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EFFECTS OF CHRONIC COPPER EXPOSURE ON DEVELOPMENT AND SURVIVAL IN THE SOUTHERN LEOPARD FROG (*LITHOBATES [RANA] SPHENOCEPHALUS*)

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Abstract—Exposure to environmental contaminants contributes to the global decline of amphibian populations. The impacts of organic contaminants on amphibians are well documented. However, substantially less is known concerning the potential effects of metals on amphibian populations. Copper (Cu) is an essential element, but it can be toxic at concentrations only slightly higher than the normal physiological range. The present study examines the effects of chronic Cu exposure on embryos and larvae of southern leopard frogs, *Lithobates (Rana) sphenocephalus*. Groups of eggs from multiple clutches were collected from two wetlands and exposed to a range of Cu concentrations (0–150 μg/L) until they reached the free-swimming stage, and then individual larvae were reared to metamorphosis. Higher Cu concentrations significantly reduced embryo survival to the free-swimming stage but did not further reduce survival to metamorphosis. Larval period was affected by Cu treatment, but the clutch from which larvae originated (i.e., parentage) explained a higher proportion of the variation. Embryo survival to hatching varied significantly among clutches, ranging from 42.9 to 79.2%. Measurable levels of Cu were found in larvae with body burdens up to 595 μg Cu/g dry mass in the 100 μg/L treatment, and larval Cu body burdens were higher than in metamorphs. The present study also demonstrated that higher initial egg density ameliorated embryo mortality at higher Cu levels and should be accounted for in future studies. Environ. Toxicol. Chem. 2012;31:1587–1594. © 2012 SETAC

Keywords—*Lithobates* (*Rana*) *sphenocephalus* Copper Amphibian Ecotoxicology Metal toxicity

INTRODUCTION

Exposure to environmental contaminants is one of many documented reasons for amphibian population declines occurring globally [1]. Amphibians are sensitive to environmental contaminants because of their highly permeable skin, unshelled eggs, and exposure to terrestrial and aquatic environments at different life stages [2,3]. Amphibians may be affected by a wide array of contaminants, including organics such as pesticides and phenols and inorganics such as metals/metalloids and fertilizers [2,3]. Many of these contaminants can be found in high concentrations in the shallow, lentic, or ephemeral wetlands in which amphibians breed and undergo larval development [4]. Numerous studies have examined the lethal or sublethal effects of organic contaminants on amphibians; these effects can include increased mortality, decreased growth rates, malformations, endocrine disruption, and immunosuppression [5]. However, substantially less is known concerning the effects of metals and metalloids on amphibian populations [6].

The metal copper (Cu) is omnipresent in the environment and occurs naturally in rock, soil, water, sediment, and, at low levels, air. Copper is an essential element crucial for many biochemical pathways [7], but it can be toxic at concentrations only slightly higher than the normal physiological range [8]. In aquatic systems, levels of Cu may be artificially elevated because of anthropogenic activities such as discharges from industries or domestic wastewater treatment centers, urban or agricultural runoff, and mining operations [9]. Copper does not break down in the environment; thus, aquatic organisms can experience both acute and chronic exposure. Copper is known to affect neuroendocrine processes negatively in fish [10] and to impair osmoregulation in fish and invertebrates [11]. In addition, Cu has been shown to have negative effects on growth and survival of numerous fish species [12]. Far less research has examined the impact of Cu on amphibians; however, the existing studies demonstrate a wide array of effects, including mortality [8,13], embryonic deformities [13,14], delayed metamorphosis [13,15], and reduced body size [13,14,16]. These studies have provided insight into the potential effects of Cu on amphibians, but they represent only four species. Reviews of Cu effects on aquatic invertebrates and fish [11] have revealed that species can vary greatly in their sensitivity to Cu exposure. Toxicity assessments that analyze effects on relevant local species are needed to adequately assess contaminant risks for a specific site or region [3].

In addition to expanding the species represented, examining potential effects of chronic exposure is also critical. Most of the studies on Cu effects only tested for toxicity to acute, short-term embryonic exposure, typically 96 h to 7 d. Short-term exposure studies may seriously underestimate the effects of Cu and other contaminants on development and survival [17]. For example, northern leopard frogs (*Lithobates pipiens*) chronically exposed to Cu survived well (>96%) during early developmental stages (Gosner Stages [GS; 18] 12–14), but less than 10% of tadpoles in the high Cu treatment survived to metamorphosis [13]. Similarly, when American toads (*Bufo americanus*) were exposed to cadmium (Cd), survival was nearly 100% during the first 4 d, but by 60 d, survival had dropped to only 22% in the highest Cd treatments [19]. Furthermore, contaminant concentrations that are sublethal during acute exposure may be lethal during chronic exposure [20]. Given the suspected role of contaminant exposure in amphibian population declines [1], examining the full range of exposure scenarios that may negatively impact a population is important.

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Contaminants can affect animals directly through mortality, but exposure also can affect populations indirectly through impaired reproduction, reduced growth rates, abnormal development, or increased susceptibility to disease [21]. These indirect effects are known to influence fitness-related traits such as survival and reproduction. Both time to and size at metamorphosis impact recruitment into the adult population [22]. A prolonged larval period can result in mortality if metamorphosis is not achieved before temporary wetlands desiccate [23]. Similarly, reduced size at metamorphosis is known to negatively affect overwintering survival, survival to reproductive maturity, and age at first reproduction [24]. Because of this cascade of potential individual-level sublethal effects on populations, examining the sublethal effects of chronic Cu exposure is important.

We examined the effects of Cu exposure on the southern leopard frog, *Lithobates (Rana) sphenocephalus*, a widespread species for which no data on Cu exposure are available. For the embryonic and larval stages (through metamorphosis), our objectives were to measure the body burdens of Cu, examine both acute and chronic Cu exposure, and quantify lethal and sublethal effects.

MATERIALS AND METHODS

Study site

The study sites consisted of two wetlands located on the U.S. Department of Energy's Savannah River Site (SRS) in Aiken County, South Carolina. The first is the H-02 wetland complex, a surface flow treatment wetland constructed in 2006 to 2007 to alleviate water quality regulatory exceedances in Cu, Zn, and pH as a result of process and storm water discharge from several industrial facilities to a nearby stream. The facility effluent water enters an equalization basin (retention pond), then is fed into two 0.25-ha constructed wetland cells, which discharge to the stream. Copper, Zn, and pH levels are highest in the retention pond and influent ends of the wetland cells but much lower by the time effluent exits the cells (G. Mills, University of Georgia, Aiken, SC, unpublished data). The second site, Ellenton Bay, is a 10-ha Carolina bay isolated wetland on the SRS without a history of Cu contamination.

Study species

Southern leopard frogs are part of a closely related species complex (*L. pipiens* complex) that collectively ranges throughout most of North and Central America [25]. Southern leopard frogs are common in the southeastern quarter of the United States, where they inhabit shallow freshwater habitats, including ephemeral wetlands, ponds, irrigation canals, and stream edges [26]. On the SRS, they are locally abundant and can breed year-round; however, they typically have two major breeding bouts per year, one in early fall and another in winter [27]. Females lay large globular egg masses that may contain hundreds to thousands of eggs. Eggs hatch within 7 to 14 d, and the larval period lasts 75 to 180 d, depending on the season of oviposition [27]. Because of their biology, southern leopard frogs have the potential to be exposed to aquatic contaminants for several months during larval development and for much of their postmetamorphic life as adults inhabiting wetland margins.

Embryo trials

All laboratory studies took place at the Savannah River Ecology Laboratory on the SRS. We conducted two separate embryo trials, one in the Savannah River Ecology Laboratory greenhouse and one in the Savannah River Ecology Laboratory animal care facility (ACF). In these experiments, we precisely controlled water chemistry variables by using a standard mix of synthetic dilution water for toxicity tests with freshwater organisms [28]—48 mg/L $NaHCO_2$, 30 mg/L $CaSO_4$, 30 mg/L $MgSO_4$, and 2 mg/L KCl added to nanopure Milli-Q water (Millipore), plus appropriate levels of Cu from a 200.4 μg/ml $CuSO_4$ solution (pH 7.2–7.4; hardness 40–48 mg/L $CaCO_3$; alkalinity 30–35 mg/L $CaCO_3$).

In the two embryo trials (greenhouse and ACF), our goal was to identify the Cu concentration(s) at which embryo mortality increased significantly compared with controls. For the greenhouse trial, we collected three egg masses from the influent end of the H-02 wetland cells on September 21, 2009, and used a factorial design with 3 clutches × 5 Cu treatments replicated four times, yielding a total of 60 containers. Trial 1 began September 23, 2009, with embryos approximately 50 h old at GS 15 to 17 [18], and ended September 29, 2009; greenhouse daily max/min temperatures ranged from 11 to 33°C during this period. For trial 2 in the ACF, we used younger embryos and more precise control of environmental conditions. We collected four egg masses from Ellenton Bay on October 16, 2009 and used a factorial design with four clutches × 5 treatments replicated four times, totaling 80 containers. Trial 2 began on October 17, 2009 with embryos approximately 34 h old at GS 10 to 13, and ended October 27, 2009. Animal care facility temperatures ranged from 18 to 21°C and the light:dark cycle was maintained at 12:12 h. In both trials, we only collected egg masses that were large (>1,000 eggs), separated from other egg masses by more than 1 m, and that, based on visual inspection, appeared healthy in terms of having well-formed eggs. We held extra eggs not needed for experiments for several days to ensure that they developed. We placed a subset of eggs from each clutch in 0.5-L containers with 400 ml synthetic water and Cu solution. Quickly counting and removing a specific number of eggs without damaging them was difficult; therefore, clutches were apportioned among containers into subsets of 9 to 47 embryos, which we subsequently counted as development occurred. Our five aqueous Cu concentrations for both trials were 0, 10, 50, 100, and 150 μg/L Cu. In both trials we randomized placement of containers on a table and inspected them daily to note and remove any nonviable or dead embryos. We did not perform water changes in the embryo trials. We ended trials 1 and 2 at GS 25 to 26, when larvae are free swimming and feeding [18].

Larval trial

For the larval trial, we used a subset of the survivors from the ACF embryo trial (trial 2). We used a factorial design of 4 clutches × 4 Cu treatments, with 12 replicates, totaling 192 containers. We ran the larval trial from October 27, 2009 to July 1, 2010. We reared larvae individually in 1-L containers (~800 ml solution) at the same Cu concentrations they experienced during the embryo trial (0, 10, 50, and 100 μg/L). We did not continue the highest Cu concentration treatment (150 μg/L), because the high mortality rate in the embryo trials yielded too few survivors. We randomized placement of all containers and inspected them daily. For the first three months, water was changed weekly and twice weekly thereafter. We performed partial water changes by removing 50% of the water along with any debris, and then added fresh synthetic water and a volume-adjusted dose of Cu to maintain the proper concentration. Initially, tadpoles were fed a size-adjusted food ration

equal to 1 g food/g body weight twice per week that ranged from 0.2 to 1.5 g per feeding. However, as larvae grew, we noticed they never ate more than 0.9 g food between feedings. To avoid fouling the water, we switched to providing 0.9 g food twice per week. We formulated a diet based on previous studies [29], with modifications suggested to improve nutritional quality (W. Hopkins, Virginia Tech University, Blacksburg, VA, personal communication). We dissolved 20 g agar and 14 g gelatin into 750 ml type I grade water (18.1 MΩ; Millipore). We then added a blended mixture of 16 g ground Sera Micron (Sera North America), 16 g TetraMin Tropical Granules (Tetra), 110 g AquaMax Grower 600 (PMI Nutrition International, LLC), and 110 g Classic Pet Rabbit Food (LM Animal Farms). We spread and cooled the mixture in plastic molds to create uniform size pellets. We reserved a sample of the food pellets for metals analysis.

We inspected containers daily for dead larvae, which we removed. For these larvae, we documented their mass, presence or absence of malformations, and date of mortality, and stored them in Whirl-Paks at −20°C for subsequent metal analysis. Malformations were present only in tadpoles and included edema and scoliosis of the tail. For individuals that survived to metamorphosis, we measured mass, snout–vent length, and when front legs appeared and the tail was resorbed. We euthanized metamorphs by immersion in 3% tricaine methanesulfonate (MS-222) and stored them at −20°C.

Metals analysis

We freeze-dried biological samples collected for metal analysis and homogenized samples greater than 250 mg dry mass in a coffee grinder. When possible we analyzed individuals separately, but in instances of small sample mass (e.g., small tadpoles or metamorphs) we combined samples having a similar exposure history. We did not use any larvae that died early in the experiment, to reduce variation in exposure time. Across the 0-, 10-, and 50-μg/L treatment groups the ages of larvae and metamorphs (GS 46) we examined ranged from 91 to 95 d and 108 to 182 d, respectively. In the 100-μg/L treatment the ranges were larger; 91 to 251 for larvae and 119 to 220 for metamorphs. In all cases we only combined individual larvae within 4 d of age and individual metamorphs within 10 d of age within a sample. We digested a subsample (~250 mg) of each homogenized sample in 10 ml trace metal grade nitric acid (70% HNO_3) using microwave digestion (MarsExpress, CEM). After HNO_3 microwave digestion, we brought samples to a final volume of 15 ml with 18 MΩ deionized water. We used inductively coupled plasma mass spectrometry (PerkinElmer) to determine concentrations of Cu in our samples as well as in certified reference material (TORT-2 and LUTS-1; National Research Council of Canada). The detection limit for Cu across seven inductively coupled plasma-mass spectrometry runs was 1.7 ± 0.84 μg/L, and the mean percent recovery for Cu in the reference tissue was $102.5 \pm 15.0\%$ for TORT-2 and $102.2 \pm 17.0\%$ for LUTS-1. We collected liver tissue from 27 metamorphs for use in a separate gene expression study; these individuals were used for analyses of survival, body size, and larval period but were excluded from analyses of final metal concentrations. To assess the ecological relevance of the Cu accumulation we observed in laboratory trials, we compared Cu body burdens in frogs for which we manipulated only aqueous Cu levels to body burdens in metamorphs collected from the field (reported in a separate study) that experienced the extremes of the Cu gradient in the H-02 treatment wetlands.

Statistical analysis

We performed all statistical tests using SAS Ver. 9.2 [30]. We used PROC GLM to test for effects of Cu concentration, clutch, and their interaction on embryo survival in our two-factor randomized block design, with initial egg number as a covariate in an analysis of covariance model. For larvae and metamorphs, we used a nonparametric log-rank test in PROC LIFETEST [31] to test the homogeneity of survival curves among treatment groups; we treated larvae that survived to metamorphosis as censored data. We tested for differences in larval survival among clutches and Cu levels with PROC LOGISTIC. For animals that reached metamorphosis, we used path analysis (PROC TCALIS) [32] to assess the effects of clutch and Cu level (exogenous variables) on larval period and body size (endogenous variables), as well as the direct effect of larval period on size at metamorphosis and the indirect effects of the exogenous variables on size as mediated through the larval period. We used the standardized path coefficients equal to the beta coefficients divided by the standard deviation of that coefficient so that they can be directly compared to assess relative magnitude of effect. We used dry mass at metamorphosis as our measure of body size, and we natural log transformed dry mass and length of larval period for all statistical analyses.

RESULTS

Copper concentrations in tissue

After combining some individuals as described, we analyzed a total of 12, 19, 19, and 17 larval samples and 4, 4, 5, and 5 metamorph samples for the 0-, 10-, 50-, and 100-μg/L treatments, respectively. Larvae that died during the experiment attained Cu body burdens up to 595 μg/g in the 100-μg/L treatment, and larval Cu levels were higher than concentrations in surviving metamorphs ($p < 0.0001$, $F_{1,\ 52} = 23.93$; Fig. 1). Copper body burdens were significantly affected by treatment

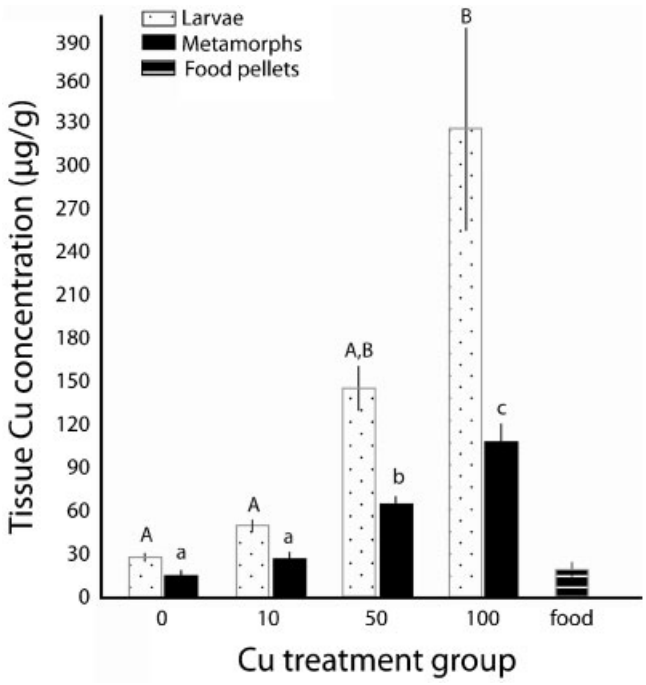

Fig. 1. Tissue concentration (μg/g dry wt) of Cu in *Lithobates sphenocephalus* larvae and metamorphs reared at four aqueous Cu concentrations. Columns marked with different letters are significantly different from each other.

($p < 0.0001$, $F_{3,\ 52} = 18.07$; Fig. 1). Overall, the Cu concentration observed in experimental animals compared favorably with the range of Cu tissue concentrations in field-collected larvae (up to 493.4 μg/g) and metamorphs (82.6 ± 8.9 to 14.4 ± 2.1 μg/g along the decreasing Cu concentration gradient), indicating that our laboratory tests were conducted at field-relevant concentrations. Food pellets fed to larvae contained 16.9 ± 0.4 μg Cu/g dry weight.

Embryo trials

We conducted two separate trials in different facilities (trial 1, greenhouse; trial 2, ACF) under different environmental conditions with *L. sphenocephalus* egg masses that were collected from two locations (trial 1, H-02 wetlands; trial 2, Ellenton Bay). Survivorship results differed between trials ($F_{1,130} = 27.4$, $p < 0.001$; Fig. 2), and we analyzed data from each trial separately.

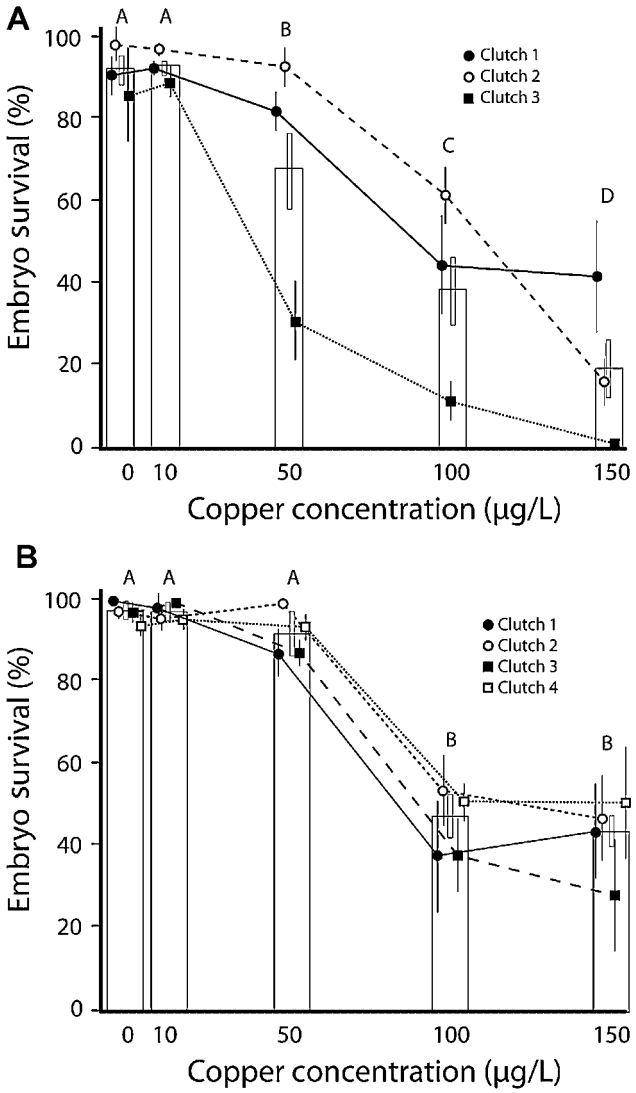

Fig. 2. Effect of aqueous Cu concentration on embryo survival (Gosner stage 25 feeding stage) in *Lithobates sphenocephalus*. Two separate trials were conducted at different times, and environmental conditions, using different source populations of clutches (trial 1, H-02 wetlands; trial 2, Ellenton Bay). Three clutches with four replicates per clutch were used in trial 1 (**A**); four clutches with four replicates were used in trial 2 (**B**). Columns represent treatment means (across clutches), and those marked with different letters are significantly different from each other. Lines indicate the differential survivorship patterns for each clutch.

For the three clutches used in trial 1, we observed a general trend of decreased survival to hatching at Cu levels above 50 μg/L ($p < 0.0001$, $F_{4,\ 52} = 51.2$; Fig. 2A). Post hoc comparisons of means showed no difference in embryo survival to hatching between 0- and 10-μg/L treatments, but a significant decline occurred among all higher Cu levels (Fig. 2). Survival also varied among clutches ($p < 0.0001$, $F_{2,\ 52} = 20.5$; Fig. 2A). Number of embryos per container ranged from 9 to 47 (mean ± SE for clutches 1 through 3 were 20.15 ± 2.31, 16.7 ± 0.99, 15.8 ± 1.04, respectively), and initial embryo number was positively related to survival ($p < 0.0003$, $F_{1,\ 52} = 15.3$), because of the significant effect of density in the 150 μg/L Cu treatment ($F_{1,10} = 14.1$, $p = 0.0038$). The interactions of treatment with clutch and treatment with initial egg number were nonsignificant and were removed from the model.

In trial 2, Cu concentration significantly affected embryo survival ($p < 0.001$, $F_{4,55} = 17.2$; Fig. 2B) in the four clutches. Mean survival at 0, 10, and 50 μg/L Cu was similar and was significantly greater than survival at 100 and 150 μg/L Cu ($p < 0.05$; Fig. 2B). Embryo survival to hatching differed among clutches ($p < 0.01$, $F_{3,\ 55} = 5.4$; Fig. 2B). Interpretation of main effects was confounded by a significant treatment by clutch interaction ($p < 0.04$, $F_{12,55} = 2.0$); in general, clutches showed similar response profiles across Cu treatments, with the exception of clutches 1 and 2 at 100 to 150 μg/L Cu. Despite a more consistent number of eggs placed in each container (range, 9–22; mean ± SE for clutches 1 through 4 were 13.35 ± 0.82, 13.30 ± 0.55, 13.20 ± 0.77, and 11.3 ± 0.56, respectively), initial egg number was positively related to embryo survival ($p < 0.01$, $F_{1,55} = 9.7$), most noticeably in the 100-μg/L treatment ($F_{1,14} = 4.5$, $p = 0.05$). A significant interaction was seen between treatment and initial egg number ($p < 0.01$, $F_{1,55} = 9.7$).

Larval trial

An error in our water change regimen resulted in the death of 34.4% of the tadpoles on day 91 of the experiment. Mortality was equivalent across Cu treatments ($p = 0.85$, $F_{3,9} = 0.27$) but was not similar among clutches ($p = 0.003$, $F_{3,9} = 10.3$). Mortality for clutch 4 (64.6%) from this event was significantly greater ($p < 0.05$) than that for clutches 1 (14.6%) or 3 (20.8%) but no different from that of clutch 2 (37.5%). For subsequent analyses, we eliminated the larvae that died on day 91 because of poor water quality.

Larval survival did not differ among Cu concentrations ($p = 0.22$, $\chi^2 = 4.43$, $df = 3$). Averaged across clutches, survival ranged from $73.1 \pm 12.9\%$ at 10 μg/L Cu to $52.1 \pm 7.1\%$ at 100 μg/L (Fig. 3). Survival curves also did not differ among Cu treatments over time ($p = 0.73$, $\chi^2 = 1.29$, $df = 3$). Survival was different among clutches ($p = 0.023$, $\chi^2 = 9.22$, $df = 3$), with clutches 1 and 2 exhibiting higher larval survival than clutch 4 ($p = 0.005$, $\chi^2 = 7.80$, $df = 1$ and $p = 0.023$, $\chi^2 = 5.17$, $df = 1$, respectively). The length of the larval period of survivors was affected by Cu level ($p < 0.0001$, $F_{3,79} = 8.87$), primarily because of increased duration of the larval period in the 100-μg/L treatment; larval period was also influenced by clutch ($p = 0.007$, $F_{3,79} = 6.25$; Table 1). Body dry mass at metamorphosis was affected by clutch ($p = 0.003$, $F_{3,79} = 5.07$) but not by Cu level ($p = 0.16$, $F_{3,79} = 1.77$; Table 1). Path analysis showed that larval period was negatively related to body size at metamorphosis, Cu level had twice the effect on larval period as clutch, and Cu level had a weak ($p = 0.06$) indirect effect on body size mediated through larval period (Fig. 4).

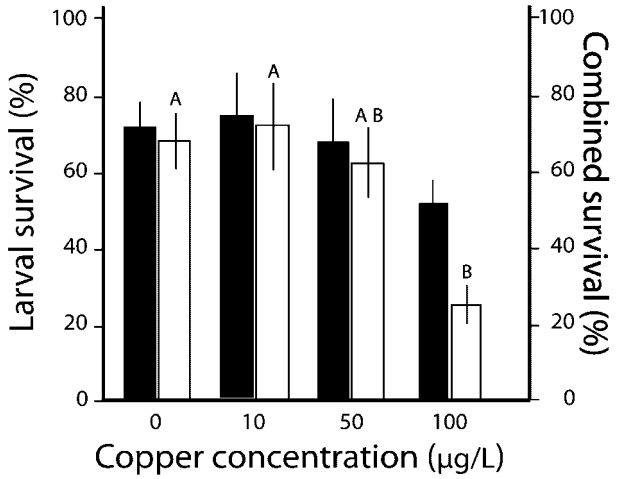

Fig. 3. Average larval survival (black bars) and combined embryonic and larval survival (white bars) for *Lithobates sphenocephalus* at four aqueous Cu concentrations. Larvae were reared individually, and the total surviving from each of four clutches was used to estimate treatment means. Columns marked with different letters are significantly different from each other.

The larvae in our trials are from the subset that survived embryonic development. For each clutch, we combined the average embryo success with the proportion of the larvae that metamorphosed at each Cu level to estimate overall survival from embryo to metamorphosis. Elevated Cu level reduced overall survival ($p = 0.0109$, $F_{3,12} = 5.80$); pairwise comparisons showed that overall survival at 100 µg/L was significantly less than at 0 or 10 µg/L (Fig. 3).

DISCUSSION

Southern leopard frog larvae and metamorphs from our experiments had measurable Cu body burdens. The amount of Cu observed in the control larvae corresponded to the amount naturally occurring in their formulated diet. Larvae from the Cu treatments had elevated Cu body burdens that fell within the range of larvae reared in the H-02 constructed wetlands (Gary L. Mills, University of Georgia, Aiken, SC, unpublished data); thus, our laboratory studies reflect field conditions present in our study system. The larval body burdens we observed are similar to those in northern leopard frogs reared under 0-, 5-, 25-, and 100-µg/L Cu treatments (Fig. 3 in Chen et al. [13]), and our whole-body estimates were intermediate between Cu levels in intestine and liver tissue in bullfrogs (*L. catesbeianus*) collected from contaminated sites [33]. We measured significantly less Cu in tissues of recent metamorphs than in larvae, a pattern noted by Roe et al. [34] for *L. sphenocephalus* at a coal fly ash-contaminated wetland, although the Cu levels in our experimentally treated animals were higher than they observed in field-collected leopard frogs. The mechanisms underlying the reduction in body burdens of Cu at metamorphosis are not completely understood [35]. During metamorphosis, substantial morphological and physiological changes and multiple possible pathways for elimination of trace elements occur [35]. Shedding of cells during tail absorption [36] and changes to the intestinal epithelium [37] can reduce burdens. Changes in the digestive tract are likely to be keys to elimination, because many trace elements bioaccumulate in the intestine [38]. Although much of the Cu is eliminated during metamorphosis, we observed significant accumulation in metamorphs, representing an important exposure pathway by which Cu is transferred from the aquatic environment to the terrestrial food web [34].

Table 1. Duration of larval period (mean days ± SE) and body size at metamorphosis (mean g dry mass ± SE) across four Cu treatments (µg/L) and four clutches

	Larval period duration	Body size
Cu treatment		
0	127.3 ± 6.4	0.135 ± 0.021
10	134.1 ± 6.9	0.152 ± 0.013
50	125.8 ± 2.9	0.153 ± 0.022
100	159.5 ± 11.4	0.137 ± 0.023
Clutch number		
1	126.9 ± 3.1	0.165 ± 0.008
2	143.8 ± 5.1	0.170 ± 0.012
3	128.5 ± 8.7	0.143 ± 0.016
4	147.4 ± 15.8	0.100 ± 0.015

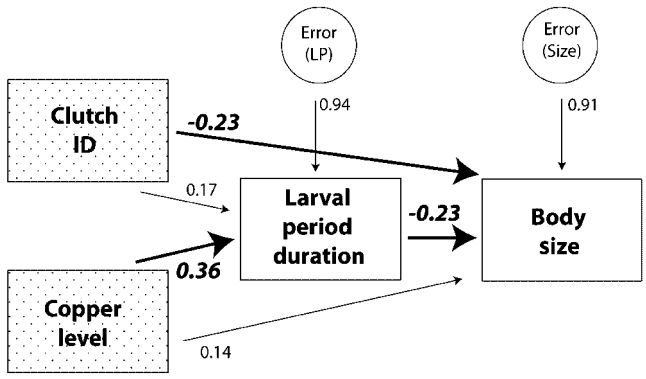

Fig. 4. Diagram of path analysis used to evaluate the relative contributions of female (i.e., clutch ID), Cu concentration, and duration of larval period on body mass at metamorphosis in *Lithobates sphenocephalus*. Arrows indicate causal paths, and line width indicates magnitude of the effect. Path coefficients for rectangles (manifest variables) are standardized partial regression coefficients; circles represent all unmeasured variables that affect larval period and body size (i.e., the error variances).

We observed a positive correlation between initial egg density and embryo survival to hatching, suggesting that detrimental effects of Cu exposure during early developmental stages may be mediated by experimental density of eggs. Importantly, density did not affect survival in our 0-µg/L treatments. Reasons for the observed density effect could be that egg jelly binds Cu and with higher numbers of eggs more total Cu is bound, thereby reducing the aqueous concentration that embryos are exposed to; that embryos bind Cu, which also would lower the remaining aqueous Cu concentration that each embryo experiences; and that clutch geometry changes with increased egg number, and more interior eggs in larger clutches may be buffered from Cu. Any mechanism that lowers Cu bioavailability to individual embryos may be most important when examining low concentrations or those close to the organism's threshold response level; we observed that a change of as little as 10 µg/L Cu can drastically affect survivorship to metamorphosis.

The fact that amphibian ecotoxicology studies use widely varying initial egg densities confounds comparisons among studies, even those examining the same species. Although many researchers standardize density within a single experiment, little standardization occurs across studies, with the exception of many Frog Embryo Teratogenesis Assay–Xenopus (FETAX) studies [39]. For example, egg densities in experiments may vary from 20 embryos in 10 ml media [40] to 14 to 20 embryos in 1.75 L media [41]. In addition to density differences, the

jelly coat is removed in FETAX [39] and some other studies [40], but not in many others [13,16,41], which can affect toxicity. Toxicological effects that are attributable to egg density make interpreting results obtained from different species or compounds difficult and may result in inaccurate risk assessments. Accounting for the potentially confounding effect of egg density would enhance our ability to make comparative assessments of species or population sensitivities to different contaminants. Future studies addressing the cause for the egg density effect on toxicity and determining whether this correlation holds true for other species and compounds are warranted.

Overall exposure to Cu significantly affected southern leopard frog embryo and larval survival and development. In both embryo trials, increased Cu concentrations led to a general decline in embryo survivorship, with a significant effect occurring at concentrations of 100 μg/L or greater. The reduced survivorship in our embryo trials is in contrast to the limited effect of elevated Cu on embryo survival in northern leopard frogs (*L. pipiens*) [13,14]. In Chen et al.'s study [13], embryos were not exposed to Cu treatment until GS 19. Earlier developmental stages such as gastrulation (GS 10–12) may be especially sensitive, perhaps by exposing embryos at GS 10 to 15 we saw a larger effect on survival. However, Lande and Guttman [16] exposed *L. pipiens* embryos immediately after fertilization to even higher Cu concentrations but still saw no effect on hatchling success (25–45% across all Cu levels). Importantly, although they used similar densities of embryos as in our study, they used more eggs (∼120) in a larger volume (1.5 L). We observed a significant reduction of Cu toxicity with increased density of embryos. The higher number of eggs used in the Lande and Guttman study may have reduced the effects of Cu if clutch geometry (see previous discussion) partly explains the increased survivorship with increasing density.

Once embryos reached the feeding stage (GS 25), Cu did not further affect survival to metamorphosis. Although a decline in survivorship was seen from 10 to 100 μg/L, the overall effect was not significant, which may be influenced by the reduced survivorship in our control (0 μg/L) treatment. The critical endpoint is whether individuals can survive to metamorphosis. By combining data from the embryo and larval trials, we see that the overall survivorship to metamorphosis is reduced in higher Cu concentrations. Copper is an essential element; mortality and malformations have been documented in *Xenopus laevis* reared in Cu-deficient medium. However, the amount of Cu in our formulated diet should be sufficient to prevent Cu deficiencies [42]. Again, our results were in contrast to those of Chen et al [13] with northern leopard frogs, in which 100 μg/L Cu significantly reduced tadpole survivorship. In their 100-μg/L treatment they saw little mortality (<4%) before hatching, but after hatching fewer than 10% of larvae survived to metamorphosis. We observe 22% survival from embryo to metamorphosis in the 100-μg/L treatment, with most mortality occurring before hatching. Thus, although the developmental stage most affected by Cu concentrations differed between the two studies, overall survivorship from embryo to metamorphosis was similar. Making comparisons with additional species is difficult because of differences in experimental design. For example, Cu treatment was not initiated until after GS 23 to 25 in studies of *Hyla versicolor* [15] or *Bufo arenarum* [8], and the concentrations of Cu used also varied widely [[8,13,15,16], present study]. Additional studies of other species with earlier exposure regimens are warranted to better understand species-level variation in sensitivities to Cu.

In addition to affecting survivorship, exposure to Cu had important sublethal effects. We observed a significantly longer time to metamorphosis in southern leopard frog tadpoles reared in 100 μg/L Cu. This effect on larval period also yielded an indirect effect of Cu on size at metamorphosis. Similar results were documented in northern leopard frogs [13]. In amphibians, size at, timing of, and survival to metamorphosis all influence recruitment into the adult population [22], and thus these attributes can be used to assess the effects of biotic and abiotic stressors on amphibian populations [20,24]. Larger size at metamorphosis can have a number of benefits, including greater overwintering success and survival to first reproduction, as well as earlier reproduction [24]. Additionally, larger females can carry more eggs, and larger males can access more females during breeding, leading to increased reproductive success [43]. Delayed metamorphosis or reduced size at metamorphosis can impact demographic processes of a population, potentially leading to declines or even local extirpations [20,44]. For amphibians breeding in temporary ponds, delayed metamorphosis can result in death because of pond desiccation, thereby limiting juvenile recruitment [23]. When average age to reproductive maturity is increased, population growth rates decrease [45]. A shift toward later reproduction in an amphibian community can alter the demographic structure of a population, resulting in a gradual population decline. Taken together, the combined effects of Cu on survival, larval period, and size at metamorphosis could have significant population-level impacts.

We designed our study to investigate variation among embryos and larvae from different clutches. This approach is atypical in amphibian ecotoxicology studies, in which mixing eggs or larvae from multiple clutches to evenly distribute the potential genetic effects is common [13,15,32,41]. In fact, in some studies determining either how many pairs of frogs were used to generate eggs or how eggs were mixed is difficult [8]. Clearly, mixing eggs from multiple clutches simplifies experimental design and analysis. However, a substantial amount of information is lost in the process. For example, independent of Cu treatment, we observed significant among-clutch variation in important endpoints such as time to and size at metamorphosis. Maternal effects are well known to affect variation in larval traits [46], so our results are not surprising. In addition, we found significant among-clutch variation in sensitivity to Cu. Bridges and Semlitsch [47] came to similar conclusions when looking at southern leopard frog sensitivities to carbaryl. They found significant among-family variation and attributed differences to additive genetic variance. The amount of among-clutch variation in sensitivity to these two very different contaminants suggests that mixing eggs from a small number of clutches will not adequately represent the breadth of variation that exists in most populations. Our results strongly reinforce Bridges and Semlitsch's [47] suggestion that any attempt to extrapolate to predict population- or species-level impacts from contaminant exposure would be misguided when only a few individuals are examined. We agree with their emphasis that if eggs are going to be mixed from multiple clutches a large number of clutches should be used, and preferably multiple populations should be examined. As pointed out by Mann [48], many amphibian ecotoxicology studies emphasize the need to understand the relationship between amphibian declines and environmental contaminants. To achieve that goal, understanding and distinguishing among individual-, population-, and species-level effects of contaminants is imperative. Conservation biologists have noted a large amount of variation seen in population declines within and among species [49] of amphibians

that inhabit physiographically similar habitats. Understanding what makes some species, populations, and individuals more resilient to anthropogenic stressors, such as environmental contaminants, is important for conservation biology and is lacking in most ecotoxicological studies.

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