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BIOACCUMULATION AND MATERNAL TRANSFER OF MERCURY AND SELENIUM IN AMPHIBIANS

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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. Strong positive relationships between THg and MMHg in tissues of all species were observed throughout. 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 with Se. In addition, Hg concentrations appeared to positively influence the amount of Se transferred from female to eggs. The present 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. Environ. Toxicol. Chem. 2010;29:989–997. © 2010 SETAC

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 with 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 because 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].

The present study 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]. The first objective was to describe the relationships between THg

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and (mono)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, the second objective of the present study 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, the 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: see Fig. 1 and 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 h) and egg masses were removed and enumerated immediately upon completion of oviposition. If pairs had not oviposted within 24 h, they were injected with human chorionic gonadotropin (females 200, males 100 IU) to induce egg laying. After 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 h 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 weight) 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 h after the sample was obtained.

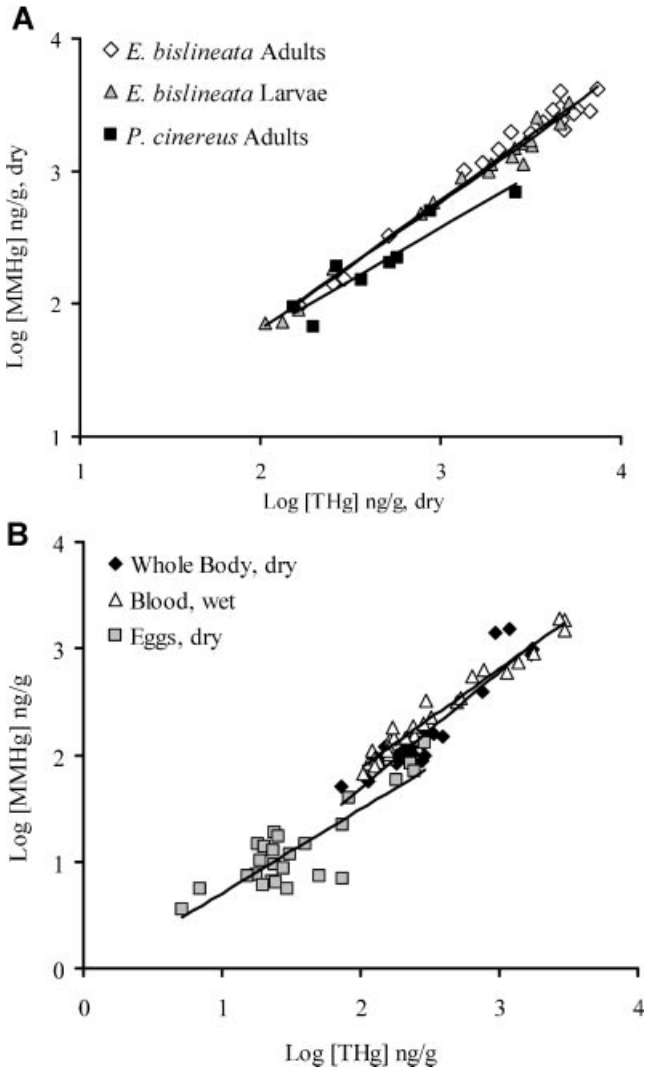

Fig. 1. Relationship between log total mercury (THg; ng/g, dry wt) concentrations and log methylmercury (MMHg; ng/g, dry wt) concentrations in (**A**) composite body samples from salamanders: adult *Eurycea bislineata* ($r = 0.986$; $p < 0.0001$; $y = 0.976x - 0.146$; $n = 20$), larval *E. bislineata* ($r = 0.993$; $p < 0.0001$; $y = 0.959x - 0.115$; $n = 19$) 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).

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 (Direct Mercury Analyzer 80, Milestone) according

Table 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[a]

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)

[a] 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.

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. [24]. The method detection limit (MDL; 3 times the 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, two to three 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 to 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°C overnight. The resulting digests were centrifuged at $\times$ 1,000 *g* for 20 min to remove any insoluble material. Aliquots of the supernatants (10 to 100 µl) were then analyzed for MMHg content using aqueous phase ethylation with room temperature precollection 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 $86.97 \pm 2.70\%$ ($n = 35$). 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 $12.28 \pm 1.99\%$ ($n = 19$). Spike recovery averaged $111.82 \pm 4.16\%$ ($n = 16$).

To determine percent MMHg, THg concentrations in the MMHg digestates were determined using a Sciex Elan DRC Plus inductively coupled plasma-mass spectrometer (ICP-MS) (PerkinElmer) according to U.S. EPA method 6020a [33]. Percent recoveries of THg in TORT-2 by this method were $107 \pm 4.40\%$ ($n = 12$). Average RPD between replicate samples was $2.81 \pm 1.31\%$ ($n = 13$). 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 $99.07 \pm 1.18\%$ ($n = 13$).

Table 2. Composite salamander samples for *Eurycea bislineata* (adults and larvae) and *Plethodon cinereus* (adults) analyzed for total mercury (by inductively coupled plasma-mass spectrometry), methylmercury, and selenium from the reference (REF) and contaminated portions of the South River (SR), Coyner Spring Park (CS), and the South Fork of the Shenandoah River (SFSR), VA, USA[a]

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

[a] Sample sizes were the same for all analyses, with exceptions for Se denoted parenthetically (). Sites where species were not found are denoted as not applicable (NA). RM = river mile downstream from contamination source.

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 fluoropolymer digestion vessels using a microwave digestion system (MARS-5, CEM) 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 ICP-MS 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* (RP*q*) 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 $102.62 \pm 0.04\%$ ($n = 17$). 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 $4.34 \pm 1.03\%$

($n = 22$) and $10.69 \pm 3.38\%$ ($n = 11$), respectively. Spike recovery averaged $114.22 \pm 4.40\%$ ($n = 23$).

Statistical analyses

To compare the analytical techniques for THg (Direct Mercury Analyzer 80 from Bergeron et al. [24] and ICP-MS [present study]) used on the same samples, the THg concentrations were regressed against one another and the slope of the relationship was compared, 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 the 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. Because transformations failed to meet the assumptions of ANOVA for THg concentrations, the Scheirer-Ray-Hare extension of the Kruskal-Wallis test [35] was performed to compare sites and tissues. Blood concentrations were reported on a wet weight basis. Because blood (wet) and body and eggs (dry) cannot be directly compared with 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 preovipositional 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 preovipositional 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), 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 percent MMHg for salamanders in the contaminated site was $61.2 \pm 3.3\%$ for *E. bislineata* adults, $56.8 \pm 2.3\%$ for *E. bislineata* larvae, and $45.7 \pm 6.6\%$ for *P. cinereus*. The small sample sizes at the reference site precluded the statistical comparison of percent MMHg with the contaminated site (Table 2). However, overall averages of percent 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 with 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 with egg concentrations. While mean THg blood concentrations were also 3.5-fold higher in the contaminated site compared with 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 three- to fourfold higher in the contaminated site compared with 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 percent MMHg concentrations between all tissues sampled ($p < 0.002$, for all); blood had the highest percent MMHg and eggs had the lowest where percent MMHg averaged

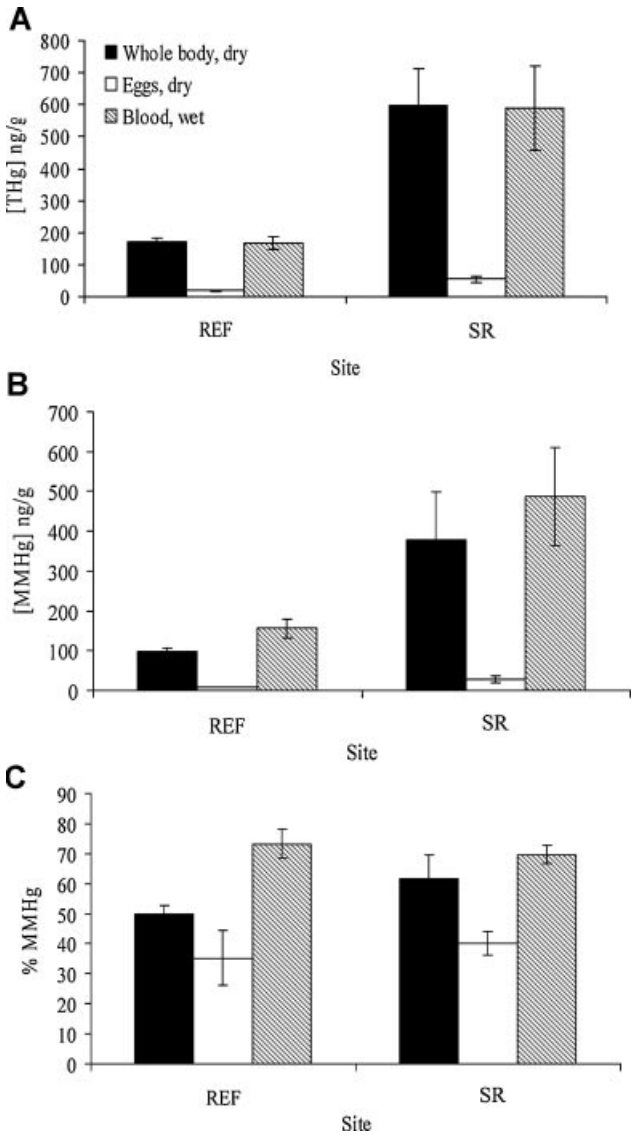

Fig. 2. (**A**) Total mercury (THg) concentrations (ng/g; mean ± 1 standard error [SE]), (**B**) methylmercury (MMHg; ng/g; mean ± 1 SE), and (**C**) percent methylmercury (% MMHg; mean ± 1 SE) in *Bufo americanus* whole body (dry wt), eggs (dry wt), and blood (wet wt) from the reference (REF) and contaminated (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 section for details.

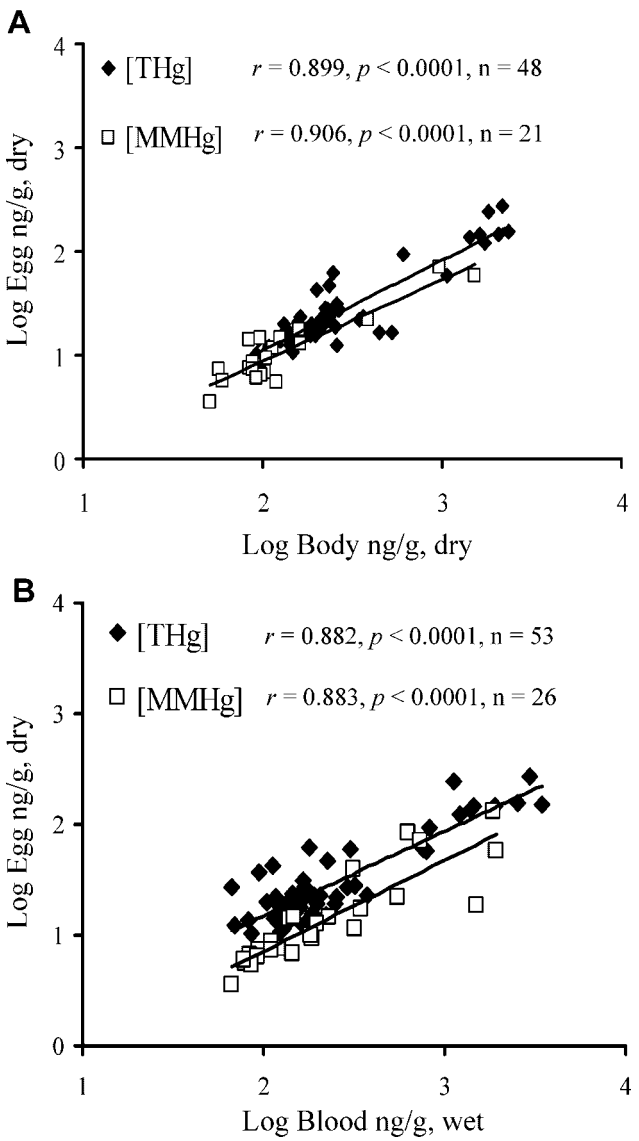

Fig. 3. Relationship of log total mercury (THg; ng/g) and log methylmercury (MMHg; ng/g) between (**A**) whole-body and egg concentrations (THg: y = 0.871x - 0.696; MMHg: y = 0.787x - 0.632) and (**B**) blood and egg concentrations (THg: y = 0.764x - 0.353; MMHg: y = 0.818x - 0.781) in *Bufo americanus* from the reference and contaminated portion of the South River (VA, USA).

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: see 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 ($r = 0.88$; $p < 0.0001$; $n = 53$) and MMHg ($r = 0.88$; $p < 0.0001$; $n = 26$) 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 to 1,171 ng/g for *P. cinereus*, 120 to 3,130 ng/g for *E. bislineata* adults, and 1,894 to 3,539 ng/g for *E. bislineata* larvae. For *E. bislineata* (adults 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: $F_{1,97} = 0.00$;

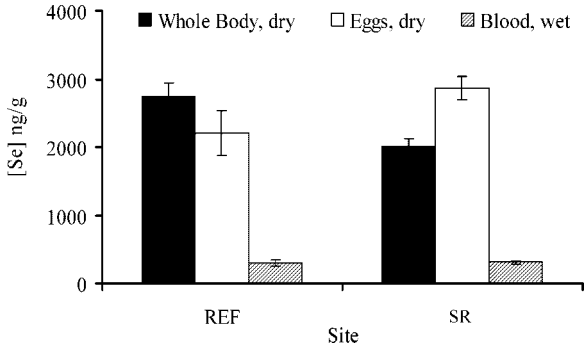

Fig. 4. Total selenium (Se) concentrations (ng/g; mean ± 1 standard error [SE]) in *Bufo americanus* whole-body (dry wt), egg (dry wt), and blood (wet wt) samples from the reference (REF) and contaminated (SR) portion of the South River, VA, USA. Blood concentrations (wet wt) were handled separately from other tissue concentrations (dry wt) in statistical models. See Methods section for details.

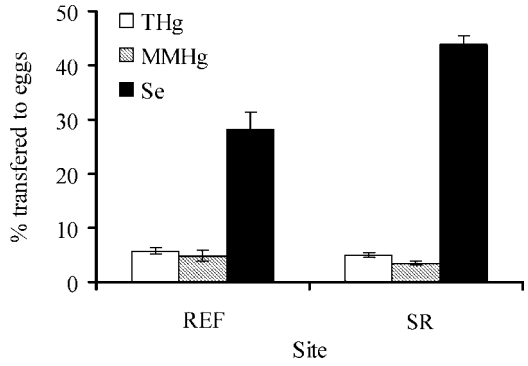

Fig. 5. Percent of *Bufo americanus* maternal body burden (preoviposition) transferred to eggs for total mercury (THg), methylmercury (MMHg), and selenium (Se) from the reference (REF) and contaminated (SR) portion of the South River (VA, USA).

$p = 0.959$; 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 preoviposition ($r = 0.32$; $p = 0.030$; $n = 47$) and postovipostion ($r = 0.46$; $p = 0.001$; $n = 48$). This relationship was also found between MMHg and Se for postovipostion females ($r = 0.51$; $p = 0.022$; $n = 20$), but not preoviposition 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 with the reference site ($44.3 \pm 1.7\%$ versus $28.2 \pm 3.1\%$, 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 $4.96 \pm 0.47\%$ and reference $5.70 \pm 0.70\%$) and MMHg (contaminated $3.39 \pm 0.31\%$ and reference $4.88 \pm 1.10\%$; $p = 0.184$). 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 postoviposition females ($r = 0.390$; $p < 0.007$; $n = 47$; Fig. 6). However, the reverse was not true; no relationship was found between the percent of Hg body burden lost to eggs at oviposition and the whole-body Se concentrations in postoviposition females ($r = 0.059$; $p = 0.689$; $n = 47$).

DISCUSSION

Mercury bioaccumulation and speciation

Compared with 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

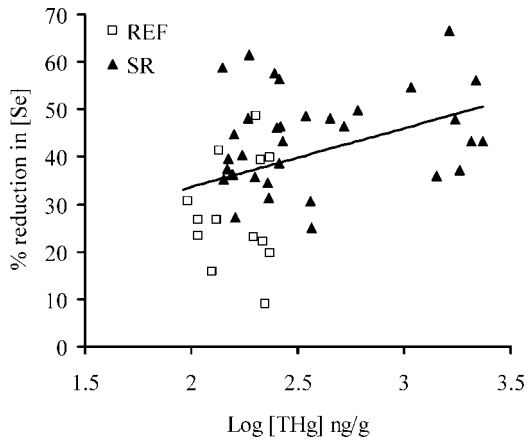

Fig. 6. Relationship between *Bufo americanus* whole-body log total mercury (THg; ng/g) concentrations in postoviposition females and the percent of selenium (Se) concentrations lost to eggs between pre- and postovipostion ($r = 0.390$; $p < 0.007$; $n = 47$) in the reference (REF) and contaminated (SR) portion of the South River (VA, USA).

example, Bank et al. [39] reported a lower THg concentrations, but higher percent MMHg for larval *E. bislineata bislineata* (northern two-lined salamanders: 73 to 97%) from Acadia National Park, Maine, USA than were found in larval *E. bislineata* in the present study (39 to 73%). For *B. americanus*, percent MMHg in blood was the highest (73.3 ± 4.8 %) of the tissues sampled. The high percent MMHg in blood is presumably due to recent dietary uptake and is similar to the percent 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 $2{,}318 \pm 156$ ng/g; *E. bislineata* larvae $2{,}550 \pm 112$ ng/g; *P. cinereus* 935 ± 243 ng/g) and preovipostion female *B. americanus* in both the reference ($3{,}860 \pm 260$ ng/g) and Hg-contaminated ($3{,}607 \pm 191$ ng/g) sites were within or close to the range considered normal background Se concentrations for amphibians and reptiles (1,000 to 3,000 ng/g, dry weight) [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 postoviposition. This relationship may have been strengthened had specific tissues been targeted for analysis because co-sequestration of these elements is known to occur in organs such as the liver and kidney [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 [37,38,45–47], it has only been examined in one other species of amphibian [19–21]. The present 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] 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 approximately 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 to 44%) than Hg to their eggs (Fig. 5). The higher transfer of Se compared with Hg is consistent with Hopkins et al. [20] who found that *G. carolinensis* transferred 53 to 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]. Selenium is incorporated into egg proteins before 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 $\geq$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 the present study system compared with 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

The present study sought to develop a better understanding of Hg bioaccumulation in amphibians by sampling three amphibian species occurring across a broad Hg contamination gradient. It 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. Maternal transfer of both Hg and Se was observed in *B. americanus*, and the first correlation between Hg concentrations in female carcass and eggs in amphibians was described. 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], the 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.

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