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معاونت بورس و امور دانشجویان خارج

به نام خداوند جان آفرین

زمانی میاسای ز آموختن   اگر جان، همی خواهی افزوختن

ز دانش در بی نیازی بجوی   وگر چند از او سختی آید به روی

(حکیم ابوالقاسم فردوسی، شاهنامه)

Pause not from learning if thou wouldst fire thy mind,

Not to do so many results in hardship Use not thy wisdom as a means of gain though,

Warner, A.G. & Warner, E., 1910. The Shahnama of Firdausi (Book of Kings) Abu'l Qasim Firdausi (935–1020 AD). Vol. I-VII. London: Kegan Paul, Trench, Trüber & Co.

تقدیم به

همسر عزیزم

و مادر مهربانم

To my dear wife and my kind mother

CONTENTS

Chapter 1

General introduction and aim of the thesis

General introduction

Man-made noise as a pollutant in natural habitats

Human activities are increasing rapidly in terrestrial and aquatic habitats. Marine and freshwater habitats are being affected by a variety of anthropogenic pollutants. Urbanization, transportation and industrialization have continuously increased ambient noise levels with different temporal and spectral patterns (Slabbekoorn et al. 2010; Radford et al. 2014). Anthropogenic noise, as an environmental pollutant, is ubiquitous in, on and near aquatic habitats and potentially may have detrimental effects on aquatic animals. Over the past few decades, public attention, activities in the field of conservation and animal welfare by non-governmental organizations (NGOs), and scientific exploration are raising awareness on the potential effects of sounds on marine mammals and fish species.

Origin of sound in aquatic habitats

Aquatic habitats, including marine and freshwater systems, are similar to terrestrial habitats in that they are filled with a variety of biotic and abiotic sound sources (Wenz 1962; Wysocki et al. 2007). Firstly, natural abiotic sound sources such as water waves and tides, surf, submarine volcanic eruptions and seismic activity are prevalent in marine habitats and riffles, waterfalls and rapids are ubiquitous in freshwater habitats. Secondly, there are also many biotic sources such as animal vocalizations, sound produced during feeding and other activities in both marine and freshwater habitats. Furthermore, sound generating human activities are responsible for so-called “anthropogenic noise”, which has spread in time and space in the last few decades and is now recognized as potential driver of environmental changes in many aquatic habitats (Slabbekoorn et al. 2010).

Next to the abundance of sounds from various sources, there are several reasons why sounds play an important role in the life of aquatic animals and why artificial elevation of ambient noise may have detrimental consequences. Firstly, sound travels almost five times faster in water than in air and therefore potentially spreads over a large area. Secondly, sound has the capacity to carry information and species may extract signals and exploit cues from ambient sounds to find prey and avoid predators, especially in dark and murky waters. Thirdly, many fish species are also able to produce sounds and use it as a tool for conspecific communication during territory defense, mate choice and reproduction. The presence of anthropogenic noise may interfere with these functions through masking, disturbance and deterrence.

Noise may affect fish species in marine and freshwater habitats

Man-made sounds are generated by a variety of human activities that vary among different waterbodies. Sound sources in marine and offshores habitats include seismic surveys, pile driving, navy sonars, shipping activities and detonation of explosives. In addition, anthropogenic noise in coastal and freshwater habitats include pumping systems, cruise vessels, motorized recreational activities, weirs and building activities (Wysocki et al. 2007). All these activities elevate ambient noise levels and potentially decrease relevant signal-to-noise ratios (typically important for both senders and receivers of signals) and relevant cue-to-noise ratios (cue reception is useful for receiver and potentially harmful for the cue-emitting animals). Consequently, anthropogenic noise elevating natural ambient noise levels may have behavioural and ecological consequences in aquatic habitats.

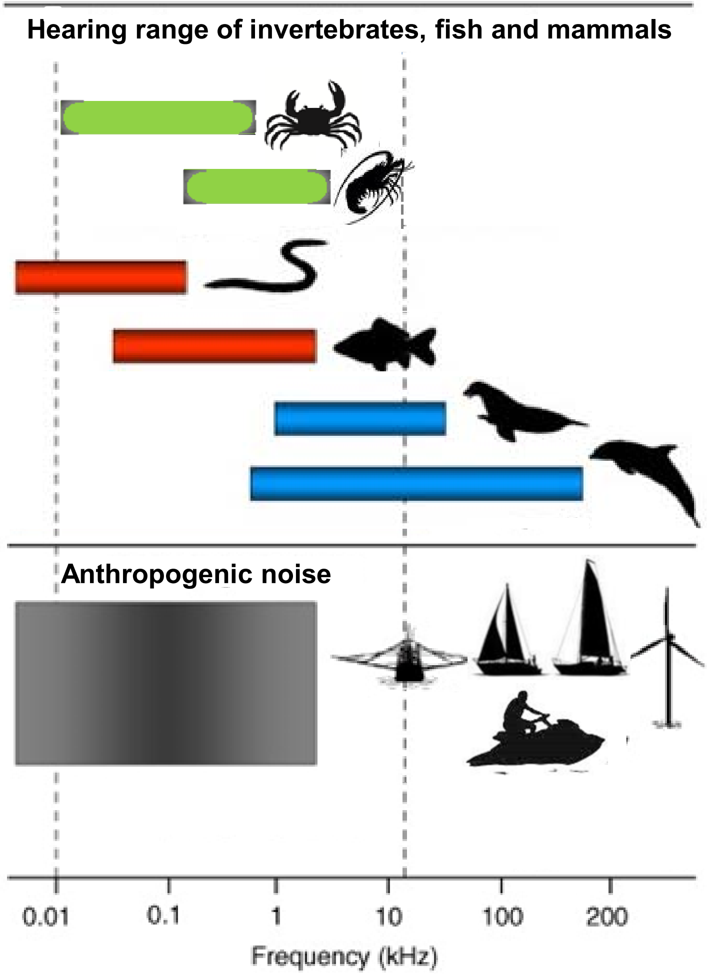

Fig. 1. Hearing range of invertebrates, fishes and mammals in aquatic habitats. The crab and prawn are representative of aquatic invertebrate species Lovell et al. (2005) and Morley et al. (2014). The eel is a representative of fish species with a bias to low-frequency sensitivity. The goldfish is a representative of the cyprinid fish, which also include the zebrafish (*Danio rerio*), that are a large relatively sensitive group of fish. Anthropogenic noise is largely overlapping the hearing range of aquatic animals and especially those of invertebrates and fishes. Modified from Slabbekoorn et al. (2010).

In natural habitats, artificially elevated ambient noise may have a variety of detrimental effects that can be described as a continuum of relative severity in sound-related effects on marine mammals and fish species. Depending on the amplitude of the sound source and the proximity of the animal, extreme levels of sound exposure may lead to elevated mortality and immediate death. Further away from loud sound sources, elevated ambient noise may still cause physical damage and physiological stress, hearing threshold shifts (permanent or temporary) , mask relevant sounds and interfere or change behavioural patterns (sound-related disturbance and deterrence). All the effects are correlated with the species-specific hearing ability of fish species, both in terms of absolute thresholds and the audible frequency range.

Very little is known about which specific sound field features are triggering changes in behaviour, especially in fish tank conditions, where sound fields can be complex. Behavioural parameters that can be used to investigate effects of sound exposure on fish are sudden rises in swimming speed, startle responses and erratic swimming movements, reduced swimming speed and freezing, going down in the water column and staying in the bottom layer, and effects on group coherence and feeding efficiency (e.g. Purser & Radford 2011; Voellmy et al. 2014; Neo et al. 2014; 2015). These measurements are all well-known indicators of physiological stress, disturbance and deterrence (see reviews: Blaser et al. 2010; Egan et al. 2009; Maximino et al. 2010).

Fish species vary in sound detection abilities

All fishes can detect sound using various sound sensitive organs (Slabbekoorn et al. 2010; Popper & Fay 2011; Ladich 2014). Unlike terrestrial animals, fish species are sensitive to the particle motion component of sound. Depending on the species-specific hearing system, they perceive sounds via different organs, including the inner ear, which consists of three semi-circular canals (utricle, saccule and lagena) and three otoliths (lapillus, sagitta and asteriscus), and peripheral structures such as the lateral line system. Moreover fishes belonging to the Ostariophysi, including zebrafish from the family Cyprinidae, are more specialized and well-known as hearing specialists, as they have a connection between swim bladder and inner ear via a set of small bones (Weberian Ossicles). Pressure fluctuations in the water cause size fluctuations of the gas-filled swim bladder. This pressure-to-motion conversion and the improved conduction via the Weberian ossicles provides fish with lower absolute sensitivity thresholds and a broader frequency hearing range. However, in contrast to the Ostariophysi families many fish species, including cichlids from the family Cichlidae, do have a swim bladder, but not that connection or other special conductors and are less specialized hearing generalists.

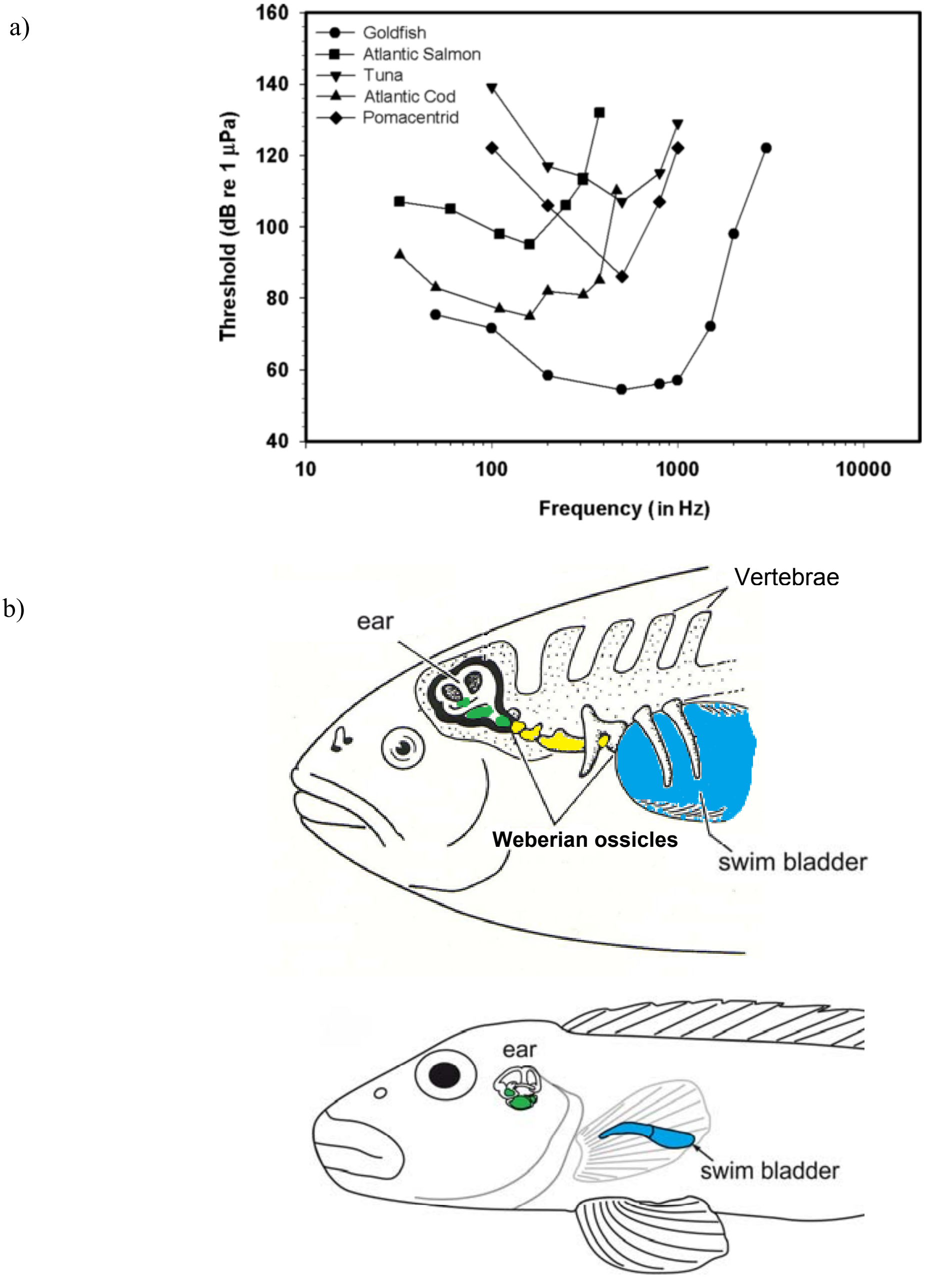

Fig. 2. a) Audiograms for a group of teleost fishes depicting the hearing thresholds across the spectral range of audible sound. Goldfish are similar in hearing abilities to zebrafish, the

model species of this thesis, while Pomacentrids are more like cichlids, also addressed in one comparative study, and have lower hearing abilities. (Audiogram originates from Popper & Schilt 2008; all data from Fay 1988). b) The fish drawings show lateral views of a species with a large swim bladder and Weberian ossicles and another species with a small swim bladder and no special adaptation to connect it to the inner ear (drawings used with permission originate from Wake 1979; Schulz-Mirbach et al. 2012). The swim bladder is shown in blue; the otoliths of the inner are shown in green and the Weberian ossicles are shown in yellow.

Multimodal complexity and ecosystem level effects

Natural habitats of fish not only vary in sound conditions but also in light conditions (Longcore & Rich 2004; Brüning et al. 2011). Fish species use their auditory and visual systems along with other environmental modality receptors for optimal perception of their surroundings (Halfwerk & Slabbekoorn 2015). They extract relevant signals and cues in this multimodal sensory context to mediate essential behaviours, including territory defense, mate choice, reproduction, finding prey and avoiding predators (Swaddle et al. 2015). Changes in artificial light levels at night are also becoming more wide-spread on a global scale and, like the impact of artificial sound, may have potentially negative consequences for fish activities and their spatial distribution (Becker et al. 2013; Swaddle et al. 2015). Elevated light levels at night have the potential to affect fish behaviour directly or indirectly when the effect of sound is altered by light level. Light pollution, like noise pollution, may go beyond single species effects. For instance, artificial lighting at night may affect biological rhythms of hormone cycles potentially leading to: higher physiological stress levels (Brüning et al. 2015), delay in dispersal timing and disrupted diel patterns in captive species (Riley et al. 2015). However, artificial lighting may also affect predator-prey interactions in coastal habitats; both large predator and small prey fish species were reported to aggregate at nocturnal light sources, which resulted in predator benefits from locally elevated prey abundance and possibly overall shifts in abundance in multiple trophic levels (Becker et al. 2013) (see Fig. 3).

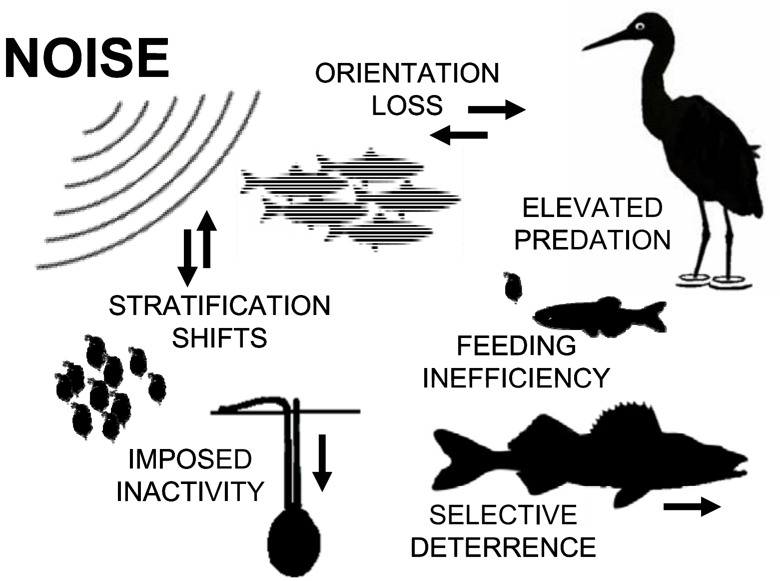

Fig. 3. Anthropogenic noise may have consequences that go beyond single species effects. Species interactions among predators and prey or among competitors may be affected in various ways and have cascading effects through different trophic levels in the underwater food chain. The figure illustrates several examples through which anthropogenic noise may cause shifts in relative species densities in the horizontal and vertical pane (Shafiei Sabet et al. 2016).

Captive fish and sound

Many fish species have been artificially introduced to confined areas for different purposes. Fishes are being used in laboratory conditions for scientific research, in aquaria and zoos for fun and entertainment, in aquaculture facilities (cages, races, pens etc.) for breeding, restockment and harvesting. For instance, in China alone there are already at least 532 species belonging to 24 families of marine fish that have been used for artificial breeding and reproduction purposes in captivity (Hong & Zhang 2003). Several fish species, including zebra fish, are used for scientific research in large numbers for a wide range of investigations in laboratories around the world. Therefore, also many fish in captivity may be continuously exposed to a variety of sound sources.

The sounds present in the breeding and rearing or experimental environment may affect production, reproductive success and potentially even non-behavioural results of any type of experiment. Sounds may not be detrimental, for instance when they learn that a particular sound, for instance from an automatic feeding system, indicates that they are likely to get food. Also, threats or uncertainties like in outdoor conditions are typically not present. However, novel sounds may induce behavioral changes due to anxiety or curiosity (Neo et al. 2015). Moreover, in aquaculture activities, sound-generating equipment may also affect both target and non-target species in surrounding marine and freshwater habitats (Lepper et al. 2004). For instance, pumping devices in aquaculture may produce high levels of background noise continuously. Also, in open water localities used for aquaculture, floating pen systems may be used in combination with commercial aquaculture acoustic devices (CAADs) that generate loud sounds to deter predator species (Lepper et al. 2004).

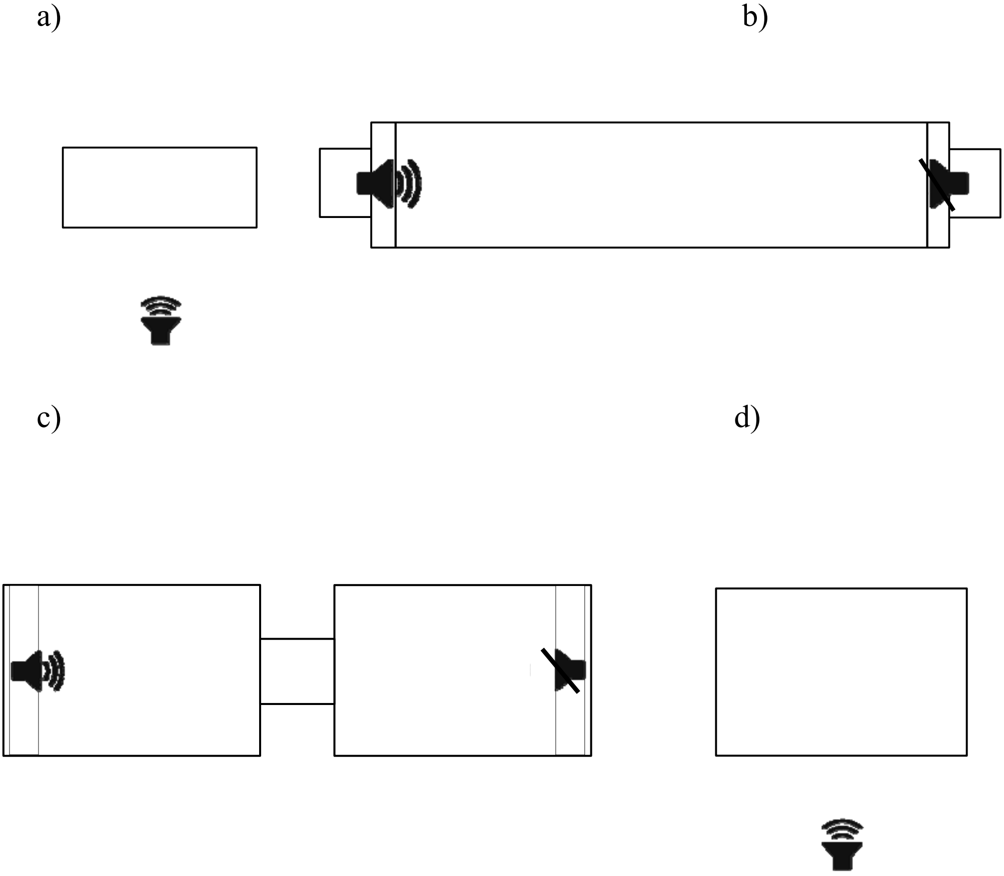

Fig. 4. Schematic overview of the four different set-ups used in this thesis in terms of relative size and shape of the experimental fish tank and the location of in air or in water speakers. (a) a small tank (Chapter 2); (b) a long tank (Chapter 3) (c) a dual- tank (Chapter 4) and (d) a standard 1 meter tank with an acoustically transparent enclosure to restrict the swimming arena for the target fish (Chapter 5).

Aim of the thesis

The aim of this thesis was to explore sound-induced behavioral changes in fish using captive zebrafish as a model species. I explored short-term behavioural parameters, which are indicators of sound-related stress, disturbance and deterrence. Several behavioural states are likely to reflect considerable changes in underlying physiology, which would be interesting and feasible to investigate for more long-term consequences, but this was beyond the scope of the current study. Here, I examined in four different studies various sound exposure treatments to provide insights that may be useful for future explorations for indoor and outdoor sound impact studies as well as for assessing animal welfare and productivity in captive situations. Furthermore, my findings may also raise awareness for sound levels in laboratories and the potential effect on reliability for fish as a model species for medical and pharmaceutical studies. I also explored the complexity of sound fields in indoor fish tanks by selecting a different set-up for each study (Fig. 4), which makes behavioural analyses and direct comparisons not only relevant within each study, but also provides insight into the role of fish tank acoustics on ‘natural’ and experimental exposure conditions.

In **Chapter 2,** I investigated how sound exposure with different temporal patterns affected swimming behaviour and foraging performance for zebrafish preying on waterfleas. In **Chapter 3,** I examined how sound exposure affected two different fish species with different hearing ability (cichlids and zebrafish) in terms of swimming behaviour and spatial distribution in a long tank set up. In **Chapter 4,** I investigated the effect of two modalities (sound and light exposure) and their potential interaction on zebrafish swimming behaviour and spatial distribution in a special dual-tank set up (c.f. Neo et al. 2014). In **Chapter 5**, I collaborated with James Campbell to explore the detailed sound field characteristics in terms of sound pressure and particle velocity that are responsible for zebrafish startle and anxiety-related response patterns. And finally, in **Chapter 6**, the general discussion and conclusion, I summarized the results of all four experiments and put them in a more general context.

Animal species

Throughout this thesis experimental sound exposure effects were assessed using the invertebrate species waterfleas (Daphnia spp) and vertebrate fish species (zebrafish and a Lake Victoria cichlid) below:

Fig. 5. Waterfleas (*Daphnia spp*) were used in the chapter 2. Waterfleas are crustaceans and a typical food item for many fish species in freshwater habitats. Crustacean are sensitive to sound in the low frequency range (Lovell et al. 2005; Montgomery et al. 2006; Mooney et al. 2010; Stanley et al. 2011; Morley et al. 2014), which they can hear through sensitivity to movement and vibration, either through the presence of a statocyst or small tentacles on their body (See Fig. 1.). The exact hearing range for waterfleas is unknown, but they are not expected to hear beyond a few hundred Hz (Picture by G. Lamers).

Fig. 6. Zebrafish (*Danio rerio*) were used in the chapter 2, 3, 4 and 5. Zebrafish are a freshwater fish species native to the flood-plains of the Indian subcontinent where they inhabit shallow and slow flowing waters (Spence et al. 2008). They are a widespread model species in a broad range of research areas such as neurophysiology, biomedicine and behavioural biology studies in laboratory conditions. As a Cyprinid, zebrafish belong to the ostariophysan teleosts, which all have a special hearing adaptation. A series of bones, the Weberian ossicles, connect the swim bladder to the inner ear and lower absolute detection thresholds and extend the spectral range. Zebrafish can therefore hear over a relatively broad frequency range between 100-4000 Hz, with sensitivity declining sharply above 2000 hz (Higgs et al. 2002). (see also Fig.2.). (Picture by S. Shafiei Sabet).

Fig. 7. Lake Victoria Cichlids (*Haplochromis piceatus*) were used in the chapter 2. Cichlids represent a non-Ostariophysi species and they are less advanced in terms of special structures for improved hearing compared to zebrafish. Cichlid hearing is therefore restricted to a lower range of frequencies and different species vary between 100-3000 Hz, with sensitivity declining sharply above 700 or 1000 Hz, depending on the species (Schulz-Mirbach et al. 2012). There is no hearing curve available for the species used for this thesis, but there are no special extensions of the swim bladder towards the inner ear, nor a particularly big or small swim bladder. Hearing sensitivity is therefore likely to be somewhere intermediate to those reported by Schulz-Mirbach et al. (2012) (Picture by A. Ekenberg).

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Chapter 2

The effect of temporal variation in sound exposure on swimming and foraging behaviour of captive zebrafish

This chapter is based on: Saeed Shafiei Sabet, Yik Yaw Neo & Hans Slabbekoorn. (2015). The effect of temporal variation in sound exposure on swimming and foraging behaviour of captive zebrafish. Animal Behaviour, 107: 49-60. doi:10.1016/j.anbehav.2015.05.022.

Abstract

Anthropogenic noise of variable temporal patterns is increasing in both marine and freshwater systems. Aquatic animals often rely on sounds for communication and orientation, which may therefore become more difficult. Predator-prey interactions may be affected by masking of auditory cues, sound-related disturbance or attentional interference. Here, we investigated the impact on both predator and prey for zebrafish (*Danio rerio*) preying on water fleas (*Daphnia magna*). We experimentally raised ambient sound levels in an aquarium and tested four sound conditions that varied in temporal pattern: continuous, fast and slow regular intermittent and irregular intermittent, which we compared to ambient sound levels with no extra exposure. We found no effects on water flea swimming speed or depth but there was an increasing number of individual zebrafish with an increased number of startle responses, especially to the intermittent sound treatments, which was also reflected in a significant increase in zebrafish swimming speed, but not in any change in zebrafish swimming depth. Discrimination in attacking edible water fleas or inedible duckweed particles was low for the zebrafish and unaffected by sound exposure, but foraging was affected in two ways: intermittent sounds delayed initial acceleration response and all treatments caused a rise in handling error. These insights confirm that elevated sound levels, and especially intermittent conditions, may affect predator-prey interactions. Our results apply to laboratory conditions but call for outdoor studies that go beyond single-species effects. If acoustic impact of human activities extends to multiple species and their interactions, natural sound conditions may turn out to be important for the stability and dynamics of aquatic ecosystems.

Keywords: behavioural impact, *Danio rerio*, foraging performance, sound pollution, species interaction, swimming behaviour, water flea.

Introduction

A variety of human activities introduce anthropogenic noise in different temporal patterns above and below the water surface in marine and freshwater systems (Andrew et al. 2002; Amoser et al. 2004; McDonaldet al. 2006). Although empirical evidence confirming short-term and especially long-term effects is still scarce, aquatic animals can be negatively affected by anthropogenic noise in many ways (Richardson et al. 1995; Popper et al. 2003; Popper et al. 2014). Masking may for example cause interference with acoustic communication, soundscape orientation, or acoustically guided predator-prey interactions, while anthropogenic noise may also cause interruption or modification of group movements, migratory activities, and courtship or other reproductive behaviours (see reviews: Slabbekoorn et al. 2010; Radford et al. 2014; Hawkins & Popper 2014).

Different taxonomic groups such as marine mammals and fish can be part of the same community, but may be affected by anthropogenic noise in different ways and to a variable extent (Weilgart 2007; Slabbekoorn et al. 2010; Popper et al. 2014; Shafiei Sabet et al. 2016). In air, it has been shown that human-induced changes in ambient noise levels can have direct and indirect effects and can lead to changes in abundance and diversity of animals and plants (Francis et al. 2009; 2011a; Francis et al. 2012a). We currently lack such insights for aquatic communities and it is clear that more data are needed that go beyond single-species effects.

There are several recent studies in various taxa which revealed an impact of artificial sound levels on predator-prey relationships. For example, Siemers & Schaub, (2010) showed that elevated sound levels may negatively affect foraging performance in bats (*Myotis myotis*) by masking auditory cues that are critical for catching invertebrate prey. Quinn et al. (2006) also reported sound-dependent changes in foraging efficiency in chaffinches (*Fringilla coelebs*) as higher ambient noise levels made them eat less and scan more. In crustaceans, Chan et al. (2010) found that boat sounds distracted hermit crabs (*Coenobita clypeatus*) in such a way that they responded less quickly to a visual stimulus indicating approaching danger. So, it appears that sound impact is widespread taxonomically, that acoustic masking or distraction can affect auditory as well as visual perception, and that anthropogenic noise may affect predator as well as prey species.

As far as we know, fish are also likely to be susceptible to the human-induced rise in underwater sound, as they are well-known to hear and use sounds for many aspects of their underwater life (Ladich 2004; Fay 2009; Slabbekoorn et al. 2010). Like in air, underwater masking effects are determined by the spectral overlap of ambient noise with biologically relevant sounds (Codarin et al. 2009; Vasconcelos et al. 2010; Gutscher et al. 2011). Independent of masking, several studies have also reported behavioural changes in response to artificial tones or wide-band sounds. For example, Andersson et al. (2007) showed several different behavioural changes in captive roach (*Rutilus rutilus*) and sticklebacks (*Gasterosteus aculeatus*) which were interpreted as species-specific responses to perceived danger of predation risk. Picciulin et al. (2010) revealed a negative impact on the time budget spent on behaviours that are critical for reproductive success in red-mouthed gobies (*Gobius cruentatus*) in their natural habitat. Sebastianutto et al. (2011) also showed that the typical outcome of acoustically mediated territorial conflicts of this species was undermined under experimentally noisy conditions. Although these studies suggest that predator-prey interactions in fish may also be affected by artificial sound exposure, this phenomenon that has potential consequences across aquatic food webs, has received relatively little attention.

Recently, a study on sticklebacks experimentally explored the impact of artificial noise on predator-prey interactions in sticklebacks catching water fleas (*Daphnia magna*). Purser & Radford, (2011) were able to show that sound playback, compared to more quiet conditions, increased the amount of errors in food-particle discrimination and food handling. Voellmy et al. (2014) showed that different species may respond differently to playback of additional ship sounds as European minnows (*Phoxinus phoxinus*) differed from sticklebacks in becoming less active and more social. These experimental data clearly show an acoustic impact on a seemingly visual task with a direct impact on fish foraging efficiency. As masking is unlikely to be important, the performance decline may be due to attentional shifts (Dukas 2002; Mendl, 1999) as found in the studies on birds and hermit crabs mentioned above (Quinn et al. 2006; Chan et al. 2010). In the experimental studies on fish (Purser & Radford 2011; Voellmy et al. 2014), it was assumed but not investigated that the effect of sound on foraging efficiency was caused by an impact on the predator and not on the prey and the relevance of temporal variation in sound characteristics (c.f. Neo et al. 2014; Neo et al. 2015) remained unexplored.

Zebrafish (*Danio rerio*) are a very suitable model system to assess behavioural changes in response to environmental conditions in general (e.g. Cachat et al. 2010; Egan et al. 2009; Gaikwad et al. 2011; Gerlai et al. 2006) and to tackle questions of sound impact on predator-prey interactions in particular. Neo et al. (2015) exposed adult zebrafish to different sound patterns and showed initial startle responses, relatively brief anxiety-related response behaviours, but no longer-lasting effects or spatial avoidance. They reported sound exposure related changes in swimming speed and group coherence, while fish moved upward in response to moderate sound levels (112 dB re 1 μPa) and downward (for brief periods) in response to higher sound levels (120-140 dB re 1 μPa). We have no insight yet into whether and how foraging behaviour in this species is affected by sound exposure (c.f. Purser & Radford 2011; Voellmy et al. 2014), but also zebrafish readily feed on live prey and provide a perfect model system to assess the impact of temporal variation in sound exposure on foraging efficiency.

Water fleas (*Daphnia* spp) are small crustaceans and important food items for many fish species in freshwater systems (e.g. Ebert 2005; Gulati 1990). They show predictable spatial behaviour by avoiding darker water areas and preferring open space (negative scototaxis and negative thigmotaxis), which probably reduces exposure to predators that may hide in the dark and in vegetation (e.g. Van Gool & Ringelberg 1995; Dodson et al. 1997). Although sensory systems for aquatic invertebrates may vary, both short-term sound effects on response behaviour to approaching predators (Chan et al. 2010) and long-term sound effects on growth and reproduction (Lagardère 1982) have been reported for example in crustaceans. Furthermore, at a larval stage, marine crustaceans have been reported to respond phonotactically to reef sounds (e.g. Radford et al. 2007; Stanley et al. 2011). Also larvae of aquatic invertebrates, of similar size as water fleas, have been shown to either increase or decrease their swimming activity in response to natural and anthropogenic sound exposure Therefore, we believe it is important to check whether or not anthropogenic noise has any effect on water flea behaviour that may have consequences for predation risk (c.f. Morley et al. 2014).

In the current study, we tested the impact of temporal variation in artificial noise exposure, mimicking temporal and spectral patterns of man-made sounds that exist in natural environments, on: 1) behaviour of water fleas (*D. magna*); 2) behaviour of zebrafish; and 3) on zebra fish preying on water fleas. We measured startle responses, swimming speed, and spatial distribution in water fleas and zebrafish. Sound treatments varied in being continuous or intermittent and the latter category in being fast or slow and in having regular or irregular intervals. We aimed for answers to the following questions: Does exposure to artificial noise reduce foraging efficiency of zebrafish hunting for water fleas? And is this impact attributable to a behavioural impact on prey, predator, or both? Furthermore, does variation in temporal patterns matter or not? We expected water flea swimming behaviour to change with the onset of sound exposure and foraging efficiency of zebrafish to be negatively affected by sound exposure through an impact on foraging performance, discrimination and handling (c.f. Purser & Radford 2011; Voellmy et al. 2014). We also expected less impact from continuous sound than from intermittent sound and less impact from regular than from irregular sound exposure.

Methods

Animal maintenance and housing

Zebrafish (adult, 4-6 month old and of the wild-type, short fin variety) were obtained from a local pet supplier in Leiden (Selecta Aquarium Speciaalzaak, who obtains stock from Europet Bernina International BV; Gemert-Bakel, The Netherlands). The fish were housed in a long stock tank (50 x 40 x 200 cm) connected to a water circulation system before being transferred individually and sequentially to the experimental set up. The fish stock was kept at 24±1°C on a 14/10 h light/dark cycle (light switched on from 06:00–20:00) and was fed on dry food twice a day (DuplaRin M, Gelsdorf, Germany). After the experiment, the exposed fish were transferred to a stock tank. Water fleas were captured in the morning (around 7:00) on the day of the experiment in which they were used. They were always captured in shallow water bodies in the southern part of Leiden (23^0 $9^{'}27^{''}$ N, 48^0 $5^{'}18^{''}$ E) by gentle pulling of a net (mesh size: 2 mm) through the water at a depth of about 30 cm. The outside water temperature ranged from 14-18^{o}C and water fleas were allowed to acclimatize gradually to the indoor water temperature of 24^{o}C over the period of one hour before use in any of the experiments. Water fleas appeared to handle the transfer to indoor conditions well and individuals compared among different sound treatments always had the same environmental background and procedural experience.

Artificial noise stimulus preparation

Four sound treatments were used with varying temporal patterns: continuous sound (CS), intermittent regular with a fast pulse rate, intermittent regular with a slow pulse rate and intermittent irregular sound, and ambient noise (AN) as a control (Fig. 1). All three intermittent sound treatments consisted of one-second pulses but differed from each other in terms of the length of the intervals without extra sound exposure. Intermittent regular noise with a fast pulse rate (1-1) consisted of 1s pulses interspersed with 1s intervals and intermittent regular noise with a slow pulse rate (1-4) consisted of 1s pulses interspersed with 4s intervals, irregular noise (1-7) consisted of 1s pulses interspersed with 1, 2, 3, 4, 5, 6 or 7s intervals in randomized sequence (using an online random number generator: http://www.random.org/), leading to a mean interval of 4s.

Continuous sound as well as sound pulses were created in Audacity (2.0.3) software, using band-filtered white noise (band-passed between 300-1500 Hz), which matches the frequency range of best hearing for zebrafish

(Higgs et al. 2002; Popper et al. 2001) and also matches in general terms with the typical wide-band sound characteristics of anthropogenic sources, such as vessels, pumping systems or pile driving (Slabbekoorn et al. 2010; Wysocki et al. 2006). The frequency range of auditory sensitivity for invertebrates varies (Morley et al. 2014) and there are no data for water fleas. However, we expect that their sensitivity could be overlapping the frequency range of fish hearing and our current stimuli. Our behavioural test will reveal whether we can exclude an impact of prey behaviour on sound-dependent foraging efficiency of the predator, but not the underlying mechanism of a potential lack of response to exposure. We used 5 ms ramps to fade in and fade out pulses for smooth transitions in the intermittent sound patterns.

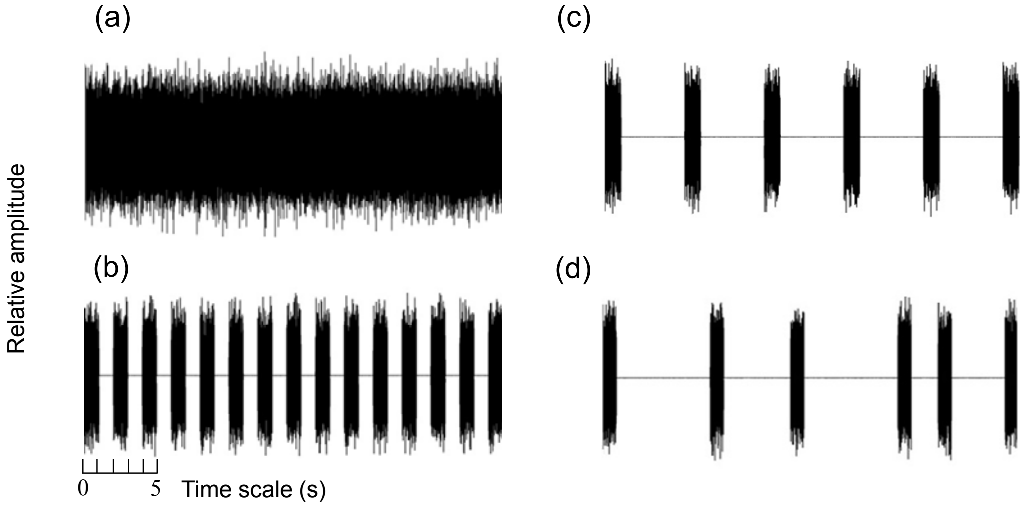

Fig. 1. Amplitude waves showing temporal variation in the four sound treatments used in the exposure experiments: (a) Continuous sound (CS). (b), Intermittent regular (1-1) with a high pulse rate of 1s sound and 1s interval. (c), intermittent regular (1-4) with a low pulse rate of 1s sound and 4s interval and (d) intermittent irregular (1-7) with 1s sound and variable intervals randomly selected from the range of 1-7s (7 different whole-second durations, on average 4s).

Experimental tank conditions

The experimental trials were conducted in a narrow subdivision (25cm×15cm×20 cm) of a larger fish tank (50cm×20cm×20cm). We reduced the swimming space by using Styrofoam dividers and we scored animal movement in two dimensions: vertical and horizontal. A black sheet of plastic covered the background of the tank to increase the contrast for the water fleas and zebrafish on video files, recorded using a 1080 P AIPTEK full HD camcorder (model H 500). The water was disconnected from the water recirculation system during the experiments and the water temperature was kept at 24.0 ºC during all trials. The sounds (WAV format, 44.1-kHz sampling rate) for all treatments were played back with a portable Tascam digital recorder (model DR-07) connected to an in-air HARMAN speaker (model EON JBL 500), which was placed at 1.5 m from the fish tank wall (long end) at the same height from the floor as the fish tank (on a separate table and on top of a Styrofoam layer to reduce transfer of sound vibrations into the floor).

In our experiments, the test animals experience variable and complex near-field conditions inherent to the fact that they are able to hear low-frequency sound of long wave lengths and that they swim in an indoor fish tank (Parvulescu 1967; Akamatsu et al. 2002). Zebrafish are cyprinids that are sensitive to the sound pressure as well as the particle motion component of sound (Fay & Popper 1974; and see Higgs et al. 2002; Bretschneider et al. 2013), while water fleas are likely only sensitive to the latter (e.g. Patek 2001; Stocks et al. 2012; Wale et al. 2013). It is therefore important for our test that both sound pressure and particle motion are elevated during experimental exposure and we therefore assessed both (definitions for our acoustic terminology follow ANSI/ASA S1.1-2013). The underwater sound pressure levels (SPL) were determined by using a High Tech hydrophone (model HTI 96 min), connected to a Marantz Solid state audio recorder (model PMD620). The hydrophone was placed in four different positions at each of which we took three measurements. We calculated the cumulative SPL within the 300-1500 Hz frequency range (rms), using a Matlab script (R2013a) calibrated for the recording set. The ambient SPL of 95 dB re 1 μPa was elevated during sound playback (continuous and intermittent treatments) to 122 dB re 1 μPa. Sound pressure levels at the bottom and close to the walls were slightly higher than in middle strata and center of the tank: mean ± SE at the bottom: 126.3 ± 0.7; in the middle: 122.4 ± 0.7; and at the surface: 121.0 ± 0.5. Spectrum levels varied due to speaker output characteristics and propagation through air into the fish tank, but sound levels were well-elevated throughout the relevant hearing range of zebrafish (see Fig. 2a and b).

We assessed the experimental elevation of the particle velocity level (PVL) by adding up the vectorial measures from 3 accelerometers, (one for each direction: X-, Y- and Z-coordinate). The accelerometers were fixed inside a custom-made transparent Plexiglas sphere (9.5 cm in diameter) with a hydrophone in the middle and suspended into the water with thin nylon wires (c.f. Bretschneider et al. 2013; van den Berg & Schuijf 1985). Accelerometers and hydrophone were connected to a digital oscilloscope: PicoScope model 3425, using a resolution of 12 bits at 20 ms/s, bandwidth 5MHz (Pico Technology, St. Neots, United Kingdom). We measured at 7 cm height from the bottom at a replicate set of seven positions in the fish tank. The ambient PVL was 165 dB ref 1 nm/s, which was elevated to 200 dB ref 1 nm/s during exposure. The spectral distribution of particle motion levels was also not flat, but PVL was elevated throughout the 300-1500 Hz range (as we were unable to calibrate absolute levels, we reported the relative levels of elevation above ambient, see Fig. 2).

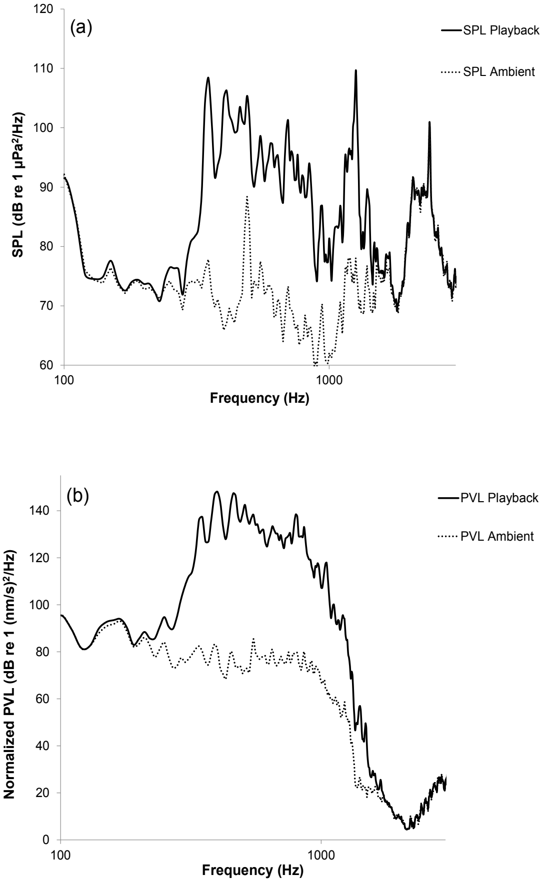

Fig. 2. (a) Spectral distribution of continuous sound pressure level in dB (re 1 μPa^2/Hz) (dotted line) ambient condition and (continuous line) sound playback) and (b) the normalized particle velocity level in dB (re 1 (nm/s)^2/Hz) in ambient condition (dotted line) and sound playback (continuous line) of continuous sound exposure as measured within the fish tank in the laboratory. The graphs show that both SPL and normalized PVL increased considerably in the same frequency range. Note that the particle velocity level concerns the sum of the root mean square averages from the vector sensors in all three X, Y and Z directions measured in the center of the tank (both SPL and PVL measurements were averaged over 10 sec and were measured in the same position at the center of the tank; 7 cm from the bottom and 10 cm from the side wall of the tank).

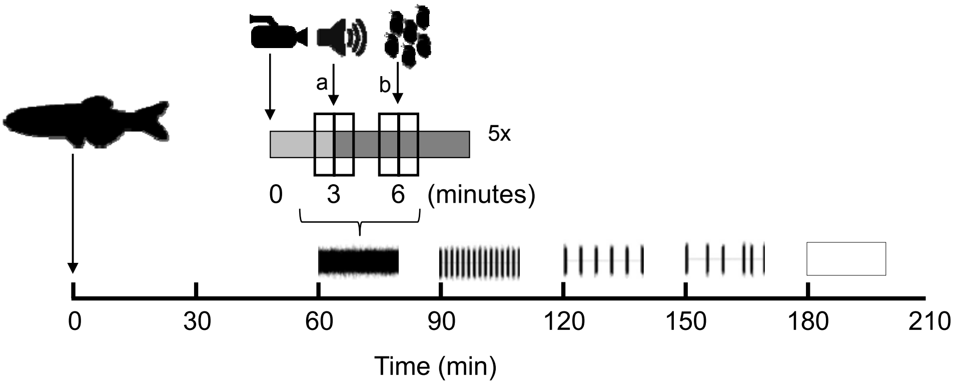

Fig. 3. Schematic representation of the exposure timeline for a single trial of an individual zebrafish. Each individual in an experimental trial was exposed to five subsequent sound treatments in randomized sequence: CS, 1-1, 1-4, 1-7, and ambient level with no extra exposure as a control (AN). Each treatment lasted for 6 min of experimental exposure through playback with our in-air speaker (preceded by 3 min of video period and followed by 18 min of interval to the video-onset of the next treatment). We assessed the behavioural impact by making comparisons between two time periods at two moments: (a) 1 min before versus 1 min after onset of sound exposure to measure variation in initial sound impact and (b) 5 seconds before and 5 seconds after introducing the 10 individual water fleas to measure variation in sound impact during foraging.

Sound impact on water fleas

We investigated sound impact on water flea swimming behaviour by tracing individuals during sound treatments in two separate tests. In the first test, a group of 10 water fleas was introduced in the experimental tank and analysed for non-targeted swimming speed difference (before sound exposure versus during sound exposure) and swimming depth difference (before sound exposure versus during sound exposure; distance to the bottom) using averages per group. In the second test, individual water fleas were introduced on the right side of a dark tank to measure the impact of sound treatment on targeted swimming speed towards the lightened left side of the tank. We randomized the order of five trials per group or individual, for the four sound treatments and one control to avoid the effect of treatment being confounded by an order effect.

In the first test, we selected 10 groups of 10 individual water fleas of equal size (~3 mm), which entered the experimental tank per group (using a pipet) after at least 1 hour of acclimatization to indoor conditions. The exposure to each of the four sound treatments (continuous and three pulsed treatments: CS, 1-1, 1-4 and 1-7) and the ambient control (AN) was recorded for 9 min per treatment (each time: 3 min before sound on-set and 6 min during sound exposure) (see Fig. 3). We analyzed swimming behaviour for all 10 individuals and compared among treatments (1 min just after sound on-set subtracted from 1 min just before sound on-set; the longer recording periods avoid an impact of observer presence during the start of the video on the selected periods for analyses). Full-tank illumination let to a range in light illuminance from 750 to 1100 lux from bottom to surface as measured by a LUNASIX F light meter (P. Gossen & Co, Erlangen made in Germany). This light condition provides sufficient visibility to allow continuous tracing of water fleas on video throughout the entire tank.

In the second test, we selected 12 water fleas which entered the experiment individually by gently pouring them into the water on the right side of the tank in dark conditions (using a pipet and after at least 1 hour of acclimatization). The top and sides of the tank were covered by a plastic black sheet for 20 cm, leaving 5 cm open on the left side as the light source to trigger positive phototaxis. We assessed targeted swimming speed by timing the duration it took each individual to cross the approximately 19 cm from the location of introduction on the right to the 5 cm lit-up area on the left.

Sound impact on zebrafish

We investigated sound impact on swimming behaviour of zebrafish by introducing 14 fish (7 males and 7 females) individually on different days into the same experimental tank as used for the water flea trials (full-tank illumination conditions). We determined zebrafish sex by coloration patterns and belly shape (Schilling 2002). After introduction into the experimental tank, each individual was allowed one hour of acclimatization before the on-set of video recording and subsequent sound exposure to the four treatments (CS, 1-1,1-4 and 1-7) and the ambient noise level (AN) as a control. All individuals were tested between 9:00 and 13:00.

Like for the water fleas, the response by the zebrafish to exposure to each of the four sound treatments (CS, 1-1, 1-4 and 1-7) and the control (AN) was recorded on video for 9 min per treatment (each time: 3 min before sound on-set and 6 min during sound exposure)
(see Fig. 3). We analyzed swimming behaviour with and without sound exposure by comparing the difference between 1 min just after sound on-set

and 1 min just before sound on-set. We determined the number of startle response differences (here defined as sudden peaks in swimming speed that were above 10 cm/s and associated with a distinct change in swimming direction), the swimming speed differences and the swimming depth differences (distance to the bottom).

Sound impact on zebra fish preying on water fleas

We investigated sound impact on foraging behaviour of zebrafish by following the test animals further during the sound exposure periods as described above. We introduced a group of 10 water fleas, again selected for equal size (~ 3 mm), together with 10 particles of duckweed leaves (~ 6-10 mm) as inedible targets, by gently pouring some water from a petri dish which contained the animals and plant particles. The visual presence of the investigator was obstructed by the non-transparent back of the experimental tank and only part of the hand was briefly in sight for the fish for all treatments. We first analyzed the initial response to water flea introduction by comparing swimming speed difference with and without sound exposure among treatments (measured by subtraction of swimming speed in the last 5 seconds before introduction from the first 5 seconds after introduction (see Fig. 3).

We subsequently measured sound impact on foraging efficiency by assessing two behavioural measures: food discrimination error and food handling error. The food discrimination error was determined by subdividing the number of attacks to inedible particles of duckweed by the total number of attacks to both the edible water fleas and the inedible particles of duckweed. The food handling error was determined by subdividing the number of unsuccessful attacks to water fleas by the total number of unsuccessful and successful attacks to water fleas. Attacks could be unsuccessful because a zebrafish pursuit and bite missed the target or because a zebrafish lost control of a captured water flea that was released again.

As we were uncertain about whether zebrafish would get saturated and less eager to forage after sequential feeding bouts, we exposed individuals to period of sounds on two subsequent days and avoided the introduction of too many water fleas on a single day. We pseudo-randomly assigned whether or not a treatment was associated with the introduction of water fleas so that two or three of the treatments received water fleas on the first day. On the second day, we introduced water fleas during treatments which had not been associated with the introduction of water fleas on the first day yet. For assessing sound impact on foraging efficiency, we analyzed only those exposure periods in which we introduced water fleas, which were sometimes on day 1 and sometimes on day 2.

Processing behavioural data

We always started video recording (Fig. 3 - shaded light grey) well before automatic sound onset and continued sound exposure and video recording (Fig. 3 - shaded dark grey) well beyond the last period used for measurements (see Fig. 3). We converted all video files of water flea and zebrafish trials by reducing the temporal resolution to 5 frames per second. Video recordings were analyzed with Logger *Pro* (Vernier Software &

Technology, Beaverton, OR, USA, version 3.6.0), quantifying startle responses and measuring swimming speed and swimming depth. We quantified startle responses as the number of sudden peaks in swimming speed that were above 10 cm/s and associated with a distinct change in swimming direction. Video recordings were also used to assess food discrimination error and food handling error. All video analyses were done without audio track and therefore blind to the treatment sequence for the observer (SSS). A portion of the data was independently scored by a second observer (YYN), which confirmed inter-observer reliability.

Statistical analyses

We compared fish behaviour al changes caused by different sound treatments (CS, 1-1, 1-4, 1-7 and AN) by testing the difference between before and during sound exposure and before and after the introduction of water fleas using one-way repeated-measures ANOVA, with sound treatment as a fixed factor. The treatment exposure sequence was randomized to avoid order effects. Although the statistical power is low due to limited sampling of each treatment in each position in the sequence, we also checked statistically for an order effect by including the position of the treatment in the trial sequence as a random factor. We did not find an order effect in any of our test results (all $P > 0.1$). Data fitted the assumptions of normality and homoscedasticity for parametric testing for all measurements (if not immediately, after the data were log-transformed), except for the number of startle responses. When sphericity could not be assumed, we used Huynh-Feldt corrections. Whenever the outcome of the repeated-measures

ANOVA was significant, Bonferroni corrected post-hoc tests were performed for pairwise comparisons among the four different sound treatments and the ambient noise control. For the difference in number of startle responses the data fitted to a Poisson distribution and we therefore used a non-parametric test (Friedman test). All tests were done by SPSS statistics for windows, version 21.0. (Armonk, NY. IBM Corp.)

Ethical note

Water fleas were allowed to acclimatize gradually to the laboratory conditions before using them in any of the experiments and showed no signs of adverse effects of the experimental conditions. Zebrafish showed only a brief startle response with the onset of the sound playbacks and did not show any sign of anxiety or unusual swimming behaviours in their holding tanks after the experiments (c.f. Neo et al. 2015). All housing and experimental conditions were in accordance with the ethical guidelines of the Association for the Study of Animal Behaviour. The experiments were only carried out after evaluation and approval of the experimental procedure (DEC no: 10060) by the Animal Experiments Committee of Leiden University (UDEC).

Results

Sound impact on water fleas

Water flea swimming behaviour appeared to slow down for the ambient noise control and to speed up slightly during the sound treatments (Fig. 4). However, individual variability was high and this pattern did not result in an effect of sound treatment, as we did not find a significant treatment effect of sound exposure on non-targeted swimming speed (repeated- measures ANOVA: $F_{4,36}$=0.919, P= 0.464) or swimming depth (repeated-measures ANOVA: $F_{4,36}$=0.208, P= 0.849). Water flea swimming speed showed highly variable patterns among and within individuals, but targeted swimming speed was twice as high compared to the non-targeted swimming speed (See table 1). However, there was also no significant effect of elevated sound levels on the targeted swimming speed for any of the treatments (repeated-measures ANOVA: $F_{4,44}$=0.624, P= 0.648).

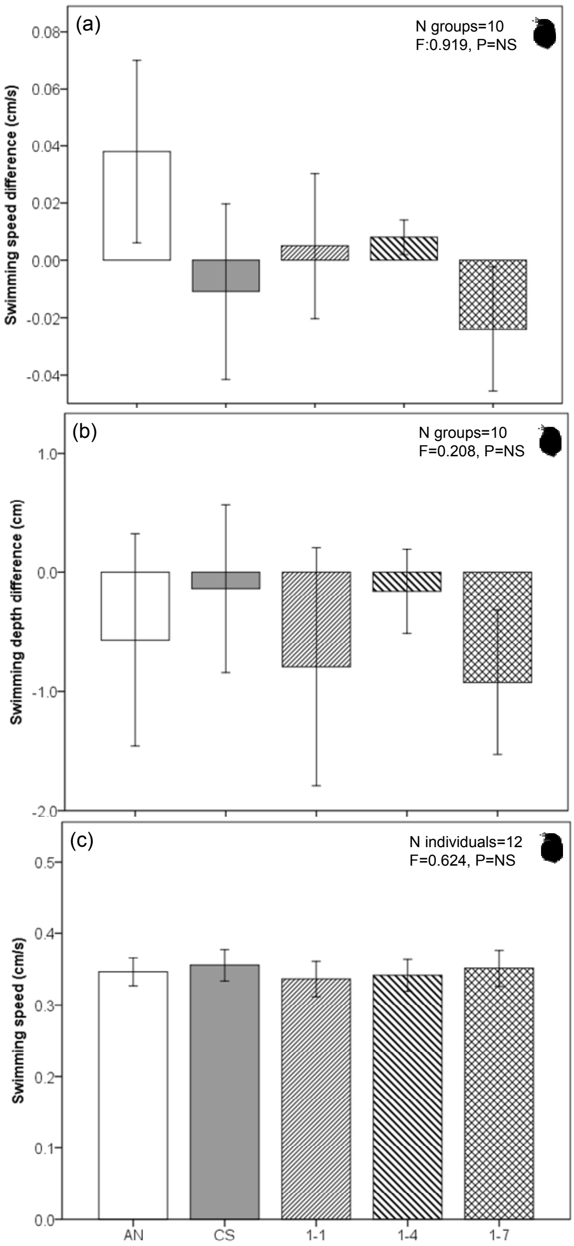

Fig. 4. Effect of sound exposure treatments on water flea behaviour: (a) water flea non-targeted swimming speed difference from the last min before to the first min during different treatments: continuous sound (CS), three intermittent treatments (1-1, 1-4 and 1-7) and ambient (AN) levels as control, (b) water flea swimming depth difference from the last

min before to the first min during the different treatments in the same test as the swimming speed score in (a). And (c) water flea swimming speed during the targeted-swimming mode (check Table 1 for absolute numbers) induced in a separate test by a light source on one side of a dark fish tank. Sample sizes were 5 x 10 = 50 individuals for (a) and (b) and 5 x 12 = 60 individuals for (c). Bars show means ± S.E.M. We found no significant effect of any sound exposure on either type of swimming speed or on swimming depth.

Sound impact on zebrafish

Sound exposure often led to an increase in the number of startle responses, sometimes in an increase in swimming speed and occasionally fish moved up in the water column (Fig. 5 a, b and c). The increase in number of startle responses upon exposure was significantly affected by treatment (Friedman chi-squared test: X^2_4=10.465, *P* =0.033). The sound-induced increase in startle responses was especially found for the intermittent exposures due to a growing number of individuals that exhibited increasingly more startle responses from CS, to 1-1, 1-4 and 1-7 (up to 19 startles, see table 1 for variation in the absolute number of startle responses among treatments). Post-hoc comparisons revealed that there was a significant difference between AN versus 1-4 (*P*=0.012) and AN versus 1-7 (*P*=0.003), and also a non-significant trend for CS versus 1-7 (*P*=0.058). There was also a significant effect of sound exposure treatment on swimming speed difference (repeated-measures ANOVA: $F_{4,52}$=3.193, *P*= 0.020). Post–hoc comparisons revealed significant differences among treatments; for AN versus 1-1 (*P*=0.025), and 1-7 (*P*=0.032), and for 1-4 versus 1-7 (*P*=0.044). There was also a non-significant trend for a difference between CS and 1-7 (*P*=0.080). Swimming depth turned out to be quite variable for the trials with sound exposures and the ambient noise control (AN) and we found no treatment effects for the difference in swimming depth between before and during sound exposure (repeated-measures ANOVA: $F_{4, 52}$=0.869, P= 0.489).

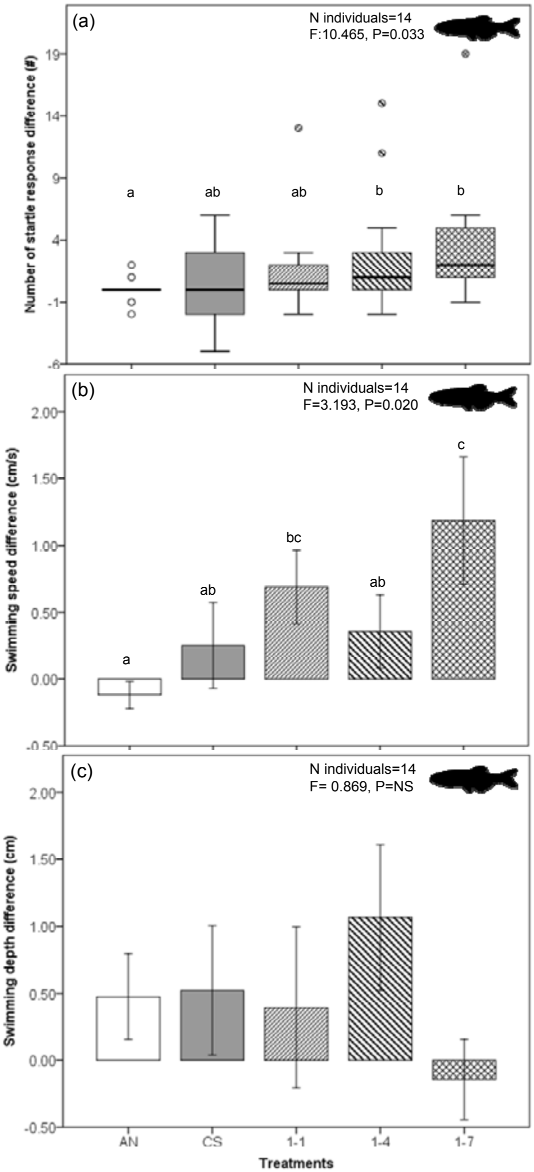

Fig. 5. Effect of sound exposure treatments on zebrafish behaviour: (a) Number of startle responses expressed as the difference between the first min during and the last min before exposure on-set: continuous sound (CS), three intermittent treatments (1-1, 1-4 and 1-7) and ambient (AN) levels as control. These count data are not normally distributed and box-

whisker plots show the median, lower and upper quartiles, extreme values and outliers (b) zebrafish swimming speed difference and (c) zebrafish swimming depth difference, both expressed as the difference between the first min during and the last min before exposure onset for the different treatments. The sample size was 14 individuals for repeated measurements on each of the four treatments and the control. Bars show means ± S.E.M. Treatments that differ significantly from one another are labeled with different letters a, b, and c ($P<0.05$). Non-significant trends ($P<0.1$) are not indicated, but just mentioned in the main text.

Sound impact on zebra fish preying on water fleas

Especially the intermittent sound exposure treatments seemed to affect zebrafish swimming in the initial response to the introduction of water fleas into the water, while there was no sound impact on food item discrimination. However, all sound treatments clearly affected food item handling (Fig. 6 a, b and c). For the initial swimming speed difference, we found a significant effect of treatments (repeated-measures ANOVA: $F_{4,52}=4.563$, $P= 0.003$). All zebrafish hunted for water fleas and increased their swimming speed with the introduction of water fleas in all treatments and the control, but this was very rapid especially for CS and to a lesser extent for AN. Post-hoc comparisons revealed significant differences among treatments for swimming speed difference between the first 5 sec after introduction and the last 5 sec before introduction of the waterfleas: CS versus 1-1(P=0.010), 1-4 (P=0.002) and 1-7 (P=0.002), but not for CS versus AN (P= 0.136). There was no significant impact of sound exposure on food discrimination error for any of the sound treatments (repeated-measures ANOVA: $F_{4,48}=0.622$, $P=0.649$). However, there was a clear and significant effect of sound treatment on food handling error (repeated-measures ANOVA: $F_{4, 52}$=4.159, *P*=0.005). Post-hoc comparisons showed a significant and indiscriminant impact for all treatments in comparison to AN as the control group; CS (*P*=0.004); 1-1 (*P*=0.006), 1-4 (*P*=0.022), and 1-7 (*P*=0.009).

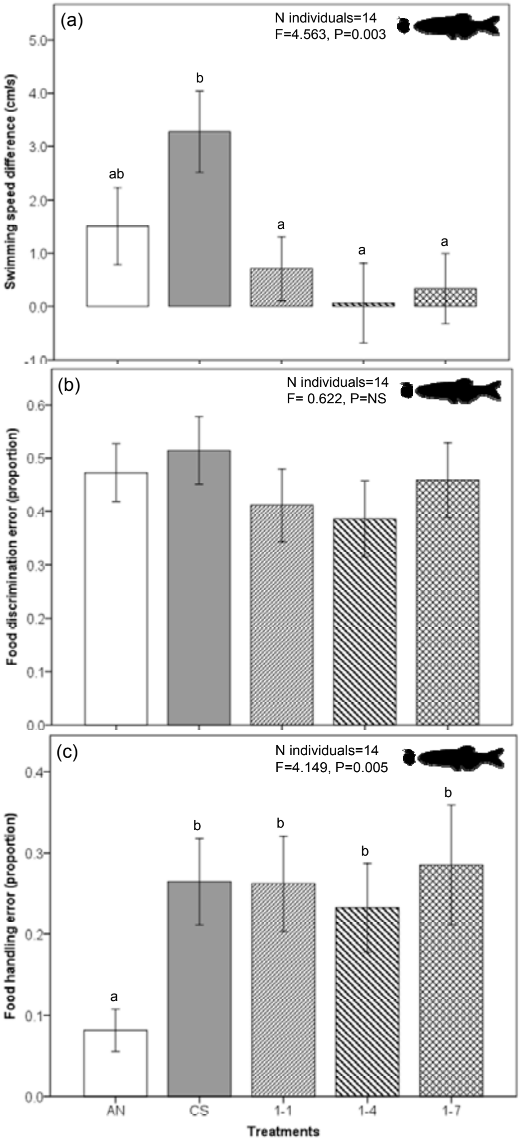

Fig. 6. Effect of sound exposure on zebrafish foraging behaviour: (a) difference in zebrafish swimming speed during sound exposure in the first 5 seconds after the introduction of water fleas subtracted from the swimming speed in the last 5 seconds before the introduction of water fleas for all treatments: continuous sound (CS), three intermittent treatments (1-1, 1-4 and 1-7) and ambient (AN) levels as control, (b) food discrimination

error as the proportion of duckweed particles attacked relative to the total number of attacks to both duckweed particles and water fleas with introduction of food items until the end of sound exposure in sequence for each zebrafish individual, and (c) food handling error as the proportion of the total of water fleas attacked that are missed or released again after initial grasping with onset of food introduction until the end of sound exposure in sequence for each zebrafish individual. Sample sizes were 14 individuals for repeated measurements on each of the four treatments and the control. Bars show means ± S.E.M. and treatments that differ significantly from one another are labeled with different letters a and b ($P<0.05$).

Discussion

We investigated potential effects of artificial noise exposure on underwater predator-prey interactions through testing the effect of temporal variation in experimental exposure on zebrafish hunting for water fleas under laboratory conditions. We were unable to detect effects of sound exposure on water flea swimming speed or depth but we found several significant effects on zebrafish. The zebrafish showed significantly more startle responses especially for two of the three intermittent sound exposures. This pattern was also reflected in an increased swimming speed for two (not the same two) of the three intermittent treatments. In contrast, there were no significant changes in zebrafish swimming depth in response to any of the treatments. Discrimination error in attacking edible water fleas or inedible duckweed particles was high and unaffected by sound exposure. However, foraging was affected in two ways: intermittent treatments significantly delayed initial acceleration response in swimming speed relative to the continuous sound exposure and all sound exposure treatments caused a significant rise in handling error.

Sound impact on foraging fish

In comparison to earlier studies on sound impact on foraging fish, some of our data are confirmative; some are contrasting, and some concern new findings. The food handling error significantly increased in all sound treatments compared to the control: like the sticklebacks, the zebrafish often missed prey in the first strike and often had problems with handling the prey item before they could swallow under noisy conditions (c.f. Purser & Radford 2011; Voellmy et al. 2014), irrespective of the temporal pattern of exposure. In the earlier studies, sticklebacks also had a problem under noisy conditions in discrimination of water fleas from other small particles that happened to float in the water, which was less under more quiet conditions. In contrast, our zebrafish were indiscriminant in all of the treatment and control conditions in attacking both edible water fleas and inedible pieces of duckweed. This species discrepancy might be due to the fact that zebrafish seem much more active, explorative, and opportunistic foragers (e.g. Grant & Kramer 1992) than the more considerate and maybe more selective sticklebacks (e.g. Matthews et al. 2010).

A new finding in our study was the immediate increase in swimming speed with the introduction of water fleas in the continuous sound treatment and to a lesser extent in the ambient noise control, while the foraging onset seemed delayed in the intermittent sound treatments. This effect may be due to masking as the introduction of water fleas may be accompanied by an auditory cue (sound associated with water fleas entering the water) that will be less easy to detect against the background of intermittent sounds than against a background of continuous sound levels. Alternatively, the intermittent sounds may have reduced the focus of the fish on foraging opportunities as they could be more aversive and may be perceived as potential danger for increased predation risk. Oswald & Robinson (2008) recently showed that aversive stimuli of mechanical, visual and chemical nature slow down foraging in zebrafish, which may also be true for acoustic stimuli depending on the sound level (Neo et al. 2015) as has also been shown for European minnows, which slow down their activities dramatically under experimental sound exposure (Voellmy et al. 2014).

We believe that the most likely explanation that can apply to both types of sound impact (the few seconds of response delay and the attack and handling problems) may be a general performance drop due to attentional shifts. This explanation has been suggested for sound-impact on non-auditory tasks in several different taxa (Chan et al. 2010; Purser & Radford 2011; Wale et al. 2013). In addition, sound exposure not only affected response latency to the water flea introduction and foraging efficiency, but also altered their swimming behaviour immediately after the on-set of the sound exposure (c.f. Neo et al. 2015). This behavioural change, which was true for relatively brief increases in swimming speed during two of the intermittent sound treatments, likely reflects the startle responses in the initial period of exposure. It seems that showing up to about five distinct startle responses in response to sudden on-set of sound exposure is a shared feature among different fish species tested in captivity. European minnows and sticklebacks had very similar amounts of startle responses compared to our zebrafish and also showed a significant increase in number from exposure to white noise to a more variable exposure type of boat noise recordings (Purser & Radford 2011; Voellmy et al. 2014).

The zebrafish moved up towards the surface during earlier sound exposure experiments (Neo et al. 2015), which was occasionally seen again but did not lead to consistent and significant treatment effects on swimming depth in the current experiments. Using an in-air speaker results in slightly higher sound levels at the bottom of the tank than in the middle and upper layers, which could trigger the fish to move upward to escape high exposure levels. However, upward swimming responses may also suggest that the on-set of sounds from experimental exposure draws attention and may induce explorative behaviour (c.f. Neo et al. 2015). However, as we did not confirm this effect in the current data-set, the intermittent treatments may here actually be responsible for missing the initial cue for the introduction of waterfleas to the water. Masking or habituation to short sound pulses may be the mechanistic explanation of this effect, while general distraction may be the explanation for an effect on general performance level from continued presence of any sound pattern (c.f. Chan et al. 2010; Purser & Radford 2011; Wale et al. 2013).

Are water fleas not affected by sound?

Our results showed that water fleas do not change their swimming behaviour in response to the current sound exposure conditions and suggest that they are not sensitive to the elevated sound levels within the target spectrum that do affect fish behaviour. This may seem surprising as there is considerable evidence that invertebrates perceive sound and use sound in social interactions, habitat defense, conspecific communication and directional orientation (Patek 2001; Popper et al. 2001; Stocks, 2012; Sueur et al. 2011; Vermeij et al. 2010). Invertebrate species are also known to be able to detect acoustic stimuli in variable ranges of the spectrum (Hughes et al. 2014; Popper et al. 2001; Stocks 2012). For example, free swimming larvae of coral reefs (*Montastraea faveolata*) were reported to exhibit both horizontal and vertical movements specifically towards playbacks of sounds recorded at reefs (Vermeij et al. 2010).

It could be that water flea behaviour is not affected by the sound exposure level that we created in our laboratory test condition, but that it would be affected at higher exposure levels. Further experiments are needed to exclude this possibility. Based on our particle motion measurements, we believe that using an in-air speaker to ensonify the experimental fish tank (as we used in our experiment) is a sufficient tool to generate high sound velocity levels. However, we may have to explore the impact of higher exposure levels in terms of particle motion by using under-water speakers. An alternative interpretation for the lack of a response in water fleas could be that the frequency range of our experimental exposure was outside their detection range. Although there are invertebrates sensitive to a wide frequency range that covers the current experimental spectrum, they may typically be more sensitive to lower frequencies (Packard et al. 1990; Lovell et al. 2005; Kaifu et al. 2008; Mooney et al. 2010). Important for our study here is that the prey is not likely to have contributed to the pattern of sound-dependent foraging efficiency of the predator.

Sound impact beyond single-species effects

It is important to realize that our results are based on tests in laboratory settings and do not allow direct extrapolation to outdoor conditions in the field (c.f. Slabbekoorn, 2016). More applied insights for outdoor conditions and data on more long-term effects that amount to fitness consequences require more and different studies (Hawkins & Popper 2014; Radford et al. 2014; Slabbekoorn et al. 2010). However, the accumulating evidence for a possible impact of sound exposure on predator foraging performance means that consequences of sound pollution in the natural environment are also likely to go beyond single-species effects (Francis et al. 2009; Francis et al. 2011b; Francis et al. 2012a; Francis et al. 2012b; Shafiei Sabet et al. 2016; Slabbekoorn & Halfwerk 2009).

Changes in foraging tendency and efficiency may directly affect relative species abundance of both predator and prey and induce changes at the community level in a similar way as with underwater light pollution (e.g. Becker et al. 2013), changes in water turbulence or flow (e.g. Powers & Kittinger 2002) or chemical pollution (reviewed in Fleeger et al. 2003). Studies on the impact of acoustic changes in air in the terrestrial environment have already confirmed such effects of anthropogenic noise at the community level (Bayne et al. 2008; Francis et al. 2009, 2011a; 2011b; Francis et al. 2012a; 2012b). Consequently, we need to be on the look-out for negative effects of anthropogenic noise on underwater food web dynamics and stability in both freshwater and marine environments.

Conclusion

Our current study does not provide evidence for an effect of artificial noise on water flea swimming behaviour, but clearly reveals an effect of experimental sound exposure on individual zebrafish swimming behaviour and foraging efficiency while hunting for water fleas. It seems that several fish species are affected by sound exposure in terms of foraging and that the impact is due to effects on the vertebrate predator instead of the invertebrate prey (Purser & Radford 2011; Voellmy et al. 2014). Furthermore, our study also reveals significant effects in terms of temporal variation, as intermittent sound treatments had stronger and different effects than continuous sound. We believe this is a relevant finding as the ‘natural’ occurrence of anthropogenic noise is characterized by highly variable conditions and intermittent sounds are almost omnipresent. Our laboratory study should not be extrapolated directly to outdoor conditions, but calls for investigation of behavioural responses of free-ranging fish to sound exposures of different temporal patterns. Also under natural conditions, anthropogenic noise may affect species interactions and may have community level consequences that are important to the stability and dynamics of aquatic ecosystems.

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Treatment:	AN	CS	1-1	1-4	1-7	Fig.
Waterflea non-targeted swimming speed (cm/s)	0.22±0.05	0.17±0.03	0.20 ± 0.03	0.14±0.02	0.14±0.02	4a
Waterflea swimming depth (cm)	7.38±4.28	3.43±2.23	6.42±1.22	7.69±1.80	8.28±1.35	4b
Waterflea targeted swimming speed (cm/s)	0.35±0.02	0.36±0.02	0.34±0.02	0.34±0.02	0.35±0.03	4c
Zebrafish number of startle response (#)	0.93±0.67	2.57±0.93	2.71±1.20	3.57±1.80	4.43±1.58	5a
Zebrafish swimming speed (cm/s)	3.16±0.33	4.06±0.30	4.01±0.24	4.16±0.481	4.49±0.57	5b
Zebrafish swimming depth (cm)	5.94±0.94	6.09±0.70	5.75±0.88	5.73±0.83	4.33±0.62	5c
Zebrafish swimming speed response to food (cm/s)	5.31±0.65	6.96±0.75	5.40±0.44	5.26±0.60	4.85±0.53	6a
Zebrafish food discrimination error (%)	0.47±0.06	0.51±0.07	0.41±0.08	0.42±0.08	0.46±0.08	6b
Zebrafish food handling error (%)	0.08±0.03	0.26±0.06	0.26±0.07	0.23±0.06	0.29±0.09	6c

Table 1. Absolute values for mean (±SE.M) behavioural measurements of waterfleas and zebrafish. Swimming speed and depth , as well as the number of zebrafish startle responses and the food discrimination and handling error, during the ambient noise control (AN) and during the different sound treatments (CS=continuous sound; 1-1=intermittent, regular, fast; 1-4= intermittent, regular, slow; 1-7=intermittent, irregular, slow). The absolute numbers correspond with the relative numbers on changes, related to the periods before and during sound on-set or before and during waterflea introduction, reported in the respective figures indicated in the column on the far right

Chapter 3

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

This chapter is based on: Saeed Shafiei Sabet, Kees Wesdorp, James Campbell, Peter Snelderwaard & Hans Slabbekoorn (in press). Behavioural responses to sound in captivity by two fish species with different hearing ability. Animal Behaviour.

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.

Key words: sound exposure, swimming behaviour, spatial avoidance, captive fish, zebrafish, cichlids, *Danio rerio*, *Haplochromis piceatus*.

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 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).

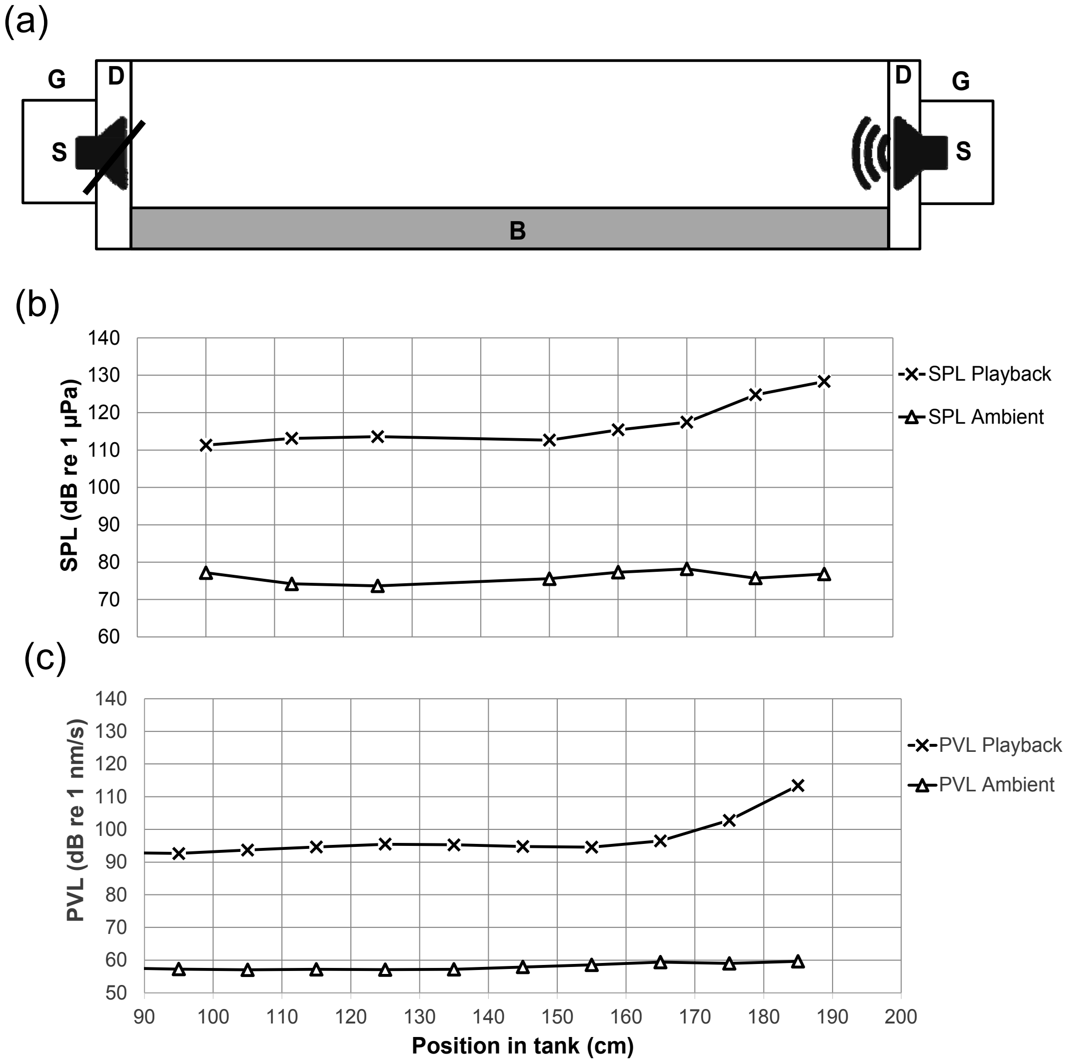

Fig. 1. a) Schematic lateral view of the experimental long tank set-up, b) Sound pressure level (SPL) and c) Particle velocity level (PVL) the target frequency range of :100-1000 Hz. Locations of the net mesh with waterproof metal frame both sides of the tank used as arena divider (D), small glass boxes 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.

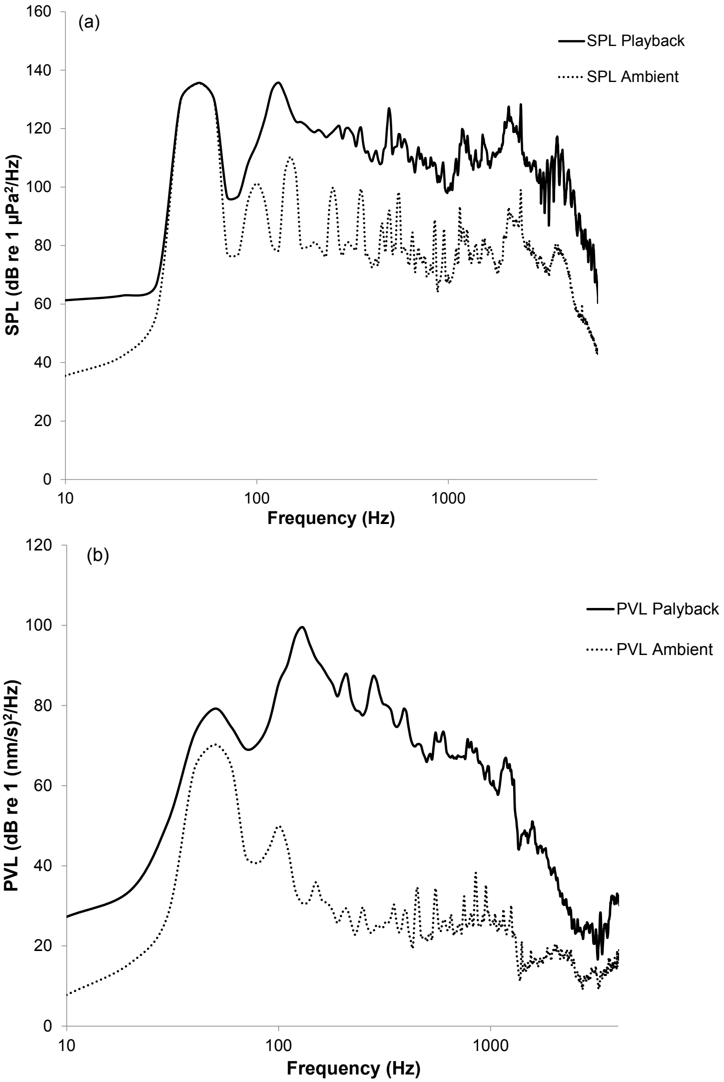

Fig. 2. Power spectral density of a) SPL (in dB re 1 µPa^2/Hz) and b) PVL (in dB re 1 (nm/s)^2/Hz) for playback and ambient conditions. Both sound pressure level and particle velocity level in ambient conditions are much lower than sound playback across the relevant frequency range (100-1000 Hz). The 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).

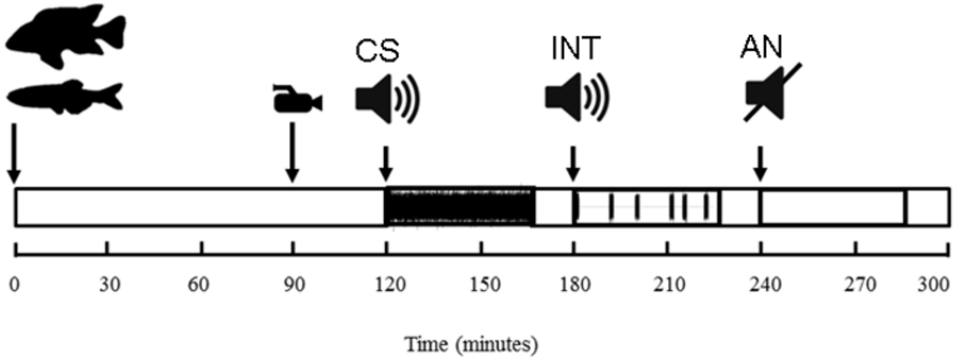

Fig. 3. Schematic view of the timeline of the whole playback procedures and fish individual release events for zebrafish and cichlids. Zebrafish and cichlids were released individually and let them to acclimatize for at least 2 h and 14 h respectively (see text for explanation). Video recording started 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).

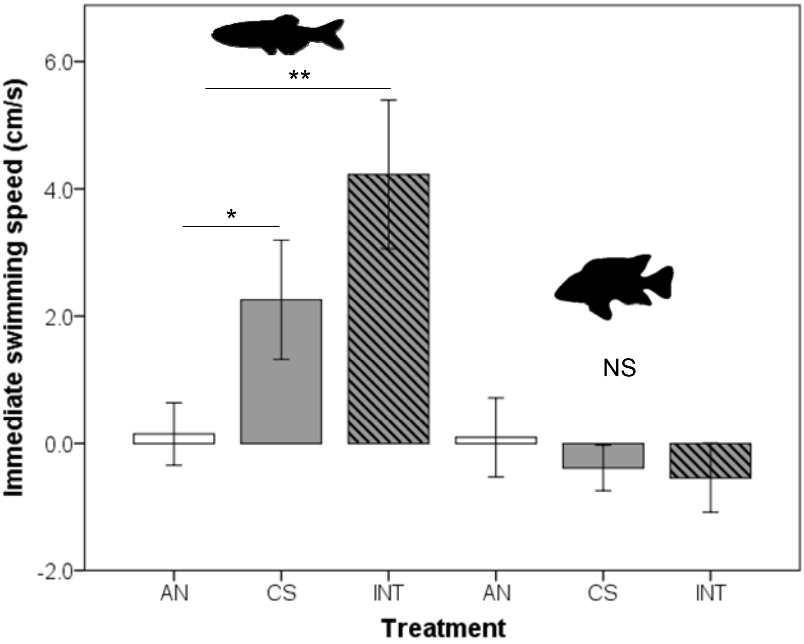

Fig. 4. Effect of sound exposure on zebrafish (n: 28) and cichlids (n: 30) brief swimming speed changes (cm/s) reflecting startle response and initial acceleration. Brief swimming speed changes were measured by subtracting the last 10 seconds before sound exposure by the first 10 seconds immediately 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).

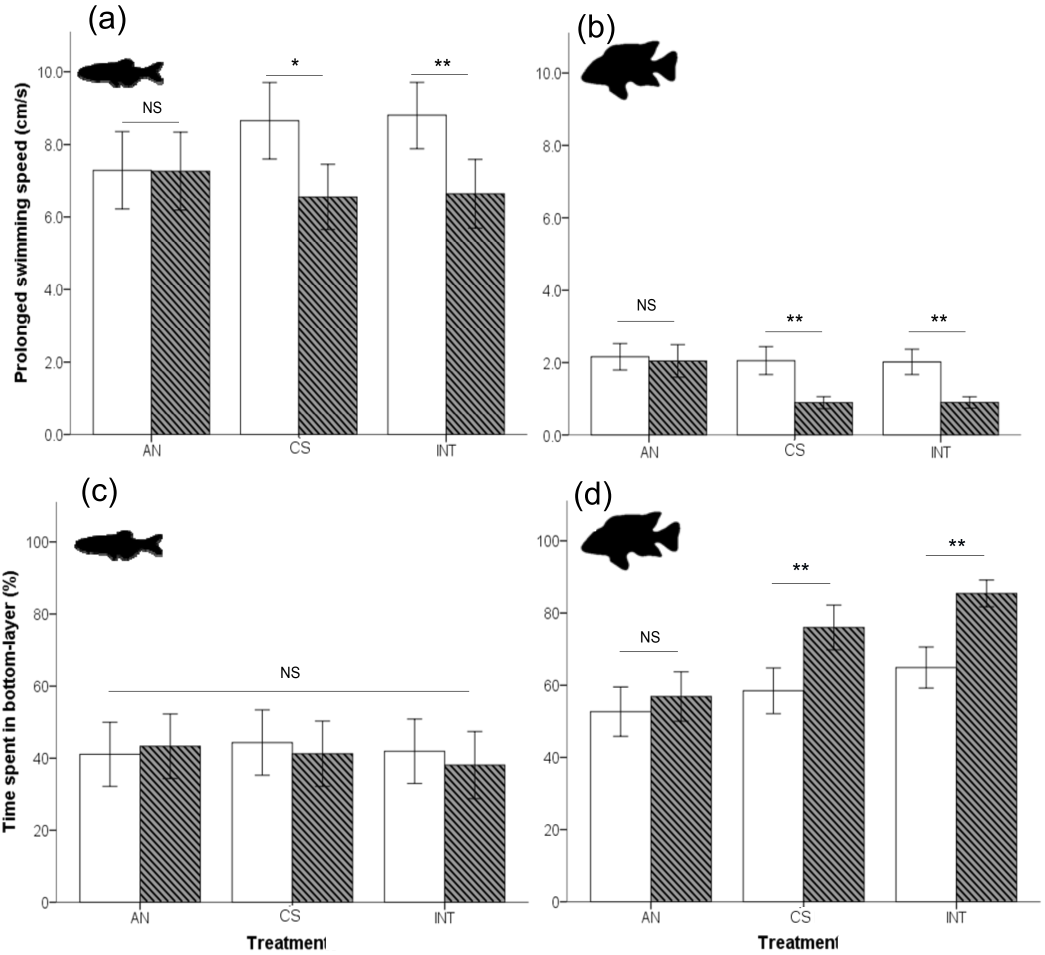

Fig. 5. Effect of sound exposure on: (a) zebrafish (n=28) and (b) cichlids (n=30) prolonged swimming speed changes (cm/s). Effect of sound exposure on the time spent in the bottom layer of the experimental tank (%) in (c) zebrafish (n=28) and (d) Cichlids (n=30). The bottom layer arena for spatial displacement 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

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).

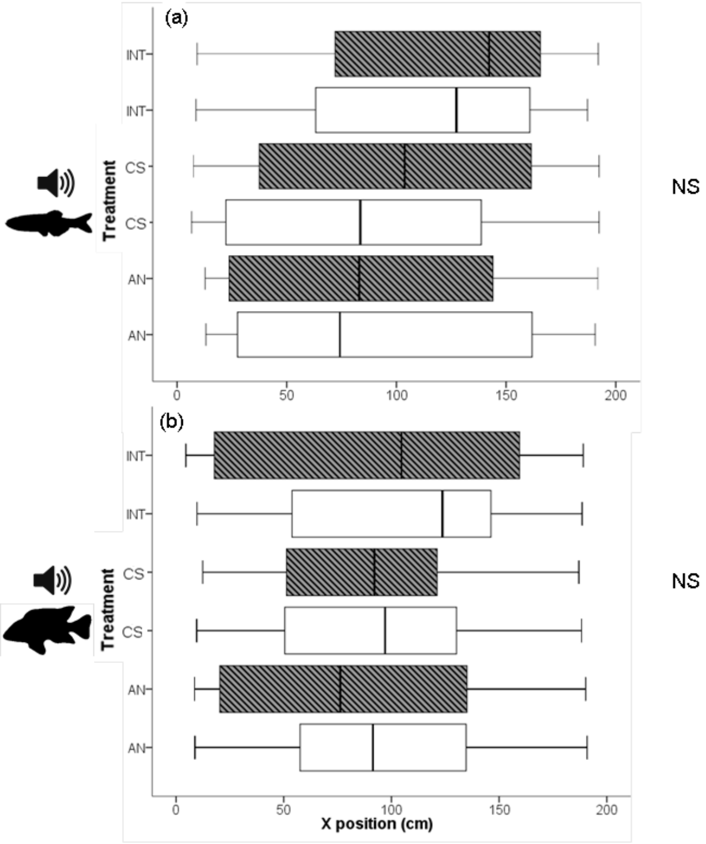

Fig. 6. Effect of sound exposure on: (a) zebrafish (n=28) and (b) cichlids (n=30) horizontal spatial displacement. Horizontal displacement was calculated from the last 15 min before sound exposure (white bars) to 15 min with immediately with on-set of sound exposure (grey bars). When sound played back 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).

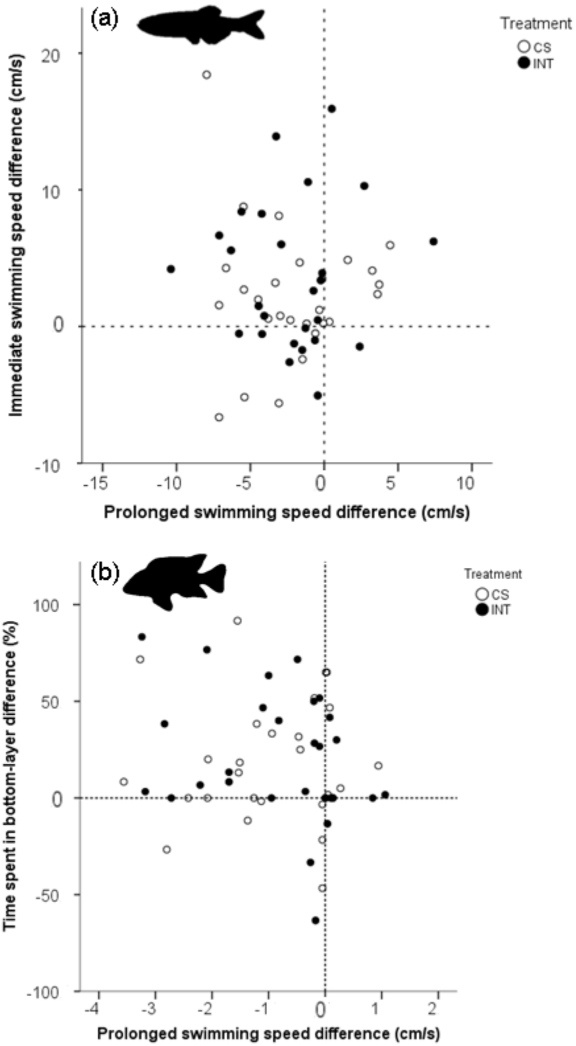

Fig. 7. (a) Lack of correlation between the difference in swimming speed (cm/s) in the brief period of 10 sec immediately after sound onset and the decrease in prolonged swimming speed (cm/s) in zebrafish (n=27). (b) Lack of correlation between the increase in time spent in the bottom layer (%) and the 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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Chapter 4

Son et lumière: sound and light effects on spatial distribution and swimming behaviour in captive zebrafish

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Abstract:

Aquatic and terrestrial habitats are heterogeneous by nature with respect to sound and light conditions. Fish may extract signals and exploit cues from both ambient modalities and they may also select their sound and light level of preference in free-ranging conditions. In recent decades, human activities in or near the water have elevated natural sound levels and also nocturnal light pollution is becoming more widespread. Artificial sound and light may cause anxiety, deterrence, disturbance or masking, but few studies have addressed in any detail how fishes respond to spatial variation in these two modalities. Here we investigated whether sound and light affected spatial distribution and swimming behaviour of individual zebrafish that had a choice between two fish tanks: a treatment tank and a quiet and light escape tank. The treatments concerned a 2 x 2 design with sound or quiet and light or dark. Sound and light treatments caused various behavioural changes in both spatial distribution and swimming behaviour. Sound exposure led to more freezing and less time spent near the active speaker. Dark conditions led to a lower number of crossings, more time spent in the upper layer and less time spent close to the tube for crossing. No interactions were found between sound and light conditions. This study highlights the potential relevance for studying multiple modalities when investigating fish behaviour and further studies are needed to investigate whether similar patterns can be found for fish behaviour in free-ranging conditions.

Keywords: anxiety-related behaviour, anthropogenic noise, artificial light, spatial distribution, swimming behaviour, zebrafish.

Introduction

Aquatic and terrestrial habitats are heterogeneous by nature with respect to ambient sound and light conditions (Endler 1992; Radford et al. 2010; Halfwerk & Slabbekoorn 2015). In the past few decades, human activities related to urbanization, industrialization and transportation are affecting these conditions with elevated levels of anthropogenic noise (Barber et al. 2010; Gage & Axel 2014; McDonald et al. 2006) and light pollution (Davies et al. 2014; Longcore & Rich 2004; Smith 2009). There is an increasing awareness that artificial fluctuations in environmental conditions affect animals and potentially reduce chances of survival and reproduction (Kight & Swaddle 2011; Radford et al. 2014; Slabbekoorn & Ripmeester 2008; Slabbekoorn et al. 2010). As animals typically rely on multiple modalities for sensory input, they can be affected via different channels and interactive effects (Halfwerk & Slabbekoorn 2015; Swaddle et al. 2015). It is therefore important to take multimodality into account to better understand the impact of artificial fluctuations in environmental conditions. However, very few such studies exist, especially addressing the impact on species from aquatic habitats.

There is a wide range of sound sources in marine and freshwater habitats with different temporal and spatial patterns. Firstly, abiotic sounds emanate from water currents and turbulence in interaction with surface, bottom structures and vegetation (e.g. Wysocki et al. 2007; Tonolla et al. 2010). Secondly, there are biotic sounds from aquatic organisms such as marine mammals, fishes and crustaceans that may generate sounds for communication or as a by-product during feeding activities (McCauley & Cato 2000; McWilliam & Hawkins 2013; Parks et al. 2014). And finally, anthropogenic noise comes from a wide variety of human activities such as seismic surveys, recreational water vehicles, pile driving and shipping (Popper & Hastings 2009; Slabbekoorn et al. 2010; Radford et al. 2014).

Also underwater light levels originate from a variety of sources with different spectral and temporal patterns. The sun, moon and stars are the prominent abiotic sources, while there are bacteria, algae and some deep water animal species that are bioluminescent, which represent biotic sources that may affect light levels locally (see e.g. Duntley 1963; Lüning & Dring 1979). Anthropogenic light may lit up waters nocturnally along urban shores, around offshore platforms and vessel-based activities, such as pile driving, seismic surveys and dredging, which may all occur 24/7. Elevated light levels at night have the potential to affect fish communities: coastal lights were reported to attract visually hunting piscivores, which altered predation pressure and thereby also abundance of prey species (Becker et al. 2013). It is also well known that fish activity levels, orientation capacities, and feeding efficiencies can be affected by light levels in both outdoor and indoor conditions (e.g. Jones 1956; Sogard & Olla 1993; Olla et al. 2000). However, insights into light-dependent spatial preferences and swimming patterns remain limited and we have no data on whether the effects of artificially elevated sound levels would vary dependent on light conditions.

Fish may be affected directly or indirectly by anthropogenic noise in various ways (Popper and Hastings 2009; Slabbekoorn et al 2010). It has been shown that very high sound levels can cause physical injuries (Halvorsen et al. 2012; Casper et al. 2013), physiological stress (Wysocki et al. 2006; Buscaino et al. 2010; Debusschere et al. 2016), and permanent or temporary threshold shifts in hearing (McCauley et al. 2003; Smith et al. 2004; Wysocki and Ladich 2005a). More moderate anthropogenic noise levels can mask relevant signals and cues (Codarin et al. 2009; Vasconcelos et al. 2007; Wysocki & Ladich 2005b), and trigger behavioural changes (Skalski et al. 1992; Picciulin et al. 2010; Handegard et al. 2014).

Spatial responses to sound that lead to approach or avoidance rely on the ability to localize the source. Fishes are known to be able to localize sound sources (Schuijf 1975; Popper & Fay 1993) and there is empirical evidence for phonotactic responses of fishes under laboratory conditions. Round gobies (*Neogobius melanostomus*), for example, showed a directional response to the playback of conspecific calls in a fish tank (Higgs et al. 2007; Rollo & Higgs 2008). Similarly, female cichlids (*Pundamilia nyererei*) preferred to associate with a male at the tank side from which they had heard conspecific sounds (Verzijden et al. 2010). Plainfin midshipman females (*Porichthys notatus*) were also attracted to the playback of conspecific male calls and were shown to be guided by the particle motion component of the sound field (Zeddies et al. 2010; 2012). Spatial avoidance in indoor tank conditions has been investigated, but there is little or no evidence for horizontal deterrence (Neo et al. 2015; Febrina et al. 2015; Shafiei Sabet et al. In Press), which is most likely due to the complex sound field of small fish tanks (Akamatsu et al. 2002).

Zebrafish (*Danio rerio*) are a very suitable species to study responses to both sound and light conditions. They are Cyprinids of standing or slow-moving water bodies, more or less densely vegetated, such as rice fields and small streams (Arunachalam et al. 2013; Engeszer et al. 2007). The hearing ability of this taxonomic group has been well-studied and is determined by the presence of otoliths and hair cells in the inner ear (yielding sensitivity to particle motion) and by the presence of a swim bladder and Weberian ossicles (yielding sensitivity to sound pressure) that serve as a pressure-to-motion converter and audio duct respectively (Higgs et al. 2003; Ladich 2014). Earlier studies have shown that sound exposure caused initial acceleration and startle responses (Neo et al. 2015; Shafiei Sabet et al. 2015) and negatively affected foraging performance in zebrafish (Shafiei Sabet et al. 2015). Furthermore, light level related behaviour is also well-studied in zebrafish and a light/dark preference test is a widely used behavioural assay to assess their anxiety level (e.g. Champagne et al. 2010; Maximino et al. 2010). However, although it seems clear that zebrafish feel more comfortable in dim conditions, many factors may modify their light level preferences (Stephenson et al. 2011) and nothing is known yet about how sound and light simultaneously affect their spatial preferences and swimming behaviour.

In this study, we investigated whether experimental sound and light exposure affected the spatial distribution and swimming behaviour of individual zebrafish (*Danio rerio*) that had a choice between two fish tanks:

a treatment tank and a quiet and light escape tank. Our research questions were the following: Firstly, do zebrafish indeed express no preference for a quiet over a noisy fish tank (as suggested by the outcome for groups in Neo et al. 2015) and do they prefer a dark over a bright fish tank? Can we find any tank preference in this dual tank set-up? And secondly, when zebrafish are in the treatment tank, do sound or light conditions affect spatial distribution and swimming behaviour, potentially revealing relative anxiety level? Thirdly, are there any interactions between sound and light for the preferences between tanks or the behaviour within the treatment tank?

Materials and methods

Animal maintenance and housing conditions

Thirty adult zebrafish (4-6 months old and of the wild-type, short-fin variety, 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) connected to a water circulation system 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 23 ^{o}C. All fish individuals were fed twice daily with dry food (DuplaRin M, Gelsdorf, Germany) and frozen Artemias (RUTO frozen fish food, The Netherlands).

Experimental Tank set up

The experiments were conducted in a dual-tank (75 cm×50 cm×50 cm each tank; water depth: ~45 cm; wall thickness: 0.8 cm) connected by a pvc tube (diameter: 12.5 cm, length: 35 cm between tanks) (c.f. Neo et al. 2015). The tanks were placed on two different trolleys with rubber wheels and on top of a layer of Styrofoam (thickness: 20 mm) to minimize transmission of environmental sound from the laboratory building. 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°C. Two underwater loud speakers (model: UW-30, Lubell Labs Columbus, OH, U.S.A.), built in portable Plexiglas frames, were placed inside of the dual tank at each far end (see Fig. 1). As a result, the swimming areas of the fish were restricted to 50 cm×50 cm× 40 cm in both sides of the dual-tank. Pre-test observations showed that zebrafish were sufficiently acclimated within 2 hours after being introduced to the test tank, (c.f. Shafiei Sabet et al. 2015; Neo et al. 2015). Consequently, the zebrafish were left exploring and habituating for at least 2 hours after being gently introduced into the fish tank. We used a standard fish net for catching and introduced them either in the right or in the left tank in randomized sequences. Trials for each individual were conducted either at 9:00 AM in the morning or 14:00 PM in the afternoon.

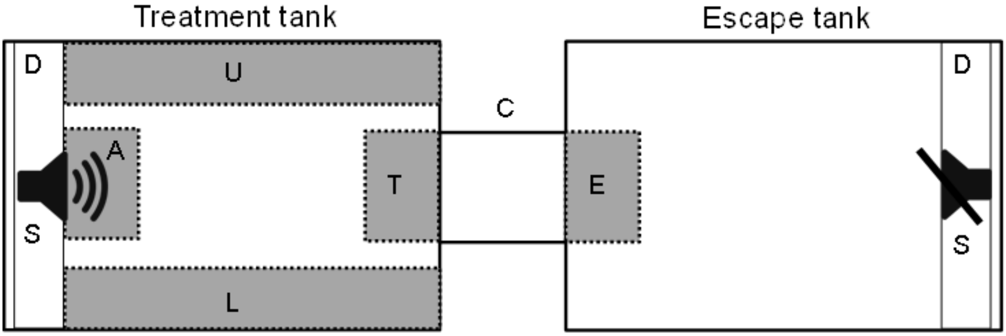

Fig. 1. Schematic view from the front of the dual-tank set up (outside measurements: 75 x 50 x 50cm each). Two underwater speakers (S) are placed on each side shielded by Plexiglas dividers to keep fish from swimming behind them (D). A sturdy pvc-tube (35 cm in length, 12.5 cm diameter) connected the two tanks (C). Grey shaded areas in the tanks indicate measurement areas: we determined the time spent in the upper layer (U), time spent in the lower-bottom layer (L), time spent close to the active speaker (A), time spent near the tube in the treatment tank (T) and time spent near the tube in the escape tank (E).

The backsides of both tanks were covered with matte plastic sheets to maximize resolution of video recordings and to enhance digital tracing. The outer sides of the tanks were also covered with black curtain textile in order to control and maximize seclusion of light. An opaque pvc plate was placed in front of the pvc crossing tube entrance for each inner side of the dual tank to prevent the fish to swim above, below or besides the pvc crossing tube and thereby exit the video observation area in another way than through entering the tube. The front sides of the tanks were left uncovered so that the camera could catch the movement of the fish for the whole period of the experiment. Above each tank a Tube Luminescent lamp was placed in the middle while the rest of the top was covered with opaque pvc plates in order to prevent light from above to illuminate the inside of the tanks. Full-tank illumination led to a range in light illuminance of 300-750 lux in the dim light condition and 1000-1500 lux in the bright light condition, measured by a LUNASIX F light meter (P. Gossen & Co, Erlangen, Germany) from bottom to surface. These light conditions provided sufficient contrast between different light treatments, while keeping enough visibility to allow continuous tracing of zebrafish on video throughout the treatment tank in both light conditions. After each experimental day, the water recirculation was switched on to maintain high water quality and consistent temperature and chemical conditions across trials.

Exposure stimuli and procedure

Sound files were created from white noise, artificially generated with Audacity (2.0.3) software (http://audacity.sourceforge.net) in WAV-file format (32 bits, 44.1 kHz sampling rate) and band-pass filtered between 100-1000 Hz (repeated 5 times with a 48 dB roll-off). We used 5ms ramps to fade in and fade out pulses for smooth transitions in the intermittent sound patterns. The experimental sound file matched the frequency range of best hearing for zebrafish (Higgs et al. 2002; Popper et al. 2001) and also matched in general terms the typical wide-band sound characteristics of anthropogenic sound sources, such as vessels, pumping systems or pile driving (Slabbekoorn et al. 2010; Wysocki et al. 2006). Subsequently, the playback files were amplified in Audacity to a maximum level, without allowing overload. Each trial consisted of the following four combinations of sound and light conditions in a random order: light-noisy (LN) with bright light conditions and sound exposure, dark-noisy (DN) with dim light and sound exposure, dark-quiet (DQ) with dim light and ambient sound conditions and light-quiet (LQ) with bright light and ambient sound conditions in the treatment tank. We investigated zebrafish spatial presences and behavioural changes with light and quiet conditions (LQ) in the escape tank. Each condition lasted 30 min followed by a 15 min break at ambient sound levels and bright light conditions. The sound treatment used in this experiment consisted of intermittent one-second pulses with irregular intervals of varying duration from 1 to 7 seconds in random sequences (mean interval of 4s) (c.f. Shafiei Sabet et al. 2015; Shafiei Sabet et al. In Press) (see Fig. 3).

The randomly selected sequences of four exposure conditions (LN, DN, DQ, LQ) included all eight combinations in such a way that each was used equally often, resulting in a in a pseudo-random design. Sound playback and light condition in each trial started either with the speaker and light session (on/off) in the left or the right tank (randomly chosen using an online random number generator: http://www.random.org/), where the speaker playing back sound was labeled the “active” speaker and the tank with varying sound and light conditions the “treatment tank”. The subsequent sound treatments for the same individual fish were played from alternating tanks. Sound files (WAV format, 44.1- kHz sampling rate) for all treatments were played back with a portable Tascam digital recorder (model DR-07) connected to the two UW30 underwater loud speakers (Lubell Labs Columbus, OH, U.S.A.). The speakers were connected to a QUAD 303 power amplifier (Mfg Co Ltd, Huntingdon England). Fish behaviour was continuously recorded using a Panasonic full HD camcorder (model HC-V500) during the entire test period.

Sound level measurements

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). Spectrum levels varied due to speaker output characteristics and propagation through the fish tank, but sound levels were well elevated throughout the relevant hearing range of zebrafish (see Fig. 2a, b). Sound pressure level (SPL) was measured using a Marantz solid state recorder (model PMD-661) in combination with a calibrated High Tech hydrophone (model HTI 96 min). 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 and Schuijf 1985; Bretschneider et al. 2013; Shafiei Sabet et al. 2015). Accelerometers were connected to a digital differential oscilloscope (PicoScope model PS3425) 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.). Measurements were taken at different locations throughout the tank with either the left, right or no speaker playing with three replicated measurements for each location.

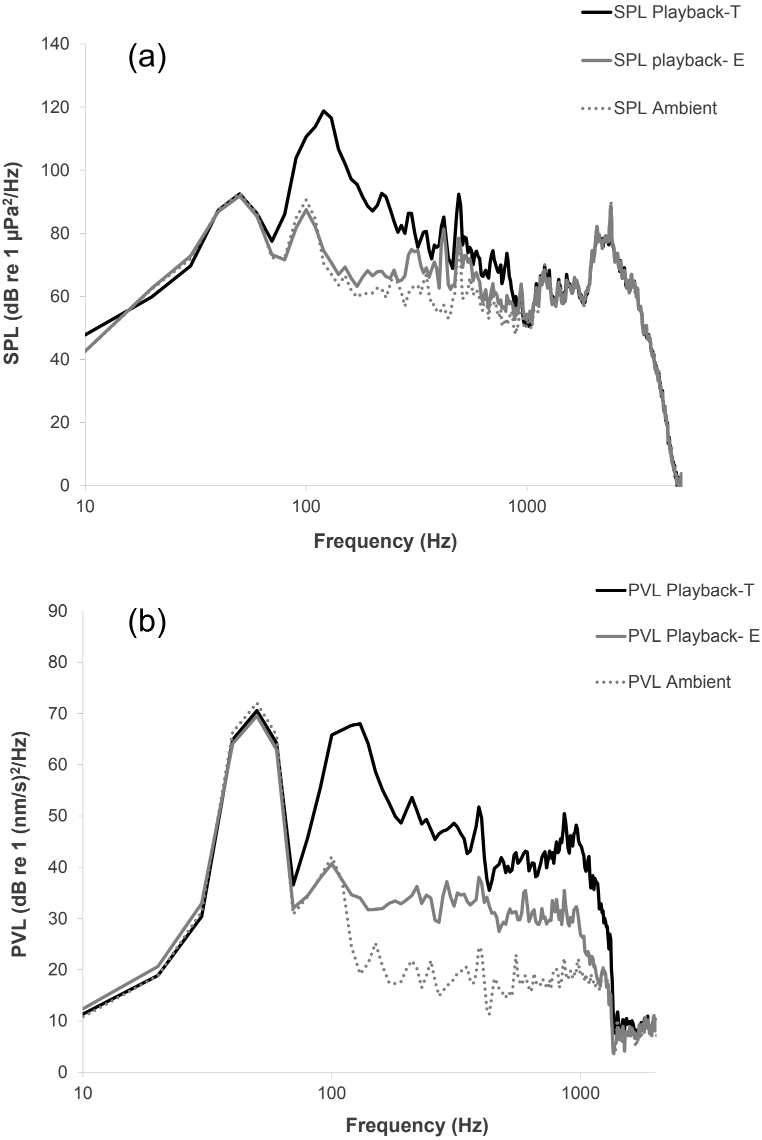

Fig. 2. Experimental sound levels as measured in locations close to the tube in the treatment tank (T), Escape tank (E) and ambient condition. Experimental elevation of SPL is between 70-800 Hz, with biggest rise between 90-250 Hz (a). Black solid line, grey line and grey dot line represent sound playback in treatment tank (T), escape tank (E) and ambient condition. Only minor leakage for SPL to other tank in narrow

bandwidth of 300-600 Hz. Experimental elevation for PVL extends over wider spectral range between 70-1050 Hz, with a big rise almost throughout the range between 90-1040Hz (b). The leakage to the other tank is more considerable in PVL as we find half of the amplitude rise in escape tank relative to exposure tank between 100-1010 Hz (dB logarithmic scale, more leakage for relatively high than low frequencies in this range).

Processing behavioural data and measurements

We converted all zebrafish video files using the AVS Video converter 8.1 into 5 frames per second (FPS) M4V file and then analyzed 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 by tracking individuals during complete trials and comparing activity just before and right after on-set of sound exposure as well as throughout the exposure period.

We assessed swimming behaviour and spatial distributions for 60 zebrafish individuals during the whole period of 30 min for each treatment. We measured how much time zebrafish spent in the treatment tank as a general and long-term tendency of spatial preference. Number of crossings between the treatment and escape tanks was used as indicator of exploratory swimming activity, time spent in upper area in the treatment tank as an indicator of curiosity (c.f. Neo et al. 2014; Neo et al. 2015; Shafiei Sabet et al. In Press). Speeding time refers to the time swimming at high speed (≥8cm/s) when present in the treatment tank, freezing time (interruption of all activities except breathing) and time spent at the bottom-layer of the treatment tank (<10 cm depth from the bottom) were measured as indicator of anxiety related and anti-predatory behaviour (Gerlai et al. 2006; Gerlai et al. 2009; Gerlai 2010; Shafiei Sabet et al. 2015). To specifically test horizontal distribution and avoidance behaviour in response to treatments, we also measured time spent close to the crossing tube (within a square of 10 cm horizontally and 20 cm vertically right in front of the tube entrance) in both the treatment and escape tanks and time spent close to the active speaker.

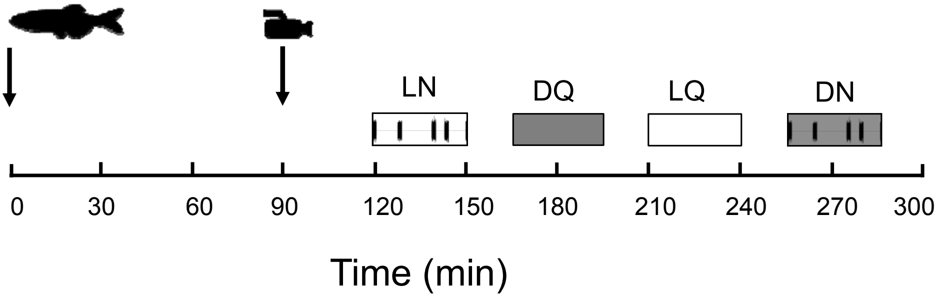

Fig. 3. Schematic representation of the exposure timeline for a single trial of an individual zebrafish in the treatment tank. Each individual in an experimental trial was exposed to four subsequent combinations of sound and light treatments in randomized sequence: LN, DQ, LQ and DN, while the escape tank was kept the same with no changes of light and quieter conditions (LQ). Each treatment lasted for 30 min of experimental exposure of sound and light. Sound exposure treatments represent playback periods through one of our two underwater speakers.

Statistics analysis

Two factorial design ANOVAs for repeated measures were applied with sound exposure and light exposure as the two main factors to test significant differences among treatments. Whenever data did not meet the assumptions for a normal distribution, we applied a transformation to avoid violations of homogeneity of variance. When the outcome of the repeated measures ANOVA was significant, Bonferroni-corrected post hoc tests were performed for pairwise comparisons among the four treatments. All tests were done using SPSS statistics for Windows, version 21.0 (IBM Corp., Armonk, NY, U.S.A.). The treatment exposure sequence was randomized to avoid order effects, but we also checked statistically for an order effect by including the position of the treatment in the trial sequence as a random factor. We did not find an order effect in any of our test results (all $P>0.1$). All video analyses were done without audio track and treatment sequence was therefore blind to the observer. To check for a possible effect of experimenter (DvD) on the behavioural measurements, half of the behavioural data set of zebrafish individuals were re-analyzed double-blind by a second experimenter (SSS) and there were no significant differences between the behavioural measurements from the two observers.

Ethical statement

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

Results

Overall, we did not find any tendency of zebrafish to spend more time in the treatment tank; zebrafish did not avoid noisy or bright light conditions in the treatment tank. There was no significant effect of sound ($F_{1,118}$=0.778, P=0.380) nor of light ($F_{1,118}$=0.173, P=0.678). For the number of crossings there was also no significant effect of sound ($F_{1,118}$=2.397, P=0.124), but the number of crossings was significantly affected by light ($F_{1,118}$=6.097, P=0.015); zebrafish showed more crossings between tanks when they were exposed to bright light in the treatment tank. There was no interaction between sound × light ($F_{1,118}$=0.037, P=0.847) (see Fig. 4b).

We found an effect of light on zebrafish time spent in the upper layer in treatment tank ($F_{1,75}$=5.066, P=0.027); zebrafish spent less time in the upper layer of the treatment tank when there was bright light. There was no effect of sound on the time spent in the upper layer ($F_{1,75}$=0.099, P=0.754). There was also no interaction of sound × light ($F_{1,75}$=2.690, P=0.105) (see Fig, 4c). Speeding time did not vary significantly with sound and light conditions in the treatment tank; the time of zebrafish swimming high speed was not affected by sound ($F_{1,75}$=1.016, P=0.317) nor by light ($F_{1,75}$=0.072, P=0.790). There was also no interaction of sound and light (all P>0.05) (see Fig. 4d). There was a significant effect of sound on freezing time in the treatment tank ($F_{1,75}$=17.521, P<0.001), but no effect of light ($F_{1,75}$=0.113, P=0.737) and no interaction of sound × light ($F_{1,75}$=0.003, P=0.955) (see Fig. 4e). The time zebrafish spent in the bottom-layer of the tank was not affected by sound ($F_{1,76}$=0.247, P=0.621) nor by light ($F_{1,76}$=0.695, P=0.407). There was also no interaction of sound × light ($F_{1,76}$=0.495, P=0.484) (see Fig. 4f).

There was no significant effect of sound treatment ($F_{1,69}$=0.158, P=0.692) and light treatment ($F_{1,69}$=0.624, P=0.432) on zebrafish time spent close to the crossing tube in the escape tank and a non-significant trend for an interaction of sound × light treatment ($F_{1,69}$=3.420, P=0.069) (see Fig. 4g). We found an effect of light on the time zebrafish spent close to the crossing tube in the treatment tank ($F_{1,76}$=10.339, P=0.002). Zebrafish spent significantly less time close to the tube in the treatment tank for both ambient and sound treatments when they were in dark conditions. There was no effect of sound on the time spent close to the tube in the treatment tank ($F_{1,76}$=0.411, P=0.523). We also did not find a significant interaction of sound × light ($F_{1,76}$=0.049, P=0.825) (see Fig. 4h). Finally, there was an effect of sound on the time zebrafish spent near the active speaker ($F_{1,75}$=23.730, P<0.001). Zebrafish spent less time close to the active speaker, when sound was played back in both light conditions. We did not find an effect of light treatment ($F_{1,75}$=0.229, P=0.634) nor an interaction of sound × light treatment ($F_{1,75}$=0.001, P=0.970) (see Fig. 4i).

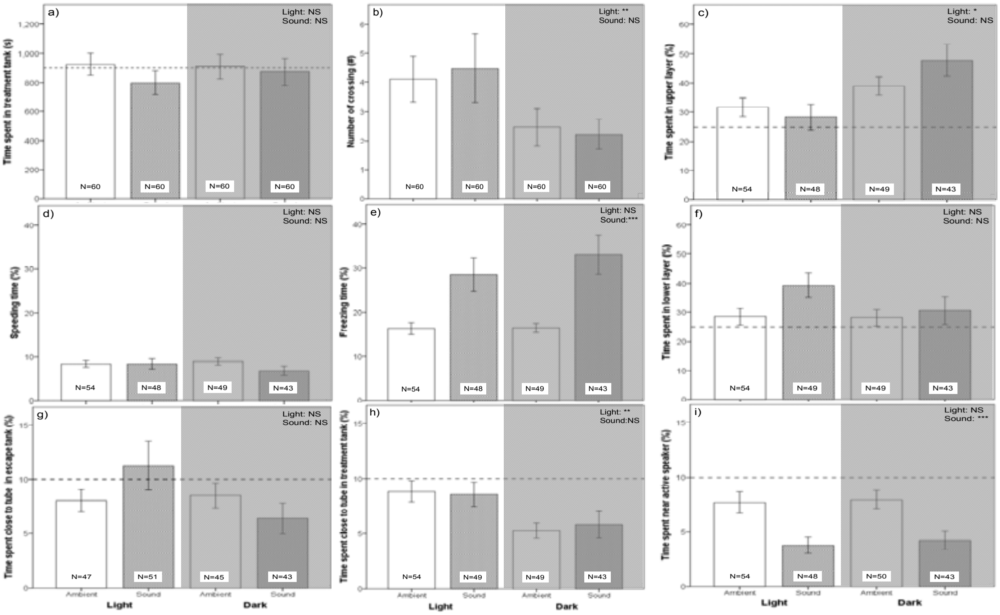

Fig. 4. Zebrafish swimming behaviour and spatial distribution changed differently by sound and light conditions among treatments. No effect of light and sound on time spent in the treatment tank (a), effect of light, but not sound on the number of crossings (b). There was an effect of light but not sound on time spent in upper layer (c), no effect of light and sound on speeding time (d), and an effect of sound but not light on freezing time (e). There was no effect of light or sound on time spent in the lower layer (f), an effect of sound bunt not light on time spent close to the tube in the escape tank (g), an effect of light but not sound on time spent close to the tube in the treatment tank (h) and an effect of sound but not light on time spent near the active speaker in treatment tank (see text for details on significance levels and statistical tests).

Discussion

We tested the effects of experimental sound and light exposure on zebrafish swimming behaviour in a dual-tank set-up. Both sound pressure and particle velocity revealed distinct sound levels in the treatment and escape tank and allowed us to test the effects of artificially elevated sound exposure under different light conditions. Firstly, we were able to confirm that these considerable sound level differences did not affect the overall time the zebrafish spent in the treatment tank. Furthermore, although dark conditions in the treatment tank reduced the crossing activity between tanks, it also did not result in a spatial bias to the dark or light tank. Secondly, the elevated sound levels clearly changed zebrafish behaviour when they were within the treatment tank; they increased freezing time and decreased the percentage of time spent near the active speaker. Dark conditions in the treatment tank also affected their behaviour and resulted in less time spent close to the tube and more time spent in the upper layer. Thirdly, we did not find any interaction effects of sound and light conditions on zebrafish behaviour.

Acoustic displacement in a fish tank

This is the second experiment in which we used our dual-tank set up to test the effect of experimental sound exposure on zebrafish spatial displacement and swimming behaviour. In the first experiment, we tested 8 groups of zebrafish (6 individual in each group) (Neo et al. 2015). In the current experiment, we tested 60 fish individually. Neither of these experiment showed a sound-dependent spatial distribution over the two tanks. We used decent sample sizes, the fish swam regularly through the crossing tube in both social and solitary conditions, and the sound conditions in the treatment tank were sufficiently loud to cause initial startle responses and significant behavioural effects that likely reflect anxiety (e.g.

proportion of freezing time). Nevertheless, we end up without any evidence for sound-related deterrence or avoidance of the noisy tank. Apparently, the experimental elevation in sound level is not sufficiently distressful to seek the exit of the noisy tank or turn around upon entry from the quiet tank. Alternatively, the fish may prefer quiet over noisy conditions, but they may be unable to detect the transition or gradient or lack the capacity to respond appropriately to express their acoustic preference. Another alternative explanation is that the noisy conditions are distressful and deterrent, but that the effect is only moderate and overruled by their explorative nature of zebrafish at least within the relatively short time-span of our experiment.

Although we did not find spatial preferences between tanks, we did find a significant spatial avoidance of the area right in front of the active speaker. This is in apparent contrast with one of our earlier studies (Shafiei Sabet et al. In Press) in which we compared the response to sound exposure of zebrafish with Lake Victoria cichlids (*Haplochromis piceatus*) while swimming in a single fish tank with an elongated shape (200-35-45 cm). Also in that study, we found startle and anxiety-related responses in both species, with zebrafish showing an initial rise in speed at the moment of sound on-set followed by an overall slow-down in swimming activity, while the cichlids just slowed down and lowered their swimming height during sound exposure (Shafiei Sabet et al. In Press). However, the playback from either the left or right end of the elongated fish tank did not yield any short- or long-term spatial displacements away from the sound source in the horizontal plane. The explanation for this discrepancy between the two studies in finding within-tank avoidance or not may be related to the sound fields in fish tanks of different lengths.

In general, we know that directional cues in sound fields are complex or completely absent in fish tanks (Parvulescu 1967; Popper & Fay 1993; Akamatsu et al. 2002), which is why we created the dual-tank set-up in the first place. We also argued for the elongated tank set-up specifically that the average level of sound pressure and particle velocity (independent of directionality) changed only slightly over the long end, except for an area in close proximity to the speaker (within 40 cm). As the fish in the elongated tank swam by far most of the time outside this close proximity area, we could not assess a potential impact of this steep sound gradient there. The length of the current treatment tank was much shorter: 75 cm for which the available swimming area was even more restricted to about 65 cm due to the underwater speaker on one side and the area shielded at the tube entrance side. As a consequence, the fish in the current experiment inherently swam much more within close proximity of the speaker, which may be the reason why we now found evidence for sound-dependent spatial avoidance for this restricted area when the speaker was active. Although there are several studies reporting phonotactic responses to playback of conspecific calls in fish tanks (Higgs et al. 2007; Rollo & Higgs 2008; Verzijden et al. 2010), we believe this is the first well-replicated study with evidence for a spatial deterrent effect for sound in a fish tank (also see Febrina et al. 2015).

Lack of light-related preference and interaction

We also did not find a light-dependent spatial tank preference in our dual-tank set-up. This may be surprising as the zebrafish did respond to light level variation in the treatment tank and the rise in the water column under dim conditions most likely reflected lower anxiety and lower perceived predation risk (c.f. Champagne et al. 2010; Maximino et al. 2010). The increase in time spent in the upper layer is reminiscent of natural cycles of vertical migration (see e.g. Rudstam & Magnuson 1985; Sogard & Olla 1993) and was likely also responsible for the decrease in time spent in front of and crossing through the tube into the light tank (although we have no explanation for the effect on reverse crossings that should have remained unaffected). Notably, results from an independent pilot study had suggested that groups of zebrafish did end up in larger numbers on the dark than on the light side of the dual-tank set-up (Neo & Slabbekoorn, unpublished data). However, also other studies have revealed variable outcomes for bright preferences in adult zebrafish. Gerlai et al. (2000) found for example a preference for brighter environments, while Serra et al. (1999) found a preference for darker environments. Stephenson et al. (2011) argued that the way of experimental manipulation (manipulation of light reflection by black or white tank walls or shielding light from above more or less) as well as variation among studies in relative light levels for the two choices of light conditions may explain the mixed results (also see Marchesan et al. 2005 for differences among species).

Although we have not shown any side preference yet, the dual tank set-up has been very successful in creating distinct spatial variation in the environmental conditions for two modalities, both independently and in concert. Creating distinct areas between which fish can freely move is easier for light levels than for sound levels. However, the current set-up is successful for both. This allowed us to show that both sound and light affect zebrafish behaviour in different ways and that there were no interactions. Light levels did not affect the nature and intensity of response patterns triggered by experimental sound exposure. Our detailed measurements now also indicated that there is some acoustic leakage from the treatment to the escape tank which varies spectrally and that leakage appears to be larger over a wider frequency range for particle velocity than for sound pressure. This does not affect our set-up dramatically, as differences between treatment and escape tank are still considerable in both sound components. However, it does indicate that sound pressure and particle motion may vary independently in complex environments, such as experimental fish tanks, but likely also in shallow water and in proximity of the natural complexity of e.g. rocky bottoms or canyon walls.

Conclusions

We were able to show that environmental conditions like sound and light levels affect fish in captivity. The freezing response and spatial avoidance of the area in close proximity to the active speaker indicated anxiety-related responses to sound exposure. Lower crossing activity and elevation in the water column in dim light conditions indicated no strong deterrent effect of bright light but a probable reduction in perceived risk during the dark conditions. The lack of sound or light dependent spatial distribution between the treatment and escape tank of the dual-tank set-up may be due to the moderate variation in sensation levels induced by the experimental manipulation or due to limitations of the set-up as a choice test. Nevertheless, we believe the dual-tank set-up has been successful in testing for independent effects and interactions for the two modalities in a well-replicated and balanced design. Although the behavioural response patterns in fish tanks may often be reminiscent of what fish would do in outdoor conditions (c.f. Neo et al. submitted), we argue that interactive effects remain a possibility and cannot be excluded for natural water bodies or for other species. Extrapolation to free-ranging fish in their natural habitat requires experimental sound exposure studies under night-time and day-time or artificially light conditions. We believe this would be a relevant exercise as many sound-generating human activities at sea or on the water, such as for example pile driving or seismic surveys, are not restricted to day-light hours.

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Chapter 5

Particle motion and sound pressure in fish tanks: a behavioural exploration of acoustic sensitivity in the zebrafish

This chapter is based on: James Campbell, Saeed Shafiei Sabet & Hans Slabbekoorn (in review). Particle motion and sound pressure in fish tanks: a behavioural exploration of acoustic sensitivity in the zebrafish. The Journal of Experimental Biology.

Abstract

There is a growing need to understand fundamental aspects of acoustic sensitivity of fish in both indoor and outdoor conditions. Many fish are kept in fish tanks for aquaculture, hobby, or for biomedical or behavioural research. These tanks can be noisy as surrounding sounds transmit easily into the water via concrete connections between the floor and the tank walls. Fish in natural water bodies are also exposed to elevated levels of anthropogenic noise at an increasing scale worldwide. Underwater sound fields can be complex, especially in fish tanks and in shallow waters, close to surface, rock or bottom. Furthermore, fish are sensitive to both particle motion and sound pressure. We here measured 1) spatial variation in artificially elevated sound levels in a relatively small fish tank, for both particle motion and sound pressure. We confirmed considerable variation over a dynamic range of 25 dB for both components and upward shifts in this range of about 10 dB when close to the tank walls or the bottom and downward shifts of about 10 dB when close to the surface. We also tested 2) whether acoustic response tendency of adult zebrafish (*Danio rerio*) correlated to the sound field conditions at their position at the moment of sound on-set. We found no correlation between the intensity, quality, or directionality of the behavioural response and the sound pressure or directivity and elipticity of particle motion. There was a negative correlation, however, between the tendency to freeze and the average particle velocity level. We suggest that our data provide a basis to further explore the acoustic world of fish in complex environments and may contribute to the study of potential welfare and conservation issues related to anthropogenic noise.

Keywords: captive behavior; experimental exposure; fish welfare; noise impact; sound measurement

Introduction

Ship traffic, wind turbines, pile driving, and seismic exploration can represent a significant component of the underwater soundscapes worldwide. As all fish are capable of detecting sound, acoustic signals and environmental cues play an important role for many fish species in the context of reproduction, orientation and predator-prey interactions (Popper & Fay 1993; Slabbekoorn et al. 2010). The sound characteristics of human activities are typically broadband, more or less temporally structured, and biased towards relatively low frequencies. There is often high structural similarity with biologically relevant sounds and large spectral overlap with the auditory sensitivity of fish. As anthropogenic sounds can be loud and propagate well through water, there is a growing concern about potentially detrimental effects and an increasing awareness about a general gap in fundamental insights about the acoustic world of fish.

To examine the acoustic world of fish and to gain understanding about the potential effects of anthropogenic noise on fishes, both outdoor and indoor experiments are employed. While outdoor experiments provide a high degree of behavioral and acoustic validity, they can be challenging to implement and have a low degree of controllability. Contrastingly, indoor experiments provide a high degree of control but suffer from a lack of acoustic and behavioral validity when compared to open water conditions (Slabbekoorn 2016). While the acoustic differences between natural water bodies and relatively small tanks have been widely acknowledged (Kaatz & Lobel 2001; Parvulescu 1967), there remains a paucity of literature examining these differences from an empirical perspective (Akamatsu et al. 2002; Kaatz & Lobel 2001). Many fish spend time in shallow waters or in close proximity to surface, rock, or bottom boundaries, where the sound fields are more complex than in far field, open water conditions. Furthermore, captive fish just experience artificial sound fields in fish tanks that can be unintentionally or experimentally noisy.

Fish can hear both the pressure and particle motion components of acoustic waves. All fish are able to detect acoustic particle motion using a specialized structure called the otolith organ within the inner ear, which is able to extract frequency and amplitude information from oscillating motions, analogous to an accelerometer (Fay 1984). Fishes possessing a swim bladder are also able to detect the pressure component of sound through pressure-to-motion conversion via the air-filled cavity of the swim bladder, which expands and contracts in response to pressure changes (Popper & Fay 2011). Specialized adaptations like the Weberian apparatus in Ostariophysians can further enhance the acoustic sensitivity to sound pressure by acting as an efficient conduit for kinetic energy between the swim bladder and the inner ear. These specialized adaptations can increase both the frequency range and absolute hearing thresholds (e.g. Schulz-Mirbach et al. 2012).

Many studies have assessed hearing thresholds and acoustic response tendencies in fish (Popper & Fay 1973; Horodysky et al. 2008). Many of these studies are done in laboratory facilities and with the fish close to the surface in a small tank which complicates the interpretation and comparison of results. It is therefore wise to treat absolute acoustic measures from such studies as study-specific and not as general truth. However, relative sensitivity information across the spectrum should also be treated with care, as this involves the outcome of overlapping ranges of perception through both particle motion and pressure, for which the sound field conditions are highly variable with dynamic ratios between the two components under typical indoor fish tank conditions (Parvulescu 1967; Rogers & Cox 1988). Some studies have compared fish hearing thresholds for particle motion and pressure by isolating these acoustic components within the experimental setup, exposing fish to acoustic signals comprised exclusively of either particle motion or pressure (Bretschneider et al. 2013; Wysocki et al. 2009). Although these studies revealed some more advanced insights into fish auditory perception, there remains especially little knowledge regarding how fish react behaviourally when exposed to variable ratios of the two components.

Although many fish do not reside in far field, open water sound conditions, this is still a useful reference for exploring more complex sound fields. In far field, open water conditions, a propagating sound shares a fixed relationship between its sound pressure and particle motion components, thus the predicted far-field particle velocity (PFV) for a given sound pressure measurement is calculated using Eqn 1:

$$PFV = \left(\frac{rms(p_{measured})}{c \cdot \rho}\right), \qquad (1)$$

where $rms(p_{measured})$ is the root mean square of the measured sound pressure over time (µPa), c is the speed of sound in water (1482 m/s), ρ is the density of water (1027 kg/m^3), and the resulting PFV is returned in µm/s.

While the relationship between sound pressure and particle motion under these conditions is generally constant, most small tank experiments are conducted in the acoustic near field due to the low frequencies of interest and relatively small dimensions of the tanks used. In the near field, sound radiates in a spherical pattern, resulting in relatively higher levels of particle motion closer to the sound source (Bretschneider et al. 2013), as compared to far-field conditions.

A critical parameter of the sound field to understand behavioural response patterns is the directionality of the particle motion (Schuijf 1975; Van den Berg & Schuijff 1985; Popper & Fay 1993; Rollo & Higgs 2008). In a boundless far field environment with a single sound source, the directionality is observed as a one-dimensional oscillation of particles along the axis parallel to the direction of the propagating wave. However, under spatially restricted conditions such as small tanks, fish are continuously exposed to reflected sound waves. When two sound waves arriving from different directions propagate through a common point, the particle motion components of both waves will combine following the rules of vector addition. Additionally, the phase difference resulting from the latency in travel times between the incident and reflected waves can cause a two- or three-dimensional oscillation of particles which can be characterized by particle ellipticity.

Current models of fish hearing are based on the assumption that fish determine the direction of sound propagation through acoustically induced otolith motion along the axis of the acoustic wave (Rollo & Higgs 2008). As points in an acoustic field with high particle ellipticity will result in otolith motion that deviates from a single axis of displacement, this suggests that particle ellipticity may undermine or contribute to the ability of fish to localize sounds by convoluting the directional component of otolith motion. To our knowledge, there is currently no literature describing particle ellipticity within the context of sound source localization by fish.

In this study, we conducted two experiments in relatively small tanks: one in which we measured particle motion and sound pressure levels to explore the relationship between the two sound components and a second to explore the potential relevance to fish. The first experiment examined how the ratio of sound pressure to particle motion in a small tank varies in response to the spatial location within the tank, as compared to theoretical open-water conditions. In the second experiment, we further examined the sound pressure and particle motion components within the context of an acoustically induced behavioural response experiment using zebrafish (*Danio rerio*). We compared the 1) occurrence, 2) intensity, and 3) direction of acoustically elicited startle/fleeing responses for individual fish to the predicted sound pressure and particle motion conditions they would have experienced at their location during the on-set of sound exposure.

Methods

Experiment 1

Experimental Setup

The experimental tank used in the present study was constructed from glass and had the following dimensions: 100 x 50 x 50cm, a wall thickness of 0.75cm, and a water depth of 40cm. The tank was positioned on a table on top of ~4cm of acoustic insulating material to reduce acoustic artifacts caused by building vibrations. Within the tank, the acoustic field was measured along a three-dimensional grid at 10cm increments using a custom-built vector sensor (c.f. Bretschneider 2013; Shafiei Sabet et al. 2015). The vector sensor was positioned along this grid using two perpendicularly oriented red lasers (λ = 635nm in air). The Perspex sphere containing the three accelerometers was hanging in the water by two nylon wires that allowed position control due to the slightly negative buoyancy of the sphere. This system allowed us to position the vector sensor within a

~1cm range of accuracy. All measured positions in this grid were at least 10cm away from the tank walls.

The tank was ensonified using a JBL EON500 in-air speaker (USA, Maximum volume, Equalizer: Boost) connected to a DR-05 handheld recorder (Tascam, USA) at a distance of 1.5m with the speaker facing the center of one of the two widest walls of the tank. During each acoustic measurement, the experimental tank was ensonified with 10 seconds of white noise. The white noise playback track was artificially generated in Audacity (http://audacityteam.org/, version 2.0.5) and a bandpass filter was applied between the frequency ranges of 100-1000 Hz. The playback volume of the in-air speaker was adjusted so that a sound pressure level (SPL) of 112dB (re 1 μPa) was measured in the center of the tank with a calibrated HTI 96-min hydrophone (High Tech, USA) connected to a DR-100MKII recorder (Tascam, USA).

In addition, a supplementary set of measurements was taken to investigate the effect of changing speaker volume where the vector sensor was placed in the vertical center of the tank, 14cm away from the wall closest to the speaker. The tank was then ensonified with the same white noise exposure 21 consecutive times, with each exposure digitally set to be 2dB quieter than the previous.

Acoustic Measurements

All sound pressure and particle motion measurements were recorded with the custom-built vector sensor and amplifier that was previously used in studies by Bretschneider et al. (2013) and Shafiei Sabet et al. (2015). This was then connected to a Picoscope 3425 USB Oscilloscope (Pico Technology, England & Wales) and data was logged from the oscilloscope using a program written in Visual Basic for Applications within Microsoft Access 2010 (Microsoft, USA).

The vector sensor was calibrated in reference to a pre-calibrated M20 directional hydrophone (Geospectrum Technologies Inc., Canada). The calibration was conducted by suspending the M20 directional hydrophone in the center of the large tank and ensonifying the tank from an in-air speaker 1.5 m away. The M20 directional hydrophone was then replaced by the custom-built vector sensor and the exposure was repeated. By comparing the resulting measurements from the two devices in the frequency domain, we were able to construct a receiver sensitivity graph for each channel of the custom-built vector sensor. As the acoustic environment in the experimental tank is prone to artifacts and the differing size of the sensors results in unequal sampling areas, a degree of inaccuracy is to be expected from this calibration method. Frequency ranges within the resulting receiver sensitivity graph that appeared to be inconsistent over repeated calibrations were discarded, resulting in a final calibrated range of 50-1000Hz.

Acoustic Analysis

All audio analyses were conducted using Matlab (Mathworks, USA, Version 8.1) with a bandpass filter applied between 100-1000Hz (the calibrated range of our vector sensor) and following the standardized definitions for each measurement as seen in Ainslie (2011), unless otherwise specified. Particle velocity measurements were reported as sound velocity level (SVL), and are defined according to Eqn 2:

$$SVL = 20 \cdot log_{10}\left(\frac{rms(u_{measured})}{u_{reference}}\right) \text{ dB}$$

(2)

where $rms(u_{measured})$ is the measured root mean square of the particle velocity over time and $u_{reference}$ is the reference particle velocity (1nm/s).

To compare SVL and SPL measurements in a context relevant to open water experiments, we examined the excess SVL. This measurement was calculated by subtracting the expected SVL under far field, open water conditions from the measured SVL in the tank as shown in Eqn 3:

$$Excess\ SVL = 20 \cdot log_{10}\left(\frac{rms(u_{measured})}{PFV}\right) \text{ dB.}$$

(3)

Under far-field open water conditions, SPL is expected to show no relationship with excess SVL, and as a result, excess SVL measurements taken in these conditions would be expected to be 0dB. Excess SVL measurements taken close to a sound source are expected to be higher than those taken further away due to near field effects of spherical sound propagation.

Statistical Analysis

All statistical analysis were carried out in R (version 3.2.2, including the packages: ggplot2, nlme, lme4, MASS, CircStats). We examined the relationship between the spatial parameters (i.e. the position of the vector sensor in the tank) of each acoustic measurement and the resulting SPL and SVL values in the experimental tank using Generalized Linear Models assuming a Gaussian error distribution. The selection of variables used in each model was determined by AIC stepwise selection (both directions). The spatial variables included in the model selection were the continuous variables: distance from the tank wall closest to the in-air speaker, distance from the closest tank wall facing the direction adjacent to sound propagation (including the second degree orthogonal polynomial), distance from the bottom of the tank and the binomial variables: close to tank bottom or water surface and close to either wall facing the direction of sound propagation. A visual examination of the residual plots for each model indicated that there were no significant deviations from the assumptions of normally distributed residuals.

For examining the relationship between Excess SVL and the spatial variables, we again used a Generalized Linear Model with assumed Gaussian error distribution. The variables used for the model selection are the same as used in the SVL/SPL comparison, except for the addition of SPL as a fixed effect and the use of Excess SVL as the responding variable.

Experiment 2

Experimental Setup

The behavioural response experiment was conducted in the same in-air speaker tank setup as in experiment 1, with the exceptions that the speaker was placed 1m away, instead of 1.5m, and a restricted swimming area measuring 24cm x 10cm x 10 cm was placed within the glass tank to constrain the fish to a small area where we had measured highly variable particle motion to sound pressure ratios (Fig. 1).

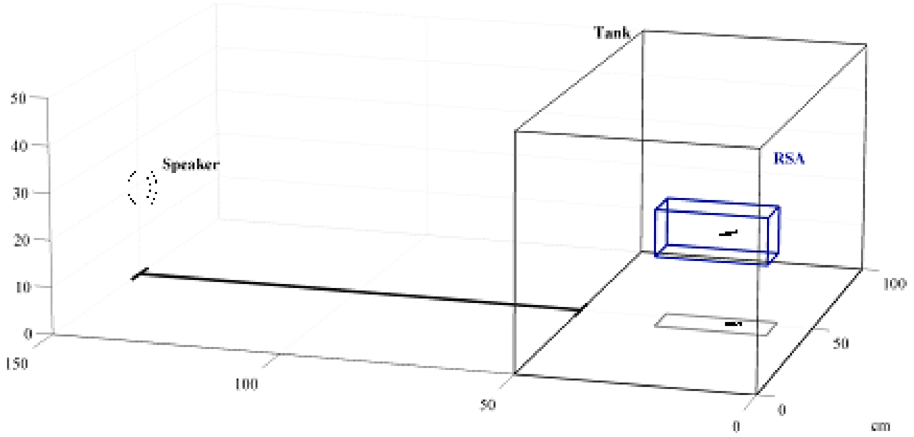


Fig. 1. Scaled 3D image of the setup used in the behavioural response study of Experiment 2. The acoustically and visually transparent restricted swimming area is labeled “RSA” and highlighted in blue.

The restricted swimming area was constructed from a rectangular iron frame with walls made of plastic wrap. Plastic wrap was chosen because of its visual and acoustic transparency. During the pilot trial, a comparison of measurements taken in the same positions both with and without the restricted swimming area surrounding the sensor resulted in no observable difference in SPL or SVL measurements. Two HC-V500 video cameras (Panasonic, Japan) set to record at 50 fps (interlaced) were placed above and to the side of the tank to obtain a dorsal and lateral view of the startles and distinct fast start responses Mirjany et al. 2011; Domenici & Blake 1997). The volume level of the DR-05 handheld recorder attached to the EOS500 loudspeaker (Maximum volume, Equalizer: Flat) was adjusted in this behavioural experiment to achieve a SPL of 120dB in the center of the tank. Playback tracks used in this experiment consisted of a one hour period of silence followed by 10 one-second pulses (white noise, 10-2000Hz) randomly distributed over a three hour period. The random placement of the pulse noises was determined by dividing the 3 hour trial period into 10 segments of 18 minutes. A pulse was then played at a randomly selected minute within each 18 minute segment.

Once the water was warmed to at least 22°C, the trials began by placing an individual into the restricted swimming area within the large tank and the playback track was started after the video cameras had begun recording. The start and end temperatures were recorded for 12 of the 14 trials and tank heaters were removed during the trials. Temperatures ranged from 22.5-24°C upon the start of each trial and the maximum drop in temperature by the end of a trial was 1.5°C. In addition, the room hosting the experiment had no windows, thus lighting conditions could be kept consistent throughout all the trials. A LUNASIX F light meter (P. Gossen & co, Erlangen, Germany) was used to measure the experimental light conditions by placing the light meter 5 cm above the water surface in the horizontal center of the tank, resulting in a light illuminance of 1290 lux. Upon the start of the playback track, we left the room and did not return until after the 4 hour trial period had ended. Because of moderate but regular background noise and vibrations due to nearby building maintenance during the morning and early afternoon, all trials were initiated between 15:45-16:40 and we only conducted one complete trial per day (one fish per day).

Behavioural Analyses

Each trial had a unique timing pattern for sound exposures and we assessed the spatial position of the fish at each pulse moment in the trial sequence. For each sound exposure, one minute of video before and after the onset of each pulse was extracted for analysis and converted to a Motion-JPEG video format (50 frames per seconds, progressive scan) using FFmpeg (https://www.ffmpeg.org/, version 2.4). Location tracking of the individuals was then conducted in Matlab using a background subtraction algorithm based on brightness values. We reviewed all video analysis data and we manually corrected tracking errors. We combined the information from the dorsal and lateral cameras to provide three-dimensional locational data for all sound exposures.

We used the video recordings to score behavioural states related to swimming speed: startle and fast start onset and freezing. The presence of distinct startles and onset of the fast start responses were defined by any sudden quick movement which followed the first and second stage motions associated with fast start responses in zebrafish (Mirjany et al. 2011). Freezing was defined by the lack of swimming activity or interruption of all activities except breathing (c.f. Shafiei Sabet et al. 2016). We scanned for fast start responses within 100 frames (2 seconds) before and after the onset of the sound exposure. In circumstances where a fast start response was suspected but not obvious to the observer, these were treated as expressing no fast start response. The sound conditions of each potential response were determined independently and after behavioural assessments and the scoring by the observer can thus be regarded blind to the treatment.

To collect more precise directional information during the startle response, the midline of the individual was traced by hand over a period of 1 second before and after the startle response. The midline was defined as a straight line drawn from the snout of the fish to the midpoint between the pectoral fins (Mirjany et al. 2011). Because of the low temporal resolution of the video footage and the relative quickness of startle reposes, the midlines could not be quantified accurately in three-dimensional space. Consequently, only the camera positioned above the tank was used to analyze the directional component of the startle responses.

Quantifying the Acoustic Field at Startle Response Locations

The acoustic field in the restricted swimming area was measured with the same calibrated vector sensor as used in experiment 1. The area enclosed by the restricted swimming cage was measured following a two-dimensional grid along 5cm increments at the center depth of the restricted swimming area (20cm). To predict the sound field characteristics of SPL, SVL, and the direction of particle motion at the exact locations of the startle responses, the grid data function in Matlab was used to conduct two dimensional linear interpolation on the measured acoustic field values (See Fig 4). Due to the flexible nature of the plastic wrap walls and the small degree of error in the video tracking, when the fish was close to the walls of the restricted swimming area during the onset of noise exposure, some interpolation points resided outside of the measured sound field and could not be interpolated. These points were excluded from the analysis.

To calculate particle ellipticity, the paired measurements of particle velocities for the X and Y channels of the vector sensor were plotted in a bivariate histogram (Fig. 2). A convex hull was then drawn around all values which were >25% of the maximum frequency in the histogram. Particle ellipticity was then calculated by comparing the length of the major axis of the convex hull to its adjacent axis using Eqn 4:

$$Particle\ Ellipticity = arctan(\frac{l_{minor}}{l_{major}}) \cdot (\frac{180}{\pi}), \qquad (4)$$

where l_{minor} and l_{major} are the lengths of the major and adjacent axes of the convex hull, respectively, and the particle ellipticity is returned in degrees. Linear interpolation was again used to predict the particle ellipticity values at the exact location of the fish during the onset of noise exposure.

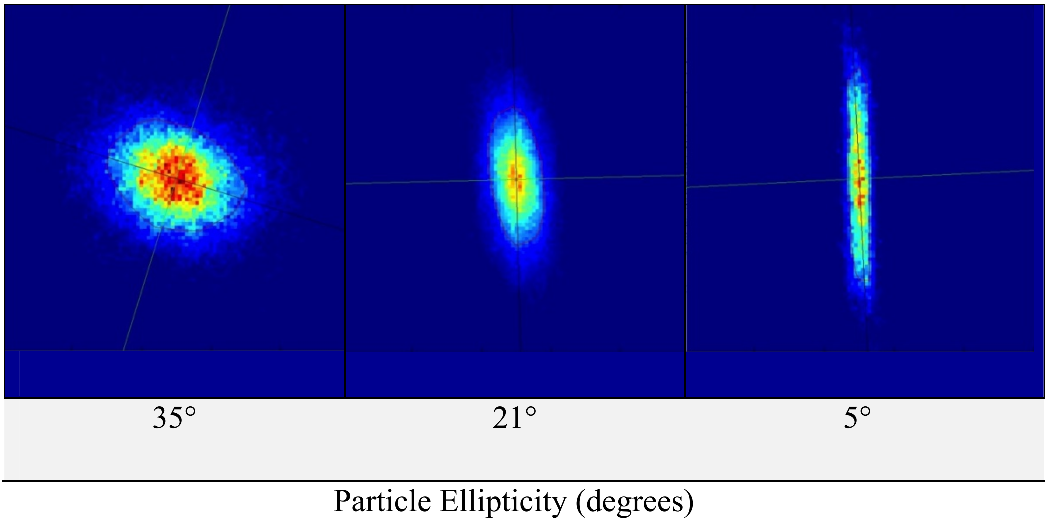


Fig. 2. Bivariate histograms of the instantaneous particle velocity along the x and y channels of the vector sensor over a period of 4 seconds during playback of white noise, band-pass filtered between 50-1000Hz. The center of each image is 0m/s for each channel and the particle ellipticity is reported in degrees. The black line represents the major axis of particle velocity while the green line represents the axis perpendicular to this major axis. A value of 45° indicates perfectly circular particle motion (the particle velocity measured along the major and adjacent axes are equal), while smaller values represent increasingly linear particle velocity.

Statistical Analysis

The effect of sound field components on the intensity of startle responses was examined with a Linear Mixed Effects Model (maximum Likelihood method) with a Gaussian Error distribution to predict the post-exposure average swimming speed and a Generalized Linear Effects Model with a Binomial error distribution to predict the probability of a freezing response within 50 seconds after the exposure. A visual check of residual plots was used to confirm that the assumptions of normally distributed residuals were met. In both models, the individual was defined as the random effect (random intercept) and the average swimming speed was calculated over a period of 10 seconds before and after the onset of noise exposure.

We determined the inclusion of the following fixed effects by AIC-score: SVL at the fish's location during the onset of noise exposure, SPL at the fish's location during the onset of noise exposure, and the average swimming speed before the onset of noise exposure. A linear regression analysis was used to explore collinearity between the paired SVL and SPL estimates, but the relationship was not significant. The fixed effect expression of freezing behavior before the onset of noise exposure was also included in model construction to distinguish between cases in which the fish was swimming normally prior to the sound exposure and then froze in response to it, as opposed to a false detection when the fish was already frozen before the exposure and remained frozen during and after the exposure.

Predicted SVL and SPL values at the individual's location during the onset of noise exposure were also compared to the occurrence of startle responses and the change in post-exposure swimming speed, but no correlations were evident. The final mixed effects models only included exposures that resulted in visible startle responses and the marginal and conditional R^2 values for each model were calculated according to

Nakagawa & Schielzeth (2013), where the marginal R^2 represents the variance explained exclusively by the fixed effects and the conditional R^2 represents the variance explained by both the fixed and random effects.

Circular statistics were employed to examine if there was a directional response related to the sound-field properties during the startle responses. The direction of escape during the fast start response over the temporal scales of 1,2,3,4, and 5 frames (Each frame is spaced 20ms apart) after an observed response was compared to the direction of particle motion analyzed over the bandwidths of 50-150Hz, 150-250Hz, 350-450Hz, and 750-850Hz. Because the mechanism which fish use to determine the acoustic directionality of particle motion is poorly understood, we treated the direction of escape as a diametrically bimodal distribution in which a value of 0 radians represents the fish swimming in either direction parallel to that of acoustic particle motion and a value of π radians as a direction perpendicular to that of particle motion.

Ethical approval

A total of 15 zebrafish were used in the experiment, one of which was exclusively used for a pilot trial and excluded from the final dataset. All experiments were performed in accordance with the Netherlands Experiments on Animals Act (DEC approval no: 13022) that serves as the implementation of the Directive 86/609/EEC by the Council of the

European Communities regarding the protection of animals used for experimental and other scientific purposes (1986).

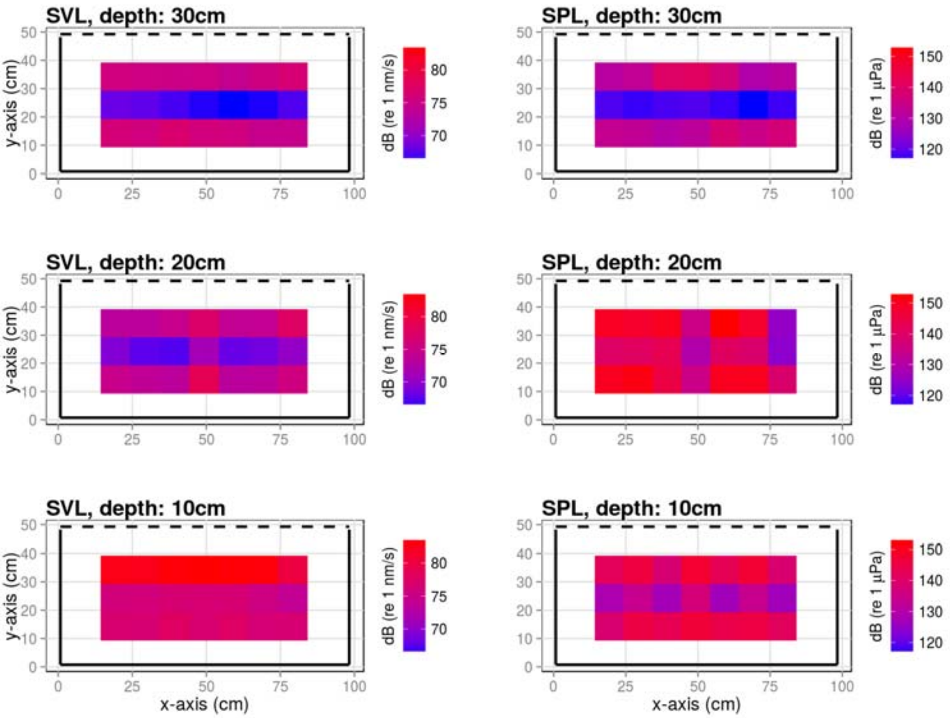
Results

Experiment 1

SVL and SPL

The SVL and SPL components of the measured sound field followed generally similar trends within the tank (Fig. 3). Both varied considerably over a dynamic range up to 15dB for SVL and 25dB for SPL and at any particular distance from the wall nearest the speaker or at any particular depth. Most notably, the sound level ranges in the center of the tank were shifted down approximately 5dB for SVL and 10dB for SPL, as compared to locations close to both tank walls. Similarly, for sound pressure the sound level range was lower for measurements close to the surface relative to in the middle and at the bottom of the water column. SVL ranges were highest at the bottom relative to both the middle and at the top of the water column. There were no significant interaction effects in the SPL model, but we found a highly significant interaction effect in the SVL model between the distance from the wall closest to the in-air speaker and the distance from the bottom of the tank ($T_{53} = -6.98$, $P = 4.86e-9$).

Trends in excess SVL measurements relative to the spatial positions within the tanks were generally similar to those observed in the SVL and SPL measurements, as the excess SVL is calculated from both SVL and SPL. In addition, SPL showed a highly significant negative correlation with excess SVL (Table 1). A supplementary set of measurements taken while the vector sensor was stationary, and the volume of the playback track was adjusted support these results (Fig. S1). Observed Excess SVL values ranged from -15.1 to 16.2 dB across all sampling positions.



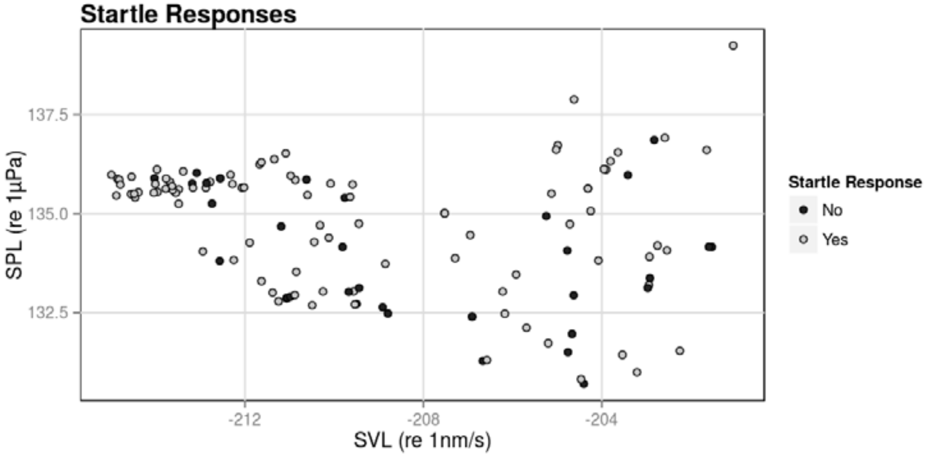


Fig. 3. Spatial variation in sound field conditions in the experimental fish tank. Rasters of the SVL and SPL measurements reflect sound field variation throughout the tank at a 10cm resolution. Black lines represent the four side walls of the tank, with the dotted line representing the wall closest to the in-air speaker.

Fig. 4. Occurrence of distinct startle and/or fast start swimming response (grey dots) and lack of any visible response (black dots) for fish in the restricted swimming area at locations with variable interpolated SVL (dB re 1 (nm/s) and SPL (dB re 1 µPa) measurements, as indicated on the x-axis and y-axis respectively. At higher SVL there is higher variability in associated SPL, but both modalities seem to vary more or less independently. There is no correlation between whether or not there is a startle response and either SVL or SPL measurements.

Experiment 2

The mixed effects model predicting post-exposure swimming speed revealed that the pre-exposure swimming speed, pre-exposure freezing behavior, and exposure number were significantly correlated with a decrease in the change of swimming speed, although a majority of the explained variance was accounted for by the random effect of the individual ($R^2_c - R^2_m = 0.28$). SPL and SVL were not significantly correlated with a change in swimming speed. The analysis results are summarized in table and illustrated in Fig. 5.

The mixed effects model predicting the probability of a freezing response within 50 seconds after noise exposure revealed that higher SVL measurements resulted in a lower probability of a post-exposure freezing response, while SPL showed no relationship. In addition, the average pre-exposure swimming speed was also negatively correlated with the probability of a freeze response. A majority of the variance was accounted for by the random effect of the individual ($R^2_c - R^2_m = 0.47$). The analysis results are summarized in table and illustrated in Fig. 5.

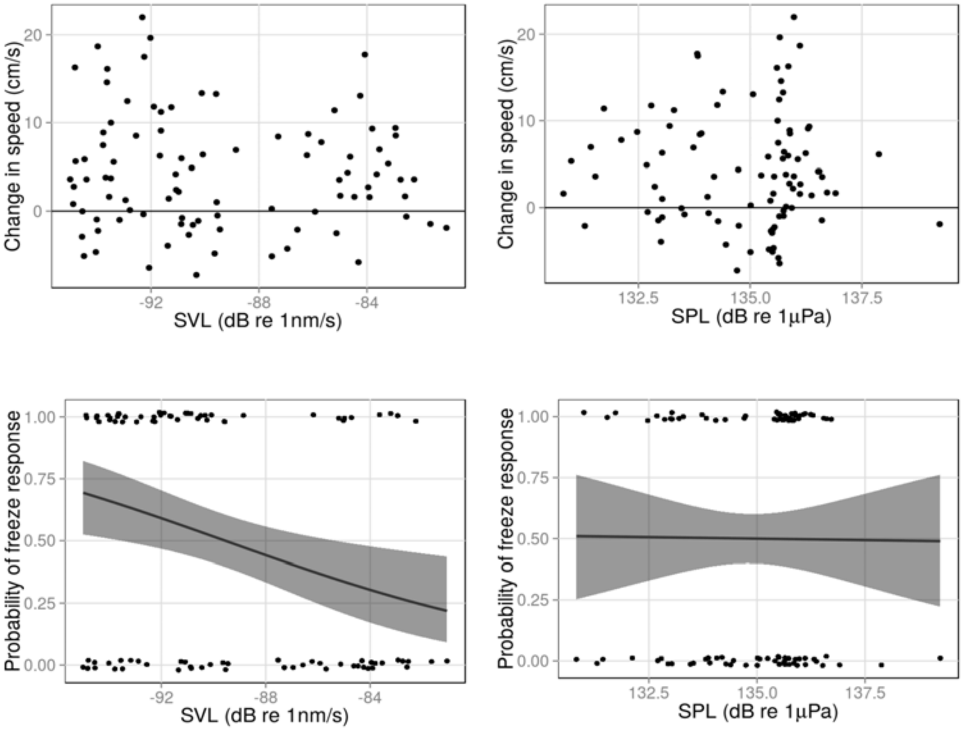


Fig. 5. Interpolated SVL (dB re 1 (nm/s) and SPL (dB re 1 μPa) values at the fast start response locations across all individuals compared to the change in swimming speed averaged over 10 seconds before and after noise exposure (top) and the probability of a freeze response within the 50 seconds after noise exposure (bottom). Y-axis variability has been added to the points on the bottom plots in addition to a LOESS curve with 95% confidence interval as a visual aid. Mixed effects models revealed that the probability of a freeze response was negatively correlated with SVL (bottom left).

Rayleigh's test (mean direction alternate hypothesis) and Watsons test of uniformity showed that the direction of escape was not significantly different than that of a uniform circular distribution, except in the temporal range of 5 frames after the first observed startle motion and over a bandwidth of 750-850Hz (Rayleigh's test: mean resultant length = 0.044, p-value = 0.011; Watsons test: $U^2 = 0.182$, p-value < 0.1). A one-tailed binomial test was then done on the non-uniform distribution to determine that there was a significant preference to escape in a direction parallel to that of particle motion ($X^2 = 2.769$, p-value = 0.048). A Watson's two-sample test was further used to check if the resulting distribution fitted a von Mises distribution, but the results were not significant.

Discussion

Our results provide new insights into the sound field complexity of relatively small fish tanks and into the challenging exploration of the link between sound field parameters and fish behaviour. In experiment 1, we showed that the SVL and SPL components of the sound fields within the experimental tank followed generally similar trends with relatively high SVL and SPL close to tank walls and relatively low SVL and SPL close to the surface. Furthermore, the excess SVL deviated well above and below open water, far field conditions, revealing considerable variation throughout the fish tank between SVL and SPL measurements taken at the same position. In experiment 2, we found a similar, highly variable pattern of acoustic measurements at spatial locations of zebrafish in the restricted swimming area, including SVL, SPL, but also sound velocity direction and ellipticity. We also found a general lack of correlations between acoustic and behavioural measurements such as speed and direction of swimming response. However, locations with higher SVL values during noise exposure were correlated with a lower probability of a post-exposure freezing response.

Fish tank acoustics

Our acoustic measurements confirmed that SPL, SVL, and excess SVL in small tanks are highly variable across spatial locations. Both, absolute levels and spatial and temporal variability stray from the theoretical values that are expected to be experienced by fish swimming in open water, far field conditions. Consequently, indoor sound field assessments and behavioural response studies can be valuable to gain fundamental understanding about underwater acoustics and insights into housing conditions of fish in captivity, but they are unlikely to shed much light on free-ranging fish in outdoor conditions. Nevertheless, as mentioned before, many fish occur in natural habitat with more complex sound fields than open water, far-field conditions. Indoor insights can therefore turn out valuable for future explorations of sound impact on fish in shallow waters, close to surface, rock or bottom.

We believe our measurements reveal several interesting findings, some of which expected and others not fully understood yet. The relatively low levels of SPL observed close to the water surface in our tank are in line with expected sound pressure release characteristics of the water-air boundary. However, we also expected relatively high levels of particle motion at the surface and that is not reflected by our measurements. This discrepancy may be caused by additive effects from the four walls and bottom as secondary sound source and the resultant patterns of reflected waves.

We also observed higher SVL and SPL values closer to the bottom and closer to either tank wall, largely independent of the speaker side. This suggests that the whole tank acts as a vibrating rigid body in response to in-air sound waves. This is not surprising as for an acoustic wave to pass from the outside air to the water within the tank, the tank walls must vibrate to transmit the acoustic energy between the two mediums. The vibrations are likely conducted among adjacent tank walls, resulting in the entire tank serving as a secondary sound source. Consequently, from the perspective of a fish within the tank, the sound field is not likely to carry much information about the location of the in-air speaker as the primary sound source.

We did find some acoustic variation in the water along the axis of sound propagation in air. Measurements taken close to both the bottom of the tank and the wall closest to the in air speaker resulted in higher SVL measurements and a significant interaction effect between horizontal and vertical variation. As this interaction effect is only visible very close to the fish tank boundaries and absent for SPL, we expect it may result from the differing area size of sampling between the hydrophone (~1-2 cm diameter) and the geophones mounted within our vector sensor (9.5cm diameter). Due to this size difference, the particle motion component of the vector sensor is sampling about 3.5 cm closer to any given sound source across all locations as compared to the paired samples from the hydrophone.

Exploring acoustic sensitivity of fish

We found our captive zebrafish to respond to sudden sound bursts of moderate levels, like in earlier experiments (Neo et al. 2014; Shafiei Sabet et al. 2015). We succeeded in triggering behavioural responses in many but not in all cases with a variety in SVL and SPL levels and variable combinations thereof. Despite reaching these experimental targets for an optimal test of whether response tendency and intensity are related to particular parameters of the local sound field, we did not find clear correlations between sound parameters and our expected behavioural response patterns.

We did, however, find one significant correlation between sound and behaviour: the probability of a freezing response was negatively correlated with the SVL at the fish's location during sound exposure. However, we believe this is in contrast with any logical expectation. Freezing responses, in concert with thrashing and erratic swimming, has been shown to be a reliable indicator of anxiety in the context of, for example, light conditions or perceived predation risk (e.g. Blaser et al. 2010; Bass & Gerlai 2008; Cachat et al. 2010) and has also been scored as such in earlier sound impact studies with this species (Shafiei Sabet et al. 2016). Consequently, if SVL was perceptually the most prominent of all sound field features and responsible for a correlation via a causal relationship, one would expect a positive correlation: higher levels triggering more freezing.

Although we are not convinced about the causal relationship of SVL and behavioural response tendency in our current study, we do see this finding as a confirmation that our set-up could work. Quantifying additional behavioral metrics, like thrashing and erratic swimming, may provide additional insights about the nature and potential for underlying physiological impact of fast swimming or freezing responses (Bass & Gerlai 2008). Integrating detailed sound field characterization and detailed behavioural assessments of free-swimming fish may yield specific correlations that indicate perceptual prominence for one among multiple audible sound parameters. This appears still quite a challenge, but also perceptual weighting studies on acoustic parameters of song in birds have only become possible after many years of methodological progress in different laboratories (e.g. Dooling & Okanoya 1995; Beckers et al. 2003; Pohl et al. 2012).

Methodological potential and problems

As we hope that our study will stimulate follow-up, we here address some methodological potential and problems in our set-up. First of all, we see potential in our approach with a restricted swimming area to keep the experimental fish in a specific part of an indoor fish tank where variation of particle motion and sound pressure levels are measurable and within certain limits. It should, however, be noted that swimming restrictions, in captivity in general and for further spatial restrictions in particular, also limit natural behavioural response patterns (Calisi & Bentley 2009; Slabbekoorn 2016; Neo et al. In Press). Our analysis of the swimming direction of startle responses, for example, yielded no relationship with the direction of the SVL component of the playback sound, except when examining the fish's location at 100ms after the startle response over a bandwidth of 750-850Hz. This result is inconclusive but potentially due to the small and rectangular shape of our experimental area: fish may have preferred to escape in the direction with the largest free area for movement which would cause a bias in escape directions (also see Shafiei Sabet et al. 2016).

As a second point, we like to draw attention to the potential for using stimuli of variable frequency to study fundamental aspects of hearing. Zebrafish are most sensitive to sound of frequencies around 800 Hz, but are likely to hear well above 1000 Hz, up to 4000 Hz (Higgs et al. 2002; Bretschneider et al. 2013). Furthermore, relative sensitivities for particle motion and sound pressure are likely to complement each other, but vary spectrally with a bias to the low end for particle motion and to the high end for sound pressure (Schulz-Mirbach et al. 2012). Future, tests could explore whether sound bursts restricted to relatively low (< 500 Hz) or relatively high (> 1000 Hz) frequencies in the audible range of zebrafish yield differential response patterns with respect to weighting of SVL and SPL. However, it should be noted that in the current study we had calibration limitations with our vector sensor that would have to be solved, as we were only able to assess particle motion levels within a range of 50-1000Hz.

As a final point, we like to highlight the phenomenon of particle ellipticity as a potentially relevant acoustic feature for auditory perception and sound-induced disturbance and deterrence in fish. The predicted levels of ellipticity at the locations of startle responses in our experimental set-up were highly variable, dependent on both spatial location and frequency range. Although the mechanism for determining directionality is not well understood in any fish species, the capacity for fish to localize a sound source based on the particle motion component of sound fields was recently nicely illustrated by a study on female midshipman fish (*Porichthys notatus*) approaching a speaker playing back a conspecific male call (Zeddies et al. 2012). We expect that higher degrees of particle ellipticity will diminish a fish's ability to localize sound sources (c.f. Rollo & Higgs 2008), thus reporting measures of particle ellipticity and incorporating them into statistical analysis may be valuable for future studies.

Conclusions

Our findings highlight the importance of reporting particle motion measurements in sound impact studies on fish. This is especially important for indoor studies in fish tanks, as we have shown that particle motion and sound pressure components do not share the same relationship in small tanks as they would in open water, far-field conditions. Furthermore, our exploration of the link between detailed characteristics of the underwater sound field and behavioural response tendencies of captive zebrafish revealed that both components of sound may be independently correlated to anxiety-related behavior such as freezing. Whether particle motion (SVL), sound pressure (SPL) or the ratio between particle velocity and sound pressure (excess SVL) are more or less prominent perceptually and responsible for specific anxiety-related, sound-induced escape or freezing behavior requires further study.

The practical challenges for further study are numerous. The lack of standardized methodology, low repeatability, and difficulty in obtaining commercially available geophones and accelerometers still remain obstacles for researchers (Radford et al. 2012; Anderson 2013). The highly complex sound field conditions (Parvulescu 1967; Akamatsu et al. 2002; Slabbekoorn 2016) also remain an issue for indoor studies in fish tanks, as should be clear from our own study. Nevertheless, we advocate the exploitation of indoor and outdoor conditions as complementary studies. Furthermore, intensive collaboration among fish biologists, acoustic engineers, and behavioural specialists remains critical for further progress in our fundamental understanding of the acoustic world of both captive and free-ranging fish (e.g. Shafiei Sabet et al. 2016. Neo et al. In Press).

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Supplementary material

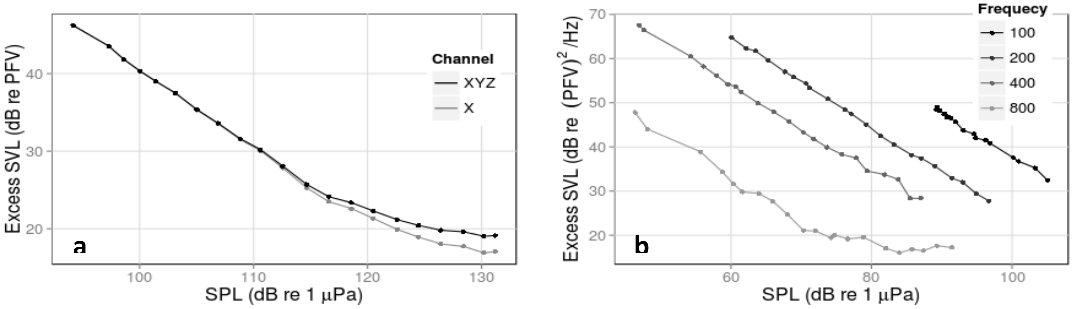

Fig. S1. The resulting excess SVL measurements from white noise playback with variable volume levels and a fixed vector sensor position: There is a negative relationship between excess SVL and SPL, independent of spatial location within the tank. The tank as ensonified with 21 white noise exposures, where each exposure was digitally adjusted to be 2dB quieter than the last. Fig. a) shows the excess particle velocity measured along the x axis (black), which is facing towards the speaker, and the summed particle velocity across all 3 channels of the vector sensor (grey). Fig. b) shows the excess particle velocity summed across all channels per selected frequency resulting from a PSD analysis (window length: 40000, window type: Hamming).

Table 1. Summary statistics for the Generalized Linear Model used to compare the spatial properties (i.e. the position of the vector sensor in the tank) and SPL of each measurement to the resulting Excess SVL. Coefficients are reported in cm, except for ordinal polynomials which are reported in transformed units to avoid the effects of covariance.

Excess SVL

Effect	**Coefficient**	***t*-value**
Intercept	159	49.6***
SPL	-1.17	59.6***
Distance from the wall closest to the in-air speaker	0.29	7.85***
Distance from a wall facing the direction adjacent to that of sound propagation: *1st degree polynomial*	-4.68	-1.91.
Distance from a wall facing the direction adjacent to that of sound propagation: *2nd degree polynomial*	-8.41	-3.43**
Distance from tank bottom	-0.189	-4.26***
Close to either wall facing the direction of sound propagation: *True*	2.17	3.37**
Close to either the bottom of the tank or the water surface: *True*	-3.05	-3.84***
Interaction: (Distance from a wall facing the direction adjacent to that of sound propagation: *2nd degree polynomial*)*(Distance from tank bottom)	0.350	3.52***
Interaction: (Distance from the wall closest to the in-air speaker)*(Distance from tank bottom)	-1.48e-2	-9.81***
Interaction: (Close to either wall facing the direction of sound propagation: *True*)*(Distance from tank bottom)	0.257	9.77***
Interaction: (Close to either wall facing the direction of sound propagation: *True*)*(Close to either the bottom of the tank or the water surface: *True*)	1.34	2.83**
Interaction: (Distance from the wall closest to the in-air speaker)*(Close to either the bottom of the tank or the water surface: *True*)	0.145	5.60***

Null Deviance: 5252.279 (n = 63); **Residual Deviance: 30.553** (n = 48)

*** p-value < 0.001; * p-value < 0.05; . p-value < 0.1

Table 2. Summary statistics for the Mixed Models comparing average swimming speed before and after noise exposure and the probability of a freeze response to SPL and SVL.

Post-exposure Average Swimming Speed			**Probability of Post-exposure Freeze response**		
Fixed effect	**Coefficient**	***t*-value**	**Fixed effect**	**Coefficient**	***z*-value**
Intercept	11.59	5.39***	***Intercept***	-22.71	-2.22**
Exposure Number	-0.51	-2.60*	SVL	-0.29	-2.45*
Average swimming speed before exposure	0.48	1.87**.**	Average swimming speed before exposure	-0.64	-2.61*
Expression of freezing behavior before exposure	-2.57	-2.13*			
	R^2_m	**R^2_c**		**R^2_m**	**R^2_c**
	0.15	0.43		0.32	0.79

*** p-value < 0.001; * p-value < 0.05; . p-value < 0.1

Table 3. Excess SVL values calculated from outdoor studies.

Excess SVL (dB ref 1nm/s)	Sound Source	Bandwidth (Hz)	Reference
11.8	Ambient Conditions	10-10000	Farina & Armelloni (2012)
10.9	Passing Boat	10-10000	-
-1.5	Ambient Conditions	200-2000	Neo et al. (In Press)
6	White noise from underwater speaker	200-2000	-

Chapter 6

General discussion

Summary of thesis results

Human generated sound (anthropogenic noise) is now widely recognized as an environmental stressor, which may affect aquatic life (Slabbekoorn et al. 2010; Radford et al. 2014). Over the last few decades, there is increasing interest of policy makers, animal welfare communities, behavioural biologists and environmental managers to understand how man-made sound may lead to negative consequences on terrestrial (Patricelli & Blickley 2006; Barber et al. 2010; Kight & Swaddle 2011) but also underwater animals (Slabbekoorn et al. 2010; Ellison et al. 2012; Williams et al. 2015). Aquatic animals can be negatively affected by anthropogenic noise in many ways (Popper et al. 2003; Popper & Hastings 2009; Slabbekoorn et al. 2010; Richardson et al. 2013). Therefore, we need to understand how anthropogenic noise may affect individuals to eventually be able to assess the impact of anthropogenic noise on populations, communities, and ecosystems. In my thesis, I have addressed several fundamental aspects of the potential impact of anthropogenic noise by experimental sound exposure studies in captive fish. Below, I first briefly summarize the findings of each of the four data chapters to then address some general concepts in a broader context.

In **Chapter 2,** I focused on the potential effects of sound exposure on predator –prey interactions in captive zebrafish preying on water fleas. I investigated how sound exposure may affect not only zebrafish as predator but also water fleas as prey. I tested sound exposure conditions that varied in temporal pattern: continuous, regular and irregular intermittent, and I also included a control condition with no additional sound exposure. I checked for a sound impact on: 1) waterflea swimming behaviour; 2) zebrafish swimming behaviour, and 3) foraging behaviour and efficiency of zebrafish hunting for waterfleas. My findings indicate: 1) no significant effects of sound exposure on waterfleas; 2) that temporal pattern affected the response to sound exposure in the fish and 3) that the detrimental impact of sound exposure on feeding efficiency was independent of temporal pattern. These data suggest that the direct impact of sound seems to be on the predator, but that will not exclude an indirect impact of sound exposure on the prey. Therefore, the impact on foraging efficiency in predator fish feeding on invertebrate prey in outside natural conditions may alter the balance in abundance between the two taxa. The results of this chapter confirm the possibility of noise impact beyond single species effects and future studies may reveal sound impact at community level under water as has been reported for terrestrial systems (Francis et al. 2009; 2012; Slabbekoorn & Halfwerk 2009). I therefore think that more studies are warranted on other species and other frequency ranges to explore the generality of findings beyond the current species and test conditions.

In **Chapter 3,** I compared the potential effects of sound exposure on two different fish species; zebrafish and cichlids, with different swimming behaviour and different hearing abilities. The findings revealed significant effects on behaviour in response to the elevated sound levels in both species, sometimes in the same way but sometimes in a different way. After the initial seconds, both species reduced their swimming speed during the "prolonged" period of sound exposure. 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. Moreover cichlids went even further 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. These responses are likely to be anxiety-related behaviour and are similar to response patterns in other species during acoustic exposure experiments (Andersson et al. 2007; Bui et al. 2013; Neo et al. 2014; Neo et al. 2015). However, we suggest that care should be taken for any interpretation in terms of relative severity for the two species. Understanding impact and underlying mechanism(s) behind the observed behavioural changes requires more studies including physiological measurements and investigations of real long-term effects (at least weeks or months and addressing development, growth, survival, reproduction).

In **Chapter 4,** I tested zebrafish behavioural changes in response to experimental sound and light conditions. My aims were to investigate the effect of two modalities and study whether sound and light exposure affect spatial distribution and swimming behaviour of zebrafish. The experimental fish had a choice between two fish tanks: a treatment tank and a quiet and light escape tank. The findings of this chapter showed that elevated sound levels did not cause any tank preference in terms of the overall time the zebrafish spent in the treatment tank. Furthermore, although dark conditions in the treatment tank reduced the crossing activity between tanks, it also did not result in a spatial bias to the dark or light tank. The elevated sound levels clearly changed zebrafish behaviour when they were within the treatment tank; they increased freezing time and decreased the percentage of time spent near the active speaker. Dark conditions in the treatment tank also affected their behaviour and resulted in less time spent close to the tube and more time spent in the upper layer. In addition, we did not find any interaction effects of sound and light conditions on zebrafish behaviour. Overall, these data suggest that each modality has its own specific and qualitatively distinct impact independent of the conditions in the other modality (see Kunc et al. 2014; Halfwerk & Slabbekoorn 2015). Dim light may be a trigger to relax and make fish less hesitant to get close to the water surface, while loud sound clearly induces anxiety-indicating interruption of activities.

In **Chapter 5**, I conducted two experiments together with MSc-student James Campbell in which we measured the acoustic field inside a standard 1-meter fish tank, including sound pressure level and sound particle velocity level. We quantified the confined area available to the fish within an enclosure cage to explore the relationship between the two sound components and the potential relevance to fish behavioural responses. The first experiment examined how the ratio of pressure to particle motion in a small enclosure cage varies in response to the spatial location within the cage, as compared to theoretical open-water conditions. In the second experiment, we further examined the pressure and particle velocity levels within the context of an acoustically induced behavioural response by zebrafish. The findings of this chapter provide new insights into the sound field complexity of relatively small fish tanks and into the challenging exploration of the link between sound field parameters and fish behaviour.

Effects of sound on feeding efficiency

I found detrimental effects of sound exposure on food intake and subsequently in overall foraging performance in captive zebrafish, which confirms the results of several other studies on different fish species (Purser & Radford 2011; Bracciali et al. 2012; Voellmy et al. 2014a; Payne et al. 2015; McLaughlin & Kunc 2015) and other vertebrates (Croll et al. 2001; Aguilar Soto et al. 2006; Miller et al. 2009) but also invertebrates (Chan et al. 2010; Wale et al. 2013; Hughes et al. 2014). These studies all show an impact of sound on non-auditory tasks, which may be caused by visual distraction or attentional shift (Mendl 1999; Dukas 2002). It is unclear whether animals can habituate to this, but it may have an impact that is easily overlooked when animals stay in a noisy area (no impact on distribution) and keep on showing natural behavior (no apparent impact on welfare or fitness consequences).

Sound, anxiety, stress and behaviour

In all four of the experimental exposure studies I have observed the same types of behavioural changes. These behaviours are typically characterized by an initial increase in swimming speed and a downward shift toward the bottom of the tank and a prolonged swimming speed decrease, which were interpreted as anxiety/fear-related behavioural responses to sound exposure (c.f. Neo et al. 2015). This interpretation was in line with reports on similar responses to chemical alarm pheromone (Egan et al. 2009) and visual threat stimuli (Bass & Gerlai 2008; Luca & Gerlai 2012a; Luca & Gerlai 2012b). Other indoor studies on other species find either the same types of responses (Pearson et al. 1992; Andersson et al. 2007; Bui et al. 2013; Neo et al. 2014; Voellmy et al. 2014b) or additional ones such as reduced food searching, lower feeding rates and increased hiding time in a shelter (Bracciali et al. 2012; Løkkeborg et al. 2012; McLaughlin & Kunc 2015).

Outdoor studies report similar (Blaxter et al. 1981) and or different fish behaviour such as sound-related horizontal escape behaviour (Ona & Godø 1990; Engås & Løkkeborg 1996; Engås & Løkkeborg 2002; Draštík & Kubečka 2005). Even though several studies have reported physiological effects of sound exposure in terms of stress-hormone levels (Santulli et al. 1999; Wysocki et al. 2006; Buscaino et al. 2010; Filiciotto et al. 2014) and also growth and survival rate (Wysocki et al. 2007; Davidson et al. 2009; Debusschere et al. 2016), there is limited data on long-term effect from studies in aquaculture (Bart et al. 2001; Smith 2004) and complete lack of data where specific behavioural response patterns are linked to physiology or long-term effects. Although it appears clear that sound exposure can induce anxiety-related responses, future studies should focus on the effects of sound exposure on both behavioral and physiological measures to explore both immediate and prolonged anxiety/fear related behavioural response in free-ranging and captive fish species.

Species comparisons

My second data chapter already stressed the fact that multiple species may be involved in impact analyses of anthropogenic noise. The third one also confirmed that two different fish species with different hearing abilities may respond to sound exposure, but in different ways. Base line differences in behavior and response, as well as direct and indirect effects of sound on species indicate the complexity of sound impact studies. It is also not clear yet to what degree fish vary individually in sensitivity to sounds in their environment and how factors such as life stage, body condition and behavioural contexts modify this sensitivity (Purser et al. 2016). Moreover, assessments of potential effects of man-made sound go beyond single species and individual fish and eventually we have to address impact in outdoor conditions at the ecosystem level (Slabbekoorn 2016).

Spatial avoidance or lack there-off

In my third and fourth data chapter I found no evidence for spatial avoidance in our long tank or in our dual tank set-up. Only in very close proximity of the active speaker in our dual tank set-up, we found evidence for a directional response away from the sound source. Field studies have reported on spatial responses during ‘natural’ occurrence of man-made sounds (Ona & Godø 1990; Engås & Løkkeborg 1996; Engås & Løkkeborg 2002; Slotte et al. 2004; Draštík & Kubečka 2005; Sarà et al. 2007; Blaxter et al. 1981; Hawkins et al. 2014; Febrina et al. 2015) and spatial avoidance may just be more difficult to induce or assess in captive conditions.

There are some studies that showed horizontal attraction to playback of conspecific sounds in fish tank conditions (Higgs et al. 2007; Rollo & Higgs 2008; Verzijden et al. 2010). This seems in contradiction with the general lack of spatial deterrence responses away from loud sound sources in the variety of fish tank conditions in my thesis. Nevertheless, the spatial avoidance of the area right in front of the active speaker in chapter 3 may reflect a capability of sound source orientation under some condition or in some parts of the fish tank that must also be the explanation for the positive phonotactic studies in captivity.

In outdoor conditions, experimental exposure studies have reported spatial avoidance, but still only to a limited extent (Neo et al. submitted). Consequently, fish tank studies may be useful for investigations on general aspects of potential impact of sound on fish, but not for spatial avoidance studies. Future studies should be done in outdoor conditions with tagged fish or penned fish. I believe such studies would yield important information because there would be less acoustic field complexity and fish in the open field are not confined and therefore may behave more naturally in response to acoustic stimuli.

Perceptual salience of sound components

In my final data chapter, I report about a first empirical exploration of both detailed acoustic properties of sound fields in relatively small fish tanks and whether it is possible to investigate the relative importance of sound components in triggering a behavioural response. I like to draw attention to the potential of using stimuli of different frequency ranges to study fundamental aspects of hearing. Zebrafish are most sensitive to sound of frequencies around 800 Hz, but are likely to hear well above 1000 Hz, up to 4000 Hz (Higgs et al. 2002; Bretschneider et al. 2013). Furthermore, relative sensitivities for particle motion and sound pressure are likely to complement each other, but vary spectrally with a bias to the low end for particle motion and to the high end for sound pressure (Schulz-Mirbach et al. 2012). I believe this concerns an area of research that could yield important insights about auditory functions in fish in general and the potential for disturbance by artificially elevated sound in particular.

My experiments in this thesis addressed fundamental issues of potential sound impact and are not directly applicable to outside conditions nor suitable to extract absolute threshold values for legislation or permits. Nevertheless, my studies are complementing growing evidence in the literature that prolonged sound exposure can also result in long-term modification of behaviour and change spatial habitat use of fishes (Bass & McKibben 2003; Wysocki et al. 2009; Slabbekoorn et al. 2010; Slabbekoorn 2016; Radford et al. 2014; Amorim et al. 2015; Ladich 2015). I believe effective management of fish stocks or wildlife areas requires many more studies, especially into chronic effects of anthropogenic noise (c.f. Slabbekoorn et al. 2010; Francis & Barber 2013; Radford et al. 2014). Policy makers have already set regulations for marine environments to safeguard a so-called good environmental status, but there are no agreements yet for freshwater habitats. This means freshwater fish in a diversity of waterbody types are more or less exposed to man-made sound without any incentive to control impact and without any protection by law. Many freshwater fish species actually have quite well-developed hearing abilities and there is no reason to believe that they are less vulnerable to detrimental effects from anthropogenic noise than their marine counterparts. I hope the studies in my thesis contribute eventually to more general awareness of potential issues with sound pollution in both marine and freshwater habitat. I am sure that, by then, more fundamental insights will come in handy for potential monitoring, protection or mitigation efforts.

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Nederlandse Samenvatting (Dutch Summary)

Samenvatting

De lawaaiige onderwaterwereld: het effect van geluid op het gedrag van zebravissen in aquaria.

Lawaai gecreëerd door mensen (antropogeen lawaai) wordt tegenwoordig wereldwijd erkend als een verstoringsbron voor het milieu, met de potentie om het onderwaterleven te beïnvloeden. In de afgelopen decennia is er een toenemende interesse van politici, dierenrechtenorganisaties, gedragsbiologen en natuurbeleidsmakers om erachter te komen hoe antropogeen lawaai zou kunnen leiden tot negatieve gevolgen voor dieren op land, maar ook onderwater. Aquatische dieren kunnen op veel manieren negatief worden beïnvloed door lawaai. Het doel van dit proefschrift was het onderzoeken van gedragsveranderingen in vissen als gevolg van blootstelling aan diverse geluiden, met zebravissen in gevangenschap als modelorganisme. Ik heb gekeken naar gedragsparameters als potentiële indicatoren voor geluid-gerelateerde stress, verstoring en verjaging.

In **hoofdstuk 2** focuste ik me op de potentiële effecten van geluidsblootstelling op predator-prooi interacties bij zebravissen in gevangenschap die op watervlooien jagen. Mijn resultaten laten zien dat: 1) er geen significant effect was van geluidsblootstelling op watervlooien; 2)

temporele patronen in geluid de respons op geluidsblootstelling in vissen beïnvloedden en 3) de negatieve invloed van geluidsblootstelling op foerageerefficiëntie onafhankelijk was van temporele patronen. Deze data suggereren dat geluid een directe invloed heeft op de predator, maar dat sluit geen indirecte invloed uit van geluidsblootstelling op de prooi. De resultaten van dit hoofdstuk bevestigen de mogelijkheid dat de invloed van geluid verder gaat dan effecten op een enkele soort, en mogelijk zullen toekomstige studies laten zien dat geluid invloed heeft op het niveau van de samenleving onderwater, zoals al gedocumenteerd is voor terrestrische systemen.

In **hoofdstuk 3** vergeleek ik de potentiële effecten van geluidsblootstelling op twee verschillende vissoorten: zebravissen en cichliden. De resultaten lieten zien dat er significante effecten van verhoogde geluidsniveaus op het gedrag van beide soorten waren. Na de eerste seconden verlaagden beide soorten hun zwemsnelheid, maar bij de start van de blootstelling gingen de zebravissen onmiddellijk sneller zwemmen.Cichliden deden dit nieten gingen soms zelfs achteruit zwemmen. Bovendien brachten cichliden significant meer tijd door in de onderste waterlaag van het aquarium gedurende de geluidsblootstelling, terwijl zebravissen op dezelfde hoogte bleven. Deze reacties zijn waarschijnlijk angstgerelateerd gedrag. Het begrijpen van de impact en onderliggende mechanisme(n) achter de geobserveerde gedragsveranderingen vergt echter meer onderzoek naar meer soorten, inclusief fysiologische metingen en studies naar echte lange-termijn effecten.

In **hoofdstuk 4** onderzocht ik gedragsveranderingen in zebravissen als reactie op experimentele geluid- en lichtcondities. De resultaten lieten zien dat verhoogde geluidsniveaus geen voorkeur voor een bepaald aquariumdeel veroorzaakten, gekeken naar de totale tijd die de zebravis in het lawaaiige gedeelte van een dubbel aquarium doorbracht. De verhoogde geluidsniveaus zorgden duidelijk voor een verandering in zebravisgedrag als ze in het lawaaiige deel van het aquarium waren: ze ‘bevroren’ vaker en brachten minder tijd door bij de actieve speaker. Donkere condities in het aquarium beïnvloeddenook hun gedrag, maar ik vond geen interactie-effecten van geluid- en lichtcondities. Bij elkaar genomen suggereren deze data dat verandering in omstandigheden op elke modaliteit zijn eigen specifieke en kwalitatief verschillende impact heeft, onafhankelijk van de condities van de andere modaliteit.

In **hoofdstuk 5** heb ik het akoestische veld in een aquarium gemeten. Ik heb de beperkte ruimte die beschikbaar was voor de vis in een dichte kooi gekwantificeerd, om de relatie tussen geluidsdruk en deeltjessnelheid, en de potentiële relevantie voor gedragsreacties van vissen te onderzoeken. Het eerste experiment bekeek hoe de verhouding van druk tot deeltjessnelheid in een kleine kooi varieert in relatie tot verschillende locaties in de kooi, vergeleken met theoretische open-watercondities. In het tweede experiment deden we verder onderzoek naar de geluidsdruk en deeltjessnelheid binnen de context van een akoestisch veroorzaakte gedragsreactie bij zebravissen. De resultaten van dit hoofdstuk geven nieuwe inzichten in de complexiteit van geluidvelden voor relatief kleine aquaria en in de uitdagende verkenning van de link tussen geluidsveld-parameters en vissengedrag.

Tot slot, mijn experimenten in dit proefschrift behandelden fundamentele kwesties met betrekking tot de potentiële impact van geluid en zijn niet direct toepasbaar voor veldcondities, noch geschikt om absolute grenswaarden te bepalen voor regelgeving of vergunningen. Desalniettemin complementeren mijn onderzoeken een groeiend bewijs in de literatuur dat blootstelling aan geluid kan leiden tot aanpassingen in gedrag en ruimtelijk habitatgebruik van vissen. Beleidsmakers hebben al regelgeving gecreëerd voor mariene gebieden om een goede milieustatus te waarborgen, maar er zijn nog geen afspraken voor zoetwater habitatten. Ik hoop dat de onderzoeken in mijn proefschrift uiteindelijk zullen bijdragen aan meer algemeen bewustzijn van potentiële problemen met geluidsverontreiniging, zowel in het marien als het zoetwater habitat.

خلاصه فارسی (Persian Summary)

خلاصه فارسی

دنیای پر سر و صدا در محیط های آبی

سابقه تحقیق

امروزه صداهای تولید شده ناشی از فعالیتهای انسانی بطور گسترده ای به عنوان آلاینده و ایجاد کننده استرس محیطی شناخته شده است که می تواند زندگی موجودات آبزی را تحت تاثیر قرار دهد. در چند دهه اخیر، علاقه رو به افزایشی در بین مجامع سیاست گذاران بهره برداری از بخش های منابع طبیعی، فعالان جمعیتهای حقوق و رفاه حیوانات، زیست شناسان علوم رفتاری و مدیران محیط های زیست جهت دانستن این موضوع که چگونه آلودگی های صوتی ناشی از فعالیت های انسانی می تواند باعث بروز اثرات منفی و زیان بار کوتاه مدت و بلند مدت بر روی جوامع جانوری خشکی و همچنین آبزیان باشد. جانوران آبزی می توانند به روش های مختلفی تحت تاثیرات مخرب آلاینده های صوتی قرار گیرند. بنابرین ضروری به نظر می رسد که در ابتدا با بررسی اثرات آلاینده های صوتی ناشی از فعالیت های انسانی بر روی موجودات آبزی بصورت انفرادی بتوانیم برآورد صحیح و درستی از اثرات صوت بصورت گسترده تر بر روی جمعیت ها، اجتماعات و اکوسیستم های متراکم جانوری داشته باشیم. هدف کلی من در این تز بررسی تغییرات رفتاری ناشی از آلاینده های صوتی الگوبرداری شده از محیط های طبیعی بر ماهی (با استفاده از ماهی زبرا فیش (گورخری) که از جمله گونه های مدل استاندارد در انجام آزمایشات در زمینه های مختلف علوم زیستی است) می باشد. در این تز من از

عوامل و فاکتورهای زیست شناسی رفتاری بعنوان شاخص های بالقوه شناسایی بروز استرس های مرتبط با صوت، اختلال های رفتاری و بازدارندگی رفتاری استفاده نمودم.

اثرات متقابل شکار- شکارگری

در فصل دوم، اثرات بالقوه در معرض قرار گرفتن صوت بر روی ماهی زبرا و دافنی آب شیرین و همچنین تعاملات متقابل شکار-شکارگری این دوگونه بررسی شد. در این بخش از تحقیق تیمارهای صوتی با الگوهای زمانی متفاوت شامل: صوت پیوسته، صوت متناوب با قاعده قابل پیش بینی تند و صوت متناوب با قاعده قابل پیش بینی کند و صوت متناوب با قاعده غیرقابل پیش بینی همچنین تیمار کنترل بدون پخش صوت انتخاب شدند. یافته های این آزمایش ها نشان داد که: ۱) پخش صوت بر روی رفتار شنای دافنی بعنوان گونه شکار تاثیر گذار نبود. ۲) الگوهای زمانی متفاوت پخش صدا بر روی رفتار شنای ماهی زبرا تاثیر معنی داری داشت. ۳) اثرات صدا بر روی کیفیت و راندمان تغذیه ماهی زبرا از دافنی مستقل از الگو های زمانی پخش صدا بود. بطور کلی، نتایج این آزمایش ها نشان داد که اثرات مستقیم پخش صدا فقط بر روی گونه شکارچی (ماهی زبرا) بوده است. البته لازم توجه است که امکان اثر غیر مستقیم پخش صدا بر روی گونه شکار (دافنی) را نمی توان نادیده گرفت. نتایج این بخش تایید کننده احتمال اثرات پخش صوت فراتر از یک گونه و بر روی گونه های مختلف جانوران می باشد. مطالعات بیشتر ممکن است نشان دهنده اثرات فزاینده پخش صوت در سطوح اجتماعات گونه های آبزیان باشد. همانطوری که این اثرات فراگونه ای صوت بر روی اجتماعات گونه های خشکی زی به اثبات رسیده است. بنابراین مطالعات بیشتر بر روی گونه های دیگر و فرکانس های صوتی قابل دریافت برای جانوران برای فهم کلیات یافته ها فراتر از شرایط فعلی گونه ها ضروری می باشد.

مقایسه دو گونه ماهی

در فصل سوم، پتانسیل اثرات صوت بر روی دو گونه مختلف ماهی، ماهی زبرا و ماهی سیکلید دریاچه ویکتوریا آفریقا، بررسی شد. این دو گونه از نظر رفتار های شنا و دامنه شنوایی کاملا متفاوت می باشند. یافته های این بخش نشان دهنده اثرات معنی دار پخش صوت بر رفتار هر دو گونه ماهی می باشد. بطوریکه این تغییرات رفتاری در بین دو گونه گاهی اوقات مشابه و گاهی اوقات کاملا متفاوت بودند. پس از گذشت اولین ثانیه ها از آغاز پخش صوت هر دو گونه رفتار کاهش سرعت شنا را در بازه زمانی بلند مدت از خودشان نشان دادند. با شروع آغازین پخش صوت سرعت شنا در ماهی زبرا در نتیجه وحشت زده شدن و یا به جهت شدت فزاینده شتاب افزایش چشمگیری نشان داد در حالیکه این واکنش رفتاری در ماهی سیکلید دیده نشد. حتی در برخی موارد ماهی سیکلید با شنای روبه عقب تغییر رفتار نشان داد. علاوه بر این، ماهی سیکلید در طول پخش هر دو تیمار صدا به قسمت های ستون پایینی آب درآکواریوم متمایل شده و زمان بسیار زیادی را در این قسمت سپری کردند. درحالی که ماهی زبرا تغییرات محسوسی را در زمینه عمق شنا در ستون آب در طول پخش هر دو تیمار صوت نداشته است. به احتمال زیاد این پاسخ های رفتاری مشاهده شده مربوط به واکنش اضطرابی ماهی می باشد و البته مشابه الگوهای پاسخ رفتاری سایر گونه ها در پاسخ به پخش صدا می باشد. اگرچه دانستن اثر و مکانیسم های خاص این تغییر رفتار های مشاهده شده نیازمند مطالعات بیشتر و گسترده بر روی گونه های متنوع و همچنین اندازه گیری فاکتور های فیزیولوژیکی و تحقیقات مداوم و طولانی تر می باشد.

نور و صدا

در فصل چهارم، تغییرات رفتاری ماهی زبرا در پاسخ به شرایط صدا و نور مورد بررسی قرار گرفت. یافته های این فصل نشان داد که افزایش میزان پخش صدا هیچ گونه ترجیح استفاده فضایی به حضور در یک آکواریوم (آکواریوم تیمار یا کنترل) توسط ماهی زبرا نشان نداد. علاوه بر این اگرچه شرایط تاریکی و نور کم در آکواریوم تیمار باعث کاهش حجم شناگری ماهی زبرا بین دو آکواریوم گردید ولی باعث گرایش فضایی ماهی زبرا به آکواریوم روشن و یا تیره نبود. البته افزایش میزان صدا بصورت کاملا واضح باعث تغییرات رفتاری ماهی زبرا بویژه زمانی که در آکواریوم تیمار بودند گردید. برای مثال زمان بی حرکت ماندن ماهی زبرا افزایش پیدا کرد و همچنین میزان درصد حضور در اطراف و نزدیک بلندگوی پخش صوت کاهش معنی داری نشان داد. ماهی زبرا در شرایط تاریکی و نور کم آکواریوم تیمار هم تغییرات رفتاری نشان داد. این رفتار ها شامل سپری کردن زمان کمتر در اطراف تیوپ اتصال دو آکواریوم و همچنین افزایش زمان سپری شده در قسمت سطوح بالای ستون آب در آکواریوم تیمار می باشد. علاوه بر این هیچگونه اثرات متقابل و بر هم کنش صدا و نور در تغییرات رفتاری ماهی زبرا مشاهده نشد. در مجموع این داده ها و نتایج پیشنهاد می کند که هر کدام از عوامل محیطی (صدا و نور) تاثیرات ویژه، منحصر به فرد و خاص خود را به طور مستقل از شرایط عامل محیطی دیگر بر رفتار ماهی اثربخش می نماید. شرایط نوری کم و تاریکی می تواند بعنوان عاملی جهت کاهش فعالیت های حرکتی ماهی باشد و در نتیجه ماهی می تواند زمان بیشتری را در قسمت های سطحی آب سپری کند. درحالی که پخش صدای شدید و بلند به طور واضح باعث ایجاد حالت اضطراب در ماهی شده که می تواند بر سایر فعالیت های ماهی نیز تاثیر گذار بوده و اختلالاتی ایجاد نماید.

پارامترهای صدا و واکنش

در فصل پنجم، به همراه دانشجوی کارشناسی ارشد زمینه فاکتورهای صوتی شامل سطوح فشار صوت و همچنین سطوح سرعت ذره صوت داخل یک آکواریوم یک متری متداول و استاندارد مورد مطالعه قرار گرفت. کمیت سطوح فشار و سطوح سرعت ذره صدا در منطقه محدود و در دسترس ماهی در قفس شناور محصور شده جهت بررسی رابطه بین دو مولفه صدا و ارتباط بالقوه آن با تغییرات رفتاری ماهی و سطوح سرعت ذرات صدا اندازه گیری شد. در آزمایش اول، چگونگی تفاوت و شیب نسبت بین فشار و سرعت ذره صوت در پاسخ به مکان های فضایی در یک قفس کوچک محصور در آکواریوم یک متری استاندارد در مقایسه با شرایط نسبی تئوری نسبت شیب بین فشار و حرکت ذره صوت در آبهای باز و آزاد محیط های آبی طبیعی مورد بررسی و مقایسه قرار گرفت. در آزمایش دوم، سطوح فشار و سرعت ذره صدا در چهارچوب تغییرات رفتاری ناشی از پخش صدا توسط ماهی زبرا مورد بررسی قرار گرفت. یافته های این فصل بینش های جدید و نوینی را در خصوص پیچیدگی میدان های صدا در آکواریوم نسبتا کوچک ماهی به همراه آورد و مطالعات بیشتر در جهت به چالش کشیدن اکتشافات جدید در خصوص ارتباط بین پارامترهای صدا و رفتار ماهی را ارایه می دهد.

نتیجه گیری کلی

در مجموع آزمایش های این تز مرتبط با مسائل اساسی تاثیر بالقوه صدا در محیط های آکواریومی و داخلی محصور می باشد. البته لازم به ذکر است که یافته های حاصل در این تز بطور کامل قابل بیان یک نتیجه گیری کلی و عمومی در شرایط محیط های طبیعی منابع آبی نمی باشد. همچنین شدت فشار صوت های بکار رفته در این آزمایش ها مناسب برای استخراج مقادیر آستانه

مطلق حساسیت گونه های استفاده شده در این آزمایش برای قانون گذاری و صدور مجوز توسط ارگان های دولتی نمی باشد. با این وجود نتایج حاصله از آزمایشات در این تز تکمیل کننده شواهد و مستندات سایر تحقیقات و پژوهش های محققین می باشد که قرار گرفتن بلند مدت در معرض پخش صوت می تواند باعث بروز اصلاحات، اختلالات و تغییرات بلند مدت رفتاری در ماهی گردیده و در نتیجه تغییر استفاده فضایی (ستون محیط های آبی) از زیست گاه های در دسترس ماهی شود. در حال حاضر سیاست گذاران مقرراتی را برای حفاظت محیط های دریایی و آبهای دور به جهت رسیدن به وضعیت زیست محیطی پایدار وضع کرده اند. اما تا کنون هیچ توافقی برای محافظت از زیستگاه های آب شیرین و آبزیان ساکن در این محیط ها وجود ندارد. این بدان معنی است که ماهیان زیستگاه های آب شیرین در تنوعی بسیار بالا از انواع محیط های آبی موجود، بدون هیچ گونه انگیزه ای جهت کنترل اثرات و بدون هیچگونه حفاظت ناشی از وضع قوانین کم و بیش در معرض آلاینده های صوتی ناشی از فعالیت های انسانی قرار دارند. این در حالی است که بسیاری از گونه های ماهیان آب شیرین در واقع دارای توانایی شنوایی کاملا توسعه یافته ای می باشند و هیچ دلیلی وجود ندارد که باور داشته باشیم که این گونه ها حساسیت و آسیب پذیری کمتری نسبت به همتایان خود در محیط های دریایی در مواجهه با اثرات مضر و مخرب آلاینده های صوتی ناشی از فعالیت های انسانی دارند. با امید به اینکه مطالعات و مجموعه تحقیقات انجام شده در این تز در نهایت به بطور کلی کمکی جهت اعتلا و آگاهی بیشتر از مسائل بالقوه آلودگی های صوتی در زیستگاه های دریایی و آب شیرین گردد. اینجانب مطمئن هستم که در آن صورت، بینش های اساسی بیشتری در خصوص اثرات آلاینده های صوتی بر روی جوامع زیستی در دسترس خواهد بود. البته این مهم به همراه پایش بالقوه نظارت، حفاظت و تلاش جهت کاهش اثرات مضر میسر خواهد بود.

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Saeed shafiei sabet

Leiden, the Netherlands,

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Curriculum Vitae

Saeed Shafiei Sabet was born on 21 September 1981 in Astaneh-ye Ashrafiyeh, Iran. In 1999 he received his diploma of natural sciences in Bandar-e Kiashahr. He received his Bachelor of Science degree (BSc) in Fisheries Science, specialized in fish nutrition, from University of Mazandaran (Sari Agricultural Recourses and Natural Sciences University). Afterward he worked for two years as fisheries advisor in the Ministry of agriculture.

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