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Exposure to dietary mercury alters cognition and behavior of zebra finches

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Abstract

Environmental stressors can negatively affect avian cognitive abilities, potentially reducing fitness, for example by altering response to predators, display to mates, or memory of locations of food. We expand on current knowledge by investigating the effects of dietary mercury, a ubiquitous environmental pollutant and known neurotoxin, on avian cognition. Zebra finches Taeniopygia guttata were dosed for their entire lives with sub-lethal levels of mercury, at the environmentally relevant dose of 1.2 parts per million. In our first study, we compared the dosed birds with controls of the same age using tests of three cognitive abilities: spatial memory, inhibitory control, and color association. In the spatial memory assay, birds were tested on their ability to learn and remember the location of hidden food in their cage. The inhibitory control assay measured their ability to ignore visible but inaccessible food in favor of a learned behavior that provided the same reward. Finally, the color association task tested each bird's ability to associate a specific color with the presence of hidden food. Dietary mercury negatively affected spatial memory ability but not inhibitory control or color association. Our second study focused on three behavioral assays not tied to a specific skill or problem-solving: activity level, neophobia, and social dominance. Zebra finches exposed to dietary mercury throughout their lives were subordinate to, and more active than, control birds. We found no evidence that mercury exposure influenced our metric of neophobia. Together, these results suggest that sub-lethal exposure to environmental mercury selectively harms neurological pathways that control different cognitive abilities, with complex effects on behavior and fitness.

Key words: animal behavior, ecotoxicology, cognition, mercury, spatial memory, zebra finch.

Environmental mercury has been steadily increasing since the beginning of the industrial revolution (Eisler 2006), accumulating in both aquatic and terrestrial systems (Cristol et al. 2008). Deleterious effects of mercury exposure are well documented in humans and wildlife, particularly birds and fish, and include behavioral, neuro-degenerative, and developmental effects (Chang 1977; Tchounwou et al. 2003; Counter and Buchanan 2004). Alternative technologies and regulations on waste disposal have likely reduced the incidence of acute mercury poisoning. However, exposure of wildlife and humans to chronic, sub-lethal levels may be increasing due to

continued use of coal for energy production and unregulated gold mining, combined with the effects of climate change which can influence temperature-dependent toxicity and impose species range shifts that alter species' exposure to mercury (McKinney et al. 2015; Pinkney et al. 2015; Richardson and Friedland 2015).

To better understand the subtle effects of sub-lethal exposure to mercury, we examined cognition and behavior in a model animal species, the zebra finch *Taeniopygia guttata*, after they had been exposed to an environmentally relevant level of mercury. Although mercury is a known neurotoxin there is surprisingly little published

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research on the effects of mercury on aspects of cognition and complex behaviors. Our first study focused on three measures of cognition (i.e., specific skill or problem solving) that have been studied previously in our model system: spatial memory, inhibitory control, and color association. While mercury causes neurodegeneration and alters neurotransmitter function in numerous species (Chang 1977), we are unaware of any previous tests of cognitive ability after experimental mercury exposure in birds. We hypothesized that all of our cognitive assays would be affected by mercury-exposure and our prediction was for a general reduction in some of the cognitive abilities tested, but we had no a priori basis for predicting which abilities would be affected most.

Our second study focused on three behavioral assays not specifically tied to a skill or problem-solving, but well-studied in zebra finches: activity level, neophobia, and social dominance. Several neurotoxins are known to alter the expression of individual behaviors (Walker 2003), but little is known about how mercuryexposure alters behavioral phenotypes. In birds, exposure to mercury has been linked with impaired motor function (Bouton et al. 1999). In humans, mercury-exposure is associated with alterations of emotional state and social tendencies (Kobal Grum et al. 2006) including depression, anxiety, and introversion (Bågedahl-Strindlund et al. 1997), and general restlessness (Michaeli-Yossef et al. 2007; Al-Batanony et al. 2013). Given these patterns, we hypothesized that mercury would influence the general behavioral phenotype of our birds. Further, we predicted that dietary mercury exposure would lead to increased activity (to parallel the restlessness data in humans); increased neophobia (informed by the observations of introversion and anxiety in humans); and decreased social dominance (in association with the decreased sociality and increased depression observed in humans). These behavioral tests correlate somewhat with suites of assays that have been developed to assess overall behavioral syndromes in zebra finches (Schuett and Dall 2009; Schuett et al. 2011), though we did not intend to assess behavioral syndromes in a comprehensive manner in this study. In both experiments, we studied captive adult zebra finches that were fed sub-lethal levels of dietary methylmercury throughout their lifetimes and compared these with the behavior of unexposed control birds.

Materials and Methods

Study species and housing

The zebra finches studied here were raised in captivity from parents that were fed ad libitum commercial pellet diet (fruit blend canary/ finch food, Zupreem, Shawnee Kansas) dosed with 1.2 µg/g methylmercury or the same diet without mercury. This amount is ecologically relevant based on the mercury levels of prey items from an industrially contaminated site in Virginia, as well as other studies globally (Cristol et al. 2008; Varian-Ramos et al. 2014). Birds of both treatments were housed in the same rooms to ensure similar ambient environmental conditions. The two diets were prepared as outlined in Varian-Ramos et al. (2013). Briefly, methylmercury chloride was converted to methylmercury cysteine to better represent the form found in natural food. This was added in aqueous solution to the pellet diet, homogenized thoroughly, and each batch tested to ensure it was within 10% of the nominal dose of 1.2 parts per million total mercury on a wet weight basis. The control diet was prepared in the same way, including addition of aqueous cysteine, and contained no detectable mercury. Cysteine was in a 1:1 ratio with mercury in the mercury-dosed food and the same amount of cysteine was present in the control food.

The parents that produced our experimental birds were paired haphazardly with non-siblings or cousins from a large, outbred domesticated colony and housed in pairs in breeding cages (approximately $0.6 \times 0.4 \times 0.4$ m) with a plastic nest box and ad libitum access to food, vitamin-enriched drinking water, bathing water, cuttlefish calcium supplement, grit, and Timothy grass hay nesting material. These pairs were maintained on a year-round 14:10 light:dark photoperiod with full spectrum lighting and at a room temperature of approximately 21 °C. They produced offspring that were separated from their parents once they reached independence, approximately 50 days after hatching, and it was these offspring that were used in the studies after they reached sexual maturity (>125 days post-hatch). These experimental birds were housed in singlesex group cages of either six individuals (in cages of approximately $0.76 \times 0.46 \times 0.46$ m) or four individuals (in cages of approximately $0.60 \times 0.41 \times 0.41$ m), except as indicated below, and maintained on the same diet as their parents. Hence, all of our mercury birds were exposed throughout their lives, including in the egg.

Throughout maturation and after completion of the study, blood total mercury level measurements were taken on all experimental birds approximately every 2 months to confirm, as in previous studies, that mercury-exposed birds had average (±SE) blood mercury concentration of 17.79 (±0.927) and control-fed birds 0.011 (± 0.002) µg/g. The food (dosed and control) and blood were tested for total mercury levels using a DMA-80 (DMA80 Direct Mercury Analyzer, Milestone, CT, USA). Both food and blood were assayed fresh (i.e., mercury values are not on a dry weight basis). Standard quality control procedures were followed for all analyses, as described previously (Varian-Ramos et al. 2013). Briefly, standard reference samples (DORM-4, DOLT-4) and machine and sample blanks were run with every 20 samples to check calibration and contamination. The instrument was recalibrated every 2 months or as necessary. Duplicate and spiked samples were run throughout the study to verify acceptable repeatability (relative percent differences <10%) and recovery rates (>95%). All procedures were approved by the Institutional Animal Care and Use Committee at the College of William and Mary and follow the same ethical justification as outlined for another study using the same colony and mercury dose (Kobiela et al. 2015).

Study 1: cognition assays

We compared the performance of the mercury-exposed subjects to controls on three assays of cognition: spatial memory, inhibitory control, and color association. All assays were video recorded with a digital video camera system to allow for later extraction of data (Lorex NR800).

Cognition assay 1: spatial memory

Twenty-eight male zebra finches (14 treatment, 14 controls) experienced a test of spatial memory. These birds were housed in large (approximately $2 \times 2.5 \times 2$ m) same-sex outdoor cages for 2 months before trials began and then moved indoors to general housing conditions (see above) for completion of the assay. Bouts of 10 trials per bird commenced at either 0800 or 1530, with no bird experiencing more than one bout of trials per day and treatment groups randomized across time of day. For bouts that started at 0800, birds were food deprived overnight. For bouts that started at 1530, birds were food deprived from 0800 to the start of the bout. Within a bout, each trial lasted 2 min with a 10 min break between each individual's trials. We performed feeding motivation checks after each bout

of trials had concluded, whereby we ensured birds were not satiated. In a motivation check a bird was observed to eat from a food cup within 2 min of presentation. Spatial memory tests were performed from June to July, 2015.

To begin a bout of trials, a bird was moved into an individual experimental cage ($0.6 \times 0.41 \times 0.41$ m) and allowed 10 min to acclimate. Within the experimental cage, we placed wooden blocks (painted uniformly white, $9 \times 9 \times 4$ cm), each with a central cylindrical well (approximately 2 cm diameter) that held 5–10 food pellets for each trial. Each bird progressed through three phases of learning followed by an unreinforced test of spatial memory.

In the first phase of learning, each bird was reinforced to feed from two baited wooden blocks. Initially, a small piece of brown paper (4 cm diameter) was placed adjacent to the food well of each block. Once a bird fed in this condition in three consecutive trials, the brown paper was positioned to half-cover the food well. Once a bird fed from the partially covered well in three consecutive trials, the paper was positioned to completely cover the food well so that the bird needed to remove it to access the food. At any stage, if a bird failed to successfully complete three consecutive trials after 20 trials, it went back to the previous stage of learning. Once a bird could successfully obtain food in five out of six consecutive trials where the food well was covered with brown paper, it progressed to the second phase.

In the second phase, the wooden blocks with covered baited food wells (as described above) were placed in each of the four corners of the experimental cage. If a bird fed from any of the four blocks during a 2 min trial, and did so in 10 cumulative trials (which could happen over more than one day), the bird progressed to the third phase of learning. If a bird failed to feed from any wooden block in 5 consecutive 2 min trials for 2 days in a row, the bird was removed entirely from the experiment.

In the third phase of learning, birds experienced four wooden blocks with covered wells (as in phase 2) but only one of the blocks was baited with food. During phase 2 we had recorded which corner was visited most, and which was avoided most. To determine which corner to bait during phase 3, we randomly selected one of the other two corners. This procedure helped to ensure that the bird was learning a new spatial memory task in phase 3. Once a baited corner was chosen for a bird, the food was always presented in that same location through this third phase of learning. Successful performance in phase 3 was defined as a bird feeding from the baited corner before attempting to feed at any other corner. Each bird progressed to the fourth, and final, phase of the experiment by succeeding in five out of six consecutive trials. A bird was removed from the experiment if it did not reach this criterion within the first 20 trials of this learning stage (2 days).

Phase 4 was an unreinforced test of spatial memory, to ensure that olfactory cues were not being used to locate the reward. The experimental cage was arranged as in phase 3, except that none of the covered wells in the wooden blocks contained food. We recorded whether each bird first went to the block location that had been baited in phase 3 (which was defined as a "pass" of the spatial memory test) or to another block location (which was defined as a "fail" of the spatial memory test).

Cognition assay 2: inhibitory control

Inhibitory control trials were conducted between 0800 and 1200 from June 5 to 22, 2015. A new group of 28 male birds were used for the inhibitory control trials (n = 14 in each group). These birds were exposed to mercury and housed indoors in the same way as

those in the spatial memory trials, and were food-deprived overnight to increase their motivation to seek food. Each trial in the inhibitory control assay lasted 5 min with an additional 5 min interval between trials for the same individual. These methods were adapted from Boogert et al. (2011).

The inhibitory control testing arena was a modified standard housing cage (approximately $0.76 \times 0.46 \times 0.46$ m) that contained a 10 cm diameter, 15 cm long, plastic tube mounted on wood for stability. We placed the tube into the bird's home cage the night before testing so that each bird could acclimate to it.

The training phase of this assay began at 0800 by placing a single bird into the test arena. After a 10 min acclimation period without the tube in the cage, the testing apparatus was introduced to the cage and birds were given 5 min to potentially gain access to the food. The plastic tube was covered with dark fabric, to render it completely opaque, and baited with 5 food pellets from the appropriate treatment. The tube was oriented so that when the bird was sitting on the start perch it could see the opaque sides and not the open ends. Hence, birds needed to explore the tube to find and access the food inside it.

Once a bird accessed the food inside the opaque tube on three consecutive 5 min trials (phase 1), the bird was moved to the second phase. If a bird did not access food in three consecutive trials of phase 1, it experienced a sequence of 5 min trials in which the food was placed just inside the tube so it was more easily visible and then was moved incrementally further inside the tube until, on the fourth of these trials, the food was again in the center of the tube. If the bird ate the food in each trial of this sequence it then repeated phase 1 (i.e., tested to see if it successfully fed in three consecutive 5 min trials).

In phase 2 of this assay we removed the opaque covering from the test apparatus so that the sides of the tube were transparent and food could be seen from the start perch through the sides of the tube. In order to pass a phase 2 trial, each bird had to inhibit their pecking response to the visual cue of food in favor of the previously learned behavior of entering the tube through the side opening. A bird passed a trial if it went directly to the opening of the transparent tube to feed without first pecking at the side—where it could see the food through the wall of the transparent tube. If a bird failed to feed within a 5-min trial or pecked at the transparent side of the apparatus before accessing the food, it was recorded as having failed. We video recorded all phase 2 trials to generate three metrics of performance per bird: latency to approach the transparent apparatus, latency to feeding from inside the apparatus, and the percentage of passed compared with failed trials.

Cognition assay 3: color association

Seventeen of the same male finches as were used in the inhibitory control trials, plus 13 replacement additional males from the same treatments, were tested for their ability to associate a particular color (orange or green) with a food reward. In phase 1 of the color association assay birds learned to remove small brown paper covers from food wells, in a similar manner as was used in the spatial memory assay. Phase 1 trials occurred between October 2015 and February 2016, and commenced at either 1000 or 1500, with 4h of food deprivation preceding every trial. Birds were trained singly, and placed in an experimental arena of the same dimensions as in the inhibitory control assay. After a 5 min acclimation period, we introduced a wooden board (2 cm thick, 22 × 22 cm) containing eight wells (1.6 cm deep × 2.5 cm diameter) drilled around the perimeter with center points 3 cm apart (cf. Boogert et al. 2008).

Four randomly selected wells contained two food pellets each from the appropriate dietary treatment. During the first stage food was visible, then it was partially occluded by a brown paper cover, and in the final stage the food well was completely covered and birds had to remove the paper from each well to access the food. To successfully complete each stage of phase 1, a bird had to feed from three out of four baited wells on three successive trials. Birds that did not feed from any wells were regressed to the previous stage.

Once a bird had learned to remove the paper covers and access food we commenced phase 2, the color association trials. Each bird was randomly assigned to associate food with either green- or orange-colored construction paper, laminated and cut to exactly cover a well. For example, a bird learning to associate orange with food received 10 trials where all baited wells were covered with orange paper and all unbaited wells were covered with green paper. Which wells were baited, and covered with the corresponding color, was determined randomly for each trial. A trial ended once the bird had uncovered all four baited wells or 2 min had elapsed. Phase 2 (color association) trials were video recorded so that we could calculate the number of baited versus unbaited wells accessed in each set of 10 trials. There was no unreinforced test of learning in this assay.

Study 2: behavioral assays

We conducted the behavioral assays between November 2012 and April 2013, studying 24 adult domesticated zebra finches (control: n=6 males and 6 females; mercury: n=6 males and 6 females). All behavioral observations were conducted between 1300 and 1600 in the same room where subjects were housed. The experimental arena was an adapted housing cage (approximately $0.8 \times 0.4 \times 4$ m) and was arranged with three wooden perches spanning the width of the cage. Two perches were 0.25 m from the cage floor while the third was 0.15 m from the floor. Zebra finches tend to compete for access to higher perches and so the difference in perch height was designed to promote agonistic activities and general activity. All trials were video recorded for later analysis.

Behavioral assay 1: activity

In the activity trials we quantified every bird's baseline activity level. Each individual was deprived of food for 1 h prior to observation and no food was available during the trial in the experimental arena. Video recording began when a bird was released into the experimental arena and continued for 10 min. Video recordings were analyzed in 5 s increments during which we noted whether a bird made at least one movement, giving a maximum possible activity score of 120 (i.e., movement in every 5 s interval across the 10 min trial). A movement was defined as a bird moving between perches or the equivalent distance (i.e., distance between perches) across the cage floor at some time during the 5 s interval. Birds were tested in a randomized order and every bird was tested twice with a 1 week interval between trials for each bird.

Behavioral assay 2: neophobia

For each bird, immediately following the activity trial we performed an assessment of the bird's latency to approach a food dish with/ without the simultaneous presentation of a novel stimulus. Similar experiments have assayed neophobia in zebra finches and other songbirds (Boogert et al. 2006; David et al. 2011). Each bird remained in the experimental arena, while we placed a small (control) food dish at one end of the arena and, in half of the trials, simultaneously placed a novel object 0.1 m from the food dish so that the

novel object was between the bird and the food. We used the following novel objects, all of which were of approximately the same size: a plastic water bottle, dog chew toy, soft toy lion, plastic toy horse, soft toy penguin (average size was $0.12 \times 0.09 \times 0.09$ m). We used a large number of novel objects to minimize problems of pseudoreplication (i.e., problems of using just one novel object). All birds experienced the neophobia trial on two separate occasions: once in the presence of a randomly assigned novel object, and once without a novel object (i.e., the food dish alone). The order of presentation of these two trials was randomized across birds. A neophobia trial lasted for 30 min during which we video recorded activity and subsequently extracted the following variables: latency to approach the perch closest to the food dish (s), latency to feed from the dish (s), number of visits to the food dish, and number of visits to the perch closest to the food dish. We calculated metrics of neophobia as the difference in behavior between when the novel object was present minus when the object was not present; hence, every individual was used as its own reference point for assessing neophobia.

Behavioral assay 3: social dominance trials

We assessed apparent social dominance of birds as they competed for access to a limited food resource. The birds were pseudo-randomly sorted into same-sex groups of six, with three control and three mercury-dosed birds in each group. Within these groups, the birds had no prior experience with each other and were not siblings. We applied three colored plastic leg bands in original combinations of yellow, white, and orange to help identify individuals. These colors are considered neutral colors that do not affect behavioral outcomes for zebra finches (Swaddle et al. 2005). Social dominance trials occurred on different days from either the activity or neophobia trials. All birds in a group were food deprived for 1 h in their home cages before each social dominance trial. The experimental arena was arranged as for the activity trials with the addition of a small food dish that contained control food pellets at one end of the cage. A 30-min social dominance trial began when all six birds were released into the experimental arena. We video recorded all interactions and noted the order in which birds fed from the food dish (Boogert et al. 2006; Val-Laillet et al. 2006; David et al. 2011), the number of aggressive interactions initiated by each bird, as well as all displacements between birds. From these latter observations we constructed a matrix of wins and losses and assigned a rank score based on wins minus losses, with lower numbers indicating higher social rank.

Statistical analyses

We performed t-tests of variables to assess whether we could reject null hypotheses associated with assays of cognition (inhibitory control, color association) and behavior (activity, neophobia, and social dominance). For the spatial memory metrics we used nonparametric Mann-Whitney U and Fisher's Exact tests to evaluate null hypotheses associated with the number of trials needed to complete the last phase of spatial learning and the percentage of "correct" responses in the unreinforced spatial memory test, respectively. In each case our null hypotheses presumed there were no systematic behavioral differences between control and mercuryexposed birds. Assumptions of symmetric error distributions and equality of variances were met in all t-tests. In analyses where we could reject null hypotheses, we inspected means, 95% confidence intervals to interpret the direction of behavioral changes induced by mercury. For the behavioral assays, we also explored a multivariate analysis (principal components analysis) that combined all

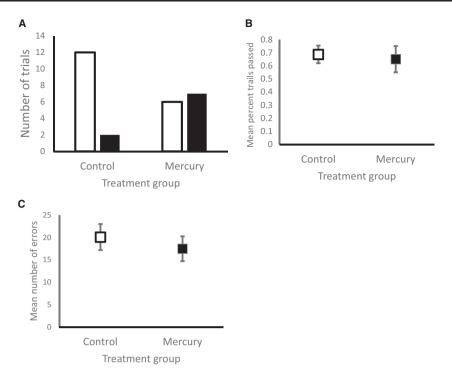


Figure 1. Summary of cognitive assays for zebra finches exposed to mercury and control diets. (A) The number of trials that were passed (hollow bars) or failed (filled bars) by birds in the final spatial memory test. (B) Mean (±95% confidence intervals) percentage of trials passed in the inhibitory control test. (C) Mean (±95% confidence intervals) number of errors made by birds during the color association learning trials.

dependent variables into a smaller number of composite components and compared scores across treatment groups using *t*-tests, and reached qualitatively similar conclusions. As principal components can be somewhat difficult to interpret from a biological point of view, we chose to present the simpler univariate analyses.

Results

Cognition assays

Birds exposed to dietary mercury required more trials than controls to complete phase 3 of the spatial memory test (Mann–Whitney U test, P = 0.021). In the unreinforced test of spatial memory, control birds were more likely to feed from the correct location than the mercury-exposed birds (Fisher's Exact test, P = 0.046; Figure 1A).

We generated three metrics of birds' performance in the inhibitory control test: latency to approach the transparent tube (apparatus), latency to feed from inside the transparent tube, and the percentage of trials passed compared with failed. Control birds did not appear to differ substantially from mercury birds in any of these metrics of inhibitory control (latency to approach apparatus, $t_{26} = 1.15$, P = 0.263; latency to feed from apparatus, $t_{26} = 0.252$, P = 0.803; percentage of trials passed, $t_{26} = 0.601$, P = 0.553; Figure 1B).

As a test of ability to associate a particular color with food reward, we compared the number of errors made by control and mercury birds during the color association learning trials. There was no indication that mercury treatment influenced this metric of cognition ($t_{27.9} = 1.29$, P = 0.207; Figure 1C).

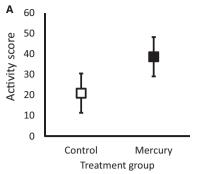
Behavioral assays

Activity scores differed between mercury and control treatments (t_{22} =2.71, P=0.013). Specifically, mercury-exposed birds showed almost twice as much activity as control birds (Figure 2A). The

mercury and control treatments did not appear to differ substantially in our metrics of neophobia (latency to approach feeder, t_{22} =0.324, P=0.749; latency to approach perch closest to feeder, t_{22} =1.89, P=0.072; number of visits to feeder t_{22} =0.133, P=0.895; and number of visits to perch closest to feeder t_{22} =0.377, P=0.710). Social dominance rank differed by mercury-exposure treatment (t_{22} =3.78, P=0.001). Inspection of mean ranks indicated that mercury-exposed birds were consistently subordinate to control birds (Figure 2B).

Discussion

Experimental exposure to dietary mercury, at a concentration similar to what a bird might experience at a heavily contaminated industrial site (Varian-Ramos et al. 2014), influenced aspects of cognition and the general behavioral phenotype of zebra finches. Interestingly, of the three assays of cognition, spatial memory was the only one that was notably affected by mercury exposure, with inhibitory control and association of a color with a food reward being relatively uninfluenced. Although our study was not designed to assess the differences in susceptibility of neural pathways to mercury, the variation we observed in assays of cognition suggest that some parts of the brain may be more affected by dietary mercury than others. There is some precedent for differential mercury accumulation in different brain centers (Choi et al. 1978; Vahter et al. 1995). Specifically, there is evidence that the hippocampus might accumulate mercury at a faster rate than the cerebral cortex (Fujimura et al. 2009) and the brain stem and cerebellum may also be more prone to mercury accumulation (Lapham et al. 1995). As spatial memory is known to be influenced by the hippocampus (Sherry et al. 1992), it makes sense that this aspect of cognition is affected by dietary mercury exposure. Although it is not clear which brain regions influence inhibitory control in birds, the right hemisphere appears to influence this aspect of cognition in



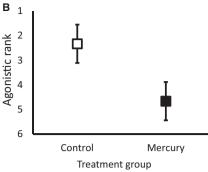


Figure 2. Mean (±95% confidence intervals) of (A) activity score and (B) agonistic rank for zebra finches from control and mercury-exposed treatments.

humans (Garavan et al. 1999). Hence, we further hypothesize that the general cerebral cortex, or at least the right hemisphere, may have lower levels of mercury accumulation than the hippocampus. There is also evidence from birds that learning of a color association activates different brain regions than spatial memory (Hampton and Shettleworth 1996), indicating that the hippocampus would not likely influence our task of color association as much as it influenced spatial memory. This observation is consistent with our emerging hypothesis that the hippocampus is particularly sensitive to dietary mercury exposure, resulting in a decrease in spatial memory ability.

Zebra finches exposed to dietary mercury throughout their lives were subordinate to, and more active than, control birds. In contrast, we had no evidence that mercury exposure influenced our metric of neophobia. Taken together, these systematic behavioral differences support our overall hypotheses and predictions concerning behavioral changes: mercury exposure induces a behavioral phenotype of hyperactivity and decreased social dominance. These changes parallel some observations of humans who have been exposed to environmental mercury—where researchers have reported episodes of depression, anxiety, low sociality, and restlessness (Bågedahl-Strindlund et al. 1997; Kobal Grum et al. 2006; Michaeli-Yossef et al. 2007; Al-Batanony et al. 2013). When we inspected the activity data more closely, we also noted, anecdotally, that mercury-exposed birds typically showed periods of almost complete inactivity followed by episodes of intense movements, further supporting the conclusion of restlessness hyperactivity. Because our activity trials were brief, we recommend a similar study over longer timelines to further explore the temporal nature of this apparent hyperactivity, including whether mercury would affect sleep patterns. We hypothesize that sleep would be disturbed under increasing mercury exposure and that disturbed sleep could lead to a number of physiological and behavioral issues for organisms (Steinmeyer et al. 2013; Lesku and Rattenborg 2014; Raap et al. 2015).

Other than supposition about daily patterns of restlessness and sleep disturbance, our anecdotal observations of behavior during social dominance trials also suggest that general sexual behavior could be altered by mercury exposure. Specifically, we noted multiple attempts by mercury-exposed males to mount other males. We did not observe any male–male mounting by control birds. A similar phenomenon was observed in juvenile male white ibis *Eudocimus albus* who had been exposed to environmentally relevant levels of methylmercury (Frederick and Jayasena 2011). It is also possible that the male–male mounting we observed was a further manifestation of restlessness and hyperactivity, which we feel could be the most parsimonious explanation in this case.

The influence of mercury on activity levels and social dominance, with no clear effect on neophobia, indicates that zebra finches are not cleanly shifting along the "bold-shy" axis of personality that has been

described in this species and others (David et al. 2011; Schuett et al. 2011). Combined with alterations of spatial memory, it appears that exposure to environmentally relevant levels of mercury is selectively altering aspects of zebra finch cognition and behavior. In addition to possible variation in sensitivity of brain centers to mercury exposure, another non-mutually exclusive explanation for the differential effects of mercury on cognition and behavior is that particular receptors may be more disrupted by mercury than others. In particular, there is some evidence that serotonin receptors, which are strongly implicated in spatial memory, are particularly sensitive to mercury contamination (Glikmann-Johnston et al. 2015).

In addition to the data presented here, we know that mercury exposure likely affects aspects of cognition and behavior in this and other birds, including singing and response to simulated predators (Hallinger et al. 2010; Kobiela et al. 2015), suggesting that mercury may be a potent depressor of cognition in wild birds. If avian cognitive ecology is disrupted by mercury, we may see long-term fitness deficits even from short-term exposure to mercury at times of neural development. For example, if spatial memory is particularly affected by mercury exposure then we predict that birds that rely on spatial memory for food caching, locating of nests in crowded colonies, and navigational cues during migration will be relatively more damaged by mercury contamination in the environment. Similarly, if mercury affects the behavior of wild bird species in a similar manner to how dietary mercury exposure affects the general behavioral phenotype of zebra finches, then we expect mercury-exposed birds to be less competitive in social and foraging situations. If these changes occur they could impose substantial fitness costs.

Overall, we found that exposure to dietary mercury, similar to levels found at a highly contaminated field site, decreased cognitive performance in a spatial memory assay and rendered birds to be somewhat hyperactive and subordinate. Taken together, these results indicate substantial sub-lethal behavioral costs to mercury exposure that might be easy to overlook in field assessments. Hence, we recommend field assays of behavioral phenotypes to more fully understand the consequences of mercury exposure.

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