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Mercury Reduces Avian Reproductive Success Through Direct
Embryotoxicity Rather Than Altered Parental Behavior

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Bachelor of Science in Biology, Virginia Tech, 2012

A Thesis presented to the Graduate Faculty
of the College of William and Mary in Candidacy for the Degree of
Master of Science

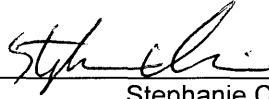
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APPROVAL PAGE

This Thesis is submitted in partial fulfillment of
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Master of Science



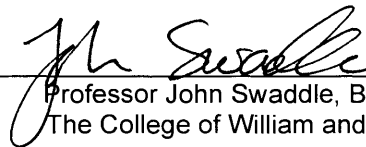
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Approved by the Committee, June, 2015



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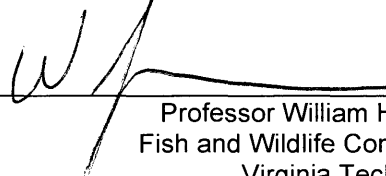
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ABSTRACT PAGE

Mercury (Hg) is an environmental contaminant that impairs avian reproduction by reducing hatching and fledging success. While direct embryotoxicity is a documented cause of reduced reproductive success, altered parental care behaviors of Hg-exposed parents could also contribute. The objective of this study was to determine how dietary Hg exposure (1.2 $\mu\text{g/g}$ wet weight in food) influenced avian parental care and associated reproductive success, utilizing zebra finches (*Taeniopygia guttata*) as a model species. Using a cross-fostering design, we isolated the influence of parental care from direct embryotoxic effects of maternally transferred Hg. Four treatments were used, with control and Hg-exposed adults raising control or Hg-exposed foster eggs. A lower percentage of Hg birds reached each reproductive stage than did control birds. Only 53% of Hg birds successfully fledged nestlings compared to 65% of control birds. Mercury-dosed parents spent less time constructing nests than control birds, and nests were lighter, possibly related to an impaired ability to bring pieces of hay through the nest box opening. However, nest temperature, incubation behavior, and provisioning rate did not differ between parental treatments. Control eggs tended to have shorter incubation periods and were more likely to hatch than Hg-treated eggs, but there was no significant effect of parental treatments on these parameters. These results suggest that embryotoxicity is the likely explanation for reduced reproductive success, rather than altered parental behavior. However, further research is warranted in field settings, where parents are exposed to greater environmental challenges, fluctuating temperatures, and limited resources.

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Introduction

Anthropogenic activity has introduced numerous xenobiotics into the environment, including increased levels of metals. One such element is mercury (Hg), which is a widespread contaminant of concern for environmental and public health. Although elemental Hg is not readily bioavailable, microbial activity converts Hg into the organic form, methylmercury (MeHg), which can bioaccumulate and biomagnify throughout food webs (Wolfe et al. 1998). Methylmercury exposure can cause direct mortality or have sublethal effects, such as reduced neural function and/or endocrine disruption, that may reduce fitness of many species of wildlife (Scheuhammer et al. 2007, Tan et al. 2009).

In wildlife, and in birds in particular, exposure to MeHg has been reported to impair reproduction (Whitney and Cristol *in prep*, Eisler 1987). For example, common loons (*Gavia immer*) with 3.0 – 4.0 µg/g Hg (hereafter ppm) in blood had 30% lower hatching success and 37% lower fledging success than loons with < 0.5 ppm in blood (Evers et al. 2001). In an experimental study, captive white ibis parents (*Eudocimus albus*) dosed with 0.3 ppm dietary MeHg had a 35% decrease in fledging success compared to controls (Frederick and Jayasena 2011). A likely cause of reduced avian reproductive success may be embryotoxicity from direct exposure to maternally transferred Hg in the egg, as the embryo is considered as a very sensitive stage to contaminant exposure (Russell et al. 1999). Some studies have reported teratogenic effects of MeHg as well

as avian embryonic mortality (Heinz et al. 2009, 2011, Rutkiewicz et al. 2013). However, reduced fledging success is a well documented negative effect of Hg-exposure and there is little evidence of Hg impacting other reproductive endpoints such as clutch size (Whitney and Cristol *in prep*, Varian-Ramos et al. 2014). Thus, it is possible that Hg is impacting multiple reproductive stages between egg laying and fledging. The deleterious effects of Hg on reproduction may manifest through impacts on parental health and behavior rather than direct embryotoxicity. In particular, altered nest construction, incubation, or food provisioning behavior could lead to lower hatching and fledging success, but there is a paucity of studies examining impacts of MeHg on avian parental care.

The major components of avian parental care involve building appropriate nests, maintaining proper incubation temperatures, and adequately provisioning young. Quality of nest construction can influence nest microclimates and the variance of incubation temperatures (Deeming 2002). Similarly, the duration and regularity of incubation behavior plays an essential role in determining the thermal environment for developing embryos and can therefore influence reproductive success. Specifically, the incubation temperature maintained by the parent(s), through behavior and insulation of nests, impacts hatching success and subsequent offspring phenotype. Recent studies have reported that a 0.9 -1.5°C decrease in mean incubation temperature can decrease hatching success and also negatively influence body size, condition, growth, metabolic rate,

immune function, thermoregulation, and stress responsiveness of avian offspring (Nord and Nilsson 2011, DuRant et al. 2012a, b, 2013).

Provisioning behavior also contributes to the quality of offspring based on the quantity and quality of food provided to developing young. In terms of reproductive measures, provisioning behavior is an important factor for determining fledging success and recruitment. For example, feeding rate positively correlated with fledgling production in blue tits (*Cyanistes caeruleus*; Mutzel et al. 2013). Similarly, quality of prey was important for house sparrow reproductive success (*Passer domesticus*; Schwagmeyer and Mock 2008); specifically, prey item size had a greater impact on chick mass and recruitment than parental feeding rate.

To our knowledge, only a few studies have reported adverse effects of Hg-exposure on avian incubation behavior, while nest building and provisioning behaviors have been entirely ignored, despite their strong influence on reproductive success. Male snow petrels (*Pagodroma nivea*) that neglected their eggs had higher blood Hg concentrations than those that fully incubated, but this relationship was not observed in females (Tartu et al. 2015). Similarly, nearly half of American kestrels (*Falco sparverius*) dosed with high dietary concentrations of MeHg (4.6-5.9 ppm) exhibited no incubation activity, in contrast to birds given control or low doses (0-3.3 ppm), but sample sizes were small (Albers et al. 2007). Lastly, Evers et al. (2008) noted that loons inhabiting high-Hg lakes left their eggs unincubated for 14% of the time sampled, in comparison to

those in low-Hg lakes, which left eggs unattended for only 1% of the time sampled. These studies did not separate the influences of parental incubation behavior from the direct effects of Hg in eggs, or other possible behavioral or physiological mechanisms. Therefore, impaired parental care, through altered nest building, incubation, and/or provisioning, may have been an alternative or additive mechanism to embryotoxicity for reduced fitness in Hg-exposed birds.

The objectives of this study were to determine if MeHg exposure alters avian parental behavior, and the relative degree to which parental behavior or embryotoxicity influences reproductive success, using zebra finches (*Taeniopygia guttata*) as a model species. Most information concerning the effects of Hg comes from aquatic and piscivorous birds due to the historical focus on Hg biomagnification in aquatic ecosystems. However, recent evidence has documented the movement of Hg into terrestrial food chains (Cristol et al. 2008). Therefore, we used the zebra finch, a domesticated terrestrial passerine native to Australia that performs well in captivity, as a model songbird species to test our hypotheses. We hypothesized that if zebra finches are exposed to dietary MeHg, then their quality of parental care would decrease. Specifically, we expected that exposed zebra finches would exhibit poor nest building, have abnormal incubation behavior resulting in lower nest temperatures, and provision nestlings less than control birds. We also hypothesized that parental care measures would influence reproductive success and offspring phenotype

more strongly than maternally deposited Hg in eggs. Specifically, we expected that impaired parental care of exposed zebra finches would be related to hatching success, body size, growth rate, and fledging success of nestlings more so than Hg-exposure of eggs.

Methods

Experimental Design and Animal Husbandry

In order to separate the contributions of any Hg effects on parental behavior from the direct effects of maternally transferred Hg on the embryo, we used two levels of treatments, the parent level and the egg level. Parents were either on a control diet and not exposed to Hg, or a contaminated diet where they were exposed with 1.2 ppm MeHg via feed and received “lifetime” exposure (continuously from *in ovo* development through maturity). Eggs were either laid by control parents or maternally exposed to Hg (laid by adult-exposed parents). Parents cross-fostered eggs from other pairs. Using this factorial design, birds were assigned to one of four treatments: 1) control parents, raising eggs from other control parents ($C_{\text{parent}}/C_{\text{egg}}$); 2) control parents raising eggs from exposed parents ($C_{\text{parent}}/Hg_{\text{egg}}$); 3) exposed parents raising control eggs ($Hg_{\text{parent}}/C_{\text{egg}}$); and 4) exposed parents raising eggs from other exposed parents ($Hg_{\text{parent}}/Hg_{\text{egg}}$). Food was prepared according to Varian-Ramos et al. (2014). Briefly, a stock solution of 40 ppm methylmercury cysteine was diluted and mixed with ZuPreem® food pellets at a 1:9 ratio by weight and homogenized with a rock tumbler for 30 minutes. Samples of every batch

were tested to confirm that concentrations were within 10% of 1.2 ppm wet weight. All birds were bred from a captive line of finches only exposed as adults and were thus the first generation of lifetime- exposed birds.

Birds were reproductively naïve and each pair was housed in its own cage, on a 14:10 light cycle, and given ZuPreem® pellets, vitamin-enhanced water, grit, cuttlefish bone for calcium supplementation, nest boxes, and ~ 14g hay daily for nesting material. Rooms were maintained at 22°C (mean: 22.4 ± 0.1°C). To minimize disturbance by investigators, processing of eggs and nestlings were done in conjunction with room cleaning and animal husbandry, from approximately 8:30-10:00AM daily.

In order to minimize any effects of direct exposure to dietary Hg in nestlings, all parents were temporarily switched to a control diet while feeding nestlings, regardless of parent or egg treatment. Thus, any offspring Hg exposure was via the egg, or residual Hg in parents' crops (see below). A pilot depuration study suggests that zebra finches switched from a 1.2 ppm MeHg diet to a control diet lose ~35% of their blood Hg in the first two weeks, but still maintain high blood Hg concentrations (~11 ppm; Whitney and Cristol, unpublished data.), so receiving a control diet for the provisioning stage (12 days) did not substantially decrease the large difference in parental Hg burden between treatment groups. To assess parental Hg concentrations, blood samples were taken from the brachial vein immediately before breeding and after nestlings had fledged.

In total, 40 control pairs and 47 Hg-pairs were bred from June 2014 to January 2015. We assessed the stage of reproduction reached by each pair. Stages included nest, eggs, nestlings, and fledglings. We defined the nest stage as building any structure out of hay, and included pairs that built nests outside of the nest box, such as in the corner of the cage. The egg stage included any pairs that laid at least one egg. We defined the nestling stage as successfully hatching at least one egg. Similarly, the fledging stage included pairs that successfully fledged at least one nestling. The number of pairs to reach each stage was divided by the number of pairs to reach the previous stage and compared between parental treatments for nest and egg stages and across parent/egg treatments for hatching and fledging success. The proportion of pairs that produced a fledgling, out of the entire number of pairs that initiated nests in each parental treatment group, was also compared.

Nest Building

Architecture of nests can influence incubation temperature, so the mass and height of nests were used as estimates of mechanical structure to determine if any differences existed in nest building between Hg-exposed and control parents. Nest boxes were weighed prior to nesting and then weighed again when the female laid her first egg. If two weeks passed without any activity in the nest box, five pieces of hay were placed in the nest box to encourage nest building. If no activity was observed after 6 weeks, the pair was replaced. Pairs received approximately 15g of

new hay per day in the bottom of their cage until the nest was completed, defined as when the female laid her first egg. Nest height was measured as the distance between the floor of the nest box and the highest point on the nest, excluding any domed roof, which was a variable feature of nest architecture. Domes that obstructed the video camera view were removed after weighing. Nest masses and heights were compared between the two parental treatments.

To observe parental behavior, each nest box lid was fitted with a small surveillance camera (LOREX, Baltimore, MD) connected to an 8-channel DVR recorder. Nests were recorded daily, undisturbed, from 7-8AM (1 hour after lights on), 12-1PM and 4-5PM everyday. One hour of nest building was analyzed per pair from approximately the same stage of nest building, when a complete layer of hay covered the floor of the nest box. One video observer (SYC), blind to treatments, recorded the number of visits to the nest, pieces of hay brought into the box, and attempts to bring hay into the nest box. Often a finch would attempt to bring in a piece of hay that would promptly fall out of the nest box. Thus, the proportion of successes (pieces of hay successfully brought in/attempts) was used as a measure of a pair's nest-building skill.

Nest Temperature and Incubation Behavior

To examine the thermal environment of the nest maintained by incubating pairs, a temperature logger (HOBO U23 Pro v2 2x, Onset Corporation, Cape Cod, MA) was placed in each nest box after the female

laid two eggs. The HOBO U23 is equipped with two metal temperature probes that recorded at 30-second intervals: one measures ambient temperature of the nest box and the second measures nest temperature. The nest temperature probe was altered by removing and replacing the metal tip of the probe with a thermistor inserted into a hollow clay egg model approximately the size of a zebra finch egg (~1.6cm length, 1.15cm diameter), and filled with wire pulling lubricant (ClearGlide, Ideal Industries, Sycamore, IL) to mimic the thermal properties of an egg (following Ardia et al. 2010). Temperature loggers were tested in artificial incubators set to 37°C, removed and equalized to room temperature. Artificial eggs reached 37°C and had approximately the same heating and cooling rates as the ambient temperature probes. Zebra finches readily accepted the egg probe and maintained normal behaviors, but in some cases adults would position the egg probe on the periphery of the clutch. Thus, the temperatures that we report are nest temperatures rather than egg incubation temperatures, in recognition of the variable location of the probe within the clutch. Clutches were standardized to four eggs, plus the artificial egg temperature probe. The incubation period (clutch completion to first egg hatching) was determined by checking nest boxes daily after 10 days (incubation period range 11-16 days (Zann 1996)). The HOBO logger was removed from the nest two days after the last nestling hatched. Mean nest temperature was calculated across the entire incubation period.

Three hours of video recorded from 7-8AM on days 2, 4, and 6 of the incubation period were analyzed for each pair to measure nest attendance (% of time on the eggs), number and duration of offbouts, and division of labor between the parents. All incubation videos were analyzed by SYC, who was blind to treatments. Additionally, since zebra finches are biparental incubators, times from the video analyses were associated with the corresponding nest temperature and used to determine average nest temperatures produced by females and males in each treatment.

Provisioning Behavior/Nestling Growth

Nests were video recorded during the provisioning phase from 7-8AM on the eighth day after hatching and videos were analyzed to determine provisioning rate, and time spent brooding or provisioning by each parent (observer Andrew Elgin was blind to treatments). We chose to analyze day 8 because chicks were large enough to clearly discern a beak-to-beak feeding event in the camera frame.

To examine growth of offspring, nestlings were banded and then measured for body mass and tarsus length (average of three successive measurements) at hatching and every 4 days until day 20. Blood samples were obtained from nestlings on day 12. Nestling body mass at day 20 and growth (change in mass over time) from day 0 to day 20 were compared among treatments. Lastly, the reproductive measures of hatching success (number of hatchlings divided by four) and fledging success (number of fledglings divided by number of hatchlings) were

compared among treatments, and related to parental care measures such as nest temperature and provisioning quantity and rate.

Nestlings in treatments Hg_{parent}/C_{egg} and Hg_{parent}/Hg_{egg} exhibited Hg concentrations that were higher than expected (see below), therefore we performed a follow up study to determine whether parents were secreting Hg into the food provisioned from their crops. We were unable to obtain large enough samples directly from adult crops to analyze for Hg, hence we collected crop contents of recently fed control nestlings raised by a subset of either control parents or Hg-exposed parents that had been taken off of Hg diets 12 days earlier.

Mercury Analysis

We analyzed total mercury (THg) in blood samples of parents at pairing and at fledging of nestlings, and in blood samples from chicks at day 12. Approximately 95% of Hg in avian blood is comprised of MeHg (Rimmer et al. 2005, Wada et al. 2009), thus THg values accurately represent concentrations of MeHg present in samples. Additionally, a subset of 40 eggs from control and Hg birds, and 10 food samples from crops of nestlings, were freeze-dried, homogenized, and analyzed for THg. Percent moisture of eggs was $82.33 \pm 0.95\%$. Samples were analyzed for THg by combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT, USA) according to U.S. Environmental Protection Agency method 7473. Samples were run with a duplicate, blank, and standard

reference materials (SRM; DOLT-4 dogfish liver and DORM-4 fish protein (National Research Council of Canada, Ottawa, ON) for quality control in every run of 20 samples. Average relative percent difference between replicate sample analyses was $4.99 \pm 1.46\%$. Mean percent recoveries of THg for the DOLT-4 and DORM-4 were $100 \pm 0.76\%$ ($n = 47$) and $99.6 \pm 0.48\%$ ($n = 47$), respectively. Unless otherwise noted, all THg concentrations are reported as wet weight (wwt).

Statistical Analyses

Analyses were performed using SAS 9.3 (SAS Institute, Cary, NC) employing two-tailed tests of significance. All variables were assessed for normality and homoscedasity prior to analyses and log-transformed if assumptions were not met.

We evaluated the effects of parental treatment on nest building using analysis of covariance (ANCOVA). Male body mass and age were included as covariates in addition to their interaction terms with treatments. We chose male mass and age as covariates because males perform the majority of the nest building by bringing hay to the nest. We also ran ANCOVAs using female body mass and age, which produced similar results.

We examined how parent-egg treatments influenced incubation temperature, behaviors, and incubation period with ANCOVA. Covariates included ambient temperature, and average body mass of males and females. Additionally, variation in the artificial eggs may have influenced

nest temperatures, so the temperature logger ID was included as a random effect. Variables related to provisioning were also analyzed using ANCOVA, with brood size on day 8 as a covariate. Growth of nestlings was analyzed using a repeated measures analysis of variance (RM-ANOVA) with treatment and day as fixed effects and brood size at each day as a covariate. When comparing responses of males and females (e.g. incubation temperature, # feedings), a nested ANCOVA was used with sex nested in treatment and mass and age as covariates. The assumption of homogeneity of slopes was met for all ANCOVAs. Covariates and interactions were removed from the final model if $p > 0.1$.

When transformation did not improve normality of variables, such as proportions (e.g. hatching success) and count data (e.g. number of days to complete a nest), we used generalized linear models (PROC GENMOD). A binomial distribution with a logit link function was applied to proportions, and a negative binomial distribution with a log link function was used for count data where the variance was larger than the mean. Mercury treatments were fixed effects and parental and egg treatments were compared in addition to parent/egg treatments. When response variables were not different across parent/egg treatments, comparing just parental treatments or egg treatments allowed us to assess whether parental behavior or maternally transferred Hg influenced the endpoint. Lastly, in order to compare parental care measures with reproductive success, we used multiple linear regressions with hatching success or

fledging success as the dependent variable and nest mass, nest height, nest temperature, number of offbouts (trips taken off the nest), and provisioning rate as independent variables.

Results

Mercury concentrations—Parents, egg, nestlings

Finches that were lifetime exposed to 1.2 ppm dietary MeHg had more than three orders of magnitude higher mean blood concentrations of THg (16.06 ± 0.38 ppm) than birds on the control diet (0.005 ± 0.0004 ppm). Likewise, eggs laid by Hg females had substantially higher concentrations of THg than eggs laid by control females (19.36 ± 1.01 ppm versus 0.02 ± 0.004 ppm). Additionally, egg concentrations positively correlated with laying female parent blood Hg (linear regression: $F_{1,12}=9.8$, $p<0.01$). Nestling blood THg concentrations differed across parent-egg treatments (Fig. 1A). As expected, nestlings in treatment $C_{\text{parent}}/C_{\text{egg}}$ had minimal blood THg (0.01 ± 0.0004 ppm) and treatment $Hg_{\text{parent}}/Hg_{\text{egg}}$ exhibited the highest blood THg concentrations (0.53 ± 0.04 ppm). Unexpectedly, treatments $C_{\text{parent}}/Hg_{\text{egg}}$ and $Hg_{\text{parent}}/C_{\text{egg}}$ had similar, intermediate THg concentrations in nestling blood ($C_{\text{parent}}/Hg_{\text{egg}}$: 0.27 ± 0.04 ppm; $Hg_{\text{parent}}/C_{\text{egg}}$: 0.36 ± 0.06 ppm), despite the fact that only $C_{\text{parent}}/Hg_{\text{egg}}$ nestlings received direct inputs of maternal Hg while in the egg (Fig. 1A). However, this result was explained by transfer of Hg body burden from parents' crops during provisioning. Control nestlings raised by contaminated parents that had been taken off of Hg-dosed food at the

time their first egg hatched, whose crops we sampled in a follow-up study, had higher Hg concentrations in their crop content than those raised by control parents (0.17 ± 0.02 ppm versus 0.004 ± 0.001 ppm, Fig. 1B).

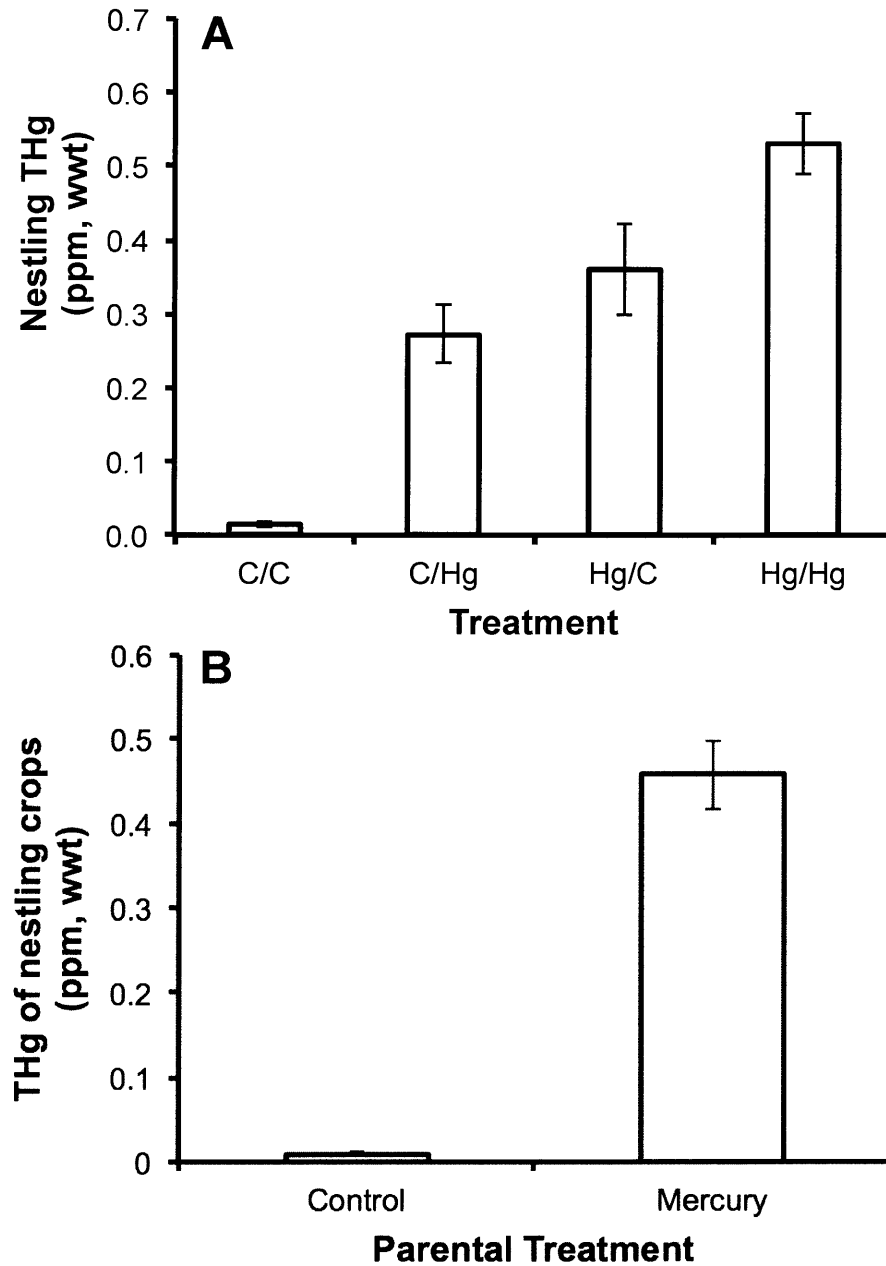


Figure 1. Total mercury (THg) concentrations of nestling zebra finches across treatments. Treatments indicate parental treatment followed by egg

treatment after the slash. A) Blood THg (wet weight) of nestlings in each treatment. B) THg concentrations of crop samples (wet weight) obtained from a separate set of control nestlings raised by either control or mercury-exposed birds.

Nest Building

Control pairs built heavier nests ($F_{3,44}=5.00$, $p=0.03$) than Hg pairs (Fig. 2A). Male age ($F_{3,44}=16.03$, $p<0.001$) and male body mass ($F_{3,44}=3.58$, $p=0.06$) influenced nest mass, such that older and heavier males tended to construct heavier nests than younger and lighter males. Adjusted mean nest masses for control and Hg pairs, corrected for male age and body mass, were $35.0 \pm 2.3\text{g}$ and $27.7 \pm 2.2\text{g}$, respectively. Control pairs also required more time than Hg pairs to complete their nests (Wald $\chi^2_{1,42}=17.07$, $p<0.001$; Fig. 2B). Again, male age (Wald $\chi^2_{1,42}=26.17$, $p<0.001$) and body mass (Wald $\chi^2_{1,42}=5.49$, $p=0.019$) influenced days to nest completion, such that older and heavier males also took longer to complete a nest than younger and lighter males. Adjusted means for days to nest completion were 3.20 ± 0.09 days and 2.54 ± 0.10 days for control and Hg birds, respectively. We found no differences between parental treatments for nest height ($F_{1,57}= 2.11$, $p=0.15$).

The proportion of successful nest-building trips (i.e., number of pieces of hay successfully brought in divided by number of total attempts) was different between the two treatment groups (Fig. 2C). Control pairs

had higher success rates compared to Hg pairs (Wald $\chi^2_{1,34}=13.11$, $p<0.001$). Neither male age nor mass were significant and were therefore removed from the model. However, this greater efficiency did not result in control pairs bringing more total hay into their nest box per hour than Hg pairs (Wald $\chi^2_{1,44}=0.72$, $p=0.39$).

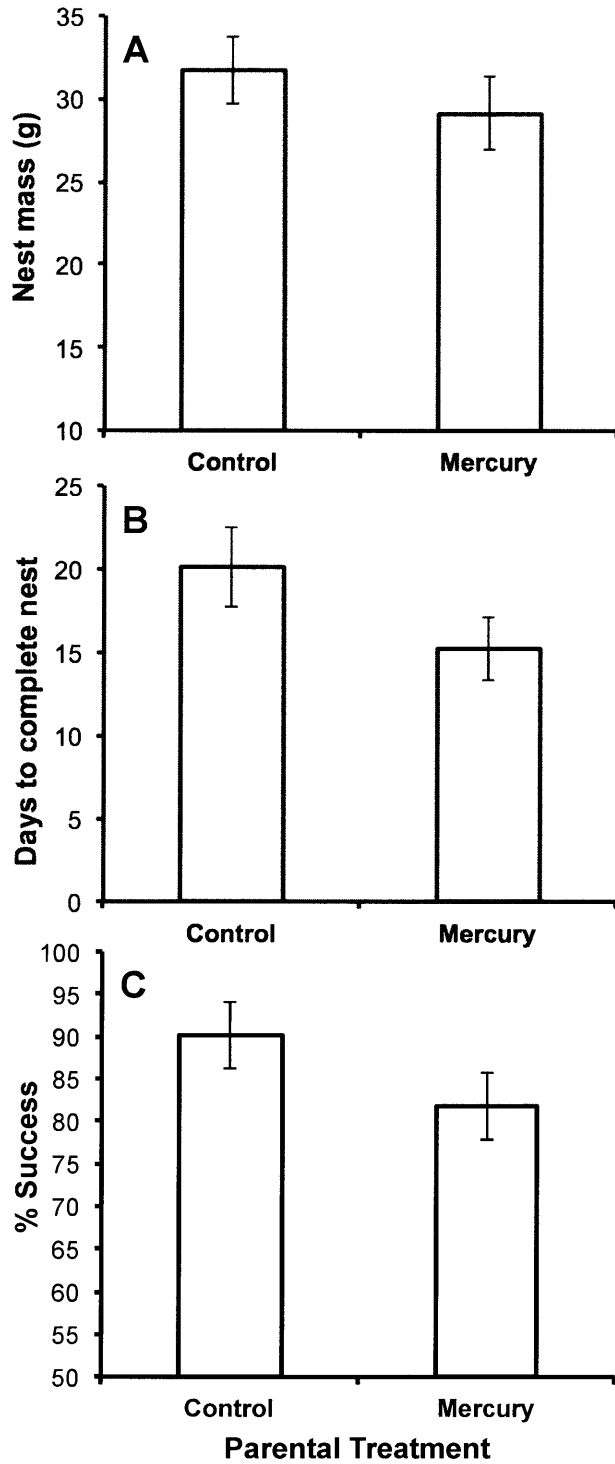


Figure 2. Comparison of parental treatment to nest-building variables in zebra finches dosed with 0.0 or 1.2ppm MeHg. Means presented are from raw data and bars represent standard error. Statistics

were run using adjusted means (ANCOVA or GzLM) A) Mean nest mass of nests built by control or mercury birds. B) Mean days to complete a nest (defined by day the female laid her first egg) for control or mercury-dosed birds. C) Proportion of successful visits with hay into the nest box by control or mercury-dosed birds.

Nest Temperature and Incubation Behavior

Nest temperature did not differ among treatments ($F_{4,56}=0.19$, $p=0.86$) or between parental treatment groups ($F_1=0.19$, $p=0.66$; Fig. 3A), but average body mass ($F_{1,60}=7.63$, $p=0.008$) and temperature logger used ($F_{1,18}=2.37$, $p=0.036$) influenced the recorded nest temperature, with heavier pairs generating warmer nest temperatures. Mercury-exposed parents produced a mean nest temperature of 33.4 ± 0.37 °C whereas control parents produced a mean nest temperature of 32.9 ± 0.41 °C. Adjusted means for each parent-egg treatment were: C/C: 32.40 ± 0.65 °C; C/Hg: 33.06 ± 0.48 °C; Hg/C: 33.12 ± 0.59 °C; Hg/Hg: 32.61 ± 0.54 °C. Additionally, total number of offbouts taken over the three hours of video analyzed did not differ across the four treatments (Wald $\chi^2_{3,52}=7.46$, $p=0.058$; Fig. 3B). Offbout duration and nest attendance were not different among parental treatments and males and females did not differ in any of the variables examined ($p>0.20$). Nest temperatures negatively correlated with incubation period (linear regression: $r^2=0.08$, $p=0.03$), but Hg exposure to parents did not affect incubation period ($F_{1,52}=0.01$, $p=0.91$;

Fig. 3C). However, Hg exposure to eggs was associated with a delay in hatching ($F_{1,52}=5.87$, $p=0.01$; Fig. 3C).

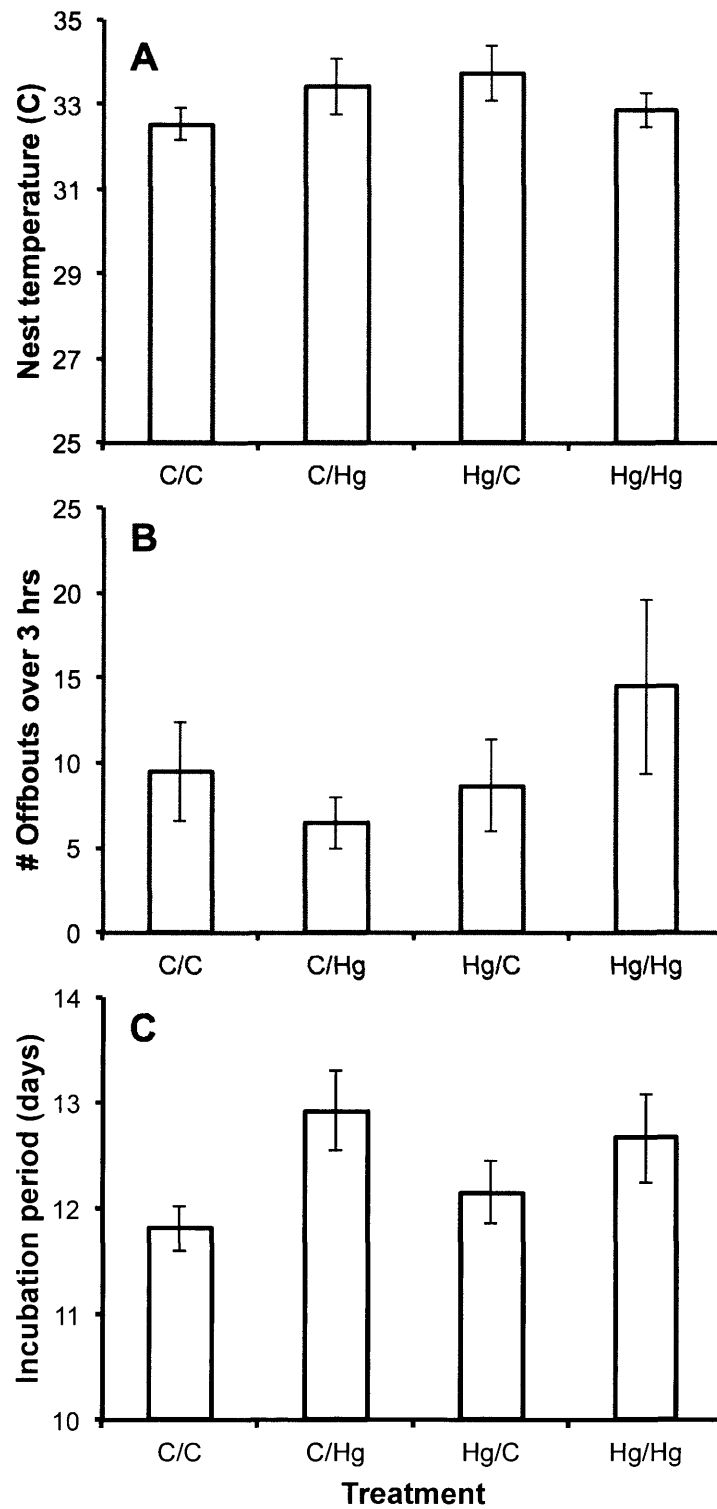


Figure 3. Comparison of variables relating to nest temperature or behavior of zebra finches in control or mercury treatments cross-fostering either control or mercury eggs. Means presented are from raw data and bars represent standard error. Statistics were run using adjusted means (ANCOVA or GzLM). Treatments indicate parental treatment followed by egg treatment after the slash. A) Mean nest temperature produced by each treatment. B) Mean for each treatment of the total number of offbouts taken by each pair over the 3-hour observation period. C) Incubation period (days from complete clutch to first egg hatching) for each treatment.

Provisioning/Nestling Growth

The number of feedings to chicks during one hour (Wald $\chi^2_{1,47} = 2.19$, $p=0.53$; Fig. 4A) and body mass of nestlings at day 20 did not differ across treatments ($F_{1,3}=0.22$, $p=0.88$; Fig. 4B). Growth, however, varied across treatments ($F_{3,48}=5.24$, $p=0.003$) and the interaction of treatment and day was statistically significant ($p=0.005$), indicating that the temporal trajectory of growth differed among treatments. Significant differences in mass were observed across treatments for days 8 ($p=0.001$) and 12 ($p<0.001$), such that nestlings in C_{parent}/C_{egg} were heaviest, and nestlings in C_{parent}/Hg_{egg} were lightest. Comparisons of parental treatments or egg treatments alone did not explain the variation ($p>0.31$).

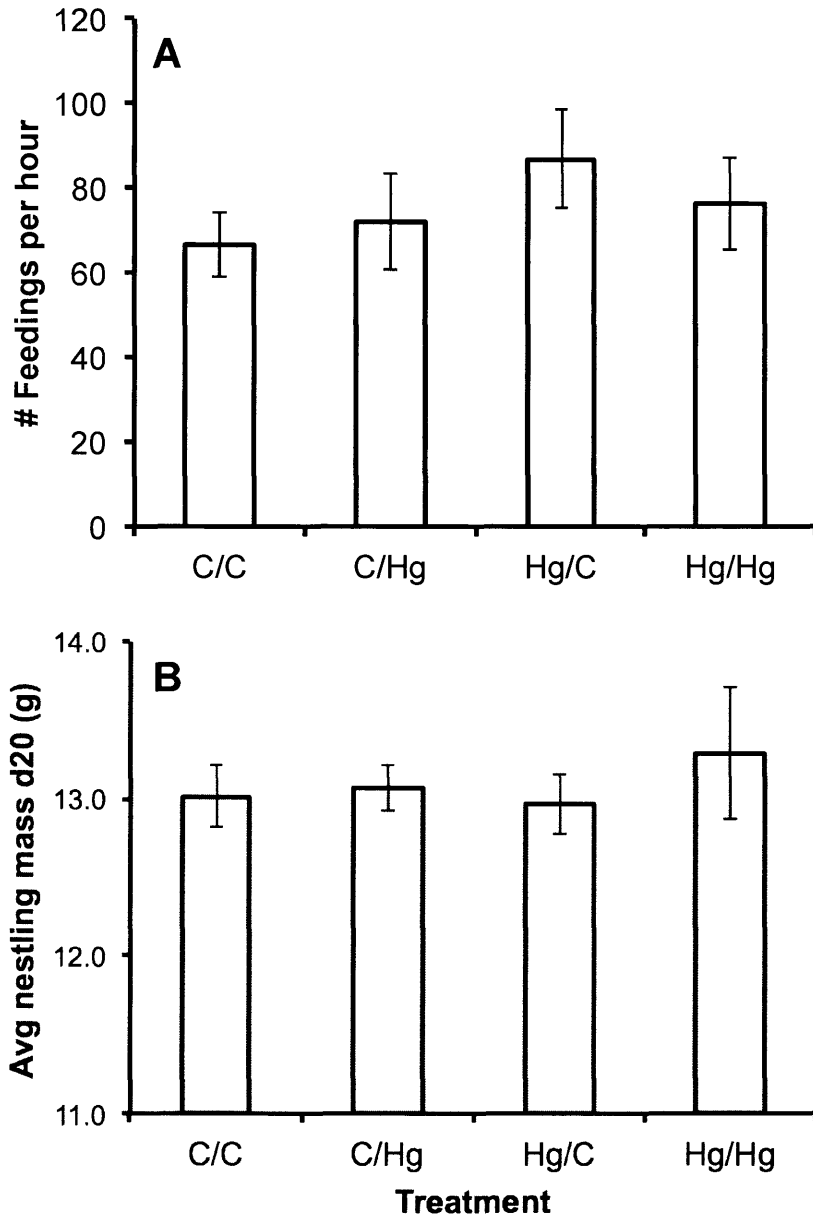


Figure 4. Comparison of provisioning and nestling variables of zebra finches in control or mercury treatments cross-fostering either control or mercury eggs. Treatments indicate parental treatment followed by egg treatment after the slash. Means presented are from raw data and bars represent standard error. Statistics were run using adjusted means (ANCOVA or GzLM) A) Mean number of feedings given to nestlings over

an hour for each treatment. B) Mean nestling mass at day 20 for each treatment.

Reproductive Success

A greater proportion of control parents initiated reproduction compared to contaminated parents (Fig. 5B). There was a 6.7% difference in pairs that successfully built nests, and a 7.7% difference in pairs that laid eggs, considered as a proportion of pairs that built nests. The net result was that only 53% of all contaminated pairs successfully raised nestlings, compared to 65% of all control pairs (Fig. 5A). Hatching success (number of hatchlings/4) and fledging success (number of fledglings/number of hatchlings) were not influenced by parental treatments ($p > 0.31$). However, a higher proportion of control eggs hatched compared to contaminated eggs (Wald $\chi^2_{1,45} = 6.82$, $p = 0.009$; Fig. 6A). Contaminated nestlings were just as likely to fledge as control nestlings (Wald $\chi^2_{1,57} = 0.52$, $p = 0.47$; Fig. 6B). In a multiple linear regression with several parental care attributes as independent variables to explain hatching success or fledging success, only treatment ($p = 0.04$) was statistically significant for influencing hatching success and no variables significantly explained fledging success ($p > 0.11$).

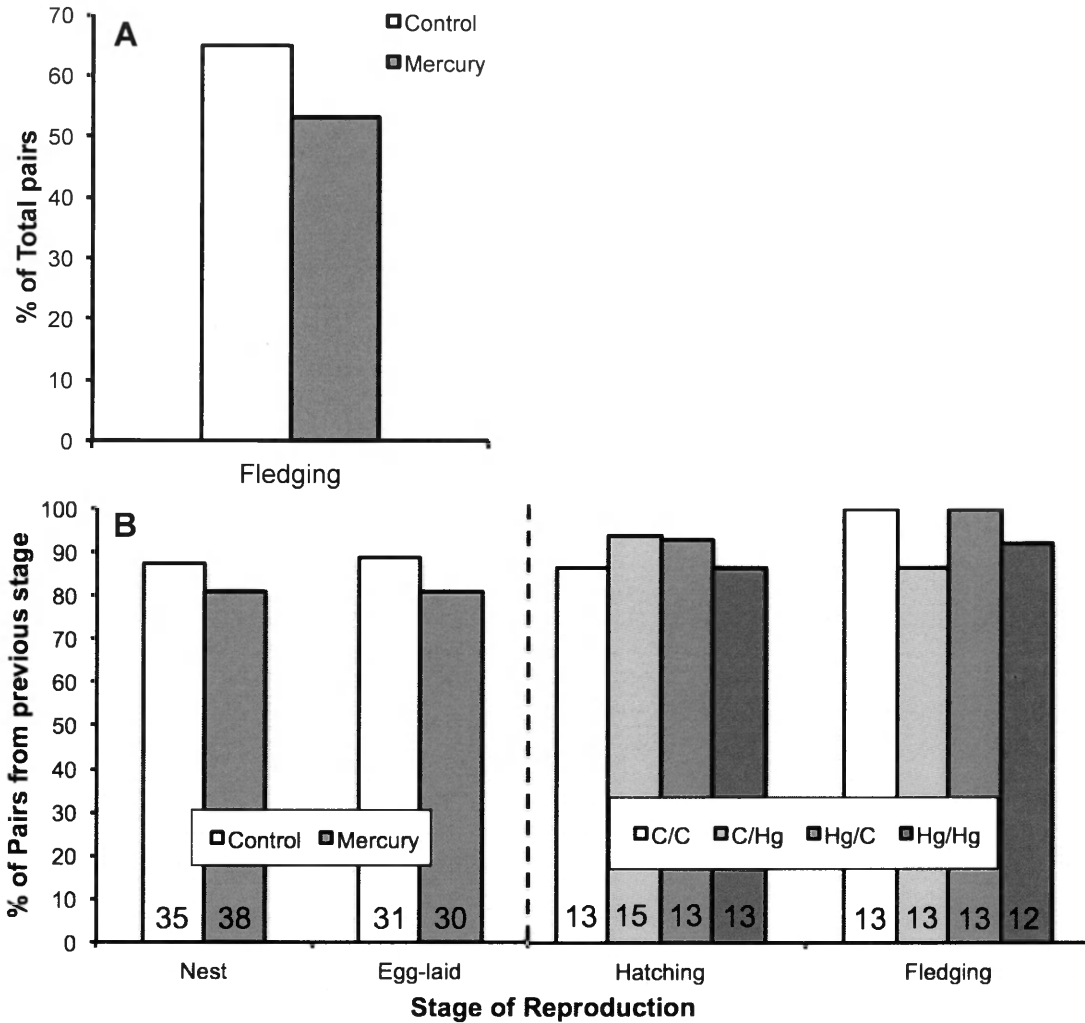


Figure 5. (A) Comparison of the proportion of total pairs of zebra finches in control (n=40) or mercury (n=47) treatments that successfully raised at least one fledgling. (B) Comparison of the proportion of pairs that reached further stages of reproduction. Proportions were calculated based on the number of pairs to reach the previous stage. Only parental treatments were compared for nest building and egg laying, and parent/egg treatments were compared for proportion of pairs to reach hatching or fledging stages. Treatments indicate parental treatment followed by egg treatment after the slash.

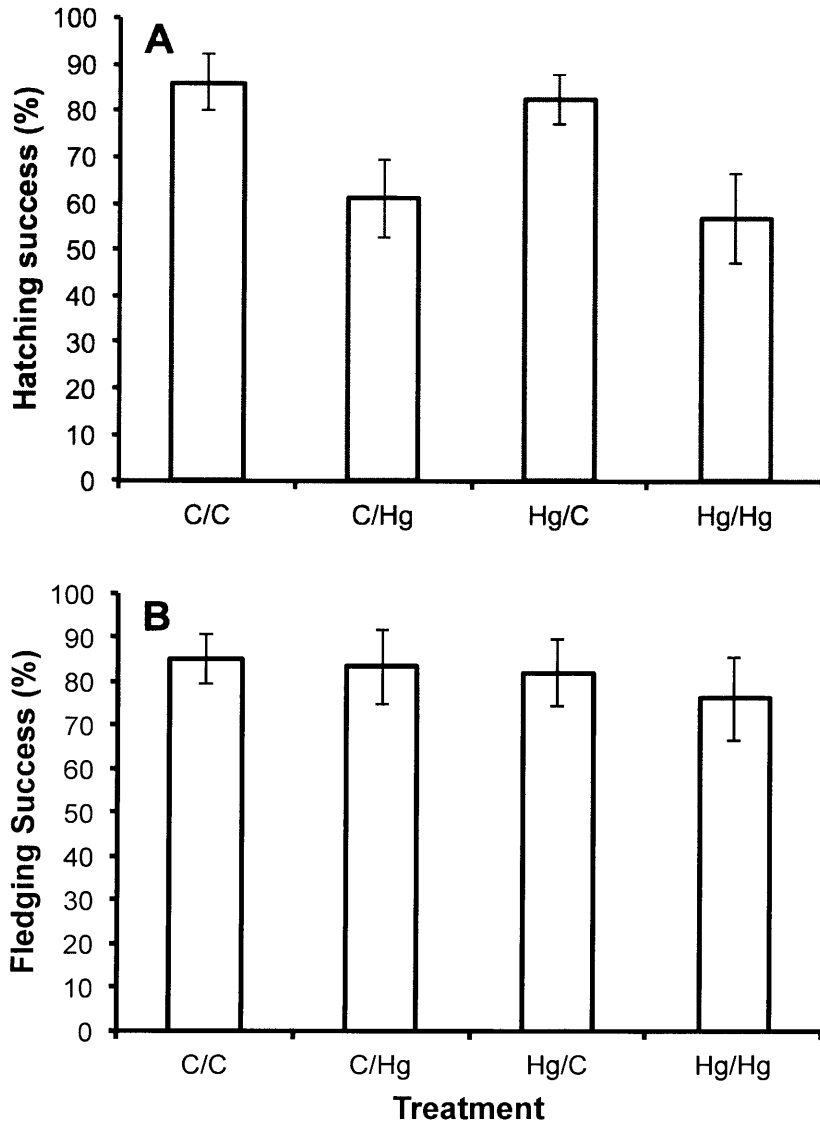


Figure 6. Comparison of reproductive success of zebra finches in control or mercury treatments cross-fostering either control or mercury eggs. Treatments indicate parental treatment followed by egg treatment after the slash. Means presented are from raw data and bars represent standard error. Statistics were run using adjusted means (GzLM) A) Mean hatching success (#hatchlings/4) for each treatment. B) Mean fledging success (#fledgings/#hatchlings) for each treatment.

Discussion

Decreased reproductive success at both the hatching and fledging stages are among the most commonly studied endpoints of Hg exposure in birds, yet parental behavioral mechanisms have been largely ignored in past studies compared to direct embryotoxicity. We evaluated the impact of Hg exposure on parental behavior in zebra finches and compared the influence of parents' care to the presence of maternally transferred Hg in the egg on reproductive success.

Reduced Reproduction

Of the 40 control pairs and 47 contaminated pairs that were set up to breed, a greater proportion of control pairs initiated nest building and laid eggs compared to Hg-exposed birds. But of the pairs that laid full clutches, the proportion of pairs to successfully hatch nestlings or fledge nestlings was similar across parent/egg treatments. The lower proportion of total contaminated pairs that reached the egg stage introduced an inherent bias to this study because the parental behavior of pairs that failed to even attempt reproduction may have been reduced, but, of course, could not be measured.

In a wild population, fewer Hg-exposed birds reproducing could also translate to decreased population size (Tartu et al. 2013, Schoch et al. 2014). Based on population modeling that used chicks fledged as the main reproductive endpoint, populations of common loons with higher Hg are experiencing a decreased growth rate compared to those with lower

Hg (Schoch et al. 2014), but this model did not take into account birds that failed to attempt reproduction. A potential explanation for the absence of reproductive initiation by contaminated birds in our study could be due to altered hormone levels that impaired normal reproductive behaviors.

Black-legged kittiwakes (*Rissa tridactyla*) that abstained from reproducing had significantly higher levels of blood Hg than those that bred (Tartu et al. 2013). Additionally, males that skipped reproduction had suppressed baseline levels of luteinizing hormone (LH), which is essential for inducing sex steroid production and reproduction (Tartu et al. 2013). Mercury is a known endocrine disruptor (Adams et al. 2009, Wada et al. 2009), yet few studies have examined the relationship between Hg and LH or prolactin, a hormone essential for incubation and provisioning.

Embryotoxicity

Embryotoxicity is a well-known effect of mercury that has been demonstrated through both dosing and egg-injection studies. Many studies refer to 0.8 ppm as the threshold for embryotoxicity, which is based on a study using mallard eggs exposed to maternally transferred Hg (Heinz 1979, Henny et al. 2002). A more recent study of the same species similarly concluded that 1 ppm Hg in the egg is likely to impact embryos (Heinz and Hoffman 2003). The mean wet weight concentration of Hg-exposed eggs in our study was 3.34 ± 0.21 ppm (range: 1.74 ppm to 5.29 ppm). These concentrations exceeded the toxic threshold proposed by Heinz, so it was not surprising that Hg-exposed eggs in our study were

18% less likely to hatch and surviving embryos took 0.82 days longer to hatch than control eggs. Similarly, Kenow et al. (2011) reported dose-dependent reductions in hatching success and increases in incubation period in common loon eggs injected with Hg. We do not know whether zebra finches are more or less sensitive to Hg than other birds, such as mallards. Based on egg injection studies of 26 bird species, the common grackle (*Quiscalus quiscula*) and tree swallow (*Tachycineta bicolor*), were the only two songbird species tested and were among the more sensitive species. Tree swallows breeding along the South River, a historically contaminated river, experienced a 10% reduction in hatching success compared to birds breeding in reference areas (Hallinger and Cristol 2011), with mean egg concentrations at that site of 0.34 ppm (range: ~0.15 ppm Hg to ~0.52 ppm, Brasso et al. 2010), which is below the toxic threshold established for mallards. It should be noted that in our study, eggs were laid by females who had themselves survived the embryotoxic effects of the same Hg dose, and thus there had been one generation of selection for Hg tolerance (Varian-Ramos et al. 2014). Therefore, it is likely that these zebra finches were more tolerant of Hg exposure compared to other zebra finches and perhaps other species of songbirds.

Despite existing research on Hg embryotoxicity, the mechanisms are still not well understood. Mutations or disruption of development are potential mechanisms because malformations have been observed in Hg-exposed eggs, and the amount of teratogenicity was dose dependent

(Hoffman and Moore 1979, Heinz et al. 2011). Malposition of embryos is another explanation, such that chicks are unable to correctly orient themselves to hatch (Herring et al. 2010). Eggs of American avocet (*Recurvirostra americana*), black-necked stilt (*Himantopus mexicanus*), and Forster's tern (*Sterna forsteri*) inhabiting Hg-contaminated San Francisco Bay were examined for malposition, and 24% of eggs that failed to hatch were incorrectly positioned. This probability of malposition was associated with an egg THg concentration of 2.41 ppm (Herring et al. 2010). Mortality of black duck (*Anas rubripes*) embryos, dosed to approximately 3 ppm through maternal transfer, was more likely to occur during the last week of incubation (Finley and Stendell 1978). Additionally, when chicken eggs were injected with Hg, high concentrations in kidneys and brains of embryos suggested that renal and neurotoxicity may have influenced mortality (Rutkiewicz and Basu 2013). Because embryos incorporate more Hg from the yolk and albumen as they grow (Heinz et al. 2008), Hg concentrations may become intolerable only at a late stage of development. Our study did not examine mechanisms of embryotoxicity, but we did assess embryos of eggs that failed to hatch. Based on visual examination, embryos were classified as: no development/infertile, little development, some development, or advanced development. This anecdotal evidence suggests that embryos did not die at a specific stage of development and rather, mortality was distributed across development. However, a more controlled study is needed to validate these results.

Overall, our results confirm that exposure to an environmentally relevant concentration of Hg reduced probability of hatching in zebra finches, even when we eliminated effects of Hg on parental behavior through a cross-fostering experimental design.

Nest Building

The main effects on parental care that we observed were during the nest-building phase of reproduction, which is an often-overlooked aspect of parental care. Contaminated parents had a lower success rate when bringing pieces of hay into their nest boxes, built lighter nests, and spent less time building them. Not only is nest building an important influence on reproductive success in birds (Lombardo 1994, Szentirmai et al. 2005), it is a behavior that involves complex cognitive abilities and specialized motor control. For example, Hall et al. (2014) quantified gene expression for the protein Fos, a molecular indicator of neuronal activity, and found that the time female zebra finches spent building nests, and the number of times males picked up nesting material, explained the variation in Fos expression in specific neuronal circuits.

Our measure of time to complete a nest was operationally defined as the interval between pairing and laying of the first egg. Also, our measure of nest quality was based on mass, rather than complexity of construction. Therefore, we can conclude that Hg-exposed finches invested less structural material and construction time in their nests, but the mechanism is unclear. Contaminated birds were less adept at bringing

pieces of hay into their nest boxes than control birds, which suggests either motor or cognitive limitations. When zebra finches were given nest boxes with either small or large holes, and the choice of short or long nesting material, males chose material that corresponded to the appropriate type of nest box (Muth and Healy 2014). Thus it is possible that in our study Hg impaired the finches' cognitive ability to identify an appropriately sized piece of hay. Alternatively, the lower success rate of bringing in hay pieces may have been due to limitations in the motor skills required to navigate a long piece of hay through a narrow opening.

Nest mass is important for the microclimate of developing eggs. Nests conserve heat and help insulate against high ambient temperatures (Rahn 1991) as well as maintain appropriate humidity levels during incubation (Rahn and Paganelli 1990, Deeming 2011). The observed difference in nest mass did not relate to decreased hatching success, but may have implications for wild birds. A poor nest construction in variable field settings might result in higher fluctuations in temperature and humidity in the nest microclimate (Conway and Martin 2000, Coe et al. 2015) and negatively impact hatching. For example, reduced hatching success observed in tree swallows breeding along the South River was associated with high ambient temperatures, but only at contaminated sites (Hallinger and Cristol 2011), indicating that weather conditions may interact with parental behavior to impact reproductive success. Alternatively, building a small nest quickly could be favorable for wild

birds. Small nests are beneficial for avoiding predators (Møller 1990) and establishing a nest quickly may allow parents to save energy that can be allocated to incubation and foraging. The observed nest building strategy may reflect Hg acting as a selection pressure, but field studies are needed to determine whether this strategy is advantageous.

Incubation

Contrary to several studies that have documented impaired incubation behavior in birds exposed to Hg (Evers et al. 2001, Albers et al. 2007, Tartu et al. 2015), we did not observe any differences in incubation. We also did not find differences in nest temperature between parental treatments or across the parent/egg treatments. However, this is the first study to our knowledge to examine how Hg exposure impacts nest temperature. Our reported mean nest temperatures are lower compared to various studies that measured egg temperatures of free-living or domestic zebra finches (El-Wailly 1966, Burley 1968, Vleck 1981, Zann and Rossetto 1991). Temperatures in those studies ranged from 35–37°C and were measured by inserting a thermocouple into a zebra finch egg and recording every 5–24 minutes, but only when parents were observed on the eggs. In contrast, our data were logged every 30 seconds, including when parents were off the eggs, and thus provide 30,000 data points per clutch, including offbouts. Although our reported nest temperatures are lower than those reported in previous zebra finch studies, hatching success was normal for control eggs (>80%) and incubation period (12-13

days) was within the range reported for zebra finches (Zann 1996). Furthermore, temperatures produced by our zebra finches (overall mean: $33.1 \pm 0.27^{\circ}\text{C}$) were comparable to those reported for tree swallows (overall mean: $33.7 \pm 0.32^{\circ}\text{C}$), using the same methodology (Coe et al. 2015) but nesting in the wild at lower ambient temperatures.

Crop Concentrations

Unexpectedly, we found that nestlings raised by contaminated parents ($\text{Hg}_{\text{parent}}/\text{C}_{\text{egg}}$) were still exposed to Hg, despite our switching parents to a control diet as soon as the first egg hatched. In fact, these control nestlings reared by Hg parents accumulated an amount of Hg comparable to that of maternally transferred Hg in the nestlings from the $\text{C}_{\text{parent}}/\text{Hg}_{\text{egg}}$ treatment. We hypothesized that finches were secreting Hg into their crops and thereby contaminating the control food provided to them before feeding it to their nestlings. To examine this phenomenon further, we conducted a follow-up study with 10 pairs from the treatments $\text{C}_{\text{parent}}/\text{C}_{\text{egg}}$ and $\text{Hg}_{\text{parent}}/\text{C}_{\text{egg}}$ in which we allowed them to raise another brood and collected crop samples from nestlings between days 11-13. Nestlings raised by contaminated parents eating control food had a mean 0.46 ± 0.04 ppm in their crop contents compared to nearly undetectable Hg in control nestlings' crops. These results support our hypothesis that nestlings were being exposed through the crop secretions of adults. This is a possible unexplored route for elimination of contaminants, beyond the well-documented routes of egg laying and molt, for species that store food

in their crops. Because males of many species feed young, this could partly explain why female birds do not always have lower Hg than their mates, despite the absence of an elimination route through eggs in males (Monteiro and Furness 2001, Robinson et al. 2012). More importantly, our findings have implications for wild birds moving from contaminated to uncontaminated sites prior to nesting. This is an additional mechanism through which parental Hg body burden could be transferred to the next generation.

Conclusion

We examined the influence of Hg-exposure on the parental care of zebra finches and also separated effects of parental behavior from potentially confounding effects of Hg embryotoxicity on reproductive success. The only effects of Hg on parental care that we detected were during the nest-building phase and these did not carry through to hatching or fledging success. Fewer contaminated parents than control parents initiated nest building and a higher percentage of total control pairs fledged offspring compared to Hg exposed birds. Of the pairs that initiated nesting, contaminated parents were less successful than control parents at bringing pieces of hay into their boxes to build a nest. These Hg-contaminated parents also built nests that were lighter, and spent less time building these nests than control parents. Contrary to our hypothesis, we observed no evidence that Hg affected parental incubation behavior. However, we found evidence that Hg exposure in eggs reduced hatching

success and increased incubation period. Overall, we confirmed that maternally transferred Hg in eggs reduced reproductive success of contaminated zebra finches, but this effect was driven by embryotoxicity rather than parental care. Ours is the first study to examine how Hg impacts several aspects of avian parental care, but additional studies are needed to understand how Hg might influence parental care in species with different mating strategies, nest architecture, and in the presence of additional environmental challenges such as food scarcity and fluctuating weather conditions.

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