Ecotoxicology (2012) 21:882–887
DOI 10.1007/s10646-012-0850-2

Use of toe clips as a nonlethal index of mercury accumulation and maternal transfer in amphibians

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Accepted: 3 January 2012 / Published online: 17 January 2012

Abstract Nonlethal indices of contaminant exposure can facilitate research on the accumulation and effects of contaminants in wildlife. Here, we tested the efficacy of using amputated toes ("toe clips"), a common byproduct when marking amphibians in population and genetic studies, to determine mercury (Hg) concentrations in amphibians. We examined total mercury (THg) concentrations in American toads (*Bufo americanus*) collected along a contamination gradient at a Hg-contaminated field site. We found significant positive correlations between toe THg and blood THg concentrations in adult males and females collected in two different years. We also found that blood and toe clips could be used to predict maternal transfer of Hg, an important mechanism of reproductive toxicity in wildlife. Maternal toe THg concentrations were more highly correlated with egg THg concentrations than were maternal blood THg concentrations. Our results indicate that amputated toes are effective for identifying Hg concentrations in amphibians.

Keywords American toad · Amphibian declines · Maternal transfer · Mercury · Metals · Nondestructive sampling

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Introduction

Mercury (Hg) is an environmental contaminant that has generated widespread concern due to its global ubiquity and known toxicity to humans and wildlife (Eisler 2006). Although naturally occurring, environmental concentrations of Hg have increased due to redistribution associated with industrial processes and atmospheric transport (Fitzgerald et al. 1998). Mercury can bioaccumulate to high levels in biota, especially in areas where Hg is as a point-source contaminant (Bergeron et al. 2010a; Hothem et al. 2010). Studies of the sources, accumulation, and toxicological effects of Hg span most taxa and include a growing body of research on wildlife (Wolfe et al. 1998; Eisler 2006; Scheuhammer et al. 2007). From a toxicological perspective, Hg is most widely known for its neurotoxicity, a characteristic responsible for altering behavior and impairing cognition in vertebrates (Wolfe et al. 1998). However, there is also growing evidence that Hg can affect the endocrine and reproductive systems of vertebrates (Scheuhammer et al. 2007; Crump and Trudeau 2009; Tan et al. 2009). Despite great interest in the behavioral, physiological, and ecological effects of Hg on wildlife, some taxa have received less investigation than others. For example, there has been comparatively little research on Hg in amphibians (Linder and Grillitsch 2000; Eisler 2006; Scheuhammer et al. 2007), despite concern over the role of environmental contaminants in global amphibian declines (Linder et al. 2003).

Amphibians can serve as important and valuable research models for understanding the ecological effects of persistent contaminants such as Hg for several reasons. First, many amphibians breed and forage in floodplain wetlands, areas that often serve as methylation hotspots for the conversion of inorganic forms of Hg to the more toxic

and bioavailable form methlymercury (Rudd 1995). Additionally, amphibians are capable of converting large proportions of consumed prey into new biological tissue due to their ectothermy and high conversion efficiencies (Pough 1980). For this reason, they may accumulate high contaminant concentrations over the course of their lives (Unrine et al. 2007). In some ecosystems, amphibians are among the most abundant vertebrates and can occur at densities of nearly 3,000 per ha (Burton and Likens 1975), amplifying the risk of transfer of accumulated contaminants to higher trophic levels as they are preyed upon by a diverse range of species. Amphibians can also play important roles in nutrient and energy flux between aquatic and terrestrial environments (Regester et al. 2006), and, subsequently, they may transport Hg they accumulated in aquatic habitats into adjacent terrestrial habitats.

Studies of contaminant concentrations in amphibians typically use destructive methods such as whole carcass or organ excision to determine tissue concentrations of the contaminant of interest, including Hg (Hopkins et al. 1998; Bank et al. 2007; Unrine et al. 2007; Hothem et al. 2010). The use of lethal methods can prove limiting in field studies of wild vertebrates in which recapture or survival estimates may be needed to determine population-level responses to environmental contaminants or other factors. Additionally, nonlethal methods are preferred when the species is rare or protected and when removal of individuals from the population may be detrimental to population persistence. This is especially important when sampling subadult and adult lifestages which have a disproportionately large influence on amphibian populations (Biek et al. 2002; Schmidt et al. 2005). Recently, Bergeron et al. (2010a) demonstrated the efficacy of cardiocentesis in anurans (frogs and toads) for determining Hg concentrations by showing that blood Hg concentrations were tightly correlated with those of the whole body. However, cardiocentesis requires skill and can be lethal if improperly performed. Thus, there is still a need for a less invasive, nonlethal method for determining Hg concentrations in amphibians. The amputation of toes (i.e. "toe-clipping") is widely used in field research to individually mark animals during studies of populations' vital rates, when collecting tissue for molecular and phylogenetic research, and for use in skeletochronology (Kalb and Zug 1990; Heyer et al. 1994). Although some studies have suggested that survival may be negatively affected by removal of more than two toes (McCarthy and Parris 2004; Waddle et al. 2008), toe-clipping of one to two toes is often well tolerated by many species (Liner et al. 2007; Phillott et al. 2007), and is a marking technique prescribed by the leading herpetological professional societies (Beaupre et al. 2004). Our primary goal in this study was to evaluate the efficacy of using toe-clips as nonlethal indicators of Hg concentrations in amphibians. Additionally, to examine the maternal transfer of Hg in amphibians, we quantified the relationship between maternal toe Hg and blood Hg concentrations and those of eggs.

Methods

Field methods

The South River in northern Virginia, USA is a tributary that flows into the South Fork of the Shenandoah River. It was historically contaminated with mercuric sulfate during industrial production of acetate fiber that occurred in Waynesboro, Virginia from 1929 to 1950 (Carter 1977). Levels of inorganic Hg (HgII) and methylmercury (MeHg) remain elevated in biota in the river and adjacent floodplain downstream from the point source. Additional site information can be found in Bergeron et al. (2007) and Bergeron et al. (2010a).

We collected a total of 34 reproductive pairs of American toads (*Bufo americanus*) by hand from along the South River floodplain both upstream and downstream of the contamination source on 10–12 April 2008 and 17–18 April 2009. Variation in the levels of environmental Hg in the upstream and downstream sites results in a broad Hg contamination gradient (Bergeron et al. 2010a). As described in Todd et al. (2011a), reproductive pairs were allowed to oviposit in the lab in individual shallow bins containing dechlorinated tap water. We next removed a small portion of each egg mass (approximately 500 eggs) and froze them for Hg analysis. In 2008, we anesthetized females ($n = 10$) after oviposition using MS-222 and collected $\sim$0.25 ml whole blood via cardiocentesis and amputated one front toe and one hind toe to individually mark each animal. We treated the remaining portion of each digit with antibiotic cream after removal of the distal parts of the digit. Eggs and amputated toes were rinsed in dechlorinated tap water before being frozen, along with blood samples, for later Hg analysis. In 2009, we used the same procedures to collect blood and toes from both males ($n = 19$) and females ($n = 24$). We measured the snout-to-urostyle length (SUL) to the nearest mm and recorded mass to the nearest mg for each animal. We released all animals at their initial point of capture after observing them for 48 h to ensure they recovered from anesthesia and the toe-clipping.

Mercury analyses

Individual toes averaged 24.6 $\pm$ 1.6 mg wet weight but both toes from each individual were combined for THg analysis. We did not measure MeHg in the toes in the

current study. In the past, MeHg concentrations of whole body, blood, and eggs in this species at this study site averaged 53.3 ± 2.3%, 71.4 ± 2.8%, and 47.8 ± 3.3% of THg, respectively (Bergeron et al. 2010b). We lyophilized and homogenized toad eggs and both front and hind toes and we report total Hg (THg) concentrations of both on a dry weight (dw) basis. Percent moisture of eggs and toes was 95.4 ± 0.2% and 54.1 ± 1.2%, respectively. We homogenized whole blood from each toad using a vortex mixer, and we report THg concentrations of blood on a wet weight (ww) basis. We analyzed subsamples (~20 mg) for THg content by combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT USA) according to U.S. Environmental Protection Agency method 7473. For quality assurance, each group of 10 to 15 samples included a replicate, blank, and standard reference material (SRM; DOLT-4 dogfish liver or DORM-3 fish protein [National Research Council of Canada (NRCC), Ottawa, ON]). We calibrated the instrument using solid SRMs (DOLT-4 and DORM-3). Method detection limits (MDLs; 3 times the standard deviation of procedural blanks) for samples were 0.33 ng, and all samples had THg concentrations that exceeded the limit. Average relative percent differences between replicate sample analyses were 4.47 ± 1.06% ($n = 14$). Mean percent recoveries of THg for the SRMs, DOLT-4 and DORM-3, were 96.68 ± 0.30% ($n = 20$) and 99.22 ± 1.57% ($n = 20$), respectively.

Statistical analyses

We used an analysis of covariance (ANCOVA) to determine whether blood Hg concentrations varied by sex or year and whether blood Hg concentrations were correlated with toe Hg concentrations. We also used a Pearson correlation of blood THg against toe THg irrespective of sex and year to generate a correlation coefficient. We used a Type II regression (reduced major axis regression) to determine the relationship between blood THg and toe THg irrespective of sex and year. We used ANCOVAs to determine whether egg THg concentrations were correlated with toe or blood THg concentrations in females, using year as an additional factor in the models. We also used separate Pearson correlations between egg THg and toe or blood THg irrespective of year to generate correlation coefficients. We again used a Type II regression to determine the relationships between egg THg and toe or blood THg irrespective of sex and year. All values were log transformed to normalize data prior to running the analyses and all regressions and analyses were conducted on the log transformed data.

A previous study on American toads along the South River showed that blood THg concentrations were highly correlated with whole body THg tissue concentrations (Bergeron et al. 2010a). We reanalyzed the data from the earlier study to generate a Type II regression equation relating blood THg to whole body THg. Next, we constructed a predictive regression between whole body THg and toe THg using the results of the Type II regression between blood THg and toe THg generated in the present study.

Results

Blood THg concentrations in American toads collected across the contaminant gradient ranged from 82–4,235 ng/g ww. Total Hg concentrations in toes ranged from 32–602 ng/g dw and eggs had THg concentrations that ranged from 15–205 ng/g dw.

Toe THg concentrations were significantly correlated with blood THg concentrations ($F_{1,47} = 122.8$, $P < 0.001$; Fig. 1). We found no effect of year, sex, or their interaction with toe THg on blood THg concentrations (year: $F_{1,47} = 0.47$, $P = 0.50$; sex: $F_{1,47} = 0.82$, $P = 0.37$, year × sex × Log(ToeTHg): $F_{2,47} = 0.16$, $P = 0.85$). The equation defining the relationship between blood THg and toe THg was

$$\text{Log(BloodTHg)} = 1.336 * \text{Log(ToeTHg)} - 0.279 \quad (1)$$

with a correlation coefficient of 0.884.

Egg THg concentrations were significantly correlated with maternal toe THg concentrations ($F_{1,29} = 92.6$, $P < 0.001$; Fig. 2a). There was no effect of year or interaction of year and toe THg concentration on egg THg

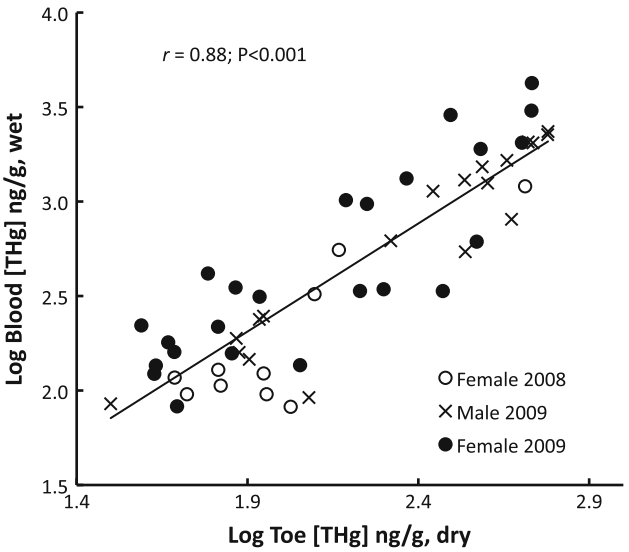

Fig. 1 Relationship between blood total mercury (THg) concentrations and toe THg concentrations in American toads collected along the Hg-contaminated South River near Waynesboro, VA, USA

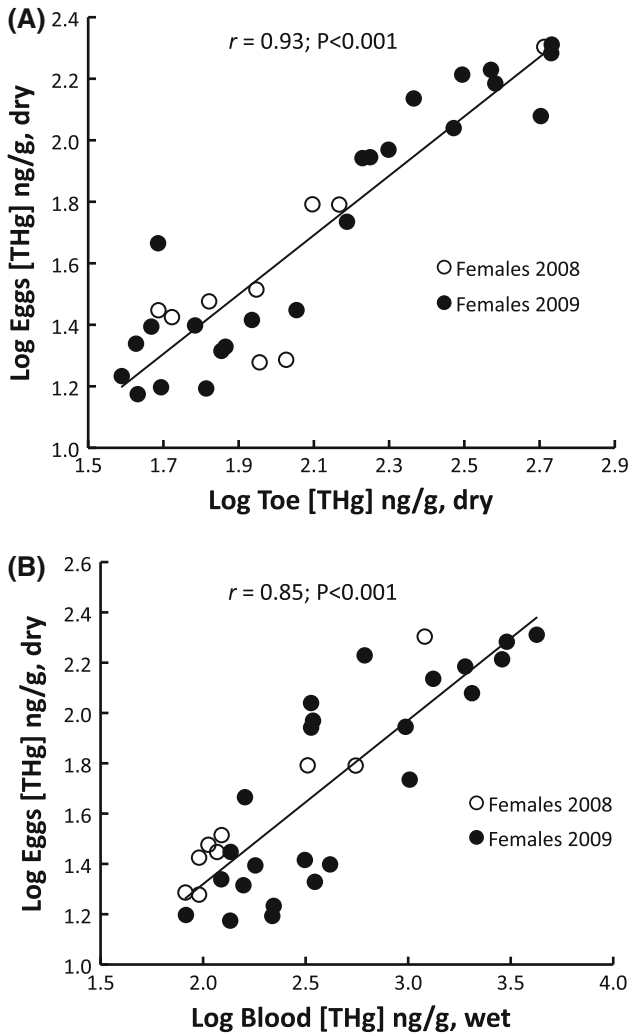

Fig. 2 Relationship between **a** maternal toe and **b** maternal blood total mercury (THg) concentrations and egg THg concentrations in female American toads collected along the Hg-contaminated South River near Waynesboro, VA, USA. Note that the axes differ

concentrations (year: $F_{1,29} = 0.1$, $P = 0.78$; year × toe: $F_{1,29} = 0.1$, $P = 0.72$). The equation defining the relationship between egg THg and toe THg was

$$\text{Log(EggTHg)} = 1.047 * \text{Log(ToeTHg)} - 0.506 \quad (2)$$

with a correlation coefficient of 0.925.

As expected based on Bergeron et al. (2010b), egg THg concentrations were significantly correlated with maternal blood THg concentrations ($F_{1,29} = 56.2$, $P < 0.001$; Fig. 2b). There was no effect of year or interaction of year and blood THg concentrations on egg THg concentrations (year: $F_{1,29} = 0$, $P = 0.96$; year × blood: $F_{1,29} = 0.2$, $P = 0.70$). The equation defining the relationship between egg THg and blood THg was

$$\text{Log(EggTHg)} = 0.732 * \text{Log(BloodTHg)} - 0.189 \quad (3)$$

with a correlation coefficient of 0.851.

Based on a Type II regression of blood and whole body THg concentrations from data collected in Bergeron et al. (2010a), and using regression (1) of blood and toe THg concentrations from above, the relationship between toe and whole body THg concentrations in American toads in this study system is predicted as

$$\text{Log(BodyTHg)} = 1.245 * \text{Log(ToeTHg)} - 0.001 \quad (4)$$

assuming no variation among years.

Discussion

There are few studies of the bioaccumulation and effects of Hg in amphibians compared to most other vertebrates (Wolfe et al. 1998; Linder and Grillitsch 2000; Eisler 2006; Scheuhammer et al. 2007). This is an important knowledge gap given concern that environmental contaminants may contribute to global amphibian declines (Sparling et al. 2010). The development of nondestructive indices for determining Hg concentrations in amphibians is therefore valuable and should aid future research. In the present study, we determined THg concentrations in the blood, eggs, and toes of American toads. Concentrations in these tissues were highly correlated across a broad gradient of Hg concentrations in field-collected animals, demonstrating their potential applicability to other study sites and systems.

Studies of the American toads examined in the present study have shown that whole body THg concentrations of ≥775 ng/g dw are sufficient to cause adverse sublethal maternal effects in offspring in laboratory studies (Bergeron et al. 2010a; Bergeron et al. 2011). Based on equation 4, this would equate to a THg concentration of 209.7 ng/g dw in toe tissue. Toes from American toads are composed mostly of bone, with small amounts of dermal tissue, tendons, and little muscle or blood. The nearly fourfold increase in THg concentrations in the whole body versus the toe is thus likely due to the tendency of Hg to accumulate in viscera and organ tissues such as the liver, rather than in bones (Weiner and Spry 1996).

Although 30–40% of the toads examined in the present study met or exceeded the threshold for maternal effects established by other studies, we did not observe here any obvious malformations or size differences between animals captured breeding at Hg-contaminated sites versus reference sites. Additionally, due to the sampling methods used in the present study, we could not estimate abundances at each of the sites and thus we do not provide direct field-based evidence of Hg impacts on the populations at these sites. However, laboratory and mesocosm studies of offspring from the animals examined in the present study demonstrated adverse effects that included smaller body

size, delayed tail resorption, increased spinal malformations, and reduced survival to metamorphosis (Bergeron et al. 2011; Todd et al. 2011a; Todd et al. 2011b). Scaling from these individual-level effects to a population-based assessment of impacts is currently the subject of ongoing work.

Bergeron et al. (2010b) previously demonstrated that female American toads will maternally transfer approximately 5% of their Hg burden to their eggs in a concentration-dependent fashion. Despite this depuration, we found no support for lower THg concentrations in females relative to males, suggesting that this slight loss of Hg via the eggs is insufficient to significantly reduce their body burdens compared to males. Alternatively, it may be that because females are larger, they consume greater amounts of contaminated prey to support their metabolic needs and so accumulate more Hg, thus counterbalancing any Hg lost through eggs. Nondestructive evidence of maternal Hg transfer in American toads was previously shown using correlations between maternal blood THg and egg THg (Bergeron et al. 2010b). Results of the present study indicate that THg concentrations in maternal toes are also correlated with egg THg concentrations. In fact, based on a comparison of the correlation coefficients in the present study, toe THg concentrations are more highly correlated with egg THg concentrations ($r = 0.925$) than are blood THg concentrations ($r = 0.851$). This may be a reflection of differences in the temporal integration of Hg accumulation in toes versus blood and the ways in which energy is partitioned into developing eggs. Specifically, blood THg concentrations may reflect more recent dietary intake whereas toe and egg THg concentrations likely represent an integration of Hg intake over a greater timeframe (e.g. Day et al. 2005).

Many nondestructive sampling techniques have been developed for assessing metal concentrations in other vertebrates, including the use of talons, feathers, and eggs in birds (Hopkins 2007), tail clips and blood in reptiles (Hopkins et al. 2001; Jackson et al. 2003; Hopkins et al. 2005; Fletcher et al. 2006), and fur and blood in mammals (Malvandi et al. 2010; Wada et al. 2010). Recent work also has identified the use of blood in anurans and tails in salamanders as nondestructive indices of Hg accumulation (Bergeron et al. 2010a). However, the use of tail clips cannot be applied to most anurans because they lack tails. Our current study instead demonstrates the viability of using toe clips to determine Hg accumulation in amphibians. This method may be preferred over blood in anurans for its possible longer-term integration and better predictive potential, relative ease of sampling, increased safety compared to cardiocentesis, and the fact that these tissues are often discarded in mark-recapture studies. Moreover, because MeHg and THg concentrations are highly correlated in amphibian tissues (Bergeron et al. 2010b), we expect that the relationships between blood, egg, whole body, and toe MeHg concentrations will be similar to the THg concentrations identified in the present study.

Despite the ease and usefulness of toe-clips as indices of Hg concentrations in amphibians, there are some possible limitations that should be considered. First, it may be necessary to validate toe and whole body Hg correlations for other species in other areas. Second, researchers must be cognizant of the size of their target amphibian species and the minimum mass requirements for Hg analysis depending on their analytical methods and the expected Hg concentrations. Such requirements may allow the use of only a single toe or may preclude the use of toes altogether if enough mass is not recoverable. Ultimately, if the use of toe clips for Hg determination is validated for other species, it will facilitate broader study of the accumulation or effects of Hg in amphibians. This will be especially true where it is preferred to keep animals alive, when toe-clipping will already be used in mark-recapture and population monitoring studies, or when repeated sampling of the individual may be desired to examine possible trends in Hg over time or with animal age.

Acknowledgments We thank S. Budischak, D. Cristol, K. Carlson-Drexler, A. Condon, M. Hepner, M. Howie, C. Ramos, J. Schmerfeld, H. Wada, and the South River Science Team for assistance and project support. We thank the landowners long the South River and the Waynesboro Parks and Recreation Department for access to sampling locations. Financial support was provided by E. I. DuPont de Nemours, Virginia Tech, and by the National Science Foundation (NSF # IOB-0615361). CMB was supported by the U.S. EPA STAR Graduate Fellowship (FP-9170040-1). EPA has not officially endorsed this publication and the views expressed herein may not reflect the views of the EPA. Research was completed with oversight from the South River Science Team, which is a collaboration of state and federal agencies, academic institutions, and environmental interests. Collecting permits and IACUC permission were obtained through WAH at Virginia Polytechnic Institute and State University.

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