Dietary Mercury Exposure in Male Zebra Finches Does Not Decrease Their Attractiveness to Females

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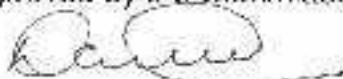August 2016

APPROVAL PAGE

This Thesis is submitted in partial fulfilment of
the requirements for the degree of

Master of Science

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Approved by the Committee, May 2016

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COMPLIANCE PAGE

Research approved by

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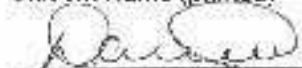_(signature)_   Signature   6-3-16
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(signature)   Daniel A. Cristol   6-3-16
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ABSTRACT PAGE

Choosing a high-quality mate contributes strongly to increased reproductive success in birds. Female birds assess quality in males, in part, via condition-dependent signals such as male songs and plumage. The production of attractive signals can be disrupted by environmental stressors, however, including environmental toxins. Mercury, a globally-increasing pollutant, is one such toxin. Mercury exposure has been shown to affect song, plumage, bill color, and mating behaviors in male birds, but the effect of these changes on the outcome of female mate choice is unknown. These effects on condition-dependent signals indicate that mercury could potentially alter males' attractiveness to females, as females use such traits to assess quality of potential mates. We sought to determine if male attractiveness to females is affected by dietary mercury exposure, using zebra finches as our model system. Males were either exposed to dietary mercury (1.2 ppm) or unexposed (0.0 ppm), and then assessed by unexposed females in three types of mate preference trials: song-only phonotaxis trials, that observed female preference for mercury-exposed or unexposed songs; two-choice association preference trials, that observed female preference for mercury-exposed or unexposed males in neighboring cages; and aviary pairing trials, in which females were given the opportunity to pair with either a mercury-exposed male or unexposed male. In phonotaxis trials and association trials, females did not spend more time near songs or males of one treatment over the other. In aviary pairing trials, females were equally likely to pair with males of either treatment. While mercury exposure is known to reduce reproductive output in zebra finches and other birds, our results suggest that females are not incorporating mercury-induced variation in male traits into their mate choice decisions. This raises questions about the future evolution of the avian mate choice system in an environment increasingly affected by toxins, as females experience fitness losses as a result of potentially poor mate choice decisions. If this is the case, then females are likely to respond to this sexual selection pressure by including toxin-mediated trait variation in their quality assessment mechanisms.

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ACKNOWLEDGEMENTS

This Master's Thesis is dedicated to my parents, Drs. Colin and Catherine Greene, who have always encouraged my love of science and the great outdoors. Thank you for your love and support!

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<u>Introduction</u>

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 benefits (those that increase fitness via the genetic quality of offspring) and direct benefits (those that increase fitness through material advantages). Making a sub-optimal mate choice, therefore, can impose a large fitness cost (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).

Such choosiness and honest-signaling have been studied extensively in sexually-dimorphic songbirds (Hill 1991, Nowicki et al. 1998, Gil and Gahr 2002, Candolin 2003, Nowicki and Searcy 2004, Andersson and Simmons 2006). Honest signals of direct benefits often include condition-dependent traits—the phenotypic expression of which are dependent on current condition—such as plumage color and song. Plumage quality is often dependent on nutritional status (especially carotenoid-intensive plumage) and can therefore reflect nutrient availability on a male's territory—a direct benefit to potential mates. For example, captive male house finches (*Haumorhous mexicanus*) fed *ad libitum* had increased carotenoid-based brightness of plumage (Hill and Montgomerie 1994), and correspondingly were preferred by female house finches (Hill 1990). This preference was beneficial to female fitness, as the more brightly colored males also exhibited higher parental

feeding rates (Hill 1991). 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, Joseph L. Woodgate 2013) and current (Schmidt et al. 2014). Quality of male song therefore can convey information about direct benefits to potential mates, including male parental feeding rate in sedge warblers (*Acrocephalus schoenobaenus*) (Buchanan and Catchpole 2000), territory quality of blue grosbeaks (*Passerina caerulea*) (Keyser and Hill 2000), and social rank in gregarious black-capped chickadees (*Poecile atricapillus*) (Otter et al. 1997). The quality of male song influences female mate choices, as females are known to pay attention to song variation (Nowicki and Searcy 2004). As condition-dependent signals reflect direct fitness benefits, females can therefore maximize these benefits by choosing mates based on these signals.

The expression of condition-dependent traits is expected to be mediated by variation in environmental conditions, such as current nutrient availability and abundance of available resources during development. However, condition-dependent signals also have the potential to be affected by unnatural environmental conditions, such as contamination with environmental pollutants. As the amount and variety of pollutants in the environment have increased significantly in the last century (Wania and MacKay 1996), their effects on birds have become a focal point for research (Scheuhammer 1987, Walker 1990, Furness 1993, Fox 1993, Ellegren et al.

1997, Grasman et al. 1998, Chen and Hale 2010), including their effects on condition-dependent signals (Møller and Mousseau 2001, 2007, Gorissen et al. 2005, Markman et al. 2008, Galván et al. 2010). One such pollutant is mercury, a potent neurotoxin and globally-increasing contaminant (Swain et al. 1992, Fitzgerald 1995, Pirrone et al. 1996) that has recently been shown to biomagnify into terrestrial systems at sub-lethal levels (Cristol et al. 2008) with potential to affect sexual selection processes in songbirds. The direct negative effects of sub-lethal mercury exposure on avian reproductive success are well-documented, most notably as decreased reproductive output across a variety of species (reviewed in Whitney and Cristol in prep.). Aside from the direct reproductive losses due to toxicity, mercury exposure alters phenotypic characteristics important to survival, including spatial memory (Bessler 2011), immune response (Lewis et al. 2013), and body mass regulation (Kobiela et al. 2015). Interestingly, mercury exposure has also been associated with changes in condition-dependent reproductive signals in birds—including altered bill coloration in zebra finches (*Taeniopygia guttata*) (Spickler 2014), altered plumage brightness in belted kingfishers (*Megaceryle alcyon*) (White and Cristol 2014), abnormal courtship display behaviors in white ibises (*Eudocimus albus)* (Frederick and Jayasena 2010), and reduced song length, pitch, and complexity of song in three species of oscine birds (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

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1989), these mercury-induced changes in the expression of signaling traits may affect a female observer's perception of a male's quality, resulting in females avoiding mercury-exposed mates.

Because mercury affects signaling traits and many other aspects of male phenotypic quality (Hallinger et al. 2010, McKay and Maher 2012, Lewis et al. 2013, White and Cristol 2014, McCullagh et al. 2015, Kobiela et al. 2015), we would expect females to perceive and respond to variation in those traits when making mate choice decisions among males with varying levels of contaminant exposure. Signals that are associated with decreased individual quality as a result of mercury exposure should be perceived as less attractive by females, if females' sensory and cognitive pathways respond to the trait variation induced by this environmental stressor. However, if individual females fail to respond to mercury-induced male trait variation during their mate choice processes, this might indicate that suitable mechanisms of detection of exposure have yet to evolve in females. This would mean that mercury could decouple signals from intrinsic quality, interfering with the honesty of the signal. This interpretation implies that a novel and fairly recent environmental stressor, such as mercury, may lead to sub-optimal pairing decisions with negative fitness effects on populations.

In this study we sought to examine the effects of dietary mercury exposure on mate choice processes of captive zebra finches, using female preference metrics as bioassays of male attractiveness. We used zebra finches as they are sexually dimorphic in both appearance and behavior, pair

quickly in captivity, and their mate choice processes have been well-studied and described (Swaddle and Cuthill 1994, Zann 1996, Rutstein et al. 2007, Riebel 2009, Tschirren et al. 2009, Griffith and Buchanan 2010, Griffith et al. 2010). Additionally, several male display traits, including bill color (Burley and Coopersmith 1987, Collins et al. 1994b, DeKogel and Prijs 1996, Simons and Verhulst 2011), cheek patch size (Naguib and Nemitz 2007), rate of courtship (Collins et al. 1994a, Collins and ten Cate 1996), and complexity of songs (Clayton and Pröve 1989, Riebel 2009), appear to be condition-dependent signals. Importantly, female preference in this species is a reliable metric for male attractiveness, as females demonstrate repeatable individual preferences and significant among-female agreement in preferences (Forstmeier and Birkhead 2004, Witte 2006, Rutstein et al. 2007). Furthermore, male zebra finches initiate courtship immediately upon introduction of a female (Silcox and Evans 1982). These qualities make the zebra finch an excellent model for answering questions about mercury's effect on mate choice processes.

The overall objective of these experiments was to understand whether mercury induces changes in male zebra finch attractiveness, as assayed through changes in females' preferences for males or their song.

First, we investigated the effects of lifetime dietary mercury exposure on the perceived attractiveness of male song in phonotaxis trials (as in Holveck and Riebel 2007), where unexposed females had the opportunity to spend time listening to unexposed versus mercury-exposed males' song. Life-

long exposure to mercury is expected to induce physiological stress that ultimately reduces attractiveness of song, as early-life stress from nutritional deprivation decreases both measured zebra finch song quality (Spencer et al. 2003) and the attractiveness of song to females (Spencer et al. 2005). Furthermore, changes in song have been detected in wild populations on contaminated sites. If females currently possess mechanisms to detect and respond to mercury-induced variation in song when choosing mates, then we predicted females would spend less time listening to mercury-exposed song. However, if female mate choice decisions are not sensitive to mercury-induced variation of signals, we expect to find no difference in female preferences.

Second, we examined the effects of mercury on overall male attractiveness through two assays: female preferences for unexposed vs. mercury-exposed males in a two-choice association apparatus (Collins et al. 1994b, Collins 1995, DeKogel and Prijs 1996, Blount et al. 2003, Witte 2006, Rutstein et al. 2007, Holveck and Riebel 2007, Swaddle and Page 2007), and actual pairing between these males and females in free-flight aviaries (Clayton 1990, Swaddle 1996, Rutstein et al. 2007). If females can detect and respond to the variation in male phenotype caused by mercury, we predict that females will demonstrate a decreased preference for mercury-exposed males in a two-choice apparatus, and will be less likely to pair with mercury-exposed males in free-flight aviaries. However, if mercury-induced variation does not affect male attractiveness to females and/or females do not use the

mercury-induced variation in their mate choice processes, they will not exhibit differences in preferences.

Methods

Zebra Finch Colony

We used a well-established, outbred colony of domestically bred zebra finches at the College of William and Mary for all experiments. Males were all wild-type and fed either an unexposed diet (0.0 parts per million [ppm] mercury) or an exposed diet (1.2 ppm mercury) of Zupreem ® commercial finch food for their entire lifetime, including in the egg, as their parents were fed the same diets. Our mercury-diet concentration of 1.2 ppm approximates the level of contamination in avian food items found in a highly contaminated area (Cristol et. al. 2008) and is therefore an ecologically-relevant dose for studying the effects of environmental pollution. All females in all experiments were fed the unexposed diet throughout life, as we wanted to focus on the effects of mercury on male attractiveness rather than the toxin's potential influence on female preferences. Prior to and during trials, all birds were kept in single-sex cages in mixed-sex rooms (unless indicated otherwise) on a 14:10 light:dark cycle at approximately 22°C and provided food and water *ad libitum*. The subset of birds kept in outdoor aviaries for pairing trials in July and August of 2015 were subject to natural lighting and weather conditions for 7 days and nights and also provided with unexposed food and water *ad libitum*. All procedures were approved by the College of William and Mary's Institutional Animal Care and Use Committee (protocol IACUC-IBC-2013-06-

02-8721-dacris). We conducted all experimental trials between May 2015 and February 2016 at the College of William and Mary.

Blood Mercury Analysis

We analyzed total mercury (THg) from blood samples taken at the time of each set of trials. Approximately 95% of mercury in avian blood is comprised of methylmercury (MeHg) (Rimmer et al. 2005, Wada et al. 2009)and therefore THg values are an accurate representation of blood MeHg concentration. THg samples were analyzed using combustion-amalgamation cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT, USA) according to U.S. Environmental Protection Agency method 7473. For quality control, before and after every 20 samples we ran duplicates, blanks, and 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 $2.49 \pm 1.54\%$. Mean percent recoveries of THg for the tuna and DORM-4 were $98.66 \pm 0.21\%$ ($n = 20$) and $95.84 \pm 0.47\%$ ($n = 20$), respectively. All THg concentrations are reported as wet weight (wwt).

Song Collection

We used audio recordings of female-directed songs that had been recorded from males of both treatments for a different study on song quality (Claire Varian Ramos, unpublished data) in 2012 and 2013. The birds that produced these songs were from the same colony and kept in the same

conditions as described above, but were from a previous generation. None of the birds recorded were used as, or directly related to, experimental subjects in the current study. Female-directed songs were recorded individually using Avisoft Recorder from 12 control males and 12 mercury males in a small cage with a novel female. All recorded sounds were manually filtered and non-song sounds were discarded. We extracted two motifs of directed song from the recordings of each male and measured motif length, peak frequency, and bandwidth using Raven Pro (1.4 build 48, Windows 64-bit version). We counted number of syllables per motif and number of unique syllables per motif visually (as in Spencer et al. 2003). Each measurement (two total, one per each motif) was averaged into one for each male.

Phonotaxis Stimuli

We randomly paired males of each treatment, creating 20 unique contests to test for female preference between songs of mercury-exposed and unexposed males. No male was used more than twice in generating the unique pairs. Each contest contained two audio tracks—an unexposed track using the two directed song motifs taken from the unexposed male assigned to that pair, and a mercury-exposed track using the two directed song motifs from the mercury-exposed male. We used the software program Audacity (2.1.1 for Windows Operating System) to edit and create song contests in which the total number of individual motifs for each treatment track was equal. Songs were organized into "bouts" (3-5 repeats of the same song) within each track, to replicate the singing patterns of live male zebra finches. Pauses

within bouts averaged 0.19s, which is the average period of silence within bouts of wild zebra finch song reported by Zann (1996).

Tracks were presented from left and right speakers (BostonBA635) placed at either end of a phonotaxis chamber (Fig. 1) in a room which had sound-dampening material affixed to the walls and floor to reduce sound reflection and reverberation. The first bout of songs played simultaneously from each track, so the females would hear them both at once. After this, bouts of each treatment alternated for 15 mins. We blocked the trials, so that in five trials, the unexposed song was played from the left first (after the initial simultaneous bout), in five the unexposed song played from the right first, in five the mercury-exposed song was played from the left first, and in five the mercury-exposed song was played from the right first.

The night before each trial, we assessed the songs in the phonotaxis chamber with a digital sound level meter (Extech 497730, set to A weighting and record Max over time), to insure a sound pressure gradient that was loudest next to the speaker at perch height and quietest at perch height at the opposite end of the chamber. The songs were switched, coming from the opposite speakers, and we recorded the decibel gradient again. Sound levels were 60.3 ± 0.74 dB SPL next to the playing speaker at perch height and 45.2 ± 0.99 dB SPL at the opposite end of the chamber at perch height.
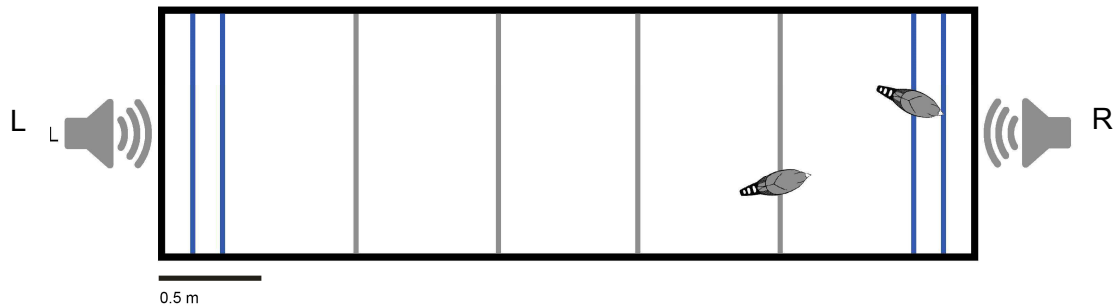
0.5 m

Figure 1. Plan view of phonotaxis chamber (measuring 46 by 229 by 46 cm) with speakers (L and R) playing alternating songs at either end. Horizontal grey bars represent perches, and blue bars represent near perches. Grey bird silhouettes represent females.

Phonotaxis Trials

Zebra finches are highly gregarious, and pilot trials indicated that females were more responsive to songs when a familiar conspecific was present in the song choice chamber. Thus, we tested two females at a time in the choice chamber and combined their responses into a single data point before analysis. The two females were former cage mates and were banded with pink or black plastic leg bands (Red Bird Products, Inc.) to enable identification during observation. We tested the song preferences of 40 females total, resulting in 20 trials (n = 20). All trials were conducted in January and February 2016.

We placed the two banded females in the phonotaxis chamber the night before a trial to acclimate overnight with food and water. Clear plexiglass dividers kept them in the central area of the chamber, allowing

them to see into the two ends of the chamber while preventing exploration. We removed food and water on the morning of the trial and allowed the females 5 mins to acclimate after we left the room. We then played the contest for 5 mins (first simultaneous, then alternating) with the plexiglass dividers still in place, to expose the females to the songs. We then removed the plexiglass dividers, and the birds were allowed 5 mins to re-acclimate and explore the entire chamber in silence. We then played the contest in its entirety (15 mins, Phase 1). During the playback, the motions of both females were observed from a live video feed outside of the room (Sony HDR-CX240). The time (in seconds) that each female spent on the perches nearest the speakers were totaled between the two females into a single data point. In addition, we recorded the first direction (left or right end of the chamber, the perches nearest a speaker) each female flew towards upon initiation of playback. After Phase 1, we replaced the food, water, and dividers for 30 mins. We then repeated the test (Phase 2) with the same playbacks, after switching the sides from which the songs were emanating to control for side preferences.

To meet the criterion for a successful trial, an individual female had to spend at least 10% of her time on the perches closest to the speakers (180 s minimum per female per trial). If one or both females failed to spend sufficient time near the speakers, that contest of songs was conducted again within a week using a new pair of females.

Association Preference in a Two-Choice Apparatus

To test for female preference for potential mates from each treatment, we randomly selected 15 males of each treatment that were all approximately the same age (within 60 days). We then paired the males randomly, generating 15 total dyads of males. At the time the trials were conducted, unexposed males had an average blood mercury level of 0.007 ± 0.001 ppm wwt. Exposed males had an average blood mercury level of 15.9 ± 0.71 ppm wwt. Female sexual experience varied, but all had previously occupied a cage with a male prior to trials. All females used were 1-3 years old. For the duration of this study, the females were kept in a room isolated from male interaction. We conducted all association preference trials in May 2015.

To determine whether females would spend more time in proximity to one or the other potential mate, we used a two-choice association apparatus similar to that in many other mate choice studies (Swaddle and Cuthill 1994, Hunt et al. 1997, Swaddle et al. 2005, Holveck and Riebel 2007, Holveck et al. 2011). We placed each male in one of two small cages next to a large central cage (Fig. 2). Males were visually separated from each other and from the large central cage by opaque dividers (Fig. 2), and allowed to acclimate to their environment for 10 min. We placed the female in the large central cage (Fig. 2) and allowed her to acclimate for 5 min. After the acclimation period, we removed the dividers between the female and the males, and began the first 30-min observation period (Phase 1). Females were free to move throughout the large central cage and associate with either male and she could not see both males at the same time from any position. Males remained

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visually isolated from one another throughout the trial. After Phase 1 of the trial, 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 mins (Phase 2) of the trial.

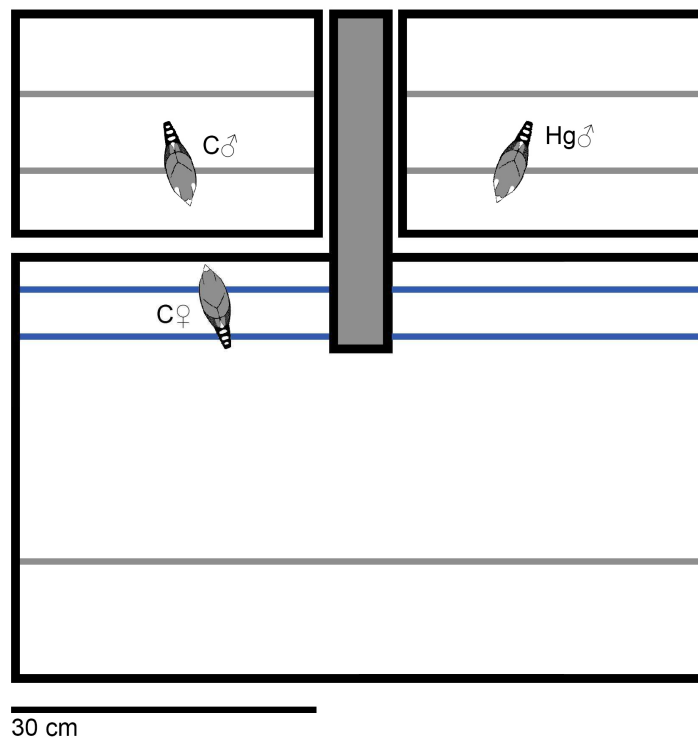Figure 2. Plan view of association preference apparatus. Blue bars are near perches where female could show interest in a male. Grey bars are neutral perches. C = unexposed, Hg = mercury-exposed.

We recorded the trials with a tripod-mounted SONY Handycam (HDR-CX240). We observed female position and behavior for each 30-min phase 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 in front of a male, facing the male (Fig. 2). Time spent in other positions (facing away from the male on near perches, on the neutral perch, on cage bottom, feeding, etc.) and other activities (on near perches but preening or sleeping) was not classified as indicating interest in a particular male. We determined female preference by totaling number of seconds spent exhibiting active interest in each male.

We presented each pair of males to three different females. We used each female subject to assess no more than two sets of males, and no female had a degree of relatedness > 0.015 to any males she assessed. In order for a female's response to qualify for inclusion in the analysis, she had to spend at least 10% of the trial time (>360 seconds) on the near perches facing either male. We summed the three females' responses into a single data point for each male in each set.

Pairing Trials

To determine if male attractiveness as assessed in a brief two-choice apparatus translated to actual choice of a mate, we used the same males that were used in the two-choice trials for week-long pairing trials in an outdoor aviary. All males were banded with either white or yellow plastic leg bands (Red Bird Products, Inc.) so they could be identified during behavioral observations. Leg band color was equalized across treatments in case color biased female preferences. At the time the trials were conducted, unexposed

males had an average blood mercury level of 0.021 ± 0.004 ppm wwt.

Exposed males had an average blood mercury level of 13.67 ± 0.81 ppm wwt.

We placed a dyad of males (one lifetime unexposed, one lifetime mercury-exposed) in outdoor aviaries with a single female (unknown to either male) for seven days. Each outdoor aviary was visually isolated from other outdoor aviaries and contained four nest boxes, a nesting material dispenser, a water bath, a large central perch, and a small perch between each nest box (Fig. 3). We conducted the aviary pairing trials in July and August 2016. The photoperiod was approximately 14:10 light:dark and the range of average daily minimum and maximum temperature and humidity was 21.5-26°C and 50-95%, respectively. Birds were provided with an unexposed diet *ad libitum* to avoid exposing unexposed birds to mercury. Previous experiments have found that blood mercury remains elevated at >50% of asymptotic level after one week (Whitney 2014), so the mercury-exposed males were still affected by their treatment, despite the temporary change in diet to unexposed food.
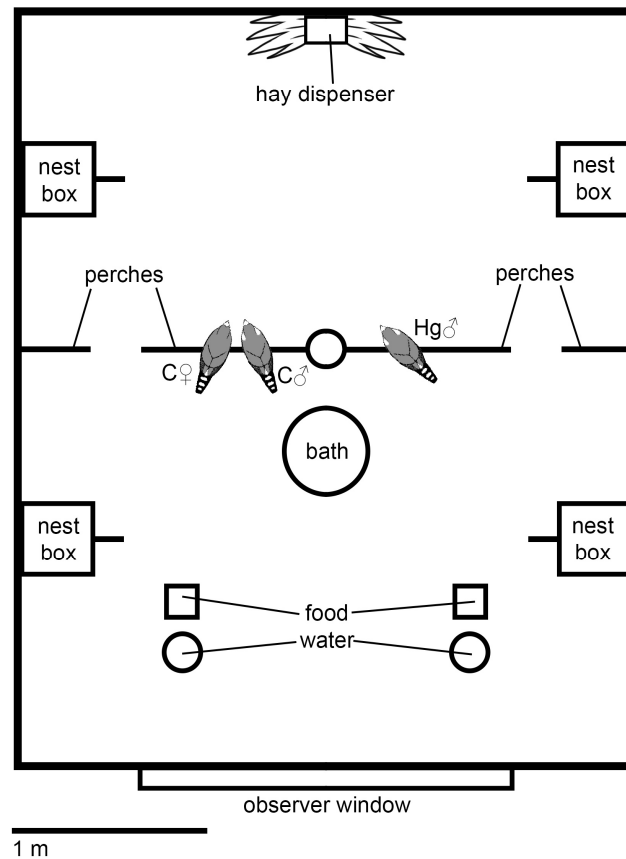
Figure 3. Plan view of aviary for pairing trials.

We released the two males into the outdoor aviary to acclimate and potentially establish territories one day before observations began. The female was placed in the aviary in a small cage during this time so that she could interact visually and audibly with males and witness interactions between them prior to her own interactions with the males. Behavioral observations began immediately after the release of the female on the following morning. Each trio of birds was observed daily for 30 mins during the period of 7:00-10:00 AM, approximately 1-3 hours after dawn, for seven consecutive days. Behaviors recorded included directed and undirected song,

displacements, time of entry and exit of next boxes, and clumping, a pair-bonding behavior typical of zebra finches in which two birds huddle together (Zann 1996). We checked nest boxes for eggs at the end of each observation period. For each behavior, the acting bird was recorded, as well as the recipient (target) bird of the interaction, if applicable. 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 the video footage to confirm recorded observations. The observer remained blind to treatment during observations and transcription of video files. At the end of the trials, the male that was permitted by the female to enter the nest box containing eggs was considered the paired male. Alternatively, in the trials where no eggs were laid, a male was considered paired if the female consistently spent time in the nest box with him. In all trials, the chosen male was apparent via constant association with the female at the next box, hay dispenser, and during feeding, though these interactions specifically were unmeasured. To determine social dominance between males, we observed ratio of displacements between males over the seven day period. A minimum of 20 social interactions between the males were required, which allowed the two males in all trials to be classified as either "dominant" or "submissive", consistent with the broader observations of the viewer. A male was considered "dominant" if he displaced other male in 15% more of their interactions than the other male over the 7-day period.

Analysis

To evaluate the phonotaxis trials, we tested the hypothesis that females would spend more time listening to unexposed songs than exposed songs with a paired t-test on the amount of time females spent near each treatment's song. 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 different treatments differed in motif length, peak frequency, bandwidth, number of notes, and number of unique notes, we performed a principle component analysis and compared means and confidence intervals of the resulting components via a multivariate general linear model where song PCAs were the dependent variables and treatment group was an among-subjects fixed factor. We then visually inspected a bivariate plot of estimated marginal means of PCAs, $\pm$ 95% confidence intervals.

For the two-choice preference trials, we tested the hypothesis that females would spend more time in front of unexposed males using a paired t-test on the amount of time females spent showing active interest on perches in front of each male.

In determining whether females preferred to mate with males of one or the other treatment, we used binomial tests. We also used binomial tests to compare whether exposed or unexposed males were more likely to be socially dominant over the other. We performed a principle component analysis to construct multivariate components that combined the behavioral metrics of clumping, time spent simultaneously in nest box, the number of

undirected and directed songs, total songs overall, the number of displacements, and the amount of aggression towards and from the female. We subsequently performed repeated-measures ANOVAs to compare the resulting components, separately, within aviaries but between treatment groups (fixed factor).

Multivariate analyses of songs and behavioral data was performed in IBM SPSS Statistics (version 23). All other statistical analyses for all experiments were completed using the statistical software R (version 3.1.1).

<u>Results</u>

Phonotaxis Trials

Females did not spend more time listening to songs of unexposed males over songs of mercury-exposed males (paired t-test, $t = 0.970$, $df = 19$, $p = 0.344$) (Fig. 4). Individual females did not overall show a preference (>20% more time on one side than the other) for either treatment (23 unexposed songs preferred, 17 mercury-exposed songs preferred, binomial test, $p = 0.426$) (Fig. 5), nor were females more likely to first approach songs of one treatment over the other (21 approached unexposed first, 19 approached mercury-exposed first, binomial test, $p = 0.875$).
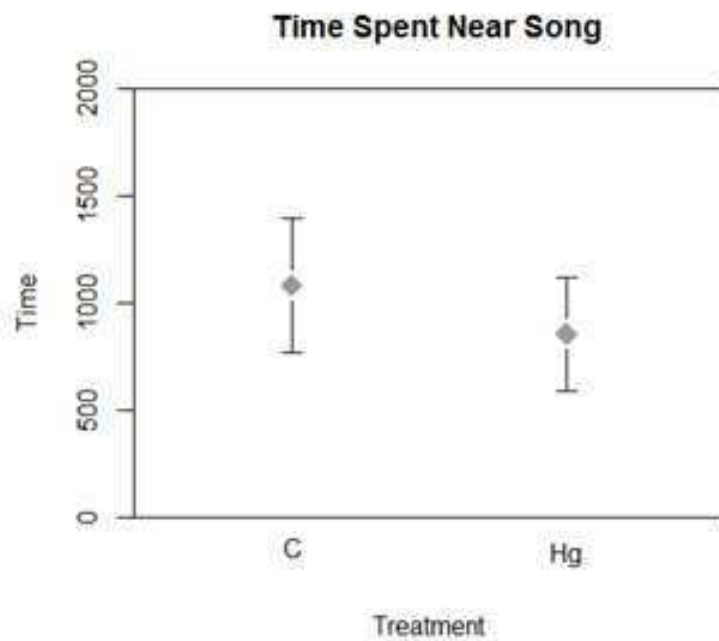
Figure 4. Mean time spent near speakers projecting songs of each treatment, in seconds. Bars represent 95% confidence intervals.

The average time spent listening to unexposed song was 1085.1 $\pm$ 159.4 s, and the average time spent listening to exposed song was 857.1 $\pm$ 135.0 s. All means reported include standard error of the mean.
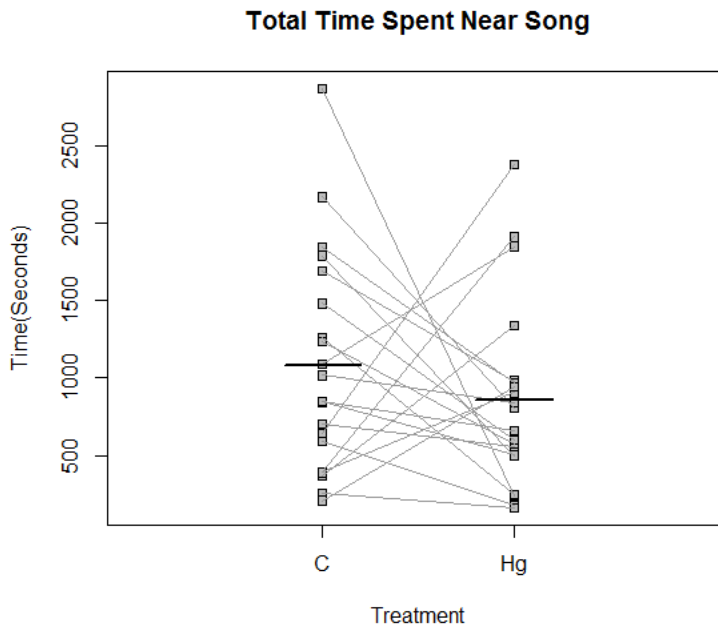
Total Time Spent Near Song

Figure 5. Total time (in seconds) each pair of females spent near speakers projecting 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 (unexposed = 1085.1 ± 159.4 s, exposed = 857.1 ± 135.0 s).

Song Analysis

We performed a principal components analysis on the five song motif metrics (average motif length, peak motif frequency, bandwidth, number of syllables, number of unique syllables), using the correlation matrix and without factor rotation, which returned two components with eigenvalues greater than 1 (Table 1). As the values of the second component (song PC2) increase peak song frequency decreases and bandwidth increases. Hence we interpreted song PC1 as describing broader and lower-frequency songs. PC1 loaded most strongly with average motif length and number of syllables

and, hence, we interpreted this component as describing longer, more complex songs.

Table 1. Summary of component scores generated by the song principal component analysis

	Song PC1	Song PC2
% variance explained	*44.6*	*21.0*
Eigenvalue	*2.23*	*1.05*
Average song length	**0.688**	0.282
Peak song frequency	-0.132	**0.764**
Bandwidth	0.231	**-0.622**
Number of Syllables	**0.946**	0.030
Number of Unique Syllables	**0.889**	0.025

The multivariate general linear model indicated that mercury exposure somewhat influenced song PC2 ($F_{1,22}$ = 2.13, P = 0.158, partial eta squared (effect size) = 8.9%) but had little influence on song PC1 ($F_{1,22}$ = 0.078, P = 0.782, partial eta squared (effect size) = 0.4%). Inspection of a bivariate plot of estimated marginal means (Table 2) of song PC1 versus song PC2, $\pm$ 95% confidence intervals indicate that mercury-exposed birds produced songs with a lower song PC2 value. Hence, mercury exposure is associated with songs that are typically narrower in bandwidth and higher in frequency. Mercury does not appear to influence song length or complexity.

Table 2 presents a summary of raw means ± confidence intervals for each song measurement.

Table 2. Raw means ± confidence intervals

	Unexposed	Mercury-Exposed
Length	0.71± 0.06	0.68 ± 0.09
Peak Frequency	3822.12 ± 351.28	4371.23 ± 504.8
Bandwidth	4407.12 ± 696.39	4148.72 ± 519.01
Number of Syllables	5.5 ± 0.58	5.25 ± 1.0
Number of Unique Syllables	4.75 ± 0.55	5.04 ± 0.88

Female Preference in a Two-Choice Association Trial

Females did not spend more time with males of one treatment over another (paired t-test, t = -1.047, df = 14, p = 0.313) (Fig. 6). The average time spent observing unexposed males was 1017.5 ± 416.3 seconds while the average number of seconds spent observing mercury-exposed males was 1220.5 ± 397.9 seconds. In the 25 trials in which a female displayed a clear preference (20% more time spent with one male than the other), females did not prefer males of one treatment (9 = unexposed, 16 = mercury-exposed, binomial test, p = 0.230) (Fig. 7).
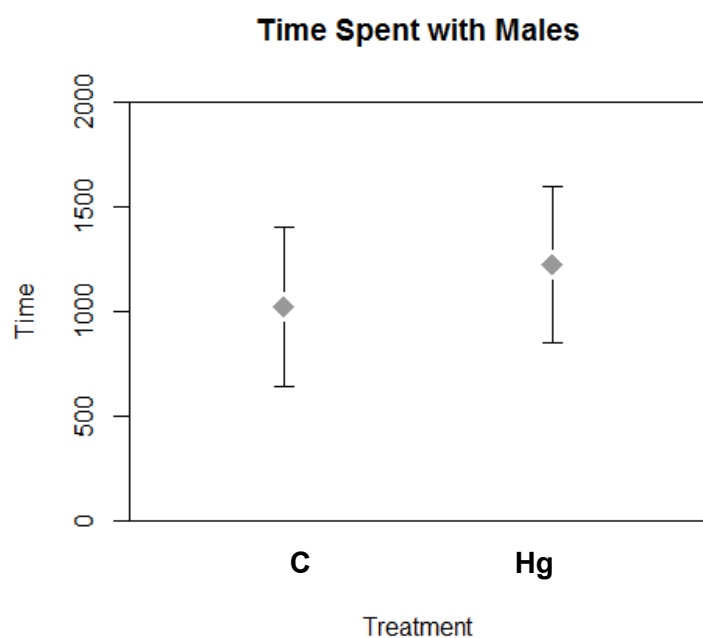
Time Spent with Males

Figure 6. Mean time (seconds) spent with each male. Bars represent 95% confidence intervals.
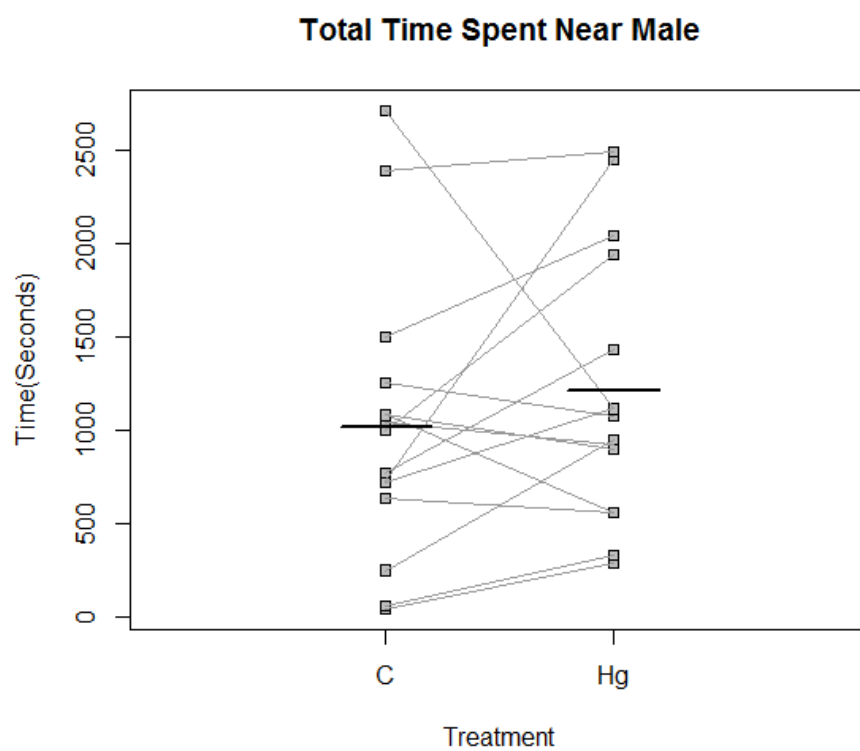
Total Time Spent Near Male

Figure 7. Total time (in seconds) females spent near males of each treatment in two-choice trials. The pairs of males presented in each trial are connected, and the black bars represent the mean number of seconds spent in showing active interest towards each male (unexposed = 1017.5 ± 416.3 s; mercury-exposed = 1220.5 ± 397.9 s).

Aviary Pairing Trials

All of the females paired with one of the males in their aviary, but this pairing was not biased toward a treatment (7 = unexposed, 7 = mercury-exposed, binomial test, p=0.999). Female pairing decisions were significantly related to apparent social dominance (displaced other male 15% more times over the 7-day period), as 12 females paired with dominant males and only 2 paired with subordinates (binomial test, p = 0.012). However, apparent social dominance was not related to treatment (unexposed dominant = 9, mercury-exposed dominant = 5, binomial test, p = 0.430). A first and last day examination of displacement ratios in each set of males indicated that several males "switched" dominance over the seven-day period. Treatment and apparent dominance on the first day of trials did not predict whether a male ultimately paired (unexposed, dominant first day and paired = 5, mercury-exposed, dominant first day and paired = 4, binomial test, p = 0.999), nor did treatment predict which male would be both dominant and paired by the last day of trials (unexposed, dominant last day and paired = 5, mercury-exposed, dominant last day and paired = 6, binomial test, p = 0.999). In only six of 14 trials was the same male dominant on the last day as on the first day, and

these were split equally between treatment (unexposed = 3, mercury-exposed = 3).

Behavioral Analyses

The principal components analysis on the eight behavioral metrics recorded returned two components with eigenvalues greater than 1 (Table 3). As the values of the first component (pairing PC1) increase, aggression both to and from the female increase along with total number of undirected songs, but the total number of displacements of the other (rival) male and total number of directed songs at the female decrease. We interpreted high values of pairing PC1 to represent unattractive behaviors. The second pairing component loaded positively with the number of directed songs, total number of songs, and the total amount of time spent in the nest box with the female. We interpreted pairing PC2, therefore, to represent attractive behaviors.

Table 3. Summary of component scores generated by the outdoor pairing principal component analysis

	Pairing PC1	Pairing PC2
% variance explained	*37.6*	*23.5*
Eigenvalue	*3.01*	*1.88*
Total contact	-0.272	0.501
Total Simultaneous time in Nest Box	-0.550	**0.602**
Total Undirected Songs	**0.732**	**0.605**
Total Female- Directed	**-0.649**	0.394

Songs

Total Song	0.468	**0.839**
Total Displacements of	**-0.675**	0.151
Other Male		
Total Aggression from	**0.716**	0.083
Female		
Total Aggression	**0.697**	-0.102
towards Female		

After performing repeated-measures ANOVAs comparing pairing PC1 and PC2, in neither model did we find evidence that mercury influenced these multivariate indices of pairing behavior (pairing PC1: $F_{1,13}$ = 0.285, P = 0.602, partial eta squared (effect size) = 2.1%; pairing PC2: $F_{1,13}$ = 0.207, P = 0.657, partial eta squared (effect size) = 1.6%).

Discussion

Mercury exposure did not influence attractiveness of either songs or males in choice trials with unexposed females, nor did it influence female mate-choice decisions in pairing trials. These findings indicate that females do not currently use mercury-induced signaling trait variation in their mate choice decisions. The lack of disruption of this presumably sexually-selected signaling system indicates that mercury exposure may uncouple phenotypic signals from quality—undermining the honesty upon which female choices rely.

As many environmental pollutants have increased substantially in recent decades (Wania and MacKay 1996), it is important to understand whether populations' existing sexually-selected mechanisms allow detection of toxin-mediated trait variation, and whether that information is integrated into mate choice strategies. Our results indicate that in domesticated zebra finches, females do not currently incorporate mercury-induced alteration of phenotypic quality into their mate choices. Assuming that zebra finches have been exposed to anthropogenic toxins 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. Possibly, inherently high-quality males are able to maintain attractive signals despite the stressor of mercury contamination, the ability to bear the burden of contamination itself a sign of quality (i.e., the handicap hypothesis). If this is the case, mercury exposure could serve to widen the apparent quality gap 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 similar effects on traits associated with male attractiveness as a result of many other developmental stressors, such as brood size and diet (DeKogel and Prijs 1996, Spencer et al. 2003, 2005) and 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 known effects on sexually selected and condition-dependent endpoints expected to play roles in mate choice. For example, zebra finches exposed to mercury have repeatedly shown detrimental changes in condition (Lewis et al. 2013, Moore et al. 2014, Henry et al. 2014). In our analysis of songs, we found that mercury-exposed males sang higher pitched songs with lower bandwidth, which is consistent with previous work in our lab detecting changes in male songs as a result of exposure (Van-Ramos, unpublished data). Mercury is known to induce variation in visual signals, as well—mercury-exposed males were found to exhibit significantly altered bill color, an important sexually-selected trait, (Spickler 2014) whereas females did not. Lastly, mercury has known effects on courtship behavior, as white ibises on a mercury-exposed diet exhibited reduced and misdirected courtship and pairing behavior (Frederick and Jayasena 2010), possibly due to endocrine-disrupting effects (Jayasena et al. 2011). Despite all of these mercury-induced changes in traits important to reproductive success, however, females did not appear to care—their preferences did not reflect treatment in any of the three types of trial.

Female preferences did however appear to relate to social dominance. Overall social dominance, as defined in this study, was significantly correlated with likelihood of pairing in the aviary trials (in 12 trials, dominant males

paired with the female, whereas in only 2 trials did subordinate males pair), providing a potential mechanism for preference in pairing decisions made by females. While overall social dominance did not significantly correlate with treatment in the current study (9 unexposed and 5 mercury-exposed, binomial test, p = 0.430), previous work in our lab found that mercury-exposed zebra finches were significantly less socially dominant (Swaddle, unpublished data). However, in the pairing trials, an examination of displacement ratios on the first and last day for each trial indicated that several males "switched" dominance over the seven-day period, and that this switching was not related to treatment. Considering dominance relationships at a finer time scale appeared to diminish the importance of social dominance in pairing, as treatment and first/last day dominance were not related to whether a male was ultimately paired. Therefore, clarifying the relationship between mercury exposure and social dominance in mate-choice situations is therefore extremely important for future research.

Evolutionary Consequences

Females, with their higher investment in reproduction, are under strong selection to choose the highest quality mates available to them according to a variety of sexual selection mechanisms. Securing either good genes (with associated indirect benefits, such as "sexy sons") and/or material advantages (with associated direct benefits, such as parental care and territory quality) is imperative to female fitness, and females need to be able to assess these qualities in males prior to investing energy in reproductive efforts. If mercury

silently decreases the actual physiological quality of males while leaving their apparent phenotypic quality intact, the sexually-selected reference points females use when assessing males in a contaminated landscape will be poorly calibrated in terms of maximizing fitness. Selection to re-calibrate this system in the presence of toxins is likely to be swift, but it may not be swift enough to keep up with the rate of human-induced environmental changes.

Because mercury was not associated with a decrease in male attractiveness to females in any behavioral context, mercury-exposed males may not lose fitness from inability to compete with unexposed males for mates. High-quality males may even stand to gain a fitness benefit from ubiquitous exposure of a shared environment, as lower-quality males may be unable to maintain attractive signals in the face of contamination, emphasizing the attractiveness of the high-quality male. However, the fact remains that mercury exposure negatively affects physical condition (Hawley et al. 2009, Wada et al. 2009, Bessler 2011, Ackerman et al. 2012, McKay and Maher 2012, Lewis et al. 2013, Whitney 2014) and reproductive output (Pollock and Machin 2007, Brasso and Cristol 2008, Frederick and Jayasena 2010, Hallinger et al. 2011, Jackson et al. 2011, Moore et al. 2014, Chin 2015, McCullagh et al. 2015) in birds. Furthermore, chronic mercury exposure may also affect male fertility in birds, as has been found in other taxa (reduced and abnormal sperm in rats, Martinez et al. 2014) and has the capacity to have epigenetic effects, as it has been shown to in other organisms (Basu et al. 2013, reviewed in 2014). Given these known and

potential issues, it remains likely that mercury affects male fitness at other points in the reproductive process.

If mercury exposure does actually decrease male quality, female birds who pair with mercury-exposed males may suffer explicit fitness costs by inadvertently making a poor mate selection. If a chosen male experiences reduced sperm count or quality as a result of exposure, her eggs may not be fertilized correctly; epigenetic effects in sperm may also interfere with the appropriate development and success of offspring. Furthermore, male quality can reflect territory quality. A female who does not differentiate between exposed and unexposed males risks pairing with a mercury-exposed male on a presumably contaminated territory, and therefore deriving all future nutrition for herself and any offspring from that contaminated territory and facing the known reproductive consequences of exposure. As wild zebra finches breed and forage in colonies rather than individuals or pairs, the issue of male territory contamination becomes irrelevant as any habitat use by one finch is generally shared by the colony. However, if we consider zebra finches in this experiment as a model for territorial species, there are serious implications for birds in which the female arrives to the breeding ground after males have established territories across an environment with a contamination gradient or patchy distribution of toxins.

Future Research

A follow up study should investigate the fitness costs of making an inappropriate choice by comparing the reproductive output of unexposed

females paired with either exposed males or unexposed males and maintained on diets that would be representative of each male's territory. This would determine whether there is a fitness loss for previously unexposed females who pair with exposed males on exposed territories. Previous experiments examining reproductive outcomes of mercury-exposed birds have involved pairs that were both members of the same treatment, rather than just the male. If fitness outcomes differ, we would predict strong selection pressure on the female for the detection of exposure in potential mates in the field.

Additionally, future mate-choice and mercury research ought to incorporate multiple male choice options for females. Because our design limited the females to just two choices in all three of the experiments, we may have eliminated more subtle effects of mercury that might emerge in an environment with multiple options. One study that might elucidate more subtle effects would be to keep groups of birds—unexposed and mercury-exposed males and unexposed females—in free-choice aviaries and track pairing latency for each male. Each female would be comparing males against all the other options, and it is possible that unexposed males would overall pair more quickly than mercury-exposed males. Furthermore, social rank in the context of a larger flock would play a role, and patterns of dominance between exposed and unexposed males may emerge alongside female preferences. This design has the benefit of more closely resembling the actual mate choice process, in which both males and females have multiple options, and are

competing with other conspecifics for the best option. Therefore, a free-choice aviary study would be most likely to determine whether mercury exposure affects patterns of pairing in this gregarious bird.

Conclusion

Females do not appear to use mercury-induced variation in phenotypic traits associated with quality in their mate choice decisions, indicating that sexual selection mechanisms do not currently reflect the variation in traits caused by anthropogenic toxins in the environment. The selective pressure for evolution of such mechanisms will increase, with the increase in mercury contamination and other pollutants in the environment. Currently, however, the absence of a detection method indicates that mercury contamination has the potential to reduce the overall fitness of individual birds in the wild, with no existing mechanism to reduce this impact through avoidance of contaminated mates. We need to know more about how contaminants affect sexual selection processes and their evolution to predict how populations will respond to increasing environmental contamination.

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