Songbird feathers as indicators of mercury exposure: patterns across feather tracts and correlations to other tissues

by
Katherine E. Low

A THESIS

submitted to

Oregon State University

Honors College

in partial fulfillment of
the requirements for the
degree of

Honors Baccalaureate of Science in Zoology
(Honors Scholar)

Honors Baccalaureate of Science in Fisheries and Wildlife Science
(Honors Scholar)

Presented May 31, 2016
Commencement June 2016

AN ABSTRACT OF THE THESIS OF

Katherine E. Low for the degree of Honors Baccalaureate of Science in Zoology and Honors Baccalaureate of Science in Fisheries and Wildlife Science presented on May 31, 2016.
Title: Songbird feathers as indicators of mercury exposure: patterns across feather tracts and correlations to other tissues.

Abstract approved: _____

W. Douglas Robinson

Monitoring mercury (Hg) exposure in avian populations is critical to understanding the effects of this neurotoxin. Avian Hg exposure is commonly evaluated by measuring Hg concentrations in internal tissues, blood, and feathers. Feathers are a popular sampling matrix due to ease of sampling and limited stress to birds. However, it remains unclear if feather Hg is representative of the Hg load in the body, which is more relevant to toxicological evaluations. Furthermore, it is unclear which feathers should be sampled, given that Hg sequestration patterns across feather tracts are poorly understood. To better understand these patterns, we tested variation in Hg concentration across five feather tracts (crown, left breast, belly, back, left flank) in 37 salvaged songbird specimens in the Thrush (N=22) and Sparrow (N=15) families. We then compared feather Hg concentrations to those of internal tissues in the same birds, to test the relationship between feather and body Hg load. Our results indicate no statistical difference in Hg concentrations across feather tracts, but a high degree of intra-individual variability. Results also suggest a high correlation between liver and muscle Hg concentrations, but weak Hg correlations between internal tissues and feathers. Based on these results, we

concluded that feathers from any of the five tracts would yield similar estimates of Hg exposure, however we recommend using a composite of various body feathers. Hg sequestration into feathers may be influenced by factors such as species and feather size, although further research into the effects of these factors is needed. Weak relationships between feather Hg and internal tissue Hg suggest that feather sampling may not be reliable for monitoring fine-scale trends of Hg exposure in songbirds. However, we show a stronger Hg correlation among internal tissues and another keratinaceous structure – toe nails which, unlike feathers, grow continuously. Further investigation into the viability of toe nails as a Hg sampling method is needed.

Key Words: mercury, songbirds, feathers, nails

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Honors Baccalaureate of Science in Zoology and Honors Baccalaureate of Science in Fisheries and Wildlife Science project of Katherine E. Low presented on May 31, 2016.

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I understand that my project will become part of the permanent collection of Oregon State University, Honors College. My signature below authorizes release of my project to any reader upon request.

Katherine E. Low, Author

Acknowledgements

I would like to thank the many people that were involved with this project, for without them, completion of my thesis would not have been possible. First, to my mentor, Dr. Doug Robinson, for taking time out of his busy schedule to guide me through this process. I thank him both for his advice and for his encouragement. Second, to my committee member, Allyson Jackson, for sparking my interest in mercury, for all of her hard work helping with study design, data collection, and data analysis, and for her unbridled support. Third, to my committee member, Dr. Don Lyons, for encouraging me to think critically about the scientific process as a whole and for his interest in and enthusiasm for this project. I would also like to thank Danielle Ramsden, for spending hours upon hours discussing results and working through 'the cloud' with me. I also thank Dr. Collin Eagles-Smith for providing access to all of the laboratory equipment used and for lending his expertise in data collection and analysis. Additionally, I would like to thank Colleen Emery, for all of her work in the early stages of this project and in feather processing, which has allowed me easy access to feather samples. I am also grateful to everyone who donated bird specimens for use in this project. Finally, I would like to thank all of the members of the Contaminant Ecology Research Lab for helping me along the way and for making lab work and data collection exponentially more entertaining.

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Introduction

Mercury (Hg) is a widespread environmental toxin that can be harmful to the overall health and reproductive viability of wildlife. This heavy metal naturally exists within the earth's crust and is released slowly into the atmosphere through natural processes such as flux from soil and outgassing from rocks (Broussard et al. 2002, Morel et al. 1998). Rapid, but temporary, influxes in atmospheric Hg naturally occur through events such as volcanic eruptions and forest fires (Morel et al. 1998). Over the last 150 years, atmospheric Hg levels have been rising steadily, and are now three times what they were in pre-industrial times. This sustained increase is due primarily to anthropogenic Hg emissions, mainly from waste incinerators, mining and pulp operations, and coal-burning power plants (Morel et al. 1998, Clarkson et al. 2003).

Whether from natural or anthropogenic sources, elemental Hg is highly volatile and remains in the atmosphere for a year, on average, during which time it can be distributed worldwide (Clarkson et al. 2003). While in the atmosphere, elemental mercury vapor (Hg0) can be oxidized into a soluble form of mercury, Hg(II), which then is returned to the earth via precipitation. In certain conditions, Hg(II) can be methylated to form methyl mercury, MeHg (Morel et al. 1998). Methylation is facilitated by microorganisms and primarily occurs in anoxic, aquatic conditions with high levels of dissolved organic matter and low pH (Furness 2010). Most MeHg is thought to be produced by sulfate-reducing (Compeau and Bartha 1985, Gilmour et al. 1992) and iron-reducing bacteria living in these environments (Fleming et al. 2006, Yu et al. 2012). MeHg is removed from the environment through demethylation reactions which, like

methylation reactions, take place mostly at the sediment-water interface and are facilitated by microbes (Gilmour et al. 1992).

Unlike elemental and inorganic forms of Hg, MeHg is biologically active. MeHg can enter the food chain by forming an uncharged, lipid-soluble, complex with a chloride (Cl-) ion, which then can pass into and out of the membranes of single-celled organisms. When these organisms are consumed, the Hg within them is easily assimilated into the tissues of the predator (Morel et al. 1998). Once ingested, MeHg absorbs into the bloodstream through the gastrointestinal tract, where it mostly binds to the protein hemoglobin in red blood cells. Once bound to red blood cells, it can circulate throughout the entire body via the bloodstream, and can even cross the blood-brain barrier (Broussard et al. 2002). Prolonged high levels of dietary Hg exposure can be detrimental to the health of an organism because Hg is a neurotoxin. Hg toxicity can affect coordination, behavior, and reproductive success. Common effects of high Hg exposure in animals include ataxia, lethargy, reduced production of sex hormones, and lowered fertility (Scheuhammer et al. 2007). Additional effects to bird populations include reduced hatching success and smaller egg and clutch sizes (Seewagen 2010). The neurotoxicity of Hg is especially a concern with species high in the food chain because MeHg biomagnifies (Morel et al. 1998).

Hg monitoring is paramount to understanding the health risks associated with Hg and to guiding policy for reducing anthropogenic Hg emissions. Animals are often used to study Hg contamination because their tissues can be easily sampled and they can provide clues to spatial trends in Hg deposition across the landscape. Furthermore, animal specimens from museums can be sampled and used to estimate historical levels of

environmental Hg, which would otherwise be difficult to measure, due to a constantly changing environment (Berg et al. 1966, Monteiro and Furness 1997). Birds in particular are useful indicators of Hg pollution because they are: (1) abundant, (2) widely distributed, (3) representative of various trophic levels, (4) and long-lived, meaning they can accumulate Hg over long periods of time (Rothschild and Duffy 2005). Although songbirds make up the majority of bird species, they often are overlooked in Hg studies, which typically have been focused on aquatic species or species that eat fish. Since most MeHg is produced in aquatic ecosystems, it was thought that only species living in these environments accumulated high levels of Hg. But recent work suggests that significant methylation may occur in terrestrial ecosystems and that MeHg can accumulate in terrestrial species, such as songbirds (Rimmer et al. 2005, Cristol et al. 2008).

Hg exposure in birds often is evaluated by measuring Hg concentrations in internal tissues, blood, or feathers. Commonly used internal tissue matrices include liver, kidney, muscle, and brain (Evers et al. 2005). Hg concentrations in the liver, in particular, correlate well to concentrations in a variety of body tissues, making liver a good predictor of Hg levels in these tissues (Gochfield 1980). The accuracy of Hg measurements in other tissues can be evaluated by comparing the Hg level in said tissue to that of the liver. Although internal tissues are most relevant to toxicological evaluations, obtaining these tissues is lethal, making them a poor sampling option for species of conservation concern. Popular nonlethal sampling methods include feather sampling and, more recently, blood sampling.

Hg concentrations in blood have been shown to relate strongly to Hg concentrations in liver and other internal tissues, suggesting that blood provides an

accurate estimate of Hg exposure (Eagles-Smith et al. 2008). Blood is particularly useful for providing information about short-term Hg exposure, as it reflects recent dietary Hg intake (Evers et al. 2005, Cristol et al. 2008). However, blood sampling still requires bird capture and handling, which can cause undue stress to birds, considering that the associated energetic costs are poorly understood. Although Hoysak and Weatherhead (1991) found that blood sampling in Brown-headed Cowbirds (*Molothrus ater*) and Red-winged Blackbirds (*Agelaius phoeniceus*) had no obvious adverse effects to reproductive success, they acknowledge that this might not be the case for all species.

Feathers offer an ideal sampling matrix because they are easy to obtain and are resistant to decay. Because feathers are inert when fully grown, feather sampling reduces both stress to birds, and risks to researchers, when compared to blood or internal tissue sampling (Bortolotti 2010). Because Hg is incorporated into feathers endogenously, its concentrations do not change significantly after feather formation is complete, even when the feather is exposed to various treatments, such as freezing (Goede 1984, Appelquist 1984). This stability allows for simple and inexpensive storage and the ability to sample from museum specimens (Monteiro and Furness 1997, Berg et al. 1966). However, relationships between Hg concentrations in feathers and internal tissues or blood are inconsistent across species (Evers et al. 1998, Hartman et al. 2013, Rimmer et al. 2005, Kahle & Becker 1999, Caldwell et al. 1999, Eagles-Smith et al. 2008, Thompson et al. 1990), leading some to doubt the accuracy of feather Hg measurements. These inconsistencies could be a result of poor correlations between feather and internal tissue Hg concentrations, or they could be a result of variability in the feathers used for analysis. Some findings suggest variation in Hg concentrations within and among feather

tracts (Lewis & Furness 1991, Honda 1986, Bortolotti 2010, Dauwe et al. 2003, Solonen & Lodenius 1990), yet there is little agreement about which feather type most accurately depicts body Hg levels, making comparisons and generalizations across studies difficult.

The purpose of this study is two-fold. First, we aim to determine if Hg concentrations in songbird body feathers correlate to Hg concentrations in internal tissues of the same birds, to determine if feathers can be used to accurately measure Hg exposure. Second, we will decipher patterns in Hg sequestration across body feather tracts to determine which of these feathers, if any, better correlate to internal tissues and, therefore, are more accurate for Hg monitoring.

Methods

Study Specimens

A total of 37 songbird specimens were donated by Chintimini Wildlife Center and by local ornithologists. All specimens died from natural causes or accidents, such as window collisions. No birds were killed for the purpose of this study. Specimens were salvaged under U.S. Fish and Wildlife Service Federal Permit #MB28361A-4 (Dr. Collin Eagles-Smith, principal permittee).

Specimens represented nine species in the Thrush (N=22) and Sparrow (N=15) families: American Robin *Turdus migratorius* (N=7), Hermit Thrush *Catharus guttatus* (N=1), Swainson's Thrush *Catharus ustulatus* (N=8), Varied Thrush *Ixoreus naevius* (N=6), Song Sparrow *Melospiza melodia* (N=2), Golden-crowned Sparrow *Zonotrichia atricapilla* (N=2), Oregon Junco *Junco hyemalis* (N=5), Spotted Towhee *Pipilo maculatus* (N=2), and Fox Sparrow *Passerella iliaca* (N=4). Birds were sexed by

plumage and by identification of sex organs during dissection. Plumage also was used to age birds, following Pyle (1997). Eleven of the specimens were female, 14 were male, and 12 were of unknown sex. Nine specimens were hatch years, 21 were after hatch year, and seven were of unknown age. Specimens were kept frozen from the time collected to the time of tissue sampling.

Sampling Procedures

Feathers were plucked from five body feather tracts: crown, left breast, belly, back, and left flank. Only feathers that did not have any skin attached to them were used for analysis. All feathers were washed with a 10% Liquinox solution, scrubbed, and then thoroughly rinsed with deionized water to remove any external contamination. Feathers then were dried at 50°C for 48 hours. After feathers were washed and dried, ten were randomly selected from each of the five feather tracts. The ten feathers from each tract were measured for length to the nearest 0.01 mm using electronic calipers (Fisher Scientific, Pittsburgh, PA). These ten feathers then were weighed together to the nearest 0.01 mg using an analytical scale (Mettler Toledo, model XS105, Columbus, OH) and stored in coin envelopes until analysis. The ten feathers from each tract were combined for Hg analysis to ensure that feather mass was suitable for obtaining an accurate Hg reading.

Toe nail samples were collected from each bird. Entire nails from the second, third, and fourth digits were cut at the base and removed from both feet. The hallux was excluded from analysis due to impossibility of sampling this nail in future field studies. To determine if Hg is evenly sequestered into toe nails, for a subset of samples, the left

third digit nail was analyzed for Hg independently and then re-composited with the other five nails, to find a Hg concentration for all six nails. This, however, was only possible in larger species, as nails of smaller species were not massive enough to obtain an accurate Hg reading if run individually. In smaller species, all six nails were run together. Before Hg analysis, all toe nails were washed using a 10% Liquinox solution, rinsed thoroughly with deionized water, and dried at 50°C for at least 48 hours. After drying, nails were weighed to the nearest 0.01 mg using an analytical scale (Mettler Toledo, model XS105, Columbus, OH).

Internal tissue samples were obtained through dissection. Before dissection, specimens were thawed at room temperature. The entire liver and portions of the left and right breast muscles were collected. Each sample was weighed to the nearest 0.1 mg using an analytical scale (Mettler Toledo, model ML104, Columbus, OH). Internal tissue samples then were dried in an oven set at 50°C for 48 hours or until mass remained constant. Once dried, samples were re-weighed to the nearest 0.1 mg. Dried samples then were homogenized by hand using a ceramic mortar and pestle and were stored in glass vials in a desiccator until Hg analysis.

Mercury Analysis

Samples were analyzed for total mercury (THg) using a Milestone DMA-80 Direct Mercury Analyzer (Milestone, Monroe, CT) at the U.S. Geological Survey, Forest and Rangeland Ecosystem Science Center (Corvallis, OR). Direct Mercury Analysis involves drying and decomposition of the sample, followed by catalytic reduction of Hg species to elemental Hg, which is then trapped by a gold amalgamator. The Hg is

measured when it passes through a single-beam atomic adsorption spectrum photometer after amalgamation. Certified reference materials (CRM), dogfish muscle and lobster hepatopancreas (DORM-2 and TORT-2, respectively), and liquid certified standards (LCS), along with boat blank and air blanks, were included in each batch to ensure accuracy of DMA results. Percent recovery of CRMs and LCS averaged 99.8% (standard deviation = 4.2%) and 99.6% (standard deviation = 3.5%), respectively.

Statistical Analysis

Because repeated freezing and thawing can affect moisture content of tissues, Hg concentrations were calculated using dry-weight of samples. In order to normalize data, all Hg data was log transformed before analysis. Simple linear regressions were used to examine pairwise relationships between tissues. Feathers were evaluated by individual feather tract and as a combined average of the five feather types.

We used a mixed linear-effects repeated measure model to test for differences in Hg concentrations across tissue types and feather tracts. Tissue or feather type was included as an independent categorical variable and individual specimen was included as a random effect variable. This same model was used to evaluate differences in feather size and feather density. Tukey HSD post hoc tests were used to determine differences between pairwise groups. Hg concentrations and feather size are reported in the text as geometric mean ± standard error. Back-transformed standard errors were estimated using the Delta method (Seber 1982). Statistical tests were conducted in JMP® 12 software (SAS Institute, Cary, NC.) and Minitab Express™ Software (Minitab Inc., State College, PA).

Results

Feather Size

Taking into account size differences across species (Linear mixed-effects model with individual bird as a random effect), feather tracts were found to differ in mass ($F_{4,143}$=83.0068, p<0.0001), length ($F_{4,141.1}$=305.5707, p<0.0001), and density ($F_{4,141}$=146.2741, p<0.0001) (Fig. 1). Crown feathers were consistently the shortest and the lightest feathers, while flank feathers were generally the longest and heaviest (Table 1). Back, breast, and belly feather tracts showed no significant difference in mass. However, back feathers were significantly longer than breast feathers. Neither of these feather tracts significantly differed from belly feathers in length. Crown and belly feathers were found to be significantly less dense (measured as feather mass divided by feather length) than feathers in the other three feather tracts.

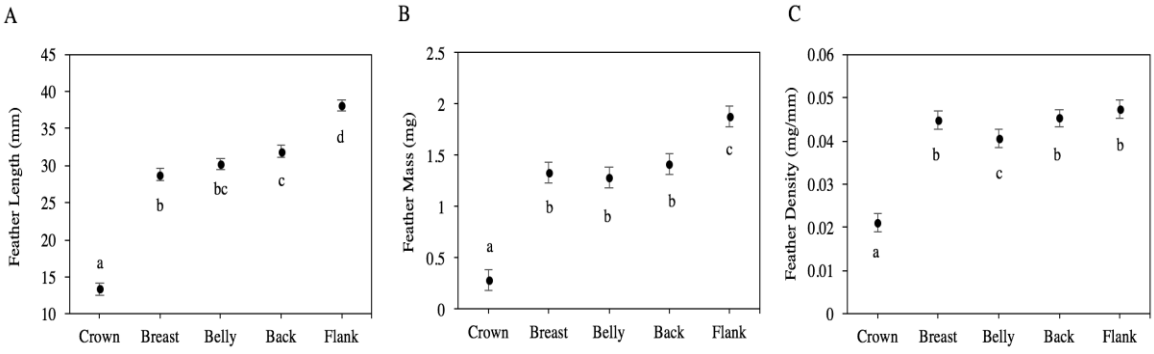

Fig. 1 Least square means of length (A), mass (B), and density (C) for each feather type, from the mixed linear-effects repeated measures model, accounting for tissue type and individual specimen effects. Error bars represent 95% confidence intervals; different letters indicate statistically significant Tukey HSD differences between pairwise groups.

Table 1. Descriptive statistics for feather mass (mg), length (mm), and density (mg/mm)

Feather Type	N	Measurement	Arithmetic Mean	Arithmetic SE	Geometric Mean	Geometric SE[a]	Min.	Max.
Crown	36	Mass	0.3018	0.0248	0.2679	0.0097	0.0830	0.5930
		Length	13.4594	0.3616	13.2920	0.1543	9.51	17.63
		Density	0.0214	0.0122	0.0202	0.0005	0.0087	0.0352
Breast	37	Mass	1.3330	0.0918	1.2092	0.0404	0.3560	2.2970
		Length	28.7840	0.6816	28.4859	0.3002	19.83	35.77
		Density	0.0448	0.0235	0.0425	0.0010	0.0180	0.0686
Belly	37	Mass	1.2789	0.0952	1.1571	0.0379	0.4560	2.5880
		Length	30.2350	0.9281	29.7167	0.4030	20.05	40.66
		Density	0.0405	0.0192	0.0389	0.0008	0.0196	0.0644
Back	35	Mass	1.4953	0.1052	1.3677	0.0440	0.4740	2.6870
		Length	31.9607	0.7728	31.6315	0.3429	23.17	39.95
		Density	0.0453	0.0235	0.0432	0.0009	0.0205	0.0719
Flank	37	Mass	1.8847	0.1475	1.6956	0.0565	0.7420	4.2550
		Length	38.1390	1.0990	37.5855	0.4631	26.30	51.89
		Density	0.0473	0.0242	0.0451	0.0010	0.0244	0.0820

[a]Calculated using the Delta method (Seber 1982)

Hg Concentrations Across Body Tissues

Our results showed no statistical difference in total Hg concentrations across body feather tracts, suggesting that feathers from any of the five tracts would give a similar estimate of Hg exposure (Linear mixed-effects model, $F_{4,141}$=1.5066, p=0.2035) (Table 2). Therefore, Hg concentrations across the five feather tracts were averaged for the remainder of the analysis, except where otherwise noted. Total Hg concentrations were found to differ between internal (liver, muscle) and keratinaceous (feather, nail) tissues ($F_{7,242.3}$=59.4830, p <0.0001) with the highest concentrations found in nails (0.5244 ± 0.0416, geometric mean ± SE) and feathers (0.5048 ± 0.0322), followed by liver (0.1640 ± 0.0162), and then by breast muscle (0.0527 ± 0.0052) (Fig. 2).

Table 2. Descriptive statistics for tissue Hg concentrations (μg g^{-1}, dw) in nine songbird species

Tissue Type	N	Arithmetic Mean	Arithmetic SE	Geometric Mean	Geometric SE[a]	Min.	Max.
Liver	34	0.3319	0.0706	0.1640	0.0162	0.0079	1.7802
Muscle	34	0.1096	0.0249	0.0527	0.0052	0.0031	0.7337
Nail	36	0.9925	0.2554	0.5244	0.0416	0.0528	8.5613
All Feathers	37	0.7861	0.1595	0.5048	0.0322	0.4148	5.0452
Crown Feather	36	0.7277	0.1532	0.4666	0.0309	0.0503	5.0245
Breast Feather	37	0.8184	0.1628	0.5198	0.0340	0.0672	4.7516
Belly Feather	37	0.7849	0.1693	0.4716	0.0329	0.0697	5.3286
Back Feather	35	0.8224	0.1770	0.5125	0.0345	0.0918	5.2916
Flank Feather	37	0.7995	0.1649	0.5005	0.0332	0.1155	4.8295

[a]Calculated using the Delta method (Seber 1982)

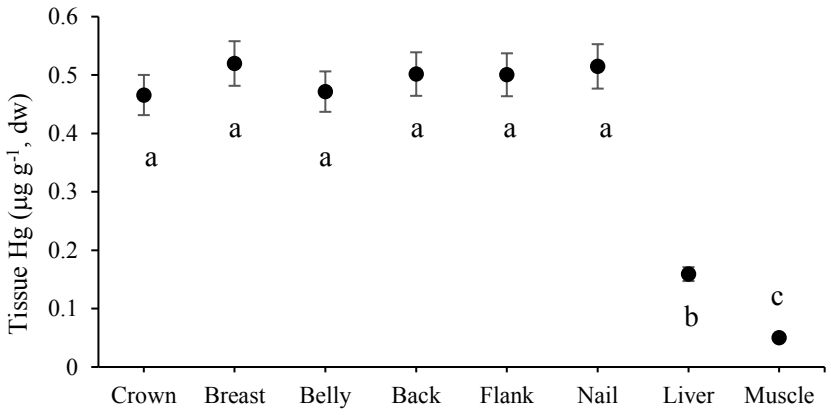

Fig. 2 Least square means of Hg concentrations (μg g^{-1}, dw) in various tissues in songbirds [back transformed μg g^{-1} dry weight (dw) from the mixed linear-effects repeated measures model, accounting for tissue type and individual specimen effects]. Error bars represent 95% confidence intervals; different letters indicate statistically significant Tukey HSD differences between pairwise groups.

Correlations Among Tissues

Pairwise relationships between each tissue type were evaluated using simple linear regressions (Table 3). All combinations showed statistically significant

correlations, however, the strength of the relationship was highly variable across tissues (Fig. 3). The strongest relationship occurred between Hg concentrations in liver and breast muscle (R^2=0.85, p<0.0001). The weakest relationships all involved feathers, with the poorest correlation occurring between Hg concentrations in feathers and breast muscle (R^2=0.13, p= 0.0351), followed by those in feathers and liver (R^2=0.24, p=0.0030), and then those in feathers and toe nails (R^2=0.31, p=0.0004). Compared to feather Hg concentrations, toe nail Hg concentrations better correlate to both muscle (R^2=0.57, p<0.0001) and liver (R^2=0.59, p<0.0001).

With all species combined, Hg concentrations in crown feathers exhibited the weakest correlations to Hg concentrations in other tissues, followed by breast feathers, then belly feathers (Table 3). Flank feather Hg concentrations best correlated to muscle Hg, while back feather Hg concentrations best correlated to Hg concentrations in the liver and nails.

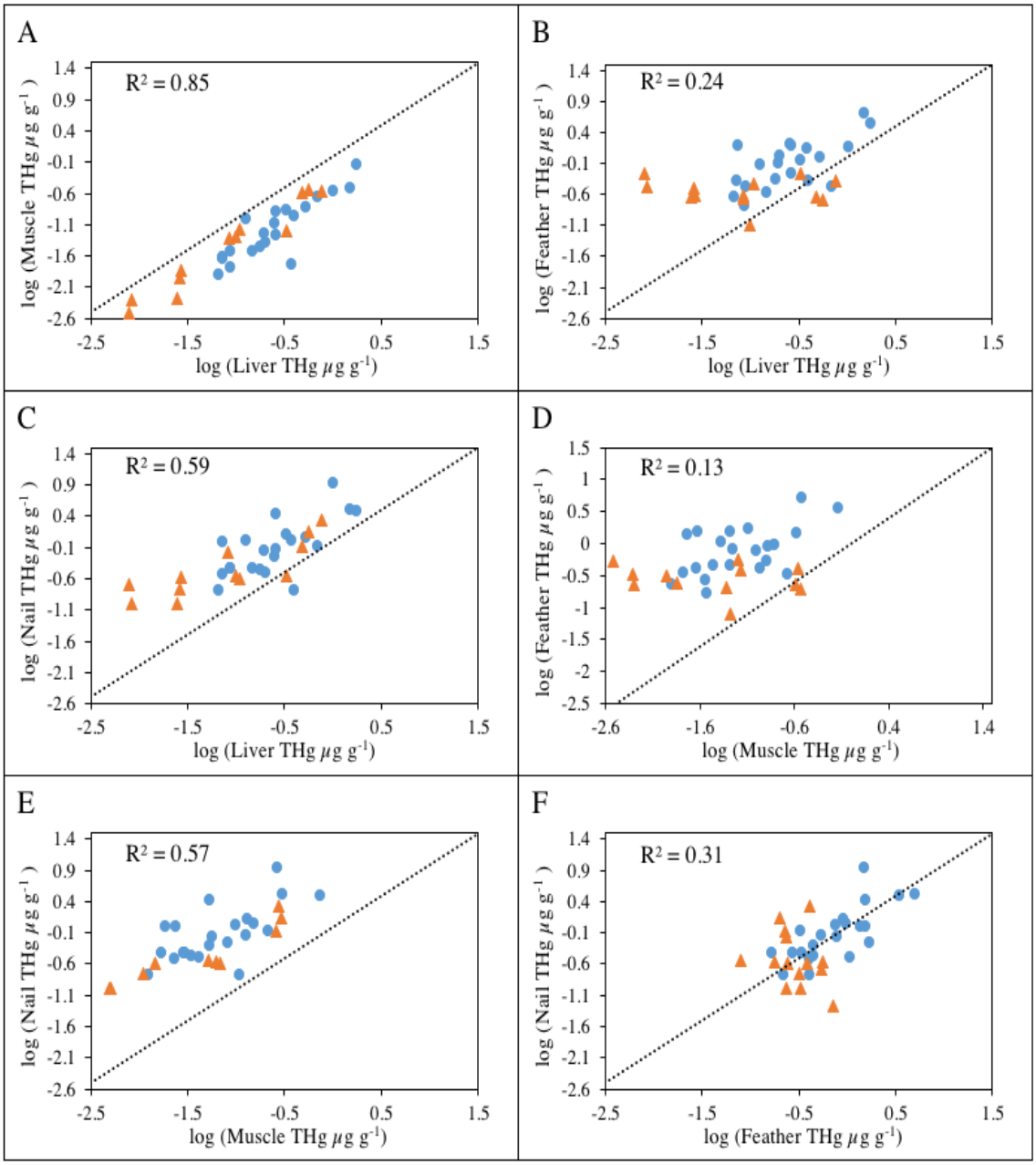

Fig. 3 Pairwise comparisons of Total Hg (THg) concentrations (measured as μg g^{-1}) in liver and muscle (A), liver and feather (B), liver and toe nail (C), muscle and feather (D), muscle and toe nail (E), and feather and toe nail (F). Blue circles represent members of the Thrush family, orange triangles represent members of the Sparrow family. Feather μg g^{-1} is an average across the five body feather tracts. Dashed line represents 1:1 relationship.

Table 3. Regression equations for pairwise relationships between THg concentrations (μg g^{-1}, dw) in various body tissues[a]

	Liver THg		Muscle THg		Nail THg	
Family	log y = b + log x	R^2	log y = b + log x	R^2	log y = b + log x	R^2
All Feather THg						
All Birds	log y = -0.0195 + 0.3398(log x)	0.24*	log y = 0.0403 + 0.2492(log x)	0.13*	log y = -0.15814 + 0.4573(log x)	0.31*
Thrushes	log y = 0.2369 + 0.5931(log x)	0.42*	log y = 0.4118 + 0.4569(log x)	0.31*	log y = -0.04385 + 0.6544(log x)	0.55*
Sparrows	log y = -0.6022 - 0.03609(log x)	0.01	log y = -0.6523 - 0.06497(log x)	0.04	log y = -0.6146 - 0.1452(log x)	0.07
Crown Feather THg						
All Birds	log y = -0.0899 + 0.2915(log x)	0.17*	log y = -0.0462 + 0.2064(log x)	0.08	log y = -0.21008 + 0.3936(log x)	0.22*
Thrushes	log y = 0.1602 + 0.5439(log x)	0.34*	log y = 0.3294 + 0.4199(log x)	0.26*	log y = -0.09809 + 0.5530(log x)	0.39*
Sparrows	log y = -0.6498 - 0.0724(log x)	0.03	log y = -0.7227 - 0.1060(log x)	0.07	log y = -0.6371 - 0.1542(log x)	0.05
Breast Feather THg						
All Birds	log y = -0.0164 +0.3313(log x)	0.22*	log y = 0.0397 + 0.2416(log x)	0.11*	log y = -0.14668 + 0.4405(log x)	0.28*
Thrushes	log y = 0.2349 +0.5867(log x)	0.38*	log y = 0.4135 + 0.4581(log x)	0.29*	log y = -0.04412 + 0.6505(log x)	0.51*
Sparrows	log y = -0.5766 - 0.0335(log x)	0.007	log y = -0.6351 - 0.0706(log x)	0.03	log y = -0.5688 - 0.1365(log x)	0.05
Belly Feather THg						
All Birds	log y = -0.0244 + 0.3728(log x)	0.24*	log y = 0.0485 + 0.2801(log x)	0.13*	log y = -0.16808 + 0.5225(log x)	0.34*
Thrushes	log y = 0.2682 + 0.6653(log x)	0.46*	log y = 0.4398 + 0.4902(log x)	0.31*	log y = -0.04447 + 0.7389(log x)	0.63*
Sparrows	log y = -0.6840 - 0.0544(log x)	0.01	log y = -0.7065 -0.0554(log x)	0.01	log y = -0.6630 - 0.1326(log x)	0.04
Back Feather THg						
All Birds	log y = 0.0170 + 0.3984(log x)	0.28*	log y = 0.0722 + 0.2703(log x)	0.13*	log y = -0.14905 + 0.4831(log x)	0.32*
Thrushes	log y = 0.2585 + 0.6380(log x)	0.46*	log y = 0.4622 + 0.4909(log x)	0.35*	log y = -0.03198 + 0.6637(log x)	0.56*
Sparrows	log y = -0.5578 + 0.0001(log x)	0.00	log y = -0.6206 - 0.0523(log x)	0.02	log y = -0.05903 - 0.1122(log x)	0.03
Flank Feather THg						
All Birds	log y = -0.0107 + 0.3579(log x)	0.26*	log y = 0.0531 + 0.2640(log x)	0.14*	log y = -0.17304 + 0.4352(log x)	0.27*
Thrushes	log y = 0.1912 + 0.5073(log x)	0.30*	log y = 0.3312 + 0.3851(log x)	0.21*	log y = -0.04834 + 0.5877(log x)	0.43*
Sparrows	log y = -0.5441 + 0.03734(log x)	0.01	log y = -0.5659 + 0.01101(log x)	0.001	log y = -0.64729 - 0.1532(log x)	0.08
Nail THg						
All Birds	log y = 0.23162 + 0.60818(log x)	0.59*	log y = 0.5217 + 0.60034(log x)	0.57*		
Thrushes	log y = 0.3131 + 0.7242(log x)	0.47*	log y = 0.6022 + 0.6183(log x)	0.43*		
Sparrows	log y = 0.0980 + 0.5063(log x)	0.69*	log y = 0.2767 + 0.51488(log x)	0.77*		
Muscle THg						
All Birds	log y = -0.56868 + 0.91377(log x)	0.84			log y = -1.04400 + 0.9505(log x)	0.57*
Thrushes	log y = -0.58758 + 0.9843(log x)	0.76*			log y = -1.08776 + 0.7100(log x)	0.43*
Sparrows	log y = -0.3835 + 0.98579(log x)	0.94*			log y = -0.7448 + 1.5130(log x)	0.77*
Liver THg						
All Birds			log y = 0.40734 + 0.92612(log x)	0.84*	log y = -0.54157 + 0.9762(log x)	0.59*
Thrushes			log y = 0.3176 + 0.77761(log x)	0.76*	log y = -0.51642 + 0.6609(log x)	0.47*
Sparrows			log y = 0.3013 + 0.95451(log x)	0.94*	log y = -0.4661 + 1.3788(log x)	0.69*

[a] Tissues listed as column headers represent x-axis, those in body of table represent y-axis. Asterisks represent relationships with significant p values.

Differences across family and species

Considerable differences exist between Hg correlations in the Thrush family and Hg correlations in the Sparrow family (Table 3). Most notably, Hg concentrations in Sparrow feathers show no correlation to Hg concentrations in internal tissues. In the Thrush family, these correlations are significantly stronger. This may suggest that correlations differ by family or by species. However, limitations imposed by small sample size prevent us from determining if the lack of Hg correlation observed between

Sparrow tissues is due to an actual difference between families, or due to shortage of data.

The intra-individual variation in feather Hg concentrations that we observed may also be dependent on species (Fig. 4). Some species exhibit consistently high variation in Hg concentrations across their feather tracts, while others species consistently show low variability. Further analysis on the effects of species is required.

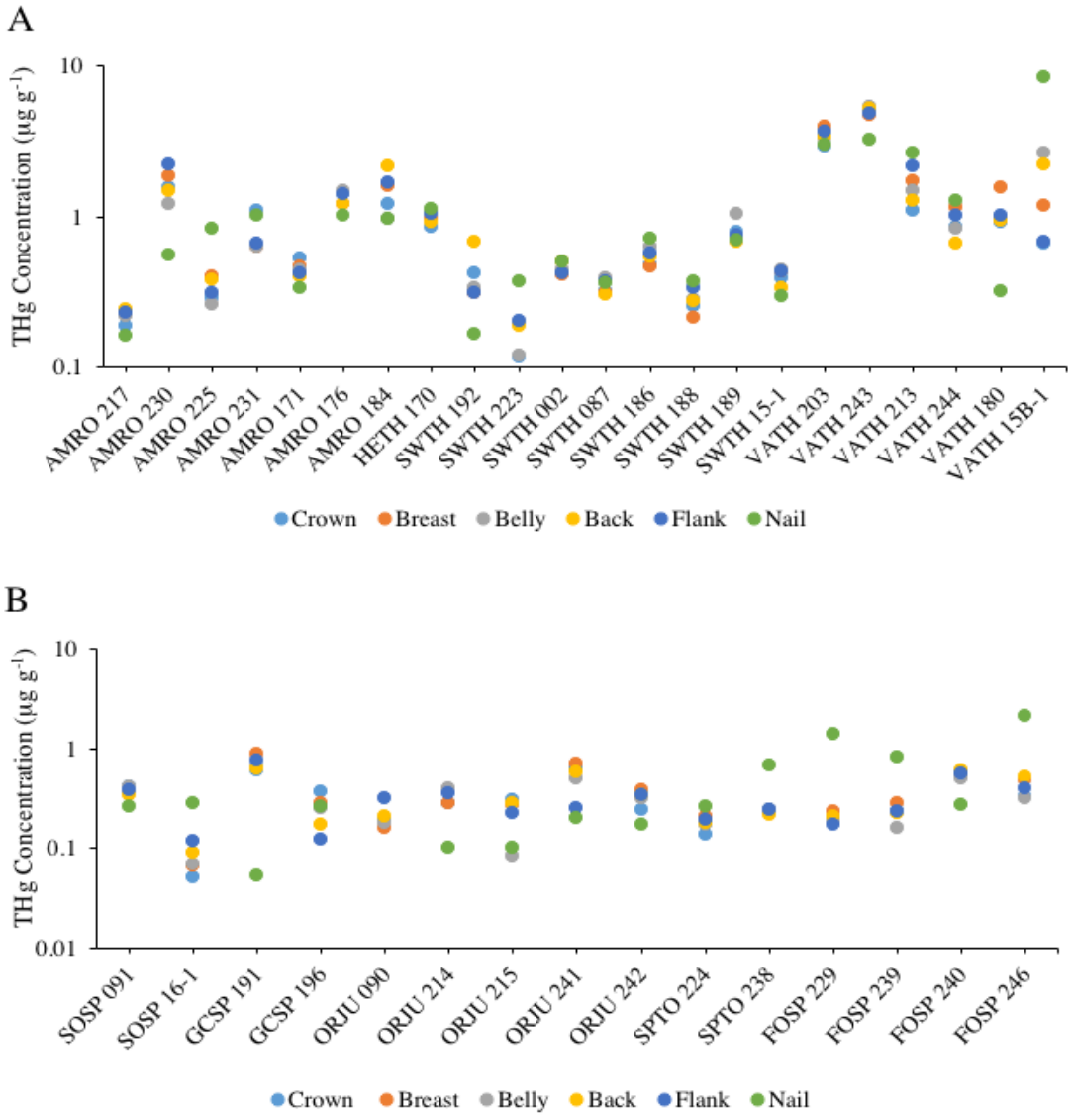

Fig. 4 Total Hg concentration (THg), measured as μg g^{-1}(shown on the log scale), of body feathers and toe nails in 22 individuals from the thrush family (A) and in 15 individuals from the sparrow family (B).

Discussion

Patterns Across Feather Tracts

Despite high variability in Hg concentrations within and among individuals, our results show no significant differences in Hg concentrations across feather tracts. Furthermore, we have found no consistent trend in the pattern of Hg sequestration across feather types. This was unexpected, considering that Hg concentrations previously have been found to differ within and among feather tracts in seabirds. The most well-documented example is the trend observed across primary feathers, where inner primaries are shown to have higher Hg concentrations compared to outer primaries (Bortolotti 2010, Lewis & Furness 1991, Furness et al. 1986). This trend is predominantly attributed to the decline of body Hg levels as molt progresses.

Since Hg is mainly excreted through feathers, and can only be incorporated into feathers as they are growing, it cannot be excreted efficiently between molting periods. Therefore, it accumulates in body tissues during this time. When molt begins, the Hg stored in internal tissues is remobilized and sequestered into feathers (Furness et al. 1986). The first feathers to regrow, therefore, are exposed to relatively higher levels of Hg, which accumulated in the body throughout the year. As molt progresses, a greater proportion of this Hg has already been incorporated into feathers, leaving less Hg available for sequestration into later-growing feathers. Therefore, inner primaries, which are the first to molt in most birds, contain higher Hg concentrations than later-growing, outer primaries.

The effect of molt order on Hg concentrations in songbird body feathers is difficult to evaluate, since molt order in these feathers is poorly understood. We might

expect, however, that the effect of molt order on body feathers is dampened, given that multiple body feather tracts grow concurrently in some species (Carravieri et al. 2014). Therefore, differences across feather tracts would be subtler. However, if molt strongly influenced Hg concentrations in feathers, we would expect to see a consistent pattern across feather tracts in members of the same species, where molt order is consistent. Our results show no such pattern (Fig. 4).

However, the lack of pattern may simply be a result of low overall Hg concentrations in body feathers. Lewis & Furness (1991) reported that, at low Hg concentrations, the pattern typically seen across primary feathers is muted, and that Hg concentrations across these feathers remained relatively constant. A concurrent study of primary feathers, taken from the same bird specimens used in this study, revealed little variation in Hg concentration across primaries, despite known molt order (Ramsden et al., unpublished data). It is possible that trends in Hg sequestration across body feathers do exist in the species examined in this study, but would only be discernable in individuals exposed to high Hg levels. Further research is needed in this area.

Using concentrations to examine trends

Although most authors agree that trends in Hg concentrations across primaries are resultant of molt order, some authors suggest alternative causes. Burger (1994) observed that this same pattern across primaries exists in species that do not follow the typical P1-P10 molt progression. Based on these observations, Bortolotti (2010) suggested that this trend is simply a result of the incorrect use of concentration measurements, in which case, actual trends across feather tracts must be evaluated in a different way. He argues that

concentration is only useful when considering elements that are essential to feather growth. These elements are incorporated into feathers in a mass-dependent fashion, where increased feather mass requires an increased amount of the element in the feather. Because Hg is not essential to feather growth, Hg level does not necessarily increase with increasing feather mass. Instead, Hg is randomly incorporated into growing feathers via the bloodstream, according to Bortolotti (2010). Feather Hg levels therefore are dependent on the amount of Hg in the blood and the amount of time that the feather is connected to the blood supply, which is determined by feather size and feather growth rate.

Despite these complications in interpretation, concentration, which is dependent on feather mass, is still applied to non-essential elements and contaminants, such as Hg. The effect is that Hg levels in more massive feathers are diluted. To correct for this, Bortolotti (2010) suggested sampling a consistent length of feather, taken from the distal end. If growth rate is constant across feathers, then the Hg in these samples would be solely indicative of the amount of Hg that was in the blood at the time of feather growth. This would help to standardize sampling procedures and allow for comparison across years and across studies. However, feather growth rates are affected by feather density (Howell 2010), body condition (Van De Wetering 2000), and potentially be sex and age (Saino et al. 2013). Furthermore, some authors report that feather mass and feather length do not increase at the same rates (Dawson 2004). Therefore, only feathers with the same mass growth rate could be compared using this method. Although mass growth rate can be calculated (Dawson 2003), it requires analysis of feather growth bars, which are difficult to see in many feathers. Such extensive analysis negates some benefits of feather

sampling by making interpretation of results difficult and by limiting generalizability across studies. Still, we think that feather size and structure may influence patterns of Hg sequestration across feather tracts. We acknowledge that the use of Hg concentrations may affect trends across feather tracts, however, without a viable alternative, we will continue to evaluate trends using Hg concentrations.

Our findings of no significant differences in Hg concentration across body feather tracts suggest that feathers from any of the five tracts would yield similar results if used to sample Hg. We do, however, recommend using a composite of feathers from various body feather tracts, to control for variability of individual feathers. We also suggest exercising caution when using feathers for toxicological evaluations. While feathers can be used to estimate rough levels of Hg exposure, we do not recommend using this tissue matrix to deduce fine-scale trends, because of weak relationships between Hg concentrations in feathers and in other tissues.

Tissue correlations

The weakest correlations between tissue Hg levels involved feathers, which suggests that feathers are poor predictors of Hg concentrations in other body tissues. Relationships are likely weakened by the unique growth pattern of feathers, compared to other tissues. At the time of sampling, internal tissue Hg concentrations represent a relatively current Hg load in the body. But Hg from feathers sampled at the same time, represent the body Hg load at the time of feather growth, which could be up to a year prior to the time of sampling. Therefore, feathers would be expected to best correlate to internal tissues during molt, when feathers are growing.

Some researchers have shown that Hg concentrations in blood and internal tissues decrease during feather molt, as Hg from these tissues is actively being sequestered into feathers (Condon & Cristol 2009, Honda 1986). Therefore, the strength of the correlation, as well as the ratio between Hg concentrations in different tissues, vary based on the time of year that tissues were sampled. Temporal fluctuations in tissue Hg correlations can be tested by grouping birds by season of mortality for analysis. However, our sample size may be too small to draw conclusions in this area. Further investigation is needed.

Nails versus Feathers

Our results support the findings of Hopkins et al. (2007), which suggest that nails better correlate to internal tissues than do feathers. This too, is likely a result of the unique pattern of feather growth. It is estimated that songbird flight feathers grow 2-5 mm/day, on average (Howell 2010). If this same growth rate were applied to body feather tracts, the largest feathers used in this study would be fully grown within four weeks, while the smallest would grow in a matter of days. Because an estimated 42-60 percent of ingested Hg is incorporated into feathers over this relatively short time span, feather Hg levels may be highly susceptible to capturing large, random fluctuations in dietary Hg intake (Lewis & Furness 1991, Condon & Cristol 2009). This could lead to inaccurate estimates of current body Hg and poor correlations to liver and muscle Hg concentrations, which reflect Hg intake over longer timespans (Stickel 1977).

In contrast, toe nails grow continuously through accretion, where keratin layers are continually added as others are worn away. Bearhop et al. (2003) found that, in five species of Palearctic songbirds, the mean nail growth rate was 0.04 (± 0.01) mm day^{-1}.

Based on this measurement, keratin deposited at the nail bed would reach the tip of the nail in 95-148 days, depending on species. However, unlike human fingernails, bird nails contain a central pulp running through the nail, meaning that in addition to growing from the proximal end, bird nails also grow from the middle. Therefore, the distal tip of the nail contains both new and old keratin, and nail samples provide an average estimate of body Hg over the last five months. Therefore, the exposure timespan reflected by toe nails is more similar to that shown by internal tissue Hg concentrations, and nail Hg is less likely to be affected by random fluctuations in Hg intake, when compared to feathers.

Alternatively, Hopkins et al. (2007) suggested that differences between feathers and nails could be driven by proteinaceous differences within these structures. Although both structures are made of keratin, nails are predominantly composed of claw-β-keratins, while feathers are mostly feather-β-keratin. Each β-keratin subfamilies code proteins with slightly different in amino acid sequences, which could affect the abundance of disulfide linkages in the keratin (Ng et al. 2014, Whitbread et al. 1990). Since Hg strongly binds to disulfide linkages (Crewther et al. 1965), the relative abundance of these linkages could affect the ability of Hg to bind to keratin. However, while discrepancies in binding ability would alter overall concentrations, it is unclear if they affect correlations between tissues.

Because toe nail Hg concentrations better correlate to those in internal tissues, nails would be more useful for deducing finer-scale trends in Hg exposure. However, we do not think that nail sampling in the field is feasible, at least not when testing for Hg in songbirds. Due to the small size of songbird nails, we used entire toe nails, and sometimes several toe nails for analysis, since small sample mass can lead to inaccurate

Hg results. In live birds, taking an entire toe nail would cut through the nail pulp, which would be painful and would cause the bird to bleed. Removing entire nails could also inhibit perching abilities of the bird, which would be detrimental to most songbirds. Therefore, we think that toe nail sampling could only be used in larger bird species, where relatively large samples could be obtained by clipping only the tip of a nail (Hopkins et al. 2007).

Conclusion

Although some species exhibit wide variation in Hg concentrations across their tissue types, Hg concentrations in keratinaceous tissues are consistently higher than those in internal tissues. Hg concentrations between nails and feathers show no statistical difference, however, nail Hg better correlates to internal tissue Hg. Weak correlations between Hg concentrations in feathers and internal tissues suggest that feathers may not be useful for examining nuanced trends in Hg exposure. However, feathers can be used to determine rough estimates of Hg levels in avian populations.

Across feather tracts, Hg concentrations do not vary significantly, suggesting that choice of feather, at least within body feather tracts, would not substantially affect results. However, due to individual feather variability and slight differences in relationships of different feather tracts to internal tissues, we suggest using a composite of various body feathers for sampling. Patterns of Hg exposure and sequestration into tissues may be influenced by a wide variety of factors, including but not limited to: molt, date of sampling, species, feather structure and feather size. Because the roles of these factors

remain poorly understood, generalization across studies is difficult and further research is needed.

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