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

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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 Specialzaak). 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 °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.

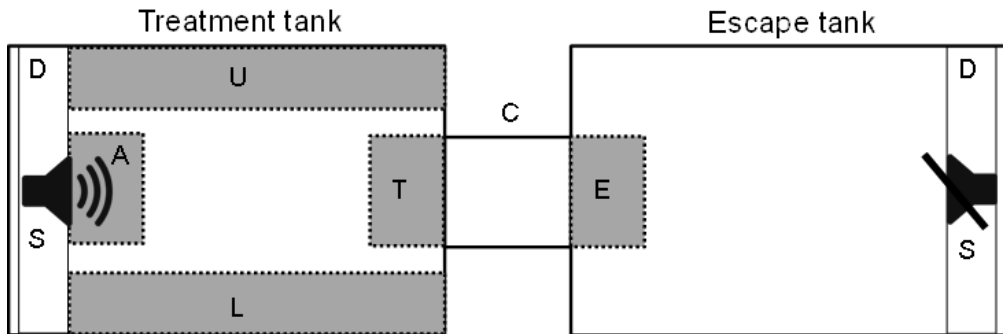


Fig. 1. Schematic view from the front of the dual-tank set up (outside measurements: 75 x 50 x 50cm each). Two underwater speakers (S) are placed on each side shielded by Plexiglas dividers to keep fish from swimming behind them (D). A sturdy pvc-tube (35 cm in length, 12.5 cm diameter) connected the 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 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.

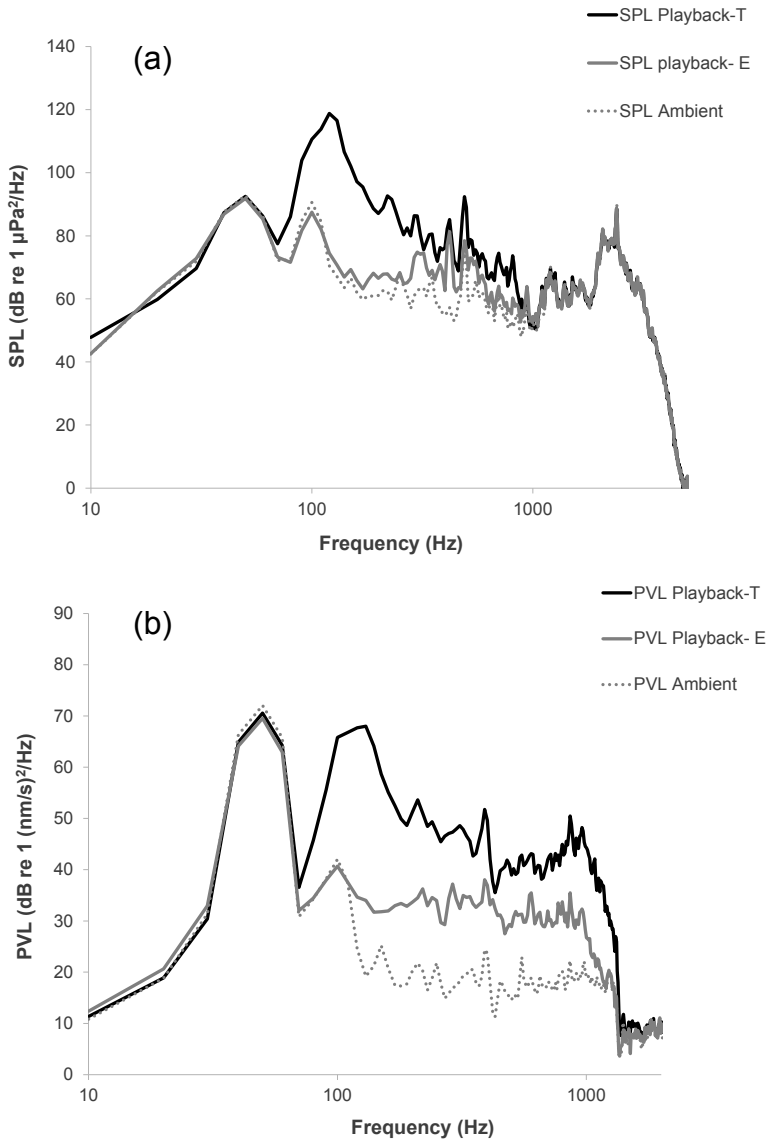


Fig. 2. Experimental sound levels as measured in locations close to the tube in the treatment tank (T), Escape tank (E) and ambient condition. Experimental elevation of SPL is between 70-800 Hz, with biggest rise between 90-250 Hz (a). Black solid line, grey line and grey dot line represent sound playback 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 onset 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 ($\geq 8\text{cm/s}$) 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 ($< 10\text{ cm}$ 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.

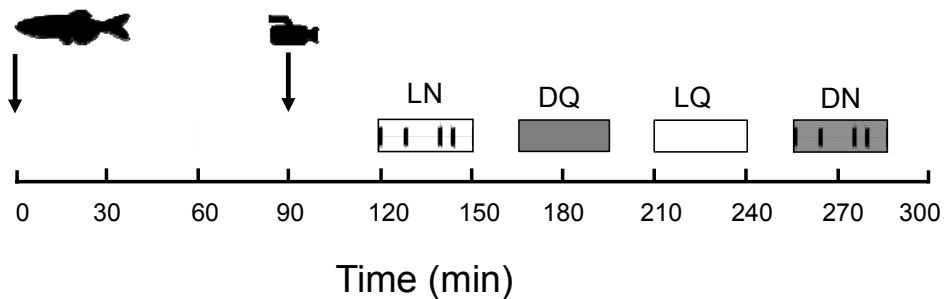


Fig. 3. Schematic representation of the exposure timeline for a single trial of an individual zebrafish in the treatment tank. Each individual in an experimental trial was exposed to four subsequent combinations of sound and light treatments in randomized sequence: LN, DQ, LQ and DN, while the escape 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 (UDEEC), (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 \times 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 \times 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 \times 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 \times 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 \times 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 \times 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 \times light treatment ($F_{1,75}=0.001$, $P=0.970$) (see Fig. 4i).

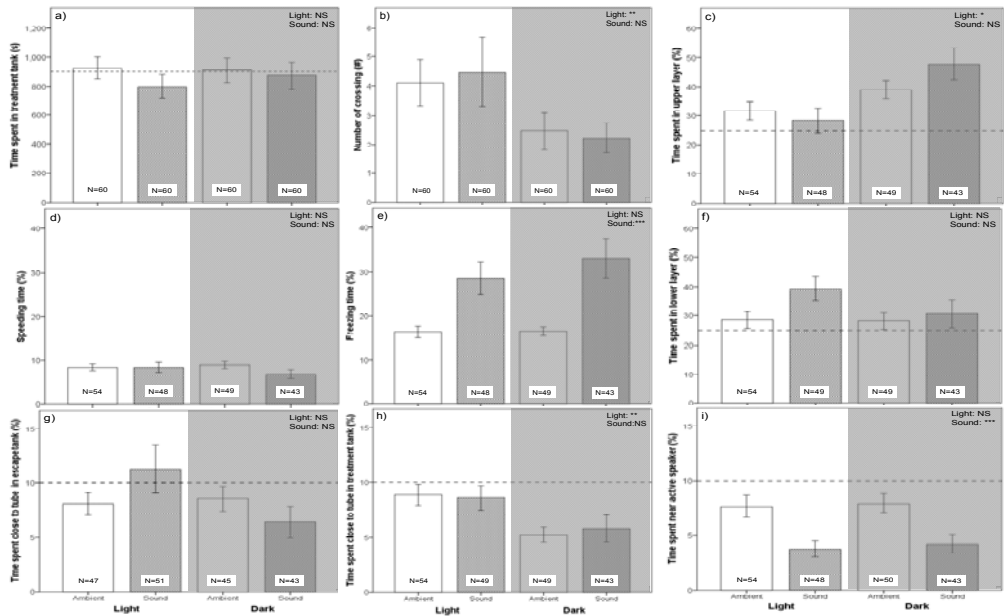


Fig. 4. Zebrafish swimming behaviour and spatial distribution changed differently by sound and light conditions among treatments. No effect of light and sound on time spent in the treatment tank (a), effect of light, but not sound on the number of crossings (b). There was an effect of light but not sound 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 but not light on time spent close to the tube in the escape tank (g), an effect of sound but not light 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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