



RESEARCH ARTICLE

## Attractiveness of male Zebra Finches is not affected by exposure to an environmental stressor, dietary mercury

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### ABSTRACT

Choosing a high-quality mate contributes to increased reproductive success in birds. Females assess quality in males, in part, via condition-dependent signals such as songs and plumage. The production of these signals of quality can be disrupted by environmental stressors, including toxic pollutants such as mercury. Mercury affects song, plumage, bill color, and courtship behaviors in male birds, but the effect of these changes on female mate choice is unknown. By affecting the condition-dependent signals that females use to assess quality, mercury could alter males' attractiveness to females. We used mate choice of female Zebra Finches (*Taeniopygia guttata*) to determine if male attractiveness to females is affected by lifetime exposure to mercury. Males were either exposed to dietary mercury or left unexposed and then assessed by unexposed females in 3 types of mate preference tests: song-playback phonotaxis (preference for audio recordings of mercury-exposed or unexposed males' songs); 2-choice social association preference (simultaneous choice between mercury-exposed or unexposed males in adjacent cages); and pairing (opportunity to pair with either a mercury-exposed male or unexposed male in an aviary). In song-playback phonotaxis and social association tests, females did not spend more time near songs or males of one treatment over the other, despite measurable differences between songs. In an aviary pairing test, females were equally likely to pair with males of either treatment. While mercury exposure is known to reduce production of offspring in Zebra Finches and other birds, our results suggest that captive female Zebra Finches may not be incorporating mercury-induced variation in male traits into their mate choice decisions. If female birds living in contaminated environments experience fitness losses as a result of potentially poor mate choice decisions, then females may eventually respond to this sexual selection pressure by including toxicant-mediated trait variation in their quality assessment mechanisms.

**Keywords:** condition-dependent signals, environmental contaminants, mate choice, mercury, reproductive behavior, song, Zebra Finch

**El atractivo de los machos de *Taeniopygia guttata* no es afectado por la exposición a un agente de estrés ambiental, el mercurio en la dieta**

### RESUMEN

La elección de una pareja de alta calidad contribuye a aumentar el éxito reproductivo en las aves. Las hembras evalúan la calidad de los machos, en parte, por medio de señales que dependen de la condición, como los cantos y el plumaje. La producción de estas señales de calidad pueden ser alteradas por agentes de estrés ambiental, incluyendo contaminantes tóxicos como el mercurio. El mercurio afecta el canto, el plumaje, el color del pico y los comportamientos de cortejo en las aves macho, pero no se conoce el efecto de estos cambios en la elección de pareja de la hembra. Al afectar las señales dependientes de la condición que usan las hembras para evaluar la calidad, el mercurio podría alterar la manera en que las hembras se ven atraídas por los machos. Usamos la elección de pareja de las hembras de *Taeniopygia guttata* para determinar si el atractivo que las hembras perciben de los machos es afectado por la exposición de por vida al mercurio. Los machos estuvieron expuestos ya sea a una dieta con mercurio o no fueron expuestos y luego fueron evaluados por hembras no expuestas en tres tipos de pruebas de preferencia de pareja: fonotaxis mediada por reproducción de cantos (preferencia por registros de audio de cantos de machos expuestos al mercurio y no expuestos); preferencia de asociación social de doble opción (selección simultánea entre machos expuestos a mercurio y no expuestos ubicados en jaulas adyacentes); y formación de parejas (oportunidad de formar pareja ya sea con un macho expuesto a mercurio o un macho no expuesto ubicados en un aviario). En las pruebas de fonotaxis mediada por reproducción de cantos y de asociación social, las hembras no gastaron más tiempo cerca de cantos o machos de un tratamiento sobre el otro, a pesar de las diferencias medibles entre cantos. En la prueba de formación de pareja en el aviario, las hembras tuvieron la misma probabilidad de formar pareja con machos de cualquiera de los tratamientos. Aunque se conoce que la exposición al mercurio reduce la producción de descendencia en *T. guttata* y otras aves, nuestros resultados sugieren que las hembras cautivas de *T. guttata* pueden

no estar incorporando en sus decisiones de elección de pareja la variación inducida por el mercurio en los rasgos de los machos. Si las hembras que viven en ambientes contaminados sufren pérdidas en la adecuación biológica como resultado de malas decisiones potenciales de elección de pareja, entonces las hembras pueden eventualmente responder a esta presión sexual de selección mediante la inclusión en sus mecanismos de evaluación de calidad la variación en los rasgos ocasionada por los tóxicos.

**Palabras clave:** canto, comportamiento reproductivo, contaminantes ambientales, elección de pareja, mercurio, señales dependientes de la condición, *Taeniopygia guttata*

## INTRODUCTION

Selection of a high-quality mate is predicted to increase individual fitness (Dawkins and Guilford 1996, Møller and Jennions 2001, Kokko et al. 2003) through both indirect and direct benefits. Making a suboptimal choice of mate, therefore, can impose a large cost to fitness (Kokko et al. 2002) resulting in strong selection on mechanisms that lead to accurate detection, perception, and selection of high-quality mates (Jennions and Petrie 1997, Candolin 2003). Similarly, there will be corresponding selection on mechanisms to signal honestly the quality of potential mates (Grafen 1990, Johnstone 1995).

In birds, honest signals of direct benefits often include condition-dependent traits—the phenotypic expression of which are dependent on current condition—such as coloration and song. Coloration of plumage and integument is often dependent on nutritional status (especially carotenoid-intensive plumage) and can therefore reflect nutrient availability in a male's home range—a direct benefit to potential mates (Hill 1991, Hill and Montgomerie 1994). Likewise, as song requires the coordination of complex neural, physiological, and mechanical components, its quality is inherently dependent on condition, both developmental (Nowicki et al. 1998; Woodgate et al. 2010, 2014) and current (Schmidt et al. 2014). The quality of male coloration and song influences female mate choices, as females are known to pay attention to both plumage (Hill 1991) and song variation (Nowicki and Searcy 2004).

The expression of condition-dependent traits is expected to be mediated by variation in environmental conditions, including unnatural environmental conditions, such as exposure to pollutants. As the amount and variety of pollutants in the environment have increased significantly in the 20th century (Wania and MacKay 1996), the effects of metals and persistent organic pollutants on birds have been a focal point for research (Scheuhammer 1987, Walker 1990, Furness 1993), including their effects on condition-dependent signals (Gorissen et al. 2005, Markman et al. 2008, Galván et al. 2010). One such pollutant is mercury, a potent neurotoxicant which has increased dramatically due to mining and industry (Swain et al. 1992, Fitzgerald 1995, Pirrone et al. 1996). Mercury has been associated with changes in condition-dependent repro-

ductive signals in birds—including altered bill coloration in Zebra Finches (*Taeniopygia guttata*; Spickler 2014), altered plumage brightness in Belted Kingfishers (*Megasceryle alcyon*; White and Cristol 2014) and Eastern Bluebirds (*Sialia sialis*; McCullagh et al. 2015), possible abnormal courtship display behaviors in White Ibises (*Eudocimus albus*; Frederick and Jayasena 2011), and reduced song length, pitch, and complexity in 3 species of songbirds (Hallinger et al. 2010). As females of some songbird species are known to prefer bright plumage (Hill 1991), high courtship display rate (Collins and ten Cate 1996), and longer, more complex songs (Clayton and Pröve 1989), these mercury-induced changes in the expression of signaling traits could affect a female observer's perception of a male's quality, resulting in females avoiding mercury-exposed mates.

We sought to examine the effects of lifetime dietary mercury exposure on mate choice processes of captive Zebra Finches, using female preference metrics as bioassays of male attractiveness. First, we investigated the effects of mercury exposure on the perceived attractiveness of male song in a phonotaxis test (as in Holveck and Riebel 2007), where songs of mercury-exposed and unexposed males were broadcast in sequence to unexposed females. We also examined the effects of mercury on overall male attractiveness through 2 assays: female preferences for unexposed versus mercury-exposed males in a 2-choice social association test with males in adjacent small cages (Collins et al. 1994, Witte 2006, Swaddle and Page 2007), and preference between these same dyads of males when females chose mates in a larger outdoor aviary (Clayton 1990, Swaddle 1996, Rutstein et al. 2007). If females can detect and respond to the variation in male phenotype caused by mercury, we predicted that females would prefer to listen to, associate with, and mate with the unexposed males in all 3 tests.

## METHODS

### Husbandry and Exposure of Zebra Finches

We used an established, outbred colony of domestically bred Zebra Finches at the College of William and Mary, Williamsburg, Virginia, USA. Wild-type males were fed either an exposed diet (1.2 mg methylmercury-cysteine per kilogram of food, also known as parts per million [ppm])

or an uncontaminated diet (0.0 ppm methylmercury, with cysteine and water added as in the exposed diet) of commercial finch food (Zupreem, Shawnee, Kansas, USA) for their entire lifetime, including exposure in the egg (their parents were fed the same diets prior to breeding). The range of mercury concentrations in avian prey items varies widely depending on location and type of prey, with vegetable matter and herbivorous invertebrates in remote terrestrial habitats often containing levels below detection, while predatory invertebrates downwind of industrial activity sometimes contain levels higher than that used in this study. Our mercury-diet concentration of 1.2 ppm wet weight approximates the average level of contamination in invertebrate prey items found at some industrially contaminated areas and results in reduction in reproductive success (~40%; global prey item concentrations reviewed in Varian-Ramos et al. 2014). All females were fed the uncontaminated diet throughout life and came from unexposed families, as we wanted to determine the effects of mercury on male attractiveness rather than the toxicant's potential influence on female preferences directly. Prior to and during trials, all birds were kept in cages (0.46 m width  $\times$  0.75 m length  $\times$  0.46 m height) with 3 other finches of the same sex in mixed-sex rooms (unless indicated otherwise) on a 14:10 light:dark cycle at ~22°C, and provided food, grit, cuttlefish calcium supplement, vitamins, and water ad libitum. We conducted all experimental trials between May 2015 and February 2016. The subset of birds kept in outdoor aviaries for mate choice trials in July–August of 2015 were subject to natural lighting and weather conditions for 7 days and nights and were provided with uncontaminated food to avoid contaminating both treatments.

To select the appropriate mercury exposure, we used dose-response curves generated for mercury-exposed Zebra Finches from the same breeding colony used for this study. We concluded that the lowest exposure providing a valid test of our hypothesis that mercury affects mate choice behavior, and thus justifies the use of animals for this study, was 1.2 ppm (as discussed further in Kobiela et al. 2015). Using lower exposure risked the use of animals for a study that would not be likely to answer the questions posed. Anecdotally, we have observed no overt signs of increased frightfulness, discomfort, or illness at exposures of 1.2 ppm.

### Blood Mercury Analysis

We analyzed total mercury from blood samples taken at the time of each set of behavioral trials. Approximately 95% of mercury in avian blood is composed of methylmercury (Rimmer et al. 2005, Wada et al. 2009) and, therefore, total mercury values are an accurate representation of blood methylmercury concentration. Samples were analyzed using combustion–amalgamation cold

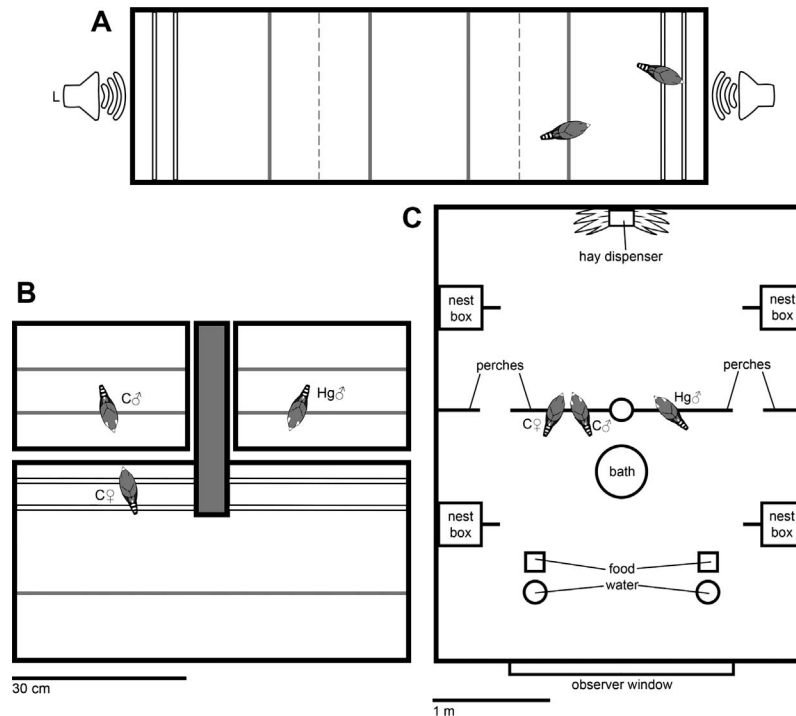
vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, Connecticut, USA) following U.S. Environmental Protection Agency method 7473. For quality control, before and after every 20 samples we ran duplicates, blanks, and 2 standard reference materials (tuna and DORM-4 fish protein, National Research Council of Canada, Ottawa, Ontario). Average relative percent difference between replicate sample analyses was  $2.49 \pm 1.54\%$  ( $n = 11$ ). Mean percent recoveries of mercury for the tuna and DORM-4 were  $98.66 \pm 0.21\%$  ( $n = 20$ ) and  $95.84 \pm 0.47\%$  ( $n = 20$ ), respectively. All mercury concentrations are reported as wet weight.

### Song Preference

We used a phonotaxis chamber similar to those used in previous song preference studies (Holveck and Riebel 2007, Woolley and Doupe 2008) to determine whether females would show a preference for songs from either mercury-exposed or unexposed males. Zebra Finches are highly gregarious, and pilot trials indicated that females were more responsive to songs when a familiar conspecific was present in the phonotaxis chamber. Thus, we placed 2 females at a time in the test apparatus and randomly chose one female's response to be used in analysis. The 2 females were former cage mates and were identified by pink or black plastic leg bands (Red Bird Products, Mount Aukumn, California, USA). We tested the song preferences of 20 females during 20 trials. No females were used more than once. All trials were conducted January–February 2016.

A single song-playback phonotaxis trial consisted of the presentation to females for 15 min of a paired playback stimulus (exposed vs. unexposed male songs), followed by a repeat presentation with the stimulus position reversed. Playbacks were presented from left and right speakers (Boston BA635, Boston Acoustics, Woburn, Massachusetts, USA) placed at either end of a phonotaxis chamber (Figure 1A, one side mercury-exposed, one side unexposed) in a room that had sound-dampening material affixed to the walls and floor to reduce reflection and reverberation. The speakers were adjusted to ensure that tracks were presented at the same sound pressure level, measured by a digital sound meter (Extech 497730, A-weighting; Extech Instruments, Nashua, New Hampshire, USA). Sound levels at perch height were  $60.3 \pm 0.74$  dBA SPL next to the playing speaker and  $45.2 \pm 0.99$  dBA SPL at the opposite end of the chamber, a realistic simulation of the volume of a proximate singing male.

We placed the 2 banded females in the phonotaxis chamber with food and water to acclimate overnight. Clear plastic dividers kept them in the central area of the chamber, allowing them to see the 2 ends of the chamber while preventing exploration. We removed food and water



**FIGURE 1.** (A) Plan view of phonotaxis chamber (measuring  $0.46 \times 2.29 \times 0.46$  m) with speakers (L and R) playing alternating songs at either end. Vertical bars represent perches, with paired bars closest to speakers representing perches on which females were classified as showing interest. Dashed lines represent position of dividers when in place before trials. (B) Plan view of social association preference apparatus. Open paired bars are nearest perches where female could show interest in a male. Solid gray single bars are neutral perches. C = unexposed, Hg = mercury-exposed, positions reversed after first 30 min. (C) Plan view of aviary for mate choice pairing trials. C = unexposed, Hg = mercury-exposed.

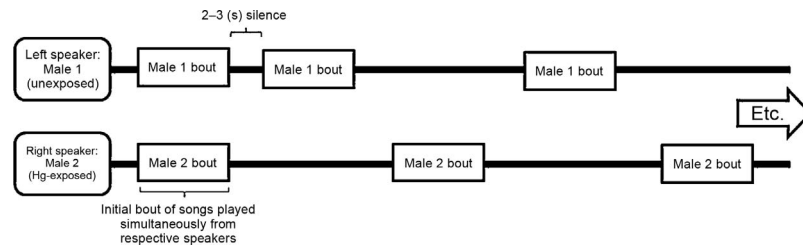
on the morning of the trial and allowed 5 min for acclimation after we departed the room. We then played the songs for 5 min (first simultaneous, then alternating) with the plastic dividers still in place, to expose the females to the songs. After removing the plastic dividers the birds were allowed 5 min to re-acclimate and explore the entire chamber in silence. We then played the song stimulus in its entirety (15 min). During the playback, the behavior of the female was observed from a live video feed outside of the room (Sony HDR-CX240; Sony, Tokyo, Japan). We noted the direction that the female flew upon initiation of audio recordings and we recorded the amount of time that the female spent on the pair of perches nearest the speakers. After the first half of the trial we replaced the food, water, and dividers for 30 min. We then completed the trial by repeating the procedure with the same playbacks and females, after switching the sides of the audio channels to control for side preferences.

To meet the criterion for a successful trial, both the focal and accompanying female had to spend at least 10% of the total 30 min on the perches nearest to the speakers (180 s minimum per female per trial; Figure 1A). To determine the minimum participation criterion for a successful trial we conducted pilot observations on a small group of

females. Three types of responses were observed using the experimental procedure outlined above: (1) unresponsiveness, (2) clear response to playing of song for much of the trial, and (3) clear responsiveness to songs for the first 10–20% of trial duration followed by lack of responsiveness. To include the latter 2 categories of females while reducing the chance that random movements by unresponsive females would affect our results, we selected the 10% activity level as our minimum. In only three trials did one of the females fail to meet this criterion. Those trials were excluded from analysis, although their inclusion did not alter conclusions.

To create the playback stimuli, we used audio recordings of female-directed songs that had been recorded from males of both mercury-exposed and unexposed treatments in a previous generation of the same colony housed under the conditions described above, in 2012–2013. None of the birds recorded were used as experimental subjects, nor were they directly related to those in the current study. Female-directed songs were recorded individually using Avisoft Recorder (Avisoft Bioacoustics, Glienicke, Germany) from 12 unexposed males and 12 mercury-exposed males in a small cage with a novel female. We randomly paired males of each treatment to create 20 unique





**FIGURE 2.** Graphical representation of playback stimuli. Whichever side the treatment came from first was switched after the first 15 min of the trial.

contrasts between a mercury-exposed and an unexposed male. No male was used more than twice, nor paired twice with the same contrasting male.

Zebra Finch males sing only one song type, composed of short sounds called syllables, given in a rapid sequence to form a motif, which is repeated several times to produce a bout of song. Males typically differ in both the structure of their individual syllables and the number of syllables in each motif. Therefore we chose 2 motifs for each male to construct playbacks—using motifs that appeared in the spectrogram to be typical but were free from background noise. Using 2 motifs per male served to minimize habituation by females to a single stimulus. All features of the selected motifs were preserved, but noise was filtered with 500 highpass filter in Audacity (2.1.1 for Windows Operating System; <http://www.audacityteam.org>).

Each stereo playback stimulus consisted of 2 audio channels (left and right), with song from an unexposed male on one channel and song from a mercury-exposed male on the other channel (Figure 2). We created bouts of 3–5 repeats of the same motif separated by silences. Silence between motifs varied but averaged 0.19 s to simulate the typical singing pattern of male Zebra Finches (Zann 1996). Bouts were separated by 2–4 s of silence.

We constructed playbacks such that audio played from the left and right simultaneously for a single bout, after which audio from each channel alternated, with songs emitting from one channel and silence emitting from the other. We used a balanced design, alternating whether the mercury-exposed songs played from the left or right and which side played first. Though bouts varied in the number of motif repetitions, the total numbers of motifs and bouts were equal across treatments within a trial.

### Comparing Songs of Mercury-exposed and Unexposed Males

To compare the songs produced by males of the 2 treatments, for each motif used from a male, we measured motif length, peak frequency, and bandwidth using Raven Pro (1.4 build 48, Windows 64-bit version; Cornell Lab of Ornithology, Ithaca, New York, USA), and we counted the

number of syllables and unique syllables, as in Spencer et al. (2003). Each measurement was averaged across the 2 motifs used per male.

### Preference among Potential Mates

To test for female preference among potential mates from each dietary treatment, we randomly selected 15 males of each treatment that were sexually mature and approximately the same age (within 60 days). We then paired the males randomly, generating 15 dyads. At the time when the trials were conducted, unexposed males had an average blood mercury level of  $0.007 \pm 0.001$  ppm, and exposed males had an average blood mercury level of  $15.9 \pm 0.7$  ppm, which is above levels generally reported, but not beyond the range of occurrence, for wild songbirds at heavily polluted sites (Cristol et al. 2008). Female sexual experience varied, but all had previously occupied a cage with a male, and all had been separated from any previous mate for at least 30 days. For the duration of this study, the females were kept in a room that housed no males. We conducted all association preference trials in May 2015, when females were 1–3 yr old.

To determine whether females would spend more time in proximity to one or the other potential mate, we used a 2-choice social association apparatus similar to that in many other mate choice studies (Swaddle and Cuthill 1994, Hunt et al. 1997, Holveck et al. 2011). We placed each male in one of two small cages adjacent to a large central cage (Figure 1B). Males were visually separated from each other and from the central cage by opaque dividers, and were allowed to acclimate to their environment for 10 min. We placed the female in the central cage and allowed her to acclimate for 5 min, after which we removed the dividers between the female and the males, and began the first 30 min observation period. The female was free to move throughout the central cage and associate with either male, but she could not see both males at the same time from any position. Males remained visually, but not acoustically, isolated from one another throughout the trial. Immediately following this initial 30 min period, we replaced the visual dividers and switched the positions of the male cages, to control for female side preferences.

After another 5 min acclimation period we removed the dividers and recorded behaviors for another 30 min of the trial.

We video-recorded the trials with a tripod-mounted SONY Handycam (HDR-CX240). We observed female position and behavior continuously for each 30 min portion of the trial. A female was considered to be actively showing interest in a male if she was standing or hopping back and forth on the perches nearest a male, and was facing the male (Figure 1B). Time spent in other positions (facing away from the male, preening or sleeping on nearest perches, any activity on the neutral perch, on cage bottom, or foraging) was not classified as indicating interest in a particular male. We determined female preference by totaling amount of time spent exhibiting active interest in each male. All females met our criterion for inclusion in the analysis, which was that she had to spend at least 10% of the trial time ( $>360$  s) on the near perches facing either male. We presented each dyad of males ( $n = 15$  total) to 3 different females, to ensure that any preferences exhibited were not idiosyncratic. Females were alone when judging males, so preferences were independent of the preferences of other females. We used each female to assess no more than 2 sets of males, never with the same “panel” of females used to judge other dyads, and no female had a degree of relatedness  $>0.015$  to any males she assessed. For 10 of 15 dyads, females preferred the same male, whereas in only 4 cases did females exhibit clear preferences for opposite males. Thus, we summed the 3 females’ responses into a single data point for each set of males ( $n = 15$  combined female).

### Mate Choice

Because preference for songs, or social association, are each components of pairing, but not the entire process, we also used the same males from the 2-choice social association tests for week-long mate choice tests in an outdoor aviary. All males wore either white or yellow plastic leg bands so they could be identified during behavioral observations. Leg band color was equalized across treatments in case it biased female preferences. At the time the trials were conducted, unexposed males had an average blood mercury level of  $0.021 \pm 0.004$  ppm, whereas exposed males had an average blood mercury level of  $13.7 \pm 0.8$  ppm.

We placed a dyad of males (one unexposed, one lifetime mercury-exposed) in each outdoor aviary ( $3.0 \times 7.2 \times 2.1$  m) with a single female (unknown to either male) for 7 days. Each outdoor aviary was visually, but not acoustically, isolated from other outdoor aviaries and contained 4 nest boxes, a nesting material dispenser, a water bath, a large central perch, and a small perch between each nest box (Figure 1C). We conducted the aviary mate choice pairing tests in July–August 2015, using the same dyads of males

as in the previous experiment, but never pairing them with females that had seen them before. In a few cases where males used in the earlier experiment were no longer available we substituted with new males. The photoperiod was  $\sim 14:10$  light:dark (similar to the indoor colony) and the ranges of average daily minimum and maximum temperature and humidity were  $21.5\text{--}26^\circ\text{C}$  and  $50\text{--}95\%$ , respectively. During their week in the outdoor aviary, birds were provided with an uncontaminated diet ad libitum to avoid exposing the unexposed female and male to mercury. Previous experiments have found that blood mercury remains elevated at  $>50\%$  of asymptotic level one week after switching from a mercury-containing diet to a control diet (Whitney and Cristol 2017a). Hence, the mercury-exposed males were still affected by their treatment despite the temporary change in diet to unexposed food.

We released the 2 males into the outdoor aviary to acclimate and potentially defend nest boxes one day before observations began. The female was placed in the center of the aviary in a small cage during this time so that she could interact visually and acoustically with males and witness interactions between them prior to her own interactions with them. Behavioral observations began immediately after the release of the female on the following morning. Each trio of birds was observed daily for 30 min during the period of 0700–1000 hours, approximately 1–3 hours after dawn, for 7 consecutive days. Our goal was to determine which male was preferred by the female. Therefore, we recorded the following: all instances of female-directed and undirected singing by each male, the amount of time the female spent in each male’s nest boxes, and the amount of time the female spent with each male engaged in clumping, which is a pair-bonding behavior in which 2 Zebra Finches huddle together (Zann 1996). We also hypothesized that differences between males in social status might be involved in the female’s choice. Thus, we recorded all displacements of one male by the other. We checked nest boxes for eggs at the end of each observation period. Observers described the actions of the birds from a blind while recording them with a SONY Handycam (HDR-CX240) and later transcribed the behaviors from the video, using it to confirm observations. The observer remained blind to treatment during observations and transcription of videos. The male that was permitted by the female to enter the nest box containing eggs was classified as the paired male. Alternatively, in the trials where no eggs were laid ( $n = 3$ ), a male was considered paired if the female consistently spent time in the nest box with him. In all trials, the chosen male was easily identifiable through constant association with the female at the nest box, hay dispenser, and food dishes.

To determine social dominance between males, we compared the number of displacements each male successfully initiated against the other over the 7-day

period. A minimum of 20 social interactions occurred between all dyads of males, with a dominant and subordinate being recognized if one had displaced the other 15% more of the time than the reverse had occurred. All dyads met this criterion ( $n = 14$ ).

### Data Analysis

**Song preference.** To evaluate the results of the auditory playback test we performed a paired  $t$ -test on the amount of time the randomly chosen female spent near songs of males from each treatment. The data conformed to the appropriate assumptions about normality and variance. We also used a binomial test to compare the count of females preferring one type of song over the other, and the count of females approaching each type of song first. To test our assumption that songs of mercury-exposed and unexposed males differed, we measured a variety of song parameters including motif length, peak frequency, bandwidth, number of syllables, and number of unique syllables, as in Spencer et al. (2003), and used these variables to perform a principal components analysis (PCA). Further, we compared the means of the resulting components via a multivariate general linear model where song PCs were the dependent variables and treatment group was an among-subjects fixed factor. We also visually inspected a bivariate plot of estimated marginal means of PCs  $\pm$  95% confidence intervals.

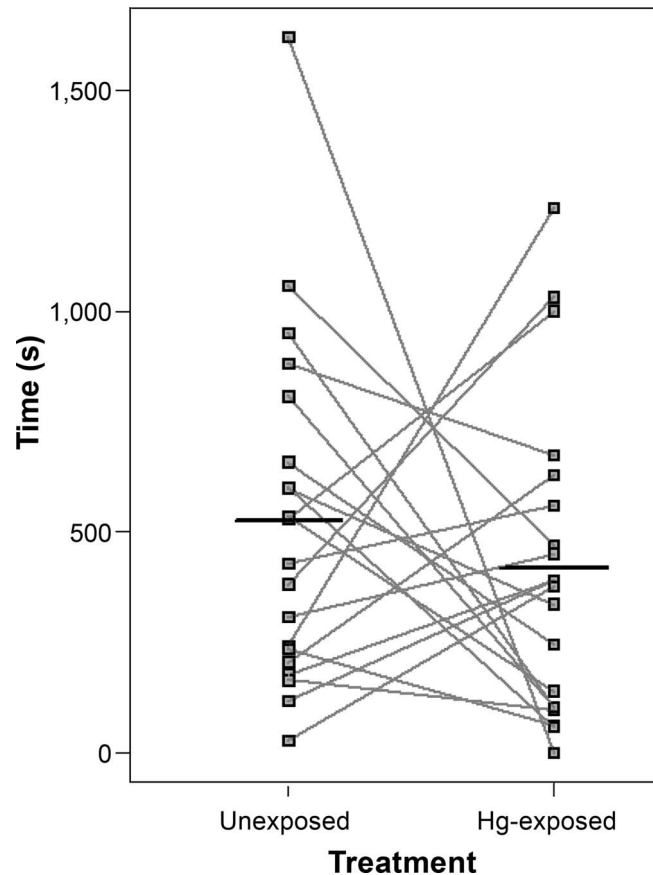
### Preference among potential mates and mate choice.

We evaluated preferences in the 2-choice social association test using a paired  $t$ -test on the total amount of time females spent showing active interest on perches in front of each male. In determining whether females preferred to pair with males of one or the other treatment in aviary trials, we used a binomial test. We also used a binomial test to compare whether exposed or unexposed males were more likely to be socially dominant over the other. Multivariate analyses were performed in SPSS Statistics (version 23; IBM, Armonk, New York, USA). All other statistical analyses were completed using the statistical software R (version 3.1.1; <https://www.r-project.org/>). All datasets conformed to the assumptions of the statistical tests we employed and we interpreted 2-tailed tests of probability throughout.

## RESULTS

### Song Preference

Females did not spend more time closer to songs of unexposed males than to songs of mercury-exposed males (paired  $t$ -test:  $t = 0.813$ ,  $df = 19$ ,  $P = 0.4263$ ; Figure 3). Individual females that showed a preference for one song type ( $>20\%$  more time in front of one speaker than the other) did not favor one treatment more often than the other (11 unexposed songs preferred, 9 mercury-exposed



**FIGURE 3.** Total time (s) each set of female Zebra Finches spent near speakers playing songs of each treatment. The pairs of songs presented in each trial are connected, and the black bars represent the mean number of seconds spent listening to each treatment.

songs preferred; binomial test:  $P = 0.824$ ). Females were no more likely to first approach songs of one treatment than the other (in 10 of these trials females approached unexposed first, while in 10 they approached mercury-exposed first; binomial test:  $P = 0.999$ ). In 15 of 20 trials both the focal and accompanying females flew to same side first. Overall, we failed to reject any null hypotheses associated with the playback phonotaxis test.

Table 1 presents a summary of raw means  $\pm$  95% confidence intervals for each song measurement. To determine whether there were differences in the songs produced by exposed and unexposed males and used for the stimuli in the playback phonotaxis trials, we performed a principal components analysis on 5 metrics for each of the motifs used (average motif length, peak motif frequency, bandwidth, number of syllables, number of unique syllables). We used the correlation matrix without factor rotation, and the analysis returned 2 components with eigenvalues greater than 1 (Table 2). PC1 loaded most strongly with average motif length and number of

**TABLE 1.** Raw means  $\pm$  95% confidence intervals for song characteristics.

	Unexposed	Mercury-exposed
Length (s)	0.71 $\pm$ 0.06	0.68 $\pm$ 0.09
Peak frequency (Hz)	3822.12 $\pm$ 351.28	4371.23 $\pm$ 504.8
Bandwidth (Hz)	4407.12 $\pm$ 696.39	4148.72 $\pm$ 519.01
Number of syllables	5.5 $\pm$ 0.58	5.25 $\pm$ 1.0
Number of unique syllables	4.75 $\pm$ 0.55	5.04 $\pm$ 0.88

syllables/unique syllables and, hence, we interpreted this component as describing longer, more complex motifs. PC2 was influenced most by peak frequency and narrowness of bandwidth. Hence we interpreted higher values for PC2 as describing songs comprised of higher-frequency motifs.

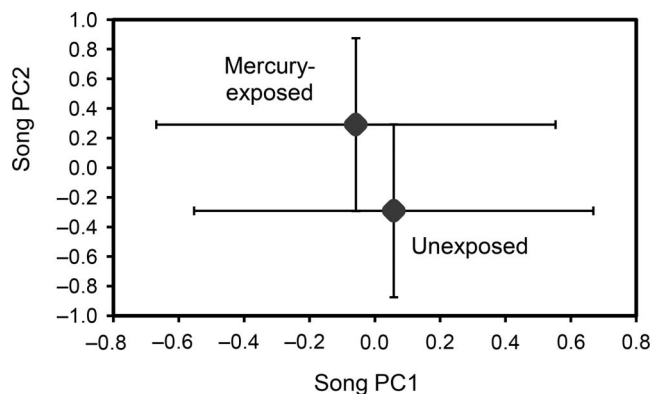
The multivariate general linear model indicated that mercury exposure influenced song PC2 somewhat ( $F = 2.13$ ,  $df = 1$  and  $22$ ,  $P = 0.158$ , partial eta squared [effect size] = 8.9%) but had little influence on song PC1 ( $F = 0.078$ ,  $df = 1$  and  $22$ ,  $P = 0.782$ , partial eta squared [effect size] = 0.4%). Inspection of a bivariate plot of estimated marginal means (Figure 4) of song PC1 versus song PC2 with 95% confidence intervals indicates that mercury-exposed birds produced songs with a higher song PC2 value. Hence, mercury exposure was associated with song motifs that were narrower in bandwidth and higher in frequency. Mercury did not appear to influence motif length or complexity (i.e. PC1) among these Zebra Finches.

### Preference among Potential Mates

Females did not spend more time with males of one treatment over another (paired  $t$ -test:  $t = -1.047$ ,  $df = 14$ ,  $P = 0.313$ ; Figure 5). An alternate way of examining this is to compare the number of males from each treatment that were preferred by females. In the 25 trials in which at least one female displayed a preference, females did not exhibit a significant preference for males of one treatment over the other (9 = unexposed, 16 = mercury-exposed; binomial test:  $P = 0.230$ ).

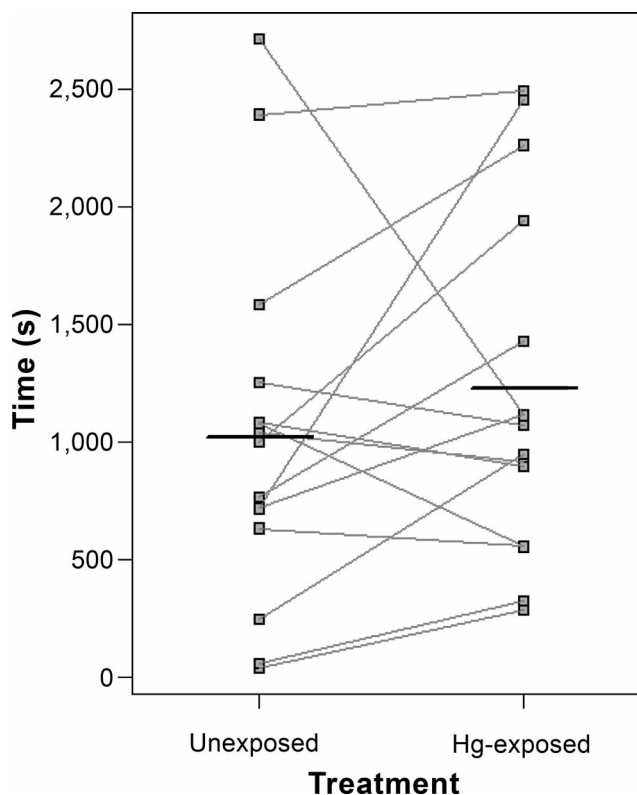
**TABLE 2.** Summary of component scores generated by the song principal component analysis; bolded values were considered to have reasonable explanatory power.

	Song PC1	Song PC2
% variance explained	44.6	21.0
Eigenvalue	2.23	1.05
Average motif length	<b>0.688</b>	0.282
Peak frequency	-0.132	<b>0.764</b>
Bandwidth	0.231	<b>-0.622</b>
Number of syllables	<b>0.946</b>	0.030
Number of unique syllables	<b>0.889</b>	0.025

**FIGURE 4.** Bivariate plot of estimated marginal means of song PCs  $\pm$  95% confidence intervals. A higher PC1 score indicates longer songs with more syllables. A higher PC2 score indicates songs with a higher pitch and narrower bandwidth.

### Mate Choice

All females paired with one of the two males in their aviary, but this pairing was not biased toward either treatment (7 = unexposed, 7 = mercury-exposed, binomial test:  $P = 0.999$ ). Female pairing decisions were significantly related

**FIGURE 5.** Total time (s) female Zebra Finches spent near males of each treatment in 2-choice trials. The dyads of males presented in each trial are connected, and the black bars represent the mean number of seconds spent showing active interest toward each male.



to apparent social dominance based on the interactions across the entire period, as 12 females paired with dominant males and only 2 paired with subordinates (binomial test:  $P = 0.012$ ). Of these 12 dominant, paired males, only 9 had been dominant on the first day of the test. Interestingly, 3 males became dominant after pairing, whereas 3 paired males became subordinate by the last day of the test. We hypothesize that there was a complex dynamic between dominance status and mating status in our test aviaries but, importantly, apparent social dominance was not related to mercury treatment (unexposed dominant = 9, mercury-exposed dominant = 5, binomial test:  $P = 0.430$ ). Examination of displacements on the first and last day for each set of males indicated that in only 6 of 14 trials was the same male dominant on the last day as on the first day, and these were split equally between treatments (unexposed = 3, mercury-exposed = 3). Thus, preferred males also tended to be dominant males, but dominance relationships were developing over the course of the test, and it does not appear that mercury exposure played a role in the outcome of male–male relationships.

## DISCUSSION

As metal and persistent organic pollutants have increased substantially in recent decades (Wania and MacKay 1996), they may be affecting phenotypes of wild animals in ways that influence reproductive success. Exposure to pollutants may render some males unattractive to females, removing them from the breeding population. Alternatively, the changes wrought by contaminants may be novel and undetectable to the perceptive mechanisms that females have evolved for mate discrimination. It is therefore important to understand whether populations' existing sexually selected mechanisms allow detection of toxicant-mediated trait variation, and whether that information is integrated into mate choice strategies. Using a colony of Zebra Finches in which female mate preferences for males has already been demonstrated (Swaddle et al. 2005), across a variety of testing environments, our results suggest that females may not currently incorporate mercury-induced alteration of phenotypic quality into their mate choices, whether evaluating songs, associating briefly with potential mates, or pairing over the course of one week. Assuming that Zebra Finches have been exposed to anthropogenic toxicants prior to and since their domestication, it could be that this exposure, and associated effects on male signals, is too recent for appropriate mechanisms of detection in the female to have evolved. It is possible that inherently high-quality males (e.g., high-functioning immune systems) are able to maintain attractive signals and overcome the stressor of mercury contamination, signaling resilience in the face of toxicant exposure. If this is the case, environmental

mercury exposure could serve to widen the quality gap apparent to females by making already low-quality males worse, while having minimal effect on high-quality males. Alternatively, the variation induced by mercury may simply not be used by females in mate choice decisions—mercury may only alter the male's signals within the normal range of female preferences. This would be a surprising explanation, as previous work has found that females do alter mate preferences when males' condition is manipulated by exposure to developmental stressors, such as brood size and diet (DeKogel and Puijs 1996; Spencer et al. 2003, 2005), or concurrent stressors, such as nutritional level (Hill 1990). Given these previous results, we would expect that females should be able to detect, perceive, and respond to the variation induced by mercury exposure as well.

Furthermore, our results are unexpected in the light of mercury's previously observed effects on sexually selected and condition-dependent endpoints expected to play roles in mate choice. For example, Zebra Finches exposed to similar concentrations of mercury have repeatedly shown detrimental changes in condition, as assessed through physiological measurements and immune functioning (Lewis et al. 2013, Henry et al. 2014, Moore et al. 2014). In our analysis of songs, we found that mercury-exposed males tended to sing higher-pitched songs with narrower bandwidth, consistent with reports of altered songs in wild mercury-exposed songbirds (Hallinger et al. 2010, McKay and Maher 2012). Mercury is known to induce variation in visual signals, as well—in a previous study mercury-exposed males exhibited bills that were demonstrably less “red,” an important sexually selected trait in Zebra Finches (Spickler 2014), whereas females' bill color was unaffected. Lastly, mercury has been implicated in altered courtship behavior, as White Ibises on a mercury-exposed diet appeared to exhibit reduced and misdirected courtship and pairing behavior (Frederick and Jayasena 2011), possibly due to endocrine-disrupting effects (Jayasena et al. 2011).

Female preferences did, however, appear to relate to social dominance. Overall social dominance was significantly correlated with likelihood of pairing in the aviary trials, providing a potential mechanism for preference in pairing decisions made by females that is in line with previous research (Otter et al. 1998). Despite this, overall social dominance did not significantly correlate with treatment, indicating that mercury did not play a role in female choice. However, an examination of displacements on the first and last day for each pairing trial indicated that several males switched dominance over the 7-day period, and that this switching was not related to treatment. Thus, it is not clear whether pairing status may have influenced dominance status, or vice versa, and further study is needed. What seems clear is that females did not

distinguish between males on the basis of their exposure to mercury.

Because mercury was not associated with a decrease in male attractiveness to females in any behavioral context that we tested, mercury-exposed males may not lose mating opportunities from their hypothesized inability to compete with unexposed males. However, the fact remains that mercury exposure negatively affects physical condition and reproductive output in birds (Whitney and Cristol 2017b)—reproductive success in captive Zebra Finches was reduced by ~40% at the same level of exposure used in this study (Varian-Ramos et al. 2014). Specifically in males, chronic exposure to mercury may affect male fertility, as has been found in other taxa (Martinez et al. 2014), and could also alter methylation patterns in sperm, which might lead to understudied epigenetic effects in zygotes (sensu Basu et al. 2014). Given these known and potential issues in males exposed to mercury, it remains likely that females would benefit from being able to discriminate exposed from unexposed mates. Female birds who do not discriminate against mercury-exposed males may suffer fitness costs via infertility or by inadvertently making a poor mate selection. While mercury exposure in females is associated with reproductive losses, the explicit effects of mercury on male avian fertility are currently unknown.

In conclusion, female Zebra Finches do not appear to use mercury-induced phenotypic variation of males in their mate choice decisions, indicating that existing sexual selection mechanisms are not sensitive to this common environmental toxicant. As pairing with mercury-exposed males may incur a fitness cost, it is surprising that we could not identify a mechanism that leads females to avoid pairing and mating with contaminated mates. It could also be that female preferences are sufficiently variable to encompass all of the male phenotypic variation induced by mercury exposure. An obvious and ecologically relevant next step would be to examine the effect of female mercury exposure on female mate preferences. A study of songbirds exposed to mercury in the environment found altered sex ratios of offspring, suggesting that females may be physiologically “aware” of their reduced condition and may adjust reproductive effort accordingly (Boulant et al. 2012). This study highlights the need to learn more about how environmental contaminants affect sexual selection processes in order to predict how populations will respond behaviorally and evolutionarily to increasing contaminant exposure.

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**Author contributions:** V.W.G., J.P.S., and D.A.C. conceived the idea, design, and experiment. V.W.G. performed the experiments, collected data, and conducted the research. V.W.G., J.P.S., and D.A.C. wrote the paper. V.W.G., J.P.S., D.L.M., and D.A.C. designed the methods. V.W.G., J.P.S., D.L.M., and D.A.C. analyzed the data. V.W.G., J.P.S., and D.A.C. contributed substantial materials, resources, or funding.

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