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EFFECTS OF CHRONIC DIETARY EXPOSURE TO TRACE ELEMENTS ON BANDED WATER SNAKES (*NERODIA FASCIATA*)

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Abstract—Little currently is known about the accumulation or effects of contaminants on reptiles. To date, most studies examining reptile exposure to trace elements report tissue burdens of field-captured animals, but seldom provide insight into the dose, duration, or mode of exposure involved. For two years, we fed juvenile banded water snakes (*Nerodia fasciata*) prey items collected from a coal ash–contaminated site that contained elevated levels of As, Cd, Cu, Se, Sr, and V. With the exception of Cu, snakes accumulated significant concentrations of elements, usually in a dose-dependent manner. Accumulation varied significantly among liver, kidney, and gonads, and in most cases between sexes. Selenium accumulation was most notable, greatly exceeding established toxicity thresholds for other vertebrates. Despite the high concentrations of pollutants accumulated, snakes exposed to the contaminated diet survived through the study and exhibited normal food consumption, growth, condition factor, overwinter survival and mass loss, metabolic rate, and gonadosomatic index. The results of this study confirm that diet can be a significant route of exposure to trace elements in snakes and indicate that further studies on snakes are warranted to better understand their responses to contaminants.

Keywords—Reptiles Snakes Trophic transfer Coal combustion Selenium

INTRODUCTION

Currently, much less is known about the accumulation and effects of contaminants in reptiles than in any other vertebrate class, making prediction of contaminant impacts on reptiles extremely difficult [1,2]. Risk predictions based on toxicity thresholds established for other vertebrates (e.g., birds and fish) may be inappropriate for many reptiles because of their unique combinations of physiological and life history characteristics (e.g., long life span, relatively small home ranges, high trophic position, and ectothermic physiology) [1,2]. Thus, it is imperative that basic toxicological information be collected for reptiles so that adequate risk assessments and conservation efforts can be initiated in the future.

Recent recognition that reptiles are grossly underrepresented in ecotoxicology, in addition to the fact that many reptile populations are declining [3], has prompted several reviews that identify immediate research needs in reptile ecotoxicology [1,2,4–7]. From these reviews, a clear consensus exists that among reptiles, turtles have received far greater attention than crocodilians and squamates (lizards and snakes). Of the few studies that have been performed on squamates, most have focused on organic contaminants [4,5]. The majority of studies on inorganic contaminants only report tissue residues in field-captured squamates, providing little information pertaining to biological effects or routes of exposure [6,8]. To date, only five studies have examined lethal or sublethal effects of inorganic contaminants on lizards and snakes [4,5,8–10].

The current study was initiated to study the accumulation and effects of inorganic contaminants on squamate reptiles under captive conditions that enabled us to control dose, duration, and mode of contaminant exposure. Specifically, we sought to expand upon two recent studies that examined accumulation and effects of trace elements on the banded water snake (*Nerodia fasciata*) [8,10]. The first of our previous studies indicated that water snakes inhabiting a coal ash disposal site had extremely high tissue burdens of Se, As, and Cd. Moreover, snakes with high tissue burdens exhibited increased standard metabolic rates (SMRs), possibly indicating that a significant physiological cost was associated with contaminant exposure [8]. In a follow-up laboratory study, we found that contaminated prey items were an important route of trace element exposure for water snakes. Snakes fed contaminated prey for one year accumulated significant quantities of As, Cd, Se, Sr, and V in their kidneys, liver, and gonads [10]. However, neither of our previous studies was designed to rigorously examine the physiological effects of chronic dietary exposure to trace elements. Therefore, in the current study we fed contaminated prey to juvenile water snakes for two years and measured trace element accumulation as well as survival, food consumption, growth, metabolic rate, condition factor, overwinter survival and mass loss, and gonadosomatic index.

MATERIALS AND METHODS

Snake collection and preexposure husbandry

To minimize genetic variation in accumulation rates and responses to contaminant exposure, we utilized a full-sibling study design. A single gravid female banded water snake (postparturition mass = 593 g) was collected from a reference site on August 13, 1997, on the Savannah River Site (SC, USA). The collection site was the same site where our previous field-

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work with water snakes was conducted (series of wetlands along Risher Pond Road); snakes and their prey at this site have very low tissue concentrations of coal ash–related trace elements [8]. The gravid female was housed in a 65-L glass tank until August 24, 1997, when she gave birth to 44 offspring. The adult female was then released at the site of collection. The mass (mean ± standard error = 4.94 ± 0.06 g), snout–vent length (SVL; mean = 16.64 ± 0.09 cm), and sex (25 females and 19 males) of each neonate was determined and each snake was assigned a unique identification number. Each neonate was then housed individually in a 5-L plastic container with a hide box and water bowl. Because snakes rely on behavioral thermoregulation, each container was partially placed on Flexwatt Heat tape (Flexwatt, West Wareham, MA, USA) to provide a thermal gradient. Snakes were housed in a temperature-controlled greenhouse (25 ± 2°C) located at the Aquatic Ecology Laboratory, a satellite facility of the Savannah River Ecology Laboratory near Aiken (SC, USA). Because snakes were housed in a greenhouse, they were exposed to the natural photoperiod during the study.

A six-month holding period was used to accustom snakes to the feeding regime and overwintering techniques. Two weeks after birth, neonates began receiving weekly rations of live mosquitofish (*Gambusia holbrooki*) and tadpoles (*Rana* spp.) that were collected from the reference site. After several weeks, previously frozen prey items were substituted for live prey. Most snakes readily ate dead prey items once they became accustomed to ingesting prey from their water bowl. In November 1997, snakes were fasted for three weeks before being placed in a dark environmental chamber for overwintering. Temperature in the chamber was decreased weekly in 2 to 3°C increments until reaching 6°C. Snakes were held at 6°C for several weeks, after which time the temperature was increased weekly in 3 to 5°C increments until reaching 25°C. After their first overwintering, snakes were returned to the greenhouse and housed individually in 38-L aquaria equipped with a hide box, water bowl, and heat source. Snakes that did not readily eat dead prey after overwintering or that appeared ill were excluded from the study. All healthy but uncooperative snakes not used in the study were released at the parental site.

Study design

On February 22, 1998, all snakes were measured (mass and SVL) and then arbitrarily assigned to one of three feeding treatments. Arbitrary treatment assignment was conducted by placing males and females in separate plastic containers and then blindly drawing snakes from the containers for each treatment. Snakes were fed weekly combinations of previously frozen fish from a reference site (Fire Pond, Savannah River Site) and a coal ash–contaminated site to create three levels of trace element exposure. A control group of snakes (n = 10) was fed only fish from the reference site. A second group of snakes (n =10) served as an intermediate-exposure treatment group (treatment 1) and was fed fish from the reference site and the coal ash–contaminated site on alternating weeks. Finally, a third group of snakes served as a high-exposure treatment group (n =10) and was fed only fish from the contaminated site (treatment 2). Each treatment group was composed of six females and four males.

All prey used in the study were captured in minnow traps, immediately placed on ice, and stored frozen for future snake feedings. Prey items included largemouth bass (*Micropterus salmoides*), bluegill sunfish (*Lepomis macrochirus*), spotted sunfish (*L. punctatus*), redbreast sunfish (*L. auritus*), dollar sunfish (*L. marginatus*), and mosquitofish. The sites where prey were collected have been the focus of much of our research, and trace element concentrations in prey items fed to snakes were recently published elsewhere [10]. Geometric least squared means of dietary trace element concentrations were calculated from representative samples of the primary prey species (n = 4/species) and are presented in Table 1. During the first and second year of the study, each snake was offered weekly prey rations of 4.55 ± 0.33 and 6.12 ± 0.20 g, respectively. Ration size for year 1 was based upon our observations of snake food consumption during the previous acclimation period. In year 2, ration size was increased by approximately 35% to accommodate snake growth. Weekly rations consisted of two to five prey items to attain the target ration mass. All snakes received a total of 67 meals of their respective food type, with snakes in the intermediate exposure receiving 32 of their meals from the contaminated site.

Table 1. Trace element concentrations (μg/g dry mass) in the experimental diet of water snakes (*Nerodia fasciata*). Geometric least squared mean is based on three prey species (n = 4/species). Each snake received 67 meals during the study. Mean concentrations and ranges for control and treatment 2 snakes were previously published in Hopkins et al. [10]. Dietary concentrations for treatment 1 snakes represent intermediate concentrations, because treatment 1 snakes received 32 contaminated and 35 uncontaminated meals (see description in text)

	Geometric least squared mean		
Element	Control	Treatment 1	Treatment 2
As	0.155	0.693	1.281
Cd	0.051	0.135	0.227
Cu	1.738	2.053	2.398
Se	0.994	11.361	22.700
Sr	128.085	198.250	274.992
V	0.163	0.938	1.786

Biological endpoints

During the study, snakes were inspected 3 to 4 d each week to monitor food consumption and to document mortality. Because prey became saturated with water after being placed in the snakes' water bowls, mass of uneaten food could not be accurately determined. However, the number of uneaten prey items was recorded every week. Growth (SVL and mass) of each snake was measured at two- to four-month intervals.

At four intervals during the study (beginning of experiment, +7.5 months, +17 months, and end of experiment) we estimated the SMR of each snake. Before metabolic rate measurements, each snake was held unfed for 7 to 10 d. Individual snakes were then placed in 1- to 4-L flasks (depending on the size of the snake) and walls of flasks were covered to prevent visual disturbances. Flasks were placed in an environmental chamber (25°C) and connected to individual channels on a computer-controlled, indirect, closed-circuit respirometer (Micro-Oxymax, Columbus Instruments, Columbus, OH, USA). The first channel for each trial was connected to a flask containing an 8.4-V battery (Procell Zinc Air Medical Battery, DA146, Duracell, Bethel, CT, USA) that consumed a known amount of oxygen per minute. Oxygen consumption for each snake was determined at 1- to 2-h intervals for at least 24 h. Because SMR is a measure of a postabsorptive ectotherm's metabolic rate at rest, the highest 50% of O_2 consumption values were removed to minimize the effect of unobserved

periods of activity on our estimates of SMR [8,11]. Standard metabolic rate of each snake was then estimated as the mean O_2 consumption rate from the lower 50% of measurements for each individual.

Because overwintering is a stressful and energetically demanding period for vertebrates, we examined overwinter survival and mass loss during two artificial hibernation periods in the laboratory. In November of each year, food was withheld from all snakes for three to four weeks before transfer to an environmental chamber. Each snake was then placed in an individual plastic container and hibernation was induced as described above. Snakes were inspected every two to three weeks to document mortality. Percent overwinter mass loss was calculated as overwinter mass loss divided by prehibernation mass multiplied by 100.

Snakes ingested their final rations at the beginning of April 2000. Seven days after this meal, final SMR was determined for each snake. Snakes were then euthanized with an overdose of anesthesia-grade ether. Final mass and SVL was determined for each snake before being dissected. Gonads, kidneys, and liver were removed from each snake. Gonads were weighed to determine gonadosomatic index (wet mass of gonad/wet mass of whole snake). A small portion of each organ was preserved for histological analysis; histological results will be reported elsewhere. The remaining portion of each organ was used for determination of trace element content.

Trace element analysis

Snake tissues were lyophilized and homogenized before being digested and analyzed for trace element concentrations in the analytical facility at the Savannah River Ecology Laboratory. Approximately 10 to 250 mg of sample was used for digestion; sample masses varied because large differences existed in organ dry masses. Nitric acid (1.0–2.5 ml) was added to samples before digestion in a microwave (CEM, Matthews, NC, USA) with heating steps of 60, 60, 70, and 80% microwave power for 10, 10, 15, and 20 min, respectively. After digestion with HNO_3, 0.25 to 0.5 ml of H_2O_2 was added to the samples and microwaved at the same power and duration as the HNO_3 digestion. After digestion, samples were brought to a final volume of 5 or 10 ml (depending on mass of the tissue) with double distilled water. Trace element analysis was performed by inductively coupled plasma–mass spectrometry (Perkin-Elmer, Norwalk, CT, USA) on samples diluted 1:1 (v/v) with double distilled water. Calibration standards covering a range of 1 to 500 μg/L were prepared daily by serial dilution of National Institute of Standards and Technology (Gaithersburg, MD, USA) traceable primary standards. Certified reference material (Tort 2, National Research Council of Canada, Ottawa, ON) and blanks were included in the digestion and analysis procedure for quality control purposes. Mean percent recoveries for trace elements in certified reference materials ranged from 97.01 to 111.75%.

Detection limits (μg/g dry mass) for trace elements varied depending on the tissue being analyzed. Mean instrument detection limits for As, Cd, Cu, Se, Sr, and V in liver were 0.026, 0.002, 0.010, 0.117, 0.002, and 0.017 μg/g, respectively. Mean detection limits for As, Cd, Cu, Se, Sr, and V in kidney were 0.044, 0.003, 0.018, 0.199, 0.003, and 0.029 μg/g, respectively. Because gonads of males and females differed substantially in mass, detection limits are provided independently for each sex. Mean detection limits for As, Cd, Cu, Se, Sr, and V in testes were 0.228, 0.017, 0.091, 1.033, 0.016, and 0.153 μg/g, respectively. Mean detection limits for As, Cd, Cu, Se, Sr, and V in ovaries were 0.061, 0.004, 0.024, 0.275, 0.004, and 0.041 μg/g, respectively.

Statistical analysis

A fully factorial multivariate analysis of variance was used to test for effects of food treatment, sex, and organ on tissue element concentrations. We log10-transformed tissue element concentrations before analysis to more closely approximate the assumptions of the model. Pillai's trace statistic was used to test the null hypothesis of no treatment, sex, or organ effects. For illustrative purposes, we then conducted individual three-way analyses of variance (ANOVAs) to examine the effect of food treatment, organ, and sex on accumulation of each trace element.

We examined the effects of food treatment on survival as well as six sublethal responses: food consumption, growth, condition factor, gonadosomatic index, overwinter mass loss, and SMR. Because survival rates were high among all treatments, we used a Fisher's exact test to compare survival among feeding treatments. Log-linear and maximum-likelihood estimates were used to compare the percentage of prey items eaten among food treatments and sexes. A two-way repeated-measures ANOVA was used to determine the effect of food treatment and sex on changes in snake SVL, mass, percent overwinter mass loss, and SMR over time. Mass, SVL, and SMR were $\log_{10}$ transformed before statistical analysis to better approximate assumptions of each model. For illustrative purposes, we also calculated percent increase in mass and SVL based on initial and final measurements of body size. To remove the effects of mass on SMR, log10-transformed oxygen consumption values were regressed against mass ($R^2 = 0.72$, $p < 0.001$). Residuals obtained from the regression model then were used as the dependent variable in the repeated-measures ANOVA model; inspection of the residual plot indicated a linear relationship between log10-transformed oxygen consumption and mass.

We used a two-way analysis of covariance to test for effects of food treatment and sex on the relationship between final mass and SVL (i.e., snake condition factor), and between final mass and gonad mass (i.e., gonadosomatic index). Our comparisons by two-way analysis of covariance are analogous to traditional comparisons of power functions among food treatments [12]. For the model investigating the relationship between final mass and SVL, SVL was included as a covariate. For the model investigating the relationship between final mass and gonad mass, final snake mass was included as a covariate. Before analysis, gonad mass and SVL were $\log_{10}$ transformed to increase linearity.

RESULTS

Snakes fed contaminated food accumulated significant quantities of all trace elements except Cu, with As, Cd, and Se exhibiting 10- to 20-fold increases above control levels in some organs (Fig. 1). Results of the multivariate analysis of variance indicated that significant interactions occurred between organ effects and both treatment and sex on trace element accumulation (Table 2). Individual ANOVAs indicated similar patterns among those elements that showed significant accumulation (Table 3). In many cases, the accumulation of trace elements in organs was linearly related to dose (Fig. 1). For As, Cd, and V, liver accumulated the highest levels, followed by lower rates of accumulation in the kidney and no

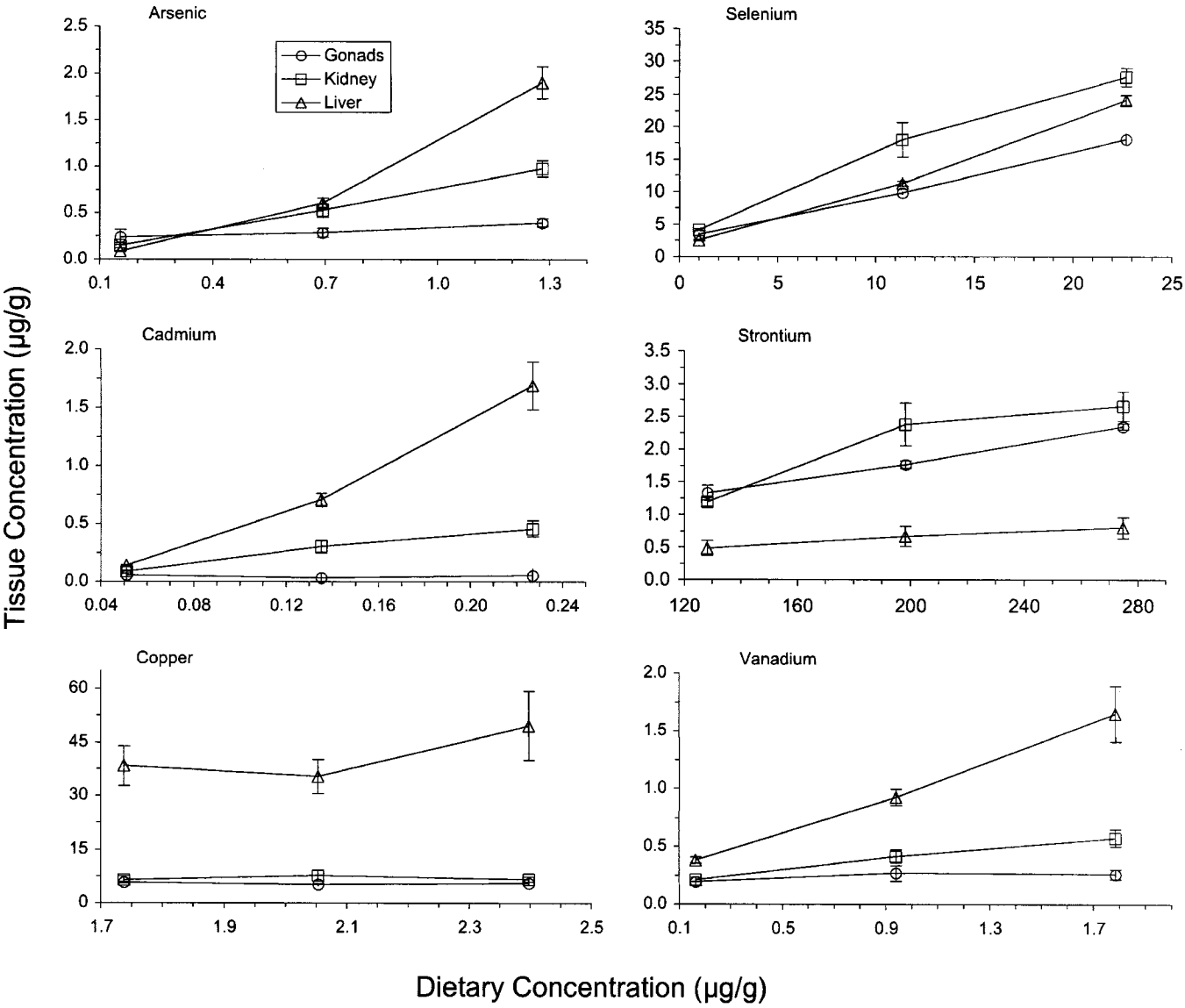

Fig. 1. Mean trace element accumulation in organs (µg/g dry mass) of banded water snakes (*Nerodia fasciata*) fed fish containing different dietary concentrations of contaminants for two years. Dietary concentrations (µg/g dry mass) are based on trace element values of prey reported in Hopkins et al. [10]. Means are based on males and females within each of the three treatment groups (control, treatment 1 [intermediate dose], treatment 2 [high dose]). Error bars represent ± 1 standard error of mean.

Table 2. Results of multivariate analysis of variance of the effects of organ, food treatment, and sex on the concentration of six trace elements in snake tissues[a]

Source[b]	Pillai's trace	*df*	*F*	*p*
Organ	1.6806	12, 130	57.01	<0.001
Treatment	1.0073	12, 130	10.99	<0.001
Organ × treatment	0.7282	24, 268	2.49	<0.001
Sex	0.1204	6, 64	1.46	0.206
Organ × sex	0.7196	12, 130	6.09	<0.001
Treatment × sex	0.1739	12, 130	1.03	0.424
Organ × treatment × sex	0.4045	24, 268	1.26	0.194

[a] *df* = degrees of freedom; *F* = *F* statistic.
[b] The times symbol (×) indicates the interaction between the two main effects in the statistical model.

accumulation in gonads. Selenium was accumulated in all organs, with kidney accumulating the highest levels. In contrast to the other elements, Sr was accumulated to a lesser extent, with the highest levels of accumulation occurring in the kidney and gonads. In general, differences in accumulation between sexes involved higher levels of accumulation of As, Cd, and V in the kidneys of females, whereas males accumulated higher levels of Se and Sr in their kidneys (Table 4). Additionally, males accumulated higher gonadal As and V levels than did females. Significant accumulation of elements in liver was similar in males and females (Table 4). A notable exception to this trend was Cu, which was consistently higher in the liver of females but did not differ statistically between sexes.

Survivorship did not differ among experimental treatments ($p = 0.99$). Only one snake died over the course of the two-year study; one male in treatment 2 stopped eating after the third week of the study and died the following week. Treatment had a significant effect on changes in SVL and mass over the course of the study, but the effects of treatment varied sig-

Table 3. Results of three-way analysis of variance of the effects of food treatment, organ, and sex on individual element concentrations in snake tissues[a]

Element	Source[b]	*df*	MS	*F*	*p*
As	Organ	2	0.3800	11.06	<0.001
	Treatment	2	4.8410	140.92	<0.001
	Organ × treatment	4	0.5340	15.54	<0.001
	Sex	1	0.0760	2.22	0.141
	Organ × sex	2	0.1280	3.72	0.029
	Treatment × sex	2	0.0160	0.46	0.633
	Organ × treatment × sex	4	0.0470	1.36	0.258
	Error	69	0.0344		
Cd	Organ	2	11.8050	120.32	<0.001
	Treatment	2	2.8610	29.16	<0.001
	Organ × treatment	4	0.4540	4.63	0.002
	Sex	1	0.0470	0.48	0.492
	Organ × sex	2	0.9580	9.77	<0.001
	Treatment × sex	2	0.0620	0.63	0.536
	Organ × treatment × sex	4	0.0290	0.29	0.882
	Error	69	0.0981		
Cu	Organ	2	5.7940	82.81	<0.001
	Treatment	2	0.0250	0.36	0.699
	Organ × treatment	4	0.0050	0.08	0.989
	Sex	1	0.0770	1.10	0.299
	Organ × sex	2	0.1350	1.93	0.153
	Treatment × sex	2	0.0250	0.35	0.704
	Organ × treatment × sex	4	0.0520	0.75	0.561
	Error	69	0.0700		
Se	Organ	2	0.2650	50.42	<0.001
	Treatment	2	5.1920	987.40	<0.001
	Organ × treatment	4	0.0420	8.05	<0.001
	Sex	1	0.0010	0.10	0.756
	Organ × sex	2	0.0160	3.14	0.050
	Treatment × sex	2	0.0120	2.31	0.107
	Organ × treatment × sex	4	0.0000	0.09	0.985
	Error	69	0.0053		
Sr	Organ	2	2.3010	187.50	<0.001
	Treatment	2	0.5780	47.10	<0.001
	Organ × treatment	4	0.0180	1.48	0.219
	Sex	1	0.0010	0.12	0.734
	Organ × sex	2	0.1360	11.04	<0.001
	Treatment × sex	2	0.0010	0.08	0.920
	Organ × treatment × sex	4	0.0090	0.74	0.567
	Error	69	0.0123		
V	Organ	2	2.1970	77.36	<0.001
	Treatment	2	1.1060	38.94	<0.001
	Organ × treatment	4	0.0900	3.14	0.020
	Sex	1	0.1580	5.56	0.021
	Organ × sex	2	0.3120	11.00	<0.001
	Treatment × sex	2	0.0190	0.68	0.509
	Organ × treatment × sex	4	0.1260	4.43	0.003
	Error	69	0.0284		

[a] *df* = degrees of freedom; MS = mean square; *F* = *F* statistic.
[b] The times symbol (×) indicates the interaction between the two main effects in the statistical model.

nificantly between sexes (Table 5 and Fig. 2). Inspection of percent change in SVL and mass during the study suggests that male control snakes grew less than males in both treatment groups (Fig. 3). In contrast, females in treatment 1 grew the most, whereas females in the control group and in treatment 2 grew to similar sizes (Fig. 3). Prey consumption rates varied significantly among treatments ($p < 0.001$), but variation among treatments was dependent on sex ($p < 0.001$). In general, males consumed a lower percentage of their prey items than females regardless of their treatment group. For both males and females, differences in consumption rates among food treatments were similar to differences in growth rates among food treatments (Fig. 3). Snout–vent length and mass were positively correlated ($F_{1,66} = 31.96$, $p < 0.001$); however, no significant effect of sex or treatment was found on the relationship between mass and SVL (i.e., snake condition; in both cases $p > 0.32$).

Although a significant treatment effect was found on SMR ($F_{2,22} = 3.74$, $p = 0.040$), it related to initial differences in oxygen consumption among food treatment groups that were maintained throughout the experiment (beginning mean SMR: control, 0.119 ml/g/h; treatment 1, 0.105 ml/g/h; treatment 2, 0.121 ml/g/h; vs final mean SMR: control, 0.032 ml/g/h; treatment 1, 0.030 ml/g/h; treatment 2, 0.037 ml/g/h). As expected, a significant effect also was found of time on oxygen consumption ($F_{3,66} = 7.47$, $p < 0.001$); as snakes grew during the study their metabolic rates (per gram of tissue) decreased.

No snakes died during the two overwinter periods. Mean

Table 4. Trace element concentrations for different food treatment, sexes, and organs of banded water snakes (*Nerodia fasciata*). Tissue concentrations are presented as mean (± standard error) μg/g dry mass

		Control		Treatment 1		Treatment 2	
Element	Organ	Female	Male	Female	Male	Female	Male
As	Gonads	0.182 (0.069)	0.325 (0.188)	0.197 (0.007)	0.415 (0.082)	0.335 (0.020)	0.520 (0.107)
	Kidney	0.117 (0.015)	0.198 (0.067)	0.615 (0.091)	0.401 (0.089)	1.055 (0.117)	0.817 (0.065)
	Liver	0.092 (0.011)	0.087 (0.013)	0.623 (0.080)	0.585 (0.069)	1.851 (0.242)	2.010 (0.226)
Cd	Gonads	0.012 (0.003)	0.122 (0.096)	0.026 (0.012)	0.041 (0.009)	0.055 (0.038)	0.059 (0.010)
	Kidney	0.082 (0.005)	0.090 (0.038)	0.398 (0.062)	0.169 (0.043)	0.573 (0.058)	0.234 (0.043)
	Liver	0.148 (0.010)	0.128 (0.010)	0.695 (0.067)	0.723 (0.111)	1.718 (0.149)	1.625 (0.622)
Cu	Gonads	5.613 (0.247)	5.699 (0.398)	5.400 (0.393)	4.695 (0.192)	5.570 (0.458)	5.299 (0.584)
	Kidney	6.009 (1.596)	7.124 (0.166)	7.768 (1.583)	7.269 (3.168)	6.475 (1.105)	6.777 (0.428)
	Liver	46.237 (7.192)	26.356 (5.598)	39.164 (6.995)	29.567 (5.710)	60.475 (12.257)	27.822 (4.128)
Se	Gonads	3.562 (0.290)	3.048 (0.172)	9.972 (0.292)	9.534 (0.267)	17.642 (1.127)	19.060 (1.074)
	Kidney	4.119 (0.240)	4.146 (0.185)	16.006 (1.459)	21.055 (6.620)	25.379 (1.195)	32.036 (1.004)
	Liver	2.628 (0.101)	2.288 (0.121)	11.630 (0.445)	10.798 (0.595)	24.076 (0.464)	24.220 (2.367)
Sr	Gonads	1.217 (0.093)	1.504 (0.234)	1.715 (0.091)	1.839 (0.406)	2.435 (0.196)	2.177 (0.289)
	Kidney	1.059 (0.042)	1.401 (0.088)	2.023 (0.297)	2.914 (0.658)	2.279 (0.089)	3.412 (0.345)
	Liver	0.584 (0.181)	0.338 (0.019)	0.765 (0.070)	0.521 (0.074)	0.836 (0.041)	0.730 (0.111)
V	Gonads	0.179 (0.040)	0.214 (0.076)	0.123 (0.022)	0.482 (0.088)	0.179 (0.026)	0.410 (0.050)
	Kidney	0.184 (0.022)	0.249 (0.021)	0.483 (0.085)	0.310 (0.045)	0.613 (0.105)	0.494 (0.075)
	Liver	0.392 (0.033)	0.366 (0.051)	0.914 (0.085)	0.939 (0.148)	1.693 (0.285)	1.558 (0.546)
Sample size (*n*)		6	4	6	4	6	3

(± standard error) percent overwinter mass loss ranged from 5.46 ± 2.04 to 8.47 ± 1.57 (among treatments) and did not vary significantly between sexes or treatments during either hibernation period (sex, treatment, and time; $p > 0.199$).

Snake mass had a significant effect on gonad size ($F_{1,66} = 4.44$, $p = 0.050$), but the relationship between gonad mass and snake mass did not vary significantly among treatments or between sexes ($p > 0.34$). A clear difference was found in gonad mass between the sexes (male mean = 0.11 g, female mean = 0.39 g), but the effect of sex on gonad size was not significant because of sex-specific differences in somatic growth (i.e., females had greater body mass and gonad mass compared to males).

Table 5. Results of repeated-measures analysis of variance of the effects of food treatment and sex on changes in snout–vent length and mass of snakes over time[a]

Source[b]	*df*	MS	*F*	*p*
Snout–vent length				
Among snakes				
Sex	1	0.1679	31.43	<0.001
Treatment	2	0.0059	1.11	0.345
Sex × treatment	2	0.0013	0.25	0.783
Error	23	0.0053		
Within snakes				
Time	9	0.4002	2,903.60	<0.001
Time × sex	9	0.0056	40.87	<0.001
Time × treatment	18	0.0005	3.47	<0.001
Time × sex × treatment	18	0.0004	2.83	<0.001
Error	207	0.0001		
Mass				
Among snakes				
Sex	1	0.5860	21.59	<0.001
Treatment	2	0.1519	5.60	0.011
Sex × treatment	2	0.0283	1.04	0.369
Error	23	0.0271		
Within snakes				
Time	9	2.9943	2,185.52	<0.001
Time × sex	9	0.0297	21.64	<0.001
Time × treatment	18	0.0044	3.19	<0.001
Time × sex × treatment	18	0.0032	2.32	0.003
Error	207	0.0014		

[a] *df* = degrees of freedom; MS = mean square; *F* = *F* statistic.
[b] The times symbol (×) indicates the interaction between the two main effects in the statistical model.

DISCUSSION

The current study demonstrates that prey items can be an important source of trace element exposure for aquatic snakes. Snakes accumulated significant quantities of As, Cd, Se, Sr,

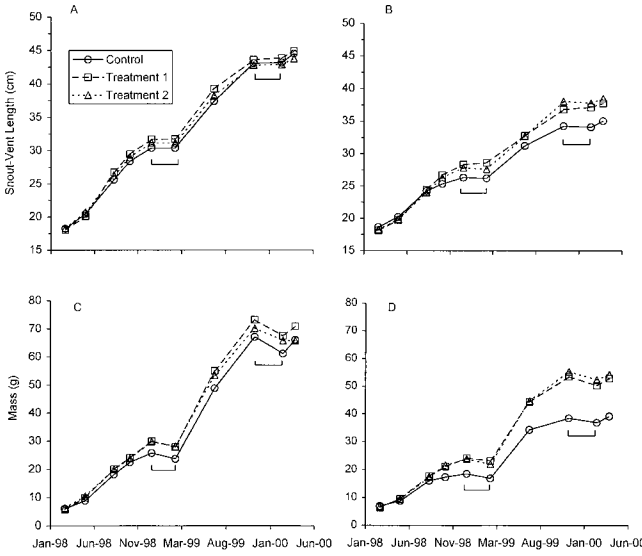

Fig. 2. Change in mean snout–vent length and mass of female (**A** and **C**) and male (**B** and **D**) banded water snakes (*Nerodia fasciata*) over the course of a two-year dietary exposure to trace elements at low (control), intermediate (treatment 1), and high (treatment 2) dietary concentrations. The two overwinter periods are indicated on the graphs with brackets and represent three-month periods of hibernation induced by placing snakes in a cold environmental chamber.

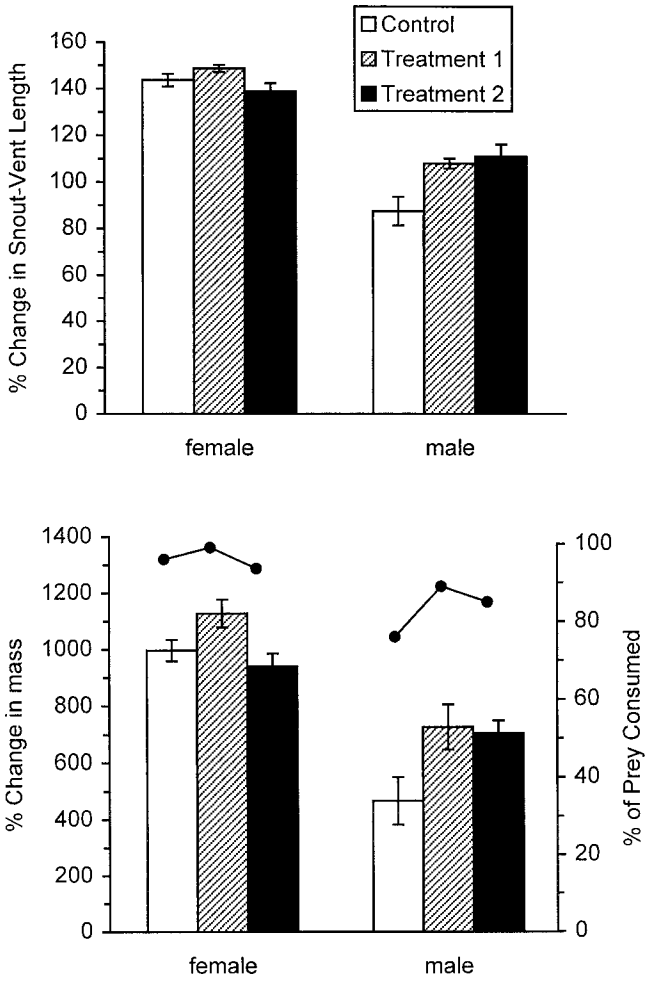

Fig. 3. Percent change in snout–vent length and mass of female and male banded water snakes (*Nerodia fasciata*) over the course of a two-year dietary exposure to trace elements at low (control), intermediate (treatment 1), and high (treatment 2) dietary concentrations. The graphs illustrate the different growth patterns of males and females on the experimental diets. Error bars represent ± 1 standard error of mean. For comparative purposes, percent of prey items consumed is presented as dotted lines on the second graph.

and V from contaminated prey, and in most cases, snakes accumulated elements in a dose-dependent manner. Our results are consistent with the findings of our recent study on adult snakes [10], the only other study to date that experimentally examined trophic uptake of inorganic contaminants in squamate reptiles. Comparisons of the two feeding experiments (previous study: adults, one-year exposure vs current study: juveniles, two-year exposure) reveal that accumulation of elements followed two patterns; for some elements final body burdens seemed to relate to the number of meals ingested and in other cases accumulation was greater in juveniles. For example, tissue concentrations of Se and Sr were usually lowest in juvenile treatment 1, intermediate in adults, and highest in juvenile treatment 2, probably resulting from the number of contaminated meals ingested by each group (32, 52, and 67 contaminated meals, respectively). In contrast, juvenile snakes in treatments 1 and 2 accumulated higher concentrations (compared to adults in previous study) of V in kidney and gonads, Cd in gonads, and As in all organs. Such differences may arise from age-specific differences in assimilation efficiency, which potentially place juvenile snakes at greater risk than adult conspecifics when exposed to trace elements in the environment.

Sex-specific differences in trace element accumulation occurred in juvenile snakes, and these differences were consistent across both treatment groups receiving contaminated prey. Although element accumulation was similar in the liver of both sexes, gonadal and renal burdens often differed substantially in males and females. For example, females accumulated As, Cd, and V concentrations in their kidneys that were up to double the concentrations found in males. In contrast, males had Se and Sr kidney burdens approximately 30 to 50% higher than in females. Additionally, As and V concentrations in testicular tissue were up to three times the levels found in ovaries. Sex-specific differences in accumulation rarely have been reported in squamate reptiles [6], making it difficult to determine what underlying processes account for such differences. Under field conditions, sex-specific differences in accumulation might arise because of sex-based differences in food preference, body size, or elimination during reproductive bouts (i.e., females that transfer contaminants such as Se to offspring may have reduced postparturition burdens). Our study design enabled us to control many of these factors, as well as genetic variability, leaving sex-specific differences in physiology and food consumption rates as possible causes for differential accumulation and contaminant partitioning among organs between sexes. Thus, our findings underscore the importance of considering sex-specific differences when designing future toxicological studies with squamate reptiles.

Selenium accumulation in both sexes resulted in tissue burdens above levels known to be toxic in other vertebrates. Selenium concentrations in liver and gonads of snakes in treatment 2 were twice the reproductive toxicity thresholds for fish proposed by Lemly [13]. Selenium concentrations in fish less than those found in treatment 2 snakes have been linked to developmental malformations resulting in high embryonic, larval, and juvenile mortality. Similarly, Se burdens much lower than those found in treatment 2 snake tissues induce a variety of abnormalities in avian species, often resulting in reproductive impairment [14,15]. Interestingly, snakes in treatment 1, which received approximately one half as many contaminated prey items as those in treatment 2, exhibited tissue burdens greater than or equivalent to reproductive toxicity thresholds for birds and fish [13,14]. Theoretically, such findings could have important implications for ecological systems impacted by anthropogenic sources of Se. Because the prey of many snakes are mobile and in some cases can move considerable distances (e.g., amphibians) [16,17], snakes and other vertebrate predators inhabiting uncontaminated sites possibly periodically ingest contaminated prey migrating from peripheral contaminated habitats. Thus, snakes do not necessarily have to inhabit a contaminated site to potentially encounter toxic levels of Se in their diet.

Despite the high concentrations of trace elements accumulated by snakes in our study, no adverse biological effects were documented. All of the biological endpoints chosen in our study are influenced by exposure to trace elements (particularly Se) in other vertebrates. However, snakes with high contaminant body burdens survived to the end of the study and exhibited normal food consumption, growth, metabolic rate, condition factor, gonadosomatic index, and overwinter survival and mass loss. Although growth was significantly influenced by treatment, feeding observations revealed that differences in growth among treatments likely arose from dif-

ferences in the quantity of food consumed, rather than differences in dietary composition.

Our studies raise the question as to whether some aquatic snakes may be more tolerant of high trace element exposure levels than other vertebrates. The suggestion has been made that many reptiles may be more sensitive to effects of organic contaminants than other wildlife, presumably because of the ectothermic physiology, apparently primitive enzyme systems, and high trophic status of reptiles [18]. However, compared to organic contaminants, much less information is available on the effects of inorganic contaminants on reptiles, making it difficult to speculate on the sensitivity of aquatic snakes to inorganic contaminants relative to other vertebrates. Taken together, the results of our field and laboratory studies suggest that extremely high body burdens of certain trace elements (higher than those found in the laboratory) may be necessary to induce adverse responses (e.g., elevated SMR) in water snakes [8,10; current study]. However, previous investigators examining the effects of coal combustion wastes on aquatic vertebrates have found that because Se, a potent teratogen, is often the major contaminant associated with coal combustion effluent, reproductive endpoints sometimes best reveal the toxicological impact of the effluent on individuals and local populations [14,19,20]. In many cases, individuals exposed to Se may appear outwardly healthy, but have greatly reduced reproductive success or even complete reproductive failure [20]. Because our studies on snakes have not yet examined the effects of coal-combustion wastes on reproductive endpoints, adverse effects may have gone undetected. Moreover, snakes fed an abundance of previously frozen prey under laboratory conditions do not endure the complex physiological challenges encountered by snakes in the field (e.g., parasitism, periodic prey unavailability, and activity costs), and therefore may only provide conservative estimates of biological responses to contaminant exposure. Future research designed to specifically evaluate the sensitivity of reptiles to Se and other inorganic contaminants will be a significant contribution to reptile toxicology and conservation efforts, especially if a variety of other biological endpoints (e.g., reproduction) are considered.

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