Exploring the behavioral, neurological, and physiological effects of methylmercury in a model songbird

by

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Dissertation Abstract

Methylmercury is a widespread environmental stressor known to disrupt reproductive and neural function of organisms even with exposure at sublethal levels. Previous studies of its effects on physiology and behavior have focused mainly on organisms tied to aquatic environments and rodents as models of human exposure. Importantly, more recent studies have shown that mercury also bioaccumulates in terrestrial food webs through emerging insects. As such, the overarching goal of this dissertation was to discern the effects of sublethal levels of mercury in songbirds, an understudied group exposed to the neurotoxicant. Using the zebra finch, *Taeniopygia castanotis*, as a model songbird, I aimed to better understand how lifelong exposure to methylmercury impacts spatial cognition and neural processes in the hippocampus, a region of the brain related to learning and memory (chapters 2 and 3). A second aim of this dissertation was to understand how chronic exposure to methylmercury across the lifespan and only during adulthood in zebra finches affects songbird physiology, specifically female reproductive physiology and endocrinology (chapters 4 and 5).

Regarding the first aim, I found that lifelong, but not developmental- or adult-only exposure, resulted in impaired spatial learning abilities and memory. Particularly, I found that while finches exposed to methylmercury their entire lives took longer to pass a spatial learning task and were more likely to return to unrewarding food locations compared to controls, they displayed neither reduced hippocampus-to-telencephalon volumes nor reduced densities of neurons in the hippocampus, a region of the brain associated with spatial cognition. Methylmercury-exposed birds, however, did surprisingly display increased expression of doublecortin, a protein expressed in immature neurons, in an area of the telencephalon that displays neurogenesis, implying migration of these neurons to the hippocampus is hindered

while neural survival possibly is not. In exploring the effects of adult methylmercury exposure on female reproductive physiology, I found no significant differences in ovarian tissue morphology, DNA damage, or estrogen production.

Regarding my second aim, I found that histologically, adult exposure appears to cause differences in ovarian follicle morphology, but this was not statistically supported by the data I was able to generate to this point. After 4 months of methylmercury exposure in adult female finches, I observed no increased in DNA damage in the ovaries of exposed finches, but I did observe a decrease in circulating estradiol on average compared to controls when accounting for measurement of ovarian tissue. Comparing this to lifelong exposure, I found no difference in circulating estradiol in zebra finches exposed to organic mercury their entire lives compared to controls, though in these exposed birds, concentration of plasma mercury negatively correlated with estradiol concentration.

That control and exposed finches have comparable neural densities but differences in spatial learning abilities indicate neural function in the hippocampus and other areas of the brain associated with spatial cognition is impaired. Based on the increase of immuno-labeling of doublecortin in a neuro-generative area of the telencephalon, I propose this impairment is occurring in the microtubules of exposed finches. Because the treatment used here is comparable to that of studies that have seen decreased reproductive success in zebra finches, methylmercury must be causing reproductive effects by other mechanisms than those explored in this dissertation. The work presented in this dissertation highlights the need for further research on the effects of sublethal methylmercury exposure in songbirds, an important group with both conservation concern and the ability to assist in bioindication of contaminants in their environments.

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"For from [Jesus Christ] and through Him and to Him are all things. To Him be the glory forever. Amen." Romans 11:36

Table of Contents

List of Tables

List of Figures

List of Abbreviations

γH2AX	anti-phospho Histone H2A.X
AI	Artificial intelligence
DAB	Diaminobenzidine
DM	Dorsomedial area of the avian hippocampus
E2	Estradiol
H&E	Hematoxylin and eosin
Hp	Hippocampus
Hg	Mercury
MeHg	Methylmercury
RADD	Repair-assisted Damage Detection
ROIs	Regions of interest
Tel	Telencephalon
UDG	Uracil DNA glycosylase

Chapter 1: General Introduction

Background and dissertation overview

Mercury in the environment

Pollution of the environment has been a growing concern since Rachel Carson's *Silent Spring* was first released in 1962. By this time, the human-agitated release of mercury into the environment via industrial and agricultural run-off had already caused public health crises in Japan and Iraq and soon focused research attention on the fate of mercury in the environment (Bakir et al. 1973, EHD 2002, Friberg and Vostal 1972, Hachiya 2012, Tsuda et al. 2009). Mercury occurs in nature in soils and rocks, predominantly in the form of various sulfides, and has been mined from primary mercury deposits for industrial use for decades (USGS 1970). Once exposed, the metal can easily enter the geochemical cycle by vaporization of metallic mercury and/or transformation into organic mercury compounds or mercury salts (Friberg and Vostal 1972). Thus, mercury has entered ecosystems at minute levels naturally from rain, erosion of soil and rock, volcanic eruptions, and natural disturbances in the earth's crust (USGS 1970); however, mining for mercury, use of it in various industries, and burning of fossil fuels and waste have increased the level of mercury in the environment to sublethal levels such that it is ubiquitous and can pose a threat to wildlife and humans (Friberg and Vostal 1972, Gustin et al. 2016, NRC 2000, Scheuhammer et al. 2007, Streets et al. 2009). Further, mercury is long-lived in the environment and easily transported atmospherically across far distances (Driscoll et al. 2013, Selin 2009), a property that shows the increasing need not only for increased control of anthropogenic mercury emissions but also for deepening our understanding of the effects of mercury from the cellular to the population level (Gustin et al. 2016, Minamata Convention on Mercury 2023).

Mercury in the terrestrial environment

Inorganic mercury in the environment is methylated to methylmercury, the bioavailable form, by microbes living in water (Friberg and Vostal 1972, Swensson and Ulfvarson 1968). From this point, methylmercury can bioaccumulate and biomagnify through the food web (Cristol et al. 2008, Driscoll et al. 2007, Eagles-Smith et al. 2016, Gardner et al. 1978, Rimmer et al. 2010, Wiener et al. 2003). Because the methylation process occurs in water, mammalian species and species closely tied to aquatic ecosystems have been most studied. Increasing evidence shows that methylmercury is a contaminant of concern in the terrestrial environment as well, even surprising distances from point source pollution sites (Bakir et al. 1973, Cristol et al. 2008, Evers and Duron 2008, Jackson et al. 2011b, Rimmer et al. 2010, Sauer et al. 2020). Given the longevity and ubiquity of methylmercury, there is need to study underrepresented taxa, notably songbirds, reptiles, and amphibians that reside in terrestrial environments (Evers and Duron 2008, Gustin et al. 2016, Wolfe et al. 1998).

Effects of methylmercury

It is well established that methylmercury is an endocrine disruptor and neurotoxicant that easily crosses the blood-brain barrier and placental barrier (Chang 1977, Swensson and Ulfvarson 1968). The primary route of exposure to methylmercury is ingestion, and absorption into the blood occurs mainly in the gastrointestinal tract, after which it accumulates in many organs, notably the liver and brain (Clarkson 1977, Finley et al. 1979, Scheuhammer 1988). At lethal doses across animal taxa, methylmercury causes spinal lesions, kidney necrosis, anorexia, numbness of extremities, ataxia, impaired vision, sensory deficits, and mood swings prior to death (Fimreite 1971, Finley et al. 1979, Heinz and Locke 1976, Montiglioa and Royauté 2014,

Nicholson & Osborn 1984, NRC 2000, Pass et al. 1975, Scheuhammer 1988, Swensson and Ulfvarson 1968). These effects are consistent with the observation that the cerebellum, visual cortex, and dorsal root ganglia are the nervous system's most sensitive methylmercury targets (Chang 1977, Kaur et al. 2012). Methylmercury also crosses the placental barrier and is teratogenic; the developing embryo is the most susceptible life stage in humans and animals due to the sensitivity of the developing brain (Bakir et al. 1973, Clarkson 1997, EHD 2002, NRC 2000).

At sublethal levels, mercury has been linked to numerous detrimental behaviors and body functions in various species both in the laboratory and in the field (Evers 2018, Scheuhammer et al. 2007, Wiener et al. 2003). As examples in birds, methylmercury suppresses immune response (Hawley et al. 2009, Lewis et al. 2013, Scheuhammer et al. 2007) and depresses growth in birds (Parkhurst and Thaxton 1973). Studies have also shown endocrine disruption (Wada et al. 2009), altered singing behavior (Hallinger et al. 2010, McKay and Maher 2012), and decreased takeoff flight performance (Carlson et al. 2014) in songbirds. Though methylmercury is known for its neurotoxicity, surprisingly little attention has been given to effects on cognition in birds (Bottini and MacDougall-Shackleton 2023), but one study did find that methylmercury exposure impaired performance on spatial memory tasks but not tasks associated with inhibitory control or color association in zebra finches (Swaddle et al. 2017; for a full review of effects of sublethal methylmercury in birds, see Whitney and Cristol 2017, and for a review of neural effects in avian brains, see Bottini and MacDougall-Shackleton 2023).

Traditionally, one of the most studied areas in regard to methylmercury's detrimental effects is reproduction (Ackerman et al. 2016b, Scheuhammer et al. 2007, Whitney and Cristol 2017, Wolfe et al. 1998). These effects include but are not limited to: reduced fertilization of

eggs (Fimreite 1971), reduced hatching success (Barr 1986, Heddle et al. 2019, Heinz 1979), increased embryonic mortality or incidence of deformities (Hoffman and Moore 1979), decreased fledging success (Varian-Ramos et al. 2014), increased likelihood of nest abandonment (Jackson et al. 2011a), and reduced egg production (Barr 1986, Heinz 1979). It is not well-understood, though, by what mechanism methylmercury decreases reproductive success in birds or other taxa. More studies on the effects of methylmercury are necessary to better predict how the toxicant could affect songbird populations, several of which are already in decline in North America (BBS 2016, Richard et al. 2021, Seewagen 2018, Whitney and Cristol 2017).

Timing of mercury exposure, durations, and concentration

Traditionally, much methylmercury research has focused on developmental exposure. At the same time, animals with limited dispersal are likely to be exposed to the toxicant throughout their lives if they are born in contaminated areas. Thus, the aim of much my dissertation is to determine what effects methylmercury has on cognition and physiology when experienced throughout life. For this reason, both the parents of birds and the birds used in the study were fed an ecologically relevant level of 1.2 parts per million (ug/g) methylmercury-cysteine dosed diet on a wet weight basis, *ad libitum*, throughout their lives, ensuring experimental birds were exposed to mercury their entire lives including *in ovo*. This level of dietary mercury-exposure was comparable to the concentration wild songbirds at industrial sites contaminated with the toxicant were exposed to through their diet (Cristol et al. 2008, Varian-Ramos et al. 2014, Abeysinghe 2017). Additionally, some birds might only experience exposure to methylmercury in adulthood while stopping at contaminated sites during migration or when their habitats

experience new contamination events. Therefore, an additional aim is to study effects of methylmercury on female reproductive physiology during chronic adult-only exposure, as little is known of exposure at this timepoint in passerines despite known reduced reproductive success due to adult exposure (Varian-Ramos et al. 2014). For these purposes, adults will be exposed for four months prior to sacrifice using the same diet as that of the lifelong exposure regime. In an experiment by Varian-Ramos *et al.* (2014), blood mercury levels zebra finches exposed via diet had plateaued at 10 weeks, thus four months was determined to be sufficient to induce any damage that might occur. In summary, lifetime exposure in this dissertation is meant to model a non-dispersing bird raised on a contaminated site, and adult-exposure is intended to model a bird that disperses to a contaminated site.

***Note on the study organism, zebra finch,* Taeniopygia castanotis (*formerly* Taeniopygia guttata castanotis)**

Generally, songbirds are excellent organisms for linking a variety of complex behaviors with neurological mechanisms, perhaps most notably vocal learning and song production (see Fee and Scharff 2010). Because songbirds naturally display an array of memory-based behaviors, they are also proposed as excellent models to understand the mechanisms and purposes of adult neurogenesis (Barnea and Pravosudov 2011). For these experiments, I propose using the zebra finch, *Taeniopygia castanotis*, as they are easily reared in laboratory settings and will allow for careful manipulation of the stressor of interest, dietary methylmercury, without the influence of other environmental variables. The behavior and ecology of this species is well known (Zann 1996), and as highly social, sexually dimorphic passerines, these organisms have been important in studying neuroscience, endocrinology, behavior, and more (see Bonoan et al. 2013, Griffith

and Buchanan 2010, Mello 2014). Additionally, this is the first passerine with a sequenced genome (Warren et al. 2010), allowing for further study of the influence of dietary methylmercury on songbird behavior, physiology, and gene expression.

Significance

In light of the ubiquity and acceleration of mercury pollution, it is vital to investigate to what extent methylmercury affects all classes of animals and their communities (Cristol and Evers 2020, Evers 2018, Montiglioa and Royauté 2014, NRC 2000). This is particularly important to study in terrestrial songbirds because they generally have been overlooked in regard to mercury studies in spite of evidence that some groups of songbirds in contaminated habitats display higher blood mercury concentrations than piscivorous bird species in the same area (Cristol et al. 2008, Evers and Duron 2008, Rimmer et al. 2010) and because anthropogenic global change is predicted to increase bioavailability of methylmercury in the terrestrial environment (Adams et al. 2020). Songbirds are proposed as sentinels for examining environmental contamination for several reasons, including the ease of biomonitoring mercury levels via collecting and analyzing feathers, blood and/or eggs (Ackerman et al. 2016a, Adams et al. 2020, Cristol and Evers 2020, Jackson et al. 2015, Wolfe et al. 1998). A report by Ackerman et al. (2016b) revealed that in many bird populations across western North America displayed blood mercury levels above toxicity thresholds, so more research is needed to understand the precise effects of methylmercury on songbird behavior and physiology to assist in biomonitoring, to inform environmental policy and future research in ecotoxicology that integrates multiple stressors, and to help predict how songbird populations will respond to increasing contamination.

It has been shown that sublethal levels of methylmercury reduces learning and memory abilities in organisms, and for birds in particular this could have profound implications for food caching, migration, song learning and production, and other important behaviors. Furthermore, methylmercury reduces reproductive success, but the mechanisms behind this phenomenon are not well-understood, even though the profound impact this could have on wildlife, especially songbird populations that are in decline. Therefore, more research is necessary to understand the mechanisms by which methylmercury influences cognition and reproduction in passerines.

Research questions

The overarching question for this research is what behavioral, physiological, and neural effects does exposure to methylmercury have in songbirds?

1. What are the effects on spatial cognition in zebra finches exposed to dietary methylmercury, and are the effects dependent on timing of exposure? – Chapter 2
2. What are the neural correlates of altered spatial cognition in lifelong-exposed zebra finches?
    a. Does methylmercury decrease hippocampus volume? – Chapter 2
    b. Does methylmercury impair neuron production, migration and/or survival? – Chapter 3
3. What effects does chronic exposure to methylmercury have on the female reproductive physiology of songbirds?
    a. Does adult exposure to methylmercury change ovarian tissue morphology? – Chapter 4

b. Does adult exposure to methylmercury induce DNA damage in the ovaries of zebra finches? – Chapter 4

c. Does adult and/or lifelong exposure to methylmercury alter circulating estrogen levels? – Chapters 4 and 5

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Chapter 2: Mercury causes degradation of spatial cognition in a model songbird species

*In review with *Ecotoxicology and Environmental Safety*

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Highlights:

- Methylmercury exposure impaired spatial memory in captive songbirds.
- Lifetime exposure was necessary to impair spatial memory.
- Exposure only during development or as an adult was not sufficient.
- Hippocampal volume was not altered in lifetime-exposed birds.

Abbreviations: MeHg, methylmercury

Keywords: cognition; developmental exposure; mercury; methylmercury; spatial memory; zebra finch

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 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.

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 2017). 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). 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 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, Sokolowski et al. 2013, 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 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 memory reported in MeHg-exposed songbirds (Swaddle et al. 2017) could be because their hippocampi are smaller or less densely packed with neurons. This causal mechanism 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.

Methods and materials

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 (Mayer et al. 2013) and MeHg and spatial memory (Swaddle et al. 2017). All finches in these studies were raised in a colony 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. 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.

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 blood plasma 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 has 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 blood concentrations for lifetime and adult-only exposures were approximately 10x the dietary concentrations (Table 1), but for the birds exposed only during development there was no more than trace MeHg in blood by the time spatial cognition tests were performed on them as adults. 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.

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)

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

Overview of training and testing for room-scale procedure (2010-2011; Figure 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. 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 (2.4L x 1.5W x 1.8H 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 (0.4L x 0.3W x 0.2H 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 flock mates, 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.

Birds were 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 sec 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.

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.

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 were never 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 shorter retention interval, it was allowed to eat for 5 min and then was returned to its home cage. After a 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.

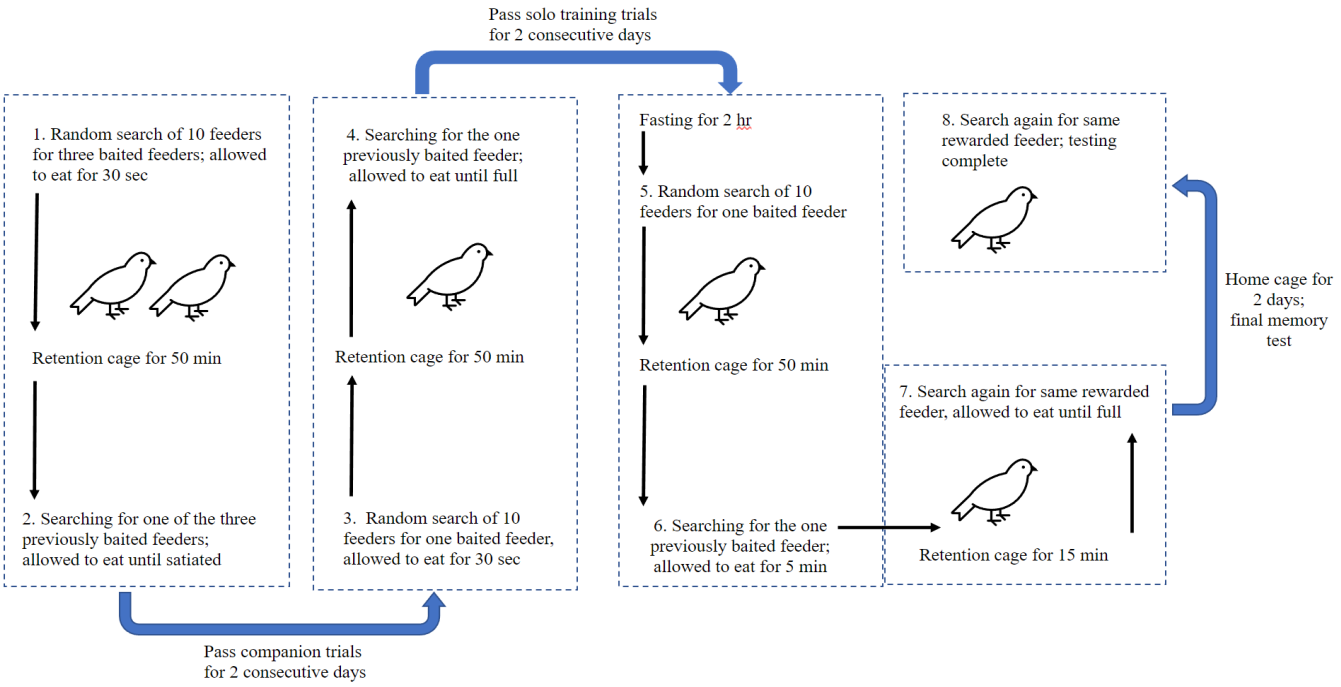

Figure 1. Zebra finches progressed through the room-scale cognition trials from training to memory test as depicted in the flow chart. Birds began with a training partner, depicted as two bird images, then completed the memory task alone, depicted as one bird.

Overview of cage-scale procedure (2015-2018; Figure 2)

Cage-scale tests were repeated in three different contexts to examine the effects of MeHg on spatial memory at a smaller spatial scale. Adapting our experiment to a smaller spatial scale following methods outlined in Hodgson *et al.* (2007) provided easier replication and finer resolution of performance and facilitated comparison of our findings to the existing literature on spatial memory in zebra finches, as well as that of MeHg in rodents. 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 (2.5L x 3W x 2.2H 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 examine any differences more closely in acquisition and learning process and in hippocampal volume and to test for interactions of treatment and sex at this spatial scale. These birds lived indoors in single-sex cages (0.6L x 0.4W x 0.4H 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.

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 x 0.09 x 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).

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.

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).

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."

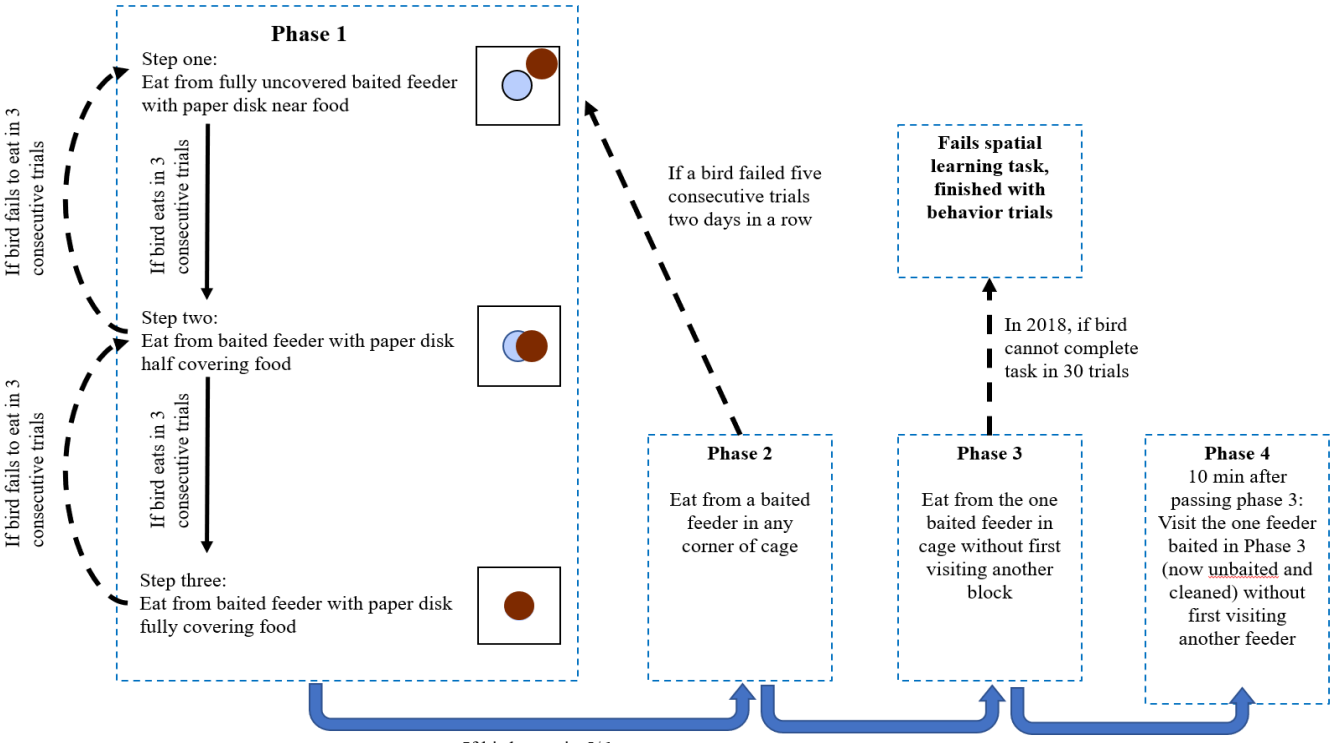

Figure 2. In the cage-scale experiments, birds progressed through four phases to assess their spatial learning and memory. The feeders are depicted as white squares. The red circle in the feeder represents a cover for the food well while a blue circle indicates a baited, uncovered food well.

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 hours following perfusion, then moved to 30% sucrose solution for 3-6 days, and quickly frozen on crushed dry ice and stored in -80°C. 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. 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.

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 to reach criterion 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 all cases), 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 proportion 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 ($P = 0.609$), and the volumes of left and right hemispheres for each individual were summed. 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 ($P > 0.15$).

Results and Discussion

Does exposure to MeHg cause impairment of spatial memory? – Room scale assessment

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 ($P > 0.5$, Fig. 3, Table 2). 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 ($P = 0.007$, Fig. 3, Table 2). 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 ($P < 0.03$, Fig. 3, Table 2). Two days after initially finding the baited feeder (a 48 hr retention interval spent in their home cage with familiar cage mates), lifetime MeHg-exposed birds again performed worse than controls, requiring 2.52x (i.e., 87%) more feeder visits as controls to find the food ($P < 0.0001$, Fig. 3, Table 2). 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, Fig. 3, Table 2).

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 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.

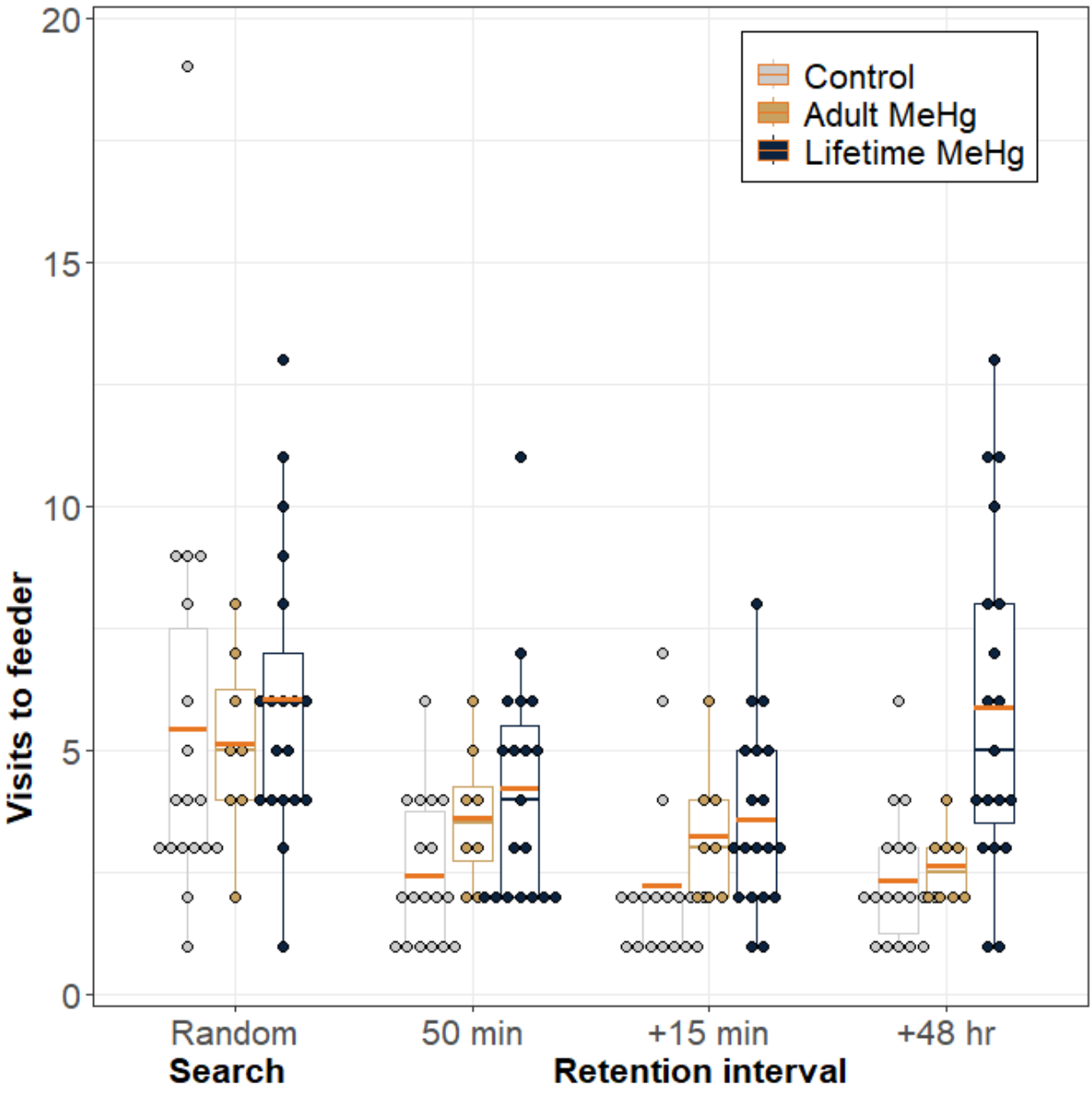

Figure 3. In room-scale spatial memory tests adult MeHg-exposed zebra finches (tan, middle bar) were not significantly worse than controls (gray, left bar) in total number of feeders visited to relocate hidden food, regardless of retention interval ($P > 0.05$). Lifetime-exposed finches (navy, right bar) 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 hours later; $P < 0.05$). Mean number of trials is denoted by an orange bar.

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*)

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).

Does exposure to MeHg cause impairment of spatial memory? – Cage scale assessment

First, in 2015, we tested lifetime MeHg-exposed birds and controls on their ability to learn and 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, Fig. 4a, Table 3), and were significantly less likely to pass the final single-trial spatial memory test ($P = 0.039$ Phase 4, Table 3). 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. We tested whether developmental exposure was sufficient to induce spatial learning impairments in 2016 and found no difference in performance between birds exposed to organic mercury only during development and control birds ($P = 0.428$, Fig. 4b, Table 3).

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, 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 ($P = 0.01$, Fig. 4c, Table 3). 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 later.

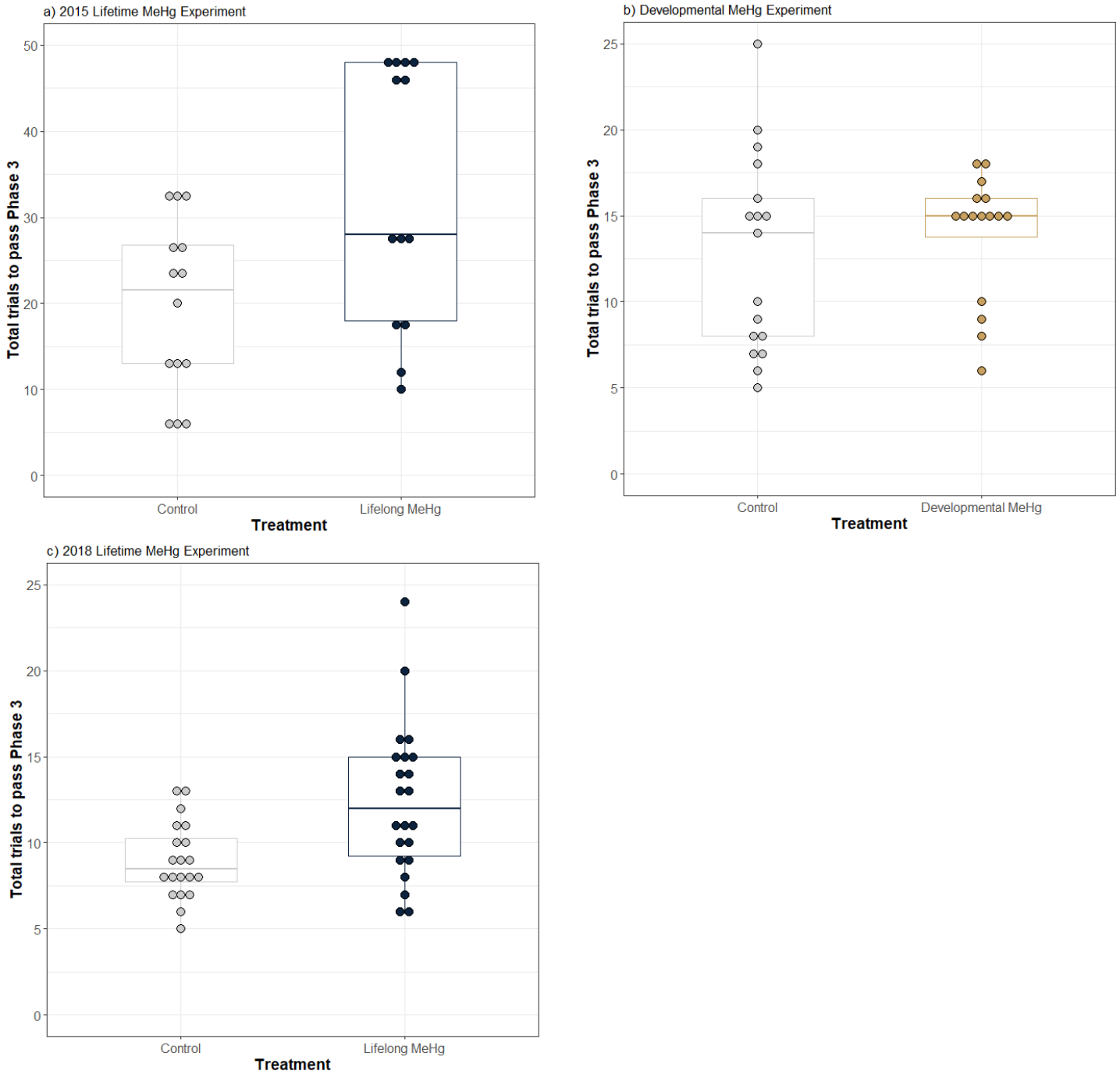

Figure 4. Number of trials required to pass Phase 3 of cage-scale spatial memory experiments for a) birds exposed to MeHg for their lifetime (2015, navy), b) birds exposed to MeHg only during development (2015-2016, tan), and c) lifetime-exposed birds (2018, navy). More trials indicate slower acquisition of the memory task. Control birds are indicated in gray in all graphs. Birds exposed throughout their lives required more trials to reach criterion for this task in both years ($P < 0.05$), whereas finches exposed only during development did not significantly differ from controls ($P = 0.428$).

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*)

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).

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, we tested for an 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) then ended MeHg exposure. 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, Table 3) or in likelihood of passing the single trial spatial memory test with the feeders unbaited to eliminate scent cues ($P = 0.309$, Table 3).

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, Sokolowski et al. 2013, Wu et al. 2016) and fish (Smith et al. 2010). It could be that differences across taxa and/or differences in method of toxicant administration drive this change in pattern.

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, treated males =290.49 mm^3, control females = 223.31 mm^3, treated females = 205.71 mm^3, $P = 0.614$; average hippocampus volume control males = 60.92 mm^3, treated males = 68.18 mm^3, control females = 41.73 mm^3, treated 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. It is possible that the mechanisms underlying the impairments in spatial cognition that we detected do involve the hippocampus in ways that do not affect volume (e.g., Behzadfar et al. 2020, Lindström et al. 1991, Liu et al. 2009). 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 (Bottini and MacDougall-Shackleton 2023).

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, Richard et al. 2021, Rosenberg et al. 2019). At the same time according to our work, 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 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). In our studies, we found neither developmental nor adult exposure by themselves affected performance on these 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. 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; however, it is encouraging that shorter exposure, even during development, did not produce the same effect. Birds that migrate into and out of contaminated habitats, or disperse widely from contaminated birthplaces, may be spared these profound deleterious effects of MeHg, as long as a large proportion of the habitats available 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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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 3: Neural correlates of suppressed spatial learning in zebra finches exposed to methylmercury

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Highlights

- We quantified neural measures in hippocampi of finches exposed to methylmercury.
- Exposed zebra finches did not display altered density of mature or immature neurons.
- Exposed birds had more immunolabeling of immature neurons in a neuro-generative area.

Abbreviations: DAB, dianinobenzidine; DM, dorsomedial area of the avian hippocampus; hp, hippocampus; MeHg, methylmercury; tel, telencephalon

Keywords: Hippocampus; methylmercury; neural density; spatial cognition; zebra finch

Abstract

Methylmercury is a persistent global contaminant that can bioaccumulate in organisms and cross the blood-brain barrier, impairing neural function. Though it is found in both the aquatic and terrestrial environments and organisms may experience lifelong exposure, most research has focused on the effects in the aquatic environment and during development. Our work focuses on understanding the effects of environmentally relevant levels of mercury on spatial learning and accompanying neural changes in captive songbirds exposed throughout their lives. Songbirds are an increasingly popular model species for toxicity studies of MeHg due to their ubiquity, conservation concerns, and known exposure to the pollutant in contaminated environments. Previous work has shown that zebra finches (*Taeniopygia castanotis*) exposed to sublethal levels of dietary MeHg throughout their lifespans exhibited impaired spatial learning and increased perseveration in a 5-phase spatial memory task but no differences in relative volumes of the hippocampus or telencephalon. As the hippocampus is a region of the brain related to spatial learning and memory, we hypothesized methylmercury may be impairing spatial memory through effects on hippocampus other than volume. To test this, we measured the density of mature and immature neurons and the percent area covered by immature neurons in the hippocampi of methylmercury exposed zebra finches that went through spatial memory trials. We observed no effects of methylmercury on neuronal density in the hippocampus but did observe increased immunolabeling of doublecortin, a protein expressed in immature neurons, in a reference area of the telencephalon known to be neuro-generative, in methylmercury-exposed birds. That birds exposed to methylmercury seem to generate more proteins related to neuronal production but display no difference in neuron number in the hippocampus suggests that cellular migration could be hindered. Furthermore, the neurons labeled in the hippocampus could display

increased damage and/or cellular death that was not detectable with our methods. These results suggest heavy metal contamination could have severe implications for songbird conservation, particularly species highly reliant on spatial memory for migration or food-caching. More work is needed to understand how lifetime methylmercury exposure impacts neural anatomy in the avian hippocampus and other areas related to spatial cognition and motivated movement. Our work shows the need for further study of the neural and behavioral effects of lifelong methylmercury exposure *in vivo* to understand the mechanisms underlying decreased spatial learning abilities in exposed birds.

Introduction

Methylmercury (MeHg) is a ubiquitous neurotoxicant that occurs as a pollutant throughout the world. Even low doses of methylmercury have neurotoxic effects, and these effects have been shown both *in vitro* and *in vivo* (Bottini and MacDougall-Shackleton 2023, Chang 1977, Nagashima 1997, Sokolowski et al. 2013, Tamm et al. 2006, Wu et al. 2016). In association with its neurotoxicity, researchers have documented changes in behavior in MeHg-exposed animals across taxa. For instance, Carolina wrens (*Thryothorus ludovicianus*) and house wrens (*Troglodytes aedon*) in MeHg-contaminated sites sing shorter, less complex songs compared to conspecifics in reference sites (Hallinger et al. 2010). Zebrafish exposed to MeHg during development displayed increased anxiety-like behaviors and impaired locomotion as adults (Glazer and Brennan 2021). Additionally, rats, as well as other mammalian groups, display reduced behavioral flexibility when exposed to MeHg (see Newland et al. 2008, Kendricks et al. 2022), and lifelong exposure to MeHg in captive zebra finches induced more

stereotyped behavior and changes in risk-reward processing under simulated predation risk (Kobiela et al. 2014). Effects of MeHg on spatial cognition in particular have also been studied. Rats exposed during development showed deficits in spatial memory as juveniles (Falluel-Morel et al. 2007) and as adolescents (Sokolowski et al. 2013, Tian et al. 2016). While the behavioral alterations caused by MeHg exposure are becoming clearer, studies linking molecular, cellular, and tissue level measurements to downstream behavior are needed (see Bottini and MacDougall-Shackleton 2023).

Both spatial learning and memory are influenced by neural processes typically associated with the hippocampus (Barnea and Nottebohm 1994, Krebs et al. 1996, Pravosudov and Omanska 2005, Sherry et al. 1992). These processes include proliferation of neural progenitor cells, differentiation and migration of immature neurons to a target site, maturation of surviving neurons, and selective formation of neural networks (Dupret et al. 2007). As neural mechanisms in the hippocampus influence spatial cognition, a question remains whether mercury exposure influences spatial cognition through damaging neurons in the hippocampus.

Many studies have focused on developmental exposure, as this is a sensitive time point for any organism (Bakir et al. 1973, EHD 2002, Kendricks et al. 2022), but environmental MeHg exposure may be lifelong or occur only during adult life. As examples, philopatric organisms at a contaminated site would remain at a polluted site, resulting in lifelong exposure to pollutants, and adult humans may not experience significant exposure until beginning an occupation that causes them to do so. Likewise, due to the ability of atmospheric mercury to travel long distances prior to precipitation (Pacyna 2020, Selin 2009), some migratory species could experience contamination at both their wintering and breeding grounds (for a review on evidence and potential effects of MeHg in migratory birds see Seewagen 2020). Recent evidence shows that

consequences of MeHg exposure only during development or adulthood are different from those of lifelong exposure (Brittain et al. in review, chapter 2). Specifically, lifelong-exposed finches, but not adult-only or developmental-only exposed birds, perform worse on spatial learning and memory tasks compared to controls (Swaddle et al. 2017, Brittain et al. in review, chapter 2). These data support the possibility of differential neurotoxic effects of MeHg depending on the timing of exposure.

To fill these knowledge gaps, we aimed to explore how lifetime methylmercury exposure impacts neural anatomy in the avian hippocampus, specifically in birds which showed impaired spatial cognition with lifelong MeHg treatment. Songbirds are growing in popularity as ecotoxicology models, as they have been shown to be exposed to MeHg through their diets at sites contaminated with MeHg (Cristol et al. 2008, Jackson et al. 2015, Zhang et al. 2022). In addition to being sentinel species for pollutants, songbirds are also good candidates for examining neurotoxic effects in juveniles as well as adults. Some species of songbirds exhibit extensive levels of neurogenesis of neurons incorporated into the hippocampus throughout their lives, displaying changes in volumes and neural densities in the hippocampus as well as other parts of the brain associated with seasonal migration, singing, and food caching (Balthazart et al. 2008, Barnea and Nottebohm 1994, LaDage et al. 2011 Smulders et al. 1995). Zebra finches (*Taenopygia castanotis*) are model songbirds with well-studied behavior and neural pathways (Zann 1996, Swaddle 2016, Mayer et al. 2013), thus we used laboratory-exposed finches to uncover the neural processes underlying changes in spatial cognition due to MeHg exposure.

We hypothesized that reduced spatial learning and memory in MeHg-exposed birds is due to suppression of migration of young neurons to hippocampi and reduced survival of the immature neurons within hippocampi, resulting in lower neuronal density. We predicted that

MeHg-exposed birds would exhibit reduced numbers of mature neurons in the hippocampus compared to control birds. We also predicted that MeHg-exposed birds would exhibit lower density of young neurons in the hippocampus compared to the control group (Table 5).

Methods and materials

Animal husbandry

Zebra finches were raised in a captive colony at William & Mary in Williamsburg, Virginia, USA. Both treatments were housed in same-sex cages (approximately 0.76 x 0.46 x 0.46) of four-to-six individuals. In the MeHg-treated group, both the parents of focal birds and focal birds themselves were fed an ecologically relevant concentration, 1.2 µg/g of MeHg-cysteine diet on a wet weight basis, *ad libitum*, ensuring the birds were exposed to MeHg for their entire lives, including *in ovo*. This level of dietary mercury-exposure was designed to be comparable to the concentration that wild songbirds might experience at industrial sites contaminated with the toxicant (Cristol et al. 2008, Varian-Ramos et al. 2014, Abeysinghe et al. 2017). Resulting blood mercury concentration in exposed birds two weeks prior to behavioral testing was 15.36 ± 3.80 µg/g (n = 15). Control birds were from lineages never exposed to MeHg, and control diet was prepared with an aqueous solution of cysteine in the same manner as the MeHg diet. Blood mercury concentration for controls was 0.13 ± 0.14 µg/g (n = 16). Average age of birds at the end of the experiment was 612.5 ± 32.0 days. All animal use was performed under protocols approved by IACUC at Auburn University (#2017-3214).

To complete behavioral testing, zebra finches were transported overnight by van from Williamsburg, Virginia, to Avian Research Laboratory 2 at Auburn University, Alabama, departing at 10:00 PM EST and arriving 7:00 AM CST, for a trip duration of 10 hours. Transport

at night, as opposed to during the day, prevented additional nutritional stress as zebra finches do not normally feed at night. Cages were covered with dark bed sheets to minimize disturbance during transport. Birds were transported at a density of approximately eight per cage (0.6 x 0.41 x 0.41m) and were given access to food in case they were able to feed. Upon arrival, birds were sorted by sex and treatment, weighed, and housed with no more than three birds per cage (0.4 x 0.5 x 0.5 m). One control male died shortly after translocation. Birds were maintained under similar conditions as they had experienced at William & Mary - they were kept in rooms approximately 21°C on a 14:10 light:dark photoperiod with full-spectrum lighting and given *ad libitum* access to vitamin-enriched water, cuttlefish beak conditioner/calcium source, digestive grit, and pelletized complete diet of the same treatment as prior to transport (fruit blend canary/finch food, Zupreem, Shawnee, Kansas, USA, prepared at William & Mary as described in Varian-Ramos et al. 2014 and shipped to Auburn University).

After an acclimation period of at least two weeks following transport, we tested spatial cognition abilities according to the procedure outlined by Swaddle et al. (2017) and Brittain et al. (in review, chapter 2). As reported in Brittain et al. (in review, chapter 2), birds exposed to dietary MeHg throughout their lives required more trials to complete a spatial learning task and were more likely to return to unrewarded locations after locating a rewarded location. Here, we correlate the spatial learning data to the neural anatomical data for these same birds. In this study, males and females did not show differences in spatial learning performance ($p > 0.1$ all spatial cognition tasks).

Brain collection

Five to seven days after finishing behavioral trials, birds were humanely sacrificed and

perfused with 4% paraformaldehyde. Brains were removed gently from the skull and fixed with 4% formaldehyde, and brains of all males that had completed the spatial learning task (n = 24) were sectioned coronally into 30µm sections and stored in cryoprotectant. Every fourth section (120µm between samples) was 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) to determine boundaries of the hippocampus and volumes of both the hippocampus and telencephalon. For immunohistochemistry, 12 equally spaced sections from throughout the hippocampus were chosen based on each individual's hippocampal measurement for histochemical analysis. Only individuals with at least 8 out of 12 sections able to be measured were included (n = 22).

Immunohistochemistry

Brain sections were incubated with primary antibodies against doublecortin (DCX, Table 4), a protein expressed in neurons younger than 25 days old in birds (Balthazart et al. 2008, Gleeson *et al.* 1999) and with NeuN (Table 4) to stain mature neurons. Prior to the assays, we optimized the concentration and incubation time of serum, primary antibody, secondary antibody, and dianinobenzidine (DAB) stain for this species (Table 4). Sectioning, mounting, and immunohistochemistry were performed blind with respect to treatment. Microscope analysis was conducted by a single blind observer.

Target	Serum	Primary	Secondary	DAB
DCX	Santa Cruz Biotech cat. SC-8066 1:1000, overnight	10% Normal Horse - Vector cat. S-2000 overnight	Horse anti-goat - Vector cat. BA-9500 1:400, 1 hr	Dianinobenzidine tablet - Sigma cat. D4418-50SET 90 sec
NeuN	EMP Millipore Corp. cat. MAB377 1:2000, overnight	10% Normal Goat - Vector S-1000 1 hr	Goat anti-mouse - Vector cat. BA-1000 1:250, 1 hr	

Table 4. We validated listed products (catalog number listed), concentrations, and incubation times of primary antibodies against DCX and NeuN, serum, secondary antibodies, and DAB stain, which were applied to tissue in that order.

Quantification of neurons

ImageJ analysis of DCX-reactive neurons

The area covered by DCX immunoreactive neurons was quantified using the thresholding feature of ImageJ from z-stacked images captured on a Nikon Ni-E motorized microscope at 40x magnification, z-stack range set to 3µm, and brightness set to 80 exp. Two areas of the hippocampus, one in the V-shaped region - hypothesized to be homologous to the mouse dentate gyrus (Atoji et al. 2016, Gupta et al. 2012) - and one in the dorsomedial (DM) region - proposed to be homologous to the mammalian Ammon's horn (Atoji et al. 2016, Rook et al. 2023) - were selected for analysis (Fig. 5a). Additionally, one area of the telencephalon, just outside the hippocampus in the lateral neurogenic zone near the ventricle, was selected for analysis as a comparison for the hippocampal areas (Fig. 5a). To ascertain if differences in thresholding values in the telencephalon were due to a change in the number of neurons, for each z-stacked image in this area, cell bodies were counted in every other complete square of a grid to ensure neurons were not counted twice (Fig. 5b and c). To explore if there were differences in the size of soma in MeHg-exposed birds, the circumference of counted cell bodies in telencephalon frames were also measured using the polygon tool in ImageJ.

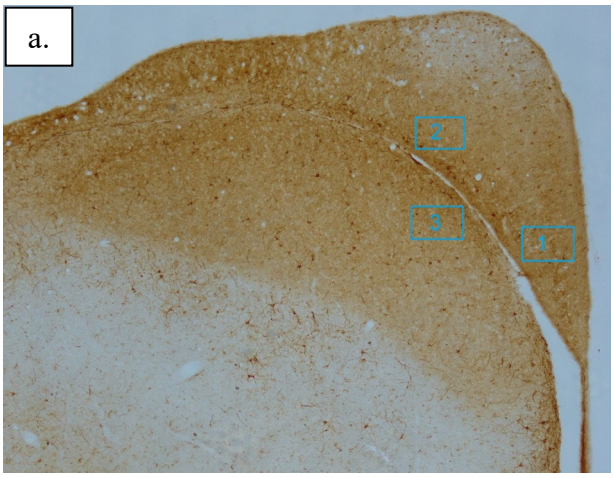
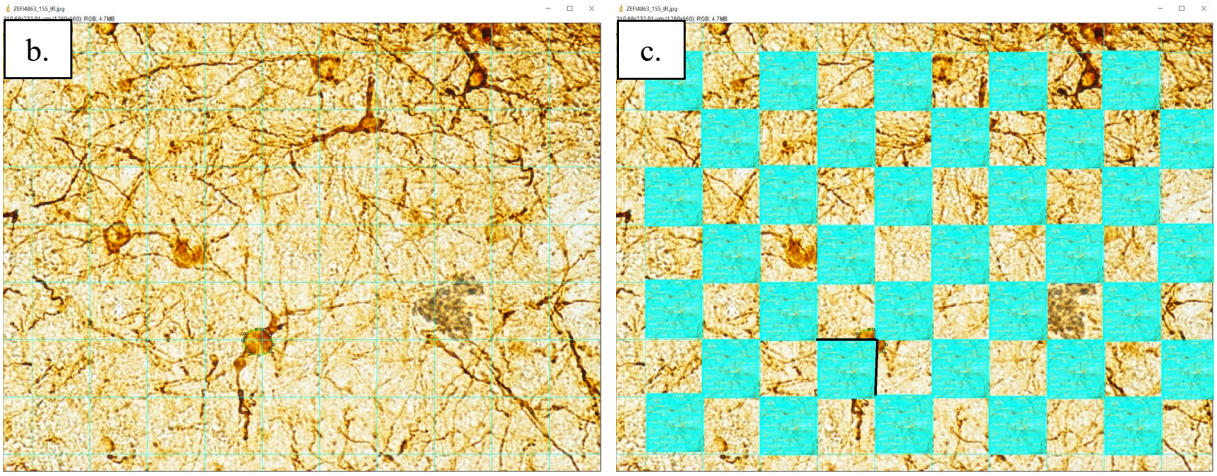


Figure 5. a - top) **-** Three areas were selected as systematically as possible for thresholding analysis of DCX-reactive neurons. Two areas of the hippocampus - one in the V, box 1, and one in the DM portion, box 2, were analyzed. One area in the telencephalon that generates neurons, box 3, was used as a reference. b - bottom left) The z-stack images captured for thresholding analysis were later used to count and measure soma. A grid was overlaid on the image. c - bottom right) The filled boxes represent counting inclusion zones, which were utilized to ensure cells were not counted twice in a frame.



Stereological analysis of NeuN- and DCX-reactive neurons

Densities of NeuN- and DCX-reactive neurons in the hippocampus were measured using a Nikon Ni-E motorized microscope with the stereological optical fractionator method (Glaser et al. 2007). With 4x objective lens, equally spaced points were systematically selected throughout the hippocampus by covering the region with a 210µm x 210µm grid digitally; however, cells in the V area were not counted, as the density was too great. Under the 63x oil lens, the microscope was moved to pre-selected points. At each point in the sampling grid, we used a 30µm x 30µm counting frame (Fig. 6). We measured the thickness of the tissue section by focusing on top and bottom boundaries in the first counting frame, disregarding cells in the top and bottom 1µm of

the section (i.e., guard zone) if the thickness was greater than 7µm. We focused on the section at 0.5µm intervals, counting the cells that came into focus that did not cross the bottom and left side of the counting square. Cell density in the hippocampus was calculated using the following formula: Total population = n x (1/ssf) x (1/asf) x (1/hsf),

where n = total number of cells counted in every section of an individual's brain, ssf = section sampling frame (i.e. range of slices divided by number of slices in that range, to give average distance between slices), asf = area sectioning frame (30µm x 30µm / 210µm x 210µm), and hsf = average optical dissector height (thickness of the individual section/average tissue thickness) across all sections of an individual's brain.

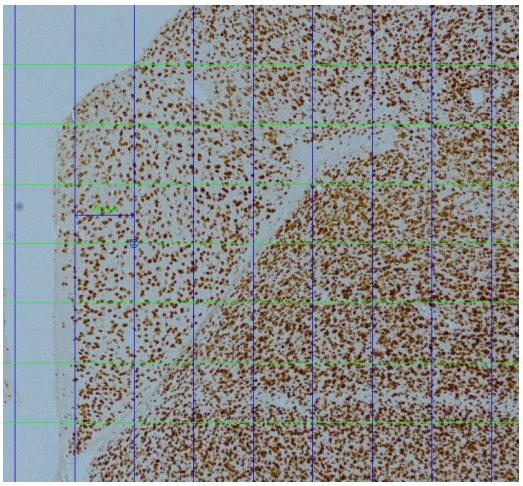


Figure 6. A grid was overlaid on the entire hippocampus and all NeuN-expressing cells within boxes that were entirely included in hp boundary were stereologically counted. The same method was used to quantify DCX density.

Statistical analysis

All analyses were performed in R (R version 4.2.2) using lme4 and lmerTest packages (Bates et al. 2015, Kuznetsova et al. 2017), and graphical figures were created using ggplot2 (Wickham 2016). DCX-reactivity and neural density in the left and right hemispheres of the brain were compared using a paired t-test. As they did not significantly differ ($p > 0.5$), the two hemispheres were averaged within individuals, and these averages used in statistical analyses.

For all variables, models with untransformed and log-transformed data were compared. Log transformation did not significantly improve model fit (AIC values < 2 for all models), so untransformed data were used. Analyses were completed with and without outliers for every variable. No patterns differed after exclusion of outliers. Data are reported with outliers included.

Linear mixed models were used to analyze the effect of MeHg on DCX coverage in the hippocampus and telencephalon with treatment and hippocampus-to-telencephalon ratio (published in Brittain et al. in review, chapter 2) as fixed effects and individual as a random effect. The effect of methylmercury on density of DCX- and NeuN-reactive neurons in the hippocampus was analyzed using linear regression. In all models, individual's mass, age at euthanasia, and position within the sequence of neuron measurements were considered as covariates. In most cases, none of these variables significantly improved model fit ($p > 0.1$) and were dropped from the model. In one case, the density of DCX-expressing neurons, the age of the bird at death significantly contributed to the model, and this was left in the statistical analysis.

To determine the underlying causes of suppressed spatial learning, which was tested in "Phase 3" of our cognition trials (Brittain et al. in review, chapter 2), we ran generalized linear models to test whether neural measures predicted ability to pass Phase 3, total trials to pass phase 3, or proportion of trials returning to an unrewarded block after locating the rewarding block, using binomial, poisson, and quasibinomial distributions, respectively. We used separate models to test if thresholded area covered by DCX neurons in the hippocampus, density of hippocampal NeuN-neurons, or density of hippocampal DCX-neurons significantly explained spatial learning measures. Behavior-neuron relationships were analyzed with and without treatment in the models. For thresholded area covered by DCX neurons in the hippocampus, region of the

hippocampus in which DCX-immunoreactivity was measured - V vs. DM - did not significantly improve model fit for any spatial learning measure ($p > 0.1$), so the coverage in the two hippocampus areas were averaged for behavior analysis. For all behavioral measures, birds' age at death, position of the test cage, and whether the bird required a repeat of early phases of testing was initially included in the model, and subsequently all removed, as none contributed significantly to model fit ($p > 0.1$).

Staining procedure	If migration is inhibited but no effect on survival	If survival inhibited
DCX (young neurons)	Staining in Hg group < control group	Hg group = control group
NeuN (mature neurons)	Staining in Hg group < control group	Staining in Hg group < control group

Table 5. Summary of predictions with regard to our immunohistochemistry procedures. We predicted that migration of immature neurons and survival of mature neurons would be hindered in treated birds.

Results

Effect of MeHg on neural measurements

To test for an effect of MeHg on percent area covered by DCX-expressing neurons we compared two areas of the hippocampus and a reference area (ventricular area of the telencephalon). Dietary MeHg exposure significantly altered area covered by DCX, but the magnitude of the effect depended on brain region (treatment x region interaction: $p < 0.001$, $t = 4.367$). Thus, we analyzed the effect of MeHg on DCX-stained areas in telencephalon and hippocampus separately. Interestingly, in the ventricular area of the telencephalon, MeHg-exposed finches displayed 1.45% more area covered in DCX-reactive neurons than controls ($p = 0.0143$, $t = 2.698$, Fig. 7b, Tables 6 and 8). This difference appeared to be due to neither an

increase in the number of soma ($p = 0.261$, $t = -1.157$, Table 6) nor the size of soma in MeHg-exposed birds; specifically, we observed no difference in the circumference of soma on average ($p = 0.854$, $t = 0.187$, Table 6) or in the largest individual soma measured ($p = 0.239$, $t = 1.216$, number of cells counted/measured included in model as covariate, Table 6).

Conversely, no significant difference in DCX-neuron-covered area was seen between treatment groups in the hippocampus overall (with values for the V and DM averaged $p = 0.257$, $t = 1.168$, Fig. 7a, Tables 6 and 8). Similarly, MeHg birds did not differ from controls in area covered by DCX-expressing neurons in either the DM ($p = 0.252$, $t = 1.181$, Table 6) or in the V ($p = 0.316$, $t = 1.030$, Table 6); there was no significant difference in the percent area covered by DCX-immunoreactive neurons between the DM and V of the hippocampus ($p = 0.837$, $t = 0.205$).

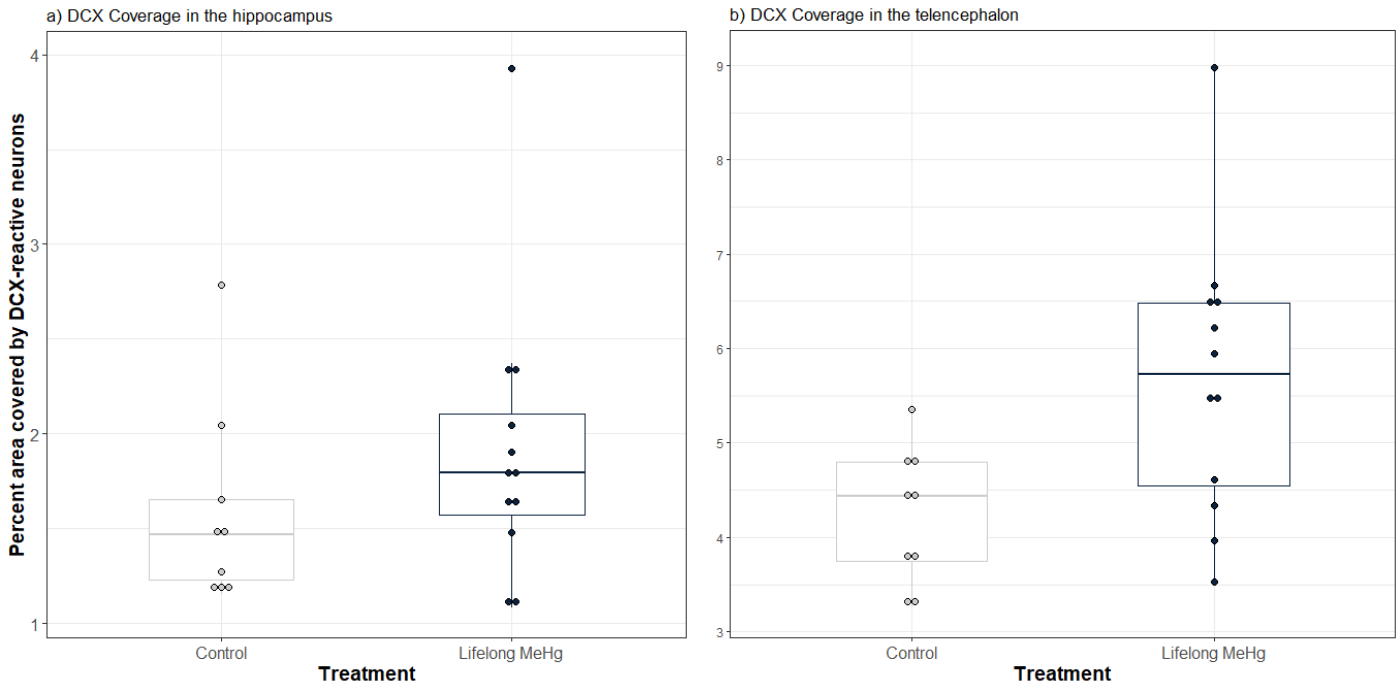


Figure 7. a - left) MeHg-exposed birds (navy, right bars) did not exhibit significantly different percent area covered by DCX-reactive neurons in the hippocampus than controls (gray, left bars; $p = 0.257$). b - right) MeHg-exposed birds had significantly higher percent area covered by DCX-reactive neurons than controls in an area of the telencephalon along the ventricles ($p = 0.0143$).

	Coverage DCX-neurons in DM			Coverage DCX-neurons in V			Average coverage DCX-neurons in hp		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.73	1.23 – 2.22	**<0.001**	1.45	0.99 – 1.91	**<0.001**	1.59	1.13 – 2.05	**<0.001**
Treatment [MeHg]	0.37	-0.28 – 1.02	0.252	0.30	-0.31 – 0.91	0.316	0.34	-0.27 – 0.95	0.257
Observations	21			21			21		
R^2 / R^2 adjusted	0.068 / 0.019			0.053 / 0.003			0.067 / 0.018		

	Coverage in telencephlon (tel) by DCX neurons			Average number cells counted in tel frames			Size largest cell body in tel frames			Average size cell bodies in tel frames		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	4.23	3.38 – 5.08	**<0.001**	68.50	61.73 – 75.27	**<0.001**	159.58	112.50 – 206.65	**<0.001**	44.50	40.36 – 48.63	**<0.001**
Treatment [1]	1.45	0.32 – 2.57	**0.014**	-5.09	-14.25 – 4.08	0.261	15.63	-11.28 – 42.55	0.239	0.50	-5.10 – 6.10	0.854
Number counted cells in tel							0.03	-0.16 – 0.21	0.762			
Observations	21			22			22			22		
R^2 / R^2 adjusted	0.277 / 0.239			0.063 / 0.016			0.083 / -0.014			0.002 / -0.048		

Table 6. Statistical summaries for models analyzing MeHg effects on DCX-coverage obtained through thresholding. Bold typeface represents statistical significance ($p < 0.05$).

We tested whether MeHg reduced the density of DCX and NeuN neurons in the hippocampus of exposed songbirds. We saw neither an effect of MeHg on the density of DCX neurons ($p = 0.53$, $t = -0.660$, Fig. 8a, Tables 7 and 8), nor NeuN-expressing neurons in the hippocampus ($p = 0.26$, $t = 1.153$, Fig. 8b, Tables 7 and 8).

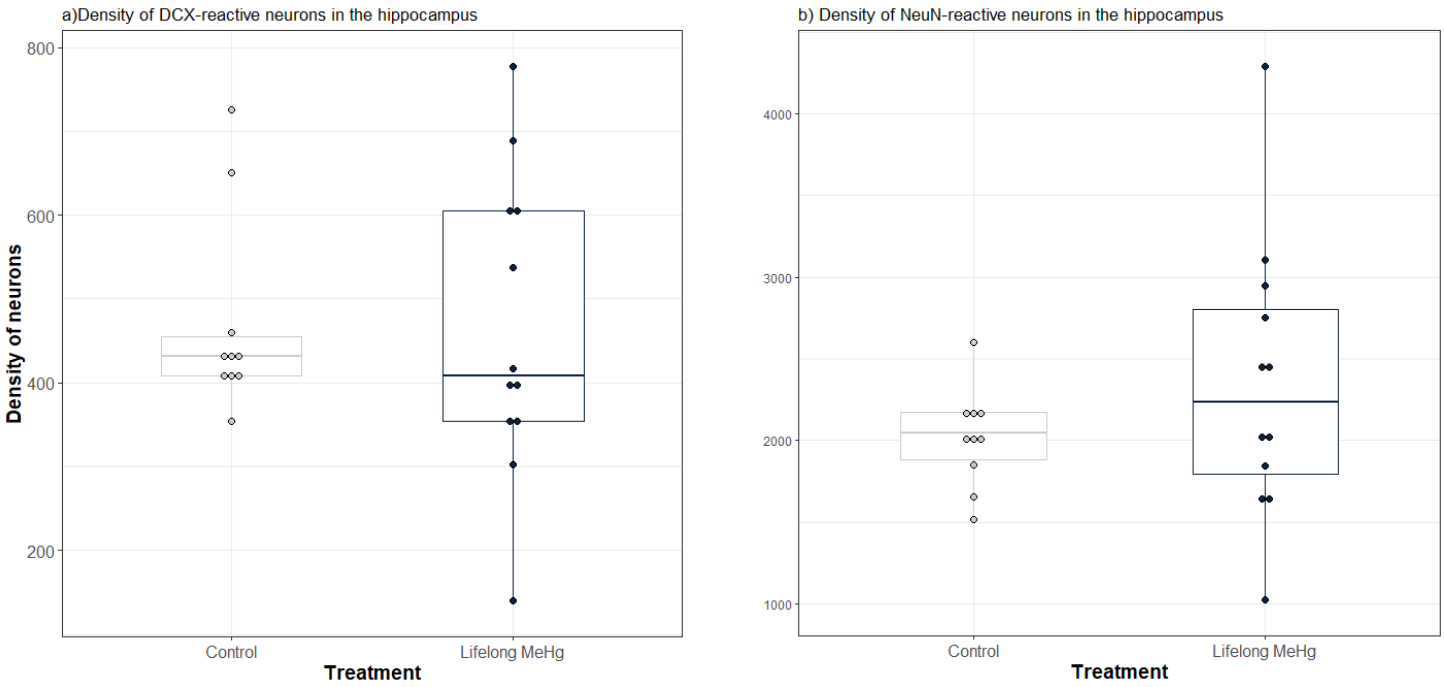


Figure 8. a - left) MeHg-exposed birds (navy, right bars) did not on average differ significantly in densities of DCX-reactive cells in the hippocampus compared to controls (gray, left bars, p = 0.53). b) Likewise, MeHg-exposed birds did not on average have significantly different densities of NeuN-reactive cells in the hippocampus compared to controls (p = 0.26).

	Density NeuN-expressing neurons			**Density DCX-expressing neurons**		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	2019.56	1579.07 – 2460.05	**<0.001**	1703.00	711.32 – 2694.67	**0.006**
Treatment [1]	329.64	-266.79 – 926.07	0.263	-63.03	-296.65 – 170.58	0.534
age at death				-2.01	-3.53 – -0.49	**0.018**
Observations	22			9		
R^2 / R^2 adjusted	0.062 / 0.015			0.636 / 0.514		

Table 7. Summary outputs for models analyzing MeHg effects on density of NeuN- and DCX-expressing neurons in the hippocampus. Bold typefaces denote statistical significance (p < 0.05).

Staining procedure	Telencephalon	Hippocampus
DCX (young neurons)	**Staining in MeHg group > control group**	MeHg group = control group
NeuN (mature neurons)	-	MeHg group = control group

Table 8. Summary of results regarding neuron staining regarding predictions. Bold typeface indicates $p < 0.05$.

Correlating neural measurements with behavior

As previously reported (Brittain et al. in review, chapter 2), MeHg-exposed birds required more trials to learn and consistently return to a rewarded location in spatial learning trials, termed "Phase 3." They also were more likely to return to unrewarded locations after locating a rewarded one in this same task. Nevertheless, no hippocampal neural measurement examined in this study correlated with these behaviors. Percent area covered by DCX-reactive neurons failed to predict ability to pass Phase 3 ($p = 0.191$, $z = 1.307$), number of trials needed to pass Phase 3 ($p = 0.153$, $z = -1.430$, Table 9), or likelihood of displaying fixation behavior as measured in these trials ($p = 0.291$, $t = -1.094$, Table 9). Furthermore, density NeuN-reactive neurons did not predict ability to pass Phase 3 ($p = 0.698$, $z = 0.388$), number of trials needed to pass Phase 3 ($p = 0.625$, $z = 0.488$, Table 9), or likelihood of displaying fixation behavior as measured in these trials ($p = 0.895$, $t = 0.135$, Table 9). Density of DCX-reactive neurons also did not predict ability to pass Phase 3 ($p=0.838$, $z = 0.205$), number of trials needed to pass Phase 3 (with treatment $p=0.295$, $z = -1.047$, Table 9), or likelihood displaying fixation behavior as measured in these trials ($p=0.747$, $t = 0.328$, Table 9). These patterns remained the same after removing treatment from the models ($p > 0.2$ for all neural measurement-behavior correlations).

	Total trials to complete phase 3									Proportion trials returning to unbaited block after locating baited block in Phase 3								
Predictors	*Incidence Rate Ratios*	*CI*	*p*	*Incidence Rate Ratios*	*CI*	*p*	*Incidence Rate Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*	*Odds Ratios*	*CI*	*p*
(Intercept)	8.08	5.19 – 12.58	**<0.001**	11.03	6.89 – 17.53	**<0.001**	11.29	7.36 – 17.55	**<0.001**	0.26	0.11 – 0.61	**0.002**	0.24	0.09 – 0.64	**0.005**	0.39	0.14 – 1.12	0.072
Treatment [1]	1.41	1.05 – 1.90	**0.024**	1.41	1.06 – 1.89	**0.018**	1.57	1.15 – 2.15	**0.005**	1.71	0.93 – 3.23	0.089	1.75	0.97 – 3.26	0.072	2.11	1.10 – 4.26	**0.030**
Density NeuN	1.00	1.00 – 1.00	0.625							1.00	1.00 – 1.00	0.893						
DCX density hp				1.00	1.00 – 1.00	0.295							1.00	1.00 – 1.00	0.743			
Average thresholding value hp							0.84	0.65 – 1.06	0.153							0.72	0.39 – 1.25	0.274
Observations	19			19			18			19			19			18		
R^2 Nagelkerke	0.404			0.448			0.512			NA			NA			NA		

Table 9. Summary of outputs for models correlating spatial learning measures with neural measures with treatment included in model. Bold typefaces denote statistical significance ($p < 0.05$).

Discussion

As MeHg-exposed finches had higher percent DCX-immunoreactive area in the telencephalon than unexposed control finches but had similar DCX coverage in the hippocampus, we hypothesize that neural migration from telencephalon to hippocampus is hindered by MeHg. The greater area of telencephalic DCX-coverage in MeHg-exposed birds could either be due to an increased production of neurons or an increased production of neurites to compensate for broken and/or poorly functioning microtubules in these neurites. The first mechanism is not consistent with the findings of this study in that we observe an increase in neither the number nor the size of DCX-expressing soma in MeHg-exposed birds compared to controls. The second mechanism is consistent with published evidence of hindered outgrowth of neurite branch lengths of migrating neurons in developmentally exposed organisms and *in vitro* studies (Fujimura et al. 2016, Nakada et al. 1981, Parran et al. 2003, Sass et al. 2001). Given the

high affinity MeHg has for binding to sulfhydryl groups, a major component of neural microtubules and membrane proteins, further work is necessary to know if lifelong MeHg exposure *in vivo* is inducing such damage in songbird cortical neurons.

MeHg also disrupts neural signal transmission via changes in neurotransmitter receptors, production, and function (van den Brink et al. 2018, Herden et al. 2008, Rutkiewicz et al. 2011, Scheuhammer et al. 2008, Yuan and Atchison 1997), and demyelination (Borg et al. 1970, Pass et al. 1975). Disruption of neural transmission and damage to neurites could also explain why previous work shows reduced spatial learning in MeHg birds without an accompanying change in neuron number. As recently reviewed by Bottini and MacDougall-Shackleton (2023), changes in N-methyl-D-aspartic acid and acetylcholinesterase functionalities are two areas that show sensitivity with MeHg exposure but are understudied in birds, particularly songbirds.

The observation of increased DCX-reactive density in the ventricular area but not the hippocampus also raises questions about energy allocation in organisms exposed to toxicants throughout their lives. MeHg-exposed birds could be expending more energy to overcompensate for neural loss in the hippocampus, yet they still are not able to learn at the same rate as unexposed birds. Neuron production and upkeep is energetically costly (Walton et al. 2012), and it has been shown that birds exposed to MeHg have reduced metabolic scopes (defined as the difference between basal metabolic rate and peak metabolic rate, Gerson et al. 2018). This added energetic demand could cause trade-offs in fitness. The cost of producing more neuron-associated proteins to compensate for reduced abilities of those neurons to migrate could be hindering other physiological processes in these birds.

Finally, we did not observe lower density of NeuN-reactive neurons in MeHg-treated birds as predicted. It could be that MeHg is inducing apoptotic and/or necrotic processes and

some dead cells were stained and counted, leading to an inflated number of NeuN-stained cells in MeHg-exposed birds (note that it is proposed that NeuN signal is reduced in apoptotic cells; see Gusel'Nikova and Korzhevskiy 2015). Quantifying apoptotic and/or necrotic cells in these tissues is necessary to confirm if this is the case.

Conclusions

While we saw neither significant effects on neural density in the hippocampus nor correlations between neuronal measurements and behavior, the hippocampus is not the only area of the brain involved in spatial cognition. Future work, for instance, could replicate these methods in the striatum, as altered reward-processing could be driving the behavior differences in MeHg-exposed birds (Newland et al. 2015). For birds in particular, MeHg-induced impaired cognition previously shown could have profound implications for food caching, migration, song learning and production, and other important behaviors, yet *in vivo* studies linking biomarkers from the molecular level to behavioral outcomes are too few to indicate a causal mechanism (Bottini and MacDougall-Shackleton 2023). We show that reduced neural density, as seen in rodent studies (Falluel-Morel et al. 2007, Sokolowski et al. 2013, Wu et al. 2016) does not appear to be the mechanism leading to impaired spatial cognition in songbirds, thus more research is still necessary to understand the mechanisms by which MeHg influences cognition and neurophysiological systems in passerines.

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CRediT authorship contribution statement

Cara N. Brittain: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, review & editing.

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Haruka Wada: Funding acquisition, Supervision, Investigation, Writing - review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 4: Ovarian and hormonal effects of chronic adult exposure to methylmercury in songbirds

*In preparation for submission to *Toxicological Sciences*

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Running head: Effects of mercury on female bird reproductive physiology

Abbreviations: AI, artificial intelligence; E2, estradiol; MeHg, methylmercury; RADD, Repair-assisted Damage Detection; ROIs, regions of interest

Keywords: estradiol, methylmercury, ovary, songbird, zebra finch

Abstract

Methylmercury is a ubiquitous contaminant long known to reduce reproductive success across taxa, yet the mechanism for the reduction is not understood. Songbird populations are proposed sentinels of mercury pollution in the environment, as their populations show sensitivity to methylmercury exposure on reproductive endpoints. We used adult-exposed female zebra finches as a model songbird to explore the effects of chronic, sublethal methylmercury exposure on ovarian morphology, DNA damage, and circulating estrogen levels, hypothesizing changes in these biomarkers could be involved in the mechanism by which methylmercury reduces reproductive success. After four months of environmentally relevant methylmercury exposure, we were able to observe morphologic changes in primary follicle ooplasm and granulosa cell layer but were unable to statistically substantiate these observations. We observed no difference in DNA damage in the ovaries of exposed finches compared to controls. We found that estradiol significantly decreased after four months of methylmercury exposure when ovary tissue area was included as a covariate. To our knowledge, ours is the first study to combine the use of artificial intelligence quantified histology, a suite of DNA damage markers, and a hormonal assay to deepen our understanding of methylmercury's effects on female reproductive physiology.

Introduction

While many pollutants plague the health of humans and wildlife today, mercury is especially concerning due to its ubiquity and longevity in the atmosphere and environment (Selin and Selin 2022). It is emitted into the atmosphere in large quantities from industrial facilities and mining, and from there, it can be carried long distances and deposited into aquatic and terrestrial environments. Once methylated by microbes, methylmercury (MeHg) bioaccumulates and

bioamplifies through the food chain. As a result, organisms, primarily those at the top of the food chain, can have mercury levels shown to lower reproductive performance, induce abnormal behaviors, and compromise physiological systems (Evers 2018, Scheuhammer et al. 2007, Wolfe et al. 1998). One group of organisms exposed to the toxicant at high levels, yet generally understudied in regard to the effects of methylmercury, is songbirds (Cristol et al. 2008, Cristol and Evers 2020). Songbirds are growing in popularity as environmental indicators; thus, it is important to understand how sublethal levels of mercury affect songbirds at the organismal and population levels (Jackson et al. 2015).

Mercury exposure decreases reproductive success in multiple ways. Specifically, mercury is linked to alteration of the timing of reproductive hormone release (Pollack et al. 2011) and lower fertility ratios (Rowland et al. 1994) in humans, reproductive disorders in cattle (Wrzecińska et al. 2021), and induction of polycystic ovary syndrome-like features in rats (Merlo et al. 2019). Proportion of wild European pond turtle (*Emys orbicularis*) gravid females negatively correlated with mercury concentration in the claws of turtles in contaminated environments (Beau et al. 2019). A similar pattern was shown in wild great egret (*Ardea alba*) colonies and captive dosed white ibises (*Eudocimus albus*), where an increase in feather MeHg content was associated with a reduction in the number of breeding pairs, even at levels lower than previously shown to reduce reproductive success (Zabala et al. 2020). In songbirds specifically, sublethal MeHg exposures reduce fledging success (reviewed in Whitney and Cristol 2017), lower breeding productivity (Heddle et al. 2020, Paris et al. 2018), and bias sex ratios in nests towards females (Bouland et al. 2012). Furthermore, MeHg concentrations linked to poor reproductive performance are seen in contaminated areas in the wild (Branco et al. 2022, Burger and Gochfeld 1997, Frederick and Jayasena 2011). Despite the overwhelming amount of

evidence that MeHg reduces reproductive success, there is little understanding of the mechanisms behind MeHg-induced reduced reproductive performance both in birds and in general, and there are relatively few studies examining the effects of mercury on female reproductive organs (Massányi et al. 2020).

MeHg's toxicity is notable in the neural system but extends to other physiological systems, such as disruption of endocrine function (Tan et al. 2009, Zhu et al. 2000), a possible mechanism for MeHg's effects on reproduction. Our group has shown that developmental mercury exposure can disrupt endocrine function of passerines, such as suppressed levels of corticosterone and thyroid hormones in nestling tree swallows living in a mercury-contaminated area (Wada et al. 2009). Decreased magnitude of adrenocortical responses with increasing blood mercury concentrations has also been noted in adult captive zebra finches (Moore et al. 2014). Although endocrine disruption by MeHg in females has been a proposed mechanism for reduced reproductive success (Bouland et al. 2012, Zabala et al. 2020), studies showing the effects of MeHg on sex steroid hormones are lacking. Estrogens are associated with several reproductive functions in birds, including regulation of calcium metabolism for shell formation, induction of its own and progesterone receptor expression, enhancing the growth of the oviduct, and control of secondary sex characteristics and sexual behaviors (Johnson 2015). As such, disruption of estrogen signaling is a plausible link between MeHg and reduced reproductive success in songbirds.

To this end, we exposed captive zebra finch (*Taeniopygia castanotis*) females to MeHg through diet and quantified ovarian morphology, DNA damage, and estrogen levels. Using zebra finches as a model songbird, we predicted that MeHg-exposed finches would sustain more DNA damage in the ovaries and have decreased circulating estrogen. As mercury is transported into

follicles of other avian species (Nishimura and Urakawa 1972), we predicted we would see greater damage in the follicles compared to other areas of the ovary.

Methods and materials

Animal care, exposure, dissection

Zebra finches breed easily in the lab setting, and their behavior, physiology, and genetics have been well-characterized, making them ideal models for controlled studies such as ecotoxicology studies (Zann 1996, Griffith and Buchanan 2010, Swaddle 2016). For this experiment, female zebra finches (N = 31) were raised in a same-sex colony at the Avian Research Laboratory 2 at Auburn University until the start of the experiment, prior to which the birds had no known previous exposure to MeHg. The birds were housed in 38 cm W x 46 cm D x 46cm H individual cages in groups of 2-3. Finches were given *ad libitum* access to water acidified with apple cider vinegar, cuttlefish bone, and their respective diets. The birds were originally maintained on blended seed (Kaytee Supreme Finch Food), and they gradually transitioned to the pelletized Zupreem fruitblend for canaries and finches (Shawnee, Kansas, USA). This occurred over the course of two weeks. For the first week, birds received a 50:50 seed:Zupreem blend. Seed was then reduced to 25% for four days and finally 0% for three days. The first two days on 100% Zupreem, the birds' health was monitored 3 times a day to ensure they were eating. Zupreem was mixed thoroughly with a solution containing water and cysteine. Additionally, the MeHg group had 1.2 µg/g MeHg added to the Zupreem on a wet-weight basis (N = 16). The dose concentration was chosen based on its environmental relevancy and known effects on reproduction (Cristol et al. 2008, Varian-Ramos et al. 2014). Fifteen birds served as controls. Consistency of the diet was ensured as outlined in Varian-Ramos *et al*. (2014). Blood

mercury level stabilizes on this diet in about 10 weeks (Varian-Ramos et al. 2014), and exposure occurred for four months after full transition to MeHg-laced diet. After four months, birds were humanely euthanized and dissected. Mass prior to euthanasia and the total time to dissect the bird was recorded. Because multiple birds were dissected in one day, the order in which a bird was dissected was also recorded.

In addition to the Zupreem diet, birds were given a supplement of BeneBac (PetAg Bene-Bac Plus Bird & Reptile Powder with FOS Prebiotic & Probiotics) mixed onto their food and vitamin drops (Wild Harvest Multi-Drops For All Birds High-Potency Vitamin Supplement) in their water once a week. Full-spectrum indoor lights came on at 7:00 AM Central Standard Time each day and were on a 14:10 light-dark cycle. All animal use was done with approval from Auburn University's IACUC (protocol #2019-3451).

Ovary slide preparation and AI ovary analysis

At the time of euthanasia, ovary mass was recorded then sections of ovaries were flash frozen in liquid nitrogen. When ovaries were prepared to be sectioned, the ovary was placed in 10% buffered formalin. Fixed sections were then paraffin-embedded and serially sectioned at 5µm. Three slides per bird were created from obtained sections. Two slides were left unstained for later DNA damage analysis, and one was stained with hematoxylin and eosin (H&E) stain.

H&E-stained slides were scanned with a Leica Versa 2000 digital slide scanner. Six birds were removed from the study because of lack of representative ovarian tissue in section (resulting in N = 13 control, 12 MeHg birds). Digital files of slides were loaded into Halo AI (Indica labs) digital pathology program. An artificial intelligence (AI) algorithm was created using nine classes of tissue and verified by an American College of Veterinary Pathologists

board certified pathologist. Tissue classes were created using terminology adapted from The Association of Avian Pathologists materials (Barnes et al. 2016). Tissues named in the algorithm are indicated in Figure 9. Area (μm^2) for each class was calculated and the percentage represented of each class was determined. Tissue classified as the "rest of the ovary" contains primary mesenchymal support tissue (stoma), blood vessels and lymphatics. Non-ovarian tissue, large vessels, kidneys, and others were excluded from analysis. The ovary, atretic follicles, vitelline follicles, follicular content, and dense bodies were utilized for subsequent analysis on DNA damage in these areas.

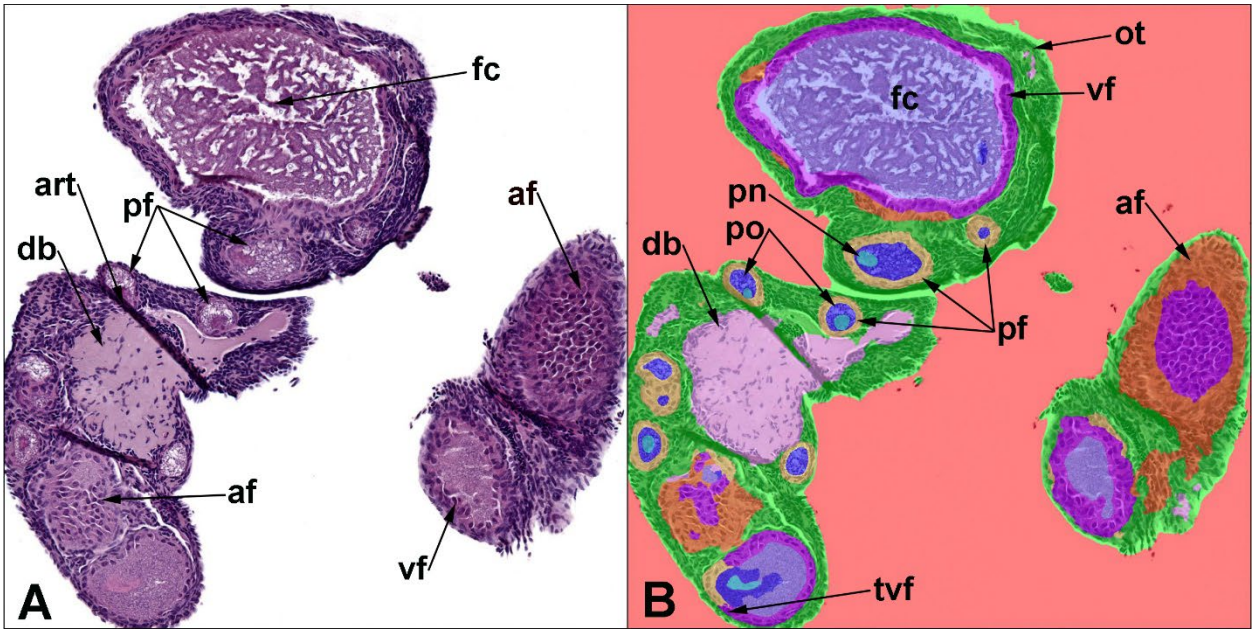

Figure 9. Images are from a control zebra finch ovary section; processing fold artifact (artifact = art) is observed in these images. - A. H&E-stained sections were used for histological analysis. B. An AI algorithm rendered color classifications of classified tissues that are listed in ascending order of prioritization. The lowest priority is the background (salmon pink), stroma of ovary medulla and cortex (lime green, ovary tissue = ot), theca and mesenchymal tissue of atretic follicles (orange-brown, atretic follicles = af), vitelline follicle granulosa cells (dark purple, vitelline follicles = vf), vitelline and atretic follicular content (lilac or light purple, follicular content = fc), post atresia follicular dense bodies (light pink, dense bodies = db), granulosa cells of primary follicle (yellow, primary follicles = pf), ooplasm of primary follicles (dark blue, primary follicle ooplasm = po) and nuclei of the primary follicle (aqua blue, nuclei of primary follicles = pn). The AI algorithm may have mixed color coding of follicles transitioning between stages (transitioning vitelline follicle = tvf).

Repair assisted damage detection (RADD)

RADD was performed as previously described in Lee *et al.* (2019). Slides of unstained ovary samples were deparaffinized, rehydrated, placed in glass Coplin jars with 200 mL of 10 mM sodium citrate (VWR, JT3646-1), and microwaved twice at 120 watts for 55 sec, with a 25 s rest between each, until the solution reached 60°C for antigen retrieval. Slides were cooled with five changes of water, briefly dried, and outlined with a PAP pen. For broad-spectrum DNA damage detection (Full RADD), all the RADD assay enzymes, Uracil DNA glycosylase (UDG), Formamidopyrimidine [Fapy]-DNA glycosylase (FPG), T4 Pyrimidine dimer glycosylase (T4PDG), 3-alkyladenine DNA glycosylase (AAG), and Endonuclease IV (EndoIV), were added to a lesion removal solution and incubated for 1 h at 37°C. The Full RADD broad-spectrum cocktail encompasses strand breaks, abasic sites, crosslinks, uracils, oxidative lesions, and alkylated bases. The gap-filling solution was added directly to the lesion removal solution and incubated for 1 h at 37°C. Then, slides were washed three times in phosphate-buffered saline (PBS, Hyclone, Logan, UT, USA, SH30028FS) for 5 min each and blocked in 2% BSA (Jackson Immuno, West Grove, PA, USA, 001-000-162) in PBS for 30 min at room temperature (RT, ~24°C). Anti-digoxigenin (Dig) antibody (Abcam, Cambridge, UK, #ab420 clone 21H8) was incubated at a dilution of 1:250 in 2% BSA in PBS at 4°C overnight. The following day slides were washed three times in PBS for 5 min each and incubated in Alexa Fluor 546 goat anti-mouse secondary (Life Technologies, Carlsbad, CA, USA, A11003) at a dilution of 1:400 in 2% BSA in PBS for 1 h at RT. For multiplexed experiments, after secondary for anti-Dig was added, the tissue sections were washed three times with PBS and then incubated with the DNA damage sensor marker anti-phospho Histone H2A.X (Ser139; γH2AX) Antibody, clone JBW301 conjugated with Alexa Fluor 647 1:750 (EMD Millipore, 05-636-AF647) in 2% BSA in PBS for

1 h at RT. Then, Hoechst 33342 (Life Technologies, Carlsbad, CA, USA, PI62249) was added at a final dilution of 1:1000 for 15 min at RT to stain the nuclei. Slides were washed three times in PBS for 5 min each, dried, mounted with coverslips using ProLong Gold Antifade reagent (Life Technologies, Carlsbad, CA, USA, P36930), allowed to dry overnight in the dark at RT, and visualized using a Nikon A1R confocal microscope or stored at 4°C until analysis.

Images were acquired using a Nikon A1r scanning confocal microscope with a PlanApochromat 10x/0.5 objective. Image acquisition settings were obtained for the Full RADD and γH2AX staining for ovary tissue and identifying gain settings that limited the number of saturated pixels. Each section was imaged at 10x with 1024 × 1024 resolution. All sections were verified as ovarian tissue by an avian pathologist prior to inclusion of the data analysis, and finch ovary tissue areas classified by AI algorithm were used to were used to define regions of interest (ROIs) to quantify damage in different classes of tissue in the ovary. The ROI size was kept consistent between birds and ovary tissue areas. Multiple ROIs for stroma of ovary medulla and cortex (ovary tissue), theca and mesenchymal tissue of atretic follicles (atretic follicles), vitelline follicle granulosa cells (vitelline follicles), and granulosa cells of primary follicle (primary follicles) were classified as described previously and the mean intensity for these areas determined for each sample. Higher fluorescent intensities indicate higher levels of overall damage.

Obtaining blood samples and estrogen ELISA

Birds were bled a total of four time points during this experiment - two days after habituation to isolated cages, after the two-week diet transition, two months after diet transition, and four months after diet transition. Starting at 8:00AM Central Standard Time for each

timepoint, brachial blood samples were obtained within three minutes of capture for each bird. After a 30 min period, in which birds were placed individually in an opaque paper bag, a second blood sample was obtained, and body mass was recorded.

We followed manufacturer instructions for the Estradiol (E2) ELISA Kit from Cayman Chemical (product no. 501890) to quantify estradiol in zebra finch plasma. Samples were randomly distributed across three plates (interplate variation = 17.72%; intraplate variation = 10.02%). Our previous data in zebra finches (unpublished) and published literature showed that estradiol levels do not significantly change within 30 minutes in other avian species (Gratto-Trevor et al. 1991, Canoine and Grinner 2005, Schoofs and Wolf 2011, Thorpe et al. 2014) so the baseline and T30 blood samples were pooled and used in the assay (N = 120). When possible, 20µL of plasma per sample was used. If 20µL was not available, the amount available was used (N = 29). For each plate, the threshold of optical density was determined by averaging three maximum binding (wells that receive only buffer, tracer, and antiserum) duplicates and subtracting two standard deviations from this mean, and the concentration of any samples with optical densities greater than these values were given the threshold limit value (4 out of 120 samples).

Statistical analysis

Treatment effects on data were compared using linear models in R (version 4.3.0). For all variables, models with all potentially explanatory variables recorded - body mass, total time to dissect the bird, dissection order, ovary mass, and eggs laid in cage over the course of the experiment - were compared. None of these variables significantly increased model explanatory

power (AICs within 2 scores) compared to a model with treatment alone as the fixed effect, thus only treatment was used as a fixed effect.

AI generated areas (μm^2) of ovary tissue, vitelline follicle granulosa cells, mesenchymal tissue of atretic follicles, follicular content, dense bodies, total area of primary follicles, granulosa cell of primary follicle, ooplasm of primary follicle, and nuclei of primary follicle for each individual were summed. To account for any difference in amount of available area to analyze between groups, the sum for each area was analyzed using linear models with the total area - sum of all areas - as a covariate. Log transforming the areas' sums significantly improved model fit and normality of residuals (AIC > 2 different compared to untransformed data), so log transformed data are reported.

As the mass of the ovary could particularly affect how much tissue was available to analyze, we initially included this variable in all models. It was removed as a covariate as it did not contribute significantly to model fit (AIC < 2 in all models compared to models only with treatment), and on average, MeHg-exposed and control birds did not differ significantly in their ovary masses ($p = 0.555$, $t = -0.600$). Sampled areas also differed arbitrarily due to position of tissue in the paraffin block resulting in non-controlled variability in total area of tissue observed histologically for each ovary.

The Full RADD, γH2AX, and UDG mean intensity values for replicate sections were averaged when available, and the final mean fluorescent intensity is reported as fluorescent intensity in arbitrary units. Linear models were used to test for differences between treatment groups' mean fluorescent intensities within each ovary area separately because areas of ovary usually differed from each other ($p < 0.05$, Table 10). DNA damage levels were different across the different regions, but this was not a function of treatment (Table 10).

	Full RADD fluorescent intensity			γH2ax fluorescent intensity			UDG fluorescent intensity		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	24387	-211260–260033	0.838	808693	591508–1025879	**<0.001**	296836	-122606 – 716279	0.163
Treatment [MeHg]	171773	-43897 – 387443	0.117	292994	104894–481094	**0.002**	127398	-258253 – 513048	0.513
Ovary tissue	1074880	7716230 – 1378130	**<0.001**	812550	548019 – 1077080	**<0.001**	1986484	1441133 – 2531835	**<0.001**
Atretic follicles	694108	394575 – 993642	**<0.001**	733129	471659–994599	**<0.001**	1167290	634217 – 1700363	**<0.001**
Primary follicles	347817	48260 – 647374	**0.023**	-186170	-489086 – 116746	0.227	386899	-151725 – 925522	0.157
Observations	93			286			90		
R^2 / R^2 adjusted	0.388 / 0.361			0.237 / 0.226			0.419 / 0.391		

Table 10. Summary of models comparing average fluorescent intensities in each area of the ovary. On average, the areas differed significantly from one another. In these models, vitelline follicle area was the reference group from which others were compared. Though primary follicle area did not differ significantly from vitelline follicle area in γH2ax or UDG fluorescent intensity, it did differ from all other areas (ovary tissue $p < 0.0001$, atretic follicles $p < 0.0001$).

To compare plasma estradiol over the four months, we used linear mixed models with bird ID and assay plate number included as random effects. As residuals were not normally distributed, plasma estradiol concentration was log transformed. We also correlated log estradiol concentration with area measured of ovary tissue and damage fluorescent intensities. Because follicles, especially smaller follicles, are the main sources of estradiol, we focused on vitelline follicles, vitelline content area, all primary follicles measures, and the total ovary area in our analyses. See Appendix 1 - Supplementary materials table 2 for analyses of quantified variables not included in manuscript. To account for total area, we divided the area by the total area measured. We used this proportion and treatment as covariates, testing for an interaction between covariates in all models and removing the interaction term if it was not significant ($p > 0.1$).

Results

Effects of chronic adult MeHg exposure on area (μm^2) of ovary tissue

We used AI algorithms to measure the area of ovarian tissue in birds exposed to MeHg in adulthood compared to unexposed birds. MeHg birds tended to have reduced areas of the ovary overall, but this was not statistically significant ($p = 0.0778$, $t = -1.850$, Fig. 10, Table 11). MeHg birds, on average, had decreased areas in the other measured portions of the ovary without considering total area measured, but this difference was not statistically significant in any portion when analyzed with total area measured as a covariate (Fig. 10, Table 11; atretic follicle area $p = 0.331$, $t = 0.995$; dense bodies area $p = 0.307$, $t = -1.048$; follicular content area $p = 0.285$, $t = -1.097$; ovary tissue area $p = 0.877$, $t = -0.157$; vitelline follicle area $p = 0.634$, $t = 0.483$). Furthermore, we tested for an effect of MeHg on the ratio of atretic follicles to vitelline follicles but did not see a significant difference ($p = 0.999$, $t = -0.001$, Table 11). Though treated birds tended to have smaller log ratios of vitelline content to vitelline follicles, they did not differ significantly from controls on average ($p = 0.0733$, $t = -1.881$, Table 11).

We examined the effects of adult MeHg exposure on the area of primary follicles, looking at individually annotated primary follicles and the total area. Though MeHg-exposed birds showed reduced areas for each variable without accounting for total area, none of these differed significantly on average compared to control birds when including total area as a covariate (Fig. 11, Table 12; nuclei $p = 0.213$, $t = -1.283$; ooplasm $p = 0.643$, $t = -0.470$; primary follicle granulosa cells $p = 0.172$, $t = 1.415$; total $p = 0.428$, $t = -0.808$). Our algorithm also counted primary follicles, nuclei within primary follicles, and primary follicle ooplasm. MeHg birds did not differ on average in the number of primary follicles ($p = 0.579$, $t = -0.563$, Table 12), primary follicular nuclei ($p = 0.219$, $t = -1.265$, Table 12), or ooplasm ($p = 0.567$, $t = -0.581$, Table 12).

We also analyzed these data after removing outliers (values <> 2SD from the treatment group mean for each variable; Appendix 1 - Supplementary materials table 1). After removing those birds, we found the same pattern as before those data points were removed in the log dense body area ($p = 0.162$, $t = -1.458$), in the log primary follicles area ($p = 0.133$, $t = 1.569$), in log ooplasm area ($p = 0.467$, $t = -0.741$), and in the log total primary follicle area ($p = 0.133$, $t = 1.569$). After removing outliers, neither ooplasm counted ($p = 0.991$, $t = 0.012$) nor nuclei counted ($p = 0.524$, $t = -0.649$) were significantly different between groups.

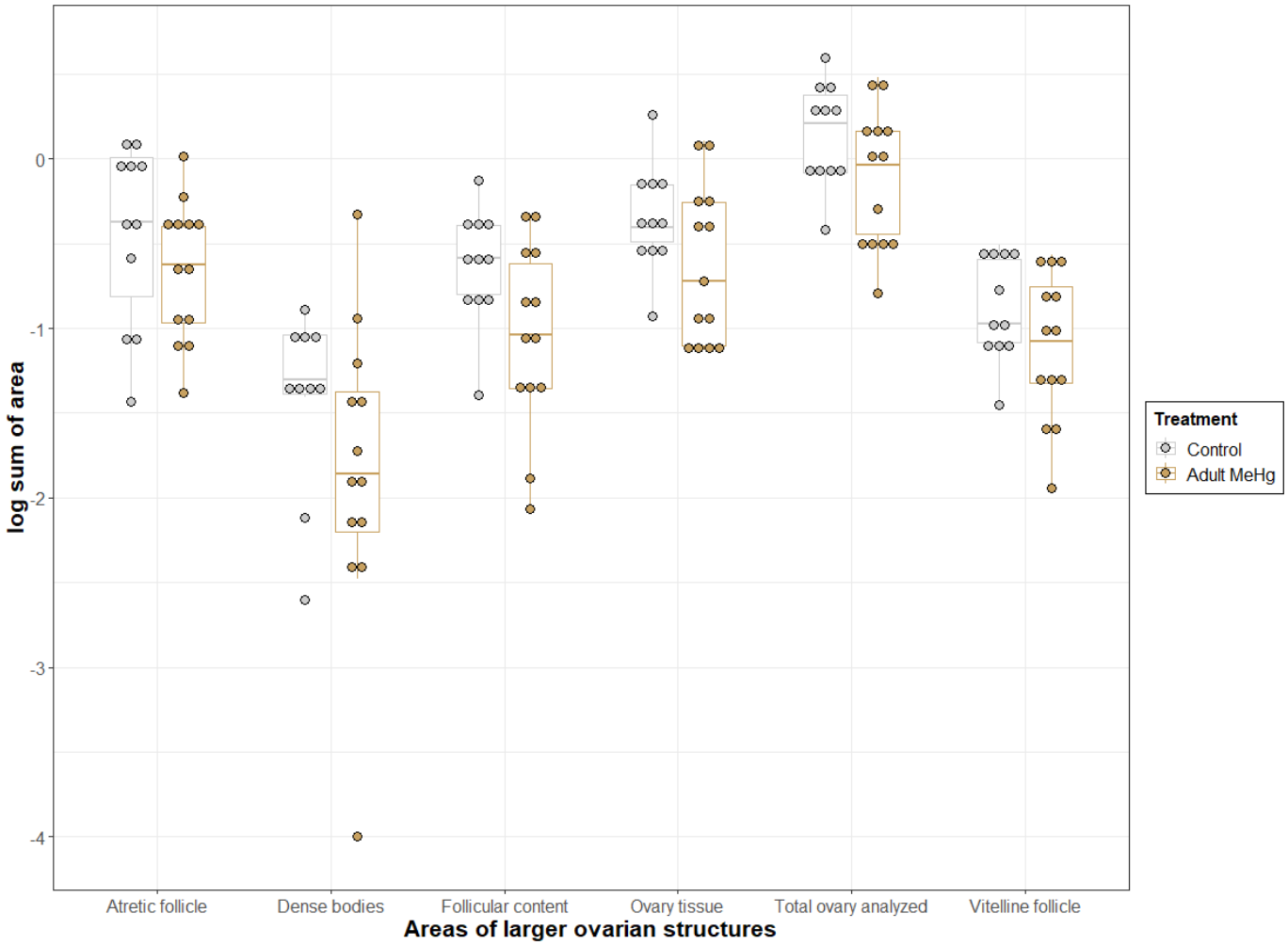

Figure 10. Generally, MeHg-exposed birds showed reduced measured area for each portion of the ovary without including total area analyzed; however, they did not differ significantly on average from control birds when accounting for total area ($p > 0.05$). Data for for all birds analyzed, including outliers, are shown. Control birds are indicated in grey (left bar at each area), and MeHg-exposed zebra finches are indicated by tan (right bar at each area).

	log Atretic follicle area			log Dense bodies area			log Ovary tissue area			log Follicular content area			log Vitelline follicle area			log Total Ovary area		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	-0.61	-0.75 – -0.47	**<0.001**	-1.52	-1.94 – -1.09	**<0.001**	-0.50	-0.62 – -0.37	**<0.001**	-0.77	-0.98 – -0.55	**<0.001**	-1.03	-1.14 – -0.92	**<0.001**	0.15	-0.08 – 0.38	0.183
Treatment [MeHg]	0.09	-0.10 – 0.29	0.331	-0.30	-0.90 – 0.30	0.307	-0.01	-0.18 – 0.16	0.877	-0.16	-0.46 – 0.14	0.285	0.04	-0.12 – 0.20	0.634	-0.27	-0.58 – 0.03	0.078
log total ovary	1.16	0.90 – 1.43	**<0.001**	1.03	0.22 – 1.84	**0.016**	0.98	0.75 – 1.21	**<0.001**	0.93	0.53 – 1.34	**<0.001**	0.95	0.74 – 1.16	**<0.001**			
Observations	24			23			24			24			24			24		
R^2 / R^2 adjusted	0.810 / 0.792			0.359 / 0.295			0.815 / 0.797			0.607 / 0.569			0.822 / 0.805			0.135 / 0.095		

	log ratio of Atretic follicles to Vitelline follicles			log ratio of Follicular Content to Vitelline follicles		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	0.45	0.26 – 0.64	**<0.001**	0.26	0.10 – 0.41	**0.002**
Treatment [MeHg]	-0.00	-0.26 – 0.26	0.999	-0.19	-0.40 – 0.02	0.073
Observations	24			24		
R^2 / R^2 adjusted	0.000 / -0.045			0.139 / 0.099		

Table 11. Summary of model outputs for the log area analyzed for the larger structures in the ovary and for the ratios of atretic follicles and follicular content to vitelline follicles. Bold typeface indicates statistical significance ($p < 0.05$).

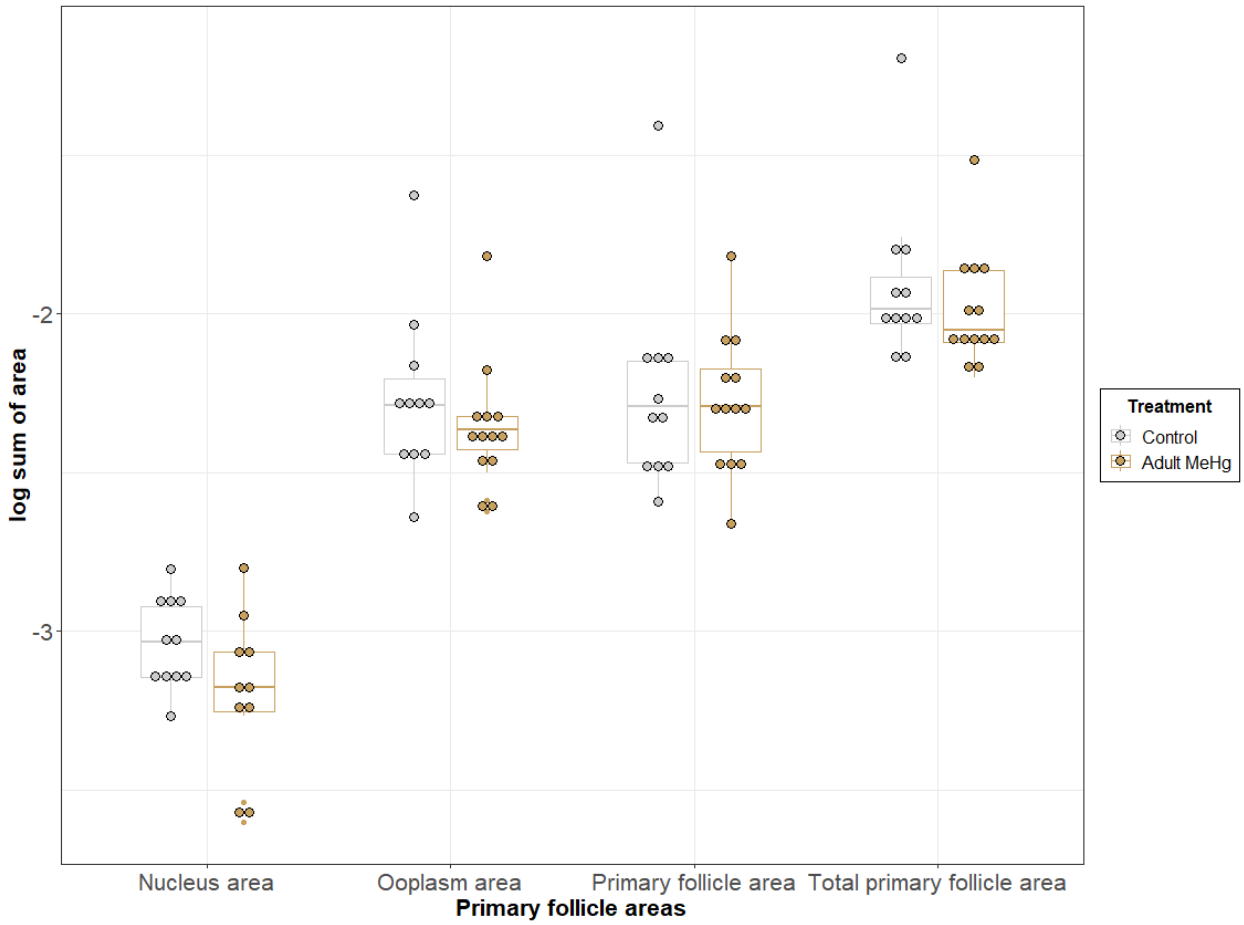

Figure 11. While on average MeHg-exposed birds displayed less area on average for most primary follicle variables when not considering total area measures, they did not differ significantly from control birds in any area of the primary follicles with total area included ($p > 0.1$ for all areas). Data for all birds analyzed, including outliers, are shown. Control birds are indicated in grey (left bar at each area), and MeHg-exposed zebra finches are indicated by tan (right bar at each area).

	log Nucleus Area			log Ooplasm Area			log Primary Follicle Area			log Total Primary Follicle Area		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	-2.80	-3.70 – -1.91	**<0.001**	-0.40	-0.76 – -0.04	**0.030**	-0.05	-0.42 – 0.31	0.763	-1.91	-2.05 – -1.77	**<0.001**
Treatment [MeHg]	-0.13	-0.33 – 0.06	0.156	-0.02	-0.10 – 0.06	0.643	0.05	-0.03 – 0.14	0.172	-0.07	-0.26 – 0.11	0.428
log total prim	0.12	-0.34 – 0.59	0.584	0.98	0.79 – 1.16	**<0.001**	1.15	0.96 – 1.34	**<0.001**			
Observations	21			24			24			24		
R^2 / R^2 adjusted	0.147 / 0.053			0.858 / 0.845			0.886 / 0.875			0.029 / -0.015		

	Primary follicles counted			Nuclei within follicles counted			Ooplasm counted		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	37.64	16.31 – 58.96	**0.001**	10.09	4.60 – 15.58	**0.001**	28.36	12.46 – 44.27	**0.001**
Treatment [MeHg]	-7.87	-36.84 – 21.11	0.579	-4.55	-12.02 – 2.91	0.219	-6.06	-27.66 – 15.55	0.567
Observations	24			24			24		
R^2 / R^2 adjusted	0.014 / -0.031			0.068 / 0.025			0.015 / -0.030		

Table 12. Summary of model outputs for the log area analyzed for each portion of the primary follicles, for the proportion of these areas to the total area, and for the counts of primary follicles, nuclei, and ooplasm. Bold typeface indicates statistical significance ($p < 0.05$).

Subjective lesion observations

Primary follicles in MeHg-exposed birds appear to have inconsistent ooplasm distribution throughout the follicle. While ooplasm in the control group has a lacy pattern with nuclear margins clearly defined, the MeHg exposure group typically had increased density of ooplasm, increased cuboidal morphology of granulosa cells and decreased definition in nuclear margins with decreased size and abundance of nuclei (Fig. 12). It is important to note, however, that these observations were not statistically analyzed.

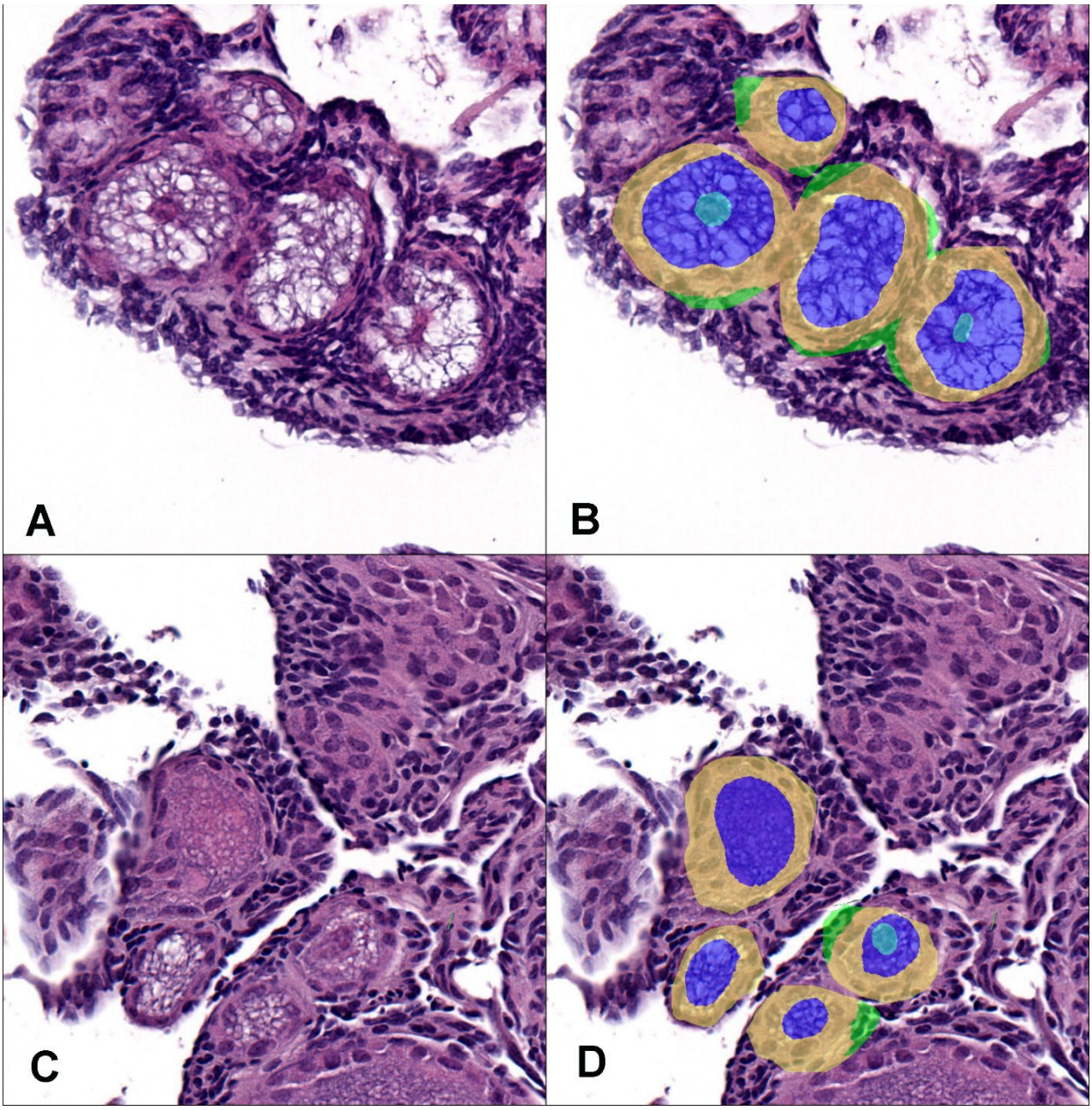

Figure 12. Finch ovary sections stained with H&E only (A and C) and with AI algorithm rendering color classifications (B and D) - A. As depicted in this representative control finch ovary section, primary follicle ooplasm (blue) in control birds appear to have consistent lacy, central, and well-defined nuclei. B. AI-classified image of the same section in 5A shows granulosa cells of primary follicles (yellow) have more thickness (not statistically analyzed) compared to MeHg exposure groups. C. As shown in this representative MeHg-exposed finch ovary section, follicular diameter in treated finch ovaries seemed reduced. Primary follicle ooplasm also appear to have increased density and loss of lacy pattern. D. AI-classified image of the same section in 5C shows granulosa cell layers of primary follicles in MeHg-exposed finch ovaries appear thickened and irregular.

Effect of chronic adult exposure to MeHg on DNA damage in the ovary

We tested whether chronic adult exposure to MeHg for 4 months would increase DNA damage in the ovaries of songbirds. RADD allows assessment of DNA lesion content by fluorescent labeling of DNA lesion sites within the genome. Tissues sections were imaged for fluorescent intensity, and ROIs for each tissue type of interest measured for fluorescent intensity. We did not observe significant differences in intensity with Full RADD, a broad spectrum measure of DNA lesions and strand breaks, between MeHg-exposed birds and control birds in the vitelline follicles ($p = 0.862$, $t = -0.176$, Fig. 13, Table 13), in the atretic follicles ($p = 0.161$, $t = 1.454$, Fig. 13, Table 13), in the primary follicles ($p = 0.545$, $t = -0.616$, Fig. 13, Table 13), or in the ovary tissue ($p = 0.200$, $t = 1.324$, Fig. 13, Table 13). In analyzing these data without outliers (Appendix 1 - Supplementary materials table 1), no significant differences were detected in average intensity in the vitelline follicles ($p = 0.452$, $t = -0.765$), or in the ovary tissue ($p= 0.481$, $t = 0.723$).

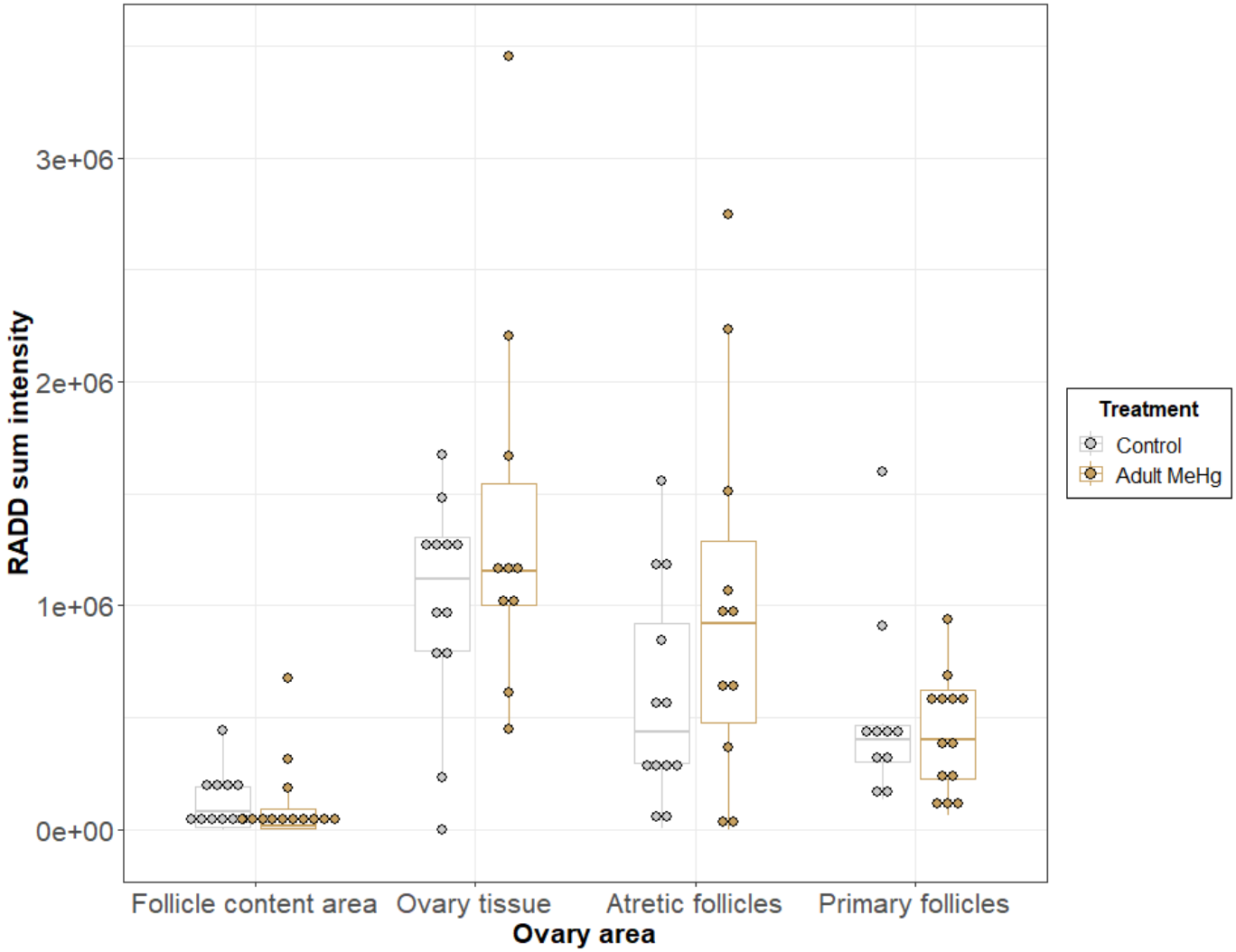

Figure 13. No significant difference in DNA lesions detected by Full RADD were observed between MeHg-exposed and control birds in any area of the ovary ($p > 0.1$ all areas). Control birds are indicated in grey (left bar at each area), and MeHg-exposed zebra finches are indicated by tan (right bar at each area). Data for all birds analyzed, including outliers, are shown.

	Vitelline follicles			**Ovary tissue**			**Atretic follicles**			**Primary follicles**		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	119818	20328–219309	**0.020**	998942	582047 – 1415838	**<0.001**	600441	186260 – 1014623	**0.007**	519738	293031 – 746446	**<0.001**
Treatment [MeHg]	-11749	-149718 – 126219	0.862	392487	-225869 – 1010842	0.200	418613	-180293 – 1017519	0.161	-89250	-390799 – 212299	0.545
Observations	25			22			23			23		
R^2 / R^2 adjusted	0.001 / -0.042			0.081 / 0.035			0.091 / 0.048			0.018 / -0.029		

Table 13. Summary of model outputs for Full RADD fluorescent intensities. Bold typeface indicates statistical significance ($p < 0.05$).

We also tested for DNA damage using the DNA damage sensing post-translation modification γH2AX. When single and double strand breaks occur, γH2AX is phosphorylated by ATM with increasing signal, indicating DNA damage. Again, we did not observe significant differences between treatment groups in fluorescent intensity in the vitelline follicles ($p = 0.301$, $t = -1.059$, Fig. 14, Table 14), in ovary tissue ($p = 0.224$, $t = 1.254$, Fig. 14, Table 14), in atretic follicles ($p = 0.222$, $t = 1.259$, Fig. 14, Table 14), or in primary follicles ($p = 0.968$, $t = -0.041$, Fig. 14, Table 14). We saw no significant differences in vitelline follicles ($p = 0.540$, $t = -0.622$), in ovary tissue ($p = 0.541$, $t = 0.666$), in atretic follicles ($p = 0.103$, $t = 1.716$), or in primary follicles ($p = 0.798$, $t = 0.259$) after removal of outliers (Appendix 1 - Supplementary materials table 1).

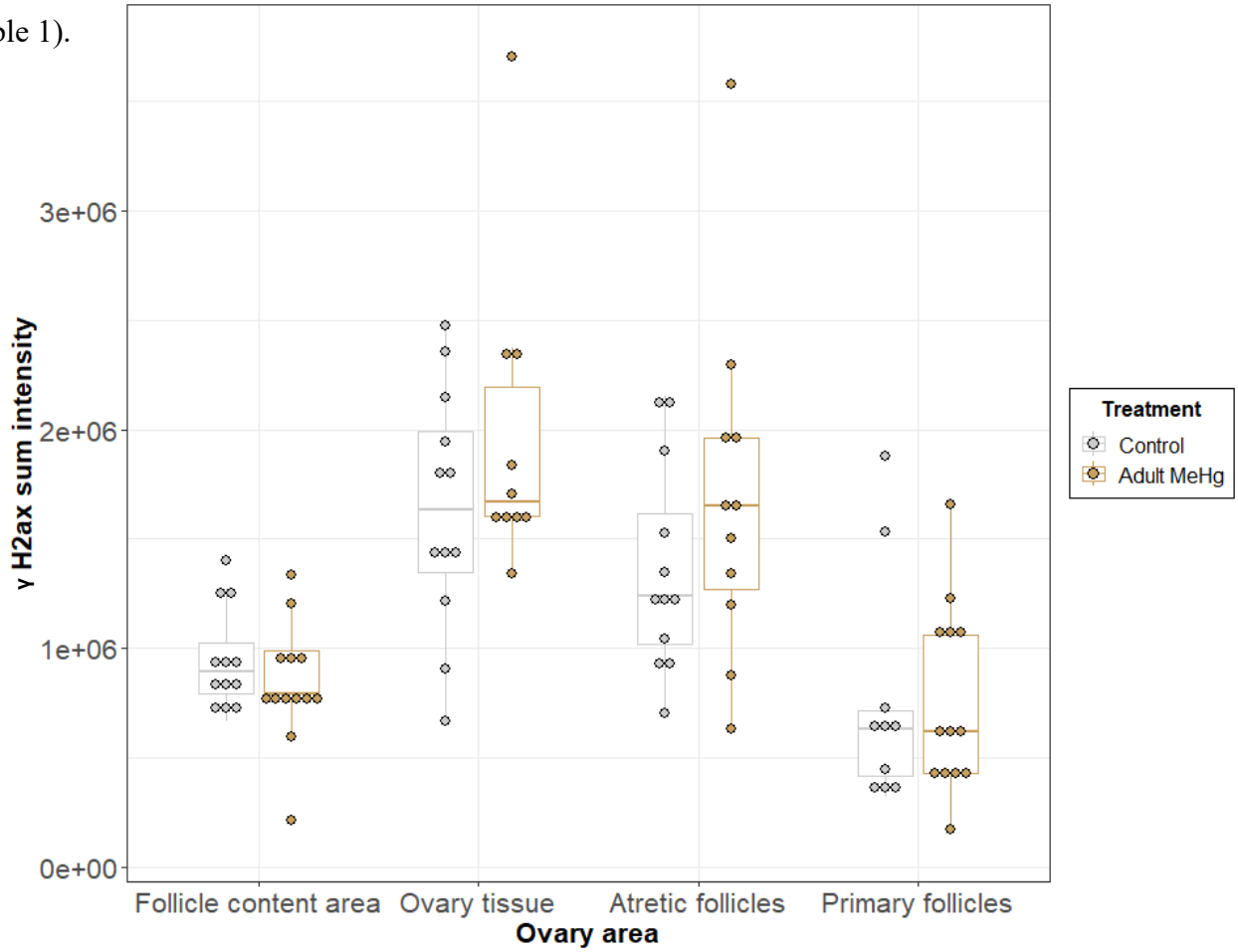

Figure 14. We observed no significant differences in γH2AX signal for any ovary area ($p > 0.1$ for all). Higher fluorescent intensity indicates higher levels of DNA damage. Control birds are indicated in gray (left bar at each area), and MeHg-exposed zebra finches are indicated by tan (right bar at each area). Data for all birds analyzed, including outliers, are shown.

	Vitelline follicles			Ovary tissue			Atretic follicles			Primary follicles		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	946945	794258 – 1099632	**<0.001**	1635437	1261776 – 2009097	**<0.001**	1357132	970318 – 1743947	**<0.001**	763837	455602 – 1072073	**<0.001**
Treatment [MeHg]	-108379	-320118 – 103359	0.301	333279	-220950 – 887508	0.224	338738	-220596 – 898071	0.222	-8103	-418094 – 401889	0.968
Observations	25			22			23			23		
R^2 / R^2 adjusted	0.046 / 0.005			0.073 / 0.027			0.070 / 0.026			0.000 / -0.048		

Table 14. Summary of model outputs for average γH2AX fluorescent intensities in different ovarian areas. Bold typeface indicates statistical significance ($p < 0.05$).

We further tested for DNA damage using uracil DNA glycosylase (UDG), which measures uracil, abasic sites, and strand breaks. Increasing intensity indicates uracil repair is not working as efficiently as compared to that of lower value intensities. Again, we did not observe significant differences between treatment groups in fluorescent intensity in ovary tissue ($p = 0.301$, $t = 1.063$, Fig. 15, Table 15) or in atretic follicles ($p = 0.717$, $t = 0.368$, Fig. 15, Table 15). In vitelline follicles, there were significant correlations with UDG mean intensity and the number order a bird was dissected in and the total time to dissect a bird ($p < 0.04$ for both variables, Table 15), but there was no significant effect of treatment on this measure of DNA damage ($p = 0.110$, $t = 1.670$, Fig. 15, Table 15). Also, there were significant relationships between UDG mean fluorescent intensity in primary follicles and ovary mass and the number of eggs laid in a cage during exposure ($p < 0.05$ for both variables, Table 15), but there was not a significant effect of treatment on this measure of DNA damage ($p = 0.993$, $t = -0.009$, Fig. 15, Table 15). We saw no significant differences in vitelline follicles ($p = 0.167$, $t = 1.441$) or in atretic follicles ($p = 0.415$, $t = -0.832$) after removal of outliers (Appendix 1 - Supplemental materials table 1).

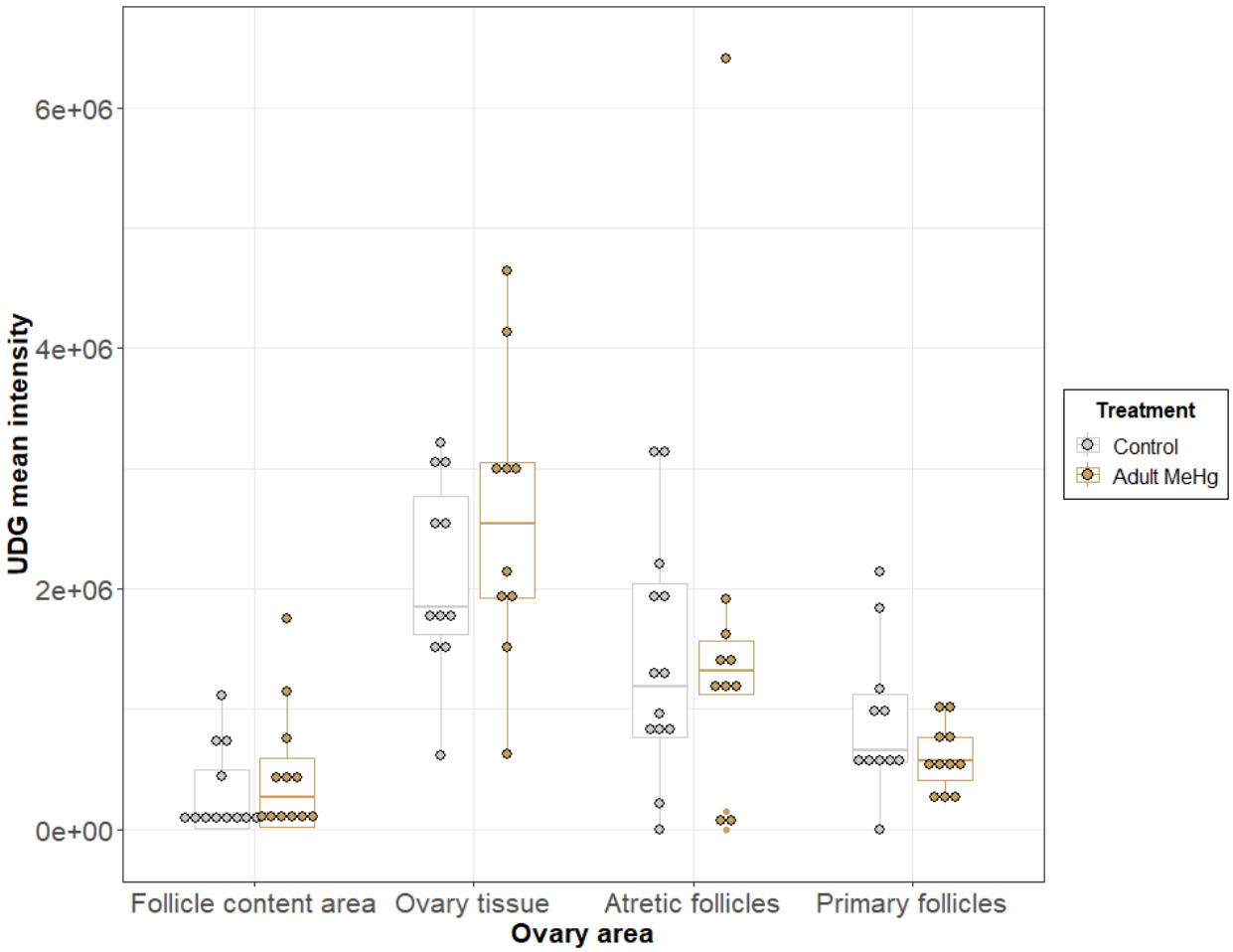

Figure 15. We observed no significant differences in UDG signal for any ovary area ($p > 0.1$ for all). Higher fluorescent intensity indicates higher levels of DNA damage. Control birds are indicated in gray (left bar at each area), and MeHg-exposed zebra finches are indicated in tan (right bar at each area). Data for all birds analyzed, including outliers, are shown.

	Vitelline follicles			**Ovary tissue**			**Atretic follicles**			**Primary follicles**		
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1530113	448637 – 2611588	**0.008**	2117432	14710501 – 2763813	**<0.001**	426247	626009 – 2226486	**0.001**	409435	112345 – 706525	**0.010**
Treatment [MeHg]	312538	-77759 – 702835	0.110	475763	-460932 – 1412458	0.301	214520	-999102 – 1428142	0.717	-1389	-318949 – 316171	0.993
Order dissected	-124146	-236009 – -12283	**0.031**									
Dissection time	-1295	-2513 – -76	**0.038**									
Ovary mass										5765577	271164 – 11259990	**0.041**
Eggs in cage										37380	4199 – 70561	**0.029**
Observations	24			21			23			21		
R^2 / R^2 adjusted	0.268 / 0.159			0.056 / 0.006			0.006 / -0.041			0.479 / 0.387		

Table 15. Summary of model outputs for average UDG fluorescent intensities in different ovarian areas. Bold typeface indicates statistical significance ($p < 0.05$).

Effects of chronic adult MeHg exposure on estrogen levels over time

We tested the effects of MeHg exposure during a 4-month chronic exposure in adulthood on log plasma estradiol concentrations (Fig. 16). We detected neither a difference between treatment groups ($p = 0.565$, $t = -0.582$, Table 16) nor between timepoints ($p = 0.762$, $t = 0.304$, Table 16). We also analyzed log estradiol concentration after removing outliers (those that were <> 2SD away from the treatment mean for each timepoint). The pattern remained the same after removing outliers where plasma estradiol did not differ between groups or over time (treatment $p = 0.490$, $t = -0.741$; time point $p = 0.623$, $t = -0.493$; Appendix 1 - Supplementary materials table 1).

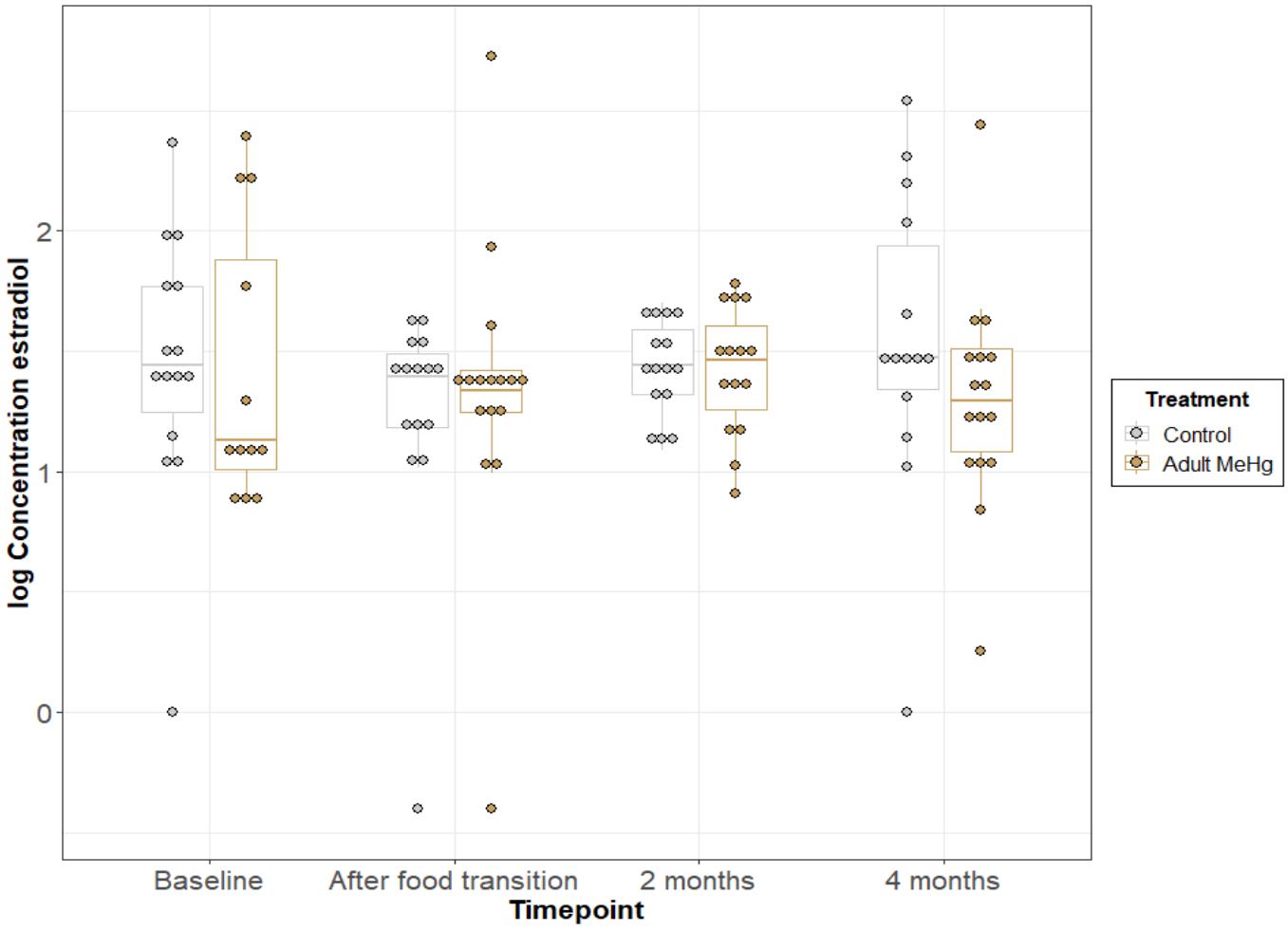

Figure 16. We did not observe any significant differences between treatment groups or over the course of the 4-month experiment. Control birds are indicated in gray (left bar at each timepoint), and MeHg-exposed zebra finches are indicated by tan (right bar at each timepoint). Data for all birds, including outliers, are shown.

	log E2 concentration		
Predictors	*Estimates*	*CI*	*p*
(Intercept)	1.45	1.24 – 1.66	**<0.001**
Treatment [MeHg]	-0.05	-0.24 – 0.14	0.595
Timepoint [after 2 week transition]	-0.13	-0.38 – 0.12	0.289
Timepoint [2 months after transition]	0.00	-0.25 – 0.25	0.997
Timepoint [4 months after]	-0.01	-0.26 – 0.24	0.931
Random Effects			
σ^2	0.23		
$\tau_{00\ ZEFI}$	0.01		
$\tau_{00\ Plate}$	0.00		
ICC	0.05		
N_{ZEFI}	31		
N_{Plate}	3		
Observations	118		
Marginal R^2 / Conditional R^2	0.017 / 0.068		

Table 16. Summary for models comparing treatment groups and timepoints with all data. Control birds at baseline are the reference group. Bold typeface indicates $p < 0.05$.

Correlating ovary morphology, DNA damage, and estradiol concentration

To understand the relationship between log estradiol concentration and ovary tissue morphology and damage, we tested for correlations between these variables. In measurements of the total ovary, we found that for every 1 μm^2 increase in total area measured, plasma estradiol concentration increased 26% ($p = 0.0255$, $t = 2.419$, Fig. 17, Table 17). Neither proportion of vitelline follicle granulosa cell area in the ovary ($p = 0.996$, $t = -0.005$, Table 17) nor proportion of vitelline content area ($p = 0.6791$, $t = 0.419$, Table 17) predicted log estradiol concentration. Looking specifically at the primary follicles, we found that for each 1% increase in proportional nuclei area, log estradiol concentration decreased significantly ($p = 0.0269$, $t = -2.379$, Fig. 18, Table 17). Other measures of primary follicles failed to predict log estradiol concentration (Table 17; total area of primary follicles $p = 0.8663$, -0.170; proportion of primary follicles area $p = 0.181$, $t = 1.384$; proportion of ooplasm $p = 0.872$, $t = -0.163$). Numbers of primary follicles ($p = 0.8411$, $t = 0.203$, Table 17), nuclei ($p = 0.651$, $t = 0.460$, Table 17), and ooplasm ($p = 0.591$, $t = 0.546$, Table 17) all failed to predict log estradiol concentration. On the other hand, with each

ovarian and primary follicular measure included, we found that MeHg-exposed birds tended to have about 50% less plasma estradiol (p-values near 0.05, Table 17). Analyzing these data without outliers did not change in pattern for any variable, but it did further confirm the treatment effect on plasma estradiol ($p < 0.05$ for all models except total ovary area, Appendix 1 - Supplementary materials table 3).

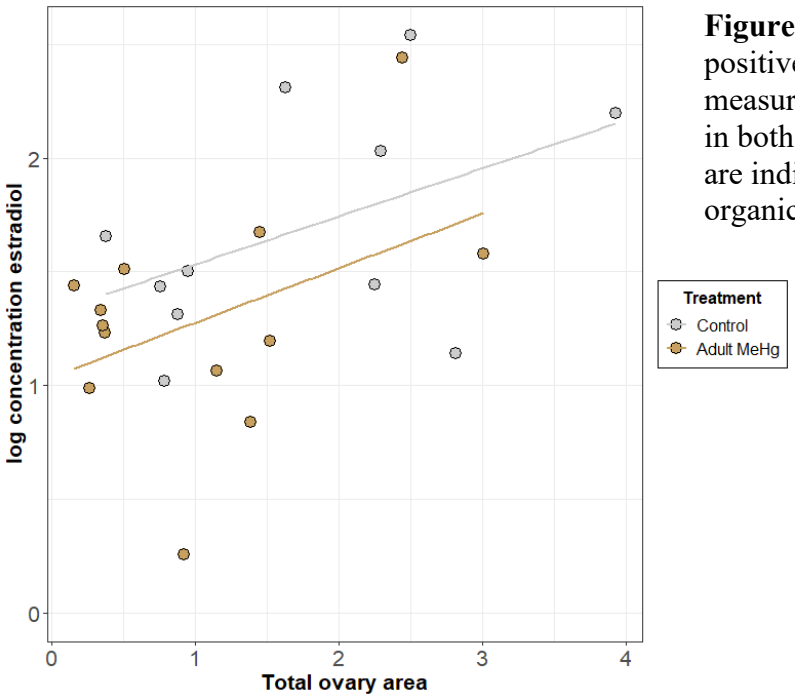

Figure 17. We observed a significant positive correlation between total ovary area measured and plasma estradiol concentration in both groups ($p = 0.0255$). Control finches are indicated in gray, and finches exposed to organic mercury are indicated in tan.

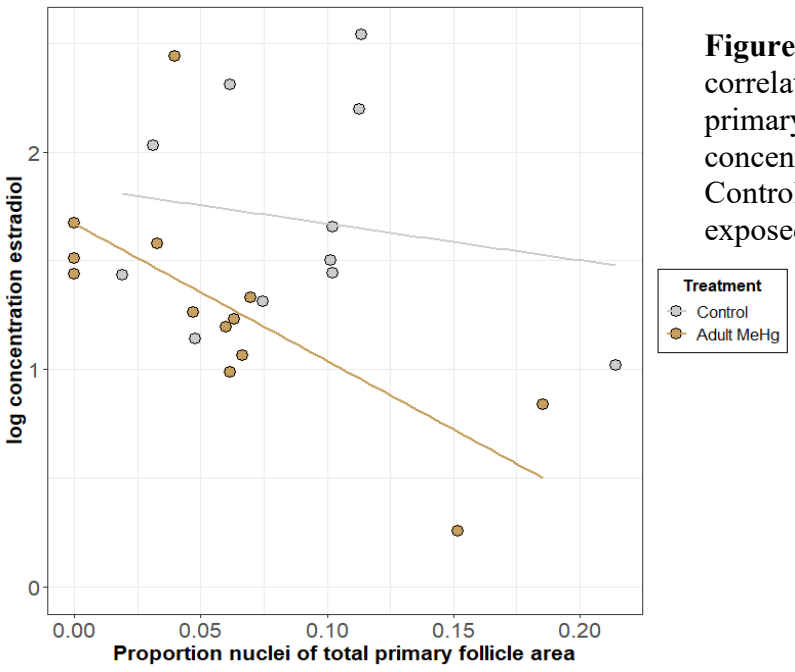

Figure 18. We found a significant negative correlation with the proportion nuclei of total primary follicle area and log estradiol concentration in both groups ($p = 0.0269$). Control finches are indicated in gray, and finches exposed to organic mercury are indicated in tan.

log Concentration Estradiol

Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.61	1.12 – 2.11	**<0.001**	1.69	1.05 – 2.33	**<0.001**	1.26	0.78 – 1.75	**<0.001**
Treatment [MeHg]	-0.39	-0.83 – 0.05	0.081	-0.40	-0.85 – 0.05	0.081	-0.24	-0.65 – 0.17	0.233
Proportion Vitelline Content	0.41	-1.64 – 2.47	0.680						
Proportion Vitelline Follicles				-0.01	-5.75 – 5.72	0.996			
Total Ovary							0.23	0.03 – 0.43	**0.026**
Random Effects									
σ^2	0.26			0.26			0.20		
τ_{00}	0.00 $_{Plate}$			0.00 $_{Plate}$			0.02 $_{Plate}$		
ICC	0.00			0.02			0.09		
N	3 $_{Plate}$			3 $_{Plate}$			3 $_{Plate}$		
Observations	24			24			24		
Marginal R^2 / Conditional R^2	0.139 / 0.141			0.132 / 0.145			0.294 / 0.360		

Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	2.07	1.63 – 2.51	**<0.001**	1.77	0.59 – 2.96	**0.005**	1.01	-0.06 – 2.08	0.062	1.71	1.26 – 2.16	**<0.001**
Treatment [MeHg]	-0.52	-0.93 – -0.11	**0.015**	-0.40	-0.84 - 0.04	0.074	-0.47	-0.90 – -0.03	**0.038**	-0.40	-0.85 – 0.04	0.074
Proportion nucleus	-4.29	-8.06 – -0.52	**0.028**									
Proportion ooplasm				-0.20	-2.73 –2.33	0.872						
Proportion primary follicle granulosa cell area							1.46	-0.75 – 3.67	0.182			
TOTAL Primary										-1.56	-20.76 –17.64	0.866
Random Effects												
σ^2	0.21			0.26			0.24			0.26		
τ_{00}	0.00 $_{Plate}$			0.00 $_{Plate}$			0.00 $_{Plate}$			0.01 $_{Plate}$		
ICC				0.01						0.02		
N	3 $_{Plate}$			3 $_{Plate}$			3 $_{Plate}$			3 $_{Plate}$		
Observations	24			24			24			24		
Marginal R^2 / Conditional R^2	0.306 / NA			0.133 / 0.144			0.200 / NA			0.133 / 0.152		

Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.66	1.24 – 2.08	**<0.001**	1.63	1.20 – 2.05	**<0.001**	1.62	1.20 – 2.04	**<0.001**
Treatment [MeHg]	-0.39	-0.83 – 0.05	0.080	-0.37	-0.82 – 0.08	0.103	-0.38	-0.82 – 0.06	0.085
Count primary follicles	0.00	-0.01 – 0.01	0.841						
Count nuclei				0.01	-0.02 – 0.03	0.651			
Count ooplasm							0.00	-0.01 – 0.01	0.592
Random Effects									
σ^2	0.26			0.26			0.26		
τ_{00}	0.01 $_{Plate}$			0.01 $_{Plate}$			0.00 $_{Plate}$		
ICC	0.02			0.02			0.01		
N	3 $_{Plate}$			3 $_{Plate}$			3 $_{Plate}$		
Observations	24			24			24		
Marginal R^2 / Conditional R^2	0.133 / 0.150			0.140 / 0.158			0.143 / 0.153		

Table 17. Summary of model outputs for correlating log estradiol concentration with given measured areas of the ovary. Bold typeface indicates statistical significance ($p < 0.05$).

DNA damage as quantified by Full RADD fluorescent intensity in the vitelline follicles did not correlate significantly with log estradiol concentration ($p = 0.154$, $t = 1.477$, Table 18), as was the case for the primary follicle area ($p = 0.513$, $t = 0.666$, Table 18). There was a marginally significant interaction ($p = 0.0592$) between γH2AX fluorescent intensity in the vitelline follicles and treatment such that the mean fluorescent intensity positively correlated with estradiol, but this effect differed between control and MeHg-exposed birds, so the groups were analyzed separately (Fig. 19). In control finches, there was a significant positive correlation between these variables ($p = 0.0225$, $t = 2.696$, Fig. 19, Table 18), but there was no relationship between these variables in MeHg-exposed birds ($p = 0.882$, $t = 0.153$, Fig. 19, Table 18). We found that log estradiol concentration positively correlated with UDG fluorescent intensity in primary follicles ($p = 0.0245$, $t = 2.451$, Fig. 20, Table 18) but had no significant relationship with UDG mean intensity in the vitelline follicles ($p = 0.455$, $t = 0.763$, Table 18). Removing outliers did not change the pattern for any of these variables (Appendix 1 - Supplementary materials table 3).

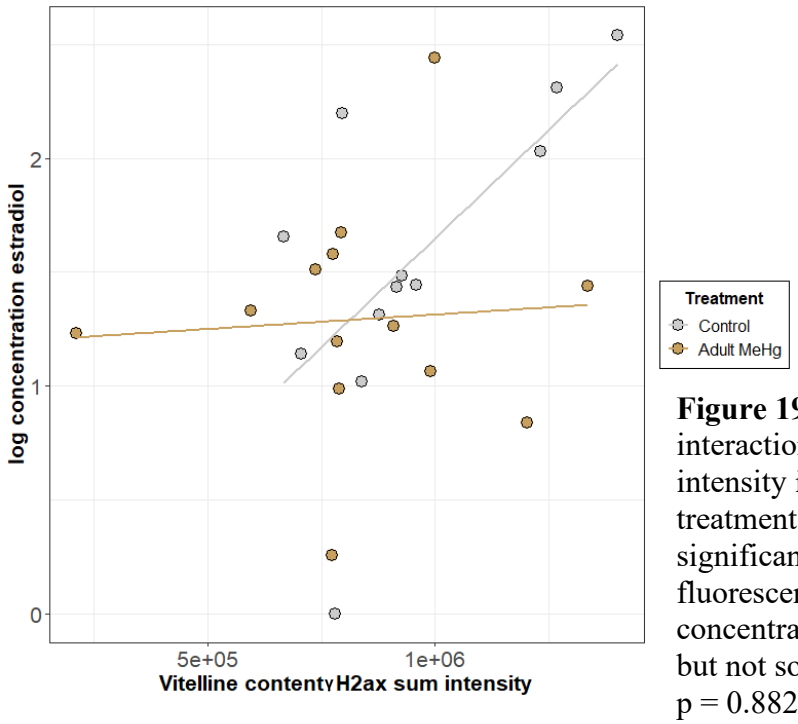

Figure 19. We saw a marginally significant interaction between γH2AX fluorescent intensity in the vitelline follicles and treatment ($p = 0.0592$), such that there was a significant positive correlation between fluorescent intensity and log estradiol concentration in controls (gray; $p = 0.0225$) but not so in the MeHg-exposed finches (tan; $p = 0.882$).

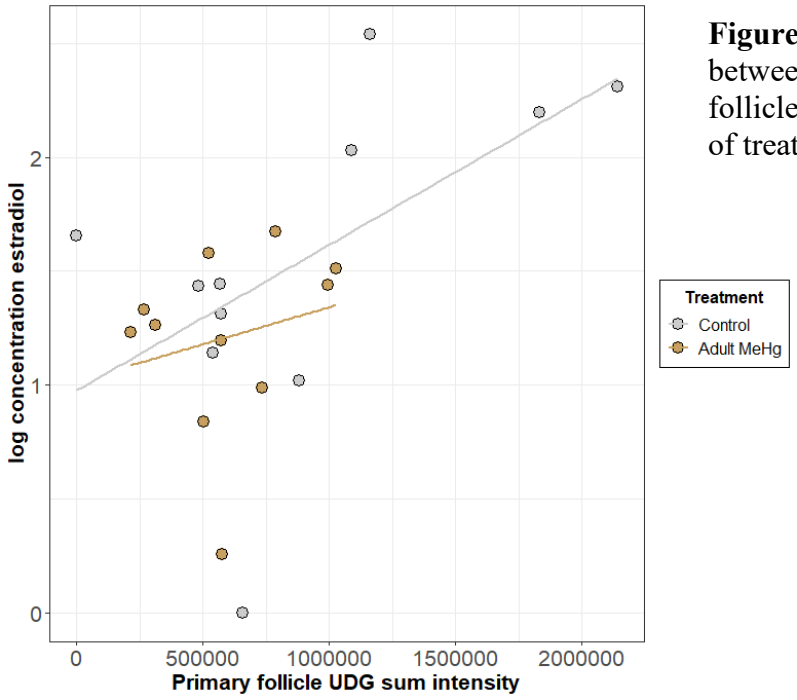

Figure 20. We observed a significant correlation between UDG fluorescent intensity in the primary follicles and log estradiol concentration independent of treatment (p = 0.0245).

log Concentration Estradiol						
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.42	1.02 – 1.81	**< 0.001**	1.39	0.78 – 2.01	**<0.001**
Treatment [MeHg]	-0.24	-0.73 – 0.24	0.312	-0.22	-0.78 – 0.33	**<0.001**
RADD intensity vitelline follicles	1.077e-06	-3.16e-07 – 2.47e-06	0.155			
RADD intensity primary follicles				2.65e-07	-4.62e-07 – 1.062e-06	0.514
Random Effects						
σ^2	0.34			0.39		
τ_{00}	0.00 $_{Plate}$			0.01 $_{Plate}$		
ICC				0.03		
N	3 $_{Plate}$			3 $_{Plate}$		
Observations	25			23		
Marginal R^2 / Conditional R^2	0.123 / NA			0.056 / 0.088		

Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	-0.26	-1.68 – 1.15	0.703	1.26	0.64 – 1.87	**<0.001**
Treatment [MeHg]	1.45	-0.31 – 3.20	0.100	-0.25	-0.79 – 0.30	0.351
γh2ax Vitelline follicles	1.91e-06	6.12e-07 – 3.21e-06	**0.012**			
γh2ax Vitelline follicles × Treatment	-1.78e-06	-3.45e-06 –1.14e-07	0.059			
γh2ax Primary Follicles				3.73e-07	-1.79e-07 – 9.20e-07	0.209
Random Effects						
σ^2	0.29			0.38		
τ_{00}	0.00 $_{Plate}$			0.00 $_{Plate}$		
ICC				0.00		
N	3 $_{Plate}$			3 $_{Plate}$		
Observations	25			23		
Marginal R^2 / Conditional R^2	0.273 / NA			0.107 / 0.108		

	log concentration E2					
Predictors	*Estimates*	*CI*	*p*	*Estimates*	*CI*	*p*
(Intercept)	1.47	1.03 – 1.92	**<0.001**	1.02	0.45 – 1.59	**0.002**
Treatment [MeHg]	-0.30	-0.82 – 0.23	0.248	-0.16	-0.65 – 0.33	0.497
UDG vitelline follicles	2.09e-07	-3.37e-07 – 7.31e-07	0.455			
UDG primary follicles				5.90e-07	1.29e-07 – 1.05e-06	**0.025**
Random Effects						
σ^2	0.36			0.27		
τ_{00}	0.03 $_{Plate}$			0.00 $_{Plate}$		
ICC	0.07			0.02		
N	3 $_{Plate}$			3 $_{Plate}$		
Observations	24			22		
Marginal R^2 / Conditional R^2	0.067 / 0.133			0.284 / 0.296		

Table 18. Summary of model outputs for correlating log estradiol concentration with DNA damage in measured estradiol-producing areas of the ovary. Bold typeface indicates statistical significance ($p < 0.05$).

Discussion

Does adult MeHg exposure alter finch ovarian morphology?

To our knowledge, ours is the first study to use AI histologic quantification on songbird ovaries. At first glance through our H&E slides with human eyes, it appeared that control birds had more healthy-looking follicles with more well-defined nuclei in the middle of the follicle, but this was not confirmed after statistical analysis of AI quantification of areas of ovarian tissue regions. AI analysis detected that MeHg-exposed birds tended to have reduced total areas overall but no reduction in any area of the ovary relative to the total area, including follicles. This result is similar to the pattern seen by Kim *et al.* (2019), where they observed no difference in the number of follicles produced in laying hens exposed to MeHg. It could be that more tissue-level disruption leads to empty space that our algorithm did not measure, as it was not ovarian tissue.

More histological analyses in general could yield great insights in understanding disruption of ovarian function when exposed to toxic and endocrine-disrupting contaminants like MeHg (Ottinger and Dean 2022). We propose AI histological analysis as a helpful tool in microscopy, reducing sampling bias. This study is only a beginning of using AI to quantify avian tissues, so continuing work is needed to refine AI measurement for bird tissue morphology.

As mercury exposure increased irregular follicular development and atretic follicles in rats (Merlo et al. 2019) and in laying hens (Ma et al. 2018), we predicted an increase in the area of atretic follicles in MeHg-exposed finches but did not observe this. There are several reasons this could be the case. Mercury chloride, the form of mercury administered in both Merlo et al. (2019) and Ma et al. (2018) may be more toxic to the ovary compared to MeHg (Tan et al. 2009). Our MeHg-exposed finches could also have a larger number of small atretic follicles. Our current data do not address this possibility. Other possible explanations are that if a follicle survives the initial toxic insult by MeHg, it is resilient against toxicity and will develop normally despite ongoing exposure and that damaged follicles were killed earlier. We were unable to differentiate these outcomes at the 4-month time point.

Does adult MeHg exposure induce DNA damage in zebra finch ovaries?

MeHg is known to induce oxidative stress and be genotoxic, damaging DNA and DNA repair proteins (reviewed in Yang et al. 2020). Yet, little work has been done on DNA damage in songbird tissues, so this area warrants more attention, especially in gonadal tissue, to further understand the mechanisms underlying reduced reproductive success in birds exposed to MeHg (Whitney and Cristol 2017). Contrary to our predictions that MeHg-exposed songbirds would sustain more DNA damage in the ovaries, we did not detect increased DNA damage in any area

of the ovary, as indicated by signal intensities for Full RADD, γH2AX, and UDG. Based on mercury transport into follicles (Nishimura and Urakawa 1972), we predicted we would see greater damage particularly in the follicles, but we did not observe a significant difference between groups. While our inability to detect differences in DNA damage could be due to our MeHg birds having less viable tissue to measure, it is also plausible the lack of damage may be reflective of adaptive repair or error-prone repair that results in somatic mutations, which could be addressed in future work by sequencing ovarian tissues to see if mutations were acquired as a result of MeHg exposure. Our study did not measure oxidative lesions or mutations, another class of DNA damage that is typically caused by MeHg exposure (reviewed in Antunes dos Santos et al. 2018).

Does adult MeHg exposure decrease circulating estrogen levels?

We predicted that MeHg-exposed finches have decreased circulating estrogen since altered estrogen production has been shown in rats and fish (reviewed in Tan et al. 2009). Looking only at circulating levels, it did not appear that four months of chronic MeHg exposure changed circulating estradiol levels in zebra finches; however, we did observe a difference in estradiol concentrations after four months of continuous MeHg exposure when adding ovary morphology as covariates, such that after four months MeHg had less estradiol than control birds. This result indicates that when studying endocrine effects, it is important to consider tissue-level effects. These females were visually and, for the most part, acoustically isolated from male finches. The overall lack of difference in estradiol between groups may be due to the lack of male signal and changes in estradiol concentrations might occur in zebra finches in a more natural social setting. Though it was not statistically significant, MeHg-exposed finches

decreased plasma estradiol in the last two months compared to previous time points, which could mean that MeHg would induce changes in estradiol with longer adult exposures.

It could also be that MeHg does not change the overall level of circulating estrogen in adult-exposed finches but rather the timing of hormone release, as is reported in healthy, premenopausal women in Pollack *et al*. (2011). A study that quantifies estrogen throughout the day and week in reproductive and non-reproductive female birds is necessary to discern if alterations in the timing of hormone release occur with MeHg exposure. This study also did not look at luteinizing or follicle stimulating hormones, which initiate signals that stimulate sex steroid production in theca cells (Johnson et al. 1996).

Does ovarian morphology and DNA damage correlate with estradiol levels, and does MeHg exposure alter this relationship?

We predicted that larger areas of ovary tissue, vitelline, and primary follicles would correlate with higher circulating estradiol levels and did see that a larger total ovary area measured correlated with higher log estradiol levels. Conversely, we found that larger proportions of nuclei to the total primary follicle measured correlated with smaller log plasma estradiol concentrations. We observed no significant relationships with either vitelline follicle areas or other primary follicles. Much work on the cellular level has shown that estrogen is primarily produced by theca cells in small follicles (Nitta et al. 1991, Robinson and Etches 1986, Tilly et al. 1991). More precise measurements of the size of individual follicles, quantification of theca cells, and larger sample sizes of small follicles in our study might have shed more light on the relationship between circulating estradiol and follicular area.

We also predicted that higher levels of DNA damage would correlate with lower circulating estradiol levels. We observed one interesting relationship in the vitelline content area, where in controls, fluorescent intensities of γH2AX were positively correlated with log estradiol concentration, but no relationship was seen in MeHg-exposed birds. Looking at UDG mean intensity in the primary follicles, we found a significant correlation between fluorescent intensity and estradiol levels regardless of treatment. Higher estrogen levels or unbalanced estrogen metabolism are reported to be associated with the risk of ovarian cancer and DNA damage to the ovary in humans (Brown and Hankinson 2015, Zahid et al. 2014), rodents (Roy and Liehr 1999), and laying hens (Hawkridge 2014), so our controls could be supporting this relationship and showing a predictable relationship in estrogen signaling, while MeHg-exposed birds are displaying an altered relationship between ovarian DNA damage and estrogen production. This relationship is altered in tree swallows exposed to environmental contamination, with birds in contaminated birds showing no differences in aromatase activity or blood DNA damage compared to birds at a reference site (Sitzlar et al. 2009). The risk of cancer is also modulated by progesterone (Ho 2003, Treviño et al. 2012), which was not quantified in this study but should be measured in future work.

Conclusions

One possible key to understanding reduced reproductive output in MeHg-exposed songbirds could be MeHg's effects on energetics. Finches exposed to MeHg have been shown to have reduced metabolic scopes resulting from increased basal metabolic rates and decreased peak metabolic rates (Gerson et al. 2019). Reduced metabolic scope can impact breeding attempts in adults as well as hatchlings who are exposed to MeHg *in ovo*. Adults could avoid

energetically costly behaviors such as breeding, nesting, and provisioning young. Hatchlings could have less energy available for pipping and begging for food. In addition to directly altering metabolic scope and energy available for the costly behaviors involved in reproduction and development, MeHg could potentially indirectly impact energy available for chicks via changes in yolk production and content. Yolk proteins synthesis is regulated in part by estrogens (Johnson 2015). By changing estrogen secretion, MeHg could subsequently change yolk deposition into eggs, impacting energy reserves necessary for hatchlings.

A final consideration is that birds more sensitive to MeHg toxicity might also be poorer breeders inherently. Work in zebra finches provides evidence that sensitivity to MeHg toxicity has a heritable genetic component that varies among individuals and families, giving the opportunity for selection to act on toxicity resistance (Buck et al. 2016, Varian-Ramos et al. 2013). Further, Elis *et al*. (2009) located five genes correlated with enhanced fertility in domesticated chickens. Future studies should determine if birds more resistant to MeHg toxicity that maintain levels of reproductive output similar to unexposed counterparts also upregulate these five fertility-linked genes.

It is important to note that while our study did not measure reproductive success, it was decreased using the same exposure dosage and duration in other studies, which observed decreased number of independent offspring produced in one year by MeHg-exposed zebra finches (Varian-Ramos et al. 2014). Thus, it is probable that our MeHg-exposed zebra finches would have displayed reduced reproductive success, and the parameters measured here either would not have explained this reduction or our data would have shown different patterns as a result of reproductive effort. While aiming to fill gaps in our knowledge of songbird ovary morphology and reproductive endocrinology following exposure to a common toxicant, our work

shows that there is still much work to do to uncover mechanisms behind reduced reproductive success in MeHg-exposed songbirds.

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CRediT authorship contribution statement

Cara N. Brittain: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, review & editing.

Dan Cristol: Funding acquisition, Visualization, Writing - review & editing.

Natalie Gassman: Investigation, Methodology, Validation, Writing - review & editing.

Kevin Lee: Methodology, Validation, Writing - review & editing.

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Haruka Wada: Funding acquisition, Supervision, Investigation, Writing - review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 5: Effect of lifelong methylmercury on circulating estrogen levels in the zebra finch, a model songbird

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Abbreviations: E2, estradiol; MeHg, methylmercury

Keywords: estradiol, methylmercury, songbird, zebra finch

Abstract

Much work has explored the neurotoxic effects of methylmercury, but exposure has also been shown to decrease reproductive success in multiple ways across taxa. We exposed zebra finches (*Taenopygia castanotis*) to environmentally relevant levels of methylmercury throughout their lives and quantified estrogen concentrations in their blood, predicting that MeHg disrupts estrogen signaling, decreasing the levels of hormone circulating in the body. We found no significant difference in average concentration of estradiol between groups. However, when

looking at mercury-exposed birds independently, there was a significant negative correlation between concentration of MeHg in the blood at time of euthanasia and estradiol. This preliminary study shows that more work is needed to understand the effects of MeHg in the endocrine system in songbirds to uncover mechanisms behind reduced reproductive success.

Introduction

Methylmercury (MeHg) is a prominent toxicant, well-known for disrupting cognition and neural function. Its effects on the neurological system have been the focus of many studies since the traumatic discovery of its neurotoxicity through multiple outbreaks of MeHg-poisoning in the mid-twentieth century (e.g., Bakir et al. 1973, Tsuda et al. 2009). Since these historic mercury poisoning outbreaks, it has been shown that MeHg has high affinity for proteins in the body and affects nearly every physiological system, accumulating in tissues throughout the body to various extents (Clarkson 1997, Finley et al. 1979, Scheuhammer 1988). Following neurotoxicity, perhaps the next most studied area regarding MeHg's toxicity is its effects on reproduction. Across taxa, at sublethal levels MeHg has been linked to altered sex ratios of offspring (Bouland et al. 2012, Matta et al. 2001, Sakamoto et al. 2001, Vorhees 1985), reduced reproductive output and fertility ratios (Beau et al. 2019, Burbacher et al. 1984, Hammerschmidt et al. 2002, Varian-Ramos et al. 2014, Zabala et al. 2020), and increased reproductive disorders (Drevnick and Sandheinrich 2003, Gerhard et al. 1998, Wrzecińska et al. 2021).

MeHg enters the food web primarily after methylation by microbes in aquatic environments, and for this reason, species associated with aquatic habitats have received more focused research attention. On the other hand, more recently, calls for study of terrestrial organisms have been increasingly answered. Particularly, there have been more efforts to

understand the effects of MeHg in avian species for their usefulness as bioindicators of pollution (Gómez-Ramírez et al. 2023). Songbirds are particularly good ecotoxicology models for their well-known neurobiology, behavior, and ubiquity (Cristol and Evers 2020, Jackson et al. 2015). The neural effects of many hormones important in reproduction, namely androgens and estrogens, in bird brains have been extensively studied, particularly in their influence on the vocal control system (Ball 1990), yet the effects of environmental contaminants on these hormones have not been given much attention. This is particularly true for estrogens, despite the key roles they play in reproduction (Wingfield and Silverin 2009).

Though MeHg has a noted effect on reproductive success at sublethal levels, the mechanism for this occurrence is not known (Diamanti-Kandarakis et al. 2009, Tan et al. 2009, Whitney and Cristol 2017). For species whose populations are in decline, like those of many songbirds, this mechanism is imperative to know in order to aid conservation and biomonitoring efforts. Some hypothesize that alteration of endocrine signaling due to MeHg exposure, particularly in females, could be a key factor leading to reduced reproductive success (Bouland et al. 2012, Drevnick and Sandheinrich 2003, Jayasena et al. 2011, Zabala et al. 2020). In this study, we hypothesized that MeHg disrupts estrogen signaling, altering the levels of the important reproductive hormone circulating in the body. A secondary aim of this study was to better understand sex differences in circulating estrogen and how male and female finches respond differently in their endocrinological responses to MeHg.

Methods and materials

Study species, animal husbandry and exposure regime

Zebra finches (*Taenopygia castanotis*) used in this study were raised in colonies at William & Mary. MeHg-exposed finches were exposed throughout their lifespans in this study, including *in ovo* via maternal deposition into the egg, as reported in Varian-Ramos *et al.* 2014. Control birds were never exposed to MeHg at any point in their lineages (average mercury blood concentration at death 0.034 ± 0.0087 µg/g, n = 12). All birds were fed a pelletized diet (Zupreem fruitblend for canaries and finches, Shawnee, Kansas, USA), mixed with a solution containing water and cysteine, with 1.2 µg/g MeHg added on a wet-weight basis for exposed birds (see Varian-Ramos et al. 2014). This dietary concentration of MeHg is an ecologically relevant level wild songbirds experience at MeHg-contaminated sites (Cristol et al. 2008, Abeysinghe et al. 2017) and a concentration that results in reduced reproductive success in this species (Varian-Ramos et al. 2014). Resulting average blood mercury levels in this study were 14.13 ± 0.47 µg/g (n = 56). In addition to *ad libitum* food, birds were given continuous access to water, oyster shell grit, and cuttlefish bone. Average age of birds at euthanasia was 470.7 ± 226.5 days. A 14:10 light:dark cycle with full spectrum indoor lights began at 8:00 Eastern Standard time each morning. All animal use was under approval of William and Mary IACUC protocol (2013-06-02-8721-dacris).

Blood sampling, estradiol quantification, and statistical analysis

Brachial blood samples were obtained within three minutes of capture for each bird. After this baseline blood sample, birds were restrained for 30 minutes in an opaque paper bag, and this blood sample was obtained (called "T30" blood sample). A blood sample was also taken the day of euthanasia to determine blood mercury concentration.

An Estradiol (E2) ELISA Kit from Cayman Chemical (product no. 501890) was validated for use with zebra finch plasma. When 25µL of baseline plasma was available, this time point and amount was used (n = 45). If this amount was not available, the baseline and T30 blood samples were pooled and used in the assay (n = 23, amount of plasma used per sample ranged from 25-50µL). Prior to analysis, we determined we could pool the samples based on estrogen levels before and after stressors in previous studies in other species (Gratto-Trevor et al. 1991, Canoine and Grinner 2005, Schoofs and Wolf 2011, Thorpe et al. 2014) and our own unpublished work during validation. Furthermore, baseline and pooled samples did not differ statistically in these samples ($p = 0.564$). For each plate, detection limit was determined by subtracting two standard deviations from the maximum binding averages in triplicates. The concentration of any samples with optical densities greater than these values were given the detection limit (9 out of 68 samples). Samples were randomly distributed throughout two plates (intraplate variation = 5.01%; interplate variation = 9.70%).

Log transformation of estradiol concentrations significantly improved model fit (AIC < 2 compared to untransformed data). Log concentration of estradiol in MeHg-exposed and control birds was compared using linear mixed models with the lme4 and lmerTest packages (Bates et al. 2015, Kuznetsova et al. 2017), and graphical figures were created using ggplot2 (Wickham 2016) in R (version # 4.3.0). A linear mixed model was used to compare estradiol concentration against concentration of blood mercury in treated birds. In all models, plate number was used as a random variable. Sex and age at euthanasia of the bird were included in initial models. Age was removed as they did not significantly contribute to model fit (age $p = 0.761$). Unless stated, sex was left in the model as a fixed effect. We analyzed our models with and without outliers.

Removing outliers only removed one MeHg-exposed bird with log estradiol concentration > mean + 2SD, and one exposed bird with blood MeHg concentration > mean + 2SD.

Results and Discussion

We predicted that MeHg-exposed zebra finches would show reduced levels of circulating estradiol due to disrupted estrogen signaling; however, we saw no significant difference in log estradiol concentrations between MeHg-exposed and control birds ($p = 0.142$, $t = 1.489$, Figure 21). This relationship seemed primarily driven by sex, as without sex in the model, MeHg-exposed birds had 29% more estradiol compared to controls, though it was not statistically significant ($p=0.0936$ $t = 1.702$). Studies looking at the influence of MeHg on hormone production and circulation in birds are lacking and the few that exist show varied results. Franceschini *et al.* (2017) found no relationship between MeHg burden and estrogen and testosterone levels, while Heath and Frederick (2005) reported a negative correlation with blood mercury and estradiol in female white ibises (*Eudocimus albus*). Jayasena et al. (2011) found that MeHg-related changes in estradiol differed across years in white ibises, but generally, exposed females displayed reduced estradiol concentrations, and exposed males displayed altered endocrine profiles that were related to altered mating behaviors. Hormone levels and heavy metal accumulation are both influenced by a number of factors such as sex, sensitivity, age, exposure dose, and breeding stage, and differences across studies could be due to these factors.

We also sought to understand if male and female finches had different levels of estradiol in their plasma, and if their hormonal response would differ following MeHg exposure. Females had 26% more circulating estradiol compared to males ($p = 0.0494$, $t = 2.003$; without treatment in the model $p = 0.0332$). Sex did not influence the effect of MeHg (p-value interaction term =

0.536) but looking only at control birds to determine baseline sex differences, we found no difference between male and female zebra finches in their log estradiol concentrations (p = 0.286, t = 1.135), meaning the overall difference in log estradiol concentrations could have been primarily driven by variation in MeHg-exposed birds.

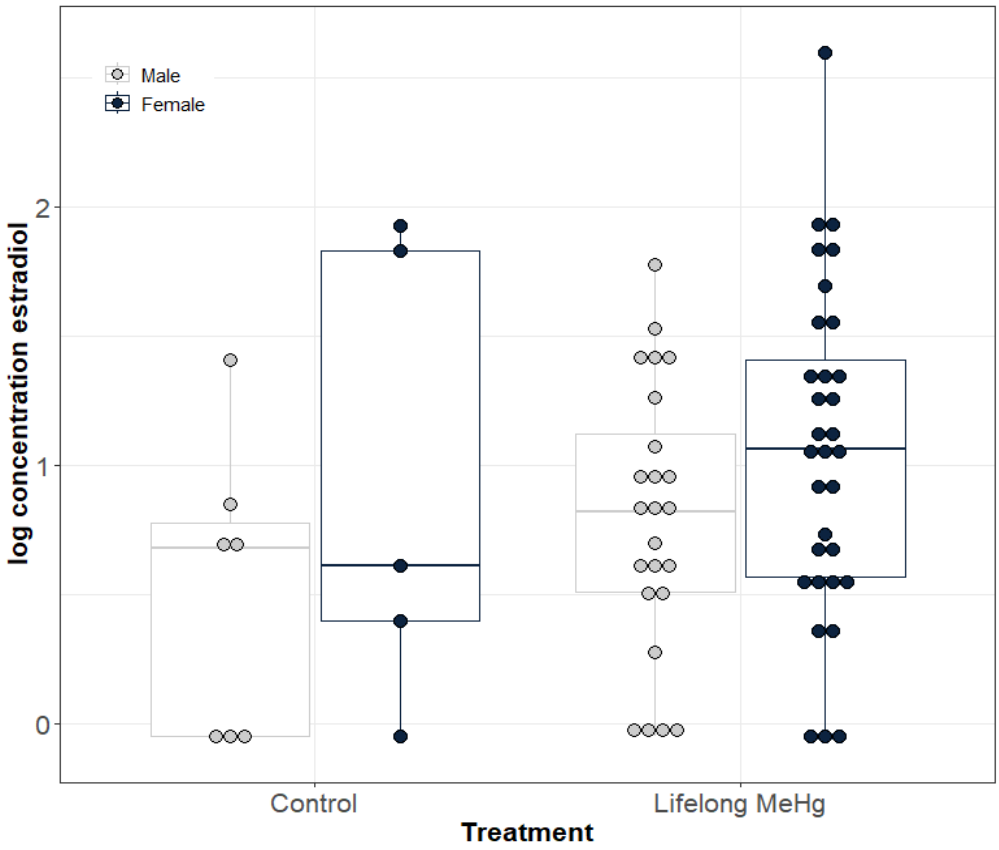

Figure 21. On average, we saw no significant difference in log E2 concentration between finches exposed to MeHg their entire lives (right bars) and controls (left bars, p = 0.142). In both groups, female birds had higher log E2 concentrations compared to male finches (p = 0.0332 in model with sex alone as a fixed effect; with treatment included in linear model p = 0.0494). There was not a significant interaction between sex and treatment (p = 0.526). Male finches are represented in gray, and female finches are represented in navy.

We correlated blood mercury levels and log estradiol and overall, found no significant relationship between these variables ($p = 0.962$, $t = -0.047$, Fig. 22a; p-value for the interaction between treatment and blood MeHg concentration = 0.299), which is not necessarily surprising given the lack of variance in plasma MeHg in controls. Alternatively, when looking only at MeHg-exposed birds, there was a significant negative correlation between blood MeHg and estradiol such that for every 1 μg/g increase in MeHg, estradiol decreased 3.6% ($p = 0.0346$, $t = -2.170$, Fig. 22b; after removal of outliers, $p = 0.0668$). Similarly, Adams *et al*. (2009) did not observe a significant linear relationship between MeHg dose and estradiol concentrations in laboratory-dosed juvenile white ibises. Rather, in Adams *et al.* (2009) a dose-response effect between MeHg and estradiol concentrations was supported, with birds administered an intermediate MeHg dose showing the greatest difference from controls compared to lower and higher doses. In our data, fitting a quadratic term to blood MeHg concentration did not significantly improve model fit (AIC scores within 2 of non-quadratic model, p-value of the quadratic term = 0.060), but this does not mean there is not a hormetic relationship. More intermediate values between 0-10 μg/g as well as MeHg concentrations beyond 25 μg/g would strengthen this relationship and improve the model.

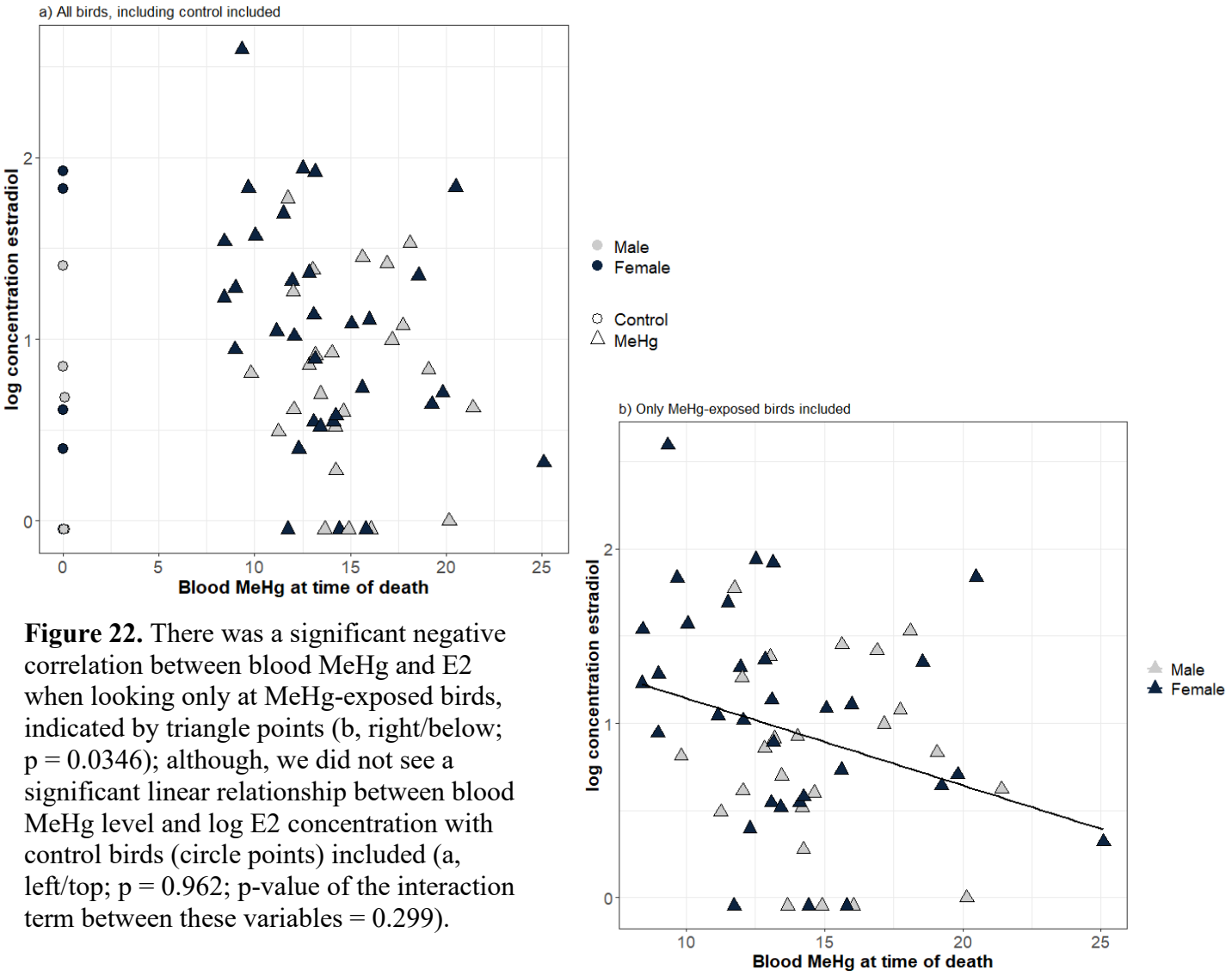

Figure 22. There was a significant negative correlation between blood MeHg and E2 when looking only at MeHg-exposed birds, indicated by triangle points (b, right/below; p = 0.0346); although, we did not see a significant linear relationship between blood MeHg level and log E2 concentration with control birds (circle points) included (a, left/top; p = 0.962; p-value of the interaction term between these variables = 0.299).

Conclusions

The results presented here show the importance of utilizing a combination of field studies that carefully quantify environmental levels of contaminants and multiple relevant biomarkers and laboratory studies using ecologically relevant exposure routes and doses to understand the impacts contaminants have on understudied organisms. While no reproductive endpoint was quantified in this study, previously the same dosing regime in captive zebra finches resulted in reduced fledging success and increased latency to renest, leading to overall reduction in number of offspring produced compared to controls (Varian-Ramos et al. 2014). As such, it is probable the birds in this study would have displayed similar reduced reproductive success, and this reduction would have been due to more than MeHg influencing estrogen signaling. Furthermore, differences in reproductive hormones might have been seen had our birds been breeding or given sexual stimuli. Our results also indicate that birds at higher trophic levels or experiencing exposures greater than those used in our study could show differences in estrogen levels compared to unexposed birds. Based on our results, more work should be conducted to elucidate the relationship between toxicant exposure and hormonal response, particularly hormones responsible for reproduction.

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CRediT authorship contribution statement

Cara N. Brittain: Conceptualization, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing - original draft, review & editing.

Dan Cristol: Funding acquisition, Writing - review & editing.

Mary Mendonça: Methodology, Validation, Writing - review & editing.

Thalia Williamson: Investigation, Writing - review & editing.

Haruka Wada: Funding acquisition, Supervision, Investigation, Writing - review & editing.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Chapter 6: General conclusions

As a result of historic outbreaks of methylmercury-induced neurotoxic disease and extensive research on exposure during development, the developing brain has long been known to be particularly vulnerable to methylmercury's neurotoxicity (Heinz and Locke 1976, Kendricks et al. 2022, Newland et al. 2008), and it has been shown that in many regards, adults are more tolerant to the contaminant's effects at sublethal levels (Kakita et al. 2000, Null et al. 1973, Weiss 2006). The results from my studies confirmed developmental exposure is necessary for precipitating adverse effects. I found that zebra finches exposed throughout their entire lives in a laboratory setting took longer to learn a spatial cognition task and performed worse on spatial memory tests on two different spatial scales. I also found that the increased number of trials required to pass the spatial learning task was due to methylmercury exposed birds being more likely to return to unrewarding food locations after locating rewarding blocks than controls. Neither adult- nor developmental-only exposure impaired spatial cognition, indicating that developmental exposure is necessary but not sufficient to induce these behavioral changes. In lifelong-exposed finches that required more trials to pass a spatial learning task, I saw no differences in hippocampus volume or in neural densities in the hippocampus. I did, however, find that methylmercury exposure increased immunolabeling of doublecortin, a protein expressed in immature neurons, in an area of the telencephalon known to be neuro-generative, though this increase was associated with neither increased neural number nor cell body size. This result suggests that finches exposed to methylmercury may be generating more microtubules, perhaps to compensate for damaged microtubules unable to assist in migration.

Similar to my neuroanatomical results, I found no significant differences in ovarian tissue morphology or DNA damage when finches were exposed during adulthood to a concentration

and duration similar to that of birds that displayed reduced reproductive success (Varian-Ramos et al. 2014). Overall, we found no changes in levels of circulating estradiol as a result of chronic adult exposure unless ovary tissue measurement was included as a covariate, in which case we found that after four months of exposure, adult zebra finches had reduced levels of estradiol compared to controls. In finches exposed to methylmercury their entire lives, we found no significant difference in log estradiol concentration between finches exposed to methylmercury their entire lives and controls. However, within the methylmercury exposed group, we did observe a significant relationship in birds exposed to methylmercury such that as blood methylmercury concentrations increased, log estradiol concentrations decreased. This indicates that lifelong exposure to methylmercury at concentrations greater than 1.2 ug/g could disrupt estrogen signaling in songbirds, reducing circulating concentrations of estradiol, an important reproductive hormone.

Though many of the predictions of my experiments were not met, results from these studies are useful in setting the stage for future work. Considering the likelihood that organisms could experience exposure only during adulthood or throughout their lives, it is vital to continue researching these exposure timepoints. For example, in songbirds, adult exposure could occur only at stops along migratory pathways or as habitats become newly contaminated and a bird does not disperse out of the contaminated habitat. Lifelong exposure could occur in songbirds that are born into contaminated sites and do not disperse from them. The results presented in this dissertation differed based on time point of exposure, confirming that timing of mercury exposure influences its consequences on behavior and physiology. These results highlight the importance of continued study of various timepoints of exposure to better understand methylmercury's effects across the lifespan and during adulthood.

This work also shows the importance of integrating a suite of biomarkers from the molecular to the organismal level in studies. Methylmercury could be disrupting physiology in ways too small to detect statistical differences but that accumulate to cause organism and population-level changes. For instance, I saw that birds exposed to organic mercury had lower mean areas measured in each segment of the ovary, but the difference was negligible statistically. Also, my data showed no effect of methylmercury on circulating estradiol concentration until accounting for measurement of ovary area, after which we found a significant decrease in log estradiol after four months of methylmercury exposure. In summary, the work presented in this dissertation underscores how much more is needed to learn about methylmercury exposure throughout life and in adulthood and that it is imperative to look across levels of organization from the molecular to organism to better comprehend the extent of organic mercury's consequences.

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Appendix 1: Supplemental materials for Chapter 4

Supplemental table 1. Summary of estimates and p-values before and after removal of outliers (outside mean ± 2SD) for the effects of treatment on area measured of given ovarian sections, DNA damage in different areas of the ovary, and log estradiol concentration. **Bold typeface** indicates statistical significance ($p < 0.05$); "~" indicates p-value < 0.1.

	Variable	Outliers removed (*df* after outliers removed)	Estimate (p-value) before outliers removed	Estimate (p-value) after outliers removed
Area larger structures of ovary	Log total ovary area	1 Control 2 MeHg (*19*)	Treatment: -0.27 (0.078)	Treatment: -0.260 (0.132)
	Log vitelline follicle area	0	-	-
	Log follicle content area	0	-	-
	Log atretic follicle area	0	-	-
	Log dense bodies area	1 Control 2 MeHg (*18*)	Treatment: -0.30 (0.307) **Log total: 1.03 (0.016)**	Treatment: -0.292 (0.162) **Log total: 0.772 (0.00934)**
	Log ovary tissue area	0	-	-
Area of primary follicle components	Log total primary follicle area	1 MeHg (*21*)	Treatment: -0.07 (0.428)	Treatment: -0.112 (0.194)
	Log primary follicle granulosa cell area	1 Control (*19*)	Treatment: 0.05 (0.172) **Log total: 1.15 (<0.001)**	Treatment: 0.0658 (0.133) **Log total: 1.221 (<0.001)**
	Log nuclei area	0	-	-
	Log ooplasm area	1 Control 1 MeHg (*19*)	Treatment: -0.02 (0.643) **Log total: 0.98 (<0.001)**	Treatment: -0.0302 (0.467) **Log total: 0.989 (<0.001)**
Counts of primary follicle components	Count primary follicles	0	-	-
	Count nuclei	1 Control (*21*)	Treatment: -4.55 (0.219)	Treatment: -1.662 (0.524)

	Count ooplasm	1 Control (*21*)	Treatment: -6.06 (0.567)	Treatment: 0.108 (0.991)
Full RADD mean intensity	Full RADD mean intensity vitelline follicles	2 Control 1 MeHg (*21*)	Treatment: -11750 (0.862)	Treatment: -29735 (0.453)
	Full RADD mean intensity primary follicles	0	-	-
	Full RADD mean intensity atretic follicles	0	-	-
	Full RADD mean intensity ovary tissue	1 Control 1 MeHg (*15*)	Treatment: 392487 (0.200)	Treatment: 132069 (0.481)
γH2AX mean intensity	γH2AX mean intensity vitelline follicles	1 MeHg (*22*)	Treatment: -108379 (0.301)	Treatment: -56221 (0.54)
	γH2AX mean intensity primary follicles	1 Control 1 MeHg (*18*)	Treatment: -8103 (0.968)	Treatment: 40229 (0.798)
	γH2AX mean intensity atretic follicles	3 MeHg (*19*)	Treatment: 338738 (0.222)	Treatment: 339212 (0.103)
	γH2AX mean intensity ovary tissue	1 MeHg (*19*)	Treatment: 333279 (0.224)	Treatment: 140593 (0.514)
UDG mean intensity	UDG mean intensity vitelline follicles	1 Control 1 MeHg (*18*)	Treatment: 312538 (0.110)	Treatment: 203134 (0.167)
	UDG mean intensity primary follicles	0	-	-
	UDG mean intensity atretic follicles	1 MeHg (*20*)	Treatment: 214520 (0.717)	Treatment: -314753 (0.415)
	UDG mean intensity ovary tissue	0	-	-
Concentration estradiol (reference =	**Log concentration E2 - baseline**	**2 Control 1 MeHg**	Treatment: -0.0563 (0.565; *df - 29*) Timepoint: 0.0121	Treatment: -0.0640 (0.465; *df = 28*) Timepoint: -0.0151 (0.623; *df*

baseline timepoint)	Log concentration E2 - after food transition	1 Control 2 MeHg	(0.762; *df - 89*)	*= 83*)
	Log concentration E2 - 2 months after transition	2 MeHg		
	Log concentration E2 - 4 months after transition	2 MeHg		

Supplemental table 2. Summary of estimates and p-values for correlation of log estradiol with components not included in main text – outliers included. **Bold typeface** indicates statistical significance ($p < 0.05$); "~" indicates p-value < 0.1.

	Variable (*df*)	Estimate	p-value
Area larger structures of ovary	Proportion atretic follicle area (*20*)	Proportion area: -0.00683 Treatment: -0.397 Interaction term: removed from model	0.993 0.0745 ~ 0.394
	Proportion dense bodies area (*20*)	Proportion area: -6.222 **Treatment: -0.851** **Interaction term: 11.348**	0.116 **0.00486** **0.0223**
	Proportion ovary tissue area (*20*)	Proportion area: -0.408 Treatment: -0.400 Interaction term: removed from model	0.648 0.072 ~ 0.744
Full RADD mean intensity	Full RADD mean intensity atretic follicles (*18)*	Intensity: -2.203e-08 Treatment: -0.230 Interaction term: removed from model	0.912 0.407 0.607
	Full RADD mean intensity ovary tissue (*18)*	Intensity: -9.759e-08 Treatment: -0.241 Interaction term: removed from model	0.647 0.414 0.791
γH2AX mean intensity	γH2AX mean intensity atretic follicles (*18)*	Intensity: -2.653e-08 Treatment: -0.230 Interaction term: removed from model	0.901 0.401 0.426

	γH2AX mean intensity ovary tissue (*18)*	Intensity: -1.265e-07 Treatment: -0.237 Interaction term: removed from model	0.593 0.419 0.759
UDG mean intensity	UDG mean intensity atretic follicles (*17)*	Intensity: 2.528e-08 Treatment: -0.202 Interaction term: removed from model	0.801 0.450 0.249
	UDG mean intensity ovary tissue (*16)*	Intensity: -8.947e-08 Treatment: -0.230 Interaction term: removed from model	0.558 0.452 0.685

Supplemental table 3. Summary of estimates and p-values before and after removal of outliers (<> 2 SD ± mean) for correlations of log E2 with all variables. Total outliers removed = outliers for the predictor and two MeHg birds that had log E2 concentrations greater than mean + 2SD.

Bold typeface indicates statistical significance ($p < 0.05$); "~" indicates p-value < 0.1.

	Variable	Total outliers removed (*df* after removal)	Estimate (p-value) before outliers removed	Estimate (p-value) after outliers removed
Area larger structures of ovary	Total ovary area	3 MeHg (*19*)	**Area: 0.232 (0.0255)** Treatment: -0.239 (0.232) Interaction term: removed from model (0.810)	Area: 0.158 (0.0804) ~ Treatment: -0.283 (0.121) Interaction term: removed from model (0.435)
	Proportion vitelline follicle area	2 MeHg (*19*)	Proportion Area: -0.0145 (0.996) Treatment: -0.396 (0.0810) ~ Interaction term: removed from model (0.813)	Proportion Area: 0.399 (0.873) **Treatment: -0.418 (0.038)** Interaction term: removed from model (0.802)
	Proportion follicle content area	3 MeHg (*18*)	Proportion Area: 0.412 (0.679) Treatment: -0.390 (0.0802) ~ Interaction term: removed from model (0.639)	Proportion Area: 0.943 (0.376) **Treatment: -0.405 (0.0374)** Interaction term: removed from model (0.812)
	Proportion atretic follicle area	3 MeHg (*18*)	Proportion Area: -0.00683 (0.993) Treatment: -0.397 (0.0745) ~ Interaction term: removed from model (0.394)	Proportion Area: 0.611 (0.456) **Treatment: -0.384 (0.0497)** Interaction term: removed from model (0.960)

	Proportion dense bodies area	2 MeHg (*19*)	Proportion Area: -6.222 (0.116) **Treatment: -0.851 (0.00486)** **Interaction term: (0.0223)**	**Proportion Area: -5.689 (0.0446)** **Treatment: -0.544 (0.00458)** Interaction term: removed from model (0.735)
	Proportion ovary tissue area	1 Control 2 MeHg (*18*)	Proportion Area: -0.408 (0.648) Treatment: -0.400 (0.072) ~ Interaction term: removed from model (0.744)	Proportion Area: -1.0145 (0.254) **Treatment: -0.404 (0.0352)** Interaction term: removed from model (0.820)
Area of primary follicle components	Total primary follicle area	2 MeHg (*19*)	Area: -1.564 (0.866) Treatment: -0.403 (0.0737) ~ Interaction term: removed from model (0.504)	Area: -0.569 (0.939) **Treatment: -0.409 (0.032)** Interaction term: removed from model (0.347)
	Proportion primary follicle granulosa cell area	2 MeHg (*19*)	Proportion Area: 1.459 (0.181) **Treatment: -0.466 (0.0364)** Interaction term: removed from model (0.201)	Proportion Area: 0.102 (0.920) **Treatment: -0.413 (0.0379)** Interaction term: removed from model (0.918)
	Proportion nuclei area	1 Control 3 MeHg (*17*)	**Proportion Area: -4.286 (0.0269)** **Treatment: -0.522 (0.0140)** Interaction term: (0.211)	Proportion Area: 4.020 0.253 Treatment: 0.118 (0.736; **without interaction p = 0.0433)** Interaction term: -9.995 (0.0751) ~
	Proportion ooplasm area	2 MeHg (*19*)	Proportion Area: -0.197 (0.871) Treatment: -0.400 (0.0735) ~ Interaction term: removed from model (0.560)	Proportion Area: 0.866 (0.394) **Treatment: -0.386 (0.0383)** Interaction term: removed from model (0.672)
Counts of primary follicle components	Count primary follicles	2 MeHg (*19*)	Count: 0.000651 (0.841) Treatment: -0.391 (0.0792) ~ Interaction term: removed from model (0.813)	Count: 0.000434 (0.879) **Treatment: -0.400 (0.0387)** Interaction term: removed from model (0.771)
	Count nuclei	1 Control 2 MeHg (*18*)	Count: 0.00569 (0.651) Treatment: -0.371 (0.103) Interaction term: removed from model (0.912)	Count: -0.01136 (0.464) **Treatment: -0.388 (0.043)** Interaction term: removed from model (0.572)

	Count ooplasm	1 Control 2 MeHg (*16*)	Count: 0.00234 (0.591) Treatment: -0.382 (0.085) ~ Interaction term: removed from model (0.560)	Count: -0.00273 (0.519) Treatment: -0.364 (0.053) ~ Interaction term: removed from model (0.221)
Full RADD mean intensity	Full RADD mean intensity vitelline follicles	2 Control 3 MeHg (*17*)	Intensity: 1.077e-06 (0.154) Treatment: -0.242 (0.311) Interaction term: removed from model (0.141)	Intensity: 3.216e-06 (0.0815) ~ Treatment: 0.165 (0.567; without interaction p = 0.449) Interaction term: -4.757e-06 (0.0676) ~
	Full RADD mean intensity primary follicles	2 MeHg (*14*)	Intensity: 2.648e-07 (0.513) Treatment: -0.225 (0.407) Interaction term: removed from model (0.490)	Intensity: 1.245e-06 (0.105) Treatment: -0.489 (0.105) Interaction term: removed from model (0.340)
	Full RADD mean intensity atretic follicles	2 MeHg (*9*)	Intensity: -2.203e-08 (0.912) Treatment: -0.230 (0.407) Interaction term: removed from model (0.607)	Intensity: 1.894e-07 (0.812) Treatment: -0.467 (0.400) Interaction term: removed from model (0.224)
	Full RADD mean intensity ovary tissue	1 Control 3 MeHg (*11*)	Intensity: -9.759e-08 (0.647) Treatment: -0.241 (0.414) Interaction term: removed from model (0.791)	Intensity: -2.534e-07 (0.579) Treatment: -0.264 (0.461) Interaction term: removed from model (0.707)
γH2AX mean intensity	γH2AX mean intensity vitelline follicles	3 MeHg (*18*)	**Intensity: 1.911e-06 (0.0121)** Treatment: 1.448 (0.0986) ~ Interaction term: -1.783e-06 (0.059) ~	**Intensity: 1.911e-06 (0.00396)** **Treatment: 1.886 (0.0346)** **Interaction term: -2.288e-06 (0.0177)**
	γH2AX mean intensity primary follicles	1 Control 2 MeHg (*15*)	Intensity: 3.729e-07 (0.208) Treatment: -0.247 (0.351) Interaction term: removed from model (0.254)	Intensity: 4.339e-07 (0.242) Treatment: -0.328 (0.202) Interaction term: removed from model (0.411)
	γH2AX mean intensity atretic follicles	5 MeHg (*14*)	Intensity: -2.653e-08 (0.901) Treatment: -0.230 (0.401) Interaction term: removed from model (0.446)	Intensity: 2.448e-07 (0.473) Treatment: -0.339 (0.317) Interaction term: removed from model (0.963)

	γH2AX mean intensity ovary tissue	3 MeHg (*16*)	Intensity: -1.265e-07 (0.593) Treatment: -0.237 (0.419) Interaction term: removed from model (0.759)	Intensity: -1.154e-07 (0.691) Treatment: -0.260 (0.378) Interaction term: removed from model (0.631)
UDG mean intensity	UDG mean intensity vitelline follicles	1 Control 3 MeHg (*17*)	Intensity: 2.085e-07 (0.455) Treatment: -0.298 (0.247) Interaction term: removed from model (0.399)	Intensity: 1.489e-08 (0.969) Treatment: -0.167 (0.512) Interaction term: removed from model (0.626)
	UDG mean intensity primary follicles	3 MeHg (*16*)	**Intensity: 5.889e-07 (0.0245)** Treatment: -0.162 (0.419) Interaction term: removed from model (0.604)	**Intensity: 6.106e-07 (0.0113)** Treatment: -0.0520 (0.808) Interaction term: removed from model (0.405)
	UDG mean intensity atretic follicles	2 MeHg (*16*)	Intensity: 2.528e-08 (0.801) Treatment: -0.202 (0.450) Interaction term: removed from model (0.249)	Intensity: 1.712e-07 (0.240) Treatment: -0.752 (0.776) Interaction term: removed from model (0.638)
	UDG mean intensity ovary tissue	2 MeHg (*16*)	Intensity: -8.947e-08 (0.558) Treatment: -0.230 (0.453) Interaction term: removed from model (0.685)	Intensity: -1.001e-07 (0.466) Treatment: -0.234 (0.424) Interaction term: removed from model (0.618)