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Effects of Cadmium on Limb Regeneration in the Northwestern Salamander *Ambystoma gracile*

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Abstract. Tests were conducted to determine the effects of cadmium on leg regeneration in larvae of the Northwestern salamander, *Ambystoma gracile*. Cadmium significantly affected limb regrowth (LOAEL = lowest observed adverse effect level) at 193.1 μg/L in a 24-day test and at 44.6 μg/L in a 10-day test. There were no significant adverse effects (NOAEL = no observed adverse effects level) at 48.9 μg/L in 24-day and 12.8 μg/L in 10-day tests.

Current water quality criteria documents contain little or no acute and chronic data for amphibians. The criteria document for cadmium (USEPA 1984) and the U.S. Fish and Wildlife Service cadmium review (Eisler 1985) contain no acute or chronic toxicity values for amphibians that were used in determining water quality criteria values for cadmium. Cadmium is highly toxic to aquatic species and wildlife associated with aquatic habitats, and contamination is widespread as a result of human activities (Eisler 1985; Francis *et al.* 1984; Freda 1991; Hall and Mulhern 1984; Linder *et al.* 1991; Nebeker *et al.* 1994; Niethammer *et al.* 1985; Power *et al.* 1989).

The ability of salamanders to regenerate limbs presents a unique opportunity to study the effects of cadmium. Data on the effects of cadmium on limb regeneration in amphibians are limited. Manson and O'Flaherty (1978) conducted long-term static exposures of adult salamanders, *Notophthalmus viridescens,* to 2.0–6.75 mg/L, and found retarded limb regeneration and limb abnormalities. Several reports deal with effects of other chemicals on limb growth and regeneration (Arias and Zavenella 1979; Chang *et al.* 1976; Scadding 1990; Zavanella *et al.* 1984; Weiss 1975).

The objectives of the present study were to determine the sublethal effects of cadmium on limb regeneration in larvae of the Northwestern salamander *Ambystoma gracile* (Baird), and to obtain NOAEL (no observed adverse effect level) and LOAEL (lowest observed adverse effects level) values based on those effects.

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Methods

Test Facility

Tests with the larval salamanders were conducted in two continuous-flow exposure systems. Test 1 had 15 38 × 55 × 35 cm glass exposure chambers (Nebeker *et al.* 1992), and Test 2 had 18 2.5-gal (10-L) aquaria for holding the test animals, including controls. Each of the randomly placed test containers received fresh well water in a flowthrough system at 300 and 125 ml/min, respectively. Water temperature was maintained at 20 ± 1°C. Mean water quality parameters during testing were: hardness, 45 mg/L; alkalinity, 39 mg/L; conductivity, 145 μS/cm; median pH, 6.8. Late spring photoperiod was maintained at 13:11 h light:dark.

Test Procedures

Larval salamanders were hatched from eggs collected from a pond in the Cascade Mountains of central Oregon. The young salamanders, 3–4 months old at the time of testing, were closely monitored, and any that showed abnormal behavior (*e.g.*, floating, not feeding, erratic swimming) or were unhealthy were not used for testing. Animals that had legs nipped off by their tank mates were collected from laboratory rearing tanks for the study. All animals used for testing had limbs missing or in various stages of regrowth, and they appeared healthy in spite of their losses. Differences in regrowth between exposed and control animals were used as an index of toxicity (Table 1). Two animals were randomly assigned to each of three replicate tanks per exposure concentration for Test 1 (Table 2); three animals were randomly assigned to each of three replicate tanks per exposure concentration for Test 2 (Table 3). The larval salamanders were fed newly hatched brine shrimp (*Artemia* sp.) during pretest rearing, and the annelid worm *Lumbriculus variegatus* during rearing and testing. Food animals were reared in the laboratory and were not analyzed for cadmium. Test animals were exposed to mean measured cadmium concentrations of 504.5, 193.1, 48.9, 15.2, and <2 μg/L during Test 1 (24-day exposure), and 535.1, 227.3, 106.3, 44.6, 12.8, and <2 μg/L in Test 2 (10-day exposure). Samples were taken two or three times a week during testing and 121 samples were used for calculating mean measured values.

Table 1. Limb regrowth values assigned to each of seven leg lengths used for estimating amount of limb regeneration in the Northwestern salamander *Ambystoma gracile*

Limb regrowth value	Leg size	Typical leg lengths[a] for an animal 55 mm long	
		Front leg (mm)	Rear leg (mm)
1	No leg	<1	<1
2	Leg visible but <½ grown	4	3
3	Leg >½ grown, not full length, w/o digits	7	4
4	Leg full length w/o digits	8	5
5	Leg full length forming digits	9	6
6	Leg, foot, digits developed; thin, fragile, little pigment	11	7
7	Leg fully developed, robust structure, good pigmentation	12	8

[a] Reared in the laboratory

Table 2. Effects of cadmium on limb regeneration in the Northwestern salamander *Ambystoma gracile* exposed for 24 days (Test 1)

Mean ±SD measured cadmium concn. (μg/L)	Test chamber	Animal	Sum of[a] initial leg values at test start (A)	Sum of[a] leg values at end of test (B)	Regrowth numerical value (B − A)	Mean ±SE regrowth numerical value
504.4 ± 136.8	1	1	—[b]	—	—	7.0 ± 0
		2	—	—	—	
	2	1	—	—	—	
		2	—	—	—	
	3	1	—	—	—	
		2	10	17	7	
193.1[c] ± 71.0	1	1	16	24	8	10.8 ± 0.7[d]
		2	12	23	11	
	2	1	21	25	4	
		2	8	21	13	
	3	1	6	23	17	
		2	11	23	12	
48.9[e] ± 25.3	1	1	12	26	14	14.8 ± 0.6[f]
		2	17	28	11	
	2	1	10	26	16	
		2	9	28	19	
	3	1	13	24	11	
		2	10	28	18	
15.2 ± 7.9	1	1	9	28	19	16.2 ± 0.5[f]
		2	12	28	16	
	2	1	9	27	18	
		2	7	23	16	
	3	1	17	28	11	
		2	11	28	17	
Control	1	1	6	28	22	17.0 ± 0.9
		2	6	27	21	
	2	1	12	24	12	
		2	16	26	10	
	3	1	10	24	14	
		2	5	28	23	

[a] Each value (Table 1) represents the sum of numbers assigned to each leg, *e.g.*, left anterior = 4, right anterior = 4, left posterior = 4, right posterior = 3, = 15, total value at start of test (A) or at end of test (B)
[b] Animals died before end of test
[c] LOAEL = lowest observed adverse effect level ($p < 0.05$)
[d] Significantly different from controls ($p < 0.05$)
[e] NOAEL = no observed adverse effect level ($p < 0.05$)
[f] Not significantly different from controls ($p < 0.05$)

Table 3. Effects of cadmium on limb regeneration in the Northwestern salamander *Ambystoma gracile* exposed for 10 days (Test 2)

Mean ±SD measured cadmium concn. (μg/L)	Test chamber	Mean ±SD of[a] initial leg values at test start (A)	Mean ±SD of[a] leg values at end of test (B)	Regrowth numerical value (B − A)	Mean ±SE regrowth numerical value
535.1 ± 47.2	1	9.7 ± 0.6	14.0 ± 1.0	4.3	5.4 ± 0.4[b]
	2	10.7 ± 3.5	17.3 ± 0.6	6.6	
	3	10.7 ± 1.1	16.0 ± 3.6	5.3	
227.3 ± 17.5	1	12.0 ± 1.7	19.3 ± 0.6	7.3	6.8 ± 0.5[b]
	2	12.0 ± 5.0	17.0 ± 3.6	5.0	
	3	9.7 ± 1.5	17.7 ± 1.5	8.0	
106.3 ± 12.0	1	13.3 ± 3.8	20.3 ± 1.1	7.0	8.6 ± 0.5[b]
	2	9.3 ± 0.6	18.7 ± 0.6	9.4	
	3	12.3 ± 5.7	21.7 ± 2.5	9.4	
44.6[c] ± 10.7	1	11.3 ± 5.5	19.0 ± 4.6	7.7	8.9 ± 0.4[b]
	2	9.5 ± 2.8	19.3 ± 0.6	9.8	
	3	11.2 ± 0.8	20.3 ± 3.2	9.1	
12.8[d] ± 9.1	1	12.5 ± 0.5	20.7 ± 1.5	8.2	10.1 ± 0.6[e]
	2	13.0 ± 4.4	23.0 ± 2.6	10.0	
	3	10.0 ± 4.0	22.0 ± 1.0	12.0	
Controls	1	10.0 ± 3.6	22.3 ± 4.5	12.3	12.6 ± 0.2
	2	10.7 ± 4.0	23.0 ± 2.6	12.3	
	3	11.0 ± 0	24.3 ± 3.2	13.3	

[a]Each value (Table 1) represents the average value from the 3 animals in each test chamber. The value for each animal is the sum of the numbers assigned to each leg, *e.g.*, left anterior = 2, right anterior = 1, left posterior = 2, right posterior = 3, = 8, total value for that animal at test start (A) or at end of test (B)
[b]Significantly different from the controls ($p < 0.05$)
[c]LOAEL = lowest observed adverse effect level ($p < 0.05$)
[d]NOAEL = no observed adverse effect level ($p < 0.05$)
[e]Not significantly different from controls ($p < 0.05$)

Chemical Analysis

Reagent grade cadmium chloride (Mallinckrodt Chemical Works,[1] St. Louis, Mo; and J.T. Baker Chemical Co., Phillipsburg, NJ), was dissolved in acidified reverse osmosis (RO) water and pumped at a constant rate into the mixing boxes of the diluter portion of the two continuous-flow test exposure facilities. Cadmium concentrations were analyzed with an ICPAES (inductively coupled plasma atomic emission spectrophotometer). Direct aspiration of sample water into the instrument was possible with no cleanup. Standards and replicates were run after every tenth sample and at the end of the batch. The detection limit for cadmium in water was 2 μg/L. Quality Assurance procedures required by ERL-Corvallis were followed during all phases of analysis.

Calculations

Mean regrowth values were calculated by first assigning each of seven leg lengths a number (Table 1), ranging from one (no leg) to seven (fully grown leg). These numbers, for each of the four legs on each animal, were then added together for each animal at the beginning and at the end of the test. The value for each animal at the beginning of Test 1 was subtracted from its regrowth value at the end of the test to estimate the amount of regrowth that had occurred. In Test 2 each animal was measured at test start and test finish, but the three animals were not marked, so mean values for the three animals from each tank at the beginning and end of test were used in data analysis. Significant differences in limb regeneration from the controls (based on regrowth values) were calculated using Dunnett's multiple-comparison procedure (Computer Sciences Corp 1988). LOAEL values, the lowest concentration of cadmium producing adverse effects (reduced leg regrowth) significantly different from controls, and NOAEL values, the highest concentration of cadmium producing no adverse effects significantly different from controls, were determined by using Dunnett's multiple-comparison procedure (Computer Sciences Corp 1988).

Results and Discussion

Limb regeneration occurred rapidly, with complete regrowth of legs occurring in some control animals within the test period. Mean limb regeneration values (Figure 1) decreased as cadmium concentration increased in both Tests 1 and 2. The values ranged from 17.0 in the controls to 7.0 at 504.5 μg/L cadmium in Test 1 (24-day exposure), and from 12.6 in the controls to 5.4 at 535.1 μg/L in Test 2 (10-day exposure). Regrowth was significantly lower than the controls ($p < 0.05$) at a concentration of 193.1 μg/L (LOAEL) in Test 1 (Table 2); no significant differences from the controls (NOAEL) were seen at 48.9 μg/L cadmium. In Test 2 (Table 3) regrowth was significantly lower than the controls at a concentration of 44.6 μg/L (LOAEL), and no significant differences from the controls (NOAEL) were seen at 12.8 μg/L. These concentrations are much lower than those of Manson and O'Flaherty (1978), who found effects of cadmium on limb regeneration and limb abnormalities with adult *Notophthalmus viridescens* in the range of 2,000–6,750 μg/L.

The NOAEL values from the two tests in the present study (48.9 and 12.8 μg/L) are above the water quality criteria value

[1]Mention of tradenames or commercial products does not constitute endorsement or recommendation.

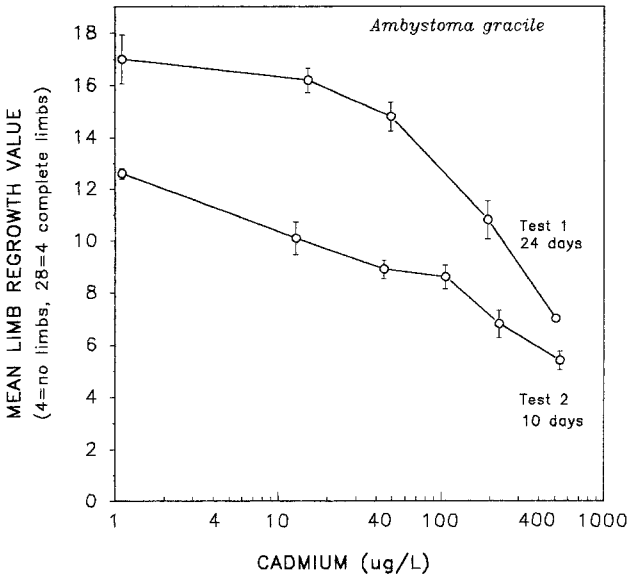

Fig. 1. Effects of exposure to cadmium for 24 days (Test 1) and 10 days (Test 2) on limb regrowth in larvae of the Northwestern salamander *Ambystoma gracile*

of 0.66 μg/L (50 mg/L hardness), but are similar to values for the more tolerant fish species tested (USEPA 1984). Nebeker *et al.* (1994) found the 96-h LC50 value for cadmium with *A. gracile* larvae to be 468 μg/L, at a water hardness of 45 mg/L, indicating that the limb regeneration data are a much more sensitive indicator of cadmium impact than acute data.

The annelid worms (*Lumbriculus variegatus*) fed to the salamanders during these two tests all died overnight in the high concentrations in Tests 1 and 2 (504 and 535 μg/L Cd, respectively), with partial mortalities in the next-to-highest concentrations (227 and 193 μg/L), indicating that they were much more sensitive to cadmium than were the salamanders. Tanks were cleaned daily and new worms added, so no long-term effects were noted. Worms were not analyzed for cadmium, but it is apparent that they would be a source of cadmium for the salamanders as they absorbed enough to kill them overnight.

In one other study dealing with the effects of chemicals on amphibian limb regeneration, Scadding (1990) showed effects of tributyltin (TBT) on growth of axolotl hind limbs and regrowth of front limbs. Limbs exposed to 1.5 μg/L TBT had slightly increased incidence of skeletal deletions; and at 5 μg/L three out of 20 limbs were complete, but almost all the limbs showed defects. No effects on forelimb regeneration were seen up to 5 μg/L, even though the concentration (5 μg/L) approached lethal levels (15 μg/L); other toxic effects were lethal at doses below those necessary to cause major limb malformations.

Amphibians are important food organisms for a large variety of fish, birds, and mammals, and have been found to be of major ecological significance in riparian and riverine habitat in the Pacific Northwest, due to high population densities attained by several species; in headwater reaches amphibians can be the dominant vertebrate predators (Bury 1988). In some communities, they predominate over birds or small mammals in terms of species and individuals or biomass; forest salamanders may exceed 3,000 individuals per ha (Bury and Raphael 1983). Amphibian densities and biomass were 10 and 4 times greater, respectively, than those reported for salmonid fishes in small streams (Bury *et al.* 1991).

Amphibians present a convenient opportunity to study the effects of cadmium on limb regeneration, although the ecological implications of inhibition of limb regeneration are unclear. This study with the Northwestern salamander (*Ambystoma gracile*) gives some evidence that the present criteria for cadmium, obtained primarily from fish and invertebrate data, will probably be protective of amphibians.

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