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Research Paper

Age-related loss of auditory sensitivity in the zebrafish (*Danio rerio*)

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ABSTRACT

Age-related hearing loss (ARHL), also known as presbycusis, is a widespread and debilitating condition impacting many older adults. Conventionally, researchers utilize mammalian model systems or human cadaveric tissue to study ARHL pathology. Recently, the zebrafish has become an effective and tractable model system for a wide variety of genetic and environmental auditory insults, but little is known about the incidence or extent of ARHL in zebrafish and other non-mammalian models. Here, we evaluated whether zebrafish exhibit age-related loss in auditory sensitivity. The auditory sensitivity of adult wild-type zebrafish (AB/WIK strain) from three adult age subgroups (13-month, 20-month, and 37-month) was characterized using the auditory evoked potential (AEP) recording technique. AEPs were elicited using pure tone stimuli (115–4500 Hz) presented via an underwater loudspeaker and recorded using shielded subdermal metal electrodes. Based on measures of sound pressure and particle acceleration, the mean AEP thresholds of 37-month-old fish [mean sound pressure level (SPL) = 122.2 dB ± 2.2 dB SE re: 1 μPa; mean particle acceleration level (PAL) = -27.5 ± 2.3 dB SE re: 1 ms^{-2}] were approximately 9 dB higher than that of 20-month-old fish [(mean SPL = 113.1 ± 2.7 dB SE re: 1 μPa; mean PAL = -37.2 ± 2.8 dB re: 1 ms^{-2}; $p = 0.007$)] and 6 dB higher than that of 13-month-old fish [(mean SPL = 116.3 ± 2.5 dB SE re: 1 μPa; mean PAL = -34.1 ± 2.6 dB SE re: 1 ms^{-2}; $p = 0.052$)]. Lowest AEP thresholds for all three age groups were generally between 800 Hz and 1850 Hz, with no evidence for frequency-specific age-related loss. Our results suggest that zebrafish undergo age-related loss in auditory sensitivity, but the form and magnitude of loss is markedly different than in mammals, including humans. Future work is needed to further describe the incidence and extent of ARHL across vertebrate groups and to determine which, if any, ARHL mechanisms may be conserved across vertebrates to support meaningful comparative/translational studies.

1. Introduction

Age-related hearing loss (ARHL), known as presbycusis, is a widespread and debilitating sensory condition that affects approximately one third of the US population between the ages of 65 and 74, and about half of the US population over the age of 75 (NIDCD, 2016). ARHL is caused by aging processes within the auditory system that can lead to (alone or in combination) loss or damage of auditory hair cells, degradation of the stria vascularis and endocochlear potential, and loss or damage of auditory afferents, among other insults (reviewed in Gates and Mills, 2005; Parham et al., 2015). Postmortem examination of human tissue has provided important insight on morphological characteristics of ARHL (e.g. Schukneckt, 1964), but fundamental constraints on mechanistic research with human subjects – paired with the complex etiology of ARHL and confounding population variables such as noise exposure (e.g., Liberman, 2017) – have necessitated the use of animal model systems for experimental studies of ARHL. To date, numerous studies have leveraged non-human mammalian models to investigate ARHL, and the use of such model systems has greatly contributed to our understanding of its causes and mechanisms (Schuknecht, 1955; Bhattacharyya and Dayal, 1989; Mills et al., 1990; Tarnowski et al., 1991; Ohlemiller, 2004; Dubno et al., 2013; Jiang et al., 2015;

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Bowl and Dawson, 2019). While non-mammalian vertebrate model systems have proven complementary to mammalian models in other domains of translational auditory research, relatively few studies have examined effects of advanced age on auditory function in non-mammalian vertebrates, and little is known about the incidence or extent of such.

One non-mammalian model system that has become increasingly popular for investigating the genetic and developmental bases of hearing is the zebrafish (*Danio rerio*) (Whitfield, 2002). Zebrafish are a genetically tractable model system with an auditory pathway that has homologies to the mammalian auditory system (Nicolson, 2005; Vanwalleghem et al., 2017; Whitfield, 2002). In addition, zebrafish are cost-effective, easy to maintain, and have a functional auditory system by 5 days post-fertilization (Whitfield et al., 2002), improving the feasibility of high-throughput studies, e.g., in the context of ototoxicity and otoprotection (Ton and Parng, 2005; Chiu et al., 2008; Coffin et al., 2010). While zebrafish exhibit key morphological and functional similarities to mammalian models in terms of auditory hair cell ontogeny and physiology, they – along with avians, anurans, and other non-mammalian auditory models – also possess the ability to *regenerate* lost auditory hair cells (reviewed in Atkinson et al., 2015). This decidedly non-mammalian trait, along with other basic mechanistic differences including relatively weak endolymphatic potentials (versus the large endocochlear potential of mammals; Koppl et al., 2018), would seem to contraindicate the use of zebrafish, and other non-mammals, for the study of ARHL. Indeed, work in avians has suggested slight (Langemann et al., 1999) or no ARHL (Krumm et al., 2017), raising the interesting possibility that auditory senescence is a uniquely mammalian condition.

However, in a zebrafish study, Wang et al. (2015) concluded that wild-type AB zebrafish exhibited age-related loss in auditory sensitivity. Specifically, Wang et al. (2015) showed using the auditory evoked potential (AEP) recording technique that, following improvement of auditory thresholds during development, adult zebrafish exhibited decreased sensitivity with increased body size. Changes in hair cell density and in *ribeye* protein expression at the hair cell synapse were also noted in larger fish (Wang et al., 2015). Using body size as a proxy for age, Wang et al. (2015) concluded that age-related loss in auditory sensitivity does occur in zebrafish, with associated inner ear morphological changes resembling some aspects of mammalian ARHL. However, many factors can affect body size during ontogeny, and the growth of zebrafish is known to be influenced by many environmental factors such as temperature, diet, animal rearing density, and other husbandry factors (Higgs et al., 2002; Vijayan et al., 1988; Fagerlund et al., 1981). Because these environmental factors can affect growth and ultimately the size of zebrafish with age, the extent and onset of the posited age-related loss of auditory sensitivity in zebrafish as reported by Wang et al. (2015) remains unclear.

The objective of this study was to characterize the auditory sensitivity of the zebrafish (*Danio rerio*) in known age groups using the AEP recording technique. We use the AB/WIK zebrafish strain, a common wild-type strain, to evaluate whether adult wild-type zebrafish exhibit age-related loss in auditory sensitivity as suggested by Wang et al. (2015), or instead exhibit resistance to ARHL as suggested in other hair cell-regenerating non-mammals (cf. Krumm et al., 2017, Langemann et al., 1999). Outbred wildtype zebrafish have an average lifespan of approximately 42 months in captivity (Gerhard et al., 2002). In the current study, we test the auditory sensitivity of adult wild-type AB/WIK zebrafish from three adult age subgroups with mean ages of 13-months, 20-months, and 37 months using the auditory evoked potential (AEP) recording technique. We interpret our findings as they relate to the potential translational application of zebrafish for the study of ARHL.

2. Materials and methods

2.1. Animals

Wild-type (AB/WIK strain) adult zebrafish ($n = 39$) were obtained from an adult zebrafish colony housed at the University of Washington. The AB/WIK strain has commonly been used in developmental and physiological zebrafish studies (e.g., Fisher et al., 2003, Bahary et al., 2004, Shima et al., 2009, Peravali et al., 2011, Kimelman, 2016, Staudacher et al., 2019). Animals used in experiments were from three different mean age groups based on the time difference between date of hatching and date of testing that included 13-month, 20-month, and 37-month-old mean-age groups. All animals were housed in 5-gallon glass aquaria exposed to 12:12 h light:dark cycle. Animals were fed daily and tank temperature was maintained at 28 ± 2° C. Animal housing and experimental procedures were approved by the University of Washington Institutional Animal Care and Use Committee.

2.2. Acoustic stimuli and calibration

Acoustic stimuli were generated by a laptop computer running a custom MATLAB (MathWorks Inc., Natick, MA, USA) script connected via USB to a digital-to-analog converter (Fireface UCX, RME, Frankfurt, Germany). All signals were synthesized at a sampling frequency of 44,100 Hz and delivered via an audio amplifier (D-75Al, Crown International Inc., Elkhart, IN, USA) connected to an underwater loudspeaker (UW-30, Telex Communications, Burnsville, MN, USA). Test stimuli were 200-ms pure tones (20-ms cosine rise/fall). For each animal, five tone frequencies were tested in random order: 115, 800, 1850, 3700, and 4500 Hz. Those frequencies were chosen to efficiently sample representative regions of the zebrafish auditory spectrum while avoiding major resonances in the test tank. At each frequency, tone presentation level was varied systematically (minimum 97 dB SPL, maximum 154 dB SPL re: 1 μPa) in 3-dB steps using the staircase method for threshold determination (see below – *Auditory evoked potential measurements*). Within each amplitude-by-frequency measurement block, the 200-ms stimulus was repeated 500 times (400 ms silence between stimuli). To reduce the effects of stimulus artifacts and limit the contribution of non-neuronal receptor potentials to AEP threshold determination, acoustic stimuli were presented from the underwater speaker with alternating polarities (0° and 180° starting-phase sines; 250 repetitions each; e.g., Kenyon et al., 1998).

Sound is a mechanical disturbance that propagates as a longitudinal wave; sound energy radiates away from the source in the form of sound pressure and particle motion (recently reviewed in Popper and Hawkins, 2019). Different from terrestrial vertebrates, which detect sound pressure, all fishes are thought to detect acoustic particle motion via the inner ear otolithic end organs (saccule, lagena, and utricle), which function as inertial accelerometers (De Vries, 1950; Popper and Fay, 2011; Sisneros and Rogers, 2016). Some fishes, including zebrafish, have also evolved a pressure mode of hearing that enables them to detect pressure-induced vibrations of the swim bladder (or other gas-filled vesicle) via a mechanical linkage to the inner ear known as the Weberian apparatus (Weber, 1819, 1820). As zebrafish are sensitive to both particle motion and pressure, stimuli and elicited responses of the present investigation were quantified in both domains.

Stimulus sound pressure level (in units of dB re: 1 μPa) at each frequency was calibrated within the test tank before each experiment using a mini-hydrophone (model 8103, Bruel and Kjaer, Naerum, Denmark) connected to a conditioning amplifier (Nexus 2692–0S1, Bruel and Kjaer, Naerum, Denmark). The mini-hydrophone was placed 8 cm above the underwater speaker (and

8.5 cm below the water surface), corresponding with the position of the fish inner ear during testing. Tones at the AEP target frequencies were presented from the underwater speaker at a nominal level of 130 dB SPL and recorded with the hydrophone. Deviations from the nominal level (i.e., differences between the desired and observed level) were computed, and necessary level correction factors applied and verified, using an automated MATLAB script.

Particle acceleration measurements (in units of dB re: 1 ms^{-2}) were conducted at the conclusion of AEP testing using a calibrated neutrally buoyant waterproofed triaxial accelerometer [PCB model VW356A, PCB Piezotronics, Depew, NY, USA; sensitivity at 100Hz: 10.42 mV/ms^{-2} (x-axis), 10.03 mV/ms^{-2} (y-axis), 10.37 mV/ms^{-2} (z-axis)], which sent signals from the accelerometer to a signal conditioner (model 482A16, PCB Piezotronics, Depew, NY, USA) that amplified (gain = x100/axis) all measurements. Amplified signals were sent to a data acquisition system (Model: NI USB-6009, National Instruments, Austin, TX, USA) and visualized using LabVIEW software (National Instruments, Austin, TX, USA). At the time of particle acceleration measurements, the accelerometer and the hydrophone were placed adjacently in the same horizontal plane of the water column (8 cm above the underwater speaker, and 8.5 cm below the water surface; accelerometer and hydrophone were 7.5 cm apart horizontally). Placement of the sensors is corresponding to the position of the fish inner ear during testing. Using a custom LabVIEW script, particle motion amplitude measurements ($V_{pk\text{-}pk}$) for each axis (*x*-, *y*- and *z*-axis) were corrected for the gain (sensitivity) of the accelerometer. The $V_{pk\text{-}pk}$ measurements for each axis (*x*-, *y*- and *z*-axis) were then used to calculate the combined magnitude vector of particle acceleration in dB scale (Eq. (1)) (Bhandiwad et al., 2017; Vasconcelos et al., 2010; Wysocki et al., 2009) as follows:

$$dB\ re:\ 1\ ms^{-2} = 20Log_{10}\left(\sqrt{x^2 + y^2 + z^2}\right) \quad (1)$$

Because we observed a linear relationship between sound pressure and particle motion at the frequencies used in threshold testing, particle acceleration level (dB re: 1 ms^{-2}) based thresholds were readily derived from the measured sound pressure level (dB re: 1 μPa) thresholds at each test frequency using best-fit linear equations. By this method, it was possible to express zebrafish AEP thresholds in terms of both sound pressure and particle acceleration.

2.3. Auditory evoked potential measurements

We used the established AEP method (e.g., Kenyon et al., 1998; reviewed in Ladich and Fay, 2013) to measure zebrafish auditory sensitivity. We refer to the AEP measurements as assessments of "auditory sensitivity" rather than "hearing" because the AEP recording technique is an electrophysiological measure of sensitivity at the level of the peripheral auditory system and not a measure of perception (cf. Ladich and Fay 2013).

The method for recording auditory evoked potentials (AEPs) in zebrafish was similar to that used recently to characterize AEPs in the plainfin midshipman fish (*Porichthys notatus*) (Brown et al., 2019). Animals were first anesthetized by immersion in a 100 ppm ethyl p-aminobenzoate (benzocaine) bath solution followed by intramuscular injections of the paralytic cistracurium besylate (2.5 mg/ml concentration) and the analgesic bupivacaine (5 mg/ml concentration). All recordings were performed in a 30 cm diameter, 26.5 cm high cylindrical Nalgene tank (similar in design to that used by Sisneros (2007) that was placed on a vibration-isolation air table and housed inside a sound attenuation chamber (Industrial Acoustics, New York, NY USA). Animals were secured in a custom-made nylon mesh sling positioned at the center of the testing tank (8.5 cm below the water surface, and 8 cm above speaker). Once in position, fish were perfused with a continuous flow of oxygenated zebrafish water (Westerfield, 1995) across the gills by a small tube inserted into the mouth from a nearby suspended reservoir (Fig. 1a). A circular opening at the level of the water surface was connected to a plastic tubing that drained excess water from the external water reservoir; this design ensured water level was consistent throughout the recording session. An underwater speaker (UW-30) was positioned in the bottom of the experimental tank and embedded in a 4 cm layer substrate of small rock. All recording and stimulus generation equipment was located outside the sound attenuation chamber. In order to determine the acoustic noise floor in the sound attenuation chamber, we took simultaneous measurements of particle acceleration and sound pressure levels. The acoustic noise floor for sound pressure level was 78.1 ± 0.3 dB re: 1 μPa and for particle acceleration level it was −67.1 ± 0.3 dB re: 1 ms^{-2}.

Acoustic noise levels in the housing aquaria at the two zebrafish holding facilities (a central holding facility at University of Washington and a temporary holding facility close to the testing site) were also measured using a mini-hydrophone (8103, Bruel and Kjaer, Naerum, Denmark), which was connected to a conditioning amplifier (gain = 100 mV/Pa, Nexus 2692–0S1, Bruel and Kjaer, Naerum, Denmark). To complete the acquisition of the background noise measurement, a TDT system III (Tucker Davis Technologies, Alachua, FL, USA) and a PC laptop (Dell precision M6800) running a custom-written MATLAB stimulus generation program (The MathWorks, Natick, MA, USA) were used. The background noise levels in the housing aquaria at the temporary housing facility and the central zebrafish holding facility at the University of Washington were 128 dB and 120 dB re: 1 μPa RMS, respectively.

AEPs were recorded using three subdermal metal electrodes (27-gage, SurePoint, Rochester Electro-Medical, Lutz, FL, USA). Each electrode lead was shielded along its full length, with only a ~1 mm exposed needle tip, inserted subcutaneously. One electrode was placed rostrally, between the nares, while a recording electrode was inserted in the midline of tested fish at the opercular opening above the otic capsule (where the inner ear is located). A third (ground) electrode was placed in water near the fish. All electrodes were connected to a head stage and differential amplifier (BMA-200, CWE Incorporated, Armore, PA, USA). All AEP signals were amplified (20,000X) and routed to a USB-connected analog-to-digital converter (Fireface UCX) that sampled the signals at 44,100 Hz.

Auditory thresholds based on sound pressure were determined by comparing the fast Fourier transform (FFT) amplitude (measured in μV) of the mean AEP response relative to a threshold derived from background noise measurements obtained from recordings in 10 dead zebrafish. These recordings, which contained only noise, were intended to capture noise variability across 10 measurement sessions and enabled us to define the system noise floor: Toward more conservative threshold judgments, we defined the system noise floor (here forward simply "the noise floor") as the mean noise FFT plus one standard deviation. For each stimulus frequency, threshold was determined as the lowest sound pressure level eliciting a "double-frequency" response at least 3 dB (i.e. twice the magnitude) above the noise floor at that frequency. The "double frequency response" is a canonical feature of fish AEPs (Ladich and Fay, 2013) thought to arise from the hair cell receptor potentials and the non-linear addition of oppositely oriented hair cell activation and subsequent firing of associated auditory afferent neurons (first described in the goldfish by Furukawa and Ishii, 1967, but also see Kenyon et al., 1998). Thus, thresholds were established according to the stimulus-evoked response versus the noise floor at 230 Hz for the 115 Hz stimulus, at 1600 Hz for the 800 Hz stimulus, etc.

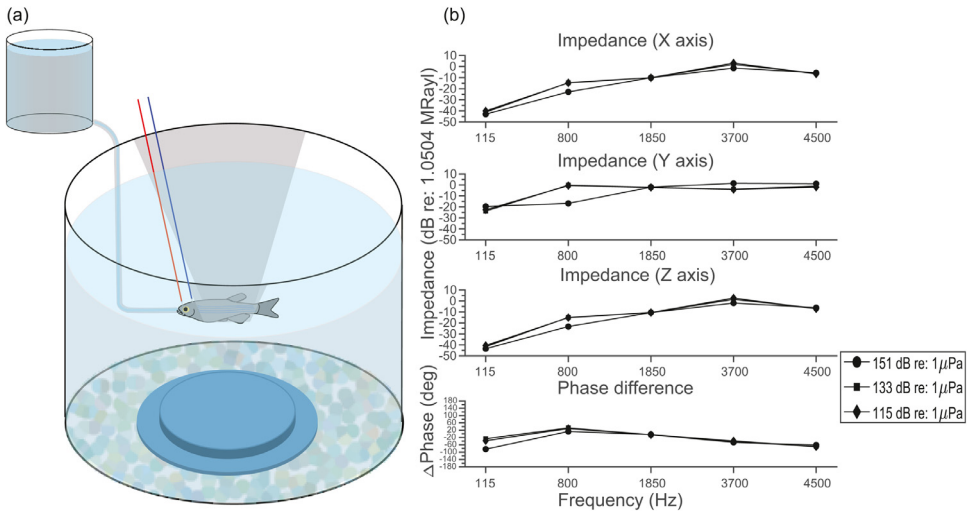

Fig. 1. (a) Schematic of the experimental setup. The animal was suspended in a mesh sling in the middle of the experimental tank (8.5 cm below water surface, and 8 cm above underwater speaker). Fresh aerated water was delivered by an elevated water reservoir with a water line that continuously provided fresh water into the fish's buccal cavity. Two electrodes were directly inserted subcutaneously into the skin on the fish's head. The positive electrode (red) was inserted between the nares on the rostral part of the head, and the negative electrode was inserted in the midline of tested fish at the opercular opening above the otic capsule (where the inner ear is located, colored in blue). The ground electrode (not shown) was placed in the tank water.
(b) Acoustic characteristics (impedance and phase difference between sound pressure and particle velocity) of the experimental tank and the speaker. *Top 3 panels*: Acoustic impedance [ratio of sound pressure (dB re: 1 μPa) to particle velocity (dB re: 1 ms^{-1})] in three axes (X, Y, Z) relative to 1.504 MRayls (the reference impedance for a free-field in fresh water at 28 °C) is plotted for all tested frequencies at three representative sound pressure levels: 115, 133, 151 dB re: 1μPa. Measurements were made using a triaxial accelerometer placed in the center of the tank and water column (8 cm above the speaker and 8.5 cm below the water surface). Multiple measurements (n = 8) for both sound pressure and particle velocity were made at each frequency and sound pressure level. Data plotted are mean ± 1 standard deviation. *Bottom panel*: Plotted are the phase differences ($\Delta\Phi_{p,v}$) between the pressure and particle velocity wave. Multiple phase difference measurements (n = 8) were made at each frequency and sound pressure level. Data plotted are mean ± 1 standard deviation.

In order to reduce the testing time for each fish, we employed a modified staircase method to determine auditory thresholds. The staircase method (Cornsweet, 1962) is an adaptive procedure, wherein the stimulus feature of interest (here, absolute sound level) is decreased when the stimulus is detected and increased when the stimulus is not detected. The staircase method has been used in a number of fish hearing studies to determine auditory thresholds (for review see Bhandiwad and Sisneros, 2016). In our experiments, the amplitude of the stimulus was adjusted in steps of 3 dB re: 1 μPa (higher or lower) based on the presence or absence of an AEP response in the previous stimulus block (Fig. 2). Depending on the frequency tested, the starting amplitude of stimulus varied from 112 – 136 dB re: 1 μPa. Pending identification of a response, the level was increased or decreased by 3 dB until a level was reached that failed to elicit a criterion response (using the double-frequency analysis described above). This procedure was completed in random order at each test frequency for each fish. In some cases for which offline analyses determined that threshold had not been reached online (i.e., that sufficient subthreshold tokens had not been presented to conclusively determine an empirical threshold), AEP thresholds were later interpolated using best linear fit between tested sound pressure levels and AEP responses, similar to previously published AEP studies (Supin et al., 2001; Brittan-Powell et al., 2002; Nachtigall et al., 2007). When interpolation was used, all available AEP responses for a tested fish (measured as the mean FFT amplitude at twice the stimulus frequency in microvolts) were plotted against all tested sound pressure levels. A best-fit linear equation was then determined to describe the relationship between sound pressure levels and AEP responses from which the AEP threshold was then interpolated. The interpolated AEP threshold for an individual fish was determined as the sound pressure level at which the best-fit line intersected with the background noise measurements that defined the threshold [i.e., twice the magnitude of the noise floor (+3 dB) at twice the corresponding stimulus frequency].

2.4. Acoustic impedance measurement

Large variation in the acoustical testing environments in which auditory physiology experiments have been performed has influenced the reported auditory capabilities of fishes (Popper et al., 2019). One way to account for such variation (and inform the interpretation of disparate results) is to report the acoustic impedance of the experimental tank environment as suggested by Popper and Fay (2011) and more recently by Popper et al. (2019). To aid in the interpretation of the present data and support its reproducibility, here we report acoustic impedance values of the tank used in the present experiments.

Acoustic impedance (Z), expressed in Rayls [1 Rayl = 1(Pa*s) m^{-1}], is the complex ratio of sound pressure to particle velocity. Here, Z measurements were made in a freshwater environment and then compared to the Z of theoretical "freshwater" ($Z_{\text{theoretical freshwater}}$ = 1.504 MRayls) in a free-field environment at 28.5° C, which was inferred from the linear relationship between water temperature and acoustic impedance (Z = 1.4074 + 0.00339 * water temperature) as demonstrated in Bradley and Wilson (1966).

The Z of our experimental tank was determined by dividing the amplitude of the pressure wave by the amplitude of the particle velocity wave. The amplitude of the particle velocity wave was calculated from the amplitude of the particle acceleration waveform (see below) measured using our 3D accelerometer. We simultaneously measured both sound pressure (dB re: 1 μPa) and particle acceleration (dB re: 1 ms^{-2}) at each of the 5 tested frequencies (115, 800, 1850, 3700, and 4500 Hz). Sound pressure and particle acceleration was measured using the same sensors and placements within the test tank described above (*Acoustic stimuli and calibration*). We measured particle acceleration along the x, y and z-axes and used these values for calculating the Z of the experimental tank. The amplified peak-to-peak (pk-pk) voltage measurements for both sound pressure and particle motion were measured and

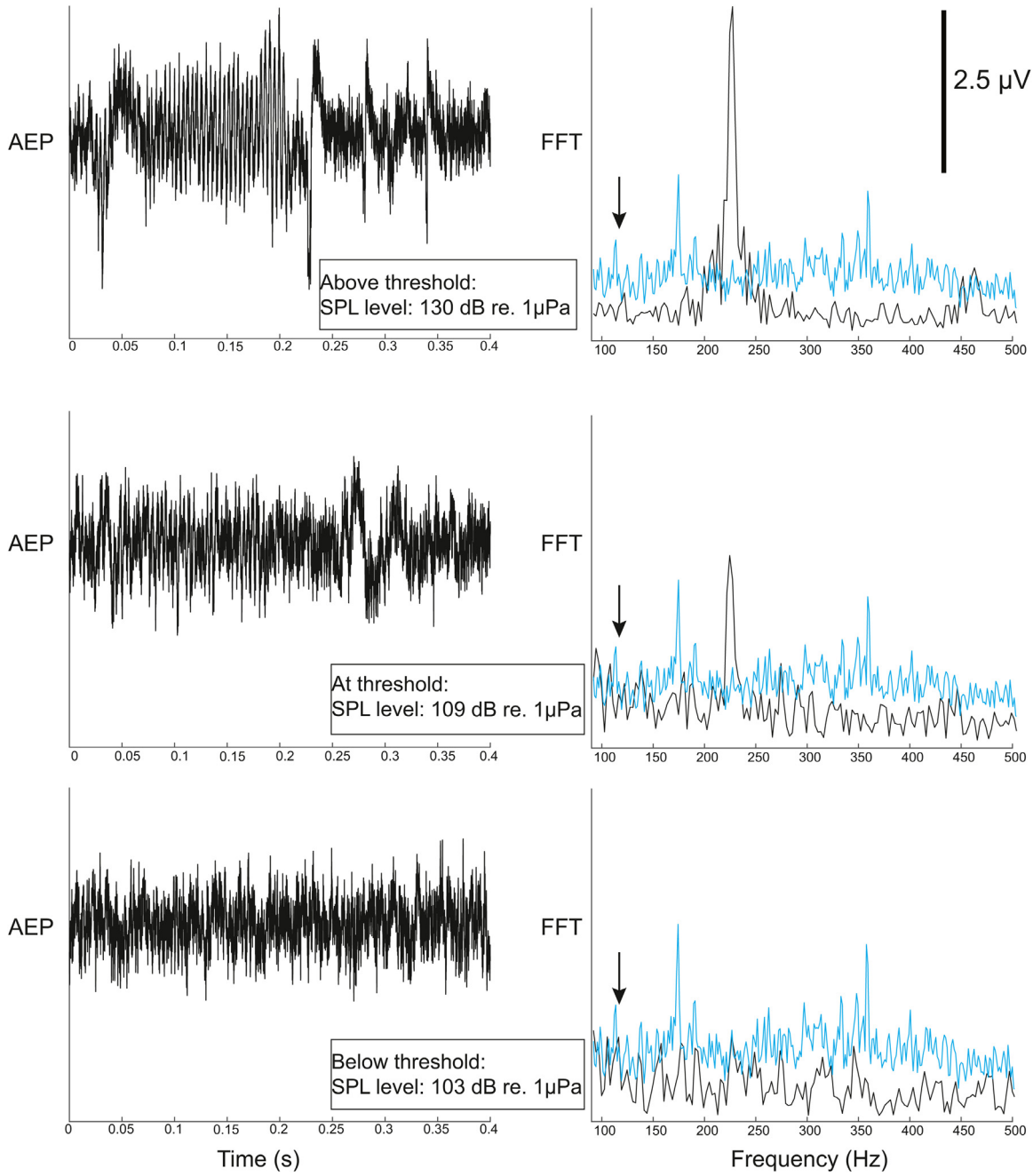

Fig. 2. Representative AEP recordings. The left panel represents the average AEP waveforms recorded from a single individual in response to 115 Hz tones at three different SPLs: 103, 109 and 130 dB re: 1 μPa (peak-to-peak measurements) with alternating polarity [refer to Methods section]. The stimulus duration was 200 ms. The right panel represents FFT of AEP waveforms (black) and FFT of background level (blue). When the stimulus is above threshold (top panel: SPL at 130 dB re: 1μPa), a prominent peak is observed in the FFT spectrum of the waveform at twice the stimulus frequency, here 230 Hz. This "double frequency response" is thought to reflect the nonlinear summation of oppositely oriented hair cell activation and subsequent firing of associated auditory afferent neurons (see text). The amplitude of the double-frequency component in the FFT is expected to decrease with decreasing stimulus amplitude (middle panel: SPL at 109 dB re: 1μPa), until its amplitude matches the background noise level (bottom panel: SPL at 103 dB re: 1 μPa). The system noise floor was defined as the mean noise FFT plus one standard deviation. For each stimulus frequency, threshold was determined as the lowest sound pressure level eliciting a "double-frequency" response at least 3 dB (i.e. twice the magnitude) above the system noise floor at that frequency. The downward pointing arrows indicate the stimulus frequency at 115 Hz.

recorded using a data acquisition system (NI myDAQ 16 bit analog-to-digital conversion at 200kS/s, National Instruments, Austin, YX, USA) that was controlled by a custom program in LabVIEW software (NI LabVIEW 2016, National Instruments, Austin, TX, USA).

In our analysis, sound pressure level (Pa) was first computed using the following equation (Eq. (2)):

$$SPL = mV\,pk - pk/sc \tag{2}$$

where sc is the scale factor (mV/Pa) from the conditioning amplifier and the $mV_{pk\text{-}pk}$ is measured peak-to-peak voltage from the recorded signal through hydrophone. Particle acceleration ($a = ms^{-2}$) was calculated using the equation (Eq. (3)):

$$a = mVpk - pk/S \tag{3}$$

where S = accelerometer sensitivity (mV/ms^{-2}) for the z-axis. The amplitude of the particle velocity waveform ($v = m/s$) was calculated from the amplitude of the particle acceleration waveform us-

ing the following equation (Eq. (4)) from Nedelec et al. (2016):

$$v = \frac{a}{2\pi f} \quad (4)$$

where f = frequency in Hz, and a = amplitude of the measured acceleration waveform via the accelerometer. The absolute magnitude of acoustic impedance [ratio of pressure to particle velocity, (Pa*s)/m] for a given frequency and sound level was then expressed logarithmically relative to the acoustic impedance of freshwater (Z = 1.504 MRayls) in the free-field with a salinity of 1500 µS/cm at 28.5 °C. We calculated the acoustic impedance using the following equation (Eq. (5)):

$$\text{dB re}: \ 1.504 \text{ MRayl} = 20*\log\left[(\text{sound pressure/particle velocity})/1.504 \text{ MRayl}\right] \quad (5)$$

and the unit of impedance was expressed in MRayls (1×10^6 Pa*s/m) (Bradley and Wilson, 1966; Erbe, 2011).

Finally, to quantify the phase of the complex acoustical impedance in our experimental tank, we characterized the phase difference ($\Delta\Phi_{p,v}$) between the particle velocity wave (v) and the pressure wave (p) in the z-axis. To assess the acoustical impedance, we measured the phase difference ($\Delta\Phi$) between the particle acceleration (a) and pressure (p), where $\Delta\ \Phi_{\mathbf{p},\boldsymbol{a}} = \Phi_{\mathbf{p}} - \Phi_{\mathbf{a}}$. In the near field of a spherical sound source, the particle acceleration (a) and sound pressure (p) are expected to be in-phase ($\Delta\Phi_{\mathbf{p},\boldsymbol{a}} = 0°$, $\Delta\ \Phi_{\mathbf{v},\boldsymbol{p}} = 90°$) (Harris and Van Bergeijk, 1962). For a sinusoidal wave, the phase of particle acceleration (Φa) will always lead the phase of particle velocity (Φv) by 90°, Φa – Φv = 90°. Therefore, the phase difference between the particle velocity (v) and pressure (p) can be determined using the following equation: $\Delta\Phi_{p,v} = \Delta\Phi_{p,a} + 90°$. Fig. 1 shows the acoustic impedance and phase characteristics of the experimental tank's acoustic environment (tank and speaker) in which the zebrafish AEPs were recorded using all the frequencies tested for three sound pressure levels: 115, 133, 151 dB re: 1 μPa.

2.5. Data analyses

Correlation between body size and age was performed using Spearman's Rho in R 3.5.2 using the *cor.test* function. Auditory threshold data were analyzed using a nonparametric 3 × 5 mixed design ANOVA (3 age groups as fixed effects, and 5 frequencies as random effects were repeated measures) to evaluate whether there were differences among the age groups. We did not make parametric assumptions in our tests, as a Shapiro-Wilk normality test results (W = 0.98, p = 0.02) did not allow us to assume normality. The nonparametric mixed design ANOVA was performed in R 3.5.2 using the f1.ld.f1 function from the nparLD package (Noguchi et al., 2012). Post-hoc comparisons were performed in the same R environment using the *pair.comparison* feature from the nparLD package (Noguchi et al., 2012). To further test effects of body size on auditory thresholds, the body size data was categorized into two classes, large (>3.3 cm SL) and small (≤ 3.3 cm SL). An additional non-parametric mixed-design ANOVA (body size as the fixed effects, and frequency tested as random effects) was carried out using the f1.ld.f1 function from the nparLD package in R 3.5.2.

3. Results

Auditory evoked potentials were recorded from 39 adult zebrafish from three age groups: 12 fish with a mean age of 13 months (age range of 369 - 419 days; mean age = 395 ± 13 SD days; mean standard length (SL) = 34 ± 3 SD mm; mean body mass (BM) = 1.07 ± 0.28 SD g), 10 fish with a mean age of 20 months (age range of 604 - 630 days; mean age = 614 ± 8 SD days; mean SL = 29 ± 0.1 SD mm; mean BM = 0.51 ± 0.08 SD g), and 17 fish with a mean age of 37 months (age range of 1024 - 1178 days; mean age = 1104 ± 36 SD days; mean SL = 32 ± 2 SD mm; mean BM = 0.72 ± 0.15 SD g). There was no correlation of age (days) with body size (SL) (Spearman's Rho, $r_s = -0.19$, p = 0.243) for the 39 adult zebrafish that comprised the three age groups (Fig. 3). The 13-month-old zebrafish (mean SL = 34 ± 3 SD mm) were in fact slightly larger than 20-month-old zebrafish (mean SL = 29 ± 1 SD mm) (t(15.58) = 4.75; p < 0.001) while 37 month-old zebrafish (mean SL = 32 ± 2 SD mm) were smaller than 13-month-old (t(23.83) = −2.565, p = 0.033) but larger than 20-month-old zebrafish (t(21.75)= 2.58; p = 0.004), suggesting that in AB/WIK zebrafish, size is not a reliable index of age (see Discussion).

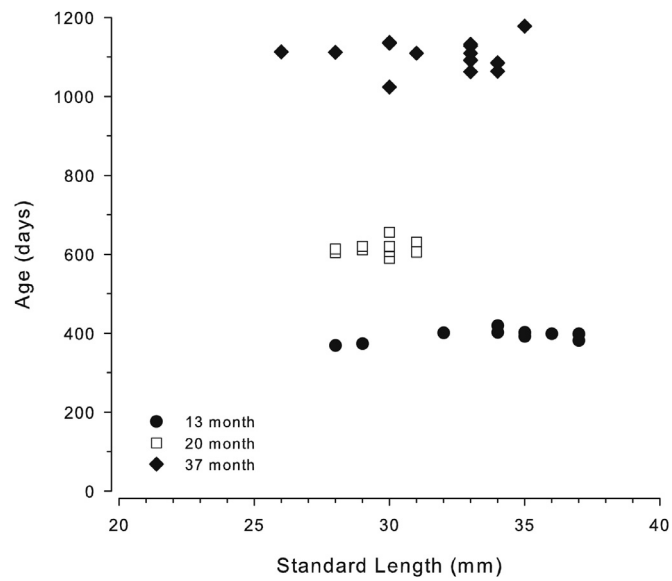

Fig. 3. Standard length (SL; unit: cm) plotted as a function of age for all individuals within the three tested age groups. The correlation between age and SL was not-significant (Spearman's Rho: $r_s = -0.23$, p = 0.14), indicating that length is an unreliable proxy for age in AB/WIK zebrafish (see text).

Auditory thresholds based on sound pressure level (SPL) were determined for the AEP responses recorded from 39 adult zebrafish in the three age groups. AEP threshold tuning curves varied across individuals but, for all three age groups, generally reached minimum thresholds (highest sensitivity) ranging from 800 Hz to 1850 Hz and gradually increased at higher frequencies, reaching maximum thresholds (lowest sensitivity) at or near 4500 Hz. Fig. 4 shows AEP thresholds in terms of sound pressure level averaged across animals for each of the 3 age groups. A 3 × 5 mixed design ANOVA for AEP thresholds in terms of sound pressure level demonstrated significant main effects of both age group (fixed effect factor, $F(1.94, \infty) = 4.71$, p = 0.025) and frequency (repeated measures factor, $F(2.7,\infty) = 39.88$, p < 0.001), while the interaction between age group and frequency was not significant ($F(4.75,\infty) = 1.56$, p = 0.170). Follow-up *post-hoc* pairwise comparisons (no pattern specified) revealed that the mean AEP thresholds of 37-month-old fish (mean = 122.2 dB ± 2.2 dB SE re: 1 μPa) were significantly higher than that of 20-month-old fish (mean = 113.1 ± 2.7 dB SE re: 1 μPa, $F(1, \infty) = 7.24$, p = 0.007) and marginally higher than that of 13-month-old fish (mean = 116.3 ± 2.5 dB SE re: 1 μPa, $F(1, \infty) = 4.41$, p = 0.052). In addition, the mean AEP thresholds of 13-month- and 20-month-old zebrafish did not differ ($F(1, \infty) = 0.68$, p = 0.410).

Auditory thresholds based on particle acceleration levels were also determined for the AEP responses recorded from the 39 adult zebrafish in the three age groups. In general, particle acceleration

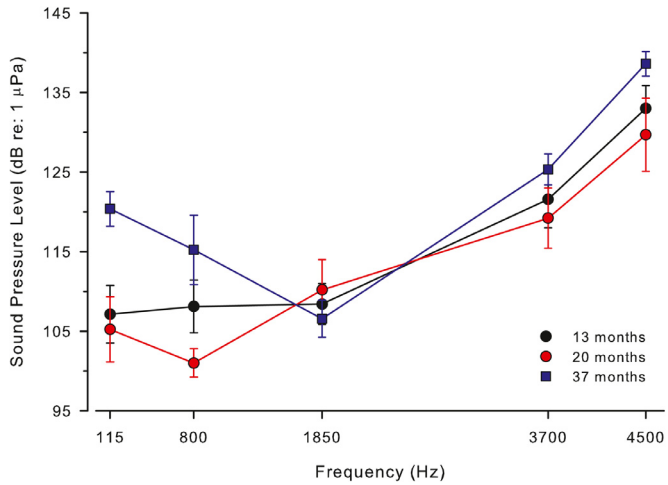

Fig. 4. Averaged auditory evoked potential tuning curves based on sound pressure level (dB re: 1 μPa). Group mean thresholds are shown for the three tested age groups of AB/WIK zebrafish: 13 months (black, n = 13), 20 months (red, n = 12), 37 months (blue, n = 16). Error bars indicate 1 standard error of the mean.

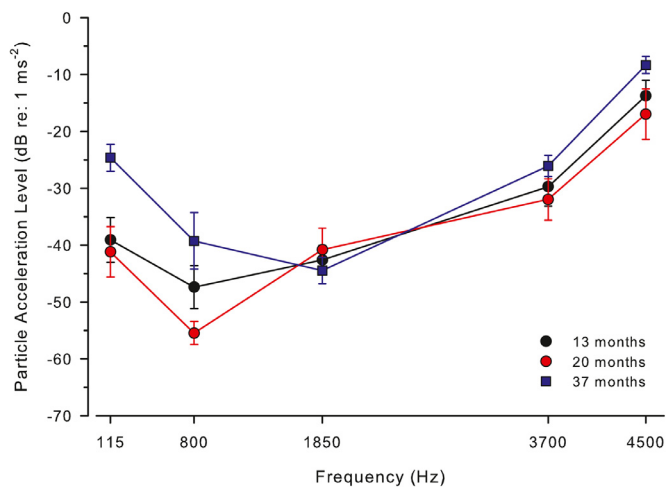

Fig. 5. Averaged auditory evoked potential tuning curves based on particle acceleration level (re: 1 ms^{-2}). Legend otherwise as in Fig. 4.

level-based AEP thresholds paralleled those observed for sound pressure level, with minimum thresholds (highest sensitivity) in the range from 800 to 1850 Hz and maximum thresholds (lowest sensitivity) at 4500 Hz (Fig. 5). Fig. 5 shows AEP thresholds in terms of particle acceleration level averaged across animals for each of the 3 age groups. Similar to sound pressure thresholds, age (F(1.96, ∞) = 3.91, p = 0.021) and frequency (F(2.7,∞) = 49.55, p < 0.001) were important factors predicting auditory thresholds based on particle acceleration level, while the interaction between age and frequency remained non-significant (F(4.76,∞) = 1.76, p = 0.120). The mean particle acceleration thresholds for 37-month-old fish (mean = −27.5 ± 2.3 dB SE re: 1 ms^{-2}) were higher than that of 20-month-old fish (mean = −37.2 ± 2.8 dB re: 1 ms^{-2}, F(1, ∞)= 7.74, p = 0.005), but were not significantly higher than that of 13-month-old fish (mean = −34.1 ± 2.6 dB SE re: 1 ms^{-2}, F(1, ∞) = 4.41, p = 0.052). Mean particle acceleration thresholds did not differ between 13-month- and 20-month-old fish (F(1, ∞) = 0.64, p = 0.423).

Furthermore, no relationship between body size and auditory thresholds (based on SPL or PAL) was observed between animals categorized into two classes: large (>3.3 cm SL) and small (≤ 3.3 cm SL). Thus, AEP thresholds (based on SPL) did not differ with body size (F(1, ∞) = 0.06, p = 0.804). A similar analysis showed that body size did not predict auditory sensitivity (thresholds based on PAL) (F(1, ∞) = 0.01, p = 0.904).

4. Discussion

4.1. Evidence for age related loss of auditory sensitivity in the zebrafish model

Over the past two decades, the zebrafish has become a powerful model system for the study of inner ear development and some forms of hearing loss (Haddon and Lewis, 1996; Whitfield, 2002). The auditory hair cells of zebrafish are homologous to those found in mammals and the early development of the zebrafish inner ear is similar to that of other vertebrates (Bang et al., 2001; Whitfield et al., 2002; Coffin et al., 2004). Zebrafish are common freshwater fishes belonging to the second-largest superorder of fishes known as Ostariophysi, which contains approximately 10,388 species or about 28% of the known fish species in the world (Nelson et al., 2016). One of the distinguishing characteristics of fishes in this superorder is the Weberian apparatus, which is comprised of modified vertebrae called Weberian ossicles that connect the anterior part of the swim bladder to the inner ear (Weber, 1819, 1820; Rosen et al., 1970; Ladich and Schulz-Mirbach, 2016). The Weberian apparatus is a derived adaptation that enables the detection of sound pressure. That is, the swim bladder of zebrafish and other ostariophysans essentially acts as a crude 'ear drum' that captures sound pressure energy and transduces it to the inner ear by the Weberian ossicles in a manner functionally similar to the middle ear apparatus of mammals. This unique anatomical feature enables detection of sounds well into the kilohertz range, higher than in most non-ostariophysan fishes and approaching the audiometric range of mammals. Zebrafish auditory function is readily evaluated using minimally invasive electrophysiological techniques including evoked potentials (as in the present study) and behavioral assays (e.g., Bhandiwad et al., 2013) routinely used in mammals. Thus, in addition to offering a powerful genetic toolkit, zebrafish audition is phenomenologically similar to that of mammalian auditory models in some important respects. However, profound and fundamental differences in other respects demand caution when considering the use of zebrafish for translational questions.

The main goal of the present study was to characterize the auditory sensitivity of wild-type zebrafish (AB/WIK) of known age groups using the AEP recording technique to evaluate whether zebrafish exhibit ARHL (cf. Wang et al., 2015), or are instead ARHL-resistant as recently suggested in other-nonmammalian vertebrates (cf. Krumm et al., 2017; Langemann et al., al.,1999; see Introduction). Based on our measures of sound pressure and particle acceleration (re. 1 μPa and 1 ms^{-2}, respectively), we found that the mean AEP thresholds of 37-month-old zebrafish (mean SPL = 122.2 dB re: 1 μPa, mean PAL = −27.5 re: 1 ms^{-2}) were approximately 9 dB higher than that of 20-month-old fish (mean SPL = 113.1 dB, mean PAL = −37.2 dB) and 6 dB higher than that of 13-month-old fish (mean SPL = 116.3 dB, mean PAL = −34.1 dB) (note: the difference between the 13-month-old and the 37-month-old groups did not reach statistical significance, p = 0.052). Although overall auditory sensitivity (gain) differed among the three age groups, the shape of the AEP audiogram was similar for all three age groups with best sensitivity between 800 Hz and 1850 Hz. Overall, the magnitude of sound pressure sensitivity loss in the oldest animals (on the order of 6–9 dB) was substantially less than that observed in aged humans and other mammals (Wang and Puel, 2020; Dubno et al., 2013), but nonzero (cf. Krumm et al., 2017) and suggestive that further study may be warranted.

The magnitude of age-related loss of auditory sensitivity observed in AB/WIK wild-type zebrafish of the present study was similar to the putative age-related loss of auditory sensitivity reported in AB wild-type zebrafish by Wang et al. (2015), who used body length as proxy for age. Wang et al. (2015) showed that the largest zebrafish size class (42–46 mm), labeled the "oldest" group, exhibited a mean threshold (124 dB re: 1 μPa) approximately 7 dB less sensitive than a putatively younger (39–40 mm) size class (mean threshold = 118 dB re: 1 μPa), which had the best auditory thresholds of five size classes tested. However, the largest zebrafish (42–46 mm) in the Wang et al. (2015) study were estimated to be only 20-months post-fertilization – an age that yielded no difference re: 13 months in our data.

Notably, we did not observe a systematic relationship between zebrafish age and body size (SL) in our study. While SL is expected to increase with age in individual fish, distributions of SL *across* individuals of the three adult age classes were largely overlapping, and the correlation between age and SL was non-significant. Additionally, we did not find any effect of body size on auditory thresholds in the AB/WIK zebrafish, similar to Higgs et al. (2002). Our data therefore suggest that size is a tenuous proxy for age across groups of zebrafish, at least in the AB/WIK strain we tested. Previously published work has also demonstrated large variations in teleost fish body size, especially when groups of fish were held in captivity (Magnuson 1962; Yamagishi, 1962, Higgs et al., 2002, Vijayan et al., 1988; Fagerlund et al., 1981). Size considerations aside, effects of age on auditory sensitivity could vary with the zebrafish strain evaluated. Recently, Monroe et al. (2016) described significant differences in auditory sensitivity among adult zebrafish of different genetic background strains – akin to auditory differences exhibited by different mouse strains (Li and Borg, 1991; Zheng et al., 1999; Ohlemiller et al., 2010). Future studies that characterize auditory sensitivity across groups of known age classes in other wild-type zebrafish strains including Tuebingen, Tupfel long-fin, WIK, and AB, would work to further elucidate the occurrence and extent of possible ARHL in zebrafish.

We used an objective method to determine AEP thresholds (Sisneros et al., 2016; Popper et al., 2019) based on quantitative assessment of response amplitude spectra (see Methods). Objective methods for determining AEP thresholds, versus, e.g., subjective evaluation of AEP waveforms (e.g., Kenyon et al., 1998; Wang et al., 2015) are expected to yield more consistent and reproducible data, and should also be employed in future studies. Other factors that can vary across experiments, such as background noise level and tank impedance, are also important to quantify (Popper et al., 2019).

4.2. Potential mechanisms for age-related loss of auditory sensitivity in zebrafish

The mechanisms responsible for the observed age-related loss of sensitivity in the present study are unknown. Wang et al. (2015) showed that the largest (putatively oldest; but see above) AB wild-type zebrafish exhibited decreased hair cell density along with possible structural changes in hair cell morphology. While zebrafish continually add hair cells throughout their lifetime in the saccular epithelia as the fish grows, age-related changes in the growth and death rates of saccular hair cells could lead to reductions in hair cell density. Previous work suggested rate of hair cell generation does not decline during development with age/size (Pinto-Teixeira et al., 2015; Higgs et al., 2002). We are not aware of other published work that performed similar measurements in the inner ear of older post-reproductive adult zebrafish (older than 24 months of age). Alternatively, the observed age-related loss in auditory sensitivity could be related to changes that occur post-synaptically at the hair cell-afferent synapse. Wang et al. (2015) quantified the expression of *ribeye b* protein - a critical component of the ribbon synapse (Sheets et al., 2011; Nicolson, 2015) - in the saccule as a proxy for synaptic ribbons and the associated afferent innervation of hair cells in the zebrafish saccule; the fluorescence signal of *ribeye b* immunostaining was reduced in the largest (putatively oldest; but see above) fish, coincident with higher AEP thresholds (i.e., decreased sensitivity). Based on their results, Wang et al. (2015) suggested that changes at both the level of the hair cell and its synapse with primary auditory neurons may contribute to age-related reductions in zebrafish auditory sensitivity. To the extent that these or related hair cell / synaptic processes resemble those observed in aging mammals (e.g., mice; Jiang et al., 2015), the zebrafish might hold promise as a model for some aspects of ARHL.

One prominent mechanism of ARHL in mammals is quite unlikely to affect zebrafish. In the cochlea, age-related degeneration of the stria vascularis can lead to a reduced endolymphatic (endocochlear) potential (EP), thereby negatively impacting the function of the cochlear amplifier (Schukneckt, 1964; termed metabolic ARHL, e.g., see Dubno et al., 2013). In mammalian models, a decrease in the EP from 90 mV (observed in animals with normal hearing) to 20 mV (evident in aging animals) is associated with a 40–50 dB loss in auditory sensitivity. This is also true for animals raised in quiet conditions, suggesting that EP degeneration occurs independent of noise exposure (Schulte and Schmiedt, 1992). Measurements of EPs in non-mammalian species are relatively sparse (reviewed in Köppl et al., 2018). In teleost fishes, as in mammals, the EP depends on the voltage difference between the endolymph and perilymph in the inner ear, but the magnitude of this difference is only ~10 mV (Enger, 1964; Katsuki et al., 1954). In mammals, the cochlear endolymph's ionic composition is thought to be maintained by the marginal cells of the stria vascularis (Marcus and Marcus, 1987; Wangemann, 1995), while in teleosts, the saccular endolymph composition is regulated by ionocytes (Mayer-Gostan et al., 1997). While it is unknown whether aging affects saccular ionocytes, and EP measurements have not been completed in zebrafish specifically, the relatively low magnitude of saccular EP measured in a limited number of teleost fishes does not suggest dramatic 'amplifying' effects as observed via the cochlear EP. Age-related decay in the saccular EP, even if it occurred, would thus be expected to yield comparatively slight effects.

One concern in the study of ARHL is the covariation of effects of age and effects of chronic noise exposure (e.g., Dubno et al., 2013; Liberman et al., 2017), a potential confound in many human studies and one incentive for the use of animal models. The noise levels in the aquaria at the two zebrafish holding facilities ranged from 120 dB to 128 dB re: 1 μPa RMS. Such noise likely arose from the operation of water pumps and other equipment external to the aquaria in both facilities. Ambient noise levels in natural zebrafish habitats range from 98 to 126 dB re: 1 μPa (in southwest India, Lara and Vasconcelos, 2019); thus, animals in the present study were reared in noise levels near the upper end of the 'natural' range. While acute exposure to high-level noise or impulses can damage hearing in fish (Scholik and Yan, 2001, 2002; Wysocki and Ladich, 2005; Popper and Hawkins, 2016; Brietzler et al., 2020), evidence for adverse effects of chronic exposure to more modest noise levels, like evidence for effects of age itself (in fish and other hair cell-regenerating non-mammals), is limited. It is nonetheless plausible that cumulative aquarium noise exposure contributed to the reduced auditory sensitivity observed in the oldest fish of the present study. Future measurements using zebrafish of varied age reared in varying levels of ambient noise could prove informative.

5. Conclusions

We have demonstrated an age-related loss of auditory sensitivity in AB/WIK wild-type zebrafish using an accurate, reproducible method for the assessment of auditory sensitivity in fishes. Old (37-month) zebrafish exhibited decreased auditory sensitivity compared to a younger group of fish (20-month-old), based on both sound pressure and particle motion levels, despite the known ability of zebrafish to regenerate hair cells. Future investigations are necessary to further establish the incidence and extent of ARHL in zebrafish, including other wild-type strains, to determine which, if any, ARHL mechanisms may be conserved to support meaningful comparative/translational studies.

Author statement

Andrew Brown, Joseph Sisneros, Ruiyu Zeng: Conceptualization, Methodology; **Andrew Brown, Loranzie Rogers:** Software; **Ruiyu Zeng:** Data curation, Investigation, Formal analysis, Validation, Writing-original draft; **Joseph Sisneros:** Supervision, Project administration; Funding acquisition; **Loranzie Rogers, Ruiyu Zeng:** Visualization; **Andrew Brown, Joseph Sisneros, John Clark, Owen Lawrence, Loranzie Rogers:** Writing – Review & Editing.

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