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The Effect of Methylmercury on The Auditory Brainstem Response in Domestic Zebra Finches (Taeniopygia Guttata)

Sarah Elizabeth Wolf
College of William and Mary, sewolf@email.wm.edu

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The Effect of Methylmercury on the Auditory Brainstem Response
in Domestic Zebra Finches (*Taeniopygia guttata*)

Sarah Elizabeth Wolf

Mason, Ohio

Bachelor of Science in Biology, Centre College, 2014

A Thesis presented to the Graduate Faculty
of the College of William and Mary in Candidacy for the Degree of
Master of Science

Department of Biology
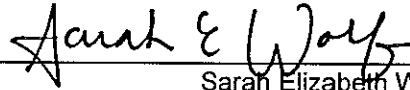
The College of William and Mary
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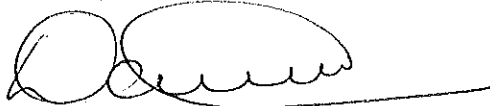
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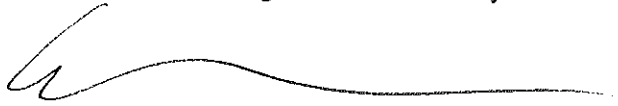
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Sarah Elizabeth Wolf

Approved by the Committee, June, 2016

_____
Committee Chair
Professor Dan Cristol, Biology
The College of William & Mary

_____
Visiting Assistant Professor William Buchser, Biology
The College of William & Mary

_____   (for J. Swaddle)
Professor John Swaddle, Biology
The College of William & Mary

_____
Professor Eric Bradley, Biology
The College of William & Mary

COMPLIANCE PAGE

Research approved by

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ABSTRACT PAGE

With global urbanization on the rise, human activities continue to threaten the functionality of auditory communication in birds through induced change to vocalizations, the acoustic landscape, and hearing ability. One human-associated pollutant potentially affecting auditory communication is mercury, which is released into the environment through industrial emissions and is correlated with markers of global climate change. Already, mercury has been implicated in mammalian high-frequency hearing impairment; however, the effect of mercury on avian hearing ability is unknown. In this study, we examined whether dietary mercury exposure affected hearing ability in domestic zebra finches using the auditory brainstem response (ABR), which measures the peripheral auditory pathway's response to sound. Our results revealed that mercury-exposed birds exhibited elevated hearing thresholds, lowered amplitudes, and longer latencies compared to non-exposed birds, all of which argue that methylmercury is degrading hearing ability in domestic zebra finches. When these effects are considered in combination with other anthropogenic stressors that are often correlated with mercury contamination in the field, the hearing impairments we document here could substantially degrade avian auditory communication in nature. This study presents the first evidence of mercury-induced hearing impairment in birds. If used as a model for vertebrate communication pathways, this and related research could elucidate how mercury-induced physiological responses associated with hearing could impact communication abilities and fitness in the field.

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Introduction

Animal communication conveys complex information essential for survival; unfortunately, global human activity appears to be slowly degrading this vital process. The increasing prevalence of urban sprawl results in anthropogenic stressors, including habitat fragmentation, introduced diseases, altered food sources, and pollution, that severely impact animal behavior and communication (Ditchkoff, Saalfeld, & Gibson, 2006). Human activity has specifically been implicated in disrupting auditory communication, in which induced change in vocalizations, landscape modifications, and hearing loss degrade the ability to send and receive information (Knight & Swaddle, 2011; Rabin, Mccowan, Hooper, & Owings, 2003).

Birds are a taxa particularly reliant on auditory communication, which facilitates detecting predators, distinguishing between individuals, advertising food locations, and choosing a mate, where the judgement of male song quality impacts territory and mating outcomes (Gil, Leboucher, Lacroix, Cue, & Kreutzer, 2004; Gill, 2006; Knudsen & Gentner, 2010; Marshall-Bal & Slater, 2004; Searcy & Brenowitz, 1988; Theunissen & Shaevitz, 2006). Within a species, natural selection typically favors a close match between signal design (i.e. auditory cues) and sensory capabilities (i.e. hearing ability) (Endler, 1992), resulting in overlap between a species' hearing range and biologically relevant cues associated with conspecific vocalizations, predators, and the environment (Endler, 1992). The process of hearing involves signal detection at the level of the ear's cochlea and signal

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processing in the auditory pathways of the brain, leading to various physiological and behavioral responses to sound (Dooling, Fay, & Popper, 2000). Under the duress of anthropogenic stressors, hearing ability could cease to match important auditory cues, perhaps resulting in higher mortality rates in affected bird populations.

Mercury is a global anthropogenic stressor correlated with the disruption of animal communication that is increasing in concentration due to industrial emissions (Burger & Gochfeld, 1997; Driscoll, Mason, Chan, Jacob, & Pirrone, 2013; Hallinger, Zabransky, Kazmer, & Cristol, 2010; Krabbenhoft & Sunderland, 2013; McKay & Maher, 2012). Released inorganic mercury can be readily converted by anaerobic bacteria into methylmercury (MeHg), which bioaccumulates and biomagnifies up food webs and readily integrates into the tissues of top predators (Hosseini, Nabavi, & Parsa, 2013; Lourenco, Tavares, del Mar Delgado, Rabaca, & Penteriani, 2011; Rimmer, Miller, McFarland, Talyor, & Faccio, 2010), leading to a variety of biological effects (Tartu et al., 2013; reviewed in Wolfe, 1998). MeHg can move from aquatic to terrestrial environments through key species utilizing both habitats, including predatory invertebrates such as spiders; therefore, terrestrial organisms can be exposed to high concentrations of MeHg in their diets (Cristol et al., 2008).

Birds exposed to MeHg in the field exhibit abnormal behaviors (Kenow, Hines, Meyer, Suarez, & Gray, 2010; Kobiela, Cristol, & Swaddle, 2015; M.C. Whitney & Cristol, in prep) including altered vocalizations (Hallinger, Zabransky, Kazmer, & Cristol, 2010; McKay & Maher, 2012; reviewed in

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Whitney & Cristol, in prep). These behavioral changes might arise from the well-documented effects of mercury on the brain, including axon degeneration (Bennett, French, Rossmann, & Haebler, 2009; Loerzel, Samuelson, & Szabo, 1999), demyelination (Evans, Garman, & Laties, 1982; Heinz & Locke, 1976), and spatial memory deficits (Bessler, 2011; unpublished data). While previous studies have correlated MeHg with changes in auditory signaling, it is currently unknown whether MeHg could also impact the reception of auditory signals. Because MeHg may impact neuron function in the avian brain, MeHg exposure could negatively affect the neuron-dependent process of hearing, here defined as a visible neuronal response to an auditory signal in the auditory pathways of the brain.

Studies have supported potential negative effects of MeHg on the auditory system in mammals, including humans (Abdel-rasul, Abu-salem, Al-batanony, Al-dalatony, & Allam, 2013; Hoshino, Ferreira, Malm, Carvallo, & Camara, 2012; Tokuomi, 1968), monkeys (Rice & Gilbert, 1992; Rice, 1998) mice (Chuu, Hsu, & Lin-shiau, 2001; Huang, Hsu, Liu, & Lin-Shiau, 2008; Huang, Liu, Hsu, & Lin-Shiau, 2011; Huang, Liu, & Lin-shiau, 2007; Wassick & Yonovitz, 1985), and rats (Igarashi, Koide, Sasaki, & Nakano, 1992). Humans exhibiting Minamata Disease show neurotoxic sensory deficits that include hearing impairment, especially at higher frequencies (Counter, Buchanan, & Ortega, 2012). High frequency hearing impairment is also exhibited by monkeys (Rice & Gilbert, 1992) and mice (Wassick & Yonovitz, 1985) exposed to MeHg during development. As these studies only

investigated MeHg's effects on hearing ability in mammals, it is important to study whether these trends are consistent in birds, a taxa heavily reliant on acoustic communication.

The objective of this study is to investigate whether MeHg affects hearing ability in the domestic zebra finch (*Taeniopygia guttata*). Here, we use the zebra finch as a model organism for studying neuron-dependent processes because of previous studies on the structures of song and auditory networks of the brain, knowledge of the song developmental process, and similarities to human speech and learning (Ackermann & Ziegler, 2013; Nowicki, Searcy, & Peters, 2002). To test hearing ability in zebra finches, we used auditory brainstem response testing (ABR), which is a class of auditory evoked responses well-known for its application to human infants but also often used as a noninvasive tool to study the functionality of the peripheral auditory system in birds (Brittan-Powell, Dooling, & Gleich, 2002; Henry & Lucas, 2010; Lohr, Brittan-Powell, & Dooling, 2013; Lucas, Freeberg, Krishnan, & Long, 2002; Noirot, Brittan-Powell, & Dooling, 2011). The ABR will record and analyze three parameters that indicate quality of hearing: (1) threshold, defined as the minimum volume required to hear a tone, (2) peak amplitude, related to the magnitude of neurons firing in response to a pitch at a specific auditory nucleus, and (3) peak latency, which is correlated with the conductance velocity of an auditory signal traveling to a specific auditory nucleus. We predicted that zebra finches exposed to dietary MeHg will show signs of hearing impairment and specifically, will exhibit increased thresholds,

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decreased amplitudes, and increased latencies, especially at higher frequencies in their hearing range.

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 <290 days post hatching (hereafter dph) (mean = 148 dph; range = 59-264 dph) and older birds of ≥335 dph (mean = 466 dph; range = 335-1455 dph) (Table 1). The birds were housed in single-sex groups of 4-6 at the College of William & Mary, where they were kept under a 14:10 light:dark photoperiod at a mean room temperature of approximately 22°C. They had ad libitum access to ZuPreem® food pellets (Shawnee, Kansas), vitamin-enhanced water, grit, and cuttlefish bone. Zebra finch diet was prepared following Varian-Ramos *et al*. (2014), in which food pellets were mixed with methylmercury cysteine or cysteine alone, resulting in two treatments: mercury (1.2ppm MeHg) and control (0ppm MeHg). All birds experienced their treatment from development inside the egg through the duration of the study. A blood sample was taken from each subject within one week of ABR testing to validate blood mercury level. On one occasion in August 2016, 25 control subjects were accidentally exposed to MeHg contaminated food for a few days, resulting in low but detectable mercury levels that reflected higher than normal MeHg levels for the control group (Table 1; Fig. S1; Jackson et al., 2011). 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.

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

Table 1. Sample size (n), age in days, and blood mercury level by treatment, sex, and age category for all zebra finches tested. Young birds were <290 days old at time of testing, and older birds were ≥335 days old. Here, control blood mercury levels are slightly elevated from an accidental mercury food mix-up in the aviary lasting several days; however, they are still representative of background mercury levels for songbirds in human-dominated landscapes (Fig. S1; Jackson et al., 2011).

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. Responses to diazepam varied; if subjects did not respond to

the sedative, we increased the dose by 0.25 mg/kg (up to 4.75 mg/kg) on subsequent trials. Subjects remained largely motionless for approximately 120 mins during data collection. After each trial, birds revived from sedation in a small cage and were returned to the aviary.

After injection, each subject was wrapped in a small towel and placed in a custom-made sound-attenuating chamber (63 x 65.5 x 54.5 cm) with a background noise floor of 37.8 dB sound pressure (Fig. 1). 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 to reduce external vibrations. During each trial we performed auditory testing on two subjects simultaneously. Each subject was positioned perpendicular to the speaker, with the bird's ear (approximately 13 cm high) level with the main element of the speaker 24 cm away. 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 (closest to speaker reference; farthest from speaker ground). Shielded active and reference electrode leads were twisted together to reduce electrical noise through common mode rejection (see Brittan-Powell et al., 2002).
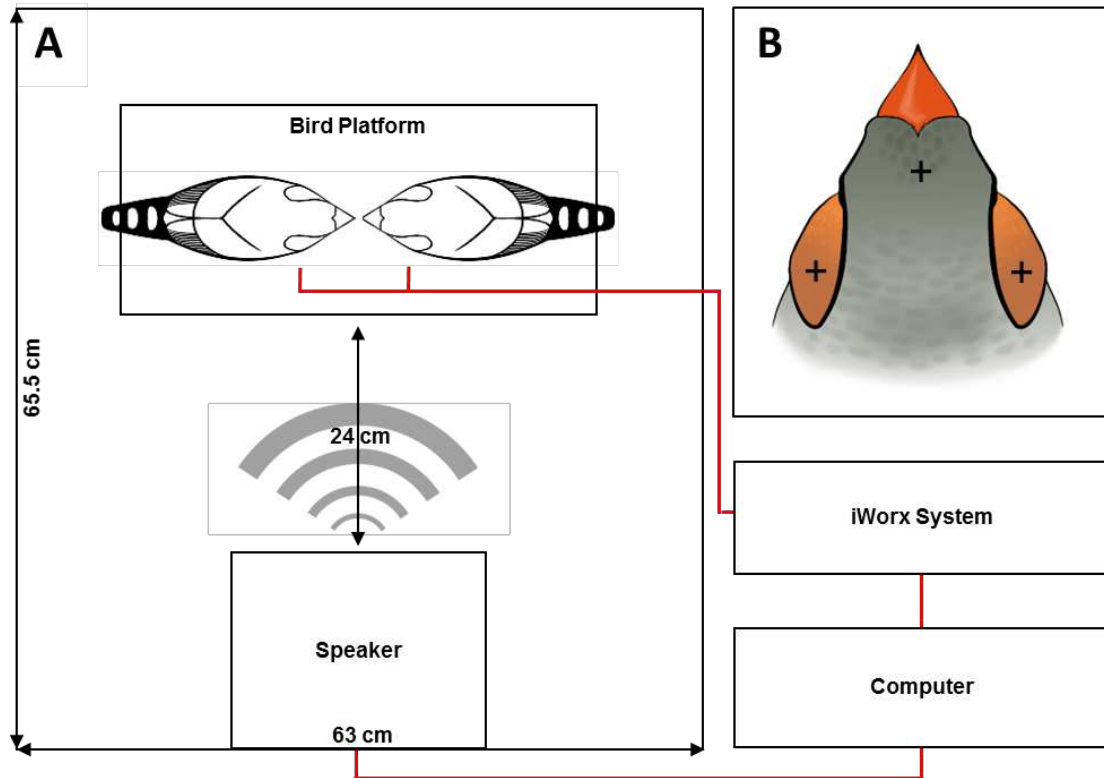
Figure 1. ABR testing setup. (A) Inside a sound-attenuating chamber, two zebra finches are attached to electrodes that record the brain's response to sound stimuli presented by the speaker. The iWorx system sends the signal to the computer to be recorded. (B) Electrodes are positioned at the apex of the head and behind each ear canal. Approximate electrode placement denoted by black + marks. Diagram not drawn to scale.

Stimuli

Subjects were presented with sound stimuli that varied in frequency and intensity and were presented using a Roland MA-74 speaker (Lake Stevens, WA, with self-contained amplifier). Each individual stimulus was 5 ms in duration (1 ms rise/fall cos^2) with a 20-ms interstimulus interval (ISI). Series of

stimuli were presented in ascending order of tonal frequency as 500, 1000, 1500, 2000, 2860, 4000, 5700, and 8500 Hz, and the accuracy and power of the peak frequencies were confirmed before ABR testing began (Fig. S2). Each frequency was presented in 1400-stimulus sets, where 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 (varied by frequency). Intensity ranges per frequency were chosen based on the floor noise level of the sound-attenuating chamber and ensured that the intensities encompassed the bird's threshold at each frequency. 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 were added together to cancel cochlear microphonic responses generated by ear hair cells). Responses were sampled at 20000 Hz for a 25 ms recording time following the onset of each individual stimulus and were bandpass filtered between 50-3300 Hz to eliminate low and high frequency noise.

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 approximate position of the bird's ear. The test tones used to calibrate intensity were identical in parameters to the stimulus presented during trials.

System Validations

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We took extensive measures to ensure that our ABR system functioned similarly to other systems described in the literature, such as the TDT modular rack-mount system (Brittan-Powell, Dooling, & Gleich, 2002). Because we only tested each bird once, it was important to validate that responses from a single day were indicative of a bird's average ABR response. In order to measure the repeatability of threshold within an individual bird, we performed ABR trials on 12 birds repeatedly for 5 days (with 1 day between each retest). We used intraclass correlation coefficients (ICC) to calculate the repeatability of ABR threshold measurements. The coefficient of variation (CV) was also calculated to determine the relative amount of dispersion in ABR latency and amplitude measures across subjects in this study.

Data Acquisition

ABR response acquisition and data storage was coordinated by an iWorx system (model iwx214; Dover, NH). Electrodes were connected to a headstage that preamplified the response signal, after which the signal was 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 (http://wmpeople.wm.edu/site/page/wjbuchser/resources) (Mcinturff & Buchser, 2015). Artifact elimination was performed prior to

averaging 1400 phase-locked stimulus responses per frequency-intensity combination (measured between 0 and 12 ms after each individual stimulus). After averaging, sets exhibiting a root mean square (RMS) higher than 2 standard deviations above the entire trial's RMS were rejected, leading to the final waveform responses, which the software analyzed to generate ABR peak amplitudes and latencies. Later analysis of amplitude responses by intensity were used to calculated threshold values.

For all trials, characteristics of peaks I and II, as well as the first trough (denoted peak –I), were described (Fig. 2). The generator of peak I is most likely the auditory nerve in birds (Brown-Borg, Beck, & Jones, 1987; Burkard, McGee, & Walsh, 1996; Hall, 1992; Katayama, 1985); however, the generator of peak II in the avian ABR waveform is as yet unknown. ABR peak amplitude is a measurement of the change in voltage from peak to trough (peaks –I and II) or base of the peak to peak (peak I). ABR latency is a measurement of the time in ms required to propagate the auditory signal to each peak or trough in the response, and was measured from stimulus presentation to each peak or trough. Lastly, threshold calculations were based on peak amplitudes for peaks I, -I, and II.

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 Studio). Resulting curves with a residual standard error above 0.4 were manually inspected for quality of fit; most of these were eliminated. We then determined the threshold value for each frequency,

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defined as the intensity at which the amplitude is 20% above the lower

asymptote of the curve.
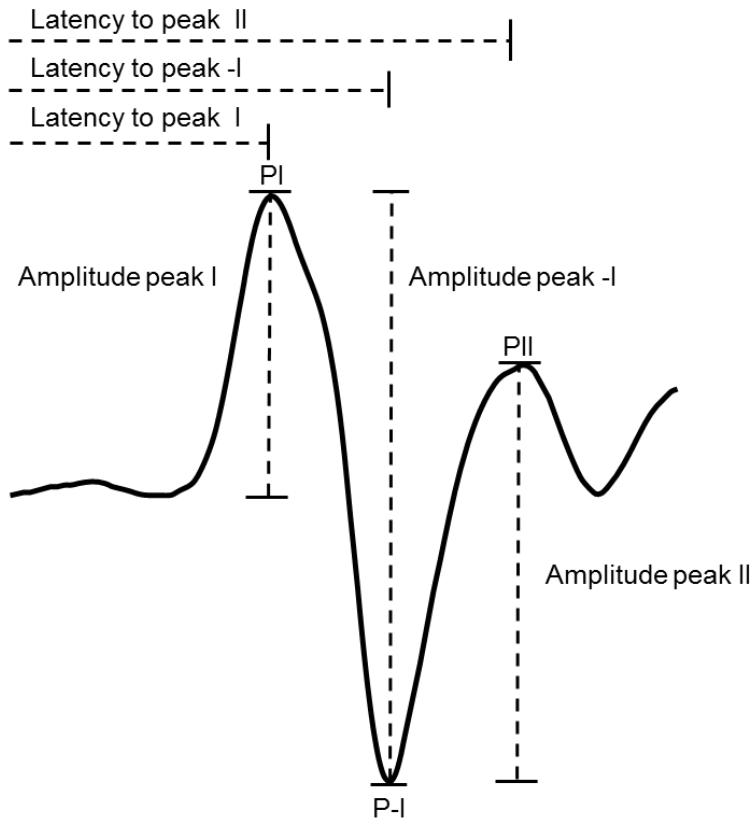
Figure 2. Schematic of latency and amplitude measurements for peaks I, -I, and II.

Mercury Analysis

We analyzed total mercury (THg) in blood samples taken from each bird

within one week after ABR testing. Because nearly all of the mercury in avian

blood is comprised of MeHg (Rimmer et al., 2005; Wada, Cristol, McNabb, &

Hopkins, 2009), THg values are an accurate representation of MeHg

concentration. THg samples were analyzed using combustion-amalgamation-cold vapor atomic absorption spectrophotometry (Direct Mercury Analyzer 80, Milestone, Monroe, CT) according to U.S. Environmental Protection Agency method 7473. Every run of 20 samples was preceded by a duplicate, a blank, and standard reference materials (SRM; tuna and DORM-4 fish protein, National Research Council of Canada, Ottawa, Ontario) for quality control in every run of 20 samples. Average relative percent difference between replicate sample analyses was 1.32 ± 0.28% (n=14). Mean percent recoveries of THg for the tuna and DORM-4 were 98.99 ± 1.42% (n = 14) and 95.99 ± 0.48% (n = 14), respectively. All THg concentrations are reported on wet weight basis. Control birds had mean ± SD blood mercury concentration, on a wet weight basis, of 0.32 ± 0.46 µg/g (range <0.01-1.99 µg/g) and mercury-exposed birds averaged 16.66 ± 4.16 µg/g (Table 1; range 9.33-27.89 µg/g).

Statistical Analysis

We performed all statistical analyses using IBM SPSS Statistics v23 employing two-tailed tests of significance. Averages are reported as estimated marginal means (i.e. means for a specific effect) while accounting for ± 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), and tonal frequency was treated as a repeated-measures fixed factor. Additionally, 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. Interaction terms included frequency × treatment, frequency × treatment × sex, and frequency × treatment × age, which examine whether mercury was having a differential effect across the frequencies we measured (i.e. high or low frequency hearing loss), and if that effect differed by age and sex.

We tested whether ABR amplitude and ABR latency were influenced by mercury exposure with two separate linear mixed models. For each, fixed factors were treatment (control vs mercury), sex, and age (young vs older), and frequency was treated as a repeated-measures fixed factor. 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. Bird was treated as a random factor. Specifically, 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. Interactions included frequency × treatment, frequency × treatment × sex, and frequency × treatment × age,

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which test whether mercury differentially affects amplitude and latency across frequencies, and if those effects differed by age and sex.

Results

ABR Quality Control

We calculated ICCs to measure the repeatability of ABR threshold measurements within individual birds, and found acceptable repeatability at 1 of 3 peaks measured. For peak -I, ICCs showed moderate to high concordance, with ICC's of tested frequencies ranging between 0.621 and 0.848; however, ICCs for peaks I and II indicated poor concordance (Table S1). The coefficient of variation (CV) was also 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. Based on ICC values, we analyzed only the ABR responses related to peak 1 (response at the auditory nerve) using measurements of amplitude and threshold from peak –I measurements and latency from peak I measurements (Fig. 1).

ABR trials resulted in stereotypical waveform morphology for individual zebra finches (Fig. 3A). Visual examination of the waveforms showed at least two replicable, prominent peaks that occurred within the first 5 ms of the response. As expected, an increase in sound intensity led to both an increase in amplitude and a decrease in latency, indicating that the response was greater for louder sounds.

Mercury exposure elevated ABR threshold

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ABR threshold responses showed the typical U-shaped response found in other avian systems (Brittan-Powell et al., 2002; Henry & Lucas, 2010; Lohr et al., 2013; Lucas et al., 2002; Noirot et al., 2011), with thresholds lower from 1500 to 2860 Hz than at higher and lower frequencies (Fig. 3B). Across frequencies, values range from 26.27 – 77.37 dB SPL. As expected with a U-shaped audiogram, thresholds differed across frequencies ($F_{7,207}$=50.697, P<0.0001) with birds displaying lowest thresholds in the most sensitive range of hearing; however, threshold responses were unexpectedly high at 2000 Hz.

We detected a weak significant effect of mercury treatment on ABR threshold (Table 2, $F_{1,789}$=6.080, P=0.014). Mercury-exposed birds exhibited higher estimated marginal means of threshold values than those of control birds, indicating that louder sound was required to initiate ABR in birds exposed to mercury. Examination of 95% CIs show that while CIs overlap, estimated marginal means per treatment fall outside of the opposite CI, supporting a statistically significant difference between treatments (Fig. 3B). Sex and age did not affect threshold values (sex: $F_{1,786}$=0.199, P=0.656; age: $F_{1,786}$=0.001, P=0.973), and no interaction terms were statistically significant, suggesting that mercury, as well as its interaction with sex and age, did not differentially affect threshold across frequencies (frequency x treatment: $F_{7,207}$=1.169, P=0.322; frequency × age × treatment: $F_{15,177}$=0.666, P=0.815; frequency × sex × treatment: $F_{15,194}$=0.537, P=0.918). Overall, our results indicated that the main effect on threshold values was exposure to mercury,

where sex and age did not significantly impact responses and there was no indication of a frequency-specific effect of mercury.
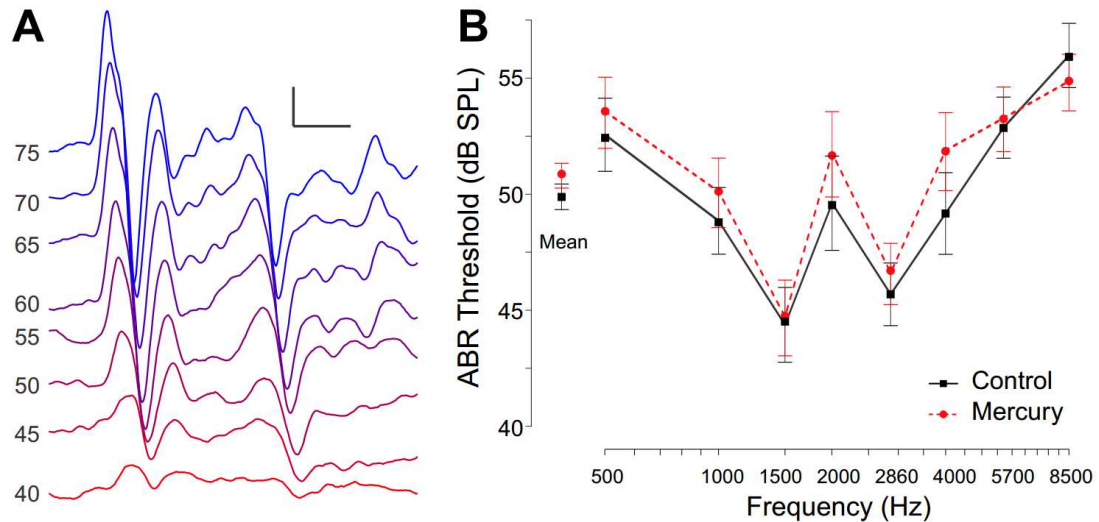
Figure 3. ABR threshold responses in zebra finches. (A) Example waveforms from an individual bird fed a normal diet at 4000 Hz as a function of time, stacked by sound intensity (dB SPL, quiet=red, loud=blue). As intensity increases, ABR amplitude of each peak increases and latency decreases. The vertical scale bar is 2 ms and the horizontal is 2 μV. (B) Estimated marginal mean of threshold values as a function of frequency in control (black solid line) and mercury-exposed birds (red dotted line). The "mean" estimated marginal means are averaged responses across all frequencies. Error bars are 95% CIs.

Lifetime mercury exposure decreased ABR peak amplitude

As intensity increased, ABR amplitude increased non-linearly for all frequencies (Fig. 4A). The slopes of the amplitude-intensity functions differed

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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; Fig. 3B). As expected, there was a significant effect of intensity at each frequency ($F_{7,8055}=35.691$, $P<0.0001$), and amplitude of the ABR response differed significantly between frequencies ($F_{7,8055}=35.691$, $P<0.0001$).

We detected a statistically significant effect of mercury exposure on ABR peak amplitude (Table 2, $F_{1,8055}=41.881$, $P<0.0001$), where mercury-exposed birds exhibited lower estimated marginal means of peak amplitude values than those of control birds (Fig. 4B). Examination of 95% CIs show no overlap in CIs between treatments. In determining whether age affected ABR amplitude, analysis showed that young birds exhibited statistically significantly higher amplitudes (M=0.969, SE=0.008) than older birds (M=0.914, SE=0.007), with non-overlapping 95% CIs ($F_{1,8055}=29.698$, $P<0.0001$, 95% CI (0.035,0.075)). However, sex did not affect amplitude ($F_{1,8055}=0.092$, $P=0.761$). While the interaction term between frequency and treatment was statistically non-significant, three-way interactions between frequency, treatment, sex, and age significantly affected peak amplitude values, suggesting an interplay between both sex and age with frequency (frequency x treatment: $F_{7,8055}=0.358$, $P=0.927$; frequency × age × treatment: $F_{15,8055}=9.927$, $P<0.0001$; frequency × sex × treatment: $F_{15,8055}=13.115$, $P<0.0001$). Thus, mercury-exposed and older birds responded less strongly to sound stimuli, with no frequency-specific effect of mercury.
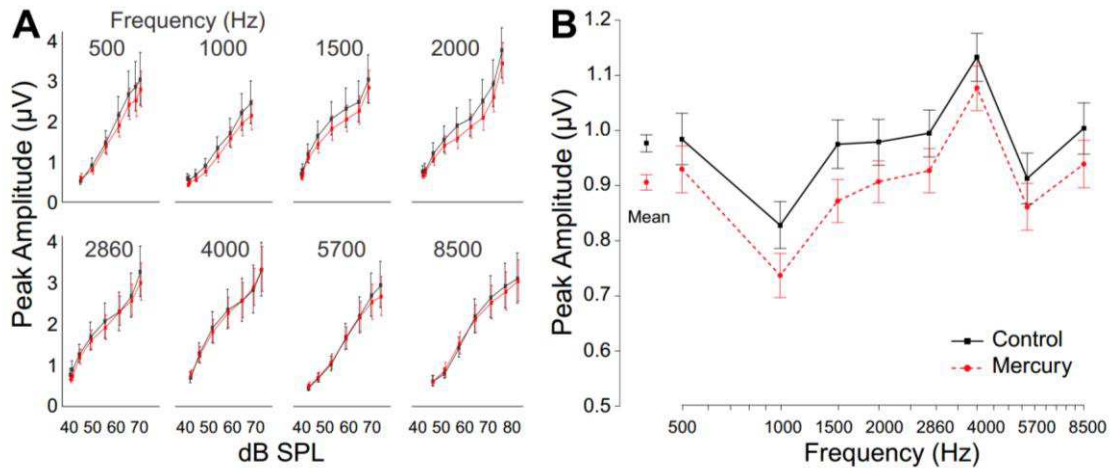
Figure 4. ABR amplitude responses in zebra finches. (A) Average amplitude as a function of intensity (dB SPL) for single frequency stimulus series. Across frequencies, as intensity increases, amplitude increases. (B) Estimated marginal means of amplitude values as a function of frequency for control (black solid line) and mercury-exposed birds (red dotted line). The "mean" estimated marginal means are averaged responses across all frequencies. All error bars are 95% confidence intervals.

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. 5A). The shortest latencies occurred at 4000 Hz (Fig. 5B), near the region of best sensitivity in ABR audiograms, which also corresponds to the highest power spectrum of zebra finch vocalizations (Hashino & Okanoya, 1989). As expected, there was a significant effect of intensity at each frequency ($F_{54,8056}=97.157$, $P<0.0001$),

and latency differed significantly between frequencies ($F_{7,8056}$=77.787,

P<0.0001).
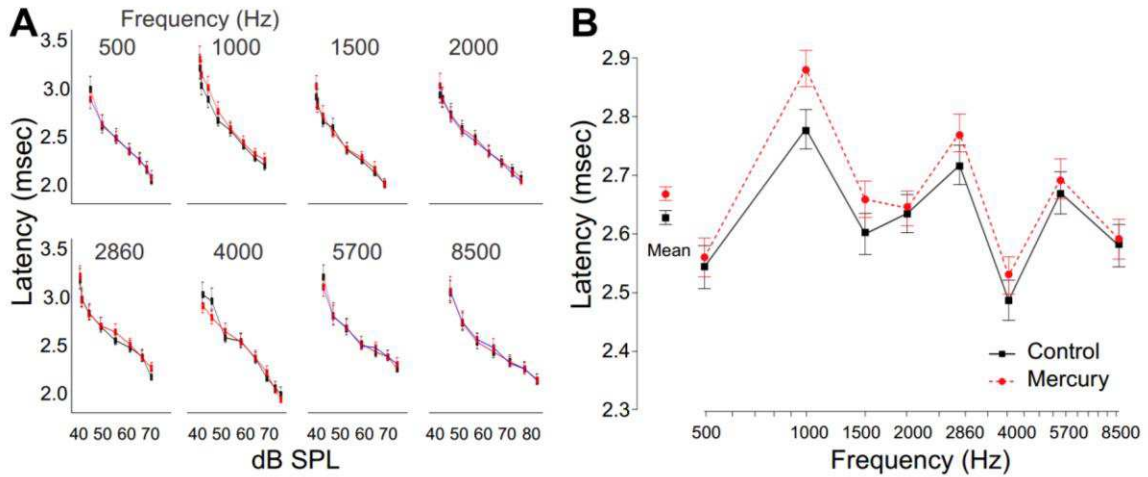
Figure 5. ABR latency responses in zebra finches. (A) Average latency as a

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) and mercury-exposed birds (red dotted line). 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 treatment on ABR

latency (Table 2, $F_{1,8056}$=21.970, P<0.0001), where mercury-exposed birds

exhibited longer estimated marginal mean latencies than control birds,

showing that auditory signal propagation to the auditory nerve took a longer

time in mercury-exposed birds (Fig. 5B). Investigating whether age impacted

ABR latencies revealed a statistically significant effect of age, in which older birds (M=2.661, SE=0.006) showed higher peak latencies than young birds (M=2.635, SE=0.006), with non-overlapping 95% CIs ($F_{1,8056}$=10.883, P=0.001, 95% CI (0.011,0.042)). While there was no significant effect of sex ($F_{1,8056}$=1.115, P=0.291), there was a weak significant frequency × sex × treatment interaction ($F_{15,8056}$=2.312, P=0.0003). All other interactions yielded non-significant results, showing that mercury, as well as its interaction with age, did not differentially affect latency across frequencies (frequency x treatment: $F_{7,8056}$=1.862, P=0.071; frequency x treatment x age: $F_{15,8056}$=0.686, P=0.801). Mercury exposure led to significant prolongation of ABR latencies; in addition, it appears that older birds showed longer latencies and there was again no frequency-specific effect.

Overall, ABR trials showed that zebra finches exposed to mercury exhibited elevated thresholds, which is tied to decreased amplitudes across all frequencies, as shown in Fig. 4A. In addition, mercury exposure led to increases in latency for peak I. Interestingly, both amplitude and latency may be impacted in later peaks of the ABR as well (visual inspection of Fig. 6A). While confidence intervals do overlap between treatments, overlap in CIs seems minimal for peak I responses (Fig. 6B).
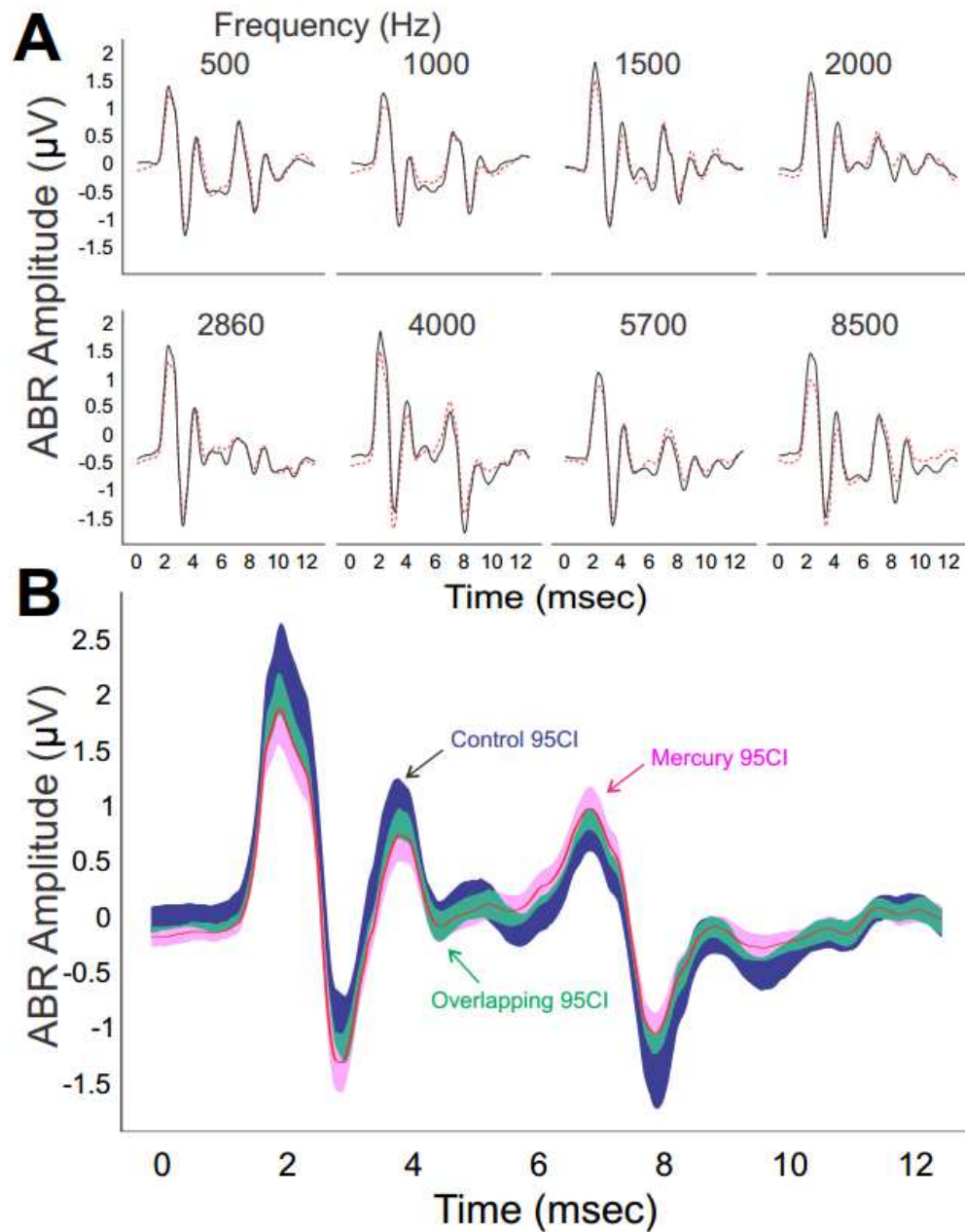
Figure 6. Consensus of ABR waveforms in response to sound intensity for various frequency stimuli in zebra finches. (A) ABR waveform responses to a 70dB stimulus, separated by frequency. Solid black lines represent the average response for all control birds and dotted red lines represent the average response for all mercury-exposed birds. (B) Magnified (or detailed)

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view of the average ABR responses to a 70dB stimulus for all birds at 4000

Hz, with 95% confidence intervals shown for each treatment. Pink indicates

non-overlapping control CIs, dark blue indicates non-overlapping mercury

CIs, and green-blue indicates overlapping CIs. Regions where the central red

line is surrounded by pink and not green-blue indicate statistically significant

areas.

Dependent Variables	Independent Variables	F_{df}	P
Threshold	Treatment	$6.080_{\ 1,789}$	**0.014**
	Frequency	$50.697_{\ 1,207}$	**<0.0001**
	Age	$0.001_{\ 1,786}$	0.973
	Sex	$0.199_{\ 1,786}$	0.656
	Frequency x Treatment	$1.169_{\ 7,207}$	0.322
	Frequency x Treatment x Age	$0.666_{\ 15,177}$	0.815
	Frequency x Treatment x Sex	$0.537_{\ 15,195}$	0.918
Amplitude	Treatment	$41.881_{\ 1,8055}$	**<0.0001**
	Frequency	$35.691_{\ 7,8055}$	**<0.0001**
	Age	$29.698_{\ 1,8055}$	**<0.0001**
	Sex	$0.092_{\ 1,8055}$	0.761
	dB SPL(Frequency)	$60.703_{\ 54,8055}$	**<0.0001**
	Frequency x Treatment	$0.358_{\ 7,8055}$	0.927
	Frequency x Treatment x Age	$9.927_{\ 15,8055}$	**<0.0001**
	Frequency x Treatment x Sex	$13.115_{\ 15,8055}$	**<0.0001**
Latency	Treatment	$21.970_{\ 1,8056}$	**<0.0001**
	Frequency	$77.787_{\ 7,8056}$	**<0.0001**
	Age	$10.883_{\ 1,8056}$	**0.001**
	Sex	$1.115_{\ 1,8056}$	0.291
	dB SPL(Frequency)	$97.157_{\ 54,8056}$	**<0.0001**
	Frequency x Treatment	$1.862_{\ 7,8056}$	0.071
	Frequency x Treatment x Age	$0.686_{\ 15,8056}$	0.801
	Frequency x Treatment x Sex	$2.312_{\ 15,8056}$	**0.003**

Table 2. Linear mixed model analysis for the main effects on threshold, amplitude, and latency of the first ABR peak. The significance of the fixed effects were assessed with Type III SS F-tests. All statistically significant P-values are bolded, using α=0.05. The degrees of freedom for the F-tests are written as subscripts after the F statistic.

Discussion

Our results indicated that mercury-exposed birds exhibited three markers of hearing impairment, namely elevated thresholds, decreased amplitudes, and extended latencies at peak I of the auditory brainstem response. Mercury-exposed zebra finches exhibited thresholds higher than non-exposed birds with an average difference of 0.959 dB SPL, suggesting that mercury induces hearing impairment such that mercury-exposed birds required louder auditory cues. While no studies have looked at the effect of mercury on the avian ABR, mercury has also induced elevated thresholds in mice (Chuu et al., 2001; Huang et al., 2008, 2011, 2007; Igarashi et al., 1992; Wassick & Yonovitz, 1985), humans (Counter et al., 2012; Tokuomi, 1968), and monkeys (Rice & Gilbert, 1992; Rice, 1998), with small to large effect sizes of 0.33-6.53, all larger than the effect size of 0.12 shown here. However, discrepancies between experimental design and animal systems may influence the degree to which mercury impacts the threshold.

Why were the absolute threshold effects in our study smaller than those in mammalian studies? Possibilities include differences in experimental

design and hearing physiology. First, mercury application between mammalian (oral gavage) and bird (dietary exposure) studies could result in mice absorbing larger doses of mercury in a relatively quick amount of time, potentially leading to more extreme effects on threshold. In addition, some discrepancies could be impacted by the length of maternal mercury deposition in mice and birds, as mouse embryos 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, bird hearing ability as measured by threshold may be more robust than that in mammals, where bird thresholds are more resilient to environmental perturbations like mercury exposure, potentially due to compensatory mechanisms available only in birds (Barbaric, Miller, & Dear, 2007). If hearing ability is robust in our finch colony, a 1.2 mg/kg dose of MeHg may be at a level where these compensatory mechanisms can still maintain mostly normal thresholds.

Mercury exposure lowered peak amplitudes by an average of 0.071 µV in exposed birds. Because ABR amplitude correlates with the number of neurons firing in response to an auditory signal (Glasscock, Jackson, & Josey, 1991), a mercury-induced decrease in ABR amplitude suggests that at the auditory nerve, fewer neurons are recruited in response to sound, though whether neuronal death or damage is occurring remains unknown. Few studies examining the effect of mercury exposure on hearing reported amplitude differences, thereby limiting our biological interpretation of a 0.071

µV change; however, significant threshold effects in previous studies imply an underlying amplitude effect as well. Despite large variation in amplitude associated with day, sex, age, and season, the present study found a significant decrease in amplitude that implies a neural mechanism.

Analysis of latency 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. 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 of ABR latency differences were 0.22 (Chuu et al., 2001) and 0.26 (Huang et al., 2008, 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 with cochlear frequency selectivity, suggesting that mercury can diminish the ability to distinguish between frequencies (Henry, Kale, Scheidt, & Heinz, 2011). This prolongation of latencies, along with increasing amplitudes, implies that mercury-induced neuronal dysfunction is resulting in both lower neuronal recruitment and slower propagation in response to an auditory signal.

Analysis of the auditory brainstem response at the auditory nerve revealed significant changes in amplitude and latency, implying that mercury

is disrupting the auditory signal at or upstream from the auditory nerve – therefore, mercury could either act on hair cells that initiate the electrical signal or neuron functionality. Mercury exposure has been implicated in the death and damage of outer hair cells of the inner ear in rats (Crofton, Ding, Padich, Taylor, & Henderson, 2000) and seals (Ramprashad & Ronald, 1977), perhaps specifically through oxidative damage and suppressed potassium currents (Clerichi, 1995). Damage or loss of hair cells would most likely require higher intensity sounds to elicit responses in the auditory brainstem, and might result in increases in absolute latency – though not interwave latency – as well as increases in threshold at the auditory nerve; however, because neuron dysfunction exhibits similar effects on ABR metrics, it is difficult to distinguish between the two alternatives. Imaging inner ear hair cells for signs of damage or loss could better determine whether hair cells are involved in mercury-induced hearing impairment in birds.

Because mercury possesses a high affinity for thiol and selenol groups, vital proteins involved in neuronal upkeep can exhibit a loss of function, resulting in the production of reactive oxygen species and excitotoxicity that lead to neuronal damage (reviewed in Farina, Rocha, & Aschner, 2011). While neurotoxicity studies in avian models reveal general mercury-induced neurological effects (reviewed in Whitney & Cristol, *in prep*), mammalian research demonstrates that mercury can disrupt mitochondrial permeability (Aschner, Syversen, Souza, Rocha, & Farina, 2007) and antioxidant enzyme function, which could exacerbate reactive oxygen species

production and make the auditory pathway inherently more susceptible to oxidative damage (Aschner et al., 2007; Farina et al., 2011; Huang et al., 2008). In addition, mercury-induced excess glutamate in the synaptic cleft and abnormal ATPase gating kinetics lead to overstimulation of neurons, after which excitotoxicity can occur (Chuu et al., 2001; Czaplinski, Abad, & Eblen-Zajjur, 2005; reviewed in Farina et al., 2011; Huang et al., 2008, 2011; Moosmayer & Anner, 1992; Nicolini et al., 2004). Similar to mercury effects on hair cells, neuronal damage would result in increases in absolute and interwave latencies, decrease in amplitude, and overall, an increase in threshold. Because our results indicate signs consistent with both hair cell and neuronal dysfunction, additional studies would be required to determine the specific mechanisms of mercury-induced hearing impairment, and whether these effects are permanent in birds.

Future studies might investigate whether these effects on threshold, amplitude, and latency are caused by birds being physiologically primed to adapt to mercury exposure. Because mercury causes a loss of function in specific proteins, mercury-exposed birds may be compensating for this loss through upregulation of mercury-targeted proteins, perhaps expressing them at excess levels that allow normal auditory functioning. Evaluating expression levels of proteins likely targeted by mercury could reveal if levels differ between exposed and non-exposed birds. In addition, studies have shown that auditory networks can be rewired to compensate for age-related hearing loss in mice (Willot, 1991; Willott, Parham, & P., 1988; Willott, 1986) and

damage to the cochlea in guinea pigs (Jenison, 1997). Evaluating peaks after peak I in the ABR could reveal whether other auditory network nuclei are compensating for, and likewise effected, by mercury exposure. Longitudinal studies observing changes in ABR for each auditory region could further elucidate this idea. Clearly, compensatory mechanisms may play a role in the hearing impairment shown in this study, underscoring that wild populations of birds, especially those newly entering a mercury-contaminated site, may react more strongly to mercury exposure than was found here.

In this study, both amplitude and latency showed age effects in which young birds exhibited higher amplitudes and shorter latencies than older birds across treatments, most likely due to natural age-related hearing loss (ARHL). ARHL affects hearing ability through the degradation of spiral ganglion neurons at the auditory nerve as well as loss of outer hair cells in the inner ear (Fujimoto & Yamasoba, 2014). Spiral ganglion degradation results from the production of reactive oxygen species, which can damage mitochondrial DNA and decrease antioxidant function in neurons (Fujimoto & Yamasoba, 2014), among other causes (Frisina, 2001). Because both ARHL and mercury-induced hearing loss seem to involve neuronal dysfunction, it would be interesting to determine if mercury exposure expedites the process of ARHL through longitudinal studies performing ABR on exposed and non-exposed birds repeatedly throughout the aging process, as was done in mercury-exposed monkeys (Rice, 1998).

Contrary to our results showing that mercury affects threshold, amplitude, and latency equally at all frequencies, mercury-exposed children (Counter et al., 2012), monkeys (Rice & Gilbert, 1992), and mice (Wassick & Yonovitz, 1985) exhibited specifically high frequency hearing impairment; however, a later study on the same monkeys revealed that the high frequency hearing impairment became more generalized across the hearing range as age increased (Rice, 1998). This disparity in frequency-specific effects could be explained by fundamental differences in hearing physiology and frequency discrimination between birds and mammals, especially due to differences in basilar papillae morphology (Dooling et al., 2000). For example, avian hair cells form a complex matrix on the basilar papilla while in mammals, hair cells are arranged in neat rows (Dooling et al., 2000). Interestingly, basal hair cells in mammalian systems, which correlate to high frequency detection, exhibit lower antioxidants, making them more susceptible to oxidative stress (Sha, Taylor, Forge, & Schacht, 2001). This frequency specific property in mammals may help explain why we observed no frequency effect in birds exposed to mercury.

Birds exposed to biologically relevant doses of mercury may suffer fitness costs if the resulting magnitude of hearing impairment disrupts the reception of important auditory signals. Our results indicated small statistically significant changes in threshold, latency, and amplitude as well as no frequency-specific effect of mercury across the hearing range of zebra finches; this small, general effect on hearing ability suggests that mercury

may impact the reception of distant vocalization signals more those nearby. Birds use perceived signal degradation to extract information about a signaler's distance, a process called ranging (Naguib & 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, Klump, Hillmann, Grießmann, & Teige, 2000; Nelson, 2000). Prolonged latencies and weaker amplitude responses suggest that weaker signals that require a quick response time, like that of a predator, would be more impacted by mercury exposure than responses to conspecific calls and interactions. Compiled with the decreased takeoff flight performance found in mercury-exposed starlings (Carlson, Cristol, & Swaddle, 2014), mercury could severely affect avian survival if it compromises the ability to respond to and flee from weak predator signals in the field.

  We cannot determine potential fitness effects of mercury exposure on avian threshold, amplitude, and latency until additional behavioral assays are performed. The current study looks at one specific physiological response in the peripheral auditory pathway of the brain; however, we cannot be sure whether these responses are strong enough to initiate further physiological responses in the body and ultimately, behavioral responses. Hearing is a multimodal process integrating auditory, visual, and other sensory cues; therefore, performing a behavioral audiogram on mercury-exposed zebra finches would better test the ultimate outcome of sound reception. Behavioral

audiograms generally show thresholds lower than those measured from an ABR, where behavioral thresholds could be 20-30 dB lower than ABR thresholds (Brittan-Powell et al., 2002; Szymanski et al., 1999; Yuen, Nachtigall, Breese, & Supin, 2005). Therefore, it is important to test behavioral responses to sound in order to investigate the ultimate fitness effects of mercury exposure on hearing.

In addition to disrupting hearing ability, mercury has also been implicated in altering song properties – vital auditory cues – in birds. Nelson's sparrows exposed to more mercury in the field exhibited songs with higher maximum frequencies and sang faster songs with shorter gap durations (McKay & Maher, 2012). In mercury-contaminated sites in Virginia, three songbird species displayed decreased tonal frequency and song complexity (Hallinger et al., 2010); however, these changes seem limited to species required to learn their songs, potentially linking mercury-induced song abnormalities with hearing impairment due to hearing's vital role in the song developmental process (Ackermann & Ziegler, 2013; Brainard & Doupe, 2002; Ota & Soma, 2014; Spencer, Buchanan, Goldsmith, & Catchpole, 2003). Mercury-induced neuronal damage may occur in both the auditory and song-learning pathways of the brain, suggesting that mercury impacts song directly through neuronal damage of song nuclei and indirectly through hearing impairment. As mercury is now tied to changes in both reception and production of auditory cues in birds, investigation of the consequences to avian communication networks is vital.

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Mercury-induced hearing impairment, when coupled with other

ecological and anthropogenic stressors, could be exacerbated to biologically

significant levels in wild bird populations. Unlike laboratory animals, wild birds

experience food limitation, adverse weather conditions, parasites and

disease, all of which could force energy allocation to survival-oriented

processes at the expense of neural upkeep associated with functional hearing

ability. Anthropogenic stressors like habitat fragmentation, diseases, and

chemical or noise pollution might worsen this effect (Laiolo, 2010). In

particular, noise pollution leads to behavioral changes (Barber, Crooks, &

Fristrup, 2009; reviewed in Ortega, 2012; Swaddle & Page, 2007),

adjustments to song in the presence of masking (Patricelli & Blickley, 2006;

Slabbekoorn & Boer-visser, 2006; Slabbekoorn & Peet, 2003; Warren,

Madhusudan, Ermann, & Brazel, 2006), and damage to the ear (reviewed in

Ortega, 2012) in birds, similar to how mercury affects both reception and

production of auditory cues. Future studies might perform ABR in mercury-

exposed birds masked with specific bandwidth noise to determine if masking

exacerbates the mercury-induced hearing impairment. Overall, the costs of

mercury-induced hearing loss must be understood in relation to other

ecological and anthropogenic forces, which in concert, may become

disastrous for avian auditory communication.

APPENDICES

Figure S1. ABR amplitude responses as a function of blood mercury level per frequency in accidentally exposed control birds with above-background blood mercury levels of 0.1-2 ppm. Lines represent linear regressions. Amplitude responses seem not to be affected in a consistent dose-dependent manner in across frequencies.
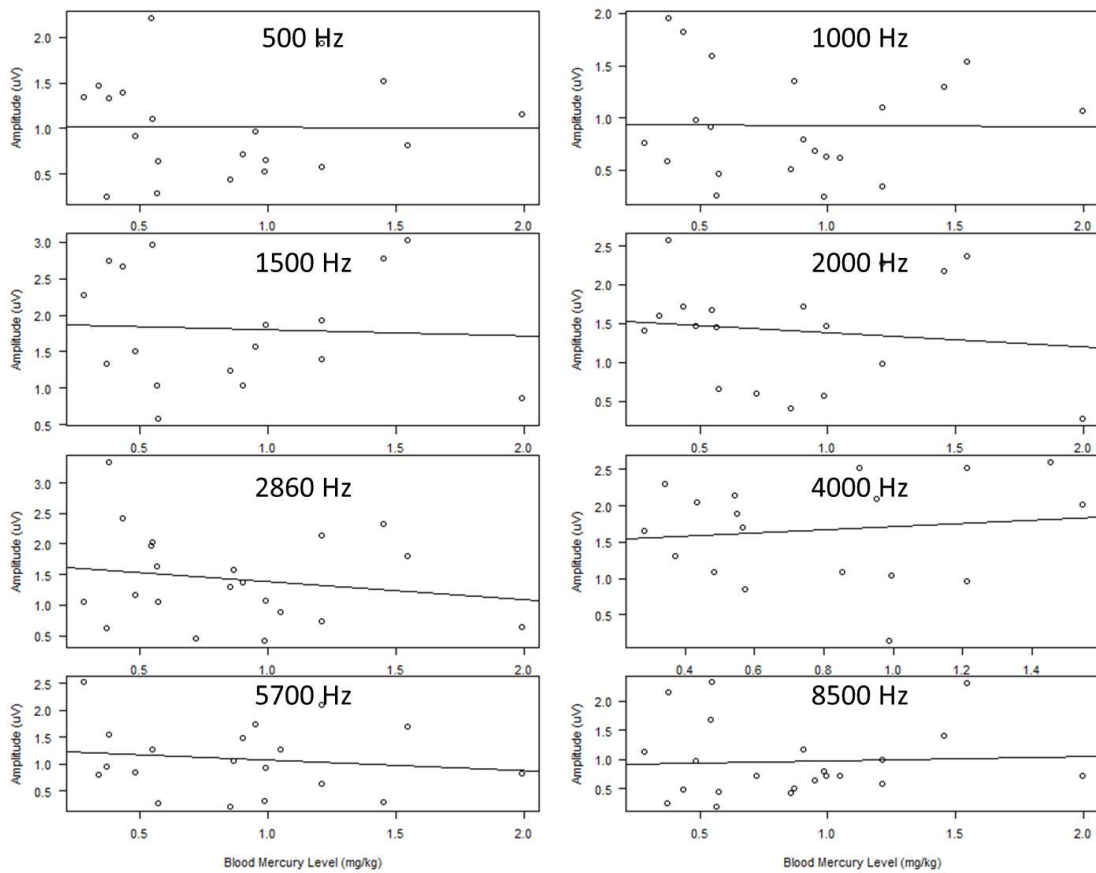
Table S1. Intraclass correlation coefficients for the repeatability of threshold values for three different auditory brainstem response peaks. The peak corresponding to that of this study's threshold values is peak –I, denoting the threshold based on amplitudes measured from the peak of peak I to the subsequent trough. The degrees of freedom for the F-tests are given as subscripts before the F statistic. Significant P-values are bolded, where α=0.05. This study was based on 12 birds (varied in sex and treatment) measured at four frequencies repeatedly, every-other-day for 10 days. We stimulated the birds using the previously described parameters and intensities.

Peak	Frequency	N	Intraclass Correlation Coefficient (95% CI)	F-statistic	P-value
I	5700	10	-0.479 (-0.839, 0.173)	$F_{9,9} = 0.352$	0.932
I	8500	9	0.312 (-0.399, 0.778)	$F_{8,8} = 1.91$	0.19
-I	1000	10	0.207 (-0.452, 0.719)	$F_{9,9} = 1.52$	0.271
-I	2860	10	0.836 (0.470, 0.956)	$F_{9,9} = 11.17$	0.001
-I	5700	11	0.621 (0.069, 0.881)	$F_{10,10} = 4.27$	0.016
-I	8500	12	0.714 (0.226, 0.908)	$F_{11,11} = 6.00$	0.003
II	2860	9	0.848 (0.466, 0.964)	$F_{8,8} = 12.17$	0.001
II	5700	9	-0.051 (-0.662, 0.600)	$F_{8,8} = 0.903$	0.556
II	8500	10	0.225 (-0.436,0.728)	$F_{9,9} = 1.58$	0.253

Figure S2. Validation of the sound stimulus used during ABR trials. We recorded the stimulus inside the sound-attenuation chamber using a Zoom H4N digital audio recorder and analyzed the recording using Audacity. (A) Validation of frequencies presented during tone train stimuli. There was no significant difference between expected and actual frequencies. (B) A spectrogram of the stimulus showed that each peak frequency was at least 10dB more powerful than the 2nd most powerful frequency in the tone train stimulus, except at 4000Hz, which was 9dB higher. (C) Power Spectrum of each tonal stimuli used in all trials

A Expected Frequency (Hz)	Actual Frequency (Hz)	Difference (Hz)	B Frequency (Hz)	2nd Highest Peak dBV (SPL)
500	464	36	500	-24.3
1000	1058	58	1000	-13.8
1500	1554	54	1500	-18
2000	2054	54	2000	-16
2860	2847	13	2860	-13
4000	4003	3	4000	-9
5700	5701	1	5700	-12
8500	8472	38	8500	-15
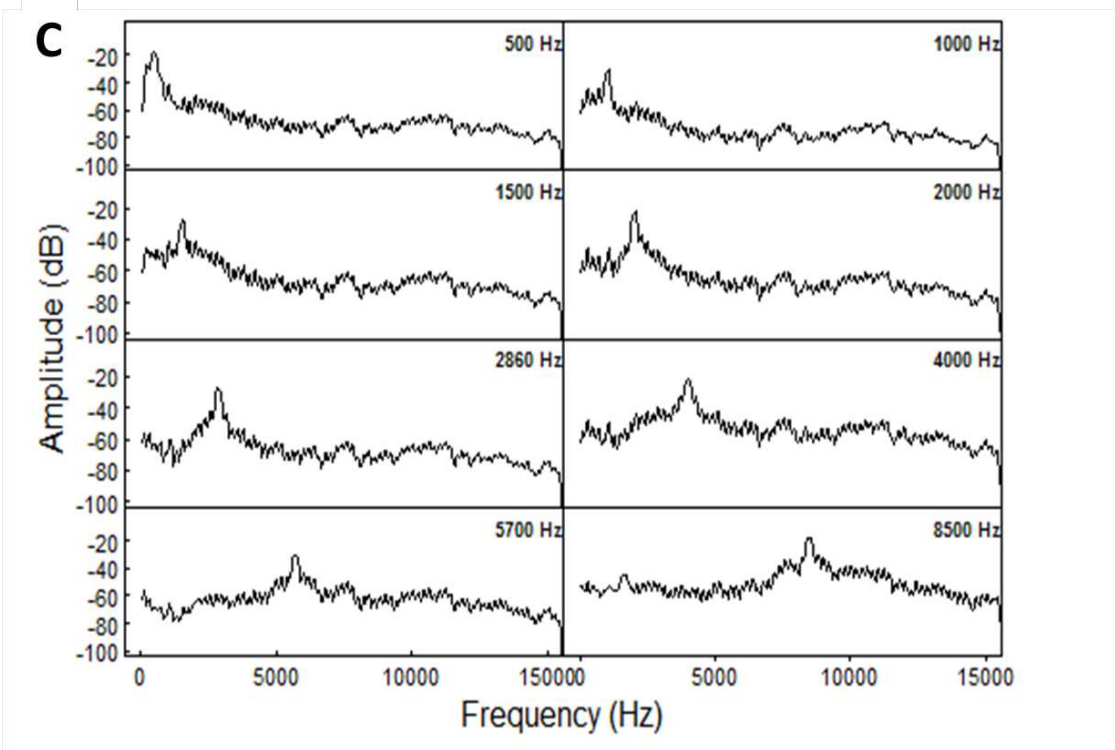
Figure S3. Average latency as a function of frequency for seven constant intensities shown for peak I responses. As expected, an increase in intensity leads to a decrease in latency. However, latency does not consistently decrease with increasing intensity. Lowest latencies occur between 1500 and 4000 Hz, corresponding to the highest sensitivity range in the zebra finch.
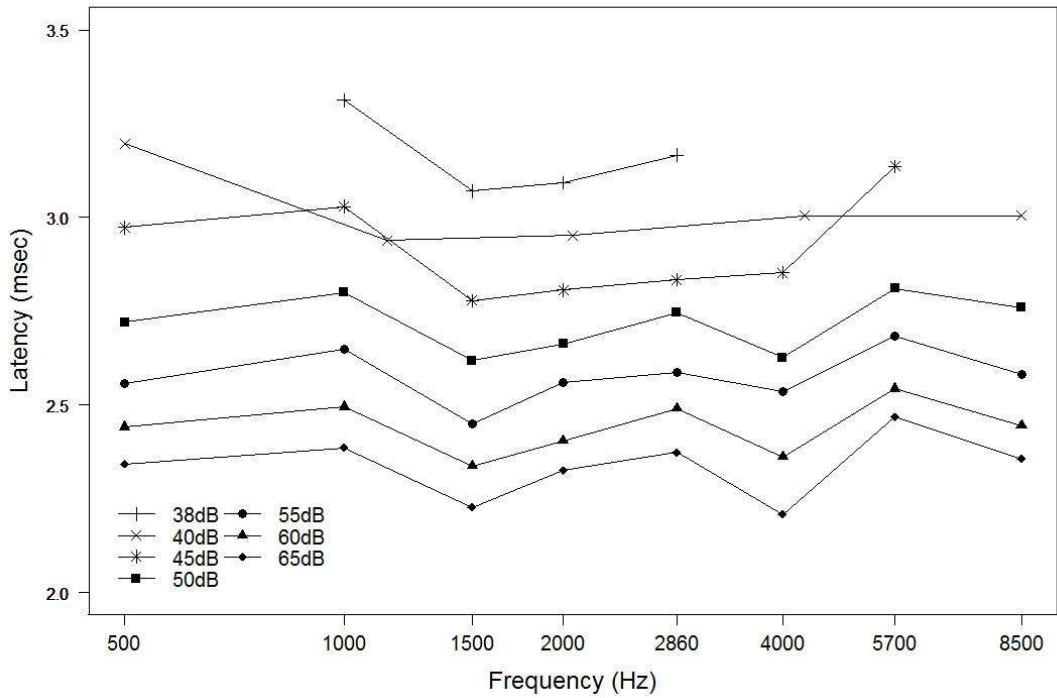
Figure S4. Average amplitude of peak –I responses as a function of intensity for tone train stimuli. As expected, when intensity increases, amplitude increases across all frequencies. Frequencies with the highest amplitudes are those corresponding to the zebra finch's highest hearing sensitivity (1500 to 4000 Hz).
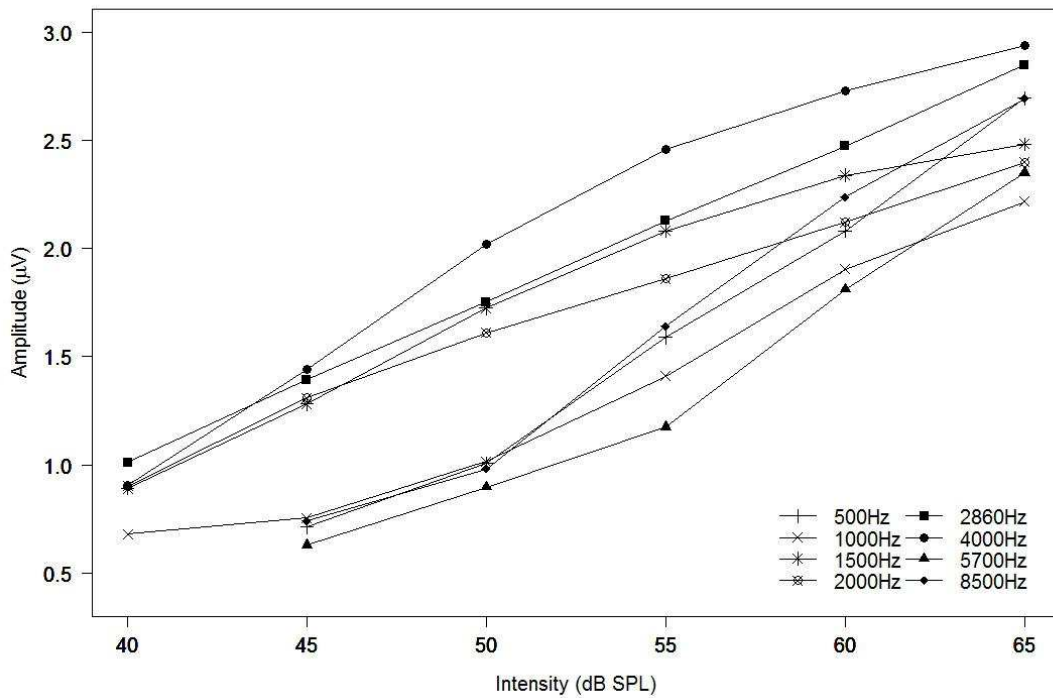
Table S2. Linear mixed model analysis for the main effects on threshold, amplitude, and latency of three ABR peaks. The significance of the fixed effects were assessed with Type III SS F-tests. All statistically significant P-values are bolded, using α=0.05. Note that inflated sample sizes may artificially lower P-values. The degrees of freedom for the F-tests are written as subscripts after the F statistic.

| Dependent Variables	Independent Variables	Peak I		Peak -I		Peak II	
		Fdf	*P*	*Fdf*	*P*	*Fdf*	*P*
Threshold	Treatment	$0.730_{1,551}$	0.393	$6.080_{1,789}$	**0.014**	$0.464_{1,695}$	0.496
	Frequency	$31.523_{7,127}$	**<0.0001**	$50.697_{1,207}$	**<0.0001**	$64.644_{7,167}$	**<0.0001**
	Age	$0.137_{1,556}$	0.712	$0.001_{1,786}$	0.973	$4.063_{1,699}$	**0.044**
	Sex	$0.078_{1,550}$	0.78	$0.199_{1,786}$	0.656	$2.643_{1,696}$	0.104
	Frequency x Treatment	$0.895_{7,127}$	0.513	$1.169_{7,207}$	0.322	$0.750_{7,167}$	0.63
	Frequency x Treatment x Age	$0.792_{16,124}$	0.685	$0.666_{15,177}$	0.815	$1.166_{15,158}$	0.304
	Frequency x Treatment x Sex	$0.646_{15,120}$	0.832	$0.537_{15,195}$	0.918	$0.803_{15,172}$	0.674
Amplitude	Treatment	$46.802_{1,8056}$	**<0.0001**	$41.881_{1,8055}$	**<0.0001**	$6.141_{1,8056}$	**0.013**
	Frequency	$37.917_{7,8056}$	**<0.0001**	$35.691_{7,8055}$	**<0.0001**	$55.600_{7,8056}$	**<0.0001**
	Age	$33.918_{1,8056}$	**<0.0001**	$29.698_{1,8055}$	**<0.0001**	$18.842_{1,8056}$	**<0.0001**
	Sex	$0.545_{1,8056}$	0.461	$0.092_{1,8055}$	0.761	$12.801_{1,8056}$	**<0.0001**
	dB SPL(Frequency)	$33.938_{54,8056}$	**<0.0001**	$60.703_{54,8055}$	**<0.0001**	$51.686_{54,8056}$	**<0.0001**
	Frequency x Treatment	$0.098_{7,8056}$	0.998	$0.358_{7,8055}$	0.927	$0.331_{7,8056}$	0.94
	Frequency x Treatment x Age	$8.287_{15,8056}$	**<0.0001**	$9.927_{15,8055}$	**<0.0001**	$8.289_{15,8056}$	**<0.0001**
	Frequency x Treatment x Sex	$15.921_{15,8056}$	**<0.0001**	$13.115_{15,8055}$	**<0.0001**	$7.821_{15,8056}$	**<0.0001**
Latency	Treatment	$21.970_{1,8056}$	**<0.0001**	$0.876_{1,8055}$	0.349	$18.408_{1,8050}$	**<0.0001**
	Frequency	$77.787_{7,8056}$	**<0.0001**	$129.877_{7,8055}$	**<0.0001**	$109.633_{7,8056}$	**<0.0001**
	Age	$10.883_{1,8056}$	**0.001**	$8.839_{1,8055}$	**0.003**	$1.041_{1,8056}$	0.308
	Sex	$1.115_{1,8056}$	0.291	$6.668_{1,8055}$	**0.01**	$14.933_{1,8056}$	**<0.0001**
	dB SPL(Frequency)	$97.157_{54,8056}$	**<0.0001**	$122.495_{54,8055}$	**<0.0001**	$222.053_{54,8056}$	**<0.0001**
	Frequency x Treatment	$1.862_{7,8056}$	0.071	$1.531_{7,8055}$	0.152	$0.386_{7,8056}$	0.911
	Frequency x Treatment x Age	$0.686_{15,8056}$	0.801	$1.554_{15,8055}$	0.078	$3.082_{15,8056}$	**<0.0001**
	Frequency x Treatment x Sex	$2.312_{15,8056}$	**0.003**	$2.648_{15,8055}$	**0.001**	$4.496_{15,8056}$	**<0.0001**

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