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Comparative Contaminant Toxicity: Are Amphibian Larvae More Sensitive than Fish?

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Because of their biphasic lifecycle, permeable eggs, skin and gills, amphibians are often considered to be sensitive to environmental contaminants (Vitt et al. 1990, Boyer and Grue 1995, Blaustein and Wake 1995). Consequently, environmental contamination has been proposed as having caused some of the widespread amphibian declines that have recently been reported (Blaustein and Wake 1990, Wake 1998, Houlahan et al. 2000). Although it has been suggested that amphibians are particularly susceptible to environmental contaminants, data for conclusively making this determination are few when compared to other taxa. In fact, a recent survey of vertebrate toxicological data reported that only 2.7% of studies included amphibians (Sparling et al. 2000).

Mayer and Ellersieck (1986) list amphibians as the least sensitive taxon out of 63 species tested with 174 different chemicals. Although most amphibian data cited in Mayer and Ellersieck include only two species from a single source (i.e., Sanders 1970). More recently, Birge et al. (2000) compared the sensitivity of several amphibian species with other commonly tested vertebrates (e.g., *Pimephales promelas*, *Oncorhynchus mykiss*) to multiple contaminants. When compared to fish, they found amphibian species more sensitive to metals in 67% of cases, and more sensitive to metals and organic chemical stresses 64% of the time. Thus, from these data it appears that amphibians are frequently, but not always, more sensitive than other vertebrate species.

In our experiment, we exposed southern leopard frog (*Rana sphenocephala*) tadpoles to five chemicals (4-nonylphenol, carbaryl, copper, pentachlorophenol, permethrin), each having a different mode of action. These chemicals have been used as model chemicals in previous studies (USEPA 1999a,b) to determine the sensitivity of fish and amphibian larvae to contaminants with differing modes of action. Southern leopard frogs are common throughout the eastern and southern U.S., often breeding in shallow pools or temporary ponds situated near or within agricultural areas. Therefore, this species may commonly come into contact with any number of chemical contaminants. Southern leopard frogs are not known to be in decline and are widely distributed across the southeastern US, and can act as a model amphibian species. The objective of our research was to determine the LC50s of southern leopard frog tadpoles and compare them with published values

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for organisms more commonly used in toxicological testing, while testing the hypothesis that amphibians are more sensitive to contaminants than fish. Furthermore, our data will add to the existing amphibian toxicological database.

MATERIALS AND METHODS

Seven masses of southern leopard frog eggs, collected in Wilson County, Tennessee, were cooled (4°C) and shipped via express mail to the USGS Columbia Environmental Research Center (CERC), in Columbia, Missouri, USA. Eggs from separate masses were pooled and placed in 30-L stainless steel tubs with flowing well-water (temperature 17°C, pH 7.8, hardness 286 mg/L as $CaCO_3$, alkalinity 258 mg/L as $CaCO_3$). After tadpoles had hatched and became free-swimming (stage 25; Gosner 1960), they were fed a 3:1 mix of TetraMin fish flakes and ground Purina rabbit chow and their chambers were cleaned every third day.

Before testing, tadpoles were acclimated to ASTM hard water (pH 8.32, hardness 171 mg/L as $CaCO_3$, alkalinity 115 mg/L as $CaCO_3$; ASTM, 1996) at 22°C for 48 hours and held without food for 24 h before the beginning of the toxicity tests. Tadpoles were tested three weeks post-hatch and were at relatively uniform sizes and developmental stages (mass = 0.05 mg $\pm$ 0.008 mg; stage = Gosner 25 (Gosner 1960).

Five chemicals, representing five modes of action were selected for toxicity tests: 4-nonylphenol (narcotic and oxidative stressor), carbaryl (acetylcholinesterase inhibitor), copper (osmoregulatory obstructer), permethrin (neurotoxin), and pentachlorophenol (oxidative phosphorylation uncoupler). The copper stock solution was formulated by dissolving copper sulfate (25.5% copper) in deionized water. All remaining stock solutions were created by dissolving the chemical in technical grade acetone. The highest test concentrations used for each of the chemicals were as follows: 4-nonylphenol (0.5 mg/L), carbaryl (28 mg/L), copper (0.5 mg/L), pentachlorophenol (0.60 mg/L), permethrin (42 μg/L). These high values were based on previous tests using these five chemicals to test sensitivity of fish and amphibian larvae (USEPA 1999a, b). Concentrations of the four organic stock solutions were confirmed using liquid chromatography at ABC Laboratories, Columbia, Missouri. The concentration of the copper stock solution was confirmed using atomic absorption spectrophotometry at the USGS, CERC. No water samples were taken during testing, therefore all reported test concentrations are based on measured stock concentrations, which were, as a percent of nominal, as follows: 4-nonylphenol (131%), carbaryl (117%), copper (63%), pentachlorophenol (96.8%), and permethrin (87.6%).

Tests were conducted in general accordance with standards set forth by ASTM (1996) and USEPA (1975), as follows. To determine LC50s, 19.6-L jars containing 15 L of ATSM hard water were placed into one of three waterbaths, and brought to 22°C. Jars were arranged in the waterbaths in a randomized block design. For each chemical, there were three replicates for each of the six test

concentrations (60% dilution series), as well as a water and an acetone control in each of the water baths. After the addition of chemical or acetone, 10 tadpoles were added to each jar. Mortality was recorded after 6, 12, 24, 48, 72, and 96 h and dead tadpoles were removed at each observation period. Dissolved oxygen was measured at 0, 48, and 96 h, and pH was measured at 0 and 96 h in the lowest, middle, and highest concentrations still containing live tadpoles. At no time did dissolved oxygen fall below recommended standards (USEPA 1975), nor did mortality exceed 10% in any control treatment. Tadpoles were not fed during the test.

All LC50s and 95% confidence intervals were calculated using either the Spearman-Karber method or nonlinear interpolation (Stephan 1977). Southern leopard frog tadpole LC50s were compared to LC50s published in several sources (USEPA 1999a, USEPA 1995, Mayer and Ellersieck 1986) for four species: rainbow trout (*Oncorhynchus mykiss*), fathead minnows (*Pimephales promelas*), bluegill sunfish (*Lepomis macrochirus*), and boreal toad tadpoles (*Bufo boreas*). We compared LC50s using means and standard errors, as presented in Sprague and Fogels (1976), to determine whether differences among species were significant.

RESULTS AND DISCUSSION

Worldwide reports of amphibian declines are increasing annually (Wake 1998, Houlahan et al. 2000). While it has been difficult to establish a cause for many of these declines, chemical contamination remains suspect. Demonstrating a link between environmental contaminants and amphibian declines has proven difficult because so little is known about the toxicity of many compounds to amphibian larvae (Sparling et al. 2000; but see Birge et al. 2000). Thus, to gain a better understanding of the extent to which chemical contaminants are contributing to amphibian declines, it is necessary to establish the toxicity of many chemicals to amphibians under standard conditions.

Table 1 reports 96-h LC50s for southern leopard frog tadpoles as well as published values for rainbow trout, fathead minnow, bluegill sunfish, and boreal toad tadpoles for each of the five chemicals we tested. The concentration of permethrin was not high enough to calculate an LC50 until 96 h.

Our experiment shows that tadpoles are significantly more tolerant to two of the five chemicals we tested than are three commonly tested fish species (i.e., fathead minnow, bluegill sunfish, rainbow trout). Tadpoles in our experiment were relatively tolerant to carbaryl and permethrin, which is in agreement with previous studies examining the toxicity of these two chemicals on tadpoles (Jolly et al. 1978, Berrill et al. 1993, Boone and Bridges 1999). Acetylcholinesterase inhibiting compounds are known to be released from some algal species (Cook et al. 1989). Thus, tadpoles may have adapted to the presence of such naturally occurring compounds in the aquatic environment and may thus show reduced responses to chemicals with a similar mode of action (e.g., carbaryl). Permethrins

are also derived from natural compounds (i.e., pyrethroids) which, potentially, may be present in the aquatic environment regularly enough for tadpoles to have built up a tolerance to such chemicals.

Table 1. 96-h LC50s for various species for five chemicals.

Chemical	S. leopard frog tadpoles	Boreal toad tadpoles[1]	Bluegill Sunfish[2]	Fathead Minnow[3]	Rainbow trout[3]
4nonylphenol (ug/L)	0.34 (0.31-0.37)	0.12 (0.09-0.15)	N/A	0.27	0.19
carbaryl (mg/L)	8.4 (7.4-9.6)	12.31 (10.3-14.7)	6.2	5.21	1.88
copper (mg/L)	0.23 (0.21-0.25)	0.12 (0.07-0.18)	7.3	0.47	0.88
PCP (mg/L)	0.14 (0.12-0.17)	0.37 (0.25-0.42)	0.192	0.25	.016
Permethrin (ug/L)	18.2	>10	6.2	9.38	3.31

Values were calculated using non-linear interpolation, moving average, or probit methods; 95% confidence intervals are in parentheses. PCP = pentachlorophenol.
[1]Data extracted from USEPA (1999a)
[2]Data extracted from Mayer and Ellersieck (1986)
[3]Data extracted from USEPA (1995)

While tadpoles were, in general, of equal or greater tolerance to organic chemicals compounds than were the three fish species, southern leopard frog tadpoles in this experiment were more sensitive to copper than fish. Similar copper sensitivity of tadpoles has been observed in other studies (Khangarot and Ray 1987, Horne and Dunson 1995, USEPA 1999a). This suggests that allowable levels of copper in the environment determined by tests using a fish species may not be protective of amphibian species. Copper is introduced into the environment through mining and industrial practices and is used as an algicide. Copper can thus potentially contaminate environments that are potentially amphibian habitats (e.g., ponds); therefore, it is possible that application of copper to such habitats may be injurious to amphibian larvae that are present. In general, amphibian larvae are vulnerable to metals because many metals are associated with acidic environments in which metals are mobilized to a greater degree than in more neutral habitats (Linder and Grillitsch 2000). Some amphibian species dwell in environments subject to rapid acidification via input from snowmelt, and are thus vulnerable because this added input coincides with migration, breeding, and larval development.

Comparison of LC50s at 24 and 96 h of exposure indicated that, generally, southern leopard frog tadpoles were significantly more tolerant to both carbaryl and permethrin when compared with other species. Overall, southern leopard

frog tadpoles were more tolerant in 48% of pairwise comparisons, while in 22.5% of comparisons there were no significant differences (Table 2). At 96 h the rank order of toxicity of compounds to southern leopard frog tadpoles, from greatest to least toxic, was roughly as follows: permethrin > copper > pentachlorophenol > 4-nonylphenol > carbaryl.

Ecotoxicological data for amphibians are not as common as for other taxa, thus it is important to determine whether a particular amphibian species will be protected at environmental concentrations deemed acceptable by using values from commonly used test species such as rainbow trout, bluegill sunfish, or fathead minnows. Using standard test species to determine the toxicity of a compound has the advantages of being readily available and easily tested. However, the ability to make inferences is often limited because little is known about how the toxicity of a compound to one species is related to toxicity in another species. Mayer and Ellersieck (1986) correlated the LC50s for several species with LC50s for three commonly tested vertebrate species (i.e., fathead minnow, rainbow trout, and bluegill) for over 200 chemicals. When high correlations were indicated, the species were assumed to be comparable in their sensitivity to the chemicals tested; therefore, comparisons between the species with high correlations may be more relevant than comparisons among weakly correlated species. The two amphibian species included in this correlation, *Pseudacris triseriata, Bufo woodhousii fowleri*, showed the highest correlation with rainbow trout while their correlations with bluegill sunfish and fathead minnows was much weaker. This suggests that rainbow trout may be the most appropriate surrogate fish species for making references to anuran tadpoles, as their LC50s for many contaminants are most similar. In our study, tadpoles were always of equal or greater tolerance than published 24 and 96-h LC50s for rainbow trout. Thus using values obtained from rainbow trout may be conservative for many chemicals and therefore protective of amphibians.

Because tadpoles are very tolerant to some compounds (e.g., carbaryl and permethrin) and sensitive to others (e.g., copper), larval amphibians should be utilized in toxicity tests that are used to determine effect concentrations for environmental contaminants. When compared with another tadpole species, the boreal toad, we found no consistent pattern of southern leopard frog tadpoles being either more or less tolerant. There is a great deal of variability among amphibian species with respect to their tolerance to a single contaminant (Birge et al. 2000, Bridges and Semlitsch 2000). Consequently, employing a single, lab-cultured species of amphibian in routine toxicity tests (e.g., *Rana pipiens*, *Xenopus laevis*) may not be adequate when determining effect concentrations for environmental contaminants that would be protective of a number of species.

LC50s for southern leopard frog tadpoles decreased at each observation period in all chemicals except 4-nonylphenol and permethrin. For 4-nonylphenol, most of the mortality occurred within 12 h, while mortality due to permethrin was not observed until 96 h. Thus, our study indicates that it is important to consider exposure time when determining the toxicity of a compound to southern leopard

frog tadpoles. For instance, because mortality was high after only 12 hours of exposure to 4-nonylphenol, pulsed exposure to this compound may be just as detrimental as a chronic exposure as both would negatively impact a population. Conversely, LC50s for permethrin could not be calculated until 96 hours because there was little or no mortality in any concentration. Thus, tadpoles may only be at risk of dying from chemical contamination when exposed chronically.

The data from our study provides information on the toxicity of five compounds to tadpoles. The fact that tadpoles were very tolerant to some compounds (e.g., carbaryl and permethrin) and sensitive to others (e.g., copper) suggests that it may be necessary to evaluate amphibians in toxicity tests when assessing the hazards of chemical substances (e.g., for Federal Insecticide, Fungicide and Rodenticide Act, Clean Water Act, Toxic Substances Control Act), rather than relying solely on the data from more commonly used surrogate species. Acute tests are relatively cheap and easy to do, yet can provide a wealth of information and can be followed up with other types of toxicity testing. While our data may be limited in applicability because of our use of a single species, it serves as a first step in evaluating sensitivity. Additional studies using chronic exposure and sublethal endpoints will be required to gain a fuller understanding of amphibian sensitivity to contaminants. We encourage the use of amphibian larvae in future toxicological testing to continue to augment the existing database. However, in light of the problem of declining populations, attention should be given to determining which common amphibian species will best serve as models for this taxon.

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