聖若瑟大學
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IMPACT OF NOISE ON EARLY DEVELOPMENT AND HEARING IN ZEBRAFISH (*Danio rerio*)

Thesis presented by

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A mi familia, en especial a mis padres, que me han dado todo.

To my family, especially my parents, who gave me everything.

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ABSTRACT

Fishes are highly specialized in extracting ecologically relevant information from their diverse acoustic habitats since early developmental stages. The zebrafish (*Danio rerio*) is a valuable and well-stablished vertebrate model for investigating hearing functioning and disorders, development of the inner ear in vertebrates including humans, drug discovery, ecotoxicology assessments and behavioral research.

Although the acoustic environment is known to shape the structure and sensitivity of auditory systems, there is no information on the natural soundscape of this species. Zebrafish are typically reared in large-scale artificial housing systems, which acoustic properties and potential effects on hearing remain largely unknown. Even though elevated levels of noise are widely present in most aquatic soundscapes and to an even greater extent in artificial environments, very limited information is known on how this important environmental stressor impacts species' development and physiology, hearing capabilities and inner ear morphology, and behaviour. Considering that noise pollution is rapidly increasing in aquatic ecosystems, causing detrimental effects on survivability and growth and altering physiology and behaviour of organisms, it is of paramount importance to assess how this stressor affects wildlife, especially in early ontogeny, a critical period for development and establishment of phenotypic traits.

For this thesis I aimed to 1) characterize the soundscape of both zebrafish natural habitats and laboratory captive conditions, and discuss possible impact on auditory sensitivity. Sound recordings were conducted in five distinct zebrafish habitats (Southwest India), from quieter stagnant environments with diverse biological/abiotic sounds to louder watercourses characterized by current and moving substrate, while artificial environmental characterization was conducted on three typical zebrafish housing systems.

In order to assess the impact of noise exposure on early development, my next goal was to 2) perform a split-brood experiment to test the effects of chronic noise exposure to increasing levels (130 and 150 dB re 1 μPa, continuous white noise) and different temporal regimes (mimicking shipping activity) on larval zebrafish in regards to general development, physiological stress, and behavioural patterns.

Finally, the last objective consisted on 3) testing the effects of chronic noise exposure on auditory sensitivity measured based on inner ear saccular microphonics and acoustic-evoked startle responses (prepulse inhibition paradigm) in larval zebrafish, as well as evaluating whether sensitivity changes were paralleled by altered inner ear morphology.

Based on bioacoustics methods, my first study found that zebrafish natural soundscape varied between 98 and 126 dB re 1 lPa in sound pressure levels. Sound spectra presented most energy below 3000 Hz and quieter noise windows were found in the noisiest habitats matching the species best hearing range. Contrastingly, recordings from zebrafish housing systems revealed higher sound levels (122–143 dB) and most energy below 1000Hz with more spectral peaks, which might cause significant impact such as auditory masking or even hearing loss.

In my second research work, the acoustic treatments did not affect general development or hatching but increased noise levels led to a significant increase in mortality of larval zebrafish. The cardiac rate, yolk sac consumption and cortisol levels increased significantly with increasing noise level at both 3 and 5 dpf (days post fertilization). Variations in noise time presentations (different random noise periods similar to shipping activity) suggested that the presence of longer silent intervals is important to down-regulate physiological stress. Moreover, 5 dpf larvae exposed to 150 dB continuous noise regimes displayed increased dark avoidance in an anxiety-related dark/light preference test and displayed a significant

impairment in spontaneous alternation behaviour (SAB) a memory and sensorimotor related behaviour.

Finally, in the last thesis goal, I found that noise-exposed specimens displayed significantly lower hair cell number and saccular epithelial area. This change in sensory morphology was paralleled by a significant decrease in inner ear saccular sensitivity at lower frequencies (100 to 200 Hz) in 5 dpf larvae. Sensorimotor hearing assessment revealed a hypersensitisation effect in noise-exposed group that displayed higher startle swimming velocity, but also significant decrease in sensitivity at 200 Hz.

Altogether, this thesis provides an important ground for future research on the adaptation of zebrafish auditory system to the natural soundscapes, and highlights the importance of controlling noise conditions in captivity systems. Furthermore, results provide first evidence of noise-induced physiological stress, anxiety-driven behaviours and memory impairment in larval zebrafish larvae, showing that both noise amplitude and timing may negatively impact key physiological and behavioural endpoints in early ontogeny. The thesis also reports new findings on how acoustic stress may impact the structure and function of the inner ear in larval fish, which was followed by decreased sensitivity in sensorimotor responses to acoustic stimuli. My research highlights the importance of investigating how altered soundscapes and associated physiological and behavioural stress may affect important sensitive windows in development and impose new evolutionary challenges under a scenario of global change.

Scientific publications

- Rafael A. Lara and Raquel O. Vasconcelos. “Characterization of the natural soundscape of zebrafish and comparison with the captive noise conditions”.

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- Rafael A. Lara and Raquel O. Vasconcelos. “Impact of noise exposure on development, physiological stress and behavioural patterns in larval zebrafish”.

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- Rafael A. Lara, Flora Gordillo-Martinez, Lukas Breitzler, Peng Sun, Fangyi Chen, Andrew H. Bass, Paulo J. Fonseca, Raquel O. Vasconcelos. “Evidence of noise-induced changes in development of the inner ear and hearing sensitivity in larval zebrafish”.

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Conference abstracts

Oral presentations

- Rafael A. Lara and Raquel O. Vasconcelos. “Assessing noise-induced stress in larval zebrafish: behavioral effects and sensory loss.”.

“The 7th Annual Meeting of the Society for Bioacoustics 2020”. Organized by The Society for Bioacoustics, Japan (SFBA).

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Most Outstanding Presentation Award.

- Rafael A. Lara and Raquel O. Vasconcelos. “Impact of noise exposure on development, physiological stress and behaviour in larval zebrafish”.

“The effects of noise in aquatic life 2019, (AN2019)”. Organizing committee: Christine Erbe, Curtin University, Australia Anthony Hawkins, Loughine Ltd., UK Arthur N. Popper, University of Maryland, USA Joseph Sisneros, University of Washington, USA Frank Thomsen, DHI A/S, Denmark Paul Lepper, Loughborough University, UK.

7-12 July 2019, Den Haag, The Netherlands.

- Rafael A. Lara and Raquel O. Vasconcelos. “Impact of noise on development and physiology of larval zebrafish”.

“The 2nd Oceanoise Asia Symposium 2018”. Organizing committee: Tomonari Akamatsu, Chair (National Research Institute of Fisheries Science, FRA), Michel Andre, co-Chair (Technical University of Catalonia), Chi Fang Chen, co-Chair (National Taiwan University), Lien Shang Chou (National Taiwan University), Shane Guan (Catholic University of America), Songhai Li (Institute of Deep-Sea Science and Engineering, Chinese Academy of Sciences), Frank Thomsen (DHI), Naoya Umeda (Osaka University), Ding Wang (Institute of Hydrobiology, Chinese Academy of Sciences).

5-7 June, 2018, Hakodate Research Centre for Fisheries and Oceans, Hokkaido, Japan.

Poster presentations

- Rafael A. Lara, Lau Ieng Hou and Raquel O. Vasconcelos. “Evaluating noise-induced stress in larval zebrafish: insights into anxiety and sensory loss”.

“ASAB winter conference 2020”. Organized by Cassandra Raby, Zoological Society of London (ZSL).

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- Rafael A. Lara and Raquel O. Vasconcelos. “Noise-induced behavioural and physiological effects in larval zebrafish”.

“XVII Congresso Anual da Sociedade Portuguesa de Etologia”. Organized by The Centre of Marine Sciences – CCMAR.

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- Rafael A. Lara and Raquel O. Vasconcelos. “Zebrafish as a model for investigating noise-Induced physiological stress and hearing loss”.

“The 11th European zebrafish meeting conference 2020”, Organizing committee: Petr Bartunek, Institute of Molecular Genetics, Prague, Czech Republic, Jana Dvorakova, C-IN, Professional Congress Organiser, Czech Republic, Jana Oltova, Institute of Molecular Genetics, Prague, Czech Republic, Sarka Simova, Institute of Molecular Genetics, Prague, Czech Republic.

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- Rafael A. Lara and Raquel O. Vasconcelos. “Light preference and spontaneous alternation behaviour as measurements of noise-induced stress in larval zebrafish”

“6th Macau International Symposium on Biomedical Sciences 2019”. Organized by the University of Macao.

12-13 June 2019. University of Macao (UMAC), Macao, China.

- Rafael A. Lara and Raquel O. Vasconcelos. “Development of zebrafish under noise conditions: insights into the natural soundscape and noise effects”.

“International Bioacoustics Congress 2018 (IBAC)”. Organized by the International Bioacoustics Society.

8-13 October 2017. Gurukul Kangri Vishwavidyalaya University, Haridwar, Uttarakhand, India.

- Rafael A. Lara and Raquel O. Vasconcelos. “Early development of the auditory sense in zebrafish: effects of hormonal levels and environmental noise conditions”

“Fish and amphibian embryos as alternative models in toxicology and teratology 2017”. Organized by the Helmholtz Centre for Environmental Research

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Figure 1.1

A) Complete life cycle of the zebrafish (Danio rerio), credit: Department of Biology. Memorial University of Newfoundland[1]. B) Adult individuals from our laboratory stock and C) embryos inside their chorion prior to hatching (2 dpf).

Figure 2.1.

Map of India (top) showing the geographical location of the different zebrafish natural habitats selected for this study in Southwest India (Karnataka state): AC – Achacanni, shallow low flow stream near Hosanagara at the border of Sharavati Valley Wildlife Sanctuary; KA – Kallahalli, natural wells connected to Kaveri river; SIS – Sidi Halla (sandy), low flow stream with sandy substrate near Shivamogga; SIR – Sidi Halla (rocky), medium flow stream with rocky substrate adjacent to SIS and SH – Shringeri, faster flow main stream of Tunga river. In all study locations, the team confirmed zebrafish (*D. rerio*) occurrence (bottom right, specimens captured at SIR).

Figure 2.2.

Three representative zebrafish housing systems (HS) considered in this study to characterize noise conditions in captivity. Red dots indicate the fish tanks selected for the recordings of sound pressure level (SPL) measurements. HS1 and HS2 - standalone systems with frame-integrated filtering and pumping system – models AAB-074-AA-A and AAB-100-AA-A,

respectively (Yakos 65, Taiwan); HS3 - multi-linking system with external WTU (Water Treatment Unit) connecting three Active Blue stand-alone racks with pumps and filters configured in an external palletized CLS (Centralized Life Support, right picture) (ZebTEC, Techniplast, Italy).

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Figure 2.4.

Comparison of sound pressure levels (linear equivalent, LZeq) between A) zebrafish natural habitats ($H(4, 27) = 19.05$; $p < 0.001$), and B) laboratory housing systems ($F(2, 18) = 15174$; $p < 0.001$). Values are based on 60s averaged measurements (LZeq), 4-6 per site. Different letters indicate statistically significant differences based on pairwise post hoc comparisons. Plots show medians and 10th, 25th, 75th and 90th percentiles as boxes and whiskers. C) Comparison between mean noise levels determined for natural habitats and artificial housing conditions ($F(1, 43) = 78.88$; $p<0.001$). Plot shows means and standard deviations.

Figure 2.5.

A) Power spectral density (PSD) of noise from three typical zebrafish housing systems (HS). Sampling frequency 44.1 kHz, FFT size 16384. B) Spectrograms of representative sound recordings from HS1 (standalone system, AAB-074-AA-A, Yakos 65, Taiwan) and HS3 (multi-linking system with external pumping/filtering units, ZebTEC, Techniplast, Italy) with FFT size 2048.

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Figure 3.1.

Comparison of mean mortality rate between treatment groups (larval zebrafish up to 5 days post fertilization) exposed to A) continuous noise at different amplitudes ($F_{(2,\ 38)}$=8.71, $p<0.001$), and B) varying noise temporal patterns ($F_{(4,\ 47)}$=3.78, $p<0.01$). Control- silent conditions, CN- continuous noise at either 130 (CN_{130}) or 150 dBre 1 μPa (CN_{150}), IN-

intermittent regime with short (IN1), medium (IN2) and long noise segments (IN3). Error bars represent 95% confidence intervals. Different letters indicate statistically significant differences between specific groups based on post hoc tests.

Figure 3.2.

Comparison of mean cardiac rate and yolk sac area of larval zebrafish with 3 and 5 dpf (days post fertilization) exposed to different noise amplitudes (A and B) and temporal patterns at 150 dB re 1 μPa (C and D). Increasing noise amplitudes induced heightened cardiac rate at 3 dpf ($F_{(2, 134)}$=4.20, p<0.05) and 5 dpf ($F_{(2, 133)}$=7.17, p<0.001), as well as a decrease in yolk sac (3 dpf - $F_{(2, 136)}$=11.96, p<0.01; 5 dpf - $F_{(2, 134)}$=16.59, p<0.01). Cardiac rate was further affected by noise temporal variation at 3 dpf ($F_{(4, 146)}$=25.36, p<0.001) and 5 dpf ($F_{(4, 143)}$=15.50, p<0.001), as well as the yolk sac 3 dpf ($F_{(4, 145)}$=13.79, p<0.001) and 5 dpf ($F_{(4, 143)}$=12.19, p<0.001). Error bars represent 95% confidence intervals. Different letters indicate statistically significant differences between specific groups based on post hoc tests. (E) Negative correlation between cardiac rate and yolk sac size (R=-0.61, N=370, p<0.001) at both 3 and 5 dpf. Solid line - best-fitted line; inner dashed lines - standard error of the mean; outer dashed lines - 95% confidence interval.

Figure 3.3.

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(CN_{150}), IN- intermittent regime with short (IN1), medium (IN2) and long noise segments (IN3). Error bars represent 95% confidence intervals. Different letters indicate statistically significant differences between specific groups based on post hoc tests.

Figure 3.4.

A) Light/dark preference assay consisting of squared plastic compartments (each 40 mm width x 40 mm length x 30 mm height) divided into two equal sized areas with distinct bottom illumination (transparent/bright versus opaque/dark). The apparatus was placed on top of a LED panel (~7000 lux). Each compartment was filled with 10 ml water and a single larval zebrafish (5 dpf) was placed in the middle of the arena and recorded for 5 min. B) Choice index for larva exposed to continuous noise (150 dB re 1 µPa) versus control conditions ($U_{(2, 173)}$=5341.50, p<0.001). Choice index was calculated as: (Time in dark–Time in light)/(Time in dark + Time in light). Individual data are presented as scatter plots and bars depict mean ± 95 % confidence intervals.

Figure 3.5.

A) Spontaneous Alternation Behaviour (SAB) assay with bottom illumination to test exploratory swimming and spatial memory. The starting arms can be used alternatively (a plastic tube blocks the entrance to the opposite arm) and converge into a perpendicular main arm that leads to a choice of alternation or same side arm. These arms lead to distinct pools of 19.50 mm^2. B) Comparison of SAB in 5 dpf under continuous noise at 150 dBre 1 µPa (CN) and control conditions ($t_{(113)}$= -4.08, p<0.001). From a total of 180 tested larvae, 115 successfully showed alternation behavior (entered the opposite side pool) within the 10-

minute recording. Individual data are presented as scatter plots and bars depict mean ±95 % confidence intervals.

Figure 3.6.

A) Diagram of the acoustic treatment tank. The tank rested on top of two granite plaques separated by anti-vibratory rubber pads. Inside, a custom-made net cylinder containing zebrafish egg/larvae was suspended 7 cm above an underwater speaker (UW30, Lubel Labs, Ohio, USA) that rested on top of a polyurethane sponge. B) Oscillogram of sound files used for playbacks. Control- silent conditions, CN- continuous noise at either 130 (CN_{130}) or 150 dB re 1 μPa (CN_{150}), IN- intermittent regime with random short noise segments (IN1): 5-12 sec duration spaced by silent intervals of 1-120 sec (total noise exposure of c. 15%); medium noise segments (IN2): 30-60 sec interspaced by 1-10 min silence (similar noise exposure to IN1); and long noise segments (IN3) of 15 min separated by 15 min silent periods (about 50 % overall noise).

Figure 4.1.

Confocal images showing hair cell bodies expressing green fluorescent protein obtained for comparison between inner ear saccular epitheliums of A) 3dpf control B) 3dpf noise exposed C) 5dpf control and D) 5dpf noise-exposed Et(krt4:GFP)sqet4 zebrafish transgenic individuals. Saccular hair cell bundle quantification was conducted by digitally marking cell bodies after which the epithelial area was also quantified, both measurements were conducted using DanioScope (Noldus Information Technology, Wageningen, Netherlands). Scale bar = 20μm.

Figure 4.2.

A) Schematic representation of the setup used to conduct the prepulse inhibition paradigm experimentation. B) Time distribution and presentation scheme of the acoustic stimulus used in the prepulse inhibition test.

Figure 4.3.

A) Image of a 5 dpf AB wild-type zebrafish embryo embedded in agarose and ready for microphonic potential recording. Image shows the recording electrode tip (RE), the stimulus probe (PP) and saccular otolith (arrow). B) Microphonic thresholds (dB) versus stimulus frequencies of 3 dpf AB wild-type zebrafish, control (green) N=14 and noise (orange) N=8. C) Microphonic thresholds (dB) versus stimulus frequencies of 5 dpf AB wild-type zebrafish, control (green) N=18 and noise (orange) N=11, showing significant differences in hearing sensitivity $F_{(4, 48)} = 14.61$, $p<0.001$. At 100 Hz (one-way ANOVA $F_{(1,23)} = 17.60$, $p<0.001$) and 200 Hz (one-way ANOVA $F_{(1,28)} = 23.84$, $p<0.001$). Error bars are 95% CI.

Figure 4.4.

PPI behavioural response curves of 5 dpf.AB wild-type zebrafish larvae in response to 100, 200 and 400Hz pulse stimulus at 240 dB re 1 m/s2. Data is presented as average swimming velocity (mm/s) vs prepulse amplitude (dB re 1 m/s^2), control (green) and noise-treated (orange). 100 Hz: dotted line; 200 Hz: dashed line; 400 Hz: continuous line. 100 Hz - $t_{(38)} = 6.55$, $p<0.001$; 200 Hz - $t_{(38)} = 8.62$, $p<0.001$ and 400 Hz - $t_{(80)} = 9.23$, $p<0.001$. At 200 Hz between 140 and 150 dB control group showed a decrease in hearing $F_{(1, 6)} = 7.46$, $p<0.05$,

which contrasted with the noise-treated specimens that showed this reduction only between 150 and 160 dB $F_{(1, 6)} = 39.71$, $p<0.001$. Error bars are SEM.

Figure 4.5.

A) Comparison between number of saccular hair cells in control and noise-exposed individuals of larval zebrafish displaying significant differences in total hair cell count at both 3dpf (one-way ANOVA $F_{(1, 39)}=14.16$, $p<0.001$) and 5dpf (one-way ANOVA $F_{(1, 30)}=19.16$, $p<0.001$). B) Comparison between area size (µm2) of saccular epitheliums in control and noise-exposed individuals displaying significant differences in area size at 3dpf (one-way ANOVA $F_{(1, 20)}=4.61$, $p<0.05$) and 5dpf (one-way ANOVA $F_{(1, 20)}=18.19$, $p<0.001$). Error bars represent 95% confidence intervals. Asterisks indicate statistically significant differences between specific groups.

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Table 1.1. General characterization of the sampling sites in Karnataka, Southwest India. (1) Recording locations were the same as selected by Arunachalam et al (2013). (2) Vegetation cover was present as dense (AC) and sparse (SIS) hanging canopy, and riparian riverside plants (AC, SIS, SIR), while it was absent at SH and KA. (3) Water flow measured in a prior study in the same recording locations during dry season; (-) designates missing values.

Table 1.2. Noise levels (LZeq) determined in five zebrafish natural habitats (Karnataka, Southwest India) and in three typical laboratory-housing systems (HS). Values are based on 4-6 averaged readings based on 60 s and are given in dB re 1 µPa. Natural habitats: SH - Shringeri, KA - Kallahalli, AC - Achacanni, SIS - Sidi Halla sandy and SIR - Sidi Halla rocky. For each HS three recording points were considered at various distances to the main noise source (water pump and filtering system): 1) 30-40 cm, 2) 140-150 cm, 3) 240-250 cm. CV - coefficient of variation.

Glossary of acronyms and abbreviations

Acoustic terminology

Lzeq - Leq equivalent continuous sound level, Z-weighted

dB – decibels

Hz – hertz

kHz - kilohertz

SPL – sound pressure levels

PSD – power spectral density

RMS – root mean square

FFT – fast Fourier transformation

dB re 1µPa – decibels relative to one micro pascal

Pa - pascal

Statistical abbreviations

CV - coefficient of variation

ANOVA – analysis of variance

SEM – Stannard error of the mean

95% CI – 95% confidence interval

SD – standard deviation

LSD – least significant difference

Methodology abbreviations

dpf – days post fertilization

hpf – hours post fertilization

HS – housing system

SH - shringeri

KA - kalahalli

AC – achacanni falls

SIS – sidihalla sandy

SIR – sidihalla rocky

C - control

CN – continuous noise

CN130 – continuous noise at 130dB

CN150 - continuous noise at 150dB

IN1 – intermittent noise 1

IN2 – intermittent noise 2

IN3 – intermittent noise 3

SAB – spontaneous alternation behaviour

SMP - saccular microphonic potentials

TTS- temporary threshold shifts

PTS – permanent threshold shifts

μm – micron

μPa - micropascal

MS-222 - tricaine methanesulfonate

PBS - phosphate buffered saline root

ms - milliseconds

cm – centimetre

CHAPTER 1 – GENERAL INTRODUCTION

UNDERWATER ACOUSTICS

Fundamentals of sound and soundscape

"One man's meat is another man's poison" and in the bioacoustics field this is highly relevant as what it is sound to one organism might be noise to another, therefore it is important to differentiate between these two concepts.

Sound is defined in ANSI/ASA S1.1-2013 R2013, 2.01[2] as: (1) oscillation in pressure, particle displacement, particle velocity, etc., propagated in a medium with internal forces (e.g., elastic or viscous), or the superposition of such propagated oscillation; or (2) auditory sensation evoked by the oscillation described in (1). Therefore, sound can be treated as a wave motion in air or any other elastic media (stimulus) or as an excitation of the hearing system mechanisms that result in the perception of sound (sensation).

On the other hand, noise is defined in ANSI/ASA S1.1-2013 R2013, 2.32 as: (1) any undesired or unwanted disturbance within a sensitive frequency band such as the unwanted electric waves in a transmission channel or device, or (2) erratic, intermittent or statistically random oscillation. While the difference between sound and noise is subject to the "ear of the beholder", sound is a precisely defined physical phenomenon with specific properties. Sound is defined and travels as a mechanical wave through mediums (fluids, gas and solids) by oscillation of its particles or by periodical variations in pressure. These waves can be longitudinal primary P-wave (fluid, gas and solid) or transverse secondary S-wave (solids). The velocity of sound propagation primarily depends on the density and elasticity of the medium, being five times higher in water than in air (1518 m/s and 343 m/s, respectively)[3].

The main acoustic features that are crucial to characterize sound waves are frequency, wavelength and amplitude. The frequency of a sound (measured in hertz – Hz) represents the number of cycles of a sound wave per unit of time (second). The frequency, commonly called sound pitch, increases as the number of cycles per second increase. In contrast, the wavelength is the spatial period of a periodic wave, for instance the distance over which the wave's cycle repeats, and it is inversely proportional to frequency. Finally, the amplitude of a sound wave, typically expressed in decibels (dB), is the measure of the height of the wave or the amount of maximum displacement of vibrating particles of the medium from their mean position when the sound is produced [4].

Propagation of these waves is affected by reflections, i.e. change in direction of a wave after interaction between two different media so that the wave returns to the origin medium; refraction, that is change in direction of a wave due to a change in its speed; transmission - movement of a sound wave through and between materials and mediums; and absorption, consisting on conversion of acoustic energy into thermal energy [5]. All these phenomena typically occur in the presence of physical barriers, which are common characteristics of natural and artificial environments.

Sound represents a very effective communication form, especially underwater, due to its fast and distant propagation. Moreover, soundscape navigation and perception based on acoustic cues is especially relevant in situations when visibility is impaired, which typically occurs in aquatic environments [6–8], this is, ambient sound conveys important information about habitat properties and quality. A few studies have already resort to soundscape measurements and characterization in order to obtain estimates of biodiversity in terrestrial ecosystems[9,10]. More recently, this methodology has also been used for underwater habitats [11–13], leading to consider soundscapes as a monitoring tool for ecosystems health. Some examples include studies by Rossi et al 2017 who reported a significant decrease in

environmental acoustically richness in habitats that suffered a regime shifts to less structural complexity [13], Bertucci et al 2015 found that an increase in coral reef coverage brought an associated increase in sound levels and variability and species diversity[14,15]. Finally, the ambient sounds of coral reef habitats have been already shown to act as an acoustic signal that attracts different reef species larvae and promotes their settlement behaviour[16–19].

As previously stated, sounds can be detected and classified by both their acoustic pressure component and by the associated particle motion, which, as we mentioned, can be quantified in terms of particle velocity, displacement or acceleration. Particle acceleration is based on vectorial quantities and therefore it carries directional information which is highly useful for acoustic localization and tracking[20]. To subtract the particle motion from the acoustic pressure component requires an accelerometer which is a set of four hydrophones assembled in the right spacing conformation and calibrated at the frequencies of interest which conducts measurements of the pressure gradient in the three orthogonal directions (X, Y, Z). These measurements of the particle motion component are of significant interest to water bioacousticians studying animals such as many species of fish that are particularly or only sensitive to particle motion[21]. In summary, there are different methodologies and sensors adopted for sound detection and characterization, including but not limited to: 1) measurements of acoustic pressure using pressure-sensitive sensors such as hydrophones; and 2) recordings of particle displacement of a fluid medium, which can be assessed as displacement, velocity and/or acceleration, using triaxial accelerometers to quantify motions in the 3D axis [22].

According to the International Organization for Standardization (ISO 12913-1:2014)[23], the soundscape or acoustic environment is the combination of all the acoustic resources, natural and artificial, within any given area as modified by the environment. However, an important distinction is to separate the broader acoustic environment from the term

soundscape, which is the component of the acoustic environment that can be perceived by organisms. The concept of soundscape refers to both the natural acoustic environment, consisting of all of the non-human biological sound sources, including animal vocalizations (biophony), and the sounds generated by non-biological natural elements, such as wind, rain, water currents (geophony), plus sounds created by human activities (anthropophony) such as industrial processes, exploration of natural resources, traffic, construction work, among others[24–27].

Soundscapes possess four measurable properties: acoustic composition, temporal patterns, spatial variability and acoustic interactions. Acoustic composition is the frequency and amplitude of all sounds occurring at any given time and location; temporal patterns refer to the periodicity in occurrence of the acoustic events; spatial variability results from the heterogeneity of the biophysical properties of the environment and their specific spatial distribution; and acoustic interactions are the diverse array of natural and human-induced interactions that occur between biophony, geophony, and anthrophony, leading to the overall soundscape.

Interestingly, the term soundscape also includes the listener's perception of sounds meaning that soundscape dynamics can drive the evolution of biological systems thus making anthropogenically induced alteration in soundscapes a key threat to biodiversity and a significant environmental change motor that can lead to associated fitness costs both directly or indirectly. Studies have found experimental evidence for negative effects of anthropogenic noise in both invertebrates and vertebrates at an individual scale in development, physiology, fitness, etc.[28–31], but also changes at community level like, altered vocal behaviour to mitigate masking[32,33], reduced abundance in noisy habitats[34,35], changes in antipredator behaviour[36,37], foraging effectiveness[38,39], parental care[40] and population settlement[16,41].

Chronic and frequent noise interferes with animals' abilities to detect important sounds, while intermittent and unpredictable noise is often perceived as a threat. For instance, the interactions between species and their competition for acoustic space influences mate selection and predator prey interactions [42], and have the potential to affect the population dynamics and community composition as reviewed by Stansfeld et al. 2003, Kunc et al. 2016 and Shannon et al. 2016 [43–45]. The disruption of the acoustic environment or soundscape that results in the alteration of the biology and natural behaviour of its inhabitants is defined as noise pollution. Anthropogenically altered soundscapes represent a key threat to biodiversity and a significant environmental driver. However, newest discoveries in noise pollution research often focus on single aspects of the phenomena (behaviour, physiology, local ecosystems, or certain taxa), thus creating a pressing need to get a holistic depiction of the effects of anthropogenic noise in aquatic ecosystems.

NOISE POLLUTION

Noise pollution as an environmental problem

From the anthropocentric perspective, noise pollution has been issued as a pervasive agent since at least the 6th century BC with the first known noise ordinance by the council of the Greek colony of Sybaris ruling that potters, tinsmiths, and other tradesmen must live outside the city walls because of the noise generated during their commercial activities. Surprisingly, it would not be until 1713, when an Italian physician ascribed the cause of the deafness of Venetian coppersmiths to the noise present during trading activities. This was the first report relating noise exposure to hearing loss[46].

The increasing levels of noise pollution are creating a serious hazard to the overall physiology and health of animals including humans [47–51]. Exposure to noise pollution is

known to affect hearing function leading to Noise-Induced Hearing Loss (NIHL) [52]. NIHL is highly dependent on the duration and amplitude of the noise exposure, whether the experience is a single traumatic event or prolonged over time (chronic) and under which time regime. This condition is commonly associated with the incidence of tinnitus, which is the perception of a sound in the absence of an external source. Psychological effects associated include depression, anxiety, worsening of psychiatric disorders, personality changes, violent behaviour and increased self-isolation [47,48,53].

Moreover, noise exposure causes a myriad of non-auditory effects including stress and anxiety disorders, annoyance, sleep disturbance, cardiovascular problems (hypertension, vasoconstriction and ischemic heart disease), endocrine disruption, and increased incidence of diabetes[47,48,50,51,54,55]. Other effects include heightened levels of stress, aggression and other anti-social behaviours [53,56], increased incidence of diabetes, changes in the immune system, teratogenicity and birth defects [48,51,57–60]. Furthermore, an increasing amount of evidence is showing that acoustic stress may lead to DNA damage, changes in gene expression and alter cellular processes related to neural, developmental, immunological and physiological functioning [45,61].

Investigators are only beginning to identify the negative implications of noise pollution on the ecosystems and biodiversity. Noise pollution is known to affect the physiology and behaviour in several taxa, including birds [62–64], mammals [65–68], amphibians [69–71] and fishes [72–76].

Nowadays, and according to the findings of the World Health Organization [77], noise pollution is the second largest environmental cause of health problems, just after air pollution. For instance, in Europe, environmental noise is estimated to cause 12.000 premature deaths and to contribute to 48.000 new cases of heart diseases per year. It is estimated that 22

million people suffer chronic high annoyance and 6.5 million people suffer chronic sleep disturbance. More than one billion people are at risk of NIHL due to recreational and occupational activities worldwide [77]. Anthropogenic noise is an increasingly pervasive form of environmental pollution increasing in both industrialized nations and developing world regions [53,78–80].

Noise sources in aquatic habitats

Sound in aquatic environments propagates five times faster than in air and is not attenuated as quickly as other signals making it an important carrier of information for communication and orientation and particularly suitable to extract information from distant sources [81]. In aquatic natural habitats, species thrive in acoustically rich and diverse environments that vary greatly in spectral composition and temporal patterns due to the interaction between abiotic (e.g. wind, waves, rain) and biotic (e.g. animal vocalizations, motion and feeding sounds) sources [82,83]. Studies investigating the acoustic characteristics of various aquatic habitats often focus on marine environments [16,84–87], specifically reefs [17,88–90]. In contrast, very little data is available on ambient noise spectra in freshwater habitats (e.g. lakes, ponds, rivers, streams) [91–93]. Characterization of natural freshwater soundscapes is of main importance, not only because information regarding spectral characterization is sparse but also because considerable differences in acoustic features have been observed across different soundscapes [94,95]. Contrastingly, studies characterizing artificial or anthropogenically noise-polluted environments are more abundant [31,90,96–98] and demonstrate that anthropogenic noise represents a soundscape interaction wherein increased anthropophony interferes with biophonic processes[69,99–101].

Anthropogenic noise in aquatic habitats is typically generated by construction work (drilling, dredging, explosions, pile driving), seismic surveys, sonar emissions,, traffic (shipping), recreational watercrafts, and other human activities [102,103]. These anthropogenic sounds differ greatly from the natural soundscape in regards to spectral composition and temporal features, occurring in either continuous or intermittent regular/random regimes.

Aquatic animals are also chronically exposed to elevated noise in aquaculture and other housing systems. In holding tanks, a great amount of high-frequency underwater noise is produced mainly by oscillating and collapsing air bubbles, electric generators, tractors and harvesters, as well as electric air/water and filter pumps, whereas low-frequency noise is mainly generated by water circulation, ground vibrations, aquarium wall vibrations and electrical pumps [29,95,104,105]. However, only few studies investigated the acoustic features of housing systems and their potential impact on animal reproduction and growth fish [29,106,107]. More attention should be given to the sound properties of artificial housing systems and their contrast to the species natural soundscapes in order to promote welfare.

Underwater noise pollution worldwide: a global environmental threat

World Health Organization (2011)[77] has addressed anthropogenic noise as a global pollutant and recognized it as one of the major disruptors that threaten the natural balance of both aquatic and terrestrial ecosystems. Only in 2018, the United Nations highlighted the need for further research and international cooperation to assess and address the potential detrimental effects of anthropogenic underwater noise pollution in all aquatic systems, as there seems to be an association between economic and human growth and rising underwater noise levels [108].

Long-term measurements from some marine areas have shown that low frequency noise levels in ocean basins have increased by at least 10 dB in a 30 year span [109,110].

However, the extent to which these trends apply to shallower continental shelf seas and freshwater environments where human activity is concentrated remains unclear due to the scarce availability of long-term datasets for these areas.

Sources of anthropogenic noise can be categorized as impulsive or continuous and each type has been linked to a particular set of detrimental effects on aquatic fauna[111]. Impulsive noise consists of brief, discrete sounds with an abrupt onset (e.g., detonations, pile driving, seismic air guns, etc.). These sources can elicit immediate acute effects on animals including but not limited to permanent or temporary auditory damage[67,112,113], impaired behaviour[31,114,115], physiological stress[30,116], physical damage[117–119] and even death[29,120]. Even though such noise sources are linked to activities that are typically subject to a legal regulatory process and license approval-controlled procedures, the reality is that, to date, the adverse effects derived from such activities remain.

Shipping and vessel traffic are the primary sources of continuous noise, which have been associated with acoustic masking of biologically relevant cues[72,121,122], behavioural disruption, heightened physiological stress and developmental impairment in aquatic organisms[36,116,123,124]. Even though continuous noise sources are typically less intense than impulsive sources, these activities extend indefinitely in time, transcending international borders. The management of traffic underwater noise requires a coordinated international effort that in most cases is highly insufficient. Policy makers are beginning to develop approaches to assess and mitigate ecological risks associated with underwater noise through legislative frameworks; however, the lack of data on current and historic noise levels remain a major constraint that limits the ability of regulators to assess the potential impact of prospective activities [125–127]. Noise pollution mitigation strategies are considered by legislative frameworks worldwide such as the US National Environment Policy Act, EU Marine Strategy Framework Directive (MSFD; Directive 2008/56/EC) and Law of the

People's Republic of China on Prevention and Control of Environmental Noise Pollution (Order No. 77, 1997).

IMPACT OF UNDERWATER NOISE ON FISH

Effects on development, physiology, and behaviour

The effects of noise on aquatic organisms depend on the characteristics of the acoustic disturbance (amplitude, spectral composition, duration, duty cycle, etc), the physical properties o the environment affecting sound propagation, and the biology and behaviour of the exposed animals. Originally, according to Verboom et al. 2005[128], the effects of noise can be issued attending to the concept "zone of influence", which varies with the distance to the sound source[129,130]. In this prediction model, five different potential zone/response can be identified:

1 - Acoustic injury: direct physical injury resulting in mortality that occur from exposure to sufficiently elevated levels of impulse sound events, which are characterized by rapid overpressure in water (e.g., pile driving, air gun explosions) [119,131,132]. High sound levels are typically limited to short distances from the sound source. Physiological injure may result indirectly from behavioural alterations (e.g., stranding and decompression sickness).

2 - Auditory physiological effects: Temporary Threshold Shifts (TTS)[133,134], hearing loss that is recovered within a given time window that varies from minutes in mammals to weeks in fish. Permanent Threshold Shifts (PTS)[131,133], where hearing loss does not recover over time. It is hypothesized that animals subject to repeated TTS could undergo PTS.

3 - Avoidance: a behaviour elicited in the presence of an acoustic event where the animal actively escapes (avoidance reflex) and moves away from the sound source[135–137].

4 - Behavioural disturbance: behavioural changes in response to underwater sound, which typically show great variability between individuals. To date there is no consensus in the scientific community on the proper sound exposure metric for assessing behavioural reactions to noise as an individual response depends on the context in which the stimulus is perceived (age, sex, behavioural state, time of exposure, proximity, etc.). Ranges over which behavioural response has been observed can be quite large, for aquatic species like marine mammals sometimes of tens of km [66,67,138].

5 - Informational and energetic masking: masking occurs when noise impedes the ability of an animal to perceive or interpret a biologically relevant signal. For this to occur the sound must be loud enough, have similar frequency domain as the signal and happen at the same time. Both anthropogenic and natural sounds can impair the individual's ability to effectively communicate, detect predators, preys, and conspecifics and to navigate through their environment (spatial orientation) [139,140]. Masking in marine mammals has been extensively investigated, in comparison, masking fish remains scarcely studied [72,74,121].

As previously stated, anthropogenic underwater noise is now recognized as a worldwide problem and as a pervasive pollutant with the potential to impact aquatic ecosystems on a global scale. Animals exposed to chronic (continuous) and/or acute (transient) noise may present heightened reduced growth rate, heightened physiological stress and metabolic rates, impaired hearing and balance, altered reproductive maturation and performance, as well as reduced foraging efficiency, cognitive responses, and predator avoidance [28,29,145–147,30,39,102,106,141–144]. Moreover, overexposure to acoustic stress may also result in reduced immune system responses, increased oxidative stress levels and DNA damage [59,148–151].

Some case studies hypothesized that noise exposure while appearing to be non-injurious due to the occurrence of habituation and desensitization, may have cumulative effects that can be identified in long term persistence scenarios and at the population level[152–154]. For instance Holmes et al 2017 [153] found that populations of damselfish exposed to boat noise displayed immediate changes in behaviour and mortality rapidly followed by a desensitization effect, suggesting that this may allow for long-term exposure to noisy environments after survival to the initial disruption.

Fishes in particular, represent the largest group of extant vertebrates that are specialized in extracting ecologically relevant information from their highly diverse acoustic habitats [8,155]. The presence of anthropogenic noise in their auditory scene thus poses unprecedented threats with diverse consequences. Increasing evidence are showing that noise exposure may lead to increased mortality due to physiological effects [29] and reduced predator avoidance [146], heightened metabolic rates [156], altered endocrine and physiological stress responses [157], impaired immune system [59,158], reduced foraging efficiency [2,39], impaired cognition [159], altered swimming behaviour [160–162], and hearing impairment [102,113,143,145].

Effects on the auditory system: inner ear and sound perception

Sound detection by fishes depends on the source level, propagation loss, background masking noise and the hearing threshold of the receiver [163,164]. Fishes can detect both acoustic components, particle acceleration and sound pressure. Sound pressure or acoustic pressure level is the localized pressure deviation from the ambient environmental pressure, caused by a sound wave while the particle component is defined as the physical velocity, displacement and/or acceleration of a given part of a fluid as it moves back and forth due to the interaction of the sound wave as it travels through the medium [165]. Only certain species that possess

accessory morphological specializations hearing such as Weberian ossicles are able to detect changes in sound pressure. These bone structures that link the swim bladder and inner ear and serve to enhance hearing by conducting pressure changes from external sound waves from the swim bladder to the inner ear. Vibrations in the swim bladder walls causes air pressure fluctuations that are transmitted to the inner ear and, thus, sensitivity is greatly enhanced in the frequency and amplitude domain. Sounds can be thus detected and encoded in regards to their temporal patterns, amplitude and spectral content, which are known to be represented in the auditory system of fishes [166].

The inner ear of fish serves a dual function for vestibular/spatial orientation and sound detection. The main peripheral auditory structures of these animals are the three otoliths endorgans with different spatial orientations (utricle, saccule, and lagena), which are present in the inner ear and are connected by semicircular canals and filled with endolymph [167,168]. Each of these endorgans contain a sensory epithelia (macula), comprised by mechanoreceptor sensory hair cells that function like mechanotransducers of acoustic/vestibular information to their neural afferents, and a calcified otolith that attaches to the epithelium via a gelatinous otolithic membrane [168,169]. The otolithic endorgans function as biological accelerometers and are sensitive to particle motion due to the difference in inertia between the sensory macula and the associated otolith [167].

Regarding the effects of noise on the hearing system, exposure to high amplitude sounds can cause a permanent or temporary hearing loss. Some anthropogenic sounds may cause temporary threshold shift (TTS), depending on a number of variables including the frequency and intensity of the sound, duration of exposure, distance to the source, etc. In fishes the physiological basis for TTS involve reversible damage to the hair cells of the inner ear as they are capable or regenerating and recover from acoustic trauma. However, fishes are still susceptible to TTS [170]. For instance, rainbow trout (*Oncorhynchus mykiss*) displayed 20

dB temporal threshold shifts after exposure to loud noise (194 dB re 1 μ Pa)[170], fathead minnows (*Pimephales promelas*) experienced same effects on thresholds after exposure to playback of boat noise (142 dB re 1 μ Pa)[113]. Although hearing thresholds returned to normal in both studies, the time required for recovery varied depending on the frequency of the sound and the duration of the exposure[113].

Additionally, acoustic trauma has been previously reported in several studies and in several fish species exposed to high amplitude sound events. This physical damage into the hair cell bundle appear as morphological anomalies (splayed bundles) and/or causing the hair cell to undergo the apoptotic process[134,171]. For instance, in a study goldfish exposed to 170 (dB re 1 μ Pa) displayed a significant hair cells loss that was accompanied by a temporal shift in hearing thresholds. However, animals recovered after 7 days post exposure and hair cells were replaced[171]. In a similar study, zebrafish exposed for 24h to white noise at various amplitudes (130, 140 and 150 dB re 1 mPa) revealed noise level dependent TTS of up to 33 dB accompanied by significant hair cells loss at the highest noise treatment. Animals, recover within 7 days (130 and 140 dB exposure) and 14 days for fish exposed to 150 dB[134] highlighting the importance of sound properties in assessing the effects on hearing.

Finally, masking occurs when noise levels interfere with the ability of the receptor to hear a sound of its interest thus effectively "hiding" vital information. This effect occurs when the noise is present at frequencies similar to those of biological relevance such as mating or territorial calls. The magnitude of the masking effect is given by the length of the time that the noise is present, amplitude level and frequency domain, for instance, low frequency anthropogenic noise below 1 kHz overlaps with the best hearing range and sounds produced by most fish species. Such is the case study of the Lusitanian toadfish's hearing which is significantly masked in the presence of ferryboat noise at multiple frequencies[33] or the significant reduction in settlement success due to the masking of relevant acoustic cues in

reef natural habitats that most coral reef fishes use to find suitable habitats which ultimately will present effects at population level[16,19,41,172].

Problems with detection, recognition and environmental navigation due to the presence of anthropogenic noise could therefore have impacts on reproductive success and fitness[173]

Implications for individuals, populations and ecosystems

Undoubtedly, the main objective of conservation policies is to protect entire ecosystems, however, these can only be protected if we understand the role of their components and the factors affecting them [174]. Despite some studies investigating on the effects of noise in individuals from specific species, reality is that there is still a lack of experimental data regarding how these impacts may translate into community and population fitness and therefore we possess limited understanding on how noise drives ecosystems dynamic. For instance, some studies have illustrated how increasing noise levels can affect predator-prey interactions [2,36,175] which directly affects the likelihood of survival, however, whether these effects translate into populations and how it affects its dynamic remains to be known. Other studies have shown that noise and other stressors can impair the ability of the genome to buffer developmental processes and protect them against environmental disturbances in fish and other species and impair early ontogeny [61,176–178]. Such early ontogeny impairments will most likely result in fitness costs and may have an impact on populations dynamic and survivability with potential implications for community structure, population resilience and dynamic and recruitment, however, the reach of this early effects remain to be studied too.

Noise pollution in a changing world: interaction between multiple environmental stressors

In addition to the increasing levels of environmental stressors, such as noise pollution, it is important to mention that these pervasive agents rarely act individually [179]. Organisms in both terrestrial and aquatic environments are simultaneously exposed to different environmental stressors and the results of multiple interactions between them. These interactions may present synergic effects heightening or creating novel negative effects. For example, it has been addressed that rising global temperatures and water acidification due to increased levels of pollutants and greenhouse emissions can act as enhancers of the negative impacts of noise pollution by altering the organoleptic properties of the water masses, which ultimately affect the sound propagation[180] and therefore impacting not only fish physiology but also acoustic reception and communication[75]. For instance, acidic waters present a lower acoustic absorption rate mostly at low frequencies, turning the environment into a "noisier" soundscape. On the other hand, the speed of sound in water increases with increasing water temperature, salinity and depth [179,180]. Once again, it is important to stress the lack of data regarding potential interactions between different factors that characterize global change. However, this is only possible once we have solid baseline information on the impact of each stressor separately. Future research would benefit from focusing on the interaction between different stressors, especially under the changing environmental conditions like the ones in effect nowadays at a global scale.

ZEBRAFISH AS A MODEL IN DEVELOPMENT, HEARING AND ECOTOXICOLOGY STUDIES

Fishes are excellent vertebrate models to address questions regarding physiological adaptations to environmental factors, as they evolved in widely diverse habitats and possess many specialized morphological features that convey improved adaptation to particular environments (Fay 2009).

The zebrafish, *Danio rerio* (Hamilton-Buchanan, 1822), is a freshwater teleost fish from the Cyprinidae family (Fig. 1.1). This species is native to South Asia where it is found in North Eastern and South Western India, Nepal, Bangladesh, South Pakistan, Northern Myanmar and Bhutan, inhabiting a variety of aquatic freshwater systems, from moderately flowing rivers to stagnant shallow clear water streams, canals, ditches, ponds and rice paddies [181,182].

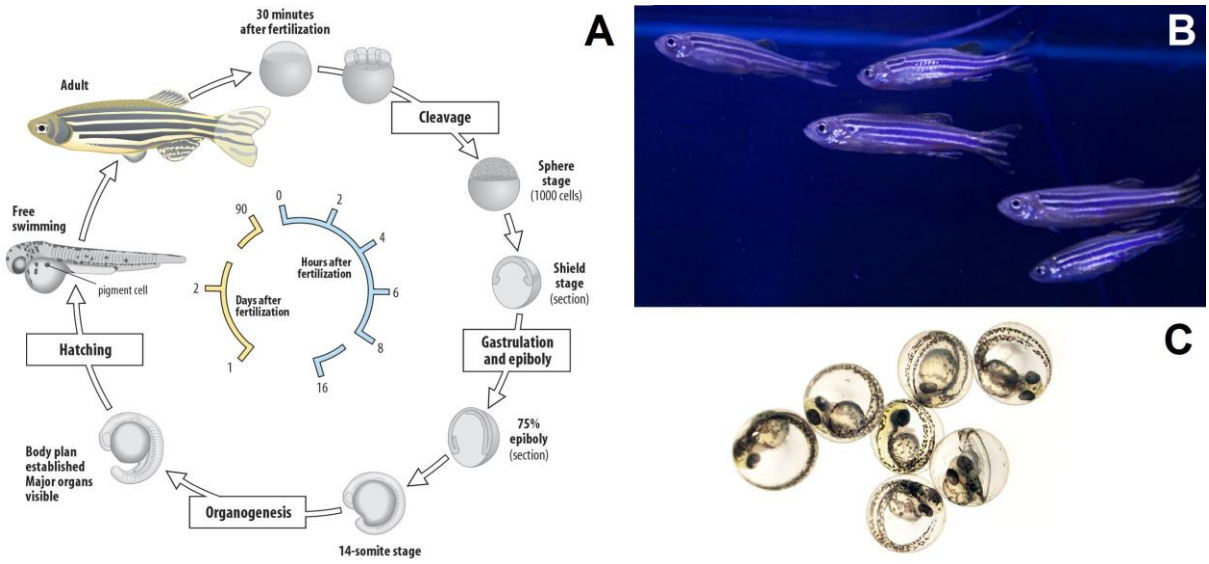

Fig. 1.1

A) Complete life cycle of the zebrafish (Danio rerio), credit: Department of Biology. Memorial University of Newfoundland[1]. B) Adult individuals from our laboratory stock and C) embryos inside their chorion prior to hatching (2 dpf).

In their natural environment, zebrafish can be found near neutral to slightly basic pH waters with temperatures ranging from 14 to 34 °C, however they are known for being somewhat resilient to variances in these conditions[183]. Zebrafish are omnivorous, primarily feeding on zooplankton, phytoplankton, insects and insect larvae being able to prey on a variety of comparatively large food sources such as worms and small crustaceans[184]. Zebrafish is a highly social species which prefers swimming in groups, a typical behaviour previously defined as shoaling[185] and described in a vast number of fish species. Its behavioural innate traits make this species especially appropriate for the analysis conducted in this thesis as the mechanisms of social behaviour in vertebrates, including humans, are not fully understood. Therefore, diseases associated with abnormal social behaviours in vertebrates and humans have been difficult to elicit and treat, behavioural research in zebrafish might provide significant insights into new treatments and procedures that could improve current knowledge and praxes.

In the wilderness they are abundant in flowing rivers, streams and still water masses. They can be found in large, tightly-aggregated groups of hundreds of individuals, as well as in small, loose shoals of merely a dozen individuals[94,186].

In the presence of males, zebrafish females are able to spawn at intervals of two to three days, laying hundreds of eggs in each batch. The approximate generation time is around three months depending on the environmental conditions. Fertilized eggs become transparent embryos, which develop rapidly, with precursors of all major organs appearing within the first 36 hours post fertilization (hpf). These features offer technical advantages for studies at multiple levels of analysis[187]. Therefore, zebrafish has become a powerful model system widely used in biomedical research, as it allows combining rapid and accessible embryogenesis, genetic and genomic tools for systematic gene discovery and analysis, and *in vivo* visualization at a cellular level in a single organism[188,189]. Indeed, larval zebrafish

became an ideal platform for studying vertebrate development and drug screening, in pre-clinical trials [190–192], rivaling with the mice model in the fields of pharmacology, teratology, cardiovascular and ecotoxicology.

The zebrafish is an otophysan fish with morphological hearing specializations connecting the inner ear and swim bladder enhancing hearing sensitivity. This species has also become a well-established organism in hearing research, specifically in studies about inner ear development, mechanisms underlying deafness and hair cell regeneration, and to test ototoxic agents and drugs for treatment of auditory impairment, thanks to a typical vertebrate inner ear at cellular level and functioning [166,168,193]. Additionally, it is commonly used for behavior and collective behavior assays [194–198].

Even though zebrafish is currently a reference model in hearing research and the acoustic environment is known to shape the structure and sensitivity of auditory systems[94,95], there is no information on the natural soundscape of this species, neither on the captive noise conditions under which specimens are maintained before being used in research laboratories.

GENERAL OBJECTIVES AND THESIS SCOPE

Anthropogenic noise of variable temporal patterns is increasing in both marine and freshwater systems. Artificial noise can cause adverse effects on organisms including impaired development, heightened physiological stress and behavioural disturbance, thus posing unprecedented risks on animal species and biodiversity. However, a lack of knowledge exists on how aquatic organism species cope with chronic exposure to noise disturbances in early ontogeny, a critical period for development and establishment of phenotypic traits.

This study relies on the zebrafish *D. rerio*, an important vertebrate model in ecotoxicology and hearing research. This species has been typically reared in large-scale housing systems in research laboratories, although the acoustic properties of these artificial environments and potential effects on hearing remain unknown.

The main objectives of this thesis were (1) to describe the soundscape of zebrafish natural habitats and compare it with the typical noise rearing conditions at research facilities; (2) evaluate the effects of chronic noise exposure to increasing levels and different temporal regimes on development, physiological stress and behaviour in larval zebrafish; and (3) determine the effect of acoustic stress on inner ear structure and hearing sense in early ontogeny.

This study expects to provide first baseline information on zebrafish habitat soundscapes and acoustic properties of typical lab housing systems, which is fundamental to evaluate the species adaptation to different acoustic environments and to promote welfare in artificial housing environments. The present work is also pioneer in assessing noise-induced physiological stress and behavioural disturbance in larval zebrafish, as well as in evaluating the impact of acoustic exposure on inner ear structure and function in a fish larva.

The research conducted relied on different methodologies from bioacoustics soundscape analysis, molecular and imaging techniques, electrophysiology and behavioural assays to achieve the goals proposed.

CHAPTER 2 – THE ACOUSTIC ENVIRONMENT OF ZEBRAFISH

Research article entitled "Characterization of the natural soundscape of zebrafish and comparison with the captive noise conditions"

Characterization of the natural soundscape of zebrafish and comparison with the captive noise conditions.

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Abstract

Zebrafish is a well-established model organism in hearing research. Although the acoustic environment is known to shape the structure and sensitivity of auditory systems, there is no information on the natural soundscape of this species. Moreover, zebrafish are typically reared in large-scale housing systems, although their acoustic properties and potential effects on hearing remain unknown. We characterized the soundscape of both zebrafish natural habitats and laboratory captive conditions, and discussed possible impact on auditory sensitivity.

Sound recordings were conducted in five distinct zebrafish habitats (Southwest India), from quieter stagnant environments with diverse biological/abiotic sounds to louder watercourses characterized by current and moving substrate sounds. Sound pressure level (SPL) varied between 98-126 dB re 1 μPa. Sound spectra presented most energy below 3000 Hz and quieter noise windows were found in the noisiest habitats matching the species best hearing range. Contrastingly, recordings from three zebrafish housing systems revealed higher SPL (122-143 dB) and most energy below 1000 Hz with more spectral peaks, which might cause significant auditory masking.

This study establishes an important ground for future research on the adaptation of zebrafish auditory system to the natural soundscapes, and highlights the importance of controlling noise conditions in housing systems.

Keywords: ambient noise, soundscape, natural habitat, hearing sensitivity, sound pressure level.

Introduction

In aquatic environments, sound acts as an efficient information carrier for fishes, which have evolved a remarkable diversity of auditory structures to enhance their hearing sense[199]. By listening to the aquatic background noise, fishes can extract critical biotic information about the presence of conspecifics and heterospecifics, including potential mates, prey and predators[163,200]. Moreover, they can also perceive important abiotic information for orientation, such as sounds derived from wind, water current, cavitation, and moving substrate[163,201]. Since fish species can detect and process both sound pressure and particle motion, perform sound source segregation and auditory scene analysis, the underwater soundscapes can be extremely complex in information, and even richer compared to terrestrial acoustic environments[6,202].

It is known that underwater soundscapes play an important role shaping auditory structures and sensitivity of fishes[203]. Several studies indicate that species are often well adapted to the lowest noise levels encountered in their natural habitats[203–205]. When background noise levels are elevated due to anthropogenic noise sources, fishes may experience physiological stress and auditory impairment, namely masking, temporary hearing loss, and damage of the sensory auditory hair cells[173,206,207].

Elevated background noise is commonly present in fish aquaculture systems. Large-scale housing systems often use equipment such as air and water pumps, filtration systems, harvesters, feeding and maintenance machinery, which produce noise especially below 1000 Hz[29,104,106]. Consequently, fish species are chronically exposed to elevated noise that is usually within their sensitive hearing ranges. Only very few studies investigated the effects of background noise from housing systems on fishes and results showed reduced egg viability and growth rates[208,209], but also no developmental and physiological stress effects[29,105]. Information on the effects of captive noise conditions on fish hearing is extremely limited.

Wysocki et al (2007)[29] did not find an impact of increasing tank noise levels from 115 to 150 dB re 1 μPa RMS on the hearing tresholds in the rainbow trout, *Oncorhynchus mykiss* (Salmonidae). However, Gutscher et al (2011)[210] investigated the effect of aquarium noise with different filtering systems on the hearing in the goldfish *Carassius auratus* (Cyprinidae), an ostariophysan species with accessory hearing structures, i.e. Weberian ossicles that link the swim bladder to the inner ear increasing auditory sensitivity and frequency range detection. The authors found considerable auditory threshold shifts (masking) by all noise types (threshold shifts of 15-19 dB) within the species best hearing range (600-1000 Hz). Therefore, it is likely that some of the published studies concerning behavioural and physiological response of fishes, including hearing sensitivity, are affected by the elevated noise conditions in the laboratory housing facilities. This might be a particular issue for those species with accessory hearing structures and enhanced auditory sensitivity.

The zebrafish, *Danio rerio* (Cyprinidae), has become an important model organism to investigate the molecular basis of inner ear development and function, human deafness and hearing regeneration[168,211,212]. This species has a typical vertebrate inner ear at the cellular level and its anatomy and development have been intensively described[168,212,213]. The zebrafish is an ostariophysan species with Weberian ossicles linking the swim bladder to the inner ear[214] with best hearing range between 600-1000 Hz[212,215]. Even though zebrafish has become a well-established model for hearing research, there is no information available on the soundscapes of its natural environment. This species is found in the North Eastern and South Western India, Nepal, Bangladesh, South Pakistan, and Northern Myanmar and inhabits diverse freshwater habitats, ranging from stagnant waters ponds to main river courses[216], which may have shaped its auditory structures and hearing abilities[203].

Moreover, zebrafish are commonly maintained in large-scale housing systems in laboratory facilities while being used for research[217]. Such environments are characterized by

elevated noise levels, probably resulting from aerators, air and water pumps, water circulation and feeding machinery[218]. The noise levels and spectral features of typical zebrafish housing systems, how they compare to the natural habitat conditions, and their potential to affect species hearing abilities have never been investigated.

The present study aimed to 1) characterize the variability of soundscapes of typical zebrafish freshwater habitats in Southwest India, from slow flow backwaters/ponds to main river courses; 2) investigate the noise conditions of typical zebrafish laboratory housing systems; and 3) compare the species auditory sensitivity with the spectral features of both natural and artificial noise environments.

Methods

Sound recordings in the natural habitats

The study area was selected based on previously reported distribution of zebrafish in Karnataka, Southwest India[181]. Among the different possible locations, we selected a variety of habitats with different hydrological traits to characterize the diversity in soundscapes (Table 1). The criteria to choose the recording locations were: selection of a site that would be representative of that specific habitat (pool, backwaters, main waterway); identification of zebrafish *D. rerio* shoals; and accessibility with the recording equipment. We also selected recording locations where prior studies[181] were conducted and further details on ecological features can be found.

In all recording locations we confirmed the occurrence of zebrafish by observation and capture using rectangular hand nets and fine mesh seines (mesh grid size varying between 1-3 mm), in collaboration with M. Arunachalam (Manonmaniam Sundaranar University, India). All sound recordings were performed under tropical dry season conditions

in the absence or with weak wind (<4km/h) and no rain. Comparing acoustic conditions between dry and wet seasons would be relevant and should be considered in future research.

The zebrafish were mostly found in shallow water masses of low flow with sand, lime, silt, and/or bedrock substrate, in small secondary or tertiary channels of a main river or in adjacent backwaters, but also along the margins of a main river. The species behaviour varied from stationary swimming compact shoals countering the water flow in the Thunga river of Shringeri (SH) to free swimming loose shoals in the riverbed pools of Kallahalli (KA) (Table 1). Ambient noise recordings and sound pressure level measurements were conducted in five distinct locations (Fig. 2.1, Table 1.1): 1) AC, Achacanni, west flowing secondary stream between waterfalls (circa 50 m away from nearest waterfall), tributary of Sharawati river near Sharawati natural reserve; 2) KA, Kallahalli, natural water pools carved in the riverbed of the south-east flowing Kaveri river; 3) SIS, Sidi Halla with sandy substrate, tertiary south-west flowing stream adjacent to paddy riversides; 4) SIR, Sidi Halla with rocky substrate, secondary south-west flowing channel in the same basin as SIS; and 5) SH, Shringeri (west flowing Thunga river), main river course with faster water flow.

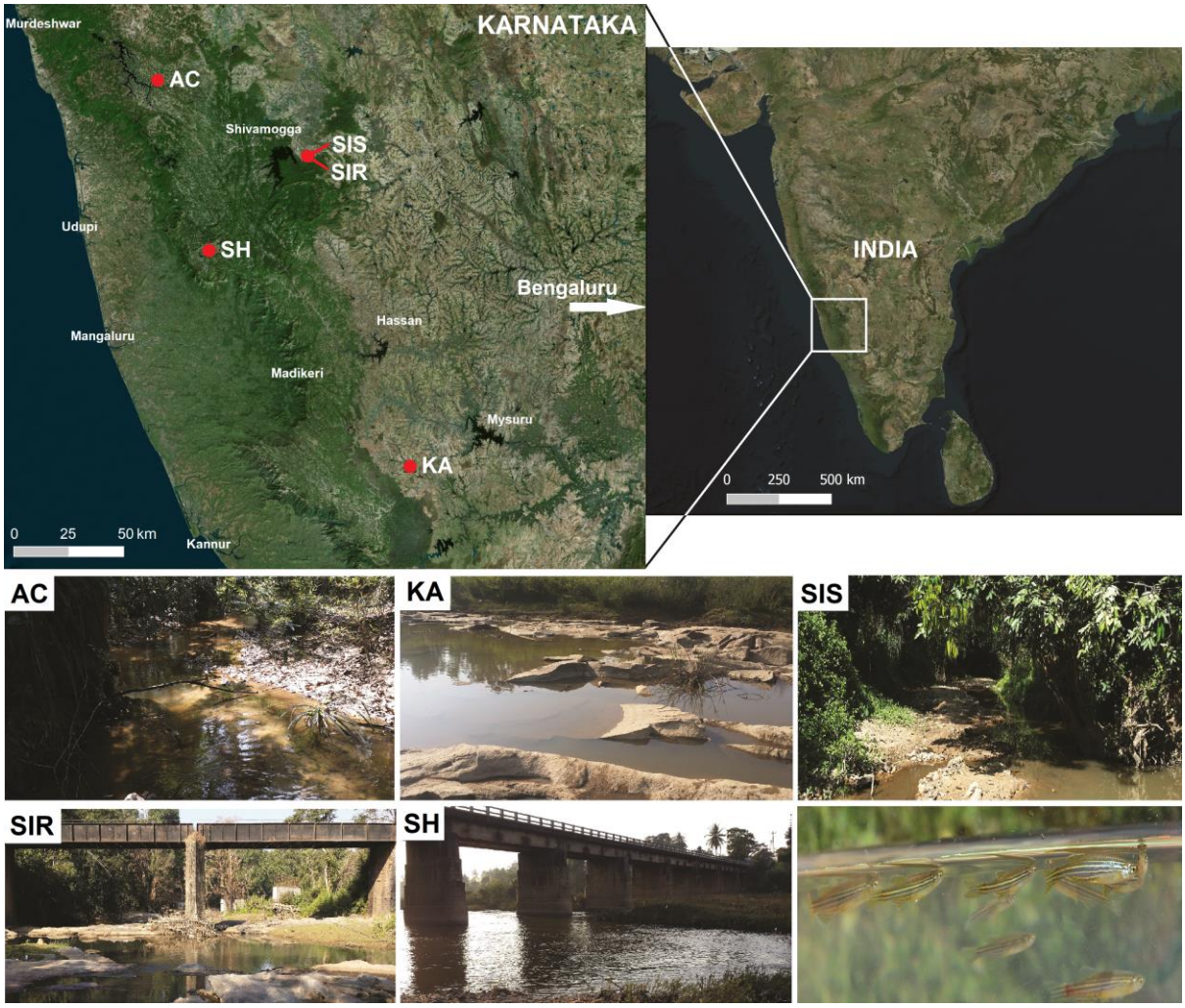

Fig. 2.1.

Map of India (top) showing the geographical location of the different zebrafish natural habitats selected for this study in Southwest India (Karnataka state): AC – Achacanni, shallow low flow stream near Hosanagara at the border of Sharavati Valley Wildlife Sanctuary; KA – Kallahalli, natural wells connected to Kaveri river; SIS – Sidi Halla (sandy), low flow stream with sandy substrate near Shivamogga; SIR – Sidi Halla (rocky), medium flow stream with rocky substrate adjacent to SIS and SH – Shringeri, faster flow main stream of Tunga river. In all study locations, the team confirmed zebrafish (*D. rerio*) occurrence (bottom right, specimens captured at SIR).

Recording locations [1]	GPS coordinates	Habitat [2]	Elevation (m)	Water temp. (°C)	Depth (cm)	Flow [3] (cm/s)	Water visibility	Substrate	Main noise sources	Zebrafish occurrence (n° individuals)
AC Achacanni	13.4848° N / 75.1034° E	Shallow and narrow second order stream	615	19.5-20.0	10-25	5.2-5.8	Clear, bottom visible	Sand, lime, silt, litter, leaves	Cavitation, moving substrate, insects, birds	Stationary compact shoals countering current (15-25)
KA Kallahalli	12.3112° N / 76.2160° E	Pool connected to main riverbed	599	27.5-28.0	20-50	5.5-6.2	Turbid, bottom not visible	Bedrock, lime, silt	Cavitation, insects	Free swimming loose shoals (5-10)
SIS Sidi Halla (sandy)	13.3945° N / 75.1822° E	Shallow third order stream	772	22.0-23.0	10-30	-	Turbid, bottom not visible	Sand, lime, silt	Moving substrate, insects, birds	Free swimming compact shoals (5-10)
SIR Sidi Halla (rocky)	13.3955° N / 75.1251° E	Narrow third order stream	771	21.0-22.0	20-35	-	Clear, bottom visible	Bedrock, gravel, sand	Water current, cavitation, moving substrate, insects	Stationary compact shoals countering current (5-10)
SH Shringeri	13.5521° N / 75.2642° E	Main river stream	680	22.0-22.5	35-50	6.3-6.8	Clear, bottom visible	Bedrock, gravel, boulders, sand	Water current, cavitation, moving substrate	Compact shoals behind substrate bulk (5-10)

Table 1.1

General characterization of the sampling sites in Karnataka, Southwest India. [1] Recording locations were the same as selected by Arunachalam et al (2013)[29]. [2] Vegetation cover was present as dense (AC) and sparse (SIS) hanging canopy, and riparian riverside plants (AC, SIS, SIR), while it was absent at SH and KA. [3] Water flow measured in a prior study[29] in the same recording locations during dry season; (-) designates missing values.

Ambient noise was recorded at a sampling rate of 44.1 kHz using a hydrophone (Aquarian Audio H2a-XLR-15, Anacortes, WA, USA; frequency range: 10-100 kHz ± 4 dB; voltage sensitivity: -180 dB re 1 V/µPa^{-1}) connected to an A/D converter phantom powered device (Edirol UA-25, Roland, Tokyo, Japan) and then to a laptop computer running Raven Pro 64 1.5 software (The Cornell Lab of Ornithology, Ithaca, NY, USA). Sound pressure levels were measured with a hydrophone (Brüel & Kjær 8101, Naerum, Denmark; frequency range: 1-80 kHz ± 2 dB; voltage sensitivity: -184 dB re 1 V/µPa^{-1}) connected to a hand-held sound level meter (Brüel & Kjær 2250). The hydrophones were attached to a pole and positioned at about 15-20 cm depth, avoiding direct contact with substrate and vegetation.

The hydrophones were positioned within the same location (<1m) where the zebrafish were previously observed.

Ambient noise recordings and Sound Pressure Level (SPL) measurements followed previously described protocols[82,83]. Sound recordings consisted of 15 min each and two consecutive recordings were conducted per site. The equivalent continuous SPL (L_{Zeq}; flat weighting: 6.3–20 kHz) averaged over 60 s was obtained six times per site, i.e. three times immediately before and after each sound recording session. L_{Zeq} (also known as L_{Leq}) is a measure of averaged energy in a varying sound field and is commonly used in environmental noise studies (ISO 1996 2003).

We considered just one sampling site per location, except in Sidi Halla (SIS and SIR), which could underestimate the potential variation within each location. However, we preferred to characterize a single sampling site per habitat where zebrafish were observed by conducting sound recordings for relatively long periods of time than usually reported, and consider a representative range of different zebrafish habitats.

Sound recordings of laboratory housing systems

We selected three typical zebrafish housing systems from different laboratory facilities in Macau, namely at the University of Saint Joseph and the University of Macau. The selected housing systems (HS) were: 1) HS1, standalone system with five double-sided shelves and frame-integrated filtering and pumping system equipped with 224 acrylic tanks (1-10L), model AAB-074-AA-A, Yakos 65, Taiwan; 2) HS2, standalone system with similar configuration to HS1 equipped with 168 acrylic tanks (3.5-10L), model AAB-100-AA-A, Yakos 65, Taiwan; and 3) HS3, multilinking system with external WTU (Water Treatment Unit) connecting three Active Blue single sided rack frames equipped with 65 acrylic tanks (1.1-8L) with pumps and filters configured in an external palletized CLS (Centralized Life

Support) unit connected to an automatic feeder (Triton), ZebTEC, Techniplast, Italy (Fig. 2.2).

Sound recordings and SPL measurements followed the same protocol abovementioned, with the exception that the hydrophones were placed in the middle of the fish tanks and submerged at 10 cm from the surface and 5 cm from the bottom. Three recording points were selected in each housing system to better characterize the noise variability attending to their distance to the main sound source, the water pump and filtering system (Fig. 2.2).

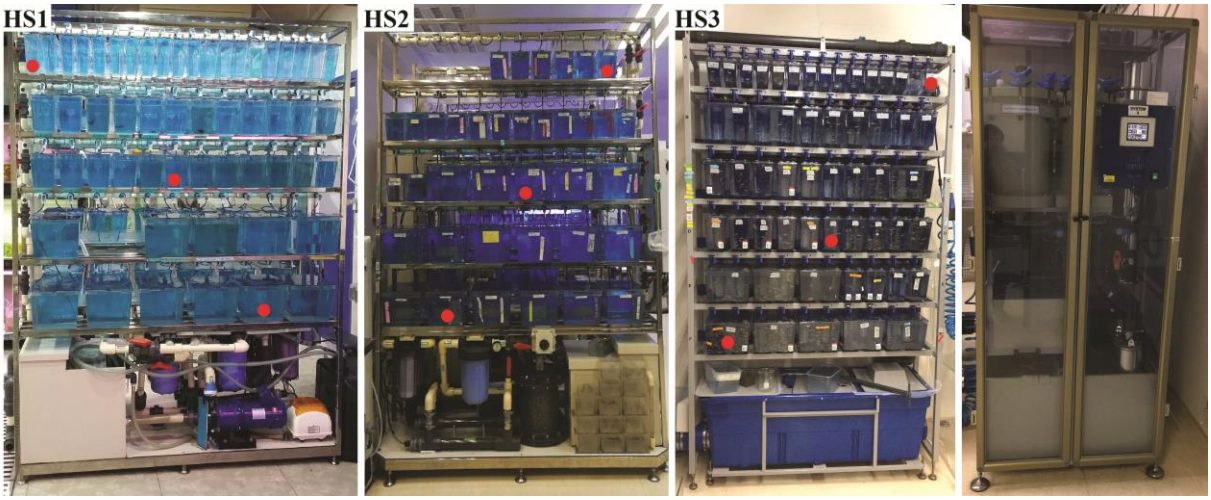

Fig. 2.2.

Three representative zebrafish housing systems (HS) considered in this study to characterize noise conditions in captivity. Red dots indicate the fish tanks selected for the recordings of sound pressure level (SPL) measurements. HS1 and HS2 - standalone systems with frame-integrated filtering and pumping system – models AAB-074-AA-A and AAB-100-AA-A, respectively (Yakos 65, Taiwan); HS3 - multi-linking system with external WTU (Water Treatment Unit) connecting three Active Blue stand-alone racks with pumps and filters configured in an external palletized CLS (Centralized Life Support, right picture) (ZebTEC, Techniplast, Italy).

SPL measurements were done in following locations for all housing systems: 10 L tank in the bottom (30 - 40 cm distance to water pump/filters); 3.5 L tank in the middle (140 - 150 cm distance), and 1 L tank at the top (240 - 250 cm distance). These locations varied in SPL but presented similar spectral composition (sound energy distribution across frequencies). Only the middle recording location was considered while comparing SPL across different housing systems and relative to field noise levels. Considering more than one HS location (at different distances to main noise source) in such analysis would have increased the SPL variability, and this would not have been consistent with what zebrafish individuals experience when they are housed in a particular location.

Sound analysis

Sound analysis was performed using Adobe Audition 3.0 (Adobe Systems Inc., San Jose, CA, USA). Natural sound files were firstly inspected regarding potential artifacts and presence of anthropogenic noise. Since the purpose of this study was to characterize the zebrafish natural soundscape, various sounds from human activities (e.g. traffic, bridge vibrations, and people talking) and other recording artifacts (hydrophone vibrating with current or touching substrate) were removed. Even though anthropogenic sounds were occasionally part of the soundscape in several locations, studying such noise sources was not the scope of the present study. A final sound file of 10-15 min was created for each habitat, providing a representative characterization of the variability of the natural soundscape with occasional low amplitude anthropogenic noise.

The relative Fast Fourier Transformation (FFT) of the final sound recordings representing each location was calculated (16384 and 2048 FFT size, overlap 50%,

Blackman–Harris window). Both power spectral density (PSD) level (given in dB re 1 µ Pa^2/Hz) and absolute sound spectra level (dB re 1 µ Pa) were determined using the averaged L_{Zeq} value calculated per site and following previously described procedures[82,219]. The PSD level was further calculated based on the equation (linearization): $Ai = 10^{(ai/10)}$, where Ai equals the linear spectral amplitude and ai is the logarithmic spectral amplitude. The values were then converted to PSD levels through the equation: PSD level (dB) = $10\times \log_{10}\left(\frac{\sqrt{Ai}}{BW}\right)^2$, where BW represents bandwidth (spectral resolution).

Statistical analysis

Noise levels (L_{Zeq}) were compared between different natural habitats with Kruskal-Wallis H tests followed by Dunn's pairwise post hoc tests to verify habitat specific differences. Comparison of noise levels between artificial housing systems was performed with One-way ANOVA, followed by post hoc Tukey tests. Overall natural and artificial noise levels were compared with a Student's t-test. Parametric tests were used only when data was normally distributed and variances were homogeneous. The statistical analysis was performed with IBM SPSS v.22 (IBM Corp., USA).

Results

Characterization of the zebrafish natural soundscapes

The zebrafish occurred in a wide range of natural acoustic environments that differed significantly in the soundscape composition, sound pressure level, and spectral features (see sound files as supplementary materials). The habitats varied from relatively quiet locations such as slow-moving streams and riverside pool sites characterized by occasional sounds

from water cavitation, moving substrate and diverse biological activity, namely Sidi Halla (SIS), Achacanni (AC) and Kallahali (KA), to noisier environments like a main river exhibiting continuous water current and moving substrate sounds (SH) - Table 1 and 2.

The biological sounds detected were mostly high-pitched and produced by insects (main energy >2000 Hz) and birds (1000-7000 Hz) in the vicinity, while the abiotic sources consisted on water flowing and cavitation (700-4000 Hz) and moving substrate (900-5000 Hz). All these different sounds consisted on discrete events that occurred several times throughout the recordings, except for the water current sounds in SH that were continuously present (Fig. 2.3).

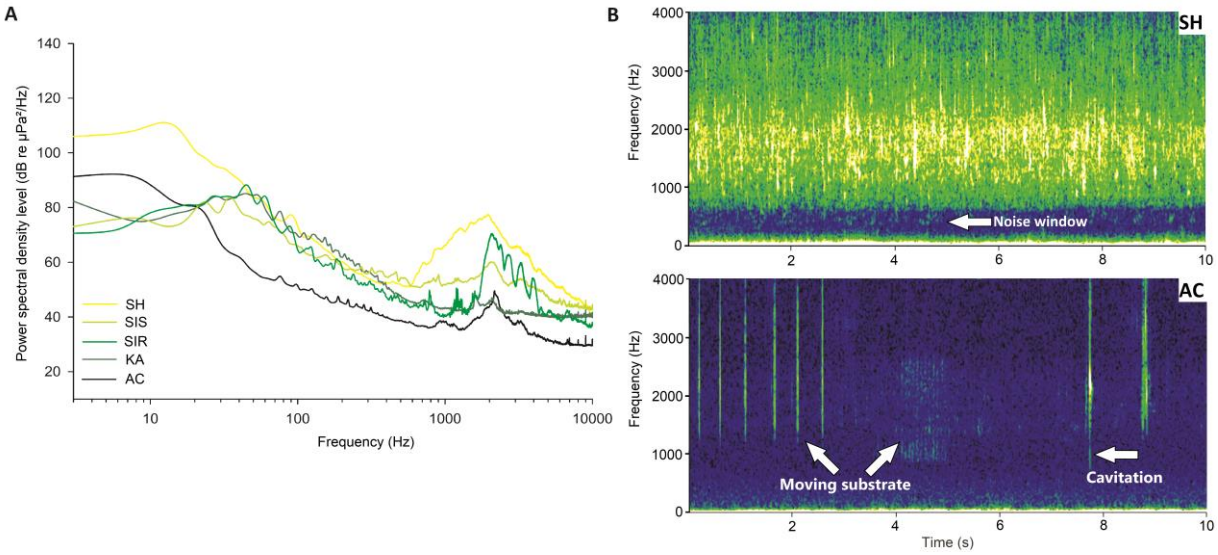

Fig. 2.3.

A) Power spectral density (PSD) of diverse zebrafish natural habitats from Southwest India (Karnataka). Sampling frequency: 44.1 kHz. FFT size 16384. B) Spectrograms of sound recordings from SH showing noise window and AC showing natural noise sources, FFT size 2048. SH, KA, AC, SIS and SIR (for abbreviation refer to Fig.2.1)

SPLs (or L_{Zeq}) varied between 102.75 ± 0.32 dB re 1 µPa (mean ± standard deviation) in a low flow small stream (SIS) to 126.08 ± 0.30 dB in a main river course (SH) (Table 1.2, Fig. 2.4).

Environment	Recording location		Mean ± SD	Min	Max
Natural	SH		126.08 ± 0.30	125.49	126.19
	KA		105.83 ± 3.63	102.42	109.53
	AC		106.24 ± 0.96	104.74	107.40
	SIS		102.75 ± 0.32	102.34	103.12
	SIR		107.38 ± 3.50	104.24	110.83
Artificial	HS1	1	145.83 ± 0.08	145.70	145.90
		2	139.17 ± 0.33	138.80	139.50
		3	145.85 ± 0.46	145.20	146.40
	HS2	1	146.63 ± 0.43	146.20	147.00
		2	135.35 ± 0.80	133.80	136.10
		3	133.40 ± 0.35	133.10	133.90
	HS3	1	126.27 ± 0.98	125.20	127.60
		2	121.03 ± 0.19	120.80	121.30
		3	119.63 ± 0.08	119.50	119.70

Table 1.2.

Noise levels (L_{Zeq}) determined in five zebrafish natural habitats (Karnataka, Southwest India) and in three typical laboratory-housing systems (HS). Values are based on 4-6 averaged readings based on 60 s and are given in dB re 1 µPa. Natural habitats: SH - Shringeri, KA - Kallahalli, AC - Achacanni, SIS - Sidi Halla sandy and SIR - Sidi Halla rocky. For each HS three recording points were considered at various distances to the main noise source (water

pump and filtering system): 1) 30-40 cm, 2) 140-150 cm, 3) 240-250 cm. CV - coefficient of variation.

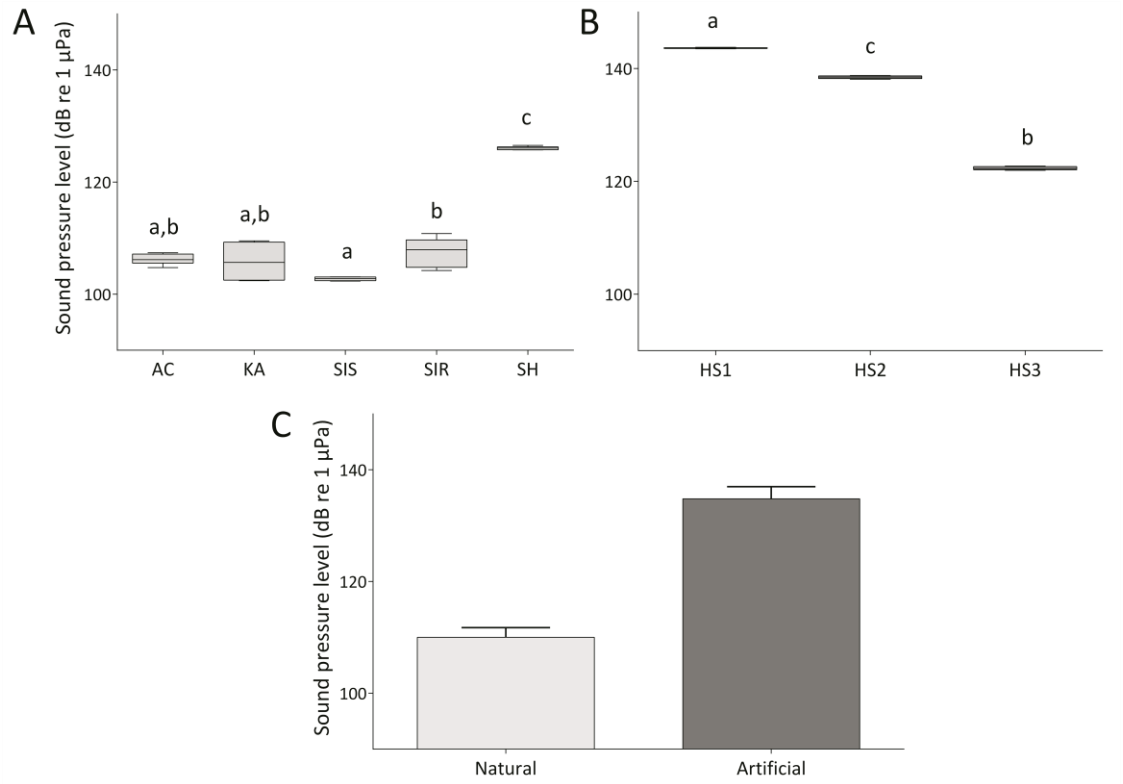

Fig. 2.4.

Comparison of sound pressure levels (linear equivalent, L_{Zeq}) between A) zebrafish natural habitats ($H(4, 27) = 19.05$; $p < 0.001$), and B) laboratory housing systems ($F(2, 18) = 15174$; $p < 0.001$). Values are based on 60s averaged measurements (L_{Zeq}), 4-6 per site. Different letters indicate statistically significant differences based on pairwise post hoc comparisons. Plots show medians and 10th, 25th, 75th and 90th percentiles as boxes and whiskers. C) Comparison between mean noise levels determined for natural habitats and artificial housing conditions ($F(1, 43) = 78.88$; $p<0.001$). Plot shows means and standard deviations.

Significant differences in SPL were found between the different recording sites ($H(4, 27) = 19.05$; $p < 0.001$). Pairwise post hoc comparisons revealed that Thunga river in Shringeri (SH) was significantly louder compared to all the other locations, as well as SIS in relation to SIR ($p < 0.05$).

The SPL variability within the same study site was the lowest at the noisier habitat, i.e. main river (SH). The difference between the minimum and maximum L_{Zeq} was 0.81 dB at SH (coefficient of variation or CV = 0.23%), whereas it was 7.11 dB at KA (CV = 3.55%). Within the same habitat type, namely the low flow streams (KA, AC, SIS), the levels differed by up to 7.19 dB. In Sidi Halla, two recording locations were considered and the presence of faster water flow and different substrate in SIR (bedrock, gravel and sand), compared to SIS (substrate sand, lime and silt), probably contributed for the increase of circa 5 dB from 102.75 ± 0.32 to 107.38 ± 3.50 dB, respectively.

The spectral profiles varied considerably between natural habitats, although they all showed a general decline in energy towards higher frequencies (Fig. 2.3). The energy decline was more gradual in the shallow streams with lower water flow (AC, KA and SIS), which presented most energy below 600-800 Hz. In the third order stream SIR, besides the higher amplitude at low frequencies, an additional spectral peak was found at 2000-4000 Hz resulting from sounds mainly produced by nearby insects. In the main river (SH), more spectral energy was observed and a steep amplitude decline or "noise window" was detected within 100-2000 Hz.

Characterization of the ambient noise of zebrafish housing systems

The two possible configurations of laboratory zebrafish housing systems were considered in this study, namely the "stand-alone system" with fish tanks, pump and filters integrated in a single rack frame (HS1 and HS2), and a "multi linking system" with multiple racks

containing fish tanks and a water deposit connected to an external enclosed module containing all pumps and filters (HS3).

The housing systems revealed SPLs ranging from 122.3 ± 0.28 dB in (HS3) to 143.6 ± 0.09 dB in (HS1) - Table 1.2, Fig. 2.4.

Significant differences in SPL were found in the middle tank of the different housing systems ($F(2, 18) = 15174$; $p < 0.001$; $p < 0.0001$ post-hoc tests between all systems). The variability of SPLs for a specific location within each system was very low, namely of 0.20-2.40 dB (CV = 0.06-0.78 %) for all the systems and recording points - Table 1.2, Fig. 2.4. The SPLs were significantly dependent on the distance to the water pump and filters for two of the three systems ($F_{(2, 54)} = 7.95$, $p < 0.05$). In both HS2 and HS3, fish were gradually exposed to higher noise levels with the proximity to these sound sources. However, in HS1 the sound level did not follow the same gradual pattern and it was lower in the middle of the rack system (139.17 dB), compared to the closest and furthest recording points in relation to the pump/filters (145.83 and 145.85 dB, respectively).

The sound spectra from the different housing systems revealed most sound energy concentrated at low frequencies below 1000 Hz and a gradual decrease towards higher frequencies (Fig. 2.5). Several conspicuous energy peaks were observed specially in HS1 at 25, 45, 95, and 140 and between 180-1200 Hz. HS2 revealed peaks at 30, 50, 100 and 280 Hz, among others. Contrastingly, HS3 revealed comparatively a more gradual decline in energy distribution towards higher frequencies.

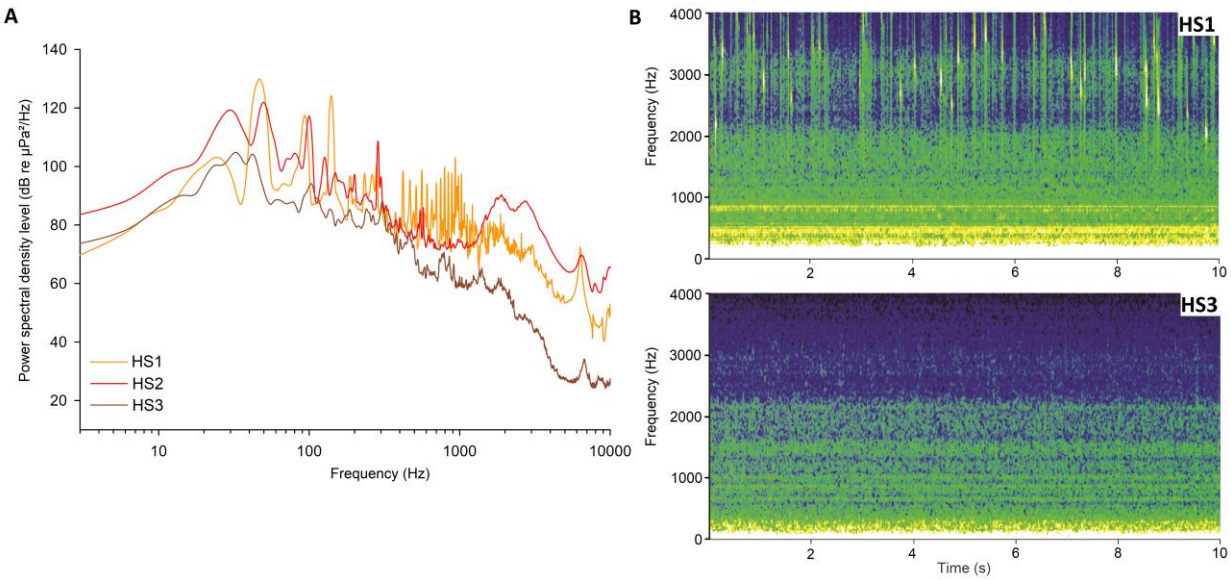

Fig. 2.5.

A) Power spectral density (PSD) of noise from three typical zebrafish housing systems (HS). Sampling frequency 44.1 kHz, FFT size 16384. B) Spectrograms of representative sound recordings from HS1 (standalone system, AAB-074-AA-A, Yakos 65, Taiwan) and HS3 (multi-linking system with external pumping/filtering units, ZebTEC, Techniplast, Italy) with FFT size 2048.

Natural versus artificial soundscapes: comparison with zebrafish hearing sensitivity

Comparison of mean SPLs between natural and artificial acoustic environments revealed overall significant differences ($F(1, 43) = 78.88$, $p < 0.001$), with lower noise levels found in the natural habitats (Fig. 2.4). However, SPL variation was comparatively higher among natural environments compared to laboratory conditions (Table 1.2).

Comparing sound spectra of both types of soundscapes revealed noticeable differences (Fig. 2.6).

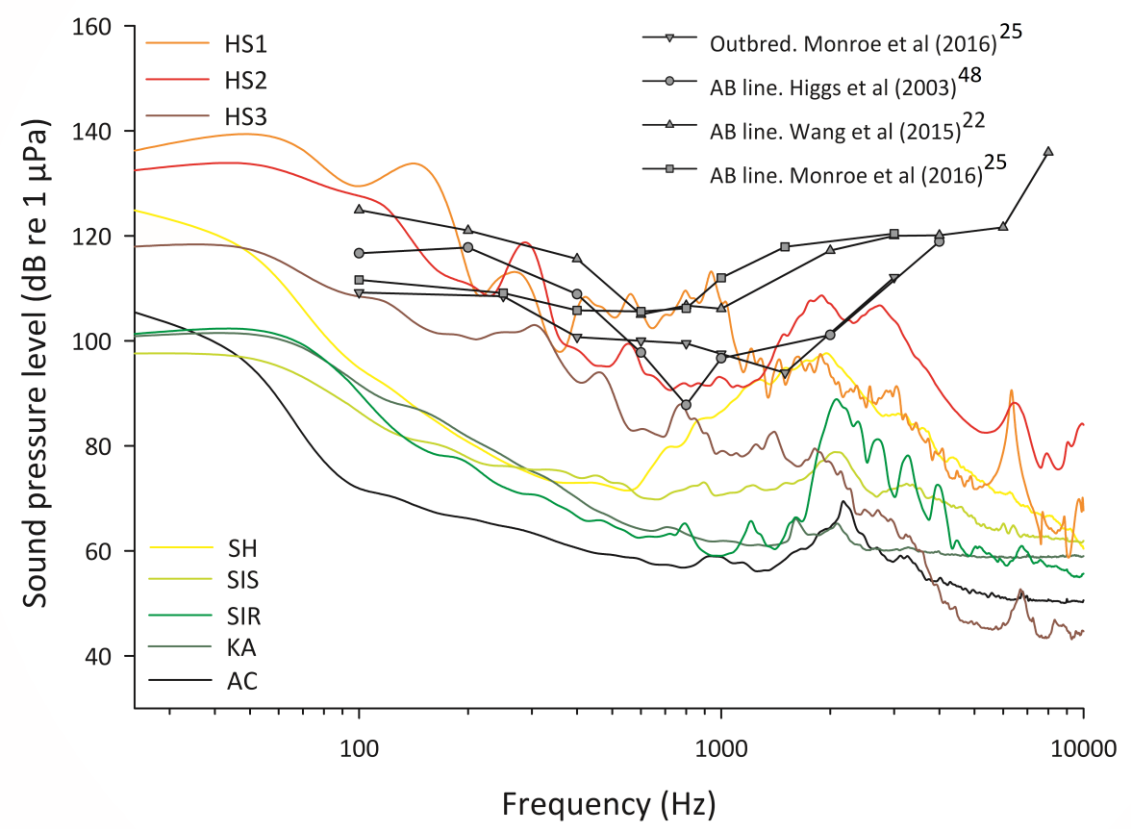

Fig. 2.6.

Sound spectra from both natural and captive noise conditions compared to zebrafish audiograms (grey bulleted lines). Mean auditory thresholds indicated are from AB wild type line[34,22,41] and wild type line from Liles Tropical Fish, Inc (Ruskin, FL)[34]. KA – Kallahalli, natural wells connected to Kaveri river AC – Achacanni, shallow low flow stream; SIS – Sidi Halla (sandy), low flow stream; SIR – Sidi Halla (rocky), medium flow stream; SH – Shringeri, fast flow Tunga river; HS1 and HS2 (standalone systems from Yakos 65, Taiwan) and HS3 (multi-linking system with external pumping/filtering units from ZebTEC, Techniplast, Italy). Sampling frequency 44.1 kHz, FFT size 2048, Blackman Harris, 50% overlap.

While the shape of the spectral profiles from natural habitats showed most energy concentrated below 600-800 Hz and an energy peak in the noisiest habitats at 1000-4000 Hz

due to diverse abiotic and biological sources, artificial housing systems presented most energy under 1000 Hz following a more irregular distribution pattern with multiple spectral peaks. Differences in sound amplitude between natural habitat and laboratory conditions were more noticeable below 1000 Hz with a variation of up to 60 dB.

Auditory sensitivity thresholds of wild type zebrafish reported in previous studies[212,215,220] are quite variable with differences of up to 22 dB throughout the frequency detection range, with higher discrepancies at 100, 800 and 1500 Hz. Comparing both types of soundscape spectral profiles with the auditory sensitivity data, revealed a significant overlap between the sound energy of the artificial housing conditions and the species hearing range (100-8000 Hz), especially for the standalone systems (HS1 and HS2). The spectral energy of these systems was up to 22.4 dB above the auditory thresholds. In contrast, the spectral profiles of most natural soundscapes were considerably below the zebrafish auditory thresholds. The fast-flowing river (SH), however, presented a conspicuous energy peak close to the lowest auditory thresholds within 800-2000 Hz. The best hearing range of the species (600-1000 Hz) matched a "noise window" within the soundscape of the noisiest habitat, but also a frequency range that exhibits the highest variability across all acoustic environments.

Discussion

To our knowledge this is the first study investigating the acoustic properties of the natural freshwater habitats of zebrafish *D. rerio*, a widely used model organism in hearing research. Moreover, we provide an important comparison between the natural soundscapes with the artificial noise conditions found in zebrafish housing systems commonly used in research facilities. Our results showed significant higher noise levels in housing systems compared to the natural environments, with potential to cause auditory masking. Additional differences were also found in sound spectral profiles and noise level variability.

Diversity in the soundscape of natural freshwater habitats

Over the past decades, there has been a growing interest on the variability of underwater soundscapes especially in marine ecosystems for commercial interests in fisheries but also for monitoring biodiversity for conservation purposes[15,86,109,121,221,222]. However, limited information is available on ambient noise from freshwater habitats[82,83,91–93,203,204].

In freshwater habitats, the ambient noise levels are usually highly dependent on the water flow strength and substrate composition. Lakes and backwaters typically present lower noise levels compared to fast-flowing waters found in streams and rivers, with noise levels that can differ more than 40 dB[82,83,92,203]. In our study, the shallow water streams with low/medium flow and backwaters presented the lowest mean SPLs (circa 103-107 dB re 1 μPa). The sound sources were mostly abiotic from water current, cavitation, and moving substrate, but also biotic from calling insects and birds. Contrastingly, the main river course at Thunga river in Shringeri (SH) showed the highest SPL (126 dB re 1 μPa), most likely due to the higher water flow, larger water volume, and significant cavitation and transportation of sediment (sand, cobble and boulders). The SPL values from quieter habitats were similar to the noise levels reported by Wysocki et al (2007)[82] for backwaters (Gänsehaufen Traverse), pond (Prellenkirchen) and stream with bedrock substrate (Schwarza) in Austria, which corresponded to 99, 98 and 110 dB re 1 μPa, respectively. In the same study, the noise levels reported for a main river course and a stream were similar to the SPL recorded in the faster-flowing Thunga river. The Triesting stream, a typical Alpine creek with cobble and boulder substrate, revealed mean SPL of 124 dB dB re 1 μPa; and the free-flowing part of the Danube river noise level of about 135 dB[82].

Other studies have reported ambient noise spectral profiles that indicate similar variability in noise levels of freshwater systems. For instance, Lugli and Fine (2003)[204]

reported differences in spectral levels (1 Hz bandwidth) in several locations within two shallow stony streams in Italy (stream Stirone and river Serchio) with maximum SPL varying between 70-80 dB re 1 μPa (quiet locations) to 100-105 dB (rapids). Additionally, Crawford et al (1997)[91] reported a noise background of about 75 dB re 1 μPa (RMS) at night in a shallow plain flood of a stream tributary of the Niger river (Mali). However, comparisons of noise levels across different studies are difficult since the mean SPL is not always described and spectral composition profiles are often given in different units and/or bandwidth.

Similar to previous studies, louder habitats, such as the Thunga river (SH), revealed lower variability in the noise levels compared to quieter environments[82,83]. Any additional noise in the soundscape in the quieter locations (including from anthropogenic sources and biological activity) contributed for a notable increase in the noise level.

Regarding noise spectral profiles, freshwater habitats such as rivers and streams typically present more energy at lower frequencies followed by a gradual noise level decline[82,83,91]. We also found a similar pattern of energy decline with increasing frequency in all zebrafish habitats investigated in this study. However, in the noisiest environment, Thunga river (SH), a "noise window" at lower frequencies was detected followed by a subsequent energy peak towards 2000 Hz. A low frequency "noise window" has been reported in previous studies of freshwater habitats. Crawford et al (1997)[91] reported a wider spectral window between 200-3000 Hz in the Niger river (Mali, Africa), followed by higher energy above 4 kHz. Lugli and Fine (2003) and Lugli (2011)[92,204] identified noise windows at 100 Hz in a stony stream, as well as, at 200-250 Hz in a vegetated spring and brackish lagoon. Wysocki et al (2007)[82] reported lower spectral levels between 200-2000 Hz in a stream (Schawarza), and a similar pattern to the spectral composition of our noisiest study site (SH) in the Danube river (close to Danube island and free-flowing area), where a steep decline in spectral level was found around 200 Hz followed by a gradual increase towards 1000 Hz.

In summary, the soundscapes of zebrafish natural habitats investigated in this study revealed considerable diversity in sound levels and spectral composition, mostly resulting from differences in abiotic sources (volume and speed of water flow with cavitation and sediment composition and transportation). These differences might be important for zebrafish orientation and sound detection in the various acoustic environments.

Ambient noise in artificial housing systems

Very limited information is known on the acoustic properties of artificial tank systems and their impact on fish behaviour, physiological stress and hearing[15,19,52,53]. But it is known that vibrations and noise may cause stress and harm aquatic animals in laboratories (NRC 2011)[225]. The studies available showed reduced fish egg viability and growth rates[208,209], but also absence of developmental and physiological stress effects in the rainbow trout (*O. mykiss*), which do not have morphological hearing specializations[29,105].

In our study we investigated the noise levels and spectral features of three typical zebrafish housing systems, including stand-alone (frame built in filters and pump) and multi linking rack units (external water treatment unit and pumps connected to racks). The SPL determined varied between 123-144 dB with significant higher noise levels in the stand-alone systems, indicating that great part of the background noise is caused by the proximity to the pumps/filters. Similar noise values were determined in other studies, although the information is scarce and difficult to compare due to distinct types of fish housing systems. For example, Gutscher et al (2011)[210] found that an earthen pond (32 x 22 m, 1.8 m depth) without operating aerators presented spectral noise levels (L_{Leq}) below 100 dB re 1 µPa; while Wysocki et al (2007)[29] reported in round fiberglass tanks (14 m diameter, 4 m depth) with recirculating system, SPL of about 149 dB re 1 µPa RMS. Additionally, Bart et al. (2001)[104] compared the acoustic properties across a wide range of fish housing systems

equipped with aeration systems and identified highest noise levels in larger fiberglass tanks (14 m diameter, 4 m depth) of about 153 dB re 1 µPa within 25-1000 Hz.

Moreover, the spectral composition of the ambient noise in the zebrafish housing systems investigated revealed most sound energy concentrated below 1000 Hz and a gradual decrease in SPL towards higher frequencies. Several energy peaks were observed between 25-1200 Hz. Such irregular spectral shape contrasted with the 'smoother' curve shape and more gradual energy decline found in natural habitats. Other studies have also reported higher sound energy <1000 Hz in artificial housing systems[95,104,210]. Such low frequency noise is usually generated by water flows, ground vibrations, tank wall vibrations and electrical pumps, while higher spectral peaks might result from oscillating and collapsing air bubbles, aeration, but also electrical motors and water pumps[104].

According to Lawrence and Mason (2015)[226], in order to minimize noise sources in a zebrafish housing system, the rack should contain dampeners on stands that support pumps or other vibratory and noisy equipment. According to the authors, the water treatment system should be isolated from the rack in a separate enclosed room. Our results showed that the system HS3 with a separate water treatment unit is significantly less noisy compared to the stand-alone systems (HS1 and HS2), although the noise levels were still well above the natural habitats with considerably more energy within the best hearing range of zebrafish.

Natural versus artificial soundscapes: potential effects on zebrafish hearing?

Zebrafish is an ostariophysan species with relatively wide frequency range detection (100 to 8000 Hz) and best hearing sensitivity at 600-1000 Hz[212,214]. This species is known to inhabit diverse freshwater habitats, ranging from stagnant waters ponds to main rivers courses[181]. In this study we confirmed the presence of zebrafish in habitats that were considerably different in noise levels and spectral composition.

In order to evaluate potential hearing adaptation of the species to the various soundscapes, we considered auditory sensitivity curves previously determined from wild type zebrafish lines[169,212,215]. We are aware of potential differences in hearing sensitivity between zebrafish in the wild compared to lines maintained in captivity and between specimens reared in different facilities and, therefore, we considered four audiograms obtained in distinct laboratories to show potential variability within the same species and due to technical differences in AEP measurements. Variation in audiograms between laboratories may also result from distinct background noise conditions and masking effects during AEP recordings, hence these data should be considered cautiously. Comparing audiograms of wild type zebrafish lines with the various habitat noise spectra showed that this species is well adapted to all freshwater environments with probably some auditory masking in the fast-flowing river (SH). The noise spectral levels in SH were right below the species auditory thresholds between 800 and 2000 Hz in two of the audiograms reported[212,220]. Previous investigation on ostariophysan species belonging to the same family (Cyprinidae) have shown that habitat noise spectral levels that were just beneath the auditory thresholds within the most sensitive frequencies induced masking effects of about 9 dB (common carp *Cyprinus carpio*[203]) and 15 dB (topmouth minnow *Pseudorasbora parva*[227]).

Interestingly, the best hearing range of zebrafish (600-1000 Hz) matched a frequency interval where ambient noise spectra varied the most, but also a quieter window in the noisiest habitats located at 100-2000 Hz. Altogether this suggests that, similar to other ostariophysan species[203], zebrafish hearing sensitivity is well adapted to detect sounds in diverse freshwater habitats with different ecological characteristics (hydrology and substrate composition) and acoustic properties since the auditory thresholds are considerably above noise spectral levels or coincide with lower energy noise windows. However, the present study did not analyse the noise spectral levels during the rainy season, which might be

considerably higher causing additional auditory masking effects. Future research should consider year-round changes of zebrafish natural soundscapes, as well as more details on habitat noise variability and relationship with hydrodynamic factors.

Furthermore, our results showed significant differences in noise levels and spectral composition between the soundscapes of natural habitats and zebrafish housing systems. Natural habitats were more variable in SPL and richer in abiotic and biotic noise sources compared to housing systems, which revealed a constant noise mostly generated by the pump and filtering equipment. Comparing noise spectral levels revealed differences up to 60 dB especially below 1000 Hz. The artificial housing systems revealed spectral noise energy up to 22 dB above the species best auditory thresholds, which most likely induces significant masking effects and maybe even hearing loss. Gutscher et al (2011)[210] showed that 119 dB noise from external filters in aquaria induced auditory threshold shifts of up to 15-19 dB in *C. auratus* (noise level was about 8 dB above baseline audiogram). Wysocki and Ladich (2005)[219] reported that 130 dB of white noise evoked auditory thresholds shifts of up to 44 dB within the best hearing range of goldfish (*C. auratus*) (noise was up to 30 dB above baseline thresholds). Moreover, elevated noise levels may also induce hearing loss[143,228]. For example, Amoser and Ladich (2003)[143] exposed two otophysine species (goldfish *C. auratus*; and the catfish *Pimelodus pictus*) to 158 dB re 1 Pa white noise and identified significant hearing sensitivity loss within the species best hearing range after 12-24h of exposure (up to 26 dB in *C. auratus* and 32 dB in *P. pictus*). However, in this study the noise level was higher compared to the SPL registered in the zebrafish housing systems and the species studied presented lower auditory sensitivities compared to zebrafish. Nevertheless, the effects of chronic exposure (since early ontogeny and across multigenerations) to noise levels found in typical artificial housing conditions remains to be investigated in otophysine species such as the zebrafish.

This study establishes an important ground for future research on the role of environmental noise shaping zebrafish hearing abilities in the wild, and highlights the importance of controlling noise conditions in fish housing systems. Elevated noise levels in zebrafish housing facilities may affect development of auditory organs and subsequently may affect studies on inner ear structure and function. Future work should investigate auditory masking effects of noise generated in zebrafish housing systems, as well as, potential hearing loss and physiological stress.

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CHAPTER 3 – IMPACT OF NOISE ON DEVELOPMENT, PHYSIOLOGY, AND BEHAVIORAL STRESS IN ADULT ZEBRAFISH

Research article entitled "Impact of noise exposure on development, physiological stress and behavioural patterns in larval zebrafish"

Impact of noise on development, physiological stress and behavioural patterns in larval zebrafish

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Abstract

Noise pollution is increasingly present in aquatic ecosystems, causing detrimental effects on growth, physiology and behaviour of organisms. However, limited information exists on how this stressor affects animals in early ontogeny, a critical period for development and establishment of phenotypic traits.

We tested the effects of chronic noise exposure to increasing levels (130 and 150 dB re 1 μPa, continuous white noise) and different temporal regimes on larval zebrafish (*Danio rerio*), an important vertebrate model in ecotoxicology.

The acoustic treatments did not affect general development or hatching but higher noise levels led to increased mortality. The cardiac rate, yolk sac consumption and cortisol levels increased significantly with increasing noise level at both 3 and 5 dpf (days post fertilization). Variation in noise temporal patterns (different random noise periods to simulate shipping activity) suggested that the presence of longer silent intervals is important to down-regulate physiological stress. Moreover, 5 dpf larvae exposed to 150 dB continuous noise displayed increased dark avoidance in anxiety-related dark/light preference test and impaired spontaneous alternation behaviour.

We provide first evidence of noise-induced physiological stress and behavioural disturbance in larval zebrafish, showing that both noise amplitude and timing negatively impact key developmental endpoints in early ontogeny.

Keywords: noise, physiological stress, cortisol, development, anxiety, zebrafish.

Introduction

Anthropogenic noise has increased unprecedentedly in the last century both on land and underwater, being considered a global environmental pollutant by international legislation [49,77,229]. Noise pollution derives mostly from traffic, industry, resource extraction, construction and recreational activities, and it is expanding in time and space with subsequent negative impacts on human health and wildlife [44,230,231]. Besides a significant increase in overall sound levels, anthropogenic noise sources add new sounds into the environment that differ greatly in spectral composition and duty cycle from the natural soundscapes [61,232]. For instance, shipping and recreational boats add a broadband noise component into the overall aquatic acoustic scene that lasts much longer compared to pile driving and seismic air guns that generate impulsive short-lasting low frequency sounds [111,138].

Repeated or chronic exposure to increased noise levels can affect how animals respond to this stressor due to mechanisms of habituation and sensitization that rely on either augmented or decreased tolerance, respectively [152,233–236]. Shifts in tolerance to noise exposure, or to any other stressor, typically depend on duration, intensity and time regime of exposure [233,237], hence identifying patterns with less impact is paramount for defining noise sustainable management and mitigation strategies.

Noise is known to cause a myriad of detrimental effects in various taxa including auditory impairment [63,70,80,238], impaired development [141,239], heightened physiological stress [61,63,71,240] and behavioural disturbance [63,67,70,232,240], thus posing unprecedented risks on species survival, biodiversity and ultimately on ecosystems health.

Considering how fast aquatic soundscapes are changing [45], it is fundamental to develop research on how different noise regimes affect development, physiology and behaviour of fish, as they are key components of most aquatic ecosystems [75]. Fishes represent the largest group of extant vertebrates that is highly adapted to extract ecologically relevant

information from their diverse acoustic habitats for orientation, conspecific social interactions, reproduction, and prey and predators detection [163,241]. The disruption of such important acoustic cues may impose driving evolutionary pressures as species either adapt or try to avoid it [75,242,243]. An increasing amount of studies are showing the negative effects of anthropogenic noise on fish species, including lowered survivability [244], impaired growth [29,235], increased physiological stress [245,246], compromised hearing [72,228], and behavioural disturbance [26,247]. The majority of these studies, however, focused on the effects of short-term exposure to intense noise amplitudes, and most of the impact is likely to derive from less noticeable mild noise levels and repeated/chronic exposure [42]. This type of exposure can introduce modifications on how species respond to the stressor due to changes across time and cumulative effects.

Only few studies have evaluated long-term noise effects on animals [40,63,70,99] and particularly in early ontogeny [64,248] a critical period for development and establishment of phenotypic traits [249]. In fish, very scarce information exists on how chronic or repeated exposure to this environmental stressor impacts species in the adult stage [107,246,250] and the effects during larval survivability [208], development and behaviour [30,235], leading to augmented physiological stress such as cardiac rate [251]. So far, only one study evaluated how prolonged noise exposure varying in regularity (regular versus random noise) can impact fish in early development [235]. This study demonstrated that two days of both regular and random noise reduced growth, while regular regime led also to faster yolk sac consumption in larval Atlantic cod *Gadus morhua*. After 16 days, all treatments converged but regular noise-exposed specimens revealed impaired body conditions that could be associated to survival-related measures (predator avoidance) during development.

Further research is needed regarding the impact of chronic noise exposure on early development using model organisms that allow for integrative studies combining

morphological, physiological, behavioural and genetic approaches. This is key to gain insights into the physiological and molecular coping mechanisms underlying acoustic stress. The zebrafish *Danio rerio* has become a reference vertebrate model in the fields of developmental biology [191], ecotoxicology [252,253] and hearing research, including studies on the mechanisms underlying deafness and hair cell regeneration [254]. This species has been further used to test the impact of noise exposure on lateral line hair cells [255] and auditory-evoked escape responses [256], but the effects of acoustic stress on development, physiology and anxiety-related behaviors remain unknown.

In the present study we performed a split-brood experiment to test the effects of chronic exposure to noise of different amplitude levels and temporal patterns (random intermittent regimes simulating shipping activity) on development, physiological stress and behavioural traits in larval zebrafish. We used ecologically-relevant mild noise levels (105/control, 130 and 150 dB), which are representative of boat noise and amplitudes found in zebrafish freshwater habitats and housing systems [94].

Results

Hatching, growth and mortality

The noise treatments used in this study did not significantly affect the hatching rate of zebrafish embryos (comparison between noise levels: $F_{(2,\ 38)}=2.80$, $p>0.05$; temporal variations: $F_{(4,\ 47)}=0.76$, $p>0.05$), nor caused any obvious developmental abnormalities. Embryos started hatching at 2 dpf (i.e. Control: 10-11%; 130 dB continuous noise (CN_{130}): 9-10%; 150 dB continuous noise (CN_{150}): 7-8%, without differences between treatments and by 3 dpf all viable specimens hatched. Furthermore, changes in both noise level and temporal patterns did not affect the total length of the larval zebrafish measured at 3 dpf (noise levels:

$F_{(2, 131)}$=0.32, p>0.05; temporal variation: $F_{(4, 147)}$=5.19, p>0.05) nor at 5 dpf (noise levels: $F_{(2, 131)}$=0.17, p>0.05; temporal variation: $F_{(4, 143)}$=0.64, p>0.05).

However, an effect of noise exposure on mortality was observed throughout the acoustic treatments for specimens under continuous noise (CN) ($F_{(2, 38)}$=8.71, p<0.001), with CN_{150} causing a significant increase compared to CN_{130} (p<0.05) and control (p<0.001) (Fig. 3.1A). Variation in the timing of acoustic disturbances also affected mortality ($F_{(4, 47)}$=3.78, p<0.01). Short (IN1) and medium (IN2) noise periods of intermittent treatments induced higher mortality rates similar to CN (IN1 vs. control: p<0.01; IN2 vs. control: p<0.05), while the treatment presenting long noise segments (IN3) did not induce significant mortality compared to control (p>0.05) (Fig. 3.1B).

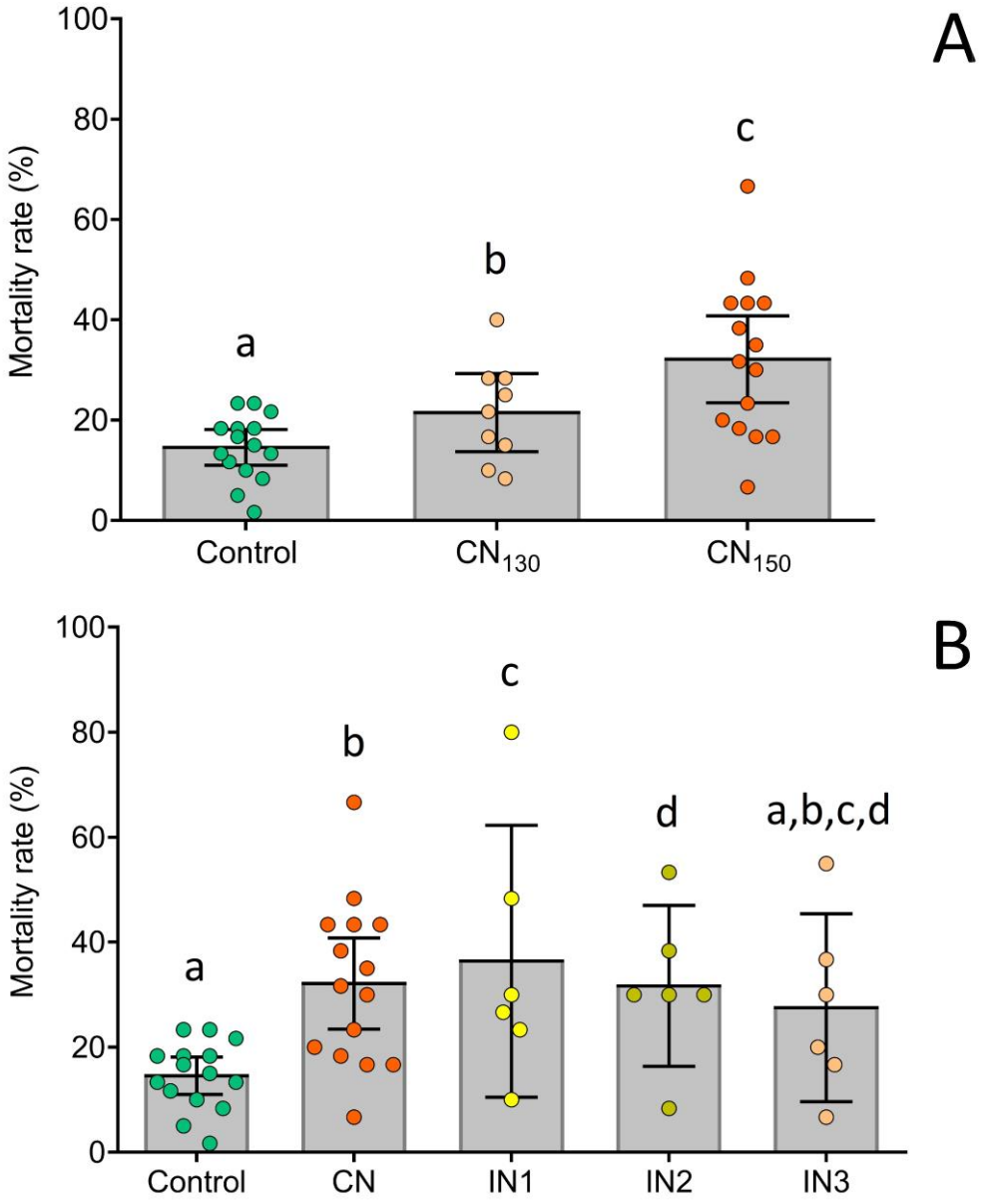

Fig. 3.1

Comparison of mean mortality rate between treatment groups (larval zebrafish up to 5 days post fertilization) exposed to A) continuous noise at different amplitudes ($F_{(2,\ 38)}$=8.71, p<0.001), and B) varying noise temporal patterns ($F_{(4,\ 47)}$=3.78, p<0.01). Control- silent conditions, CN- continous noise at either 130 (CN_{130}) or 150 dBre 1 μPa (CN_{150}), IN- intermittent regime with short (IN1), medium (IN2) and long noise segments (IN3). Error bars represent 95% confidence intervals. Different letters indicate statistically significant differences between specific groups based on post hoc tests.

Physiological stress indicators

Cardiac rate, yolk sac consumption and cortisol levels increased significantly with increasing noise level, which clearly indicated higher physiological stress. The cardiac rate of larval zebrafish at 3 dpf was about 173 ± 30 bpm (mean ± SEM, standard error of the mean; bpm, beats per minute) for control and increased to 191 ± 60 bpm under playback of CN_{150}, whilst at 5 dpf it was around 203±40 (control) and increased to 224 ± 50 bpm under CN_{150}. A significant increase was verified with increasing noise level in both 3 dpf ($F_{(2, 134)}$=4.20, $p<0.05$) and 5 dpf larvae ($F_{(2, 133)}$=7.17, $p<0.001$) with CN_{150} causing the highest differences compared to control ($p<0.001$) (Fig. 3.2A).

Similarly, the yolk sac consumption increased significantly with noise level at both 3 dpf ($F_{(2, 136)}$=11.96, $p<0.01$) and 5 dpf larvae ($F_{(2, 134)}$=16.59, $p<0.01$), with CN_{150} also inducing the highest differences ($p<0.001$) (Fig. 3.2B).

Variation in the timing of the acoustic disturbances caused different effects on both cardiac rate and yolk sac consumption, suggesting that noise temporal regime is important to regulate physiological stress and depletion of embryonic endogenous energy reserves. The cardiac rate was significantly affected by noise time variations at both 3 dpf ($F_{(4, 146)}$=25.36, $p<0.001$) and 5 dpf ($F_{(4, 143)}$=15.50, $p<0.001$), with continuous noise causing the highest impact compared to intermittent treatments ($p<0.001$) (Fig 3.2C). IN3 induced consistently the lowest impact among intermittent regimes at both 3 and 5 dpf ($p<0.01$ and $p<0.05$ respectively). Additionally, significant differences in the yolk sac consumption were found between these noise treatments at 3 dpf ($F_{(4, 145)}$=13.79, $p<0.001$) and 5 dpf ($F_{(4, 143)}$=12.19, $p<0.001$) (Fig. 3.2D). CN induced the highest yolk sac consumption compared to intermittent noise groups at 5 dpf.

Overall, the cardiac rate was negatively correlated with yolk sac size (R= -0.61, N=370, p<0.001), meaning that individuals with higher cardiac rate also consumed their nutritional and energy reserves faster (Fig. 3.2E).

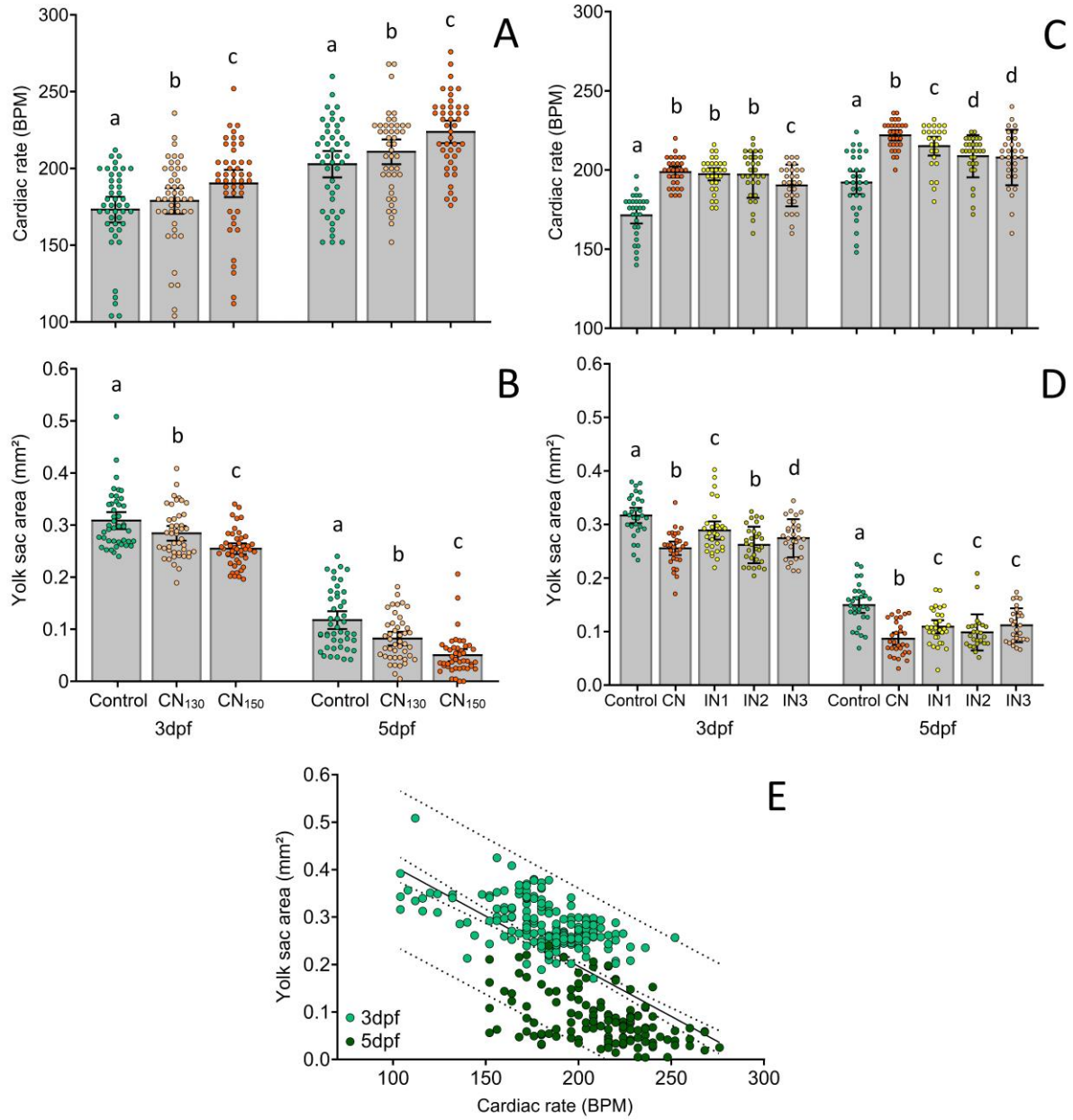

Fig. 3.2

Comparison of mean cardiac rate and yolk sac area of larval zebrafish with 3 and 5 dpf (days post fertilization) exposed to different noise amplitudes (A and B) and temporal patterns at 150 dBre 1 µPa (C and D). Increasing noise amplitudes induced heightened cardiac rate at 3

dpf ($F_{(2, 134)}$=4.20, p<0.05) and 5 dpf ($F_{(2, 133)}$=7.17, p<0.001), as well as a decrease in yolk sac (3 dpf - $F_{(2, 136)}$=11.96, p<0.01; 5 dpf - $F_{(2, 134)}$=16.59, p<0.01). Cardiac rate was further affected by noise temporal variation at 3 dpf ($F_{(4, 146)}$=25.36, p<0.001) and 5 dpf ($F_{(4, 143)}$=15.50, p<0.001), as well as the yolk sac 3 dpf ($F_{(4, 145)}$=13.79, p<0.001) and 5 dpf ($F_{(4, 143)}$=12.19, p<0.001). Error bars represent 95% confidence intervals. Different letters indicate statistically significant differences between specific groups based on post hoc tests. (E) Negative correlation between cardiac rate and yolk sac size (R=-0.61, N=370, p<0.001) at both 3 and 5 dpf. Solid line - best-fitted line; inner dashed lines - standard error of the mean; outer dashed lines - 95% confidence interval.

Noise-induced physiological stress was confirmed through whole-body cortisol quantification. Cortisol levels increased significantly with noise amplitude at both 3 dpf ($F_{(2, 35)}$=4.84, p<0.05; CN_{150} higher than control p<0.01) and 5 dpf ($F_{(2, 29)}$=4.37; p<0.05; both CN_{130} and CN_{150} higher than control p<0.05 and p<0.01 respectively) (Fig. 3.3A). Variation in the noise time regime caused changes in cortisol, however, they were not statistically significant - 3dpf ($F_{(4, 45)}$=2.23, p>0.05); 5 dpf ($F_{(4, 35)}$=2.65, p>0.05) (Fig. 3.3B).

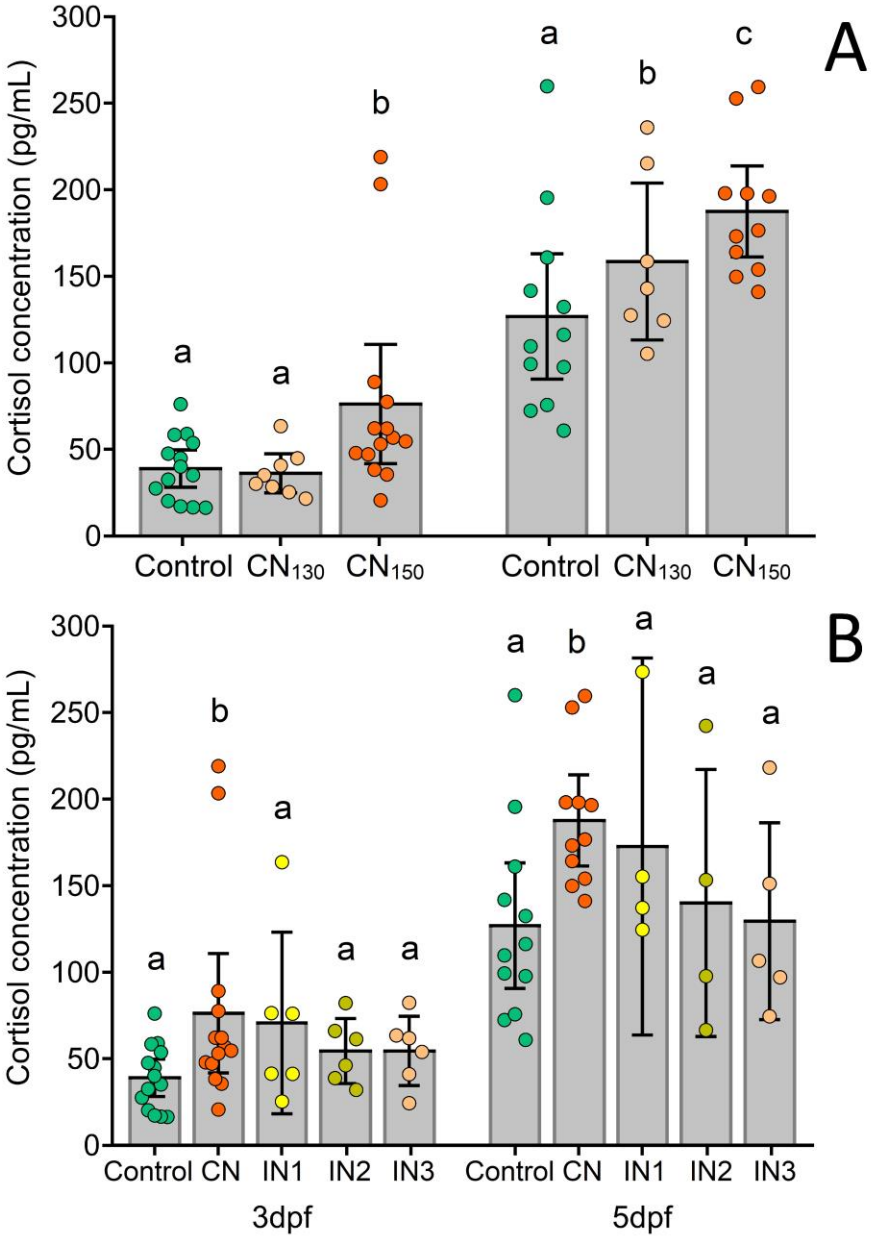

Fig. 3.3

Whole-body cortisol levels from larval zebrafish exposed to (A) continuous noise at different amplitudes (3 dpf: $F_{(2, 35)}$=4.84, p<0.05; 5 dpf: $F_{(2, 29)}$=4.37, p<0.05) and B) varying noise temporal patterns at 150 dB re 1 µPa (3 dpf: $F_{(4, 45)}$ =2.23, p>0.05; 5 dpf: $F_{(4, 35)}$=2.65, p>0.05). Control- silent conditions, CN- continous noise at either 130 (CN_{130}) or 150 dBre 1 µPa (CN_{150}), IN- intermittent regime with short (IN1), medium (IN2) and long noise segments (IN3). Error bars represent 95% confidence intervals. Different letters indicate statistically significant differences between specific groups based on post hoc tests.

Behavioural patterns

In order to assess potential changes at the behavioural level, 5 dpf larvae exposed to 150 dB continuous noise (CN_{150}) were further tested using an anxiety-related dark/light preference test (Fig. 3.5A). Specimens exposed to the acoustic stressor exhibited stronger dark avoidance or scotophobia, as measured based on a choice index ($U_{(2, 173)}$=5341.50; $p<0.001$) (Fig. 3.5B).

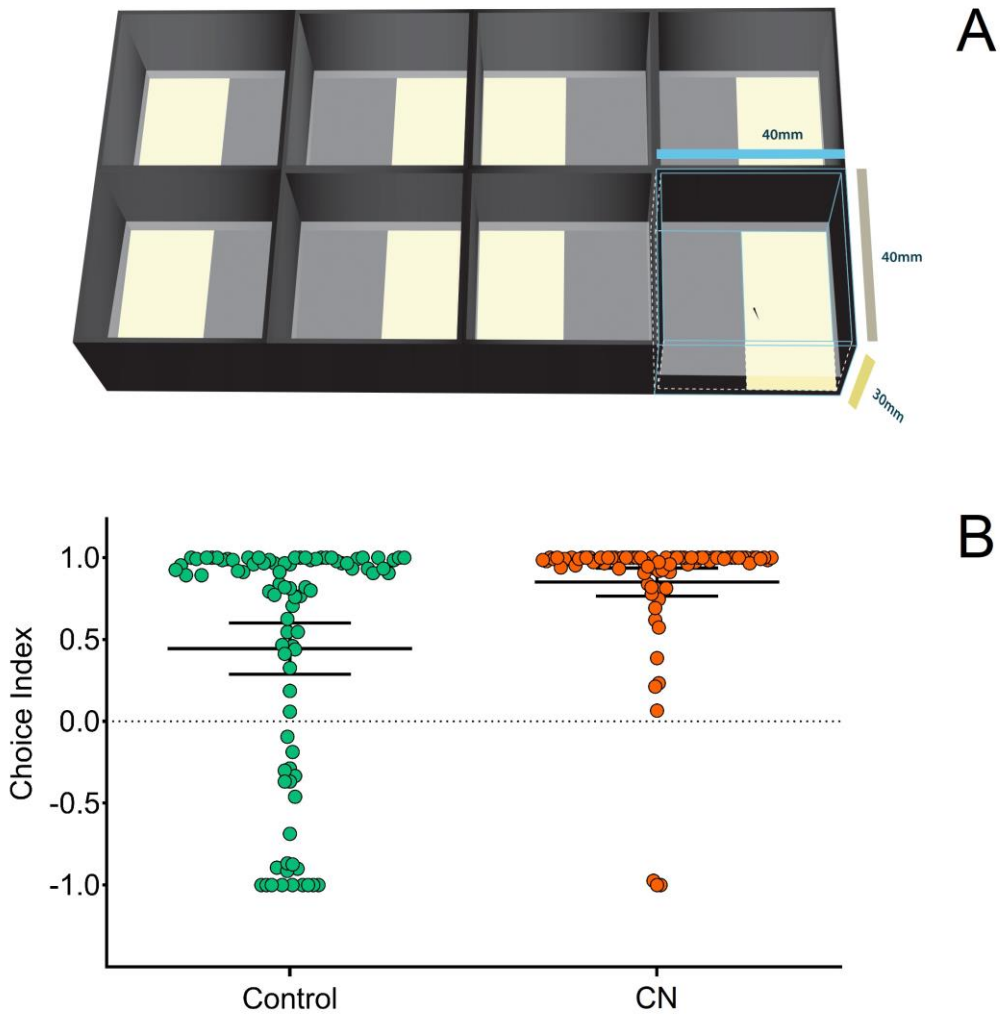

Fig. 3.4

A) Light/dark preference assay consisting of squared plastic compartments (each 40 mm width x 40 mm length x 30 mm height) divided into two equal sized areas with distinct

bottom illumination (transparent/bright versus opaque/dark). The apparatus was placed on top of a LED panel (~7000 lux). Each compartment was filled with 10 ml water and a single larval zebrafish (5 dpf) was placed in the middle of the arena and recorded for 5 min. B) Choice index for larva exposed to continuous noise (150 dBre 1 µPa) versus control conditions ($U_{(2, 173)}$=5341.50, p<0.001). Choice index was calculated as: (Time in dark–Time in light)/(Time in dark + Time in light). Individual data are presented as scatter plots and bars depict mean ± 95 % confidence intervals.

Larval zebrafish (5 dpf) were also tested regarding exploratory behaviour and mnestic capabilities using the Spontaneous Alternation Behaviour (SAB) assay (Fig. 3.6A). The SAB assay revealed that 70% of larvae reared under silent control conditions exhibited normal swimming alternation, as opposed to only 34% from the noise treatment group (CN_{150}) ($t_{(113)}$ = -4.08, p<0.001) (Fig. 3.6B). The swimming patterns of these specimens were investigated in an open field arena, which revealed a reduction in covered area for the noise-treated larvae ($t_{(89)}$=7.33,p<0.001).

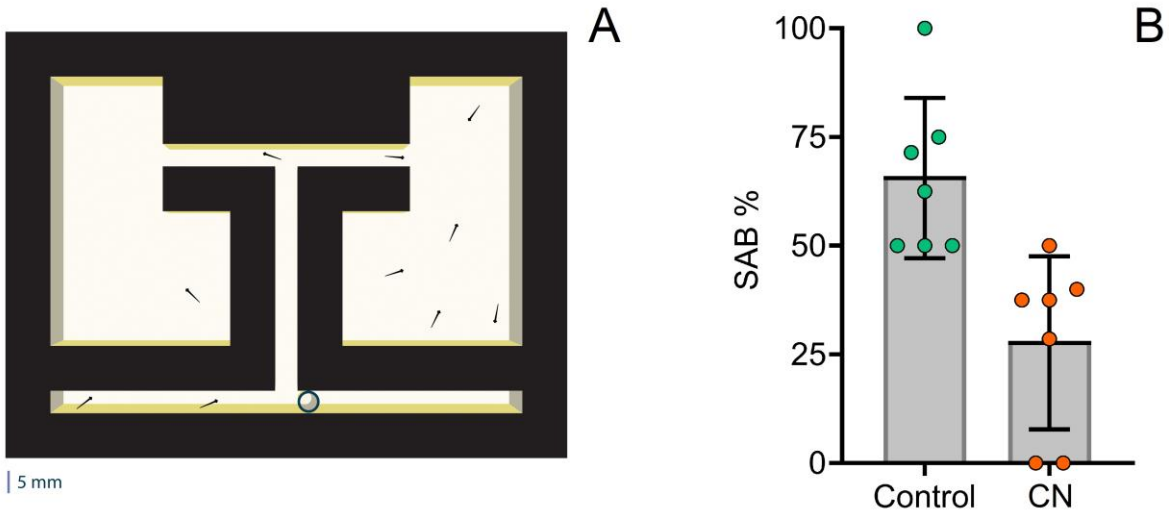

Fig. 3.5

A) Spontaneous Alternation Behaviour (SAB) assay with bottom illumination to test exploratory swimming and spatial memory. The starting arms can be used alternatively (a

plastic tube blocks the entrance to the opposite arm) and converge into a perpendicular main arm that leads to a choice of alternation or same side arm. These arms lead to distinct pools of 19.50 mm^2. B) Comparison of SAB in 5 dpf under continuous noise at 150 dBre 1 μPa (CN) and control conditions ($t_{(113)}$= -4.08, $p<0.001$). From a total of 180 tested larvae, 115 successfully showed alternation behavior (entered the opposite side pool) within the 10-minute recording. Individual data are presented as scatter plots and bars depict mean ±95 % confidence intervals.

Discussion

To our knowledge this is the first study assessing the impact of noise exposure on larval zebrafish (*Danio rerio*), a reference model organism in ecotoxicology. We provide evidence of noise-induced physiological and behavioural disturbance in larval zebrafish, and demonstrate that both amplitude and timing of the acoustic stressor may impact key health-related endpoints in early ontogeny.

The acoustic treatments considered in the present study did not affect hatching or general development but higher noise levels (130 and 150 dB re 1 μPa) led to increased mortality (from 14-15% in control up to 32-33% at the highest sound level). Very limited information exists on the impact of noise on fish hatching success and survival. Banner and Hyatt (1973) found significant lethal effects on fish embryos (*Cyprinodon variegatus*, Cyprinodontidae) exposed to higher noise conditions in a tank system with two distinct acoustic zones, but no effect was detected at the post hatching stage (>24 hpf) [208]. Bruintjes and Radford (2014) evaluated the impact of small motorboat noise (127 dB re 1 μPa RMS) on a cichlid fish (*Neolamprologus pulcher*) and did not find an effect on hatching success or fry survival [257].

Previous studies described mixed results in regards to the impact of noise on fish growth. Banner and Hyatt (1973) reported noise-induced decreased growth in larvae during the first 11-15 days post-hatch [208], whereas Bruintjes and Radford (2014) described an absence of effects on body length or weight after 4 weeks post-hatch [257]. Both Davidson et al (2009) and Nedelec et al (2015) found that increased noise levels significantly reduced development in different fish species (rainbow trout *Oncorhynchus mykiss* [105] and Atlantic cod *Gadus morhua* [235], however this was followed by a catch-up growth at later stages.

In the present study, the intermittent treatment with many onsets of acoustic disturbance (IN1, characterized by 5-12 sec noise and 1-120 sec silence, about 15% total noise) caused higher mortality up to 33%, similar to continuous noise exposure. This was significantly above baseline levels in control group (15%) and contrasted with the IN3 treatment with prolonged noise presentations (total 50% noise presence) but fewer onsets that caused 24% in mortality. IN2 (with similar noise exposure to IN1 but extended silent periods of 1-10 min) caused about 32%. The relevance of the timing of acoustic exposure has received limited attention but increasing evidence points towards a significant impact on development and physiological stress in different fish species [235,258]. Nedelec et al (2015) reported no difference in body length of larval codfish exposed to either regular or random playback of ship noise (average 15 min noise playback/hour) [235]. The body width-length ratio, however, declined over the course of the study and the greatest decline was registered under regular noise treatment at 16 days post-hatch. These larvae were easier to catch in a predator-avoidance experiment, demonstrating that the timing of acoustic disturbance can impact survival-related measures during development. Additionally, Neo et al (2014) assessed the impact of continuous versus intermittent regular (0.1s noise plus 0.9 s silence) noise on seabass adults (*Dicentrarchus labrax, Moronidae*) and found that intermittent exposure resulted in slower normal behaviour recovery compared to continuous treatment [258].

The present study also found heightened cardiac rate, yolk sac consumption, and cortisol levels at both 3 and 5 dpf zebrafish treated with elevated noise levels, which is a clear indication of noise-induced physiological stress. The control cardiac rate values varied between 150 and 220 (3 and 5 dpf, respectively), which is comparable to prior studies on the same model organism [259–261]. We verified an increase of about 10% in both ontogenetic stages (3 dpf: from 173 to 191 bpm; 5 dpf: 203 to 224 bpm) under increased noise conditions (CN_{150}). Simpson et al (2005) and Jain-Schlaepfer et al (2018) reported first evidences of higher cardiac activity due to anthropogenic noise in fish larvae [251,262]. The authors found that the embryos (staghorn damselfish *Amblyglyphidodon curacao* and clownfishes *Amphiprion spp.*) increased their heart rate (up to 5%) in the presence of increased levels of shipping noise, and that the physiological impact depended on the engine type.

Increased cardiac activity represents an adrenergic stress response, which is typically responsible for activating metabolic pathways and mobilizing energy to cope with potential challenges [263–267]. In this case, the perceived challenge consisted on acoustic disturbance, which is not a life-threatening situation. Hence, the energy depletion due to acoustic stress might be detrimental to the embryos that could otherwise use it for survival–related developmental processes.

To our knowledge, the present study is the first to measure cardiac rate and related it with endogenous embryonic energy substrates (yolk sac size) in fish larvae. We provide evidence that these variables are significantly correlated. In addition, we confirmed the heightened physiological stress in noise-exposed larvae by measuring their cortisol levels that were significantly above control groups. Chronic exposure to an environmental stressor may interfere with resource allocation from reserves maintenance to activation of the adrenal system, resulting in allostatic load [268]. Our study shows that larval zebrafish under noise exposure (CN_{150}) consume their yolk sac 18% (at 3 dpf) and 58% (at 5 dpf) faster compared

to baseline conditions. Considering that noise-exposed individuals showed similar development (body size) to control group, we predict that the increased yolk consumption is not being invested in a faster development but reflects additional survival costs to cope with acoustic stress. The increased mortality registered in the noise-treated group further supports this hypothesis. Nedelec et al (2015) also reported an effect of shipping noise on yolk sac consumption in larval codfish [235]. Larvae exposed to regular noise used their yolk sac 29% faster after 2 days of exposure and had a lower body width-length ratio after 16 dph (days post hatching) compared to specimens raised in quieter ambient noise conditions. Other studies have used the yolk sac size of larval fish to assess the impact of environmental stress [269–272], and likewise showed that variables like salinity, temperature, light conditions and even maternal stress, can lead to a significant impact on the yolk sac absorption rate and also composition.

Our work provides first evidence of noise-induced increase in whole-body cortisol levels in a larval fish. Cortisol elevation due to environmental stress, such as salinity, temperature, light conditions, acidity, mechanical disturbance, has been previously observed in larval zebrafish. For instance, Bait et al 2016 reported an elevation in cortisol/protein of about 65% under cold conditions, 40% under UV light and almost 100% under mechanical disturbance. While measuring units widely vary between studies, cortisol levels are consistently within a range of 5-10 pg/larva (whole body, 3-7 dpf range) for control and 200-250 pg/larva for stressed individuals. This represents a similar increase age-dependent range of ~10-45%, compared to the change recorded in the present study [273–277].

Variation in the temporal patterns of intermittent treatments caused different effects on cardiac rate, yolk sac consumption, and nearly on cortisol levels (although not statistically significant), suggesting that time regime is important to down-regulate physiological stress. Overall, continuous noise exposure induced the highest cardiac rate and yolk consumption

compared to intermittent treatments, whilst IN3 characterized by longer noise playback but also prolonged silent intervals caused less impact on these variables compared to control and other IN treatments. Moreover, cortisol levels were generally elevated in noise-exposed groups, and the highest level reached under continuous noise treatment. Although not significantly different, IN3 induced lower cortisol level compared to IN2. Similar to Nedelec (2015), we hypothesize that longer time intervals during random disturbances allow for recovery, compensation and/or habituation in larval fish, and that the total duration of noise exposure is less crucial compared to the time regime adopted (number of onsets of acoustic disturbance and silent intervals) [235].

The present study also investigated the impact of noise exposure (CN_{150}) at the behavioural level in larval zebrafish. We focused on 5 dpf, a developmental stage when larvae acquire full motility, display active feeding behavior and danger/predator avoidance, suggesting that simple neural circuitries for processing reward and aversion are already functional [39,278]. We tested the effect of CN_{150} treatment on 5 dpf larvae using the anxiety-related light/dark preference test, which has been widely used to test stress and anxiety in mammals [279] and zebrafish [276,280–283]. Our results indicate increased darkness aversion (scotophobia) in noise-exposed larvae, suggesting that such environmental stressor elicits anxiety behaviour. Similarly, Bai et al (2016) reported that heat, cold and UV treatment significantly enhanced darkness aversion in larval zebrafish [276]. The authors also found that treatment with two anxiolytics with different pharmacokinetics (chlordiazepoxide, a GABAergic benzodiazepine and buspirone, a serotonin agonist) attenuated this behavior, which confirmed that such pattern was anxiety-driven. Future research should consider testing the effect of different anxiolytics on noise-treated zebrafish and evaluate light/dark preference at different development stages.

Finally, this study also showed that noise-exposed 5 dpf larvae (under CN_{150}) displayed impaired innate Spontaneous Alternation Behaviour compared to control individuals. Bögli *et al*, (2017) [284] effectively established the presence of SAB in larval zebrafish (6 dpf) suggesting the presence of early mnestic capabilities. At this developmental stage (4 to 5 dpf), the hippocampal-like pallium develops [285], and this brain structure is known to be related with navigation and spatiotemporal sensing in fishes [286,287]. Other studies have investigated the effect of acoustic experience in memory function. For instance, Cheng et al (2011)[288] reported impaired learning and memory capabilities in mice after exposure to white noise at 80 dB SPL for 2 hours per day for a 6-week period, which caused an increase in enzymes levels and reactive compounds in different brain structures including the hippocampus. Other authors have reported changes at early ontogeny of postnatal mice and rats further supporting our discoveries regarding early impact of noise in menstic and sensorimotor capabilities [289,290].

Additionally, the overall locomotor activity of these specimens was further investigated and a significant reduction in covered area was observed for the noise-treated larvae. This results are in line with Bhandiwad et al 2018 that investigated the effect of noise exposure (white noise at 20 dB re 1 ms^{-2}) on larval zebrafish (5-7 dpf) and identified a significant decrease in total distance compared to control [256]. Further studies are needed to confirm whether impaired SAB resulted from spatial memory dysfunction and/or changes in the exploratory behaviour that are anxiety-driven or related to motor function.

In summary, we provide first evidence of noise-induced physiological stress and behavioural disturbance in larval zebrafish, showing that increased noise amplitude and changes in the noise temporal regimes can induced higher cardiac rate and activate the adrenal system leading to increased cortisol levels and depletion of embryonic endogenous energy reserves. Intermittent sounds with short duration, such as those commonly found in

aquatic systems with elevated traffic activity from small motor boats, speed boats and personal water crafts [98,223,291–293], may have a stronger physiological and behavioural consequences on fish, including higher mortality, physiological dysfunctions and behavioural alterations, compared to regimes of similar or higher overall noise exposure but less number of noise onsets and longer silent periods.

Future studies are required to evaluate whether noise-induced energetic costs may result in carryover effects to subsequent life stages and fitness. We show that larval zebrafish can be established as a high-throughput platform for fast screening of acoustic disturbances and their biological impact at developmental, physiological and molecular levels. Furthermore, we highlight the importance of noise regularity and its consideration for noise management and mitigation strategies.

Methods

Fish husbandry and sampling

Zebrafish eggs were obtained from wild type adults (AB line) purchased from China Zebrafish Resource Center (CZRC, China) and reared in our research facilities at the University of Saint Joseph, Macao. Stockfish were maintained in a standalone housing system (model AAB-074-AA-A, Yakos 65, Taiwan) with filtered and aerated water (pH balanced 7-8; 400-550 µS conductivity) at 28±1 °C and under a 12:12 light/dark cycle. For each experimental trial, eggs were collected within 2 hpf (hours post fertilization) from 2 to 6 breeding tanks (each tank containing about 10 females and 5 males). Collected eggs were mixed, distributed into 3 or 5 groups of 50 specimens, and each group allocated to a different treatment tank (see details below). This split-brood approach was adopted to minimize potential differences related to egg quality/viability.

Egg survivability was evaluated during the first 48 hpf after examination of the batches at a

fixed time in the morning (between 10-11 am). Morphological and physiological data was consistently collected at the same time in the morning at two developmental stages, 3 and 5 dpf. These stages of development were selected since at 3 dpf embryos already have a functioning inner ear [294], and at 5 dpf specimens already exhibit active feeding and sensorimotor behaviors, such as auditory-evoked escape responses, that are affected by noise exposure [256].

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 (China), license AL017/DICV/SIS/2016.

Experimental design and acoustic treatments

A total of 15 experimental trials consisting of simultaneous treatments of either different noise amplitudes or varying temporal patterns (at the same noise level each) were conducted. The acoustic treatments 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 to maintain appropriate water quality similar to stock conditions. Each treatment tank was mounted on top of Styrofoam boards placed over two granite layers (1.5 cm thick) spaced by rubber pads to reduce non-controlled building vibrations. Eggs were placed inside a custom-made cylindrical fine-mesh netbox (5 cm diameter, 6 cm high) suspended at ~7 cm above an underwater speaker (UW30, Lubel Labs, Ohio, USA) that rested on top of a sponge base to minimize transmission of playback vibrations into the tank bottom (Fig. 3.7A). Speakers were connected to audio amplifiers (ST-50, Ai Shang Ke,

China) that were connected to laptops running Adobe Audition 3.0 for windows (Adobe Systems Inc., USA). A total of 5 experimental tanks were used alternately for the different treatments across the various trials. In **"**control**"** groups, the amplifier connected to the speaker was switched on but without playback, reaching a background noise level varying between 103 and 108 dB re. 1 µPa.

Sound treatments consisted of white noise low-pass filtered at 1500 Hz and adjusted to the tank acoustic properties using Adobe Audition software tools to deliver a relative flat spectrum. To test for the effects of amplitude on larval zebrafish, two sound files of continuous white noise at different amplitudes or Sound Pressure Levels (SPLs), namely 130 dB (CN_{130}) and 150 dB re. 1 µPa (CN_{150}), were generated and played back in loop. These noise levels were similar to those found in freshwater aquatic habitats characterized by anthropogenic noise activity such as shipping [93,121] and noise conditions in certain zebrafish housing systems [94]. To evaluate the impact of different noise temporal regimes, three additional sound files of intermittent noise and 60 min duration were generated and played back in loop at 150 dB re 1 µPa (Fig. 3.7B): IN1- short noise segments of 5-12 sec duration spaced by silent intervals of 1-120 sec (60 noise events per hour) reaching an overall noise exposure of about 15%, designed to mimic intense boat traffic noise as described in Nichols et al 2015 [291]; IN2- medium noise segments of 30-60 sec interspaced by 1-10 min silence, with 15 noise segments per hour and similar overall noise exposure to IN1; and IN3- long noise segments of 15 min separated by 15 min silent periods (about 50 % overall noise), which followed a prolonged shipping activity as described by Nedelec et al (2015) [235] (Fig. 3.7B). All noise presentations contained a logarithmic fade-in and fade-out ramps of 10% of the noise presentation. We used random exposure regimes since it reflects better an acoustic environment characterized by traffic noise and it is known to cause physiological stress in fish [235,291], and affect specifically zebrafish behaviour [295].

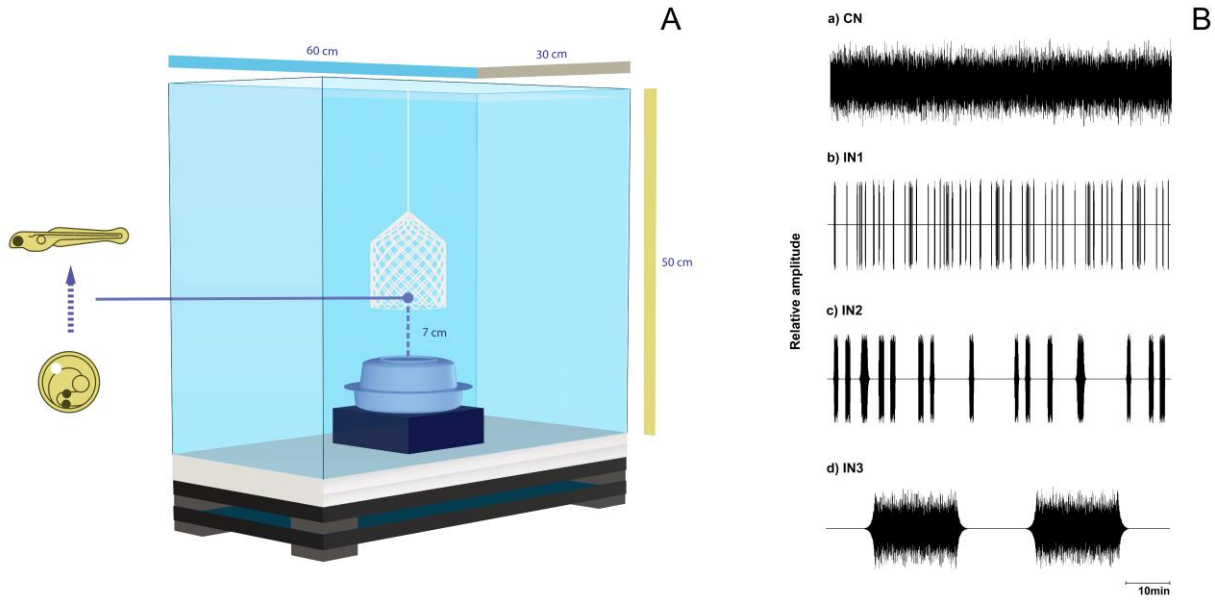

Fig. 3.6

A) Diagram of the acoustic treatment tank. The tank rested on top of two granite plaques separated by anti-vibratory rubber pads. Inside, a custom-made net cylinder containing zebrafish egg/larvae was suspended 7 cm above an underwater speaker (UW30, Lubel Labs, Ohio, USA) that rested on top of a polyurethane sponge. B) Oscillogram of sound files used for playbacks. Control- silent conditions, CN- continuous noise at either 130 (CN_{130}) or 150 dB re 1 µPa (CN_{150}), IN- intermittent regime with random short noise segments (IN1): 5-12 sec duration spaced by silent intervals of 1-120 sec (total noise exposure of c. 15%); medium noise segments (IN2): 30-60 sec interspaced by 1-10 min silence (similar noise exposure to IN1); and long noise segments (IN3) of 15 min separated by 15 min silent periods (about 50 % overall noise).

Noise levels were calibrated before each treatment so that the intended sound level (LZS, RMS sound level obtained with slow time and linear frequency weightings: 6.3 Hz-20 kHz) were reached at the bottom of the net box (~7 cm distance from the speaker) using a hydrophone (Bruel&Kjær 8104, Naerum, Denmark; frequency range: 1–80 kHz ± 2 dB; voltage sensitivity: -184 dB re 1V/lPa^{-1}) 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, sensitivity 1–3 kHz, GeoSpectrum Technologies, Dartmouth, Canada). The particle acceleration sensor was placed horizontally with the acoustic center also positioned at about 7 cm from the speaker in the position occupied by the netbox containing the specimens. Particle acceleration level determined for the highest sound level (150 dB re 1 µPa) in the vertical axis consisting of 120 dB/s^2. A decrease of 20 dB in sound pressure resulted in similar variation in acceleration. The sound playbacks generated most energy in the vertical axis compared to the other orthogonal directions. The calibration was conducted using a Matlab script (paPAM) based on Nedelec et al. (2016) [296].

Morphological and cardiac rate measurements

The impact of the acoustic treatments on morphological development and cardiac rate of larval zebrafish was assessed based on 5 specimens per treatment in each experimental trial. The specimens were lightly sedated using low concentration (0.004%) of MS-222 buffered with sodium bicarbonate to quantify cardiac activity following previously described procedures [297], and then observed under a stereomicroscope (Stemi 2000CS, Carl Zeiss, Jena, Germany) connected to a digital camera (Axiocam, ICc3, Carl Zeiss) and a desktop running Zen 2.3 Lite (Carl Zeiss). Photographs were taken from each specimen in lateral position for determining total body length and yolk sac size based on Teixidó *et al* 2019 [298], followed by a video of 60 sec focusing on the cardiac area. Both photographs and videos were analysed

using specific tools for morphological analysis and automatic detection of cardiac activity of DanioScope software (Noldus Information Technology, Wageningen, Netherlands). The yolk sac size was defined as the lateral area (xy coordinates) delimitated manually. The cardiac rate detection was also done manually to validate the software automatic measurements and whenever the video quality was not appropriate.

Cortisol quantification

The analyses were conducted based on 10 larvae collected as one sample from the different treatments. We followed previously described procedures by Bai et al 2016 [276] adapted to improve extraction process. Specimens were euthanized in MS-222 300mg/L (Thermo Fisher Scientific INC, Massachusetts, USA), excess water was removed, and individuals frozen at -80°C in collection tubes. Prior to analysis, samples were thawed by adding 150 µl of ice-cold Millipore Ultrapure water to each tube and homogenized with a hand-held pellet mixer for 20 seconds. Immediately, 450 µl of pure methanol were added to each sample and vortexed at max speed for 30 sec. Samples were placed at 4°C for 1 hour, vortexed again, and centrifuged at 3000 g for 10 min at 4°C. The liquid phase was carefully collected and transferred to a new vial. The methanol extraction was again repeated following same procedure and the liquid phase again collected into the same vial. Liquid nitrogen-evaporated samples were reconstituted in 500 µl of enzyme immunoassay buffer and kept at -80°C for less than 24 h, and vortexed prior to assay. Samples were tested in duplicate using a colorimetric cortisol enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI, USA).

Light/Dark choice assay

Larval zebrafish (5 dpf) exposed to either continuous noise (CN_{150}) or control conditions were tested with the anxiety-related light/dark preference assay, which is a known

behavioural paradigm to assess environmental stress in larval zebrafish [276]. Increased percentage of time spent in the light side is assumed to reflect increased anxiety in this model organism, which was previously found to prefer light or show dark avoidance (scotophobia) [299]. The light/dark preference apparatus was designed based on Bai et al (2016) [276] and consisted on eight individual squared transparent plastic compartments (4.0 cm length x 4.0 cm width x 3.0 cm height), each divided into two equal sized areas with distinct bottom illumination conditions - transparent/bright and opaque/dark (Fig. 3.5A). The apparatus was placed on top of a LED panel covered with a white translucent glass to provide uniform illumination (~7000 lux). The sides of the compartments were covered with black vinyl tape to control for external visual interferences. Each compartment was filled with 10 ml of system water at about 28 °C (water depth: ~5 mm). A single larva was carefully placed with a plastic pipette in the middle region between the two areas in each of the four middle compartments and a 5 min video covering the testing area was recorded. A total of 11 trials with four simultaneous test compartments were conducted. The videos were analysed regarding the time spent in each area and a Choice Index calculated: time spent in darkness − time spent in light)/(time spent in darkness + time spent in light). Meaning that (0) reflects no preference, (−1) larva spent all the time swimming in the light zone, and (1) all the time in the dark zone (Fig. 3.5B).

Spontaneous alternation behaviour assay

Larval zebrafish were further tested regarding the Spontaneous Alternation Behaviour (SAB), which consists on the tendency of animals to alternate their movement directions in consecutive turns when navigating through their environment. The SAB was assessed in a 3D printed T-maze designed based on Bögli and Huang (2017) [300] and consisted in two starting arms converging into a main arm that bifurcated in the end into two goal arms, each leading

to a pool (Fig. 3.6A). Starting and main arms had a length of 5.0 cm, while goal arms measured 2.5 cm. All arms had a width of 0.5 cm and depth of 1.0 cm, and the goal arenas were 19.50 cm^2. The maze was illuminated from below by placing it on top of a LED panel**.** For each of the 9 trials, 10 larvae were tested simultaneously. The maze was prefilled with fresh system water at 28°C (to a depth of about 0.7-0.75 cm) and the intersections between the two starting arms and the main arm were both initially closed with plastic tubes. Testing larvae were carefully placed simultaneously using a plastic pipette in one of the starting arms and, after 5 min of acclimation, the respective tube was removed to start the trial. Successful entry was counted when a larva fully entered one of the goal arenas within the 10 min of the recording. In the case of returning to the main arm and/or entering the second goal arena after a prior successful goal arm entry, only the first entry was counted. In order to evaluate potential changes in motor behaviour due to acoustic stress, another set of specimens subject to the same treatment/control (10-15 per trial) were analysed regarding the total swimming area in petri dish (8 cm diameter) based on video analysis of the area covered using a digitally overlayed fine grid.

Data analysis

Comparisons of hatching rate, total length, mortality, cardiac rate, yolk sac size and cortisol levels between groups exposed to different noise levels and temporal patterns variations were based on One-way ANOVA tests. These were followed by LSD multiple comparison post hoc tests to verify pairwise differences. The relationship between cardiac rate and yolk sac size was further assessed with a Pearson's correlation coefficient.

The potential effects of the batch/trial on batch quality, such as embryo length and mortality, were assessed through mixed models containing "batch" as a random factor, and no interaction between these variables was detected.

Differences in light/dark preference were quantified based on a Choice Index and compared between treatment groups based on a two-tailed Man-Whitney U test. Finally, the presence of spontaneous alternation behaviour and the total swimming area were compared between noise-exposed and control groups using 2-tailed t-tests.

The 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 SPSS v26 (IBM Corp. Armonk, NY, US) and Statistica 10 for Windows (Dell Software, Inc., Round Rock, T, USA).

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Authors' contribution statement

R.V. conceived the idea and conceptualized the research. Both authors contributed to the design of the methodology. R.L. assembled and carried out the experiments, conducted data acquisition and analysis. Both authors contributed critically to the interpretation the results and wrote the manuscript.

CHAPTER 4 – EFFECTS OF NOISE ON THE AUDITORY SYSTEM DURING DEVELOPMENT

Research article entitled "Evidence of noise-induced changes in development of the inner ear and hearing sensitivity in larval zebrafish"

Auditory sensitivity loss correlates with inner ear hair cell decrease in larval zebrafish under noise conditions

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Abstract

Elevated levels of noise are widely present in most aquatic soundscapes and to an even greater extent in artificial environments, however, very limited information is known on how this important environmental stressor, impacts species' hearing capabilities and inner ear morphology during early ontogeny. Fishes are highly specialized in extracting ecologically relevant information from their diverse acoustic habitats since early developmental stages. The zebrafish (*Danio rerio*) is a valuable vertebrate model for investigating hearing functioning and disorders and development of the inner ear in vertebrates and humans. In this study, we performed a 5-day split-brood experiment to test the effects of chronic noise exposure to environmentally relevant noise amplitude levels (150dB re 1 μPa, continuous white noise) on larval wild-type (AB) zebrafish inner ear morphology (hair cell count, epithelial size and hair cell density) and auditory sensitivity measured from two different approaches 1) Physiological (Saccular Microphonic Potentials) and 2) Behavioral (Prepulse Inhibition paradigm). Noise exposed groups displayed a significantly lower hair cell count and epithelial area size (μm^2). Acoustic treatment considered in this study elicited a significant increase in hearing threshold at lower frequencies in 5 dpf larvae. Contrastingly, sensorimotor hearing assessment (PPI) revealed a hypersensitisation effect in noise-exposed animals that displayed higher startle swimming velocity (mm/s). Synchronous occurrence of hearing impairment and hypersensitization to noise has never been previously reported.

Keywords: hearing, inner ear, electrophysiology, hair cell, prepulse inhibition, confocal

Introduction

Noise pollution has increased unprecedentedly during the last century both on land and underwater habitats [24,78,301]. Anthropogenic noise sources introduce a significant increase in overall sound amplitude and adds new components into the environments that differ greatly in spectral composition and frequency range from the natural soundscapes [302,303] with effects extending from megafauna [304,305] to the base of the trophic chain [306], including critically endangered, commercially valuable and ecosystem mediating species [115,147,307].

Mounting scientific evidence links noise exposure to a set of detrimental effects on both humans and wildlife including but not limited to altered physiology [42,61,80,308], impaired development and fitness [28,29,105,141,245,309], heightened physiological stress [147,228,310,311], behavioural disturbances [16,135,312,313] and pose a serious hazard to the auditory system of animals including humans [25,134,230,248,314].

A considerable number of studies have shown the negative effects of noise on fish species in both the wilderness [42,44,102] and aquaculture systems [29,61,105,223,315]. However, the majority of these studies focused on the effects of acute exposure to intense noise amplitudes but the more significant impact on aquatic fauna is likely to derive from less noticeable effects of chronic noise exposure [145] such as the conditions reported in anthropogenically noise-polluted areas[97,98,316,317] and aquaculture and housing systems [29,94,104,136].

Precisely, these increasing levels of noise are creating a serious hazard to the auditory system and the overall physiology and health condition of animals including humans [48,49,51,79]. Overexposure to elevated noise levels may affect inner ear sensory receptors, resulting in neuropathy and cell death [318–320] and leading to temporary or permanent Noise-Induced Hearing Loss (NIHL)[52]. The consequences of acoustic trauma on the peripheral system can ultimately induce changes at the morphological, physiological and functional levels of the

central auditory pathways [321,322]. Impaired auditory function due to noise exposure may also result in changes in sensorimotor behaviors. Hickox and Liberman (2014)[323] reported that mice exposed to 94-100 dB noise for 2 h increased their thresholds in regards to acoustic startle responses, prepulse inhibition, and activation of auditory processing pathways, while also showed behavioral hyperactivity.

Mammalian models have long been used to investigate the effects of noise on the auditory system[65,324–327]. Mounting evidence shows that the molecular and cellular mechanisms associated to 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 are complex and might differ in cell death signaling and homeostatic pathways[328,329]. Overall, there is 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 candidate protective targets and potential therapeutic agents[330,331].

The zebrafish (*Danio rerio*) has become an important model organism to investigate hearing and mechanisms of inner ear development of vertebrates. This species offers several technical advantages as it allows combining rapid and accessible embryogenesis, genetic and genomic tools, and *in vivo* visualization at a cellular level in a single organism[166,191,332–335]. The development and anatomy, including of the inner ear, have been intensively described[213]. Larval zebrafish at 5 days post-fertilization possess a fully functioning auditory system similar to other vertebrates[155,167] and with homologies to the mammalian auditory pathways[336]. Moreover, larval zebrafish also show a robust acoustic startle response that is easy to quantify[215,256] and that is under control by relatively simple neural circuitry[337,338]. Based on these features, zebrafish is considered a tractable model

system that can be used for testing the impact of noise overexposure on the auditory sensory morphology and sensorimotor behavioral response pathways.

Only few studies have evaluated long-term noise effects on animals [70,99,339] and particularly in early ontogeny [64,248], a critical period for development and establishment of phenotypic traits [249]. The relationship between inner ear structure and auditory function following acoustic trauma has been poorly examined in fishes [143,171,320,340,341], contrary to other vertebrates such as birds and mammals [131,319,342]. To our knowledge, there is no information on the effects of noise exposure on the inner ear and associated hearing loss in a larval fish. A recent study by Bhandiwad et al. (2018)[256] evaluated the impact of long-term noise on auditory-evoked startle response and hearing sensitivity in larval zebrafish with 5-7 days post fertilization. The authors observed significant noise-induced decrease in startle response threshold and hypersensitization to startle-inducing stimuli. These observations, however, were not related to changes in absolute hearing thresholds (based on prepulse inhibition) 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 (5 days post fertilization) through recordings from saccular hair cells and sensorimotor responses to acoustic stimuli based on Prepulse Inhibition assay (PPI). We further aimed to relate noise-induced sensory loss to potential changes on inner ear saccular morphology. We hypothesized that acoustic stress would induce hypersensitisation and auditory threshold shifts, along with potential changes at the inner ear hair cell number.

Methods

Zebrafish embryos: husbandry and sampling

Zebrafish eggs were obtained from wild type adults (AB line) and Et(krt4:GFP)^{sqet4} (ET4) transgenic zebrafish with hair cells in the inner ear and lateral line expressing green fluorescent protein (GFP) [343,344] purchased from China Zebrafish Resource Center (CZRC, China) and reared at our research facilities of the University of Saint Joseph, Macao. Stockfish were maintained in a standalone housing system (model AAB-074-AA-A, Yakos 65, Taiwan) with filtered and aerated water (pH balanced 7-8; 400-550 µS conductivity) at 28±1 °C and under a 12:12 light/dark cycle. For each experimental trial, eggs were collected within 2 hpf (hours post fertilization) from 2 to 6 breeding tanks (each tank containing about 10 females and 5 males). Collected eggs were mixed, distributed into 2 groups of 50 specimens, and each group allocated to both control and sound exposure tank. This split-brood approach was adopted to minimize potential differences related to egg quality/viability. At 3 dpf, embryos already have a functioning inner ear [294] and at 5 dpf specimens already exhibit sensorimotor behaviors, such as auditory-evoked escape responses, that are affected by noise exposure [256].

Individuals selected for inner ear imaging were consistently collected and immediately processed at the same time in the morning for the two developmental stages considered in the study, namely at 10:00 am at 3 and 5 dpf (days post fertilization). Individuals selected for behavioural hearing assessment were collected and immediately subjected to one of the two methodologies (electrophysiological or behavioural).

Experimental design and acoustic treatments

A total of 9 experimental trials consisting on chronic exposure to white noise at 150 dB re. 1 µPa were conducted. The acoustic treatment was carried in a glass tank (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 within 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 to maintain appropriate water quality similar to stock conditions. Each treatment tank was mounted on top of Styrofoam placed over two granite plates (1.5 cm thick) spaced by rubber pads to reduce non-controlled transmission of building vibrations. Eggs were placed inside a custom-made fine-mesh cylindrical netbox (5 cm diameter, 6 cm high) suspended at ~7 cm above an underwater speaker (UW30, Lubel Labs, Ohio, USA) that rested on top of a sponge base to minimize transmission of playback vibrations into the tank floor – see Lara and Vasconcelos 2021(a)[178] for a detailed graphical depiction of the acoustic setup. Speakers were connected to audio amplifiers (ST-50, Ai Shang Ke, China) that were connected to laptops running Adobe Audition 3.0 for windows (Adobe Systems Inc., USA). In **"**control**"** groups, the amplifier connected to the speaker was switched on but without playback, reaching a background noise level varying between 103 and 108 dB re. 1 µPa.

Sound treatment consisted on white noise low-pass filtered at 1500 Hz, adjusted to the tank acoustic properties using Adobe Audition software tools to deliver a relative flat spectrum and played back in loop at 150 dB re. 1 µPa. These noise levels were representative of freshwater aquatic habitats characterized by anthropogenic noise activity such as shipping [93,121] and noise conditions found in common zebrafish housing systems [94].

Noise levels were calibrated before each treatment so that the intended sound level (LZS, RMS sound level obtained with slow time and linear frequency weightings; flat

weighting: 6.3 Hz-20 kHz) were reached at the bottom of the net box (~7 cm distance from the speaker) using a hydrophone (Bruel & Kjær 8104, Naerum, Denmark; frequency range: 1–80 kHz ± 2 dB; voltage sensitivity: -184 dB re 1V/lPa^{-1}) connected to a hand-held sound level meter (Bruel & Kjær model 2250).

Additionally, the acoustic treatments were calibrated with a tri-axial accelerometer (M20-040, sensitivity 1–3 kHz, GeoSpectrum Technologies, Dartmouth, Canada). The particle acceleration sensor was placed horizontally with the acoustic center positioned at about 7 cm from the speaker in the position occupied by the netbox containing the specimens. Particle acceleration levels were determined in the three orthogonal directions and for the two noise amplitudes used. The sound playbacks generated most energy in the vertical axis compared to the other two orthogonal directions where resulted in similar variations to the ones obtained in sound pressure levels. Particle acceleration level determined for the highest sound level (150 dB re 1 µPa) determined in the the vertical axis consisted of 120 dB/s^2. A decrease of 20 dB in sound pressure resulted in similar variation in acceleration. The sound playbacks generated most energy in the vertical axis compared to the other orthogonal directions. The calibration was conducted using a Matlab script (paPAM) based on Nedelec et al. (2016) [296].

Inner ear saccular sensory epithelia imaging and hair cell quantification.

To quantify saccular hair cell bundles, Et(krt4:GFP)^{sqet4} (ET4) transgenic zebrafish were selected for optimal fluorescence [294,344] under fluorescent microscope followed by euthanasia in 300 mg/L of Tricaine Methanesulfonate (MS-222, Thermo Fisher Scientific INC, Massachusetts, USA) and immediately fixed in 4% fresh paraformaldehyde (PFA) at 4°C overnight. Next day samples were rinsed in phosphate-buffered saline (PBS) 3 times for 10

min each. To visualize the entire saccular epithelium, the otolith was dissolved in 1% (3 dpf) and 2% (5 dpf) Triton X-100 (Sigma-Aldrich, St. Louis, MO) for up to 24h at 4°C. Animals were positioned laterally in custom prepared microscope slides (3 vinyl-layered microscope slides with two squared holes containing a drop of Vectashield anti-fading solution (Vector Laboratories, Burlingame, CA) and a single larva each, then covered and sealed with a microscopy coverslip). A z-stack of images of GFP-labelled saccular hair cells were taken and 3D reconstructed into a single file using a STELLARIS 5 LEICA confocal unit with a 488 nm laser line (Leica Microsystems, Wetzlar, Germany) (Fig. 4.1). Quantitative evaluation of cell number was determined by counting and measuring hair cells bundles in whole 3D reconstructed saccular epitheliums and the area measured using DanioScope imaging software (Noldus Information Technology, Wageningen, Netherlands).

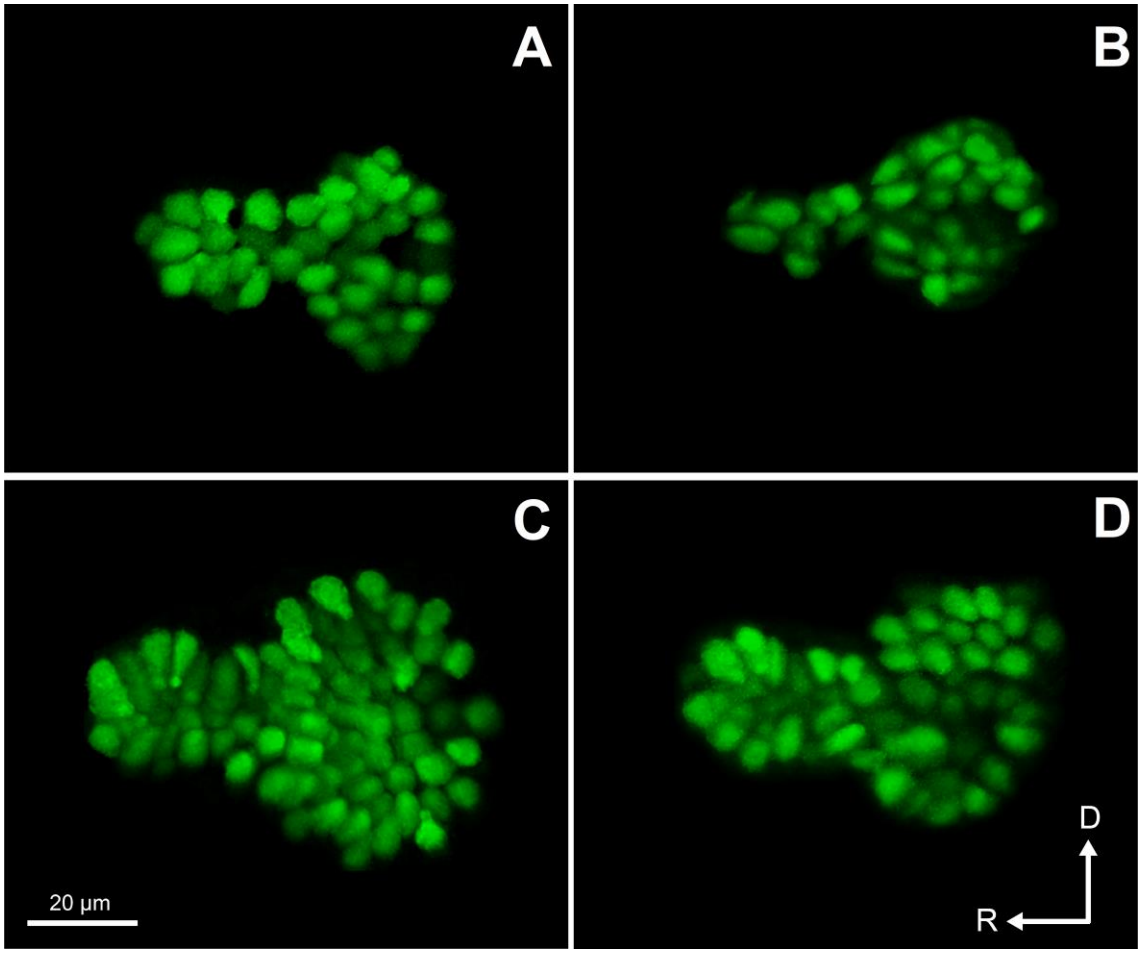

Fig. 4.1

Confocal images showing hair cell bodies expressing green fluorescent protein obtained for comparison between inner ear saccular epitheliums of A) 3dpf control B) 3dpf noise exposed C) 5dpf control and D) 5dpf noise-exposed Et(krt4:GFP)sqet4 zebrafish transgenic individuals. Saccular hair cell bundle quantification was conducted by digitally marking cell bodies after which the epithelial area was also quantified, both measurements were conducted using DanioScope (Noldus Information Technology, Wageningen, Netherlands). Scale bar = 20µm.

Saccular microphonic potential recordings

Electrophysiology setup and mounting

The electrophysiology rig for microphonic potential recordings was composed of a Zeiss Examiner A1 microscope (Carl Zeiss AG, Oberkochen, Germany) equipped with a N-achroplan 40× (NA=1.0) water immersion lens. Accessory devices included a joystick controlled manual micromanipulator (DC3001L) three-dimensional hydraulic micromanipulator (World Precision Instruments, WPI, Sarasota, FL) for holding the stimulus probe that was connected to a piezoelectric actuator with its own amplifier mounted on a MS-314 manipulator (Marzhauser Wetzlar, Germany) and a joystick controlled manual micromanipulator (DC3001R) three-dimensional hydraulic micromanipulator for holding the recording and grounding electrode (Ag/AgCl2 electrode). These devices rested on top of a 9101– 02-46 anti-vibration table (Kinetic System, Boston, MA). The whole recording setup was placed inside a custom-built, electrically and acoustically shielded audiometric booth (IAC120A3-53, iac acoustics, Dongguan, China). Outside of the booth a SR830 DSP lock in amplifier (Standford Research Systems, Novato, CA, US), a programmable signal filter system (SR650, Stanford Research Systems, USA), a preamplifier (model 5A, Getting Instruments, USA) and a UTD-2062CE digital storage oscilloscope, were mounted in a single unit and connected to a dedicated computer.

Microphonic potentials were recorded at 26℃ from the otic vesicles of non-anesthetized AB wild type zebrafish at 3 and 5 days postfertilization (dpf). Embryos were mounted laterally on top of a drop of 0.5% low-melting point agarose in 35 mm microscopy dishes so the otic capsule stayed outside the agarose. To prevent dry out during recordings and unify the medium, an aliquot of embryo buffer was added to the dish.

Stimulus probe and piezoelectric actuator

Stimulus probes with a tip size of 50μm in diameter were made from glass capillaries (OD=1.50 mm, ID=0.86 mm, World Precision Instruments, WPI, Sarasota, FL, US) using a P-1000 electrode puller (Sutter Instrument Company, Novato, CA, USA) and the tips were flame-blunted prior to use. The probe displacement driven by the piezoelectric actuator (Piezosystem Jena, Inc., Hopedale, MA, US) was first calibrated at the selected frequencies (100, 200, 300, 400 and 500 Hz) under the Zeiss Examiner A1 microscope with a high-speed camera (FASTEC-IL5-254, Fastec Imaging, San Diego, CA). The probe tip was placed in contact with the skin and the saccular otolith at the posterior end of the otic vesicle and provided linear oscillatory motion along an axis ~20 degrees off the longitudinal axis of the fish body [294,344]. Additional parameters such as angle, location and size of the stimulus probe that might have affected microphonic responses were kept consistent among trials.

Recording and reference electrodes

Recording electrodes were sharp micropipettes made from WPI glass capillaries (OD=1.0 mm, ID=0.50 mm) with the aforementioned microelectrode puller. They were filled with a 3M KCL solution and selected with an impedance from 2 to 8 MΩ. The recording electrode was then mounted in an electrode holder (Digitimer, Welwyn, UK) and secured by a joystick controlled manual micromanipulator used to push forward the electrode tip in order to penetrate the wall of the otic vesicle of the zebrafish. An Ag/AgCl reference electrode was introduced in the medium at the border of the 35 mm dish containing the mounted larva. The recording electrode tip was always positioned pointing at the centre of the saccular otolith, then lowered and advanced until reaching ~1 μm underneath the otolith (Fig. 4.3A).

Recording system and data acquisition

Stimulus signals were generated in 100Hz increments from 100 to 500 Hz, using a custom MATLAB script (MathWorks, MA, USA). Stimulus signals were delivered to the piezoelectric actuator that drove the stimulus probe frequencies and presented in random order during the recording session. Stimuli were initially presented at an amplitude of 0.04 mV and then decreased in 0.005 mV steps until a threshold was reached. Each stimulus was repeated eight times at a rate of one every 1 s for a duration of 500 ms, which yielded stimulus cycles of 50, 100, 150, 200 and 250 for 100, 200, 300, 400 and 500 Hz respectively.

Microphonic potential responses were preamplified 10 times (model 5A, Getting Instruments, USA), and high pass filtered at 12 kHz (SR650, Stanford Research Systems, USA). Each response was averaged across eight repetitions. Thresholds were defined as the lowest stimulus amplitude that evoked a response with peak at 2F, which was greater than 2 standard deviations (SD) of background noise. When responses were not present at 0.04 mV, stimulus amplitudes were gradually increased in 0.01 mV steps, until a threshold could be determined.

Prepulse inhibition paradigm.

Experimental setup

To measure acoustically-triggered startle responses on zebrafish larvae (5 dpf) we used the custom-made apparatus developed and validated by Wang et al 2017 [345] (Fig. 4.2A).

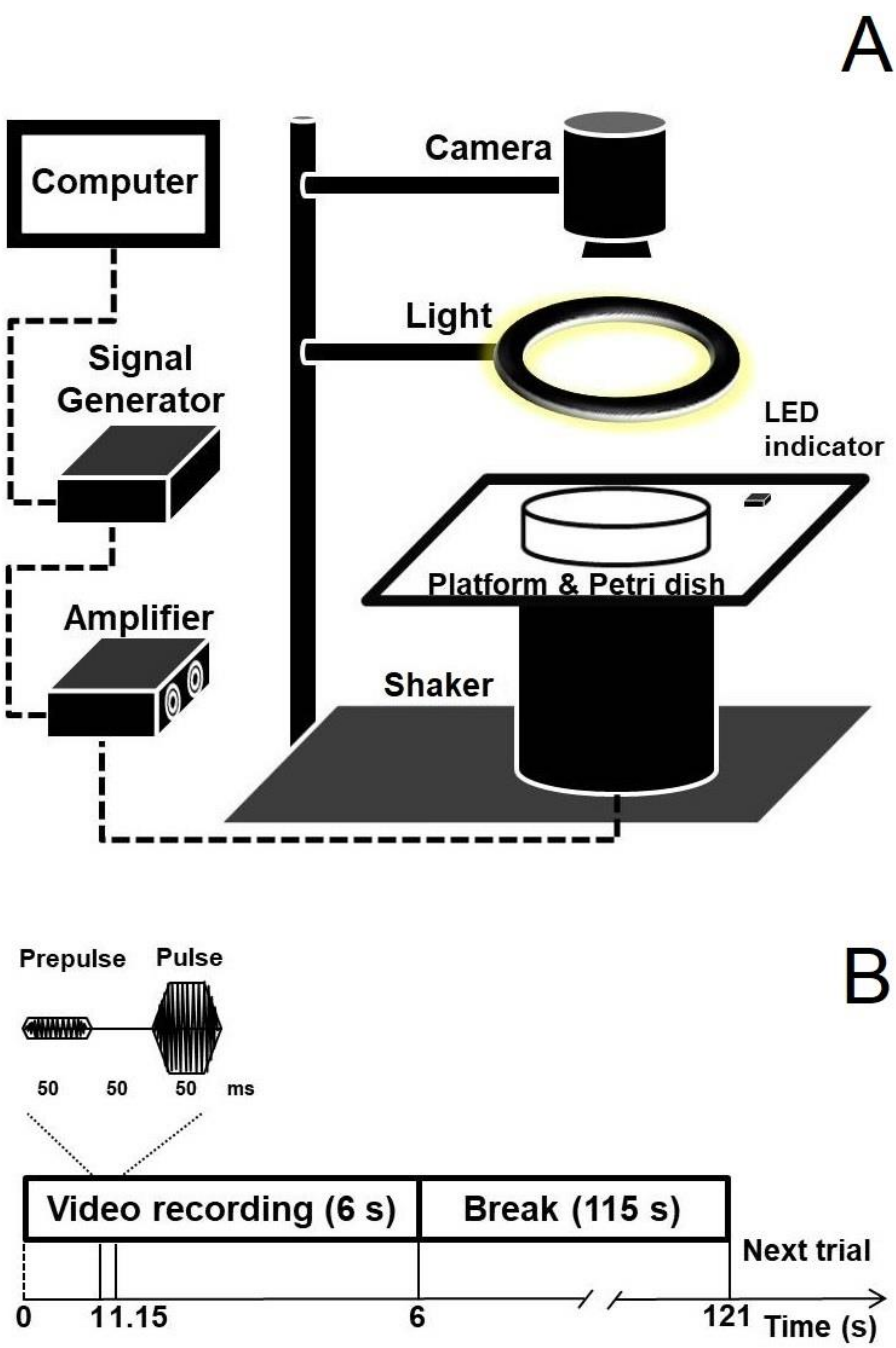

Fig. 4.2

A) Schematic representation of the setup used to conduct the prepulse inhibition paradigm experimentation. B) Time distribution and presentation scheme of the acoustic stimulus used in the prepulse inhibition test.

Groups of 20 larvae were gently pipetted into a 3D-printed petri dish size platform (8 cm) and contained within a thin layer (2 mm) of water to assure an even focus and magnification across individuals. The dish was illuminated directly from above with a light guide ring panel (infrared wavelength at 850 nm, model HA92123, Feiye, Guangzhou, China) to provide evenly distributed illumination necessary for optimal image processing. A high-speed digital camera system CS-S6-6C12WFBR, 120fps, 1080p (EZVIZ, Hangzhou, China) was mounted on top of the LED ring and suspended independently of the setup to avoid interferences. Acoustic vibrations at the desired frequency and amplitudes were scripted using a QT Platform (The QT Company, Espoo, Finland) and created by an electrical signal generator (model AUDIO-V1.0.3-20181028, designed by Shenzhen University, Shenzhen, China) connected to an amplifier (model TPA-2578AY, Weiliang, Foshan, China) that drove the mini vibrator (frequency range from 60Hz to 20KHz, model QY50R-Z, Haoshengyuan Inc, Dongguan, China) which was finally coupled with the platform to deliver vibratory stimulus at different amplitudes and frequency into the Petri dish. The particle acceleration of the water surface was calibrated for frequency and amplitude with a laser Doppler interferometer (model OFV-505, Polytec GmbH, Waldbronn, Germany) to ensure linearity and consistency. This step provided a direct measurement of the stimulus applied to the fish based on the amplitude of vibrations as previously described by Wang et al 2017 [345] in order to control the electrical signal input using the custom-made ZFishPPI V4.0.1 software (Qt Platform, The Qt Company, Espoo, Finland).

Acoustic stimulus

Prepulse stimulus array ($-\infty$, −33, −28, −23, and −19 dB re 1 ms^{-2}) corresponding to (0, 130, 140, 150, 160 dB re 1µPa) consisted on tone bursts of 50 ms, followed by 50 ms break after

which the pulse tone burst of 50 ms (100 Hz, 200 Hz, and 400 Hz at 230 dB re 1 ms^{-2}) was delivered. Each stimulus level was repeated 20 times with an interval of 120 seconds between presentations in order to avoid habituation and achieve statistical significance (Fig. 4.2B). We decided to test for 400 Hz as it has been previously reported to be the plateauing point for threshold responsiveness [346], additionally 100 Hz and 200 Hz were further tested due to significant differences found in electrophysiological hearing thresholds assessment.

Data collection and analysis

For the acoustic startle characterization, six seconds of video were recorded for each stimulus level at 120 frames per second (8.3·ms per frame, 0.707 mm/pixel). Average swimming velocity (mm/s) was calculated based on X-Y coordinates displacement, of each individual, subtracted frame by-frame from the high-speed HD video recordings. A total of 651 frames were analysed.

Potential differences in startle responses could also result from generalized hyperactivity. In order to test for generalized hyperactivity, noise-exposed and control fish were tested in an open field test and recorded for 10 min at 26°C in the absence of auditory stimuli. All noise-exposed fish were tested immediately after cessation of noise exposure. Videos were analyzed using Ethovision XT (Noldus Technologies, Wageningen, Netherlands) and total distance moved and time spent moving were measured for each group.

Ethical statement

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 (China), license AL017/DICV/SIS/2016. Larvae were anesthetized (0.004%) and sacrificed (300mg/L) using Tricaine Methanesulfonate (MS-222, Thermo Fisher Scientific INC, Massachusetts, USA) following ZFIN protocols.

Statistical analysis

Parametric tests were used only when data was normally distributed and variances were homogeneous. Quantification of differences in number of hair cell bundles between control and noise exposed animals' saccular epithelia was based on One-way ANOVA tests. Differences in saccular epithelia area size between control and noise exposed group were quantified following One-way ANOVA tests.

Quantification of differences in hearing sensitivity based on saccular microphonic potentials were based on repeated measures ANOVA.

Differences in auditory evoked behavioural responses (PPI) were determined using chi square tests while decrease in responsiveness between prepulse amplitudes was conducted using a within-group One-way ANOVA test.

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 SPSS v26 (IBM Corp. Armonk, NY, US) and Statistica 10 for Windows (Dell Software, Inc., Round Rock, T, USA).

Results

Saccular hair cell sensitivity

Microphonic potentials were recorded from saccular hair cells in both 3 and 5 dpf larvae (Fig. 4.3A). The threshold data are reported as dB relative to the minimum stimulus output of our experimental apparatus (0.004V from lock-in amplifier), which was similar to the baseline noise level measured from a dead fish or no specimen in the recording setup. The auditory responses varied between 27 dB at 100 Hz down to 1.9 dB at 500 Hz at 3dpf, and between 27 dB and 3.5 dB at 5 dpf. Significant differences in microphonic responses were found between these two developmental stages $F_{(3, 22)} = 7.38$, $p<0.01$

Comparison between noise-exposed group and control revealed no significant differences in the microphonic responses at 3dpf zebrafish larvae $F_{(4, 40)} = 1.63$, $p>0.05$ (Fig. 4.3B). However, significant impairment in auditory sensitivity was identified at 5 dpf in noise exposed specimens ($F_{(4, 48)} = 14.61$, $p<0.001$) (Fig. 4.3C). Significant differences were found in thresholds at 100 and 200 Hz; $F_{(1, 22)} = 17.603$, $p<0.001$ and $F_{(1, 27)} = 23.844$, $p<0.001$ respectively. (Fig. 4.3C).

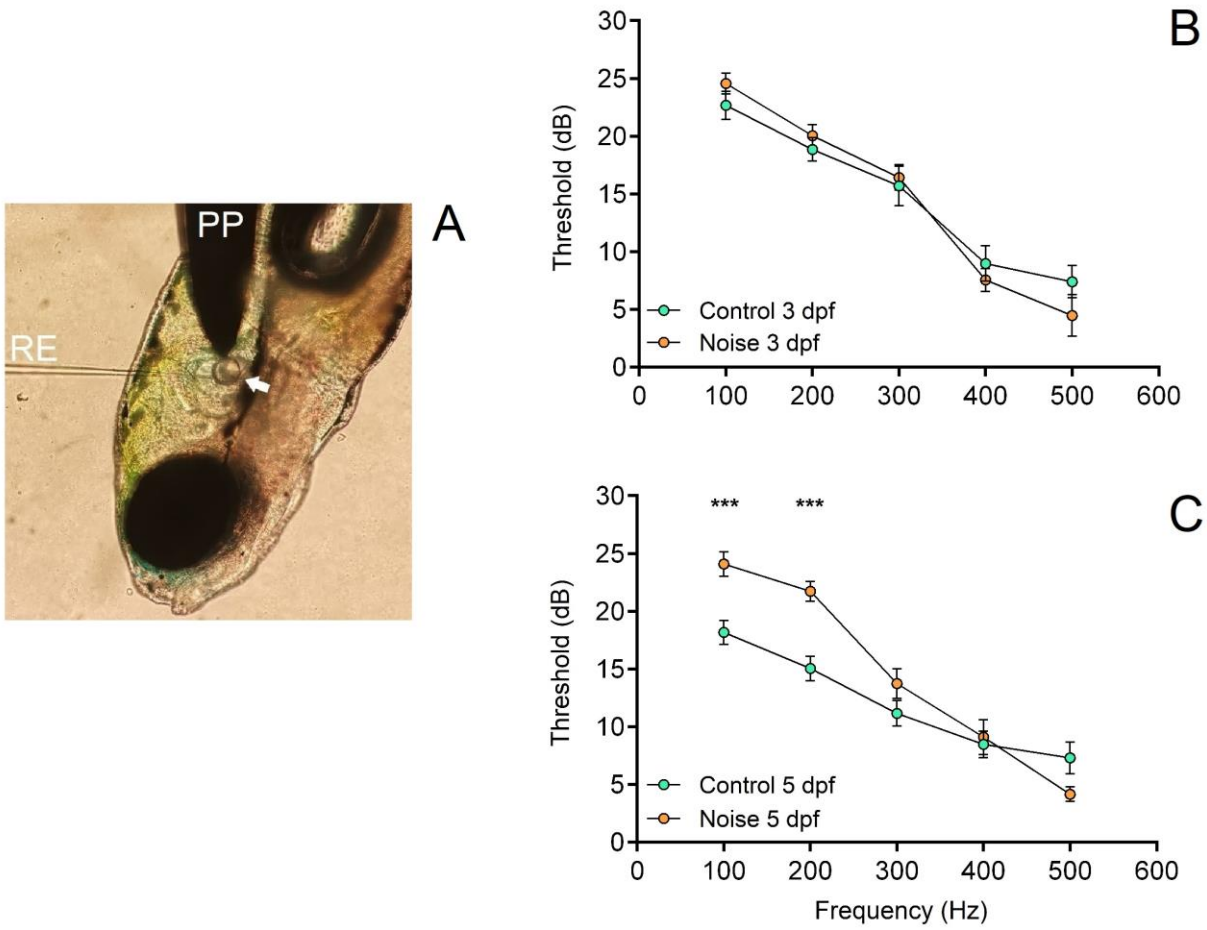

Fig. 4.3

A) Image of a 5 dpf AB wild-type zebrafish embryo embedded in agarose and ready for microphonic potential recording. Image shows the recording electrode tip (RE), the stimulus probe (PP) and saccular otolith (arrow). B) Microphonic thresholds (dB) versus stimulus frequencies of 3 dpf AB wild-type zebrafish, control (green) N=14 and noise (orange) N=8. C) Microphonic thresholds (dB) versus stimulus frequencies of 5 dpf AB wild-type zebrafish, control (green) N=18 and noise (orange) N=11, showing significant differences in hearing sensitivity $F_{(4, 48)} = 14.61$, $p<0.001$. At 100 Hz (one-way ANOVA $F_{(1,23)} = 17.60$, $p<0.001$) and 200 Hz (one-way ANOVA $F_{(1,28)} = 23.84$, $p<0.001$). Error bars are 95% CI.

Auditory sensitivity based on startle responses

Quantification of the swimming speed of acoustic evoked startle responses in 5 dpf revealed a significant increase in noise-exposed specimens at all frequencies tested: 100 Hz - $t_{(38)} = 6.55$, $p<0.001$; 200 Hz - $t_{(38)} = 8.62$, $p<0.001$ and 400 Hz - $t_{(80)} = 9.23$, $p<0.001$ (Fig. 4.4).

Comparison of auditory response to the different frequencies across the various prepulse amplitudes revealed significant decrease (threshold) at 200 Hz between 140 and 150 dB in control group dB $F_{(1, 6)} = 7.46$, $p<0.05$, which contrasted with the noise-treated specimens that showed significant reduction only between 150 and 160 dB $F_{(1, 6)} = 39.71$, $p<0.001$. These results suggest a ~10 dB difference in acoustic evoked startle response thresholds at 200 Hz.

At 100 Hz, noise-exposed specimens revealed similar thresholds as in 200 Hz assay, but control group failed to show typical prepulse inhibition, while at 400 Hz, both experimental groups showed significant decrease between 150 and 160 dB ($F_{(1, 6)} = 4.2393$, $p>0.05$) and ($F_{(1, 12)} = 4.1556$, $p>0.05$) respectively, suggesting that the acoustic treatment did not affect auditory thresholds. (Fig. 4.4)

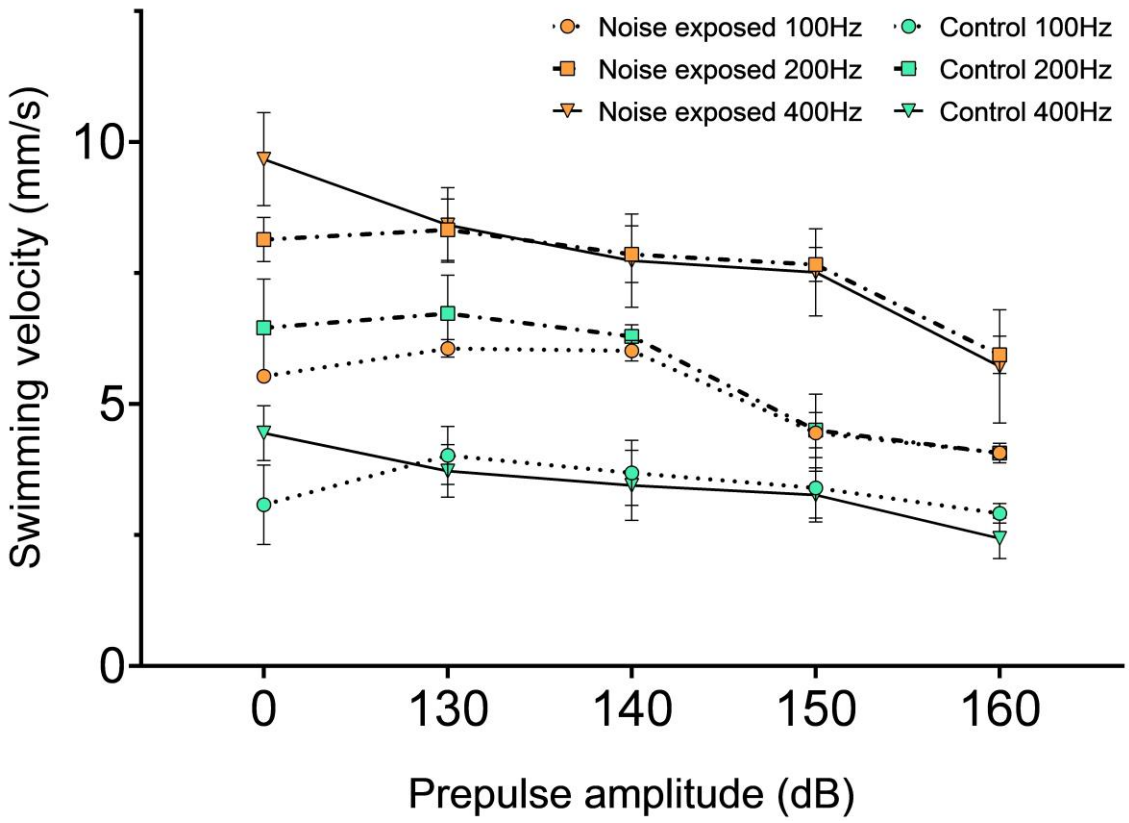

Fig. 4.4

PPI behavioural response curves of 5 dpf.AB wild-type zebrafish larvae in response to 100, 200 and 400Hz pulse stimulus at 240 dB re 1 m/s2. Data is presented as average swimming velocity (mm/s) vs prepulse amplitude (dB re 1 m/s^2), control (green) and noise-treated (orange). 100 Hz: dotted line; 200 Hz: dashed line; 400 Hz: continuous line. 100 Hz - $t_{(38)}$ = 6.55, $p<0.001$; 200 Hz - $t_{(38)}$ = 8.62, $p<0.001$ and 400 Hz - $t_{(80)}$ = 9.23, $p<0.001$. At 200 Hz between 140 and 150 dB control group showed a decrease in hearing $F_{(1, 6)}$ = 7.46, $p<0.05$, which contrasted with the noise-treated specimens that showed this reduction only between 150 and 160 dB $F_{(1, 6)}$ = 39.71, $p<0.001$. Error bars are SEM.

We disregarded potential differences in startle responses from generalized hyperactivity after testing the swimming patterns of these specimens in an open field arena, which revealed a reduction in covered area for the noise-treated larvae ($t_{(89)}=7.33, p<0.001$).

To test whether decreases in startle threshold were due to generalized increase in locomotor activity, noise-exposed and control fish were tracked for 10 min immediately after cessation of treatment and locomotor activity was recorded. We observed an increase in locomotor activity (% of time spent moving during the 10 min recording) in noise-exposed animals (51.95%) being control (33.32%), $F_{(1, 52)} = 19.188$, $p<0.001$, Together, these results indicate that noise-exposed fish were more active than the control group in a normal locomotor activity test.

Inner ear saccular morphology

In order to evaluate whether auditory sensitivity changes could result from differences in the inner ear hair cells, we investigated the number and saccular epithelial area of zebrafish at 3 and 5 dpf exposed to the same experimental conditions aforementioned.

The number of saccular hair cells increased significantly with age for control (one-way ANOVA $F_{(1,32)} = 38.23$, $p<0.001$) and in the noise exposed group (one-way ANOVA $F_{(1,37)} = 22.19$, $p<0.001$)Although the general shape of the saccular epithelia did not change, the acoustic treatment caused a significant reduction in hair cell bundles at both developmental stages (3 dpf: ($F_{(1, 39)}=14.16$, $p<0.001$); 5 dpf: ($F_{(1, 30)} = 19.16$, $p<0.001$) (Fig. 4.5A). Additionally, epithelial area of noise-exposed individuals was significantly smaller compared to control at 3 dpf (one-way ANOVA $F_{(1, 19)} = 4.71$, $p<0.05$) and 5 dpf (one-way ANOVA $F_{(1, 19)} = 18.19$, $p<0.001$) (Fig. 4.5B). The hair cell density, however, did not reveal differences between treatments (3 dpf: $F_{(1, 19)} = 2.56$, $p>0.05$; 5 dpf: $F_{(1, 19)} = 3.19$, $p>0.05$).

Finally, we compared average epithelial growth between noise-exposed and control group revealing similar results with no statistically significant differences ($F_{(1, 19)} = 4.03$, $p>0.05$).

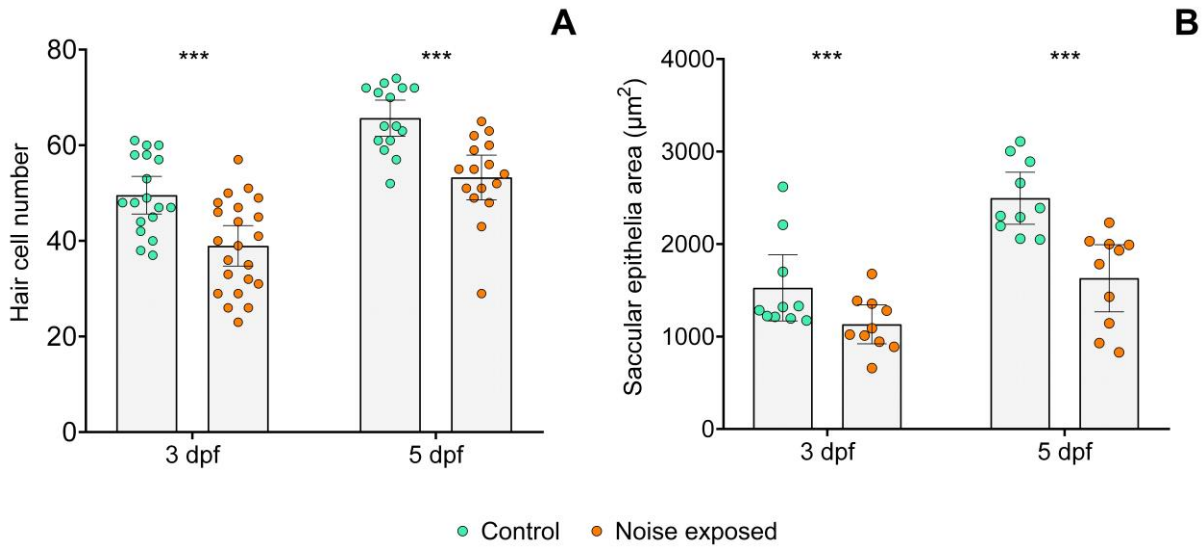

Fig. 4.5

A) Comparison between number of saccular hair cells in control and noise-exposed individuals of larval zebrafish displaying significant differences in total hair cell count at both 3dpf (one-way ANOVA $F_{(1, 39)}=14.16$, $p<0.001$) and 5dpf (one-way ANOVA $F_{(1, 30)}=19.16$, $p<0.001$). B) Comparison between area size (µm2) of saccular epitheliums in control and noise-exposed individuals displaying significant differences in area size at 3dpf (one-way ANOVA $F_{(1, 20)}=4.61$, $p<0.05$) and 5dpf (one-way ANOVA $F_{(1, 20)}=18.19$, $p<0.001$). Error bars represent 95% confidence intervals. Asterisks indicate statistically significant differences between specific groups.

Discussion

In the present study we provide first evidence of noise-inducted decrease in inner ear saccular sensitivity and morphology in a fish larva. These findings were paralleled by hypersensitization to noise stimuli and decreased sensitivity in startle responses. We relied on larval zebrafish (*Danio rerio*) as our model species, a reference organism in hearing research and ecotoxicology.

Saccular hair cell sensitivity

We first assessed hearing sensitivity in 3 and 5 dpf wildtype zebrafish larvae using the microphonic potential recording method, which has been confirmed as a reliable and powerful tool to effectively assess hearing function and sensitivity of zebrafish embryos and juveniles [347–349]. Saccular microphonic potentials were recorded from the otic capsule of zebrafish larvae as early as 3 dpf, a time point by which the auditory circuit is functional [349].

Fish can detect auditory stimuli via particle motion and/or sound pressure (Popper and Fay, 2011). We used a vibratory particle motion-dependent stimulus to stimulate the opening of mechanotransduction channels that lead to the encoding of auditory stimuli by hair cells in the inner ear saccule [294,347,349]. Particle motion is the ideal stimulus for studying inner ear function at this early stage in ontogeny since the zebrafish larvae is not yet capable of detecting the sound pressure component but only direct inertial stimulation [350]. This only happens after 56 dpf, when specialized bones (Weberian ossicles) are fully developed and connect the swimbladder to the inner ear (Grande and Young, 2004)

Our study shows a decrease in saccular auditory thresholds with development from 3 to 5 dpf as the hair cell number increases, which has also been reported in prior studies [294]. Moreover, we found a significant decrease in saccular auditory sensitivity of up to 6 dB at low

frequencies (100 Hz to 200 Hz) in 5 dpf individuals. Rohmann et al 2014 also found changes of similar magnitude (up to 3.5 dB) in auditory thresholds of larval zebrafish subject to morpholino oligonucleotides injection to alter expression o duplicate genes coding for the pore-forming **α**-subunits of BK channels [351].

Other studies have identified similar impact of noise exposure on hearing sensitivity in fish [228,340,352], including zebrafish (Breitzler et al., 2020). These studies typically relied on AEP recordings to evaluate changes in overall auditory sensitivity due to noise treatment. In the present study we show an impact of acoustic stress on the auditory sensitivity at the receptor level.

Given the extended use of this model in a wide range of biomedical studies, including hearing research, and considering that they are usually kept in artificial housing systems where noise levels and particle motion component might be similar to the acoustic treatment used in this study [94], we highlight the potential interference of housing acoustic conditions in the sensory development of this model system.

Auditory sensitivity based on startle responses

We further investigated the effect of noise exposure on hearing from a behavioural approach based on startle responses to acoustic stimuli. There has been a recent increase in the interest in animal models of hyperacusis and hypersensitization [323,353,354], including zebrafish [256]. Hyperacusis, a condition in which tolerance to sounds of moderate and high intensities is diminished, has been reported in previous studies [256,355], moreover, our result are consistent with observations by Salloum et al 2014[355] regarding the developmental stage at which larvae present hypersensitization.

Considering results obtained in the electrophysiological hearing assessment, the noise-induced sensitization is not likely to be only due to changes in hearing thresholds but also related to the auditory-evoked escape response as observed by Bhandiwad et al 2018[256], this is further supported by the significant decrease in responsiveness observed at 200 Hz between 140 and 150 dB prepulse level (control) and 150 to 160 dB prepulse level (noise), suggesting an impairing effect in absolute hearing thresholds. The reasons for hyperacusis occurrence are multiple and complex and remain largely unknown. However, some factors have been identified to be associated with this phenomenon such as increased loudness perception and heightened sensitivity to sound. Increased sound sensitivity is a symptom that has recently been demonstrated in animals [356,357].

In our study, larval zebrafish also exhibited a significant decrease in habituation to startle-inducing stimuli with an average increase of 45% in the swimming velocity after acoustic stimulation. Even though this increase in noise sensitivity was more pronounced at 400 Hz, we observed the same tendency at 100 Hz and 200 Hz, frequencies at which our model was displaying a significantly impaired hearing sensitivity. The combination of the shift in hearing thresholds at lower frequencies and a decrease in the auditory-evoked behavioural response suggest a significant impairment of the hearing capabilities with complex sources and pathways.

It is worth to mention that, different studies have reported different results regarding this methodology, with some studies reporting enhanced startle reflex [323] like our assessment, others reporting reduces responses [358], and others reporting little to no change in the magnitude of the acoustic startle reflex following noise exposure [359].

The behaviourally observable startle reflex is easily replicable but dependent on multiple factors like the developmental stage of the model, the amplitude, frequency and delay of the

prepulse, the amplitude and frequency of the stimulus pulse, the post-exposure delay and the timing and context of the measurements. therefore, caution should be exercise when interpreting our data.

Inner ear morphology

In order to evaluate whether differences found in hearing sensitivity were related to alterations in the inner ear morphology, we further investigated possible noise-induced changes in the zebrafish saccular epithelia, which is considered the main hearing endorgan of teleosts [199]. We analysed saccular epithelia of transgenic Et(krt4:GFP)^{sqet4} (ET4) zebrafish line, which presents the same number of saccular hair cells and identical auditory sensitivity as wild-type AB zebrafish during the first week of development [344].

The numbers reported in this study regarding total number of hair cell bundles in control group are consistent with observations by Lu and Desmidt (2013) and Yao and Desmidt (2016) [294,344].

We found decreased hair cell number at both 3 and 5 dpf in noise-treated specimens, as well as reduced saccular sensory epithelial area. Similarly, Uribe et al., (2018) also found reduced hair cell number in larval zebrafish neuromasts exposed to acoustic trauma induced by underwater cavitation[255].

Noise exposure is known to cause impaired growth and developmental abnormalities in early ontogeny [141], cause tissue damage in the inner ear [119] and central auditory pathways [360]. Overexposure to increased noise levels is also known to induce impaired synaptic connections, reduced blood flow, increased level of reactive oxygen species (ROS), mitochondrial alterations (e.g. mitochondrial DNA deletion), apoptotic and necrotic cell

death [328], and alter the physiological properties and biochemical functions of the hair cells [361,362]. All these consequences and their potential interaction may have contributed for the changes in auditory function.

Few studies have investigated how noise exposure affects inner ear in early development [124] , including in zebrafish [294], hence additional research is needed to elucidate the relationship between sensory morphology and auditory function under acoustic stress.

In summary, these experiments suggest a complex and multiscale effect of noise exposure on the neural circuits mediating auditory-evoked behaviours and hearing sensitivity in larval zebrafish.

Acknowledgments

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GENERAL DISCUSSION

This thesis provides (1) first insights into the natural soundscape of our model system, the zebrafish *D. rerio*, which has never been investigated. Furthermore, the work provided a comparison of the acoustic properties of natural habitat with artificial housing systems commonly used in laboratory facilities in which the species is typically reared for research purposes. Based on bioacoustics methods, my first study found that zebrafish natural soundscape presented most energy below 3000 Hz and quieter noise windows were found in the noisiest habitats matching the species best hearing range. This confirms that the hearing sensitivity of zebrafish is well adapted to the acoustic properties present in their natural environments. Contrastingly, recordings from zebrafish housing systems revealed higher sound levels and most energy below 1000Hz with more spectral peaks, which might cause significant impact such as auditory masking or even hearing loss. This research provided an important ground for future research on the adaptation of zebrafish auditory system to the natural soundscapes, and highlights the importance of controlling noise conditions in captivity systems.

Additionally, I also provide evidence in a second study of (2) the impact of noise exposure to increased amplitudes and different time regimes on larval zebrafish. We showed the occurrence of noise-induced physiological and behavioural disturbance and demonstrate that both amplitude and timing of presentation of the acoustic stressor may impact key health-related endpoints during early ontogeny.

Finally, in the last chapter I demonstrate (3) first data on the impact of noise exposure on inner ear saccular morphology and sensitivity in combination with noise-induced changes in behavioural responses to noise such as hypersensitization in startle response speed and decreased sensitivity.

Altogether, my research highlights the importance of investigating how altered soundscapes may impact key physiological and sensory-related endpoints in early ontogeny, and impose new evolutionary challenges under a scenario of global change.

Research questions answered

Chapter 1

1.1 How are the acoustic properties of the natural habitat of the zebrafish?

Our study provided the first acoustic characterization of the natural habitat of the zebrafish, adding to the bibliography the soundscape in which this widely used model thrives.

In freshwater habitats, the ambient noise levels are usually highly dependent on the water flow strength, substrate composition and acoustic external event. In our study, the shallow water streams with low/medium flow and backwaters presented the lowest SPLs with most sound events deriving from abiotic sources, (water current, cavitation, and moving substrate) but also biotic sources like birds calls and insects. On the other hand, the river course environment showed the highest sound pressure levels due to the higher water flow, larger water volume, and significant cavitation and transportation of sediment. The SPL values from quieter habitats were similar to the noise levels reported by previous studies [82,83,92]. Moreover, the noise levels reported for a main river course and a stream were similar to the ones recorded in our study[82]. Some studies have reported ambient noise spectral profiles within the range reported at this study [91,204]. However, comparisons of noise levels across different studies proved to be difficult given the scarcity of this studies. In concurrence with previous studies, louder natural habitats, revealed lower variability in the noise levels compared to quieter environments[82,83].

Finally, regarding the spectral profiles, freshwater habitats such as rivers and streams typically present more energy at lower frequencies followed by a gradual noise level

decline[82,83,91]. However, we found a “sound window”, at lower frequencies, in the noisiest environment. A low frequency “noise window” has been previously reported in previous studies of freshwater habitats [9182].

In summary, the soundscapes of zebrafish natural habitats investigated in this study revealed significant diversity in sound levels and spectral composition. Moreover, any additional noise in the soundscape at these quieter habitats (including from anthropogenic sources) contributed for a notable increase in the overall noise level. These differences might be important for zebrafish orientation and sound detection in the various acoustic environments.

1.2 How are the acoustic properties of the captive conditions at laboratories?

In our study we also investigated the noise levels and spectral features of three typical zebrafish housing systems following the same methodology that we used for natural environments. The SPLs determined were significantly higher in these artificial builds, indicating that great part of the background noise is caused by the proximity of the pumps, filters, piping, enhanced water flow, ground vibrations, tank wall vibrations and electrical devices, oscillating and collapsing air bubbles, aeration, etc. Moreover, the spectral composition of the ambient noise in the zebrafish housing systems investigated revealed most sound energy concentrated below 1000 Hz (coinciding with the zebrafish best hearing range) and a gradual decrease in SPL towards higher frequencies. Similar noise values were determined in other studies, although the information is scarce and difficult to compare due to distinct types of fish housing [29,95,104,210].

Our results showed that system with a separate water treatment unit is significantly less noisy compared to the stand-alone, although the noise levels were still well above the natural habitats with considerably more energy within the best hearing range of zebrafish.

According to Lawrence and Mason (2015)[226], in order to minimize noise sources in a zebrafish housing system, the rack should contain dampeners on stands that support pumps or other vibratory and noisy equipment

But it is known that vibrations and noise may cause stress and harm aquatic animals in laboratories (NRC 2011)[225]. The studies available showed reduced fish egg viability and growth rates[208,209], but also absence of developmental and physiological stress effects in the rainbow trout (*O. mykiss*), which do not have morphological hearing specializations[29,105].

1.3 Are these conditions within the perception range of the zebrafish?

The zebrafish is an ostariophysan species with relatively wide frequency range detection (100 to 8000 Hz) and best hearing sensitivity at 600-1000 Hz[212,214,363].

We evaluated hearing adaptation of the species to the various soundscapes, by overlapping auditory sensitivity curves previously determined from wild type zebrafish lines[169,212,215]. We considered four audiograms obtained in distinct laboratories to show potential variability within the same species and due to technical differences in measurements. Comparing audiograms of wild type zebrafish lines with the various habitat noise spectra showed that this species is well adapted to all freshwater environments with probably some auditory masking in the fast-flowing environment.

Interestingly, the best hearing range of zebrafish (600-1000 Hz) matched a frequency interval where ambient noise spectra varied the most, but also a quieter window in the noisiest habitats. Altogether this suggests that, similar to other ostariophysan species[203], zebrafish hearing sensitivity is well adapted to detect sounds in diverse freshwater habitats with different ecological characteristics and acoustic properties. However, the artificial

housing systems revealed spectral noise energy well above the species best auditory thresholds, which most likely induces significant masking effects and possibly hearing loss.

The effects of chronic exposure (since early ontogeny and across multiple generations) to noise levels found in typical artificial housing conditions remained to be investigated until the realization of this study.

Logically, once we could 1) characterized both soundscapes in which the zebrafish can be found, natural habitat and artificial housing conditions, 2) assessed that the artificial soundscapes were widely different from what we observed in their natural habitat and 3) confirmed that this noise conditions were well within our model's perception range; the next step was to experimentally investigate the possible effects of this heightened and different noise conditions, at which the zebrafish is exposed in artificial concealing environments at laboratories and research institutions, on the biology of this animal, this included: physiology, morphology, hatching and survivability, development, hearing capabilities and sensitivity, memory and behaviour.

Chapter 2

2.1 What are the effects of chronic artificial noise exposure on the physiology of the zebrafish?

The acoustic treatments considered in the first part of our study (see chapter 3) led to a significant increase in mortality (up to 32-33%). Very limited information exists to this date on the impact of noise on fish hatching success and survival [208,257] with some studies only reporting effects on growth and development [105,235].

Our study additionally found heightened cardiac rate, faster yolk sac consumption, and higer cortisol levels at both 3 and 5 dpf zebrafish treated with elevated noise levels,

which is a clear indication of noise-induced physiological stress. The cardiac rate values obtained are comparable to prior studies on the same model organism [251,259–262] We verified an increase of about 10% in both ontogenetic stages (3 dpf: from 173 to 191 bpm; 5 dpf: 203 to 224 bpm) under increased noise conditions.

Our study also showed that larval zebrafish under noise exposure consume their yolk sac 18% (at 3 dpf) and 58% (at 5 dpf) faster compared to baseline conditions. Other studies have used the yolk sac size of larval fish to assess the impact of environmental stress [269–272].

Increased cardiac activity represents an adrenergic stress response, which is typically responsible for activating metabolic pathways and mobilizing energy to cope with potential challenges [263–267] which in the case of our study consisted on acoustic disturbance. Hence, the energy depletion due to acoustic stress might be detrimental to the embryos that could otherwise use it in other processes. We further provided evidence that these variables are significantly correlated.

In addition, we confirmed the heightened physiological stress in noise-exposed larvae by measuring their cortisol levels that were significantly above control groups confirming that chronic noise exposure interfere with resources allocation from reserves maintenance, activation of the adrenal system and results in an allostatic load [268].

Finally, our work provided first evidence of noise-induced increase in whole-body cortisol levels and while measuring units widely vary between studies, cortisol levels were comparable with previous studies [273–277].

2.2 How does this impact chronic exposure compare with impact from noise presented in different temporal variations?

Our study showed that intermittent treatments with multiple onsets of acoustic disturbance caused higher mortality of up to 33%, similar to continuous noise exposure, this contrasted

with the treatment with few prolonged noise presentations that caused significantly less mortality rate. The relevance of the timing of acoustic exposure has received limited attention but increasing evidence points towards a significant impact on development and physiological stress in different fish species [235,258].

Variations in the temporal patterns of intermittent treatments caused different effects on cardiac rate, yolk sac consumption, and cortisol levels, suggesting that time regime is important to down-regulate physiological stress. Overall, continuous noise and the treatment with multiple acoustic presentations induced the highest cardiac rate and yolk sac consumption; while the treatment characterized by longer noise presentations and prolonged silent intervals caused less impact on these variables compared to control and other intermittent treatments. Moreover, cortisol levels generally spiked in noise-exposed groups.

We hypothesize that longer time intervals during random disturbances allow for recovery, compensation and/or habituation in larval fish, and that the total duration of noise exposure is less crucial compared to the time regime adopted (number of onsets of acoustic disturbance and silent intervals) [235].

2.3 Is there any effect of chronic noise exposure on sensorimotor traits?

Our study also investigated the impact of noise exposure at behavioural level in larval zebrafish from two different approaches. We focused on 5 dpf, a developmental stage when larvae acquire full motility, display active feeding behavior and danger/predator avoidance, suggesting that simple neural circuitries for processing reward and aversion are already functional [39,278]. We tested the effect of chronic noise on 5 dpf larvae using the anxiety-related light/dark preference test [276,279–283]. Our results indicated an increased darkness aversion (scotophobia) in noise-exposed embryos, suggesting that such environmental stressor elicits anxiety behaviour

Finally, this study also showed that noise-exposed animals displayed an impaired innate Spontaneous Alternation Behaviour compared to control individuals. Bögli *et al*, (2017) [284] effectively established the presence of SAB in larval zebrafish (6 dpf) suggesting the presence of early mnestic capabilities. At this developmental stage (3 to 8 dpf) an hippocampal like pallium structure develops [285], which is a related with navigation and spatiotemporal sensing in fishes [286,287].

Additionally, the overall locomotor activity of these specimens was further investigated and a significant reduction in covered area was observed for the noise-treated larvae.

For the last part of our noise impact assessment study, we wanted to provide first evidence of impact of noise exposure on inner ear morphology and hearing sensitivity and possible hypersensitization/desensitization to noise stimuli in larval zebrafish (*Danio rerio*). We assessed concurrence of noise-induced inner ear physiological alteration accompanied by hearing sensitivity impairment and hypersensitization in larval zebrafish and demonstrate that exposure to environmentally relevant noise levels may impact key sensory-related endpoints in early ontogeny.

Chapter 3

3.1 Is there any impact of chronic noise exposure on zebrafish hearing sensitivity?

Given the extended use of this model in a wide range of research, including hearing, and considering that they are usually kept in artificial housing systems where noise levels and particle motion components are significantly similar to the acoustic stimulus used in this experiment [94], we first assessed hearing sensitivity in 3 and 5 dpf AB wildtype zebrafish larvae using the microphonic potential recording method[347–349].

We found a significant impact on hearing sensitivity at low frequencies (100 Hz to 200 Hz) in 5 dpf individuals which concurs with the best hearing range at this developmental stage [364].

Additionally, we investigated hearing capabilities from a behavioural approach by using the prepulse inhibition method [256,323,353,354]. We assessed a significant increase in the reactivity of noise exposed animal (hyperacusis), noise-induced sensitization have been reported in previous studies [256,355]. This in combination with the significant decrease in responsiveness observed at 200 Hz between 140 and 150 dB prepulse level (control) and 150 to 160 dB prepulse level (noise), suggests an impairment in absolute hearing thresholds.

In our study, larval zebrafish also exhibited a significant decrease in habituation to startle-inducing stimuli following noise over-exposure with an average increase of 45% in the swimming velocity after acoustic stimulation, this results have been previously reported in other studies [356,357]. It is worth to mention that, different studies have reported different results regarding this methodology, with some studies reporting enhanced startle reflex [323] like our assessment, others reporting reduces responses [358], and others reporting little to no change in the magnitude of the acoustic startle reflex following noise exposure [359].

3.2 Is the reduction in hearing sensitivity related with morphological changes in inner ear?

In order to confirm that differences found in hearing sensitivity were related with alterations in the inner ear sensory epithelia, we investigated for possible noise-induced changes in the zebrafish inner ear saccular epithelia which is regarded as the main hearing endorgan [199]. By comparing saccular epithelia images of transgenic Et(krt4:GFP)^{sqet4} (ET4) zebrafish larvae we reported a significant reduction of the total number of ciliary bundles in combination with

a significantly reduced saccular epithelia area in noise-exposed animals which might be correlated with observed shifts in hearing thresholds.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

The acoustic environment is known to impact animal's physiology, development, and behavior, as well as, the evolution of inner ear structures and the hearing sense. Noise pollution is an increasing environmental problem, acting as a stressor on organisms at many levels. The impact of noise exposure on animals' auditory system and development is far from understood, and very limited knowledge is available on fish that, contrary to mammals, can regenerate inner ear hair cells and represent the largest extant group of vertebrates.

Fishes are excellent vertebrate models to address questions regarding physiological adaptations to environmental stressors, as they evolved in widely diverse habitats, possess many specialized morphological features, and offer technical advantages for studies at multiple levels of analysis. In particular, the zebrafish *Danio rerio* is a powerful model system widely used in biomedical research, as it allows one to combine embryology, genetics and *in vivo* visualization at a cellular level in a single organism. Although it does not possess outer or middle ears, zebrafish have a typical vertebrate inner ear at the cellular level (hair cells), and the development and anatomy have been intensively documented.

The present thesis relied on zebrafish as a key vertebrate model system to investigate the species adaptation to the environmental noise conditions and the effects of noise exposure on development, physiological stress, hearing and behavior. The work shows that larval zebrafish can be established as a high-throughput platform for fast screening of the impact of acoustic disturbances.

Furthermore, my research highlights the importance of investigating how altered soundscapes and associated physiological and behavioural stress may affect important sensitive windows in development and impose new evolutionary challenges under a scenario of global change. Understanding how changes in the environment affect organismal responses and their adaptive potential is paramount in the current scenario of global change [365,366]. Fish species are increasingly being challenged by changes in their sensory environments due to anthropogenic activities and climate change [75,367]. The effects of these changes on their sensory systems, and the ultimate consequences for fitness and evolution, have only recently received attention [366]. Environmental disturbance on one sensory channel may trigger compensation through other senses [75,368]. Yet surprisingly few studies have examined how environmental pressures impact different sensory systems and how these systems respond through genetic adaptation and/or plasticity [366].

The acoustic environment may act as environmental pressure on phenotypic plasticity leading to sensorial and behavioural changes. During ontogeny, the acoustic environment is known to shape the development of the auditory system and perceptual acoustic preferences in various taxa e.g. [124,369–371]. Despite the growing literature on how the acoustic environment impacts sensory systems and communication in late diverging vertebrates [124], very scarce information exists on how aquatic soundscapes may shape the auditory function and the consequences for environmental adaptation in fishes (e.g. [372]. Considering how fast aquatic acoustic environments are changing it is paramount to address these issues in fish, as they are key components of most aquatic ecosystems [75].

Similarly to the evolutionary divergence reported for the visual system in different fish species due to changes in visual conditions in the natural habitats [373,374], it is likely that the auditory system will also exhibit rapid evolutionary responses to human acoustic disturbances [375], but this has yet to be investigated.

The present thesis relied on zebrafish as a key vertebrate model system to investigate the species adaptation to the environmental noise conditions and the effects of noise exposure on development, physiological stress, hearing and behavior. The work shows that larval zebrafish can be established as a high-throughput platform for fast screening of the impact of acoustic disturbances. Building on the research questions that were raised in this thesis and the respecting findings, novelties and limitations, the following issues should be addressed in future research:

- Develop a detailed characterization of the natural soundscape in freshwater systems in India where zebrafish can be found, including a comparison between wet and dry seasons, and auditory sensitivity between different wild populations.
- Investigate the auditory masking effects caused by maintaining zebrafish under captive noise conditions in typical laboratory facilities. Besides, the potential hearing loss and physiological stress should be assessed by rearing the species under different housing systems.
- Assess the impact of different noise regularity, including regular and random regimes, on auditory sensitivity, physiological stress (including oxidative stress) and behavioural endopoints in both larval and adult zebrafish.
- Evaluate whether acoustic stress induces energetic costs in early ontogeny that may result in carryover effects to subsequent life stages and/or fitness. This could be investigated by exposing embryos to different acoustic conditions and evaluating the effects later throughout life including reproduction success, and potential consequences for the following generations.
- Investigate the potential noise-induced changes in gene expression patterns in zebrafish inner ear sensory hair cells and brain macroareas.

- Evaluate noise induced hearing loss in zebrafish throughout life, including potential interaction/cumulative effects with age-related hearing impairment.

Moreover, the research conducted with zebrafish should be complemented with studies on wild fish, both freshwater and marine, and including species that rely on acoustic communication for social interactions, in order to confirm coping molecular and physiological mechanisms and adaptive responses to real-world noise disturbances.

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