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Summary

There is concern about the potential adverse effects of pile-driving sounds, generated during the construction of offshore wind farms, on marine fauna. Recently, several studies have addressed short-term, physical effects of pile-driving sound on fish (larvae), but long-term effects or delayed effects have not been investigated yet. We examined long-term effects of larval exposure to pile-driving sound on mortality and growth in European sea bass (*Dicentrarchus labrax*) and found no significant differences between exposed fish and control group fish up to 255 days post-exposure. We also examined potential delayed effects of larval exposure on behavioural responses to sound in the juvenile life phase. There was some evidence for such effects, but the effects found were not straightforward and not consistent over all experiments: Under elevated background noise larval exposed fish appeared to be less sensitive to a sound signal than control group fish, while under ambient background noise they might be more sensitive to a sound signal than control group fish. These effects were only observed in experiments with a group of 4 fish, not in experiments with a single fish.

1   Introduction

1.1   Dutch context

In the Netherlands, pile-driving for the construction of offshore wind farms was limited to the period July - December. This precautionary management measure was installed partly because of potential adverse effects of pile-driving sounds on fish larvae. Negative effects on fish larvae may impact juvenile fish in Natura 2000 areas, thus affecting food availability for birds and marine mammals. The Natura 2000 areas are protected under the EU Birds and Habitats directives.

For the Appropriate Assessment of Dutch offshore wind farms, a modelling study was carried out to estimate the effect of pile-driving sound on the number of plaice (*Pleuronectes platessa*), common sole (*Solea solea*), and herring (*Clupea harengus*) larvae that reach the Dutch Natura2000 sites (Prins et al. 2009). For this, an existing larval transport model (Bolle et al. 2009) was expanded with an assumption on larval mortality caused by pile-driving sound, that was roughly based on interim criteria published in 2009 (Oestman et al.). Subsequently, based on expert-judgment, the model results were extrapolated to other fish species and older life stages in an attempt to assess the effect of offshore pile-driving on the overall prey availability for birds and marine mammals in Natura2000 sites (Bos et al. 2009). This extrapolation indicated that a reduction of more than 5% might occur for seven important prey species. These findings contributed to the decision for implementing a mitigation rule on the period of the year in which pile-driving is allowed. However, the Appropriate Assessment was hampered by lack of knowledge. Little was known about the vulnerability of fish eggs and larvae to pile-driving sound and the spatial scale at which mortality or injury may occur (Popper & Hastings 2009).

To address this knowledge gap, controlled exposure experiments were included in the 'Shortlist Masterplan Wind' (SMW) and 'Voortzetting Uitvoering Masterplan' (VUM) research programmes, both financed by the Dutch government. A device was developed specifically for the purpose of exposure of fish larvae to pile-driving sounds, as high intensity, low frequency impulsive sounds are distorted in aquaria and small basins. These studies focussed on potential lethal effects of pile-driving sounds in fish larvae (Bolle et al. 2012, Bolle et al. submitted a).

Additional research was commissioned by Eneco as compliance to the conditions under their permit for wind farm construction. The first project focussed on sub-lethal effects of exposure to pile-driving sound in juvenile fish; injury assessments were carried in collaboration with American colleagues (Bolle et al. submitted b). The current study was the second project commissioned by Eneco and focussed on delayed effects of larval exposure to pile-driving sound.

1.2   Scientific context

The rapid increase of offshore wind farms has led to an urgent need to acquire more knowledge on the ecological consequences of offshore wind farm construction and operation (Inger et al. 2009). Concern exists about the potential adverse effects of sounds associated with these activities, in particular the loud impulsive sounds generated by pile-driving during the construction of wind farms. Loud impulsive sounds have the potential to kill or injure fishes (Popper & Hastings 2009). Until recently, very little was known about the sound levels at which physical damage may occur in fish, especially in fish larvae (Popper & Hastings 2009). Fish larvae are planktonic and have limited capabilities of avoiding sound and may therefore be more vulnerable to sound exposure than juvenile and adult fish.

Previously, interim criteria for non-auditory tissue damage in fish due to pile-driving sounds were formulated (Oestman et al. 2009). These criteria included a cumulative sound exposure level (SEL_{cum}) threshold of 183 dB re 1 μPa^2s for fish of mass < 2 g and of 187 dB re 1 μPa^2s for fish of mass ≥ 2 g. Since then, several experimental studies have been carried out to examine the effects of pile-driving sounds on fish, indicating that the SEL_{cum} thresholds for injuries or death are considerably higher than the interim criteria suggested. Controlled exposure experiments in a laboratory setting showed no lethal effects up to 10 days after exposure to 206-216 dB re 1 μPa^2s SEL_{cum} for different larval stages of 3 fish species with different types of swim bladders (Bolle et al. 2012, Bolle et al. submitted a). Field experiments corroborated these findings; no lethal effects were observed up to 14 days after exposure for early juveniles exposed to 215-222 dB re 1 μPa^2s SEL_{cum} (Debusschere et al. 2014). Injury assessments, carried out for juvenile fish exposed to pile-driving sound in a laboratory setting, revealed an onset of injuries at 204-210 dB re 1 μPa^2s SEL_{cum} for five fish species with a swim bladder (Halvorsen et al. 2012a, Halvorsen et al. 2012b, Caspar et al. 2013, Bolle et al. submitted b). No injuries were observed in a flatfish species without a swim bladder exposed to 216 dB re 1 μPa^2s SEL_{cum} (Halvorsen et al. 2012b). Recovery from injuries was examined in three species and evidence of healing was observed within 10-13 days post-exposure, for fish exposed to 207-217 dB re 1 μPa^2s SEL_{cum} (Caspar et al. 2012, Caspar et al. 2013, Bolle et al. submitted b). These new insights were incorporated in recently published sound exposure guidelines (Popper et al. 2014) and, compared to the interim criteria of 2009, higher SEL_{cum} thresholds for pile-driving sounds were proposed: 207 dB re 1 μPa^2s if the swim bladder is involved in hearing, 210 dB re 1 μPa^2s if the swim bladder is not involved in hearing and >219 dB re 1 μPa^2s if no swim bladder is present.

To date, no studies have yet addressed long-term (>15 days) or delayed effects of early-life exposure to pile-driving sound. Exposure to sound may cause hearing damage. However, fish, unlike mammals, have the ability to replace or repair sensory hair cells that have been damaged (Popper & Hastings 2009). Permanent threshold shifts due to damage of the sensory hair cells are therefore unlikely. But recent auditory research in humans showed that cochlear neurons are actually more vulnerable and that even temporary threshold shifts cause permanent damage to neurons (Liberman, 2015). This effect had never been discovered before because the tissue degeneration was not visible until 6 months after exposure. The neural damage does not affect the audiogram, but it is expected to impair sound signal detection in noise.

Early-life exposure may also cause physiological effects later in life. Tsalafouta et al. (2015) showed fish larvae to be vulnerable to stress, and that stressors may negatively affect the development of the larvae. This same study showed that fish stressed in the larval stage have a higher cortisol level when stressors were introduced again in later stages of the development, compared to fish with little stress in the larval stage. Early-life stress may lead to differences in general anxiety levels and sound exposure related response tendencies between exposed and non-exposed fish

In this study, we examined whether larval exposure to pile-driving sound affects larval and juvenile mortality and growth rates and behavioural responses to sound in the juvenile life phase of European sea bass (*Dicentrarchus labrax*). Behavioural responses to sound are important for many fish species in their natural surroundings, as survival often depends on behavioural responses to sound (e.g. predator avoidance, prey detection, soundscape habitat selection) (Slabbekoorn and Bouton 2008, Slabbekoorn et al. 2010).

In principle, any sound within the hearing range of sea bass could have been chosen to examine potential alterations of behavioural responses to sound in the juvenile life phase. We used pile-driving sound (at sound levels that evoke behavioural responses, not physical damage). Firstly, because pile-driving sound is a broadband sound, with most energy within the hearing spectrum of sea bass (Kastelein et al. 2008, Ainslie et al. 2009). Secondly, because intermittent sound, such as pile-driving sound, is expected to provoke a stronger response than continuous sounds (Neo et al. 2014). Thirdly, because this enabled examination of behavioural responses to pile-driving sound, which in itself is a relevant research question.

1.3   Aim (assignment)

The aim of this study was to examine long-term effects of exposure to high-intensity pile-driving sound in the larval life phase. The main goal was to investigate delayed effects on behavioural responses to sound in the juvenile life phase. Additional goals were to examine long-term effects on larval and juvenile mortality and growth rates. The fish species used in this study was European sea bass (*Dicentrarchus labrax*).

2 Materials and Methods

2.1 Sea bass rearing and transfer

European sea bass is an important species in both the fishing and aquaculture industry. This fish species has a physoclistous swim bladder (no connection with the gut) in the adult life phase. Like most physoclistous fish, sea bass is physostomous (swim bladder has a connection with the gut) in the early larval phase. The swim bladder is initially inflated by passage of air from the gut, through the pneumatic duct, to the swim bladder. Initial inflation occurs between 7 and 16 days after hatching (DAH) when reared at 13-14 ^{o}C (Moretti et al., 1999). The larval exposures to pile-driving sound were carried out at 40 DAH by which time the pneumatic duct is closed. The closed swim bladder of physoclistous fish is expected to make them more vulnerable to pressure differences, such as sound pressure exposure, than physostomous fish (Halvorsen et al. 2012b).

Sea bass eggs were purchased from a commercial hatchery in France (Ecloserie Marine de Gravelines) and reared to 40 DAH in larval rearing tanks (75x75 cm, height=40 cm) at IMARES (IJmuiden, The Netherlands), according to guidelines from the hatchery. Water temperature was slowly raised (0.5 ^{o}C per day) from the temperature in the hatchery (14 ^{o}C) to the ambient temperature in the laboratory (18 ^{o}C). The larvae started feeding at 9 DAH, when the yolk-sac was absorbed. The larvae were fed live artemia (*Artemia salina*), in excess, twice during daytime. The remaining artemia were flushed out of the larval rearing tanks at night. Gas-filled swim bladders were observed in all larvae examined (random samples from the stock of larvae, n=39).

At 40 DAH, the larval sound exposures were carried out. Seventeen groups of approximately 100 larvae per group were included in the experiments. Subsequently, the 17 groups were held separately in small aquaria (30x50 cm, height=30 cm) until 69 days after exposure (109 DAH). These aquaria were blinded to minimise disturbance. From 40 DAH onwards, the fish were fed dry feed pellets, three times a day by hand to satiation.

At 109 DAH (69 days after exposure), the fish (by then juveniles) were transferred to 17 larger aquaria (70x70 cm, height=45 cm). The fish were sedated (with ethylene glycol monophenyl ether) before transferring them to enable measurements (see below). The juveniles were fed to satiation with dry feed pellets, they were fed throughout the day using a belt feeder.

At 295 DAH (255 days after exposure), 12 fish were randomly selected from each of the 17 groups. The fish were sedated (with ethylene glycol monophenyl ether) to avoid inadvertently choosing the slowest swimming fish of the group. Fish showing any external malformations were excluded from the selection. Almost 1 week later (at 301 DAH), the selected fish were transferred to SEAMARCO (Wilhelminadorp, the Netherlands) for the juvenile behavioural experiments. The fish were transported in large sturdy bags with pure oxygen above the water and with each of the original groups in a separate bag. All fish survived the transport without any visible harm. At SEAMARCO, the fish were stored in 3 round tanks (diameter 2.2 m, depth 1 m). Each tank contained 5 or 6 creel nets, and each net contained one of the original groups. The fish were fed the same dry pellets as at IMARES.

The tanks at SEAMARCO were outdoors, with a flow-through system that was connected to a marine inlet (the Oosterschelde). Consequently, water temperature ranged from 9 ^{o}C in early April, when the fish were transferred from IMARES to SEAMARCO, to 15 ^{o}C at the end of May, when the experiments were completed. In the 3 week period before the transfer, the fish were slowly acclimated from the temperature at IMARES (18 ^{o}C) to the initial temperature at SEAMARCO (9 ^{o}C).

At IMARES, the larvae and juveniles were held at a day-night cycle of 14 hours of light and 10 hours of darkness. At SEAMARCO, the fish experienced a natural day-night cycle, which ranged from 13 hours light and 11 hours darkness in early April to 16 hours light and 8 hours darkness towards the end of May.

2.2   Larval treatments

Fish larvae were exposed to pile-driving sound in the 'larvaebrator'. This device was developed to enable exposure of fish larvae to high intensity, low frequency impulsive sounds in a laboratory setting (see Bolle et al. 2012 for a detailed description). It was inspired by an existing laboratory set-up for larger fish called the fishabrator or HICI-FT (Halvorsen et al. 2012a, Martin & Rogers 2008).

A sound signal recorded at 100 m distance from the pile during the construction of the OWEZ wind farm in the North Sea (4 m diameter steel monopile, at a water depth of approximately 20 m, with a hammer strike energy of approximately 800 kJ) was played-back in the larvaebrator. Sound spectra recordings show that the main energy of underwater pile-driving sound is generated in the 50 Hz to 1 kHz bands (Ainslie et al. 2009). The playback sound was limited to this frequency band, to avoid excitation of spurious resonances in the larvaebrator. The playback level was quantified in terms of zero-to-peak pressure level (L_{z-p} in dB re 1 μPa^2), single-strike sound exposure level (SEL_{ss} in dB re 1 $\mu Pa^2 s$) and cumulative sound exposure level (SEL_{cum} in dB re 1 $\mu Pa^2 s$) as defined by Ainslie (2011). Further characteristics of the original and reproduced signals (such as frequency spectra) have previously been published by Bolle et al. (2012, submitted a).

Only one sound exposure was included in the larval treatments: 999 strikes (1 pulse per second) of the OWEZ@100m sound signal. This was the highest exposure included in the previous larvaebrator studies (Bolle et al. submitted a, Bolle et al. submitted b). Average playback levels (± s.d.) were: L_{z-p} = 208 (±1) dB re 1 μPa^2, SEL_{ss} = 184 (±1) dB re 1 $\mu Pa^2 s$ and SEL_{cum} = 214 (±1) dB re 1 $\mu Pa^2 s$.

Seventeen groups were included in the larval treatments: 9 exposure groups and 8 control groups in randomised order. We intended to use 18 groups based on the capacity at IMARES for rearing the larvae to juveniles, but we had to drop one group due to insufficient numbers of larvae. The groups were numbered 1 to 17 to refer to the order in the treatment sequence (group 1-2=control, 3=exposure, 4=control, 5=exposure, 6=control, 7-8=exposure, 9=control, 10-12=exposure, 13-14=control, 15-16=exposure, 17=control). The control groups underwent exactly the same procedures as the exposure groups, except exposure to pile-driving sound. All groups were treated on one day (40 DAH). Each group consisted of approximately 100 larvae and all larvae within a group were treated simultaneously. The exact numbers were counted after the treatment, before placing them in the small aquaria (see above).

2.3   Physical effects

As stated before, the main goal of this study was to examine effects of larval exposure on behavioural responses to sound in juveniles. Therefore, the fish were disturbed as little as possible to maximise survival until the juvenile experiments. This limited the scope of physical measurements. Nevertheless, long-term effects of larval exposure on mortality and growth could be examined at two points in time: 69 and 255 days after exposure (i.e. the days that the fish were sedated, see above).

The number of surviving larvae in each group was counted at 0, 69 and 255 days after exposure. Mortality was estimated separately for two periods: 0 – 69 days after exposure (mainly larval mortality) and 69-255 days after exposure (juvenile mortality). Separation of these periods is necessary because, in fish, larval mortality is generally much higher than juvenile mortality.

The lengths of all surviving fish were measured at 69 and 255 days after exposure. It was impossible to measure the larvae on the day of exposure without causing extreme mortality. Random samples were taken from the stock and we therefore assume no differences between the groups in mean length on the day of exposure. Other samples from the stock indicated that larvae of this age (40 DAH) were 16.8 ± 0.8 mm (n=39). Separation of growth in two periods was not possible, because the fish were not marked individually. Instead, differences in total growth were examined at 69 and 255 days after exposure.

During the length measurements, malformations were observed in all groups. Spinal deformation was most frequently observed followed by uncovered gills. The proportion of malformed fish was much higher in the first two groups (28-45% at 255 days after exposure) than in the other groups (4-15% at 255 days after exposure). Apart from the first two groups, no correlation between sequence and proportion malformed was observed.

Spinal deformation strongly affected fish length. These fish were 'hunch-backed', showing limited growth in anterior-posterior direction compared to the dorsal-ventral axis. Therefore, the first 2 groups (both control groups) were eliminated from the length analysis. No correlation between proportion malformed and mortality appeared to occur and all groups were included in the mortality analysis.

The persons who counted and measured the surviving fish at 69 and 255 days after exposure did not know which of the groups had been exposed to pile-driving sound.

2.4 Effects on behavioural responses to sound

2.4.1 Sounds

A sound signal recorded at 800 m distance from the pile during the construction of the OWEZ wind farm in the North Sea (i.e. the same pile-driving operation where the sound signal was recorded that was used in the larval exposures). The sound file consisted of 5 pulses from one pile (normalized and filtered by TNO). The amplitude was scaled to the required sound levels during the juvenile experiments.

In Audacity, segments of 1.293 seconds were cut from the original file (exactly 1 pulse) and saved. Only one of the 5 pulses was used within one experiment day in the case of single pulse exposures (see the experimental design below), and the 5 pulses were used in random order on consecutive days. The whole sound file containing 5 pulses was used in the case of extended exposures (see the experimental design below).

All sounds were played from a laptop running Ultrasonic player recorder (v 1.1). The sound went into a mixer, which enabled us to introduce white noise to simulate elevated background noise. The white noise was generated with a white noise generator (HP 33120A). The white noise passed a 2 kHz low pass filter before entering the mixer. The sounds from the mixer went through a power amplifier (Macro-tech 5000 VZ, Crown Audio, Elkhart, US) to the transducer (LL-1424HP, Lubell Labs, Columbus, US).

The play-back levels of pile-driving sound were measured in the research pool at the beginning of the study period. The sound measurement equipment consisted of three hydrophones (Brüel & Kjaer (B&K) – 8106) with a multichannel high frequency analyser (B&K PULSE - 3560 D) and a laptop computer with B&K PULSE software (Labshop version 12.1; sample frequency used: 524288 Hz). Before analysis the recordings were high-pass filtered (cut-off frequency 100 Hz; 3rd order Butterworth filter; 16 dB/octave) to remove low-frequency sounds made by water surface movements. The system was calibrated with a pistonphone (B&K - 4223).

The sound levels of the pile-driving pulses and the elevated background noise to be included in the main experiments were determined during pilot experiments (with fish that were not used during the main experiments).

The sound levels of the pulses were determined by starting at the maximum level of the amplifier and reducing the level until no reaction of the fish was observed. This was tested 7 times and resulted in 6 levels with a difference of 6 dB between the levels:
single-strike sound exposure levels (SEL_{ss}) = 128, 134, 140, 146, 152, 158 dB re 1 μPa^2s
zero-to-peak pressure levels (L_{z-p}) =   149, 155, 161, 167, 173, 179 dB re 1 μPa^2

The background noise (white noise level in mV), that was needed to reduce behavioural responses, was determined at the second loudest level of the pile-driving sound (SEL_{ss} = 152 dB re 1 μPa^2s), which usually caused behavioural reactions under ambient background noise. The output of the noise generator was increased with 100 mV steps once per 10 min and pile-driving sounds were played at the end of each 10 min period. This was tested 4 times. The outcome was that at 600 mV ~50% of the fish reacted, compared to almost 100% under ambient background noise.

2.4.2   Experimental arena

The research was done in an outdoor rectangular basin (7 x 4 x 2 m) with a roof (9 x 6 m) (see Kastelein et al. 2008 for a more detailed description of the basin). Two nets were placed within the basin (each approximately 1.1 x 1.1 m and 1.6 m deep). A white canvas tarp covered the bottom and three sides of the net. This prevented visual stimuli from affecting the fish in the other net and also provided sufficient contrast for video analysis. A research cabin next to the basin contained all necessary equipment for the video recordings and sound playback.

The transducer was 3.5 meters away from the net. The cameras were lowered 2.1 meters away from the net. There were 2 cameras for each nets (Figure 1). The lowest cameras were GoPro Hero cameras. The top cameras were Conrad underwater colour cameras. Above the water, in the middle of the nets, were 2 cameras (Conrad underwater colour cameras). The four Conrad cameras were connected to four different laptops, which recorded the behaviour of the fish.
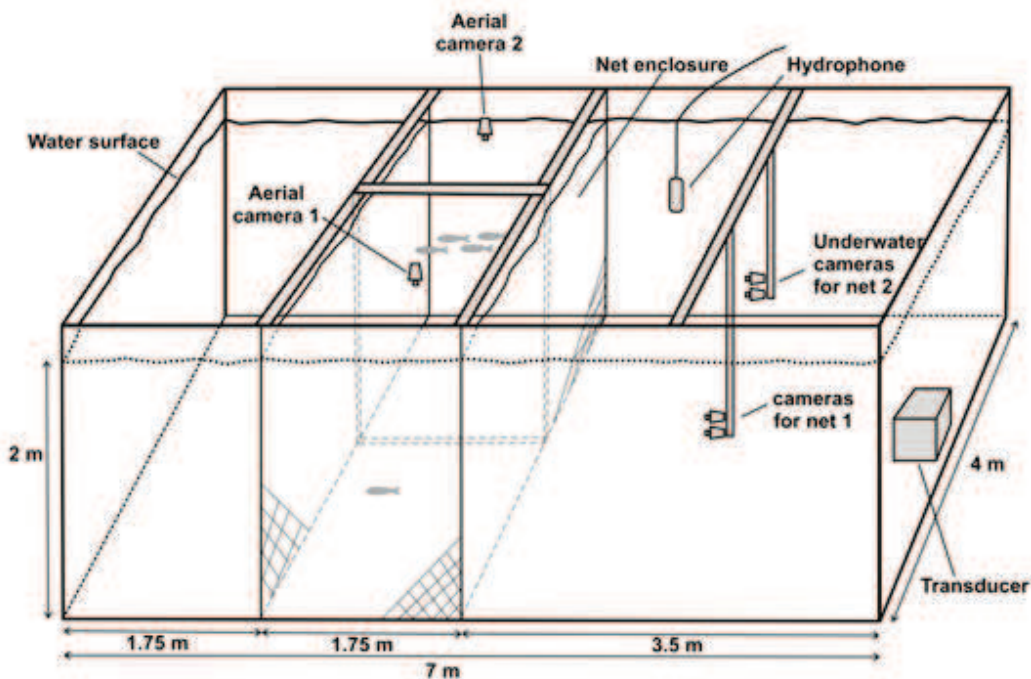

Figure 1   *The research basin used for the juvenile fish experiments. The two nets were 3.5 meter away from the transducer. One net contained 1 fish, the other net contained 4 fish. The hydrophone shown was used to add the sounds to the video recordings.*

2.4.3   Experimental design

Behavioural responses to sound were examined using (1) single pile-driving pulses at varying sound levels, (2) single pile-driving pulses at varying sound levels together with white noise at a fixed level, (3) 'extended' pile-driving sound (1 pulse per 1.3 s for 30 min) at a fixed sound level. The single sound pulses were included to examine sound thresholds and dose-effect relationships for startle responses. White noise was included to simulate elevated background noise for examination of signal detection in noise. The extended sound was included to examine anxiety behaviour.

Behavioural responses were tested for a group of 4 fish (cf. Neo et al. 2014) and for single fish. Juvenile sea bass exhibit social behaviour and may therefore respond differently to stimuli when they are alone or in a group.

Five fish from each of the original larval groups were tested each day. One fish was put in one net and 4 fish were put in the other net (Figure 1). The fish were placed in the experimental set-up at the end of the afternoon and acclimated overnight. In the morning of the following day the behavioural response tests were carried out.

All three test were performed each day (with the same 5 fish from one of the original larval groups): the dose-response test at ambient background noise, the dose-response test at elevated background noise and the anxiety test using extended pile-driving sound (Figure 2).

The dose-response tests consisted of 6 steps of 6 dB: SEL_{ss} = 128, 134, 140, 146, 152, 158 dB re 1 μPa^2s (see above). Each sound level was played twice. The order of the steps was randomized. A recovery period of approximately 5 min between the pulses was considered to be sufficient, based on the pilot experiments, in which we estimated the time required for the fish to return to baseline swimming behaviour after a response to sound to be about 2 min. The period between 2 sound pulses was randomised between 4 and 6 min (with steps of 20 seconds), to minimise potential expectation patterns in the fish. Rstudio (version 0.98.1091) was used for the randomisation.

The difference between the two dose-response tests was the presence or absence of elevated background noise. Both dose-response tests were performed each day, but the sequence alternated between days. The level of the white noise was slowly increased and reached its maximum level 15 min before the first sound pulse was played. Two hours of ambient background noise was maintained between the two tests (Figure 2).

The anxiety test was done 2 hours after the last dose-response test. The test contained three periods of 30 min: pre-exposure, exposure and post-exposure. The pre-exposure period was for acquiring baseline behaviour. The exposure period for examining response to sound and potential habituation. Neo et al. (2014) showed that recovery during sound exposure is caused by habituation, not by fatigue or sensory adaptation. The post-exposure period was to observe (further) recovery of behaviour. Directly after the post-exposure period, the fish were replaced with a new group of fish (Figure 2).

Procedures were fine-tuned during pilot experiments with fish that were not used during the main experiment. No behavioural responses were observed when turning off the water pump, when switching on the acoustic equipment including the 1 kHz calibration tone, or when lowering and activating the cameras. The pump was turned off during the behavioural response tests to minimise ambient background noise. Turning off the water pump and calibration of the acoustic equipment was done 15-30 min before the first test of the day. The cameras were lowered 15 min before each dose-response test and at the beginning of the 30 min pre-exposure period of the anxiety test.
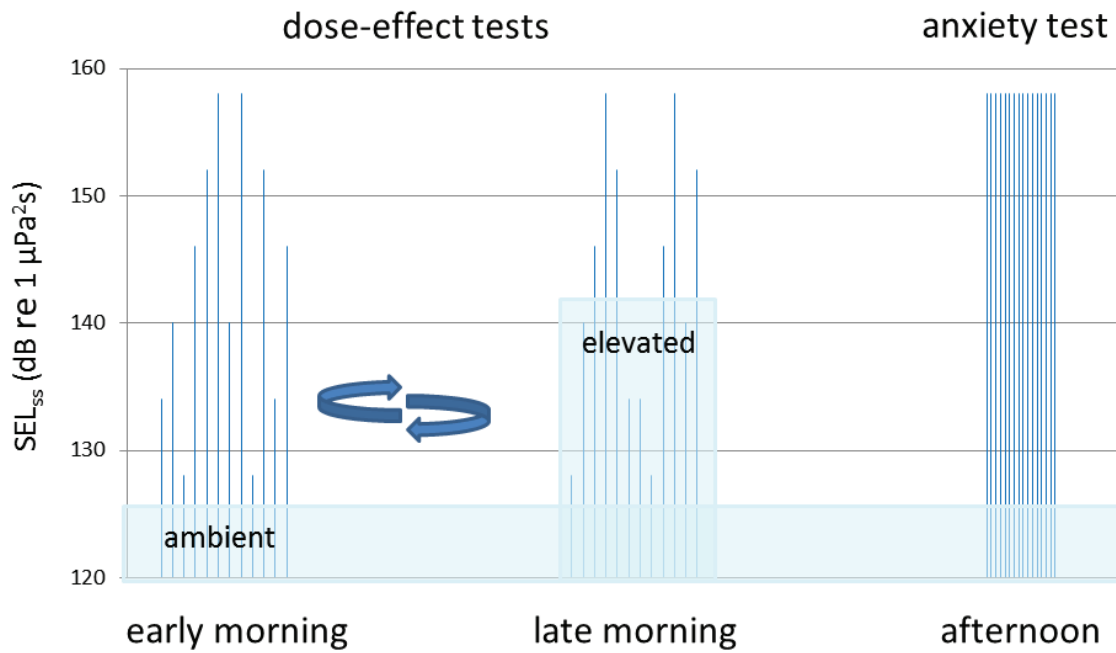
Figure 2 *Scheme illustrating the sequence of behavioural response tests and sound exposures. The sequence of ambient and elevated background noise was alternated between days.*

2.4.4 Behavioural observations

All tests were video recorded and analysed by human eye (1 analyst), using windows media player. The original plan was to automate the analysis with motion tracking software, like EthoVision, but the contrast of the fish on the video recordings was not high enough for these techniques. To prevent bias when analysing, it was unknown which groups were exposed to pile-driving sounds in the larval stage. For the same reason, all videos were analysed without sounds.

The dose-response videos were analysed for sudden movements and changes in swimming behaviour, further referred to as startles. This was done independently for the single fish and the four fish. If such movements were observed, the time and the number of fish which had startled, was noted. These times were later compared to the times that sound was played.

The videos recorded during the anxiety tests with extended pile-driving sound were analysed for swimming depth. Sea bass typically dive to deeper water when anxious. The depth of the fish was determined every three minutes for a period of one hour. This period consisted of 15 minutes of pre-exposure, the full 30 minutes of exposure and 15 minutes of post-exposure. Depth was determined based on the depth layer in which the fish was located. The net was divided into four depth layers (the nets were 1.6 m deep, thus each layer was 0.4 m). If a fish was on the border of two depth layers, its swimming direction was taken into account. This gave sufficient insight into the depth layer the fish was moving towards.

2.5   Statistical analyses

The mortality data were binomially distributed and analysed using a generalised linear mixed model (R, glmer function in lme4 package, family=binomial, link=logit). The 17 larval groups were included in the model as a random effect. The explanatory variable of interest is factor treatment (control or exposure). We furthermore included the sequence of the experiments (larval group number as continuous variable) to examine if this might have had an effect on mortality. The data were analysed separately for 2 periods: 0-69 days after exposure and 69-255 days after exposure.

The length data were normally distributed and analysed using a linear mixed effects model (R, lmer function in lme4 package). The random and fixed effects included in the model were the same as in the analysis of the mortality data. The data were analysed separately for 69 days after exposure and 255 days after exposure.

The startle response data in the juvenile experiments were binomially distributed and analysed using a generalised linear mixed model (R, glmer function in lme4 package, family=binomial, link=logit). The startle data from the single fish tests (presence-absence data) and four fish tests (proportional data) were analysed separately. The 17 experiment days were included in the model as random effect, because measurements with the same fish or group of 4 fish were carried out during each day. Furthermore, each day corresponded with each original larval group. The fixed effects included in the model were sound level (as a continuous variable), factor background noise (ambient or elevated) and factor larval treatment (control or exposed). The variable sound level was rescaled (from SEL_{ss} to difference between SEL_{ss} and maximum SEL_{ss}) to avoid problems in the convergence of the statistical model.

2.6   Ethics statement

The experiments were performed in accordance with the Dutch Experiments on Animals Act. The applications were approved by the DEC of Wageningen UR: part 1 (larval exposures): 2013173.b, part 2 (juvenile experiments): 2014187.b.

3 Results

3.1 Physical effects

3.1.1 Mortality

Mortality was not significantly different between the control groups and the exposed groups, not in the first period after treatment (0-69 days, mainly larval mortality), nor in the second period (69-255 days, juvenile mortality) (Table 1, Figure 3).

The sequence of the larval treatments had a significant effect on larval mortality in the first period (up to 69 days after treatment) (Table 1). Larval mortality decreased from group 1 to group 17. This sequential effect was no longer observed between 69 and 255 days after exposure.

Table 1. Mortality. Analysis of variance (single term deletion, Chi^2 test), variance of random effect and estimates of fixed effects with standard error (logit scale), for probability of death modelled as a function of treatment, sequence and random group effect.

Period	0-69 days after exposure (mainly larval mortality)				69-255 days after exposure (juvenile mortality)			
Analysis of variance	df	AIC	Chi^2	p-value	df	AIC	Chi^2	p-value
<none>		110				94		
treatment	1	109	0.096	0.757	1	92	0.012	0.913
sequence	1	127	18.927	<0.001				
Random effect	n	variance			n	variance		
group	17	0			17	0.124		
Fixed effects		estimate	se			estimate	se	
intercept		0.157	0.110			-2.033	0.197	
treatment exposed		0.031	0.100			0.029	0.266	
sequence		-0.044	0.010					

Model selection was based on Akaike information criteria (AIC); treatment was not removed from the model as this is the explanatory variable of interest.
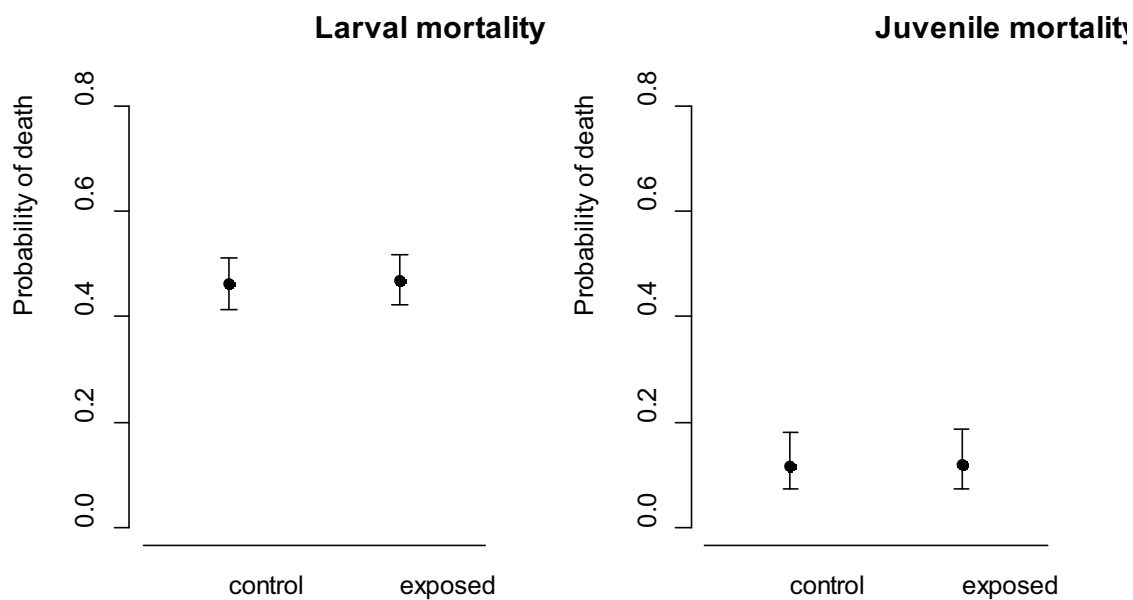
Larval mortality   **Juvenile mortality**

Figure 3   *Model estimates (with 95% confidence interval) of probability of death for control and exposed groups, in the period 0-69 after exposure (mainly larval mortality) and in the period 69-255 days after exposure (juvenile mortality).*

3.1.2 Growth

Fish were slightly larger in the exposed groups than in the control groups (Figure 4), but the differences were not significant (Table 2).

The sequence of the larval exposures had a significant effect on length in the first period after treatment (Table 2). Length increased from group 3 to group 17 (group 1 and 2 were omitted from this analysis, see methods). This sequential effect was no longer observed at 255 days after exposure.

Table 2. Growth. Analysis of variance (single term deletion, Chi^2 test), variance of random effect and estimates of fixed effects with standard error, for length modelled as a function of treatment, sequence and random group effect.

Period	69 days after exposure				255 days after exposure			
	(early juveniles)				(juveniles)			
Analysis of Variance	df	AIC	Chi^2	p-value	df	AIC	Chi^2	p-value
<none>		5427				6575		
treatment	1	5426	0.874	0.350	1	6575	2.246	0.134
sequence	1	5430	4.898	0.027				
Random effect	n	variance			n	variance		
group	15	2.667			15	0		
Fixed effects		estimate	se			estimate	se	
intercept		48.4	1.4			171.2	1.2	
treatment-exposed		0.8	1.0			2.3	1.6	
sequence		0.2	0.1					

Model selection was based on Akaike information criteria (AIC); treatment was not removed from the model as this is the explanatory variable of interest.
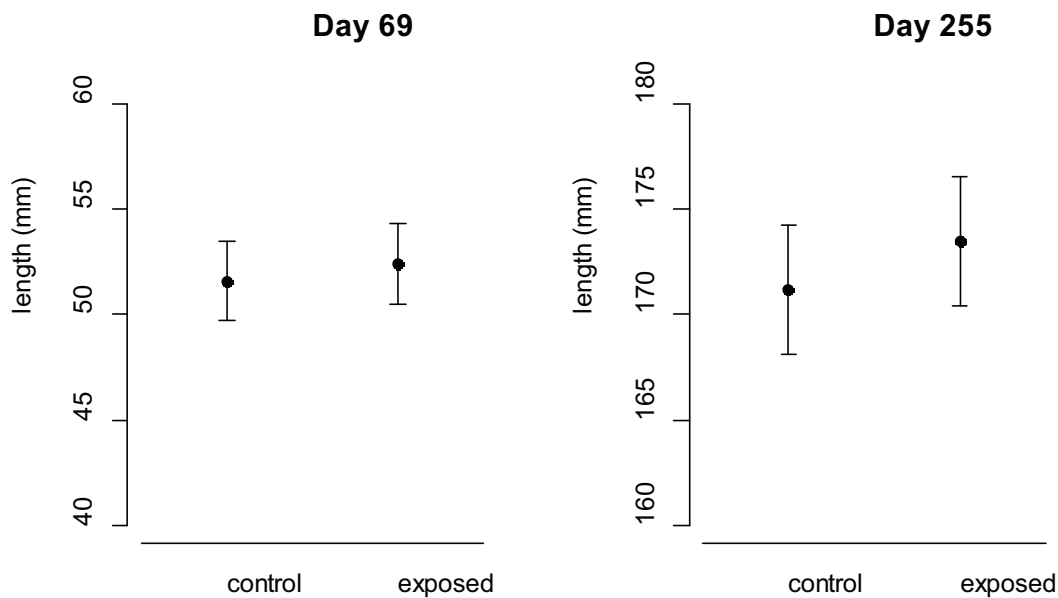
Figure 4   *Model estimates of length (with 95% confidence interval), for control and exposed groups, at 69 days after exposure (early juveniles) and 255 days after exposure (juveniles).*

3.2   Effects on behavioural responses to sound

3.2.1   Startle responses

The dose-response tests showed a significant increase in startle probability with increasing sound level (Figure 5, Table 3 and 4).

In the single fish tests, startle probability was significantly lower at elevated background noise than at ambient background noise. Larval treatment (control or exposed) did not have a significant effect on startle probability (Table 3, Figure 5a-b).

In the four fish tests, startle response appeared to be influenced by (interactions between) sound level, background noise (ambient or elevated) and larval treatment (control or exposed) (Table 4). The shape of the dose-response curve was significantly steeper for exposed fish than for control group fish (Figure 5c-d). Furthermore the interaction between larval treatment and background noise was significant (Table 4). Startle probability was reduced by elevated background noise in the case of exposed fish, whereas no clear differences were observed between elevated and ambient background noise in the case of control group fish (Figure 5c-d).
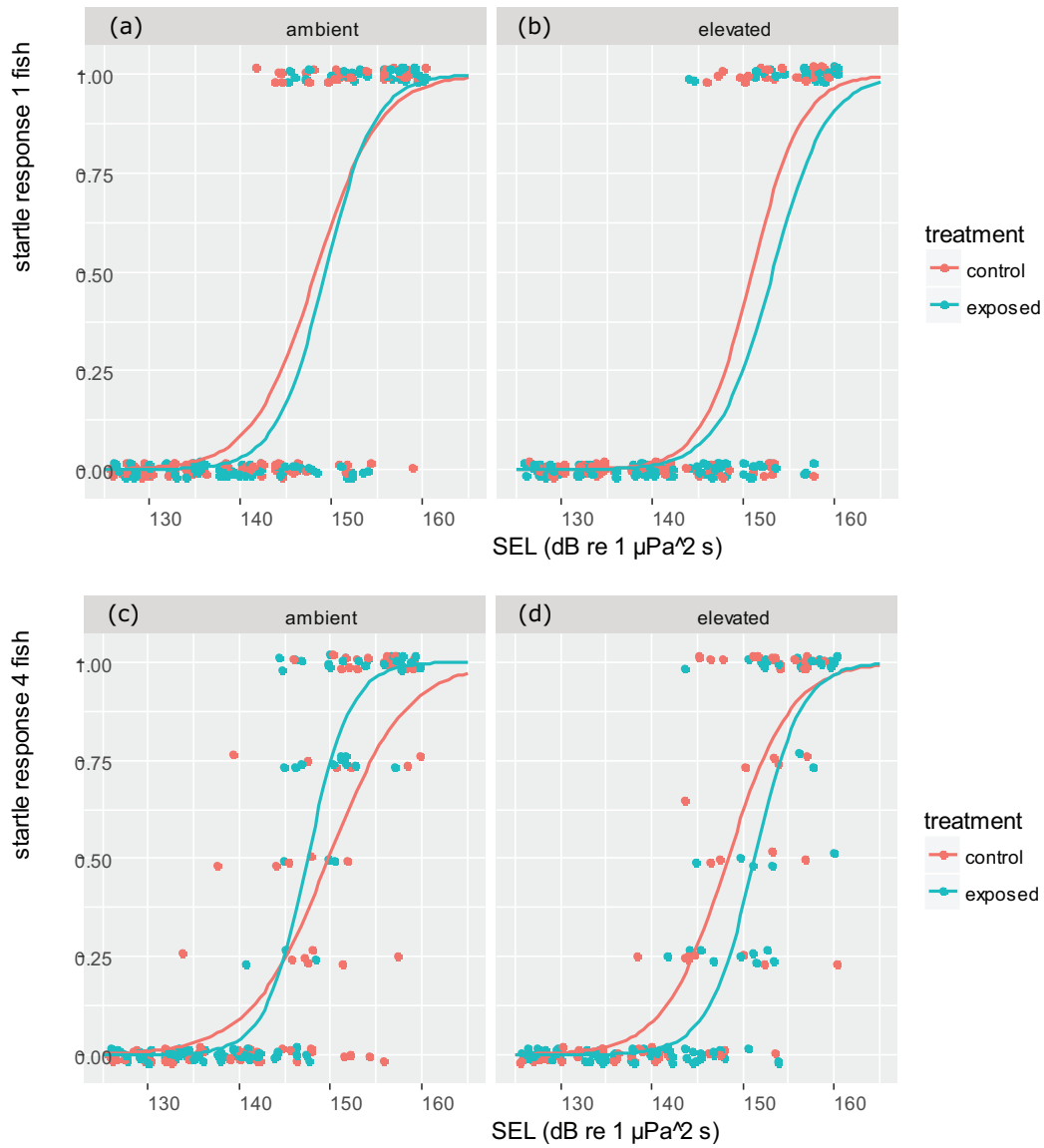
Figure 5 *Startle response observations with binomial smoother under ambient (a, c) and elevated background noise (b, d) for the single fish tests (a, b) and the 4 fish tests (c, d).*

Table 3. Startle response 1 fish. Analysis of variance (single term deletion, Chi^2 test), variance of random effect and estimates of fixed effects (logit scale) for startle response modelled as a function of sound level, background noise, larval treatment and random effect.

Analysis of Variance	df	AIC	Chi^2	p-value
<none>		211		
sound level	1	496	286.6	<0.001
background	1	220	10.98	0.001
treatment	1	210	1.051	0.305

Random effect	n	variance		
	17	0.974		

Fixed effects	estimate	se	z-value	p-value
intercept	4.256	0.694	6.137	<0.001
sound level	0.386	0.045	8.562	<0.001
background-elevated	-1.257	0.398	-3.161	0.001
treatment-exposed	-0.633	0.612	-1.034	0.301

Model selection was based on Akaike information criteria (AIC); treatment was not removed from the model as this is the explanatory variable of interest.

Table 4. Startle response 4 fish. Analysis of variance (single term deletion, Chi² test), variance of random effect and estimates of fixed effects (logit scale) for startle response modelled as a function of sound level, background noise, larval treatment and random effect.

Analysis of Variance	df	AIC	Chi^2	p-value
<none>		583		
sound level * treatment	1	598	17.50	<0.001
background * treatment	1	610	28.90	<0.001

Random effect	n	variance		
	17	0.700		

Fixed effects	estimate	se	z-value	p-value
intercept	2.674	0.427	6.268	<0.001
sound level	0.287	0.022	12.803	<0.001
background-elevated	0.362	0.254	1.421	0.155
treatment-exposed	2.520	0.679	3.709	<0.001
sound level * treatment-exposed	0.165	0.041	3.978	<0.001
background-elevated * treatment-exposed	-2.067	0.398	-5.191	<0.001

Model selection was based on Akaike information criteria (AIC).

3.2.2 Swimming depth

In the 4 fish tests, mean swimming depth showed a sharp increase at the onset of extended pile-driving sound (Figure 9b). Subsequently, mean swimming depth decreased slowly during the 30 min period of extended pile-driving sound and appeared to decrease more quickly after pile-driving sound ceased. No clear differences in mean swimming depth between larval exposed and control group fish were observed, not in the initial response to sound, nor during the 45 min period thereafter. A difference was however observed in the variability of behaviour (Figure 9d). Variance between groups declined strongly at the onset of pile-driving sound, for both the exposed and control groups; all fish dived to the same depth. Then, for the control groups, variance quickly increased again, back to the level of variance before pile-driving sound. This clearly took longer for the exposed groups.

The results of the single fish tests were quite different (Figure 9a, c). Firstly, no clear response to the onset of extended pile-driving sound was observed. Secondly, throughout the 60 min observation period, mean water depth was less and variance was higher for control group fish than exposed fish.
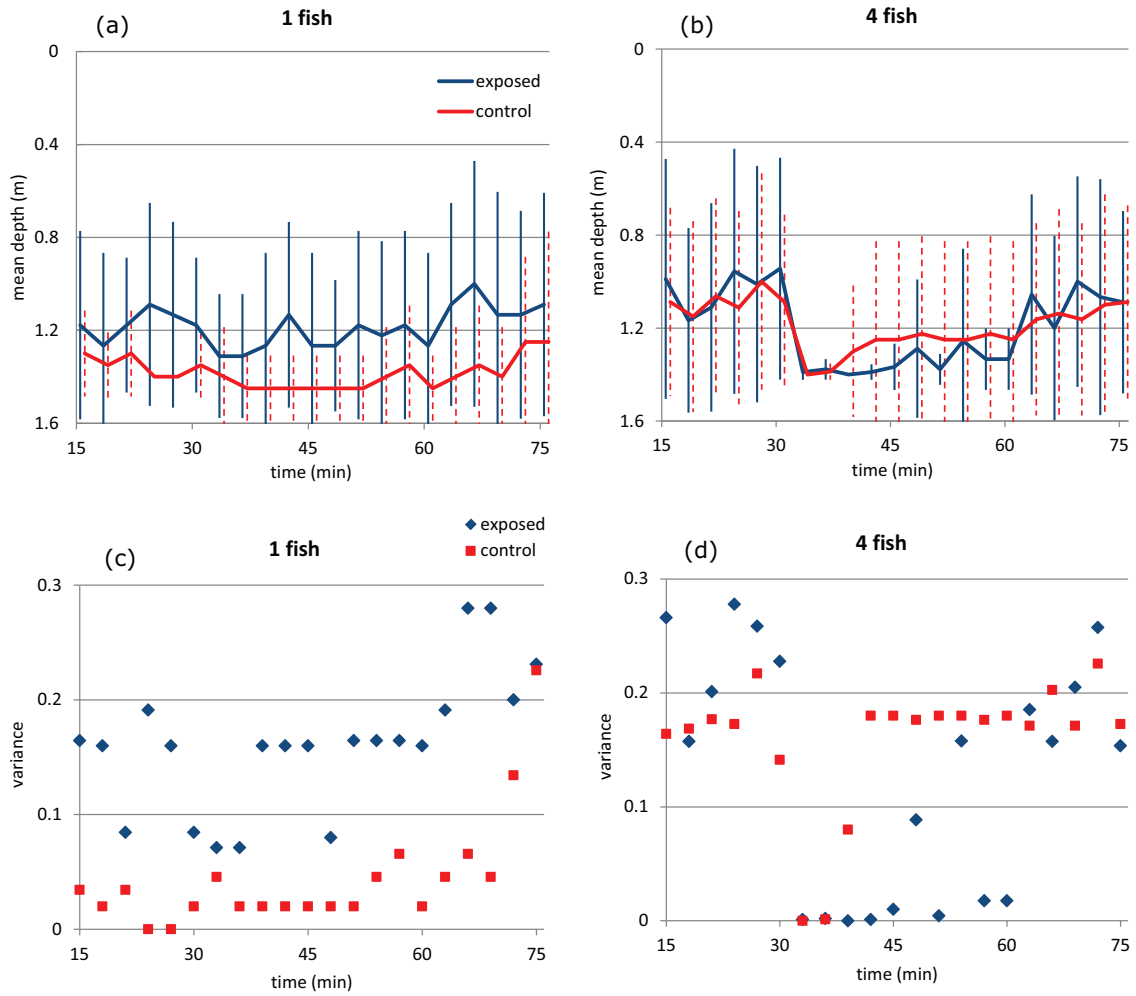
Figure 6 *Mean swimming depth (± s.d.) of single fish (a) and groups of 4 fish (b) and variance in swimming depth of single fish (c) and groups of 4 fish (d) over time. Extended pile-driving sound was turned on at 33 (± 3) min and turned off at 63 min (± 3). The net in the pool was 1.6 m deep, observations consisted of the position of individual fish in 1 of 4 depth layers.*

4 Discussion

4.1 Physical effects

Previously, the effect of larval exposure to pile-driving sound on short-term mortality (7-10 days post exposure) was examined in 3 different species including sea bass and no significant differences between control and exposed groups were found (Bolle et al. 2012, Bolle et al. submitted a). Exposure of juvenile sea bass to pile-driving sound also did not significantly affect short-term mortality (13-14 days post exposure) (Debusschere et al. 2014, Bolle et al. submitted b). The present study showed that even long-term mortality, up to 255 days post-exposure, is not significantly affected by larval exposure to pile-driving sound.

Long-term effects on growth were not observed either. No significant differences in length between exposed and control groups were observed at 69 or 255 days post-exposure.

Malformations, such as spinal deformations and uncovered gills, frequently occur in aquaculture fish (e.g. Moretti et al. 1999, Grotmol et al. 2005). The presence of malformations was much higher in the first two groups than in the other groups. This might indicate that we inadvertently selected weaker animals (slower swimming larvae) from the stock at the onset of the larval treatments. Sequence was a significant term in the statistical analyses of the first period after exposure, both for mortality and length. The decreased mortality and increased length with sequence also indicated that we might have selected weaker animals from the stock first. The statistical significance tests of the effect of larval treatment (exposed or control) on growth and mortality were corrected for this sequential effect. To avoid any bias during the selection of fish for the behavioural experiments, the fish were sedated.

4.2 Effects on behavioural responses to sound

There was some evidence for delayed effects of larval exposure on behavioural responses in juveniles. However, the effects found were not straightforward and not consistent over all experiments. We will try to speculate about potential underlying mechanisms, but we argue anyway that follow-up studies are both needed and warranted to confirm the impact and reveal the causal mechanism.

In the dose-response experiments with 4 fish we found a significant effect of larval treatment. Fish from the larval exposed groups showed a significantly lower startle probability at elevated background noise compared to ambient background noise, while fish from the control group showed little difference. Also, the exposed fish appeared to be more sensitive to sound signals at higher sound levels under ambient background noise. So, under elevated background noise the exposed fish appeared to be less sensitive and under ambient background noise they might be more sensitive than control group fish. The results of the single fish experiments however showed no significant effect of treatment.

The anxiety experiments with 4 fish did not show clear differences in mean swimming depth between larval exposed and control group fish, but a difference in the variability of swimming behaviour was observed. Exposed fish showed reduced variability for a longer period after the onset of pile-driving sound than control group fish, indicating that exposed fish might be more anxious. Again, these results were not confirmed by the single fish experiments.

Higher sensitivity can emerge due to sensitization, but this is typically a result of repeated exposure to the same stimulus and not the result of an exposure early in development. Higher sensitivity may also result from elevated stress levels during larval exposure and consequent development of a more anxious behavioural phenotype. Tsalafouta et al. (2015) showed that fish stressed in the larval stage have a higher cortisol level when stressors were introduced again later in life, compared to fish with little stress in the larval stage.

Lower sensitivity could emerge through any kind of detrimental effect on hearing ability due to larval exposure to high-intensity pile-driving sound. We only observed lower sensitivity of larval exposed fish in the case of elevated background noise. These results may point towards neural damage due to larval exposure. Liberman (2015) observed sound-induced damage to the cochlear neurons in humans and hypothesised that this will impair sound signal detection in noise.

This study provides an indication that early-life exposure to high-intensity sound may affect behavioural responses later in life. It furthermore shows that behavioural responses are dependent on group size in the case of social fish such as juvenile sea bass. Further research is required to confirm the results of this study and to reveal the underlying mechanisms.

Acknowledgments

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Quality Assurance

IMARES utilises an ISO 9001:2008 certified quality management system (certificate number: 187378-2015-AQ-NLD-RvA). This certificate is valid until 15 September 2018. The organisation has been certified since 27 February 2001. The certification was issued by DNV Certification B.V. Furthermore, the chemical laboratory of the Fish Division has NEN-EN-ISO/IEC 17025:2005 accreditation for test laboratories with number L097. This accreditation is valid until 1th of April 2017 and was first issued on 27 March 1997. Accreditation was granted by the Council for Accreditation. The scope can be found at the website of the Council for Accreditation (www.rva.nl).

Justification

Report C037/16
Project Number: 4302505602

The scientific quality of this report has been peer reviewed by a colleague scientist and the head of the department of IMARES.

Approved: Cindy van Damme
                 Researcher

Signature:
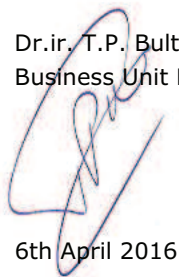
Date: 6th April 2016

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