Mercury Bioaccumulation and Adverse Reproductive Effects in Snapping Turtles Inhabiting a Historically Contaminated River

Brittney Cole Hopkins

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William A. Hopkins
Ignacio T. Moore
Dana M. Hawley

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Abstract

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Mercury (Hg) is a global pollutant that has received much attention due to its ability to bioaccumulate, biomagnify, and maternally transfers in humans and wildlife. In vertebrates, exposure to Hg can impair growth, alter behavior and morphology, decrease survival, and reduce reproductive success. Unfortunately, most ecotoxicology studies euthanize animals to quantify the concentrations of Hg bioaccumulation and in doing so eliminate the ability to relate Hg accumulation to observed effects. The development of non-destructive sampling techniques is a critical step for sustainable monitoring of Hg bioaccumulation and associated effects because it eliminates adult harvest, enables repeated sampling of the same individual over time, and allows the collection of larger sample sizes. My research aimed to develop and validate non-destructive sampling techniques for assessing Hg bioaccumulation, maternal transfer, and consumption risks in a long-lived aquatic omnivore, the common snapping turtle (*Chelydra serpentina*). I collected blood, nail, muscle, and egg tissues from turtles inhabiting an Hg contaminated gradient at a historically contaminated river, the South River, located in central Virginia. In my first chapter, I developed mathematical models describing relationships between the four tissues sampled and in doing so, described important demographic, spatial, and temporal factors that influence Hg bioaccumulation in turtles that may be important for ecological risk assessment and consumption. Additionally, I found that my mathematical models were applicable to other Hg contaminated locations in Virginia. In my second chapter, I examined the effects Hg bioaccumulation and maternal transfer has on turtle reproduction. I collected and incubated eggs from gravid females from reference and contaminated sites and quantified embryonic morality, infertility, and hatching success of each clutch, and assessed all hatchlings and dead embryos for gross morphological malformations. I found that Hg exposure negatively influenced hatching success through increased egg infertility and embryonic mortality. Taken together, my results are applicable to a wide array of systems where biomonitoring and assessing the ecological and consumption risk of contamination in turtles needs to be accomplished in a sustainable and conservation-minded fashion.

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Attribution

Chapter 1 was co-authored with my advisor, William A. Hopkins, and a technician Mark J. Hepner. Mark Hepner was a vital component in data collection. All co-authors provided input on the design of the experiment and use of lab/field equipment. Myself and Dr. Hopkins edited the chapter.

Chapter 2 was co-authored with my advisor, William A. Hopkins, a fellow graduate student Devin K. Jones, and a post-doctoral researcher John D. Willson. All co-authors provided input on the design of the experiment, aided in data collection, use of lab/field equipment, and edited the chapter.

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Introduction

Anthropogenic environmental changes such as habitat destruction, pollutants, and climate change are hypothesized causes of species population declines, extinction, and the increased prevalence of emerging diseases (Lande 1998; Daszak et al. 2001). However, conservation science is lagging behind the high rate of anthropogenic change that is currently threatening biodiversity. For example, the numbers of studies that have addressed the issue of pollution in no way stand up to its perceived threat on biodiversity (Lawler et al. 2006). This is troublesome considering within the past 5 years, the U.S. alone has faced several catastrophic environmental disasters that have again brought environmental pollution to the forefront of political debate, public awareness, and conservation concern. Recent mass contamination events like the Tennessee Valley Authority coal ash spill in Kingston, TN and the British Petroleum oil spill in the Gulf of Mexico, have threatened ecological resources and human health on an unprecedented scale. In efforts to provide additional information on the adverse consequences that pollutants can have on wildlife, my master's research sought to document current concentrations and associated effects of a ubiquitous heavy metal contaminant that can persist within affected organisms and their environment.

Mercury. Mercury (Hg) has received significant attention from the scientific community because of its widespread environmental abundance and associated toxicity to humans and wildlife (Scheuhammer et al. 2007; Fitzgerald et al. 1998; Clarkson 1990). Mercury is a naturally occurring heavy metal stored within the earth's crust, but it can enter the environment in high concentrations through mining and other industrial processes. In the U.S., the most common sources of Hg are emissions from power plants, incinerators, water treatment plants,

and manufacturing facilities (Schmeltz et al. 2011). Although Hg emissions within the U.S. have been reduced by 95% over the past 20 years, coal burning power plants still emit over 50 tons of Hg each year which pose serious local, regional, and global ecological risks (Schmeltz et al. 2011). Emission sources facilitate the deposition of Hg into the environment through atmospheric deposition, point source contamination, and improper disposal (Carlsen et al. 2006; Hageman et al. 2006; Lorey and Driscoll 1999; Muller et al. 2002). Once introduced into the environment, Hg can cycle through terrestrial and aquatic ecosystems. Anaerobic sulfate-reducing bacteria in aquatic sediments mediate the methylation of Hg into a more bioavailable and toxic form, methylmercury (MeHg), which can subsequently be accumulated by biota (Lindqvist 1991; Watras and Bloom 1992). In 2009, the U.S. Environmental Protection Agency (EPA) released a survey that reported detectable Hg in fish tissue from all U.S. lakes that were sampled (n=500). From these samples, they estimated that approximately 49% of lakes across the U.S. contain fish with tissue Hg burdens exceeding the 0.3 ppm health screening limit associated with human health risks brought about by Hg toxicity (e.g., neurological damage; USEPA 2009).

Impacts of Mercury on wildlife. Methylmercury can bioaccumulate over an individual's lifetime and biomagnify as it transfers from lower trophic level organisms to higher trophic-level predators. The biochemical transmission of MeHg between trophic levels causes high Hg burdens to accumulate in long-lived predators (Lindqvist 1991; Watras & Bloom 1992). Methylmercury's toxic effects have been well documented in both field sampling and laboratory experiments in multiple taxa. In fish, MeHg exposure has been shown to impair growth, behavior, gonad development, and sex hormone production (Crump & Trudeau 2009; Drevnick et al. 2003; Friedmann et al. 1996; Weber et al. 2003). Due to MeHg's ability to be transferred

through the food web, similar impairments have been documented in several piscivorous species (reviewed in Scheuhammer et al. 2007). In aquatic mammals, such as mink and otter, MeHg can disrupt neurochemistry, causing adverse neurological effects such as seizures, limb paralysis and death (Wiener 2003, Sleeman et al. 2010). Adverse effects of Hg are not only apparent in aquatic systems, but can affect terrestrial species as well. Insectivorous and piscivorous bird species can accumulate high concentrations of Hg through dietary intake and associated effects have included decreased yearly survival, inhibited immunocompetence, altered hormone profiles, and reduced reproductive success (Hallinger et al. 2011, Hawley et al. 2009, Wada et al. 2009, Brasso et al. 2008, Barr 1986). Additionally, numerous studies have demonstrated MeHg's influence on reproduction in both terrestrial and aquatic oviparous species causing embryotoxicity, thus decreasing hatching success in fish, amphibians, and birds (Bergeron et al. 2011a; Hammerschmidt et al., 2002; Jackson et al. 2011).

The toxic effects of Hg exposure in reptiles have received less attention from the scientific community compared to fish, birds, amphibians, and aquatic mammals. Only recently have reptiles, specifically aquatic turtles, been utilized to monitor bioaccumulation in Hg contaminated areas (Bergeron et al. 2007; Golet & Haines 2000; Turnquist et al. 2011; Blanvillain et al. 2007). Although several of these studies have described Hg tissues concentrations in turtles (Golet & Haines 2001; Blanvillain et al. 2007; Turnquist et al. 2011, Hopkins et al. In review), no studies have related these tissue relationships to the effects of Hg bioaccumulation and maternal transfer on turtle reproduction.

Maternal Effects and Transfer. Maternal effects are defined as non-genomic female traits that influence offspring phenotype (Mousseau & Fox 1998; Wolf & Wade 2009). These maternal traits contribute to variation in offspring development, phenotype, and fitness

(Bernardo 1996; Badyaev & Uller 2009). In reptiles, common maternal effects include nest site selection and hormone and nutrient allocation to eggs (Shine & Harlow 1996; Warner et al. 2007). In addition to these natural physiological and behavioral factors, harmful substances (e.g., contaminants) can be maternally transferred to offspring and their deleterious effects on offspring development and survival are well documented in many oviparous species (Hopkins et al. 2006; Hammerschmidt et al. 2002; Alvarez et al. 2006; Kelly et al. 2008, Eisenreich et al. 2009; Bergeron et al. 2010, 2011a,b). Recently, several studies have focused on the maternal transfer of Hg and its influence on offspring phenotype (Jackson et al. 2011; Bergeron et al. 2010, 2011a,b; Todd et al. 2011).

Mercury can maternally transfer from mother to ova (Bergeron et al. 2010; Bargar et al. 2001; Guirlet et al. 2008) and cause lethal and sublethal effects on developing offspring. Bergeron et al. (2010 & 2011a) found that female toads were capable of transferring ~5% of their Hg body burden to their eggs which was sufficient to subsequently reduce embryonic hatching success. Similar effects have also been observed in birds and fish, where females exposed to Hg had lower nest success and reproductive output (Hallinger et al. 2011; Brasso et al. 2008; Barr 1986; Hammerschmidt et al. 2002). Along with reducing hatching success, Hg is also known to cause sublethal effects in exposed individuals. Alvarez et al. (2006) reported compromised behavioral performance in Atlantic croaker larvae hatched from eggs produced by adults exposed to MeHg. Additionally, Bergeron et al. (2011b) found that maternal transfer of Hg caused sublethal effects in developing amphibian larvae including reductions in body size and swimming performance along with an increase in time to metamorphic climax. These lethal and sublethal alterations in offspring phenotype caused by maternally transferred Hg could have implications for fitness and serve as a source for phenotypic variation within populations living in contaminated areas.

Study Species. My focal study species was the common snapping turtle (*Chelydra serpentina*). The snapping turtle occurs across much of the central and eastern half of the United States and extends into the southern most parts of eastern Canada (Ernst 2008). Snapping turtles are long-lived, large-bodied, semi-aquatic turtles that primarily occupy streams, rivers, ponds, and lakes. Females migrate to open terrestrial areas with soft soils and minimal vegetation in order to construct nest chambers by using their hind feet. Once nest construction is complete, females then continuously lay their entire clutch, cover the eggs and return to the aquatic habitat. Snapping turtles exhibit temperature-dependent sex determination in which incubation temperatures ranging from ~21.4˚C to ~27.8˚C predominately produce male offspring and temperatures below and above that range give rise to females (Janzen 2008). Female turtles reach reproductive maturity anywhere from 7-15 years of age and produce a single clutch per year with an average size ranging from 26 to 55 eggs (Miller et al. 1989). However, clutch size and sexual maturity are highly dependent on body size and latitudinal location (Congdon et al. 2008). Nesting phenology is also highly dependent on latitude, with turtles at lower latitudes nesting earlier than those at higher latitudes. Rainfall and soil moisture are also key components in determining the nesting season as these factors are important in female nesting behavior and can influence embryonic development (Congdon et al. 2008). In Virginia, nesting typically occurs between mid-May through the end of June (Mitchell 1994; Hopkins and Hopkins, pers observations).

Biomonitoring. Turtles serve as excellent model organisms for monitoring contaminants because of their unique suite of ecological and life-history attributes. Because they are long-lived and often an apex predator, the common snapping turtle is an excellent model organism to study

the bioaccumulation and magnification of Hg. For these reptiles, accumulation of Hg in target tissues (e.g., brain, liver, kidney, muscle, egg) could reach levels that are associated with both sublethal and lethal effects in other aquatic species. Unfortunately, most ecotoxicological studies kill turtles to sample target tissues (Aguirre et al. 1994; Lamb et al. 1995; Golet & Haines 2001) and therefore lack the ability to relate accumulated toxicant levels to the physiological effects that may arise from such exposure.

With many turtle populations declining (Gibbons et al. 2000; Buhlmann et al. 2009), euthanizing adults for toxicity sampling is neither sustainable nor conservation-minded, and efforts must be made to implement new nondestructive methods (Hopkins 2006). Although nondestructive indices have been used to measure contaminant levels in turtle blood and nails, a study has yet to develop non-invasive techniques that relate these values with accumulated tissue burdens (e.g., muscle tissue) and reproductive effects. The successful development of nondestructive methods will allow researchers to simultaneously collect larger sample sizes while minimizing effects on wild turtle populations. Additionally, for bioaccumulative contaminants, nondestructive sampling techniques will enable investigators to repeatedly sample the same individual in order to monitor its exposure throughout its lifetime. Sampling multiple tissues will allow researchers to build mathematical correlations between less invasive samples (e.g., nail and blood) to concentrations that can be accumulated in tissues (e.g., muscle and eggs) that are often relevant to overall health, reproductive success, and transgenerational effects attributable to maternal transfer (Hopkins 2006).

Human Consumption. Mercury toxicity is not only limited to wildlife but can pose severe health risks to humans. The majority of human exposure to Hg occurs through the uptake of contaminated fish and seafood (Clarkson 1990, Fitzgerald & Clarkson 1991). Exposure to

high dietary concentrations of Hg is associated with several health problems in humans such as heart attacks and cardiovascular disease (Gullar et al. 2002; Roman et al. 2011). However, exposure to Hg is not only limited to dietary intake. Pregnant women can maternally transfer Hg across the placenta, causing impairment of infant motor control (May 2000). Mercury poisoning in children can result in negative effects on development of attention span, language, and memory, causing permanent life-long damage (Grandjean et al. 1999; Swain et al. 2007). With over 3,700 fish consumption advisories within the U.S., the likelihood of humans consuming Hg is a reality (USEPA 2010).

According to the USEPA (2010) there are 3,710 listed Hg-related fish consumption advisories that encompass 1,143,327 affected river miles and 16, 396,422 lake acres that have some degree of fish consumption restrictions. Clearly, Hg contamination is a significant threat that has affected multiple communities across the U.S. In order to protect local communities, states issue their own safety guidelines and commonly post warning signs along rivers indicating safe fish consumption limits. However, these state guidelines frequently ignore other species that may be targets for human consumption and bioaccumulate high levels of Hg such as birds, mammals, and turtles. Although areas of Hg contamination occur across the U.S., currently, there are only three states within the U.S. that have identified the consumption of snapping turtles to be potentially hazardous to human health and implemented consumption restrictions where appropriate (Pennsylvania Fish & Boat Commission 2012; Ohio Environmental Protection Agency 2009; New York State Department of Health 2011). For many regions, turtle consumption can be common and humans may be at risk of serious health problems if consumption guidelines and public education are not implemented.

Turtle Conservation. Over millions of years, turtles have evolved a unique suite of life-history traits including delayed sexual maturity, long life-spans, and low natural adult mortality (Andrews 1982; Shine and Iverson 1995). However, these traits may be maladaptive to turtle populations facing multiple modern day stressors such as increased harvesting, habitat fragmentation, emerging infectious diseases, and pollution (Turtle Conservation Fund 2002). For example, it is estimated that over 10 million adult turtles are harvested annually and traded on the global market for human consumption or medicinal purposes (Van Dijk et al. 2000). As a consequence, turtle harvests have led to dramatic declines in turtle populations, particularly those native to Asia (Altherr and Freyer, 2000; Gibbons et al. 2000; Cheung and Dudgeon 2006). However, within the U.S., turtle harvest rates are largely unknown and research has yet to examine whether current national and/or local harvesting limits are sustainable for turtle populations. For instance, in Virginia, citizens holding a fishing license can harvest up to 15 adult snapping turtles a day, without any size, sex, or seasonal restrictions. Turtle populations are dependent upon the annual survival of adults, especially females, and are unable to withstand chronic adult mortality (Congdon et al. 1987, 1994). Additionally, many turtle populations may be faced with more than one anthropogenic stressor, such as pollution and overharvesting, which may have adverse consequences on populations. These anthropogenic disturbances, compounded with naturally low hatchling survivorship and nest survival may leave turtle populations limited in their ability to withstand and respond to the growing threat of environmental pollution and human disturbances. Thus, scientific investigations on turtles must be conducted in a sustainable and conservation-minded manner without compromising biomonitoring and risk assessment efforts.

Assessment of Bioaccumulation and Reproductive Effects of Mercury. Broadly, I had two main objectives for my Master's research. First, I sought to develop and validate non-destructive sampling techniques for assessing Hg bioaccumulation, maternal transfer, and consumption risks in turtles so that future monitoring studies can be performed sustainably. From these tissues, I also described demographic, spatial, and temporal factors that influence Hg bioaccumulation in turtles. Second, based on the known deleterious reproductive effects associated with Hg exposure, I hypothesized that exposure to excessive Hg concentrations would negatively affect turtle reproduction and development. Specifically, I predicted that Hg exposure would negatively influence hatch success, malformation frequency, mortality, egg fertility, and clutch characteristics (e.g., clutch size, egg mass, and clutch mass).

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Chapter 1: Non-destructive Techniques for Describing Patterns of Mercury Bioaccumulation, Consumption Risks, and Maternal Transfer in a Freshwater Turtle

Co-authors: Mark J. Hepner, William A. Hopkins

Abstract

Mercury (Hg) is a global pollutant that has received much attention due to its ability to persist within the environment and biota. Currently, most ecotoxicology studies euthanize animals to quantify the concentrations of Hg bioaccumulation. The development of non-destructive sampling techniques is a critical step for sustainable spatial and temporal monitoring of Hg accumulation because it eliminates adult sacrifice, enables repeated sampling of the same individual over time, and allows the collection of larger sample sizes. In this study, we collected blood, nail, muscle, and egg tissues from snapping turtles (*Chelydra serpentina*) inhabiting an Hg contaminated gradient at a historically contaminated river located in central Virginia and formulated mathematical models describing relationships between tissues. From these tissues, we also describe important demographic, spatial, and temporal factors that influence Hg bioaccumulation in turtles. Additionally, in order to validate our mathematical models, we sampled two additional Hg contaminated locations in Virginia. As predicted, turtles inhabiting contaminated areas accumulated higher levels of Hg in their tissues than reference individuals. However, all Hg tissue concentrations were strongly and positively correlated with each other (in all cases: $p < 0.001$). Body size significantly influenced bioaccumulation of Hg in muscle but the nature of this effect was dependent upon site, with muscle Hg concentrations increasing with body size within contaminated individuals but did not change with size within the two reference sites. After correcting for body size, females had on average 93.9%, 6.3%, and 15.3% higher Hg in muscle than males from the two reference sites along the Middle and South River and the

contaminated site, respectively. Additionally, we found that tissue relationships developed from the two other validation sites did not significantly differ from those generated from the historically contaminated site. The models provided herein will be useful for a wide array of systems where biomonitoring of turtles needs to be accomplished in a sustainable and conservation-minded fashion.

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Keywords: Mercury, tissue, consumption, non-destructive, turtle

Introduction

Despite the variety and quantity of contaminants that continue to be released into the environment (Joyce and MacDonald 2010; USEPA 2011), pollution remains one of the most understudied anthropogenic stressors threatening biodiversity (Lawler et al. 2006). Industrial point sources, improper waste disposal, and atmospheric transport have facilitated the global distribution of harmful toxicants (Lorey and Driscoll 1999; Carlsen et al. 2006; Hageman et al. 2006; Muller et al. 2002). The heavy metal mercury (Hg) is of particular concern due to its widespread prevalence and deleterious effects on humans and wildlife (Schuster et al. 2002; Scheuhammer et al. 2007). Within the U.S., Hg is released into the environment by various anthropogenic sources, with coal burning power plants contributing to the majority of emissions (Schmeltz et al. 2011). Ultimately, Hg is deposited into aquatic ecosystems such as lakes, rivers, and estuaries where sedimentary sulfate-reducing bacteria mediate methylation of Hg into its more toxic form, methylmercury (MeHg) (Lindqvist et al. 1991; Watras and Bloom 1992). Methylmercury is of particular ecological concern because it bioaccumulates over an individual's lifetime and biomagnifies within food webs (Lindqvist et al. 1991; Watras and Bloom 1992).

The toxic effects of Hg have been well documented in vertebrates. Due to the ability of Hg to be transferred and magnified through trophic interactions, severe physiological impairments have been documented in several invertivorous and piscivorous species (reviewed in Scheuhammer et al. 2007). In fish, Hg exposure can impair growth, behavior, gonad development, and sex hormone production (Friedmann et al. 1996; Drevnick et al. 2003; Weber et al. 2003; Crump & Trudeau 2009). In birds, high concentrations of Hg are associated with decreased yearly survival, inhibited immunocompetence, altered hormone profiles, and reduced reproductive success (Hallinger et al. 2011, Hawley et al. 2009, Wada et al. 2009, Brasso & Cristol 2008, Barr 1986). For apex aquatic mammal species, exposure to Hg is linked to adverse neurological effects such as seizures, limb paralysis and death (Wiener 2003; Sleeman et al. 2010). Additionally, Hg can be maternally transferred from female to offspring (Bergeron et al. 2010a) and several studies have demonstrated the negative influence of Hg on reproduction in oviparous vertebrates through reducing female egg laying (Barr 1986; Hammerschmidt et al. 2002) and decreasing hatching success (Wiener 2003; Bergeron et al. 2011).

Turtles have been proposed as excellent model species for monitoring Hg contamination in aquatic ecosystems because of their ecological and life-history attributes (Meyers-Schone & Walton 1994; Golet & Haines 2001). Many turtle species are long-lived, occupy a wide-range of habitats, occur in high densities in a variety of aquatic habitats, and feed at high trophic levels (Iverson 1982). Additionally, turtles and their eggs are common food items for predatory wildlife (Mitchell 1994, Ernst et al. 1994), and are utilized as a human food source in many regions (Klemens et al. 1995; Green et al. 2010), extending the threat of Hg exposure to organisms that eat turtles.

The development of non-destructive sampling methods would facilitate sustainable monitoring of spatial and temporal Hg patterns within turtles inhabiting polluted areas. Because

many turtle populations are limited in their capacity to withstand declines in adult survivorship (Congdon et al. 1987, 1994), euthanizing adult turtles for monitoring Hg exposure and accumulation is not a sustainable sampling option and efforts must be made to develop new nondestructive methods while still providing accurate toxicant exposure data. Non-destructive sampling also enables repeated sampling of the same individual over time, critical for understanding temporal changes in Hg accumulation and exposure (Hopkins et al. 2005, 2007). In addition, developing mathematical correlations between less invasive tissues and target tissues that are often relevant to overall health, reproductive success, and transgenerational effects (i.e. maternal transfer; Hopkins 2006) may eliminate the need for sacrifice of adults and eggs.

Although previous studies have describe correlations between Hg tissues concentrations in turtles (Golet & Haines 2001; Blanvillain et al. 2007; Turnquist et al. 2011) no study has yet described the relationship between easily obtained tissues and those relevant to trangenerational effects and consumption risks. Additionally, no previous study has validated their tissue relationships to other Hg contaminated sites, an important step towards understanding whether relationships developed at one site are broadly applicable. Therefore, our study sought to address two main objectives. First, we sought to develop and validate non-destructive sampling techniques for assessing Hg bioaccumulation, maternal transfer, and consumption risks in turtles so that future monitoring studies can be performed sustainably. Second, we aimed to describe demographic, spatial, and temporal factors that influence Hg bioaccumulation in turtle tissues along a wide gradient of contamination.

Methods

Study species. The common snapping turtle (*Chelydra serpentina*) is widely distributed across eastern and central North America and inhabits a wide array of freshwater habitats. These

reptiles possess several traits, including longevity, large body-size, and high trophic level feeding habits, that make them susceptible to bioaccumulation and biomagnification of contaminants such as Hg (Ernst 2008). Within snapping turtles, adult males asymptote at a larger body size than most females, creating a distinct body size dimorphism between the sexes (Gibbons and Lovich 1990). Female snapping turtles lay a single large clutch (averaging 26 – 55 eggs) per year (Miller et al. 1989). Gravid females migrate to open terrestrial areas with soft soils and minimal vegetation in order to construct nest chambers into which they deposit their entire clutch before returning to the aquatic habitat. In Virginia, nesting typically occurs between mid-May and the end of June (Mitchell 1994).

Study Site. We studied Hg bioaccumulation in snapping turtles inhabiting the South River, located near Waynesboro, VA, USA. From 1929-1950, Hg was released into the South River from an industrial plant manufacturing acetate fiber using a mercuric sulfate catalyst (Carter 1977). An extensive gradient of Hg contamination has been documented along the South River, with water, sediment and animal tissue concentrations increasing downstream from the contamination source (Murphy 2004; Southworth et al. 2004; Bergeron et al. 2007; Brasso & Cristol 2008, Bergeron et al. 2010). A previous study found that blood Hg concentrations in turtles downstream of the contamination source were up to 108-fold higher than those collected upstream of the source as well as those collected from a nearby reference river (Bergeron et al. 2007).

From April-July in 2010 and 2011, we collected snapping turtles at various locations upstream and downstream of the industrial plant located on the South River and at several sites along the nearby Middle River (Figure 1). Some of these sites had been sampled in a previous study on turtles (Bergeron et al. 2007), but additional sites were added in this study due to

increased accessibility to properties and identification of additional areas of suitable turtle habitat. In order to include the extensive Hg contamination gradient present at the river, we sampled a total of eleven sites on the South River located between 2 and 22 (SR 2-22) river miles downstream of the contamination source (SR 0). Multiple sites ranging from 1.5-4 miles upstream of SR 0 were used as a reference (SR-ref) along with additional reference sites located on the Middle River (MR-ref), a nearby tributary of the South Fork of the Shenandoah River that joins the South River at Port Republic, Virginia, USA. Turtle movement between contaminated and reference sites along the South River is limited by Rife Loth dam located approximately one mile upstream of SR 0.

Additionally, in order to understand the extent of Hg migration into other rivers and provide validation for our non-destructive tissue models, we sampled at two locations downstream (approximately 30 and 45 miles downstream of the source) of the confluence of the South River and the South Fork of the Shenandoah. Blood and muscle tissues were the only tissues analyzed for THg concentrations from this sampling area. In order to further validate the mathematical relationships generated from tissues sampled from turtles inhabiting the South and Middle Rivers, we collected turtles from another population inhabiting an Hg contaminated site in Southwestern Virginia, the North Fork Holston River. From 1950 to 1972, a chloralkali plant, created a 30 hectare disposal pond filled approximately 24 meters deep with wastes containing Hg. This disposal area has since been identified by the Environmental Protection Agency (EPA) as a superfund site and is the primary point-source of Hg to the North Fork Holston River. In late July 2011, we collected turtles at various sites located both upstream (reference) and downstream of the source (contaminated) along the North Fork Holston River. Because sampling at the North Fork Holston River occurred after turtle reproduction had ceased, we were unable to sample eggs from that location.

Turtle collection and tissue sampling. We collected snapping turtles using baited hoop traps (Memphis Net and Twine, Memphis, TN, USA) set in suitable microhabitats along the river banks and baited with a mixture of sardines, corn, and/or chicken livers. Typically, 8-20 traps were set at a given site depending on habitat and the size of the sampling reach. We checked traps daily until a site failed to produce new captures (typically within 3-4 days), at which time traps were removed and reset at a different site. All turtles were transported to the field station where they were measured to the nearest cm (carapace length, carapace width, and plastron length), weighed, sexed by visual examination of cloacal position, and permanently marked along their marginal scutes according to a three scute code, previously used by Bergeron et al. (2007), for future identification. We removed 2-4 small (2-3 mm) nail samples from the tips of the left and right hind claws of each turtle using canine nail clippers and drew a 1-mL blood sample from either the caudal vein or the cervical sinus. Nail and blood samples were placed separately in 1.5 mL eppendorf tubes and stored at -20˚C prior to Hg analyses. In order to determine accumulated Hg in turtle muscle tissue, we removed a small biopsy from the ventral-lateral aspect of the tail following administration of a local anesthetic (Lidocane). The biopsy site was then sutured with 2-3 stitches using clear Polydioxanone monofilament material (3/8cm) and a topical antibiotic was applied to reduce risk of infection. Gravid female turtles were weighed prior to induction of egg laying (see below), but all other procedures were delayed until after oviposition to reduce handling stress. After all samples were collected, turtles were released at their point of capture.

Egg Collection. Upon capture, we physically palpated female turtles for the presence of shelled eggs. We weighed gravid females and placed them individually within 100 gal plastic cattle

tanks inside the field house. Each tank was filled with ~20 gal of dechloraminated water. We injected gravid females intraperitoneally with 40 mg/kg of oxytocin solution every 24 hrs for three consecutive days to induce oviposition. We removed deposited eggs within 3 hrs and marked and measured (length, width, and mass) each egg. Completion of oviposition was confirmed by radiographs taken by qualified technicians at the Wildlife Center of Virginia, Waynesboro, VA. Once oviposition was complete, 3 eggs per clutch were frozen at -20°C to determine egg THg concentration and account for any intra-clutch differences.

Mercury Analysis. We lyophilized and homogenized muscle and eggs and report their THg concentrations on a dry weight (dwt) basis. We homogenized whole blood using a vortex mixer and we report THg concentrations in blood on a wet weight (wwt) basis. We washed nail clippings by placing them in a sterilized tube with 10mL solution of 15:1 deionized water to ethanol and sonicated for 20 minutes. After sonication we discarded the solution and allowed nails to air dry on a clean laboratory bench and report THg concentrations on a fresh weight basis (fwt). A homogenized sample of three randomly selected eggs per clutch was used to determine egg Hg concentrations (Bishop et al. 1995). Percent moisture was 77.3 ± 0.24% (mean ± 1 standard error of the mean hereafter) for muscle and 75.5 ± 0.18% for eggs. Samples were analyzed for THg by Dr. Dan Cristol (College of William and Mary, Williamsburg, Va) by combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT, USA) according to U.S. Environmental Protection Agency (U.S. EPA) method 7473 (USEPA 1998). For quality assurance, each group of 20 samples included a replicate, blank, and standard reference material (SRM; DOLT-4 dogfish liver, DORM-3 fish protein (National Research Council of Canada (NRCC), Ottawa, ON)). Method detection limits (MDLs; 3 times the standard deviation of procedural blanks) for samples were

0.0042 mg kg^{-1} (ppm), and all samples had THg concentrations that exceeded the limit. Average relative percent differences (RPD) between replicate sample analyses were 8.38% ± 1.25% (*n*=60). Mean percent recoveries of THg for the DOLT-4 and DORM-3 ranged from 99.77 ± 0.26 % to 102.08 ± 0.36 %, respectively.

Nail clippings were analyzed by the Center for Environmental Sciences and Engineering, University of Connecticut. Samples were digested and analyzed using cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80) according to U.S Environmental Protection Agency (U.S. EPA) method 245.6 (USEPA 1991). For quality assurance, we used control samples consisting of calibration verifications and blanks, spikes, duplicates, and standard reference material (SRM; DOLT-4 dogfish liver, DORM-3 fish protein). Limit of detection averaged 0.083 ppm and all samples had THg concentrations that exceeded these limits. Average RPD between replicate sample analyses were 0.5% ± 2.4% (*n*=13). Mean percent recoveries of THg for the DOLT-4 and DORM-3 were 95.0 ± 1.6 % to 94.2 ± 2.3%, respectively. Calibration verification and laboratory control sample recoveries of THg averaged 104.7± 0.6% and 103.7± 1.2%, respectively. Matrix spike recovery averaged 108.2± 4.4%.

A subset of 12 homogenate muscle and egg samples were analyzed for Hg speciation (MeHg/HgII) by Quicksilver Scientific using high pressure liquid chromatography (Method QS–LC/CVAF–001). To account for any intra-clutch differences in Hg, homogenate samples comprised of three randomly selected eggs per clutch were used for egg MeHg analysis. Due to small sample mass, homogenate samples of three individual muscle samples (pooled by site, body size, and sex) were used for muscle MeHg analysis. A combination of blanks (3), SRM's (2:TORT-2, and DOLT-4), laboratory control samples (1), matrix spikes (2) and sample duplicates (2) were used for quality control. Limit of detection for HgII and MeHg was $2.10E^{-7}$ mg mL^{-1} for egg and 3.0 E^{-4} mg kg^{-1} for muscle and all samples had Hg concentrations that

exceeded these limits. Average RPD between replicate sample analyses were 9.55 ± 3.65% for Hg II and 3.05 ± 2.85% for MeHg. Percent recovery for HgII/MeHg for the TORT-2, DOLT-4, and laboratory control samples were 106.6/109.8%, 96.0/91.3%, and 112.6/111.5% respectively. Average matrix spike recoveries of HgII and MeHg were 111.8± 0.8% and 107.9± 1.9%, respectively.

Statistical Analyses. All analyses were performed using SAS 9.1 (SAS Institute, Inc, Cary, NC, USA) or Microsoft Excel with significance assessed at $\alpha \leq 0.05$. We detected no difference in THg concentrations within blood, muscle, or egg between 2010 and 2011 captures and therefore these years were pooled for all subsequent analyses (in all cases $p \geq 0.13$). When appropriate, THg concentrations were $\log_{10}$-transformed to improve normality and homoscedasticity of variance. Initial models included all interactions between independent variables and covariates, but non-significant interactions ($p \geq 0.10$) were dropped from final models.

We used Pearson correlation coefficients and linear regressions to assess relationships between blood, nail, egg, and muscle tissues from individual turtles collected from the South and Middle Rivers and our two validation sites, the North Fork Holston River and the South Fork Shenandoah River. We then used an analysis of covariance (ANCOVA) to verify that the slope and y-intercept of the tissue models generated from the South and Middle River were similar at the two validation sites. In addition, we replicated the analysis using a truncated South/Middle River data set to only include Hg values that correspond with range observed at the two validation sites.

Subsites sampled along the Middle River, in addition to those sampled up and downstream of the source on the South River, are not independent of one another and are collectively referred to as SR-Ref (South River reference), MR-Ref (Middle River reference),

and SR-Cont (South River contaminated) for all statistical comparisons of total Hg in turtle tissues among sites. In order to understand the spatial and temporal variation in Hg exposure, we evaluated differences in blood THg concentrations between turtles collected in our earlier survey in 2006 (Bergeron et al. 2007) and 2010-11 (this study) using a two-way ANCOVA on rank-transformed data (Conover & Iman 1982) with year and site as main effects and carapace length as a covariate. Since blood was the only tissue sampled in our previous study in 2006 (Bergeron et al. 2007), we were only able to test for temporal differences using THg concentrations in blood. We report mean THg blood values in Figure 2 for visual representation of spatial and temporal variation that exist between our study and those collected by Bergeron et al. (2007) in 2006. Additionally, we used a Tukey-Kramer method to determine differences in mean blood THg between years within each of the three sites.

In order to determine if demographic factors should be considered when assessing consumption risk, we tested for effects of body size and sex on THg bioaccumulation in muscle tissue using a two-way ANCOVA with site and sex as the main effect and carapace length as a covariate.

Results

Mercury concentrations and non-invasive sampling. Total Hg concentrations in tissues of turtles collected from the Middle and South Rivers ranged from 0.008- 4.992 ppm (wwt) in blood, 0.037-32.288 ppm (dwt) in muscle, 0.151-166.109 ppm (fwt) in nail and 0.009-6.065 ppm (dwt) in eggs. A smaller range in Hg concentrations was observed for tissues sampled from turtles collected from the two validations sites. Total Hg concentrations in tissues of turtles collected from the North Fork Holston River ranged from 0.033-1.011 ppm (wwt) in blood, 0.185-7.334 ppm (dwt) in muscle, and 0.814-32.333 ppm (fwt) in nail. Turtles collected from the

South Fork of the Shenanadoah (10-23 miles downstream of the South River confluence) exhibited THg tissue concentrations ranging from 0.162-1.77 ppm (wwt) in blood and 0.123-12.199 ppm (dwt) in muscle. In all cases, tissues sampled from turtles were strongly and positively correlated with one another (Table 1; all $p < 0.001$). Models describing relationships between tissues generated from turtles sampled from the North Fork Holston and the South Fork Shenandoah Rivers did not differ significantly from those of the South and Middle Rivers (Figure 2; in all cases $p > 0.13$).

Methylmercury concentrations of egg and muscle tissues collected from turtles inhabiting portions of the South and Middle Rivers ranged from 25.8-77.7% and 86.6-96.3% respectively. As egg THg concentrations increased so did %MeHg in eggs (Table 1; $r^2 = 0.52$, $p = 0.007$). However, there was not a significant correlation between THg and %MeHg for muscle tissue sampled (Table 1: $p = 0.13$).

Spatial and Temporal Patterns. Blood THg concentrations differed greatly among the Middle River reference, South River reference, and South River contaminated sites (Figure 3a, site: $F_{2,460} = 48.81$, $p < 0.001$), with the contaminated site (SR-Cont) having the highest mean THg concentrations (1.723 ± 0.061 ppm, wwt) followed by South River reference site (0.123 ± 0.151 ppm, wwt) then Middle River reference site (0.026 ± 0.117 ppm, wwt). A broad range of THg concentrations were detected with levels being lowest in the Middle River and upstream of the source then gradually increasing after the source and later peaking downstream (Figure 3b). In general, blood THg increased from 2006 to 2010-2011 (year: $F_{1,420} = 17.12$, $p < 0.001$) but this effect was dependent upon site (site x year: $F_{2, 420} = 3.64$, $p = 0.027$) (Figure 3a). Post-hoc analysis revealed no differences in mean blood THg concentrations between those published by Bergeron et al. (2007) and current concentrations for the Middle and South River reference sites

(in both cases $p \geq 0.993$) but significant differences were detected between the two sampling time frames within the contaminated site ($p < 0.001$). Within the contaminated site, blood THg concentrations increased from 16.0 to 219.4% from 2006 to 2010-11, depending on river mile (Figure 3b).

Consumption Risks. Body size (carapace length) significantly influenced THg concentrations in muscle (two-way ANCOVA, carapace length: $F_{1, 169} = 9.11$, $p = 0.003$, Figure 4), but the nature of this effect was dependent upon site (carapace length x site $F_{2,169} = 5.87$, $p = 0.003$). Muscle THg concentrations increased with body size for individuals collected from the contaminated site (SR-Cont; $p < 0.001$) but did not change with size within the two reference sites (MR-Ref or SR-Ref; in both cases $p \geq 0.16$). Additionally, muscle THg concentrations differed significantly between sexes after correcting for body size (two-way ANCOVA, sex: $F_{1,169} = 12.21$, $p < 0.001$). Total Hg values for females were 93.9%, 6.3%, and 15.3% higher in muscle than males from the two reference sites along the Middle and South River and the contaminated site, respectively (Figure 5).

Discussion

Our results demonstrate that minimally invasive tissues can be used to sustainably monitor Hg bioaccumulation and predict Hg concentrations maternally transferred to eggs, which has implications for turtle health and reproduction. Total Hg concentrations in all turtle tissues were strongly and positively correlated with each other. This is consistent with previous studies that showed similar correlations between tissues sampled in both snapping turtles and other herpetofauna (Golet & Haines 2001; Bergeron et al. 2010a, 2010b; Turnquist et al. 2011). However, the models presented here are the first to be validated by sampling individuals

inhabiting other Hg contaminated sites. We demonstrate that our mathematical models describing the relationships between tissues sampled from turtles inhabiting the South River are applicable to those from other Hg contaminated sites, providing a strong foundation for future sustainable monitoring programs. Our results also reveal several important spatial, temporal, and demographic patterns in bioaccumulation that may be important factors to consider when evaluating consumption risks, and developing future monitoring, restoration, and mitigation programs for Hg contaminated systems.

Turtles collected from the contaminated portion of the South River had significantly higher THg tissue concentrations than individuals collected from reference sites. Total Hg concentrations in blood, muscle and nail of adult turtles collected from the contaminated portion of the South River are the highest ever reported in turtles and surpass tissue concentrations that are associated with severe neurological, physiological, and reproductive effects observed in other aquatic animals (reviewed in Scheuhammer et al. 2007). For example, blood THg concentrations in turtles inhabiting contaminated areas of the South River ranged from 0.012-4.992 ppm (wwt). Comparatively, common loons (*Gavia immer*) with blood THg concentrations above 3.0 ppm (wwt) were reported to have reduce fledging success and those exceeding 4.0 ppm (wwt) were associated with reduced reproductive effort, elevated stress hormones, asymmetry in flight feathers, and altered breeding behaviors (reviewed in Scheuhammer et al. 2007). Muscle THg concentrations in turtles inhabiting contaminated areas of the South River ranged from 0.10-32.29 ppm (dry), with 86.6-96.3% being in the more toxic form of MeHg. In fish, muscle THg concentrations reaching ~30 ppm (dwt), which falls within the upper end of the THg in reported here in turtle muscle, is associated with reduced growth and survival (Wiener 1996).

Our study is the first to rigorously demonstrate Hg can maternally transfer from female to eggs in turtles. Maternally transferred THg concentrations in egg were found to range from 0.04-

6.06 ppm (dwt), with MeHg comprising 29.5-77.7% of the THg within the egg. Although the effects of maternally transferred Hg on turtle reproduction are still unknown, a recent study in Carolina wrens (*Thryothorus ludovicianus*) showed a 50% reduction in nest success when egg THg concentrations reached ~2.15 ppm (dwt) (Jackson et al. 2011a). In free-living common loons, egg THg concentrations of ~2.5 ppm (dwt) are associated with impaired hatchability and embryotoxicity (Barr 1986). In amphibians, Bergeron et al. (2011) showed that maternally transferred egg THg concentrations of ~1.7 ppm (dwt) were associated with a 25% reduction in hatching success of American toads (*Bufo americanus*). Based on these studies, it is possible that the concentrations of Hg we document in eggs may lead to deleterious consequences on hatching success or other aspects of reproduction (e.g., malformation frequency).

We identified several important spatial and temporal patterns in Hg bioaccumulation within tissues sampled from turtles inhabiting the South and Middle Rivers. Total Hg concentrations in turtle blood slowly increased downstream of the Hg source, finally peaking ~20 miles downstream and then declining. In addition, THg concentrations in blood collected from contaminated turtles have significantly increased since 2006, while Hg concentrations in reference turtles have remained relatively low and unchanged. Similar spatial and temporal trends have been reported within other South River biota in recent years (e.g., fish, amphibians, birds, invertebrates: Southworth et al. 2004; Murphy 2004; Bergeron et al. 2007; Brasso & Cristol 2008; Bergeron et al. 2010b; Jackson et al. 2011b). Although there is no evidence that additional Hg has been emitted from the original point source in recent decades, increases in Hg within biota may be due to several factors that influence the fate of Hg within this systems. For example, Hg has been found to persist within the South River floodplain and therefore be may be re-circulated by the combination of flood events, bank erosion, and anthropogenic and agricultural disturbances (Rhoades et al. 2009; Newman et al. 2011; Eggleston 2009). Gradual

reintroduction of Hg from large expanses of the floodplain may also help explain why Hg levels peak in turtles ~ 20 miles downstream of the point source.

Body size significantly influenced THg concentration in muscle but this effect was dependent upon site, with Hg concentrations increasing with carapace length for individuals collected within the contaminated site but not within the two reference sites. Previous studies have found a similar pattern, with Hg concentrations increasing with respect to body size in turtles and other aquatic species (Driscoll et al. 1994; Kenyon et al. 2001; Bergeron et al. 2007; Turnquist et al. 2011). In turtles, the pattern of increasing Hg concentrations within larger individuals is most likely a consequence of age, as larger turtles are more likely to be older, and therefore have had more time to bioaccumulate Hg than those of smaller size classes. In contrast, two previous studies found no relationship between body size (mass) and Hg concentrations (Helwig et al. 1983; Golet & Haines 2001). We believe this discrepancy is a result of extensive range of Hg concentrations in the South River. Mercury concentrations reported in these two studies were fairly low and fell with the range of concentrations we observed at the South River reference site, where body size did not influence Hg concentration.

We found that female snapping turtles had higher tissue Hg concentrations than males at a given size. This pattern is counter to some studies that suggest females should have lower Hg body burdens since they maternally transfer Hg to their eggs, thus providing females with an additional excretion pathway (Meyers-Shone & Walton 1994). Instead, we believe higher Hg concentrations present in female tissue may be due sexual dimorphism in size and growth (Christainsen & Burken 1979). Snapping turtles exhibit a strong body size dimorphism, in which adult females grow not only at a slower rate but also asymptote at a smaller size compared to males which rely on their size to fight and defend territories (Christainsen & Burken 1979; Galbraith 1987). Given this growth pattern, it is likely that females, although smaller, may be

older and therefore have accumulated more Hg than males of similar size. Alternatively, the sexes may differ in feeding rates and/or feeding ecology, but little is known about sex-specific differences in feeding ecology of snapping turtles.

Our study provides a foundation for future studies intending to use snapping turtles to monitor exposure, bioaccumulation, and maternal transfer of contaminants. Future research is needed to thoroughly understand the effects that Hg is having on turtles and the risk that Hg bioaccumulation is posing to wildlife and humans that consume turtles. Although Hg concentrations observed in turtle muscle and egg in some cases exceed those associated with negative effects in other species, our comparisons remain speculative because the actual Hg concentrations associated with adverse effects in turtles remain unknown. Taken together, all previous studies linking Hg exposure to effects demonstrate a large range in Hg sensitivity among different species, reinforcing the need for future research to determine the sensitivity of snapping turtles to Hg (Bergeron et al. 2011; Jackson et al. 2011a; Barr 1986; Scheuhammer et al. 2007; Crump & Trudeau 2009). Additionally, the rate at which turtles are harvested and consumed by wildlife predators and humans from the South River remains unknown. In order understand the health risks of consuming contaminated turtles, future research is needed to determine human and wildlife consumption patterns of adult turtles and eggs.

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Table 1.1. Relationships in total mercury (THg) concentrations among tissues in *Chelydra serpentina* collected from the South River (SR), Middle River (MR), North Fork Holston River (NFHR), and South Fork Shenandoah River, VA, USA. All regressions were calculated from raw data reported in ppm (mg kg^{-1}). Egg and muscle THg values are reported on a dry weight basis whereas nails and blood are reported on a fresh and wet weight basis, respectively.

Site	Regression	Slope	Intercept	*r*	*p*-value	*n*
SR/MR	THg Blood v. THg Muscle	5.726	0.546	0.95	< 0.001	170
NFHR	THg Blood v. THg Muscle	6.347	0.019	0.96	< 0.001	23
SFSR	THg Blood v. THg Muscle	5.430	0.293	0.86	< 0.001	12
SR/MR	THg Blood v. THg Nail	26.50	1.769	0.89	< 0.001	131
NFHR	THg Blood v. THg Nail	29.38	0.067	0.94	< 0.001	23
SR/MR	THg Nail v. THg Muscle	0.178	1.113	0.92	< 0.001	112
NFHR	THg Nail v. THg Muscle	0.201	0.196	0.95	< 0.001	23
SR/MR	THg Nail v. THg Egg	0.035	0.391	0.92	< 0.001	95
SR/MR	THg Blood v. THg Egg	1.129	0.311	0.92	< 0.001	95
SR/MR	THg Muscle v. %MeHg Muscle	0.172	92.241	0.21*	0.133	12
SR/MR	THg Egg v. %MeHg Egg	6.975	37.517	0.52*	0.008	12

*denotes an r^2 value

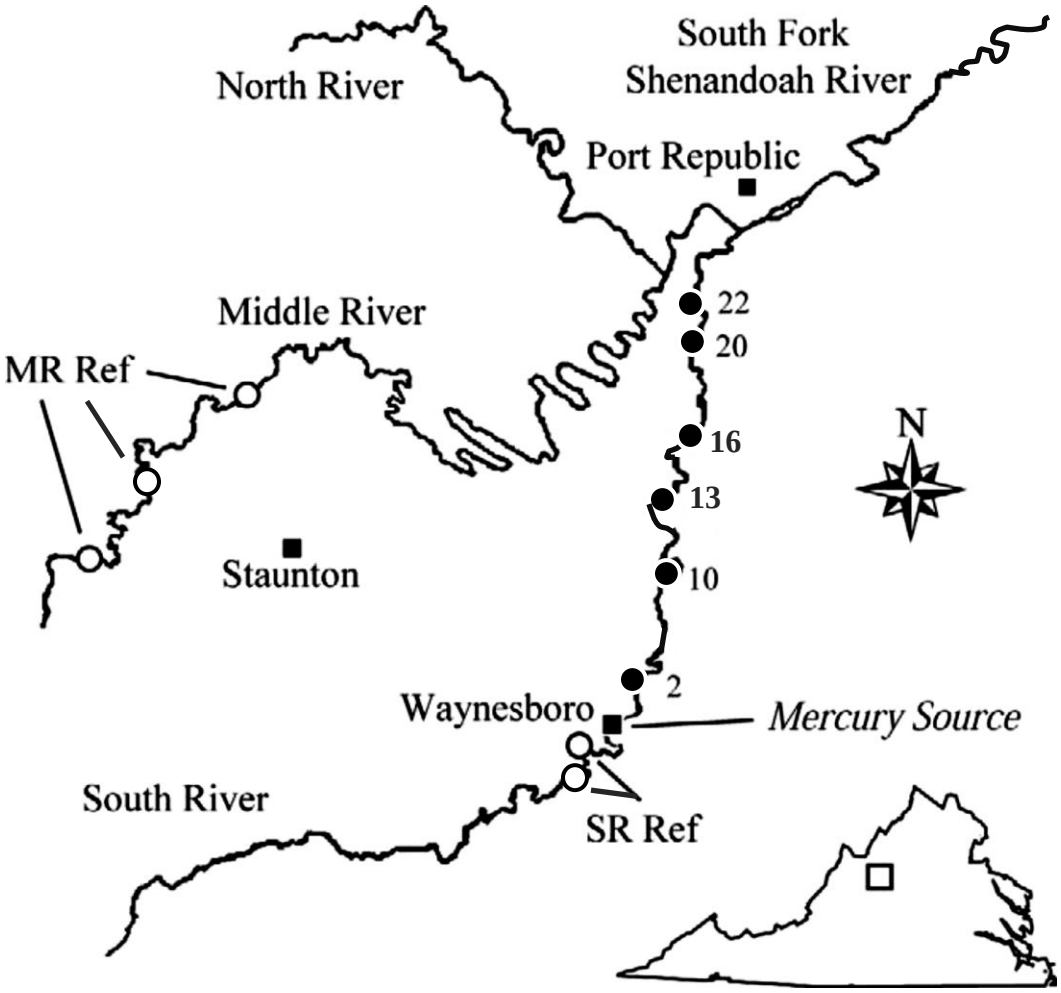


Figure 1.1. Turtle collection locations along the South River (SR) and Middle River (MR), located in central VA, USA. Subsites sampled along the contaminated portion of the South River are by closed symbols and labeled by river mile downstream of the contamination source. Open symbols represent reference sites that were sampled upstream of the source along the SR and at additional locations along the MR. Note that the river flows from south to north.

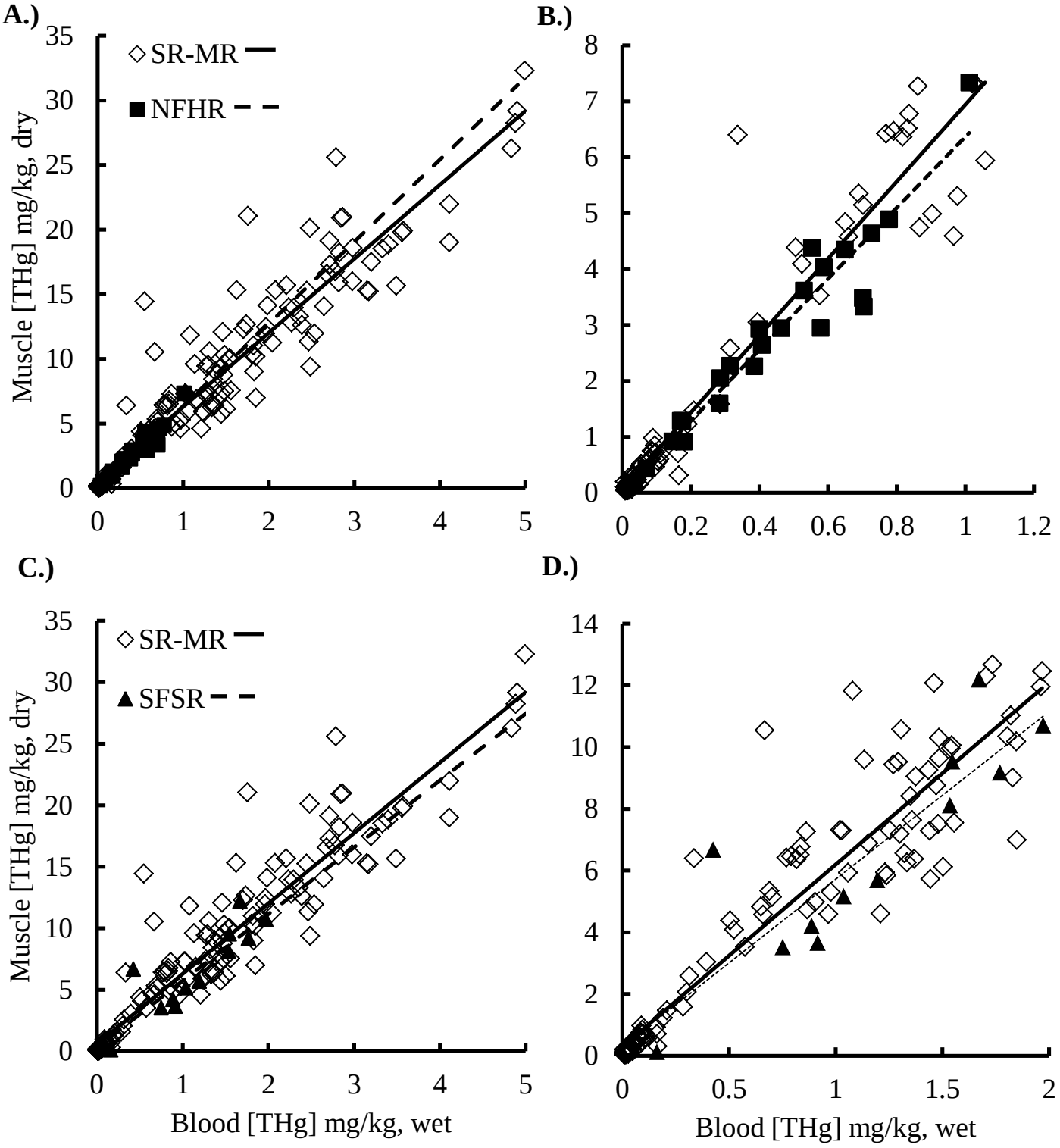
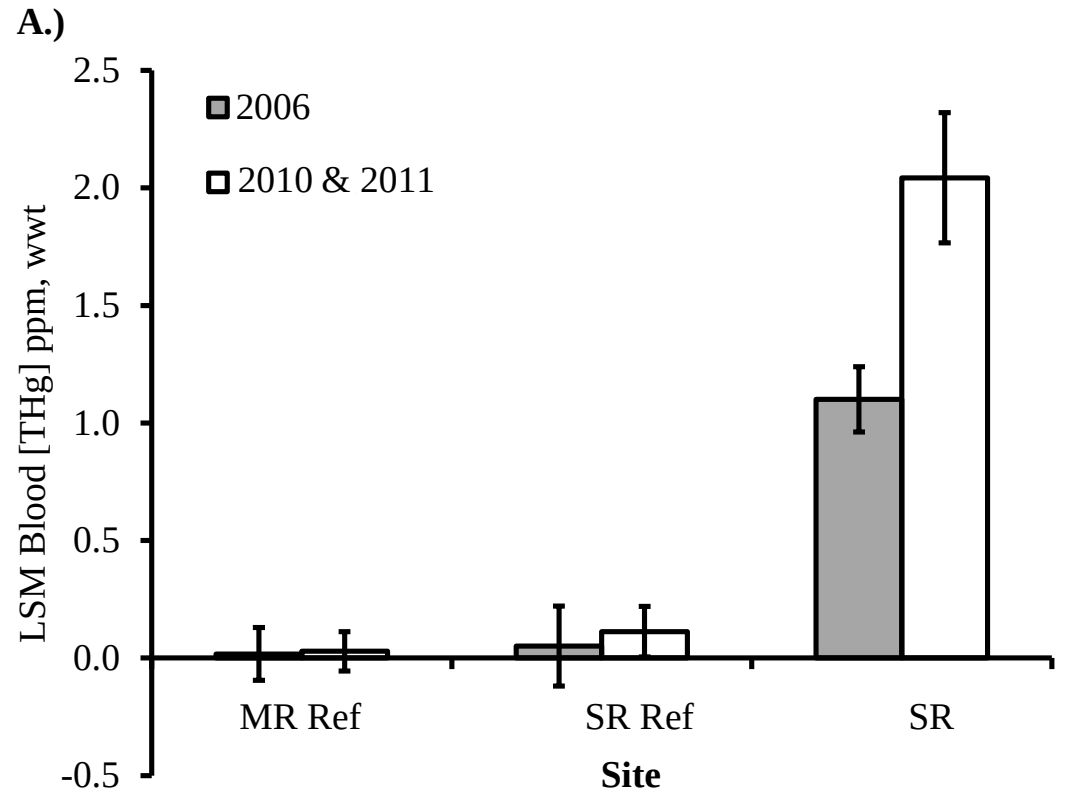

A.)
SR-MR
NFHR
Muscle [THg] mg/kg, dry
B.)
C.)
SR-MR
SFSR
Muscle [THg] mg/kg, dry
Blood [THg] mg/kg, wet
D.)
Blood [THg] mg/kg, wet

Figure 1.2. Validations of the mathematical relationship observed between blood (wet weight) total mercury (THg) and muscle (dry weight) THg of *Chelydra serpentina* collected from: A.) the South and Middle Rivers (SR-MR; y=5.723x +0.573, $r = 0.96$, $p < 0.001$, n= 170) and the North Fork Holston River (NFHR; y = 6.347x + 0.019, $r = 0.96$, $p < 0.001$, n= 23), VA, USA, B.) truncated blood THg values for South and Middle Rivers (SR-MR; y=6.881x +0.058, $r = 0.97$, $p < 0.001$, n=88) that fall within the range of concentrations observed on the North Fork Holston River (NFHR; y = 6.347x + 0.019, $r = 0.96$, $p < 0.001$, n= 23) C.) the South and Middle Rivers (SR-MR; y=5.723x +0.573, $r = 0.96$, $p < 0.001$, n= 170) and the South Fork Shenandoah River (SFS; y = 5.4301x + 0.2927, $r = 0.86$, $p < 0.001$, n= 12), VA, USA, and D.) truncated blood THg values for the South and Middle Rivers (SR-MR; y=5.912x + 0.281, $r = 0.93$, $p < 0.001$, n=127) that fall within the range of concentrations observed on the South Fork Shenandoah River (y = 5.4301x + 0.2927, $r = 0.86$, $p < 0.001$, n= 12).


A.)
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
2006
2010 & 2011
LSM Blood [THg] ppm, wwt
MR Ref
SR Ref
SR
Site

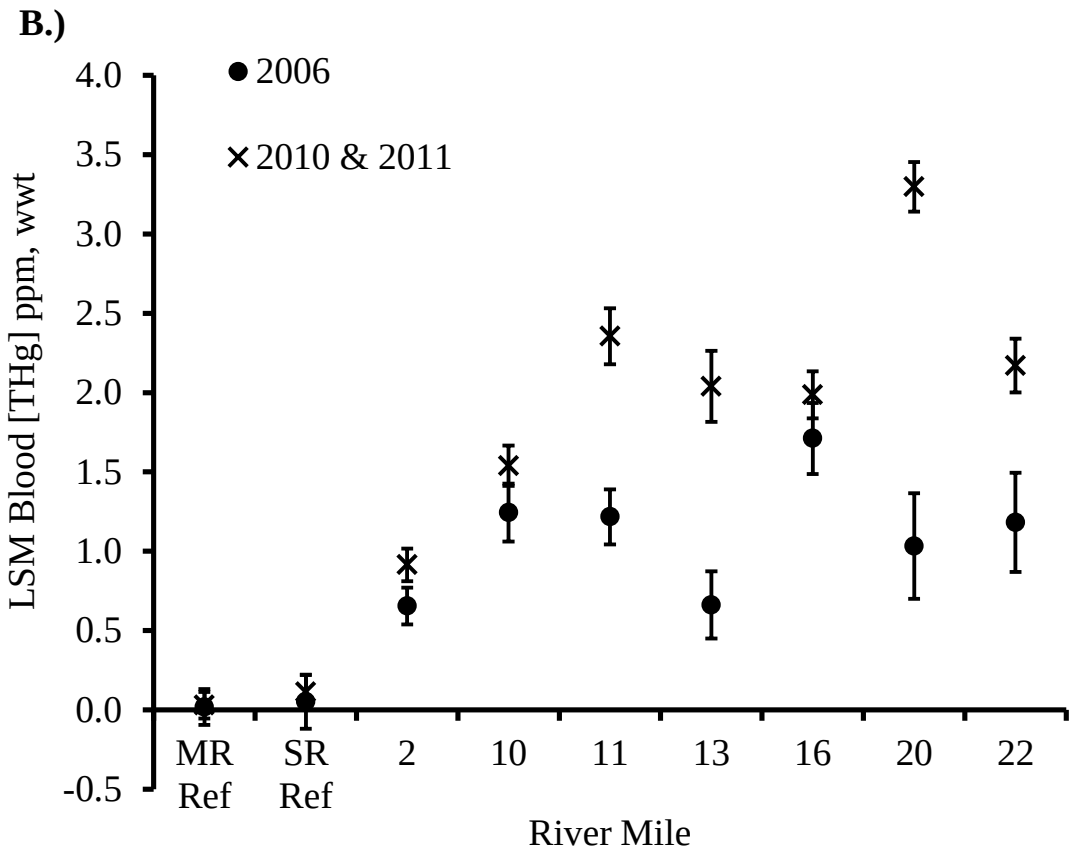

B.)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
2006
2010 & 2011
LSM Blood [THg] ppm, wwt
MR Ref
SR Ref
2
10
11
13
16
20
22
River Mile

Figure 1.3. Average total mercury (THg) concentrations in blood of *Chelydra serpentina* collected from 2006 and 2010/11 sampling periods inhabiting A.) areas of the Middle River reference site (MR-Ref), upstream of the Hg source on the South River (SR-Ref), and downstream of the Hg source on the South River (SR Cont). B.) Spatial representation of average THg concentrations at the two reference sites (MR Ref & SR Ref) and the various sub-sites downstream of the Hg source (RM 0) along the South River (River Mile 2-22), VA, USA, from the 2006 and the 2010-11 sampling periods. All values shown are least-square means corrected for body size (carapace length) ± 1 S.E.

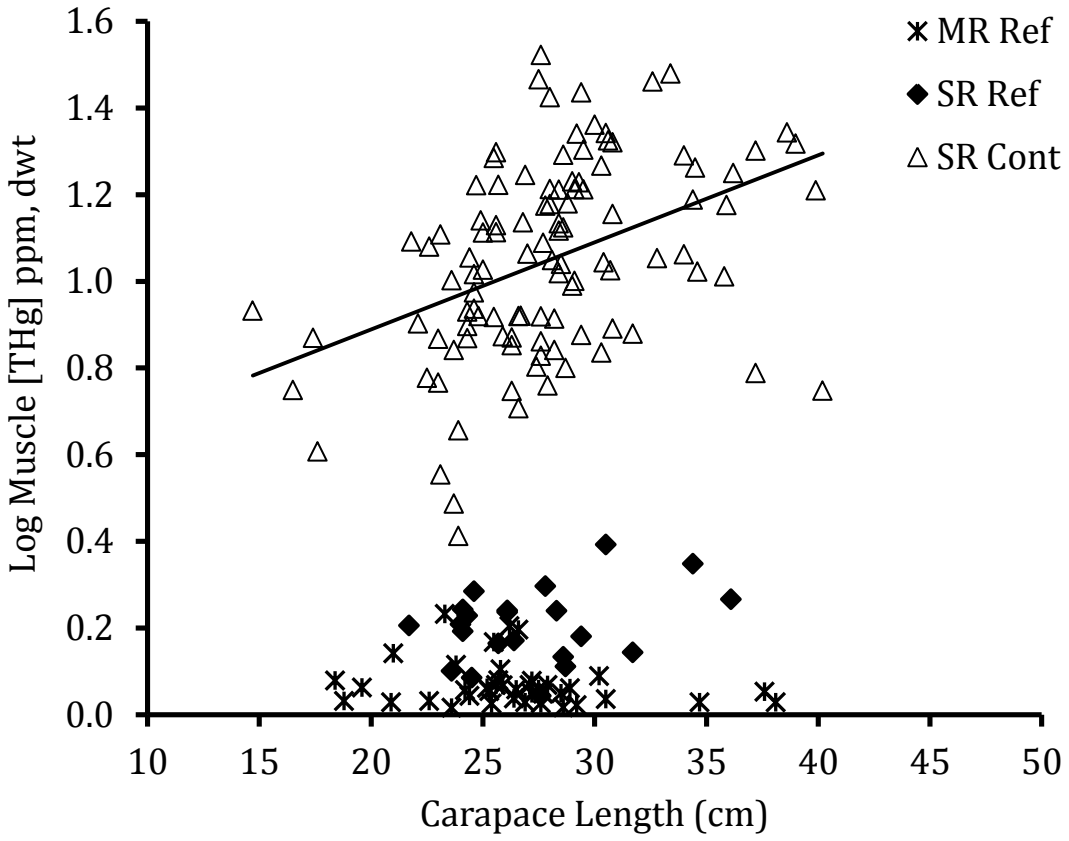


Figure 1.4. Relationships between carapace length and total mercury (THg) in muscle (dry weight) tissue of *Chelydra serpentina* collected from two reference sites on Middle River (MR Ref; n= 41) and the South River (SR-Ref; n=22), and the contaminated portion of the South River (SR-Cont; $y= 0.0201x +0.4869$, $R^2= 0.17$, $p < 0.001$, n= 108), VA, USA. Total Hg in turtle muscle did not increase with increasing body size with the two reference sites ($p > 0.16$), but did for individuals collected within the contaminated site ($p < 0.001$).

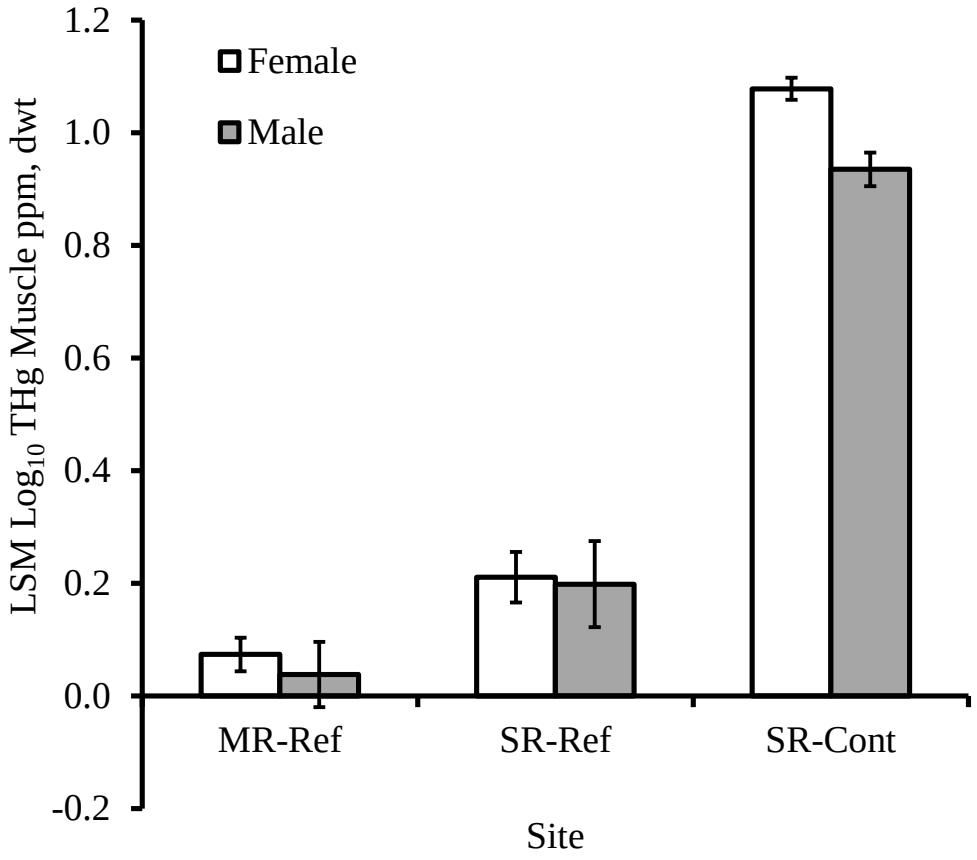


Figure 1.5. Total mercury (THg) concentrations in male and female *Chelydra serpentina* muscle tissue (dry weight) collected from the two reference sites along the Middle River (MR-Ref) and upstream of the source on the South River (SR-Ref), and the contaminated site (SR-Cont), located downstream of the Hg source, VA, USA. Values shown are least-squares means (±1 standard error) corrected for body size (carapace length).

Chapter 2: Mercury Exposure Has Negative Consequences on Turtle Reproduction

Co-authors: Devin K. Jones, J. D. Willson, William A. Hopkins

Abstract

In addition to the suite of beneficial resources (e.g. antibodies, hormones, and nutrients) that females allocate to their offspring, research has demonstrated the maternal transfer of a wide variety of harmful contaminants. For example, the maternal transfer of mercury (Hg), a common and highly toxic environmental contaminant, has been shown to negatively influence several reproductive parameters in birds, fish and amphibians. Additionally, Hg can bioaccumulate and biomagnify within food webs, exposing long-lived apex predators to high levels of Hg. Based on these observations, we evaluated the consequences of maternally transferred Hg on a long-lived aquatic omnivore. We collected eggs and tissues from gravid female snapping turtles (*Chelydra serpentina*) along an Hg contaminated gradient at a historically contaminated river in central Virginia. We incubated eggs in the laboratory, quantified embryonic morality, infertility, and hatching success of each clutch, and assessed all hatchlings and dead embryos for gross morphological malformations. As predicted, female turtles inhabiting contaminated areas accumulated high levels of Hg in their tissues, and female Hg concentrations were strongly and positively correlated with Hg levels in their eggs. We found that Hg negatively influenced hatching success through increased egg infertility and embryonic mortality. However, we found no effect of Hg on gross malformation frequency, clutch size, egg mass, or clutch mass. This is the first study to demonstrate direct effects of maternally transferred Hg on hatchling survival in turtles and our results suggest that Hg may also affect other female physiological factors important for reproduction.

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Keywords: Maternal effects, maternal transfer, mercury, reproduction, turtle

Introduction

Maternal effects play a key role in determining offspring phenotype and are often a reflection of a female's behavior, physical condition, and/or physiology. In oviparous species, important maternal effects can include nest site selection, incubation temperature, and the maternal transfer of resources (Bernardo 1996). However, these female contributions are often dictated by the maternal environment (Rossiter 1996) and can vary along a broad spectrum of beneficial to deleterious outcomes for offspring phenotype. For example, in addition to the suite of natural resources (e.g., antibodies, hormones, and nutrients) that females allocate to their offspring, research has demonstrated that females can maternally transfer a wide-variety of harmful toxicants, negatively affecting offspring phenotype (Eisenreich et al. 2009, Bergeron et al. 2011a&b, Hopkins et al. 2006).

Mercury (Hg), a ubiquitous heavy metal contaminant, can be maternally transferred from female to offspring and has received significant attention due to its widespread prevalence, bioaccumulative and biomagnifiying properties, and known toxicity in humans and wildlife (Mason et al. 1996; Fitzgerald et al. 1998; Scheuhammer et al. 2007; Harada 1995). The effects of Hg on reproduction and offspring phenotype have recently been documented for several oviparous species. For example, female American toads (*Bufo americanus*) collected from an Hg contaminated site transferred approximately 5% of their Hg body burden to their offspring, resulting in reduced hatching success (Bergeron et al. 2010, 2011a). Alternatively, Hammerschmidt et al. (2002) found that dietary Hg did not affect hatching success in fathead minnows (*Pimephales promelas)* but instead impaired reproduction by decreasing egg production. Along with reductions in offspring viability, maternally transferred Hg has also been associated with sublethal consequences. For instance, successfully hatched toad larvae exposed to maternally transferred Hg exhibited latent sublethal effects, including reduced body size,

impaired swimming performance, and increased time to metamorphic climax (Bergeron et al. 2011b). In fish, Alvarez et al. (2006) reported adverse effects on behavioral performance in Atlantic croaker (*Micropogonias undulates*) larvae hatched from eggs laid by Hg contaminated adults. While the effects of maternally transferred Hg have been demonstrated in several vertebrate species, nothing is known about the reproductive effects of Hg contamination in turtles.

In this study, we used common snapping turtles (*Chelydra serpentina*) inhabiting a historically Hg contaminated river to investigate whether Hg exposure can affect reproduction in adult females. Snapping turtles possess a suite of attributes (e.g., longevity, aquatic habitat preferences, and high trophic-level feeding habits) that make them highly susceptible to Hg bioaccumulation. In fact, a recent study found that muscle Hg concentrations of turtles inhabiting contaminated portions of the same river were on average 72-fold higher than those of turtles collected from nearby reference sites (Hopkins et al. In prep). Based on these findings and the known deleterious reproductive effects of Hg, we hypothesized that exposure to excessive Hg concentrations would negatively affect turtle reproduction and development. Specifically, we predicted that Hg exposure would negatively influence hatch success and clutch characteristics (i.e., clutch size, egg mass, clutch mass), and increase malformation frequency, embryonic mortality, and egg infertility. Additionally, we sought to develop non-destructive sampling techniques for assessing bioaccumulation and maternal transfer of Hg in female turtles so that future studies can be performed without sacrificing mature adults or viable eggs.

Methods

Sample Collection Methods. From April-July 2010 and 2011, we collected gravid female snapping turtles at various locations upstream and downstream of a historic Hg contamination

site along the South River, VA, USA, and at a nearby uncontaminated reference river, using baited hoop traps (see Hopkins et al. 2010 for additional information). All female turtles were physically palpated for the presence of shelled eggs and gravid females were transported back to the field house. We weighed gravid females to the nearest 0.10 kg and placed them separately in egg laying chambers consisting of 100 gal Rubbermaid© tanks filled with ~ 20 gal of decholoraminated water. We intraperitoneally injected gravid females with 40 mg/kg of oxytocin solution every 24 hrs for three consecutive days to induce egg laying. We removed deposited eggs within 3 hrs, weighed each egg to the nearest 0.01 g, measured egg length and width to the nearest 0.1 mm, and marked each egg according to female ID, and lay sequence. Completion of oviposition was confirmed by radiographs taken by qualified technicians at the Wildlife Center of Virginia, Waynesboro, VA. Three randomly selected eggs per clutch were frozen and later homogenized to determine egg Hg concentration (Bishop et al. 1995). After oviposition, we measured carapace length, carapace width, and plastron length of each female to the nearest mm using oversized calipers and individually marked each individual by filing three marginal scutes (Bergeron et al. 2007). We removed 2-4 small (2-3 mm) nail samples from the tips of the left and right hind claws of each turtle using canine nail clippers and took a 1-mL blood sample from the caudal vein. Nail and blood samples were stored separately in 1.5 mL eppendorf tubes at -20˚C prior to Hg analyses. In order to determine accumulated Hg in turtle muscle tissue, we removed a small biopsy from the ventral-lateral aspect of the tail following administration of a local anesthetic (Lidocane). We sutured the biopsy site with 2-3 stitches using clear Polydioxanone monofilament suture material (3/8 cm) and applied a topical antibiotic to reduce risk of infection. Following sample collection we released turtles at their point of capture.

Egg Incubation. We transported the remaining eggs to Virginia Tech where they were placed in plastic containers with vermiculite (1:1 water, vermiculite), capped with a perforated lid, and set inside styrofoam hovabators (Model #1602N; G.Q.F. Manufacturing Company, Savannah, GA, USA). Ibutton loggers (DS1923, Embedded Data Systems, KY, USA) were placed within each incubator so that temperature and humidity levels could be continuously monitored and adjusted in order to achieve a target incubation temperature of 25 ˚C (producing all-male clutches; Yntema 1976) and a relative humidity of 85%. Actual mean incubation temperature and relative humidity achieved for each incubator averaged 25.3± 0.1˚C and 86.0±0.8%, respectively. Approximately every 2 weeks, we candled eggs to assess development. Dead embryos were removed from the incubator, dissected, staged according to Yntema (1968) and examined for gross malformations according to Bell et al. (2006). Once hatchlings started to pip, a small perforated plastic cup was placed over each egg so that hatchlings could be identified after emergence.

Mercury Analysis. We lyophilized and homogenized muscle and eggs and report THg concentrations on a dry weight (dwt) basis. Whole blood from each turtle was homogenized using a vortex mixer and we report THg concentrations in blood on a wet weight (wwt) basis. We washed nail clippings by placing them in a sterilized tube with 10mL solution of 15:1 deionzied water to ethanol and sonicating for 20 minutes. After sonication we discarded the solution and allowed nails to air dry on a clean laboratory bench and report THg concentrations on a fresh weight basis (fwt). Percent moisture was 77.2 ± 0.19% (mean ± 1 standard error of the mean hereafter) for muscle and 75.5 ± 0.18% for eggs. Muscle, blood and egg samples were analyzed for THg at the College of William and Mary, Williamsburg, Va, by combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80,

Milestone, Monroe, CT, USA) according to U.S. Environmental Protection Agency (U.S. EPA) method 7473 (USEPA 1998). For quality assurance, each group of 20 samples included a replicate, blank, and standard reference material (SRM; DOLT-4 dogfish liver, DORM-3 fish protein (National Research Council of Canada (NRCC), Ottawa, ON). Method detection limits (MDLs; 3 times the standard deviation of procedural blanks) for samples were 0.0042 mg kg^{-1} (ppm), and all samples had THg concentrations that exceeded the limit. Average relative percent differences (RPD) between replicate sample analyses were 8.38% ± 1.25% (*n*=60). Mean percent recoveries of THg for the DOLT-4 and DORM-3 ranged from 99.77 ± 0.26 % to 102.08 ± 0.36 %, respectively.

Nail samples were analyzed by the Center for Environmental Sciences and Engineering, University of Connecticut, and we report THg on a wwt basis. Samples were digested and analyzed using cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80) according to U.S Environmental Protection Agency (U.S. EPA) method 245.6 (USEPA 1991). For quality assurance, we used control samples consisting of calibration verifications and blanks, spikes, duplicates, and standard reference material (SRM; DOLT-4 dogfish liver, DORM-3 fish protein). Limit of detection averaged 0.083 ppm and all samples had THg concentrations that exceeded these limits. Average RPD between replicate sample analyses were 0.5% ± 2.4% (*n*= 13). Mean percent recoveries of THg for the DOLT-4 and DORM-3 were 95.0 ± 1.6 % to 94.2 ± 2.3%, respectively. Calibration verification and laboratory control sample recoveries of THg averaged 104.7± 0.6% and 103.7± 1.2%, respectively. Matrix spike recoveries average 108.2± 4.4%.

A subset of homogenate egg samples representing 12 individual clutches were analyzed for MeHg by Quicksilver Scientific using high pressure liquid chromatography (Method QS–LC/CVAF–001). A combination of blanks (3), SRM's (2:TORT-2, and DOLT-4), a laboratory

control samples, a matrix spike, and a sample duplicate were used for quality control. Limit of detection was $2.10E^{-7}$ mg mL^{-1} for egg and all samples had Hg concentrations that exceeded these limits. Relative percent difference between replicate sample analyses was 9.55 ± 3.65% for Hg II and 3.05 ± 2.85% for MeHg. Percent recovery for HgII/MeHg for TORT-2, DOLT-4, and laboratory control samples were 106.6/109.8%, 96.0/91.3%, and 112.6/111.5%, respectively. Matrix spike recovery of HgII and MeHg was 112.6% and 109.7%, respectively.

Statistical Analysis. We used SAS 9.1 (SAS Institute, Inc, Cary, NC, USA) or Microsoft Excel for all statistical analyses and assessed significance at $\alpha \leq 0.05$. When appropriate, THg concentrations were $\log_{10}$-transformed to improve normality and homoscedasticity. Initial models included all interactions between independent variables and covariates, but non-significant interactions were dropped from final models. Subsites sampled along the South and Middle Rivers are not independent of one another and are collectively defined as SR-Ref (South River reference), MR-Ref (Middle River reference), and SR-Cont (South River contaminated) for all statistical comparisons of turtle tissue Hg concentrations between sites.

First, we examined relationships between THg concentrations in female blood, muscle, and nail tissues to THg concentrations in her eggs using Pearson correlation coefficients and linear regressions. In order to determine the amount of egg THg available in the more toxic form of MeHg, we examined the relationship between THg and %MeHg in a subset of egg samples and analyze the data using an analysis of variance (ANOVA). Since Hg can bioaccumulate within muscle tissue over an individual's lifetime, we examined the relationship between body size and THg concentrations in muscle tissue at each site using analysis of covariance (ANCOVA), with site as a main effect and size (carapace length) as a covariate.

Some of the eggs collected for this experiment were not fully calcified upon deposition and were therefore not viable. Out of the 90 females, eight (3 reference, 5 contaminated) deposited completely non-viable clutches of eggs and six (3 reference, 3 contaminated) deposited partially not-viable clutches. We attribute this to premature induction of oviposition on our part, and therefore do not include these full or partial clutches in analyses of hatching success or frequency of embryo mortality, infertile eggs, or malformations. Using generalized linear models for mixed distributions (SAS PROC GLIMMIX), a procedure capable of modeling non-continuous distributions, we tested for effects of maternally transferred egg THg on hatch success, malformation frequency, mortality during development, and infertility using clutch as the statistical unit. However, since these models explained little of the variation in the dataset, we also present a more conservative analysis using the same mixed model (PROC GLIMMIX) approach, but with site (contaminated vs. references) as the main effect, rather than egg THg. Since all dead eggs were dissected and staged during development, we compared the proportion of embryos that died within each categorical stage (early, middle, and late) between sites using non-parametric Kruskal-Wallis tests (PROC NPAR1WAY).

We examined the influence of THg muscle concentrations on maternal investment since female snapping turtles are thought to rely on energy reserves stored in muscle and fat for reproduction (Bonnet et al. 1998; Congdon et al. 2008). We tested for effects of THg in female muscle tissue on clutch size, egg mass, and clutch mass using a multivariate analysis of variance (MANOVA). Due to strong effects of female body size on investment (Congdon and Gibbons 1985), we corrected for body size by using residuals of from regressions of carapace length on respective reproductive measures (i.e., clutch size, egg mass, clutch mass) as the dependent variable in this analysis. Because some females may not deposit their entire clutch upon induction, in order to accurately measure clutch characteristics, we used radiographs to measure

withheld eggs in order to calculate mean egg mass and total clutch mass, and determine true clutch size.

Results

In total, we collected 2,579 eggs produced from 90 clutches laid by gravid female snapping turtles collected from the South and Middle Rivers. Total Hg concentrations in tissues of gravid female snapping turtles collected from the South and Middle Rivers ranged from 0.008-4.992 ppm (wwt) in blood, 0.052-32.288 ppm (dwt) in muscle, 0.151-161.109 ppm (fresh weight, fwt) in nail, and 0.009-6.605 ppm (dwt) in egg. In all cases, tissue concentrations of Hg were strongly correlated with one another (Figure 1; all $p < 0.001$). Egg THg significantly correlated with female muscle THg, suggesting that females transfer Hg to their offspring (Figure 1d; $p < 0.001$). Methylmercury concentrations of eggs collected from turtles inhabiting portions of the South and Middle Rivers ranged from 25.8-77.7%. As egg THg increased so did the %MeHg in eggs (Figure 2; $r^2 = 0.52$, $p = 0.008$).

Body size significantly influenced muscle THg, but this effect was dependent upon site (ANCOVA: site x carapace length: $F_{2, 77} = 4.47$, $p = 0.015$, site: $F_{2, 77} = 0.30$, $p = 0.739$; carapace length: $F_{1, 77} = 1.64$, $p = 0.204$, Figure 3). Specifically, muscle THg concentrations increased with body size for females collected from the contaminated site (SR-Cont; $p < 0.001$) but did not change with size within the two reference sites (MR-Ref or SR-Ref; in both cases $p \geq 0.12$).

Total Hg in eggs significantly influenced most reproductive parameters. Egg THg negatively influenced hatch success (PROC GLIMMIX; $F_{1, 80} = 91.07$, $R^2 = 0.07$, $p < 0.001$, Figure 4b), frequency of embryos that died during development ($F_{1, 80} = 27.72$, $R^2 = 0.10$, $p < 0.001$, Figure 5b), and frequency of unfertilized eggs ($F_{1, 80} = 63.17$, $R^2 = 0.07$, $p < 0.001$, Figure 6b). Examining the data by site, rather than in relation to egg THg, revealed a similar pattern

(Figures 4a, 5a, & 6a). Site significantly influenced hatch success ($F_{2, 79}$= 40.22, $p < 0.001$, Figure 4a), with clutches from females collected from the contaminated site averaging 11.3-12.4% lower hatch success than those collected from the two reference sites. Site also influenced the frequency of embryos that died during development ($F_{2, 79}$= 15.24, $p < 0.001$, Figure 5a) and frequency of unfertilized eggs ($F_{2, 79}$= 19.70, $p < 0.001$, Figure 6a), with clutches from females collected at the contaminated site averaging 153.5-425.1% and 48.5-174.3% more deaths and unfertilized eggs, respectively, than clutches laid by females collected from the two reference sites. Stage of embryonic mortality did not differ between sites (in all cases: $p > 0.37$, Table 1), with the highest proportion of embryonic mortality occurring during early development for all sites. Frequency of malformations was generally low (MR-Ref: 3.06±1.51%; SR-Ref: 0.48±0.43%; SR-Cont: 1.48±0.50%) and was not influenced by either egg THg ($F_{1, 80}$= 2.84, p = 0.096) or site ($F_{2, 79}$= 0.80, p = 0.452). After correcting for body size, female THg in muscle did not influence clutch size, clutch mass, or egg mass (MANOVA: $F_{2, 88}$= 1.19, p = 0.32).

Discussion

Exposure to maternally-derived contaminants can have a profound influence on offspring phenotype and act as a significant source of variation in free-living populations. Our study is the first to describe the maternal transfer of Hg and its associated reproductive effects in a turtle species. Total concentrations of Hg in muscle tissue of female turtles inhabiting the contaminated portions of the South River averaged 33 to 80-fold higher than those of females collected from the two reference sites. Additionally, average THg concentrations in eggs laid by contaminated females were 30 to 85-fold higher than those of eggs produced by females collected from the two reference sites. Mercury negatively influenced hatching success through increased infertility and

embryonic mortality. However, THg concentrations did not significantly influence malformation frequencies or female clutch characteristics (e.g., clutch size, egg mass, and clutch mass).

Egg THg was negatively correlated with hatching success through increased egg infertility and embryonic mortality. Clutches collected from females inhabiting contaminated portions on the South River had 48.6-174.3% and 153.6-425.1% higher frequencies of infertility and embryonic mortality, respectively, compared to clutches laid by females collected from the two reference sites. We hypothesize two mechanisms that may be responsible for the reductions in egg viability. First, bioaccumulation of Hg within adult reproductive tissues may impair physiological functions essential for reproduction. Here we have shown that contaminated female turtles retain high concentrations of Hg within their tissues, which may lead to deleterious effects on female reproductive physiology. Although we did not detect a significant effect of female Hg on maternal investment (i.e., clutch mass, egg mass, clutch size), disruptions in reproduction may occur through other mechanisms, such as disruption in reproductive hormones or egg quality. A recent review by Tan et al. (2009) concluded that many vertebrate species bioaccumulate Hg in their reproductive tissues, resulting in gross morphological malformations and the disruption of important reproductive hormones. For example, in fish, impaired neurochemistry adversely affected reproduction through impaired egg production, gonadal development, fecundity and fertility (Dey and Bhattacharya 1989; Hammerschmidt et al. 2002). Additionally, Hg-induced reproductive impairment is not limited to females, as Hg has been shown to alter testosterone levels, sperm production, and morphology in male fish (reviewed in Tan et al. 2009). Therefore, egg infertility may be driven by female or male reproductive impairment through Hg's disruption of essential physiological functions important in gametogenesis and fertilization. Second, reductions in hatching success were also attributed to embryonic mortality, suggesting that egg Hg can have direct effects on embryonic development.

Mercury is an embryotoxicant that is known to reduce hatchability and cause malformations in multiple vertebrate species (reviewed in Scheuhammer et al. 2007). For example, eggs laid by female American kestrels (*F. sparverius*) exposed to dietary Hg showed decreased hatching and lower fledging rates that those produced by mothers who were not exposed during nesting (Albers et al. 2007). We observed a similar pattern of reproductive impairment in turtles, with clutches laid by females collected from Hg contaminated sites having higher rates of embryonic mortality than those of females collected from reference areas. Together, our results suggest that maternally transferred Hg is more embryotoxic than teratogenic in snapping turtles, as malformation frequencies were uniformly low across sites and were not correlated with egg THg.

Decreases in turtle egg viability due to Hg exposure may have negative consequences for turtle populations inhabiting Hg contaminated areas. Traditionally, turtle population dynamics are thought to be highly dependent upon the survival of adults, particularly mature females, whereas egg and hatchling survival are generally thought to have relatively little influence on populations (Congdon et al. 1987, 1994). However, population models produced by Cunningham and Brooks (1996) suggest that the importance of annual hatchling survival increases in cases where adult survival is compromised. For adult turtles inhabiting contaminated portions of the South River, muscle Hg concentrations fall well within the range that induce lethal and sublethal effects in other aquatic species (Hopkins et al. In prep; reviewed in Scheuhammer et al. 2007). Furthermore, even if Hg exposure does not significantly affect adult survival, many anthropogenic sources of mortality, such as road mortality and harvest, may compromise adult survival (Gibbs & Shriver 2002; Gibbons et al. 2000). Thus, decreases in hatching success may have negative implications for turtle populations inhabiting Hg contaminated areas where adult survival is also compromised by Hg or other factors. Ultimately, threats to adult and hatchling

survivorship, compounded with naturally low hatchling recruitment and nest survival, may leave snapping turtle populations limited in their ability to withstand and respond to Hg contamination.

Although we demonstrate deleterious effects of Hg on turtle reproduction, the importance of these effects to turtle populations is still unknown. We propose three activities that would aid in maintaining healthy turtle populations in contaminated areas and contribute to our understanding of how Hg might influence turtle population dynamics. First, our results enable immediate initiation of sustainable programs for monitoring exposure of turtles to Hg. We show that female Hg tissue concentrations strongly correlate to concentrations in eggs, allowing future researchers to estimate egg concentrations that would be expected to have adverse effects from relatively easy to collect non-destructive tissues (i.e., blood and nail). Second, in order to accurately assess the influence of impaired reproduction on turtle population viability, factors influencing adult survival must be understood. Throughout their range, snapping turtles are frequently harvested for human consumption, but the rate and frequency of turtle harvest is largely unknown. For turtle populations facing Hg contamination, adult harvest in conjunction with reduced egg viability may drastically influence demographics and ultimately population persistence. Finally, protection and/or construction of nesting habitat may help mitigate the negative reproductive effects in Hg contaminated areas. Nest failure due to predation can range from 30-100% in turtles, and other factors such as flooding, erosion, low nest temperatures, and nest destruction can further increase rates of nest failure (Congdon et al. 2009). If appropriate nesting habitat is readily available and protected from nest predators, the negative reproductive effects elicited by maternally transferred Hg could be offset.

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Table 2.1 Proportion of embryos that died at a given stage during development from eggs collected from female snapping turtles (*Chelydra serpentina*) inhabiting the Middle River (MR-Ref) and upstream of the contamination source on the South River (SR-Ref), and downstream of the Hg contamination source along the South River (SR-Cont).

	MR-Ref	**SR-Ref**	**SR-Cont**
Number of total clutches collected	23	10	59
Number of clutches with embryonic mortality	15	4	33
Proportion of embryos that died during development	0.07	0.05	0.16
Stage of embryonic mortality			
Early	0.38	0.75	0.66
Middle	0.29	0.00	0.15
Late	0.33	0.25	0.19

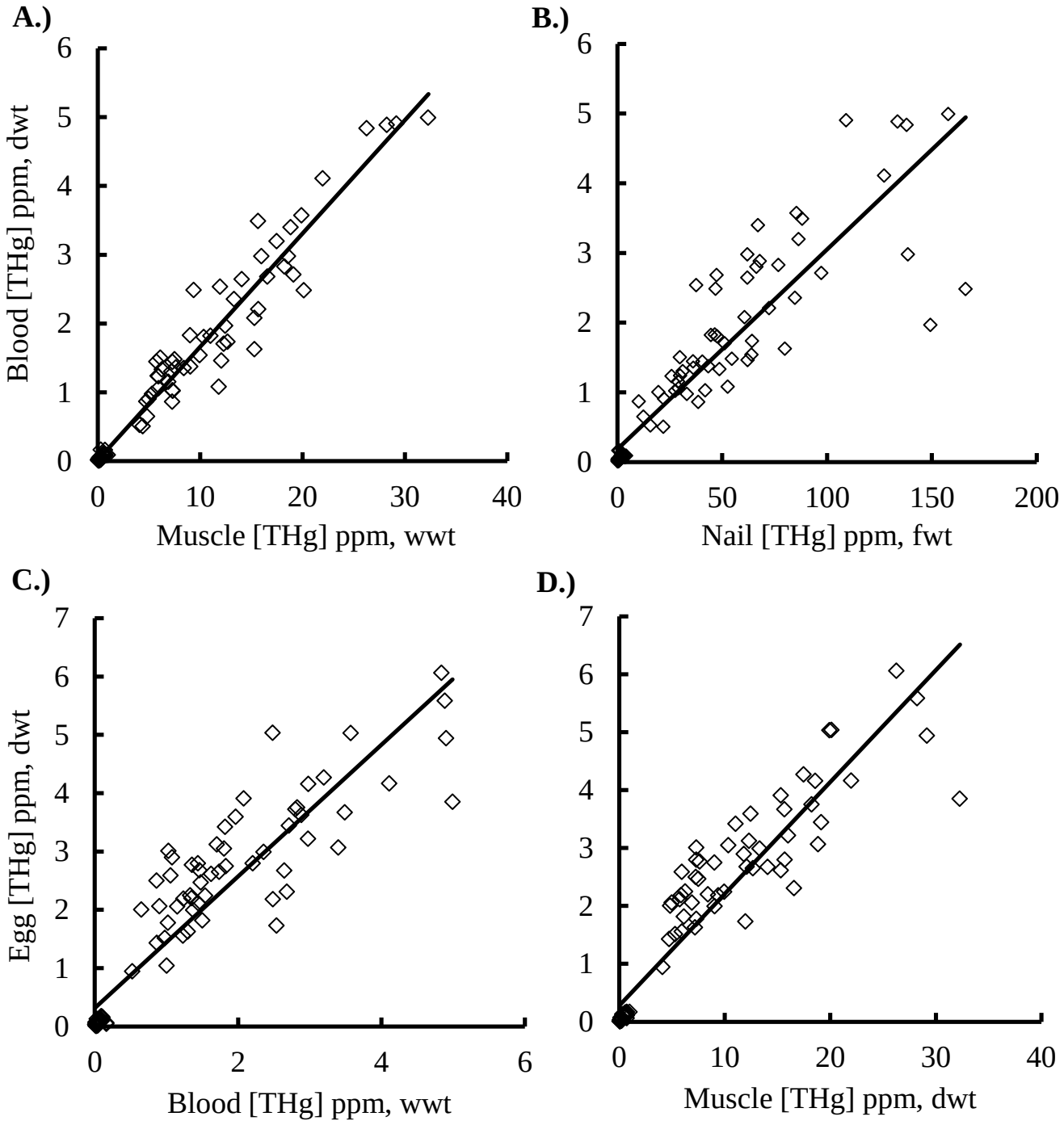

Figure 2.1 A.) Relationship between muscle (dry weight) total mercury (THg) and blood (wet weight) THg of gravid female *Chelydra serpentina* collected from the South and Middle Rivers (SR-MR; y= 0.1648x +0.0152, $r = 0.90$, $p < 0.0001$, n= 92) VA, USA B.) Relationship between blood THg and nail (fresh weight) THg of gravid female *Chelydra serpentina* collected from the South and Middle Rivers (SR-MR; y= 0.0287x +

0.1835, r =0.86, p < 0.0001, n=95) VA, USA C.) Relationship between blood THg and egg (dry weight) THg of gravid female *Chelydra serpentina* collected from the South and Middle Rivers (SR-MR; y=1.1298x +0.3117, r = 0.92, p < 0.0001, n= 95), VA, USA. D.) Relationship between muscle THg and egg THg of *Chelydra serpentina* collected from the South and Middle Rivers (SR-MR; y=0.1928x + 0.2855, r = 0.93, p < 0.0001, n=92).

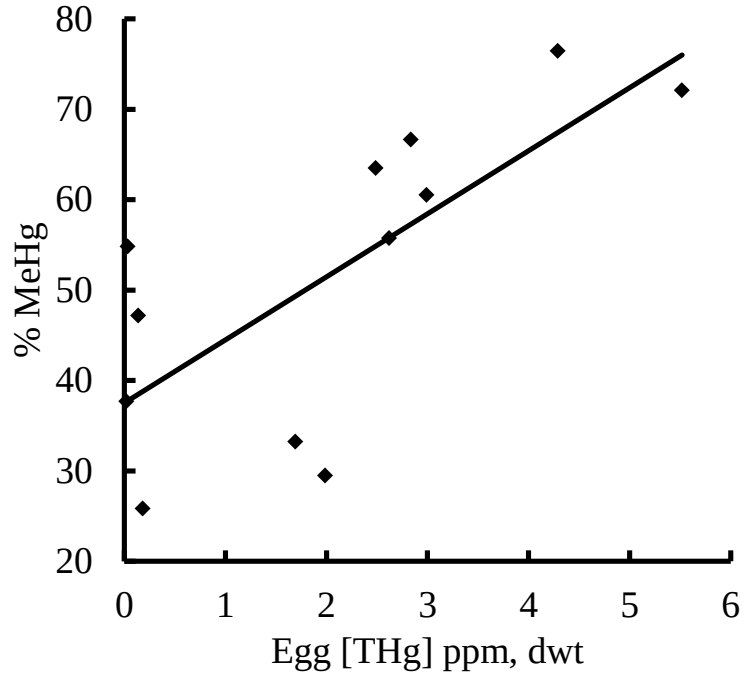

Figure 2.2 Relationship between % Methylmercury (MeHg) and egg total mercury (THg) (dry weight) in eggs laid by gravid females *Chelydra serpentina* collected from the South and Middle Rivers ($y= 6.975x + 37.517$, $R^2 = 0.552$, $p = 0.008$, n= 12) VA, USA.

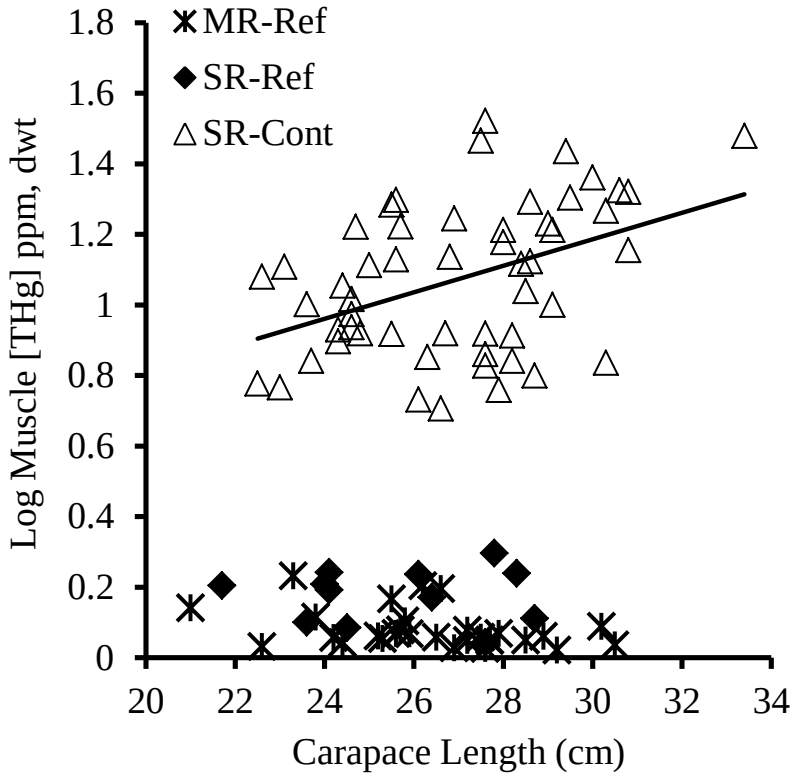

Figure 2.3 Relationships between carapace length and total mercury (THg) in muscle (dry weight) tissue of gravid *Chelydra serpentina* collected from the two reference sites on Middle River (MR Ref; n=29) and the South River (SR-Ref; $y = 0.0375x + 0.0616$, $R^2 = 0.18$, n=12), and the contaminated portion of the South River (SR-Cont; n= 54), VA, USA. Total Hg in turtle muscle did not increase with increasing body size at the two reference sites ($p > 0.16$), but did for individuals collected at the contaminant site ($p < 0.001$).

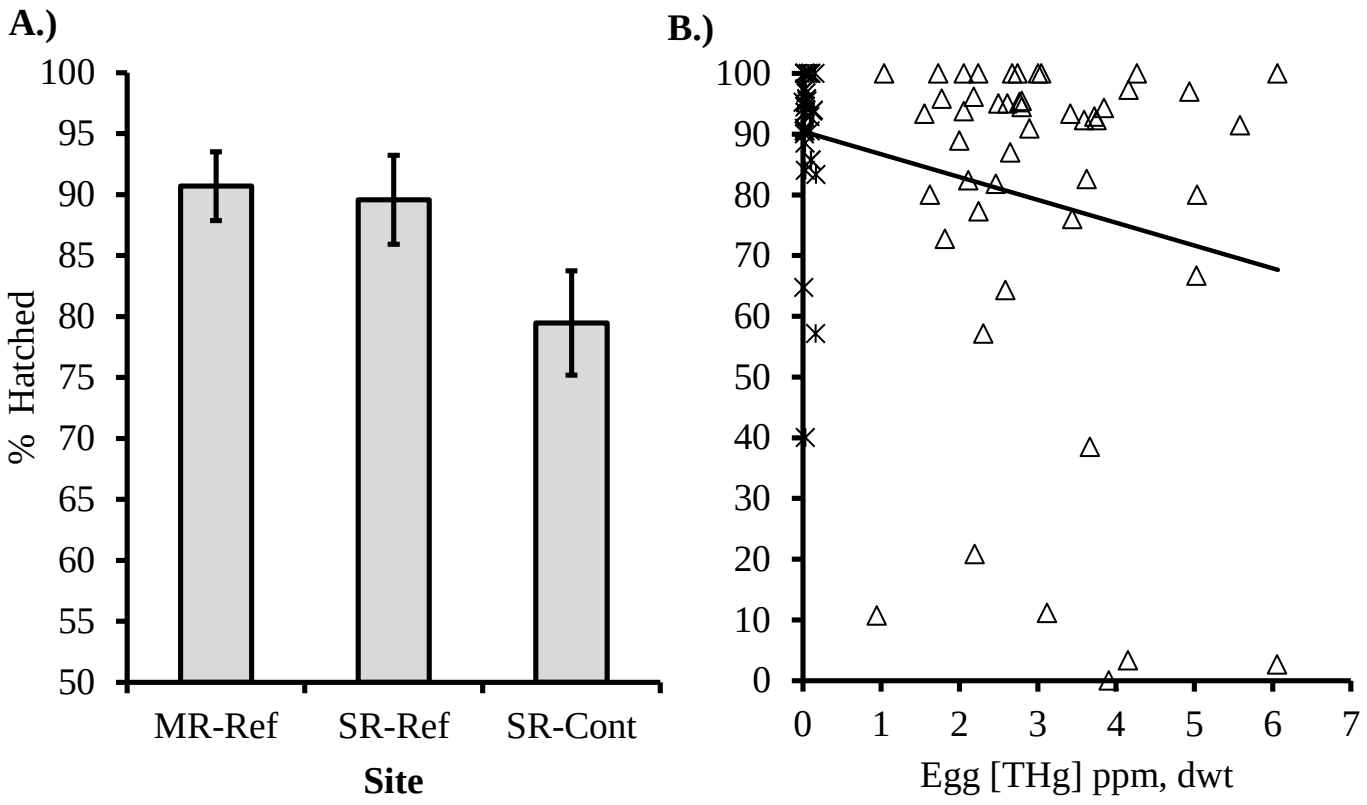

Figure 2.4 (A.) Mean (±1 SE) hatching success (% hatched within each clutch; n=82) of clutches laid by female *Chelydra serpentina* from the two reference sites (Middle River [MR-Ref] and upstream of the Hg source on the South River [SR-Ref]), and the contaminated portion of the South River (SR-Cont), VA, USA. (B.) Relationship between hatch success and total mercury in egg (dry weight) from turtles collected from reference areas (Ref: asterisks) along the Middle River and upstream of the source on the South River and those collected downstream of the source on the South River (Cont: open triangles).

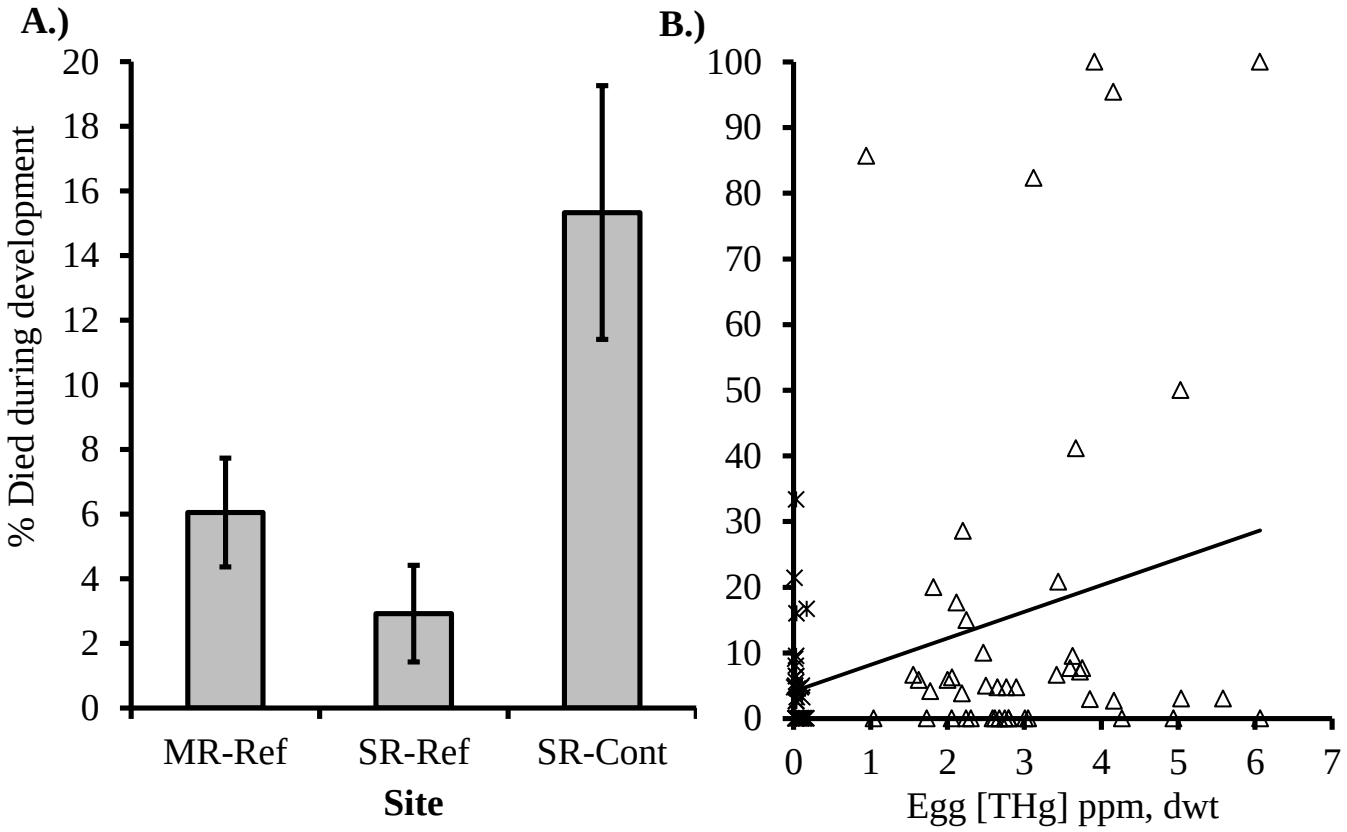

Figure 2.5 (A.) Mean (±1 SE) frequency of embryo mortality (% died during development; n=82) of clutches laid by female *Chelydra serpentina* from the two reference sites (Middle River [MR-Ref] and upstream of the Hg source on the South River [SR-Ref]), and the contaminated portion of the South River (SR-Cont), VA, USA. (B.) Relationship between embryo mortality (% died during development; n=82) and total mercury in egg (dry weight) from turtles collected from reference areas (Ref: asterisks) along the Middle River and upstream of the source on the South River and those collected downstream of the source on the South River (Cont: open triangles).

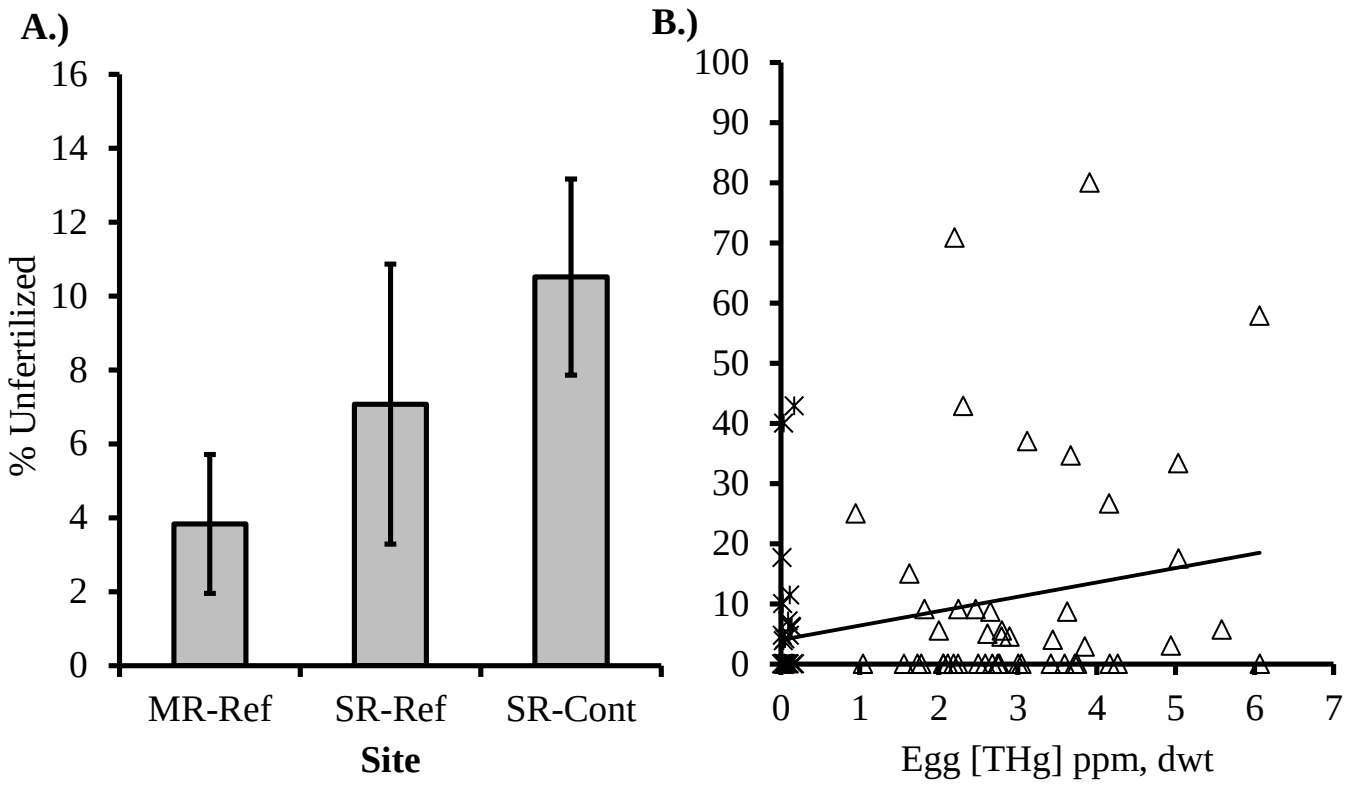

Figure 2.6 (A.) Mean (±1 SE) frequency of infertility (% unfertilized within each clutch; n=82) of clutches laid by female *Chelydra serpentina* from the two reference sites (Middle River [MR-Ref] and upstream of the Hg source on the South River [SR-Ref]), and the contaminated portion of the South River [SR-Cont], VA, USA. (B.) Relationship between of frequency of infertility (% unfertilized within each clutch; n=82) and total mercury in egg (dry weight) from turtles collected from reference areas (Ref: asterisks) along the Middle River and upstream of the source on the South River and those collected downstream of the source on the South River (Cont: open triangles).

Discussion & Conclusions

The ability of mercury (Hg) to bioaccumulate, bioamplify, and maternally transfer enables it to persist within food webs for extended periods of time and influence future generations. Although the U.S. has seen dramatic reductions of Hg emission from anthropogenic sources within the past 15 years (Schmeltz et al. 2011), Hg concentrations reported in fish and other aquatic biota exceed dietary thresholds considered potentially harmful to humans and wildlife throughout much of the nation (USEPA 2011). For example, according to the most recent listing published by the United States Environmental Protection Agency (USEPA), the number of Hg consumption advisories within the U.S. has risen from 3,361 in 2008 to 3,710 in 2010. Additionally, all 50 states are currently listed to have at least one localized fish consumption advisory, with 24 states listing statewide advisories. Thus, in addition to causing deleterious health problems for humans and wildlife (reviewed in Scheuhammer et al. 2007; Harada 1995), Hg contamination also diminishes the use and economic benefits of local fisheries, tourism, and other recreational activities gleam from functional stream, rivers, and lakes (Swain et al. 2007). However, although the deleterious consequences of Hg contamination are well documented in fish, reptiles have received significantly less attention and the physiological, ecological, and human risks associated Hg exposure and bioaccumulation in these organisms remain largely unknown.

My research utilized descriptive field studies to examine the factors influencing Hg bioaccumulation in free-living adult turtles and document the associated effects on reproduction. Using the extensive Hg gradient present along the contaminated portion of the South River and uncontaminated reference sites, I was able to collect blood, muscle, nail, and egg tissues from nearly 1,000 adult snapping turtles that represented a robust distribution of turtle body sizes for both sexes. From these data, I described important spatial, temporal, and demographic factors

that influence Hg concentrations in turtle tissues that should be considered by the South River Science Team and its collaborators when assessing consumption risk or working towards mitigation and restoration in areas of Hg contamination (Chapter 1). In addition, using turtle tissues collected from the South River and other sites, I developed and validated mathematical models that will enable future researchers to predict Hg bioaccumulation and maternal transfer in muscle and egg tissues using minimally invasive tissues (Chapter 1). Lastly, I collected 97 gravid females that produced over 2,500 eggs and provide the first line of evidence that female snapping turtles do maternally transfer Hg to their eggs which is associated with declines in egg viability (Chapter 2).

Human Consumption Risks. My study reports Hg concentrations in muscle tissue sampled from adult turtles inhabiting contaminated portions of the South River to be the highest ever reported in turtles within the United States. Not only are these concentrations associated with deleterious fitness consequences (reviewed in Scheuhammer et al. 2007; Bergeron et al 2011a), but they also pose serious risk to humans that may be harvesting contaminated turtles as a food resource. For example, in 2000, the EPA released a risk assessment that listed monthly meal (fish) consumption guidelines for a suite of environmental contaminants. Using two equations that accounted for consumer body mass, meal size, and Hg fish concentration, a consumption chart was generated that listed the number of allowable meals per month for various Hg fish concentrations (USEPA 2000). For MeHg, monthly fish muscle MeHg concentrations exceeding 1.9 ppm (wwt) were not acceptable for consumption whatsoever due to risk of mercury poisoning (USEPA 2000). In my study, I observed THg concentrations in muscle tissue collected from South River contaminated turtles to average 2.64 ± 0.15 ppm (wwt), with 93.36± 0.78% of the THg in the MeHg form. Taken together, consuming turtles from the

contaminated portion of the river may carry serious health risks, as MeHg exposure has been shown to impair vision, coordination, speech, and hearing in adults and cause severe neurological development in fetuses, infants, and young children (Gullar et al. 2002; Roman et al. 2011, May 2000; Harada 1995). However, there are no local or statewide regulatory guidelines that provide safe consumption limits for eating turtles collected from the contaminated portions of the South River or other Hg contaminated areas in Virginia. In addition, according to Virginia state law, citizens holding a fishing license may harvest up to 15 snapping turtles a day. Ideally, my graduate research will act as a foundation for setting consumption limits for turtles not only on the South River, but throughout the state of Virginia, and my nondestructive sampling methods can be used to monitor Hg bioaccumulation in a sustainable manner.

Mercury & Turtle Life-history. My research demonstrated differential reproductive success between reference and Hg contaminated females, where egg viability decreased as maternally transferred Hg concentrations increased. Because females living in Hg contaminated areas are producing fewer surviving offspring that can potentially contribute to the gene pool, Hg exposure is directly affecting the fitness of adult turtles and their offspring inhabiting contaminated areas. Additionally, my research did not quantify sublethal phenotypic effects that may be occurring in the surviving offspring. Mercury can cause a suite of sublethal effects such as decreased immunocompetence, altered hormone profiles, and impaired locomotor performance and behavior (Hawley et al. 2009, Tan et al. 2009; Bergeron et al. 2011b; Alvarez et al. 2006). Such effects may act as a significant source of phenotypic variation for turtle populations exposed to contamination, however, a study has yet to address whether Hg can produce sublethal effects in adult turtles or their offspring. Measuring the associated sublethal

effects induced by Hg will be important for understanding turtle population demographics if affected offspring are unable to reach adulthood or successfully reproduce later in life.

Although Hg significantly affected egg viability, egg Hg concentrations explained very little of the variation in hatching success (Chapter 2). Weak coefficients of determination were associated with all regressions describing reproductive parameters in relation to egg THg concentrations (in all cases $R^2 < 0.10$). Because egg Hg alone did not account for much of the variability in reproductive parameters, I recommend that future research be geared towards accounting for other factors that may contribute to the observed variation. For instance, although I implemented a standardized incubation regime for all eggs, I was unable to account for or quantify other factors that are known to influence turtle hatching success, such as egg composition (e.g., lipids, micronutrients, hormones) or site variation in resource availability. Both of these factors are discussed below.

In addition to being embryotoxic, Hg may indirectly affect offspring phenotype and/or survival as Hg exposure may alter the quantity of micronutrients and hormones that females can allocate to their eggs. For example, lipids are the primary energy component of the yolk (Kraemer and Bennett 1981, Finkler et al 2002) and are critical for turtle embryo and hatchling survival. Embryos and hatchlings rely solely on the yolk to meet the energetic demands necessary for embryonic development, hatching, activity, maintenance, and growth until the hatchling is able to leave the nest and capture its first meal item (Congdon & Gibbons 1985, 1990; Nagel et al. 1998; Rowe et al. 1995). However, Hg exposure has been shown to influence female lipid levels in several fish species. For instance, female Bronze featherbacks (Notopterus notopterus), walking catfish (*Clarias batrachus*), and snakehead murrel (*Channa punctatus*) exposed to mercury experienced decreases in lipid levels within the ovaries (Verma & Tonk 1983; Ram & Sathyanesan 1985; Kirubagaran & Joy 1995). Based on these observations, Hg

alteration of lipid availability may have consequences for vitellogenesis and therefore affect female and offspring fitness. Additionally, micronutrients and hormones, such as selenium and corticosterone, are important for embryonic and hatchling quality and development but availability can be affected by Hg exposure (Tan et al. 2009, Cuvin-Aralar et al. 1991; Khan et al. 2009, Watanabe 2002). Therefore, understanding Hg's effects on micronutrients and yolk composition of eggs may help explain the large variation observed in hatching success and potentially elucidate an additional mechanism by which Hg affects offspring phenotype.

While my research showed that female turtles maternally transfer Hg to their eggs, habitat variables that influence resource availability may create differences in maternally transferred Hg among sites and year. Variation in the quality and quantity of resources accessible for reproduction may lead to differences in where energy is being mobilized (e.g., energy stores v. dietary intake). Female snapping turtles are thought to rely on energy reserves stored in muscle and fat bodies for vitellogenesis (Bonnet et al. 1998; Congdon et al. 2008) and start producing follicles a year prior to ovulation (White & Murphy 1973). Because Hg preferentially binds to sulfur groups that are commonly found in muscle proteins (Gallagher & Lee 1980), the majority of Hg being maternally transferred from females to the egg is most likely mobilized from muscle during vitellogenesis. However, limitations on resource availability may force females to finish egg production following overwintering when fat reserves have been depleted, therefore shifting their energetic reliance on dietary intake. Additionally, the large temporal range in which females are allocating resources to their eggs may give rise to variation in the concentration of Hg being maternally transferred and its derivation. Therefore, differential resource limitations between years or sites may lead to increased variability in reproductive success brought about by maternally transferred Hg. Future research is needed to determine

whether variation in resource availability would significantly alter maternally transferred Hg concentrations through changes in the derivation of resources that are allocated to eggs.

Conclusion. While the lethal and sublethal effects of Hg exposure are documented in multiple birds and fish species (reviewed in Scheuhammer et al. 2007), only recently has the influence of Hg exposure on adult and offspring survival and phenotype begun to be understood and emphasized in herpetofauna (Bergeron et al. 2011a,b). As a result, little is known about the effects Hg bioaccumulation and maternal transfer has on the physiology and reproductive success of most reptile species. Understanding the factors that influence Hg are important for assessing the health of turtle populations living in Hg contaminated areas and also protecting humans and other predatory wildlife that are consuming turtles. Like many other persistent contaminants, elimination of Hg from the environment is currently not feasible and requires continual monitoring of concentrations within polluted areas. The non-destructive sampling techniques I developed will be critical for periodically monitoring Hg bioaccumulation in turtles in order to revise consumption guidelines, reassess ecological risk, and quantify the current effects on turtle reproduction and the will allow sampling to be performed sustainably.

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