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EGG-LAYING SEQUENCE INFLUENCES EGG MERCURY CONCENTRATIONS AND EGG SIZE IN THREE BIRD SPECIES: IMPLICATIONS FOR CONTAMINANT MONITORING PROGRAMS

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Abstract: Bird eggs are commonly used in contaminant monitoring programs and toxicological risk assessments, but intraclutch variation and sampling methodology could influence interpretability. The authors examined the influence of egg-laying sequence on egg mercury concentrations and burdens in American avocets, black-necked stilts, and Forster's terns. The average decline in mercury concentrations between the first and last eggs laid was 33% for stilts, 22% for terns, and 11% for avocets, and most of this decline occurred between the first and second eggs laid (24% for stilts, 18% for terns, and 9% for avocets). Trends in egg size with egg-laying order were inconsistent among species, and overall differences in egg volume, mass, length, and width were <3%. The authors summarized the literature, and among 17 species studied, mercury concentrations generally declined by 16% between the first and second eggs laid. Despite the strong effect of egg-laying sequence, most of the variance in egg mercury concentrations still occurred among clutches (75–91%) rather than within clutches (9%–25%). Using simulations, the authors determined that accurate estimation of a population's mean egg mercury concentration using only a single random egg from a subset of nests would require sampling >60 nests to represent a large population (10% accuracy) or ≥14 nests to represent a small colony that contained <100 nests (20% accuracy). *Environ Toxicol Chem* 2016;35:1458–1469. Published 2015 Wiley Periodicals Inc. on behalf of SETAC. This article is a US Government work and, as such, is in the public domain in the United States of America.

Keywords: Bird Egg-laying order Egg-laying sequence Egg size Mercury

INTRODUCTION

Bird eggs are commonly the focus of mercury monitoring programs [1,2], because eggs are more easily sampled than other bird tissues and egg mercury concentrations often represent local contamination [3], are highly related to mercury contamination of the parents [1,4], and relate directly to the potential risk of reproductive impairment [5]. Typically, 1 egg per clutch is collected from several clutches to monitor mercury contamination [2,6], and it is assumed that a single egg represents mercury contamination within each clutch and that the combination of these single eggs from multiple nests represents population-level exposure. Yet, because females can reduce their body burdens of mercury by depurating methylmercury (MeHg) into their eggs, subsequently laid eggs may have lower mercury concentrations [7]. Therefore, the potential exists for substantial intra-clutch variation in egg mercury concentrations, which might hinder the interpretability of mercury concentrations derived from sampling a single egg from a clutch.

In addition to the implications for mercury monitoring programs, understanding how egg mercury concentrations vary with egg-laying order has direct relevance to the toxicological risk of mercury contamination to embryos. Although there is substantial variation among bird taxa, eggs laid earlier in the laying sequence can be larger and have more essential nutrients and antioxidants, and the chicks often have higher growth and survival rates than chicks from eggs laid later in the laying sequence, especially among precocial species [8,9]. Yet, if the first laid eggs also have higher mercury concentrations, this general pattern in birds' life histories might be disrupted in highly contaminated ecosystems, because mercury can negatively affect egg hatchability [10,11] and subsequent chick health and survival [12–16].

We conducted a detailed assessment of the influence of egg-laying order on mercury concentrations and burdens in bird eggs. We collected original data from 3 species of waterbirds to examine trends within individual species, and we also summarized the peer-reviewed literature and estimated the general decline in egg mercury concentrations with egg-laying order among species. Because diet plays a large role in resulting mercury concentrations in bird eggs [17,18], we examined effects of egg-laying order on egg mercury concentrations within 3 species of birds that forage in different guilds and at different trophic levels. Specifically, American avocets (*Recurvirostra americana*) and black-necked stilts (*Himantopus mexicanus*) consume mostly aquatic invertebrates, whereas the diet of Forster's tern (*Sterna forsteri*) consists mainly of fish. To assess whether observed changes in egg mercury concentrations could have been influenced by changes in egg size, we simultaneously examined intraclutch variation in egg mercury concentrations and egg size. Lastly, we evaluated whether sampling 1 egg from a clutch is adequate for contaminant monitoring purposes when the goal is to examine mercury contamination levels within a population or within the clutch itself.

MATERIALS AND METHODS

Egg collection, dissection, and processing

American avocet (hereafter "avocet"), black-necked stilt (hereafter "stilt"), and Forster's tern (hereafter "tern") nests were monitored in San Francisco Bay, California, USA, following the methods of Ackerman et al. [19]. We entered

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nesting areas weekly throughout the nesting season from April to July 2007 and marked each newly initiated nest with a uniquely numbered flag. Newly initiated nests that contained only 1 egg at the initial visit were considered for inclusion in the present study. The single egg in the nest was floated to confirm that the embryo's age was 0 d [20] and numbered with a permanent marker. We returned to each of these nests daily until the full clutch had been completed, each time numbering with a permanent marker the newly laid egg that was added to the clutch. Birds typically laid a new egg every 1 d to 2 d, and we waited for a consecutive 2 d after the clutch was suspected to have been completed before collecting the full clutch. Depredated nests were excluded. Average clutch sizes are 3.8 in avocets, 3.8 in stilts, and 2.4 in terns [19; J.T. Ackerman et al., unpublished data]. Eggs were placed in egg cartons and stored on wet ice until transport back to the laboratory, where they were stored in a refrigerator until dissection.

During egg dissection, refrigerated eggs were allowed to warm to room temperature before egg length and width were measured to the nearest 0.01 mm using digital calipers (Fowler) and total egg weight (including eggshell) was weighed to the nearest 0.01 g on a digital balance (Ohaus Adventurer Pro; Ohaus). Using clean, stainless steel instruments, we cut a hole approximately 15 mm in diameter in the wide end of each egg and removed the entire contents into a sterile 30-mL or 60-mL jar. Egg content (without eggshell) was then weighed with a digital balance to the nearest 0.01 g, and egg contents were stored at –20 °C until processing and mercury determination.

During processing, eggs were thawed at room temperature, and then the entire egg contents were dried at 50 °C for 48 h or until completely dried. To determine moisture content, we reweighed dried egg contents with a digital balance to the nearest 0.0001 g (Ohaus Adventurer Balance, model AR064; Ohaus). Dried egg contents were then ground to a powder using a spice grinder with stainless steel blades, followed by further grinding by hand in a mortar and pestle. Processed egg samples were stored in a desiccator until mercury determination.

Mercury determination

We determined total mercury (THg) concentration in eggs and used it as an index of MeHg concentrations, because most (96%) of the mercury in bird eggs is in the more toxic MeHg form [6]. We determined THg concentrations in egg contents (without the eggshell) on a DMA-80 Direct Mercury Analyzer (Milestone) following US Environmental Protection Agency method 7473 [21], using an integrated sequence of drying, thermal decomposition, catalytic conversion, and then amalgamation, followed by atomic absorption spectroscopy. Quality assurance measures included analyses of at least 2 certified reference materials (either dogfish muscle tissue, dogfish liver, or lobster hepatopancreas certified by the National Research Council of Canada), 2 system and method blanks, 3 continuing calibration verifications, 2 duplicates, and 2 spiked duplicates per batch. Recoveries (mean ± standard error [SE]) were 101.7 ± 0.9% ($n = 21$) for certified reference materials, 101.9 ± 1.0% ($n = 44$) for calibration verifications, and 105.1 ± 1.8% ($n = 30$) for matrix spikes. Relative percent difference was 3.5 ± 0.5% ($n = 29$) for duplicates and 4.4 ± 1.3% ($n = 15$) for matrix spike duplicates.

Total mercury concentrations in eggs were reported on a fresh wet weight basis. To do so, we determined THg concentrations in eggs on a dry weight basis and then converted them into fresh wet weight egg concentrations using individual-specific moisture content of the egg contents and egg morphometrics following the methods of Ackerman et al. [6] and egg densities specific to these bird species (J.T. Ackerman et al., unpublished data). We also calculated the total burden of THg in each egg by multiplying the egg THg concentration on a dry weight basis by the total dry weight of the egg contents (without eggshell), as very little (<3%) of the whole egg's mercury burden occurs in the eggshell [22–24].

Statistical analyses

For each species, we used separate linear mixed-effects models to examine the variation in either egg THg concentration ($\log_e$-transformed), THg burden, volume, mass, length, or width with the fixed effect of position of the egg in the laying sequence (egg-laying order 1–4) and clutch identification as a random effect. This model structure statistically nested individual eggs within their clutch. The Satterthwaite method was used to estimate the degrees of freedom. Tukey's honestly significant difference tests ($\alpha < 0.05$) were then used to specifically compare differences among egg number in the laying sequence. Unless otherwise noted, we report model-based, back-transformed least-squares means ± SEs. Back-transformed SEs were approximated using the delta method [25] when a $\log_e$-transformation was implemented

$$\mathrm{SE}(\hat{v}) \approx e^{\hat{\mu}} \times \mathrm{SE}(\hat{\mu}) \tag{1}$$

where $\hat{\mu}$ is the least-squares mean $\log_e$-transformed egg THg concentration in the sampled population, $\hat{v}$ is the back-transformed least-squares mean, and $\mathrm{SE}(\hat{\mu})$ and $\mathrm{SE}(\hat{v})$ are their respective standard errors.

Egg sampling simulations and sample size estimates

We determined whether sampling only a single egg per clutch would accurately represent mean egg THg concentrations for each species' population and for clutches themselves by conducting simulations ($n_{\mathrm{sim}} = 1000$) where 1 egg was randomly sampled from each of the clutches used in the present study. Simulations were performed separately for each species. For the population-level comparison, we compared the mean $\log_e$-transformed THg concentration for each of the randomly sampled single egg simulations with the mean $\log_e$-transformed THg concentration for the entire collection of eggs for each species. For the clutch-level comparison, we compared the $\log_e$-transformed THg concentration of each randomly selected egg from a given clutch to the mean $\log_e$-transformed THg concentration of its entire clutch.

For the species-specific population-level analysis, we estimated the amount of error in the geometric mean egg THg concentration (which is the back-transformation of the mean $\log_e$-transformed egg THg concentration) when sampling a single egg from the clutch rather than sampling the complete clutch as the coefficient of variation (*CV*) defined as the SE divided by the geometric mean

$$CV(\hat{v}) = \frac{\mathrm{SE}(\hat{v})}{\hat{v}} \approx \frac{e^{\hat{\mu}} \times \mathrm{SE}(\hat{\mu})}{e^{\hat{\mu}}} = \mathrm{SE}(\hat{\mu}) \tag{2}$$

where $\hat{\mu}$ is the mean $\log_e$-transformed egg THg concentration in the sampled population and $\hat{v}$ is the back-transformed $\hat{\mu}$ (i.e., $e^{\hat{\mu}}$). We estimated $\mathrm{SE}(\hat{\mu})$ by the root mean squared error (RMSE), which represents the standard deviation (SD) of the estimated means (from simulations where 1 random egg per

clutch was sampled) and was calculated using Equation 3

$$\mathrm{SE}(\hat{\mu}) \approx \mathrm{RMSE}_{\mathrm{popn}} = \sqrt{\frac{\sum_{\mathrm{sim}} \left(\bar{x}_{\mathrm{sim}} - \mu_{\mathrm{popn}}\right)^2}{n_{\mathrm{sims}}}} \quad (3)$$

where $\bar{x}_{\mathrm{sim}}$ is the mean $\log_e$-transformed egg THg concentration of each simulation (sim $= 1, \ldots, n_{\mathrm{sims}}$), n_{sims} is the number of simulations (i.e., 1000), and μ_{popn} is the mean $\log_e$-transformed egg THg concentration of all eggs in the population. The *CV* when presented on a percentage basis represents the standard error as a percentage of the mean and, given the properties of the SD for normal distributions, can be interpreted as the percentage deviation within which approximately two-thirds of the simulation means (where 1 random egg per clutch was sampled, i.e., the sampled population's mean) will occur from the overall population mean (where all eggs from all clutches were sampled, i.e., the entire population's actual mean). We multiplied *CV* by the *Z*-score of 1.96 (for $\alpha = 0.05$) to approximate the percentage deviation within which sampled means would occur 95% of the time. To validate this method, we compared $1.96 \times CV$ to the empirically derived 95th percentile of the 1000 simulated percent deviations, and the differences were <2% in all cases; therefore, we present only the former method.

Likewise, we approximated *CV* and derived percentage deviation analogously for the clutch-level analysis using

$$\mathrm{RMSE}_{\mathrm{clutch}} = \sqrt{\frac{\sum_{\mathrm{sim}} \sum_{\mathrm{clutch}} \left(x_{\mathrm{sim,clutch}} - \mu_{\mathrm{clutch}}\right)^2}{n_{\mathrm{sims}} \times n_{\mathrm{clutches}}}} \quad (4)$$

where $x_{\mathrm{sim,clutch}}$ is the $\log_e$-transformed egg THg concentration for the randomly sampled egg from the clutch (clutch $= 1, \ldots, n_{\mathrm{clutches}}$) in simulation (sim $= 1, \ldots, n_{\mathrm{sims}}$), n_{clutches} is the total number of clutches sampled for a given species, n_{sims} is the number of simulations (i.e., 1000), and μ_{clutch} is the mean $\log_e$-transformed egg THg concentration of the clutch (clutch $= 1, \ldots, n_{\mathrm{clutches}}$).

Lastly, we estimated the sample size of nests (where a single egg is collected) that would be necessary to achieve 95% confidence that the mean egg THg concentration is within 10% of the actual population mean egg THg concentration. By expressing SE as the variance in $\log_e$-transformed egg THg concentrations among eggs sampled (S^2) divided by the number of clutches sampled (n), and incorporating an adjustment for finite population size [26], we can express *CV* as a function of n and then solve for n for a given *CV*

$$CV(\hat{v}) = \frac{\mathrm{SE}(\hat{v})}{\hat{v}} \approx \frac{e^{\bar{\mu}} \sqrt{\frac{S^2}{n} \times \frac{N-n}{N}}}{e^{\bar{\mu}}} \quad (5)$$

$$n = \frac{S^2 N}{(CV)^2 N + S^2} \quad (6)$$

where n is the number of clutches required to be sampled (where a single egg is collected), N is the size of the population from which the clutches were sampled, and S^2 was approximated by the sample variance in $\log_e$-transformed egg THg concentrations among eggs sampled, averaged across simulations, when a single egg was randomly sampled from each clutch.

In Equation 6, if we set *CV* to 0.1 and assume the variance obtained from the simulations is the variance within a population, then n represents the number of nests required to be sampled from the population (1 egg randomly sampled per clutch) to be within 10% of the actual mean of the population 68% of the time (i.e., probability distribution within 1 SD of the mean). For a more robust estimate of n, we reduced *CV* by a factor equal to the *Z*-score of 1.96 (for $\alpha = 0.05$)

$$n_{10\%} = \frac{S^2 N\delta}{\left(\frac{0.1}{1.96}\right)^2 N\delta + S^2} \quad (7)$$

where $n_{10\%}$ represents the number of nests required to be sampled from the population to be within 10% of the actual mean of the population 95% of the time, δ is the average clutch size for each species, and thus $N\delta$ approximates the total number of eggs in the population.

This provides a convenient estimate when sampling a colony or wetland site with a smaller or finite population size. In this equation, it is possible for $n_{10\%}$ to exceed the maximum sample size; if this occurs, then even when sampling 1 egg from all possible nests the probability of estimating the actual mean to within 10% accuracy will be less than 95% and sampling more than 1 egg from some nests might be necessary to reach greater statistical power. In situations where the population is much greater than the number of sampled nests, such as region-wide contaminant monitoring, the appropriate equation with a large or undefined population is

$$n_{10\%} = \frac{S^2}{\left(\frac{0.1}{1.96}\right)^2} \quad (8)$$

Literature review

We conducted a literature review and summarized all of the prior studies that had investigated egg mercury concentrations in relation to egg-laying order. We then calculated the percent decline in egg THg concentrations between the first egg laid in the clutch and all subsequently laid eggs within the clutch. We included clutch sizes up to 11 eggs in the table (but no data were available for the ninth egg in a clutch), although 1 supplemental feeding study continued to document the decline in egg THg concentrations until the 31st consecutively laid egg, and these data were included as a footnote. Although it would be preferable to calculate the decline in egg THg concentrations within the same clutch and then average these values among clutches (similar to the approach we used for Table 1), these data were not available in the literature. Instead, most authors reported average egg THg concentrations by egg-laying order for all clutches combined. Therefore, Table 2 compares mean egg THg concentrations by position of the egg within the clutch for each study. As a consequence, the results for the present study's species were slightly different between Tables 1 and 2 because of the differences in mathematical approaches. When they were reported, we kept data within each study separated by study site, clutch size, egg component, or supplemental feeding group (for dosed birds). Not all studies reported mean egg THg concentrations in text or tabular form; instead, some reported them only as figures. In these 3 cases [18,22,27], we extracted the data visually from the figures; therefore, these studies' results should be considered as approximations.

RESULTS

We collected 84 complete clutches ($n = 31$ avocets, $n = 14$ stilts, $n = 39$ terns) of known egg-laying order from 3 waterbird

species ($n = 263$ eggs). Total Hg concentrations (mean ± SD) in eggs were 0.27 ± 0.09 μg/g fresh wet weight in avocets ($n = 123$ eggs), 0.68 ± 0.38 μg/g fresh wet weight in stilts ($n = 53$ eggs), and 1.27 ± 0.55 μg/g fresh wet weight in terns ($n = 87$ eggs). Egg length and width were 49.67 ± 1.63 mm and 34.41 ± 0.80 mm in avocets, 44.03 ± 1.42 mm and 30.77 ± 0.71 mm in stilts, and 43.07 ± 1.78 mm and 30.32 ± 0.73 mm in terns. Egg volume was 27.48 ± 1.69 cm^3 in avocets, 19.72 ± 1.06 cm^3 in stilts, and 19.28 ± 1.26 cm^3 in terns. Predicted fresh egg mass was 30.48 ± 1.82 g in avocets, 21.79 ± 1.16 g in stilts, and 21.19 ± 1.36 g in terns.

Egg mercury by egg-laying sequence

Total Hg concentrations in eggs differed with egg-laying order for avocets ($F_{3,89.02} = 9.63$, $p < 0.0001$; Figure 1A), stilts ($F_{3,36.00} = 63.03$, $p < 0.0001$; Figure 1B), and terns ($F_{2,47.00} = 24.19$, $p < 0.0001$; Figure 1C). Total Hg burdens in eggs also differed with egg-laying order for avocets ($F_{3,89.03} = 8.71$, $p < 0.0001$; Figure 1D), stilts ($F_{3,36.00} = 57.47$, $p < 0.0001$; Figure 1E), and terns ($F_{2,46.77} = 30.00$, $p < 0.0001$; Figure 1F). For each species, the first egg laid had the highest THg concentrations and THg burdens. The second and third eggs laid, and the fourth in the case of avocets, did not differ from each other in THg concentrations or THg burdens in avocets or terns. In stilts, the second egg laid had higher THg concentrations and THg burdens than the fourth egg laid, but the third and fourth eggs laid did not differ.

The proportional change in THg concentrations between consecutively laid eggs differed for avocets ($F_{2,60.06} = 3.57$, $p = 0.03$; Table 1), stilts ($F_{2,26.14} = 15.10$, $p < 0.0001$; Table 1), and terns ($F_{1,30.4} = 10.20$, $p = 0.003$; Table 1). The proportional change in THg burdens between consecutively laid eggs also differed for stilts ($F_{2,26.48} = 10.04$, $p = 0.001$; Table 1) and terns ($F_{1,37.6} = 5.06$, $p = 0.03$; Table 1) but not avocets ($F_{2,60.11} = 0.65$, $p = 0.53$; Table 1). The largest proportional decline in egg THg concentrations and THg burdens occurred between the first egg laid and the second egg laid (Table 1) and averaged –24.2% for stilts (range, –11.7% to –41.6%), –18.0% for terns (range, +59.0% to –42.4%), and –9.0% for avocets (range, +12.7% to –33.7%). Further, the average decline in THg concentrations between the first egg laid and the last egg laid was 32.7% for stilts, 22.0% for terns, and 10.9% for avocets. Total Hg concentrations in eggs increased with egg-laying order in 3 tern and 6 avocet clutches (Figure 2). After excluding these 9 clutches, the proportional change in THg concentrations between the first egg laid and the second egg laid was not related to THg concentrations in the first egg laid for avocets ($F_{1,23} = 0.73$, $p = 0.40$), stilts ($F_{1,12} = 0.15$, $p = 0.71$), or terns ($F_{1,34} = 1.75$, $p = 0.19$). This indicated that the relative decline in egg THg concentrations between sequentially laid eggs was not larger for the more contaminated clutches.

Although THg concentrations in eggs differed significantly with egg-laying order, most of the variance in egg THg concentrations occurred among clutches (91% in avocet, 86% in stilt, and 75% in tern) compared to within clutches (9% in avocet, 14% in stilt, and 25% in tern; Figure 2). The *CV* in egg THg concentrations within a clutch was not related to the geometric mean egg THg concentration in the same clutch for avocets ($F_{1,29} = 1.43$, $p = 0.24$), stilts ($F_{1,12} = 0.02$, $p = 0.88$), or terns ($F_{1,37} = 0.31$, $p = 0.58$), such that the variability (relative to the mean) in egg THg concentrations within a clutch did not increase at higher egg THg concentrations.

Egg size by egg-laying sequence

Egg morphometrics did not consistently differ with egg-laying order. Egg volume differed with egg-laying order for avocets ($F_{3,89.10} = 5.53$, $p = 0.002$; Figure 3A) and stilts ($F_{3,36.06} = 2.94$, $p = 0.05$; Figure 3B) but not significantly for terns ($F_{2,50.16} = 1.66$, $p = 0.20$; Figure 3C). The first egg laid tended to have a smaller volume than the second egg laid for avocets and a smaller volume than the third egg laid for stilts. Predicted fresh egg mass differed with egg-laying order only for avocets ($F_{3,89.10} = 5.04$, $p = 0.003$; Figure 3D) and not for stilts ($F_{3,36.05} = 2.69$, $p = 0.06$; Figure 3E) or terns ($F_{2,49.88} = 2.39$, $p = 0.10$; Figure 3F). For avocets, the first egg laid tended to have a smaller mass than the second egg laid, but the first, third, and fourth eggs laid did not differ. Egg length differed with egg-laying order only for stilts ($F_{3,36.40} = 3.33$, $p = 0.03$; Figure 4B) and not for avocets ($F_{3,89.15} = 0.87$, $p = 0.46$; Figure 4A) or terns ($F_{2,48.44} = 0.58$, $p = 0.56$; Figure 4C). For stilts, the first egg laid tended to be shorter than the fourth egg laid, but the first, second, and third eggs laid did not differ. Egg width differed with egg-laying order for avocets ($F_{3,89.14} = 5.92$, $p = 0.001$; Figure 4E) but not stilts ($F_{3,36.20} = 2.43$, $p = 0.08$; Figure 4D) or terns ($F_{2,48.48} = 2.29$, $p = 0.11$; Figure 4F). For avocets, the fourth egg laid tended to be narrower than the second or third

Table 1. Least squares mean and standard error (SE) percent change in egg total mercury (THg) concentrations and egg THg burdens with egg-laying order for American avocets, black-necked stilts, and Forster's terns nesting in San Francisco Bay, California, USA[a]

		Egg THg concentration (μg/g fresh wet wt)			Egg THg burden (μg)		
Species	Consecutively laid eggs compared	Mean difference between eggs	SE difference between eggs	Significantly different groups	Mean difference between eggs	SE difference between eggs	Significantly different groups
Avocet	Egg 1 vs egg 2	−9.0%	2.4%	B	−6.3%	2.5%	A
Avocet	Egg 2 vs egg 3	−1.4%	2.4%	A,B	−2.2%	2.5%	A
Avocet	Egg 3 vs egg 4	−0.5%	2.4%	A	−3.0%	2.6%	A
Stilt	Egg 1 vs egg 2	−24.2%	2.4%	B	−21.6%	2.5%	B
Stilt	Egg 2 vs egg 3	−6.8%	2.4%	A	−5.7%	2.5%	A
Stilt	Egg 3 vs egg 4	−5.5%	2.8%	A	−7.3%	2.8%	A
Tern	Egg 1 vs egg 2	−18.0%	2.8%	B	−18.6%	2.9%	B
Tern	Egg 2 vs egg 3	1.9%	5.9%	A	−3.7%	6.1%	A

[a]The mean difference between eggs represents differences in consecutively laid eggs within the same clutch and specifically compares THg concentrations and THg burdens in the first egg laid to those in the second laid egg, the second egg laid to the third egg laid, and the third egg laid to the fourth egg laid. Different letters denote significant ($p < 0.05$) differences between means within each species for egg THg concentrations and burdens separately.

Table 2. Literature review documenting the decline in egg total mercury (THg) concentrations with egg-laying order[a]

Species	Mean egg THg conc. in egg 1	Units	Eggs compared in laying sequence: Egg 1 vs egg 2	Egg 1 vs egg 3	Egg 1 vs egg 4	Egg 1 vs egg 5	Egg 1 vs egg 6	Egg 1 vs egg 7	Egg 1 vs egg 8	Egg 1 vs egg 10	Egg 1 vs egg 11	Significant decline?	Study notes	Ref
American avocet (*Recurvirostra americana*)	0.27	μg/g fresh wet wt	−10%	−11%	−13%	—	—	—	—	—	—	Yes	Wild birds	Present study
Black-necked stilt (*Himantopus mexicanus*)	0.77	μg/g fresh wet wt	−25%	−30%	−34%	—	—	—	—	—	—	Yes	Wild birds	Present study
Forster's tern (*Sterna forsteri*)	1.32	μg/g fresh wet wt	−20%	−17%	—	—	—	—	—	—	—	Yes	Wild birds	Present study
Common tern (*Sterna hirundo*)	0.65	μg/g dry wt	−11%	−14%	—	—	—	—	—	—	—	No	Wild birds; Maryland site	[39]
Common tern	0.59	μg/g dry wt	−34%	−14%	—	—	—	—	—	—	—	No	Wild birds; Massachusetts site	[39]
Arctic terns (*Sterna paradisaea*)	2.11	μg/g dry wt	−22%	—	—	—	—	—	—	—	—	Yes	Wild birds	[40]
Long-tailed duck (*Clangula hyemalis*)	0.85	μg/g dry wt	—	—	—	—	—	—	—	−48%	—	No	Wild birds	[40]
Common eider (*Somateria mollissima*)	0.86	μg/g dry wt	—	—	−28%	—	—	—	—	—	—	Yes	Wild birds; high arctic site; assumed clutch size of 4 eggs	[40]
Common eider	0.50	μg/g dry wt	—	—	−30%	—	—	—	—	—	—	Yes	Wild birds; low arctic site; assumed clutch size of 4 eggs	[40]
Common loon (*Gavia immer*)	0.69	μg/g wet wt	−20%	—	—	—	—	—	—	—	—	Yes	Wild birds	[41]
Wood duck (*Aix sponsa*)	0.34	μg/g wet wt	−20%	−24%	−34%	−37%	−43%	−40%	−43%	—	—	Yes	Wild birds; egg albumen	[22][d]
Wood duck	0.05	μg/g wet wt	−2%	−13%	−4%	−15%	−23%	−11%	−11%	—	—	Yes	Wild birds; eggshell	[22][d]
Wood duck	0.07	μg/g wet wt	−40%	−51%	−57%	−51%	−51%	−61%	−58%	—	—	No	Wild birds; egg yolk	[22][d]
Mallard (*Anas platyrhynchos*)	7.00	μg/g wet wt	—	−40%	—	—	—	−50%	—	—	−83%[c]	Not tested	Lab dosed birds[b]; 5 μg/g wet wt	[18][d]
Mallard	18.00	μg/g wet wt	—	−42%	—	—	—	−46%	—	—	−67%[c]	Not tested	Lab dosed birds[b]; 10 μg/g wet wt	[18][d]
Mallard	31.00	μg/g wet wt	—	−6%	—	—	—	−40%	—	—	−58%[c]	Not tested	Lab dosed birds[b]; 20 μg/g wet wt	[18][d]
Herring gull (*Larus argentatus*)	0.43	μg/g wet wt	−14%	−28%	—	—	—	–	—	—	—	Yes	Wild birds	[42]
Common tern	0.59	μg/g wet wt	−22%	−27%	—	—	—	–	—	—	—	Yes	Wild birds	[42]
Oystercatcher (*Haematopus ostralegus*)	0.22	μg/g wet wt	−5%	−9%	—	—	—	–	—	—	—	No	Wild birds	[42]
Audouin's gull (*Larus audouinii*)	5.69	μg/g dry wt	−18%	–	—	—	—	–	—	—	—	No	Wild birds; 2 egg clutches; egg albumen	[31]
Audouin's gull	0.51	μg/g dry wt	−33%	—	—	—	—	—	—	—	—	No	Wild birds; 2 egg clutches; egg yolk	[31]
Audouin's gull	0.13	μg/g dry wt	0%	—	—	—	—	—	—	—	—	No	Wild birds; 2 egg clutches; eggshell	[31]
Audouin's gull	6.57	μg/g dry wt	−11%	−16%	—	—	—	—	—	—	—	Yes	Wild birds; 3 egg clutches; egg albumen	[31]
Audouin's gull	0.43	μg/g dry wt	−9%	−5%	—	—	—	—	—	—	—	No	Wild birds; 3 egg clutches; egg yolk	[31]
Audouin's gull	0.19	μg/g dry wt	−5%	−11%	—	—	—	—	—	—	—	No	Wild birds; 3 egg clutches; eggshell	[31]
Audouin's gull	5.23	μg/g dry wt	−9%	—	—	—	—	—	—	—	—	No	Wild birds; 2 egg clutches; egg contents	[43]
Audouin's gull	0.22	μg/g dry wt	0%	—	—	—	—	—	—	—	—	No	Wild birds; 2 egg clutches; eggshell	[43]
Audouin's gull	6.01	μg/g dry wt	−13%	−32%	—	—	—	—	—	—	—	Yes	Wild birds; 3 egg clutches; egg contents	[43]
Audouin's gull	0.27	μg/g dry wt	−22%	−48%	—	—	—	—	—	—	—	Yes	Wild birds; 3 egg clutches; eggshell	[43]

(*continued*)

Table 2. (*Continued*)

Species	Mean egg THg conc. in egg 1	Units	Eggs compared in laying sequence: Egg 1 vs egg 2	Egg 1 vs egg 3	Egg 1 vs egg 4	Egg 1 vs egg 5	Egg 1 vs egg 6	Egg 1 vs egg 7	Egg 1 vs egg 8	Egg 1 vs egg 10	Egg 1 vs egg 11	Significant decline?	Study notes	Ref
Tree swallow (*Tachycineta bicolor*)	0.36	μg/g wet wt	−6%	−8%	−4%	−1%	−6%	−18%	—	—	—	No	Wild birds	[27][d]
Great tit (*Parus major*)	0.03	μg/g dry wt	113%	−71%	−26%	48%	52%	—	—	—	—	No	Wild birds	[28]
Zebra finch (*Taeniopygia guttata*)	8.38	μg/g dry wt	−18%	−18%	−25%	−28%	—	—	—	—	—	Yes	Lab dosed birds; 0.3 μg/g wet wt	[4]
Zebra finch	15.50	μg/g dry wt	−5%	−9%	−16%	−18%	—	—	—	—	—	Yes	Lab dosed birds; 0.6 μg/g wet wt	[4]
Zebra finch	29.89	μg/g dry wt	−14%	−16%	−15%	−21%	—	—	—	—	—	Yes	Lab dosed birds; 1.2 μg/g wet wt	[4]
Zebra finch	55.86	μg/g dry wt	−12%	−10%	−17%	−21%	—	—	—	—	—	No	Lab dosed birds; 2.4 μg/g wet wt	[4]
Average (egg contents or albumen; only significant results included)			−16%	−21%	−24%	−26%	−43%	−40%	−43%	—	—			

[a]All comparisons are relative to the first egg laid in the clutch. Egg order number 9 was excluded because no data were available.
[b]Heinz and Hoffman's [18] methylmercury dosing was stopped after the 15th egg was laid. We considered "egg number 1" to be the 15th egg, which was the last egg that was laid before the female was switched off the methylmercury-contaminated diet.
[c]Heinz and Hoffman [18] continued to document the decline in egg THg concentrations until the 31st consecutively laid egg. The total decline in egg THg concentrations in the 31st egg laid relative to the first egg laid was 98% in the 5 μg/g wet weight treatment group, 96% in the 10 μg/g wet weight treatment group, and 95% in the 20 μg/g wet weight treatment group.
[d]Data were extracted from published figures.

egg laid, but not the first egg laid. However, these egg morphological differences were all small (≤3%).

Most of the variance in egg morphometrics occurred among clutches for avocets (volume, 70%; mass, 68%; length, 65%; width, 61%) and terns (volume, 60%; mass, 57%; length, 66%; width, 67%). For stilts, egg volume and egg mass varied more among clutches (volume, 64%; mass, 64%) than within clutches, but egg length and egg width were less variable among clutches (length, 40%; width, 37%) than within clutches.

Egg sampling simulations and sample size estimates

Using simulations, we investigated how sampling only a single egg from clutches would influence estimates of mean egg THg concentrations for each species' population and the clutches themselves. For the clutch-level comparison, we found that sampling only 1 egg per clutch would result in an egg THg concentration that will be within 21.3% of the actual clutch's mean for avocets, within 34.2% of the mean for stilts, and within 28.3% of the mean for terns 95% of the time. For the population-level comparison, we found that randomly sampling only 1 egg per clutch, rather than sampling the entire clutch, from every nest that we collected would have resulted in a mean egg THg concentration that was within 3.8% for avocets, 9.4% for stilts, and 4.7% for terns of the actual mean of all eggs in our sampled population 95% of the time. If only a subset of the nests in the population was sampled, we estimated that it would require sampling a single (random) egg from 65 avocet, 111 stilt, and 58 tern nests to be within 10% of the actual population's mean egg THg concentration 95% of the time, when the actual population is large and undefined (Figure 5A). To be within 20% of the actual population's mean egg THg concentration 95% of the time, it would require sampling a single egg from only 16 avocet, 28 stilt, and 15 tern nests (Figure 5A).

We also estimated the number of nests that would need to be sampled when the actual population size was relatively small, such as a specific colony or wetland site. When the population size is small, it would require sampling fewer nests to estimate the actual population's mean egg THg concentration. For example, it would require randomly sampling a single egg from 55 avocet, 86 stilt, and 47 tern nests to be within 10% of the actual colony's mean egg THg concentration when the colony size is 100 nests (Figure 5B). For small populations <100 nests, it would require sampling a substantial proportion (>50%) of nests to be within 10% of the actual colony's mean egg THg concentration (Figure 5B). Moreover, there are several instances in which sampling a single egg from every nest in a small colony will not produce a mean egg THg concentration that is accurate to within 10% of the actual mean (Figure 5B), and it would require sampling more than 1 egg per clutch to further reduce this error. To illustrate, sampling a single egg from every clutch in a colony with 20 tern nests would result in an estimated mean egg THg concentration that is expected to be within only 14% of the actual colony's mean 95% of the time (Figure 5B). To be within 20% of the actual population mean egg THg concentration 95% of the time, it would require sampling a single egg from only 15 avocet, 24 stilt, and 13 tern nests when the colony size is 50 nests and 16 avocet, 26 stilt, and 14 tern nests when the colony size is 100 nests (Figure 5B). Thus, unless a substantial proportion of the population is sampled, estimated mean egg THg concentrations for small colonies will be less precise, likely to only be within 10% to 25% of the actual mean, and will depend on the number of nests sampled

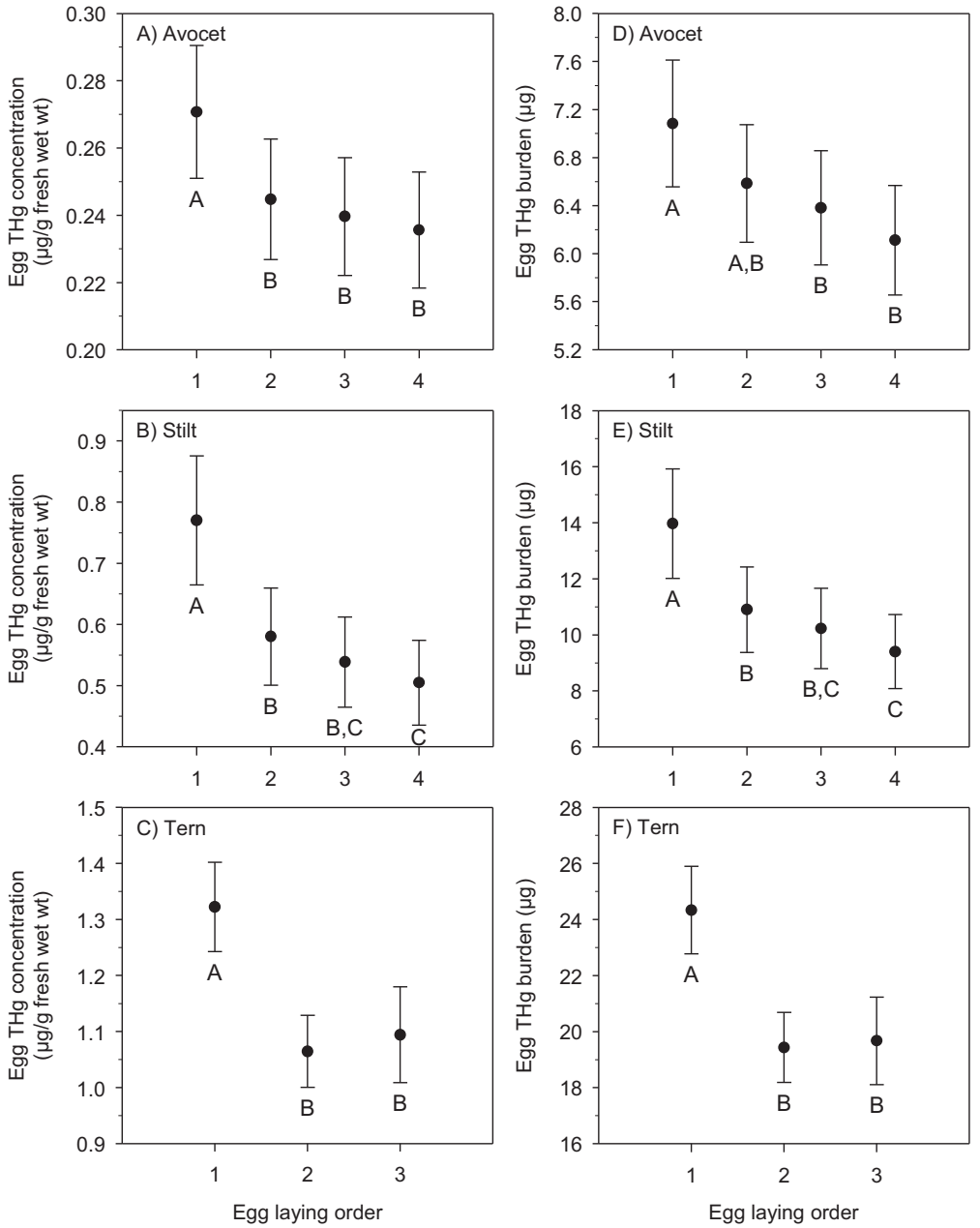

Figure 1. Mean ± standard error total mercury (THg) concentrations (**A–C**) and THg burdens (**D–F**) in eggs declined with egg-laying order in American avocets (**A**, **D**), black-necked stilts (**B**, **E**), and Forster's terns (**C**, **F**) nesting in San Francisco Bay, California, USA. Different letters below data points denote significant ($p < 0.05$) differences among egg positions in the laying order.

and the variance in egg THg concentrations within the population (Figure 5B).

DISCUSSION

Total Hg concentrations and THg burdens in bird eggs strongly differed with egg-laying order. The first egg laid had the highest THg concentrations and THg burdens in each species. Egg THg concentrations and burdens decreased in the second egg laid and thereafter did not change or declined only slightly. The average decline in THg concentrations between the first egg laid and the last egg laid was 33% for stilts, 22% for terns, and 11% for avocets; and most of this decline occurred between the first and second eggs laid (24% for stilts, 18% for terns, and 9% for avocets).

We also reviewed the literature and found that the decline in egg THg concentrations with egg-laying order was largely a result of the decline in THg concentrations between the first and second eggs laid (Table 2). Among the 17 species studied, THg concentrations in eggs (egg contents or albumen) generally declined by 16% between the first and second eggs laid (Table 2); thereafter, small successive declines in egg THg concentrations with egg-laying order generally stabilized by the fourth egg laid in species with larger clutch sizes (e.g., Kennamer et al. [22]). However, birds that were heavily dosed as part of a laboratory study continued to show large declines in THg concentrations between successively laid eggs after the MeHg supplement was removed from their diet [18]. Nearly all of the 13 studies documented a decline in egg THg concentrations with egg-laying order (Table 2). However,

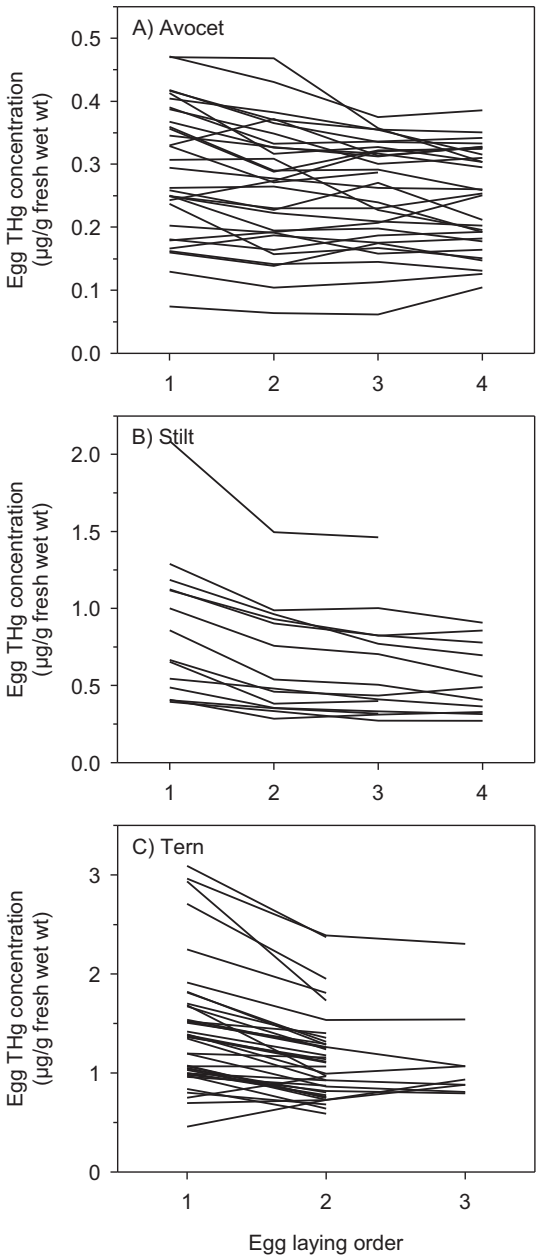

Figure 2. Total mercury (THg) concentrations in sequentially laid eggs within the same clutch for American avocets (**A**), black-necked stilts (**B**), and Forster's terns (**C**) nesting in San Francisco Bay, California, USA. Each line connects sequentially laid eggs within the same clutch.

songbirds were notable in that egg THg concentrations generally did not appear to decline with egg-laying order. In particular, the 2 songbird studies conducted on wild populations of tree swallows (*Tachycineta bicolor*) [27] and great tits (*Parus major*) [28] found no difference in egg THg concentrations and egg-laying order. In contrast, a study on captive zebra finches (*Taeniopygia guttata*) that were chronically dosed with MeHg through their diet did show a substantial decline in egg THg concentrations with egg-laying order [4]. Studies examining THg concentrations in egg yolk, and sometimes eggshell, also did not generally show a significant decline in THg concentrations with egg-laying order (Table 2). This result is still consistent with an effect of egg-laying order on whole-egg THg concentrations because most MeHg within the egg is contained within the albumen [18,22,23,29,30], and a declining trend of THg concentrations in egg albumen with egg-laying order was still found in these studies [22,31].

The relative change in egg THg concentrations between sequentially laid eggs was not related to the THg concentration in the initial egg within the clutch. Because egg THg concentrations directly relate to MeHg contamination of the female [1,4], this indicates that the overall contamination level of the mother did not influence the proportional change in THg concentrations between sequentially laid eggs. This result is similar to that of Ou et al. [4]; however, Kennamer et al. [22] found a more pronounced decline in albumen THg concentrations between sequentially laid wood duck (*Aix sponsa*) eggs when the clutches contained higher THg concentrations.

The mechanism causing egg THg concentrations to decline with egg-laying order is likely a decline in the female's body burden of MeHg with sequentially laid eggs. For example, it is estimated that female birds can eliminate approximately 20% to 40% more MeHg than males because of their ability to transfer MeHg into eggs [7,32,33]. However, changes in egg size with egg-laying order might be another potential mechanism that could contribute to variable egg THg concentrations within the egg-laying sequence. Egg size can influence the THg burden in an egg and may also influence the THg concentration in an egg if egg composition changes with egg size. For example, larger eggs often have proportionally more water (in semialtricial and semiprecocial birds) or yolk lipids (in precocial birds) than smaller sibling eggs within the same clutch (reviewed by Williams [8]). Importantly, intraclutch variation in egg size is rarely associated with differences in protein content among eggs [8], and MeHg in eggs is primarily associated with proteins within the albumen fraction of the egg, rather than the yolk [30,34]. Because it is necessary to report contaminant concentrations in bird eggs on a fresh wet weight basis [6,35], larger eggs could have lower estimated THg concentrations because of a proportionately larger mass of egg components that are not as highly associated with MeHg (i.e., water or yolk lipids). We therefore examined how egg size changed with egg-laying order.

The first egg laid was typically smaller (by volume and predicted fresh egg mass) than the second egg laid in avocets, but this trend was not significant in stilts. In terns, the first and second laid eggs were similar in size and the third egg laid tended to be smaller, although this trend also was not significant. In general, trends in egg size with egg-laying order were inconsistent among species, and the overall differences in egg sizes observed within a species were $<3\%$. Thus, we conclude that egg size likely played little role in the observed decline of egg THg concentrations with egg-laying order.

Although the vast majority (89%) of egg THg concentrations declined with egg-laying order, there were 6 of 31 avocet clutches and 3 of 39 tern clutches where THg concentrations in eggs increased with egg number in the laying sequence (Figure 2). This tended to occur when initial egg THg concentrations were relatively low, and it may indicate that females had moved into more contaminated wetlands to nest and were rapidly accumulating higher MeHg concentrations through their diet than they had been exposed to previously. Indeed, the wetlands where these increasing trends occurred were known to have relatively high mercury concentrations in birds [36,37], and captive bird studies have demonstrated that exposure to a new diet supplemented with MeHg will result in rapidly increasing THg concentrations in sequentially laid eggs [18,38].

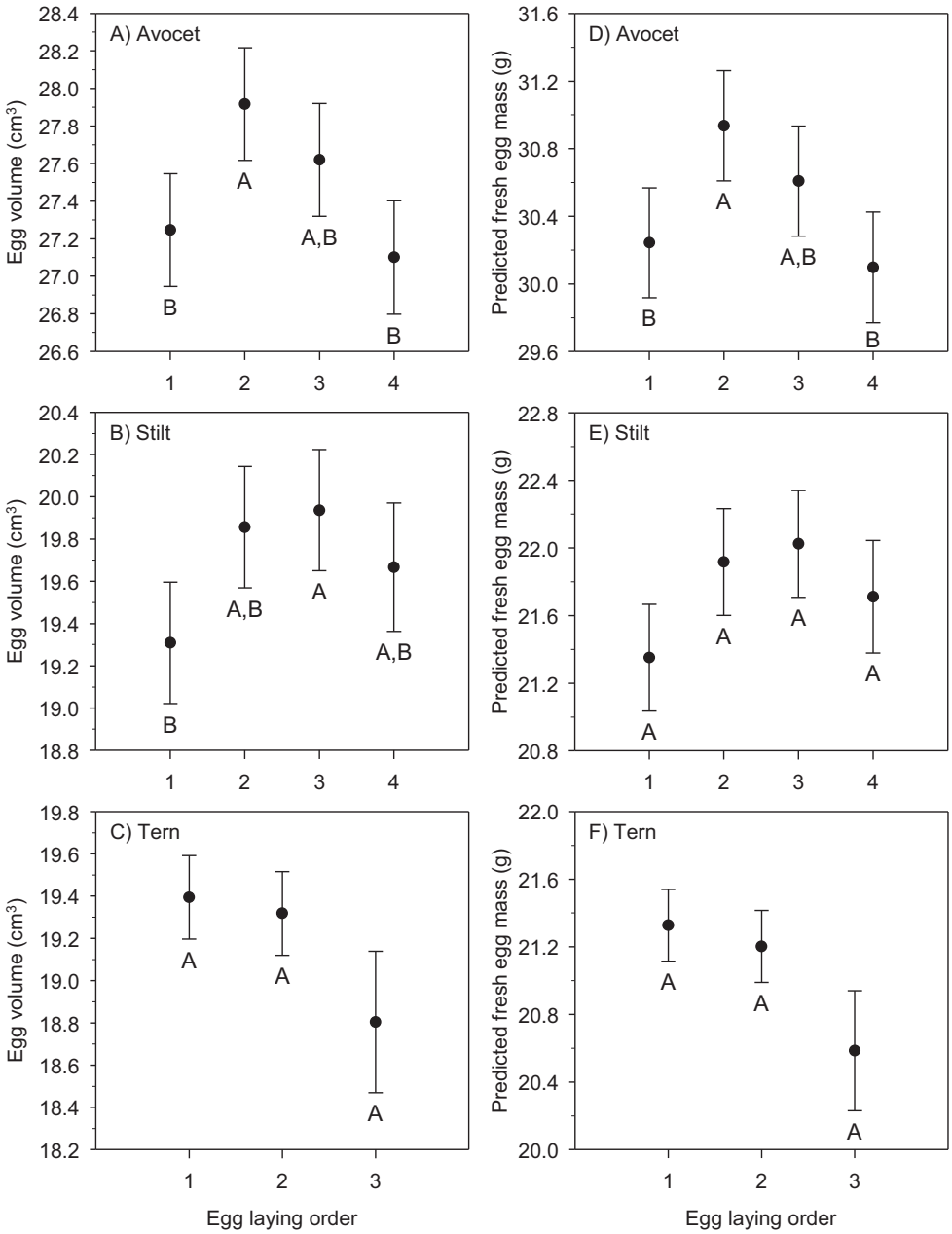

Figure 3. Mean ± standard error egg volume (**A–C**) and predicted fresh egg mass (**D–F**) versus egg-laying order in American avocets (**A**, **D**), black-necked stilts (**B**, **E**), and Forster's terns (**C**, **F**) nesting in San Francisco Bay, California, USA. Different letters below data points denote significant ($p < 0.05$) differences among egg positions in the laying order.

Despite the strong effect of egg-laying order on THg concentrations in eggs, most of the variance in egg THg concentrations still occurred among clutches (75–91%) rather than within clutches (9–25%). Also, THg concentrations in eggs within a clutch were no more variable (relative to the clutch mean) at higher overall mercury levels than at lower mercury levels. Both of these results support the use of sampling a single egg from clutches for monitoring contaminant levels in populations. We tested this directly by simulating the collection of only 1 egg from a nest and found that sampling 1 egg randomly from every nest, instead of sampling the entire clutch, would result in a highly accurate (within 4–9%) mean egg THg concentration. However, sampling 1 egg from every nest in a population is typically not possible. We therefore estimated the required sample size of nests to obtain a desired level of accuracy when only a subset of the population is sampled. When the population is large and generally undefined, we estimated that it would require sampling 1 egg from more than 60 nests to accurately estimate the population's mean egg THg concentration. The specific number of nests differed among species and depended on the variance in the population's egg THg concentrations. For example, the variance in stilt egg THg concentrations was much higher than the variance in avocet or tern eggs; therefore, the number of nests required to be sampled was much larger for stilts (111 nests) than avocets (65 nests) or terns (58 nests). When the population size is smaller and known, such as a specific colony or within a wetland site, it would require sampling fewer total nests, but a larger proportion of the population, to accurately estimate the population's mean egg THg concentration. Nearly all nests would need to be sampled in

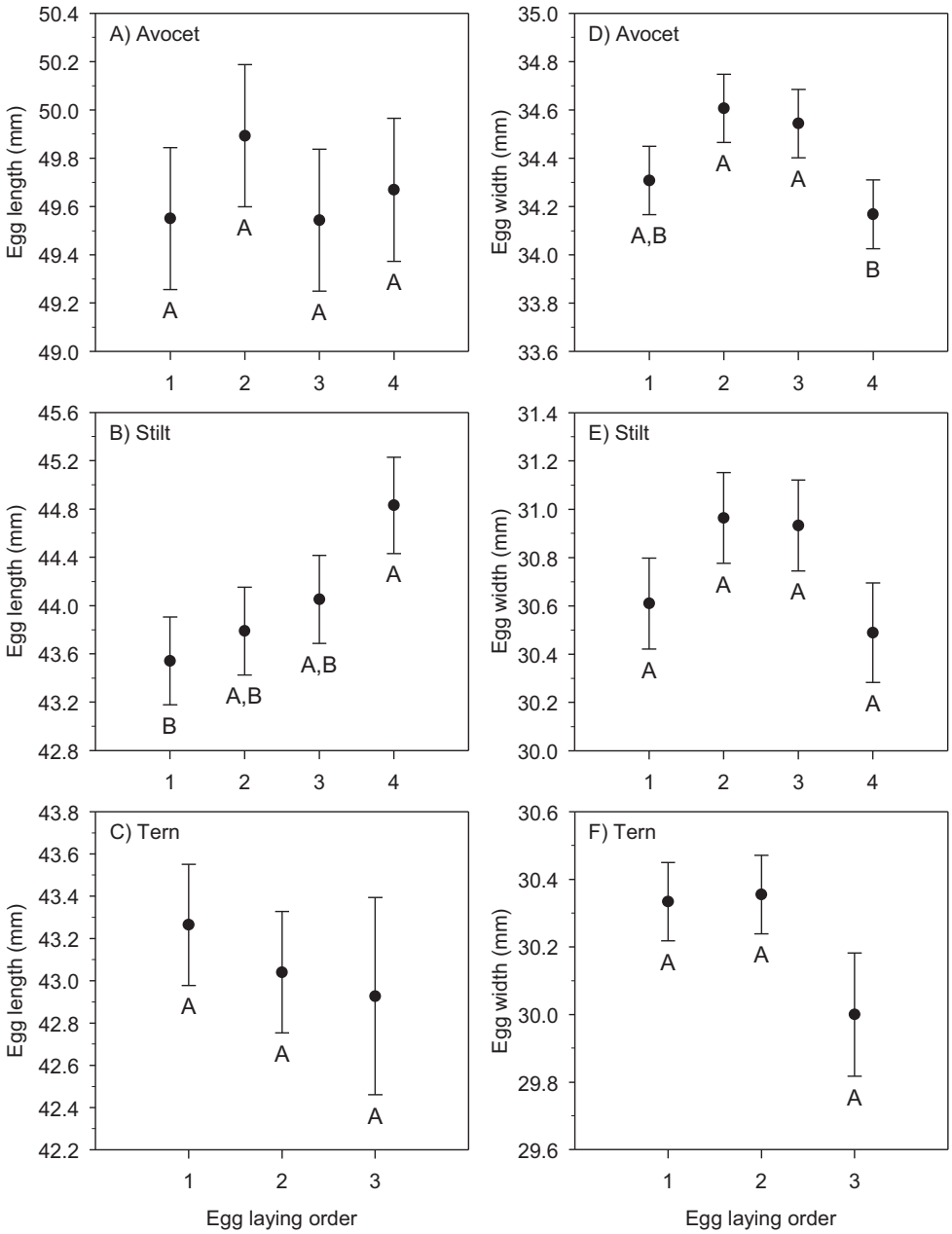

Figure 4. Mean ± standard error egg length (**A–C**) and egg width (**D–F**) versus egg-laying order in American avocets (**A**, **D**), black-necked stilts (**B**, **E**), and Forster's terns (**C**, **F**) nesting in San Francisco Bay, California, USA. Different letters below data points denote significant ($p < 0.05$) differences among egg positions in the laying order.

small colonies with <50 nests, and >50% of nests would need to be sampled in colonies up to 100 nests. Therefore, estimated mean egg THg concentrations for small populations with <100 nests are unlikely to be accurate to within 10% of the actual mean with sample sizes typical of most contaminant monitoring programs and, instead, are more likely to be accurate to within 20% of the actual mean. To be within 20% of the actual population mean egg THg concentration would require sampling 1 egg randomly from only ≥14 nests, a much more practical goal for contaminant monitoring programs, when the colony size is 100 nests or fewer. In summary, to accurately estimate a population's mean egg THg concentration using only a single random egg from a subset of nests would require sampling >60 nests to represent a large population (with 10% accuracy; Figure 5A) or ≥14 nests to represent a specific colony or wetland site that contained a population of <100 nests (with 20% accuracy; Figure 5B). Similar sample size requirements would be necessary for other bird species or populations where variance in egg THg concentrations is comparable to that of any of the 3 species in the present study. Sampling fewer eggs is an option for contaminant monitoring studies comfortable with lower accuracy, and Figure 5 can be used to estimate the accuracy of the mean as a function of the sample size.

We also tested whether sampling a single egg from a clutch would adequately represent the toxicological risk of mercury to the clutch itself. Sampling only 1 egg randomly per clutch would result in an egg THg concentration that will be within 21% to 34% of the actual clutch's mean egg THg concentration. This error is the result of the large intraclutch variation in egg THg concentrations caused mainly by the position of the egg

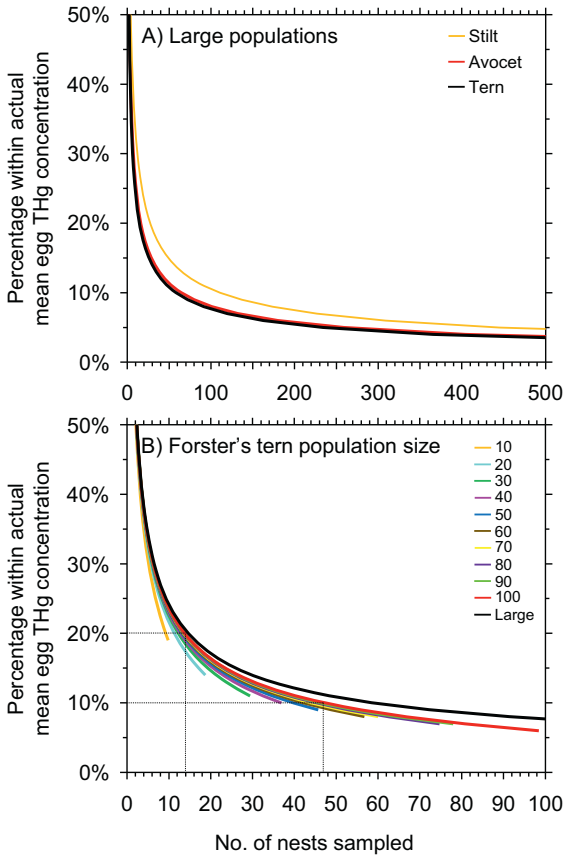

Figure 5. Relationship between sample size of nests (1 egg randomly sampled per clutch) and percentage error in the geometric mean egg total mercury (THg) concentration. The *y* axis represents the 95% confidence that the estimated geometric mean egg mercury concentration is within a specific percentage of the actual population's geometric mean egg mercury concentration, based on simulations that randomly sampled a single egg from 84 nests of 3 species. (**A**) Estimated number of nests that would need to be sampled (1 egg randomly sampled per clutch) when the population size is large and undefined for black-necked stilts (orange curve), American avocets (red curve), and Forster's terns (black curve). (**B**) Estimated number of nests that would need to be sampled (1 egg randomly sampled per clutch) when the population size is smaller and known, such as a specific colony or wetland site, for Forster's terns. Different-colored lines represent population sizes from 10 nests to 100 nests, and the black line represents a large or undefined population size without the finite population size adjustment (i.e., the black lines in the top and bottom panels are the same). Curves stop at the point where an egg has been sampled from every nest in the population; thus, it would require sampling more than 1 egg per clutch from every nest in the population to further reduce the error that is associated with the estimated mean. Stippled lines in (**B**) highlight the number of Forster's tern nests that would be required to be sampled to be within 10% (47 nests) and 20% (14 nests) of the actual population's mean egg mercury concentration when the colony size is 100 nests (red curve).

within the laying sequence. Thus, studies using a single egg's THg concentration to represent the mean THg concentration in the clutch should either account for egg-laying order (statistically or methodologically, by sampling a fixed egg position) or increase their sample size of nests to overcome the uncertainty associated with high intraclutch variation.

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Data availability—Data are available upon request to the authors (jackerman@usgs.gov).

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