

Short Communication

RELATIONSHIP BETWEEN LAYING SEQUENCE AND MERCURY CONCENTRATION
IN TREE SWALLOW EGGS

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Abstract—When female birds lay eggs, some of their body burden of mercury is eliminated into each egg, potentially leading to declining mercury across the clutch. However, there was no decline in mercury with laying sequence in clutches of tree swallow (*Tachycineta bicolor*) eggs at a mercury-contaminated site, presumably due to daily replenishment of mercury in females during laying. Sampling just one egg from the nest provided an accurate measure of clutch mercury contamination. Environ. Toxicol. Chem. 2010;29:1155–1159. © 2010 SETAC

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INTRODUCTION

The risk of mercury exposure to wildlife has steadily risen throughout the 20th century due to increased anthropogenic mercury emissions transported and deposited around the globe [1]. Determining mercury availability using birds as biomonitors has historically required invasive sampling of internal organs, however, recent studies have produced comparable results using less invasive sampling methods [2,3]. Tissues such as feathers, blood, and eggs are becoming more commonly used as bioindicators of mercury contamination [2,4]. Egg mercury levels are significantly correlated with female blood mercury levels, as well as prey mercury concentrations, and are therefore equally informative of local contamination [4]. Because embryonic birds are sensitive to small amounts of mercury [5], sampling of eggs is often a component of ecological risk assessment [6].

Egg laying provides a route for elimination of mercury in female birds through which mercury is deposited into the egg albumen, shell, and yolk [7]. As a result, females may decrease the fitness of their offspring [8]. Understanding the way birds allocate mercury into each egg according to laying sequence is essential for estimating risk of exposure. Females may deplete their body burden of a contaminant before the clutch is complete, in which case later eggs would be less contaminated. As mercury can negatively affect egg size, embryonic growth, and the hatchability of eggs [4,5], later eggs could be at lower risk of ill effects at contaminated sites. Most research on intraclutch variation has focused on large piscivorous birds that lay small clutches of eggs. These species, such as the common tern (*Sterna hirundo*), herring gull (*Larus argentatus*), and common loon (*Gavia immer*) allocate the most mercury into the first egg laid and significantly less into each successive egg [4,9].

Significant intraclutch variation was detected in all of these studies, with the first egg having at least 25% more mercury than the second or third.

A similar pattern of intraclutch variation was found in wood ducks (*Aix sponsa*), with mercury levels in the shell and albumen decreasing from the first to fifth egg laid [7]. Wood ducks are primarily herbivorous, but females also consume insects during the breeding season [7]. Thus, intraclutch variation in mercury level does not appear to be specific to piscivorous birds and occurs in species with large or small clutches of eggs. Only one study has addressed intraclutch variation of mercury in songbirds; insectivorous great tits (*Parus major*) eliminated a similar amount of mercury into each egg within their clutches of 4 to 11 eggs [2]. However, the authors suggested that the level of mercury contamination on their study site was too low to draw a definite conclusion. Indeed, others have proposed that lower mercury levels could lead to decreased intraclutch variation [2,4].

Though researchers have long relied on obligate piscivorous species to monitor environmental mercury availability, recent studies have indicated similar risk and exposure to mercury in insectivorous birds [10] leading to an increase in use of these species as biomonitors [10–12]. In particular, tree swallows (*Tachycineta bicolor*) are becoming more commonly used as biomonitors of environmental contaminants due to their ready use of nest boxes placed in suitable habitat and their hardiness in the face of human disturbance [13]. Tree swallows forage within 400 m of their nests during the breeding season, eating a mix of terrestrial and emergent aquatic insects and thereby serving as a means of quantifying local mercury availability [14]. As insectivorous birds become more commonly used for biomonitoring, it will be useful to understand how they allocate mercury across the clutch.

The purpose of the present study was to investigate intraclutch variation in insectivorous songbirds using the tree swallow as a model species. Eggs were collected from an experimental tree swallow population established along the mercury-contaminated South River in Virginia, USA. The main objective of the present study was to determine if intraclutch variation is large enough to require knowledge of the laying

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sequence when interpreting results of eggs sampled for mercury. If tree swallows exhibit consistent intraclutch variation in mercury, then monitoring the laying sequence prior to egg collection, or collecting entire clutches, would be necessary for interpreting data. On the other hand, if there is little variation, collecting a single egg may be sufficient.

METHODS

A nest box trail (~600 boxes) was established in the Shenandoah Valley (VA, USA) (38.06°N, -78.88°W) in 2005 to study legacy industrial mercury contamination along a 40-km stretch of the South River. Released from 1929 to 1950, the source of the mercury was mercuric sulfate used as a catalyst to produce acetate fibers in a Waynesboro (VA, USA) manufacturing plant. Two adjacent tributaries with no known history of mercury contamination, the Middle and North Rivers, served as reference sites (see map in Cristol et al. [10]). Tree swallows arrived on the breeding grounds in mid-March to early April. Beginning in April 2008, nest boxes were monitored daily for signs of imminent egg laying. As there was ongoing research on survivorship of returning, banded birds at this study site, only nests of females that had not previously nested on the site were used in the present study. Using a pencil, the shells of the first and all subsequent eggs were numbered on the day they were laid. (Tree swallows lay one egg each day in the early morning.) Clutches were considered complete when no new eggs were found in the nest for two mornings in a row. Entire clutches were then collected: 15 contaminated and 15 reference. All eggs were collected May 4 to May 10, 2008. Upon collection, each egg was placed in a sterile glass jar, kept on ice for 5 to 7 h, weighed to the nearest 0.001 g, and measured (length and breadth) to the nearest 0.01 mm using dial calipers. Egg volume (V) was calculated using the following formula: $V = LB^2 \cdot 0.51$, where L = length, B = breadth, and 0.51 is the volume coefficient constant [15]. The egg contents (albumen and yolk) were extracted from the shell using an 11-mm stainless steel sterile surgical blade and emptied into a sterile glass jar. The egg contents were weighed to the nearest 0.001 g and were frozen at -25°C for approximately 30 d until analysis.

The adult female was captured and bled on the day of egg collection at all but three of the nests. Blood was collected using a small gauge (26G, 1.3 cm) needle to puncture the cutaneous ulnar vein. A total of 100 μ l of blood was collected into three heparinized capillary tubes, sealed with Crito-caps[®] (McCormick Scientific) and placed in a 10-cc vacutainer (Becton Dickinson[®]) to prevent breakage. Blood samples were kept on ice for 5 to 7 h, after which they were frozen for approximately 30 d at -25°C until analysis.

Measurement of total mercury was used as a proxy for the highly bioavailable methylmercury based on the premise that nearly all mercury in avian egg is methylmercury [16]. To confirm this for our site, the proportion of total mercury that was methylmercury in tree swallow eggs was determined from the same study site. Methylmercury was analyzed by Quicksilver Scientific using acidic thiourea leaching and mercury-thiourea liquid chromatography coupled to cold vapor atomic fluorescence spectrometry, which separates monomethyl (CH_3Hg^+) from mercuric (Hg^{II}) mercury by the charges on their respective thiourea complexes. Online cold-vapor generation follows separation with an absolute instrument detection limit of 0.40 pg for CH_3Hg^+ . It was previously demonstrated that methylmercury constitutes >95% of total mercury in swallow blood at this site (see Wada et al. [17]).

Total mercury analysis took place at the College of William and Mary (Williamsburg, VA, USA) using cold vapor atomic absorption spectroscopy performed on a Milestone DMA 80. Prior to mercury analysis, each egg was freeze-dried for 24 h and then re-weighed. The yolk and albumen were homogenized using a glass stirring rod. A duplicate aliquot (~0.02 g) of each egg was analyzed to assess repeatability. Each set of 20 samples was preceded and followed by two method blanks, a sample blank, and two samples of each standard reference material (DORM-3, DOLT-3, fish protein and dogfish liver certified reference materials, respectively, provided by National Research Council Canada). All mercury levels have been reported on a wet weight basis to be comparable with the literature, and all results are means \pm SD. The wet weight concentration of egg samples was calculated from the dry weight results using the following formula: wet weight = dry weight Hg \cdot (1 - [% moisture/100]) [4]. Mean percent recoveries of the standard reference materials were $100.14 \pm 1.14\%$ (DORM-3) and $100.62 \pm 1.30\%$ (DOLT-3). Relative percent difference between 174 pairs of duplicate samples was $1.72 \pm 1.81\%$. Detection limit of the assay was 0.003 to 0.006 μ g.

SPSS software (SPSS, Version 16.0) was used to perform the statistical analyses. Though all eggs were analyzed for mercury, clutches ranged from five to seven eggs, with only a limited number of sixth and seventh eggs. Thus, all analyses were carried out only on the first five eggs in a clutch, although means for the sixth and seventh eggs are shown in the appropriate figure. The relationship between laying order and egg volume was determined for eggs within a clutch using a one-way analysis of variance (ANOVA). Intraclutch comparisons of mercury levels were conducted using nonparametric Friedman ANOVA and Wilcoxon signed rank tests. The relationship between a female's blood mercury level and the mercury level in her clutch was determined using linear regression analysis. Statistical significance was defined as $p < 0.05$.

RESULTS

Average mercury level in the first five eggs of clutches from contaminated sites (0.34 ± 0.12 ppm, $n = 75$) was significantly higher than those from reference areas (0.04 ± 0.01 ppm, $n = 75$; $F_{1,149} = 440.32$, $p < 0.0001$). Methylmercury comprised $96.5 \pm 1.1\%$ ($n = 19$ eggs from different nests) of total mercury in eggs from these study sites. Eggs in the contaminated and reference areas did not differ in volume ($F_{1,144} = 0.44$, $p = 0.51$), nor was there a relationship between laying sequence and egg volume ($F_{4,144} = 1.26$, $p = 0.29$). A female's blood mercury level was significantly, positively correlated with the average mercury of the eggs in her clutch ($r^2 = 0.87$, $p < 0.0001$; Fig. 1). Despite elevated mercury levels in the eggs from contaminated sites, there was little intraclutch variation (Friedman ANOVA, $X^2 = 4.693$, $p = 0.32$); more specifically there was no difference in the mercury level of the first egg laid and the fifth ($W = 55.0$, $p = 0.78$; Fig. 2). Clutches in reference areas exhibited a decrease in mercury from the first laid egg to the fifth (Friedman ANOVA, $X^2 = 20.37$, $p < 0.0001$). However, though the difference between egg 1 and 5 was statistically significant ($W = 5.0$, $p = 0.002$), the difference in mercury concentrations was <2% and may not have been biologically significant.

To determine whether sampling fewer eggs from each clutch would have provided an adequate estimate of mercury level for the entire clutch, a re-sampling procedure (using only the first

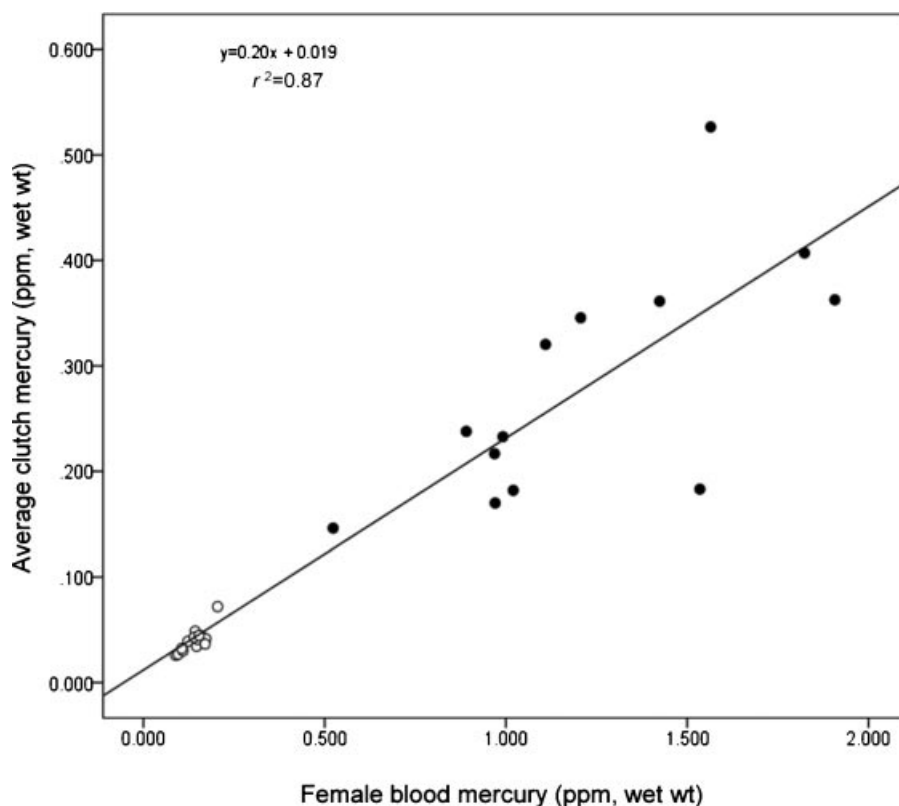


Fig. 1. A female swallow's blood mercury level was significantly, positively correlated with the average mercury level of eggs in her clutch (linear regression $y = 0.20x + 0.019$, $r^2 = 0.87$, $p < 0.0001$). Closed circles = contaminated ($n = 13$), open circles = reference ($n = 14$). Mercury levels reported as parts per million (ppm), wet weight.

five eggs as in other analyses) was carried out in which the mercury level of one randomly selected egg from each clutch was used instead of the whole clutch. This was repeated 1,000 times and the average mercury level was calculated and compared to the mercury level calculated from all five eggs. On

contaminated sites, sampling one randomly selected egg from each nest would have provided an estimate that was within 10% of the true mean 100% of the time, within 5% of the true mean 87% of the time, or within 1% of the true mean 27% of the time. The results from re-sampling reference clutches were within a percentage point of those for contaminated clutches.

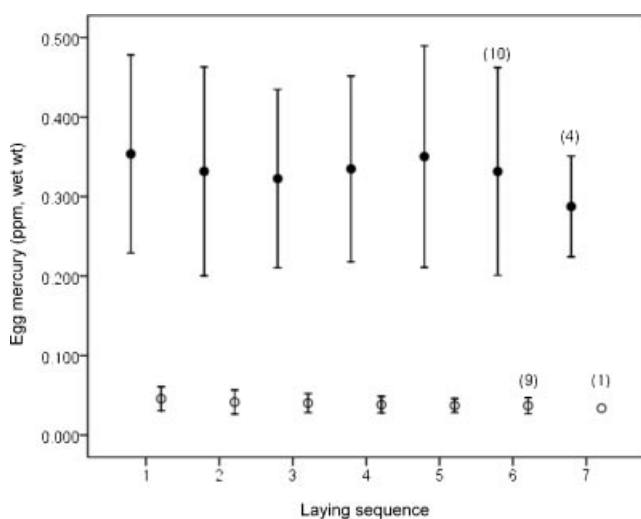


Fig. 2. There was little intraclutch variation among the first five eggs in contaminated nests (closed circles; Friedman analysis of variance [ANOVA], $X^2 = 4.693$, $p = 0.32$). Among the first five eggs in reference nests (open circles), mercury levels were slightly, though significantly, lower in later eggs (Friedman ANOVA, $X^2 = 20.37$, $p < 0.0001$). Error bars indicate standard deviation; sample size was 15 for each point for eggs 1 to 5, sample sizes for eggs 6 and 7 given above data points. Mercury levels reported as parts per million (ppm), wet weight.

DISCUSSION

Previous studies on piscivorous birds have reported that mercury levels are lower in eggs laid later in the clutch [4,9,18]. At the contaminated site along the South River (VA, USA), tree swallow eggs did not differ in mercury concentration whether they were laid first or fifth and there was no apparent trend of decreasing mercury in consecutively laid eggs. The present study is the first to report a lack of intraclutch variation in free-living songbirds with exposure to high levels of dietary mercury (average female blood mercury, contaminated: 1.23 ± 0.40 ppm, reference: 0.14 ± 0.03 ppm). The only other study addressing intraclutch variation of mercury in a songbird, the great tit, also found similar mercury levels across the eggs in a clutch [2], though the eggs sampled for that study had low levels of mercury (median concentration 0.26 ppm, dry wt) comparable to the reference clutches in the present study. There was a slight (<2%) decrease in mercury from the first to fifth egg on the reference sites, which may represent elimination of mercury accumulated on the tree swallows' wintering grounds. In studies of piscivorous birds with significant intraclutch variation, the difference in mercury between first and later (usually second or third) eggs has exceeded 25% [4,9,18], an order of magnitude greater

than the effect on reference sites in the present study. It has been suggested that the contribution of methylmercury stored in muscle and other tissues is minimal compared to the contributions from dietary uptake during egg development [4]. In the present study, the result of no laying sequence effect on egg mercury at contaminated sites suggests that dietary mercury can offset any depuration of body burden mercury into songbird eggs. If the slight laying sequence effect found at reference sites is robust, this would suggest that depuration of body burden mercury occurs in songbirds, but is not an important route.

Laboratory dosing studies on mallards (*Anas platyrhynchos*), chickens (*Gallus gallus*), and Japanese quail (*Coturnix coturnix*) have demonstrated that egg mercury concentrations increase in a dose-dependent manner in response to increasing dietary methylmercury [8,19,20]. Mercury has been detected in eggs within 2 d of a single oral dose of methylmercury in chickens [19]. These studies also reported significant declines in egg mercury within days of the cessation of dietary mercury administration. Heinz and Hoffman [8] reported similar patterns of mercury loss among clutches of mallard eggs after 15 d of dietary mercury exposure. Laboratory dosing studies such as these are important in understanding depuration rates and the toxicodynamics of mercury elimination through egg laying after short-term dietary exposure. However, free-living birds, such as the swallows in the present study, may experience continuous dietary exposure to mercury and their ability to reduce body burden through egg laying may be negated.

In species that lay small clutches of one to three eggs, such as gulls and loons, the difference in mercury between the first and last egg laid can exceed 25% [4,9,18]. Therefore, laying sequence would represent a serious confounding factor in predicting the effect of mercury on young and in a researcher's ability to estimate local mercury availability through sampling less than complete clutches. The onset of dietary mercury uptake in relation to the beginning of egg production must also be considered, particularly when comparing resident to migratory species on a contaminated site [7]. Swallows nesting along the South River (VA, USA) experienced continuous exposure to mercury for several weeks or more prior to egg laying, but spent most of the year at less contaminated sites at the other end of their migratory route.

As this is only the second study to have addressed the effect of laying sequence on mercury concentrations in songbird eggs, it is worth looking for similar patterns with other pollutants. Van den Steen et al. [21] published an overview of the literature on studies addressing laying sequence effects on the concentration of a variety of organic pollutants (including dichlorodiphenyltrichloroethane [DDT], dichlorodiphenyldichloroethylene [DDE], polybrominated diphenyl ether [PBDE], organochlorines pesticide [OCP], and polychlorinated biphenyl [PCB]) and found no clear pattern of diminishment across the clutch. Songbirds used as biomonitors in the reviewed studies included great tits, European starlings (*Sturnus vulgaris*), prothonotary warblers (*Protonotaria citrea*), and blue tits (*Cyanistes caeruleus*) with clutch sizes ranging from 5 to 12 eggs. One recent study using blue tits reported a significant effect of laying sequence on PBDE, OCP, and PCB concentrations in which the average difference between the first laid eggs (eggs 1 and 2) and later laid eggs (eggs 9 to 12) was >30%, but other studies have failed to find patterns of contaminant decline across clutches of songbird eggs [21].

The findings of the present study suggest that the manner in which songbirds eliminate mercury into their eggs could be

different than that of other birds. Further investigation into the disparities between groups associated with egg formation, the metabolism of dietary mercury, and the maternal tissue source of egg mercury would be beneficial. Clutch size, relative egg size, and home range size might also be factors influencing the homogeneity of intraclutch contaminant concentrations. Regardless of the mechanism, the present study has demonstrated through a simple re-sampling procedure that for tree swallows at contaminated or reference sites, any one egg within the clutch can provide an accurate assessment of mercury contamination for the whole clutch. Collecting one egg at random from each nest is faster, less expensive, and less disruptive to the population than determining laying sequence or analyzing entire clutches.

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