Bioaccumulation of methylmercury in wood frog and spotted salamander eggs, larvae, and adults in Vermont vernal pools

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**Introduction**

Vernal pools are temporary to semi-permanent, isolated wetlands occurring in shallow depressions that typically fill during the spring and/or fall and dry during summer or in drought years (Calhoun and deMaynadier 2009). In the Northeastern and North Central United States and adjacent Canada, vernal pools are relatively widespread and abundant in forested landscapes (Colburn 2004, Van Meter et al. 2008, Faccio et al. 2016), and provide critical breeding habitat for amphibians, such as wood frogs (*Lithobates sylvatica*) and *Ambystomid* salamanders (Semlitsch and Skelly 2009), as well as numerous invertebrate taxa adapted to temporary waters (Colburn et al. 2009). Globally, amphibians are among the most imperiled vertebrate groups, due to widespread population declines and species extinctions (Wake and Vredenburg 2008, Adams et al. 2013). In the Northeastern United States, 70% of vernal pool-breeding amphibians are considered moderate- to high-priority species of regional conservation concern (NEPARC 2010), underscoring the importance of these keystone ecosystems to maintaining viable populations of at-risk species.

Vernal pools may also be hotspots for accumulation of mercury (Hg), and its more bioavailable and toxic form, methylmercury (MeHg), which can bioaccumulate through foodwebs reaching levels that cause reproductive and neurological effects in top predators (Scheulhammer 2007). Mercury (Hg) contamination via atmospheric deposition, originating primarily from coal-fired power plants and industrial incinerators, is widespread in the Northeast (Miller et al. 2005), and hotspots with enhanced deposition and biological uptake have been identified throughout the region (Evers et al. 2007). In the Northeast, hotspots are often associated with forested regions with an abundance of wetlands, which facilitate the conversion of Hg to MeHg by anaerobic bacteria (Driscoll et al. 2007, Benoit 2003).

Landscape plays a critical role in Hg accumulation, methylation and mobilization in forest floors. A variety of landscape characteristics common to vernal pools are associated with enhancing Hg deposition. First, vernal pools are typically found embedded in a forest matrix where leaves in the canopy scavenge Hg from the atmosphere and subsequently transfer it to forest soil in throughfall and litterfall (Lindberg et al. 1995, Rea et al. 1996). Second, Hg transport from the forest floor is greatest along shallow hydrologic flowpaths (Grigal 2002, Galloway and Branfireun 2004), such as surface waters that typically fill most vernal pools. And third, the hydrologic and biogeochemical properties of vernal pools provide ideal conditions for production of the sulfate-reducing bacteria responsible for Hg methylation (Wiener et al. 2003). These include water level fluctuations and periodic wetting, low dissolved oxygen, high dissolved organic carbon (DOC), and low pH, all of which increase methylation efficiency (Benoit et al. 2003), suggesting that vernal pools are potential hotspots for MeHg production and bioaccumulation in fauna. Biota in vernal pools, especially higher trophic level taxa such as amphibian larvae, may transport Hg to terrestrial systems via metamorphosis. The wide abundance of larvae across vernal pools in the Northeast, may also make them valuable bioindicators for monitoring MeHg loading, bioavailability, and ecological impacts in these sensitive environments (Evers, 2016).

Few studies have investigated Hg in vernal pools (Brooks et al. 2012, Loftin et al. 2012, Benoit et al. 2013, Davis 2013) and only Brooks et al. (2012) and Loftin et al. (2012) evaluated Hg concentrations in amphibians, with both studies sampling developing wood frogs. We found no studies reporting on Hg concentrations in adult wood frogs or vernal pool-breeding *Ambystomid* “spotted” salamanders at any life stage. In Maine vernal pools, total Hg (THg) concentrations in wood frog larvae were correlated with THg in pool water, which increased during April to June when amphibian embryos and larvae were developing (Loftin et al. 2012). In New York and Vermont pools, Davis (2013) found that THg, MeHg, and the percent Hg present as MeHg (%MeHg) in pool water also increased during the spring, reaching 43% to 58%. Methylation efficiencies, estimated as %MeHg, exceeding 10% have been linked to elevated levels of MeHg in biota (Krabbenhoft et al. 1999), suggesting that amphibian metamorphs may export significant MeHg into surrounding terrestrial systems, especially given the significant amount of biomass that emerging amphibians contribute into forested uplands surrounding vernal pools (Windmiller 1996, Berven 2009). In addition, water from pools in coniferous forests were found to have higher THg than those surrounded by hardwoods (Loftin et al. 2012), which is consistent with higher throughfall and Hg capture by coniferous trees (Risch et al. 2012).

As part of a broader study to evaluate the role of landscape characteristics and land-use on the production and transfer of MeHg in Vermont vernal pools, this study focused on the bioaccumulation of MeHg in wood frog and spotted salamander (*Ambystoma maculatum*) eggs, larvae, and adults, and investigated relationships among MeHg and Hg, water chemistry (pH, DOC,), and forest cover type surrounding pools. We hypothesized that MeHg in amphibian larvae would increase as they developed, and that predatory salamander larvae, which feed at a higher trophic level, would have higher concentrations of MeHg than omnivorous wood frog larvae due to biomagnification. We also hypothesized that water in pools surrounded by conifers would have higher Hg concentrations compared to those in deciduous stands, and that this would correlate with amphibian Hg burdens. And finally, because MeHg bioaccumulates and is not easily eliminated from the body (Bergeron et al. 2010a), we hypothesized that longer-lived spotted salamander adults would have higher MeHg concentrations compared to shorter-lived wood frog adults.

**Methods**

Study Sites

For this study, six vernal pools (three located in coniferous forests and three in deciduous forests) were selected from the Vermont Vernal Pool Mapping Project database (Faccio et al. 2013) (Table 1). All pools were located in east-central Vermont (N43°53’, N43°42’; W72°27’, W72° 16’), with four pools located in south-eastern Orange Co., and two in northeastern Windsor Co. The largely forested, rural landscape is characterized by moderate hills with numerous wetlands and vernal pools. The forest in this region is actively logged second-growth dominated by mixed northern hardwoods of maple (*Acer* spp.), American beech (*Fagus grandifolia*), birch (*Betula* spp.), ash (*Fraxinus* spp.), eastern hemlock (*Tsuga canadensis*), and pine (*Pinus* spp.). All pools were located in interior forests ≥140 m from the nearest road or building.

Water Sampling

Water samples for Hg analysis were collected using clean Hg sampling techniques, and were filtered to 0.2 µm, preserved in 0.5% HCl, and stored in the dark prior to analysis. Independent water samples at each pool were collected for total suspended solids and ancillary water chemistry analyses, while temperature, pH, and oxidation reduction potential were measured at the site. Water sampling was initiated at all pools immediately following ice-out in April, 2015, when water levels were at or near peak. Sampling continued monthly through July until pools were dry (all pools were dry by August)

Amphibian Sampling

Following spring immigration to pools for breeding, adult wood frogs and spotted salamanders were captured (n=4/pool/spp.) between 20 Apr and 12 May, 2015 using dip nets or funnel traps that were partially submerged 16-24 hours prior. Salamanders (n=8) at four pools were found by turning over logs around pool edge. After each individual was measured (snout-to-vent length, and total length), weighed to the nearest 0.1 g, and sexed (based on external characteristics), a tissue and blood sample was collected for MeHg and Hg analysis. Tissue samples from wood frogs consisted of a ca. 4-7 mm toe-clip, distal to the webbing, of the 4th (longest) toe from a single hind foot. Frog digits were anesthetized prior to amputation using topical lidocaine, and the wound was treated with antibiotic cream afterward. From each frog, a 30-60 µl blood sample was collected in a 75 µl heparinized capillary tube by puncturing the facial vein with a 30 gauge needle, following the methods outlined in Forzan et al. (2012). Capillary tubes were sealed on both ends with Critocaps®. Tissue samples were collected from adult spotted salamanders by amputating ca. 1-2 cm of tail tip using either a scalpel or surgical scissors. Blood samples (<50 μl) were collected from tail wounds using heparinized capillary tubes. Salamander tails were anesthetized prior to amputation using topical lidocaine, and the wound was treated with antibiotic cream afterward. All tissue and blood sampling was conducted in the field, and animals were held for a short period (ca. 15-80 minutes) before being released back to point of capture.

Wood frog and spotted salamander embryos were collected for Hg analysis between 9 and 12 May, 2015. Wood frog egg samples were only collected at three pools (n=4/pool; 12 samples total), while salamander samples were collected at all six pools (n=4/pool; 24 samples total). A sample consisted of 10-15 embryos collected from a single egg mass; for a total of 40-60 embryos/species/pool.

Wood frog and spotted salamander larvae were collected for Hg analysis between 12 May and 8 July, 2015. Using dip nets, larvae were collected during two sampling periods; the first (12 May to 6 June) was ca. 1 week post-hatching, the second (6 to 8 July) was ca. 5-8 weeks post-hatching. Larvae were placed in ziplock bags filled with pool water and returned to the lab where they were examined under a dissecting scope, inspected for developmental abnormalities, Gosner stage (Gosner 1960) established for wood frogs, and then frozen. At each pool, four early- and late-stage wood frog larvae were collected for a total of 48 samples. Early-stage tadpoles were between Gosner stage 22 and 25, while late-stage were between Gosner stage 26 and 39. A total of 27 spotted salamander larvae were collected at five pools, including early-stage larvae (n=12) at three pools, and late-stage larvae (n=15) at five pools.

We followed clean-Hg sampling protocols to prevent contamination of field samples. Sterile gloves were used when collecting field samples, and all samples were placed in sterile ziplock bags or plastic containers that had been acid-washed and transported to field sites in clean plastic bags. We also followed guidelines for decontamination of boots, nets, and other field equipment to minimize the risk of spreading Chytridiomycosis and Ranavirus between pools (NEPARC 2014).

Chemical Analyses

Total Hg and MeHg measurements were conducted at the Dartmouth Trace Element Analysis Core Facility. Blood samples were transferred from capillary tubes to acid-washed, pre-weighed 2 mL centrifuge tubes. Samples were spiked with appropriate amounts of enriched isotope standards (Me201Hg and 199Hg), then diluted with 0.5 mL double-distilled 2M HNO3 (Fisher, Trace Element Grade), and heated to 90 °C on a heating block for 2 h (Rahman et al., 2014). Toe and tail clips, embryos and larvae were freeze dried and weighed into glass vials (IChem, Certified 300 series). Samples were spiked with enriched isotope standards, and diluted with 1-3 mL 4M HNO3, then heated to 60 °C overnight. Aliquots (0.1 – 0.5 mL) of blood and tissue extracts were transferred to 40 mL brown glass vials, and diluted with ultrapure water (>18 MΩ cm–1; produced by PurelabPlus water purifier, US Filter, MA, USA). To each vial, 0.3 mL citrate buffer was added, then samples were neutralized to pH 4.5 with potassium hydroxide (KOH). Finally, 40 µL ethylating reagent was added and vials were filled to the brim with water. Samples were analyzed for MeHg and inorganic Hg by species-specific isotope dilution gas chromatography – inductively coupled plasma mass spectrometry (GC-ICP-MS), using an automated MERX-M (Brooks Rand Instruments, Seattle, USA) interfaced with an Element 2 ICP-MS(Thermo, Bremen, GE) (Taylor et al., 2008; 2011). Note that Aqueous MeHg and inorganic Hg standard recoveries for blood analyses were 113±5% and 106±6% (n=2), respectively. Recoveries relative to certified reference values for NIST 2976 Mussel (National Institute of Standards and Technology; Gaithersburg, MD) were 110±1% for MeHg (certified 28 ng/g; *n*=4) and 113±3% for THg (61 ng/g; *n*=4).

Water samples for MeHg analysis were weighed into 40 mL brown glass vials, and spiked with Me201Hg. Samples were buffered and pH adjusted, then ethylated, as described above. Water samples were analyzed by direct ethylation GC-ICP-MS (Jackson et al., 2009). Samples for THg analysis were weighed into 40 mL clear glass septa-lid vials (Brooks Rand Instruments), and digested chemically with bromine monochloride (BrCl) overnight. Samples were then neutralized with hydroxylamine hydrochloride (NH2OH·HCl) and Hg was reduced to Hg0 with stannous chloride (SnCl2). Water samples were analyzed by cold vapor atomic fluorescence spectrometry, in a method similar to EPA 1631, using a Merx-T automated system (Brooks Rand Instruments). The relative percent difference (RPD) for duplicates samples was 7±5% (n=5) for MeHg, and 12±6% (n=5) for THg analyses. Recoveries of aqueous standards were 106±2% (*n*=3) and 101±7% (*n*=3) for MeHg and THg, respectively.

Statistical Analyses

To examine differences in pool basin morphology and water chemistry metrics by forest cover type, and to examine differences in THg, MeHg, and % MeHg concentrations between species within life stages, we used t-tests, or where data failed to normalize, non-parametric Mann-Whitney tests. Significant differences were assigned for *P*≤0.05.

We used generalized linear mixed models to examine causes of variation in MeHg loads in eggs, larvae, and adult tissue samples. We ran separate analyses for eggs and larvae, and for adults because we believed that the causes of variation likely differed between the juvenile and adult life stages. In particular, we expected that MeHg in eggs and larvae might reflect conditions in the breeding pool, especially levels of MeHg in the water, whereas we had no reason to expect a correlation between MeHg in adults and MeHg levels in the water in breeding pools because of the short duration of time that adults spend in the breeding pool. Because sample sizes for adult blood (n=35) were considerably smaller than for tissue (n=49), we used tissue mercury as the measure for adult variation.

For both analyses, we treated the pool (n=6) as a random effect to account for the nested nature of our sampling, in which MeHg levels were estimated from multiple individuals from the same pool. For eggs and larvae, we considered a suite of models constructed from four variables that we believed might reasonably influence levels of methylmercury in the amphibians: species, under the expectation that dietary differences among larval wood frogs (primarily herbivorous) and larval spotted salamanders (predatory) might contribute to differences in methylmercury accumulation; habitat around the pool, because pools surrounded by coniferous forest may have higher levels of MeHg and differences in water chemistry (DOC, pH)\; life stage (egg, early-stage larvae, or late-stage larvae) because later stages of development have both more time to accumulate MeHg and perhaps more exposure to MeHg through consumption of prey items; and finally levels of MeHg in the water, as this is presumablya primary source of MeHg. From this set of variables, we constructed a candidate set of models that included all combinations of the variables. In addition, we included three models with an interaction between species and life stage, allowing for the possibility that wood frogs and spotted salamanders might accumulate MeHg at different rates owing to differences in diet. This resulted in a total of 18 unique models: all subsets of the global model including species, habitat, life stage, and water methylmercury, plus the three models from this group that included main effects of life stage and species with an added interaction term between species and life stage.

For adults, we used the same approach to construct a candidate set of models but included only two variables: species and habitat. We did not consider MeHg in the water of the vernal pool as a predictor of MeHg in adults because we assumed that adults had a short duration of exposure to the pool and that it was thus unlikely that MeHg in adults could be linked plausibly toMeHg in the water. This left us with three possible models: the combined effect of species and habitat plus two models that considered each variable in isolation.

We fit each model using the lmer function in R package lme4 (Bates et al. 2015). We then used the R package AICcmodavg (Mazerolle 2017) to rank each model based on Akaike’s Information Criteria (AIC). We chose to use AIC as the ranking variable, rather than the small-sample correction (AIC­­c), because of the uncertainty in determining the effective sample size for mixed-effects models (Bolker et al. 2009). However, when we ranked models according to AIC­­c, setting the effective sample size (using the nobs argument in AICcmodavg) to either the number of pools (n=6) or the total number of observations, the results were qualitatively similar. We assessed the importance of each predictor variable based on the model-averaged regression coefficient and its unconditional 95% confidence interval. When averaging the coefficients, we used only the subset of models in which each term appeared because the inclusion of models with an interaction term precludes the use of shrinkage methods, which include all models in the averaging procedure (Mazerolle 2017). We note that disagreement exists among statisticians regarding the validity of generating inference from model-averaged coefficients (Bolker et al. 2009, Cade 2015); however, our findings were the same whether relying on model-averaged coefficients or model-averaged predictions as the basis of inference about methylmercury levels in the amphibians. In the primary critique of using model-averaged coefficients as the basis of inference, Cade (2015:2381) notes that “model averaging the predicted responses…can be used to indirectly explore model relationships”. As such, we believe that the concordance between these two approaches suggests our findings are robust to any putative shortcoming of model-averaged regression coefficients. We assessed goodness-of-fit using R2 values estimated by the r.squaredGLMM function in the R package MuMIn (Barton 2018). We used residual plots as described by Pinheiro and Bates (2000) to examine the validity of model assumptions.

**Results**

There were no differences in mean pool size (area, perimeter), maximum water depth, or pool elevation between forest cover types (Table 1). However, several water chemistry metrics differed significantly between pools in deciduous and coniferous stands: pools in coniferous stands had lower pH (mean coniferous = 5.16, deciduous = 6.21), greater DOC (mean coniferous = 17.46 mg/L, deciduous = 11.90 mg/L), lower conductivity (mean coniferous = 17.53 µS/cm, deciduous = 37.13 µS/cm), and greater aluminum concentrations (mean coniferous = 152.45 µg/L, deciduous = 61.62 µg/L) (Table 2). Additionally, coniferous pool water had greater concentrations of THg (mean coniferous = 5.56 ng/L, deciduous = 3.05 ng/L) and MeHg (mean coniferous = 1.19 ng/L, deciduous = 0.56 ng/L) compared to deciduous pool water (Table 2). Temporally, during the inundation period (April through July), increases in water MeHg coincided with increases in temperature and decreases in dissolved oxygen (Figure 1).

Mercury in Amphibians

Eggs and larvae of wood frog and spotted salamander accumulated MeHg at different rates (Table 3; Figure 2B), such that models without the interaction term between species and life stage received no support in the model-selection process (Table 4). The global model (which included species, habitat, life stage, and MeHg in the water column) provided a good fit to the data (R2 = 0.92). Although each of the four predictor variables appeared among the top-ranked models, model-averaged regression coefficients for both habitat (*b*habitat = 0.16, 95% CI = -0.04 – 0.35) and water MeHg (*b*water MeHg= -0.04, 95% CI = -0.21 – 0.13) were not distinguishable from zero.

Indeed, MeHg loads in eggs and larvae were best explained by the effect of species, life stage, and the interaction between these two variables (Figure 3). Levels of both total mercury and methylmercury in eggs of both species were low but increased substantially among both early- and late-stage larvae. Early salamander larvae had significantly greater THg concentrations compared to early wood frog tadpoles (t = 8.37, *P*<0.001), but THg increased three-fold in late-stage wood frog tadpoles, while it dropped slightly in older salamander larvae (Fig. 2A). Spotted salamander larvae had significantly greater concentrations of MeHg at both early- (U = 85.6, *P*<0.001) and late-stages (t = 4.7, *P*<0.001) than did wood frogs (Fig. 2B). The proportion of total mercury that was comprised of methylmercury (e.g. %MeHg; MeHg/THg) was significantly higher among spotted salamander eggs (t = 2.27, *P* = 0.03), early larvae (t = 12.84, *P*<0.001), and late larvae (t = 19.56, *P*<0.001) compared to wood frogs (Figure 2C). In both wood frogs and spotted salamander adults, %MeHg was significantly greater in blood samples than in tissue (wood frog, t = 8.98, *P*<0.001; spotted salamander, t = 5.24, *P*<0.001), while there was no difference in % MeHg in blood or tissue between species (Figure 2C). Although habitat did not explain variation in MeHg loads among samples of embryos or larvae, spotted salamander larvae and embryos of both species averaged higher MeHg in deciduous pools (Table 3), but our sample sizes were quite small.

Surprisingly, predicted MeHg loads in adult tissue from both species were substantially lower than predicted levels among late-stage larvae of both species (wood frog adults and late-stage larvae, respectively: 35.8 ng/g [95% CI = 27.6 – 46.7] versus 83.0 ng/g [66.4 – 103.8]; spotted salamander adults and late-stage larvae, respectively: 70.8 ng/g [54.1 – 92.8] versus 195.4 ng/g [151.5 – 252.0]).

Variation in MeHg levels among adult tissue was explained by species, with wood frogs having substantially lower levels than spotted salamanders (*b*Wood Frog  = -0.66, 95% CI = -0.99 - -0.34) (Figure 4). Habitat (forest type) had no discernible effect on MeHg levels in adult salamanders or frogs (*b*habitat = -0.02, 95% CI = -0.21 – 0.17). Most of the observed variance in MeHg loads among adults remained unexplained by the global model (R2 = 0.27).

**Discussion**

Our results support those of others (Brooks et al. 2012, Loftin et al. 2012, Benoit et al. 2013, Davis 2013) in demonstrating that vernal pools provide highly suitable conditions for MeHg production and bioaccumulation in amphibian larvae, which may then be exported to terrestrial systems via metamorphosis and direct predation from the pools?. Mean MeHg levels in amphibian embryos were similar among the two species (wood frog = 5.4 ng/g; spotted salamander = 3.5 ng/g), but bioaccumulated rapidly in salamander larvae (mean = 237.6 ng/g ±18.5 SE), which was significantly greater compared to wood frog tadpoles (mean = 62.5 ng/g ±5.7 SE) (Table 3; Figure 2). Methylmercury levels in adult tissue samples were significantly greater in spotted salamanders (mean = 79.9 ng/g ±8.9 SE) compared to woodfrogs (mean = 47.7 ng/g ±9.7 SE) (Table 3; Figures 2, 4). These results appear to represent the first to quantify Hg concentrations in spotted salamander eggs, larvae, and adults, and in adult wood frogs.

Methylmercury in Amphibian Eggs

Methylmercury concentrations in amphibian embryos were relatively low compared to larval and adult burdens (Table 3), but were 3 to 4 orders of magnitude greater than water MeHg levels (Table 2), suggesting that female amphibians may have depurated some of their Hg burdens during oviposition. This is consistent with Bergeron et al. (2010b) who reported that American toads (*Anaxyrus americanus*) transferred approximately 5% of their Hg burdens to their eggs, and that concentrations of THg and MeHg in eggs were positively correlated with concentrations in both maternal blood and whole-body, indicating that Hg depuration to eggs is related to Hg levels of the female. Larvae that are exposed to maternal Hg may be at greater risk of sublethal or lethal effects, especially if they are also exposed to dietary Hg. Previous studies have shown that maternal transfer of Hg in American toads had greater negative effects on larval health than dietary Hg, including delayed metamorphosis, smaller body size, and increased prevalence of spinal malformations in metamorphs (Bergeron et al. 2011, Todd et al. 2011a, b). However, Bergeron et al. (2011) found that exposure to both dietary and maternal Hg combined can cause significant mortality at metamorphosis. It is important to note that mean MeHg concentrations in that study were an order of magnitude greater than wood frog tadpoles in our study, and that mortality only occurred during metamorphic climax, supporting the hypothesis that metamorphosis may be a vulnerable period for Hg-exposed amphibians, as Hg may be remobilized from regressing muscle tissue into critical organs during tail resorption (Unrine et al. 2004).

Methylmercury in Amphibian Larvae

Concentrations of MeHg increase substantially between embryo and larval stages, emphasizing the role of feeding on MeHg accumulation. Higher levels of MeHg in salamander larvae are explained by the more predatory feeding habits of these species relative to wood frogs.

Although we found significantly higher levels of MeHg in water samples from pools located in coniferous stands, forest cover type did not explain variation in MeHg loads among samples of amphibian larvae. This may be due to our small sample sizes, or the complexities of MeHg bioavailability, which can be affected by differences in water chemistry, such as DOC concentration and character (Ortega 2018).In addition to water chemistry, other factors can affect the accumulation of MeHg in amphibian larvae, including diet and feeding niche (Eisler 2006), density of larval amphibian populations (Todd et al. 2011), species composition of pool leaf litter (Stephens et al. 2013), and whether or not there was maternal transfer of Hg to eggs (Bergeron et al. 2011). Among wood frog larvae, mean concentrations of MeHg in this study were ~100% twice as high? higher than those reported for larvae from Massachusetts pools, and ~50% 0.5times? higher than Minnesota pools (Brooks et al. 2012). THg concentrations in wood frog larvae reported from pools at Acadia National Park, Maine ranged from 15.2-54.2 ng/g ww (Loftin et al. 2012), which, assuming a moisture content of 80%, would equal 76-271 ng/g dw, similar to results in our study (range = 25.4-454.9 ng/g dw).

It is unknown whether the bioaccumulation of MeHg that we detected in amphibian larvae, especially in spotted salamanders, could have sublethal effects on fitness traits such as reduced body size, increased tail resorption time, and reduced locomotor performance. Marbled salamander (*Ambystoma opacum*) larvae experienced 50% mortality with THg levels of 103 ng/g (Sparling et al. 2000), approximately 2.5 times lower than the THg concentrations of ~260 ng/g that we found in spotted salamander larvae. In a laboratory study investigating the effects of dietary Hg on developing wood frogs, Wada et al. (2011) did not observe any adverse effects on larval development, size at metamorphosis, survival, or hopping performance at MeHg concentrations 2-3 times those that we detected in wood frogs. However, differences in dietary selenium concentrations between tadpoles raised in a controlled environment verses those developing in natural systems could alter Hg toxicity (Ralston et al. 2007). While the existing literature on Hg toxicity thresholds among amphibians is limited, it does appear that interspecific sensitivity to Hg is highly variable. Southern leopard frog tadpoles (*Lithobates sphenocephalus*) suffered lower survival, decreased tail resorption rates, and lower metamorphic success at MeHg concentrations of ~28 ng/g dw (Unrine et al. 2004), while American toad tadpoles showed impaired growth at MeHg concentrations of ~500 ng/g dw (Bergeron et al. 2011). Additionally, considerable genetic variation in sensitivity to toxicants can occur within amphibian populations (Bridges and Semlitsch 2001), which could potentially confound observed differences between species.

Methylmercury in Adult Amphibians

We could not find any published studies that investigated Hg concentrations or toxicity thresholds for adult *Ambystomid* salamanders or wood frogs. Among caudate, Burke et al. (2010) showed that behavioral responses of adult two-lined salamanders (*Eurycea bislineata*) with mean THg concentrations of ~4,500 ng/g dw from a Hg contaminated site had lower speeds of locomotion, diminished responsiveness, and reduced ability to capture prey compared to an uncontaminated reference site. THg concentrations in red-backed salamanders (*Plethodon cinereus*) have been reported from three different geographic areas in the eastern U.S. Whole-body concentrations of THg from red-backed salamanders in Virginia (Bergeron et al. 2010a) and the Catskill Mountains, NY (Townsend and Driscoll 2013) were similar to THg concentrations in tail-clips from salamanders in the Green Mountains, Vermont (Rimmer et al. 2010) (mean values were 100 ng/g dw; 127 ng/g dw, 110 ng/g dw, respectively). These results were similar to mean THg in adult *A. maculatum* tail-clips in this study (118+/-XX ng/g dw). Although *P. cinereus* is terrestrial throughout its entire life cycle, it occupies similar habitats as adult *A. maculatum* and co-occurring populations would likely be exposed to similar dietary Hg concentrations through atmospheric deposition. Still, we would expect adult spotted salamanders to bioaccumulate more Hg than red-backed salamanders due to increased exposure during their aquatic larval stage (Todd et al. 2012), longer life span (Flageole and Leclair 1992), and higher trophic position resulting from larger prey size. Although Hg samples from salamander tail-clips have been shown to correlate strongly with whole-body Hg, it is not a 1:1 relationship and can vary substantially between species (Pfleeger et al. 2016). Therefore, comparison of tail and whole-body Hg concentrations between- and within-species must be interpreted cautiously.

Data on mercury concentrations in adult anurans is lacking, hence there were few relevant studies with which to compare our results. MeHg in American toad blood from an uncontaminated site in Virginia (Bergeron et al. 2010b) was about three times higher than wood frog blood in the present study. Since MeHg in blood is comprised of both a transient component which reflects recent dietary intake, and a more stable component related to life-time accumulation (Day et al. 2005), it seems unlikely that wood frogs in our study would have accumulated any recent dietary Hg since they were captured within days of emerging from winter dormancy and do not forage during spring migration and breeding (Wells and Bevier 1997). Therefore, early spring may represent the lowest blood Hg levels of the year among spring-breeding amphibians that do not begin foraging until after the breeding season, and may be more representative of life-time Hg accumulation. It is also possible that blood Hg concentrations could decline during extended periods of heat and drought when terrestrial amphibians estivate. Studying stranded loggerhead sea turtles (*Caretta caretta*), Day et al. (2005) found significantly lower blood Hg levels in a severely emaciated individual with no fat stores compared to other stranded loggerheads, suggesting that a cessation of dietary input of prey resulted in a decline in blood Hg concentration. Therefore, it may be important to know the precise timing of blood collection and whether animals recently emerged from hibernation or estivation when comparing blood Hg concentrations between studies.

As expected, predaceous salamander larvae accumulated MeHg more rapidly than omnivorous wood frog tadpoles, and in both species aquatic larval stages had higher Hg concentrations than the terrestrial adults. Following metamorphosis, juveniles and adults of both species are quite vagile and primarily occupy terrestrial habitats (Faccio 2003, Baldwin et al. 2006) and change their food sources. Even though Hg bioaccumulates and is not easily eliminated from the body, the lower concentrations that we observed in adults may be due to dilution over time as they feed in a terrestrial-based food web. If that is the case, and assuming that both species eliminate MeHg from the body at similar rates, it suggests that the significantly greater MeHg concentrations that we detected in adult spotted salamanders compared to adult wood frogs may be due to the fact that salamander metamorphs started off terrestrial life with higher Hg burdens because they accumulate Hg more rapidly as larvae than wood frog tadpoles. It is also possible that since *Ambystoma* are much longer-lived than wood frogs they have more time over which to accumulate Hg. Further research is needed to determine the time frame over which Hg levels decline following metamorphosis. We hypothesize that Hg concentrations remain relatively stable during the first few weeks or months following metamorphosis, then steadily decline due to dilution during the juvenile stage as amphibians grow to adult size (1-2 years for wood frogs; 3-4 years for spotted salamanders), eventually reaching equilibrium that is dependent on Hg intake from prey (Carrier et al. 2001). Given the large amount of amphibian biomass that can be exported from vernal pools following a successful breeding season (Windmiller 1996, Berven 2009), and the wide range of predators that may consume juvenile amphibians (Mitchell et al. 2009), vernal pools have the potential to export a significant amount of MeHg from the aquatic system to the terrestrial food web.

Percent MeHg

Spotted salamander eggs and larvae had significantly higher percent MeHg compared to wood frogs of the same life stage (Figure 2C). Although eggs of both species were collected on the same date at each pool, we do not know the date of oviposition, nor did we attempt to age embryos. Therefore, it is possible that salamander eggs were in the water longer and had more time over which to accumulate MeHg. It is also possible that female salamanders transferred a higher percentage of MeHg to their eggs than did wood frogs. Among larvae, we suspect that dietary differences between predatory salamanders and herbivorous wood frogs accounted for the difference in %MeHg, since predators may be more efficient at assimilating MeHg due to partitioning of MeHg in their prey (Wada et al. 2011). It is interesting to note however, that THg increased almost three-fold between early- and late-stage wood frog tadpoles, while %MeHg declined about 20% (Figure 2A, C). The observed increase in THg was largely due to high concentrations in late-stage larvae collected at two pools located in coniferous stands. Late-stage larvae from pools SDF516 and SDF951 had mean THg concentrations of 424 ng/g (range = 363-562 ng/g), while larvae from the four other pools had mean THg concentrations of 231 ng/g (range = 106-352 ng/g). Still, the ~30% MeHg that we found in late-stage wood frog larvae was consistent with the 25% MeHg that Wada et al. (2011) measured among wood frog tadpoles at Gosner stage 42 that were fed low Hg diets.

Among adult amphibians, blood had significantly higher %MeHg in both species (spotted salamander mean = 89.2 ±2.2%; wood frog mean = 94.3 ±1.7%) compared to tail-clip (mean = 70 ±2.4%) and toe (mean = 73 ±1.6%) samples (Figure 2C). Bergeron et al. (2010b) found that American toad blood had the highest %MeHg of any tissues sampled (73%), which they attributed to recent dietary uptake. As discussed above, it seems unlikely that the high proportion of MeHg that we measured in adult amphibian blood was due to recent dietary intake since neither species is known to feed during immigration to breeding pools or during breeding. Wood frogs metabolize glycogen reserves in their abdominal muscles during the breeding period (Wells and Bevier 1997), while spotted salamanders presumably metabolize fat reserves stored in their tails and abdomen (Fitzpatrick 1976). It is possible that metabolizing fat and/or muscle following winter dormancy could mobilize Hg stored in these tissues, thereby elevating Hg in the blood (Day et al. 2005).

Conclusions

This research demonstrates that vernal pools are important hotspots of MeHg bioaccumulation, and biota from the pools may be vectors of MeHg to the terrestrial ecosystem. Water from pools located in coniferous stands had higher THg and MeHg concentrations, higher DOC, and lower pH compared to pools in deciduous stands. Although forest cover type was insufficient to explain the variation in Hg concentrations among amphibian eggs or larvae, our sample sizes were quite small. In order to better understand the role that land-cover/land-use plays in methylmercury production and bioaccumulation in vernal pool fauna, future studies should include more robust sampling from a greater number of sites. We found that spotted salamander larvae rapidly accumulated MeHg concentrations (1.5 to 2 orders of magnitude greater than their eggs), while wood frog larvae accumulated MeHg more moderately (one order of magnitude greater than their eggs), suggesting that predators that feed on amphibian larvae in these ephemeral systems may be exposed to relatively high concentrations of MeHg. The abundance and high levels of MeHg in larvae suggest these to be potentially important bioindicators for monitoring loading and bioavailability of MeHg in these sensitive ecosystems. Although MeHg concentrations were 2 to 3 times lower in adult amphibians compared to their aquatic larvae, spotted salamander adults had higher MeHg levels than wood frog adults. It is unknown how rapidly mercury levels decline following metamorphosis, but since mercury is difficult to flush from the body we suspect that it declines slowly due to dilution as amphibians grow to adult size. This suggests that vernal pool amphibians may export significant mercury from an aquatic system to terrestrial predators including snakes, birds, and small to medium-sized mammals. Future research that quantifies mercury concentrations among amphibian metamorphs and juveniles will help elucidate cycling and trophic transfer of mercury from aquatic to terrestrial systems.

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