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RELATIONSHIPS AMONG DEVELOPMENTAL STAGE, METAMORPHIC TIMING, AND CONCENTRATIONS OF ELEMENTS IN BULLFROGS (*RANA CATESBEIANA*)

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Abstract—We collected bullfrog (*Rana catesbeiana*) larvae from a coal combustion waste settling basin to investigate the effects of developmental stage and timing of metamorphosis on concentrations of a series of trace elements in bullfrog tissues. Bullfrogs at four stages of development (from no hind limbs to recently metamorphosed juveniles) and bullfrogs that metamorphosed in the fall or overwintered in the contaminated basin and metamorphosed in the spring were analyzed for whole-body concentrations of Al, V, Cr, Ni, Cu, As, Pb, Cd, Zn, Ag, Sr, and Se. After the effects of dry mass were removed, tissue concentrations of six elements (Al, V, Cr, Ni, Cu, As, and Pb) decreased from the late larval stage through metamorphosis. Decreases in concentrations through metamorphosis ranged from 40% for Cu to 97% for Al. Tissue concentrations of these elements were also similar or higher in spring; Al and Cr concentrations were 34 and 90% higher in the spring, respectively, whereas As, Ni, Cu, and Pb concentrations were <10% higher. Concentrations of Cd, Se, and Ag varied among seasons but not among stages; Cd and Ag concentrations were 40 and 62% lower, respectively, and Se concentrations were 21% higher in spring. Concentrations of Zn varied only among stages; concentrations decreased gradually through late larval stage and then increased through metamorphosis. Concentrations of Sr varied among stages, but this variation was dependent on the season. Concentrations of Sr were higher in larval stages during the spring, but because concentrations of Sr increased 122% through metamorphosis in the fall and only 22% in the spring, concentrations were higher in fall metamorphs when compared with spring metamorphs. Our results indicate that metamorphosis and season of metamorphosis affects trace element concentrations in bullfrogs and may have important implications for the health of juveniles and the transfer of pollutants from the aquatic to the terrestrial environment.

Keywords—Coal combustion waste Amphibians Trace elements Metamorphosis Larval period

INTRODUCTION

In aquatic systems, bioaccumulation and effects of trace elements are influenced by the chemical properties of specific ions; environmental conditions (e.g., pH, E_H, and dissolved organic carbon); their interactions with specific biochemical functional groups (e.g., sulfhydryl, amino, carboxyl, hydroxide, oxide); and the ecology and life stage of individual organisms. Models have been developed to predict element speciation, bioavailability, and bioaccumulation as a function of aqueous chemistry and cell surface interactions with element ions [1]. However, the influence of variations in autecology and life stage (i.e., larvae, juvenile, and adult forms) on contaminant bioaccumulation has received less attention. This is particularly true in the case of animals with complex life cycles, which undergo large rearrangements in morphology and ontogenetic changes in physiology and habitat requirements.

With growing concern over amphibian population declines [2–4] and the occurrence of high rates of malformations in some amphibian populations [5,6], both of which have been linked to pollution of aquatic environments in specific cases [7], it is important to understand how accumulation and elimination of pollutants is affected by variations in autecology and ontogeny. Most anurans have complex life cycles [8] involving egg fertilization and embryo and larval development in aquatic habitats. After varying periods of time, larvae metamorphose and may spend significant portions of their remaining lives in terrestrial habitats, returning seasonally to the aquatic habitat to breed. Metamorphosis in anurans involves resorption of the tail, development of the front and hind limbs, and large changes in most organ systems [9]. Furthermore, the timing of reproduction and the duration of the aquatic larval stage can vary greatly among anuran species, conspecific populations, and even individuals within populations.

The bullfrog (*Rana catesbeiana*), a common frog throughout much of North America, has one of the longest and most variable larval stages of any anuran. The duration of the bullfrog's larval stage can vary from several months to years. Breeding occurs over a two-to-three month period during the summer [10]. In southern latitudes, varying proportions of larvae may undergo metamorphosis at the end of their first summer, whereas others may remain in the pond (i.e., overwinter) until the following spring before metamorphosing [11]. In northern latitudes, overwintering may be obligatory and larval periods may last two or more years [10,12].

In this paper we report trace element concentrations in bullfrog larvae and recently metamorphosed juveniles (metamorphs) that did or did not overwinter in a coal ash–polluted settling basin. Extensive previous work on bullfrogs has been conducted at the coal ash–polluted site [6,13–17], and observations from the field suggested that differences in body burdens of contaminants might occur based on season and developmental stage [18]. We collected bullfrogs at four stages of development, in both fall and spring, and analyzed whole-

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body concentrations of 12 trace elements. Our objectives were to investigate changes in trace element concentrations in larvae through metamorphosis and the influence of overwintering on tissue concentrations. We specifically ask three questions. First, how do whole-body trace element concentrations change through metamorphosis? Second, how do trace element concentrations vary with timing of metamorphosis? Third, do different elements show similar patterns of change in concentration among stages and seasons of metamorphosis?

MATERIALS AND METHODS

Study site description

We collected bullfrog larvae from a secondary settling basin that receives sluiced coal ash from an electrical power generation plant on the Department of Energy's Savannah River Site, Aiken County (SC, USA). Coal ash is sluiced from the power plant to a primary basin where surface water then enters a secondary basin and swamp before draining into the Savannah River via Beaver Dam Creek (Aiken County; for a map of the study site see Hopkins et al. [6]). As a result of this process, at least 18 elements are higher in the sediments of the settling basins than they are in nearby, uncontaminated wetlands [19–21]. Furthermore, bullfrog larvae and juveniles from the settling basin have elevated tissue concentrations of As, Cd, Cr, Cu, Se, Sr, and V compared with tissue concentrations of individuals from reference sites [6,13,14]. Exposure of developing larvae to elevated concentrations of trace elements results in sublethal changes in physiology, developmental morphology, and performance [6,13–16]. Because we focused on the dynamics of bioaccumulated trace elements, larvae were not collected from reference sites for this study. For comparisons of tissue, water, and sediment concentrations between reference sites and the contaminated study site, we refer readers to other studies [6,13–17,22,23].

Larval collection and handling

During the fall (September–December) of 1998 and spring (May) of 1999 we used minnow traps to collect larvae at four different stages of development. Developmental stages were based on those of Gosner [24]. Individuals at the no hind limb (NHL) stage were all Gosner stage 25. Early hind limb (EHL) individuals ranged from Gosner stages 31 to 35. Late hind limb (LHL) individuals were either Gosner stages 39 or 40, and early front limb development (EFL) individuals were all Gosner stage 42. All larvae were returned to the laboratory where they were housed in plastic containers containing aged tap water. Larvae in NHL, EHL, and LHL stages were held in the laboratory for 48 h to allow their guts to clear, then sacrificed (overdose of MS 222) and stored frozen at −40°C until analyzed for trace elements. Within each developmental stage we attempted to collect larvae of similar size in the fall and spring because we were interested in relationships among developmental stage, season of metamorphosis, and trace element concentrations independent of allometric relationships among trace element accumulation and size.

Larvae in the EFL stage were returned to the laboratory and held at room temperature in individual 2.5-L containers filled with aged tap water until they had completed metamorphosis (complete tail resorption). Approximately 50% of the water in each container was changed every 48 h. A piece of sponge was placed in each container to allow metamorphosing larvae to leave the water. Bullfrogs were not fed during metamorphosis because feeding ceases once front limbs emerge and individuals do not begin feeding again until metamorphosis is complete [25]. Over the course of the study, 74% of the 58 EFL-stage larvae survived through complete tail resorption. Following metamorphosis, juveniles were sacrificed and stored as described above for larvae.

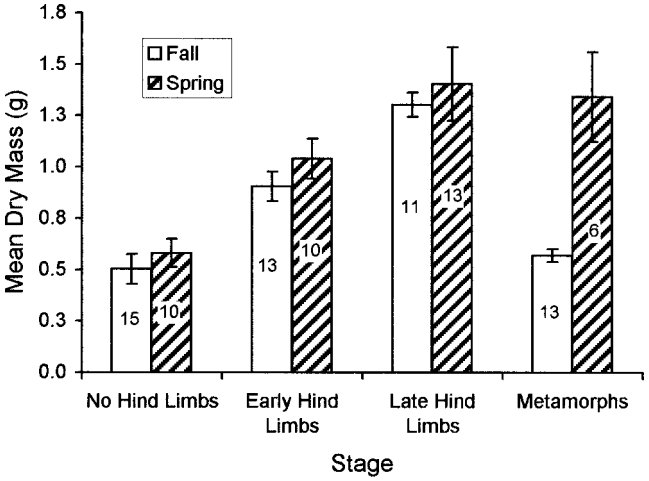

Fig. 1. Mean dry mass of bullfrog larvae and metamorphs collected in the fall and the spring from a coal combustion waste-settling pond in South Carolina, USA. Numbers in bars are sample sizes.

Trace element analysis

Whole larvae and juveniles were lyophilized, homogenized, and shipped to the Soil Science Center, University of Georgia (Athens, GA, USA), where they were analyzed for 12 trace elements (Al, V, Cr, Ni, Cu, As, Pb, Cd, Zn, Ag, Sr, and Se). We chose this group of trace elements because they have been documented as occurring in relatively high concentration in sediment of the basin or they have received attention in the recent toxicological literature. Procedures follow those detailed in U.S. Environmental Protection Agency Methods [26]. Samples were digested with HNO_3/H_2O_2 before they were analyzed by inductively coupled plasma mass spectroscopy (see Fig. 1 for sample sizes). All concentrations are expressed in micrograms per gram (μg/g) dry mass. Instrument detection limits ranged from 0.006 to 0.918 ppb and recovery of standards ranged from 91 to 112%.

Data Analyses

The goals of our analyses were to investigate the effects of developmental stage and overwintering on whole-body concentrations of trace elements. Because some of the trace elements we analyzed might show allometric relationships with size [27], we first investigated correlations between trace element concentrations and dry mass and removed these effects. We then determined differences and similarities among trace elements at different developmental stages and seasons.

To investigate similarities in patterns of accumulation among trace elements, and potential confounding effects of body size, we constructed a matrix of Pearson correlation coefficients and their associated individual significance tests. We included dry mass in these analyses as a measure of size. Because individual tests of correlation coefficients were not independent, we used a sequential Bonferroni correction to maintain the experiment-wide error rate at $p < 0.05$. In all samples, trace element levels were above detection limits with

Table 1. Minimum, maximum, mean, and standard deviation (SD) of trace element tissue concentration in larvae and recently metamorphosed juvenile bullfrog (*Rana catesbeiana*) from a coal ash contaminated site in South Carolina, USA[a]

Element	Minimum	Maximum	Mean	SD
Al	20.59	9424.23	3549.86	2393.64
V	0.88	50.94	15.91	9.37
Cr	0.79	21.38	7.79	4.90
Ni	1.49	133.69	27.11	22.97
Cu	24.94	250.16	100.48	46.51
Zn	59.70	607.33	134.85	67.72
As	4.90	73.21	29.17	15.62
Se	7.55	59.09	28.48	8.24
Sr	20.62	248.32	87.03	36.52
Ag	BDL	1.08	0.11	0.15
Cd	1.21	13.66	3.74	1.67
Pb	0.04	46.04	7.20	6.23

[a] For a breakdown of concentrations by stage and season see Figure 4. All values are micrograms per gram (μg/g) dry mass. BDL = below detection limits.

the exception of Ag. In <5% (4 of 91) of the samples, Ag tissue concentrations were below detection. For statistical analyses we replaced these values with half of the detection limit for Ag (0.005 μg/g). Dry mass and trace element tissue concentrations were $\log_{10}$ transformed before analysis.

To investigate differences in concentrations of trace elements among developmental stages and overwintered and nonoverwintered individuals, we used two-way analysis of variance (ANOVA) of principle components analysis (PCA) scores for each larva and juvenile. PCA was used to summarize relationships among trace element concentrations and to remove the effects of dry mass on trace element tissue concentrations. PCA identifies a number of orthogonal axes in multivariate data sets and expresses these as linear combinations of the original variables; often a few axes account for the majority of the variation in the original data set [28]. Use of PCA as a preliminary data processing step offered two advantages. First, by including dry mass in the PCA we were able to identify an axis that summarized variation in trace element concentrations with size and a number of other axes that summarized variation in trace element concentrations independent of size. Second, because each axis is independent of the other axes by definition, our ANOVA models meet the assumption of independence of individual analyses. We used SAS® (SAS Institute, Cary, NC, USA) to conduct a PCA of the correlation matrix and estimate PCA scores for individual larvae and juveniles. To aid in interpretation of the resulting PCA axes, we used a varimax rotation of the original PCA axes. We used a scree plot to decide on the number of axes to include in ANOVA. A fully factorial two-way ANOVA model was used to test for differences among seasons and stages in PCA scores. When models indicated significant differences among stages, but no significant interaction of stage with season, we used Tukey's standardized range test to compare means among stages. All ANOVAs were conducted with SAS.

RESULTS

Mean dry mass of sampled larvae and metamorphs differed significantly among stages and seasons (two-way ANOVA; $p < 0.001$ in both cases), but differences among seasons were dependent on stage ($p = 0.009$ for the interaction term). As expected, the mean size of larvae was lowest for the NHL stage and highest for the LHL stage, with EHL stage larvae being intermediate in size (Fig. 1). Although larvae collected in the fall were slightly smaller than larvae collected in the spring, there were no significant differences between fall and spring in NHL, EHL, or LHL stage larval dry mass (*t*-tests with correction for heterogeneity of variance; $p > 0.301$). In contrast, juveniles resulting from metamorphosis of EFL larvae collected in the fall were significantly (*t*-tests with correction for heterogeneity of variance; $p = 0.004$) smaller than juveniles resulting from metamorphosis of EFL larvae collected in the spring.

Minimum, maximum, mean, and standard deviation of trace element concentration for all organisms analyzed are given in Table 1. With the exception of Zn, all trace element concentrations showed weak ($-0.26 > r < 0.27$) and insignificant correlations with dry mass (Table 2). Overall, Zn exhibited a significant negative correlation with dry mass (Table 2). Among individual trace elements, Al, V, Cr, Ni, Cu, As, and Pb concentrations showed strong significant positive correlations with each other ($r \geq 0.71$; Table 2). Among other trace elements, significant correlations were weaker (Table 2). Cadmium showed positive relationships with Ni, Cu, As, Pb, and Zn. Additionally, Zn concentrations were positively correlated with Cu and Cd. Strontium concentrations were negatively correlated with Al and Ni and positively correlated with Se concentrations.

Inspection of the scree plot of the eigenvalues from PCA of the dry mass and trace element tissue concentration correlation matrix suggested retention of seven PCA axes for further analysis. Eigenvalues on the first seven axes were

Table 2. Correlation of trace element concentrations in tissues of larva and recently metamorphosed juvenile bullfrog (*Rana catesbeiana*) from a South Carolina, USA pond contaminated with coal combustion waste. Numbers are Pearson's correlation coefficients and those in italics are significant ($p < 0.05$) after a sequential Bonferroni correction for multiple comparisons

	Dry mass	V	Al	As	Pb	Cr	Ni	Cu	Cd	Ag	Se	Sr
V	−0.15											
Al	−0.10	*0.95*										
As	−0.13	*0.93*	*0.90*									
Pb	−0.19	*0.91*	*0.92*	*0.87*								
Cr	0.27	*0.83*	*0.87*	*0.75*	*0.78*							
Ni	0.04	*0.88*	*0.90*	*0.83*	*0.85*	*0.77*						
Cu	−0.21	*0.71*	*0.68*	*0.73*	*0.71*	*0.61*	*0.70*					
Cd	−0.23	0.30	0.28	*0.40*	*0.35*	0.05	*0.40*	*0.56*				
Ag	−0.26	0.10	0.04	*0.47*	0.15	−0.09	0.13	0.33	0.13			
Se	−0.09	−0.12	−0.22	−0.02	−0.21	−0.10	−0.19	0.07	−0.06	0.25		
Sr	−0.20	−0.31	*−0.39*	−0.20	−0.32	−0.20	*−0.35*	0.03	−0.03	0.27	*0.63*	
Zn	*−0.65*	0.16	0.07	0.24	0.25	−0.10	0.12	*0.44*	*0.62*	0.24	0.25	0.27

Table 3. Loadings of trace elements on the first seven principle component axes resulting from principle component analysis of trace element concentrations in tissues of larva and recently metamorphosed juvenile bullfrogs (*Rana catsbeiana*) from a South Carolina, USA pond contaminated with coal combustion waste. Trace elements are ordered in the table to reflect the groups of metals discussed in the text. For clarity, only loadings >0.25 or <−0.25 are shown

	Factor						
Variable	I	II	III	IV	V	VI	VII
Dry mass		0.968					
V	0.971						
Al	0.966						
As	0.932						
Pb	0.920						
Cr	0.890						
Ni	0.878						
Cu	0.678						
Cd			0.880	0.290			
Ag				0.949			
Se					0.951	0.282	
Sr					0.360	0.892	
Zn		−0.416	0.335				0.778
% Of variance explained	44.2	9.6	8.8	8.6	8.6	7.6	6.0

>0.77, whereas eigenvalues on the remaining axes were <0.42. The first seven axes accounted for 93% of the variation in the data set. Loadings on PCA axis I indicated that this axis summarized the strong positive correlations among Al, V, Cr, Ni, Cu, As, and Pb (Table 3). Principle components analysis axis II was related to variation in dry mass and associated variation in Zn. Principle components analysis axes III through VII were related to variation in Cd, Ag, Se, Sr, and Zn, respectively, with low loadings of other trace elements reflective of weak correlations among concentrations of Cd, Se, Ag, Sr, Zn, and other elements.

Table 4. Results of two-way ANOVA of the effects of season of metamorphosis and developmental stage on principle components analysis (PCA) scores of individual larva and juvenile bullfrogs (*Rana catesbeiana*)

Source	*df*	MS[a]	*F*	*p*
PCA I (Al, V, Cr, Ni, Cu, As and Pb)				
Stage	3	22.28	179.61	<0.001
Season	1	2.97	23.92	<0.001
Stage × season	3	0.07	0.60	0.619
PCA III (Cd)				
Stage	3	1.22	1.81	0.152
Season	1	31.35	46.63	<0.001
Stage × season	3	0.56	0.84	0.477
PCA IV (Ag)				
Stage	3	0.12	0.12	0.948
Season	1	4.93	4.92	0.029
Stage × season	3	0.56	0.56	0.643
PCA V (Se)				
Stage	3	1.72	1.89	0.138
Season	1	8.39	9.21	0.003
Stage × season	3	1.33	1.46	0.232
PCA VI (Sr)				
Stage	3	1.47	1.87	0.141
Season	1	4.22	5.38	0.023
Stage × season	3	3.41	4.35	0.007
PCA VII (Zn)				
Stage	3	2.81	3.00	0.035
Season	1	0.65	0.69	0.408
Stage × season	3	0.62	0.66	0.581

[a] MS = Mean squares.

Mean scores on PCA axis I differed significantly among seasons and stages (Table 4). Principle components analysis axis I scores were higher for larvae and juveniles during the spring (Fig. 2), reflecting higher concentrations of Cr and Al in overwintered individuals (Fig. 3). Aluminum and Cr concentrations were 34 and 90% higher in the spring, respectively, whereas As, Ni, Cu, and Pb concentrations were <10% higher. Among larval stages, mean PCA I scores were similar and did not differ significantly ($p > 0.05$; Fig. 2). However, mean PCA I scores were significantly ($p < 0.05$) lower for metamorphs when compared with all larval stages (Fig. 2), reflecting decreases in Al, V, Cr, Ni, Cu, As, and Pb concentrations through metamorphosis (Fig. 3). Decreases in mean concentrations of these six elements through metamorphosis range from 40% for Cu to 97% for Al. We did not analyze PCA II scores because they were related mainly to size. Mean PCA III, IV, and V scores differed significantly among seasons, but not among stages (Table 4; Fig. 2). Higher PCA III and IV scores among individuals collected in the fall reflected 40 and 62% higher mean fall concentrations of Cd and Ag, respectively (Fig. 3). In contrast, mean PCA V scores were higher in the spring, reflecting 21% higher mean Se concentrations in the spring. Mean PCA VI scores varied significantly among seasons, but this variation was dependent on stage (i.e., there was a significant stage × season interaction; Table 4). Mean PCA VI scores were higher during the spring for all larval stages, but lower in the spring among metamorphs (Fig. 2), reflecting differences in mean Sr concentrations among seasons and stages. Concentrations of Sr were higher in larval stages during the spring, but because concentrations of Sr increased 122% through metamorphosis in the fall, concentrations among metamorphs were higher in the fall (Fig. 3). Finally, mean PCA VII scores varied significantly among stages, but not among seasons (Table 4). In general, mean PCA VII scores declined from the NHL stage through the LHL stage and then increased again through metamorphosis (Fig. 2), reflecting changes in Zn concentrations independent of dry mass (Fig. 3).

DISCUSSION

At metamorphosis, bullfrog larvae (and most anurans) cease eating as their bodies undergo massive reconstruction from an

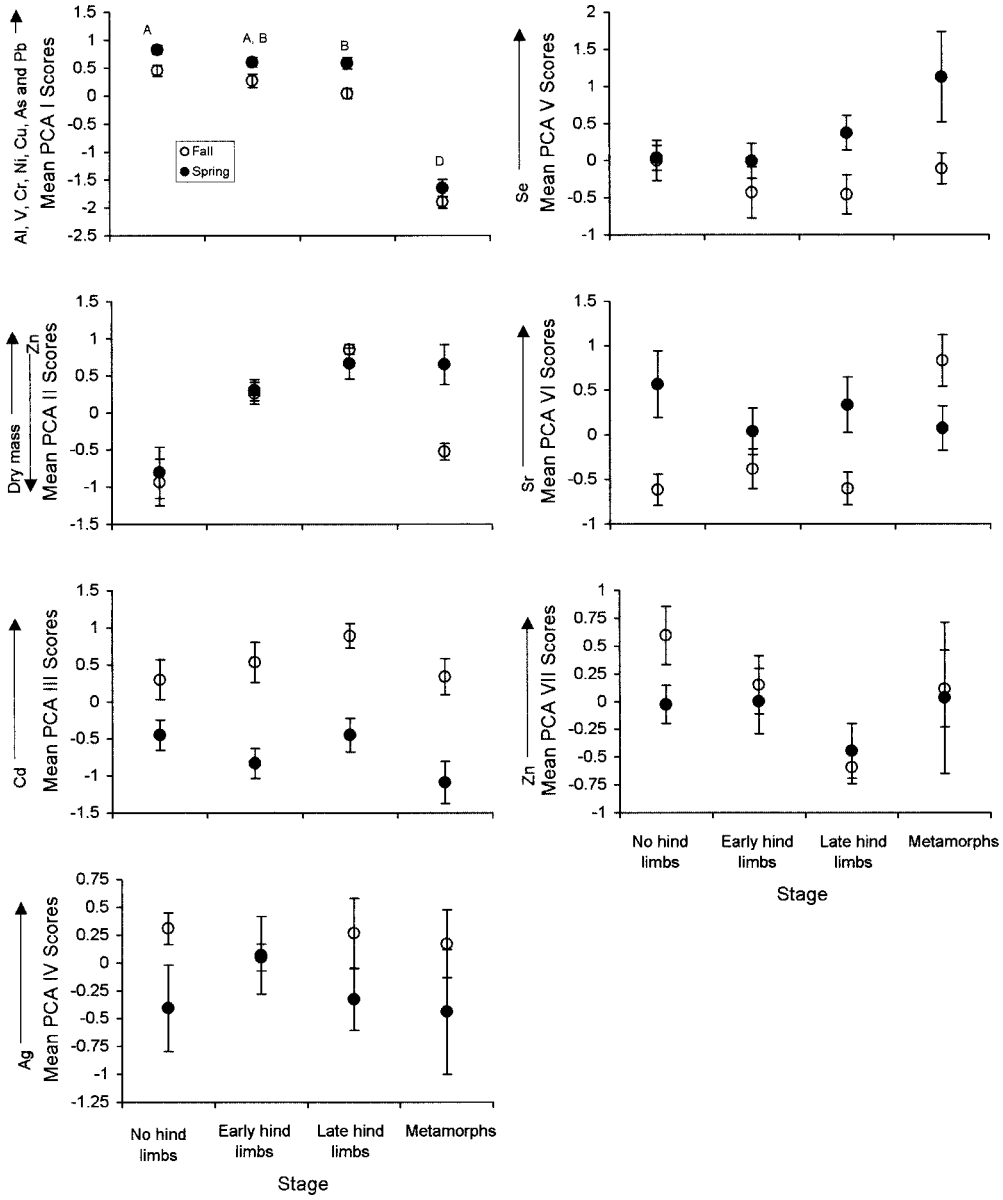

Fig. 2. Changes in bullfrog trace element principle components analysis (PCA) scores among developmental stage for larvae metamorphosing in the fall (open circles) and spring (closed circles). Error bars are ±1 SEM. Means with different letters above them are significantly different (Tukey's post hoc comparison; $p < 0.05$). In the plot with no letters there was not a significant effect ($p > 0.05$) of stage on PCA scores.

aquatic larva to a semiaquatic metamorph [25]. During this morphological reconstruction, trace elements bioaccumulated during the larval period can either be eliminated or retained and redistributed throughout the metamorph's tissues. Moreover, those elements that are retained may become concentrated in tissues because of mass loss resulting from lack of feeding and high energetic costs associated with metamorphosis [29,30]. Among the trace elements we investigated, we observed different patterns in trace element retention and elimination through metamorphosis as suggested by differences in average tissue trace element concentration between LHL stage larvae and metamorphs. Whole-body concentrations of V, Al, As, Pb, Cr, and Ni drop substantially through metamorphosis; in all cases, concentrations in recent metamorphs were 3 to 38 times lower than concentrations in LHL stage larvae (just before metamorphosis). Copper concentrations also dropped through metamorphosis, but only declined by approximately 65%. In contrast, tissue concentrations of Cd, Ag, Se, and Zn varied little among stages. Tissue concentrations of Sr were similar among stages for larvae and recent metamorphs in the spring, but were greater than two times higher among fall metamorphs when compared with LHL larvae in the same season.

Because of the extensive changes in morphology and physiology of anurans during metamorphosis, a number of processes may have contributed to differences among trace element elimination through metamorphosis. Possible elimination routes include excretion of bile from the gall bladder, secretion from the hepatopancreas, intestinal mucosa and kidneys, and shedding of apoptotic cells. Shedding of degenerated cells occurs during tail absorption [31] and remodeling of the intestinal epithelium [32]. Remodeling of the intestines may be particularly important as some elements tend to bioaccumulate in the intestine to a greater extent when compared with other body parts or organs of larvae [22,23]. For bullfrogs, Burger and Snodgrass [22] reported digestive tract tissue concentra-

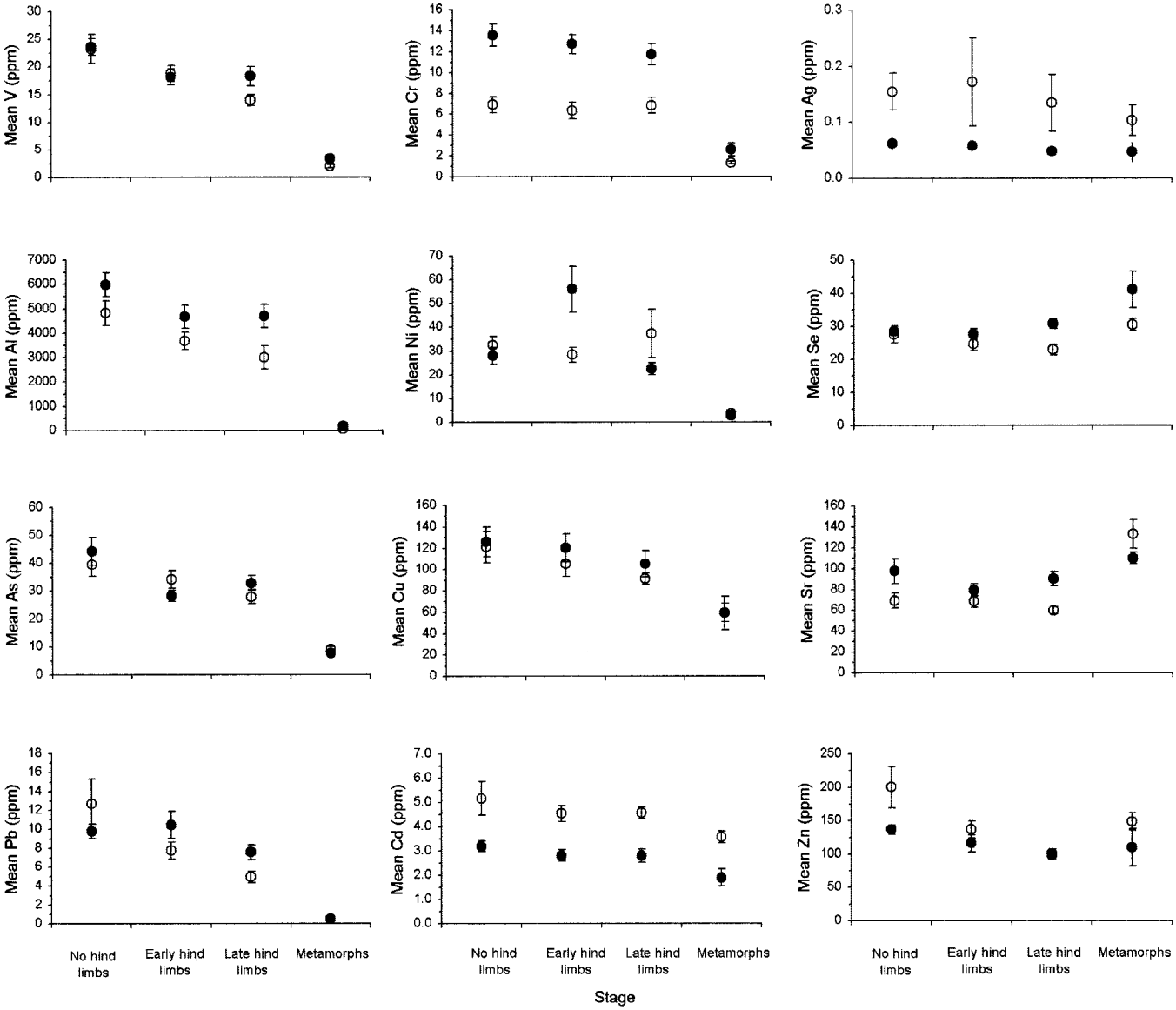

Fig. 3. Changes in trace element concentrations among stages for larvae metamorphosing in the fall (open circles) and spring (closed circles). Error bars are ±1 SE.

tion factors (calculated as concentration in specific tissues divided by total body burden) of 8.87, 7.75, and 5.56 for Pb, Cr, and As, respectively. Concordantly, we observed substantial decreases in concentrations of Pb, Cr, and As through metamorphosis. In contrast, among the larval bullfrogs studied by Burger and Snodgrass, digestive tract tissue concentration factors for Cd and Se were 4.69 and 2.72, respectively. We observed only a slight decrease in Cd concentrations and a slight increase in Se concentrations through metamorphosis (Fig. 3). These comparisons suggest that trace elements that are concentrated mainly in the gut are more likely to be eliminated during metamorphosis.

Although trace elements associated with the digestive tract are likely eliminated during metamorphosis due to restructuring of the digestive system, it remains unclear where in the digestive tract these elements are sequestered during the larval period. The duration of our depuration period was selected to maximize clearing of the digestive tract while minimizing changes in concentration of trace elements in other body compartments resulting from weight loss [22]. Small amounts of sediment (i.e., several grains) sometimes remain in the digestive tract of tadpoles even after a 48 h depuration period (J.W. Snodgrass and W.A. Hopkins, personal observation). Thus it is possible that some trace elements associated with remaining sediments are included in body measures of trace element concentration. However, Sparling and Lowe [33] reported significantly higher concentrations of a number of trace elements (including Be, Cd, Cu, Fe, Mg, Mn, Ni, Sr, V, and Zn) in the digestive tract of green frog (*Rana clamitans*) larvae when compared with sediments at the site of collection, suggesting some elements may be bound to or incorporated into cells and tissues of the digestive tract. Thus the role of the digestive tract of larval anurans in sequestering trace elements and affecting incorporation into other body parts of both larvae and juveniles warrants further investigation.

Ultimately, the relative bioactivities of trace elements are determined by ionic characteristics. For example, the ionic characteristics of trace elements are often associated with their relative toxicity (e.g., [34–36]). Specifically, characteristics such as ion electronegativity, absolute value of the first hy-

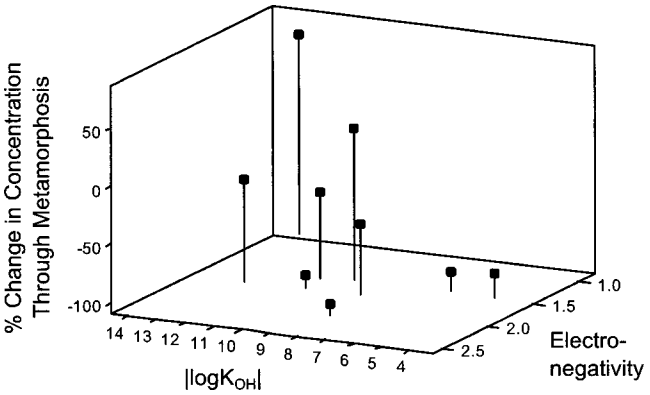

Fig. 4. Relationship between percent change in trace element concentration through metamorphosis and ion electronegativity and the first hydrolysis constant ($|\log K_{OH}|$). Values of ion characteristics are from McCloskey et al. [32]. Only those elements for which values were available are plotted.

drolysis constant ($|\log K_{OH}|$), and the softness index (i.e., [coordinate bond energy of the metal fluoride − coordinate bond energy of the metal iodide] / coordinate bond energy of the metal fluoride) are associated with relative toxicity of trace element ions. These properties are believed to reflect the tendency of ions to bind to biomolecules, which ultimately influences their bioaccumulation and toxic potential. Moreover, ionic properties may influence where specific elements are sequestered within the organism, which in turn may interact with changes in organ systems during metamorphosis in determining which elements are eliminated and which are retained in the resulting juvenile form. Although a formal investigation of relationships between ion characteristics and trace element concentrations in larvae is beyond the scope of this investigation, a plot of percent change in trace element concentration through metamorphosis against ion electronegativity and $|\log K_{OH}|$ (Fig. 4) suggests ion characteristics play a role in determining the fate of trace elements during bullfrog metamorphosis. Figure 4 suggests that elements such as Sr and Zn, with relatively low electronegativity and high $|\log K_{OH}|$ values, are retained through metamorphosis, whereas elements such as Cr and Pb, with the opposite characteristics, are lost. These relationships suggest that ions with a high affinity for intermediate and soft ligands are more likely to be retained through metamorphosis. Although it remains unclear if these specific properties are involved, or if other correlated properties are responsible, the ability of Se and Sr to replace S and Ca, respectively, in biological molecules [37,38] appears to result in retention of these trace elements through metamorphosis. Selenium and strontium were the only two trace elements we measured that exhibited their highest mean concentrations in metamorphs (Fig. 3).

We also observed differences in element concentration between seasons, but again the direction of these differences varied by element. Tissue concentrations of Al, Se, and Cr were higher among larval stages during the spring. In contrast, tissue concentrations of Cd and Ag were higher among all stages during the fall (Fig. 3). Additionally, differences among seasons in Sr concentrations were dependent on stage (as discussed previously). An increase from fall to spring may be interpreted as a result of longer exposure time for larvae that overwinter. Because the bullfrog larvae rarely overwinter more than one year in our study area, a maximum estimate of exposure for fall metamorphs is approximately four to seven months if eggs were laid in early May (earliest month of breeding in our study area), and we collected resulting EFL-stage larvae between September and December. A minimum exposure duration estimate for spring metamorphs is approximately nine months if eggs were laid in August (the last month of breeding in our study area), and we collected the resulting EFL larvae in May. These estimates suggest that at a minimum spring metamorphs were exposed approximately two months longer than fall metamorphs. However, most elements showed no consistent difference in concentrations between seasons, and some (Cd and Ag) even decreased from fall to spring. Therefore, differences in the types and quality of resources, environmental conditions between seasons, or other factors affecting bioavailability may be at least partially responsible for the difference we observed in tissue concentrations of Cd and Ag. Water concentrations of trace elements in the contaminated system we studied are generally low, indicating that the majority of trace elements are accumulated via ingestion of contaminated food and sediments [17,21]. Since uptake of trace elements by organisms in this system is likely linked to food ingested, bioavailability of elements to consumers is at least partially determined by the types and quantities of resources available. Resources in aquatic communities can differ dramatically among seasons, and thus the quantities and chemical species of trace elements consumed may differ as well. Consumption of resources is determined by larval physiology and behavior, which also likely vary seasonally due to differences in temperature, dissolved oxygen, and predation pressure. For example, the ability of larvae to sequester or excrete elements may differ due to seasonal variation in environmental factors (e.g., temperature [39,40]) that affect larval physiology. Future studies that examine seasonal differences in bioavailability of trace elements and the effects of differences in food resources and physical environments on toxico-kinetics will be of value for predicting risk to overwintering individuals as well as to species that breed at different times of the year.

Elimination or retention of trace elements through metamorphosis not only has important implications for the health of recent metamorphs, but also determines the quantities of contaminants leaving the aquatic environment and entering terrestrial food webs. Juvenile and adult amphibians are often abundant in aquatic and terrestrial systems and are important prey items in the diets of birds and reptiles. Because body burdens of trace elements acquired during the aquatic larval phase, such as Se and Sr, are retained through metamorphosis, they may be transferred into terrestrial food webs from wetland systems where they are sequestered. Thus studies are needed to gain a fundamental understanding of how variations in autecology and life stage of amphibians influence trace element accumulation and elimination, and ultimately determine rates of transfer of pollutants from wetland systems to terrestrial food webs. Our results provide a first step in addressing these questions, and laboratory experiments that control exposure conditions and duration will be a valuable next step.

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