



Male zebra finches exposed to lead (Pb) during development have reduced volume of song nuclei, altered sexual traits, and received less attention from females as adults

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ABSTRACT

Lead (Pb) is a pervasive global contaminant that interferes with sensitive windows for neurological development and causes oxidative damage to tissues. The effects of moderate and high exposure to Pb have been well-studied in birds, but whether low-level early-life exposure to Pb influences adult phenotype remains unclear. Female songbirds use a male's song and coloration to discriminate between high- and low-quality males. Therefore, if early-life exposure to Pb disrupts song learning ability or shifts the allocation of antioxidant pigments away from colorful secondary sexual traits, male birds exposed to Pb may be less attractive to females. We exposed developing zebra finches (*Taeniopygia guttata*) to Pb-contaminated drinking water (100 or 1000 parts per billion [ppb]) after hatching (days 0–100). Once male finches reached adulthood (120–150 days post hatch), we measured song learning ability, coloration of bill and cheek patches, and volume of song nuclei in the brain. We also measured female preference for Pb-exposed males relative to control males. Finally, we measured motoric and spatial cognitive performance in male and female finches to assess whether cognitive traits differed in their sensitivity to Pb exposure. Male zebra finches exposed to 1000 ppb Pb had impaired song learning ability, reduced volume of song nuclei, bills with less redness and received less attention from females. Additionally, Pb exposure impaired motoric performance in both male and female finches but did not affect performance in a spatial cognitive task. Adult finches exposed to Pb-contaminated water had higher blood-Pb levels, though in all cases blood-Pb levels were below $7.0 \mu\text{g dL}^{-1}$. This study suggests that low-level exposure to Pb contributes to cognitive deficits that persist into adulthood and may indirectly influence fitness by altering secondary sexual traits and reducing male attractiveness.

1. Introduction

Early-life exposure to lead (Pb) can interfere with sensitive windows for learning and development by causing neurobehavioral impairments and oxidative damage to tissues (Williams et al., 2018; McCabe, 1979). For this reason, reducing environmental Pb contamination has been a priority for both wildlife conservation and human disease prevention (Frank et al., 2019; Pokras and Kneeland, 2008). The toxic effects of moderate and high levels of Pb have been particularly well-studied in birds because they are susceptible to Pb exposure via ingestion of expended Pb-shot ammunition and discarded fishing tackle (Arnemo

et al., 2016; Fisher et al., 2006; Haig et al., 2014). However, birds may also be exposed to low levels of Pb in water contaminated by industrial processes, legacy contamination, or damaged municipal infrastructure (Olson et al., 2017; Beyer et al., 2004; Scheuhammer and Templeton, 1998; Pain et al., 1998). Low-level Pb exposure is unlikely to directly impact nestling survival and growth (Chatelain et al., 2018; Eeva et al., 2014), but early-life exposure to Pb can have both proximate physiological effects and long-term consequences for adult fitness. Great tit (*Parus major*) nestlings orally dosed with Pb (15 or $30 \mu\text{g g}^{-1}$ body mass) exhibited lower hematocrit and heterophil:lymphocyte ratio, suggesting that developmental exposure to Pb causes physiological damage

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(Markowski et al., 2019). Additionally, herring gull chicks (*Larus argentatus*) exposed to Pb via injection (0.1 and 0.2 mg g⁻¹ body mass) exhibited altered behavior (Burger and Gochfeld, 1995). Finally, correlative studies of free-living birds found low-level Pb exposure is associated altered movement behavior, increased aggression, and reduced life-time breeding success (Fritsch et al., 2019; McClelland et al., 2019; Provencher et al., 2016). While these studies indicate Pb exposure can cause both proximate physiological effects as well as shifts in behavior and life-history traits, the underlying mechanisms linking physiological damage to organismal effects remain unclear. In this study, we investigated how early-life exposure to Pb contaminated water may indirectly influence reproduction by altering the expression of secondary sexual traits, impairing cognitive performance, and reducing the ability of males to attract mates.

Characterizing the effects of early-life Pb exposure on coloration and song is critical for understanding the relationship between Pb exposure during development and the ability to attract a mate in adulthood. Female songbirds often select mates based on preferences for male song and coloration of ornaments (e.g., bill and plumage) because these traits are condition-dependent, allowing females to distinguish between low- and high-quality males (Hill, 1990; Nowicki et al., 1998). Adverse developmental conditions can influence both song quality and coloration (Simons et al., 2012b; Spencer and MacDougall-Shackleton, 2011). Male songbirds learn their songs by imitating tutors, and early-life challenges (e.g., malnutrition) can impair neurological processes underlying this song learning, making adult song an indicator of developmental condition (Spencer et al., 2003, 2005; Buchanan et al., 2004; Nowicki et al., 2002, 1998). Similarly, carotenoids and melanins determine the coloration of ornaments that are important for a male's ability to attract females (Hill, 1990; Von Schantz et al., 1999; Griffith et al., 2006; Naguib and Nemitz, 2007), and recent environmental conditions such as nutrition and stress can influence coloration by shifting the availability and allocation of pigments for ornament coloration (Rosenthal et al., 2012; Blount et al., 2003; McGraw and Ardia, 2003; Naguib and Nemitz, 2007). Therefore, both song and coloration are vulnerable to adverse environmental conditions, reflect a male's quality, and influence reproductive success.

Although Pb is associated with a variety of cognitive impairments in murine systems (e.g., spatial learning, attentional deficits, motor coordination) (e.g., Kuhlmann et al., 1997; Morgan et al., 2001; Luthman et al., 1992), less is known about how Pb affects vocal learning because learned vocal communication is absent in model organisms used for toxicity testing (e.g., fish and rodents) (Arriaga and Jarvis, 2013). Furthermore, it is unclear whether exposure to Pb causes global cognitive impairment or whether certain cognitive traits are more sensitive to Pb exposure. Similar to studies in mammals, exposure to high levels of Pb can impair motoric performance in birds, suggesting the mechanism of action responsible for the impairment of certain cognitive modalities is similar across birds and other model study systems. For instance, herring gull chicks exposed to Pb via intraperitoneal injection (100 mg kg⁻¹) had impaired motor coordination (Burger and Gochfeld, 2005). However, song learning appears to be an independent cognitive process (i.e., cognitive module) (Searcy and Nowicki, 2019) and may be more sensitive to Pb exposure, raising the potential for low-level Pb exposure to differentially affect song learning compared to other cognitive traits (e.g., spatial or motoric cognitive performance).

Male songbirds learn their songs early in life during a sensitive window of neurological development (Immelman, 1969). Similar to human language development, song learning involves memorizing and replicating song produced by adult tutors (Bolhuis and Gahr, 2006; Doupe and Kuhl, 1999). Song crystallization occurs at the completion of the critical learning period and, in close-ended learners, an individual's song remains stable throughout adulthood. In field studies, adult birds exposed to heavy metals have altered song characteristics, which may be indicative of neurological damage that impairs a bird's ability to memorize and replicate a song (Gorissen et al., 2005; McKay and Maher,

2012; Hallinger et al., 2010). However, it can be difficult to disentangle whether altered song in wild birds exposed to metals is an indirect effect of a low-quality environment (e.g., poor nutrition) or a direct effect of heavy metals interfering with neurological development (Koivula et al., 2011; Eeva et al., 2003).

Song learning relies upon an extended period of postnatal neurogenesis and circuit remodeling among three key nuclei of the song learning brain circuit: HVC, Area X, and lateral magnocellular nucleus of the anterior nidopallium (LMAN) (Jarvis, 2007; Scharff and Nottebohm, 1991). Birds with reduced volume of these song nuclei have impaired song learning ability and less attractive songs (Airey and DeVoogd, 2000; DeVoogd et al., 1993), and previous studies found these song nuclei are sensitive to environmental contaminants (e.g., pesticides, polychlorinated biphenyls [PCBs], flame retardants) (Iwaniuk et al., 2006; Eng et al., 2018; Hoogesteijn et al., 2008). However, no studies have examined whether developmental Pb exposure influences the volume of song nuclei.

In addition to neurotoxic effects, Pb exposure may alter allocation of pigments to colorful ornaments important for attracting mates (Geens et al., 2009; Koivula and Eeva, 2010; McGraw, 2003). Carotenoid and melanin pigments are directly or indirectly related to antioxidant defense mechanisms. For instance, birds adaptively reallocate carotenoids towards protection against pro-oxidants (Rosenthal et al., 2012; Tomášek et al., 2016; Hill and Johnson, 2012), though the strength of carotenoids as antioxidants has been questioned (Costantini and Møller, 2008). Nonetheless, mallard ducks (*Anas platyrhynchos*) and red-legged partridges (*Alectoris rufa*) exposed to Pb exhibited increased levels of circulating carotenoids and reduced coloration of carotenoid-based features (Vallverdú-Coll et al., 2015, 2016). Additionally, in zebra finches (*Taeniopygia guttata*), the ketocarotenoid pigment astaxanthin determines bill redness and is controlled by cytochrome P450 genes, which are also important detoxification enzymes (Mundy et al., 2016). Therefore, exposure to Pb may alter carotenoid coloration by causing a reallocation of carotenoids towards antioxidant defense as well as redirecting cytochrome P450 enzyme activity towards detoxification and away from astaxanthin synthesis.

In addition to carotenoids, melanin is a predominate pigment in feathers and has two forms: eumelanin (gray-black plumage coloration) and pheomelanin (orange-red plumage coloration). Cysteine is essential for the endogenous synthesis of pheomelanin but not eumelanin (Benathan et al., 1999). Because the antioxidant glutathione is the main intracellular reservoir of cysteine (Wu et al., 2004), the balance between eumelanin and pheomelanin coloration is dependent on a bird's oxidative status. That is, oxidative stress depletes glutathione concentrations, resulting in less cysteine for pheomelanin synthesis. In several cases, birds exposed to heavy metals exhibited lower glutathione concentrations (Espín et al., 2014b; Włostowski et al., 2010), but it is worth noting that glutathione concentrations increased in other cases (Mateo et al., 2003; McGowan and Donaldson, 1986; Congiu et al., 2000). If Pb-induced oxidative stress increases glutathione metabolism, then the available cysteine for pheomelanin production will be limited, resulting in a shift towards plumage with less red saturation. Although the effects of heavy metals on eumelanin coloration have been previously studied (Dauwe and Eens, 2008; Giraudeau et al., 2015; McGraw, 2003; Chatelain et al., 2014), the effect of Pb exposure on pheomelanin coloration is unclear.

In this study, we examined whether early-life exposure to Pb influences adult phenotype using the zebra finch model to measure song learning, volumes of brain nuclei, and coloration in males. We predicted that male finches exposed to Pb would have smaller song nuclei and impaired song learning. Additionally, male zebra finches have pheomelanin-based orange cheek patches and carotenoid pigmentation determines bill redness, so we predicted Pb-exposed males would have cheek patches with less red saturation (decreased pheomelanin pigmentation) and bills with less red saturation (decreased carotenoid pigmentation). To test whether Pb-induced changes to these traits

influence a male's ability to attract a mate, we measured female preference for control or Pb-exposed males. We predicted Pb-exposed males would receive less attention from females because females prefer males with redder bills and more attractive songs (Spencer et al., 2005; Simons and Verhulst, 2011). Finally, we measured motoric and spatial learning in male and female finches to assess whether cognitive traits differed in their sensitivity to Pb exposure. Because song learning appears to be an independent cognitive process (i.e., cognitive module) (Searcy and Nowicki, 2019), we predicted Pb exposure would differentially affect cognitive traits.

2. Methods

2.1. Animal husbandry

Laboratory-reared zebra finches were provided water and millet seed ad libitum, and aviary conditions were maintained at 24–27 °C, 15–48% humidity, and a 14:10 light:dark photoperiod. All rearing and experimental procedures were approved by Virginia Tech Institutional Animal Care and Use Committee.

2.2. Pilot dosing study

We conducted a pilot study to confirm Pb-treated water increased blood-Pb levels in finches. First, we prepared a stock solution of 100,000 parts per billion (ppb) with Pb acetate (99.999% purity; Sigma) and millipore water, then we further diluted the stock solution with deionized water to nominal target concentrations of 100 and 1000 ppb. We supplied each cage with two water dispensers, both containing either Pb-treated water or tap water (control) to ensure all birds ($n = 2$ males and 2 females per treatment) had adequate access to water. Water dispensers prevented birds from bathing in Pb-treated water. 100% renewal of water bottles occurred every 48 h to maintain Pb concentrations. Adult finches had continuous access to Pb-treated water for 150 days. At the end of the dosing period, we collected 50 μ l of blood via venipuncture of the alar vein for blood-Pb analysis. Blood aliquots were shipped on ice to Dartmouth Trace Element Analysis Core and Pb concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS) (U.S. EPA, 2007). Unfortunately, one control female sample was lost during the sample preparation procedure (control: $n = 3$).

2.3. Offspring Pb exposure

We exposed zebra finch offspring to drinking water with 100 or 1000 ppb Pb or a control (deionized water) after hatching and throughout the juvenile song learning period (days 0–90). Prior to Pb exposure, we paired 8 male and 8 female finches without previous exposure to Pb in $45 \times 45 \times 45$ cm cages and provided pairs with a nest box, nesting materials, mashed hard-boiled eggs, millet spray, as well as Pb-free water and millet seed. For Pb-exposed broods, we replaced Pb-free water with Pb-treated water (100 ppb or 1000 ppb; see Section 2.2. for preparation and maintenance of nominal concentrations) just prior to eggs hatching. Nestlings were likely exposed to Pb indirectly via parents prior to fledging, though we did not quantify nestling exposure. Offspring had direct access to Pb-treated water after fledging (ca. 18 days post hatch) and until day 98 ± 6 (mean $\pm$ SD) post hatch. The passive dosing design reduced the potential for confounding handling effects, which can occur when birds are dosed by gavage (e.g., Goodchild et al., 2020). However, this design did not allow us to vary treatments within broods because an entire cage had access to Pb-treated water; we accounted for potential brood effects in the statistical analysis (see Section 2.9). In order to achieve an adequate sample size, we transferred offspring from each brood to group housing with a single adult male (i.e., tutor) when they reached nutritional independence (ca. 35 days post hatch) but while song learning was ongoing (Riebel et al.,

2002) to allow control breeding pairs to produce a second brood, which was treated with Pb. Tutors received the same treatment as the offspring. In total, 11 experimental clutches were reared across the 3 treatments. Sample sizes varied with brood size and sex ratio within each nest and are specified below.

We collected mass for male and female offspring on days 10, 20, 30, 40, and 50 post hatch and at adulthood (117 ± 13 days [mean $\pm$ SD]). We conducted motoric and spatial learning trials for males and females on day 125 ± 13 (mean $\pm$ SD) post hatch. For males, we measured song on day 117 ± 13 post hatch, bill and cheek patch coloration on day 142 ± 17 , and female preference for control or Pb-exposed males on day 145 ± 15 (mean $\pm$ SD). We euthanized males and collected brains on day 154 ± 17 (mean $\pm$ SD).

2.4. Male song

We recorded songs from all male offspring (control: $n = 10$; 100 ppb: $n = 6$; 1000 ppb: $n = 7$) and their tutors by temporarily housing them in individual cages within sound attenuation chambers (IAC acoustics, Bronx, NY). It was not possible to assign birds to tutors in a balanced design because each cage of subjects was assigned a specific water treatment. However, tutors were selected to have similar song complexity, and we confirmed that tutors' songs were not impaired by exposure to Pb by comparing tutors' own songs before and after treatment (see below). Only male zebra finches produce song, so we did not measure vocalizations of females. Spontaneously produced songs were recorded using cardioid dynamic microphones (Shure SM57) attached to an 8-line recording interface (AudioBox 1818VSL, PreSonus, USA) and a laptop running Sound Analysis Pro bioacoustics recording software.

We compared spectrograms of the songs of male offspring to those of their tutors in order to assess song learning. One experienced researcher who was blind to treatment selected 6–10 single motifs from songs of each tutor (adult motifs mean $\pm$ SD: 8.7 ± 1.6) and each experimental offspring (offspring motifs mean $\pm$ SD: 9.4 ± 0.2). We always selected the most common motif and normalized amplitudes of all motif samples using Audacity® (Version 2.4.2). We then compared tutor and song motifs using the batch similarity process (with asymmetric comparison) in Sound Analysis Pro to examine how much of the tutor's song was represented in each pupil's song (Tchernichovski et al., 2000). This analysis produced three measures: (1) similarity is the percentage of the tutor song that is matched in pupil song based on significant similarity in five acoustic features (pitch, FM, AM, Wiener entropy, and goodness of pitch) assessed over 70 ms intervals, (2) accuracy is the frame-by-frame similarity of these matching segments, and (3) sequential match compares the sequences of the matching segments in the tutor and pupil songs.

2.5. Song nuclei volume

We measured the volume of song nuclei according to Sewall et al. (2018). Birds were first euthanized with isoflurane, then we rapidly removed the brain, separated the hemispheres, flash froze the tissue on dry ice and stored all tissue at -80 °C until processing. We sectioned both hemispheres from each bird in the sagittal plane at 40 μ m using a cryostat (set to -22 °C) and mounted sections on glass microscope slides (superfrost plus, Fischer). Every third section was Nissl stained (13.8 ± 1.2 sections per bird). Specifically, we immersed the frozen slides in 4% paraformaldehyde and then serially dehydrated the tissue in ethanol before staining with thionin, removing excess stain in an ethanol wash, clearing the tissue in xylenes, and affixing coverslips. We captured gray scale images of each brain region using an AxioCam MR camera attached to a Zeiss Axioimager microscope (Zeiss, USA). We imaged the entire telencephalon using a high-resolution scanner (Epson V500 at 1200 dpi resolution) and the song control nuclei using the 5 $\times$ objective (50 $\times$ magnification). We located each brain region of interest with reference to an avian brain atlas (Nixdorf-Bergweiler and Bischof, 2007). A single

researcher blind to treatment used ImageJ to trace the boundaries of HVC, Area X, LMAN in both hemispheres, and the entire telencephalon. We calculated the volume (summed areas $\times$ distance between sections) of each brain region as a proportion of the volume of the telencephalon for each subject (hereafter referred to as relative volume) (Sewall et al., 2018). Some brains were being used for other analyses, thus we collected brains opportunistically and have a smaller sample size ($n = 5$ per treatment).

2.6. Cognitive tasks

2.6.1. Motoric task

We placed two 2.5 cm $\times$ 2.5 cm $\times$ 2 cm blocks with a center hole of 1.2 cm diameter and 1 cm depth in each subject's cage to allow the birds to habituate to the testing apparatus for 24 h (*sensu* Campbell et al., 2017; control: $n = 11$; 100 ppb: $n = 15$; 1000 ppb: $n = 12$). During trials, the holes were covered with lids consisting of a blue plastic disc (diameter 1.5 cm) with a rubber bumper affixed to one side to weigh the lid down. The motoric learning task consisted of a shaping procedure in which birds were trained to pull the lids off of the wells in order to retrieve a food reward. This was done at three levels: level 1, with the lid next to the well; level 2, with the lid half-covering the well; and level 3, with the lid covering the well. Birds advanced to the next level when they successfully completed three consecutive trials at each level. Birds were given a maximum of 60 trials to successfully complete all three levels. The total number of trials required for each bird to successfully complete all three levels of the motoric learning task was summed across days. Birds were returned to group housing with their tutor after completing the motoric learning task (a maximum of 3 days).

2.6.2. Spatial task

48 h before spatial testing began, we moved adult offspring from their cages to individual housing (30 $\times$ 30 $\times$ 40 cm cage) for a four-corner spatial task (*sensu* Campbell et al., 2017; control: $n = 12$; 100 ppb: $n = 16$; 1000 ppb: $n = 12$). First, birds' preferences for specific corners of their testing cages were determined and then their ability to learn and remember the location of a food reward hidden in an unpreferred corner was assessed. To test for corner preferences, four blocks were baited with food, the wells covered with lids, and the blocks placed in the corners of the cage for 10 consecutive trials. The corners that were visited first, the most often, and the least were not chosen to be baited during the spatial task. During the spatial task a specific corner of the cage was selected to be baited in every trial, requiring the bird to remember the location of the food reward. A bird passed a trial if the first well it uncovered was the well containing the food reward. If the bird failed the trial, the bird was given until the end of the 2 min trial period to visit other corners in order to find the baited corner. A bird passed the spatial learning task when it visited the baited corner first in six of seven consecutive trials. The number of trials required to finish the spatial learning task was summed across days.

2.7. Bill and cheek patch coloration

We quantified reflectance of the bill and cheek patch directly from each male (control: $n = 14$; 100 ppb: $n = 6$; 1000 ppb: $n = 7$), using a JAZ reflectance spectrophotometer and SpectraSuite software (Ocean Optics Dunedin, FL). A 200-micron fiber optic probe was held in a sheath that excluded ambient light and maintained a constant distance from the bill or cheek of 5.5 mm. All reflectance measurements were made relative to a white standard (Labsphere, North Sutton, NH). We made four measurements each of the cheek patch and bill, lifting and replacing the probe between measurements to assess slightly different areas of each region. We used the program CLR version 1.05 (Montgomerie, 2006, 2008) to extract color variables. We focused on hue, red saturation, and total brightness for the bill and cheek patch. For hue, we identified the wavelength which corresponded to the point that was

midway between the peak and minimum reflectance. Red saturation was calculated as the sum of the percent reflectance from 605 to 700 nm divided by the total reflectance (brightness) from 320 to 700 nm and total brightness as the sum of the reflectance values from 320 to 700 nm.

2.8. Mate preference

We assessed female preference for control males or males exposed to either 100 or 1000 ppb Pb in a two-choice test by comparing the relative amount of time and number of visits control females ($n = 12$) made to adult males that were reared under each treatment. Each control female was presented with a control male ($n = 12$) and a male exposed to 100 ppb ($n = 5$) or 1000 ppb ($n = 7$) by housing her in a cage (90 $\times$ 45 $\times$ 45 cm) supplied with a viewing perch the length of the cage. Stimulus males were placed individually in adjacent cages (45 $\times$ 45 $\times$ 45 cm) and were visually isolated from each other. We conducted mate preference trials in a room that was isolated from the main colony. The day before the trial we placed females without previous interaction with experimental males in focal cages between 1600 h and 1700 h. The following morning we video-recorded females for 30 min without stimulus males present to confirm females were behaving normally, then we placed males in their respective cages between 0900 h and 1030 h. Immediately after control and Pb-exposed males were placed in their respective cages, we gently flushed the female between the left and right sides of her cage to ensure the female was aware that males were present in both stimulus cages. Each trial began immediately after researchers left the room. During female preference trials, we were unable to attribute vocalizations to specific males, but we did confirm that males produced song during trials. We measured female preference for control or Pb-exposed males as the proportion of time the female spent on the viewing perch looking at a stimulus male. Additionally, we counted the number of female visits to viewing areas. To account for individual variation in female activity, we calculated visits to a stimulus male as a proportion of the total number of visits to the viewing perch (*sensu* Spencer et al., 2005). Females were most actively observing males during the first 15 min of the trial, therefore we recorded female behavior continuously during this period. We analyzed videos for female behavior using BORIS version 7.8.2 (Friard and Gamba, 2016).

2.9. Statistics

All analyses were conducted in R (version 3.6.3). We analyzed the effects of Pb treatment and sex on blood-Pb levels with a linear model (LM). We did not have a sufficient sample size to test the sex $\times$ treatment interaction, thus this term was excluded from the model. Nestling growth was analyzed with a linear mixed model (LMM) with time, treatment, sex, and brood size as fixed effects and parents and individual as nested random effects. We added a log function to the time term in the model, which performed better than the model fitting a linear relationship ($\Delta AIC = -118.7$). We calculated estimated marginal means for mass at each timepoint using the 'emmeans' package. The effects of treatment on male song learning (percent similarity, accuracy, sequential match; see Section 2.5) were analyzed using LMMs, with tutor as a random effect. To analyze the effects of Pb exposure on the number of trials until success for the motoric and spatial tasks, we used generalized linear models (GLMs) with parents and sex of nestling as covariates and a Poisson distribution with a log link-function for count data. In the motoric learning dataset, two birds failed to complete the motoric task by a predetermined threshold of 60 trials and were removed from the dataset (1 bird in control group, 1 bird in 100 ppb treatment). We analyzed bill and cheek patch coloration by conducting a principle components analysis (PCA) of brightness, red saturation, and hue for each region and compared 95% confidence intervals for the first 2 components. Additionally, we used a GLM to test for significant differences in bill and cheek patch color PC1 among treatments. We also analyzed each color variable independently use LMs (see Supplemental

Information). For female preference, we arcsine transformed proportional preference (time spent looking at male and number of visits to the viewing areas) and analyzed each measure of female preference for control or Pb-exposed male (100 or 1000 ppb) using separate LMMs for each two-choice test (i.e., control vs. 100 ppb Pb was analyzed separately from control vs. 1000 ppb Pb), with female as a covariate and side of cage (right or left) of stimulus males as a random effect. We analyzed the volume of brain regions using GLMs to test the main effect of Pb treatment. We used the 'nlme' package (Pinheiro et al., 2020) for mixed effects models and the 'car' package (Fox and Weisberg, 2019) to test for main effects of GLMs and LMMs (i.e., analysis of deviance) using likelihood ratios. For all analyses, we checked residuals for normality and equal variance.

3. Results

3.1. Pilot dosing

Blood-Pb levels were significantly higher in adult finches exposed to Pb-treated water (Fig. 1; LM: $R^2 = 0.73$; treatment: $F_{2,7} = 9.04$, $p = 0.01$). Birds exposed to 1000 ppb Pb had blood-Pb levels that ranged from 2.62 to 6.83 $\mu\text{g}/\text{dL}$, which was significantly higher than birds exposed to 100 ppb Pb ($|t| = 3.09$, $p = 0.02$) and controls ($|t| = 4.02$, $p = 0.005$). There was not a significant difference in blood-Pb levels between birds exposed to 100 ppb compared to the control ($|t| = 1.18$, $p = 0.28$). Males treated with 1000 ppb appeared to have higher blood-Pb levels compared to females, but overall, there was not a significant difference in blood-Pb levels between sexes ($F_{1,7} = 1.83$, $p = 0.22$).

3.2. Offspring growth

Pb treatment did not cause mortality, and we did not observe impaired flying, perching, or other signs of disorientation in offspring exposed to Pb. Growth was similar across treatments (Fig. 2). Although offspring mass varied by the interaction of treatment $\times$ time (Fig. 2; LMM: treatment $\times$ time: $\chi^2 = 11.93$, $\text{df} = 2$, $p = 0.003$; treatment: $\chi^2 = 1.01$, $\text{df} = 2$, $p = 0.60$; time: $\chi^2 = 259.63$, $\text{df} = 1$, $p < 0.001$), the only significant difference in mass among birds exposed to Pb and the control occurred on day 10 post hatch (100 ppb: $t = -2.58$, $p = 0.04$; 1000 ppb: $t = -1.41$, $p = 0.39$; for all other time points $|t| < 1.49$, $p > 0.36$). Growth did not vary by sex ($\chi^2 = 2.24$, $\text{df} = 1$, $p = 0.13$) or brood size (range 1–4 nestlings brood $^{-1}$; $\chi^2 = 5.97$, $\text{df} = 3$, $p = 0.11$).

3.3. Male song

Male offspring exposed to Pb-treated water had poorer imitation of tutor song by one measure; they had lower % sequential match compared to controls (Fig. 3; LMM: $\chi^2 = 8.49$, $\text{df} = 2$, $p = 0.01$). Specifically, males exposed to 1000 ppb Pb had a significantly lower %

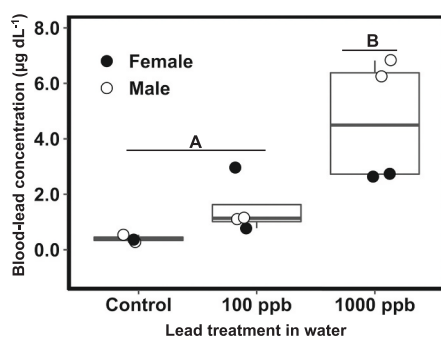


Fig. 1. Blood-Pb levels in adult zebra finches exposed to deionized water (control) or water treated with 100 ppb or 1000 ppb Pb for 150 days. Significant differences ($p < 0.05$) are denoted by different letters.

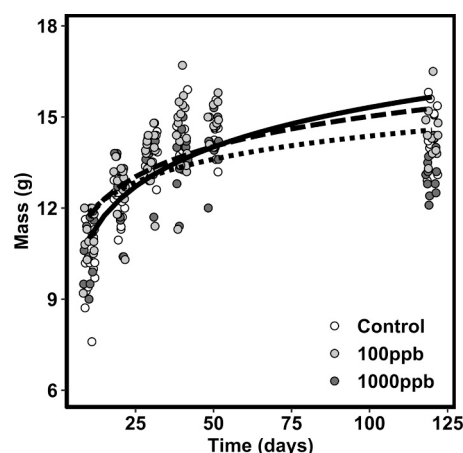


Fig. 2. Individual masses and trendlines of zebra finches exposed to deionized water (control; solid line) or water treated with 100 ppb (dashed line) or 1000 ppb (dotted line) Pb from 0 to 100 days post hatch. Offspring mass did not differ across treatments.

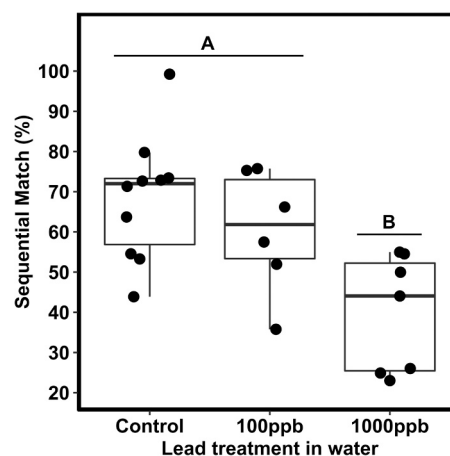


Fig. 3. Song learning in zebra finches exposed to deionized water (control) or water treated with 100 ppb or 1000 ppb Pb from 0 to 100 days post hatch. Significant differences ($p < 0.05$) are denoted by different letters.

sequential match compared to controls ($t = -2.67$, $p = 0.045$), whereas there was not a difference between finches exposed to 100 ppb and controls ($t = -0.92$, $p = 0.64$). Furthermore, males exposed to 1000 ppb Pb appeared to have a lower % sequential match compared to males exposed to 100 ppb Pb, though this difference was not significant ($t = -2.37$, $p = 0.078$). Pb exposure did not affect percent similarity (LMM: $\chi^2 = 0.13$, $\text{df} = 2$, $p = 0.94$) or accuracy (LMM: $\chi^2 = 3.33$, $\text{df} = 2$, $p = 0.19$). For all models, brood size was not a significant covariate (LMM: in all cases $\chi^2 < 2.20$, $p > 0.14$). A comparison of tutor song recordings at the start and end of the study demonstrates that Pb treatment did not influence tutor song characteristics (% similarity: 90.7 ± 6.4 ; accuracy: 79.3 ± 3.3 ; % sequential: 80.4 ± 6.3 [mean $\pm$ SD]).

3.4. Song nuclei volume

There was a significant effect of Pb exposure on relative LMAN volume (Fig. 4B; LM: $R^2 = 0.68$, $F_{2,12} = 12.49$, $p = 0.001$), specifically there was a decrease in relative LMAN volume for birds exposed to either 100 ppb ($t = -4.70$, $p < 0.001$) or 1000 ppb ($t = -3.81$, $p = 0.003$) compared to controls but there was not a difference between 100 ppb and 1000 ppb treatments ($t = 0.90$, $p = 0.39$). Additionally, birds exposed to either dose of Pb appeared to have smaller relative Area X

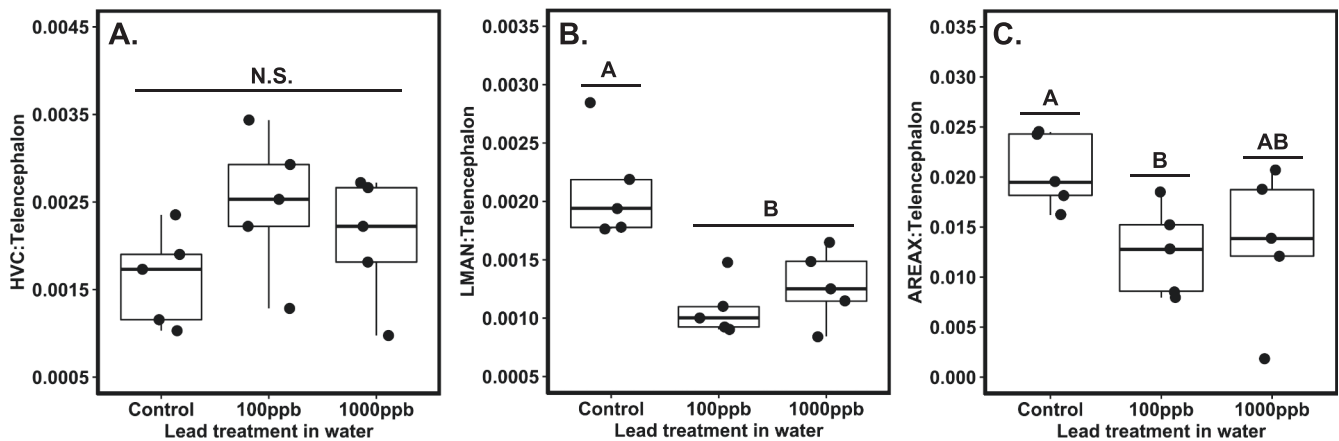


Fig. 4. Volume of the HVC (A) lateral magnocellular nucleus of the anterior nidopallium (LMAN) (B) and Area X (C) relative to telencephalon volume in zebra finches exposed to deionized water (control) or water treated with 100 ppb or 1000 ppb Pb from 0 to 100 days post hatch. Significant differences ($p < 0.05$) are denoted by different letters. (N.S. = nonsignificant).

volume (Fig. 4C; LM: $R^2 = 0.35$, $F_{2,12} = 3.23$, $p = 0.08$), but this effect was only significant for birds exposed to 100 ppb Pb compared to the control (100 ppb: $t = -2.32$, $p = 0.04$; 1000 ppb: $t = -2.07$, $p = 0.06$) Pb treatment did not affect relative HVC volume (Fig. 4A; LM: $R^2 = 0.23$, $F_{2,12} = 1.83$, $p = 0.20$).

3.5. Cognitive tasks

There was a dose-dependent increase in the number of trials required for birds to complete the motoric task (Fig. 5B; GLM: $\chi^2 = 3.91$, $p = 0.048$). Exposure to both 100 and 1000 ppb Pb resulted in birds requiring more trials to complete the motoric task compared to the control (100 ppb: $z = 1.93$, $p = 0.05$; 1000 ppb: $z = 3.16$, $p = 0.002$), and birds exposed to 1000 ppb Pb took longer to complete the motoric task compared to birds exposed to 100 ppb Pb ($z = 1.99$, $p = 0.047$).

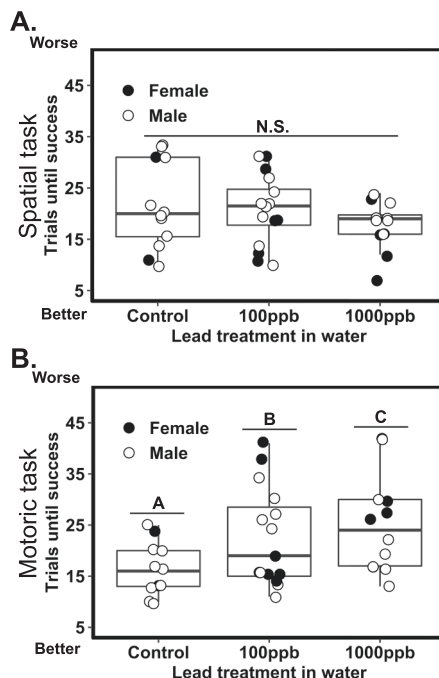


Fig. 5. Performance in spatial (A) and motoric (B) tasks for zebra finches exposed to deionized water (control) or water treated with 100 ppb or 1000 ppb Pb from 0 to 100 days post hatch. Finches that took more trials until success performed poorer in cognitive tasks. Significant differences ($p < 0.05$) are denoted by different letters. (N.S. = nonsignificant).

There was not a significant effect of sex on motoric task performance ($z = 1.41$, $p = 0.16$). Exposure to Pb did not affect performance in the spatial task (Fig. 5A; GLM: treatment: $\chi^2 = 2.60$, $df=1$, $p = 0.11$; sex: $\chi^2 = 2.31$, $df = 1$, $p = 0.13$).

3.6. Bill and cheek patch coloration

The first two dimensions of the bill coloration PCA explained 95.14% of the total variance (PC1: 63.20% of variance, $SD = 1.90$; PC2: 31.85% of variance, $SD = 0.96$). Bill brightness loaded positively on PC1, whereas bill hue and red saturation loaded negatively on PC1, such that higher PC1 values represent greater reflectance, while lower values represent more red pigmentation and darker orange/red. A comparison of PC1 residuals among treatments revealed a significant effect of treatment on bill coloration (Fig. 6A; LM: $R^2 = 0.36$, $F_{2,24} = 6.67$, $p = 0.005$). Specifically, birds exposed to 1000 ppb Pb had a significantly higher PC1 score (i.e., greater reflectance, but lower hue and red saturation; also see [Supplemental Information](#)) compared to controls ($t = 3.61$, $p = 0.001$) and birds exposed to 100 ppb Pb ($t = 2.48$, $p = 0.03$). Furthermore, a plot of PC1-PC2 coordinates revealed that the 95% confidence intervals of birds exposed to 1000 ppb Pb and control birds did not overlap (Fig. 6B). Finches exposed to 100 ppb Pb did not differ from controls in PC1 scores ($t = 0.59$, $p = 0.56$).

For the PCA of cheek patch coloration, the first two dimensions explained 96% of the total variance (PC1: 74.63% of variance, $SD = 1.50$; PC2: 20.71% of variance, $SD = 0.79$). Cheek patch hue and red saturation loaded positively on PC1, whereas brightness loaded negatively on PC1 such that higher PC1 scores reflected cheek patches with darker orange coloration. A comparison of PC1 residuals among treatments revealed a significant effect of treatment on cheek coloration (Fig. 6C; LM: $R^2 = 0.29$, $F_{2,23} = 4.60$, $p = 0.02$; [Supplemental Information](#)). Specifically, birds exposed to 1000 ppb Pb had cheek patches with greater hue and red saturation than birds exposed 100 ppb ($t = 3.03$, $p = 0.006$), but the difference in cheek patch coloration among birds exposed to either Pb treatment compared to the control was not significant (LM: 100 ppb: $t = -1.86$, $p = 0.07$; 1000 ppb: $t = 1.73$, $p = 0.10$). 95% confidence intervals based on a plot of PC1-PC2 coordinates further supports separation between birds exposed to 1000 ppb and birds exposed to 100 ppb Pb, but the 95% confidence interval of control birds overlapped with birds exposed to both 100 ppb and 1000 ppb Pb (Fig. 6D).

3.7. Mate choice preference

When female preference for control relative to Pb treated males was

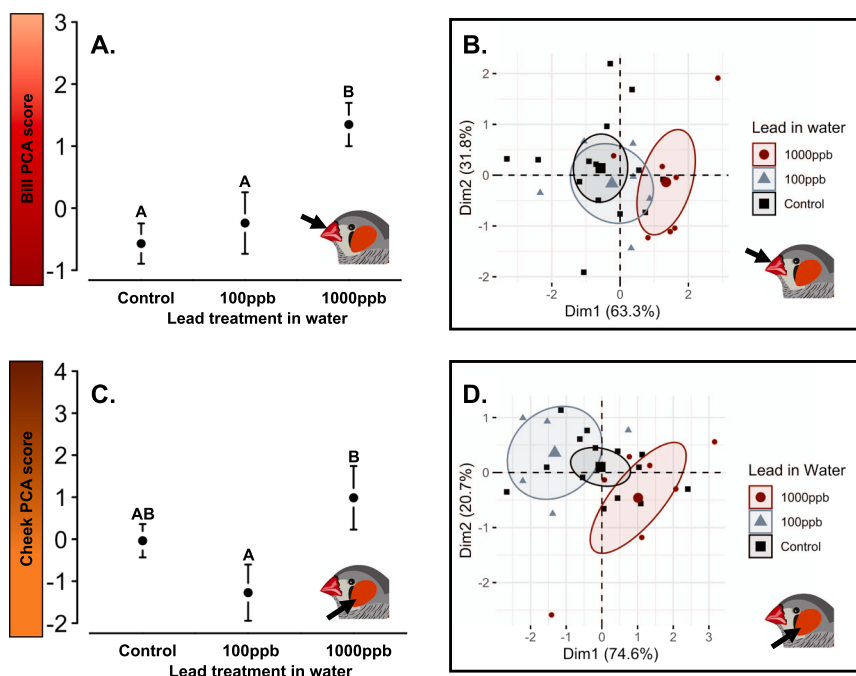


Fig. 6. Coloration of zebra finches exposed to deionized water (control) or water treated with 100 ppb or 1000 ppb Pb from 0 to 100 days post hatch. The first dimension of a principle components analysis (PCA) for bill (A) and cheek patch (C) coloration (mean $\pm$ std. error) are shown. Different letters denote significant ($p < 0.05$) differences between treatments. PCA plots of dimensions 1 and 2 for bill (B) and cheek patch (D) coloration. Ellipses represent 95% confidence intervals for each treatment.

calculated as a proportion of total visits to viewing areas, females were less likely to visit males exposed to 1000 ppb Pb compared to control males (Fig. 7; LMM: $t = -275$, $p = 0.04$). However, the difference in the proportion of time females spent in the viewing area in front of males exposed 1000 ppb Pb compared to control males was not statistically significant (Fig. 7; LMM: $t = 1.91$, $p = 0.11$). Females appeared to visit males exposed to 100 ppb Pb more than controls, but this trend was not significant (LMM: $t = 1.95$, $p = 0.12$). Further, there was not a significant difference in the amount of time females spent in front of controls compared to males exposed to 100 ppb Pb (LMM: $t = 3.06$, $p = 0.06$).

4. Discussion

Pb-contaminated surface waters pose an ecological hazard to wildlife, including songbirds. Surface water Pb concentrations at polluted industrial and mining sites typically range from 10 to 150 ppb and in some cases exceed 10,000 ppb (Palumbo-Roe et al., 2012; Lee et al., 2005; Concas et al., 2006). While birds living in urban and industrial

areas exhibit elevated blood-Pb levels (e.g., Cai and Calisi, 2016), the consequences of sublethal Pb levels are not yet fully understood. We found that male zebra finches exposed to 1000 ppb Pb exhibited bills with less red saturation, impaired song learning, and received less attention (number of visits) from females. These results support our hypothesis that Pb exposure early in life compromises the development of secondary sexual traits, with consequences for mate attraction. The specific mechanism by which Pb exposure impairs song learning appears to be through reducing the volume of LMAN and Area X within the song learning circuit in the brain (Fig. 4). In the pilot dosing experiment, we confirmed that adult zebra finches exposed to Pb-treated water had higher blood-Pb levels than controls, though in all cases blood-Pb levels were below $7.0 \mu\text{g dL}^{-1}$. Offspring blood-Pb levels likely differed from adults in the dosing study (developing vertebrates absorb Pb more readily than do adults; e.g., Ballew and Bowman, 2001; Lidsky and Schneider, 2003); nonetheless, elevated blood-Pb levels in adults validates that the Pb treated water resulted in Pb uptake. Collectively, our results suggest exposure to environmentally relevant Pb concentrations early in life alters male coloration and compromises song learning and may indirectly influence fitness by reducing a male's ability to attract a mate later in life. Determining the consequences of these effects for wild populations requires future studies to confirm female preference for unexposed males in free-living populations. Furthermore, our study primarily focused on the effects of Pb on male development, but exposure to Pb may also impair the ability of females to differentiate between high- and low-quality males. If Pb-exposed females do not preferentially choose higher quality males, then it is feasible that sink population dynamics could result. Therefore, to better understand the population-level consequences of Pb contamination on mate selection and fitness in songbirds, future research should investigate whether Pb interferes with female mate choice.

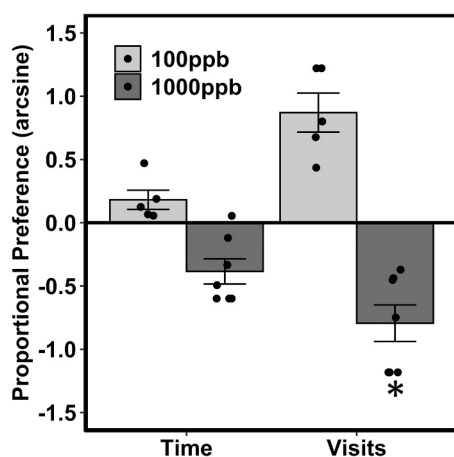


Fig. 7. Female preference for male zebra finches exposed to drinking water treated with either 100 ppb or 1000 ppb Pb from 0 to 100 days post hatch relative to control males. Asterisks denote significant ($p < 0.05$) differences between birds exposed to Pb and paired controls.

4.1. Effects of Pb on brain and behavior

Male zebra finches exposed to 1000 ppb Pb had a lower % sequential match to tutors' songs (i.e., poorer imitation of the temporal order of sounds), whereas exposure to 100 ppb Pb did not affect any measures of song learning. This difference may partially explain why females were less attracted to males exposed to 1000 ppb Pb but did not exhibit a clear

preference between controls and males exposed to 100 ppb Pb (discussed below). Our results are similar to previous findings that male zebra finches exposed to adverse developmental conditions (e.g., nutritional limitation or elevated corticosterone as nestlings) have less attractive songs and receive less attention from females (Nowicki et al., 1998; Spencer and MacDougall-Shackleton, 2011; Lampe and Espmark, 2002), though it is worth noting that developmental stress does not always translate to rearing poor quality offspring (Crino et al., 2014). Adult tutors showed no evidence of song impairment, which is consistent with previous studies demonstrating song production is stable after crystallization (Nottebohm, 1968; Immelman, 1969). Together these findings suggest birds exposed to Pb during a sensitive developmental window early in life have compromised adult song quality because their ability to learn a tutor's song is impaired.

Song learning involves behavioral traits (e.g., attentiveness) as well as neurological processes (Chen et al., 2016; Doupe et al., 2004), so a reduction in sequential match in birds exposed to Pb could reflect altered behavior (e.g., attention deficit) or be caused by damage to neural circuits. Indeed, Pb exposure has been shown to interfere with attentional processes (Brockel and Cory-Slechta, 1998) as well as neural development (Neal and Guilarte, 2010). We quantified the effects of Pb on the volumes of the song control nuclei and found that birds exposed to Pb had reduced LMAN and Area X volume. However, the study was not designed to evaluate attentional processes underlying learning and we did not measure offspring behavior (e.g., attention to tutor) (Chen et al., 2016). Our study provides evidence that Pb exposure impairs song learning through damage to the song learning circuit in the brain, but future studies are needed to examine additional mechanisms that could impair song outcomes, as well as the specific neurological processes that are impacted. Additionally, the song control nuclei experience a period of rapid neural growth early in development (10–30 days post hatch), followed by neural pruning and circuit strengthening (30–90 days post hatch) (Doupe and Kuhl, 1999). The next step in this research is to identify the developmental window and underlying neural processes that are impacted by Pb.

Exposure to 1000 ppb Pb also impaired cognitive performance in the motoric task, but not the spatial task. Our results are consistent with human studies showing that early-life Pb exposure causes motor learning deficits (Dietrich et al., 1993; Wasserman et al., 2000). Motor function is primarily regulated by the cerebellum (Spence et al., 2009), which is also integral to song learning (Pidoux et al., 2018). Therefore, the mechanism by which Pb impairs song learning may not be isolated to neurological damage to song nuclei. Conversely, spatial learning involves the hippocampus and is independent of song learning (Bailey et al., 2009; Mayer and Bischof, 2012). Unlike the song learning circuit, the hippocampus shows adult neurogenesis, which may permit greater recovery from neural insults (Gahr et al., 2002). Our study suggests song learning is more vulnerable to postnatal Pb exposure than other cognitive learning modalities (e.g., spatial learning) because the growth and remodeling of song circuits occurs postnatally and damage to that process cannot be repaired after this window of neural plasticity ends at maturity.

Similar to our results, Ruuskanen et al. (2015) did not find differences in spatial learning in great tit (*Parus major*) nestlings exposed to Pb. However, several studies with murine systems indicate that exposure to Pb causes spatial learning deficits, though the results vary across studies and Pb doses are often 2–4 orders of magnitude greater than the doses used in our study, ranging from 100 to 1500 parts per million (e.g., Jett et al., 1997; Kuhlmann et al., 1997; Guilarte et al., 2003; Gilbert et al., 2005). Differences in the timing of developmental exposure to Pb may also contribute to discrepancies among studies. For instance, Kuhlmann et al., 1997 found that male rats exposed to Pb during gestation and lactation (via maternal exposure) had impaired spatial learning, but spatial learning was not affected by post-weaning Pb exposure. Our passive dosing design is comparable to real-world exposure scenarios, yet limited our control over when during development

nestlings were exposed to Pb because exposure to Pb was dependent on parental feeding prior fledging. Therefore, nestlings may have avoided sufficient Pb exposure prior to fledging to impair spatial learning.

In our study, both parents and offspring were exposed to Pb-treated water and we did not measure parental behavior. Therefore, we are unable to ascertain whether the cognitive effects observed in zebra finch offspring were caused directly by exposure to Pb or indirectly via Pb exposure modifying parental behavior (e.g., decreased nest attendance). However, offspring growth was similar across treatments, suggesting similar parental feeding rates. Importantly, offspring had direct access to Pb-treated water after fledging and during the critical window of song learning (20–90 days post hatch). Although we are unable to distinguish direct and indirect effects of exposure to Pb, taken as whole, our results suggest birds inhabiting Pb-contaminated environments may exhibit impaired song learning and motoric performance.

4.2. Effects of Pb on coloration

In our study, zebra finches exposed to 1000 ppb Pb exhibited bills with less red saturation but appeared to have increased red saturation of the cheek patch. These two ornaments rely upon different pigments, change over different time periods, and are correlated with different aspects antioxidant defense strategies. Consequently, it is unsurprising that Pb impacted these two pigments and thus coloration of the respective ornaments differently (Dauwe and Eens, 2008; Grunst et al., 2020). Bill color is determined by carotenoid availability, and carotenoids are directly or indirectly involved in the antioxidant defense system (Simons et al., 2012b; Hill and Johnson, 2012; Mundy et al., 2016). Elevated oxidative stress can result in a rapid (hours-days) reallocation of carotenoids away from bill pigmentation and towards defense against pro-oxidants produced by Pb exposure (Rosenthal et al., 2012; Eeva et al., 1998). Indeed, previous studies found birds exposed to Pb exhibited elevated oxidative stress (Mateo et al., 2003; Espín et al., 2014a) as well as increased circulating carotenoids and reduced carotenoid-dependent coloration (Vallverdú-Coll et al., 2016, 2015; Vermeulen et al., 2015). Additionally, it is possible that Pb-induced oxidative stress results in reduced astaxanthin (the red pigment in zebra finch bills) metabolism caused by a shift of cytochrome P450 enzymes towards detoxification, rather than ketocarotenoid synthesis. Therefore, zebra finches exposed to Pb in our study may have bills with less red saturation due to a reallocation of carotenoids towards antioxidant defense systems or shifts in P450 enzyme activity. Future studies measuring oxidative stress and P450 enzyme activity in birds exposed to Pb could directly test this hypothesis.

Unlike bill coloration that can change rapidly, changes in cheek patch coloration are dependent on new feather growth. Zebra finches develop their adult plumage between 50 and 100 days post hatch, thus male birds in our study were exposed to Pb while they grew their adult cheek patches. We predicted male zebra finches exposed to Pb would have cheek patches with less red saturation due to depletion of glutathione, the primary intracellular pool for cysteine required for pheomelanin synthesis (Galván et al., 2012), yet we observed the opposite trend. Zebra finches exposed to 1000 ppb Pb exhibited increased cheek patch red saturation compared to finches exposed to 100 ppb, but not compared to the control. While both glutathione and pheomelanin require the amino acid cysteine, high intracellular concentrations of glutathione shift melanin production from eumelanin to pheomelanin (Galván and Alonso-Alvarez, 2008). Some studies found that birds exposed to Pb exhibited higher glutathione concentrations, likely as an adaptive response to limit oxidative damage (Mateo et al., 2003; McGowan and Donaldson, 1986; Congiu et al., 2000). An increase in glutathione could lead to greater pheomelanin production and a more saturated cheek patch, whereas a reduction in glutathione may increase coloration of black, eumelanin-based traits. Indeed, several studies observed shifts in the coloration of black traits in free-living birds inhabiting metal contaminated sites (Chatelain et al., 2014; Dauwe and

Eens, 2008; Giraudeau et al., 2015, but see Grunst et al., 2020). We did not measure coloration of black plumage in zebra finch offspring, so we were unable to determine whether exposure to Pb altered black coloration. Our study provides further evidence of a relationship between exposure to toxic metals and altered melanin feather coloration; however, more research is required to understand the interaction between Pb and the underlying physiological mechanisms controlling feather coloration.

Carotenoid- and melanin-dependent coloration are indicators of physiological condition, and coloration of secondary sexual traits influences female mate preference. For instance, in many species of birds, females prefer males with more carotenoid coloration (Hill, 1990). Although there is some disagreement over the strength of bill redness as a sexual signal in zebra finches, females appear to prefer males with bills that have greater red saturation (Simons et al., 2012a, 2012b, but see Collins et al., 1994; Forstmeier and Birkhead, 2004). Likewise, female zebra finches prefer males with larger cheek patches (Tschirren et al., 2012; Burley et al., 2018; Naguib and Nemitz, 2007), but the importance of cheek plumage saturation and reflectance is less clear (e.g., Jerónimo et al., 2018; Price and Burley, 1994). Because male coloration is important for attracting female mates, shifts in pigmentation may contribute to male zebra finches exposed to Pb being less attractive to females.

4.3. Effects of Pb on female preference

Control female zebra finches visited males exposed to 1000 ppb Pb less often than control males. However, there was no evidence that control females avoided males exposed to 100 ppb Pb, and there was even a trend for females to spend more time near those males compared to the control. These results suggest exposure to 1000 ppb Pb indeed altered the ornaments that females use to assess potential mates. However, these effects were not observed in males exposed to 100 ppb Pb. Importantly, there were significant differences in song learning and bill coloration between males exposed to 1000 ppb Pb and controls, whereas neither song learning nor bill coloration differed between males exposed to 100 ppb Pb and controls. This supports our hypothesis that females will be less attracted to Pb exposed males because of impacts on their coloration and song. However, our design does not allow us to determine the differential importance of plumage, bill color or song traits for attracting female attention.

Reduced female attraction to Pb-exposed males has implications for male fitness. Previous studies found low-level exposure to Pb did not impact reproductive success as measured by nestling hatching success and survival when parents or nestlings were dosed (Chatelain et al., 2018; Eeva et al., 2014). However, these experimental designs did not examine indirect effects of Pb on reproductive success mediated by female preference. Our study is the first to look at female birds' preferences for Pb-exposed males relative to control males, but there is evidence of other contaminants (e.g., methylmercury) altering mate choice in white ibises (*Eudocimus albus*) (Frederick and Jayasena, 2011 but see Greene et al., 2018). Additionally, studies in other taxa found Pb exposure disrupts mate choice. For instance, female fruit flies (*Drosophila melanogaster*) were less likely to mate with Pb-exposed males compared to control males (Peterson et al., 2017), and there is robust evidence for Pb exposure disrupting reproductive and courtship behaviors in fish and rodents (Quintão et al., 2018; Alados and Weber, 1999; Sant'Ana et al., 2001). Our study focused on traits important for attracting mates, but many songbirds also exhibit courtship and pair bonding behaviors (e.g., dancing, allopreening, clumping, nest building) and future studies should investigate whether exposure to Pb influences these behaviors.

4.4. Concluding remarks

While much of the previous work examining the effects of Pb

exposure on the reproductive success of birds has focused on clutch size and nestling survival, our data suggest Pb exposure may indirectly affect fitness by altering secondary sexual traits important for attracting mates. Specifically, we found evidence of Pb exposure reducing the volume of song learning circuits in the brain and impairing song learning in zebra finches. Additionally, developmental Pb exposure altered adult coloration of males. Consequently, developmental exposure to Pb had implications for male attractiveness as an adult. Although Pb has been recognized as a neurotoxicant for centuries, the effects of Pb exposure on vocal communication remains understudied because many laboratory models (e.g., fish, rodents) produce limited vocalizations and do not show imitative vocal learning. In contrast, complex vocalizations are integral to songbird ecology and future studies are needed to (i) identify the specific neural mechanisms affected by Pb exposure, (ii) link neurotoxicity to vocal learning outcomes, and (iii) assess the consequences of these effects on lifetime reproductive success. It is worth noting that some caution should be exercised when extrapolating these effects to other songbirds due to interspecific variation in sensitivity to toxic metals and differences in sexual traits and reproductive strategies. Additionally, there is considerable spatial and temporal variation in Pb surface water concentrations within water catchments (e.g., Palumbo-Roe et al., 2012; Concas et al., 2006). Nonetheless, although this study employed experimental manipulation of Pb in water, wild birds are also exposed to Pb through soil and air. Thus, though birds in urban and industrial areas are likely at greatest risk of Pb exposure and sublethal consequences for reproductive success, more work in this area is needed to capture the range of potential fitness outcomes for songbirds exposed to Pb.

CRedit authorship contributions statement

CGG: data analysis and manuscript preparation; MLB: manuscript preparation and data collection; IVD: data collection; FC: data collection; SL: data collection; KBS: experimental design and manuscript preparation.

Declaration of Competing Interest

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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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2020.111850.

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