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Estimating mean lethal concentrations of three nitrogenous compounds for larvae of the Iberian waterfrog, *Pelophylax perezi* (Seoane, 1885)

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Abstract: The sensitivity of *Pelophylax perezi* larvae from a natural population located in the Segura River basin (southeastern Spain) to three nitrogenous compounds (NH_4Cl, $NaNO_2$ and $NaNO_3$) was analysed. Larval mortality was significantly increased by raising concentrations and exposure time to these compounds. LC_{50} values obtained for NO_2^- and NO_3^- ions are in all cases higher than the peak concentrations found in the environment but this is not the case for NH_4^+ ion, for which LC_{50} values obtained are lower than the concentrations found in the field. This may be a threat to populations of *P. perezi*, a species highly sensitive to NH_4^+ pollution, which could be exposed to lethal concentrations of the NH_4^+ ion and, therefore, be potentially suffering a decline as a consequence of eutrophication.
Key words: lethal concentration, nitrogenous compounds, *Pelophylax perezi*, tadpole.

Resumen: Estimación de las concentraciones letales medias de tres compuestos nitrogenados para larvas de la rana común, *Pelophylax perezi* (Seoane, 1885). – Se analizó la sensibilidad de larvas de *Pelophylax perezi* procedentes de una población natural localizada en la cuenca del río Segura (sureste de España) a tres compuestos nitrogenados (NH_4Cl, $NaNO_2$ y $NaNO_3$). La mortalidad larvaria aumentó con el incremento en las concentraciones y en el tiempo de exposición a estos compuestos. Los valores LC_{50} obtenidos para los iones NO_2^- y NO_3^- son en todos los casos superiores a las concentraciones máximas registradas en la naturaleza aunque éste no es el caso para el ión NH_4^+, para el que los valores LC_{50} obtenidos son inferiores a los detectados en el campo. Este hecho puede amenazar a las poblaciones de *P. perezi*, especie altamente sensible a la contaminación por NH_4^+, que podrían estar expuestas a concentraciones letales del ión NH_4^+ y sufrir potencialmente una regresión como consecuencia de la eutrofia.
Palabras clave: compuestos nitrogenados, concentración letal, larva, *Pelophylax perezi*.

INTRODUCTION

Habitat destruction and degradation have been described as one of the major threats currently faced by amphibians (STUART *et al.*, 2004). Such degradation may be a consequence of habitat fragmentation, the alteration and suppression of natural ecosystem processes, introduction of exotic species and the presence of pollutants (DODD & SMITH, 2003). Among the latter, fertilizers, which have been used intensively in last decades, are considered to have a potential impact on amphibian populations because they concentrate in waterbodies located within agricultural areas (BERGER, 1987, 1989; HAMER *et al.*, 2004; MASSAL *et al.*, 2007). Experimental approaches have revealed their

negative effect on amphibian survival and life history traits (e.g., ORTIZ *et al.*, 2004). However, the effects that pollution may have on amphibian species are not well known, and so it becomes a matter of conservation priority to determine the level of potential tolerance to fertilizers that amphibian species may withstand by conducting standardized toxicological experiments (MARCO & ORTIZ-SANTALIESTRA, in press).

LC_{50} (mean lethal concentration) assays are the most common tests used to determine the sensitivity of a species to a pollutant (BRIDGES & SEMLITSCH, 2001). These assays determine, by using different concentrations of a chemical, the concentration at which 50% of a test population dies. As a consequence, naturally occurring concentrations of such chemical in the environment that equal or exceed the LC_{50} value may lead to population extinction. Additionally, and although the concentrations necessary to induce direct mortality may be higher than actual concentrations in the environment (BOONE & BRIDGES, 2003), LC_{50} values can be used to establish sublethal levels of a pollutant and to study their effects (BRIDGES, 1999).

With regard to fertilizers, LC_{50} assays have been performed for different nitrogenous compounds in several species of amphibians (see review in MARCO & ORTIZ-SANTALIESTRA, in press). One of the major conclusions of these studies is the large inter- and intraspecific variation described in relation to the LC_{50} value of nitrite and nitrate (HECNAR, 1995; MARCO *et al.*, 1999; SHINN *et al.*, 2008). This highlights the importance of determining the actual sensitivity to nitrogenous compounds of a broad range of species to determine their potential impact on amphibian populations and communities.

The aim of this study was to analyze the sensitivity to nitrogenous pollutants of a larval population of *Pelophylax perezi* by determining the LC_{50} values for ammonium, nitrite and nitrate compounds. This species is an European waterfrog whose distribution ranges through the Iberian Peninsula and southern France (LLORENTE & ARANO, 1997). It mainly inhabits permanent waterbodies (DÍAZ-PANIAGUA, 1990), especially those showing high riparian vegetation cover (EGEA-SERRANO *et al.*, 2005). Because these permanent waterbodies may hold high concentrations of nitrogenous compounds as a result of farming practices and urban sewage (one of the main nitrogen sources in the environment) (e.g., RITTER & BERGSTROM, 2001), aquatic stages of *P. perezi* may potentially be threatened by nitrogen pollution, as suggested for nitrite by the results of MACÍAS *et al.* (2007). This hypothesis contradicts claims that this species is tolerant to pollution (LLORENTE *et al.*, 2002). So, a great effort needs to be made to accurately assess the actual sensitivity of larvae of *P. perezi* to nitrogenous compounds.

MATERIALS AND METHODS

We sampled five different egg masses obtained from a natural *P. perezi* population located in the Segura River basin (southeastern Spain, U.T.M. 30SWH, 1197.92 m a.s.l.), not exposed to eutrophication (e.g., < 2.1 mg NO_3^- / L, unpublished data). The samples belonging to the five clutches were pooled to increase the probability of analysing a representative sample of the genetic variation within the population. Embryos were reared in the laboratory in 12 L glass aquaria, at roughly 25° C, in aerated dechlorinated tap water. When embryos reached Gosner developmental stage 25 (GOSNER, 1960) (total length: 10.79 ± 0.18 mm, n = 230), they were transferred to clear, food-quality, 1 L plastic beakers containing 0.5 L of test solution. Ammonium, nitrite and

nitrate solutions were prepared from NH_4Cl, $NaNO_2$ and $NaNO_3$, respectively. Treatments consisted of the following nominal increasing concentrations: 0, 15, 30, 60 and 120 mg NH_4Cl / L; 0, 500, 1000, 5000, 10 000 and 20 000 mg $NaNO_2$ / L; 0, 2000, 5000, 10 000 and 20 000 mg $NaNO_3$ / L. Each beaker, containing six haphazardly chosen larvae, was randomly assigned to one of the previous treatments. Each treatment was replicated three times in the case of NH_4Cl and twice for $NaNO_2$ and $NaNO_3$ due to a shortage of tadpoles.

The experiment consisted of static renewal tests (STEPHEN, 1975). Water treatments were renewed daily and dead animals were removed from the experimental units to avoid oxygen depletion. Beakers were loosely closed to avoid water evaporation and ammonium volatilization.

TABLE 1. Cumulative number of dead *Pelophylax perezi* tadpoles recovered following 24, 48, 72 and 96 h of exposure. Summary statistics for repeated measures ANOVAs for the effects of the three nitrogenous compounds tested on larval mortality over time are shown.

TABLA 1. Número acumulado de larvas de *Pelophylax perezi* muertas a las 24, 48, 72 y 96 h de exposición. Se presentan los estadísticos correspondientes a los ANOVAs de medidas repetidas que analizaron los efectos de los tres compuestos nitrogenados sobre la mortalidad larvaria a lo largo del tiempo.

Nitrogenous compound	Concentration (mg/L)	n	Time 24-h	48-h	72-h	96-h
NH_4Cl						
Concentration: $F_{4,10} = 491.73$, $p < 0.001$						
Time $F_{3,30} = 34.10$, $p < 0.001$						
Concentration x Time: $F_{12,30} = 22.60$, $p < 0.001$						
	0	18	0	0	0	0
	15	18	0	0	0	0
	30	18	0	0	0	0
	60	18	0	0	2	12
	120	18	9	16	18	18
$NaNO_2$						
Concentration: $F_{5,6} = 58.57$, $p < 0.001$						
Time: $F_{2,12} = 85.02$, $p < 0.001$						
Concentration x Time: $F_{10,12} = 29.74$, $p < 0.001$						
	0	12	0	0	0	–
	500	12	0	0	2	–
	1000	12	1	1	3	–
	5000	12	10	10	12	–
	10 000	12	12	12	12	–
	20 000	12	12	12	12	–
$NaNO_3$						
Concentration: $F_{4,5} = 46.87$, $p < 0.001$						
Time: $F_{2,10} = 25.86$, $p < 0.001$						
Concentration x Time: $F_{8,10} = 9.76$, $p < 0.001$						
	0	12	0	0	0	–
	2000	12	0	1	1	–
	5000	12	0	1	11	–
	10 000	12	1	11	12	–
	20 000	12	12	12	12	–

Tadpoles were fed dried dog chow, of which a food pellet (250-350 mg) was added each day. Tadpoles were observed each 12 h over a 96 h period for mortality in the case of NH_4Cl treatments. As regards $NaNO_2$ and $NaNO_3$, the observation period was limited to 72 h.

To assess the effect of the concentrations used in this study (independent variables) on larval mortality (dependent variable: number of dead tadpoles in each beaker), repeated measures ANOVAs were performed separately for each nitrogenous compound. Probit analysis was used to determine LC_{50} for NH_4^+, NO_2^- and NO_3^- ions during 24, 48, 72 and 96 h exposure. Data were transformed logarithmically. Statistical analyses were performed using SPSS® statistical package v. 11.0 and a significance level of 5% was selected.

RESULTS

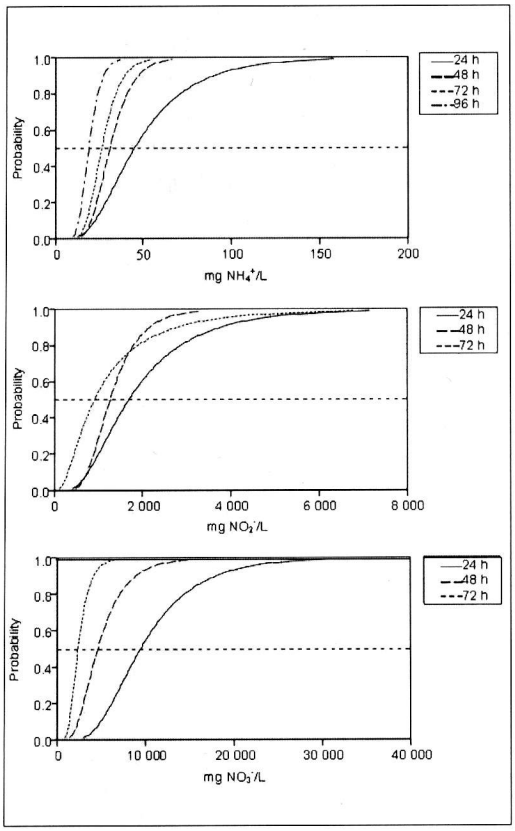
Table 1 shows the number of tadpoles that died as a consequence of exposure to the different treatments. No mortality was recorded for the control treatment. For all three nitrogenous compounds, both increasing concentrations and exposure times increased larval mortality ($p < 0.001$). Nevertheless, for NH_4Cl no larval mortality was recorded for concentrations lower than 60 mg NH_4Cl / L. The significant concentration x time interactions (Table 1) revealed that the effects produced by the concentrations used for the three nitrogenous compounds differed between observation times. So, the highest concentrations produced the highest larval mortality earlier than the remaining treatments.

The results of the probit analyses are shown in Table 2. LC_{50} values decreased at each observation period for all the nitrogenous compounds. Figure 1 shows dose-response curves for larvae of *P. perezi* exposed to NH_4^+, NO_2^- and NO_3^- ions. In the case of NH_4^+ and NO_3^-, the concentration needed to kill 100% of the larvae decreased with time. However, in the case of NO_2^-, the concentration needed to kill 100% after 48 h of exposure was lower than that observed after 24 and 72 h.

TABLE 2. Results of the probit analysis for ions NH_4^+, NO_2^- and NO_3^- following 24, 48, 72 and 96 h of exposure. SE: standard error. LC_{50}: mean lethal concentration. CI: confidence interval.
TABLA 2. Resultados de los análisis probit para los iones NH_4^+, NO_2^- y NO_3^- a las 24, 48, 72 y 96 h de exposición. SE: error estándar. LC_{50}: concentración letal media. CI: intervalo de confianza.

Nitrogenous compound	Time (h)	Probability of mortality (PM)	SE on slope	LC_{50}	
				LC_{50} (mg/L)	95% CI
NH_4^+	24	PM = -7.10 + 4.23 $\log_{10}([NH_4^+]+1)$	1.51	45.39	34.96-106.12
	48	PM = -10.56 + 7.06 $\log_{10}([NH_4^+]+1)$	1.78	31.37	25.79-38.24
	72	PM = -10.47 + 7.34 $\log_{10}([NH_4^+]+1)$	1.80	26.69	22.24-32.51
	96	PM = -10.31 + 8.02 $\log_{10}([NH_4^+]+1)$	2.15	19.27	16.03-22.90
NO_2^-	24	PM = -12.03 + 3.72 $\log_{10}([NO_2^-]+1)$	0.80	1697.60	1077.15-2552.54
	48	PM = -17.25 + 5.56 $\log_{10}([NO_2^-]+1)$	1.78	1270.90	848.90-3038.71
	72	PM = -7.92 + 2.67 $\log_{10}([NO_2^-]+1)$	0.62	914.59	579.60-1526.53
	96	–	–	–	–
NO_3^-	24	PM = -18.23 + 4.59 $\log_{10}([NO_3^-]+1)$	1.38	9440.12	6967.97-13959.02
	48	PM = -16.41 + 4.48 $\log_{10}([NO_3^-]+1)$	1.04	4611.02	2299.23-9404.95
	72	PM = -17.98 + 5.33 $\log_{10}([NO_3^-]+1)$	1.46	2381.53	1697.45-3230.75
	96	–	–	–	–

FIGURE 1. Dose-response curves for mortality of *Pelophylax perezi* larvae exposed to NH_4^+, NO_2^- and NO_3^-. The LC_{50} value for each time interval is graphically represented by the dotted line in each plot.
FIGURA 1. Curvas dosis-respuesta para la mortalidad de larvas de *Pelophylax perezi* expuestas a NH_4^+, NO_2^- y NO_3^-. El valor LC_{50} para cada intervalo de tiempo está representado en cada gráfico por la línea punteada.



DISCUSSION

The results obtained indicate that *Pelophylax perezi* tadpoles were negatively affected by exposure to nitrogenous compounds. Nevertheless, the LC_{50} values obtained suggest some degree of tolerance to the nitrogenous compounds tested. The LC_{50} values obtained for NO_2^- and NO_3^- ions are in all cases higher than the peak concentrations naturally occurring in localities in the Segura River basin where breeding populations of the studied species have been detected (e.g., 74.35 mg NO_2^- / L and 332.74 mg NO_3^- / L) (M.L. Suárez, personal communication). This suggests that the present results are not suitable for determining the effect of exposure to these nitrogenous compounds on *P. perezi* in natural settings. Thus, examining the possible sublethal effects of NO_2^- and NO_3^- ions on the studied species would be more ecologically relevant, since sublethal levels of fertilizers have been shown to affect parameters such as time to hatching (DE WIJER *et al.*, 2003; MEREDITH & WHITEMAN, 2008; SHINN *et al.*, 2008), rates of morphological abnormalities (KRISHNAMURTHY *et al.*, 2008; SHINN *et al.*, 2008), activity levels (HECNAR, 1995; XU & OLDHAM, 1997; SHINN *et al.*, 2008), feeding (BAKER & WAIGHTS, 1994; HECNAR, 1995; XU & OLDHAM, 1997), and habitat use (HUEY & BEITINGER, 1980; MARCO & BLAUSTEIN, 1999), which may indirectly affect larval size or lead to larval mortality (DE WIJER *et al.*, 2003; SHINN *et al.*, 2008; MEREDITH & WHITEMAN, 2008). Nevertheless, as regards the NH_4^+ ion, the LC_{50} values obtained for each observation period corresponded to lower concentrations than those found in the field in the study area (e.g., 154.6 mg NH_4^+/L) (M.L. Suárez, personal communication), which suggests that *P. perezi* populations may be naturally exposed to lethal concentrations of the NH_4^+ ion and, therefore, be potentially suffering a decline as a consequence of eutrophication.

Our LC_{50} estimates confirm the prediction that the risk of dying from a pollutant increases both as the concentration of the toxicant raise (WATT & JARVIS, 1997; SHINN *et al.*, 2008) and with longer exposure times as it has been previously described for different amphibian species (MARCO *et al.*, 1999; SCHUYTEMA & NEBEKER, 1999a, b; SPARLING & HARVEY, 2006; SHINN *et al.*, 2008). This

fact evidences that mortality was not restricted to initial exposure to the different chemicals, but was due to a chronic effect of continuous exposure.

Significant interspecific variation concerning amphibian larvae tolerance to fertilizers has been described (e.g., HECNAR, 1995; MARCO *et al.*, 1999). However, most experiments differed greatly in the tested conditions (Table 3) that largely affect tadpole responses. For instance, larval sensitivity to nitrogenous pollutants varies greatly with exposure time (MARCO *et al.*, 1999; SCHUYTEMA & NEBEKER, 1999a, b; SPARLING & HARVEY, 2006), developmental stage (ORTIZ-SANTALIESTRA *et al.*, 2006), chemical compound employed (SCHUYTEMA & NEBEKER, 1999a, b) and experimental venue (A. Egea-Serrano, M. Tejedo & M. Torralva, unpublished data). Therefore, any reliable comparison between species has to take account of such differences in experimental settings. Despite these drawbacks our results suggest that *P. perezi* is less tolerant to NO_3^- ion than *Xenopus laevis* and *Pseudacris regilla*, but more resistant than other ranids such as *Rana clamitans* or *R. pipiens*. Interestingly, *P. perezi* is generally

TABLE 3. LC_{50} values of different chemical forms of ammonium, nitrite and nitrate reported in the literature for larval amphibians. References (in brackets), 1: MARCO *et al.* (1999); 2: HECNAR (1995); 3: XU & OLDHAM (1997); 4: SCHUYTEMA & NEBEKER (1999a); 5: SPARLING & HARVEY (2006); 6: SHINN *et al.* (2008); 7: Present data. *Developmental stages *sensu* GOSNER (1960).

TABLA 3. Valores LC_{50} de diferentes formas químicas de amonio, nitrito y nitrato presentados en la bibliografía para larvas de anfibios. Referencias (entre paréntesis), 1: MARCO *et al.* (1999); 2: HECNAR (1995); 3: XU & OLDHAM (1997); 4: SCHUYTEMA & NEBEKER (1999a); 5: SPARLING & HARVEY (2006); 6: SHINN *et al.* (2008); 7: Este trabajo. * Estadios de desarrollo *sensu* GOSNER (1960).

Species	Gosner stage*/Age	Days of exposure	Nitrogen source	LC_{50} (Reference)
Ambystoma gracile	Newly hatched	4	$NaNO_2$	6.24 mg NO_2^-/L (1)
	Newly hatched	7	$NaNO_2$	5.06 mg NO_2^-/L (1)
	Newly hatched	15	$NaNO_2$	3.32 mg NO_2^-/L (1)
	Newly hatched	15	KNO_3	103.6 mg NO_3^-/L (1)
Bufo americanus	25	4	NH_4NO_3	60.2-174 mg NO_3^-/L (2)
Bufo boreas	Newly hatched	4	$NaNO_2$	> 23.0 mg NO_2^-/L (1)
	Newly hatched	7	$NaNO_2$	17.7 mg NO_2^-/L (1)
	Newly hatched	15	$NaNO_2$	5.75 mg NO_2^-/L (1)
Bufo bufo	32-35	4	NH_4NO_3	1704 mg NO_3^-/L (3)
	32-35	7	NH_4NO_3	1837 mg NO_3^-/L (3)
Bufo calamita	25	15	$NaNO_2$	> 24.64 mg NO_2^-/L (6)
Hyla meridionalis	25	5	$NaNO_2$	383.59 mg NO_2^-/L (6)
	25	7	$NaNO_2$	143.20 mg NO_2^-/L (6)
	25	10	$NaNO_2$	65.7 < LC50 < 104.0 mg NO_2^-/L (6)
	25	15	$NaNO_2$	> 49.29 mg NO_2^-/L (6)
Hyla regilla	Newly hatched	4	$NaNO_2$	18.07 mg NO_2^-/L (1)
	Newly hatched	7	$NaNO_2$	11.8 mg NO_2^-/L (1)
	Newly hatched	15	$NaNO_2$	4.04 mg NO_2^-/L (1)
Pelophylax perezi	14-18	15	$NaNO_2$	16.4 < LC50 < 49.3 mg NO_2^-/L (6)
	25	6	$NaNO_2$	419.24 mg NO_2^-/L (6)
	25	7	$NaNO_2$	151.16 mg NO_2^-/L (6)
	25	10	$NaNO_2$	< 16.43 mg NO_2^-/L (6)
	18-19	10	$NaNO_2$	48.0 mg NO_2^-/L (6)

.../...

TABLE 3. (cont.)

Species	Gosner Stage*/Age	Days of exposure	Nitrogen source	LC_{50} (Reference)
Pelophylax perezi	18-19	12	$NaNO_2$	7.15 mg NO_2^-/L (6)
	18-19	16	$NaNO_2$	< 1.64 mg NO_2^-/L (6)
	25	4	NH_4Cl	19.27 mg NH_4^+/L (7)
	25	3	$NaNO_2$	914.59 mg NO_2^-/L (7)
	25	3	$NaNO_3$	2381.53 mg NO_3^-/L (7)
Pseudacris regilla	26-27	4	NH_4SO_4	148.24 mg NH_4^+/L (4)
	26-27	10	NH_4SO_4	115.33 mg NH_4^+/L (4)
	26-27	4	NH_4NO_3	599.6 mg NO_4^+/L (4)
	26-27	10	NH_4NO_3	244.5 mg NO_3^-/L (4)
	26-27	4	$NaNO_3$	7749.1 mg NO_3^-/L (4)
	26-27	10	NaNO3	1178.9 mg NO_3^-/L (4)
Pseudacris triseriata	25	4	NH_4NO_3	75.3 mg NO_3^-/L (2)
Rana aurora	Newly hatched	4	$NaNO_2$	18.37 mg NO_2^-/L (1)
	Newly hatched	7	$NaNO_2$	13.14 mg NO_2^-/L (1)
	Newly hatched	15	$NaNO_2$	3.91 mg NO_2^-/L (1)
Rana clamitans	25	4	NH_4NO_3	143.5 mg NO_3^-/L (2)
Rana pipiens	25	4	NH_4NO_3	100.1 mg NO_3^-/L (2)
	25	4	NH_4HCO_3	37.1 mg NH_4^+/L (5)
	25	7	NH_4HCO_3	15.6 mg NH_4^+/L (5)
	25	4	NH_4ClO_4	57.9 mg NH_4^+/L (5)
	25	7	NH_4ClO_4	29.9 mg NH_4^+/L (5)
Rana pretiosa	Newly hatched	4	$NaNO_2$	22.4 mg NO_2^-/L (1)
	Newly hatched	7	$NaNO_2$	4.27 mg NO_2^-/L (1)
	Newly hatched	15	$NaNO_2$	1.87 mg NO_2^-/L (1)
	Newly hatched	15	KNO_3	72.85 mg NO_3^-/L (1)
Xenopus laevis	26-27	4	NH_4SO_4	173.57 mg NH_4^+/L (4)
	26-27	4	NH_4Cl	163.93 mg NH_4^+/L (4)
	26-27	10	NH_4SO_4	58.5 mg NH_4^+/L (4)
	26-27	10	NH_4Cl	82.19 mg NH_4^+/L (4)
	26-27	4	NH_4NO_3	446 mg NO_3^-/L (4)
	26-27	10	NH_4NO_3	243,3 mg NO_3^-/L (4)
	26-27	4	$NaNO_3$	7332.8 mg NO_3^-/L (4)
	26-27	10	$NaNO_3$	5474.6 mg NO_3^-/L (4)

less tolerant to NH_4^+, but exhibits high resistance to increased concentrations of NO_2^- (Table 3). Inter- and intraspecific physiological studies concerning the effectiveness of detoxification pathways for nitrogenous ions are needed to explain this disparity.

Finally, the results presented in this study must be considered preliminary for establishing the effects of nitrogenous compounds on *P. perezi* natural populations. Previous studies show the existence of both intraspecific differences (SHINN *et al.*, 2008), and even local genetic adaptation (JOHANSSON *et al.*, 2001; EGEA-SERRANO *et al.*, 2009) to different chemical water stressors. In addition, ontogenetic differences in sensitivity to fertilizers have been described in amphibians (ORTIZ-SANTALIESTRA *et al.*, 2006) and the lethal effects of the exposure to nitrogenous compounds detected in laboratory experiments may be significantly higher than in more natural conditions (A.

Egea-Serrano, M. Tejedo & M. Torralva, unpublished data). Furthermore, pollutant exposure could produce sublethal effects on amphibian larvae (BAKER & WAIGTHS, 1994; HATCH & BLAUSTEIN, 2000; JOHANSSON *et al.*, 2001; DE WIJER *et al.*, 2003; ORTIZ *et al.*, 2004; KRISHNAMURTHY *et al.*, 2008; MEREDITH & WHITEMAN, 2008; SHINN *et al.*, 2008; EGEA-SERRANO *et al.*, 2009) that could have important implications on population viability (SMITH, 1987; SEMLITSCH *et al.*, 1988). These aspects point to the importance of conducting further studies to establish the actual sensitivity of different developmental stages and populations of *P. perezi* to nitrogen compounds.

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