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Stable mercury isotopes in songbirds and their prey

UNDERSTANDING SOURCES OF METHYLMERCURY IN SONGBIRDS WITH STABLE MERCURY ISOTOPES: CHALLENGES AND FUTURE DIRECTIONS

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Abstract

Mercury (Hg) stable isotope analysis is an emerging technique that has contributed to a better understanding of many aspects of the biogeochemical cycling of Hg in the environment. However, no study has yet evaluated its usefulness in elucidating the sources of methylmercury (MeHg) in songbird species, a common organism for biomonitoring of Hg in forested ecosystems. In the present pilot study, we examined stable mercury isotope ratios in blood of 4 species of songbirds and the invertebrates they are likely foraging on in multiple habitats in a small watershed of mixed forest and wetlands in Acadia National Park in Maine (USA). We found distinct isotopic signatures of MeHg in invertebrates (both mass-dependent fractionation [as δ^{202}Hg] and mass-independent fractionation [as Δ^{199}Hg]) among 3 interconnected aquatic habitats. It appears that the Hg isotopic compositions in bird blood cannot be fully accounted for by the isotopic compositions of MeHg in lower trophic levels in each of the habitats examined. Furthermore, the bird blood isotope results cannot be simply explained by an isotopic offset as a result of metabolic fractionation of δ^{202}Hg (e.g., internal demethylation). Our results suggest that many of the birds sampled obtain MeHg from sources outside the habitat they were captured in. Our findings also indicate that mass-independent fractionation is a more reliable and conservative tracer than mass-dependent fractionation for identifying sources of MeHg in bird blood. The results demonstrate the feasibility of Hg isotope studies of songbirds but suggest that larger numbers of samples and an expanded geographic area of study may be required for conclusive interpretation.

Keywords: Songbird, Wetland, Methylation, Isotopic fractionation, Trophic transfer

This article includes online-only Supplemental Data.

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INTRODUCTION

Mercury (Hg) is a global pollutant, and has been shown to be a potent neurotoxin and endocrine disruptor, thus posing a significant risk to human and wildlife health [1]. Many anthropogenic sources (e.g., coal combustion, artisanal gold mining, and biomass burning) can emit Hg to the atmosphere, and deposited Hg (mainly inorganic) can be microbially converted to highly toxic methylmercury (MeHg) in low oxygen environments such as wetlands [2].

Monitoring of Hg bioavailability in the environment can be carried out using birds, because they effectively integrate MeHg from their prey items over spatial and temporal scales, and thus their Hg levels in certain nonintrusive tissue types (e.g., blood) are good indicators of current body burdens of MeHg in birds [3] as well as ambient levels of MeHg in the surrounding habitats [4]. Because of their mixed diets, songbirds are useful for determining MeHg availability in ecosystems integrating both terrestrial and aquatic habitat types, but the mixed sources of MeHg likely introduce unknown variations in blood MeHg levels [5]. Understanding the sources of MeHg in songbird blood is important to understanding their role as a biomonitor of MeHg, as well as accurately assessing the risk of MeHg to the health of songbird populations in natural ecosystems.

We suggest that stable Hg isotope analyses can provide a more in-depth understanding of the sources of MeHg to songbirds, because the isotope ratios can act as a direct tracer of both sources and chemical transformations of Hg in the environment prior to bioaccumulation [6]. There are 2 types of isotopic fractionation associated with stable Hg isotopes: mass-dependent fractionation and mass-independent fractionation [6]. The mass-dependent fractionation type can

result from a wide variety of biogeochemical processes, while large-magnitude mass-independent fractionation is known to occur most commonly through photochemical reactions such as the photodemethylation of MeHg [6]. Our previous studies in a semiremote watershed in northern California showed that mass-dependent fractionation values of MeHg in stream food webs differed significantly from terrestrial food webs throughout the watershed [7,8]. Because isotopic fractionation of MeHg during trophic transfer is negligible, as demonstrated in a controlled study with fish [9], we may directly compare the isotopic signatures of MeHg in organisms across trophic levels and habitats to understand the trophic transfer pathways for MeHg [7,8,10,11]. These previous findings showed that stable Hg isotopes can be a useful tool for understanding the origins of MeHg exposure among and within food webs.

In the present pilot study, we used stable Hg isotopes to examine the sources of MeHg in 4 different species of songbirds and their prey in Acadia National Park, Maine, USA. Mercury contamination is widespread in many freshwater ecosystems in Acadia National Park, with higher than average levels of Hg in biota at various trophic levels [12]. Elevated MeHg levels in Acadia National Park are mainly because of the fact that wetlands occur in approximately 20% of the area [13] and low-oxygen soils in wetlands are known to promote Hg methylation [2]. The study area includes complex landscapes with multiple ecotones (Figure 1). We hypothesized that songbirds would show Hg isotopic signatures similar to prey items in the habitat where they were captured. Because Hg cycling differs among different habitats [7], we expected to see different isotopic compositions of MeHg in birds among the different study habitats in Acadia National Park. Habitat-specific MeHg isotope signatures were established using vegetation and invertebrate samples found within each site, following an approach we developed previously in other natural ecosystems [7,8]. It is recognized that some biota samples (e.g., leeches) may not

be the ultimate prey for the songbird species. However, they are likely to obtain MeHg from the same source(s) as the actual prey items for the songbirds, and thus it is justified to collect these food web items for isotopic analysis of MeHg. We collected bird blood samples without euthanizing the sampled individuals and analyzed them for total Hg concentration and stable Hg isotope ratios (if sample mass was sufficient), with the assumption, verified by other studies, that most blood Hg is in the form of MeHg (e.g., 89–100%) [14,15]. We report on stable Hg isotopes in 3 interconnected aquatic habitats and in songbird blood samples at Acadia National Park. We discuss what this technique tells us about where songbirds are exposed to MeHg and we also discuss the utility of Hg isotopes as a bioindicator for Hg pollution in complex landscapes such as Acadia National Park.

MATERIALS AND METHODS

Study sites and sample collection

Sampling sites were selected in distinct habitats across the Aunt Betty Pond and Richardson Brook watersheds in Acadia National Park (Figure 1). In the summer of 2012 we collected a variety of biota samples at Aunt Betty Pond (site A, a marshy pond with an average depth of ~1 m, a maximum depth of ~2 m, and extensive coverage by aquatic vegetation) and 3 sampling sites upstream of Aunt Betty Pond, including Marsh (site B), Beaver Pond impoundment (site C), and Upland Forest (site D; Figure 1). Previous studies have shown that Aunt Betty Pond (site A) is where the highest levels of Hg in invertebrates (as food bolus for tree swallows) and bird (tree swallows) tissues were found, compared with other sites investigated in Acadia National Park [16]. Marsh (site B) is connected with Aunt Betty Pond (site A) through Chasm Brook, at a distance of approximately 350 m. Marsh (site B) has open water but also has extensive growth of sedges and rushes. Beaver Pond impoundment (site C) is located further

upstream of Marsh (site B), but is within a densely forested area. The sizes of individual ponds vary widely, but in most cases Beaver Pond impoundments are shallow (<50 cm deep) with very muddy bottoms. Upland Forest (site D) is located at an upland location near site C, and we believe that its location is far enough from aquatic habitats (>200 m) to minimize lateral inputs of aquatic MeHg via insect movements into the forests [8].

Macroinvertebrates were sampled and composited at each site, and fish larvae were sampled at sites A and B when encountered (Table 1). In most cases, samples were caught using tweezers and/or nets, and pitfall traps for site D. Foliage samples were only collected at site D. All non-bird biota samples were placed in new polypropylene centrifuge vials (Corning) and stored in a cooler and later in a freezer at –20 °C.

A total of 49 songbird individuals representing 15 species (see the complete list in the Supplemental Data, Table S1) were captured by mist net using playback recordings of conspecific songs, and species, age, and sex were determined by external characteristics at 4 different locations including site A-B (2 locations, near sites A and B), site C, and site D (Figure 1). Bird blood samples were collected by puncturing the cutaneous ulnar vein with a sterile 26- to 28-gauge needle and placing a heparinized capillary tube at the puncture site to gather 30 to 150 μL of blood (<1% of bird weight [17]), and birds were released unharmed. All samples were kept on ice during sampling, and then frozen until processing and analysis.

Sample processing and Hg analyses

All non-bird biota samples were frozen, lyophilized, homogenized (either by mortar and pestle or mixer-mill), and analyzed for total Hg and MeHg concentrations using cold vapor atomic fluorescence spectrometry (Supplemental Data, Part I). All bird blood samples were frozen and thawed, and then a fraction was weighed and analyzed for total Hg concentrations

using a direct mercury analyzer (Supplemental Data, Part II). Selected samples of non-bird biota samples (n = 19) and bird blood samples (n = 20; representing 4 dominant species) were thermally combusted and prepared for stable Hg isotope ratios using multicollector–inductively coupled plasma–mass spectrometry (Supplemental Data, Part III).

As demonstrated in previous studies, inorganic Hg (Hg(II)) and MeHg often have different isotopic compositions (both mass-dependent fractionation and mass-independent fractionation) even within the same ecosystem [7,8]; thus we cannot simply compare stable Hg isotope ratios among all food web members of different trophic levels because of the mixing of inorganic Hg and MeHg in their tissues [7]. Because of the variability of the fraction of total Hg as MeHg (i.e., %MeHg) in many invertebrate samples analyzed in the present study, we estimated endmember MeHg isotopic compositions in mass-dependent fractionation as $\delta^{202}Hg_{MeHg}$, and mass-independent fractionation of odd-mass isotopes as $\Delta^{199}Hg_{MeHg}$ for each biota sample (Supplemental Data, Part IV) by extrapolating data with variable %MeHg to a pure MeHg endmember value using an approach we developed and applied previously [7,8]. It should be noted that mass-dependent fractionation of MeHg isotopes can occur through many different biogeochemical reactions while significant mass-independent fractionation of odd-mass MeHg isotopes (e.g., >+0.4‰) is believed to be exclusively because of photodemethylation of MeHg [18]. We used habitat-specific mean isotopic values of inorganic Hg from aquatic or forest food webs to estimate the isotopic composition of MeHg in invertebrate samples from each habitat. Estimates of the isotope ratios of MeHg in invertebrates could then be directly compared between samples, and with values in bird blood, which has previously been shown to be close to 100% MeHg [14,15].

Statistical analyses

Linear regression analyses were performed using SigmaPlot 13.0 (Systat), while statistical tests comparing slopes of regression lines and one-way analysis of variance (ANOVA) followed by Tukey's post hoc test were performed using Prism 7.02 (GraphPad). The significance level for all statistical analyses was set at $\alpha = 0.05$.

RESULTS AND DISCUSSION

Hg levels in food webs and bird bloods among habitats

There is a large range of total Hg and MeHg concentrations among all macroinvertebrates and fish larvae. Below, we will focus on our results for MeHg concentrations, because it is the bioavailable form of Hg to songbirds. We found that MeHg concentrations were significantly higher ($p < 0.05$) in biota samples (including samples with %MeHg > 40%) from site C (Beaver Pond impoundments) than the other 3 habitats (sites A, B, and D), and there were no significant differences ($p > 0.05$) in MeHg concentrations among samples collected from sites A, B, and D (Table 1); these findings are consistent with previous studies in Acadia National Park demonstrating that wetlands represent the dominant sources of MeHg to the local food webs [12,16]. At site C, both predatory dragonfly larvae and herbivorous amphipods contained elevated MeHg concentrations. These organisms live on surface soils or burrow into the anoxic soils in Beaver Pond impoundments and may be directly exposed to diets with elevated MeHg concentrations.

Birds captured from the 3 locations (Figure 1) had variable levels of Hg in their blood, ranging from 74.7 to 880 ng/g wet weight. In parallel to the differences we observed in non-bird biota samples among sites, we also found that bird blood Hg was significantly different among these habitats, with Hg in bird blood samples collected at site C (mean ± standard deviation = 375 ± 167 ng/g wet wt) being significantly higher ($p < 0.05$) than Hg in bird blood samples

collected at site A-B (273 ± 118 ng/g wet wt) and site D (188 ± 99 ng/g wet wt), while we found no significant differences in Hg in bird blood samples collected from site A-B and site D (p > 0.05; one-way ANOVAs, followed by Tukey's post hoc analysis; Figure 2). Therefore, these findings suggest that site C (Beaver Pond) may be a location of enhanced MeHg production in our study watersheds.

Isotopic compositions of MeHg in non-bird biota

Even though our study sites are fairly close to one another, we found distinct isotopic signatures of MeHg (after accounting for different %MeHg) [7,8] among the 3 interconnected aquatic habitats (Aunt Betty Pond, site A; Marsh, site B; and Beaver Pond impoundments, site C). For the first time we observed the natural occurrence of negative $\Delta^{199}Hg_{MeHg}$ (mass-independent fractionation; from –0.23 to –0.11‰) for MeHg in 3 invertebrate samples collected in site C, and the mass-independent fractionation values of their MeHg was similar to that of inorganic Hg in foliage samples we collected from Acadia National Park (from –0.34 to –0.15‰; Figure 3). Previously, negative mass-independent fractionation of MeHg isotopes had been thought to represent MeHg in the environment prior to significant photodemethylation, but it had not been directly measured [10]. The isotopic compositions of Hg in foliar samples at Acadia National Park are quite similar to those of foliar Hg isotopes in other locations in North America [19].

Methylation of inorganic Hg has been shown to either produce MeHg with lower mass-dependent fractionation [20,21] than the bulk inorganic Hg substrate or MeHg with higher mass-dependent fractionation than the pool of inorganic Hg available for methylation [22]. Janssen et al. [22] recently found that there was an isotopically distinct pool of bioavailable Hg(II) (higher mass-dependent fractionation) in their incubation experiments and that this pool of intracellular

Hg(II) was preferentially methylated compared with the bulk Hg(II) added to the culture. With further so-called dark microbial demethylation (mediated by *mer b*), mass-dependent fractionation of the final pool of bioavailable MeHg should have higher values**<ZAQ;1>** [6]. However, mass-independent fractionation has not been observed during methylation because of the absence of photochemical processes [6]. The Hg isotopic variation reveals that the biogeochemical processes occurring within the Beaver Pond soils are ideal for Hg methylation because little or no light penetrates the organic-rich saturated soils, and the abundance of decomposed organic matter provides an ideal substrate for anaerobic methylation of inorganic Hg [23,24]. Thus, no change in mass-independent fractionation values in MeHg compared with inorganic Hg from foliage (the main substrate of Hg methylation) would be expected in this setting, because inorganic Hg is microbially methylated in the dark. These results provide a sound explanation for why beaver ponds are often hotspots for MeHg [23], because of extensive methylation but perhaps more importantly as a result of the near absence of photodemethylation in the soil horizons under the dense canopy [18]. This finding suggests that rates of photodemethylation as well as dark microbial demethylation might be as important as methylation rates for creating MeHg hotspots in these ecosystems.

As water flows downstream for approximately 0.7 km from site C to site B (Marsh), MeHg (if derived from site C) in biota becomes elevated and positive in mass-independent fractionation values ($\Delta^{199}Hg_{MeHg}$ increased from –0.2 to +0.5‰; Figure 3). One exception to this pattern was a composited sample of fish larvae collected from site B that had $\Delta^{199}Hg_{MeHg}$ of +1.0‰; we speculate that the fish larvae might have recently migrated from other downstream locations (e.g., site A, where MeHg had more positive mass-independent fractionation values,

see next paragraph) or that they consumed prey either near the surface of the water or from terrestrial inputs [8].

A little further downstream (~0.3 km of stream length) at site A, we found slightly higher mass-independent fractionation values (average of +0.9‰ of $\Delta^{199}Hg_{MeHg}$) in MeHg from food webs within the pond (Figure 3). Exceptions to these values included leeches and water beetles that we caught near the water surface, and thus they may have consumed different diets (e.g., at different water depths in the pond, and so with different mass-independent fractionation values). The mismatch among food web members within a single small habitat suggests that movement and integration of external diets may be common for aquatic food web members in heterogeneous landscape ecosystems such as Acadia National Park. Such a scenario may be analogous to the situation in upstream small tributaries in a montane watershed in northern California that we studied previously [8]. In addition, we found that the trend of isotopic compositions of MeHg (i.e., the slope of $\Delta^{199}Hg/\delta^{202}Hg$) in each of the 3 aquatic habitats generally fit with the laboratory-derived photodemethylation relationships for experiments conducted with dissolved organic carbon (DOC) concentrations between 1 and 10 mg C/L (denoted as dashed lines in Figure 3; based on previous controlled experiments [25], because DOC levels at Acadia National Park sites measured in another study were between 1 and 10 mg C/L [26].

Isotopic compositions of MeHg in songbird blood

To our knowledge we are reporting the first measurements of stable Hg isotope ratios in blood samples from songbird species (a recent study measured Hg isotopes in blood from seabirds [27]). We found that both mass-dependent fractionation and mass-independent fractionation values varied widely among the 20 songbird blood samples (out of a total of 49

samples for total Hg analysis) collected over 3 sampling locations in a relatively small sampling area at Acadia National Park (Table 2 and Figure 4). Among the 20 blood samples, δ^{202}Hg (mass-dependent fractionation) ranged from –1.95 to –0.08‰, while Δ^{199}Hg (mass-independent fractionation) ranged from +0.06 to +1.31‰. The ranges for both δ^{202}Hg and Δ^{199}Hg in bird blood samples were relatively larger than those for invertebrates and fish larvae found in nearby aquatic and terrestrial habitats (after estimation of MeHg isotope ratios), for which $\delta^{202}Hg_{MeHg}$ ranged from –1.23 to –0.06‰, and $\Delta^{199}Hg_{MeHg}$ ranged from –0.23 to +1.17‰. Notably, we did not observe any bird blood samples with negative mass-independent fractionation values such as we observed in macroinvertebrates from site C, but one of the bird samples (bird #15, common yellowthroat) had a mass-independent fractionation value of nearly 0 (+0.06 ‰); this bird was caught at site C, which was the location where macroinvertebrates had shown slightly negative $\Delta^{199}Hg_{MeHg}$ values (Table 1 and Figure 3).

We found that the slope of $\Delta^{199}Hg/\delta^{202}Hg$ among bird blood samples (shown as the red solid line in Figure 4) was significantly different (p = 0.0014; and the 95% confidence intervals are non-overlapping) from the slope associated with invertebrates and fish collected from the 3 aquatic habitats and the upland forest (shown as the blue dashed line in Figure 4), revealing some unexplained offset of MeHg isotope values between bird blood and their potential prey in the study habitats. The difference between these 2 slopes is much larger for low mass-dependent fractionation and mass-independent fractionation values, while the difference is nonexistent when both mass-dependent fractionation and mass-independent fractionation values are higher (Figure 4).

We attempted to correlate the isotopic compositions (both mass-dependent fractionation and mass-independent fractionation) of Hg in bird blood with those of habitat-averaged MeHg

from non-bird biota samples at the bird sampling locations (sites A-B, C, and D; Figure 5). We found no significant correlations ($p > 0.05$) in δ^{202}Hg (mass-dependent fractionation) between bird blood and biota samples, but interestingly we found a significant correlation ($p < 0.05$) in Δ^{199}Hg (mass-independent fractionation) between bird blood and biota samples (Figure 5), suggesting that mass-independent fractionation could be a more reliable tracer of habitat MeHg sources in bird blood: mass-independent fractionation appears to be the more powerful tracer because feeding and metabolic activities do not cause mass-independent fractionation [6], and thus mass-independent fractionation values of MeHg isotopes are a better tracer than mass-dependent fractionation between bird blood and their potential prey. However, as shown in Figure 4, we did not observe that the relationships for mass-independent fractionation between bird blood and biota samples were on a 1:1 line, indicating that there are other confounding factors affecting this trend. Nevertheless, within each site, there were highly variable δ^{202}Hg and Δ^{199}Hg values in bird blood, implying that birds collected at each sampling point could have obtained MeHg from different sources (or habitats) before arriving at the sampling points (i.e., a much wider spatial scale, which has yet to be defined).

These stable Hg isotope data, along with future studies, can help us better define which songbird species are the best indicators of MeHg levels in specific types of habitat and should improve our understanding of which habitats (e.g., beaver pond impoundments) are the major sources of MeHg in complex terrestrial/aquatic habitats such as Acadia National Park. Further studies and more extensive sampling (e.g., larger sample size) will be required to better understand these complexities.

Potential explanations for the variations of mass-dependent fractionation in songbird blood

In areas with many habitat interfaces such as Acadia National Park, we found that birds caught locally often did not have blood Hg isotope values representing local sources (prey) of MeHg, emphasizing the point that bird studies on contaminant availability need to consider the spatial extent of animal movements, the heterogeneity of landscapes/habitats, and the integration and uptake of contaminants along flight paths. Also, there could be large intraspecific and interspecific differences in these variations that need to be taken into account. Other factors that may cause mass-dependent fractionation of Hg isotopic differences could be related to physiological stressors such as nutritional stress [28,29]. Also, tissue-specific isotopic compositions of Hg should be fully examined, rather than exclusively blood, as in the present study. A previous study found that organs in birds could have very different turnover rates of carbon isotopes, following the order: liver > blood > muscle > bone collagen [30], with blood displaying relatively quick turnover, implying that the history reflected in blood Hg is relatively short term (e.g., within 10–20 d), whereas other organs such as feathers may contain Hg acquired over longer time periods [3].

Because of its extensive biomagnification, MeHg is a health concern for high-trophic-level wildlife including birds, reptiles, and mammals [1]. Noninvasive sampling is often used to collect samples from these animals—including blood and feathers from songbirds [14], blood and toenails from turtles [31], fur from mink and river otter [32], and blood, urine, and hair from humans [33]. It has been reported that there can be significant mass-dependent fractionation for stable isotopes of MeHg that occurs once prey is assimilated. For example, Laffont et al. [34] found that Hg in hair samples of native people in the Bolivian Amazon who subsist on fish is enriched by +2.0‰ in δ^{202}Hg relative to the fish they consume, and Sherman et al. [35] and Li et al. [36] found similar results in North American fish eaters. These studies indicate that there is

internal fractionation of Hg isotopes in humans, and this same phenomenon has also been observed in seabirds off the coast of the northeastern United States [27]. Other studies of Hg isotopes in wildlife have documented 1 to 2‰ higher δ^{202}Hg values in bird eggs and in the hair of seals and whales compared with their respective diets [37,38], and this pattern has been attributed to kinetic fractionation of δ^{202}Hg via internal demethylation of MeHg. Exposure of MeHg in wildlife occurs through diet, and it is rapidly distributed in the blood to target organs, including liver, brain, kidney, and muscle, where it accumulates over time.

Previous bird studies (that did not use Hg isotopes) demonstrated substantial demethylation of MeHg in the livers of birds [39], which could lead to mass-dependent fractionation of Hg isotopes [27]. Overall, blood is an excellent predictor of Hg concentrations in internal tissues, but in contrast, feathers are relatively poor indicators [40]. Blood distributes MeHg throughout the body tissues and represents a dynamic equilibrium between dietary Hg assimilation and tissue Hg redistribution [41]. The timing of feather production and organ growth [40] may potentially cause variability of Hg isotope values in the blood in addition to the different diets (and MeHg isotope ratios) consumed by the individual birds.

CHALLENGES AND RESEARCH NEEDS

The present pilot study has demonstrated that there is a relatively weak match in δ^{202}Hg and Δ^{199}Hg values of MeHg between bird blood and their potential prey (whole-body values) at the time of capture in the present study at Acadia National Park (Figures 4 and 5). The weak match may be the result of a variety of factors, including internal fractionation of MeHg isotopes (for δ^{202}Hg) within the bird body, or it could indicate some external MeHg assimilated by the birds that is acquired from habitats outside the current sampling area (e.g., from sites they migrated from). Internal fractionation and migratory movements may both contribute to some of

the mismatch in Hg isotope compositions between bird blood and their potential prey, and will require further investigation.

Despite these challenges, we have shown that our approach of using stable Hg isotopes appears to be feasible for investigation of the sources of MeHg in songbird species, and blood appears to be an appropriate tissue for this investigation. The dynamic nature of MeHg exposure in these animals implies that a larger scale of sampling over longer periods will be necessary to better evaluate this isotopic method for assessing MeHg bioaccumulation in songbirds, and perhaps other avian species and wildlife.

Many questions remain regarding the use of stable Hg isotope analyses for elucidating the sources of MeHg in songbird species inhabiting complex landscapes such as our study sites at Acadia National Park. We suggest that future studies examine 4 aspects of Hg isotope systematics in birds: 1) changes in isotopic values of individual birds through time and space, 2) internal redistribution and demethylation within individual birds, 3) Hg transfer to eggs and accompanying fractionation, and 4) Hg transfer to feathers and accompanying fractionation.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at DOI: 10.1002/etc.3941

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Data availability—Most of the data in this manuscript are reported in the manuscript and/or Supplemental Data.

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Figure 1. Locations of sampling sites in the Acadia National Park. Triangle denotes non-bird biota sampling and star denotes bird sampling at Aunt Betty Pond (site A, without bird collection), Marsh (site B, without bird collection), Beaver Pond (site C, with bird collection), and Forest (site D, with bird collection). Bird sampling was performed at 2 locations between site A and site B, and is denoted as a single site A-B.

Figure 2. Box plot (including outliers) of total mercury (Hg) data in all bird blood samples (n = 49; note that only 20 samples were analyzed for Hg isotopes) among the 3 sampling sites. Means for a treatment are not significantly different ($p > 0.05$) if they bear the same letter.

Figure 3. Stable mercury (Hg) isotope compositions in invertebrates and fish among the 3 interconnected aquatic habitats and upland forest (after estimation of methylmercury [MeHg] isotope ratios; site A: Aunt Betty Pond; site B: Marsh; Site C: Beaver Pond impoundments; site D: Upland Forest), and inorganic Hg in foliage collected at site D. Dashed lines represent photodemethylation effects from a controlled experiment conducted at dissolved organic carbon levels of 1 and 10 mg C/L (the latter having the steeper slope; based on Bergquist and Blum [25]). Error bars represent external analytical reproducibility (2 standard deviation). MDF = mass-dependent fractionation; MIF = mass-independent fractionation.

Figure 4. Stable mercury (Hg) isotope compositions of bird blood (assumed to be 100% as methylmercury [MeHg]), and the regression lines for both the plotted bird blood data and non-bird data plotted in Figure 3. Note that we excluded one data point from the non-bird data (i.e.,

leech from site A), because its mass-dependent fractionation value is a marginal outlier (through Thompson Tau test). Bird ID is listed in Table 2. Error bars represent external analytical reproducibility (2 standard deviation). MDF = mass-dependent fractionation; MIF = mass-independent fractionation.

Figure 5. Relationship between bird blood mercury (Hg) isotope data and those of mean Hg isotope data in each habitat where bird samples were collected (site A-B, site C, site D). (**A**) Mass-dependent fractionation (MDF). (**B**) Mass-independent fractionation (MIF). Data are denoted as bird species; CY = common yellowthroat; HT = hermit thrush; BV = blue-headed vireo; RV = red-eyed vireo. Note that all non-bird data at sites A and B are included for comparing bird blood samples from site A-B. Regression line is shown as red solid line. Dashed shaded line indicates 1:1 relationship.

<<ENOTE>>AQ1: Is the sense of the sentence OK as edited? Added word "values".

Table 1. Data on composite foliage, invertebrates, and fish larvae samples collected in summer 2012 from 4 study habitats in Acadia National Park[a]

te D	Sample common name	Total Hg (ng/g dry wt)	MeHg (ng/g dry wt)	%MeHg	δ^{202}Hg(‰) [MDF]	Δ^{199}Hg(‰) [MIF]	$\delta^{202}Hg_{MeHg}$ (‰) [MDF]	$\Delta^{199}Hg_{MeH}$ (‰) [MIF
	Leech	133	95.0	72	–0.55 (0.08)	+0.47 (0.07)	–0.06	+0.55
	Water beetles	176	137	78	–1.21 (0.08)	+0.48 (0.07)	–1.04	+0.54
	Damselfly adults	184	158	86	–0.84 (0.14)	+0.81 (0.09)	–0.67	+0.85

Dragonfly adults	236	241	100	–0.52 (0.08)	+0.76 (0.07)	–0.52	+0.76
Fish larvae	660	620	94	–0.84 (0.08)	+0.86 (0.07)	–0.78	+0.88
Leech	39.2	16.4	42	nd	nd		
Hemiptera	102	81.6	80	nd	nd		
Damselfly adults	123	97.6	79	nd	nd		
Water beetles	137	116	85	–1.02 (0.08)	+0.49 (0.07)	–0.88	+0.53
Dragonfly larvae	172	168	98	–0.71 (0.08)	+0.34 (0.07)	–0.68	+0.35
Megaloptera larvae	210	170	81	nd	nd		
Fish larvae	260	254	98	–0.50 (0.08)	+1.17 (0.07)	–0.46	+1.17
Dragonfly adults	334	282	84	–0.92 (0.08)	+0.34 (0.07)	–0.76	+0.38
Megaloptera adults	406	391	96	–0.88 (0.08)	+0.50 (0.07)	–0.85	+0.51
Damselfly adults	469	449	96	–1.20 (0.29)	+0.09 (0.21)	–1.17	+0.10
Amphipod	474	307	65	–1.22 (0.08)	–0.23 (0.07)	–0.91	–0.11
Megaloptera larvae	582	472	81	–1.34 (0.08)	–0.28 (0.07)	–1.23	–0.23
Dragonfly larvae	950	785	83	–1.32 (0.08)	–0.20 (0.07)	–1.22	–0.15
Birch foliage[b]	18.0	0.29	2	–2.39 (0.14)	–0.15 (0.09)		
Cedar foliage[b]	26.7	0.21	1	–2.14 (0.14)	–0.34 (0.09)		
Ground beetles	50.3	40.8	81	–0.75 (0.29)	+0.87 (0.21)	–0.46	+0.95
Slug[b]	116	9.8	8	nd	nd		
Earthworm[b]	142	9.1	6	–1.62 (0.14)	–0.28 (0.09)		
Spiders (mixed)	341	158	46	–0.26 (0.14)	+0.43 (0.09)		

[a] The site, common name, total mercury (Hg), methylmercury (MeHg), %MeHg, mass-dependent fractionation before ($\delta^{202}Hg$) and after estimation of values for MeHg ($\delta^{202}Hg_{MeHg}$),

and mass-independent fractionation before ($\Delta^{199}Hg$) and after estimation for MeHg ($\Delta^{199}Hg_{MeHg}$). Values in parentheses represent external analytical reproducibility (2 standard deviation) associated with isotopic measurements; the values were based on isotopic measurements at different final Hg concentrations [14].

[b] Not included in statistical analyses comparing MeHg contents among habitats.

MDF = mass-dependent fractionation; MIF = mass-independent fractionation; nd = not determined.

Table 2. Individual bird blood data[a]

ID	Site	Species	Sex	Age	Total Hg (ng/g wet wt)	$\delta^{202}Hg$ (‰) [MDF]	$\Delta^{199}Hg$ (‰) [MIF]
1	A-B	Hermit thrush	M	SY	153	–0.88 (0.29)	+0.88 (0.21)
2	A-B	Hermit thrush	M	AHY	357	–1.00 (0.14)	+0.78 (0.09)
3	A-B	Hermit thrush	M	AHY	174	–0.34 (0.14)	+1.30 (0.09)
4	A-B	Hermit thrush	M	ASY	343	–0.34 (0.14)	+1.09 (0.09)
5	A-B	Hermit thrush	M	AHY	225	–0.08 (0.14)	+1.31 (0.09)
6	A-B	Common yellowthroat	M	ASY	335	–1.23 (0.14)	+0.60 (0.09)
7	A-B	Common yellowthroat	M	ASY	438	–1.11 (0.14)	+0.43 (0.09)
8	A-B	Common yellowthroat	M	ASY	337	–1.10 (0.29)	+0.44 (0.21)
9	A-B	Blue-headed vireo	F	AHY	344	–0.36 (0.08)	+1.12 (0.07)
10	A-B	Blue-headed vireo	F	AHY	540	–0.34 (0.14)	+1.05 (0.09)
11	A-B	Blue-headed vireo	U	AHY	493	–0.84 (0.08)	+0.83 (0.07)

12	C	Red-eyed vireo	M	AHY	370	–1.43 (0.14)	+0.55 (0.09)
13	C	Red-eyed vireo	M	AHY	337	–1.21 (0.29)	+0.68 (0.21)
14	C	Red-eyed vireo	M	AHY	426	–0.73 (0.08)	+0.73 (0.07)
15	C	Common yellowthroat	F	ASY	880	–1.56 (0.08)	+0.06 (0.07)
16	C	Hermit thrush	M	SY	331	–1.95 (0.14)	+0.48 (0.09)
17	C	Hermit thrush	M	ASY	220	–0.45 (0.14)	+0.85 (0.09)
18	D	Red-eyed vireo	F	AHY	355	–0.39 (0.14)	+1.01 (0.09)
19	D	Hermit thrush	M	SY	213	–0.47 (0.29)	+1.26 (0.21)
20	D	Hermit thrush	F	ASY	133	–1.62 (0.29)	+0.81 (0.21)

[a] Site of collection, species, sex, age, total mercury (Hg) concentrations in blood, mass-dependent fractionation (δ^{202}Hg), and mass-independent fractionation (Δ^{199}Hg). Values in parentheses represent external analytical reproducibility (2 standard deviation) associated with isotopic measurements; the values were based on isotopic measurements at different final Hg concentrations [14]. For samples only analyzed for total Hg in blood, refer to the Supplemental Data, Table S1.

MDF = mass-dependent fractionation; MIF = mass-independent fractionation; U = unidentified as to male or female; SY = second year; AHY = after hatching year; ASY = after second year.

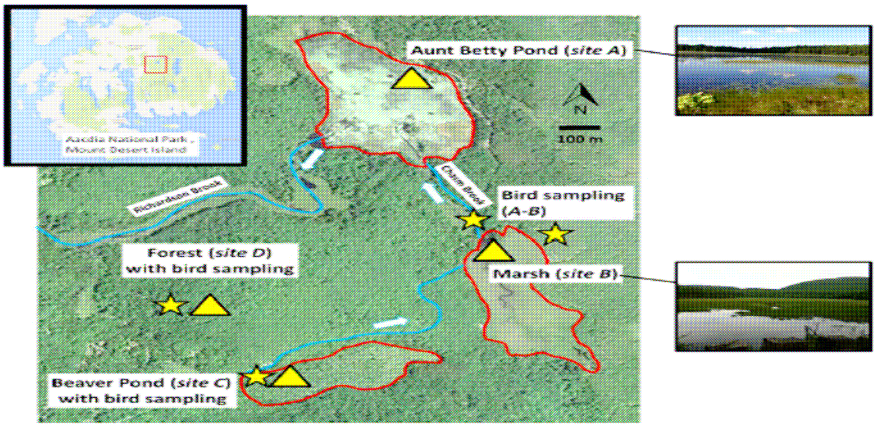

Fig. 1

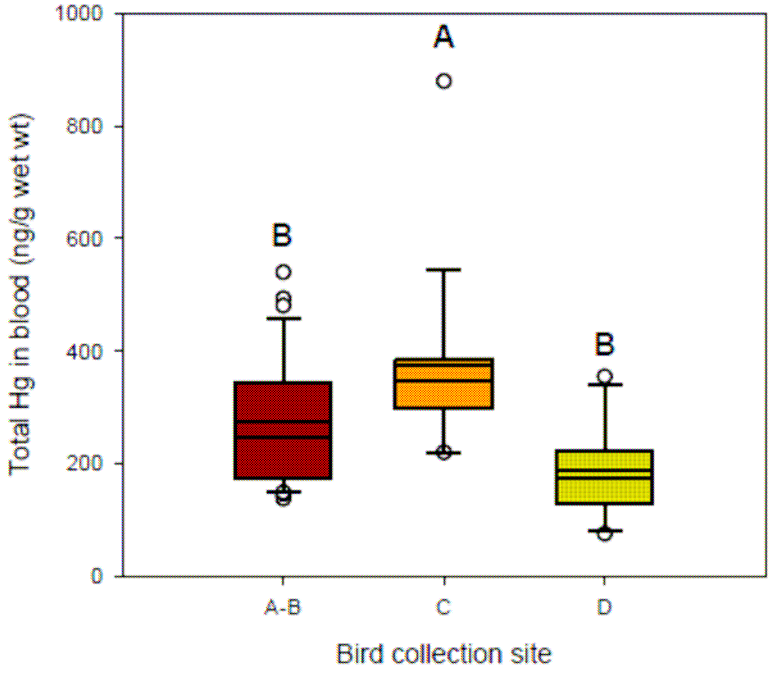
1000
800
600
400
200
0
Total Hg in blood (ng/g wet wt)
A
B
B
A-B
C
D
Bird collection site

Fig. 2

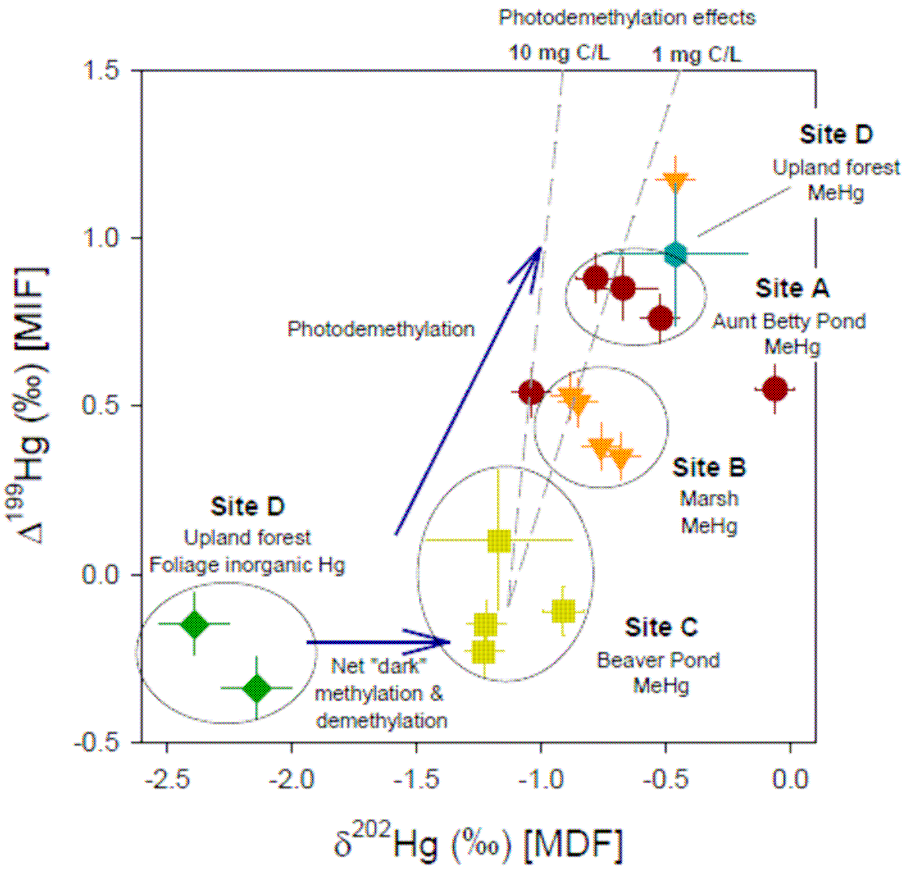
Photodemethylation effects
10 mg C/L
1 mg C/L
Site D
Upland forest
MeHg
Site A
Aunt Betty Pond
MeHg
Photodemethylation
Site B
Marsh
MeHg
Site D
Upland forest
Foliage inorganic Hg
Site C
Beaver Pond
MeHg
Net "dark"
methylation &
demethylation
1.5
1.0
0.5
0.0
-0.5
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
Δ199Hg (‰) [MIF]
δ202Hg (‰) [MDF]

Fig. 3

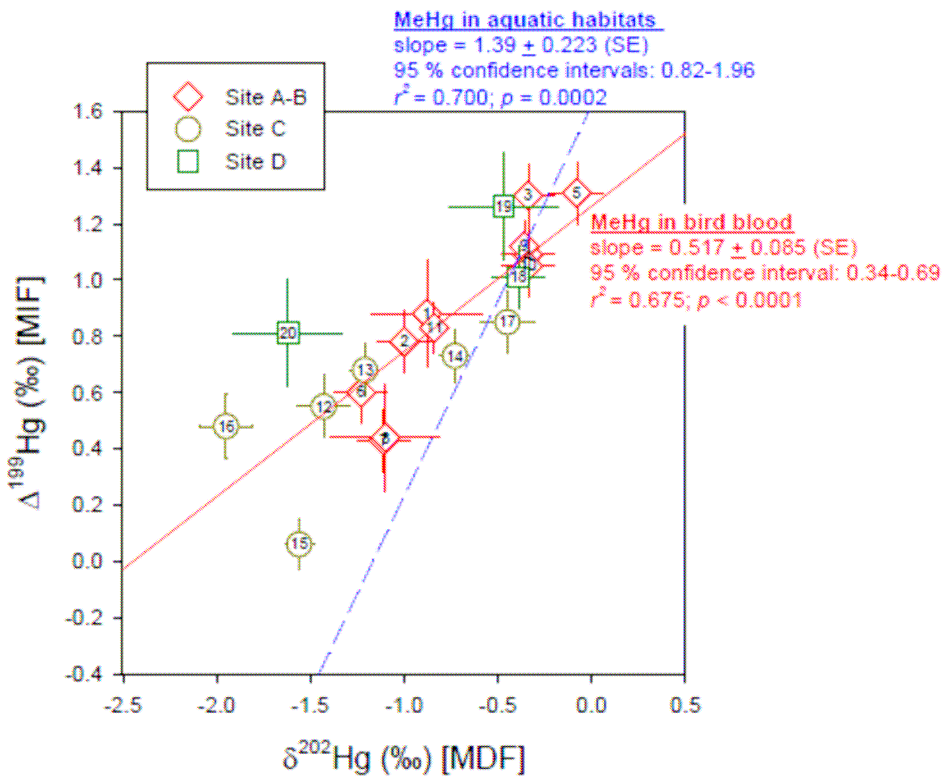
MeHg in aquatic habitats
slope = 1.39 ± 0.223 (SE)
95 % confidence intervals: 0.82-1.96
r² = 0.700; p = 0.0002
Site A-B
Site C
Site D
MeHg in bird blood
slope = 0.517 ± 0.085 (SE)
95 % confidence interval: 0.34-0.69
r² = 0.675; p < 0.0001
Δ199Hg (‰) [MIF]
δ202Hg (‰) [MDF]
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-2.5
-2.0
-1.5
-1.0
-0.5
0.0
0.5

Fig. 4

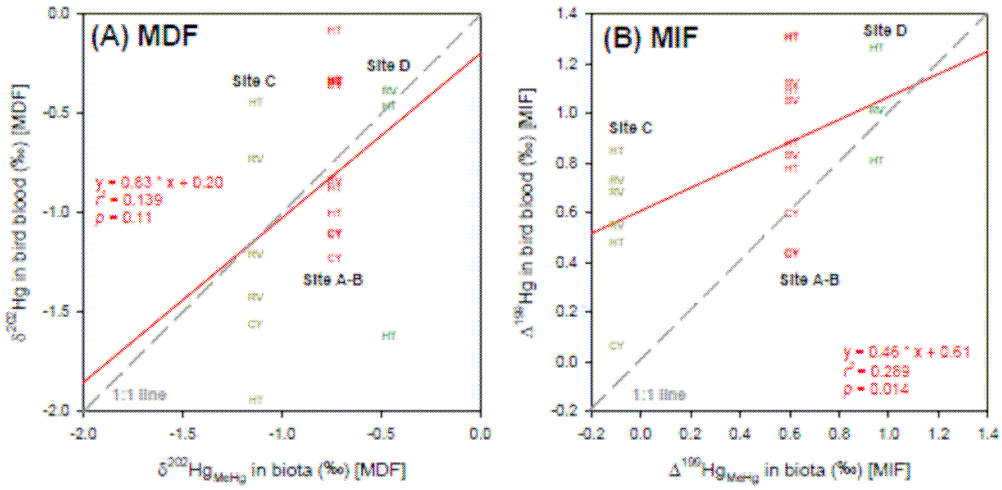
(A) MDF
Site C
Site D
Site A-B
y = 0.63 * x + 0.20
r² = 0.139
p = 0.11
1:1 line
δ202Hg in bird blood (‰) [MDF]
δ202HgMeHg in biota (‰) [MDF]
(B) MIF
Site C
Site D
Site A-B
y = 0.46 * x + 0.61
r² = 0.269
p = 0.014
1:1 line
Δ199Hg in bird blood (‰) [MIF]
Δ199HgMeHg in biota (‰) [MIF]

Fig. 5