Swimming bass under pounding bass : fish response to sound exposure

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CHAPTER 1

General Introduction

Acoustic world of fish

Fish live in a world that is not silent. On the contrary, they experience an acoustic world that is brimming with sounds, produced by natural processes, such as winds and surges, and by various aquatic animals, from invertebrates to mammals[1,2]. Fish can hear low frequency sounds (typically below 4 kHz) and make use of them[3]. For example, they may use sounds in their environment as a guide to navigate among different habitats[4–6]. They may also use sounds generated by their predators and prey to avoid or locate them[7,8]. Moreover, some fish species produce calls to repel competitors or attract potential mates[9–11]. Since the underwater world contains a large amount of acoustic information, which propagates more effectively underwater than information in other sensory modalities, fish depend considerably on sounds for survival.

Due to their dependence on sounds, fish may be particularly sensitive to changes in their acoustic world. Since the 1900s, the acoustic world of fish has been altered by a new prominent sound source: human activities[12]. These activities include commercial shipping, offshore construction, sonar exploration, seismic shootings and underwater explosions, which generate a cacophony of high-intensity sounds. At close range, these sounds may damage the auditory tissue of fish[13,14] or deafen them temporarily[15,16]. When fish are further away from the sound source, more moderate sounds they experience may mask important acoustic cues[17,18]. Moreover, the sounds may also alter the behaviour of the fish, changing their swimming patterns[19], disrupting spawning activities[20] and impairing territorial defence[21]. Furthermore, sound exposure may reduce fish efficacy in foraging[22,23] and avoiding predators[24,25]. There are currently growing concerns that these changes may threaten the health of fish stocks and eventually disrupt the stability of ecosystems (Fig. 1).

Challenges in understanding behavioural impacts

In order to regulate man-made sound productions and mitigate behavioural impacts, regulators and developers need to be able to assess potential impacts effectively. The assessments of the severity of behavioural impacts are not straightforward and are often problematic. For example, current guidelines predict severity based on the sound level and the duration of an exposure, using several standard metrics, such as sound pressure level (SPL) and sound exposure level (SEL)[26,27]. Although such metrics have been shown to correlate with physical injuries and temporary hearing loss[13,14], they may not be adequate in assessing behavioural impacts. These metrics do not sufficiently account for variation in relevant acoustic features, such as the temporal structure of sound exposure. The temporal variation in diverse man-made sounds are reflected in their intermittency, pulse shape, pulse repetition interval and interval regularity (Fig. 2). Since the temporal characteristics of sounds can be perceived by fish to gather important information about their environment[28,29], fish may be particularly sensitive to specific acoustic features. This feature-dependent sensitivity may in turn determine their susceptibility to certain sound exposures.

Behavioural studies on sound impacts can sometimes carry implications that are difficult to assess because of generalisation uncertainties inherent to different experimental approaches. These approaches can be divided into three categories: 1) laboratory, 2) field and 3) semi-natural, each with their pros and cons[30–32] (Fig. 3). For example, indoor tank set-ups enable researchers to control and manipulate experimental conditions to examine causal relationships between behavioural changes and specific factors of interest. However, these studies may not

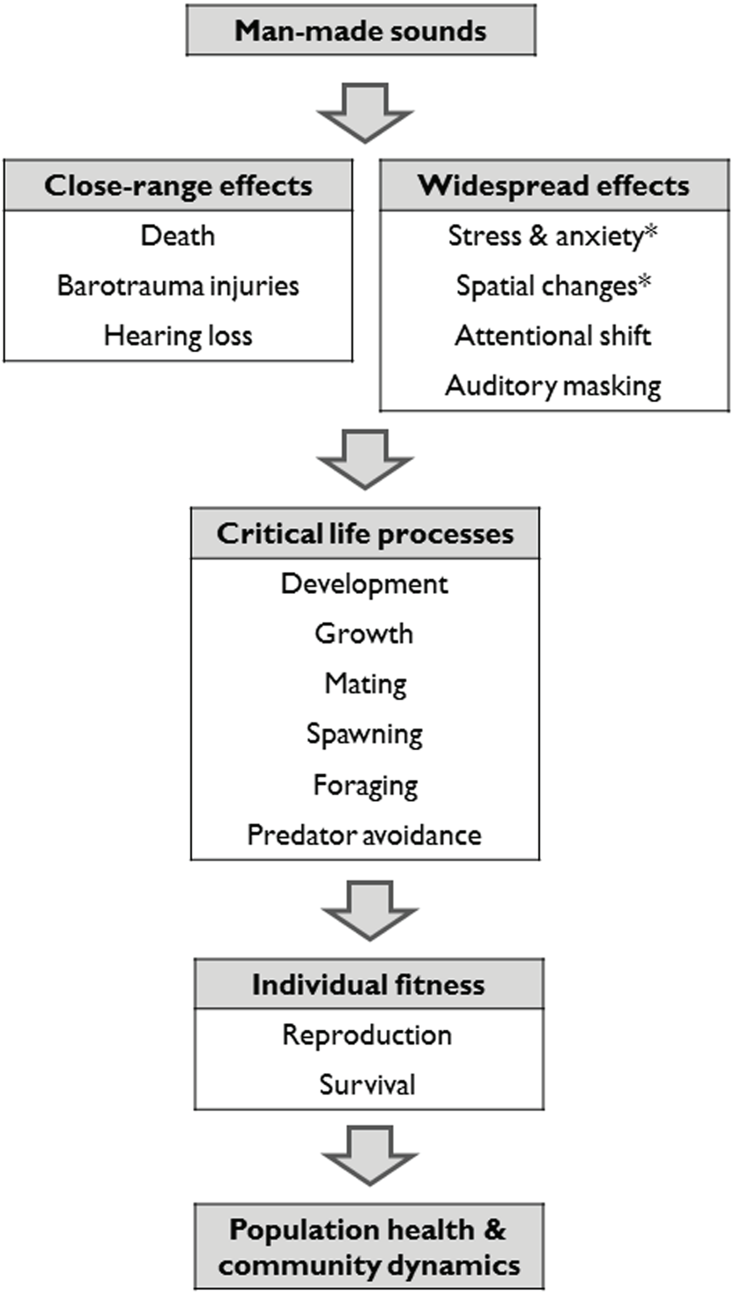


Fig. 1 Diagram showing the different potential effects of man-made sounds. Asterisks (*) indicate the effects that are examined in this thesis.

reflect the acoustic fields in natural waters and the behavioural changes observed may differ considerably from wild fish. On the other hand, field studies on free-ranging fish can provide direct evidence of behavioural impacts. However, such outdoor studies, when properly replicated and controlled, can be exceedingly costly and logistically challenging. Bridging the laboratory and field approaches, a semi-natural approach may be an optimal compromise, as it offers some control over the experimental design while keeping the experimental setting natural. Nonetheless, for proper interpretation of findings, it is crucial to determine which sound impacts are generalisable among these approaches and which are not. To test for generalisability, these approaches should be compared directly using the same experimental design and study species. Such comparisons will provide insights into the underlying mechanisms of the impacts, which are instrumental to devising mitigating strategies.

Translating findings from behavioural research to management decisions can be tricky, since animal behaviour is inherently complex (Fig. 1). Behavioural complexity is often reflected in highly variable behavioural read-outs, which can be due to personality differences among individuals, as well as context-dependent behavioural response[33]. For example, behavioural phenotypes of fish (e.g. bold vs shy) may determine their responsiveness towards an acoustic stressor. In addition, fish response towards sound exposure may be modulated by various factors, such as time of day, temperature, light level and tide condition. Moreover, the behavioural changes may be permanent or temporary. The persistence of these changes needs to be examined, because fish may recover from or habituate to sound exposure. The behavioural recovery may be influenced by some acoustic or ecological factors, and carry management implications[34]. Furthermore, the implications of behavioural studies are sometimes difficult to ascertain because some behavioural changes may not directly indicate fitness consequences. However, these behavioural changes may reflect physiological stress or compromised energetics, which may in turn affect growth and reproduction, but these effects still need to be demonstrated.

Scope of the thesis

This thesis was part of a larger project entitled 'The effects of underwater noise on fish and marine mammals in the North Sea', funded by the Dutch National Ocean and Coastal Research Programme (NWO-ZKO). The project consisted of three subprojects with complementary aims: 1) generating sound maps to understand the distribution and composition of sounds in the North Sea, 2) relating the distribution of man-made sounds to the distribution of marine mammals and their exploitation of natural resources, and 3) assessing experimentally the impacts of different types of man-made sounds on fish behaviour. The latter was my project and I collaborated with Őzkan Sertlek from the first project to determine the relevant man-made sounds and acoustic parameters to be tested, and collaborated with Geert Aarts from the second project to understand the distributional relationship between fish and their predators, marine mammals under sound exposure.

In this thesis, I used the European seabass (*Dicentrarchus labrax*) as a model species (Fig. 4). European seabass are a very good model species to represent fish in the North Sea, since their ecology and hearing ability are generally similar to many other important commercial fishes, such as cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*), whiting (*Merlangius merlangus*) and pollock (*Pollachius pollachius*)[35]. Like these species, European seabass live and feed near

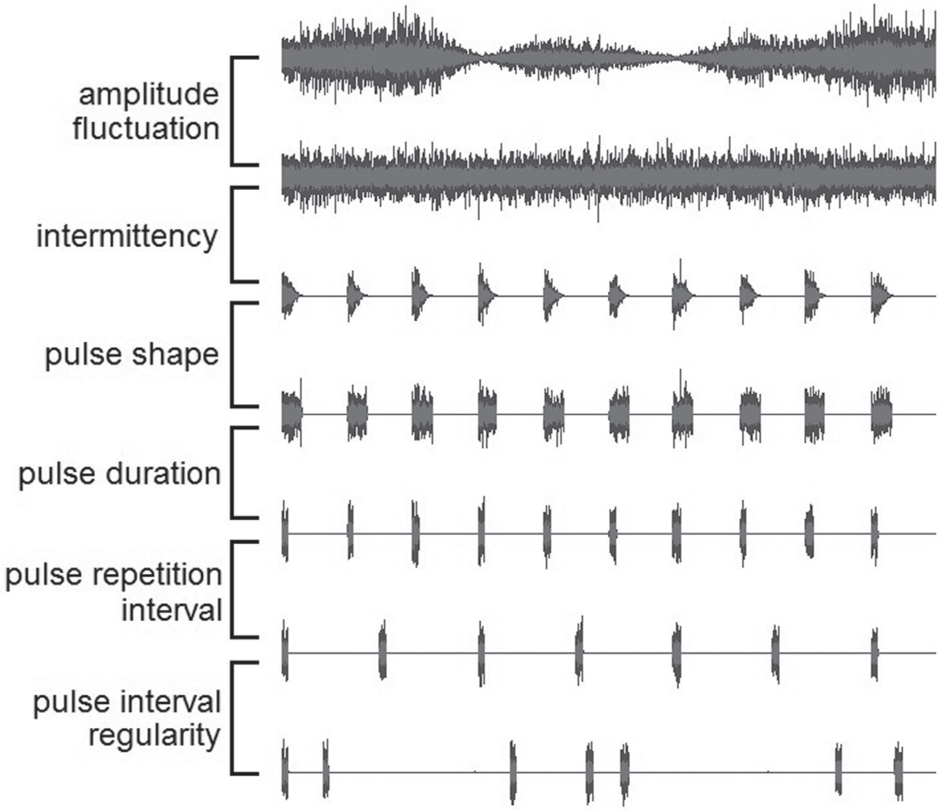


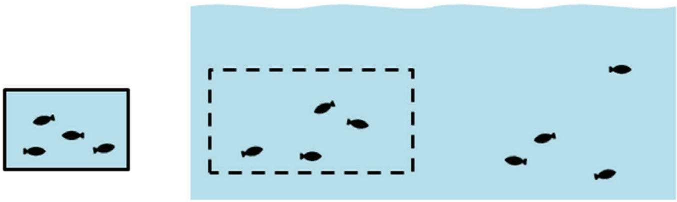
Fig. 2 Comparisons between acoustic waveforms showing different temporal parameters.

the sea bottom. They hear best below 1000 Hz using their otoliths and aided by the presence of swim bladder, without other accessory hearing apparatus[35–37]. Based on their auditory morphology, they possibly perceive both sound pressure and particle motion, although the latter component may be perceptually more dominant3. The fish are economically important and are widely farmed in Europe. They occur naturally throughout most of European coasts, including the North Sea, the Mediterranean Sea and the Black Sea[38]. Since these waters are full of human activities, the fish experience a great deal of man-made sounds. They are sensitive to man-made sounds and are known to respond with clear movement changes36. With the rise of underwater noise pollution, as well as the increasing pressure from commercial fishing[39], European seabass may suffer negative consequences that could disrupt the fish stocks.

Using hatchery-reared European seabass, I looked into how their swimming patterns changed upon sound exposure and how the changes could be associated with stress and anxiety. Next, I examined relevant acoustic parameters that contribute to noise impacts. More specifically, I focused on the influence of the temporal structure of sound. I teased apart different temporal parameters with a bottom-up approach, in which I artificially generated sound treatments that differed only in the parameters of interest, while keeping all other acoustic conditions the same. With this approach, I was able to conduct behavioural assays that revealed the relative impact strengths of different acoustic parameters. My findings have important implications for assessing noise impacts and devising exposure schemes.

In addition, I used the same experimental design to conduct experiments both in an indoor basin as well as an outdoor floating pen. The basin set-up offered much control of the experimental conditions to reveal high-resolution behavioural changes and potential impact differences caused by the temporal structure of sound. On the other hand, the semi-natural set-up offered more natural acoustic conditions and behavioural response, while allowing me to examine how noise impact may be influenced by other environmental factors, such as temperature, tide and time of day. By comparing findings from these two approaches, I demonstrated the generalisability of the behavioural changes. Moreover, the comparison may also provide insights into the underlying mechanisms of noise-induced behavioural changes.

This thesis contains six chapters. This general introduction chapter (chapter 1) is followed by two indoor experiments (chapter 2 & 3), two outdoor experiments (chapter 4 & 5), and a general discussion (chapter 6). **Chapter 2** examines how European seabass change their swimming patterns in an indoor basin upon sound exposure, and how sound intermittency and amplitude fluctuation affect the behavioural recovery. **Chapter 3** describes how the pulse repetition interval of impulsive sounds affects the immediate and delayed behavioural changes of European seabass. **Chapter 4** assesses the efficacy of a ‘ramp-up’ procedure and the influence of sound intermittency and pulse interval regularity in a semi-natural setting, while comparing findings with the two previous indoor basin studies. **Chapter 5** demonstrates if European seabass habituate to repeated sound exposures and whether sound exposure at night affects fish differently than during the day. **Chapter 6** summarises findings from previous chapters and provides guidelines for future research.



	Laboratory	Semi-natural	Field
Acoustic validity	LOW	HIGH	HIGH
Behavioural validity	LOW	MEDIUM	HIGH
Generalisability	LOW	MEDIUM	HIGH
Controllability	HIGH	MEDIUM	LOW
Replicability	HIGH	MEDIUM	LOW
Practicability	HIGH	MEDIUM	LOW

Fig. 3 Comparisons among three categories of experimental approaches: laboratory, semi-natural and field [adjusted after 32].

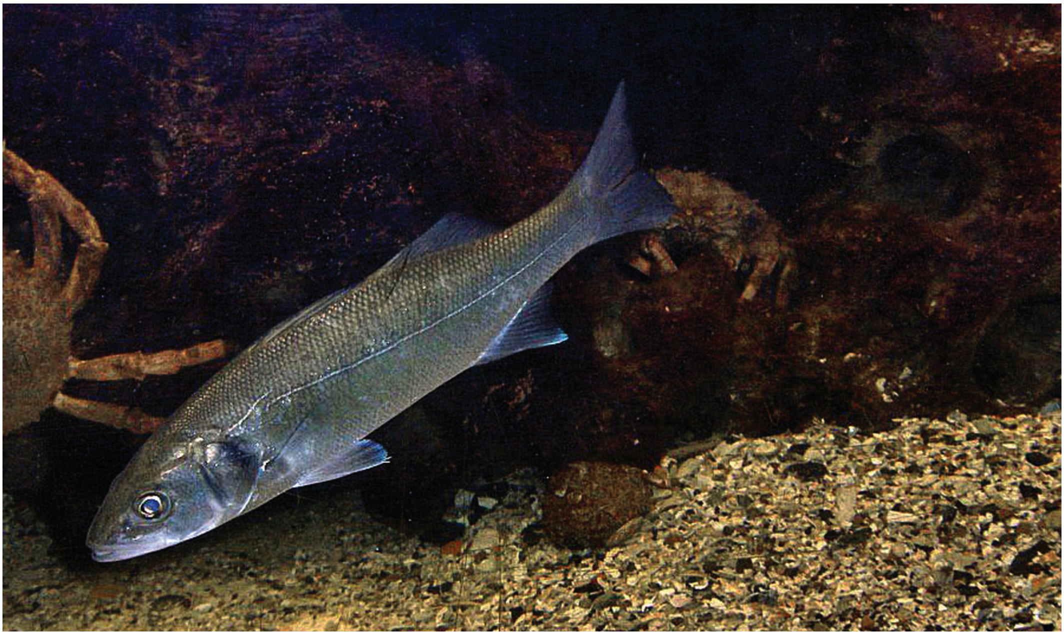


Fig. 4 European seabass (*Dicentrarchus labrax*).

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CHAPTER 2

Temporal structure of sound affects behavioural recovery from noise impact in European seabass

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2014. **178**: 65–73.

Abstract

Human activities in and around waters generate a substantial amount of underwater noise, which may negatively affect aquatic life including fish. In order to better predict and assess the consequences of the variety of anthropogenic sounds, it is essential to examine what sound features contribute to an impact. In this study, we tested if sounds with different temporal structure resulted in different behavioural changes in European seabass. Groups of four fish were exposed in an outdoor basin to a series of four sound treatments, which were either continuous or intermittent, with either consistent or fluctuating amplitude. The behavioural changes of the fish were analysed by a video-tracking system. All sound treatments elicited similar behavioural changes, including startle responses, increased swimming speed, increased group cohesion and bottom diving. However, with all other sound conditions being the same, intermittent exposure resulted in significantly slower behavioural recovery to pre-exposure levels compared to continuous exposure. Our findings imply that the temporal structure of sound is highly relevant in noise impact assessments: intermittent sounds, such as from pile driving, may have a stronger behavioural impact on fish than continuous sounds, such as from drilling, even though the latter may have higher total accumulated energy. This study urges regulatory authorities and developers to pay more attention to the influence of temporal structure when assessing noise impacts. However, more studies are needed to examine other sound parameters and to determine the generality of our observations in other species and in other outdoo water bodies.

Introduction

The underwater world is filled with a variety of biotic and abiotic sounds. In fact, these natural sounds are often so prominent that they have interfered with the underwater acoustic communication by the navy since the early 1900s[1]. However, as human exploitations of the marine environment increased over the years, a cacophony of anthropogenic sounds has also been introduced underwater through commercial shipping, offshore construction, sonar exploration, seismic surveys and underwater explosions. This change in the underwater acoustic scene may be posing a threat to marine life[2,3]. Consequently, underwater noise pollution has been listed in the European Union's Marine Strategy Framework Directive 2008/56/EC as one of the descriptors for achieving good environmental status, despite a considerable deficiency of empirical data.

In comparison to sea mammals, relatively few noise impact studies exist on fish, despite their high diversity, abundance, and economical importance[4,5]. All fish species studied to date can hear and many may use sound for habitat selection[6,7], conspecific communication[8,9] and predator-prey interactions[10,11]. Acoustic signals are especially effective over long distances or under low-visibility conditions. However, the biologically relevant sounds used by fish often overlap with anthropogenic noise, which typically also consists of relatively low frequencies[2,12]. This spectral overlap suggests that fish may be especially vulnerable to human-induced elevation of underwater noise levels.

Anthropogenic noise can be loud and localized or more moderate but widespread: both may affect fish differently. For example, several exposure experiments with high-intensity sounds, such as those resembling pile driving or explosions, have reported auditory tissue damage[13–17] or temporary hearing loss[18–22]. The exposure levels in these studies were usually very high, which in practice only happen when fish are in the immediate proximity of loud sound sources. In this regard, more moderate but widespread noise could be more critical to population and ecosystem stability as it covers wider areas affecting larger numbers of fish[2].

In particular, fish exposed to more moderate noise may also take an active role and alter their behaviour in response, which may alleviate some but induce other problems[23]. Anthropogenic sounds have, for example, been shown to disrupt spawning events[24], affect territorial dynamics[25] and reduce feeding efficacy[26]. Moreover, after seismic airgun shootings, fishing vessels have experienced significant catch reductions, suggesting active avoidance of the noise source by fish[27,28]. Many fish species also show startle responses[29] at the onset of noise exposure[26,30–33] and some dive to greater depth[34–38]. However, behavioural observations in these studies usually only lasted for several minutes and we still lack critical insights into the persistence of behavioural changes over longer periods[39], which may be related to long-term effects on growth and body condition e.g. [40,41].

The behavioural impact of anthropogenic sounds may not only be determined by their mere presence and level, but also by the frequency range, amplitude fluctuation and temporal structure of the sounds that arrive at a fish[2,42]. It is crucial to study these sound features explicitly because feature-dependent perceptual sensitivity may determine fish susceptibility to specific noise exposures. Among these sound features, little is known about the influence of temporal structure on noise impact but see [14,43], even though fish are known to be sensitive to the temporal characteristics of sounds, which may carry important information[44,45]. In terms of temporal structure, anthropogenic sounds vary with regard to intermittency (whether continuous or intermittent/impulsive), pulse duration, pulse repetition rate and pulse regularity. For example, seismic airgun and pile-driving noise are intermittent while wind turbine and ship noise are continuous. Moreover, sound amplitude may be fluctuating or consistent over time depending on the characteristics or movements of the sound sources. Hence, to assess the potential impact of anthropogenic noise, we need to understand what sound features actually contribute to the impacts.

In this study, we investigated whether intermittency and amplitude fluctuation of noise exposure contribute to behavioural changes and recovery in the European seabass (*Dicentrarchus labrax*), an important commercial fish species. The fish were exposed to artificially generated sounds resembling man-made noise, and their swimming patterns were analyzed with movement-tracking software.

Materials and methods

STUDY SPECIES

The European seabass is a demersal species that is commonly found in shallow waters in the North Sea and the Mediterranean Sea. It is an oceanodromous species and can tolerate a wide range of temperature and salinity[46]. The juveniles form schools but the adults are less gregarious and may shoal loosely with fewer individuals[46]. The species is known to hear best below 700 Hz[31] and has no accessory hearing organs besides the otoliths and the swim bladder.

ANIMAL MAINTENANCE

The European seabass used in this study came from a commercial hatchery (Ecloserie Marine, Gravelines, France) and were about 35 cm in total body length and 350 g in weight. The fish were kept in round polyester holding tanks (2.2m in diameter, 1m deep) before and after the test trials at the Sea Mammal Research Company (SEAMARCO) in Wilhelminadorp, The Netherlands. Water was refreshed continuously with a recirculating system connected to the nearby Oosterschelde estuary. The fish were fed Neo Grower Extra Marin pellets (Le Gouessant Aquaculture, Lamballe, France) every other day based on the temperature-dependent prescription by the manufacturer. Water temperature varied from 9 to 16 °C throughout the one-and-a-half-month experimental period (May-June 2012). All experiments were performed in accordance with the Dutch Experiments on Animals Act (DEC approval no: 12026) which serves as the implementation of the Directive 86/609/EEC by the Council of the European Communities regarding the treatment of animals used for scientific purposes.

EXPERIMENTAL ARENA

The experiment was conducted in a large outdoor rectangular basin (7 x 4 x 2 m) equipped with a water recirculating system at SEAMARCO [see 31 for details]. Next to the basin, there was a research cabin containing sound generating and monitoring equipment and video recording and monitoring equipment. During the exposure trials, fish were placed in a white nylon net enclosure (1.6 x 1.6 x 2.0 m) in the basin to ensure full coverage by two video cameras (Lanmda, China) for observation (Fig. 1). White tarps were positioned at the bottom and the background to ensure sufficient contrast in video images, without causing abnormal swimming behaviour in the fish.

TREATMENT SERIES

The fish were subjected to a series of four sound treatments: continuous consistent (CC), continuous fluctuating (CF), intermittent

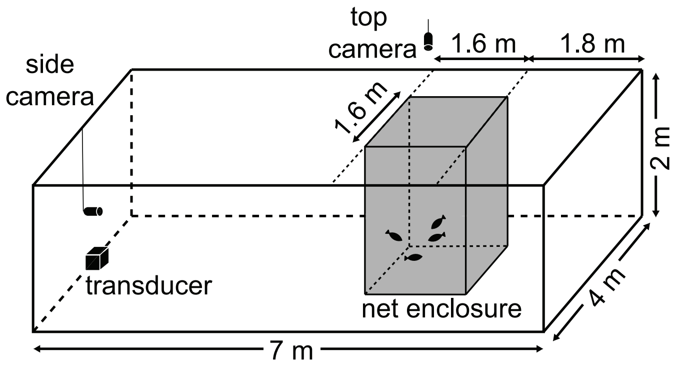


Fig. 1 Experimental arena in the outdoor basin at SEAMARCO. The transducer for playback is indicated on the left near the bottom and the net enclosure with the restricted swimming space for the four fish is indicated in grey.

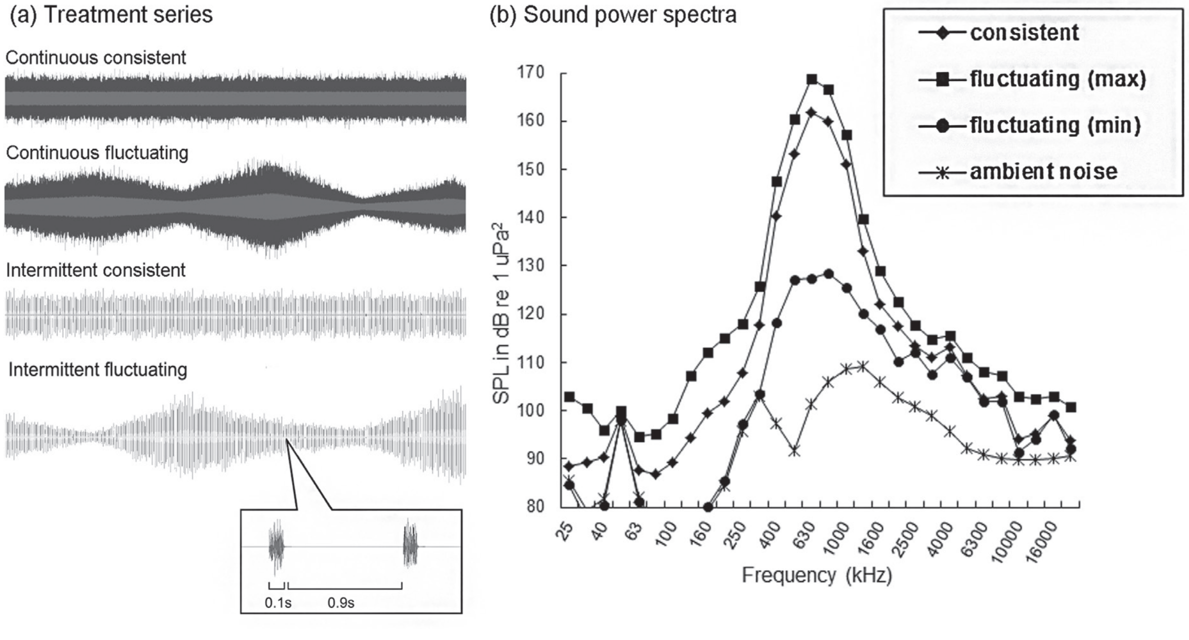


Fig. 2 (a) Time-domain waveforms showing 5 min of the four treatments exposed to each group of fish and a 2-second magnification of the intermittent treatments. (b) Frequency-domain power spectra in 1/3 octave bands of the four conditions measured for their SPL. The SPLs of consistent treatment, the maximum and minimum amplitude of fluctuating treatment and ambient noise are 165, 172, 134 and 115 dB re 1μPa respectively.

consistent (IC) and intermittent fluctuating (IF) (Fig. 2a). The treatments vary only in terms of the two temporal parameters of interest, i.e. intermittency and amplitude fluctuation, and have all other sound parameters (e.g. frequency bandwidth, start amplitude, average amplitude and exposure duration) kept the same. The continuous treatments consisted of elevated noise levels without interruption, while the intermittent treatments consisted of 0.1-second noise pulses interspersed with 0.9-second silent intervals, with a regular repetition rate of 1 pulse per second, comparable to the range of pile-driving sounds. The continuous and intermittent treatments were played back at either a consistent (CC & IC) or a fluctuating amplitude level (CF & IF). In order to vary only the temporal parameters of interest in the treatments while keeping all other sound parameters constant, the sound samples were created in Audition 3.0 (Adobe, San Jose, US) using filtered brown noise (bandpassed: 300-1000 Hz) and played back with an underwater transducer (LL-1424HP, Lubell Labs, Columbus, US) from a laptop connected to an attenuator (AS-2008-2, SEAMARCO, Harderwijk, Netherlands), a pre-amplifier (SEAMARCO, Harderwijk, Netherlands), a power amplifier (Macrotech 5000 VZ, Crown Audio, Elkhart, US) and a bandwidth filter (KrohnHite Corporation, Brockton, US).

Prior to the exposure trials, the sound pressure level (SPL) of the treatments was determined in the experimental basin with a calibrated system consisting of a broad-band hydrophone (8101, Brüel & Kjær, Denmark; frequency range 0–100 kHz), a voltage amplifier system (TPD, TNO, Netherlands; frequency range 0–300 kHz) and a personal computer with spectral analysis software (Cool Edit Pro, Syntrillium Software Corp, US; sample frequency 11–96 kHz, frequency range 0–48 kHz, df = 15–115 Hz). The SPL was calculated as the root mean square of sound pressure over 10 s. Four conditions were measured: the ambient noise level without playback, the CC treatment, the maximum amplitude of the CF treatment and the minimum amplitude of the CF treatment (same level as when no sound was played but with the transducer switched on). Since the intermittent treatments were created from the continuous treatments in the computer program, their equivalent SPL was assumed to be the same as their corresponding continuous treatments.

Measurements made at 56 points in the basin (in a 7 x 4 x 2 grid) showed that the sound pressure field was rather homogenous in the whole basin (± 0.8 dB). The ambient SPL in the experimental basin was 115 dB re 1µPa. When the transducer was switched on, the SPL increased to 134 dB re 1µPa, even though no sound was played, due to a low humming sound made by the transducer. However, this increase in SPL did not trigger any fish response during the trials. The SPL of the consistent amplitude treatments was 165 dB re 1µPa and the SPL of fluctuating amplitude treatments changed gradually every minute between random levels ranging from 134 to 172 dB re 1µPa.

Spectral investigation confirmed that most of the sound energy during experimental exposure was concentrated between 250 and 1600 Hz (Fig. 2b), around the best hearing sensitivity for seabass [31,47]. Due to technical limitations, measurements were only conducted for SPL and not for particle motion. Both sound components are probably perceived by European seabass, but particle motion may be perceptually more dominant[48], although there is still a general lack of insight about the relative contributions of both sound components to hearing in most species[49,50]. The ratio of sound pressure to particle motion in our basin is probably not constant like in the far-field at open sea[51]. Nonetheless, we believe this potential acoustic heterogeneity is not a concern for our bio-assay, as we use broadband sound treatments that differed only in the temporal structure. We expect that the temporal variation in SPL that we measured reflect the temporal variation in particle motion, which makes our set-up valid for comparing the effects of sound temporal structure and relative amplitude variation.

EXPERIMENTAL SET-UP

We tested twelve groups of four fish, where each group was exposed to all four treatments (48 fish, N = 12). The order of the treatments per fish group followed an incomplete counterbalanced design. At least 17 h prior to the trials, each fish group was transferred to the experimental basin to allow acclimatization. The fish acclimatized quickly to the conditions in the experimental arena after being introduced in groups of four from their holding tanks. Within one or two hours, they swam at a relaxed speed throughout the experimental arena. We conducted two trials per day: one in the morning and one in the afternoon, with a break of at least three hours in between. The trials consisted of 30 min of silence before and after 30 min of sound exposure. 30 min before each trial, the transducer and the lights above the experimental basin were turned on (to ensure good video quality), while the oxygen pump and the water recirculating system were turned off (to minimise ambient noise). Water temperature (9-16 °C), light intensity (320-1280 lux), wind speed (13-35 kmh^{-1}) and weather condition (sunny, cloudy or rainy) were recorded before the start of the trials, to be used as covariates in the data analysis. During the trials, no external anthropogenic noise or disturbance was allowed near the study area.

BEHAVIOURAL OBSERVATION & ANALYSIS

Fish behaviour was video-recorded during the entire exposure sessions (30 min before, 30 min during and 30 min after sound exposure). The recordings from the top camera were used for the analysis of startle responses, defined as sudden changes in swimming direction accompanied by acceleration. Four 5-minute fragments were cut from the recordings: right before sound exposure ('before'), at the start of sound exposure ('during 1'), right before the end of sound exposure ('during 2') and right after sound exposure ('after'). The number of all startle responses exhibited by the four fish was then scored manually, blind to the observer (J.S.) for the treatment type and exposure period. For the analysis of other behavioural parameters, the full recordings from the side camera were analysed with tracking software, Logger Pro 3.8.5.1 (Vernier Software & Technology, Beaverton, US), which assigned a pair of coordinates to each fish in a trial every second by on-screen visual tracking and manual cursor placement. The coordinates were then used to calculate swimming depth, group cohesion (the average distance between pairs of individuals) and swimming speed.

STATISTICS

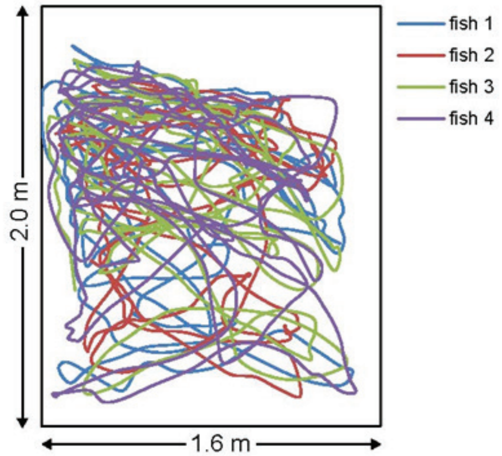
Startle responses were only observed at the onset of sound exposure and the effect of treatments on the number of startle responses was analysed with the Friedman test. To test for the magnitude of change in swimming depth and group cohesion during the trials, we compared four 5-minute period bins from our data set: 'before', 'during 1', 'during 2' and 'after' (like the analysis of startle responses). Two-way repeated measures ANOVAs were then performed, with treatment and period as the withinsubject factors. For swimming speed, the analysis was the same, but the period bins used were 10 s instead of 5 min due to the transient nature of the increase in speed. We also analysed the recovery time of behavioural changes, which was defined as the time that the fish took to revert back to the pre-exposure swimming depth and group cohesion. The 5-minute average of swimming depth and group cohesion before exposure was used as a baseline to compare with the 5minute moving averages (shifting forward every second) during and after exposure, to see when the baseline was reached again. If the baseline was not reached after 60 min (30 min after sound exposure), the recovery time was counted as 60 min (occurred in 4 out of 48 trials for swimming depth and 2 out of 48 trials for group cohesion). For swimming speed, the analysis was the same, but the averages used were 10 s instead of 5 min. The effects of temporal parameters on the recovery time were then analysed with twoway repeated measures ANOVAs, with intermittency and amplitude fluctuation as the withinsubject factors. The data of the recovery time were log-transformed to fulfil the assumptions of normality and homoscedasticity. When sphericity could not be assumed in a repeated-measures ANOVA, Huynh-Feldt correction was used. For ANOVAs with a significant outcome, Bonferroni-corrected posthoc tests were conducted.

Results

Startle responses were seen in 41 of the 48 trials and typical only in the first 10 s of sound exposure (all within period 'during 1', except in 2 of the 48 trials where it happened also in period 'after'). The number of startle responses in the first 5 min of exposure did not differ among treatments of different temporal structure (Friedman's test: $X^2_3 = 1.991$, $P = 0.574$). Fish typically dived to the bottom during the first 5 min of exposure (Fig. 3), which is supported by a significant drop in swimming depth of 32.19 ± 19.62 (SD) cm (repeated-measures ANOVA: $F_{3,27} = 25.457$, $P < 0.001$; Bonferroni-corrected *post-hoc*: 'during 1' compared to three other periods, all Ps < 0.005). However, there were no significant effects of treatment and treatment-period interaction ($F_{3,27} = 1.094$, $P = 0.368$; $F_{5.7,51.4} = 1.132$, $P = 0.357$ respectively). The distance between individuals also became 7.01 ± 8.00 (SD) cm closer during the exposure, leading to a significantly tighter group cohesion (repeated-measures ANOVA: $F_{3,27} = 11.078$, $P < 0.001$; Bonferroni-corrected *post-hoc*: 'during 1' compared to three other periods, all Ps < 0.05). Again, no significant effects of treatment and treatment-period interaction were found ($F_{3,27} = 0.441$, $P = 0.726$; $F_{9,81} = 0.643$, $P = 0.757$ respectively). Besides, group cohesion did not correlate with swimming depth ($R^2 = 0.06$). Furthermore, a significant increase in swimming speed of 53.99 ± 42.84 (SD) cms^{-1} was also observed at the onset of sound exposure (repeated-measures ANOVA: $F_{1.60,17.65} = 35.330$, $P < 0.001$; Bonferroni-corrected *post-hoc*: 'during 1' compared to three other periods, all Ps < 0.05).

Besides analysing the magnitude of the behavioural changes, we also examined the recovery of these behavioural patterns (Fig. 4). We found that the recovery time (log-transformed) of swimming depth was affected by sound intermittency (Fig. 5), with intermittent treatments showing 19.74 ± 20.70 (SD) min longer recovery time than continuous treatments (repeated-measures ANOVA: $F_{1,11} = 23.569$, $P = 0.001$). However, it was not influenced by amplitude fluctuation (repeated-measures ANOVA: $F_{1,11} = 0.046$, $P = 0.835$). In contrast, the recovery time (log-transformed) of group cohesion was not significantly influenced by sound intermittency (repeated-measures ANOVA: $F_{1,11} = 0.001$, $P = 0.978$) and amplitude fluctuation (repeated-measures ANOVA: $F_{1,11} = 4.264$, $P = 0.063$), although the latter was a non-significant trend with a difference of 9.01 ± 21.93 (SD) min. The recovery time (log-transformed) of swimming speed was also not significantly influenced by sound intermittency (repeated-measures ANOVA: $F_{1,11} = 0.107$, $P = 0.749$) and amplitude fluctuation (repeated-measures ANOVA: $F_{1,11} = 1.353$, $P = 0.269$).

(a) Before sound exposure



(b) During sound exposure

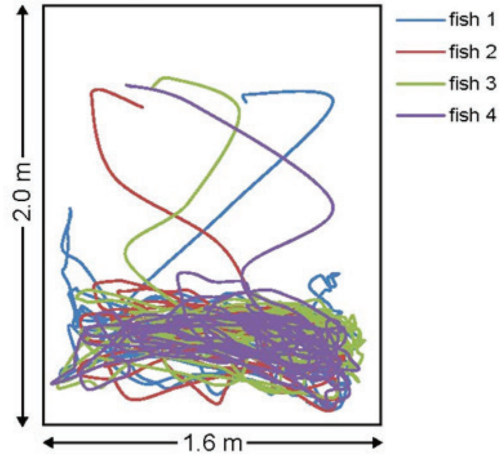
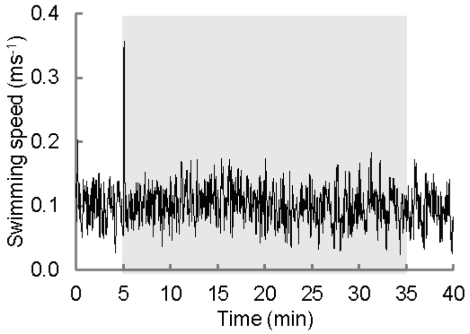
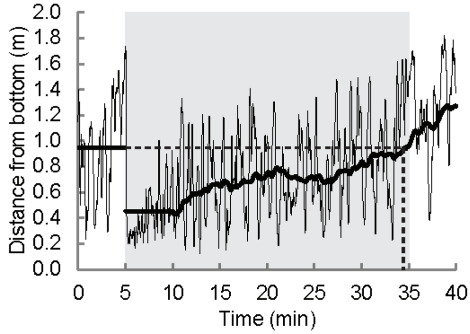


Fig. 3 Typical swimming track images of four fish within the net enclosure viewed from the side camera (a) 5 min before and (b) 5 min at the start of sound exposure (example of an IF treatment trial). The fish occupy the whole study arena before sound exposure but dive to the bottom at the onset of sound exposure and stay there for the next 5 min.

(a) Swimming speed



(b) Swimming depth



(c) Group cohesion

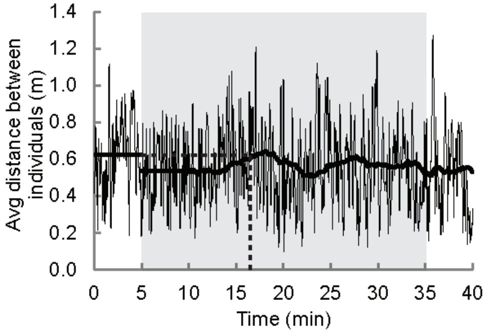
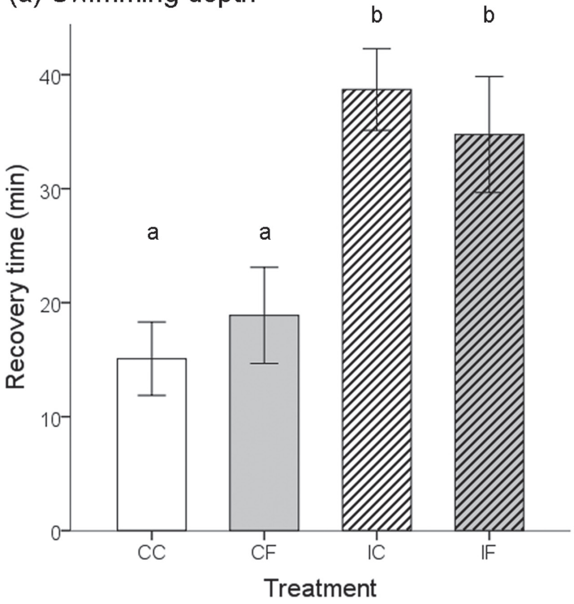


Fig. 4 Time series showing typical (a) swimming speed, (b) swimming depth and (c) group cohesion of fish 5 min before, 30 min during (shaded) and 5 min after sound exposure (example of an IC treatment trial). In (b) & (c), the thick line in the first 5 min is the average level before sound exposure; the thick line thereafter is the 5-minute moving averages shifting forward every second; the dashed line is the trace of how recovery time is determined.

(a) Swimming depth



(b) Group cohesion

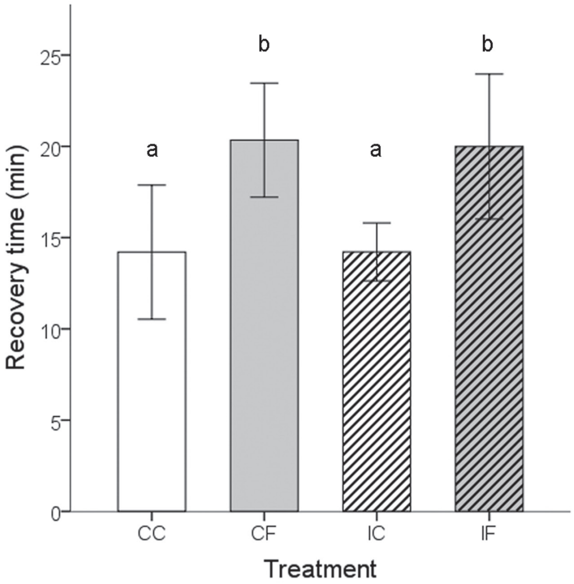


Fig. 5 Recovery time (± SE) of (a) swimming depth and (b) group cohesion of fish. The recovery time of swimming depth is significantly longer for intermittent treatments than for continuous treatments (a's and b's reflect statistically different groups, $P = 0.001$). For the recovery time of group cohesion, there is a trend for the recovery time of amplitude fluctuation to be longer than amplitude consistency (a's and b's reflect statistical trend for being different groups, $P = 0.063$). Note that the graphs are not log-transformed but do show the same patterns after transformation.

Discussion

Our results revealed that all the different noise treatments initially resulted in a similarly strong response with behavioural changes that are typically associated with fright and anxiety[52–54]. However, intermittent exposure prolonged the recovery of swimming depth significantly compared to continuous exposure.

BEHAVIOURAL RESPONSES RELATED TO ANXIETY?

Caution is necessary when making direct comparisons between studies in captivity and in the wild, as there are important behavioural and acoustic differences[55]. However, the nature of the behavioural changes observed in our study on captive European seabass mirrors earlier observations on other captive, semi-wild, or wild species. For example, several studies have shown that fish dive deeper upon noise exposure[34–38]. Considering that our experimental basin had a rather homogenous sound pressure field independent of depth, we believe that the response of our seabass is not an active avoidance of the sound source but a typical anxiety-related reaction. Many fish species have been reported to dive to greater depth when subjected to various stressors such as predation cues[56,57], novel environments[58–60], chemical pollution[61] and anxiogenic drugs[53,58]. Moreover, two field studies have shown that vessel noise induced more immediate and prominent fish movements in the vertical axis down the water column than in the horizontal axis away from the vessel[34,37]. Therefore, diving behaviour may be a good indicator, in both indoor and outdoor settings, of a primary stress response, by which pelagic and demersal fish species may try to escape danger and minimize potential risks of predation.

Changes in group cohesion as a result of anthropogenic noise exposure have also been documented in previous studies[35,62]. Some species may form tighter shoals[35] while others may decrease school coordination[62]. Both directions of change may reflect an anxiety-driven response related to the perception of potential danger[63]. The difference in response may be related to the variation in test conditions, group sizes[64] or species-specific escape strategies that may be different for shoaling and highly coordinated schooling aggregations[65].

We also observed startle responses and sudden accelerations of fish at the onset of exposures, which have been reported by many other researchers[26,30–33,35]. These erratic movements in the form of rapid flight reflexes are mediated by Mauthner cells in the hindbrain and were an anti-predation strategy that have been associated with anxiety[29,58]. They have also been shown to co-occur with an increase in lactate and haematocrit levels in our study species, the European seabass, when exposed to artificial sound[66]. Although we did not take any physiological measurements, the behavioural changes observed in our study could have been accompanied by an increase in anxiety-related hormones (e.g. cortisol) upon noise exposure, as reported in several other studies[20,67–69]. However, the correlation between behaviour and stress physiology is not always clear[70].

BEHAVIOURAL RECOVERY FROM NOISE EXPOSURE

Our experimental noise exposure resulted in a highly consistent initial behavioural response among all four treatments. However, the recovery time varied significantly among treatments, allowing the investigation into the relative impact of the temporal variation in sound exposure. Three mechanisms may explain the behavioural recovery observed in our study: 1) habituation, 2) sensory adaptation and 3) motor fatigue[71]. Habituation happens in the central nervous system, filtering out irrelevant stimuli and allowing animals to focus selectively on biologically important stimuli; sensory adaptation happens in sensory organs as a result of decreased sensitivity induced by acute stimulations, e.g. temporary threshold shift (TTS); motor fatigue happens in muscles due to exhaustion.

We believe that the first explanation, habituation, is most likely the dominant determinant for the behavioural recovery during the noise exposure in our study. One of the characteristics of habituation is that higher stimulation rates result in faster habituation[72,73]. The faster recovery rate for continuous noise (constant stimulation, thus high stimulation rate) compared to intermittent noise (relatively low stimulation rate) in our study, implicitly suggest that the recovery was possibly due to habituation. Furthermore, we observed fast inter-trial recovery, where a successive noise exposure after 3 h induced the same level of behavioural changes as the previous exposure. This renders sensory adaptation a less likely explanation for the behavioural recovery, as TTS would usually take days to weeks to recovery[20,21,74]. Even if TTS did occur, it was probably not strong enough to hinder fish hearing of excessive sounds. Motor fatigue may have been responsible for the rapid recovery (< 20 s) of swimming speed[75], but less likely for swimming depth and cohesion, as these parameters are generally not associated with extensive muscle use. However, further investigations are necessary before we can be more conclusive about the mechanism underlying the behavioural recovery in our assay[72].

Our results also seem to suggest qualitatively different influence of the two temporal parameters tested: intermittent treatments affected the recovery time of swimming depth, while fluctuating treatments did not, but they tended to affect the recovery time of group cohesion. This finding suggests that different stress responses exhibited by fish may be determined by specific acoustic characteristics of a sound stressor. For example, compared to the continuous treatments, the fluctuating treatments had an unpredictable rise and fall in amplitude that may resemble more natural and biologically relevant sounds, such as an approaching predator. These fluctuations may have been perceived as potentially more dangerous, which made the fish stay close together for longer. When Speedie & Gerlai[76] exposed zebrafish (*Danio rerio*) to an alarm pheromone, the fish also showed tighter group cohesion without spending more time close to the tank bottom like when exposed to other stressors[56,58]. However, more studies are needed in order to shed light on the potential relationship between qualitatively different behavioural responses and stressor types.

MANAGEMENT IMPLICATIONS

Our findings call for a reconsideration of current practices in noise impact assessments, which typically only consider the standard metrics, such as SPL, sound exposure level (SEL) and exposure duration, but not the temporal patterns of noise exposure. Although these metrics may be important in determining the impacts of high-intensity exposure in terms of auditory tissue injury and temporary hearing loss[16,17,21], here we show that they are not always sufficient when assessing the behavioural impact of exposure to more moderate levels. For example, while having comparable SPL, our intermittent treatments with a 10% duty cycle would only accumulate ~10% of the energy of the continuous treatments, hence in principle had about 10 dB lower cumulative SEL (SEL_{cum}) than the continuous treatments. Yet, our results revealed that the intermittent treatments prolonged the behavioural recovery for twice as long as the continuous treatments, suggesting that noise treatments with a lower

SEL_{cum} may sometimes lead to a stronger behavioural impact. Since the behavioural impact of anthropogenic noise is more widespread than physical harm[2], temporal structure of sound should be considered more prominently in noise impact assessments and taken into account when devising mitigating measures.

Our findings suggest that noise impact on fish may be alleviated by modifying the temporal pattern of exposure schemes. For example, offshore piling may become more fish-friendly by using more continuous drilling techniques instead of the conventional impulsive pile-driving methods. Drilling may reduce not only the risk of physical impact for fish near the construction site due to lower SPL and SEL[77], but also the extent of behavioural impact for fish in a much larger area through faster habituation and recovery of natural activities. Because of the different impact strengths related to the temporal structure of sound exposure, the thresholds of detrimental effects on fish behaviour are possibly also lower for intermittent sounds compared to continuous sounds, although it is still not easy to suggest any quantitative adjustments.

However, at this early stage of research, considerable care should be exercised when assessing management implications[78]. On the one hand, even though all the behavioural changes observed in our study point to anxiety and may involve stressful physiological changes, little is known about whether these behavioural changes will result in fitness consequences through reduced survival, growth and reproduction. On the other hand, even in the absence of direct behavioural reactions, subtle aspects of behaviour such as the time spent on nest guarding, may still be affected[39], potentially leading to significant consequences for life-time reproductive success.

We also need to be cautious with the extrapolation of data from captive to wild animals[55,79]. Wild fish have, for example, the potential to swim away from the sound source, which could result in a different kind of behavioural response. Furthermore, wild fish have been shown to be more vulnerable to some stressors than captive ones[80,81], suggesting that behavioural impact of noise exposure may be stronger for wild fish. Absolute threshold levels assessed under captive conditions are therefore likely to have limited value in the wild and we emphasize that the value of our findings is in the relative impact of the different treatments varying in the temporal patterns of noise exposure.

CONCLUSIONS

Our study provides clear evidence of the contribution of sound temporal pattern to the behavioural recovery of fish after noise disturbance. Many fundamental questions remain, but we argue that the insights from our study provide important implications for underwater noise impact assessments. We believe that temporal variations are critical when assessing or predicting the severity of anthropogenic noise impact on fish behaviour and may have applied value for devising mitigating measures.

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CHAPTER 3

Impulsive sounds change European seabass swimming patterns: Influence of pulse repetition interval

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Abstract

Seismic shootings and offshore pile-driving are regularly performed, emitting significant amounts of noise that may negatively affect fish behaviour. The pulse repetition interval (PRI) of these impulsive sounds may vary considerably and influence the behavioural impact and recovery. Here, we tested the effect of four PRIs (0.5-4.0s) on European seabass swimming patterns in an outdoor basin. At the onset of the sound exposures, the fish swam faster and dived deeper in tighter shoals. PRI affected the immediate and delayed behavioural changes but not the recovery time. Our study highlights that (1) the behavioural changes of captive European seabass were consistent with previous indoor and outdoor studies; (2) PRI could influence behavioural impact differentially, which may have management implications; (3) some acoustic metrics, e.g. SEL_{cum}, may have limited predictive power to assess the strength of behavioural impacts of noise. Noise impact assessments need to consider the contribution of sound temporal structure.

Introduction

The ever increasing global energy demand has led to extensive exploitation of seas and oceans for both fossil and sustainable energy resources[1]. Related human activities, such as seismic surveys and offshore constructions for wind farms and oil rigs, generate a substantial amount of noise in the underwater environment. This introduction of anthropogenic noise into the underwater acoustic scene may post a threat to aquatic life, including fish, causing a range of negative effects, from physical injuries in close range, to behavioural changes further away from the sound sources[2–4]. To ensure the stability of marine ecosystems under increased pressure of ocean exploitation, it is important to understand whether and how underwater anthropogenic noise may affect fish behaviour, which in turn may have consequences on fish populations.

Whether behavioural changes will result in negative fitness consequences, depends partly on whether fish habituate to the noise exposures and recover from the changes. However, behavioural observations in previous noise impact studies generally did not last long enough to show recovery after initial behavioural changes[5–8,but see 9]. Moreover, a recovery or a decrease in response does not necessarily denote habituation, where the animals hear selectively while filtering out repeated or irrelevant sound signals in the background[10]. A decrease in behavioural response could also be attributed to 1) sensory adaptation, i.e. the sensitivity of the hearing organs is reduced by loud exposures, leading to temporary threshold shift (TTS), or 2) motor fatigue, i.e. animals become unresponsive due to exhaustion[11]. It is crucial to determine the mechanism of such behavioural recovery since the different mechanisms vary in their ecological implications.

Underwater noise impact assessments are complex also because anthropogenic noise shows a variety of amplitudinal, spectral and temporal variations. Of these, the temporal structure of sound is rarely studied, even though it may play a crucial role in triggering behavioural response in fish[9,12]. For example, Neo *et al*[9] showed that European seabass (*Dicentrarchus labrax*) recovered more slowly from impulsive sounds than from continuous sounds (despite the former having lower accumulated sound pressure level), after exhibiting consistent initial behavioural changes upon noise exposures. Considering that impulsive sounds differ in various temporal features, there is a need for systematic studies addressing other temporal parameters, such as pulse repetition interval, pulse repetition regularity, pulse duration and pulse shape (including rise time).

Among these temporal parameters, pulse repetition interval (PRI), which can also be expressed in pulse repetition rate (PRR, where PRR = 1 / PRI), is rather variable among the current practices in pile driving and seismic surveys. PRI generally varies between 1 – 4 s[13] for pile driving and 5 – 15 s[14] for seismic surveys. Different PRIs have been shown to influence the habituation rate to repeated sound stimuli in zebrafish and rats[15,16]. However, it is unclear if PRI also contributes to fish habituation to impulsive anthropogenic sound exposures, such as pile driving and seismic shootings.

In this study, we used a similar setup as in Neo *et al*[9] to answer two questions: 1) How do impulsive sounds of different PRIs (0.5 s, 1.0 s, 2.0 s, 4.0 s) affect the swimming patterns and behavioural recovery of European seabass? 2) Can the behavioural recovery be attributed to habituation? We expected larger PRIs to prolong the behavioural recovery and the recovery be attributed to habituation.

Materials and methods

ANIMAL MAINTENANCE

The European seabass (mixed sex; 20-25 cm in total body length) came from a commercial hatchery (Ecloserie Marine, Gravelines, France) and were kept in four round holding tanks (diameter: 2.2 m; depth: 1 m) before and after the test trials at the Sea Mammal Research Company (SEAMARCO) in Wilhelminadorp, The Netherlands. Water was refreshed continuously with a recirculating system connected to the nearby Oosterschelde marine inlet and the water temperature varied from 4 to 12 °C throughout the experimental period (May-June 2013). Fish were fed pellets (Le Gouessant Aquaculture, Lamballe, France) every other day based on a temperature-dependent prescription. All experiments were performed in accordance with the Dutch Experiments on Animals Act and approved by the Animal Experiments Committee at Leiden University (DEC no: 13023).

EXPERIMENTAL ARENA

The experiment was conducted in a large outdoor rectangular basin (7 x 4 x 2 m) equipped with a water recirculating system at SEAMARCO. During the exposure trials, fish were put in a white nylon net enclosure (4 x 1.6 x 2 m) to ensure full coverage by an underwater video camera for observation (Fig. 1). A white tarp was placed at the bottom and in the background to ensure sufficient contrast in video images, without disrupting the normal swimming behaviour of the fish. Beside the basin, there was a research cabin containing sound generating and video monitoring equipment.

TREATMENT SERIES

We exposed the fish to a series of four regularly repeated impulsive sound treatments differing in PRI: 0.5 s, 1.0 s, 2.0 s and 4.0 s (Table 1). The pulse duration of all the treatments was the same, which was around 0.15 s (Fig. 2a). The pulses were created in Adobe Audition 3.0 using filtered brown noise (bandpassed: 200-1000 Hz; matching the hearing range of European seabass) and played back with an underwater transducer (LL-1424HP, Lubell Labs, Columbus, US) from a laptop through a power amplifier (Macrotech 5000 VZ, Crown Audio, Elkhart, US). The whole experimental arena had a very homogenous sound pressure field during the playback of broadband sounds[9]. The average root-mean-square sound pressure level (SPLrms) before the exposure (ambient) in the experimental basin was 104 dB re 1µPa, which was comparable to the ambient noise levels of our measurements in the Oosterschelde marine inlet. To quantify the amplitude level of the impulsive sound treatments, single-strike sound exposure level (SEL_{ss}) and zero-to-peak sound pressure level (SPL_{z-p}) were measured (Table 1). The amplitude levels were chosen to represent received level of pile driving at a range of around 50-100 km according to ideal cylindrical spreading. Spectral investigation confirmed that most of the sound energy of the pulses was concentrated between 200 and 1000 Hz (Fig. 2b).

Particle motion may be perceptually dominant in European seabass hearing[17], but we were unable to measure this. However, we believe that the lack of this information is not a concern in this study, since our aim was not to assess absolute threshold levels that can be extrapolated to outside conditions. Our main interest was to compare the effects of PRI on behavioural response while keeping other acoustic parameters constant.

EXPERIMENTAL SET-UP

We tested twelve groups of four fish, where each group was exposed to all four treatments (N = 12, 48 fish). The order of the treatments per fish group followed an incomplete counterbalanced design (12 of 24 possible orders), to minimise the potential 'carry-

Table 1. Relevant acoustic parameters of the four sound treatments: pulse repetition interval (PRI), pulse repetition rate (PRR), exposure duration, average zero-to-peak sound pressure level ($SPL_{z\text{-}p}$), average single-strike sound exposure level (SEL_{ss}), number of pulse and average cumulative sound exposure level (SEL_{cum}).

Treatment no	PRI (s)	PRR (s^{-1})	Duration (min)	Avg $SPL_{z\text{-}p}$	Avg SEL_{ss}	Pulse no	Avg SEL_{cum}
1	0.5	2.00	60	158	140	7200	179
2	1.0	1.00	60	158	140	3600	176
3	2.0	0.50	60	158	140	1800	173
4	4.0	0.25	60	158	140	900	170

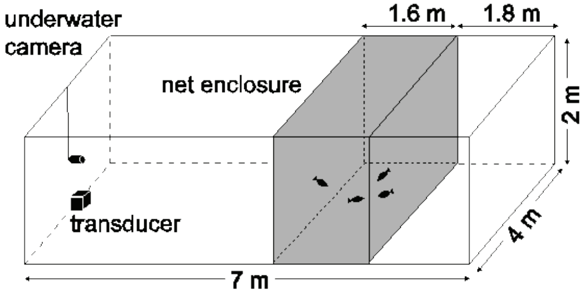
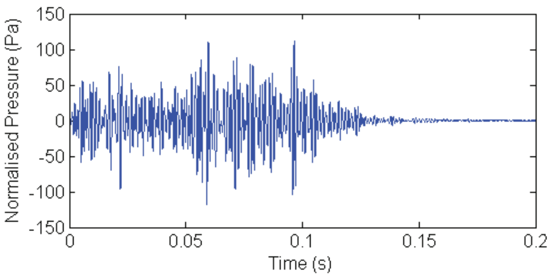


Fig. 1 Experimental basin at SEAMARCO. Shaded area is the net enclosure with restricted swimming space for four fish.

(a) Time domain waveform



(b) Power density spectra

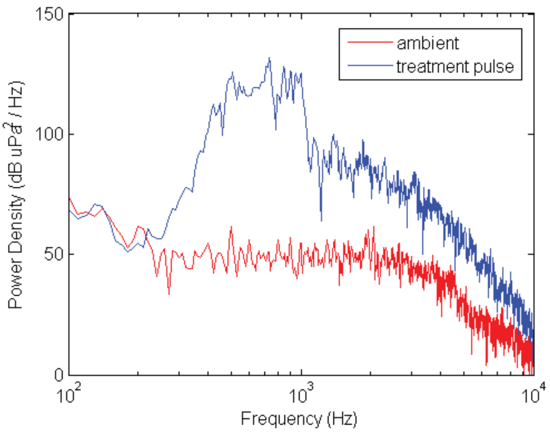


Fig. 2 (a) Time domain waveform of a single pulse used in the treatments and (b) power density spectra of the same pulse and the ambient noise before the playback of the pulse.

over' effect due to sequential exposures. At least 17 h prior to the trials, each fish group was transferred to the experimental basin to allow acclimatisation. 30 min before each trial, the transducer and the lights above the experimental basin were turned on. We conducted two trials per day: one in the morning and one in the afternoon, with a break of at least three hours in between. There was no external anthropogenic noise or disturbance near the study area during the trials. The trials consisted of 10 min of pre-exposure silence and 60 min of sound exposure. Based on pilot and previous studies[9], we expected the fish behaviour to recover within 60 min of sound exposure. Right after the playback of the 60-min treatment sound, a 2-second 600Hz tone (SEL_{ss} = 156 dB re 1µPa, $SPL_{z\text{-}p}$ = 157 dB re 1µPa) was played back to test for stimulus specificity of the behavioural recovery, which was to demonstrate that the recovery was due to habituation and not sensory adaptation or motor fatigue[10]. The fish were expected to show startle response again upon presentation of the tone, if they had only habituated to the repeated impulsive treatment sound and could still hear and react to a novel sound stimulus.

BEHAVIOURAL OBSERVATION & ANALYSIS

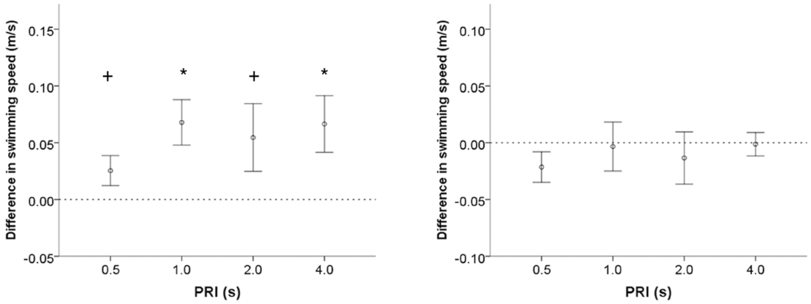
The fish were video-recorded during the entire exposure sessions (10 min before, 60 min during and 10 s after sound exposure). The full video recordings were analysed with tracking software, Logger Pro 3.8.5.1 (Vernier Software & Technology, Beaverton, US), with manual placement of all fish coordinates every second. The coordinates were then used to calculate the swimming speed, the swimming depth and the group cohesion (average inter-individual distance).

STATISTICS

To test for the change in swimming depth and group cohesion during the trials, we conducted two-way repeated measures ANOVAs comparing three 5-minute bins of exposure sequence from our data set: 5 min right before sound exposure ('before'), the first 5 min of exposure ('start') and the last 5 min of exposure ('end'). The bin length was chosen based on a previous study with comparable setup[9]. Both exposure sequence and treatment were treated as within-subject (repeated) factors. If sphericity cannot be assumed, Huynh-Feldt correction was used. To test for the change in swimming speed, we used the same procedure but the bins were 10 s instead of 5 min, in order to capture the transient nature of speed change. The order effect was also tested in the model as a covariate but was subsequently excluded when it showed no correlation.

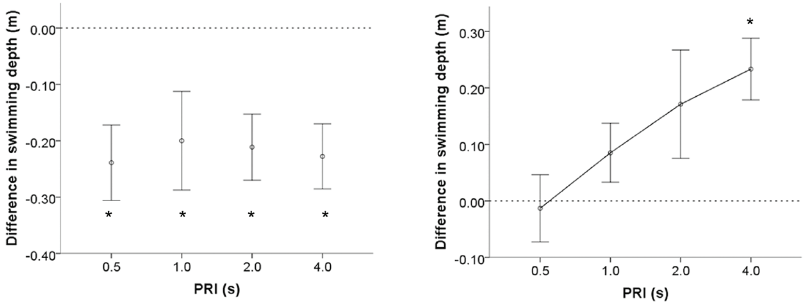
To understand the interaction of the two factors in the previous test and find out if the behavioural changes varied systematically across treatments of different PRIs, we performed repeated measures linear contrast analyses to compare the difference before and at the start of exposure, as well as before and at the end of exposure for the three parameters above. This statistical test has a higher power than omnibus ANOVA tests, given that we had a priori expectation that our variables correlated in a linear manner. We also performed one-sample *t*-tests to see if each difference mentioned was significantly larger than 0.

We also analysed the recovery time of the behavioural changes, which was defined as the time that the fish took to revert back to the pre-exposure swimming depth and group cohesion. The 5-minute average of swimming depth and group cohesion before exposure was used as a baseline to compare with the 5minute moving averages (shifting forward every second) during exposure, to see when the baseline was reached again. If the baseline was not reached by the end of the trial, the recovery time was counted



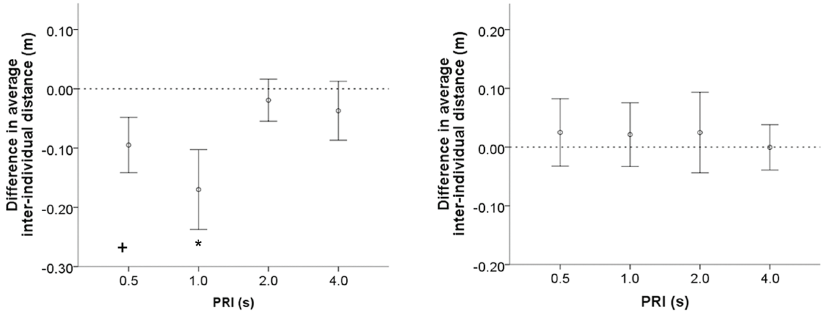
(a) Swimming speed | before - start

(b) Swimming speed | before - end



(c) Swimming depth | before - start

(d) Swimming depth | before - end



(e) Group cohesion | before - start

(f) Group cohesion | before - end

Fig. 3 The difference in swimming speed, swimming depth and group cohesion (mean ± SE) before and at the start of the exposure, and before and at the end of the exposure, for the four different pulse repetition interval (PRI) treatments. (a) Swimming speed increases in all the treatments at the start of the exposure, (b) and resumes back to baseline level at the end of the exposure. (c) The change in swimming depth is the same for all treatments at the start of the exposure, (d) but the difference in swimming depth between before and the end of the exposure is positively correlated with the PRI (linear contrast analysis: $F_{1, 11} = 9.754$, $P = 0.01$). (e) The difference in group cohesion before and at the start of the exposure is significant only for PRI 1.0 s, (f) while there is no significant difference before and at the end of the exposure for all four treatments. An asterisk (*) denotes a significant difference from 0 ($P \leq 0.05$) and a plus (+) denotes a non-significant trend ($0.05 < P \leq 0.1$).

as 60 min (occurrence frequency: 2/48 for swimming depth and 2/48 for group cohesion). The recovery time of swimming speed was analysed in the same manner but with 10-second averages. To compare the effect of different treatments on recovery time, we performed non-parametric Friedman test, which accounted for the repeated nature of the treatments. To demonstrate that the behavioural recovery observed was due to habituation and not sensory adaptation or motor fatigue, we compared 10-second bins of swimming speed before and after the start of sound treatments, and before and after the playback of a 600-Hz tone right after the sound treatments.

Results

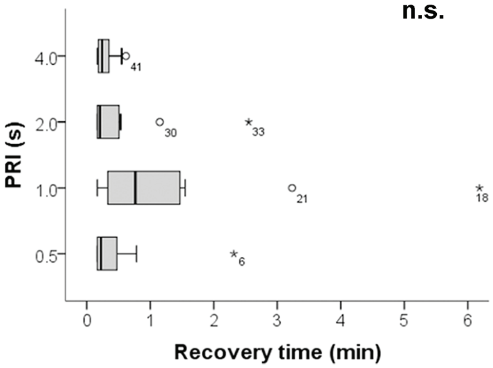
The fish significantly increased in swimming speed (two-way rANOVA: $F_{2,\ 22} = 12.108$, $P < 0.001$; Bonferroni-Holm *post-hoc*: 'before' vs 'start', $P = 0.008$) and swam to significantly greater depth at the start of the exposure (two-way rANOVA: $F_{2,\ 22} = 28.121$, $P < 0.001$; Bonferroni-Holm *post-hoc*: 'before' vs 'start', $P = 0.005$) in significantly tighter shoals (two-way rANOVA: $F_{2,\ 22} = 6.886$, $P = 0.005$; Bonferroni-Holm *post-hoc*: 'before' vs 'start', $P < 0.05$). By the end of the exposure, swimming speed and group cohesion recovered to pre-exposure level (Bonferroni-Holm *post-hoc*: 'before' vs 'end', $P = 1.000$ & $P = 0.454$ respectively) but swimming depth became even shallower than pre-exposure level (Bonferroni-Holm *post-hoc*: 'before' vs 'end', $P < 0.01$). The analysis also showed that for swimming depth, there was a non-significant trend on the interaction between PRI and the 5-min exposure sequence (Huynh-Feldt corrected two-way rANOVA: $F_{4.7,\ 51.8} = 2.136$, $P = 0.079$).

The magnitude of change from before to the start of exposure did not vary systematically across the four PRIs for swimming speed, swimming depth and group cohesion (linear contrast analysis: $F_{1,\ 11} = 1.232$, $P = 0.\ 291$, $F_{1,11} = 0.015$, $P = 0.\ 904$; $F_{1,\ 11} = 0.\ 063$, $P = 0.128$ respectively) (Fig. 3). When testing for PRI-specific effects in the difference of swimming speed, the difference was significantly larger than 0 for PRI 1.0 s and 4.0 s (one-sample *t*-test: $t_{11} = 3.388$, $P = 0.006$; $t_{11} = 2.666$, $P = 0.022$ respectively) but a non-significant trend for PRI 0.5 s and 2.0 s (one-sample *t*-test: $t_{11} = 1.921$, $P = 0.081$; $t_{11} = 1.832$, $P = 0.094$ respectively). For swimming depth, the difference was significant for all the treatments (one-sample *t*-test: $-3.944 \leq t_{11} \leq -2.285$, all *P*s < 0.05). For group cohesion, only PRI 1.0 s was significantly larger than 0 (one-sample *t*-test: $t_{11} = -2.523$, $P = 0.028$), while PRI 0.5 s was a non-significant trend (one-sample *t*-test: $t_{11} = -2.035$, $P = 0.067$).

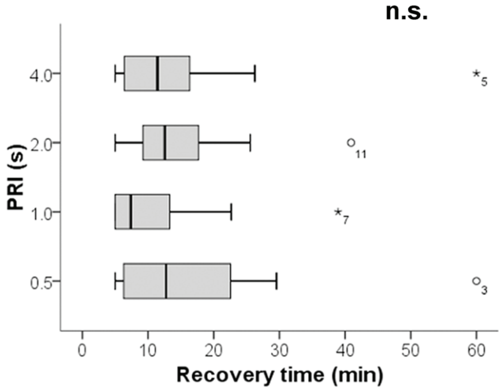
For the difference between before and the end of the exposure, swimming speed and group cohesion was the same across all treatments (linear contrast analysis: $F_{1,11} = 1.021$, $P = 0.\ 334$; $F_{1,\ 11} = 0.\ 133$, $P = 0.\ 722$ respectively), but swimming depth showed a significant positive linear effect (linear contrast analysis: $F_{1,\ 11} = 9.754$, $P = 0.\ 01$). The difference with baseline ranged from no significant difference for PRI 0.5 s (one-sample *t*-test: $t_{11} = -0.218$, $P = 0.831$), 1.0 s (one-sample *t*-test: $t_{11} = -0.218$, $P = 0.831$) and 2.0 s (one-sample *t*-test: $t_{11} = -1.630$, $P = 0.131$), to a significant difference of 0.23 ± 0.19 (SD) m for PRI 4.0 s (one-sample *t*-test: $t_{11} = 4.285$, $P = 0.001$).

We found that the fish habituated to the treatment sounds within an hour (Fig. 4), but the habituation time did not vary significantly among all treatments, for swimming speed (Friedman test: $X^2_3 = 5.625$, $P = 0.131$), swimming depth (Friedman test: $X^2_3 = 2.806$, $P = 0.423$) and group cohesion (Friedman test: $X^2_3 = 1.216$, $P = 0.749$). When comparing

(a) Recovery of swimming speed



(b) Recovery of swimming depth



(c) Recovery of group cohesion

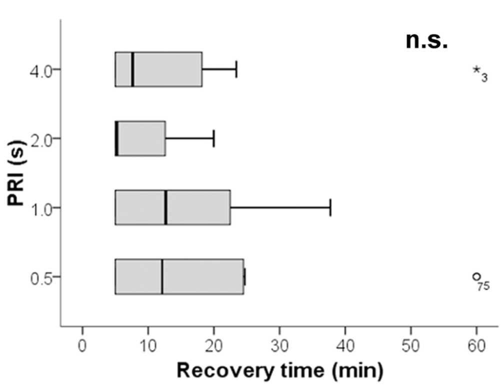


Fig. 4 Boxplots showing the recovery time of (a) swimming speed, (b) swimming depth and (c) group cohesion for the four pulse repetition interval (PRI) treatments. The fish habituate to the sound exposure within the same time for the four treatments.

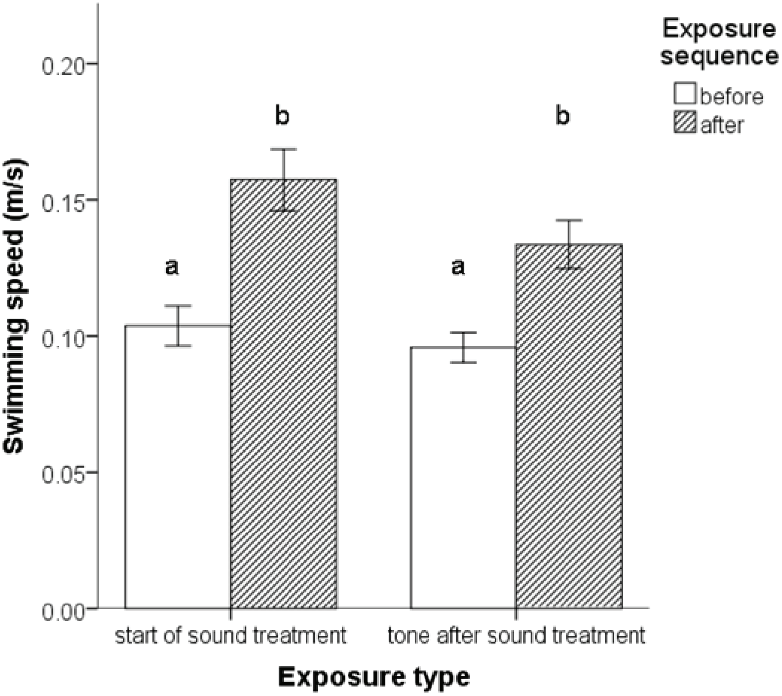


Fig. 5 Average swimming speed (± SE) before and after the exposure of two different acoustic stimuli. The swimming speed changes in the same way during the playback of 600 Hz tone compared to the playback of sound treatments, indicating that the fish could still hear and react to a novel acoustic stimulus after having habituated to the treatment exposure.

the swimming speeds before and after the first pulse of sound treatments, and before and after the playback of a 600-Hz tone right after the sound treatments, both exposure types resulted in a rapid increase in swimming speed (startle response) (two-way rANOVA: $F_{1,46} = 32.377, P < 0.001$ for exposure sequence; $F_{1,46} = 1.997, P = 0.164$ for exposure type; $F_{1,46} = 0.669, P = 0.418$ for the interaction of the two factors), indicating that at the end of the trials, the fish could still hear the tone and react to it to the same degree as to the treatment sound at the start of the trials (Fig. 5). The PRI of the treatment did not influence the speed change caused by the 2-second tone (two-way rANOVA: $F_{1,44} = 16.192, P < 0.001$ for exposure sequence; $F_{1,44} = 0.147, P = 0.931$ for the interaction of exposure sequence and PRI).

Discussion

The European seabass in this study swam to the bottom of the basin and formed tighter shoal upon exposure to impulsive sounds. All treatments seemed to be equally effective in eliciting an initial response of increased swimming speed and bottom diving, independent of the PRI, except for group cohesion, which was less affected by the slower than the faster pulse rates. Over time, they recovered by swimming higher up in the water column and shoaling less tightly again. Recovery occurred at the same rate regardless of the PRI of the sound treatments. While swimming speed and group cohesion reverted back to the baseline level and remained there till the end of the exposure, swimming depth went beyond baseline level by the end of the exposure depending on the PRI of the sound treatments: the longer the PRI, the shallower the fish swam. We also demonstrated that the behavioural recovery could be attributed to habituation to the sound treatments.

IMMEDIATE BEHAVIOURAL CHANGES

The startle response, bottom diving and shoaling behaviour observed at the start of sound exposure in this study mirror a previous study using larger European seabass (35 cm) in a smaller experimental enclosure[9], although the magnitude of changes was smaller in the current study. The observed behavioural changes were also consistent with several other studies conducted on other captive or wild species using various sound sources[5–7,18,19]. These behaviours have typically been associated with fright and anxiety in neurophysiological studies[20–22], and each of them is likely related to specific stress-related contexts9. These behavioural changes may incur short-term energetic costs to the fish, but it is still unknown if they will lead to disruptions of other important behaviours and have long-term consequences on fish populations.

EFFECTS OF PULSE REPETITION INTERVAL

Contrary to our expectation, PRI of sound exposure had no influence on the habituation rate of the fish. This may be either because the range of PRIs tested in this study was not large enough to provide sufficient resolution to yield an observable difference in habituation rate, or because our setup did not allow inter-pulse spontaneous recovery, like in other studies that tested different PRIs on sound habituation[15,16]. Nevertheless, our fish appeared to perceive the difference of the four PRIs tested, since they changed their group cohesion at the start of the sound exposure depending on the PRI and showed a PRI-dependant swimming depth difference at the end of the sound exposure. The correlation of PRI and 'post-habituation' swimming depth suggests that PRI of impulsive sound exposure can cause fish swimming behaviour to deviate from baseline level, although the underlying mechanisms are still unknown.

One possible explanation is that the fish swam higher up in the water column due to reduced general wariness as a result of attentional shift caused by the constant input of the habituated sounds, despite immediate stress-related response at the onset of exposure[20,21,23]. Although the fish might be in a lower anxiety state, there could be other negative consequences during noise exposure, such as masking of important acoustic cues and distraction from potential predators and prey[23,24]. Another explanation is that the observation was in fact habituation that proceeded beyond the baseline and reached a different asymptotic level[10,25,26]. This implies that the treatments with larger PRIs in our study resulted in longer habituation time, which is in agreement with the literature[10,27]. However, it is unclear if the elevated swimming depth would eventually revert back to the baseline, as this potential recovery could not be determined in our experimental setup, where a 600 Hz tone was played right after the treatment trials and the fish readily dived to the bottom regardless of the PRIs.

IMPLICATIONS FOR NOISE IMPACT ASSESSMENTS

Our findings suggest the potential contribution of PRI of impulsive sound exposure in changing the swimming patterns and perhaps the state of wariness of fish during sound exposures. This effect was seen at the start of the exposure for group cohesion and the end of the exposure for swimming depth, but not for the habituation rate to the sound exposure. However, the mechanisms of the observed effects are unclear, which renders the interpretation problematic. Therefore, caution is necessary when translating this finding to management strategies.

Nevertheless, our results suggested that the treatment with 0.5 s PRI had the least impact on fish swimming depth compared to other treatments with larger PRIs, even though its SEL_{cum} and number of strikes were the highest. This implies that some standard metrics such as SEL_{cum} and the number of pulse strikes may have limited predictive power for assessing potential behavioural impacts of impulsive sounds[28]. This is in agreement with the results of Neo *et al*[9], where impulsive sound treatments prolonged the behavioural recovery of European Seabass for twice as long compared to continuous sound treatments (with about double the SEL_{cum}). In view of this, our results agreed with Halvorsen et al[29], who investigated the thresholds for impulsive sounds to cause barotrauma injuries in Chinook salmon (*Oncorhynchus tshawytscha*), in rejecting the 'Equal Energy Hypothesis', which states that the same amount of acoustic energy will cause the same level of impact severity, regardless of how the energy is distributed in time[30,31].

In regard to relevant metrics for noise assessment on behavioural impacts, we believe that the qualitative characteristics of sound, such as various temporal structure parameters, including PRI, may be very useful[9]. Therefore, more temporal parameters, such as pulse repetition regularity, pulse duration and pulse shape, still need to be studied, so that some well-grounded quantification metrics and assessment methods can eventually be developed, which will provide practical information to inform management decisions regarding anthropogenic noise impacts.

CONCLUSION

Our study suggests that different PRIs of impulsive sounds may affect the immediate or delayed behavioural changes of fish differentially, without influencing the rate of the behavioural recovery. It is unclear whether these differences in behavioural changes would matter when it comes to the fitness consequences of the fish, especially in the wild. Nevertheless, despite the uncertainties, our findings provide insights into the relative impact strength of PRI, and highlight the importance of future studies conducted in the field, examining the temporal variations of sound exposures in assessing impact severity.

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CHAPTER 4

Sound exposure changes European seabass behaviour in a large outdoor floating pen: Effects of temporal structure and a ramp-up procedure

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Abstract

Underwater sound from human activities may affect fish behaviour negatively and threaten the stability of fish stocks. However, some fundamental understanding is still lacking for adequate impact assessments and potential mitigation strategies. For example, little is known about the potential contribution of the temporal features of sound, the efficacy of ramp-up procedures, and the generalisability of results from indoor studies to the outdoors. Using a semi-natural set-up, we exposed European seabass in an outdoor pen to four treatments: 1) continuous sound, 2) intermittent sound with a regular repetition interval, 3) irregular repetition intervals and 4) a regular repetition interval with amplitude 'ramp-up'. Upon sound exposure, the fish increased swimming speed and depth, and swam away from the sound source. The behavioural readouts were generally consistent with earlier indoor experiments, but the changes and recovery were more variable and were not significantly influenced by sound intermittency and interval regularity. In addition, the 'ramp-up' procedure elicited immediate diving response, similar to the onset of treatment without a 'ramp-up', but the fish did not swim away from the sound source as expected. Our findings suggest that while sound impact studies outdoors increase ecological and behavioural validity, the inherently higher variability also reduces resolution that may be counteracted by increasing sample size or looking into different individual coping styles. Our results also question the efficacy of 'ramp-up' in deterring marine animals, which warrants more investigation.

Introduction

The rise of underwater noise pollution resulting from human activities at seas may threaten the health and stability of fish populations[1–6]. This concern needs to be corroborated by understanding how critical fish behaviours change in response to the exposure of man-made noise[1,5]. For example, man-made noise has been shown to affect fish by changing their swimming patterns[7–11], territorial dynamics[12], antipredator vigilance[13,14], foraging efficacy[15–19] and other fitness-related activities[20,21]. These studies were conducted using different sound sources, which reflected the diversity of man-made noise sources in reality, and varied in their spectral, amplitudinal and temporal characteristics[1]. Different acoustic features likely differ in their relative importance in exerting behavioural effects, but such findings cannot be properly interpreted without deeper fundamental understanding[5,6].

It was only recently that the temporal characteristics of sound were shown to affect the on-set and recovery of behavioural changes for fish[7,8]. For example, the behavioural recovery of captive European seabass (*Dicentrarchus labrax*) in a large basin was faster when exposed to continuous sound than to impulsive sound[7]. In addition, impulsive sound exposure induced initial and delayed behavioural changes that were influenced by the pulse repetition interval (PRI)[8]. Moreover, amplitude fluctuations were shown to affect shoaling behaviour of the seabass[7]. The latter effect is interesting as amplitude fade-in, usually called 'ramp-up' or 'soft-start', is widely recognised and has been applied as a mitigation strategy[5,6,22,23]. A gradual rise in sound level, before a pile-driving or seismic shooting operation at full power, is assumed to drive away marine mammals and fish, in order to prevent injuries caused by intense sound exposure close to the sound source. However, the efficacy of the procedure still needs to be demonstrated[24].

Behavioural studies often carry implications that are difficult to ascertain because of interpretation discrepancies and generalisation uncertainties inherent to different experimental approaches. For example, tank-based and laboratory studies examining the behavioural impact of sound on captive fish have methodological advantages but also apparent extrapolation limitations[5,25–27]. Such confined set-ups have high internal validity but lack ecological validity, wherein the acoustic fields likely differ from natural waters in a complex and unpredictable manner[28], and the fish behaviour different and more constrained than in the wild[4,5]. However, this concern has not been substantiated with empirical evidence showing in what ways these limitations result in different behavioural observations between tank-based and open-water studies. Comparisons of behavioural responses to the same stimuli in the same social setting in both tank-based and open-water conditions could improve the external validity of test results and may provide additional insights into the underlying mechanisms[29,30].

Field studies on free-ranging animals have the highest ecological validity, but conducting well-replicated and well-controlled sound exposure studies at sea is exceedingly costly and logistically challenging. Moreover, discrepancies between contradictory results from different field studies can often not be sufficiently explained [see 9], due to unknown and potentially confounding or modulating factors. Consequently, a semi-natural approach with semi-controlled setting and a size-appropriate enclosure in the fish natural environment may sometimes be an optimal compromise[26,27].

In this study, we used European seabass in a large floating pen in a man-made cove within a tidal marine inlet, to test the impacts of sound exposure with different temporal structures. We tested four sound treatments varying in intermittency (continuous vs impulsive), repetition interval regularity and the presence of 'ramp-up' to test the following hypotheses: 1) Upon sound exposure, fish change their swimming speed, swimming depth, group cohesion and swim further away from the sound source; 2) the behavioural changes are affected by the different temporal structures, including intermittency, repetition interval regularity and the presence of 'ramp-up'; 3) the behavioural changes are in agreement with previous indoor studies which had the same experimental design[7,8].

Materials and methods

ANIMAL MAINTENANCE

Mixed-sex European seabass from a hatchery (Ecloserie Marine de Gravelines, France) with a total body length of about 30 cm were used in this study[7,8]. Before and after the experiment, the fish were kept in two cylindrical holding tanks (Ø 3.5 m, depth 1.2 m) in an 8:16 dark-light cycle at Stichting Zeeschelp research institute in Zeeland, the Netherlands. The water in the holding tanks was continuously refreshed with water from the nearby Oosterschelde marine inlet and the water temperature varied from 17 to 22 °C throughout the experimental period (June-August 2014). The fish were fed pellets (Le Gouessant Aquaculture, France) every other day based on a temperature-dependent prescription. All experiments were in accordance with the Dutch Experiments on Animals Act and approved by the Animal Experiments Committee at Leiden University (DEC approval no: 14047).

EXPERIMENTAL ARENA

The experiment was conducted in the Jacobahaven, a man-made cove in the Oosterschelde. The cove is about 200 m wide, 300 m long and 2-5 m deep depending on tides. It has a level and muddy bottom. The water is relatively calm in the summer and is home to wild European seabass. No external boat traffic is allowed within about 2 km of the cove, making it quiet and ideal for noise impact studies.

In the middle of the Jacobahaven, a floating island consisting of two platforms (Fig. 1) was constructed from a modular floating system (Candock, Canada) and anchored to the sea bottom with dead weights, chains and stretchable bungee ropes that kept the island in place at all tides. The octagonal platform (Ø 11.5-12.5 m) supported a custom-made octagonal net (volume 334 m^3), in which test fish were held during sound exposures; the square working platform supported a work tent (4 x 5 m), which protected all equipment from the weather and served as a working space during the experiment. The two platforms were kept at 0.5 m distance from each other to minimise unwanted noise transmission from the working platform to the octagonal platform during sound exposure. The working platform was detachable from the octagonal platform, and for every quarter of the total trials, it was repositioned at another orthogonal arm of the octagonal platform. The use of four different positions facing the four cardinal directions was intended to minimise the influence of extraneous factors (e.g. seabed topography, tide flows) on fish swimming patterns.

TREATMENT SERIES

We exposed the fish to a series of four sound treatments: continuous, impulsive regular, impulsive irregular and impulsive regular with 'ramp-up' (Fig. 2a). In order to vary only the temporal parameters of interest in the treatments while keeping all other sound

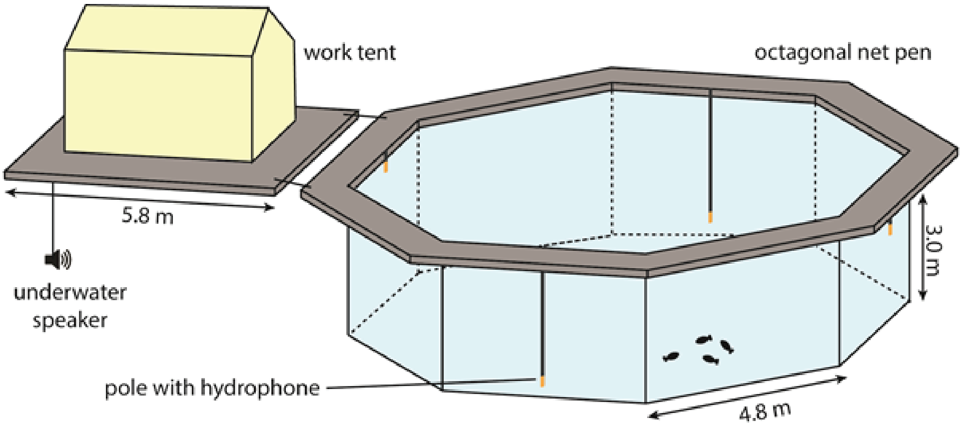


Fig. 1 Floating island where experiment was conducted. The square working platform is connected to the octagonal platform by two ropes, leaving a gap of 0.5 m between the two platforms. On the working platform, there is a work tent (5 x 4 m). The underwater speaker hangs on the far end of the working platform at a depth of 2.2 m. The distance of the underwater speaker and the closest side of the net is 7.8 m. The four poles with hydrophones are responsible for tracking the four test fish via acoustic telemetry. Two of the hydrophones are placed near the surface and the other two near the bottom. The distance between adjacent hydrophones is 8.7 m.

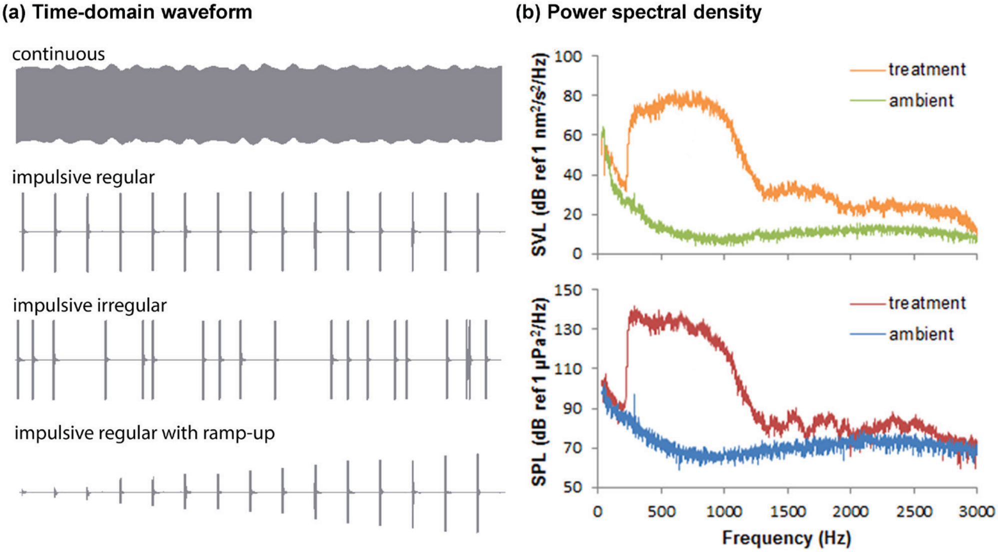


Fig. 2 (a) Time-domain waveforms showing 30 s of the four treatments exposed to each group of fish. Note that the rate of amplitude change for impulsive regular with 'ramp-up' is adjusted for illustration purposes; the original exposure consists of 20 min of 'ramp-up' followed by 50 min of exposure at the same amplitude as the other treatments. (b) Power spectral density plots of measurements in the middle of the octagonal net showing SPL and SVL of 1 s continuous noise and their respective ambient levels. Most energy concentrates between 200 and 1000 Hz as intended, overlapping with the hearing range of European seabass. The original spectra contained a dent between 500 and 1000 Hz, which was caused by the splitting of the original sound files for the avoidance of overload during measurements. The dent was removed in post-processing to reflect the actual acoustic conditions during the exposure trials.

parameters constant, the sound treatments were created in Audition 3.0 (Adobe, San Jose, US) using filtered brown noise (bandpassed: 200-1000 Hz; matching the hearing range of European seabass[31,32]). The continuous treatment consisted of uninterrupted sound elevation with constant amplitude. The other three impulsive treatments consisted of a pulse train with 0.1 s pulses, repeated at either a regular PRI (pulse repetition interval) of 2 s, or an irregular PRI of 0.2 - 3.8 s (random; average 2 s). The 'ramp-up' treatment consisted of 20 minutes of fade-in from ambient level to the same amplitude as the other treatments. All sound samples were played back with an underwater transducer (LL-1424HP, Lubell Labs, Columbus, US) from a laptop through to a power amplifier (DIGIT 3K6, SynQ) and a transformer (AC1424HP, Lubell Labs).

To examine the soundscape of the whole experimental arena, we measured both sound pressure level (SPL) and sound velocity level (SVL). These metrics relate to sound pressure and particle motion, which are both involved in fish hearing, although their relative importance are not always clear. The rms SPL and SVL of the continuous treatment were measured over 1 s at 360 points - three depths (0.5, 1.5 & 2.5 m) x 120 points - within the octagonal net (Fig. 3a). These measurements were performed during both ebb and flow tides for all four positions of the working platform, totalling eight sets of 360 measurements. The measurements were made using the M20 particle motion sensor (GeoSpectrum Technologies, Canada), whose pre-amplifier was powered by a 12 V car battery. The sensor was connected to a current-to-voltage convertor (GeoSpectrum Technologies, Canada) that gave an output of four channels: three for the 3D particle velocity directions (u, v & w) and one for sound pressure. These channels were connected to a laptop via an oscilloscope (PicoScope 3425, Pico Technologies, UK) and data were logged at 40 kHz using a script in Microsoft Access and subsequently analysed with MATLAB.

Measurements closer to the speaker caused signal overload. To avoid signal overload, we split the original sound file into two files of 200-560 Hz and 560-1000 Hz bandwidth, and redid the measurements. The readouts of the two files were merged during post-processing (Fig. 2b). The splitting caused a dent in the power spectra around the splitting frequency, leading to a slight underestimation of amplitude level (consistent throughout all measurements and not reflecting the actual exposure conditions). The mean rms SPL and SVL of the ambient noise were 108 dB re 1 µPa and 47 dB re 1 nm/s respectively. The mean rms SPL and SVL for the continuous treatment were 163-169 dB re 1 µPa and 101-105 dB re 1 nm/s respectively (the range indicates values from the furthest to the nearest points from the speaker within the experimental arena). For the impulsive treatments, the mean zero-to-peak SPL (SPL_{z-p}) and SVL (SVL_{z-p}) were 180-192 dB re 1 µPa and 124-125 dB re 1 nm/s respectively; the mean single-strike sound exposure level (SELss) and velocity exposure level (VEL_{ss}) were 156-167 dB re 1 $\mu Pa^2 s$ and 99-100 dB re 1 nm^2/s respectively.

EXPERIMENTAL DESIGN

Sixteen groups of four fish (64 fish, N = 16) were used and each group was exposed to all four sound treatments sequentially in two days; the exposure order followed an incomplete counterbalanced design (16 of 24 possible orders), to minimise potentially confounding effect of the treatment orders. Each group of fish was transferred to the floating pen in a black plastic container (56x39x28 cm) enriched with oxygen (OxyTabs, JBL, Germany) and allowed to acclimatise for at least eight hours. At least 30 min before the start of each trial, researchers arrived on the floating island by a small motorised rubber boat. The

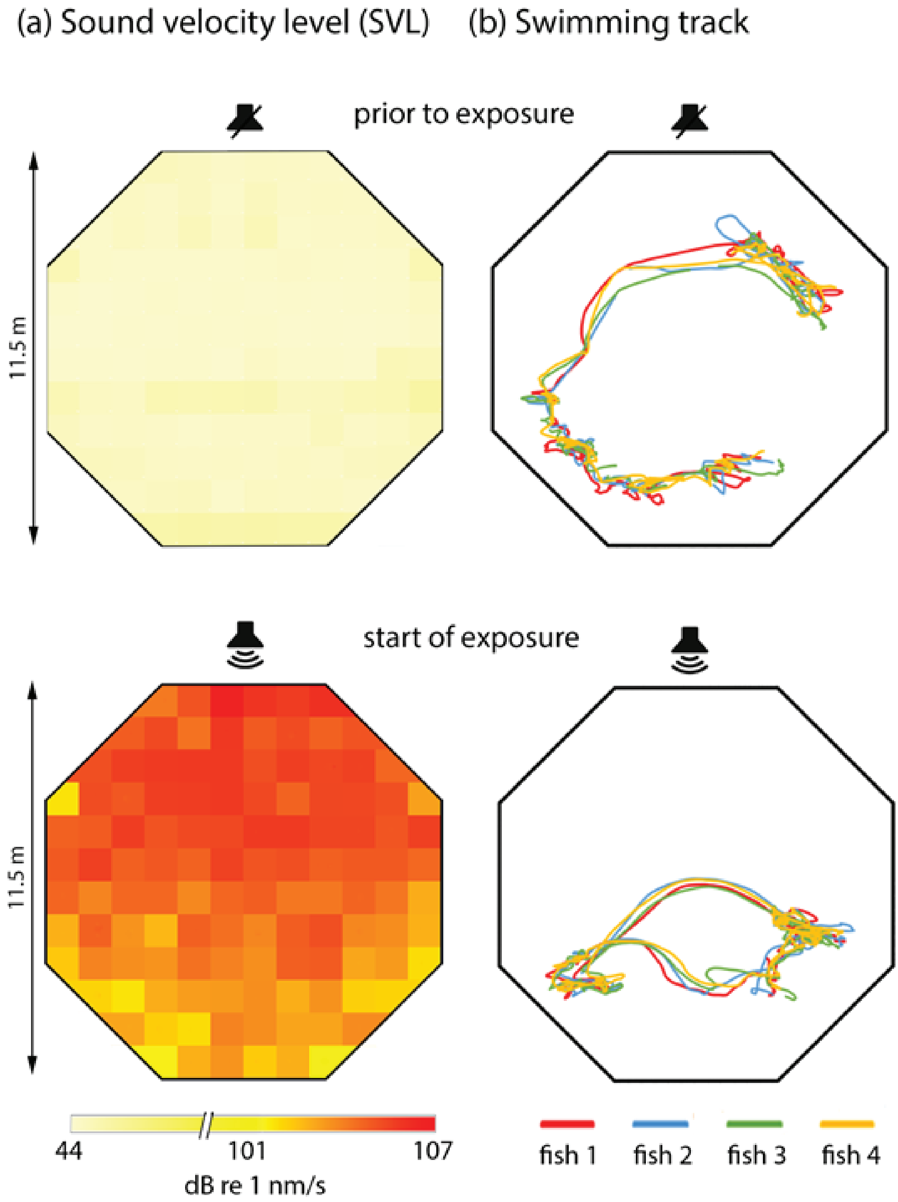


Fig. 3 (a) 2D soundscape maps in sound velocity level (SVL) prior (ambient) and at the start of sound exposure, measured at 1.5 m water depth. The speaker is 7.8 m away from the experimental arena, making the experimental arena outside the postulated acoustic nearfield < 7.5 m (sound treatments had minimum frequency 200 Hz). There is a clear amplitude gradient, also in sound pressure level (not shown). (b) Aerial view of swimming tracks of four fish 10 min prior and 10 min at the start of sound exposure. The fish swim around the periphery of the whole study arena before sound exposure but swim away from the speaker at the onset of sound exposure.

arrival triggered slight behavioural changes but the fish recovered within 30 min, before the start of the trial. We conducted two trials per day, one during ebb tide (starting 1.5 h after the high tide) and one during flood tide (ending 1.5 h before the high tide), ensuring that the water depth was always between 3-4 m during the trials. Each trial lasted for 1.5 h and comprised 60 min of sound exposure and 15 min of silence before and after, except for trials with 'ramp-up', where the exposure consisted of 20 min of 'ramp-up' plus 50 min of standard sound exposure (overall energy equalled 60 min standard exposure). During the exposure trials, the researchers stayed in the work tent and did not set foot on the octagonal platform. Light intensity, weather condition and water temperature were recorded during each trial and subsequently used as covariates in the statistical analyses. After each group of fish went through four trials, they were transferred back to the onshore holding tank.

ACOUSTIC TELEMETRY

The swimming patterns of the fish were studied with a 3D telemetry system using acoustic tags (Model 795-LG, HTI, US). The tags were programmed with a programmer (Model 490-LP, HTI, US) to emit 307 kHz pings (inaudible to the fish) of 0.5 ms at four different PRIs (985, 995, 1005 and 1015 ms), in order to identify the four fish in a group. The fish were tagged externally, directly under the first and second dorsal fin[cf. 33]. After tagging, the four fish were kept in a rectangular recovery tank (1.20 x 1.00 x 0.65 m), which was continuously refreshed with filtered water from the Oosterschelde. The fish stayed in the recovery tank for at least two days before being transferred to the outdoor pen. The pings emitted from the fish were received by four hydrophones (Model 590-series, HTI, US) attached to the octagonal platform (Fig. 1). The signals were then digitised by an acoustic tag receiver (Model 291, HTI, US) connected to a laptop. The digital data were subsequently processed by computer programs MarkTags v6.1 & AcousticTag v6.0 (HTI, US) into 3D coordinates (x, y, z), with a temporal resolution of one position every second for all four fish (position accuracy = ± 0.5 m). The 3D coordinates were used to calculate four behavioural parameters: swimming speed, swimming depth, average inter-individual distance (group cohesion) and distance from the speaker.

STATISTICS

To test for the changes in the behavioural parameters during the trials, we used linear mixed models to compare four 5-minute bins in the exposure sequence from our data set: the 5 min right before sound exposure ('before'), the first ('start') and the last 5 min of exposure ('end'), and the 5 min right after exposure ('after')[cf. 7]. Both exposure sequence and treatment were treated as repeated variables, with covariance structure defined as compound symmetry. We used the same procedure for swimming speed but the bins were 1 min instead of 5 min in order to capture the transient speed change. To understand the impact differences between the treatments, we subsequently ran the same test for each treatment separately, treating exposure sequence as a repeated variable. In addition, we compared the difference of the behaviours before and at the start of exposure between impulsive regular and the other three treatments separately, treating treatment as a repeated variable. We subsequently performed one-sample *t*-tests to see if the calculated differences were significantly larger than 0. In all tests, tide (ebb/flood), water temperature, light level and trial order were fitted as covariates. To select for the best model, irrelevant variables were omitted from the model through backward stepwise selection based on Akaike information criteria.

All posthoc tests and multiple comparisons were corrected using the Holm-Bonferroni method.

We also analysed the recovery time of the behavioural changes, which was defined as the time that the fish took to revert back to the pre-exposure level. The 'before' bin was used as a baseline to compare with 5minute moving averages during exposure shifting forward every second, to see when the baseline was reached again. If the baseline was not reached by the end of the trial, the recovery time was counted as 60 min (occurrence frequency: 3/64 for swimming depth, 9/64 for average inter-individual distance and 8/64 for distance from speaker). To compare the difference in recovery time between impulsive regular and the other three treatments separately, we used linear mixed models like above on ranked data, since the original data were not normally distributed.

Results

BEHAVIOURAL CHANGES

We were able to generate high-resolution swimming tracks of four fish for all trials (see Fig. 3b). At the start of the exposure, the fish increased the swimming speed (linear mixed model: $F_{3,237} = 4.978$, $P = 0.002$; Holm-Bonferroni *post-hoc*: 'start' vs 'before', 'end' & 'after' $P = 0.026$, 0.007 & 0.007 respectively) and swimming depth (linear mixed model: $F_{3,240} = 3.913$, $P = 0.009$; Holm-Bonferroni *post-hoc*: 'start' vs 'before', 'end', 'after', all Ps < 0.001), and swam further from the speaker (linear mixed model: $F_{3,240} = 2.654$, $P = 0.049$; Holm-Bonferroni *post-hoc*: 'start' vs 'before', 'end' & 'after' $P = 0.021$, 0.068 & 0.099 respectively), without changing the group cohesion. All the interaction terms between exposure sequence and treatment were not significant and were removed from the final models.

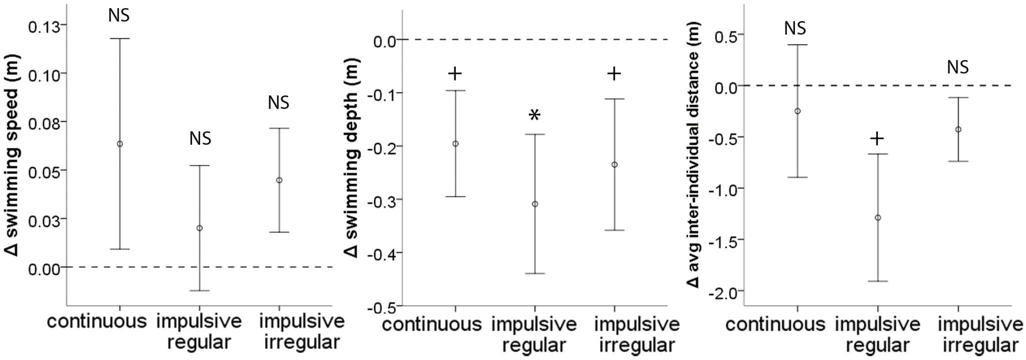
When the treatments were analysed separately, swimming speed did not increase significantly for all treatments, except for the continuous treatment, although the change was not significant in the *post-hoc* test after correcting for multiple testing (linear mixed model: $F_{3,48} = 4.910$, $P = 0.005$; Holm-Bonferroni *post-hoc* Ps>0.1). Swimming depth increased consistently across all treatments (linear mixed model: $F_{3,48} = 3.144$, $P = 0.034$ for continuous; $F_{3,48} = 5.141$, $P = 0.004$ for impulsive regular; $F_{3,49} = 4.277$, $P = 0.009$ for impulsive irregular; $F_{3,48} = 5.702$, $P = 0.002$ for impulsive regular with ramp-up; all Holm-Bonferroni *post-hoc:* 'start' vs 'before', 'end', 'after', all Ps < 0.05) but distance from speaker did not increase significantly for any of the treatments. The group cohesion increased significantly for impulsive regular (linear mixed model: $F_{3,43} = 3.916$, $P = 0.015$; Holm-Bonferroni *post-hoc*: 'start' vs 'before', 'end' & 'after' $P = 0.026$, 0.032 & 0.134) and continuous treatment, although *post-hoc* test did not reveal a significant change for the continuous treatment (linear mixed model: $F_{3,47} = 4.639$, $P = 0.006$; Holm-Bonferroni *post-hoc* Ps>0.1). The increase in group cohesion was not significant for impulsive irregular treatment and impulsive regular with ramp-up.

TREATMENT EFFECTS

Intermittency

Comparing between continuous and impulsive regular treatment, the increase in swimming speed, swimming depth and group cohesion did not differ significantly from each other, although the increase in swimming depth and group cohesion seemed larger in impulsive sound (Fig. 4). This was suggested by one-sample *t*-tests, where for swimming depth, there was a significant difference from zero for impulsive treatment and a non-significant trend for continuous treatment ($t_{15} = -2.362$, $P = 0.032$; $t_{15} = -1.773$, $P = 0.096$ respectively); and for group cohesion, there was a non-

(a) Behavioural changes



(b) Recovery time

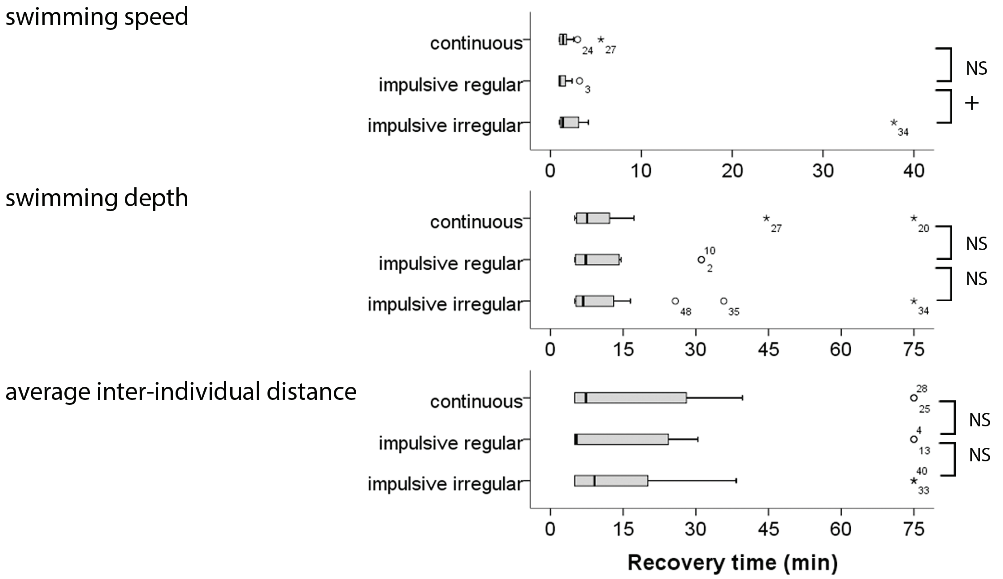


Fig. 4 (a) Changes in swimming speed, swimming depth (from net bottom) and average inter-individual distance (mean ± SE) from before to start of exposure for continuous and impulsive regular treatments. (b) Recovery time of swimming speed, swimming depth and average inter-individual distance for continuous and impulsive regular treatments. An asterisk (*) denotes a significant difference from 0 ($P \leq 0.05$), a plus (+) denotes a non-significant trend ($0.05 < P \leq 0.1$) and NS denotes non-significance ($P > 0.1$).

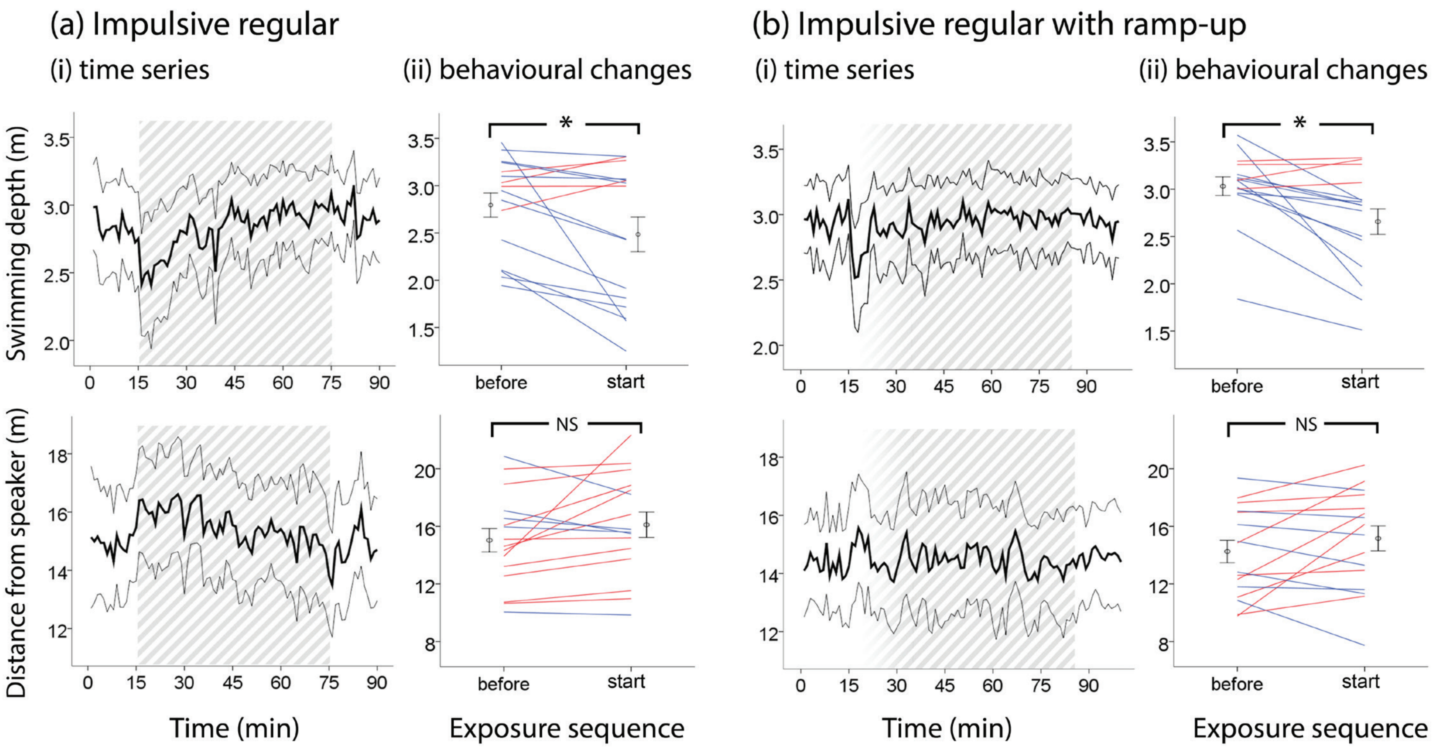


Fig. 5 Comparisons between (a) the absence and (b) presence of 'ramp-up', showing (i) the time series plots of the whole exposure period (with 95% confidence interval) and (ii) the changes in swimming depth (from net bottom) and distance from speaker (mean ± SE) for all 16 fish groups. In the time series plots, the shaded area indicates noise exposure; in the behavioural changes plots, red lines indicate increases while blue lines indicate decreases for the different groups. An asterisk (*) denotes significance ($P \leq 0.05$) and NS denotes non-significance ($P > 0.1$).

significant trend for impulsive treatment and no significant difference for continuous treatment (t_{14} = -1.815, P = 0.091; t_{14} = -0.114, P = 0.911 respectively). Contrary to the previous study, the recovery time of continuous and impulsive treatment did not differ significantly for swimming depth or group cohesion.

Interval regularity

Behavioural changes caused by the impulsive irregular treatment did not significantly differ from the regular treatment. Subsequent one-sample *t*-tests for irregular treatment yielded a non-significant trend in swimming depth (t_{15} = -1.905, P = 0.076) and no significant difference in group cohesion (t_{14} = -1.378, P = 0.191), which contrasted with the regular treatment in the same way as continuous treatment (see previous paragraph). However, for distance from speaker, the increase was significantly larger than zero for the irregular treatment, but not for the regular treatment (one-sample *t*-test: t_{15} = 2.595, P = 0.020; t_{15} = 1.744, P = 0.102 respectively). Furthermore, the irregularity of PRI did not significantly affect the recovery of swimming depth, group cohesion and distance from speaker, although there was a non-significant trend that the recovery of swimming speed was prolonged (linear mixed model: $F_{1,15}$ = 6.346, P = 0.071).

'Ramp-up' procedure

'Ramp-up' caused diving behaviour already within the first 5 min, similar to the treatment without it (Fig. 5). Within this period, the amplitude levels were still much lower than the full standard levels. Exposures at these sound levels triggered behavioural changes not significantly different from the treatment without the 'ramp-up', which had constant sound levels from the exposure on-set that were at least 16 dB (up to 31 dB) higher. The 'ramp-up' procedure also did not affect the recovery of the behavioural changes.

Discussion

In the current study, we could observe detailed swimming patterns of fish in a large floating pen in outdoor conditions. Upon sound exposure, the fish swam faster, deeper, and further away from the speaker. Within 30 min, most fish returned to their baseline behaviour. Despite some noticeable patterns, sound intermittency and interval regularity did not significantly influence fish response and recovery. In addition, the 'ramp-up' procedure triggered a behavioural response as immediate as when the procedure was absent, but did not make fish move away from the speaker. Some fish even seemed to approach the sound source, at least in the beginning. In general, the fish response was qualitatively similar to earlier indoor experiments, but the behavioural changes and recovery in the current study were more variable. We also found horizontal avoidance behaviour that was absent in the previous studies.

NO INFLUENCE OF TEMPORAL STRUCTURES

In contrast to our expectations, the impulsive treatment did not prolong the recovery of swimming depth as in previous indoor study[7]. One explanation may be that the current set-up allowed the fish to swim away from the speaker to quieter areas. As a result, the fish had some control over the sound exposure levels they experienced, and increased the variability of their swimming depth such that this differential impact on recovery was invisible. It should be noted that the absence of a significant difference does not necessarily mean the absence of an effect, because individuals may respond to sound using different coping strategies[34,35], e.g. freeze versus flight, and the causal relationship between sound exposure and behavioural changes may be moderated by some unknown environmental factors[29]. These context-dependent effects of noise exposure can only be answered with more well-controlled studies[4,26].

Consistent with a previous study conducted on groups of five zebrafish in aquaria[36], we found no significant effects of repetition interval regularity. Irregular pulses could be less predictable, potentially resulting in higher anxiety response as well as slower habituation[35,37]. However, evidence for these effects were so far only found in an indoor study on individual zebrafish, where the speed change was higher upon exposure to irregular sounds[17]. The influence of pulse repetition regularity may be too subtle to show in groups of fish where behavioural responses are strongly influenced by group dynamics. This implies that pulse repetition regularity might be less important in inducing behavioural impacts, at least within the temporal resolutions used in the current study (random PRI range: 0.2-3.8 s) and the previous study (random PRI range: 1-17 s)[36].

EFFICACY OF 'RAMP-UP'

To our knowledge, the inclusion of 'ramp-up' procedure allowed us to test for its efficacy on fish for the first time. The 'ramp-up' procedure caused fish to dive deeper without delay, which implies that the fish were sensitive to the presence of impulsive sound already at relatively low sound levels. However, when it comes to avoidance of the sound source, the effect was not very clear. When all treatments were analysed together in the statistical model, the avoidance effect was significant; but when analysed separately, the effect was not significant for any treatment. It can be seen in Fig. 5(a, ii) & (b, ii) that not every group of fish responded to the noise exposure by swimming away from it. A smaller but considerable proportion of the groups even initially approached the speaker, possibly due to sound-independent swimming pattern where the fish continuously circled the pen periphery, or due to a phonotactic response, potentially driven by curiosity for novel sounds[22,38]. Therefore, contrary to our expectation, 'ramp-up' may not necessarily drive fish away from ensuing intense noise exposure and some fish may actually stay where they are or even swim closer to the noise source.

Furthermore, the gradual increase in sound level of the 'ramp-up' procedure may allow the fish to habituate to the sound exposure more easily[37,39] and stay within the exposure area without avoidance behaviour. This suggests that 'ramp-up' procedure may actually reduce the effect of horizontal displacement of fish, instead of inducing deterrence as intended. In view of this, ramp-up procedures may actually be used to prevent distribution changes of fish if an exposed site is critical for foraging or breeding. However, fish may consequently experience other negative effects resulting from the ensuing noise exposure, such as physiological stress[40,41], auditory masking[42] and attentional shifts[43]. The effect of 'ramp-up' has also been suggested to be species-dependent, as some species are more mobile or more ready to swim from one area to another[44]. However, empirical data on more species is still unavailable. Therefore, extrapolating our findings to other species or other 'ramp-up' procedures is unadvisable at this stage. More studies are needed to test the efficacy of different 'ramp-up' procedures, which should not only consider a gradual rise in amplitude, but also a start with slower pulse repetition rates or 'ramp-up' in other relevant temporal characteristics.

FROM INDOOR TO OUTDOOR

One of the explicit aims of the current study was to compare findings from this study with a previous indoor study 7. The indoor study was conducted within a net enclosure (1.6 x 1.6 x 2 m) in a large basin (7 x 4 x 2 m), using a very similar experimental design and exposure scheme on captive European seabass of similar sizes. Two of the four sound treatments used were similar to the current study: continuous versus impulsive regular. The indoor study reported increased swimming speed, swimming depth and group cohesion upon sound exposures, irrespective of the treatment types. However, the impulsive treatment caused swimming depth to recover twice as slowly compared with the continuous treatment. The study successfully highlighted the relatively stronger impact of impulsive sound, but had extrapolation limitations because 1) the natural swimming behaviour of fish might be constrained by the small experimental enclosure and 2) the acoustic characteristics in the basin were quite different from natural waters, such as the lack of natural acoustic gradient due to near-field effects and reverberation, and the potentially complex sound pressure and particle motion ratios in the basin.

Despite apparent differences between the experimental arenas, the current study found comparable immediate behavioural changes. The increase in swimming depth was especially clear, implying that it is a robust indicator for behavioural impact, while other read-outs, such as group cohesion, may only become informative with the high resolution of indoor studies. Social effects that explain group cohesion are possibly lessened in the outdoor conditions due to the large experimental arena and the inherently reduced mutual visibility. In addition, we also showed that the fish avoided the sound source by swimming away from it (Fig. 3). The absence of this behaviour in the previous indoor study confirmed that either the artificial sound fields or the spatial restrictions in tanks could prevent some response patterns from emerging[45]. In view of this, the behavioural validity of any studies conducted in a confined or unnatural setting (especially the absence of effects), needs to be assessed critically before implications for noise impact assessments can be drawn. This can be achieved by comparing studies across different contexts from laboratory to field, to find out what behavioural parameters are generalisable, and whether they depend on specific contexts. For example, diving behaviour occurs in indoor studies conducted in reverberant enclosures without acoustic gradient[7,8,46] and has typically been associated with anxiety across contexts[47–52]. By knowing this, diving behaviour in outdoor studies[53–55] can be interpreted, at least partly, as related to anxiety instead of acoustic avoidance by vertical displacement. In any case, researchers, as well as regulators, should never take the findings of a single study at face value, but advice management decisions based on studies over a variety of contexts and approaches.

Performing indoor studies using robust behavioural parameters can increase their external validity, so that researchers can take advantage of the high controllability and practicality of tank-based set-ups to support outdoor experiments, which are typically more challenging to perform[4,26]. Currently, most studies so far were conducted on captive fish from a hatchery, which may be less or differently affected by environmental stressors than wild fish[56,57], making the observed response levels potentially less strong than in the wild. Moreover, there is still a need in determining which behavioural parameters may lead to long-term consequences on fish populations.

CONCLUSION

The findings from our semi-natural set-up successfully breached the extrapolation gap between laboratory and field studies. We showed that certain behavioural changes were qualitatively consistent with previous indoor studies, while horizontal avoidance behaviour only occurred outdoors. In addition, the outdoor conditions increased the variability of the behavioural response and did not reveal the effects of different temporal structures. Furthermore, we showed that a ‘ramp-up’ procedure had unexpected results, where fish startled already at the start of the ‘ramp-up’, without swimming away from the speaker. This observation implies that the 'ramp-up' procedure may affect fish behaviour, but not necessarily have the mitigation effect that is generally assumed.

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CHAPTER 5

European seabass respond more strongly to sound exposure at night and habituate over repeated sessions

Submitted as:
Neo YY, Hubert J, Bolle L, Winter HV & Slabbekoorn H.

Abstract

Offshore pile driving can take place day and night in series of repeated sessions of high-intensity pulse trains interspersed with relatively quiet intervals. The sound exposure may negatively affect aquatic animals, including fish. The effects may be context-dependent and vary with time of day, depending on the diel cycles of physiology and behaviour. Most studies to date have investigated behavioural changes within an exposure session, but the impacts and potential habituation to repeated sessions are unknown. Here, we exposed European seabass (*Dicentrarchus labrax*) in an outdoor pen to a series of eight impulsive sound exposure sessions over two days. Baseline behaviour before sound exposure was different between day and night; at night, the fish generally swam slower and closer to the surface in a looser shoal. Upon sound exposure, the fish increased their swimming speed, swimming depth and group cohesion, and the changes were more prominent at night. Furthermore, the fish also showed inter-trial habituation as they changed their swimming depth less with subsequent exposures. Although the observed behavioural changes do not directly imply fitness or population consequences, our findings suggest that sound impacts may be stronger at night than during the day for some fish species. Moreover, our results imply that habituation should be taken into account for appropriate sound impact assessments and potential mitigating measures.

Introduction

The increasing global energy demand has prompted the energy industries to construct more oil platforms and renewable energy farms at sea. The constructions typically involve pile driving, which produces a large amount of high-intensity impulsive sound that may negatively affect aquatic animals, including fish[1–3]. For example, fish in the vicinity of the sound source may suffer from barotrauma injuries[4–6]. However, only a small proportion of fish populations are usually close enough to receive such high-intensity sound, and they typically recover from the injuries within a few weeks[6,7]. In view of this, the potential effects of more moderate sound levels further from the sound source may be more problematic, as it is unclear how they may change the behaviour of large numbers of fish in vast areas[1,8].

In response to impulsive sound exposure, fish have been shown to change their swimming behaviour. They typically swim faster, deeper and further away from a sound source, in a tighter shoal[9–12]. It is important to understand how these changes are modulated by different acoustic parameters or environmental factors, so that potential impacts may be reduced by adjusting the current pile-driving procedures or mitigating measures. For example, a recent study revealed that European seabass recovered more slowly from impulsive sound than from continuous sound, highlighting the relevance of intermittency difference between impact-hammered pile-driving methods and more continuous drilling techniques[10]. However, given that behavioural changes are often complex and context-dependent, there is currently still need for studies testing more factors that potentially influence sound impacts[8].

Pile driving is often conducted day and night[13,14], while to date, all studies investigating underwater sound impacts were conducted during the day. Since pile driving is experienced by fish throughout their diel cycles, the sound-induced effects may also vary depending on the time of the day like with other external stressors. For example, when subjected to air exposure (lifted out of water), nocturnal green sturgeon (*Acipenser medirostris*) and Gilthead sea bream (*Sparus aurata L.*) increased plasma cortisol more at night than during the day[15,16]. In contrast, nocturnal Senegalese sole (*Solea senegalensis*) was more affected during the day[17]. It is unknown how the time of the day may influence the effects of sound exposure in a diurnal species such as the European seabass (*Dicentrarchus labrax*).

Furthermore, pile-driving operations usually consist of series of multiple exposure sessions (up to 70 sessions) over several weeks or months[13,14]. However, the impacts of sound on fish behaviour have mainly been studied within a single exposure session[18]. It is unknown if repeated exposure sessions may cause behavioural effects to accumulate, leading to stronger impact, or to diminish through habituation[19–21]. Intra-trial habituation has been shown in European seabass as their behaviour recovered to the baseline level within 30 min during sound exposure[9,10]. However, whether the fish also show inter-trial habituation over repeated trials, still needs to be demonstrated.

In this study, we exposed European seabass to a series of eight sound exposures in a large outdoor floating pen throughout the diel cycle of the fish. We expected that the fish change behaviour upon sound exposure and that the behavioural changes depend on the time of the day. We also expected that behavioural changes diminish with subsequent exposures.

Materials and methods

ANIMAL MAINTENANCE

We used mixed-sex European seabass that came from a hatchery (Ecloserie Marine de Gravelines, France) and measured about 30 cm in total body length. They all had experienced an identical series of sound exposures at least three weeks earlier in a previous experiment, using the same set-up as the current experiment[22]. The fish were kept in two cylindrical holding tanks (Ø 3.5 m, depth 1.2 m) in an 8:16 dark-light cycle at Stichting Zeeschelp, the Netherlands. The holding tanks were continuously refreshed with water from the nearby Oosterschelde marine inlet, which had a temperature ranging from 14 to 19 °C throughout the experimental period (August-October 2014). The fish were fed pellets (Le Gouessant Aquaculture, France) every other day based on a temperature-dependent prescription. The experiment was approved by the Animal Experiments Committee (DEC) of Leiden University (DEC approval no: 14047).

EXPERIMENTAL ARENA

The experiment took place in the Jacobahaven, a man-made cove in the Oosterschelde. The cove is about 200 m wide, 300 m long and 2-5 m deep depending on tides. It has a level and muddy bottom. The water is calm in summer and inhabited by wild migratory European seabass from April to November. The cove is free from boat traffic within its 2 km vicinity, making it quiet and ideal for noise impact studies.

In the middle of the Jacobahaven, we constructed a floating island (Fig. 1) using a modular system (Candock, Canada) and anchored it to the sea bottom with a stretchable system that kept the structure in place at all tides. The structure consisted of an octagonal and a square platform. The octagonal platform (Ø 11.5-12.5 m) carried an octagonal net (volume 334 m^3), where test fish were held during sound exposure trials. The square working platform held an underwater speaker suspended in water, and supported a work tent (4 x 5 m) that protected the equipment from the weather and served as a working space during the experiment. The work tent was supplied with electricity via an underwater cable from Stichting Zeeschelp. The two platforms were kept at 0.5 m distance from each other to minimise unwanted sound transmission from the working platform to the octagonal platform during sound exposure. The working platform was detachable from the octagonal platform, and for every quarter of the total trials (every four fish groups), it was repositioned at another orthogonal arm of the octagonal platform. This procedure ensured that observed behavioural changes would be explained mainly by sound exposure, instead of the effects of extraneous factors, such as seabed topography and tide flows.

SOUND TREATMENT

We exposed the fish to a one-hour impulsive sound treatment consisting of 0.1 s pulses, repeated at a regular repetition interval of 2 s. The sound sample was created in Adobe Audition 3.0 using bandpassed brown noise within 200-1000 Hz, matching the hearing range of European seabass[23,24]. It was played back with an underwater transducer (LL-1424HP, Lubell Labs, Columbus, US) from a laptop through to a power amplifier (DIGIT 3K6, SynQ) and a transformer (AC1424HP, Lubell Labs).

Prior to the start of the experiment, the amplitude levels of the sound treatment were measured at 360 points within the octagonal net (120 points at 0.5, 1.5 & 2.5 m depth). The measurements were made using an M20 particle motion sensor (GeoSpectrum Technologies, Canada), which was connected to a current-to-voltage convertor that gave an output of four channels: one for sound pressure and three for the 3D particle velocity directions (u, v & w). The data output was logged at 40 kHz on a laptop via an oscilloscope (PicoScope 3425, Pico Technologies, UK), using a Microsoft Access script. The data was subsequently analysed in MATLAB. The analysis revealed a clear gradient in amplitude levels from the furthest to the nearest points from the speaker within the experimental arena. The mean zero-to-peak sound pressure level (SPL_{z-p}) and sound velocity level (SVL_{z-p}) were 180-192 dB re 1 µPa and 124-125 dB re 1 nm/s respectively (the range reflects values from the furthest to the nearest points from the speaker). In addition, the mean single-strike sound exposure level (SEL_{ss}) and velocity exposure level (VEL_{ss}) were 156-167 dB re 1 µPa^{2}s and 99-100 dB re 1 nm^2/s respectively.

EXPERIMENTAL DESIGN

Sixteen groups of four fish (N = 16, 64 fish) were exposed to the impulsive sound treatment for eight times sequentially in two days (Fig. 2). Each group of fish was transferred to the octagonal pen in a black plastic container (56x39x28 cm) enriched with oxygen (OxyTabs, JBL, Germany) and allowed to acclimatise for at least eight hours. Half of the groups started with the first trial of the exposure series during the day and the other half at night. The exposures took place during ebb tide (starting 1.5 h after the high tide) and flood tide (ending 1.5 before the high tide), when the water depth was between 3-4 m for all the trials. Due to the tides, a subsequent trial started either 3 h or 7.5 h (alternating) after the end of the previous trial. Each trial lasted for 1.5 h and comprised 60 min of sound exposure and 15 min of silence before and after. During each trial, we recorded the light intensity, the weather condition and the water temperature, which were subsequently used as covariates in the statistical analyses. After each group of fish went through the series of eight trials, they were transferred back to the onshore holding tank.

ACOUSTIC TELEMETRY

We studied the swimming patterns of the fish with a 3D telemetry system using acoustic tags (Model 795-LG, HTI, US). The tags were programmed (Model 490-LP, HTI, US) to emit 307 kHz pings (inaudible to the fish) of 0.5 ms at four different repetition intervals (995, 1005, 1015 and 1025 ms), in order to identify the four fish in a group. The fish were tagged externally, directly under the first and second dorsal fin[25]. After tagging, the four fish were kept in a rectangular recovery tank (1.20 x 1.00 x 0.65 m), which was continuously refreshed with filtered water from the Oosterschelde. The fish stayed in the recovery tank for at least two days before being transferred to the outdoor pen. In the octagonal pen, the pings emitted from the fish were received by four hydrophones (Model 590-series, HTI, US) attached to the platform (Fig. 1). The signals were then digitised by an acoustic tag receiver (Model 291, HTI, US) connected to a laptop. The digital data were subsequently processed with computer programs MarkTags v6.1 & AcousticTag v6.0 (HTI, US) into 3D coordinates (x, y, z), with a temporal resolution of one position every second for all four fish. The 3D coordinates were then used to calculate four behavioural parameters: swimming speed, swimming depth, average inter-individual distance (group cohesion) and distance from the speaker[cf 22].

STATISTICS

We first examined the 5-minute bin of the behavioural parameters right before the sound exposure, to see if baseline behaviours varied depending on the exposure sequence (order) and the time of the day. We categorised the time of the day into 'day' or 'night', depending on whether the trial started before or after the sunrise/sunset of the day. We modelled the baseline behaviours using linear mixed model, treating group as subject variable with random effects and exposure sequence as repeated variable with an AR(1) covariance

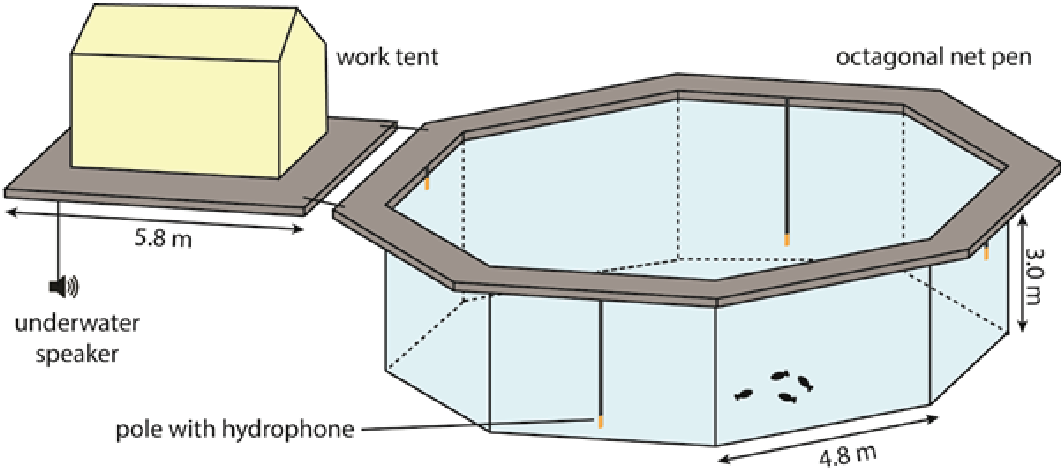


Fig. 1 Schematic of floating island where experiment was conducted. The underwater speaker hangs on the far end of the working platform at a depth of 2.2 m. The distance of the underwater speaker and the closest side of the net is 7.8 m. The four poles with hydrophones are responsible for tracking the four test fish via acoustic telemetry.

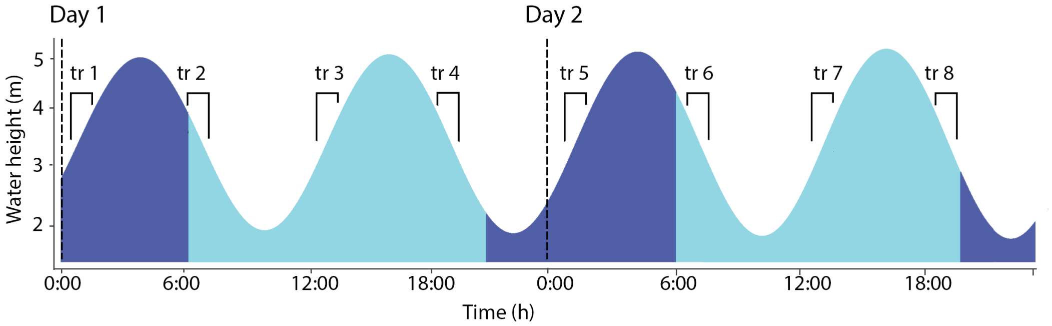


Fig. 2 Tide table showing the sound trial exposure scheme. All eight trials take place over two days when the water height is 3-4 m. Dark blue indicates night time and light blue indicates day time.

structure. In addition, we treated time of day as an explanatory factor, and tide, water temperature and exposure sequence as covariates. We selected the best model using backward stepwise method based on Akaike information criteria. Subsequently, we used the same procedure to model the behavioural changes caused by the sound exposure, which were the differences between the 5-minute bins right before and at the start of sound exposure. We also performed one-sample *t*-tests to see if the calculated differences were significantly larger than zero.

Results

We compared the pre-playback baseline behaviour of the fish between day and night (69 and 59 trials respectively) (Fig. 3a). At night, the fish swam significantly slower (linear mixed model: $F_{1,94} = 5.312$, $P = 0.023$) in groups with significantly lower cohesion (linear mixed model: $F_{1,98} = 13.799$, $P < 0.001$). There was a non-significant trend that they also swam higher up in the water column (linear mixed model: $F_{1,107} = 3.014$, $P = 0.085$), at similar distance from the speaker. Upon sound exposure, the increase in group cohesion was significantly larger at night (linear mixed model: $F_{1,89} = 3.954$, $P = 0.050$) (Fig. 3b). There was also a non-significant trend that the increase in swimming speed was also larger at night (linear mixed model: $F_{1,95} = 3.671$, $P = 0.058$). Subsequent one-sample *t*-tests showed that only increases in swimming speed and swimming depth at night were significantly larger than zero (one-sample *t*-test: $t_{57} = 3.782$, $\mathrm{P} < 0.001$; $t_{57} = -2.008$, $P = 0.049$ respectively). There was also a non-significant trend that increase in group cohesion at night was larger than zero (one-sample *t*-test: $t_{53} = -1.716$, $P = 0.092$). Within the 60 min exposure trials, all the behavioural changes reverted back to baseline levels, indicating intra-session habituation[9,10]. For inter-session habituation, we found that changes in swimming depth diminished significantly with subsequent exposure sessions (linear mixed model: $F_{1,57} = 4.002$, $P = 0.050$) (Fig. 4). For group cohesion, we found significant interaction between the time of the day and the trial order (linear mixed model: $F_{1,86} = 4.353$, $P = 0.040$), which was due to a subtle decline in response over time at night and a change in response from less to more cohesion during daytime.

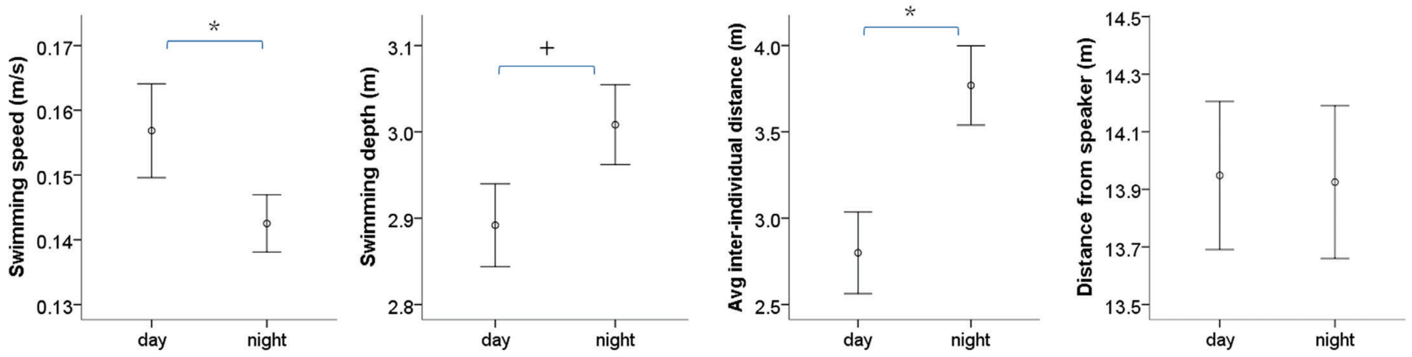
Discussion

Using a semi-natural set-up, we showed that European seabass swimming patterns varied throughout the diurnal cycle. Comparing baseline behaviour at night to during the day, the fish tended to swim slower, nearer to the surface, in a looser shoal. When exposed to sound, the fish increased their swimming speed, swimming depth and group cohesion. These changes were stronger at night. For all the behavioural changes, the fish recovered to baseline levels within an exposure session, indicating intra-session habituation. Furthermore, with subsequent sound exposure sessions, the fish gradually reduced the change in swimming depth, indicating inter-trial habituation.

STRONGER RESPONSE AT NIGHT

European seabass in our study showed clear diurnal swimming patterns. Such daily behavioural rhythm has also been shown in the dusky grouper (*Epinephelus marginatus*) and the yellowfin tuna (*Thunnus albacares*), where the fish swam closer to the surface at night[26,27]. This daily rhythmicity in movement is possibly linked to the daily rhythmicity in several hormones and metabolites[28–32]. For example, our study species, the European seabass has been shown to have daily variation in plasma glucose, insulin and cortisol[33,34]. The daily peaks of these parameters depend on whether the species is diurnal or nocturnal.

(a) Baseline behaviour



(b) Behavioural changes

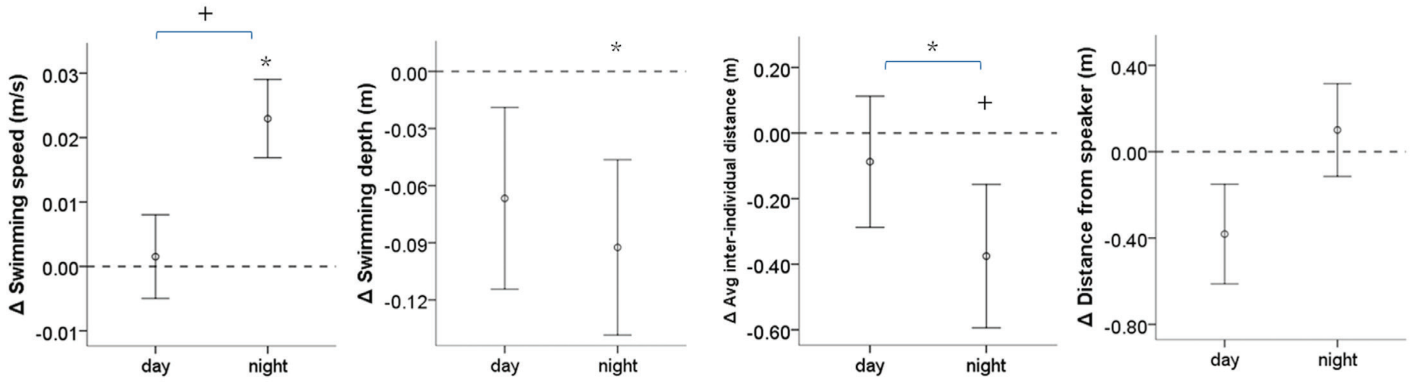


Fig. 3 (a) Baseline behaviour (mean ± SE) during the day and during the night for swimming speed, swimming depth (from bottom), average inter-individual distance and distance from the speaker. (b) Behavioural changes from before to the start of sound exposure during the day and during the night. An asterisk (*) denotes a significant difference ($P \leq 0.05$) and a plus (+) denotes a non-significant trend ($0.05 < P \leq 0.1$). The symbol between the bars indicates a difference between day and night, and the symbol above the bars indicates a difference from zero.

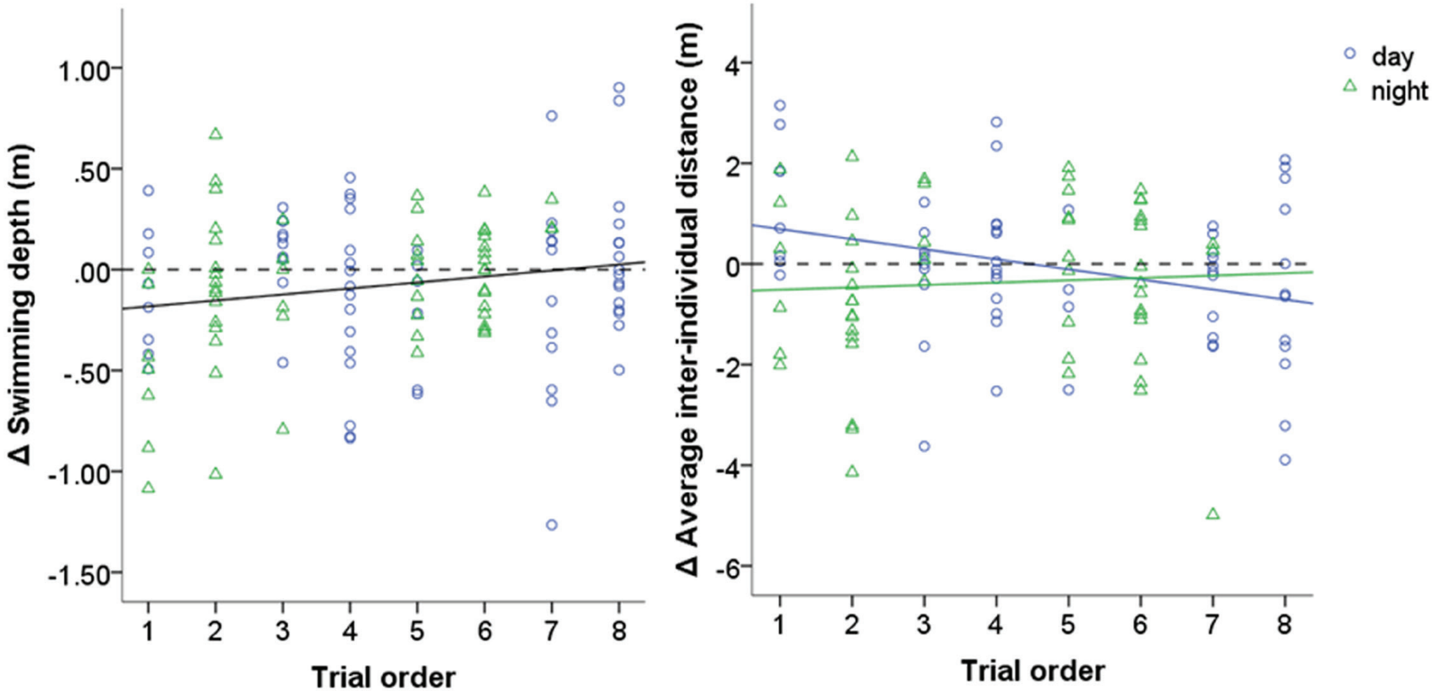


Fig. 4 Change in swimming depth and average inter-individual distance throughout the series of eight trials. The change in swimming depth diminishes with subsequent trials, indicating inter-trial habituation. The influence of trial order on the change in group cohesion is different between day and night.

Diurnal species typically produce most cortisol at the start of the day, while nocturnal species at the start of the night[15,28,35]. Such hormonal fluctuations are also affected by feeding and external stressors[15,28,31].

Upon sound exposure, European seabass in our study showed stronger behavioural changes at night compared to during the day. The influence of the time of the day on stress response has been shown in three nocturnal fish species subjected to air exposure[15–17]. Two of the species showed stronger cortisol increase at night and one during the day, suggesting that daily variation in sensitivity to stressors is species-specific. The mechanism of such differential sensitivity is still unknown, although it may be related to potential daily rhythm in the sensitivity of the glands in the hypothalamic–pituitary–adrenal (HPA) axis to corticotropin-releasing (CRH) or adrenocorticotropic hormone (ACTH)[36,37]. Rhythmicity in stress sensitivity may allow fish to effectively handle different daily activities and challenges.

The response to sound exposure during the day was particularly small compared to a previous experiment conducted two months earlier using the same setup on the same fish[22]. In the previous experiment, the fish were exposed to a series of four sound treatments varying in their temporal structures (one of the sound treatments was re-used in the current study), which took place during the day over a two-day period. This prior experience may have induced anticipation in the fish to the ensuing sound exposure in the current study, yielding lower response levels, especially during the day. Nevertheless, the fish still responded strongly to sound exposure at night, potentially because they were woken up from their resting or sleep-like state[38,39]. Such disruption can be particularly harmful to the fish as they may perform worse in daily activities. For example, when subjected to unpredictable chronic stress at night compared to during the day, zebrafish (*Danio rerio*) learned less well in an inhibitory avoidance task[40].

Despite low response levels during the day, our observation suggests that sound exposure at night may have more impact on European seabass. Although night exposure can be avoided by changing the schedules of current pile-driving practices, any modifications require careful considerations, as some species within an affected area may actually be more sensitive to stress during the day[17]. Nonetheless, our findings suggest that the responsiveness of fish to sound exposure may be affected by the natural rhythms in physiology as well as the environmental contexts. Consequently, such factors should also be considered when evaluating the efficacy and potential impacts of the schedule of a pile-driving operation.

INTER-SESSION HABITUATION

European seabass not only habituated to sound exposure within a session, they also habituated over subsequent sessions. Such inter-session habituation has been previously reported in zebrafish and the common cuttlefish exposed to repeated sound tones[41,42]. The zebrafish reduced the distance moved during startle response, while the cuttlefish reduced the probability of jetting and inking. In the current study, the European seabass reduced the change in swimming depth at the onset of sound exposure. Compared to intra-session habituation, the inter-session habituation was less prominent. For example, inter-session habituation only occurred with swimming depth, but not for the other test parameters. The lack of inter-session habituation in other parameters suggests that the fish may not have completely habituated to repeated exposures. However, it can also be explained by the more variable nature of these responses.

It is debatable whether habituation is necessarily beneficial to the fish under sound exposure[43]. On the one hand, habituation may reduce spatial and distributional changes, which is critical when a site is crucial for foraging or spawning. On the other hand, habituation may also cause fish to stay within an affected area, while still causing physiological stress[44,45], auditory masking[46] and attentional shifts[47–49]. Nevertheless, knowing that fish can habituate to repeated sound exposures, regulators and developers may be able to control the habituation rate by altering the trial intervals[9,50,51] or the interval regularity of repeated trials[18,49]. The effectiveness of such modifications still needs to be demonstrated in future studies.

CONCLUSION

Our study showed that European seabass responded more strongly to sound exposure at night and they habituated to repeated exposures. These findings demonstrate that environmental context and exposure experience may modulate sound impact on fish due to noisy human activities. Consequently, mitigation efforts aiming at minimising sound impact should take these factors into account when devising pile-driving operations. Although the implementation of implied refinements may still be problematic in the field, our study provides insights and empirical evidence that certainly help inform mitigating strategies.

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CHAPTER 6

Synthesis & General Discussion

This thesis set out to investigate potential modulating factors that influence behavioural impacts of underwater man-made sounds on European seabass. The investigation comprised four complementary experiments. The first two experiments were performed in an indoor basin, where groups of European seabass were exposed to a series of four sound treatments and their behaviour was analysed with a video-tracking system. I first examined the influence of sound intermittency and amplitude fluctuation on the behavioural impacts (chapter 2). I found the fish to recover from behavioural changes within an exposure session, so I subsequently investigated if the recovery was due to habituation, while testing the influence of pulse repetition interval on sound impacts (chapter 3). The next two experiments were performed in a large outdoor floating pen using the same experimental design, while the fish swimming trajectories were visualised with a 3D acoustic telemetry system. Using this semi-natural set-up, I examined the efficacy of a 'ramp-up' procedure, as well as the effects of sound intermittency and pulse interval regularity (chapter 4). Next, I tested whether European seabass habituated to repeated exposure sessions, and whether sound exposures at different times of the day affected the behavioural response (chapter 5).

Influence of temporal structure of sound

In the two indoor basin studies, I showed that the temporal structure of sound influenced the behavioural impacts of sound exposure. In the first experiment where I tested sound intermittency and amplitude fluctuation, the fish swimming depth recovered more slowly under impulsive sound than under continuous sound (chapter 2). Moreover, there was a trend that group cohesion recovered more slowly under fluctuating amplitude than under consistent amplitude. In the second experiment, I showed that longer pulse repetition interval of impulsive sound caused fish to swim higher up in the water column after the end of sound exposure (chapter 3). Although the mechanisms for these differential effects are unknown, I showed that European seabass are sensitive to temporal characteristics of sound exposure and may behave differently depending on what sound they are exposed to. However, the temporal effects were not as clear when we tested sound intermittency and pulse interval regularity in the outdoor setting (chapter 4). Although impulsive sound seemed to affect fish behaviour more strongly compared to continuous sound, the effects were not statistically significant.

Nonetheless, this thesis shows that behavioural impacts cannot be sufficiently explained by the standard acoustic metrics (e.g. SPL or SEL), which only consider the sound level and duration. In contrast, the severity of sound impacts may be influenced by the temporal structure of sound. As a result, temporal structure needs to be assessed when evaluating potential impacts, by for example assigning appropriate weighting to different temporal parameters. The temporal structure of sound exposure may also be used to devise mitigating strategies. For example, our results suggest that fish may habituate more easily to continuous pile-drilling than impulsive hammer pile-driving, implying that the former method may be more favourable. However, since temporal parameters tested so far are qualitative in nature, impact assessments may still be complicated. To overcome this complication, some quantitative temporal parameters still need to be developed and tested, such as using temporal entropy or kurtosis[1]. Furthermore, the timescale of temporal variability may also influence sound impacts but still largely unexplored. For example, although pulse interval irregularity of 2 s on average did not influence behavioural impacts (chapter 4), exposure interval irregularity of 1 h on average has been shown to affect the growth of larval Atlantic cod (*Gadus morhua*)[2].

Bridging indoor and outdoor studies

In both the basin and net pen studies, the fish changed their swimming behaviour upon sound exposure. The immediate behavioural changes were rather consistent between the two settings, where the fish startled and dived deeper in a tighter shoal. However, with the outdoor net pen set-up, I revealed sound source avoidance that was absent in the basin studies (chapter 4). The absence of this horizontal avoidance behaviour in the basin set-up could be due to the restricted space or a lack of directional cues in the sound field of the experimental arena. Comparing these two approaches allowed us to evaluate which behaviours are more robust and generalisable than others. For example, the immediate diving behaviour, which is typically associated with anxiety[3], seems to occur readily in all experiments. Using such robust behavioural response, noise impact studies can maintain behavioural validity when conducted indoors, which are generally easier to control and perform than outdoor experiments. On the other hand, some behaviours may be hindered by the size of the experimental enclosure and have limited extrapolative values. Therefore, findings from indoor set-ups can only be extrapolated to outdoor conditions after deliberate evaluation.

All the experiments in this thesis only tested hatchery-reared European seabass in confined environments. The behavioural repertoire of the fish might differ from free-ranging fish, as fish in the wild have a very different experience with their environment and may respond to an acoustic stressor differently[4,5]. Therefore, future studies should reveal whether wild-caught and free-ranging fish react in a similar way as what has been observed in this thesis. Furthermore, the fish in this thesis were exposed to sound playbacks that were artificially generated and acoustically different from real man-made sounds. There is a need to test the impacts of man-made sounds in situ in order to reveal the generalisability of the observed behavioural responses in this thesis. Nonetheless, the acoustic characteristics of natural outdoor conditions may still vary considerably, from open water to shallow water, coral reefs and rocky habitats. In this thesis, I measured the soundscapes of my set-ups, and revealed that sounds produced under tank-based and open-water conditions varied substantially in the ratios of sound pressure and particle motion. The interplay between sound pressure and particle motion may play an important role in fish hearing, although the exact contribution of each component is still largely unclear[6]. Hence, there is a need for future studies to describe the variability of the relationship between sound pressure and particle motion in various natural or unnatural environments, and how it influences fish acoustic sensitivity.

Efficacy of ‘ramp-up’ procedure

‘Ramp-up’ procedures have often been implemented before pile driving and airgun shootings as a mitigating measure, in order to repel marine mammals and fish from the loud sound source. However, the efficacy of such practice was only tested for the first time in our study (chapter 4). I used a ‘ramp-up’ procedure that gradually increased amplitude from the ambient level to the standard exposure level over 20 min. The onset of the ‘ramp-up’ caused the fish to change behaviour in the same way as when they were exposed to sound treatment directly without a ‘ramp-up’. However, the fish did not swim away from the sound source as expected. Moreover, they seemed to habituate to the sound more quickly. These observations suggest that a ‘ramp-up’ may not necessarily achieve its conventional goal in deterring fish from the proximity of an impact site. In fact, our findings suggest that a 'ramp-up' may enhance fish habituation to the sound exposure.

The failure of 'ramp-up' in repelling fish may result in negative consequences, such as hearing loss or acoustic masking. However, this absence of spatial deterrence may sometimes be favourable, especially if the site is critical for foraging or mating. Therefore, mitigating strategies of either increasing deterrence or enhancing habituation should always be critically evaluated before being implemented. Nevertheless, different 'ramp-up' scenarios may vary in their efficacies. These still need to be tested using 'ramp-up' procedures of different temporal structures, such as a decrease in pulse repetition intervals, different rates of amplitude rise, different lengths and starting sound levels of the 'ramp-up'.

Habituation to sound exposure

Upon sound exposure, European seabass typically increased their swimming speed, swimming depth and group cohesion. Within the 30 or 60 min exposure trials, the fish behaviour recovered back to baseline levels. This recovery was shown to be habituation instead of sensory adaptation or motor fatigue, as the fish could still respond to novel acoustic stimuli (chapter 3). Habituation is a simple form of learning that helps animals ignore irrelevant stimuli in order to focus selectively on biologically significant ones[7,8]. Apart from intra-session habituation, I also revealed inter-session habituation, where the fish habituated to repeated exposure trials within eight sessions over two days (chapter 5). Although both intra-session and inter-session habituation may serve the same adaptive function[8–10], they likely reflect different neurobiological processes. Intra-session habituation is related to working memory and adjustability to the surrounding, whereas inter-session habituation measures long-term memory of previous exposure[8,11].

Although habituation may mean that fish become less disturbed by the sounds, it does not necessarily entail the absence of negative impacts. Ongoing sound exposure may still cause chronic stress[12,13], acoustic masking[14,15] and attentional shift[16,17]. These impacts may in turn affect other critical life processes, such as foraging and anti-predatory behaviour. Future studies need to explore how such activities are influenced by long-term sound exposure. Moreover, it will be useful to know if wild fish growing in the presence of man-made sounds also suffer fitness consequences. Whether habituation leads to positive, neutral or negative fitness consequences still needs to be demonstrated. In each scenario, habituation may be deliberately enhanced or prevented as a mitigating strategy by manipulating the temporal characteristics of sound exposure.

Sound exposure at night

Offshore pile driving can take place day and night, exposing fish to sound throughout their diurnal cycles. I exposed European seabass to a series of eight sound exposures over two days, where 46% of the trials took place at night (chapter 5). Comparing baseline swimming patterns before sound exposure at night to during the day, the fish typically swam slower, closer to the surface and less close to each other. These behaviours were probably related to the resting or sleep-like state of the European seabass. When exposed to sound, the behavioural changes were larger at night than during the day, which was either due to sleep disruption or previous experience with sound exposure.

The diel variations in sound impact sensitivity may differ between diurnal and nocturnal species. This species-specific effect warrants more noise impact studies on various species groups. Moreover, since fish alter their behaviour and physiology depending on their experience with the environments, their sensitivity and responsiveness to sound exposure may also vary accordingly. Fish with different ontogenetic backgrounds, such as hatchery-reared or wild-caught, still need to be compared to see how their prior experiences affect their vulnerability to sound exposure. These differences should also be considered when assessing sound impacts and devising mitigating strategies. Moreover, wildlife management should also take into account how sound impacts and human interventions may affect the whole assemblage of fish community.

Future research

The four experiments in this thesis showed that behavioural assessments of sound impacts are more complex than previously assumed. While this thesis answered many important fundamental questions, it also revealed other critical gaps in our knowledge. Many new questions can only be answered with continued interdisciplinary collaborations. As a successful attempt, this thesis collaborated with two other subprojects under a larger project entitled 'The effects of underwater noise on fish and marine mammals in the North Sea', funded by the Dutch National Ocean and Coastal Research Programme (NWO-ZKO). By collaborating with underwater acousticians (primary researcher: Őzkan Sertlek), we can now use sound maps and propagation models to assess the area and diversity of fishes experiencing man-made noise pollution[18,19]. Furthermore, collaboration with marine mammal researchers (primary researcher: Geert Aarts) has inspired the use of individual-based models to evaluate survival and distributional changes of fish upon sound exposure, as well as predator-prey interactions between fish and marine mammals[20,21].

This thesis also highlights the need for more sound impact studies looking beyond effects at individual level, and examining potential impacts at population, community and ecosystem levels. It is crucial to study impacts at different ecological levels, since it would provide insights into effects at different scales and potentially aid in choosing the right focus for wildlife conservation or stock management. For example, animal welfare biologists may be concerned about the different coping strategies of a species to noise pollution, while fisheries biologists may be more interested in the health and stability of fish stocks as a whole. Furthermore, the links between the different ecological levels need still to be explored, in order to improve our understanding of the underlying mechanisms and the scale of underwater sound impacts.

Besides extending our understanding by viewing from broader perspectives, it is also useful to zoom into the mechanistic relationship between various acoustic parameters and sound sensitivity. In fact, our understanding of fish hearing is still rather limited[6,22]. Most audiograms that have been developed so far still suffer from several limitations, such as the exclusion of infrasound sensitivity (< 100 Hz), measurements at various background noise levels, and acoustically and behaviourally unnatural experimental settings[22,23]. Furthermore, most hearing studies focused on the pressure component of sound, while ignoring the particle motion component that is the principal hearing component for many species. There is currently a need to discern the interplay between sound pressure and particle motion in fish hearing. Only then can we use the acoustic information at a particular site to predict the susceptibility of a particular species. Although many questions remain, this thesis addressed an important area that was previously unexplored, and thereby opened up many venues for future research.

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Dutch Summary
Nederlandse Samenvatting

De akoestische wereld van vissen

Vissen leven niet in een stille wereld. Ze leven in een akoestische wereld, boordevol met geluiden afkomstig van zowel natuurlijke processen als onderwaterdieren, van pistoolgarnalen tot zingende walvissen. Vissen kunnen geluiden met een lage frequentie horen en er ook gebruik van maken. Zo kunnen ze geluiden gebruiken om een geschikte leefomgeving en prooien te vinden, en predatoren uit de weg te gaan. Sommige soorten kunnen ook zelf geluiden maken om concurrenten weg te jagen of potentiele partners aan te trekken. De onderwaterwereld bevat dus een grote hoeveelheid akoestische informatie en dit verspreidt zich onderwater effectiever dan visuele, geur- en tactiele signalen.

Door hun afhankelijkheid van geluiden is het aannemelijk dat vissen impact ondervinden van een nieuwe prominente bron van geluid: menselijke activiteiten. Hieronder vallen commerciële scheepvaart, offshore bouw, sonaronderzoek, seismische exploratie en onderwaterexplosies, dit genereert een kakofonie van geluiden met een hoge intensiteit. Vissen relatief dicht bij deze geluidsbronnen kunnen beschadigingen in hun gehoororganen oplopen of tijdelijk doof worden. Verder van de geluidsbron is de intensiteit van het geluid lager, maar beslaat het geluid wel een veel groter gebied. Hier kan het geluid het gedrag van vissen verstoren of veranderen, waardoor ze mogelijk minder effectief foerageren of predatoren vermijden. Er zijn groeiende zorgen dat deze veranderingen de gezondheid van vispopulaties kan schaden en de stabiliteit van ecosystemen kan verstoren.

De invloed van geluid op gedrag begrijpen

Het beoordelen van de ernst van de gevolgen van onderwatergeluid is niet eenvoudig. Ten eerste zijn huidige beoordelingen over het algemeen gebaseerd op de intensiteit en de duur van een geluidsblootstelling, terwijl dit mogelijk niet de juiste parameters zijn om de impact op het gedrag te voorspellen. Andere akoestische parameters - zoals de temporele structuur van geluid - zijn mogelijk belangrijker, maar dit moet eerst onderzocht worden. Ten tweede kan het lastig zijn om gedragsonderzoeken naar de impact van geluid te extrapoleren naar de situatie in het wild, omdat er voor verschillende onderzoeken verschillende methodes worden gebruikt. Zo worden er onderzoeken uitgevoerd in het laboratorium, het veld en in semi-natuurlijke opstellingen, elk met hun eigen voor- en nadelen. De resultaten van de verschillende opstellingen moeten met elkaar worden vergeleken, om te bepalen welke effecten van geluid gegeneraliseerd kunnen worden, en hoe de resultaten zijn beïnvloed door de onderzoeksmethode. Ten derde kan het lastig zijn om onderzoeksresultaten te vertalen naar managementimplicaties, omdat gedrag erg complex is. De complexiteit van gedrag wordt vaak duidelijk door de hoge variatie in de gevonden resultaten. Dit kan veroorzaakt worden door verschillende persoonlijkheden van de individuen en een omgevingsafhankelijke reactie. Daarnaast is het mogelijk dat niet elke gedragsverandering direct consequenties voor de fitness heeft, desalniettemin kunnen deze gedragingen wel op fysiologische stress of een veranderde energiehuishouding wijzen.

Om deze uitdagingen aan te gaan heb ik eerst twee experimenten uitgevoerd in een bassin. Hier heb ik groepen van Europese zeebaarzen blootgesteld aan een serie van geluiden. De zwempatronen van de vissen zijn geanalyseerd met behulp van een videovolgsysteem. In het eerste experiment heb ik de impact van continue met onderbroken geluiden vergeleken, met een ofwel continue of fluctuerende geluidssterkte (hoofdstuk 2). Uit de resultaten bleek dat elk type geluid ervoor zorgde dat de vissen sneller, dieper en dichter bij elkaar gingen zwemmen. De vissen herstelden echter wel binnen een uur na de start van de geluidsblootstelling, dit hield in dat hun zwempatroon weer hetzelfde was als voor de start van het geluid. Interessant was dat de vissen twee keer zo langzaam herstelden van de onderbroken geluiden als van de continue geluiden. Hierna heb ik onderzocht of het herstel optrad door habituatie (gewenning), doordat de vissen niet meer reageerden omdat ze tijdelijk doof waren geworden, of omdat ze fysiek waren uitgeput, door het geluid en hun eigen reactie. Ik heb ook bekeken of verschillende intervallengtes tussen opeenvolgende geluidspulsen de impact van geluidsblootstellingen beïnvloedden (hoofdstuk 3). Ik heb aangetoond dat het herstel werd veroorzaakt door habituatie en dat langere pulsintervallen ervoor zorgden dat vissen na de geluidsblootstelling hoger in de waterkolom gingen zwemmen.

Hierna heb ik twee experimenten uitgevoerd in een groot drijvend net met behulp van dezelfde onderzoeksmethodiek als de voorgaande experimenten. De 3D zwempatronen van de vissen zijn deze keer inzichtelijk gemaakt met behulp van akoestische chips. Gebruikmakend van deze opstelling in semi-natuurlijke proefopstelling heb ik eerst de effectiviteit van een 'ramp-up' procedure getest. Een dergelijke procedure wordt vaak gebruikt vóór heiwerkzaamheden en seismische exploratie (met airgun) om zeezoogdieren en vissen weg te jagen en te voorkomen dat ze gewond raken door de luide geluiden. Ik heb ook opnieuw continu met onderbroken geluid vergeleken en nu ook het effect van regelmatig met onregelmatig onderbroken geluid (hoofdstuk 4). In de drijvende set-up reageerden de vissen op een vergelijkbare manier op het geluid als in het bassin, behalve dat de vissen nu ook wegzwommen van de geluidsbron. Hoewel de vissen reageerden op de start van de 'ramp-up', zwommen ze niet weg van de geluidsbron, terwijl dit wel algemeen werd aangenomen. Er waren geen duidelijke verschillen tussen de reacties op continu en onderbroken geluid of tussen regelmatig en onregelmatig onderbroken geluid. Hierna heb ik onderzocht of er habituatie optrad bij Europese zeebaarzen tijdens herhaalde blootstellingen van hetzelfde geluid, en of geluidsblootstellingen op verschillende tijden van de dag de vissen anders beïnvloedden (hoofdstuk 5). Ik heb aangetoond dat er habituatie optrad bij de blootstelling van acht achtereenvolgende blootstellingen gedurende twee dagen en dat ze 's nachts sterker op het geluid reageerden dan overdag.

Managementimplicaties en verder onderzoek

De vier experimenten in dit proefschrift laten zien dat het beoordelen van de impact van geluidsblootstellingen op gedrag complexer zijn dan hiervoor werd aangenomen. Ten eerste wordt de impact op gedrag beïnvloed door de temporele structuur van het geluid. Daarom moet er bij de beoordeling van potentiele impact van geluid en het toepassen van mitigatiestrategieën rekening worden gehouden met de temporele structuur van het geluid. Ten tweede wennen vissen waarschijnlijk aan geluidsblootstellingen (het geluid van menselijke activiteiten), maar of gewenning leidt tot een positief of negatief effect op fitness moet nog worden aangetoond. Ten derde kunnen sommige gedragingen die in aquaria en bassins worden waargenomen wel gegeneraliseerd worden naar het open water, terwijl andere gedragingen erg verschillen of afwezig kunnen zijn in begrensde omgevingen. Bevindingen verkregen in aquaria en bassins kunnen dus alleen geëxtrapoleerd worden naar het open water na weloverwogen evaluatie. Ten vierde worden vissen niet per se verjaagd door een 'ramp-up' procedure terwijl dit wel algemeen werd aangenomen. Als laatste kan de impact op het gedrag afhankelijk zijn van het moment op de dag. Dit kan gerelateerd zijn aan het dag/nacht ritme van de vissen. Regelgeving, bedoelt om de effecten van geluid te verminderen, zou hier rekening mee moeten houden, dit zou ook afgestemd moeten worden op de aanwezige soorten.

Hoewel dit proefschrift veel fundamentele vragen heeft beantwoord, heeft het ook belangrijke gaten in onze kennis blootgelegd. Het is bijvoorbeeld nog onbekend of wildvang vissen en vissen in het veld op dezelfde manier reageren als vissen van een kwekerij. Vissen in het wild hebben een andere interactie met hun omgeving en reageren mogelijk anders op een akoestische stressor. Daarnaast vertonen vissen mogelijk ook ander gedrag in een niet-afgesloten omgeving. Met experimenten in situ, waar vissen aan het geluid van echte menselijke activiteiten worden blootgesteld, kan aangetoond worden in hoeverre de gedragsveranderingen in dit proefschrift gegeneraliseerd kunnen worden. Verder moeten simpele gedragsveranderingen gekoppeld kunnen worden aan (mogelijke) veranderingen in overlevingskans, voortplantingssucces en de stabiliteit van een populatie. Dit moet gedaan worden door middel van verder onderzoek of modeleren. Hoewel er nog steeds veel vragen zijn, is er met dit proefschrift een belangrijk en onbekend terrein verkend en heeft het voor veel nieuwe aanknopingspunten voor verder onderzoek gezorgd.

Chinese Summary
中文概括

魚类声感世界

鱼类的生长环境并非寂静无声。反之，海底世界充满了种种自然现象及水生动物的发声，如鼓虾慑猎时巨螯速合的声响以及鲸群用以沟通的鲸歌。鱼类能听见低频率的声音,并利用声音寻找合适生境、搜寻猎物和避开捕食者。一些鱼种也能发声，以吸引配偶或击退竞争者。因为海底大量的声音信息比视觉、嗅觉及触觉的信号在水中更容易传播，鱼类更得依赖其听觉以得生存。

由于依赖听觉，鱼类也可能被一个新兴的非自然声源所影响——人类活动。这些活动包括船务运输、海上工程、声纳探索、地震勘探以及海底爆破。所产生的噪音在近距离内能直接杀死鱼群、损坏其听觉组织或将它们暂时性的震聋；在远距离能改变鱼类的行为，减低它们觅食与避免被捕的效力。而让人担忧的是这些改变有可能威胁渔业资源的可持续性及生态系统的稳定性。

了解噪音对鱼类行为的影响

评估噪音的严重性并非直截了当，也存在种种困难。一、当前的评估指南主要以噪音的音量与长短为基准，但这些标准并无法充分地反映噪音对鱼类行为的影响。其他声音度量，如声音的瞬时构造，虽然可能更为重要，但却未经测试。二、行为研究对噪音影响所发表的结果不容易推测鱼类在野生状态下的行为改变，因为各别的研究使用不同的实验方法。这些方法可以是实验室内、野外实地或半自然式的研究，也各有其利与弊。各个方法的研究结果须要直接地比较，才能推定各种观察结果的概括性，以及实验条件对结果的影响。三、将研究结果转化为管理决策并非易事，毕竟动物行为本质上是极为复杂的。行为的复杂性反映在行为数据上极大的变异性，而此变异性来自个体间性格的差异以及行为反应受环境的影响。此外，有些行为改变并不直接表示动物的存活以及繁殖受到影响，尽管这可能造成生理压力和精力受损。

为了克服这些挑战，我首先在一方人工水池里进行了两个实验，对来自养殖场的欧洲鲈鱼播放一系列的噪音，并以视频跟踪系统分析鱼群的游动。第一个实验比较连续性与脉冲性的噪音，以及稳定与变动音量的影响（第二章）。当鱼群听见噪音时，它们马上加速并聚合游向更深处，反应了对噪音感受胁迫。然而，鱼群在噪音播放的一小时内就恢复了开始时的行为。有意思的是鱼群在脉冲性噪音下的恢复时间是连续性噪音下的两倍。接着，我检验此行为的恢复是否是因为鱼群对噪音持续地刺激形成了习惯化，而非因被噪音震聋或耗尽精力而停止反应。此外，我也测试脉冲之间的间歇长短是否调节噪音的影响(第三章)。从这个实验，我示证了行为的恢复是由习惯化所引起，而当脉冲的间歇越长，鱼群在噪音停止后则游得越靠水面。

我接下来使用相同的实验设计在一个悬浮于海面的大型网箱进行了另两个实验，并利用三维声波遥测技术分析鱼群的游动模式。我利用此半自然式的设置来测试噪声淡入的功效，既是一般海上工程或地震勘探前以渐增音量来驱赶海洋动物以防受巨响伤害的程序。同时，我也再次比较连续性与脉冲性的噪音，以及规律与不规律脉冲间歇的影响(第四章)。在这露天的网箱中，鱼群行为的改变与之前在水池内大致相似，但它们却同时远离了声源。然而，虽然鱼群在淡入程序一开始就受惊，它们并没如预计般游开。此外，此实验装置并没有显示连续性与脉冲性噪音之间，以及规律与不规律脉冲间歇之间对鱼群的影响有明显差异。接着，我检测欧洲鲈鱼是否对多次噪音的播放也会形成习惯化，而且对噪音的反应会否因播放的时段而有所不同（第五章）。我发现鱼群在两天内对连续八次噪音播放的反应逐渐减少，而晚上的反应比白天来得大。

对生态管理及后续研究的启发

本论文内的四个实验显示了噪音对鱼类行为的影响比事先预想的还复杂。首先，鱼类行为的改变受噪音的瞬时构造影响。因此，噪音的瞬时构造在评估噪音的严重性及策划纾缓措施时须被考量。二、一些室内实验所观察到的行为改变可被外推至户外环境， 但一些行为则在局限空间内无法显示或与野外有所不同。因此，实验室内的研究结果须经仔细审核后才可外推至大自然。三、噪声淡入的程序不一定如一般所预计般能驱赶鱼群。一些淡入程序不但无效，更可能适得其反。四、鱼类会对反复的噪音习惯化，但这对鱼的影响是好是坏目前还无以定夺。最后，鱼类因昼夜节律的关系而对噪音的反应会因时段而有所不同。这些差异须受重视，生态管理才能更为有效。

本论文不但解答了许多重要的问题，也揭露了我们目前对此课题了解的不足。比方说，我们依然不知道野外的鱼类对噪音的反应是否与本论文来自养殖场的欧洲鲈鱼相同。野生的鱼经历了不一样的环境，所以对噪音的反应也可能不同。而且，自由游动的鱼群所显示的行为模式与在局限空间内被测试的鱼群也可能有别。除此之外，我们须要在人类海洋活动现场进行测试，以示证本论文所观察的鱼类行为改变的概括性。另外，为了了解行为上的改变是否影响鱼群的存活与繁殖，以及其种群的稳定性，我们还需要更多的后续研究。虽然未解的问题还很多，本论文探索了一个先前未知的领域， 因而开拓了许多新的研究方向。

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