Methylmercury Bioaccumulation in Spotted Salamanders (Ambystoma maculatum) in Southern Ohio

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Methylmercury Bioaccumulation in Spotted Salamanders
(Ambystoma maculatum) in Southern Ohio

A thesis submitted in partial fulfillment of the requirements
for the degree of Master of Science

By

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2017
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Abstract

Walker, Rachel Alex. M.S., Department of Earth & Environmental Sciences, Wright State University, 2017. Methylmercury Bioaccumulation in Spotted Salamanders (Ambystoma maculatum) in Southern Ohio

Mercury (Hg) is a volatile element increasing in concentration in the environment as a result of anthropogenic emissions. Microorganisms can transform mercury into monomethylmercury (MMHg), the form of Hg that bioaccumulates, biomagnifies, and can harm humans and wildlife. Most studies of MMHg bioaccumulation in wildlife have focused on aquatic organisms due to consumption of fish being the primary route of human exposure to MMHg. However, organisms in terrestrial ecosystems also are exposed to MMHg that may impact ecosystem biodiversity, food-web dynamics, and organisms and ecosystem health. I investigated bioaccumulation and maternal transfer of MMHg in spotted salamanders (Ambystoma maculatum) captured from two locations in southern Ohio in 2016. Total length, weight, sex, and whole-body concentrations of MMHg were determined for 159 organisms. Spotted salamanders in southern Ohio bioaccumulated MMHg to concentrations (mean = 93 ± 33 ng/g dry wt.) comparable to those is other salamander species in other locations. MMHg concentrations in spotted salamander carcasses were unrelated to organism size. MMHg was maternally transferred to eggs, but concentrations in eggs were not strongly correlated with concentrations in associated maternal carcasses.
MMHg concentrations in the distal 4 cm of tail were positively correlated with concentrations in spotted salamander carcasses, which provides a non-destructive sampling method for future screening and biomonitoring MMHg concentrations in these organisms. Due to their ubiquity, spotted salamanders may be useful bioindicators of MMHg bioaccumulation and cycling in forested ecosystems of southern Ohio, and by extension, other terrestrial ecosystems that they inhabit.
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Acknowledgements

I would like to thank Wright State University for their support over the past two years and Dr. Chad Hammerschmidt for his advice and guidance to complete this research. Thank you to Alison Agather for spending time with me to ensure I was analyzing accurately, and Kelly Muterspaw for assistance in the lab, as well.

Furthermore, I would like to extend a thank you to all those who helped me sample salamanders during those cold and rainy nights in late February to early March: Dane Boring, Erica Strope, Heather Perusini, Andrew Parent, Ryan Shell, Katelynn Alcorn, Kortney Mullen, Kyle McGeary, Taylor Bernhard, Ashlynn Boedecker, Justyna Hample, and Mike Zimmerman.

I am grateful to Kipp Brown and Joe Letsche for teaching me all they know about salamanders and how to sample them.

To the best people, and even better friends, I could ever dream of spending so much time with at Wright State University’s Earth and Environmental Sciences Department, thank you for your support. Also, thank you to my father (Eddy), mother (Mel), sister (Lauren), and my dogs (Roxie and Ike) for the emotional support through graduate school.

Lastly, thank you Andrew Parent for your devotion and for always keeping me on my toes.
I. Introduction

Mercury (Hg) is a volatile element that is increasing in concentration in the environment as a result of anthropogenic emissions (Fitzgerald et al., 2007; Fitzgerald et al., 1998), dominantly from fossil fuel combustion (Hammerschmidt, 2011; Streets et al., 2011). Natural emissions from volcanoes, crustal degassing, and forest fires also contribute to the atmospheric reservoir of Hg (Fitzgerald and Lamborg, 2002). Once emitted, gaseous elemental Hg (Hg\(^{0}\)) is readily transported over long distances in the atmosphere, where it has a residence time of about one year (Lamborg et al. 2002). Mercury that is deposited to aquatic and terrestrial systems can be transformed to monomethylmercury (MMHg), CH\(_3\)Hg\(^{+}\), by microorganisms such as sulfate- and iron-reducing bacteria (Compeau & Bartha, 1985; Gilmour et al., 1992; Kerin et al., 2006; Fleming et al., 2006; Gilmour et al., 2013). MMHg is toxic, bioaccumulates in heterotrophic organisms mostly through their diet (Hall et al., 1997; Tsui and Wang, 2004), and biomagnifies in food webs (Hammerschmidt and Fitzgerald, 2006), resulting in top predators having the greatest risk of harm from MMHg exposure (Evers et al., 2008). Most studies of MMHg bioaccumulation have focused on marine and freshwater organisms due to consumption of fish being the primary route of human exposure to MMHg (Fitzgerald and Clarkson, 1991; Sunderland, 2007). However, organisms in terrestrial ecosystems also are exposed to MMHg that may impact ecosystem biodiversity and health (Cristol et al., 2008).
Amphibians are imperative to aquatic and terrestrial ecosystems they populate. Spotted salamanders, specifically, have a crucial ecological role regarding food web energy and nutrient contribution, therefore providing ecosystem stability. Populations of salamanders are important to ecosystem nutrient distribution and stability for numerous reasons (Davic and Hartwell, 2004). As mid-trophic positioned opportunistic predators of worms, and different arthropods, including spiders and flies (Petranka et al., 1998; Wyman, 1998; Pfingsten et al., 2013; Beard, 2002), spotted salamanders may control species diversity and ecosystem processes among grazers and detritivores. Breeding migrations between aquatic and terrestrial ecosystems allow nutrients to flow between the two landscapes. Largely influenced by rain, terrestrial adult salamanders migrate as far as about 1.6 km to breed in vernal pools and wetlands (Pauly et al., 2007). Many consumers of salamanders, including raccoons, snakes, minks, otters, wading birds, and turtles (Petranka, 1998; Bergeron, 2010a), obtain energy and nutrients from salamanders.

Salamanders are often viewed as indicator, or representative, species (Pfingsten et al., 2013) for specific ecosystems because they are sensitive to environmental changes due to their thin skin that readily absorbs toxins. In addition to their sensitivity, some salamander species can be used to monitor stressors within both aquatic and terrestrial ecosystems because they have dual life stages that couple the two habitats (Davic & Hartwell, 2004). Spotted salamanders begin life strictly as aquatic organisms during a gilled-larval stage (Pfingsten et al., 2013), later undergoing complete metamorphosis in which the
larvae lose gills, develop eyelids and bones, and become terrestrial organisms, no longer requiring bodies of water to function (Pfingsten et al., 2013). Other salamander species, such as red-backed (*Plethodon cinereus*; Appendix Figure A1) and two-lined salamanders (*Eurycea bislineata*; Appendix Figure A2), are either lungless or gilled, and require moisture for respiration processes.

Not only does sensitivity to environmental changes make salamanders an indicator species for ecosystems, salamanders also populate ecosystems in large numbers. In the Hubbard Brook experimental forest of New Hampshire, for example, five species of salamanders had a combined average density of 2,950 individuals per hectare and combined average biomass of 1.8 kg/ha wet weight (Burton, 1975; Davic and Hartwell, 2004). At the peak of bird breeding season in the forest, the combined biomass of the salamanders was estimated to be 2.6× that of birds and equal to the areal mass of small mammals (Davic & Hartwell, 2004). Similar salamander abundances have been observed in an isolated wetland in South Carolina (Gibbons et al., 2006).

The Ambystomidae family, commonly referred to as mole salamanders, spend most their adult lives underground and under organic matter on forest floors. Mole salamanders are migratory, exhibiting short distance movements across various landscapes (up to ~1.6 km; Pauly et al. 2007). Mass migrations of mole salamanders occur in late spring for reproduction, most often to small bodies of water such as wetlands and vernal pools that do not contain fish that could consume breeding adults, their eggs, and offspring. Of the 25 species of salamanders in Ohio, eight species are mole salamanders, one of which is
considered endangered, the blue-spotted salamander (*Ambystoma laterale*; Appendix Figure A3). One of the most abundant species in Ohio is the spotted salamander (*Ambystoma maculatum*; Appendix Figure A4), which is distributed throughout all of Ohio and most of the eastern United States (Appendix Figure A5).

Spotted salamanders are mid-level consumers. Adults have a diverse diet consisting of forest-floor invertebrates, including earthworms, slugs, snails, spiders, and other insects (Downs, 1989; Petranka, 1998; Wyman, 1998; Beard, 2002; Pfingsten et al., 2013). Mole salamander larvae are opportunistic consumers of water beetles, tadpoles, zooplankton, and other salamander larvae (Walls and Jaeger, 1987; Brodman, 1995, 1996).

Global populations of amphibians have been rapidly declining due to habitat loss and degradation; wetlands are the most threatened ecosystem type (Dahl, 1990) and sensitive to pollutants such as Hg (Lacerda and Fitzgerald, 2001). Spotted salamanders require wetlands and small bodies of water to complete their lifecycle. It is possible that ground-foraging organisms, such as spotted salamanders, can be exposed to MMHg from the forest floor as it biomagnifies through terrestrial food webs (Hall et al., 2005; Rimmer et al., 2010; Townsend et al., 2013). However, little is known about MMHg bioaccumulation in amphibians and no studies have examined MMHg bioaccumulation in spotted salamanders.
II. Research Objectives

Due to the ubiquity and abundance of spotted salamanders, they may be a useful bioindicators of MMHg in terrestrial ecosystems of Ohio. I examined MMHg in individual adult salamanders from varying locations in southern Ohio to better understand geographical variations of concentrations and relationships to morphological characteristics of the organisms. Furthermore, I and for the first time, I examined whether spotted salamanders maternally transfer MMHg to their eggs. Lastly, a non-destructive sampling method was developed for estimating MMHg in spotted salamanders from concentrations in the distal portions of their tails.
III. Background

3.1. Hg Transfers from Aquatic to Terrestrial Food Webs

Monomethylmercury is transferred between aquatic and terrestrial food webs. Small bodies of water, such as vernal pools and ephemeral wetlands, have a potential for Hg methylation and transfer of MMHg between aquatic and terrestrial ecosystems via emigrating amphibians (Loftin et al., 2012; Townsend et al., 2013). MMHg has the potential to transfer from aquatic to terrestrial food webs, by bioaccumulating in terrestrial organisms that forage on aquatic organisms. Conversely, terrestrially derived MMHg can be transferred to aquatic food webs via oviposition during breeding in water. Acidic freshwater ecosystems, such as wetlands that are exposed to high temperatures and have high dissolved organic carbon (DOC) concentrations, facilitate MMHg bioaccumulation and biomagnification (Bank et al., 2005; Gilmour and Henry, 1991).

Concentrations of total Hg (THg) and MMHg have been measured in a variety of organisms occupying different trophic positions in Acadia National Park, Maine, including salamanders. Microorganisms at the base of food webs in Acadia National Park had MMHg concentrations ranging from 4.6 to 21 ng/g wet weight and THg concentrations from 23 to 76 ng/g wet weight (Burgess, 1997), assuming a 90% water content by mass. Amphibians that are consumers of microorganisms had average THg concentrations of $66 \pm 3$ ng/g wet wt. in
northern two-lined salamanders (*Eurycea bislineata*; *n* = 116), 25 ± 2 ng/g wet wt. in green frog tadpoles (*Rana clamitans*, *n* = 61; Bank et al., 2005) and 19 ± 1 ng/g wet wt. in bullfrog tadpoles (*Rana catosbeiana*, *n* = 31; Bank et al., 2004). It is possible that two-lined salamanders had greater concentrations of THg because they were exposed to Hg over a longer period of time than either frog species; two-lined salamanders remain in their larval stage for 1 to 3 years.

Piscivorous bird species from Mount Desert Island, Maine, such as bald eagles (*Haliaeetus leucocephalus*) and common loons (*Gavia immer*), and mammals such as mink (*Mustela vison*) and river otters (*Lutra canadensis*), also indicate transfer of MMHg from aquatic systems to terrestrial organisms in Acadia National Park due to their diet consisting of mostly fish and other small carnivores (Bank et al. 2007). Amphibians are important prey of mink and otter (Clavero & Delibes, 2005; Lake et al., 2007; Bergeron et al., 2010a) and could be contributing MMHg to terrestrial food webs accordingly.

Moreover, populations of northern dusky salamanders (*Desmognathus fuscus fuscus*; members of the Plethodontidae family, otherwise known as lungless salamanders; Appendix Figure A6) have been declining in Acadia National Park. One hypothesis for the decline of dusky salamanders is increased MMHg exposure from consumption of northern two-lined salamanders and other contaminated organisms (Bank et al., 2006). Adult and larval northern two-lined salamanders sampled from headwater streams in Acadia National Park had elevated levels of MMHg, potentially contributing to bioaccumulation of MMHg in northern dusky salamanders (Bank et al., 2005, 2006).
Bank et al. (2005) examined Hg bioaccumulation in northern two-lined salamanders from Acadia National Park and found that MMHg was the dominant form of Hg, comprising 73–97% of THg ($n = 8$ per stream, $n = 4$ streams; THg = $66 \pm 3$ ng/g). Most THg bioaccumulated in two-lined salamanders in Acadia National Park is thought to occur during the aquatic, larval stage, which can last from 1–3 years. They observed no significant correlations between THg concentrations and either the length or weight of larvae. Larval and adult two-lined salamanders had significantly more THg concentrations than juvenile brook trout sampled from the same stream ($P = 0.003$; Bank et al., 2005).

3.2. Effects of Hg on Amphibians

When exposed to dietary Hg, as HgCl$_2$, southern leopard frogs (*Rana sphenocephala*) exhibited malformations during development (Unrine et al., 2004). Tadpoles were exposed to environmentally realistic concentrations of Hg-contaminated aufwuchs (periphyton and other benthic food sources common to a typical anuran diet) amended with HgCl$_2$. Total Hg concentrations in aufwuchs treatments ranged from 14 ng/g wet weight in the control (no added Hg) to 110 (low treatment), 370 (medium), and 860 ng/g (high). The concentration of THg in the high aufwuchs treatment was 2× greater than that measured in aufwuchs collected from the environment. Time to metamorphosis, size at metamorphosis, metamorphic success, and growth and development during the larval period (60–254 d) were examined after tadpoles were exposed to aufwuchs treatments.
Unrine and colleagues (2004) monitored tadpoles every 1–2 days for food consumption, abnormalities, and survival. The number of days to Gosner Stage (GS; Gosner, 1960) 39, GS 42, and GS 46 were counted, with GS 46 denoted as completion of metamorphosis. Larvae survival decreased with greater Hg exposure from aufwuchs (P = 0.04). Survival of tadpoles that consumed the control diet was 88% (one tadpole was injured and therefore removed) and the low treatment had a survival of 100%. The medium and high treatments both had survival rates of 72% with tadpole death occurring after GS 39, when hind legs were developed (60–90 d).

Malformations were observed in tadpoles exposed HgCl$_2$-contaminated aufwuchs, and the number of malformation increased with THg concentration in aufwuchs (Unrine et al., 2004). Malformations were defined in this study to be scoliosis, microptalmia, micromelia, and ectromelia in the tibia and fibulae of both hind legs. Tadpoles that consumed the high Hg diet were, on average, 39% heavier than control tadpoles (P = 0.02): tadpoles exposed to the high-Hg treatment consumed entire rations, potentially as a stress response (Carr, 2002; Unrine et al., 2004). Tadpoles exposed to the high treatment took longer to reach GS 39 (P = 0.03), GS 42 (P = 0.03), and complete GS 46 (P = 0.02) relative to controls.

Burke et al. (2010) observed adverse effects in northern two-lined salamanders collected from a Hg-contaminated (n = 15 individuals) compared to a reference site (n = 19) in the South River, Virginia. In a behavior and performance study, observed responsiveness to prodding and travel time of adult
salamanders from the contaminated site was significantly different than that of salamanders from the reference site. However, in a second trial of the same study, there was not a significant difference in responsiveness between salamanders from the contaminated and reference sites. The authors observed that individual motivation to feed, not efficiency at catching prey (i.e., flightless fruit flies), was significantly different between contaminated and reference sites. Salamanders from the contaminated site (4500 ± 350 ng/g dry wt) contained significantly more THg than those from the reference site (250 ± 26 ng/g, P < 0.001).

3.3. Maternal Transfer of Hg and Effects of Hg on Offspring

Maternal transfer of contaminants, such as Hg, can cause adverse effects, and sometimes mortality, to developing embryos. Due to their feeding behaviors, amphibian larvae readily accumulate Hg, which can cause malformations during metamorphosis. American toads (Bufo americanus) sampled from a Hg-contaminated site maternally transferred Hg to their offspring in proportion to concentrations in the maternal carcass: both THg ($r = 0.89, P < 0.0001, n = 48$) and MMHg ($r = 0.91, P < 0.0001, n = 21$) concentrations were positively correlated between maternal whole-body and egg samples (Bergeron, 2010).

Twenty-seven reproductive pairs of American toads were sampled from the South River, Virginia, upstream (reference) and downstream (contaminated) of a Hg point source, to examine the effects of dietary THg exposures on larval development from reference and contaminated sites (Bergeron et al., 2011).
After breeding, reference and contaminated clutches of eggs were separated and larvae were fed diets of varying concentrations of Hg, as Hg$^{2+}$, for up to 28 days (control = 10 ± 1 ng/g dry wt.; low = 2500 ± 60 ng/g; high = 10100 ± 2300 ng/g; $n$ = 25 per treatment). Concentrations of THg in larval toads differed significantly between reference (21 ± 1 ng/g dry wt.) and contaminated egg clutches (149 ± 18 ng/g dry wt.; $P$ = 0.006). Dietary and maternal exposure significantly affected growth and development of larvae until front limb emergence (GS 42; diet, $P$ = 0.02; maternal Hg exposure, $P$ = 0.03), and diet had a significant effect on organisms mass at GS 42 ($P$ = 0.004) and GS 46 (tail resorption; $P$ = 0.018). Hg exposure had no effect on duration of the larval period ($P$ = 0.79), however, larvae fed the high Hg diet were 16% smaller than those fed the control diet, and larvae from contaminated mothers were 10% smaller at GS 42 than those from reference mothers. It also took contaminated larvae longer to resorb their tails and THg exposure had negative effects on developing larvae locomotion.

Additionally, survival decreased during the metamorphic climax from dietary and maternal Hg exposure, and the combination of maternal exposure and high Hg diet led to a 50% reduction in metamorphic success, and a 125% greater mortality compared to larvae from reference mothers raised on a control diet.

An increased duration of metamorphic climax can be detrimental to amphibians in the environment due to an increased risk of vulnerability to predation, and a decrease of immunological and energetic functions (Burke et al. 2010). Smaller amphibian size can negatively impact survival, reproduction, and fecundity (Liang et al., 1994; Bergeron, 2011). During tail resorption by toads, Hg
remobilizes from tail into circulation, making metamorphic climax a particularly sensitive stage in development (Bergeron, 2011).

3.4. Non-Destructive Sampling of Salamanders

Most salamanders use tail autonomy as an escape mechanism, in which the organism can release its tail to later be regenerated with either little or no effect on lifestyle. Accordingly, non-destructive sampling techniques that subsample a portion of the tail have been developed for some salamander species. Bergeron et al. (2010a) sampled red-backed and northern two-lined salamanders from sites upstream and downstream of a Hg-contaminated source in the South River, Virginia, and examined Hg concentrations in whole bodies and tails. They observed that THg concentrations were positively correlated between tails (dependent variable) and whole bodies in both northern two-lined \( (r = 0.99, P < 0.0001, n = 51, \text{slope} = 0.985) \) and red-backed salamanders \( (r = 0.97, P < 0.0001, n = 24, \text{slope} = 0.783) \). The differences in regression slopes between the two species indicates that tail-body Hg relationships are salamander species specific.

Non-destructive sampling techniques also were developed for THg in four salamander species in the Pacific Northwest region of North America (Pfleeger et al., 2016). These species included northwestern salamander \( (A. gracile, n = 133) \), long-toed salamander \( (A. marcodactylum, n = 11) \), rough-skinned newt \( (Taricha granulosa, n = 39) \), and coastal giant salamander \( (Dicamptodon tenebrosus, n = 42) \). Salamander tails were clipped 4 cm from the distal portion of
the tail, and cut into segments of 0–1 (i.e., tip), 1–2, and 2–4 cm. Concentrations of THg in all three segments of tails were positively correlated with the corresponding THg whole-body concentration (P < 0.0001). However, correlations and regression slope values differed among species and among tail portions within species, suggesting that tail:body THg ratios differ among salamander species and sampling methods. The strongest correlation between concentrations in tail segments and whole bodies was different for each species of salamander. Optimal tail clip segments were 0–1 cm \( (R^2 = 0.92) \) for northwestern salamander, 1–2 cm for long-toed salamander \( (R^2 = 0.87) \) and rough-skinned newt \( (R^2 = 0.86) \), and 2–4 cm for coastal giant salamander \( (R^2 = 0.97) \); Pfleeger et al., 2016).
IV. Methods

4.1 Area of Study

Spotted salamanders ($n = 159$) were sampled from year-round ponds located in Ross and Highland Counties, in southern Ohio. The majority of the salamanders were collected from Buzzard’s Roost Nature Preserve near the city of Chillicothe, Ross County ($n = 135$). Buzzard’s Roost Nature Preserve is ~9 km from downtown Chillicothe and is at a greater elevation than most of the surrounding area (Appendix Figure A7). This location was selected because the preserved land and its wildlife population is distant from direct anthropogenic disturbances. Three pools located within the preserve were identified as Sites A, B, and C. Sites A and B are old agricultural ponds, surrounded mostly by maintained landscape, including mowed grass, a roadway, and man-made structures, and all are adjacent to deciduous forest. Site C also is an old agricultural pond but differs from Sites A and B because it is secluded and surrounded entirely by dense forest. One side of the Site C pond is a pine stand that has existed for ~25 yrs. None of the ponds are thought to contain fish nor have either stream water inputs or outputs.

The fourth sampling pond (Site D; $n = 24$) is located in Hillsboro, Highland County, Ohio, about 64 km southwest of the other three sites (Appendix Figure A8). The pond at Site D is surrounded by deciduous forest and near a conservation grassland. The pond at Site D also remains filled with water year-
round and does not contain fish; however, it has inlet and outlet streams.

4.2 Salamander Sampling

Salamanders from Buzzard’s Roost Nature Preserve (Sites A, B, and C) were sampled overnight on February 24 and March 4, 2016. Sampling at Site D in Highland County occurred on March 10, 2016. Sampling events immediately followed thunderstorms when air temperatures were between ~4–10 °C and breeding migrations were occurring. Instead of one mass breeding migration, multiple smaller migrations occurred in 2016 due to unseasonably warm temperatures during the first three months of the year. Males were first to migrate to the sampling sites in Buzzard’s Roost Nature Preserve, later followed by females, as indicated by no females being sampled and observation of spermatophores during the first sampling day (February 24). At Buzzard’s Roost, salamanders were sampled with either unbaited funnel traps (Micacchion, 2011), gloved hands, or dip nets. Site D was completely flooded; therefore, all individuals were sampled with funnel traps placed at the sampling site before storm activity. Funnel traps were recovered after the storms, and no salamanders were observed either within or around the pond after removal of the traps from the water.

4.3 Sample Preparation and Analysis

Salamanders were euthanized in the field with an overdose of tricaine methane sulfonate, placed in separate and uniquely identified zip-type plastic
bags, and stored on dry ice until they were transported to Wright State University. At the university, salamanders were stored at −20 °C until they were thawed and measured for total length (TL) and weighed for whole-body wet mass. The distal 4 cm of tail was dissected from each salamander with a clean stainless steel blade and weighed. Egg sacs (including ovary tissue) were removed from gravid females and weighed. Tails, egg sacs, and carcasses were stored in separate plastic tubes and bags and refrozen before each sample was lyophilized to a constant dry weight and water content was determined by mass difference. Dried salamander carcasses were ground to a powder and homogenized with a stainless-steel blender.

MMHg was determined after sample digestion in dilute HNO₃. For homogenized carcasses, a representative subsample (0.1–0.2 g) of each organism was accurately weighed into an acid-cleaned 15-mL tube to which was added 7 mL of 4.57 M HNO₃ and then allowed to digest in a 60 °C covered water bath for 12 h (Hammerschmidt and Fitzgerald, 2006). Dried whole tail clips (0.106–0.267 g) and egg sacs (0.151–0.200 g) were digested similarly.

MMHg in samples was measured by gas-chromatographic cold vapor atomic fluorescence spectrometry (GC-CVAFS; Tseng et al., 2004). An aliquot of digestate was added to reagent-grade water (nominal resistivity > 18 MΩ-cm) in a UConn-type bubbler (Lamborg et al., 2012), acidity was neutralized with KOH, pH was buffered to 5 with acetate, and sample MMHg was derivatized with sodium tertraethylborate. Derivatized MMHg (as methylethylmercury) was
purged from solution with high-purity N₂, concentrated on Tenax, and quantified by GC-CVAFS (Tseng et al., 2004).

Sample MMHg was quantified after calibration with procedural blanks and standard solutions that went through the digestion process. All blanks contained undetectable amounts of MMHg. Biological reference materials TORT-2 and TORT-3 (both lobster hepatopancreas, National Research Council of Canada) were digested and analyzed with each batch of salamander samples, and about 10% of samples were either digested in replicate (carcasses only) or analyzed in replicate. The mean measured concentration of MMHg in TORT-2 (n = 6) was 150 ± 4 ng/g (certified range = 139–165 ng/g) and measured MMHg in TORT-3 (n = 18) averaged 130 ± 3 ng/g (certified range = 125–149 ng/g). Reproducibility among samples digested in triplicate averaged 2.8% relative standard deviation (n = 8 sets of replicates). Analytical precision averaged 3.6 relative percent difference among 16 samples analyzed in duplicate. The estimated method detection limit for MMHg was less than 0.1 ng/g dry wt.

All statistical analyses were performed with SigmaPlot 12.3. Assumptions of normality and homoscedasticity were tested with Shapiro-Wilk’s tests, and Dunn’s and Holm Sidak methods. Analysis of variance (ANOVA) was used to compare total length, whole body mass, and MMHg concentration among sampling locations and linear regression was used to determine a correlation between whole body samples and corresponding tail clips.
V. Results and Discussion

5.1 MMHg Bioaccumulation in Spotted Salamanders

There were no significant differences in MMHg concentration between male and female spotted salamanders in this study. Females were collected from each of the four sites but there were far fewer females ($n = 15$) than males ($n = 144$). Among all sites, MMHg concentrations were not different between males and females ($t$-test, $P = 0.14$). The absence of differences between sexes allowed for males and females to be combined for statistical purposes.

Spotted salamanders sampled from southern Ohio bioaccumulated MMHg. The mean ($\pm$ SD) measured concentration of MMHg in spotted salamanders ($93 \pm 33$ ng/g dry wt.) was comparable to those of other salamander species sampled in other locations. Red-backed salamanders collected from the Catskill Mountains, New York contained MMHg concentrations ranging from 55 to 122 ng/g dry wt., and MMHg concentrations increased with elevation (Townsend et al., 2013). In contrast, adult two-lined salamanders sampled from Acadia National Park had average THg concentrations of $66 \pm 3$ ng/g wet wt. (Bank et al., 2005). Northern dusky salamanders in Acadia National Park, Maine are hypothesized to have elevated levels of THg because their
carnivorous diet consists largely of larval and adult two-lined salamanders (Bank et al., 2006).

MMHg concentrations in carcasses of adult spotted salamanders were independent of total length (Figure 1) and whole-body wet weights (Figure 2). Accordingly, size is not a useful proxy for MMHg concentration in bodies of spotted salamanders, at least at these locations. The average total length of spotted salamanders differed between Sites B and C (P = 0.03; Table 1). Average salamander lengths at Sites A and B were not different from either each other or Sites C and D. Among all spotted salamanders, total length averaged 17.8 cm (range =14.7–21.6 cm). Average whole-body wet weights of spotted salamanders were not different among sites (P = 0.22). Among all salamanders, whole-body wet weight averaged 19.54 g (range =11.72–35.25 g).

The absence of a relationship between MMHg concentration and either total length or whole-body weight is consistent with findings from other salamander species at different locations. Total Hg concentrations in red-backed salamanders were weakly related to body mass in the northeastern United States (r = 0.35, P < 0.0001, slope = 0.78; Townsend and Driscoll, 2013). Additionally, THg concentrations in larval two-lined salamanders were unrelated to either total length or body weight in Acadia National Park (Bank et al., 2005). Because there were no relationships between MMHg concentration and either length or mass of spotted salamanders in this study, size is not a suitable proxy for MMHg concentration. The absence of a relationship between MMHg concentration and length in spotted salamanders is contrary to relationships commonly observed
between the two variables in many species of freshwater (Lange et al. 1994) and marine fish (Barber et al., 1972).

Average concentrations of MMHg in adult spotted salamander carcasses differed among some of the sampling sites (Table 1). Concentrations of MMHg in spotted salamanders sampled from Sites A and B were greater than those in salamanders sampled from Sites C and D (P < 0.05). However, concentrations in spotted salamanders sampled from Site A were not different from those at Site B. Likewise, mean MMHg concentrations did not differ between Sites C and D. Site C was approximately 64 km from Site D and both ponds were near forests. Because stomatal uptake may attenuate Hg deposition to forested ecosystems (Browne and Fang, 1978; St. Louis et al., 2001; Tabatchnick et al., 2012), it is possible that spotted salamanders populating Sites C and D had less exposure to atmospherically deposited Hg via their ground-foraging diet. Furthermore, salamander larvae that bioaccumulate high concentrations of Hg in breeding pools through environmental exposure to Hg after oviposition (Wolfe et al., 1998; Bergeron et al. 2011) could have varying MMHg concentrations depending on the amount of tree coverage over the bodies of water they are developing in during metamorphosis.

Sites A and B had greater average MMHg concentrations in salamander carcasses than either Sites C or D, possibly because Sites A and B have more exposure to direct atmospheric Hg deposition. The absence of MMHg concentration differences in salamanders between Sites A and B may be a result of the same spotted salamander population using both ponds, which were less 1
km apart and within the migration ranges of spotted salamanders (up to ~1 km; Petranka, 1998). If Sites A and B were the same population, then it is possible that the salamanders were consuming similar prey items, which could differ from prey items, and their associated MMHg concentrations, consumed by salamanders sampled at Sites C and D.

Moreover, because spotted salamanders demonstrate indeterminate growth (Perrin and Sibley, 1993), the age of salamanders cannot be determined from size alone. All salamanders sampled in this study were deemed to be adults based on the physiological function of breeding, not size. It can be assumed that smaller salamanders are younger than the larger ones, as with most organisms. However, for the purpose of this study, age was not determined. Future studies should consider methods to measure age to examine whether MMHg in salamanders varies as a function of age, as it does in fish (e.g., Lange et al., 1994).
Figure 1. Relationship between methylmercury concentration in spotted salamander carcasses and whole-body total length.
Figure 2. Relationship between methylmercury concentration in spotted salamander carcasses and whole-body wet weight.

\[
y = -0.1829x + 96.962 \\
R^2 = 0.00046 \\
P = 0.219
\]
Table 1. Summary statistics (mean ± SD) of carcass (without eggs and tail tip) methylmercury concentration and total length, water content, and whole-body wet weight of whole salamanders among sampling sites.

<table>
<thead>
<tr>
<th>Site</th>
<th>n</th>
<th>Total length (cm)</th>
<th>Water content (%)</th>
<th>Whole-body wet mass (g)</th>
<th>Carcass MMHg (ng/g dry wt.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>37</td>
<td>17.6 ± 1.0</td>
<td>78.8 ± 4.4</td>
<td>18.37 ± 4.31</td>
<td>106 ± 29</td>
</tr>
<tr>
<td>B</td>
<td>36</td>
<td>17.4 ± 1.2</td>
<td>77.5 ± 1.7</td>
<td>18.33 ± 4.50</td>
<td>112 ± 43</td>
</tr>
<tr>
<td>C</td>
<td>61</td>
<td>18.1 ± 1.1</td>
<td>76.6 ± 1.9</td>
<td>19.39 ± 3.05</td>
<td>80 ± 24</td>
</tr>
<tr>
<td>D</td>
<td>24</td>
<td>17.7 ± 1.3</td>
<td>76.7 ± 1.7</td>
<td>19.28 ± 4.39</td>
<td>78 ± 26</td>
</tr>
</tbody>
</table>
5.2. MMHg Maternal Transfer in Spotted Salamanders

MMHg transfer from mother to egg was examined in 11 gravid females (Figure 3). MMHg concentrations in spotted salamander egg sacs (i.e., eggs + ovary) ranged from 5 to 28 ng/g dry wt. and averaged 18% (range, 4–27%) of the concentration in maternal carcasses. There was not a strong correlation between MMHg concentrations in the egg sacs and those in the associated mother. Fertilization of spotted salamander eggs is internal, therefore whether the eggs had been fertilized was based on whether the eggs had a clear, gelatinous coating. Only three of the eleven egg sacs appeared to have been fertilized. It was not certain that eggs were completely removed from the egg sacs during dissection, and inclusion of some or all of the ovarian tissue could have contributed to the wide range of percentages of maternally transferred MMHg. While partitioning of MMHg between and eggs and ovaries is unknown for salamanders, MMHg concentrations in ovaries of freshwater fish, for example, yellow perch (Perca flavescens), are much greater than those in eggs (Hammerschmidt et al., 1999).

Maternal transfer of MMHg can cause adverse effects on developing larvae (Bergeron, 2009), and salamander eggs and larvae are a source of nutrients for many consumers in forested ecosystems. Because this is the first time maternal transfer of MMHg has been examined in salamanders, future research should seek to develop a method of egg removal from salamander egg sacs for improved determination of maternal transfer of MMHg to eggs only.
Although American toads (*Bufo americanus*) were found to maternally transfer Hg to their offspring (Bergeron, 2010), the collection of eggs from spotted salamanders differs in that spotted salamander eggs are internally fertilized, therefore requiring dissection to obtain the eggs. Spotted salamanders are an ideal amphibian species to study maternal transfer because they are fertilized internally and not potentially compromised by environmental exposures after oviposition.
Figure 3. Relationship of concentrations of methylmercury in carcasses and eggs of gravid spotted salamanders
5.3. Non-Destructive Sampling Method for Spotted Salamanders

A non-destructive sampling method was developed for spotted salamanders. MMHg concentration in tail clippings were positively correlated with those in adult carcasses of spotted salamanders (Figure 4). This relationship suggests that future research on MMHg in spotted salamanders may not require destructive sampling to examine bioaccumulation. However, additional analyses of paired carcasses and tail clips would likely strengthen the relationship and reduce uncertainty. Previous studies have found different slopes between salamander species, therefore a positive correlation for one species is not indicative of another (Bergeron et al., 2010a; Pfleeger et al., 2016). However, by comparing the 4 cm tail clips to the corresponding body sample, a positive correlation indicates that for spotted salamanders, removing the distal portion of the tail for analysis can be used to predict the MMHg concentration in carcasses.

Future studies should determine if varying the length of tail removal from spotted salamanders could either improve the strength of the tail-to-carcass MMHg correlation or if the slopes of the correlations vary according to tail clip length. It is possible that a smaller length of tail clip could result in a stronger correlation with concentrations in carcasses. Pfleeger et al. (2016) found varying optimal lengths among salamander species, two of which were Ambystomids. Stronger relationships between Hg concentrations in bodies and tails were observed when a shorter length of tail, the distal either 1 or 2 cm, was removed.

Because spotted salamanders do not visibly show any external indication of previous tail loss, it is unclear whether MMHg concentrations in tail slips are
representative of those in the carcass if the tail had previously been regrown.

Given that bone does not bioaccumulate MMHg to concentrations comparable to those in soft tissue, it is possible that when a tail is regenerated, MMHg within the body is remobilized to the tip of the tail. Future research should consider variation in MMHg concentration between original and regenerated tails.
Figure 4. Relationship between methylmercury concentrations in carcasses and tails of spotted salamanders (n = 30 organisms, $R^2 = 0.69$, tail = 0.59(carcass) + 12.077).
5.4. **Toxicological Significance**

The observed breeding migrations of spotted salamanders during early spring 2016 in both counties indicated that the species has a large biomass within the forested ecosystems in those areas, and therefore can be used as a terrestrial bioindicator in southern Ohio. Spotted salamanders bioaccumulated MMHg, which has implications for biomagnification within terrestrial food webs. Because spotted salamanders are mid-trophic level predators of many organisms, they share a similar diet to other mid-level consumers that potentially could be exposed to MMHg through their diet. Spotted salamanders also are prey items for a variety of predators and are a vector for MMHg biomagnification.

Spotted salamanders sampled from southern Ohio had wet-weight MMHg concentrations (mean = 21.2 ± 8.6 ng/g) that are well below toxicity thresholds for MMHg in freshwater fish. Sublethal effects, such as decreased reproduction and damage to cells and tissues, in freshwater fish are associated with whole-body MMHg concentrations exceeding a 300–700 ng/g wet weight threshold (Sandheinrich and Wiener, 2011). However, little is known about the toxicological effects of MMHg in wildlife and concentrations associated with toxicity (Scheuhammer et al., 2007). Because toxicological thresholds for MMHg in amphibians are not well-developed, more research should be done on behavioral, physiological, and sublethal physical and biochemical changes of amphibians.

Although spotted salamanders from southern Ohio are below sublethal toxicity thresholds established for MMHg in fish, salamanders can be a vector of
MMHg to predators, particularly during breeding migrations from late winter to early spring. Amphibians compose a large portion of mink and otter diet (Clavero & Delibes, 2005; Lake et al., 2007; Bergeron et al., 2010a), it is possible that predatory mammals could be exposed to dietary MMHg. Otters fed a MMHg-contaminated diet of fish developed anorexia and ataxia at a lowest observed adverse effect level (LOAEL) dose of 90 ng/g dry weight (O’Connor and Neilsen, 1981), which is well within the range of concentrations in my study.

Due to the potential for MMHg to have deleterious effects on amphibians, such as lack of motivation to feed, developmental malformations, and prolonged metamorphosis (Unrine et al. 2004; Burke et al. 2010), more research is needed on MMHg bioaccumulation and toxicity, particularly in industrious regions where point sources of Hg to the environment may exacerbate MMHg production and bioaccumulation. Spotted salamanders can be a useful bioindicator for forested ecosystems in Ohio because of their nearly ubiquitous distribution in the state. Research on MMHg bioaccumulation and toxicity in spotted salamanders can help pave a way toward better understanding MMHg biomagnification in terrestrial food webs and serve as an additional avenue toward conservation of amphibians.
Literature Cited


Appendix

Figure A1. Adult red-backed salamanders (*Plethodon cinereus*). Photo credit: Virginia Herpetological Society.

Figure A2. Adult northern two-lined salamander (*Eurycea bislineata*). Photo credit: Virginia Herpetological Society.
Figure A3. Adult blue spotted salamander (Ambystoma laterale). Photo credit: New Hampshire Fish and Game.

Figure A4. Adult spotted salamander (Ambystoma maculatum). Photo credit: Ohio Department of Natural Resources.
Figure A5. Spotted salamander distribution (International Union for the Conservation of Nature Red List).

Figure A6. Adult northern dusky salamander (*Desmognathus fuscus fuscus*). Photo credit: Greg Lipps, Ohio Amphibians.
Figure A7. Sampling Sites A, B, and C in Buzzard's Roost Nature Preserve, Chillicothe, Ross County, Ohio.

Figure A8. Conservation and surrounding rural land at sampling Site D in Hillsboro, Highland County, Ohio.