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Mercury bioaccumulation and *Hepatozoon* spp. infections in two syntopic watersnakes in South Carolina

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Abstract

Mercury (Hg) is a ubiquitous environmental contaminant known to bioaccumulate in biota and biomagnify in food webs. Parasites occur in nearly every ecosystem and often interact in complex ways with other stressors that their hosts experience. *Hepatozoon* spp. are intraerythrocytic parasites common in snakes. The Florida green watersnake (*Nerodia floridana*) and the banded watersnake (*Nerodia fasciata*) occur syntopically in certain aquatic habitats wetlands in the Southeastern United States. The purpose of this study was to investigate relationships among total mercury (THg) concentrations, body size, species, habitat type and prevalence and parasitemia of *Hepatozoon* spp. infections in snakes. In the present study, we sampled *N. floridana* and *N. fasciata* from former nuclear cooling reservoirs and isolated wetlands of the Savannah River Site in South Carolina. We used snake tail clips to quantify THg and collected blood samples for hemoparasite counts. Our results indicate a significant, positive relationship between THg and snake body size in *N. floridana* and *N. fasciata* in both habitats. Average THg was significantly higher for *N. fasciata* compared to *N. floridana* in bays (0.22 ± 0.02 and 0.08 ± 0.006 mg/kg, respectively; $p < 0.01$), but not in reservoirs (0.17 ± 0.02 and 0.17 ± 0.03 mg/kg, respectively; $p = 0.29$). Sex did not appear to be related to THg concentration or *Hepatozoon* spp. infections in either species. We found no association between Hg and *Hepatozoon* spp. prevalence or parasitemia; however, our results suggest that species and habitat type play a role in susceptibility to *Hepatozoon* spp. infection.

Keywords: trace elements; snake; diet; hemoparasite; habitat; reptile

Introduction

Mercury (Hg) is a ubiquitous environmental contaminant of particular concern for human and wildlife health because of its toxicity and persistence in the environment (Rice et al. 2014; Eagles-Smith et al. 2018). Although Hg occurs naturally (i.e., volcanic emission, forest fires, volatilization), anthropogenic activities such as mining, fossil fuel combustion, waste incineration, gold processing facilities, cement production, and caustic soda production through chlor-alkali processes have increased its mobilization and bioavailability to biota (Wang et al. 2004; Pacyna et al. 2006; Schneider et al. 2013). Methylmercury, the most toxic form of Hg, can persist in an environment for long periods of time, and is easily transferred through diet, especially in aquatic food webs (Burger et al. 2001; Hogan et al. 2007; Chumchal et al. 2011; Haskins et al. 2021a). Methylmercury uptake in wildlife occurs primarily through diet and can become widely distributed in body tissues such as liver and muscle (Green et al. 2010; Azevedo et al 2012; Drewett et al. 2013; Moore et al. 2022).

Bioaccumulation of Hg has been documented in a wide range of taxa (Wolfe et al. 1998; Nilsen et al. 2017; Rodriguez-Jorquera et al. 2017) and biomagnification has been observed in numerous aquatic and terrestrial systems, with the highest concentrations of Hg often occurring in top predators (Burger et al. 2001; Rimmer et al. 2010; Carrasco et al. 2011; Chumchal et al. 2011). The harmful effects of Hg include reduced reproductive success (Heinz 1979; Hopkins et al. 2013; Thompson et al. 2018), endocrine impairment (Dieter and Ludke 1975; Wolfe et al. 1998; Lemaire et al. 2021), overt neurotoxicity, and death (Wren et al. 1987; Heinz 1996; Scheuhammer et al. 2007). Furthermore, even if concentrations of Hg are low or exposure is chronic, less obvious sublethal effects can occur. Mercury exposure may compromise an organism's immune function, leaving them more susceptible to disease and parasite infection (Lafferty and Kuris 1999; Sures 2004; Martin et al. 2010; Marcogliese and Pietrock 2011). For example, a study investigating associations between Hg and parasite infection in common loons (*Gavia immer*) found prevalence and intensity of *Leucytozoon* parasites increased with blood Hg concentrations (Weinandt 2006). More recently, Borchert et al. (2019) found positive associations between Hg concentrations and endoparasite abundance in raccoons (*Procyon lotor*) from South Carolina and Georgia. However, a contrasting effect is also possible; parasites or their vectors may be more sensitive to contaminants than the primary hosts (Sures 2004, Martin et al. 2010, Brown et al. 2022), leading to lower parasite occurrence within a polluted system.

Despite a growing interest in the use of herpetofauna as ecological receptors, reptiles are included less often in environmental monitoring and risk assessment compared to other vertebrate taxa (i.e., birds, mammals, fish, amphibians; Hopkins 2000; Campbell and Campbell 2001; Sparling et al. 2010; Burger et al. 2017; Haskins et al. 2019a,b). Snakes in particular are well-suited for studying the accumulation and effects of contaminants, as they are relatively long-lived and exclusively carnivorous, often serving as mid-level to top predators in the ecosystems they inhabit (Gibbons and Dorcas 2004; Burger et al. 2017; Haskins et al. 2019a). Snakes also have relatively small home ranges and are likely to remain in a contaminated area throughout their lifespan (Bauerle et al. 1975; Beaupre and Douglas 2009; Drewett et al. 2013), subjecting them to chronic contaminant exposure. Collectively, these life history traits combine to make snakes a reliable bioindicator of environmental health (Campbell and Campbell 2001; Burger et al. 2017; Haskins et al. 2019a). In particular, snakes that feed primarily on aquatic prey appear to be more susceptible to accumulating contaminants compared to those that feed on terrestrial prey (Burger et al. 2006; Chumchal et al. 2011; Drewett et al. 2013).

Differences in habitat association and feeding ecology are likely to influence patterns of Hg accumulation in wildlife (Weiner et al. 2003; Lemaire et al. 2018). The bioavailability of Hg can depend on many biogeochemical processes that occur within a particular aquatic habitat

(Gilmour et al. 1992). Isolated Carolina bays found throughout the Coastal Plain of the southeastern United States have been found to exhibit high concentrations of Hg despite a lack of proximity to a point-source (Snodgrass et al. 2000a). This is likely due to several characteristics found in Carolina bays that are associated with increased Hg biomethylation rates, such as fluctuating water levels, high dissolved organic carbon, and low pH (Snodgrass et al. 2000a; Unrine et al. 2005). Aquatic habitats may also vary in prey sources available to aquatic snakes (Durso et al. 2013), which may in turn impact the bioavailability of Hg (Lemaire et al. 2018). Snakes living in aquatic habitats with shorter hydroperiods may rely more on amphibian prey sources, while diets of snakes living in larger more permanent bodies of water may include both fish and amphibians (Durso et al. 2013). Closely related syntopic species occupying habitats with broader prey resources may also partition into various trophic niches, which may in turn lead to differences in Hg bioaccumulation between species.

Habitat type and feeding ecology may also play a role in exposure to parasites. Hemogregarines of the genus *Hepatozoon* spp. are a common intraerythrocytic parasite of snakes (Smith 1996; Telford et al. 2001). Infections with *Hepatozoon* spp. parasites in aquatic snakes usually occur as a result of the ingestion of an infected intermediate host (e.g., frog or lizard) but may also be transmitted through a bite from an invertebrate vector (i.e., mosquito, leech) (Smith 1996; Telford et al. 2001). Interestingly, fish—a common staple of some watersnake diets—do not appear to be a major intermediate host of *Hepatozoon* spp. parasites (Smith 1996). Thus, the prey assemblage within a habitat is likely to affect a snake's susceptibility to *Hepatozoon* spp. infection—with infections more common in snakes that often feed on anuran prey items. Snakes inhabiting wetlands with fewer fish will be reliant on more available amphibian prey sources, potentially influencing exposure to hemoparasites and prevalence of parasitic infections (Tomé et al. 2012). Bioaccumulation of a contaminant such as Hg can directly impact a host's ability to fight off parasitic infections leading to increased prevalence and intensity of infections (Weinandt 2006). Furthermore, exposure to Hg can affect host-parasite dynamics by altering behavior and reproductive patterns of vectors and hosts, indirectly influencing transmission of parasites (Borchert et al. 2019).

In the present study, we evaluated how Hg accumulation varies with habitat type and between two species of aquatic snakes. We were also interested in elucidating how species, body size, habitat and Hg concentrations impact prevalence and parasitemia of *Hepatozoon* spp. in syntopic species. Our specific objectives were to (1) examine the relationship between tail total Hg (tail THg) and snake body size, (2) compare tail THg between species and aquatic habitat types, (3) compare *Hepatozoon* spp. infections between watersnake species and aquatic habitat types, and (4) determine associations between tail THg and *Hepatozoon* spp. infections. To meet our objectives, we sampled two syntopic species of watersnakes—the Florida green water snake (*Nerodia floridana*) and banded watersnake (*Nerodia fasciata*)—from isolated Carolina bays and a former nuclear cooling reservoir of the Savannah River Site in South Carolina. We used snake tail clips as a non-lethal sampling method to quantify THg and examined blood smears to determine *Hepatozoon* spp. prevalence and parasitemia. We predicted tail THg would increase with body size in both *N. floridana* and *N. fasciata*. Based on their suspected differences in diet, we predicted that in the reservoirs, *N. floridana* adults, which prey on fish when available, would accumulate higher concentrations of THg compared to *N. fasciata*. In contrast, we predicted THg concentrations would be similar in the two species in Carolina bays, where diet may be more likely to rely on amphibians and overlap between snake species. We hypothesized that *Hepatozoon* spp. infections would be more common in *N. fasciata* based on a higher reliance of amphibian prey items in their diet. We expected that *Hepatozoon* spp. infections would be more common for both species in isolated wetlands, where amphibian prey sources are more plentiful compared to fish. Finally, we predicted that snakes with higher tail THg would be more likely to be infected with *Hepatozoon* spp.

Methods

Study sites

The Savannah River Site (SRS) is a 780 km^2 United States Department of Energy property in west-central South Carolina. Due to past activities associated with production of nuclear weapons on the SRS, legacy heavy metals and radionuclides remain in some aquatic habitats, particularly in former nuclear cooling reservoirs. The SRS is home to a diversity of aquatic habitat types including multiple cooling reservoirs totaling 2000 hectares (ha) and more than 300 relatively unimpacted isolated wetlands and ponds (Schalles et al. 1989; White and Gaines 2000). Most of the isolated wetlands have no history of direct Hg inputs but may receive the contaminant through atmospheric deposition and runoff (Snodgrass et al. 2000a; Unrine et al. 2005). In the present study, we sampled snakes from two aquatic habitat types: a former nuclear cooling reservoir and two Carolina bays.

We collected snakes from Pond B (Fig. 1; 33°17'37.5"N 81°32'50.7"W), an 87 ha cooling reservoir with a maximum depth of 12 m constructed in 1961 to serve as a secondary cooling system for nuclear production reactors (Parker et al. 1973). The Savannah River borders the western edge of the SRS and was used as a water source for filling the constructed reservoirs and to cool nuclear reactors during operation. However, the Savannah River was contaminated with mercury by an upstream chloro-alkali plant and introduced Hg into the cooling reservoirs on the SRS (Kvartek et al. 1994; Sugg et al. 1995). Pond B received thermal effluent from one of the nuclear reactors (R Reactor) until it was shut down in 1964. Pond B water levels are now maintained exclusively by precipitation and groundwater seepage (Kennamer et al. 2005). Contaminant levels in Pond B fish have been monitored by numerous studies since the reservoir was created, with at least 11 species of fish documented (Parker et al. 1973; Whicker et al. 1990; Fulghum et al. 2019). Several studies have reported concentrations of Hg in wildlife inhabiting Pond B (Sugg et al. 1995; Gaines et al. 2002; Kennamer et al. 2005; Haskins et al. 2019), which along with radiocesium (^{137}Cs), is the primary contaminant of concern in the reservoir.

We also collected snakes from two isolated Carolina bays, Craig's Pond and Sarracenia Bay (Fig. 1; 33°17'06.2"N 81°28'39.3"W and 33°17'05.0"N 81°28'22.4"W, respectively), which have no history of Hg inputs resulting from SRS operations. Craig's Pond is the largest Carolina bay (78.2 ha) on the SRS and is semi-permanent, only drying during extended droughts (Davis and Janacek 1997). Craig's Pond also has one external and two internal ditches which allow fish to invade it during high water levels. Due to its long hydroperiod, Craig's Pond has comparatively high diversity of amphibians and smaller fish species compared to other SRS bays (Snodgrass et al. 2000). Despite being much smaller, Sarracenia Bay (4.0 ha) also has an extended hydroperiod (Janacek and Davis 1997). Because of their close proximity (approximately 200 m apart), the two bays can become connected. Prior studies have documented Hg concentrations in wildlife inhabiting these two sites as well as other Carolina Bays on the SRS (Snodgrass et al. 2000; Unrine et al. 2005; Borchert et al. 2019). Since animals can easily move between the two wetlands, we chose not to examine these two sites independently, and hereafter refer to them collectively as "bays."

Study species

The Florida green watersnake (*N. floridana*) and the banded watersnake (*N. fasciata*) are two of the nine species of *Nerodia* found in North America. Both species occur sympatrically in some parts of Florida, Georgia, and South Carolina, and they can occur syntopically in some wetlands, but relative abundance of each species varies with habitat type. *Nerodia floridana* prefer permanent lentic habitats such as reservoirs, lakes, and some isolated wetlands with extended hydroperiods (Gibbons and Dorcas 2004). In contrast, *N. fasciata* is a habitat generalist occupying wetlands with a wide range of hydrological conditions. Among aquatic habitats of the SRS in South Carolina, where the two species co-occur, *N. fasciata* is one of the most abundant snake species except in the large reservoirs, where *N. floridana* is more common. Factors that likely contribute to this pattern include interspecific differences in drought response (Willson et al. 2006; Vogrinc et al. 2018) and diet (Durso et al. 2011).

The diets of *N. floridana* and *N. fasciata* may overlap, especially as juveniles when both rely heavily on amphibians and small fish (Gibbons and Dorcas 2004; Willson et al. 2006). However, as adults, the diet of *N. floridana* consists more heavily of fish (when fish are available), while *N. fasciata* diet has a wider range of aquatic prey (Gibbons and Dorcas 2004; Durso et al. 2013). The feeding ecology of *N. fasciata* has been studied more extensively, with several studies reporting an ontogenetic shift from smaller prey (e.g., mosquitofish, tadpoles) as juveniles to large anurans (e.g., ranid frogs and toads) as adults (Mushinsky et al. 1982; Vincent et al. 2007). At the SRS, *N. floridana* are more likely to be detected in permanent wetlands containing fish, while *N. fasciata* are more common in less permanent wetlands (Durso et al. 2011).However, in wetlands without fish but where *N. floridana* and *N. fasciata* co-occur, both snake species may rely heavily on the same prey, including larval or paedomorphic mole salamanders (*Ambystoma talpoideum*; Durso et al. 2013).

Data and sample collection

We used a combination of plastic minnow traps and funnel traps to capture snakes from bays (16 May - 11 June 2017) and Pond B reservoir (11 June - 1 August 2018). We arranged 20 trap arrays around the water's edge of each aquatic habitat and checked traps daily (Seigel et al. 1995, Willson et al. 2006). Each array consisted of one funnel trap and four minnow traps, which we positioned 2-3 m apart in shallow water, with 3-5 cm of the trap remaining above the water level. We transported all captured snakes to the Savannah River Ecology Laboratory for processing, which included measuring mass (to nearest 1 g), snout-vent length (SVL; to the nearest 1 mm) and permanently marked each individual by injection of a passive integrated transponder (PIT) tag into the coelomic cavity (AVID, Norco, CA, USA; Gibbons and Andrews 2004). We determined sex by examining tail morphology and/or probing the cloaca (Fitch 1960).

We removed approximately 1.0 cm of tail tip from each snake for the quantification of total Hg (THg, using tail Hg as a proxy for whole-body Hg; Hopkins et al. 2001). We recorded the wet weight of each tail clip to the nearest 0.001 g (Sartorius Research Analytical Balance R160D, Goettingen, Germany) and stored tail clips at -70ºC until subsequent analysis. We collected blood from the caudal vein with a 25 G needle and syringe and created blood smears on microscope slides to be used for *Hepatozoon* spp. hemoparasite counts. We fixed slides with 100% methanol and stained with modified Wright-Giemsa (Diff-Quik, PolySciences Inc., Warrington, PA, USA) to facilitate visualization of erythrocytes and hemoparasites. We released snakes at their location of capture within 24-48 hours of sample collection. We released any recaptured snakes immediately after capture.

Mercury quantification

We dried tail clips in an oven for a minimum of 24 hours at 50˚C and recorded dry weight (d.w.) of each tail clip to the nearest 0.001 g (Mettler-Toledo AX504 Delta Range, Columbus, OH, USA). We quantified tail THg using thermal decomposition, catalytic conversion, amalgamation, and cold vapor atomic absorption spectrophotometry by a DMA-80 Tri-cell Direct Mercury Analyzer (Milestone, Shelton, CT, USA). Our THg analysis used a modified version of the USEPA method 7473 (USEPA 1998). We ran two blanks and two standard reference material checks, TORT-3 lobster hepatopancreas and PACS-2 marine sediment (National Research Council of Canada, Ottawa, ON), before sampling began and after every 10 samples. The detection limit for THg in tail tissue was 4.36×10^{-4} mg/kg dry mass. Average percent recoveries (± SE) for TORT-3 and PACS-2 (both n = 12) reference materials were 98.8% ± 4.76% and 101.9% ± 1.39%, respectively. We present all THg concentrations as mg/kg on a dry weight basis (Table 1; see Supplementary Table 1 for average wet weight, percent moisture, and wet/dry factor).

Hemoparasite counts

We scanned blood smears in a zig-zag manner using a standard light microscope (Zeiss Axioscope 50, Jena, Germany) at 1000x magnification using oil immersion. We counted a total of 8000 erythrocytes and recorded the number of cells infected with *Hepatozoon* spp. for each slide (Davis and Sterrett 2011). Although several species of *Hepatozoon* may infect a single species of *Nerodia*, we did not identify hepatozoa beyond the genus nor did we assess developmental stage. We determined prevalence as the proportion of individual snakes infected with at least one *Hepatozoon* spp. We calculated parasitemia (i.e., the percentage of erythrocytes infected with *Hepatozoon* spp.) using the following equation: Parasitemia= (Number of *Hepatozoon* spp. infected erythrocytes/8000 erythrocytes) *100.

Statistical analyses

We conducted all statistical analyses using program R (R Core Team 2022). We tested data for normality and homogeneity of variances using Shapiro-Wilks test of normality and Bartlett's test, respectively. We log-transformed any data that did not meet the assumptions of normality. If data transformations did not result in meeting model assumptions, the data were evaluated for outliers using the interquartile range rule. We compared THg concentrations and average *Hepatozoon* spp. parasitemia in *N. floridana* and *N. fasciata* from bays and Pond B reservoir using an analysis of covariance (ANCOVA), with species and habitat as the independent variables and SVL as the covariate. We used logistic regression to determine the importance of species, habitat, and individual-level factors (SVL, sex) in predicting the probability of *Hepatozoon* spp. infections in *N. floridana* and *N. fasciata*. We used Akaike information criterion (AIC) values to select the most supported among candidate models using an information-theoretic approach (Burnham and Anderson 1998, Burnham and Anderson 2004).

Results

During the summers of 2017 and 2018, we captured a total of 112 snakes. However, we were unable to obtain tail clips to test for Hg from seven of the captured snakes. A further five snakes were determined to be outliers due to size or THg concentration and were removed from further analyses in models. The remaining snakes sampled consisted of 34 *N. fasciata* and 10 *N. floridana* in bays and 18 *N. fasciata* and 38 *N. floridana* from Pond B reservoir (for summary information see Table 1). Overall mass for *N. fasciata* ranged from 14.0 to 266.0 g (mean = 89.12 ± 9.7 g), while mass of *N. floridana* ranged from 36.0 to 278.0 g (mean = 102.2 ± 8.0 g). Overall SVL for *N. fasciata* ranged from to 255 – 695 mm (mean = 425 ± 15.8 mm), while SVL of *N. floridana* ranged from 346 to 696 mm (mean= 474 ± 11.5 mm). An analysis of variance (ANOVA) comparing average SVL between sites indicated no significant difference in size between sites. However, a two sample t- test comparing average size between species indicated a significant difference in SVL between *N. floridana* and *N. fasciata*, with *N. floridana* averaging slightly larger (t_{98}=-2.48, $p < 0.01$).

Tail Total Mercury Concentrations

Average tail THg for all 100 snakes captured was 0.17 ± 0.008 mg/kg dry weight (range: 0.02-0.38 mg/kg d.w.; Table 1). There was a significant, positive trend of increasing tail THg with body size (SVL) for *N. floridana* and *N. fasciata* from both the bays and the former nuclear cooling reservoir (Fig. 2). Average tail THg was highest in *N. fasciata* from Carolina bays (0.25 ± 0.02), followed by *N. floridana* (0.17 ± 0.007) and *N. fasciata* (0.17 ± 0.005) from the reservoir, with the lowest occurring in *N. floridana* from Carolina bays (0.08 ± 0.007) (Fig. 3). The effect of sex on average tail THg was evaluated but did not differ significantly for either species and was excluded from further models. There were significant differences in average tail THg among groupings (species + habitat type) after controlling for the effect of SVL (ANCOVA: $F_{3,98} = 19.16$, $p < 0.001$). When comparing within habitat type, average tail THg was significantly higher in *N. fasciata* than *N. floridana* from bays ($t = -7.43$, $p < 0.001$) but differences between the species were not significant in snakes collected from the reservoir ($t = -1.56$, $p = 0.39$; Fig. 3). Within species, there was no significant difference in average tail THg between reservoir and bay for *N. fasciata* ($t = -0.86$, $p = 0.82$); however, average tail THg was significantly higher in *N. floridana* collected from reservoirs than those collected from bays ($t = 5.39$, $p < 0.001$; Fig. 3).

Hepatozoon spp. Prevalence and Parasitemia

We obtained blood smears from 100 snakes, in which overall prevalence of *Hepatozoon spp*. infections was 43% (43/100). Infections by *Hepatozoon* spp. were more prevalent in snakes of both species when captured in bays compared to when captured from the reservoir and were more common in *N. fasciata* than *N. floridana* in both habitats (Table 1). Overall average parasitemia was 4.32 ± 0.97 and was higher in both species from the bays compared to those from the reservoir. After controlling for body size, parasitemia varied significantly between habitats and species (ANCOVA: $F_{3,96} = 1.36$, $p < 0.001$). Posthoc comparisons of means revealed average parasitemia for *N. fasciata* from the bays was significantly higher than *N. fasciata* from the reservoir ($t = -3.79$, $p < 0.001$) and from *N. floridana* from both the bays ($t = - 4.08$, $p < 0.001$) and the reservoir ($t = -6.43$, $p < .001$). Average parasitemia for *N. floridana* from the reservoir was not significantly different from *N. fasciata* from reservoir ($t = -0.90$, $p = 0.53$) or *N. floridana* from bays ($t = -0.09$, $p = 0.99$) (Table 1). The most parsimonious models predicting probability of *Hepatozoon* spp. infection in watersnakes included habitat, species, and SVL (Table 2), all of which were significant predictors of infection (Table 3). Based on the best supported model, *N. fasciata* were more likely to be infected by *Hepatozoon* spp. compared to *N. floridana*, there was a weakly positive effect of size on infection probability and infections are more common in bays compared to the reservoir (Table 3).

Discussion

As expected based on known habitat affinities for the two species (Gibbons and Dorcas 2004), including from prior research on the SRS (Durso et al. 2011, 2013), we captured more *N. floridana* in the former nuclear cooling reservoir and more *N. fasciata* in the Carolina bays. As has been previously reported for THg in snakes, we found higher tail THg concentrations in larger individuals of each species, and this pattern was observed in both habitat types. A positive relationship between body size and Hg levels has been consistently documented across taxa (Bergeron et al. 2007; Loseto et al. 2008; Staudinger 2011), including several species of snakes (Rainwater et al. 2005; Lemaire et al. 2018; Rumbold and Bartoszek 2019) and specifically in watersnakes (Drewett et al. 2013; Haskins et al. 2019a; Haskins et al. 2021a; Haskins et al. 2021b, Brown et al. 2022). Within species, the relationship between body size and tail THg was stronger for *N. fasciata* from the reservoir and *N. floridana* from the bays, where fewer of each species were captured. However, body size explained only a moderate amount of the variation in tail THg for both *N. fasciata* and *N. floridana* in each habitat. Thus, additional factors are likely impacting the bioaccumulation of THg in *Nerodia* within our systems.

The observed patterns of interspecific variation between *N. fasciata* and *N. floridana* were in contrast to our expectations. We expected concentrations of tail THg to be similar between species in Carolina bays, where dietary resources between the two species would be more likely to overlap (Durso et al. 2013) and more distinct in the former nuclear cooling reservoir, with *N. floridana* accumulating more THg due to a diet expected to consist more heavily of fish. In contrasts, tail THg concentrations were similar between *N. floridana* and *N. fasciata* in the reservoir but differed significantly in Carolina bays. Our results likely reflect both ontogenetic shifts in diet as well as the size distribution of snakes sampled from each habitat type. The diet of *N. fasciata* has been more thoroughly documented than that of *N. floridana*. However, Mushinsky et al. (1982) studied the diet of both *N. fasciata* and Mississippi green watersnake (*N. cyclopion*)—a sister taxa to *N. floridana*. After reaching 500 mm SVL, *N. fasciata* exhibited a distinct shift in diet from small fish prey (*Gambusia* spp. and *Fundulus* spp.) to larger anuran prey items (*Rana* [*Lithobates*]spp. *and Bufo* [*Anaxyrus*] *spp.).* In contrast, *N. cyclopion* continued to contain high proportions of small fish even after reaching larger size classes (>500 mm SVL) (Mushinsky et al. 1982). We caught very few individuals of either species over 500 mm within the former nuclear cooling reservoir; thus, it is possible that both species in Pond B are often feeding on small fish prey items, which are abundant in the reservoir. We did observe multiple regurgitations of fish by *N. floridana*, and fish and frogs by *N. fasciata*; however, we did not force regurgitations for each snake captured. Thus, it is possible the diets of *N. fasciata* and *N. floridana* in Pond B overlap much more than expected over the size of the snakes we were able to sample, which could potentially contribute to the similar tail THg concentrations in both species in the reservoir.

Furthermore, our method of trapping may have limited our ability to observe interspecific differences in tail THg in snakes from the reservoir. We only sampled in the shallow edges of the littoral zone and were not able to sample deep water habitat where larger snakes from both species could be feeding (Aresco and James 2005). Pond B is larger and deeper in comparison to the Carolina bays; thus, there is an increased possibility for inter- and intraspecific spatial partitioning of foraging locations. While the use of funnel and minnow traps is generally accepted as the best method for capturing aquatic snakes (Seigel et al. 1995, Willson et al. 2006, Vogrinc et al. 2018), trap size may have also hindered our ability to capture individuals of both species in larger size classes. For example, Willson et al. (2008) found that conventional aquatic traps may not be useful for capturing *Nerodia* over 800 mm SVL. Modifying trapping efforts to gather samples from a broader range of snake sizes, particularly those from larger size classes, may help elucidate patterns of THg accumulation in both *N. fasciata* and *N. floridana*.

Unexpectedly, *N. fasciata* exhibited significantly higher average tail THg compared to *N. floridana* in Carolina bays. Again, this is likely related to the abundance of certain prey items and the differences in body size of species captured. In the Carolina bays we captured more *N. fasciata* over 500 mm, which have likely shifted to a diet consisting of more large anuran prey items (Mushinsky et al. 1982). We caught few *N. floridana* over 500 mm in the bays, and those individuals may be feeding on lower trophic prey items (e.g., salamander larvae, small fish, and tadpoles). Anecdotally, we observed several *N. floridana* and *N. fasciata* captured in bays regurgitate larval mole salamanders (*Ambystoma talpoideum*) after capture, while several large *N. fasciata* regurgitated adult and larval southern leopard frogs (*Rana [Lithobates] sphenocephala*). Previous research in SRS Carolina bays has documented significantly higher levels of whole-body THg in *R. sphenocephala* tadpoles (mean= 2.5 mg/kg) compared to larval *A. talpoideum* (mean= 1.0 mg/kg: Lance et al., unpublished data). Thus, if *N. floridana* are relying more often on *A. talpoideum* and *N. fasciata* are consuming more *R. sphenocephala*, it is reasonable to suspect tail THg concentrations would be higher in *N. fasciata* as a result. Further research into dietary partitioning of sympatric watersnake species and its relationship to Hg bioaccumulation is needed to further support this hypothesis.

Differences in factors affecting the bioavailability of Hg between habitat types potentially played a role in the pattern of tail THg concentrations we observed. Biota, including fish and anurans, from Carolina bays on the SRS have been documented to have elevated levels of Hg despite having no inputs of the contaminant beyond atmospheric deposition and run-off (Snodgrass et al. 2000a; Unrine et al. 2005). As noted in those previous studies, increased water fluctuation, higher dissolved organic carbon, lower pH, and anoxic conditions associated with Carolina bays likely lead to an increased bioavailability of Hg. The relative stability of water levels in Pond B and the decades that have passed since Hg-contaminated Savannah River water was introduced into Pond B have likely decreased bioavailability in the reservoir (Sugg et al. 1995; Kennamer et al. 2005). Increased bioavailability could explain higher concentrations of THg in *N. fasciata* living in bays compared to those from the reservoir. However, THg was higher in *N. floridana* from the reservoir compared to those from the bay. A potential explanation for this could be related to the higher trophic prey that are available in the reservoir compared to the bay. The larger *N. floridana* living in the reservoir have the ability to prey upon centrarchid fish (bass, sunfish) which are not found as commonly in the bays and have generally been shown to have higher THg relative to lower trophic prey items (Eagles-Smith et al. 2008; Chumchal et al. 2011). Additionally, Hg concentrations in wetlands can show variation among years (Morris et al. 2022). Due to logistical constraints, our sites were sampled in different years and we were not able to assess potential temporal variation in Hg dynamics in our study systems.

Overall, the THg concentrations in snakes from both the former nuclear cooling reservoir and Carolina bays on the SRS we sampled were relatively low compared to levels documented in other regions globally (see Drewett et al. 2013; Haskins et al. 2019a). One of the highest documented concentrations of THg in snake tail tips was observed in *N. sipedon* from Hg-contaminated parts of the South River in Virginia (13.84 mg/kg d.w.; Drewett et al. 2013) and was well above the highest concentration documented in our study (0.62 mg/kg, d.w.). In fact, the average tail THg observed in *N. fasciata* from Carolina bays was less than the average tail THg of *N. sipedon* from uncontaminated reference sites in the South River study (Drewett et al. 2013). While levels of THg observed in our study appear to be relatively low, little is known of thresholds for toxic effects of Hg on snakes (Haskins et al. 2019a).

Species, habitat type, and size (SVL) were the most important factors contributing to probability of *Hepatozoon* spp. infections in the snakes sampled in this study. Tail THg did not appear to be an important factor contributing to *Hepatozoon* spp. infections. A similar result of no association between Hg concentrations and hematozoa infections has been observed in nestling wading birds (Bryan et al. 2015). The importance of species and habitat type in probability of *Hepatozoon* spp. are likely reflective of dietary differences between species and prey resources between sites. Infections by *Hepatozoon* spp. in snakes generally occur through the ingestion of an initial vertebrate host, which for *Nerodia* spp., is often an anuran (Smith et al. 1994; Smith 1996). The overall high prevalence (32/37 individuals infected) and parasitemia in *N. fasciata* from Carolina bays could speak to their reliance on anuran prey items in comparison to their conspecific *N. floridana* – even at sites where they co-occur. Moreover, we observed higher prevalence and parasitemia for both species living in Carolina bays, where anurans are a more common prey source (Durso et al. 2013). Thus, it is not surprising that *Hepatozoon* spp.

infections are more common in snakes captured in Carolina bays. Interestingly, body size appeared to have a weak effect on probability of infection, with larger snakes being more likely to have *Hepatozoon* spp. detected. However, snakes of larger size classes (>500mm SVL) were underrepresented in our study. We must keep in mind that there were unmeasured factors that could impact *Hepatozoon* spp. infection prevalence and parasitemia. For example, additional contaminants with the potential to affect infection rates may be found in our study sites but were not measured during this study (Brown et al. 2022). Furthermore, several invertebrates may serve as initial vectors for *Hepatozoon* spp., including mosquitoes, arthropods, and annelids (Telford et al. 2001) and their presence within study sites and relationship to study species was not measured. Our analyses were limited in that we only considered one representative site for each habitat type. Even similar aquatic habitat types can differ considerably in size, biota, water chemistry and hydrology, among other factors (Schalles et al. 1989, Snodgrass et al. 2000b). Thus, a comparison across a broader array of wetlands is merited to better elucidate patterns of *Hepatozoon* spp. infections in aquatic snakes.

Conclusions

The limited research on the effects of Hg accumulation in snakes has yielded varying results in terms of potential health consequences. Some studies indicate that snakes are relatively tolerant to Hg (Wolfe et al. 1998; Chin et al. 2013a, Haskins et al. 2022), while others suggest the possibility of detrimental effects (Bazar et al. 2002; Chin et al. 2013b). We documented the bioaccumulation of THg and *Hepatozoon* spp infections in *N. fasciata* and *N. floridana* inhabiting both a former nuclear cooling reservoir and two Carolina bays on the Savannah River

Site in west-central South Carolina. Concentrations of THg in snake tail tips were relatively low and did not appear to be related to *Hepatozoon* spp infections in *N. fasciata* and *N. floridana* in this study. The monitoring of *Hepatozoon* spp. infections was perhaps the most informative in terms of revealing the potential dietary differences between species and habitat types due to the primary route of infection occurring through the consumption of amphibians rather than fish.

Our findings suggest that habitat and associated prey availability can be important factors influencing bioaccumulation of environmental contaminants and exposure to infections by hemoparasites, such as *Hepatozoon* spp. in snakes. Our results also suggest snakes feeding more often on anurans may be more susceptible to *Hepatozoon* spp. infections. Future studies examining the relationships between habitat, diet, and *Hepatozoon* spp. infections are warranted and should include a wider array of aquatic habitats and more species of aquatic snakes. The isolated Carolina bays and former nuclear cooling reservoirs of the SRS offer an excellent opportunity to study environmentally relevant concentrations of contaminants and subsequent effects in snakes and other taxa. Future studies could benefit by determining diet of captured snakes, measuring additional factors that could influence endpoints, and incorporating stable isotopes analysis to further investigate the effect of trophic dynamics on Hg accumulation and *Hepatozoon* spp. susceptibility in watersnakes.

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Declarations

Ethical approval

Snakes were collected under South Carolina Department of Natural Resources Collection Permit #'s SC-04-2017, SC-06-2018 and were handled and processed in accordance with University of Georgia's IACUC Animal Use Protocol # A-201602-006-A3.

Competing interests

The authors declare no competing interests.

Authors' contributions (CRediT format)

M. Kyle Brown: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing - Original draft, Writing - Review & Editing

David L. Haskins: Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Visualization, Writing - Review & Editing

Melissa A. Pilgrim: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing - Review & Editing

Tracey D. Tuberville: Conceptualization, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Visualization, Writing - Review & Editing

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Availability of data

Data are available upon request from the corresponding author.

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Table 1 Average (± 1 SE) snout vent length (SVL), mass, total mercury concentration (THg) in tail tips and prevalence (number of individuals infected / number of individuals sampled) and parasitemia(per 8000 erythrocytes) of *Hepatozoon* spp. infections in *Nerodia fasciata* and *N. floridana* from a former nuclear cooling reservoir and two Carolina bays of the Savannah River Site in Aiken, South Carolina. Sex ratios are reported below sample size (n). Ranges of all values are reported in parentheses below respective means.

Species and Site	***n***	**SVL (mm)**	**Mass (g)**	**Tail THg (mg/kg)**	***Hepatozoon* Prevalence**	***Hepatozoon* Parasitemia**
Reservoir						
N. floridana	38 (23F:15M)	475 ± 14 (346-696)	100 ± 9 (36-278)	0.17 ± 0.03 (0.09-0.32)	0.05 (2/38)	0.001 ± 0.001 (0.00-0.04)
N. fasciata	18 (10F:8M)	371 ± 19 (262-551)	55 ± 12 (16-200)	0.17 ± 0.02 (0.10-0.38)	0.42 (8/18)	0.98 ± 0.56 (0.00-9.90)
Bays						
N. floridana	10 (6F:4M)	469 ± 19 (366-560)	107 ± 14 (58-194)	0.08 ± 0.006 (0.05-0.10)	0.50 (5/10)	0.12 ± 0.07 (0.00-0.11)
N. fasciata	34 (13F:21M)	453 ± 21 (255-695)	107 ± 13 (18-266)	0.22 ± 0.02 (0.02-0.38)	0.85 (29/34)	12.06 ± 2.47 (0.00-47.30)

Table 2 Twelve candidate logistic regression models to predict the probability of *Hepatozoon* spp. infection in Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*Nerodia fasciata*) captured from a former nuclear cooling reservoir (Pond B) and Carolina bay system (Craig's Pond and Sarracenia Bay) on the Savannah River Site, South Carolina. The most parsimonious model is indicated in bold. Parameters included: snout-vent length (SVL), Habitat (Reservoir vs. Bay), log-transformed tail THg, and species (*N. floridana* vs. *N. fasciata*). Model values presented include log-likelihood, model degrees of freedom (K), Akaike Information Criterion (AIC), delta AIC (ΔAIC), and the weight of each model (AIC_{Wt}). *$p < 0.05$

Model	**Log-likelihood**	**K**	**AIC**	**ΔAIC**	**AIC_{Wt}**
Species + Habitat + SVL	**-37.82**	**4**	**83.65**	**0.00**	**0.67**
Species + Habitat + SVL + log(THg)	-37.74	5	85.48	1.83	0.27
Habitat + Species + log(THg)	-40.73	4	89.46	5.81	0.04
Species + Habitat	-42.54	3	91.08	7.44	0.02
Habitat + log(THg)	-45.43	3	96.85	13.20	0.00
Habitat + log(THg) + SVL	-45.32	4	98.64	14.99	0.00
Species + SVL	-47.56	3	101.12	17.47	0.00
Habitat + SVL	-49.26	2	104.52	20.87	0.00
Habitat	-50.80	2	105.60	21.95	0.00
Species	-53.44	2	110.89	27.24	0.00
log(THg)	-67.66	2	139.32	55.68	0.00
SVL	-69.95	2	143.90	60.26	0.00

Table 3 Summary of the most parsimonious model predicting the probability of *Hepatozoon* spp. infection for Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*Nerodia fasciata*) captured from a former nuclear cooling reservoir and Carolina bay system of the Savannah River Site, SC. Model parameters, estimates, and associated *p*-values are displayed

Parameter(s)	**Estimates**	**z-value**	***p*-value**
Model intercept	-1.54	-1.225	0.22
Species: *N. floridana*	-2.99	-4.11	<0.001
Habitat: Reservoir	-2.45	-4.11	<0.001
SVL	0.01	2.72	0.006

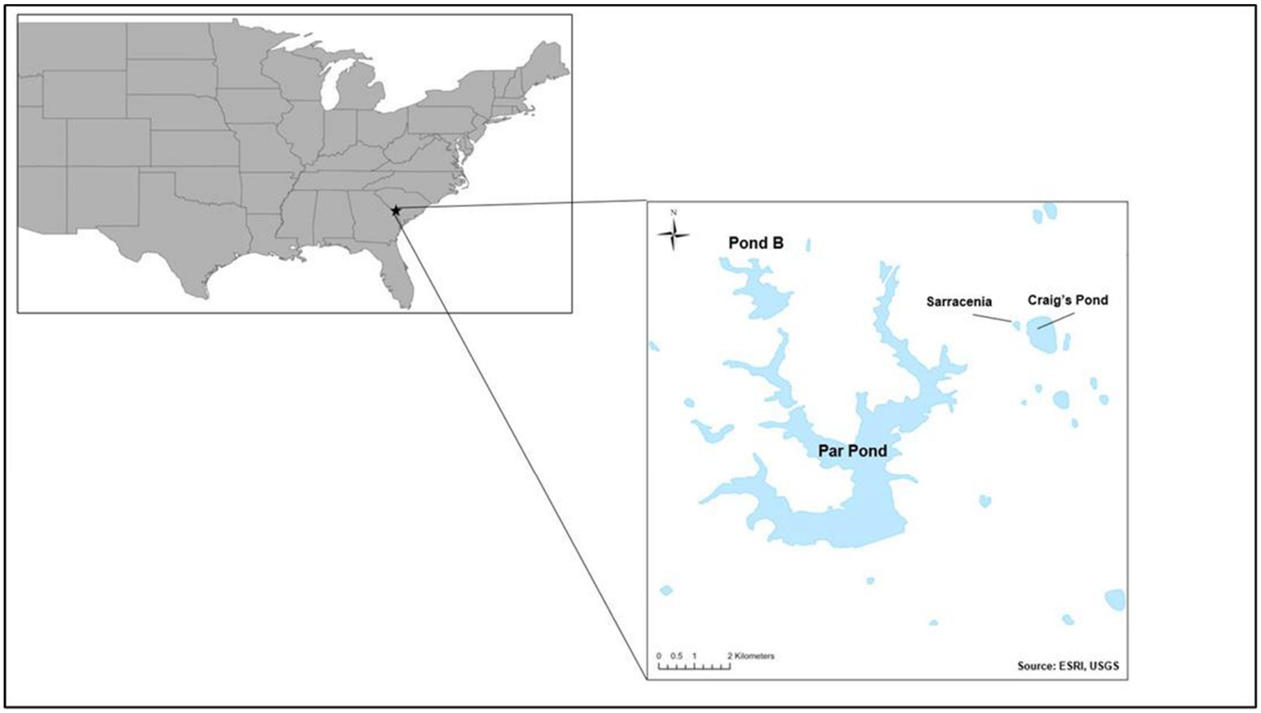

Fig. 1 Map of study sites sampled on the Savannah River Site near Aiken, South Carolina. Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*N. fasciata*) were collected from two Carolina bays (Craig's Pond and Sarracenia Bay) and a former nuclear cooling reservoir (Pond B) in the summers of 2017 and 2018 (Basemap: Esri, USGS)

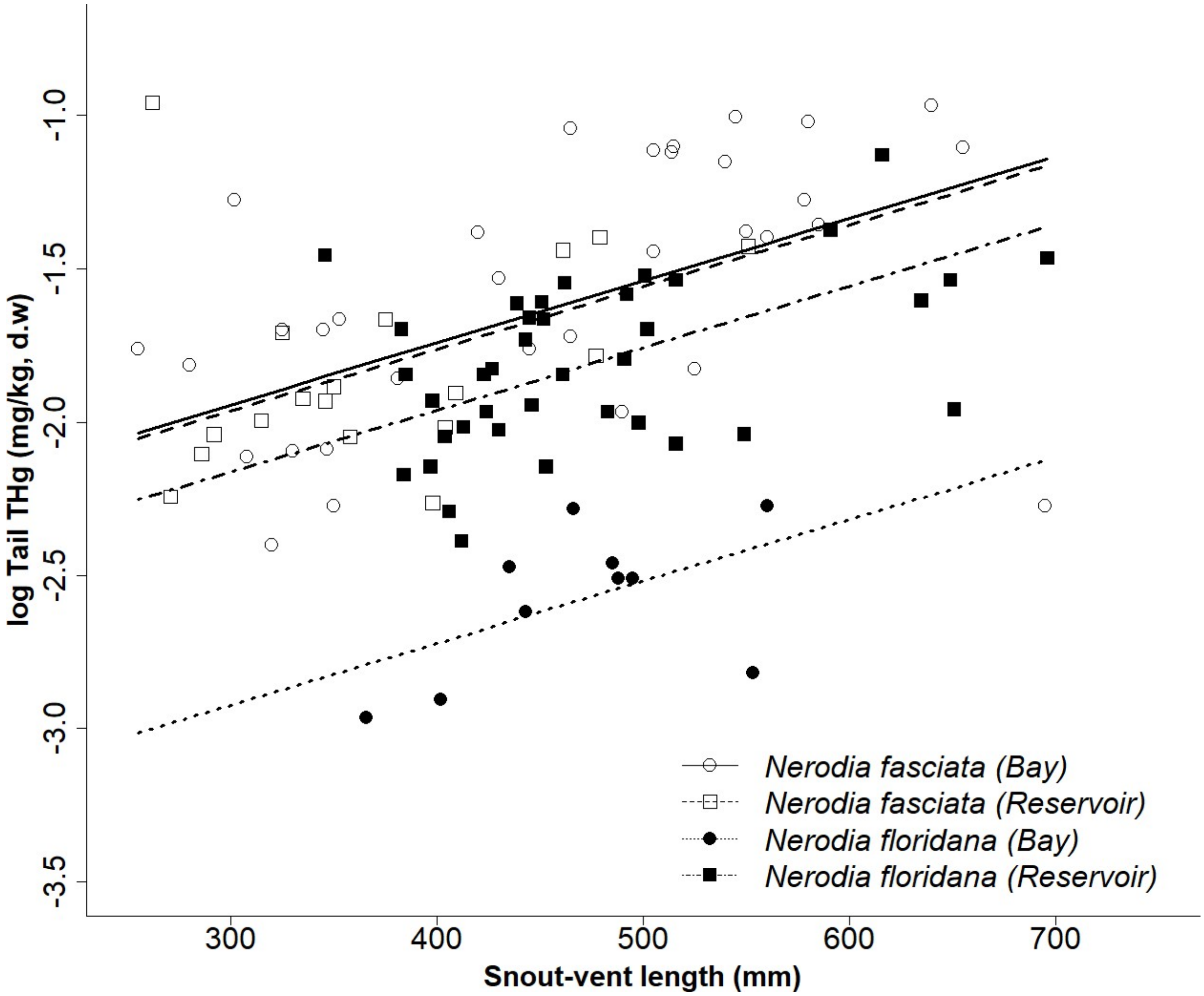

Fig. 2 The relationship between snout-vent length and tail total mercury (mg/kg, dry weight) for Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*N. fasciata*) from two Carolina bays (open shapes; Craig's Pond and Sarracenia Bay) a former cooling reservoir (filled shapes; Pond B) on the Savannah River Site near Aiken, SC. All groupings of snakes exhibited significant and positive associations ($r^2 = 0.15$-0.53, all $p < 0.05$) between snout-vent length and tail total mercury.

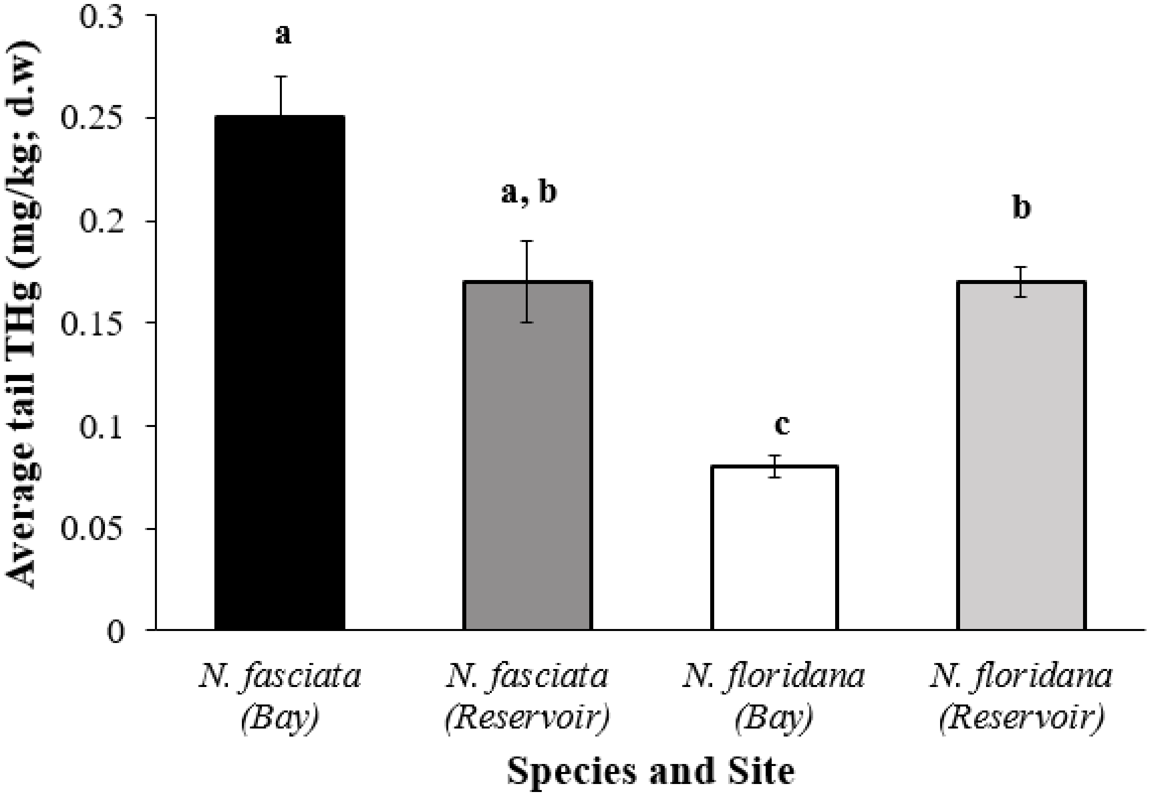

Fig. 3 Average (±1SE) tail total mercury for Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*N. fasciata*) living in a former nuclear cooling reservoir and isolated Carolina bays of the Savannah River Site, Aiken, SC. Letters indicate statistical significance based on posthoc Tukey HSD test from Analysis of Covariance

Supplemental Table 1: Average (± 1 SE) percent moisture content, wet:dry factor values, and total mercury (THg, mg/kg wet weight) for tail tissues (n=100) from Florida green watersnakes (*Nerodia floridana*) and banded water snakes (*Nerodia fasciata*) collected from a former nuclear cooling reservoir and two Carolina bays on the Savannah River Site, South Carolina. Ranges of all values are reported in parentheses below respective means.

Percent moisture	Tail THg (mg/kg; dry weight)	Wet:Dry ratio	Tail THg (mg/kg; wet weight)
62.07 ± 0.31 (56.32 – 80.30)	0.17 ± 0.008 (0.02 – 0.38)	2.66 ± 0.03 (2.28 – 5.08)	0.06 ± 0.003 (0.003 – 0.16)