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Noise-Induced Hearing Loss in Zebrafish: investigating structural and functional inner ear damage and recovery

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Abstract

Exposure to continuous moderate noise levels is known to impair the auditory system leading to Noise-Induced Hearing Loss (NIHL) in animals including humans. The mechanism underlying noise-dependent auditory Temporary Threshold Shifts (TTS) is not fully understood. In fact, only limited information is available on vertebrates, such as fishes, that share homologous inner ear structures to mammals and have the ability to regenerate hair cells. The zebrafish *Danio rerio* is a well-established model in hearing research providing an unmatched opportunity to investigate the molecular and physiological mechanisms of NIHL at the sensory receptor level. Here we investigated for the first time the effects of noise exposure on TTS and functional recovery in zebrafish, as well as the associated morphological damage and regeneration of the inner ear saccular hair cells.

Adult specimens were exposed for 24h to white noise at various amplitudes (130, 140 and 150 dB re. 1 μ Pa) and their auditory sensitivity was subsequently measured with the Auditory Evoked Potential (AEP) recording technique. Sensory recovery was tested at different times post-treatment (after 3, 7 and 14 days) and compared to individuals kept under quiet lab conditions. Results revealed noise level-dependent TTS up to 33 dB and gradual increase in response latency. Recovery of hearing function occurred within 3 days for fish exposed to 130 and 140 dB noise levels, while fish subject to 150 dB only returned to baseline thresholds after 14 days. Hearing impairment was accompanied by significant loss of hair cells only at the highest noise treatment. Full regeneration of the sensory tissue (number of hair cell receptors) occurred within 7 days, which was prior to functional recovery.

We provide first baseline data of NIHL in zebrafish and validate this species as an effective vertebrate model to investigate the impact of noise exposure on the structure and function of the adult inner ear and its recovery process.

Keywords: noise, sound pressure level, hearing sensitivity, temporary threshold shift, hair cells, fish.

Introduction

The increasing levels of noise pollution are creating a serious hazard to the auditory system and the overall physiology and health condition of animals including humans (Hammer et al., 2014; Passchier-Vermeer & Passchier, 2000; Sørensen et al., 2013; WHO, 2015). Exposure to elevated background noise levels is known to affect hearing function leading to noise-induced hearing loss (NIHL) (Nelson, 2005), with a worldwide estimation of more than one billion people at risk of NIHL due to recreational and occupational activities (WHO, 2015; Mirza et al., 2018).

Mammalian and avian models have long been used to investigate the effects of noise on the auditory system (Clark et al., 1974; Cotanche et al., 1987; Davis, 1953; Hultcrantz et al., 1979; Kujawa & Liberman, 2009; Valero et al., 2017). Over the last decades several studies using these model organisms have shown that overexposure to noise can lead to either short term attenuation of hearing acuity, commonly referred to as temporary threshold shifts (TTS), and/or permanent threshold shifts (PTS) (Benitez et al., 1972; Carder & Miller, 1972; Le et al., 2017; Syka & Popelář, 1980). The molecular and cellular mechanisms associated to NIHL are similar to those described for age-related and drug-induced hearing loss, including loss of hair cells and synaptic connections (Hamernik et al., 1989; Johnsson & Hawkins 1972; Kujawa & Liberman, 2009; McFadden et al., 1997), reduced blood flow (Browning et al., 1986; Le Prell et

al., 2007; Prazma et al., 1987), increased level of reactive oxygen species (ROS) (Seidman et al., 2004; Yamane et al., 1995), mitochondrial DNA alterations (Seidman et al., 1997; Kurabi et al., 2017), and apoptotic and necrotic cell death (Hu et al., 2000; Hu et al., 2008; Yang et al., 2004). However, recent investigations suggest that the underlying mechanisms of the different types of acquired hearing loss are complex and might differ in cell death signaling and homeostatic pathways (Wong & Ryan, 2015; Yang et al., 2015). Such differences are most likely compromising the attempts to identify common pharmacological treatments (Bielefeld, et al., 2008; Le Prell et al., 2011; Sha et al., 2012).

Further research is needed using alternative vertebrate models such as fish. Since the molecular events and developmental processes underlying auditory function are highly conserved in vertebrates (Fritzsche & Beisel, 2003), the study of a model belonging to an early diverging vertebrate lineage is relevant for a fundamental understanding of the inner ear ability to cope with environmental stressors. Moreover, contrary to mammals, fish can spontaneously regenerate auditory hair cells, which may provide important insights into the molecular and cellular mechanism of inner ear regeneration (Lanford et al., 1996; Monroe et al., 2015).

Noise exposure is known to affect fish auditory system, causing hearing loss (Scholik & Yan, 2001; Popper et al., 2005; Smith et al., 2004a), auditory masking (Fay et al., 1978; Vasconcelos et al., 2007, Wysocki & Ladich, 2005), and inner ear hair cell loss and damage (Enger, 1981; Smith et al., 2006; Sun et al., 2011). Other physiological stress effects include increased cortisol levels (Smith et al., 2004a), elevated cardiovascular rate (Graham & Cooke, 2008), reduced egg survival (Banner & Hyatt, 1973), and/or reduced growth and reproductive rates (Lagardère, 1982). Since fish species are generally able to regenerate the auditory periphery, most studies only report

TTS. These studies indicated significant TTS mostly within the species best hearing range following exposure to white noise at amplitudes between 140-170 dB re. 1 μ Pa for 24 or 48 h (goldfish *Carrasius auratus*, Amoser & Ladich, 2003; Popper & Clarke, 1976; Smith et al., 2004a, 2004b; catfish *Pimelodus pictus*, Amoser & Ladich, 2003; fathead minnow *Pimephales promelas*, Scholik & Yan, 2001). These studies also showed that the time course of recovery was dependent on the noise level, exposure duration, and induced TTS.

The structural damage of the fish inner ear saccule, which is probably the main auditory endorgan in this taxon (Ladich & Schulz-Mirbach, 2016), has also been assessed in a few prior investigations. For instance, Smith et al. (2006) exposed *C. auratus* to white noise (170 dB re. 1 μ Pa for 48h) and identified localized damage in the central and caudal regions of the saccular epithelia. Schuck & Smith (2009) used zebrafish *Danio rerio* to evaluate the effects of exposure to a pure tone (100 Hz at 179 dB re. 1 μ Pa for 36 h), and reported significant loss and damage in the hair cells of the caudal region of the saccular epithelia. Hair cells showed either partial recovery after 8 days (Smith et al., 2006), or complete return to baseline levels after 14 days (Schuck & Smith, 2009). However, as these studies considered very high noise levels unlikely to be encountered by fishes in their natural habitats for extended periods of time, the consequences of prolonged exposure to moderate noise levels remain unclear.

Only a few studies have analysed both hearing loss and inner ear structural damage in fish (e.g. Faucher et al., 2009; Smith et al., 2006; Smith et al., 2011; Uribe et al., 2013), and only one has looked at recovery from noise. Smith et al. (2006) exposed *C. auratus* to noise (170 dB re. 1 μ Pa for 48h), inducing saccular hair cell bundle loss and TTS ranging from 13- 20 dB at all measured frequencies. Although hearing thresholds partially recovered after 7 days, both auditory thresholds and hair cell

number were still significantly elevated from control levels and no assessment was done beyond this time period. Further research is needed in this taxon to evaluate the effects of acoustic stress on both auditory sensitivity and sensory hair cell receptors, as well as on the mechanisms underlying its recovery process.

The zebrafish *D. rerio* (Cyprinidae) is an otophysian teleost with morphological hearing specializations connecting the inner ear and swim bladder enhancing hearing sensitivity. This species has become a well-established model organism in hearing research to investigate inner ear development, hair cell regeneration, and to test ototoxic agents and drugs for treatment of auditory impairments (Bever & Fekete 2002; Chiu et al., 2008; Ou et al., 2010; Varshney et al., 2016; Wang et al 2015; Whitfield 2002).

In the present study we investigated for the first time the functional NIHL and sensory recovery in the zebrafish model, as well as the underlying morphological damage and regeneration of the inner ear saccular sensory receptors. We used relatively mild noise levels of 130, 140 and 150 dB as acoustic treatments, which may reflect the noise amplitude found in human-altered environments including housing systems (Lara & Vasconcelos, 2019).

Methods

Test animals and husbandry

Wild type adult zebrafish (AB line) were initially obtained from China Zebrafish Resource Center (CZRC, China) and reared in our zebrafish facility at the University of Saint Joseph, Macao. The subjects used in this study were 44 adult zebrafish with a total length between 2.8-3.5 mm and about 5 months old. Test subjects were initially maintained in 10 L tanks in a standalone housing system (model AAB-074-AA-A, Yakos 65, Taiwan) with filtered and aerated water (pH balanced 7-8; 400-550 μ S

conductivity) at 28 ± 1 ° C and under a 12:12 light: dark cycle. Fish were fed twice a day with live artemia and dry powder food (Zeigler, PA, USA).

All experimental procedures complied with the ethical guidelines enforced at the University of Saint Joseph and were approved by the Division of Animal Control and Inspection of the Civic and Municipal Affairs Bureau of Macao, license AL017/DICV/SIS/2016.

Noise treatments and recovery

Prior to noise exposure, all test subjects were transferred for a minimum of 7 days to 4 L isolation glass tanks that were placed in a quiet lab environment with Sound Pressure Level (SPL) ranging between 103-108 dB re 1 μ Pa (based on LZS, i.e. RMS sound level obtained with slow time and linear frequency weightings; flat weighting: 6.3–20 kHz; see below SPL measurement equipment). These tanks had no filtering system but were subject to frequent water changes, and the light, temperature and water quality were kept similar to the stock conditions.

After this period the subjects were transferred to acoustic treatment glass tanks (dimensions: 50 cm length x 30 cm width x 25 cm height; 38 L), where they were exposed to white noise (bandwidth: 100-1500 Hz) at 130 dB, 140 dB or 150 dB re. 1 μ Pa SPL for 24 h (supplementary Fig. 1). Three acoustic tanks were used simultaneously for this purpose. They were mounted on top of Styrofoam and two granite plates (1.5 cm thick) spaced by rubber material to reduce non-controlled transmission of building vibrations. The noise playbacks were generated via underwater speakers (UW30, Electro-Voice, MN, USA) that were positioned vertically in one side of the treatment tanks using a custom built Styrofoam holder that prevented direct contact with the tank walls. The speakers were connected to amplifiers (ST-50, Ai

Shang Ke, China) that were connected to laptops running Adobe Audition 3.0 for windows (Adobe Systems Inc., USA). The tanks were used alternately for the different treatments across the various trials. In the “control” treatment tank, the amplifier connected to the speaker was switched on but without playback.

During noise exposure, individual fish were placed in a net box (dimensions: 15x15x15 cm, mesh size 1 mm) that was suspended underwater in front of the speaker at 2 cm distance. Sound recordings and SPL measurements were made with a hydrophone (Brüel & Kjær type 8101, Naerum, Denmark; frequency range: 1-80 kHz $\pm$ 2 dB; voltage sensitivity: -184 dB re. 1 V μ Pa-1) connected to a hand-held sound level meter (Brüel & Kjær type 2270). Noise levels were calibrated before each treatment so that the intended sound level (LZS) was reached at the center of the fish holding net box. A variation in SPL of $\pm$ 8 dB was registered in the closest and farthest points (in relation to the speaker). The sound spectra of the noise treatments were relatively flat and presented similar shape across the different noise levels, which can be seen in supplementary Fig. 2. Calculation of power spectra followed previously described procedures (Amoser & Ladich, 2010).

Following noise treatments, fish were either used for auditory sensitivity measurements or placed in recovery tanks (4 L), where they remained 3, 7 or 14 days post treatment (depending on the noise level) before being tested for auditory recovery.

Auditory sensitivity measurements

Auditory Evoked Potential (AEP) recordings were conducted immediately after noise treatments (day 0) and/or at specific recovery times. The AEP recording technique adopted followed previously described setups and procedures (Vasconcelos & Ladich, 2007; Wang et al., 2015). The recordings were conducted in a rectangular plastic water

194 tank (50 cm length $\times$ 35 cm width $\times$ 23 cm height) equipped with an underwater speaker
195 (UW30) positioned in the bottom and surrounded by fine sand, and a custom-built
196 sound stimulation system with enhanced performance at lower frequencies (< 300 Hz)
197 mounted in the center of the front tank wall. This stimulation system was similar to a
198 previously described setup (Vasconcelos et al., 2011) and consisted of a vibrating
199 plexiglass disc (diameter 6 cm) driven by a mini-shaker (Brüel & Kjær 4810) kept
200 outside of the tank and fixed to the table top. The disc was placed inside of the tank and
201 attached to a stainless steel rod that crossed the tank wall through a water restraining
202 flexible rubber spring, which not only prevented water drainage but also kept the rod
203 horizontally positioned. The experimental tank was placed on top of an anti-vibration
204 air table (Vibraplane, KS kinetic systems, MA, USA), which was housed in a walk-in
205 soundproof chamber (2.13 x 2.13 x 2.0 m, 120a-3, IAC Acoustics, North Aurora, IL,
206 USA) constructed as a Faraday cage.

207 Adult zebrafish were slightly anaesthetized until they lost balance in 0.12g/L
208 tricaine methanesulfonate bath (MS-222, Arcos Organics, NJ, USA) buffered with equal
209 amount of sodium bicarbonate. Specimens were then positioned in a custom-designed
210 sponge holder with a fine net covering the upper body, where they remained
211 immobilized without further anesthesia. The fish were able to recover from anesthesia
212 in the recording tank and breathe normally during experiments. The holder was
213 positioned underwater so that the fish head was just below the water surface aligned
214 with the center of the underwater loudspeaker at 8 cm distance, and facing the
215 stimulation disc (at 7 cm distance). The recording tank was filled with water from the
216 zebrafish rack system that was aerated or changed between experiments and kept at $25 \pm$
217 1° C.

Two stainless steel electrodes (0.40 mm diameter, 13 mm length, Rochester Electro-Medical, Inc., FL, USA) isolated with nitrocellulose ethyl acetate polish (except for the tips) were used for recordings. The recording electrode was placed firmly against the skin over the brainstem region, while the reference electrode was placed on the side of the body. A stereomicroscope (Stemi 2000CS, Zeiss, Jena, Germany) was used for positioning electrodes using micromanipulators.

Both sound stimuli presentation and AEP recordings were accomplished using the workstation from TDT (Tucker-Davis Technologies, Alachua, FL, USA). The AEP recording was fed into a low impedance headstage (RA4LI, TDT) connected to a pre-amplifier (RA4PA, TDT, 20x amplification), band-pass filtered (0.1-1kHz), and digitized (16 bit, +/- 4 mV). The output was then sent to a Multi-I/O processor (RZ6, TDT). Sound stimuli and AEP recordings were controlled with SigGen and BioSig TDT software.

Stimuli consisted of tone bursts of 100, 200, 400, 600, 800, 1000, 2000, 3000, 4000, 6000, and 8000Hz, presented randomly, with 20 ms duration and 2 ms rise/fall time. Tone stimuli ranged from 2 (100 Hz) up to 160 (8000 Hz) complete cycles and were at least presented 1000 times, half at opposite polarities (180° phase shifted). Prior to each experiment, a hydrophone (Brüel & Kjær 8101) connected to a sound level meter (Brüel & Kjær 2270) was used for calibrating the stimuli at the position that would be occupied by the fish head in the recording tank. For each frequency, tones were initially presented at 140 dB and then decreased in 2.5 dB steps. The auditory threshold was defined as the lowest SPL at which a visible and repeatable AEP response was identified in at least two averaged waveforms (Fig. 1). This criterium has been widely used in audiometry studies with fish species, including zebrafish (Monroe et al 2016; Wang et al 2015).

About half of the zebrafish adults used in this study were tested twice in terms of auditory sensitivity, namely after noise treatment and a recovery time. No effect on auditory sensitivity was detected due to the repeated AEP recording procedure and/or confinement in the recovery tanks. This was clearly indicated by the “control” fish (not subject to noise playback treatments) that were measured twice and presented similar auditory thresholds.

Fluorescent staining and microscopy

The specimens used for observation of inner ear sensory epithelia were dissected immediately following either AEP measurements or noise exposure/recovery periods without prior assessment of hearing function. Fish were euthanized with a MS-222 overdose (300 mg/L) buffered with sodium bicarbonate. We followed the methods previously described to extract zebrafish inner ear sensory epithelia (Liang & Burgess 2009) and stain fish saccular hair cells (Chaves et al., 2017). The fish heads were cut, the ventral part of the skull capsule was cracked open, and then fixed in 4% paraformaldehyde (96% extra pure, Acros Organics, Geel, Belgium) at 4°C overnight. After fixation, the saccules were removed and either rinsed and stored in PBS for later dissection or used immediately and dissected in a PBS bath under a dissecting stereomicroscope (Stemi 2000CS, Zeiss).

In order to stain the hair cells (HC), saccular epithelia were rinsed 3 times for 5 minutes with PBS and incubated in Alexa Fluor 488 phalloidin (diluted 1:1000; Invitrogen, ThermoFisher Scientific, Waltham, USA) for 30 minutes. Epithelia were then rinsed 3 times for 10 minutes in PBS and finally whole mounted with Fluoromount-G (Southern Biotech, Birmingham, USA) and cover slipped.

Tissue samples were initially observed under an epifluorescence microscope (DM6000B, Leica Microsystems CMS GmbH, Buffalo Grove, IL, USA) equipped with a Leica digital camera (DFC450), and then further analyzed using a confocal laser scanning (TCS SP8, Leica Microsystems CMS GmbH). Analysis of tissue images was performed with both Leica Application Suite software X (LAS X, Leica Microsystems CMS GmbH) and Image J (version 1.52i, National Institutes of Health, Maryland, USA).

HC bundle density was quantified throughout the saccular epithelia in four different non-overlapping squared regions of $2500\ \mu\text{m}^2$ (based on Smith et al., 2006). Locations were selected at 5%, 25%, 50%, and 75% across the length of the rostral-caudal axis of the sacculle. All HC bundles that were within or overlapping the outline of the square regions were considered in the HC counts.

Data analysis and statistics

Auditory sensitivity thresholds and TTS were determined for each individual for all test frequencies. TTS were calculated based on the difference between the measured auditory threshold after noise treatment and the mean value from baseline control group at a particular frequency. The effects of noise treatment and recovery time on auditory thresholds were tested with one-way repeated measures analysis of variance (RM ANOVA). The noise treatment (or recovery time) was considered a between-subject factor, while the different frequencies were the repeated measures (within-subject factor). RM ANOVA was also adopted to evaluate the effects of noise level on TTS. The overall TTS was determined based on a mean value from all TTS across different frequencies for each treatment group. The effect of noise level on the overall TTS was tested with one-way ANOVA.

The peak latency of auditory responses was determined for each individual at 1000 Hz at 120 dB stimulation. This measure was determined based on the time interval between the tone stimulus onset and the maximum peak of the AEP response (Fig. 2). The impact of noise treatment and recovery time on the peak latency was tested also with one-way ANOVA.

To test the effects of noise and recovery time on hair cell bundle quantity, two-way ANOVAs were performed with treatment and epithelial region as factors.

All ANOVAs were followed by Tukey HSD pairwise comparison post hoc tests to verify pairwise differences.

The relationship between peak latency of auditory responses and TTS was determined with Pearson correlation.

All assumptions for parametric analyses were confirmed through the inspection of normal probability plots and by performing the Levene's test for homogeneity of variances. All statistical tests were performed using Statistica 13 for Windows (Dell Software, Inc., Round Rock, T, USA).

Results

Effects of noise on auditory sensitivity and sensory recovery

The zebrafish tested in the present study presented an overall sensitivity bandwidth of 100 - 8000 Hz. However, since auditory responses to 6000 and 8000 Hz tone stimuli were not consistently present in all recordings, they were not considered for further analysis. The baseline auditory thresholds, i.e. from fish without noise treatment, were consistently the lowest at 1000 Hz (81.3 ± 1.0 dB, mean $\pm$ SEM, standard error mean; 77.5-85.0 dB range) and the highest at 4000 Hz (122.8 ± 1.5 dB; 112.5-125.0 dB) – see Fig. 3a.

Exposure to acoustic treatments for 24 h induced changes in the latency of auditory responses measured at the maximum peak of the AEP curve (at 1000 Hz, 120 dB stimulation). Latency increased significantly with increasing noise levels ($F_{3, 26}=13.80$, $p<0.001$) – Fig. 2. An increment in the response latency of 0.40 ± 0.11 , 0.65 ± 0.12 and 0.96 ± 0.15 ms (mean $\pm$ SEM) was observed for 130, 140 and 150 dB noise exposure, respectively.

The acoustic treatments induced a significant noise level-dependent elevation in auditory thresholds ($F_{3, 27}=73.9$, $p<0.001$) – Fig. 3a. There was a significant interaction between the noise level and frequency tested, meaning that noise causes different changes depending on the frequency ($F_{21, 289}=3.13$, $p<0.001$).

Noise treatments induced significant TTS, which increased with increasing noise level ($F_{2, 17}=43.79$, $p<0.001$). The TTS obtained for the treatment groups varied depending on the frequency ($F_{14, 119}=1.90$, $p<0.05$) (Fig. 3b). While fish under 140 and 150 dB noise treatments showed significant TTS at most frequencies (post hoc test $p<0.001$; except at 100 and 4000 Hz for 140 and 150 dB treatments respectively), subjects in 130 dB treatment only exhibited significantly elevated thresholds at 2000 Hz ($p<0.001$). Mean TTS (averaged across frequencies) increased significantly with exposure to higher noise levels ($F_{2, 17}=43.79$, $p<0.001$), namely mean TTS of 7.36 ± 0.64 , 13.26 ± 0.80 , and 21.5 ± 0.83 dB (mean $\pm$ SEM) were observed for individuals exposed to 130, 140 and 150 dB respectively (Fig. 3c). Furthermore, the magnitude of TTS was positively correlated with the peak latency of auditory responses ($r=0.68$, $n=16$, $p<0.01$) (Fig. 3d), i.e. the larger the threshold shift, the more delayed is the auditory response.

Recovery of the hearing function after noise-induced hearing loss was also investigated in the zebrafish. Complete recovery was achieved within 3 days for fish

exposed to 130 dB ($F_{7, 70}=0.58$, $p>0.05$) and 140 dB ($F_{7, 84}=0.38$, $p>0.05$), with auditory thresholds returning to baseline levels at all frequencies (Fig. 4a,b). In contrast, auditory thresholds of subjects treated with 150 dB noise were still elevated after 7 days ($F_{7, 77}=2.40$, $p<0.05$), and only returned to baseline levels when measured after 14 days ($F_{7, 77}=0.71$, $p>0.05$) (Fig. 4c).

The peak latency also showed a time-dependent recovery. For instance, fish exposed to 150 dB noise still showed a significantly elevated response delay of 0.39 ± 0.21 ms (mean $\pm$ SEM) after 7 days recovery (significant difference to baseline levels: $F_{1, 13}=5.81$, $p<0.05$), while no difference to the baseline response latency was found after 14 days post treatment ($F_{1, 13}=1.44$, $p>0.05$).

Effects of noise on saccular sensory epithelia and regeneration

Detailed morphological analysis of the saccular epithelia immediately following noise exposure revealed evidences of hair cell damage, namely splayed, ragged and fused stereocilia, reduced number or complete absence of stereocilia, lesions, shorter and thinner hair cell bundles (Fig. 5). Such tissue damage occurred considerably more often in subjects treated with 150 dB (re. 1 μ PA) noise, compared to the other groups. However, objective identification of these types of cellular damage was difficult and the quantification of structural damage was determined based on the number of hair cell bundles.

Noise induced significant hair cell loss ($F_{3, 132} = 98.12$, $p<0.001$), but this effect was only significantly detected in specimens treated with 150 dB noise. The greatest loss was observed in the 25% region along the rostral-caudal axis of the saccular epithelia, with 27% of hair cell bundles missing after noise exposure ($p<0.001$). The 75% region also exhibited a significant reduction in hair cell bundles of 15% ($p<0.05$)

(Fig 6a, b). Although a reduction of hair cell bundles in the center of the saccular epithelia (50% region) was observed, results were not significantly different from the control group ($p>0.05$).

Morphological recovery of the saccular epithelia was assessed in specimens exposed to 150 dB noise, since this treatment group showed significant hair cell loss. The number of hair bundles increased significantly after 7 days recovery from noise exposure ($F_{3, 183} = 199.5$, $p<0.001$). Hair cell bundles in both regions revealing damage returned to baseline levels, with increases of 25% ($p<0.01$) and 16% ($p=0.05$) at the 25% and 75% regions, respectively (Fig. 6c).

Discussion

This study is the first to demonstrate Noise-Induced Hearing Loss (or NIHL) based on changes in the auditory sensitivity in zebrafish, which is a well-established model organism in hearing research. Continuous white noise exposure for 24 h led to increased latency of auditory responses and decreased sensitivity. The magnitude of these changes as well as the recovery time (of both structural and functional levels) were noise-level dependent. Furthermore, we show first evidence that noise exposure can cause significant TTS without loss of auditory receptors in fish. Hearing loss was only accompanied by hair cell loss at 150 dB (re. 1 μ Pa), suggesting that other molecular and cellular mechanisms might be involved during mild hearing loss at lower noise levels (130 and 140 dB).

Noise-induced hearing loss in zebrafish

In this study the best hearing sensitivity of zebrafish was observed within 600-1000 Hz, with mean auditory thresholds around 82-84 dB (re. 1 μ Pa). These thresholds

were about 20-30 dB lower than previously reported audiograms for other AB lines (Monroe et al., 2016; Wang et al., 2015). Such differences might be related to the acoustic isolation period adopted in this study before audiometry, different setup configurations (i.e. shorter distance between subject and underwater speaker in our system that may result in higher particle motion component), distinct setup calibration procedures and SPL determination, and probably sensitivity differences between AB lines. Nevertheless, the overall shape of our baseline audiogram and best response frequencies were similar to prior studies using similar AEP recording procedures (Monroe et al., 2016; Wang et al., 2015). We have also adopted a criterion for threshold determination based on the visual inspection of the AEP responses, which may be more subjective than alternative methods including comparison between AEP amplitude and controlled residual noise to set specific threshold criteria (Xiao & Braun, 2008). Future research may consider alternative objective threshold determination techniques to reduce interobserver variability and facilitate comparison of auditory threshold data in future studies.

Exposure of zebrafish to 24 h of continuous white noise at moderate levels (130-150 dB) significantly elevated auditory thresholds at most frequencies and within the hearing range where the species is most sensitive. The effects of noise exposure on TTS were significantly dependent on the noise level. The same phenomenon has been described in other otophysian fish species, which possess auditory accessory hearing structures, and were exposed to white noise of similar amplitudes and duration. Scholik & Yan (2001) exposed fathead minnows (*P. promelas*) to white noise (142 dB re. 1 μ Pa for 24h) and measured TTS of 11-20 dB within the best hearing sensitivity range (800-2000 Hz). This is comparable to our data in zebrafish, where 140 dB noise induced TTS of 15-19 dB for those same frequencies. Amoser & Ladich (2003) treated goldfish *C.*

auratus and catfish *P. pictus* with white noise at 158 dB re 1 μ Pa (for 12 and 24h) and registered TTS of up to 26 dB (*C. auratus*, between 800-1000 Hz) and 32 dB (*P. pictus*, at 4000 Hz) with larger hearing loss within their most sensitive frequency ranges. Moreover, Smith et al. (2004b) also exposed goldfish to white noise but at a higher sound level (170 dB re. 1 μ Pa, 24h) and observed TTS of around 30-35 dB between 800-2000 Hz. Similar TTS of about 20-32 dB was observed in our study in zebrafish exposed to the highest noise treatment (150 dB).

The hearing frequencies mostly affected by noise exposure are usually those where the fish species is most sensitive (Scholik and Yan, 2001; Amoser & Ladich, 2003; Smith et al. 2004b). In our study, the frequencies that were more affected due to increased noise conditions lied between 600 and 2000 Hz, which corresponds to zebrafish best hearing range. Interestingly, 2000 Hz exhibited the largest TTS for all noise treatments (average of 16.5, 19.8 and 31.5 dB for 130, 140 and 150 dB, respectively) and this frequency did not correspond to the lowest auditory thresholds (about 12 dB higher than 1000 Hz). Although it is unclear why TTS would be highest at this frequency, similar observations have been reported and discussed in prior studies. Smith et al. (2004b) described a similar inconsistency in the relationship between best hearing thresholds in goldfish and the TTS measured at 1000 and 2000 Hz. Although baseline hearing thresholds in his study increased considerably by about 10 dB from 1000 to 2000 Hz, the magnitude of TTS at these two frequencies was roughly the same. These authors have suggested a potential explanation based on the asymmetry of auditory filters. Fish may have multiple narrow-band detection channels or filters that are tuned to specific frequency bandwidths. Noise-induced TTS are probably mediated by these filters, operating at specific frequency ranges and being differentially influenced by background noise. According to Fay & Megela Simmons (1999), the

auditory filter bandwidth increases with frequency, which would allow more acoustic energy to affect the auditory system inducing greater TTS at higher frequencies. The reduced TTS at 4000 Hz can be explained based on the lower species auditory sensitivity and noise playback amplitude at this particular frequency.

In addition, this study quantified changes in auditory response latency due to noise exposure in the zebrafish. This parameter may be a fast measure to predict changes in auditory thresholds since it is significantly correlated with the magnitude of TTS (Fig. 3d). Similarly, a delay in the onset of auditory responses has been reported for zebrafish after exposure to low intensity noise level (115 dB re. 1 μ Pa) or ototoxic chemicals such as gentamicin (Bellah et al 2016).

A few studies have also reported increases in AEP latency related to NIHL in birds, as well as in mammals such as rats, guinea pigs and humans (Gourévitch et al., 2009; Le Prell, 2019; Lucas et al., 2002, Sohmer et al., 1991), although shifts were generally smaller. Gourévitch et al. (2009) for instance found increases in AEP latency of up to 0.5 ms in noise-exposed guinea pigs (5 kHz, 120 dB SPL for 1-4 hours) revealing auditory threshold shifts of up to 40 dB. Changes in AEP latency of around 3 ms, as shown in our study, were only observed when threshold shifts were over 60 dB.

Effects of noise exposure on TTS have been more widely described in other model species, including birds (Adler et al., 1993; Pugliano et al., 1993; Ryals et al., 1999) and mammals (Campo et al., 1991; Canlon et al., 1987; Melnick, 1976), and results also showed TTS dependent on noise treatment level. Compared to fishes, bird and mammal species exhibit greater increase in TTS with increasing noise exposure levels above baseline audiograms (reviewed in Smith et al 2004b).

In addition, the results from this study also raise concerns regarding the potential impact of the acoustic conditions found in zebrafish housing systems of research

facilities. A recent study by Lara and Vasconcelos (2019) investigated the soundscape of various zebrafish natural freshwater habitats and compared it with the acoustic conditions found in typical laboratory housing systems. According to this study, the noise amplitude in these captive conditions reached levels of 123-144 dB (re. 1 μ Pa), with most sound energy concentrated at frequencies corresponding to the best hearing range of this species. The spectral sound levels of such artificial conditions were up to 60 dB higher compared to the natural habitats. Combined with the present study, these findings indicate that zebrafish held in typical housing systems might often be exposed to prevailing noise levels that most likely induce masking and even hearing loss.

Noise-induced damage of inner ear sensory epithelia

Immediately following noise exposure, zebrafish inner ear revealed significant damage of the sensory epithelia (hair cell loss) but only in the 150 dB treatment. The hair cell loss of these specimens was observed in all epithelial regions analyzed, but more significantly in the rostral area (25% region showing 27% reduction) and caudal (75% region with 15% reduction). The lack of significant hair cell loss in the middle epithelial area (50% region) was probably related to the lower bundle density typical of this location.

Few studies have looked at the location of noise-induced damage in fish auditory endorgans. Enger (1981) reported evidence of frequency discrimination at the peripheral level by showing that low frequency tones mainly damaged hair cells from the caudal region of the saccule in the codfish (*Gadus morhua*), whilst higher frequencies primarily produced damage in the rostral region. Smith et al. (2011), investigating the tonotopic organization in the goldfish saccule, also found that lower and higher frequencies induced localized damage in caudal and rostral regions, respectively.

Schuck & Smith (2009) observed that a 100 Hz tone produced significant hair cell bundle loss mostly in the caudal region of the zebrafish saccule. Our study is consistent with these previous findings, since we found more damage in the rostral region of the zebrafish saccular epithelia and higher TTS was found at the frequencies that are known to affect this region of the epithelium (800 and 2000 Hz; Smith et al., 2011).

Besides hair bundle loss, we also observed other types of tissue damage that are characteristic of noise-induced trauma to the inner ear sensory epithelia. Instances of lesions, splayed and fused stereocilia, reduction in stereocilia density, cuticular plates with missing hair cells, and shortened and thinned hair bundles were further examples of the damage observed. Similar injuries have been reported by Schuck & Smith (2009) in zebrafish saccular epithelia immediately following noise exposure (pure tone 100 Hz, 179 dB re 1 μ Pa, during 36h). Similar damage was also observed in a number of mammalian studies focusing on cochlear epithelia damage induced by acoustic trauma, namely missing hair cells, lesions, and sprayed fused stereocilia (e.g. Engström et al., 1983; Fukushima et al., 1990; Liberman & Beil, 1979; Saunders et al., 1985; Thorne et al., 1984).

An important finding of this study was the identification of the noise conditions that induced altered auditory sensory function in zebrafish without obvious morphological damage to the sensory receptors (i.e. at 130 and 140 dB noise levels). It is possible that the changes in auditory sensitivity were in part due to subtler types of structural damage, but more likely caused by other molecular and cellular mechanisms. However, a detailed analysis of the ultrastructure of hair cell receptors would be needed in order to fully determine the noise amplitude that initiates morphological damage. Noise-induced hearing loss is known to be associated with loss of synaptic connections, reduced blood flow, increased level of reactive oxygen species (ROS), and disrupted

mitochondrial function (Hamernik et al., 1989; Kurabi et al., 2017; Le Prell et al., 2007; Yamane et al., 1995). Further research on the functional implications of these potential alterations is being conducted in zebrafish in order to reach a better understanding of the underlying mechanisms of auditory impairment.

Functional and structural recovery after noise exposure

Hearing sensitivity of all zebrafish tested in this study returned to baseline levels following acoustic stress. The time course for sensory recovery depended on the noise amplitude. Auditory thresholds of 130 and 140 dB noise-exposed fish returned to baseline after 3 days, while thresholds of 150 dB noise-treated individuals recovered only after 14 days. Smith et al. (2004b) exposed goldfish to long-term white noise (21 days, 160-170 dB) and yielded similar results to our study. The auditory thresholds for noise-exposed subjects were still significantly elevated after 7 days and only returned to pre-exposure levels after 14 days. In contrast, a study that evaluated the impact of white noise (142 dB, 24h) in the fathead minnow, reported a significant elevation in auditory thresholds in all frequencies tested (800, 1000, 1500, 2000 Hz), two of which (1500 and 2000 Hz) did not reach baseline levels even after 14 days of recovery (final TTS of 6-12 dB) (Scholik & Yan, 2001). Altogether, these findings suggest that auditory recovery from acoustic trauma might depend on multiple factors, including species hearing abilities, and that more research is needed focusing on how different fish species cope with different types of acoustic stress.

The only noise treatment that induced significant damage of the inner ear saccular epithelia (measured based on hair cell bundle loss) was 150 dB. Full regeneration of auditory receptors was achieved after 7 days post treatment, which was prior to full auditory sensory recovery. Smith et al. (2006) investigated auditory

recovery in the goldfish following exposure to white noise (at 142 dB re. 1 μ Pa for 48h) and verified that sensitivity thresholds significantly recovered after 7 days, which was paralleled by an increase in hair cell bundles. However, this study did not consider enough time for full functional and morphological recovery. The present work provides a more complete picture on the time course of full auditory recovery following acoustic stress in a key model fish species.

Previous studies in other taxa such as birds have found that, following moderate acoustic trauma, most of the functional recovery occurred within the first week, before new hair cells have formed and become mature (Niemiec et al., 1994; Saunders et al., 1996). Functional recovery in these studies was relatively independent from morphological recovery of the sensory tissue. These findings suggest that other factors such as the connection between the hair cells and the tectorial membrane may play an important role during recovery of hearing capabilities in birds (McFadden & Saunders, 1989). Furthermore, loss of supporting cells and impairment of synaptic connections between hair cells and afferent and efferent neurons have also been reported as a result of acoustic overexposure (Cotanche & Dopyera, 1990; Ryals & Dooling, 1996; Yang et al., 2015).

Several studies have investigated the regenerative abilities of hair cells in various model systems. Hair cells are known to regenerate through cell proliferation or transdifferentiation of supporting cells (Burns & Corwin, 2013; Corwin & Oberholtzer, 1997; Rubel et al., 2013; Warchol, 2010). In particular, the zebrafish lateral line has been the focus of several investigations aiming to evaluate the regeneration abilities of hair cells after ototoxic chemical exposure (Harris et al., 2003; Hernandez et al., 2007; Williams & Holder, 2000). Literature on hair cell regeneration following chemically-induced hair cell damage is more common in this model species, and several signaling

pathways have been described (details in Head et al., 2013; Jacques et al., 2014; Moon et al., 2011). These signaling pathways are also implicated in hair cell proliferation and transdifferentiation in mammalian hair cell development (Li et al., 2015; Lin et al., 2011; Shi et al., 2013).

Only a few investigations have looked at regeneration in the zebrafish inner ear and after noise treatment (Schuck & Smith, 2009; Sun et al., 2011; Liang et al., 2012). Schuck & Smith (2009) demonstrated that following noise exposure, cell proliferation increases leading to newly formed saccular hair cells. Liang et al. (2012) generated a list of candidate genes involved in inner ear hair cell regeneration, and identified a key pathway implicated in hair cell regeneration through stem cell activation, cell differentiation, and cell division. Sun et al. (2011) found that growth hormone promotes hair cell regeneration in noise damaged zebrafish saccules by stimulating cell proliferation and suppressing apoptosis.

Future work should extend our findings by evaluating noise-induced changes in gene expression and synaptopathy mechanisms in the zebrafish inner ear saccule, as well as their association to specific auditory impairments. Altogether, this study established an important baseline data of NIHL in zebrafish for further research on the cellular and molecular mechanisms underlying different degrees of hearing loss.

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Figures Captions

Fig 1. Examples of Auditory Evoked Potential (AEP) response curves of adult zebrafish in response to 1000 Hz tone stimuli presented in decreasing amplitudes and measured either after lab silent conditions (control) or following 24h of white noise at 150 dB re. 1 μ Pa. Auditory thresholds (asterisks) were determined as the lowest stimuli amplitude at which a response curve pattern could be repeatedly observed. Scales in voltage were adjusted to improve visibility and correspond to the AEP curves presented below.

Fig 2. A) Mean Auditory Evoked Potential (AEP) response curves of adult zebrafish under 1000 Hz tone stimuli (at 120 dB re. 1 μ Pa) after treatment with different white noise amplitudes. Arrowheads indicate the peak of each AEP and respective latency in regards to the stimulus onset. Red dotted line denotes the AEP response peak in the control group for comparison purposes. Note the increase in response latency with exposure to higher noise amplitude. B) Mean peak latencies $\pm$ standard error of the mean (SEM) in response to 1000 Hz tone stimuli (at 120 dB re. 1 μ Pa) for the different noise treatments.

Fig 3. A) Mean auditory thresholds $\pm$ standard error of the mean (SEM) of zebrafish after 24h of either lab silent conditions (control, c. 105 dB re. 1 μ Pa) or white noise exposure (at 130, 140, and 150 dB). Noise induced significant changes in the auditory thresholds ($F_{21, 189}=3.13$, $p<0.001$). B) Mean Temporary Threshold Shifts (TTS) $\pm$ SEM for the different treatment groups. TTS increased significantly with increasing noise level ($F_{14, 119}=1.90$, $p<0.05$). TTS measures from “Repeated Control” group (control specimens that were measured twice) confirm that no significant effect on auditory sensitivity was detected due to repeated AEP recordings and/or confinement in the recovery tanks. C) Variation in overall mean TTS $\pm$ SEM (averaged across frequencies) for the different noise treatments ($F_{2, 17}=43.79$, $p<0.001$). D) Correlation between TTS

and respective peak latency (obtained under 1000Hz tone stimuli, 120 dB) for the different treatments ($r=0.68$, $n=16$, $p<0.01$).

Fig 4. Mean auditory thresholds $\pm$ standard error of the mean (SEM) of zebrafish measured after A) 130 dB re. 1 μ Pa noise treatment, and respective 3 days post recovery; B) 140 dB noise treatment, and 3 days post recovery; and C) 150 dB noise treatment, and 7/14 days post recovery. Auditory thresholds returned to baseline levels for 130/140 dB noise treatments after 3 days (130 dB: $F_{7, 70}=0.58$, n.s.; 140 dB: $F_{7, 84}=0.38$, n.s.), while for 150 dB they only returned to baseline levels after 14 days ($F_{7, 77}=0.71$, n.s.).

Fig 5. Phalloidin-stained hair cells from zebrafish saccular epithelia (25% region) following 24h of a) lab silent conditions (control, ca 105 dB re. 1 μ Pa), b) 140 dB noise exposure, c) 150 dB noise exposure and d) 7 days recovery post 150 dB noise treatment. Only exposure to 150 dB noise caused a clear reduction in hair cell bundles and signs of scarring (white arrowhead), cuticular plates with very short or missing hair bundles (red arrow), and thinned or fused hair cell bundles (white arrow). Other types of damage such as disordered hair bundles with splayed stereocilia (red arrowheads) could be observed in epithelia from all treatments.

Fig 6. A) Diagram of a representative inner ear saccular epithelium from a zebrafish adult showing the four regions defined for hair cell bundle quantification. D, dorsal; R, rostral. B) Variation in hair cell bundle density of control and treatment groups following noise exposure across the four regions of the saccular epithelia ($F_{3, 132} = 98.12$, $p<0.001$). Only 150 dB re. 1 μ Pa noise-exposed epithelia showed a significant reduction in hair cell bundles compared to control in the regions 25% and 75% ($p<0.05$). C) Hair cell bundle density for fish immediately following 150 dB noise treatment and after 7 and 14 days recovery. Hair cell bundle quantity returned to baseline levels after 7 days post treatment with no significant difference compared to control at the regions 25% and 75% ($p>0.05$).

Supplementary Fig 1. Experimental playback setup. Tanks used for noise exposure were mounted on top of a Styrofoam plate and two granite plates (1.5 cm thickness). Granite

plates were spaced by rubber material. The underwater speaker (UW30, Electro-Voice, MN, USA) was mounted vertically using a custom-built Styrofoam holder. Fish were placed inside a net box, which was suspended underwater in front of the speaker.

Supplementary Fig 2. Power spectra of sound recordings relative to 130, 140 and 150 dB re. 1 μ Pa white noise playbacks used as noise treatments. Sound recordings and Sound Pressure Level measurements were carried out in the center of the fish holding net box placed underwater in front of the underwater speaker. Sampling frequency: 44.1 kHz; FFT size: 512.

Highlights:

- Noise-induced hearing loss and subsequent recovery were investigated for the first time in zebrafish, an important model in hearing research.
- Continuous white noise exposure for 24h led to increased latency of auditory responses and decreased sensitivity.
- Auditory impairment was followed by significant damage of the inner ear saccular sensory tissue and loss of hair cells at 150 dB re 1 μ Pa.
- The magnitude of structural and functional changes, as well as the respective recovery time, was noise-level dependent.
- Full regeneration of the sensory tissue (based on the number of hair cells) occurred prior to auditory sensitivity recovery.