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Measurement of Hearing in the Atlantic salmon (*Salmo salar*) using Auditory Evoked Potentials, and effects of Pile Driving Playback on salmon Behaviour and Physiology

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Introduction

Anthropogenic (human-generated) noise is widespread in aquatic environments and is increasing in prevalence and intensity (Hildebrand, 2005). Aquatic noise-generating activities include high intensity air guns used for seismic exploration, chronic low-frequency noise from shipping, and noise produced during construction and operation of offshore energy installations (Hildebrand, 2005; McDonald *et al*., 2006; Normandeau Associates 2012). The noise produced during such activities is very different to sounds that typically arise from natural sources (Hildebrand, 2009; Popper & Hastings, 2009; Normandeau Associates 2012; Radford *et al*., 2014). The continued development of Marine Renewable Energy Devices in Scottish coastal waters is recognised as an important step in the UK's drive for clean, low carbon energy. The unavoidable noise caused by such developments, principally pile-driving used in the construction of offshore windfarms, has raised concerns about the potential impact of noise on sensitive marine species. As a result, energy entering the sea in the form of anthropogenic noise has been included in the European Commission Marine Strategy Framework Directive, providing a statutory responsibility for member states to monitor sound levels. However, knowledge on hearing abilities of potential receptor species and potential impacts of underwater noise on marine organisms is incomplete.

To date, human-induced underwater noise has been shown to cause widespread effects on marine organisms (Tyack, 2008; Slabbekoorn *et al*., 2010; Normandeau

Associates Inc 2012). Close to a strong impulsive sound source, physical injuries and mortality can occur in some species (Halvorsen *et al.,* 2012). Anthropogenic noise has the potential to alter an individual's physiology, causing stress, and shifts in hearing thresholds in a number of species (Smith *et al*., 2004; Wysocki *et al*., 2006; Slabbekoorn *et al*., 2010; Simpson *et al*., 2015). Behavioural level responses are most likely to occur further from the sound source, and as such, behavioural changes may be most detrimental for populations due to the spatial scale over which effects can occur (Normandeau Associates 2012, Simpson *et al*., 2015). Behavioural level changes due to anthropogenic noise have been observed in regard to foraging, communication and anti-predator behaviours (Wysocki *et al*., 2006; Slabbekoorn *et al*., 2010; Purser & Radford, 2011, Simpson *et al*., 2015).

To assess hearing abilities of fish, Simpson has developed a mobile Auditory Evoked Potentials (AEP) electrophysiology system that can be taken to locations where fish are held, thus maximising application of this audiometric approach while minimising stress to focal animals from transportation. This approach was used to determine the hearing thresholds of a number of cohorts of Atlantic salmon.

Given the potential widespread range for behavioural impacts of anthropogenic noise, we considered the impact of noise on movement patterns in Atlantic salmon (*Salmo salar*). Atlantic salmon are known to detect low frequency acoustic stimuli below 380 Hz (Hawkins & Johnstone, 1978), coinciding with the dominant frequencies produced during impact piling operations (100 Hz to 2 kHz; Bailey *et al*., 2010; Hawkins *et al*., 2015). The construction noise from offshore windfarms, therefore, has the potential to interact with the two migratory stages of the Atlantic salmon life cycle, through salmon avoiding noisy locations (region where noise from the source is above ambient ocean levels; Nedwell & Mason, 2012). For adult Atlantic salmon, the concern centres upon impact pile driving in coastal regions delaying or preventing the migration to natal rivers, with potential consequences on spawning. Following this, we considered whether changes in movement behaviour were a consequence of an altered physiological state, measuring changes in dissolved oxygen consumption, a proxy for metabolic rate, for use as an indicator of stress (Barton, 2002, Simpson *et al*., 2015).

In this study, we adapted established methodologies to consider the potential impact of pile driving noise on Atlantic salmon behaviour and physiology (see Simpson *et al*., 2015). As a starting point to address these questions, we performed carefully controlled laboratory based experiments using underwater playback. Although the stimulus produced within the laboratory setup can't accurately represent the sound field from a real noise source in an open soundscape (Normandeau Associates

2012), the use of the aquarium facilities enabled the tight control of potential confounding variables (Bruintjes & Radford 2014; Simpson *et al.,* 2015), and allowed testing of potential impacts in controlled conditions. We investigated the behavioural and physiological impact of the additional noise of piling driving compared to ambient control conditions.

This report is divided into two sections:

Part 1: Audiometry, using Auditory Evoked Potentials (AEP) to Determine Hearing Thresholds of a Number of Cohorts of Salmon

Stephen D Simpson and Rick Bruintjes

Part 2: The Impact of Pile-Driving Playback on the Behaviour and Physiology of Atlantic salmon (*Salmo salar*)

Harry Harding, Andrew N Radford, Stephen D Simpson

Ethics Statement

Hearing assessments were carried out with the approval of the University of Exeter Ethical Review Committee (2013/247), under Home Office licences (Project Licence PPL 30/2860, Personal Licences PIL 60/10650 (Steve Simpson) and PIL 30/9624 (Rick Bruintjes)and in consultation and with regular monitoring from MS pathologist Dr David Bruno. Training and accreditation in AEP electrophysiology had been previously undertaken by Steve Simpson under the guidance of Dr Dennis Higgs (University of Windsor, Ontario). All behavioural and physiological experimental procedures were approved by the University of Exeter Ethics Committee (2013/247) and the Marine Scotland Science named vet, and were deemed by the Home Office as being below the level of severity that would require licensing, although an appropriate licence was in place (PPL 30/2860). Prior to each experiment, fish were given time to recover from transfer and to acclimatise to the experimental set ups. Fish husbandry was in accordance with the standard operating procedures of the Marine Scotland (MS) Marine Laboratory.

Part 1)

Audiometry, using Auditory Evoked Potentials (AEP) to Determine Hearing Thresholds of a Number of Cohorts of Salmon

Stephen D Simpson and Rick Bruintjes

Methodology

Study Fish and Holding Conditions

Three groups of fish were tested:

1) Wild Post-Smolt: Ten post-smolt collected in traps in the River Tay as seaward migrating smolt and held for one year in a 1.5 m diameter circular tank indoors (LT1 Tank 3). Standard Length (SL) 273.5 ± 11.3 mm (mean ± SE) and Fork Length (FL) 288.7 ± 12.1 mm.

2) Captive Post-Smolt: Ten post-smolt reared since hatching in captivity from wild stock eggs and held outdoors in a 1.5 m diameter circular tank outdoors (Outdoor Tank 16). SL 294.0 ± 11.4 mm, FL 312.0 ± 11.4 mm.

3) Captive Adults: Ten adult salmon reared since hatching in captivity from wild stock eggs and held in a 15 m long tank (Dumbell). SL 379.0 ± 15.0 mm, FL 401.5 ± 15.7 mm.

All holding tanks had running seawater and aeration via airstones, and temperature was a constant 10°C. The indoor tanks had a 9:15 hours Light:Dark lighting regime with low intensity green lamps, while the outside tank received ambient mid-March daylight conditions of 8:16 L:D.

Acoustic conditions in the holding tanks (water 1 m depth, recording 10 cm above bottom where salmon usually resided) were measured using a calibrated omnidirectional HTI-96-MIN hydrophone (frequency response = 2 Hz - 30 kHz, voltage sensitivity = -165 dB re 1 V/µPa; High Tech, Inc., Gulfport, Mississippi) and a Sony PCM-M10 24-Bit recorder (96 kHz sampling rate; Sony Corporation, Tokyo, Japan). Root Mean Square (RMS) noise levels, analysed using SASLab Pro v4.5.2 (Avisoft Bioacoustics, Berlin), were 127.8 (LT1 Tank 3), 127.0 (Outdoor Tank 16) and 134.5 (Dumbell) dB re 1 µPa (1 sec averaging), with levels only ~1 dB lower

when analysed within the range 0-1 kHz indicating most of the noise was low frequency.

Hearing Sensitivity Tests

To minimise stress, fish were transferred from the holding tanks in nets submerged in buckets prior to handling during preparation of the fish in the AEP apparatus. The captive adult fish were immersed in a bin with a nonlethal dose of buffered tricaine methanesulfonate (MS222) to induce temporary mild anaesthesia (70 mg/l MS222, fish immersed for around one minute until balance was lost) and revived (>five minutes) in fresh seawater (aerated between each testing period) prior to taking AEP measurements. Testing took 30-45 minutes per fish, and no mortality or obvious injury resulted from the experimental procedures; after testing fish were sacrificed by a Home Office Schedule 1 method (fatal blows to the head). All experiments took place in a quiet room in the MS Science Laboratory in the daytime (0800-1800) during 11-15 March 2013.

Acoustic Stimulation and Data Acquisition

To assess hearing sensitivity of fish from the three test cohorts, we used the Auditory-Evoked Potentials (AEP) technique, which is a non-invasive electrophysiological measure of the synchronized brain response to auditory stimuli (e.g., Corwin *et al.,* . 1982; Kenyon *et al.,* . 1998). Hearing assessments were conducted on each fish only once. During testing, fish were wrapped in soft knotless fine mesh and secured in a rigid harness suspended 10 cm below the surface of the water in the centre of a 1 m diameter circular fibreglass tank (water depth 1 m). Stainless steel recording electrodes (0.4 mm diameter; Spes Medica S.r.l., Battipaglia, Italy) were insulated except at the tips with vinyl paint, and one was inserted subcutaneously at the front of the dorsal fin (reference electrode) while the recording electrode was inserted subcutaneously at the dorsal midline 2 mm posterior to the cranium. A third non-insulated grounding electrode was placed in the water 5 cm from the fish.

Sound Stimulus

The sound stimulus presented to fish was a repetitive 20 ms pure tone, with a 2 ms Hanning window gate at the beginning and end to avoid loss of quality due to the rapid firing of the speaker. The signal file was developed in SigGenRZ (Tucker-Davis Technologies, Alachua, Florida), with test frequencies of 100, 200, 300, 400, 500, 600, 700 and 800 Hz. Signals were sent from BioSigRZ to a TDT RZ6 Multi-I/O

processor, amplified with a Phonic Max 500 amplifier (120W; Phonic Corporation, Taipei, Taiwan) and played through an underwater speaker (UW30; Lubell Laboratories, Inc., Columbus, Ohio) suspended 57 cm below the fish and facing upwards from the centre of the tank.

To calibrate the received sound pressure levels of stimuli at each frequency and test level, multiple recordings were made in the test tank with the calibrated hydrophone fixed in the same position as the head of the fish would be located during testing, and RMS received levels (re 1 µPa, averaged over the 10ms in the centre of each pulse) were derived in SASLab Pro. Peaks of the focal frequencies were all at the frequencies as intended, and first harmonics of the test frequencies were all >20 dB below the focal frequency.

Electrophysiology

The AEPs at each frequency and signal level were collected from the fish via a TDT Medusa RA4LI headstage and RA4PA preamplifier, based on averages of 200 repeats of 40 ms recordings including the 20 ms tones repeating at a rate of 10 times per second. The brain signal was amplified with a gain of 20 and was low-pass filtered at 3 kHz, high-pass filtered at 10 Hz, and notch filtered at 50 Hz using BioSigRZ software. Averaged AEPs were digitised and stored.

The sound pressure levels presented at each frequency were attenuated in 6 dB steps until the stereotypical AEP responses were lost into background myogenic noise. This measure was based on visual assessment of the AEP signal, which is most commonly used in AEP studies (Hall 1992; Kenyon *et al.,* 1998) and which gives results similar to those of statistical methods (Mann *et al.,* 2001; Brittan-Powell *et al.,* 2002). For each fish, the AEP threshold was defined at each frequency as the lowest sound level that gave a defined repeatable response.

Due to the limitations of our experimental set up and in line with recent AEP studies (e.g., Halvorsen *et al.,* 2009), we measured the acoustic pressure component of the presented sounds, and thus give hearing thresholds in terms of sound pressure (dB re 1 µPa RMS, 10 ms measured in the centre of the pulse). Due to morphological limitations, it is likely that Atlantic salmon respond predominantly to the particle motion component of the presented sounds. Thus, we would caution the interpretation of thresholds in terms of absolute hearing abilities. Nevertheless, the use of this approach for comparative studies of hearing between the three study groups is valid, and with further experimentation to investigate behavioural thresholds and/or open water AEP measurements with the system calibrated using

an *in situ* accelerometer it would be possible to relate these initial thresholds to hearing thresholds based on particle motion and provide a likely spatial scale of detection of anthropogenic noise (e.g., pile driving).

Results

Auditory thresholds based on acoustic pressure were recorded at 100, 200, 300, 400, 500, 600, 700 and 800 Hz for each group of salmon to provide a complete AEP audiogram (Figure 1). The mean AEP threshold value at each frequency and size distributions of fish in each group of individuals for each experimental group is presented in Table 1.

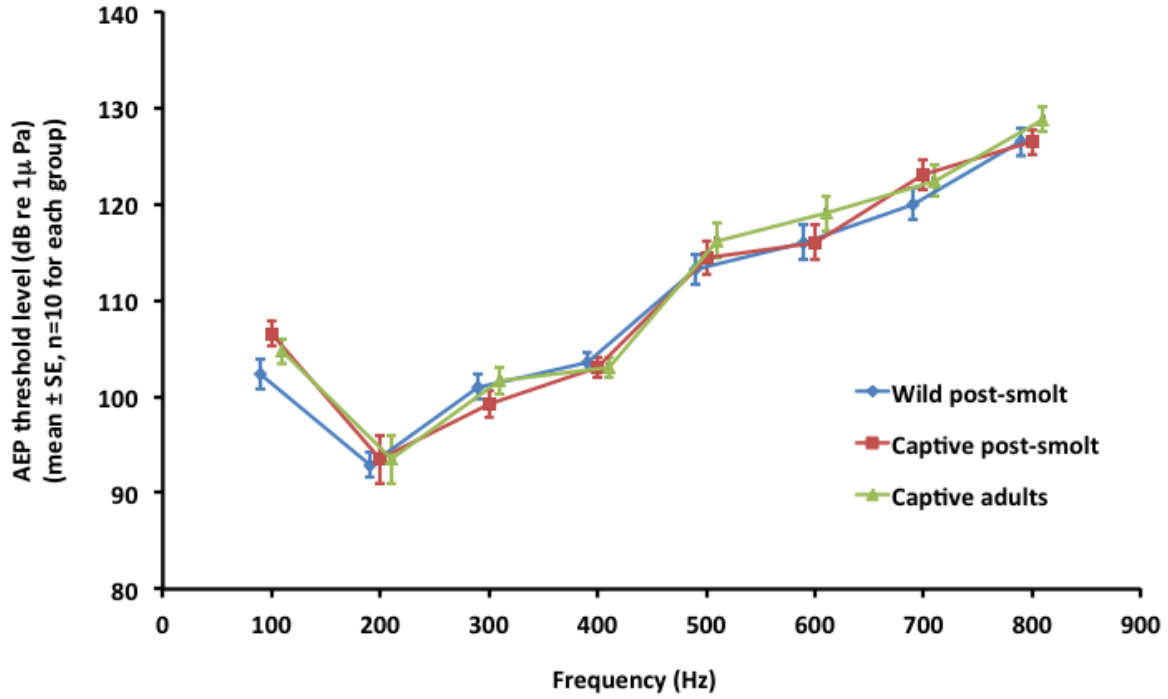

Figure 1: Audiograms based on mean (± SE) minimum received levels (dB re 1µPa) that elicited a characteristic auditory evoked potential. Three groups of Atlantic salmon (*Salmo salar*) were tested (n = 10 in each case): Wild post-smolt, Captive post-smolt, Captive adults. See Table 1 for mean values and size distributions of fish.

Discussion

This study generally concurs with the previous findings of Hawkins & Johnstone (1978) that Atlantic salmon (*Salmo salar*) does not appear to have sensitive hearing relative to many other marine fish species, including gadoids (e.g., Atlantic cod, *Gadus morhua*) and clupeids (e.g., herring, *Clupea harengus*). This is likely due to a lack of secondary hearing modifications linking the swimbladder to the auditory system. We found evidence of a response to sounds at higher frequencies (400-800 Hz) than had been previously published for this species (Figure 2), although hearing up to 600 Hz has recently been reported in the Chinook salmon (*Oncorhynchus tshawytscha*; Halvorsen *et al.,* 2009) using the same method as this current study.

Table 1
Mean (± SE) auditory evoked potentials thresholds, measured in the pressure domain (dB re 1µPa), for Atlantic salmon (SL = standard length; FL = fork length).

Group:	Wild Post Smolt	Captive Post Smolt	Captive Adults
n:	10	10	10
100 Hz	102.3 ± 1.3	106.5 ± 1.5	104.7 ± 1.3
200 Hz	92.9 ± 1.4	93.5 ± 1.3	93.5 ± 2.5
300 Hz	101 ± 1.8	99.2 ± 1.3	101.6 ± 1.4
400 Hz	103.6 ± 1.8	103 ± 1	103 ± 1
500 Hz	113.2 ± 1.7	114.4 ± 1.6	116.2 ± 1.8
600 Hz	116 ± 1.3	116 ± 1.8	119 ± 1.8
700 Hz	120 ± 1.3	123 ± 1.6	122.4 ± 1.6
800 Hz	126.4 ± 1.4	126.4 ± 1.4	128.8 ± 1.3
SL (mm)	273.5 ± 11.3	294.0 ± 11.4	379.0 ± 15.0
FL (mm)	288.7 ± 12.1	312.0 ± 11.4	401.5 ± 15.7

Exploring Discrepancies between Previously Published Audiograms for Atlantic and Chinook Salmon

We found salmon had slightly less sensitive hearing at 100 Hz than reported in Hawkins & Johnstone (1978), with the earlier study suggesting threshold values 10 dB below those measured here, but found more sensitive hearing than the earlier study at frequencies >200 Hz. Since the five fish in the Hawkins & Johnstone study (320-360 mm) were larger than our two post-smolt groups, but smaller than our captive adults (see Table 1), it does not seem likely that size or ontogeny explains the difference in audiograms. Further, since we have used our AEP apparatus to measure goldfish hearing (hearing specialists), giving thresholds at low frequency that concur with many published audiograms, we do not attribute these differences to poor performance of the equipment used here.

Thus we suggest that there are three possible reasons for the differences between our results and those of Hawkins & Johnstone:

1. Since AEP measurements can be ± 20 dB different to measured behavioural and physiological thresholds, the two sets of audiograms may be within the margin of error in the 100-300 Hz range since the two studies use very different approaches. Further studies that combine AEP measurements with behavioural and/or physiological thresholds should explore whether the differences are experimental or are biologically meaningful based on different

measures of response.

2. The logistically simpler modern AEP set up uses smaller speakers (UW30 in this case) compared to the Dyna-Empire J9 and J11 speakers used in the field in the Hawkins & Johnstone study. This will significantly reduce the particle motion component of the broadcast signal, meaning that the current AEP measurements may be conservative at low frequencies. While such large speakers could not be employed in a tank environment, we have discussed the future use of larger speakers in the 10 m diameter tank at the MS Science Laboratory, and ultimately also in open water to measure *in situ* AEPs, where we would combine measurements of received levels using hydrophones (pressure) and accelerometers (particle motion).

3. The fish in the Hawkins & Johnstone study were reared at the Invergarry Hatchery before being transferred as smolts to an open water sea pen in Loch Ailort where they were grown out. Thus the ambient noise levels in the open water holding pen prior to testing would have been very much lower than those experienced by the three groups in the current study which were held in large tanks at the Marine Scotland Science Laboratory with high levels of low-frequency noise (Root Mean Square (RMS) noise levels: 127.0-134.5 dB re 1 µPa; 1 sec averaging). The fish tested in the present study had either been kept in captivity from hatching (Captive post-smolts, Captive adults) or else in captivity for at least one year (Wild post-smolts) following capture in the wild. It is possible that prolonged culture of fish in noisy conditions may have brought about a temporary or permanent threshold shift, particularly as much of the noise in the holding tanks was in the frequency range of greatest sensitivity (<300 Hz). To address this issue we suggest the use of fish collected recently from the wild, such as in Halvorsen *et al.,* (2009) which found higher sensitivity in Chinook salmon (Figure 2), should be used, with fish kept in open water environments or quietened tanks after capture prior to testing.

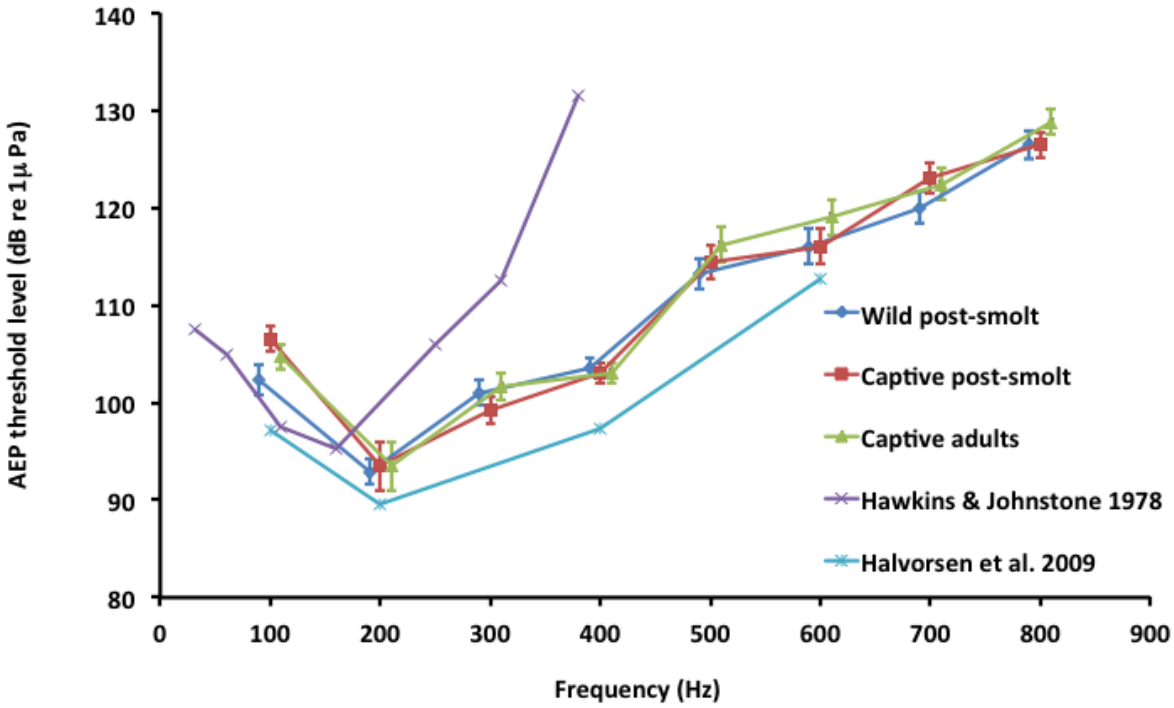

Figure 2: Figure 2 redrawn with previously published hearing thresholds transformed into dB re 1µPa by Nedwell *et al.,* (2004) from Hawkins & Johnstone (1978), and with AEPs for Chinook salmon measured using similar approach to the current study (Halvorsen *et al.,* 2009). The earlier field-based approach identified hearing thresholds at low frequencies (<200 Hz) slightly below those in the present study, but underestimated hearing sensitivity in higher frequencies (>200 Hz). Present study shows mean (± SE) for ten individuals, while the Hawkins & Johnstone data shows a mean from five fish and Halvorsen *et al.,* study shows a mean from ten wild fish.

General Conclusions

The cohorts of Atlantic salmon held at the MS Science Laboratory, which have been reared in captivity since hatching or since collection as smolt from the River Tay, appear to have hearing abilities that generally concur with previous studies. Thus they, and similar fish, will provide a valuable model system for (a) testing impacts of noise on hearing (e.g., temporary threshold shifts dues to pile driving noise), (b) exploring relationships between electrophysiological, physiological and behavioural thresholds of hearing, and (c) studying impacts of noise on physiological (e.g., metabolic rate, opercular beat rate) and behavioural (e.g., foraging, anti-predator, movement) performance.

Part 2

The Impact of Pile-Driving Playback on the Behaviour and Physiology of Atlantic salmon (*Salmo salar*)

Harry Harding, Andrew N Radford, Stephen D Simpson

Methodology

Study Species and Holding Conditions

1. Behavioural Experiment - 40 adult Atlantic salmon raised in captivity at the Marine Laboratory following collection as smolts in the wild (trials 1–16) or from a farm (Landcatch Ltd., Ormsary, trials 17-20). The salmon were housed in two outdoor tanks (tanks 2 and 20, respectively). Wet weight was 3595.10 g ± 151.67 (mean ± SE); TL (Total length) 677.63 mm ± 9.04. The outdoor tanks received ambient lighting conditions during the experimental period (May – June).

2. Physiological Experiment - 26 marine-phase salmon that had been fertilised and hatched at the Marine Laboratory from eggs and milt from broodstock at Aultbea, were housed in one indoor tank (tank room two) for the duration of the experiment (June-September). The indoor tank was lit by low intensity green lamps. Wet Weight was 1319.48 g ± 62.82; TL 480.17 mm ± 5.37.

All holding tanks received running seawater with a constant temperature of 10˚C, with aerators maintaining dissolved oxygen levels. Acoustic conditions in the holding tanks were measured using a calibrated omnidirectional HTI-96-MIN hydrophone (frequency response = 2–30,000 Hz, sensitivity = -165 dB re 1V/µPa), positioned 10cm above the bottom of the tank (water depth: 1 m), and a Sony PCM-M10 24-bit recorder (96 kHz sampling rate). Root Mean Square (RMS) noise levels, analysed using SASLab Pro v4.5.2 (Avisoft Bioacoustics, Berlin) were 134.54 (Outdoor tank two) and 130.77 (Indoor tank 2) dB re 1µPa (full spectra, 1 sec averaging, 15 sec recording).

Playback Tracks and Experimental Design

Behaviour and Physiology

Our primary aim was to test the impact of additional noise (playback of impact pile driving) on behaviour and physiology relative to responses of individuals raised in the same conditions but experiencing control conditions (playback of ambient harbour noise). Any subsequent effect found is thus likely due to the additional noise and not from captivity. A representative collection of both pile driving and ambient playback tracks were used for the experiments. Three ambient tracks were recorded from three major UK harbours (as described in Simpson *et al*., 2015). Three unique five minute individual sub-sections from each sound file were created using Audacity 2.0.3 (http://audacity.sourceforge.net), generating nine ambient harbour tracks (A1, A2, A3, B1, B2, B3, C1, C2, C3) and three pile driving tracks (P1, P2, P3). Ambient sound files were matched with the three pile driving tracks to produce nine individual pile driving sound files overlaid with ambient harbour noise (e.g. A2 + P1).

For both experiments the order of the trials and sound files to be used were randomised. The pile driving and ambient treatments were randomly assigned in pairs for each two day period to ensure that no treatment was tested consecutively for more than two days. We used an independent measures for the behavioural trials with 40 fish divided into the two sound exposure groups (ambient control vs. pile driving), with two fish participating in each trial. The control group consisted of ambient-only playback tracks, whereas the piling treatment used both pile driving and ambient sound files. The total duration of the trial was five hours with the first hour providing a pre-exposure 'baseline' with ambient only playback in both treatment groups. Following this, randomised playback tracks alternated between the two ends of the experimental tank. The end at which the inital piling track was played was alternated between the ten treatment trials (Table 2) and the trial order was subsequently randomised. Sound files were randomly assigned to each trial, dependent upon treatment, from groups containing tracks from harbours A+B, C+A, B+C. Within the groups of sound files, the specific track to be used for each hour of the experiment was randomly selected. This ensured appropriate degrees of freedom when analysing the data. The randomised combinations and use of different playback tracks within each trial avoided pseudo-replication. The origin of the fish was counter balanced between the two treatments to prevent bias of origin influencing any behavioural responses.

Table 2
Experimental design and sound file combinations for the behavioural experiment.

			Exposure							
			Left end pod				Right end pod			
Trial	Treatment	Pre-exposure (1hr)	1hour	2hour	3hour	4hour	1hour	2hour	3hour	4hour
1	Pile Driving	A1	C2	P3+C1	C3	P3+A1	P1+C2	A2	P2+C3	A3
2	Control	C1	A3	A3	C3	C1	A1	A2	C2	C2
3	Pile Driving	C3	B1	P3+C1	B2	P2+B3	P3+B1	C1	P2+C3	C2
4	Control	B3	C2	B2	B1	B3	C3	C2	B2	C1
5	Control	B3	B2	B1	A3	B1	B2	A1	A2	A2
6	Pile Driving	A3	P2+A3	B3	P3+A1	A2	A1	P1+A2	B1	P1+B2
7	Pile Driving	C2	P3+C1	C1	P2+C3	B2	B1	P1+C2	B3	P2+B3
8	Control	A2	A3	C1	C2	A1	A1	A3	A2	C3
9	Control	B2	C1	C2	B1	C3	C3	B3	B2	C1
10	Pile Driving	A2	C1	P1+A2	C2	P1+C2	P2+A3	C3	P2+C3	A3
11	Control	B1	A2	A1	A3	A1	B2	B3	B2	A2
12	Pile Driving	B1	C1	P2+B3	B3	P3+C1	P2+C3	C2	P1+B2	B2
13	Control	A3	A2	C2	A1	C1	C3	C1	C2	A2
14	Pile Driving	C1	P3+A1	A2	P2+A3	A1	A3	P1+A2	C2	P1+C2
15	Pile Driving	B1	P3+A1	A2	P2+A3	A1	B2	P1+B2	A3	P1+A2
16	Control	C1	C3	B2	C3	B1	C2	B3	C2	B1
17	Pile Driving	C1	P1+C2	A1	P3+C1	A2	A3	P3+A1	C2	P2+C3
18	Control	B3	A1	A1	A3	A2	A2	B2	B3	B1
19	Pile Driving	B1	B2	P1+A2	B3	P3+B1	P2+A3	A1	P2+B3	A3
20	Control	C2	C2	A3	C1	C1	A2	C3	A2	A1

We used an independent measures design for the physiology study with 26 fish divided into the two treatment groups. We used a single track per trial in a testing block of 18 tracks (9 ambient and 9 pile driving tracks) (Table 3). Playback tracks were randomly assigned.

Table 3
Sound file combinations and trial order for the physiology experiment.

Trial	Treatment	Sound file
1	Pile Driving	A1+P3
2	Control	C2
3	Pile Driving	B2+P1
4	Control	A2
5	Control	C3
6	Pile Driving	C3+P2
7	Pile Driving	C1+P3
8	Control	B2
9	Pile Driving	A3+P2
10	Control	A1
11	Pile Driving	A2+P1
12	Control	A3
14	Control	B3
13	Pile Driving	B3+P2
15	Pile Driving	C2+P1
16	Control	B1
18	Control	C1
17	Pile Driving	B1+P3
19	Pile Driving	A1+P3
20	Control	B3
21	Pile Driving	A3+P2
22	Control	A3
24	Control	C3
23	Pile Driving	C1+P3
25	Pile Driving	B2+P1
26	Control	A1

Pressure Levels

For the behavioural experiment, we used two Dyna-Empire J9 sound projectors (referred to as J9-1, J9-2) each connected to a 40 watt mini amplifier (LP-2020A + Lepai Tripath class-T Hi-Fi audio mini amplifier). The Pile driving (RMS log) noise levels 15 cm from the speakers, analysed using SASLab Pro v5.2.07 (Avisoft Bioacoustics, Berlin), were 153.68 (J9-1) and 149.40 (J9-2) dB re 1 µPa (one second averaging). The ambient RMS noise levels were 133.66 (J9-1) and 126.72 (J9-2) dB RMS re 1µPa (one second average). RMS noise levels on a single hammer strike were 162.31 (J9-1) and 160.01 (J9-2) dB re 1 µPa (10 ms average, one second recording, maximum pressure level). RMS noise levels at 13 meters from the speakers were 128.26 (J9-1) and 125.32 (J9-2) dB re 1 µPa (one second averaging), with levels of a single hammer strike at 139.51 (J9-1) and 130.52 (J9-2) dB re 1 µPa (10 ms average, one second recording, maximum pressure level). The background RMS noise level of the dumbbell tank was 118.59 dB re 1µPa (one second average). All measurements were taken from 15 second recordings, excluding the individual hammer strike measurements. We used SASLab Pro v5.2.07 (Avisoft Bioacoustics, Berlin) to determine the spectral densities of the recordings described above (Power spectrum, level units averaged; FFT size 1024, Hamming evaluation window, 43 Hz resolution, 12 second recording, piling track had ten strikes 1.2 seconds for each strike) (Figure 3). We displayed the spectral content of the same piling track recorded at increasing distances along the dumbbell tank (Figures 4, 5).

For the physiological experiment, we used a Lubell LL916C underwater speaker connected to a 40 watt mini amplifier (LP-2020A + Lepai Tripath class-T Hi-Fi audio mini amplifier). Sound recordings were taken inside the chamber with the hydrophone positioned 20 cm off the base of the tank. The speaker was positioned 2 m from the chamber suspended off the gantry of the annular tank at a depth of 50 cm. RMS (Log) noise levels were 152.58 (Pile Driving), 125.84 (Ambient) and 122.17 (Background) dB re 1 µPa (one second average, 15 seconds recording). The RMS (Log) noise level on a single hammer strike was 164.33 dB re 1 µPa (10 ms average, one second recording, maximum pressure level). Using SASLab Pro v5.2.07 (Avisoft Bioacoustics, Berlin) we determined the spectral densities of each recording described above, including the outdoor holding tank (tank two) and background noise of the annular tank (Power spectrum, level units averaged; FFT size 1024, Hamming evaluation window, 43 Hz resolution, 12 second recording, piling track: ten strikes with 1.2 seconds for each strike) (Figure 6).

Particle Motion

Using a calibrated M30 accelerometer (sensitivity 0–3 kHz), we assessed the particle acceleration levels produced from the loudspeakers in both experimental tanks. The accelerometer was used to take measurements from both speakers in the dumbbell tank at increments of two meters at a depth of 730 mm to assess the propagation of the particle motion element of the sound field. Individual recordings were cut using Audacity 2.0.3 (http://audacity.sourceforge.net) to produce 12 seconds of ambient and 12 seconds (10 hammer strikes, 1.2s for each strike to remove background noise) of the pile driving sound files for each of the three axes of measurement (x, y and z). The subsequent recordings were used to determine the power spectral density (Figures 7-9). One minute complete recordings for both ambient and piling tracks were analysed using broadband analysis to determine the average particle acceleration level (sampling rate 44100 Hz, Hamming window, one minute recording, 0-3kHz bandpass filter). Particle acceleration levels for both J9 sound projectors along with the levels achieved along the dumbbell tank are displayed in Table 4.

Table 4

Particle acceleration levels (PAL) (channel XY) (dB re (1µm/s^2) (sampling rate 44100Hz, Hamming window, one minute recording, 0-3kHz bandpass filter) of both ambient and pile driving playback at various distances along the dumbbell tank from both J9-sound projectors.

		PAL (dB re (1 µm/s^2)	
Distance (m)	Playback track	J9-1	J9-2
0.1	Ambient	91.7	82.29
2.75	Ambient	90.55	81.21
4.75	Ambient	84.43	81.32
6.75	Ambient	86.25	81.80
8.75	Ambient	86.07	81.72
10.75	Ambient	84.79	81.63
12.75	Ambient	84.35	81.72
0.1	Pile Driving	101.67	96.83
2.75	Pile Driving	95.22	87.65
4.75	Pile Driving	85.90	81.73
6.75	Pile Driving	86.32	81.96
8.75	Pile Driving	86.68	82.02
10.75	Pile Driving	84.94	81.74
12.75	Pile Driving	84.76	81.50

During the physiological experiment we recorded particle accelerations both outside the chamber at 2 m from the speaker and again in the middle of the chamber at a depth of 44.5 cm. For spectral analysis ten seconds of each recording was used (ten hammer strikes, one second for each strike to remove background noise) (Figure 10). One minute complete recordings were used for broadband analysis to calculate the average particle acceleration level. The particle acceleration level (channel XY) recorded inside the respirometry chamber for pile driving and ambient playback tracks were 107.15 and 89.89 (dB re (1µm/s^2) respectively (sampling rate 44100Hz, Hamming window, one minute recording, 0-3kHz bandpass filter).

Behavioural Experiment

We assessed the impact of additional pile driving noise on the avoidance response of Atlantic salmon. To consider how pile driving may affect movement behaviour, we determined the duration of time, as a percentage, that both salmon collectively spent within the four quadrants of the experimental tank over each hour of the 5-hour trial.

Blue and green low intensity lights were used to illuminate the dumbbell tank (Figure 11). To prevent visual cues from influencing behaviour, a black ceiling to floor curtain was used to separate the tank from the rest of the main tank room. Two Dyna-Empire J9 sound projectors were positioned at opposite ends of the dumbbell tank. Both speakers were suspended at a depth of 700 mm (total tank depth; 830 mm) and centralised on the midline of each end ‘pod’ 10 cm from the tank wall.

The dumbbell tank was divided into four quadrants; two end pods (3 m diameter) and two sections of the middle corridor (total length 7 m). Soundproof baffles were incorporated into the corridor of the dumbbell tank to aid sound attenuation of the noise stimulus. For the pile driving treatment this created an environment with a reduced sound pressure level in the opposite end of the tank to the speaker playing pile driving. To remove potential bias resulting from differences between speakers, the two sound systems were swapped between ends after every two trials. Three overhead CCTV video cameras, attached to the ceiling above the tank, were used to record fish movement throughout the arena. The camera feeds were passed through a ‘Sprite SX 9-channel digital multiplexer to split the display on the monitor into separate camera feeds. The multi-screen video was recorded directly onto a Panasonic DMR-EX86 DVD recorder hard drive and backed up onto DVD for archiving and analysis.

Two adult Atlantic salmon were placed into the dumbbell tank between 15:00 and 16:30 the day before the trial to allow fish to recover from the stress of transfer. The experiment began between 09:30 and 10:00 the next morning providing an

acclimation time of 17-19 hours. The water supply to the dumbbell tank was switched off thirty minutes prior to the experiment starting, to allow time for the water level to reduce below the outlet. This avoided any flow of water circulating around the tank, which may have influenced any behaviour observed. The video recording was started and after two minutes the pre exposure (ambient) sound files were started from both speakers at the same time. The playback files from both speakers were changed to either a different ambient or pile driving track after one hour. In the pile driving treatment one speaker played an ambient track whilst the speaker at the opposite end of the tank played pile driving. Subsequently on every hour following this change, finishing after four hours, the playback files were switched between ends. The fish were terminated using a Home Office Schedule 1 method (fatal blow to the head) after the experiment and subsequently weighed and measured.

The position of the two salmon was scored every minute throughout the duration of the trial. Analysis of video was conducted without volume, so 'blind' to the treatment. A fish was defined as being contained within a quadrant when the majority of their total body length was within the particular quadrant. Positional scoring was used to calculate a combined percentage of time spent within the four quadrants for each hour of the trial for both fish. We calculated the total proportion of time, across the four hour exposures, that the fish spent within the near end pod (pod with speaker playing piling track) and far end pod (pod with speaker playing ambient track). In the ambient trials we used the accompanying pile driving treatment in the block of two to define which end pod was "near" and "far". We determined the percentage difference in time spent in the near end pod between the pre-exposure hour and first exposure hour. This was used to assess the influence of the pre-exposure hour on the remaining trial period. The near end pod of the first hour in the pile driving trials was used to define the near end for the pre-exposure hour.

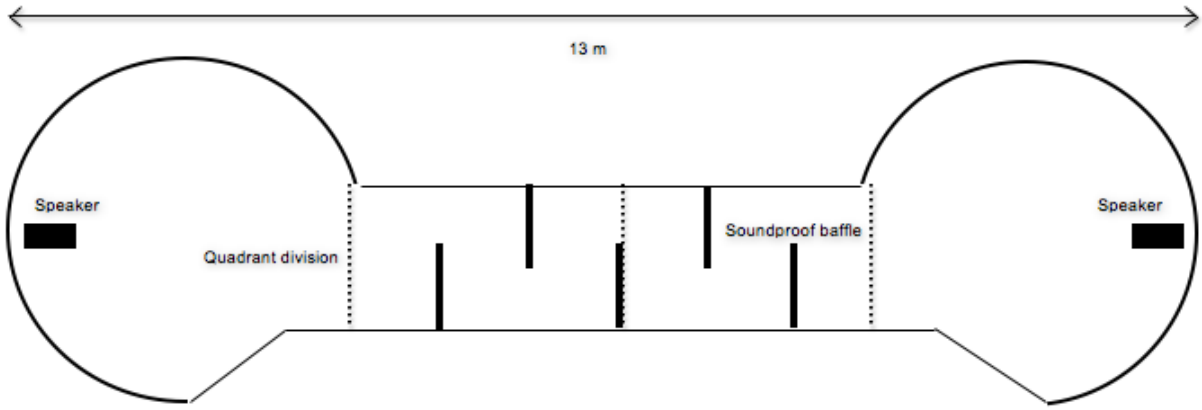

Figure 11: A Schematic of the dumbbell tank used for the behavioural experiment showing the approximate positions of the soundproof baffles, quadrant divisions (dashed lines) and speakers.

Physiological Experiment

As per previous studies, we used routine metabolic rate as an indicator of stress (Barton, 2002; Simpson *et al*., 2015) to investigate whether altered movement patters (behaviour) are a consequence of an altered physiological state. To assess active metabolic rate we determined the dissolved oxygen depletion for an individual fish over a two-hour trial duration. We subsequently calculated an individual's oxygen consumption rate relative to body mass in fish exposed to either pile driving noise, or an ambient control. The respirometry chamber was submerged within the annular tank. A Lubell LL916C underwater speaker, suspended from the gantry, was positioned 2 m from the chamber at a depth of 50 cm. The main tank room containing the annular tank (10 m diameter; total depth 1.06 m) was lit with white lights that were switched on between the hours of 08:00 and 17:00 each day. The respirometry system was tested over a two hour preliminary trial without a fish present to assess the effectiveness of the system and determine the influence of any microbial activity on our recorded dissolved oxygen levels (Figure 16 in supplementary information).

For each trial, a test salmon was transferred into the respirometry chamber between 15:00-17:00 the day before the trial was due to begin. Mesh was then cable-tied on to the top of the chamber to contain the fish during the overnight acclimation period. An Eheim universal pump (2014 EHEIM GmbH & Co. KG) was left running to help maintain a constant supply of oxygenated water. Following the acclimation period, the mesh was removed and the chamber lid bolted down. During this time, the Eheim pump continued to circulate oxygenated water from the annular tank into the chamber. An HD megapixel camera mounted on a Start 75 tripod was permanently attached to the lid to maintain the same camera position for each trial. The video

recorder was started once the lid was secure. After 2 minutes, the pump inlet hose was connected to the chamber outlet to close the system. When the system was closed, water from the chamber was circulated through a watertight container, with a dissolved oxygen probe attached (Handheld dissolved oxygen meter - HI-9146N, Hanna Instruments Ltd.), and back into the test chamber. This provided a constant flow of water through the system and ensured that the water within the chamber was well mixed, thus preventing bias in the oxygen measurements resulting from localised depletions of dissolved oxygen. After a further two minutes, the exposure playback track (pile driving or ambient) was initiated. This point represented time zero of data collection.

In order to determine overall dissolved oxygen usage during the trial, dissolved oxygen concentration (ppm), oxygen saturation (%), and temperature of the water was recorded every 150 seconds. On completion of the trial the video recording was stopped and the Eheim pump disconnected, to pump in oxygenated water from the annular tank. The chamber lid was subsequently removed and the fish taken out. The fish was terminated using a Home Office Schedule 1 method (fatal blow to the head) before being weighed and measured.

The video was recorded directly onto a Panasonic DMR-EX86 DVD recorder during the trial before being backed up onto DVD for archiving and data analysis. During the trial, if the dissolved oxygen concentration dropped below a cut off limit of 6 mg/L the trial was stopped and the fish removed. Throughout the trials the limit of 6mg/l was never reached. The video of each trial was used to record the activity level of each fish. The overall time the fish spent moving around the tank was used to calculate a percentage activity level.

The initial and final dissolved oxygen concentrations from each trial were used to determine the oxygen consumption rate for each fish. The total volume of the respirometry system was 303 litres. Dissolved oxygen consumption rate was calculated by converting the change in oxygen (ppm) over the duration of the trial into micromoles of oxygen used per gram of tissue per hour (µmoles/g/h). A seawater salinity of 35 (g/l), along with the known average temperature for each trial, was used to calculate water density. A specific density per trial in g/cm^3 was then used to determine accurate concentrations of dissolved oxygen. These were applied to each trial to report the total dissolved oxygen consumed relative to the mass of each fish per hour. Oxygen consumption rate for each fish was plotted against its corresponding activity assessment. The gradient of the subsequent slope was then multiplied with the activity of the trial. The resultant values, representing an estimated oxygen consumption rate when the fish was active, were subtracted from the original consumption rates. This produced dissolved oxygen consumption rates

factoring in variances in activity level between trials. Mean activity levels were calculated for both treatments to monitor for bias.

Statistical Analysis

All statistical analysis was carried out in R (Version 3.2.2). Dependent upon assumptions, either a two samples t-test or Wilcoxon rank sum test was used to analyse the behavioural experiment and mean relative oxygen consumptions/activity levels. A Linear Regression model was used for the analysis of relative oxygen consumption and activity level.

Results

Behavioural Experiment

Fish in the control treatment spent significantly more time at either end of the dumbbell tank during the 4-hour exposure compared with fish in the pile-driving treatment (Figure 12). Capturing combined fish positions every minute showed that control fish spent an average of 45.00% of the exposure period in the near pod, compared to 34.06% for fish played pile driving (Wilcoxon rank sum test (W: 23.5; p-value: 0.049). In addition, the control fish spent 44.58% of the trial duration at the far end pod, whereas the fish in the piling treatment spent an average of 32.83% (Wilcoxon rank sum test (W: 18; p-value: 0.017). However, there was no statistical difference in time spent at the near end pod between the pre-exposure ambient hour and first hour of the experimental sound treatment. In the ambient treatment the average change in percentage time spent at the near end pod was -3.33%, whereas in the pile driving treatment it was 5.67% (Figure 13; two-sample t-test (t: 1.664; df: 18; P-value: 0.113).

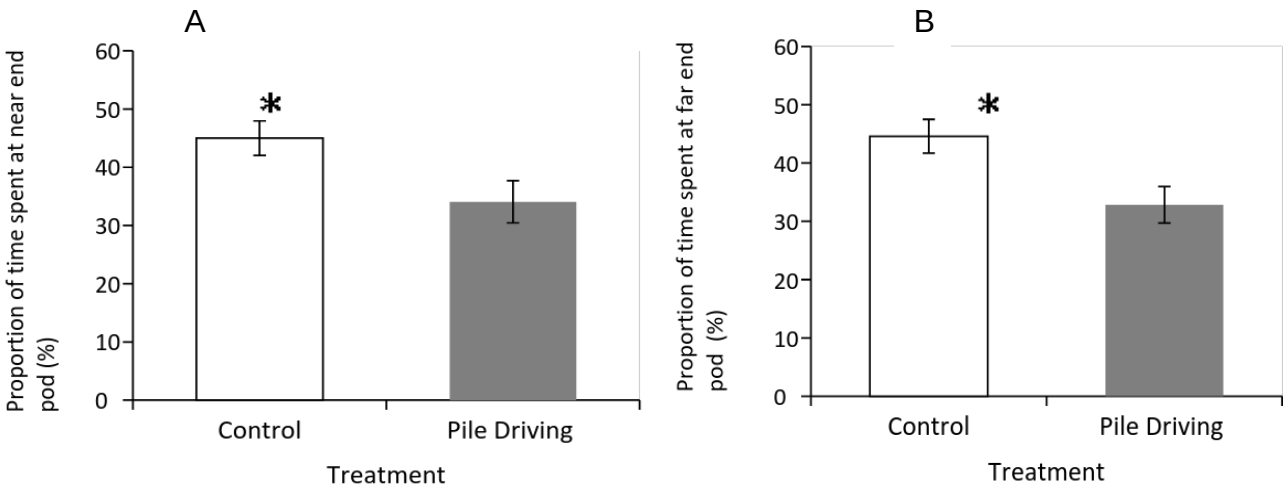

Figure 12: **A**; Proportion of time both fish spent in the near end pod (pod with speaker playing pile driving) over the four hour exposure period (Wilcoxon rank sum test (W: 23.5; p-value: 0.049). Near end pod in the ambient trials was defined using the respective piling trial in the block of two. **B**; Proportion of time both fish spent in the far end pod (pod furthest from the speaker playing pile driving) over the four hour exposure period (Wilcoxon rank sum test (W: 18; p-value: 0.017). Control: weight 3578.58 g ± 206.38 (mean ± SE); TL 677.25 mm ± 12.14; N = 10. Pile Driving: weight 3611.63 g ± 227.63, TL 678.00 mm ± 13.70; N = 10.

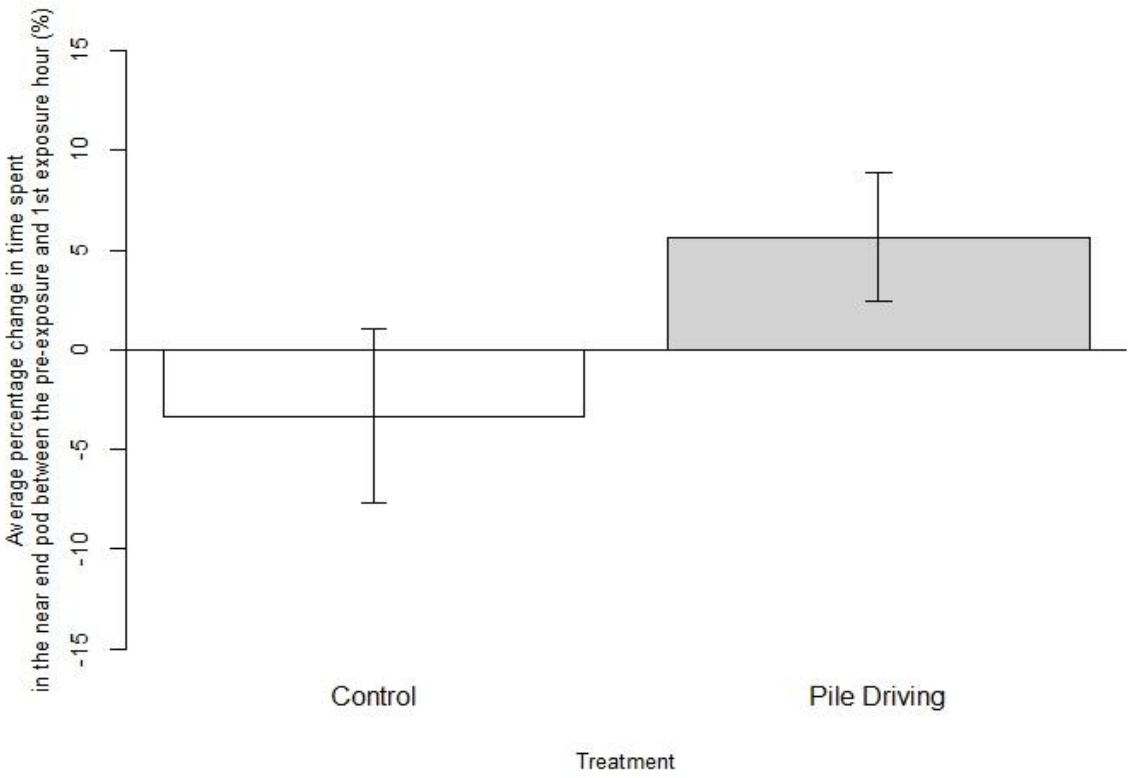

Figure 13: Average percentage change between the pre-exposure hour and first exposure hour in the proportion of time fish spent at the near end speaker. Control: weight 3578.58 g ± 206.38 (mean ± SE); TL 677.25 mm ± 12.14; N = 10. Pile Driving: weight 3611.63 g ± 227.63, TL 678.00 mm ± 13.70; N = 10. Two-sample t-test (t: 1.664; df: 18; P-value: 0.113).

Physiological Experiment

Activity level was shown to be a strong predictor of oxygen consumption rate for individual fish (Regression analysis; R^2=0.549, $F_{(1,21)}$=25.55, p-value= <0.005, N=23, Figure 14A). The resultant linear model was used to factor out activity from the oxygen consumption rates. In both treatments, activity level was a strong predictor of dissolved oxygen consumption (Ambient; Regression analysis: R^2: 0.664; $F=17.76_{(1,9)}$, p-value= 0.0023; N=11; Pile driving; Regression analysis: R^2=0.470, $F=8.86_{(1,10)}$, p-value= 0.0139; N=12; Fig. 14B). Fish exposed to pile driving noise had no statistical difference in oxygen consumption rate compared to the control treatment (two sampled t.test: t= 0.62, df=21, N=23, p-value=0.539; Figure 15). There was no statistical difference in mean activity level between treatment groups (Wilcoxon rank sum test: W = 59, N=23, p-value=0.695). Three trials were dropped from the analysis due to a lack of fish activity data, two from the ambient exposure group and one from the pile driving treatment. Figure 16 (supplementary

information) displays the dissolved oxygen concentration and saturation recorded in the respirometry chamber over a 2 hour duration without a fish present.

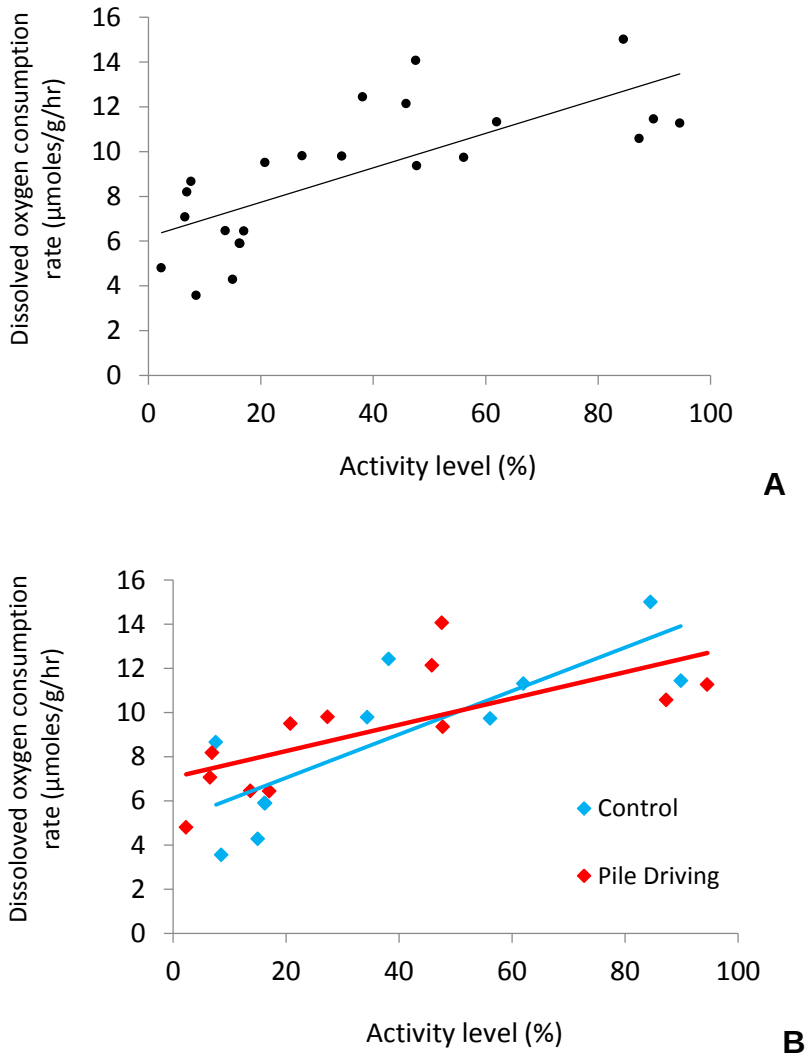

Figure 14: A; Dissolved oxygen consumption rate (µmoles/g/hr) against activity level (duration of trial spent moving~ percentage time over trial duration). Weight 1319.48 g ± 62.82 (mean ± SE); TL 480.17 mm ± 5.37; Regression analysis; R^2=0.549, $F_{(1,21)}$=25.55, p-value= <0.005, N=23. **B**; Dissolved oxygen consumption rate (µmoles/g/hr) against activity level (duration of trial spent moving ~ percentage time over trial duration) of both treatment groups (Ambient control, Pile Driving). Ambient: Weight 1329.97 g ± 97.66; TL 485.73 mm ± 7.33; N = 11; Regression analysis: R^2: 0.664; $F_{(1,9)}$=17.76, p-value= 0.0023. Pile driving: Weight 1309.86 g ± 84.63; TL 475.08 mm ± 7.81; N = 12; Regression analysis: R^2=0.470, $F=8.86_{(1,10)}$, p-value= 0.0139).

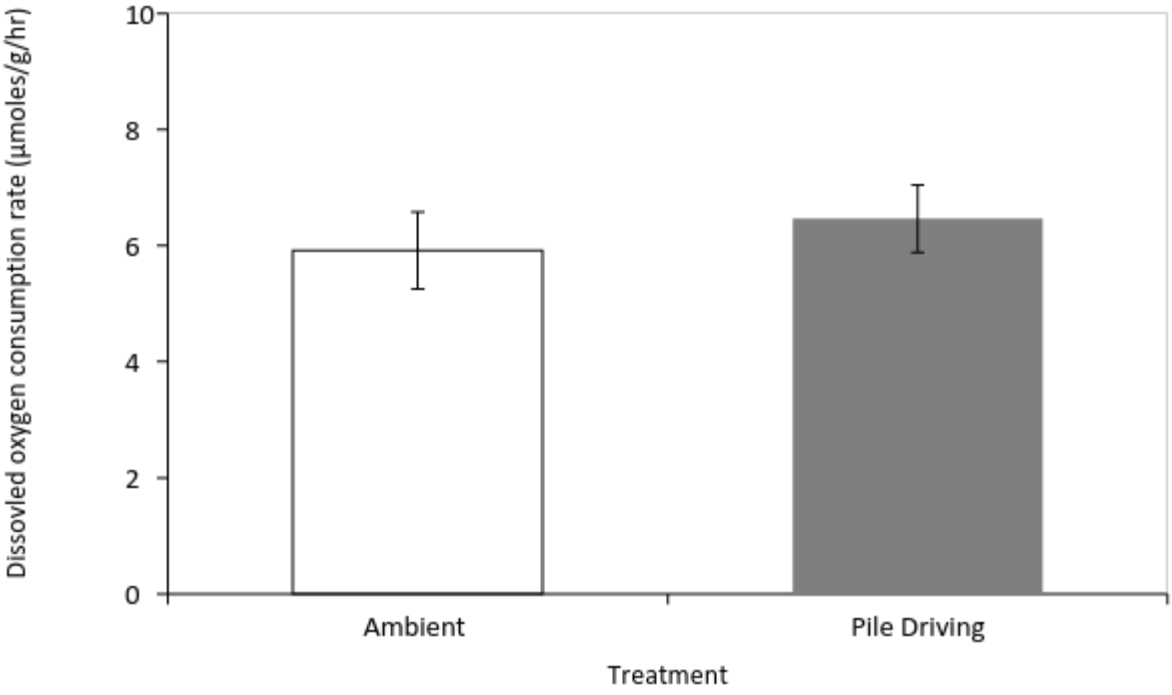

Figure 15: Average oxygen consumption rate relative to body mass of marine phase Atlantic salmon (*Salmo salar*) exposed to pile driving noise against an ambient control, with activity factored in. Ambient: 5.91 µmoles/g^{-1}/hr^{-1} ± 0.66 (mean ± SE); Weight 1329.97 g ± 97.66; TL 485.73 mm ± 7.33; Activity level 38.97 % ± 9.06; N = 11). Pile driving: 6.46 µmoles/g^{-1}/hr^{-1} ± 0.58); Weight 1309.86 g ± 84.63; TL 475.08 mm ± 7.81; Activity level 34.80 % ± 8.89; N = 12). Two sampled t.test: t= 0.62, df=21, N=23, p-value=0.539.

Discussion

In the behavioural experiment we found salmon in the ambient control treatment spent significantly more time at either end pod of the dumbbell tank compared to the piling treatment. However, despite the observation we found no difference between the proportion of time spent at the near end pod during the pre-exposure hour and the first exposure hour. The behaviour observed in the pre-exposure hour tended to match the movement captured in the rest of the trial. This strongly suggests that the additional noise of the piling is not driving the observed differences in behaviour between the two treatments. Additionally, there was no clear evidence of a startle response in relation to playback of individual hammer strikes (H Harding pers. obs.). In a previous study, although using a different noise stimulus, juvenile Atlantic salmon failed to display avoidance behaviours in response to a 150 Hz sound, 30 dB above defined awareness reaction thresholds (Knudsen *et al*., 1992). Similarly, juvenile coho salmon displayed no avoidance behaviour from exposure to a real impact-piling event when positioned in cages close to the noise source (Ruggerone *et al.,* 2008).

The physiological experiment has shown marine-phase Atlantic salmon do not experience a change in active metabolic rate (AMR), using oxygen consumption as a proxy, when exposed to pile driving noise (Figure 15). Using an alteration in AMR as an indicator of stress, this would suggest the cohort of Atlantic salmon used here did not perceive the pile driving playback noise as a stressor. In contrast, other anthropogenic noise stimuli, such as ship noise, have been shown to be a stressor in a number of other species (Wysocki *et al*., 2006; Slabbekoorn *et al*., 2010), which can lead to alterations in metabolic scope and subsequent consequences on predator avoidance and immune system functioning (Barton, 2002, Simpson *et al*., 2015). One explanation for this may centre on Atlantic salmon hearing ability. Compared to other teleost fish, including Atlantic cod (*Gadus morhua*) and herring (*Clupea harengus*), Atlantic salmon are particularly sound insensitive lacking specialist hearing mechanisms (Chapman & Hawkins, 1973; Hawkins & Johnstone 1978). The lack of such mechanisms reduces the fish's sensitivity and bandwidth to detect a noise stimulus, resulting in a poorer ability to distinguish specific acoustic cues from background noise (Hawkins & Johnstone, 1978; Popper & Fay, 1993; Kenyon *et al*., 1998; Radford *et al*., 2012). The nature of salmon hearing would, therefore, suggest a subdued or lack of response to specific noise stimuli and is consistent to the data we present here. In a closely related species, the brown trout (*Salmo trutta*), no observable changes in behaviour were recorded from exposure to a real piling event (average noise level 134 re 1 µPa, peak), supporting responses seen in our study (Nedwell *et al*., 2003).

The lack of significant response seen in the respirometry experiment is not attributed to poor functioning of the chamber. The effectiveness of the respirometry equipment, system and validation of the oxygen consumption data is provided by the recorded activity level being a strong predictor of oxygen consumption rate (Figure 14A). Due to the size of the respirometry chamber, the individual fish was allowed to move in the arena during the trial. This posed a potential problem of varying activity levels influencing the calculated oxygen consumption rate. Despite this, activity was balanced between treatments, suggesting that activity level was not related to the sound stimulus presented (Figure 14B). The barrier of the respirometry chamber wall was not shown to have reduced the particle motion component of the sound field (Figure10). We recorded particle acceleration levels (Channel XY) of 107.15 (dB re (1µm/s^2) inside the chamber compared to 106.79 (dB re (1µm/s^2) outside.

The suite of different experimental measures used in this study enabled us to assess organism effects upon multiple levels. If a fish perceives a stimulus as a stressor then a number of physiological response mechanisms are triggered; if the physiological responses become maladaptive, then to maintain 'normal' functioning,

changes in behaviour will occur with the aim to reduce exposure to the stressor (*Schreck et al*., 1997; Barton, 2002). We used active metabolic rate to determine whether altered movement patterns are a potential consequence of an altered physiological state (Barton, 2002; Simpson *et al*., 2015). The absence of a stress response from exposure to piling playback, therefore, compares well with there being no change in movement behaviour (avoidance response).

In this study we fully characterised both elements of the sound field (pressure and particle motion) for each experiment. This provided unique data on fish responses to defined particle motion values; such data are not often included when investigating the effects of anthropogenic noise on marine organisms. Despite continued developments to characterise particle motion in the field, recordings from a range of noise sources under varying environmental conditions are still required (Merchant *et al*., 2015). In light of this, we are not able to make direct comparisons of our recorded levels of particle motion to real noise sources.

Using captive fish in tank-based experiments provided the opportunity to minimise confounding variables and collect accurate data, free from a complex mix of variables present in fieldwork (Halvorsen *et al.,* 2012; Simpson *et al*., 2015). However, another possible explanation for the lack of either a behavioural or physiological response may be due to the environmental conditions the fish were raised. The present study used marine-phase Atlantic salmon with all cohorts of fish tested kept in captivity since hatching or brought to the aquarium as smolt. The acoustic history of the fish may have contributed to the biological responses observed. Studies have shown temporary changes in hearing thresholds, for a range of species with varying hearing abilities, from exposure to increased continuous noise levels (Smith *et al., 2004; Wysocki & Ladich 2004,* Halvorsen *et al*., 2009*)*. Conversely, exposure to a continuous 150 dB re 1µPa RMS ambient noise for eight months produced no changes in hearing sensitivity in rainbow trout (*Oncorhynchus mykiss*) (Wysocki *et al*., 2007). There is currently a paucity of data on whether noise from hatchery systems can cause permanent changes in hearing thresholds in captive reared Atlantic salmon. Further research is needed to identify how hearing sensitivities of such fish compare to wild conspecifics of the same development stage. This will aid in determining how useful captive fish are as a model for wild Atlantic salmon responses to an acoustic disturbance. The use of captive reared salmon in this study means we are unable to extrapolate our results to wild populations.

In tank-based studies it is not possible to fully replicate a natural sound field due to reflections and reverberations around the aquaria (Okumura *et* al., 2002). The playback of pile driving we presented shared similar acoustic characteristics to the

real stimuli, including rapid rise times and maximum pressure levels below 1kHz (Normandeau Associates, 2012; Radford *et al*., 2014). The design of the experiment enabled any differences observed during the trials to be attributed to the additional noise of the pile driving. Therefore, these experiments provided an initial assessment for the potential impact of pile driving playback on captive reared Atlantic salmon.

Further research is required to determine both the influence of pile driving on wild fish and a comparative analysis of the responses of other species to the same sound stimulus. It is very difficult to extrapolate behavioural responses, such as movement, from fish held in small arenas (Popper & Hastings, 2009). This study made use of the large dumbbell tank at the Marine Laboratory to provide a large arena to infer behavioural responses to pile driving. However, in order to provide meaningful, robust data for marine regulators biological response measures are needed from exposures to a real noise source in an open soundscape. This study represents a key stepping stone towards such field-based experiments.

Acknowledgments

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References

Bailey H, Senior B, Simmons D, Rusin J, Picken G, Thompson PM (2010) Assessing underwater noise levels during pile-driving at an offshore windfarm and its potential effects on marine mammals. *Marine Pollution Bulletin*, **60**, 888-897.

Barton BA (2002) Stress in Fishes: A diversity of responses with particular reference to changes in circulating corticosteroid. *Integrative and comparative biology*, **42**, 517-525.

Brittan-Powell EF, Dooling RJ, Gleich O (2002) Auditory brainstem responses in adult budgerigars (*Melopsittacus undulatus*). Journal of the Acoustical Society of America 112:999–1008

Bruintjes R & Radford AN (2014) Chronic playback of boat noise does not impact hatching success or post-hatching larval growth and survival in cichlid fish. *PeerJ 2*:e594, https://doi.org/10.7717/peerj.594.

Chapman CJ & Hawkins AD (1973) A field study of hearing in the cod, *Gadus morhua* L. *Journal of comparative physiology*, **85**, 147-167.

Corwin JT, Bullock TH, Schweitzer J (1982) The auditory brainstem response in five vertebrate classes. Electroencephalography and Clinical Neurophysiology 54:629–641

Hall JW (1992) Handbook of auditory evoked responses. Allyn and Bacon, Boston

Halvorsen MB, Wysocki LE, Stehr CM, Baldwin DH, Chicoine DR, Scholz NL, Popper AN (2009) Barging effects on sensory systems of Chinook salmon smolts. *Transactions of the American Fisheries Society*, **138**, 777–789.

Halvorsen MB, Casper BM, Woodley CM, Carlson TJ, Popper AN (2012) Threshold for onset of injury in Chinook salmon from exposure to impulsive pile driving sounds. *PLoS ONE*, **7(6)**, 1-11.

Hawkins AD, Pembroke AR, Popper AN (2015) Information gaps in understanding the effects of noise on fishes and invertebrates. *Reviews in Fish Biology Fisheries*, **25**, 39-64.

Hawkins AD & Johnstone ADF (1978) The hearing of the Atlantic salmon, *Salmo salar*. *Journal of fish biology*, ***13***, 655-673.

Hildebrand JA (2009) Anthropogenic and natural sources of ambient noise in the ocean. *Marine Ecology Progress Series*, **395**, 4–20.

Hildebrand JA (2005) Impacts of Anthropogenic Sound. *Marine Mammal Research: conservation beyond crisis* (eds Reynolds JE, Perrin WF, Reeves RR, Montgomery S, Ragen TJ), 101-124. The Johns Hopkins University Press, Baltimore, Maryland.

Kenyon TN, Ladich F, Yan HY (1998) A comparative study of hearing ability in fishes: the auditory brainstem response approach. *Journal of comparative physiology A*, **182**, 307-318.

Knudsen FR, Enger PS, Sand O (1992) Awareness reactions and avoidance responses to sound in juvenile Atlantic salmon, *Salmo salar*. *Journal of fish biology*, **40**, 523-534.

Mann D, Higgs D, Tavolga W, Souza M, Popper AN (2001) Ultrasound detection by clupeiform fishes. Journal of the Acoustical Society of America 109:3048–3054

McDonald MA, Hildebrand JA, Wiggins SM (2006) Increases in deep ocean ambient noise in the Northeast Pacific west of San Nicholas Island, California. *Journal of the Acoustical Society of America*, **120**, 711–718.
Merchant ND, Fristrup KM, Johnson MP, Tyack PL, Witt MJ, Blondel P, Parks SE (2015) Measuring acoustic habitats. *Methods in Ecology and Evolution*, **6**, 257-265.

Nedwell J, Turnpenny A, Langworthy J, Edwards B (2003) Measurements of underwater noise during piling at the Red Funnel Terminal, Southampton, and observations of its effect on caged fish. *Subacoustech Ltd*. Document reference: 558 R 0207.

Nedwell JR, Edwards B, Turnpenny AWH, Gordon J (2004) Fish and marine mammal audiograms:
A summary of available information. Report Reference: 534R0214, Subacoustech Ltd, Hampshire UK

Nedwell JR & Mason TI (2012) Modelling of noise during impact piling operations at the Neart na Gaoithe Offshore wind farm in the Firth of Forth. *Subacoustech Environmental Ltd.* Subacoustech Environmental report No. E297R0106.

Normandeau Associates, Inc. (2012) Effects of noise on fish, fisheries, and invertebrates in the U.S. Atlantic and Arctic from energy industry sound-generating activities. A literature synthesis for the U.S. Dept. of the Interior, Bureau of Ocean Energy Management. Contract # M11PC00031, 1-361 pp. Available at: http://www.data.boem.gov/homepg/data_center/other/espis/espismaster.asp?appid=1 (accessed 14 December 2015).

Okumura T, Akamatsu T, Yan, HY (2002) Analyses of small tank acoustics: Empirical and theoretical approaches. *Bioacoustics: the international journal of animal sound and its recording,* **12(2-3)**, 330-332.

Popper AN & Fay RR (1993) Sound detection and processing by fish: critical review and major research questions. *Brain, Behaviour and Evolution,* **41**, 14-38.

Popper AN, Halvorsen MB, Kane AE, Miller DL, Smith ME, Stein P, Wysocki LE (2007) The effects of high-intensity, low-frequency active sonar on rainbow trout. Journal of the Acoustical Society of America 122:623–635

Popper AN, Hastings MC (2009) The effects of anthropogenic sources of sound on fishes. *Journal of Fish Biology,* **75**, 455-489.

Purser J & Radford AN (2011) Acoustic noise induces attention shifts and reduces foraging performance in Three-Spined Sticklebacks (*Gasterosteus aculeatus*). *PLoS ONE,* **6(2)**, 1-8.

Radford CA, Montgomery JC, Caiger P, Higgs DM (2012) Pressure and particle motion detection thresholds in fish: a re-examination of salient auditory cues in teleosts. *The journal of experimental biology,* **215**, 3429-3435.

Radford AN, Kerridge E, Simpson SD (2014) Acoustic communication in a noisy world: can fish compete with anthropogenic noise? *Behavioural Ecology*,**00(00)**, 1-9.

Ruggerone GT, Goodman S, Miner R (2008) Behavioural Responses and Survival of Juvenile Coho Salmon Exposed to Pile Driving Sounds. *Natural Resources Consultants, Inc.* 1-42 pp. Available at: http://home.comcast.net/~ruggerone/FishTerminalPileDriveStudy.pdf (accessed 04 February 2016).

Schreck CB, Olla BL, Davis MW (1997) Behavioural responses to stress. In: Fish stress and health in aquaculture. *Society for experimental biology seminar series (*eds Iwama GK, Pickering AD, Sumpter JP, Schreck CB), **62,** 145- 170. Cambridge University Press, Cambridge, UK.

Simpson SD, Purser J, Radford AN (2015) Anthropogenic noise compromises antipredator behaviour in European eels. *Global Change Biology*. Doi: 10.1111/gcb.12685.

Slabbekoorn H, Bouton N, van Opzeeland I, Coers A, ten Cate C, Popper AN (2010) A noisy spring: the impact of globally rising underwater sound levels on fish. *Trends in Ecology and Evolution,* **25(7)**, 419-427.

Smith ME, Kane, AS, Popper AN (2004) Noise-induced stress and hearing loss in goldfish (*Carassius auratus*). *The journal of Experimental Biology*, **207**, 427-435.

Tyack P (2008) Implications for marine mammals of large-scale changes in the marine acoustic environment. *Journal of Mammalogy*, **89**, 549–558.

Wale MA, Simpson SD, Radford AN (2013) Noise negatively affects foraging and antipredator behaviour in shore crabs. *Animal behaviour,* **86**, 111-118.

Wysocki LE, Davidson III JW, Smith ME, Frankel AS, Ellison WT, Mazik PM, Popper AN, Bebak J (2007) Effects of aquaculture production noise on hearing, growth, and disease resistance of rainbow trout *Oncorhynchus mykiss*. *Aquaculture,* **272**, 687-697.

Wysocki LE, Dittami JP, Ladich F (2006) Ship noise and cortisol secretion in European freshwater fishes. *Biological conservation,* **128**, 501-508.

Wysocki LE & Ladich F (2004) Hearing in Fishes under Noise Conditions. *Journal of the association for research Otolaryngology*, **6**, 28-36.

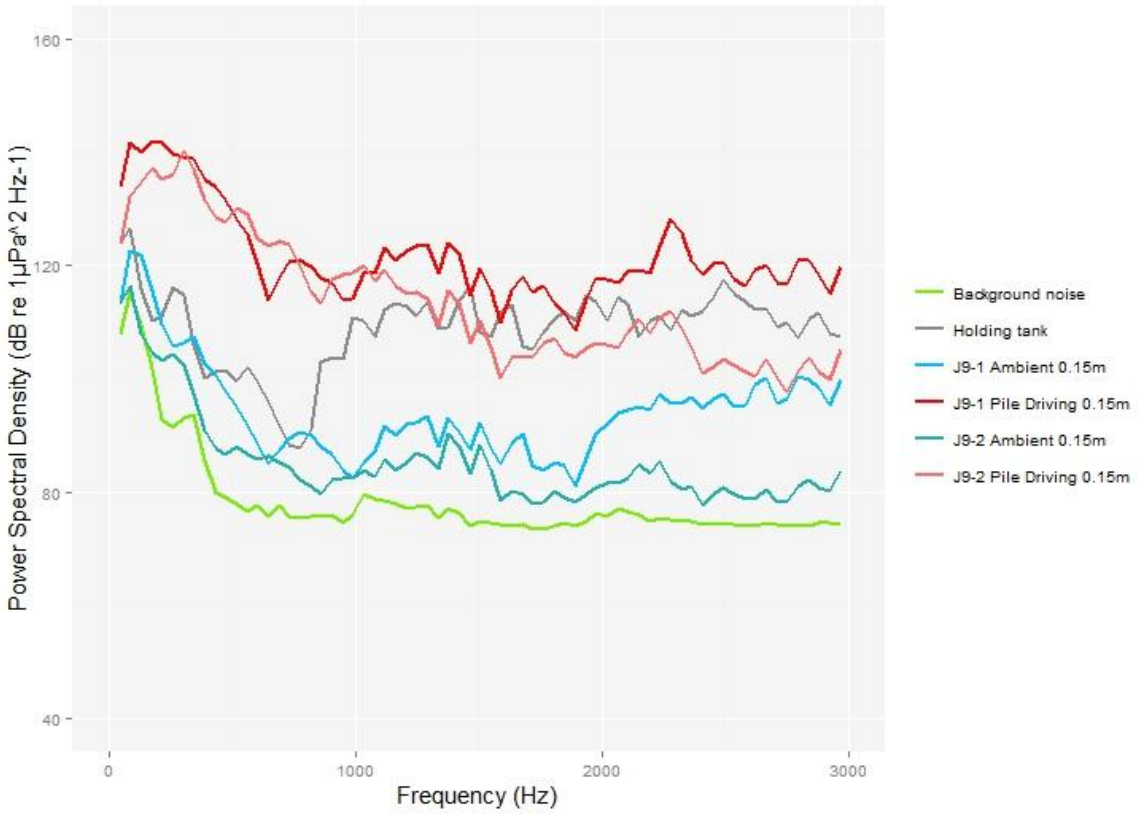


Figure 3: Power spectral densities of pile driving and ambient playback (both J9 sound projectors), background noise of the dumbbell tank and fish holding tank. The hydrophone was positioned 15 cm from the speaker. Fast Fourier Transform (FFT) analysis of sound recordings, using SASLab Pro v4.5.2 (Avisoft Bioacoustics, Berlin), power spectral analysis (level units, averaged), Hamming evaluation window, FFT size 1024, 43 Hz resolution), recordings averaged from 12 s samples, piling track: 10 hammer strikes, 1.2 seconds for each strike. The background holding tank recording was made with the hydrophone positioned off the bottom of the tank.

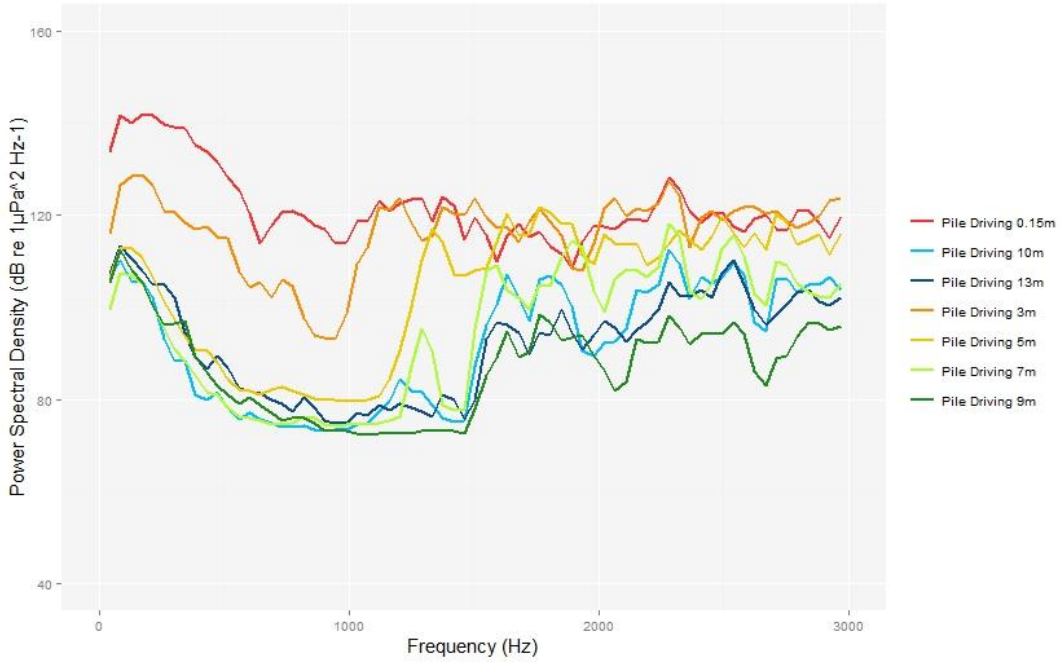


Figure 4: Power spectral densities of pile driving playback from one of the J9 sound projectors (J9-1). The hydrophone was positioned at various intervals from the speaker (0.15, 3, 5, 7, 9, 10, 13m). Fast Fourier Transform (FFT) analysis of sound recordings, using SASLab Pro v4.5.2 (Avisoft Bioacoustics, Berlin), power spectral analysis (level units, averaged), Hamming evaluation window, FFT size 1024, 43 Hz resolution), recordings averaged from 12 second samples, 10 hammer strikes, 1.2 seconds for each strike.

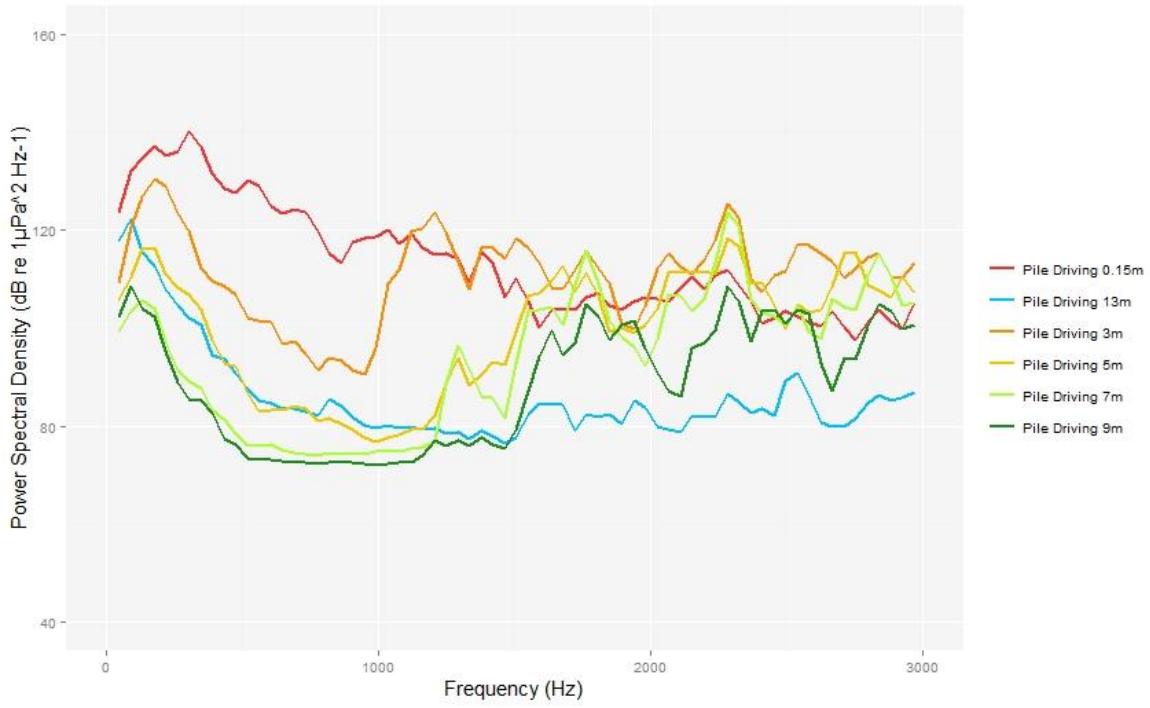


Figure 5: Power spectral densities of pile driving playback from one of the J9 sound projectors (J9-2). The hydrophone was positioned at various intervals from the speaker (0.15, 3, 5, 7, 9, 13m). Fast Fourier Transform (FFT) analysis of sound recordings, using SASLab Pro v4.5.2 (Avisoft Bioacoustics, Berlin), power spectral analysis (level units, averaged), Hamming evaluation window, FFT size 1024, 43 Hz resolution), recordings averaged from 12 s samples, 10 hammer strikes, 1.2 seconds for each strike.

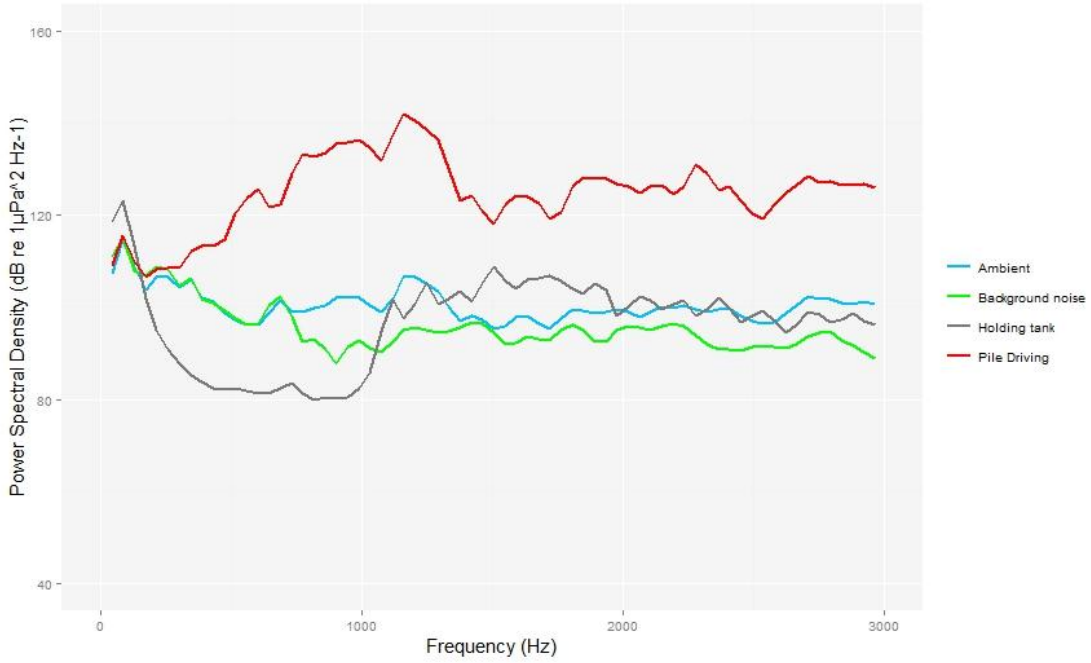


Figure 6: Power spectral densities of recordings made of both playback files (ambient and pile driving), background noise of the annular tank and tank two (holding tank) (averaged from 12 second recordings). The background holding tank recording was made with the hydrophone positioned off the bottom of the tank. All recordings were made using a Sony linear PCM recorder with an HTI hydrophone positioned within the respirometry chamber 20cm from the bottom. The chamber was positioned two meters from the speaker (Lubell LL916C). Fast Fourier Transform (FFT) analysis of sound recordings, using SASLab Pro v4.5.2 (Avisoft Bioacoustics, Berlin), power spectral analysis (level units, averaged), Hamming evaluation window, FFT size 1024, 43 Hz resolution, 12 second recording, piling track: 10 hammer strikes, 1.2 seconds for each strike).

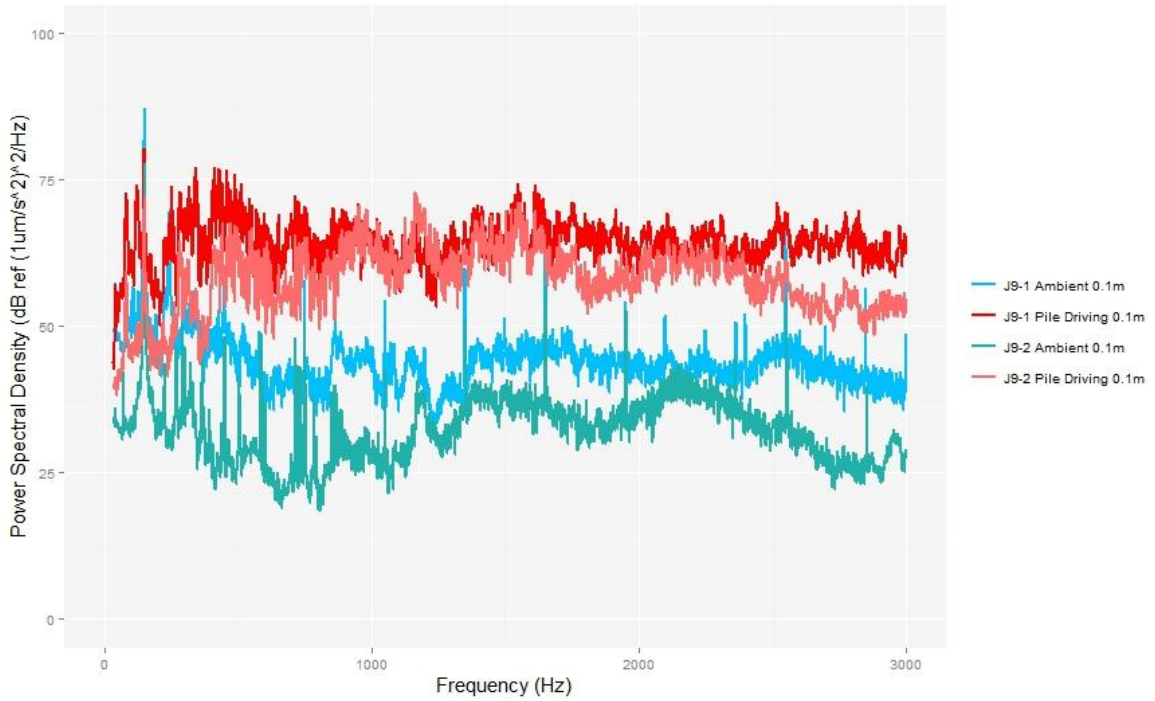


Figure 7: Power spectral densities (Channel XY) of ambient (0.1m) and pile driving (0.1m) playback in the dumbbell tank from both J9 sound projectors used in the experiment. Measured using a M30 calibrated accelerometer. Ambient measurements made from 12 s of ambient recording. Pile driving measurements made from 12 seconds of recording (10 hammer strikes, 1.2 seconds for each strike). Analysis performed using MatLab (R2013a; 8.1.0.604; MathWorks Inc. Sampling rate 44100Hz, Hamming window, Bandpass filter 0-3kHz).

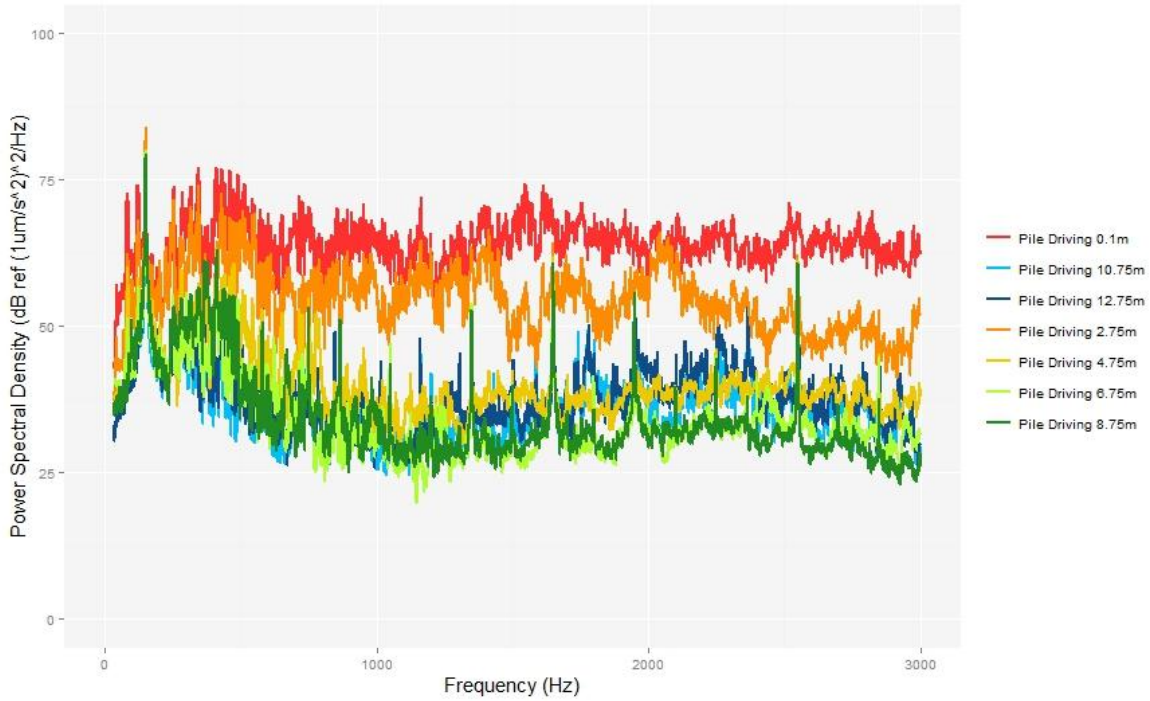


Figure 8: Power spectral densities (Channel XY) of pile driving playback measured at 0.1, 2.75, 4.75, 6.75, 8.75, 10.75, 12.75m along the dumbbell tank (J9-1). Measured using a M30 calibrated accelerometer. Ambient measurements made from 12 s of ambient recording. Pile driving measurements made from 12 seconds of recording (10 hammer strikes, 1.2 seconds for each strike). Analysis performed using MatLab (R2013a; 8.1.0.604; MathWorks Inc. Sampling rate 44100Hz, Hamming window, Bandpass filter 0-3kHz).

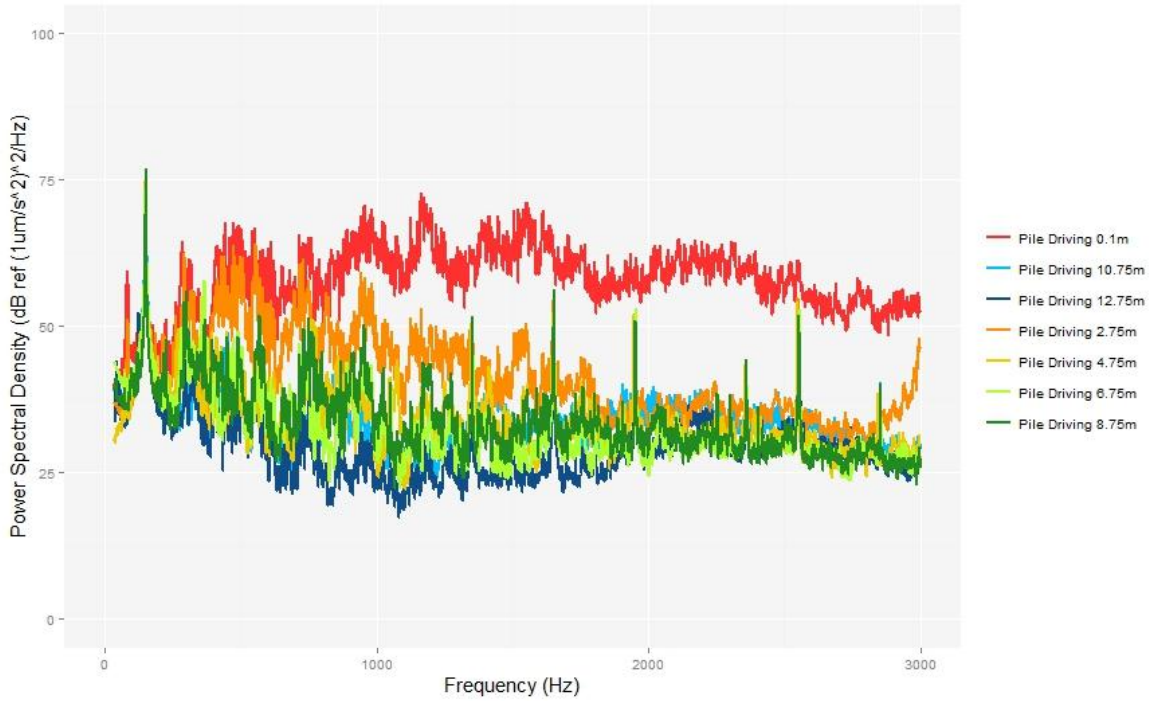


Figure 9: Power spectral densities (Channel XY) of pile driving playback measured at 0.1, 2.75, 4.75, 6.75, 8.75, 10.75, 12.75 m along the dumbbell tank (J9-2). Measured using a M30 calibrated accelerometer. Ambient measurements made from 12 s of ambient recording. Pile driving measurements made from 12 seconds of recording (10 hammer strikes, 1.2 seconds for each strike). Analysis performed using MatLab (R2013a; 8.1.0.604; MathWorks Inc. Sampling rate 44100Hz, Hamming window, Bandpass filter 0-3kHz).

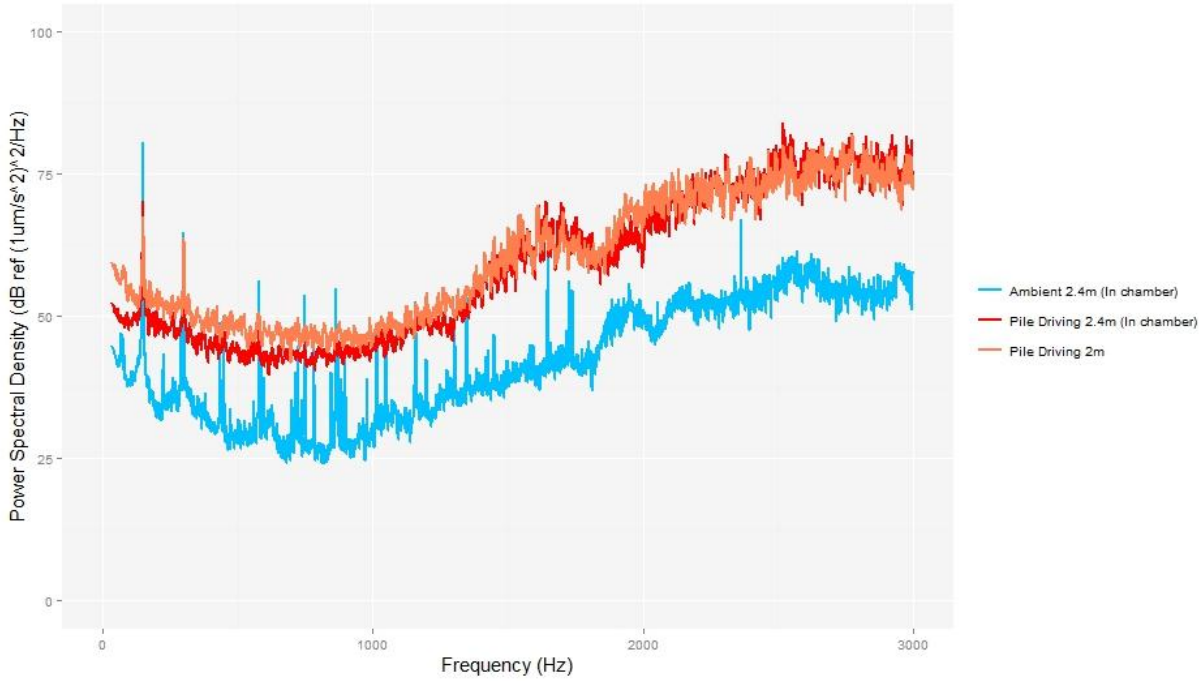
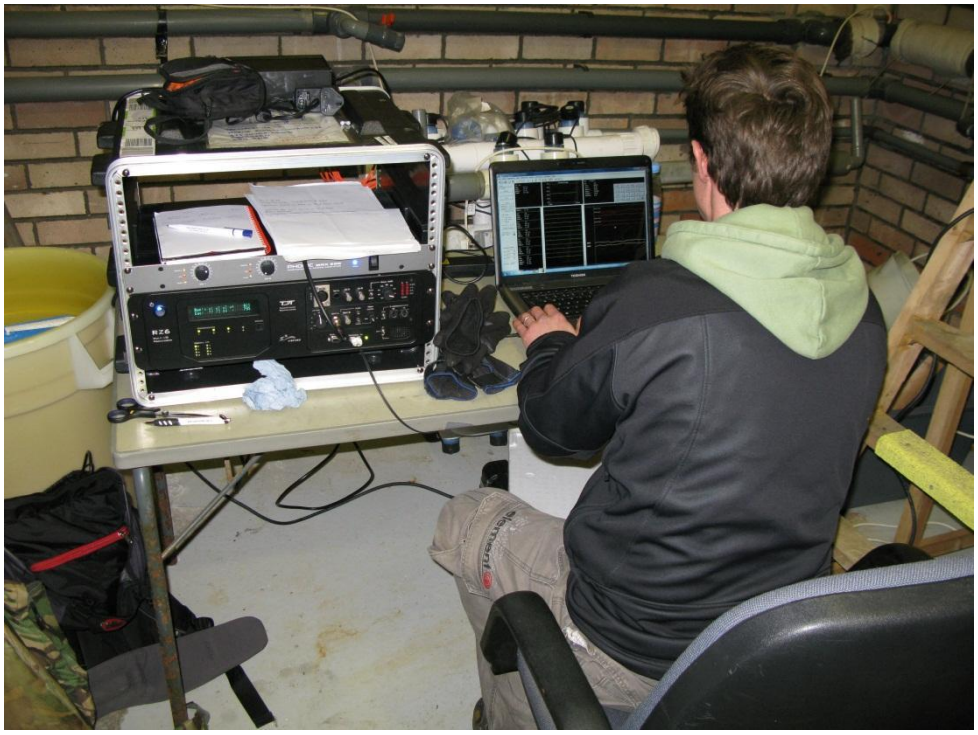


Figure 10: Power spectral density (Channel XY) of ambient and pile driving playback in the respirometry experiment. Measured using a M30 calibrated accelerometer positioned within the respirometry chamber 20 cm from the base of the tank (2.4 m from speaker) and next to the chamber 2 m from the Lubell LL916C loud speaker. The chamber was positioned two meters from the speaker (Lubell LL916C). Ambient measurements made from 10 s of ambient recording. Pile driving measurements made from 10 hammer strikes (10 s recording, 1 s from each strike). Analysis performed using MatLab (R2013a; 8.1.0.604; MathWorks Inc. Sampling rate 44100Hz, Hamming window, Bandpass filter 0-3kHz).

Images of the AEP set up in Operation

Above: Complete set up of the AEP equipment.



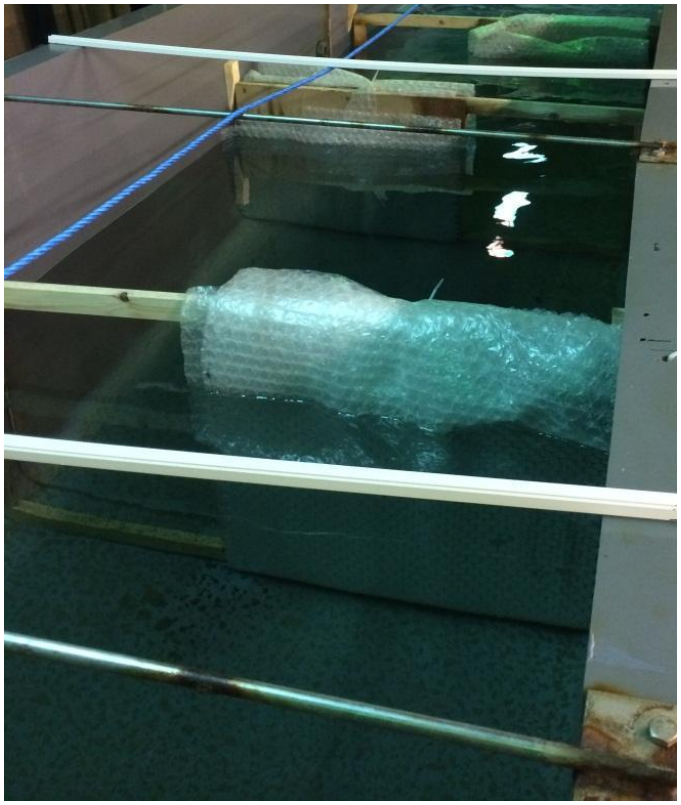
Above: Signal generation and data acquisition equipment.

Above: 1 m diameter fibreglass tank with fish AEP apparatus set up.

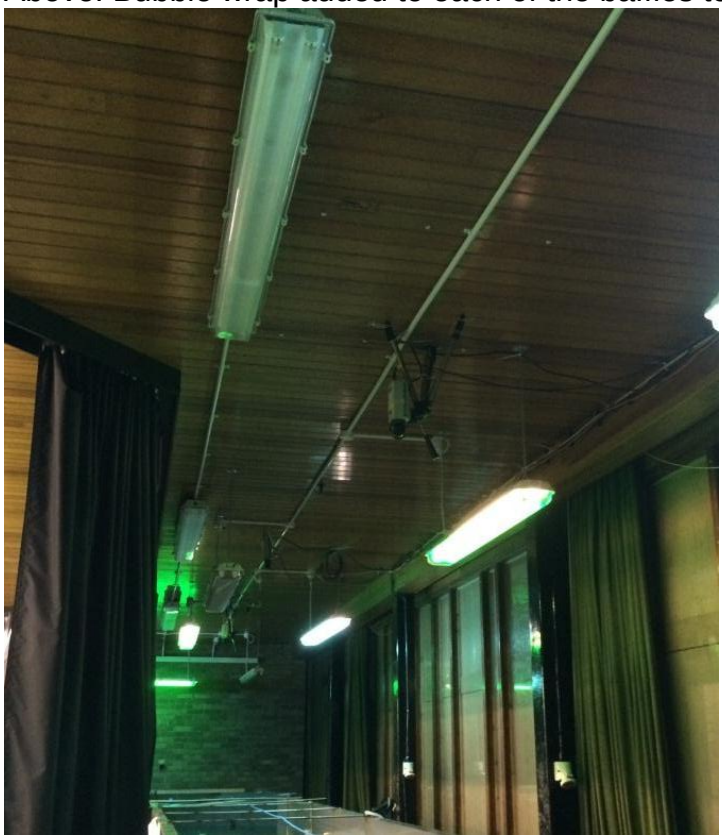
Images of Behavioural Experiment

Above: Soundproof baffles under construction for the dumbbell tank.

Above: Soundproof baffles installed in the dumbbell tank raceway prior to additional bubble wrap being added.



Above: Bubble wrap added to each of the baffles to aid in sound attenuation.



Above: Three CCTV video cameras attached to the ceiling above the dumbbell tank to record fish movement.

Images of Physiological Experiment

Above: Respirometer in the annular tank with the HD megapixel camera positioned above the chamber to film the fish.

Above: Respirometer positioned in the annular tank 2 meters from the speaker, with the pumping system to circulate water in the chamber in place.

Supplementary Information

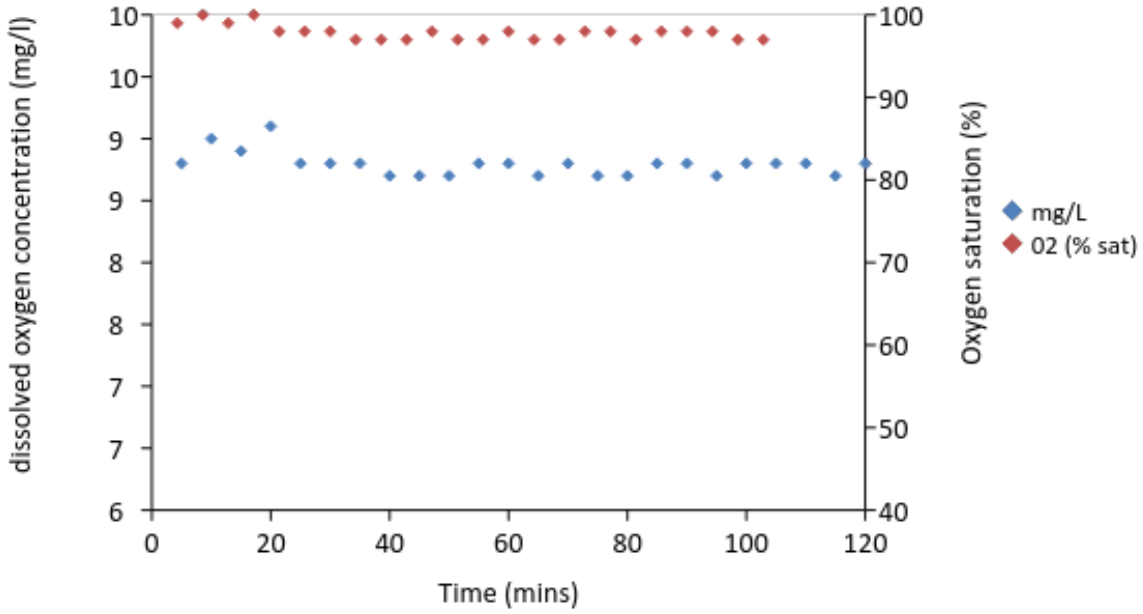


Figure 16: Dissolved oxygen concentration and oxygen saturation measured over two hours in the sealed respirometer without a fish present.

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