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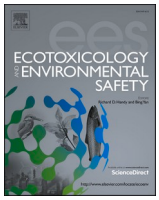

Mercury causes degradation of spatial cognition in a model songbird species

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ABSTRACT

Mercury is a widespread pollutant of increasing global concern that exhibits a broad range of deleterious effects on organisms, including birds. Because the developing brain is well-known to be particularly vulnerable to the neurotoxic insults of mercury, many studies have focused on developmental effects such as on the embryonic brain and resulting behavioral impairment in adults. It is not well understood how the timing of exposure, for example exclusively *in ovo* versus throughout life, influences the impact of mercury. Using dietary exposure to environmentally relevant methylmercury concentrations, we examined the role that timing and duration of exposure play on spatial learning and memory in a model songbird species, the domesticated zebra finch (*Taeniopygia guttata castanotis*). We hypothesized that developmental exposure was both necessary and sufficient to disrupt spatial memory in adult finches. We documented profound disruption of memory for locations of hidden food at two spatial scales, cage- and room-sized enclosures, but found that both developmental and ongoing adult exposure were required to exhibit this behavioral impairment. Methylmercury-exposed birds made more mistakes before mastering the spatial task, because they revisited unrewarded locations repeatedly even after discovering the rewarded location. Contrary to our prediction, hippocampal volume was not affected in birds exposed to methylmercury over their lifetimes. The disruption of spatial cognition that we detected is severe and would likely have implications for survival and reproduction in wild birds; however, it appears that individuals that disperse or migrate from a contaminated site might recover later in life if no longer exposed to the toxicant.

1. Introduction

Mercury is a naturally occurring element released into the global atmosphere both by natural phenomena, such as erosion and volcanism, and by anthropogenic activities, including mining and combustion of fossil fuels (Pacyna et al., 2016; Obrist et al., 2018; Outridge et al., 2018; Edwards et al., 2020). Inorganic mercury can be biomethylated by microorganisms, particularly in moist habitats, to a highly bioavailable form, methylmercury (MeHg). MeHg bioaccumulates and magnifies up trophic levels in the food web, with plants and herbivores having the lowest concentrations and top predators like birds containing the highest concentrations (Ackerman et al., 2016; Knutsen and Varian-Ramos, 2020). MeHg can have adverse effects on the behavior, physiology, and reproductive success of species at higher trophic levels, including birds (Whitney and Cristol, 2017a). While most studies of environmental MeHg have focused on aquatic predators that feed atop piscivorous food webs, MeHg also bioaccumulates in terrestrial songbirds via their consumption of predatory invertebrates, such as spiders (Cristol et al., 2008).

Exposure to widespread pollutants such as MeHg as well as other forms of anthropogenic habitat degradation (e.g. Eeva et al., 2012) is contributing to dramatic population declines of many songbird and other wildlife species (Rosenberg et al., 2019), regardless of feeding guild (Richard et al., 2021). To effectively understand the problem of MeHg pollution, one must understand both the amount of MeHg necessary to cause harm and how the duration or timing of exposure

Abbreviations: MeHg, methylmercury.

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influences deleterious outcomes. Understanding the full effects of MeHg on birds, including effects of non-lethal exposure, may assist efforts to reverse some of these population threats.

Among its effects, MeHg is a noted neurotoxicant, especially during early development of the nervous system (Castoldi et al., 2008). In mammals, MeHg exposure can lead to degradation of cells and morphological changes in the hippocampus (Kakita et al., 2000; Sokolowski et al., 2011), with effects on spatial memory later in life (Falluel-Morel et al., 2007; Wu et al., 2016), including in humans exposed through their occupations (Powell, 2000). In birds, exposure to MeHg affects cognition of captive zebra finches (*Taeniopygia guttata castanotis*) at dietary concentrations similar to highly contaminated industrial sites, causing a decline in performance on a spatial memory assay, but not a comparable assay of memory for non-spatial information (Swaddle et al., 2017). As the hippocampus is a region of the brain important in cognition, including spatial memory in birds (Sherry and MacDougall-Shackleton, 2015) and both field and lab studies have shown that bird species that perform better on tests of recall for spatial locations of hidden food have hippocampal regions that are relatively larger or denser with neurons (Krebs et al., 1996; Cristol et al., 2003), reduced spatial learning and memory reported in MeHg-exposed songbirds (Swaddle et al., 2017) could be due to their hippocampi being smaller or less densely packed with neurons, or altered in other ways. This causal link between mercury, spatial memory and hippocampus has yet to be explored.

The objective of our study was to evaluate the effects of exposure to MeHg on spatial learning and memory of zebra finches in a variety of contexts. We investigated spatial memory at two spatial scales, a room-scale or cage-scale arena, and exposure to MeHg according to three timing regimes, either entire lifetime (including *in ovo*), only during adult life (>150 days old), or only during development (*in ovo* through 50 days old). We hypothesized that 1) exposure to environmentally relevant levels of MeHg would impair performance on tests of spatial recall; 2) developmental exposure (<50 days old) would be necessary and sufficient to cause impairment; and 3) hippocampal volume would be reduced in birds exposed to MeHg.

2. Material and methods

2.1. Study species and husbandry

The zebra finch is a suitable model system for studying the effects of toxicants on spatial memory due to its success in captivity and well-studied neurobiology and behavior (Zann, 1996; Griffith et al., 2017), including links already established in this species between spatial memory and the hippocampus (Watanabe 2004) and MeHg and spatial memory (Swaddle et al., 2017). It should be noted that while zebra finches are granivorous, dietary MeHg accumulates in tissue similarly across avian taxa (Wiener et al., 2002). Exposure in these studies is still broadly relevant to exposure in the wild via arthropods or fish and physiological effects of experimental exposure of granivorous zebra finches to mercury has generally matched results from insectivorous songbirds measured in the field (discussed in Caudill et al., 2015). All finches in these studies were raised in aviaries at William & Mary in Williamsburg, Virginia, USA. Control birds were from lineages never exposed to dietary MeHg (at least since the inception of this colony in 2004). Adult-only exposed birds were also from lineages unexposed to MeHg. In the case of *in ovo* exposure, parents were exposed as adults to induce maternal deposition of MeHg into eggs (Ackerman et al., 2019). To reduce inbreeding, cousins or closely related birds were never paired. Colony reproductive success was comparable to that of other research colonies (Griffith et al., 2017). Experiments were performed at William & Mary except for those in 2018, for which the finches were raised in the same colony but then transported overnight by automobile to the Avian Research Laboratory 2 at Auburn University, Auburn, Alabama, USA for behavioral testing after acclimation.

All birds were fed a pelletized diet (fruitblend for extra small birds, Zupreem, Shawnee, Kansas, USA). Home cages contained ad libitum food, water with mineral/vitamin supplement, oyster shell grit, and cuttlefish for calcium and beak maintenance. Full spectrum indoor lights were on a constant 14:10 light:dark cycle with lights on at 08:00 Eastern Standard Time, and birds housed in groups outdoors were on a natural cycle that was similar. All animal use was performed under protocols approved by IACUC at William & Mary and/or Auburn University between 2009 and 2019.

2.2. Overview of mercury-exposure regimes

We compared the effects of three different combinations of timing and duration of MeHg exposure: 1) exposure starting *in ovo,* via maternal deposition by exposed parent, and continuing via diet through the time of testing until death (hereafter "lifetime" exposure); 2) exposure via diet only after sexual maturity at ~150 days, continuously dosing for at least 3 months prior to testing (hereafter "adult" exposure); and 3) exposure *in ovo*, via maternal deposition, followed by dietary exposure during only the first 50 days after hatching (hereafter "developmental" exposure). Both control and MeHg-exposed diets were prepared by thoroughly mixing food pellets with a solution containing water and cysteine, with MeHg added to the desired concentration for diets of exposed birds (described fully in Varian Ramos et al., 2014). Each batch of MeHg-treated food was tested to ensure that it was within 10% of the nominal concentration, and bird whole blood was sampled for total mercury periodically to ensure against accidental contamination of controls (mercury analysis by atomic absorbance spectroscopy using a direct mercury analyzer (Milestone DMA80, Sorisole, Italy) as described in more detail in Varian Ramos et al., 2014; conservative quality assurance benchmarks were met for recovery and repeatability in all studies).

Birds were fed a continuous diet of 1.2 μg/g MeHg throughout their exposure period (1.0 μg/g in 2010–2011 study only). This level of dietary mercury-exposure was designed to be ecologically relevant to the concentration that wild songbirds experience at mercury-contaminated industrial sites (Cristol et al., 2008; Varian-Ramos et al., 2014; Abeysinghe et al., 2017), and similar dietary exposures in the wild have been associated with altered singing behavior (Hallinger et al., 2010; McKay and Maher, 2012), increased probability of nest abandonment (Barr, 1986; Jackson et al., 2011), and decreased provisioning effort (Merrill et al., 2005). When exposure was *in ovo*, parents were fed a continuous diet of 1.2 μg/g MeHg for at least 3 months so that the contaminant would be deposited into eggs by the female. Resulting adult whole blood concentrations for lifetime and adult-only exposures were approximately 10x the dietary concentrations (Table 1). For the birds exposed only during development there was no more than trace MeHg in whole blood by the time spatial cognition tests were performed on them as adults, as a result of depuration, for example by the liver and through

Table 1
Year, timing of MeHg exposure, spatial scale of memory test, and mean (± SEM) whole blood MeHg at time of testing for zebra finches in each experiment. "Trace" MeHg concentration indicates a mean whole blood total mercury value below minimum laboratory detection level of 0.005–0.01 μg/g.

Experiment	Timing of exposure	Spatial scale	Control: total mercury (μg/g) at time of testing (n)	Exposed: total mercury (μg/g) at time of testing (n)
2010–2011	Lifetime	Room	trace (10)	14.66 ± 2.84 (21)
2010–2011	Adult	Room	trace (8)	11.53 ± 1.94 (8)
2015	Lifetime	Cage	0.09 ± 0.06 (11)	15.14 ± 3.47 (12)
2015–2016	Developmental	Cage	trace (21)	trace (19)
2018	Lifetime	Cage	0.13 ± 0.14 (8)	15.36 ± 3.80 (32)

deposition into new feathers (Whitney and Cristol, 2017b). In all experiments, MeHg-exposed birds were tested alongside a control group that was exposed to the same diet and husbandry but with no intentional exposure to MeHg. At time of testing, all birds were sexually mature (at least 125 days post hatch), and ages were kept as consistent as possible between treatment groups.

2.3. Overview of training and testing for room-scale procedure (2010–2011; Fig. 1)

To determine whether exposure to MeHg affected memory for locations of hidden food at a spatial scale in which birds had to fly between locations and there were many options to remember, we designed a challenging test with 10 available feeders among which to search for the reward. Subjects used in the room-scale experiment were either lifetime exposed, adult exposed, or control (Table 1). In overview, birds located food on the first day by randomly searching feeders and upon finding the reward were tested after a moderate (50 min) retention interval, then again on the same day after an additional short (15 min) retention interval, and finally after a long (48 hr) retention interval. The rewarded location did not move across the three retention periods, so subjects gained additional experience with each of the retests and were expected to have performed better as a result. Latency to perch on the first feeder (motivation check) and number of incorrect feeders visited before relocating the reward were recorded. Training and testing took place between October 2010 and August 2011 (control n = 18, lifetime exposed n = 21, adult exposed n = 8).

Each potential feeder position was assigned a number using a grid of coordinates so that numerous unique feeder arrangements could be generated randomly and each sequential training trial was different. None of the feeder arrangements during training were the same as those in the later memory tests. Experiments were performed in an arena the size of a small room (2.4 L x 1.5 W x 1.8 H m), which contained three fixed spatial cue objects (water dish, grit cup and colored flag), 10 feeders mounted on any of 32 pre-selected positions on the walls and floor, and a central perch. Two visually isolated retention cages (0.4 L x 0.3 W x 0.2 H m) were accessible through remotely operated sliding doors and allowed the observer to introduce birds into, or lure birds out of, the test arena without direct handling by darkening the occupied enclosure and illuminating the desired destination.

Every experimental subject was randomly matched with a companion bird of the same sex to increase the speed with which these highly gregarious birds explored the room. Prior to training, both birds were placed into a similar, adjacent room for two days of acclimation to greater flight space, fewer flockmates, and more dispersed feeders. The companion was not released during later training trials or testing trials but was housed in this adjacent room within acoustic contact to reduce fear in the test subject. Birds were observed by an experimenter through one-way reflective glass.

Each bird was placed into one of the two retention cages positioned on opposite sides of the arena and deprived of food for 2 hrs in order to motivate them to search for food during the trial. The bird was then released from the first retention cage into the arena and observed checking feeders until it located the one feeder of 10 that contained food. The bird was then allowed to eat at this baited feeder for 30 s before room lights were turned off and the light in the other retention cage was turned on to induce the bird to exit the room without finishing the food. The subject was then held in the lighted retention cage without food. During this retention interval, all 10 feeders in the arena were swapped out for identical-looking feeders to remove any cues that were not spatial. The main perch, on which birds landed when entering the arena, was rotated clockwise 90 degrees to prevent “traplining” and any food or feces on the floor was swept away so as not to serve as clues to the rewarded feeder. Following the 50-min retention interval the bird was released back into the lit arena to relocate the baited feeder. Upon locating the baited feeder, the bird was allowed to eat from the baited feeder for 5 min as a reward.

2.3.1. Details of training for room-scale procedure

Training trials occurred every other day for a given individual. Birds were first trained with companions and then trained alone. Initially during training three of the 10 feeders in the arena contained food to increase the bird’s chance of associating a feeder with reward. Each bird received the same one-time arrangements of feeders in the same order. To pass a companion training trial the subject bird had to locate food in one of the three baited feeders within 45 min of initial release, and then after a 50-min retention interval it had to relocate the same baited feeder within 20 min (only one of the three feeders baited in the first step remained baited following the retention interval). After passing two companion training trials, birds graduated to solo training trials, in which only one randomly-selected feeder contained food, and they had to relocate this feeder without their companion within 20 min after the 50-min retention interval. Once two solo training trials were passed on successive training days, memory testing commenced the following day. Using these methods, birds were trained that only one feeder contained food, that the same feeder position always contained the food during both random search and memory test portions of a given day, and that different feeder arrangements on different days signified a change in the rewarded location.

2.3.2. Details of testing for room-scale procedure

For memory tests, a novel arrangement of 10 feeders was selected at random from a pool of options that had never been used in training. Each subject experienced three tests on one feeder arrangement but with different retention intervals. The first day of testing was the same as a solo training trial except for the addition of another, shorter (15 min) retention interval after the food was relocated the first time. When the bird relocated the food after the second, shorter retention interval, it was allowed to eat for 5 min and then was returned to its home cage. After a much longer retention interval (48 hr) it was tested one more time with the same feeder arrangement and food location. During trials, number of feeder visits required to locate the baited feeder and latency to visit the first feeder were recorded by an observer blind to treatment.

2.4. Overview of cage-scale procedure (2015–2018; Fig. 2)

Cage-scale tests were repeated in three different contexts to examine the effects of MeHg on spatial memory at a smaller spatial scale. In overview, experimental trials took place in four phases. Phase 1 was a 3-step shaping phase for the bird to acquire the motor skills for the task. Phase 2 was a bias assessment phase to allow researchers to identify and avoid pre-existing spatial preferences. Phase 3 was a spatial learning task for the bird to learn the constant position of hidden food, and finally, Phase 4 was a test of spatial memory on the learned location.

In June-August 2015, we validated the use of the cage-scale spatial memory test as a substitute for a room-scale test with lifetime exposed male adults (n = 14) housed in a large outdoor aviary (2.5 L x 3 W x 2.2 H m). These lifetime exposed birds were compared to a group of male controls (n = 14) of the same age range housed in the adjacent outdoor cage. Then, from September 2015 to December 2016, we compared a group of developmentally exposed adult male zebra finches (n = 19) to similar-aged controls (n = 21). In 2018, we repeated the cage-scale procedure with lifetime exposed zebra finches (n = 32), and a control group (n = 32) of both sexes, to more closely examine any differences in acquisition and learning process and in hippocampal volume and to test for interactions of treatment and sex at this spatial scale. The reason that females were added to the study in 2018 is because we were going to examine the brain for the first time and expected that sex differences in the hippocampus or reference areas might be important. These birds lived indoors in single-sex cages (0.6 L x 0.4 W x 0.4 H m) in groups of four to six, which was the same type of cage used during training and testing in 2018. Birds lived in their test cage, with the same neighbors

and visual cues around the room, for the entire period they were being trained and tested.

Before each batch of cage-scale trials began we moved equal numbers of birds from each treatment group into their test cages (same dimensions and layout as home cages) where they would live alone, but in visual and auditory proximity to others, for one week. To prevent visual distractions, an opaque divider was placed between cages during trials. This divider was removed after completion of trials each day to allow these gregarious birds to see one another when not being trained or tested. As much as possible, observers were blind to treatment. Before trials, we deprived birds of food to motivate them to find the reward. Deprivation lasted either overnight for trials beginning at approximately 08:00 the following morning, or from 09:00–15:00 for trials run at 15:00 (first 2015 experiment only). Each trial lasted 2 min, there was an interval of approximately 10 min between consecutive trials of a given individual, and no bird experienced more than 10 trials per day. Motivation checks were performed after each day of testing or after a bird did not feed for five consecutive trials. The motivation check consisted of returning each bird's food dish to its cage and observing whether it fed within 2 min. If the bird fed within 2 min it "passed" the motivation check; conversely, if the bird did not feed within the 2 min, which was extremely rare, it "failed" the motivation check and data from that bird on that day were discounted. If a bird failed five consecutive trials two days in a row, the bird was retrained more than a month later or, in the rare case it had already failed and been retrained (control n = 3, MeHg-exposed n = 4), the bird was removed from the experiment.

2.4.1. Phase 1 of cage-scale procedure

Initially in the cage-scale procedure, the birds progressed through Phase 1, a three-step phase designed to acclimate and train them to remove paper covers from and feed from white-painted wooden feeders (0.09 ×0.09 ×0.04 m), each having a central cylindrical well holding a few ZuPreem food pellets during food-baited trials. In step one of Phase 1, we placed a single feeder in the middle of the cage and a paper disc adjacent to the food well so that food was visible and birds could familiarize themselves with the feeder and paper. In step two of Phase 1, we covered half of each food well with a paper disc. A bird progressed through steps one or two after successfully feeding from the feeder in three consecutive trials. If a bird progressed out of step one, but failed to feed on three consecutive trials of step two, it was sent back to step one for a second attempt. In step three of Phase 1, we entirely covered the food wells with the paper discs, such that a bird had to move the paper with its bill to see and then eat the food within the well. A bird progressed out of step three and the entire Phase 1 shaping procedure after successfully feeding in five of six consecutive trials in this step. We considered Phase 1 trials to be consecutive even if trials occurred over two sequential days (i.e., the last trial of day one could be consecutive with the first trial of day two).

2.4.2. Phase 2 of cage-scale procedure

Phase 2 was designed to identify whether birds displayed any preferences in selection of feeding locations, so that favored or disfavored locations could be avoided during Phases 3 and 4. We presented each bird with four baited feeders, placed in each of the four corners of the cage, and we covered each of the four food wells with a paper disc. We deemed a 2 min trial a "pass" if the bird fed from any of the four feeders. When a bird accumulated 10 cumulative passes (on the same day or across two days), it progressed to Phase 3. As in the other phases, the bird was already housed in the test cage so that its initial position was on a self-selected perch near the top of the cage.

2.4.3. Phase 3 of cage-scale procedure

The goal of Phase 3 was for the bird to learn which one corner had the food reward. We arranged the experimental cage as in Phase 2, except we placed food in only one of the four feeders rather than in all four. To account for any biases towards feeding locations that we had observed during Phase 2, we did not bait the corners the bird had visited the most or the least times. We flipped a coin to select which of the remaining two corners would be baited in Phase 3. Once we determined the location of the baited corner for an individual bird, that location remained constant throughout Phases 3 and 4. Hence, we reinforced each bird to feed from just one location, but the location of the baited corner differed among birds. We deemed a trial a "pass" if the bird mounted and pulled the cover off the baited feeder before any unbaited feeder. A bird progressed to the final spatial memory test by passing five out of six consecutive Phase 3 trials on the same day. Birds were given up to 30 trials to pass Phase 3 (except in the first 2015 experiment, when birds were given as many trials as needed to pass).

2.4.4. Phase 4 of cage-scale procedure

Finally, in Phase 4 we tested birds in one non-reinforced spatial memory trial occurring 10 min after they graduated from Phase 3. We arranged the experimental cage as in Phase 3, except that we baited none of the feeders to prevent any use of odor to find food. If a bird first mounted the feeder which had previously been baited in Phase 3, we deemed the test a "pass." If the bird approached another feeder or failed to touch any feeder, we deemed the test a "fail."

2.5. Brain volume analysis

In 2018, 5–7 days after finishing behavioral trials, lifetime exposed and control birds were humanely euthanized via inhalation of isoflurane, and immediately perfused with heparinized 0.1 M phosphate-buffered saline (PBS; pH 7.4; 5000 IU/mL) followed by 4% buffered paraformaldehyde. Brains were removed gently from the skull and stored in 4% buffered paraformaldehyde for 24 h following perfusion, then moved to 30% sucrose solution for 3–6 days, and quickly frozen on crushed dry ice and stored in − 80 ℃. We sectioned the brains of all birds that participated in Phase 3 (n = 46) coronally into 30 μm sections and stored them in cryoprotectant. For Nissl staining, the sections were mounted on slides, stained with 1% thionin solution, serially dehydrated through graded alcohols, placed in Neo-Clear (Merck KGaA, Darmstadt, Germany), and coverslipped using permount (Fisher Scientific Company, Fair Lawn, New Jersey, USA). The hippocampus proper and telencephalon areas were measured in every fourth section (120 μm between samples) in accord with stereotaxic axes described in Nixdorf-Bergweiler and Bischof (2007). For males, telencephalon area was measured from scanned slides using ImageJ, and hippocampus area was measured with a Nikon Ni-E motorized microscope. Song nuclei area in males was also measured according to these methods as reference regions to test for systemic effects of MeHg on songbird brain volume. For females, area of telencephalon and hippocampus was measured using the polygon tool in QuPath on images of slides scanned at 10x on an Olympus VS200 Slide Scanner. Area was then converted to volume using the cone frustum equation (Smith et al., 1995). Sectioning, mounting, and measuring were performed blind with respect to treatment. For each sex, area measurement was conducted by a single observer.

2.6. Statistical analysis

All analyses were performed and graphical figures created in R (version 4.2.2, packages: lme4, lmerTest, ggplot2; Bates et al., 2015, Kuznetsova et al., 2017, Wickham, 2016). We used generalized linear models with treatment as a fixed variable to analyze the effect of MeHg on spatial cognition in both room-scale and cage-scale experiments. The same behavioral variables were used across data sets, where comparable data existed. Counts (number of trials required or failed in acquisition of cognitive tasks) were analyzed using a quasi-Poisson distribution to account for overdispersion of the residuals. Likelihood of passing or failing a spatial memory test was examined via binomial logistic regression. For experiments in which both sexes were used, we initially included sex in models, but because sex did not significantly improve

model fit ($P > 0.05$), this variable was dropped.

In order to better understand whether MeHg was causing birds to fixate on unrewarded feeders, in 2018 cage-scale experiment, we recorded which unbaited feeders birds visited after locating the baited feeder in Phase 3. The proportion of number of erroneous visits after locating the baited feeder out of total visits to reach criterion was analyzed with a binomial logistic regression.

To determine if motivation differed between groups, latency to feed was analyzed via linear regression with treatment as a fixed variable. In room-scale experiments we examined the total latency time between opening the retention cage and the bird's first contact with any feeder, summed across the entire testing sequence. In cage-scale experiments we examined the average time during Phase 3 from start of trial to mounting the first feeder. We also examined if impaired motor responses could account for differences in performance between groups by analyzing the number of trials a subject required to graduate to Phase 2 of the cage-scale experiments, which is when the bird needed to use a pecking motion to remove a paper cover and no spatial cognition was involved. Number of trials for this portion was analyzed using quasi-Poisson distribution with treatment as a fixed effect.

For brain volume analyses we first used a t-test to compare volumes of the left and right hemispheres of the brain. On average, they did not differ ($P = 0.609$), so the volumes of left and right hemispheres for each individual were summed and treated as one variable. Linear models were used to test for treatment effects on telencephalon volume (the larger area of the brain within which the hippocampus is contained). Likewise, we used linear models to test for treatment effects on hippocampus volume, using telencephalon volume as a covariate to account for overall brain size. Subject body mass and age at death were initially included as covariates but were removed as neither significantly explained brain volume ($P > 0.15$).

2.7. Results and discussion

2.7.1. Does exposure to MeHg cause impairment of spatial memory?

2.7.1.1. Room-scale spatial memory. To determine whether exposure to MeHg, over a lifetime or only during adult life, reduced spatial memory for locations of food, we carried out a room-scale experiment in 2010–2011. During the initial random-searching portion of the test, in which each bird had to identify one baited feeder among 10, performance did not differ between treatment groups ($P > 0.5$, Table 2, Fig. 1). This was expected because any MeHg-related differences in spatial cognition would not be reflected during this random-searching portion of the test. After the first retention interval (50 min), lifetime MeHg-exposed birds performed significantly worse than controls when trying to relocate the baited feeder, visiting 1.72x (i.e., 53%) more feeders as controls to relocate the food ($P = 0.007$, Table 2, Fig. 3). After a second retention interval (15 more min), lifetime MeHg-exposed birds continued to perform significantly worse than controls, requiring 1.61x (i.e., 46%) more feeder visits to relocate the food ($P < 0.03$, Table 2, Fig. 1). Two days after initially finding the baited feeder (a 48 hr retention interval spent in their home cage with familiar cagemates), lifetime MeHg-exposed birds again performed worse than controls, requiring 2.52x (i.e., 87%) more feeder visits as controls to find the food ($P < 0.0001$, Table 2, Fig. 3). Adult-exposed subjects tended not to relocate rewarded feeders as well as controls, but the difference was not statistically supported after any retention interval ($P > 0.1$ all retention intervals, Table 2, Fig. 1). Further, we observed a statistically significant interaction between treatment and retention intervals ($P < 0.05$, see supplemental Fig. S1 to compare mean group performance) such that control and adult-exposed birds on average decreased in number of visits to feeders in each retention interval compared to the random search phase ($P < 0.05$ all retention intervals). At the same time, finches exposed to MeHg their entire lives on average decreased visits to feeders in the first and second retention intervals compared to the random search phase ($P < 0.05$) but in the final retention interval visited the same amount of feeders as during the random search phase ($P = 0.860$).

Table 2
Statistical comparisons of behavioral responses of zebra finches during room-scale spatial memory tests in 2010–2011. Number of feeders visited were analyzed using quasi-Poisson distribution specified generalized linear models, and total latency was analyzed using linear models. Asterisks indicate level of statistical significance in comparison with control treatment (*<0.05, **<0.01, ***<0.001).

Response (*df*)	Mean ± SE (*test stat*)
Number of feeders visited during initial random searching (*44*)	Control = 5.4 ± 1.2 Adult MeHg = 5.1 ± 1.3 (*-0.226*) Lifetime MeHg = 6.1 ± 1.2 (*0.536*)
Number of feeders visited after 50-min retention interval (*44*)	Control = 2.4 ± 1.2 Adult MeHg = 3.6 ± 1.3 (*1.624*) Lifetime MeHg = 4.2 ± 1.2 * * (*2.855*)
Number of feeders visited after additional 15- min retention interval (*44*)	Control = 2.2 ± 1.2 Adult MeHg = 3.2 ± 1.2 (*1.463*) Lifetime MeHg = 3.6 ± 1.2 * (*2.319*)
Number of feeders visited after 48 hr retention interval (*44*)	Control = 2.3 ± 1.2 Adult MeHg = 2.6 ± 1.3 (*0.399*) Lifetime MeHg = 5.9 ± 1.2 * ** (*4.635*)
Total latency (s) between release and landing on first feeder (*42*)	Control = 2926.5 ± 709.5 Adult MeHg = 4055.3 ± 1279.1 (*0.882*) Lifetime MeHg = 1113.8 ± 990.1 (*-1.831*)

To determine whether the observed difference between groups was the result of a difference in motivation to search for food we compared the total latency between leaving the retention cage and landing on any feeder, summed across all of the trials. Total latency did not differ significantly between treatment groups ($P > 0.07$, Table 2), but lifetime exposed birds began to search for food in 0.38x (i.e., 90%) less total time than controls across all trials. Because lifetime exposed birds were not slower (and in fact tended to be faster) to look for food there is no indication that their reduced spatial memory performance was due to reduced motivation.

2.7.1.2. Cage-scale spatial memory. In order to provide easier replication and finer resolution of performance and to facilitate comparison of our findings to the existing literature on spatial memory in zebra finches, as well as that of MeHg in rodents, we adapted our experiment to a smaller spatial scale (Hodgson et al., 2007). First, in 2015, we tested lifetime MeHg-exposed birds and controls on their ability to remember which corner of their cage held the food. These lifetime-exposed birds required 1.66x (i.e., 66%) more trials to learn where the food was ($P = 0.014$, Phase 3 total trials, Table 3, Fig. 4a), and were significantly less likely to pass the final single-trial spatial memory test ($P = 0.039$). This was consistent with the result of our 2010–2011 room-scale study, which showed lifetime MeHg exposure significantly reduced the ability of zebra finches to remember where they have previously found a food reward. The effect size of MeHg exposure on spatial memory was smaller at the reduced spatial scale of the 2015 room-scale experiment, perhaps because the spatial memory test was easier, for example involving only one-third the number of unbaited feeders and reduced retention interval.

To examine the possible mechanism underlying reduced spatial cognition in lifetime MeHg-treated birds, we repeated the study in 2018 but focused particularly on mechanics of acquisition of spatial memory,

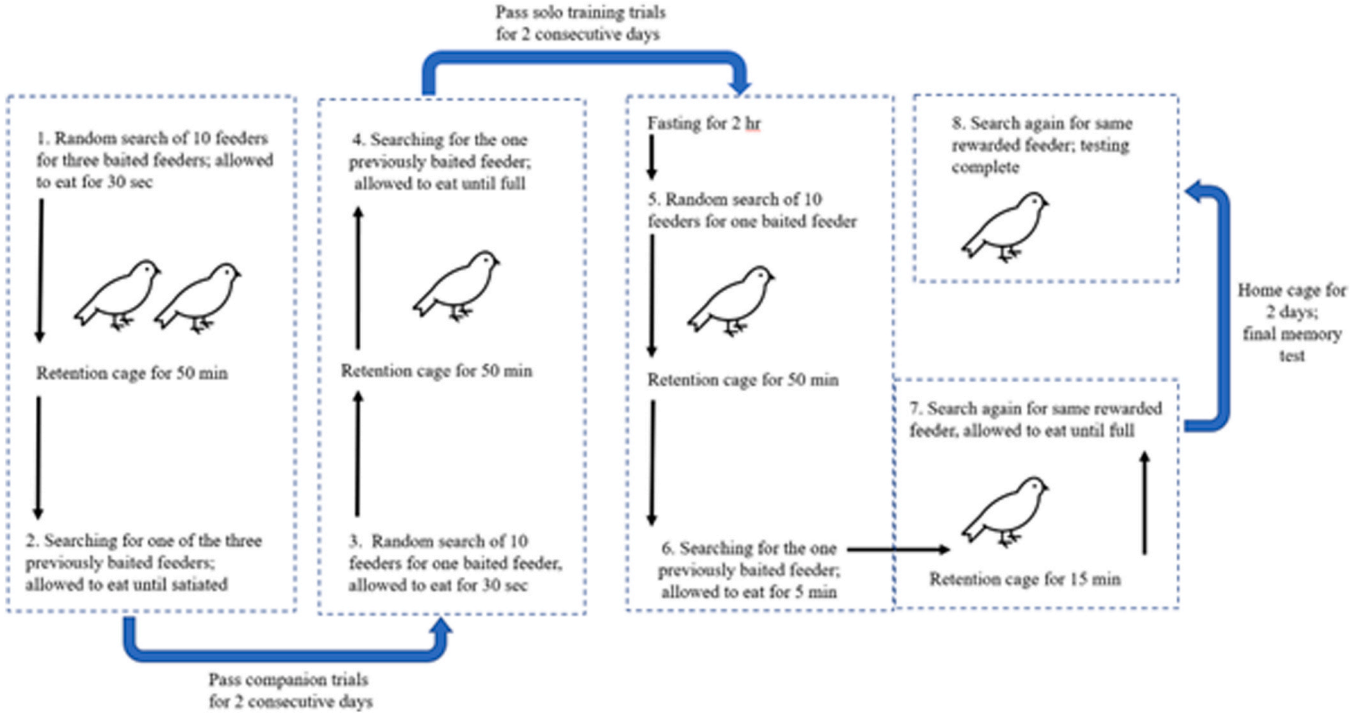

Fig. 1. Zebra finches progressed through the room-scale cognition trials from training to memory test as depicted in the flow chart. Birds began with a training partner, depicted as two bird images, then completed the memory task alone, depicted as one bird.

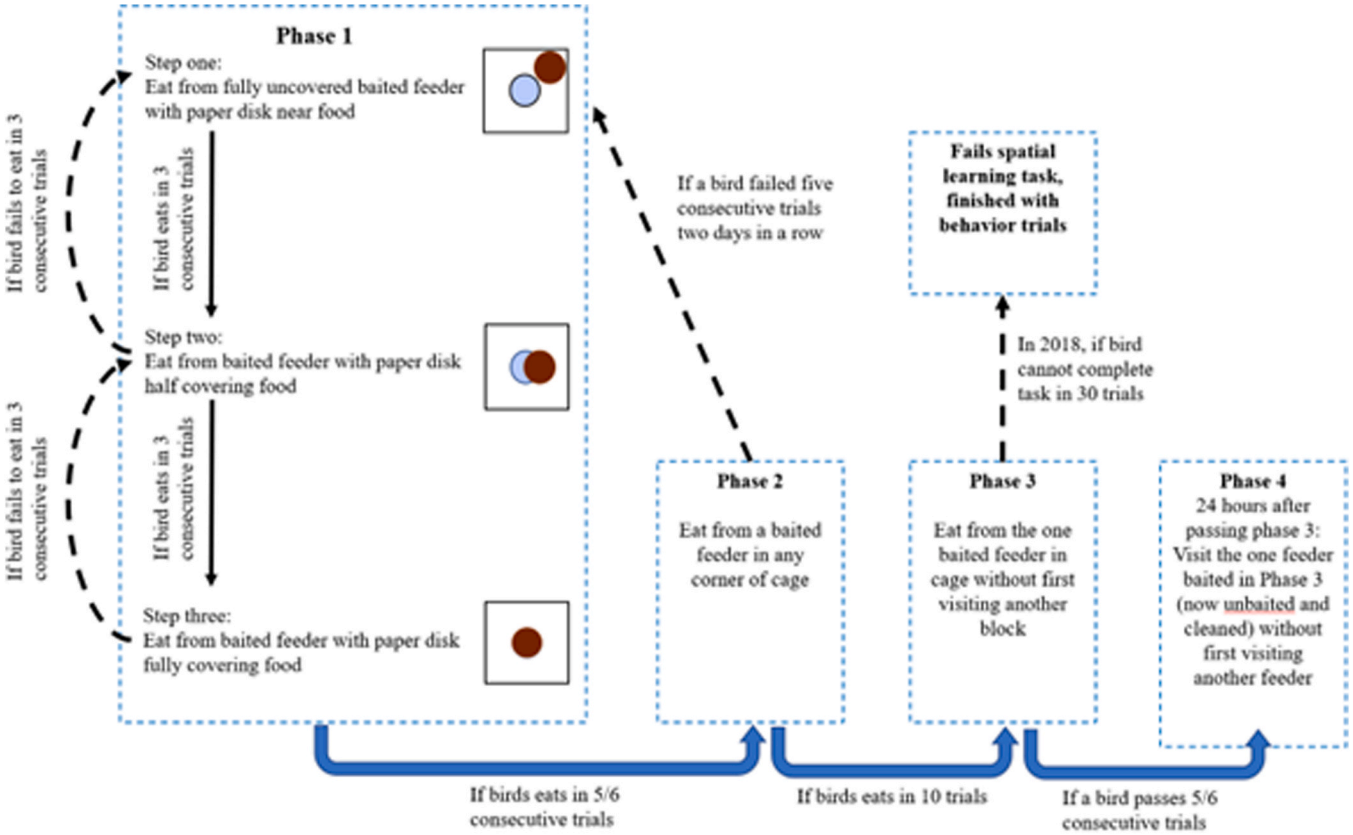

Fig. 2. In the cage-scale experiments, birds progressed through four phases to assess their spatial learning and memory. The feeders are depicted as white squares. The red circle in the feeder represents a cover for the food well while a blue circle indicates a baited, uncovered food well.

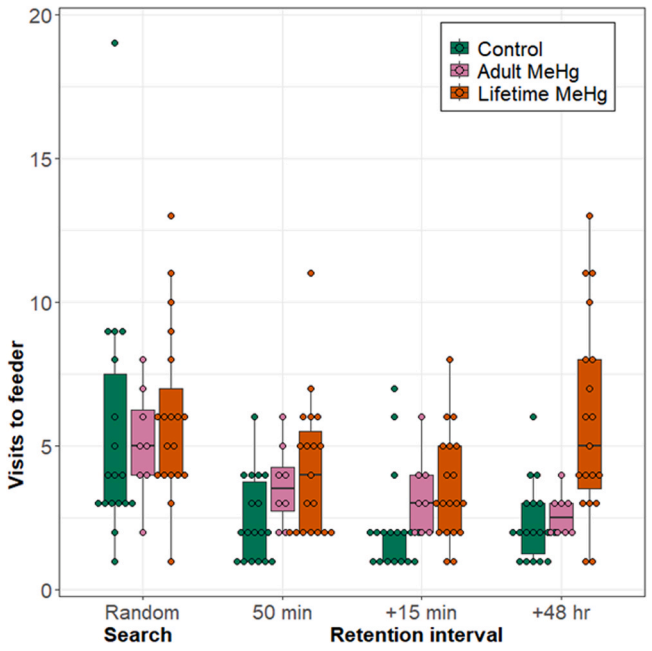

Fig. 3. In room-scale spatial memory tests adult-MeHg-exposed zebra finches (pink) were not significantly worse than controls (green) in total number of feeders visited to relocate hidden food, regardless of retention interval ($P > 0.05$). Lifetime exposed finches (orange) on average visited the same number of feeders during the random search phase but significantly more incorrect feeders than controls at all three retention intervals (X-axis legend: Random Search = non-spatial memory task to identify baited feeder, 50 min = 50 min-retention interval after Random Search, +15 min = 15 min additional retention interval after baited feeder is relocated, +48 hr = searching for same feeder 48 h later; $P < 0.05$).

which occurs during Phase 3 of the cage-scale procedure. Lifetime MeHg-exposed birds required 1.39x (i.e., 32%) more visits to feeders to graduate from this phase. In other words, lifetime MeHg-exposed birds required significantly more repetition to learn where the food was located than did control birds ($P = 0.01$, Table 3, Fig. 4c). Specifically, these birds were more likely to erroneously return to an unbaited feeder after discovering and eating from the baited feeder, thereby extending the number of trials required to graduate from this Phase 3. This difference between groups was statistically significant, such that MeHg-exposed birds were 1.37x (i.e., 32%) more likely to return to unbaited feeders after locating the baited feeder ($P = 0.022$, Table 3).

To determine whether differences in learning in the 2018 study were related to indirect effects of MeHg, such as on hunger or coordination, we compared several measures of performance that do not require spatial cognition. First, we compared number of trials required to learn the cover-removal task by each treatment group in the third step of Phase 1. There was no difference between lifetime exposed and control birds in the number of trials required to learn this task, which entails associative learning and motor skills but no spatial memory ($P = 0.840$, Table 3). To examine whether there were differences in motivation, we compared latency to mount the first feeder in Phase 3 (whether baited or not) and detected no significant difference ($P = 0.900$, Table 3), indicating that MeHg-exposed and control birds had a similar level of motivation to search for food (<10% mean difference). There were multiple points during the experiment when a bird could fail to meet criterion and be removed from further study (17 birds out of 64 failed out), yet there was no difference between treatment groups in the likelihood of failing out ($P = 0.78$).

In summary, MeHg causes impairment of spatial memory in that we found significant decreases in spatial cognitive abilities in finches exposed to MeHg their entire lives. This could be due to effects on hippocampal processes (Falluel-Morel et al., 2007; Wu et al., 2016) or, because our results indicate increased fixation behavior, alterations in reward processing (Newland et al., 2015), as discussed further.

Table 3
Statistical comparisons of spatial learning and memory tests using cage-scale arenas in zebra finches in 2015–2018. Total trials to pass a phase were analyzed using quasi-Poisson distribution-specified generalized linear models, proportion of trials returning to unrewarding feeders after locating rewarding feeder in Phase 3 was analyzed using a binomial-specified generalized linear model, and total latency was analyzed using linear models. Asterisks indicate level of statistical significance in comparison with control treatment (*<0.05, **<0.01). Results are presented with each experimental year analyzed separately. To see analysis of groups across experiment years, see supplemental Fig. S2.

Cage-scale tests	Response (*df*)	Mean ± SEM (*t-value*)
Lifetime MeHg exposure (2015)	Total trials required to pass Phase 1 (*25*)	C = 17.2 ± 1.1 MeHg = 18.3 ± 1.1 (*0.52*)
	Total trials required to pass Phase 2 (*25*)	C = 10.4 ± 1.0 MeHg = 12.3 ± 1.1 * (*3.05*)
	Total trials required to pass Phase 3 (*26*)	C = 19.6 ± 1.2 MeHg = 32.5 ± 1.2 * (*2.634*)
	Log odds ratio for pass/fail Phase 4 (*26*)	MeHg 7.0 ± 2.8 times more likely to fail * (*-2.059*)
Developmental MeHg exposure (2015–2016)	Total trials required to pass all three steps of Phase 1 (*36*)	C = 16.2 ± 1.1 MeHg = 16.8 ± 1.1 (*0.559*)
	Total trials required to pass cover removal (step three) of Phase 1 (*33*)	C = 5.8 ± 1.1 MeHg = 5.8 ± 1.1 (*0.122*)
	Total trials required to pass Phase 2 (*36*)	C = 10.2 ± 1.0 MeHg = 10.7 ± 1.0 (*1.064*)
	Total trials required to pass Phase 3 (*32*)	C = 12.8 ± 1.1 MeHg = 13.9 ± 1.1 (*0.686*)
	Log odds ratio for pass/fail Phase 4 (*32*)	MeHg 3.8 ± 3.4 times more likely to fail (*-1.076*)
Lifetime MeHg exposure (2018)	Total trials required to pass Phase 1 (*42*)	C = 16.9 ± 1.1 MeHg = 16.8 ± 1.1 (*-0.076*)
	Total trials required to pass cover removal (step three) of Phase 1 (*42*)	C = 6.7 ± 1.1 MeHg = 6.9 ± 1.2 (*0.203*)
	Total trials required to pass Phase 2 (*49*)	C = 11.4 ± 1.1 MeHg = 10.3 ± 1.1 (*0.559*)
	Total trials required to pass Phase 3 (*41*)	C = 8.9 ± 1.1 MeHg = 12.5 ± 1.2 * * (*3.245*)
	Proportion of trials returning to unbaited feeder in Phase 3 (*44*)	C = 0.27 ± 0.53 MeHg = 0.36 ± 0.55 * (*2.295*)
	Latency (s) to visit first food feeder in Phase 3 (*47*)	C = 41.6 ± 20.9 MeHg = 45.4 ± 29.8 (*0.127*)

2.7.2. Is the effect of MeHg on spatial cognition dependent on timing of exposure?

In the room-scale test of spatial memory (2010–2011), the effects seen among lifetime-exposed birds were not evident among birds exposed only as adults. Adult-only exposed birds did not significantly differ from controls in the number of feeders visited when initially searching for the rewarded location ($P = 0.46$), after the 50-min retention interval ($P = 0.73$), after an additional 15 min-retention interval ($P > 0.99$), or after two additional days in the home cage ($P = 0.39$; Fig. 3). As the results of both room-scale tests were analyzed together (they were carried out in sequence without a break), we tested for an

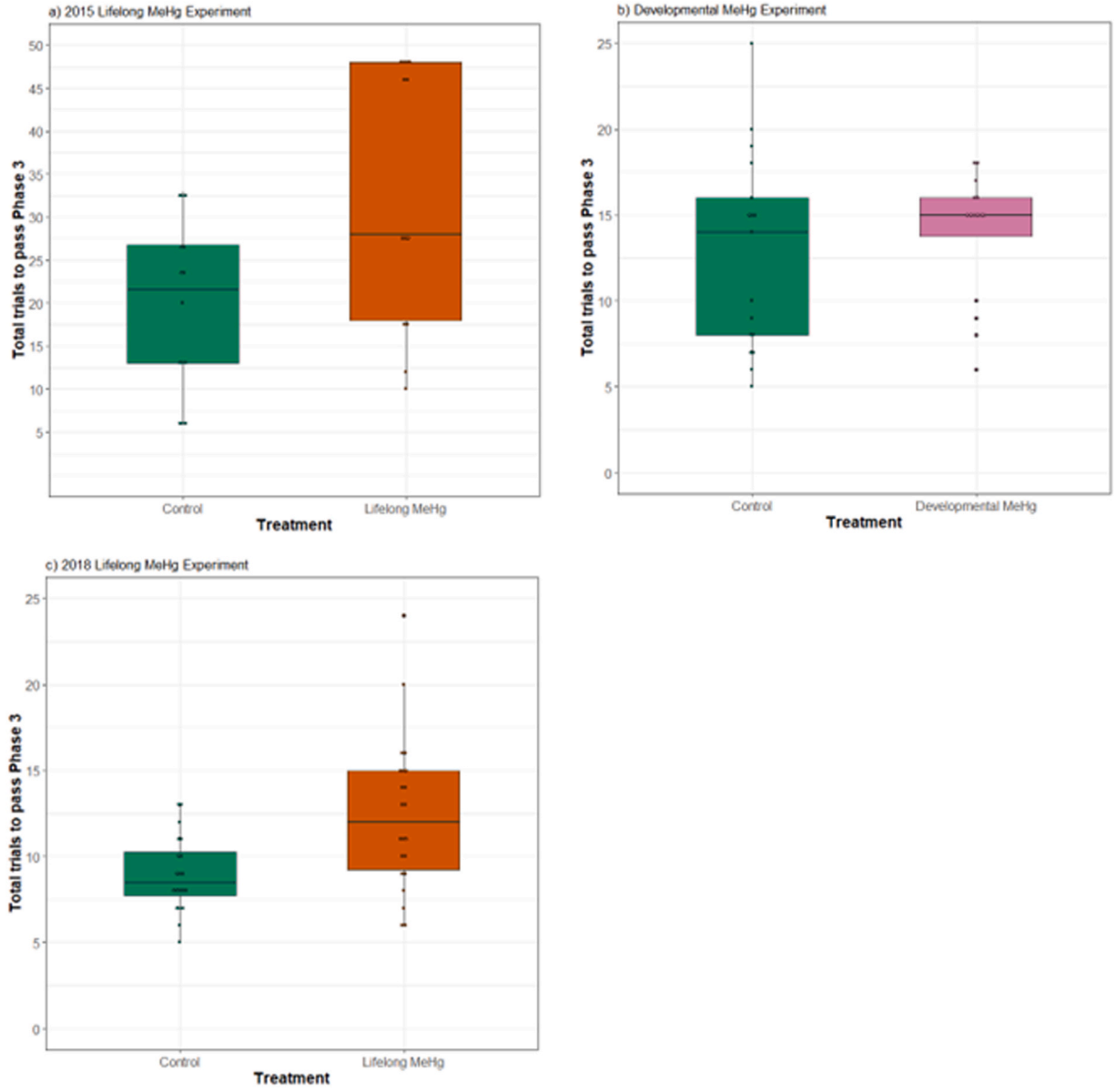

Fig. 4. Number of trials required to pass Phase 3 of cage-scale spatial memory experiments for a) birds exposed to MeHg for their lifetime (2015, orange), b) birds exposed to MeHg only during development (2015–2016, pink), and c) lifetime-exposed birds (2018, orange). More trials indicates slower acquisition of the memory task.

interaction between MeHg treatment and timing of exposure. We found significant interactions between treatment and timing of exposure such that the negative effect of MeHg on spatial memory performance was dependent on timing of exposure, whether after the 50-min retention interval ($P = 0.028$), the additional 15-min retention interval ($P = 0.014$), or the 48-hour retention interval ($P = 0.002$). These results confirmed that MeHg exposure throughout finches lives is necessary to elicit the detrimental effect on spatial memory. To determine whether developmental exposure by itself was sufficient to cause deficits in spatial cognition, we exposed parents to MeHg in 2016 so that their offspring would be exposed from conception through independence (Day 50), and then ended MeHg exposure for the rest of their lives. We tested these birds as adults with only traces of mercury in their blood on a cage-scale test. There was no difference between developmentally MeHg-exposed birds and controls when learning the food location during Phase 3 ($P = 0.428$; Fig. 4b) or in likelihood of passing the single trial spatial memory test with the feeders unbaited to eliminate scent cues ($P = 0.309$).

Thus, we found no evidence that exposure to MeHg during early life left residual deleterious effects on adult spatial memory, contrary to our hypothesis. It appears that both early and ongoing MeHg exposure are necessary to produce effects on spatial memory that are detectable on the relatively small spatial scales we examined. This was especially surprising given effects of developmental MeHg exposure on spatial cognition in rodent models (Falluel-Morel et al., 2007; Wu et al., 2016). Birds are more visually oriented than rodents, and rodent studies tend to inject MeHg rather than administering via diet, so differences could be due to these differences across taxa and/or due to differences in toxicant administration.

2.7.3. Is the effect of MeHg on spatial cognition due to smaller hippocampal volume?

In the 2018 cage-scale study, birds exposed to lifetime MeHg did not differ from control birds in telencephalon volume or hippocampus volume (average telencephalon volume control males = 285.78 mm^3, MeHg exposed males = 290.49 mm^3, control females = 223.31 mm^3,

MeHg exposed females = 205.71 mm^3, $P = 0.614$; average hippocampus volume control males = 60.92 mm^3, MeHg exposed males = 68.18 mm^3, control females = 41.73 mm^3, MeHg exposed females = 39.93 mm^3, $P = 0.237$). There was a significant difference between sexes in the telencephalon such that on average male telencephalon volume was 73.54 mm^3 larger than that of female telencephalon volume ($p < <0.001$). Thus, it appears that differences in spatial learning that resulted from MeHg exposure in this study were not due to gross morphological changes in the volume of the hippocampus, although this does not rule out a role for the hippocampus in mediating the effects of MeHg in spatial memory, for example changes in neuronal migration or connectivity. Similarly, we observed no significant effects of MeHg on volumes of the song nuclei (supplemental Table S1). It is possible that the mechanisms underlying the impairments in spatial cognition that we detected do involve the hippocampus and other brain regions associated with these behaviors in ways that do not affect volume (e.g. Behzadfar et al., 2020). Future work in birds and other organisms should examine neurotransmitter release and reuptake, rates of apoptosis and cell migration, and other cellular and molecular markers of neurotoxicity in the hippocampus and the telencephalon broadly (Bottini and MacDougall-Shackleton, 2023).

3. Conclusions

The combined effects of degradation of habitats and global change are placing greater strain on organisms such as songbirds, requiring more behavioral flexibility to survive (Hooper et al., 2013; Moe et al., 2013). At the same time, as demonstrated by the present study, MeHg is altering spatial cognition in songbirds, reducing their abilities to remember where they have previously located food or other rewarding stimuli, leading to wasted effort searching repeatedly at unrewarded locations. Both mechanisms are consistent with the demonstrated effect of MeHg on behavior in primates and rodents (Newland et al., 2015). This cognitive deficit occurred in zebra finches at both small (0.25 m^2) and moderate (6.5 m^3) spatial scales and was robust across multiple studies. We suggest that the resulting behavioral rigidity and inability to recall and relocate important resources could have profound conservation implications if it is occurring in populations of wild animals exposed to environmental mercury pollution. Specifically, the effects of mercury on spatial cognition could include reduced ability to relocate food sources, areas of high predation risk, potential mates and rivals, nests, or habitat patches previously visited for breeding, wintering, or migration.

The lingering effects of exposure to mercury during development have been demonstrated before across a wide range of organisms, including reproductive success in zebra finches (Paris et al., 2018), auditory and visual function in nonhuman primates (Rice, 1998; Rice and Hayward, 1999), perseveration in rodents (Newland and Rasmussen, 2000; Paletz et al., 2007), and quality-of-life-related aging processes of humans born in Minamata, Japan (Kinjo et al., 1993). However, neither developmental nor adult exposure by themselves affected performance on the present spatial memory tests. This finding indicates that in terms of spatial cognition, birds appear to recover from the effects of mercury exposure, at the environmentally relevant concentration we tested, early in life and do not experience a spatial memory deficit when exposure is limited to a portion of their adult lives. It should be noted that the lack of detection of effects of developmental-only or adult-only exposure to MeHg may be the result of limited sample size and a small number of cognitive endpoints, and the effects of both exposures should be investigated further. The effect of MeHg on memory that we found in lifetime-exposed birds may have been fatal outside of captivity due to the need to accurately learn and recall spatial information about resources and threats. Nonetheless, it is encouraging that shorter exposure, even during development, may not produce the same effect. Birds that migrate into and out of contaminated habitats, or disperse widely from contaminated birthplaces, could be spared these profound deleterious effects of MeHg, as long as a large proportion of the available habitat remains free of contaminants. Mercury exposure can impair migration behavior critical to many species through numerous mechanisms (Seewagen, 2020). The findings of this study underscore the need to further investigate whether global mercury pollution is rendering wild birds incapable of learning and remembering important spatial information and the mechanisms by which this phenomenon could be occurring.

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CRediT authorship contribution statement

Cara N. Brittain: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Amanda M. Bessler:** Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing. **Andrew S. Elgin:** Investigation, Writing – review & editing. **Rachel B. Layko:** Investigation, Writing – review & editing. **Sumin Park:** Investigation, Writing – review & editing. **Shelby E. Still:** Investigation, Writing – review & editing. **Haruka Wada:** Funding acquisition, Supervision, Investigation, Writing – review & editing. **John P. Swaddle:** Formal analysis, Funding acquisition, Investigation, Writing – review & editing. **Daniel A. Cristol:** Conceptualization, Formal analysis, Funding acquisition, Investigation, Methodology, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2023.115483.

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