AMPHIBIAN METAMORPHOSIS AND JUVENILE TERRESTRIAL PERFORMANCE FOLLOWING CHRONIC CADMIUM EXPOSURE IN THE AQUATIC ENVIRONMENT

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The undersigned, appointed by the Dean of the Graduate School, have examined the dissertation entitled

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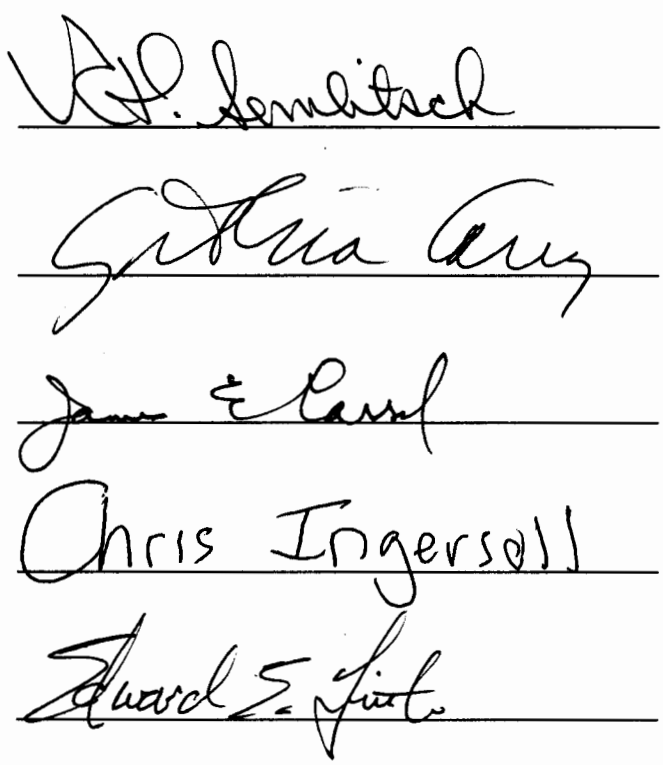

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ABSTRACT

Effective amphibian conservation requires an understanding of how populations respond to specific natural and anthropogenic factors. Chemical contaminants are among the known sources of acute and chronic stress and have been linked with habitat degradation and amphibian declines. Manipulative experiments were conducted to investigate the performance (i.e., growth, survival) of amphibians that metamorphose from chemically-contaminated aquatic breeding sites. The heavy metal cadmium (Cd) was selected as the focal contaminant because it bioaccumulates, is highly toxic, and occurs in polluted water bodies around the world. Effects of larval exposure to Cd on amphibian metamorphosis and juvenile performance were tested using the southern leopard frog (*Rana sphenocephala*) and American toad (*Bufo americanus*), two common North American habitat generalists that utilize Cd-contaminated breeding sites. It was expected that Cd exposure would decrease the growth and survival of tadpoles and juveniles because Cd affects the physiological processes and body condition of amphibians and other taxa.

In the first experiment, southern leopard frog and American toad tadpoles were reared separately in cattle tank mesocosms (1325-L volume) that had been dosed once at 0, 5, 18, 60, or 200 µg Cd/L. The experimental endpoints were survival, mass and age at metamorphosis, and whole body Cd content. Both species had a decrease in survival and increase in larval period and Cd content with increasing aqueous Cd concentration. However, southern leopard frog mass increased and American toad mass decreased with Cd exposure.

A subset of metamorphs from the first experiment was subsequently reared in terrestrial enclosures (2 m^2) located in a field along a forest edge. Metamorphs were obtained from all five Cd concentrations (American toads) or just the 0, 5, and 18 µg Cd/L treatments (southern leopard frogs). Juveniles were monitored for survival and growth in their first autumn and the following spring. There were no significant effects of Cd exposure history on the mass or growth rate of either species, but there a deleterious effect on American toad survival. Southern leopard frogs from the 18 µg Cd/L concentration were larger than the controls throughout the study.

A laboratory experiment was conducted on the terrestrial performance of southern leopard frog juveniles that metamorphosed from cattle tank mesocosms dosed once with 0, 5, or 18 µg Cd/L. Individuals were kept in separate containers for two months and fed either a low or high level diet of mealworms (*Tenebrio molitor*). Overall survival was 99%, indicating neither Cd nor diet were lethal. Initial differences in mass among Cd treatments were maintained within each diet level and those on the high diet weighed more than twice those on the low diet. The effect of Cd exposure history on juvenile mass depended on food abundance.

A fourth study was conducted to determine the combined effects of Cd concentration and interspecific competition on amphibian metamorphosis. Southern leopard frogs were the focal species and American toads were the competitor. Metamorphs were collected from cattle tank mesocosms that had been dosed once at 0, 5, or 18 µg Cd/L. Cadmium exposure decreased survival, increased mass and age at metamorphosis, and resulted in significant body burdens for the southern leopard frogs. Interspecific competition from the American toads increased survival and shortened the

larval period, decreased mass at metamorphosis, and had no effect on contaminant uptake. The effect of Cd on metamorph age depended on whether American toads were present.

A subset of southern leopard frog metamorphs from the fourth study was reared in terrestrial enclosures (9 m^2) in two habitat types for the final experiment. Enclosures were located in deciduous forests or open fields and juvenile growth and survival was determined in the first autumn following metamorphosis. Cadmium exposure history affected growth rate, mass, and survival. Initial differences in mass due to larval Cd exposure were maintained over time in each habitat type, but growth was highest in field enclosures and survival was highest in forest enclosures. The forest sites were cooler and wetter, which may have improved juvenile survival.

This research suggests that aquatic breeding sites polluted with Cd produce fewer, older, and contaminated amphibian metamorphs relative to uncontaminated sites. Those that survive to metamorphosis do not appear to be hindered by reduced terrestrial survival or growth as juveniles. However, Cd exposure influenced juvenile performance through interactions with terrestrial habitat quality. Assessments of the effects of contaminants on amphibians that incorporate multiple routes of exposure and other potential stressors may produce different outcomes than assessments that only manipulate the aquatic concentration.

CHAPTER 1

THE EXPERIMENTAL FRAMEWORK

The advancement of human civilization has largely depended upon the utilization of naturally occurring elements and the creation of new chemical compounds. Bronze brought tools and weapons to a new level, with iron and steel came technology and industrialization, and pesticides and fertilizers were dominant players in the Green Revolution. The benefits of such utilization were clearly seen, and embraced for seemingly improving the human condition. However, as 16th century European physician Paracelsus observed, "All things are poison and not without poison; only the dose makes a thing not a poison" (Institute of Medicine 2004). The Earth contains 92 natural elements, and populations of organisms have evolved to withstand or require the levels present in their habitats. But when concentrations increase due to human extraction, individuals and species are not always capable of tolerating the exposure. Many compounds were created to serve as poisons for particular organisms, but toxicity is rarely limited to targeted species. It was not until Rachel Carson's book *Silent Spring* was published in 1962 that a large public audience was made aware of the potential for chemical contamination to have harmful and complex effects on the environment (Carson 1962). The rich flora and fauna of the Earth were identified as innocent bystanders whose fate can be documented by their silence and absence. Following World War II, toxicology was expanded to include studies of toxic effects on non-human animals. A

further progression came in the form of ecotoxicology, which was coined in 1969 by Truhaut as an extension of toxicology that includes the effects of contaminants on populations and their habitats. Since then, there has been much research on the effects of contaminants on wildlife, particularly fishes and birds (Sparling et al. 2000). However, given the number of chemicals in the world today, the complexity of the environment, and the potential for interactions between chemicals and abiotic and biotic factors, the gap between what we know and what we should know is tremendous.

Amphibians have received relatively little attention by the toxicology community, which may be due to the secretive nature of amphibians. However, amphibians are important components of trophic food webs, and in some ecosystems amphibian biomass equals or exceeds that of small mammals or birds (Burton and Likens 1975). The number of toxicological studies on amphibians has increased dramatically in the last fifteen years, a likely result of concern about global declines in their populations (Sparling et al. 2000). Herpetologists from around the world met at the first World Congress of Herpetology in 1989 and discussed story after story of declining and disappearing populations (Wake 1998). Subsequent studies of amphibian population data revealed that major declines began to occur in the late 1950's (Houlahan et al. 2000) and that 43% of species are declining relative to only 0.5% that are increasing (Stuart et al. 2004). Amphibians appear to be disappearing at a higher rate than mammals or birds (Stuart et al. 2004). There are many proposed causes for the declines and it appears that there is no single culprit. Chemical contamination of habitats is one of the proposed causes (Wake 1998) and is thought to cause deformities (Ouellet et al. 1997), intersex gonads (Reeder et al. 1998, Hayes et al. 2003), and sex-ratio reversal (Reeder et al. 1998). Contaminants have

also been implicated in the loss of amphibian abundance or diversity at some field sites (Beyer et al. 1985, Kucken et al. 1994, Lambert 1997, Bishop et al. 1999, Davidson et al. 2001). A multiple stressor approach is now being adopted by the amphibian conservation community, including toxicologists, and deleterious effects have been found when amphibians are exposed to both contaminants and other potential stressors (Zaga et al. 1998, Kiesecker 2002).

The vast majority of amphibian toxicology research has been conducted in the laboratory using embryos or tadpoles. Chemical concentration is usually the only factor manipulated, and the most common endpoint is mortality. Laboratory studies are popular because they have a long and respected history, variables such as temperature can be controlled, physical disturbances are minimized, supplies and equipment are accessible, and data can be easily interpreted. Laboratory research has been important for determining mechanisms of action and has allowed for comparisons of relative sensitivity among taxa. Limited data (e.g., Hall and Swineford 1980, Schuytema et al. 1991) indicate that the early, aquatic amphibian life stages may be more sensitive to contaminants than the older, post-metamorphic life stages. Exposure for embryos and larvae may be at higher concentrations and more unavoidable than is the case for terrestrial life stages, given the breeding season of many species coincides with snowmelt or the application of agricultural chemicals, both of which may result in a pulse of chemicals entering breeding sites. Studies that manipulate only the chemical concentration are useful for determining direct toxicity, but other factors such as predation may influence the response of amphibians to contaminants (Relyea and Mills

2001). Mortality has definite fitness consequences, but sublethal endpoints such as growth and behavior may also have important effects.

Although laboratory tests have increased our understanding of the effects of contaminants on amphibians, studies in experimental mesocosms that incorporate direct and indirect effects and examine lethal and sublethal responses are needed. Complex systems are necessary to predict impacts on natural populations because of the ability of biotic and abiotic factors to influence amphibian ecology as well as contaminant distribution and toxicity. Sublethal responses are not as perilous as death, but do indicate a change which could render individuals more susceptible to other stressors or impact their ability to grow and reproduce. Chemical contaminants can adversely affect behavior, reproduction, growth, morphology, physiological processes, and organ function. Expected environmental concentrations cause sublethal effects more often than they cause lethality. Emphasis must also be placed on the metamorph, juvenile, and adult life stages, given reproduction is impossible without their survival and evidence that populations are declining due to a loss of adults (Carey et al. 2001). Analyses have determined it is the loss of post-metamorphic life stages that has the largest impact on amphibian populations (Biek et al. 2002, Vonesh and De la Cruz 2002). It is important to not only look at terrestrial contamination, but also the performance of amphibians that have metamorphosed from contaminated breeding sites. There is far too much uncertainty associated with using early life stage data to predict long-term effects on individuals and populations. Finally, it is important to conduct tests using native species, especially when there is concern that contamination is affecting a particular species in the wild. The African clawed frog (*Xenopus laevis*) has been touted as a model species for

laboratory studies, but is native to South Africa and fully aquatic. Assessments of multiple species have demonstrated that there can be large interspecific differences in sensitivity (Birge et al. 2000). Therefore, it should not be assumed that effects (and their magnitude) observed in one species will also be found in another.

The majority of amphibian toxicology research has been on metals, non-chlorinated pesticides, and acidification (Sparling et al. 2000). There has been less work with polycyclic aromatic hydrocarbons (PAHs), organochlorines (OCs), and polychlorinated biphenyls (PCBs) (Sparling et al. 2000), perhaps in part because the latter two are banned in some countries. Metals are of interest because they bioaccumulate and affect physiological processes such as enzyme function. Uptake may occur by oral, dermal, and pulmonary exposure. Elimination of metals from the body of an exposed organism can be a slow process, so that repeated exposure results in increased concentration over time (i.e., age-accumulation). Metals are also persistent in the environment and unlike many pesticides, do not degrade or transform. Metals are among the most frequently cited pollutants present at contaminated sites and can cause habitat degradation and alteration. Considerable work has been done on metal toxicity and bioaccumulation with a vast array of amphibian species (e.g., Khangarot and Ray 1987, Birge et al. 2000). However, as has proven to be the trend, the focus has been on acute, aqueous exposures, early life stages, and mortality. Expanding upon this body of knowledge with mesocosm and field studies that use multiple life stages and environmentally-relevant concentrations and exposure scenarios would increase our understanding of the impacts metals have on natural populations.

For my dissertation research I chose to study the effects of larval exposure to cadmium (Cd) on amphibian metamorphosis and subsequent terrestrial performance (i.e., growth, survival). Cadmium was selected because it is a U.S. Environmental Protection Agency contaminant of concern that is frequently found at contaminated sites and has the potential to bioaccumulate and alter food chains (Eisler 1985). It is mined with zinc and lead, and is used in industrial products and fertilizers. It occurs in water bodies and partitions into algae and other tadpole food resources, thus potentially resulting in oral, dermal, and gill exposure. Numerous laboratory studies of Cd effects on larval amphibians exist, and many have shown that bioaccumulation can be rapid and result in lethality or sublethal effects that are potentially detrimental to fitness. I have found only one study in which amphibian larvae were exposed to Cd in outdoor aquatic microcosms (Lefcort et al. 1998), but the concentrations tested were what would be found at a Superfund site and survival to metamorphosis was subsequently low. Because no research has been done on post-metamorphic amphibians, the fate of those surviving Cd exposure as larvae is unknown. Amphibians will breed in sites contaminated with Cd, but impacts on amphibian populations remain unclear and assessment is complicated by the fact that other metals and contaminants are usually present (Pollio 2001, Rowe et al. 2001).

My experimental approach acknowledges that contaminants are present within complex ecological systems. My work adds to our existing knowledge of Cd by using environmentally relevant concentrations ($\leq$200 μg/L) in a simulated pond exposure scenario and assessing the responses of multiple life stages. I conducted larval exposures in polyethylene cattle tank mesocosms because they have been used successfully in

ecological and ecotoxicological studies (Rowe and Dunson 1994, Boone and James, in press). The mesocosms effectively simulate natural ponds by including substrate (i.e., leaves), plankton, and periphyton, as well as a natural photoperiod and temperature fluctuations. Surviving metamorphs were subsequently reared in the laboratory or in terrestrial enclosures until the autumn or following year. Terrestrial enclosures, like the cattle tanks, have the benefit of a more natural setting and prey base than the laboratory, and are subject to changing weather and other biotic and abiotic factors. Experimental enclosures have also greatly enhanced our understanding of amphibian ecology and responses to habitat contamination (Boone and James, in press). A laboratory component was added because stochastic events and habitat heterogeneity can have significant and misleading effects on outdoor studies. Toxicology research benefits greatly from a multi-tiered approach that extends from the laboratory to free-ranging populations (Thompson 2004). Multiple life stages (tadpole, metamorph, juvenile) were monitored to have a more complete understanding of the effects of aquatic Cd contamination on amphibians. It is often the case that researchers project effects on older life stages based on larval or metamorphosis data. Ecological studies are sometimes cited that correlate large body mass at and early timing of metamorphosis with increased fitness (e.g., Semlitsch et al. 1988). However, exposure to a contaminant, particularly one that bioaccumulates, may influence these apparent trends. The monitoring of post-exposure terrestrial performance in a natural, outdoor setting is something that has been done only once before with the short-lived insecticide carbaryl (Boone, in press).

Experiments were conducted that increased sequentially in complexity. The two amphibian species tested were the American toad (*Bufo americanus*) and southern

leopard frog (*Rana sphenocephala*). They were selected because they are common, widespread species that utilize contaminated habitats in the United States (Pollio 2001). One bufonid and one ranid were chosen because of evidence that the taxa differ in sensitivity (Birge et al. 2000). Several studies have examined the ecology of these species, so there is a good literature base from which to draw. Some laboratory-based information is available on Cd toxicity for American toads (Birge et al. 2000, James and Little 2003), but there are no published studies on southern leopard frogs. Therefore, toxicity studies of these species are warranted. In Year 1, I reared the two species in separate cattle tanks and exposed them to one of five concentrations of Cd. Metamorphs were then kept in enclosures at one field site until the following year. In Year 2, I only worked with southern leopard frogs, and exposed them in cattle tanks to the three lowest Cd concentrations tested in Year 1. Following exposure, metamorphs were raised for two months in the laboratory on a low or high diet level, so that both laboratory and field data would be available for assessment of terrestrial performance and so that the role of food availability could be evaluated. In Year 3, southern leopard frogs were exposed to the year 2 Cd concentrations, but were reared with and without American toads to examine the effects of interspecific competition. Resulting southern leopard frog metamorphs were placed in terrestrial enclosures in two habitat types and recovered in the autumn.

There were many benefits to the approach I took in conducting amphibian toxicity research. By using cattle tanks, Cd was allowed to partition into and influence different media, making the exposure scenario more environmentally realistic compared to typical laboratory tests. The addition of a competitor to the aquatic environment likewise increased realism given that interspecific competition is prevalent in amphibian

communities and may affect metamorphic traits. Competitive interactions can in turn be affected by contamination (Mills and Semlitsch 2004). By using terrestrial enclosures, metamorphs were subject to natural factors such as predation, starvation, desiccation, and competition. Performance was assessed in two habitat types in order to understand the influence of the terrestrial environment on amphibian responses to larval Cd exposure. Certain habitat types may be more challenging than others and thus the consequences of larval exposure history may play out differently in different habitats. Research on multiple life stages that incorporates habitat complexity in both the aquatic and terrestrial environment is critical for more accurately predicting the effects of contamination on amphibians.

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CHAPTER 2

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

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 (1325-L polyethylene cattle tanks) that simulated natural ponds. Both species exhibited a decrease in survival with increasing initial nominal aqueous Cd concentration. When survival was included as a covariate, cadmium treatment did not influence mass at metamorphosis for either species, 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 accumulate a higher concentration of Cd compared to the southern leopard frogs at a given exposure. Most of the Cd partitioned out of the water column within seven days of dosing 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.

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 to inhabit the terrestrial environment. Characteristics of the breeding site can have important effects on metamorphosis and early life stage survival, and aquatic contamination may contribute to reduced amphibian abundance and species richness at some field sites (Kucken et al. 1994, Bishop et al. 1999). Amphibians have been found in wastewater treatment wetlands, farm ponds, mining sites, and other contaminated habitats. Amphibians are unlikely to escape environmental degradation because they have high breeding-site fidelity and low mobility.

Laboratory tests have been important for understanding direct effects of contaminants on amphibians. However, these studies rarely examine indirect effects (Mills and Semlitsch 2004), and may greatly overestimate (Thompson et al. 2004) or underestimate (James and Little 2003) responses that would occur in natural conditions because they tend to be acute (usually less than seven days), 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 when the exposure is chronic (James and Little 2003) or an additional stressor is present (Relyea and Mills 2001). Dietary uptake of contaminants can also be important

but realistic dietary exposure regimes are rarely tested with amphibians in the laboratory. Alternatives to traditional testing are needed to better understand amphibian responses in contaminated habitats and routes of uptake. Mesocosms (e.g., polypropylene cattle tanks) provide the benefits of a more realistic aquatic environment while still maintaining relatively controlled conditions (Rowe and Dunson 1994). Investigators have used cattle tanks to study amphibian ecology (Wilbur 1987, Rowe and Dunson 1994) and ecotoxicology (Rowe and Dunson 1994, Mills and Semlitsch 2004). 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 media 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 (Mills and Semlitsch 2004). Unfortunately, the interpretation of cattle tank study results can be more difficult than those obtained in the laboratory, and natural disasters and heterogeneity among experimental units are more prevalent.

The heavy metal cadmium (Cd) is one of the contaminants that amphibians encounter at breeding sites. 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; USEPA 2001), but concentrations can be well above that in industrial and mining areas (Barks 1977) and may exceed 200 μg/L (LeJeune et al. 2000). Cadmium is more bioavailable in soft water (Canton and Sloof 1982), 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 (Canton and Sloof 1982,

Nebeker et al. 1995, Loumbourdis et al. 1999) and survival (Canton and Sloof 1982, Nebeker et al. 1995, Lefcort et al. 1998, Loumbourdis et al. 1999). However, Cd can also have hormetic effects (James and Little 2003). Significant Cd uptake by tadpoles from water can occur within 24 h (Dobrovoljc et al. 2003) and may increase with the length of exposure (Dobrovoljc et al. 2003) and aqueous Cd concentration (Nebeker et al. 1995, Loumbourdis et al. 1999). 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 (Lefcort et al. 1998). Larval amphibians collected from contaminated field sites have possessed in excess of 13 μg Cd/g whole body dry mass (Snodgrass et al. 2003), while those exposed in the laboratory can withstand at least 60 μg Cd/g (Loumbourdis et al. 1999).

Cadmium added to the water column can partition into periphyton and plankton (Stephenson and Turner 1993), altering the food source of amphibians. Periphyton often contains many times the concentration of aqueous Cd (Besser et al. 2001) and when Cd is experimentally added to water, uptake by periphyton can reach equilibrium in a matter of days (Stephenson and Turner 1993). Phytoplankton abundance may increase in Cd-contaminated systems (deNoyelles et al. 1980, Kettle and deNoyelles 1986) because of alteration of the zooplankton community and reduced grazing. However, Cd can also inhibit some phytoplankton species and change species composition (deNoyelles et al. 1980, Kerrison et al. 1988). These complex responses to Cd by aquatic communities reinforce the need for mesocosm and field studies (see also Wojtaszek et al. 2004).

Reliance on laboratory data alone to predict the impacts of Cd in the environment should be avoided (Kay 1985).

The need for more environmentally relevant tests of Cd effects on amphibians is indicated from laboratory toxicity data and 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 sphenocephala*). These common United States species were chosen because they are habitat generalists that will breed in contaminated sites (Pollio 2001) and have not previously been tested for their metamorphic responses to Cd outside of the laboratory (American toads) or at all (southern leopard frogs). One bufonid and one ranid were selected because previous toxicology studies indicate ranids may be relatively more sensitive (Birge et al. 2000). 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 usually take at least fifteen days longer to metamorphose but do so at approximately ten 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. Because this area has been set aside for ecological research since 1938 and has no known on-site sources of contamination, ambient Cd concentrations were not determined. Three southern leopard frog (*Rana sphenocephala*) egg masses were collected from one pond and portions of five American toad (*Bufo americanus*) egg strings were collected from the other pond. Oviposition occurred within 48 h of collection. Eggs were transported in plastic buckets with source pond water to a laboratory at the USGS Columbia Environmental Research Center (CERC) in Columbia, MO, USA. Eggs hatched in the buckets and daily partial water changes were made using well water diluted with deionized water (hardness ≈ 60 mg/L as $CaCO_3$). This allowed 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, 1325-L volume; Behlen PolyTuff, Columbus, NE, USA) were set up to simulate natural ponds. The tanks 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 $CaCO_3$) was 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 by raking the forest floor in an oak-hickory-maple stand at the Baskett Wildlife Research Area, and was in various states of decay but largely consisted of dry, intact leaves. Equal-volume aliquots of plankton and algae from several ponds in Boone County natural areas were

added to the tanks approximately every five days for a total of four inoculations. Concentrated samples were obtained by pouring buckets of pond water through a 63-μm 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 predaceous invertebrates. These methods were based on well-established procedures for amphibian cattle tank studies (Wilbur 1987, Boone and Semlitsch 2001). However, it should be recognized that variation in the creation of the larval environment among studies may influence contaminant effects.

A stock solution was made by adding Cd (certified American Chemical Society $CdCl_2 \cdot 2.5H_2O$ crystals; Fisher Scientific, Fairlawn, NJ, USA) to deionized water, and its 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 μg Cd/L. These concentrations are considered the treatment levels even though they decreased over time. Stock solution was first added to a plastic watering can containing water from the respective tank, and then the contents were evenly sprinkled 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 (Gosner 1960). Clutches of each species were combined and only free-swimming tadpoles that appeared healthy were used. 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 (Brockelman 1969, Petranka 1989) and the species were reared separately to assess the effects of Cd on populations instead of communities. Tank replication was as follows for the different Cd concentrations (μg/L): American toads- 0 (n = 3), 5 (n = 3), 18 (n = 3), 60 (n = 3), 200 (n = 4); southern leopard frogs- 0 (n = 4), 5 (n = 4), 18 (n = 4), 60 (n = 5), 200 (n = 5). Replication was unequal among concentrations and species so that enough metamorphs would be obtained for use in a subsequent study (Chapter 3). The amphibian species and Cd treatment level for each tank were assigned 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; Gosner 1960) 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 (≈ 50 mg/L as $CaCO_3$), uncontaminated well water that was changed (100%) daily. There were still southern leopard frog tadpoles present on August 12 when tanks were drained and the litter carefully sorted through for remaining survivors. It was assumed that any unrecovered amphibians had died, although it is possible that some were not detected during the daily checks or at the end of the study.

Some of the southern leopard frog and American toad metamorphs were sampled for whole body Cd analysis once 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. There was a shortage of metamorphs from the 60 μg Cd/L concentration, 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 previously described. Amphibians were stored individually at –15 C until they were freeze-dried and shipped for Cd analysis.

Mesocosm sampling techniques

All tanks were sampled throughout the study 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, June 14. On June 14, only the southern leopard frog tanks were sampled because most American toads had metamorphosed. A cylindrical polyvinyl chloride (PVC) sampling device was used to obtain a 1-L water sample from four predetermined locations within a tank. These four liters 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 mL of tank water 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*

(American Public Health Association et al. 1998). 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 0.1N 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, irrespective of species. Each tank contained eight 7.5 x 12.5 cm PVC 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 partitioning of Cd from water into a tadpole 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 CERC (unfiltered water) and the Mississippi State Chemical Laboratory (periphyton and amphibian tissue; 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 once the study ended. Differences among treatment levels in the number of survivors that metamorphosed indicate effects on rate of development (Mills and Semlitsch 2004). Therefore, percent metamorphosis was calculated as the number of individuals that reached at least stage 42 (Gosner 1960) (i.e., metamorphs) divided by the number surviving. Cadmium treatment effects on percent survival and metamorphosis were determined with 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 there were no American toad tadpoles left at the end of the experiment (i.e., toad percent metamorphosis was 100% for every Cd treatment level). Analysis of covariance (ANCOVA) 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 (Boone and Semlitsch 2001). 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 (SAS Institute 1989). 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, there is the possibility of dependence and an increase in the experiment-wide probability of a type I error. Therefore, we determined whether there was a significant relationship 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 (SAS Institute 1989).

RESULTS

Mesocosm sampling

Five measures of tank water quality were determined approximately every 10 d and the means and ranges, respectively, 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 $CaCO_3$/L, 27 - 70 mg $CaCO_3$/L), hardness (49 mg $CaCO_3$/L, 32 - 73 mg $CaCO_3$/L). Aqueous Cd concentrations 24 h after dosing were 40-59% of nominal, but proportional to the intended dose (Figure 1a). A rapid drop in concentration has been documented in other studies using experimental ponds (Kettle and deNoyelles 1986), and is likely due to partitioning into other media. Cadmium in the

water column decreased over time, so that by seven days after dosing (or two days before tadpole addition), less than 10% of the initial nominal concentration remained (Figure 1a). The Cd content of periphyton significantly increased 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 non-control tanks was lower 43 d after dosing relative to 18 d (Figure 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 μg Cd/L = 51,000, 18 μg Cd/L = 142,308, 60 μg Cd/L = 444,444, 200 μg Cd/L = 1,391,813.

American toads

The mean responses of American toads to chronic Cd exposure are reported in Table 1. There was a significant inverse correlation 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, while age at metamorphosis significantly increased (Table 2). Mass decreased 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 result 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 μg/L, according to the Tukey's multiple comparisons tests (Table 1). The few metamorphs produced at

the two highest Cd levels were very small and lethargic. The whole body Cd concentration found in exposed metamorphs was significantly higher than the controls (Tables 1 and 2), and as high as 380 μg/g dry weight was detected in one sample. There was a significant effect of Cd ($F = 4.50$, $df = 4$, $p = 0.0245$), time ($F = 42.67$, $df = 4$, $p < 0.0001$), and their interaction ($F = 3.57$, $df = 16$, $p = 0.0005$) on phytoplankton abundance. Phytoplankton increased with time and was greatest in the two highest Cd treatment levels (Figure 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 existed between mass, age, and percent metamorphosis, 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). Survival in the 18, 60, and 200 μg/L treatments was significantly higher than the controls (Table 1). The highest Cd level had very few survivors, many of which were still tadpoles when the experiment ended. Without the use of survival as a covariate, age at metamorphosis was still non-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). There was a highly

significant effect of Cd, time, and their interaction (all p's <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 (Figure 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 initial nominal aqueous concentration of 18 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. Exposed metamorphs of both species possessed very high amounts of Cd on a whole body dry weight basis (≥11 μg/g), and American toads always had higher body burdens than southern leopard frogs at a given exposure level. It has previously been documented that different amphibian species in the same field exposure scenario will accumulate different amounts of a contaminant (Westerman et al. 2003). Differences between the two species in contaminant uptake and retention may help explain differences in some of the other responses. Survival of both species was approximately 90% in the controls and was significantly less in the two highest Cd treatment levels. 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 at each of the four Cd levels. The study was terminated after 103 d, at which time all American toads had metamorphosed or died but 36 southern leopard frog tadpoles were found. Cadmium treatment did not significantly affect percent metamorphosis in either species, but percent metamorphosis for the

southern leopard frogs 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 result suggests direct Cd toxicity influenced the age of American toads but not southern leopard frogs. Control toads metamorphosed almost twice as quickly as those at 200 μg Cd/L. The non-significant 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 species difference in mass response may be attributable to the greater mortality experienced by southern leopard frogs compared with American toads. Perhaps selection was strong enough that only the most robust southern leopard frogs survived. It has been 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 (Rowe et al. 1998). 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 (Wilbur 1977). 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 (Nebeker et al. 1995, Irving et al. 2003). It is also 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 2004). Southern leopard frogs feed predominantly on periphyton and phytoplankton whereas American toads are bottom-feeders and primarily consume periphyton and detritus (Wilbur 1987), but relative consumption rates for either species are unknown.

The longer larval period of the southern leopard frogs relative to the American toads would seemingly 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 (Westerman et al. 2003). 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 prior to metamorphosis (Snodgrass et al. 2003) 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 (Breden 1988). Southern leopard frogs and American toads

had no or low breeding success in ponds contaminated with Cd and other metals (Pollio 2001), and 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 (Semlitsch et al. 1988). Metamorphosing later means less time in the terrestrial environment to feed and grow before the first winter. A prolonged larval period could also 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) were also 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 concentrations in the three highest treatment levels were far higher than is typically found in the environment, where levels are usually <50 μg Cd/g (dry mass; Besser et al. 2001). The Cd content of phytoplankton was not determined, but accumulation does occur (Conway 1978). Hence, although the aqueous concentrations of Cd declined over time, the tadpoles consumed highly contaminated food. There were also measurable Cd treatment differences in phytoplankton abundance. Treatment levels did not differ from each other during the pre-dose sampling, but did differ when sampled after dosing but prior to 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 μg 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 previously observed in the laboratory (James and Little 2003). In this experiment, tadpoles were reared in glass jars at a density of 1/L and exposed to 0, 5, 54, or 540 μg 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 μg Cd/L. Excluding the highest concentration, the relationship to Cd treatment level for mass and age at metamorphosis was positive and negative, respectively, an opposite finding to what was observed in the cattle tanks. While the laboratory results indicate that Cd ≤54 μg/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, and 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 was likely across the skin and gills and exposure had a hormetic effect up to a point, indicating 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 media and both direct and indirect effects occur (see also Mills and Semlitsch 2004). The two studies combined strongly suggest that in situ and natural field studies are also needed to assess risk because environmental complexity can have important consequences and is difficult to manipulate (see also Thompson 2004).

This research documented effects of aqueous Cd treatment on amphibian survival, metamorphic traits, and the larval environment. However, the current U.S. federal water quality criteria of 0.15 µg Cd/L may be adequately protective of American toads and southern leopard frogs given significant effects were not observed below 18 µg Cd/L. 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 to 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.

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

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

ND = not determined

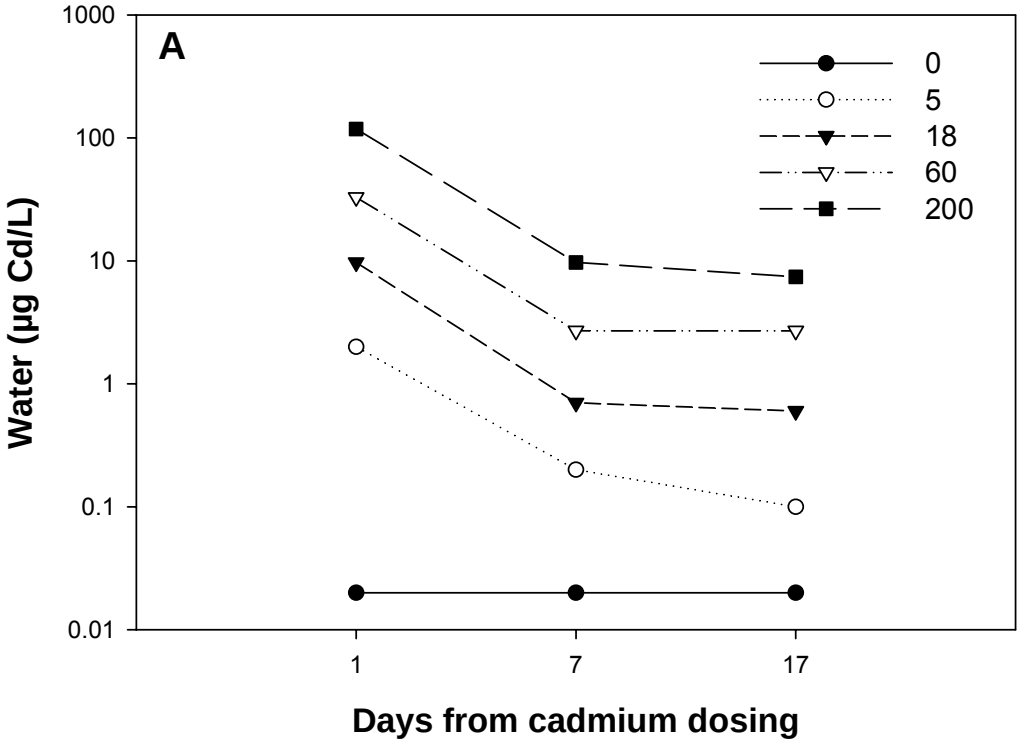
[a] Differing letters within species in a given row indicate significant differences due to cadmium concentration according to the Tukey's test (p <0.05).

[b] n = 1.

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 sphenocephala*) metamorphs

Response variable	Source	*MS*	*df*	*F*	*p*
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		

Figure 1. Mean cadmium concentration of (a) the water column and (b) periphyton in mesocosms containing American toad (*Bufo americanus*) or southern leopard frog (*Rana sphenocephala*) tadpoles. Each line represents a different initial nominal aqueous cadmium dose (μg/L) and means were derived from two tanks per cadmium treatment level.


A
1000
100
10
1
0.1
0.01
Water (µg Cd/L)
1
7
17
Days from cadmium dosing
0
5
18
60
200

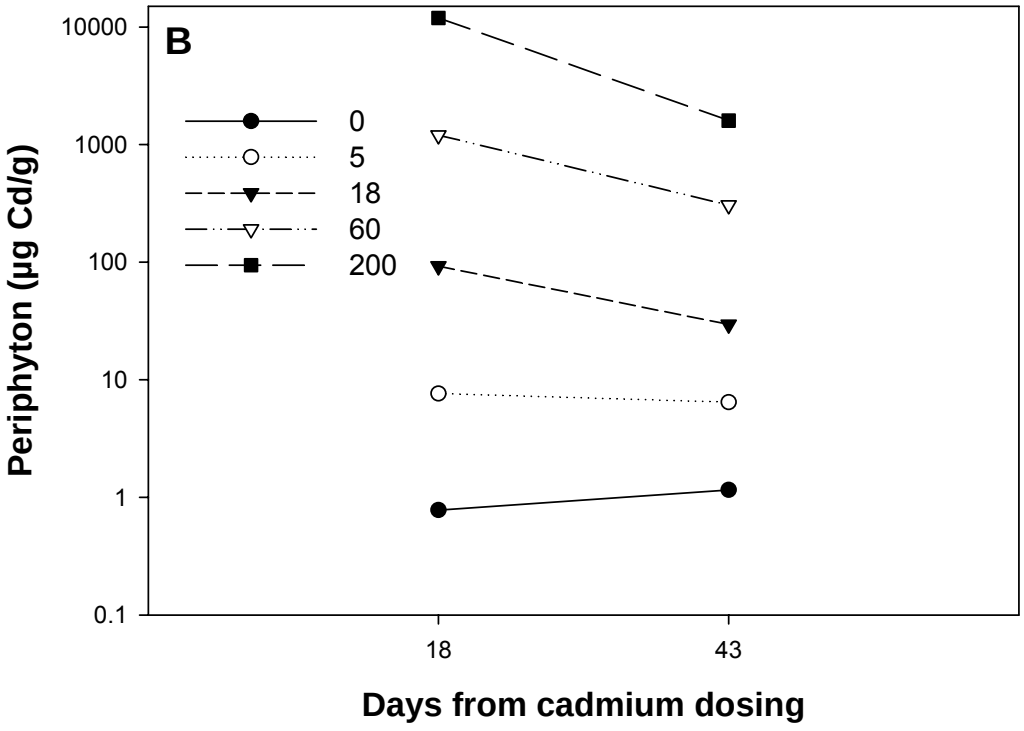
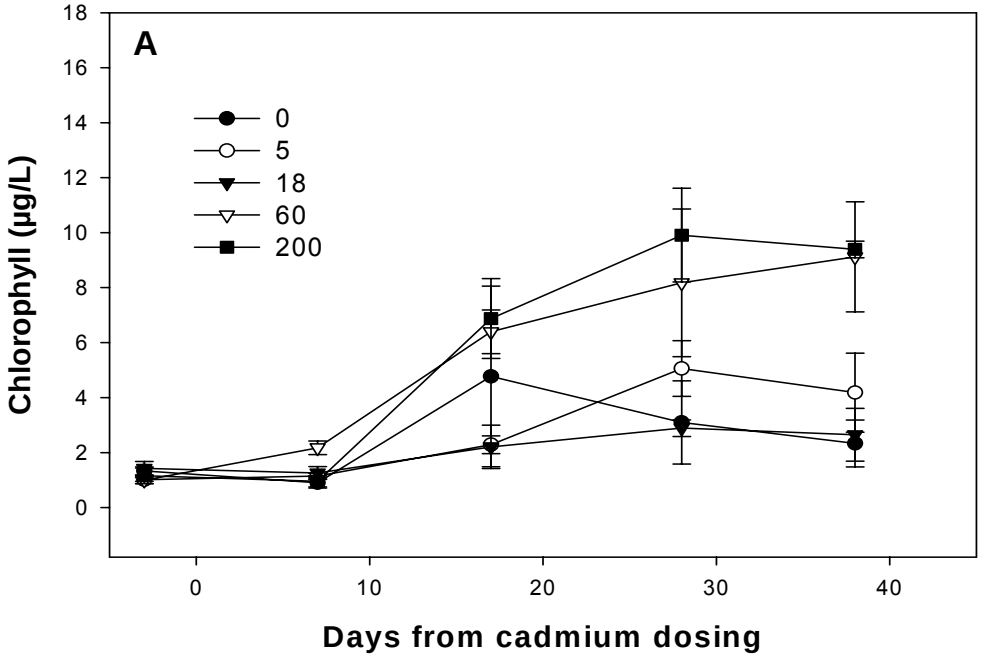
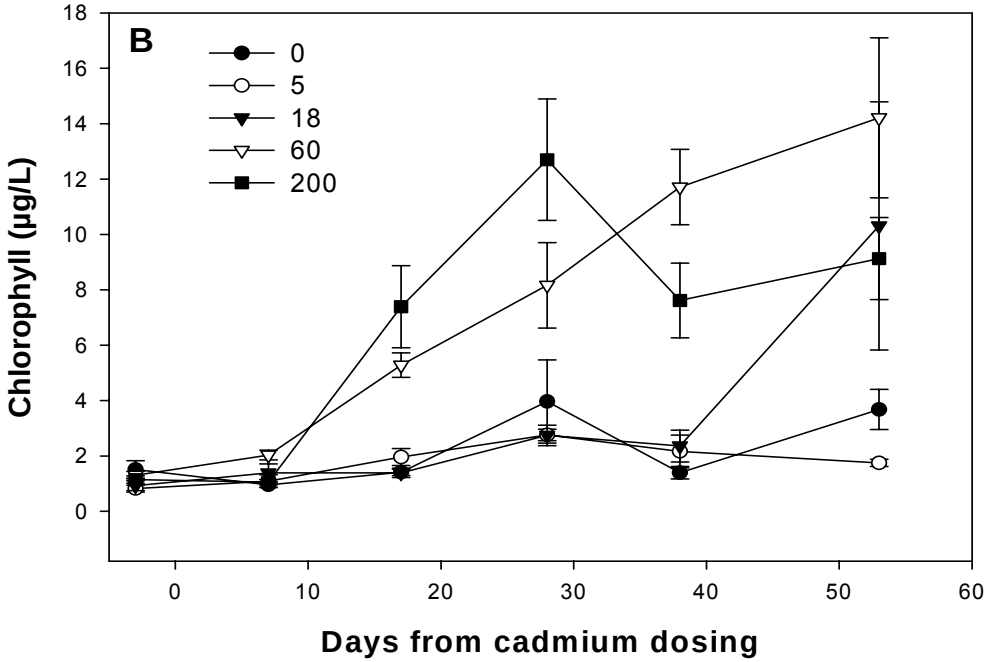

B
10000
1000
100
10
1
0.1
Periphyton (µg Cd/g)
18
43
Days from cadmium dosing
0
5
18
60
200

Figure 2. Mean (± standard error) phytoplankton abundance (measured as chlorophyll A) over time in mesocosms dosed once with one of five nominal concentrations of aqueous cadmium (μg/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.


A
0
5
18
60
200
Chlorophyll (µg/L)
Days from cadmium dosing


B
0
5
18
60
200
Chlorophyll (µg/L)
Days from cadmium dosing

CHAPTER 3

TERRESTRIAL PERFORMANCE OF AMPHIBIANS IN EXPERIMENTAL ENCLOSURES AFTER CHRONIC LARVAL EXPOSURE TO CADMIUM

ABSTRACT

Amphibians that survive larval exposure to contaminants can possess a body burden or altered metamorphic traits upon emergence into the terrestrial environment. Toxicology studies of the effects of specific contaminants on amphibians should therefore assess multiple life stages, both during and after exposure. American toad (*Bufo americanus*) and southern leopard frog (*Rana sphenocephala*) larvae were raised separately through metamorphosis in outdoor aquatic mesocosms (1325-L polyethylene cattle tanks) that had been dosed once with 0, 5, 18, 60, or 200 μg Cd/L. Survivors of each species were then stocked separately in 2-m^2 terrestrial enclosures at a density of 3.5/m^2. American toads from all five treatments were stocked, but high larval mortality of the southern leopard frogs at 60 and 200 μg Cd/L prevented stocking at these concentrations. Survival, mass, and growth rate were monitored as indicators of performance in the autumn after metamorphosis and the following spring. Monitoring was extended until the next autumn for southern leopard frog juveniles. It was hypothesized that cadmium (Cd) body burdens obtained as larvae would impair terrestrial performance. In the spring, American toad survival in the two highest Cd treatments was 0% and there was a decrease in survival with increasing concentration, but initial

treatment differences in mass had disappeared. Southern leopard frog survival through the first autumn was identical among treatments, while spring survival was somewhat higher in the 5 μg Cd/L treatment (49%) relative to the 0 and 18 μg Cd/L treatments (37%). There were no significant treatment differences in growth rate, mass, or survival through the second autumn, but those from the 18 μg Cd/L treatment remained the largest throughout the study. Southern leopard frog mortality was highest during overwintering, whereas American toads died primarily during their first growth period from summer to autumn. Larval exposure to Cd at the concentrations tested can influence both larval and juvenile survival such that particularly contaminated sites may be population sinks. However, initial treatment differences in metamorph mass were overcome with time and Cd body residues did not directly alter performance. This study demonstrates the importance of using more than one species and observing multiple life stages when determining contaminant toxicity.

INTRODUCTION

Amphibian larvae in chemically-contaminated aquatic breeding sites can have altered growth (Boone et al. 2004) and increased mortality (Rowe et al. 2001), body residues (Hopkins et al. 2000), maintenance costs (Rowe et al. 1998a), physical deformities (Rowe et al. 1998b, Hopkins et al. 2000), and abnormal behavior (Hopkins et al. 2000) relative to larvae in uncontaminated conditions. Those that survive to metamorphose may be impaired due to stress from contamination affecting physiological processes, organ function, disease resistance, behavior, and morphology. In addition, juveniles may be smaller (Bridges 2000, Chapter 2 or James et al., in press) and hence

more subject to predation, starvation, and desiccation in terrestrial habitats (Zug and Zug 1979, Newman and Dunham 1994, Scott 1994) or have a reduced potential to reproduce (Semlitsch et al. 1988). However, sometimes contaminant exposure results in larger size or earlier metamorphosis (Boone and Semlitsch 2001, James and Little 2003, James et al., in press), both of which are advantageous because they are linked to earlier reproduction and higher fitness (Smith 1987, Semlitsch et al. 1988, Berven 1990). Larger mass at metamorphosis is also associated with greater lipid stores (Scott 1994), better locomotion (John-Alder and Morin 1990), larger juvenile body mass, and higher juvenile survival (Morey and Reznick 2001). Unfortunately, assessments of the effects of aquatic contamination on larval amphibians almost always stop before or at metamorphosis (but see Rowe et al. 2001, Rehage et al. 2002, Boone, in press). As a result, it is generally unknown whether those that survive contaminant exposure experience poorer performance (i.e., growth, survival) and lower fitness as juveniles and adults. Some investigators project potential costs based on data from tadpoles or metamorphs. A better understanding of the role that aquatic contamination plays in amphibian populations can be achieved by monitoring amphibians post-metamorphosis.

Many amphibian species migrate into the terrestrial environment shortly after metamorphosing from aquatic habitats. Juveniles travel from a few to several hundred meters from the breeding site and spend the rest of the active period foraging. Growth is essential because hibernation success is enhanced by large body size (Tester and Breckenridge 1964) and energy stores (Pinder et al. 1992). Natural mortality is high in the early terrestrial life stages (Breden 1988) and population dynamics is greatly influenced by post-metamorphic survival (Biek et al. 2002). Hence, any change in

juvenile body condition, size, or growth period resulting from aquatic contamination may increase the chance that a breeding site becomes a population sink.

The ability to monitor individuals over time is a powerful tool for detecting treatment effects. Unfortunately, amphibian juveniles are usually too small for direct tracking and drift fences may be ineffective due to escape or highly-seasonal migrations. One viable option is the use of terrestrial enclosures. Amphibians may be individually marked and stocked at known densities, so that indicators of performance can be determined at pre-selected times. Enclosures have been used in ecological (Pechmann 1995, Parris 2001) and toxicological (Hopkins et al. 1997,1998, Oldham et al. 1997, Laposata and Dunson 2000, Boone, in press) studies. Enclosures have the benefit of incorporating some natural complexity and the fluctuation of abiotic and biotic variables, thereby enhancing environmental realism relative to the laboratory. The incorporation of factors such as competition and desiccation is important because of their potential to influence contaminant effects (Mills and Semlitsch 2004). Unfortunately, there is also greater opportunity for stochastic events to disturb experiments and for habitat heterogeneity to mask treatment differences.

Of the toxicology experiments conducted in outdoor terrestrial enclosures, only one (Boone, in press) assessed amphibians after aquatic exposure; the others addressed terrestrial contamination. Boone (in press) concluded that larval exposure to the short-lived insecticide carbaryl had no significant impact on juvenile growth and survival, and that metamorphic traits may not be reliable predictors of future success. However, there was evidence that juveniles from carbaryl treatments grow faster than controls (Boone, in press). The type of contaminant tested and its mode of action and persistence may

greatly influence the responses observed in juvenile performance studies. When the contaminant of interest bioaccumulates, there is the potential for harmful sublethal toxicity and resulting lower body condition, or even mortality, long after the actual environmental exposure is over (Pascoe and Shazili 1986). Therefore, deleterious effects of exposure that extend over multiple life stages could be expected for persistent contaminants such as heavy metals.

In the spring of 2002, American toad (*Bufo americanus*) and southern leopard frog (*Rana sphenocephala*) larvae were reared in outdoor mesocosms (1325-L cattle tanks) that had been dosed once with 0, 5, 18, 60, or 200 μg Cd/L (James et al., in press). Both species experienced reduced survival and delayed metamorphosis with increasing aquatic cadmium (Cd) concentration (James et al., in press). However, they had opposite responses in mass at metamorphosis; mass decreased (American toads) or increased (southern leopard frogs) with increasing Cd (James et al., in press). It was determined that Cd did not affect mass directly, but rather indirectly through changes in survival (i.e., density). There was a general trend of metamorph whole body Cd residues increasing with aquatic concentration, and of American toads containing more residues than southern leopard frogs at a given exposure level (James et al., in press). The body burdens of exposed organisms ranged from 9 to 380 μg Cd/g (dry weight). The results of this study indicated that the body residues and metamorphic traits of individuals of both species had been affected by larval exposure to Cd. The possession of a Cd body burden can directly influence numerous organs (e.g., liver, kidney, skin, spleen) and physiological processes (e.g., enzyme activity, vitellogenesis) (Eisler 1985, Pramoda and Saidapur 1986). Larger or smaller mass at metamorphosis may increase or decrease the

chance of survival, respectively (Morey and Reznick 2001). A delay in metamorphosis means individuals will have less time to obtain adequate energy reserves for overwintering. Therefore, there might be differences in juvenile terrestrial performance attributable to Cd contamination of the larval environment.

A subsample of the metamorphs from the 2002 aquatic mesocosm study were raised in uncontaminated terrestrial enclosures. The first objective of the enclosure study was to determine if juveniles from different larval Cd treatments differ in post-metamorphic growth and survival. The second objective was to determine whether direct Cd stress alone affects post-metamorphic growth and survival. An assessment of the second objective requires correcting for differences in mass and time to metamorphosis because of their potential influence on the terrestrial response variables. Also of interest was whether American toads and southern leopard frogs differ in their responses. Interspecific differences were expected because species often vary in sensitivity (Birge et al. 2000), uptake, distribution, exposure period, and exposure route (Snodgrass et al. 2004). Natural interspecific variation in juvenile growth rates may also influence the effect of larval exposure history on terrestrial performance (Werner 1986), given bufonids and ranids metamorphose at approximately 1% and 10% of adult size, respectively. Individuals were monitored before and after the first overwintering. It was hypothesized that individuals exposed to Cd as larvae would be more stressed and subsequently experience higher mortality and slower growth compared to unexposed individuals. Of the two species, it was expected that American toads would have the worst terrestrial performance given that they naturally have a lower aerobic capacity (Pough and Kamel 1984) and smaller size at metamorphosis than the southern leopard

frogs. Furthermore, larval Cd exposure widened the difference between the two species in mass at metamorphosis, and American toads had higher average Cd body burdens than did the southern leopard frogs (James et al., in press).

MATERIALS AND METHODS

Larval exposure

American toad and southern leopard frog metamorphs were obtained from outdoor aquatic cattle tank mesocosms that had been dosed once with Cd (details in James et al., in press). In spring 2002, an array of mesocosms (1325-L volume, 1.83-m diameter) was set up in a mown field at the USGS Columbia Environmental Research Center (Columbia, MO, USA). Each mesocosm contained approximately 950 L of water, 1 kg of deciduous leaf litter, and an algal and plankton community, and was covered by a lid to prevent colonization by predators and competitors. When there was visible periphyton growth on the walls, mesocosms were dosed once at 0, 5, 18, 60, or 200 μg Cd/L. Nine days later, 50 southern leopard frog or 120 American toad hatchlings were added to separate mesocosms. Larvae were allowed to grow undisturbed until metamorphosis and fed on the naturally occurring food resources. The Cd initially added to the water column partitioned into the periphyton and other potential resources, so that the larvae were exposed via both water and food. When individuals reached developmental stages 42 to 46 (Gosner 1960), they were captured and transported to a laboratory where they remained until complete tail resorption. Metamorphs that had resorbed their tails were fed fruit flies or pinhead crickets and were kept in the laboratory for up to 8 d before being released into the enclosures.

Experimental design

Prior to release, amphibians were weighed ("summer mass") and toe-clipped so that individuals could be measured over time. Only two toes were removed from each amphibian because of evidence that return rate decreases with the number of toes cut (McCarthy and Parris 2004). Gross toe and limb malformations were noted during toe clipping. However, a rigorous examination of subtle aberrations was not performed so reported malformation rates are likely underestimated. American toads were stocked from June 10 to July 3, and southern leopard frogs from June 26 to July 10. Stocking did not occur all at once in order to reflect individual, species, and treatment differences in time to metamorphosis. To minimize initial stress, enclosures that were stocked on a given evening were soaked with water immediately before the metamorphs were released. There were seven metamorphs of a particular larval Cd treatment and species in each enclosure. The number (n) of replicate enclosures for each Cd treatment were as follows: American toads: 0 (n = 5), 5 (n = 5), 18 (n = 5), 60 (n = 2), 200 (n = 3) μg Cd/L; southern leopard frogs: 0 (n = 5), 5 (n = 5), 18 (n = 5) μg Cd/L. Unfortunately, there were not enough southern leopard frogs from the two highest treatments to stock enclosures. A total of ten enclosure blocks (35 enclosures) were used; each block contained only one species and the enclosures were randomly assigned different larval Cd treatments. Species were assigned to every other block to maximize interspersion.

The terrestrial performance experiment took place in a field at the University of Missouri Research Park Greenhouse facility (Columbia, MO, USA). The enclosures were located along a deciduous forest edge where the vegetation consisted primarily of fescue grass (*Festuca* spp.), bluegrass (*Poa* spp), crabgrass (*Digitaria ischaemum*),

bristlegrass (*Setaria viridis*), fleabane (*Erigeron annuus*), aster (*Aster simplex*), and copperleaf (*Acalypha virginica*). The enclosures (1 x 2 m) were made of sheet metal walls buried approximately 0.7 m deep and stood 0.6 to 0.8 m above the ground. Ten blocks of four adjacent enclosures were separated from each other by 1 m. These enclosures have previously been used for terrestrial research on the overwintering survival and growth of *Bufo* and *Rana* spp., and stocking densities were comparable to the present study (Parris 2001, Boone, in press). Enclosures were covered with 0.6-cm nylon mesh (Delta Net & Twine, Greenville, MS, USA) clamped on with binder clips to prevent juvenile escape and predator or competitor entry. There was no supplemental feeding but invertebrate prey could get through the mesh. Each enclosure contained a central pit (45-cm across, 45-cm deep) filled with moist deciduous leaves and covered by a square piece of plywood. The pit provided refuge for the amphibians and was also a source of prey. Additional refuges were made by creating a 45^{o} angle tunnel (10-cm long x 2.5-cm wide) in each of the four corners. The vegetation was clipped to approximately 20 cm before the metamorphs were added and again when it had grown nearly as tall as the enclosure walls. During the hot summer months, each enclosure was sprayed with a garden hose for a count of 60 if it had not rained in seven days.

The juveniles were left undisturbed until autumn, when the enclosures were searched for survivors and recovered individuals were weighed. Enclosures were searched on four separate days in a six-day period, with no individuals being found on the last day. More leaf litter was added to the central pits so that the amphibians could burrow during cold weather. All amphibians were returned to their original enclosures and allowed to overwinter. The mesh covers were kept off because of snow. In spring

2003, the enclosures were searched once again and the criterion for stopping was three consecutive days of not recovering any individuals. There were enough southern leopard frogs alive in each of the three Cd treatments that a subset was randomly reassigned to enclosures until autumn 2003. Restocking occurred at a density of three per enclosure and four enclosures were used per treatment. The enclosures were randomly selected from the blocks that had previously contained American toads, to minimize the chance of previously undetected southern leopard frogs confounding the results. The refugia in the corners were deepened to 24 cm and the central pits received new leaf litter. The covers were not reinstated out of concern that the mesh size might inhibit colonization by larger insect prey, which the southern leopard frogs would presumably need as they grew in size. Toes were reclipped if necessary but new toes were not cut. The enclosures were thoroughly searched in autumn 2003, and the study was concluded after three consecutive searches of no recoveries.

Statistical analysis

Separate analyses were conducted on the two amphibian species for growth and survival in autumn 2002 and spring 2003 (hereafter referred to as "year 1"). An additional analysis was conducted on the growth and survival of the southern leopard frog juveniles that were reassigned to enclosures in spring 2003 and recovered in autumn 2003 (hereafter referred to as "year 2"). Indicators of performance were mass, growth rate, and survival as determined in the autumn and spring. Autumn and spring survival were computed as the number of individuals recovered divided by the number initially stocked. Overwinter survival was the number recovered in the spring divided by the number recovered in autumn. Autumn and spring growth rate were calculated as (present

mass – summer mass)/(days in enclosure), and overwinter growth rate was (spring mass – autumn mass)/(days overwintered). In the case of the year 2 southern leopard frogs, the spring mass was considered the initial mass, and growth rate and survival pertained to the spring to autumn active period. Growth rate was not calculated based on individual growth rates due to difficulty reading some of the toe clips, particularly those of the American toads. Mean values were generated for each replicate enclosure because individuals within an enclosure are not independent. The experimental array was divided into blocks to account for spatial differences in habitat quality. Repeated measures (year 1) and univariate (year 2) analyses of variance were performed on least squares means to determine whether Cd exposure had a significant effect on terrestrial performance. The computation of survival and growth rate for the year 1 repeated measures analysis was based on two time intervals: summer to autumn, and autumn to spring (i.e, overwinter). Analyses were done both with and without covariates, to test direct Cd stress and larval exposure history effects, respectively. The covariates selected were time of addition to the enclosures ("start day;" year 1 only) and initial mass because they confound Cd treatment. In the case of significance, a Tukey's studentized range test was conducted to more closely examine differences among concentrations. Normality and homogeneity of variance were checked with the Shapiro-Wilk and Bartlett's tests, respectively. Mass and growth rate were logarithmically transformed and survival was arcsine square root transformed. Pearson correlation matrices were generated to determine significant correlations among all measured variables. Correlations between mass and growth rate in the same season are not reported because growth rate is computed based on mass and hence the two are always significantly correlated. Significance was set at $\alpha = 0.05$, type

III sum of squares was used to account for unequal replication, and analyses were performed with SAS (SAS Institute 1989).

RESULTS

American toad juveniles (year 1)

Survival was significantly affected by Cd, but not time or the time*Cd interaction (Table 1). However, when initial differences in mass and start day were accounted for by using them as covariates, Cd was no longer significant ($F = 1.21$, $df = 4$, $p = 0.3718$). The majority of mortalities occurred before autumn, but overwinter mortality was also high (Table 2, Figure 1a). Of the toads in the two highest Cd treatments, only one survived to autumn and none were alive in spring (Table 2). Spring survival was very low across all treatments (4%, n = 6 individuals) and there was a trend of decreasing survival with increasing Cd concentration (Table 2). The initial, summer mass of American toads was significantly different among larval Cd treatments ($F = 32.61$, $df = 4$, $p < 0.0001$), with a general trend of decreasing mass with increasing Cd (Table 2). American toads in the two highest concentrations were less than half the size of the controls (Table 2), and notably weak and inactive. In the autumn, American toads in the 5 μg Cd/L treatment were approximately half the size of those in the other treatments, which may have been a tradeoff of higher survival (Table 2). By the following spring, mean mass differences had been reduced to <0.2 g among the three surviving treatment groups (Table 2). Because of high mortality and resulting inadequate replication, mass and growth rate were statistically analyzed only for the control and 5 μg Cd/L treatments, and start day (covariate) and block were dropped from the model. Cadmium treatment

did not affect mass from summer to spring, but the effect of time was significant and indicates mass changed over time (Table 1). Likewise, time but not Cd had a significant effect on growth rate (Table 1). The highest growth occurred from summer to autumn, and total growth rate as determined in the spring was 0.010 to 0.011 g/day across treatments (Table 2). The addition of initial mass as a covariate resulted in no significant effect of Cd, time, or their interaction for both mass and growth rate. There were many significant correlations among variables, including positive relationships between summer mass and autumn survival ($r = 0.46$) and autumn and spring mass ($r = 0.96$), and a negative relationship between autumn growth rate and survival ($r = -0.63$) (Table 3). Somewhat weaker positive correlations were found between summer mass and spring survival ($r = 0.43$, $p = 0.0576$), autumn and overwinter growth rate ($r = 0.84$, $p = 0.0762$), and autumn and spring survival ($r = 0.39$, $p = 0.0929$). When toes were originally clipped, the number of individuals with toe or limb malformations increased with larval Cd concentration. Of the 13 to 35 American toads examined per treatment, malformation rates for the 0, 5, 18, 60, and 200 µg Cd/L treatments were 3%, 40%, 63%, 100%, and 100%, respectively. The most common malformations observed were fused or missing toes, and these were seen in both the forelimbs and hindlimbs. The limbs of some individuals ended as stumps.

Southern leopard frog juveniles (year 1)

Time but not Cd had a significant effect on survival (Table 4). Addition of covariates to the model resulted in time as no longer significant ($F = 0.14$, $df = 1$, $p = 0.7191$). Survival was identical and high (86%) across treatments in the autumn. Most mortality occurred during the overwinter period, when survival ranged from 40 to 51%

(Table 5, Figure 1b). As of spring, control and 18 µg Cd/L treatment southern leopard frogs had the same survival (37%), which was somewhat less than the 5 µg Cd/L treatment (49%). Upon addition to the enclosures, there was a significant increase in mass with increasing aquatic Cd concentration ($F = 170.98$, $df = 2$, $p < 0.0001$). Southern leopard frogs from the 18 µg Cd/L treatment weighed the most initially and maintained this advantage in the autumn and spring (Table 5). However, neither Cd nor the time*Cd interaction had a significant effect on mass (Table 4). Time was significant (Table 4) and resulted from a steady increase in mass over the course of the study (Table 5). When covariates were added to the model (i.e., start day and initial mass were accounted for), time remained significant ($F = 12.65$, $df = 1$, $p = 0.0379$) and there was also a significant time*Cd interaction ($F = 19.54$, $df = 2$, $p = 0.0190$) that suggests the effect of Cd depends on time. By the spring, mass and growth rate were similar among treatments, but southern leopard frog controls had grown by 5.1-fold relative to their summer mass, compared with only 3.6-fold and 2.8-fold for the 5 and 18 µg Cd/L treatments, respectively (Table 5). Nevertheless, Cd had no effect on growth rate, but time did affect growth rate (Table 4). Interestingly, when covariates were part of the model, the time*Cd interaction was significant ($F = 26.61$, $df = 2$, $p = 0.0123$) and time was not ($F = 5.05$, $df = 1$, $p = 0.1103$). Significant positive correlations included summer and autumn mass ($r = 0.71$), autumn growth rate and overwinter survival ($r = 0.60$), autumn growth rate and spring survival ($r = 0.54$), and autumn and spring survival ($r = 0.52$) (Table 6). There were also strong positive relationships between start day and summer mass ($r = 0.51$, $p = 0.0516$), autumn mass and overwinter survival ($r = 0.45$, $p = 0.0957$), and autumn mass and spring survival ($r = 0.46$, $p = 0.0856$). Only 1 of 105 (1%) southern

leopard frogs examined during toe clipping were noted to have a toe or limb malformation, which is an expected background level (Burkhart et al. 2000).

Southern leopard frog juveniles (year 2)

No significant effects of Cd, time, or their interaction were found for any of the response variables, without (Table 7) or with the use of covariates. When southern leopard frogs were reassigned to enclosures in spring 2003 at a lower density, there were no significant differences in mass ($F = 2.81$, $df = 2$, $p = 0.1374$), although there was a trend for mass to increase with Cd treatment (Table 8). By the next autumn, the 18 µg Cd/L treatment southern leopard frogs were on average at least 2 g heavier than those in the other two treatments, and had the fastest growth rate but the lowest survival (Table 8). There were no significant correlations among variables, but there was an inverse relationship between survival and autumn mass ($r = -0.53$, $p = 0.0865$).

In some cases individual growth could be monitored over time because each southern leopard frog had a unique toe clip. There were 21 frogs alive at the end of the study that had complete mass data sets (i.e, no missing data cells). Of these, seven were controls and eight and six were from the 5 and 18 µg Cd/L treatments, respectively. Mean mass was determined for each concentration and plotted over time (Figure 2). The plot demonstrates that individuals that metamorphose at a large size can maintain or increase the size advantage through their second growing season.

DISCUSSION

Reductions in abundance and species richness have been documented in natural amphibian communities that breed in waters contaminated with metals and agricultural

chemicals (Kucken et al. 1994, Bishop et al. 1999). Causality is usually attributed to poor recruitment from early life stage mortality and assessment of only the embryos and tadpoles is often defended based on evidence that younger life stages are more sensitive to contaminants (Bishop et al. 1999). As a result, little is known about the quality and success of the terrestrial phase of amphibians that survive contaminant exposure as larvae. However, poor terrestrial performance could also play a role in changes observed in amphibian populations occupying contaminated sites.

Research was conducted on the terrestrial performance of two biphasic amphibian species following chronic larval exposure to environmentally realistic concentrations of Cd. Growth and survival were monitored because they are considered phenotypic indicators of fitness. Natural mortality is high and generally less than 10% of free-ranging juveniles survive to the spring following metamorphosis (Breden 1988, Biek et al. 2002). The relatively protective environment of enclosures seems to improve survival (e.g., 33 - 43% survival; Parris 2001), which was the case in the present study for southern leopard frogs but not American toads. American toads had poorer survival than the southern leopard frogs, and most toad mortality occurred during the first growth period post-metamorphosis. Low survival may have been due to their small size at metamorphosis, which could make them susceptible to dehydration, predation, or starvation because of gape limitation (Zug and Zug 1979). Emigrating American toad metamorphs choose forest over old-field habitat (Rothermel and Semlitsch 2002), which suggests the disadvantages of being small are greater in open habitats such as where the present study's experimental enclosures were located. By the first autumn after metamorphosis, American toads had increased at least ten-fold in size, which enabled

rapid compensation for initial size differences due to larval Cd treatment and was likely important for overwinter survival. Terrestrial density-dependence was suggested by a significant negative correlation between autumn mass or growth rate and survival. In the spring, mass and growth rate were very similar among treatments, and there was a trend of decreasing survival with increasing larval Cd exposure concentration. Survival was 0% for those in the 60 and 200 μg Cd/L concentrations, which suggests aquatic habitats contaminated with ≥60 μg Cd/L are population sinks because of low larval (James et al., in press) and juvenile survival. Survival was the only terrestrial endpoint significantly affected by Cd treatment. However, Cd did not appear to have any direct effects on juvenile American toad performance apart from those accounted for by using start day and initial mass as covariates.

In contrast to the American toads, southern leopard frog juveniles (year 1) had very high survival through the autumn (86%) and experienced the greatest mortality during overwintering. Autumn growth rate and survival across Cd treatments were equivalent or nearly so, but the control southern leopard frogs lagged behind in mass by at least 0.4 g. Spring survival, mass, and growth rate were similar among treatments. Initial differences in mass were gradually made up for in the terrestrial environment, with mass increasing by a total of approximately five-, four-, and three-fold in the 0, 5, and 18 μg Cd/L treatments, respectively. The ability of smaller southern leopard frogs (i.e., those from the 0 and 5 μg Cd/L treatments) to catch up may have been due to any of the following: 1) more time in the terrestrial environment, 2) a statistically insignificant but faster growth rate, and 3) less Cd stress. Previous research has demonstrated that initial mass differences can be narrowed due to higher growth rates in smaller metamorphs

during the initial weeks after metamorphosis (Goater 1994). There were no significant differences among larval Cd treatments in terrestrial performance, and direct effects of Cd were not found. Because juveniles were monitored through their second autumn, a better assessment was made of the long-term impacts of larval contaminant exposure than was possible for the American toads. There was considerable mass gain (>9 g) during the second growth period and some individuals showed signs of reproductive maturity (e.g., dark thumb pads). Survival exceeded 50% in all treatments and would have been higher had there not been nearly complete mortality in one spatial block. Southern leopard frogs in the 18 μg Cd/L treatment continued to have the highest mean mass, but also had the lowest survival. If density-dependence was occurring in the enclosures such that competition for food limited body mass, then perhaps the lower survival in the 18 μg Cd/L treatment released individuals from competition and resulted in the higher mass. Density-dependence was also suggested by the strong inverse relationship between survival and final (autumn) mass. However, once again there were no significant differences among Cd treatments for any of the response variables, and no direct effects of Cd. The individual body mass data further indicate Cd stress did not impair growth because the control southern leopard frogs remained the smallest throughout the study.

The only measured effect of larval exposure to Cd for either species was on American toad survival, which was explained by significant treatment differences in metamorph mass and time to metamorphosis. However, in non-toxicological studies it has generally been shown with numerous amphibian species both free-ranging (Smith 1987, Semlitsch et al. 1988, Berven 1990, Scott 1994) and in captivity (Goater 1994, Morey and Reznick 2001) that effects of the larval environment on metamorphic traits

persist through adulthood. There is a tendency for metamorphs larger than other members of their cohort to remain relatively large through the first year or adulthood, and to experience higher survival. This even occurs in toads that have initial mass differences of <0.2 g (Goater 1994). Therefore, barring direct effects of Cd, the same trend was expected for this study, but did not clearly or consistently occur. For example, the southern leopard frogs from the 18 μg Cd/L treatment were statistically the largest as metamorphs, but after the second growth season did not significantly differ in mass or survival from the other treatments despite still having the largest mean mass. Likewise, the control American toads were largest initially but not by the study's end, although they did have insignificantly higher survival than those exposed to Cd. In contrast, the 21 southern leopard frogs followed over time maintained mass differences among treatments. The reasons for these inconsistent observations are unknown but likely include terrestrial density dependence (i.e., competition), direct Cd effects, inadequate replication, and habitat heterogeneity. It is often assumed that metamorphic traits are indices of adult fitness and that alteration of these traits influences population dynamics (Wilbur 1980). However, the terrestrial environment can also play an important role (Scott 1994).

When assessing the effects of contaminants on amphibians, it is best to test several species simultaneously because of interspecific response differences (Birge et al. 2000, Snodgrass et al. 2004). For example, of all the significant correlations found among variables, American toads and southern leopard frogs only had two in common: overwinter growth rate and spring growth rate, and overwinter survival and spring survival. Sensitivity to contamination may depend on factors such as life history

characteristics, ontogenetic development rate, and microhabitat use. American toads normally metamorphose at about 10% the size of southern leopard frogs and are less developed physically. However, American toad juveniles have a tremendous growth rate which exceeds that of the southern leopard frogs, as was demonstrated in this study. Differences in initial mass or preferred terrestrial habitat may explain why the two species differed in timing of mortality. In the autumn and spring, American toad survival was less than half that of the southern leopard frogs, even in the controls. This might suggest that American toads naturally have lower survival in open habitats, or perhaps in all habitat types, which may explain their relatively large clutch sizes and selection of forest as emigrating juveniles (Rothermel and Semlitsch 2002). When considering the 0, 5, and 18 µg Cd/L treatments only, American toad survival was always lowest in the 18 µg Cd/L treatment. This trend was present but not as well established in the southern leopard frogs. Both species were able to overcome significant initial treatment differences in mass because of similar growth rates across treatments. Any effects from a Cd body burden likely became less important over time for both species because of considerable increase in mass and associated dilution of the Cd concentration in tissues. Tadpoles exposed sequentially to contaminated and clean water have also exhibited recovery (Dobrovoljc et al. 2003). Although American toads presumably possessed higher initial body burdens (James et al., in press), it appears that the two species are comparable in their sensitivity to Cd at the concentrations tested in common (i.e., 5 and 18 µg/L). This may be further evidence that direct Cd toxicity was not significantly harmful to the juveniles of either species. If all Cd was bound by metallothionein, it would have little effect even though present in the tissues (Suzuki and Kawamura 1984).

Exposures of early life stage amphibians to aquatic Cd contamination have demonstrated that this heavy metal bioaccumulates and reduces larval growth and survival (Nebeker et al. 1995, Loumbourdis et al. 1999). It was hypothesized that stress from body residues would impair terrestrial performance, but direct effects were not found. The only significant treatment effect was for American toad survival, and this was explained by treatment differences in metamorphic traits. American toads and southern leopard frogs that survive exposure appear capable overall of normal growth and can overcome initial mass differences. A lack of carryover effects was also found for the non-bioaccumulating insecticide carbaryl (Boone, in press) and the bioaccumulating pesticide triphenyltin (Rehage et al. 2002). Unfortunately, high American toad mortality and the inability to stock southern leopard frogs from the 60 and 200 μg Cd/L treatments prevented more robust assessments of performance. This study also stopped short of actual reproduction, which might be a sensitive and important endpoint given Cd affects vitellogenesis (Pramoda and Saidapur 1986) and spermatogenesis (Kasinathan et al. 1987). Those that survive Cd exposure may pass on their body burden to their offspring via maternal transfer (Lienesch et al. 2000) and their predators (Eisler 1985). Nevertheless, given the present results and the effects of Cd on larval mortality (James et al., in press), it is likely that low recruitment is the dominant means by which aquatic Cd contamination affects amphibian populations. It is a widespread and historical belief that amphibian populations are regulated primarily by the early life stages and conditions in the aquatic environment (Wilbur and Collins 1973, Berven 1990). Successful recruitment is necessary to counteract high natural juvenile mortality and years when breeding sites dry before metamorphosis.

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Table 1. Results of univariate analyses of variance for mass, growth rate, and survival of American toad (*Bufo americanus)* juveniles reared in terrestrial enclosures (year 1)

Response variable	Source	*MS*	*df*	*F*	*p*
Mass[a]	cadmium	0.067	1	0.13	0.7547
	error	0.521	2		
	time	0.203	1	18.18	0.0509
	time*cd	0.005	1	0.44	0.5739
	error (time)	0.011	2		
Growth rate[a]	cadmium	0.005	1	0.00	0.9514
	error	0.978	2		
	time	4.400	1	29.95	0.0318
	time*cd	0.071	1	0.48	0.5599
	error (time)	0.147	2		
Survival	block	48.384	4	2.29	0.1251
	cadmium	82.556	4	3.91	0.0327
	error	21.133	11		
	time	3.656	1	0.24	0.6369
	time*cd	10.320	4	0.67	0.6292
	error (time)	15.514	11		

[a] analysis based only on the 0 and 5 μg Cd/L treatments.

Table 2. Mean (±1 standard error) mass and growth rate for American toad (*Bufo americanus)* juveniles reared in 2-m^2 terrestrial enclosures at an initial summer density of 3.5/m^2 (year 1)

Initial aquatic concentration (µg Cd/L)	Summer mass[a] (g)	Autumn mass (g)	Spring mass (g)	Autumn growth rate (g/day)	Overwinter growth rate (g/day)	Spring growth rate (g/day)
0	0.151A	3.251	3.572	0.025	0.004	0.011
	(± 0.006)	(± 0.619)	(± 0.474)	(± 0.005)	(± 0.002)	(± 0.001)
5	0.118B	1.864	3.603	0.014	0.006	0.011
	(± 0.006)	(± 0.443)	(± 1.836)	(± 0.004)	(± 0.004)	(± 0.006)
18	0.130AB	3.685	3.486[b]	0.029	0.005[b]	0.010[b]
	(± 0.007)	(± 0.842)	(—)	(± 0.007)	(—)	(—)
60	0.077C	—	—	—	—	—
	(± 0.008)	(—)	(—)	(—)	(—)	(—)
200	0.072C	3.177[b]	—	0.030[b]	—	—
	(± 0.004)	(—)	(—)	(—)	(—)	(—)

[a] capital letters within the column indicate significant differences according to the Tukey's test (p <0.05).

[b] measurement based on 1 individual.

Table 3. Significant correlations ($\alpha = 0.05$) among variables for American toad *(Bufo americanus)* juveniles (year 1)

Response variables	**n**[a]	***r***	***p***
start day, summer mass	20	-0.83	<0.0001
start day, autumn survival	20	-0.54	0.0150
summer mass, autumn survival	20	0.46	0.0412
autumn mass, autumn survival	14	-0.61	0.0209
autumn mass, spring mass	5	0.96	0.0103
autumn mass, spring growth rate	5	0.95	0.0116
autumn growth rate, autumn survival	14	-0.63	0.0149
autumn growth rate, spring mass	5	0.96	0.0082
autumn growth rate, spring growth rate	5	0.96	0.0093
overwinter growth rate, spring growth rate	5	0.96	0.0107
overwinter survival, spring survival	20	0.90	<0.0001

[a] n = number of enclosure means evaluated.

Table 4. Results of univariate analyses of variance for mass, growth rate, and survival of southern leopard frog (*Rana sphenocephala)* juveniles reared in terrestrial enclosures (year 1)

Response variable	**Source**	***MS***	***df***	***F***	***p***
Mass	block	0.043	4	0.70	0.6228
	cadmium	0.035	2	0.57	0.6002
	error	0.062	5		
	time	1.061	1	53.77	0.0007
	time*cd	0.057	2	2.90	0.1457
	error (time)	0.020	5		
Growth rate	block	0.221	4	1.10	0.4476
	cadmium	0.095	2	0.47	0.6495
	error	0.201	5		
	time	3.246	1	19.57	0.0069
	time*cd	0.258	2	1.56	0.2983
	error (time)	0.166	5		
Survival	block	0.389	4	1.89	0.2051
	cadmium	0.053	2	0.26	0.7775
	error	0.206	8		
	time	2.582	1	15.37	0.0044
	time*cd	0.024	2	0.14	0.8701
	error (time)	0.168	8		

Table 5. Mean (±1 standard error) mass and growth rate for southern leopard frog (*Rana sphenocephala)* juveniles reared in 2-m^2 terrestrial enclosures at an initial summer density of 3.5/m^2 (year 1)

Initial aquatic concentration (µg Cd/L)	Summer mass[a] (g)	Autumn mass (g)	Spring mass (g)	Autumn growth rate (g/day)	Overwinter growth rate (g/day)	Spring growth rate (g/day)
0	0.848A	2.256	4.357	0.013	0.010	0.011
	(± 0.025)	(± 0.107)	(± 0.557)	(± 0.001)	(± 0.003)	(± 0.002)
5	1.134B	2.650	4.105	0.014	0.006	0.010
	(± 0.067)	(± 0.227)	(± 0.273)	(± 0.002)	(± 0.001)	(± 0.001)
18	1.642C	3.002	4.610	0.014	0.008	0.010
	(± 0.100)	(± 0.208)	(± 0.784)	(± 0.002)	(± 0.002)	(± 0.002)

[a] capital letters within the column indicate significant differences according to the Tukey's test (p <0.05).

Table 6. Significant correlations (α = 0.05) among variables for southern leopard frog *(Rana sphenocephala)* juveniles (year 1)

Response variables	**n**[a]	***r***	***p***
summer mass, autumn mass	15	0.71	0.0030
autumn growth rate, overwinter survival	15	0.60	0.0189
autumn growth rate, spring survival	15	0.54	0.0395
autumn survival, spring survival	15	0.52	0.0462
overwinter growth rate, spring growth rate	12	0.95	<0.0001
overwinter survival, spring survival	15	0.97	<0.0001

[a] n = number of enclosure means evaluated.

Table 7. Results of univariate analyses of variance for autumn mass, growth rate, and survival of southern leopard frog (*Rana sphenocephala)* juveniles reared in terrestrial enclosures (year 2)

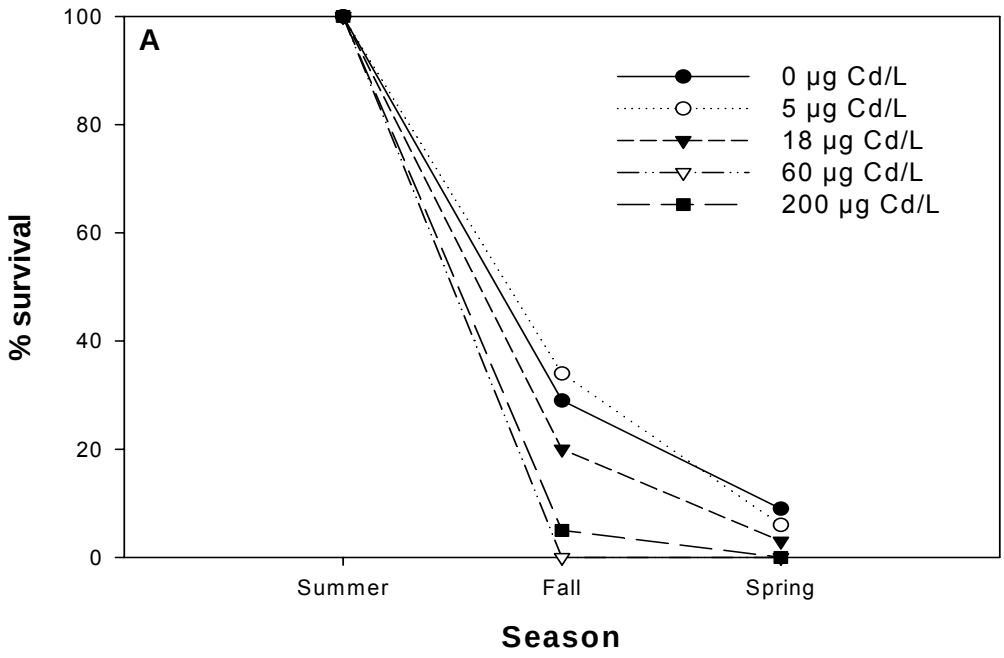
Response variable	**Source**	***MS***	***df***	***F***	***p***
Mass	block	0.127	3	5.27	0.0525
	cadmium	0.047	2	1.94	0.2385
	error	0.024	5		
Growth rate	block	0.261	3	4.77	0.0627
	cadmium	0.056	2	1.03	0.4229
	error	0.055	5		
Survival	block	0.769	3	14.61	0.0036
	cadmium	0.221	2	4.20	0.0723
	error	0.053	6		

Table 8. Mean (±1 standard error) mass, growth rate, and survival for southern leopard frog (*Rana sphenocephala*) juveniles reared in 2-m^2 terrestrial enclosures at an initial (spring) density of 1.5/m^2 (year 2)

Initial aquatic concentration (µg Cd/L)	Spring mass[a] (g)	Autumn mass (g)	Autumn growth rate (g/day)	Autumn survival (%)
0	3.838A	14.188	0.068	75
	(± 0.340)	(± 2.360)	(± 0.016)	(± 16)
5	4.281AB	13.750	0.061	83
	(± 0.335)	(± 1.651)	(± 0.010)	(± 17)
18	4.746B	16.193	0.073	58
	(± 0.174)	(± 1.419)	(± 0.009)	(± 21)

[a] capital letters within the column indicate significant differences according to the Tukey's test (p <0.05).

Figure 1. Mean survival over time for (a) American toad (*Bufo americanus)* and (b) southern leopard frog (*Rana sphenocephala)* juveniles reared in terrestrial enclosures at an initial density of 3.5/m^2 after chronic larval exposure to different concentrations of cadmium.


A
100
80
60
40
20
0
% survival
0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
60 µg Cd/L
200 µg Cd/L
Summer
Fall
Spring
Season

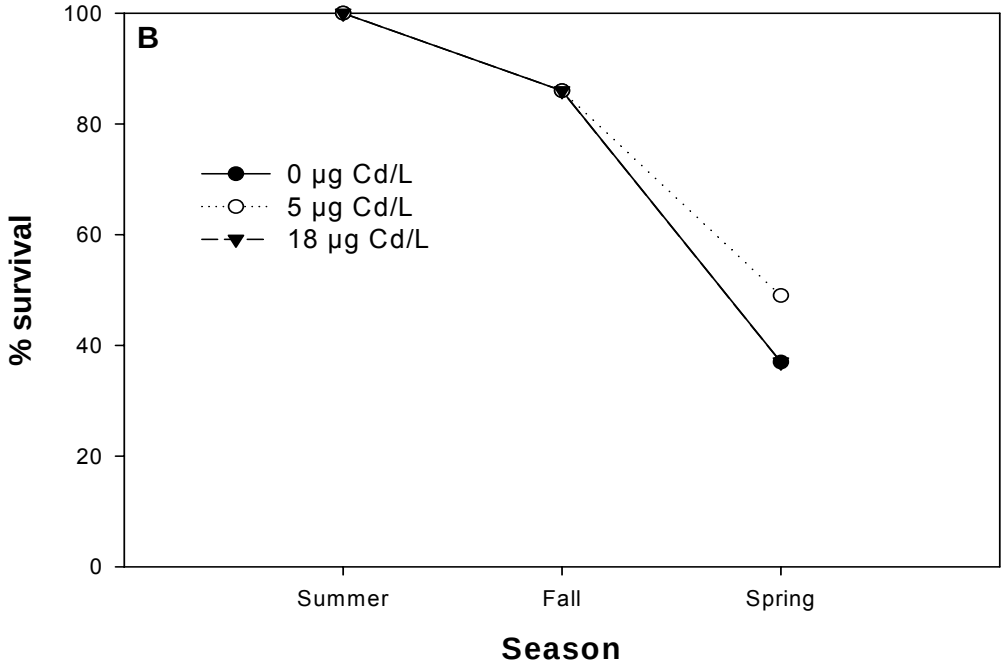
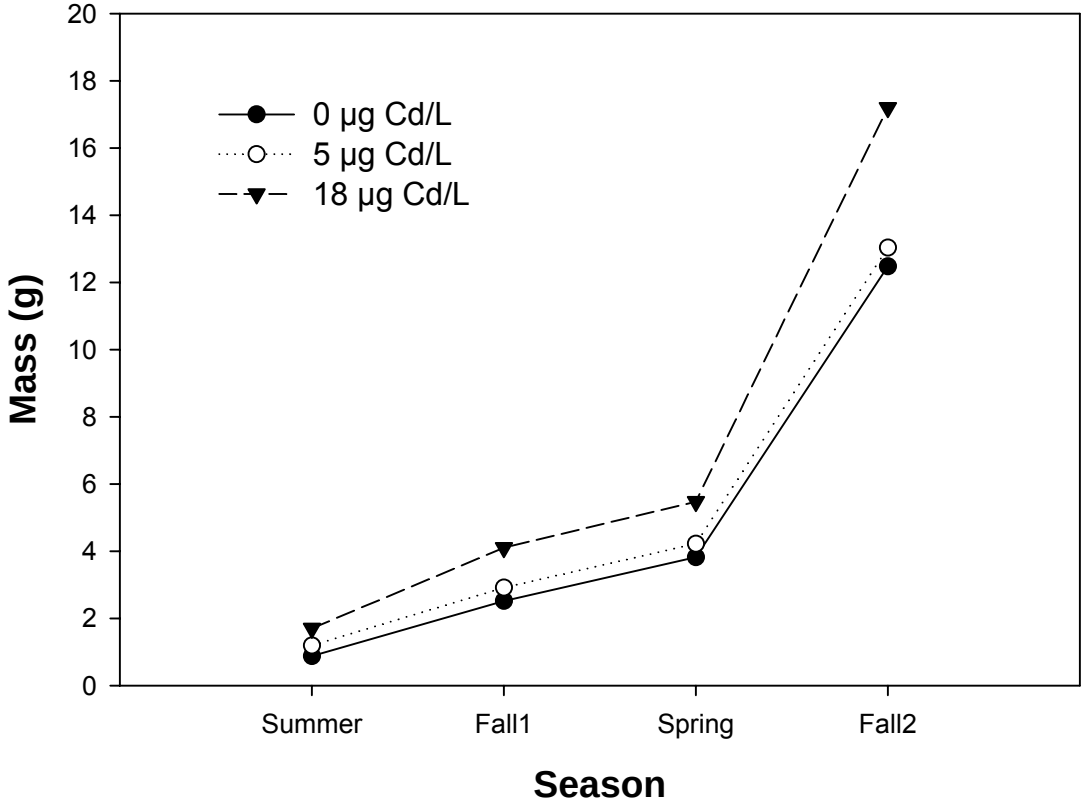

B
100
80
60
40
20
0
% survival
0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
Summer
Fall
Spring
Season

Figure 2. Mean mass change over time for individual southern leopard frog (*Rana sphenocephala)* juveniles during the first two terrestrial growth periods after chronic larval exposure to different concentrations of cadmium.


20
18
16
14
12
10
8
6
4
2
0
Mass (g)
0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
Summer
Fall1
Spring
Fall2
Season

CHAPTER 4

COMBINED EFFECTS OF CONTAMINANT EXPOSURE HISTORY AND FOOD AVAILABILITY ON THE TERRESTRIAL PERFORMANCE OF SOUTHERN LEOPARD FROG (*RANA SPHENOCEPHALA*) JUVENILES

ABSTRACT

Little is known about the terrestrial performance (i.e., growth, survival) of amphibians that metamorphose from contaminated breeding sites. An investigation was carried out to determine the combined effects of larval cadmium (Cd) exposure history and terrestrial food availability on the performance of southern leopard frog (*Rana sphenocephala*) juveniles. Individuals collected from outdoor aquatic mesocosms (1325 L) dosed once at 0, 5, or 18 µg Cd/L were monitored for two months in the laboratory. Initial mass increased with increasing Cd concentration. Terrestrial food availability was manipulated by rearing juveniles on a low or high diet of mealworms (*Tenebrio molitor*). Juvenile survival was 99%, indicating that neither diet nor Cd had lethal effects. Mass and growth rate were significantly affected by diet and time, and initial differences in mass among Cd concentrations within each diet level were maintained. A significant interaction between Cd and diet for juvenile mass after correcting for initial mass differences suggests there are direct effects of Cd on mass and that the response of mass to Cd depends on food availability in the terrestrial environment. There is clearly a link

between the larval environment and terrestrial performance, and terrestrial conditions are also highly influential.

INTRODUCTION

The majority of the literature on amphibian ecotoxicology has focused on early life stage responses to aquatic contamination. There is limited evidence that pre-metamorphic amphibians are the life stages most sensitive to contaminants (Hall and Swineford 1980, Schuytema et al. 1991) and there are links between poor embryonic development at contaminated sites and the loss of amphibian density and diversity (Bishop et al. 1999). However, regardless of relative sensitivities, population viability depends on the success of all life stages. Assessments should be conducted on multiple life stages to increase the understanding of the effects of particular contaminants on amphibian species. The current emphasis on aquatic exposure studies with tadpoles could be capitalized upon in this regard by rearing individuals to metamorphosis, and then monitoring the performance of juveniles. Such monitoring would ideally occur through reproductive maturity, given that life history characteristics such as fecundity and time to maturity affect population persistence (Stearns 1992). In the event this is not possible, monitoring over a shorter period such as the first growth season would also provide important information. Post-metamorphosis studies are particularly needed when metamorphic traits such as mass are affected, or when the contaminant bioaccumulates in amphibian tissue.

One bioaccumulating contaminant that affects amphibian larval development and metamorphosis is the heavy metal cadmium (Cd). Tadpoles have been collected from

field sites possessing more than 13 μg Cd/g (whole body dry weight; Snodgrass et al. 2003), and uptake has also been documented in the laboratory (Nebeker et al. 1995, Loumbourdis et al. 1999) and outdoor mesocosms (Lefcort et al. 1998, Chapter 2 or James et al., in press). Cadmium exposure can result in malformations (Herkovits et al. 1997) and altered growth (Nebeker et al. 1995, Loumbourdis et al. 1999, James and Little 2003, James et al., in press). Latent effects such as mortality also occur after the actual exposure is over (Pascoe and Shazili 1986). Cadmium can exceed 60 μg/L in the water column (Barks 1977) and also accumulates in periphyton (Besser et al. 2001), a major tadpole food source. Amphibians will breed in areas contaminated with Cd and other metals, but breeding success can be quite poor (Pollio 2001, Rowe et al. 2001). The post-metamorphic performance of those that survive early life stage Cd exposure is unknown. However, deleterious latent effects could occur given Cd accumulates in organs (e.g., liver, kidney) and alters physiological processes (e.g., enzyme and membrane function) (Eisler 1985).

A study was conducted to assess the performance (i.e., growth, survival) of southern leopard frog (*Rana sphenocephala*) juveniles that had been chronically exposed to Cd in outdoor aquatic mesocosms. This common species of the southeastern United States is considered a habitat generalist and will breed in Cd-contaminated water bodies (Pollio 2001, Burger and Snodgrass 2001). Following metamorphosis, individuals migrate from the breeding site into the surrounding terrestrial habitat, where they spend the summer and early fall foraging. The objective of the study was to determine whether the performance of juveniles exposed to Cd as larvae differed from unexposed juveniles. Cadmium may affect juvenile performance by changing metamorphic traits such as size

at metamorphosis, or by directly altering physiological processes and organ function (Eisler 1985). In this study there were two main questions: 1) does larval exposure to Cd affect juvenile performance? and 2) does Cd have direct effects on juvenile performance? Analysis of question 1 does not involve correcting for differences in metamorphic traits, but rather acknowledges their potential to influence performance. The analysis of question 2 corrects for such initial differences and focuses on Cd effects that occur post-metamorphosis. When southern leopard frog juveniles move into the terrestrial environment, they are subject to multiple potential stressors such predation, disease, and starvation. The role or importance of these natural factors may depend on contaminant exposure history (Taylor et al. 1999). Therefore, the diet level fed to the juveniles was manipulated to understand whether food availability influences the potential for Cd larval history to affect terrestrial performance. A resulting third question was asked: is there an interaction between Cd and diet, or, do the effects of Cd on juvenile performance depend on diet level?

MATERIALS AND METHODS

Study organisms

The juveniles used for this study were obtained from outdoor aquatic mesocosms (1325-L volume, 1.83-m diameter) that had been dosed once at the initial nominal concentrations of 0 (control), 5, or 18 µg Cd/L. The mesocosms were located in a mown field at the USGS Columbia Environmental Research Center (Columbia, MO, USA). Each mesocosm simulated a natural pond in that it contained approximately 950 L of softened well water (≈ 50 mg/L as $CaCO_3$), 1 kg of deciduous leaf litter, and a plankton

and algal community. Mesocosms were first filled with water in March 2003 and were dosed with Cd in mid-April. Four days after dosing, 50 southern leopard frog (*Rana sphenocephala*) tadpoles at developmental stage 25 (Gosner 1960) were added to each mesocosm and allowed to grow undisturbed through metamorphosis. This density is within the range of natural field densities (Petranka 1989). The tadpoles were collected as eggs from five clutches found in rain-fed ponds at the University of Missouri Baskett Wildlife Research Area and Mark Twain National Forest (Boone County, MO, USA). Metamorphs were captured in July and allowed to completely resorb their tails in the laboratory. Mass was determined following tail resorption, and is hereafter referred to as initial mass. All individuals used in this study were collected within a seven-day period. After initial mass was recorded, juveniles were transferred to a laboratory at the University of Missouri where the feeding study occurred from July 8 to September 11. The laboratory was maintained at 23 to 26 C and a 14:10 (light:dark) photoperiod, which was provided by a combination of standard fluorescent and broad-spectrum grow lights.

Experimental design

Each southern leopard frog juvenile was placed in its own container and is considered an independent replicate. The containers were plastic faunaria (19-cm long x 12-cm wide x 13-cm high; Hagen Corporation, Mansfield, MA, USA) that had lids with air slots and a hinged door for easy access by the investigators. Within each container was 730 g of dry, fine-grained silica sand (2.5-cm deep), a Petri dish with carbon-filtered tap water, a food cup, and a 7-cm-long piece of black polyvinyl chloride (PVC) pipe (3.8-cm diameter) that served as a refuge. The containers were positioned on four carts that had three shelves, and each shelf contained two replicates of each treatment. Treatments

were randomly assigned to shelf positions, and individual juveniles were randomly assigned to treatments. Containers were checked daily for mortalities and fresh water was provided if needed. Feces were removed twice weekly, and water dishes were cleaned weekly. Half-way through the study (August 12), containers were rinsed and received new sand, and the water dishes and refuges were scrubbed clean.

The two manipulated factors were larval Cd exposure history (0, 5, 18 μg Cd/L) and juvenile diet (low, high), for a total of six treatments. Each treatment was replicated 22 times and there was a total of 132 juveniles in the study. The low and high diet levels were 6% and 18% of individual body mass, meaning each juvenile was fed 6% or 18% of its mass over the course of one week. These feeding levels were selected to represent a maintenance and ad libitum diet, respectively, and were based on metabolic rate data (Gatten et al. 1992) and the energy density of the diet. However, it was sometimes observed that all mealworms in the high diet were eaten. Juveniles were fed three times weekly until August 11, and twice weekly thereafter. The diet consisted solely of mealworms (*Tenebrio molitor*) that were kept in plastic containers containing high-calcium cricket feed (Fluker's, Port Allen, LA, USA) mixed with calcium powder (Tetrafauna ReptoCal, Tetra, Inc., Blacksburg, VA, USA). Mealworms are an adequate diet for amphibians (Claussen and Layne 1983). When juveniles were fed, mealworms were weighed out in separate cups for each southern leopard frog. Juveniles were weighed approximately every two weeks, and mealworm rations were based on these masses in order to account for juvenile growth. The masses of both the southern leopard frogs and the mealworms were wet weight, and no attempt was made to drain the bladder of the juveniles before weighing. At the end of each week, uneaten mealworms were

removed from containers, but data on the number or mass of remaining mealworms were not recorded. The research ended 60 d after the last juveniles were initiated into the study. Although individuals began the study on different days due to differences in time to metamorphosis, the mean start days and mean study durations of the six treatments were within 0.5 d of each other.

Statistical analysis

The response variables were survival, mass, and growth rate, and the independent variables were Cd and diet. Each southern leopard frog was considered a replicate. The mass of each individual was recorded a total of five times, resulting in four sequential time intervals. Total percent mass increase was the final mass divided by the initial mass, multiplied by 100%. Growth rate was computed as change in mass over change in time, and was calculated for each time interval. Total growth rate was calculated by dividing the total change in mass by the total time between the initial and final mass determinations. Changes in mass and growth rate over time were analyzed with repeated measures analysis of variance and covariance on least squares means, using initial mass as the covariate to account for initial differences in mass. Analysis of variance was performed on initial mass to determine the effect of larval Cd exposure. Mass and growth rate were log-transformed to improve homogeneity of variance and normality, which were checked with the Bartlett and Shapiro-Wilk tests, respectively. Relationships between initial mass, final mass, and total growth rate were assessed using the Pearson correlation. Significance was set at $\alpha = 0.05$ and analyses were conducted with SAS (SAS Institute 1989). Survival was not statistically analyzed because it was high across all treatments.

RESULTS

Survival

One hundred thirty of the 132 (99%) southern leopard frog juveniles survived the study. Of the two that died, one was from the 0 µg Cd/L, low diet treatment and one was from the 5 µg Cd/L, low diet treatment. A third juvenile (0 µg Cd/L, high diet) was found with an open wound, which was perhaps obtained from a sharp edge on the pipe refuge. Because of this injury, the individual was removed from its container and euthanized.

Mass

Initial mass was significantly affected by larval Cd exposure ($F = 93.15$, $df = 2$, $p < 0.0001$) but did not differ among diet levels ($F = 1.52$, $df = 1$, $p = 0.2200$). Initial mass increased with increasing aqueous Cd concentration, and juveniles in the 18 µg Cd/L treatment were almost twice as large as the controls (Figure 1). Cadmium affected mass at metamorphosis indirectly by increasing mortality and subsequently releasing larvae from competition (James et al., in press). After being placed in the terrestrial containers, juvenile mass increased over time, particularly in the high diet treatments (Figure 1). The repeated measures analysis of mass over time showed a significant effect of Cd, diet, and time (Table 1). However, when initial mass was corrected for (i.e., initial mass was a covariate), diet and time remained significant, Cd became non-significant ($F = 0.04$, $df = 2$, $p = 0.9607$), and the Cd*diet interaction became significant ($F = 10.42$, $df = 2$, $p < 0.0001$). At the end of the study, individuals fed a low diet were only 112 to121% of their initial mass, compared with 235 to 272% for those on the high diet (Table 2). Mean

total mass gain ranged from 0.1356 g (0 µg Cd/L, low diet) to 2.6166 g (18 µg Cd/L, high diet) (Table 2). Initial trends in differences in mass among Cd concentrations were maintained throughout the study at both diet levels (Figure 1), which is reflected in the significant positive correlation between initial and final mass ($r = 0.56$, $p < 0.0001$).

Growth rate

Growth rate was significantly affected by Cd, diet, and time (Table 1). However, when initial mass was included as a covariate, Cd became non-significant ($F = 0.81$, $df = 3$, $p = 0.4452$). The least growth occurred in the first time interval for all six treatments (Table 2). Juveniles in the high diet treatments all had the greatest growth during the third time interval (Table 2). Mean total growth rate ranged from 0.0021 g/day (0 µg Cd/L, low diet) to 0.0410 g/day (18 µg Cd/L, high diet) and was greatest in the high diet treatments (Table 2). The difference in mean total growth rate among juveniles on different diets but from the same Cd concentration was greater than 5-fold (Table 2). Within each diet level, mean total growth rate increased with increasing Cd concentration (Table 2). There were significant positive correlations between total growth rate and initial mass ($r = 0.17$, $p = 0.0523$) and total growth rate and final mass ($r = 0.91$, $p < 0.0001$). Growth rate was approximately parallel among Cd concentrations within each diet level (Figure 1).

DISCUSSION

This study demonstrates that effects of the larval environment on metamorphic traits can carry over into the terrestrial environment by influencing juvenile mass and growth rate. Initial differences in mass due to larval Cd treatment (initial mass: 18 > 5 >

0 µg Cd/L) were maintained through the first two months of the terrestrial life stage. When initial mass differences were not corrected for, juvenile mass and growth rate were significantly affected by larval exposure to Cd. When initial mass differences were corrected for, there was no significant effect of Cd alone on mass and growth rate. However, there was an interaction between Cd and diet for mass, which suggests Cd has direct effects on juvenile mass. Cadmium influences juvenile growth rate indirectly through the initial mass differences, which in turn can be explained by larval survival differences due to direct Cd toxicity. The extremely high juvenile survival (99%) demonstrates that those who survive Cd exposure as larvae at the levels and conditions tested do not suffer elevated mortality as juveniles due to direct Cd-induced changes in body condition.

Juvenile mass and growth rate were also significantly affected by diet level, with or without correcting for initial mass. Individuals from a given Cd concentration grew approximately twice as large on the high diet relative to those on the low diet. There was a significant Cd by diet interaction for mass when initial mass was a covariate. This resulted from 0 and 5 µg Cd/L treatment juveniles on the high diet growing large enough to exceed the mass of the 18 µg Cd/L metmorphs on the low diet. Therefore, initial mass differences can be overcome if adequate food resources are found, and Cd treatment effects on juvenile mass are influenced by diet level. Juvenile performance clearly depends on the quality of both the larval and terrestrial environments. This is perhaps most clearly elucidated by the >3 g difference in mean final mass between the 0 µg Cd/L, low diet (1.2698 g) and the 18 µg Cd/L, high diet (4.6168 g) juveniles.

This study adds to an existing body of evidence that effects of the larval environment carry over into the terrestrial environment and impact post-metamorphic performance. In natural, uncontaminated populations, individuals with larger mass at metamorphosis reach reproductive maturity sooner and at a larger size (Smith 1987, Semlitsch et al. 1988, Berven 1990, Scott 1994). Resulting fitness benefits include greater likelihood of surviving to reproductive maturity (Smith 1987), as well as greater female fecundity and male mating success (Davies and Halliday 1977). Data from natural populations have been corroborated by mesocosm and laboratory research that shows initial size advantages can be maintained over time (Goater 1994, Goater and Vandenbos 1997, Morey and Reznick 2001, Relyea and Hoverman 2003) and that larger metamorphs have a greater probability of surviving (Goater 1994, Morey and Reznick 2001). Even the southern leopard frogs possessing a Cd body burden were able to maintain mass differences, presumably because any Cd-induced impairment was of far less consequence than body size or because there was selection in the larval stage for those with superior genotypes. Given the similar results from the number of species and terrestrial conditions represented in the studies cited above, the evidence appears strong that metamorphic traits are linked with adult fitness and that amphibian populations are largely regulated by the larval environment (Wilbur and Collins 1973, Wilbur 1980).

However, the terrestrial environment also plays some role in the regulation of amphibian populations (Werner 1986, Scott 1994, Pechmann 1995). This should be expected given metamorphs can be <1% of adult body mass and the time to reproductive maturity may exceed five years. Contrary to the previously cited studies, there is evidence from terrestrial enclosures that initial differences in mass can be made up for

within one year of metamorphosis (Beck and Congdon 1999, Boone, in review), a possible result of terrestrial density dependence. Predation rates and competition for resources (e.g., food, refugia) can be density-dependent (Pechmann 1995). Food availability in the terrestrial environment has the potential to influence juvenile performance (Morey and Reznick 2001). Competition is likely to be particularly high when individuals first metamorphose because of elevated density around the breeding site (Cohen and Alford 1993, Beck and Congdon 1999). Individuals occupying habitats where prey are low in abundance or of poor nutritional quality may experience slow growth and delayed maturity, regardless of size at metamorphosis (Goater 1994, Morey and Reznick 2001, Relyea and Hoverman 2003). The contribution of starvation to juvenile mortality is unknown relative to other potential causes, but competition for resources and resulting slower growth has been documented in amphibians reared in terrestrial enclosures at different densities (Pearson 1957, Cohen and Alford 1993). Under conditions of abundant food, amphibians have the benefit of greater body size, lipid levels, and clutch size, and earlier reproduction than occurs when food is more limiting (Scott and Fore 1995). Because initial differences in mass among individuals may change depending on relative energy consumption rates, food availability can have a large influence on the degree to which the larval environment affects juvenile performance (Morey and Reznick 2001).

Phenotypic plasticity of developmental traits such as mass and growth rate may be induced by changes in the environment. Predicting the consequences of environmental alteration and fluctuation is particularly difficult in amphibians, given many species are biphasic and have complex habitat use and reproductive behaviors. Predictions can be

made more confidently when relationships are established between successive life stages. In this study, mass at metamorphosis was correlated with the mass of two-month-old juveniles, even in treatments in which individuals had been exposed to Cd as larvae. Hence, it appears that individuals robust enough to survive larval exposure will also do well in the terrestrial environment and may even experience enhanced fitness because of a larger initial mass. There have been very few studies in which amphibians reared in contaminated water have been monitored post-metamorphosis, but they indicate terrestrial performance is not significantly affected by larval exposure history (Plowman et al. 1994, Rowe et al. 2001, Rehage et al. 2002, Boone, in review). However, these and the present study were limited in the number and range of exposure concentrations tested and individuals were not monitored through sexual maturity. Complete life cycle studies are necessary given Cd (Lienesch et al. 2000) and many other contaminants can have deleterious effects on reproduction. The ability of some contaminants to damage amphibian organs and physiology or cause malformations also warrants further investigation of latent effects of aquatic contamination on terrestrial life stage performance. Such investigation is necessary if we are to understand whether contaminant exposure influences amphibian population dynamics and persistence.

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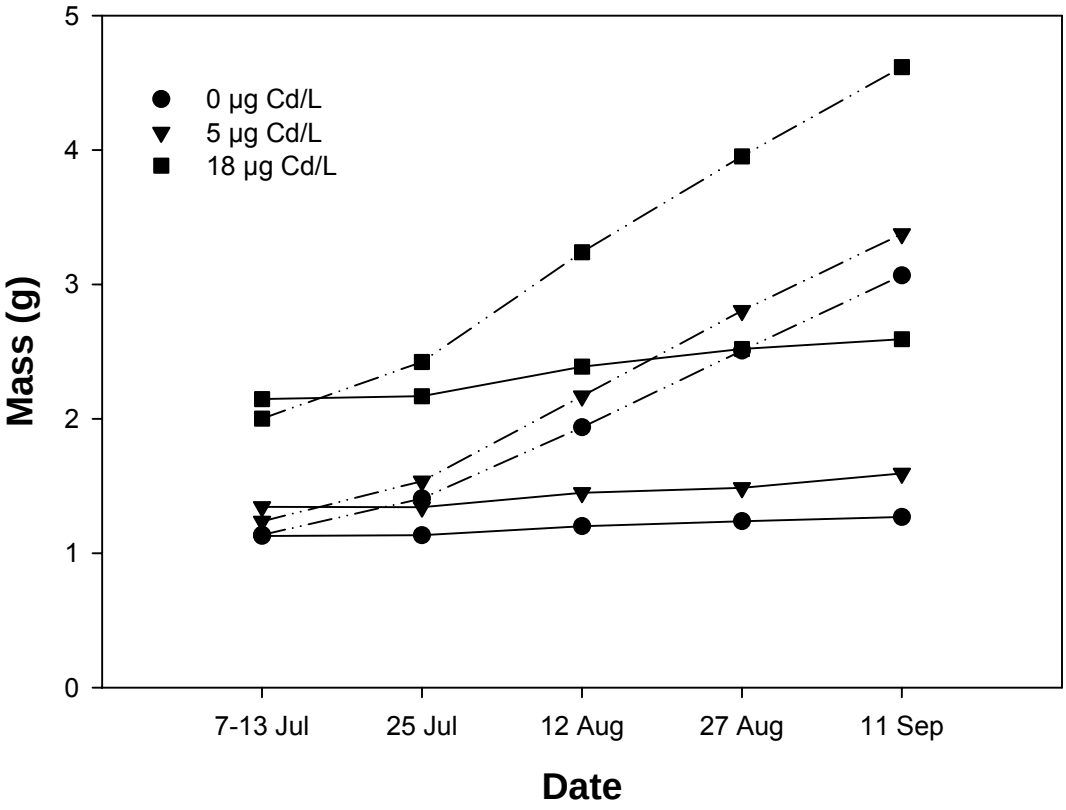
Table 1. Analysis of variance table for responses of southern leopard frog *(Rana sphenocephala*) juveniles reared on two diet levels for two months after chronic larval exposure to cadmium

Response variable	**Source**	***MS***	***df***	***F***	***p***
Mass	cadmium	16.355	2	77.89	<0.0001
	diet	29.257	1	139.32	<0.0001
	cadmium*diet	0.505	2	2.41	0.0944
	error	0.210	123		
	time	4.617	3	1576.83	<0.0001
	error (time)	0.003	369		
Growth rate	cadmium	0.155	2	13.77	<0.0001
	diet	8.438	1	747.82	<0.0001
	cadmium*diet	0.011	2	1.02	0.3642
	error	0.011	123		
	time	0.281	3	37.12	<0.0001
	error (time)	0.008	369		

Table 2. Mean growth (±1 standard error) of southern leopard frog (*Rana sphenocephala)* juveniles reared in individual containers for two months on low or high diets after chronic larval exposure to cadmium

Aquatic concentration (µg Cd/L)	Growth rate #1 (g/day)	Growth rate #2 (g/day)	Growth rate #3 (g/day)	Growth rate #4 (g/day)	Total growth rate (g/day)	Total mass gain (g)	Total mass increase (%)
low diet							
0	-0.0001	0.0038	0.0025	0.0015	0.0021	0.1356	112
	(± 0.0012)	(± 0.0010)	(± 0.0009)	(± 0.0016)	(± 0.0004)	(± 0.0253)	(± 2)
5	-0.0003	0.0042	0.0024	0.0072	0.0035	0.2217	114
	(± 0.0014)	(± 0.0014)	(± 0.0013)	(± 0.0021)	(± 0.0008)	(± 0.0529)	(± 3)
18	0.0013	0.0122	0.0088	0.0049	0.0070	0.4460	121
	(± 0.0019)	(± 0.0020)	(± 0.0025)	(± 0.0024)	(± 0.0007)	(± 0.0415)	(± 2)
high diet							
0	0.0175	0.0294	0.0380	0.0358	0.0302	1.9201	269
	(± 0.0013)	(± 0.0017)	(± 0.0020)	(± 0.0039)	(± 0.0013)	(± 0.0810)	(± 6)
5	0.0192	0.0351	0.0424	0.0381	0.0336	2.1393	272
	(± 0.0023)	(± 0.0023)	(± 0.0032)	(± 0.0045)	(± 0.0023)	(± 0.1516)	(± 6)
18	0.0267	0.0454	0.0475	0.0443	0.0410	2.6166	235
	(± 0.0023)	(± 0.0029)	(± 0.0050)	(± 0.0041)	(± 0.0022)	(± 0.1378)	(± 8)

Figure 1. Change in body mass over time for southern leopard frog (*Rana sphenocephala*) juveniles exposed to three concentrations of cadmium as larvae before being fed low (solid) or high (dashed) diet levels for a two-month period.


0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
Mass (g)
0
1
2
3
4
5
7-13 Jul
25 Jul
12 Aug
27 Aug
11 Sep
Date

CHAPTER 5

CADMIUM CONTAMINATION AND INTERSPECIFIC COMPETITION INFLUENCE THE SURVIVAL AND METAMORPHIC TRAITS OF SOUTHERN LEOPARD FROGS (*RANA SPHENOCEPHALA*)

ABSTRACT

Amphibian tadpoles occupying chemically-contaminated breeding sites are simultaneously faced with other potential stressors, such as interspecific competition. Given the complexity of the larval environment, studies that simulate natural conditions are needed to understand the effects of contamination on natural populations and communities. This study manipulated the heavy metal cadmium (Cd; initial nominal concentrations 0, 5, or 18 µg/L) and interspecific competition (presence or absence of American toad [*Bufo americanus*] tadpoles) in outdoor mesocosms (1325-L polyethylene cattle tanks) containing southern leopard frog (*Rana sphenocephala*) tadpoles. Cadmium exposure resulted in decreased survival, increased mass and time to metamorphosis, and elevated Cd body burdens for southern leopard frogs. Southern leopard frogs that competed with American toads had increased survival, a shorter larval period, decreased mass at metamorphosis, and no change in Cd body burdens relative to frogs reared without toads. A lack of interactions between Cd and interspecific competition suggests they largely act independently of each other. Direct toxicity is likely the primary explanation for the Cd effects, and food limitation is suspected for the effects of

interspecific competition. Water bodies contaminated with Cd at the concentrations tested may produce fewer, older, and larger southern leopard frog metamorphs, and population decline or loss could eventually occur. However, the complex and dynamic nature of natural systems may render such predictions tenuous.

INTRODUCTION

The role that contamination plays in amphibian larval development and metamorphosis has predominantly been assessed in isolation of other potential stressors (Boone and Semlitsch 2002). However, when factors such as hydroperiod, competition, predation, ultraviolet radiation, and acidification are also included, the magnitude and directionality of the response variables can be quite different than when contamination is examined alone (Zaga et al. 1998, Relyea and Mills 2001, Boone and James 2003, Mills and Semlitsch 2004). A contaminant may affect or be affected by abiotic and biotic factors in the environment, and significant interactions or enhanced toxicity may result. Laboratory studies have contributed substantially to understanding how amphibian responses to contamination are influenced by such factors. However, because the laboratory environment is usually not complex and kept somewhat constant over time, realism is potentially compromised. For instance, the influence of the presence of a predator may be overestimated in the laboratory considering the likelihood that the water is clear and well lit (instead of shaded and murky) and good hiding places are few and artificial. Likewise, competition may not occur as it would normally if food is provided ad libitum and chemical cues are lost with water renewals (Broomhall and Shine 2003).

Given that amphibian breeding sites are dynamic and complex systems, it is imperative to build on laboratory work by conducting mesocosm and field studies (Thompson 2004).

Cattle tank mesocosms have been used for many ecological and ecotoxicological studies with amphibians (see review in Boone and James, in press). Tanks are created to simulate natural ponds through the inclusion of substrate, plankton, and algae, and are subject to a normal photoperiod and fluctuating temperatures. The incorporation of variation in primary productivity, light intensity, temperature, dissolved oxygen, and other water quality characteristics is important because these factors can influence contaminant effects and organism responses (Lahr 1997). The environment should be self-sustaining in that supplemental feeding is not necessary and amphibian tadpoles can forage on a preferred diet. Dosing tanks with a contaminant results in exposure not only for the amphibians, but the rest of the aquatic community as well. Any changes in this community that result may influence tadpole behavior and life history traits, and the effects can be positive or negative (Mills and Semlitsch 2004). Bioaccumulating contaminants will partition into different environmental media, resulting in uptake by tadpoles via more than one route, which in turn affects distribution within the body (Wren et al. 1995). When other factors are manipulated in addition to contamination, the system quickly becomes quite complex as components influence and interact with each other. Fortunately, tanks allow for more highly replicated and less heterogeneous experiments than would be possible in natural ponds (Wilbur 1987). However, tanks include only some of the habitat complexity, environmental processes, and biota found in lentic systems (Williams et al. 2002), and exposure concentrations are difficult to maintain.

Interspecific competition is among the most influential regulators of larval amphibian communities (Wilbur 1987). It is typical for more than one species to utilize a given breeding site, and some species will oviposit simultaneously or within days of each other. The intensity of competition is a function of the relative timing of breeding among species (Alford and Wilbur 1985), as well as body size and resource and microhabitat use. Competition occurs through exploitation of the same food resources and the release of growth inhibitors (Steinwascher 1978). Species interactions depend on the food web structure and habitat disturbance (e.g., pond drying) (Wilbur 1987). When predators are absent, competition in natural systems is thought to slow growth and increase the chance of death from desiccation through lengthening the larval period (Wilbur 1987).

This study was conducted to assess the combined effects of interspecific competition (American toad [*Bufo americanus*] tadpoles) and contaminant exposure (cadmium [Cd]) on the survival and metamorphic traits of southern leopard frogs (*Rana sphenocephala*). American toads and southern leopard frogs have spatially and temporally overlapping breeding seasons. The tadpoles of both species forage on periphyton and suspended algae (phytoplankton), but American toads may on average feed relatively lower in the water column and along the bottom (Wilbur 1987). Southern leopard frogs usually require more than 45 d to metamorphose and weigh ≈ 1 to 3 g, compared with American toads that metamorphose relatively quickly (≈ 30 d) and at a small size (≈ 0.1 - 0.3 g). In uncontaminated conditions, southern leopard frogs have lower survival, smaller mass at metamorphosis, and a longer larval period when American toads are present than when they are absent (Alford and Wilbur 1985).

Cadmium is a non-essential heavy metal that can have deleterious effects on tadpole survival (Nebeker et al. 1995, Lefcort et al. 1998), growth (Nebeker et al. 1995, Loumbourdis et al. 1999), behavior (Lefcort et al. 1998), and morphology (Herkovits et al. 1997). Bioaccumulation is rapid and can increase with age as long as exposure continues (Dobrovoljc et al. 2003, Milton et al. 2003). Significant uptake occurs from water and food and increases with the exposure concentration (Nebeker et al. 1995). The distribution and fate of Cd in the environment depends on local conditions and processes (Eisler 1985). Cadmium in the water column will partition into other media, including tadpole food resources (Rowe et al. 2001). Background aqueous concentrations are generally less than 0.5 μg Cd/L and the United States federal water quality chronic criterion is 0.15 μg Cd/L (at 50 mg/L hardness; USEPA 2001). However, concentrations may exceed 60 μg Cd/L at some field sites (Barks 1977). Southern leopard frogs and American toads will breed in Cd-contaminated water bodies (Pollio 2001), but population- and community-level responses have received little attention. Almost all that is known about Cd effects on tadpoles is based on laboratory research that ended before metamorphosis and manipulated only the exposure concentration.

The objective of this study was to improve our understanding of the effects Cd has on larval amphibian populations by using cattle tanks as simulated ponds where interspecific competition is present. The measured response variables were percent survival, percent metamorphosis, mass at metamorphosis, time to metamorphosis, and whole body Cd concentration. Changes in the aquatic community were documented by monitoring the abundance of phytoplankton and periphyton, as well as the Cd content of

periphyton. Resource availability may both explain and be due to amphibian responses to Cd and interspecific competition.

MATERIALS AND METHODS

Study organisms

Eggs were collected from four southern leopard frog (*Rana sphenocephala*) and four American toad (*Bufo americanus*) clutches found in late April 2004. The eggs had been oviposited in rain-fed ponds at the Baskett Wildlife Research Area (Boone County, MO, USA), where there are no known sources of contamination. The eggs were transported in pond water to the USGS Columbia Environmental Research Center, where they were kept in plastic containers (5.5-L volume) in a laboratory (65 C, 14L:10D photoperiod). Partial water changes were made every one to two days with pond water and softened well water to prevent fouling and allow acclimation to the testing water. Once tadpoles were free swimming, they were fed ground fish flakes ad libitum (TetraMin®, Blacksburg, VA, USA).

Experimental design

A rectangular array of 29 polyethylene cattle tank mesocosms (1325-L volume, 1.83-m diameter; Behlen PolyTuff, Columbus, NE, USA) was set up in March 2004 on a mown field at the USGS Columbia Environmental Research Center. Tanks were filled with ≈ 950 L of softened well water (hardness ≈ 55 mg/L as $CaCO_3$) and then 1 kg of deciduous leaf litter was added. The leaves had been raked from the forest floor of an oak-hickory-maple stand at the Baskett Wildlife Research Area. Once the leaves had settled to the bottom, tanks were inoculated with equal volumes of concentrated plankton

and algae approximately every five days for a total of five inoculations. The concentrates were obtained by straining pond water through a zooplankton net (63 μm) at 16 ponds at the Baskett Wildlife Research Area and adjacent Mark Twain National Forest. Eight flat polyvinyl chloride (PVC) tiles (94 cm^2) were suspended from a plastic-coated wire across each pond, evenly spaced and <3 cm below the surface of the water. The tiles would later be scraped for periphyton to determine Cd bioaccumulation. A screen lid was placed over each tank to prevent the colonization of predaceous insects and anuran competitors.

Tanks were dosed with Cd in late April (day 0) using a concentrated stock solution (certified American Chemical Society $CdCl_2 \cdot 2.5H_2O$ crystals dissolved in deionized water; Fisher Scientific, Fairlawn, NJ, USA) to achieve initial nominal aqueous concentrations of 0 (control), 5, and 18 μg/L. Cadmium was administered by adding water from the respective tank into a plastic watering can along with the appropriate stock volume, and then sprinkling the contents evenly over the surface of the tank. Control tanks did not receive stock solution but were otherwise treated the same way so that all tanks would be disturbed equally. The watering can was rinsed between tanks and tanks were dosed in the order of increasing concentration. Eight days later healthy and free-swimming tadpoles of both species were added to the tanks. Fifty southern leopard frogs were placed in all 29 tanks, and 120 American toads were added to 20 of these tanks. This resulted in a total of six treatments, and the number of replicates (i.e., tanks) for each treatment was as follows: 0 μg Cd/L, interspecific competition (n = 4); 0 μg Cd/L, no interspecific competition (n = 3); 5 μg Cd/L, interspecific competition (n = 5); 5 μg Cd/L, no interspecific competition (n = 3); 18 μg Cd/L, interspecific competition

(n = 11); 18 μg Cd/L, no interspecific competition (n = 3). There was uneven replication so that enough metamorphs would be obtained for use in a subsequent terrestrial performance study (Chapter 6).

Tadpoles were not monitored until the onset of metamorphosis (developmental stage 42; Gosner 1960), at which time they were captured by dip-netting and transported to a laboratory. The first metamorph (American toad) was observed on day 27, and thereafter tanks were checked daily. Individuals with tails longer than ≈ 2 mm were kept in the laboratory in plastic containers with softened well water. Mass was determined once the tail had been resorbed (stage 46). A subset of metamorphs was randomly selected for determination of whole body Cd content. A sample consisted of two metamorphs of the same species pooled together from a given tank, and two tanks were randomly selected from each treatment for each species for a total of 18 samples (American toad: n = 6, southern leopard frog: n = 12). Samples were stored in plastic freezer bags at −15 C. The experiment was terminated on day 91, 83 d after tadpole addition. Tanks were drained and the leaf litter carefully searched so that remaining tadpoles could be recovered, staged, and weighed.

Tank sampling

Tanks were monitored during the study to characterize the larval environment. Each tank was sampled for water quality (temperature, dissolved oxygen, pH, hardness, alkalinity) and phytoplankton and periphyton abundance (as chlorophyll A) 3 d before Cd dosing and also on days 22 (all but pH, hardness, alkalinity) and 43. A PVC water sampler was used to obtain a 1-L sample from each of four locations within every tank. The four liters were combined in a polyethylene bucket and a 500-mL sample was

subsequently removed, placed in a polyethylene bottle, and stored on ice for analysis later that day. To minimize cross-contamination, the sampler was rinsed between tanks and each tank had a separate, permanently assigned bucket. Temperature and dissolved oxygen were measured directly from the water column, but the other three water quality variables were determined in the laboratory using 50 mL of the collected water. An additional 100 mL was used for phytoplankton abundance analysis; after running the water through a glass fiber filter (47 μm), the filter was placed in buffered acetone and refrigerated overnight. The next day a 7 mL sample was analyzed for chlorophyll before and after acidification using a fluorometer (665 - 870 nm; Turner Designs 10-AU, Sunnyvale, CA, USA). Periphyton abundance was determined by scraping a 5.7 cm^2 area on the side of each tank below the water line with a razor blade and depositing the collected material on a glass fiber filter. The chlorophyll content was determined in the same way as described above for phytoplankton.

Two tanks were randomly selected from each treatment for Cd determination. With one exception, the selected tanks were the same as those from which amphibian tissue samples were obtained. Cadmium in the water column was assessed on days 1 (24 h after dosing), 8 (tadpoles stocking day), and 26 by taking a ≈ 20 mL sample from 4 L collected. Water samples were acidified with a drop of 100% HNO_3 and kept at room temperature in plastic scintillation vials. Periphyton Cd content was monitored on day 26. Periphyton was scraped from both sides of 4 PVC tiles with a razor blade, placed in polyethylene bottles, and frozen at −15 C.

Cadmium analyses were performed by Severn Trent Laboratory Burlington (Colchester, VT, USA) using inductively coupled plasma-mass spectrometry (ICP-MS

X5; Thermo Electron Corporation, Madison, WI, USA). Water samples were filtered through a 0.45 µm filter before direct analysis, and the periphyton and amphibian tissue samples were digested in nitric acid and hydrogen peroxide. Quality control for all samples included spikes (77 - 119%), controls (92 - 96%), reference standards (95 - 106%), and blanks (free of contamination; ≤0.2 µg Cd/L or µg/g). Periphyton and amphibian tissue had been freeze-dried and the reported values are based on dry weight.

Statistical analysis

The unit of replication was the individual tank and analyses were performed on tank means. Days to metamorphosis (hereafter "age") was the number of days from addition to the tank to complete tail resorption. Metamorphs (stage ≥42) and tadpoles were considered survivors. Percent survival (hereafter "survival") was calculated as the number of survivors divided by the number initially stocked. It was assumed that all individuals not recovered had died. Percent metamorphosis (hereafter "metamorphosis") was metamorphs divided by survivors, and was defined in this way as a second indicator of the length of the larval period. Lower metamorphosis would indicate a prolonged larval period and older age. Cadmium and interspecific competition were the two main effects, and their interaction was also tested. A significant interaction would suggest that the main effects do not act independently of each other and that differences exist among the six treatments. Southern leopard frog metamorphosis and survival were analyzed with a ranked analysis of variance (ANOVA). Southern leopard frog mass at metamorphosis (hereafter "mass") and age were initially analyzed with a ranked multivariate analysis of covariance (MANCOVA) because there was a significant correlation (p <0.05) between the two endpoints as determined with the Pearson

Correlation. The MANCOVA was significant for the main effects so subsequent ranked ANCOVAs were performed. Survival was used as the covariate because mass and age are often influenced by survival due to density dependence (Wilbur 1997). American toad metamorphosis was analyzed with a ranked ANOVA, and survival with ANOVA. Mass and age were analyzed with MANOVA followed by individual ANOVAs, because of a significant correlation between the two endpoints. Cadmium was the class variable, and survival was not used as a covariate for mass or age because of a significant interaction between survival and Cd. The Cd content of periphyton and amphibian tissue was analyzed with a ranked ANOVA. Periphyton and phytoplankton abundance were analyzed with a repeated measures ANOVA, with Cd and interspecific competition as the main effects and interactions included. When significant differences were found in amphibian responses due to Cd, LSD multiple comparisons tests were conducted to examine which treatments differed. Analyses were conducted using the GLM procedure and Type III sum of squares in SAS (SAS Institute 1989) and significance was set at $\alpha = 0.05$.

RESULTS

Southern leopard frogs

A total of 890 southern leopard frogs metamorphosed by the time the study ended. Survival in the highest Cd treatment (18 µg Cd/L) was significantly less than the other treatments (Tables 1 and 2). Survival was lowest in tanks without interspecific competition from American toads (Tables 1 and 2). Although there was not a significant interaction among these main effects, differences in survival due to interspecific

competition appeared to depend on Cd (Table 1). Southern leopard frogs in the 18 µg Cd/L, no interspecific competition treatment had by far the lowest survival (19%). Metamorphosis was not influenced by Cd or interspecific competition and always exceeded 80% (Tables 1 and 2). The MANCOVA for mass and age was significant for Cd (Wilks' Lambda = 0.3426, $F_{4,42} = 7.44$, $p = 0.0001$) and interspecific competition (Wilks' Lambda = 0.6382, $F_{2,21} = 5.95$, $p = 0.0090$), but not the interaction (Wilks' Lambda = 0.7542, $F_{4,42} = 1.59$, $p = 0.1947$). The univariate analyses indicated mean body mass increased with Cd concentration (Tables 1 and 2); southern leopard frogs that had not been exposed to Cd were approximately half the size of those in the 18 µg Cd/L treatments (Table 1). Interspecific competition decreased mass by at least 0.3 g (Tables 1 and 2). Age also increased with Cd concentration, and tanks with American toads produced younger metamorphs (Tables 1 and 2). The effect of interspecific competition on the length of the larval period depended on (i.e., significant main effects interaction) and increased with Cd concentration (Tables 1 and 2). The delaying effect of Cd on the larval period as determined by age is further supported by a trend for metamorphosis to decrease with Cd concentration (Table 1). The Cd body burdens of the metamorphs increased with increasing initial aquatic concentration (Tables 1 and 2), and the most contaminated sample was 8.7 µg Cd/g. However, interspecific competition did not influence Cd body burdens, and there was not a significant interaction between Cd and interspecific competition (Table 2). At the end of the study, 101 (7% of initial) tadpoles were recovered, weighed, and staged, and the mean values are reported in Table 3.

American toads

There were 1377 American toad metamorphs by the end of the study. Control survival was 75%, but dropped significantly by approximately 20% in the two Cd concentrations (Tables 4 and 5). Metamorphosis exceeded 94% in all treatments and did not differ among them (Tables 4 and 5). The MANOVA of mass and age was significant for Cd (Wilks' Lambda = 0.3047, $F_{4,32} = 6.49$, $p = 0.0006$). The univariate analyses indicated Cd had a significant effect on age and mass, but there was not a linear dose response (Tables 4 and 5). Metamorphs from the 5 μg Cd/L treatment were the oldest, and metamorphosed over a week later than and at 78% the mass of the controls (Table 4). Control American toads had the highest survival and largest mass (Table 4). Whole body Cd content increased with aquatic concentration (Tables 4 and 5), and the most contaminated sample was 16.0 μg Cd/g. At the conclusion of the study, only 1% (n = 31) of those initially stocked were still tadpoles. Mean values for mass and developmental stage are reported in Table 3.

Larval environment

Cadmium in the water column dropped quickly and measured ≤6% of the initial nominal concentrations by the time the tadpoles were added eight days after dosing (Figure 1). Such a rapid drop is not unusual when mesocosms are dosed only once with Cd (Kettle and deNoyelles 1986). Some of the Cd partitioned into the periphyton, resulting in substantial contamination of a primary tadpole food source (Figure 2). It is typical for periphyton to contain many times the concentration of water in experimental (Selby et al. 1985) and natural (Besser et al. 2001) systems. There was a significant effect of aquatic Cd ($F = 34.91$, $df = 2$, $p = 0.0005$) on periphyton contamination, but no

effect of interspecific competition ($F = 0.73$, $df = 1$, $p = 0.4265$) or their interaction ($F = 0.73$, $df = 2$, $p = 0.5214$). Phytoplankton was not measured for Cd content, but was likely contaminated as well (Conway 1978). The means and ranges, respectively, of the water quality variables for all 29 tanks were: temperature (21.8 C, 15.8 - 28.0 C), dissolved oxygen (5.4 mg/L, 3.4 - 8.8 mg/L), pH (7.4, 7.2 - 7.9), hardness (43 mg $CaCO_3$/L, 38 - 46 mg $CaCO_3$/L), alkalinity (34 mg $CaCO_3$/L, 30 - 42 mg $CaCO_3$/L). Phytoplankton abundance was significantly affected by Cd ($F = 7.56$, $df = 2$, $p = 0.0030$), time ($F = 64.82$, $df = 2$, $p < 0.0001$), and their interaction ($F = 7.68$, $df = 4$, $p < 0.0001$), but not interspecific competition ($F = 1.05$, $df = 1$, $p = 0.3167$) and there was not a significant Cd*competition interaction ($F = 0.20$, $df = 2$, $p = 0.8199$). Phytoplankton abundance measured lowest on the first sampling date (Figure 3), probably in part because phytoplankton was only just beginning to establish and grow in the tanks following the inoculations. On the final sampling date, 5 d before the first southern leopard frog metamorph was collected and when 85% of the total American toad metamorphs had transformed, phytoplankton was least abundant in the 18 µg Cd/L tanks (Figure 3). Mean phytoplankton abundance for all sampling dates combined was: 0 µg Cd/L = 13.2 µg/L, 5 µg Cd/L = 8.5 µg/L, 18 µg Cd/L = 7.5 µg/L. Periphyton abundance was significantly affected by Cd ($F = 8.79$, $df = 2$, $p = 0.0015$) and time ($F = 68.53$, $df = 2$, $p < 0.0001$) but not interspecific competition ($F = 2.90$, $df = 1$, $p = 0.1018$). Of the possible interactions, time*Cd ($F = 5.76$, $df = 4$, $p = 0.0008$) and time*competition ($F = 3.23$, $df = 2$, $p = 0.0493$) were significant. Like the phytoplankton, the lowest measured values occurred on the first sampling date (Figure 4). The last time periphyton was sampled, there was a trend of increasing abundance with increasing initial aqueous Cd concentration and there

was more periphyton in tanks without interspecific competition (Figure 4). Mean total periphyton abundance over the course of the study was: 0 μg Cd/L = 0.5 μg/cm^2, 5 μg Cd/L = 0.9 μg/cm^2, 18 μg Cd/L = 1.2 μg/cm^2.

DISCUSSION

Amphibian reproductive success and metamorphic traits are partially a function of the quality of the breeding site. Factors such as competition, predation, hydroperiod, and chemical contamination can be highly influential, and may even interact with each other (Boone and James 2003, Mills and Semlitsch 2004). Assessments of the effects of contaminants on amphibian populations should therefore include the manipulation of other important variables at realistic levels. Outdoor mesocosms facilitate this, and incorporate indirect contaminant effects on the community (Mills and Semlitsch 2004). Exposure to Cd and interspecific competition with American toads had both harmful and beneficial effects on southern leopard frog larval development in cattle tank ponds. Considering the general lack of interactions between Cd and interspecific competition, they appear to largely act independently of each other under the conditions tested.

Cadmium contamination of the aquatic environment resulted in lower survival, larger mass at metamorphosis, a longer larval period, and elevated whole body Cd residues for southern leopard frogs relative to uncontaminated conditions. Reduced survival indicates that either direct Cd toxicity or Cd alteration of the environment (i.e., indirect effect) was deleterious to the tadpoles, and therefore implies that the larval environment was in some way unfavorable. Direct toxicity likely resulted in the mortalities, given the presence of Cd in metamorph tissue and given that resource

limitation does not appear to have been a factor as indicated by larger mass at metamorphosis and greater periphyton abundance with increasing aquatic contamination. Lower density due to reduced survival likely freed up food resources enough that tadpoles prolonged metamorphosis so a larger mass was achieved. The existence of a tradeoff between mass and age is well-documented in larval amphibians, and the onset of metamorphosis appears to be a function of recent growth history, density, and the minimum and maximum size at which transformation can occur (Wilbur and Collins 1973). When density is low, there is a tendency for metamorphosis to occur closer to the maximum mass physiologically possible (Wilbur and Collins 1973). The significant effect of Cd on mass after survival was corrected for as a covariate indicates Cd also had a direct effect on mass. The increase in mass with Cd concentration may be attributed to hormesis, the stimulation of growth that is directly induced or results from compensation after the disruption of homeostasis (Calabrese and Baldwin 2002). Hormesis is an adaptive response to stress induced by low chemical concentrations (Calabrese and Baldwin 2002). Cadmium has hormetic effects in many organisms, including amphibians (James and Little 2003) and insects (Nascarella et al. 2003). Cadmium also had a direct effect on metamorph age. Exposure was chronic and via more than one medium because Cd partitioned from the water into the food base at high concentrations and the contamination persisted throughout the larval period. It might therefore be expected that metamorphosis would occur in the shortest time and at the smallest mass possible, in order to move to a better environment (Wilbur and Collins 1973). However, there can be significant variation in Cd uptake among individuals (Dobrovoljc et al. 2003), potentially rendering some more sensitive than others. It is possible that the survivors of exposure

accumulated relatively little Cd or were genetically superior compared to those that died. Other chronic studies that used Cd as the sole contaminant found that metamorphosis was quicker and at a larger size relative to controls (laboratory: James and Little 2003), that there was no effect on mass or the length of the larval period (outdoor microcosm: Lefcort et al. 1998), or that metamorphosis was delayed (laboratory: Flament et al. 2003). The inconsistent results among studies are certainly troubling and may be attributable to a number of variables, including primary exposure route and food quality and abundance.

The viability of southern leopard frog populations that breed in Cd-contaminated sites may be relatively poor in comparison with populations at uncontaminated sites. Lower tadpole survival means there will be fewer metamorphs that have a chance to become reproductive adults. Provided terrestrial life stage survival is density-independent, smaller cohorts would presumably eventually result in smaller breeding populations and eventual extinction. Lower reproductive success is particularly detrimental to amphibians given high rates of mortality persist throughout their life cycle (Breden 1988). There is also evidence of a tradeoff between contaminant tolerance and performance in uncontaminated conditions (Semlitsch et al. 2000), such that those that survive contaminant exposure best may be less fit in more competitive environments. An increase in age at metamorphosis means populations may be at greater risk of mortality from desiccation when breeding in temporary water bodies, and metamorphs have less time to forage before the winter. However, given that individuals exposed to Cd also metamorphosed at a larger size, earlier metamorphosis at a smaller size should be possible, provided Cd has no sublethal effects on the physiology of transformation. Finally, a Cd body burden may lower body condition through organ dysfunction and

altered physiology because Cd accumulates in organs (e.g., liver, kidney) and binds to and alters enzymes, DNA, cellular membranes, and other molecules (Eisler 1985). Contaminated amphibians can experience reduced spermatogenesis (Kasinathan et al. 1987) or transfer Cd residues to their offspring (Lienesch et al. 2000) or predators. The concentrations found in the southern leopard frogs were within the range of that found in wild-caught amphibians (Hall and Mulhern 1984). All of these conclusions are based on the result that metamorphs were indeed produced in all treatments. However, complete reproductive failure documented at some field sites where Cd and other metals are present (Pollio 2001, Rowe et al. 2001) can quickly decimate short-lived amphibian species.

When southern leopard frogs had to compete with American toads, survival was higher and metamorphs were younger, but metamorphs were also smaller in mass. This result was the case for controls, but even more so for those exposed to Cd. In another cattle tank study conducted without Cd but using these same two species and identical addition dates, southern leopard frogs metamorphosed smaller and later and had lower survival relative to frogs raised without toads (Alford and Wilbur 1985). Such effects are an expected outcome of interspecific competition and density that commonly occurs in larval amphibians (Wilbur 1977, Wilbur 1987). The present study indicates contamination can change the nature of species interactions because interspecific competition had effects on survival and age that were opposite what is expected in uncontaminated conditions. The smaller size and earlier metamorphosis found with interspecific competition suggest metamorphosis was a better alternative than remaining in the aquatic environment (Wilbur and Collins 1973). This is particularly interesting

considering the American toads metamorphosed on average at least 25 d sooner than did the southern leopard frogs, and indicates effects of the toads were long-lasting (see also Alford and Wilbur 1985). Higher southern leopard frog survival in the presence of American toads resulted in higher density and probably contributed to lower mass via competition mechanisms such as lower per capita food resources. Neither phytoplankton nor periphyton abundance was affected by interspecific competition, so total consumption appears to have been the same despite differences in total amphibian density. Food resources may have been more limiting for the southern leopard frogs when American toads were present due to higher inter- and intra-specific competition than when the toads were absent. Limited food resources may have resulted in smaller southern leopard frog mass and prompted earlier metamorphosis. The smaller mass may have also been due to the release of chemical inhibitors, given interference can supplant exploitation mechanisms of competition as relative food availability decreases (Steinwascher 1978). Both food limitation and chemical inhibition are very important in structuring the competitive relationships among species, and failure to incorporate them can result in no significant effects of interspecific competition in the presence of a contaminant (Broomhall and Shine 2003). The reasons for increased survival when American toads were present remain unclear. Perhaps the American toads somehow improved the quality of the aquatic environment despite using food resources. The American toads may have enhanced nutrient cycling and consequently increased the productivity or quality of food consumed by southern leopard frogs (Alford and Wilbur 1985). Or, given the effect of interspecific competition on survival was greatest in Cd-treated tanks, perhaps the improvement was in the form of American toads making the

environment less toxic through Cd consumption. Indeed, American toads contained a higher average body burden than did southern leopard frogs, and tanks (18 µg Cd/L treatment only) with American toads had lower Cd residues in the periphyton. With the exception of mass, southern leopard frogs actually did better with the American toads than when alone.

The abundance of periphyton and phytoplankton were monitored periodically to understand conditions in the larval environment. However, the results are a complex product of both direct Cd toxicity on the periphyton and phytoplankton, as well as indirect effects of Cd on components of the aquatic environment (e.g., tadpoles, zooplankton). The few studies that have been conducted to assess the toxicity of Cd to periphyton indicate biomass is reduced (Nayar et al. 2003) and phytosynthesis inhibited (Hill et al. 2000). In more natural systems where other organisms are present, there is no effect (Selby et al. 1985, Brooks et al. 2004) or growth is stimulated (Brooks et al. 2004). Knowledge of the effects of Cd on phytoplankton appears limited to studies that included other organisms, and results are mixed. Cadmium exposure can reduce (Marshall and Mellinger 1980, Kerrison et al. 1988) or increase (Kerrison et al. 1988) photosynthesis and primary production, as well as change species composition (deNoyelles et al. 1980). In the present study, the average abundance relative to initial nominal Cd concentration was periphyton: 18 > 5 > 0 µg Cd/L and phytoplankton: 0 > 5 > 18 µg Cd/L, which suggests an inverse relationship between the two. Phytoplankton and periphyton are indeed known to compete in aquatic communities, and relative abundance depends on the presence of predators such as zooplankton and larval amphibians (Leibold and Wilbur 1992). Southern leopard frogs consume periphyton, which frees phytoplankton from

competition (Leibold and Wilbur 1992). Therefore, high southern leopard frog survival should be associated with enhanced phytoplankton and suppressed periphyton, which is what the above trends indicate given survival decreased with increasing Cd.

As was the case for the southern leopard frogs, Cd exposure lowered American toad survival. Controls not only had the highest survival, but also the largest mass and youngest age. This confirms laboratory research on other species that has found Cd causes mortality, stunts growth, and delays development (Nebeker et al. 1995, Loumbourdis et al. 1999, Flament et al. 2003), but contradicts a laboratory study on American toads in which Cd increased mass, decreased the larval period, and had no effect on survival at constant aqueous concentrations of ≤54 µg Cd/L (James and Little 2003). Variation can be expected, however, because the present study was of a far more complex nature than those conducted in the laboratory, and the exposure concentrations and routes were different. Given that total tadpole density was less and periphyton abundance was more in the Cd treatments, it was probably direct toxicity instead of food limitation or competitive interference that resulted in the lower survival and mass. However, food might have been limited in the sense that dietary Cd inhibits feeding (Irving et al. 2003). Interestingly, it was the American toads in the 5 µg Cd/L treatment that had the poorest performance, indicating toxicity does not always increase with concentration. The presence of southern leopard frogs may have played a role in the magnitude and direction of American toad responses. Southern leopard frogs use both chemical interference and aggressive behavior to decrease the growth and survival of other species (Faragher and Jaeger 1998). The survival of southern leopard frogs exceeded that of the American toads in all Cd treatments by at least 10%, which suggests

American toads are more sensitive to Cd, are inferior competitors, or both. Response variation among and within species are expected given differences in genetics, life history traits, behavior, and biogeography (Bridges and Semlitsch 2000).

The initial nominal aquatic concentrations of 5 and 18 µg Cd/L are within the range of what is found in the environment, but are higher than what is typically found at contaminated sites (e.g., Besser et al. 2001). However, by the time the tadpoles were added these concentrations had dropped to below 1 µg Cd/L, which is more representative of field exposures. Cadmium partitioned into periphyton at a mean concentration of 11 and 128 µg/g dry weight for the 5 and 18 µg Cd/L treatments, respectively. Similar or higher concentrations (≤884 µg Cd/g dry weight) in periphyton have been documented at contaminated field sites (Farag et al. 1998, Besser et al. 2001). For example, streams containing an average of 1.2 µg Cd/L had periphyton measuring 31 µg Cd/g (Besser et al. 2001). Laboratory studies of strictly aqueous exposure scenarios do not indicate that Cd decreases tadpole survival or delays growth at concentrations below 54 µg Cd/L (James and Little 2003). Therefore, it is likely that oral uptake by ingestion of Cd-contaminated food contributed greatly to bioaccumulation and subsequent responses. Regulatory monitoring that samples only aqueous Cd may not be protective of amphibians and other herbivorous organisms because of the potential for Cd to biomagnify from water into primary producers.

This study adds to a growing body of literature in which outdoor mesocosms were used to assess the responses of amphibian populations or communities to aquatic contamination (see review in Boone and James, in press). Simulated ponds depict environmental exposure scenarios more accurately than laboratory studies by including a

complex food web and ecological factors such as interspecific competition. Direct toxicity may occur, particularly with persistent, bioaccumulating contaminants (Lefcort et al. 1998), but sometimes the major effect of a contaminant is alteration of the aquatic environment (Boone and James 2003, Mills and Semlitsch 2004). As was found in the present study, contamination can alter the magnitude and direction of metamorphic traits, and species differ in their sensitivity (Boone and Semlitsch 2002, Boone and James 2003). Interspecific competition has important effects of its own, which can be influenced by contamination (Mills and Semlitsch 2004). Contaminants may decrease interspecific competition by lowering density via mortality (Britson and Threlkeld 2000) and increasing prey (Boone and Semlitsch 2002), or intensify competition through reduction of food resources (Mills and Semlitsch 2004). Both contamination and interspecific competition interact with numerous other potential stressors present in amphibian breeding habitats (Mills and Semlitsch 2004).

It is very important to incorporate ecology into toxicological research. The complex dynamics that rule the natural world render assessments based only on a single factor (i.e., the contaminant) highly questionable (Boone and Semlitsch 2002). Even studies that manipulate a few factors in relatively realistic conditions may fall short of providing predictive power. For instance, the results of the present study likely would have changed somewhat if the two species had not been added synchronously (Alford and Wilbur 1985). The conservation of amphibian populations relies on our ability to distinguish causal agents from all other variables amidst natural fluctuations (Pechmann et al. 1991), but we are faced with the daunting fact that most agents have accomplices, which in turn depend on characteristics of the environment. Indeed, the environment is

complex and dynamic enough that every population faces unique challenges and conditions. Manipulative, multi-factor studies are necessary for establishing relationships, but should not take the place of site-specific research when particular populations are considered at risk.

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Table 1. Mean (±1 standard error) percent survival and metamorphosis, mass, age, and cadmium content of southern leopard frog (*Rana sphenocephala*) metamorphs reared with and without interspecific competition from American toads (*Bufo americanus*)

	American toads present			**American toads absent**		
	Initial nominal aqueous concentration (μg Cd/L)					
	0	**5**	**18**	**0**	**5**	**18**
Survival (%)	87.5	84.8	64.7	83.3	62.7	19.3
	(± 3.1)	(± 2.2)	(± 2.8)	(± 11.7)	(± 3.3)	(± 1.8)
Metamorphosis (%)	93.8	89.1	87.6	93.5	90.0	80.4
	(± 2.3)	(± 3.3)	(± 1.6)	(± 3.9)	(± 3.9)	(± 8.4)
Mass (g)	0.873	1.121	1.649	1.190	1.604	2.360
	(± 0.056)	(± 0.082)	(± 0.079)	(± 0.174)	(± 0.028)	(± 0.366)
Age (d)	58.0	61.8	66.0	58.7	65.8	77.6
	(± 2.1)	(± 0.6)	(± 0.8)	(± 1.1)	(± 0.9)	(± 1.6)
Cd content (μg/g)	0.2	3.4	6.0	0.2	2.9	6.3
	(—)	(—)	(—)	(—)	(—)	(—)

Table 2. Results of univariate analyses of (co)variance for percent survival and metamorphosis, mass, age, and cadmium content of southern leopard frog *(Rana sphenocephala*) metamorphs

Response variable	Source	*MS*	*df*	*F*	*p*
Survival	cadmium	529.3992	2	21.29	<0.0001
	competition	380.6089	1	15.31	0.0007
	cadmium*competition	47.5533	2	1.91	0.1705
	error	24.8680	23		
Metamorphosis	cadmium	205.1804	2	2.97	0.0710
	competition	5.4493	1	0.08	0.7812
	cadmium*competition	17.5028	2	0.25	0.7781
	error	68.9943	23		
Mass	survival (covariate)	77.9647	1	4.92	0.0372
	cadmium	96.7427	2	6.10	0.0078
	competition	85.9802	1	5.42	0.0295
	cadmium*competition	0.6977	2	0.04	0.9570
	error	15.8566	22		
Age	survival (covariate)	2.4970	1	0.13	0.7181
	cadmium	289.5068	2	15.50	<0.0001
	competition	140.9185	1	7.54	0.0118
	cadmium*competition	65.4282	2	3.50	0.0478
	error	18.6782	22		
Cadmium content	cadmium	57.0000	2	12.67	0.0070
	competition	0.0000	1	0.00	1.0000
	cadmium*competition	1.0000	2	0.22	0.8070
	error	4.5000	6		

Table 3. Mean ±1 SE mass and developmental stage of southern leopard frog *(Rana sphenocephala*) and American toad (*Bufo americanus*) tadpoles recovered at the end of the study when tanks were drained

	Initial nominal aqueous concentration (µg Cd/L)		
	0	**5**	**18**
Southern leopard frogs			
American toads present			
Mass (g)	2.722 ± 0.289	2.052 ± 0.163	2.630 ± 0.103
Stage	39.3 ± 0.6	37.1 ± 0.5	37.3 ± 0.6
American toads absent			
Mass (g)	2.542 ± 0.288	2.643 ± 0.140	1.935 ± 1.782
Stage	37.4 ± 1.9	36.9 ± 0.3	40.0 ± –[a]
American toads			
Mass (g)	0.150 ± –	0.129 ± 0.008	0.101 ± 0.007
Stage	36.0 ± –	35.7 ± 0.3	35.6 ± 0.9

[a] no standard error because tadpoles were collected from only one tank.

Table 4. Mean (±1 standard error) percent survival and metamorphosis, mass, age, and cadmium content of American toads (*Bufo americanus*) reared in interspecific competition with southern leopard frogs (*Rana sphenocephala*)[a]

	Initial nominal aqueous concentration (µg Cd/L)		
	0	**5**	**18**
Survival (%)	74.8A	53.5B	54.9B
	(± 10.8)	(± 4.4)	(± 2.9)
Metamorphosis (%)	99.7	94.7	98.7
	(± 0.3)	(± 2.3)	(± 0.6)
Mass (g)	0.081A	0.063B	0.068B
	(± 0.004)	(± 0.002)	(± 0.003)
Age (d)	27.9A	35.6B	29.5A
	(± 1.0)	(± 1.3)	(± 0.8)
Cd content (µg/g)	1.1A	9.3AB	13.7B
	(± 0.1)	(± 0.2)	(± 2.4)

[a] Different letters within rows indicate significant differences due to cadmium concentration according to the LSD test ($p < 0.05$).

Table 5. Results of univariate analyses of variance for percent survival and metamorphosis, mass, age, and cadmium content of American toad *(Bufo americanus)* metamorphs

Response variable	**Source**	***MS***	***df***	***F***	***p***
Survival	cadmium	0.0664	2	4.10	0.0352
	error	0.0162	17		
Metamorphosis	cadmium	44.8153	2	1.76	0.2016
	error	25.4335	17		
Mass	cadmium	0.0004	2	3.97	0.0386
	error	0.0001	17		
Age	cadmium	82.2794	2	11.47	0.0007
	error	7.1764	17		
Cadmium content	cadmium	8.0000	2	16.00	0.0251
	error	0.5000	3		

Figure 1. Concentration of cadmium in the water column over time in tanks with (solid lines) and without (dashed lines) interspecific competition. The legend indicates the initial nominal aqueous cadmium concentrations.

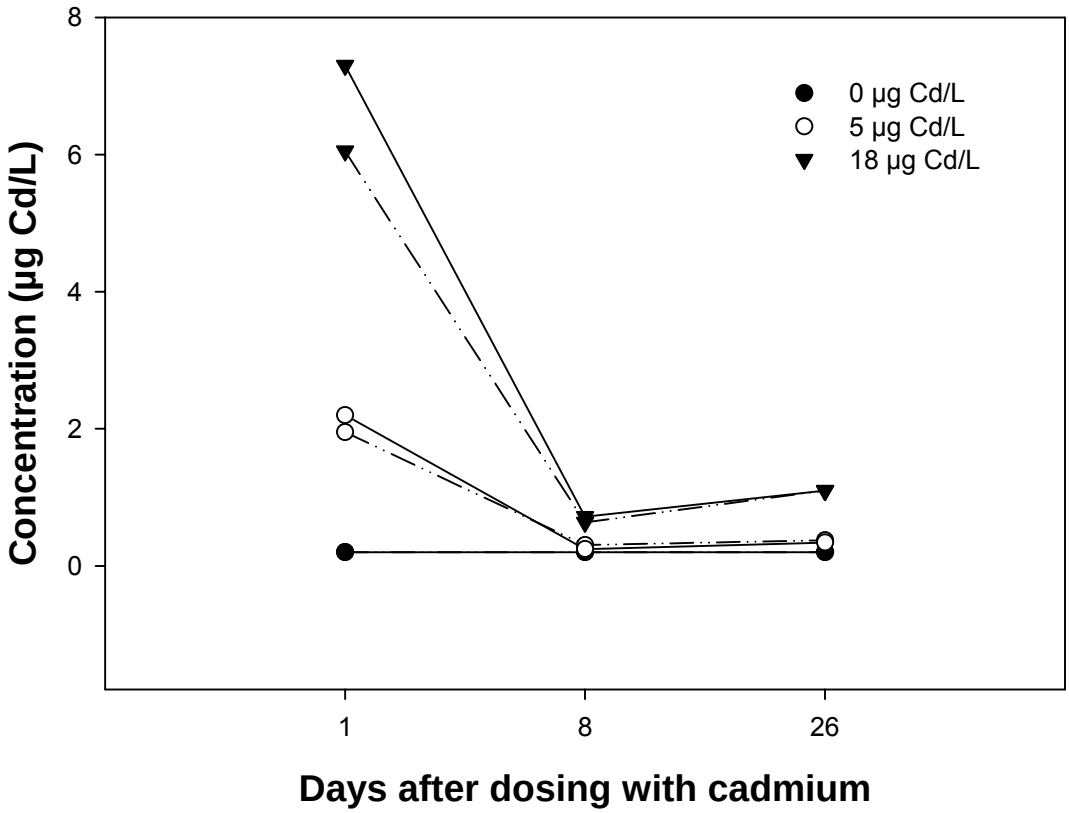

8
6
4
2
0
Concentration (µg Cd/L)
0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
1
8
26
Days after dosing with cadmium

Figure 2. Periphyton bioaccumulation of cadmium in tanks with and without interspecific competition at 26 days after dosing. The initial nominal aqueous cadmium concentrations are indicated.

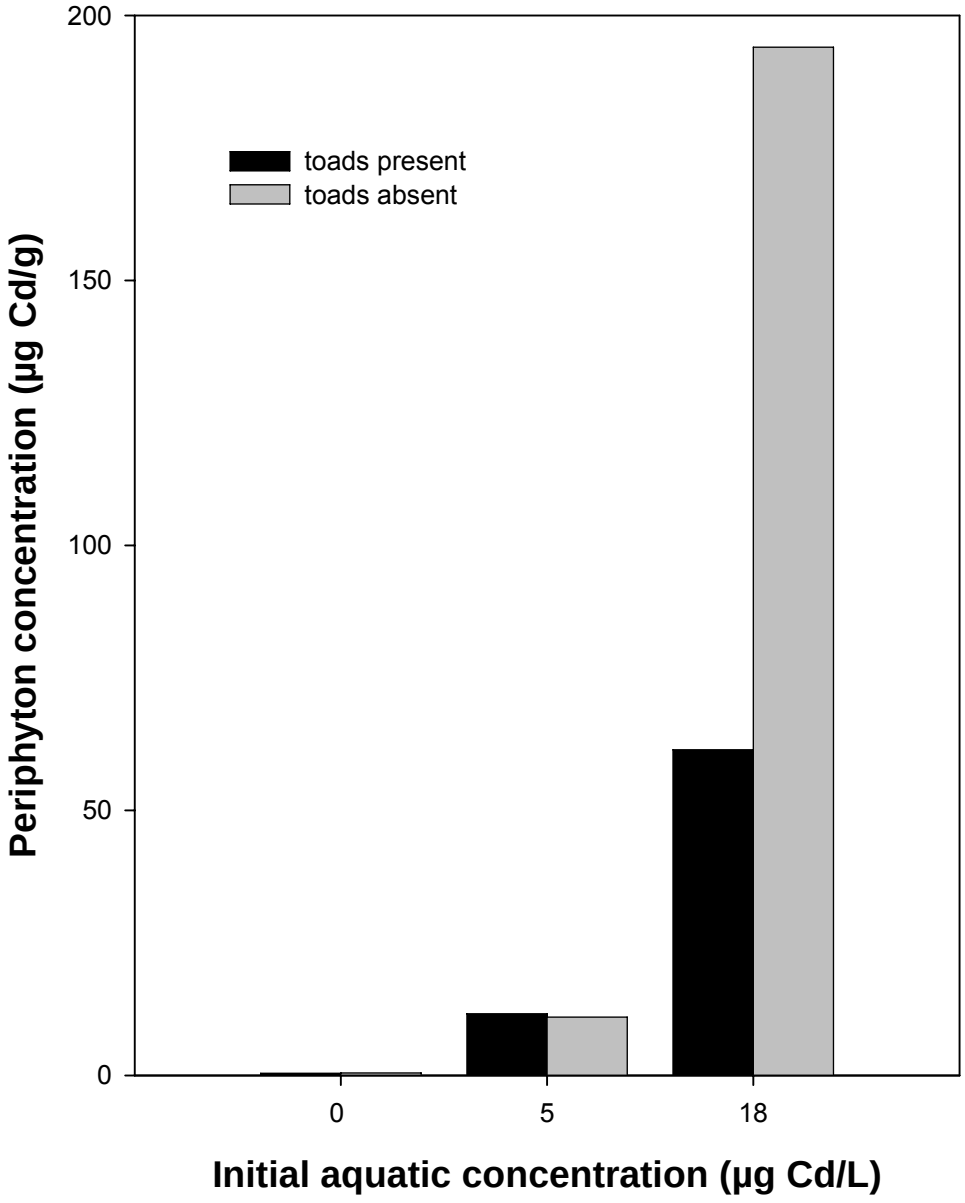

toads present
toads absent
Periphyton concentration (µg Cd/g)
200
150
100
50
0
0
5
18
Initial aquatic concentration (µg Cd/L)

Figure 3. Phytoplankton abundance (as chlorophyll A) over time in tanks with (solid lines) and without (dashed lines) interspecific competition. The legend indicates the initial nominal aqueous cadmium concentrations. Error bars represent ±1 standard error.

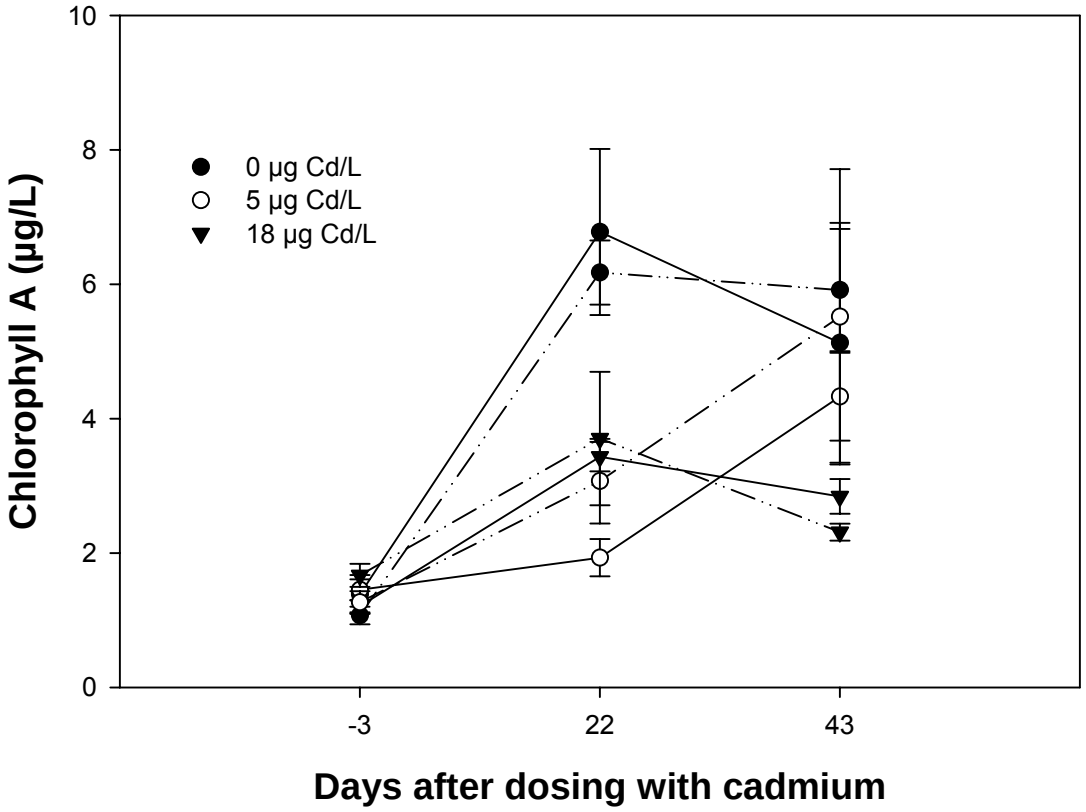
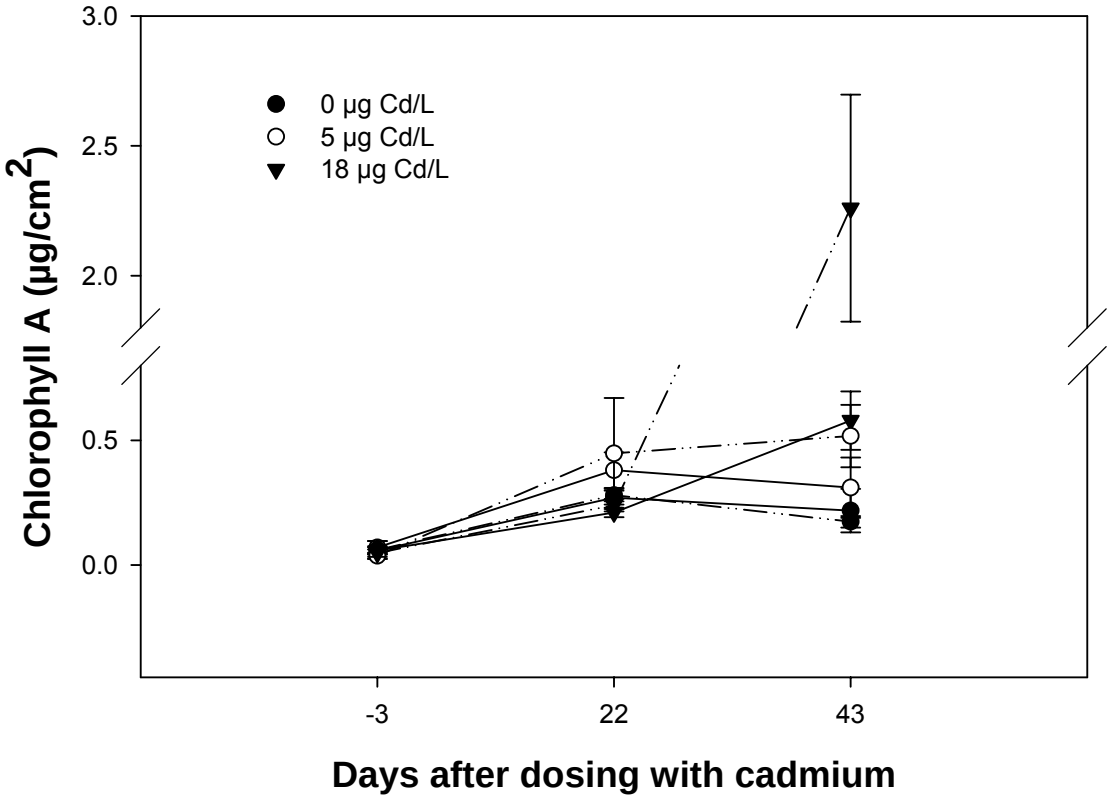

10
8
6
4
2
0
Chlorophyll A (µg/L)
0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
-3
22
43
Days after dosing with cadmium

Figure 4. Periphyton abundance (as chlorophyll A) over time in tanks with (solid lines) and without (dashed lines) interspecific competition. The legend indicates the initial nominal aqueous cadmium concentrations. Error bars represent ±1 standard error.


3.0
2.5
2.0
0.5
0.0
0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
Chlorophyll A (µg/cm2)
-3
22
43
Days after dosing with cadmium

CHAPTER 6

TERRESTRIAL PERFORMANCE OF SOUTHERN LEOPARD FROGS (*RANA SPHENOCEPHALA*) IN TWO HABITAT TYPES AFTER CHRONIC LARVAL EXPOSURE TO CADMIUM

ABSTRACT

Chemical contamination and physical alteration of the environment can each cause changes in the abundance and diversity of amphibian populations. However, these factors are typically considered in isolation using single life stages. A study was conducted to determine the terrestrial performance (i.e., growth, survival) of southern leopard frog (*Rana sphenocephala*) juveniles that had been chronically exposed to one of three concentrations of the heavy metal cadmium (initial nominal 0, 5, or 18 μg Cd/L) as larvae. Juveniles were reared in terrestrial enclosures within deciduous forest and open field habitats through their first growth season (summer to autumn). The effect of Cd on terrestrial survival depended on habitat type; survival increased with Cd level in the field enclosures and decreased with Cd level in the forest enclosures. Terrestrial survival was 73% in the forest enclosures and 54% in the field enclosures, but the difference was not significant. The trend for summer mass to increase with Cd concentration was maintained through autumn, and individuals in the field enclosures were heavier than their counterparts in the forest. Growth rate was likewise significantly affected by habitat type and Cd concentration. The forest enclosures had higher soil moisture and relative

humidity, and cooler air temperatures, which should be beneficial to amphibians. Larval exposure to Cd had latent effects on survival, but also conferred the advantage of a larger size at metamorphosis that remained through autumn. Southern leopard frog juveniles had higher survival in deciduous forest habitats, but slower growth was a tradeoff. Efforts to manage this species should minimize the conversion of deciduous forests to open fields around aquatic breeding sites.

INTRODUCTION

Aquatic and terrestrial habitat quality can determine the demographics and persistence of amphibian populations (Kucken et al. 1994, Rothermel 2003). The chemical contamination and physical alteration of habitat are among the leading proposed causes of worldwide amphibian declines (Collins and Storfer 2003). Although the impacts of habitat contamination and physical alteration on amphibians are typically considered separately, many natural populations must face both at once or sequentially. Physical habitat alteration has caused the decline of many amphibian populations and the driving mechanisms are often well understood (Collins and Storfer 2003). For instance, forest clear-cutting results in the removal of canopy and understory cover, soil compaction, and drier and hotter conditions, which can lead to the loss or migration of local populations (Petranka et al. 1994). Contaminants can be found in remote and relatively pristine areas, but higher concentrations potentially harmful to flora and fauna are more likely to occur closer to the vicinity of human settlement. However, chemical contamination has not been clearly linked with many instances of amphibian population decline and the potential mechanisms are more complex and may take longer to manifest

(Collins and Storfer 2003). In natural systems it is often hard to separate contaminant effects from other factors, particularly when responses are delayed (Mann et al. 2003). Concentrations are often not high enough to produce direct lethality, but sublethal effects may occur that increase susceptibility to other potential stressors (Carey and Bryant 1995). It is particularly hard to determine the impacts of contamination and physical habitat alteration on biphasic amphibians because of their complex habitat use. Different life stages not only occupy different micro- or macro-habitats, but also vary in their sensitivity to environmental variables (Schuytema et al. 1991).

Amphibian ecotoxicology research has focused primarily on aquatic contamination and early life stages (Sparling et al. 2000a). This can largely be attributed to the belief that embryos and larvae are more sensitive than juveniles and adults, which has been verified in a few laboratory studies (Hall and Swineford 1980, Schuytema et al. 1991). It is also traditionally thought that populations are primarily regulated by the aquatic environment due to high rates of mortality, although this may not be true (Biek et al. 2002, Vonesh and De la Cruz 2002). Pesticides and metals are the most thoroughly investigated contaminants (Sparling et al. 2000b). Documented environmental concentrations can result in mortality, altered growth, elevated maintenance costs, malformations, hermaphroditism, behavioral changes, and body residues in larvae (Rowe et al. 1998, Hayes et al. 2002, Boone and James 2003, Broomhall and Shine 2003, Bridges et al. 2004). Metamorphic traits such as mass and age at metamorphosis may also be affected (Boone and James 2003). Most research suggests that aquatic contamination has no or only sublethal impacts on amphibians at concentrations typically found in the environment (Pauli et al. 1999, Wojtaszek et al. 2004). However, sublethal

toxicity may contribute to lethality, lower body condition, or altered behavior in a multiple stressor scenario, even after exposure has ended (Broomhall and Shine 2003). Given that potential stressors such as predation, competition, disease, and desiccation are persistent threats, sublethal responses should be considered highly important. Changes in amphibian species abundance and diversity have been documented in areas where breeding sites are contaminated (Kucken et al. 1994, Bonin et al. 1997, Bishop et al. 1999). Causality is usually attributed to low recruitment because of contaminant-associated early life stage mortality. However, the loss of terrestrial life stages following metamorphosis is another possibility. A reduction in juvenile body condition due to larval exposure history or the quality of the surrounding terrestrial environment may result in increased mortality or delayed maturity and subsequent population declines.

Following metamorphosis, biphasic amphibians leave the breeding site and move into the surrounding terrestrial environment. Upland habitats are utilized for foraging, migration, dispersal, aestivation, and hibernation. Particularly fossorial species will not return to aquatic areas until maturation, which may take several years, and then leave again shortly after breeding. Unfortunately, buffer zones around water bodies are often not required or are of insufficient width to encompass core habitats (Semlitsch and Bodie 2003). Species diversity and abundance at wetlands depends on the composition of the adjacent landscape (Houlahan and Findlay 2003, Porej et al. 2004). Any changes in connectedness and quality that reduce post-metamorphic survival, growth, or time to maturity could have important impacts on fitness and population persistence (Rothermel 2003). Land alteration has been associated with less diverse amphibian communities (Hecnar 1997) and favors those species that can adapt to or tolerate disturbance (Collins

and Storfer 2003). Land use change may also influence terrestrial permeability and the maintenance of metapopulation dynamics (Marsh and Trenham 2001, Chan-McLeod 2003). One of the most common types of land change is the conversion of forests into fields for grazing animals or open space. Such conversion is likely detrimental to forest-dependent amphibians, beneficial to species found in open environments, and may be of neutral or beneficial consequence to habitat generalists (Delis et al. 1996). However, research has focused primarily on forest management, cropland agriculture, and urbanization, and has neglected pastures, old fields, and other open spaces dominated by grasses and forbs (but see Rothermel and Semlitsch 2002, Rothermel 2004). Relative to forests, fields are characterized by wider temperature ranges, higher winds, greater sun exposure, and denser ground cover. All of these characteristics may be detrimental to amphibians because of their limited mobility and high rates of evaporative water loss (Rothermel and Semlitsch 2002), and may increase the likelihood of predation (Rohr and Madison 2003).

It is unknown whether metamorphs emerging from chemically contaminated breeding sites are more sensitive to terrestrial cover type than those with contaminant-free larval histories. Mechanisms for increased sensitivity could include an altered age or size at metamorphosis, or poor body condition due to body residues that affect organs and physiological processes. The harmful potential of residues is obvious, and both smaller body size and slower development have been associated with delayed maturity and poorer fitness (Smith 1987, Semlitsch et al. 1988). The future of those surviving metamorphosis from a contaminated environment is sometimes predicted based on metamorphic traits (e.g., Boone and James 2003). Unfortunately, the accuracy of these

predictions remains largely untested because almost all early life stage studies stop before or at metamorphosis. Laboratory and single-habitat-type enclosure studies indicate no difference in the terrestrial performance (i.e., growth, survival) or locomotory capacity of juveniles with different exposure histories (Rowe et al. 2001, Rehage et al. 2002, Boone, in press). However, larval exposure to some contaminants may cause persistent malformations (Plowman et al. 1994) or greater risk of terrestrial desiccation (Rohr and Palmer 2005). Recent evidence suggests amphibian population dynamics are sensitive to juvenile survival rates (Trenham et al. 2000, Biek et al. 2002, Vonesh and De la Cruz 2002). Therefore, ecotoxicological research should examine not only the pre-metamorphic period, but also the subsequent juvenile stage. The first growth season may be the most important because it is when terrestrial mortality appears to be greatest (Breden 1988, Rothermel 2003).

Research was conducted on the terrestrial performance of southern leopard frog (*Rana sphenocephala*) juveniles in outdoor enclosures after chronic larval exposure to the heavy metal cadmium (Cd). The enclosures were located in deciduous forests and open fields. Southern leopard frogs occupy the southeastern United States and appear to be habitat generalists because of their association with deciduous and coniferous forests, pastures, mown fields, agricultural areas, and residential neighborhoods (Mitchell 1986, Delis et al. 1996, Hanlin et al. 2000, Zampella and Bunnell 2000, Donnelly et al. 2001). Southern leopard frogs will breed in metal-contaminated water bodies and larvae can obtain significant body burdens (Burger and Snodgrass 2001, Pollio 2001). Recruitment from particularly impacted sites can be quite low (Pollio 2001) and the fate of those surviving to metamorphosis is unknown. Cadmium is a U.S. Environmental Protection

Agency contaminant of concern that is a by-product of zinc, lead, and copper production and known for its toxicity to wildlife at concentrations found in the environment due to human activities (Eisler 1985). Wetlands and other water bodies may become contaminated with Cd from industrial effluent, mining runoff, aerial deposition from smelters, and runoff from agricultural fields on which sewage sludge or phosphate fertilizers are applied (Eisler 1985). Southern leopard frogs chronically (stages 25 - 42; Gosner 1960) exposed to Cd in outdoor aquatic mesocosms (1325-L polyethylene cattle tanks) experience lower survival, higher whole body Cd residues, larger metamorphic mass, and a longer larval period with increasing aquatic concentration (Chapter 5). The larger mass results from lower survival and subsequent competitive release, and from direct toxicity that stimulates growth (Chapter 5). While a larger body mass may be beneficial (Morey and Reznick 2001), a delay in emergence (Semlitsch et al. 1988) and the possession of a Cd body burden (Hopkins et al. 1997) could hinder terrestrial success. Cadmium not only alters metamorphic traits, but also accumulates in organs and disrupts physiological processes (Eisler 1985). Therefore, survivors of Cd exposure may have lower success in the terrestrial environment relative to amphibians from uncontaminated sites. One very pragmatic way of determining the terrestrial performance of juvenile amphibians in a natural setting is to rear them in outdoor enclosures. Enclosures have been used by both ecologists (Pechmann 1995, Altwegg 2003) and ecotoxicologists (Hopkins et al. 1997, Laposata and Dunson 2000) to answer questions about the success of post-metamorphic amphibians in terrestrial habitats. Confinement to a specific area provides greater experimental control than would occur in a tracking study. Individuals can be monitored at predetermined times and their habitat use and density are known.

The objective of the study was to determine whether the growth rate, mass, and survival (i.e., performance) of juveniles are influenced by larval exposure to Cd, terrestrial habitat type, or their interaction through the first growth season. It was predicted that individuals reared in uncontaminated larval conditions would have better terrestrial performance because of an absence of body burdens and a longer time to forage in the enclosures. However, the smaller mass at metamorphosis of unexposed individuals may result in higher mortality or poorer growth (Morey and Reznick 2001); young, small juveniles are very susceptible to desiccation, starvation, and predation (Ray 1958, Freeland and Kerin 1991). Although southern leopard frogs are habitat generalists, it was predicted that their performance would be better in the forest sites because of the milder climatic conditions and because the juveniles were obtained from eggs oviposited in forest ponds. This study was intended to provide data for both contaminant risk assessors and land managers interested in conserving habitat for southern leopard frogs.

MATERIALS AND METHODS

Study organisms

The southern leopard frog (*Rana sphenocephala*) metamorphs used for this study were obtained from aquatic outdoor mesocosms that had been dosed with Cd (Chapter 5). The mesocosms (1325-L volume polyethylene cattle tanks; Behlen PolyTuff, Columbus, NE, USA) were set up in March 2004 on a mown field at the USGS Columbia Environmental Research Center (Columbia, MO, USA). Each mesocosm contained approximately 950 L of softened well water (hardness ≈ 55 mg $CaCO_3$/L), 1 kg of deciduous leaf litter, and algae and plankton inoculated from natural ponds. After

periphyton was observed growing on the walls, mesocosms were dosed once in late April at the initial nominal aqueous concentrations of 0, 5, and 18 μg Cd/L. These concentrations represent the Cd treatment levels that will be referred to throughout the rest of the chapter. Eight days later, each mesocosm received 50 southern leopard frogs and 120 American toads (*Bufo americanus*) that were at developmental stage 25 (Gosner 1960). The tadpoles had hatched in the laboratory from eggs collected out of forest ponds at the University of Missouri Thomas Baskett Wildlife Area (Boone County, MO, USA). The American toads were added to provide an additional source of stress in the form of interspecific competition, which frequently occurs among these two spring-breeding species. The tadpoles were allowed to grow undisturbed until metamorphosis, when the metamorphs were collected and transported to a laboratory. When tail resorption was complete (i.e., Gosner stage 46), southern leopard frogs were weighed and toe-clipped. Two toes on different feet were removed from each southern leopard frog, and frogs assigned to the same terrestrial enclosure had unique toe clips. Southern leopard frogs from different Cd treatments were kept in separate moist plastic containers until there were enough to stock an enclosure. Most southern leopard frogs were in the laboratory no more than three days after being toe-clipped.

Experimental design

The enclosures were located at the 911-ha Thomas Baskett Wildlife Area. This property has been used for ecological research since 1938 and consists of deciduous forests with sparse ground vegetation, and open fields maintained by mowing and controlled burns. The predominant deciduous tree species were black hickory (*Carya texana*), white oak (*Quercus alba*), and black maple (*Acer nigrum*). The open fields were

dominated by little bluestem (*Andropogon scoparius*), lespedeza (*Lespedeza virginica),* and common ragweed (*Ambrosia artemisiifolia*). A total of 24 enclosures were built in autumn 2002; half were in deciduous forest habitat and half in open field habitat (hereafter, "forest" and "field"). Enclosures were located >40 m from any habitat edges. There were two sites within each habitat type, and each site contained two blocks of three adjacent enclosures. Within each site, the two blocks were located 20 to 40 m apart. Each square enclosure was approximately 9 m^2 and the walls were made of aluminum flashing (91-cm wide) buried approximately 45-cm deep and standing as tall. The corners were attached to a piece of angle iron and untreated wooden stakes helped support the walls. On top of the walls was a 10-cm lip made of hardware cloth and glued to the flashing with silicone. Half of the lip (i.e., 5 cm) was on the inside of the enclosures to prevent southern leopard frogs from climbing out, and the other half was on the outside to prevent predators and competitors from climbing in. However, given the height of the walls and the fact that the enclosures were not covered, trespassing was possible although predators or their sign were not observed in the enclosures. The enclosures were open-bottomed such that the floor was the natural substrate. Invertebrate prey could move in and out so there was no supplemental feeding. Each enclosure contained one soil moisture probe buried just below the surface (Watermark; Irrometer Company, Inc., Riverside, CA, USA). Halfway between the two blocks at each site was a weather station that consisted of a rain gauge and a temperature and relative humidity sensor (Hobo® Pro Series; Onset Computer Corporation, Bourne, MA, USA). The rain gauge and sensor stood 120 and 45 cm above ground, respectively. Soil moisture and rainfall data were collected once weekly, and the Hobo sensors took hourly readings.

Each enclosure was stocked with ten southern leopard frog metamorphs from a single Cd treatment, for an initial density of 1.1/m^2. This is within the range of natural terrestrial densities for juvenile amphibians, which can exceed 50/m^2 in the vicinity of breeding sites (Beck and Congdon 1999). Within each block, the three enclosures were randomly assigned a larval Cd treatment. Metamophs were added in the early evening from June 18 to July 3, 2004. It took over two weeks to obtain enough metamorphs from the mesocosms to stock all the enclosures because of Cd treatment, mesocosm, and individual differences in time to metamorphosis. Instead of stocking all metamorphs simultaneously, the variation in larval development period was incorporated as part of the study. Stocking order was randomly determined among the four sites and among the blocks at each site. One block from each site was stocked initially, and then the site stocking order was rerandomized. After stocking was completed, the metamorphs were left undisturbed until autumn. The sites were visited weekly to take the environmental measurements and to trim vegetation within approximately 0.6 m of the walls. Trimming was necessary to prevent the amphibians from escaping, particularly in the field enclosures where the vegetation exceeded 2 m in height. Beginning September 29, southern leopard frog juveniles were recaptured using both funnel traps (2 per enclosure) and hand-searches. To facilitate detection, forest leaf litter was carefully sorted through and deposited outside the enclosures, and field vegetation was cut to ground-level with hedge shears and likewise removed. The study was terminated after three consecutive nights of no trap captures followed by a day (October 15) of time-constrained searches during which no juveniles were found. All recovered southern leopard frogs were individually identified and measured for mass.

The diversity and abundance of invertebrate prey were determined over the course of a 48 h time interval on July 19 to 21 and September 20 to 22, 2004. These determinations were made in the area around each block so that sampling would not deplete invertebrate populations within the enclosures. One pitfall trap (0.47-L plastic freezer container) was buried flush with the ground at six points around each block and at a distance of 4 m from the enclosure walls. All eight experimental blocks were sampled in the same fashion both times. When traps were initially installed, attempts were made to minimize disturbance to the surrounding soil and litter. At the beginning of a sampling period, traps were cleared of debris and provided with 200 mL of soapy water. After 48 h, the water was poured through a tea strainer and remaining invertebrates were removed with forceps and placed in a 40 mL glass vial with 80% EtOH. Specimens ≥2 mm were later classified to Order using a dissecting microscope and all individuals were measured to the nearest millimeter. Data were pooled within habitat type and across time, and calculations were made of ordinal diversity, abundance, and biomass. Estimates of biomass were made using the formula: mg dry weight = $0.0305(\text{length in mm})^{2.62}$, taken from Rogers et al. (1976).

Statistical analysis

Enclosures were considered replicates because southern leopard frogs within an enclosure were not independent of each other. Mean values of individual responses were generated for each enclosure. Each spatial block of three adjacent enclosures was analyzed as a statistical block. Terrestrial duration was the number of days juveniles were in the enclosures. The initial mass of southern leopard frogs upon addition to the enclosures was considered "summer mass" and the final mass upon recapture and

removal from the enclosures was "autumn mass." Survival was calculated as the number recovered in autumn divided by the number initially stocked (i.e., 10). Growth rate was the change in mass (autumn mass – summer mass) divided by terrestrial duration. Survival, summer mass, autumn mass, and growth rate were analyzed with analysis of variance using Proc Mixed (SAS Institute 1989) and habitat type and Cd level as the main effects. Interactions between habitat and Cd were tested to determine whether the two main effects act dependently (i.e., significant interaction) or independently (i.e., non-significant interaction). A second analysis of variance was performed for mass and growth rate using survival as a covariate to account for possible terrestrial density effects on growth (see Rothermel 2003). Analyses were of type III sum of squares and least squares means to account for covariance. When significant effects were found, the LSD multiple comparisons test was performed to determine differences among treatments. To meet the assumptions of homogeneity of variance and normality, summer and autumn mass were logarithmically-transformed, growth rate was square root transformed, and survival was arcsine square root transformed (Zar 1999). Because of the likelihood that endpoints were correlated with each other, relationships were determined with the Pearson Correlation. The environmental variables were also statistically analyzed, but habitat type was considered the only main effect. Habitat differences in soil moisture were determined with a repeated measures mixed model analysis of variance because there were repeated measurements for each enclosure within the eight spatial blocks. Habitat differences in daily mean and maximum temperature, daily mean and minimum relative humidity, and mean weekly rainfall were determined with repeated measures analysis of variance (Proc GLM) because at each time interval there were two

measurements for each habitat type. All analyses were conducted with SAS (SAS Institute 1989) and significance was set at $\alpha = 0.05$.

RESULTS

Southern leopard frogs

Control metamorphs were stocked on average four and six days before those in the 5 µg Cd/L and 18 µg Cd/L treatments, respectively. The range in timing of stocking reflects larval Cd treatment differences in time to metamorphosis (see Chapter 5). Mean terrestrial duration was 102 (0 µg Cd/L), 98 (5 µg Cd/L), and 96 (18 µg Cd/L) days (Table 1). There were no instances of southern leopard frogs trespassing into adjacent enclosures during the terrestrial period. Therefore, the results are not biased by the loss or gain of conspecifics. Of the 240 individuals initially added to the enclosures, 153 (64%) were recovered in autumn. Of these, 88 (58%) were from forest enclosures and 65 (42%) were from field enclosures, for an overall survival rate of 73% in forest and 54% in field (Table 1). However, survival did not differ significantly among the habitat types ($F = 2.23$, $df = 1$, $p = 0.1860$) nor among Cd levels ($F = 0.02$, $df = 2$, $p = 0.9809$), but there was a significant habitat*Cd interaction ($F = 4.69$, $df = 2$, $p = 0.0313$). The interaction resulted from survival increasing with Cd level in field enclosures and decreasing with Cd level in forest enclosures. Summer mass increased with increasing Cd level ($F = 291.54$, $df = 2$, $p < 0.0001$) such that those from the 18 µg Cd/L concentration were more than twice as large as controls (Figure 1). Summer mass did not differ among habitat types ($p = 0.6283$) and there was not a habitat*Cd interaction ($p = 0.9153$), so initial spatial biases did not exist. By autumn, the trend for mass to increase

with Cd remained ($F = 15.76$, $df = 2$, $p = 0.0006$) in both habitat types (Figure 1). Southern leopard frogs reared in field enclosures were far larger than their counterparts in forest enclosures ($F = 11.69$, $df = 1$, $p = 0.0142$; Figure 1). Mean treatment values ranged from 3.3 g (controls in forest) to 8.6 g (18 µg Cd/L in field) (Figure 1). However, the interaction was not significant ($p = 0.1973$), indicating the effect of Cd on autumn mass was independent of habitat type. Terrestrial growth rate was also greatest in the field enclosures ($F = 12.52$, $df = 1$, $p = 0.0122$), differed among Cd levels ($F = 4.56$, $df = 2$, $p = 0.0361$), and was not influenced by an interaction between Cd and habitat ($p = 0.2746$) (Table 1). There was a tendency for growth rate to increase with larval Cd treatment (Table 1). Significant positive correlations were found between summer and autumn mass ($r = 0.43$, $p = 0.0383$) and autumn mass and growth rate ($r = 0.98$, $p < 0.0001$). Negative correlations existed between survival and autumn mass ($r = -0.41$, $p = 0.0503$) and survival and growth rate ($r = -0.41$, $p = 0.0534$). However, when survival was used as a covariate to account for possible density-dependent effects on growth, there was no significant change in the results for mass (habitat: $p = 0.0197$, Cd: $p = 0.0013$, interaction: $p = 0.2397$) or growth rate (habitat: $p = 0.0175$, Cd: $p = 0.0541$, interaction: $p = 0.2884$).

Environmental variables

Soil moisture was measured in each enclosure a total of 12 times while the southern leopard frogs were present. Soil moisture ranged from 0 (saturated; lowest reading possible with meter) to 199 (dry; highest reading possible) centibars. The mean moisture across the study duration was greater in the forest enclosures (28 centibars) than in the field enclosures (36 centibars) ($F = 14.49$, $df = 1$, $p = 0.0003$). Moisture tended to

be highest in the forest enclosures in the summer and in the field enclosures in the autumn (Figure 2). The soil was relatively moist over time because of periodic rehydration from rainfall (Figure 2). Weekly rainfall exceeded 0.1 cm in all but four weeks in August and September. Mean weekly rainfall did not differ among habitat types ($F = 1.00$, $df = 1$, $p = 0.4226$), and mean total rainfall was probably slightly less in the forest sites (27.3 cm) than in the field sites (30.7 cm) because trees intercepted falling precipitation. The average daily maximum temperature was lower in the forest sites (26.0 C) than the field sites (33.6 C) ($F = 221.95$, $df = 1$, $p = 0.0045$), but there was no habitat difference in mean temperature (20.5 C and 20.9 C, respectively). Similarly, the average daily minimum relative humidity was higher in the forest sites (63.8%) relative to the field sites (44.0%) ($F = 37.08$, $df = 1$, $p = 0.0259$), but mean relative humidity was the same in both habitats (85.2% and 83.4%, respectively). Overall, the forest sites had cooler (Figure 3) and wetter (Figure 4) air than did the field sites.

A total of 3313 invertebrates was collected during both sampling periods (Table 2). Of these, 1773 (54%) were in the field sites and 1540 (46%) were in the forest sites. The ordinal diversity was similar in the forest (n = 17) and field (n = 15) sites (Table 2). The three most abundant Orders in each habitat type were (in descending order): field - Isopoda, Collembola, Hymenoptera; forest - Collembola, Hymenoptera, Diptera. Isopods were the most common invertebrate in the field sites, but were not found in the forest sites. Mean mass of individuals in all orders combined was 3.7 mg (field) and 8.7 mg (forest), and the total mass of all individuals captured was 6512 mg (field) and 12879 mg (forest). However, mass estimates were underestimated and possibly biased because mass was not determined for those individuals that were dismembered (i.e., only part of

the body was found; field = 19, forest = 52). Only 10 to 11 earthworms (Haplotaxida) were captured in each habitat type, but two were not measured from the field sites. Given the large contribution to total mass by earthworms (Table 2), another data set was made without them. This resulted in a mean mass for all orders combined of 3.3 mg (field) and 3.7 mg (forest), and a total mass of 5777 mg (field) and 5478 mg (forest). However, forest total mass was likely more underestimated than field total mass because a greater number of individuals were not measured.

DISCUSSION

Pond-breeding amphibians naturally experience wide fluctuations in population size such that local extinction and recolonization are common (Hecnar and M'Closkey 1996, Semlitsch et al. 1996, Skelly et al. 1999). The existence of anthropogenic stressors and habitat alteration in both aquatic and terrestrial systems in addition to the natural suite of environmental factors adds layers of complexity and makes it very difficult to determine reasons for amphibian population declines. Reductions in amphibian abundance are frequently attributed to characteristics of the aquatic breeding site such as the presence of predators, a shortened hydroperiod, or poor water quality. Reproductive success and the presence of potential stressors can be determined because offspring are confined to the breeding site, which is often small in area. Given the short lifespan of most amphibians, no or low recruitment for successive years is of obvious detriment (Semlitsch et al. 1996). It is less common for amphibian declines to be attributed to the disappearance of juveniles and adults in the terrestrial environment, but there have been many instances in which these older life stages have failed to appear at breeding sites for

unknown reasons (Pounds and Crump 1994, Scott 1994), are observed dead or dying around aquatic habitats (Bradford 1991), or die during terrestrial overwintering (Resetarits 1986). Relative to the aquatic environment, little is known about the mechanisms that cause terrestrial mortality and poor body condition (Clarke 1977). This is primarily because of a historical focus on reproduction and recruitment in aquatic habitats, but can also be attributed to the difficulty of monitoring free-ranging post-metamorphic amphibians in terrestrial habitats. Most tracking studies follow adults during or after breeding and do not last more than a few months (e.g., Richter et al. 2001), which renders valuable but incomplete information. Herpetologists are just beginning to conduct manipulative research on the relatively more sensitive juvenile life stage and the potential for carryover effects from the aquatic environment (Rohr and Palmer 2005).

In this study, juvenile southern leopard frogs that had been exposed to Cd as larvae had a larger mass at metamorphosis (i.e., summer mass) than unexposed frogs, but less time in the terrestrial environment by four to six days. However, the delay in metamorphosis did not appear to negatively influence growth given that initial trends in mass differences were maintained through autumn and growth rate tended to increase with Cd level. Other studies have found that individuals large at metamorphosis remain relatively large through their first year or even adulthood (Scott 1994, Morey and Reznick 2001). If body condition was significantly impaired by sublethal Cd stress, the initial mass trends were expected to have flattened out as control and low Cd (5 μg/L) juveniles caught up. Given this did not occur in either habitat type and growth rate was actually lowest in the controls, it appears that those that survive larval Cd exposure at the

levels and conditions tested are able to effectively forage and grow in the terrestrial environment. The relatively poor growth of the controls may have been due to their small body size if gape limitation or reduced capture ability limited feeding rates or if susceptibility to dehydration curtailed activity. It was also expected that juveniles with a Cd exposure history would have lower survival, but this occurred only in the forest enclosures. The transition from an herbivorous to a carnivorous diet upon metamorphosis may have reduced latent Cd toxicity effects. Invertebrates contain high amounts of calcium in their exoskeletons, and calcium reduces Cd uptake in fishes (Hollis et al. 2000) and zooplankton (Stephenson and Mackie 1989). Although the southern leopard frogs were not exposed to new sources of Cd while in the terrestrial enclosures, the intake of calcium from their diet may have had some protective effect.

Interestingly, trends in survival among Cd treatments differed between the habitat types. In the forest enclosures, survival decreased with increasing Cd level, while in the field enclosures the opposite was true. Smaller individuals are generally more subject to desiccation (Ray 1958) and predation (Berven 1990), but small size appeared to be more of a detriment in the field enclosures. Conditions may have been mild enough in the forest sites for size to not be a strong factor for survival. However, it is surprising that smaller southern leopard frogs would have higher survival under any conditions, which suggests Cd may have had some effect. Climatic and moisture conditions were harsher in the field sites relative to the forest sites, so the benefit of a large size may have predominated over any deleterious Cd effects for amphibians in field enclosures. Habitat differences in temperature may have influenced the responses of amphibians to Cd exposure. Warmer temperatures in the field sites could have decreased Cd potency,

considering the median lethal concentration of Cd to amphibian tadpoles (Ferrari et al. 1993) and fishes (Yang et al. 1996) increases at higher temperatures and Cd redistribution and excretion rate increases with temperature in fishes (Douben 1989). However, there is also evidence from fish that Cd exposure adversely affects the ability to withstand high temperatures (Carrier and Beitinger 1988). It is clearly important to consider multiple terrestrial conditions when predicting or assessing amphibian responses to environmental contaminants.

The lack of deleterious effects from Cd exposure are in general agreement with the no-effect findings of the few previous studies that have monitored amphibians after metamorphosis from contaminated aquatic conditions (Rowe et al. 2001, Rehage et al. 2002, Boone, in press). This is surprising given the numerous organs and physiological processes Cd influences (Eisler 1985), and the potential for latent effects (Pascoe and Shazili 1986). Larval mortality in the 5 and 18 μg Cd/L treatments was elevated relative to the controls, and survivors possessed an average of 3.4 and 6.0 μg Cd/g (whole body dry weight), respectively (Chapter 5). Therefore, it was predicted that the survivors of aquatic exposure would exhibit some signs of toxicity. Perhaps these individuals successfully activated detoxifying mechanisms (e.g., metallothionein) to sequester Cd residues or possessed superior genes. The lack of reduced terrestrial growth suggests that Cd actually benefited southern leopard frogs because larger individuals have increased locomotory performance (Goater et al. 1993), habitat permeability (Chan-McLeod 2003), and fecundity (Davies and Halliday 1977). However, although Cd-contaminated breeding sites may produce larger metamorphs, there will be fewer of them (Chapter 2 or James et al., in press). A reduction in survival has negative population-level

consequences, whereas enhanced growth is an individual-level response. Whether the advantages of a larger mass outweigh the disadvantages of poorer recruitment is unknown but likely depends on terrestrial conditions. Survivors of Cd exposure also carry with them a body burden that may hinder growth and survival under more natural and stressful conditions, and impair reproduction through effects on spermatogenesis (Kasinathan et al. 1987) and oogenesis (Lienesch et al. 2000).

Juvenile southern leopard frogs in forest enclosures had higher survival but slower growth rates and smaller autumn mass compared to juveniles in field enclosures. The difference in survival among habitat types was not significant due to high within-treatment variation, despite being almost 20%. Nevertheless, higher forest survival was expected because of the milder climatic conditions. The forest sites had cooler mean temperatures, lower maximum temperatures, higher mean and minimum relative humidity, and moister soils than the field sites. The forest and field sites had similar mean climatic conditions, but differed greatly in maximum temperature and minimum relative humidity. It was more common for forest soils to be wetter than field soils during the first half of the study, which may have been influential because natural amphibian mortality generally decreases with age and size (Clarke 1977, Breden 1988). As ectotherms with highly permeable skin, amphibians are very sensitive to dry conditions and temperature extremes. Free-ranging amphibians seek cool, moist terrestrial refuges during periods of inactivity, and the successful location of suitable microhabitat may influence survival (Seebacher and Alford 2002). Metamorph mortality can increase with maximum temperatures (Cohen and Alford 1993). A close relative of the southern leopard frog, *Rana pipiens*, has a preferred body temperature of 24 to 31 C

(Fitch 1956), which was exceeded more often in the field sites than the forest sites. A dehydration trial conducted within 15 km of the present study found that the evaporative water loss of juvenile spotted salamanders (*Ambystoma maculatum*) was higher in open fields than deciduous forests (Rothermel and Semlitsch 2002), thereby further suggesting forests may provide better habitat. Indeed, when given the choice of forest or field substrate in the laboratory, juvenile southern leopard frogs strongly selected the forest side (James et al., unpublished data). The choice test used substrates from two of the four enclosure sites, and was conducted to complement the findings of this study. It was presumed that the particular habitat chosen would confer greater fitness. Similarly, spotted salamanders select (Rittenhouse et al. 2004) and have higher survival in (Rothermel 2003) deciduous forests.

The forest enclosures proved better than the field enclosures for survival but worse for growth. Slower growth may have resulted from greater competition among individuals for resources such as food and shelter because there were more survivors. Effects of density on juvenile growth have been documented in other terrestrial enclosure (Cohen and Alford 1993, Pechmann 1994) and field (Gray and Smith 2005) studies. Although the stocking density fell within the natural range, density may have been relatively high given the distance from potential breeding sites (>20 m), duration of the study, and increasing age of the juveniles over time. Amphibians typically have small summer home ranges (Bellis 1959), but under natural conditions they can move in search of new resources as others are depleted. Nevertheless, density-dependence probably did not contribute substantially to the habitat differences in growth because the use of survival as a covariate for southern leopard frog mass and growth rate did not influence

the outcome of statistical analyses of habitat type effects. The lower abundance of invertebrate prey in the forest also could have decreased growth. Southern leopard frogs are opportunistic, generalist predators that will feed on most moving organisms that can fit in their mouths. Stomach content analyses have revealed the presence of numerous invertebrate taxa, most notably Araneae (spiders), Coleoptera (beetles), and Orthoptera (grasshoppers, crickets) (Kilby 1945, Forstner et al. 1998). The forest sites contained more Coleoptera and Orthoptera but fewer Araneae than did the field sites. However, the pitfall traps were likely ineffective in capturing grasshoppers, which were far more common in the field sites than the forest sites (*personal observation*). Given the large size of grasshoppers relative to the other invertebrates captured, a sampling effort that caught all potential prey might have resulted in the field sites having even more invertebrate biomass and abundance than the forest sites compared to what was documented. If this was indeed true, habitat differences in invertebrate biomass may partially explain why the southern leopard frogs in the field enclosures were larger than those in the forest. Higher air temperatures in the field sites may be yet another explanation for why amphibian growth was better in the field enclosures. Digestion, feeding rates, and weight gain improve with increased body temperature (Freed 1980).

Predictions of performance in the terrestrial environment are often based on metamorphic traits. A larger size at metamorphosis is sometimes associated with a larger size and earlier age at maturity, but greater survival may or may not occur (Smith 1987, Semlitsch et al. 1988, Scott 1994). In this study, the mass of southern leopard frog metamorphs was positively correlated with autumn mass but not survival. This provides further evidence that size advantages can be maintained over time and within different

habitat types. Correlations in size between metamorphs and adults appear particularly strong in ranids (Werner 1986), perhaps because metamorphs weigh approximately 10% of adult mass. However, relative size relationships among members of a cohort may change depending on the ability of individuals to find prey and other resources (Goater 1994, Morey and Reznick 2001). This ability may be partially a function of terrestrial habitat type and associated environmental characteristics. While metamorph mass was determined by larval treatment, juvenile mass was determined by both mass at metamorphosis and terrestrial habitat type. Therefore, the extent to which metamorphic traits influence juvenile traits appears to depend on the quality of both the aquatic and terrestrial environment.

The protection of adequate terrestrial habitat around aquatic sites has frequently been neglected, to the detriment of biphasic taxa such as amphibians that depend on habitat complementation (Semlitsch and Bodie 2003). Habitat alteration causes physical, chemical, and biological changes that subsequently influence the ecology, behavior, and demography of amphibians. Unfortunately, the ability of amphibians to respond and adapt to such changes may be poor relative to other organisms because amphibians have specific microhabitat requirements, physiological constraints, limited mobility, and high site fidelity. However, juvenile amphibians will choose to occupy specific habitats, and selected habitats are presumably associated with greater fitness benefits (deMaynadier and Hunter 1999, Rothermel and Semlitsch 2002). Environmental variables such as soil moisture and forest patch size influence amphibian distribution and abundance across landscapes (Wyman 1988, Kolozsvary and Swihart 1999). As habitat generalists, southern leopard frogs are found in a diversity of habitats that range greatly in abiotic and

biotic factors. Nevertheless, performance may be better in some habitats than others. Southern leopard frogs in deciduous forests may take longer to reach maturity than their counterparts in open fields because juvenile growth is slower. This in turn may increase the chance of mortality before breeding occurs. Habitat differences in growth are an individual-level effect, but habitat differences in survival are a population-level effect. Natural juvenile annual survival appears to be only approximately 20 to 40% (Kelleher and Tester 1969, Clarke 1977) and population dynamics are most sensitive to the alteration of postmetamorphic survival rates (Biek et al. 2002, Vonesh and De la Cruz 2002), so maximizing terrestrial survival is critical. Although not statistically significant, survival in the forest enclosures was higher than survival in the field enclosures by 19%. Therefore, minimizing the conversion of deciduous forests into open fields has benefits for population size and persistence.

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Table 1. Mean (±1 standard error) autumn survival, growth rate, and terrestrial duration for southern leopard frog (*Rana sphenocephala*) juveniles reared in 9-m^2 terrestrial enclosures at an initial density of 1.1/m^2

Aquatic concentration (µg Cd/L)	Survival[a] (%)	Growth rate[a] (g/day)	Terrestrial duration (days)
Forest			
0	85 A	0.023 A	103
	(± 9)	(± 0.003)	(± 2)
5	73 AB	0.025 A	98
	(± 9)	(± 0.004)	(± 2)
18	63 AB	0.035 A	97
	(± 9)	(± 0.008)	(± 2)
Field			
0	40 B	0.049 A	101
	(± 15)	(± 0.002)	(± 3)
5	58 AB	0.069 B	97
	(± 3)	(± 0.012)	(± 1)
18	65 A	0.069 B	95
	(± 16)	(± 0.015)	(± 1)

[a] capital letters within the column indicate significant differences according to the LSD test (p <0.05).

Table 2. Total number of individuals captured from each invertebrate Order and their mean and total mass, with each habitat type reported separately and sample dates pooled

	FIELD			*FOREST*		
Order	**Total number**	**Mean mass (mg)**	**Total mass (mg)**	**Total number**	**Mean mass (mg)**	**Total mass (mg)**
Acarina	2	0.19	0.38	0	—	—
Anoplura	0	—	—	1	0.19	0.19
Araneae	59	1.64	96.48	38	2.97	109.82
Blattodea	0	—	—	3	53.46	160.38
Chilopoda	0	—	—	3	38.17	76.34
Coleoptera	77	18.99	1424.51	118	10.87	1195.62
Collembola	388	0.34	131.79	541	0.54	282.46
Dermaptera	2	338.66	677.32	1	0.54	0.54
Dilopoda	0	—	—	1	25.28	25.28
Diptera	93	0.51	47.15	184	1.39	235.62
Haplotaxida	10	91.89	735.14	11	672.79	7400.73
Hemiptera	20	2.32	46.32	11	0.94	9.37
Homoptera	44	1.19	50.98	35	0.79	26.72
Hymenoptera	368	1.84	669.91	498	2.62	1298.84
Isopoda	654	2.31	1510.86	0	—	—
Lepidoptera	13	54.94	659.32	9	58.43	525.89
Opiliones	5	0.26	1.30	2	2.59	5.18
Orthoptera	37	15.33	459.87	83	20.61	1524.99
Thysanoptera	1	0.54	0.54	0	—	—
Thysanura	0	—	—	1	0.54	0.54

Figure 1. Initial (summer) and final (autumn) body mass of southern leopard frog (*Rana sphenocephala*) juveniles reared in terrestrial enclosures at an initial density of 1.1/m^2 following chronic exposure to one of three concentrations of cadmium as larvae. Solid lines represent forest sites, and dotted lines represent field sites.

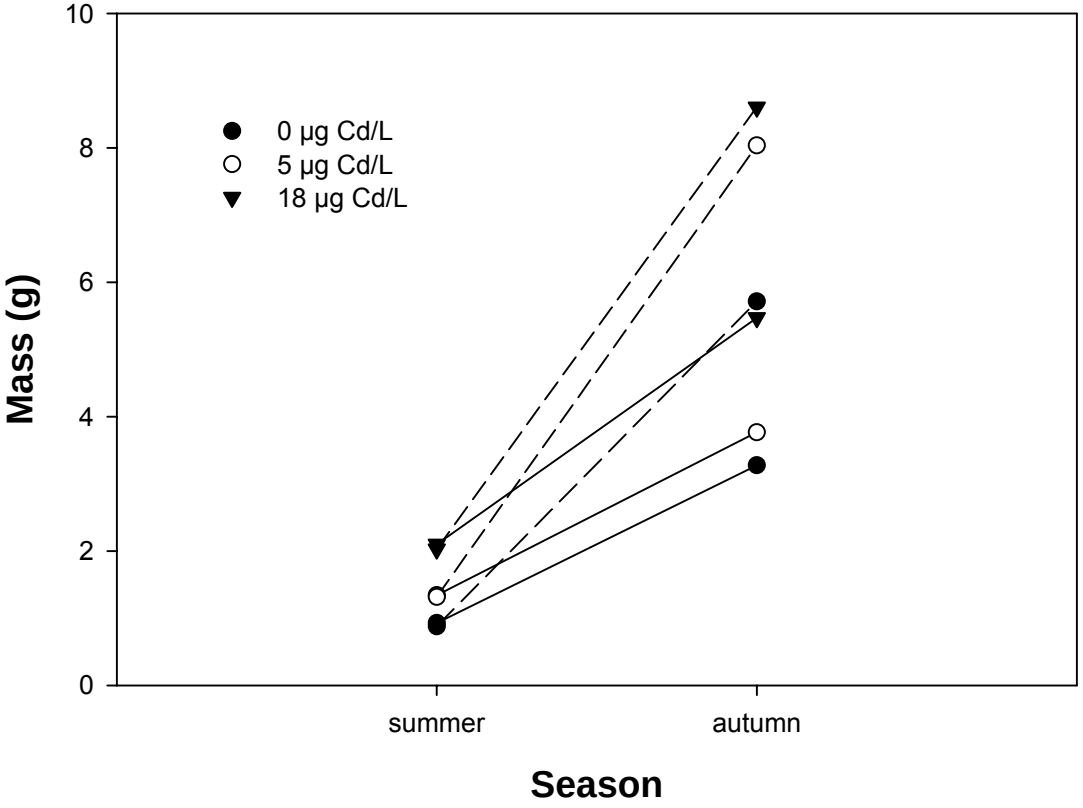

10
8
6
4
2
0
Mass (g)
0 µg Cd/L
5 µg Cd/L
18 µg Cd/L
summer
autumn
Season

Figure 2. Mean rainfall (dotted lines) and soil moisture (solid lines) in the forest and field sites where southern leopard frog (*Rana sphenocephala*) juveniles were reared in terrestrial enclosures. Higher soil moisture readings (in centibars) indicate lower hydration.

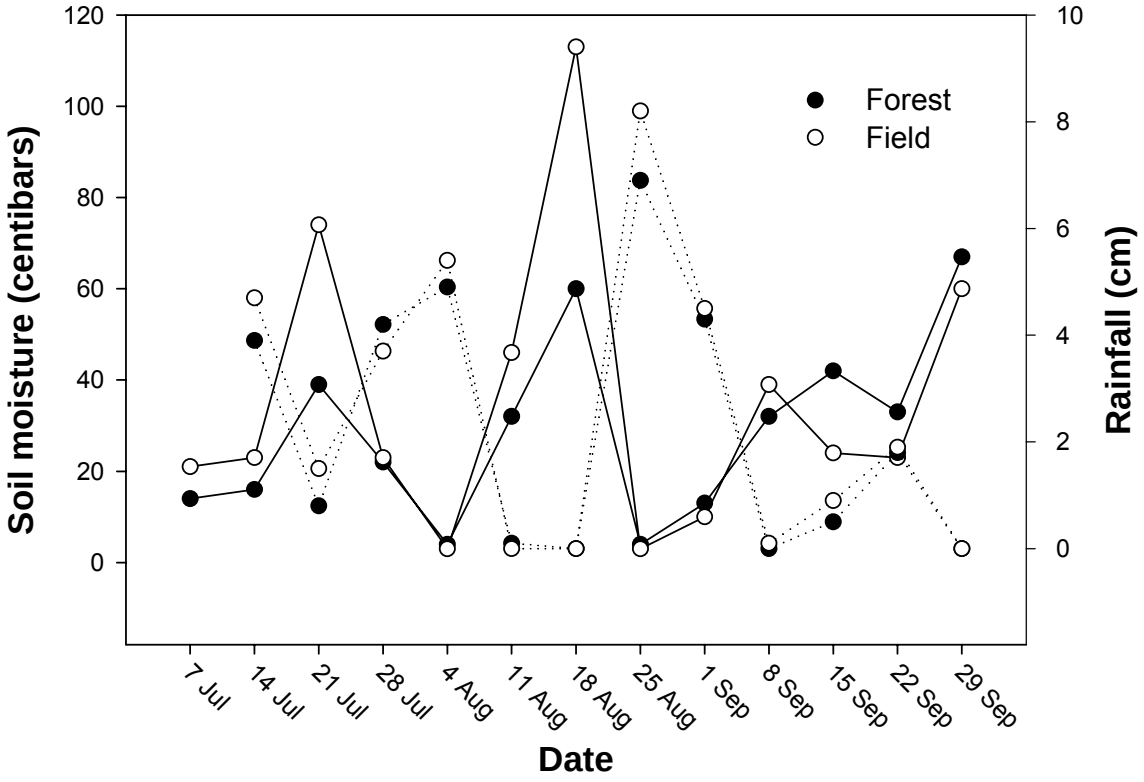

Soil moisture (centibars)
Rainfall (cm)
Date
Forest
Field
120
100
80
60
40
20
0
10
8
6
4
2
0
7 Jul
14 Jul
21 Jul
28 Jul
4 Aug
11 Aug
18 Aug
25 Aug
1 Sep
8 Sep
15 Sep
22 Sep
29 Sep

Figure 3. Average daily mean (solid lines) and maximum (dotted lines) air temperature in the forest and field sites where southern leopard frog (*Rana sphenocephala*) juveniles were reared in terrestrial enclosures.

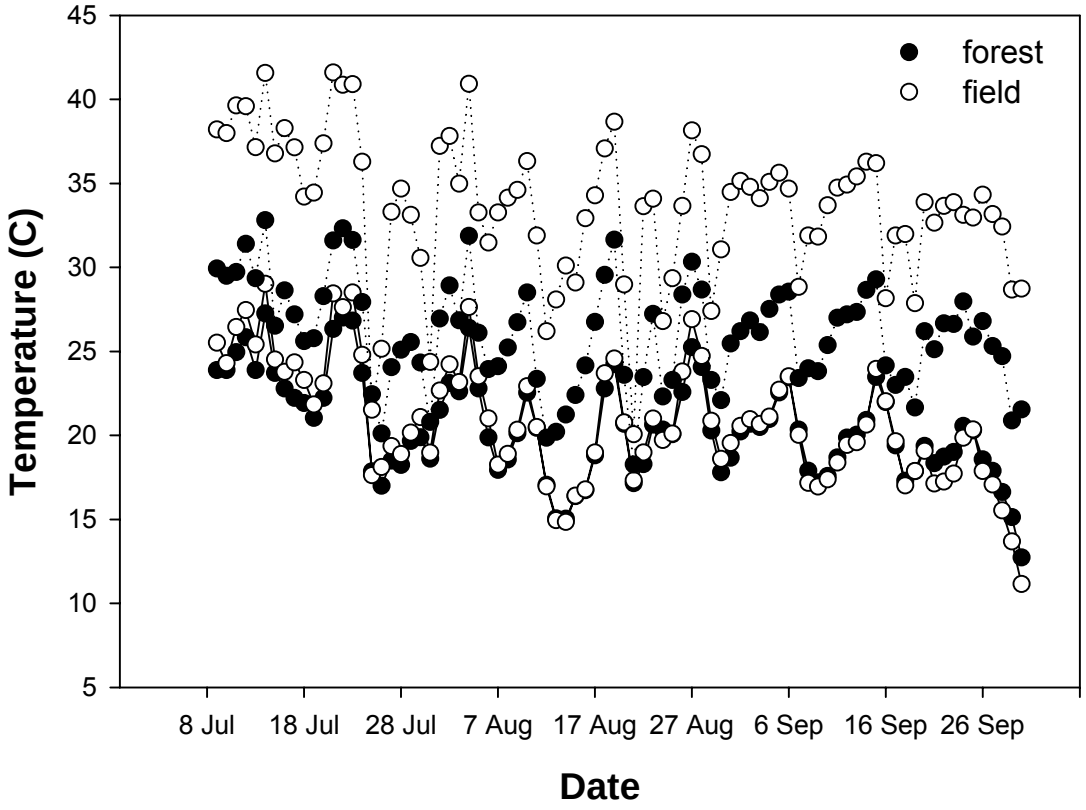
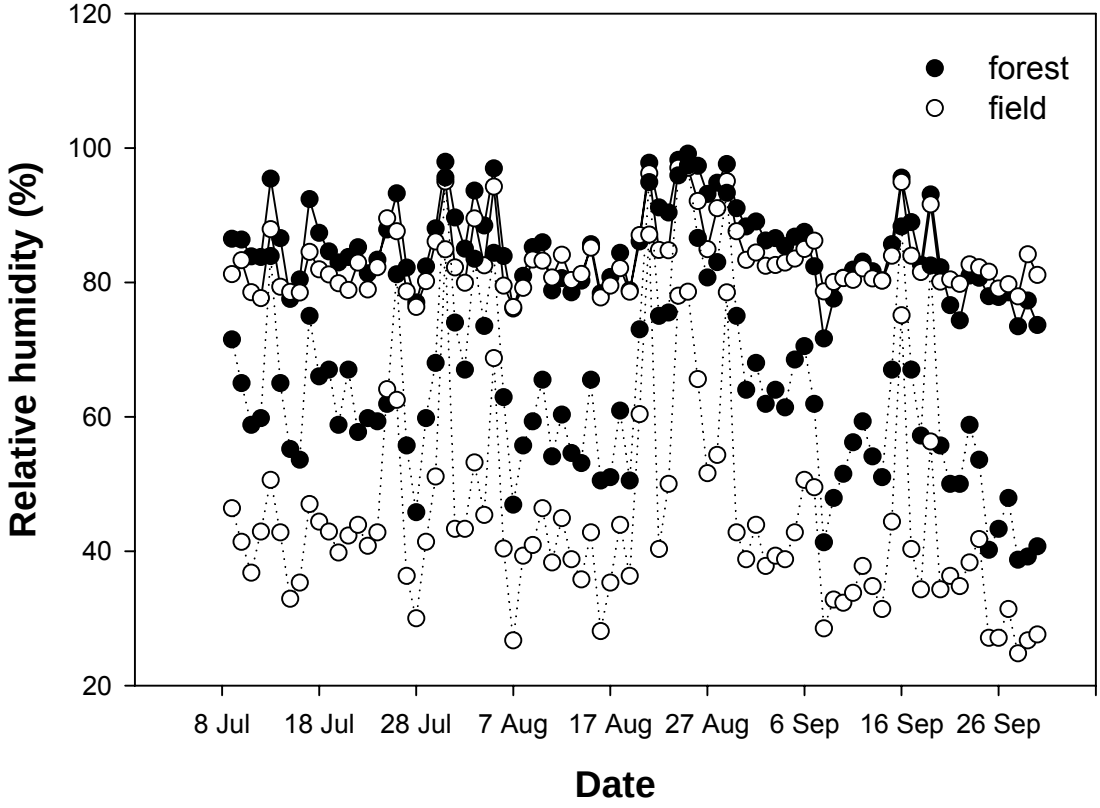

45
40
35
30
25
20
15
10
5
Temperature (C)
forest
field
8 Jul
18 Jul
28 Jul
7 Aug
17 Aug
27 Aug
6 Sep
16 Sep
26 Sep
Date

Figure 4. Average daily mean (solid lines) and minimum (dotted lines) relative humidity in the forest and field sites where southern leopard frog (*Rana sphenocephala*) juveniles were reared in terrestrial enclosures.


120
100
80
60
40
20
Relative humidity (%)
forest
field
8 Jul
18 Jul
28 Jul
7 Aug
17 Aug
27 Aug
6 Sep
16 Sep
26 Sep
Date

CHAPTER 7

CONCLUSIONS, IMPLICATIONS FOR AMPHIBIAN CONSERVATION, AND FUTURE RESEARCH DIRECTIONS

INTRODUCTION

Documenting and understanding the effects of the chemical contamination of the environment is a significant and ongoing challenge for research ecotoxicologists worldwide. Not only are there thousands of natural and man-made chemicals in current use, new contaminants are continually proposed for registration. The determination of causality and effect is complicated by the fact that the natural world is complex, dynamic, and subject to intricate interactions among its innumerous components. Each contamination scenario is different and may render unique outcomes for the focal species of concern. Practicality mandates that natural systems be simplified in order for general patterns to be discerned. Laboratory studies have done much to improve our understanding of which contaminants are harmful to organisms. Mesocosm studies can be used to make similar determinations, and may also incorporate direct and indirect effects, multiple routes of uptake, and interactions between contaminants and other potential stressors.

Pond-breeding anurans are at particular risk of contaminant exposure and toxicity because of their reproductive biology, physiology, microhabitat requirements, herbivorous and carnivorous diet, and biphasic life cycle. Amphibians are poorly studied

relative to other taxa, but have received increased attention since it has become apparent that populations are declining worldwide (Sparling et al. 2000). Most of the contaminant research on amphibians has been conducted with the egg and larval stages, to the neglect of the older life stages whose survival may be relatively more important (Biek et al. 2002, Vonesh and De la Cruz 2002). Instead of projecting outcomes to individuals or populations based on data obtained at or before metamorphosis, studies should include both aquatic and terrestrial life stages so that more accurate assessments are made. Research should also take a multi-tiered approach that spans from the laboratory to wild populations. The incorporation of natural processes and interactions can provide information that is not easy to obtain in the laboratory.

CONCLUSIONS SUMMARY

Aquatic mesocosm, terrestrial enclosure, and laboratory tests were conducted with the heavy metal cadmium (Cd) and two amphibian species (southern leopard frogs [*Rana sphenocephala*], American toads [*Bufo americanus*]) to determine the effect of this contaminant on larval and juvenile life stages. Exposures were limited to the aquatic environment so that potential latent effects could be assessed in terrestrial juveniles. It was concluded that tadpoles exposed to Cd have reduced survival, elevated Cd body burdens, delayed metamorphosis, and increased or decreased mass relative to unexposed tadpoles. The effects on metamorphic traits are due to a combination of direct toxicity and reduced survival. Those that survive Cd exposure and metamorphose into the terrestrial environment do not experience deleterious latent effects in the form of reduced juvenile growth or survival. However, the effects of Cd on juvenile performance

depended on terrestrial habitat quality. Cadmium in aquatic habitats appears to affect amphibian populations primarily through a reduction in larval survival. Larval mortality is naturally very high and any decrease in survival due to contamination could result in population declines. The existing published literature on Cd effects on tadpoles likewise suggests Cd bioaccumulates and causes mortality. However, this research added to the literature by demonstrating lethality at lower concentrations than are typically tested (e.g., Nebeker et al. 1995). This research also demonstrated that Cd-induced mortality can affect metamorphic traits, that Cd alters amphibian food resources, that oral uptake of Cd may be more important than gill and dermal uptake from water, and that the effects of Cd on tadpoles may depend on other potential stressors such as interspecific competition. There are no existing publications on juvenile growth and survival following Cd exposure as tadpoles.

MANAGEMENT IMPLICATIONS SUMMARY

Water bodies contaminated with Cd will likely have reduced larval amphibian recruitment relative to uncontaminated sites if the concentrations in the water and periphyton are equal to or greater than what was documented in Chapters 2 and 5 (see figures of Cd concentrations). This could eventually result in the decline or loss of resident amphibian populations. Managers may improve conditions with restoration projects that remove contaminated substrates, add amended soils, create or widen a vegetated terrestrial buffer, and reduce or eliminate Cd inputs.

QUESTIONS, CONCLUSIONS, AND IMPLICATIONS

The main research questions and resulting conclusions and implications are below:

I. What are the metamorphic responses of amphibians that have been chronically exposed to Cd in the breeding site?

CONCLUSIONS: Both southern leopard frogs and American toads experienced reduced survival, a delay in larval development, and absorbed significant amounts of Cd in their body tissue. However, southern leopard frogs had a larger mass at metamorphosis while American toads had a smaller mass. Cadmium uptake likely occurs from the water column and food resources.

IMPLICATIONS: Amphibian breeding sites that are contaminated with Cd may have lower recruitment due to decreased survival and failure to metamorphose before the hydroperiod ends. Those that survive to metamorphosis may be hindered by a smaller size and a Cd body burden. Overall this may result in local population declines.

II. What is the terrestrial performance (i.e., growth, survival) of amphibian juveniles that have metamorphosed from Cd-contaminated breeding sites relative to juveniles from uncontaminated sites?

CONCLUSIONS: Southern leopard frogs with a larval Cd exposure history did not have reduced growth or survival relative to unexposed individuals. There were initial differences in mass at metamorphosis due to Cd exposure, and juveniles maintained these mass differences through at least their first autumn in the terrestrial environment. Cadmium exposure decreased survival in American toads only if initial treatment differences in mass were ignored. Initial mass differences in American toads due to Cd effects on mass at metamorphosis disappeared by the first autumn.

IMPLICATIONS: Cadmium-exposed southern leopard frogs may breed earlier and have larger clutches, given that a large mass at metamorphosis is often correlated with a large mass and earlier age at maturity (Smith 1987, Semlitsch et al. 1988). American toads may experience elevated mortality in the terrestrial environment, which would result in fewer surviving to breed. Both species possess Cd residues in their tissues, which may influence performance in more stressful scenarios or be passed on to offspring and predators.

III. Do potential stressors such as habitat alteration, food resource availability, and interspecific competition affect the responses of amphibians that were chronically exposed to Cd as larvae?

CONCLUSIONS: The effect of Cd exposure history on the survival of juvenile southern leopard frogs depended on terrestrial habitat type. Considered alone, terrestrial habitat significantly affected juvenile growth. The effect of Cd exposure history on the growth

of juvenile southern leopard frogs depended on terrestrial food resource availability. Food resource availability alone had a significant effect on juvenile southern leopard frog growth. Interspecific competition with American toads interacted significantly with larval Cd treatment to affect the larval period of southern leopard frogs. Interspecific competition alone significantly affected southern leopard frog survival, larval period, and mass.

IMPLICATIONS: Habitat alteration, food resource availability, and interspecific competition are among the many factors that regulate amphibians and potentially influence the responses of amphibians to contaminants. Therefore, toxicity tests that manipulate only the contaminant or the contaminant and a few other factors may miscalculate the responses of natural amphibian populations to contaminants. Risk assessors should consider the potential role and influence of multiple factors when determining risk.

IV. Do amphibian species differ in their responses to exposure to Cd?

CONCLUSIONS: When southern leopard frogs and American toads were reared in separate cattle tanks, the two species had similar trends in their metamorphic responses with the exception of mass, for which they had opposite trends. For any given Cd treatment, southern leopard frogs had lower survival but American toads had higher Cd uptake. The delay in metamorphosis due to Cd treatment relative to the controls was greatest in the American toads. Metamorphs from this experiment were subsequently

reared in terrestrial enclosures and the species were once again kept apart. When only the metamorphs from the 0, 5, and 18 μg Cd/L treatments are compared, there were no significant effects of Cd on survival or growth of either species, but initial mass differences due to Cd treatment were maintained more so in the southern leopard frogs than the American toads through the first autumn. When the two species were reared together in cattle tanks that had been dosed with Cd, the directionality of the responses was essentially the same as when the two were reared apart, but the linearity of the dose response was not as strong (particularly for the American toads). The southern leopard frogs had higher survival than the American toads within each Cd treatment, but the trend remained for American toads to contain relatively higher tissue concentrations of Cd.

IMPLICATIONS: Previous research suggests bufonids are relatively more tolerant of contaminants than are ranids (Birge et al. 2000). It was therefore expected that the southern leopard frogs would exhibit more sublethal and lethal effects in response to larval Cd exposure. Interestingly, American toads that survive to metamorphosis accumulate more Cd than southern leopard frogs, which may indicate they can withstand higher concentrations before death occurs. However, it appears that no one species is particularly and regularly more sensitive than the other. The fact that American toads had higher survival than the southern leopard frogs when reared alone, but lower survival when reared together, confirms that amphibian responses change depending on the components of the environment. Species differences in size at metamorphosis is another important consideration; it is likely that the southern leopard frog juveniles were more capable of maintaining initial size differences than were the American toads because they

metamorphose at a larger percent of adult mass. There were enough differences between the two species that assessments based on single-species data are likely inadequate to predict the responses of all amphibians to Cd contamination. Variation in life history characteristics, physiology, and behavior probably make a large contribution to species differences in susceptibility to contaminants.

V. Can the juvenile performance of amphibians following metamorphosis from a Cd-contaminated breeding site be predicted from laboratory studies?

CONCLUSIONS: In the laboratory study, larval Cd exposure history had a significant effect on juvenile southern leopard frog growth but not survival. The large (9 m^2) terrestrial enclosure study determined that Cd influenced growth and survival. Initial treatment differences in mass due to Cd exposure were maintained in both studies through the first autumn.

IMPLICATIONS: It appears that laboratory studies can provide valuable insights into how Cd larval history influences juvenile amphibian performance in the terrestrial environment through the first autumn. The similarity in mass between those fed high-level (ad libitum or nearly so) diets in the laboratory and those reared in enclosures suggests that food is plentiful in the terrestrial environment and that starvation may be uncommon during the active period.

FUTURE DIRECTIONS

The questions above were answered for Cd and two amphibian species, but these same questions should be asked for other contaminants and species from other taxa (e.g., hylids, ambystomatids). There should also be more studies examining the responses of amphibians to both contaminants and common and persistent stressors, because of the documented importance of habitat quality and other environmental factors. Far more attention needs to be given to contaminant effects in terrestrial life stages. Likewise, more studies need to assess chronic exposure to federal water quality criteria concentrations for specific contaminants. The following areas are in particular need of research:

- Latent effects of larval contaminant exposure in terrestrial life stages, with assessments made of major contaminant classes (e.g., carbamates, organophosphates, organochlorines, polycyclic aromatic hydrocarbons)
- Combined effects of aquatic and terrestrial contaminant exposure
- Effects of contaminant exposure on age, size, and fecundity at maturity
- In situ aquatic and terrestrial studies, preferably using large experimental units so that multiple life stages and seasons can be studied
- Monitoring of free-ranging populations to determine correlations between contamination and endpoints such as mass, hatching success, recruitment, and annual survival rates
- Maternal transfer of contaminants to offspring and their success
- Effects of aerial pesticide and fire retardant spraying on terrestrial amphibians and their prey

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VITA

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