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Exposure to Dietary Methyl-Mercury Solely during Embryonic and Juvenile Development Halves Subsequent Reproductive Success in Adult Zebra Finches

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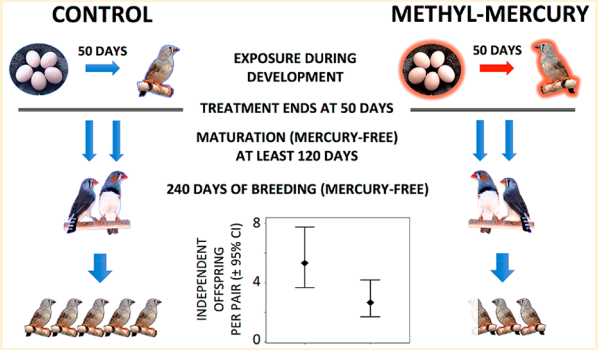
Supporting Information

ABSTRACT: Long-term exposure to methyl-mercury has deleterious effects on avian reproduction. However, little is known about whether exposure to mercury solely during embryonic and juvenile development can have long-lasting effects on subsequent reproductive performance as adults. Birds that hatch on contaminated sites but disperse elsewhere will be exposed only during development. Hence, it is important to understand the reproductive consequences of avian exposure to methyl-mercury during early life. Accordingly, in this experiment, domesticated zebra finches (*Taeniopygia guttata*) were exposed to dietary methyl-mercury (1.20 μg/g wet weight) from conception through independence (50 days post-hatching). Following maturity, developmentally exposed and control finches were paired within treatment groups and allowed to breed repeatedly for 8 months. Developmentally exposed pairs hatched 32% fewer eggs and produced 50% fewer independent juveniles despite transferring only traces of mercury into their offspring. This is the first example of mercury-related reproductive declines in birds not exposed to mercury during breeding. The magnitude of reproductive decline was similar to that of zebra finches exposed to methyl-mercury during the breeding process. Bird populations breeding in contaminated habitats may suffer from a 2-fold fitness cost of mercury exposure; adult exposure compromises parents' reproduction, while offspring exposure results in reduced reproduction in the next generation.



INTRODUCTION

Mercury (Hg) in the biosphere is readily converted by bacteria to methyl-mercury (hereafter referred to as MeHg), which is highly bioavailable and can disrupt the functions of vital physiological and neural systems in a broad variety of taxa.[1−3] When MeHg is ingested, it readily crosses cell membranes and the blood−brain barrier and can biomagnify to toxic concentrations at higher trophic levels in ecosystems.[1,3,4] Exposed populations of animals at higher trophic levels, including terrestrial songbirds, can accumulate detrimental and sometimes lethal amounts of MeHg.[1,3−5] Even at sublethal exposures, MeHg can have significant fitness consequences for many avian species, including suppression of breeding (e.g., refs 6−11). Exposure to dietary MeHg may affect avian reproduction through many pathways, including disruption of endocrine system functions,[12,13] alteration of behaviors associated with pairing and parenting,[8,10,14] and direct embryotoxicity acting in ovo.[15] In combination with human-induced habitat degradation and global climate change, MeHg pollution may exacerbate bird population declines and further threaten endangered populations.[5,16]

Songbirds, a taxon of economic, ecological, and conservation importance,[17] suffer deleterious Hg-related reproductive declines.[18] For example, Carolina wrens (*Thryothorus ludovicianus*) with blood total Hg in the range of 1−4 μg/g wet weight (ww) exhibited an increased rate of nest abandonment and reduced reproductive success at sites contaminated by industrial Hg.[6] Tree swallows (*Tachycineta bicolor*) with parental blood total Hg averaging 3.0 μg/g ww exhibited a 20% reduction in the number of offspring produced.[11] Although correlational field studies such as these have shown reproductive declines, causation cannot be directly attributed to Hg because polluted areas may also differ in other ways that affect the birds.

Controlled dosing studies using domestically bred zebra finches (*Taeniopygia guttata*) have demonstrated experimentally that MeHg causes reproductive deficits in adult songbirds. Finches had a 42% reduction in reproductive output (number of independent offspring produced in one year) when birds were fed a diet of 1.20 μg/g ww MeHg during breeding, resulting in mean Hg blood levels of approximately 16.5 μg/g ww.[7] The reproductive decline was similar whether zebra finches were exposed to MeHg only during breeding (hereafter

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adult exposure) or throughout embryonic and juvenile development and for their entire subsequent lives (hereafter lifetime exposure). Because Hg is known to disrupt endocrine and neurological functions,[1] which have critical roles in development,[2,19] Hg-related developmental stress may have far-reaching consequences for later-life fitness.[19] Exposure during critical periods in development may alter developmental trajectories and later-life fitness,[20,21] with impairments that could differ in kind and scope from ones borne of acute, adult exposure.[22,23] However, the question of whether exposure to MeHg that occurs only during development can result in lingering effects on subsequent breeding success as adults has not been answered. This question is ecologically important because many birds hatch on contaminated sites but disperse or migrate to uncontaminated areas before reaching full reproductive adulthood.

Songbirds can rapidly depurate MeHg from their bodies,[24] hence, it is not clear whether MeHg exposure that occurs only during early life development will have effects on reproductive success of the exposed individuals once they have matured into adults, long after exogenous MeHg exposure has ceased. Avian embryos might be expected to be more sensitive to MeHg exposure than adults,[15,23] but recent MeHg egg-injection studies with zebra finch eggs failed to detect differences between MeHg-exposed birds and controls in important reproductive metrics during adulthood, such as latency to breeding, clutch size, and offspring hatching or survivorship.[25] The only lingering effect of MeHg detected in adults was a significant increase in the volume of the adult telencephalon of the exposed birds' brains.[26] The injection of MeHg directly into the albumen of the egg, as in these two cited studies, allows for precise regulation of the amount of toxicant present in the egg but may not expose the embryo to MeHg in the same way as occurs when a female naturally deposits MeHg into an egg.[15] We used a different method of early MeHg-exposure, allowing maternal transfer into the egg by dosing parents and continuing exposure of nestlings after hatching by providing dosed food. In this way, we simulated what occurs when birds breed on a Hg-contaminated site.

Specifically, we exposed an initial generation of breeding zebra finches (generation F0) to environmentally relevant[7] dietary levels of MeHg (1.20 μg/g ww), which ensured maternal deposition of the toxicant into the egg and initiated exposure of the F1 embryo. F1 nestlings remained exposed through their diets until 50 days after hatching, and then were switched permanently to an MeHg-free diet. Following sexual maturation, F1 birds were paired with members of their own treatment group. Their reproductive success was monitored and compared to that of pairs raised under identical conditions but with no history of exposure to MeHg. Our objective was to understand whether songbirds with a history of developmental-only exposure to MeHg suffered permanent fitness reduction, even when exposure to a dietary source of MeHg had ceased long before breeding. Based on the neurological and endocrine effects of MeHg reported in the literature, we hypothesized that developmentally exposed birds would experience altered reproduction later in life due to the latent effects of developmental exposure. Specifically, we predicted that early-life exposure to MeHg (until 50 days post-hatching) would delay pair formation and nesting, reduce hatching success and offspring survival (despite no direct Hg exposure in the offspring generation), and result in production of fewer fledglings and offspring that survive to independence.

EXPERIMENTAL SECTION

All research was conducted at The College of William and Mary aviaries in Virginia, United States, between 2015 and 2017. The study complied with the recommendations of the National Institutes of Health Guide of the Care and Use of Laboratory Animals. All procedures and protocols were approved and overseen by The College of William and Mary's Institutional Animal Care and Use Committee (IACUC 2012-05-23-7982). Birds were kept under constant, monitored environmental conditions (14:10 light/dark photoperiod at approximately 23 °C), in cages housed in a single room, with ad libitum access to food, water enriched with vitamins (Vitasol), oyster shell grit, and a cuttlefish calcium supplement. To confirm that our avian husbandry was comparable with that of other colonies, we compared three measures of reproductive performance of control pairs to averages reported for domestic zebra finch colonies.[27] Proportion of hatchlings to fledge was 18% lower in this study than the published average, while proportion of females to fledge offspring was 10% higher, and the proportion of females that laid a clutch was similar to the reported averages.

Experimental Treatments. Control birds were fed a commercial pelletized diet (Zupreem FruitBlend). Exposed birds were fed the same diet dosed with 1.20 μg/g wet weight (ww) MeHg-cysteine, prepared as described for previous studies (see ref 7 and Figure S1), equivalent to 1.39 μg/g on a dry weight (dw) basis). The MeHg dosing concentration of 1.20 μg/g ww was chosen because it is high enough to have a documented effect on reproduction yet sublethal for zebra finches and is similar to concentrations in predatory arthropod prey items (up to 1.24 μg/g dw) eaten by songbirds living in forests and grasslands downstream of a heavily contaminated industrial site in Virginia.[4]

The subjects that were the focus of the reproductive study here, F1 birds, were produced by F0 breeding pairs and reared by foster parents to allow the same F1 breeding pairs to switch diets and produce eggs for both control and MeHg treatments. The effects of potential genetic differences between birds in sensitivity to Hg were minimized by dividing siblings across the two treatments. F0 breeding pairs produced offspring for one treatment, and, after a transition period of at least 8 weeks to allow for MeHg depuration, produced offspring for the other treatment. Whether F0 genetic parents produced their earlier clutch on a control or MeHg diet was randomized, therefore spreading any effects of clutch order among treatments. Fostering was accomplished by substituting clutches produced by F0 parents for those of foster parents at a similar nesting stage. F1 birds in the control treatment were the offspring of F0 breeding pairs and foster parents on a diet without MeHg. After hatching, control F1 subjects were reared by foster pairs (n = 17) on the same MeHg-free diet until independence (50 days post-hatching). F1 birds in the MeHg-exposed treatment were the offspring of F0 breeding pairs fed a 1.20 μg/g ww MeHg diet, and were reared by foster pairs (n = 18) exposed to the same MeHg diet until independence. When F1 birds completed their treatments, 50 days after hatching, they were moved from their foster parents' cages into single-sex cages of birds of a similar age from both treatments and fed a control diet for the rest of their lives (Figure 1).

This experimental design does not control for possible effects of differential incubation or rearing quality between control and MeHg-exposed foster parents. Such effects could have affected

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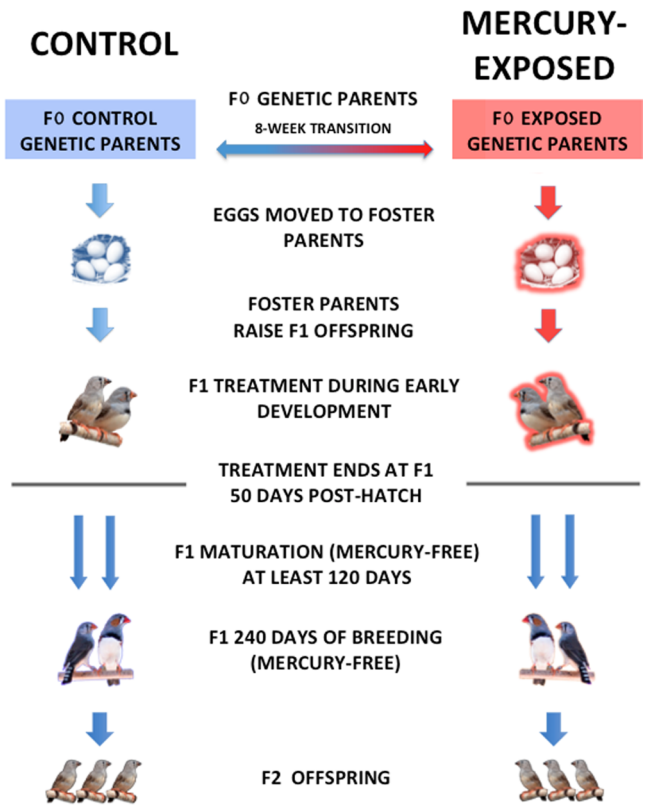

Figure 1. Process of producing control and exposed F1 birds from genetic F0 parents. F1 birds were the offspring of the same F0 genetic parents in each of the two treatments: control or mercury-exposed. Eggs were moved from genetic parents to foster parents on the same treatment. F1 birds were reared by foster parents until the end of the treatment period, 50 days post-hatching. F1 birds remained unpaired for at least 120 days before breeding.

juvenile F1 birds independent of the direct effects of early exposure to dietary MeHg that are the subject of this study. A previous study conducted on the same flock, using similar rearing conditions and the same dietary concentration of MeHg, did not detect effects of MeHg dosing on parental incubation or provisioning behaviors,[28] indicating that any such effects in the current study were likely insubstantial. Nevertheless, to address the possibility of rearing effects, we compared the performance of F1 control birds to that of an additional treatment group that was raised identically to controls until independence but then received MeHg exposure for the same length of time as the MeHg group in this study. These late-developmental exposure birds were siblings of the control and MeHg-exposed birds and were raised concurrently by the same set of foster parents but exposed to MeHg only from 50 through 114 days post-hatching. Thus, any effects of late-developmental exposure to MeHg cannot be mediated through Hg effects on genetic or foster parents. This group of late-development exposed siblings exhibited a reduction in key reproductive metrics that is comparable to reductions observed in the exposed group in this study. The results of this comparison (see Figure S2 for a detailed review of the late-exposure group's reproductive performance) were consistent with the conclusion of the present study because they indicate that effects of developmental exposure to MeHg on reproductive capacity later in life manifest independently from any effect of mercury on parent/foster behavior.

Pairing. F1 pairs were composed of males and females from the same treatment group (control or MeHg-exposed), in which the two individuals were no more than 0.125 related (mean relatedness ± SD: control 0.021 ± 0.019, n = 25; exposed 0.030 ± 0.025, n = 34). Within each treatment, pairs were composed of unique combinations of F0 parental lineages (i.e., if members of one pair were birds A and B, no sibling of A was paired with a sibling of B). Age differences within and between pairs were minimized by avoiding pairing unusually young or old birds together. The average age of each pair was kept within 300−530 days post-hatching (mean days ± SD: control, 369 ± 55; exposed, 390 ± 64). To initiate pairs, a male and female were placed in a 40 cm × 60 cm × 36 cm wire cage and provided with a wooden nest box measuring 12 cm × 7 cm × 18 cm. After a 1 day acclimation period, nesting material was provided and the process of data collection began, with that point defined as day 0 of the first nesting cycle. A total of 25 control pairs and 34 exposed pairs were established during the study.

Measures of Nesting, Reproduction, and Survival. Immediately following pair formation (day 0), we collected the following breeding and reproductive data through daily nest checks: (i) latency to lay the first egg in a nest constructed in the nest box, measured in days; (ii) clutch size (in which a clutch is defined as a group of eggs that were laid consecutively with no more than 4 days between subsequent eggs); (iii) interclutch interval (defined as the time interval, in days, between the end of one nesting cycle and the laying of a first egg in the next cycle); (iv) hatching success (proportion of eggs in each cycle from which nestlings emerged completely); (v) fledging success (probability of nestling surviving for 20 days after hatching); (vi) offspring success (probability of offspring surviving for 48 days after hatching, which is when they can live independently of parents); (vii) pair survival (probability that both members of a pair lived through the entire 8 month breeding period); and (viii) reproductive output (number of 48 day old offspring produced per pair over an 8 month breeding period). This latter metric served as our ultimate fitness measure and was a cumulative summation of the other components of pair reproductive success that encompassed all reproductive metrics while also accounting for pair survival.

With the beginning of data collection (day 0) or a new nesting cycle, pairs were provided with approximately 20 g of Timothy and Orchard grass hay for nest building, which was replenished every day until an egg was laid inside the nest box. If a pair did not initiate breeding (i.e., no eggs were laid in the nest) in the first 60 days after pairing, the pair was separated and its members put back into single-sex cages. Eggs laid in nest boxes were numbered with a marker (Sharpie, thin) for identification. The corresponding chicks had their back feathers colored for identification (Crayola Broad Line Markers, nontoxic) until 24 days after hatching, when they were banded with uniquely numbered metal bands. All eggs and offspring were handled by researchers wearing nitrile gloves. A nesting cycle was considered complete when all offspring died or all surviving offspring reached independence. Pairs were not allowed to raise more than one clutch in any given cycle, even in those rare cases in which additional eggs were laid before fledglings gained independence. Most data collection was performed by O.J.P., with a minority (approximately 10%) performed by trained assistants.

Mercury Analysis. Analysis of diet, blood, and eggs was for total Hg content using a Milestone DMA-80 with extensive

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quality controls (see the Supporting Information). Because the pelletized diet contained only MeHg, and avian blood and egg Hg is consistently ~95% MeHg,[29] total Hg is a suitable proxy for MeHg. Each batch of food (made approximately weekly) was tested to ensure that it was within 5% of the target dose of 1.20 μg/g ww. Blood samples were taken from breeding pairs at the time of pairing to establish the initial MeHg blood concentration, and also once per month from a random sample of 20% of pairs, to ensure that accidental MeHg exposure had not occurred. To determine the amount of MeHg depurated into eggs, without affecting our measurements of reproductive success, we collected one egg from any female that laid outside of a nest box, a behavior observed in pairs with unfinished nests or offspring approaching independence. Sampled eggs were frozen whole, freeze-dried, and assayed for total Hg on a dw basis, and then these measurements converted to ww based on the moisture content of each egg.

Statistical Analysis. To examine whether early developmental exposure to MeHg influenced metrics of reproductive success in the breeding pairs, our analysis focused on the effects of the treatment on each of the measures of reproductive success: latency to lay the first egg, clutch size, interclutch interval (latency periods of pair, in days, from the end of one breeding cycle to the laying of the first egg in the next cycle), hatching success (proportion), fledging success (proportion), offspring success (proportion), pair survival, and total reproductive output over 8 months. For each analysis, we fitted a general or generalized linear model with treatment type as a fixed effect. Additional co-variates were included in models if they were significantly linearly correlated with the dependent variable (Pearson's correlation coefficient, $P < 0.05$), and the AICc was no more than 2 points above that of the model with treatment type as the only main effect. A comparison of clutch sizes was performed using a generalized linear mixed model with a Poisson distribution, logit link, a co-variate of clutch number, and a random effect of pair, with PROC GLIMMIX in SAS 9.4. A comparison of interclutch intervals was performed using a generalized linear mixed model with a Poisson distribution, logit link, and a random effect of pair. To compare hatching success, which is a proportion measure, we used a generalized linear mixed model with a binomial distribution, logit link, and a random effect of pair, with PROC GLIMMIX in SAS. A comparison of reproductive output was performed using a generalized linear model with a Poisson distribution and a log link function, with PROC GENMOD in SAS. Residual over-dispersion was adjusted by estimating the Pearson dispersion parameter and inflating the covariance matrix of the parameter estimates by this factor.[30] Welch's t tests were used to test for differences in natural log transformed blood and egg Hg concentrations using PROC TTEST in SAS.

Comparisons of time-to-event measures, latency to breed, fledging success, and offspring success were analyzed using Cox Proportional Hazards Models with PROC PHREG in SAS. Proportional hazards assumptions were tested using SAS PROC PHREG and the R package Survival. In the analysis of fledging and offspring success, the experimental units, F2 offspring, were hierarchically derived from individual F1 parental pairs, which were the subjects of this study. Therefore, siblings were included within a parental pair shared frailty (a random factor that accounts for characteristics that are shared by a group of subjects with non-independent survival times).[31] It is important to assess the level of dependence between pairs of reproductive parameters to identify predictive relationships, as well as extraneous factors that may impact earliest-occurring variables initially, and later-occurring variables indirectly. Correlations between variables were tested and no unexpected dependencies were identified. The sample size obtained for pairs that underwent the initial successful event of egg-laying was larger than that for events subsequent to egg-laying because some pairs did not complete all of these, i.e., may not have hatched any nestlings or may have died before completing 8 months of breeding.

RESULTS

Tissue Hg Concentrations. We measured total Hg concentration in the blood of all F1 birds in the middle of the treatment period (25 days post-hatching, shortly after fledging) and at the end of the treatment period (50 days post-hatching, shortly after independence). At 25 days after hatching, the juvenile F1 finches that would eventually become the control breeding pairs in this study had a geometric mean of 0.004 μg/g ww (95% CI of 0.002−0.007 μg/g ww, range of 0.000−0.050 μg/g ww), compared to 7.411 μg/g ww (95% CI of 7.000−7.850 μg/g ww, range of 4.627−12.088 μg/g ww) for their MeHg-exposed counterparts ($t = -27.83$, df = 36.9, $P < 0.001$, $n_{control} = 37$, $n_{exposed} = 48$; Figure S3). At 50 days after hatching, the control birds had a geometric mean of 0.005 μg/g ww (95% CI of 0.004−0.007 μg/g ww, range of 0.000−0.038 μg/g ww), compared to 10.498 μg/g ww (95% CI of 9.926−11.103 μg/g ww, range of 6.607−16.548 μg/g ww) for the exposed group ($t = -57.16$, df = 47.2, $P < 0.001$, $n_{control} = 44$, $n_{exposed} = 52$; Figure S4). By the time of pairing, Hg concentration in the blood of the F1 zebra finches in both treatments was approximately 2 orders of magnitude below typical reproductive effects levels[32] for bird blood (control geometric mean = 0.010 μg/g ww, 95% CI of 0.008−0.013 μg/g ww, range of 0.003−0.067 μg/g ww, $n = 43$; exposed geometric mean = 0.014 μg/g ww, 95% CI of 0.011−0.019 μg/g ww, range of 0.003−0.233 μg/g ww, $n = 64$; $t = -1.89$, df = 104.9, $P = 0.06$; Figure S5). Eggs laid by F1 finches in both control and exposed treatments also had low Hg concentrations (control geometric mean = 0.0013 μg/g ww, 95% CI of 0.0010−0.0018 μg/g ww, range of 0.0013−0.0022 μg/g ww, $n = 5$; exposed geometric mean = 0.0039 μg/g ww, 95% CI of 0.0021−0.0070, range of 0.0013−0.0409 μg/g ww, $n = 12$; Figure S6). Mean Hg concentrations were higher in eggs from exposed females ($t = -3.16$, df = 14.7, $P = 0.01$) but still 2−3 orders of magnitude lower than reported in ovo effects thresholds in songbirds (2.7 μg/g ww for a 20% effect concentration in zebra finches but as low as 0.6 μg/g ww for no observed adverse effect concentration in tree swallows, *Tachycineta bicolor*; ref 32). Therefore, we believe in ovo exposure to MeHg is unlikely to have produced negative effects on F2 offspring during this study. A small sample of F2 nestlings of Hg-exposed pairs, at 24 days after hatching, also had low blood Hg concentrations (mean = 0.0032 μg/g ww, 95% CI of 0.0010−0.0050 μg/g ww, $n = 3$).

Nesting, Reproduction, and Survival. Exposed pairs had a lower probability of initiating breeding than control pairs, but the difference was not statistically significant ($\chi 2 = 2.61$, df = 1, $P = 0.11$, hazard ratio = 0.60, 95% CI 0.32−1.11, $n_{control} = 25$, $n_{exposed} = 34$; Figure S7). This result can be interpreted as a 62.5% chance that a given control pair would initiate breeding before an exposed pair.[31]

Developmental exposure did not significantly affect clutch size ($t = 0.75$, df = 96, $P = 0.46$; control mean = 4.43 eggs, 95%

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CI of 3.93−4.98 eggs, n = 20 pairs; exposed mean = 4.72 eggs, 95% CI of 4.17−5.34 eggs, n = 21 pairs; Figure S8). The effect of nesting cycle order on clutch size was marginally significant (F-value = 2.97, P = 0.09), indicating a possible 2.77% increase in clutch size with each subsequent nest cycle. Interclutch intervals of the treatments did not differ (t = −0.82, df = 99, P = 0.41; control mean = 4.45 days, 95% CI of 3.25−6.62 days, n = 19; exposed mean = 3.66 days, 95% CI of 2.77−5.21, n = 16). Exposed pairs had statistically significantly lower hatching success than controls (t = −3.05, df = 97, P = 0.003; Figure 2).

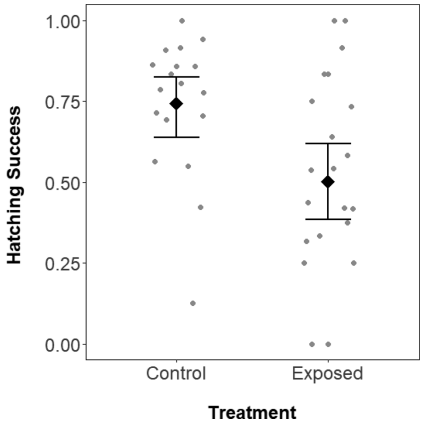

Figure 2. Differences in proportional hatching success (eggs hatched divided by eggs laid) between control (n = 18) and mercury-exposed (n = 21) zebra finch pairs, with 95% confidence intervals. Black diamonds represent treatment means from the generalized linear mixed model. Gray dots represent the mean hatching success of individual pairs.

The hatching success of exposed pairs (mean = 0.50, 95% CI of 0.39−0.62, n = 21) was 32% lower than that of control pairs (mean = 0.74, 95% CI of 0.64−0.83, n = 18). An analysis of hatching success that excluded the few birds in the exposed treatment with lingering elevated Hg in blood at the time of pairing did not alter conclusions (see Figure S9).

There was no statistically significant difference in the probability of survival of offspring to 48 days post-hatching (offspring success) ($\chi 2$ = 0.23, df = 1, adjusted P = 0.14, hazard ratio = 1.21, 95% CI of 0.55−2.66, $n_{control}$ = 18 pairs and 197 offspring, $n_{exposed}$ = 19 pairs and 182 offspring; Figure 3) or to age 20 days (fledging success) ($\chi 2$ = 0.30, df = 1, adjusted P = 0.13, hazard ratio = 1.28, 95% CI of 0.54−3.03, $n_{control}$ = 18 pairs and 198 offspring, $n_{exposed}$ = 19 pairs and 183 offspring; Figure 3). In both analyses, the effect of breeding pair (shared frailty) was significant (P < 0.001), indicating that there was substantial variation within treatment groups in the ability of pairs to raise offspring.

The reproductive output of exposed pairs (mean = 2.64 offspring, 95% CI of 1.67−4.15, n = 22) was statistically significantly lower than that of controls (mean = 5.31 offspring, 95% CI of 3.65−7.73, n = 16) across the 8 month breeding period ($\chi 2$ = 5.43, df = 1, P = 0.02; Figure 4). On average, exposed pairs produced 50.4% fewer independent offspring than controls. It is regrettable that some breeding pairs in the exposed treatment retained Hg levels above those of controls at the time of pairing, but an analysis of reproductive output that excluded these few birds with lingering elevated Hg in blood at the time of pairing did not alter conclusions (see Figure S9).

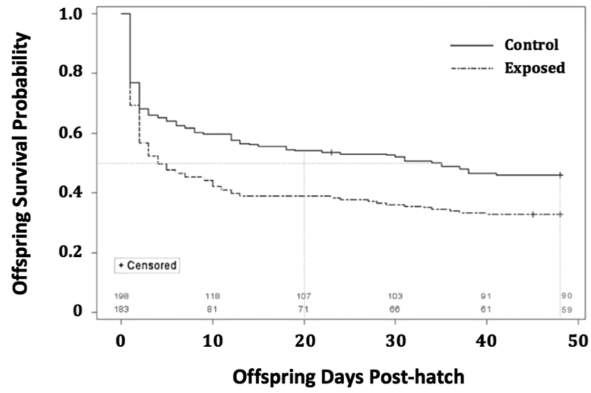

Figure 3. Differences in the survival of offspring from control pairs and mercury-exposed pairs represented by Kaplan−Meier survival curves (the plot does not reflect shared frailty effects). The numbers above the x-axis indicate living offspring of control pairs (upper row) and exposed pairs (lower row) at each time point. The thin gray lines point to day 20 and day 48 time points, corresponding to the metrics of fledging and offspring success. The horizontal gray line indicates the median survival time of the two treatments. Individuals whose survival could not be tracked beyond a given point on the timeline were removed (censored) from the analysis (indicated by a cross on the survival curve).

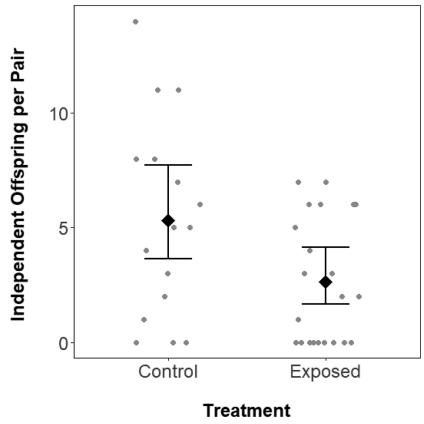

Figure 4. Differences in reproductive output (number of independent offspring produced in 8 months) between control (n = 16) and mercury-exposed (n = 21) zebra finch pairs, with 95% confidence intervals. Black diamonds represent treatment means from the generalized linear model. Gray dots represent the reproductive output of individual pairs.

DISCUSSION

Birds exposed to dietary MeHg during embryonic development and fed MeHg contaminated food until independence had notably reduced reproductive success in adulthood, even though they were not exposed to a dietary source of MeHg during adult life. The mean hatching success of exposed pairs was only 68% that of controls, and the total reproductive output of exposed pairs was half that of controls. While the deleterious effect of MeHg on avian reproduction is not a novel finding, this is the first study to report that exposure to MeHg solely during embryonic (in ovo maternal deposition) and juvenile development (to 50 days after hatching) is sufficient, when reared by exposed parents, to generate similar suppression of reproduction as was observed in the same species exposed to MeHg throughout their entire lives.[7] Given that only very low concentrations of MeHg were present in

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developmentally exposed birds while they were breeding (similar to the MeHg concentration of control birds and orders of magnitude below known effects thresholds), our findings indicate that embryonic and juvenile development is particularly sensitive to MeHg-exposure and causes lasting damage to systems regulating reproduction later in life. While it is important to note that these effects cannot be definitively teased apart from any possible effects of MeHg dosing on incubation and provisioning behaviors of foster parents, a previous study conducted on the same flock, using the same concentration of MeHg, found no such effects,[28] thus, any differences in rearing environment due to MeHg-exposed foster parents likely did not contribute much to the disparity in later reproductive performance of control and exposed pairs. Additionally, we raised another cohort of finches with control parents and control foster parents but with exposure to Hg that began after independence and ran for the same duration as the Hg-exposed birds in this study and found strong deleterious effects of Hg on adult reproductive success in those birds as well. Based on those additional studies (ref 28 and Figure S2), we are confident that the results of this study indicate the direct effects of early developmental exposure to Hg on later reproductive success.

The most-comprehensive measure of fitness in our study was the final count of independent offspring produced per pair in 8 months of breeding. Exposed pairs produced on average 50% fewer independent offspring than controls during this period. Thus, the consistently poorer performance of the exposed treatment at each step of the reproductive process, although not statistically different for some metrics, compounded into a large reduction in fitness relative to controls. This 8 month breeding period was sufficiently long for some pairs to successfully rear four clutches, as all offspring that hatched within the 8 month period were included in the data even if they did not reach independence until afterward.

In addition to its disruptive effects on the growth and maturation of the central nervous system,[2] and general neurotoxicity, MeHg also acts as an endocrine disruptor.[33] For example, disruption of appropriate development of the endocrine system could have serious effects on somatic functions and eventual reproductive phenotypes.[34] Our results contrast with a recent study in which MeHg was injected directly into eggs of zebra finches and no differences were detected in reproductive success of these birds once they became parents.[25] This disparity in the results of the two experiments, both of which examined delayed effects of early exposure to MeHg, may stem from any of several methodological differences: timing of exposure to the toxicant (in ovo only versus in ovo plus 50 days), the differential effect of MeHg when injected into an egg by researchers versus maternal transfer through dietary exposure, and/or differential behavior of foster parents (e.g., reduced incubation and provisioning in Hg-exposed foster pairs), although the latter is unlikely considering previous results[28] and additional data provided here (see the Supporting Information S2). It may be the case that neuroendocrine or other systems related to reproduction are most sensitive during the period of rapid growth and development after hatching. During the egg-injection studies, molting of natal down would likely have reduced MeHg concentrations soon after hatching.[35,36] Alternatively, MeHg injected into albumen may not have the same effect on the developing brain as that which is maternally transferred into the egg. We are not aware of studies that directly compare the relative toxicity of the same concentration of MeHg when injected directly into the egg versus maternal transfer, but injections are generally thought to be more toxic.[15] Despite the potential greater toxicity of in ovo injections, developmentally exposed birds in the current study exhibited greater reproductive declines than did in ovo injected birds,[26] suggesting that greater sensitivity of post-hatching developmental processes to MeHg exposure may best explain the discrepancy between study results.

A previous study on the same colony used for the present study found that zebra finches exposed to the same concentration of MeHg (1.20 μg/g ww) throughout their lives experienced an approximately 25% reduction in fledging success and 40% reduction in output of independent offspring regardless of whether exposure was for their entire lifetime (including in ovo) or only during breeding.[7] The close match in results between lifetime exposure, adult exposure, and the developmental exposure used in the present study (23% reduction in fledging success and 50% reduction in output) further indicates that MeHg exposure during development inflicts permanent damage on reproduction-related neuroendocrine systems. Exposure during the breeding process may create the same degree of impairment in different ways, such as through increased oxidative stress on parents[37] or direct embryotoxicity after maternal transfer of MeHg.[15]

The effects we have detected of developmental MeHg exposure on adult reproduction are important in the context of avian conservation; a population would require substantially more time and effort to replace itself, if that were even possible, given the 50% reduction in reproduction that we documented here. Clearly, that degree of reproductive suppression could have considerable effects on population viability even if adult birds are never directly exposed to MeHg. In addition, if the population under consideration was a migratory songbird, in which migration can increase rates of mortality by factors of 4–15,[38−40] many developmentally exposed individuals would not survive to make the additional breeding attempt(s) that may be necessary to sustain the population. Thus, the level of reproductive depression produced by the early-life exposure to MeHg may have an especially high impact on vulnerable migratory songbird populations with limited breeding seasons and short lifespans.

Of course, any extrapolation of our results to wildlife management should be applied with caution due to the use of a caged, domesticated songbird model and the relatively high levels of dietary MeHg exposure in this study. Mean blood Hg concentration in exposed juveniles in this study, around the time of fledging, was 7.57 μg/g ww, an order of magnitude higher than levels reported in wild eastern bluebird (*Sialis sialis*) fledglings at a contaminated site (0.52 $\pm$ 0.36 μg/g ww).[41] These levels are approximately an order of magnitude higher than mean levels for three species of yearling songbirds captured across a range of habitats in eastern North America (Carolina wren, *Thryothorus ludovicianus*, 0.68 μg/g ww; red-winged blackbird, *Agelaius phoeniceus*, 1.45 μg/g ww; and Louisiana waterthrush, *Parkesia motacilla*, 0.73 μg/g ww)[5] and are 2−3 times higher than maximum blood Hg levels reported for these species (Carolina wren, 3.93 μg/g ww; red-winged blackbird, 2.89 μg/g ww; and Louisiana waterthrush, 2.32 μg/g ww). However, our estimate of reproductive suppression due to early life exposure to MeHg may be an underestimate because zebra finches have low sensitivity to Hg exposure relative to other species,[32] and our captive birds had many challenges

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removed that would likely have exacerbated the effects of MeHg exposure in a wild population, such as food shortages and predators.

The results of our study indicate that it is important to consider whether organisms were ever exposed to Hg, not just whether they are currently exposed to the toxicant. If we had measured the blood and egg Hg concentrations of our breeding (F1) pairs, as one might in a field study, we would not have predicted any negative consequences because Hg levels were low by this point in their lives, yet their fitness was effectively halved because of an early-life history of Hg exposure. Refocusing on the history of exposure to Hg emphasizes the need for longitudinal and transgenerational studies in ecotoxicology, which often fall outside the scope of rapid environmental assessments. While such studies are logistically and financially more-challenging, our data suggest that sampling breeding birds at one point in time may not provide a sufficiently meaningful estimate of risk.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b04752.

Additional experimental details and data from an additional treatment group of birds exposed to mercury later in development. Figures showing differences in reproductive output, tissue Hg levels in individual birds and eggs, latency in laying the first egg in the first clutch, and mean clutch sizes. (PDF)

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Notes

The authors declare no competing financial interest.

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NOTE ADDED AFTER ASAP PUBLICATION

This paper was published ASAP on February 5, 2018, with an error in Figure 1. The corrected version was reposted on February 14, 2018.

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