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CRITICAL PERIOD OF SENSITIVITY FOR EFFECTS OF CADMIUM ON FROG GROWTH AND DEVELOPMENT

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Abstract—Cadmium is a ubiquitous pollutant in aquatic environments that can alter organismal physiology and ecology. Previous experiments found that ecological Cd exposures increased the growth and development of two North American anurans. However, the generality of these effects among species, the time period over which they occur, and the mechanisms responsible remain conjectural. The goal of the present study was to determine the critical period of sensitivity of *Rana pipiens* exposed to ecologically relevant levels of Cd. We exposed tadpoles to Cd (0 [control], 1.0, and 10.0 μg/L) from Gosner stage (GS) 25 to metamorphic climax. We assessed effects of Cd on amphibian length, survival, and development during premetamorphosis (GS 25–30) and prometamorphosis (GS 31–42). After 14 d of exposure, we staged tadpoles and recorded snout–vent length. Tadpoles were then pooled according to treatment and stage (GS $\leq$ 29 or GS $\geq$ 30) and allowed to undergo metamorphic development. Tadpoles exposed to 10 μg/L were significantly larger and more advanced in development by 14 d. Survival to forelimb emergence exceeded 90% in all treatments, and time to metamorphic climax was not different from that in controls. Body burdens of Cd were positively correlated with increasing treatment. Early amphibian development (premetamorphosis) was shown to be the critical period of sensitivity for growth and development. Whereas the freshwater criterion for Cd appears to be protective for survival, a lack of knowledge remains about the sublethal effects of chronic exposures of metal pollutants, especially as they relate to tissue concentrations at various stages of amphibian life history.

Keywords—Sublethal Cadmium Amphibian Tadpoles Development

INTRODUCTION

Since the mid-1990s, the U.S. Environmental Protection Agency (EPA) has been developing bioassays to screen chemicals for the capacity to alter normal endocrine pathways. One of these screens uses amphibian metamorphosis to assess a chemical's ability for disruption of the thyroid hormone (TH) axis. Environmental pollutants such as flame retardants, rocket propellants, and heavy metals such as cadmium have been found to be antagonistic and agonistic to TH synthesis [1,2].

Cadmium is a ubiquitous heavy metal naturally averaging 0.18 mg/kg in the earth's crust [3]. Cadmium becomes concentrated by anthropogenic activity, however, and enters the environment from various sources, such as mining wastes, burning of fossil fuels, zinc refining, electroplating processes, iron and steel production, landfill leaching, fertilizers, and pesticides [4]. In wetlands impacted by industrial processes, surface waters have been reported with Cd concentrations as high as 703 μg/L [5]. Whereas average concentrations of Cd in surface waters of the United States have been reported to approximate 1.0 μg/L [4], other, more recent reports suggest that dissolved Cd in most surface waters usually does not exceed 1.0 μg/L [6,7] (http://pubs.water.usgs.gov/sir20065245/). In wetlands affected by highway runoff, Cd concentrations can reach 60.0 μg/L [4], and those found near mining sources can exceed 15 μg/L [7]. In aquatic systems, organisms are predominantly exposed to Cd via transport across the dermis and gill membranes as well as through dietary uptake [8], where it can readily bioconcentrate and accumulate to levels 4,000-fold greater in whole-body residues compared with those observed in the environment [9]. Consequently, even when Cd concentrations fall below the established safety criterion for aquatic organisms, the concentrations within amphibian larvae can bioaccumulate to levels that are 3,000-fold greater than those in the surrounding environment [10]. Moreover, because amphibian larvae and adults serve as prey for many fish, birds, and mammals, Cd bioaccumulation also may influence contaminant transfer throughout the food web.

Previous studies have demonstrated altered growth and development in native amphibians chronically exposed to low, environmentally relevant concentrations of Cd. Northern leopard frogs (*Rana pipiens*) [10] and American toads (*Bufo americanus*) [11] exposed to 5.0 and 54 μg/L, respectively, grew larger and developed faster relative to controls. Dosed *R. pipiens* tadpoles reached metamorphosis, on average, 20 d sooner than the controls. This response is surprising given how amphibians that develop and metamorphose earlier tend to be smaller in length and mass [12]. This observation has led to our hypothesis that Cd may affect the TH axis. In amphibians, metamorphosis is hormonally driven and is characterized by a significant rise in TH synthesis that causes the morphological reorganization of structures and physiological systems [13]. Whereas early larval growth and limb development stages

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(premetamorphosis, Gosner stages [GS] 25–30) are unresponsive to TH, later developmental stages (prometamorphosis, GS 31–42) and metamorphic climax can be accelerated with application of endogenous TH [14]. Additional administration of glucocorticoids (i.e., ''stress hormones'') can further accelerate metamorphic development during prometamorphosis by synergizing with TH, significantly decreasing the time required to complete metamorphosis [15].

Endocrinological responses to Cd are diverse. Cadmium binds to estrogen receptors in breast cancer cell lines and induces estrogen-regulated gene expression in ovariectomized rats [16]. It also reduces TH in chickens [17], decreases pituitary hormone secretions through lipid peroxidation [18], and increases plasma cortisol in rainbow trout (*Oncorhynchus mykiss*) at environmentally relevant Cd concentrations [19]. Thus, although Cd can influence endocrine functions, its effects are varied, and the mechanisms through which such interactions occur have remained ambiguous. Because the growth stages of amphibians, which have been well-characterized, are influenced primarily by TH only during certain developmental phases (prometamorphosis rather than premetamorphosis), they offer an ideal study organism in which to examine whether the effects of Cd are dependent on TH.

The aim of the present study was to identify the critical period of sensitivity of Cd-altered growth and development in *R. pipiens* larvae while further elucidating overall dose–response patterns. We hypothesized that the effects of Cd occurred during TH-dependent growth and development (prometamorphic stages). In particular, we sought to determine whether the acceleration of growth from Cd exposure occurred during developmental stages independent of TH (premetamorphic development) or during those dependent on TH (prometamorphic stages). Also, because growth effects were manifest at 5 μg/L in our previous study, with increased mortality at 20 μg/L, we predicted that *R. pipiens* larvae exposed to 10 μg/L would exhibit faster growth and increased bioaccumulation but no significant increase in mortality relative to controls.

MATERIALS AND METHODS

Solution preparation

Stock solution was prepared by dissolving cadmium chloride (99.3% Cd; Sigma-Aldrich) in ultrapure water (Millipore) to a final volume of 1.0 L. Each treatment solution was prepared by diluting the stock solution (1.0 ml delivered with calibrated pipette) with ultrapure water to achieve the appropriate Cd exposure concentrations (1.0 and 10 μg/L). Final solutions were ultraviolet light–sterilized and had pH 7.5 to 7.8, a total hardness of 170 mg/L $CaCO_3$, and a temperature of 23°C.

Diet preparation

Tadpole diet was prepared by embedding ground rabbit chow in a matrix of agar and gelatin [20]. Diets were prepared by mixing 250 g of dried ground rabbit chow (Harlan-Teklad), 20 g of agar, and 14 g of gelatin in 1,000 ml of reverse-osmosis water. The mixture was heated to 100°C and then allowed to cool and congeal. Diets were prepared weekly and stored frozen at −20°C or at 4°C for later use.

Larval exposures

Seven clutches of *R. pipiens* embryos were obtained from Mid Mississippi Biological Supply in April 2005 and were acclimated in laboratory water for 72 h before exposure. On reaching GS 25 [21], 300 healthy tadpoles were pooled (~43 tadpoles/clutch) and randomly distributed into twenty 20.8-L glass aquaria (40 × 20 × 25 cm) and dosed with 1.0 or 10.0 μg/L of Cd. We established 10 control tanks and five tanks for each treatment, with 15 tadpoles per tank and a total water volume of 18.9 L. Tadpoles were maintained in continuously aerated, ultraviolet light–sterilized, laboratory-prepared water (pH 7.5–7.8; total hardness, 170 mg/L as $CaCO_3$; 23°C) at a 14:10-h light:dark photoperiod in a climate-controlled animal facility throughout the experiment. Each day, tanks received a 50% water change followed by the respective treatment and the provisioning of diet. Mortality was monitored daily for each tank during water changes. Larvae were fed ad libitum. Multiple blocks of food were added to each tank, and we could assess relative amounts not consumed, confirming ad libitum feeding.

After 14 d of exposure, all tadpoles from each tank were measured, and snout–vent length (SVL) and developmental stage (i.e., GS) was recorded. Tadpole and metamorph length were measured ventrally from the tip of the snout to the posterior margin of the vent. Tadpoles were dip-netted and placed, without anesthesia, into a V-shaped measuring board. Because the shape of the measuring board enables it to hold water, tadpoles lie on their lateral surfaces and can remain partially submerged, thus minimizing stress. Measurements were taken to the nearest 0.01 mm with a Fisherbrand Traceable Digital Calipers (Fisher Scientific). Tadpoles within each treatment were then separated and pooled according to developmental stage (GS $\leq$ 29 or GS $\geq$ 30); for the purposes of the present study, only tadpoles at GS 30 or later were redistributed among 20.8-L aquaria and allowed to progress through development (n = 3 tanks for the control and 2 tanks each for the 1.0 and 10.0 μg/L treatments). This was done because we sought to decouple the effects of Cd on the pre- and prometamorphic periods of development, and stage-matching individuals from each treatment effectively functioned to ''reset'' the clock by removing stage-specific differences having originated during premetamorphosis. Previous studies [10] indicated that it would be possible to bias the results of the present study by selecting only the faster developers in each treatment, but by using only tadpoles at GS 30 or later, we could determine whether the effects of Cd on growth and development were specific to a particular developmental period while simultaneously maximizing the number of animals needed to conduct the experiment. Furthermore, by keeping animals under constant Cd exposure throughout their development, we ensured that the current results would be comparable to those of other chronic exposure studies of Cd and amphibians [10,11].

At GS 42, metamorphosing tadpoles were removed from their respective tanks and housed individually (115 × 51 × 45 mm) without food in plastic tackle boxes (Plano Molding). Animals were maintained in 3 ml of water, which was changed every other day until GS 46 or complete tail resorption. On survival to GS 46, each metamorphic frog was measured (SVL) and examined for malformations. Other endpoints recorded were time to metamorphosis, time to reach metamorphic climax (complete tail resorption), and survival during metamorphosis (GS 42–46). On exposure day 75, two or three tadpoles were netted and removed from each replicate in all treatments to provide tissue mass for analysis of Cd residue. All remaining tadpoles were allowed to metamorphose, with tadpole exposures ending on day 88 and the final remaining, metamor-

Table 1. Survival of all tadpoles to exposure day 14, from Gosner stage 30 (exposure day 14) to forelimb emergence, and to metamorphic climax[a]

	Survival (%)		
Cadmium (μg/L)	To day 14	To forelimb emergence	To metamorphic climax
0 (control)	98.0 ± 0.1	98.0 ± 0.0	75.9 ± 0.1
1.0	98.7 ± 0.1	100.0 ± 0.0	61.3 ± 0.0
10.0	97.3 ± 0.1	93.5 ± 0.0	69.0 ± 0.0
Generalized estimating equations	−0.036 ± 0.095 (Wald = 0.144, $p = 0.704$)	−0.99 ± 0.07 (Wald = 824.34, $p < 0.05$)	−5.93 ± 0.12 (Wald = 2,316.84, $p < 0.05$)

[a] Survival values are presented as the mean ± standard error. Generalized estimating equation values are presented as the estimate ± standard error.

phosing frog exposures ending on day 98. Metamorphic frogs and tadpole larvae were killed humanely, with regard for the alleviation of suffering, using 0.5 g/L of MS-222 (tricaine methanesulfonate; Western Chemical). Tadpoles removed for tissue Cd analysis were frozen at −80°C and freeze-dried (~1 g of whole-tadpole dry mass was selected from each tank). Whole-tadpole body burdens were analyzed by inductively coupled plasma–mass spectroscopy after an open-vessel HNO_3 hot-plate digestion at the Soil and Plant Analysis Lab of the University of Wisconsin–Extension (Madison, WI, USA). Analysis of exposure concentrations and submitted standards also was performed by inductively coupled plasma–mass spectroscopy. Treatment water was measured twice throughout the study. Nominal 1.0 and 10.0 μg/L Cd treatment concentrations, measured once during the study at 0 and 24 h postdosing, were measured at 1.61 and 1.09, and at 7.59 and 8.13 μg/L, respectively. Nominal concentrations are referred to throughout unless otherwise indicated.

Statistical analyses

Because the experiments were nested in structure, with multiple tadpoles grouped within treatment aquaria, forms of analysis were used that explicitly incorporated the likelihood of correlated responses of tadpoles housed within the same aquarium. This allowed analyses to maximize statistical power while still satisfying assumptions of the analyses. For continuous response variables, including SVL and days required to reach a particular developmental stage, we used mixed-model analyses with Cd dosage as a fixed effect and tadpole subjects nested within aquarium, which was a random effect. For the categorical response variable of survival (0 vs 1), we used generalized estimating equations (GEEs). The GEEs are an extension of the generalized linear model that allow correlations among observations from the same subject (e.g., individuals within the same container) and that use an iterative approach to estimate the correlations among observations [22,23]. We used the procedure ''geepack'' [24] in the statistical package R (R Core Development Team 2005, http://www.r-project.org). We used unstructured covariance matrix in all analyses and $\log_{10}$-transformed, continuous-response variables before analyses. Tadpoles removed for metal analysis at the experiment's end were not included in the survival analysis as reaching forelimb emergence (GS 42) or completing tail resorption (GS 46). The relationship between measured Cd content in tadpoles and in water was analyzed by least-squares regression of log-transformed values performed with SYSTAT® 11 (Systat Software).

RESULTS

Tadpole survival

Tadpoles at GS 25 were exposed to Cd for 16 weeks or until complete tail resorption (GS 46). Tadpole survival to day 14 was not significantly different among treatments (GEE analysis: −0.036 ± 0.095 [estimate ± standard error], Wald = 0.144, $p = 0.704$). Survival in stage-matched tadpoles from day 14 to forelimb emergence (GS 42) or complete tail resorption (GS 46) varied significantly when compared to controls (GS 42 survival: −1.99 ± 0.07, Wald = 824.34, $p < 0.05$; GS 46 survival: −5.93 ± 0.12, Wald = 2316.84, $p < 0.05$) (Table 1).

Growth, metamorphosis, and malformations

Based on the mixed-model analysis, tadpole growth (SVL: $F_{2,17.19} = 16.681$, $p < 0.001$) (Fig. 1a) and development (GS: $F_{2,292.001} = 6.568$, $p < 0.01$) (Fig. 1b) were significantly enhanced by Cd exposure at day 14. Only tadpoles in the 10 μg/L treatment, after 14 d of exposure, had reached more developmentally advanced stages (10 μg/L: Student's t $\text{test}_{1,292.001} = 3.159$, $p = 0.460$; 1 μg/L: Student's t $\text{test}_{1,292.001} = -0.627$, $p = 0.531$) and were larger than tadpoles from controls (10 μg/L: Student's t $\text{test}_{1,17.313} = 5.401$, $p < 0.001$; 1 μg/L: Student's t $\text{test}_{1,17.025} = -0.137$, $p = 0.893$). After stage-matching, tadpoles were not significantly different in developmental stage as a function of Cd treatment (mixed-model analysis: $F_{2,143} = 0.782$, $p = 0.460$) but were still significantly larger (mixed-model analysis: $F_{2,19.188} = 7.763$, $p < 0.005$). Tadpoles exposed to 10 μg/L were larger than control animals in the GS ≤ 29 and the GS ≥ 30 groups, stage-matched tadpoles (10 μg/L: Student's t $\text{test}_{1,16.101} = 3.115$, $p = 0.007$). The SVL (mean ± standard error) in GS 30 tadpoles were 20.7 ± 0.3, 20.1 ± 0.4, and 22.7 ± 0.5 mm in the control, 1, and 10 μg/L treatment, respectively. Time to reach forelimb emergence, however, was not significantly altered by Cd exposure, even among the larger tadpoles exposed to 10 μg/L (median ± standard error: 64 ± 3, 61 ± 3, and 64 ± 1 d in the control, 1, and 10 μg/L treatment, respectively; mixed-model analysis: $F_{2,4.202} = 0.758$, $p = 0.523$). Cadmium exposure had a marginally significant effect on total days to tail resorption (forelimb emergence to complete tail resorption; mixed-model analysis: $F_{2,66} = 3.023$, $p = 0.06$). At completion of metamorphosis, mean SVL also was significantly different (mixed-model analysis: $F_{2,65} = 4.345$, $p = 0.017$). Only a single limb malformation (hind limb hyperextension in a control tadpole) was recorded.

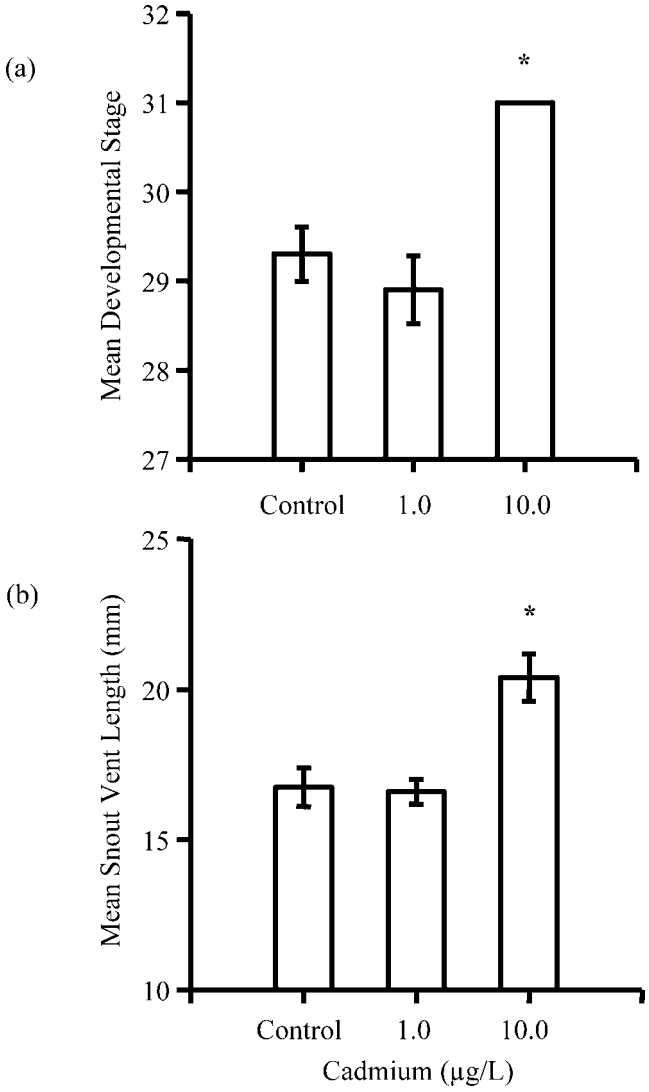

Fig. 1. Developmental stage (**a**) and snout–vent length (**b**) of *Rana pipiens* tadpoles after 14 d of cadmium exposure (n = 10 control tanks, 5 tanks for each treatment, and 15 tadpoles for each tank). Values are presented as the mean ± standard error. Asterisks indicate significant difference from control ($p < 0.05$).

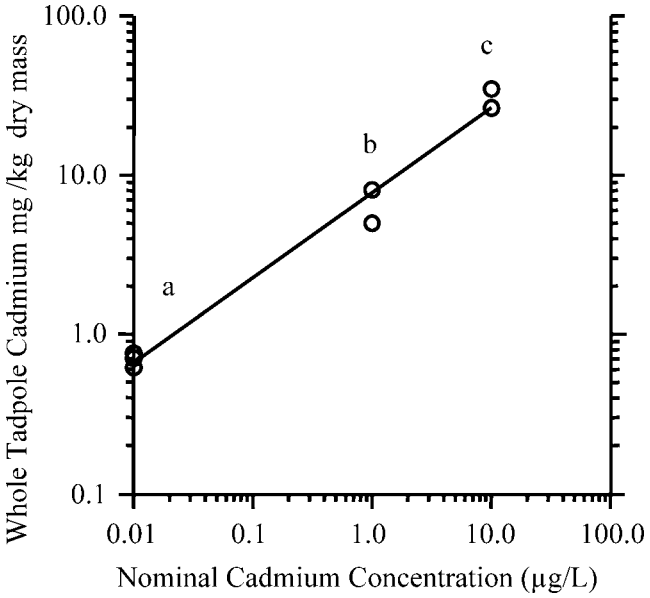

Fig. 2. Correlation between cadmium concentrations in *Rana pipiens* tadpoles (mg/kg dry mass) and nominal cadmium concentrations (µg/L) after 75 d of exposure. Each data point represents the analysis of approximately 1 g of dried tadpoles from each tank (n = 3 measures for control tanks and 2 measures for each treatment). The solid line shows the linear least-squares regression line of the log-transformed cadmium concentrations in tadpoles ($r = 0.99$, $p < 0.001$). Treatments labeled with different letters were significantly different from each other (Tukey's test: $p < 0.05$).

Whole-body and aqueous Cd analysis

Whole-body Cd concentrations in tadpoles exposed for 75 d were positively associated with nominal Cd concentrations in water (Tukey's test: $r = 0.99$, $p < 0.001$) (Fig. 2).

DISCUSSION

Growth and development

The present study demonstrates that the critical period of sensitivity for positive effects on growth is premetamorphosis (GS <31), a period of amphibian development that is characterized by its insensitivity to TH [25]. Previous research influenced the initial hypotheses that the effects of Cd occurred during prometamorphosis (GS > 30) [10]. After 14 d of exposure to 10 µg/L of Cd, however, tadpoles were 25% larger than control animals, and 66% of the those tadpoles had reached more advanced stages of development (GS > 30), compared with only 33% in control and the 1 µg/L treatment. Even after stage-matching tadpoles to normalize developmental stage before initiating prometamorphic exposures, the tadpoles exposed to Cd at 10 µg/L were still significantly larger, although at completion of metamorphosis, frog length was similar for each treatment. In the present and previous *R. pipiens* studies [10], no significant effects of size after metamorphosis were reported, because both the control tadpoles and those treated with low doses of Cd metamorphosed at the same size. This effect may be attributable to reaching an optimal size before initiating metamorphosis [26].

The effects of Cd on development also were consistent with those found in studies using *B. americanus* [11]. In both our *R. pipiens* studies and the *Bufo* sp. studies, larvae chronically exposed to Cd reached metamorphosis sooner than controls. The mechanism responsible for these effects, however, is largely unknown. Amphibian metamorphosis is mediated by hormonal signaling; the most notable is TH. Before the present study, the possibility of Cd positively altering the thyroid–pituitary axis during prometamorphosis was considered, thereby explaining the more rapid development in chronically exposed larvae. Had this been the case, then based on traditional amphibian metamorphosis studies in which TH treatment during prometamorphosis decreases the time to metamorphosis [27], exposure to Cd during this sensitive period (prometamorphosis) would have been expected to similarly shorten this developmental window. Instead, by decoupling Cd exposure and larval development period, the present study found that the developmental promoting effects of Cd occur during premetamorphosis rather than during prometamorphic stages.

Below are two proposed, alternative hypotheses regarding the influences of Cd on amphibian growth and development during premetamorphosis. The induction of metamorphosis in amphibians by TH is mediated by the transactivation of TH receptors [13]. Whereas TH receptors can bind as monodimers or homodimers to thyroid-response elements on DNA activation sites, they are bound most efficiently as heterodimers

with retinoic-X receptors (RXR). In *Xenopus laevis*, there are three RXR genes: α, β, and γ. All receptors are expressed during premetamorphosis, even as early as fertilization, with RXR-γ expression peaking with the start of hind limb development (Nieuwkoop Faber stage 45 in *Xenopus* sp. [28], GS 25 in *Rana* sp.) and in parallel with the expression of TH receptors (for a recent review of TH induced metamorphosis, see Furlow and Neff [29]). Cadmium, a well-known teratogen of mammals [30], has been shown to interact with retinoic acid, altering limb development in mice [31]. Few studies have been directed at the interactions of RXR and heavy metals.

Recent studies concerning the effects of Cd on estrogen receptor, insulin-like growth factor (IGF), and IGF-binding proteins (IGFBP) also may provide insight regarding the positive effects of Cd on growth and development. Messenger RNA expression of IGFBP-1 was induced by Cd exposure in HepG2 cell culture [32]. Cadmium also stimulated mitogenic growth factors and kinase activity in vitro, by an estrogen receptor α–dependent mechanism, leading to increased cell proliferation in a breast cancer cell line [33]. Although the literature regarding these effects is not conclusive, because oral exposure of rats to Cd decreased levels of IGF-I and IGFBP-3 [34], the results of other in vivo studies suggest that effects of Cd on gonadotropins may be dose dependent. At the lower Cd concentrations, orally exposed male rats had increased pituitary hormone excretions, including prolactin, thyroid-stimulating hormone, and ACTH, whereas higher concentrations had negative effects [35]. Additional research is needed to elucidate possible endocrine-modulating effects of Cd on humans and wildlife.

The positive growth and development associated with Cd may be a function of the length of exposure and the Cd body burden. Further chronic exposure studies initiated with nonexposed, GS 31 tadpoles will be necessary to confirm the lack of effects on growth and development during TH-induced metamorphosis, because this could elucidate whether these effects are dependent on body burden. It is possible that other physiological mechanisms, such as those that increase feeding behavior or other endocrine and growth factor expression, may be altered by Cd accumulation [36].

Bioaccumulation

A previous study found significantly higher mortality as well as retarded growth and development in tadpoles with a mean tissue concentration of 66.4 mg/kg dry weight at seven weeks of exposure to Cd at 20 μg/L [10]. *Rana ridibunda* larvae subchronically exposed to Cd experienced similarly enhanced mortality when mean tadpole tissue concentrations exceeded 40 mg/kg dry weight [37]. Mean tadpole tissue concentrations of *R. pipiens* tadpoles in the present study never exceeded 31 mg/kg dry weight in the highest treatment group, helping to explain the absence of negative effects on growth or development and the more than 90% survival to forelimb emergence across all treatments. James et al. [38], however, reported that Southern leopard frog (*Rana sphenocephala*) larvae experienced significant mortality when chronically exposed in a mesocosm to a single dose of Cd at 60 μg/L. This result was not necessarily unexpected, because total hardness in that study was 60 mg/L as $CaCO_3$ and because water hardness is decreased as Cd toxicity is increased. What was interesting is that tadpoles probably ingested most of the Cd through the diet and that mean whole-body burdens in the tadpoles that survived to complete tail resorption (GS 46) were 25.5 mg/kg dry weight. In the same study, no effects on survival were reported in *B. americanus* with mean whole-tadpole tissue concentrations of 30 mg/kg dry weight. Route of exposure may be a significant factor in amphibians, because Cd appears to be mobilized differentially if absorbed across gills and skin or through the gastrointestinal tract [38,39]. In the present study, tadpoles were exposed primarily to Cd across the gills and dermally, with a small fraction probably occurring orally, through binding of Cd to food and possible reingestion of feces. Based on these few studies, it appears that the sensitivity to Cd exposure and the rate of bioaccumulation vary among species and that tissue concentrations in excess of 30 mg/kg likely pose a significant health risk to some North American frogs and toads. Amphibian larvae in the field may reach this critical body burden, because environmental concentrations of Cd have been measured at 10 to 20 μg/L in considerably softer water [40].

The current U.S. EPA water-quality safety value for chronic Cd exposure is protective of *R. pipiens* larvae at 0.4 μg/L (when corrected for a water total hardness of 170 mg/L as $CaCO_3$), but it is conceivable that adverse effects, such as impacts on reproductive tissues, may be found in juvenile and adult amphibians or in their predators inhabiting those contaminated sites. We emphasize the need for additional ecologically relevant field studies [38] to evaluate tissue concentrations and potential significance of Cd exposure in naturally occurring aquatic food webs.

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