Noise-induced hearing loss correlates with inner ear hair cell decrease in larval zebrafish

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Summary statement

Increased noise in artificial enclosures can negatively impact zebrafish embryos' hearing capabilities at physiological and cellular level with observable behavioural implications.

Abstract

Anthropogenic noise can be hazardous for the auditory system and wellbeing of animals, including humans. However, very limited information is known on how this global environmental pollutant affects auditory function and inner ear sensory receptors in early ontogeny. The zebrafish (*Danio rerio*) is a valuable model in hearing research, including to investigate developmental processes of the vertebrate inner ear. We tested the effects of chronic exposure to white noise in larval zebrafish on inner ear saccular sensitivity and morphology at 3 and 5 days post fertilization (dpf), as well as on auditory-evoked swimming responses using the prepulse inhibition paradigm (PPI) at 5 dpf. Noise-exposed larvae showed significant increase in microphonic potential thresholds at low frequencies, 100 and 200 Hz, while PPI revealed a hypersensitisation effect and similar threshold shift at 200 Hz. Auditory sensitivity changes were accompanied by a decrease in saccular hair cell number and epithelium area. In aggregate, the results reveal noise-induced effects on inner ear structure-function in a larval fish paralleled by a decrease in auditory-evoked sensorimotor responses. More broadly, this study highlights the importance of investigating the impact of environmental noise on early development of sensory and behavioural responsiveness to acoustic stimuli.

Introduction

Increasing levels of noise pollution are considered a potential threat to the auditory system and overall physiological condition of animals, including humans (Hammer, Swinburn and Neitzel, 2014; Krug *et al.*, 2015; Peris, 2020). Overexposure to elevated sound levels may affect inner ear sensory receptors, resulting in neuropathy and/or cell death and leading to temporary or permanent Noise-Induced Hearing Loss (NIHL) (Hu, Henderson and Nicotera, 2002; Kurabi *et al.*, 2017; Zheng and Zuo, 2017). The effects of acoustic trauma on the auditory periphery can induce, in turn, changes in the central auditory system at morphological, physiological and functional levels (Wang, Hirose and Liberman, 2002; Eggermont, 2015). Impaired auditory function due to noise exposure may also result in changes in sensorimotor behaviors. For instance, Hickox and Liberman (2014) (Hickox and Liberman, 2014) reported that mice exposed to 94-100 dB re 20 μPa noise for 2h showed increased thresholds in acoustic startle responses,

prepulse inhibition, and activation of auditory processing along with behavioral hyperactivity.

Increasing evidence shows that the molecular and cellular mechanisms associated with NIHL are similar to those described for age-related and drug-induced hearing loss, although recent investigations also suggest that the different types of acquired hearing loss might differ in cell death signaling and homeostatic pathways (Wong and Ryan, 2015; Yang, Schrepfer and Schacht, 2015). Overall, there is a substantial lack of information on the onset and progression of noise-induced hair cell degeneration, as well as on the mechanisms of synaptopathy and recovery. Although neurotrophins have shown promising regenerative functions after acoustic trauma, more research is needed on potential protective targets and therapeutic agents (Ton and Parng, 2005; Le Prell *et al.*, 2007).

Although mammals have long been used to investigate how noise impacts the auditory system (Ketten, 1992; Ketten *et al.*, 1998; Rabin, Coss and Owings, 2006; Kujawa and Liberman, 2009; Valero *et al.*, 2017), zebrafish (*Danio rerio*) have become an important model to investigate the mechanisms of inner ear development, hair cell regeneration, and to screen for ototoxicity (Brignull, Raible and Stone, 2010; Stawicki *et al.*, 2015; Wang *et al.*, 2017; Breitzler *et al.*, 2020). Larval zebrafish at 5 days post-fertilization (dpf) possess a functioning auditory system with processing pathways of auditory information (medial octavolateral nucleus, the torus semicircularis, the medial hindbrain, and the thalamus) that overall resemble those found in adult fish and mammals (Vanwalleghem, Heap and Scott, 2017). Moreover, larval zebrafish show a robust acoustic startle response that is easy to quantify (Monroe *et al.*, 2016a; Bhandiwad *et al.*, 2018) and controlled by well-stablished neural circuitry (Korn and Faber, 2005; Tabor *et al.*, 2014). Based on these features, larval zebrafish are considered a tractable model system that can be used for testing the impact of acoustic trauma on auditory-dependent sensorimotor function and behavior.

Few studies have evaluated long-term noise effects on animal health (Dooling and Popper, 2007; Alimohammadi *et al.*, 2018; Simmons and Narins, 2018), and even less have focused on early critical periods for the development and establishment of adult phenotypic traits (Bureš, Popelář and Syka, 2017a; Dorado-Correa *et al.*, 2018; Mueller, 2018; Erbe *et al.*, 2019; Lara and Vasconcelos, 2021). Compared to other vertebrates, especially birds and mammals (Perry, 1998; Dooling and Popper, 2007; Ketten, 2008, 2012; Ortega, 2012; Erbe, Dunlop and Dolman, 2018), the relationship between inner

ear structure and auditory function following acoustic trauma has been scarcely examined in fishes (Scholik and Yan, 2001; Smith, Kane and Popper, 2004; Smith *et al.*, 2006). To our knowledge, there is no information on the effects of noise exposure on the inner ear and associated hearing loss in larval fish. This is a particularly important issue to address given that increasing evidence show that fish rely on acoustic cues from the soundscape to localize suitable habitats for settlement (Simpson *et al.*, 2004; Leis and Lockett, 2005a; Montgomery *et al.*, 2006; Vermeij *et al.*, 2010; Parmentier *et al.*, 2015) and that anthropogenic noise may disrupt habitat identification and impair orientation at early life stages (Simpson *et al.*, 2005; Caiger, Montgomery and Radford, 2012; Holles *et al.*, 2013; Holmes *et al.*, 2017).

In this regard, a recent study by Bhandiwad et al. (2018) (Bhandiwad *et al.*, 2018) evaluated the impact of long-term noise on auditory-evoked startle responses in larval zebrafish at 5-7 dpf. The authors observed significant noise-induced increases in startle response thresholds and hypersensitization to startle-inducing stimuli. These observations, however, were not related to changes in absolute auditory thresholds (determined based on prepulse inhibition behavioral assay), but were specific to auditory-evoked escape responses.

The goal of the present study was to test the effect of chronic noise exposure on auditory sensitivity of larval zebrafish through both evoked potential recordings from population of hair cells in the inner ear saccule, which plays a major role in hearing in this species and other teleosts (Lu and Desmidt, 2013), and by measuring sensorimotor responses to acoustic stimuli based on prepulse inhibition assay (PPI). We further aimed to relate noise-induced sensory loss to potential changes in saccular morphology. We hypothesized that acoustically induced stress would induce hypersensitisation (Bhandiwad *et al.*, 2018) and auditory threshold shifts, along with changes in saccular hair cell number (Monroe *et al.*, 2016a; Breitzler *et al.*, 2020).

Materials and methods

Zebrafish: husbandry and sampling

Zebrafish eggs were obtained from either wild type adults (AB line) or Et(krt4:GFP)^{sqet4} (ET4) adults with GFP expression in hair cells (Yao *et al.*, 2016) initially purchased from China Zebrafish Resource Center (CZRC, China) and reared at the research facilities of the University of Saint Joseph, Macao. These two zebrafish

lines are known to have equivalent auditory sensitivity and inner ear morphology at the larval stage (Monroe *et al.*, 2016b). Stockfish were maintained in a standalone housing system (model AAB-074-AA-A, Yakos 65, Taiwan) with filtered and aerated water (7-8 pH; 400-550 µS conductivity) at 28 ± 1 °C, subjected to a 12:12 light/dark cycle and under acoustic features as described by Lara et al (2019) (Lara and Vasconcelos, 2019). For each experimental trial, eggs were collected within 2 hpf (hours post fertilization) from 2 to 6 breeding tanks, each containing about 10 females and 5 males. Collected eggs were mixed and randomly distributed into 2 groups of 50 each. Each group was allocated to either noise treatment or silent condition (control).

Nine experimental trials (acoustic treatments) were conducted for saccular potential recordings and morphological analysis. For these tests, larval zebrafish were consistently collected between 10-11 am at two developmental stages, 3 and 5 dpf. These developmental stages were selected since at 3 dpf embryos already have a functioning inner ear (Lu and Desmidt, 2013) and at 5 dpf specimens exhibit auditory-evoked escape responses that are affected by previous noise exposure (Bhandiwad *et al.*, 2018). At the end of the experimental trials, specimens were euthanized in 300 mg/L of tricaine methanesulfonate (MS-222, ThermoFisher Scientific INC, Waltham, Massachusetts, USA) based on Strykowski and Schech (2015) (Strykowski and Schech, 2015). For the PPI assay, only specimens with 5 dpf were used. A total of 14 trials were conducted - 7 trials per frequency tested (100 and 200Hz) with 10 individuals per group. After data collection, individuals were either euthanized (noise treated group) or returned to stock conditions (control).

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

Acoustic treatments

Acoustic treatments followed Lara and Vasconcelos 2021 (Lara and Vasconcelos, 2021) and were carried in glass tanks (60 cm length × 30 cm width × 50 cm height) equipped with top built-in illumination (~7000 Lux in a 12:12 light/dark cycle) and covered with a styrofoam structure to control for light, temperature and noise conditions. No filtering system was used to avoid additional noise, but complete water changes were carried between trials. The treatment tanks were mounted on top of styrofoam boards placed

over two granite plates (1.5 cm thick) spaced by rubber pads to reduce transmission of external vibrations. Eggs were placed inside a custom-made fine-mesh cylindrical netbox (5 cm diameter, 6 cm high) that was suspended at ~7 cm above an underwater speaker (UW30, Lubell Labs, Columbus, OH, USA) that rested on top of a sponge in the tank bottom. Speakers were connected to audio amplifiers (ST-50, Ai Shang Ke, China), which were connected to a laptop running Adobe Audition 3.0 for windows (Adobe Systems Inc., San Jose, CA, USA). In the control group, the amplifier connected to the speaker was switched on but without playback, reaching a Sound Pressure Levels (SPL) of 103-108 dB re 1 µPa, LZS, RMS sound level obtained with slow time and linear frequency weightings: 6.3 Hz–20 kHz.

In the noise treatment group, fish were continuously exposed to white noise at 150 dB re 1 µPa, an amplitude level representative of freshwater habitats characterized by anthropogenic noise activity such as shipping (Amoser, Wysocki and Ladich, 2004; Codarin *et al.*, 2009) and noisier zebrafish housing systems (Lara and Vasconcelos, 2019). This noise level is also known to affect survivability, induce physiology stress and cause anxiety-like responses in larval zebrafish (Lara and Vasconcelos, 2021). The acoustic playback consisted of white noise low-pass filtered at 1500 Hz and adjusted to compensate for the frequency response of the loudspeaker and the tank acoustic properties using Adobe Audition software tools to deliver a relatively flat spectrum. Noise level was calibrated before treatment so that the intended sound level (LZS, RMS sound level; slow time and linear frequency weightings; flat weighting: 6.3 Hz-20 kHz) was reached at the bottom of the net box (~7 cm distance from the speaker) using a hydrophone (Bruel & Kjær 8104, Naerum, Denmark; frequency range: 0.1 Hz-120 kHz; sensitivity: -205 dB re. 1V/µPa ± 3 dB) connected to a hand-held sound level meter (Bruel & Kjær model 2270). Additionally, the acoustic treatments were calibrated with a tri-axial accelerometer (M20-040, frequency range 1–3 kHz, GeoSpectrum Technologies, Dartmouth, Canada) that was placed horizontally with the acoustic center at about 7 cm from the speaker in the position later occupied by the netbox containing the specimens. The sound playbacks generated most energy/particle motion in the vertical axis reaching circa 120 dB re 1 m/s^2, which was calculated based on Matlab script paPAM (Nedelec *et al.*, 2016).

Inner ear saccular potential recordings

Microphonic potential recordings from the saccule followed procedures initially described by Sisneros (2009) and Vasconcelos et al. (2011) (Sisneros, 2009; Vasconcelos *et al.*, 2011), and adapted to larval zebrafish by Rohmann et al. (2014) (Rohmann *et al.*, 2014).

Individual larval zebrafish were paralysed in 20 µl of 1 mg/ml α-Bungarotoxin (Life Technologies, ThermoFisher Scientific INC, Massachusetts, USA) in Hank's solution and then mounted laterally embedded in 0.5% agarose on top of a 35 mm microscopy dish, with otic capsule positioned outside the agarose. The specimen was covered in Hank's solution containing 0.0002% methylene blue and the dish placed on a fixed stage microscope (Axio Examiner A1, Carl Zeiss meditec AG, Jena, Germany) equipped with 10x N-Achroplan water immersion objectives. The recording platform rested on top of an air table (Kinetic System, Boston, MA, USA) inside of a walk-in soundproof chamber (IAC120A3-53, IAC Acoustics, Dongguan, China), while the remaining audiometry setup was located outside. All recordings were obtained at room temperature (20-23°C).

The stimulus probe consisted of a metal needle with a tip of approximately 50 µm diameter, which was positioned at the posterior edge of the left otic capsule along the posterior edge of the saccular otolith, and provided a linear oscillatory motion along an axis ~20 degrees off the longitudinal axis of the specimen (Lu and Desmidt, 2013; Yao *et al.*, 2016) (Fig. 1A). The position of the stimulus probe remained consistently the same across all trials. Vibratory stimuli were achieved by driving the probe with a piezoelectric actuator (Piezosystem, Jena, Germany) controlled by a lock-in amplifier (SR830, Standford Research Systems, Sunnyvale, CA, USA) through a custom written MATLAB software (MathWorks, Inc., Natick, MA, USA) modified after Rohmann and Bass (2011) (Rohmann and Bass, 2011). Stimuli consisted of 500 ms bursts of 100 to 400 Hz (in 100 Hz increments, presented randomly) followed by an interstimulus interval of 1 s, and were repeated eight times.

The linear motion of the probe was calibrated under the Zeiss microscope with a high-speed camera (FASTEC-IL5-254, Fastec Imaging, San Diego, CA, USA). The displacement of the probe tip was extracted from the high-speed videos, extrapolated to the X-Y plane to check for oscillatory movement of the probe using a custom written

MATLAB script. This information was used to determine threshold values in dB re 1 μm based on the actual probe displacement at the threshold level.

Microphonic potentials were recorded with glass microelectrodes (2 to 8 MΩ) filled with 3M KCl and positioned approximately in the middle of the saccular epithelia. A reference electrode (Ag/AgCl) was placed in the medium at the border of the dish. The recorded signals were preamplified (model 5A, Getting Instruments Inc., San Diego, CA, USA), high pass filtered and further amplified (SR650, Stanford Research), then fed into the lock-in amplifier for analog-to-digital conversion and processing, and finally analyzed on a desktop computer. At each stimulation frequency, the stimulus amplitude was increased until the mean of the eight microphonic potentials responses was greater than two standard deviations above the mean response to background noise with the lock-in amplifier power set to the minimum. The threshold data was reported as dB relative to the minimum stimulus output of the setup (0.004 V from lock-in amplifier). The noise recorded with the stimulation set to the minimum was similar to the "responses" measured if either a dead fish or no fish was placed in the recording dish.

Prepulse inhibition assay (PPI)

Acoustic startle responses from 5 dpf zebrafish were determined with the apparatus developed by Wang et al 2017 (Wang *et al.*, 2017) (Fig. 2). A total of 14 trials were conducted. Groups of 10 larvae were gently pipetted into a 3D-printed dish platform (8 cm diameter) containing system water (approx. 2 mm depth). The dish was illuminated from above with a LED ring (infrared wavelength at 850 nm, model HA92123, Feiye, Guangzhou, China) and the larvae behaviour was recorded with a digital camera (CS-S6-6C12WFBR, 4K HD, EZVIZ, Hangzhou, China) that was suspended above of the light ring. The test frequencies and amplitudes were defined using a QT Platform script (The QT Company, Espoo, Finland) that controlled the signal generator (model AUDIO-V1.0.3-20181028, designed by F.C., Southern University of Science and Technology, Guangdong, China) connected to an amplifier (model TPA-2578AY, Weiliang, Foshan, China) that drove a mini vibrator (frequency range: 60 Hz -20 KHz, model QY50R-Z, Haoshengyuan Inc, Dongguan, China). The particle acceleration at the water surface was initially calibrated with a laser Doppler interferometer (model OFV-505, Polytec GmbH, Germany) (Wang *et al.*, 2017). Prepulse stimulation

consisted of tone bursts of 50 ms at 100 and 200 Hz (frequencies that previously showed differences in microphonic potential recordings), and varying particle acceleration levels (-∞, −35, −30, −25, -20 and -15 dB re 1 ms^{-2}). The startle responses were induced with pure tones of 50 ms at the same frequencies as the prepulse stimulus but at 29 and 25 dB re 1 ms^{2} for 100 and 200 Hz, respectively, and delivered after a 50 ms interval (Fig. 2B). Stimulation was repeated 10 times at each level with 120 s intervals between presentations to avoid habituation. Six-second videos (120 frames per second, 8.3 ms per frame, 0.707 mm/pixel) were recorded per prepulse stimulus level and the average swimming velocity was tracked and calculated from each individual fish's displacement in the X-Y coordinates by subtracting sequential frames of the video recording (Wang *et al.*, 2017). For each treatment group, the startle responses (quantified as swimming velocity) to successive and increasing prepulse amplitudes allowed to determine the amplitude level that caused a significant decrease in the motor response (prepulse inhibition). This prepulse amplitude level was compared between treatment groups (see statistical analysis).

Potential differences in startle responses could derive from differences in swimming patterns and motor abilities, thus a separate set of 15 specimens from each noise exposure and control groups were recorded for 10 min in an open field consisting of a petri dish (equivalent size to the PPI dish) at 28°C in the DanioVision chamber (Noldus Technologies, Netherlands). A total of 9 open field recordings were conducted. Videos were analysed using Ethovision XT (Noldus Technologies, Netherlands) and total distance moved and time spent moving were measured for each group.

Inner ear saccular sensory analysis

The inner ear saccular hair cell bundles of larval ET4 subject to noise versus control conditions were morphologically analysed. After euthanasia, specimens were immediately fixed in 4% paraformaldehyde (PFA) at 4 °C overnight. In the following day, samples were rinsed in PBS 3 times for 10 min. To visualize the entire saccular epithelia, the otoliths from 3 and 5 dpf larvae were dissolved in either 1% or 2% Triton X-100 (Sigma-Aldrich, St. Louis, Missouri, USA), respectively, for up to 24 h at 4 °C. Samples were subsequently rinsed in PBS and then mounted laterally in microscope slides containing squared holes previously prepared with vinyl tape and containing Vectashield anti-fading solution (Vector Laboratories, Burlingame, California, USA).

Samples were visualized under a confocal laser scanning microscope (Stellaris 8, Leica Microsystems, Buffalo Grove, Illinois, USA) with a 488 nm laser line (Leica Microsystems, Wetzlar, Germany). Imaging was based on a z-stack of 45 images (spanning approximately 181 μm, 4 μm per image) and 3D reconstruction analysis was performed using Leica LAS X 3.0.14 software (Leica Microsystems). Quantification of saccular hair cell number was made by counting the hair cells bundles in the whole epithelia (Fig. 4), and the epithelial area measured using Leica LAS X 3.0.14 software.

Statistical analysis

Differences in inner ear sensitivity based on saccular microphonics potentials between the two developmental stages, and between noise-exposed versus control groups, were tested with repeated measures ANOVA, using noise or age as between-subject factor, while the different frequencies were the repeated measures (within-subject factor).

Differences in PPI responses between treatments were also determined based on repeated measures ANOVA, with noise as a between-subject factor and prepulse amplitude as repeated measures. Only the trials that revealed a significant decrease in response to increasing prepulse amplitude (prepulse inhibition) were considered for the analysis, which was firstly verified based on one-way ANOVAs for each group and frequency separately.

The variables related to larval behavioural patterns, i.e. total swimming distance and time spent moving, were compared with one-way ANOVA tests between treatment and control groups. Comparison of the inner ear morphological features (hair cell number and epithelial area) was also carried with one-way ANOVAs

ANOVAs were followed by LSD multiple comparison post hoc tests to verify for pairwise differences. Parametric tests were used since data were normally distributed and variances were homogeneous. Assumptions for parametric analyses were confirmed through the inspection of normal probability plots and by the Levene test. Statistical analyses were performed using SPSS v26 (IBM Corp. Armonk, New York, USA).

Results

Inner ear saccular hair cell sensitivity

Microphonic potentials were recorded from saccular hair cells in both 3 and 5 dpf larvae (Fig. 1B and C, respectively) and displayed an age-related enhancement in sensitivity of up to 4 dB (at 100 and 200 Hz). The microphonic thresholds for control groups at 3 dpf

decreased from 27 ± 4 to 18 ± 5 dB re 1µm (mean ± standard error of the mean) from 100 to 400 Hz. At 5 dpf, thresholds decreased from 23 ± 4 to 17 ± 4 dB at the same frequencies. Significant differences in auditory thresholds were found between these two developmental stages ($F_{(1, 24)} = 10.05$, $p=0.004$).

Noise treatment did not cause significant changes in the microphonic responses at 3 dpf ($F_{(1, 10)} = 0.31$, $p>0.05$; Fig. 1B), but a noise-induced sensitivity loss was found at 5 dpf ($F_{(1, 12)} = 8.12$, $p<0.001$; Fig. 1C). Increased thresholds ranged from 30 ± 3 to 16 ± 3 dB at 3 dpf. At 5dpf, they ranged from 29 ± 3 to 18 ± 5 dB (between 100-400 Hz). The significant differences found at 5 dpf were identified at both 100 Hz ($F_{(1, 22)} = 17.60$, $p<0.001$) and 200 Hz ($F_{(1, 27)} = 23.84$, $p<0.001$), with threshold shifts of up to 6 dB and 7 dB, respectively.

Auditory-evoked sensorimotor responses

Noise exposure induced a significant increase from 3.56 ± 0.19 mm/s (control; mean ± standard error of the mean) up to 5.26 ± 0.21 mm/s (noise exposed) at 100 Hz; and from 4.34 ± 0.24 mm/s (control) to 7.54 ± 0.21 mm/s (noise exposed) at 200 Hz, corresponding to increments of 34.9 and 60.9 %, respectively (Fig 3). This hypersensitization was significant at both test frequencies (100 Hz: $F_{(1, 30)} = 21.36$, $p<0.001$; Fig. 3A; 200 Hz: $F_{(1, 37)} = 25.00$, $p<0.001$; Fig. 3B).

Comparing the startle responses across increasing prepulse amplitudes (PPI) revealed auditory sensitivity loss associated with noise exposure. At 200 Hz the startle responses significantly decreased between -30 and -25 dB re 1 ms^{-2} prepulse amplitude for the control group ($F_{(1, 38)} = 8.21$, $p<0.01$), which contrasted with the noise-treated specimens that showed significant reduction only between -25 and -20 dB re 1 ms^{-2} ($F_{(1, 38)} = 4.93$, $p<0.001$). These results indicated a noise-induced 5 dB shift in response thresholds at 200 Hz.

At 100 Hz, however, noise treatment did not elicit a significant change in response thresholds at neither control ($F_{(1, 33)} = 0.28$, $p>0.05$) or noise treated group $F_{(1, 37)} = 1.47$, $p>0.05$ (Fig. 3A).

Additionally, to test whether larval general locomotor activity was affected by the acoustic treatment, specimens were observed in an open field arena. Noise-exposed larvae showed significantly lower swimming speed ($F_{(1, 191)} = 14.25$, $p<0.001$) and time

spent swimming ($F_{(1,\ 191)}$ = 1.829, p<0.001), suggesting additional alterations in the locomotor capabilities.

Inner ear saccular morphology

In order to evaluate whether auditory sensitivity changes were associated with differences in inner ear morphology, we investigated saccular hair cell number and epithelial area of 3 and 5 dpf zebrafish exposed to the same aforementioned conditions (Fig. 4).

The number of saccular hair cells increased significantly with age both in control (from 49 to 65, a 33 % increment; $F_{(1,32)}$ = 38.23, p<0.001) and noise exposed (from 38 to 53, a 39 % increment; $F_{(1,37)}$ = 22.19, p<0.001) groups. Although the general shape of the epithelia did not change, acoustic treatment caused a significant reduction at 3 dpf (about 10-11 less bundles, 21 % reduction; $F_{(1,\ 39)}$=14.16, p<0.001) and at 5 dpf (12-13 less bundles / 19 % reduction; $F_{(1,\ 30)}$ = 19.16, p<0.001) (Fig. 5A). Additionally, saccular epithelial area also decreased 23% at 3 dpf ($F_{(1,\ 19)}$ = 4.71, p<0.05) and 35 % at 5 dpf ($F_{(1,\ 19)}$ = 18.19, p<0.001) (Fig. 5B).

Consistent with the parallel changes in hair cell number and epithelia area, hair cell density did not reveal differences between noise-treated and control group (3 dpf: $F_{(1,\ 19)}$ = 2.56, p>0.05; 5 dpf: $F_{(1,\ 19)}$ = 3.19, p>0.05). Finally, there were no age-related differences in hair cell number and epithelial growth between the two experimental groups ($F_{(1,\ 19)}$ = 4.03, p>0.05).

Discussion

To our knowledge, the present study provides first evidence that exposure to increased noise levels can impact auditory sensitivity and the amount of auditory hair cell receptors in larval fish, and that such sensory loss correlates with a behavioural hypersensitization. We relied on larval zebrafish (*Danio rerio*) as our model system, showing that this species can be used to evaluate the impact of noise on auditory function in early ontogeny.

Noise-induced changes in inner ear function and structure

We assessed inner ear saccular sensitivity in 3 and 5 dpf zebrafish larvae based on microphonic potential recordings, a reliable method to assess peripheral auditory function in zebrafish at these developmental stages (Lu and Desmidt, 2013; Rohmann *et*

al., 2014) when the auditory pathways are already functional (Tanimoto *et al.*, 2011; Vanwalleghem, Heap and Scott, 2017).

The results demonstrate an age-related enhancement in saccular sensitivity of up to 4 dB (at 100 and 200 Hz) accompanied by 33 % increase in hair cell number. These findings are similar to the studies by Lu and DeSmidt (2013) (Lu and Desmidt, 2013) and Yao and DeSmidt (2016) (Yao *et al.*, 2016) that showed an improvement of 8 and 4 dB at 100 and 200 Hz, respectively, along with 34% hair cell increment between 3 and 5dpf.

In the present study, we show that chronic exposure to elevated noise levels (150 dB re 1 µPa, white noise) causes hearing loss of up to 6-7 dB in larval zebrafish at 5 dpf. The lack of noise-induced threshold shifts at 3 dpf might be related to differences in hair cell sensitivity, inner ear development, and/or the overall duration of the acoustic treatment. A few studies have identified noise-induced auditory threshold shifts in fish species at the adult stage (Scholik and Yan, 2001; Amoser and Ladich, 2003; Smith, Kane and Popper, 2004; Popper *et al.*, 2005), including zebrafish (Breitzler *et al.*, 2020). Yet, these studies typically relied on auditory evoked potential recordings that measure overall sensitivity of both peripheral and central auditory pathways, showing threshold increases up to 30 dB re 1 µPa. Here, we show the impact of the acoustic environment on sensitivity at the sensory receptor level of the inner ear saccule, which is considered to serve mainly a hearing function in most teleosts (Popper and Fay, 1973, 1993; Schuck and Smith, 2009).

In order to evaluate whether differences in the saccular sensitivity were related to changes in the sensory epithelia, we investigated possible noise-induced effect in the hair cell number. Noise exposure induced a 21% and 19% decrease in saccular hair cell number at 3 and 5 dpf, respectively. Such changes were not related to changing hair cell density, but to an overall reduction in sensory epithelial size (23% and 35% less in total area at 3 and 5 dpf, respectively). Similarly, Uribe et al. (2018) (Uribe *et al.*, 2018) found saccular and lateral line hair cell damage in 6 dpf zebrafish induced by underwater cavitation producing high intensity broadband sounds. According to these authors, acoustic exposure to circa 186 dB re 1 µPa, RMS for 120 min, reduced saccular hair cells by 14% and lateral line hair cells by 30% which was recovered after 72 h post-exposure. Our findings show that noise exposure affects inner ear development potentially damaging hair cells in larval zebrafish, when accessory hearing structures

are not yet present and auditory stimulation most likely comes from particle motion (Grande and Young, 2004). Future studies should evaluate the potential impact of noise on otolith development, integrity of otolith membrane, and quantify hair cell death and damage.

Schuck and Smith (2009) (Schuck and Smith, 2009) and Breitzler et al (2020) (Breitzler *et al.*, 2020) reported noise-induced damage of saccular hair cells in adult zebrafish. Exposure to 100 Hz pure tone at 179 dB re 1 µPa for 36 h resulted in 43% hair cell loss in the posterior region of the saccule (Schuck and Smith, 2009), whereas white noise treatment at 150 dB re 1 µPa for 24 h caused 15% hair cell loss in adult zebrafish. Other studies have also investigated the impact of acoustic trauma on the inner ear of adult fish from various species and reported noise-level dependent saccular hair cell loss (Schuck and Smith, 2009; Monroe, Rajadinakaran and Smith, 2015).

Only a few studies have evaluated the effect of chronic noise treatments in early ontogeny (Bureš, Popelář and Syka, 2017b, 2017a; Dorado-Correa *et al.*, 2018), which is a critical window for the establishment of phenotypic traits (Mueller, 2018). Bureš et al. (2017) (Bureš, Popelář and Syka, 2017a) reported frequency-dependent neuronal alterations in sound intensity representation in adult rats (strain Long-Evans) that were briefly exposed to noise (up to 80 dB SPL) in early ontogeny, while Dorado-Correa et al. (2018) reported faster telomere loss in juvenile zebra finches (*Taeniopygia guttata*) exposed to traffic noise (65 and 85 dB re 20 µPa). To our knowledge, the present work is the first study reporting the impact of the acoustic environment on the inner ear and associated sensitivity loss in a larval fish.

By listening to the aquatic background noise, fishes can extract critical biotic information about the presence of conspecifics and heterospecifics, and perceive important abiotic information for orientation (Popper and Fay, 1993; Lagardere *et al.*, 1994; Ladich and Schulz-mirbach, 2013). More specifically, larval fish rely on acoustic cues to detect suitable habitats for settlement and the presence of anthropogenic noise may interfere habitat identification and impair orientation (Simpson *et al.*, 2004; Leis and Lockett, 2005b; Montgomery *et al.*, 2006; Parmentier *et al.*, 2015).

Noise is known to cause impaired growth and development in early ontogeny (de Soto *et al.*, 2013), tissue damage in the inner ear (Casper *et al.*, 2013) and molecular and cellular changes along the auditory pathway (Lim, 1986; Bohne, Harding and Lee,

2007; Yang, Schrepfer and Schacht, 2015; Mancera *et al.*, 2017), which may have also contributed to the decreased saccular sensitivity observed in our study.

The molecular mechanisms underlying NIHL and their impact on the functional relationship between structure and function remain to be more fully explored in this model species.

Noise effects on acoustic startle responses

We additionally investigated the effects of acoustic overexposure on the sensory-motor responses to acoustic stimuli in larval zebrafish at 5 dpf. We found that continuous exposure to 150 dB re 1 µPa white noise induced a generalized hypersensitization of the acoustic startle responses as observed by a significant increase in swimming velocity (up to 41%). Such hypersensitisation was not influenced apparently by alteration in total locomotor activity since the swimming speed and the time spent swimming were actually lower in noise treatment groups compared to control.

The hypersensitisation observed in this study is similar to the startle-inducing hypersensitisation registered in prior studies using fish species (Purser and Radford, 2011; Bhandiwad *et al.*, 2018). Purser and Radford (2011) (Purser and Radford, 2011) exposed adult three-spined stickleback (*Gasterosteus aculeatus*) to recreational boat noise conditions (white noise, 100-1000 Hz at 128 dB SPL) and identified a twofold increase in the number of startle responses to broadband stimuli. Bhandiwad et al. (2018) (Bhandiwad *et al.*, 2018) exposed 5-7 dpf larval zebrafish to white noise at 20 dB re 1 m/s^2 using a one-dimensional shaker and also found significant hypersensitisation of startle responses in noise-treated specimens. Similar hypersensitivity was also observed in rodent species (hamster LVG strain and CBA/CaJ male mice) after acoustic overexposure (Chen *et al.*, 2013; Hickox and Liberman, 2014), suggesting that noise-induced sensitisation of sensory-motor responses might be a common effect among vertebrates.

We report that noise caused a significant increase in absolute PPI thresholds in larval zebrafish at 200 Hz. Prepulse inhibition is thought to be regulated by GABAergic and glycinergic interneurons in the zebrafish hindbrain that inhibit the firing activity of Mauthner cells and receive direct input from primary VIIIth nerve afferents (Weiss, Preuss and Faber, 2008). Noise exposure could potentially affect this neuronal pathway (Bhandiwad *et al.*, 2018), yet the results presented here suggest that sensitivity loss is

likely related to decreased number of the hair cells and probably cell damage, but this requires further confirmation.

Bhandiwad et al. (2018) also tested the effect of noise on auditory sensitivity in larval zebrafish at 5-7 dpf, but did not report significant noise-induced changes in absolute PPI thresholds. However, these authors used lower stimulation frequencies, 30 and 90 Hz, and, even though not significant, the average absolute thresholds were higher in the noise-treated group. Studies using the PPI methodology to test the effects of noise treatments on acoustic startle responses, have shown different results, ranging from increased response (Hickox and Liberman, 2014; Wang *et al.*, 2017) up to small or even reduced response magnitude of the acoustic startle reflex (Weiss, Preuss and Faber, 2008).

We report noise-induced hearing loss using both electrophysiology recordings and behavioural tests in a larval fish, with both experimental approaches identifying a significant increase in auditory thresholds at 200 Hz. The lack of significant changes at 100 Hz using the PPI assay is similar to the findings reported at even lower frequencies by Bhandiwad et al. (2018). The difference between the results obtained with the two audiometry systems might be related to limitations of the PPI setup that induced higher variability in acoustic startle responses and less overall inhibition ≤100 Hz, as well as to differences that are specific to the type of response measured (peripheral sensory versus behavioural).

Further research is necessary to confirm the causes of sensory loss at the physiological and behavioural levels. Altogether, the present work strongly suggests that zebrafish is a tractable model to investigate noise-induced perceptual disorders, and that underlying changes in the auditory system and behaviour related to acoustic trauma might be conserved across vertebrates. Given the extended use of zebrafish in biomedical research, including hearing studies, and considering that this species is typically raised under noisy captive conditions (Lara and Vasconcelos, 2019), our results also highlight the potential interference of the acoustic conditions on the sensory-cognitive development of this model system. Previously, we showed that larval zebrafish subject to the same noise treatment (150 dB white noise) presented heightened physiological stress, anxiety-related behaviour, and impaired spontaneous alternation behaviour (Lara and Vasconcelos, 2021). We now show that noise conditions may affect the detection of

auditory stimuli at a receptor cellular level with potential consequences for environmental sensory adaptation. Future studies should investigate this phenomenon in natural fish populations and the ecological and evolutionary implications. Further research should also rely on the technical advantages of zebrafish to investigate whether noise exposure in early ontogeny has carryover effects to subsequent life stages and transgenerational consequences.

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Competing interests

No competing interests declared.

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Figures

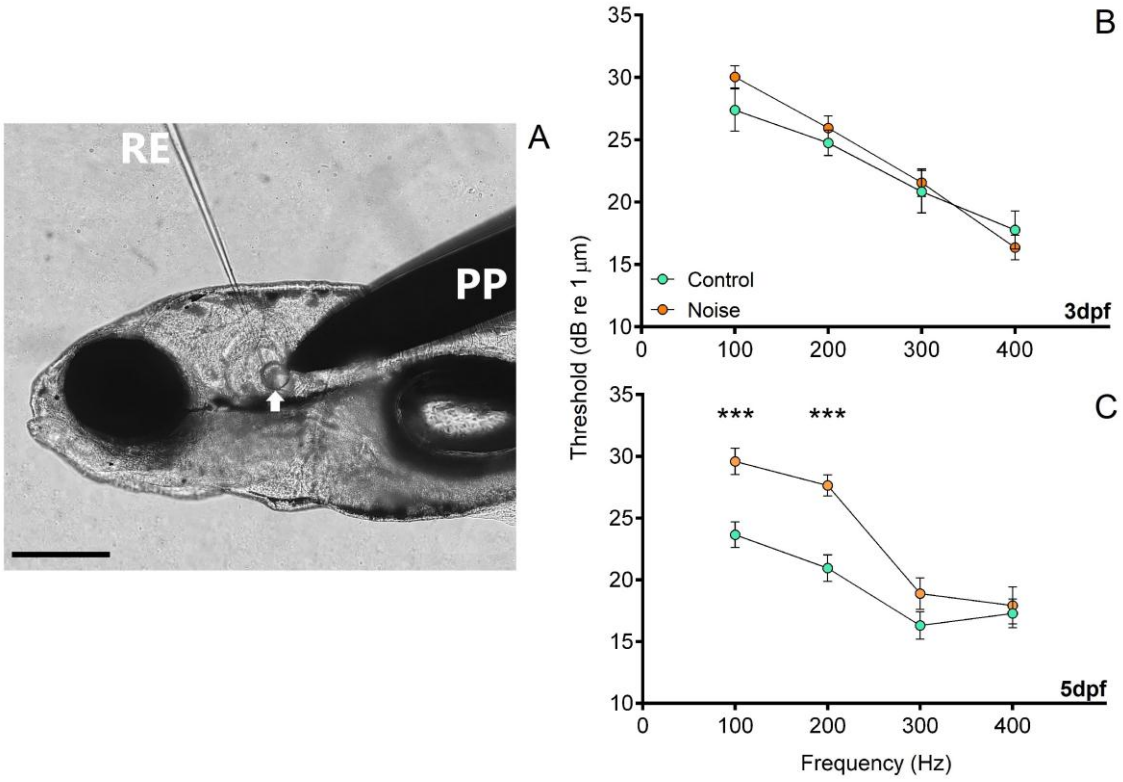

Figure 1. Microphonic potential recordings from saccular end organ of inner ear in zebrafish larvae. A) Image of a 5 days post-fertilization (dpf) zebrafish mounted in agarose for microphonic potential recordings. Image shows the recording electrode (RE) tip placed underneath the saccular otolith (arrow), and stimulus probe (PP) touching the posterior edge of the otolith. Scale bar is 200 µm. B) Microphonic thresholds obtained across the various test frequencies (Hz) from 3 dpf zebrafish - control (green, N=14) and noise-treated (orange, N=8). C) Microphonic thresholds from 5 dpf zebrafish - control (green, N=18) and noise-treated (orange, N=11), showing significant differences at 100 Hz and 100 Hz ($F_{(1, 22)}$ = 17.60, p<0.001) and 200 Hz ($F_{(1, 27)}$ = 23.84, p<0.001). Values are mean ± 95% confidence intervals. Bars are SEM, *** p<0.001

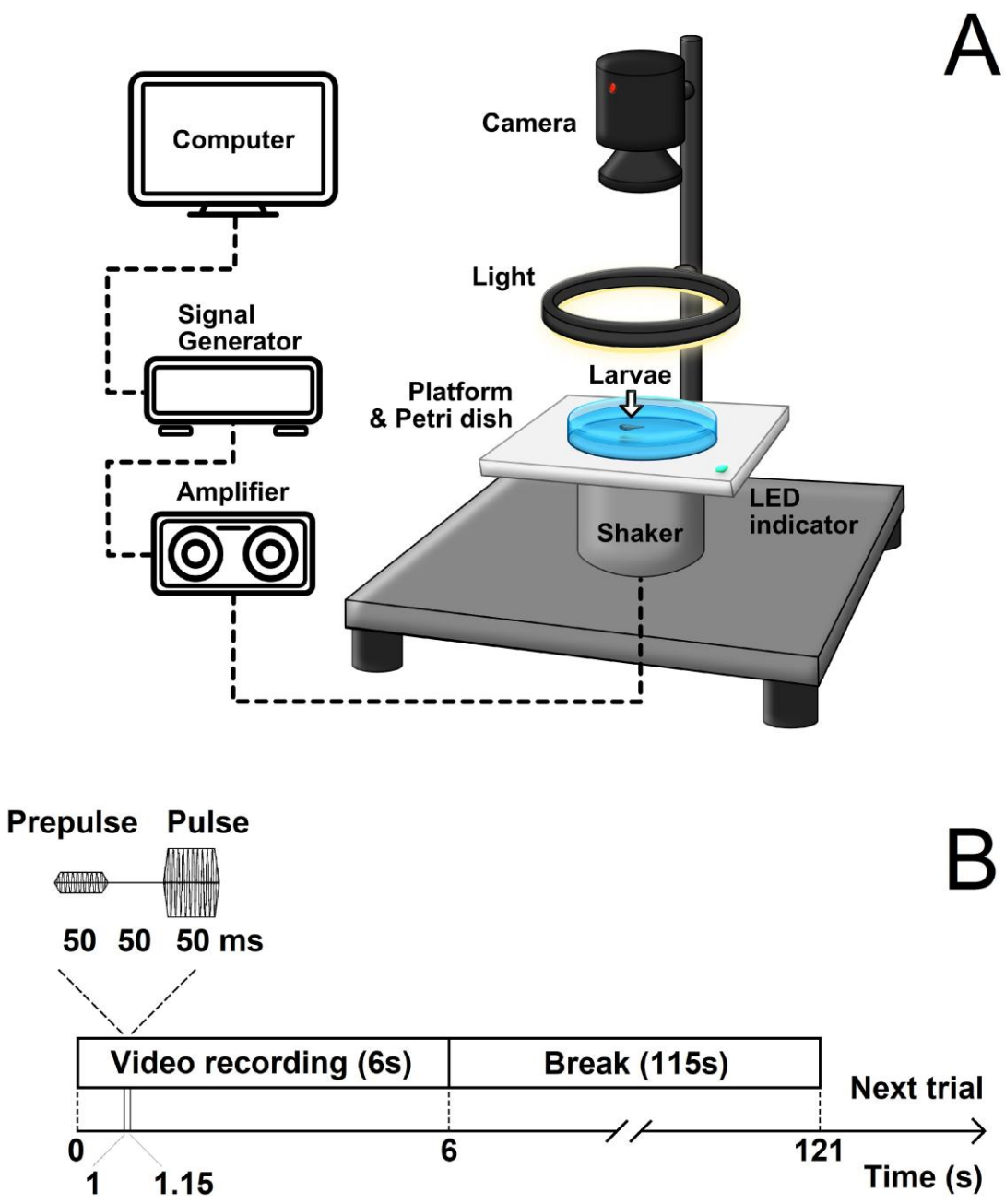

Figure 2. Prepulse inhibition assay (PPI). A) Schematic representation of the setup used to conduct the PPI recordings. B) Time presentation and recording protocol used in the PPI test.

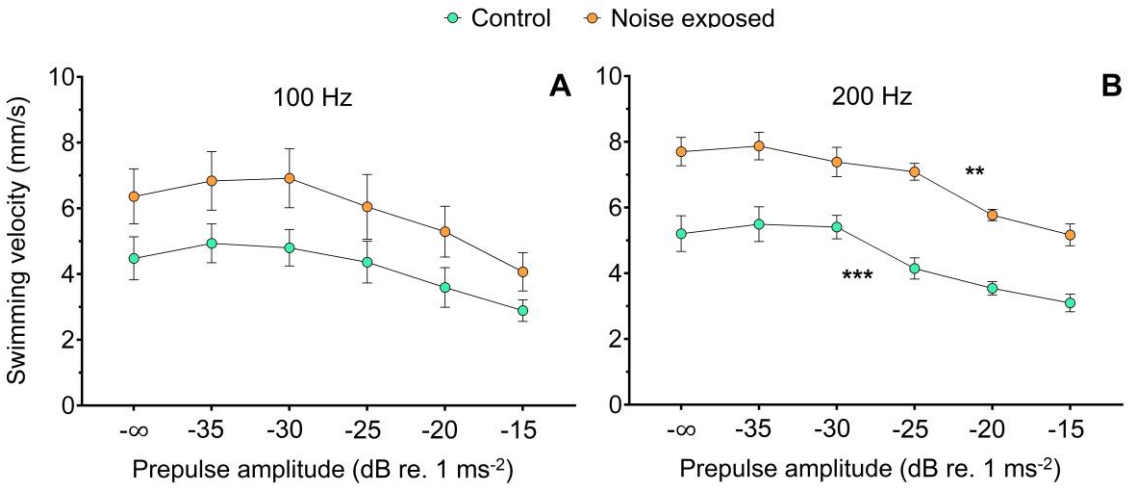

Figure 3. Prepulse inhibition assay (PPI) response curves (N=20/trial, 5 dpf) in response to 100 and 200 Hz stimulus. Data is presented as mean swimming velocity (mm/s) ± Standard Error of the Mean in response to various prepulse amplitudes for control (green) and noise-treated (orange) larvae. At 200 Hz, a significant decline in responsiveness was found in control group between -30 and -25 dB re 1 ms^{-2} ($F_{(1,38)}$ = 8.21, p=0.006), while in the noise treated group the decline was only found between -25 and -20 dB re 1 ms^{-2} ($F_{(1,38)}$ = 4.93, p<0.001). At 100 Hz, no significant changes in response thresholds were observed. Bars are SEM.** p<0.01; *** p<0.001

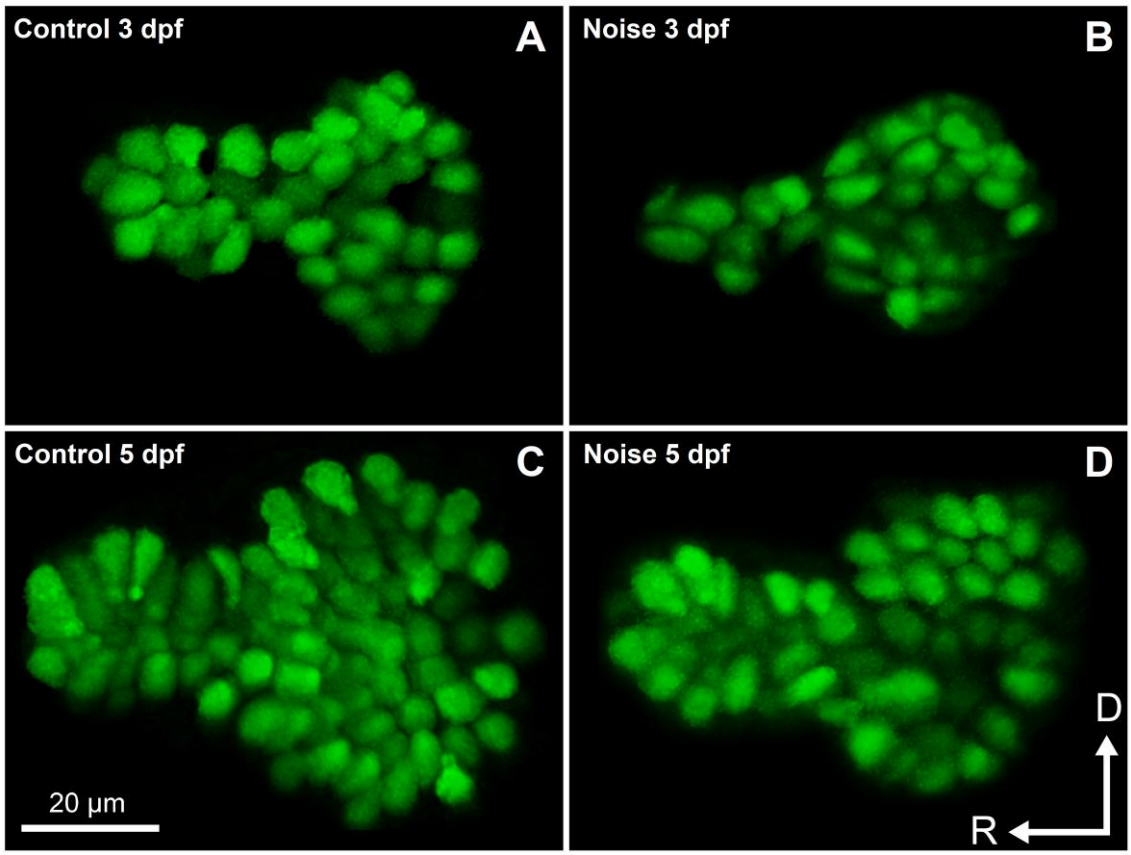

Figure 4. Representative examples of confocal images showing the whole saccular sensory epithelia from Et(krt4:GFP)sqet4 (ET4) zebrafish larvae with 3 dpf after (A) control and (B) noise conditions, and with 5 dpf after (C) control and (D) noise treatment. Green areas are green fluorescent protein (GFP)-labeled hair cell bodies. Background obscured for visual clarity. Scale bar represents 20 µm. R- Rostral, D- Dorsal.

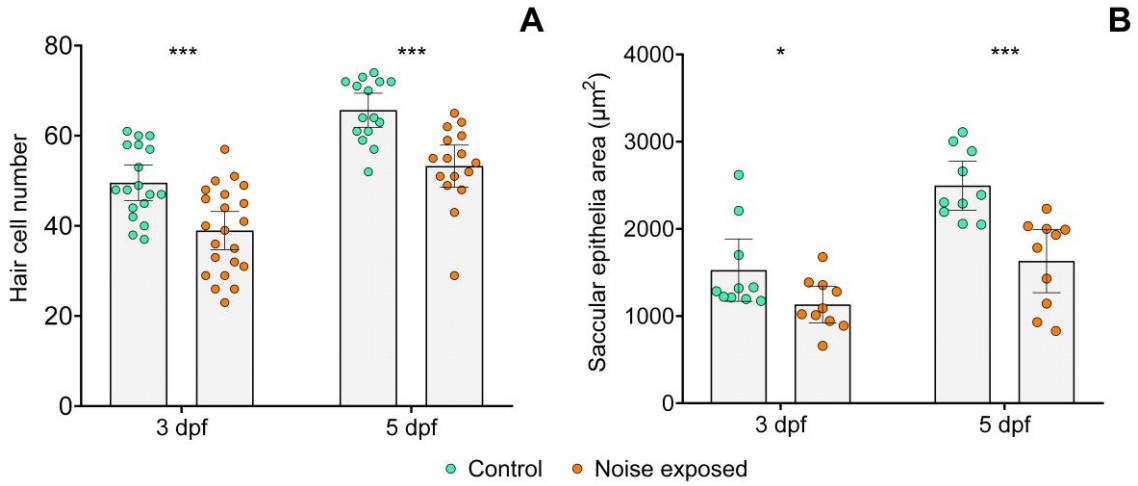

Figure 5. A) Comparison of the number of saccular hair cells between control and noise-exposed larval zebrafish at 3 dpf ($F_{(1, 39)}$=14.16, p<0.001) and 5 dpf ($F_{(1, 30)}$=19.16, p<0.001). B) Comparison of the saccular epithelia area between control and noise-exposed 3dpf ($F_{(1, 19)}$ = 4.71, p<0.05) and 5dpf ($F_{(1, 20)}$=18.19, p<0.001). Values are means ± 95% confidence intervals. * p<0.05; *** p<0.001.