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INTER- AND INTRASPECIFIC VARIATION IN MERCURY BIOACCUMULATION BY SNAKES INHABITING A CONTAMINATED RIVER FLOODPLAIN

DAVID V.V. DREWETT,† JOHN D. WILLSON,†‡ DANIEL A. CRISTOL,§ STEPHANIE Y. CHIN,† and WILLIAM A. HOPKINS*†
†Department of Fish and Wildlife Conservation, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, USA
‡Department of Biological Sciences, University of Arkansas, Fayetteville, Arkansas, USA
§Department of Biology, College of William and Mary, Williamsburg, Virginia, USA

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Abstract—Although mercury (Hg) is a well-studied contaminant, knowledge about Hg accumulation in snakes is limited. The authors evaluated Hg bioaccumulation within and among four snake species (northern watersnakes, *Nerodia sipedon*; queen snakes, *Regina septemvittata*; common garter snakes, *Thamnophis sirtalis*; and rat snakes, *Elaphe obsoleta* [*Pantherophis alleghaniensis*]) from a contaminated site on the South River (Waynesboro, VA, USA) and two nearby reference sites. Total Hg (THg) concentrations in northern watersnake tail tissue at the contaminated site ranged from 2.25 to 13.84 mg/kg dry weight (mean: 4.85 ± 0.29), or 11 to 19 times higher than reference sites. Blood THg concentrations (0.03–7.04 mg/kg wet wt; mean: 2.24 ± 0.42) were strongly correlated with tail concentrations and were the highest yet reported in a snake species. Within watersnakes, nitrogen stable isotope values indicated ontogenetic trophic shifts that correlated with THg bioaccumulation, suggesting that diet plays a substantial role in Hg exposure. Female watersnakes had higher mean THg concentrations (5.67 ± 0.46 mg/kg) than males (4.93 ± 0.49 mg/kg), but no significant differences between sexes were observed after correcting for body size. Interspecific comparisons identified differences in THg concentrations among snake species, with more aquatic species (watersnakes and queen snakes) accumulating higher mean concentrations (5.60 ± 0.40 and 4.59 ± 0.38 mg/kg in tail tissue, respectively) than the more terrestrial species, garter snakes and rat snakes (1.28 ± 0.32 and 0.26 ± 0.09 mg/kg, respectively). The results of the present study warrant further investigation of potential adverse effects and will aid in prioritizing conservation efforts. Environ. Toxicol. Chem. 2013;32:xx–xx. © 2013 SETAC

Keywords—Reptiles Heavy metals Pollution Trophic position Stable isotopes

INTRODUCTION

Although the ecological value of reptiles has historically been underappreciated, recent recognition of global reptile declines has led to a newfound enthusiasm for reptile conservation [1]. Conservation efforts have prompted research illustrating the critical ecological functions that reptiles perform [2] and have suggested that they are excellent biological indicators of ecosystem health [3]. Despite increasing research, reptiles remain one of the most understudied vertebrate groups [4]. Among reptiles, snakes are a diverse but particularly understudied group, comprising approximately 39% of all reptiles with more than 3,100 named species worldwide [1]. Snakes inhabit a wide variety of habitats, and because they often occur at high densities and serve as both predators and prey, they can be vital components of food webs [2]. Despite the importance of snakes, population assessments have been conducted on relatively few snake species, and consistent methods for monitoring and management have not been developed [5]. Six major factors have been identified as primary threats to snakes and other reptiles: habitat loss, invasive exotic species, disease, unsustainable harvest, global climate change, and environmental pollution [6].

Heavy metal contaminants such as mercury (Hg) are a major concern for conservation of biodiversity because of their toxicity and tendency to persist in the environment [7,8]. Although Hg occurs naturally, anthropogenic activities have led to a number of novel environmental sources, including point-source emissions from chloralkali incinerators, mining, waste incineration, coal combustion, and other industrial activities [9]. Point-source emissions are particularly dangerous because they are usually associated with high levels of contamination at localized scales, often in aquatic systems [10]. After deposition in an aquatic system, Hg can persist in its inorganic form (Hg) or be methylated by aquatic anaerobic microorganisms to its more bioavailable organic form, methylmercury (MeHg). Methylmercury is very stable and is known to bioaccumulate and biomagnify through successive trophic levels [11]. The primary route of Hg exposure in wildlife is diet, and because of MeHg biomagnification, apex predators can be exposed to high concentrations [8]. Mercury exposure can cause direct mortality or severe damage to tissues, especially those of the nervous system [11]; these effects may influence vital rates important to population viability, such as survival, reproduction, and growth rates [8].

Although there have been many studies examining Hg in fish, birds, and mammals, knowledge about Hg accumulation in snakes is limited. Snakes exhibit a suite of natural and life history traits that make them particularly susceptible to adverse effects of environmental contaminants [12]. All snakes are carnivorous; and as secondary, tertiary, and apex predators, they are particularly vulnerable to biomagnification of MeHg [13]. Snakes are also long-lived and inhabit relatively small home ranges, making them vulnerable to chronic local contaminant exposure [14]. Other aspects of snake ecology provide excellent opportunities to evaluate ecological factors that influence Hg bioaccumulation. Overall, snakes consume a wide array of prey taxa (e.g., birds, small mammals, fish, amphibians, reptiles, and invertebrates), but many snake species are dietary specialists. Within species, snakes can exhibit ontogenetic shifts in prey size

* To whom correspondence may be addressed (hopkinsw@vt.edu).
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or type as they grow [15]. This variation in diet among and within snake species affords opportunities to evaluate specific hypotheses about how diet affects Hg exposure and accumulation. Finally, variation in habitat preferences (e.g., aquatic vs terrestrial) among snake species makes it possible to evaluate contaminant exposure across habitat types [16].

We examined Hg bioaccumulation in four snake species inhabiting an Hg-contaminated river floodplain in Virginia, USA: northern watersnakes (*Nerodia sipedon*), rat snakes (*Elaphe obsoleta* [*Pantherophis alleghaniensis*]), queen snakes (*Regina septemvittata*), and common garter snakes (*Thamnophis sirtalis*). These species differ in feeding ecology and habitat use, providing an excellent opportunity to investigate factors that influence Hg concentrations within and among snake species. We examined Hg concentrations in snake tail tissue relative to their size, sex, diet, trophic position (as inferred by nitrogen stable isotope composition), and location along a contamination gradient. Specifically, we evaluated the following four hypotheses. First, snakes accumulate Hg, and Hg concentrations in snake tissues reflect their spatial location along the contamination gradient. Second, within species, ontogenetic shifts in diet from low trophic-level prey to higher trophic-level prey expose larger snakes to increased Hg bioaccumulation. Third, adult males of species with female-biased sexual size dimorphism (e.g., northern watersnakes) exhibit higher Hg levels than females because males are older at a given size and females may excrete Hg through maternal transfer to their offspring. Finally, snake species that primarily forage on aquatic prey (northern watersnakes and queen snakes) exhibit higher Hg levels than species that primarily forage terrestrially (garter snakes and rat snakes); within the two foraging groups, however, species that consume higher trophic-level prey exhibit higher Hg concentrations.

MATERIALS AND METHODS

Study site

We examined Hg bioaccumulation in snakes inhabiting the South River in Waynesboro, Virginia, USA (Fig. 1). An acetate fiber production plant contaminated the South River with point-source Hg emissions between 1929 and 1950, and Hg levels currently remain high in the system [17,18]. An extensive contamination gradient exists in the South River, ranging from low Hg levels upstream of the contamination source to extremely high levels downstream of the source. Previous studies have found that Hg concentrations in water, sediment, and biota peak approximately 25 km downstream from the contamination source [19,20] and continue to be elevated above background levels far downstream [21].

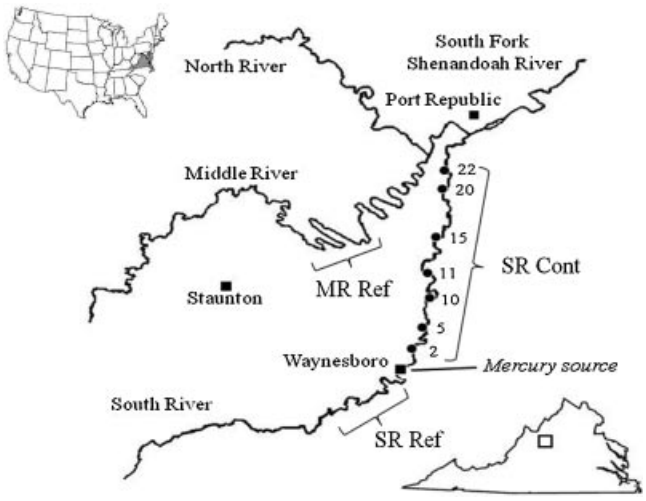

Fig. 1. Sampling sites along the South and Middle Rivers in the Shenandoah Valley, Virginia, USA. The Middle River and the portion of the South River upstream of the contamination source served as reference sites (MR Ref and SR Ref, respectively), and the contaminated site (SR Cont) was divided into several subsites downstream from the source. Numbers refer to river miles downstream from the contamination source. Note that the river flows from south to north.

Study species

We targeted four species of snakes for the present study. These species were selected because they differ substantially in feeding ecology and habitat use, presenting an opportunity to examine multiple factors that may influence Hg accumulation.

Northern watersnakes (*N. sipedon*) are habitat generalists but are particularly abundant in rocky, shallow water habitats. Northern watersnakes are primarily piscivorous and exhibit female-biased sexual dimorphism in body size [22]. Queen snakes (*R. septemvittata*) also occupy waterways with rocky banks but are dietary specialists, feeding almost exclusively on soft-shelled (freshly shed) crayfish [22]. Rat snakes (*E. obsoleta* [*P. alleghaniensis*]) inhabit a variety of wooded habitats and are highly arboreal, but they also travel and forage on the ground. Rat snakes feed primarily on terrestrial endotherms, especially birds and small mammals [22]. Lastly, common garter snakes (*T. sirtalis*) are habitat and dietary generalists, feeding primarily on earthworms and amphibians along forest edges, in fields, and especially around bodies of water [22].

Field sampling of snakes

We hand-captured snakes from suitable habitats at several sites upstream and downstream of the point of Hg contamination on the South River (hereafter referred to as river mile [RM] 0) during May through July 2011. Downstream from the Hg source (hereafter referred to as SR Cont), we collected snakes from five subsites between RM 1 and RM 22 (Fig. 1). We also collected snakes from two reference sites: a 20-km stretch of the South River upstream from the manufacturing plant (SR Ref) and the Middle River (MR Ref), which is approximately 25 km northwest of the South River and joins the South River to form the South Fork of the Shenandoah River at Port Republic, Virginia, USA. Every snake that was encountered was captured and processed. A Garmin global positioning system unit (Garmin International) was used to obtain geospatial coordinates for each captured snake.

Because northern watersnakes are common in the area, we captured approximately the same number at each of the five subsites along the contamination gradient and used this species for our intraspecific comparisons. For this species, we sought to collect approximately the same number of adult males, adult females, and juveniles. Male and female watersnakes were classified as adults at snout-vent lengths (SVLs) of 430 and 560 mm, respectively [22]. Our collection of other species targeted 10 individuals of each species from a section of the contamination gradient (RM 9–22). Because there were no intraspecific analyses (e.g., examination of sex or size effects) within queen snakes, rat snakes, or garter snakes, we sampled only adult snakes of these species.

Once the snakes were captured, we determined sex by examination of tail morphology and/or probing the cloaca and measured snakes for SVL (millimeters from snout tip to cloaca) by stretching the snake along a meter stick, tail length

(millimeters from cloaca to tail tip), and mass. For snakes with a mass greater than 20 g, we collected a 0.2- to 1-ml blood sample from the caudal vein for blood mercury analysis. We also collected a tail clip of approximately 1 cm (containing skin, muscle, and bone) from each snake as a sample for nitrogen stable isotope composition and Hg analyses. Previous studies have demonstrated that snake tail clips are reliable indicators of Hg concentrations in internal tissues [23–25] and that they are useful in stable isotope dietary analyses [26]. After collection, we stored samples at −80°C for analysis. To avoid resampling previous captures, we marked snakes individually by branding posterior ventral scales [27] before releasing them at the point of capture.

Sample preparation

Prior to analyses, we removed any superficial contamination by gently scrubbing each tail sample with a thin-fibered plastic brush, rinsing with Millipore water, and transferring to a clean Eppendorf vial. We used a different brush to wash samples from each species and site. Within species, we washed the brush with a 1% Alconox detergent solution and rinsed thoroughly with Millipore water between each sample. Following the washing of tail samples, we lyophilised them and shipped them to the College of William and Mary (Williamsburg, VA, USA) for total mercury analysis and to the University of Arkansas (Fayetteville, AR, USA) for stable isotope analysis.

Mercury analyses

We analyzed tail samples from 106 individual snakes and blood samples from 25 individuals for total Hg (THg) content by combustion amalgamation cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer-80; Milestone) according to U.S. Environmental Protection Agency method 7473 [28]. For quality assurance, control samples included replicate, blank, and standard reference material (DOLT-4 dogfish liver, DORM-3 fish protein; National Research Council of Canada) in every batch of approximately 10 samples. Method detection limits (threefold the standard deviation of procedural blanks) for samples ranged from 0.0054 to 0.0114 mg/kg (ppm), and all samples had THg concentrations that exceeded those limits. The average relative percentage difference between replicate sample analyses was 13.35 ± 10.86% (mean ± standard error throughout). Mean recovery percentages of THg for the DOLT-4 and DORM-3 were 101.82 ± 0.16% ($n = 36$) and 101.90 ± 0.46% ($n = 36$), respectively. Results are presented on a dry weight basis for tail samples and a wet weight basis for blood samples, in accordance with similar recent literature. Moisture percentages for tail tissues were 61.06 ± 0.59% in watersnakes, 64.88 ± 0.79% in queen snakes, 67.91 ± 0.89% in garter snakes, and 60.20 ± 1.29% in rat snakes.

A subset of tail samples ($n = 24$) was analyzed for MeHg percentages and reanalyzed for THg by Quicksilver Scientific using high-pressure liquid chromatography (Method QS–LC/CVAF–001). A combination of blanks, standard reference materials TORT-2 and DOLT-4 (N = 2), matrix spikes (N = 2), and a five-point blank calibration curve (N = 1) were used for quality control. The detection limit for MeHg was 5.0×10^{-11} mg/kg for tail tissues, and all samples had Hg concentrations that exceeded that limit. Average relative percentage differences between replicate sample analyses were 1.2 ± 0.02% for THg and 2.1 ± 0.21% for MeHg. The recovery percentages for THg and MeHg for the TORT-2 and DOLT-4 standard reference materials were 105.0 and 104.7% for TORT-2 and 91.3 and 85.1% for DOLT-4, respectively. Average matrix spike recoveries of THg and MeHg were 89.7 ± 0.49% and 88.8 ± 1.08%, respectively.t

Stable isotope analysis

Nitrogen stable isotope composition was determined for a subset of tail samples from RM 9 through RM 22 of the contaminated portion of the South River. Sample ^{15}N isotope values were measured on a Finnigan Delta Plus continuous flow isotope–ratio mass spectrometer and elemental analyzer located at the University of Arkansas Stable Isotope Laboratory (Fayetteville, AR, USA). Stable isotope ratios are reported in per mill units (‰) using δ notation

$$\delta^{15}\mathrm{N} = \left[\left(\mathrm{R}_{\mathrm{sample}}/\mathrm{R}_{\mathrm{standard}}\right) - 1\right] \times 10^3$$

where R_{sample} refers to the ratio of ^{15}N to ^{14}N in the sample, and $R_{standard}$ refers to the ratio of ^{15}N to ^{14}N in atmospheric N_2 gas.

Statistical analyses

We performed all statistical analyses using SAS 9.1 (SAS Institute) or Microsoft Excel, and a α value of 0.05 was used to assess statistical significance. When appropriate, we log- or rank-transformed data to improve normality and homoscedasticity.

We evaluated spatial variation in THg concentrations in tail tissue of adult northern watersnakes after correcting for sex and body size using a two-way analysis of covariance (ANCOVA) on rank-transformed data, with site and sex as main effects and SVL as a covariate. Contaminated subsites along the South River could not be treated independently of one another and are, therefore, collectively referred to as the South River contaminated site (SR Cont; RM 1–22).

We examined the influence of demographic factors (sex and size) and diet on THg bioaccumulation using northern watersnakes because sample sizes were highest for this species. To reduce the influence of spatial variation on THg concentrations in this analysis, we focused on snakes from a six-mile (RM 16–22) river reach in the most highly contaminated region of SR Cont. We determined the relationship between SVL and THg bioaccumulation in tail tissue using ANCOVA, with sex as the main effect and SVL as a covariate. We used stable nitrogen isotope ($\delta^{15}N$) analyses to infer ontogenetic dietary shifts by examining $\delta^{15}N$ values in relation to SVL. Stable isotopes have been proposed as useful tools for examining relative trophic position [29,30], and studies have demonstrated their reliability as trophic indicators in snakes [26]. We evaluated demographic effects on $\delta^{15}N$ values of tail tissue using ANCOVA, with sex as the main effect and SVL as a covariate. We examined the relationship between trophic position and THg bioaccumulation by regressing THg values against $\delta^{15}N$ values for both sexes combined. Finally, to facilitate comparisons to studies of other vertebrates of the South River, for which blood has been the primary sample tissue, we assessed the relationship between blood and tail THg concentrations in a subset of northern watersnakes ($n = 25$) collected from the South and Middle Rivers using linear regression.

To determine whether differing habitat associations and feeding ecologies influenced THg bioaccumulation among the four snake species, we conducted a multispecies comparison on a subset of adult snakes from RM 9 through RM 22 of SR Cont. We inferred the relative trophic position of each species by examining $\delta^{15}N$ values of tail tissue. We used a one-way

Table 1. Body sizes and total mercury (THg) concentrations in tail tissue and blood of northern watersnakes (*Nerodia sipedon*) collected from the contaminated portion (river miles 1–22) of the South River (SR Cont), and the South River and Middle River reference sites (SR Ref and MR Ref), Virginia, USA[a]

				Tail THg (mg/kg, dry wt)			
	No.	SVL (mm)	Mass (g)	MR Ref	SR Ref	SR Cont	Blood THg (mg/kg, wet wt)
Total	80	587 ± 19 (270–895)	229 ± 22 (15–710)	0.29 ± 0.01 (0.23–0.37)	0.49 ± 0.07 (0.16–0.92)	4.85 ± 0.29[b] (2.25–13.84)	2.24 ± 0.42[d] (0.03–7.04)
Adult male	25	512 ± 12 (435–655)	96 ± 7 (49–170)	0.30 ± 0.02 (0.25–0.32)	0.39 ± 0.04 (0.19–0.52)	4.93 ± 0.49[b] (2.68–10.19)	1.78 ± 0.87[d] (0.05–7.04)
Adult female	39	727 ± 14 (562–895)	392 ± 25 (115–710)	0.28 ± 0.02 (0.23–0.37)	0.64 ± 0.15 (0.16–0.92)	5.67 ± 0.46[b] (2.83–13.84)	2.80 ± 0.57[d] (0.03–5.32)
Juvenile	16	360 ± 22 (270–540)	40 ± 8 (15–110)	—	—	3.30 ± 0.15[c] (2.25–4.21)	1.34 ± 0.43[d] (0.26–2.35)

[a]Means ± standard error, with ranges in parentheses.
[b]River miles 1–22.
[c]River miles 16–22.
[d]River miles 1–22, MR Ref, SR Ref; ($n = 25$); males ($n = 10$); females ($n = 15$).
SVL = snout-vent length.

analysis of variance (ANOVA) on rank-transformed $\delta^{15}N$ values, followed by a Tukey–Kramer adjustment for multiple comparisons to assess differences among species. However, a stable isotope study of the South River food web [31] indicated that baseline (primary producer) $\delta^{15}N$ values differed substantially between aquatic (periphyton: 0.61‰) and terrestrial (violet, grass, honeysuckle: 8.53‰) habitats. Thus, for comparison, we present estimated $\delta^{15}N$ values for each snake species corrected for baseline differences in nitrogen stable isotope composition in the appropriate habitat, but we did not evaluate these estimates statistically. Finally, we assessed among-species differences in THg concentrations in tail tissue using a one-way ANOVA on log-transformed THg data, followed by a Tukey–Kramer adjustment for multiple comparisons.

RESULTS

We captured, marked, and collected tissue samples from 106 snakes of four species from the South and Middle Rivers, in Virginia, USA (Tables 1 and 2). Overall, THg concentrations at the contaminated site were highest in tail tissues of watersnakes, ranging from 2.25 to 13.84 mg/kg (dry wt), followed by queen snakes (1.90–6.00 mg/kg, dry wt), common garter snakes (0.08–2.53 mg/kg, dry wt), and rat snakes (0.05–0.89 mg/kg, dry wt). The percentage of THg consisting of MeHg in a subset of tail samples (dry wt) for each species collected from the highly contaminated portion of the South River (RM 9–22) ranged from 87.1 to 95.5% ($n = 12$) for watersnakes, 87.7 to 93.9% ($n = 4$) for queen snakes, 57.6 to 95.7% ($n = 4$) for garter snakes, and 78.7 to 88.9% ($n = 4$) for rat snakes.

We used a subset of watersnake samples (Table 1) to evaluate the relationship between THg concentrations in blood and tail tissues. The THg concentrations in blood of watersnakes positively correlated with THg concentrations in tail tissue (Fig. 2; $r^2 = 0.92$, $p < 0.01$). Although we did not analyze blood samples from all individuals, the positive relationship we found allowed us to estimate that blood values would exceed 10.0 mg/kg (wet wt) for the individuals with the highest tail THg concentrations.

Examination of spatial patterns of THg bioaccumulation focused on adult watersnakes captured at all sites along both rivers ($n = 64$). After correcting for sex and body size effects, we observed that THg concentrations in tail tissues of adult watersnakes differed significantly among sites (Fig. 3 and Table 1; ANCOVA, site: $F_{4,59} = 63.37$, $p < 0.01$). Watersnakes from SR Cont had the highest mean THg concentrations in tail tissue, followed by SR Ref watersnakes and MR Ref watersnakes (Table 1). Although subsites could not be treated independently of one another in our statistical comparisons, we observed a large range in THg concentrations within SR Cont (Fig. 3), with the highest levels recorded between RM 15 and RM 16 (3.79–13.84 mg/kg, dry wt). Mercury concentrations appeared to decrease downstream of this peak, but remained well above reference concentrations.

Our intraspecific analysis of the effect of size and sex was restricted to watersnakes of all ages sampled within the most contaminated section of SR Cont (RM 16–22; $n = 42$). Body size had a significant influence on $\delta^{15}N$ values, with larger individuals having enriched $\delta^{15}N$ values relative to smaller individuals (Fig. 4A; ANCOVA, SVL: $F_{2,39} = 12.35$, $p < 0.01$); however, sex did not influence $\delta^{15}N$ values after accounting for body size differences (sex: $F_{2,39} = 12.35$, $p = 0.57$). Body size also had a positive influence on THg concentrations in tail tissue of watersnakes (Fig. 4B; ANCOVA, SVL: $F_{2,39} = 21.64$, $p < 0.01$). Although female watersnakes had the highest overall

Table 2. Body sizes and total mercury (THg) concentrations in tail tissue of adults of four snake species collected from the contaminated portion (river miles 9–22) of the South River, Virginia, USA

	No.	SVL (mm)[a]	Mass (g)[a]	Tail THg (mg/kg, dry wt)[a]
Northern watersnake (*Nerodia sipedon*)	36 (22 female, 14 male)	647 ± 21 (435–850)	277 ± 33 (55–710)	5.60 ± 0.40 (2.68–13.84)
Queen snake (*Regina septemvittata*)	9 (6 female, 3 male)	445 ± 28 (265–560)	64 ± 11 (10–125)	4.59 ± 0.38 (1.90–6.00)
Garter snake (*Thamnophis sirtalis*)	7 (7 female)	505 ± 33 (378–590)	97 ± 16 (39–150)	1.28 ± 0.32 (0.08–2.53)
Rat snake (*Elaphe obsoleta*)	10 (5 female, 5 male)	1218 ± 87 (775–1590)	614 ± 111 (125–1250)	0.26 ± 0.09 (0.05–0.89)

[a]Means ± standard error, with ranges in parentheses.
SVL = snout-vent length.

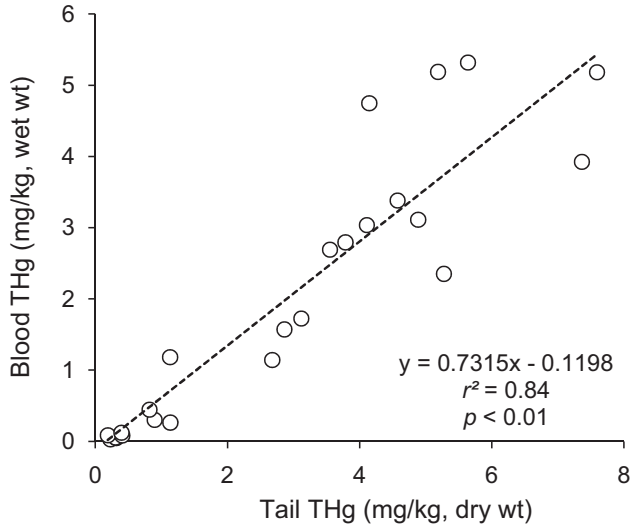

Fig. 2. Relationship between tail total mercury (THg) and blood THg concentrations in northern watersnakes (*Nerodia sipedon*) from the South and Middle Rivers, Virginia, USA.

THg concentrations, concentrations did not differ between similarly sized males and females (Fig. 4B; sex: $F_{2,39} = 21.64$, $p = 0.51$). Tail THg concentrations positively correlated with $\delta^{15}N$ values (Fig. 4C; $r^2 = 0.33$, $p < 0.01$).

Our interspecific comparisons focused on tail tissue from adult individuals of all four species collected from RM 9 through RM 22 within SR Cont (Table 2). Within this region, $\delta^{15}N$ values differed significantly among species (Fig. 5A; ANOVA, $F_{3,48} = 87.67$, $p < 0.01$) with watersnakes having the most enriched mean $\delta^{15}N$ values, followed by queen snakes, rat snakes, and garter snakes. Pairwise comparisons revealed that all species differed significantly from one another ($p < 0.01$), except for garter snakes and rat snakes ($p = 0.55$). After accounting for differences in baseline (primary producer) $\delta^{15}N$ values, differences among species were much less pronounced (Fig. 5B). The THg concentrations in tail tissues differed significantly among the four species (Fig. 5C; ANOVA,

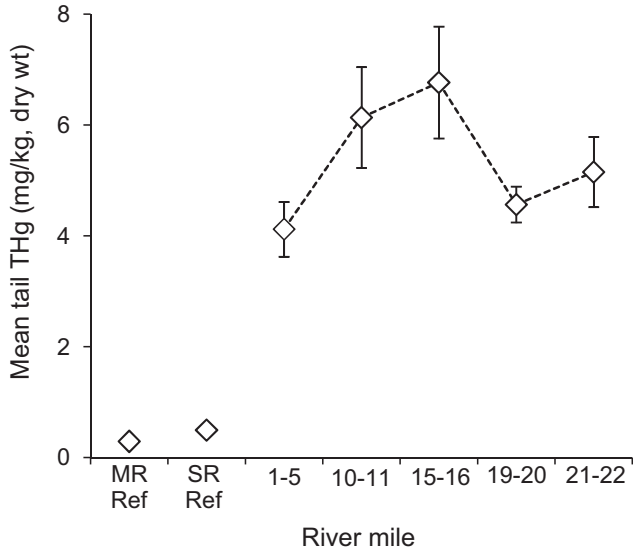

Fig. 3. Spatial variation in total mercury (THg) concentrations in adult northern watersnakes (*Nerodia sipedon*) from the South River (SR) and Middle River (MR), Virginia, USA (two-way analysis of covariance; site: $F_{4,59} = 63.37$, $p < 0.01$). Tail THg concentrations (mg/kg, dry wt; mean ± SE) in watersnakes from the two reference sites (MR Ref and SR Ref) and the subsites along the contaminated section (river miles 1–22) of the South River (SR Cont). Lines connecting points are for visual display, and do not represent connectivity between the means.

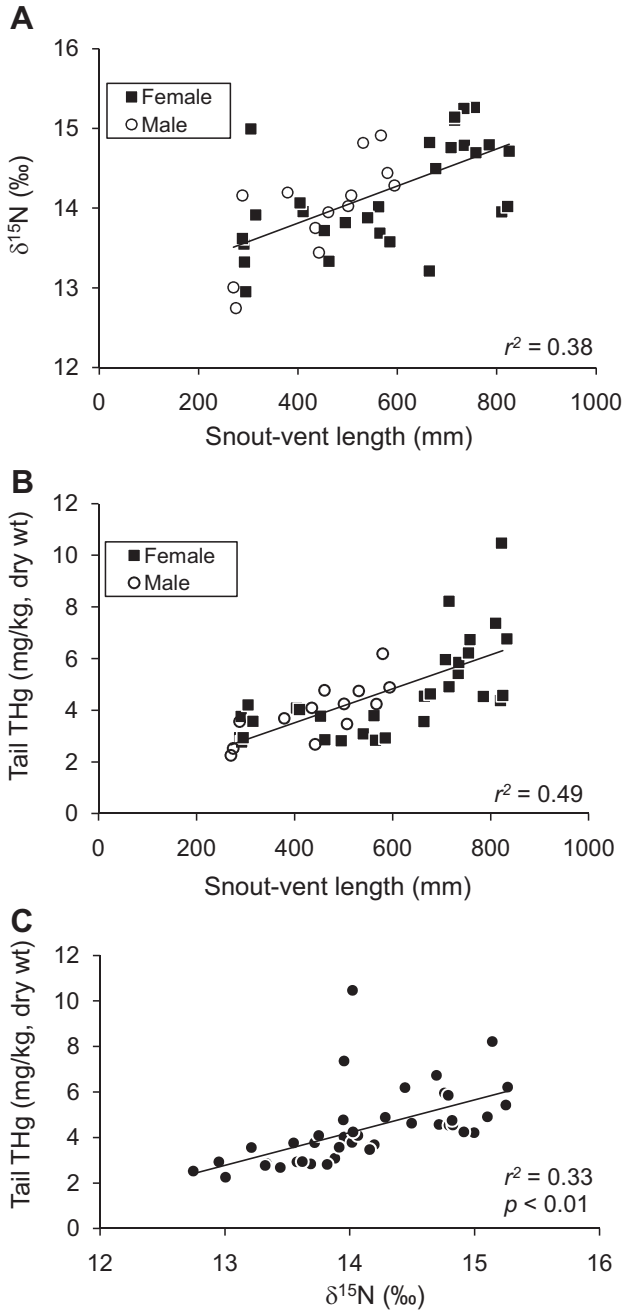

Fig. 4. Relationships between demographic and dietary attributes and total mercury (THg) bioaccumulation in northern watersnakes (*Nerodia sipedon*) from the highly contaminated portion (river miles 16–22) of the South River, Virginia, USA. (**A**) Relationship between snake snout-vent length (SVL) and $\delta^{15}N$ values for male and female watersnakes (two-way analysis of covariance; SVL: $F_{2,39} = 12.35$, $p < 0.01$, $r^2 = 0.38$; sex: $F_{2,39} = 12.35$, $p = 0.57$). (**B**) Relationship between SVL and tail THg concentrations (two-way analysis of covariance; SVL: $F_{2,39} = 21.64$, $p < 0.01$; sex: $F_{2,39} = 21.64$, $p = 0.51$). (**C**) Relationship between $\delta^{15}N$ values and THg concentrations. Black circle symbol in **C** represents both male and female snakes.

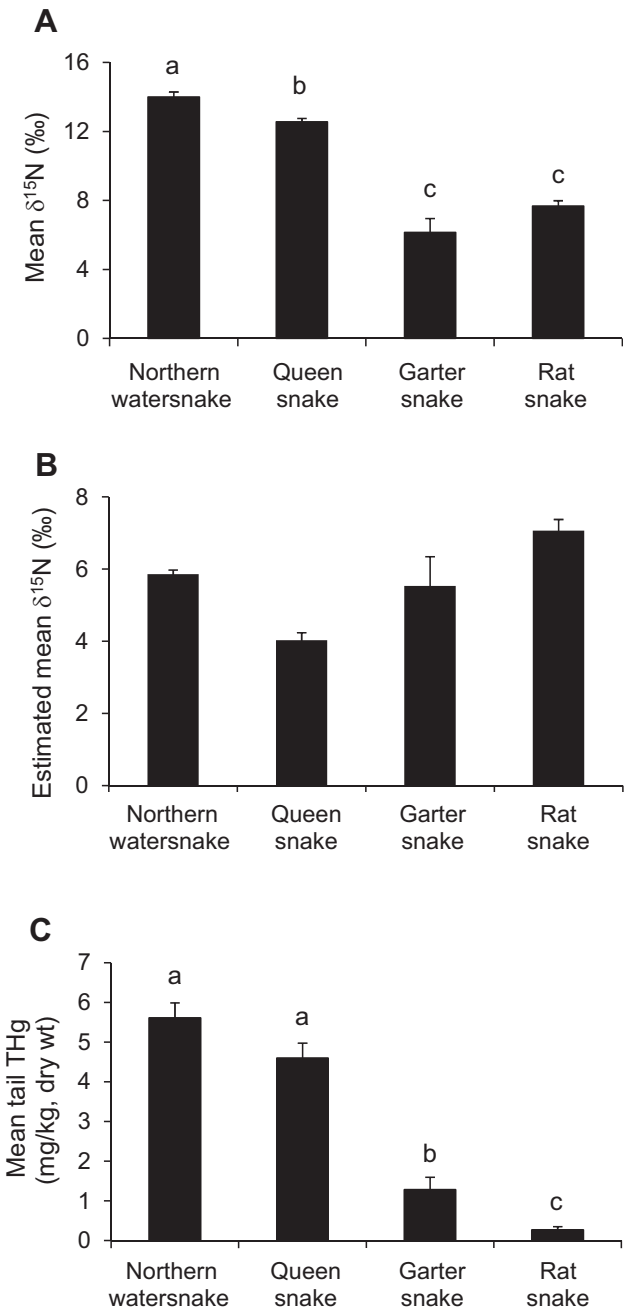

Fig. 5. Interspecific differences in $\delta^{15}N$ values and THg concentrations in tail tissue of adults of four snake species (northern watersnake, *Nerodia sipedon*; queen snake, *Regina septemvittata*; common garter snake, *Thamnophis sirtalis*; and rat snake, *Elaphe obsoleta*) collected from river miles 9 through 22 of the contaminated portion of the South River, Virginia, USA. (**A**) Nitrogen stable isotope composition of tail tissues (one-way analysis of variance; $F_{3,48} = 87.67$, $p < 0.001$). (**B**) Estimated $\delta^{15}N$ values of snake species, corrected for differences in baseline (primary producer) nitrogen stable isotope composition (terrestrial baseline: 0.613‰; aquatic baseline: 8.528 ‰; [28]). (**C**) Total mercury (THg) concentrations in tail tissues (one-way analysis of variance; $F_{3,60} = 89.91$, $p < 0.01$). Letters above bars (a, b, and c) represent statistically significant differences ($p < 0.05$) between species (e.g., a is statistically different from b). Data are presented as means (± standard error) and letters above bars represent statistically significant ($p < 0.05$) groupings.

$F_{3,60} = 89.91$, $p < 0.01$). Pairwise comparisons demonstrated that THg levels of all species differed significantly from one another ($p < 0.01$), except for watersnakes and queen snakes ($p = 0.61$).

DISCUSSION

We examined bioaccumulation of THg within and among four snake species from an Hg-contaminated river floodplain and two nearby reference sites in Central Virginia, USA. To our knowledge, the THg concentrations that we observed in watersnake tissues are the highest reported for a snake species and are among the highest reported for reptiles [25,32,33]. The spatial variation in snake THg concentrations corresponds with the well-documented contamination gradient [20] present at the South River and identifies areas where snakes may be at highest risk of Hg bioaccumulation. The results of the present study also indicate that body size and diet are key factors influencing Hg accumulation in snakes, but that sex differences are not significant after accounting for body size. Finally, by examining multiple snake species with different habitat and feeding ecologies at a single site, we show that aquatic snakes are exposed to the highest levels of Hg bioaccumulation, presumably as a result of feeding on aquatic prey, which tend to have higher Hg concentrations than terrestrial prey at the South River [31]. Our results provide information critical for understanding which snake species, guilds, or demographic groups may be at highest risk of Hg exposure at the South River or other aquatic sites contaminated with Hg.

Spatial variation and mercury concentrations

We observed a large range of THg concentrations in tail tissue of adult watersnakes from the South and Middle Rivers (Table 1). The THg concentrations at reference sites were low relative to the contaminated site. Downstream from the contamination source (RM 1–22), THg concentrations in watersnake tail tissues gradually increased, peaking around RM 15 to RM 16 and thereafter declining to levels similar to those observed upstream of the peak. A similar spatial pattern has been observed in other biota inhabiting the South River (e.g., fish, amphibians, birds, turtles, invertebrates [20,21,34,35]).

Blood THg concentrations provide the best opportunity to compare our results with those of other studies. The mean THg concentrations in watersnake blood are, to our knowledge, the highest reported for a snake species, and the mean concentrations in tail tissue are among the highest reported for any reptile. For example, northern watersnakes from the Oak Ridge Reservation in eastern Tennessee (USA), which was historically contaminated with Hg between the mid-1950s and early 1960s, had blood THg concentrations between 0.14 and 0.82 mg/kg (wet wt) [32]. Cottonmouths (*Agkistrodon piscivorus*) and diamondback watersnakes (*Nerodia rhombifer*) from an insecticide-contaminated site in Texas had mean blood THg concentrations of 0.01 and 0.15 mg/kg (wet wt), respectively [36]. Murray et al. [33] found mean blood THg concentrations of 0.12 mg/kg (dry wt) in cottonmouths, 0.38 mg/kg in banded watersnakes (*Nerodia fasciata*), and 0.61 mg/kg in brown watersnakes (*Nerodia taxispilota*) from a site in South Carolina (USA) contaminated with metals associated with nuclear production. Finally, Jagoe et al. [37] found that the mean THg concentration in blood of American alligators (*Alligator mississippiensis*) from the same contaminated South Carolina site was 2.19 ± 0.38 mg/kg (wet wt).

The blood THg concentrations we report for watersnakes (Table 1) are also among the highest reported for South River vertebrates with aquatic diets. For example, watersnake blood concentrations are higher than those of common snapping turtles (*Chelydra serpentina*; levels within SR Cont of 1.0–3.4 mg/kg, wet wt; B.C. Hopkins, 2012, Master's thesis, Virginia

Polytechnic Institute and State University, Blacksburg, VA, USA), and are similar to those of belted kingfishers (*Megaceryle alcyon*) from contaminated subsites along the South River (mean: 3.35 ± 2.67 mg/kg, wet wt; [35]). Thus, watersnakes may be among the vertebrates that are most at risk of Hg accumulation at the South River and other contaminated habitats.

Intraspecific variation

Total Hg concentrations in tail tissue of watersnakes increased with body size (Fig. 4B). In species exhibiting indeterminate growth, it is common for larger individuals to have higher concentrations of bioaccumulative contaminants than smaller conspecifics [38]. For example, Wylie et al. [13] found that SVL of giant garter snakes (*Thamnophis gigas*) was positively correlated with Hg concentrations in liver tissue. Similarly, Rainwater et al. [25] found that Hg concentrations in liver, kidney, and tail clips positively correlated with SVL and body mass in cottonmouths. Total Hg concentration in blood of common snapping turtles (*C. serpentina*) from the South River has also been found to correlate positively with body size [20]. This can be a result of age, as larger individuals are usually older and, thus, have had more time to bioaccumulate Hg than younger, smaller individuals. Alternatively, intraspecific variation in diet may explain positive relationships between Hg and body size. As gape-limited predators (e.g., those that cannot mechanically reduce prey size prior to ingestion), many snakes undergo dietary shifts as a result of ontogenetic increases in gape, which enables them to consume larger prey— and, in some cases, different prey species—as they grow [15]. Differences in prey size and prey species composition between large and small individuals have been observed in northern watersnakes [39], as well as in other watersnake species [15]. We investigated possible ontogenetic shifts in watersnake diet at the South River by using nitrogen stable isotope composition to assess relative trophic position in relation to snake body size. Body size correlated positively with $\delta^{15}N$ values, indicating that watersnakes exhibit ontogenetic transitions in feeding ecology that are related to body size (Fig. 4A). In conjunction with a positive correlation between $\delta^{15}N$ and THg values, these data suggest that, as a watersnake grows, it is exposed to increasing levels of Hg bioaccumulation through consumption of larger and/or higher trophic-level prey, such as centrarchid fish or catfish, which can have elevated Hg concentrations compared with smaller species, such as minnows [30,31,40–42]. Although the present study was not designed to comprehensively evaluate diet, our anecdotal observations support this conclusion. Several small watersnakes had consumed small fish (e.g., darters, *Etheostoma flabellare*; minnows, *Campostoma* spp., *Cyprinella anomalum, Rhinichthys cataractae*; and sculpins, *Cottus* sp.), whereas two of the largest individuals had consumed large bullhead catfish (*Ameiurus* sp.).

Bioaccumulation of heavy metals has been found to differ between the sexes of some animals, possibly as a result of maternal transfer of contaminants by females to their offspring, differential growth patterns among the sexes, inherent physiological differences, and/or differences in feeding ecology or behavior that affect contaminant accumulation [43,44]. Previous studies of contaminants in snakes have yielded inconsistent results on the topic of sex differences. Mercury concentrations in tissue of giant garter snakes (*T. gigas*) did not differ between sexes [13]. Conversely, Rainwater et al. [25] found that male cottonmouths exhibited significantly higher Hg concentrations in liver and kidney tissue than females. Campbell et al. [32] found that male northern watersnakes had significantly higher Hg concentrations in muscle than females, although their data were not corrected for body size. In the present study, female watersnakes had the highest mean and maximum THg concentrations in tail tissue (Table 1); but females tended to be larger than males, and our isotope data suggest that these larger females fed at higher trophic levels than males. We found no significant differences in $\delta^{15}N$ values or THg concentrations between similarly sized male and female watersnakes (Fig. 4A and B). Thus, within species, trophic position is likely a driving factor of Hg bioaccumulation; after correcting for body size, similar dietary habits between sexes explain the lack of differential Hg exposure. Likewise, although female northern watersnakes most likely maternally transfer some of their Hg body burden to their offspring, the results of the present study suggest that the transferred amount is not sufficient to cause sex differences in THg concentrations of tissues such as blood and tail.

Interspecific variation

Aquatic snake species (watersnakes and queen snakes) had significantly higher THg concentrations in tail tissue than more terrestrial species (garter snakes and rat snakes; Fig. 5C). Although aquatic species also had higher $\delta^{15}N$ values, this difference was likely attributable to differences in baseline $\delta^{15}N$ values between terrestrial and aquatic habitats (e.g., $\delta^{15}N$ values of primary producers). Newman et al. [31] reported that $\delta^{15}N$ values of aquatic periphyton and terrestrial producers at the South River differed by approximately 8 ‰, which corresponds well with the difference we observed (~8 ‰) between aquatic and terrestrial snakes. Thus, after correcting for differences in baseline $\delta^{15}N$ values, trophic levels of terrestrial and aquatic snakes were similar (Fig. 5B), leaving habitat ecology as the likely primary factor influencing Hg exposure and bioaccumulation. Aquatic prey tend to have higher THg concentrations at a given trophic level than terrestrial prey because Hg is often emitted into and/or most concentrated and bioavailable in aquatic systems [31]. As a result, aquatic snakes bioaccumulate the highest Hg concentrations. Within the aquatic species, trophic position appears to be a strong driver of Hg bioaccumulation. The slightly higher THg concentrations and $\delta^{15}N$ values of watersnakes, relative to queen snakes, reflected the relative isotopic and Hg composition of their primary prey, fish and crayfish, respectively [18,31]. Within the more terrestrial species, however, garter snakes had substantially higher mean THg concentrations (1.28 mg/kg) than rat snakes (0.26 mg/kg; Fig. 5C), despite lower relative trophic position. It is likely that the diet of garter snakes at the South River is composed primarily of earthworms, which have high Hg concentrations despite their relatively low trophic status [45]. Alternatively, rat snakes may be consuming mostly eggs and nestling birds, which have lower Hg levels than adults, or migratory bird species, which likely have lower Hg levels than residents [35].

CONCLUSIONS

Although the recent direction of snake ecotoxicology appears promising, we still have large gaps in our basic understanding of factors that influence contaminant exposure, accumulation, and effects. The results of the present study suggest that the habitat ecology of aquatic snakes at the South River puts them at the greatest risk of excessive Hg exposure. The THg concentrations in tail tissue of watersnakes and queen snakes are the highest reported for snake species and are among the highest reported in

numerous wildlife species studied at the South River. Although aquatic snakes had the highest Hg levels, garter snakes also had relatively high levels, suggesting that some primarily terrestrial species may also be at risk because they consume prey that contain high Hg concentrations (e.g., earthworms). Because snakes often attain high densities and serve as important prey for other animals, high Hg concentrations in snakes may have significance for the health of other wildlife. For example, predators (e.g., predatory birds, mammals, and other snakes) that eat aquatic-feeding snakes may be exposed to high Hg concentrations. The foundational information we provide will be useful to wildlife managers in prioritizing conservation efforts at the South River and other contaminated sites. Finally, high contaminant concentrations may pose serious health threats to snakes, but relatively few studies have examined possible effects. Future studies are warranted to investigate adverse effects of Hg accumulation in snakes and to determine whether these effects threaten the health and persistence of snake populations.

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