

## Research Article

# Methylmercury Exposure Reduces the Auditory Brainstem Response of Zebra Finches (*Taeniopygia guttata*)

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### ABSTRACT

Mercury contamination from mining and fossil fuel combustion causes damage to humans and animals worldwide. Mercury exposure has been implicated in mammalian hearing impairment, but its effect on avian hearing is unknown. In this study, we examined whether lifetime dietary mercury exposure affected hearing in domestic zebra finches (*Taeniopygia guttata*) by studying their auditory brainstem responses (ABRs). Zebra finches exposed to mercury exhibited elevated hearing thresholds, decreased amplitudes, and longer latencies in the ABR, the first evidence of mercury-induced hearing impairment in birds. Birds are a more appropriate model for the human auditory spectrum than most mammals because of similarities in frequency discrimination, vocal learning, and communication behavior. When mercury is considered in combination with other anthropogenic stressors such as noise pollution and habitat alteration, the hearing impairments we document here could substantially degrade avian auditory communication in wild birds.

**Keywords:** ABR, amplitude, hearing, hearing impairment, latency, toxicology, ecotoxicology, threshold shift

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### INTRODUCTION

Mercury is a concern for many humans who eat fish or are exposed occupationally, especially as mercury pollution is readily transported across the globe and expected to increase due to changes in mercury cycling associated with climate change drivers, including temperature, hydrology, and emissions (Krabbenhof and Sunderland 2013). Mercury causes a suite of human health effects, most notably neurotoxicity in the offspring of mothers exposed during pregnancy, but even at low levels can cause renal, cardiac and immune problems, and hearing impairment.

The effect of mercury on hearing has been studied intensively in mammals, including humans (Tokuomi 1968; Hoshino et al. 2012; Abdel-rasul et al. 2013), monkeys (Rice and Gilbert 1992; Rice 1998), mice (Wassick and Yonovitz 1985; Chuu et al. 2001; Huang et al. 2007; Huang et al. 2008; Huang et al. 2011), and rats (Igarashi et al. 1992), where mercury exposure can lead to impairment of high frequency hearing (Wassick and Yonovitz 1985; Rice and Gilbert 1992; Counter et al. 2012). In order to investigate the mechanisms and consequences of mercury-induced hearing impairment, avian model systems may represent the human system better than non-human mammals because of the parallels between human and avian vocal learning and auditory processing, including hearing-dependent vocal learning (Wooley 2013).

For both humans and birds, it has been hypothesized that mercury pollution disrupts auditory communication by causing hearing loss (Rabin et al. 2003). Chemical pollutants and metal toxins including mercury are associated with changes in bird song (Gorissen et al. 2005; DeLeon et al. 2013). In particular, mercury exposure has been linked with alterations in tonal frequency, complexity and quantity of male song

(Hallinger et al. 2010; McKay and Maher 2012). These song-related deficits might arise from mercury-induced changes to the songbird's brain. Specifically, axon degeneration (Loerzel et al. 1999; Bennett et al. 2009) and demyelination (Heinz and Locke 1976; Evans et al. 1982; Carpinelli et al. 2014) occur in the song-production pathways, and this type of axonal damage could also be present in the auditory pathway.

Mercury exposure may lead to hearing loss by impairing the structural and functional integrity of the inner ear and neuronal auditory pathways. Mercury has been shown to damage outer hair cells in rat cochleas (Crofton et al. 2000) and seals (Ramprasad and Ronald 1977), perhaps indirectly through mercury-induced oxidative damage (Clerichi 1995; Huang et al. 2008; Huang et al. 2011; Farina et al. 2011) or suppressed potassium currents, as shown in guinea pigs (Liang et al. 2003). Rodent studies show several mechanisms of mercury exposure that can alter neuron functionality, including disruption of mitochondrial permeability (Aschner et al. 2007) and antioxidant enzyme function (Aschner et al. 2007; Huang et al. 2008; Farina et al. 2011), glutamate excitotoxicity (reviewed in Aschner et al. 2007; reviewed in Farina et al. 2011), and abnormal ATPase gating kinetics (Anner and Moosmayer 1992; Chuu et al. 2001; Czaplinski et al. 2005; Huang et al. 2008; Huang et al. 2011). As avian hearing physiology exhibits similarities to that of mammals, it is reasonable to predict that mercury may disrupt these processes in birds as well.

The objective of this study was to investigate whether mercury affects hearing ability in the domestic zebra finch (*Taeniopygia guttata*). Prior research on the zebra finch auditory neural networks and song developmental processes, as well as parallels between bird song and human speech, make the zebra finch an appropriate model organism for this question (Nowicki et al. 2002; Ackermann and Ziegler 2013). To test hearing ability in zebra finches, we used auditory brainstem response (ABR) testing, which has been used to study the functionality of the peripheral auditory system in birds (Brittan-Powell

et al. 2002; Lucas et al. 2002; Brittan-Powell et al. 2005; Köppl and Gleich 2007; Henry and Lucas 2010; Noirot and Brittan-Powell 2011; Lohr et al. 2013; Crowell et al. 2015). The present study assessed zebra finches exposed to mercury over their entire lifetime, to test whether they, like mammals, exhibit mercury-induced hearing loss.

## METHODS

### Subjects

We collected auditory data from 146 lab-bred zebra finches (105 males, 41 females) between June 15, 2015, and March 28, 2016, including young birds of <265 days post-hatching (hereafter dph) (mean = 148 dph; range = 59–264 dph) and older birds of  $\geq 335$  dph (mean = 466 dph; range = 335–1455 dph) (Table 1). We expected little effect of age group on baseline hearing sensitivity, as juvenile and adult sensitivity can become indistinguishable shortly after fledging (Brittan-Powell et al. 2002). The birds were housed in single-sex groups of 4–6 at the College of William & Mary aviary, where they were kept under a 14:10 light/dark photoperiod at a mean room temperature of approximately 22 °C, with average noise levels of 75–80 dBA sound pressure (SPL). They had ad libitum access to food pellets (ZuPreem, Shawnee, Kansas), vitamin-enhanced water, grit, and cuttlefish calcium supplement. Zebra finch diet was prepared following Varian-Ramos et al. (2014), in which food pellets were mixed with aqueous methylmercury cysteine or cysteine alone, resulting in two treatments: mercury (1.2 ppm mercury,  $n = 78$ ) and control (0 ppm mercury,  $n = 68$ ). This amount is ecologically relevant based on the mercury levels of prey items from an industrially contaminated site in Virginia, as well as other studies globally (Cristol et al. 2008, reviewed in Varian-Ramos et al. 2014). Birds on the mercury treatment were exposed from development inside the egg (via maternal deposit of dietary mercury) until the age at time of testing. All experi-

**TABLE 1**

Sample size ( $n$ ), age in days, and blood mercury level by treatment, sex, and age category for all zebra finches tested

Treatment	Sex	Age category	$n$	Mean age (days)	Blood Hg level (mg/kg)
Control	Male	Young	22	163.9 $\pm$ 10.6	0.415 $\pm$ 0.018
		Older	25	518.0 $\pm$ 52.4	0.336 $\pm$ 0.017
	Female	Young	4	109.3 $\pm$ 6.2	0.015 $\pm$ 0.0002
		Older	17	457.7 $\pm$ 7.1	0.249 $\pm$ 0.014
Mercury	Male	Young	33	223.3 $\pm$ 16.1	16.452 $\pm$ 0.138
		Older	25	483.4 $\pm$ 31.7	17.701 $\pm$ 0.159
	Female	Young	17	126.8 $\pm$ 4.2	15.858 $\pm$ 0.139
		Older	3	371.3 $\pm$ 40.1	14.945 $\pm$ 0.023

Young birds were <290 days old at time of testing, and older birds were  $\geq 335$  days old. Age and blood mercury (Hg) levels are reported  $\pm$  standard error

mental procedures were in accordance with the Animal Care and Use Committee (protocols IACUC-2014-02-28-9273-wjbuchser and IACUC-IBC-2013-06-02-8721-dacris) at the College of William & Mary.

### General Auditory Test Procedure

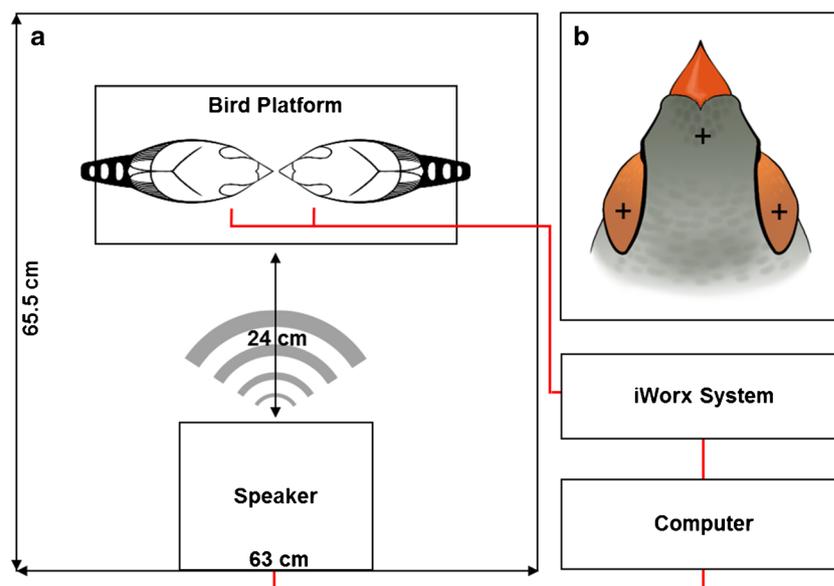
Prior to ABR testing, birds were sedated with an intramuscular injection of diazepam (Hospria Inc., Lake Forest, IL) at 4 mg/kg body mass, which was further diluted with sterile saline so that the total injection volume remained around 0.2 mL. This is standard in avian studies, and we used a minimal dose to reduce any effects on ABR (Brittan-Powell et al. 2008; Prather 2012). Sedation response to diazepam varied; if subjects did not rapidly become motionless, the bird was retested on a later date and the dose was increased by 0.25 mg/kg (up to 4.75 mg/kg) on subsequent trials. Subjects remained largely motionless for approximately 120 min during data collection. After each trial, birds were allowed to recover from sedation in a small cage before being returned to the aviary. Any retesting, due to incomplete sedation or equipment errors during a trial, did not occur until at least 1 week after the initial ABR trial to avoid cumulative sedation effects of diazepam. We observed return to normal behavior within 24 h in all cases.

After injection, each subject was wrapped in a small towel and placed in a custom-made sound-attenuating chamber (63 × 65.5 × 54.5 cm) with a background noise floor of 37.8-dB sound pressure. The chamber walls (5.75 cm thick) were composed of plywood, foam, and fabric inserts, and additional foam was

placed under the lip of the chamber. During each trial, we performed auditory testing on two subjects simultaneously. Each subject was positioned perpendicular to the speaker, so that the bird's ear was level with the main element of the speaker 24 cm away (Fig. 1). Standard platinum alloy subdermal needle electrodes (Grass F-E2; Warwick, RI) were placed just under the skin at the vertex (active) and directly behind each ear canal (reference electrode was placed at the ear closest to speaker; ground electrode was farthest from speaker). Shielded active and reference electrode leads were twisted together to reduce electrical noise through common mode rejection (see Brittan-Powell et al. 2002).

### Stimuli

Subjects were presented with frequency-specific stimuli of 500, 1000, 1500, 2000, 2860, 4000, 5700, and 8500 Hz using a speaker (Roland MA-74 with self-contained amplifier, Lake Stevens, WA). The accuracy and power of the peak frequencies presented were confirmed prior to trials. Each individual stimulus was 5 ms in duration (1 ms rise/fall  $\cos^2$ ) with a 20-ms interstimulus interval (ISI). Each frequency was presented in 1400-stimulus sets, where different sets differed by intensity. Intensities presented per frequency began at 40–45 dB and increased in 5 dB steps to a maximum of 70–75 dB. ABR results presented here represent two replicate trials (performed back-to-back), each of which is the average response of 1400 stimulus presentations per frequency-intensity combination (700 averages for each polarity/phase



**FIG. 1.** ABR testing setup. **a** Inside a sound-attenuating chamber, two zebra finches are attached to electrodes that record the brain's response to sound stimuli presented by the speaker. The iWorx system sends the signal to the computer to be recorded. **b** Electrodes are positioned at the apex of the head and behind each ear canal. Approximate electrode placement denoted by *plus symbols*. Diagram not drawn to scale.

were added together to cancel cochlear microphonic responses). Responses were sampled at 20 kHz and were bandpass filtered between 50–3300 Hz.

Stimulus intensities were calibrated in the sound-attenuating chamber using a Mastech MS6700 digital sound level meter (fast-weighting A scale; Pittsburgh, PA), which was placed at the same distance from the speaker as the bird's ear. We took extensive measures to ensure that our ABR system functioned similarly to other systems described in the literature. Twelve birds were used specifically for this task, and each was subjected to five ABR tests, performed every other day for 5 days. These ABR trials resulted in stereotypical waveform shape and as expected, an increase in sound intensity led to both an increase in amplitude and a decrease in latency. The coefficient of variation (CV) was calculated and used to determine the relative amount of dispersion in ABR latency and amplitude measures, resulting in CV's of 10 and 40–45 %, respectively, which are similar to values reported by Brittan-Powell et al. (2002). We also used intraclass correlation coefficient (ICC) repeatability analysis to determine that the peak I thresholds were indeed significantly repeatable (1000 Hz  $p = 0.271$ , 2860 Hz  $p = 0.001$ , 5700 Hz  $p = 0.016$ , 8500 Hz  $p = 0.003$ ), meaning that for peak I, an individual's response to testing on 1 day would be more similar to its response on a future day than would that of another subject.

## Data Acquisition

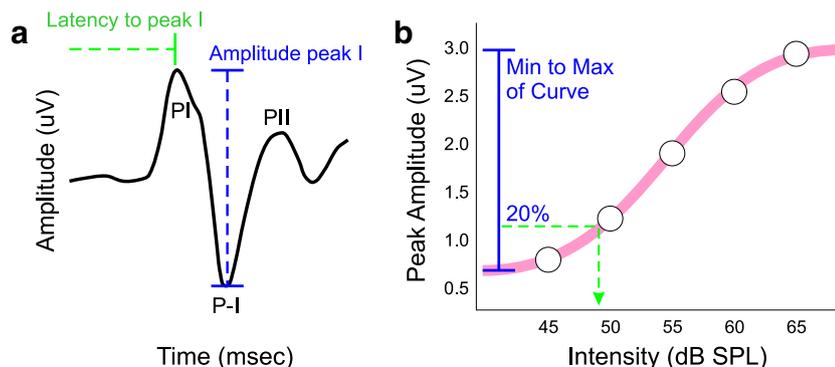
ABR response acquisition and data storage was coordinated by an iWorx system (model iwx214; Dover, NH). Electrode signals were preamplified then filtered and digitized by the recording unit. All trials were saved in files containing both the ABR responses and temporal synchronization information. ABR waveforms were extracted from each file using

proprietary open-source software (Supplemental Material) (Mcinturff and Buchser 2016). Artifact elimination was performed prior to averaging 1400 phase-locked stimulus responses per frequency-intensity combination.

For all trials, only characteristics of the ABR peak I are included (Fig. 2a). The generator of peak I is most likely the auditory nerve in birds (Katayama 1985; Brown-Borg et al. 1987; Hall 1992; Burkard et al. 1996); however, the generator of subsequent peaks in the avian ABR waveform are, as yet, unconfirmed. ABR peak I amplitude is a measurement of the change in voltage from the first waveform peak to the subsequent trough. ABR latency was defined as the time between stimulus presentation and peak I. Amplitude-intensity functions generated for each peak and frequency combination were used to estimate threshold using a non-linear least squares fit (implemented with R). We then determined the threshold value for each frequency, defined as the intensity at which the amplitude is 20 % above the lower asymptote of the curve (Fig. 2b). These thresholds were then visually inspected to check for errors.

## Mercury Analysis

We analyzed total mercury in blood samples taken from each bird within a week of ABR testing (Table 1). Methods followed Jackson et al. (2011), that is, samples were analyzed using combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT) according to US Environmental Protection Agency method 7473. Nearly all of the mercury in avian blood is comprised of methylmercury (Rimmer et al. 2005; Wada et al. 2009); thus, total mercury values are an accurate representation of methylmercury concentration.



**FIG. 2.** Measurement of ABR characteristics. **a** Schematic demonstrating how latency and amplitude measurements were taken for peak I of the zebra finch ABRs. **b** Schematic for how the threshold was computed after the peak amplitudes were fit to a sigmoid curve. In this case, the result would be a threshold of 49 dB.

## Statistical Analysis

We performed statistical analyses using IBM SPSS Statistics v23 employing two-tailed tests of significance. Results are reported as estimated marginal means, which are the mean responses for each response parameter adjusted for covariates in the model, with error bars representing 95 % confidence intervals (CI) unless otherwise noted. We assessed all response variables for normality and homoscedasticity prior to analysis. ABR threshold and ABR latency were normally distributed, and ABR amplitude was  $\ln + 1$  transformed to attain normality.

To examine whether mercury exposure influenced ABR threshold, we performed a linear mixed model. Fixed factors were treatment (control vs mercury), sex, and age (young vs older), while tonal frequency was treated as a repeated-measures fixed factor. Additionally, individual bird was treated as a random factor to account for between-subject variation in response. Specifically, we interpreted the main effect of treatment to determine whether mercury exposure led to significant changes in threshold values, and examined whether sex and age affected the response regardless of treatment.

We tested whether ABR amplitude and ABR latency were influenced by mercury exposure with two separate linear mixed models as described above. In addition, we treated intensity nested within frequency as a fixed factor to validate that increasing intensity leads to higher amplitudes and lower latencies across frequencies. We interpreted the main effect of treatment to determine whether mercury exposure led to significant changes in amplitude and

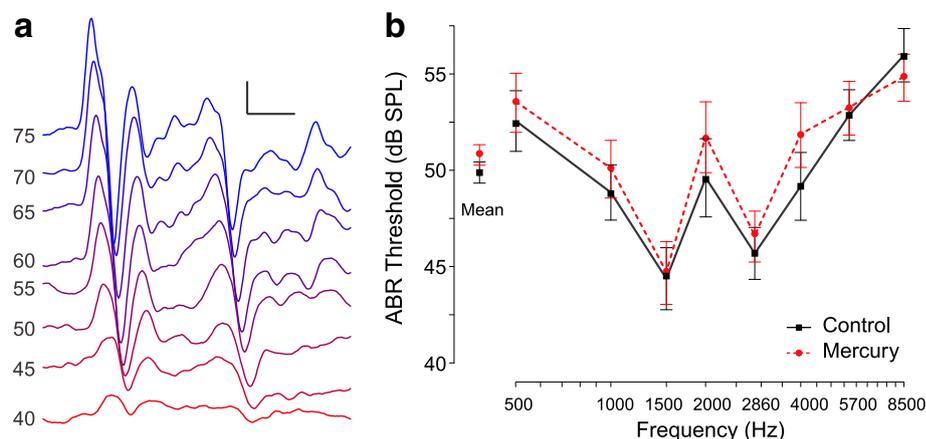
latency, and examined whether sex and age independently affected each response.

## RESULTS

### Mercury Exposure Elevated ABR Threshold

ABR waveforms (Fig. 3a) and threshold curves (Fig. 3b) showed the typical response found in other avian systems (Brittan-Powell et al. 2002; Lucas et al. 2002; Henry and Lucas 2010; Noirot and Brittan-Powell 2011; Lohr et al. 2013), with thresholds lower from 1500 to 2860 Hz than at higher and lower frequencies. Across frequencies, values spanned from 40.6–60.9 dB SPL (5th to 95th percentile). As expected with any audiogram, thresholds differed across frequencies ( $F_{7, 207} = 50.7$ ,  $P < 0.0001$ , other statistical tests listed in Table 2 (a)) with birds displaying lowest thresholds in the most sensitive range of hearing. Threshold responses were lower than expected at 1500 Hz, most likely due to the acoustics of the sound chamber. Results within each frequency are consistent, so it has no effect on the rest of the analysis.

We detected a significant effect of mercury treatment on ABR threshold (Table 2 (a)). Mercury-exposed birds exhibited elevated estimated marginal means of threshold values (averaged across dB SPL, sex, and age per frequency) relative to those of control birds (Fig. 3b), indicating that louder sound was required to initiate ABR peaks in birds exposed to mercury. Sex and age did not affect threshold values (Table 2 (a)), and no interaction terms were statisti-



**FIG. 3.** ABR threshold responses in zebra finches. **a** Representative waveforms from an individual bird (fed a control diet) at 4000 Hz as a function of time, stacked by sound intensity (dB SPL, quiet = red, loud = blue). As sound intensity increases, ABR amplitude of each peak increases and latency decreases. The horizontal scale bar is 2 ms and the vertical bar is 2 μV. **b** Estimated marginal means of

threshold values as a function of frequency in control (black solid line,  $n = 51$ ) and mercury-exposed birds (red dotted line,  $n = 60$ ). The “mean” estimated marginal means are averaged responses across all frequencies. Error bars are 95 % CIs.

TABLE 2

Linear mixed model analysis for the main effects on threshold, amplitude, and latency of the first ABR peak

Measure	Predictors	$F_{df}$	$P$
(a) Threshold	Treatment	6.080 (1,789)	<b>0.014</b>
	Frequency	50.697 (1, 207)	<b>&lt;0.0001</b>
	Age	0.001 (1,786)	0.973
	Sex	0.199 (1,786)	0.656
	Frequency × treatment	1.169 (7,207)	0.322
	Frequency × treatment × age	0.666 (15,177)	0.815
	Frequency × treatment × sex	0.537 (15,195)	0.918
(b) Amplitude	Treatment	41.881 (1,8055)	<b>&lt;0.0001</b>
	Frequency	35.691 (7,8055)	<b>&lt;0.0001</b>
	Age	29.698 (1,8055)	<b>&lt;0.0001</b>
	Sex	0.092 (1,8055)	0.761
	dB SPL (frequency)	60.703 (54,8055)	<b>&lt;0.0001</b>
	Frequency × treatment	0.358 (7,8055)	0.927
	Frequency × treatment × age	9.927 (15,8055)	<b>&lt;0.0001</b>
(c) Latency	Frequency × treatment × sex	13.115 (15,8055)	<b>&lt;0.0001</b>
	Treatment	21.970 (1,8056)	<b>&lt;0.0001</b>
	Frequency	77.787 (7,8056)	<b>&lt;0.0001</b>
	Age	10.883 (1,8056)	<b>0.001</b>
	Sex	1.115 (1,8056)	0.291
	dB SPL (frequency)	97.157 (54,8056)	<b>&lt;0.0001</b>
	Frequency × treatment	1.862 (7,8056)	0.071
Frequency × treatment × age	0.686 (15,8056)	0.801	
Frequency × treatment × sex	2.312 (15,8056)	<b>0.003</b>	

The significance of the fixed effects were assessed with type III SS  $F$ -tests (significant at  $\alpha = 0.05$ , highlighted in bold font). The degrees of freedom for the  $F$ -tests are written as subscripts after the  $F$  statistic

cally significant, suggesting that mercury did not differentially affect threshold across frequencies and neither did sex or age (Table 2 (a)).

### Mercury Exposure Decreased ABR Peak Amplitude

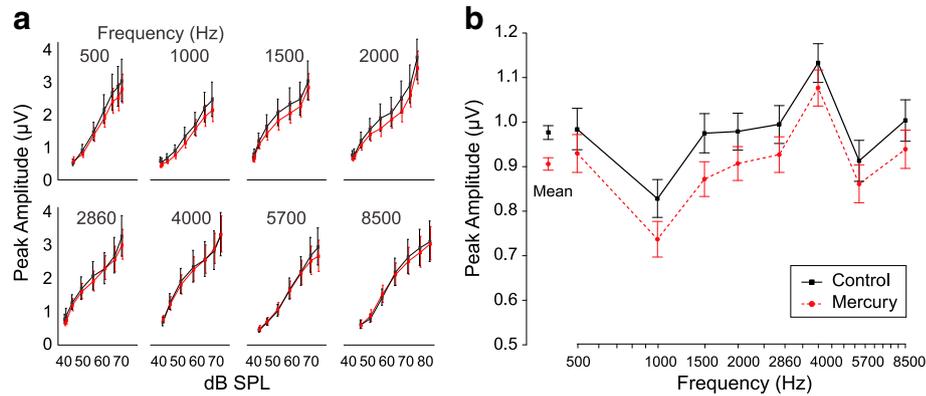
We next asked whether the amplitude of the waveform changes with mercury exposure. As expected, with increasing sound stimulus intensity, ABR amplitude increased (non-linearly) for all frequencies (Fig. 4a). The slopes of the amplitude-intensity functions differed by frequency, with slopes at mid-range frequencies moderately steeper than those higher and lower. The highest average amplitudes across all birds were observed near the range of best hearing in zebra finches (i.e., 4000 Hz; averaged across dB SPL, Fig. 4b). As expected, there was a significant effect of intensity at each frequency ( $F_{54,8055} = 60.7$ ,  $P < 0.0001$ ), and amplitude of the ABR response differed significantly between frequencies ( $F_{7,8055} = 35.7$ ,  $P < 0.0001$ ).

We detected a robust and statistically significant effect of mercury exposure on ABR peak amplitude ( $F_{1,8055} = 41.8$ ,  $P < 0.0001$ , Table 2 (b)), such that mercury-exposed birds exhibited lower estimated marginal means of peak amplitude values (averaged across dB SPL, sex, and age per frequency) than those of control birds (Fig. 4b). In determining whether age affected ABR amplitude, analysis showed that young

birds exhibited significantly higher amplitudes (mean =  $0.969 \pm 0.035$  [95 % CI], SE = 0.008) than older birds (mean =  $0.914 \pm 0.075$  [95 % CI], SE = 0.007), with non-overlapping 95 % CIs ( $F_{1,8055} = 29.7$ ,  $P < 0.0001$ ). The effect of age considered outside of the model was minimal (Fig. 5). However, sex did not affect amplitude (Table 2 (b)). While the interaction term between frequency and treatment was not statistically significant, three-way interactions between frequency, treatment, sex, and age did appear to affect peak amplitude values, suggesting an interplay between both sex and age with frequency (Table 2 (b)). Thus mercury-exposed birds had a dampened response to the sound stimuli, with no frequency-specific effect of mercury.

### Mercury-Exposed Birds Exhibited Extended ABR Latency

Latency of ABR responses decreased non-linearly as a function of increasing sound intensity across all frequencies (Fig. 6a). The shortest latencies occurred at 4000 Hz (Fig. 6b), near the region of best sensitivity in ABR audiograms, which also corresponds to the highest power spectrum of zebra finch vocalizations (Hashino and Okanoya 1989). As expected, there was a significant effect of intensity at each frequency ( $F_{54,8056} = 97.2$ ,  $P < 0.0001$ , Table 2 (c)), and latency differed significantly among frequencies ( $F_{7,8056} = 77.8$ ,  $P < 0.0001$ ).



**FIG. 4.** ABR amplitude responses in zebra finches. **a** Average amplitude as a function of intensity (dB SPL) for single frequency stimulus series. Across frequencies, as intensity increases, amplitude increases. **b** Estimated marginal means of amplitude values as a function of frequency for control (black solid line,  $n = 65$ ) and

mercury-exposed birds (red dotted line,  $n = 75$ ). Estimated marginal means are averaged responses across dB SPL, sex, and age. The “mean” estimated marginal means are averaged responses across all frequencies. All error bars are 95 % confidence intervals.

We detected a statistically significant effect of mercury treatment on ABR latency ( $F_{1,8056} = 21.97$ ,  $P < 0.0001$ , Table 2 (c)), where mercury-exposed birds exhibited longer estimated marginal mean latencies (averaged across dB SPL, sex, and age per frequency) than control birds, likely the result of auditory signal propagation to the auditory nerve requiring more time in mercury-exposed birds (Fig. 6b). Investigating whether age impacted ABR latencies revealed a statistically significant effect of age, in which older birds (mean =  $2.66 \pm 0.011$  [95 % CI]) showed longer peak latencies than young birds (mean =  $2.64 \pm 0.042$  [95 % CI]), with non-overlapping 95 % CIs ( $F_{1,8056} = 10.9$ ,  $P = 0.001$ , Table 2 (c)). Age had little effect outside the model (Fig. 5). While there was no significant main effect of sex, there was a notable interaction of mercury and sex on the latency for individual frequencies ( $P = 0.0003$ , Table 2 (c)). No other interactions yielded statistically significant results (Table 2 (c)). Thus, mercury exposure led to significant prolongation of ABR latencies.

The ABR waveforms contain other information, but there were no clear or consistent impacts on the rest of the waveform morphology (Fig. 7). Overall, ABR trials showed that zebra finches exposed to mercury exhibited elevated thresholds, which is tied to decreased amplitudes across all frequencies. In addition, mercury exposure led to increases in latency for peak I.

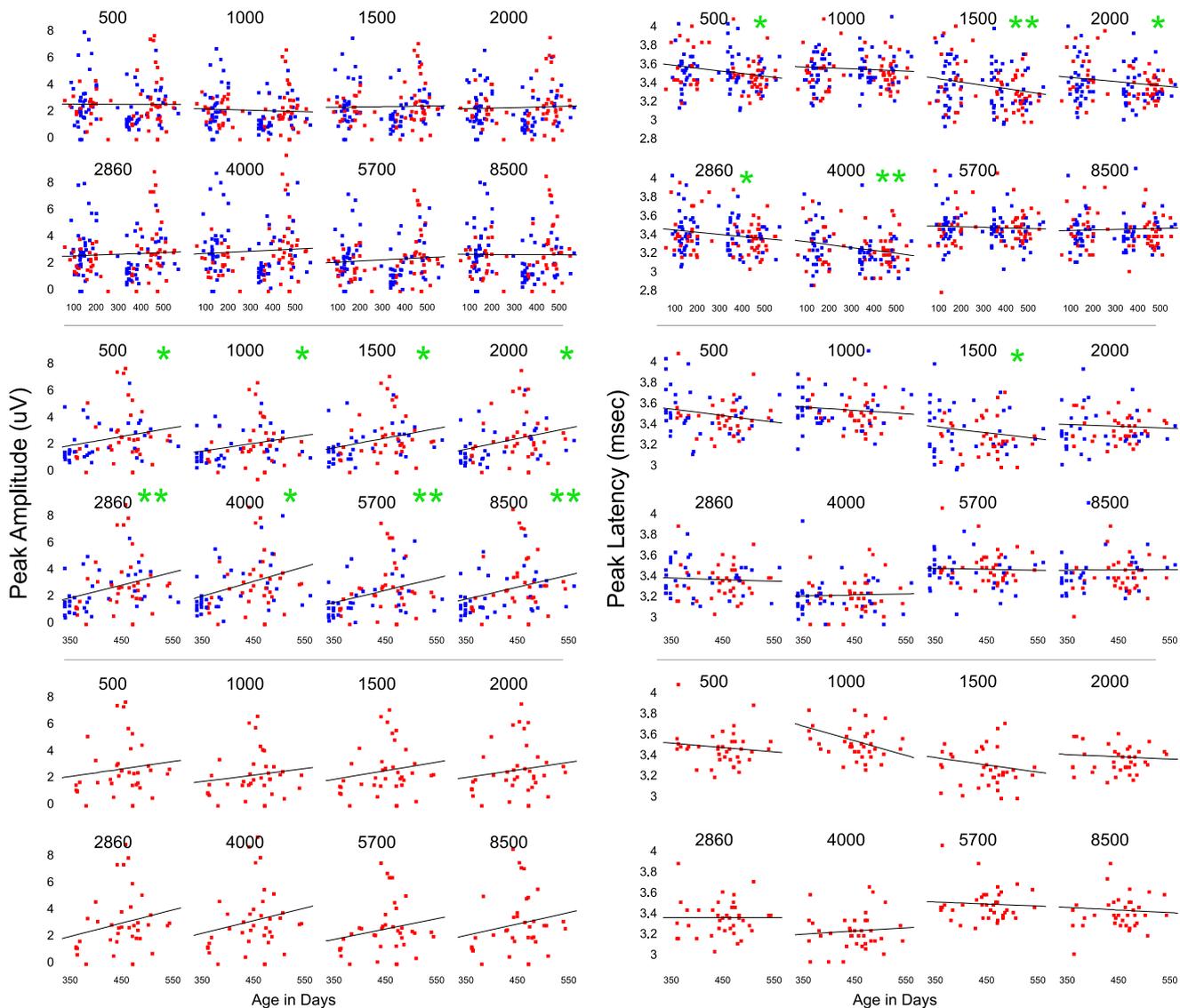
## DISCUSSION

In this study, we investigated whether lifetime mercury exposure in the domestic zebra finch, like that in mammals, causes mercury-induced hearing impairment. Mercury-exposed birds exhibited three markers of hearing impairment, namely elevated thresholds,

decreased amplitudes, and prolonged latencies at peak I of the ABR, presenting the first evidence of mercury-induced hearing impairment in birds.

Mercury-exposed zebra finches had higher thresholds than non-exposed birds by an average of 0.959 dB SPL in our experimental conditions, indicating that mercury-exposed birds required slightly louder auditory cues to elicit a hearing response. While no other studies have looked at the effect of mercury on the avian ABR, mercury induces elevated thresholds in mice (Wassick and Yonovitz 1985; Igarashi et al. 1992; Chuu et al. 2001; Huang et al. 2007; Huang et al. 2008; Huang et al. 2011), humans (Tokuomi 1968; Counter et al. 2012), and monkeys (Rice and Gilbert 1992; Rice 1998). To compare our mercury effect with those from other species, we used data from the published papers cited in the previous sentence to calculate Cohen’s  $d$  (Cohen 1977) coefficient ( $d = \text{mean of control} - \text{mean of mercury} / \text{pooled standard deviation}$ ). We used the threshold value at the mid-range frequency. The range of effect sizes was 0.33–6.53, all larger than the effect size of 0.12 shown here.

Mercury exposure lowered peak amplitudes by an average of 0.071  $\mu\text{V}$  in exposed birds. Because ABR amplitude correlates with the number of neurons firing in response to an auditory signal (Glasscock et al. 1991), a mercury-induced decrease in ABR amplitude suggests fewer neurons or lower firing rate in the auditory nerve. Few studies examining the effect of mercury exposure on hearing reported amplitude differences, thereby limiting our biological interpretation of a 0.071  $\mu\text{V}$  change. However, despite the typical large variation in amplitude associated with day, sex, age, and season (Lucas et al. 2002), we found a significant decrease in amplitude that implies a neural mechanism for mercury-induced hearing impairment.

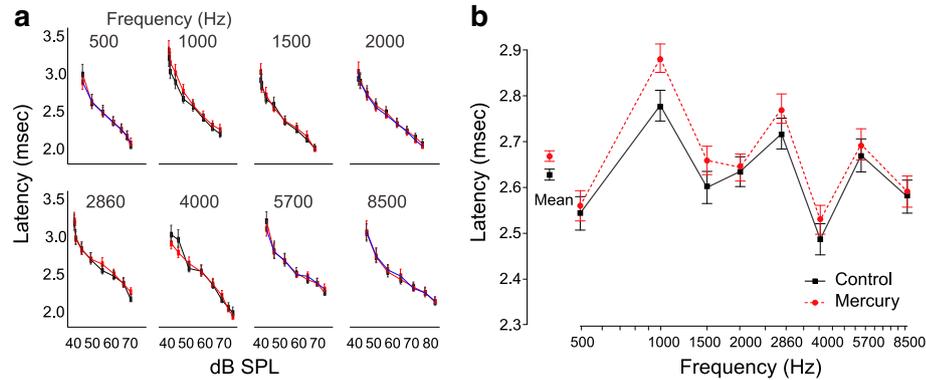


**FIG. 5.** ABR peak amplitude and latency is generally not related with age. Peak amplitude and latency are plotted from 65 dB stimulus for control (red) and mercury-treated (blue) birds by age in days. On the first row, both treatments and all ages from 100 to 600 days old are included. Second row, only “older” birds, at least

1 year old. Third row, only control birds (red) with the same ages as the second row. A linear regression was performed on each dataset independently, with the result indicated by the line overlaid across the points. Significant relationship is indicated by green asterisks.

Analyses of latency data revealed that mercury-exposed birds exhibited increased latencies at the auditory nerve, where latency represents the time it takes to propagate an auditory signal to a specific region of the brain. This latency difference could be a direct correlate to the lower observed amplitude or could be a separate mechanism. The average difference in estimated marginal means between treatments was 0.04 ms, suggesting that the conductance of the signals through the peripheral auditory pathway was slowed in mercury-exposed birds. Mercury has also increased latencies in mice (Chuu et al. 2001) and humans, in which the effect sizes (Cohen’s  $d$ ) of ABR latency differences were 0.22

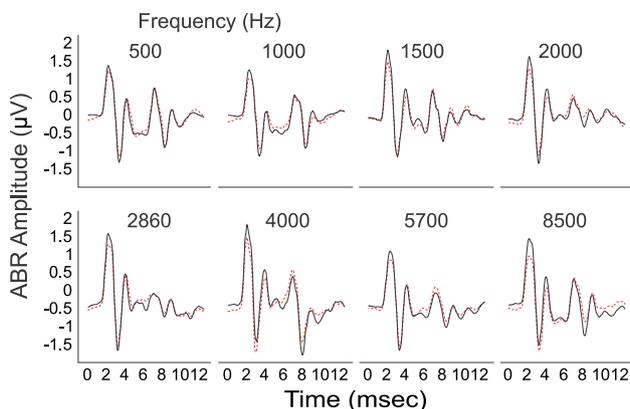
(Chuu et al. 2001) and 0.26 (Huang et al. 2008; Huang et al. 2011) in mice. In the current study, exposed birds exhibited prolonged latencies with an effect size of 0.14, similar to studies on mammals. Interestingly, prolonged latencies have been found to correlate negatively with cochlear frequency selectivity, suggesting that mercury can diminish the ability to distinguish between frequencies (Henry et al. 2011). This prolongation of latencies, which is expected with lower amplitudes, implies that mercury-induced neuronal dysfunction is resulting in both lower neuronal recruitment and slower propagation (i.e., axon degeneration or myelin loss) in response to an auditory signal.



**FIG. 6.** ABR latency responses in zebra finches. **a** Average latency as a function of sound intensity for single frequency tone trains. For all frequencies, an increase in intensity leads to a decrease in latency. **b** Estimated marginal mean latencies as a function of frequency for control (black solid line,  $n = 65$ ) and mercury-

exposed birds (red dotted line,  $n = 75$ ). Estimated marginal means are averaged responses across dB SPL, sex, and age. The “mean” estimated marginal means are averaged responses across all frequencies. All error bars are 95 % confidence intervals.

Why were the mercury effects in our study smaller than those in mammalian studies? Possibilities include differences in experimental design or hearing physiology. First, differences in mercury application between mammalian (oral gavage) and bird (dietary exposure) studies could result in mice absorbing larger doses of mercury in a relatively short amount of time, potentially leading to more extreme effects on threshold. In addition, the discrepancy could be impacted by the length of maternal mercury deposition in mice and birds, as mouse embryos, unlike birds in eggs, are continually exposed to new sources of maternal mercury throughout development. Time and length of exposure is also inconsistent between studies and could contribute to differences in effect size. Lastly, birds may have compensatory mechanisms not present in mammals (Barbaric et al. 2007), for example more robust hair cell regeneration (Brigande and Heller 2009).



**FIG. 7.** Consensus of ABR waveforms in response to sound intensity for various frequency stimuli in zebra finches. ABR waveform responses to a 70 dB stimulus, separated by frequency. Solid black lines represent the average response for all control birds ( $n = 63$ ), and dotted red lines represent the average response for all mercury-exposed birds ( $n = 74$ ).

Birds exposed to environmentally relevant doses of mercury may suffer if the hearing impairment disrupts the reception of important auditory signals. The general diminution of hearing ability across frequencies that we found suggests that mercury pollution may impact the reception of distant vocalizations more than those nearby, though the degree to which a signal is lost may be impacted by signal and environmental properties, warranting further investigation. Birds use perceived signal degradation to extract information about a signaler’s distance, a process called ranging (Naguib and Wiley 2001). Ranging incorporates auditory and visual cues, knowledge of the habitat, and an estimate of the sound source direction into a refined perception of where the signaler, in this case perhaps a competing male or predator, is located in the environment (Naguib et al. 2000; Nelson 2000). Combined with the decreased takeoff flight performance found in mercury-exposed starlings (Carlson et al. 2014), mercury could affect avian survival if it compromises the ability to respond to and flee from quiet or distant predator signals in the field. Especially when coupled with other stressors such as noise pollution and alteration of the acoustic properties of the landscape through habitat degradation, mercury-induced hearing loss could have major survival consequences.

In addition to disrupting hearing ability, mercury has also been implicated in altering song properties in birds. Songbirds exposed to more mercury at breeding sites exhibited changes in song tonal frequency, speed, gap durations, and complexity (Hallinger et al. 2010; McKay and Maher 2012). Interestingly, these changes were only found in species that actively learn song, suggesting that mercury might have its effects on song through the disruption of learning. As hearing plays a vital role in the song learning process, mercury-induced hearing impairment could also potentially affect the quality of male song (Brainard and

Doupe 2002; Spencer et al. 2003; Ackermann and Ziegler 2013; Ota and Soma 2014). Mercury-induced neuronal or axonal damage may occur in both the auditory and song-learning pathways of the brain; thus, mercury could impact song directly through neuronal damage of song nuclei and indirectly through hearing impairment.

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## COMPLIANCE WITH ETHICAL STANDARDS

All experimental procedures were in accordance with the Animal Care and Use Committee (protocols IACUC-2014-02-28-9273-wjbuchser and IACUC-IBC-2013-06-02-8721-dacris) at the College of William & Mary.

*Conflict of Interest* The authors declare that they have no conflict of interest.

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