

METAMORPHOSIS OF TWO AMPHIBIAN SPECIES AFTER CHRONIC CADMIUM EXPOSURE IN OUTDOOR AQUATIC MESOCOSMS

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(Received 9 November 2004; Accepted 31 January 2005)

Abstract—Amphibian larvae at contaminated sites may experience an alteration of metamorphic traits and survival compared to amphibians in uncontaminated conditions. Effects of chronic cadmium (Cd) exposure on the metamorphosis of American toads (*Bufo americanus*) and southern leopard frogs (*Rana sphenocephala*) were determined. The two species were reared separately from shortly after hatching through metamorphosis in outdoor mesocosms (1,325-L polyethylene cattle tanks) that simulated natural ponds and enhanced environmental realism relative to the laboratory. Both species exhibited a decrease in survival with increasing initial nominal aqueous Cd concentration. Cadmium treatment did not influence mass at metamorphosis for either species when survival was included as a covariate, but increased the age at metamorphosis for the American toads. The whole body Cd content of metamorphs increased with aqueous Cd treatment level for both species, and the American toads tended to possess more elevated residues. Cadmium quickly partitioned out of the water column and accumulated in and altered the abundance of the tadpoles' diet. Cadmium-contaminated sites may produce fewer metamorphs, and those that survive will metamorphose later and contain Cd. Interspecific differences in the response variables illustrate the importance of testing multiple species when assessing risk.

Keywords—*Bufo americanus* Cadmium Mesocosm Metamorphosis *Rana sphenocephala*

INTRODUCTION

Chemicals potentially harmful to amphibians have been found in the most remote montane lakes to highly urban streams. Most amphibians spend their embryonic and larval periods in aquatic habitats before metamorphosing into the terrestrial environment. Characteristics of the breeding site can have important effects on metamorphosis and early life-stage survival, and aquatic contamination is believed to have reduced amphibian abundance and species richness at some field sites [1,2]. Amphibians have been found in wastewater treatment wetlands, farm ponds, mining sites, and countless other contaminated habitats. Amphibians are unlikely to escape environmental degradation because they have high breeding-site fidelity and low mobility.

Laboratory tests have been crucial for understanding direct effects of contaminants on amphibians. However, these studies rarely examine indirect effects [3] and may greatly overestimate [4] or underestimate [5] responses that would occur in natural conditions because they tend to be acute (usually less than 7 d), maintained at a constant exposure concentration, and involve a single treatment variable (contaminant concentration) and route of exposure (across the skin or gills from water). Mortality can increase significantly when the exposure is chronic [5] or an additional stressor is present [6]. Dietary uptake of contaminants also can be important, but is difficult to manipulate realistically in the laboratory. Alternatives to traditional testing are needed to determine more accurately amphibian responses in contaminated habitats and routes of uptake. Mesocosms such as cattle tanks provide the benefits of a more realistic aquatic environment while still maintaining relatively controlled conditions [7]. Numerous investigators

have used cattle tanks to study amphibian ecology [7,8] and ecotoxicology [3,7]. The addition of litter (dead leaves or grass), plankton, and periphyton to cattle tanks subject to natural climatic conditions allows contaminants to partition into several mediums and incorporates environmental complexity and fluctuation. Contamination may act both directly on larval amphibians through dermal and oral uptake, and indirectly by altering the aquatic community [3]. Unfortunately, the interpretation of cattle tank study results can be more difficult than those obtained in the laboratory.

One of the most toxic metals amphibians encounter is cadmium. Aquatic habitats become polluted with Cd from terrestrial runoff, aerial deposition, and the release of effluent directly into water bodies. The chronic criterion for Cd as set by the U.S. Environmental Protection Agency is 0.15 µg/L (at 50 mg/L hardness; [9]), but concentrations may exceed 200 µg/L in some areas [10]. Cadmium is more bioavailable in soft water [11], so species such as amphibians that breed in rain-fed pools may be at increased risk. Larval amphibians reared in Cd-contaminated water can experience reduced growth [11–13] and survival [11–14]. However, Cd also can have hormetic effects [5]. Significant Cd uptake by tadpoles from water can occur within 24 h [15] and may increase with the length of exposure [15] and aqueous Cd concentration [12,13]. Frog tadpoles reared outdoors in small aquatic mesocosms containing leaf litter, sand, algae, and zooplankton dosed once with 100 µg Cd/L contained an average of 2.2 µg Cd/g whole body wet weight after three weeks, and only 25% survived to metamorphosis [14]. Larval amphibians collected from contaminated field sites have possessed in excess of 13 µg Cd/g whole body dry mass [16], and those exposed in the laboratory can withstand at least 60 µg Cd/g [13].

Cadmium added to the water column can partition into periphyton and plankton [17], polluting and altering the food

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source of amphibians. Periphyton often contains many times the concentration of aqueous Cd [18] and, when Cd is added experimentally to water, uptake by periphyton quickly can reach equilibrium [17]. Phytoplankton abundance may increase in Cd-contaminated systems [19,20] because of alteration of the zooplankton community and reduced grazing. However, Cd also has been shown to inhibit some phytoplankton species and change species composition [20,21]. These complex responses to Cd by aquatic communities reinforce the need for mesocosm and field studies (see also Wojtaszek et al. [22]), and the uncertainties in relying solely on laboratory data to predict the impacts of Cd in the environment [23].

The need for more environmentally relevant tests of Cd effects on amphibians is indicated from laboratory toxicity data and because of the potential for Cd to alter amphibian food resources. A cattle tank study was conducted to determine the effects of chronic Cd exposure on the metamorphosis of American toads (*Bufo americanus*) and southern leopard frogs (*Rana sphenoccephala*). These common U.S. species were chosen because they are habitat generalists that will breed in contaminated sites [24] and have not been tested previously for their metamorphic responses to Cd outside of the laboratory (American toads) or at all (southern leopard frogs). The selection of one bufonid and one ranid was deliberate because previous toxicological work indicates ranids may be relatively more sensitive [25]. These species are both herbivorous and have overlapping breeding seasons, but differ greatly in their normal average size at metamorphosis and length of larval period. Southern leopard frogs take longer to metamorphose, but do so at approximately 10 times the size of American toads and have more advanced ontogenetic development. Differences in behavior, physiology, and life history may result in differences in sensitivity to contamination.

MATERIALS AND METHODS

Study organisms

Study organisms were obtained on April 23 and 24, 2002, from two rain-fed forest ponds at the University of Missouri Baskett Wildlife Research Area in Boone County (MO, USA). This area has been set aside for ecological research since 1938 and has no known on-site sources of contamination, so ambient Cd concentrations were not determined. Three southern leopard frog (*Rana sphenoccephala*) egg masses were collected from one pond and portions of five American toad (*Bufo americanus*) egg strings were collected from the other pond. Oviposition had occurred within 48 h of collection. Eggs were transported in plastic buckets with source pond water to a laboratory at the U.S. Geological Survey Columbia Environmental Research Center (CERC) in Columbia (MO, USA). Eggs were allowed to hatch in the buckets, and daily partial water changes were made using well water diluted with deionized water (hardness = 60 mg/L as CaCO₃). This enabled the organisms to acclimate to the water being used for the exposure study. Once tadpoles were free swimming, they were fed ground fish flakes (TetraMin[®], Blacksburg, VA, USA).

Experimental mesocosms

Thirty-eight polyethylene cattle tanks (1.83-m diameter, 1,325-L volume; Behlen PolyTuff, Columbus, NE, USA) were set up to simulate natural ponds. They were positioned in a roughly rectangular array on a mown field at CERC. Approximately 950 L of well water diluted with deionized water to

a nominal hardness of 60 mg/L (as CaCO₃) were added to the tanks March 11 to 16, 2002, which resulted in a water depth of approximately 0.5 m. Immediately afterwards, 1 kg of deciduous leaf litter was added to each tank and allowed to settle to the bottom. The litter was collected from an oak-hickory-maple forest at the Baskett Wildlife Research Area. Equal volume aliquots of plankton and algae from several ponds in Boone County natural areas were added to the tanks approximately every 5 d for a total of four inoculations. Concentrated samples were obtained by pouring buckets of pond water through a 63- μ m plankton towing net. Collections occurred at a total of seven ponds, with different sites visited each day. For the duration of the study, each tank was covered with a lid made of fiberglass screen to prevent colonization by predeceous invertebrates. These methods were based on well-established procedures for amphibian cattle tank studies [8,26]. However, it should be recognized that variation in the creation of the larval environment might influence contaminant effects.

A stock solution was made by adding Cd (certified American Chemical Society CdCl₂·2.5H₂O crystals; Fisher Scientific, Fair Lawn, NJ, USA) to deionized water, and its exact concentration was confirmed before dosing the tanks. On April 22, 2002, appropriate volumes of the stock were added to the tanks to result in initial nominal aqueous concentrations of 0 (control), 5, 18, 60, and 200 μ g Cd/L. These concentrations are considered to be the treatment levels, although they did not remain constant over time. Stock solution first was added to a plastic watering can containing water from the respective tank, and then the contents were sprinkled evenly onto the surface of the tank water. Control tanks received the same treatment but without any stock solution added to the watering can, so that the level of physical disturbance would be the same among treatments.

Tadpoles were added to the experimental ponds on May 1, 2002, which was a few days after hatching and when individuals were at developmental stage 25 [27]. We combined the clutches within each species and used only free-swimming tadpoles that appeared healthy. Assignment of individual tadpoles to tanks was done haphazardly. Of the 38 total tanks, 22 each received 50 southern leopard frogs and 16 each received 120 American toads. Stocking rates were based on natural densities [28,29], and the species were reared separately to assess the effects of Cd on amphibian populations instead of communities. Tank replication was as follows for the different Cd concentrations (μ g/L): American toads, 0 ($n = 3$), 5 ($n = 3$), 18 ($n = 3$), 60 ($n = 3$), and 200 ($n = 4$); southern leopard frogs, 0 ($n = 4$), 5 ($n = 4$), 18 ($n = 4$), 60 ($n = 5$), and 200 ($n = 5$). Replication was unequal among concentrations and species so that enough metamorphs would be obtained for use in a subsequent study. The amphibian species and Cd treatment level for each tank were determined randomly.

The first metamorph (American toad) was observed on May 31, 2002. Thereafter, the tanks were checked daily and individuals with at least one emerged forelimb (stages 42–46) [27] were collected. Those with tails less than 2 mm long were weighed to the nearest 0.1 mg after being blotted dry. Metamorphs still needing time for tail resorption were kept on a laboratory bench in slanted plastic containers with softened well water that was changed daily. On August 12, when tanks were drained and the litter was sorted carefully for remaining individuals, there still were southern leopard frog tadpoles present. It was assumed that any unrecovered amphibians had

died, although it is possible that some were not detected both during the daily checks and at the study's end.

Some of the southern leopard frog and American toad metamorphs were sampled for whole body Cd analysis after they had been weighed. For the 0-, 5-, 18-, and 200- μg Cd/L treatment levels, two individuals from each of three randomly selected tanks were pooled into one sample for Cd analysis. From the 60- μg Cd/L concentration, there was a shortage of metamorphs, so only two samples were obtained for each species, and the two southern leopard frogs pooled for each sample may or may not have come from the same tank. Not enough southern leopard frogs survived in the 200- μg Cd/L treatment level to submit tissue for analysis, but American toads were sampled as described previously. Amphibians were stored individually at -15°C until they were freeze-dried and shipped for Cd analysis.

Mesocosm sampling techniques

All tanks were sampled regularly for water quality, Cd, and phytoplankton abundance. Sampling was conducted on the following dates: April 19 (pre-Cd dosing), April 23 (post-Cd dosing; only aqueous Cd concentration determined), April 29 (pre-tadpole addition), May 9, May 20, May 30, and June 14. On June 14, only the southern leopard frog tanks were sampled because most American toads had metamorphosed. A cylindrical polyvinyl chloride sampling device was used to obtain a 1-L water sample from four predetermined locations within a tank. These 4 L were combined in a 19-L polyethylene bucket. In order to prevent cross-contamination, each tank was assigned its own bucket and the sampling device was rinsed between tanks. Five hundred milliliters were placed in a plastic bottle and stored in a cooler with ice. The contents of the bottle were used for phytoplankton (as chlorophyll *a*), Cd, and water quality analyses. Water quality parameters included temperature, dissolved oxygen, pH, alkalinity, and hardness. Aqueous Cd was determined in two randomly selected tanks from each treatment level, irrespective of amphibian species. Approximately 20 ml of tank water were added to a plastic scintillation vial, acidified with HNO_3 , and stored at room temperature until analysis. Chlorophyll analysis was performed according to procedures in Standard Methods [30]. Chlorophyll was measured by filtering 100 ml of tank water through a glass fiber filter, refrigerating (4°C) the filter overnight in buffered acetone, and analyzing a 7-ml sample of the extract before and after acidification with 0.1 N HCl using a Turner Designs 10-AU fluorometer (665–870 nm; Sunnyvale, CA, USA).

Periphyton was sampled on May 10 and June 4 in two tanks randomly selected from each Cd treatment level, regardless of species. Each tank contained eight 7.5- \times 12.5-cm polyvinyl chloride tiles hung equidistant along a crosswire with fishing line, so that the tiles were submerged just below the water surface. The tiles had been added to the tanks on March 30 and 31 in order to be colonized by periphyton and to gauge environmentally relevant partitioning of Cd from water into the tadpoles' food source. Both sides of these tiles were scraped using a razor blade, and the contents were placed in a plastic bottle for Cd determination and stored at -15°C .

Cadmium samples were analyzed by the Columbia Environmental Research Center (unfiltered water only; Columbia, MO, USA) and the Mississippi State Chemical Laboratory (periphyton and amphibian tissue only; Mississippi State, MS, USA). Cadmium in the water column was determined using inductively coupled plasma mass spectrometry (Sciex Elan

6000, Perkin-Elmer, Boston, MA, USA). Periphyton and amphibian tissue samples were freeze-dried, block digested in HNO_3 , and analyzed with atomic absorption (Solaar M6, TJA Solutions, Franklin, MA, USA). Quality control included duplicates, blanks, spikes, and certified standard reference solutions. The standards were within 12% of nominal concentrations, recovery from spikes ranged from 96 to 100%, and the contribution from blanks was insignificant.

Statistical analysis

Age at metamorphosis was recorded as the number of days from the time tadpoles were added to the tanks to the time metamorphs were weighed. Percent survival was the number surviving (i.e., tadpoles plus metamorphs) divided by the number initially stocked, and was determined only after the study was terminated. Differences among treatment levels in the number of survivors that metamorphosed indicate effects on rate of development [3]. Therefore, percent metamorphosis was calculated as the number of individuals that reached at least stage 42 [27] (i.e., metamorphs) divided by the number surviving. Cadmium treatment effects on percent survival and metamorphosis were determined with analysis of variance (ANOVA) (American toads) or a ranked ANOVA (southern leopard frogs). A ranked ANOVA was performed because transformations failed to meet ANOVA assumptions. Percent metamorphosis was analyzed only for the southern leopard frogs because no American toad tadpoles were left at the end of the experiment (i.e., toad percent metamorphosis was 100% for every Cd treatment level). Analysis of covariance was used to determine the effect of Cd treatment on mass and age at metamorphosis, with percent survival as the covariate. The covariate adjusts for the varying density of larvae due to differential survival from Cd exposure, and should be used because density can affect mass and timing of metamorphosis [26]. Cadmium content detected in metamorphs (whole body) and periphyton was analyzed with ANOVA. Phytoplankton abundance was analyzed with repeated measures ANOVA, and the reported significance values were adjusted based on the Huynh-Feldt Epsilon when the assumption of type H covariance was not met [31]. To improve the assumptions of homogeneity of variance and normality, mass, age, phytoplankton, and Cd content were log-transformed and American toad survival was arcsine square root transformed. Variance and normality were checked with the Bartlett and Shapiro-Wilk tests, respectively. When multiple response variables are taken from single individuals, the possibility of dependence and an increase in the experiment-wide probability of a type-I error exist. Therefore, we determined if a significant relationship existed between the response variables mass, age, and percent metamorphosis for each species using the Pearson correlation. In order to better understand which levels of Cd had significant effects on the metamorph response variables, the Tukey's studentized range test was performed when the main effect was significant. All analyses were on type-III sum of squares to account for unequal replication. Statistical significance was set at $\alpha = 0.05$ and analyses were conducted with the software SAS® [31].

RESULTS

Mesocosm sampling

Five measures of tank water quality were determined approximately every 10 d and the means and ranges, respectively,

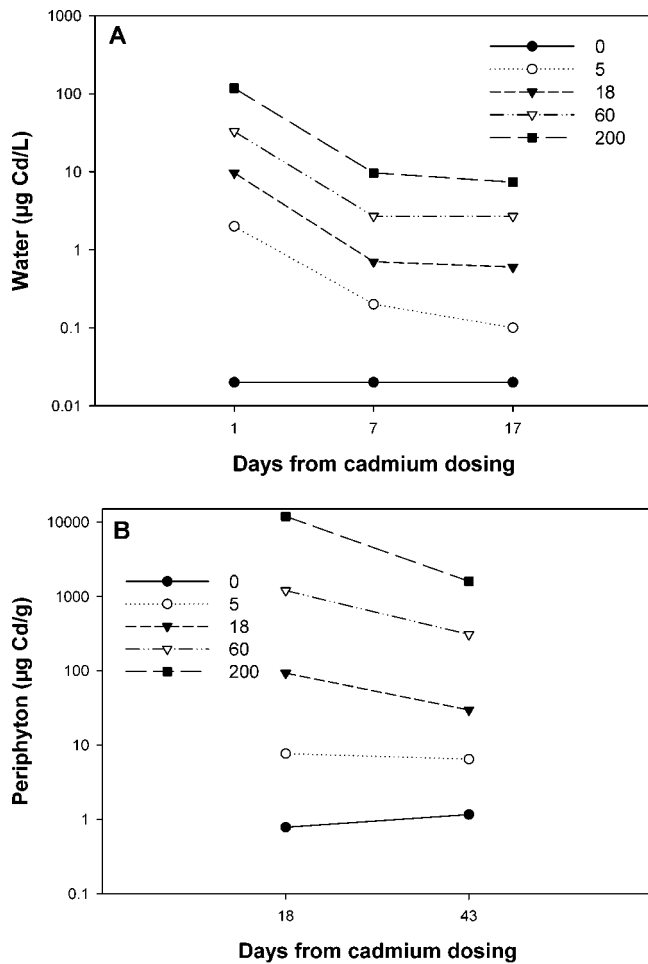


Fig. 1. Mean cadmium concentration of (A) the water column and (B) periphyton in mesocosms containing American toad (*Bufo americanus*) or southern leopard frog (*Rana sphenoccephala*) tadpoles. Each line represents a different initial nominal aqueous cadmium dose ($\mu\text{g/L}$), and means were derived from two tanks per cadmium treatment level.

across both species and all Cd levels were temperature (23.2°C, 14.4–31.8°C), dissolved oxygen (7.0 mg/L, 2.7–11.3 mg/L), pH (7.6, 7.2–8.7), alkalinity (42 mg CaCO₃/L, 27–70 mg CaCO₃/L), and hardness (49 mg CaCO₃/L, 32–73 mg CaCO₃/L). Aqueous Cd concentrations 24 h after dosing were 40 to 59% of nominal, but proportional to the intended dose (Fig. 1A). A rapid drop in concentration has been documented in other studies using experimental ponds [19], and likely is due to partitioning into other mediums. Cadmium in the water column decreased over time so that, by 7 d after dosing, less than 10% of the initial nominal concentration remained (Fig. 1A). The Cd content of periphyton increased significantly with initial nominal aqueous Cd concentration (day 18: $F = 55.56$, $df = 4$, $p = 0.0002$; day 43: $F = 114.36$, $df = 4$, $p < 0.0001$), and contamination in the noncontrol tanks was lower 43 d after dosing relative to 18 d (Fig. 1B). Bioconcentration factors were generated for each treatment level by dividing the concentration of Cd in periphyton 18 d after dosing by the average of the aqueous Cd concentrations on days 7 and 17 after dosing, and were as follows: 5 $\mu\text{g Cd/L} = 51,000$, 18 $\mu\text{g Cd/L} = 142,308$, 60 $\mu\text{g Cd/L} = 444,444$, and 200 $\mu\text{g Cd/L} = 1,391,813$.

American toads

The mean responses of American toads to chronic Cd exposure are reported in Table 1. A significant correlation was found between age and mass ($r = -0.838$, $p < 0.0001$), so alpha was lowered to 0.025 for these variables by dividing $\alpha = 0.05$ by 2 (2 representing the two correlated variables). The controls had the highest survival, largest mass at metamorphosis, shortest larval period, and lowest Cd body burden (Table 1). Percent survival decreased significantly with increasing Cd concentration, and age at metamorphosis significantly increased (Table 2). Mass had a trend of decreasing with increasing Cd, but the treatment effect was not significant when survival was included as a covariate (Table 2). Without the covariate, both mass ($F = 12.05$, $df = 4$, $p = 0.0005$) and age ($F = 50.73$, $df = 4$, $p < 0.0001$) were significantly affected by Cd treatment. This indicates density dependence and that Cd affected these response variables by altering survival. It appears that Cd became particularly toxic at some level greater than 18 $\mu\text{g/L}$. The few metamorphs produced at the two highest

Table 1. Mean percent survival and metamorphosis, mass, age, and cadmium content of American toad (*Bufo americanus*) and southern leopard frog (*Rana sphenoccephala*) metamorphs.^a Numbers in parentheses represent one standard error, and original stocking densities per tank were $n = 120$ and $n = 50$ for each species, respectively

	Initial nominal aqueous concentration ($\mu\text{g Cd/L}$)									
	American toads					Southern leopard frogs				
	0	5	18	60	200	0	5	18	60	200
Survival (%)	88.6A (± 3.9)	80.6A (± 5.8)	70.0A (± 10.6)	18.6B (± 10.0)	16.0B (± 4.9)	90.5A (± 5.9)	75.0AB (± 5.4)	44.0B (± 9.1)	11.6C (± 5.7)	1.2D (± 0.5)
Metamorphosis (%)	100.0 (± 0.0)	100.0 (± 0.0)	100.0 (± 0.0)	100.0 (± 0.0)	100.0 (± 0.0)	98.6 (± 1.4)	85.1 (± 8.1)	94.4 (± 4.3)	87.5 (± 7.7)	60.0 (± 24.5)
Mass (g)	0.145 (± 0.014)	0.110 (± 0.002)	0.101 (± 0.011)	0.078 (± 0.007)	0.072 (± 0.006)	0.926 (± 0.071)	1.053 (± 0.041)	1.528 (± 0.124)	1.826 (± 0.248)	1.519 ^b — ^c
Age (d)	33.5A (± 0.3)	37.2A (± 0.5)	38.3A (± 1.0)	50.6B (± 2.9)	63.6C (± 3.0)	64.3 (± 4.9)	65.6 (± 3.2)	73.6 (± 1.3)	74.9 (± 3.8)	70.0 ^b —
Cd content ($\mu\text{g/g}$)	1.7A (± 0.5)	16.7B (± 4.3)	31.0BC (± 12.5)	235.5D (± 144.5)	80.3CD (± 3.7)	0.5A (± 0.2)	14.0B (± 2.3)	11.0B (± 2.0)	25.5B (± 4.5)	—

^a Differing capital letters within species indicate significant differences due to cadmium concentration according to the Tukey test.

^b $n = 1$.

^c Not determined

Table 2. Results of univariate analyses of (co)variance for percent survival and metamorphosis, mass, age, and cadmium content of American toad (*Bufo americanus*) and southern leopard frog (*Rana sphenoccephala*) metamorphs^a

Response variable	Source	<i>MS</i>	<i>df</i>	<i>F</i>	<i>p</i>
American toads					
Survival	Cadmium	0.5228	4	19.08	<0.0001
	Error	0.0274	11		
Mass	Survival (covariate)	0.0003	1	1.57	0.2388
	Cadmium	0.0004	4	2.16	0.1476
	Error	0.0002	10		
Age	Survival (covariate)	0.0117	1	3.11	0.1085
	Cadmium	0.0334	4	8.86	0.0025
	Error	0.0038	10		
Cadmium content	Cadmium	8.7617	4	30.03	<0.0001
	Error	0.2918	9		
Southern leopard frogs					
Survival	Cadmium	201.0625	4	46.03	<0.0001
	Error	4.3676	17		
Metamorphosis	Cadmium	19.8938	4	0.51	0.7288
	Error	38.9662	17		
Mass	Survival (covariate)	0.0138	1	0.41	0.5357
	Cadmium	0.0488	3	1.44	0.2831
	Error	0.0338	11		
Age	Survival (covariate)	0.0221	1	2.28	0.1595
	Cadmium	0.0033	3	0.34	0.7972
	Error	0.0097	11		
Cadmium content	Cadmium	9.0045	3	69.27	<0.0001
	Error	0.1300	7		

^a *df* = degrees of freedom; *MS* = mean sum of squares; *F* = value of *F* test statistic; *p* = probability of *F* value.

Cd levels were very small and lethargic and seemed to be in poor condition. The whole body Cd concentration found in metamorphs showed a general trend of significantly increasing with treatment level (Tables 1 and 2), and as high as 380 µg/g dry weight was detected in one sample. The Cd ($F = 4.50$, $df = 4$, $p = 0.0245$), time ($F = 42.67$, $df = 4$, $p < 0.0001$), and their interaction ($F = 3.57$, $df = 16$, $p = 0.0005$) had a significant effect on phytoplankton abundance. Phytoplankton increased with time and was greatest in the two highest Cd treatment levels (Fig. 2A).

Southern leopard frogs

The mean responses of southern leopard frogs to chronic Cd exposure are reported in Table 1. Only one individual in the highest Cd treatment level (200 µg/L) survived long enough for mass and age at metamorphosis to be determined. Therefore, this concentration was not included when analyzing Cd effects on these two response variables. No significant correlations between mass, age, and percent metamorphosis were found, so alpha was not adjusted. Increasing Cd treatment level resulted in lower rates of survival and metamorphosis, and larger and older metamorphs (Table 1). However, these trends were statistically significant only for survival (Table 2). The highest Cd level had very few survivors, many of which still were tadpoles when the experiment was terminated. Without the use of survival as a covariate, age at metamorphosis still was not significant ($F = 2.40$, $df = 3$, $p = 0.1191$), and mass significantly increased with Cd level ($F = 11.89$, $df = 3$, $p = 0.0007$). Exposed southern leopard frogs possessed a significant amount of Cd (Tables 1 and 2), and the highest detected concentrations were from the highest of the sampled treatment levels (60 µg/L). A highly significant effect was found of Cd, time, and their interaction (for all, $p < 0.0001$) on phytoplankton abundance. As was found with the American

toads, phytoplankton generally increased with time and was greatest in the most contaminated tanks (Fig. 2B).

DISCUSSION

Changes in larval life-history traits and whole body Cd concentration were observed in American toads and southern leopard frogs at and above the lowest initial nominal aqueous concentration tested (5 µg Cd/L). The two species generally had the same directional trends in their responses to chronic Cd exposure as tadpoles, but there were interspecific differences. Noncontrol metamorphs of both species possessed very high amounts of Cd on a whole body dry weight basis, and American toads always had higher body burdens than southern leopard frogs at a given exposure level. It has been documented previously that different amphibian species in the same field-exposure scenario will accumulate different amounts of a contaminant [32]. Differences between the two species in contaminant uptake and retention may help explain differences in some of the other response variables. For both species, survival was approximately 90% in the controls and declined with increasing Cd treatment level, so that at the highest initial nominal dose (200 µg/L) survival was only 1% (southern leopard frogs) and 16% (American toads). Southern leopard frogs experienced higher mortality than American toads in each of the four Cd levels. The study was terminated after 103 d, at which time 36 southern leopard frog tadpoles were found. Cadmium treatment did not significantly affect percent metamorphosis, but percent metamorphosis was highest and lowest in the control and 200 µg Cd/L treatment levels, respectively. Age at metamorphosis increased with increasing Cd treatment level for both species, although the trend was significant only for the American toads when density dependence was accounted for by using survival as a covariate. This suggests direct Cd toxicity influenced the age of American toads but not southern

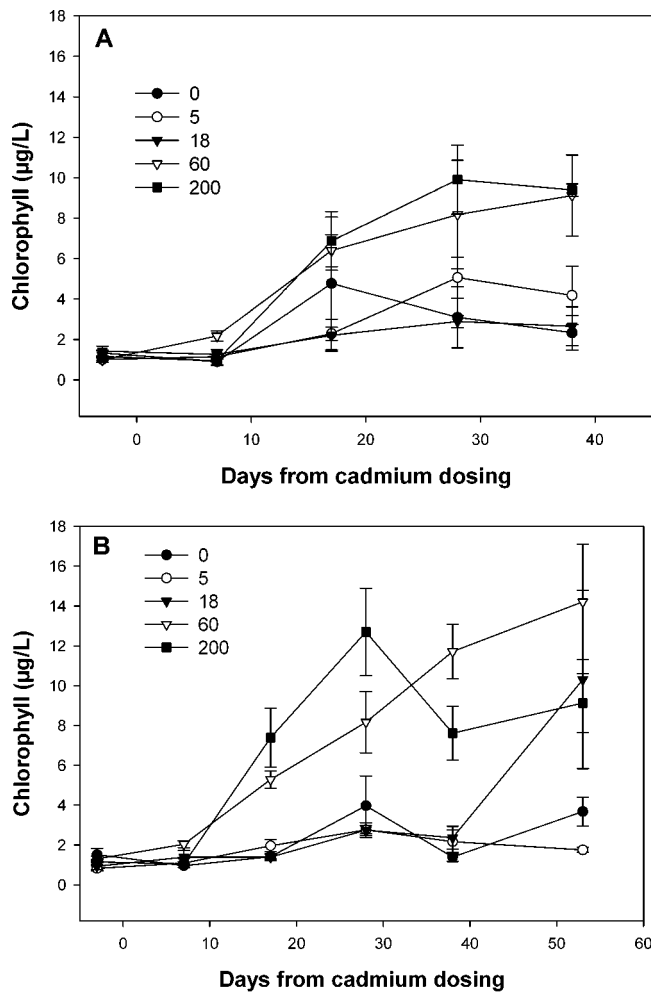


Fig. 2. Mean (\pm standard error) phytoplankton abundance (measured as chlorophyll *a*) over time in mesocosms dosed once with one of five nominal concentrations of aqueous cadmium ($\mu\text{g/L}$) and containing (A) American toad (*Bufo americanus*) or (B) southern leopard frog (*Rana sphenocephala*) tadpoles. Means were generated from all replicate tanks, which ranged in number from three to five.

leopard frogs. Control toads metamorphosed almost twice as quickly as those at 200 $\mu\text{g Cd/L}$. The nonsignificant results for both percent metamorphosis and age at metamorphosis provide strong evidence that direct Cd toxicity does not affect the development rate of southern leopard frogs. Southern leopard frog mass increased and American toad mass decreased with increasing Cd treatment level, but these trends were insignificant with survival as a covariate. Dropping survival as a covariate for analyses on the mass and age at metamorphosis of both species resulted in lower *p* values, which suggests Cd affected these response variables through density dependence and competitive release.

The noteworthy difference in mass response may be attributable to the greater mortality experienced by southern leopard frogs. Perhaps selection was strong enough that only the most robust southern leopard frogs survived. In another study it was suggested that tadpoles (*Rana catesbeiana*) from a site contaminated with metals were larger than those from a reference site because of reduced survival and competition for resources [33]. However, it is unlikely that differences in density via survival explain why the two species showed opposite trends in mass, because American toads metamorphose at a larger size with decreased density in uncontaminated conditions [34].

Another explanation could be that American toads are subject to more sublethal Cd stress than are southern leopard frogs. American toad metamorphs contained a higher Cd tissue burden than did southern leopard frogs, despite a shorter larval period. Cadmium may have impaired normal feeding behavior and harmed the digestive system [12,35]. It also is possible that the two amphibian species differ in their food preferences and that increasing Cd increasingly reduced the abundance or quality of the primary food source of the American toads (see also Mills and Semlitsch [3]). Southern leopard frogs predominantly feed on periphyton and phytoplankton; American toads are bottom-feeders and primarily consume periphyton and detritus [8], but relative consumption rates for either species are unknown.

The longer larval period of the southern leopard frogs seemingly would translate into a higher body burden of Cd. This was not the case, and may be because southern leopard frogs absorb less Cd from the environment than American toads due to differences in foraging behavior, physiology, excretion, or uptake [32]. Another reason could be a decrease in Cd in the food resources over time, which was documented in the periphyton. Hence, during the course of the larval period, American toads might have been exposed to higher concentrations on average than were the southern leopard frogs. However, it is possible that tadpoles experience peak whole body concentrations before metamorphosis [16] and that analyses of metamorphs instead of tadpoles is misleading when comparing uptake by the two species.

Negative effects of Cd on larval life-history traits could pose important risks to exposed amphibian populations. In uncontaminated conditions, less than 1% of eggs may survive to reproductive age [36]. Southern leopard frogs and American toads had no or low breeding success in ponds contaminated with Cd and other metals [24], which is not surprising given the present study's results. Reduced survival to metamorphosis due to aquatic pollution means that even fewer individuals will metamorphose and have a chance to become reproductive adults. Alterations in age and size at metamorphosis can have important impacts on adult fitness. Metamorphs that emerge larger and sooner are more likely to reach reproductive maturity at a faster rate and larger size [37]. Metamorphosing later means less time in the terrestrial environment to feed and grow before the first winter. A prolonged larval period also could result in desiccation in ephemeral pools, where a few days can be the difference between life and death.

The food sources of the tadpoles (i.e., periphyton, phytoplankton) also were affected by Cd. Cadmium accumulation in periphyton was substantial and proportional to the initial aqueous concentrations. The bioconcentration factors ranged from 51,000 to over one million, and increased with aqueous Cd treatment level. The Cd content of phytoplankton was not determined, but accumulation does occur [38]. Hence, although the aqueous concentrations of Cd declined over time, the tadpoles had to consume highly contaminated food. Also, there were measurable Cd treatment differences in phytoplankton abundance. Treatment levels did not differ from each other during the predose sampling, but did differ when sampled after dosing but before tadpole stocking. American toad and southern leopard frog tanks exhibited the same trend of phytoplankton increasing with Cd treatment level and then decreasing to an amount similar to the controls at 200 $\mu\text{g Cd/L}$. After tadpole addition, phytoplankton abundance tended to be highest in the two most-contaminated concentrations. Cadmium may have

affected phytoplankton directly and indirectly via altering grazer (e.g., zooplankton, tadpoles) or competitor (e.g., periphyton) abundance.

The responses of American toads to Cd differed from what was observed previously in the laboratory [5]. In 2001, tadpoles were reared in glass jars at a density of 1/L and exposed to 0, 5, 54, or 540 $\mu\text{g Cd/L}$. These concentrations were maintained chronically by redosing after water changes. Timing of exposure and water hardness were similar among the studies, but the laboratory water was kept at approximately 23°C, the aqueous Cd concentration did not drop over time, the tadpole density was higher, and the sole food source was fish flakes provided ad libitum. In the laboratory, percent survival was close to 100% for the three lowest concentrations, but dropped to 22% at 540 $\mu\text{g Cd/L}$. Excluding the highest concentration, the relationship to Cd treatment level for mass and age at metamorphosis was positive and negative, respectively; this is opposite to what was observed in the cattle tanks. Although the laboratory results indicate that Cd $\leq 54 \mu\text{g/L}$ has no effect on survival and seemingly confers the benefits of larger and younger metamorphs, the outdoor mesocosm study demonstrates that a single dose of Cd is lethal at far lower levels than was demonstrated in the laboratory and has detrimental sublethal effects. Direct comparison of the two studies is difficult because they differ greatly in abiotic and biotic characteristics, exposure routes, and concentrations. However, the two studies complement each other and provide a broader understanding of amphibian responses to Cd. In the laboratory study, the primary route of uptake likely was across the skin and gills, and exposure had a hormetic effect up to a point. This shows that aqueous Cd has direct effects on amphibian development. The mesocosm study indicates that responses may be quite different in more complex systems where there are multiple exposure mediums and both direct and indirect effects occur (see also Mills and Semlitsch [3]). The two studies combined strongly suggest that in situ and natural field studies also are needed to assess risk because environmental complexity can have important consequences but is difficult to manipulate (see also Thompson [39]).

This research documented effects of aqueous Cd treatment on amphibian survival, metamorphic traits, and the larval environment at environmentally realistic concentrations. The use of outdoor aquatic mesocosms enhanced environmental realism and furthered our understanding of the responses of two species for which published Cd toxicity data have been generated only in the laboratory (American toads) or not at all (southern leopard frogs). Interspecific differences were documented, which reinforces the need to incorporate multiple species when designing experimental studies to assess risk or determine causes of amphibian decline. Risk assessments that use these results should consider that the aqueous concentrations referred to are the initial nominal doses, the aqueous concentrations decreased over time, and Cd partitioned into a primary amphibian food source at high concentrations.

Acknowledgement—This manuscript was improved by the comments of N. Mills, S. Storrs, and three anonymous reviewers; M. Boone, N. Mills, and C. Witte kindly provided experimental methodology advice. Thanks to E. Brunson and D. Pestka for field assistance and T. May for Cd analysis. This research was funded by the Declining Amphibian Populations Task Force (Seed Grant), the Conservation Federation of Missouri, and the Conservation Foundation of Missouri Charitable Trust (Charles P. Bell Conservation Scholarship).

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