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Sexually selected traits as bioindicators: exposure to mercury affects carotenoid-based male bill color in zebra finches

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Abstract

To examine whether sexually selected traits are particularly sensitive bioindicators of environmental toxicants, we assessed the effects of exposure to environmentally relevant dietary concentrations of the pollutant methylmercury on pigment coloration in zebra finches (*Taeniopygia guttata*). First, we tested whether effects of methylmercury on coloration were influenced by timing of exposure. Birds were either exposed developmentally (up to 114 days after hatching), as adults (after reaching sexual maturity), or for their entire life. Bill coloration, which is a carotenoid-based, sexually selected trait, was less red in males with lifetime exposure to methylmercury, compared to controls. Neither adult, nor developmental exposure influenced bill color in adult males, with the possible exception of early exposure of nestlings. Among females, where bill color is not under strong sexual selection, neither lifetime nor adult exposure to methylmercury affected bill color. For males and females, there was no effect of either lifetime or adult methylmercury exposure on coloration of back feathers, which is a non-sexually-dimorphic, melanin-based trait that is not likely the result of sexual selection. This study is a comprehensive experimental test of the proposal that sexually selected traits may be particularly useful bioindicators of the stress imposed by environmental toxins such as methylmercury.

Keywords Bioindicator · Carotenoid · Coloration · Methylmercury · Sexual selection · Zebra finch

Introduction

Traits exposed to strong sexual selection may be particularly sensitive to environmental stressors during development (Hill 1995; Møller and Swaddle 1997; Spencer and MacDougall-Shackleton 2011). A sexually selected trait can be more costly to develop and maintain, as the increased costs of the trait may enforce honest signaling of genetic and/or phenotypic quality (Johnstone 1995; Kuijper et al. 2012), which has led to suggestions that such traits will be sensitive bioindicators of environmental conditions during development or the production of the ornament (Hill 1995; Lifshitz and Clair 2016; Gore et al. 2018). However, evidence for such claims does not appear to be consistent across taxa or traits (Moore et al. 2015) and there is limited experimental support for the predicted patterns (Bjorksten et al. 2000).

Experimental application of environmental toxicants has indicated that certain sexually selected traits or behaviors may be particularly affected by contaminants, either through disruption of endocrine pathways (Shenoy 2012; Bertram et al. 2015), or as others have hypothesized, oxidative stress may be a mediating pathway through which contaminants disrupt signaling (Marasco and Costantini 2016). There is also research suggesting that developmental or early-life exposure to stressors can have lasting effects on the fitness of an individual (Spencer and MacDougall-Shackleton 2011; Paris et al. 2018). Though we did not assess reproductive output in this study, the developmental stress hypothesis can be applied to coloration that influences mating outcomes—where exposure during a key window of early life can result in downstream changes to color, particularly carotenoid-based traits where the ability to assimilate pigments into integument could be disrupted by developmental stress (Walker et al. 2013).

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Many avian species rely heavily on coloration, which is critical for camouflage, thermoregulation, species recognition, dominance interactions, and mate attraction (Hill and McGraw 2006). There are several studies indicating associations between environmental contaminants and changes in coloration of sexually selected traits of birds (Bortolotti et al. 2003; Dauwe and Eens 2008; Giraudeau et al. 2015), but most existing evidence is based on field correlations, thereby not explicitly demonstrating that contaminants directly influence color production (but see Vallverdú-Coll et al. 2015), nor the magnitude of any such effect. Several recent examples involve the pollutant methylmercury (hereafter Hg). Both belted kingfishers (*Megaceryle alcyon*) and eastern bluebirds (*Sialia sialis*) living on Hg-contaminated sites had increased brightness of their blue feathers when compared with birds from reference sites, possibly due to a decrease in the light-absorbing melanin layer present in structurally blue feathers, which would allow more light to be reflected (White and Cristol 2014; McCullagh et al. 2015). The concentration of Hg was negatively correlated with color brightness of yellow in feathers of great tits (*Parus major*), likely indicating decreased carotenoids in these feathers (Giraudeau et al. 2015).

In zebra finches (*Taeniopygia guttata*), the main carotenoid-mediated coloration is bill color, which is a sexually-dimorphic trait under sexual selection because female zebra finches prefer to mate with males that have redder bills (Burley and Coopersmith 1987; Simons and Verhulst 2011). Based on previous studies of avian exposure to Hg, we hypothesized that coloration of zebra finch bills would be affected by exposure to Hg and, specifically, that coloration traits known to be under sexual selection would be more sensitive to Hg exposure than traits not involved in mating behaviors. Therefore, we also tested the effect of Hg exposure on melanin-based coloration in the gray/brown feathers of the zebra finch back, which are not known to be under sexual selection. We tested these effects by experimentally manipulating the Hg content of the diet of captive zebra finches. We also explored whether developmental exposure to dietary Hg influenced the traits more than exposure after the birds reached sexual maturity. To do this we examined effects of exposing birds to Hg either from conception to independence, conception to near sexual maturity, independence to near sexual maturity, sexual maturity through adulthood (adult exposure), or throughout their entire lives (lifetime exposure).

Materials and methods

Animal care

Experiments were conducted at the William & Mary aviary in Virginia, USA, between May 2012 and July 2016. Domesticated wild-type zebra finches were housed in single-sex indoor cages (~0.5 × 1.0 × 0.5 m) in groups of 3–6 on a 14:10 light:dark photoperiod at 18–20 °C. All birds were provided ad libitum access to food, vitamin-enriched water, grit, and cuttlefish calcium supplement. A 10% lutein and 1% zeaxanthin powder (FloraGlo Lutein, Kemin Industries Inc., Des Moines, IA) was also mixed with drinking water at 7 μg/mL to provide plant carotenoids in an amount representing approximately 10% of what is found in commercial seed-based diets (McGraw and Ardia 2004). We intended for birds to be carotenoid-limited so as to maximize the probability of observing variation in carotenoid-based coloration patches. No carotenoid supplement was used for the experiment involving Hg-exposure early in development because these birds were also being used for additional studies, producing an even more carotenoid-limited situation for the birds.

A commercial pelletized bird food (Zupreem FruitBlend for very small birds) was dosed with aqueous methylmercury-cysteine, representing 10% of the weight of the food, and was thoroughly mixed using a rock tumbler. Hg exposures spanned the range of concentrations (0.3–2.4 μg/g wet weight, dry weight range: 0.4–2.8 μg/g), which included environmentally relevant exposures expected for birds near industrial point sources, as described in detail in Varian-Ramos et al. (2014). To reduce the amount of animal use, not all Hg exposures were used in all experiments, as noted. The control (0.0 μg/g) birds received in their diet 10% water and cysteine without Hg to ensure equivalency with Hg-dosed diets. To ensure the concentration of Hg consumed was close to the intended dose, 10 food samples were analyzed for Hg content from every batch prepared and the batch was rejected if it varied by more than 10% from the nominal dose.

Hg exposure to examine bill coloration

To accomplish our goal of examining the effect of Hg-exposure on bill coloration we examined the coloration of adult bills using three different exposure scenarios: developmental (3 different timing scenarios), adult (i.e., no Hg exposure until after sexual maturity), or lifetime (i.e., both developmental and adult). As noted below, some experiments included both sexes whereas others included only males, in an effort to reduce animal use. For the same reason, not all levels of Hg exposure were used with each set of birds. Treatments and exposures are detailed in Table 1.

Developmental exposure

To create young birds that had been exposed to Hg at different stages of development, but not during adult life, we started with birds from lineages never exposed to Hg and mated pairs of unrelated birds (no siblings or cousins)

Table 1 Overview of Hg exposure treatments used to test effects on coloration of red bill or gray/brown back feathers

Bill color[a]		Feather color[b]	
Timing of Hg exposure	Exposure (μg/g)	Timing of Hg exposure	Exposure (μg/g)
Development			
Early: egg + nestling	1.2		
Late: juvenile	1.2		
Both: early + late	1.2		
Adult (after sexual maturity)	0.6 or 1.2	Adult (after sexual maturity)	0.6 or 1.2
Lifetime (Development + Adult)	0.3, 0.6, 1.2 or 2.4	Lifetime (Development + Adult)	0.6 or 1.2

[a]Trait under sexual selection

[b]Trait under natural selection

exposed to no Hg (control) or 1.2 μg/g Hg, for 10 weeks prior to breeding. This duration of exposure was sufficient to allow blood Hg levels to asymptote. This treatment resulted in eggs of Hg-exposed pairs receiving maternally-transferred Hg (see Ou et al. 2015 for Hg concentrations resulting in zebra finch eggs on this dose). However, to create three different developmental exposure scenarios and a control group, we also used the same parents to produce eggs while eating undosed food, with at least 10 weeks for Hg depuration allowed between treatments. This switching of parental treatments allowed us to split siblings into control or Hg-exposure treatments. Once these eggs were laid they were taken from their biological parents and given to foster parents on the same exposure to Hg. By using foster parents to raise young from many different pairs of parents we partially decoupled effects of parental genetics from those of parental care.

Foster parents incubated eggs and fed nestlings for their first 50 days after hatching, so if an egg was laid by a control or Hg-exposed pair, the hatchling had the same exposure treatment as the parents for at least 64 days, including 14 days in the egg and 50 days as a nestling/fledgling. Upon reaching the age of 50 days after hatching, the juveniles were randomly assigned to either a control or a Hg treatment for the next 64 days of life. This resulted in four treatment groups: the first (control) received no Hg exposure ever ($n = 24$), the second (early) was exposed in the egg (~14 days) and during the first 50 days after hatching, but not thereafter ($n = 51$), the third (late) was exposed only during their second 64 days after hatching ($n = 25$), and the final treatment (both: early + late) was exposed in the egg and during their first 114 days after hatching ($n = 41$). Control and Hg-exposed birds were housed in the same rooms to avoid potential effects of pseudoreplication due to room effects. For this portion of the study, on developmental exposure, we used only males to simplify analysis. Birds were bred in batches over several years to generate the necessary sample size, but the bill coloration of these developmentally exposed birds was measured at one time to reduce measurement error. Thus, birds were measured at a variety of ages, but always after they had reached sexual maturity (>120 days; mean age of measurement: 388.1 ± 16.5 days, range: 122–788 days). Because bill color can vary rapidly with conditions, but there were constraints on how many birds could be bred and housed at one time, we decided that measuring birds during a narrow time period with similar environmental conditions was preferable to measuring them at similar ages over a long period of time.

Adult exposure

To produce the adult-exposed treatment, a different group of sexually-mature zebra finches (>120 days of age, range: 123–712 days) were assigned randomly across three treatment groups and housed in single-sex social groups of three birds per cage (0.0 μg/g Hg: 9 males, 9 females; 0.6 μg/g Hg: 9 males, 9 females; 1.2 μg/g Hg: 9 males, 9 females). We included no more than two related individuals (parent, offspring, or siblings) in each treatment to reduce confounding factors of genetic similarity. Ten weeks prior to the start of the study, birds were converted from a whole seed diet (Volkman Avian Science Super Finch Blend) to pelletized food (ZuPreem, as described above) with one of three levels of Hg (0.0, 0.6, or 1.2 μg/g Hg). Initial blood samples were tested to ensure that birds had not been exposed to Hg prior to beginning the experiment. Weekly blood samples (<30 μL) were collected on a rolling basis for Hg analysis to detect any accidental Hg exposure. After 10 weeks, blood Hg levels had stabilized and experimental data collection began.

Lifetime exposure

To create the lifetime-exposed treatment, offspring were obtained from parents who were exposed to 0.0, 0.6, or 1.2 μg/g Hg, as in the developmental exposure study above. Parents were exposed through the entire breeding process,

and then their young were kept throughout life on the same exposure for use in the current study. Foster parents were not used for the lifetime exposure study, nor were parents switched between treatments. Such a precaution against maternal effects would be desirable out of an abundance of caution, but we deemed it to be unnecessary because we have not detected any effects of these concentrations of Hg-exposure on incubation or parental care in our colony of zebra finches (Chin et al. 2017). Juveniles were removed from parents upon independence (~50 days after hatching) and placed temporarily in large, single-sex outdoor aviaries before being assigned to treatments and moved into the smaller, indoor cages. To reduce confounding factors due to relatedness, no more than two offspring of each sex from a single breeding pair were used in this study. The resulting sample size in each treatment of lifetime-exposed birds was: 0.0 µg/g Hg: 9 males, 9 females; 0.6 µg/g Hg: 9 males, 9 females; 1.2 µg/g Hg: 9 males, 8 females.

We then replicated the lifetime exposure study a second time using five dietary Hg exposures instead of three, for finer resolution and to examine whether there was a dose-response. We raised male zebra finches (N = 68) from parents fed Hg at one of the following exposures: 0.0 µg/g Hg (n = 13), 0.3 µg/g Hg (n = 13), 0.6 µg/g Hg (n = 12), 1.2 µg/g Hg (n = 12), or 2.4 µg/g Hg (n = 12). When the juveniles reached independence (46 days old for this portion of the study) they were removed from their natal cages, housed in single-sex cages of 3–5 birds, and provided with the same diet as their parents. Two or fewer siblings were used from each pair, but in three cases a third sibling was added to increase sample sizes. At sexual maturity (120–150 days), we measured bill color using the reflectance spectrophotometric techniques described below.

Hg exposure to examine feather coloration

To determine the effect of Hg on color of newly grown feathers in sexually mature birds, we collected gray/brown back feathers from both the adult-exposed (0.0, 0.6, or 1.2 µg/g Hg) and the first group of lifetime-exposed (0.0, 0.6, or 1.2 µg/g Hg) birds. Because feathers only incorporate Hg from the circulatory system while they are growing, we had to pluck existing feathers, potentially grown prior to Hg exposure or sexual maturity, so that new ones would grow while the birds were sexually mature and consuming Hg in their diet, close to the date of bill measurements. Prior to sampling, a patch of ~45 feathers was removed from the interscapular region of the spinal feather tract to remove feathers that had grown under prior Hg exposures. After allowing all feathers in this region to fully regrow (~6 weeks), 15 feathers were removed from the center of the regrown patch to ensure that they were newly grown feathers, and stored in glassine envelopes for later mounting and color analysis using the reflectance spectrophotometric techniques described below.

Measuring color with spectrometry

We measured the reflectance of bills with a USB2000 UV-VIS portable reflectance spectrometer with a PX-2 pulsed xenon lamp (Ocean Optics Inc., Dunedin, FL). We took five measurements of bill reflectance from each bird on a single day, after they had reached sexual maturity, aiming the probe at the upper surface of the mandible between the nares. Measurement repeatability was high for a given bird (relative percent differences ≤1% for all variables). The five measurements were averaged to minimize the small measurement error. Feather reflectance was measured with the same technique, with each sample of 15 feathers mounted on standardized black card stock in a way that mimics how they lay in an overlapping pattern on the back of a bird. All reflectance curves, for bills and feathers, were processed using the Java-based CLR program (supplied by Robert Montgomerie, Queen's University) to generate metrics of bill color variation: hue, saturation, and brightness (Table 2). Because zebra finch back feathers are monochromatic, we only assessed feather brightness.

Hg analysis

Because birds of all dietary treatments were housed in the same rooms to avoid pseudoreplication, monthly blood samples were collected on a rolling basis to detect any accidental exposure of treatment groups to the incorrect diet. Approximately 30 µL were collected with a heparinized microcapillary tube via brachial vein by puncture with a 30-gauge needle. All samples were tested using a direct Hg analyzer (DMA-80, Milestone Inc., Shelton, CT). For approximately every 20 samples, we ran a duplicate blood sample, three method blanks, and two certified

Table 2 Equations used to calculate color variables from raw reflectance spectra

	Color metric	Equation	Tissue analyzed
Equation A	Brightness	[a] $B = \sum_{\lambda R700}^{\lambda R450} Ri$	Bill and feather
Equation B	Saturation	$S = (\sum_{\lambda 700}^{\lambda 610} Ri)/B$	Bill
Equation C	Hue	[b] $H = \lambda_{R450\text{-}700}$	Bill

Brightness is a measure of how much light is reflected off of the bill or feather and measured by the spectrometer, while saturation is the measure of how much of that light falls into the "red" spectrum—between 605 and 700 nm. Hue is the peak wavelength of light, where the most light is reflected. All variables were calculated by the CLR program supplied by Robert Montgomerie, Queen's University

[a] R_i is the proportion of light reflected at the ith wavelength (λi)

[b] λ is the wavelength at maximum reflectance in the given range of wavelengths

Table 3 Average blood Hg levels for adult-exposed and lifetime-exposed adults (both first and second repeats of study) within approximately one month of color sampling

Treatment	0.0 µg/g	0.3 µg/g	0.6 µg/g	1.2 µg/g	2.4 µg/g
Development	0.00–0.05	–	–	4.63–16.55	–
Adult	$0.01_{0.002}$	–	$8.32_{0.237}$	$16.50_{0.407}$	–
Lifetime (1st)	$0.01_{0.001}$	–	$10.65_{0.231}$	$18.62_{0.467}$	–
Lifetime (2nd)	$0.07_{0.022}$	$4.14_{0.219}$	$7.95_{0.280}$	$14.02_{0.547}$	$30.03_{1.038}$

For developmentally exposed birds, only trace quantities of Hg remained in blood during adult life, when they were sampled, but range of blood concentrations measured 25-50 days after hatching are reported here (from Paris et al. 2018). Mean blood levels are presented in µg/g wet weight with subscripted standard errors

standard reference materials (DORM-3 and DOLT-4, National Research Council of Canada). Recovery of standards and spiked matrix, repeatability of duplicates, and adherence to the stated dietary exposures were all within normally accepted limits for within-lab quality assurance, and have been published previously, as these same birds were used for multiple studies (Varian-Ramos et al. 2014 for adult and lifetime exposure experiments, Paris et al. 2018, for developmental exposure experiments).

Data analyses

All statistics were conducted using SPSS v23 (IBM Inc., Armonk, New York) employing two-tailed tests of probability throughout where statistical significance was interpreted as $P \leq 0.05$. Bill coloration datasets, including brightness, saturation, and hue, were reduced into a tristimulus color score using Principal Components Analysis (PCA). Males and females were analyzed separately because zebra finches are highly sexually dimorphic (see Supplemental Material). Developmentally exposed birds were also analyzed separately because the color of the pelletized food (ZuPreem) they received appeared to have been changed by the manufacturer over the course of the studies reported here, and we did not supplement developmentally exposed birds with additional carotenoids due to constraints of other studies for which they were used. Both factors could influence overall bill color relative to those birds in the lifetime and adult-exposure experiments.

In general, the color variables from bills loaded heavily onto a single component (PC1), accounting for the majority of the total variation. As PC1 increased, saturation and hue increased and brightness decreased, indicating that bills with high PC1 scores were more red-saturated, more concentrated in the red portion of the color spectrum, and less bright across the whole spectrum. For detailed interpretation of the PCA for each sex and study, see Supplemental Material.

In order to determine if Hg affected bill coloration of lifetime, adult-exposed, and developmental-exposed birds (males and females separately), PC scores for bills were analyzed using linear models where treatment was included as a fixed effect. We did not include family as a random variable in these analyses as few families were represented repeatedly within our final data sets. Additionally, removing the repeated representatives of each family did not alter the results qualitatively.

Results

Hg exposure

Experimental exposure to dietary Hg resulted in consistent biomagnification in blood, and each doubling of Hg exposure approximately doubled the circulating blood Hg concentration (Table 3). Birds exposed to Hg before their first 100 days of life and not thereafter had only trace quantities of Hg in their blood by the time we measured their bill color during adult life, and their blood Hg concentrations during the period of developmental exposure has been reported in more detail previously (Paris et al. 2018).

Bill coloration

Developmental exposure

Exposure to Hg (1.2 µg/g) only during the developmental period, ceasing before sexual maturity, did not systematically alter the coloration of male zebra finch bills in adult life ($F_{3,\ 136} = 0.071$, $P = 0.976$; Fig. 1). As the main analysis did not indicate robust differences across all four treatment groups in this analysis we did not explore post-hoc tests, although a visual inspection of means and associated confidence intervals (Nakagawa and Cuthill 2007) suggests that the early-exposed birds may have had a different bill color than the other treatments (Fig. 1). This weak difference in bill coloration of males exposed early in development resulted in bills that were lower in saturation and hue and higher in brightness.

Adult versus lifetime exposure

Male bill coloration was not impacted by diet treatment in adult-exposed birds (PC1, $F_{2,\ 24} = 0.083$, $P = 0.921$), but was marginally affected in adult males with lifetime exposure to dietary Hg (Fig. 2). When we used 0, 0.6, and

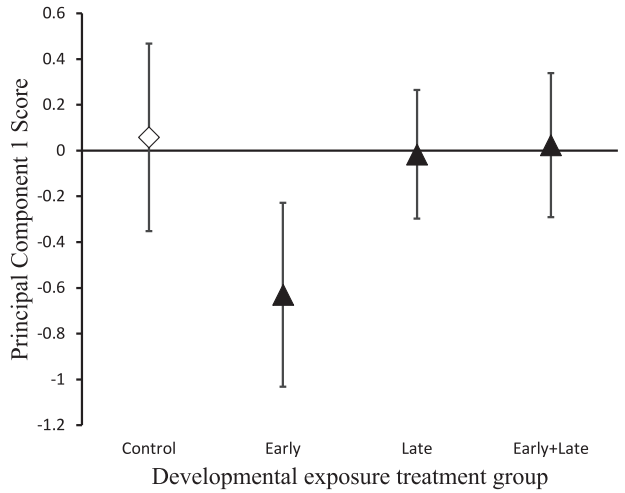

Fig. 1 Red bill coloration of adult male zebra finches with previous developmental exposure to Hg. Control = birds that were never exposed to dietary Hg; Early = birds that were exposed to Hg in the egg and for 50 days after hatching; Late = birds that were exposed to Hg between days 50 and 114 after hatching; and Early + Late = birds that were exposed during both the early and late periods. Displayed are the estimated marginal means with 95% confidence intervals

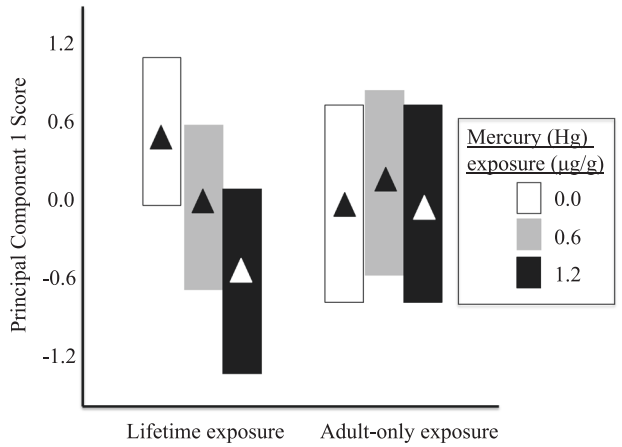

Fig. 2 Lifetime mercury (Hg) exposure significantly impacted bill coloration in male zebra finches, with controls having more saturated, redder bills with lower brightness. Male bill coloration was not affected by Hg treatment in birds exposed to mercury only as adults. Rectangles indicate 95% confidence limits of principal component scores and triangles indicate means. Note that confidence limits of the 1.2 µg/g lifetime Hg treatment do not overlap the mean for the control treatment

1.2 µg/g Hg treatments there was marginal statistical support for a difference in male bill color across treatment groups (PC1, $F_{2,\ 26} = 2.78$, $P = 0.083$; Fig. 2). The difference in bill color PC1 indicates that lifetime exposure to Hg resulted in bills that had lower hue values, were less red saturated, and had higher overall brightness scores than bills of control birds (Fig. 2). Post-hoc Tukey tests among the lifetime-exposed birds indicated that males fed 0.6 µg/g Hg

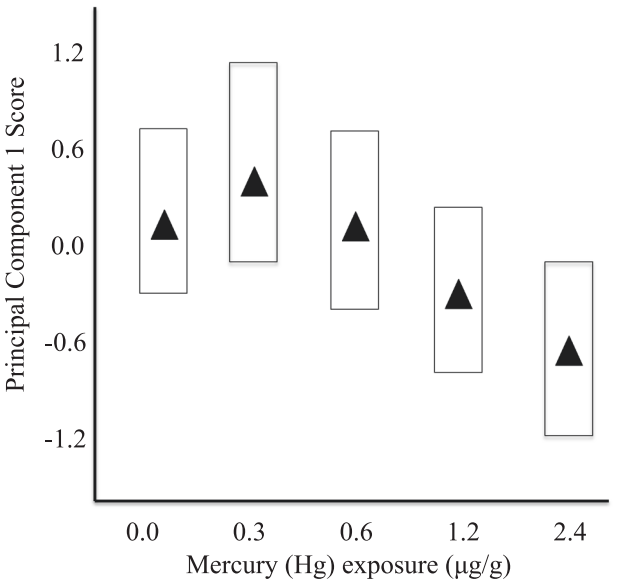

Fig. 3 In a replication of the lifetime mercury (Hg) exposure experiment with more levels of Hg exposure, male zebra finches with higher Hg exposure had brighter but less red-saturated bills. Rectangles indicate 95% confidence limits of principal component scores and triangles indicate means. Note that confidence limits for 2.4 µg/g Hg treatment do not overlap means of the lowest three exposure levels

did not differ from controls ($P = 0.413$), but that males fed with 1.2 µg/g Hg were more different to controls ($P = 0.068$). Examination of Fig. 2 indicates that the 95% confidence interval around the control group overlapped slightly with the 95% confidence interval around the mean of the 1.2 µg/g exposed birds. We observed similar patterns when we repeated the experiment with the set of adult-exposed males that were exposed to a more fine-tuned set of Hg treatment levels including a higher concentrations of dietary Hg (i.e. 0, 0.3, 0.6, 1.2, 2.4 µg/g Hg) (PC1, $F_{4,62} = 2.25$, $P = 0.075$; Fig. 3). Post-hoc Tukey tests revealed that the strongest difference lay between the 0.3 and 2.4 treatment groups ($P = 0.062$) while all other comparisons had no statistically supported differences ($P > 0.3$). As before, males exposed to higher concentrations of dietary Hg had bills that were lower in hue and saturation but marginally higher in brightness scores. Examination of Fig. 3 indicates that the 95% confidence intervals around the 2.4 µg/g group overlapped somewhat with the 95% confidence intervals of the three lowest treatments, including controls. Taken together, these two experiments support a conclusion that lifetime exposure to dietary Hg alters bill color of adult male zebra finches in subtle ways, resulting in bills that are slightly less intensely red and brighter; though we note that there is only marginal statistical support for these effects.

Female bill coloration was apparently unaffected by exposure to Hg, whether they were adult-exposed (PC1, $F_{2,\ 23} = 0.627$, $P = 0.543$; PC2, $F_{2,\ 23} = 0.634$, $P = 0.540$) or lifetime-exposed (PC1, $F_{2,\ 24} = 0.838$, $P = 0.445$).

Feather coloration

Feather brightness did not differ between Hg treatments for adult-exposed adults (males: $F_{2,\ 22} = 1.16$, $P = 0.332$; females: $F_{2,\ 22} = 0.355$, $P = 0.705$) or lifetime-exposed adults (males: $F_{2,\ 23} = 0.111$, $P = 0.895$; females: $F_{2,\ 23} = 0.830$, $P = 0.450$), despite the fact that all feathers were grown while the Hg-exposed birds were consuming dietary Hg.

Discussion

The objective of this study was to determine whether dietary exposure to Hg altered the sexually selected bill coloration of zebra finches, whether the effect was also present in a non-sexually selected feather color trait, and if the timing of exposure was important. Bill color, a dimorphic trait that can be affected by conditions during development in zebra finches (Bolund et al. 2010), was marginally affected by lifetime exposure to Hg in that males that experienced higher dietary concentrations of Hg developed bills that were less intensely red and were somewhat brighter overall. In contrast, exposure to Hg affected neither the same bill color trait in females, nor the non-sexually selected gray/brown feather colors in either sex. Our results thus indicate that this particular sexually selected trait can be an indicator of contaminant exposure in male zebra finches, although the strength of this effect was not strong at the sample size of this study.

Intriguingly, exposure to dietary Hg only during adult life did not affect male bill color, suggesting that exposure to the stressor of Hg earlier in life, during some stage of development, is necessary for an effect of Hg on bill coloration later in life. However, our experiment in which we exposed males to Hg only during development, and not during adult life, rendered puzzling results that require further investigation. Specifically, when Hg exposure occurred in ovo and during the first 50 days after hatching (egg, nestling and fledgling stages, our early treatment group) then bill color tended to be altered many months later, consistent with the findings of the lifetime exposure study, although not a statistically significant result. The bill color in early-exposed birds changed in the same manner as with lifetime exposure—the bills were less intensely red and brighter. However, exposure to dietary Hg at a later stage in development (juvenile stage, 50–114 days after hatching, our late treatment), or in both time windows (egg, nestling, fledgling and juvenile stages), did not alter bill color later in life, relative to controls. We did not predict this pattern. It may be that there is an early-life developmental process that is sensitive to Hg and switches birds on to a different developmental trajectory than if exposure is continued for longer. Selective mortality due to mercury during the follicular or egg stage, which may have gone undetected, could have also played a role in changing the phenotypic characteristics of birds in different treatments. In previous studies we have shown that there is greater mortality of embryos or nestlings in groups exposed to higher concentrations of mercury (Varian-Ramos et al. 2014; Paris et al. 2018). These explanations are speculative but the suggestive result that early exposure to Hg alone may alter later expression of a sexually selected trait underscores the importance of investigating contaminant effects on very early life stages and fitness-related traits that are expressed much later in life.

Bill redness is often positively selected for by female zebra finches through mate preferences (although female preference for redder bills is not universal across studies, Simons and Verhulst 2011). Bill color is influenced by the allocation of carotenoids from the diet into pigment production (Burley and Coopersmith 1987; Blount et al. 2003a; McGraw et al. 2003; McGraw and Toomey 2010). Hence, in this system, we have found experimental support for the overall hypothesis that sexually selected traits are relatively more sensitive indicators of developmental and current environmental conditions than non-sexually selected traits. This may occur because sexually selected traits are costly to produce and maintain relative to other traits (Johnstone 1995; Kuijper et al. 2012) but some have reported that sexually selected traits in zebra finches are no more susceptible to developmental perturbations than naturally selected traits (Bolund et al. 2010). To our knowledge, this is the first experimental evidence that Hg plays a causal role in reducing the quality of a sexually selected ornament; albeit a weak effect in our study. A previous study in which red-legged partridges (*Alectoris rufa*) were fed with lead shot, found that lead exposure actually increased sexually selected red coloration in males during the breeding season, but these measurements were not performed using spectroscopy and interpretation of "redness" is therefore difficult (Vallverdú-Coll et al. 2015). Our results do not appear to be tightly dose-dependent, but they are consistent with a negative response of red saturation across a range of dietary Hg-exposure treatments that spans the range of environmentally relevant Hg exposures. Interestingly, in a separate study we found that lifetime exposure to similar levels of dietary mercury as used in this study does not appear to reduce the attractiveness of male zebra finches to female conspecifics (Greene et al. 2018). Hence, even though bill color is changed slightly, it may not be sufficient to alter the outcomes of sexual selection processes in this species.

Although we did not specifically explore the mechanisms through which bill redness was altered by lifetime exposure to Hg, we hypothesize that this arose through alteration of carotenoid allocation to bill pigments. Carotenoids are proposed to be antioxidant molecules with immune boosting properties

and are common components of sexually selected ornaments in birds (Hill and McGraw 2006). Male and female zebra finches both accumulate carotenoids in their bills, but males accumulate them to a greater extent. Thus, the reduction in color saturation is consistent with the hypothesis that male finches stressed by Hg were less able to allocate carotenoids for use in bill ornamentation, possibly due to Hg-induced immune and oxidative stress (Lewis et al. 2013; Henry et al. 2015), which is known to decrease carotenoid color quality (Svensson and Wong 2011, Marasco and Costantini 2016). Our finding of likely decreased redness of male bill color is consistent with a recent correlative study showing a negative relationship between Hg and carotenoid-based feather color in wild great tits (Giraudeau et al. 2015), suggesting that carotenoid-disruption could be a widespread consequence of Hg exposure.

In other studies, zebra finches challenged as adults through immune or nutritional stresses have exhibited decreases in bill coloration (McGraw et al. 2003; McGraw and Ardia 2003; Blount et al. 2003b; McGraw and Ardia 2004; Naguib and Nemitz 2007). This pattern was not observed in our study, as bill color was not affected by 0.6 or 1.2 µg/g dietary Hg in adult-exposed birds. The adult-exposed birds ingested Hg for approximately seven months, and had comparable circulating blood Hg concentrations to lifetime-exposed birds. These observations suggest that some form of developmental switch is altered during early-life exposure, which then makes the bird more sensitive to a later perturbation. This interpretation is different from the developmental stress hypothesis, or a mechanism involving current stress conditions. Both hypotheses need to be combined to produce a developmental switch hypothesis, which is what we propose here. Our observation (Fig. 1) that bill color of birds exposed only at the earliest stages of development also tended to be affected by Hg suggests the possibility that certain developmental exposure windows and durations may lead to lingering effects on a sexually selected trait in the adult. We feel there is much more to be learned about how the exact timing and duration of exposure to stressors, such as contaminants, influences phenotypes and fitness-related traits.

Hg is known to hinder tyrosinase, a critical enzyme in the melanin production pathway, by competitively inhibiting the binding of the copper cofactor (Lerner 1952), potentially leading to a decrease in melanin production. Despite this, back feather melanization in zebra finches, as assessed by reflectance spectrometry, did not differ across Hg treatments regardless of the exposure mode (lifetime or adult). This result is in contrast to studies on two species studied in the wild that exhibited greater reflectance in structural blue color underlain with melanin in feathers from birds at mercury-contaminated sites (White and Cristol 2014; McCullagh et al. 2015). We hypothesized that the effects of mercury would be greater in sexually selected red bill coloration than in non-sexually selected gray/brown back feathers. Our results are consistent with this hypothesis, and we conclude that this is because there are no honesty-reinforcing mechanisms regulating the production of color in the feathers, in either males or females. A logical follow-up study would be to examine the effect of mercury on a sexually selected feather color in a bird.

Our results suggest that populations of birds born at a contaminated site would suffer greater effects on coloration than would dispersers arriving as adults, and that effects on coloration may be expressed most readily in sexually selected ornaments. Caution must be exercised in extrapolating these results to wild populations, as not only will different species vary in their response to Hg, but wild birds face many additional stressors to which the captive birds in this study were not exposed. We also must remark that the effects of lifetime exposure to mercury on bill color were weak, but consistent across our different experiments. Future work should determine if the coloration effects on lifetime-exposed birds can be remediated through removal of Hg from the diet, thereby simulating either a cleanup effort at a contaminated site or dispersal of birds away from the site of contamination.

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Compliance with ethical standards

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

Ethical approval Experiments were in accordance with the Guide of the Care and Use of Laboratory Animals of the National Institutes of Health, and were approved and overseen by The College's Institutional Animal Care and Use Committee (IACUC-IBC-2010-05-03-6516-dacris). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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