Individual and Interactive Effects of Maternally- and Trophically-Derived Mercury on Early Amphibian Development

Christine Marie Bergeron

Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Doctor of Philosophy
In
Fisheries and Wildlife

William A. Hopkins, Chair
C. Andrew Dolloff
Marcella J. Kelly
Christopher L. Rowe
Jill C. Sible

November 4, 2011
Blacksburg, VA

Keywords: amphibian, American toad, mercury, maternal transfer, latent effects

Individual and Interactive Effects of Maternally- and Trophically-Derived Mercury on Early Amphibian Development

Christine Marie Bergeron

Abstract

Mercury (Hg) is an important environmental contaminant due to its global distribution, tendency to bioaccumulate, and toxicity to wildlife. However, Hg has received little attention in amphibians compared to other vertebrates, despite the fact that amphibian population declines have been documented worldwide and environmental contaminants are believed to contribute to some declines. During my dissertation research, I used a pluralistic approach which combined field studies and manipulative laboratory and mesocosm experiments to examine the bioaccumulation and ecological effects of environmentally relevant Hg exposure routes acting at various early life stages in amphibians. By collecting amphibians in the field at the Hg-contaminated South River, VA, I confirmed that amphibians exhibiting different life histories and occupying different ecological niches (*Plethodon cinereus*, *Eurycea bislineata*, and *Bufo americanus*) can bioaccumulate sufficient levels of Hg to warrant concern (Chapter 2) and female *Bufo americanus* transfer accumulated Hg to their eggs (Chapter 3). Maternal transfer of contaminants is a parental effect which typically has negative consequences for offspring because early development is a critical organizational period in the ontogeny of vertebrates. Through laboratory observations and mesocosm experiments, I examined the short and long-term effects of maternal contaminant exposure on offspring, and found the negative effects of maternal Hg exposure manifested either immediately at the embryonic stage or later during the larval stage, depending on the year in which the study was conducted (Chapters 4 and 5). Lastly, using a factorial laboratory experiment, I examined whether the latent effects of maternal transfer of contaminants manifests differently depending on the environment in which offspring develop,

and found both maternal and dietary Hg exposure independently produced negative, but different, sublethal effects on larval development. Most importantly, maternal exposure to Hg combined with high dietary Hg exposure later in ontogeny had a lethal effect in larvae (Chapter 6). This study is one of the first to demonstrate that the latent effects of maternally transferred contaminants may be exacerbated by further exposure later in ontogeny, findings that may have important implications for both wildlife and human health.

Acknowledgements

Few people could go through this endeavor alone, and I am certainly not one of them! There are so many people to acknowledge for their assistance getting me to this point, whether it was through their support, encouragement, interest, knowledge, curiosity, patience, skill, and more. Most importantly, I thank my dissertation advisor, Dr. William Hopkins. Bill is an excellent mentor and I have learned so much from him both about science and the rigors of academia. He knew when to push and prod and when to sit back. He provided unwavering support throughout my tenure at Virginia Tech and I appreciate that he believed in my abilities until I believed in them myself. Bill graciously provided both professional and personal encouragement and opportunities to gain teaching and mentoring experience to prepare me for future directions. I also thank my committee members, Drs. Andrew Dolloff, Marcella Kelly, Christopher Rowe, and Jill Sible for their valuable input and advice on my dissertation research.

My research would not have been possible without the support of the South River Science Team, a collaboration of state and federal agencies, academic institutions, and environmental interests. Specifically, I acknowledge Drs. Ralph Stahl and Erin Mack from E.I. DuPont de Nemours and John Schmerfeld from U.S. Fish and Wildlife Service for understanding the importance of this research. Our work on the South River would not have been as successful from the start without the generous help of Dr. Daniel Cristol from the College of William & Mary and his lab (Ariel White, Mikaela Howie, Anne Condon, Scott Friedman, and Rebecka Brasso) who shared all their hard earned knowledge of land owner access and support with us. I also thank the land owners along the South River and the Waynesboro Parks and Recreation Department for access to sampling locations.

I thank my Master's advisor, Dr. Robert Mason, for giving me the opportunity to learn how to analyze mercury (among other things). The ability to analyze my own samples for mercury was vital in the beginning of my project. I thank Dr. Jason Unrine, an incredible scientist and collaborator, for agreeing to work with me at the Savannah River Ecology Laboratory (SREL) where I learned even more about mercury! He has supreme patience for working with the part-time chemist in me. Also while at SREL, I thank Heather Brant and Diane Addis for their assistance with analyses and J.D. Willson for always happily providing housing for me. In the past few years, Dan Cristol has been kind enough to allow me to analyze samples in his laboratory, often at very short notice and even after I broke his mercury analyzer!

There are so many post docs, technicians, and undergrads to thank for their assistance in the field. Besides assisting with research, these people helped me keep my sanity while waiting and waiting for the toads to breed, frantically counting thousands of eggs, and running around at night in the rain! In general order of field seasons: Jerry Husak, Cathy Bodinof, Haruka Wada, Sarah Budischak, John Burke, Katie McCaleb, Brain Todd, Mark Hepner, and Jake McPherson. You made most of it fun and suffered with me through the pain! Thank you!

Back at Virginia Tech, I am thankful for the support and friendships that have developed in the Hopkins Lab. Thank you for making each day at the lab enjoyable, for challenging me to be a better scientist, for helping me to not think about science every waking moment of the day, and for being there to celebrate life's events (big and small). From the start, I enjoyed learning the ins and outs of our new program with Sarah DuRant and Sarah Budischak. We grew a great deal together. For all the smiles through the years, also thanks to Brittney Hopkins, Devin Jones, Haruka Wada, Brian Todd, J.D. Willson, John Burke, Amanda Wilson, Chad Stachowiak, and de-facto lab members Jessica Homyack, Tom Gorman, and Kristine Grayson.

Going back much further, I would like to acknowledge two previous mentors without whom I may not be where I am today. I was not even considering a future in science until my high school biology teacher, Mrs. Donna DerKinderen took a keen interest in me, and I may have ended up in a very different field of biology if my undergrad academic advisor, Dr. Owen Sholes, had not encouraged me to explore environmental studies options despite the fact that I "probably wouldn't be able to swim with the dolphins". It is because of these two that I have always had an interest in teaching and working closely with undergraduates, and I thank them for seeding that passion and for their continued interests in my endeavors to this day.

I am thankful to have many friends spanning the country and their support has been important through this process, but two people have REALLY been there through the thick and thin of it all. Sabrina Zadrozny has encouraged and supported me, and has been one of my biggest cheerleaders through all the growing and changing from our early days at Assumption College to now. Carrie Miller has been my "mercury guru" from our time living, working, and socializing together in Maryland, and was always willing to listen to the successes and failures while providing sound advice since she has been through it all before.

Lastly, my entire family and my husband, Jody Callihan, deserve many thanks for seeing me to this point. I thank my parents especially for never questioning the sometimes seemingly endless student status, for always encouraging me to take the path that would lead to a fulfilling career, and for supporting me in countless ways along the way! I thank Jody for being the one to know exactly what I was going through every day, for celebrating the highs and picking me up from the lows, and for weathering all the mundane and wonderful moments in between. We have survived many miles and overcome many obstacles to be where we are today and I look forward to navigating the next many years "wherever life may bring us" side by side.

Attribution

I am first author for Chapters 2-6 and all of my co-authors were a part of the Department of Fish and Wildlife Conservation, Virginia Tech at the time the research was conducted unless otherwise noted.

I co-authored Chapters 2 and 3 with my advisor, William Hopkins, a field technician, Catherine Bodinof, and a collaborator, Jason Unrine (Department of Plant and Soil Sciences and The Tracy Farmer Institute for Sustainability and the Environment, University of Kentucky). Catherine Bodinof aided in data collection and reviewed the chapter. All other co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

I co-authored Chapter 4 with my advisor, William Hopkins, field technicians, Catherine Bodinof and Sarah Budischak, a post-doctoral researcher, Haruka Wada, and a collaborator Jason Unrine. Catherine Bodinof, Sarah Budischak, and Haruka Wada aided in data collection and reviewed the chapter. All other co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

I co-authored Chapter 5 with my advisor, William Hopkins and a post-doctoral researcher, Brian Todd. All co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

I co-authored Chapter 6 with my advisor, William Hopkins, a post-doctoral researcher, Brian Todd, a technician, Mark Hepner, and a collaborator, Jason Unrine. Mark Hepner aided in data collection and reviewed the chapter. All other co-authors provided input on the design of the experiment, use of lab/field equipment, and edited the chapter.

Table of Contents

List of Tables

Chapter 2: Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians.

Chapter 3: Bioaccumulation and maternal transfer of mercury and selenium in amphibians.

List of Figures

Chapter 2: Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians.

Chapter 3: Bioaccumulation and maternal transfer of mercury and selenium in amphibians.

Chapter 4: Counterbalancing effects of maternal mercury exposure during different stages of early ontogeny in American toads.

Chapter 1: Introduction

Amphibians are the most imperiled group of terrestrial vertebrates on earth, and population declines have been documented worldwide. Over 40% of amphibian species are threatened with extinction and amphibian populations are declining more rapidly than either birds or mammals (Hoffmann et al., 2010). Habitat loss and degradation are the greatest threats, impacting almost 4,000 species of amphibians. The next most common threat, impacting nearly 1,000 amphibian species, is environmental contamination (IUCN, 2011). Other factors that influence populations include emerging diseases, invasive species, overexploitation, and global climate change (Alford, 2010, Corn, 2000, Stuart et al., 2004). With several factors contributing to declines, individuals may be exposed to multiple anthropogenic stressors over their lifetime, making conservation of amphibians difficult to address.

Amphibians can play a critical role in transferring energy and nutrients through food webs and therefore are important components of many aquatic and terrestrial communities (Beard et al., 2002, Hopkins, 2007, Wyman, 1998). As ectotherms, amphibians are very efficient at converting ingested energy into biomass (Grayson et al., 2005, Pough, 1980), and as a result, can often occur in extremely high densities. For example, in Virginia, certain species of amphibians represent the single most abundant vertebrate in many aquatic and terrestrial communities, with densities exceeding 40,000 individuals per hectare in some cases (Jaeger, 1980). Due to their abundance and high conversion efficiencies, amphibians often serve as vital links for energy and nutrient flow between low and high trophic levels. These characteristics also suggest that amphibians may have high rates of contaminant bioaccumulation compared with other animals of similar trophic positions (Unrine et al., 2007). In addition, the complex lifecycles of many amphibians potentially make them important in transferring contaminants

from aquatic to terrestrial food chains (Roe et al., 2005, Snodgrass et al., 2003), and place them at risk of environmental contaminants in both aquatic and terrestrial habitats.

Environmental contaminants may have strong impacts on amphibian populations by negatively altering their food resources and causing immune dysfunction, infertility, developmental malformations, feminization, and endocrine disruption (Corn, 2000). Early ecotoxicological studies focus on concentrations of contaminants in the environment and tissue residues rather than their biological effects (Sparling and Lowe, 1996). Accumulation of contaminants does not always translate into lethal or even detrimental effects for organisms, but contaminant concentrations in biota are powerful measures when combined with a knowledge of biological effects that directly affect or have a strong influence on fitness of individuals. For example, pollutant-induced alterations in morphology may be sublethal to larvae, but may ultimately decrease the recruitment of individuals to the terrestrial environment (Rowe et al., 2001). These biological effects can provide the clearest link between responses in individuals and effects at the population and community levels, which are the targets of most conservation programs (Hopkins, 2006). Unfortunately, population-level manipulations are generally logistically impossible, especially with suitable replication. Therefore, important individual-level parameters (i.e., growth, survival, reproduction), known to be important contributors to population-level processes, are often measured.

Although the accumulation of trace elements has been well documented in tissues and organs of amphibians (see Linder and Grillitsch, 2000), fewer studies have made the connection between tissue concentrations and their potential biological effects. However, laboratory, mesocosm, and field studies of trace element contamination in larvae of several amphibian species have documented effects on: survival (James et al., 2005, Lefcort et al., 1998, Rowe et

al., 1998, Snodgrass et al., 2005); growth and development by way of developmental abnormalities, altering size at metamorphosis, or delaying metamorphosis (Hopkins et al., 2000, James et al., 2005, Lefcort et al., 1998, Roe et al., 2006, Snodgrass et al., 2005, Snodgrass et al., 2004); energy acquisition through oral abnormalities and allocation by increasing maintenance costs (Rowe et al., 1996, Rowe et al., 1998); and behavior and performance which may impair the ability to detect or evade predators (Hopkins et al., 2000, Lefcort et al., 1998, Raimondo et al., 1998). These sublethal effects can influence survival to metamorphosis which is an important component of population viability for many pond-breeding amphibians. The number of surviving metamorphs influences the number of individuals recruited to the adult population (Beebee et al., 1996, Berven, 1990). In fact, Rowe et al. (2001) observed complete failure in the recruitment of toads (*Bufo terretris*) from a coal ash contaminated wetland and argued that contaminated aquatic systems may act as sinks for amphibian populations. Alteration in age and size at metamorphosis due to contaminants can also have important impacts on adult fitness because metamorphs that emerge larger or earlier are more likely to reach reproductive maturity faster and at a larger size (Semlitsch et al., 1988, Smith, 1987). In addition, a prolonged larval period increases the duration of exposure to aqueous contaminants, risk of desiccation in ephemeral ponds, and can result in less time to feed and grow in the terrestrial environment prior to overwintering (Roe et al., 2006).

Unfortunately, there is a paucity of information on effects of contaminants on amphibian reproduction, an endpoint that directly influences the viability of populations (Hopkins et al. 2006). Maternal transfer of contaminants may be an important mechanism of impaired reproductive success in amphibians because early development is a critical period in ontogeny and adverse effects of maternally-derived contaminants during this sensitive stage have been

well documented in other vertebrates. For example, in many animals, maternal transfer of contaminants reduces reproductive success through embryoic mortality and malformations (e.g., Di Giulio and Tillitt, 1999). In amphibians, most of the information regarding the effects of contaminants on early development is from aqueous exposure of embryos to contaminants, often in environmentally unrealistic concentrations (Linder and Grillitsch, 2000). To date, only four studies have documented maternal transfer of contaminants in amphibians (Hopkins et al., 2006, Kadokami et al., 2004, Kotyzova and Sundeman, 1998, Wu et al., 2009). Kadokami et al. (2004) and Wu et al. (2009) demonstrated the occurrence of maternal transfer of organochlorine pesticides, dioxins, and PCBs in *Rana ornativentri* and polybrominated diphenyl ethers (PBDEs) in *Rana limnocharis,* respectively; however, they did not investigate the biological effects of maternal transfer. The other two studies examined the relationship between maternal transfer of contaminants and embryonic development or reproductive success. In aqueous laboratory exposures, adult *Xenopus laevis* (African clawed frogs) maternally transferred cadmium (Cd) to their eggs, resulting in a residue-dependent increase in developmental malformations (Kotyzova and Sundeman, 1998). In a field study, *Gastrophryne carolinensis* (eastern narrow-mouth toads) from an industrial site maternally transferred high concentrations of selenium (Se) and strontium (Sr) to their eggs, associated with a 19% reduction in offspring viability (Hopkins et al., 2006). There are few studies examining the potential for latent effects of maternal exposure to contaminants beyond embryonic development in vertebrates; however, there is growing evidence that early disruptions in nervous, endocrine, or immune system development due to maternally-derived contaminants (Guillette et al., 1995) can also have effects that are expressed later in ontogeny (e.g., Eisenreich et al., 2009, Nye et al., 2007). The goal of this dissertation was to further examine both the immediate and latent or long-term effects of maternal contaminant

exposure in amphibians to assess whether maternal exposure may be a possible contributing mechanism leading to amphibian declines.

Study objectives

Using a pluralistic approach that integrated field surveys and laboratory and mesocosm techniques, I investigated the following objectives in order to evaluate the individual and interactive effects of mercury (Hg) from maternal and dietary sources on female reproductive success and embryonic and larval survival and development:

1) To determine whether life history strategies and feeding ecologies influence Hg exposure and accumulation in amphibians (Chapter 2).

2) To develop nondestructive methods for assessing Hg bioaccumulation in amphibians (Chapter 2).

3) To determine whether amphibians maternally transfer Hg to their offspring in a tissue-residue dependent manner (Chapter 3).

4) To determine whether maternal transfer of Hg negatively impacts reproductive success by affecting hatching success and embryonic development in a tissue-residue dependent manner (Chapters 4 & 5).

5) To determine whether embryos that successfully hatch from Hg-exposed females experience latent effects of maternal transfer later in larval development that decreases the number and size of individuals recruited to the local population (Chapters 4 & 5).

6) To determine whether larvae from Hg-exposed females experience additive or synergistic negative effects of continued Hg exposure during the larval period through dietary intake (Chapter 6).

To evaluate these objectives, I studied the effects of Hg bioaccumulation and maternal transfer in American toads (*Bufo americanus*) inhabiting the highly Hg-contaminated South River, VA, USA.

Study species

I chose American toads as a model species because they are ubiquitous throughout much of eastern North America (Lannoo, 2005), and they have closely related congeners with similar ecologies world-wide. American toads are not considered to be under threat of extirpation (Lannoo, 2005); however, they do undergo fluctuations in population size (Hecnar and McLoskey, 1996), and Bufonidae is one of four families to "contribute overwhelmingly to the total number of declining [amphibian] species" (Stuart et al., 2004). In addition, American toads exhibit the classic complex life cycle typical of many temperate anurans. As adults, they inhabit a variety of terrestrial habitats, but they require cover objects with moist microhabitats, an abundant supply of insects and other small invertebrates as prey, and shallow bodies of water to breed (e.g., ephemeral wetlands, drainage ditches, shallow portions of streams with low flow). Larval American toads are omnivorous grazers that consume algae, suspended organic matter, detritus, and decomposing animal material (Lannoo, 2005). At the South River, VA, American toads occur in sufficient abundance to study maternal transfer and reproductive effects, are easy to capture and breed readily in captivity, and produce large clutches of eggs (~6,000 per female), allowing large sample sizes for experiments and laboratory analyses.

Mercury

Mercury is an important environmental contaminant due to its global ubiquity, toxicity, and tendency to bioaccumulate. The regulatory and scientific focus on Hg in aquatic ecosystems has been motivated largely by the human health risks of consuming contaminated fish, as this is the primary route of human exposure to methymercury (MMHg) (Clarkson, 1990, Fitzgerald and Clarkson, 1991), a highly toxic and bioaccumulative form of Hg (Fitzgerald et al., 1998). While the study does not include Virginia, Facemire et al. (1995) states that Hg contamination is the

most serious environmental contamination threat to fishery and wildlife resources in the southeastern United States (AL, AR, FL, GA, KY, LA, MS, NC, SC, and TN). All 50 states have Hg advisories in at least some of their water bodies with 27 states having statewide advisories for all freshwater systems and 13 states having statewide advisories in their coastal waters (USEPA, 2009b). In addition, Hg was detected in every fish sample collected from 500 U.S. lakes in a 2009 U.S. Environmental Protection Agency study. From these samples, it was estimated that nearly 50% of the lakes nationwide contain fish with Hg concentrations above the 300 ppb health screening value associated with potential human health risks (USEPA, 2009a).

Natural or anthropogenic sources of mercury loading in aquatic ecosystems can result from either atmospheric deposition or point source emissions. Due to anthropogenic activities, atmospheric input of Hg has tripled over the past 150 years (Mason et al., 1994). Atmospheric deposition results in widespread distribution of Hg due to long range airborne transport (Fitzgerald et al., 1998), and point source emissions are often associated with high contamination levels at a localized scale (e.g., within riverine or lacustrine systems). The majority of Hg released into the environment is inorganic, yet the more toxic and bioaccumulative form is MMHg (Fitzgerald et al., 1998). Production of MMHg is primarily a biologically-mediated reaction by anaerobic sulfate –reducing bacteria in aquatic sediment (Benoit et al., 1999, Benoit et al., 2003). Thus, knowledge of the total Hg (THg) concentration in the environment is inadequate to accurately evaluate its toxicity. In fact, in many cases, even knowing both the THg and MMHg concentrations in biota is not adequate to predict toxicity because the bioavailability and toxicity of Hg depends upon its specific chemical form and other biogeochemical properties (e.g., pH, dissolved organic matter, presence of other metals) of the environment.

Mercury tends to accumulate in aquatic habitats where it readily bioaccumulates and can biomagnify in aquatic food webs, especially as MMHg (Hill et al., 1996, Watras and Bloom, 1992). Lower trophic level organisms are important in transferring Hg throughout the food web, especially since the greatest bioconcentration of MMHg occurs between water and phytoplankton (Lindqvist et al., 1991, Mason et al., 1996). In fact, MMHg concentrations in fish can be six to seven orders of magnitude higher than MMHg concentrations in the surrounding surface waters (Bowles et al., 2001). Mercury accumulation in wetlands threatens critical breeding and foraging habitats for many fish and wildlife species. The primary route of Hg exposure is through the diet (Hall et al., 1997, Unrine and Jagoe, 2004), and consumption of diets with environmentally realistic concentrations of Hg result in a large range of toxic effects in fish and wildlife, including behavioral, developmental, neurological, hormonal, and reproductive changes (Crump and Trudeau, 2009, Eisler, 2006, Scheuhammer et al., 2007, Tan et al., 2009, Weiner and Spry, 1996, Wolfe et al., 1998). Due to the neurotoxic, teratogenic, and endocrine-disrupting nature of Hg, subtle effects on behavior and reproduction may occur at concentrations well below levels associated with overt toxicity and death (Scheuhammer, 1991, Weiner and Spry, 1996, Weis, 2009). Indeed, reproductive success is the demographic parameter expected to be most affected by exposure to Hg in fish and birds (Crump and Trudeau, 2009, Scheuhammer et al., 2007, Weiner and Spry, 1996).

The adverse effect of Hg accumulation in amphibians has received little attention compared to fish, birds, and mammals (Eisler, 2006, Linder and Grillitsch, 2000, Scheuhammer et al., 2007, Wolfe et al., 1998). Several studies have investigated aqueous Hg toxicity (see Linder and Grillitsch, 2000; table 7-6) and Hg bioaccumulation (Bank et al., 2005, Bank et al., 2007, Bradford et al., 2011, Gerstenberger and Pearson, 2002, Hothem et al., 2010, Ugarte et al.,

2005, Unrine and Jagoe, 2004, Unrine et al., 2004, Unrine et al., 2005, Weir et al., 2010) in amphibians. However, very little is known about the effects of Hg exposure in amphibians (Eisler, 2006, Linder and Grillitsch, 2000, Wolfe et al., 1998). Kanamadi and Saidpur (1991) and Punzo (1993) both found significant decreases in ovary weight when adult *Rana cyanophlyctis* and *R. heckscheri*, respectively, were exposed to aqueous inorganic Hg. Birge et al. (2000) also reported impaired gamete function and reduction in early life stages with gonadal Hg residues of 0.49 μg g^{-1} in the South African clawed frog (*Xenopus laevis*). However, in one recent study, amphibians were found to be sensitive to Hg exposure during the critical periods of ontogeny of larval development and metamorphic climax. Unrine and colleagues found adverse effects of environmentally realistic dietary Hg exposure on survival, development, and growth of southern leopard frog larvae (*Rana sphenocephala*) (Unrine and Jagoe, 2004, Unrine et al., 2004). The results from these studies are more ecologically realistic than previous studies using aqueous Hg exposure since dietary Hg is likely the most important exposure route. These data indicate that Hg pollution, in concentrations associated solely with atmospheric deposition, has the potential to negatively impact amphibian populations by decreasing the number and quality of offspring recruited to the terrestrial environment (Unrine et al., 2004). In addition, maternal transfer of Hg in amphibians is a concern since early developmental stages in aquatic biota are particularly sensitive to Hg (Birge et al., 2000, Weiner et al., 2003).

Study site

Although Hg is ubiquitous due to long range transport from atmospheric deposition (Fitzgerald et al., 1998), point source emissions can result in high localized Hg concentrations. For example, my study site encompasses a 30 mile segment of the South River, VA, USA which was historically (1929-1950) contaminated with mercuric sulfate used by a plant manufacturing

acetate fiber in Waynesboro, VA, USA (Carter, 1977). The use of Hg was terminated in the 1950s, but Hg concentrations currently remain high in the river (Southworth et al., 2004). A Hg contamination gradient along the river spans from low concentrations (upstream from the point source), presumably attributed to atmospheric deposition and geologic sources, to extremely high Hg levels downstream from the source (up to 191 ng/L unfiltered river water; Southworth et al., 2004). Previous studies have revealed orders of magnitude differences in biota tissue Hg concentrations from several species between the upstream (reference) and downstream (contaminated) sites (e.g., fish (Southworth et al., 2004, G.W. Murphy, 2004, Master's thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA), birds (Cristol et al., 2008), turtles (Bergeron et al., 2007), and bats (Wada et al., 2010)). This continuum of Hg contamination makes this site an ideal model ecosystem for my research because it allows inferences to be drawn at other Hg contaminated sites that fall within this broad range.

Literature cited

Alford, R.A., 2010. Declines and the global status of amphibians. Pages 13-45 in Sparling DW, Linder G, Bishop CA, Krest SK, eds. Ecotoxicology of amphibians and reptiles, 2nd edition. Boca Raton, FL: CRC Press.

Bank, M.S., Loftin, C.S., Jung, R.E., 2005. Mercury bioaccumulation in northern two-lined salamanders from streams in the northeastern United States. Ecotoxicology 14, 181-191.

Bank, M.S., Crocker, J., Connery, B., Amirbahman, A., 2007. Mercury bioaccumulation in green frog (Rana clamitans) and bullfrog (Rana catesbeiana) tadpoles from Acadia National Park, Maine, USA. Environmental Toxicology and Chemistry 26, 118-125.

Beard, K.H., Vogt, K.A., Kulmatiski, A., 2002. Top-down effects of a terrestrial frog on forest nutrient dynamics. Oecologia 133, 583-593.

Beebee, T.J.C., Denton, J.S., Buckley, J., 1996. Factors affecting population densities of adult natterjack toads *Bufo calamita* in Britain. Journal of Applied Ecology 33, 263-268.

Benoit, J.M., Gilmour, C.C., Mason, R.P., Heyes, A., 1999. Sulfide controls on mercury speciation and bioavailability to methylating bacteria in sediment pore waters. Environmental Science & Technology 33, 1780-1780.

Benoit, J.M., Gilmour, C.C., Heyes, A., Mason, R.P., Miller, C.L., 2003. Geochemical and biological controls over methylmercury production and degradation in aquatic ecosystems. Pages 262-297 in Cai C, Braids, OC, ed. Biogeochemistry of Environmentally Important Trace Elements, vol. 835.

Bergeron, C.M., Husak, J.F., Unrine, J.M., Romanek, C.S., Hopkins, W.A., 2007. Influence of feeding ecology on blood mercury concentrations in four species of turtles. Environmental Toxicology and Chemistry 26, 1733-1741.

Berven, K.A., 1990. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology 71, 1599-1608.

Birge, W.J., Westerman, A.G., Spomberg, J.A., 2000. Comparative toxicology and risk assessment of amphibians. Pages 727-792 in Sparling DW, Linder G, Bishop, CA, ed. Ecotoxicology of Amphibians and Reptiles. Pensacola, FL: SETAC Press.

Bowles, K.C., Apte, S.C., Maher, W.A., Kawei, M., Smith, R., 2001. Bioaccumulation and biomagnification of mercury in Lake Murray, Papua New Guinea. Canadian Journal of Fisheries and Aquatic Sciences 58, 888-897.

Bradford, D.F., Kramer, J.L., Gerstenberger, S.L., Tallent-Halsell, N.G., Nash, M.S., 2011. Mercury in tadpoles collected from remote alpine sites in the southern Sierra Nevada

mountains, California, USA. Archives of Environmental Contamination and Toxicology DOI 10.1007/s00244-011-9674-y.

Carter, L.J., 1977. Chemical-plants leave unexpected legacy for two Virginia rivers. Science 198, 1015-1020.

Clarkson, T.W., 1990. Human Health Risks from Methylmercury in Fish. Environmental Toxicology and Chemistry 9, 957-961.

Corn, P.S., 2000. Amphibian declines: Review of some current hypotheses. Pages 663-696 in Sparling DW, Linder G, Bishop, CA, ed. Ecotoxicology of Amphibians and Reptiles. Pensacola, FL, USA: SETAC Press.

Cristol, D.A., Brasso, R.L., Condon, A.M., Fovargue, R.E., Friedman, S.L., Hallinger, K.K., Monroe, A.P., White, A.E., 2008. The movement of aquatic mercury through terrestrial food webs. Science 320, 335.

Crump, K.L., Trudeau, V.L., 2009. Mercury-induced reproductive impairment in fish. Environmental Toxicology and Chemistry 28, 895-907.

Di Giulio, R.T., Tillitt, D.E., 1999. Reproductive and developmental effects of contaminants in oviparous vertebrates. Pensacola, FL: SETAC Press.

Eisenreich, K.M., Kelly, S.M., Rowe, C.L., 2009. Latent mortality of juvenile snapping turtles from the upper Hudson River, New York, exposed maternally and via the diet to polychlorinated biphenyls (PCBs). Environmental Science & Technology 43, 6052-6057.

Eisler, R., 2006. Mercury Hazards to Living Organisms. Boca Raton, FL: CRC Press.

Facemire, C., et al., 1995. Impacts of Mercury Contamination in the Southeastern United-States. Water Air and Soil Pollution 80, 923-926.

Fitzgerald, W.F., Clarkson, T.W., 1991. Mercury and Monomethylmercury - Present and Future Concerns. Environmental Health Perspectives 96, 159-166.

Fitzgerald, W.F., Engstrom, D.R., Mason, R.P., Nater, E.A., 1998. The case for atmospheric mercury contamination in remote areas. Environmental Science & Technology 32, 1-7.

Gerstenberger, S., Pearson, R., 2002. Mercury concentrations in bullfrogs (Rana catesbeiana) collected from a southern Nevada, USA, wetland. Bulletin of Environmental Contamination and Toxicology 69, 210-218.

Grayson, K.L., Cook, L.W., Todd, M.J., Pierce, D., Hopkins, W.A., Gatten, R.E., Dorcas, M.E., 2005. Effects of prey type on specific dynamic action, growth, and mass conversion efficiencies in the horned frog, *Ceratophrys cranwelli*. Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology 141, 298-304.

Guillette, L.J., Crain, D.A., Rooney, A.A., Pickford, D.B., 1995. Organization versus activation - The role of endocrine-disrupting contaminants (EDCS) during embryonic development in wildlife. Environmental Health Perspectives 103, 157-164.

Hall, B.D., Bodaly, R.A., Fudge, R.J.P., Rudd, J.W.M., Rosenberg, D.M., 1997. Food as the dominant pathway of methylmercury uptake by fish. Water Air and Soil Pollution 100, 13-24.

Hecnar, S.J., McLoskey, R.T., 1996. Regional dynamics and the status of amphibians. Ecology 77, 2091-2097.

Hill, W.R., Stewart, A.J., Napolitano, G.E., 1996. Mercury speciation and bioaccumulation in lotic primary producers and primary consumers. Canadian Journal of Fisheries and Aquatic Sciences 53, 812-819.

Hoffmann, M., et al., 2010. The Impact of Conservation on the Status of the World's Vertebrates. Science 330, 1503-1509.

Hopkins, W.A., 2006. Use of tissue residues in reptile ecotoxicology: A call for integration and experimentalism. Pages 29-51 in Gardner S, Oberdorster, E., ed. New Perspectives: Toxicology and the environment, Volume 3: Reptile Toxicology. London: Taylor and Francis Publishers.

Hopkins, W.A., 2007. Amphibians as models for studying environmental change. Ilar Journal 48, 270-277.

Hopkins, W.A., Congdon, J., Ray, J.K., 2000. Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. Environmental Toxicology and Chemistry 19, 862-868.

Hopkins, W.A., DuRant, S.E., Staub, B.P., Rowe, C.L., Jackson, B.P., 2006. Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. Environmental Health Perspectives 114, 661-666.

Hothem, R.L., Jennings, M.R., Crayon, J.J., 2010. Mercury contamination in three species of anuran amphibians from the Cache Creek Watershed, California, USA. Environmental Monitoring and Assessment 163, 433-448.

IUCN. 2011. IUCN Red List of Threatened Species. Version 2011.1. http://www.iucnredlist.org. Downloaded on 16 June 2011. Report no.

Jaeger, R.G., 1980. Microhabitats of a terrestrial forest salamander. Copeia, 265-268.

James, S.M., Little, E.E., Semlitsch, R.D., 2005. Metamorphosis of two amphibian species after chronic cadmium exposure in outdoor aquatic mesocosms. Environmental Toxicology and Chemistry 24, 1994-2001.

Kadokami, K., Takeishi, M., Kuramoto, M., Ono, Y., 2004. Maternal transfer of organochlorine pesticides, polychlorinated dibenzo-p-dioxins, dibenzofurans, and coplanar polychlorinated biphenyls in frogs to their eggs. Chemosphere 57, 383-389.

Kanamadi, R.D., Saidapur, S.K., 1991. Effect of Sublethal Concentration of Mercuric-Chloride on the Ovary of the Frog Rana-Cyanophlyctis. Journal of Herpetology 25, 494-497.

Kotyzova, D., Sundeman, F.W., 1998. Maternal exposure to Cd(II) causes malformations of *Xenopus laevis* embryos. Annals of Clinical and Laboratory Science 28, 224-235.

Lannoo, M., 2005. Amphibian declines: The conservation status of United States species. Berkeley, CA: University of California Press.

Lefcort, H., Meguire, R.A., Wilson, L.H., Ettinger, W.F., 1998. Heavy metals alter the survival, growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (Rana luteiventris) tadpoles. Archives of Environmental Contamination and Toxicology 35, 447-456.

Linder, G., Grillitsch, B., 2000. Ecotoxicology of metals. Pages 325-459 in Sparling DW, Linder G, Bishop, CA, ed. Ecotoxicology of Amphibians and Reptiles. Pensacola, FL: SETAC Press.

Lindqvist, O., Johansson, K., Aastrup, M., Andersson, A., Bringmark, L., Hovsenius, G., Hakanson, L., Iverfeldt, A., Meili, M., Timm, B., 1991. Mercury in the Swedish environment - Recent research on causes, consequences and corrective methods. Water Air and Soil Pollution 55, R11-&.

Mason, R.P., Fitzgerald, W.F., Morel, F.M.M., 1994. The biogeochemical cycling of elemental mercury - Anthropogenic influences. Geochimica et Cosmochimica Acta 58, 3191-3198.

Mason, R.P., Reinfelder, J.R., Morel, F.M.M., 1996. Uptake, toxicity, and trophic transfer of mercury in a coastal diatom. Environmental Science & Technology 30, 1835-1845.

Nye, J.A., Davis, D.D., Miller, T.J., 2007. The effect of maternal exposure to contaminated sediment on the growth and condition of larval Fundulus heteroclitus. Aquatic Toxicology 82, 242-250.

Pough, F.H., 1980. Advantages of Ectothermy for Tetrapods. American Naturalist 115, 92-112.

Punzo, F., 1993. Effect of Mercuric-Chloride on Fertilization and Larval Development in the River Frog, Rana-Heckscheri (Wright) (Anura, Ranidae). Bulletin of Environmental Contamination and Toxicology 51, 575-581.

Raimondo, S.M., Rowe, C.L., Congdon, J.D., 1998. Exposure to coal ash impacts swimming performance and predator avoidance in larval bullfrogs (Rana catesbeiana). Journal of Herpetology 32, 289-292.

Roe, J.H., Hopkins, W.A., Jackson, B.P., 2005. Species- and stage-specific differences in trace element tissue concentrations in amphibians: implications for the disposal of coal-combustion wastes. Environmental Pollution 136, 353-363.

Roe, J.H., Hopkins, W.A., DuRant, S.E., Unrine, J.A., 2006. Effects of competition and coal-combustion wastes on recruitment and life history characteristics of salamanders in temporary wetlands. Aquatic Toxicology 79, 176-184.

Rowe, C.L., Hopkins, W.A., Coffman, V.R., 2001. Failed recruitment of southern toads (Bufo terrestris) in a trace element-contaminated breeding habitat: Direct and indirect effects that may lead to a local population sink. Archives of Environmental Contamination and Toxicology 40, 399-405.

Rowe, C.L., Kinney, O.M., Fiori, A.P., Congdon, J.D., 1996. Oral deformities in tadpoles (*Rana catesbeiana*) associated with coal ash deposition: Effects on grazing ability and growth. Freshwater Biology 36, 723-730.

Rowe, C.L., Kinney, O.M., Nagle, R.D., Congdon, J.D., 1998. Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. Physiological Zoology 71, 27-35.

Scheuhammer, A.M., 1991. Effects of acidification on the availability of toxic metals and calcium to wild birds and mammals. Environmental Pollution 71, 329-375.

Scheuhammer, A.M., Meyer, M.W., Sandheinrich, M.B., Murray, M.W., 2007. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. Ambio 36, 12-18.

Semlitsch, R.D., Scott, D.E., Pechmann, J.H.K., 1988. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. Ecology 69, 184-192.

Smith, D.C., 1987. Adult recruitment in chorus frogs - Effects of size and date at metamorphosis. Ecology 68, 344-350.

Snodgrass, J.W., Hopkins, W.A., Roe, J.H., 2003. Relationships among developmental stage, metamorphic timing, and concentrations of elements in bullfrogs (Rana catesbeiana). Environmental Toxicology and Chemistry 22, 1597-1604.

Snodgrass, J.W., Hopkins, W.A., Jackson, B.P., Baionno, J.A., Broughton, J., 2005. Influence of larval period on responses of overwintering green frog (Rana clamitans) larvae exposed to contaminated sediments. Environmental Toxicology and Chemistry 24, 1508-1514.

Snodgrass, J.W., Hopkins, W.A., Broughton, J., Gwinn, D., Baionno, J.A., Burger, J., 2004. Species-specific responses of developing anurans to coal combustion wastes. Aquatic Toxicology 66, 171-182.

Southworth, G.R., Peterson, M.J., Bogle, M.A., 2004. Bioaccumulation factors for mercury in stream fish. Environmental Practice 6, 135-143.

Sparling, D.W., Lowe, T.P., 1996. Environmental hazards of aluminum to plants, invertebrates, fish, and wildlife. Pages 1-127. Reviews of Environmental Contamination and Toxicology, Vol 145, vol. 145.

Stuart, S.N., Chanson, J.S., Cox, N.A., Young, B.E., Rodrigues, A.S.L., Fischman, D.L., Waller, R.W., 2004. Status and trends of amphibian declines and extinctions worldwide. Science 306, 1783-1786.

Tan, S.W., Meiller, J.C., Mahaffey, K.R., 2009. The endocrine effects of mercury in humans and wildlife. Critical Reviews in Toxicology 39, 228-269.

Ugarte, C.A., Rice, K.G., Donnelly, M.A., 2005. Variation of total mercury concentrations in pig frogs (*Rana grylio*) across the Florida Everglades, USA. Science of the Total Environment 345, 51-59.

Unrine, J.M., Jagoe, C.H., 2004. Dietary mercury exposure and bioaccumulation in southern leopard frog (*Rana sphenocephala*) larvae. Environmental Toxicology and Chemistry 23, 2956-2963.

Unrine, J.M., Jagoe, C.H., Hopkins, W.A., Brant, H.A., 2004. Adverse effects of ecologically relevant dietary mercury exposure in southern leopard frog (*Rana sphenocephala*) larvae. Environmental Toxicology and Chemistry 23, 2964-2970.

Unrine, J.M., Hopkins, W.A., Romanek, C.S., Jackson, B.P., 2007. Bioaccumulation of trace elements in omnivorous amphibian larvae: Implications for amphibian health and contaminant transport. Environmental Pollution 149, 182-192.

Unrine, J.M., Jagoe, C.H., Brinton, A.C., Brant, H.A., Garvin, N.T., 2005. Dietary mercury exposure and bioaccumulation in amphibian larvae inhabiting Carolina bay wetlands. Environmental Pollution 135, 245-253.

USEPA. 2009a. The national study of chemical residues in 1032 lake fish tissue. Washington, D.C., USA: U.S. Environmental Protection Agency, Office of Water. Report no.

USEPA. 2009b. 2008 Biennial National Listing of Fish Advisories: Technical Fact Sheet, EPA-823-F-09-007. Washington D.C., USA: U.S. Environmental Protection Agency. Report no. EPA-823-F-09-007.

Wada, H., Yates, D.E., Evers, D.C., Taylor, R.J., Hopkins, W.A., 2010. Tissue mercury concentrations and adrenocortical responses of female big brown bats (Eptesicus fuscus) near a contaminated river. Ecotoxicology 19, 1277-1284.

Watras, C.J., Bloom, N.S., 1992. Mercury and methylmercury in individual zooplankton - Implications for bioaccumulation. Limnology and Oceanography 37, 1313-1318.

Weiner, J.G., Spry, D.J., 1996. Toxicological significance of mercury in freshwater fish. Pages 297-340 in Beyer WN, Heinz GH, Redmon-Norwood AW, eds. Environmental contaminants in wildlife: Interpreting tissue concentrations. Boca Raton, FL: Lewis Publishers.

Weiner, J.G., Krabbenhoft, D.P., Heinz, G.H., Scheuhammer, A.M., 2003. Ecotoxicology of Mercury. Pages 409-463 in Hoffman DJ, Rattner BA, Burton TM, Cairns J, eds. Handbook of Ecotoxicology (2nd ed). Boca Raton, FL: CRC Press.

Weir, S.M., Halbrook, R.S., Sparling, D.W., 2010. Mercury concentrations in wetlands associated with coal-fired power plants. Ecotoxicology 19, 306-316.

Weis, J.S., 2009. Reproductive, developmental, and neurobehavioral effects of methylmercury in fishes. Journal of Environmental Science and Health Part C-Environmental Carcinogenesis & Ecotoxicology Reviews 27, 212-225.

Wolfe, M.F., Schwarzbach, S., Sulaiman, R.A., 1998. Effects of mercury on wildlife: A comprehensive review. Environmental Toxicology and Chemistry 17, 146-160.

Wu, J.P., Luo, X.J., Zhang, Y., Chen, S.J., Mai, B.X., Guan, Y.T., Yang, Z.Y., 2009. Residues of Polybrominated Diphenyl Ethers in Frogs (*Rana limnocharis*) from a Contaminated Site, South China: Tissue Distribution, Biomagnification, and Maternal Transfer. Environmental Science & Technology 43, 5212-5217.

Wyman, R.L., 1998. Experimental assessment of salamanders as predators of detrital food webs: effects on invertebrates, decomposition and the carbon cycle. Biodiversity and Conservation 7, 641-650.

Chapter 2: Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians

Christine M. Bergeron
Co-Authors: Catherine M. Bodinof, Jason M. Unrine, and William A. Hopkins

Formatted for and used with permission by:
Environmental Toxicology & Chemistry, Vol 29, No. 4, pp 980-988.

Abstract

Mercury (Hg) is an important environmental contaminant due to its global distribution, tendency to bioaccumulate, and toxicity in wildlife. However, Hg has received little attention in amphibians compared to other vertebrates. Amphibians vary widely in life history strategies and feeding ecologies which could influence Hg exposure and accumulation. To determine whether species and life stage affects Hg bioaccumulation, we collected adults from three species (*Plethodon cinereus, Eurycea bislineata,* and *Bufo americanus*) and larvae from the latter two species along a contamination gradient on the South River (VA, USA). Total Hg (THg) concentrations in the contaminated site were 3.5 to 22 times higher than in the reference site. We found differences in THg concentrations in amphibians that were consistent with their habitat requirements and feeding preferences. In general, adults (3453 ± 196 ng/g, dry mass) and larvae (2479 ± 171 ng/g) of the most river-associated species, *E. bislineata,* had the highest THg concentrations, followed by *B. americanus* tadpoles (2132 ± 602 ng/g), while adults of the more terrestrial *B. americanus* (598 ± 117 ng/g) and *P. cinereus* (583 ± 178 ng/g) had the lowest concentrations. In addition, nondestructive sampling techniques, which are non-existent for small amphibians, were developed. For the salamander species, THg concentrations in tail tissue were strongly correlated ($r \geq 0.97$) with the remaining carcass. A strong positive correlation (r =

0.92) also existed between blood and whole body THg concentrations in *B. americanus*. Our results suggest that amphibians and their terrestrial predators may be at risk of Hg exposure in this system and that nondestructive methods may be a viable sampling alternative that reduces impacts to local populations.

Keywords: Mercury, Bioaccumulation, Amphibian, Nondestructive tissues

Introduction

Mercury (Hg) is an important environmental contaminant due to its global ubiquity, tendency to bioaccumulate, and toxicity in wildlife. Atmospheric deposition results in widespread distribution of Hg due to long range airborne transport [1]. Mercury tends to accumulate in wetlands where it readily bioaccumulates and can biomagnify in aquatic food webs, especially as methylmercury (MMHg) [2,3]. Mercury accumulation in wetlands threatens critical breeding and foraging habitats for many fish and wildlife species. The primary route of Hg exposure is through the diet [4,5], and consumption of diets with environmentally realistic concentrations of Hg result in a large range of toxic effects in fish and wildlife, including behavioral, developmental, neurological, hormonal, and reproductive changes [6-9].

Mercury accumulation in amphibians has received little attention compared to fish, birds, and mammals [6,9,10]. Amphibians play a critical role in transferring energy and nutrients through food webs and therefore are important components of aquatic and terrestrial communities [11,12]. As ectotherms, amphibians are efficient at converting ingested energy into biomass [13,14] and as a result, can often occur in extremely high densities. For example, in Virginia, certain species of amphibians represent the single most abundant vertebrate in many

aquatic and terrestrial communities, exceeding 40,000 individuals per hectare [15]. Due to their abundance and high conversion efficiencies, amphibians often serve as vital links for energy and nutrient flow between low and high trophic levels. These characteristics also suggest that amphibians may have high rates of contaminant bioaccumulation compared with other animals of similar trophic positions. In addition, the complex lifecycles of many amphibians potentially make them important in transferring contaminants from aquatic to terrestrial food chains [16,17], especially for Hg since evidence suggests that most Hg accumulated by larvae is retained through metamorphosis [4,18].

Although Hg is ubiquitous due to long range transport from atmospheric deposition [1], point source emissions can result in high localized Hg concentrations. For example, the South River, VA, USA was historically (1929-1950) contaminated with mercuric sulfate used by a plant manufacturing acetate fiber in Waynesboro, VA, USA [19]. The use of Hg was terminated in the 1950s, but Hg concentrations currently remain high in the river [20]. This system contains a wide gradient of contamination between locations upstream and downstream from the point source. Previous studies reveal orders of magnitude differences in tissue Hg concentrations from several species between the upstream (uncontaminated reference) and downstream (contaminated) sites (e.g., fish [20, G.W. Murphy, 2004, Master's thesis, Virginia Polytechnic Institute and State University, Blacksburg, VA, USA], birds [21], turtles [22]).

The first objective of the present study was to determine whether Hg accumulation and risk of Hg exposure differs among amphibian species and stages. To accomplish this objective we collected three species of adult amphibians, *Eurycea bislineata cirrigera*, *Plethodon cinereus*, and *Bufo americanus,* which exhibit different life histories and occupy different ecological niches within the South River system. For *E. bislineata cirrigera* and *B. americanus*, we also

collected larvae since accumulation of contaminants has been shown to differ among life stages [16]. The second objective was to determine whether spatial patterns of Hg accumulation in these amphibian species were similar to other taxa along the South River. The final objective was to develop nondestructive methods for assessing Hg bioaccumulation in amphibians which will be critical for future studies of amphibians on the South River, as well as in other localities where Hg contamination is a concern. To meet our objective, we determined whether Hg concentrations in tissues that could be sampled non-lethally were correlated with carcass Hg concentrations. Blood samples were collected from *B. americanus*, but the small body size of the salamander species precluded this technique. Many salamander species, including *E. bislineata cirrigera* and *P. cinereus*, exhibit tail autotomy as a natural defensive mechanism to prevent capture by predators [23] so we collected and analyzed body and tail tissue separately for these species.

Materials and Methods

Species natural history

Mercury bioaccumulation patterns observed in amphibians should reflect local conditions due to their movement patterns. In general, terrestrial stages of amphibians are not as mobile compared to many other vertebrates, and many exhibit high fidelity to breeding and over-wintering habitats [24]. From several studies, Wells [24] summarizes that routine movement during their active season averages less than 10m for terrestrial salamanders and less than 15m for anurans. In the present study, *B. americanus* is likely the most mobile species as females have been shown to disperse up to 1 km from their breeding pond [25].

The three amphibians studied in the South River are common, widespread species that exhibit ecological and life history traits encompassing most of the North American amphibian species. The two salamanders are both from the family Plethodontidae, *Eurycea bislineata cirrigera* (hereafter *E. bislineata*; Southern two-lined salamander) and *Plethodon cinereus* (red-backed salamander) and the anuran, *Bufo americanus* (American toad) is from the Bufonidae family. *Eurycea bislineata* inhabit streams from Virginia to the Gulf Coast. Adults can be found beneath rocks and logs along the margins of rocky streams, but also in the forest farther from shore. The larval stage is fully aquatic and lasts 1-3 years. They reside beneath cover objects until foraging at night. Both adults and larvae consume a variety of small invertebrates [23]. The second salamander species, *Plethodon cinereus*, inhabits the forest litter environment in deciduous, northern conifer, or mixed habitats from North Carolina to the Canadian Maritime Provinces and west to Minnesota. Many individuals remain underground, but when surface active, they remain under cover (i.e., logs, rocks, leaf litter) during the day and emerge at night to forage. *Plethodon cinereus* are top predators within detrital food webs and prey mainly on small invertebrates. *Plethodon cinereus* bypasses an aquatic larval stage and reproduces through direct development [23,26]. *Bufo americanus* are found throughout eastern North America to the edge of the coastal plain. The terrestrial habitat of adult *B. americanus* is diverse, but they require cover objects with moist microhabitats, abundant supply of insects and other small invertebrates as prey, and shallow bodies of water to breed (e.g., ephemeral wetlands, drainage ditches, shallow portions of streams with low flow). *Bufo americanus* tadpoles are omnivorous grazers and will consume algae, suspended organic matter, detritus, and decomposing animal material [26].

Animal collection

Amphibians were collected from multiple sites upstream and downstream of the Hg contamination source (river mile [RM] 0) along the South River (Fig. 1) in all available and accessible habitats for each species. Adult and larval *E. bislineata* were captured by turning rocks, logs and leaf litter along banks within 3m of the edge of the water and in shallow margins of the river while using a mesh dip net to collect individuals. *Eurycea bislineata* were sampled at two reference locations, a South River reference site, 5 river miles upstream from the Hg source (SR REF; RM -5), and Coyner Springs Park (Parks and Recreation, Waynesboro, VA; CS REF), an upstream tributary to the South River. The aquatic portion of the reference subsites are separated from contaminated subsites by Rife Loth dam in Waynesboro, VA, USA, upstream from the source of contamination. In the contaminated portion of the South River, eight subsites were sampled downstream from the Hg source (RM 2, 5, 9, 11, 13, 16, 20, and 22) and are collectively referred to as the contaminated site. An additional site was sampled approximately 10 miles after the South River joins the South Fork of the Shenandoah River (SFSR; RM 34) (Fig. 1, Table 1).

Adult *P. cinereus* were captured by turning rocks and logs in the riparian zone along the South River. The distribution of *P. cinereus* along the South River was patchy and thus they were only successfully collected at SR REF (RM -5) and three subsites within the contaminated reach (RM 1, 14, and 20) (Fig. 1, Table 1). Collection localities ranged from 5 to 30 m from the edge of the river.

Both species of salamanders were captured over a two week period in May 2007. Upon capture, salamanders were given a unique identification code and placed in a Ziploc bag with water and/or a wet paper towel. A Garmin (Olathe, KS USA) hand-held global positioning

system (GPS) unit was used to obtain geospatial coordinates for each individual captured. Salamanders were transported to the laboratory and held for 48 hrs to void their gut contents and then sacrificed using an overdose of buffered tricaine methane sulfonate (MS-222). Mass, total length (TL) and snout-vent length (SVL) were obtained. To standardize sampling for this study, the tail of adult salamanders was removed just posterior to the vent using a sterile blade. In future studies, where salamanders are to be released, tail autonomy could be induced by gently restraining the animal by the tail. Adult bodies and tails and whole body larvae were frozen separately until analysis.

Breeding pairs of *B. americanus* were captured over 4.5 weeks in March and April 2007 from breeding ponds located in or near the flood plain of the South River. Ponds were located within 30 to 180 m of the river. Toads were collected from the reference site encompassing two subsites on the South River (SR REF; RM -1.7 and -5) and five subsites in the contaminated reach (RM 2, 5, 9, 16, and 20) (Fig. 1, Table 1). Females were given a unique identification code and pairs were placed in individual breeding containers with water. Again, geospatial coordinates for each pair were acquired. The pairs were transported to the laboratory and their eggs were obtained for research on reproductive effects in females (results to be reported elsewhere). After oviposition, the male was released at the site of capture and the female was held for 48 hrs to void her gut contents. Mass and SVL were recorded. Before sacrificing the female with an overdose of MS-222, approximately 0.6 ml of blood was withdrawn by cardiac puncture (or decapitation after sacrifice) using a 1-ml heparinized syringe. Female toads and blood samples were frozen until analysis.

In May 2007, *B. americanus* tadpoles were collected from the same ponds where breeding pairs were captured. Approximately 20 tadpoles between Gosner stages 28 and 32

(before forelimb emergence) [27] were collected from each subsite. Tadpoles were held for 48 hrs to void their gut contents, sacrificed with an overdose of MS-222, weighed, and frozen until analysis. Before analysis, 4-7 tadpoles were pooled to create 3-4 composite samples per site (Table 1). When different developmental stages of tadpoles existed within a site, the stages were distributed among composites as equally as possible.

Total mercury analysis

Adult bodies, larval bodies, and salamander tails were lyophilized and homogenized separately and Hg concentrations are reported on a dry wt basis. Whole blood from adult *B. americanus* was homogenized using a vortex mixer and Hg concentrations are reported on a wet wt basis. Whole body percent moisture, calculated from weights before and after lyophilization, was similar for all species (adult *E. bislineata* 73.9 ± 0.5%; larval *E. bislineata* 76.0 ± 0.3%; *P. cinereus* 75.9 ± 0.4%; *B. americanus* 77.8 ± 0.4%). Subsamples (20-150 mg) were analyzed for total Hg (THg) content by combustion-amalgamation-cold vapor atomic absorption spectrophotometry (DMA 80, Milestone, Monroe, CT USA) according to U.S. Environmental Protection Agency (U.S. EPA) method 7473 [28]. For quality assurance, each group of 10 to 15 samples included a replicate, blank, and standard reference material (SRM; TORT-2 lobster hepatopancreas, DOLT-2 dogfish liver [National Research Council of Canada, Ottawa, ON] or SRM 966 Toxic Metals in Bovine Blood Level 2 [National Institute of Standards and Technology, Gathersburg, MD USA]). The instrument was calibrated using solid SRMs (TORT-2 and DORM-2, dogfish muscle [National Research Council of Canada, Ottawa, ON]). Method detection limits (MDLs; 3 times standard deviation of procedural blanks) for samples depended on sample mass and were calculated separately for each observation based on sample mass.

Method detection limits ranged from 5.80 ng/g to 48.04 ng/g, and all samples had THg concentrations that exceeded the limit. Average relative percent difference (RPD) between replicate sample analyses was 3.59 ± 0.51% (n=46; mean ± 1 standard error of the mean hereafter). Mean percent recoveries of THg for the standard reference materials TORT-2, DOLT-2, and SRM 966 were 89.66 ± 0.01 % (n =46), 100.99 ± 0.01 % (n =8), and 92.11 ± 0.04 % (n =8), respectively.

Statistical analyses

Subsites within the reference and contaminated reaches of the river could not be treated independently of one another in our spatial comparisons and are collectively referred to as the reference site and contaminated site, respectively. Since sampling SFSR occurred 10 miles after the confluence (RM 34, Fig. 1), it was also treated as a separate site. In addition, species could not be compared statistically because each species occurred at different subsites (Table 1) and Hg accumulation patterns may have depended upon which subsites were sampled.

To compare THg concentrations among reference and contaminated sites and stages within each species, the assumptions of analysis of variance (ANOVA) were first tested. Data were $\log_{10}$-transformed to meet ANOVA assumptions or analogous nonparametric tests were employed. For *E. bislineata* a two-way ANOVA was performed to compare sites and stages followed by Tukey's pairwise comparisons. A one-way ANOVA was performed for *P. cinereus* to compare sites. Since transformations failed to meet the assumptions of ANOVA for *B. americanus* data, we performed a Scheirer-Ray-Hare extension of the Kruskal-Wallis test [29] to compare sites and stages. Pearson correlation coefficients were used to assess relationships between salamander bodies and tails as well as between *B. americanus* whole bodies and blood.

All analyses were performed with SAS 9.1 (SAS Institute, Cary, NC, USA), and an α value of 0.05 was used to assess statistical significance.

Results

Effects of site and stage on mercury accumulation

A large range in THg concentrations was observed among species and stages (Figs. 2-4), with greater concentrations for all species and stages within the South River downstream from the source of contamination (Figs. 2A, 3A, and 4A). Although subsites within the reference and contaminated reaches of the South River could not be treated independently of one another in our statistical comparisons, Figures 2B, 3B, and 4B illustrate the spatial trends downstream from the contamination source. In the contaminated site, *E. bislineata* adults and larvae had the highest THg concentrations, followed by *B. americanus* tadpoles, while terrestrial adult *B. americanus* and *P. cinereus* had the lowest concentrations.

For *E. bislineata*, THg concentrations were influenced both by site ($F_{2,96} = 23.42$, $p < 0.001$) and stage ($F_{1,96} = 410.89$, $p < 0.001$). No interaction was detected between site and stage ($p = 0.270$), indicating similar patterns between stages at different sites. Indeed, THg concentrations in adult *E. bislineata* were 1.4 to 2.1-fold higher than larval concentrations at each site. Pairwise comparisons revealed significant differences in THg concentrations ($p < 0.001$) between all three sites (Fig. 2A). Total Hg concentrations in adults and larvae at the contaminated site were 15 and 22-fold higher than the reference site and 2.9 and 4.3-fold higher than the SFSR site, respectively. Despite being 34 miles downstream from the original Hg source, total Hg concentrations in adult and larval *E. bislineata* were 5.2 and 5-fold higher in the SFSR than the reference site, respectively.

Total Hg concentrations were 5.4-fold higher in *P. cinereus* from the contaminated site compared to the reference site ($F_{1,22} = 11.83$, $p = 0.0023$) (Fig. 3A). Similarly, THg concentrations were 3.5 and 4.0-fold higher in adult and larval *B. americanus* in the contaminated site than the reference site, respectively ($p < 0.005$). Stage also differed ($p < 0.005$) between *B. americanus* adults and larvae (Fig. 4A), but the influence of stage was opposite of that observed in *E. bislineata*. Total Hg concentrations in larval toads were 3.2 and 3.6-fold higher than adults at the reference and contaminated sites, respectively. No interaction was detected between site and stage ($p > 0.975$) for *B. americanus*.

Relationships between bodies and non-lethal samples

There was a strong positive relationship between THg concentrations in salamander bodies and tails for both *E. bislineata* ($r = 0.994$, $p < 0.0001$, $y = 0.9848x - 0.1191$) (Fig. 5a) and *P. cinereus* ($r = 0.973$, $p < 0.0001$, $y = 0.7825x + 0.3413$) (Fig. 5b). The slope of the regression for salamander bodies and tails was significantly steeper for *E. bislineata* than *P. cinereus* (ANCOVA, $p < 0.001$). A strong positive relationship was also found for THg concentrations between adult bodies and blood in *B. americanus* ($r = 0.916$, $p < 0.0001$, $y = 0.9771x - 0.0422$) (Fig. 6). The slopes of all relationships did not differ significantly from 1.0 (t test, $p > 0.1$ for all).

Discussion

Amphibians inhabiting the contaminated portion of the South River had elevated Hg concentrations in their tissues compared to conspecifics from the reference site. Total Hg concentrations were low in the reference site for all species, and THg concentrations for both

stages of *E. bislineata* in the reference site were comparable to concentrations of the closely related *E. bislineata bislineata* found in Shenandoah and Acadia National Parks [30]. In contrast, peak whole body THg concentrations in amphibians from the contaminated site were the highest reported for amphibians in the literature [6,10]. Adult THg concentrations ranged up to 5,785 ng/g in *E. bislineata*, 3,386 ng/g in *P. cinereus*, and 2,350 ng/g in *B. americanus,* and approximately 50% (mean range 46-60%) of this was MMHg [31]. Unfortunately, little research has focused on the effects of Hg in amphibians, especially through dietary exposure. However, Unrine et al. [32] found adverse effects on development and decreased metamorphic success in *Rana sphenocephala* at much lower THg tissue concentrations (~240 ng/g) compared to the THg concentrations found in amphibians from the South River. On the other hand, several studies have focused on Hg concentrations known to affect reproductive success in fish. Beckvar et al. [33] used the data from ten studies to create a whole-body tissue threshold-effect level of 1,000 ng MMHg /g, dry weight (assuming 80% moisture) for juvenile and adult fish based on sublethal endpoints (growth, reproduction, development, and behavior). In our study, 91% of *E. bislineata* adults, 11% of *B. americanus* adults, and < 1% of *P. cinereus* adults from the contaminated site reached MMHg concentrations above the threshold-effect level for fish [33].

Amphibians are important prey for a variety of organisms along the South River. Many organisms such as raccoons, snakes, screech owls, wading birds, turtles, and predatory fish (e.g., bass and sunfish) are known to eat various lifestages of these amphibian species [23]. Because of their ability to occur in high densities and their high conversion efficiencies, amphibians may serve as critical trophic links for energy, nutrient flow, and Hg to predators. The most well studied mammalian species in terms of Hg are the mink and otter which both include amphibians as an important component of their diet [34,35]. Several studies on these species indicate that

neurotoxicity and death can occur with the consumption of diets containing Hg (as MMHg) ≥ 1,000 ng/g wet wt [36]. In addition, the U.S. EPA estimates that the lowest observable adverse effect level (LOAEL) for mink is 180 ng /g body wt / day or 1100 ng/g wet wt MMHg in the diet [37]. Several studies have observed reduced reproductive success and juvenile survival in birds with environmentally realistic concentration of Hg in their diet. The piscivorous common loon (*Gavia immer*), which eats amphibians and fish that eat amphibians [38], had reduced reproductive success with diets that contained Hg (as MMHg) > 300 ng/g wet wt [39]. More recently, a study by Burgess and Meyer [40] suggests loon productivity decreases by 50% when fish THg concentrations in the diet were 210 ng/g wet wt and failed when THg concentrations were 410 ng/g wet wt. Bouton et al. [41] fed captive-raised great egret chicks (*Ardea albus*), another piscivorous species, diets of 500 ng/g wet wt MMHg and found the chicks displayed decreased appetite and strength, altered activity and maintenance behavior, and reduced motivation to hunt live prey. The authors suggest that these effects may result in reduced juvenile survival in wild birds. In addition, diets containing MMHg concentrations of 3,000 ng/g dry wt in mallard ducks (*Anas platyrhynchos*) [42] and black ducks (*Anas rubripes*) [43] impaired reproductive success by reducing egg production, hatching success, and embryo and duckling survival. Individual amphibians at the South River with the highest THg concentrations, mainly *E. bislineata*, approach the mink LOAEL and Hg concentrations known to affect reproduction in ducks. However, 80% of adult and 50% of larval *E. bislineata* surpassed the dietary threshold for reproductive success in loons. Thus, the Hg concentrations in the present study support the notion that amphibians, as important components of aquatic and terrestrial communities, potentially transfer Hg within and between these environments and pose significant health risks to their predators.

Species and stage differences

The magnitude of Hg accumulation in amphibians appeared dependent on habitat use and feeding behavior. Within the contaminated portion of the river, we observed that both stages of *E. bislineata* had the highest average THg concentrations in the present study (adults 3,453 ± 196 ng/g; larvae 2,479 ± 171 ng/g). *Eurycea bislineata* is the most river-associated species and remains carnivorous throughout its complex lifecycle. Like *E. bislineata, P. cinereus* is carnivorous throughout its life, but *P. cinereus* is a direct developer in the terrestrial environment. Both of the more terrestrial species, *P. cinereus* (583 ± 178 ng/g) and *B. americanus* (598 ± 117 ng/g), had the lowest average THg concentrations in the present study. Thus, our results suggest that individuals of these species encounter reduced amounts of bioavailable Hg because they may be more closely tied to terrestrial than aquatic food webs. Future studies using stable isotopes may allow us to disentangle these relationships and better understand ecological risk in relation to amphibian feeding ecology [22].

We found interesting ontogenetic changes in Hg accumulation in both species with complex lifecycles, but the opposite patterns were observed for *E. bislineata* and *B. americanus* even though the larval stages of both species had similar THg concentrations at the contaminated site (larvae: *E. bislineata* 2,479 ± 171 ng/g and *B. americanus* 2,132 ± 602 ng/g). For *E. bislineata*, adults had higher THg concentrations than larvae at all sites except RM 22. Since Hg accumulates over time and is not well eliminated from the body, older and larger organisms often have proportionally higher THg concentrations than younger and smaller conspecifics. For example, THg concentrations often correlate with age, body mass, or length in fish [6,8]. Adult *E. bislineata* were significantly larger than the larvae ($p = 0.004$) in both the contaminated (adults: 0.81 ± 0.03 g, larvae: 0.66 ± 0.04 g) and reference (adults: 0.80 ± 0.06 g, larvae: 0.63 ±

0.05 g) site. Because the older and larger adults had higher Hg concentrations than larvae, adults may have simply been exposed to Hg at similar rates but for a longer time. This is plausible because all of the adults we sampled were collected within 3m of the river, where aquatic prey can remain a significant portion of their diet [23]. Alternatively, adults could be ingesting larger prey or prey at higher trophic levels with higher Hg concentrations, or a larger proportion of the more bioavilable Hg form, MMHg. Again, stable isotopes could provide critical insights into whether accumulation differences between stages were attributable to ontogenetic shifts in feeding preferences or exposure duration (e.g., [22]).

The opposite ontogenetic Hg accumulation pattern from *E. bislineata* was observed in *B. americanus* where the aquatic tadpoles had higher THg concentrations than the terrestrial adults. Although tadpoles are omnivorous grazers, this observation is consistent with the literature that many pond-breeding larvae are excellent accumulators of trace metals because they are closely associated with the benthos [16,44]. While many metals, including some Hg, may be bound to the gut epithelium in tadpoles [17,18], evidence suggests that Hg becomes mobilized during metamorphic climax and is retained in amphibian tissues after metamorphosis is completed [4,18]. After metamorphosis, *B. americanus* juveniles and adults are highly terrestrial and mobile [25]. Thus, the reduced body concentrations of adults are likely caused by a dilution of the Hg body pool as they consume prey items from the terrestrial environment. Alternatively, because of their mobility, it is possible that some of the adults we sampled were immigrants from surrounding uncontaminated habitats that were only recently exposed to Hg.

Spatial differences

Mercury accumulation in amphibians followed the same spatial pattern as observed in birds, fish, and turtles along the South River, which generally increased for several miles downstream from the point source before peaking between miles 10 and 20 and decreasing or remaining high until the confluence with the South Fork of the Shenandoah River [20,22, G.W. Murphy, 2004, Master's thesis]. Although it is not currently known why Hg concentrations peak downstream from the source, it is theorized that historical deposition in the cobble bed within the river and/or flood plain, coupled with current erosion patterns, may provide a continual and dispersed source of Hg loading to the river. This pattern was the strongest for *E. bislineata* which is most closely associated with the river. The patchy availability of suitable habitat for *P. cinereus* due to deforestation along the South River precludes descriptions of spatial patterns for this species. A notable exception to the pattern was at RM 16, where *B. americanus* adults and larvae had THg concentrations comparable to the reference site. The breeding pond at RM 16 was 60 m from the South River, but was elevated above the 100 year flood plain. In comparison, the breeding pond at RM 20 had the highest THg concentrations, was 40 m from the river, and within the 2 year flood plain. These site differences suggest the risk of Hg exposure in pond-breeding amphibians is strongly influenced by Hg deposition in the South River floodplain from flood events. In addition, our observations suggest that larvae in these breeding ponds may be good bioindicators of localized Hg contamination and bioavailability because they are confined to natal wetlands and integrate Hg from the aquatic environment.

Nondestructive sampling

Our study illustrates the utility of two nondestructive sampling techniques for determining whether amphibians have a history of exposure to Hg. Total Hg concentrations between bodies and tails of both salamanders and between blood and whole body in *B. americanus* were highly correlated. Importantly, the large range in Hg contamination at the South River allows inferences to be drawn at other Hg-contaminated sites that fall within this broad range.

A variety of nondestructive sampling techniques have been developed for reptiles, but not for amphibians. For example, concentrations of metals and metalloids in skin, tails and/or blood are predictive of whole-body or target tissue concentrations in snakes and lizards [e.g., 45-47]. In contrast, amphibians are typically sacrificed for whole-body contaminant analysis because nondestructive techniques have not been developed. This practice is not conservation- minded nor is it convenient for continued sampling at sites over protracted timescales because of the impact it could have on local populations. Cardiac puncture is a recommended method for blood collection in anurans [48] and can be used repeatedly on the same individuals [49]. Although tail loss in salamander species can decrease fitness through several routes such as reduction in courtship success, locomotive performance, growth rate, or reproductive success (reviewed in [50]), the tail is regenerated over time. Thus, the impact of tail sampling on local populations is minimized compared to the lethal alternative. Improved analytical techniques may allow smaller quantities of tail tissue to be analyzed and decrease the impact it has on the individuals sampled.

While the correlations with nondestructive tissues provide a predictive index of Hg concentrations at the South River, they should be validated at other Hg contaminated sites for these three species and closely related species. For example, the slopes of the correlations

between body and tail differed between the two salamander species indicating one relationship is not necessarily predictive for all salamander species. In addition, blood is often correlated with contaminant concentrations in whole body or target organs and is commonly used as a nondestructive sampling technique for other vertebrates (e.g., [45,46,51]). However, blood THg concentrations may reflect recent dietary intake while muscle and other tissues represent a more consistent integration of Hg intake over time [52].

Conclusions

A common theory regarding Hg in wildlife is that piscivores and other top predators feeding in the aquatic food chain have the greatest risk of Hg exposure and toxicity [6,7]. Our study demonstrated that amphibians occupying both aquatic and terrestrial habitats in a contaminated site accumulated Hg in concentrations that exceed those of the reference site by up to an order of magnitude. In some cases, Hg concentrations in amphibians exceeded threshold concentrations for adverse effects in juvenile and adult fish, indicating amphibians may be at risk of Hg exposure and toxicity. In addition, Hg concentrations in amphibians exceeded dietary levels known to cause adverse effects in some mammals and birds, indicating that predators of amphibians may also be at risk. Because the South River has a large range in Hg concentrations, it affords the opportunity to quantify threshold concentrations for adverse effects in the field. The development of thresholds at this model system may assist in risk assessments and management decisions for other Hg-contaminated sites with concentrations within this broad range. In addition, the nondestructive sampling techniques we developed will be most useful if they can ultimately be used to predict health risks to individuals, such as adverse reproductive effects.

Acknowledgements

We thank the South River Science Team for their support and the landowners along the South River and the Waynesboro Parks and Recreation Department for access to sampling locations. D. Addis, H. Brant, S. Budischak, J. Callihan, S. DuRant, S. Orlofske, B. Todd and H. Wada provided field and laboratory assistance. Collection of animals was in conformance with appropriate permits, and sample methods were in compliance with Virginia Polytechnic and State University's animal care and use protocols. Financial support to WAH was provided by E. I. DuPont de Nemours, startup funds from Virginia Polytechnic and State University, and by the National Science Foundation (NSF # IOB-0615361). Additional support was provided to CMB in a Seed Grant from The World Conservation Union Species Survival Commission Amphibian Specialist Group.

Literature Cited

1. Fitzgerald WF, Engstrom DR, Mason RP, Nater EA. 1998. The case for atmospheric mercury contamination in remote areas. *Environ Sci Technol* 32: 1-7.
2. Watras CJ, Bloom NS. 1992. Mercury and methylmercury in individual zooplankton - Implications for bioaccumulation. *Limnol Oceanogr* 37: 1313-1318.
3. Hill WR, Stewart AJ, Napolitano GE. 1996. Mercury speciation and bioaccumulation in lotic primary producers and primary consumers. *Can J Fish Aquat Sci* 53: 812-819.
4. Unrine JM, Jagoe CH. 2004. Dietary mercury exposure and bioaccumulation in southern leopard frog (*Rana sphenocephala*) larvae. *Environ Toxicol Chem* 23: 2956-2963.
5. Hall BD, Bodaly RA, Fudge RJP, Rudd JWM, Rosenberg DM. 1997. Food as the dominant pathway of methylmercury uptake by fish. *Water, Air, Soil Pollut* 100: 13-24.

6. Eisler R. 2006. *Mercury Hazards to Living Organisms*. CRC Press, Boca Raton, FL.

7. Scheuhammer AM, Meyer MW, Sandheinrich MB, Murray MW. 2007. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. *Ambio* 36: 12-18.

8. Weiner JG, Spry DJ. 1996. Toxicological significance of mercury in freshwater fish. In Beyer WN, Heinz GH,Redmon-Norwood AW, eds, *Environmental contaminants in wildlife: Interpreting tissue concentrations.* Lewis Publishers, Boca Raton, FL, pp 297-340.

9. Wolfe MF, Schwarzbach S, Sulaiman RA. 1998. Effects of mercury on wildlife: A comprehensive review. *Environ Toxicol Chem* 17: 146-160.

10. Linder G, Grillitsch B. 2000. Ecotoxicology of metals. In Sparling DW, Linder G, Bishop, CA, eds, *Ecotoxicology of Amphibians and Reptiles.* SETAC Press, Pensacola, FL, pp 325-459.

11. Wyman RL. 1998. Experimental assessment of salamanders as predators of detrital food webs: effects on invertebrates, decomposition and the carbon cycle. *Biodiversity and Conservation* 7: 641-650.

12. Beard KH, Vogt KA, Kulmatiski A. 2002. Top-down effects of a terrestrial frog on forest nutrient dynamics. *Oecologia* 133: 583-593.

13. Burton TM, Likens GE. 1975. Energy flow and nutrient cycling in salamander populations in Hubbard Brook Experimental Forest, New Hampshire. *Ecology* 56: 1068-1080.

14. Grayson KL, Cook LW, Todd MJ, Pierce D, Hopkins WA, Gatten RE, Dorcas ME. 2005. Effects of prey type on specific dynamic action, growth, and mass conversion efficiencies

in the horned frog, *Ceratophrys cranwelli*. *Comparative Biochemistry and Physiology a-Molecular & Integrative Physiology* 141: 298-304.

15. Jaeger RG. 1980. Microhabitats of a terrestrial forest salamander. *Copeia*: 265-268.

16. Roe JH, Hopkins WA, Jackson BP. 2005. Species- and stage-specific differences in trace element tissue concentrations in amphibians: implications for the disposal of coal-combustion wastes. *Environ Pollut* 136: 353-363.

17. Snodgrass JW, Hopkins WA, Roe JH. 2003. Relationships among developmental stage, metamorphic timing, and concentrations of elements in bullfrogs (*Rana catesbeiana*). *Environ Toxicol Chem* 22: 1597-1604.

18. Unrine JM, Jagoe CH, Brinton AC, Brant HA, Garvin NT. 2005. Dietary mercury exposure and bioaccumulation in amphibian larvae inhabiting Carolina bay wetlands. *Environ Pollut* 135: 245-253.

19. Carter LJ. 1977. Chemical-plants leave unexpected legacy for two Virginia rivers. *Science* 198: 1015-1020.

20. Southworth GR, Peterson MJ, Bogle MA. 2004. Bioaccumulation factors for mercury in stream fish. *Environmental Practice* 6: 135-143.

21. Cristol DA, Brasso RL, Condon AM, Fovargue RE, Friedman SL, Hallinger KK, Monroe AP, White AE. 2008. The movement of aquatic mercury through terrestrial food webs. *Science* 320: 335.

22. Bergeron CM, Husak JF, Unrine JM, Romanek CS, Hopkins WA. 2007. Influence of feeding ecology on blood mercury concentrations in four species of turtles. *Environ Toxicol Chem* 26: 1733-1741.

23. Petranka JW. 1998. *Salamanders of the United States and Canada*. Smithsonian Institution Press, Washington, D.C.

24. Wells KD. 2007. *The Ecology and Behavior of Amphibians*. The University of Chicago Press, Chicago, Il.

25. Forester DC, Snodgrass JW, Marsalek K, Lanham Z. 2006. Post-breeding dispersal and summer home range of female American toads (*Bufo americanus*). *Northeastern Naturalist* 13: 59-72.

26. Lannoo M. 2005. *Amphibian declines: The conservation status of United States species*. University of California Press, Berkeley, CA.

27. Gosner KL. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. *Herpetologica* 16: 183-190.

28. U.S. Environmental Protection Agency. 1998. Method 7473: Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrometry. Washington, D.C., USA.

29. Sokal RR, Rohlf FJ. 1995. *Biometry: Principles and practice of statistics in biological research*. W.H. Freeman and Company, New York.

30. Bank MS, Loftin CS, Jung RE. 2005. Mercury bioaccumulation in northern two-lined salamanders from streams in the northeastern United States. *Ecotoxicology* 14: 181-191.

31. Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA. *In press*. Bioaccumulation and maternal transfer of mercury and selenium in amphibians. *Environ Toxicol Chem*.

32. Unrine JM, Jagoe CH, Hopkins WA, Brant HA. 2004. Adverse effects of ecologically relevant dietary mercury exposure in southern leopard frog (*Rana sphenocephala*) larvae. *Environ Toxicol Chem* 23: 2964-2970.

33. Beckvar N, Dillon TM, Read LB. 2005. Approaches for linking whole-body fish tissue resides of mercury or DDT to biological effects thresholds *Environ Toxicol Chem* 24: 2094-2105.

34. Clavero M, Prenda J, Delibes M. 2005. Amphibian and reptile consumption by otters (*Lutra lutra*) in a coastal area in southern Iberian Peninsula. *Herpetological Journal* 15: 125-131.

35. Lake JL, Ryba SA, Serbest J, Brown CF, Gibson L. 2007. Mercury and stable isotopes of carbon and nitrogen in mink. *Environ Toxicol Chem* 26: 2611-2619.

36. Weiner JG, Krabbenhoft DP, Heinz GH, Scheuhammer AM. 2003. Ecotoxicology of Mercury. In Hoffman DJ, Rattner BA, Burton TM,Cairns J, eds, *Handbook of Ecotoxicology (2nd ed).* CRC Press, Boca Raton, FL, pp 409-463.

37. U.S. Environmental Protection Agency. 1997. Mercury Study Report to Congress. EPA-452/R-97-004. US EPA Office of Air, Washington, D.C.

38. Terres JK. 1980. *The Audubon Society encyclopedia of North American birds.* Alfred A. Knopf, New York.

39. Barr JF. 1986. Population dynamics of the common loon (*Gavia immer*) associated with mercury-contaminated waters in Northwestern Ontario. *Canadian Wildlife Service Occasional Paper No 56*: 25.

40. Burgess NM, Meyer MW. 2008. Methylmercury exposure associated with reduced productivity in common loons. *Ecotoxicology* 17: 83-91.

41. Bouton SN, Frederick PC, Spalding MG, McGill H. 1999. Effects of chronic, low concentrations of dietary methylmercury on the behavior of juvenile great egrets. *Environ Toxicol Chem* 18: 1934–1939.

42. Heinz GH, Locke LN. 1976. Brain lesions in mallard ducklings from parents fed methylmercury. *Avian Diseases* 20: 9-17.

43. Finley MT, Stendell RC. 1978. Survival and reproductive success of black ducks fed methylmercury. *Environ Pollut* 16: 51-64.

44. Unrine JM, Hopkins WA, Romanek CS, Jackson BP. 2007. Bioaccumulation of trace elements in omnivorous amphibian larvae: Implications for amphibian health and contaminant transport. *Environ Pollut* 149: 182-192.

45. Hopkins WA, Roe JH, Snodgrass JW, Jackson BP, Kling DE, Rowe CL, Congdon JD. 2001. Nondestructive indices of trace element exposure in squamate reptiles. *Environ Pollut* 115: 1-7.

46. Burger J, Campbell KR, Campbell TS, Shukla T, Jeitner C, Gochfeld M. 2005. Use of skin and blood as nonlethal indicators of heavy metal contamination in northern water snakes (*Nerodia sipedon*). *Arch Environ Contam Toxicol* 49: 232-238.

47. Fletcher DE, Hopkins WA, Saldana T, Baionno JA, Arribas C, Standora MM, Fernandez-Delgado C. 2006. Geckos as indicators of mining pollution. *Environ Toxicol Chem* 25: 2432-2445.

48. Wright KM, Whitaker BR. 2001. *Amphibian medicine and captive husbandry*. Krieger Publishing Company, Malabar, FL.

49. Hopkins WA, Mendonca MT, Congdon JD. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. *General and Comparative Endocrinology* 108: 237-246.

50. Bernardo J, Agosta SJ. 2005. Evolutionary implications of hierarchical impacts of nonlethal injury on reproduction, including maternal effects. *Biological Journal of the Linnean Society* 86: 309-331.

51. Golet WJ, Haines TA. 2001. Snapping turtles (*Chelydra serpentina*) as monitors for mercury contamination of aquatic environments. *Environ Monit Assess* 71: 211-220.

52. Day RD, Christopher SJ, Becker PR, Whitaker DW. 2005. Monitoring mercury in the loggerhead sea turtle, *Caretta caretta*. *Environ Sci Technol* 39: 437-446.

Table 2.1: Individual sample sizes for total mercury (THg) analyses in the three amphibian species, *Eurycea bislineata* (adult and larvae), *Plethodon cinereus* (adult), and *Bufo americanus* (adult and larvae), at the reference and contaminated subsites of the South River (SR) and the South Fork of the Shenandoah River (SFSR) VA, USA. RM = river mile from contamination source.

Site	*E. bislineata* adult	*E. bislineata* larvae	*P. cinereus* adult	*B. americanus* adult	*B. americanus* larvae*
Reference Subsites					
SR REF	5	5	6	13	7
CS REF	5	5	-	-	-
Reference (totals)	10	10	6	13	7
Contaminated subsites					
SR RM 1	-	-	6	-	-
SR RM 2	5	5	-	1	4
SR RM 5	5	5	-	12	4
SR RM 9	5	5	-	12	4
SR RM 11	1	1	-	-	-
SR RM 13	5	5	-	-	-
SR RM 14	-	-	6	-	-
SR RM 16	5	5	-	4	4
SR RM 20	5	5	6	6	3
SR RM 22	5	5	-	-	-
South River (totals)	36	36	18	35	19
South Fork Shenandoah River					
SFSR RM 34	5	4	-	-	-
Species Total	51	50	24	48	26

* composite samples of 4-7 tadpoles each

Figure 2.1: Sampling locations of the three amphibian species studied (Ba = *Bufo americanus*; Eb = *Eurycea bislineata*; Pc = *Plethodon cinereus*) along the South River (SR), Coyner Springs Park (CS), and South Fork of the Shenandoah River (SFSR) of the Shenandoah Valley (VA, USA). Numbers refer to river miles downstream from contamination source (river mile 0). Open symbols represent reference sites and closed symbols represent contaminated sites. Note that the South River flows south to north.

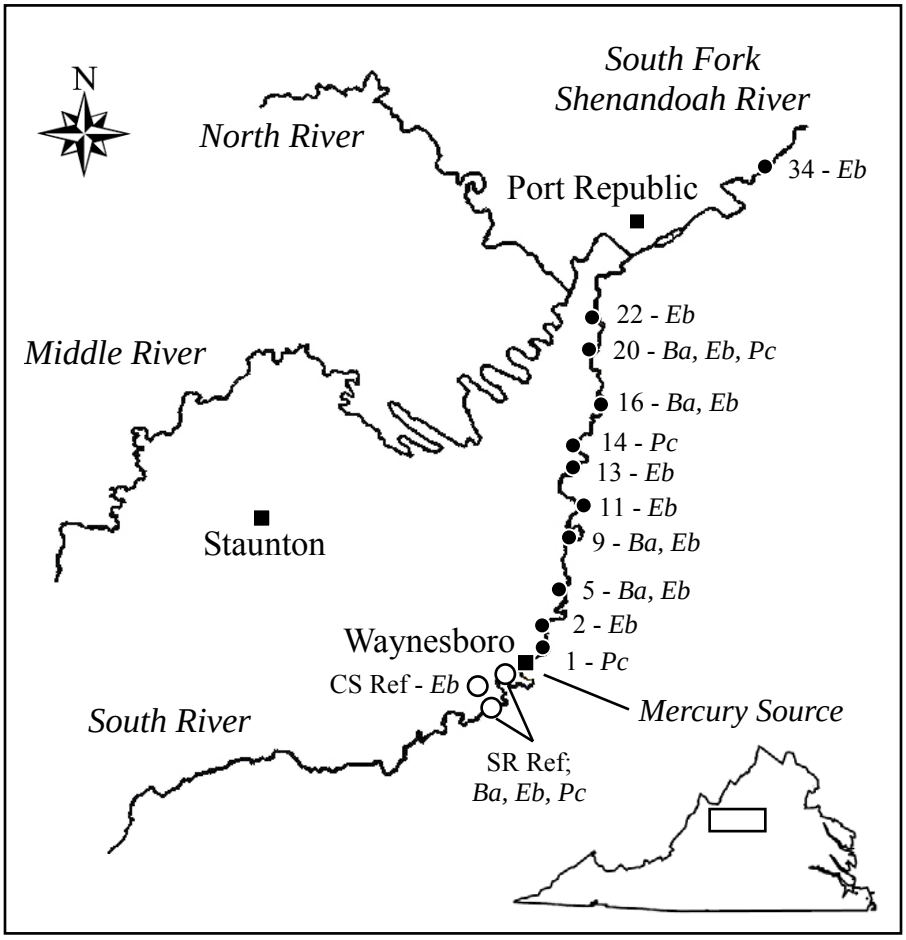


Figure 2.2: (**A**) Whole body total mercury (THg) concentrations (ng/g, dry mass; mean ± 1 standard error [SE]) in adults and larval *Eurycea bislineata* from the reference (REF) and contaminated portion of the South River (SR) and the South Fork of the Shenandoah River (SFSR). (**B**) Whole body THg (ng/g, dry mass; mean ± 1 SE) in adult and larval *E. bislineata* at the reference and contaminated subsites along SR and SFSR. Numbers represent river miles downstream from the source of contamination (river mile 0). Lines connecting points are for visual presentation and do not reflect connectivity among means. Symbols are staggered for clarity.

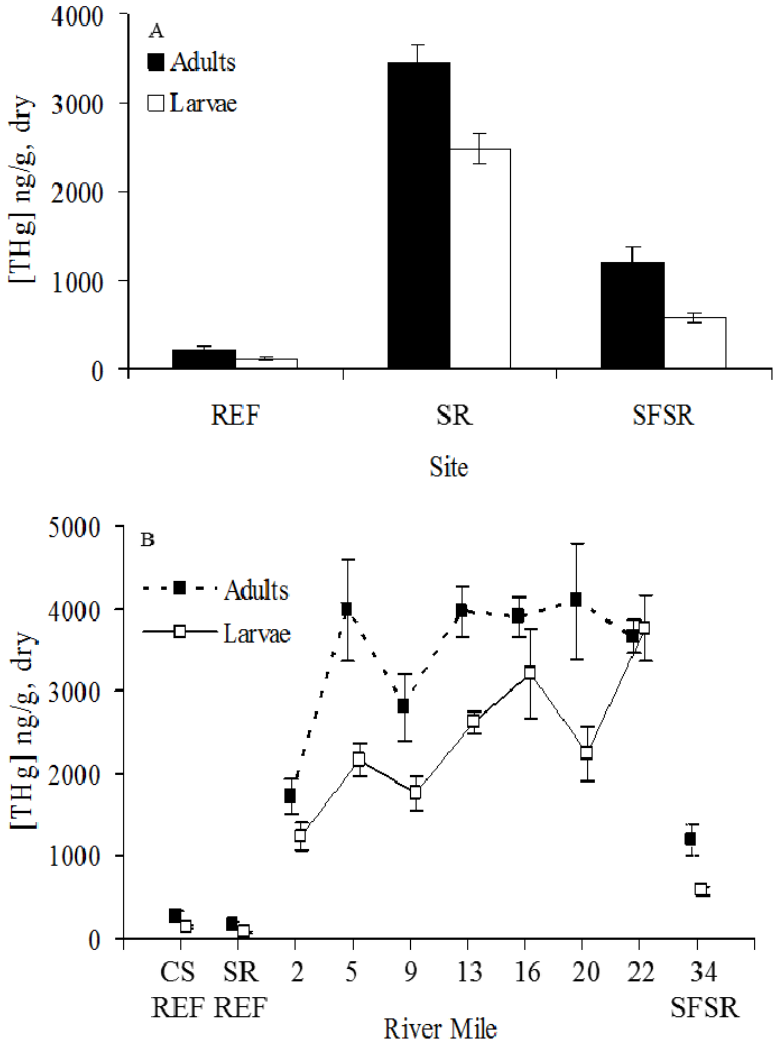


Figure 2.3: (**A**) Whole body total mercury (THg) concentrations (ng/g, dry mass; mean ± 1 standard error [SE]) in adult *Plethodon cinereus* from the reference (REF) and contaminated portion of the South River (SR). (**B**) Whole body THg (ng/g, dry mass; mean ± 1 SE) in adult *P. cinereus* at the reference and contaminated subsites along SR. Numbers represent river miles downstream from the source of contamination (river mile 0). Lines connecting points are for visual presentation and do not reflect connectivity between means.

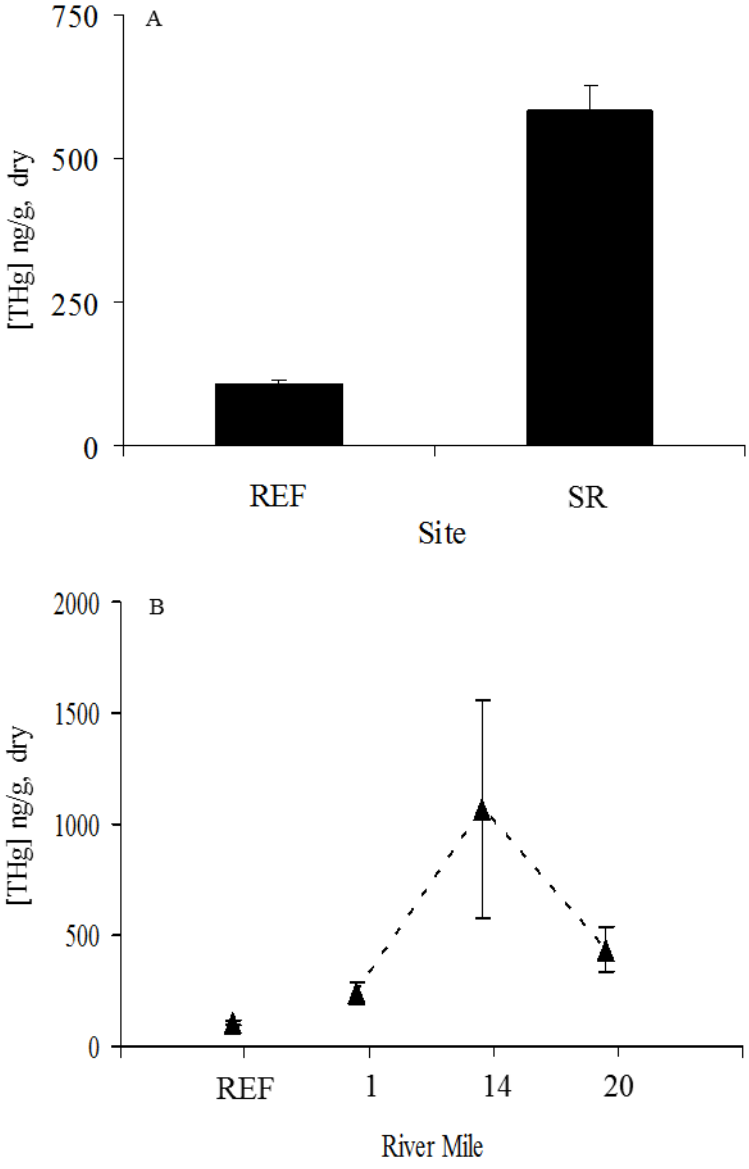


Figure 2.4: (**A**) Whole body total mercury (THg) concentrations (ng/g, dry mass; mean ± 1 standard error [SE]) in adults and larval (tadpoles; composite samples) *Bufo americanus* from the reference (REF) and contaminated portion of the South River (SR). (**B**) Whole body THg (ng/g; mean ± 1 SE) in adults and tadpoles *B. americanus* at the reference and contaminated subsites along SR. Numbers represent river miles downstream from the source of contamination (river mile 0). Lines connecting points are for visual presentation and do not reflect connectivity among means. Symbols are staggered for clarity.

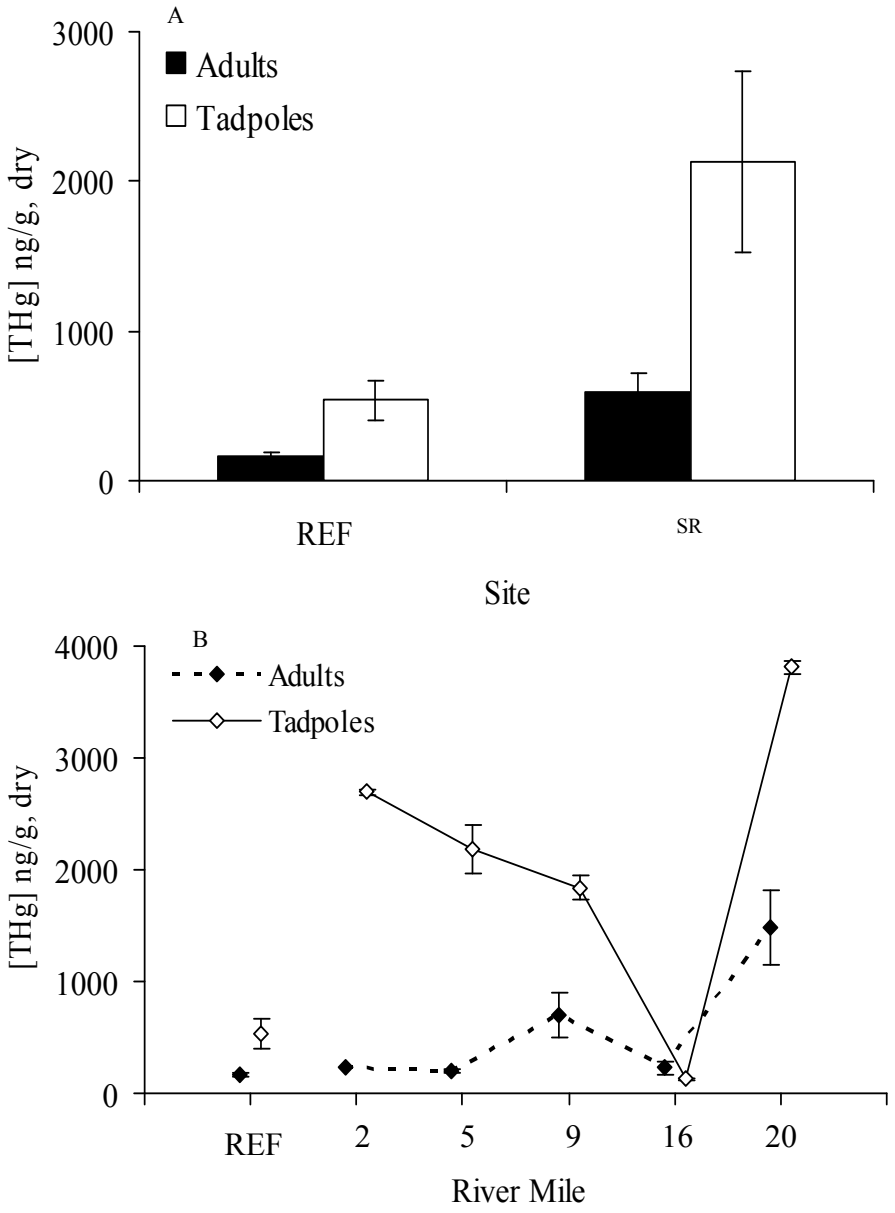


Figure 2.5: Relationship between log body total mercury (THg; ng/g, dry mass) concentrations and log tail THg concentrations in (**A**) adult *Eurycea bislineata* and (**B**) adult *Plethodon cinereus* from the reference (REF) and contaminated (SR) portion of the South River (VA, USA).

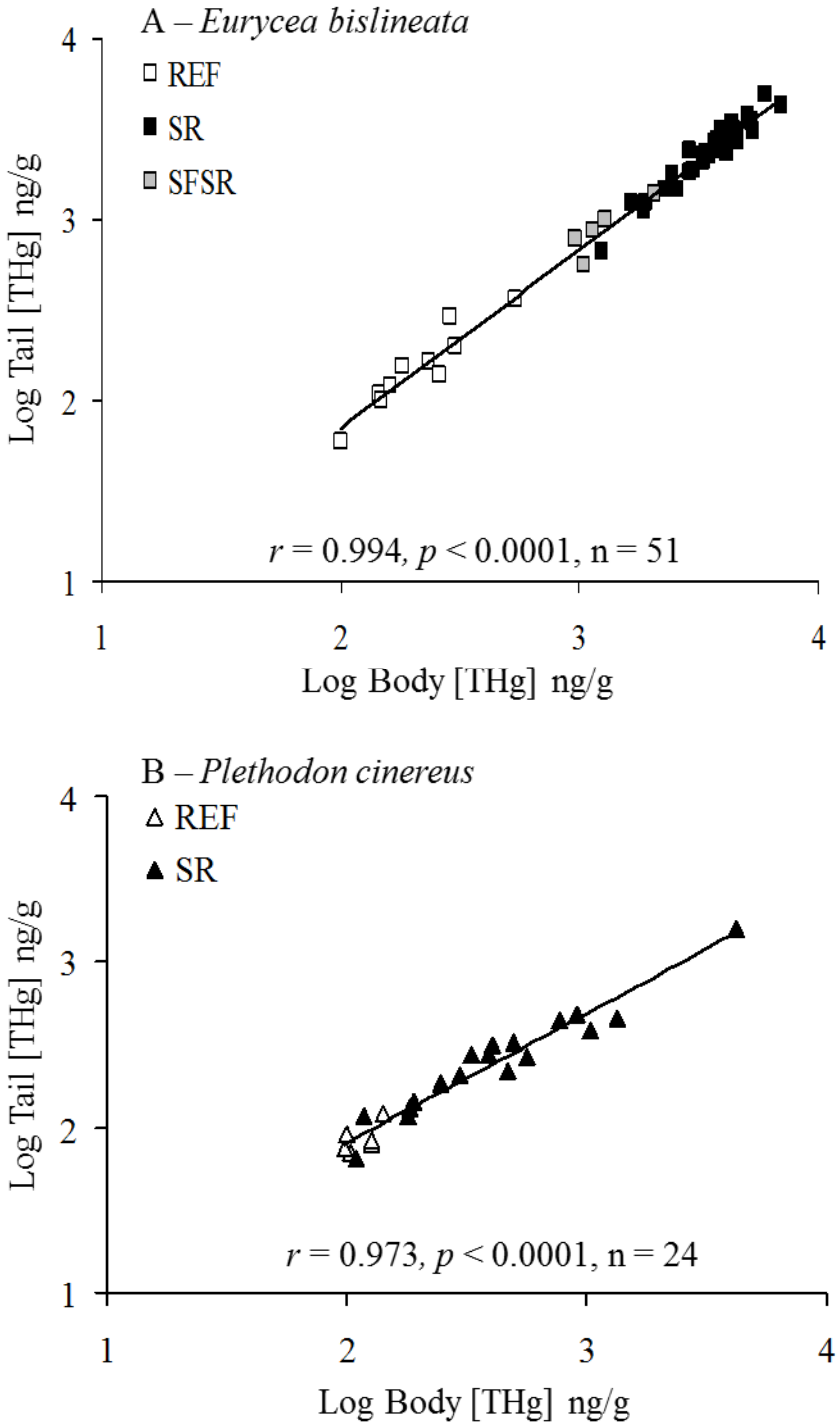
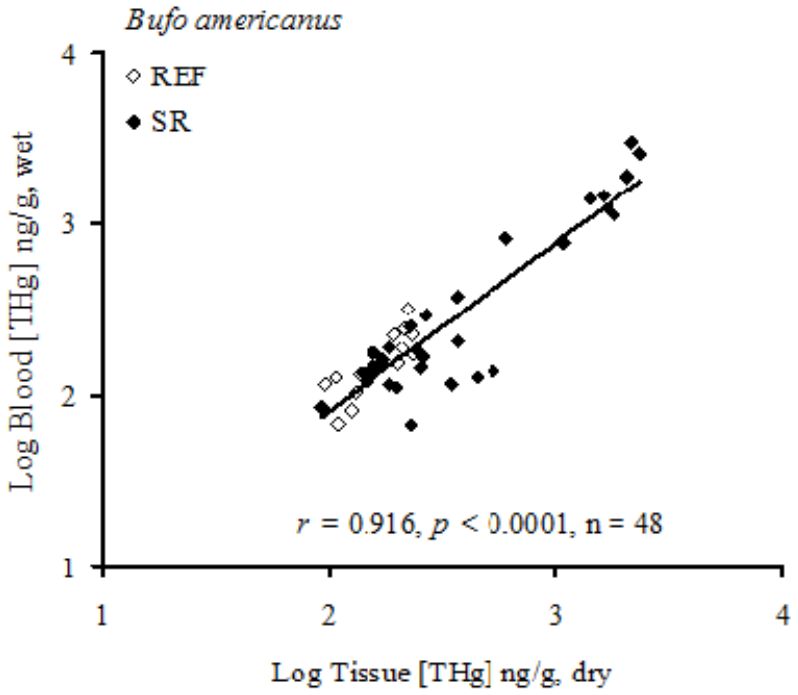


Figure 2.6: Relationship between log whole body total mercury (THg; ng/g, dry mass) concentrations and log blood THg (wet mass) concentrations in adult *Bufo americanus* from the reference (REF) and contaminated (SR) portion of the South River (VA, USA).



Chapter 3: Bioaccumulation and maternal transfer of mercury and selenium in amphibians

Christine M. Bergeron
Co-Authors: Catherine M. Bodinof, Jason M. Unrine, and William A. Hopkins

Formatted for and used with permission by:
Environmental Toxicology & Chemistry, Vol. 29, No. 4, pp. 989-997.

Abstract

Amphibian population declines have been documented worldwide and environmental contaminants are believed to contribute to some declines. Maternal transfer of bioaccumulated contaminants to offspring may be an important and overlooked mechanism of impaired reproductive success that affects amphibian populations. Mercury (Hg) is of particular concern due to its ubiquity in the environment, known toxicity to other wildlife, and complex relationships with other elements, such as selenium (Se). The objectives of the present study were to describe the relationships between total Hg (THg), methlymercury (MMHg), and Se in three amphibian species (*Plethodon cinereus, Eurycea bislineata cirrigera,* and *Bufo americanus*) along a Hg-polluted river and floodplain, and to determine if *B. americanus* maternally transfers Hg and Se to its eggs in a tissue residue-dependent manner. Total Hg and MMHg concentrations in all species spanned two orders of magnitude between the reference and contaminated areas while Se concentrations were generally low in all species at both sites. We observed strong positive relationships between THg and MMHg in tissues of all species. Both Hg and Se were maternally transferred from females to eggs in *B. americanus*, but the percentage of the females' Hg body burden transferred to eggs was low compared to Se. In

addition, Hg concentrations appeared to positively influence the amount of Se transferred from female to eggs. Our study is the first to confirm a correlation between Hg concentrations in female carcass and eggs in amphibians and among the first to describe co-transference of Se and Hg in an anamniotic vertebrate. The results suggest future work is needed to determine whether maternal transfer of Hg has transgenerational implications for amphibian progeny.

Keywords: Amphibians, Mercury, Methylmercury, Selenium, Maternal transfer

Introduction

Amphibian population declines have been documented worldwide [1] and environmental contaminants are believed to contribute to some declines [2]. Several studies have examined the impact of trace element concentrations on larval amphibians, and have found effects on survival [3-5], growth and development [3-8], energy acquisition and allocation [9,10], and behavior or performance [3,7,11]. One trace element that is of particular concern is mercury (Hg), due to its ubiquity in the environment and known toxicity to other wildlife. However, the toxic effects of Hg bioaccumulation in amphibians has received little attention compared to fish, birds, and mammals [12-15]. In one recent study, Unrine et al. [16] found adverse effects of environmentally realistic dietary Hg exposure on survival, development, and growth of amphibian larvae. These data indicate that Hg pollution, in concentrations associated with atmospheric deposition only, has the potential to negatively impact amphibian populations and suggests the importance of further research in this area.

Trophic uptake of contaminants throughout ontogeny, ultimately leading to maternal transfer of bioaccumulated contaminants to offspring, may be an important mechanism of

impaired reproductive success in amphibians. Maternal transfer of Hg in amphibians is a concern since early developmental stages in aquatic biota are particularly sensitive to Hg [17,18]. In amphibians, most of the information regarding the effects of contaminants on development is from aqueous exposure of embryos to contaminants, often in environmentally unrealistic concentrations [14]. To date, only three studies have documented maternal transfer of contaminants in amphibians [19-21], and two of these examined its relationship to development and reproductive success [20,21]. In aqueous laboratory exposures, adult *Xenopus laevis* (African clawed frog) maternally transferred cadmium (Cd) to their eggs, resulting in a residue-dependent increase in developmental malformations [21]. In a field study, *Gastrophryne carolinensis* (eastern narrow-mouth toad) from an industrial site maternally transferred high concentrations of selenium (Se) and strontium (Sr) to their eggs and exhibited a 19% reduction in offspring viability [20].

In the present study, we sought to develop a better understanding of Hg bioaccumulation in amphibians by sampling amphibians inhabiting the South River (VA, USA). The South River was historically (1929-1950) contaminated near Waynesboro, VA with mercuric sulfate [22] and despite terminating the use of Hg at the manufacturing plant in the 1950s, the Hg concentrations remain high in the system [23]. Indeed, the highest whole body total Hg (THg) concentrations in amphibians (*Eurycea bislineata cirrigera*) from the contaminated site [24] were the highest reported for amphibians in the literature [13,14]. Our first objective was to describe the relationships between THg and methylmercury (MMHg) in three amphibian species (*Plethodon cinereus, Eurycea bislineata cirrigera,* and *Bufo americanus*) which exhibit different life histories and occupy different ecological niches within the South River [24]. Describing the speciation of Hg in amphibian tissues is important because the bioavailability and toxicity of Hg

depends upon its specific chemical form. The majority of Hg released into the environment is inorganic, yet the most toxic and bioaccumulative form is MMHg [25]. Production of MMHg is primarily a biologically-mediated reaction by anaerobic sulfate –reducing bacteria in aquatic sediment [26,27]. Thus, knowledge of the THg concentration in the environment is inadequate to accurately evaluate its toxicity. In fact, in many cases, even knowing both the THg and MMHg concentrations in biota is not adequate to predict toxicity because Hg can interact with other trace elements in the environment. In particular, Se is known to have protective effects against Hg toxicity, possibly by redistributing Hg to less sensitive tissues (e.g., muscle) or assisting in sequestration of Hg as Hg-Se in target organs (e.g., liver and kidney) [28]. Thus, our second objective was to describe the relationships between Hg (THg and MMHg) and Se in the three amphibian species. Because there has been little research devoted to the maternal transfer of contaminants in amphibians, our third and final objective was to determine if *B. americanus* maternally transfers Hg (THg and MMHg) and Se to their eggs in a tissue residue-dependent manner.

Materials and Methods

Three species of amphibians, *E. bislineata cirrigera* (hereafter *E. bislineata*; Southern two-lined salamander; adults and larvae), *P. cinereus* (red-backed salamander; adults), and *B. americanus* (American toad; adults) were collected from multiple sampling locations upstream and downstream of the Hg contamination source (river mile [RM] 0) along the South River in spring 2007 as described in Bergeron et al. ([24]; Fig 1, Table 1). A subset of the individuals (toads) or pooled samples (salamanders) were analyzed for THg, MMHg, and Se (Tables 1 and 2).

Breeding and egg collection in Bufo americanus

To determine if *B. americanus* females maternally transfer Hg to their eggs, we generally followed the methods of Hopkins et al. [20]. Adult male and female *B. americanus* were collected in amplexus from breeding ponds and transported to the laboratory. They were placed in 8 L plastic bins containing a small amount of dechlorinated tap water and allowed to breed. Each bin was slightly slanted to create one end with water while the other end remained dry. Breeding pairs were frequently monitored (every 2 to 4 hours) and egg masses were removed and enumerated immediately upon completion of oviposition. If pairs had not oviposted within 24 hours, they were injected with human chorionic gonadotropin (females 200 IU, males 100 IU) to induce egg laying. Once eggs were counted, a subset from each egg mass was frozen for Hg and Se analyses, the males were released at their point of capture, and the females were held for 48 hrs to void gut contents. Before sacrificing the females with an overdose of buffered tricaine methane sulfonate (MS-222), approximately 0.6 ml of blood was obtained by cardiac puncture (or decapitation after sacrifice) using a 1-ml heparinized syringe. Five females from RM 9 were the exception (Table 1), where approximately 0.25 ml of blood (< 1 % body wt) was collected. Cardiac puncture can be used repeatedly on the same individuals [29] and these females were not sacrificed but instead released at their point of capture 24 hrs after the sample was obtained.

Sample preparation and analyses

Total mercury analysis

Total Hg analysis for *B. americanus* whole body, blood, and egg samples was performed using combustion-amalgamation-cold vapor atomic absorption spectrometry (DMA 80,

Milestone, Monroe, CT, USA) according to U.S. Environmental Protection Agency (U.S. EPA) method 7473 [30]. Details of quality control parameters and detection limits for body and blood are discussed in Bergeron et al. (this issue). The method detection limit (MDL; 3 times standard deviation of procedural blanks) for egg samples was 0.05 ng (n=14).

Mercury speciation analysis

A subset of samples from Bergeron et al. [24] for all species were analyzed for MMHg and reanalyzed for THg (Table 1). Because of the limited mass of salamander tissue available for analyses, 2-3 individuals (excluding tails which were used in THg analyses in Bergeron et al. [24]) within a site were pooled to create two composite samples per species per site for MMHg (Table 2). Lyophilized samples of bodies and eggs and whole blood samples (50 – 300 mg) were extracted in sealed 15 ml polypropylene centrifuge tubes containing 2 to 8 ml 4.5 M trace metal grade HNO_3 at 60 ºC overnight. The resulting digests were centrifuged at x1000 g for 20 min to remove any insoluble material. Aliquots of the supernatants (10 – 100 µl) were then analyzed for MMHg content using aqueous phase ethylation with room temperature pre-collection followed by gas chromatography and cold vapor atomic fluorescence spectrometry (GC-CVAFS) according to the methods of Liang et al. [31] as modified by Hammerschmidt and Sandheinrich [32]. Standard reference materials (SRM; TORT-2 [lobster hepatopancreas; National Research Council of Canada, Ottawa, ON]), blanks, duplicate samples, and samples spiked with standards were processed identically and analyzed simultaneously with the samples. Mean recoveries of MMHg for TORT-2 were 86.97 ± 2.70% (n=35). The estimated MDL for MMHg was 0.29 ng/ml and all samples had MMHg concentrations above the MDL. The average relative percent

difference (RPD) between replicate samples analyzed was 12.28 ± 1.99% (*n*=19). Spike recovery averaged 111.82 ± 4.16% (*n*=16).

In order to determine % MMHg, THg concentrations in the MMHg digestates were determined using a Sciex Elan DRC Plus inductively coupled plasma mass spectrometer (ICP-MS; PerkinElmer, Norwalk, CT, USA) according to U.S. EPA method 6020a [33]. Percent recoveries of THg in TORT-2 by this method were 107 ± 4.40% (*n*=12). Average RPD between replicate samples was 2.81 ± 1.31% (*n*=13). The estimated MDL depended on sample mass and ranged from 1.16 to 12.69 ng/g. All samples had THg concentrations that exceeded the MDL. Mean spike recovery was 99.07 ± 1.18% (*n*=13).

Selenium analysis

Selenium concentrations were determined for all *B. americanus* samples (body, blood, and eggs) and the salamander composite samples analyzed for MMHg with a few exceptions (Tables 1 and 2). Lyophilized samples of bodies and eggs and whole blood samples (approximately 300 mg) were digested in 5 ml trace metal grade HNO_3 in flouropolymer digestion vessels using a microwave digestion system (MARS-5, CEM, Matthews, NC, USA) according to U.S. EPA method 3052 [34]. After digestion, the samples were brought to a final volume of 15 ml with >18 MΩ deionized water. Analytical method blanks and the SRM TORT-2 were included in each digestion batch. Selenium analysis was performed on diluted samples according to a modification of U.S. EPA method 6020a [33]. The method was modified by using an ICPMS equipped with a dynamic reaction cell (DRC). The DRC parameters were optimized to minimize the instrumental detection limit. The cell was pressurized with 0.4 ml/min of ultra high purity CH_4 and the rejection parameter q (RPq) was set to 0.6. Intensities

were monitored for both m/z=80 and m/z=82 and the net intensities for both masses were within 10% of each other. Calibration was performed using the method of standard addition. Mean recovery of Se for TORT-2 was 102.62 ± 0.04% (*n*=17). The estimated MDL for Se was 1.82 ng/ml for body and eggs and 0.014 ng/ml for blood. All samples had Se concentrations that exceeded the limit. Average RPD between analytical replicate samples and method replicate samples was 4.34 ± 1.03% (*n*=22) and 10.69 ± 3.38% (*n*=11), respectively. Spike recovery averaged 114.22 ± 4.40% (*n*=23).

Statistical analyses

To compare the analytical techniques for THg (DMA 80 from Bergeron et al. [24] and ICP-MS [present study]) used on the same samples, we regressed the THg concentrations against one another and compared the slope of the relationship, which was not significantly different from one (*t* test, $p > 0.2$, *n*=86).

Methylmercury and Se in salamander composite samples could not be compared statistically between sites due to small sample sizes in the reference site (see Bergeron et al. [24] for THg concentrations by site). Pearson correlation coefficients were used to assess relationships between log-transformed concentrations of THg and MMHg in salamander samples. The slopes of these relationships were compared using analyses of covariance (ANCOVA). Pearson correlation coefficients were also used to examine the relationships between Se and THg or MMHg.

For *B. americanus*, Pearson correlation coefficients were used to assess relationships between log-transformed concentrations of THg and MMHg among tissue samples (whole body, blood, and eggs). The slopes of these relationships were compared using ANCOVA. Pearson

correlation coefficients were also used to assess several relationships for Se, including between Se concentrations in different *B. americanus* tissues (whole body, eggs, and blood) and between Se and THg or MMHg for body, eggs, and blood. Subsites could not be treated independently of one another in our spatial comparisons due to their close proximity and are collectively referred to as either the reference or contaminated site for comparisons using analysis of variance (ANOVA). The assumptions of ANOVA were verified to compare Se, THg, and MMHg between sites and among body and egg tissues and appropriate transformations were performed when needed. For Se (log-transformed) and MMHg (log-transformed) data, two-way ANOVAs were performed to compare site and tissue followed by Tukey's pairwise comparisons. Since transformations failed to meet the assumptions of ANOVA for THg concentrations, we performed a Scheirer-Ray-Hare extension of the Kruskal-Wallis test [35] to compare sites and tissues. Blood concentrations were reported on a wet wt basis. Since blood (wet) and body and eggs (dry) cannot be directly compared to one another, one-way ANOVAs were used to compare concentrations of Se (log-transformed), THg (inverse-transformed), and MMHg (inverse-transformed) in blood between sites. For percent MMHg, two-way ANOVAs were performed to compare among all tissues (body, egg, and blood) and between sites followed by Tukey's pairwise comparisons.

To further assess whether THg, MMHg, and Se were maternally transferred in *B. americanus*, total pre-ovipositional body burdens were constructed using individual tissue (whole body and eggs) concentrations and tissue dry masses following the methods of Hopkins et al. [20,36]. The percentages of total pre-ovipositional body burdens deposited in eggs were then calculated and compared between sites and metal species using ANOVA followed by Tukey's pairwise comparisons.

All analyses were performed with SAS 9.1 (SAS Institute, Cary, NC, USA), and an α value of 0.05 was used to assess statistical significance.

Results

Mercury speciation

Total Hg and MMHg concentrations were positively correlated in the composite body samples for the two salamander species (Fig. 1a), *E. bislineata* (adult: $r = 0.99$, $p < 0.0001$, $n = 20$ and larvae: $r = 0.99$, $p < 0.0001$, $n = 19$) and *P. cinereus* ($r = 0.93$, $p < 0.001$, $n = 8$). The slopes of the regressions of THg and MMHg for *P. cinereus* and *E. bislineata* adult and larvae were similar (ANCOVA, $F_{2,41} = 1.95$, $p = 0.155$). The average % MMHg for salamanders in the contaminated site was 61.2 ± 3.3% for *E. bislineata* adults, 56.8 ± 2.3% for *E. bislineata* larvae, and 45.7 ± 6.6% for *P. cinereus*. The small sample sizes at the reference site precluded the statistical comparison of % MMHg with the contaminated site (Table 2). However, overall averages of % MMHg in the reference site were similar to the contaminated site, ranging from 47% for *P. cinereus* to 62% for *E. bislineata* larvae.

For *B. americanus*, THg and MMHg were positively correlated in whole body ($r = 0.93$, $p < 0.0001$, n = 26), blood ($r = 0.98$, $p < 0.0001$, n = 32), and egg ($r = 0.86$, $p < 0.0001$, n = 32) samples (Fig. 1b). The slope of the regressions for the different tissues were significantly different from one another (ANCOVA, $F_{2,79} = 4.01$, $p = 0.022$). Visual inspection revealed that the slope for the relationship between THg and MMHg in eggs was less than that of body and blood (Fig 1b). Mean THg concentrations (Fig. 2A) were 2.9 and 3.5-fold higher for *B. americanus* egg and body tissues, respectively, in the contaminated site compared to the reference site (site: $H = 5.73$, $p < 0.025$). Within each site, there were significant differences in THg concentrations among body and egg tissues (tissue: $H = 55.04$, $p < 0.001$), but the pattern

was similar at both sites (tissue X site interaction: $H = 0.080$, $p > 0.75$). Specifically, THg concentrations were up to 11-fold higher in body when compared to egg concentrations. While mean THg blood concentrations were also 3.5-fold higher in the contaminated site compared to the reference site, the high variance in the contaminated site resulted in no statistical significance between sites ($F_{1,51} = 3.17$, $p = 0.081$, power = 0.42). Likewise, MMHg concentrations (Fig. 2B) were 3 to 4-fold higher in the contaminated site compared to the reference site for egg and body tissues (site: $F_{1,49} = 6.66$, $p = 0.013$). Methylmercury concentrations in body tissues were 11.4 and 13.5-fold higher than egg tissues in the reference site and contaminated site, respectively (tissue: $F_{1,49} = 83.85$, $p < 0.0001$; tissue X site: $F_{1,49} = 0.06$, $p = 0.805$). Similar to THg, blood MMHg concentrations were not significantly different between sites ($F_{1,30} = 1.03$, $p = 0.318$, power = 0.17). Percent MMHg (Fig. 2C) in *B. americanus* did not differ between the reference and contaminated site (site: $F_{1,76} = 0.73$, $p = 0.396$), but did differ among tissues sampled (tissue: $F_{2,76} = 24.04$, $p < 0.0001$; tissue X site: $F_{2,76} = 0.68$, $p = 0.509$). Pairwise comparisons revealed significant differences in % MMHg concentrations between all tissues samples ($p < 0.002$, for all); blood had the highest % MMHg and eggs had the lowest where % MMHg averaged 53.3 ± 2.3% for whole body, 71.4 ± 2.8 % for blood, and 47.8 ± 3.3% for egg samples.

A significant positive relationship between THg concentrations in whole body and blood was reported in Bergeron et al. ([24]; Fig. 6). Similarly, there was a positive relationship between whole body MMHg and blood MMHg in *B. americanus* (data not shown; $r = 0.91$, $p < 0.0001$, $n = 27$). For both THg and MMHg, body and egg concentrations were positively correlated (THg; $r = 0.90$, $p < 0.0001$, $n = 48$ and MMHg; $r = 0.91$, $p < 0.0001$, $n = 21$) (Fig. 3A) demonstrating that maternal transfer of Hg occurs in *B. americanus*. Similar positive relationships existed between blood and egg THg ($r^2 = 0.88$, $p < 0.0001$, $n = 53$) and MMHg (r^2

= 0.88, $p < 0.0001$, $n = 26$) concentrations (Fig. 3B). For both of these relationships (egg and body, egg and blood) the slopes of the regressions for THg and MMHg were not significantly different (ANCOVA, F values < 0.71, p values > 0.40 for both).

Selenium concentrations

Selenium concentrations were generally low across all species and sites. In salamanders, Se concentrations ranged from 395 – 1,171 ng/g for *P. cinereus*, 120 – 3,130 ng/g for *E. bislineata* adults, and 1,894 – 3,539 ng/g for *E. bislineata* larvae. For *E. bislineata* (adult and larvae) and *P. cinereus*, no relationships between THg or MMHg and Se in composite samples were observed (data not shown; $r < 0.33$, $p > 0.198$, for all relationships).

For *B. americanus*, Se concentrations in body and egg tissues differed by site, but this was dependent on the interaction with tissue (site: $F_{1,97} = 0.01$, $p = 0.908$; tissue: $F_{1,97} = 0.00$, $p = 0.959$; tissue X site: $F_{1,97} = 14.23$, $p = 0.0003$) (Fig. 4). In the reference site, Se concentrations were 25% higher in whole body than eggs. The reverse pattern was observed in the contaminated site where Se concentrations in eggs were 42% higher than whole body. Blood Se concentrations were similar in the contaminated (309.3 ± 18.9 ng/g) and reference site (300.1 ± 51.8 ng/g; $F_{1,44} = 0.42$, $p = 0.521$). Unlike Hg, there was no relationship between Se concentrations in female body and egg ($r = 0.15$, $p = 0.317$, $n = 48$). In contrast, there were significant positive relationships between Se concentrations in blood and body ($r = 0.51$, $p < 0.001$, $n = 46$) and blood and eggs ($r = 0.43$, $p = 0.005$, $n = 48$). Total Hg and Se concentrations were negatively correlated with one another in female whole bodies both pre-oviposition ($r = 0.32$, p = 0.030, $n = 47$) and post-ovipostion ($r = 0.46$, $p = 0.001$, $n = 48$). This relationship was also found between MMHg and Se for post-ovipostion females ($r = 0.51$, $p = 0.022$, $n = 20$), but

not pre-oviposition females ($r = 0.33$, $p = 0.157$, $n = 20$). There was no relationship between egg Se and THg ($r = 0.14$, $p = 0.322$, $n = 53$) or MMHg ($r = 0.04$, $p = 0.864$, $n = 26$), nor was there a relationship between blood Se and THg ($r = 0.03$, $p = 0.508$, $n = 48$) or MMHg ($r = 0.16$, $p = 0.436$, $n = 28$).

Maternal transfer of mercury and selenium

For *B. americanus*, the percentage of females' body burden transferred to her eggs for THg, MMHg, and Se differed by site, but this was dependent on the element (site X element: $F_{2,108} = 14.83$, $p < 0.0001$) (Fig 5). The interaction was caused by maternal transfer of a greater percentage of Se in the females from the contaminated site compared to the reference site (44.3 ± 1.7% vs. 28.2 ± 3.1%, respectively). Pairwise comparisons revealed differences between the percentage of females' Se and THg or Se and MMHg body burden transferred to her eggs ($p < 0.0001$, for both). However, there were no differences in the percent of females' body burden transferred between THg (contaminated 4.96 ± 0.47% and reference 5.70 ± 0.70%) and MMHg (contaminated 3.39 ± 0.31% and reference 4.88 ± 1.10%; $p = 0.184$). There was a significant positive relationship between the percent of Se body burden lost to eggs at oviposition and the whole body THg concentrations in post-oviposition females ($r = 0.390$, $p < 0.007$, $n = 47$; Fig 6). However, the reverse was not true; we found no relationship between the percent of Hg body burden lost to eggs at oviposition and the whole body Se concentrations in post-oviposition females ($r = 0.059$, $p = 0.689$, $n = 47$).

Discussion

Mercury bioaccumulation and speciation

Compared to birds, mammals, and fish, there is a paucity of data for amphibians regarding Hg bioaccumulation [12-15]. The South River is a useful system to investigate Hg relationships in amphibians because both THg and MMHg concentrations in all three amphibian species form a wide distribution, spanning two orders of magnitude between the lowest and highest concentrations at the reference and contaminated site. Concentrations of THg were strongly correlated with MMHg in whole bodies for all species, as well as in all tissues sampled in *B. americanus*, suggesting that MMHg concentrations in amphibians can be predicted from THg concentrations. Similar correlations have been observed for carcasses and tissues in other organisms (e.g., [37,38]). While this relationship may hold true at other locations, the slope is likely to change due to the differences in THg concentrations in various systems. For example, Bank et al. [39] reported a lower THg concentrations, but higher % MMHg for larval *E. bislineata bislineata* (northern two-lined salamanders: 73-97%) from Acadia National Park, ME, USA than were found in larval *E. bislineata* in the present study (39-73%). For *B. americanus*, % MMHg in blood was the highest (73.3 ± 4.8 %) of the tissues sampled The high % MMHg in blood is presumably due to recent dietary uptake and is similar to the % MMHg in blood reported for other organisms (e.g., turtles [40] and birds [41]).

Selenium concentrations and relationships with mercury

Selenium concentrations were generally low among species at the contaminated and reference sites. Unlike Hg, Se is physiologically important to organisms because it is required for the synthesis of the essential amino acid selenocysteine; however, there is a narrow range between dietary concentrations that are nutritious and those that are toxic [13,42]. The whole body concentrations of Se in salamanders from the contaminated site (*E. bislineata* adults 2,318

± 156 ng/g; *E. bislineata* larvae 2,550 ± 112 ng/g; *P. cinereus* 935 ± 243 ng/g) and pre-ovipostion female *B. americanus* in both the reference (3,860 ± 260 ng/g) and Hg-contaminated (3,607 ± 191 ng/g) sites are within or close to the range considered normal background Se concentrations for amphibians and reptiles (1,000-3,000 ng/g, dry wt) [43], indicating little influence of urbanized areas, such as Waynesboro,VA. Significant positive relationships were observed between Se concentrations in *B. americanus* blood and whole body and blood and eggs, however, the relationships were considerably weaker than for Hg (in all cases, $r \leq 0.51$). Hopkins et al. [20] described a functional relationship between Se concentrations in female amphibian carcasses and their eggs spanning two orders of magnitude. This relationship was not observed in the present study, most likely because we encountered a narrower range in Se concentrations. However, the concentrations of Se for females and eggs in the present study are comparable to the values predicted by the functional relationship reported in Hopkins et al. [20].

In general, the interaction between Hg and Se is believed to be antagonistic with low levels of Se in the environment providing some protection against Hg toxicity; however the mechanisms for protection are not fully understood [28]. No correlations were observed between Hg and Se concentrations in *B. americanus* blood or eggs. However, a weak, negative relationship was observed between Hg and Se concentrations in female whole bodies both pre- and post- oviposition. This relationship may have been strengthened had we targeted specific tissues for analysis because co-sequestration of these elements is known to occur in organs such as the liver and kidney (e.g., [28,44]).

Maternal transfer of mercury and selenium in Bufo americanus

Maternal transfer of contaminants may be an important and overlooked mechanism of impaired reproductive success in amphibians [20]. While the transfer of Hg from a female to her offspring has been confirmed in several fish and bird species (e.g., [37,38,45-47]), it has only been examined in one other species of amphibian [19-21]. Our study is the first to describe a correlative relationship between female and egg Hg concentrations. Similar to the functional relationships observed for several fish species [32,37,45,48], concentrations of THg and MMHg in eggs were positively correlated with the concentrations in the maternal carcass in *B. americanus* (Fig. 3A), clearly indicating that Hg transfer to the eggs is related to Hg exposure of the female. In addition, concentrations of THg and MMHg in eggs were correlated with maternal blood concentrations (Fig. 3B). These relationships suggest that blood can provide nondestructive predictions of not only whole body concentrations ([24], Fig. 6) but also egg concentrations. Conversely, Hg concentrations in eggs may be used to estimate female whole body Hg concentrations.

In *B. americanus,* egg laying does not appear to be a major elimination route for Hg as only ~5% of the maternal Hg body burden was transferred to her eggs (Fig. 5). This is consistent with studies involving several fish species [32,37,45,46,49] where generally a small percentage (< 10%) of female Hg body burdens is transferred to the eggs. In contrast, females transferred considerably more of their body burden of Se (28-44%) than Hg to their eggs (Fig. 5). The higher transfer of Se compared to Hg is consistent with Hopkins et al. [20] who found that *G. carolinensis* transferred 53-54% of Se in the maternal carcass to the eggs at both Se-contaminated and reference sites. These differences in maternal transfer of Hg and Se were noteworthy. Unlike highly lipophilic contaminants like organochlorines, Hg and Se do not

partition with tissue lipid, but can be incorporated into amino acids through their affinity for sulfhydyl groups and molecular mimicry (Hg) or substitution for sulfur (Se) [50-52]. Se is incorporated into egg proteins prior to their transport into the egg, which gives Se an efficient route of entry [50]. In addition, Hammerschmidt and Sandheinrich [32] found that the maternal diet during oogenesis, and not the maternal body burden remobilized from somatic tissues, was the primary source of Hg in fish eggs. These attributes of Se and Hg may account for the differences in the proportion of the female's body burden passed onto her eggs.

The present study is among the first to describe co-transference of Se and Hg in an anamniotic vertebrate. Interestingly, the percentage of Se maternally transferred by females from the Hg-contaminated site was 60% higher than that transferred by females at the reference site. These results suggest that the female's Hg body burden might positively affect the amount of Se transferred to her eggs (Fig. 6). However, the dynamic factors controlling maternal transfer of these two elements appear to be influenced by whether Hg or Se is elevated in the environment. In a Se-polluted system where environmental concentrations of Hg were low (the opposite scenario of the current study), Hopkins et al. [20] suggested that females transferring ≥20 μg/g of Se to their eggs transferred very little Hg to their eggs. In contrast, the Se concentrations were orders of magnitude lower in our study system compared to Hopkins et al. [20] and Se in the female carcass was not related to the amount of Hg transferred to the eggs. Heinz and Hoffman [47] found a similar interaction between Hg and Se in feeding studies with *Anas platyrhynchos* (Mallard ducks). Adult ducks were fed either equal concentrations of Hg, Se, or Hg and Se. The addition of dietary Se had little effect on accumulation of Hg in eggs and liver, but they found a considerable increase in Se concentrations in these tissues when Hg was supplemented. In addition, Prati et al. [53] used *Xenopus laevis* embryos in Hg toxicity bioassays where no Se was

added. However, they observed the Hg-exposed embryos had a significant increase in Se uptake over control embryos. While the mechanisms of maternal transfer of contaminants are not clear [54], elevated Hg at the contaminated site may have facilitated the transfer of a larger percentage of Se to *B. americanus* eggs. This enhanced maternal transfer of Se in the Hg-contaminated site may be due to formation of equimolar Hg - Se complexes which bind to proteins in blood [55]. The toxicological implications of the co-transference of Hg and Se remain unclear, but future studies should be designed to examine the interactive effects of these maternally transferred elements on developing embryos.

Conclusions

In the present study, we sought to develop a better understanding of Hg bioaccumulation in amphibians by sampling three amphibian species occurring across a broad Hg contamination gradient. We demonstrated that amphibians from the South River possess some of the highest THg concentrations documented in amphibians to date, but other factors such as the proportion of THg that is methylated and whether Se is co-sequestered with Hg can play a role in the potential toxic effects of Hg. For whole body samples of all amphibians in the Hg-contaminated and reference site, average percent MMHg ranged from 45 to 61% and Se concentrations were within the range considered normal background concentrations. Few studies have examined the maternal transfer of bioaccumulated contaminants to offspring in amphibians. We observed maternal transfer of both Hg and Se in *B. americanus,* and described the first correlation between Hg concentrations in female carcass and eggs in amphibians. There also appeared to be interactions between these two elements that influenced the amount of Se transferred to the egg. Even though contaminated sites often contain mixtures of contaminants, little information exists

regarding the maternal transfer of both Hg and Se in any organism. Clearly, additional work is needed to understand the interactive dynamics of co-transfer of Se and Hg and whether this influences toxicological outcomes. Together with previous work [20], our results suggest that maternal transfer of contaminants in amphibians deserves further study, particularly in relationship to reproductive success of adults and the growth and development of offspring.

Acknowledgements

We thank the South River Science Team for their support and the landowners along the South River and the Waynesboro Parks and Recreation Department for access to sampling locations. D. Addis, H. Brant, S. Budischak, J. Callihan, S. DuRant, S. Orlofske, B. Todd and H. Wada provided field and laboratory assistance. Collection of animals was in conformance with appropriate permits, and sample methods were in compliance with Virginia Polytechnic and State University's animal care and use protocols. Financial support to WAH was provided by E. I. DuPont de Nemours, startup funds from Virginia Polytechnic and State University, and by the National Science Foundation (NSF # IOB-0615361). Additional support was provided to CMB in a Seed Grant from The World Conservation Union Species Survival Commission Amphibian Specialist Group.

Literature Cited

1. Stuart SN, Chanson JS, Cox NA, Young BE, Rodrigues ASL, Fischman DL, Waller RW. 2004. Status and trends of amphibian declines and extinctions worldwide. *Science* 306: 1783-1786.
2. Corn PS. 2000. Amphibian declines: Review of some current hypotheses. In Sparling DW, Linder G, Bishop, CA, eds, *Ecotoxicology of Amphibians and Reptiles.* SETAC Press, Pensacola, FL, USA, pp 663-696.
3. Lefcort H, Meguire RA, Wilson LH, Ettinger WF. 1998. Heavy metals alter the survival, growth, metamorphosis, and antipredatory behavior of Columbia spotted frog (*Rana luteiventris*) tadpoles. *Arch Environ Contam Toxicol* 35: 447-456.
4. James SM, Little EE, Semlitsch RD. 2005. Metamorphosis of two amphibian species after chronic cadmium exposure in outdoor aquatic mesocosms. *Environ Toxicol Chem* 24: 1994-2001.
5. Snodgrass JW, Hopkins WA, Jackson BP, Baionno JA, Broughton J. 2005. Influence of larval period on responses of overwintering green frog (*Rana clamitans*) larvae exposed to contaminated sediments. *Environ Toxicol Chem* 24: 1508-1514.
6. Snodgrass JW, Hopkins WA, Broughton J, Gwinn D, Baionno JA, Burger J. 2004. Species-specific responses of developing anurans to coal combustion wastes. *Aquatic Toxicology* 66: 171-182.
7. Hopkins WA, Congdon J, Ray JK. 2000. Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. *Environ Toxicol Chem* 19: 862-868.

8. Roe JH, Hopkins WA, DuRant SE, Unrine JA. 2006. Effects of competition and coal-combustion wastes on recruitment and life history characteristics of salamanders in temporary wetlands. *Aquatic Toxicology* 79: 176-184.

9. Rowe CL, Kinney OM, Fiori AP, Congdon JD. 1996. Oral deformities in tadpoles (*Rana catesbeiana*) associated with coal ash deposition: Effects on grazing ability and growth. *Freshwater Biology* 36: 723-730.

10. Rowe CL, Kinney OM, Nagle RD, Congdon JD. 1998. Elevated maintenance costs in an anuran (*Rana catesbeiana*) exposed to a mixture of trace elements during the embryonic and early larval periods. *Physiological Zoology* 71: 27-35.

11. Raimondo SM, Rowe CL, Congdon JD. 1998. Exposure to coal ash impacts swimming performance and predator avoidance in larval bullfrogs (*Rana catesbeiana*). *J Herpetol* 32: 289-292.

12. Scheuhammer AM, Meyer MW, Sandheinrich MB, Murray MW. 2007. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. *Ambio* 36: 12-18.

13. Eisler R. 2006. *Mercury Hazards to Living Organisms*. CRC Press, Boca Raton, FL.

14. Linder G, Grillitsch B. 2000. Ecotoxicology of metals. In Sparling DW, Linder G, Bishop, CA, eds, *Ecotoxicology of Amphibians and Reptiles.* SETAC Press, Pensacola, FL, pp 325-459.

15. Wolfe MF, Schwarzbach S, Sulaiman RA. 1998. Effects of mercury on wildlife: A comprehensive review. *Environ Toxicol Chem* 17: 146-160.

16. Unrine JM, Jagoe CH, Hopkins WA, Brant HA. 2004. Adverse effects of ecologically relevant dietary mercury exposure in southern leopard frog (*Rana sphenocephala*) larvae. *Environ Toxicol Chem* 23: 2964-2970.

17. Birge WJ, Westerman AG, Spomberg JA. 2000. Comparative toxicology and risk assessment of amphibians. In Sparling DW, Linder G, Bishop, CA, eds, *Ecotoxicology of Amphibians and Reptiles.* SETAC Press, Pensacola, FL, pp 727-792.

18. Weiner JG, Krabbenhoft DP, Heinz GH, Scheuhammer AM. 2003. Ecotoxicology of Mercury. In Hoffman DJ, Rattner BA, Burton TM,Cairns J, eds, *Handbook of Ecotoxicology (2nd ed).* CRC Press, Boca Raton, FL, pp 409-463.

19. Kadokami K, Takeishi M, Kuramoto M, Ono Y. 2004. Maternal transfer of organochlorine pesticides, polychlorinated dibenzo-p-dioxins, dibenzofurans, and coplanar polychlorinated biphenyls in frogs to their eggs. *Chemosphere* 57: 383-389.

20. Hopkins WA, DuRant SE, Staub BP, Rowe CL, Jackson BP. 2006. Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. *Environmental Health Perspectives* 114: 661-666.

21. Kotyzova D, Sundeman FW. 1998. Maternal exposure to Cd(II) causes malformations of *Xenopus laevis* embryos. *Annals of Clinical and Laboratory Science* 28: 224-235.

22. Carter LJ. 1977. Chemical-plants leave unexpected legacy for two Virginia rivers. *Science* 198: 1015-1020.

23. Southworth GR, Peterson MJ, Bogle MA. 2004. Bioaccumulation factors for mercury in stream fish. *Environmental Practice* 6: 135-143.

24. Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA. *In press*. Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians. *Environ Toxicol Chem*.

25. Fitzgerald WF, Engstrom DR, Mason RP, Nater EA. 1998. The case for atmospheric mercury contamination in remote areas. *Environ Sci Technol* 32: 1-7.

26. Benoit JM, Gilmour CC, Heyes A, Mason RP, Miller CL. 2003. Geochemical and biological controls over methylmercury production and degradation in aquatic ecosystems. In Cai C, Braids, OC, eds, *Biogeochemistry of Environmentally Important Trace Elements.* 835. pp 262-297.

27. Benoit JM, Gilmour CC, Mason RP, Heyes A. 1999. Sulfide controls on mercury speciation and bioavailability to methylating bacteria in sediment pore waters. *Environ Sci Technol* 33: 1780-1780.

28. Cuvin-Aralar MLA, Furness RW. 1991. Mercury and selenium interaction - a review. *Ecotoxicol Environ Saf* 21: 348-364.

29. Hopkins WA, Mendonca MT, Congdon JD. 1997. Increased circulating levels of testosterone and corticosterone in southern toads, *Bufo terrestris*, exposed to coal combustion waste. *General and Comparative Endocrinology* 108: 237-246.

30. U.S. Environmental Protection Agency. 1998. Method 7473: Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrometry. Washington, D.C., USA.

31. Liang L, Bloom NS, Horvat M. 1994. Simultaneous determination of mercury speciation in biological-materials by GC/CVAFS after ethylation and room-temperature precollection. *Clin Chem* 40: 602-607.

32. Hammerschmidt CR, Sandheinrich MB. 2005. Maternal diet during oogenesis is the major source of methylmercury in fish embryos. *Environ Sci Technol* 39: 3580-3584.

33. U.S. Environmental Protection Agency. 1998. Method 6020a: Inductively Coupled Plasma - Mass Spectrometry. Washington, D.C., USA.

34. U.S. Environmental Protection Agency. 1996. Method 3052: Microwave assisted acid digestion of siliceous and organically based matrices. Washington, D.C., USA

35. Sokal RR, Rohlf FJ. 1995. *Biometry: Principles and practice of statistics in biological research*. W.H. Freeman and Company, New York.

36. Hopkins WA, Staub BP, Baionno JA, Jackson BP, Talent LG. 2005. Transfer of selenium from prey to predators in a simulated terrestrial food chain. *Environ Pollut* 134: 447-456.

37. Hammerschmidt CR, Wiener JG, Frazier BE, Rada RG. 1999. Methylmercury content of eggs in yellow perch related to maternal exposure in four Wisconsin lakes. *Environ Sci Technol* 33: 999-1003.

38. Scheuhammer AM, Perrault JA, Bond DE. 2001. Mercury, methylmercury, and selenium concentrations in eggs of common loons (*Gavia immer*) from Canada. *Environ Monit Assess* 72: 79-94.

39. Bank MS, Loftin CS, Jung RE. 2005. Mercury bioaccumulation in northern two-lined salamanders from streams in the northeastern United States. *Ecotoxicology* 14: 181-191.

40. Bergeron CM, Husak JF, Unrine JM, Romanek CS, Hopkins WA. 2007. Influence of feeding ecology on blood mercury concentrations in four species of turtles. *Environ Toxicol Chem* 26: 1733-1741.

41. Rimmer CC, McFarland KP, Evers DC, Miller EK, Aubry Y, Busby D, Taylor RJ. 2005. Mercury concentrations in Bicknell's thrush and other insectivorous passerines in Montane forests of northeastern North America. *Ecotoxicology* 14: 223-240.

42. Ohlendorf HM. 2003. Ecotoxicology of Selenium. In Hoffman DJ, Rattner BA, Burton GA,Cairns J, eds, *Handbook of Ecotoxicology, 2nd edition.* CRC Press, Boca Raton, FL, pp 465-500.

43. U.S. Department of the Interior. 1998. National Irrigation Water Quality Program Information Report No. 3, Guidelines for Interpretation of the Biological Effects of Selected Constituents in Biota, Water, and Sediment: Selenium. U.S. Department of the Interior (Bureau of Reclamation, U.S. Fish and Wildlife Service, U.S. Geological Survey, Bureau of Indian Affairs): 139-184.

44. Hopkins WA, Hopkins LB, Unrine JM, Snodgrass J, Elliot JD. 2007. Mercury concentrations in tissues of osprey from the Carolinas, USA. *J Wildl Manage*.

45. Johnston TA, Bodaly RA, Latif MA, Fudge RJP, Strange NE. 2001. Intra- and interpopulation variability in maternal transfer of mercury to eggs of walleye (*Stizostedion vitreum*). *Aquatic Toxicology* 52: 73-85.

46. Latif MA, Bodaly RA, Johnston TA, Fudge RJP. 2001. Effects of environmental and maternally derived methylmercury on the embryonic and larval stages of walleye (*Stizostedion vitreum*). *Environ Pollut* 111: 139-148.

47. Heinz GH, Hoffman DJ. 1998. Methylmercury chloride and selenomethionine interactions on health and reproduction in mallards. *Environ Toxicol Chem* 17: 139-145.

48. Drevnick PE, Horgan MJ, Oris JT, Kynard BE. 2006. Ontogenetic dynamics of mercury accumulation in Northwest Atlantic sea lamprey (*Petromyzon marinus*). *Can J Fish Aquat Sci* 63: 1058-1066.

49. Niimi AJ. 1983. Biological and toxicological effects of environmental contaminants in fish and their eggs. *Can J Fish Aquat Sci* 40: 306-312.

50. Unrine JM, Jackson BP, Hopkins WA, Romanek C. 2006. Isolation and partial characterization of proteins involved in maternal transfer of selenium in the western fence lizard (*Sceloporus occidentalis*). *Environ Toxicol Chem* 25: 1864-1867.

51. Ballatori N. 2002. Transport of toxic metals by molecular mimicry. *Environmental Health Perspectives* 110: 689-694.

52. Simmons-Willis TA, Koh AS, Clarkson TW, Ballatori N. 2002. Transport of a neurotoxicant by molecular mimicry: the methylmercury-L-cysteine complex is a substrate for human L-type large neutral amino acid transporter (LAT) 1 and LAT2. *Biochemical Journal* 367: 239-246.

53. Prati M, Gornati R, Boracchi P, Biganzoli E, Fortaner S, Pietra R, Sabbioni E, Bernardini G. 2002. A comparative study of the toxicity of mercury dichloride and methylmercury, assayed by the Frog Embryo Teratogenesis Assay-Xenpus (FETAX). *Alternatives to Laboratory Animals* 30: 23-32.

54. Kleinow K, Baker J, Nichols J, Gobas F, Parkerton T, Muir D, Monteverdi G, Mastrodone P. 1999. Exposure, uptake, and disposition of chemicals in reproductive and developmental stages of selected oviparous vertebrates. In Di Giulio RT,Tillitt DE, eds, *Reproduction and developmental effects of contaminants in oviparous vertebrates.* SETAC Press, Pensacola, FL, pp 9-111.

55. Yoneda S, Suzuki KT. 1997. Detoxification of mercury by selenium by binding of equimolar Hg-Se complex to a specific plasma protein. *Toxicology and Applied Pharmacology* 143: 274-280.

Table 3.1: Individual sample sizes for *Bufo americanus* whole body, blood, and eggs from the reference and contaminated portions of the South River (SR), VA, USA. The first number denotes the number of samples analyzed for total mercury (THg; combustion-amalgamation-cold-vapor atomic absorption spectrophotometry) and selenium (Se). Where sample sizes differed for Se, the sample size for Se is indicated in brackets [ ]. Sample sizes for methylmercury (MMHg) analyses are denoted in parentheses ( ). RM = river mile downstream from contamination source.

Site	Body	Blood	Eggs
Reference Site	13 (10)	13 (10)	13 (8)
Contaminated subsites			
SR RM 2	1 (1)	1 (1)	1 (1)
SR RM 5	12 (5)	12 [11] (5)	12 (4)
SR RM 9	12 (1)	17 [13] (6)	17 (5)
SR RM 16	4 (4)	4 (4)	4 (4)
SR RM 20	6 (6)	6 (6)	6 (4)
Contaminated sites (totals)	35 (17)	40 [35] (22)	40 (18)
Total Analyzed	48 (27)	53 [48] (32)	53 (26)

Table 3.2: Composite salamander samples for *Eurycea bislineata* (adult and larvae) and *Plethodon cinereus* (adult) analyzed for total mercury (THg; by inductively coupled plasma mass spectrometry [ICP-MS]), methylmercury (MMHg), and selenium (Se) from the reference and contaminated portions of the South River (SR), Coyner Spring Park (CS), and the South Fork of the Shenandoah River (SFSR), VA, USA. Sample sizes were the same for all analyses, with exceptions for Se denoted parenthetically ( ). RM = river mile downstream from contamination source.

Site	*E. bislineata* adult	*E. bislineata* larvae	*P. cinereus* adult
Reference subsites			
SR REF	2	2	2
CS REF	2	2	-
Reference (totals)	4	4	2
Contaminated subsites			
SR RM 1	-	-	2
SR RM 2	2	2	-
SR RM 5	2 (1)	1 (0)	-
SR RM 9	2	2	-
SR RM 11	2	2	-
SR RM 13	2	2	-
SR RM 14	-	-	2 (1)
SR RM 16	2	2	-
SR RM 20	2	2	2
SR RM 22	2	2	-
South River (totals)	16 (15)	15 (14)	6 (5)
South Fork Shenanndoah River			
SFSR RM 34	2	2	-
Species Total	20 (19)	19 (18)	8 (7)

Figure 3.1: Relationship between log total mercury (THg; ng/g, dry wt) concentrations and log methylmercury (MMHg; ng/g, dry wt) concentrations in (**A**) composite body samples from salamanders: adult *Eurycea bislineata* ($r = 0.986$, $p < 0.0001$, $y = 0.976x - 0.146$, $n = 20$), larval *E. bislineata* ($r = 0.993$, $p < 0.0001$, $y = 0.959x - 0.115$, $n = 19$) and adult *Plethodon cinereus* ($r = 0.932$, $p < 0.001$, $y = 0.797x + 0.185$, $n = 8$) and (**B**) *Bufo americanus*: whole body (dry wt; $r = 0.932$, $p < 0.0001$, $y = 1.094x - 0.501$, $n = 26$), blood (wet wt; $r = 0.984$, $p < 0.0001$, $y = 0.928x + 0.022$, $n = 32$), and egg (dry wt; $r = 0.860$, $p < 0.0001$, $y = 0.786x - 0.091$, $n = 32$) samples in the reference and contaminated portion of the South River (VA, USA).

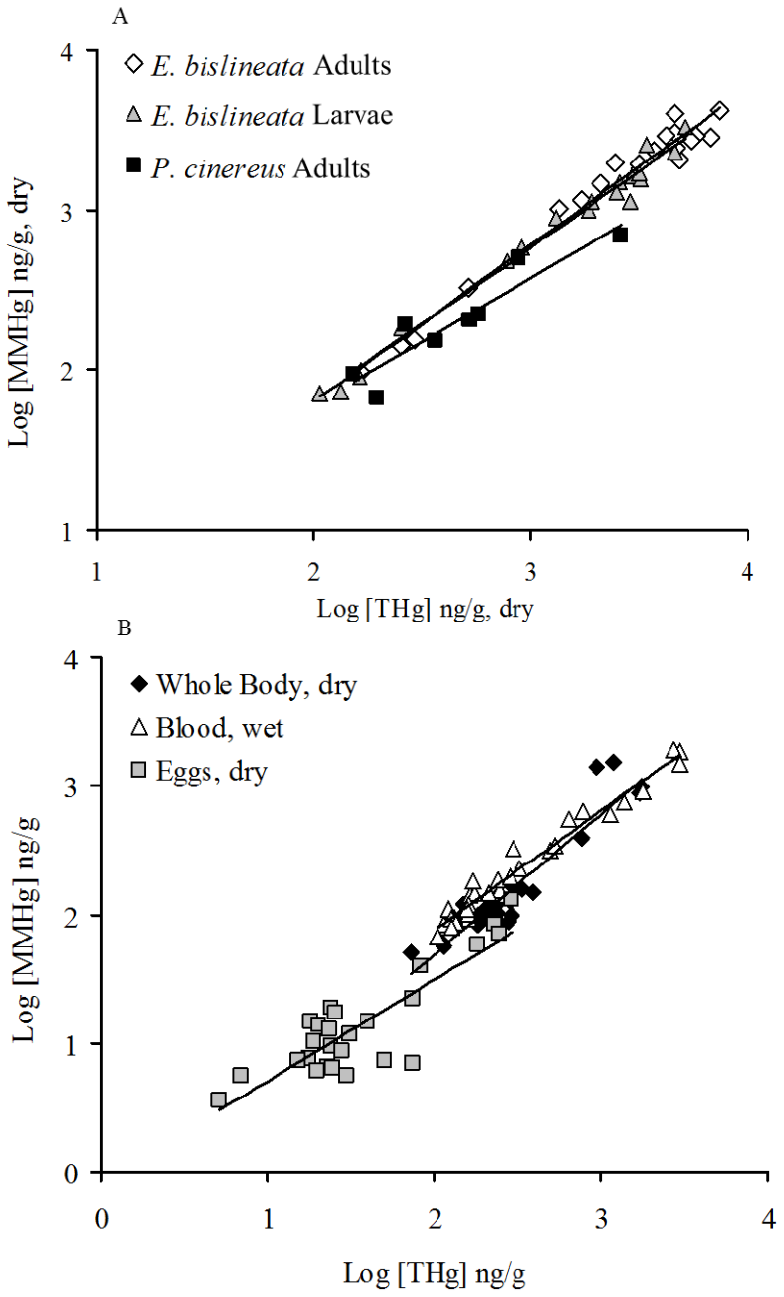


Figure 3.2: (**A**) Total mercury (THg) concentrations (ng/g; mean ± 1 standard error [SE]), (**B**) methylmercury (MMHg; ng/g; mean ± 1 SE), and (**C**) percent methylmercury (% MMHg; mean ± 1 SE) in *Bufo americanus* whole body (dry wt), eggs (dry wt), and blood (wet wt) from the reference (REF) and contaminated (SR) portion of the South River, VA, USA. For THg and MMHg, blood concentrations (wet wt) were handled separately from other tissue concentrations (dry wt) in statistical models. See methods for details.

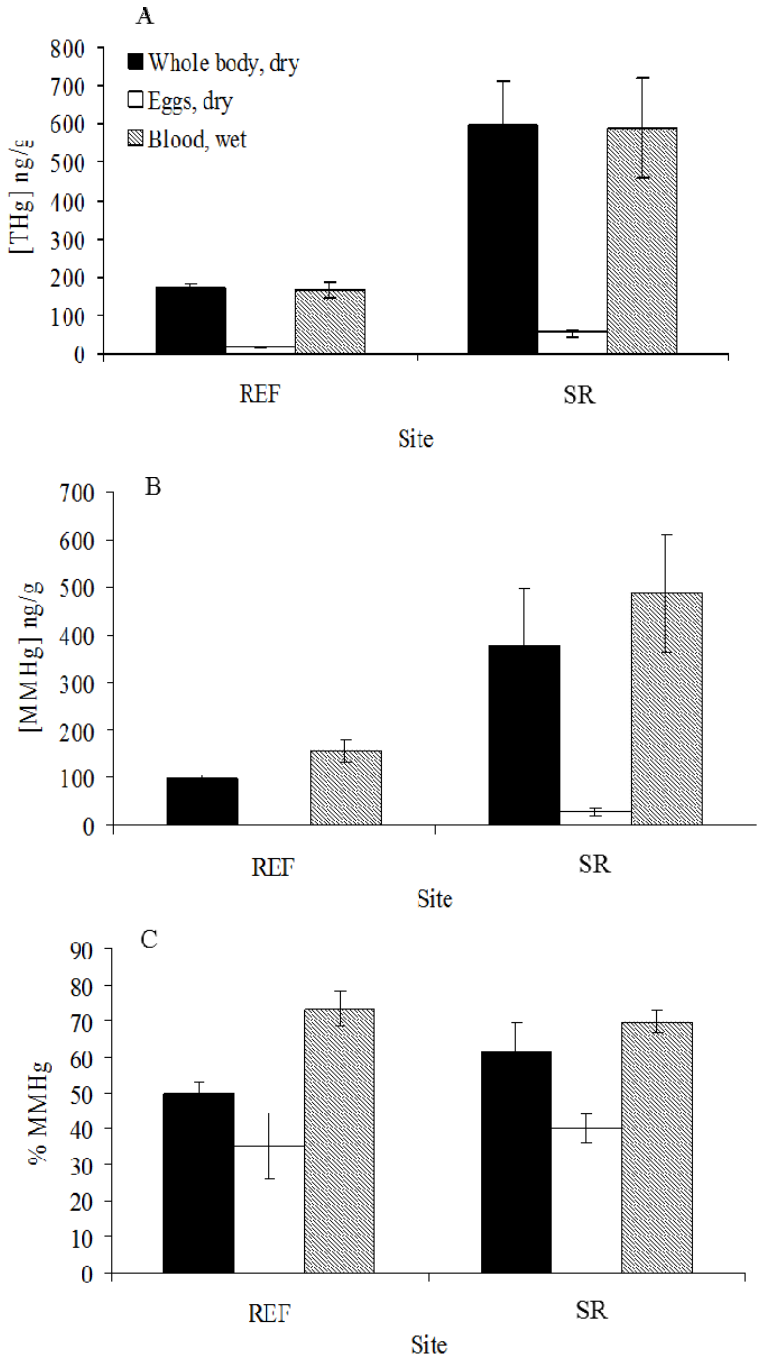


Figure 3.3: Relationship of log total mercury (THg; ng/g) and log methylmerury (MMHg; ng/g) between (**A**) whole body and egg concentrations (THg: y = 0.871x - 0.696; MMHg: y = 0.787x - 0.632) and (**B**) blood and egg concentrations (THg: y = 0.764x - 0.353; MMHg: y = 0.818x - 0.781) in *Bufo americanus* from the reference and contaminated portion of the South River (VA, USA).

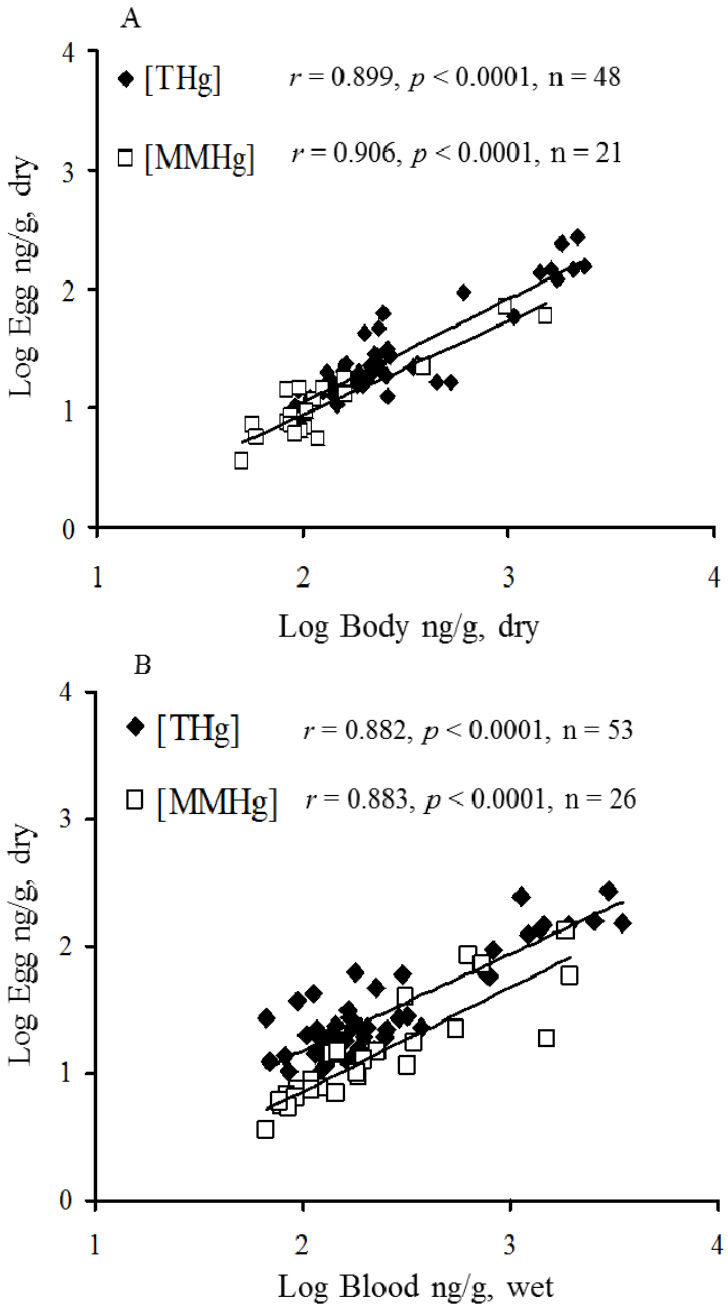


Figure 3.4: Total selenium (Se) concentrations (ng/g; mean ± 1 standard error [SE]) in *Bufo americanus* whole body (dry wt), egg (dry wt), and blood (wet wt) samples from the reference (REF) and contaminated (SR) portion of the South River, VA, USA. Blood concentrations (wet wt) were handled separately from other tissue concentrations (dry wt) in statistical models. See methods for details.

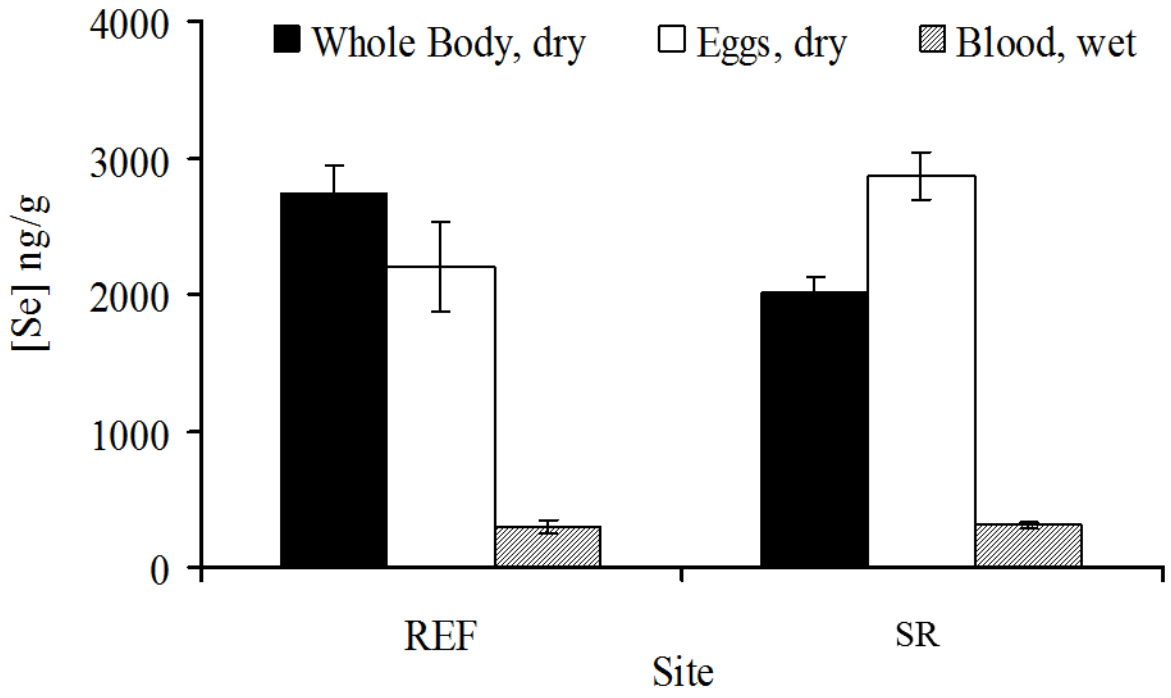


Figure 3.5: Percent of *Bufo americanus* maternal body burden (pre-oviposition) transferred to eggs for total mercury (THg), methylmercury (MMHg), and selenium (Se) from the reference (REF) and contaminated (SR) portion of the South River, VA, USA.

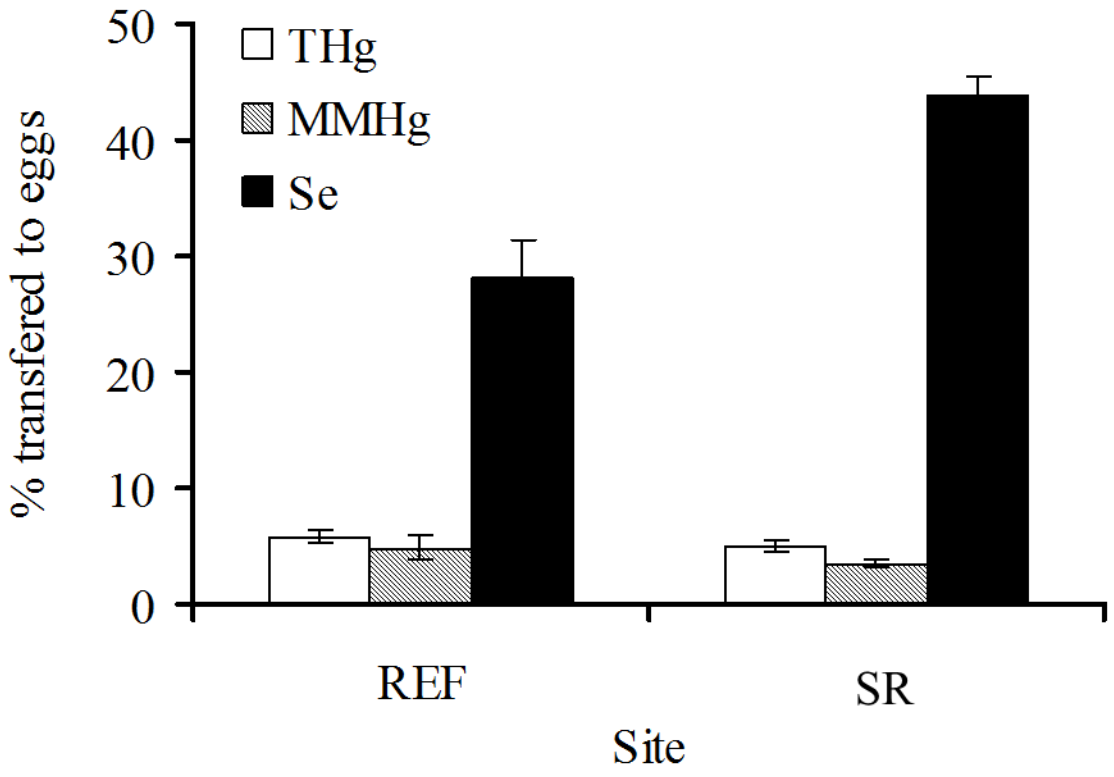
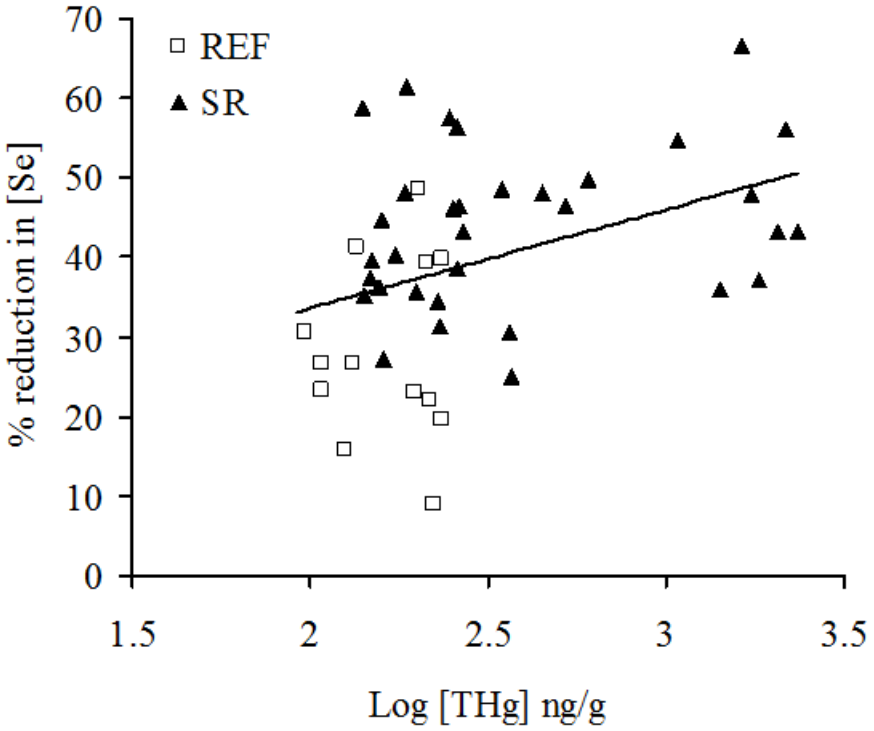


Figure 3.6: Relationship between *Bufo americanus* whole body log total mercury (THg; ng/g) concentrations in post-oviposition females and the percent of selenium (Se) concentrations lost to eggs between pre- and post-ovipostion ($r = 0.390$, $p < 0.007$, $n = 47$) in the reference (REF) and contaminated (SR) portion of the South River (VA, USA).



Chapter 4: Counterbalancing effects of maternal mercury exposure during different stages of early ontogeny in American toads

Christine M. Bergeron
Co-Authors: William A. Hopkins, Catherine M. Bodinof, Sarah A. Budischak, Haruka Wada, and Jason M. Unrine

Formatted for and reprinted from *Science of the Total Environment,* 409, Christine M. Bergeron, William A. Hopkins, Catherine M. Bodinof, Sarah A. Budischak, Haruka Wada, and Jason M. Unrine, Counterbalancing effects of maternal mercury exposure during different stages of early ontogeny in American toads, pp. 4746-4752, Copyright 2011, with permission from Elsevier.

Abstract

Maternal transfer of environmental contaminants is a disadvantageous parental effect which can have long-lasting implications for offspring fitness. We investigated the effects of mercury (Hg) on the reproductive success of female amphibians and the subsequent effects of maternal transfer on the development of their offspring. American toads (*Bufo americanus*) maternally transferred Hg to their eggs, and there was a negative relationship between Hg concentrations and the percentage of viable hatchlings produced in clutches. However, when we continued to monitor larvae that successfully hatched, we found 21% greater metamorphic success in larvae from Hg-exposed mothers compared to reference larvae. The negative effect in the embryonic stage and positive effect in the larval stage counterbalanced one another, ultimately resulting in no difference in predicted terrestrial recruitment, regardless of maternal Hg exposure. Our findings demonstrate that maternal effects on survival manifesting at different stages in ontogeny have the potential to produce complicated outcomes.

Keywords: Maternal effects; Amphibian; Mercury; Maternal transfer

Introduction

The condition or physiological state of parents can be affected by the environment, and these effects can be transmitted to offspring (usually by the mother) in the form of nutrition, hormones, and antibodies (Bernardo, 1996). Parental effects on offspring fitness are highly context-dependent and can be positive or negative based on different environmental circumstances (Rossiter, 1996). However, exposure to environmental contaminants is one of the most obvious disadvantageous parental effects. Contaminants may directly affect parental health, fertility, or fecundity, but females may also maternally transfer bioaccumulated contaminants to developing embryos. In the majority of circumstances that have been investigated, maternal transfer of contaminants is deleterious to offspring due to the effects of transferred contaminants on key organizational events that occur early in ontogeny (Russell et al., 1999). For example, rapid declines of North American raptor and piscivorous bird populations in the mid-20th century were largely attributed to the pesticide dichlorodiphenyltrichloroethane (DDT) and its metabolites which caused eggshell thinning and subsequently reduced hatching success (Blus, 1996). In addition to immediate effects, parental effects can have long-term consequences (Lindstrom, 1999). For instance, alligators maternally exposed to endocrine-disrupting chemicals from Lake Apopka, FL experienced low reproductive success as a result of reduced hatching success and increased juvenile mortality, along with abnormal development of the endocrine and reproductive systems (Guillette et al., 2000). In humans, prenatal exposure in females to the synthetic estrogen diethylstilbestrol (DES) increased the risk of vaginal clear cell adenocarcinoma, abnormalities of the reproductive tract, and infertility (Swan, 2000). However, unlike the effects of maternal exposure of DDT on hatching success in birds, the effects of DES are latent and manifested only at the onset of offspring

adolescence, another critical period in ontogeny (Swan, 2000). These examples highlight the range in timing of the expression of deleterious effects that maternal exposure to chemicals can have on offspring.

Compared to other vertebrate classes, few studies have investigated the effects of contaminants on reproduction in amphibians, even though environmental contamination is one of several factors suspected of contributing to worldwide amphibian population declines (Corn, 2000). In particular, maternal exposure and transfer of contaminants may be important mechanisms of impaired reproductive success in amphibians, but most of the information regarding the effects of contaminants on amphibian development is from aqueous exposure of embryos (Linder and Grillitsch, 2000). In amphibians, developmental processes dominate the embryonic stage, however, considerable growth and development continues during the larval stage, culminating in metamorphosis. Only two studies have examined the effects of maternal transfer of contaminants on amphibian offspring (Hopkins et al., 2006; Kotyzova and Sundeman, 1998), and there are few studies of the potential for latent effects of maternal exposure to contaminants beyond embryonic development. Much like DES, it is possible that the effects of maternal transfer of contaminants could manifest during critical developmental periods weeks to months after hatching (Budischak et al., 2008; Rohr and Palmer, 2005).

The current study sought to investigate the effects of maternal exposure to mercury (Hg) on the reproductive success of female amphibians across a large Hg-contamination gradient and the subsequent development of their offspring through metamorphosis at the extremes of this gradient. Mercury is an environmental contaminant of global concern due to its ubiquity, toxicity, and ability to bioaccumulate in animals, especially as (mono)methylmercury (MMHg) (Fitzgerald et al., 1998; Mason et al., 1996). Due to the neurotoxic, teratogenic, and endocrine-

disrupting nature of Hg, subtle effects on behavior and reproduction may occur at concentrations well below levels associated with overt toxicity and death (Scheuhammer, 1991; Weiner and Spry, 1996). Indeed, reproductive success is the demographic parameter expected to be most affected by exposure to Hg in fish and birds (Crump and Trudeau, 2009; Scheuhammer et al., 2007; Weiner and Spry, 1996). Although there is little information about the effects of Hg in amphibians, larval development and metamorphic climax are both stages during ontogeny when amphibians are known to be sensitive to Hg exposure (Unrine et al., 2004). In a previous study, we determined that *Bufo americanus* (American toads), one of the most common amphibians inhabiting the floodplain of the historically Hg-contaminated South River, VA, USA (Carter, 1977), maternally transferred Hg to their eggs (Bergeron et al., 2010a). Here, we predicted offspring from female *B. americanus* collected at Hg-contaminated sites would be negatively affected by the maternal transfer of Hg through both decreased embryonic viability (i.e., decreased hatching success and increased frequency of morphological abnormalities) and decreased metamorphic success due to the latent effects of Hg during the larval stage, thus decreasing the female's overall reproductive success.

Material and Methods

Breeding and egg collection

We captured amplexing pairs of *B. americanus* in March and April of 2007 (n = 53) and 2008 (n = 30) from breeding pools located within 180 m of the South River, along a broad contamination gradient upstream (river mile [RM] -1.7 and -5; n = 24) and downstream (RM 2, 5, 9, 16, and 20; n = 59) of a Hg contamination source (RM 0; see Bergeron et al., 2010b for additional information). The South River was historically contaminated with mercuric sulfate

used by a manufacturing plant in Waynesboro, VA (Carter, 1977) and an analysis of surface water and sediment at the South River and the reference sites confirmed that Hg was the primary contaminant while organochlorine pesticides, polycyclic aromatic hydrocarbons, and other trace metals, such as cadmium, copper, chromium, lead, selenium, and zinc, were generally low (URS, 2007).

To determine whether Hg maternally transferred to eggs in *B. americanus* has any effect on reproductive success and development, we followed the methods of Hopkins et al. (2006) and Bergeron et al. (2010a). Briefly, amplexing pairs of *B. americanus* were transported to the laboratory where they were allowed to breed in dechlorinated tap water. We recorded mass and snout-vent length (SVL) of females after oviposition. In 2007, we held most females for an additional 48 hrs to void gut contents, then collected blood and sacrificed them with an overdose of buffered tricaine methane sulfonate (MS-222) to examine relationships between Hg in female carcasses and blood/eggs (see Bergeron et al., 2010a; 2010b). In a parallel study we determined that the percentage of Hg that was methylated (MMHg) in female carcass, blood, and eggs was 53.3 ± 2.3%, 71.4 ± 2.8%, and 47.8 ± 3.3% (mean ± 1 standard error hereafter), respectively (Bergeron et al., 2010a). Thus, in the present study, we report only total Hg (THg) concentrations for these tissues. By establishing mathematical relationships between Hg in female blood and carcass in this supporting study (Bergeron et al., 2010a), we were also able to avoid sacrificing females in 2008 and instead analyzed blood for Hg analysis. Females and males were individually marked by toe clipping and released at their point of capture.

Embryonic developmental assessment

After determining the clutch size of each female, we allocated subsets from each clutch for Hg analyses, hatching and morphological assessments, and mesocosm experiments (2008 only). To assess hatching and morphological development, subsets of 500 eggs from 52 females were allowed to develop to hatching (~ Gosner stage [GS] 20) at 17-20°C in ~3 L of dechlorinated tap water. Hatchlings from each subset were counted to quantify hatching success, and then fixed in formalin and stored in 70% ethanol. We classified each hatchling as either morphologically "normal" or "abnormal" according to the methods of Bantle et al. (1991) using a dissecting microscope. Morphological abnormality classifications included edema or swelling, craniofacial malformations, and/or four types of axial malformations (dorsal flexure, lateral flexure, wavy tail, and axial shortening). All morphological assessments were performed blind to female identity. Finally, we calculated the overall viability of embryos in each clutch by combining hatching success and the frequency of abnormalities (assuming abnormal hatchlings were not viable) (Hopkins et al., 2006).

Latent effects on larvae

To examine latent effects of maternally-derived Hg on larval traits and recruitment (i.e., successful metamorphosis) at the extremes of the Hg-contamination gradient, we established replicated (n = 12) outdoor aquatic mesocosms in 1,500 L polyethylene stock tanks at Virginia Tech in Blacksburg, VA. On February 29, 2008, we filled the mesocosms with approximately 475 L of well water and 475 L of dechlorinated city water. No Hg was added to any of the mesocosms. To provide nutrients, each mesocosm received 1 kg of air-dried deciduous leaf litter (50:50 poplar and oak mix) and 17 g of finely ground Purina Rabbit Chow® (St. Louis, MO,

USA). To initiate algal and periphyton growth, we added 2 L of filtered water to each mesocosm from two ponds within Montgomery County, VA on three separate dates before March 14, 2008. To decrease the variability in initial phytoplankton communities, portions of water were repeatedly exchanged among mesocosms prior to the addition of hatchlings. We covered mesocosms with black mesh lids to provide shade and exclude predators and competitors.

After embryos hatched, "normal" hatchlings were added to the mesocosms where they remained until the initiation of metamorphic climax. Because female blood and egg THg concentrations are closely correlated (Bergeron et al., 2010a), we used female blood THg to initially characterize clutches as either reference (female blood THg concentrations < 250 ng/g, wet weight) or maternally Hg-exposed (hereafter, Hg-exposed; female blood concentrations > 1,000 ng/g, wet weight). These threshold concentrations represented the extremes of the Hg-contamination gradient as females from the reference sites did not exceed 250 ng/g THg in their blood and the upper quartile of blood from females at the South River exceeded 1000 ng/g THg. On April 9, 2008 clutches (reference $n = 6$, Hg-exposed $n = 3$) within each group were combined to homogenize genetic variation to avoid confounding mesocosm effects with clutch effects (Boone and James, 2005). Next, equal densities of 100 hatchlings were added to each randomly chosen mesocosm ($n = 6$ mesocosms/group). We monitored mesocosms daily and moved metamorphosing individuals (≥ GS 42) into the lab to complete metamorphosis. At the time of front limb emergence, we placed individuals in separate 500 ml cups with ~20 ml mesocosm water and a dry area to climb onto during tail resorption. We checked each metamorphosing tadpole once a day for mortality or completion of tail resorption (GS 46). All surviving metamorphosed toads were weighed and measured, euthanized with buffered MS-222, and then frozen for subsequent Hg analyses.

Estimating terrestrial recruitment

We estimated overall recruitment to the terrestrial environment as the percent of a clutch to metamorphose for the reference and Hg-exposed clutches by using a simple algorithm:

$$\text{Estimated recruitment (\%)} = \frac{\text{clutch size} * \text{\% viable} * \text{\% metamorphic success}}{\text{clutch size}}$$

The model incorporates clutch size and associated viability for each female sampled and relies on the mean metamorphic success from the two mesocosm groups (Hg-exposed or reference) to estimate terrestrial recruitment. We made the following assumptions: 1) hatchlings with morphological abnormalities were not viable; 2) the percent viability for the 500 egg subset is representative of the entire clutch; 3) the metamorphic success of pooled clutches from reference and Hg-exposed mesocosms at the extremes of the Hg-contamination gradient (45.3% and 54.5%, respectively) is indicative of metamorphic success of individual clutches from reference and Hg contaminated sites. While we fully recognize that this estimate is overly simplistic and does not account for individual clutch effects on larval development or important ecological factors such as density-dependence and competition, it is a useful first order approximation of metamorph production for each female.

Sample preparation and mercury analyses

We lyophilized and homogenized adult carcasses (2007), eggs (2007 and 2008), and metamorphs from the mesocosm experiment (2008) and we report THg concentrations on a dry wt basis. Whole blood from each adult *B. americanus* was homogenized using a vortex mixer

and we report THg concentrations in blood on a wet wt basis. Percent moisture was 77.8 ± 0.4% (mean ± 1 standard error of the mean hereafter) for female carcasses, 96.3 ± 0.2% for eggs, and 87.1 ± 0.1% for metamorphs. We analyzed subsamples (20-150 mg) for THg content 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 10 to 15 samples included a replicate, blank, and standard reference material (SRM; TORT-2 lobster hepatopancreas, DOLT-2 dogfish liver, DOLT-3 dogfish liver, DORM-3 fish protein [National Research Council of Canada (NRCC), Ottawa, ON] or SRM 966 Toxic Metals in Bovine Blood Level 2 [National Institute of Standards and Technology, Gathersburg, MD USA]). We calibrated the instrument using solid SRMs (TORT-2 and DORM-2 dogfish muscle [NRCC], or DOLT-3 and DORM-3). Method detection limits (MDLs; 3 times the standard deviation of procedural blanks) for samples were 0.95 ng, and all samples had THg concentrations that exceeded the limit. Average relative percent differences (RPD) between replicate sample analyses were 5.98 ± 0.75% (n=75). Mean percent recoveries of THg for the SRMs ranged from 89.66 ± 0.01 % to 106.87 ± 0.51 %.

Statistical analyses

We examined the relationship between female, blood, or egg THg concentrations and female body size on clutch size using multiple regression analyses. We used linear regression to examine the relationship between female body size and THg concentrations in tissues. We used analysis of variance (ANOVA) to test for differences in female body size and clutch size by year.

To assess the effects of Hg on embryonic development, we used linear regression to describe the relationships between female body THg concentrations (log-transformed) in 2007 (the only year we sacrificed females) and hatching success, the frequency of abnormalities, or viability (all angular-transformed). We used analysis of covariance (ANCOVA) to compare relationships between blood or egg THg concentrations (log-transformed) and hatching success, the frequency of abnormalities, or viability (all angular-transformed) between years (2007 and 2008). Although female body size (SVL) did not influence hatching success or viability, there was a weak tendency for it to influence abnormalities ($r = 0.243$, $p = 0.083$), where smaller females produced more abnormal individuals. Thus, to correct for body size, we used the residuals of SVL and the frequency of abnormalities in the linear regression and ANCOVA analyses.

To compare development of larvae in mesocosms from reference and Hg-exposed females, we used multivariate analysis of variance (MANOVA) with several parameters describing traits of recruits to the terrestrial environment. These endpoints included: SVL and condition ($mass/SVL^3*10^5$) at the completion of metamorphosis (GS 46), days until the beginning of metamorphoic climax (GS 42), days to complete metamorphic climax (from GS 42 to 46), and the percentage of individuals to complete metamorphosis. Mean responses of each mesocosm were used in all statistical analyses. Lastly, we used linear regression to examine the relationship between THg concentrations in eggs and blood and estimated recruitment.

We performed all analyses with SAS 9.1 (SAS Institute, Cary, NC, USA) and used $\alpha = 0.05$ to determine statistical significance.

Results

Effects of mercury on clutch characteristics

There were no significant effects of female, egg, or blood THg concentrations on clutch size ($p > 0.244$, for all), but female mass and SVL were both positively correlated with clutch size ($p < 0.0001$, for all). Females were larger in 2008 (70.5 ± 2.8 g, 87.17 ± 1.58 mm) than in 2007 (56.1 ± 2.1 g, 80.38 ± 0.74 mm; $F_{1,80} > 16.68$, $p < 0.0001$, for both), resulting in larger clutch sizes in 2008 (mean, SE: 7,495 ± 495) compared to 2007 (6,078 ± 366; $F_{1,80} = 5.28$, $p = 0.024$). In 2007, there was no relationship between female body THg concentrations and SVL ($r^2 = 0.022$, $p = 0.319$, n = 48). However, with both years combined, female SVL was positively correlated with blood and egg THg concentrations ($r^2 = 0.073$, $p = 0.013$, n = 83 and $r^2 = 0.089$, $p = 0.006$, n = 83, respectively), suggesting a trend towards larger females accumulating and transferring more Hg to their eggs.

Effects of mercury on embryonic development

The percentage of embryos that successfully hatched (clutch range: 0.6-100%) decreased with increasing THg concentrations. This relationship was best explained by the correlation with female body THg concentrations in 2007 (Fig 1a; $r^2 = 0.472$, $p < 0.001$, $n = 23$). A strong negative relationship was also found between maternal blood THg and hatching success (Fig 1b; $F_{1,53} = 13.11$, $p < 0.001$) and between egg THg and hatching success (Fig 1c; $F_{1,53} = 19.27$, $p < 0.001$). For blood and eggs, the effect of THg on hatching success was consistent across years ($F < 0.93$, $p > 0.339$, for all year and THg by year combinations).

We assessed the morphology of newly hatched embryos from 52 clutches ($n = 20,551$ hatchlings). Contrary to our predictions, the frequency of abnormalities (clutch range: 0.2 –

56%) decreased as THg concentrations increased in female body (Fig 2a; $r^2 = 0.297$, $p = 0.013$), blood (Fig 2b; $F_{1,48} = 7.35$, $p = 0.009$), and eggs (Fig 2c; $F_{1,48} = 9.15$, $p = 0.004$). For blood and eggs, the effect of THg on the frequency of abnormalities was consistent across years ($F < 2.10$, $p > 0.154$, for all year and THg by year combinations). Of the total abnormalities observed, 83% were axial, 12% were craniofacial, 3.2% were edema, and 4.4% were considered "other" which included abnormal gut coiling, reduced tail margins, and no head.

The decrease in the frequency of abnormalities with increasing THg concentrations was not sufficient to offset the effect of decreased hatching success on overall hatchling viability (clutch range: 0.4 – 95%), which decreased with increasing THg concentrations in female body (Fig 3a: $r^2 = 0.292$, $p = 0.014$, $n = 20$), blood (Fig 3b: $F_{1,48} = 5.55$, $p = 0.023$), and eggs (Fig 3c: $F_{1,48} = 6.88$, $p = 0.012$). Again, for blood and eggs, the effect of THg on viability was consistent across years ($F < 0.37$, $p > 0.544$, for all year and THg by year combinations).

Effects of maternal mercury on larval development

The mean THg concentrations in the blood of females from the reference and contaminated site used in the mesocosm experiment were 181.6 ± 28.0 and 2,122.0 ± 480.4 ng/g, respectively. The corresponding mean THg concentrations in the eggs were 29.8 ± 3.5 and 286.1 ± 38.3 ng/g for the reference and contaminated clutches, respectively. The overall MANOVA model describing metamorphic responses to maternal Hg exposure extremes at the South River revealed a significant difference between the Hg-exposed and reference groups (Pillai's Trace = 0.80, $F_{5,6} = 4,76$, $p = 0.042$). Component ANOVAs revealed no difference ($p > 0.133$, for all) between the reference and Hg-exposed groups in SVL (mm: 13.8 ± 0.2 and 13.0 ± 0.5), body condition (9.0 ± 0.2 and 9.4 ± 0.3), days to the start of metamorphic climax (47.8 ± 0.5 and 49.0

± 0.6), and days to complete metamorphic climax (5.2 ± 0.1 and 5.3 ± 0.1), respectively. Although the significance of the MANOVA may be attributable to the variance properties of the combined endpoints rather than a single strong effect on any single endpoint, the most notable difference was a 21% decrease in metamorphic success in the reference group (45.3 ± 3.8%) compared to the Hg-exposed group (54.5 ± 2.9%; p = 0.084). Because individuals were not subjected to additional exposure of excessive Hg during larval development, THg concentrations (dry weight) in metamorphs were low and did not differ between individuals exposed as embryos to reference and high maternal Hg (42.1 ± 2.2 ng/g versus 43.1 ± 2.4 ng/g; $F_{1,10} = 0.09$, $p = 0.776$).

We found no significant relationship between the THg in the eggs or maternal blood and estimated recruitment (Fig 4; $r^2 = 0.024$, $p = 0.273$ and $r^2 = 2.8\text{x}10^{-5}$, $p = 0.970$, respectively), suggesting that the negative effect of maternal Hg exposure on the embryonic stage was offset by enhanced survival during the larval stage, resulting in no effect on the number of juveniles entering the terrestrial environment.

Discussion

Individuals can be directly affected by their environment, but also indirectly affected by the environment in which their parents and even their grandparents inhabited (Bernardo, 1996; Mousseau and Fox, 1998; Rossiter, 1996). Because these environmental factors can influence offspring phenotypes, there are widespread implications for both wildlife and human health. For instance, parental exposure to environmental contaminants or poor nutritional state can have negative, transgenerational effects on adult offspring in terms of disease susceptibility and reproductive success (Anway and Skinner, 2006; Bateson et al., 2004). In some cases, there are

compensatory mechanisms within an individual to offset the effects of the maternal environment either during the embryonic stage or later in development. For example, in mice, developmental exposure to the endocrine-active chemical, bisphenol A (BPA) modified the epigenome via DNA methylation. However, maternal nutrient supplementation negated the hypomethylating effect of BPA (Dolinoy et al., 2007). Alternatively, individuals that experience nutritional deficits during early development can compensate by accelerating growth in a later stage if conditions improve, however this compensatory growth can be associated with long-term costs in the adult stage (Metcalfe and Monaghan, 2001). Interestingly, here we demonstrate a counterbalancing effect within an amphibian population. Although we found a negative effect of maternal Hg exposure on amphibian reproduction through reduced embryonic viability, the effect was counterbalanced by relatively high metamorphic success in surviving larvae from Hg-exposed females at the extreme of the Hg-contamination gradient. Based on a simple model that combines survival from these two stages of ontogeny, we observed no net effect of maternal Hg exposure on estimated terrestrial recruitment, suggesting the counterbalancing effect could have important ecological consequences for the population.

Maternal exposure to environmental contaminants can negatively affect reproduction by influencing a variety of factors including egg production, embryo viability, and successful offspring development. Maternal Hg exposure did not affect female clutch size in *B. americanus*, even at the elevated concentrations of Hg documented in this study. This stands in contrast to other studies that have found impaired ovary development, egg production, and spawning success in females of oviparous species exposed to Hg, particularly in fish (reviewed in Crump and Trudeau, 2009; Tan et al., 2009). Alternatively, even though female *B. americanus* only transfer a small proportion (~5%) of their pre-ovipositional Hg body burden to

their eggs (Bergeron et al., 2010a), the resulting Hg concentrations in eggs appear to be sufficient to decrease hatching success. Although correlative, this relationship provides compelling evidence that female amphibians can maternally transfer a contaminant and influence the survival of offspring in the early stages of development.

Offspring that successfully hatch may be at a functional disadvantage due to the effect of maternal exposure to contaminants on embryonic neurodevelopment or morphology. Contrary to the prevailing body of literature on Hg and many other contaminants (e.g., Weis and Weis, 1991) as well as our predictions, we found that the frequency of abnormalities decreased with increasing Hg concentrations. Our findings suggest that Hg induces mortality rather than a teratogenic effect in amphibian embryos from high Hg clutches. Consequently, we hypothesize that a greater proportion of lower quality embryos hatched successfully in clutches from reference mothers, resulting in a greater proportion of abnormal hatchlings. However, the decrease in the frequency of abnormalities in high Hg clutches was not sufficient to offset the effect of decreased hatching success, and ultimately, overall hatchling viability decreased with increasing Hg concentrations. Interestingly, the strongest correlation between Hg concentrations in all the tissues sampled (maternal carcass, maternal blood, and eggs) and the embryonic developmental endpoints (hatching success, frequency of abnormalities, and viability) were with maternal carcass. We expected the strongest correlations to be with Hg concentrations in eggs, assuming the direct effect of transferred Hg causes the reduction in viability. The stronger relationship between female Hg concentrations and embryonic development suggests that the effects of Hg on the female's reproductive axis or other aspects of her physiology might contribute to the observed embryonic effects. As a result, exposure to Hg could lead to suboptimal egg quality or maternal deposition of other hormones (e.g., stress hormones) into the

egg. For example, Verboven et al. (2009) found egg quality in Glaucous Gulls (*Larus hyperboreus*) exposed to persistent organic pollutants may be affected by the direct maternal transfer of pollutants to eggs and indirectly through changes in egg size and composition (lipid and water content).

Few studies have investigated the long-term effects of maternal transfer of contaminants on offspring development despite the fact that important organizational events often occur early in development. This is one of the first studies in amphibians to examine potential long-term or latent effects of maternal contaminant exposure during the larval period. We found no difference in body size or condition, time to metamorphosis, or days to complete tail resorption between the metamorphs from reference or Hg-exposed groups. However, contrary to our predictions, we found that larvae from Hg-exposed group actually had greater metamorphic success than larvae from reference group. Based on these observations, we hypothesize that the poorest quality embryos in the clutch were eliminated due to elevated maternal Hg exposure and the surviving larvae were more robust than larvae from the reference clutches, which were not subjected to similar selective pressures. In our simplified developmental environments (i.e., excluding predators and heterospecific competitors), the "selection" by Hg for more robust larvae appears to be advantageous. However, Hg is a neurotoxicant with known effects on behavior and performance, even when maternal exposure is the only exposure to Hg. For example, larval Atlantic croaker (*Micropogonias undulates*) from parents fed MMHg-contaminated diets showed reduced performance including altered swimming behavior and startle response (Alvarez et al., 2006). Thus, it remains unknown how amphibian larvae maternally-exposed to Hg would respond to predators or intense competition.

To assess the overall impact of maternal Hg exposure in *B. americanus* on the number of surviving metamorphs, we used a simple model to estimate recruitment to the terrestrial environment. We found that the negative effect of maternal Hg exposure on viability in the embryonic stage was counterbalanced by the selection for more robust larvae in the Hg-exposed group, resulting in no differences in recruitment across a wide range of Hg concentrations. This counterbalancing effect is a significant finding because it suggests that, assuming all else is equal, maternal Hg exposure ultimately has little effect on amphibian juvenile recruitment, which can later influence the future reproducing population (Beebee et al., 1996; Berven, 1990). However, while juvenile recruitment may be unaffected, we did not investigate the potential impact of maternal Hg exposure on the population through changes in genetic variability. For example, the selection for robust larvae in the Hg-exposed group may drive evolution in ways that are not adaptive in terms of resistance to natural stressors (e.g., predation or disease) (Medina et al., 2007). In addition, it is possible that the counterbalancing effect may produce variable results under natural conditions because we began the larval experiment with equal densities of hatchlings in each of the simulated ponds (mesocosms) and did not account for known density-dependent interactions in amphibian larvae. For example, in natural conditions, if hatching success was reduced in Hg-contaminated ponds, the density of surviving larvae would be reduced, releasing them from competitive pressure and potentially increasing their metamorphic success beyond that observed in this experiment compared to larvae from reference ponds. Because competition is an important factor influencing metamorphic success of amphibians (e.g., Semlitsch and Caldwell, 1982; Wilbur, 1977), demographic population modeling (e.g., Vonesh and De la Cruz, 2002) will ultimately be a more informative method to estimate recruitment provided that juvenile and adult parameters can be obtained or estimated.

The maternal environment can greatly influence reproductive conditions and both the immediate and long-term development of offspring. Our findings shed further insight into the effects of maternal contaminant exposure on reproductive success and are among the first to correlate contaminant concentrations in the field with deleterious effects (reduced hatchling viability) on amphibian reproduction. In addition, comparatively few studies investigate the long-term consequences of maternal transfer of contaminants. Our work demonstrates that maternal effects which manifest at different stages in ontogeny have the potential to offset one another. This is of broad importance because it suggests that advantageous or disadvantageous parental effects on survival during early life stages may be counterbalanced in a later stage, resulting in no net effect on recruitment to the adult population. However, because maternal effects are highly context-dependent, future studies should account for differing environmental circumstances. For example, an important next step will be to incorporate greater environmental complexity by determining whether the latent effects of Hg are altered under more environmentally realistic situations where individuals are required to compete for resources with heterospecifics or avoid predators. In addition, larval amphibians are highly efficient at accumulating Hg compared to other life stages (Bergeron et al., 2010b), and exposure to dietary Hg alone may have negative effects on larval recruitment (Unrine et al., 2004). Thus, it is important to determine whether maternally-derived Hg interacts additively or synergistically with larval dietary exposure to negatively impact the number and size of individuals recruited to the local population.

Acknowledgements

We thank the landowners along the South River and the Waynesboro Parks and Recreation Department for access to sampling locations, and D. Cristol, J. Schmerfeld, H. Brant, J. Burke, J. Callihan, S. DuRant, and S. Orlofske for their support and field and/or laboratory assistance. We thank B. Todd, C. Rowe, and J. Willson for reviewing earlier drafts of this manuscript. Collection of animals was in conformance with appropriate permits, and sample methods were in compliance with Virginia Polytechnic and State University's animal care and use protocols. Research was completed with oversight from the South River Science Team which is a collaboration of state and federal agencies, academic institutions, and environmental interests. Financial support to WAH was provided by E. I. DuPont de Nemours, startup funds from Virginia Polytechnic and State University, and by the National Science Foundation (NSF # IOB-0615361). During the preparation of this manuscript, CMB was supported by the U.S. EPA STAR Graduate Fellowship (FP-9170040-1). The information presented here has not been subjected to review by the supporting agencies and no official endorsement should be inferred.

Literature Cited

Alvarez MD, Murphy CA, Rose KA, McCarthy ID, Fuiman LA. Maternal body burdens of methylmercury impair survival skills of offspring in Atlantic croaker (*Micropogonias undulatus*). Aquat Toxicol 2006; 80: 329-337.

Anway MD, Skinner MK. Epigenetic transgenerational actions of endocrine disruptors. Endocrinology 2006; 147: S43-S49.

Bantle JA, Dumont JN, Finch R, Linder G. Atlas of abnormalities: A guide for the performance of FETAX. Stillwater, OK: Oklahoma State Publications Department, 1991.

Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, et al. Developmental plasticity and human health. Nature 2004; 430: 419-421.

Beebee TJC, Denton JS, Buckley J. Factors affecting population densities of adult natterjack toads *Bufo calamita* in Britain. J Appl Ecol 1996; 33: 263-268.

Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA. Bioaccumulation and maternal transfer of mercury and selenium in amphibians. Environ Toxicol Chem 2010a; 29: 989-997.

Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA. Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians. Environ Toxicol Chem 2010b; 29: 980-988.

Bernardo J. Maternal effects in animal ecology. Am Zool 1996; 36: 83-105.

Berven KA. Factors affecting population fluctuations in larval and adult stages of the wood frog (*Rana sylvatica*). Ecology 1990; 71: 1599-1608.

Blus LJ. DDT, DDD, and DDE in Birds. In: Beyer WN, Heinz GH, Redmon-Norwood AW, editors. Environmental contaminants in wildlife: Interpreting tissue concentrations. Lewis Publishers, Boco Raton, FL, 1996, pp. 49-71.

Boone MD, James SM. Aquatic and terrestrial mesocosms in amphibian ecotoxicology. Applied Herpetology 2005; 2: 231-257.

Budischak SA, Belden LK, Hopkins WA. Effects of malathion on embryonic development and latent susceptibility to trematode parasites in ranid tadpoles. Environ Toxicol Chem 2008; 27: 2496-2500.

Carter LJ. Chemical-plants leave unexpected legacy for two Virginia rivers. Science 1977; 198: 1015-1020.

Corn PS. Amphibian declines: Review of some current hypotheses. In: Sparling DW, Linder G, Bishop, CA, editor. Ecotoxicology of Amphibians and Reptiles. SETAC Press, Pensacola, FL, USA, 2000, pp. 663-696.

Crump KL, Trudeau VL. Mercury-induced reproductive impairment in fish. Environ Toxicol Chem 2009; 28: 895-907.

Dolinoy DC, Huang D, Jirtle RL. Maternal nutrient supplementation counteracts bisphenol A-induced DNA hypomethylation in early development. Proceedings of the National Academy of Sciences of the United States of America 2007; 104: 13056-13061.

Fitzgerald WF, Engstrom DR, Mason RP, Nater EA. The case for atmospheric mercury contamination in remote areas. Environ Sci Technol 1998; 32: 1-7.

Guillette LJ, Crain DA, Gunderson MP, Kools SAE, Milnes MR, Orlando EF, et al. Alligators and endocrine disrupting contaminants: A current perspective. Am Zool 2000; 40: 438-452.

Hopkins WA, DuRant SE, Staub BP, Rowe CL, Jackson BP. Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. Environ Health Perspect 2006; 114: 661-666.

Kotyzova D, Sundeman FW. Maternal exposure to Cd(II) causes malformations of *Xenopus laevis* embryos. Annals of Clinical and Laboratory Science 1998; 28: 224-235.

Linder G, Grillitsch B. Ecotoxicology of metals. In: Sparling DW, Linder G, Bishop, CA, editor. Ecotoxicology of Amphibians and Reptiles. SETAC Press, Pensacola, FL, 2000, pp. 325-459.

Lindstrom J. Early development and fitness in birds and mammals. Trends Ecol Evol 1999; 14: 343-348.

Mason RP, Reinfelder JR, Morel FMM. Uptake, toxicity, and trophic transfer of mercury in a coastal diatom. Environ Sci Technol 1996; 30: 1835-1845.

Medina MH, Correa JA, Barata C. Micro-evolution due to pollution: Possible consequences for ecosystem responses to toxic stress. Chemosphere 2007; 67: 2105-2114.

Metcalfe NB, Monaghan P. Compensation for a bad start: grow now, pay later? Trends Ecol Evol 2001; 16: 254-260.

Mousseau TA, Fox CW. Maternal Effects as Adaptations. New York: Oxford University Press, 1998.

Rohr JR, Palmer BD. Aquatic herbicide exposure increases salamander desiccation risk eight months later in a terrestrial environment. Environ Toxicol Chem 2005; 24: 1253-1258.

Rossiter MC. Incidence and consequences of inherited environmental effects. Annu Rev Ecol Syst 1996; 27: 451-476.

Russell RW, Gobas F, Haffner GD. Maternal transfer and in ovo exposure of organochlorines in oviparous organisms: A model and field verification. Environ Sci Technol 1999; 33: 416-420.

Scheuhammer AM. Effects of acidification on the availability of toxic metals and calcium to wild birds and mammals. Environ Pollut 1991; 71: 329-375.

Scheuhammer AM, Meyer MW, Sandheinrich MB, Murray MW. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. Ambio 2007; 36: 12-18.

Semlitsch RD, Caldwell JP. Effects of density on growth, metamorphosis, and survivorship in tadpoles of *Scaphiopus holbrooki*. Ecology 1982; 63: 905-911.

Swan SH. Intrauterine exposure to diethylstilbestrol: Long-term effects in humans. Apmis 2000; 108: 793-804.

Tan SW, Meiller JC, Mahaffey KR. The endocrine effects of mercury in humans and wildlife. Crit Rev Toxicol 2009; 39: 228-269.

Unrine JM, Jagoe CH, Hopkins WA, Brant HA. Adverse effects of ecologically relevant dietary mercury exposure in southern leopard frog (*Rana sphenocephala*) larvae. Environ Toxicol Chem 2004; 23: 2964-2970.

URS. Evaluation for Removal of Organochlorine Pesticides, Polycyclic Aromatic Hydrocarbons, and Trace Metals Analyses in the Phase I System Characterization, South River, VA. Appendix F, Phase I, Year 1 Progress Report: Ecological Study of the South River and a Segment of the South Fork Shenandoah River, Virginia. Contract No.: 18985057.70030. 2007.

USEPA. Method 7473: Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrometry, Washington, D.C., USA, 1998, pp. 1-15.

Verboven N, Verreault J, Letcher RJ, Gabrielsen GW, Evans NP. Differential investment in eggs by Arctic-breeding Glaucous Gulls (Larus hyperboreus) exposed to persistent organic pollutants. Auk 2009; 126: 123-133.

Vonesh JR, De la Cruz O. Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. Oecologia 2002; 133: 325-333.

Weiner JG, Spry DJ. Toxicological significance of mercury in freshwater fish. In: Beyer WN, Heinz GH, Redmon-Norwood AW, editors. Environmental contaminants in wildlife: Interpreting tissue concentrations. Lewis Publishers, Boca Raton, FL, 1996, pp. 297-340.

Weis P, Weis JS. The developmental toxicity of metals and metalloids in fish. In: Newman MC, McIntosh AW, editors. Metal Ecotoxicology: Concepts and Applications. Lewis Publishers, Chelsea, MI, 1991, pp. 145-169.

Wilbur HM. Density-dependent aspects of growth and metamorphosis in *Bufo americanus*. Ecology 1977; 58: 196-200.

Figure 4.1: Relationship between log total mercury (THg; ng/g) concentration and the percentage of embryos that successfully hatched (angular transformation) in A) female body (dry weight; 2007 only), B) female blood (wet weight) and C) eggs (dry weight) from the reference (open symbols) and contaminated (closed symbols) portion of the South River (VA, USA) in 2007 (circle symbols) and 2008 (square symbols). Note that the y-axis is expressed as an angular transformation of the percent hatching.

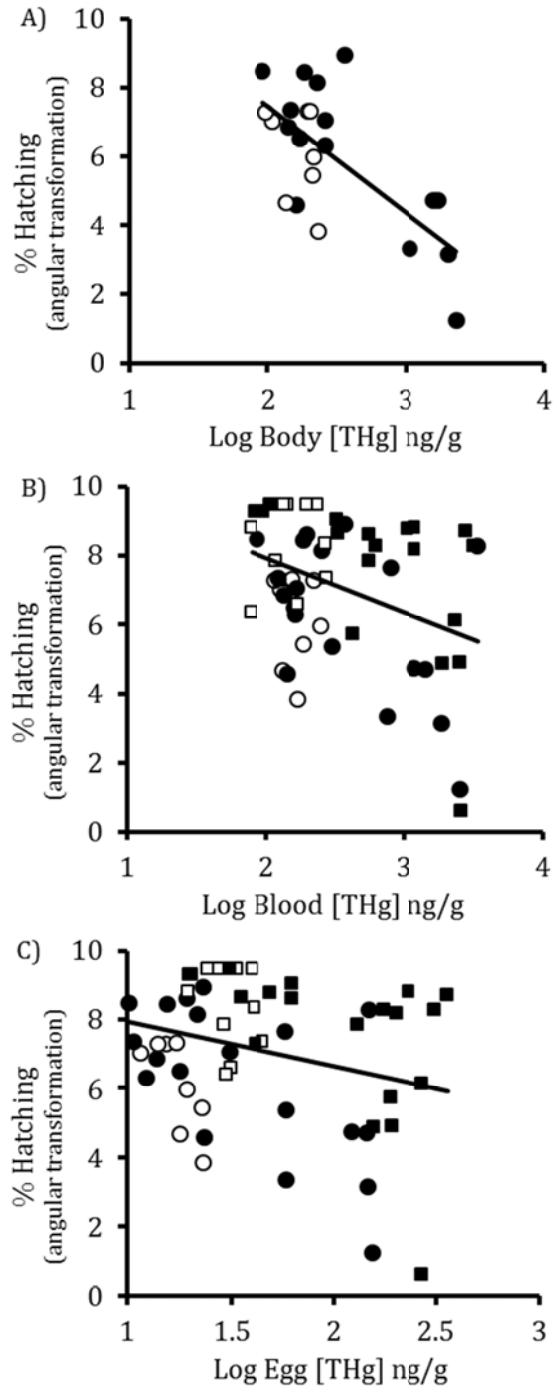


Figure 4.2: Relationship between log total mercury (THg; ng/g) concentration and the percentage of abnormal hatchlings (angular transformation) corrected for body size (snout-vent length; SVL) in A) female body (dry weight; 2007 only), B) female blood (wet weight) and C) eggs (dry weight) from the reference (open symbols) and contaminated (closed symbols) portion of the South River (VA, USA) in 2007 (circle symbols) and 2008 (square symbols).

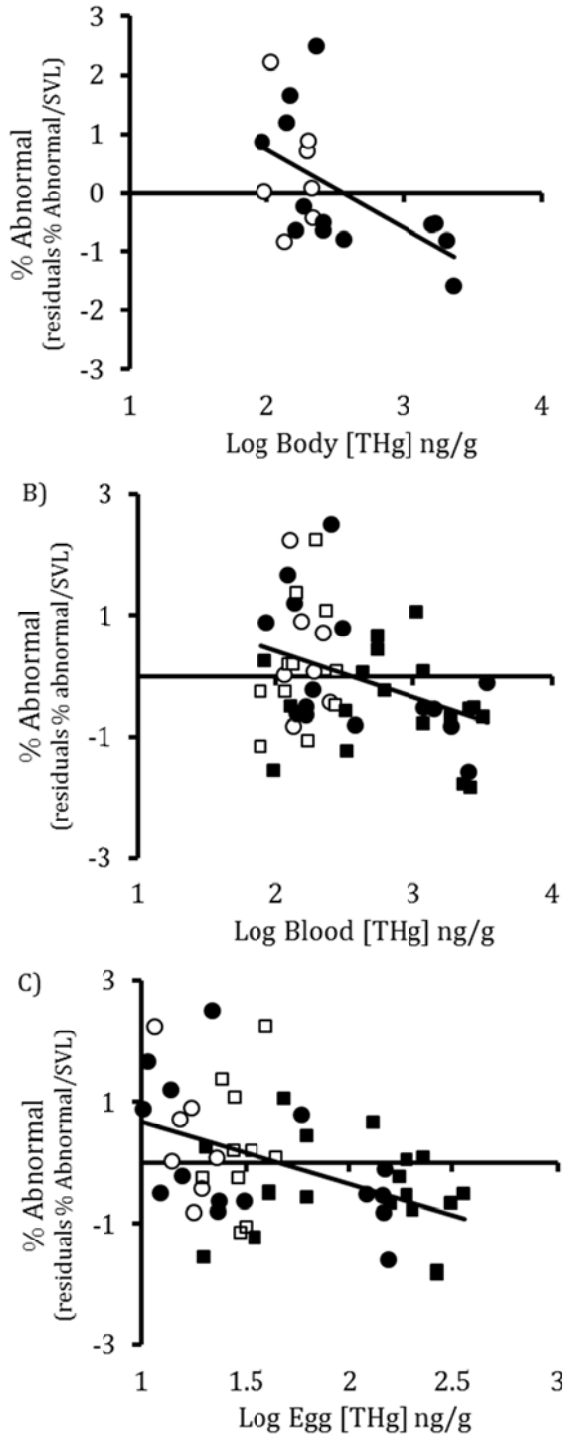


Figure 4.3: Relationship between log total mercury (THg; ng/g) concentration and the percentage of viable hatchlings (angular transformation) in A) female body (dry weight; 2007 only), B) female blood (wet weight) and C) eggs (dry weight) from the reference (open symbols) and contaminated (closed symbols) portion of the South River (VA, USA) in 2007 (circle symbols) and 2008 (square symbols). Overall viability of embryos in each clutch was estimated by combining hatching success and the frequency of abnormalities (assuming abnormal hatchlings were not viable). Note that the y-axis is expressed as an angular transformation of the percent viable.

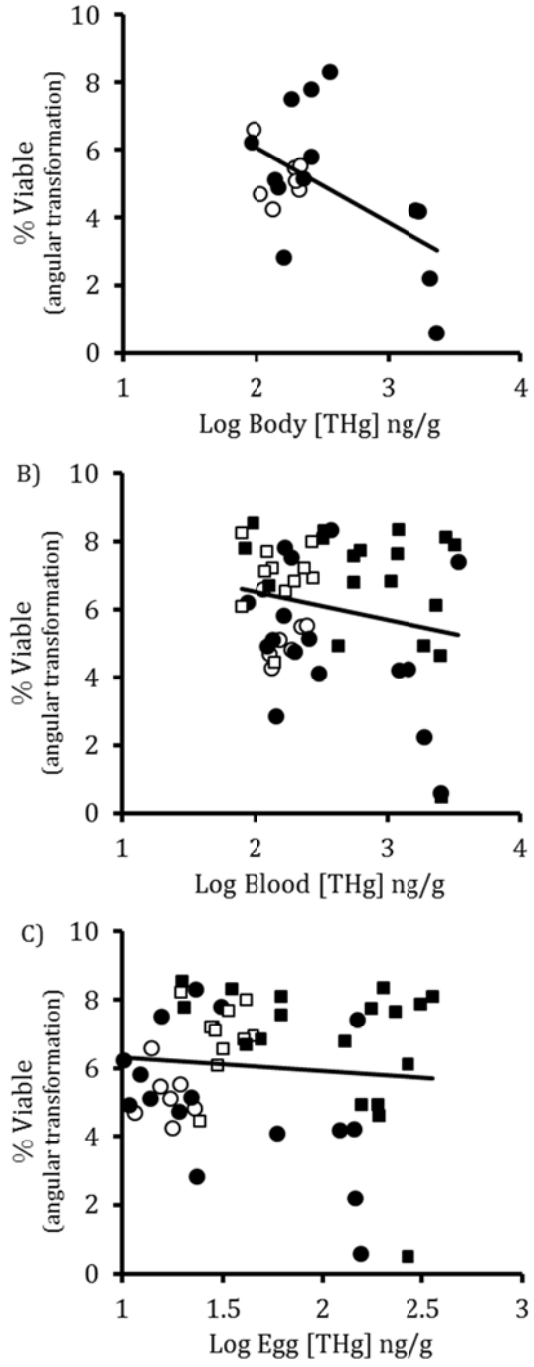
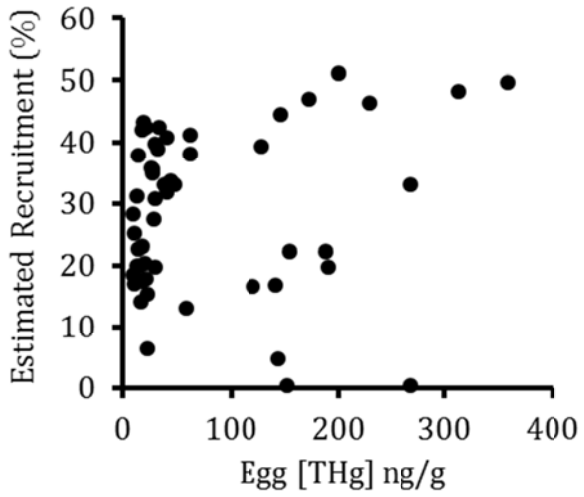


Figure 4.4: Relationship between total mercury (THg; ng/g, dry weight) concentrations in eggs and estimated recruitment (%) ($r^2 = 0.024$, $p = 0.273$).



Chapter 5: Inter-annual variation in the ontogenetic onset of adverse effects of maternal transfer in amphibians

Christine M. Bergeron
Co-Authors: Brian D. Todd and William A. Hopkins

Formatted for and submitted to *Environmental Toxicology & Chemistry*

Abstract

Maternal transfer of contaminants often results in negative consequences. We examined the effects of maternal mercury (Hg) exposure in amphibians and found that negative effects manifested either immediately at the embryonic stage or later during the larval stage, providing an unexpected demonstration of annual variation in the ontogenetic onset of maternal effects. However, the net result was no considerable difference in estimated recruitment in any year.

Keywords: amphibians, latent effects, maternal transfer, mercury

Introduction

Maternal effects occur when the physiology or behavior of a mother influences the phenotype of her offspring. These effects can influence offspring size, performance, and survival [1]. Maternal effects can greatly affect offspring during early ontogeny [2], when key organizational events shape developmental trajectories [3]. Maternal effects can also manifest at later stages of development [4] and may be influenced by environmental factors, having widespread implications for both wildlife and human health. For example, poor pre-breeding nutritional state in female zebra finches (*Taeniopygia guttata*) resulted in latent effects on

offspring fecundity; upon reaching adulthood, offspring from nutritionally-stressed females had lower fecundity compared to offspring from females in a better nutritional state [5]. Similarly, in humans, poor nutrition or exposure to environmental contaminants *in utero* may increase later susceptibility as adults to reproductive dysfunction and diseases such as hypertension, coronary heart disease, and type II diabetes [6, 7].

Maternal effects on offspring fitness are highly context-dependent and can be positive or negative depending on environmental circumstances [8]. However, the maternal transfer of environmental contaminants is a maternal effect which typically has negative consequences for offspring because early development is a critical organizational period in the ontogeny of vertebrates [e.g., 9]. Latent effects of maternally-derived contaminants are less well known and are beyond the scope of most studies to date. However, increasing evidence suggests that maternal contaminant exposure can have either latent lethal [e.g., 10] or sublethal [e.g., 11, 12-15] effects on offspring due to early disruptions in nervous, endocrine, or immune system development [16].

Maternal effects that influence fecundity or survival may ultimately affect population dynamics [17]. However, the immediate or latent maternal effects of contaminant transfer on demographic characteristics are poorly understood, especially in vertebrates with complex lifecycles. In a recent study, we investigated the effects of maternal transfer of mercury (Hg), an environmental contaminant of global concern, on the reproductive success of female amphibians and the embryonic development of their offspring in two consecutive years [18]. We found that American toads (*Bufo americanus*) maternally transferred Hg to their eggs, which had a negative effect on embryonic development in both years [19]. However, this negative effect was offset by relatively high larval survival in embryos of Hg-exposed females at the high end of a Hg-

contamination gradient [18]. Thus, we observed no net deleterious effect of maternal Hg exposure on estimated terrestrial recruitment. Here, we revisit our results from the two prior years, in light of newly collected data on embryonic and larval development, to examine how maternal effects that manifest in different life stages interact. These new analyses and newer data provide an unexpected demonstration of the annual variation in directionality and timing of the maternal effects of Hg.

Materials & Methods

We captured amplexing pairs of American toads in March and April 2007 (*n*=53), 2008 (*n*=30), and 2009 (*n*=27) from breeding pools located along the floodplain of the South River, VA, USA. Toads were collected across a contamination gradient upstream (reference sites: river mile [RM] -1.7 and -5; *n*=30) and downstream (contaminated sites: RM 2, 5, 9, 16, and 20; *n*=80) from a historic Hg source (RM 0; see Fig 1 in [20]), resulting in a broad range of Hg concentrations in females and their eggs. Methods used to determine the effects of maternally transferred Hg on toad embryos and larvae are reported in Bergeron et al. [18]. Briefly, once eggs were laid under controlled conditions, we determined clutch size and allocated subsets of eggs from each clutch for Hg analysis, embryonic development, and larval development experiments (larval experiments in 2008 and 2009 only; for details see [14, 18]). Subsets of 500 eggs were removed from 81 clutches and allowed to hatch to evaluate embryonic development. Hatchlings were counted to quantify hatching success and were then individually preserved and examined under dissecting microscope for morphological abnormalities. Finally, we calculated overall viability as the product of hatching success and frequency of hatchlings with normal gross morphology (assuming abnormal hatchlings were not viable). Embryonic viability was thus

the overall percentage of embryos with normal morphology that hatched successfully for each clutch (as described in [21]).

Analyses of embryonic development in 2007 and 2008 were originally provided in Bergeron et al. [18]. Here, we include newly collected data from 2009 and use separate analyses of covariance (ANCOVA) to test for an effect of egg THg concentrations (log-transformed) on hatching success, frequency of abnormalities, and viability (all angular-transformed) among years. Although hatching success and overall embryonic viability showed no relationship with maternal body size (snout-vent length; SVL), there was a negative relationship between maternal body size and the frequency of abnormalities ($r = 0.29$, $p = 0.01$). Thus, to correct for body size, we used the residuals from the relationship between SVL and the frequency of abnormalities as a covariate in the ANCOVA analysis.

In separate experiments in 2008 and 2009, we examined the latent effects of maternally-derived Hg on larval survival through the completion of metamorphosis using experimental mesocosms (~1000 L of water) where larvae were raised in equal densities (100 larvae per mesocosm) [14, 18]. Offspring in the larval experiments were derived from the same clutches used in the aforementioned embryonic viability assays. In both years, we selected hatchlings that were morphologically normal and introduced them into mesocosms ~4 days post hatch. Each group of 100 larvae was evenly drawn from clutches of either reference or maternally Hg-exposed mothers from the extremes of the Hg-contamination gradient. No Hg was added to mesocosms.

Here, we calculated metamorphic recruitment as the product of embryonic viability of each clutch and mean larval survival of each maternal treatment (reference or maternally Hg-exposed) for both 2008 and 2009 to estimate metamorphic recruitment to the terrestrial

environment (see [18]). We used an ANCOVA to compare estimated recruitment between years using egg THg concentrations as a covariate.

Results

The percentage of embryos that successfully hatched decreased with increasing THg concentrations in 2007 and 2008, but not in 2009 (THg: $F_{1,75} = 11.64$, $p = 0.001$; year: $F_{2,75} = 3.01$, $p = 0.055$; interaction: $F_{2,75} = 4.86$, $p = 0.010$). Contrary to our predictions, the frequency of abnormalities decreased as THg concentrations increased in 2007 and 2008, but there was no relationship between THg concentrations and abnormalities in 2009 (THg: $F_{1,70} = 4.64$, $p = 0.035$; year: $F_{2,70} = 4.44$, $p = 0.015$; interaction: $F_{2,70} = 3.34$, $p = 0.041$). The effect of THg concentrations in eggs on overall embryonic viability was significant (Fig 1a; $F_{1,70} = 5.43$, $p = 0.023$). Embryonic viability differed by year ($F_{2,70} = 4.92$, $p = 0.010$), but we did not find an interaction between THg and year ($F_{2,70} = 1.73$, $p = 0.185$). The combined effect in 2007 and 2008, when reduced hatching success from Hg was not completely offset by fewer abnormalities, was an overall decrease in embryonic viability as egg THg concentrations increased. However, in 2009, embryonic viability was high across the entire gradient of egg THg concentrations.

Our evaluation of potential latent effects of maternal Hg transfer revealed annual differences in larval survival from the mesocosm experiments. In 2008, the year in which we observed a negative effect of Hg on embryonic viability, larval survival was greater in maternally-exposed offspring compared to those from reference mothers (Fig 1b). Conversely, in 2009, when embryonic viability was high regardless of Hg concentrations, larval survival was lower in maternally-exposed offspring compared to those from reference mothers (Fig 1b). Subsequently, we found a significant effect of year on recruitment (Fig 2; $F_{1,49} = 7.27$, $p <$

0.001), but no relationship between egg THg concentration and recruitment ($F_{1,49} = 0.45$, $p = 0.504$).

Discussion

Maternal effects can act directly on early offspring development and the immediate consequences to these offspring are often examined because of the relative ease of shorter-term studies at this stage. In some cases, maternal effects are strong in early development but subsequently decline [2] or even reverse direction through ontogeny [18, 22]. In other cases, maternal effects may be latent, not appearing until sometime later in ontogeny [4]. In the present study, the negative effects of maternal Hg exposure were present in all years, but manifested either immediately at the embryonic stage or later during the larval stage, depending on the year. In 2008, when embryonic viability was negatively affected by maternal Hg, the overall impact on estimated terrestrial recruitment was offset by greater larval survival from Hg-exposed mothers. In contrast, in 2009, when embryonic viability was high across the gradient of maternal Hg concentrations, subsequent survival of larvae from Hg-exposed mothers was lower than in reference larvae. Nevertheless, the net result was ultimately no considerable difference in estimated terrestrial recruitment in either of the two years because effects during embryonic and larval development offset one another.

The expression of a maternal effect can be context-dependent and may vary based on environmental circumstances. For example, the maternal effect of smaller initial body size in the amphibian *Bombina orientalis* resulted in smaller size at metamorphosis and longer larval period when reared in a low quality environment (food limited), but had little effect on larval and metamorphic traits when reared in a high quality environment (fed ad libitum) [23]. We observed

annual variation in the developmental stage most affected by maternal exposure to Hg which may have resulted from differing environmental or physiological circumstances between years. Specifically, in 2007 and 2008, early embryonic development was negatively affected by maternal Hg and larval survival of maternally exposed animals offset this decrease. In contrast, in 2009, early embryonic development was unaffected by maternal Hg but reduced larval survival of maternally exposed animals offset the earlier lack of effects. This annual variation in expression of maternal effects did not stem from differences in Hg because the range in Hg concentrations was similar among years (Fig 1a). Thus, differences in maternal influences may be attributable to some other aspect of female and/or egg quality. For example, we found an annual difference in female post-oviposition body condition (mass/SVL^3; $p = 0.026$) where females in 2009, the year when embryonic viability was high regardless of Hg concentrations, were 5 to 8% lower than in 2007 and 2008, respectively. Additionally, there was a marginally significant difference in clutch size among years (LS means corrected for body size, $p = 0.057$; 2007: 6,742 ± 320; 2008: 7,452 ± 410; 2009: 6,001 ± 444). The lower body condition and clutch size in 2009 suggests that females could have invested more in each of their offspring that year, potentially resulting in higher overall embryonic viability and larval survival than in previous years. Ultimately, the mechanism for the annual variation in the stage affected by maternal Hg exposure remains unclear. However, examining other aspects of egg quality from contaminated females, and how it influences offspring quality, is an important area for future research.

The cumulative impact of a maternal effect on a cohort of individuals [24] or the stage affected in organisms with complex lifecycles [25] has the potential to influence population dynamics. In our study, the ontogenetic timing of adverse maternal effects at the individual-level varied, but certain effects can contribute more to population-level processes than others. For

example, Vonesh and De la Cruz [25] modeled the impact of declines in each life stage of two amphibian species and found adult densities in both species were more sensitive to changes in juvenile survival than to embryonic survival. We found that regardless of which stage was affected by maternal Hg exposure, there was little difference in estimated terrestrial recruitment across a wide range of Hg concentrations. However, our larval experiments did not account for density-dependent interactions in amphibian larvae because they were initiated with equal densities of hatchlings in each of the simulated ponds (mesocosms). Because intraspecific competition among larvae is an important factor affecting larval survival in many amphibians [26, 27], it is likely that the effects of maternal Hg exposure may be annually exaggerated or dampened in the field depending on which life stage is most strongly affected. For example, when maternal transfer of Hg affects the embryonic stage only, the density of surviving larvae may be reduced, resulting in minor or even positive effects on recruitment due to reduced intraspecific competition in the larval stage.

The influence of maternal effects such as egg size and oviposition site has been widely studied in amphibians [28, 29], but our findings further demonstrate the importance of examining maternal effects at multiple developmental stages and quantifying inter-annual variation in their expression. Although the effect of maternal exposure to contaminants has received little attention in amphibians, there is growing evidence that contaminants negatively affect early amphibian development [18, 21] and contaminants are implicated as a contributing factor in worldwide amphibian declines [30]. However, relating effects on early development to population declines remains a major challenge in amphibian conservation [31]. Integrative studies examining the immediate and latent effects of maternal contaminant transfer, in the context of ecologically

relevant natural factors, such as competition and predation, are necessary to understand how such effects translate to population dynamics and long-term viability.

Acknowledgments

We thank the landowners along the South River and Waynesboro Parks and Recreation for access to sampling locations. We thank J. Schmerfeld, D. Cristol, K. Carlson-Drexler, A. Condon, M. Howie, C. Ramos, C. Bodinof, S. Budischak, J. Burke, J. Callihan, S. DuRant, M. Hepner, M.K. McCaleb, J. McPherson, and H. Wada for their support and field or laboratory assistance. This manuscript was improved by the comments of J.D. Willson. Research was completed with oversight from the South River Science Team which is a collaboration of state and federal agencies, academic institutions, and environmental interests. Collection of animals was in conformance with appropriate permits, and sample methods were in compliance with Virginia Tech's animal care and use protocols. Financial support was provided by E. I. DuPont de Nemours, the National Science Foundation (NSF # IOB-0615361), and a U.S. EPA STAR Graduate Fellowship (FP-9170040-1) to C.M.B. EPA has not officially endorsed this publication and the views expressed herein may not reflect the views of the EPA.

Literature Cited

1. Mousseau TA, Fox CW (1998) *Maternal Effects as Adaptations* (Oxford University Press, New York).
2. Lindholm AK, Hunt J, Brooks R (2006) Where do all the maternal effects go? Variation in offspring body size through ontogeny in the live-bearing fish Poecilia parae. *Biol Lett* 2(4):586-589.

3. Bernardo J (1996) Maternal effects in animal ecology. *Am Zool* 36(2):83-105.

4. Lindstrom J (1999) Early development and fitness in birds and mammals. *Trends Ecol Evol* 14(9):343-348.

5. Gorman HE, Nager RG (2004) Prenatal developmental conditions have long-term effects on offspring fecundity. *Proceedings of the Royal Society of London Series B-Biological Sciences* 271(1551):1923-1928.

6. Bateson P, Barker D, Clutton-Brock T, Deb D, D'Udine B, Foley RA, Gluckman P, Godfrey K, Kirkwood T, Lahr MM, McNamara J, Metcalfe NB, Monaghan P, Spencer HG, Sultan SE (2004) Developmental plasticity and human health. *Nature* 430(6998):419-421.

7. Heindel JJ (2007) Role of exposure to environmental chemicals in the developmental basis of disease and dysfunction. *Reprod Toxicol* 23(3):257-259.

8. Rossiter MC (1996) Incidence and consequences of inherited environmental effects. *Annu Rev Ecol Syst* 27:451-476.

9. Di Giulio RT, Tillitt DE (1999) *Reproductive and developmental effects of contaminants in oviparous vertebrates* (SETAC Press, Pensacola, FL).

10. Eisenreich KM, Kelly SM, Rowe CL (2009) Latent mortality of juvenile snapping turtles from the upper Hudson River, New York, exposed maternally and via the diet to polychlorinated biphenyls (PCBs). *Environ Sci Technol* 43(15):6052-6057.

11. Yoshida M, Shimizu N, Suzuki M, Watanabe C, Satoh M, Mori K, Yasutake A (2008) Emergence of delayed methylmercury toxicity after perinatal exposure in metallothionein-null and wild-type C57BL mice. *Environ Health Perspect* 116(6):746-751.

12. Steinberg RM, Walker DM, Juenger TE, Woller MJ, Gore AC (2008) Effects of perinatal polychlorinated biphenyls on adult female rat reproduction: Development, reproductive physiology, and second generational effects. *Biol Reprod* 78(6):1091-1101.

13. Nye JA, Davis DD, Miller TJ (2007) The effect of maternal exposure to contaminated sediment on the growth and condition of larval Fundulus heteroclitus. *Aquat Toxicol* 82(4):242-250.

14. Todd BD, Bergeron CM, Hepner MJ, Burke JN, Hopkins WA (2011) Does maternal exposure to an environmental stressor affect offspring response to predators? *Oecologia* 166(1):283-290.

15. Bergeron CM, Hopkins WA, Todd BD, Hepner MJ, Unrine JM (2011) Interactive effects of maternal and dietary mercury exposure have latent and lethal consequences for amphibian larvae. *Environ Sci Technol* 45:3781-3787.

16. Guillette LJ, Crain DA, Rooney AA, Pickford DB (1995) Organization versus activation - The role of endocrine-disrupting contaminants (EDCS) during embryonic development in wildlife. *Environ Health Perspect* 103:157-164.

17. Beckerman AP, Benton TG, Ranta E, Kaitala V, Lundberg P (2002) Population dynamic consequences of delayed life-history effects. *Trends Ecol Evol* 17(6):263-269.

18. Bergeron CM, Hopkins WA, Bodinof CM, Budischak SA, Wada H, Unrine JM (2011) Counterbalancing effects of maternal mercury exposure during different stages of ontogeny in American toads. *Sci Total Environ* 409:4746-4752.

19. Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA (2010) Bioaccumulation and maternal transfer of mercury and selenium in amphibians. *Environ Toxicol Chem* 29(4):989-997.

20. Bergeron CM, Bodinof CM, Unrine JM, Hopkins WA (2010) Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians. *Environ Toxicol Chem* 29(4):980-988.

21. Hopkins WA, DuRant SE, Staub BP, Rowe CL, Jackson BP (2006) Reproduction, embryonic development, and maternal transfer of contaminants in the amphibian *Gastrophryne carolinensis*. *Environ Health Perspect* 114(5):661-666.

22. Metcalfe NB, Monaghan P (2001) Compensation for a bad start: grow now, pay later? *Trends Ecol Evol* 16(5):254-260.

23. Parichy DM, Kaplan RH (1992) Maternal effects on offspring growth and development depend on environmental quaility in the frog *Bombina orientalis*. *Oecologia* 91(4):579-586.

24. Lindstrom J, Kokko H (2002) Cohort effects and population dynamics. *Ecol Lett* 5(3):338-344.

25. Vonesh JR, De la Cruz O (2002) Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. *Oecologia* 133(3):325-333.

26. Semlitsch RD, Caldwell JP (1982) Effects of density on growth, metamorphosis, and survivorship in tadpoles of *Scaphiopus holbrooki*. *Ecology* 63(4):905-911.

27. Wilbur HM (1977) Density-dependent aspects of growth and metamorphosis in *Bufo americanus*. *Ecology* 58(1):196-200.

28. Kaplan RH (1998) Materal effects, developmental plasticity, and life history evolution: An amphibian model. *Maternal Effects as Adaptations*, eds Mousseau TA & Fox CW (Oxford University Press, New York), pp 244-260.

29. Resetarits WJ (1996) Oviposition site choice and life history evolution. *Am Zool* 36(2):205-215.

30. Sparling DW, Linder G, Bishop CA, Krest SK (2010) *Ecotoxicology of Amphibians and Reptiles, 2nd edition* (SETAC Press, Pensacola, FL) p 944.

31. Beebee TJC, Griffiths RA (2005) The amphibian decline crisis: A watershed for conservation biology? *Biol Conserv* 125(3):271-285.

Figure 5.1: A. Relationship between total mercury (THg; ng/g) concentrations in eggs (dry weight) and embryonic viability. B. Larval survival (%) of offspring from either reference (REF) or Hg-exposed females (Hg) in 2008 and 2009.

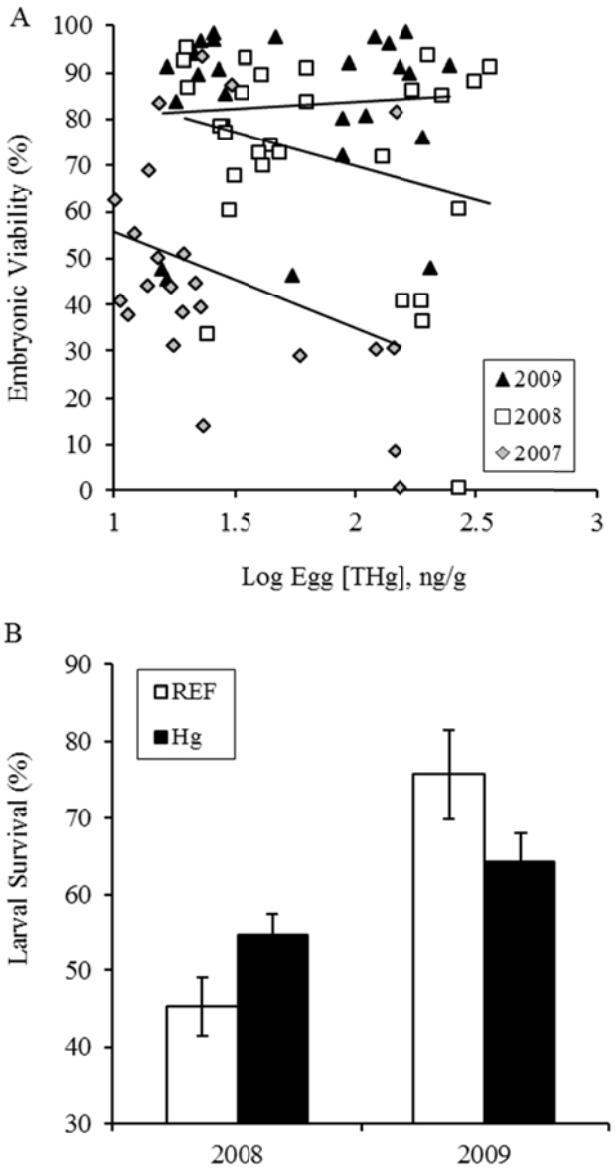
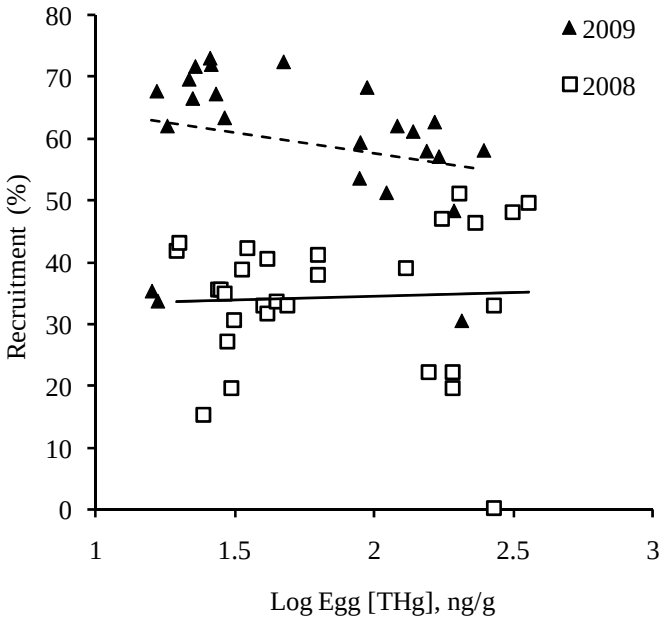


Figure 5.2: Relationship between total mercury (THg; ng/g, dry weight) concentrations in eggs and overall recruitment.



Chapter 6: Interactive effects of maternal and dietary mercury exposure have latent and lethal consequences for amphibian larvae

Christine M. Bergeron
Co-Authors: William A. Hopkins, Brian D. Todd, Mark J. Hepner, and Jason M. Unrine

Formatted for and reprinted with permission from
Environmental Science & Technology 45, 3781-3787.

Abstract

Organisms born into the same contaminated environment as their parents can be exposed both maternally and environmentally to contaminants, potentially placing them at greater risk of adverse effects than when exposed via either of the two pathways independently. We examined whether embryonic exposure to maternally-derived mercury (Hg) interacts with dietary exposure to negatively influence larval development in American toads (*Bufo americanus*). We collected eggs from breeding pairs at reference and Hg-contaminated sites and monitored performance, development, and survival of larvae fed three experimental Hg diets (total Hg: 0.01, 2.5, and 10 μg/g). The negative sublethal effects of maternal and/or dietary Hg manifested differently, but maternal Hg exposure had a greater overall influence on offspring health than dietary exposure. However, the combination of sublethal effects of the two exposure routes interacted with lethal consequences; larvae exposed to maternal Hg and high dietary Hg experienced 50% greater mortality compared to larvae from reference mothers fed the control diet. This study is the first to demonstrate that the latent effects of maternally transferred contaminants may be exacerbated by further exposure later in ontogeny, findings that may have important implications for both wildlife and human health.

Keywords: mercury, maternal transfer, amphibians, latent effects

Introduction

Early development is a critical period in ontogeny of vertebrates and adverse effects of maternally-derived contaminants during this sensitive stage have been well documented. For example, in many animals, maternal transfer of contaminants reduces reproductive success through embryo mortality and malformations (e.g., (1)). Due to early disruptions in nervous, endocrine, or immune system development (2), there is growing evidence that maternally-derived contaminants can also have effects that are expressed later in ontogeny. These latent adverse effects may be lethal, as in the case of snapping turtles (*Chelydra serpentina*), where maternal transfer of polychlorinated biphenyls (PCBs) reduced survival over the first 14 months of life (3). Alternatively, latent effects of maternal transfer of contaminants may be sublethal. The effects of maternally-derived contaminants that occur long after hatching or birth are beyond the scope of most studies, but these latent adverse effects may have important implications for both wildlife and human health.

The latent effects of maternal transfer of contaminants may manifest differently depending on the environment in which offspring develop. Offspring of organisms that forage in one location but breed in another, such as migratory birds and many amphibians, may be exposed to maternally transferred contaminants, but may or may not be further exposed to contaminants after the embryonic stage. For example, migratory white-faced ibis (*Plegadis chihi*) nesting at Carson Lake, NV, USA show reduced reproductive success with elevated egg concentrations of dichloro-diphenyldichloroethylene (DDE), but prey on the nesting grounds contained little DDE (4). However, prey in wintering habitats had elevated DDE levels, suggesting that the main source in offspring was maternal transfer from energy stores gathered at

the wintering grounds. Conversely, organisms born into the same contaminated environment as their parents can be exposed both maternally and environmentally to contaminants, potentially placing them at greater risk of adverse effects than when exposed via the two pathways independently.

There has been little research on the combined effects of multiple exposure routes through early development. However, Nye et al. (5) recently investigated the effects of maternal and direct exposure to sediments contaminated with polycyclic aromatic hydrocarbons (PAHs) from Elizabeth River, VA, USA in larvae of the estuarine fish, *Fundulus hereroclitus.* The effect of maternal exposure on growth and body condition in larvae (14 days post-hatch) was stronger than direct larval exposure (5). In another recent study, Eisenreich et al. (3) found a strong negative maternal influence of PCBs on survival of juvenile snapping turtles, but dietary PCB exposure only reduced metabolic rates. Both studies observed latent effects of maternal contaminant transfer, but neither observed interactive effects of the multiple exposure routes. Additional studies are needed to evaluate the relative contributions of maternally- and environmentally-induced variation in offspring phenotype (6) and to ensure that latent and potential interactive effects are not overlooked.

We investigated the individual and interactive effects of maternally- and trophically-derived mercury (Hg) contamination on larval development in the American toad (*Bufo americanus*). Mercury is a contaminant of global concern due to its ubiquity, toxicity, and ability to bioaccumulate and biomagnify, especially in the form of (mono)methlymercury (MMHg) (7, 8). Early life stages can be particularly sensitive to Hg exposure and subtle sublethal effects on behavior and reproduction can occur at concentrations much lower than lethal effects due to the neurotoxic and endocrine-disrupting nature of Hg (9, 10). Although there is a paucity of

information regarding the effects of Hg on amphibians compared to other vertebrates (11), amphibian larvae are highly efficient at accumulating elevated concentrations of trace elements, including Hg, due to their feeding ecology and ectothermy (12). In a previous study, we determined female American toads from a historically Hg-contaminated site maternally transferred Hg to their eggs (13), disrupting early development of embryos (14), but the effects of maternal transfer of Hg could also manifest during critical developmental periods weeks to months after hatching. In an additional field study, we found that larvae bioaccumulated very high concentrations of Hg in ephemeral breeding pools (15), suggesting significant environmental exposure occurs after maternal transfer. However, adult American toads have the opportunity to oviposit in either contaminated or uncontaminated pools because they are highly mobile for amphibians and can travel up to 1 km from their breeding pool (16). Thus, their offspring have the potential to experience maternal, environmental (e.g., aqueous or dietary), or both routes of exposure. Here, we used field-collected adults and an experimental feeding study to test the hypothesis that trophic exposure to Hg during the larval period has a negative effect on the development and performance of larvae from Hg-exposed females.

Materials and Methods

Field collection

On 17 and 18 April 2009, we collected 27 reproductive pairs of American toads from breeding pools along the South River (VA, USA) floodplain at three locations (river mile [RM] - 1.7, 9, and 20), upstream and downstream from a Hg contamination source (RM 0; see (15) for additional information). An analysis of surface water and sediment at the South River confirmed that Hg was the primary contaminant while organochlorine pesticides, polycyclic aromatic

hydrocarbons, and other trace metals were generally low (17). We transported amplexing pairs into the laboratory and placed them in a shallow bin with dechlorinated tap water to allow them to breed. The next morning, we removed the adult toads from the bins and counted the eggs. We froze a small portion of each egg mass (~500 eggs) for subsequent Hg analysis. Following the methods of Bergeron et al. (15), we collected ~0.25 mL whole blood from each anesthetized mother via cardiocentesis and released them at their capture location within 24 h.

Experimental design

We used a 2 X 3 factorial design to test the individual and interactive effects of maternally- and trophically- derived Hg on the larval development of American toads. Experimental diets consisted of a dry feed mix spiked with or without Hg (inorganic [HgII] and organic [MMHg]; Alfa Aesar), and suspended in an agar-gelatin mixture similar to the diet formulated by Unrine and Jagoe (18). The resulting diet was in a semi-solid matrix which allowed the larvae to graze naturally while preventing the diet from dissolving. We conducted a preliminary study to determine if Hg was leaching from the food to the water and found Hg concentrations in the water were below the detection limit of 50 ng/L. The target total Hg (THg) concentration for the low Hg treatment was 2.5 µg/g, dry weight (dw) (2.75% MMHg). This concentration corresponds to approximately twice the highest measured THg concentrations in the guts of larval leopard frogs (*Rana sphenocepala*) from ephemeral wetlands in southeastern USA receiving Hg solely from atmospheric deposition (19). The target THg concentration for the high Hg treatment was 10 µg/g, dw (1.05% MMHg). This corresponds to the upper limits of Hg concentrations found in periphyton at the Hg-contaminated South River (K.R. Tom, 2008, Master's thesis, The College of William and Mary, VA). Percent MMHg in periphyton is generally low (<15 %) (e.g., 18, 20, 21). Thus, we used the equations in Unrine et al. (20) to

determine the MMHg concentrations in this study. See *SI Materials and Methods* for details of the experimental diet formulation.

To determine which eggs to allocate to our experimental groups from the 27 collected clutches, we used the known strong correlation between female blood THg concentrations and those of eggs (13). The reference and maternally-Hg exposed (hereafter: Hg-exposed) groups included hatchlings from females with blood THg concentrations < 250 and >1,000 ng/g, wet weight (ww) and originated from five and six combined clutches, respectively. On 28 April 2009, at ~4 days post-hatch, we mixed free swimming hatchlings with normal morphology across clutches within each maternal Hg group (reference and Hg-exposed) to homogenize genetic variation. We then randomly allocated the hatchlings among three diet treatments (control, low Hg, and high Hg; n=25/treatment). We placed one larva into each of the 150 4-L polypropylene aquaria containing 3 L of dechlorinated tap water. We individually weighed larvae every 9 days to document growth and increase food rations accordingly. We fed larvae 6% of their body weight per day (wet weight basis), and raised them under a 12L:12D photoperiod. At the beginning of the experiment, air temperature was 18ºC and was increased weekly in 0.5ºC increments until 20ºC was reached and maintained for the remainder of the study. Every third day, we replaced 50% of the water in each aquarium. At this time, we siphoned out accumulated feces and uneaten food and provided fresh food.

After the larvae had fed on the experimental diets for 26 to 28 days (Gosner stages [GS] 26-30), we conducted a swimming performance test to determine if speed or responsiveness to stimuli differed among the six treatment combinations using methods similar to Hopkins et al. (22). We tested 50 larvae per day for three consecutive days (23-25 May 2009), ensuring a

representative daily sample of each treatment combination. See *SI Materials and Methods* for details of the larval swimming performance experiment.

We inspected larvae daily for developmental stage and mortality. As larvae neared metamorphosis, they were checked at 12 h intervals for front limb emergence (GS 42), completion of tail resorption (GS 46), or mortality. At the time of front limb emergence, larvae were removed from aquaria, weighed and measured, and individually placed in 500 mL cups with ~20 mL water and a clean unbleached paper towel to allow them to climb out of the water following complete tail resorption. The first larva reached metamorphic climax on 15 June 2009 and the last completed metamorphosis on 22 July 2009. In addition to quantifying the proportion of individuals that successfully completed metamorphosis in each treatment, we also measured mass and size at metamorphosis (GS 46) and the amount of time to reach metamorphic climax (GS 42) and complete metamorphosis (GS 46). Toads surviving through metamorphosis were humanely euthanized with buffered tricaine methane sulfonate (MS-222) 24 h after completion of metamorphosis and then frozen for analyses of Hg in tissue.

Mercury analyses

In a previous study (13), percent MMHg of the total Hg burden in female toad blood and eggs from the same study site was 71.4 ± 2.8% and 47.8 ± 3.3% (mean ± 1 standard error hereafter), respectively. Thus, in the present study, we analyzed only THg for these tissues. We homogenized whole blood from each female toad and report THg concentrations of blood on a wet weight basis. We lyophilized and homogenized eggs and report THg concentrations of eggs on a dry weight basis. Percent moisture of eggs was 95.4 ± 0.2%. We analyzed subsamples (~20 mg) for THg content by combustion-amalgamation-cold vapor atomic absorption

spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT, USA). See *SI Materials and Methods* for method details and quality assurance.

We measured both Hg(II) and MMHg in the experimental diet (n = 3 samples/diet level) and metamorphs (n = 3 composite samples/treatment). We lyophilized and homogenized the samples and report Hg concentrations on a dry weight basis. Percent moisture of the diet and metamorphs was 58.6 ± 0.4% and 90.4 ± 0.3%, respectively. Samples were then analyzed by Quicksilver Scientific (Lafayette, CO). See *SI Materials and Methods* for method details and quality assurance.

Statistical analyses

We used nonparametric Mann-Whitney U tests to compare blood and egg THg concentrations between reference and Hg-exposed females because data were non-normally distributed. We used a multivariate analysis of variance (MANOVA) to test for effects of diet, maternal Hg exposure, and their interaction on MMHg and Hg(II) concentrations in metamorphs.

We used a MANOVA to test the effects of diet, maternal Hg exposure, and their interaction on the mass (g) of tadpoles at GS 42 and the time (d) to reach GS 42 (larval duration). We log transformed larval duration to normalize the data to meet assumptions for analyses of variance. Because several animals died during metamorphosis before reaching GS 46, we used a separate MANOVA to test the effects of diet, maternal Hg exposure, and their interaction on the mass of tadpoles at GS 46 and days required to complete tail resorption (GS 42-46). We inverse transformed mass at GS 46 and log transformed days for tail resorption to normalize data and meet statistical assumptions.

Initial iterations of a repeated measures analysis of covariance (ANCOVA) model to test for differences in larval swimming performance among treatments revealed that larvae swam similarly in all three laps and the main effects did not differ over time (swimming speed: $P \geq 0.094$ for all; responsiveness: $P \geq 0.271$ for all). Thus, we used ANCOVA to test for effects of maternal and trophic exposure and their interaction on the average of the three laps for the performance endpoints of time and responsiveness, using developmental stage and mass as covariates.

We used animals fed control diet from reference mothers to determine expected survivorship under optimal conditions. We subsequently examined both metamorphic success (i.e., survivorship to GS 46) and survivorship during metamorphic climax (GS 42 to 46) in our five experimental crosses using chi-square tests of independence to determine whether the observed number of animals surviving in an experimental cross differed from that expected under optimal conditions. Lastly, we used logistic regression to examine the relationship between survival and mass of larvae (GS 42) from Hg-exposed and reference mothers.

Results

Mercury concentrations

We allocated eggs to maternal experimental groups based on the THg concentrations in female blood, and we found a significant difference in the blood THg concentrations of reference females (160 ± 18.6 ng/g, ww) compared to Hg-exposed females (2,250 ± 490 ng/g, ww; $Z = -2.74$, P=0.006). We later analyzed eggs from each clutch for THg concentrations, and the expected significant difference was confirmed between clutches from reference females (20.6 ± 1.3 ng/g, dw) and Hg-exposed females (149 ± 17.9 ng/g, dw; $Z = -2.71$, P=0.006). Total Hg

concentrations in diets (dw) were 0.010 ± 0.001 μg/g (56.7 ± 5.5% MMHg), 2.50 ± 0.06 μg/g (3.19 ± 0.03% MMHg), and 10.1 ± 2.27 μg/g (1.05 ± 0.01% MMHg) for the control, low Hg, and high Hg treatments. We found a significant effect of diet on tissue concentrations of Hg in metamorphic toads in our overall MANOVA (Fig 1; diet: $F_{4,24}$=184, P<0.001). However, there was no effect of maternal Hg exposure on Hg-tissue concentrations of metamorphs (maternal Hg exposure: $F_{2,11}$=1.39, P=0.29; interaction: $F_{4,24}$=1.08, P=0.39). Component ANOVAs revealed that diet had a significant effect on accumulation of both MMHg (Fig. 1; $F_{2,12}$=494, P<0.001) and Hg(II) (Fig. 1; $F_{2,12}$=318, P<0.001) in metamorphic toads. As expected, mean percent MMHg decreased in metamorphic toads with increasing diet concentration (Fig. 1). Post-hoc Tukey's tests revealed that Hg-tissue concentrations in metamorphs differed among all three diets for both MMHg and Hg(II) (P-values <0.001).

Biological responses to maternal and dietary mercury

Survival was high in larvae until the onset of metamorphic climax (n = 20, 23, 24 for larvae from reference mothers fed control, low Hg, and high Hg diet, respectively, and n = 23, 24, 24 for larvae from Hg-exposed mothers fed control, low Hg, and high Hg diet, respectively). Both dietary and maternal Hg exposure significantly affected growth and development of larvae until the onset of metamorphic climax but there was no interaction effect in the overall MANOVA (GS 42; diet: $F_{4,264}$=2.97, P=0.02; maternal Hg exposure: $F_{2,131}$=3.70, P=0.027; interaction: $F_{4,264}$=1.56, P=0.19). Component ANOVAs revealed that diet had a significant effect on mass at GS 42 (Fig. 2a; $F_{2,132}$=5.77, P=0.004) but not on the duration of larval period (Fig. S1; $F_{2,132}$=0.23, P=0.79). Post-hoc Tukey's tests showed that mass at GS 42 differed significantly only between the control diet and high Hg diet (P=0.004). On average, animals fed

high Hg diet were 16% smaller than those fed control diet. Maternal Hg exposure also had a significant effect on mass at GS 42 ($F_{1,132}$=5.61, P=0.019) but not on the duration of larval period ($F_{1,132}$=1.34, P=0.25). On average, animals from contaminated mothers were 10% smaller at GS 42 than those from reference mothers.

Survival decreased during metamorphic climax (n = 12, 11, 13 metamorphs from reference mothers fed control, low Hg, and high Hg diet, respectively, and n = 14, 10, 6 metamorphs from Hg-exposed mothers fed control, low Hg, and high Hg diet, respectively). Both dietary and maternal Hg affected toads at the completion of metamorphosis but there was no interaction effect in the overall MANOVA (GS 46; diet: $F_{4,120}$=2.56, P=0.042; maternal Hg exposure: $F_{2,59}$=8.28, P=0.001; interaction: $F_{4,120}$=1.21, P=0.31). Component ANOVAs revealed that diet had a significant effect on mass at GS 46 (Fig. 2b; $F_{2,60}$=4.27, P=0.018) but not on days to complete tail resorption (Fig. 2c; $F_{2,60}$=1.51, P=0.23). Post-hoc Tukey's tests showed that mass at GS 46 differed significantly only between the control diet and high Hg diet (P=0.016), with animals fed high Hg diet being 21% smaller at GS 46 than those fed control diet. In contrast, maternal Hg exposure had a significant effect on days for tail resorption (Fig 2c; $F_{1,60}$=15.7, P<0.001) but no effect on mass at GS 46 (Fig 2b; $F_{1,60}$=0.38, P=0.54). Larvae from contaminated mothers took 14% longer to fully resorb their tails than did those from reference mothers.

We found maternal Hg exposure, but not dietary exposure, affected average swimming speed of larvae, and we found no evidence of an interaction between these two modes of exposure (Fig. 3a; maternal Hg exposure: $F_{1,133}$=5.86, P=0.017; diet: $F_{2,133}$=1.61, P=0.203; interaction: $F_{2,133}$=0.769, P=0.465). Larvae from contaminated mothers took 11% longer to traverse 1 m than did those from reference mothers, even after correcting for size and

developmental stage. Likewise, maternal Hg exposure, but not dietary exposure, affected average larval responsiveness, and we found no evidence of an interaction between modes of exposure (Fig. 3b; maternal Hg exposure: $F_{1,133}$=13.9, P<0.001; diet: $F_{2,133}$=0.432, P=0.650; interaction: $F_{2,133}$=0.835, P=0.436). Larvae from contaminated mothers had to be prompted to swim 34% more often than those from reference mothers.

Whereas both maternal and dietary Hg exposure independently produced sublethal effects, the combination of the two exposure routes was lethal. The combination of maternal Hg exposure and high Hg diet led to a 50% reduction in metamorphic success (survivorship to GS 46; Fig. 4a; χ^2=5.77, P=0.016) compared to larvae from reference mothers that were raised on control diet as an optimal scenario (all other experimental crosses: χ^2 values <0.7, P-values >0.4). Importantly, larval survival was high in all treatments (ranging from 80-96%) until the onset of metamorphic climax. However, during the critical period of metamorphic climax (from GS 42 to GS 46), the combination of maternal Hg exposure and high Hg diet led to 125% greater mortality (Fig 4b; χ^2=12.3, P<0.001) compared to larvae from reference mothers that were raised on control diet (all other experimental crosses: χ^2 values <0.3, P-values >0.06). Lastly, mortality of small individuals was higher than large individuals from Hg-exposed mothers (P=0.047, β=6.87), but not from reference mothers (P=0.20) during metamorphic climax.

Discussion

Depending on their life history, organisms may be at risk of one or more routes of contaminant exposure during early development. Our study is one of the few to investigate the individual and interactive effects of maternally- and environmentally- derived contaminants on early vertebrate development. Mercury-exposed female American toads transferred ~14 times

more Hg to their eggs than reference females, and larvae were efficient at accumulating Hg from their diet regardless of maternal origin. The whole-body THg concentrations in metamorphs at the completion of metamorphosis were ~800 and ~1,700 ng/g for the low and high diets, respectively, which is similar to concentrations found in free-ranging American toad larvae from contaminated portions of our study site (~2,100 ng/g at GS 28-32) (15). We found both maternal and dietary Hg exposure independently produced negative, but different, sublethal effects on larval development. Most importantly, the latent effects of maternal exposure to Hg combined with high dietary Hg exposure later in ontogeny had lethal effects in larvae.

The transfer of Hg from mother to offspring and resulting effects have been well documented in several species (11), but less is known about effects that manifest in offspring later in life. We found maternal exposure negatively affected growth, duration of metamorphic climax, and swimming performance in American toad larvae. Larvae from Hg-exposed mothers were smaller than larvae from reference mothers at the onset of metamorphic climax. However, maternal Hg exposure did not affect mass at the completion of metamorphosis due to the high mortality of small individuals from Hg-exposed mothers, but not from reference mothers, during metamorphic climax. In addition, maternal Hg exposure increased the duration of metamorphic climax, a period of increased vulnerability for immunological (23), energetic (24), and ecological (25) reasons, by ~ 1 day compared to larvae from reference mothers which may increase mortality risk in natural settings. Unrine et al. (26) also found increased tail resorption time in leopard frog larvae fed Hg diets and suggested that Hg may inhibit the thyroid axis, which is known to play an important role in metamorphic climax (27). Lastly, swimming performance was negatively affected by maternal Hg exposure which could impair foraging efficiency and increase predation risk in nature. Mercury is known to affect behavior and performance in fish

(28), but only one study has investigated the effects of maternal transfer of Hg on behavior (29). Our study is one of the first to investigate the effects of Hg on performance in amphibians (but see (30)). Here, metamorphs from Hg-exposed mothers did not have elevated tissue concentrations due to dilution of maternally transferred Hg during growth. In addition, effects of maternal Hg-exposure on performance were independent of larval body size and developmental stage. Because Hg is a neurotoxicant and suspected endocrine disruptor (9, 10), the effects we observed were likely caused by physiological or neurological changes during embryonic development but were detectable during later larval development.

Dietary exposure alone negatively affected larval size at the onset and completion of metamorphic climax, but did not affect any other measured traits. Individuals fed the high Hg diet were smaller than those fed the control diet at GS 42 and 46, respectively. Reduced amphibian size at metamorphosis is linked to adverse effects on fitness-related traits, including survival, body size/age at first reproduction, and fecundity (e.g., 31) . Because of the strong influence of size, an important next step will be to determine whether small body size at metamorphosis in Hg-exposed individuals affects post-metamorphic growth and survival in the terrestrial environment. Interestingly, in a similar study investigating the effects of dietary Hg on leopard frog larvae (18, 26), adverse effects on development and decreased survival were observed at THg concentrations (236 and 412 ng/g) much lower than the concentrations where effects were observed in our study. These differences may be due to differences in sensitivity or length of larval period between species.

We demonstrated that the sublethal, latent effects of maternal Hg exposure interacted with the sublethal effects of high dietary Hg exposure to reduce survival in amphibian larvae. The combination of maternal and dietary exposure to environmentally relevant Hg

concentrations had lethal consequences; larvae experienced a 125% increase in mortality during metamorphic climax and a 50% decrease in overall metamorphic success compared to larvae from reference mothers fed the control diet. Until the onset of metamorphic climax, larval survival in all treatments was high, but metamorphic climax is a critical and sensitive stage in the life history of amphibians. Our findings support the hypothesis that metamorphic climax may be a period which is sensitive to the remobilization of Hg from tissues into circulation during tail resorption (26) due to the extensive morphological, physiological, and behavioral changes that occur as animals prepare for the transition to terrestrial life. Further, timing of mortality can have important implications for amphibian population dynamics. In some cases, mortality of eggs or larvae can have slight, or even positive effects on amphibian populations because they release surviving larvae from the detrimental effects of intraspecific competition (e.g., 32). In our study, mortality occurred during metamorphic climax, suggesting larvae in the natural environment may suffer from both density-dependent effects of competition and density-independent effects of contaminant exposure.

Although immediate effects of the maternal transfer of contaminants on early development are well studied, there are fewer investigations into potential latent and long term effects, and even fewer on interactions with environmental exposures through ontogeny. We found the majority of the significant sublethal effects in amphibians resulted from maternal Hg exposure, and the latency of these maternal effects is not surprising due to the effects of transferred contaminants on key organizational events that occur early in ontogeny (1). However, these negative maternal effects are of great importance to the field of ecotoxicology because studies often examine the effects of environmental contaminants using organisms from reference sites, ignoring maternal contaminant exposure. Our findings emphasize the importance

of investigating environmentally relevant routes of contaminant exposure over multiple early life stages because studies based on single routes of exposure or single life stages may underestimate the severity of adverse effects, potentially having widespread implications for both wildlife and human health (33). Futhermore, future studies investigating a greater number of intermediate concentrations of maternal and dietary Hg exposures in amphibians could help to identify the lowest exposure concentrations causing interactive effects and potentially aid in identifying the route of toxicity. In our study, amphibian larvae were raised individually, but further studies are necessary to determine whether embryonic exposure to maternally-derived Hg and/or larval dietary exposure have different effects in more complex environments, such as in the presence of competitors or predators. Additionally, investigating how different contaminant exposure routes impact the terrestrial juvenile stage will ultimately aid in predicting their effects on amphibian population dynamics.

Acknowledgements

We thank the landowners long the South River and the Waynesboro Parks and Recreation Department for access to sampling locations, J. Schmerfeld, D. Cristol, K. Carlson-Drexler, A. Condon, M. Howie, C. Ramos, J. Burke, J. Callihan, S. DuRant, M.K. McCaleb, J. McPherson, and H. Wada for their support, field and/or laboratory assistance, and C. Shade of Quicksilver Scientific for MMHg analysis. This manuscript was improved by the comments of J. Willson. Collection of animals was in conformance with appropriate permits, and sample methods were in compliance with Virginia Polytechnic and State University's animal care and use protocols. Financial support was provided by E. I. DuPont de Nemours, the National Science Foundation (NSF # IOB-0615361), and a U.S. EPA STAR Graduate Fellowship (FP-9170040-1) to C.M.B. EPA has not officially endorsed this publication and the views expressed herein may not reflect

the views of the EPA. Research was completed with oversight from the South River Science Team which is a collaboration of state and federal agencies, academic institutions, and environmental interests.

Supporting Information Available

Further details are provided of the experimental diet formulation, swimming performance experiment, and methods and quality assurance for THg and MMHg analyses. Figure S1 depicts larval duration of American toads from all treatments. This information is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited

(1) Di Giulio, R. T.; Tillitt, D. E. *Reproductive and developmental effects of contaminants in oviparous vertebrates*. SETAC Press: Pensacola, FL, 1999.

(2) Guillette, L. J.; Crain, D. A.; Rooney, A. A.; Pickford, D. B. Organization versus activation - The role of endocrine-disrupting contaminants (EDCS) during embryonic development in wildlife. *Environ. Health Perspect.* **1995,** *103*, 157-164.

(3) Eisenreich, K. M.; Kelly, S. M.; Rowe, C. L. Latent mortality of juvenile snapping turtles from the upper Hudson River, New York, exposed maternally and via the diet to polychlorinated biphenyls (PCBs). *Environ. Sci. Technol.* **2009,** *43* (15), 6052-6057.

(4) Yates, M. A.; Fuller, M. R.; Henny, C. J.; Seegar, W. S.; Garcia, J. Wintering area DDE source to migratory white-faced ibis revealed by satellite telemetry and prey sampling. *Ecotoxicology* **2010,** *19* (1), 153-162.

(5) Nye, J. A.; Davis, D. D.; Miller, T. J. The effect of maternal exposure to contaminated sediment on the growth and condition of larval Fundulus heteroclitus. *Aquat. Toxicol.* **2007,** *82*(4), 242-250.

(6) Bernardo, J. Maternal effects in animal ecology. *Am. Zool.* **1996,** *36* (2), 83-105.

(7) Fitzgerald, W. F.; Engstrom, D. R.; Mason, R. P.; Nater, E. A. The case for atmospheric mercury contamination in remote areas. *Environ. Sci. Technol.* **1998,** *32* (1), 1-7.

(8) Mason, R. P.; Reinfelder, J. R.; Morel, F. M. M. Uptake, toxicity, and trophic transfer of mercury in a coastal diatom. *Environ. Sci. Technol.* **1996,** *30* (6), 1835-1845.

(9) Weiner, J. G.; Spry, D. J. Toxicological significance of mercury in freshwater fish. In *Environmental contaminants in wildlife: Interpreting tissue concentrations*, Beyer, W. N.; Heinz, G. H.;Redmon-Norwood, A. W., Eds. Lewis Publishers: Boca Raton, FL, 1996; pp 297-340.

(10) Scheuhammer, A. M. Effects of acidification on the availability of toxic metals and calcium to wild birds and mammals. *Environ. Pollut.* **1991,** *71* (2-4), 329-375.

(11) Scheuhammer, A. M.; Meyer, M. W.; Sandheinrich, M. B.; Murray, M. W. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. *Ambio* **2007,** *36* (1), 12-18.

(12) Unrine, J. M.; Hopkins, W. A.; Romanek, C. S.; Jackson, B. P. Bioaccumulation of trace elements in omnivorous amphibian larvae: Implications for amphibian health and contaminant transport. *Environ. Pollut.* **2007,** *149*, 182-192.

(13) Bergeron, C. M.; Bodinof, C. M.; Unrine, J. M.; Hopkins, W. A. Bioaccumulation and maternal transfer of mercury and selenium in amphibians. *Environ. Toxicol. Chem.* **2010,** *29* (4), 989-997.

(14) Bergeron, C. M.; Bodinof, C. M.; Budischak, S. A.; Wada, H.; Unrine, J. M.; Hopkins, W. A. Counterbalancing effects of maternal mercury exposure during different stages of ontogeny. *Environ. Pollut.* **In review**.

(15) Bergeron, C. M.; Bodinof, C. M.; Unrine, J. M.; Hopkins, W. A. Mercury accumulation along a contamination gradient and nondestructive indices of bioaccumulation in amphibians. *Environ. Toxicol. Chem.* **2010,** *29* (4), 980-988.

(16) Forester, D. C.; Snodgrass, J. W.; Marsalek, K.; Lanham, Z. Post-breeding dispersal and summer home range of female American toads (*Bufo americanus*). *Northeast. Nat.* **2006,** *13* (1), 59-72.

(17) URS 2007. *Evaluation for Removal of Organochlorine Pesticides, Polycyclic Aromatic Hydrocarbons, and Trace Metals Analyses in the Phase I System Characterization, South River, VA. Appendix F, Phase I, Year 1 Progress Report: Ecological Study of the South River and a Segment of the South Fork Shenandoah River, Virginia.* Report No.: _____. Contract No.: 18985057.70030.

(18) Unrine, J. M.; Jagoe, C. H. Dietary mercury exposure and bioaccumulation in southern leopard frog (*Rana sphenocephala*) larvae. *Environ. Toxicol. Chem.* **2004,** *23* (12), 2956-2963.

(19) Unrine, J. M.; Jagoe, C. H.; Brinton, A. C.; Brant, H. A.; Garvin, N. T. Dietary mercury exposure and bioaccumulation in amphibian larvae inhabiting Carolina bay wetlands. *Environ. Pollut.* **2005,** *135* (2), 245-253.

(20) Bell, A. H.; Scudder, B. C. Mercury accumulation in periphyton of eight river ecosystems. *Journal of the American Water Resources Association* **2007,** *43* (4), 957-968.

(21) Tom, K. R.; Newman, M. C.; Schmerfeld, J. Modeling mercury biomagnification (South River, Virginia, USA) to inform river management decision making. *Environ. Toxicol. Chem.* **2010,** *29* (4), 1013-1020.

(22) Hopkins, W. A.; Congdon, J.; Ray, J. K. Incidence and impact of axial malformations in larval bullfrogs (*Rana catesbeiana*) developing in sites polluted by a coal-burning power plant. *Environ. Toxicol. Chem.* **2000,** *19* (4), 862-868.

(23) Todd, B. D. Parasites lost? An overlooked hypothesis for the evolution of alternative reproductive strategies in amphibians. *Am. Nat.* **2007,** *170* (5), 793-799.

(24) Beck, C. W.; Congdon, J. D. Energetics of metamorphic climax in the southern toad (*Bufo terrestris*). *Oecologia* **2003,** *137*, 344-351.

(25) Arnold, S. J.; Wassersug, R. J. Differential predation on metamorphic anurans by garter snakes (Thamnophis) - Social-behavior as a possible defense. *Ecology* **1978,** *59* (5), 1014-1022.

(26) Unrine, J. M.; Jagoe, C. H.; Hopkins, W. A.; Brant, H. A. Adverse effects of ecologically relevant dietary mercury exposure in southern leopard frog (*Rana sphenocephala*) larvae. *Environ. Toxicol. Chem.* **2004,** *23* (12), 2964-2970.

(27) Denver, R. J.; Boorse, G. C.; Glennemeier, K. A. Endocrinology of complex life cycels: Amphibians. In *Hormones, Brain and Behavior*, Pfaff, D. W.; Arnold, A. P.; Etgen, A. M.; Fahrbach, S. E.;Rubin, R. T., Eds. Academic Press, Inc.: San Diego, CA, 2002; pp 469-513.

(28) Weis, J. S. Reproductive, developmental, and neurobehavioral effects of methylmercury in fishes. *Journal of Environmental Science and Health Part C-Environmental Carcinogenesis & Ecotoxicology Reviews* **2009,** *27* (4), 212-225.

(29) Alvarez, M. D.; Murphy, C. A.; Rose, K. A.; McCarthy, I. D.; Fuiman, L. A. Maternal body burdens of methylmercury impair survival skills of offspring in Atlantic croaker (*Micropogonias undulatus*). *Aquat. Toxicol.* **2006,** *80* (4), 329-337.

(30) Burke, J. N.; Bergeron, C. M.; Todd, B. D.; Hopkins, W. A. Effects of mercury on behavior and performance of nothern two-lined salamanders (*Eurycea bislineata*). *Environ. Pollut.* **2010,** *158*, 3546-3551.

(31) Semlitsch, R. D.; Scott, D. E.; Pechmann, J. H. K. Time and size at metamorphosis related to adult fitness in *Ambystoma talpoideum*. *Ecology* **1988,** *69* (1), 184-192.

(32) Vonesh, J. R.; De la Cruz, O. Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. *Oecologia* **2002,** *133* (3), 325-333.

(33) Heindel, J. J. Role of exposure to environmental chemicals in the developmental basis of disease and dysfunction. *Reprod. Toxicol.* **2007,** *23* (3), 257-259.

Figure 6.1: Whole body mercury (Hg) concentrations (ng/g, dry weight; inorganic mercury [HgII] and methylmercury [MMHg]) and the percent of the total Hg that is MMHg (% MMHg) in American toad metamorphs from the 2 X 3 factorial design of maternal Hg exposure (reference [Ref] and Hg-exposed [Hg]) and the three diet treatments (control, low Hg, and high Hg). Error bars represent the standard error of the mean.

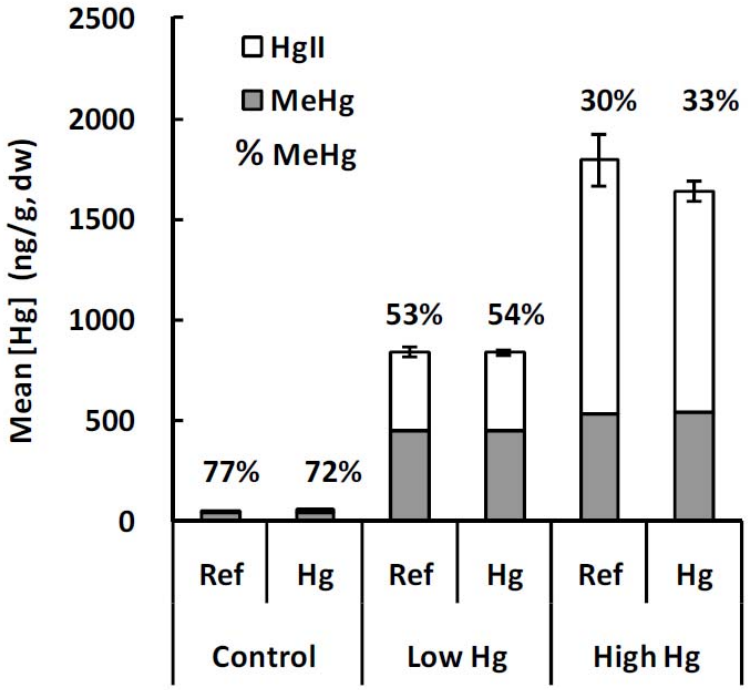


Figure 6.2: Components of the multivariate analysis of variance for American toad larvae from the 2 X 3 factorial design of maternal mercury (Hg) exposure (reference females and Hg-exposed females) and the three diet treatments (control, low Hg, and high Hg). (A) Mass at Gosner stage (GS) 42, front limb emergence (B) Mass at GS 46, completion of metamorphosis, (C) Tail resorption time (GS 42 to GS 46). Error bars represent the standard error of the mean.

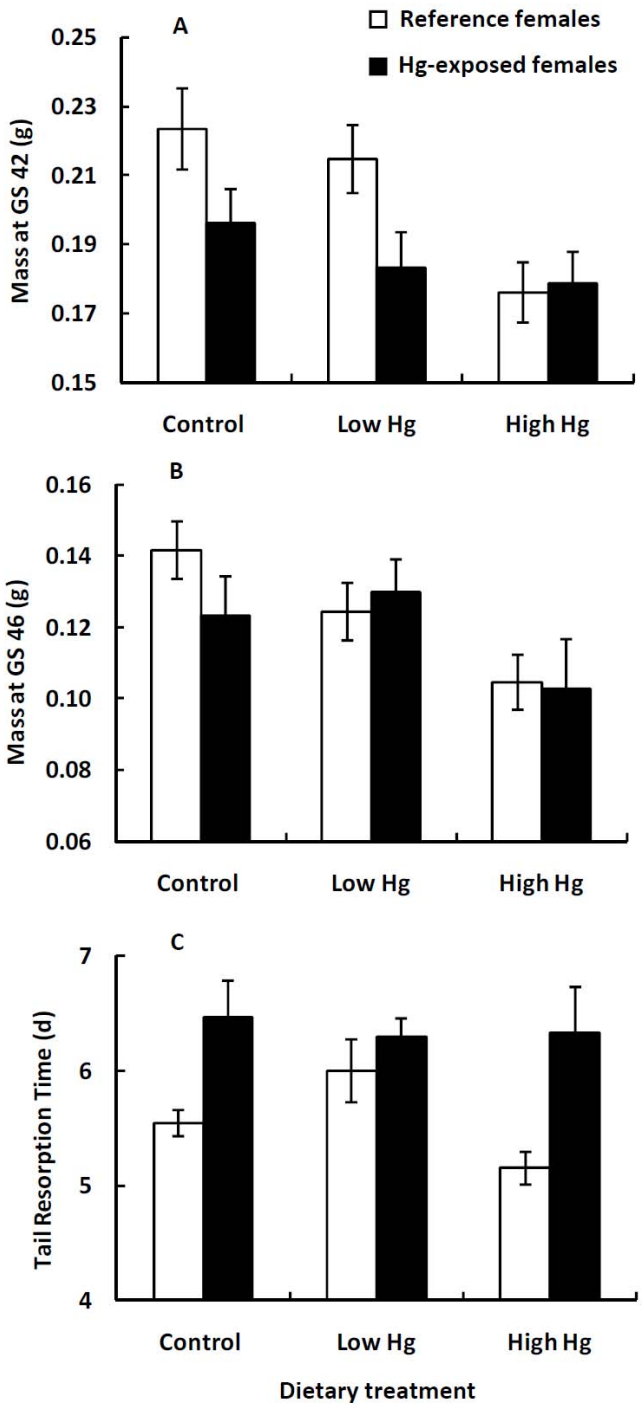


Figure 6.3: Larval swimming performance of American toads from the 2 X 3 factorial design of maternal mercury (Hg) exposure (reference females and Hg-exposed females) and the three diet treatments (control, low Hg, and high Hg). (A) velocity (m/s) and (B) responsiveness (prods/m). Error bars represent the standard error of the mean.

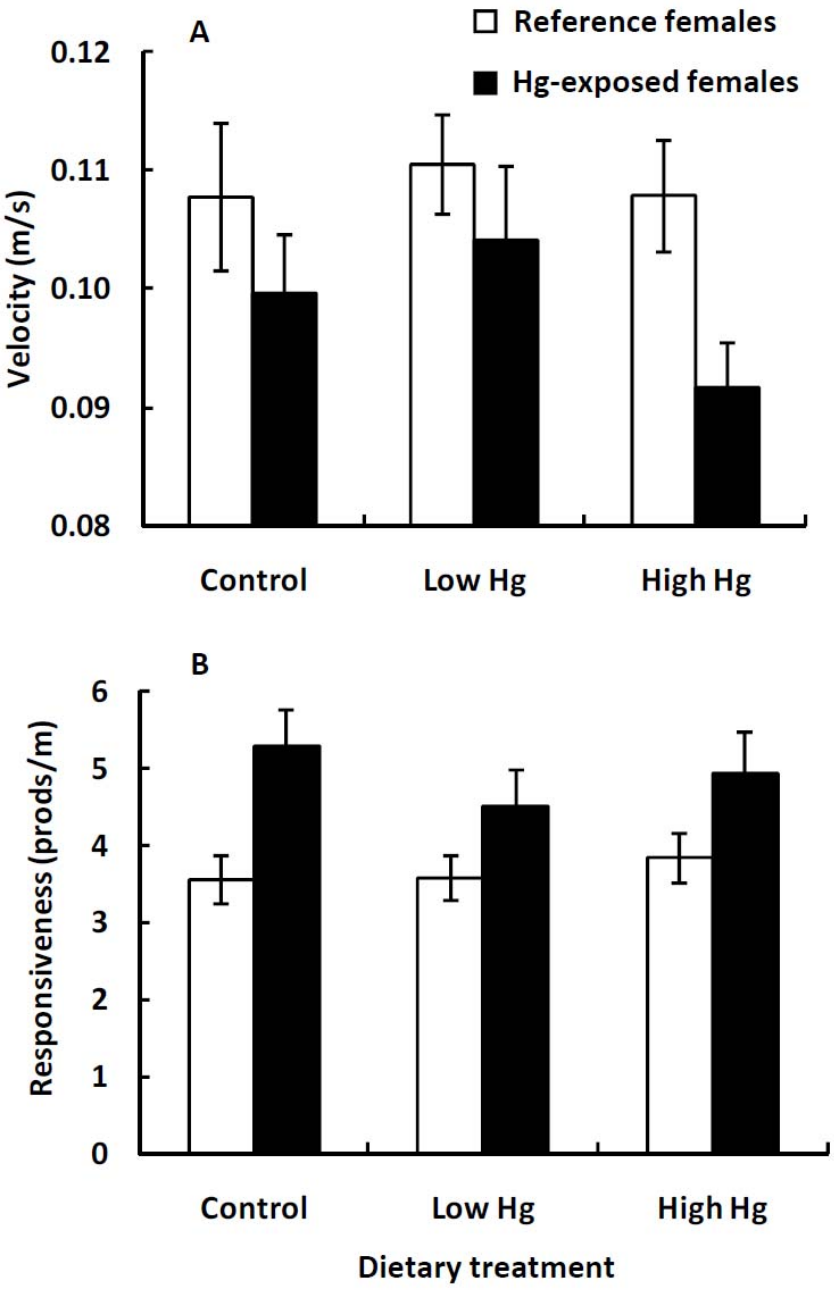
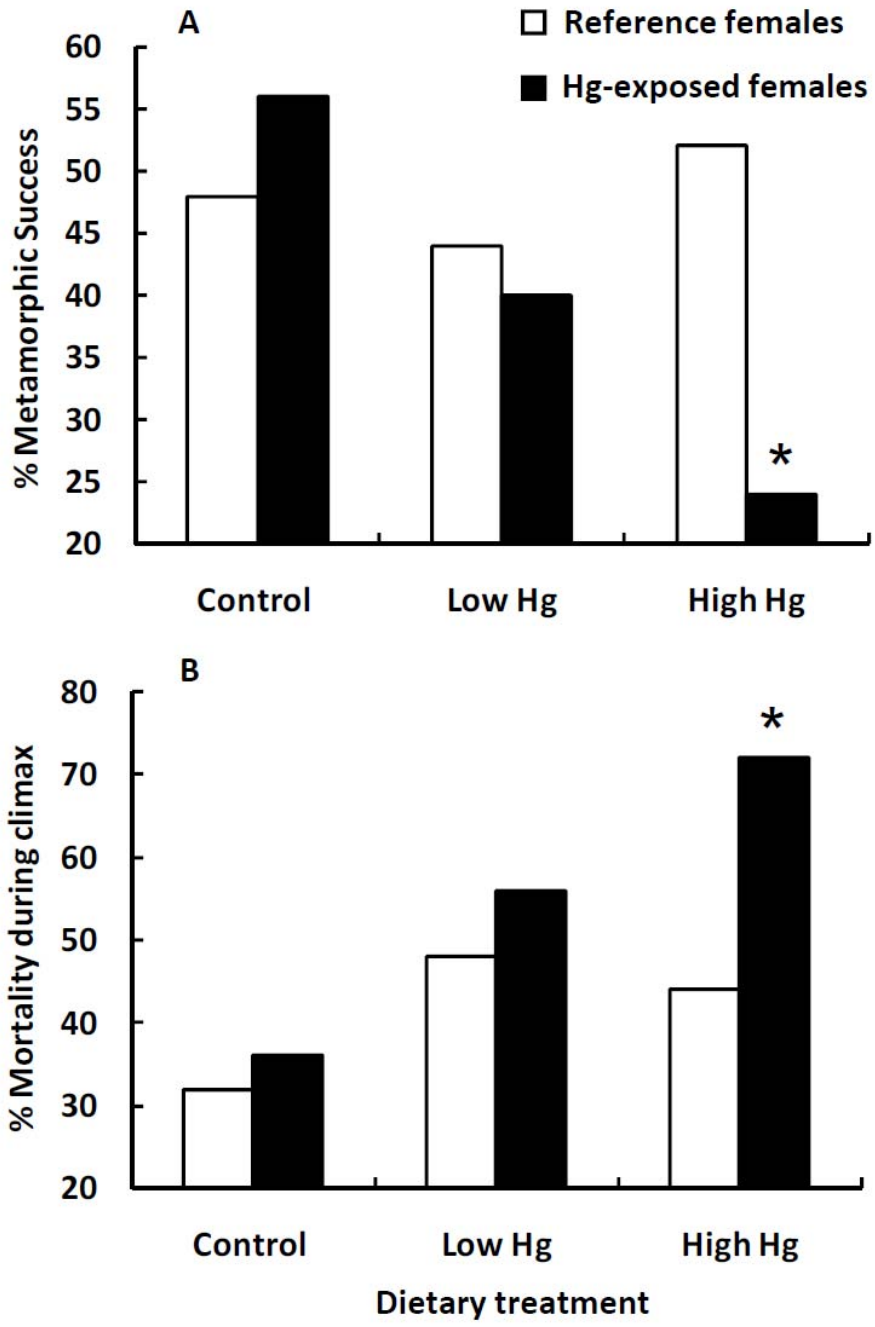


Figure 6.4: (A) Percent of American toads successfully completing metamorphosis and (B) the percent of mortalities during metamorphic climax from the 2 X 3 factorial design of maternal mercury (Hg) exposure (reference females and Hg-exposed females) and the three diet treatments (control, low Hg, and high Hg). Asterisks (*) denote a significant difference from the treatment reference females fed control diet.



Supporting Information Materials and Methods

Experimental diet formulation

We attained total mercury (THg) concentrations in the diets by adding mercury (II) chloride and methylmercury (II) chloride based on equations in Unrine and Jagoe (1). Briefly, we ground and homogenized dry components (vitamin-enriched rabbit pellets [Classic Blend Rabbit Food, L/M Animal Farms, Pleasant Plain, OH, USA; 218 g], trout pellets [Aquamax Grower 600, PMI Nutrition International, Brentwood, MO, USA; 218 g], fish flakes [TetraMin, Tetra, Blacksburg, VA, USA; 32 g], and algae powder [SeraMicron, Sera, Abington, PA; 32 g]). We added ethanol (95%) with or without Hg to the dry components, homogenized, and dried the mixture under a fume hood. Once dry, we combined nanopure water (750 ml), agar (20 g), and gelatin (14 g) while stirring and heating the solution to ~70ºC on a hot plate. We poured this solution over the dry components, mixed until homogenized, and cooled. We stored experimental diets in a -80ºC freezer. We prepared uniform rations by pressing the thawed diet out of a syringe and cutting into equal lengths of known masses. The coefficient of variation of mass for diet pieces averaged 4.9%.

Larval swimming performance

To assess swimming performance (swimming speed and responsiveness to stimuli), we used a 1 m long and 3.6 cm wide polyurethane racetrack with 1 cm demarcations down its length, filled with dechlorinated water at a depth of 2 cm. The experiment began by placing an individual larva in the racetrack corridor covered with a dark, inverted cup. After a 1 min acclimation period, we lifted the cup, and recorded the start time when the larva began forward progress. Individuals completed three consecutive trials with a 1 min acclimation period between each trial. We defined swimming as any conspicuous tail movement, and when forward motion

stopped, we gently prodded larvae by touching their tails with a blunt probe to stimulate movement. We recorded all trials with a digital video camera (30 frames/s). After recording, an observer blind to treatments viewed and analyzed trials using Adobe® Premiere Pro CS3. To determine overall swimming performance, we calculated speed as the time required to swim one complete m (m/sec) and responsiveness as number of prods required for the larva to reach 1 m. We calculated time to 1 m as net movement and only included portions of the trial during which the larva was swimming.

Total mercury analysis

We analyzed samples for THg content 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 (2). For quality assurance, each group of 10 to 15 samples included a replicate, blank, and standard reference material (SRM; DORM-3 fish protein and DOLT-3 or DOLT-4 dogfish liver [National Research Council of Canada, Ottawa, ON]). We calibrated the instrument using solid SRMs (DORM-3 and DOLT-3 or DOLT-4). Method detection limits (MDLs; 3 times standard deviation of procedural blanks) for samples were 0.39 ng, and all samples had THg concentrations that exceeded the limit. Average relative percent differences (RPD) between replicate sample analyses were 7.42 ± 2.74 % (n=11). Mean percent recoveries of THg for the SRMs, DORM-3, DOLT-3 and DOLT-4 were 107.82 ± 1.15 % (n=23), 99.66 ± 0.51 % (n=12), and 99.46 ± 0.53 % (n=10), respectively.

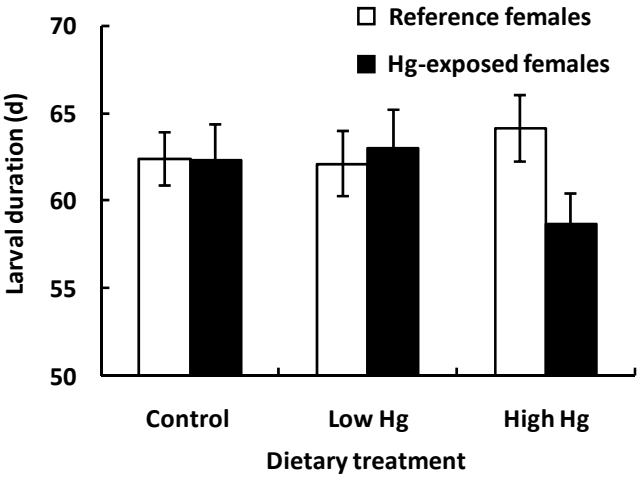
Methlymercury analysis

We analyzed samples for methlymercury **(**MMHg) using acidic thiourea leaching and Hg-thiourea liquid chromatography coupled to cold vapor atomic fluorescence spectrometry (HgTu/LCCVAFS) (3). This method separates monomethyl (CH_3Hg^+) from mercuric (HgII) mercury by the charges on their respective thiourea complexes; online cold-vapor generation follows separation with an instrument detection limit of 4 pg/g for MMHg and 7 pg/g HgII (for a 100 mg sample). For our samples, average RPD between replicate sample analyses were 3.94 ± 0.69 % for MMHg and 6.26 ± 2.22 % for HgII (*n*=6). Mean percent recoveries of MMHg and HgII for matrix spikes were 92.85 ± 0.58 % and 103.40 ± 2.50 % (n=6), respectively. Also, mean percent recoveries for the SRMs, DOLT-3 (n=3) and BCR-463 (tuna fish [Institute for Reference Materials and Measurements, Geel, Belgium]) (n=3) were 98.97 ± 4.32 % and 99.00 ± 4.54 % for MMHg and 104.43 ± 5.81 % and 101.37 ± 4.46 % for THg.

Supporting Information References

1. Unrine JM, Jagoe CH (2004) Dietary mercury exposure and bioaccumulation in southern leopard frog (*Rana sphenocephala*) larvae. *Environ Toxicol Chem* 23(12):2956-2963.
2. USEPA (1998) Method 7473: Mercury in solids and solutions by thermal decomposition, amalgamation, and atomic absorption spectrometry. (Washington, D.C., USA), pp 1-15.
3. Shade CW (2008) Automated simultaneous analysis of monomethyl and mercuric Hg in biotic samples by Hg-thiourea complex liquid chromatography following acidic thiourea leaching. *Environ Sci Technol* 42(17):6604-6610.

Figure 6.S1: Larval duration (days from the beginning of experiment to GS 42, front limb emergence) in American toad larvae from the 2 X 3 factorial design of maternal mercury (Hg) exposure (reference females and Hg-exposed females) and the three diet treatments (control, low Hg, and high Hg).



Chapter 7: Conclusions and Future Directions

Amphibian declines have been documented worldwide and have been touted as the Earth's sixth mass extinction (Wake and Vredenburg, 2008). Environmental contamination ranks second only to habitat loss and degradation as the most common risk to amphibian populations (IUCN, 2011). Since the early 1990s, when the problem of global amphibian declines was initially recognized, there has been an increase in the number of studies of amphibians and contaminants (Sparling et al., 2010). However, studies on the ecological effects of contaminants in amphibians have focused primarily on organic pollutants (e.g., pesticides, herbicides, and fungicides) and there has been comparatively little research on the ecological effects of metals (Hopkins and Rowe, 2010). While modern synthetic chemicals are often formulated to target specific organisms and degrade in the environment, metal contamination is often the widespread result of human industrial activity and metals usually do not readily degrade in the environment (Hopkins and Rowe, 2010). These factors can result in risk of chronic metal exposure in wildlife. One such metal of concern is mercury (Hg) due to its ubiquity, toxicity, and ability to bioaccumulate and biomagnify in food webs. However, the toxic effects of Hg bioaccumulation in amphibians has received little attention compared to fish, birds, and mammals (Scheuhammer et al., 2007).

During my dissertation research, I used a pluralistic approach which combined field studies and manipulative laboratory and mesocosm experiments to examine the bioaccumulation and ecological effects of environmentally relevant Hg exposure routes acting at various early life stages in amphibians. By collecting amphibians in the field at the Hg-contaminated South River, VA, I confirmed that amphibians can bioaccumulate sufficient levels of Hg to warrant concern

(Chapter 2) and females maternally transfer accumulated Hg to their eggs (Chapter 3). Through laboratory observations and mesocosm experiments, I examined the short and long-term effects of maternal contaminant exposure on offspring, and found the negative effects of maternal Hg exposure manifested either immediately at the embryonic stage or later during the larval stage, depending on the year in which the study was conducted (Chapters 4 and 5). Lastly, using a factorial laboratory experiment, I examined whether the latent effects of maternal transfer of contaminants manifests differently depending on the environment in which offspring develop, and found both maternal and dietary Hg exposure independently produced negative, but different, sublethal effects on larval development. Most importantly, maternal exposure to Hg combined with high dietary Hg exposure later in ontogeny had a lethal effect in larvae (Chapter 6).

Important implications from this research

Although ecological research often focuses on effects of aqueous or dietary exposure to a contaminant, the maternal effects of contamination represent an additional important route for toxicological effects in organisms. However, there are few studies which account for parental exposure in their design, despite the fact that maternal transfer of contaminants can have negative effects on vertebrate embryonic development. In fact, many researchers go to great lengths to find "clean" or "pristine" sites to obtain their study species and then apply their treatments. I observed adverse lethal and sublethal effects of maternal Hg exposure throughout early amphibian development. This research, along with other recent studies (e.g., Eisenreich et al., 2009, Nye et al., 2007) which find strong effects of maternal contaminant exposure compared to dietary or direct environmental exposure, stresses the importance of considering parental

conditions to prevent potentially underestimating contaminant effects. Conversely, another important option to consider is that maternal exposure to Hg could convey increased resistance to Hg pollution as observed in some wild fish populations (Crump and Trudeau, 2009). In addition, few studies focus on the latent or long-term effects of contaminant exposure early in development and how this exposure may interact with exposure pathways later in life. In amphibians, I found maternal Hg exposure interacted with high dietary exposure during the larval phase to have a lethal effect in late-stage larvae. This research supports the growing literature in fish, wildlife, and humans on the long-term importance and broad implications of maternal effects, especially the maternal transfer of contaminants.

A major challenge in environmental toxicology and conservation is making the connection between laboratory findings and effects observed in wild populations. For amphibians, the complex life cycles of many species raise unique, context-dependent challenges regarding contamination in aquatic verses terrestrial habitats. Despite these challenges, I was able to gain environmentally realistic information regarding the effects of Hg on amphibians by using a varied approach. Through a combination of field and laboratory approaches, I was able to explore the linkages between Hg body burdens and effects at environmentally realistic Hg concentrations and influences on individual fitness through multiple life stages. This holistic perspective resulted in data that could be used to model the effects of Hg on amphibian populations. In future research, pluralistic and interdisciplinary approaches similar to the ones used in this dissertation will be required to solve the multifaceted conservation problems facing amphibians.

Recent directions using this research as a foundation

There is great need for future research in amphibian ecotoxicology concentrating on the terrestrial life stages of amphibian development. Most studies on amphibians and contaminants have focused solely on the aquatic portion of the amphibian life cycle because embryos and larvae are easily maintained and manipulated in the laboratory, and it is often assumed that sublethal effects observed in larvae persist to affect survival and reproduction in the terrestrial stages (Todd et al., *In press*). The embryonic and larval stages are important considering the developmental changes occurring during this time period, and studies often find that early life-stages are most sensitive to contaminants. However, studies that concentrate solely on the aquatic life stages may overlook latent effects of contaminants that appear later in ontogeny after previous exposure (e.g., Budischak et al., 2008, Rohr and Palmer, 2005). In addition, amphibians, like many organisms, have evolved life history tactics with large variability in survival of early life-stages. For these species, survival in the juvenile or adult stage, may be most important to population dynamics and viability (Biek et al., 2002, Schmidt et al., 2005, Vonesh and De la Cruz, 2002), but few ecotoxicological studies with amphibians have extended into the terrestrial stage (but see Boone, 2005). My dissertation research laid the foundation for a terrestrial enclosure study (Todd et al., *In press*) examining whether maternal or dietary Hg exposure in larval American toads had any adverse effects in the terrestrial environment following metamorphosis or the onset of latent effects not previously observed. Todd et al. (*In press*) found the size difference at metamorphosis in animals exposed to maternal Hg exposure persisted for one year in the terrestrial environment indicating that adverse effects of Hg observed in larval amphibians may persist to affect later terrestrial life stages. However, no novel adverse effects developed during a year of growth in an uncontaminated terrestrial environment

and no overall differences in survival to one year of age were observed. These results reiterate the importance of maternal Hg exposure and the need to consider the maternal effects of maternal contaminant exposure in amphibian ecotoxicology.

Additionally, a major challenge in amphibian conservation is relating adverse effects observed at individual life stages to population dynamics and viability (Beebee and Griffiths, 2005). Like this dissertation research, most ecotoxicological risk assessments measure effects on individual organisms (e.g., growth, survival, or reproductive output) at single life stages, but the ultimate goal of these assessments is generally to maintain viable populations (Kramer et al., 2011). A key issue regarding the lack of studies is that conducting replicated experiments at the population level is generally not logistically feasible. However, developing mechanistic linkages across multiple levels of biological organization (from the molecular level to ecosystem level) would allow us to comprehensively understand how organisms are affected by environmental contaminants. Recently, Willson et al. (*In review*) used a demographic population model to investigate the effects of Hg acting throughout the ontogeny of American toads on their population dynamics by synthesizing the field, laboratory, mesocosm, and terrestrial enclosure studies from my dissertation research (Chapters 3-6) and studies conducted by Todd et al. (2011a, *In press*, 2011b). To our knowledge, this is the first study to model individual-level effects of contaminants detected experimentally to amphibian population dynamics. Model simulation and sensitivity analyses demonstrated that exposure to Hg through maternal transfer or larval diet, alone, had minor effects on population viability (population size and extinction probability). However, the elevated mortality at metamorphic climax observed in individuals exposed to Hg both maternally and trophically results in reduced adult population size and increased extinction risk. These results suggest that the timing of effects may have important

consequences for population dynamics and further emphasizes the importance studying the post-metamorphic life stages in amphibians.

Future directions

There are many important questions that remain unanswered and potential future directions for research regarding the accumulation, maternal transfer, and adverse effects of Hg in amphibians to be resolved in other systems. One important question that may inform the potential effectiveness of remediation efforts because it could span multiple environments (i.e., aquatic versus terrestrial) includes whether females transfer Hg from their body burden stores or from recent dietary uptake while provisioning their eggs. Hammerschmidt and Sandheinrich (2005) found that the maternal diet of adult fathead minnows (*Pimephales promelas*), and not the adult body burden, was the principle source of Hg in fish eggs. However, the maternal source may vary depending on species even within a vertebrate class. For example, the pattern of accumulation and maternal transfer may differ for wood frogs (*Rana sylvatica*) that lay their eggs very soon after coming out of hibernation compared to green frogs (*Rana clamitans*) that lay their eggs much later in the season. Experimentally, this could be investigated using a diet study by feeding adults different stable isotopes of Hg before and after overwintering and observing which isotope is transferred to their eggs, however, the feasibility of this may be low as wild amphibians may not readily breed and produce eggs in captivity.

We also know very little about species sensitivity differences to Hg toxicity among amphibians. For example, whole-body Hg concentrations in larval wood frogs exceeded those associated with increased mortality, malformation, and/or delayed development in American toads (this study) and southern leopard frogs, *Rana sphenocephala* (Unrine et al., 2004),

however no adverse effects of Hg on development, survival, performance, or whole-body thyroid hormone concentrations were observed in wood frogs (Wada et al., 2011). I hypothesized that the effects observed from the maternal transfer of Hg in American toads may be conservative compared to other species, but we did observe negative effects of maternal transfer in the embryonic and larval stages. However, while Hg concentrations were higher in the eggs of spotted salamanders (*Ambystoma maculatum*), there was no observed negative effects of maternal transfer in spotted salamanders (Bergeron and Hopkins, unpublished data). This was surprising because spotted salamanders have a longer lifespan so they can accumulate more Hg and have a much longer embryonic period allowing the transferred Hg potentially more time to disrupt embryonic development. The limited data available for Hg effects on amphibian species suggest there may be large interspecies variation in Hg sensitivity that needs further research attention.

In general, the importance of maternal effects on offspring health and development has received considerably more attention recently (Mousseau et al., 2009), but we still know very little about how potential paternal effects may affect offspring. In my research, I examined the effects of maternal transfer of Hg on female reproductive success and the subsequent development of their offspring, ignoring any effects of contaminant exposure on males. Disruption of testicular function has been observed in fish exposed to environmentally relevant concentrations of Hg (Crump and Trudeau, 2009), but the question of whether contaminant exposure affects male reproductive success and their offspring remains largely unexplored in fish and wildlife. There is evidence to suggest that contaminant exposure in males deserves further research attention. For example, Marteinson et al. (2010, 2011) found adult male American kestrels (*Falco sparverius*) exposed embryonically to environmentally relevant polybrominated

diphenylethers (PBDEs) affected their reproductive physiology and behavior which lead to decreased reproductive success when paired with unexposed females. In amphibians, experimental crosses with contaminated females and males paired with reference females and males may help disentangle negative maternal versus paternal effects due to contaminant exposure.

Few studies of maternal effects in vertebrates have followed offspring over time or through critical life history transitions, especially under varied ecological or environmental conditions typical of natural habitats. Thus, another area of research that warrants further attention is the effect of biotic and abiotic factors on the toxicity of Hg. For example, in a companion study, Todd et al. (2011a) fed amphibian larvae raised communally greater food rationing (9% of body mass per day) than those raised individually (6% of body mass per day) in Chapter 6, and found larvae feed greater amounts of food attained larger sizes and accumulated more Hg. However, only a marginal effect of diet on survival was observed compared to the study in Chapter 6 where significantly increased mortality was observed in the interaction of maternally exposed larvae fed high dietary Hg. This greater food rationing may have masked some of the physiological deficits that were evident with more restrictive rationing. An experiment that examined differing levels of ration size and con-specific competition would further inform whether these factors affect Hg toxicity. In addition to food availability and competitors, the effects of predators (but see Todd et al., 2011b) and other factors that affect timing of development (i.e., temperature) on Hg toxicity require more attention.

For conserving species, it is important to determine how Hg affects populations, but it is also important to examine processes within the individual level of organization to gain a better understanding of the mechanisms of Hg toxicity. Historically, research on the health-effects of

Hg has focused on neurological outcomes of exposure, but recent research has explored the effects of Hg on the endocrine system (Tan et al., 2009). Weiner and Spry (1996) concluded that reduced reproductive success was the most plausible effect of Hg on wild-fish populations at current exposure levels in aquatic ecosystems, but the mechanistic effects of Hg on reproduction remain unclear (Crump and Trudeau, 2009). Whether the mechanisms of toxicity are similar for amphibians and fish or other wildlife is unknown. An important question to explore in in all of these animals is how maternal transfer of Hg may affect the development of sensitive physiological systems such as the nervous, immune, and especially endocrine system since Hg is a suspected endocrine disrupter.

This research lends further support that the maternal environment can greatly influence reproductive conditions and both the immediate and long-term development of offspring. Amphibians are excellent model organisms for examining developmental effects of contaminants in vertebrates and are valuable in investigating the growing number of contaminants of concern with potential reproductive effects in fish, wildlife, and humans. I observed that maternal transfer of the environmental contaminant, Hg, interacting with later environmental exposure may be one mechanism of impaired reproductive success contributing to worldwide amphibian population declines. While I found negative sublethal effects of Hg from both maternal and dietary exposure separately, it was only the combination of the two exposure routes that produced drastic lethal effects. This is important information when considering conservation efforts of biphasic organisms like amphibians. Negative effects on individuals and potentially the population could be mitigated by restoring one habitat (e.g., terrestrial or aquatic) and eliminating one route of exposure. Because amphibians are important components of many

terrestrial and aquatic ecosystems, it is important to continue investigating the negative impacts of contaminants on amphibian declines. A future research priority should be to investigate whether environmentally realistic concentrations of other persistent, bioaccumulative contaminants produce similar results singularly and in environmentally relevant combinations.

Literature Cited

Beebee, T.J.C., Griffiths, R.A., 2005. The amphibian decline crisis: A watershed for conservation biology? Biological Conservation 125, 271-285.

Biek, R., Funk, W.C., Maxell, B.A., Mills, L.S., 2002. What is missing in amphibian decline research: insights from ecological sensitivity analysis. Conservation Biology 16, 728-734.

Boone, M.D., 2005. Juvenile frogs compensate for small metamorph size with terrestrial growth: Overcoming the effects of larval density and insecticide exposure. Journal of Herpetology 39, 416-423.

Budischak, S.A., Belden, L.K., Hopkins, W.A., 2008. Effects of malathion on embryonic development and latent susceptibility to tremadoe parasites in ranid tadpoles. Environmental Toxicology and Chemistry 27, 2496-2500.

Crump, K.L., Trudeau, V.L., 2009. Mercury-induced reproductive impairment in fish. Environmental Toxicology and Chemistry 28, 895-907.

Eisenreich, K.M., Kelly, S.M., Rowe, C.L., 2009. Latent mortality of juvenile snapping turtles from the upper Hudson River, New York, exposed maternally and via the diet to polychlorinated biphenyls (PCBs). Environmental Science & Technology 43, 6052-6057.

Hammerschmidt, C.R., Sandheinrich, M.B., 2005. Maternal diet during oogenesis is the major source of methylmercury in fish embryos. Environmental Science & Technology 39, 3580-3584.

Hopkins, W.A., Rowe, C.L., 2010. Interdisciplinary and hierarchical approaches for studying the effects of metals and metalloids on amphibians. Pages 325-336 in Sparling DW, Linder G, Bishop CA, eds. Ecotoxicology of Amphibians and Reptiles, 2nd edition. Pensacola, FL: SETAC Press.

IUCN. 2011. IUCN Red List of Threatened Species. Version 2011.1. http://www.iucnredlist.org. Downloaded on 16 June 2011. Report no.

Kramer, V.J., Etterson, M.A., Hecker, M., Murphy, C.A., Roesijadi, G., Spade, D.J., Spromberg, J.A., Wang, M., Ankley, G.T., 2011. Adverse outcome pathways and ecological risk assessment: Bridging to population-level effects. Environmental Toxicology and Chemistry 30, 64-76.

Marteinson, S.C., Bird, D.M., Shutt, J.L., Letcher, R.J., Ritchie, I.J., Fernie, K.J., 2010. Multi-generational effects of polybrominated diphenylethers exposure: Embryonic exposure of male American kestrels (Falco sparverius) to DE-71 alters reproductive success and behaviors. Environmental Toxicology and Chemistry 29, 1740-1747.

Marteinson, S.C., Kimmins, S., Bird, D.M., Shutt, J.L., Letcher, R.J., Ritchie, I.J., Fernie, K.J., 2011. Embryonic Exposure to the Polybrominated Diphenyl Ether Mixture, DE-71, Affects Testes and Circulating Testosterone Concentrations in Adult American Kestrels (Falco sparverius). Toxicological Sciences 121, 168-176.

Mousseau, T.A., Uller, T., Wapstra, E., Badyaev, A.V., 2009. Evolution of maternal effects: past and present. Philos Trans R Soc Lond B 364.

Nye, J.A., Davis, D.D., Miller, T.J., 2007. The effect of maternal exposure to contaminated sediment on the growth and condition of larval Fundulus heteroclitus. Aquatic Toxicology 82, 242-250.

Rohr, J.R., Palmer, B.D., 2005. Aquatic herbicide exposure increases salamnder desiccation risk eight months later in a terrestrial environment. Environmental Toxicology and Chemistry 24, 1253-1258.

Scheuhammer, A.M., Meyer, M.W., Sandheinrich, M.B., Murray, M.W., 2007. Effects of environmental methylmercury on the health of wild birds, mammals, and fish. Ambio 36, 12-18.

Schmidt, B.R., Feldmann, R., Schaub, M., 2005. Demographic processes underlying population growth and decline in *Salamandra salamandra*. Conservation Biology 19, 1149-1156.

Sparling, D.W., Linder, G., Bishop, C.A., Krest, S.K., 2010. Ecotoxicology of Amphibians and Reptiles, 2nd edition. Pensacola, FL: SETAC Press.

Tan, S.W., Meiller, J.C., Mahaffey, K.R., 2009. The endocrine effects of mercury in humans and wildlife. Critical Reviews in Toxicology 39, 228-269.

Todd, B.D., Bergeron, C.M., Hepner, M.J., Hopkins, W.A., 2011a. Aquatic and terrestrial stressors in amphibians: a test of the double jeopardy hypothesis based on maternally and trophically derived contaminants. Environmental Toxicology and Chemistry 30, 2277-2284.

Todd, B.D., Willson, J.D., Bergeron, C.M., Hopkins, W.A., *In press*. Do effects of mercury in larval amphibians persist after metamorphosis? . Ecotoxicology DOI 10.1007/s10646-011-0768-0.

Todd, B.D., Bergeron, C.M., Hepner, M.J., Burke, J.N., Hopkins, W.A., 2011b. Does maternal exposure to an environmental stressor affect offspring response to predators? Oecologia 166, 283-290.

Unrine, J.M., Jagoe, C.H., Hopkins, W.A., Brant, H.A., 2004. Adverse effects of ecologically relevant dietary mercury exposure in southern leopard frog (*Rana sphenocephala*) larvae. Environmental Toxicology and Chemistry 23, 2964-2970.

Vonesh, J.R., De la Cruz, O., 2002. Complex life cycles and density dependence: assessing the contribution of egg mortality to amphibian declines. Oecologia 133, 325-333.

Wada, H., Bergeron, C.M., McNabb, F.M.A., Todd, B.D., Hopkins, W.A., 2011. Dietary mercury has no observable effects on thyroid-mediated processes and fitness-related traits in wood frogs. Environmental Science & Technology 45, 7915-7922.

Wake, B.D., Vredenburg, V.T., 2008. Are we in the midst of the sixth mass extinction? A view from the world of amphibians. Proceedings of the National Academy of Sciences 105, 11466–11473.

Weiner, J.G., Spry, D.J., 1996. Toxicological significance of mercury in freshwater fish. Pages 297-340 in Beyer WN, Heinz GH, Redmon-Norwood AW, eds. Environmental contaminants in wildlife: Interpreting tissue concentrations. Boca Raton, FL: Lewis Publishers.

Willson, J.D., Hopkins, W.A., Bergeron, C.M., Todd, B.D., *In review*. From individual-level effects to population-level responses: The missing link in amphibian ecotoxicology. Proceedings of the Royal Society B.