

# Tissue mercury concentrations and adrenocortical responses of female big brown bats (*Eptesicus fuscus*) near a contaminated river

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**Abstract** Much of the research on mercury (Hg) in wild vertebrates has focused on piscivores and other animals at high trophic levels. However, recent studies indicated that insectivorous terrestrial vertebrates may also be at risk. In the present study, we examined blood and fur Hg concentrations as well as the adrenocortical responses of insectivorous big brown bats (*Eptesicus fuscus*) near the Hg-contaminated South River, VA and a nearby reference area. Baseline glucocorticoids and adrenocortical responses to handling have been widely used to assess the influence of environmental stressors because plasma glucocorticoids rise in response to various physical, psychological, and physiological challenges. Female bats captured at the contaminated site had 2.6 times higher blood and fur Hg concentrations than those captured at the reference site (blood: 0.11 vs. 0.04  $\mu\text{g/g}$  wet weight; fur: 28.0 vs. 10.9  $\mu\text{g/g}$  fresh weight). Fur Hg concentrations at the contaminated site were higher than most wild omnivorous and carnivorous mammals reported in the

literature. Although fur and blood Hg concentrations were tightly correlated, fur Hg concentrations averaged 260 times higher than concentrations in blood. This suggests that fur may be an important depuration route for bats, just as it is in other mammals. Despite the high Hg concentrations in bat tissue, we did not observe any site difference in adrenocortical responses. Our results suggest that the bats at the contaminated site were exposed to Hg concentrations below those causing adverse effects on their adrenal axis.

**Keywords** Mercury · Cortisol · Fur: blood ratio · Insectivore · *Eptesicus fuscus*

## Introduction

Mercury (Hg) is a heavy metal that acts as a neurotoxicant impairing motor functions as well as reproductive systems (Eisler 2006). It biomagnifies as it moves up the food chain, posing significant health risks to high trophic level organisms, especially piscivorous species (Scheuhammer et al. 2007; Wolfe et al. 2007). However, recent studies suggest that the risk of Hg exposure can extend to invertivorous species. This may be particularly true for certain predators such as spiders, amphibians, birds, and bats inhabiting forested habitats that have received Hg from nearby aquatic systems or by high levels of atmospheric deposition (Bergeron et al. 2010a, b; Clark and Shore 2001; Cristol et al. 2008; Rimmer et al. 2005; Wada et al. 2009).

Adverse effects of Hg on invertivores have recently been observed along the Hg-contaminated South River, VA, USA. A portion of the river near Waynesboro, VA, was polluted by use of mercury sulfate in a nearby factory

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more than 50 years ago (Carter 1977). Yet persistent Hg exposure continues to have sublethal, adverse effects on organisms in the area today. For example, American toads (*Bufo americanus*), a species that feeds on insects in the South River floodplain, maternally transfer Hg to their offspring, and tissue Hg concentrations of females are negatively correlated with offspring hatching success (Bergeron et al. 2010a, b; Bergeron et al., unpublished). Contrary to conventional perception, Hg concentrations in avian invertivores can equal (Cristol et al. 2008) or exceed (Evers et al. 2005) those of piscivores. Consequently, tree swallow nestlings at the South River contaminated sites show altered plasma corticosterone and thyroid hormone concentrations that are important for development, metabolism, and coping with stress (Wada et al. 2009). Interestingly, mercury concentrations in these studies were lower than levels associated with physiological effects in previous studies for avian piscivores (Kenow et al. 2007; Scheuhammer et al. 2007; Spalding et al. 2000). Thus, there is a clear need for additional studies on physiological effects of Hg on non-piscivorous species.

To determine if adverse effects of Hg on invertivorous wildlife extends to mammals, we sampled big brown bats (*Eptesicus fuscus*) at a reference and contaminated site near the South River. Many bats, including big brown bats, may be exposed to pesticides and heavy metal pollution by consuming insects from contaminated rivers and fields (Kurta 1999; Linzey 1998). However, little is known about the effects of Hg in bats. There are advantages to using some bat species for contaminant studies (Hickey and Fenton 1996; Kurta 1999) because they (1) have a long lifespan (9–23 years, Kunz and Fenton 2003), (2) live in close proximity to humans and industrial areas, (3) consume 40–100% of their body mass each night, and (4) often forage in areas (e.g., over streams) where emerging aquatic insects may provide contaminant subsidies to terrestrial consumers (Baxter et al. 2005; Hickey and Fenton 1996; Kurta 1999; Nakano and Murakami 2001; Walters et al. 2008). We quantified bats' tissue Hg concentrations and cortisol responses to standardized capture and handling protocol (Wingfield 1994). One of the first signs of distress in animals is a rise in plasma glucocorticoids (cortisol and corticosterone). Thus, baseline glucocorticoids and adrenocortical responses to handling have been widely used to assess the influence of environmental stressors such as weather, human disturbance, and pollution on animals (Hopkins et al. 1997; Romero and Wikelski 2001; Walker et al. 2005). Based on recent findings with swallows exposed to Hg (Franceschini et al. 2009; Wada et al. 2009), we hypothesized that female bats along the Hg-contaminated South River, VA, would have higher baseline cortisol and/or suppressed adrenocortical responses compared to individuals at a nearby reference site.

## Materials and method

### Study species and site

Big brown bats are found throughout the United States, Mexico, and parts of Canada (Kurta 1999; Linzey 1998). These 14–21 g bats consume insects weighing 10% or more of their body mass per hour (Kurta 1999). During lactation, female bats can consume approximately 100% of their body weight per night. Big brown bats of Virginia can mate in autumn and spring and migrate short distances to buildings or caves where they hibernate (Linzey 1998). Despite mating in autumn, fertilization does not occur until spring and females give birth to one to two young in June. Big brown bats also undergo molt in early summer (Phillips 1966). After mating, pregnant females congregate to form maternal colonies where they roost together in barns, bridges, and other crevices.

Adult post-lactating female big brown bats were captured in August, 2007 from a barn 29.0 km downstream of the Hg-contamination source near the South River, VA, (designated as the contaminated site) and a barn 9.1 km upstream of the contamination source in Waynesboro, VA (reference site), also near the South River, VA. The barn in the contaminated site was 1.0 km from the river and the barn of the reference site was 7.3 km from the river. Both barns were near residential areas, cow pastures, and corn fields. The roosting sites of maternal colonies were either known previously or located by radio-tracking females caught in mist nets on the South River.

### Tissue sampling

Female bats were captured from roosts using a harp trap suspended in their flight path. They were caught between 2015 and 2100 hours as they initially left the roosting sites to feed. This allowed us to control for the effects of feeding and time of day on plasma cortisol levels (Reeder et al. 2004). Blood samples were obtained by puncturing patagium and uropatagium veins with a 27 1/2 gauge needle, and then collecting it into heparinized capillary tubes. Fur samples were cut from the back and abdomen with clean stainless steel scissors and collected directly into ziplock bags.

Adrenocortical responses were determined using a repeated measures design (similar to the protocol described by Wingfield 1994). Baseline blood samples for cortisol were collected within three minutes of bats hitting the harp trap. Individuals were then restrained in an opaque cloth bag for 27 min and a second blood sample was collected from each individual 30 min post-capture. After obtaining the second blood sample, body mass and forearm length were measured and reproductive condition (pregnant,

lactating, post-lactating, or non-reproductive) was determined by physical examination. The amount of fur around the nipples was used to distinguish between post-lactating and non-reproductive females. All bats were confirmed to be post-lactating. Blood samples were kept on ice and brought back to the field station. Usually within 6 h of sample collection, samples were centrifuged for 8 min (at 12,000 rpm (15,300×g) at room temperature), and plasma was separated and frozen immediately at  $-20^{\circ}\text{C}$ .

#### Plasma cortisol assays

Plasma cortisol concentrations were determined using enzyme immunoassay (EIA) kits (Cayman Chemical, Cat No. 582121.1). Each plate contained standards ranging from 7.82 to 10,000 pg/ml, additional 500 pg/ml standards for assessing inter-plate variability in triplicate, and bat plasma samples in duplicate. The assay was first optimized for the species (for details, see Wada et al. 2007). In the optimization assays, pooled plasma was stripped of endogenous hormone, spiked with a known amount of cortisol (250 pg/ml), then serially diluted from 1:50 to 1:500 (1:50, 100, 200, 300, 400, and 500). At the end of the assay, plates were read at 405 nm with a plate reader (SpectraMax Plus 384, Molecular Devices) and cortisol concentrations of each dilution were compared with the buffer containing the cortisol spike only. Optimization assays showed that 1:200 or higher dilution eliminated the interference between plasma and the assay. Thus individual plasma samples were diluted 1:200 in the cortisol assay. Plasma samples were haphazardly distributed across plates. Detection limit of this assay was 1.11 ng/ml (determined as two standard deviations above the total binding wells which received only buffer, tracer, and antiserum). Intra- and inter-plate variation were 14.5 and 0.96%, respectively.

#### Mercury analysis

Blood and fur samples were analyzed for total mercury concentrations using a direct Hg analyzer DMA 80 at Texas A&M Trace Element Research Laboratory. Blood samples were heated at  $850^{\circ}\text{C}$  and combusted. Mercury atoms were trapped using a gold column and released into atomic absorption cell where they were detected (USEPA 1998). Fur samples were digested with  $\text{HNO}_3$ , HCl, and  $\text{H}_2\text{O}_2$  prior to Hg analysis. The digested samples were diluted with deionized water then analyzed by atomic absorption. Geometric means of weight of blood and fur samples were 19.6 and 8.27 mg, respectively. Although we do not know the % methylmercury (MeHg) in bat blood or fur, studies reported to date show that mammalian blood and fur contain >80% MeHg (Cernichiari et al. 1995;

Porcella et al. 2004). Thus we believe that the vast majority of Hg detected in our samples was methylated. Data are presented as wet weight for blood (ww) and fresh weight (fw) for fur Hg concentrations. The average detection limit was  $0.0025 \mu\text{g/g}$  ww for blood and  $0.0987 \mu\text{g/g}$  fw for fur. Average relative percent differences of duplicate samples were  $5.4 \pm 1.9\%$  (blood) and  $17.8 \pm 5.5\%$  (fur), and mean percent recoveries for the mercury spiked samples were  $99 \pm 1.3\%$  (blood) and  $110 \pm 2.0\%$  (fur). Average percent recoveries for the standard reference materials, DOLT-3 and DORM-2, were  $98.2 \pm 0.9$  and  $96.4 \pm 0.5\%$ , respectively for blood samples. The average recovery of DORM-2 for fur samples was  $61.2 \pm 0.9\%$ .

#### Data analysis

Statistical analyses were performed using JMP 5.0.1. Prior to each analysis, data were tested for equal variance and normality. To meet assumptions of the models, all data were log-transformed except for fold-increase data which were arcsine square root transformed. Site differences in blood and fur Hg concentrations as well as fold-increase in cortisol were analyzed using one-way ANOVAs. The effect of site on plasma cortisol levels before and after restraint was compared using repeated-measures ANOVA. The relationship between blood and fur Hg was determined using Pearson correlation after log-transformation. Parameters of adrenocortical responses (baseline, stress-induced levels of cortisol, and fold increase) were also regressed against blood and fur Hg concentrations using linear regression. Comparisons were considered statistically significant when  $p \leq 0.05$ . Data are presented as mean  $\pm$  1 SE.

## Results

#### Mercury concentrations in reference and contaminated sites

Bats at the contaminated site had 2.6 times higher blood and fur Hg concentrations compared to the reference site (blood:  $F = 48.39$ ,  $p < 0.001$ ; fur:  $F = 20.80$ ,  $p < 0.001$ ; Table 1). Blood and fur Hg concentrations were positively correlated with each other ( $y = 274.27x - 0.82$ ,  $r = 0.870$ ,  $p < 0.001$ ; Fig. 1). However, Hg concentrations were 260 times higher in fur than in blood.

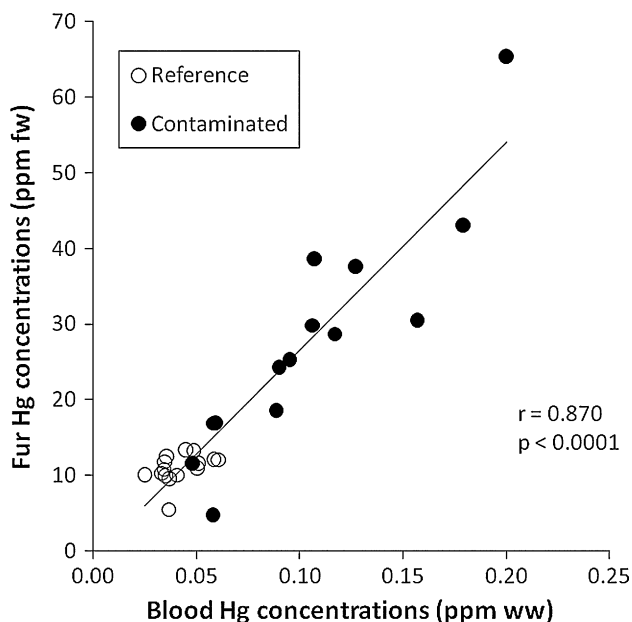
#### Adrenocortical responses in post-lactating bats

Baseline cortisol levels were similar in post-lactating females at each site (Table 1), but ranged from 21 to 214 ng/ml. All bats showed a robust adrenocortical

**Table 1** Blood and fur Hg concentrations as well as adrenocortical responses of adult female big brown bats (*Eptesicus fuscus*) from reference and Hg-contaminated sites along the South River, Virginia, USA

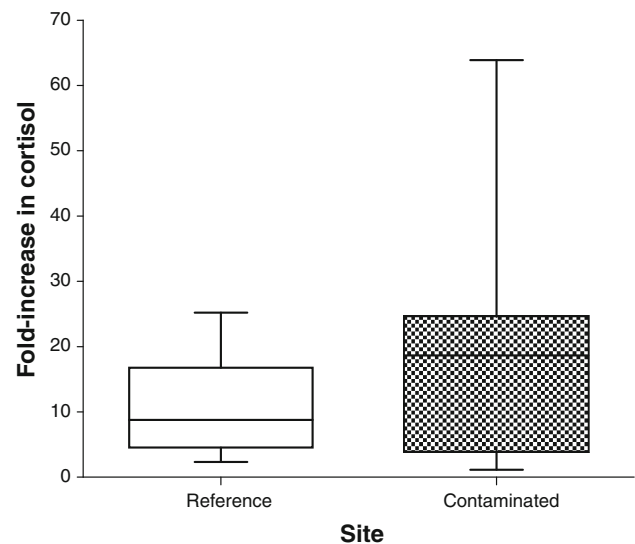
	Reference site	Contaminated site
Mercury		
Blood ( $\mu\text{g/g ww}$ )	$0.042 \pm 0.003$	$0.110 \pm 0.012$
Fur ( $\mu\text{g/g fw}$ )	$10.94 \pm 0.50$	$28.01 \pm 4.06$
Cortisol (ng/ml)		
Baseline (<3 min)	$83.81 \pm 11.46$	$66.29 \pm 14.81$
Post-handling (30 min)	$833.68 \pm 184.88$	$733.42 \pm 131.94$

Values represent mean concentrations  $\pm 1$  SE in reference and contaminated sites. Adrenocortical responses include plasma cortisol concentrations before and after 30 min of restraint. Sample size for all categories is 15/site, except for fur concentrations in the contaminated site ( $n = 14$ )



**Fig. 1** The relationship between blood and fur Hg concentrations ( $\mu\text{g/g}$ ) in adult female big brown bats (*Eptesicus fuscus*) along the South River, Virginia, USA. Open and filled circles represent bats from reference and contaminated sites, respectively

response following 30 min of restraint (restraint:  $F = 4.70$ ,  $p < 0.0001$ ), with their stress-induced levels ranging from 77 to 2.718 ng/ml. However, site had no effect on cortisol levels, and there was no significant interaction between site and the effect of restraint (site:  $F = 0.03$ ,  $p = 0.349$ ; restraint \* site:  $F = 0.01$ ,  $p = 0.593$ ). Bats in the contaminated site had slightly higher fold increase in cortisol than bats from the reference site, however, the difference was not significant ( $F = 1.30$ ,  $p = 0.263$ , power = 0.20; Fig. 2). Regression analyses of Hg and cortisol concentrations showed that neither blood nor fur Hg



**Fig. 2** Adrenocortical responses of adult female big brown bats (*Eptesicus fuscus*) from reference and Hg-contaminated sites along the South River, Virginia, USA. The figure represents mean fold-increase in cortisol  $\pm 1$  SE after 30-min restraint. Boxes represent 25th to 75th percentiles with a line at the median. Whiskers represent the minimum and maximum values.  $n = 15$ /site

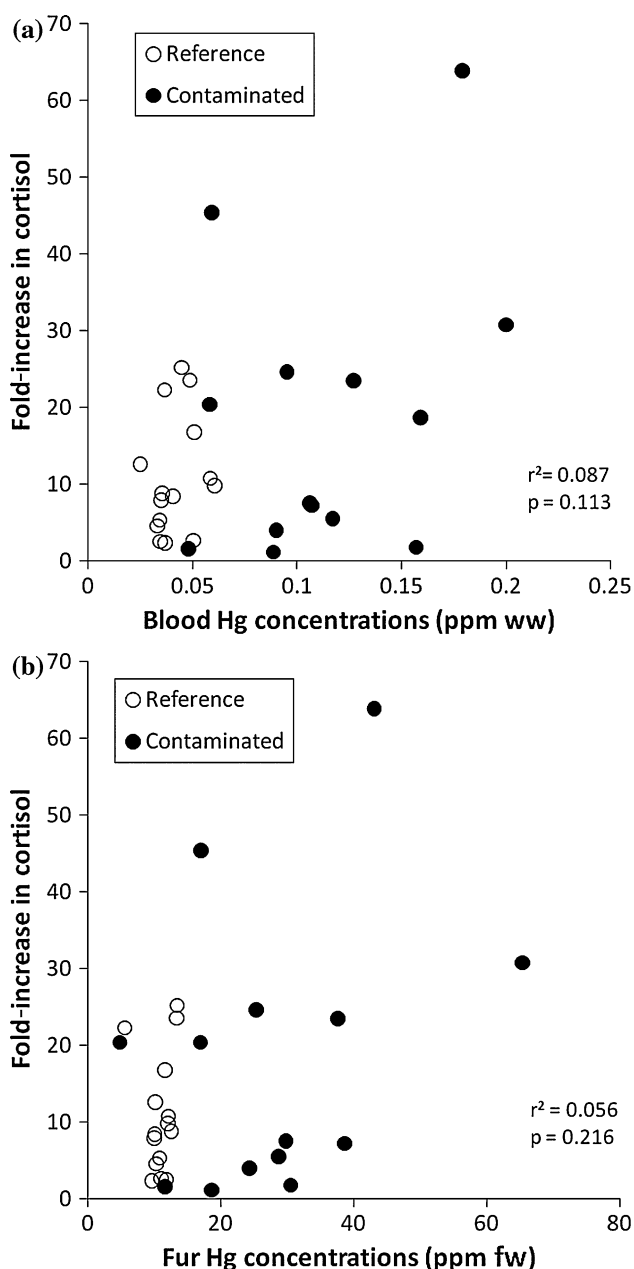
concentrations were correlated with baseline cortisol, stress-induced cortisol, or fold-increase in cortisol (in all cases  $r^2 < 0.10$ ,  $p > 0.11$ ; Fig. 3a, b).

## Discussion

Female big brown bats captured near a Hg-contaminated portion of the South River, VA had significantly higher fur and blood Hg concentrations compared to the nearby reference site. Furthermore, fur and blood Hg concentrations were positively correlated with each other, but fur Hg concentrations were 260 times higher than blood concentrations. Females from both sites showed similar, robust adrenocortical responses to capture and handling stress. Although the responses did not differ between sites, fold-increase in corticosterone at the contaminated site was 1.7 times higher and more variable than at the reference site (97% coefficient of variation compared to 71%, respectively).

### Characterizing bat tissue Hg levels

Despite the proximity to the Hg-contaminated South River, blood Hg concentrations of big brown bats captured at the contaminated site were lower than other animals at the site. Total blood Hg concentrations at the contaminated site ranged from 0.05 to 0.20  $\mu\text{g/g ww}$ , averaging 0.11  $\mu\text{g/g ww}$ . These blood concentrations were more than an order of magnitude less than those of birds sampled along the



**Fig. 3** Linear regression between tissue concentrations and fold-increase in cortisol after 30-min of restraint in adult female big brown bats (*Eptesicus fuscus*) along the South River, Virginia, USA. **a** Blood Hg concentrations ( $\mu\text{g/g ww}$ ), **b** Fur Hg concentrations ( $\mu\text{g/g fw}$ ). Open and filled circles represent bats from reference and contaminated sites, respectively

same contaminated reaches of the South River. For example, insectivorous tree swallows (*Tachycineta bicolor*), Carolina wrens (*Thryothorus ludovicianus*), and red-eyed vireos (*Vireo olivaceus*) had average total blood Hg concentrations of 3.66, 4.49, and 6.72  $\mu\text{g/g ww}$ , respectively (Cristol et al. 2008). In fact, bat blood Hg concentrations at the contaminated site were actually comparable to those of birds at the reference sites (average for 18

species of reference birds = 0.18  $\mu\text{g/g ww}$ ). Furthermore, the blood concentrations observed in bats were also lower than blood concentrations of omnivorous turtles captured in the South River (painted turtles (*Chrysemys picta*), stinkpots (*Sternotherus odoratus*), and snapping turtles (*Chelydra serpentina*)) (Bergeron et al. 2007). When compared to previous dosing studies on mammals, the blood Hg concentrations observed in our study were within the range of concentrations associated with normal behavior and survival in captive mink (Basu et al. 2006) and lower than levels that caused loss in appetite, weight, and mortality in captive harp seal (Ronald et al. 1977), visual disturbance in monkeys (Suzuki 1979), neural impairment and numbness in humans (Suzuki 1979; Takizawa 1979), and fatality in other mammals (Ronald et al. 1977; Takizawa 1979).

In contrast to blood, fur Hg concentrations in bats were high and average Hg concentrations were among the highest observed in wild mammals to date, exceeding toxicity thresholds (11  $\mu\text{g/g}$ , USEPA 1997). Bats' fur Hg concentrations at the contaminated site ranged from 4.8 to 65.4  $\mu\text{g/g fw}$ , averaging 28.0  $\mu\text{g/g fw}$ . These concentrations were higher than semiaquatic herbivorous Muskrats (*Ondatra zibethicus*) at a Tennessee Hg-contaminated site (3.9  $\mu\text{g/g dw}$ , Stevens et al. 1997) and omnivorous raccoons (*Procyon lotor*) near the Hg-contaminated Savannah River, SC (9.9  $\mu\text{g/g ww}$  with the highest individual having 51.0  $\mu\text{g/g}$ , Cumbie and Jenkins 1975). Interestingly, Miura et al. (1978) and Hickey et al. (2001) also found relatively high fur Hg concentrations in insectivorous Chiroptera (reaching 33 and 13  $\mu\text{g/g dw}$ , respectively). The levels observed in bat fur at the South River were higher than fur Hg concentrations associated with decline in stress tolerance and swimming ability in wild mice (Burton et al. 1977) and more than an order of magnitude higher than fur methylmercury levels found to cause anorexia, ataxia, and death in captive mink (Wobeser et al. 1976). Furthermore, average fur Hg concentrations at our contaminated site were above the proposed "at-risk" fur concentrations (total Hg of 12.6  $\mu\text{g/g ww}$ ) for Florida panthers (Newman et al. 2004; Roelke et al. 1991).

Relatively low blood Hg concentrations in big brown bats despite their proximity to the Hg-contaminated river suggest that bats can eliminate a high proportion of the Hg they ingest. Specifically, a high fur: blood Hg ratio of 260 indicates that fur is an important route of Hg elimination for big brown bats. Although we did not measure the oral intake of Hg, our findings imply that big brown bats at our study site are exposed to high levels of Hg but able to excrete a great deal of the Hg into fur, possibly keeping other tissue levels of Hg low. The essential role of fur and hair in depurating Hg has been documented in other mammals exhibiting similar or lower fur/hair: blood Hg



ratios compared to big brown bats. For instance, harp seals dosed with 25 mg MeHgCl/kg body wt/day had a fur:blood Hg ratio of 18.2 (Ackerman et al. 2008). In the wild, fur/hair:blood Hg ratios ranged from 50 in Pacific harbor seals living in central and north California (Brookens et al. 2007) to 106 in polar bears in Southern Beaufort Sea (Cardona-Marek et al. 2009). In humans, the hair:blood ratio was shown to vary with age and averaged around 250 (Budtz-Jorgensen et al. 2004; Cernichiari et al. 2007).

The cause for the interspecies variation in fur/hair to blood Hg ratios is unknown; however the ratios are generally higher in mammals than comparable feather:blood ratios in birds. At the South River, feather Hg concentrations in Carolina wrens (*Thryothorus ludovicianus*), eastern screech-owl (*Megascops asio*), red-bellied woodpecker (*Melanerpes carolinus*) were approximately twice their respective blood Hg concentrations (Cristol et al. 2008). One possibility for the observed difference between bats and birds at the South River is that blood Hg concentrations in big brown bats may have been reduced due to maternal transfer of Hg to their young. Female bats sampled in this study were all post-lactating which may have eliminated some portion of their Hg burden across the placenta and during lactation. In comparison, Hg samples in birds were comprised of both sexes due to lack of sex differences in blood Hg concentrations (Cristol et al. 2008). However, it is unlikely that this is the only explanation because a relatively small proportion of Hg can be transferred to young via lactation (Sundberg et al. 1998). Another possibility for the difference in tissue Hg concentrations in bats and birds at the South River is the timing of tissue sampling in relation to molt. Both taxa molt once a year, however bats were sampled 2–3 months after molt had taken place while birds were sampled 1–4 months prior to annual molt (Cristol et al. 2008). Nonetheless, it is doubtful that maternal transfer and molting patterns fully explain the pronounced interspecies variation in fur/feather:blood ratios between birds and bats. Thus, future studies are needed to determine the cause of interspecies variation in fur:blood or feather:blood ratios.

#### Adrenocortical responses of big brown bats in Hg-contaminated areas

In comparison to previous studies on fish and birds (Bleau et al. 1996; Friedmann et al. 1996; Kirubakaran and Joy 1991; Wada et al. 2009), we did not observe any site difference in adrenocortical responses of big brown bats, despite the fact that their fur Hg concentrations were above suggested adverse effect levels. Blood Hg concentrations, which represent the circulating fraction of Hg that can reach target organs or be excreted, were also slightly higher than blood Lowest Observed Adverse Effects Levels

(LOAELs) set by USEPA (MeHg: 0.044 µg/g, USEPA 1997; derived from hair Hg that caused developmental effects in children of mothers who ingested Hg). Laboratory and field studies clearly show that Hg can alter the HPA axis of other animals. For instance, juvenile rainbow trout (*Oncorhynchus mykiss*) exposed to inorganic or organic Hg had elevated cortisol within 4 h, and returned to baseline levels by 7 days post-exposure (Bleau et al. 1996). On the other hand, fish treated with Hg for 90 or 180 days via diet or water decreased plasma cortisol and showed enlarged pituitary adrenocorticotrophic hormone (ACTH, hormone that stimulates glucocorticoids secretion from adrenals) cells, a sign of increased ACTH secretion (Friedmann et al. 1996; Kirubakaran and Joy 1991). Similarly, tree swallow nestlings near the Hg contaminated South River showed altered adrenocortical responses (Wada et al. 2009). The adrenocortical responses were enhanced during early stages of nestling development but were suppressed in 13–17 day-old nestlings. One possible reason for Hg having effects on birds, but no detectable effects on the adrenal axis of bats at the same site, is that relatively high depuration rates in bats compared to birds may buffer bats from excessive physiological exposure to Hg. Relatively low circulating concentrations of Hg in blood and high fur:blood Hg ratio support this possibility. In other words, invertivorous bats and birds at the same site may be exposed to similar levels of Hg, however bats are able to eliminate higher proportion of Hg through fur than birds through feathers, minimizing their tissue exposure to Hg. Yet target organ concentrations of Hg in bats remain a major unknown that needs to be evaluated to test this hypothesis.

Our findings indicate that bats can be exposed to very high Hg concentrations in the environment, but the physiological fate and effects of ingested Hg remains poorly understood. We suggest that important next steps in ecotoxicological research on Hg in bats are to determine (1) whether depuration into fur reduces Hg accumulation in internal organs, and (2) the blood and fur Hg concentrations associated with altered reproduction and survival. In recent years, large mortality events have likely reduced bat populations in eastern North America because of white-nose syndrome (Blehert et al. 2009) and wind energy facilities (Arnett et al. 2008). Future investigations examining the role of Hg exposure in bat reproduction and survival is therefore even more critical for conserving sustainable bat populations in eastern North America.

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