The noisy underwater world : the effect of sound on behaviour of captive zebrafish

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Citation

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Chapter 3

Behavioural responses to sound in captivity by two fish species with different hearing ability

Abstract:

Anthropogenic noise with variety of temporal and spectral patterns is increasing in, on and near aquatic environments. Artificially elevated ambient sound levels in natural conditions can have various detrimental effects on fish, such as temporary or permanent hearing loss, masking of relevant acoustic signals and cues or behavioural changes that may have fitness consequences. Also captive fish are often exposed to noisy conditions, which may have consequences for production in aquaculture, biases in scientific results in laboratories or welfare in hobby aquaria. However, we still have limited insight into how fish cope with artificial sound exposure and how species differ in sensitivity. Here, we compared zebrafish (*Danio rerio*) and cichlids (*Haplochromis piceatus*), for which the former is sensitive to lower absolute thresholds and wider spectral ranges than the latter. Experimental sound exposure induced a prolonged swimming speed reduction (during 1 min exposure) for both species in captive conditions. Furthermore, zebrafish showed clear startle response behaviour with the onset of the sound exposure leading to a brief increase in swimming speed, which was not found for the cichlids. Neither species showed spatial shifts away from the active speaker in the horizontal plane, but cichlids shifted downward to spend more time in the bottom area of the fish tank after the onset of sound exposure, while zebrafish retained their average swimming height during the same exposure levels. Our results show that sound exposure can cause both similar as well as species specific

responses in two fish species and that these responses are not obviously related to differences in their hearing ability.

Introduction

Human activities have acoustically changed aquatic environments over the past decades and anthropogenic noise is now recognized as a ubiquitous pollutant (Radford et al. 2014; Slabbekoorn et al. 2010). Shipping activities, wind farm operation, pile-driving, seismic surveys, naval sonars and fisheries activities are all accompanied by the introduction of both intended and unintended anthropogenic sounds in the water. Consequently, anthropogenic noise comes in many forms and can vary greatly in both temporal and spectral patterns. Although we know that sounds can play an important role for fish in natural habitats (Montgomery et al. 2006; Radford et al. 2007), we still have little understanding of the potentially negative consequences of noise pollution for aquatic life. While field studies in open water conditions are challenging to implement (Slabbekoorn 2016), studies in tanks have only just started to reveal e.g. the importance of temporal variation in sound exposure (Neo et al. 2014) and variation in disturbance tendency among species (Shafiei Sabet et al. 2015; Voellmy et al. 2014b)

Ambient noise may be abundant in marine and freshwater habitats without human presence. Common contributors to the natural acoustic environment include: biotic sounds produced by animals during mating and shoaling behaviour (Ladich 1997; Radford et al. 2008; Radford et al. 2010), abiotic sounds produced by geological and physical events such as seismic activity (Montgomery et al. 2006; Radford et al. 2007; Tolimieri et al. 2000), windy conditions and water currents (Tonolla et al. 2010). All these

sounds are potentially audible and useful to aquatic life. Some fish species, for example, use auditory cues for conspecific communication (Crawford et al. 1986; Myrberg et al. 1986) migratory orientation (Parmentier et al. 2015; Slabbekoorn et al. 2010), group cohesion (Staaterman et al. 2014), courtship and mate choice behaviour (Ladich 2004; Amorim 2006). Consequently, anthropogenic noise may also be audible and deter, disturb or mask relevant acoustic signals and cues (Slabbekoorn et al. 2010). However, although there is an increasing awareness of the potentially detrimental effects of anthropogenic noise on the behaviour of free ranging fish, there still remains a paucity of empirical evidence on the subject.

So far, a limited number of studies have reported on fish responses in the wild and only for a limited number of anthropogenic noise sources and these reports are often anecdotal or without replication. For instance, vessel noise was reported to change both the schooling structure and swimming behaviour of pelagic tuna (*Thunnus thynnus*) (Sarà et al. 2007) and air gun shooting during seismic survey made various fish species swim away from the sound source and down the water column (Engås & Løkkeborg 1996; Slotte et al. 2004). Moreover, short impulsive pile driving sounds caused to different behavioural changes; schools dispersal or density changes of sprat (*Sprattus sprattus*) whereas depth changes of mackerel (*Scomber scombrus*) (Hawkins et al. 2014). A study on roach (*Rutilus rutilus*) and rudd (*Scardinius erythrophthalmus*) reported on interruption of spawning activities by a fast-moving power-boat (Boussard 1981), while boat noise also reduced outside-burrow activities of red-lip gobies (*Gobius cruentatus*) and disturbed nest-care activities in damselfish (*Chromis*

chromis) (Picciulin et al. 2010). However, understanding the effect of noise on fish behaviour through studies in natural habitats is challenging as replication with fish of known background is hard to achieve and species may vary in their behavioural response (Slabbekoorn 2016).

Noise impact studies in indoor conditions provide the possibility to manipulate the experimental environment, to control the test group of subjects and to achieve sufficient replication. Studies on captive fish have revealed, for example, that acoustic over-exposure can cause temporary or permanent hearing loss (Amoser et al. 2004; Popper et al. 2005; Smith 2004). Also more moderate but realistic anthropogenic noise levels have been tested in the laboratory and have been shown to mask relevant acoustic signals and cues (Codarin et al. 2009; Vasconcelos et al. 2007) and to elicit anti-predator behaviour (Bruintjes & Radford 2013; Voellmy et al. 2014b; Simpson et al. 2015) and to reduce foraging performance (Purser & Radford 2011; Voellmy et al. 2014a; Shafiei Sabet et al. 2015). However, studies on noise-dependent spatial avoidance, such as done on several terrestrial animals (Knutson & Bailey 1974; MacKenzie et al. 1993; McAdie et al. 1993; O'connor et al. 2011; Schaub et al. 2008), are difficult on captive fish. Fish tanks yield obvious limitations for escape behaviour and sound field conditions are complex and different from outdoor conditions (Slabbekoorn 2016).

Although spatial avoidance or phonotaxis may not be expected from captive fish within the confinement and complex sound field of a fish tank (Parvulescu 1967; Akamatsu et al. 2002), there are a few studies that have

addressed this issue (see e.g. Neo et al. 2015; Febrina et al. 2015).

Horizontal displacements have been used to infer the ability of localization of sound sources under natural conditions in the wild (Popper & Fay 1993; Tolimieri et al. 2000; Fay & Popper 2005), but several studies have shown that also captive fish can localize sound sources and reveal positive phonotaxis in the horizontal plane (Higgs et al. 2007; Rollo & Higgs 2008; Verzijden et al. 2010). Vertical displacements may be another relevant spatial read-out that may indicate an anxiety-related response (Pearson et al. 1992; Brown et al. 2006; Luca & Gerlai 2012; Neo et al. 2014), providing a tool to study the effects of temporal variety in sound exposure or differences among different fish species.

In this study, we investigated how sound exposure affects two fish species with different swimming behaviour and different hearing abilities. We selected zebrafish (*Danio rerio*) and Lake Victoria cichlids (*Haplochromis piceatus*) as they represent fish with distinct swimming tendencies and hearing abilities and they were readily available. Zebrafish are typically swimming continuously, often with quick turns and frequent changes in speed, but always with a forward pace (see e.g. Cachat et al. 2010; Neo et al. 2015). Cichlids are much slower swimmers in general and alternate swimming bouts with periods of no movement (see e.g. Heuts 1999; Estramil et al. 2014). Zebrafish have Weberian ossicles that provide a lower absolute threshold and a wider spectral range of auditory sensitivity compared to Lake Victoria cichlids (Kenyon et al. 1998; Higgs et al. 2002; Ladich & Fay 2013), which vary in hearing sensitivity due to variation in

swim bladder size and position, but do not have the more advanced hearing aids of cyprinid fishes (Popper & Fay 1993; Schulz-Mirbach et al. 2012).

Our aims were to test how continuous and intermittent sound exposure changes swimming speed and spatial behaviour in a long fish tank in which sound is played from one or the other side. We compared baseline behaviour for individual fish of both species and tested differences in swimming speed in brief periods around sound onset (reflecting startle responses and sudden acceleration) as well as prolonged changes in swimming speed. In addition, we tested sound-related spatial variation by measurement of horizontal and vertical displacements. Moreover, we tested for internal consistency in swimming behaviour among behavioural measurements for which sound exposure had a significant impact. We expected no sound impact on horizontal displacement (c.f. Estramil et al. 2014; Neo et al. 2015), but we did expect anxiety-related vertical displacement (c.f. Gerlai 2010; Voellmy et al. 2014b) that could be correlated to an initial speeding response and to slowing down in the long-term. We further expected that differences in the behavioural effects of sounds that are well within the audible range for both species are not necessarily related to their relative hearing abilities.

Materials and Methods

Study Species and Housing Condition

Thirty adult wild type zebrafish (*Danio rerio,* sex ratio ~ 1:1) were obtained from our own breeding stock (Sylvius laboratory, Leiden University), which originated from fish stocks from Europet Bernina International BV (Gemert-Bakel, The Netherlands), bought at a local pet supplier (Selecta Aquarium Speciaalzaak). All zebrafish were housed in a 400-litre glass holding tank (200 cm ×40 cm ×50 cm; water depth: 40 cm; wall thickness: 4 mm) on a 14 h light: 10 h dark cycle (light switched on at 6:00 and switched off at 20:00) and with the water temperature kept at 24°C. Zebrafish have their peak hearing sensitivity around 800 Hz (Higgs et al. 2002).

Thirty adult wild type Lake Victoria cichlids (*Haplochromis piceatus*, sex ratio ~ 1:1) were taken from our own breeding stock (Sylvius laboratory, Leiden University, third generation in captivity), which originated from wild-caught fish imported from Tanzania. All cichlids were housed in a 300-litre glass holding tank (150 cm ×40 cm ×50 cm; water depth: ~40 cm; wall thickness: 4 mm), also on a 14 h light: 10 h dark cycle and with the water temperature kept at 24°C. Fish holding tanks were connected to a central water recirculation system (Fleuren & Nooijen, Nederweert, The Netherlands). All fish individuals for both species were fed twice daily with dry food (DuplaRin M, Gelsdorf, Germany) and frozen Artemias (RUTO frozen fish food, The Netherlands). *H. piceatus* has not been tested for hearing sensitivity, but cichlids with a range of swim bladder

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sizes and shapes varied in peak sensitivity between 200-500 Hz (Schulz-Mirbach et al. 2012). We inspected size and position of the swim bladder in a dead specimen of *H. piceatus* and no extreme morphology was observed and measures appeared well within the range of the three cichlid species tested by Schulz-Mirbach et al. (2012). Ambient noise conditions (around 95 dB re 1 µPa) were similar for both species as their holding tanks were on the same type of tables and in the same room.

Experimental tank and set-up

The experiments were conducted in a rectangular glass tank (200 cm ×35 cm ×45 cm; water depth: ~35 cm; wall thickness: 1 cm). The tank was placed on a steady table on top of a layer of Styrofoam (thickness: 20 mm) to minimize transmission of environmental sound from the laboratory building (Fig. 1a). The water recirculation was controlled by an Eheim water pump Type 2115 (made in Germany), which was always switched on except during the experiment. The air temperature in the experiment room was kept at 24°C and the water temperature in the tank was kept at 23±1°C.

Two underwater loud speakers UW-30, Lubell Labs Columbus, OH, U.S.A. were embedded in the tank walls at each far end of the tank (in direct contact with the tank water on the inside and surrounded by water-filled glass extension boxes (25 cm ×20 cm ×20 cm) on the outside). The speakers were connected to a QUAD 303 power amplifier (Mfg Co Ltd, Huntingdon England). A stainless steel frame with a fine-meshed net was placed on both

sides of the tank at 5 cm from each speaker to keep the fish from swimming to the side of or below the speakers.

Behavioural experiments were performed after the fish had acclimated to the test tank. Acclimated refers to the fish swimming freely in the tank, making explorative rounds above the bottom layer, without freezing bouts or rapid turns and erratic swimming tracks (for zebrafish, see Neo et al. 2014; Shafiei Sabet et al. 2015; and for cichlids, see Verzijden et al. 2010; Estramil et al. 2014). Pre-test observation showed that cichlids required more time than zebrafish to swim freely and show state of explorative swimming and we therefore left them in the tank overnight to test them in the following morning. Zebrafish were sufficiently acclimated within 2 hours after being introduced to the test tank, swam freely in whole arena of the tank, and were tested after the cichlids in the afternoon. Consequently, individual cichlids and zebrafish were gently introduced into the fish tank using a fish net and kept in there for at least 14 hours and 2 hours respectively.

Trials for each individual per species were conducted at the same time of day (9:00 for cichlids and 14:00 for zebrafish). In this way, we avoided the confounding effect of diurnal activity cycles within a species, but inherently introduced a confounding effect in testing time of day between species. Testing both species at the same time of day would have been better, but would also have taken much longer for the overall testing period, which was not feasible, and maybe would have introduced another variable in fish age or testing time in the year. We decided on the current

compromise as we expected intra-specific variation over the day to be smaller than inter-specific variation irrespective of time of day. Independent data from a study on just zebrafish (Shafiei Sabet et al. in press) indeed revealed no significant differences in the tendency to respond to sound between morning and afternoon exposures (n = 17 zebrafish tested in the morning, n= 18 tested in the afternoon, $P > 0.1$ for immediate (10 sec) and prolonged (1 minute) swimming speed).

Sound Stimulus Preparation and Acoustic Measurements

Sound files were created from white noise, artificially generated with Audacity (version 2.0.3, http://audacity.sourceforge.net) in WAV-file format (32 bits, 44.1 kHz sampling rate) and band- pass filtered between 100-1000 Hz. We decided to use this artificial stimulus as it is a crude spectral reflection of all broadband sounds in nature, allows easy replication, and avoids typical problems of pseudoreplication with one or few natural outside recordings (see e.g. Slabbekoorn & Bouton 2008). Subsequently, the playback files were amplified in Audacity to a maximum level, without allowing overload. Each trial consisted of the following three playback components played in a random order, with each component lasting 45 minutes followed by a 15 minute break of ambient noise: Ambient noise with the speaker switched on but without sound playback (AN); continuous playing back of sound (CS), and intermittent irregular white noise (INT), consisting of one-second pulses at intervals of random duration varying from 1 to 7 seconds (labelled 1-7 in our previous study in which we used more intermittent sound stimuli of different temporal patterns (Shafiei Sabet

et al. 2015). The randomly selected sequences included all six combinations in such a way that each was used equally often: AN-CS-INT, AN-INT-CS, INT-AN-CS, INT-CS-AN, CS-INT-AN, and CS-AN-INT, resulting in a full factorial design (Fig. 3).

Sound playback in each trial started either with the speaker on the left side or on the right side of the experimental tank (randomly chosen), where the playback speaker was labelled the "active" speaker. The subsequent sound treatments were played from alternating sides of the tank, one speaker at a time. Sound files were played back with a portable Tascam (model DR-07) and amplified with a power amplifier (Quad 303). Fish behaviour was continuously recorded using a Panasonic full HD camcorder (model HC-V500) during the entire test period.

In order to check if there was a sound gradient in the experimental tank, the sound pressure level (SPL) was measured using a Marantz solid state recorder (model PMD-661) in combination with a High Tech hydrophone (model HTI 96 min). Measurements were taken at different locations throughout the tank with either the left, right or no speaker playing (Fig. 1b&c). Both recorder and hydrophone were calibrated (Netherlands Organisation for Applied Scientific Research). Underwater particle velocity was measured using a calibrated vector sensor comprised of three orthogonally placed geophones, (X-, Y- and Z-coordinate), mounted inside a transparent Plexiglas sphere (9.5 cm in diameter; c.f. van den Berg & Schuijf 1985; Bretschneider et al. 2013; Shafiei Sabet et al. 2015). Accelerometers were connected to a digital differential oscilloscope

(PicoScope model 3425) and the particle velocity levels per measurement location were calculated by taking the root mean square of the velocity data received by each geophone over the measurement period, and then summing the results for each geophone using vector addition. All acoustic calculations were done in MatLab (version R2013a, Mathworks, Natick, MA, U.S.A.). The sound pressure level and particle velocity level were measured in 15 cm distance from one side of the long tank, 15 cm distance from the active speaker horizontally and 20 cm distance from the bottom vertically (Fig. 2a&b). We used three replicate measurements for each location. Both sound pressure and particle motion were elevated during experimental exposure and we therefore assessed both (definitions for our acoustic terminology follow ANSI/ASA S1.1, 2013).
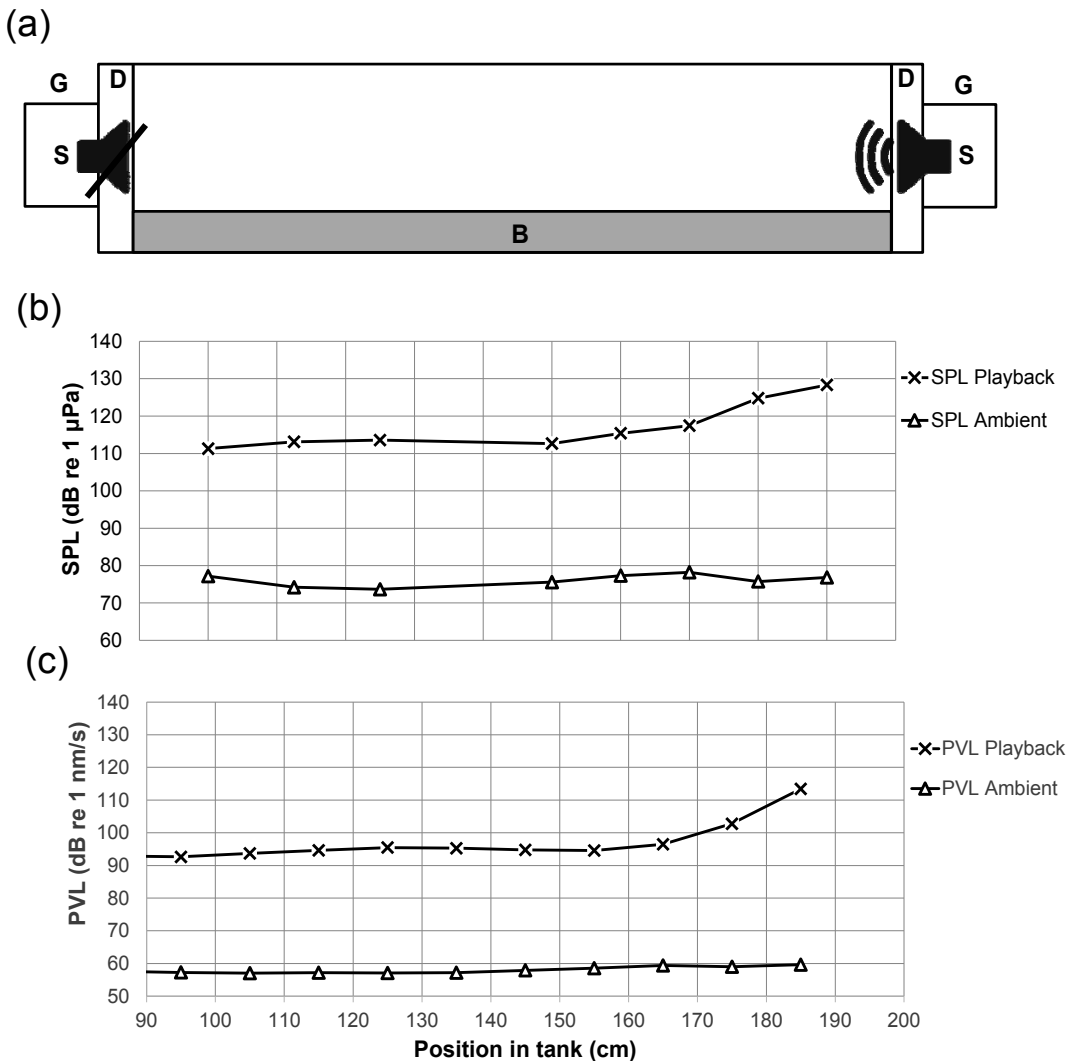
Fig. 1. a) Schematic lateral view of the experimental long tank set-up, b) Sound pressure level (SPL) and c) Particle velocity level (PVL) the target frequency range of :100-1000 Hz. Locations of the net mesh with waterproof metal frame both sides of the tank used as arena divider (D), small glass boxes connected to the each side of the long tank designed to submerge the both side of the underwater speakers in water (G), the UW30 underwater speakers attached to the each side of the long tank (S) and the area in the tank referred to as bottom layer to assess the behavioural displacement vertically (B). b) Sound pressure level (SPL) (dB re 1 µPa) profile of both playback and ambient conditions across the long tank

emanated from the active speaker positioned on the right side to the middle of the long tank (1m), and c) Particle velocity level (PVL) (dB re 1 (nm/s) profile of both playback and ambient conditions across the long tank emanated from the active speaker positioned on the right side to the middle of the long tank (1m). For B and C all sound pressure level and particle velocity level averaged across all frequencies.
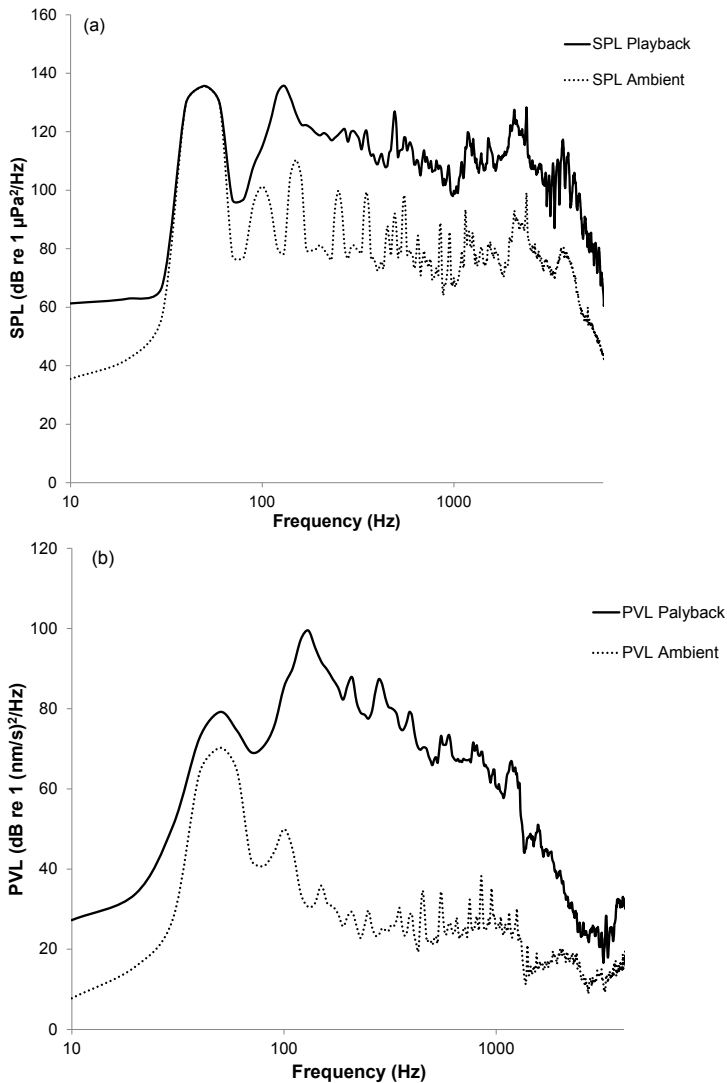
Fig. 2. Power spectral density of a) SPL (in dB re 1 $\mu Pa^2/Hz$) and b) PVL (in dB re 1 $(nm/s)^2/Hz$) for playback and ambient conditions. Both sound pressure level and particle velocity level in ambient conditions are much lower than sound playback across the relevant frequency range (100-1000 Hz). The sound pressure level and particle velocity level were measured 15 cm from one side of the long tank, 15 cm from the active speaker horizontally and 20 cm distance from the bottom vertically. The solid black line on the

graphs show playback measurements and the grey dot lines show ambient measurements. The frequency range of the artificially elevated sound overlaps well the peak hearing sensitivity of both zebrafish (around 800 Hz, See Higgs et al. 2002) and cichlids species (200-500 Hz, See Schulz-Mirbach et al., 2012).
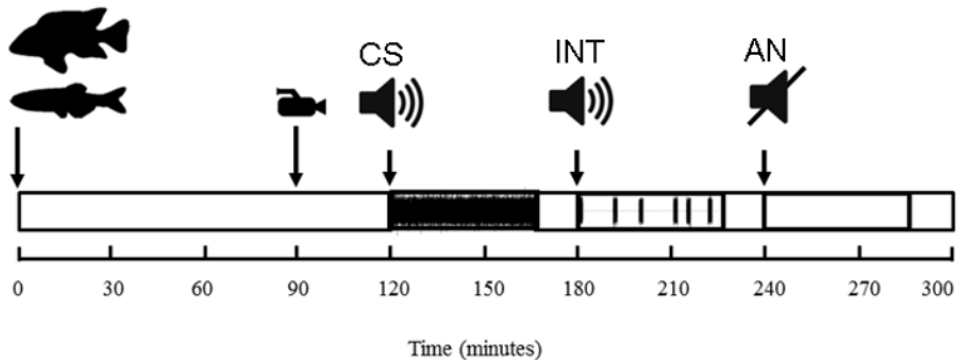

Fig. 3. Schematic view of the timeline of the whole playback procedures and fish individual release events for zebrafish and cichlids. Zebrafish and cichlids were released individually and let them to acclimatize for at least 2 h and 14 h respectively (see text for explanation). Video recording started 30 min before the first exposure in each trial to exclude any influence of the presence of a human observer. The sequence of the trial on the figure for example indicated: CS-INT-AN, CS: continuous sound exposure, INT: intermittent irregular sound exposure (randomized sound pulses composed of 1-7s with 1s silence interval) and AN: ambient sound as control.

Processing of Behavioural Data and Measurements

All zebrafish video files were converted by AVS Video converter 8.1 into a 5 frame-rate per second (FPS) M4V file. Converted video files were then analysed with the Matlab custom-written script to trace individual fish automatically in Matlab 2013a. This tracking system allowed us to

precisely quantify the swimming behaviour and spatial pattern of the experimental fish. We used a different method for assessing behaviour of cichlids as their swimming speed was often too slow for the automatic processing. Therefore, we converted cichlid video files using the AVS Video converter 8.1 into one-frame per second rate MOV file and analysed movements and displacements manually with the same method we used in an earlier study (Shafiei Sabet et al. 2015) by Logger Pro (version 3.6.0, Vernier). We investigated sound-induced changes in both species by tracking individuals during complete trials and comparing activity just before and right after onset of sound exposure as well as throughout the exposure period. The treatment exposure sequence was randomized to avoid order effects. We also checked statistically for an order effect by including the position of the treatment in the trial sequence as a random factor, butdid not find an order effect in any of our test results (all P>0.1). All video analyses were done without audio track and therefore blind to the treatment sequence for the observer (S.S.S.). Inter-observer reliability was tested and confirmed by reanalysis of half of the behavioural data set by a second observer (K.W.).

We assessed brief changes in swimming speed that may indicate a startle response or just sound-induced acceleration (c.f. Neo et al. 2014; Shafiei Sabet et al. 2015) and we quantified this parameter by subtracting the swimming speed of the individuals during 10 sec right after onset of the sound by the swimming speed during the 10 sec immediately before onset of the sound for both species. We assessed the changes in prolonged swimming speed for all sound exposures in a similar way by comparing 1

min periods before and after sound onset for both species, but we depicted and tested absolute levels per species to allow better insight in actual swimming speeds for baseline and during exposure. We also analyzed the time that fish spent in the bottom layer (0-5 cm) of the tank (see Fig. 1a) in this way for the period of 1 min before onset of the sound and 1 min right after onset of the sound. Furthermore, we tested for shifts in spatial behaviour by assessing horizontal displacements for even longer periods of 15 min before and after onset of the sound for both species. When behavioural changes were significant, we tested for individual consistency in each species by exploring correlations among parameters.

Statistical Analysis

Behavioural data were analyzed in SPSS version 21.0 (Armonk, NY. IBM Corp.), using analysis of variance (ANOVA) for repeated measures. We used an initial two factorial design analysis with sound treatment as a "within-subject factor" and species as a "between-subject factor", while including possible interactions to test for significant effects on the difference in swimming speed in the brief periods right after and before sound onset. Subsequently, we used again ANOVA repeated measures for separate species-specific analyses to test for the effects of sound exposure (before and after the start of the relevant sound treatment) and treatment, while including possible interactions. We chose this approach for prolonged swimming speed, time spent in the bottom layer and spatial behaviour changes because we believe absolute values of these parameters are

important and stand out more from relative values in which are comparable for other studies. A Huynh-Feldt correction was performed when sphericity could not be assumed in the repeated measures ANOVA. Bonferroni corrected Post-hoc tests were performed when ANOVA test results were significant. A Pearson correlation was used as follow-up test to analyse a possible correlations between parameters that were significantly affected in each species.

Ethical Statement

All housing, experimental conditions and procedures were in accordance with the ethical guidelines of the association for the Study of Animal Behaviour in the Netherlands. The experiments were only carried out after an evaluation and approval by the Animal Ethics Committee of Leiden University (UDEC), (DEC #: 13022). In both species, fish were tested individually only once they were acclimated to the experimental set-up. At the end of the test, individual fish of each species were transferred to the stock tank and resumed normal activities. All fish used in this experiment were kept in order to produce new generations for future research.

Results

Sound impact on immediate and brief changes in swimming speed

Immediate and brief changes in swimming speed were affected by sound exposure for zebrafish but not for cichlids (See fig. 4). There was a statistically significant species difference ($F_{1, 56}$=18.379, P=0.001) and a non-significant trend for an effect of sound treatment ($F_{2, 112}$=2.959, P=0.056). There was a significant interaction effect for species × treatment ($F_{2, 112}$=5.553, P=0.005). Post-hoc comparisons revealed that for zebrafish there were significant differences for both treatments in comparison to AN as the control group; (AN: CS, P=0.026) and (AN: INT, P=0.001) and a non-significant trend for a difference between the two sound treatments (CS: INT, P=0.055). In the other word, irrespective to the sound temporal patterns, both sound treatments (CS and INT) have increased zebrafish immediate swimming speed as startle response changes in comparison with (AN) as control treatment. For cichlids, there was no significant variation among any of the treatments (AN: CS, P=0.592; AN: INT, P=0.559; CS: INT, P=0.875).
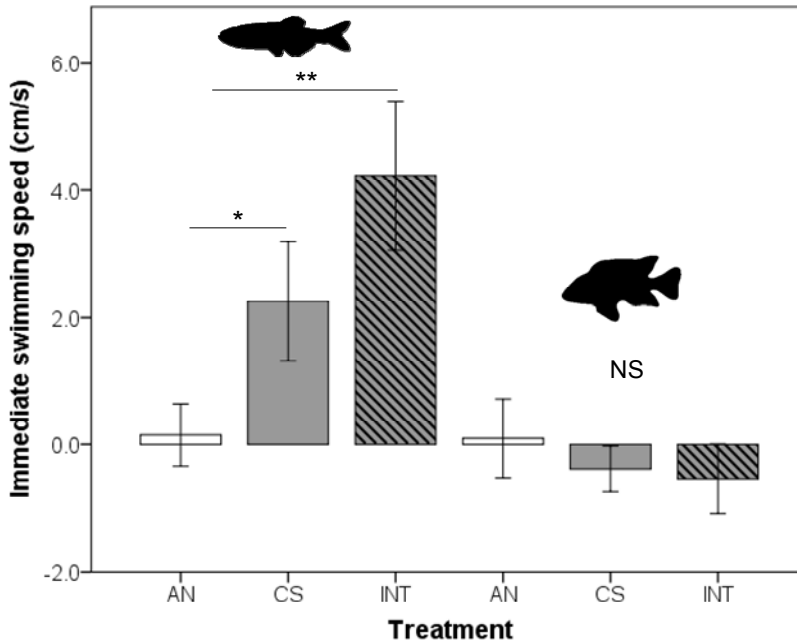
Fig. 4. Effect of sound exposure on zebrafish (n: 28) and cichlids (n: 30) brief swimming speed changes (cm/s) reflecting startle response and initial acceleration. Brief swimming speed changes were measured by subtracting the last 10 seconds before sound exposure by the first 10 seconds immediately after onset of sound exposure. Abbreviation of treatments: AN: ambient noise with no sound as a control, CS: continuous sound and INT: intermittent irregular sound (randomized sound pulses composed of 1-7s with 1s silence interval). White bars represent ambient condition with no sound playback as control (AN), grey bars show continuous sound treatment (CS) and grey hatched bars display intermittent irregular sound treatment (INT). Bars show means ± S.E.M. and significant differences are indicated as ** ($p<0.01$),* ($p<0.05$) and NS (not significant; $p>0.1$).

Baseline swimming and sound impact on prolonged swimming speed

The zebrafish average baseline swimming speed (~ 8 cm/s) was four times higher than the cichlid swimming speed (~ 2 cm/s) and the baseline swimming speed was significantly different between species ($F_{1,52}$= 55.965, P=0.001) (See fig. 5a). In both zebrafish and cichlids, sound exposure led to a reduction in prolonged swimming speed, irrespective of the temporal pattern of the sound stimulus (CS: continuous and INT: intermittent). In zebrafish, there was a significant effect of sound exposure ($F_{1, 27}$=13.518, P=0.001), no overall effect of treatment ($F_{2, 48}$=0.135, P=0.874), but a significant interaction for exposure × treatment ($F_{2, 54}$=5.453, P=0.007). Post-hoc pairwise comparisons revealed significant effects of exposure with a reduction of prolonged swimming speed for CS (P=0.004) and INT (P=0.002) and no significant effect for the AN control (P=0.948). In cichlids, prolonged swimming speed was also significantly affected by sound exposure ($F_{1, 29}$=31.256, P=0.001), with no effect of treatment ($F_{2, 58}$=1.396, P=0.256), but with a significant interaction for exposure × treatment ($F_{2, 58}$=3.316, P=0.043), (See fig. 5b). Pairwise comparisons revealed a significant effects in a way of reduction of prolonged swimming speed for CS (P < 0.001) and INT (P < 0.001) but no significant effect for the AN control (P=0.279).

Sound impact on time spent in the bottom layer

Both species responded to sound exposure, although the patterns were not similar. Zebrafish did not change allocation of time spent in the bottom layer of the tank, while cichlids did change their vertical distribution and spent more time in the bottom layer of the tank after onset of the sound exposure (See fig. 5c and 5d). In zebrafish, there was no overall effect of exposure ($F_{1, 26}=0.223$, $P=0.641$), treatment ($F_{1.676, 43.584}=0.293$, $P=0.709$), or an interaction for exposure × treatment ($F_{1.469, 38,200}=0.857$, $P=0.401$). In cichlids, we did find a significant exposure effect ($F_{1, 27}=15.308$, $P=0.001$) and a treatment effect ($F_{2, 54}=7.806$, $P=0.001$) in a way with onset of sound exposure cichlids spent more time in the bottom-layer for both sound treatments (CS and INT), but no significant interaction for exposure × treatment ($F_{2, 54}=2.197$, $P > 0.10.121$).
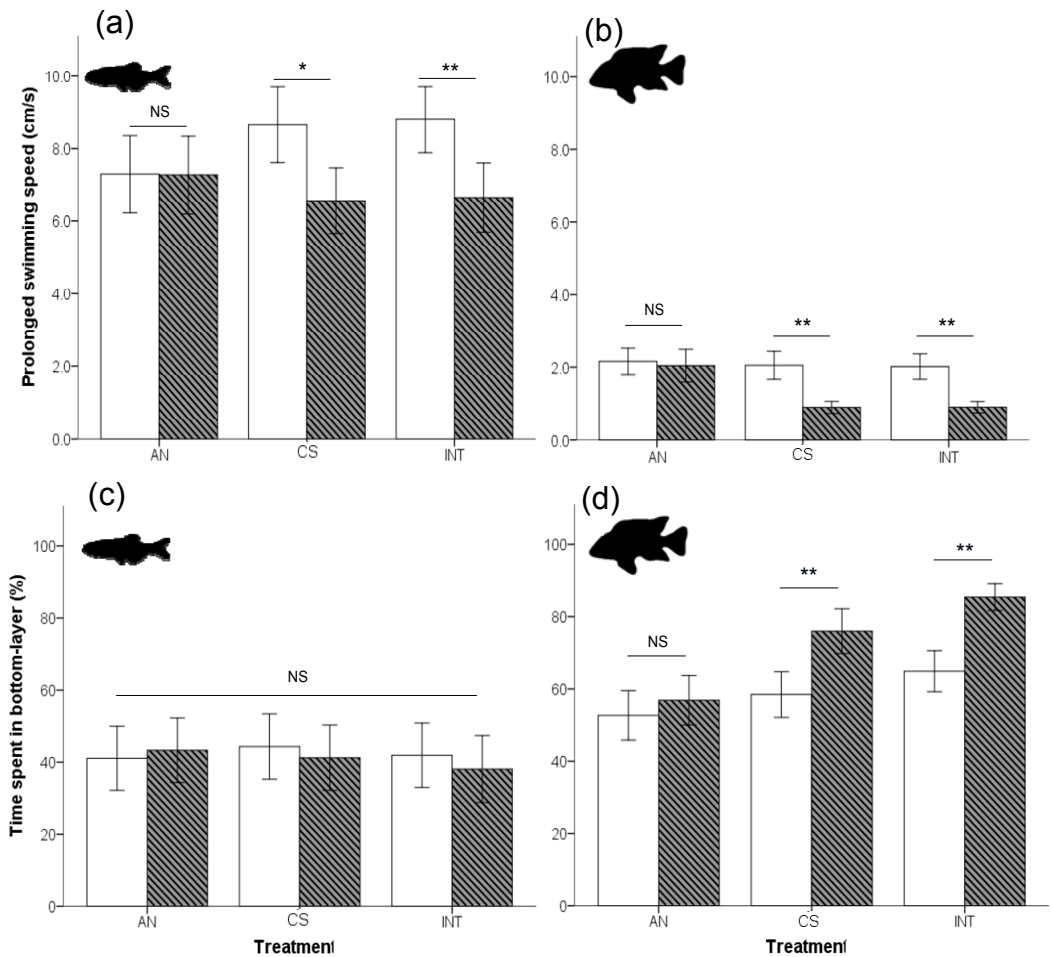
Fig. 5. Effect of sound exposure on: (a) zebrafish (n=28) and (b) cichlids (n=30) prolonged swimming speed changes (cm/s). Effect of sound exposure on the time spent in the bottom layer of the experimental tank (%) in (c) zebrafish (n=28) and (d) Cichlids (n=30). The bottom layer arena for spatial displacement was defined as the bottom layer with 5 cm vertical distance from the bottom of the tank. Prolonged swimming speed changes and time spent in the bottom layer was calculated from the last 1 min before sound exposure (white bars) to 1 min with immediately with on-set of sound exposure (grey bars). Abbreviation of treatments: AN: ambient noise with no sound as a control, CS: continuous sound and INT: intermittent irregular sound (randomized sound pulses composed of 1-7s with 1s silence

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interval). Bars show means ± S.E.M. and significant differences are indicated as ** (p<0.01), * (p<0.05) and NS (not significant; p>0.1).

Sound impact on spatial behaviour in the horizontal plane

We did not find sound-related horizontal displacement for zebrafish or for cichlids. The pattern of horizontal distribution did not vary significantly among treatments (See fig. 6a and 6b). We did find large variation for horizontal distribution across the long tank in both species, but there was no indication of an effect of localized sound playback. In zebrafish, there was no effect of exposure ($F_{1, 26}$=1.146, P=0.294) and no treatment effect ($F_{1.50, 38.997}$=1.136, P=0.317) or interaction for exposure × treatment ($F_{1.548, 40.238}$=1.073, P=0.337). In cichlid, there was no exposure effect ($F_{1, 28}$=3.445, P=0.074) and no treatment effect ($F_{2, 56}$=0.331, P=0.719) or interaction for exposure × treatment ($F_{2, 56}$=0.314, P=0.732).
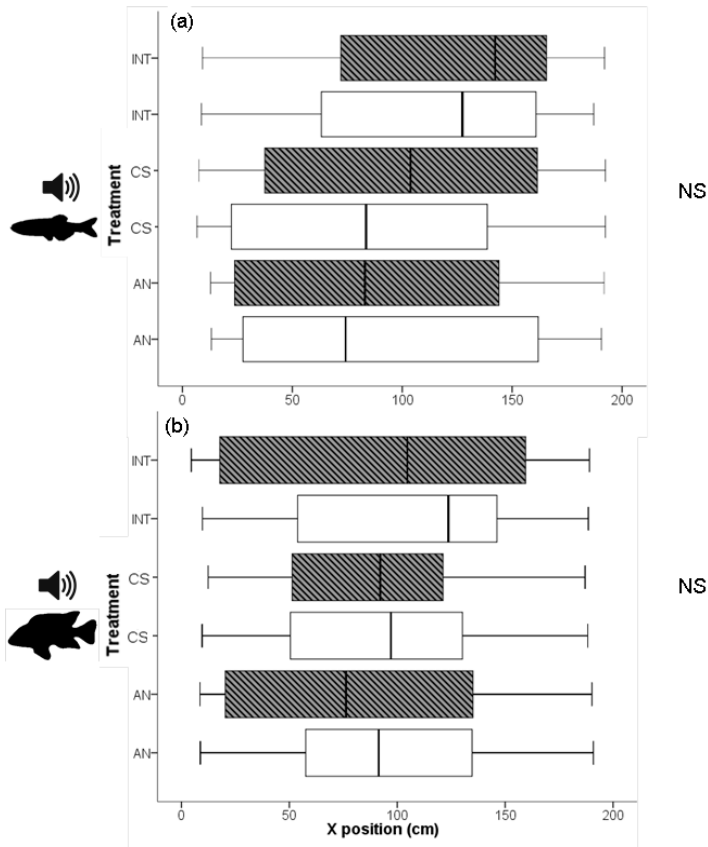
Fig. 6. Effect of sound exposure on: (a) zebrafish (n=28) and (b) cichlids (n=30) horizontal spatial displacement. Horizontal displacement was calculated from the last 15 min before sound exposure (white bars) to 15 min with immediately with on-set of sound exposure (grey bars). When sound played back from right speaker the spatial displacement data were filliped over to the left side direction. Abbreviation of treatments: AN: ambient noise with no sound as a control, CS: continuous sound and INT: intermittent irregular sound (randomized sound pulses composed of 1-7s with 1s silence interval). Bars show means± S.E.M and significant differences are indicated as ** (p<0.01), * (p<0.05) and NS (not significant; p>0.1). The UW30 underwater speaker played back from the left side.

Individual variation in response strength in different parameters

We did not find consistent patterns in the individual response tendencies for different parameters. There was no correlation between the significant increase in swimming speed in the brief period after sound onset and the significant reduction in prolonged swimming speed (for both CS and INT) in zebrafish (r=10.012, n=54, p=0.934) (See fig. 7a and 7b). We also did not find a correlation between the significant increase in time spent in the bottom layer and the significant reduction in prolonged swimming speed (for both CS and INT) in cichlids (r=-0.157, n=56, p=0.248).
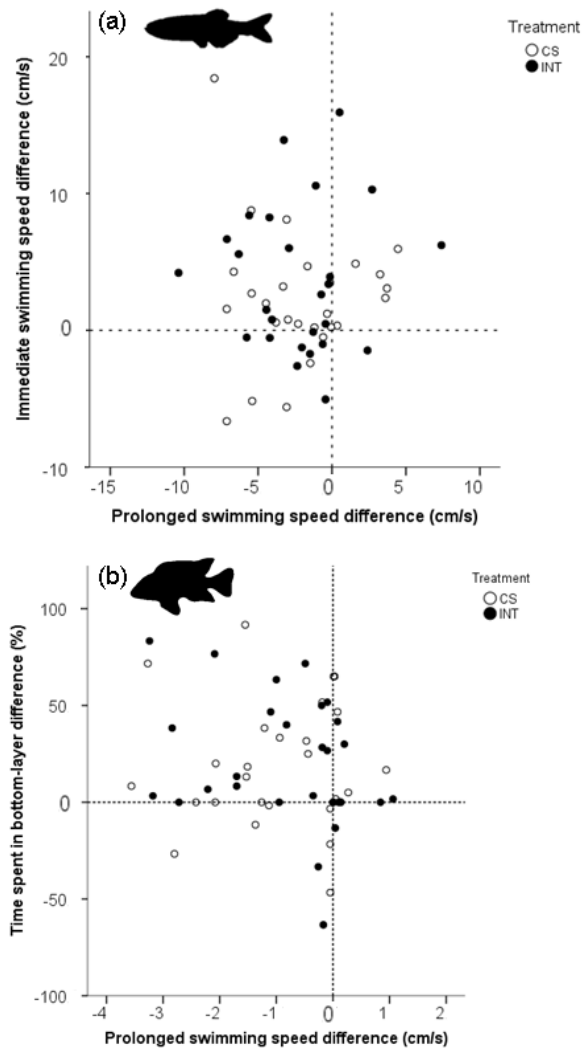
Fig. 7. (a) Lack of correlation between the difference in swimming speed (cm/s) in the brief period of 10 sec immediately after sound onset and the decrease in prolonged swimming speed (cm/s) in zebrafish (n=27). (b) Lack of correlation between the increase in time spent in the bottom layer (%) and the decrease in prolonged swimming speed (cm/s) in cichlids (n=28). Black and white circular dots show (CS) and (INT) treatments respectively. Abbreviation of treatments: CS: continuous sound and INT: intermittent irregular sound.

Discussion

Our results showed significant effects on behaviour in response to the experimentally elevated sound levels in both species: they were already different in baseline behaviour, but showed both similarities and discrepancies in response patterns. In zebrafish, the baseline swimming speed before any sound exposure was four times higher than in cichlids (Fig. 5a &b) and they also spent less time in the layer close to the bottom compared to cichlids (Fig 5c &d). At the onset of sound exposure the zebrafish immediately increased their swimming speed due to startle or initial acceleration responses, which were not observed for cichlids, which occasionally even started to swim backwards. The brief swimming speed changes of zebrafish also tended to be more affected by the intermittent than the continuous sound exposure. After the initial seconds, both species reduced their swimming speed during the "prolonged" period of sound exposure and cichlids went even more down the water column and spent significantly more time in the bottom layer of the tank during both sound exposure conditions, while zebrafish remained at the same level. We found no effects of the sound exposure on the horizontal distribution for neither of the fish species. Finally, we found no correlations among behavioural parameters that showed significant changes: there was no correlation between the initial and brief change in swimming speed and the change in prolonged swimming speed for zebrafish and no correlation between time spent in the bottom-layer and prolonged swimming speed for cichlids.

Sound exposure induced anxiety-related behaviour

The initial increase in swimming speed for zebrafish, the downward shift towards the bottom of the tank for cichlids and the decrease of prolonged swimming speed for both species are behavioural responses that are not unexpected and can probably be best interpreted as induced by anxiety. Similar response patterns were reported in previous sound exposure studies on zebrafish (*Danio rerio*) (Neo et al. 2015), sea bass (*Dicentrarchus labrax*) (Neo et al. 2014), Atlantic salmon (*salmo salar*) (Bui et al. 2013), roach (*Rutilus rutilus*) and three-spined sticklebacks (*Gasterosteus aculeatus*) (Andersson et al. 2007). Furthermore, for zebrafish it was shown that moderate sound pressure levels (112 dB re 1 µPa) induced initial increases in swimming speed and upward shifts towards the surface as well as increases in group cohesion for the socially tested individuals, but that higher levels (122 dB re 1 µPa) induced the above-mentioned behavioural changes (Neo et al. 2015).

Studies with other stimuli that are likely to trigger anxiety, such as chemical and visual indicators of the presence or approach of a predator induced similar behaviours in several different captive and free-ranging fish species (c.f. Dill 1974a; 1974b; Wisenden & Sargent 1997; Vilhunen & Hirvonen 2003; Wisenden et al. 2008; Voellmy et al. 2014b), including zebrafish (e.g. Speedie & Gerlai 2008; Gerlai et al. 2009) and cichlids (e.g. Vavrek & Brown 2009). Consequently, responses such as startles, moving down the water column, overall slow-down in activities, reduced feeding

rates and increased hiding time in a shelter are all likely due to an increase in perceived predation risk and may be adaptive under natural conditions.

Lack of horizontal avoidance in a fish tank

Zebrafish and cichlids did not show any consistent spatial changes in the horizontal plane that could indicate acoustic avoidance. In very specific sound exposure conditions to the left or right side of individual fish, there is evidence that both goldfish (*Caracius auratus*) and cichlids (*Haplochromis burtoni*) are able to respond in a lateral fashion away from the direction of the sound source (Canfield & Rose 1996). However, our results on general exposure of captive but free-swimming fish are in line with other earlier studies (Kastelein et al. 2007; Kastelein et al. 2008; but also see Febrina et al. 2015). Captive conditions may just limit directional escape options and prevent swimming away from the sound source. It might also be that there were no directional sound cues in our experimental tank: sound pressure and particle velocity declined steeply, but only in close proximity to the speaker and in most areas of the long fish tank sound levels were rather similar. Furthermore, these particle velocity levels concern averaged levels in all directions and reflections and near-field sound conditions may render the directional cues from particle motion in the different directions unpredictable and chaotic (Parvulescu 1967; Popper & Fay 1993; Akamatsu et al. 2002). Alternatively, it might be that there were sufficient sound cues

but that they were not detected by the fish or did not induce any biased directional response. In an earlier exposure study with zebrafish, discrete acoustic compartments in a dual tank set-up also did not affect spatial distribution among quiet and noisy compartments (Neo et al. 2015).

It may therefore be concluded that, for one reason or the other, sound may induce anxiety related responses but that horizontal escape behaviour that is reported for free-ranging fish (Blaxter et al. 1981; Olsen et al. 1983; Ona & Godø 1990; Engås et al. 1996: Engås & Løkkeborg 2002; Draštík & Kubečka 2005) is not a typical response behaviour in captive conditions. This seems in contrast with some studies carried out in captivity that focused on possible attraction to sound sources. Laboratory tank-based experiments showed that the round goby (*Neogobius melanostomus*) was attracted to conspecific sound (Higgs et al. 2007; Rollo & Higgs 2008) and female Lake Victoria cichlids (*Pundamilia nyererei*) seemed attracted to the tank side of sound playback when exposed to conspecific calls in concert with the visual presence of life males (Verzijden et al. 2010). When exposed to just conspecific sound, the cichlids did not show any phonotactic response any more (Estramil et al. 2014). More analyses of both deterrent and attractant effects are needed together with more detailed measurements on local variability of sound field conditions to really understand what cues fish could be responding to in natural and captive conditions (cf. Zeddies et al. 2010; Zeddies et al. 2012).

Interpretation of species differences

Although zebrafish and cichlids responded partly similar, there were also differences that we may try to interpret and explain. Initial acceleration and startles are reflex behaviours that occur in response to stimuli that signal potential danger (Dill 1974b; Wisenden et al. 2008; Gerlai et al. 2009; Gerlai 2010; Voellmy et al. 2014b) and that may save a fish from a predator attack (Wisenden et al. 1995; Gotz & Janik 2011; Luca & Gerlai 2012). Swimming down the water column, as we only found for the cichlids here, is a very general anxiety-related behaviour that may be longer lasting and may therefore also interfere for longer with other activities such as exploration, feeding, and social interactions (Gerlai 2010). However, it remains difficult to interpret the cause or consequence of one response (e.g. initial but brief speeding/startle) as more or less severe than the other (e.g. longer lasting shift downward towards the bottom) and neither of them was correlated at the individual level with prolonged slow-down of activity. Maybe more physiological measures, such as breathing rate, heart beat, or cortisol concentrations could provide more insight into the relative severity of a behavioural impact (see e.g. Santulli et al. 1999; Wysocki et al. 2006; Barcellos et al. 2007; Graham & Cooke 2008; Cachat et al. 2010; Debusschere et al. 2016).

Zebrafish have better hearing sensitivity than cichlids, both in terms of absolute thresholds as well as in terms of spectral range (Fay & Popper 1974; Higgs et al. 2002), and this may be an explanation for their higher tendency to startle in response to sounds of the current experimental

exposure level. However, it may also be that they have a more pelagic and erratic style of exploration and a more dynamic style of interaction with their environment that explains the threshold differences between the two species. Zebrafish have been observed to go down the water column in response to higher sound levels in a previous experiment (Neo et al. 2015) and this behaviour is also for this species a well-known anxiety-indicating read-out (Luca & Gerlai 2012; Speedie & Gerlai 2008). Consequently, the fact that with the current experimental exposure conditions cichlids do go down but zebrafish do not may imply that the sounds are perceived as more threatening by the cichlids than by the zebrafish, while the opposite would have been expected if audibility played a role. However, it may also be that the perceived threat levels are the same for both species, but that at these moderate levels cichlids seek shelter close to the bottom (or rock in their natural environment of Lake Victoria) while zebrafish would seek cover horizontally among vegetation or shoal members (Lawrence 2007; Engeszer et al. 2007; Spence et al. 2008). Again, we probably need more insight into the underlying physiology to understand the relative level of anxiety and to understand species differences in the potential consequences of such behavioural effects of sound exposure.

Conclusions

We tested the effect of experimental sound exposure on swimming behaviour and spatial distribution in captive fish using two species with different hearing abilities. Both species detected our sound stimuli playback

and changed their behaviour in ways that suggested an anxiety-related response. Species differences were also found, but we argue that any interpretation of relative severity of impact is premature and requires more studies including physiological measurements. The lack of spatial avoidance behaviour in captive conditions is likely due to limitations for behavioural responses in captivity or to sound field conditions that are complex and unlike open-water conditions. Consequently, a horizontal displacement seems not a useful read-out for any noise impact study in captivity. Furthermore, our results clearly demonstrate that hearing abilities probably play a minor or no role in explaining behavioural effects to audible levels of sound exposure. Consequently, in cases where reliable hearing curves for particular species exist (e.g. Chapman & Hawkins 1973; Sand & Karlsen 1986), these may be useful for determining detection levels and audibility ranges for sounds in natural conditions, but these will not be helpful to predict behavioural effects.

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