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Intraspecific and Interspecific Tolerance to Copper Sulphate in Five Iberian Amphibian Species at Two Developmental Stages

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Abstract Intraspecific and interspecific variations have been observed across many taxa with respect to resistance to natural environmental stressors. It has already been well documented that amphibians are sensitive to habitat degradation and are regarded as bioindicators of aquatic and agricultural ecosystems. In southern Spain, different toxic substances, including copper sulphate, which is used to control *Cycloconium oleaginum* (Fungi, Ascomycota, Venturiaceae) during spring and autumn, are used in intensive olive tree agriculture. In this context, many wetlands are affected by a diffuse pollution process. Thus, toxicological studies using different species living in wetlands surrounded by agricultural activity are needed to understand the alterations suffered by these ecosystems. To achieve this understanding, individuals of five amphibian species (*Bufo bufo*, *Epidalea calamita*, *Discoglossus jeanneae*, *Pelobates cultripes*, and *Pelophylax perezi*) at Gosner developmental stages 19 and 25 were exposed to different copper sulphate concentrations in 96 h acute toxicity tests. Exposure to copper sulphate had a negative effect on total larval length reached at the end of the experimental period and generated approximately 30% of growth reduction respect to control treatments. *P. perezi* was the most tolerant species studied and showed no mortality at the maximum concentration tested (0.20 mg Cu L^{-1}), whereas the most sensitive species (*B. bufo*, *E. calamita*, and *D. jeanneae*) showed approximately 90% mortality at the same concentration. These results indicates that the sole presence in wetlands of *P. perezi*, the most abundant species in southeast of Iberian Peninsula, might be correlated with its high tolerance to agrochemicals.

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Environmental contamination in general, resulting from escalating human numbers and activities combined with inappropriate environmental management, has increased, thus creating growing pressures on the Earth's resources and life-support systems (Herkovits et al. 2002). Chemical contaminants are not homogeneous stressors because their presence in the environment varies across temporal and spatial scales. Variation in different organisms' responses to a contaminant substance can thus arise from differential exposure resulting from variability in the presence of the contaminant (Bridges and Semlitsch 2000). Intraspecific and interspecific variation has been observed across many taxa with respect to resistance to natural environmental stressors (Arad et al. 1993). These variations can help us understand how contaminants affect the ecosystem. It has already been well documented that amphibians are sensitive to habitat degradation and are regarded as bioindicators of aquatic and agricultural ecosystems (Schuytema and Nebeker 1999; Pollet and Bendell-Young 2000; Marco et al. 2001; Tejedo 2003). Thus, they have been used as typical test animals in evaluating the effects of chemicals on aquatic and agricultural ecosystems (Cooke 1973, 1977; Sundaram 1995). Some amphibians that breed opportunistically in aquatic habitats embedded in agricultural landscapes are sensitive to pollutants not only during the terrestrial adult phase but also during the aquatic embryonic and larval stages (Greulich and Pflugmacher 2003). Their eggs and tadpoles may be exposed to environmental contaminants at some stage during development (Bridges and Boone 2003).

Moreover, amphibians absorb many toxic substances through the epithelium. In some species, eggs larvae and newly metamorphosed amphibians are more sensitive to poor water quality than later age classes (Vitt et al. 1990), thus showing intraspecific tolerance to toxic substances.

In southern Spain, different toxic substances, including copper sulphate, which is used to control *Cycloconium oleaginum* (Fungi, Ascomycota, Venturiaceae) during spring and autumn, are used in intensive olive tree agriculture (Junta de Andalucía 2008). This heavy metal has two oxidation states: $Cu^{1+}$and Cu^{2+}. The Cu^{2+} ion is the most environmentally relevant form to aquatic systems and is generally considered the most toxic form to aquatic life (Lenwood et al. 1998). Exposure to high copper concentrations can affect populations and individuals at morphological, physiological, biochemical, or genetic levels (Troncoso et al. 2000). Thus, toxicological studies using different species living in wetlands surrounded by agricultural activities are needed to understand the alterations suffered by these ecosystems.

Recent studies have shown that eggs and larvae of the Natterjack toad (*Epidalea calamita*) are sensitive to copper (García-Muñoz et al. 2009). Previous works carried out in southern Spain demonstrated that wetlands surrounded by olive groves have lower amphibian richness than those that do not undergo agricultural activity on their drainage basin (García-Muñoz et al. 2010). Copper sulphate applications in olive tree agriculture occur mainly during spring and autumn; thus, the first spring rains increase copper by way of drainage into bodies of water used for amphibian reproduction. Moreover, copper concentrations of approximately 0.04 mg Cu L^{-1} were detected in some wetlands in this area (García-Muñoz et al. 2009). In this sense, it is interesting to note that the field application rate used in olive cultivation is in the range of 1875 to 3750 mg Cu L^{-1} (De Liñán 1997).

Taking into account all previous comments, it is possible to hypothesize that the most common species in highly altered wetlands (García-Muñoz et al. 2010), *Pelophylax perezi*, might be correlated with its high tolerance to agrochemicals. Although laboratory conditions do not show real ecosystem conditions because copper sulphate toxicity in ecosystems is difficult to demonstrate, experimental tests can contribute to its understanding. Thus, to assess the degree of variation in response to agrochemicals, five Iberian amphibian species at two different developmental stages were used in experiments with copper sulphate, a fungicide used in olive tree culture, to detect different tolerance responses among and within different species of amphibians.

Materials and Methods

Study Species

Five anuran species were used in the present study: *Bufo bufo* (Bb), *E. calamita* (Ec), *Discoglossus jeanneae* (Dj), *Pelobates cultripes* (Pc), and *P. perezi* (Pp). The amphibians were collected from different wetlands (Universal Transverse Mercator (UTM) for: *B. bufo*: 30S 436040 and 4169244; *E. calamita*, *D. jeanneae*, and *P. cultripes*: 30S 448100 and 4220986; and *P. perezi*: 30S 426744 and 4175154) situated in the Alto Guadalquivir region (southern Spain), which does not have a known history of pollution. Amphibian populations were not affected by the collected samples. At least six different clutches (50 eggs/clutches; early developmental stage) were collected from each species (Gosner stages 10 to 12 [Gosner 1960]).

Experimental Procedure

Test Organisms

Egg masses were brought into the laboratory immediately after collection and placed (100 eggs from the same species/aquarium) in different 20-L aquariums that were filled with water from a spring with no known pollution history (pH 7.2 to 7.8; alkalinity 170 to 250 mg L^{-1}). The eggs were kept at 20°C (±0.5°C) under a 12:12-h light-to-dark cycle in a temperature-controlled chamber. The eggs and resulting larvae were allowed to develop to Gosner stages 19 and 25, before each acute toxicity tests. We selected these Gosner stages for different reasons. Gosner stage 19 was selected because (1) the heart of the new hatchling starts to beat and (2) the protective structure of eggs disappears. Gosner stage 25 was selected because (1) tadpoles acquire free-swimming abilities; (2) tadpoles begin eating; and (3) larvae remain for a longer time in this stage (i.e., Gosner stage 25) while their weight increases (García-Muñoz et al. 2009).

Acute Toxicity Tests

Static toxicity experiments were conducted for all species in 1-L vessels using animals at two developmental stages (Gosner stages 19 and 25). Copper sulphate ($CuSO_4{\cdot}5H_2O$; Sigma-Aldrich Química, S.A., Madrid, España) was used to prepare concentrations for use in acute-toxicity tests. To ensure accuracy of the dose, copper concentrations in each experimental vessel were analyzed with a photometer (Filterphotometer PF11; Macherey-Nagel) according a colorimetric technique (detection limit 0.04 mg Cu^{2+} L^{-1}). Copper was not detected in the manantial water used to prepare the different test concentrations. Ten individuals were placed in each vessel (1000 mL, 15 cm Ø). Six

replicates for each concentration, including control treatments (i.e., without copper), were maintained in a temperature-controlled chamber at 20°C under a 12:12-h light-to-dark cycle. No statistical differences were detected among copper-concentration replicates.

Two different sets of toxicity tests were conducted. The aim of the first was to determine the LC_{50} for each species at each developmental stage. The second one was developed to evaluate intraspecific and interspecific tolerance responses. The copper concentrations used were different in each set of experiments.

LC_{50} Experiments

LC_{50} values in each species at each developmental stage were determined using the following nominal copper-concentration ranges:

- *B. bufo*: Gosner stages 19 (Bb19) 0.05 to 0.20 mg Cu L^{-1} and 25 (Bb25) 0.07 to 0.20 mg Cu L^{-1}
- *E. calamita*: Gosner stages 19 (Ec19) 0.05 to 0.20 mg Cu L^{-1} and 25 (Ec25) 0.07 to 0.20 mg Cu L^{-1}
- *D. jeanneae*: Gosner stages 19 (Dj19) 0.04 to 0.20 mg Cu L^{-1} and 25 (Dj25) 0.06 to 0.20 mg Cu L^{-1}
- *P. cultripes*: Gosner stages 19 (Pc19) 0.10 to 0.40 mg Cu L^{-1} and 25 (Pc25) 0.10 to 0.50 mg Cu L^{-1}
- *P. perezi*: Gosner stages 19 (Pp19) 0.30 to 0.45 mg Cu L^{-1} and 25 (Pp25) 0.50 to 1.00 mg Cu L^{-1}.

Tolerance-Response Experiments

Three different nominal copper concentrations (0.08, 0.10, and 0.20 mg Cu L^{-1}) were selected to analyzed intraspecific and interspecific tolerance in all species at both developmental stages. In both sets of experiments, individuals were visually examined once every 24 h. Larval mortality was monitored, and dead larvae were removed every 24 h. Water temperature (20°C ±0.5°C) and pH (7.20 and 7.80) were checked daily. No statistical differences among treatments within the same experiment were detected in pH or water temperature. The experiments were concluded after 96 h of exposure. At the end of the experimental period, larval stage and total length were recorded for each surviving individual.

Developmental Stage and Growth

At the end of the experimental period (i.e., 96 h), individuals were placed in Petri dishes filled with water (1 cm deep). Then they were photographed individually against a grid paper using a CANON S40 camera. Images were downloaded into a computer and magnified on the computer screen. Total length (from snout to tail tip) was measured for all individuals using the ruler function (accurate to 0.01 mm) of the Image-J program (http://rsbweb.nih.gov/ij/).

Statistical Analysis

LC_{50} values were estimated using linear functions relating log-transformed copper concentration to probit-transformed mortality (Abel and Axiak 1991). The LC_{10} and LC_{90} values were also calculated using the same functions.

In tolerance-response experiments, interspecific comparisons were performed using a general linear model (GLM), with cumulative mortality rates (arcsin of square root transformed) as the dependent variable and copper concentration, developmental stage, species, and exposure time (24, 48, 72, and 96 h) as the categoric variables. These were used to determine the influence of copper sulphate concentration on Gosner developmental stage, species, and time.

For intraspecific comparison, different GLMs for all species were performed, with cumulative mortality rates (arcsin of square root transformed) as the dependent variable and copper concentration, developmental stage, and exposure time as the categoric variables. A posthoc Tukey test for pairwise comparisons was used to detect significant differences.

Because different species at different stages showed different growth rates, multiple regression was used for all species, with larval length (log transformed) as the dependent variable and copper exposure as the categoric variable, to determine the effects of copper sulphate on larval size in both stages. The regression slope gives a value that can be used to compare the different effects that copper sulphate exposure generates in different species at different stages. Stage differences among concentrations were analyzed using nonparametric χ^2 test.

Kolmogorov-Smirnoff Z-tests were conducted before general linear modelling to confirm normality of the transformed mortality rates. In all cases, Z-tests resulted in $p > 0.05$. Statistica 7 software for Windows (StatSoft, Inc.; http://www.statsofl.com) was used for statistical analysis.

Results

Acute-Toxicity Tests

No mortality occurred in any controls during the experimental period in both experiments.

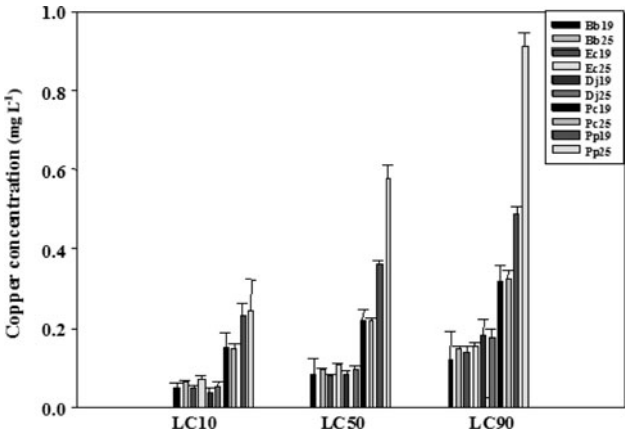

Fig. 1 LC_{10}, LC_{50}, and LC_{90} values obtained at both developmental stages (Gosner stages 19 and 25) in Bb, Ec, Dj, Pc, and Pp tadpoles. Copper concentrations are expressed in mg Cu L^{-1}

LC_{50} Experiments

LC_{10}, LC_{50}, and LC_{90} values obtained at both developmental stages and in all species are shown in Fig. 1. Total mortality resulted in *B. bufo, E. calamita*, and *D. jeanneae* at both Gosner stages using concentrations >0.20 mg Cu L^{-1}. In *P. cultripes,* total mortality resulted using concentrations >0.40 mg Cu L^{-1}, but *P. perezi* showed total mortality at concentrations >0.50 mg Cu L^{-1} for Gosner stage 19 and at 1.00 mg Cu L^{-1} for Gosner stage 25.

Tolerance-Response Experiments

Mortality rates after 24, 48, 72, and 96 h of copper exposure at both Gosner stages and at all copper concentrations are shown in Figs. 2, 3, 4 and 5 for *B. bufo*, *E. calamita, D. jeanneae*, and *P. cultripes*, respectively. Table 1 lists the results of interspecific general linear modelling. The results showed significant different response (mortality) between species. No mortality occurred in any vessels with *P. perezi* at both developmental Gosner stages. The most sensitive species was *D. jeanneae* at both Gosner stages. In the same group was *E. calamita*, at both Gosner stages, and *B. bufo* at Gosner stage 25. *B. bufo* at Gosner stage 19 was relatively less sensitive than those at Gosner stage 25. In contrast, *P. cultripes* at both Gosner stages showed an intermediate sensitivity to copper sulphate exposure, whereas *P. perezi* at both developmental Gosner stages showed the highest tolerance to copper exposure. The results of copper-exposure experiments permitted us to group species and stages according to different sensitivities. It was possible to distinguish three groups: (1) the most sensitive species were *B. bufo*, *E. calamita*, and *D. jeanneae* at both Gosner stages; (2) the intermediately sensitive species was *P. cultripes* at both Gosner stages; and (3) the least sensitive species was *P. perezi* at both Gosner stages.

Table 2 lists statistical differences in tolerance between *B. bufo*, *E. calamita*, *D. jeanneae*, and *P. cultripes* at both Gosner stages. Intraspecific analysis also confirmed *P. perezi* to be the most tolerant species because it showed no mortality at any concentration tested. No statistical differences were found in tolerance *between E. calamita* and *P. perezi* at both Gosner stages. In species comprising the most sensitive group, mortality was slightly higher at Gosner stage 25 than at stage 19 during the first 48 h (Figs. 2, 3, 4, and 5). However, in the last 72 to 96 h of exposure, all species at Gosner stage 19 were slightly more sensitive at the highest (0.10 and 0.20 mg Cu L^{-1}) than at the lowest copper concentration (0.08 mg Cu L^{-1}).

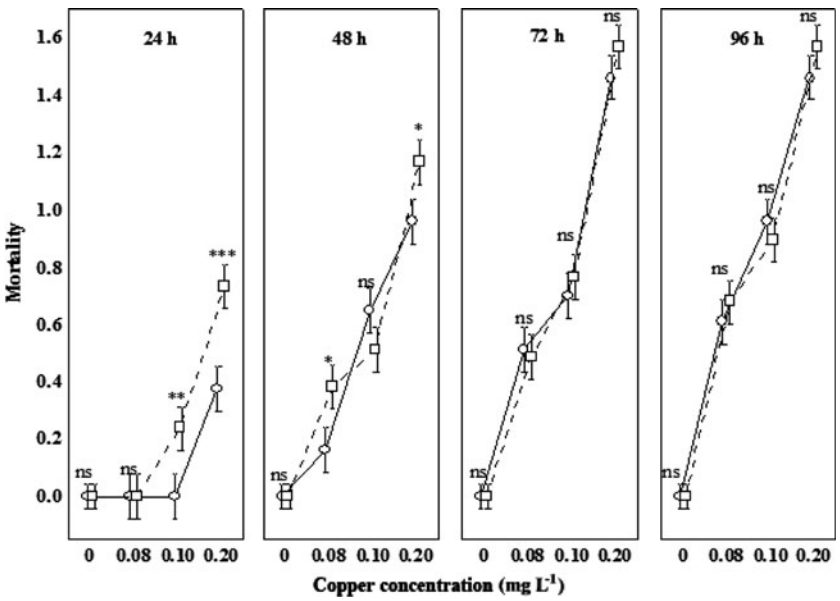

Fig. 2 Bb mortality (arcsin square root transform), at 24, 48, 72, and 96 h, including both Gosner stages (*circle* = stage 19; *square* = stage 25), at different copper sulphate concentrations (0, 0.08, 0.10, and 0.20 mg Cu L^{-1}; vertical bars denote CI 95%). Results of Tukey test to compare mortality among stages are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant

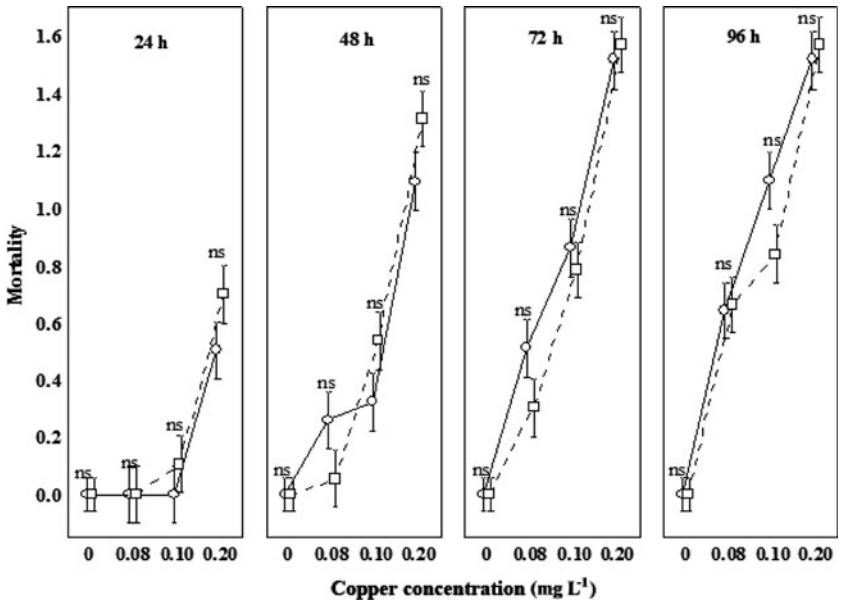

Fig. 3 Ec mortality (arcsin square root transform) at 24, 48, 72, and 96 h, including both Gosner stages (*circle* = stage 19; *square* = stage 25), at different copper sulphate concentrations (0, 0.08, 0.10, and 0.20 mg Cu L^{-1}; vertical bars denote CI 95%). Results of Tukey test to compare mortality among stages are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant

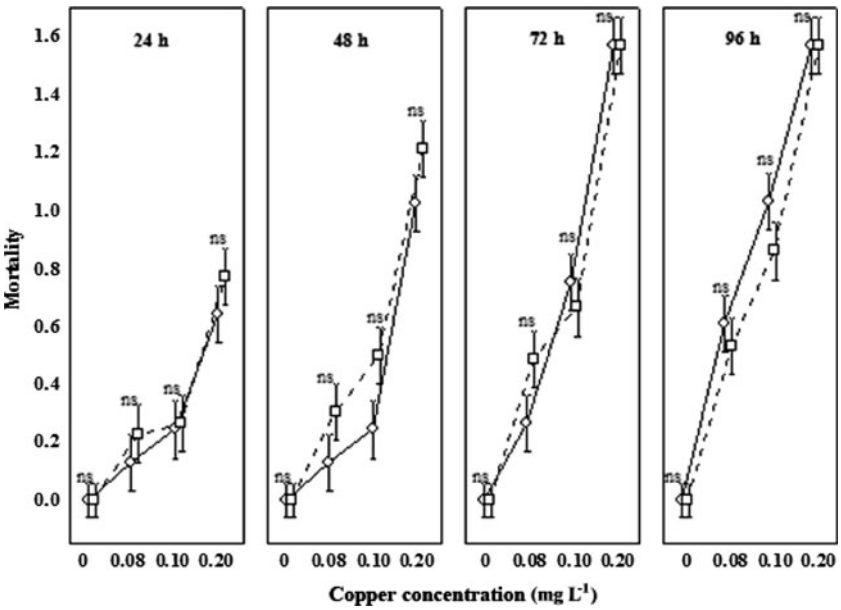

Fig. 4 Dj mortality (arcsin square root transform) at 24, 48, 72, and 96 h, including both Gosner stages (19, *circle*; and 25, *square*), at different copper sulphate concentrations (0, 0.08, 0.10, and 0.20 mg Cu L^{-1}; vertical bars denote CI 95%). Results of Tukey test to compare mortality among stages are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant

Development Stages and Growth

All larvae from control treatments reached Gosner stage 25 at the end of the experiments initiated at Gosner stage 19, whereas the maximum copper concentration tested (0.2 mg L^{-1}) completely interrupted development in *B. bufo* and *E. calamita*. In the experiments initiated at Gosner stage 19, significant differences were found in the stage reached by larvae in controls compared with other treatments in four of the five species, with being *D. jeanneae* the exception (Bb19 [χ^2 0.000, *df* 15], Ec19 [χ^2 0.008, *df* 21], Dj19 [χ^2 0.604, *df* 4], Pc19 [χ^2 0.000, *df* 9], and Pp19 [χ^2 0.001, *df* 12]). In the experiments initiated at Gosner stage 25, no statistical differences were found in the stage reached at the end of the experiment in larvae of any species.

Copper sulphate exposure also resulted in decreased larval growth in all species at both developmental stages (Table 3; Figs. 6 and 7 for Gosner stages 19 and 25, respectively). Control treatments showed the highest mean larval sizes at the end of all experiments. Experiments initiated at Gosner stage 19 showed greater growth reduction than those initiated at stage 25 in all species. *B. bufo* and *E. calamita* in both stages, and *D. jeanneae* in stage 25, showed the highest slope gradients, which means that exposure impacts in more acute in the growth of these species.

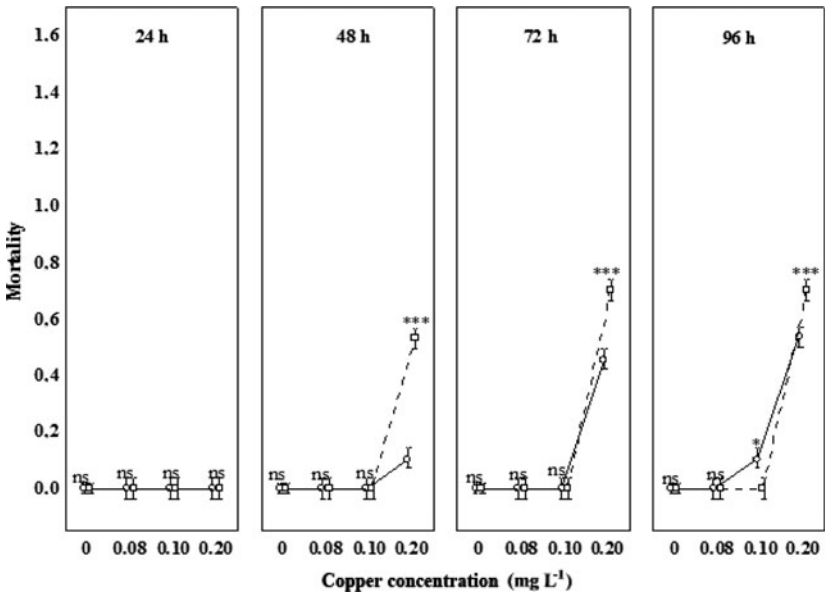

Fig. 5 Pp mortality (arcsin square root transform) at 24, 48, 72, and 96 h, including both Gosner stages (*circle* = stage 19; *square* = 25), at different copper sulphate concentration (0, 0.08, 0.10, and 0.20 mg Cu L^{-1}; vertical bars denote CI 95%). Results of Tukey test to compare mortality among stages are shown. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; ns = not significant

Table 1 Results of interspecific GLM[a]

Source of variation	*df*	MS	*F*	*p*
Concentration	3	39.872	4775.773	0.000
Species	4	16.102	1928.627	0.000
Stage	1	0.337	40.348	0.000
Time	3	8.115	971.944	0.000
Concentration * species	12	4.356	521.766	0.000
Concentration * stage	3	0.241	28.917	0.000
Species * stage	4	0.052	6.283	0.000
Concentration * time	9	1.885	225.808	0.000
Species * time	12	0.912	109.227	0.000
Stage * time	3	0.123	14.676	0.000
Concentration * species * stage	12	0.045	5.381	0.000
Concentration * species * time	36	0.220	26.404	0.000
Concentration * stage * time	9	0.041	4.900	0.000
Species * stage * time	12	0.034	4.093	0.000
Concentration * species * stage * time	36	0.031	3.749	0.000
Error	1272	0.008		

[a] With mortality (arcsin square root transform) as the dependent variable and copper concentration, species, stage, and time as the categoric variables

Discussion

Copper is present in many aquatic ecosystems of Europe and the Iberian Peninsula. Its widespread use has generated much research on its effect in aquatic systems (Real et al. 2003). Data on mean annual copper concentration in Spain ranged from 0.0013 to 0.0175 mg L^{-1} (Junta de Sanejament 1998), with values $\leq$0.1 mg L^{-1} occasionally detected in extremely polluted streams (Armengol et al. 1993). In southern Spain, copper concentrations measured in the Guadalquivir basin ranged from 0.001 to 0.007 mg L^{-1} (Mendiguchía 2005). In the upper zone of the Guadalquivir basin, where olive cultivation represents 78% of the total agriculture area, some wetlands surrounded by this human activity showed copper concentrations of approximately 0.04 mg L^{-1} (García-Muñoz et al. 2009). The concentrations used in this study gave us an accurate picture of the possible effect of copper in amphibian populations.

The results obtained in this study showed that *B. bufo*, *E. calamita, D. jeanneae, P. cultripes*, and *P. perezi* at two developmental Gosner stages were sensitive to copper sulphate exposure. The values obtained in LC_{50} experiments showed that *P. perezi* at Gosner stage 25 was the most tolerant species tested. *P. cultripes* at both Gosner stages exposed to copper sulphate showed intermediate LC_{50} values. The lowest LC_{50} values were noted by *B. bufo*, *E. calamita*, and *D. jeanneae* at both Gosner stages. In contrast, tolerance-response experiments confirmed the most sensitive species and stages. These results are interesting because *P. perezi* is the most common amphibian in agricultural landscapes and in wetlands surrounded by this type of land use (mainly olive tree agriculture) in southern Spain, whereas the most sensitive species have disappeared in the recent years from these wetlands (García-Muñoz et al. 2010). In addition, *D. jeanneae* showed a dramatic decrease in the southeastern Iberian Peninsula. The results obtained in this study are also interesting because *D. jeanneae* was the most common amphibian in Jaén province 10 years ago, but currently it is the scarcest species in this area (database of

Table 2 Results of intraspecific GLMs[a]

Species	Source of variation	*df*	MS	*F*	*p*
Bb	Concentration	3	17.377	1871.509	0.000
	Stage	1	0.299	32.233	0.000
	Time	3	4.163	448.391	0.000
	Concentration * stage	3	0.119	12.772	0.000
	Concentration * time	9	0.862	92.842	0.000
	Stage * time	3	0.044	4.772	0.003
	Concentration* stage * time	9	0.045	4.880	0.000
	Error	256	0.009		
Ec	Concentration	3	18.857	1223.526	0.000
	Stage	1	0.003	0.177	0.675
	Time	3	4.521	293.370	0.000
	Concentration * stage	3	0.105	6.810	0.000
	Concentration * time	9	0.958	62.138	0.000
	Stage * time	3	0.067	4.374	0.005
	Concentration * stage * time	9	0.054	3.528	0.000
	Error	256	0.015		
Dj	Concentration	3	19.350	1309.323	0.000
	Stage	1	0.129	8.755	0.003
	Time	3	2.821	190.858	0.000
	Concentration * stage	3	0.045	3.056	0.029
	Concentration * time	9	0.696	47.081	0.000
	Stage * time	3	0.116	7.852	0.000
	Concentration * stage * time	9	0.039	2.671	0.006
	Error	256	0.015		
Pc	Concentration	3	1.878	906.253	0.000
	Stage	1	0.114	54.996	0.000
	Time	3	0.316	152.496	0.000
	Concentration * stage	3	0.152	73.224	0.000
	Concentration * time	9	0.267	128.697	0.000
	Stage * time	3	0.032	15.533	0.000
	Concentration * stage * time	9	0.027	13.190	0.000
	Error	248	0.002		

[a] With mortality (arcsin square root transform) as dependent variable and copper concentration, stage and time as categoric variables for all species, except for *P. perezi*, which showed no mortality in both stages during the experimental period

the Association Giennense of Herpetology). The existence of diverse factors, such as land use changes, a huge increase in the rate of xenobiotic products used (MMA 2007), and regional variations in pond community structure, among others, create difficulties in defining and attributing cause-and-effect to decreased amphibian populations (Mann et al. 2009). Laboratory conditions do not show the real effects on ecosystems but can help us to determine what is happening in natural ecosystems.

In contrast, it is well known that pollutants can decrease the growth of tadpole and amphibian larvae (Griffis-Kely 2007; Shin et al. 2008). In the present study, copper sulphate exposure caused decreased larval growth and development in all species at both Gosner stages studied. Decreased growth rate has been correlated with longer developmental time and smaller size at metamorphosis (e.g., Breden and Kelly 1982). This effect on growth and development might have large repercussions in conservation of the most sensitive species (*B. bufo, E. calamita*, and *D. jeanneae*). Because these species use small and temporary wetlands that suffer rapid desiccation processes (Sinsch 1998), increased development time affects the total number of individuals that can successfully complete metamorphosis. In contrast, the most tolerant species tested in this study, i.e., *P. cultripes* and *P perezi*, use permanent water bodies. These species remain in the aquatic phase for a longer time, and the possible effects of long-term copper exposure could decrease their apparent tolerance as well as magnify the sublethal effects of copper exposure. Altwegg and Reyer (2003) demonstrated that smaller larval size implies rapid metamorphosis leads, which are associated with lower survival and fecundity. Relyea and Diecks

Table 3 Multiple regression results for all species[a]

Species	Stage	Source of variation	Beta	SE of beta	B	SE of B	*t*	*n*	*p*
Bb	19	Concentration	–0.842	0.115	–0.091	0.012	–7.307	22	0.000
	25	Concentration	–0.735	0.196	–0.048	0.013	–3.751	12	0.003
Ec	19	Concentration	–0.811	0.122	–0.099	0.015	–6.648	22	0.000
	25	Concentration	–0.603	0.230	–0.042	0.016	–2.621	12	0.022
Dj	19	Concentration	–0.874	0.130	–0.061	0.009	–6.717	14	0.000
	25	Concentration	–0.488	0.212	–0.029	0.013	–2.306	17	0.034
Pc	19	Concentration	–0.632	0.149	–0.046	0.011	–4.243	27	0.000
	25	Concentration	–0.793	0.140	–0.062	0.011	–5.671	19	0.000
Pp	19	Concentration	–0.746	0.105	–0.054	0.008	–7.086	40	0.000
	25	Concentration	–0.845	0.123	–0.052	0.008	–6.878	19	0.000

[a] Total length (log transform) was the dependent variable, and copper concentration (0, 0.08, 0.10, and 0.20 mg Cu L^{-1}) was the categoric variable

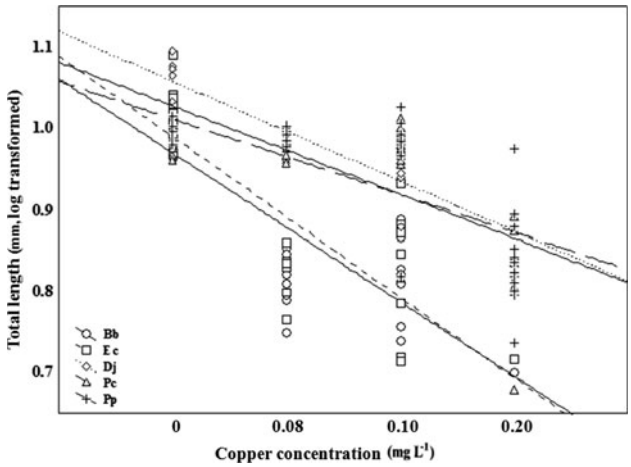

Fig. 6 Result of multiple regression for all species (Bb, Ec, Dj, Pc, and Pp) for total length reached (log transformed) by the end of the experimental period (i.e., 96 h) in larvae exposed in Gosner stage 19. Copper concentration expressed in mg Cu L^{-1}. The different lines represent the multiple regression slopes for each species

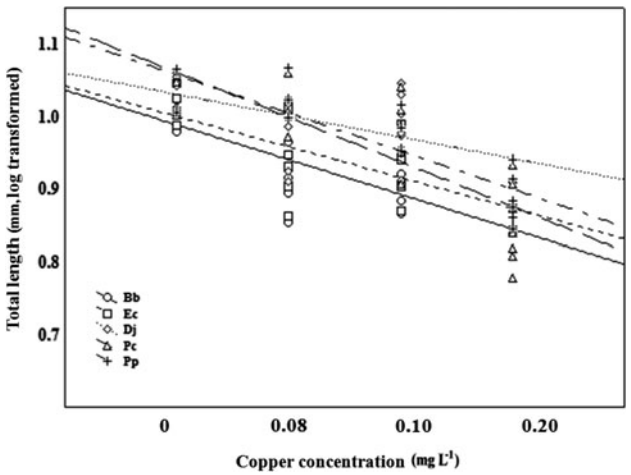

Fig. 7 Result of multiple regression for all species (Bb, Ec, Dj, Pc, and Pp) for total length reached (log transformed) by the end of the experimental period (i.e., 96 h) in larvae exposed in Gosner stage 25. Copper concentration expressed in mg Cu L^{-1}. The different lines represent the multiple regression slopes for each species

(2008) observed that smaller size observed in frogs sampled in contaminated regions might be caused by the physiological effects of pesticides on growth or by decreased feeding caused by poorer food quality. It is interesting to note that the present study, using growth as the end point, showed specific sensitivity not only in terms of survival but also in terms of ecophysiological response to exposure, thus increasing and strengthening the concept of sensitivity. In addition, copper's negative effects were noted during the present experiments, as was observed by the detection of large-scale skin epithelial damage, which probably disturbs osmotic equilibrium and energy expenditure (Prosser 1991). Moreover, copper exposure also induces lower capacity and less efficiency in larval escape behaviour (García-Muñoz et al. 2009). Regarding the lack of effect by copper exposure on development detected in Gosner stage 25 experiments, it is interesting to note that the experimental period (96 h) might not have been long enough to note any effects on larvae in this developmental phase. Similarly to other amphibians, larvae of these species remain in Gosner stage 25 for a longer period while their weight increases (García-Muñoz et al. 2009).

Recent studies have hypothesized that chemical contaminants are in some way responsible for decreased amphibian populations (Sparling et al. 2001; Marco 2002; Blaustein et al. 2003; Greulich and Pflugmache 2003; Davidson 2004; Relyea 2005; García-Muñoz et al. 2009). Available toxicity literature on amphibians exposed to metals indicates that copper sulphate 96 h LC_{50} values calculated in tadpoles ranged from 0.04 to 5.38 mg L^{-1} (Linder and Grillitsch 2000). Studies of other aquatic organisms show 96 h LC_{50} values ranging from 0.06 to

6.68 mg L^{-1} (see, among others, Bridges et al. 2002; Khangarot and Battish 1994; Parra et al. 2005).

Only a few amphibian species inhabit the Guadalquivir valley (Ceacero et al. 2007). The most abundant species in this region, *P. perezi*, was the most tolerant species tested in this work. The use of widely distributed and abundant species, similar to *P. perezi* (García-París et al. 2004), in toxicologic studies has been criticized because probably their wide distribution can also be related to their higher resistance to pollution (Marco 2003). There is a wide variation in tolerance levels among amphibians, even between closely related species (Bridges and Semlitsch 2000; Bridges 2000). Therefore, to protect a wider range of species in the community, legal copper sulphate restrictions must take into account toxicity information from the most sensitive species and not from the most tolerant ones. However, laboratory conditions may not always represent what it is happening in natural conditions, and further studies are required to understand the ways in which these species can be affected by other contaminant used in agriculture.

Although conclusions drawn from studies of only a few species cannot show the full effects of potentially harmful chemicals to amphibians in general (McDiarmid and Mitchell 2000), the present results could give an accurate picture of what is happening in small wetlands and will provide increased understanding of the ecology of temporary wetlands (Blaustein and Schwartz 2001). However, further research on sublethal effects and comparative sensitivity to key pesticides, as well as field mesocosm experiments, must be conducted.

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