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# Continuous exposure to mercury during embryogenesis and chick development affects later survival and reproduction of zebra finch (*Taeniopygia guttata*)

Cybele Heddle[1] · John E. Elliott[1,2] · Tanya M. Brown[1] · Margaret L. Eng[1,3] · Marie Perkins[4,5] · Niladri Basu[4] · Tony D. Williams[1]

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## Abstract

Methylmercury (MeHg) is a global environmental contaminant that bioaccumulates and has multiple toxic modes of action. Aquatic species have traditionally been the focus of wildlife toxicological research on mercury, but terrestrial organisms, including passerine birds, can be exposed to similarly elevated levels of MeHg. In this study we exposed a model passerine, the zebra finch (*Taeniopygia guttata*), to MeHg in ovo, as chicks only, or with a combined 'in ovo + chick' treatment. We isolated exposure to specific developmental stages through the use of egg injections (3.2 µg Hg/g egg) and controlled oral dosing of chicks (0.24 µg Hg/g bw/day from day 1 to day 30). In ovo exposure to MeHg reduced hatching success, but there was no effect of MeHg on chick growth. We found that in ovo only or chick only exposure did not have long-term effects, but there was some evidence for longer-term effects of combined 'in ovo + chick' exposure on post-fledging survival and potentially sex-biased survival which resulted in very few 'in ovo + chick' exposed females surviving to breed. These females also had lower overall breeding productivity that was mainly due to lower hatching success of their offspring, not lower chick-rearing success. We found no effect of treatment on clutch size or latency to laying among females that did lay eggs. Our study suggests that combined embryonic and nestling MeHg exposure has compounding latent effects on productivity, likely through a mechanism that influences the ability of females to lay fertile eggs that hatch.

**Keywords** Methylmercury · Passerine · Hatching success · Survival · Reproduction · Courtship behaviour

## Introduction

Since the onset of industrialisation, both diffuse and point-source emissions of mercury have contributed to a two to three-fold increase in the levels detected in the atmosphere, soil and water (Gobeil et al. 1999; Driscoll et al. 2013; Lamborg et al. 2014). While mercury can exist in many valence states, natural biological processes result in the methylation of mercury into its most toxic form, methylmercury (MeHg). MeHg is persistent, bioaccumulative, and can biomagnify through food webs (Wiener et al. 2003; Eagles-Smith et al. 2009; Elliott and Elliott 2016; Eagles-Smith et al. 2018). Methylmercury is also a potent neurotoxin, and avifauna exposed to sub-lethal but environmentally relevant levels have shown physiological and behavioural changes that may affect reproduction and survival (Heinz and Locke 1976; Heinz 1979; Scheuhammer et al. 2007). In controlled feeding experiments juvenile great egrets (*Ardea alba*) exposed to environmentally relevant levels of MeHg

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✉ John E. Elliott
john.elliott@canada.ca

[1] Biological Sciences, Simon Fraser University, Biological Sciences, Burnaby, BC V5A 1S6, Canada

[2] Environment and Climate Change Canada, Ecotoxicology and Wildlife Health Directorate, Delta, BC V4K 3N2, Canada

[3] Toxicology Centre, University of Saskatchewan, Saskatoon, SK S7N 5B3, Canada

[4] Faculty of Agricultural and Environmental Sciences, McGill University, Montreal, QC H9X 3V9, Canada

[5] Harvard John A. Paulson School of Engineering and Applied Sciences, Harvard University, Cambridge, MA 02138, USA

showed a reduced ability to forage effectively (Bouton et al. 1999), and white ibis (*Eudocimus albus*) exposed to environmentally relevant concentrations of MeHg were more likely to engage in same-sex pairings—a phenomenon unknown in wild populations of this species with no exposure to the pollutant (Frederick and Jayasena 2010).

The bulk of wildlife research on the effects of MeHg have focused on aquatic species largely due to the tendency of the compound to biomagnify to high concentrations in freshwater and marine food webs (Lavoie et al. 2013), but research has shown that terrestrial organisms, including passerines, may potentially be exposed to equivalently elevated concentrations of MeHg (Cristol et al. 2008; Jackson et al. 2015; Yu et al. 2016). There is also increasing evidence linking environmentally relevant levels of MeHg to negative effects on terrestrial passerines (Whitney and Cristol 2017a). In a laboratory study, zebra finches fed environmentally relevant levels of MeHg during breeding showed significant reproductive impairment (Varian-Ramos et al. 2014). There is also evidence that some life stages are more sensitive to the toxic effects of MeHg than others. A recent study showed a reproductive decline in zebra finches (*Taeniopygia guttata*) exposed to MeHg only during developmental stages (in ovo to 50 days post hatch) (Paris et al. 2018). In a different study, zebra finches exposed to MeHg in ovo only had reduced hatching success but there was no apparent change in chick growth or survival post-hatch between treated and untreated chicks (Yu et al. 2016); there was, however, a later neuroanatomical change in sexually mature males that had been exposed to MeHg only as embryos (Yu et al. 2017). In a parallel study, zebra finch chicks were dosed for 21 days post-hatch and no differences were found in the breeding behaviour or reproductive success between treated or control finches (Morran et al. 2018). Growing chicks may be protected from the effects of elevated mercury exposure by sequestering MeHg into their feathers (Spalding et al. 2000; Ackerman et al. 2011; Rutkiewicz et al. 2013; Whitney and Cristol 2017b) and 'diluting' their body burden through somatic growth.

To date most studies on effects of MeHg on passerines and other bird species have focused on single life-stages (Heinz et al. 2009; Ackerman et al. 2011; Morran et al. 2018; Yu et al. 2016) or chronic, life-long-exposure (Hester et al. 1978; Fimreite and Karstad 1971; Kenow et al. 2003; Frederick et al. 2011). Few studies have compared effects of single life-stage exposures to ongoing exposure spanning multiple life-stages. Such studies could help identify the critical timing and duration of MeHg exposure to cause effects. In the present study we evaluated the acute and long-term effects of MeHg exposure in ovo only (embryonic exposure), as chicks only (post-hatching exposure), and with an 'in ovo + chick' treatment (embryonic and post-hatching exposure). The aims of the present study were to determine if terrestrial passerines have a developmental life-stage that is most sensitive to MeHg exposure, assess the potential for cumulative effects associated with exposure during more than one developmental life stage, and investigate the effects of different exposure scenarios on breeding success. Specifically, we investigate the effects of a combined in ovo and post-natal MeHg dosing, a situation that might represent the most realistic pattern of exposure for numerous, free-living bird species.

## Materials and methods

### Zebra finch husbandry

This project was conducted at the Animal Care Facility at Simon Fraser University in Burnaby, British Columbia, Canada. Non-breeding birds were housed in single-sex double sized cages (100 × 39 × 43 cm), with a maximum of 10 birds per cage. Non-breeding birds were provided with mixed seeds (panicium and white millet 1:2; 11.7% protein, 0.6% lipid and 84.3% carbohydrate by dry mass), water, grit, cuttlefish bone *ad libitum*, and a multivitamin supplement once per week. Food and water were changed daily. Animal rooms were maintained at a consistent temperature (19–25 °C) and humidity (35–55%) with a constant photoperiod of 14L:10D. All experimental work was conducted by trained individuals under a Simon Fraser University Animal Committee Permit (1070B-08) according to the guidelines of the Canadian Committee on Animal Care.

### Zebra finch breeding protocols

Male and female zebra finches were taken from stock cages and were randomly paired with preference given to experienced birds (those that had successfully reared chicks in the past). Because there were limited successful breeding pairs, some inexperienced birds were also used. Experienced and inexperienced birds were equally distributed between the four treatment groups. Breeding pairs were housed in individual cages (51 × 39 × 43 cm), each with an external nest box (14 × 14.5 × 43 cm). Each nesting box was filled with fresh hay for nesting material. In addition to the standard diet (above), breeding pairs were supplemented with egg food (hard-boiled chicken eggs including shells, breadcrumbs and cornmeal; approximately 20% protein, 7% lipid) for the duration of the breeding process (pairing until chicks were 30 days of age). The females and chicks in the F2 breeding trial were not provisioned with egg food. Pairs that did not successfully lay eggs within 15 days of pairing were separated, and females re-paired with a new male. Nest boxes were monitored daily for egg laying, and upon

laying all new eggs were weighed and numbered with a fine-tipped marker for recognition. Nest boxes were checked twice daily (morning and afternoon) starting at 12 days post first egg to monitor for hatching. Broken eggs were not included in fertility calculations.

At hatching, each chick was marked by plucking down feathers on different areas of the body for individual identification within the nest. Chicks were weighed (±0.01 g) daily, starting on the day of hatching. Plastic weigh boats were used to weigh younger chicks, and older chicks capable of flying were weighed in a felt bag. Tarsus length was taken on day 30. Chicks were banded with an aluminium or split plastic band on approximately day 10, enabling recognition of individual chicks after the loss of the initial down feathers. Once chicks reached 30 days of age they were considered 'independent' and were removed from the breeding cage. Chicks were then placed in regular non-breeding cages (100 × 39 × 43 cm), and small dishes with extra seed were placed at the bottom of the cages to ensure that those chicks that had not learned to use the feeder had access to food. Once chicks reached 60 days post-hatch, sexual dimorphism became apparent and chicks could be segregated and placed into single-sex cages. While chicks were segregated by sex, they were randomly assigned to cages so that all treatments were found in each cage. At a minimum of 90 days post-hatch, reproduction and courtship experiments were run on females and males respectively to investigate adult phenotypic quality.

## MeHg solution preparation

For solution preparation, methylmercury (II) chloride (MeHg chloride PESTANAL® analytical standard from Sigma-Aldrich; CAS: 115-09-3) was dissolved in double deionised water. Solutions were prepared and stored in new glassware that had been autoclaved, rinsed three times with acetone and hexane and then washed in nitric acid using the following procedure: clean lab ware was fully submerged in a dilute nitric acid tray containing 1.5 % HNO3 (Sigma-Adlrich; CAS: 7697-37-2) for at least 8 h. Glassware was then manually triple rinsed four times with Reverse Osmosis (RO) water, and then air-dried in a still-air hood. When completely dry, glassware was covered with Parafilm™. Two stock solutions were prepared; one for egg injection and one for chick dosing via pipette. The final analyzed concentration of the egg stock solution was 1.6 µg Hg/µl (2 µg MeHgCl/µl), and the final analyzed concentration of the chick stock solution was 0.96 µg Hg/µl (1.20 µg MeHgCl/µl). These concentrations were confirmed as total mercury concentrations (THg) at the Faculty of Agricultural and Environmental Sciences at McGill University in Montreal, QC, Canada, using a Nippon Instruments MA-300 in accordance with the EPA Method 7473 (U.S. EPA 2007), as detailed in Perkins et al. (2017).

## Egg dosing

Eggs in each clutch (Table 1) were randomly assigned to either: 1) control (vehicle-injected); 2) chick only (vehicle injected), or 3) MeHg injected treatments. Before dosing, eggs were weighed (±0.0001 g). Egg MeHg doses were based on the previous study of zebra finch embryotoxicity by Yu et al. (2016). The egg stock solution contained 1.6 µg Hg/µl. At the first signs of fertility, eggs were injected with a single dose of 2 µl of stock solution per gram of egg, resulting in a nominal dose of 3.2 µg Hg/g egg. Eggs were injected using 10 µl Hamilton syringes (Gastight 1700 Series) and sterile 26-gauge beveled needles. The needle was pushed through the side of the shell and the dose was injected into the albumen. The methods follow those used by Yu et al. (2016) and as described in Winter et al. (2013). The injection hole was sealed with cyanoacrylate glue

**Table 1** Hatching success and survival (of hatched chicks) across the four treatment groups. Hatching success was lower in eggs treated in ovo with MeHg, compared with control (vehicle-injected) eggs

| | Number and % of chicks per treatment | | | |
|---|---|---|---|---|
| | Control | In ovo only | Chick only | Combined (in ovo + chick dosed) |
| Number of eggs treated | 26 | 36 | 30 | 37 |
| Number hatched | 25 (96%) | 24 (67%)* | 27 (90%) | 25 (68%)* |
| Number survived (21 days) | 25 (100%) | 19 (79%) | 25 (93%) | 24 (96%) |
| Number survived (30 days) | 23 (92%) | 19 (79%) | 24 (89%) | 22 (88%) |
| Number survived (90 days) | 21 (84%) | 17 (71%) | 20 (74%) | 18 (72%) |
| Number survived (90 days; male/female) | 9 / 12 | 9 / 8 | 13 / 7 | 12 / 6 |
| Number of females surviving to breed (90–120 days) | 12 | 8 | 7 | 6 |
| Number of males surviving to breed (120–140 days) | 9 | 9 | 13 | 12 |

Asterisk indicates a significant difference

(Loctite Gel Control), and once the glue was dry the egg was returned to the nest. Remaining eggs in the clutch were injected with the vehicle (water) using the same method. After returning eggs to the nest, each egg was monitored to record hatching success and teratogenicity (i.e., deformities).

## Chick dosing

Chick dosing methods follow Morran et al. (2018). Clean stock birds were paired, and their nests were monitored closely to identify which chicks hatched from which eggs. Chicks ($n = 101$; see Table 1) were assigned to one of four treatment groups: (1) MeHg dosed in ovo (embryonic exposure); (2) MeHg dosed only as a chick (post-hatching exposure); (3) MeHg dosed both in ovo and as a chick (embryonic and post-hatching exposure); or (4) control (no dose), assigned randomly, but contingent on the egg treatment. Chicks from the embryonic and postnatal exposure treatment (the 'combined' treatment, $n = 26$) and the postnatal exposure group (the 'chick' treatment, $n = 26$) were dosed with MeHg from day 1 (24 h after hatching) to day 30. The target MeHg doses for the chicks in this study used previous dietary MeHg doses developed by Morran et al. (2018), which were based on previous laboratory and field studies of environmentally relevant dietary MeHg doses (Heinz et al. 2009; Varian-Ramos et al. 2013). Our targeted dose for chicks was based on the originally intended targeted 'high' dose (0.27 µg/g body weight (bw)) in the study by Morran et al. (2018), which was based on a seed-dosing study by Varian-Ramos et al. (2014). In the present study, the measured concentration of our stock solution was 0.96 µg/µl, which was diluted by a 1:3 ratio to give a dosing solution of 0.24 µg Hg/µl and chicks received 1 ul/g bw per day, pipetted directly into their gape. Chicks were dosed early in the day (07.30–10.30 h) prior to provision of egg food. Chicks were weighed every day to determine the precise dose and volume of water vehicle for dosing (±0.01 g), and to monitor effects of MeHg on growth.

## Blood samples

Blood samples were collected from chicks at 30 days post-hatch from the brachial vein of the wing using heparinized capillary tubes. Blood was transferred to microcentrifuge tubes and immediately frozen −20 °C until analysis. A pilot study was run to test MeHg dosing methods. A single P2 feather was taken from seven birds at 30 days when blood was sampled, and these were placed in individual labelled envelopes and shipped with the blood samples for analysis. All samples were analyzed on a Nippon Instruments MA-300 in accordance with the U.S. Environmental Protection Agency Method 7473, as detailed in Rutkiewicz and Basu (2013). Standard Reference Materials (SRMs) were measured each day of analysis to determine validity of the calibration curves. The SRMs for this study were Dorm-4 (Fish Protein; National Research Council of Canada), Human Hair 13 (National Institute for Environmental Studies, Japan), and two human blood standards (QMEQAS10B-09 and QMEQAS09B-05, Institut National de Santé Publique du Québec). One SRM and an empty quartz boat were run at least every nine samples, and one replicate blood sample was included at least every nine samples. Precision (reproducibility) was measured by comparing within-day and between-day replicate analysis of SRMs. The method detection limit (mean + 5 × SD) was 0.21 ng THg, and the mean analytical accuracy (102%) and precision (4.0%) across batch runs were acceptable.

## Assessment of adult phenotype: male (F2) courtship trials

Male courtship behaviour was assessed following the methods outlined by Yu et al. (2017), and Morran et al. (2018) and described in Zann (1996). In brief, males were placed in a cage with an untreated, experienced female for a 10 min mating trial and courtship behaviour of the male was recoded as: a) number of bill wipes (male wiping his bill on the perch); b) number of follows (male follows when the female moves between perches or between the bottom of the cage and a perch); c) number of unsuccessful mounts; d) number of successful mounts (male is able to make cloacal contact); e) time to first mount and f) if the male attempted to court the female by showing any of the aforementioned behaviours (yes or no). If the males failed to engage in courtship behaviours, they were exposed to an alternate female the following day. The female's response to the male was recorded on a scale of one to five with one meaning she avoided the male or showed no apparent response to courtship efforts (i.e., turned head away, flew away) and five meaning she allowed him to make cloacal contact. All males were tested twice.

## Assessment of adult phenotype: female (F2) breeding trials

At 90+ days of age, females were paired with a random, unrelated, clean experienced male under the same conditions as described above for breeding pairs. If a female did not lay eggs within 15 days of pairing, she was un-paired and labelled as a 'non-breeder'. All eggs were checked for signs of fertility and infertile eggs or eggs that showed signs of fertility but failed were noted. For the remaining females (those that laid eggs within 15 days), laying interval (number of days between pairing and first egg), clutch size, mean egg mass, brood size at hatch, brood size at 21 days,

and brood size at 30 days were recorded. For those that successfully raised chicks, the resulting chicks were weighed (±0.01 g) and tarsus measurements (±0.01 mm) were taken 30 days post-hatch.

### Statistical analysis

Statistical analyses were conducted in R (Version 3.4.1, packages used: nlme, lsmeans, lme4, lmerTest, multcomp, plyr, ggplot2) or SAS (Version 9.4, procedures used: glimmix, mixed, glm, logistic). Data were tested for normality and heteroscedasticity. Post-hoc tests for differences between means were adjusted for multiple comparisons using the Tukey-Kramer method. All values are presented as mean ± standard error of the mean (SEM), and statistical significance for all tests was set at $p < 0.05$. The mean blood mercury concentration of each treatment group was compared using a single-factor completely random design ANOVA. Egg hatching success and the proportion of chicks surviving to maturity was modelled with generalised linear mixed models (GLMM) using a binomial distribution and a logit link with the pair (nest) as a random factor. Goodness of fit was confirmed using the ratio of the chi-square statistic and its degrees of freedom (close to 1). The Hosmer-Lemeshow goodness-of-fit test (Hosmer and Lemeshow 1989) was used to determine whether the data adequately fit the logistic function; a $p$ value > 0.05 indicates adequate fit. Mean chick mass was compared between treatments using linear mixed-effects models, correcting for egg mass (covariate) and blocking by nest (random factor).

Treatment effects of treatment on continuous variables (latency to breed, clutch size, brood size at hatch, brood size at fledge) were tested using general linear models. Nested continuous variables (egg mass, chick mass) were tested using linear mixed models with nest as a random factor. Binomial variables such as the proportion of females that laid eggs, the proportion of fertile eggs, hatching success and fledging success (coded as 0 = died, 1 = survived) were compared among the treatments using logistic regression with proportion of events blocked by female. Adequate fit was confirmed by Hosmer-Lemeshow goodness-of fit tests. Offspring mass by age (i.e., growth) was compared between treatments using linear mixed-effects models, correcting for egg mass (covariate) and blocking by female (random factor). Female birds that laid eggs that failed to hatch were included in fertility assessments, but birds that failed to nest and lay eggs were excluded from these analyses. For analysis of male courting behaviours, only those that invited the female to court (i.e., performed a courtship behaviour) were used in the analysis. The data for some courtship behaviours (e.g., number of bill wipes) was normally distributed and was therefore analyzed using a Pearson's Chi2 test. The data for other behaviours were non-normally distributed and were analyzed using a Kruskal-Wallace test.

## Results

### Total blood and feather mercury levels

Blood THg concentrations in chicks sampled at 30 days post-hatching were higher in the chick only and the combined 'in ovo + chick' treatment groups compared with the in ovo and control groups ($p < 0.0001$; Fig. 1, Table S1). Blood THg concentrations in chicks did not differ between the in ovo and control treatment groups ($p = 0.74$) and between the chick only and combined 'in ovo + chick' treatment groups ($p = 0.17$, Fig. 1). The average THg concentration (626.5 ± 88.4 ng/g dry weight (dw), $n = 2$) in the feathers of birds from the combined 'in ovo + chick' treatment group was 38-fold greater than the mean THg concentration (16.5 ± 4.3 ng/g dw, range 10–29 ng/g dw, $n = 4$) in the vehicle-dosed P2 feathers for the control birds from the pilot study (Table S2).

### Hatching success

A total of 154 eggs were laid by 58 pairs of the F1 (unexposed) generation of zebra finches in this experiment. Of these, 17 were infertile (17/155; 11%), 7 were broken during handling (7/155; 4.5%), and 129 showed signs of fertility (131/155; 84.5%). Seventy-three fertile eggs were injected with methylmercury, 56 fertile eggs (30 chick only, 26 control) were injected with the vehicle (double deionised water) used to dissolve the methylmercury chloride. There was a treatment effect on hatching success ($F_{2,96} = 4.98$, $p = 0.009$; egg mass was controlled for, though this term was not significant in the model, $p = 0.42$). Hatching success was lower in eggs treated in ovo with MeHg (67%),

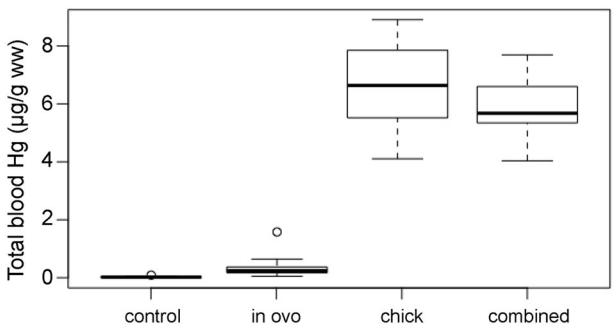


**Fig. 1** Total mercury concentrations (THg µg/g wet wt) in blood of zebra finch chicks at 30 d posthatch (dph) for the four treatment groups. The thick line represents the median blood THg concentration, and the outer squares of the boxplot represent the and 1st and 3rd quartiles

compared with control (96%) and chick only (90%) (vehicle-injected) eggs ($p < 0.05$). Hatching success did not vary between the control eggs and chick only eggs ($p = 0.42$; Table 1). The $\chi^2$/df ratio was close to 1 (0.92) indicating adequate fit of the model without residual overdispersion.

## Chick growth

There was no effect of treatment on growth, with no interaction between treatment and age for chick mass ($F_{12,426} = 0.75$, $p = 0.700$). Body mass significantly increased with age ($F_{4,\ 426} = 4689$, $p < 0.001$), but there was no effect of treatment on overall mass ($F_{3,426} = 1.76$, $p = 0.15$). Post-hoc multiple comparisons confirmed there were no differences in chick mass at any age (0, 5, 10, 21, 30 days) among different treatments (Tukey test, $p > 0.05$ in all cases).

## Survival

There was no treatment effect on the proportion of hatched chicks that survived to fledging (21 days post-hatch) ($F_{3,68} = 1.08$, $p = 0.362$; Table 1), and there was no treatment effect on post-fledging survival to indepencence (30 days post-hatch) ($F_{3,68} = 0.51$, $p = 0.678$) or maturity (90 days post-hatch) ($F_{3,68} = 0.46$, $p = 0.712$). However, mean hatchling survival to 90 days was highest in the control treatment group (84%) compared to 'in ovo + chick' (72%),'in ovo' (71%), and 'chick only' (74%) (Table 1). There were no overall treatment effect on sex ratio of the chicks surviving to 90 days among all four treatments ($\chi^2 = 2.92$, d.f. = 2, $p = 0.23$), likely due to low statistical power. However, pooling 'low THg' birds (control and in ovo) and 'high THg' birds (chick and 'in ovo + chick') there was a marginally non-significant sex-bias at 90 days. Only 13/38 high THg birds were female (34%) compared with 20/38 low THg birds that were females (53%, $\chi^2 = 2.62$, d.f. = 1, $p = 0.11$).

## Breeding success of F2 females

Although similar numbers of birds survived to independence (day 30) across treatments, only $n = 6$ females in the combined 'in ovo + chick' treatment survived to breeding (Table 2). Only 4/6 'in ovo + chick' females laid eggs and these females only produced two fertile eggs which, in turn, gave rise to a two chicks surviving to fledging.

There was no treatment effect on the interval between pairing of the females and the time taken to lay the first egg ($F_{3,24} = 0.64$, $p = 0.596$). Clutch size did not vary across treatments ($F_{3,29} = 0.36$, $p = 0.78$, including all females that were paired for breeding (Table 2). Similarly, the proportion of females that laid eggs did not vary across treatments ($\chi^2 = 0.898$, d.f. = 3, $p = 0.826$); however, fertility of eggs was significantly affected by maternal treatment ($\chi^2 = 20.4$, d.f. = 3, $p = 0.0001$), with eggs of 'in ovo + chick' exposed mothers having the lowest fertility (10%) and eggs from control and chick only females having the highest fertility (55% and 67%, respectively). Considering all eggs that were laid (and coding success to hatching or fledging as 0/1) there was a significant effect of treatment on hatching success ($\chi^2 = 16.0$, d.f. = 3, $p = 0.001$; Fig. 2). Hatching success was lowest (10.5%) in the 'in ovo + chick' treated females with only 2/16 eggs producing a chick at fledging (Fig. 2). Brood size at hatch was lower in the 'in ovo + chick' and 'in ovo' groups compared to the controls and

**Table 2** Female breeding success across the four treatment groups. A total of 33 female chicks survived until breeding. Mean clutch size did not differ between treatment groups, but low fertility resulting in low hatching success in eggs of birds exposed to MeHg in ovo led to low overall numbers of offspring surviving to independence (day 30)

| | Female breeding success by treatment | | | |
|---|---|---|---|---|
| | Control | In ovo only | Chick only | Combined (in ovo + chick dosed) |
| Number of females (Total) | 12 | 8 | 7 | 6 |
| Number of females (Laying) | 12 | 7 | 5 | 4 |
| Number of eggs laid[a] | 54 (51) | 30 (29) | 28 | 18 (19) |
| Number hatched | 25 | 6 | 17 | 2 |
| Mean clutch size (all females; ± SE) | 4.5 (±0.4) | 3.8 (±0.8) | 4 (±1.1) | 3.3 (±1.4) |
| Mean brood size at hatching (all females; ± SE) | 2.1 (±0.4) | 0.8 (±0.3)* | 2.4 (±1) | 0.3 (±0.2)* |
| Mean brood size at fledging (all females; ± SE) | 2.1 (±0.4) | 0.8 (±0.3) | 1.9 (±1) | 0.3 (±0.2) |
| Number of offspring surviving to day 30 | 25 | 6 | 13 | 2 |
| Chick mass at 30 days (g) | 13.5 (±0.1) | 13.7 (±0.3) | 12.8 (±0.3) | 13.1 (±1.1) |

Asterisk indicates a significant difference

[a]Number of eggs after removing broken eggs in brackets

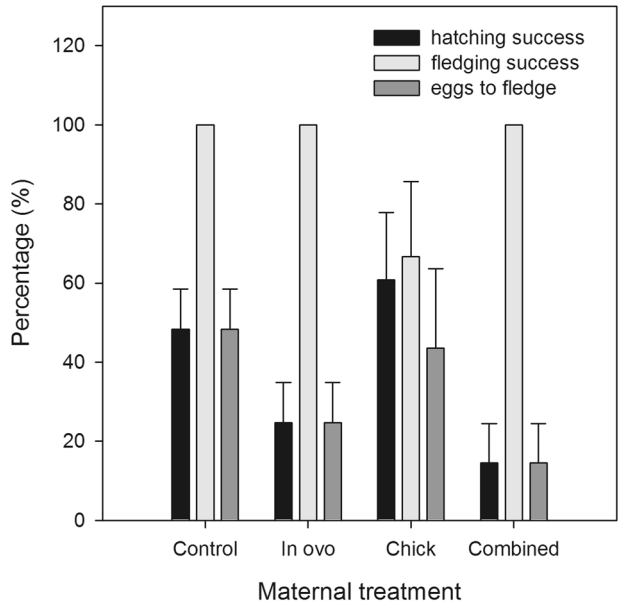


**Fig. 2** Mean hatching success (percentage of eggs to hatch), fledging success (percentage of chicks to fledge), and percentage of eggs to fledge (%) of F2 females differed across treatments with success being lowest in the 'in ovo + chick' combined treatment group. Fledging success was high across all treatment groups, and patterns are driven by low fertility of eggs from females in treatment groups with in ovo exposure

chick only groups ($F_{3,29} = 2.92$, $p = 0.050$; Table 2). Very few offspring died between hatching and fledging ($n = 4$, all in 'chick'-treated females, average fledging success of hatched eggs = 92%) so consequently fledging success did not differ across maternal treatments ($\chi^2 = 3.87$, d.f. = 3, $p = 0.275$). The proportion of all eggs that produced offspring that survived to fledging was lowest in the 'in ovo + chick' treatment group ($\chi^2 = 11.5$, d.f. = 3, $p = 0.009$), an effect that was primarily contributed to by the low hatching success (Fig. 2). Brood size at fledge was also lowest in the combined and in ovo groups, however the difference was not significant ($F_{3,29} = 2.24$, $p = 0.105$). Eggs from mothers exposed in ovo only were larger than eggs from control birds ($F_{3,104} = 3.45$, $p = 0.019$; pair as random factor), although chick size at hatch did not vary among treatments ($F_{3,30} = 0.84$, $p = 0.481$), and there was no difference among treatments in chick mass at independence (30 days post-hatching; $F_{3,27} = 2.47$, $p = 0.083$, Table 2).

### F2 male courtship trials

Thirty-six males were tested in mating trials across four treatments; $n = 9$ for the 'control', $n = 10$ for 'in ovo', $n = 8$ for the 'chick' and $n = 9$ for the 'combined' treatments (Table S4). Twenty-six (59%) males showed positive attempts to court during their first introduction to the females, and an additional 8 males attempted their first courtship during a second breeding trial, resulting in a total of 34 (77%) successful pooled trials. Ten males did not engage in any courtship behaviour in any trial; 6 were from the 'chick', 3 from 'combined' and 1 from the 'control' treatments (Table S4). There was a treatment effect on the number of males engaging in courtship behaviour (Fisher's Exact Test, $p = 0.0014$), with the 'chick' treatment males showing a lower inclination to engage in courtship behaviour compared to the other groups (Table S4).

The data for the males that engaged in courtship in the second trial was pooled with the successful first trials for subsequent analysis to maximise the data set available for analysis ($n = 34$ males). There was no treatment effect on the frequency of 'follow' behaviours ($F_{3,30} = 1.03$, $p = 0.39$), 'bill wipes' ($F_{3,30} = 0.16$, $p = 0.93$), the number of mount attempts ($\chi^2 = 3.95$, d.f. = 3, $p = 2.67$), successful mount attempts ($\chi^2 = 4.80$, d.f. = 3, $p = 0.19$) or the time taken to mount females ($\chi^2 = 0.93$, d.f. = 3, $p = 0.81$) by the male birds (Table S4). There was no treatment effect on the response of the females to the male birds ($\chi^2 = 0.11$, d.f. = 3, $p = 0.99$) (Table S4).

## Discussion

We exposed a model terrestrial passerine species, the zebra finch, to environmentally relevant concentrations of MeHg by (1) injecting eggs with MeHg in a water vehicle to simulate maternal transfer and embryonic exposure to MeHg, and (2) dosing of chicks orally from hatching until fledging to simulate provision of food by parents until chicks were ready to disperse from the nest. Through these controlled dosing methods we were able to isolate exposure to specific life stages, allowing us to evaluate the influence of different timing and durations of exposure to MeHg during early developmental stages. In addition to measuring acute effects (hatching success, growth, nestling survival), we also measured long-term effects (adult survival, reproductive success, mating behaviour) to identify whether developmental exposure to MeHg can have latent consequences for terrestrial songbirds. A recent study in zebra finches (Paris et al. 2018) also evaluated the long-term effects of MeHg exposure that was restricted to the developmental stage (embryo to 50 days post-hatch). In the previous study, all embryos were exposed via maternal transfer, and chick exposure was through the diet, meaning that the parents were simultaneously exposed, and possible indirect effects (e.g. via parental behaviour) could not be ruled out (Paris et al. 2018). Despite methodological differences, our results are largely consistent with Paris et al. (2018), which found reduced reproductive success in exposed birds once they reached maturity. In addition, we were able to identify that the combined'in ovo + chick'

exposure treatment has compounding long-term effects compared to exposure at a single life stage.

The blood THg concentrations in the present study were within the target range for each of the treatments and reflect environmentally relevant concentrations. The blood THg concentrations in the in ovo treatment chicks (0.3 ± 0.08 µg/g) were similar to concentrations reported in Yu et al. (2016), who had blood THg levels of 0.066 ± 0.015 µg/g in the highest dosed zebra finch cohort at 30 days post-hatch. A wide range of passerine species from across the north-east of North America had mean blood THg between 0.044–1.060 µg/g (Jackson et al. 2015), which are within the range of the blood THg levels seen in the control (0.03 µg/g) and in ovo treatment (0.31 µg/g) in the present study. The blood THg concentrations in the birds from the 'chick only' and the combined 'in ovo + chick' treatments (~6 µg/g) were, however, consistent with Paris et al. (2018) mercury exposed 25 day old juvenile F1 finches (7.4 µg/g) and more in-line with the higher range of blood THg concentrations measured in wild birds at heavily contaminated sites. Tree swallows at such contaminated sites in the northeastern United States had blood mercury in the range of 3.56 ± 2.41 µg/g (Brasso and Cristol 2008), while free-living Black-footed Albatross (*Phoebastria nigripes*) had blood THg of up to 6.4 µg/g (Finkelstein et al. 2007). These concentrations have also been associated with sub-lethal but sensitive endpoints such as fertility and courtship behaviour in free-living avifauna (Schoch et al. 2014; Fuchsman et al. 2017; Evers et al. 2008). The levels of THg seen in the feathers of the 'combined' dosed birds (627 ng/g) were within the range of THg seen in birds in the wild. For example, a range of gull species sampled on the Southern Baltic coast in Poland had feather mercury levels between 79.0–9186 ng/g (Szumiło-Pilarska et al. 2017), and tree swallows living at mercury impacted sites had feather THg levels of 13,550 ± 6940 ng/g (Brasso and Cristol 2008).

MeHg is excreted into feathers as young birds are growing during the nestling and fledgling phases, and that reduction in circulating and tissue MeHg appears to act as a protective mechanism for growing passerines. Increased THg concentrations in P2 feathers were found in birds that were dosed in ovo and also as chicks, and is consistent with MeHg being excreted in feathers during rapid growth periods and with findings of previous studies (Yu et al. 2016; Morran et al. 2018).

In ovo exposure to MeHg (3.2 µg Hg/g egg) was associated with reduced hatching success (67–68%) in exposed group vs 96% and 90% in the control and chick only egg groups, respectively, however no post hatching effects were found for growth, mating behaviour, reproduction, or survival. These results mirror previous lab-based findings on zebra finches which showed that in ovo exposure to 3.2 µg Hg/g egg) reduced hatching success (Yu et al. 2016), yet no adverse effects were found for the hatched chicks for growth, mating behaviour or survival (Yu et al. 2016, 2017). There is extensive evidence linking increased exposure to MeHg in ovo with reduced hatching success in avifauna (Heinz et al. 2009; Kenow et al. 2011; Rutkiewicz et al. 2013; Yu et al. 2016). Heinz et al. (2009) found that both mallard and chicken (*Gallus gallus*) egg hatching rates were affected at MeHg concentrations of 1 µg/g ww, and that survival dropped significantly at 1.6 µg/g ww (Hester et al. 1978; Heinz 1979). Common Loons showed a reduced hatching rate when eggs were injected with 1.3 µg/g ww of MeHg (Kenow et al. 2011). Conversely, low level exposure to MeHg has been found to result in a hormotic response, with mallards (*Anas platyrhynchos*) exposed to 0.5 µg/g $bw^{-1}$ of MeHg producing larger clutches containing heavier chicks than those in the control group (Heinz et al. 2010). Methylmercury is a neuroteratogen in mammals, with foetal exposure causing changes to neuronal structure, gross brain structure and an overall reduction in brain weight (Hulla 2014). In birds, egg failure may also be attributed to the teratogenic effects of MeHg (Heinz et al. 2011). To date, no studies have determined the mechanism by which maternal transfer reduces embryotoxicity (G. Heinz, *pers. comm.*). The findings that egg injections enhance MeHg toxicity when compared to maternally transferred MeHg (Heinz et al. 2009), should be taken into consideration when extrapolating from the results of this study to the potential impacts of MeHg egg exposure on wild bird populations (Heinz et al. 2009, 2011).

We found no growth-related effects for any of the treatment groups. These results are consistent other bird studies which found no change in growth when zebra finch and common loons were exposed to environmentally relevant levels of MeHg (Morran et al. 2018; Yu et al. 2016; Kenow et al. 2003). The putative mechanism of action for MeHg growth suppression in birds has been linked to behaviour; organisms exposed to elevated levels of MeHg may experience appetite suppression (Frederick et al. 2011). For example, chickens provisioned with water containing 500 mg/L of MeHg had reduced growth that was linked to a reduced appetite (Hester et al. 1978). Red-tailed Hawk (*Buteo jamaicensis*) chicks had reduced growth when provisioned with food containing 10 mg/g of MeHg (Fimreite and Karstad 1971).

In the present study the birds in the exposed treatment groups had lower mean survival rates to sexual maturity compared to the 'control' group. While differences were not statistically significant, this might still have pertinent population-level effects on wild birds, especially when coupled with the trend for sex-biased survival and reduced fertility we observed (see below). There are limited studies assessing the long-term impacts of MeHg exposure to bird survival in the wild, mainly because of challenges in

determining exposure and effects in wild birds. Other studies which have used life-long or single life-stage exposures to MeHg have found mixed results regarding altered survival or avian fledging rates largely due to the wide range of exposure regimes and study designs used. Frederick et al. (2011) fed white ibises (*Eudocimus albus*) up to 0.3 MeHg µg/g ww and found that survival was lower in the control and high-dosed birds when compared to the low or medium-dosed birds, suggesting a possible hormetic effect. Whereas, Ackerman et al. (2008a, b) found no apparent relationship between blood THg and survival for the first 35 days post-hatch in the Forster's terns (*Sterna forsteri*), and only weak evidence of a relationship between Hg exposure and the survival of fledgling American avocets (*Recurvirostra americana*) and black-necked stilts (*Himantopus mexicanus*). Morran et al. (2018) exposed zebra finch chicks with up to 0.15 µg/g $bw^{-1}$ MeHg for 21 days post-hatch and found no significant effects on survival between treatment groups, which suggests that in ovo exposure coupled with chick exposure, as in our study, might be important.

In addition to low hatching success and lower post-fledging survival we found some evidence for sex-biased survival: the sex ratio of birds reaching sexual maturity (90 days) was male-biased in the 'chick' and 'combined' treatment groups when compared to the sex ratios of both the 'control' and 'in ovo' chicks. This suggests birds may have sex-based differences in MeHg sensitivity. However, we cannot be certain if this sex ratio bias reflects a difference in M:F ratio at egg-laying, or sex differences in survival since we could not sex birds until 35–40 days post-hatching. Few environmental toxicology studies have tested for sex differences in MeHg toxicity, despite evidence across multiple species that males and females have differing sensitivities to this organometallic compound and to other trace metals (Haber and Jennings 1964; Hirayama and Yasutake 1986; Vahter et al. 2007). Androgens have been implicated in the higher levels of MeHg seen in the urine of male mice when compared to female mice (Hirayama and Yasutake 1986), and sex-related differences in the patterns of nephrotoxicity have been observed in rats exposed to MeHg (Haber and Jennings 1964). Robinson et al. (2012) undertook a meta-analysis of THg concentrations in birds of both sexes and found that female birds of all species had lower overall THg body burdens when compared to males which they attributed to maternal transfer of some of the THg body burden into eggs.

As a consequence of decreased survival and the sex-bias at maturity very few females survived to breed in the 'in ovo + chick' treatment, and these females showed reduced fertility: only 4/6 females laid eggs, and these females had lower hatching and fledging success. Lower productivity was mainly due to lower hatching success, with only 2/16 eggs hatching, not due to lower chick-rearing success, and we found no effect of treatment on clutch size or latency to laying among females that did lay eggs. Those observations are consistent with field studies which have reported reduced female fertility with increased levels of THg. Wild female tree swallows near contaminated sites had a mean blood THg concentration of 3.56 µg/g and reduced hatching success. Wild Carolina wrens (*Thryothorus ludovicianus*) had blood THg levels between 1.96 to 3.38 µg/g and concurrent reduced female fertility in the group of birds exposed to higher THg concentrations, and wild common loons (*Gavia immer*) which had mean blood THg concentrations of 3.0 µg/g and associated impaired reproductive success (Evers et al. 2008; Jackson et al. 2011).

Males exposed to MeHg as chicks only showed a lower inclination to engage in courtship behaviour when compared to the other treatment groups. These results are consistent with observations from previous studies which found that exposure to MeHg may adversely impact male pairing efforts (Heinz 1979; Frederick and Jayasena 2010; Heinz et al. 2009). White ibis exposed to environmentally relevant levels of MeHg had an increased number of same sex pairings and reduced clutch success (Frederick and Jayasena 2010), and songbirds living in mercury-impacted areas had a reduced song complexity, potentially reducing their perceived fitness by females (Hallinger et al. 2010). Results from the present study, however, differ from Morran et al. (2018) which found no evidence of a change in male courtship behaviour in zebra finches exposed to MeHg as chicks. That could quite likely be due to the higher (3.3-fold) dose given to the chicks in the present study compared with the highest exposure group dose given to chicks in Morran et al. (2018).

In summary, we confirmed that in ovo exposure to MeHg reduces hatching success, and we found some evidence for longer-term effects of combined 'in ovo + chick' exposure on post-fledging survival, potentially sex-biased survival and fertility (reduced hatching success in adult females treated as chicks). Our results are largely consistent with findings from a recent study by Paris et al. (2018) which found that zebra finch exposed to relatively high concentrations of MeHg during embryonic and juvenile development had reduced reproduction (although both the male and female in the breeding pairs were exposed). They also found other similar effects as in our study, including a non-significant trend for MeHg-exposed pairs to have a lower probability of initiating breeding, no treatment effect on clutch size, reduced hatching success in MeHg-exposed pairs, and no difference in probability of survival of offspring to 48 days post-hatching. These two studies therefore suggest that MeHg exposure during early developmental stages in songbirds alters reproduction in later generations likely through a mechanism that influences female fertility,

i.e. the ability to lay fertile eggs that hatch. That could be a result of increased oxidative stress on parents (Henry et al. 2015) or through direct embryotoxicity after maternal transfer of MeHg (Heinz et al. 2009). Elevated environmental levels of MeHg have been implicated in the decline of a range of species, and animal and human studies have shown that MeHg has a deleterious effect on the endocrine system and on reproductive success (Zhu et al. 2000; Tartu et al. 2013; Varian-Ramos et al. 2014). There is some evidence that THg may suppress the ability of the pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH) (Tartu et al. 2013). Gonadotropin-releasing hormone (GnRH) in the hypothalamus may also be suppressed in the presence of THg (Tartu et al. 2013). Both of those mechanisms are likely to impact reproductive performance of birds. Other mechanisms that may impact productivity include behavioural changes; for example, White Ibises (*Eudocimus albus*) had lower fecundity, which was attributed to an increasing predisposition towards same sex pairing in birds with higher MeHg body burdens (Frederick and Jayasena 2010). The physiological and/or behavioural basis of long-term effects of MeHg should be investigated further in future studies.

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## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Ethical approval** All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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