology; Embryonic survival; Fertilizers; Parental cares

1. Introduction

Aquatic stages of many amphibian species are naturally exposed to a high mortality due to predation or pond drying (e.g. Wilbur and Collins, 1973). Furthermore, most amphibian species do not provide parental care to their eggs (Duellman and Trueb, 1994), which contributes to decreased survival during the embryonic stage (Trivers, 1972). However, some species show behavioural patterns that can indirectly affect embryonic survival. For example, females of *Triturus* species lay between 100 and 500 eggs during their breeding season (Miaud, 1995). Eggs are laid individually on aquatic plant leaves and wrapped using the leaf as an additional protective envelope (Díaz-Paniagua, 1989; Griffiths, 1995; Miaud, 1995). This behaviour supposes a reproductive investment that, without any further parental care, can increase embryonic survival by protecting eggs from mechanical damage (Ward and Sexton, 1981), predation by aquatic invertebrates or adult newts (Miaud, 1994), or direct impact of ultraviolet radiation (Marco et al., 2001).

Many amphibian species suffer increased mortality, especially during their aquatic stages, due to habitat acidification or water pollution (e.g. Rouse et al., 1999; Gibbs et al., 2005). Ammonium nitrate is one of the most commonly used chemical fertilizers in the world. Its application on crop fields usually results in an increase in environmental levels of nitrogen ions in water bodies near the point of application (Vitousek et al., 1997). Environmental concentrations of nitrate and ammonium cause deleterious effects, such as high mortality or delayed growth rates, in embryos and larvae of several amphibian species (e.g. Hecnar, 1995; Ortiz et al., 2004). Furthermore,

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excessive acidification in water bodies caused by atmospheric deposition of nitrate and sulphate ions from industrial areas (Vertucci and Corn, 1996) directly kills larvae (Horne and Dunson, 1995) or increases lethal effects of some other stressors such as nitrate pollution or ultraviolet radiation (Long et al., 1995; Hatch and Blaustein, 2000). Decrease of pH in natural areas exposed to acid rain makes freshwater ecosystems unsuitable for amphibian breeding and thus produces local extinctions of some populations (e.g. Beebee et al., 1990). Both excess nitrogen and the acidification of water bodies have been proposed as possible causes for the decline of amphibian populations (Blaustein et al., 2003). Although nothing is known about how ammonium nitrate or low water pH may affect amphibian reproductive behaviours, previous work has demonstrated that some behavioural patterns of adult urodeles may be altered by these kinds of pollution (e.g. Wyman and Hawksley-Lescault, 1987; Ortiz-Santaliestra et al., 2005).

Dwarf newt (*Triturus pygmaeus*) is an endemic species of the south-western Iberian Peninsula. The species inhabits areas from sea level to 1450 m, in a wide range of habitats, from grasslands to forest of cork trees, holm oaks and gall-oaks. It appears indistinctly on siliceous and calcareous soils. It uses both temporal and permanent lentic waters for breeding (García-París et al., 2004). Its western populations do not present problems of conservation. However, eastern populations are endangered due to loss of water bodies, water pollution from agricultural activities and predation by exotic species such as red crayfish (*Procambarus clarkii*) (see García-París et al., 2004 for review). Its oviposition process starts when the female selects a submerged plant for egglaying; she places herself over a plant leaf and bends it with her hind limbs while the egg is being deposited; finally, the egg sticks to and is wrapped by the leaf (see Díaz-Paniagua, 1989 for details). The impacts to which females could be exposed during this process, such as predation or water pollution, might alter the oviposition behaviour and its final result; eggs could thus lose the protection given by the leaf, which would increase embryonic mortality (Miaud, 1995).

We hypothesize that acidification or nitrogen excess in aquatic environments could alter the oviposition behaviour of *T. pygmaeus*. Furthermore, since the protection given by plant leaves might indirectly protect embryos from pollutant effects by reducing egg surface area exposed to contaminated water, toxicant-mediated alteration of oviposition behaviour may affect embryonic survival. The aim of this study is therefore to assess the effects of ammonium nitrate and water acidification on oviposition behaviour of *T. pygmaeus* females and the possible negative effect of the alteration of this behaviour on embryonic survival.

2. Material and methods

We conducted two different static renewal experiments to assess the effects of ammonium nitrate and water acidification on oviposition behaviour and embryonic survival of *T. pygmaeus*. In the oviposition experiment, we exposed adult females during the oviposition period in order to assess the effects of ammonium nitrate and water acidification on this specific behaviour. Instead of natural opaque leaves, we used, as wrapping substrate, strips of transparent plastic film because they prevented females from eating their own eggs. Females were unable to break off plastic strips and eat the piece containing the egg, as they do when eggs are wrapped by plants (Marco, 2004). In the embryonic development experiment, we exposed eggs laid on plants in non-polluted waters to ammonium nitrate and low pH. We kept half of these eggs wrapped and carefully unwrapped the other half in order to compare embryonic survival and hatching traits between wrapped and unwrapped eggs.

2.1. Chemical exposure

Water temperature and pH were checked daily during the experiments. Differences among treatments in water temperature and pH were checked with one-way analyses of variance (ANOVAs). For both experiments females or eggs were exposed to three treatments: control (no contaminants added), ammonium nitrate (nominal concentration of 90.3 mg $N{-}NO_3NH_4$/L), and acid (nominal pH 4.00 ± 0.10, obtained after the addition of sulphuric acid). 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 crop fields (Scholefield et al., 1996). In this treatment, we used 99% pure ammonium nitrate (Merck KGaA®, Darmstad, Germany) to prepare a stock solution that we pipetted in every tank assigned to the ammonium nitrate treatment in order to get the experimental level. The frequency of water renewal was every four days in both experiments. Degradation of ammonium and nitrate was expected to occur within this period, as previous experiments developed with similar methodology have demonstrated (Marco et al., 1999). This design would represent a "pulse" exposure (Hatch and Blaustein, 2000), similar to what happens in the amphibian breeding sites, where peak levels occur just after fertilizer runoff and then decrease with the passage of time.

The nominal level of pH was selected because we expected that a quick fluctuation to less acidic levels would happen. Thus, this initial level would be the minimum value in the variation range of pH during the experiments. Levels of pH ranging from 4 to 5 have been reported in several areas of Europe and North America in which amphibians inhabit (e.g. Freda and Dunson, 1986; Räsänen et al., 2003), and some amphibian species usually breed in ponds with pH levels around 4 (Warner and Dunson, 1998). We did not use buffers to get the nominal pH level because they can present specific effects that could not be controlled during the experiments. As a significant oscillation in pH values was expected in a relatively short time, we made daily readjustments of pH levels to the nominal value when necessary. Sulphuric acid (10%; Fluka Chemie AG®, Buchs, Switzerland) was pipetted into tanks in order to get the selected pH. The quantity of 10% sulphuric acid needed to get the final level varied between 0.04 and 0.05 mL per tank. At the beginning of the experiments, sulphuric acid was first added to the water, and animals were subsequently placed inside tanks once the nominal pH level had been confirmed. When pH needed to be readjusted during the experiments, 10% sulphuric acid was carefully dripped and pH was checked after every drop in order to avoid an excessive acidification that could have affected the animals. Sulphuric acid was used for acidification because it is the major component of acidity in acid rain.

2.2. Oviposition experiment

Fifty-four *T. pygmaeus* females were collected in January 2002 from several ponds in Almonte, Huelva (south-western Spain). Díaz-Paniagua (1979) analyzed the water chemistry of 12 of the ponds used by this population for breeding. The author obtained a mean pH level of 7.17 and mean concentrations of nitrate and nitrite of 8.24 and 2.31 g/L, respectively (which probably were 8.24 and 2.31 mg/L). To our knowledge, this is the only *T. pygmaeus* population whose breeding ponds have been chemically characterized, so we do not have comparative data. Females were taken to the laboratory where they were exposed to a natural sunlight photoperiod at temperatures ranging daily from 12 to 18 °C. Just after collection, females were placed individually in 3.4 L tanks with 2 L of dechlorinated tap water. Inside each tank we submerged six transparent plastic film strips (110 mm long and 7 mm wide) that females used as oviposition substrate. These film strips were easy to bend due to their soft texture and females did not show any problem in manipulation of plastics for oviposition. Furthermore, strip measures were selected in order to simulate plant leaves where eggs are usually laid (Díaz-Paniagua, 1989). Plastic strips were tied to a stainless metallic wire to prevent them from floating. Each tank was randomly assigned to one of the three treatments: control, ammonium nitrate, and acid water (18 replicates per treatment). The females were kept in experimental tanks for seven days. No differences among treatments were observed in females' snout–vent length ($F_{2,51} = 0.185$; $P = 0.832$) or mass ($F_{2,51} = 0.246$; $P = 0.783$).

We checked the number of eggs laid in every tank daily. We also recorded whether the eggs were properly wrapped in the film, attached to the film but unwrapped or free in the water. The percentage of females that laid eggs in each treatment, number of eggs that females laid per day and the percentage of wrapped eggs in each tank were calculated. We analyzed whether the number of eggs that each female laid per day was correlated with the female's body size. We used a chi-square test to determine the influence of ammonium nitrate and low pH on the proportion of females laying eggs. To determine the effects of the fertilizer and the acidification on the oviposition process we used one-way ANOVAs with the number of eggs laid per female and day (log transformed) and percentage of wrapped eggs (arcsin of square root transformed) as dependent variables.

2.3. Embryonic development experiment

In January 2004 we collected 20 *T. pygmaeus* females from the same population as that used for oviposition experiment. The newts were placed in an 11 L tank with 8 L of dechlorinated tap water and exposed in the laboratory to similar temperature and photoperiod conditions as before. Plants of *Mentha pulegium*, the species most often used by *T. pygmaeus* for oviposition (Díaz-Paniagua, 1989), were fixed to a sandy substrate on the bottom of the tank and females were allowed to lay eggs during a two-day period. Percent of wrapped eggs in this case was similar to what was measured in control females of the oviposition experiment, which demonstrates that plastics used in the previous experiment constitute a suitable substrate for oviposition. At the end of this time, half of the eggs laid on each plant were carefully unwrapped. The plants with wrapped and unwrapped eggs were placed in 12 experimental tanks of 3.4 L volume with 2 L of dechlorinated tap water in such a way that each experimental tank contained 10 wrapped and 10 unwrapped eggs. Plants were fixed to the bottom of the tank as described above in order to keep the eggs underwater. Each tank was randomly assigned to one of the three treatments (control, ammonium nitrate, and low pH), so each treatment was replicated four times. Eggs were exposed until their eclosion or death. All the individuals had hatched or died at day 25 of experiment.

Embryos were checked individually inside each tank and effects of ammonium nitrate or acidification on wrapped and unwrapped eggs were distinguished. On hatching, each individual was staged in accordance with Harrison's stages (Harrison, 1969) and its total length (mouth to tail tip) was taken with a digital calliper to the nearest 0.01 mm. At the end of the experiment, mean time to eclosion, total length, and stage at eclosion were calculated separately for wrapped and unwrapped eggs in each tank. To determine the sensitivity of embryos to pollutants and if this sensitivity was affected by oviposition behaviour we used a two-way ANOVA with the mortality rate at the end of the experiment (25 days, arcsin of square root transformed) as dependent variable and the treatment and the wrapped or unwrapped condition as the categorical factors. We did not analyze the mortality rates prior to the end of this experiment because we could not definitely know if wrapped eggs were dead or alive until they hatched or were infected by fungus. The use of plants in this experiment was preferred because our objective was to analyze the protective effects of the natural egg envelop; however, it had the inconvenience that egg observation through the envelopes was not possible. In some cases, when we wanted to know the mortality rates at a given moment, we estimated them from the observation of individuals during the subsequent days. To assess differences between sensitivity of wrapped and unwrapped eggs in a specific treatment, we conducted one-way ANOVAs separately for each treatment, with the mortality rate (arcsin of square root transformed) as dependent variable, and the wrapped or unwrapped condition as factor. Variables concerning hatching traits were analyzed by two-way ANOVAs using the same methodology as for survival.

3. Results

3.1. Oviposition experiment

Water temperature varied inside its natural daily range from 12.3 to 17.1 °C. Mean water temperature was 14.6 °C. Water pH in the control and ammonium nitrate treatments varied from 6.80 to 7.60 (mean pH 7.31). No differences among treatments were detected either in water temperature ($F_{2,18} = 0.003$; $P = 0.997$) or, excluding acid treatment, in water

Table 1
Descriptive statistics of the variables measured during the oviposition experiment

	Control		Ammonium nitrate		Acid		ANOVA		
	Mean (±S.D.)	CI (95%)	Mean (±S.D.)	CI (95%)	Mean (±S.D.)	CI (95%)	*F*	d.f.	*P*
Day	1.64 (±0.94)	1.17–2.10	1.07 (±0.88)	0.63–1.51	1.02 (±0.89)	0.58–1.47	2.160	2, 51	0.126
Wrapp	90.1 (±15.9)	82.0–98.3	78.4 (±28.7)	61.1–95.8	19.2* (±27.0)	3.0–35.5	33.224	2, 40	<0.001
Attach	1.0 (±3.0)	−0.5–2.5	3.6 (±5.7)	0.1–7.1	2.2 (±5.8)	−1.3–5.7	1.054	2, 40	0.358

Day: number of eggs laid per female and day. Wrapp: percent of wrapped eggs. Attach: percent of eggs attached to the film. Results of one-way analyses of variance (ANOVAs) to compare variables among treatments are shown. S.D.: standard deviation; CI: confidence interval.
* Treatments are significantly different from controls as revealed by post hoc tests.

pH ($F_{1,12}=0.002$; $P=0.969$). In the acid water treatment, pH ranged from 3.91 to 4.78, with a mean value of 4.26.

Ammonium nitrate and low pH did not produce mortality or any apparent effect on exposed females, except skin moulting of three individuals exposed to low pH, and one individual in the other two treatments. The number of eggs laid per female and day was not influenced either by female snout–vent length ($r=0.060$; $n=54$; $P=0.669$) or mass ($r=0.251$; $n=54$; $P=0.068$). Forty-three of the 54 females laid eggs; the percentage of females laying eggs was 94% in controls, while in the other two treatments it was 72%. No significant differences among treatments were observed in percent of females laying eggs ($\chi^2=3.653$; $n=3$; $P=0.161$).

Number of eggs laid per female and day was higher in controls than in the other two treatments. However, no significant differences among treatments were observed for this variable (Table 1). Oviposition behaviour was altered by low pH exposure. The percentage of wrapped eggs was significantly lower in females exposed to acid water than in controls (Table 1). In the ammonium nitrate treatment, although the proportion of wrapped eggs was also lower than in controls, post hoc test (HSD Tukey) did not show significant differences among these two treatments. Non-wrapped eggs were mainly laid directly on the bottom of the tank. The proportion of eggs attached to the plastic strips was similar in all three treatments and statistically non-different from zero (Table 1), so we did not consider this variable relevant.

3.2. Embryonic development experiment

Variation in water temperature was 12.3–17.7 °C, with an average water temperature of 14.9 °C. Water pH in the control and ammonium nitrate treatments ranged from 6.67 to 7.56 (mean pH 7.26). No differences among treatments were detected in water temperature ($F_{2,72}=0.006$; $P=0.994$) or pH ($F_{1,48}=0.040$; $P=0.843$). Finally, in the acid water treatment, pH varied from 3.89 to 5.04, with an average value of 4.56.

During the first six days of exposure, estimated mortality in all three treatments was less than 5%, so we rejected a possible effect on fecundity rate that could have partially explained the embryonic mortality. We found a high mortality rate in all three treatments after 25 days of experiment (control: 62.5%, ammonium nitrate: 66.7%, low pH: 56.6%). This high mortality is suspected to be related with a genetic, species-based reason that will be discussed later. No effects of ammonium nitrate or acid water on embryonic survival were observed at the end of the experiment ($F_{2,9}=0.251$; $P=0.783$). Time of eclosion was not affected by exposure to ammonium nitrate or low pH, but size at eclosion and stage at eclosion significantly varied among treatments (Table 2). Mean body length at eclosion was 9.33 (±0.22) mm in controls, 8.90 (±0.53) mm in larvae exposed to low pH, and 8.10 (±0.14) mm in individuals exposed to ammonium nitrate. According to Harrison stages, average stage at eclosion was 41.35 (±0.27) in controls, 41.49 (±0.35) in the acid water treatment, and 41.05 (±0.20) in the ammonium nitrate

Table 2
Results of two-way analyses of variance (ANOVAs) to assess the effects of ammonium nitrate or low pH (treatment), and the wrapped or unwrapped condition of the eggs, on time to eclosion, length at eclosion and stage at eclosion of *T. pygmaeus* embryos during the embryonic development experiment

Variable	Source of variation	Mean squares	d.f.	*F*	*P*
Time to eclosion	Treatment	$5.154e^{-4}$	2	1.282	0.303
	Wrapping condition	$8.718e^{-5}$	1	12.095	0.647
	Treatment × wrapping	$8.621e^{-4}$	2	3.742	0.148
	Error	$4.020e^{-4}$	17		
Length at eclosion	Treatment	3.496	2	0.217	0.001
	Wrapping condition	$1.324e^{-2}$	1	0.046	0.833
	Treatment × wrapping	$7.118e^{-2}$	2	0.097	0.784
	Error	0.289	17		
Stage at eclosion	Treatment	$3.708e^{-5}$	2	2.145	0.045
	Wrapping condition	$9.626e^{-7}$	1	0.246	0.759
	Treatment × wrapping	$2.533e^{-5}$	2	2.557	0.107
	Error	$9.907e^{-6}$	17		

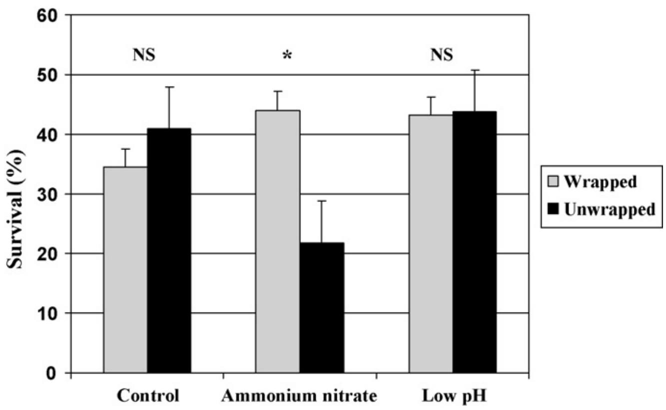

Fig. 1. Mean survival rates at eclosion (±S.D.) of *T. pygmaeus* wrapped and unwrapped embryos exposed to ammonium nitrate and low pH. Results of one-way analyses of variance (ANOVAs) per treatment to compare survival among wrapped and unwrapped embryos are shown (NS, $P > 0.05$; $^{*}P < 0.05$).

treatment. Post hoc test revealed that only ammonium nitrate significantly affected these hatching traits.

Both in control and low pH treatments, mortality of unwrapped did not differ significantly from that observed in wrapped eggs (Fig. 1). However, an interaction between the exposure to the contaminants and egg wrapping was detected at the end of the experiment. In the ammonium nitrate treatment, significant differences between wrapped and unwrapped egg mortalities were observed ($F_{1,6} = 8.027$; $P = 0.030$). After 25 days of exposure in this treatment, survival of unwrapped eggs hardly surpassed 20%, while eggs that were protected by the leaves presented a survival rate close to 50% (Fig. 1). Individual length and developmental stage at eclosion and time to eclosion did not differ between wrapped and unwrapped individuals in any of the three treatments (Table 2).

4. Discussion

The only detected response of females to experimental conditions was some skin moulting. Moulting frequency in amphibians is controlled by thyroid hormones (Larsen, 1976), and some environmental factors such as water temperature (Stefano and Donoso, 1964) or photoperiod (Taylor and Ewer, 1956) may affect duration of the intermoult period. However, nothing is known on how ammonium nitrate or water pH may affect moulting frequency. Nevertheless, Larsen (1976) pointed out that the time elapsing between two moults in some amphibians varied from a few days to a few weeks. The moulting frequency observed in our seven-day experiment (5 out of 54 newts) would thus be inside this range. Moreover, skin moulting was observed in all three treatments, so it was probably a normal response to experimental manipulation of newts and not an effect of the chemical exposure.

Our results show that low pH strongly altered the wrapping behaviour of *T. pygmaeus* females. The percentage of wrapped eggs was clearly lower in females exposed to acid water than in controls. The primary mechanism of acid toxicity in amphibians that could have led to the alteration of oviposition process appears to be the disruption of osmoregulation (Rowe and Freda, 2000) and consequent inhibition of sodium uptake (McDonald et al., 1984). This alteration in the sodium balance can force newts to increase energetic costs and alter respiratory function, which would induce females to gulp air with a greater frequency and thus prevent the oviposition process being completed between two respiratory pulses (Díaz-Paniagua, 1989).

Females of *Triturus* species present the wrapping behaviour as a mechanism of egg protection (Díaz-Paniagua, 1989; Miaud, 1994), so the alteration of oviposition can indirectly affect embryonic survival. Eggs that are laid on the bottom of the ponds can be buried in the mud and they can die from asphyxiation. Laying eggs on plant leaves prevents them from becoming buried (Griffiths, 1995). Marco et al. (2001) demonstrated that wrapped eggs of *Triturus marmoratus* exposed to intensities of ultraviolet radiation that produced high mortality in unwrapped eggs responded to the treatment as if they were free from the radiation impact. Even in the absence of external pressures, those species that naturally present a lower proportion of wrapped eggs, such as *Mesotriton alpestris* or *Lissotriton helveticus*, suffer a higher embryonic mortality than species, such as *Triturus cristatus*, which are very efficient in wrapping their eggs (Miaud, 1994).

In spite of altering behaviour, water acidification did not inhibit females laying eggs and hardly reduced the number of egg-laying events per female and day. An alteration that induces females to forego the wrapping of their eggs but not prevent them from laying them altogether could be especially relevant in ecological terms. Females could be expected to adapt their oviposition timing to the environmental conditions (Baker, 1992) and thus lay eggs when or where water pH is less dangerous. However, *T. pygmaeus* females did not recognize acid water as a potential risk and, thus, were not inhibited from laying eggs. Nevertheless, we exposed females during 7 days, and the duration of the oviposition period in this population is between 75 and 90 days (Díaz-Paniagua, 1979), so this conclusion should be taken carefully. Whiteman et al. (1995) reported that males of some *Ambystoma tigrinum* populations were unable to discriminate breeding habitats where their embryos would be exposed to a high mortality as a consequence of the low water pH. On the other hand, some adult amphibians have been shown to avoid acidic waters (e.g. Vatnick et al., 1999), but the specific mechanism of pH assessment is unknown. Nevertheless, in our study the pH level that impaired oviposition behaviour did not affect embryonic survival or development.

Another possible effect of low water pH or ammonium nitrate on oviposition could come from the effects on plants used for egglaying. Díaz-Paniagua (1986) described that *T. pygmaeus* females are in some way flexible in their oviposition substrates if plants with wide and flexible leaves are available. Although nothing is known on how water acidification may affect these potential oviposition substrates, plants of *M. pulegium* used in the embryonic development experiment did not show visible effects after 25 days of exposure to ammonium nitrate or acidification. We can thus conclude that the potential effects of low pH on laying substrates are little relevant in comparison with the direct alteration of oviposition behaviour and the consequent decrease of egg survival probabilities discussed above.

Sensitivity of embryos exposed during the embryonic development experiment was very high. Nevertheless, we must consider that death of half of the embryos in newt species of the genus *Triturus* is attributed to an unusual chromosomal alteration that arrests embryonic development (Horner and MacGregor, 1985). Chromosome 1 in these species is heteromorphic, and this heteromorphism is maintained by a balanced lethal system that arrests development of the embryos that are homozygous for this chromosome (MacGregor and Horner, 1980). This syndrome appears between the stages 28 and 34 (myotomes to tailbud stages), which in our experiment would correspond to days 13–18 of exposure. Estimated mortality in our experiments during the few days when eggs were at the developmental stages affected by the syndrome was over 40% in all three treatments. Many of these embryos that died during the theoretical period of manifestation of the syndrome showed the typical symptoms described by Wallace (1987) in affected *T. cristatus*: dorsal blisters over the head and spinal cord, reduced tail development, and absence of the two pigmented dorsal stripes.

These observations would support the reported existence of the developmental arrest syndrome in the population tested (Marco et al., 2005). One question arising from the analysis of the developmental arrest syndrome is how an apparently maladaptive character has been selected and maintained during the evolution. Several studies have demonstrated, however, that this chromosomal lethal system is well balanced and so has allowed it to persist in these species (Sims et al., 1984; Wallace, 1994). Its main advantage would be the selection of heterozygosis, thus ensuring a higher genetic variability in the affected species, which ultimately favours the biological efficiency of organisms.

Ammonium nitrate reduced length and stage at eclosion of *T. pygmaeus* embryos. The effects of ammonium nitrate on amphibian embryos have already been demonstrated. For example, Watt and Jarvis (1997) observed a high mortality of *L. helveticus* embryos exposed during their development to ammonium nitrate at 70 mg $N–NO_3NH_4$/L. Hecnar (1995) reported, for four amphibian species, medium lethal concentrations between 27.2 and 78.6 mg $N–NO_3NH_4$/L after only 96 h of exposure. In fishes, there is evidence that nitrate inhibits iodine uptake by the thyroid gland, which may reduce growth (Crow et al., 2001). Watt and Oldham (1995) described a growth delay in larval *Triturus vulgaris* produced by sub-lethal levels of the fertilizer. Ortiz et al. (2004) observed how ammonium nitrate levels similar to what we have used in our study reduced embryonic and larval growth in six amphibian species.

We did not find effects of water acidification on embryonic survival. Nevertheless, some previous studies have demonstrated that this kind of pollution may affect species of urodela. Larval salamanders of the genus *Ambystoma* exposed to the acid pH level existing in their natural habitats (between 4.0 and 6.5) suffered altered swimming activity, increased risk of predation, and significant mortality (Kutka, 1994; Horne and Dunson, 1995). Griffiths et al. (1993) demonstrated that *L. helveticus* and *T. vulgaris* embryos incubated under acidic conditions (pH 5.5) hatched at an earlier stage of development, at a smaller size, and earlier than controls.

The results of the embryonic development experiment revealed that wrapped eggs were more tolerant than unwrapped to ammonium nitrate exposure. We are unaware of the existence of published literature concerning protective effects of wrapping behaviour against chemical exposure. Since the gelatinous envelope of the egg does not seem to prevent chemical diffusion of environmental nitrate or ammonium (Schuytema and Nebeker, 1999), the higher proportion of egg surface in direct contact with water would increase the ratio of absorption of these ions by unwrapped eggs, thus provoking a higher risk of suffering the toxic effects than if these eggs were totally protected from the chemical by a vegetable envelope. Nevertheless, since ammonium nitrate did not affect oviposition behaviour, we should be cautious when considering the real impact of the increased mortality reported for unwrapped eggs.

Chemical contamination of water bodies might be contributing to the decline of some amphibian species (Blaustein et al., 2003). Nevertheless, mortality rates shown by some species in experimental tests are not the only way that water pollution could contribute to the decline. Sub-lethal levels of several pollutants could affect vital behaviour, indirectly decreasing survival probabilities of the individuals and, thus, producing effects at a population level.

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