SETAC PRESS

Environmental Toxicology and Chemistry, Vol. 30, No. 12, pp. 2771–2777, 2011

Printed in the USA
DOI: 10.1002/etc.674

STAGE-DEPENDENT SUSCEPTIBILITY TO COPPER IN *RHINELLA ARENARUM* EMBRYOS AND LARVAE

CAROLINA M. ARONZON,† MARIA TERESA SANDOVAL,‡ JORGE HERKOVITS,*† and CRISTINA S. PÉREZCOLL§
†Programa de Seguridad Química, Instituto de Ciencias Ambientales y Salud (ICAS), Fundación PROSAMA, Buenos Aires, Argentina
‡Laboratorio de Herpetología, Facultad de Ciencias Exactas y Naturales y Agrimensura, Universidad Nacional del Nordeste, Corrientes, Argentina
§Instituto de Investigación e Ingeniería Ambiental, Universidad Nacional de San Martín, Buenos Aires, Argentina

(*Submitted* 10 *May* 2011; *Returned for Revision* 28 *June* 2011; *Accepted* 9 *August* 2011)

Abstract—Copper toxicity in different embryonic and larval stages of the common South American toad *Rhinella arenarum* was evaluated by means of continuous and 24-h pulse treatments in 12 different developmental stages. Lethal concentrations (LC) of 10, 50, and 90% of continuous treatment with Cu from early blastula (S.4), complete operculum (S.25), and hind limb bud (S.28) stages were plotted from 24 to 168 h, resulting from S.4 in a 24-h LC50 of 137 μg Cu^{2+}/L and a 168-h LC50 of 19.5 μg Cu^{2+}/L. This result was in agreement with pulse treatments that showed a high resistance to Cu at blastula and gastrula stages, whereas the organogenic period, between muscular response (S.18) and open mouth (S.21), was very susceptible to this metal. Continuous treatments from S.25 showed no significant differences along exposure time (168-h LC50 = 51 μg Cu^{2+}/L), but in the case of S.28 toxicity increased slightly from a 24-h LC50 of 138.6 μg Cu^{2+}/L to a 168-h LC50 of 104 μg Cu^{2+}/L, pointing out that, although the larval period was significantly more resistant to Cu, there was also a remarkable stage-dependent susceptibility to this metal. Copper teratogenic potential was approximately two, and main adverse effects were reduced body size, axial flexure, microcephaly, acephaly, mouth malformations, agenesis of or underdeveloped gills, agenesis of or underdeveloped tail, and hydropsy. The results are discussed considering Cu toxicity mechanisms, an evolutionary perspective, and environmental protection. Environ. Toxicol. Chem. 2011;30:2771–2777. © 2011 SETAC

Keywords—Amphibian embryos Copper Teratogenesis Stage-dependent toxicity AMPHITOX

INTRODUCTION

Copper (Cu) is an essential trace element for all living systems, crucial for many cellular processes and metabolism, such as production of enzymes associated with elastin and collagen synthesis, melanin production, and the integrity of the central nervous system [1]. Nonetheless, Cu concentrations as low as 1 to 20 μg/L, slightly higher than the pristine concentrations, might bring about adverse effects for aquatic organisms, in both invertebrates and vertebrates [2]. Copper exposure in fish has been shown to affect reproductive strategies, swimming speed, immunity, metabolism, enzyme activities, ionic regulation, and epithelial cells in gills and intestine [3]. Copper levels in water are increased by anthropogenic activities, such as mines, domestic and industrial discharges, agricultural chemical applications, animal feed additives, and soil erosion [2]. Other sources of contamination include the textile industry, petroleum refining, manufacture of Cu compounds, building siding and roofs, automobile brakes, tires, oil leakage, and road surface materials.

Amphibians are considered keystone members of ecosystems and vital links in food chains. The decline of amphibian populations and the large number of malformations found in many geographic regions has caused increasing concern [4]. Some studies indicate that this could be related to their high susceptibility to contaminants, particularly during early life stages [5], in that this susceptibility has been reported with diverse physicochemical agents such as metals [6,7], pesticides [8,9], industrial [10] and pharmaceutical [11] chemicals, and ultraviolet B radiation [12,13]. In addition, the risk for adverse effects might be enhanced by the preference to breed in shallow, lentic, or ephemeral water bodies in which pollutants might be concentrated.

Most toxicity studies in early life stages explore adverse effects in acute exposure conditions, such as the cases reported for Cu in *Rana sphenocephala*, *Bufo boreas,* and *Rana catesbeiana*, with 96-h lethal concentration (LC)50 of 230 μg/L, 120 μg/L, and 2,400 μg/L, respectively [14,15]. Teratogenic effects of Cu, such as embryonic deformities, decreased growth rate, and delayed metamorphosis [16], as well as behavioral changes [17], have been reported.

Toxicity studies performed by treating embryos and larvae during specific developmental stages allow the evaluation of stage-dependent susceptibility and the potential adverse effects related to specific cell differentiation and morphogenic processes, providing possible explanations based on well-known developmental features, toxicity mechanisms, and evolutionary perspective [12,18–20]. The main aim of the present study was to evaluate the toxic effects of Cu on the South American toad *Rhinella arenarum* at different embryonic and larval stages, reporting lethality and malformations at optical and electronic microscopic levels. The results are discussed in relation to environmental concentrations of Cu, its toxicity mechanisms, and an evolutionary perspective.

MATERIALS AND METHODS

Rhinella arenarum *embryos*

Rhinella arenarum adults weighing approximately 200 to 250 g were obtained in Lobos (Buenos Aires Province, Argentina: 35°11′S; 59°05′W), a presumably pristine region. Ovulation of *R. arenarum* females was induced by means of an

* To whom correspondence may be addressed (herkovit@retina.ar).
Published online 14 September 2011 in Wiley Online Library (wileyonlinelibrary.com).

intraperitoneal injection of a suspension of one homologous hypophysis in 1 ml of amphibian embryo–larval toxicity test (AMPHITOX) solution (AS) per female. Oocytes were fertilized in vitro with sperm suspensions in AS. The AS composition was (in mg/L) Na^+ 14.75, Cl^- 22.71, K^+ 0.26, Ca^{2+} 0.36, HCO_3^- 1.45. After fertilization, embryos were kept in AS at 20 ± 2°C until they reached the stage required for each experimental protocol. The stage of embryos and larvae were defined according to Del Conte and Sirlin [21] and Echeverria y Fiorito de López [22], respectively. Embryos used before hatching (muscular response stage [S.18]) were dejellied by means of a 2-min treatment with 2% thioglycolic acid solution, neutralized at pH 7.2 with 1.35 ml of saturated NaOH solution every 100 ml in AS, and then thoroughly washed.

Test solutions

A Cu stock solution of 1.5 g/L was prepared by directly weighing and dissolving the corresponding mass of $CuCl_2 \cdot 2H_2O$ (purity 99%, lot 11570; Riedel-de Haën) in distilled water. Hydrochloric acid was added until reaching pH 1.9 for conservation purposes. Test solutions, ranging in concentrations between 3 and 375 μg Cu^{2+}/L, were prepared by diluting a secondary stock solution of 30 mg Cu^{2+}/L in AS. Experimental Cu solutions were measured four times with a PerkinElmer atomic absorption spectrophotometer. The error between nominal and measured concentrations did not exceed 5%.

Experimental protocol

Rhinella arenarum embryos and larvae were continuously treated with Cu from early blastula (S.4), complete operculum (S.25), and hind limb bud (S.28) developmental stages for acute (96 h) and short-term chronic (168 h) exposures. For the stage-dependent susceptibility studies, embryos and larvae at blastula (S.4), gastrula (S.11), neural plate (S.13), neural fold (S.14), tail bud (S.17), muscular response (S.18), gill circulation (S.20), open mouth (S.21), opercular folds (S.23), right operculum closed (S.24), complete operculum (S.25), and hind limb bud (S.28) stages were 24-h pulse exposed to Cu.

For each experimental condition, triplicate batches of 10 embryos or larvae (S.28) were placed in covered 10- or 20-cm-diameter glass petri dishes containing 40 or 200 ml AS with different Cu concentrations, respectively. Simultaneously, control embryos or larvae were maintained in AS without additions. Test solutions were renewed every other day, and temperature was maintained at 20 ± 2°C. For the stage-dependent susceptibility studies, after exposure, individuals were thoroughly washed with 200 ml AS and were maintained in petri dishes in 40 or 200 ml AS for 168 h. Lethal and sublethal effects were evaluated every 24 h, and dead individuals were removed. Larvae from S.25 onward were fed with balanced fish food TetraColor® ad libitum for 24 h every other day. The bioassays were replicated up to seven times by using embryos and larvae from different couples.

Teratogenic effects were studied with a Zeiss Stemi DV4 stereoscopic microscope. Photographs of embryos and larvae were digitally recorded with a Sony DSC-S90 camera mounted on a Zeiss Stemi DV4 stereoscopic microscope. For ultrastructural observations, embryos were fixed in 4% formol, dehydrated in a gradient of acetone, prepared by means of the critical-point technique, and viewed in a JEOL 5800LV scanning electron microscope.

Statistical analysis

Lethality data were analyzed statistically by the U.S. Environmental Protection Agency Probit Program [23]. Toxicity profiles (TOPs), as isotoxicity curves [24], were plotted based on the LC10, LC50, and LC90 values at different times. To establish statistical differences between the LC50 values obtained, a comparison was made, considering the difference statistically significant when the higher LC50 and the lower LC50 ratio exceeded the critical value (95% confidence interval) established by the American Public Health Association [25].

RESULTS

Lethality

Rhinella arenarum susceptibility to Cu during embryo and larval development was determined by means of different experimental protocols. As a general pattern, the initial developmental stages exhibited a high resistance to Cu, whereas the organogenic period showed a very high susceptibility to this metal. Then, the resistance to Cu increased significantly as development progressed through the larval stages.

Figures 1, 2, and 3 show the TOP curves obtained by means of Cu continuous treatments (168 h) of *R. arenarum* from early blastula (S.4), complete operculum (S.25), and hind limb bud (S.28) stages, respectively. In the case of embryos exposed from early blastula (S.4) stage, Cu toxicity significantly increased from an LC50 of 137 (116.6–158.6) μg Cu^{2+}/L at blastula–gastrula to an LC50 of 19.5 (16.2–24.4) μg Cu^{2+}/L at complete operculum (S.25) stage. The toxicity slope was more pronounced from a 48-h LC50 value of 108.1 (90.2–127) to a 72-h LC50 of 42.7 (34–52.9) μg Cu^{2+}/L, corresponding to neural tube (S.16) and muscular response (S.18) stages, respectively. It is noteworthy that the confidence interval (CI) of LC50 overlapped the CI of LC10 and LC90 at the last embryonic stages (Fig. 1). In continuous treatments from complete operculum (S.25) or the end of embryonic development onward, Cu toxicity showed no significant differences along exposure time (24-h LC50 = 54 [43–63] μg Cu^{2+}/L and 168-h LC50 = 51 [45–57] μg Cu^{2+}/L; Fig. 2). Continuous exposure from hind limb bud stage (S.28) onward with a 24-h LC50 of 138.6 (137–150) μg Cu^{2+}/L registered a significant increase in toxicity, with a final 168-h LC50 of 104 (100–110) μg Cu^{2+}/L (Fig. 3);

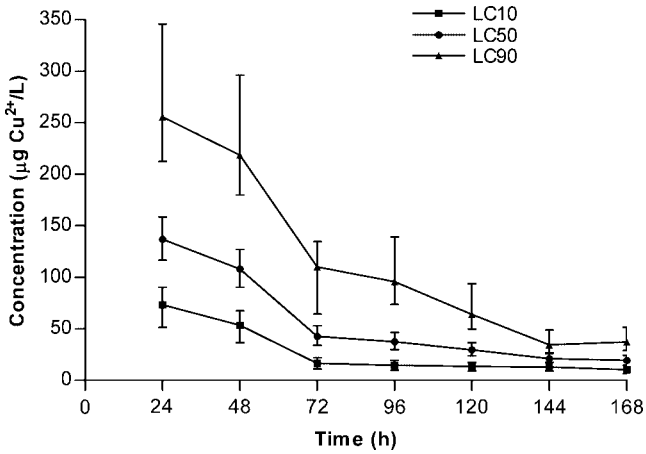

Fig. 1. Toxicity profile curves of Cu representing the lethal concentrations (LCs) 10, 50, and 90% in *Rhinella arenarum* embryos from early blastula stage (S.4) from 24 h up to 168 h. Bars show 95% confidence intervals.

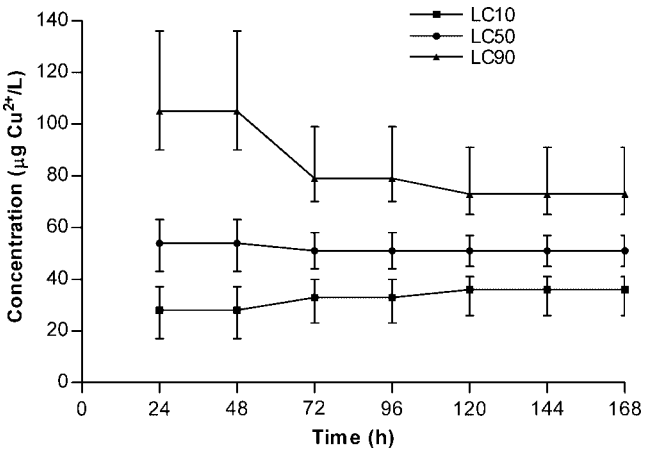

Fig. 2. Toxicity profile curves of Cu representing the lethal concentrations (LCs) 10, 50, and 90% in *Rhinella arenarum* larvae from complete operculum stage (S.25) from 24 h up to 168 h. Bars show 95% confidence intervals.

however, this late larval stage was approximately 2.5 times more resistant to Cu than S.25.

The remarkable increase in susceptibility to Cu by expanding the exposure from early blastula stage (S.4) during the embryonic period might result from a stage-dependent susceptibility to this metal. This presumption was confirmed by means of 24-h pulse exposures at 12 developmental stages. In Figure 4, the Cu 24-h LC10, LC50, and LC90 values of blastula (S.4), gastrula (S.11), neural plate (S.13), neural fold (S.14), tail bud (S.17), muscular response (S.18), gill circulation (S.20), open mouth (S.21), opercular folds (S.23), right operculum closed (S.24), complete operculum (S.25), and hind limb bud (S.28) stages are plotted. The embryos at the beginning of their development (blastula [S.4] and gastrula [S.11]) exhibited the highest resistance to Cu (LC50 = 154 [124–176] μg Cu^{2+}/L). There was a significant increase in Cu toxicity from neural fold (S.14) to muscular response (S.18) stages (LC50 = 19.3 [17.4–22.5] μg Cu^{2+}/L). S.18 until open mouth (S.21) were the most susceptible stages to Cu during the whole of embryonic development (S.20, LC50 = 17 [15.8–18.4] μg Cu^{2+}/L). From the opercular folds stage (S.23) onward, the resistance to Cu increased gradually until hind limb bud (S.28) stage, with an LC50 of 138.61 (128.59–150.3) μg Cu^{2+}/L.

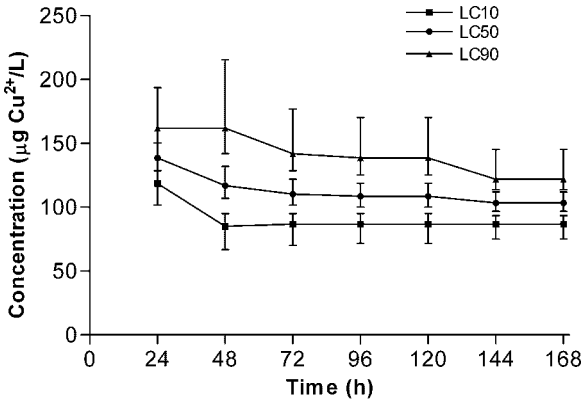

Fig. 3. Toxicity profile curves of Cu representing the lethal concentrations (LCs) 10, 50, and 90% in *Rhinella arenarum* larvae from the hind limb bud development stage (S.28) from 24 h up to 168 h. Bars show 95% confidence intervals.

Sublethal effects

Embryos continuously treated from blastula stage (S.4) onward showed abnormalities directly related to concentration and exposure time. Copper concentrations higher than 180 μg Cu^{2+}/L stopped embryo development at blastula (S.4) or initial gastrula (S.11) stages, resulting in cellular dissociation and yolk exudation to the perivitelline space. In treatments up to 105 μg Cu^{2+}/L, the embryos reached tail bud stage (S.17), but 30% of them had morphological anomalies, in most cases in the abdominal region. Embryos treated from 45 to 105 μg Cu^{2+}/L did not develop beyond the open-mouth stage (S.21), whereas treatments with 15 μg Cu^{2+}/L had as a developmental limit the opercular folds stage (S.23). The main malformations exhibited by embryos were delayed development, reduced body size, axial incurvations, microcephaly, acephaly, microphthalmia, and underdeveloped gills and caudal fin (Fig. 5a–d). The teratogenic potential of Cu, estimated as the ratio between the no-effect-concentration (NOEC) value for lethality (15 μg Cu^{2+}/L) and malformations (7.5 μg Cu^{2+}/L) at 168 h, was approximately two.

The stage-dependent susceptibility study performed by means of 24-h pulse exposures to Cu at 12 developmental stages allowed us to identify the range of concentrations resulting in malformations as well as the main adverse effects caused by the metal at each developmental stage evaluated. Table 1 summarizes the main malformations and abnormalities observed in embryos and larvae exposed to 24-h pulse exposure to Cu at different developmental stages during the whole embryonic and early larval period. Copper treatments from blastula (S.4) until tail bud stage resulted in teratogenic effects affecting even late developmental stages (Fig. 5f–i). The most severe adverse effects in embryos were irregular shapes, severe cellular dissociation, and yolk exudation. Embryos at later developmental stages exhibited microcephaly, underdeveloped gills, microphthalmia or eye agenesis, mouth and adhesive structure malformations, failure in the opercular closure, underdeveloped tail and caudal fin, axial flexures, abdominal edemas, and gut agenesis in a concentration–adverse effect relationship.

Exposure to Cu from muscular response (S.18) to complete operculum (S.25) stage also showed severe morphological alterations, but adverse effects were limited to the structures developed in the given embryonic stage (Table 1). Those embryos and larvae exhibited reduced body size, delayed development, severe gill and abdominal dropsy, abnormal gut development, and underdeveloped tail.

Neurotoxic effects such as erratic swimming and spasmodic movements were registered from opercular fold stage (S.23) onward, even if individuals had been exposed at earlier developmental stages. Those adverse effects were observed with Cu concentrations between 3.75 and 7.5 μg Cu^{2+}/L.

DISCUSSION

The present results show the high Cu toxicity to embryos and larvae of *R. arenarum*, a widely distributed South American amphibian species, both in continuous and in pulse exposures at different developmental stages. As a whole, embryos at blastula (S.4) and gastrula (S.11) stages showed the highest resistance to Cu; the organogenic stages were the most susceptible, and during the last embryonic and larval stages the resistance to Cu increased gradually up to values obtained in the blastula and gastrula. This pattern within the embryonic stages has also been found with other metals, such as Pb [18] and Ni [20], and organic substances such as 2,4-dichlorophenoxyacetic acid

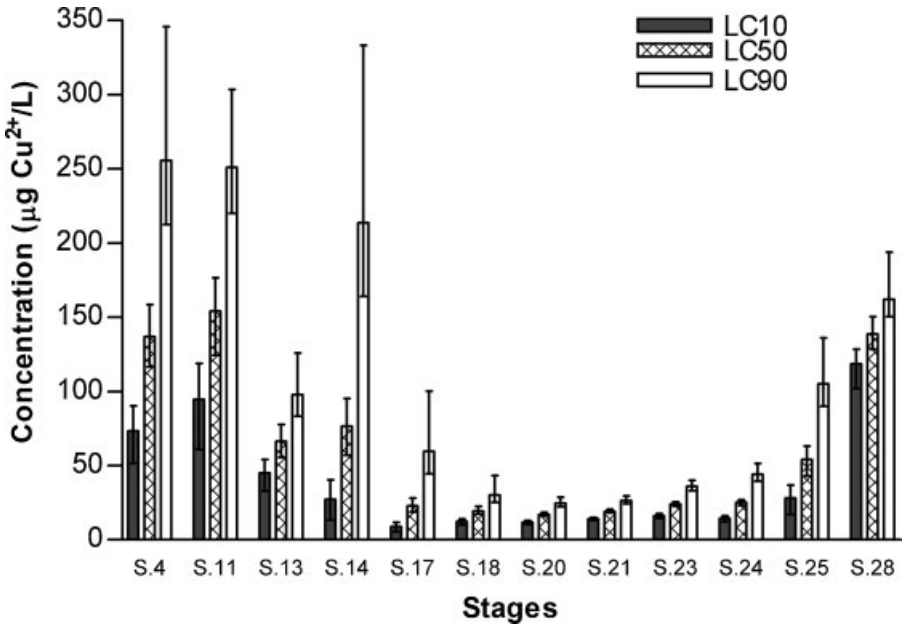

Fig. 4. The 24-h lethal concentrations (LCs) 10, 50, and 90% in Cu pulse exposure in *Rhinella arenarum* embryos at 12 different developmental stages.

[26], confirming as a general concept that organogenic stages are very susceptible to noxious agents. Because the organogenic stages were the most susceptible to Cu, the fact that jelly coats might reduce the toxicity of environmental agents at embryonic stages prior to hatching [27] does not affect the main purpose of this study, which focuses on identifying the most susceptible developmental stages of amphibian to Cu for protection purposes.

The TOP curves report the concentrations exerting the same degree of adverse effects for different exposure periods [24], so this might be relevant for incorporation in all toxicity studies. In the case of early life stages of *R. arenarum*, the significant increase in Cu toxicity from an LC50 of 137 to an LC50 of 42.7 and then to an LC50 of 19.5 μg Cu^{2+}/L at blastula (S.4), neural tube (S.17) and complete operculum (S.25) stages, respectively, anticipated a remarkable stage-dependent susceptibility to Cu, confirmed in the present study by means of pulse exposure experiments. Conversely, Cu toxicity in continuous treatment from complete operculum stage (S.25) onward did not significantly change along the exposure time (168 h), confirming previous results [24], in which Cu at this larval stage achieves its maximal toxicity within the initial 24 h of exposure. This pattern of fast maximal toxic effect found for Cu is similar to the results reported for Cd [19], whereas the toxicity of Ni significantly increased by expanding the evaluation period over 96 h, even if the metal exposure had been for only 24 h [20]. At advanced larval stages (S.28), the increase in the resistance to Cu was significant, up to 2.5 times higher than complete operculum stage (S.25); nevertheless, Cu toxicity increased toward metamorphosis. It is important to note the multiple overlap among the confidence limits of LC50 with LC10 and LC90 (Fig. 1), which implies that, for certain developmental stages, the exposure to Cu concentrations of about LC10 or LC50 may represent a risk for 50% or 90% of the population, respectively. Similar results were obtained for other chemicals in certain exposure conditions [24,28]. In the case of Cu, these facts are of special concern because of the very small difference from the LC10 to the LC50 or from the LC50 to the LC90 in certain developmental stages.

Most toxicity studies focus on acute effects in a predetermined period of the life cycle, as the cases reported for *Rana sphenocephala*, *Bufo boreas,* and *Rana catesbeiana* larvae with a 96-h LC50 of 230 μg/L, 120 μg/L, and 2,400 μg/L of Cu, respectively [14,15]. For tadpole stages, *R. arenarum* had approximately the same susceptibility as *B. boreas*. However, based on the stage-dependent susceptibility reported here, the susceptibility for *R. arenarum* was approximately six times higher than the toxicity value reported for *B. boreas.* Therefore, an approach evaluating the susceptibility at all developmental stages would be of great relevance for species protection purposes. However, differences in the reported Cu toxicity may be partially related to the Cu salt employed and the temperature and salinity in the toxicity tests conditions. For example, the sulfate salt may be less toxic than the chloride form, perhaps because of the higher solubility of the chloride form [2]. Moreover, high concentrations of other ions in the maintaining solution compete with Cu for binding and uptake, reducing the Cu uptake, bioaccumulation, and toxicity [29]. It has been reported that FETAX and AMPHITOX tests have different conditions of salinity and temperature in the maintaining media [30], which might modify toxicity results.

In comparison with other metals, the present results show that Cu was the most toxic for *R. arenarum* embryos followed by Cd [19], Ni [20], Al [31], and Pb [18]. Copper also caused relevant teratogenic effects in all the developmental stages evaluated. Its teratogenic potential in continuous treatments from blastula to complete operculum stage was about two; values higher than 1.5 indicate a high risk for embryos to be malformed in the absence of significant embryonic lethality [32]. Thus, in the case of Cu, it is meaningful to incorporate teratogenesis as a relevant endpoint for risk assessment purposes. As reported for lethality, the teratogenic effects produced by Cu also had a stage-dependent susceptibility pattern, the organogenic stages being most susceptible to this metal. The reduced body size produced by Cu at all the developmental stages evaluated (Table 1) has been also reported for *Rana pipiens* [16,17] and may be considered as a nonspecific adverse effect of toxic agents on early life stages [18,20]. The fact that

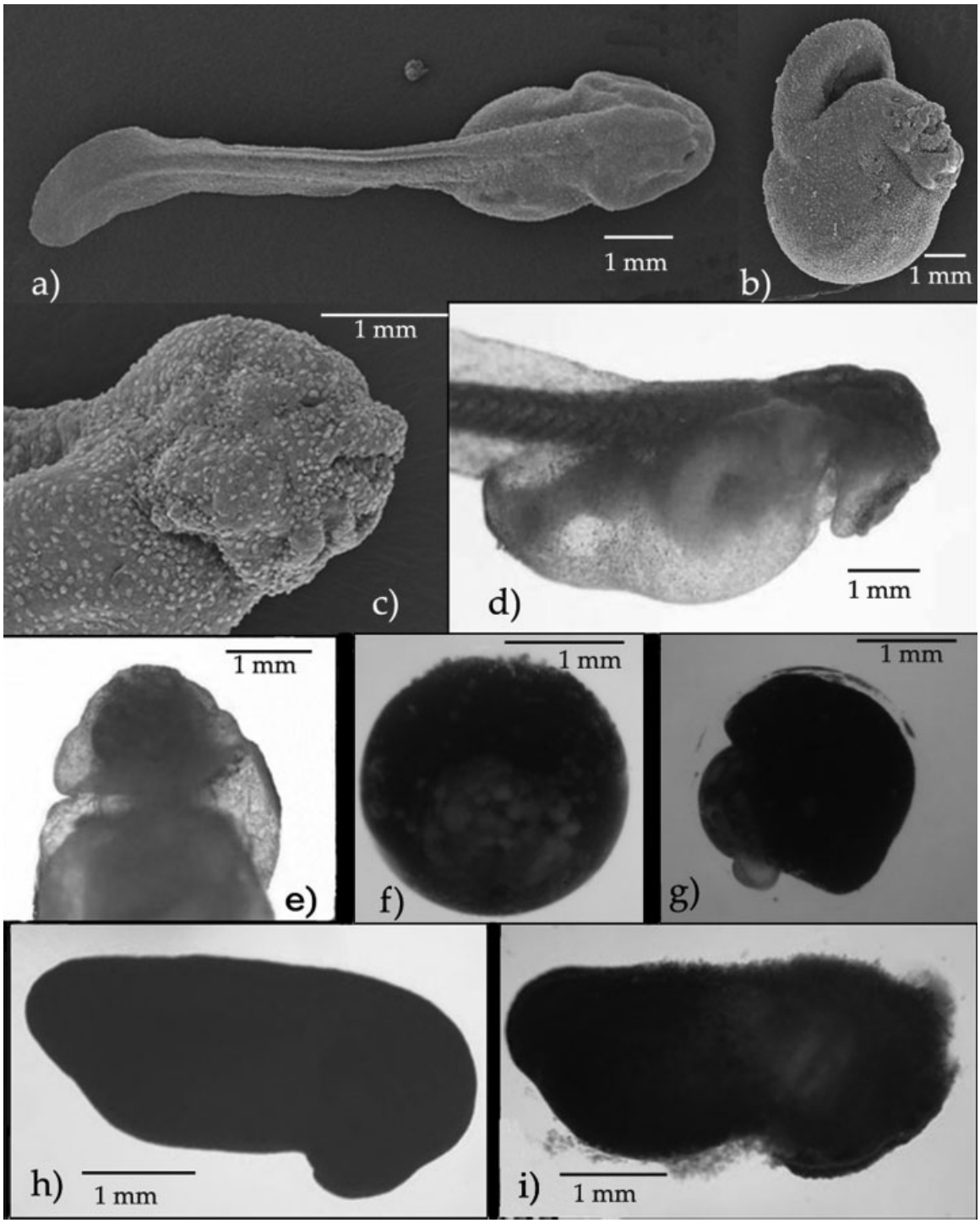

Fig. 5. Examples of scanning electron and optical microscopic views of malformations produced by Cu in *Rhinella arenarum* embryos and larvae. Continuous treatments from blastula stage: control at S.25 (**a**); 15 μg Cu^{2+}/L continuous treatments (**b–e**): Delayed development, reduced body size, microcephaly, gill agenesis, and axial flexures (b); mouth and gill malformations (c); abdominal and cardiac edema, abnormal gut development (d and e); 24-h pulse exposure to Cu (**f–i**): 180 μg Cu^{2+}/L at blastula (f) and 7 μCu^{2+}/L at gastrula stage, resulting in cellular dissociation and failure in the gastrulation process (g); control embryo at tail bud stage (h); and embryo exposed at neural stages to 3 μg Cu^{2+}/L with remarkable cellular dissociation (i).

certain malformations such as axial flexures and hydropsy were obtained at all developmental stages seems to indicate that Cu might produce some adverse effects in spite of the exposure period. Other malformations, such as persistent yolk plug, are related exclusively to the developmental stage at which the morphogenic and/or cellular differentiation features occur, in this case during the gastrula stage. This is partially confirmed, considering that Cu exposure during the initial developmental stages resulted in similar malformations, such as cephalic abnormalities (microcephaly/acephaly), mouth malformation, agenesis/underdeveloped gills and tail, axial flexures, hydropsy, and reduced body size. In the case of slight effects, embryos in later stages recovered to some extent during later developmental stages. This result may be considered within the general concept of regulation and recovery capacity during early life stages [33]

Table 1. Malformations produced by 24-h Cu pulse exposure in different embryo–larva stages of *Rhinella arenarum*

		Neural stages	Organogenesis			
Blastula (S.3–4)	Gastrula (S.11)	S.13; S.14	S.17	S.18; S.20; S.21	S.23	S.24; S.25
Cephalic abnormalities (microcephaly/acephaly)	Persistent yolk plug Cephalic abnormalities (microcephaly/acephaly)	Cephalic abnormalities (microcephaly/acephaly)	Cephalic abnormalities (microcephaly/acephaly)			
Mouth malformation	Mouth malformation	Mouth malformation	Mouth malformation	Mouth malformation		
Agenesis/underdevelopment of gills	Agenesis/underdevelopment of gills	Agenesis/underdevelopment of gills	Agenesis/underdevelopment of gills	Agenesis/underdevelopment of gills		
			Failed at operculum closure	Failed at operculum closure	Failed at operculum closure	
Agenesis/underdevelopment of tail	Agenesis/underdevelopment of tail	Agenesis/underdevelopment of tail	Agenesis/underdevelopment of tail	Agenesis/underdevelopment of tail	Agenesis/ underdevelopment of tail	
Axial flexure	Axial flexure	Axial flexure	Axial flexure	Axial flexure	Axial flexure	Axial flexure
Hydropsy	Hydropsy	Hydropsy	Hydropsy	Hydropsy	Hydropsy	Hydropsy
			Gut agenesis	Gut agenesis	Gut agenesis	Gut agenesis
Reduced body size	Reduced body size	Reduced body size	Reduced body size	Reduced body size	Reduced body size	Reduced body size

The usual explanation of the remarkable stage-dependent susceptibility to chemical agents focuses on the complexity achieved in cell differentiation and morphogenic processes as development advances [34]. The biochemical toxicity of Cu, when it exceeds homeostatic control, is derived from its effects on the structure and function of biomolecules such as DNA, proteins, and membrane molecules or through oxygen-radical mechanisms [35]. In the case of hydropsy, the documented disruption of Na/K ATPase (the ''sodium pump'') by Cu could be directly related to this particular adverse effect [36]. Moreover, the high susceptibility to Cu in gill circulation stage (S.20) might be related to the hydropsy and epithelial lifting reported for fish gills, probably initiated also by the inhibition of Na/K ATPase. The moderate hypoxia resulting from gill injury probably also contributes to Cu toxicity [3]. The cellular dissociation observed from gastrula (S.11) to tail bud (S.17) stages seems to reflect the interference of Cu with Ca homeostasis [36], resulting in cellular adhesivity reduction. The erratic swimming may be related to neurotoxic effects [16,17], possibly associated with acetylcholinesterase (AChE) inhibition as has been reported for fish [37].

An evolutionary perspective could also contribute to a rational explanation for the stage-dependent susceptibility to Cu reported in the present study. In fact, living organisms at ontogenic stages could be considered as biomarkers of environmental signatures during the evolutionary process of their phylogenetic ancestors [38]. From this perspective, the ancestors of free-living organisms were exposed to harsh environmental conditions, including metal availability, as has been reported for the anoxic period in Earth history based on geochemical studies [39]. The initial anaerobic stages of *R. arenarum* embryos reflect the environmental conditions of these ancient times [38] and therefore could be associated with high resistance to metals and physical agents [12,20], including the results of the present study of Cu toxicity. The gradual increase in free oxygen from approximately 2.4 billion years ago onward may be reflected during ontogenesis by the gradual increase in oxygen consumption as the embryo develops [38]. The associated increase in oxidative stress enhanced by transition metals such as Cu could explain the higher susceptibility to this chemical during organogenic stages. The adaptation process of living organisms to oxidative stress during the phylogenetic process could be reflected during ontogenesis by the increased resistance to Cu as embryo–larva development advances.

Compared to Cu toxicity for fishes, ranging mainly between 340 and 10,000 μg/L, and for fresh water invertebrates, ranging between 54 and 1,700 μg/L [2], the high susceptibility of amphibian embryos to this heavy metal is remarkable; the LC50 reported in the present study for the most susceptible developmental stage of *R. arenarum* was for S.20 a 24-h LC50 of 17 (15.8–18.4) μg Cu^{2+}/L. Moreover, in unpolluted freshwater bodies, Cu concentrations range between 1 and 20 μg/L, and, as a result of anthropogenic impacts, Cu concentrations might rise to even severalfold higher, as in the case of the Reconquista River, an urban ecosystem of Buenos Aires Province with values from 27 to 64 μg/L [40]. Consequently, our results on the susceptibility of *R. arenarum* to Cu point out that the toxicity reported for the Reconquista River might be at least partially related to the high level of Cu pollution in that urban ecosystem [30].

Acknowledgement—The present study was supported by PIP-2317 grant from the Scientific Council of Science and Technology of Argentina

(CONICET), Fundación Pro Salud y Medio Ambiente, and Universidad Nacional de San Martín (grant SA 08-02). We thank Josefina Ramon for her helpful assistance with assays. C. Aronzon has received a fellowship from the CONICET.

REFERENCES

1. O'Dell BL. 1990. Copper. In Brown M, ed, *Present Knowledge in Nutrition*, 6th ed. International Life Sciences Institute Nutrition Foundation, Washington, DC, USA, pp 261–267.
2. World Health Organization. 1998. *Environmental Health Criteria 200: Copper*. The International Programme on Chemical Safety, Geneva, Switzerland.
3. Handy RD. 2003. Chronic effects of copper exposure versus endocrine toxicity: Two sides of the same toxicological process? *Comp Biochem Physiol A Mol Integr Physiol* 135:25–38.
4. Wake DB, Vredenburg VT. 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. *Proc Natl Acad Sci U S A* 105:11466–11473.
5. van der Schalie WH, Gardner HS Jr, Bantle JA, De Rosa CT, Finch RA, Reif JS, Reuter RH, Backer LC, Burger J, Folmar LC, Stokes WS. 1999. Animals as sentinels of human health hazards of environmental chemicals. *Environ Health Perspect* 107:309–315.
6. Pérez-Coll CS, Sztrum AA, Herkovits J. 2008. Nickel tissue residue as a biomarker of sub-toxic exposure and susceptibility in amphibian embryos. *Chemosphere* 74:78–83.
7. Pérez-Coll CS, Herkovits J, Fridman O, Daniel P, D'Eramo JL. 1999. Metallothionein induction and cadmium uptake in *Bufo arenarum* embryos following an acclimation protocol. *Environ Pollut* 106:443–448.
8. Mann RM, Hyne RV, Choung CB, Wilson SP. 2009. Amphibians and agricultural chemicals: Review of the risks in a complex environment. *Environ Pollut* 157:2903–2927.
9. Brodeur JC, Svartz G, Perez-Coll CS, Marino DJG, Herkovits J. 2009. Comparative susceptibility to atrazine of three developmental stages of *Rhinella arenarum* and influence on metamorphosis: Non-monotonous acceleration of the time to climax and delayed tail resorption. *Aquat Toxicol* 91:161–170.
10. Mann RM, Bidwell JR. 2000. Application of the FETAX protocol to assess the developmental toxicity of nonylphenol ethoxylate to *Xenopus laevis* and two Australian frogs. *Aquat Toxicol* 51:19–29.
11. Pérez-Coll CS, Herkovits J. 2004. Lethal and teratogenic effects of naringenin evaluated by means of an amphibian embryo toxicity test (AMPHITOX). *Food Chem Toxicol* 42:299–306.
12. Castañaga LA, Asorey CM, Sandoval MT, Perez-Coll CS, Argibay TI, Herkovits J. 2009. Stage dependent teratogenic and lethal effects exerted by ultraviolet B radiation on *Rhinella (Bufo) arenarum* embryos. *Environ Toxicol Chem* 28:427–433.
13. Bancroft BA, Baker NJ, Blaustein AR. 2007. A meta-analysis of the effects of ultraviolet B radiation and its synergistic interactions with pH, contaminants, and disease on amphibian survival. *Conserv Biol* 22:987–996.
14. Ferreira CM, Lombardi JV, Machado-Neto JG, Bueno-Guimarães HM, Soares SR, Saldiva PH. 2004. Effects of copper oxychloride in *Rana catesbeiana* tadpoles: Toxicological and bioaccumulative aspects. *Bull Environ Contam Toxicol* 73:465–470.
15. Bridges CM, Dwyer FJ, Hardesty DK, Whites DW. 2002. Comparative contaminant toxicity: Are amphibian larvae more sensitive than fish? *Bull Environ Contam Toxicol* 69:562–569.
16. Chen T, Gross JA, Karasov WH. 2007. Adverse effects of chronic copper exposure in larval northern leopard frogs (*Rana pipiens*). *Environ Toxicol Chem* 26:1470–1475.
17. Redick MS, La Point TW. 2004. Effects of sublethal copper exposure on behavior and growth of *Rana pipiens* tadpoles. *Bull Environ Contam Toxicol* 72:706–710.
18. Pérez-Coll CS, Herkovits J. 1990. Stage dependent susceptibility to lead in *Bufo arenarum* embryos. *Environ Pollut* 63:239–245.
19. Pérez-Coll CS, Herkovits J. 1996. Stage-dependent uptake of cadmium by *Bufo arenarum* embryos. *Bull Environ Contam Toxicol* 56:663–669.
20. Sztrum AA, D'Eramo JL, Herkovits J. 2011. Nickel toxicity in embryos and larvae of the South American toad: Effects on cell differentiation, morphogenesis, and oxygen consumption. *Environ Toxicol Chem* 30:1146–1152.
21. Del Conte E, Sirlin L. 1951. The firts stages of *Bufo arenarum* development. *Acta Zool Lilloana* 12:495–499.
22. Echeverria DD, Fiorito de López LE. 1981. Estadios de la metamorfosis en *Bufo arenarum* (Anura). *Biol Acuat* 1:15–23.
23. U.S. Environmental Protection Agency. 1988. *Users Guide for a Computer Program for PROBIT Analysis of Data from Acute and Short-Term Chronic Toxicity Test with Aquatic Organisms*. Biological Methods, Environmental Monitoring and Support, Laboratory, Cincinnati, OH, USA.
24. Herkovits J, Helguero LA. 1998. Copper toxicity and copper–zinc interactions in amphibian embryos. *Sci Total Environ* 221:1–10.
25. American Public Health Association. 1980. *Standard Methods for the Examination of Water and Wastewater*. American Water Works Association, Water Pollution Control, Federation, and American Public Health Association, Washington, DC.
26. Aronzon C, Sandoval M, Herkovits J, Pérez-Coll C. 2011. Stage-dependent toxicity of 2,4-dichlorophenoxyacetic on the embryonic development of a South American toad, *Rhinella arenarum*. *Environ Toxicol* 26:373–381.
27. Edginton A, Rouleau C, Stephenson G, Boermans H. 2007. 2,4-D butoxyethyl ester kinetics in embryos of *Xenopus laevis*: The role of the embryonic jelly coat in reducing chemical absorption. *Arch Environ Contam Toxicol* 52:113–120.
28. Herkovits J, Pérez-Coll C. 2000. Evaluation of nickel–zinc interactions by means of bioassays with amphibian embryos. *Ecotoxicol Environ Saf* 45:266–273.
29. Santore RC, Di Toro DM, Paquin PR, Allen HE, Meyer JS. 2001. Biotic ligand model on the acute toxicity of metals. 2. Application to acute copper toxicity in freshwater fish and *Daphnia*. *Environ Toxicol Chem* 20:2397–2402.
30. Herkovits J, Pérez-Coll CS. 2003. A customized set of toxicity tests employing amphibian embryos. In Linder GL, Krest S, Sparling D, Little EE, eds, *Multiple Stressor Effects in Relation to Declining Amphibian Populations*. STP 1443. American Society for Testing and Materials, West Conshohocken, PA, pp 46–60.
31. Herkovits J, Herkovits FD, Pérez-Coll C. 1997. Identification of aluminium toxicity and aluminium–zinc interaction in amphibian *Bufo arenarum* embryos. *Environ Sci* 5:57–64.
32. Amercian Society for Testing and Materials. 1993. Standard guide for conducting the frog embryo teratogenesis assay—*Xenopus* (FETAX). In *Standards on Aquatic Toxicology and Hazard Evaluation*. Philadelphia, PA, pp 1199–1209.
33. Herkovits J. 1977. Shape regulation capacity during development: recovery of embryos developing notwithstanding asymmetry until the neurula stage. *Acta Embryol Exp* 1:3–10.
34. Saxen L. 1976. Mechanism of teratogenesis. *J Embryol Exp Morphol* 36:1–12.
35. Valko M, Rhodes CJ, Moncola J, Izakovic M, Mazura M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 160:1–40.
36. Grosell M, Wood CM. 2002. Copper uptake across rainbow trout gills: mechanisms of apical entry. *J Exp Biol* 205:1179–1188.
37. Vieira LR, Gravato C, Soares AMVM, Morgado F, Guilhermino L. 2009. Acute effects of copper and mercury on the estuarine fish *Pomatoschistus microps*: Linking biomarkers to behaviour. *Chemosphere* 76:1416–1427.
38. Herkovits J. 2006. Evoecotoxicology: Environmental changes and life features development during the evolutionary process—The record of the past at developmental stages of living organisms. *Environ Health Perspect* 114:1139–1142.
39. Konhauser K, Pecoits E, Lalonde S, Papineau D, Nisbet E, Barley M, Arndt N, Zahnle K, Kamber B. 2009. Oceanic nickel depletion and a methanogen famine before the great oxidation event. *Nature* 458:750–753.
40. Ferrari L, de la Torre FR, Demichelis SO, García ME, Salibián A. 2005. Ecotoxicological assessment for receiving waters with the premetamorphic tadpoles acute assay. *Chemosphere* 59:567–575.