

**BIOACCUMULATION OF MERCURY AND RADIOCESIUM IN
NERODIA FLORIDANA: STANDARD METABOLIC RATE, HEMOPARASITE
INFECTION, AND INTERSPECIFIC COMPARISONS TO NERODIA FASCIATA**

by

MARTY KYLE BROWN

(Under the Direction of Tracey D. Tuberville and Robert Bringolf)

ABSTRACT

Mercury (Hg) and radiocesium (^{137}Cs) are well-known environmental contaminants with the potential to impact the health of humans and wildlife. Despite having several ecological characteristics conducive to studying environmental contamination, snakes have rarely been included in biological monitoring efforts of polluted sites. I investigated the accumulation of Hg and ^{137}Cs and associations between contaminants and sublethal effects, standard metabolic rate and hemoparasite infections, in Florida green watersnakes (*Nerodia floridana*) on the United States Department of Energy's Savannah River Site (SRS). I also compared Hg accumulation and hemoparasite infection between *N. floridana* and banded watersnakes (*N. fasciata*), in former nuclear cooling reservoirs and Carolina bays of the SRS. My results demonstrate the use of watersnakes as ecological indicators and suggest interspecific differences, and that habitat and subsequently prey resources can be important determinants of accumulation of environmental contaminants and exposure to infections by hemoparasites.

INDEX WORDS: Watersnake, *Nerodia*, Reptile, sublethal effects, Natricine, *Hepatozoon*, Radionuclides, Contamination, Savannah River Site

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Anthropogenic actions have significantly altered many ecosystems globally leading to numerous impacts on wildlife. For example, the increase in environmental pollution associated with anthropogenic activities has been identified as one of the major factors contributing to the decline of vertebrate species worldwide (Gibbons *et al.* 2000, Ceballos *et al.* 2017, Goudie 2018). Contaminants can have direct impacts on the health of wildlife and can potentially lead to an increased susceptibility to other stressors, such as disease, predation, and parasitism (Relyea and Mills 2001; Kiesecker 2002; Martin *et al.* 2010; Hanlon and Parris 2014). Watersnakes (*Nerodia spp.*) are high trophic predators with links to both terrestrial and aquatic food webs and can be used as indicators to study the fate of environmental contaminants in an affected ecosystem (Hopkins 2000, Campbell and Campbell 2001, Campbell *et al.* 2005).

Mercury (Hg) and radiocesium (^{137}Cs) are long-lived environmental contaminants with the potential to bioaccumulate in biota and biomagnify up trophic levels (Drewett *et al.* 2013; Sundbom *et al.* 2003). Bioaccumulation of contaminants, such as Hg or ^{137}Cs , occurs in an organism when uptake and assimilation of a substance occurs at a faster rate than elimination, leading to gradual accumulation in the cells and tissues of the organism. Biomagnification refers to the increasing concentration of a contaminant with successive trophic positions in a food web (Barron *et al.* 2004). Compared to other vertebrates such as fish and amphibians, relatively few studies have investigated the effects of contaminant accumulation in reptiles, especially snakes (but see Hopkins *et al.* 2001, Hopkins and Winne 2006, Chin *et al.* 2013a, Chin *et al.* 2013b);

thus, determining associations between contaminant load and measures of health in watersnakes from Hg and ^{137}Cs contaminated sites will contribute new data to the field of ecotoxicology in reptiles.

As a result of past activities associated with nuclear weapons production and site operations, the Department of Energy's Savannah River Site (SRS) in Aiken, SC offers a unique opportunity for long-term environmental monitoring in areas that have a history of legacy Hg and ^{137}Cs contamination. While beneficial, environmental monitoring can be costly and time consuming. Sampling taxa from different trophic levels and life histories is important for getting a picture of the health of the entire ecosystem; however, it is usually not practical to select multiple species from a single genus. Thus, it can be critical to choose the most appropriate species for use in environmental assessments (Carignan and Villard 2002, Bal *et al.* 2018).

Snakes as Ecological Receptors

Despite a growing interest in the use of herpetofauna as ecological receptors, reptiles still lag behind other vertebrate species (i.e., birds, mammals, fish, amphibians) when it comes to studies examining the accumulation and biological effects of contaminants (Hopkins 2000, Campbell and Campbell 2001, Sparling *et al.* 2010, Burger *et al.* 2017). Snakes in particular have several ecological characteristics and life history traits that make them potentially susceptible to the deleterious consequences of contaminant exposure and accumulation. Snakes are relatively long-lived and exclusively carnivorous (Gibbons and Dorcas 2004). Like other animals of high trophic status, snakes often serve as top predators in the ecosystems where they occur and have been shown to bioaccumulate harmful contaminants through their prey (Fontenot *et al.* 2000, Chumchal *et al.* 2011, Cusaac *et al.* 2016, Burger *et al.* 2017). Additionally, snakes have small home ranges and are likely to remain in a contaminated area throughout their lifespan

(Baurle *et al.* 1975, Beaupre and Douglas 2009, Drewett *et al.* 2013), subjecting them to chronic contaminant exposure. Collectively, these traits can lead to snakes being a reliable indicator of environmental health (Campbell and Campbell 2001, Burger *et al.* 2017).

Mercury

Mercury (Hg) is one of the most well-described and pervasive environmental contaminants. Mercury emission occurs naturally (i.e., volcanic emission, forest fires, volatilization), but anthropogenic activities including mining, fossil fuel combustion, coal power plants, gold processing facilities, and cement production have drastically increased mobilization and bioavailability of the contaminant (Pacyna *et al.* 2006, Lamborg *et al.* 2014). Aquatic environments have a long history of receiving point-source Hg pollution. This is particularly true for rivers near industrial facilities that produced chlorine and caustic soda through the chlor-alkali process prior to 1987 (Wang *et al.* 2004). Mercury has been shown to bioaccumulate in a wide range of taxa (Wolfe *et al.* 1998, Nilsen *et al.* 2017, Rodriguez-Jorquera *et al.* 2017). Biomagnification of Hg has been documented extensively in both aquatic and terrestrial food webs, with the highest concentrations of contaminants almost always documented in top predators (Cabana and Rasmussen 1994, Atwell *et al.* 1998, Burger *et al.* 2001, Rimmer *et al.* 2010, Carrasco *et al.* 2011, Chumchal *et al.* 2011).

Environmental mercury occurs in several forms, but methylmercury (MeHg) is the form most commonly assimilated and harmful to biota. The bioavailability of MeHg can be impacted by many factors, most notably those that affect methylation rates. Elevated water temperatures, low pH, anaerobic conditions, and high dissolved organic carbon concentrations increase the rate of Hg methylation (Schneider *et al.* 2013). Bacteria that are responsible for the methylation of Hg reside primarily in the sediment of aquatic environments. Wetlands with fine-grained

sediments are often associated with anoxic conditions that can increase rates of biomethylation (Butler *et al.* 2007). Methylmercury is a known neurotoxin with the potential to impact neurological function (Wren *et al.* 1987, Heinz 1996, Burger 2006), physiology (Dieter and Ludke 1975, Wolfe *et al.* 1998), behavior (Chang and Annau 1984, Chin *et al.* 2013), and ability to produce viable offspring (Heinz 1979, Thompson *et al.* 2018) in a wide range of vertebrate species.

While MeHg is the primary concern regarding Hg accumulation in vertebrates, most studies examining Hg in snakes have reported total mercury (THg) concentrations (e.g., Chin *et al.* 2013b, Drewett *et al.* 2013, Lemaire *et al.* 2018). This is due in part to the relative ease of measuring THg in comparison to MeHg, which requires more in depth and costly techniques to determine Hg speciation (Bloom 1992). In addition, MeHg comprises the majority of assimilated THg. For example, MeHg has been documented to comprise 95% and 94% of THg in fish and snapping turtles, respectively (Bloom 1992, Turnquist *et al.* 2011). Similarly, 87.1 to 95.5 % of the THg concentration in tail tips of northern watersnakes (*N. sipedon*) collected from the South River in Virginia was made up of MeHg (Drewett *et al.* 2013). Therefore, THg appears to be a suitable surrogate for MeHg in most vertebrate taxa.

Mercury Accumulation and Effects in Snakes

Although there has been increased interest in Hg accumulation and effects in reptiles, studies involving snakes are fewer than those involving turtles or crocodylians (Schneider *et al.* 2013). Mercury has been shown to bioaccumulate (Campbell *et al.* 2005, Drewett *et al.* 2013), biomagnify (Chumchal *et al.* 2011), maternally transfer (Chin *et al.* 2013a), and impact offspring behavior (Chin *et al.* 2013b) in snakes. For example, neonatal banded watersnakes (*N. fasciata*) born from mothers captured in a Hg-contaminated river exhibited lower motivation to feed and

impaired striking efficiency (Chin *et al.* 2013b), which can have impacts on acquiring prey items and the ability to deter predators. A consistent finding among snake studies is a greater propensity for Hg accumulation in snakes feeding on aquatic prey compared to terrestrial feeding species—indicating that feeding ecology can play a role in susceptibility to Hg exposure (Burger *et al.* 2006, Drewett *et al.* 2013). Drewett *et al.* (2013) investigated Hg accumulation patterns in four species of snakes in a Hg-contaminated river system—northern watersnakes (*N. sipedon*), queen snakes (*Regina septemvittata*), rat snakes (*Pantherophis alleghaniensis*), and common garter snakes (*Thamnophis sirtalis*). They concluded that the primarily aquatic feeding species (*N. sipedon* and *R. septemvittata*) accumulated significantly higher levels of Hg compared to the primarily terrestrial feeding species (*T. sirtalis* and *P. alleghaniensis*). Additionally, Burger *et al.* (2006) found that brown watersnakes (*N. taxispilota*), which feed almost exclusively on catfish (*Ictalurus spp.*), had higher levels of Hg in blood compared to the cottonmouth (*A. piscivorous*), which has more of a generalist diet that includes amphibians, fish, small mammals, and other snakes.

Radiocesium (¹³⁷Cs)

Though not as globally ubiquitous or well-known as Hg, radiocesium (¹³⁷Cs) is a contaminant associated with nuclear energy and weapons production that is of great concern. A byproduct of the process of nuclear fission, ¹³⁷Cs is a long-lived (T_{1/2}=30.2 years) gamma-emitting radionuclide that has been shown to bioaccumulate in a range of taxa (Brisbin *et al.* 1974, Kennamer *et al.* 1998, Leaphart 2017) and, in some systems, biomagnify (Sundbom *et al.* 2003, Hakanson and Fernandez 2001). Radiocesium has been released into the environment due to fallout associated with nuclear weapons testing, waste from nuclear power generation and weapons productions, and accidents at nuclear power plants (Smith and Beresford 2005)). The

most familiar incidents associated with ^{137}Cs contamination are accidents that occurred at nuclear power plants in Chernobyl, in the USSR and Fukushima, Japan, in 1986 and 2011, respectively. Radiocesium is an analog for potassium (K) and can be incorporated into K transport systems as an organism assimilates food (Mettler *et al.* 2007). Once ingested by an organism, ^{137}Cs competes with K^+ for membrane protein carriers and can be incorporated in place of K^+ by membrane proteins that function in the growth and maintenance of biomass (Hakanson and Fernandez, 2001). Relman *et al.* (1957) investigated the accumulation of Cs^+ and K^+ in the skeletal muscles of frogs. During the study, researchers inhibited the activity of the Na^+/K^+ pump and determined that Cs^+ accumulation was also inhibited. Therefore, the primary mechanism of ^{137}Cs uptake appears to be through active transport pathways, such as the Na^+/K^+ pump (Relman *et al.* 1957). Once incorporated into potassium pathways, ^{137}Cs primarily accumulates in skeletal muscles (Peters *et al.* 1999). Studies of channel catfish (Peters *et al.* 1999), mourning doves (Kennamer *et al.* 1998) and slider turtles (Scott *et al.* 1986) have consistently shown the highest concentrations of ^{137}Cs accumulation occurring in muscle compared to other locations throughout the body.

Chronic low-dose exposure to ^{137}Cs can lead to several deleterious sub-lethal effects that include damage to genetic material (Iyer and Lehnert 2000, Sokolov and Neumann 2016). Exposure to ^{137}Cs can lead to direct and indirect effects that are caused by gamma ray emissions. Direct effects occur when gamma emissions strip hydrogen atoms from nearby DNA molecules, resulting in single and double-strand breakage (Shugart *et al.* 1989). Indirect effects occur when gamma rays pass through water molecules in the cell leading to the creation of hydroxyl radicals. Hydroxyl radicals are highly reactive and can cause damage to DNA if located in close proximity (Sevilla *et al.* 2016). A study of a population of yellow-bellied slider turtles

(*Trachemys scripta*) living in a ^{137}Cs contaminated reservoir (Pond B) on the Savannah River Site in Aiken, SC and a non-contaminated reference site yielded evidence for genetic effects of chronic exposure to low-levels of ^{137}Cs in a reptile species (Lamb *et al.* 1991). Flow cytometric assays showed increased intercellular variation in turtles from Pond B, indicating possible DNA abnormalities. Evidence of aneuploidy mosaicism was also observed in two Pond B turtles and may have resulted from prolonged exposure to low-level ^{137}Cs (Lamb *et al.* 1991).

Radiocesium and Snakes

While snakes are underrepresented in studies examining Hg accumulation, there are even fewer studies investigating ^{137}Cs contamination, and the few that exist were conducted decades ago (Brisbin *et al.* 1974; Staton *et al.* 1974). A study conducted in 1974 at the SRS examined levels of ^{137}Cs in aquatic and terrestrial snakes from a contaminated effluent stream and nuclear reactor cooling reservoir in comparison to those living in two unaffected reference sites (Brisbin *et al.* 1974). The results showed that both terrestrial and aquatic snakes captured in the vicinity of the effluent stream and reservoir had ^{137}Cs body burdens significantly distinguishable from the background levels, indicating snakes can be considered reliable indicators of ^{137}Cs contamination. Interestingly, ^{137}Cs levels found in a brown watersnake (*N. taxispilota*) from the effluent stream were the highest reported for any wild vertebrate predator at the time (38 Bq/g). Similar to results with Hg, snakes from the cooling reservoir known to primarily feed on aquatic prey items had significantly higher ^{137}Cs body burdens compared to terrestrial feeding species captured near the reservoir; however, there was no significant difference in body burden based on feeding type for snakes captured in or near the vicinity of the effluent stream (Brisbin *et al.* 1974). More recently, Leaphart (2017) investigated the biomagnification of ^{137}Cs in cottonmouths (*A. piscivorous*) collected from the R-Canal system at the SRS – an area where

^{137}Cs was directly released from a reactor. Cottonmouths had lower average body burdens of ^{137}Cs than lower trophic species (e.g., mosquitofish, bullfrogs, mole salamander larvae), which can possibly be attributed to cottonmouths including both terrestrial and aquatic prey sources rather than strictly aquatic prey.

Contaminant Bioaccumulation and Standard Metabolic Rate

Contaminants can have impacts at the individual and population level (Wolfe *et al.* 1998, Köhler and Triebkorn 2013), yet studies investigating the biological effects of contaminants on reptiles are scarce (Wolfe *et al.* 1998, Hopkins 2000). In some cases, reptiles appear to be more resilient to exposure compared to amphibians, birds, and mammals (Chin *et al.* 2013a, Gibbons *et al.* 2015). For example, garter snakes (*Thamnophis sirtalis*) dosed with MeHg up to 200 ug/g exhibited no observable toxic effects (Wolfe *et al.* 1998). Additionally, northern watersnakes (*N. sipedon*) exposed at 0, 0.1 and 10 ug/g MeHg were found to have no significant difference in levels of the stress hormone corticosterone (CORT) or changes to leukocyte differentials (Cusaac *et al.* 2016). However, reptiles exposed to contaminants or with elevated contaminant body burdens have shown either elevated (Hopkins *et al.* 1999) or depressed (Nagle *et al.* 2001, Sasaki *et al.* 2016, Cochran *et al.* 2018) standard metabolic rates (SMRs). For example, at a coal combustion waste contaminated wetland on the SRS, resident banded water snakes (*N. fasciata*) were found to exhibit SMRs 32% higher than those from reference sites (Hopkins *et al.* 1999). Similar results have been documented for crayfish and bullfrogs exposed to coal combustion waste (Rowe *et al.* 1998, Rowe *et al.* 2001). In contrast, SMR's for garter snakes (*T. sirtalis*) living in barren habitats of Canada contaminated with mining pollution, such as sulfur and other metal particulates, exhibited a depressed SMR along with poor body condition (Sasaki *et al.* 2016). However, because contaminant loads were not directly measured in the study, the association between depressed SMR and contaminants can only be speculated.

Standard metabolic rates are considered to be a measurement of an organism at rest, when only the most basic energy demands for survival are met (Rowe *et al.* 1998, Holliday *et al.* 2009). Stressors such as contaminants, environmental extremes, parasites, and disease can alter an individual's activity level, ability to harvest resources and assimilate food items, in addition to triggering energetically costly cellular repair mechanisms (Congdon *et al.* 2001, Hopkins *et al.* 2003). Thus, an elevated SMR may be indicative of an organism experiencing some type of physiological stress. Alternatively, depressed SMRs could be an indicator of illness or reduced food intake (Holliday *et al.* 2009, Sasaki *et al.* 2016). Presumably, snakes living in environments where contaminants are bioavailable must use valuable energy resources to combat the deleterious effects caused by constant exposure, leaving less resources available for growth, development and reproduction.

Contaminant Bioaccumulation and Hemoparasites

Elevated body burdens of contaminants such as Hg and ^{137}Cs may make hosts more susceptible to parasitism if host immunological capabilities are negatively affected (Lafferty and Kuris 1999, Martin *et al.* 2010, Marcogliese and Pietrock 2011). However, a contrasting effect is also possible; parasites or their vectors may be more sensitive to contaminants than the primary hosts, leading to lower prevalence (i.e. percentage of individuals infected) within a polluted system (Sures 2004, Martin *et al.* 2010). Hemogregarines are the most common intraerythrocytic hemoparasites found in reptiles and watersnakes are documented hosts of hemogregarines of the genus *Hepatozoon* (Smith 1996, Telford *et al.* 2001). Infections of *Hepatozoon* parasites in snakes primarily come from ingestion of an infected intermediate host (e.g., frog or lizard) but may also stem from direct infection after a bite from an invertebrate host (i.e., mosquito, leech) (Smith 1996, Telford *et al.* 2001). Interestingly, fish do not appear to be a major intermediate host of *Hepatozoon* parasites (Smith 1996). Thus, feeding ecology and prey availability can also

play a role in a snake's susceptibility of *Hepatozoon* infection—with more chances for infection occurring when feeding on anuran rather than fish prey items. Habitat type is another factor that can impact prevalence of hemoparasite infection in addition to contaminant bioavailability. Snakes inhabiting wetlands with fewer fish will likely shift to the more available amphibian prey sources, potentially impacting exposure to contaminants and hemoparasites.

Study System: Former Nuclear Cooling Reactors and Carolina Bays on the SRS

The Savannah River Site (SRS) is a 780 km² United States Department of Energy property located in west-central South Carolina. Due to past activities associated with production of nuclear weapons on the SRS, there is a history of Hg and ¹³⁷Cs contamination in some aquatic habitats, specifically in former nuclear cooling reservoirs—most notably Par Pond (1068 ha.) and Pond B (87 ha.). The SRS is located near the Savannah River and downstream of a Hg-cell type chlor-alkali plant that was active during the 1970's (Kvartek *et al.* 1994). During the active years of nuclear reactors on the SRS, Hg contaminated water from the Savannah River was pumped in to cool the nuclear reactors and ultimately ended up in the associated cooling reservoirs. Most radiocesium present within the Par Pond reservoir system was received in cooling water contaminated by a leaking fuel element in nearby R reactor from 1963-1964 (Whicker *et al.* 1990, Gaines *et al.* 2005). Despite radionuclide contamination at the SRS occurring more than 50 years ago, ¹³⁷Cs is still present in some former cooling reservoirs at levels of concern due to a 30.1 year physical half-life.

In addition to the manmade reservoir system, the SRS is home to more than 200 isolated wetlands ranging from 0.1-58 ha. in size and with varying hydroperiods (Schalles *et al.* 1989, Davis and Janacek 1997). While the manmade reservoirs account for 2000 ha. of open water, isolated Carolina bays are the primary natural lentic habitats on the SRS and are characterized by

an egg shape and high levels of water fluctuation (Schalles *et al.* 1989, White and Gaines 2000). All SRS Carolina bays dry out periodically, with the smallest only holding water during the wet season, while larger bays—such as the 57 ha. Craig’s Pond—only dry during prolonged drought (Schalles *et al.* 1989, Gibbons *et al.* 1976). Within and around aquatic habitats, over 100 species of herpetofauna occur on the SRS, including five species of watersnakes (*Nerodia spp.*; Gibbons and Semlitsch 1991).

Study Species: *Nerodia floridana* and *Nerodia fasciata*

The Florida green watersnake (*N. floridana*) and the banded watersnake (*N. fasciata*) are two of the five watersnake species found at the SRS. Although they can occur sympatrically, the relative abundance of each species varies with habitat type. Permanent lentic habitats such as reservoirs, lakes, and some isolated wetlands with extended hydroperiods are the preferred habitat for *N. floridana* (Gibbons and Dorcas 2004). In contrast, *N. fasciata* is a habitat generalist occupying wetlands with a wide range of hydrological conditions. In most aquatic habitats of the SRS, *N. fasciata* is 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). Three studies spanning pre-, during and post- severe droughts of a wetland at the SRS during the last three decades indicated the abundance of *N. fasciata* and *N. floridana* are heavily impacted by drought; however, *N. fasciata* is more likely to leave a drying wetland and quicker to rebound in post-drought years (Seigel *et al.* 1995, Willson *et al.* 2006, Vogrinc *et al.* 2018). In contrast, *N. floridana* are rarely found exiting drying wetlands and take many years to recolonize habitats after water returns (Seigel *et al.* 1995, Willson *et al.* 2006, Vogrinc *et al.* 2018). The diets of both species may overlap, especially as small juveniles when both tend to prey on

amphibians and occasionally 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 prey including fish, anurans, salamanders, and tadpoles (Gibbons and Dorcas 2004, Durso *et al.* 2013). The feeding ecology of *N. fasciata* has been studied more extensively, with numerous studies reporting an ontogenetic shift from tadpoles and small fish prey (e.g., mosquitofish, topminnows) as juveniles to large anurans (e.g., ranid frogs and toads) as adults (Mushinsky *et al.* 1982, Vincent *et al.* 2007).

Not surprisingly, *N. floridana* are more likely to be detected in more permanent wetlands containing fish, while *N. fasciata* are more likely to be detected in less-permanent wetlands lacking fish (Durso *et al.* 2011). However, in wetlands without fish, both species may rely heavily on the same prey, including larval or paedomorphic mole salamanders (*Ambystoma talpoideum*; Durso *et al.* 2013). Thus, in addition to ontogenetic shifts, diet will depend on prey availability within a wetland. Feeding ecology is likely to influence both patterns of contaminant accumulation (Lemaire *et al.* 2018) and prevalence of hemoparasite infections (Tomé *et al.* 2012). The differences in feeding ecology and behavior between *N. floridana* and *N. fasciata* may lead to one species being more sensitive to contaminant bioaccumulation and/or parasite infections. Therefore, the suitability of a particular species for environmental monitoring may be context-dependent.

Study Objectives

My masters thesis research focused on the bioaccumulation of Hg and ¹³⁷Cs in two species of watersnakes (*N. floridana* and *N. fasciata*) inhabiting the a USDOE property with a legacy of contamination associated with nuclear weapons production during the Cold War era. The ultimate goal for this research was to gain a better understanding of the factors associated

with contaminant accumulation in watersnakes, including body size, habitat, species, sex, SMR, and hemoparasite load. In Chapter 2, I will present a study examining the bioaccumulation of Hg and ^{137}Cs in *N. floridana* inhabiting three former nuclear cooling reservoirs with varying contamination histories. I measured contaminant body burdens using non-lethal techniques—whole-body counts for ^{137}Cs and tail tips for total Hg quantification. To determine the potential sublethal effects of contaminant exposure, I investigated associations between contaminant burdens and standard metabolic rate. I also examined prevalence and parasitemia rates of the intraerythrocytic hemoparasite *Hepatozoon* in *N. floridana* in association with burdens of Hg and ^{137}Cs , along with other factors such as body size and sex. I hypothesized higher contaminant burdens would be associated with elevated SMR and an increase in *Hepatozoon* prevalence and parasitemia. In Chapter 3, I compared Hg burdens, as well as *Hepatozoon* prevalence and parasitemia in *N. floridana* and sympatric congener *N. fasciata* between two distinct habitats—a former nuclear cooling reservoir and uncontaminated Carolina bays. I hypothesized that *N. floridana* would exhibit higher body burdens of Hg in reservoirs where feeding ecology is more likely to differ between species; however, I expected burdens of Hg to be similar in isolated Carolina bays where diets of the species are more likely to overlap. In comparing contaminant burdens and *Hepatozoon* prevalence and parasitemia, I hoped to determine if differences in ecology between species has the potential to make one or the other more sensitive to contaminant accumulation and subsequently a more reliable indicator of Hg and/or ^{137}Cs for environmental monitoring. Furthermore, this research will provide novel information about the utility of watersnakes as useful bioindicators for studying the uptake and fate of environmental contaminants. Collectively, this research seeks to provide novel information on the accumulation and sublethal effects associated with the exposure to Hg and ^{137}Cs in two watersnake species that

play the role of both predator and prey in aquatic and surrounding terrestrial habitats that they inhabit.

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CHAPTER 2

ASSOCIATIONS AMONG MERCURY AND RADIOCESIUM BODY BURDENS, STANDARD METABOLIC RATE AND *HEPATOZOON* INFECTIONS IN FLORIDA GREEN WATERSNAKES (*NERODIA FLORIDANA*) FROM THE SAVANNAH RIVER SITE, SC

Introduction

The production of nuclear weapons and the use of nuclear power as an alternative to the burning of fossil fuels presents an interesting conflict between national security, renewable energy and the risks associated with environmental contamination. Nuclear weapons may serve as a powerful deterrent to global conflict (Sagan 1994), but the bombing of Hiroshima and Nagasaki during World War II serve as a reminder of the potential for devastating effects on humans and the environment (Douple *et al.* 2011, Kamiya *et al.* 2015). Nuclear power is viewed by many as a cleaner, more sustainable source of energy and a safer alternative to the use of fossil fuel based energy (Brook and Bradshaw 2015, Buongiorno *et al.* 2019). However, there is a potential for events that can introduce persistent, harmful contaminants into the environment on a global scale (Steinhauser *et al.* 2014). The high-profile accidents at Chernobyl (Ukraine, 1986) and Fukushima (Japan, 2011) introduced radionuclides, including cesium (^{137}Cs , ^{134}Cs), strontium (^{90}Sr , ^{89}Sr), hydrogen (^3H), iodine (^{131}I , ^{129}I) and plutonium ($^{238-242}\text{Pu}$), into widespread regions across the northern hemisphere (Steinhauser *et al.* 2014). Some radionuclides, notably ^{137}Cs (half-life or $t_{1/2}$: 30.1 years) and ^{90}Sr ($t_{1/2}$: 28.9 yrs), can persist for many years after initial release and may cause long-lasting deleterious effects on exposed biota

(Sugg *et al.* 1995, Møller *et al.* 2013). Furthermore, nuclear reactors are sometimes powered by coal combustion facilities that emit additional harmful contaminants such as mercury (Hg) and other toxic metals. Contaminants associated with nuclear production may pose risks to both human and environmental health, even many years after introduction into the environment (Hinton *et al.* 2007). Thus, the continued monitoring of contaminant levels across taxa and ecosystems is needed to gain a better understanding of the fate and potential risk associated with the production of nuclear weapons and use of nuclear power.

Radiocesium (^{137}Cs) is a well-described environmental contaminant closely associated with the production of nuclear power and weapons. The accidents at nuclear plants in Chernobyl and Fukushima brought awareness of ^{137}Cs to the general public and generated substantial interest in its fate and environmental impact. With a physical half-life of roughly 30 years, ^{137}Cs is a gamma-emitter and can persist in water, air, and sediment for decades after release into the environment (Kennamer *et al.* 1998). An example of the long-lasting effects of ^{137}Cs fallout comes from Møller *et al.* (2013), who conducted a census of birds and insects inhabiting areas surrounding Chernobyl 25 years after the accident, discovering decreased abundances in areas with high legacy contamination. Like Hg, ^{137}Cs has been shown to bioaccumulate in wildlife (Brisbin *et al.* 1974, Kennamer *et al.* 1998, Leaphart 2017) and biomagnify in certain food webs (Zhao *et al.* 2001, Sundbom *et al.* 2003). Radiocesium is an analog for potassium and can be incorporated into potassium transport systems as organisms assimilate food (Mettler *et al.* 2007). The harmful impacts associated with ^{137}Cs exposure include acute radiotoxic and chronic sublethal effects. Acute effects vary from extreme radiation sickness and death associated with exposure to ionizing radiation (Djomina and Barilyak 2010) to increases in oxidative stress (Møller *et al.* 2005) and compromised immune function (Camplani *et al.* 1999). Damage to DNA

can result from acute or chronic ^{137}Cs exposure (Lamb *et al.* 1991, Sugg *et al.* 1995) and lead to deleterious mutations in populations over a long period of time, impacting levels of stress in individual organisms and potentially reducing overall fitness.

Mercury has been extensively studied as an environmental contaminant, much more so than ^{137}Cs . Since the beginning of the industrial revolution (late 1700s), anthropogenic activities have increased concentrations of Hg cycling globally (Lamborg *et al.* 2014). Although Hg occurs naturally (i.e., through volcanic emission, forest fires, volatilization), human activities including mining, fossil fuel combustion, gold manufacturing facilities, caustic soda produced through chlor-alkali processes and cement production have drastically increased Hg mobilization and bioavailability to biota (Wang *et al.* 2004, Pacyna *et al.* 2006, Schneider *et al.* 2013). Once released into the environment, elemental Hg can be transformed by sediment dwelling anaerobic, sulfur-reducing bacteria into the more toxic and bioavailable methylmercury (MeHg)—especially in aquatic habitats. When ingested via contaminated food items, Hg can become widely distributed throughout an organism and accumulate in various tissues, such as liver and muscle, over time (Green *et al.* 2010, Azevedo *et al.* 2012, Drewett *et al.* 2013). Mercury has been shown to bioaccumulate, increasing in concentration in an organism over time, in a wide range of taxa (Wolfe *et al.* 1998, Nilsen *et al.* 2017, Rodriguez-Jorquera *et al.* 2017). Biomagnification of Hg, an increase in concentration as trophic level increases, has also been documented in numerous aquatic and terrestrial systems, with the highest concentrations of Hg almost always documented in top predators (Cabana and Rasmussen 1994, Atwell *et al.* 1998, Burger *et al.* 2001, Rimmer *et al.* 2010, Carrasco *et al.* 2011, Chumchal *et al.* 2011). Mercury bioaccumulation has been shown to cause a variety of harmful effects across taxa, from reduced reproductive success (Heinz 1979, Thompson *et al.* 2018) and endocrine impairment (Dieter and

Ludke 1975, Wolfe *et al.* 1998) to overt neurotoxicity and death (Wren *et al.* 1987, Heinz 1996, Scheuhammer *et al.* 2007).

Among vertebrates, reptiles are understudied in the field of ecotoxicology compared to other taxa (i.e. fish, birds, mammals) despite having many attributes that make them ideal candidates for environmental monitoring (Hopkins 2000, Campbell and Campbell 2001, Sparling *et al.* 2010, Burger *et al.* 2017). Snakes in particular are relatively long-lived, strictly carnivorous, and have smaller home ranges compared to other vertebrate taxa (Burger 1992, Hopkins *et al.* 1999, Gibbons and Dorcas 2004, Haskins *et al.* 2019). Thus, they are generally exposed to bioavailable contaminants within a habitat throughout their lives, potentially making them a reliable indicator of contamination within a particular location and useful for studying the accumulation and impacts of chronic exposure (Bauerle *et al.* 1975, Beaupre and Douglas 2009, Drewett *et al.* 2013). Although not often used as indicators of environmental contamination, snakes have been shown to bioaccumulate both Hg (Campbell *et al.* 2005, Drewett *et al.* 2013) and ^{137}Cs (Brisbin *et al.* 1974, Staton *et al.* 1974). Compared to terrestrial snakes, semi-aquatic snakes (e.g., watersnakes) have been shown to bioaccumulate higher levels of both Hg and ^{137}Cs (Burger *et al.* 2006, Drewett *et al.* 2013). For example, northern watersnakes (*Nerodia sipedon*) and queen snakes (*Regina septemvittata*) accumulated significantly higher levels of Hg compared to terrestrial feeding rat snakes (*Pantherophis alleghaniensis*) and common garter snakes (*Thamnophis sirtalis*) in a contaminated area of the South River in Virginia (Drewett *et al.* 2013). Similarly, ^{137}Cs concentrations were higher in primarily aquatic feeding snakes compared to terrestrial feeding species living in or near a former nuclear cooling reservoir on the Savannah River Site in South Carolina, and were the highest concentrations documented in any wild vertebrate at the time of the study (38 Bq/g, Brisbin *et al.* 1974). Thus, semi-aquatic snakes

appear to be important indicators of Hg and ^{137}Cs levels in contaminated water systems. Furthermore, many watersnake (*Nerodia* spp.) species feed on fish that are favorites of sustenance anglers and have the potential to be used as an ecological receptor to evaluate the risks of trophic transfer of Hg and ^{137}Cs from fish to consumers.

Several studies have quantified contaminant levels in snakes (Brisbin *et al.* 1974, Staton *et al.* 1974, Bagshaw and Brisbin 1985, Hopkins *et al.* 1999, Fontenot *et al.* 2000, Burger *et al.* 2006, Murray *et al.* 2010, Lemaire *et al.* 2018). However, relatively few have measured health related endpoints in association with contaminant load (but see Hopkins *et al.* 1999, Hopkins and Winne 2006, Murray *et al.* 2010, Chin *et al.* 2013a, b, Cusaac *et al.* 2016). Standard metabolic rate (SMR) is considered to be a measurement of an organism's oxygen (O_2) consumption at rest, when only the most basic energy demands for survival are met (Rowe *et al.* 1998, Holliday *et al.* 2009). Thus, an altered SMR associated with contaminant exposure could be indicative of an organism experiencing associated physiological stress (Calow and Sibly 1990, Congdon *et al.* 2001). The effects of Hg or ^{137}Cs on SMR are not well understood and studies of the relationship between Hg and SMR in fish have yielded conflicting results (Tatara *et al.* 2001, Hopkins *et al.* 2003). Tatara *et al.* (2001) evaluated the effects of acute Hg exposure in laboratory-reared mosquitofish (*Gambusia holbrooki*) and observed an increase in SMR with higher doses of Hg; however, Hopkins *et al.* (2003) observed no relationship between Hg and SMR in a study of *G. holbrooki* chronically exposed in mesocosms.

Reptiles exposed to elevated levels of other contaminants have commonly exhibited significantly altered SMRs compared to reptiles from uncontaminated reference sites (Hopkins *et al.* 1999, Holliday *et al.* 2009, Sasaki *et al.* 2016). For example, banded water snakes (*Nerodia fasciata*) living in a coal combustion waste contaminated wetland were found to exhibit SMRs

32% higher than those from reference sites (Hopkins *et al.* 1999). Increased metabolic rates in association with contaminant exposure could indicate that resources are being diverted from growth, development and reproduction, thereby affecting the overall fitness of an organism. Alternatively, depressed SMRs were observed in garter snakes (*Thamnophis sirtalis*) exposed to contaminants including sulfur and other metal particulates that originated from mining activities (Sasaki *et al.* 2016). Depressed SMRs may be indicative of disease or prolonged lack of food, which may also be linked to concentrations of contaminants in an ecosystem (Holliday *et al.* 2009, Sasaki *et al.* 2016). Regardless of direction, metabolic perturbations associated with contaminant exposure have the potential to upset the balance of energy allocation within an organism.

Contaminant exposure may also impact an organism's immune function, leading to lower resistance to disease and parasite infections (Martin *et al.* 2010, Marcogliese and Pietrock 2011). Elevated body burdens of contaminants such as Hg and ¹³⁷Cs may make hosts more susceptible to parasitism if host immunological capabilities are negatively affected (Lafferty and Kuris 1999, Morley 2012). Immunosuppression has been documented in wildlife living in areas impacted by radionuclide contamination (Lourenço *et al.* 2011, Kesäniemi *et al.* 2019) and Hg has been negatively correlated with leukocyte counts in loggerhead sea turtles (*Caretta caretta*; Day *et al.* 2007). Alternatively, parasites or their vectors may be more sensitive to contaminants than the primary hosts, leading to lower parasite occurrence within a polluted system (Lefcort *et al.* 2002, Sures 2004, Martin *et al.* 2010). For example, a study of an aquatic snail and parasite community found that heavy metal contamination may have an indirect positive effect on host snail populations by reducing parasite diversity and abundance (Lefcort *et al.* 2002). Animals are often exposed to multiple disturbances of both natural and anthropogenic origins, and research

involving the effects of multiple stressors on reptiles is rare compared to other taxa (Sparling *et al.* 2010). Thus, there is a need for further research to understand relationships between contaminants, reptiles, and their parasites.

Study Objectives

Our goals were to investigate the bioaccumulation of Hg and ^{137}Cs in a semi-aquatic snake and evaluate associations with sublethal health endpoints. Our specific objectives were to: (1) determine relationships between body burdens of contaminants and body size, capture locations, and sex, and (2) assess the relationships between contaminant body burdens and sublethal health metrics in Florida green watersnakes (*Nerodia floridana*). Snakes were collected from three former nuclear cooling reservoirs on the Savannah River Site in South Carolina with varying contamination histories of Hg and ^{137}Cs . Non-destructive indices were used to quantify ^{137}Cs body burden (whole-body counts) and total Hg (tail clips). Sublethal endpoints measured included SMR, and prevalence and parasitemia of *Hepatozoon* spp., an intraerythrocytic parasite common in snakes. For each endpoint measured, the relationship between capture site, sex, and body size was also evaluated. This research has the potential to provide novel information on the factors that predict Hg and ^{137}Cs accumulation and subsequent associations with health metrics in aquatic snakes. Because the majority of the contamination in our study system occurred more than 30-50 years ago, our study will also provide valuable information on the environmental fate of legacy contaminants associated with nuclear production.

Methods

Study system

The Savannah River Site (SRS) is a 780 km² United States Department of Energy property located in west-central South Carolina. After its creation in the 1950's, access to the

land encompassed by the SRS became restricted to public access and agricultural lands were converted back to forest (Workman and McLeod 1990, White and Gaines 2000). Along with reservoirs constructed for nuclear operations, the SRS is home to extensive stream systems and more than 200 isolated wetlands (Schalles *et al.* 1989), providing freshwater for terrestrial, semiaquatic, and aquatic wildlife species. As a result, the SRS has become a refuge to an abundance of plants and wildlife, which have benefitted from the lack of anthropogenic disturbance and habitat fragmentation within the site's borders (White and Gaines 2000, Kilgo and Blake 2005). However, there is a history of Hg and ^{137}Cs contamination in some SRS habitats, specifically in former nuclear cooling reservoirs, which cover 2000 ha of the site. The Par Pond reservoir system was used to cool thermal effluent from two of the site's five reactors (all now no longer operational) and consists of canals and several cooling reservoirs. Of the former nuclear cooling reservoirs sampled in this study, Par Pond is the largest (1068 ha), followed by Pond B (87 ha) and Pond 2 (23 ha) (Figure 2.1).

From 1958 until 1964, Par Pond received effluent water directly from R-Reactor. Beginning in 1961, cooling water from R-Reactor was sent through a new canal system that included R-canal and Pond B, until R-Reactor was decommissioned in 1964. Effluent was contaminated with ^{137}Cs as a result of faulty fuel rods, and peak release of contaminated cooling effluent occurred in 1963 and 1964, with an estimated 5.7×10^{12} Becquerels (Bq) introduced into the Par system (Ashley and Zeigler 1980). Another reactor, P-Reactor, was constructed in 1961 and discharged into a system of canals and ponds, including Pond 2 and Par Pond, until 1988 when the reactor was closed (Halverson and Noonkester 1998). While there have been documented releases of ^{137}Cs from R-reactor to both Pond B and Par Pond, there have been no documented ^{137}Cs releases from P-reactor directly into Pond 2. However, secondary ^{137}Cs

contamination of Pond 2 due to recirculated water from Par Pond and prior releases from R-reactor has been documented (Whicker *et al.* 1990, Halverson and Noonkester 1998). A gamma-detecting ^{137}Cs -detecting flyover in 1991 (Feimster 1993) and a subsequent 1998 survey indicated Pond B had the highest levels of surface contamination compared to Par Pond and Pond 2, suggesting that ^{137}Cs levels and bioavailability may be elevated in the Pond B reservoir.

Mercury was used or generated as part of site operations of the SRS, and prior to 1986 there were few regulations that required the SRS to track the quantities of Hg released, except in the uranium separation and tritium facilities. Most of the Hg used on-site was recycled or disposed of in seepage basins, underground waste tanks, or solid waste disposal facilities (Kvartek *et al.* 1994). The combustion of coal used for steam energy at the SRS also released into the atmosphere and surrounding waters an estimated 600 kg of Hg prior to 1974 and 556 kg from 1980-1993. Additionally, a Hg-cell type chlor-alkali plant off-site and upstream of the SRS discharged up to 5 kg of Hg per day into the Savannah River in the 1970's, eventually reducing operational discharges to 0.11 kg per day (Kvartek *et al.* 1994). The Savannah River borders the western edge of the SRS and water from the river, contaminated with Hg by upstream sources, was pumped to cool SRS nuclear reactors during production. This water was then discharged into the cooling reservoir systems, including Par Pond, Pond B, and Pond 2.

Study Species

The Florida green watersnake (*Nerodia floridana*) is one of five species of *Nerodia* found at the SRS and is the largest watersnake found in North America, reaching a snout-vent length of 76-180 cm and a mass up to 1880 g (Gibbons and Dorcas 2004, Buhlmann *et al.* 2005). *Nerodia floridana* may be dietary generalists as juveniles, feeding on anurans, salamanders, and small fish, but switch to a diet consisting mostly of fish as adults (Gibbons and Dorcas 2004, Vogrin

et al. 2013). Compared to other aquatic snakes, *N. floridana* appear to be poor overland dispersers and are heavily impacted by drought; the species was extirpated from Ellenton Bay (an isolated wetland on the SRS) for many years after prolonged droughts (Seigel *et al.* 1995, Willson *et al.* 2006, Vogrinc *et al.* 2018). Although it is scarcely found among the smaller, less permanent bodies of water of the SRS, *N. floridana* is a common inhabitant of the former nuclear cooling reservoirs, potentially making them an ideal species for long term environmental monitoring of Hg and ^{137}Cs at the SRS.

Data collection

Snakes were captured using a combination of plastic minnow and metal funnel traps. Traps were set in 20 arrays around the water's edge at each of the three reservoirs. Each array consisted of one funnel trap and four minnow traps, positioned 2-3 m apart in shallow water, with 3-5 cm of the trap remaining above the water level. Traps were checked each morning between 0700 and 1100 hours from 10-30 June 2016. Captured snakes were transported in snake bags back to the Savannah River Ecology Laboratory for initial processing, which included determining mass and giving the individual a unique identification number.

Whole-body ^{137}Cs determination

Whole-body ^{137}Cs counts were obtained within 48 hours of capture with a 10.2 cm x 15.2 cm NaI(Tl) gamma detector (Bicron Model 6H3Q/5; Bicron, Torrington, CT, USA) coupled to a computer equipped with gamma spectroscopy software (Canberra Genie, Canberra Industries, Meriden, CT, USA), which used a counting window of 596-728 kiloelectron volts (keV) to record total absorptions of ^{137}Cs at 662 keV. The instrument was calibrated daily using a traceable ^{137}Cs calibration chip (Gamma Reference Disc Source Set, Catalogue No. NES-101S, ^{137}Cs disc; New England Nuclear, Boston, MA, USA) and adjusting the system amplifier gain

control to center on the disc-generated peak of channel 331, which corresponded to 661.7 keV. Before each series of whole-body counts, a background count was taken by placing an empty holding container into the detector for 30 minutes. Whole-body counts of individual snakes were acquired by placing the snake in a holding container for a counting period of 15 minutes. Aqueous standards containing known ^{137}Cs quantities were counted to produce background corrected count rates (counts per second) that were used to produce mass specific yield counts. The mass specific yield counts were then used to create a predictive equation of expected yields for varying sample mass ($\text{yield}=0.4449*\text{mass}^{(-0.343)}$) (Kennamer *et al.* 1998). Whole-body counts of snakes were adjusted for background rate and were used with mass specific yields to determine whole-body ^{137}Cs (Bq). We then converted whole-body ^{137}Cs to Bq/g by dividing by the mass of the snake. Minimum Detectable Concentrations of ^{137}Cs counts were calculated by the equation described in Currie (1968).

Standard metabolic rate measurements

Snakes were housed at the Savannah River Ecology Laboratory's Animal Care Facility for 7-10 days and food was withheld to ensure that snakes were post-absorptive and SMR would not be affected by digestion of food items. Standard Metabolic Rate (SMR) was measured in respirometry trials using a flow-through respirometer (Field Metabolic System, FMS; Sable Instruments, Las Vegas, NV). Respirometry trials ran from 0700-1500 hours, the period in which *N. floridana* were predicted to be least active (Gibbons and Dorcas 2004) and at 27°C, a temperature shown to be preferable by closely related *Nerodia* (Lillywhite 1987, Mills 2002). A maximum of three snakes were run per trial, with each snake housed in individual plastic metabolic chambers and placed inside the FMS cooler, which was dark to minimize visual disturbances to snakes during respirometry trials. Each chamber had an 'in' and 'out' flow airline

and individuals experienced constant airflow, with flow rates ranging from 30 to 150 mL/min depending on snake mass. An empty chamber was also included in each trial to serve as a baseline. Snakes were allowed to acclimate to their chambers for 75 minutes prior to the 30 min period during which O₂ consumption (VO₂; mL O₂/hr) was recorded. Individual snakes were monitored sequentially and baseline measurements were collected from the empty chamber every hour to allow for corrections to VO₂ data due to lag and drift. Resting VO₂ values were calculated from raw metabolic data with ExpeData-P Data Analysis Software (Sable Systems, Las Vegas, NV, USA).

Morphometrics and sample collection

After SMR trials, snake sex was determined by examining tail morphology and/or probing the cloaca. Snout-vent length (SVL; length from tip of snout to cloaca) and tail length (TL; length from cloaca to tail tip) were measured to the nearest 0.1 cm. Female snakes were assessed for gravidity by gentle palpation of the abdomen. For the quantification of total Hg (THg), snake tail clips were used as a proxy for whole-body Hg (see Hopkins *et al.* 2001). Approximately 1.0 cm of tail tip was removed from each snake, unless the snake was missing a substantial amount of its tail (within ~1.0-1.5 cm from vent) before capture. The wet weight of each tail clip was recorded to the nearest 0.001 g (Sartorius Research Analytical Balance R160D, Goettingen, Germany) and tail clips were stored at -70°C until subsequent analysis. During the process of collecting tail clips, blood was collected from the cut tail by syringe with a 25 G needle and used to create smears on microscope slides for *Hepatozoon* counts. Slides were fixed with 100% methanol and stained with modified Wright-Giemsa (Diff-Quik, PolySciences Inc., Warrington, PA, USA) to facilitate visualization of erythrocytes and hemoparasites.

Mercury quantification

Tail clips were removed from the freezer and dried in an oven for a minimum of 24 hours at 50°C. Dry weight of each tail clip was recorded to the nearest 0.001 g (Mettler-Toledo AX504 Delta Range, Columbus, OH, USA). Total mercury (THg) quantification in tail clips was accomplished using decomposition, catalytic conversion, amalgamation, and atomic absorption spectrophotometry by a DMA-80 Tri-cell Direct Mercury Analyzer (Milestone, Shelton, CT, USA). For quality assurance, two blanks and two standard reference material checks, TORT-3 lobster hepatopancreas and PACS-2 marine sediment (National Research Council of Canada, Ottawa, ON), were run on the machine before sampling began and after every ten samples. To clean the DMA-80 and ensure proper Hg analysis, flour, nitric acid, and three blanks were run after every 20 samples. The detection limit for THg in tail tissue was defined as three times the standard deviation of the procedural blanks (0.0000475 ppm dry mass). The average percent recoveries for TORT-3 and PACS-2 reference materials were 106.0% (range: 102.9-109.8%, n=9) and 96.7% (range: 89.0-110.3%, n=9), respectively. All THg values are reported in mg/kg dry weight.

Hemoparasite counts

Blood smears were scanned in a zig-zag manner using a standard light microscope (Zeiss Axioscope 50, Jena, Germany) at 1000x magnification using oil immersion. A total of 8000 erythrocytes and the number of cells infected with *Hepatozoon* spp. were counted for each slide. Although several species of *Hepatozoon* may infect a single species of *Nerodia*, hepatozoa were not identified beyond the genus or developmental stage in this study. Prevalence is presented as the ratio of individuals infected with at least one *Hepatozoon* to those with no observed infected cells. Parasitemia is the percentage of cells with a *Hepatozoon* in an infected individual and was

calculated using the following equation: Parasitemia= (Number of *Hepatozoon* infected erythrocytes/8000 erythrocytes) *100.

Statistical analyses

Statistical analyses were performed using program R (R Core Team, 2018). Data were tested for normality using Shapiro-Wilks test of normality, and homogeneity of variances was assessed using Bartlett's test or Levene's statistic. Data not meeting the assumptions of normality were log-transformed prior to analysis. Preliminary Pearson's correlations were determined among variables to assess for issues of multi-collinearity. If any two variables had correlations where $r \geq 0.80$, one of the variables was excluded from final models. Analysis of covariance (ANCOVA) was used to test for differences in tail THg and whole-body ^{137}Cs among sites, while controlling for the effect of snake body size (SVL).

A logistic regression was used to evaluate the relationships between site- and individual-level factors associated with presence of *Hepatozoon* infections in *N. floridana*. Multiple linear regression models were used to determine the relationships of factors associated with response variables of THg and whole-body ^{137}Cs burdens. Variables in the models included mass, site, sex, and interaction between site and mass. Separate linear mixed effects models were used to investigate associations between the sublethal effects of interest (i.e., oxygen consumption (VO_2) or *Hepatozoon* parasitemia as response variables) and potential explanatory variables, including mass, whole-body ^{137}Cs , tail THg, site, and sex. Snake ID was included as a random effect if it improved relative standard error (RSE) of the model. Some of the variables (i.e., SMR, ^{137}Cs , THg, SVL) were transformed to meet assumptions of models used but are presented in tables and graphs as untransformed raw data. In all cases a logarithmic transformation was used, with the exception being ^{137}Cs , which was scaled by adding 0.1 (to prevent log-transformation of

negative values) and then log-transformed. A quadratic effect of mass on VO_2 was evaluated in preliminary models but did not improve RSE and was excluded from further consideration. Akaike information criterion (AIC) values were used to select the most supported among candidate models based on an information-theoretic approach (Burnham and Anderson 1998, Burnham and Anderson 2004).

Results

A total of 78 *N. floridana*—11 from Par Pond, 23 from Pond B and 44 from Pond 2—were captured from 10-30 of June 2016. Of the snakes captured, 56% were male (n=44) and 44% (n= 34) were female. Female *N. floridana* ranged in mass from 18.0 - 545.0 g (mean \pm 1 SE = 179.0 ± 26.9 g), males ranged from 22.0 – 154.0 g (mean \pm 1 SE = 65.9 ± 6.3 g). Female *N. floridana* ranged in SVL from 29.0 – 82.0 cm (mean = 54.0 ± 3.1 cm), males ranged from 30.0 – 61.5 cm (mean = 41.7 ± 1.3 cm). Analysis of variance on log-transformed SVL indicated *N. floridana* captured from Par Pond had significantly higher average SVL compared to snakes from Pond B which were significantly larger than snakes from Pond 2 (Table 2.1; $F_{2,73} = 16.73$, $p < 0.001$).

Contaminant Burdens: Radiocesium

Whole-body ^{137}Cs burdens were transformed for statistical analysis but averages (mean \pm 1 SE) presented in text and tables are based on raw data to facilitate comparisons. Average whole-body ^{137}Cs for all 78 *N. floridana* captured was 0.23 ± 0.08 Bq/g (range: 0.00-1.02 Bq/g). Average whole-body ^{137}Cs was higher in females (0.30 ± 0.06 Bq/g) than in males (0.17 ± 0.05 Bq/g), but differences were not statistically significant after controlling for the effect of body size (ANCOVA: $F_{1,74} = 0.61$, $p = 0.43$). Snakes from Pond B had the highest average whole-body ^{137}Cs burdens, which were significantly higher than those from Par Pond (Tukey's HSD: $t = 8.21$,

$p < 0.001$) or Pond 2 (Tukey's HSD: $t = 14.03$, $p < 0.001$) after controlling for the effect of SVL (Table 2.1, ANCOVA: $F_{2,74} = 106.94$, $p < 0.01$). Whole-body ^{137}Cs was significantly and positively correlated with snake body size at both Par Pond (Pearson's $r = 0.75$, $p < 0.01$) and Pond B (Pearson's $r = 0.53$, $p < 0.01$), but not at Pond 2 (Figure 2.2). Based on AIC and model weight, the most parsimonious model for whole-body ^{137}Cs burdens in *N. floridana* included site (Par Pond, Pond 2, Pond B), mass, and the interaction of site and mass (Table 2.2) – all of which were significant predictors of whole-body ^{137}Cs (Table 2.3). However, sex was also included in a model with $< 2 \Delta\text{AIC}$ compared to the best supported model.

Contaminant Burdens: Mercury

Average tail THg for all 78 *N. floridana* captured was 0.33 ± 0.03 mg/kg dry weight (range: 0.16-2.10 mg/kg). There was a trend of increasing tail THg with body size (Figure 2.3). After controlling for the effect of body size, average tail THg in females (0.42 ± 0.07 mg/kg) and males (0.26 ± 0.01) was not significantly different (ANCOVA: $F_{1,74} = 1.16$, $p = 0.28$). Tail THg was highest in snakes from Par Pond but was not significantly different among sites after controlling for the effect of body size on average tail THg (Table 2.1, ANCOVA: $F_{2,74} = 1.84$, $p = 0.16$). There was a significant, positive relationship between tail THg and SVL (Figure 2.3, Pearson's $r = 0.52$, $p < 0.01$). Based on AIC and model weight, the most parsimonious model predicting THg burden in *N. floridana* tail tips included only mass as a significant predictor (Table 2.4, Table 2.5). Mass was also the only variable of significance in the next three most supported models, all $> 2 \Delta\text{AIC}$ compared to the best supported model.

Hepatozoon Prevalence and Parasitemia

Blood smears were obtained from 67 snakes (21 from Pond B, 42 from Pond 2, 4 from Par Pond). Due to low sample size, blood smears from Par Pond snakes were not considered

further. Mean prevalence of *Hepatozoon* spp. in all snakes sampled from Pond 2 and Pond B was 58.7% (37/63). Prevalence of *Hepatozoon* infection was higher in Pond 2 than in Pond B (73.8% vs. 28.6%). Likewise, mean parasitemia was higher in snakes from Pond 2 than Pond B (0.13 ± 0.04 , 0.03 ± 0.02 , respectively) but differences were not significant after controlling for snake body size (SVL; ANCOVA: $F_{1,60}=1.36$, $p=0.24$). The most parsimonious models predicting *Hepatozoon* spp. infection (0=no, 1=yes) in *N. floridana* from Pond 2 and Pond B included ^{137}Cs , mass, site, and the interaction of ^{137}Cs and mass (Table 2.6). However, only ^{137}Cs and the interaction of ^{137}Cs and mass were significant predictors (Table 2.7). Based on the best supported model, the probability of *Hepatozoon* spp. infection decreased significantly as ^{137}Cs whole body burdens increased (Figure 2.4).

Standard Metabolic Rates

Individual VO_2 values were log-transformed for models but presented as averages (mean ± 1 SE) of raw data in text and tables/figures to facilitate comparisons. Overall, VO_2 for all 78 *N. floridana* ranged from 0.04 - 56.75 mL O_2/hr (mean: 12.57 ± 1.16 mL O_2/hr). There was a significant, positive relationship between VO_2 and mass (Figure 2.5, Pearson's $r=0.80$, $p < 0.01$). Average VO_2 appeared to differ among sites, with snakes from Par Pond exhibiting the highest average VO_2 compared to those from Pond B or Pond 2 (Table 2.1). However, an ANCOVA of log-transformed VO_2 indicated no significant difference among sites (ANCOVA: $F_{2,74}= 2.18$, $p=0.12$), but mass was a significant covariate ($F_{2,74}= 37.67$, $p < 0.01$), with VO_2 increasing with increasing mass. Of the models we investigated, the most parsimonious model predicting VO_2 in *N. floridana* included mass and ^{137}Cs body burdens (Table 2.8), with only mass as a significant predictor but with the effect of ^{137}Cs approaching significance (Table 2.9). Mass was also the

only significant predictor in the next three most supported models with $<2 \Delta\text{AIC}$ compared to the best supported model.

Discussion

The observed among-reservoir variation in ^{137}Cs body burdens in *N. floridana* from Pond B, Par Pond and Pond 2 was consistent with the known contamination histories of the three former nuclear cooling reservoirs. Pond B was the recipient of $\sim 5.7 \times 10^{12}$ Bq of ^{137}Cs discharged from R-Reactor between 1961-1964 (Carlton *et al.* 1992), whereas Par Pond and Pond 2 received less contaminated effluent (Ashley and Zeigler 1980, Whicker *et al.* 1990, Sugg *et al.* 1995). From 1961-1964, water contaminated with ^{137}Cs from R-reactor would have first flowed into Pond B before reaching Par Pond and Pond 2 (Figure 2.1). Thus, it is likely that differences in whole-body ^{137}Cs patterns in *N. floridana* are related to the effluent flow from R-Reactor to the reservoirs. Furthermore, the overall persistence of ^{137}Cs in Pond B has been documented with longer ecological half-lives found in resident fish compared to those from Par Pond (Paller *et al.* 1999). More recently, Fulghum *et al.* 2019 found higher concentrations of ^{137}Cs in littoral zone fish from Pond B compared to fish from other canals and ponds closer on the effluent path to R-reactor. Similarly, the higher burdens of ^{137}Cs documented in *N. floridana* from Pond B seem to reflect an increased bioavailability and persistence of the contaminant in the reservoir. Our findings are also consistent with those observed in alligators at the SRS, with individuals from Pond B exhibiting higher body burdens of ^{137}Cs compared to those living in Par Pond (Tuberville, *unpublished data*).

In contrast to differences observed for whole-body ^{137}Cs in *N. floridana*, patterns of THg in snake tail tips were similar among reservoirs. Age of a reservoir has been shown to influence patterns of THg accumulation in fish (Willacker *et al.* 2016), with maximum concentrations

occurring 5-13 years after impoundment (Schetagne and Verdon 1999). Par Pond, Pond B and Pond 2 were all created between 1958 and 1961; thus, with respect to reservoir age, it is not surprising that snakes from all reservoirs might have similar Hg concentrations. Although differences were not statistically significant, the highest tail THg was observed in *N. floridana* from Par Pond, which may be related to a more recent influx of water from the Savannah River. In 1991, water levels in Par Pond were lowered for three years to make repairs to a retention dam (Kennamer *et al.* 1998). After repairs were completed, water from the Savannah River was used to refill Par Pond, potentially introducing and redistributing Hg in the reservoir. In contrast, Pond B and Pond 2 have not received water from the Savannah River since reactors were closed, and these reservoirs have remained relatively undisturbed since 1964 (Sugg *et al.* 1995) and 1988, respectively (Halverson and Noonkester 1998). Because we captured only 11 snakes from Par Pond, our ability to detect THg differences in *N. floridana* among reservoirs may have been hindered by a small sample size. Thus, further sampling is warranted to elucidate any potential differences in THg in *N. floridana* among SRS reservoirs.

Not surprisingly, capture site and individual body size were important predictors of whole-body burdens of ^{137}Cs . Body burdens increased with body size for *N. floridana* captured from Pond B and Par Pond, but not *N. floridana* from Pond 2, likely reflecting the lower bioavailability of ^{137}Cs in Pond 2. The observed relationship is consistent with previous work showing increases in ^{137}Cs with body size in fish (Rowan *et al.* 1998), and slider turtles in Pond B (Peters and Brisbin 1996). Interestingly, our results in *N. floridana* contrast a previous finding of no relationship between body size and ^{137}Cs in cottonmouths (*Agkistrodon piscivorus*) captured from R Canal, which is in the immediate vicinity of Pond B (Leaphart 2017). The conflicting results between studies could be related to differences in feeding ecology of the study

species, as *N. floridana* are primarily piscivorous (i.e., consume mostly fish), while *A. piscivorus* are more generalist in nature (Ditmars 1912). Both species experience ontogenetic shifts in diet but *N. floridana* prey on aquatic prey throughout their lives, while *A. piscivorus* diet may shift to include more terrestrial prey (e.g., small mammals, birds, other snakes) once snakes reach larger body sizes (Eskew *et al.* 2009). Thus, at sites where ^{137}Cs is primarily in the aquatic environment (as in our study system), *N. floridana* are likely exposed to similar levels of ^{137}Cs in prey throughout their life, but *A. piscivorus* exposure to ^{137}Cs may diminish with age.

Concentrations of THg in tail tips increased with increasing body size in *N. floridana* from all three reservoirs sampled. Similar trends of a strong positive relationship between body size and THg have been observed for Northern watersnakes (*N. sipedon*) in Virginia (Drewett *et al.* 2013), cottonmouths (*A. piscivorus*) in Texas (Rainwater *et al.* 2005), Burmese pythons (*Python bivittatus*) in Florida (Rumbold and Bartoszek 2019) and viperine snakes (*Natrix maura*) in Europe (Lemaire *et al.* 2018). Increasing THg burdens with increasing body size does provide support for bioaccumulation (Wolfe *et al.* 1998). However, the positive relationship could also be attributed to ontogenetic shifts in diet and metabolism differences between juvenile and adult snakes (Peters and Brisbin 1996, Drewett *et al.* 2013). Generally, body size is correlated with age in snakes (Halladay and Verrell 1988); thus, age is also a likely factor determining THg as older individuals have had more time to accumulate Hg (Hopkins *et al.* 2013). Interestingly, concentrations of THg in tails of *N. floridana* in the present study are similar to or lower than those observed in Drewett *et al.* (2013) in *N. sipedon* from reference sites in the South River of Virginia. Overall, tail THg in *N. floridana* from former nuclear cooling reservoirs of the SRS are relatively low compared concentrations obtained in other Hg contaminated areas around the

globe (see Drewett *et al.* 2013, Haskins *et al.* 2019), and several decades have passed since the influx of Hg into the reservoirs via Savannah River water.

We expected to find an increase in hemoparasite prevalence and parasitemia in association with increasing body burdens of contaminants but this was not supported by our results. Our analyses gave no indication of associations between *Hepatozoon* spp. infections and tail THg. In direct contrast to our hypotheses, we did find that *Hepatozoon* spp. infections may decrease with increasing ¹³⁷Cs body burdens in *N. floridana*. Infections were much more common for snakes captured from Pond 2 compared to Pond B and the probability of *Hepatozoon* spp. infection decreased with increasing ¹³⁷Cs. Our results suggest that ¹³⁷Cs could be impacting *Hepatozoon* spp. infections in *N. floridana* either by directly harming the parasite, or indirectly by affecting the parasite's vectors or initial vertebrate hosts (e.g., anurans).

Decreases in parasite infections have been reported in other taxa exposed to contaminants. For example, heavy metal pollution was linked to a decrease in the abundance of acanthocephalan, cestode, and digenean parasites in fish living in contaminated environments (Lafferty 1997). In another example, Krivolutsky and Pokarzhevsky (1992) documented an increase in caterpillar survival with increasing radioactive contamination and a decrease in parasitic infections. Thus, low-level radionuclides could provide an indirect, positive effect on the health of wildlife with regard to parasite loads.

Few field studies examining the relationships between radionuclides and parasite infections in vertebrates exist; however, results similar to ours were observed by Wilbur *et al.* (1994) in pocket gophers that had lower prevalence and sporulation of the protozoan *Eimeria jemezi* when living in radionuclide contaminated areas compared to reference sites (Wilbur *et al.* 1994). Similarly, prevalence of the protozoan *Sarcocystis* spp. in white-footed mice living in

^{137}Cs contaminated habitats at the Oak Ridge National Laboratory (ORNL) was reduced compared to those from reference sites (Childs and Cosgrove 1966). In contrast to our results, Love *et al.* (2017) observed a positive correlation in radiation exposure and parasite loads of *Coccidia* in gray wolves (*Canis lupus*) and raccoon dogs (*Nyctereutes procyonoides*) living in the Chernobyl Exclusion Zone; however, it must be noted that carnivores in Chernobyl and their parasites are exposed to higher external doses of gamma radiation in addition to any bioaccumulated ^{137}Cs . The potential for varying effects of radioactive contamination on parasite-vector-host dynamics is not surprising given the complexity of the relationships and interactions between organisms and the biotic and abiotic factors in their environment. Many protozoan parasites such as *Hepatozoon* spp. have complex lifecycles which consists of multiple vectors and several potential vertebrate hosts before reaching a final host (Smith 1996, Telford *et al.* 2001). Thus, further studies would be needed to determine if decreases in *Hepatozoon* spp. infections in *N. floridana* are the result of negative impacts on the parasites, vectors or initial hosts.

Although neither site nor body size (i.e., mass) were significant predictors of *Hepatozoon* spp. infection probability, ontogenetic shifts in diet of *N. floridana* and differences in prey base among sites likely impact *Hepatozoon* spp. infection probability. Ontogenetic shifts in the diet of *N. floridana* are not well understood, but anuran tadpoles are a readily available food source that can be consumed by smaller juvenile *N. floridana*. Anurans are more likely to harbor *Hepatozoon* spp. compared to fish (Smith 1996), which are more commonly consumed by adult *N. floridana*. Furthermore, Pond 2 is a smaller body of water with fewer species of fish (Bennett and McFarlane 1983), and resident *N. floridana* may rely on amphibians to a greater extent compared to those from the larger Pond B. Thus, irrespective of the higher ^{137}Cs levels at Pond B

reservoir, *N. floridana* in living there may be less likely to become infected with *Hepatozoon* spp. through their diet compared to those living in Pond 2.

Metabolic rate in *N. floridana* increased with body mass. A positive relationship between body size and SMR is a common phenomenon reported for snakes (Hopkins *et al.* 1999, McCue and Lillywhite 2002, Dorcas *et al.* 2004) and other taxa (Sims 1996, Homyack *et al.* 2010). Although support was weak, ^{137}Cs was included in the most supported model predicting SMR, suggesting contaminant burdens, in addition to mass, could be associated with SMR in *N. floridana*. Four of the five highest SMR measurements came from snakes living in Pond B, where ^{137}Cs body burdens were the highest. In one of the only prior studies examining SMR in snakes exposed to contaminants, Hopkins *et al.* (1999) found that *N. fasciata* from habitats with coal combustion waste exhibited higher SMR compared to snakes from reference sites. Although effects of chronic exposure to bioaccumulated ^{137}Cs are poorly understood in vertebrates, accumulation has been shown to be associated with increased DNA damage in channel catfish (*Ictalurus punctatus*; Sugg *et al.* 1995) and turtles residing in Pond B (Bickham *et al.* 1988). If similar DNA damage is occurring in *N. floridana* exposed to ^{137}Cs , it is likely that the continuous repair of damaged DNA by energetically costly repair mechanisms could increase SMR. Furthermore, Sugg *et al.* (1995) also detected a positive relationship between Hg accumulation and DNA double strand breaks in largemouth bass. Thus, although concentrations of THg in tails of *N. floridana* from Pond B are relatively low, there is the potential for Hg accumulation to contribute to genotoxic damage synergistically with ^{137}Cs and subsequently elevate SMR at sites where environmental Hg is higher than at our study sites.

Little is known about the effects of prolonged exposure to ^{137}Cs and Hg in wildlife, especially reptiles and specifically snakes (Hopkins 2000, Campbell and Campbell 2001,

Haskins *et al.* 2019). Our work indicates that higher ^{137}Cs body burdens in particular may be related to an increase in SMR but a decrease in the probability of *Hepatozoon* spp. infections in *N. floridana* inhabiting former nuclear cooling reservoirs. The varying associations between contaminant burdens and measures of sublethal health effects highlight the complexity of studying the sublethal effects of anthropogenic stressors on wildlife populations. Furthermore, the few documented health effects of long-term dietary exposure to ^{137}Cs in animals typically involves the eventual development of cancers (ATSDR 2004). While measuring SMR in organisms from contaminated habitats may provide some indication of physiological stress in the animal, investigating additional endpoints such as those that may indicate the presence of cancer may provide information on the full extent of health effects of contaminant exposure. This may prove difficult in studies focused on wild populations of cryptic animals, such as snakes, because typical capture methods (e.g., trapping, hand grabs) may not provide an accurate representation of the entire population. Since burdens of Hg and ^{137}Cs in our sites were relatively low, future observational studies into the bioaccumulation and sublethal effects of Hg and ^{137}Cs should target areas with a more recent history of contamination events (e.g., Fukushima for ^{137}Cs in particular). Experimental, lab-based studies into the sublethal effects of ^{137}Cs and Hg dosing lab-reared snakes with environmentally relevant concentrations of contaminants could also provide valuable information into the toxicological consequences of exposure, as well as the specific mechanisms associated with adverse effects.

A limitation of our work was the scope of contaminants measured. While we focused on ^{137}Cs and Hg, the primary contaminants known to be of concern in our study system, there are likely other contaminants found in these former nuclear cooling reservoirs. Prior studies from these same sites have measured additional radionuclides (^{244}Cm , ^{241}Am , $^{235,238}\text{U}$, ^{60}Co ,

^{231,232,234}Th, ^{239,240}Pu, ⁷Be, ⁹⁰Sr; Lamb *et al.* 1991, Halverson and Noonkester 1998, Whicker *et al.* 1999) and heavy metals (Cd, Cr, Pb, Mn, Se; Burger *et al.* 1997) in resident biota. Burger *et al.* (1997) determined levels of selenium to be elevated in doves feeding on Par Pond lake beds, and Lamb *et al.* (1991) found Pond B slider turtles that exhibited DNA abnormalities had higher burdens of ⁹⁰Sr compared to ¹³⁷Cs. Most contaminant research at the SRS has focused on radionuclides and heavy metals, while information on organic pollutants is lacking or absent entirely. Thus, we cannot assume that unmeasured contaminants have no impact on SMR and *Hepatozoon* infections in *N. floridana* examined in this study.

Our study indicates the potential of *N. floridana* as a reliable indicator of ¹³⁷Cs and Hg contamination in former nuclear cooling reservoirs of the SRS. With the exception of ongoing atmospheric deposition of Hg and run-off, the majority of contamination at our sites, occurred 30 to 60 years ago. While levels of THg in *N. floridana* were relatively low in comparison to others observed globally, whole-body burdens of ¹³⁷Cs in *N. floridana* from Pond B were well above minimum detectable concentrations, and 16 of 23 snakes captured there had body burdens that were above the European Economic Communities guideline for ¹³⁷Cs content in fresh meat (0.600 Bq/g). Watersnakes are not a commonly consumed food item but this finding does speak to the persistence of ¹³⁷Cs at levels of concern in a lentic, freshwater ecosystem. Thus, there is a continued need for monitoring the former nuclear cooling reservoirs of the SRS, even though contamination happened decades prior. *Nerodia floridana* tend to occur in large, permanent bodies of water capable of supporting fish and the former nuclear cooling reservoirs of the SRS provide valuable habitat for the species. Our work indicates that *N. floridana* readily accumulate Hg and ¹³⁷Cs that can be measured through non-lethal techniques and are captured with relative

ease. Thus, for future monitoring and risk assessment efforts at the SRS, *N. floridana* should be considered as a suitable ecological receptor species.

Despite a history of contamination associated with nuclear weapons productions, the snakes sampled from former nuclear cooling reservoirs our work overall exhibit only modest burdens of ^{137}Cs and Hg. These reservoirs provide important habitat to an impressive array of wildlife, including *N. floridana*. While abundant in the state of Florida, the disjunct South Carolina population of *N. floridana* is listed as a species of concern due to a fragmented distribution and habitat loss (Bennett and Buhlmann 2015). However, *N. floridana* is the most commonly encountered aquatic snake at our study sites on the SRS. Because *N. floridana* is a poor overland disperser and slow to recolonize sites from which they have become extirpated, one of the biggest threats to the species is anthropogenic alterations to aquatic habitats (e.g., the ditching and draining of wetlands for agricultural purposes), along with drought (Seigel *et al.* 1995, Willson *et al.* 2006, Vogrinc *et al.* 2018). Public access to aquatic habitats within the boundaries of the SRS has been restricted since the site's inception in the early 1950's (White and Gaines 2000). Thus, the limited anthropogenic disturbance to aquatic habitats on the SRS has likely been to the benefit of an array of wildlife species, including *N. floridana*, and may offset any detrimental effects associated with the legacy Hg and ^{137}Cs contamination.

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Table 2.1: Comparison of sample sizes (n), snout-vent length (SVL), whole-body radiocesium (^{137}Cs), tail total mercury (THg), oxygen consumption (VO_2), and *Hepatozoon* prevalence and parasitemia for Florida green watersnakes (*Nerodia floridana*) captured from three former nuclear cooling reservoirs on the Savannah River Site, South Carolina. Values are reported as means \pm 1 SE (ranges are presented below means in parentheses). All snakes were captured from 10-30 of June 2016. Prevalence and parasitemia for snakes from Par Pond were excluded due to low sample size.

Parameter	Par Pond	Pond B	Pond 2
<i>n</i> (sample size)	11	23	44
SVL (cm)	63.4 \pm 4.8 (40.0-82.0)	51.5 \pm 2.8 (30.0-75.0)	40.6 \pm 1.6 (29.0-81.0)
^{137}Cs (Bq/g)	0.10 \pm 0.02 (0.02-0.25)	0.67 \pm 0.05 (0.0-1.02)	0.03 \pm 0.02 (0.0-0.92)
THg (mg/kg dw)	0.56 \pm 0.18 (0.19-2.10)	0.29 \pm 0.03 (0.16-0.70)	0.29 \pm 0.03 (0.17-1.36)
VO_2 (mL O_2 /hr)	19.70 \pm 4.14 (6.13-56.79)	16.66 \pm 2.33 (4.15-36.49)	8.65 \pm 0.98 (0.04-31.41)
<i>Hepatozoon</i> Prevalence	NA	23.8% (5/21)	73.8% (31/42)
Parasitemia	NA	0.03 \pm 0.02 (0.00-0.53)	0.12 \pm 0.04 (0.00-1.25)

Table 2.2: Seven candidate models to explain variation in log-transformed whole-body (^{137}Cs) in Florida green watersnakes (*Nerodia floridana*) captured from three former nuclear cooling reservoirs on the Savannah River Site, South Carolina. The most parsimonious model is indicated in bold. Parameters included: mass, site (PAR Pond, Pond B, Pond 2), sex and the interaction between site and mass. 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}).

Model	Log-likelihood	K	AIC	ΔAIC	AIC_{wt}
Site + Mass + Site*Mass	-40.72	7	95.44	0	0.37
Site + Mass	-42.99	5	95.99	0.54	0.28
Site + Mass + Sex + Site* Mass	-40.41	8	96.82	1.38	0.18
Site + Mass + Sex	-42.88	6	97.76	2.32	0.12
Site	-45.66	4	99.32	3.87	0.05
Mass	-98.36	3	202.72	107.28	0.00
Sex	-99.87	3	205.73	110.29	0.00

Table 2.3: Summary of the most parsimonious model explaining variation in log-transformed whole-body ^{137}Cs for *N. floridana* captured from Par Pond, Pond B, and Pond 2 of the Savannah River Site, SC. Model parameters, estimates, and associated p-values are displayed.

Parameter(s)	Estimates	t-value	p-value
Model intercept	-1.60	-16.43	<0.0001
Site (PAR Pond)	-0.36	-2.15	<0.05
Site (Pond 2)	-0.54	-4.95	<0.0001
Mass	-0.0012	2.42	<0.05
PAR Pond:Mass	9.91×10^{-5}	0.15	0.87
Pond 2: Mass	-0.0013	-1.95	0.05

Table 2.4: Seven candidate models to explain variation in log-transformed tail THg in Florida green watersnakes (*Nerodia floridana*) captured from three former nuclear cooling reservoirs on the Savannah River Site, South Carolina. The most parsimonious model is indicated in bold. Parameters included: mass, site (PAR Pond, Pond B, Pond 2), sex and the interaction between site and mass. Model values presented include log-likelihood, model degrees of freedom (K), Akaike Information Criterion (AIC), delta AIC (Δ AIC), and the weight of each model (AIC_{wt}).

Model	Log-likelihood	K	AIC	Δ AIC	AIC_{wt}
Mass	-34.23	3	74.46	0	0.62
Site + Mass	-33.42	5	76.83	2.36	0.19
Site + Mass + Sex	-32.74	6	77.47	3.01	0.14
Site + Mass + Site * Mass	-33.20	7	80.41	5.94	0.03
Site + Mass + Sex + Site * Mass	-32.48	8	80.95	6.49	0.02
Sex	-47.67	4	103.34	28.87	0.00
Site	-50.46	3	106.92	32.45	0.00

Table 2.5: Summary of the most parsimonious model explaining variation in log-transformed tail THg (mg/kg; dry weight) in *N. floridana* captured from Par Pond, Pond B, and Pond 2 of the Savannah River Site, SC. Model parameters, estimates, and associated p-values are displayed.

Parameter(s)	Estimates	t-value	p-value
Model intercept	-1.52	-25.67	<0.0001
Mass	0.002	6.55	<0.0001

Table 2.6: Ten candidate logistic regression models to predict the probability of *Hepatozoon* spp. infection in Florida green watersnakes (*Nerodia floridana*) captured from three former nuclear cooling reservoirs on the Savannah River Site, South Carolina. The most parsimonious model is indicated in bold. Parameters included: mass, site (Par Pond, Pond B, Pond 2), whole-body ^{137}Cs , tail THg, sex and the interaction between ^{137}Cs and mass. 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}).

Model	Log-likelihood	K	AIC	ΔAIC	AIC_{wt}
$^{137}\text{Cs} + \text{Mass} + ^{137}\text{Cs}*\text{Mass}$	-32.28	4	72.56	0.00	0.35
^{137}Cs	-34.40	2	72.80	0.24	0.31
$^{137}\text{Cs} + \text{Mass} + \text{Site} + ^{137}\text{Cs}*\text{Mass}$	-32.22	5	74.44	1.88	0.14
$^{137}\text{Cs} + \text{Mass}$	-34.40	3	74.80	2.24	0.12
$^{137}\text{Cs} + \text{Mass} + \text{Site}$	-34.40	4	76.79	4.23	0.04
$^{137}\text{Cs} + \text{THg} + \text{Mass} + \text{Site}$	-34.38	5	78.76	6.20	0.02
Mass + Site	-36.53	3	79.07	6.50	0.01
Mass + THg + Site	-36.41	4	80.83	8.26	0.01
Mass + Site + Sex	-36.43	4	80.85	8.29	0.01
Mass + THg	-40.23	3	86.47	13.91	0.00

Table 2.7: Summary of the most parsimonious model predicting the probability of *Hepatozoon* spp. infection for *N. floridana* captured from Pond B and Pond 2 of the Savannah River Site, SC. Model parameters, estimates, and associated p-values are displayed.

Parameter(s)	Estimates	z-value	p-value
Model intercept	1.60	3.20	<0.01
Mass	-0.006	-1.18	0.23
¹³⁷ Cs	-5.71	-3.33	<0.001
¹³⁷ Cs: Mass	0.02	2.00	<0.05

Table 2.8: Ten candidate models to explain variation in log-transformed VO₂ (ml O₂/hr) in Florida green watersnakes (*Nerodia floridana*) captured from three former nuclear cooling reservoirs on the Savannah River Site, South Carolina. The most parsimonious model is indicated in bold. Parameters included: mass, site (Par Pond, Pond B, Pond 2), sex, whole-body ¹³⁷Cs burden, and tail THg. Model values presented include log-likelihood, model degrees of freedom (K), Akaike Information Criterion (AIC), delta AIC (ΔAIC), and the weight of each model (AICWt).

Model	Log-likelihood	K	AIC	ΔAIC	AIC _{wt}
¹³⁷Cs + Mass	-99.26	4	207.92	0.00	0.24
¹³⁷ Cs + THg + Mass	-99.44	5	208.88	0.96	0.15
Mass + Site	-99.54	5	209.09	1.17	0.14
Mass + Site + Sex	-98.69	6	209.39	1.47	0.12
Mass	-101.78	3	209.57	1.65	0.11
THg + Mass	-100.95	4	209.91	1.99	0.09
THg + Mass + Site	-99.07	6	210.14	2.22	0.08
¹³⁷ Cs + Mass + Site	-99.51	6	211.03	3.11	0.05
¹³⁷ Cs + THg + Mass + Site	-99.05	7	212.09	4.17	0.03
Site	-110.38	4	228.76	20.84	0.00
Sex	-115.66	3	237.31	29.39	0.00

Table 2.9: Summary of the most parsimonious model explaining variation in log transformed VO₂ (ml O₂/hr) for *N. floridana* captured from Par Pond, Pond B, and Pond 2 of the Savannah River Site, SC. Model parameters, estimates, and associated p-values are displayed.

Parameter(s)	Estimates	t-value	p-value
Model intercept	1.49	10.15	<0.00001
Mass	0.005	1.89	<0.00001
¹³⁷ Cs	0.58	5.53	0.062

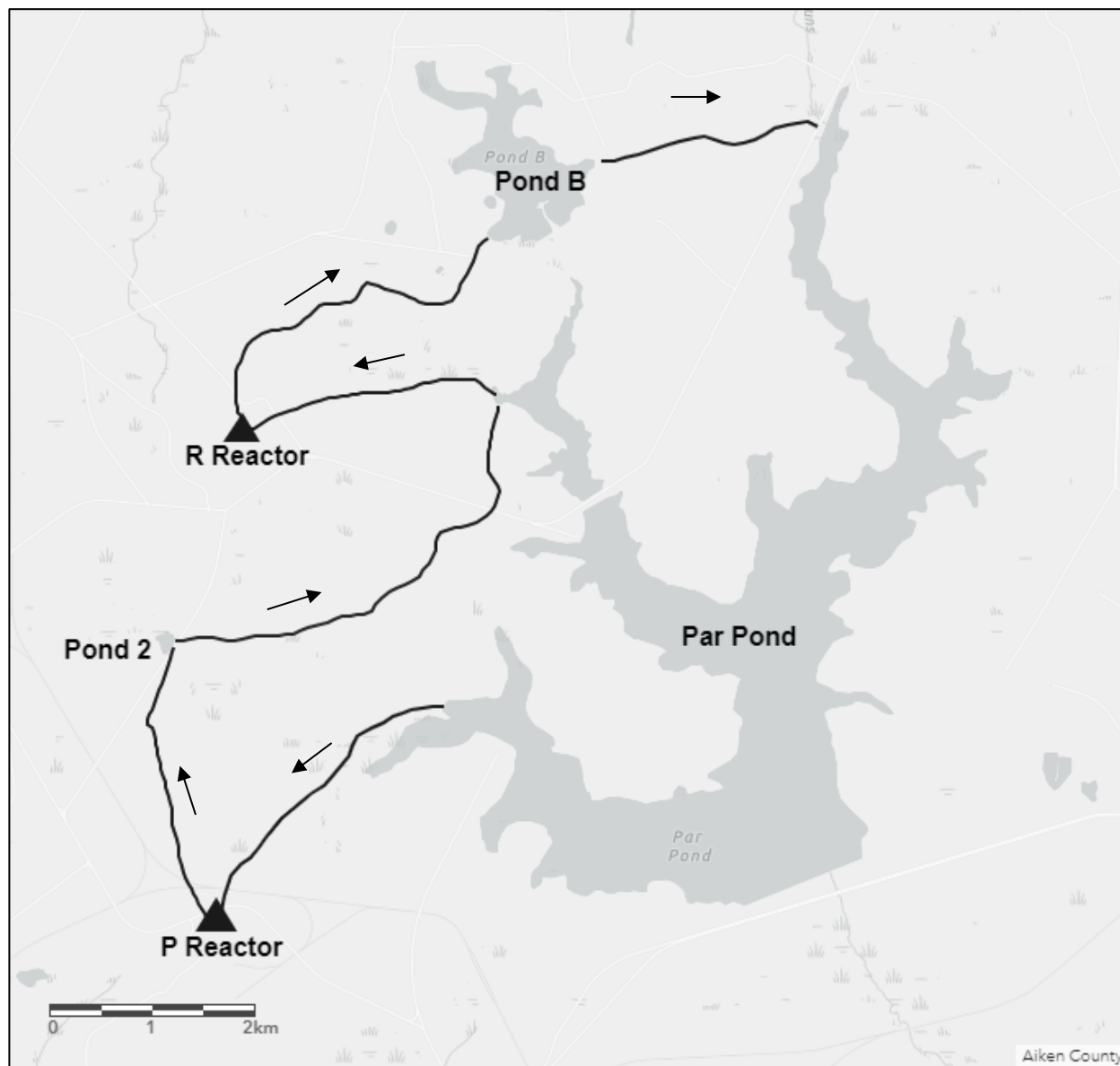


Figure 2.1. Map of Par Pond nuclear cooling reservoir system of the Savannah River Site near Aiken, South Carolina, USA. Sampling for *Nerodia floridana* in this study occurred at Par Pond, Pond B, and Pond 2 in the summer of 2016. Solid black lines indicate canal system through which effluent from reactors flowed to reservoirs. Arrows indicate direction of effluent flow. Basemap: Esri Light Gray Canvas, HERE, Garmin, METI/NASA, USGS, EPA, NPS, USDA.

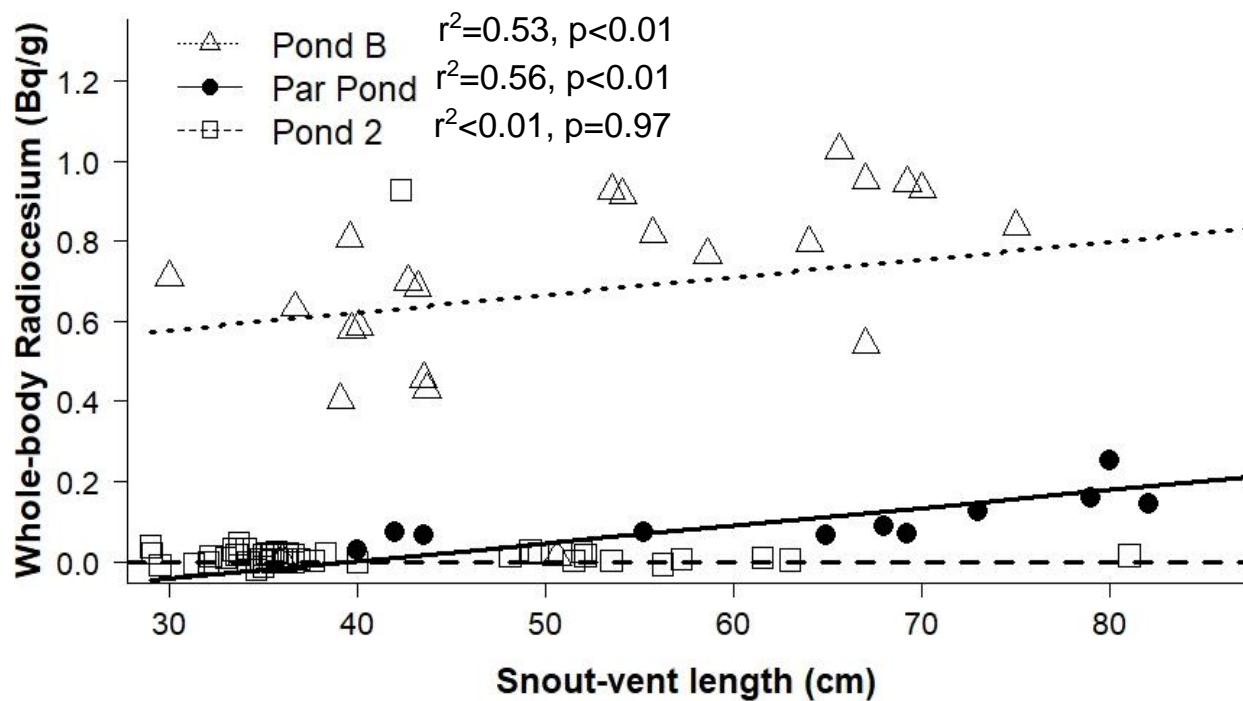


Figure 2.2. The relationship between whole-body radiocesium (^{137}Cs ;Bq/g) and snout-vent length (SVL;cm) for *N. floridana* (n=78) captured from three former nuclear cooling reservoirs (Par Pond (n=11), Pond B (n=23), and Pond 2 (n=44)) of the Savannah River Site near Aiken, South Carolina.

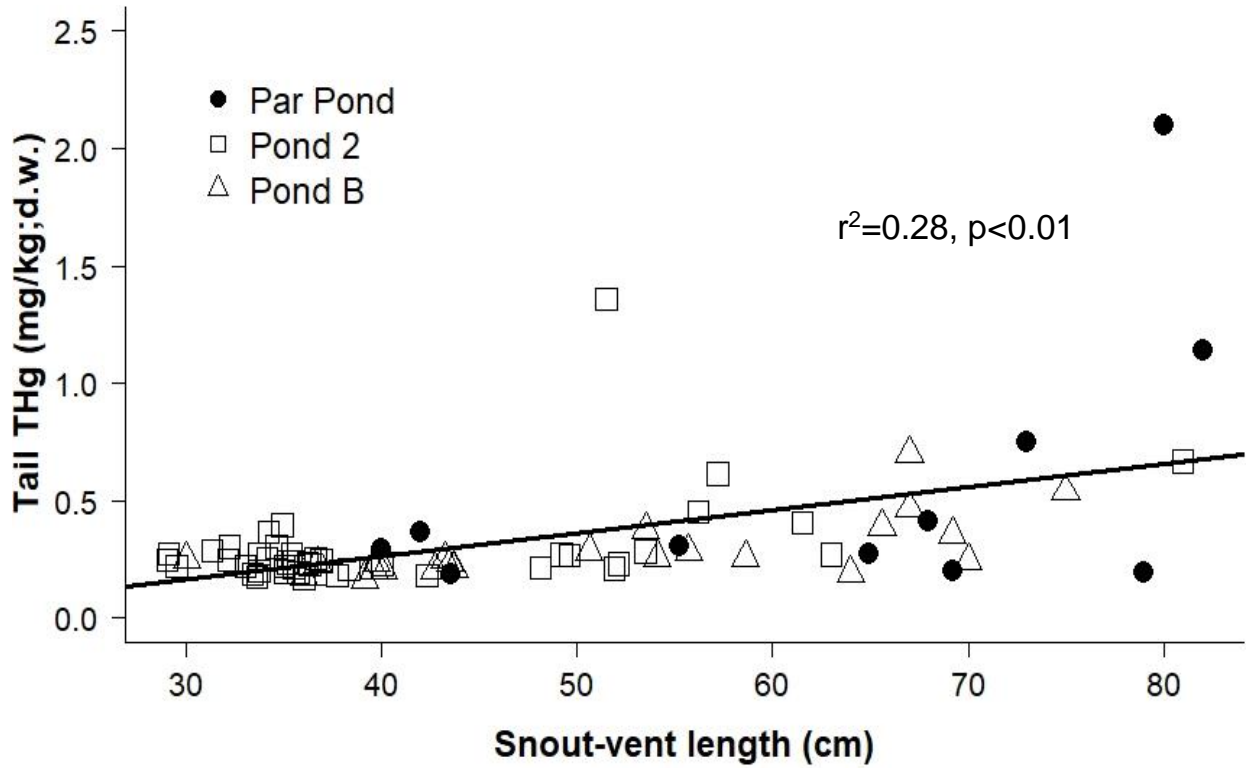


Figure 2.3. The relationship between tail total mercury (THg) and snout-vent length (SVL) for *N. floridana* (n=78) captured from three former nuclear cooling reservoirs (Par Pond, Pond B, and Pond 2) of the Savannah River Site near Aiken, South Carolina.

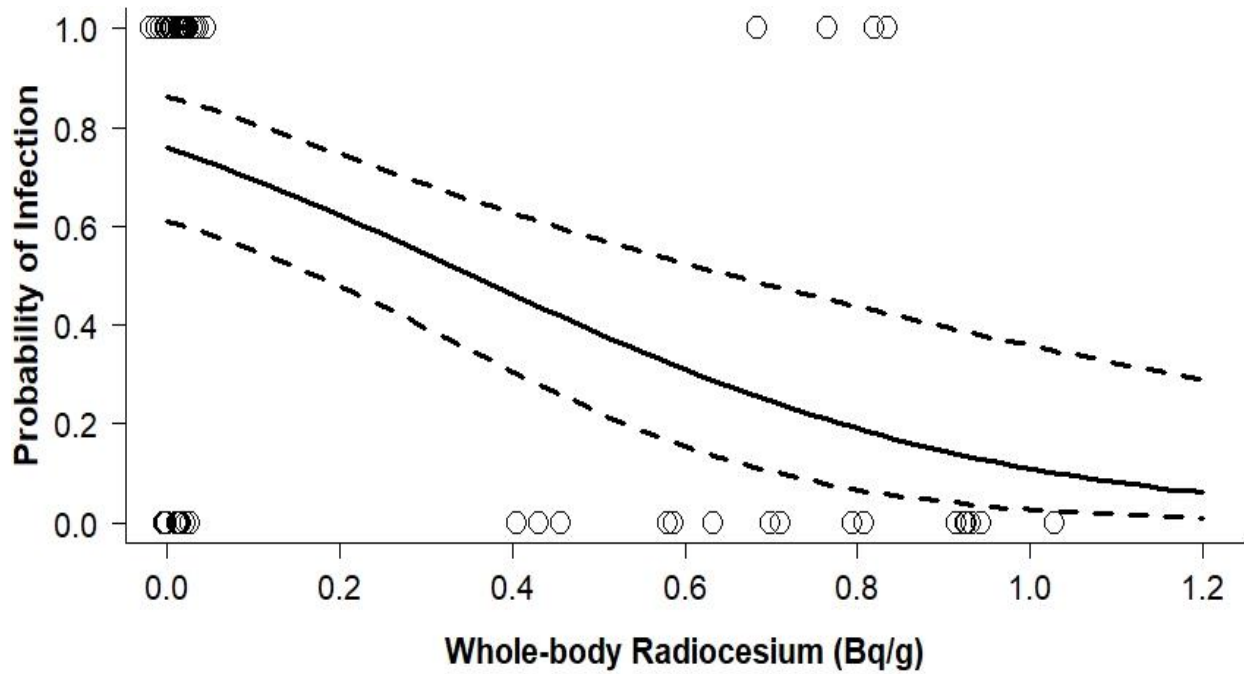


Figure 2.4. The predicted probability of *Hepatozoon* spp. infection ($\pm 95\%$ CI) for *N. floridana* of the Savannah River Site near Aiken, South Carolina as a function of whole-body radiocesium (Bq/g) burden.

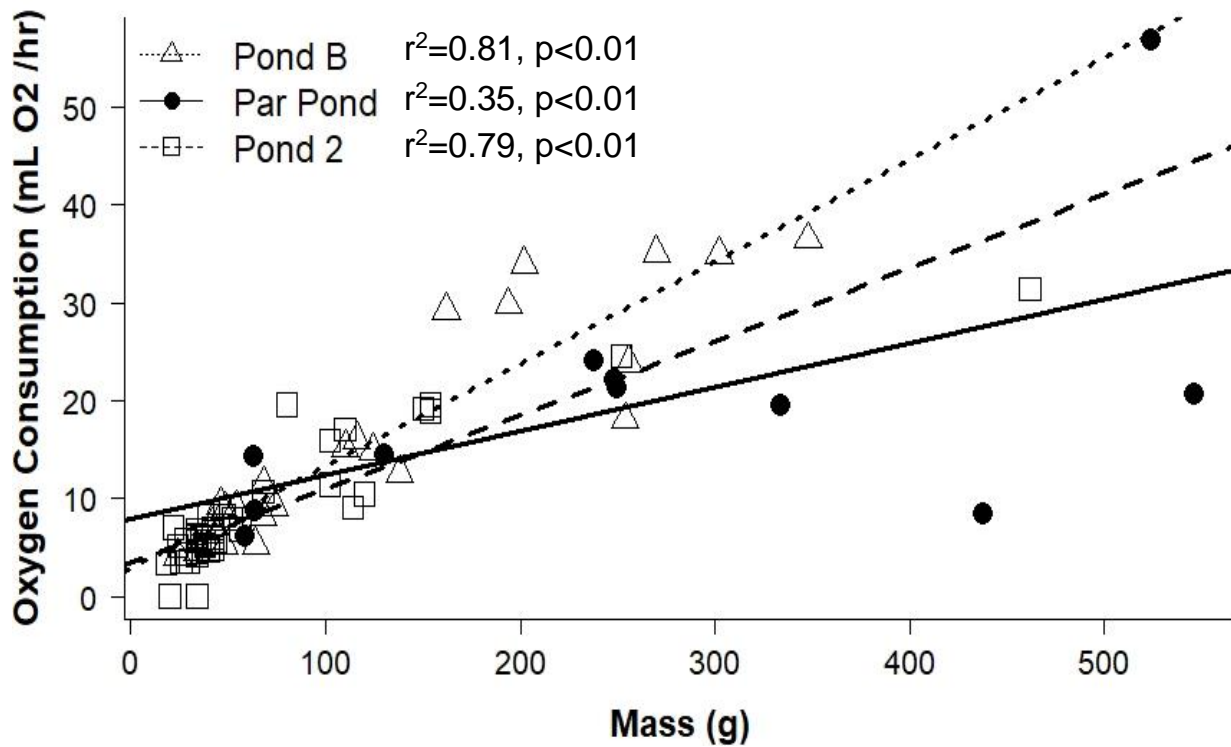


Figure 2.5. The relationship between standard metabolic rate (VO_2 , ml O_2 /hr) and mass for *N. floridana* (n=78) captured from three former nuclear cooling reservoirs (Par Pond (n=11), Pond B (n=23), and Pond 2 (n=44)) of the Savannah River Site near Aiken, South Carolina.

CHAPTER 3:
**INTERSPECIFIC COMPARISONS OF MERCURY ACCUMULATION AND
HEPATOZOON INFECTIONS IN FLORIDA GREEN WATERSNAKES (*NERODIA
FLORIDANA*) AND BANDED WATERSNAKES (*NERODIA FASCIATA*) INHABITING
THE SAVANNAH RIVER SITE.**

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. Mercury occurs naturally (i.e., volcanic emission, forest fires, volatilization), but 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). Inorganic mercury (Hg^0 , Hg^{2+}) can be transported long distances in the atmosphere before being deposited on the earth's surface and into bodies of water, making emissions a global concern (Zhang *et al.* 2009). Biotransformation of inorganic Hg into the more bioavailable organic methylmercury (MeHg) can occur via anaerobic, sulfur-reducing bacteria that reside in the sediments of aquatic habitats. Methylmercury is 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). When consumed and assimilated through diet, Hg (primarily in the form of MeHg) can become widely distributed throughout an organism

and accumulate in various tissues, such as liver and muscle, over time (Green *et al.* 2010, Azevedo *et al.* 2012, Drewett *et al.* 2013).

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 (Cabana and Rasmussen 1994, Atwell *et al.* 1998, Burger *et al.* 2001, Rimmer *et al.* 2010, Carrasco *et al.* 2011, Chumchal *et al.* 2011). The harmful effects of Hg have been documented across taxa and include reduced reproductive success (Heinz 1979, Thompson *et al.* 2018), endocrine impairment (Dieter and Ludke 1975, Wolfe *et al.* 1998), overt neurotoxicity, and death (Wren *et al.* 1987, Heinz 1996, Scheuhammer *et al.* 2007). Furthermore, even if concentrations of Hg are low or exposure occurs over a long period of time, less obvious sublethal effects may occur (Martin *et al.* 2010, Chin *et al.* 2013b). Mercury exposure may compromise an organism's immune system, leaving them more susceptible to disease and parasite infection (Lafferty and Kuris 1999, Sures 2004, Martin *et al.* 2010, Marcogliese and Pietroock 2011). For example, wild mink (*Mustela vison*) with high levels of accumulated Hg were found to have an increased chance of infection by the giant kidney worm (*Dioctophyma renale*) (Klenavic *et al.* 2008). High blood mercury levels have also been associated with increased prevalence and higher intensity of *Leucytozoon* infections in common loons (Weinandt 2006). Additionally, Borchert *et al.* (2019), found positive associations between Hg concentrations and parasite 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), leading to lower parasite occurrence within a polluted system. For example, heavy metal pollution has been

linked to a decrease in the abundance of acanthocephalan, cestode, and digenean parasites in fish living in polluted environments (Lafferty 1997).

Despite a growing interest in the use of herpetofauna as ecological receptors, reptiles are included less often in environmental monitoring and risk assessments 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). Additionally, snakes typically have small home ranges and are therefore 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 traits combine to make snakes a reliable indicator of environmental health (Campbell and Campbell 2001, Burger *et al.* 2017, Haskins *et al.* 2019a).

Watersnakes (*Nerodia* spp.) appear to be more susceptible to accumulating higher levels of contaminants compared to terrestrial snakes and other vertebrate predators living in contaminated areas. Accumulation of Hg has consistently been found to be highest in snakes feeding primarily on aquatic prey (Burger *et al.* 2006, Chumchal *et al.* 2011, Drewett *et al.* 2013). Drewett *et al.* (2013) reported significantly higher levels of Hg in semi-aquatic northern watersnakes (*Nerodia sipedon*) and queen snakes (*Regina septemvittata*) compared to terrestrial feeding rat snakes (*Pantherophis alleghaniensis*) and garter snakes (*Thamnophis sirtalis*) in a Hg-contaminated area of the South River in Virginia. Though work examining the sublethal effects of Hg is rare, snakes in general may be relatively resilient (Wolfe *et al.* 1998, Chin *et al.*

2013a); however, some evidence suggests watersnakes may experience detrimental impacts of accumulating Hg at environmentally relevant, sublethal concentrations (Chin *et al.* 2013b).

Compared to litters born to mothers from reference sites, neonate banded watersnakes (*N. fasciata*) born from mothers captured in a Hg-contaminated river exhibited lower motivation to feed and impaired striking efficiency (Chin *et al.* 2013b), which could compromise fitness by reducing their ability acquire prey items and deter predators.

Differences in habitat selection and feeding ecology are likely to influence patterns of contaminant 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). For example, 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 may include both fish and amphibians (Durso *et al.* 2013). Closely related species occupying habitats with broader prey resources may also partition into various trophic niches, which may in turn lead to certain species accumulating higher concentrations of Hg. In a recent study in Europe, Lemaire *et al.* (2018) found Hg concentrations to be significantly higher in fish-eating populations of the viperine snake (*Natrix maura*) compared to populations feeding primarily on

frogs. Thus, even within a single species, differences in feeding ecology can play a role in Hg accumulation. Further research examining multiple species in differing habitats is needed to have a better understanding of the factors influencing Hg accumulation in aquatic snakes.

Habitat type and feeding ecology may also play a role in a snake exposure to parasitic infections. Hemogregarines of the genus *Hepatozoon* are a common intraerythrocytic parasite of snakes (Smith 1996, Telford *et al.* 2001). Infections of *Hepatozoon* 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* parasites (Smith 1996). Thus, the prey assemblage within a habitat is likely to impact a snake's susceptibility of *Hepatozoon* 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 impacting exposure to hemoparasites and prevalence of parasite infections (Tomé *et al.* 2012).

Study Objectives

The goal of this research was to determine if differences between species and aquatic habitat type are associated with differences in Hg accumulation in aquatic snakes. We were also interested in elucidating how species, body size, habitat and mercury burden are related to prevalence and parasitemia of *Hepatozoon* spp. Our specific objectives were to (1) examine the relationship between tail total Hg (THg) and snake body size, (2) compare tail THg between species and aquatic habitat type, (3) compare *Hepatozoon* infections between watersnake species and aquatic habitats, and (4) determine associations between tail THg and *Hepatozoon*

infections. To meet our objectives, we sampled two sympatric species of watersnakes with differences in feeding ecology and abundance within aquatic habitats—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 to non-destructively quantify THg and examined blood smears to determine *Hepatozoon* prevalence and parasitemia. We hypothesized that tail THg would increase with body size in both *N. floridana* and *N. fasciata*. Based on their suspected differences in diet, we anticipated in the reservoirs, *N. floridana*, which as adults prey on fish when available, would accumulate higher burdens of THg compared to *N. fasciata*, but that THg burdens would be similar in the two species in Carolina bays, where diet would be more likely to overlap. We hypothesized that *Hepatozoon* infections would be more common in *N. fasciata* based on a higher reliance of amphibian prey items in their diet. Finally, we anticipated that *Hepatozoon* infections would be more common for both species in isolated wetlands, where amphibian prey sources are more plentiful compared to fish.

Methods

Study sites

The Savannah River Site (SRS) is a 780 km² United States Department of Energy property located near Aiken, South Carolina. Due to past activities associated with production of nuclear weapons on the SRS, the site has legacy contamination of heavy metals and radionuclides in some aquatic habitats, specifically in former nuclear cooling reservoirs. The SRS is home to a diversity of wetlands including, 2000 ha of cooling reservoirs, and more than 200 smaller bodies of water, including many isolated wetlands (Schalles *et al.* 1989, White and Gaines 2000). Most of the outlying, 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).

We collected *N. floridana* and *N. fasciata* from Pond B, an 87 ha cooling reservoir constructed in 1961 to serve as a secondary cooling system for nuclear production reactors. 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 production. 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 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). Several studies have documented 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, that have no history of Hg inputs resulting from SRS operations. The 78.2 ha Craig's Pond is the largest Carolina bay on the SRS and is in close proximity to the much smaller Sarracenia Bay (4.0 ha) (Davis and Janecek 1997, Gaines *et al.* 2005). During periods of high rainfall, the two bays can become connected. Because the two wetlands are sometimes connected, and because animals can easily move between wetlands, we chose not to examine the two Carolina bay 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, but relative abundance of each species varies with habitat type. Permanent lentic habitats such as reservoirs, lakes, and some isolated wetlands with extended hydroperiods are the preferred habitat for *N. floridana* (Gibbons and Dorcas 2004). In contrast, *N. fasciata* is a habitat generalist occupying wetlands with a wide range of hydrological conditions. At the Savannah River Site (SRS) in South Carolina where the two species co-occur, *N. fasciata* is 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 impact of prolonged drought on aquatic snakes has been studied over several decades at the SRS (Seigel *et al.* 1995, Willson *et al.* 2006, Vogrinc *et al.* 2018). While both *N. fasciata* and *N. floridana* experience population declines due to prolonged droughts, the former appears to be relatively resilient and repopulates wetlands quickly after they refill (Willson *et al.* 2013, Vogrinc *et al.* 2018). In contrast, *N. floridana* are extirpated for years after droughts have subsided, potentially due to an aversion to disperse overland during times of environmental stress (Vogrinc *et al.* 2018).

The diets of both species may overlap, especially as juveniles when both tend to prey 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 prey that can include fish, salamanders, frogs and tadpoles (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 tadpoles and small fish prey (e.g., mosquitofish, topminnows) as juveniles to large anurans (e.g., ranid frogs and toads) as adults (Mushinsky *et al.* 1982, Vincent *et al.* 2007). Not surprisingly, *N. floridana* are more likely to be detected in more permanent wetlands containing fish, while *N. fasciata* are more common in less-permanent wetlands lacking fish (Durso *et al.* 2011). However, in wetlands without fish, both 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. 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 initial processing, which included measuring mass (to nearest 1.0 g) and giving each individual a unique identification number. We determined sex by examining tail morphology and/or probing the cloaca. We measured snout-vent length (SVL; length from tip of snout to cloaca) and tail length (TL; length from cloaca to tail tip) to the nearest 1.0 mm by stretching the snake along a meter stick.

We used Hg in snake tail clips as a proxy for whole-body Hg (see Hopkins *et al.* 2001). We removed approximately 1.0 cm of tail tip from each snake for the quantification of total Hg (THg). 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 -70°C 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* 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.

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 decomposition, catalytic conversion, amalgamation, and atomic absorption spectrophotometry by a DMA-80 Tri-cell Direct Mercury Analyzer (Milestone, Shelton, CT, USA). 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), on the machine before sampling began and after every 10 samples. We ran flour, nitric acid and 3 blanks after every 20 samples to ensure proper Hg analysis. The detection limit for THg in tail tissue was 0.000436 ppm dry mass. Average percent recoveries for TORT-3 and PACS-2 reference materials was 98.8% (range: 89.8-106.4%) and 101.9% (range: 83.8-137.4%), respectively. We present all THg concentrations as mg/kg on a dry weight basis.

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. Although several species of *Hepatozoon* may infect a single species of *Nerodia*, we did not identify hepatozoa beyond the genus or assess developmental stage. We determined prevalence as the proportion of individual snakes infected with at least one *Hepatozoon*. We calculated parasitemia (i.e., the percentage of erythrocytes infected with a *Hepatozoon*) using the following equation: Parasitemia= (Number of *Hepatozoon* infected erythrocytes/8000 erythrocytes) *100.

Statistical analyses

We conducted all statistical analyses using program R (R Core Team, 2018). We tested data for normality and homogeneity of variances using Shapiro-Wilks test of normality and Bartlett's test, respectively. We log-transformed data that did not meet the assumptions of normality and used nonparametric analyses of untransformed data when data were not normally distributed after transformation. We compared body burdens of THg and average *Hepatozoon* parasitemia in *N. floridana* and *N. fasciata* from bays and Pond B reservoir using an analysis of covariance (ANCOVA), with grouping (species and habitat) as the independent variable and SVL as the covariate. We used a logistic regression to determine the importance of species, habitat, and individual-level factors (SVL, sex) in predicting the occurrence of *Hepatozoon* 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

We captured 37 *N. fasciata* and 10 *N. floridana* in bays and 19 *N. fasciata* and 38 *N. floridana* from Pond B, during the summers of 2017 and 2018. Of *N. fasciata* captured 26 were female and 30 were male, while 29 of *N. floridana* captured were female and 19 were male. Overall mass for *N. fasciata* ranged from 14.0 to 672.0 g (mean= 108.0 ± 15.4 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 240 – 765 mm (mean= 437 ± 17.6 mm), while SVL of *N. floridana* ranged from 346 to 696 mm (mean= 474 ± 11.5 mm). An analysis of variance (ANOVA) on log-transformed SVL of both species from both habitats indicated a statistically significant difference in SVL

among groups (Table 3.1; $F_{3,100} = 3.57, p < 0.01$). However, Tukey's HSD test for multiple comparisons only indicated a significant difference in SVL between *N. floridana* and *N. fasciata* from Pond B, with *N. floridana* averaging a slightly larger SVL (Table 3.1; $p < 0.01$).

Contaminant Burdens: Mercury

Tail THg concentrations were log-transformed for statistical analysis but averages (mean ± 1 SE) presented in text and tables are based on raw data to facilitate comparisons. Average tail THg for all 104 snakes captured was 0.19 ± 0.01 mg/kg dry weight (range: 0.02-0.63 mg/kg d.w.). There was a significant, positive trend of increasing tail THg with body size for *N. floridana* and *N. fasciata* from both the bays (Figure 3.2) and the former nuclear cooling reservoir (Figure 3.3). There was no significant difference in average tail THg between male and female snakes after controlling for body size (ANCOVA: $F_{1,102} = 1.16, p = 0.41$); thus, sex was not considered in further models. 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) (Figure 3.4). 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 collected from the reservoir were not significant ($t = -1.56, p = 0.39$; Figure 3.4). 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 ($t = 5.39, p < 0.001$; Figure 3.4).

Hepatozoon Prevalence and Parasitemia

We obtained blood smears from 104 snakes. Overall prevalence of *Hepatozoon* spp. infections in snakes sampled was 47.1% (49/104). Infections by *Hepatozoon* spp. were more prevalent in both species of snake from bays compared those from the reservoir, and were more common in *N. fasciata* than *N. floridana* in both habitats (Table 3.2). Overall average parasitemia per 8000 erythrocytes 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: $F_{4,99} = 1.36$, $p < 0.001$; Table 3.2). 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 both *N. floridana* from bays ($t = -4.08$, $p < 0.001$) and the reservoir ($t = -6.43$, $p < 0.001$). 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 3.2). The most parsimonious models predicting *Hepatozoon* spp. infection (0=no, 1=yes) in watersnakes included habitat, species, and SVL (Table 3.3), all of which were significant predictors of infection (Table 3.4). Based on the best supported model, the probability of *Hepatozoon* spp. infection was less probable for *N. floridana* compared to *N. fasciata*, more common in bays compared to the reservoir, and slightly increased with body size (Table 3.4).

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.* 2004, Haskins *et al.* 2019a). Within species, the relationship between body size and body burden was stronger for *N. fasciata* from the reservoir and *N. floridana* from the bays. 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, it is likely several variables are impacting the bioaccumulation of THg in *Nerodia* within our systems.

The observed results of interspecific variation between *N. fasciata* and *N. floridana* were counter to our hypotheses. 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 contrast, tail THg burdens 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* spp. and *Bufo* 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). The maximum SVL recorded for *N. fasciata* and *N. floridana* is 1588 mm and 1880 mm, respectively. 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 feeding often on small fish prey items that 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 shallow edges 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, thus these individuals are likely younger and feeding mostly on lower trophic prey items (e.g., salamander larvae, small fish, tadpoles). Anecdotally, we observed many *N. floridana* and small *N. fasciata* regurgitate larval mole salamanders (*Ambystoma talpoideum*) after capture, while several large *N. fasciata* regurgitated adult and larval southern leopard frogs (*Rana sphenocephala*). Previous research at the 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. Diet partitioning between sympatric species of watersnakes warrants further investigation.

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 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 level 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 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).

Overall, the THg concentrations in snakes from both the former nuclear cooling reservoir and Carolina bays on the SRS that we sampled were relatively low compared to levels documented in other regions affected by contamination (see Drewett *et al.* 2013, Haskins *et al.* 2019). 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 highest average tail THg observed in *N. fasciata* from Carolina bays was less than the average tail THg of *N. sipedon* from South River reference sites (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 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 associated with *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 to *Hepatozoon* spp. infection probability are possibly reflective of dietary differences. 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 speaks to their reliance on anuran prey items in comparison to their conspecific *N. floridana*. Moreover, we observed higher prevalence and parasitemia for both species living in Carolina bays, where anurans are the more dominant prey item (Durso *et al.* 2013). Thus, it is not surprising that snakes living in the Carolina bay are more likely to have *Hepatozoon* spp. infections. However, we must keep in mind that there were unmeasured factors that could impact *Hepatozoon* infection prevalence and parasitemia. As previously mentioned, radiocesium (¹³⁷Cs) is an additional contaminant of concern in the former nuclear cooling reservoir. Although it was not considered in this study, preliminary analysis indicates that ¹³⁷Cs burdens could be related to a decreased incidence of *Hepatozoon* spp. infections living in Pond B (see Chapter 2). Likewise, 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, comparisons across a broader array of wetlands is merited to better elucidate patterns of *Hepatozoon* spp. infections in aquatic snakes.

We found an increase in probability of *Hepatozoon* spp. infections to be weakly related to an increase in body size. In contrast, Madsen *et al.* (2005), found probability of hematozoan infections decreased with body size in water pythons. While most *Hepatozoon* spp. infections in *Nerodia* spp. occur via prey ingestion, water pythons are often infected by mosquito vectors (Madsen *et al.* 2005). Thus, it is possible that a shift to prey items that are more likely to serve as an intermediate host for *Hepatozoon* spp. (e.g., ranid frogs) is responsible for more instances of

Hepatozoon spp. infections in larger snakes, specifically *N. fasciata*, in our study. Recent sampling of several additional Carolina bays on the SRS indicated similar high prevalence of *Hepatozoon* spp. infections in *N. fasciata* and another anuran eating watersnake species, the plain-bellied watersnake (*N. erythrogaster*) (M.K. Brown, unpublished data).

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), while others suggest the possibility of detrimental effects (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. While THg concentrations in snake tail tips were relatively low, they were comparable to levels linked to deleterious effects in other taxa. Concentrations of THg in snake tail tips also 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 research further demonstrates the potential for watersnakes to serve as environmental indicators of Hg contamination in aquatic ecosystems. Our findings suggest that habitat and associated resources available for prey can be important determinants of exposure to environmental contaminants and exposure to infections by hemoparasites, such as *Hepatozoon* spp.. 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 and incorporating stable isotopes analysis to further investigate the effect of trophic dynamics on Hg accumulation and *Hepatozoon* susceptibility in watersnakes.

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Table 3.1: Comparison of sample sizes (n), snout-vent length (SVL), mass, and sex ratio for Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*Nerodia fasciata*) from a former nuclear cooling reservoir (Pond B) and two Carolina bays (Craig’s Pond and Sarracenia Bay) on the Savannah River Site, South Carolina. Values are reported as means \pm 1 SE (ranges are presented below means in parentheses). Snakes from bays were captured in summer 2017 and snakes from Pond B were captured in summer 2018.

Species and Site	n	Mass (g)	SVL (mm)	Sex
Pond B				
<i>N. floridana</i>	38	101 \pm 9.5 (58-194)	476 \pm 13.8 (346-696)	F: 23 M: 15
<i>N. fasciata</i>	19	74 \pm 22.2 (16-420)	392 \pm 26.7 (262-749)	F: 11 M: 8
Carolina Bays				
<i>N. floridana</i>	10	107 \pm 14.0 (58-194)	469 \pm 19.3 (366-560)	F: 6 M: 4
<i>N. fasciata</i>	37	125 \pm 20.0 (14-672)	461 \pm 22.1 (240-765)	F: 15 M: 22

Table 3.2. Prevalence (number of individuals infected / number of individuals sampled) and average parasitemia (± 1 SE, range; per 8000 erythrocytes) of *Hepatozoon* spp. infections in *Nerodia fasciata* and *Nerodia floridana* from a former nuclear cooling reservoir and two Carolina bays of the Savannah River Site in Aiken, South Carolina.

Species and Site	n	Prevalence	Parasitemia
Pond B Reservoir			
<i>N. floridana</i>	38	0.05 (2/38)	0.001 \pm 0.001 (0.00-0.04)
<i>N. fasciata</i>	19	0.42 (8/19)	1.04 \pm 0.54 (0.00-9.90)
Carolina Bays			
<i>N. floridana</i>	10	0.50 (5/10)	0.12 \pm 0.07 (0.00-0.11)
<i>N. fasciata</i>	37	0.86 (32/37)	11.57 \pm 2.30 (0.00-47.30)

Table 3.3: 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 a 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: 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 (Δ AIC), and the weight of each model (AIC_{wt}).

Model	Log-likelihood	K	AIC	Δ AIC	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 3.4: 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 a Carolina bay system of the Savannah River Site, SC. Model parameters, estimates, and associated *p*-values are displayed.

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

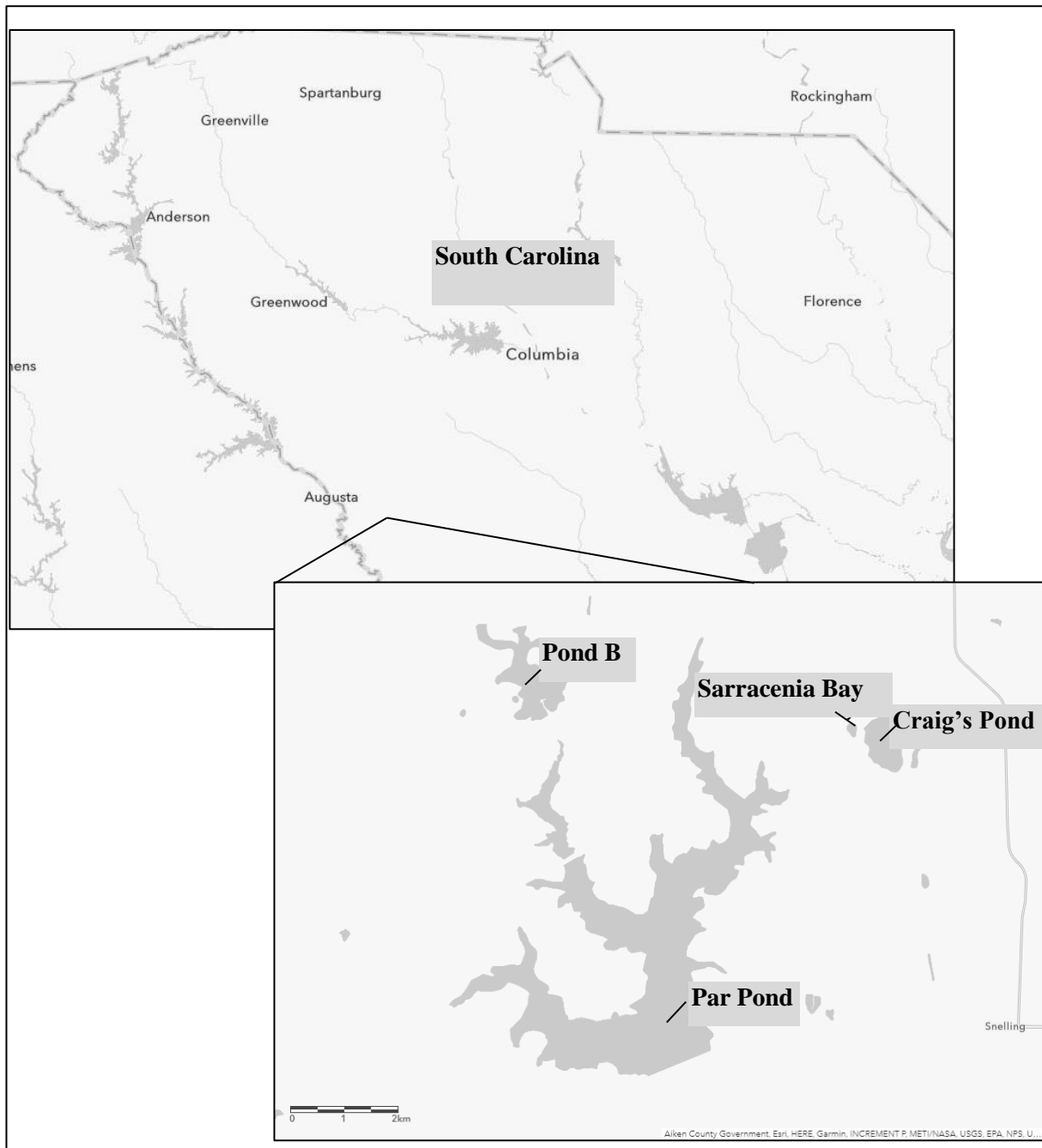


Figure 3.1. Map of study sites sampled on the Savannah River Site near Aiken, South Carolina. Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*N. fasciata*) were collected from two Carolina bays (Craig’s Pond and Sarracenia Bay) and a former nuclear cooling reservoir (Pond B) in the summers of 2017 and 2018 (Basemap: Esri Human Geography Map, HERE, Garmin, METI/NASA, USGS, EPA, NPS, USDA).

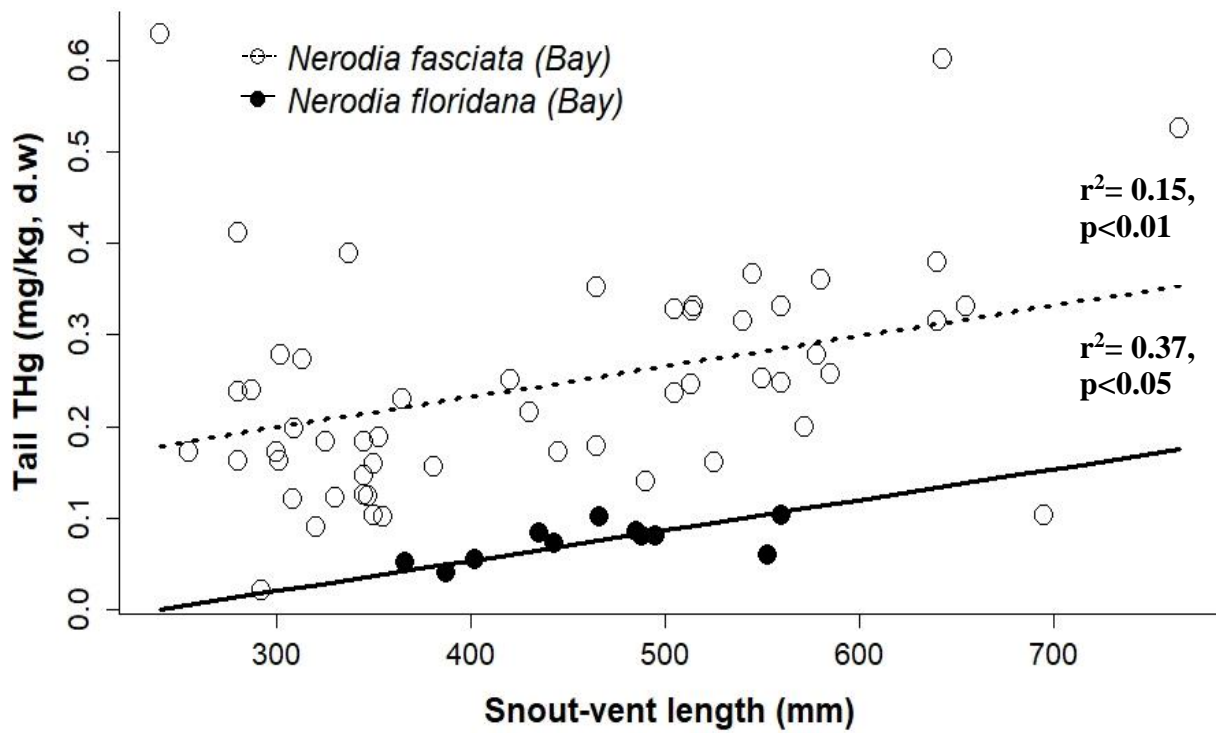


Figure 3.2: The relationship between snout-vent length and tail total mercury (mg/kg, dry weight) for Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*N. fasciata*) from two Carolina bays, Craig’s Pond and Sarracenia Bay, on the Savannah River Site near Aiken, SC.

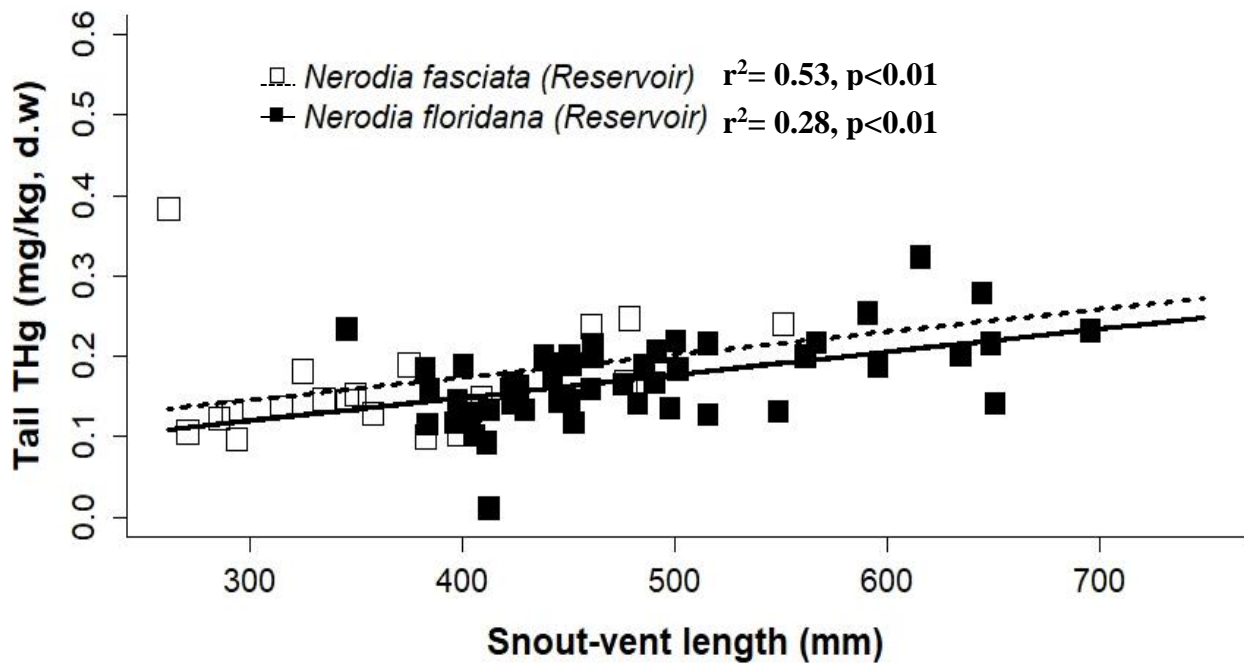


Figure 3.3: The relationship between snout-vent length and tail total mercury (mg/kg, dry weight) for Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*N. fasciata*) from a former nuclear cooling reservoir, Pond B, on the Savannah River Site near Aiken, SC.

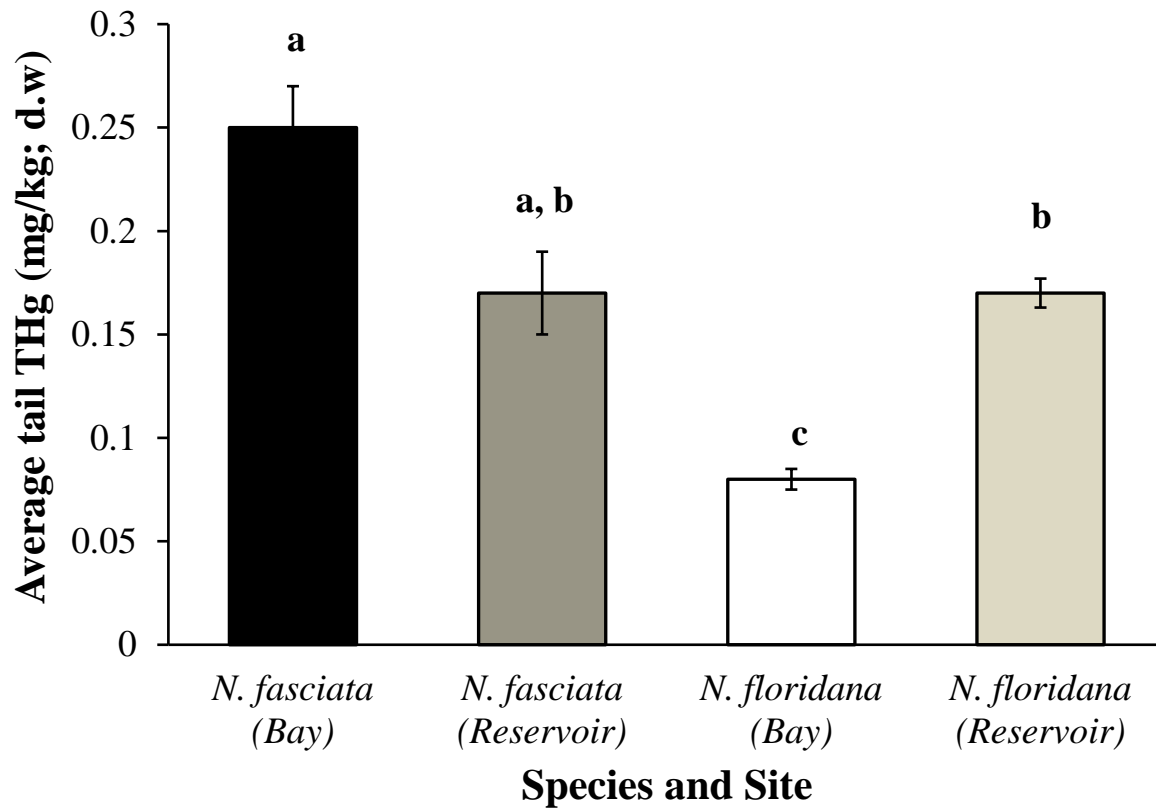


Figure 3.4. Average ($\pm 1SE$) tail total mercury for Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*N. fasciata*) living in a former nuclear cooling reservoir and isolated Carolina bays of the Savannah River Site, Aiken, SC. Letters indicate statistical significance.

CHAPTER 4

CONCLUSIONS

Environmental pollution associated with anthropogenic activities has been identified as one of many factors contributing to the global decline of vertebrate species (Gibbons *et al.* 2000, Ceballos *et al.* 2017, Goudie 2018). Mercury (Hg) and radiocesium (^{137}Cs) are well-known environmental contaminants associated with energy and weapons production that have the potential to negatively impact the health of both humans and wildlife (Wolfe *et al.* 1998, Møller *et al.* 2005, Scheuhammer *et al.* 2007, Djomina and Barilyak 2010). The bioaccumulation of contaminants, such as Hg and ^{137}Cs , can lead to direct impacts on the health of organisms and can lead to an increased susceptibility to other stressors such as disease and parasitism (Relyea and Mills 2001, Hanlon and Parris 2014). Despite having several ecological and life history characteristics conducive to studying environmental contamination (e.g., relatively long-lived, high site fidelity, and high trophic status), snakes have rarely been included in biological monitoring and risk assessments of polluted sites (Hopkins *et al.* 1999, Beaupre and Douglas 2009). In particular, semi-aquatic snakes, such as watersnakes (*Nerodia*) may be more susceptible to accumulating elevated levels of contaminants (Brisbin *et al.* 1974, Drewett *et al.* 2013).

For my research, I sampled Florida green watersnakes (*Nerodia floridana*) and banded watersnakes (*Nerodia fasciata*) from former nuclear cooling reservoirs and isolated Carolina bay wetlands of the United States Department of Energy's Savannah River Site (SRS) near Aiken, South Carolina. The aquatic habitats sampled had varying histories of Hg and ^{137}Cs

contamination associated with past nuclear weapons productions at the SRS. The overall goals of my research were to: (1) gain a better understanding of the factors associated with the accumulation of Hg and ^{137}Cs in aquatic snakes, (2) to compare accumulation of Hg between two species (*Nerodia floridana* and *Nerodia fasciata*) inhabiting two habitat types (nuclear cooling reservoir and Carolina bays) and (3) determine the relationships between body burdens of Hg and ^{137}Cs and sublethal health endpoints, standard metabolic rate (SMR) and infections by the intraerythrocytic hemoparasite, *Hepatozoon* spp in aquatic snakes. In measuring contaminant burdens and associations with SMR and *Hepatozoon* spp. infections in aquatic snakes, I hoped to contribute novel information on the accumulation of Hg and ^{137}Cs in reptiles and determine if health effects were apparent in snakes, decades after nuclear production activities ceased at the SRS.

In Chapter 2, I focused on Florida green watersnakes (*N. floridana*) living in three former nuclear cooling reservoirs with varying histories of ^{137}Cs and Hg contamination. As expected, *N. floridana* from Pond B, the site with the most history of radionuclide contamination, had the highest body burdens of ^{137}Cs . My results also indicated a significant, positive relationship between body size and ^{137}Cs , which is consistent with previous studies documenting the bioaccumulation of ^{137}Cs in aquatic ecosystems (Hakanson and Fernandez 2001, Sundbom *et al.* 2003). I also found that capture site and body size were important determinants of ^{137}Cs burdens. Interestingly, my results contrasted with recent research which found no relationship between body size and burdens of ^{137}Cs in cottonmouths (*Agkistrodon piscivorus*) inhabiting another nearby contaminated location on the SRS (Leaphart 2017). However, the disparity between study results is possibly linked to species differences in ontogenetic dietary shifts and the degree to which the species feed on aquatic versus terrestrial prey. Further investigations into ^{137}Cs

accumulation in several snake species is warranted to determine if interspecific differences in ecology and life history impact contaminant exposure. In contrast to my results for ^{137}Cs , site-level differences in THg in snake tail tips were not apparent, likely due to overall low levels of THg persisting at those sites. Overall, there was a positive relationship between tail THg and body size, which is consistent with other studies investigating the accumulation of Hg in snakes (Rainwater *et al.* 2005, Drewett *et al.* 2013, Lemaire *et al.* 2018, Rumbold and Bartoszek 2019). Not surprisingly, the only significant factor predicting tail THg in *N. floridana* was snake body size.

A surprising result of my research was the persistence of ^{137}Cs in snakes living in the Pond B reservoir. Most of the contamination occurring in the former nuclear cooling reservoirs occurred between 1964-1965 (Sugg *et al.* 1995), over 50 years prior to this study. However, body burdens of ^{137}Cs in *N. floridana* from Pond B was still at levels of potential concern (>0.6 Bq/g; EEC limit for fresh meat). Semi-aquatic snakes from Par Pond collected between 1971-1972, less than a decade after contamination, were reported to have an average ^{137}Cs body burden of 57.0 pCi/g (Brisbin *et al.* 1974). In comparison, *N. floridana* collected from Par Pond showed a substantial decrease in ^{137}Cs burden to 2.4 ± 4.3 pCi/g (converted from Bq/g). Comparatively, body burdens of ^{137}Cs in *N. floridana* from Pond B are still at a relatively high average of 18.6 ± 14.0 pCi/g in 2016, highlighting the bioavailability and persistence of ^{137}Cs in the Pond B reservoir 50+ years after contamination occurred.

In contrast to expectations, I did not find any positive associations between *Hepatozoon* spp. infections and body burdens of Hg and ^{137}Cs in *N. floridana*. However, increasing whole-body ^{137}Cs was associated with decreased probability of *Hepatozoon* spp. infections, suggesting that the parasite, vectors, or initial vertebrate host could be more susceptible to the harmful

effects of ^{137}Cs compared to *N. floridana*. Additional research is needed to further elucidate the role of ^{137}Cs in the parasite-vector-host relationship between *Hepatozoon* spp. and *N. floridana*, as there are many other factors besides contaminant burden that could be influencing our results (e.g., prey availability, habitat characteristics). However, the results of my research indicate the potential for low-level ^{137}Cs to decrease *Hepatozoon* spp. infections in *N. floridana*. A link between ^{137}Cs and decreases in parasitism has been suggested by previous studies (Childs and Cosgrove 1966, Wilbur *et al.* 1994) of mammalian taxa, but to our knowledge this is the first study to present evidence in a reptilian species.

While there was not strong support for an association between contaminant burdens and SMR, some of the highest SMR rates were exhibited by *N. floridana* from Pond B—the site where ^{137}Cs body burdens were the highest on average. Higher body burdens of ^{137}Cs have been associated with DNA damage in fish (Sugg *et al.* 1995) and turtles (Bickham *et al.* 1988) living in the Pond B reservoir. If similar damage is occurring to the DNA of *N. floridana* living in Pond B in 2016, it is possible that energetically costly DNA repair mechanisms could result in elevated SMR. Research by Sugg *et al.* (1995) also found an association between Hg concentrations and DNA double strand breaks in bass living in the contaminated reservoir. Thus, even though THg concentration in tails of *N. floridana* from Pond B were relatively low, it is possible that Hg and ^{137}Cs are synergistically causing genotoxic damage that leads to an elevated SMR. Further investigations including more sublethal health endpoints (e.g., DNA double-strand breakage) could be useful in further elucidating the effects of ^{137}Cs and THg on *N. floridana* residing in the former nuclear cooling reservoirs of the SRS.

In Chapter 3 of my thesis, I compared tail THg and *Hepatozoon* spp. infections in *N. floridana*, and the sympatric congener *N. fasciata*, living in the Pond B reservoir and two isolated

Carolina bays on the SRS. I was interested in determining if differences in habitat affinity and feeding ecology between the two species would translate to differences in tail THg and prevalence and parasitemia of *Hepatozoon* spp. infections. Both *N. floridana* and *N. fasciata* were captured in the reservoir and bays, with *N. floridana* occurring more commonly in Pond B and *N. fasciata* more common in bays. We found a positive relationship between body size and tail THg in both species from both habitats. However, the observed interspecific variation in THg was counter to expectations. Based on expected dietary overlap in bays and less overlap in Pond B, we expected THg burdens to be similar between species in the bays and different in the reservoir. In contrast, burdens of THg were higher for *N. fasciata* in the bay compared to *N. floridana* and similar between the two species living in Pond B. Ontogenetic shifts in diet, size-classes, and number of species captured from each site are likely related to the observed results. The Pond B reservoir has a higher abundance and diversity of fish species that may be a convenient food resource for both species. The Carolina bays are inhabited by more amphibian prey resources including frogs, tadpoles, and salamander larvae, the latter of which is an important resource for both species (Durso *et al.* 2011, 2013). However, prior investigations into ontogenetic shifts in diet indicate larger *N. fasciata* in the bays are likely consuming large anuran prey items (e.g., ranid frogs; Mushinsky *et al.* 1982). Within species, we found that *N. fasciata* from the bays had higher average THg compared to those from the reservoir. However, the inverse was true for *N. floridana* with those from the reservoir exhibiting higher THg than those from the bay. Again, the observed differences are likely related to diet and body size of snakes captured in each location.

The results from my investigation into *Hepatozoon* spp. infections were most likely related to habitat-based interspecific differences in feeding ecology. Infections with *Hepatozoon*

spp. were less prominent for both species residing in the former nuclear cooling reservoir and more likely in the Carolina bays. Infections by *Hepatozoon* spp. in aquatic snakes often stems from the consumption of an initial vertebrate host, which in the case of *Nerodia* is often a frog (Smith *et al.* 1994, Smith 1996, Tomé *et al.* 2012). Prevalence and parasitemia were higher for *N. fasciata* compared to *N. floridana* in the reservoir and were by far the highest for *N. fasciata* living in the bays likely reflecting a reliance on amphibian prey items. While diet and habitat appear to be the most important predictors of *Hepatozoon* spp. infections in the two *Nerodia* sampled in my research, we cannot eliminate the possibility that ^{137}Cs burdens of snakes played a role, as suggested in the results from Chapter 2. Interestingly, we did not find a relationship between tail THg and *Hepatozoon* spp. infections. Levels of Hg observed in snake tail tips in my research were relatively low compared to similar surveys elsewhere (Drewett *et al.* 2013, Haskins *et al.* 2019), and a few studies investigating the harmful effects of Hg on snakes (Wolfe *et al.* 1998, Chin *et al.* 2013) suggests snakes may have some tolerance to the contaminant. Thus, it is possible that THg concentrations I observed may be below thresholds that would cause detrimental effects, or that snakes living in SRS reservoirs and Carolina bays may have developed a tolerance to THg.

Despite a history of contamination associated with past nuclear weapons productions at the SRS, the former nuclear cooling reservoirs I sampled overall retain only modest levels of ^{137}Cs and Hg. These reservoirs provide important habitat to an impressive array of wildlife, including *N. floridana*. Additionally, the over 200+ Carolina bays of the SRS provide critical habitat for an immense biomass of biota, including reptiles and amphibians. While commonly found throughout most of its range in the state of Florida, the disjunct South Carolina population of *N. floridana* is listed as a species of concern due to a fragmented distribution and habitat loss

(Bennett and Buhlmann 2015). However, *N. floridana* is commonly encountered in the former nuclear cooling reservoirs of the SRS. Because *N. floridana* is a poor overland disperser and slow to recolonize sites from which they have become extirpated, one of the biggest threats to the species is anthropogenic alterations to aquatic habitats (e.g., the ditching and draining of wetlands for agricultural purposes), along with drought (Seigel *et al.* 1995, Willson *et al.* 2006, Vogrinc *et al.* 2018). Access to the aquatic habitats within the boundaries of the SRS has been restricted since the site's inception in the early 1950's (White and Gaines 2000). Thus, anthropogenic disturbance to aquatic habitats of the SRS has been limited, likely to the benefit of an array of wildlife species, including *N. floridana*, and may offset any detrimental effects associated with the legacy Hg and ^{137}Cs contamination.

My research demonstrates the potential of watersnakes to serve as environmental indicators of Hg and ^{137}Cs contamination in aquatic ecosystems. My findings indicate that habitat and subsequently resources available for prey can be important determinants of exposure to environmental contaminants and exposure to infections by hemoparasites, such as *Hepatozoon* spp.. My results suggest that species 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 and incorporating stable isotopes analysis to further investigate the effect of trophic dynamics on contaminant accumulation and *Hepatozoon* susceptibility in watersnakes.

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