

1 **European seabass respond more strongly to noise exposure at night and**  
2 **habituate over repeated trials of sound exposure**

3

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9

10 Running title: Seabass habituate to repeated sound exposures

11

12 Main finding in two lines:

13 Seabass behaviour in a pen varied between day and night. Responses to sound were stronger  
14 at night and seabass showed inter-trial habituation over eight repeated sound exposures in  
15 two days.

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25 **Abstract**

26 Aquatic animals live in an acoustic world, prone to pollution by globally increasing noise  
27 levels. Noisy human activities at sea have become widespread and continue day and night.  
28 The potential effects of this anthropogenic noise may be context-dependent and vary with the  
29 time of the day, depending on diel cycles in animal physiology and behaviour. Most studies  
30 to date have investigated behavioural changes within a single sound exposure session while  
31 the effects of, and habituation to, repeated exposures remain largely unknown. Here, we  
32 exposed groups of European seabass (*Dicentrarchus labrax*) in an outdoor pen to a series of  
33 eight repeated impulsive sound exposures over the course of two days at variable times of  
34 day/night. The baseline behaviour before sound exposure was different between day and  
35 night; with slower swimming and looser group cohesion observed at night. In response to  
36 sound exposures, groups increased their swimming speed, depth, and cohesion; with a greater  
37 effect during the night. Furthermore, groups also showed inter-trial habituation with respect  
38 to swimming depth. Our findings suggest that the impact of impulsive anthropogenic noise  
39 may be stronger at night than during the day for some fishes. Moreover, our results also  
40 suggest that habituation should be taken into account for sound impact assessments and  
41 potential mitigating measures.

42

43 **Keywords:** anthropogenic noise, *Dicentrarchus labrax*, diurnal cycle, fish behaviour, field  
44 study, impulsive sound series, inter-trial habituation

45

## 46 **Introduction**

47 Increasing global energy demand has prompted the energy industry to construct more oil  
48 platforms and wind farms at sea. These offshore activities produce a variety of anthropogenic  
49 noises, which range from continuous sounds produced by ship traffic and windfarm operation  
50 to high-intensity impulsive sounds from seismic surveys and pile driving. Especially,  
51 impulsive sounds, which occur at both day and night (Leopold & Camphuysen, 2008; Brandt  
52 *et al.*, 2011), have been suggested to negatively affect fishes (Popper & Hastings, 2009a,  
53 2009b; Slabbekoorn *et al.*, 2010).

54 Fish in close proximity to a loud impulsive sound source may suffer from barotrauma injuries  
55 (Halvorsen *et al.*, 2012; Casper *et al.*, 2013a, 2013b). In laboratory settings fish are reported  
56 to recover from such injuries within a few weeks (Casper *et al.*, 2012, 2013b), but this may  
57 be different for free-ranging fish that need to find food and flee for predators. However,  
58 although physical damage may appear a severe impact, it only concerns a small proportion of  
59 the fish population that is close enough to receive such high-intensity sound. In view of this,  
60 the farther-ranging behavioural effects of impulsive sounds at moderate levels may be more  
61 concerning for fish populations (Slabbekoorn *et al.*, 2010; Hawkins *et al.*, 2014a).

62 In response to impulsive sound exposures, fish have been shown to change their  
63 swimming behaviour; typified by swimming faster, deeper, in a tighter shoal and further  
64 away from a sound source (Hawkins *et al.*, 2014b; Neo *et al.*, 2014, 2015, 2016). Such  
65 behavioural responses were actually found to be stronger for impulsive sounds compared to  
66 continuous sounds (Neo *et al.* 2014). Groups of European seabass (*Dicentrarchus labrax*)  
67 took longer to return to baseline swimming depth in response to impulsive sounds than to  
68 continuous sounds, while it took longer to return to baseline group cohesion levels when the  
69 exposures (either impulsive or continuous) had variable amplitude, as opposed to constant.

70 These results highlight the biological relevance of sound intermittency and reveal the  
71 limitations of using exclusively sound level or sound exposure level to predict response  
72 tendency or disturbance potential of aquatic animals.

73 Additionally, while the majority of studies investigating behavioural effects of  
74 underwater sound have been conducted during the day, impulsive sounds can be experienced  
75 by fish throughout their diel cycle which may affect their response level, like with other  
76 external stressors. For example, when subjected to air exposure (lifted out of the water),  
77 nocturnal green sturgeon (*Acipenser medirostris*) and Gilthead sea bream (*Sparus aurata* L.)  
78 increased plasma cortisol more at night than during the day (Lankford *et al.*, 2003; Vera *et*  
79 *al.*, 2014). In contrast, nocturnal Senegalese sole (*Solea senegalensis*) were more affected  
80 during the day (López-Olmeda *et al.*, 2013). It is currently unknown how the time of day may  
81 influence the effects of sound exposure in diurnal species such as the European seabass.

82 Furthermore, impulsive sounds from seismic surveys or pile-driving may be repeated,  
83 with breaks of inactivity, for several weeks or months (Leopold & Camphuysen, 2008;  
84 Brandt *et al.*, 2011). Despite this, the impacts of sound on fish behaviour have mainly been  
85 studied within a single exposure session and there are a few cases in which the effects of  
86 repeated exposures were tested. Nedelec *et al.* (2016) showed that the Threespot dascyllus  
87 (*Dascyllus trimaculatus*) increased hiding behaviour during playback of boat noise, but the  
88 effect was no longer significant after one and two weeks of repeated exposures. In another  
89 study, larval Atlantic cod (*Gadus morhua*) revealed no experience-related variation in  
90 responsiveness in a predator-avoidance test between different rearing noise treatments  
91 (Nedelec *et al.*, 2015). Besides these studies, there is little evidence as to whether repeated  
92 exposure sessions cause behavioural responses to accumulate, potentially leading to stronger  
93 responses through sensitization (e.g. Götz & Janik, 2011), or diminish through habituation  
94 (Groves & Thompson, 1970; Grissom & Bhatnagar, 2009; Rankin *et al.*, 2009). Earlier

95 studies have already shown evidence for intra-trial habituation of European seabass to  
96 intermittent sounds (Neo *et al.*, 2014, 2015), but inter-trial habituation over repeated trials for  
97 this species has yet to be demonstrated.

98         In the current study, we exposed groups of European seabass each to a series of eight  
99 sound exposures in a large outdoor floating pen throughout the diel cycle of the fish. We  
100 aimed to answer the following questions: Do seabass vary consistently in swimming  
101 behaviour over the day? Does a sound-induced change in behaviour depend on whether it is  
102 night or day? Finally, do seabass habituate to repeated exposures of the same sound stimulus?  
103 We expected that the fish would change behaviour upon sound exposure and that the  
104 behavioural changes would depend on the time of the day. We also expected that behavioural  
105 changes would diminish over subsequent exposures.

106

## 107 **Materials and methods**

### 108 ANIMAL MAINTENANCE

109 We used hatchery-raised European seabass (from Ecloserie Marine de Gravelines, France),  
110 approximately 30 cm in length. Before testing, the fish were kept in a cylindrical holding tank  
111 (Ø 3.5 m, depth 1.2 m) at Stichting Zeeschelp, the Netherlands where the dark-light cycle  
112 was identical to the outdoor conditions. The holding tanks had a continuous inflow of fresh  
113 seawater from the nearby Oosterschelde estuary and water temperatures ranged from 14 to 19  
114 °C during the experimental period (August-October 2014). We fed the seabass three times a  
115 week with food pellets (Le Gouessant Aquaculture, France), for which amounts were  
116 determined by fish number and size and adjusted based on the water temperature. Although  
117 previous experience does not affect the validity of the current test for fading responsiveness  
118 from the first to the last of a new series of sound exposures, we like to mention that the

119 animals were also used in a previous experiment (Neo *et al.*, 2016). In that experiment, they  
120 were exposed to four sound exposures, of which one was identical to the sound exposures in  
121 the current experiment. The time between the previous and the current experiment was at  
122 least three weeks. These experiments were ethically evaluated and approved by the Animal  
123 Experiments Committee (DEC) of Leiden University (DEC approval no: 14047).

124

## 125 EXPERIMENTAL ARENA

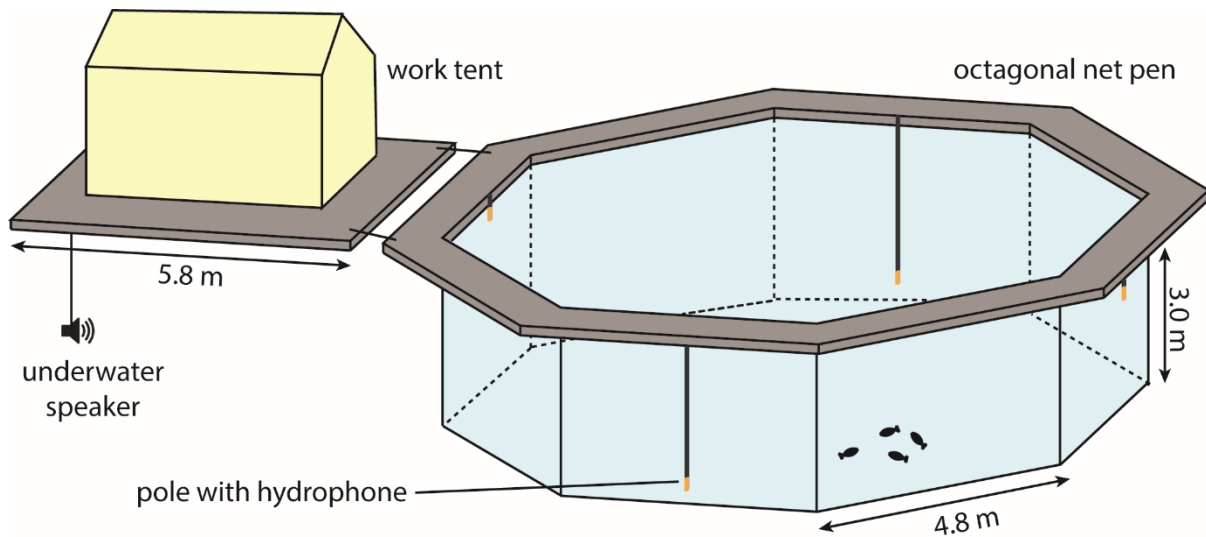
126 The experiments were conducted in the Jacobahaven, an artificial cove located at the opening  
127 of the Oosterschelde, an estuary of the North Sea. The cove is about 200 m by 300 m in size  
128 and 2-5 m deep depending on tides with bottom sediment consisting of mud and sand. The  
129 water in the cove is relatively calm due to surrounding dams and a pier which shield the  
130 Jacobahaven from wind. Additionally, no boat traffic is allowed within 1 km of the cove,  
131 resulting in minimal levels of underwater anthropogenic noise, making it ideal for sound  
132 impact studies.

133         We constructed a floating platform (Fig. 1) in the center of the Jacobahaven using a  
134 modular floating dock system (Candock, Canada). We anchored it to dead weights on the  
135 bottom with an elastic cable system that kept the platform in place at all tides. The  
136 construction consisted of an octagonal walkway surrounding the pen and a square working  
137 platform for storing equipment tied to the outer perimeter of the walkway. The octagonal  
138 walkway held a net of 3 m depth and a diameter of 11.5-12.5 m (volume 334 m<sup>3</sup>) where test  
139 fish were held during experimental exposures. The working platform carried an underwater  
140 speaker at 2.2 m depth, and supported a work tent (4 x 5 m) that shielded the equipment from  
141 weather and served as office space. The work tent was supplied with electricity via an  
142 underwater cable from Stichting Zeeschelp. We maintained a distance of 0.5 m between the

143 platform and walkway using a physical buffer of soft buoys to minimise unwanted sound  
144 transmission from activity at the working platform to the net pen. Additionally, the working  
145 platform could be moved and reattached to one of four positions with respect to the octagonal  
146 walkway (North, East, South, and West). Every four trials, the working platform (i.e. the  
147 experimental sound source) was repositioned to the next position along the walkway, to  
148 control of the potential effects of consistent spatial preference in the experimental area across  
149 trials.

150

151 Figure 1



152

153 Fig. 1. Schematic of the floating platforms. The underwater speaker was suspended at  
154 the center of the far edge of the working platform. The distance from the underwater speaker  
155 to the closest side of the net was 7.8 m. The four hydrophones attached to the poles were used  
156 to track the test fish via telemetry.

157

158 SOUND TREATMENT

159 We exposed the groups of fish eight times to a 1-h impulsive sound treatment consisting of  
160 0.1 s pulses, repeated at a regular repetition interval of 2 s. The sound sample was created in  
161 Adobe Audition 3.0 using band-passed brown noise within 200-1000 Hz (48 dB rolloff per  
162 octave). This range matches the spectral range of highest hearing sensitivity for European  
163 seabass (Lovell, 2003; Kastelein *et al.*, 2008). However, it should be noted that these  
164 audiograms are based on sound pressure only and the methods of both papers have important  
165 limitations (cf. Ladich & Fay, 2013; Sisneros *et al.*, 2016). The sound was played back with  
166 an underwater speaker (LL-1424HP, Lubell Labs, Columbus, US) from a laptop through a  
167 power amplifier (DIGIT 3K6, SynQ) and a transformer (AC1424HP, Lubell Labs).

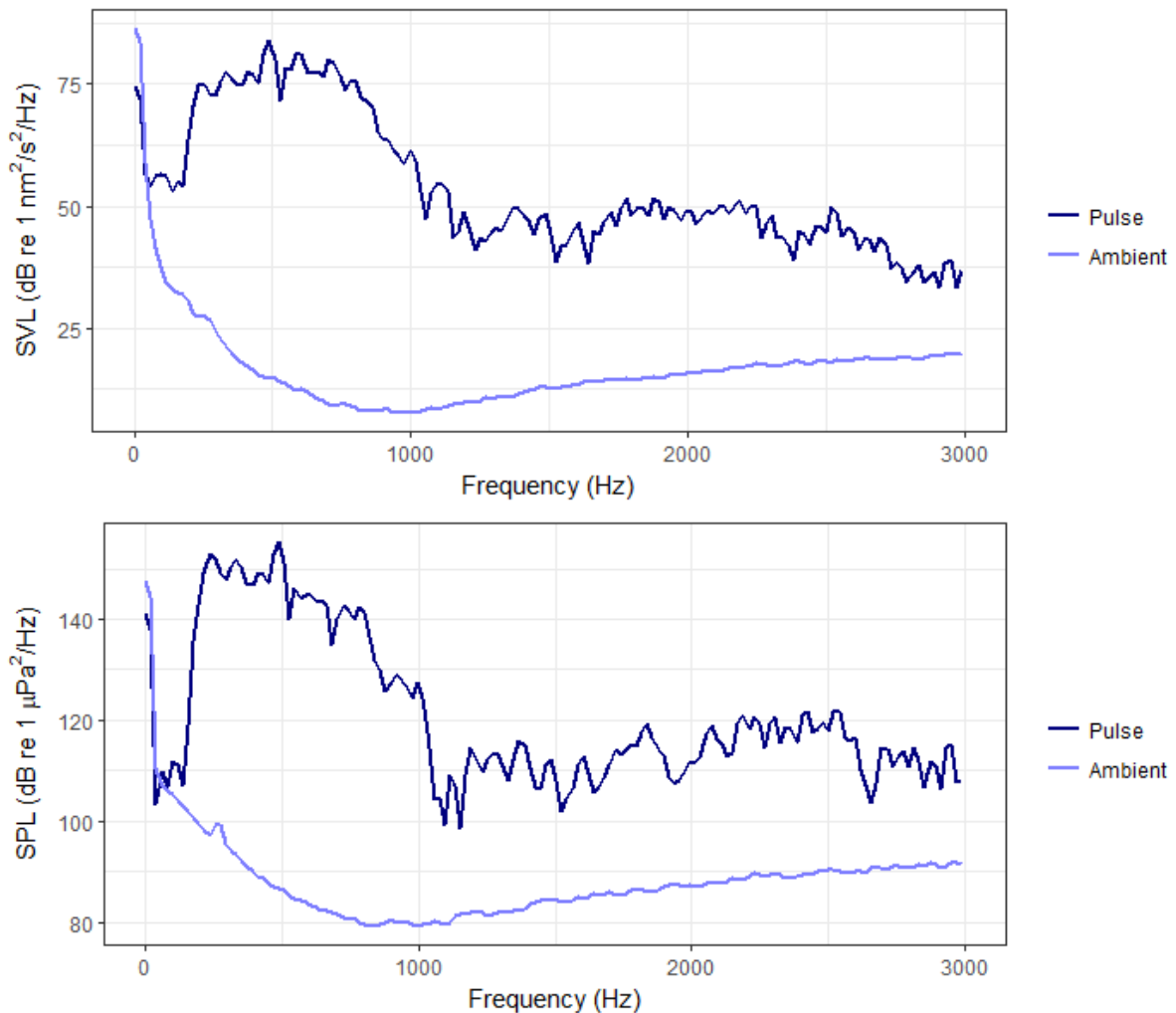
168 The amplitude levels of the sound treatment were measured at 360 points along a  
169 uniformly spaced three-dimensional grid within the octagonal net (120 points at 0.5, 1.5 &  
170 2.5 m depth) prior to the start of the experiment. These measurements were repeated with all  
171 four working platform (i.e. speaker) positions during both flow and ebb tide (8 replicate sets).  
172 We measured the sound pressure levels (SPL) and sound velocity levels (SVL) using a M20  
173 particle motion sensor (GeoSpectrum Technologies, Canada). The sensor was comprised of  
174 three orthogonal accelerometers and a hydrophone. The data output was logged at 40 kHz on  
175 a laptop via an oscilloscope (PicoScope 3425, Pico Technologies, UK) using an application  
176 written in Microsoft Access via Visual Basic for Applications. The data were subsequently  
177 analysed in MATLAB using a 200-1000 Hz bandwidth filter and power spectral density plots  
178 were generated using R (Fig. 2). For the sound velocity measurements, we calculated the  
179 root-mean-square, zero-to-peak and single strike energy of sound velocity for each  
180 accelerometer channel then combined the values using vector addition to result in an  
181 omnidirectional measure of particle motion which was comparable to SPL. We then averaged  
182 these values with respect to their positions relative to the working platform (8 replicates per  
183 aggregate) to calculate the presumed average sound gradient over all experimental trials. The



184 results revealed a clear gradient in amplitude levels with an increasing distance from the  
185 speaker within the experimental arena. The mean zero-to-peak sound pressure level ( $SPL_{z-p}$ )  
186 and sound velocity level ( $SVL_{z-p}$ ) were 180-192dB re 1  $\mu\text{Pa}$  and 124-125dB re 1 nm/s,  
187 respectively. In addition, the mean single-strike sound exposure level ( $SEL_{ss}$ ) and velocity  
188 exposure level ( $VEL_{ss}$ ) were 156-167 dB re 1  $\mu\text{Pa}^2\text{s}$  and 99-100 dB re 1  $\text{nm}^2/\text{s}$  respectively.

189

190 Figure 2



191

192

193 Fig. 2. Power spectral density (PSD) plots of sound velocity level (SVL, top) and sound  
194 pressure level (SPL, bottom) of a single pulse and the ambient condition in the pen. These

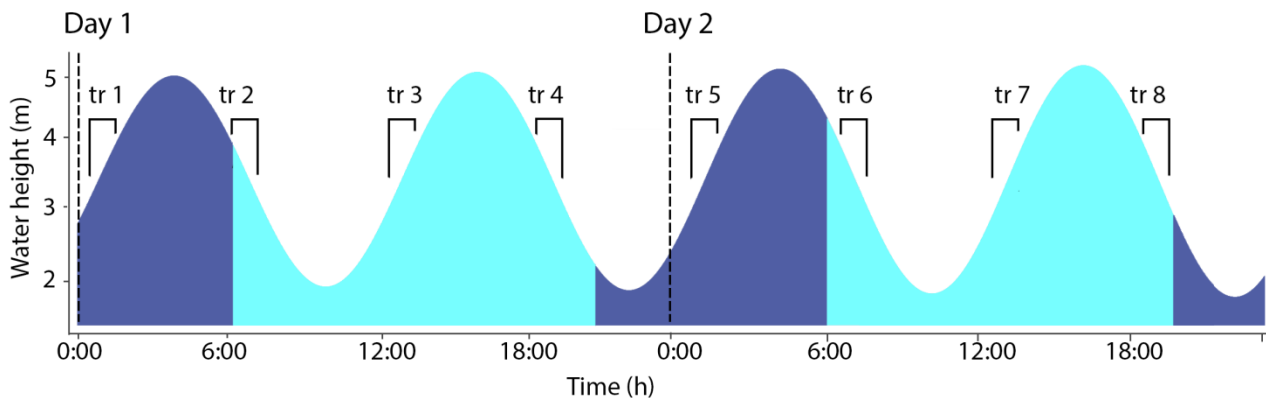
195 PSD's were made using a sound recording in the pen at 17.5 m from the speaker and 1.5 m  
196 depth. For generating the PSD's, we used a window length of 2048 with a Hamming window  
197 type.

198

## 199 EXPERIMENTAL DESIGN

200 We exposed each of sixteen groups of four fish ( $N = 16$ , 64 fish) to an impulsive sound  
201 treatment eight times during two consecutive days (Fig. 3). Each group of fish was  
202 transported to the net pen in a black plastic container (56x39x28 cm) with oxygen tablets  
203 (OxyTabs, JBL, Germany) to ensure sufficient oxygen levels. The fish were allowed to  
204 acclimate for at least 20 h before the start of the first exposure. Half of the groups started with  
205 the first trial of the exposure series during the day and the other half at night. The exposures  
206 took place during ebb tide (starting 1.5 h after the high tide) and flood tide (ending 1.5 before  
207 the high tide), when the water depth ranged between 3 and 4 m for all the trials. Due to the  
208 tides, a subsequent trial started either 3 h or 7.5 h (alternating) after the end of the previous  
209 trial. Each trial lasted for 1.5 h and consisted of 60 min of sound exposure and 15 min of  
210 silence before and after. We arrived at the platform 30 min before the start of the trial, where  
211 we would then record the light intensity, weather condition and the water temperature, which  
212 were used as covariates in the statistical analyses. During the trial, we waited quietly at the  
213 working platform until after the last exposure, where we then lifted the net pen, caught the  
214 fish with a scoop net and transported the group of fish back to the onshore holding tank.

215



217

218 Fig. 3. Tide table showing the sound trial exposure scheme. All eight trials took place over  
 219 two days when the water depth was 3-4 m. Dark blue indicates night time and light blue  
 220 indicates day time.

221

## 222 ACOUSTIC TELEMETRY

223 We analysed the swimming patterns of the four seabass individuals per trial with 3D  
 224 telemetry using acoustic tags (Model 795-LG, HTI, US). We set the tags to emit 0.5 ms long  
 225 pings of 307 kHz (inaudible to the fish) at different repetition intervals (995, 1005, 1015 and  
 226 1025 ms) in order to identify the four unique swimming tracks. The fish were externally  
 227 tagged under the first and second dorsal fin (cf. FISHBIO, 2013). Tags were reused and a  
 228 maximum of 8 fish were tagged at any given time: We tagged the next group of individuals  
 229 while the current group was still in the experimental trial. After the tagging procedure, the  
 230 fish were kept in a recovery tank (1.20x1.00x0.65 m), which had a continuous inflow of fresh  
 231 seawater from the Oosterschelde. The fish were allowed to recover for at least two days  
 232 before being transported to the floating pen. In the pen, the pings from the acoustic tags were  
 233 recorded by four hydrophones (Model 590-series, HTI, US) attached to the octagonal

234 walkway (Fig. 1). The signals were then processed by an acoustic tag receiver (Model 291,  
235 HTI, US) and transferred to a connected laptop. The data were further processed with  
236 software from the manufacturer (MarkTags v6.1 & AcousticTag v6.0, HTI, US). This  
237 resulted in 3D positions per each individual per approximately 1 s intervals. The positional  
238 information was then used to calculate the group behavioural parameters: swimming speed,  
239 swimming depth, average inter-individual distance (group cohesion) and distance from the  
240 speaker (*cf* Neo et al., 2016).

241

## 242 STATISTICS

243 We first examined behavioural parameters in a 5 min segment immediately before the onset  
244 of each sound exposure to see if baseline behaviours varied depending on the exposure  
245 sequence (order) and the time of the day. We categorised the time of the day into ‘day’ or  
246 ‘night’, depending on whether the trial started before or after the sunrise/sunset of the day.  
247 We modelled the baseline behaviours using a linear mixed effects model, treating the group  
248 ID as a random effect and exposure sequence (1-8) and time of day (day/night) as continuous  
249 and categorical fixed effects, respectively. In addition, we also used time of day, tide, and  
250 water temperature as additional fixed effects covariates. We selected the best model using  
251 backward stepwise selection based on Akaike information criteria (AIC). Subsequently, the  
252 same modelling procedure was applied to the behavioural changes caused by the sound  
253 exposure, where the responding variable was instead the change in swimming behaviour  
254 values between the 5 minute segments immediately before and after the onset of each sound  
255 exposure. We also performed one-sample t-tests to see if the calculated differences were  
256 significantly larger than zero.

257

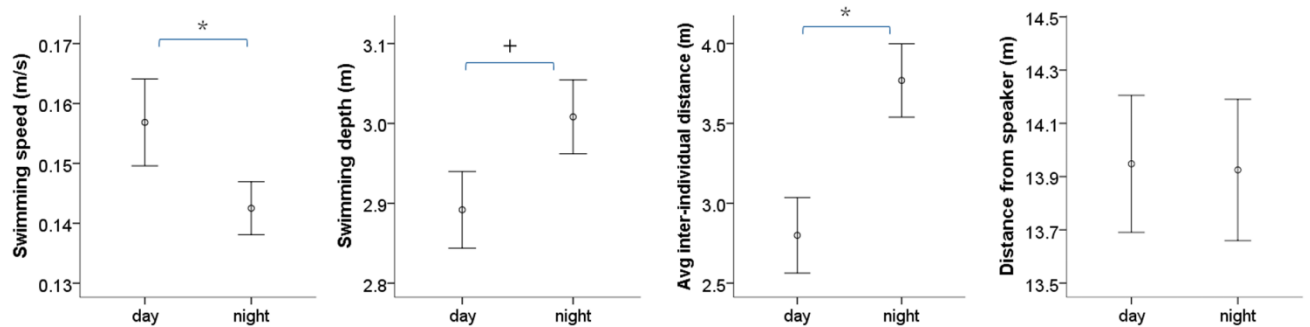
## 258 **Results**

259 We compared the pre-playback baseline behaviour of the fish between day and night (69 and  
260 59 trials respectively) (Fig. 4a). At night, the fish swam significantly slower (linear mixed  
261 model:  $F_{1,94} = 5.312$ ,  $P = 0.023$ ) in groups with significantly lower cohesion (linear mixed  
262 model:  $F_{1,98} = 13.799$ ,  $P < 0.001$ ). There was a non-significant trend that they also swam  
263 higher up in the water column (linear mixed model:  $F_{1,107} = 3.014$ ,  $P = 0.085$ ), at similar  
264 distance from the speaker. Upon sound exposure, the increase in group cohesion was  
265 significantly larger at night (linear mixed model:  $F_{1,89} = 3.954$ ,  $P = 0.050$ ) (Fig. 4b). There  
266 was also a non-significant trend that the increase in swimming speed was also larger at night  
267 (linear mixed model:  $F_{1,95} = 3.671$ ,  $P = 0.058$ ). Subsequent one-sample t-tests showed that  
268 only increases in swimming speed and swimming depth at night were significantly larger  
269 than zero (one-sample t-test:  $t_{57} = 3.782$ ,  $P < 0.001$ ;  $t_{57} = -2.008$ ,  $P = 0.049$  respectively).  
270 There was also a non-significant trend that increase in group cohesion at night was larger  
271 than zero (one-sample t-test:  $t_{53} = -1.716$ ,  $P = 0.092$ ). Within the 60 min exposure trials, all  
272 the behavioural changes reverted back to baseline levels, indicating intra-session habituation  
273 (Neo *et al.*, 2014, 2015, 2016). For inter-session habituation, we found that changes in  
274 swimming depth diminished significantly with subsequent exposure sessions (linear mixed  
275 model:  $F_{1,57} = 4.002$ ,  $P = 0.050$ ) (Fig. 5). For group cohesion, we found significant  
276 interaction between the time of the day and the trial order (linear mixed model:  $F_{1,86} = 4.353$ ,  
277  $P = 0.040$ ), which was due to a subtle decline in response over time at night and a change in  
278 response from less to more cohesion during daytime.

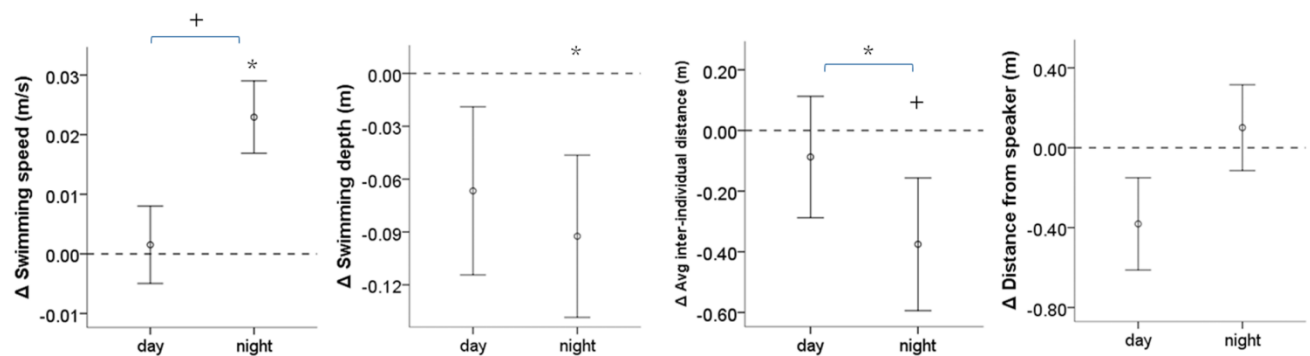
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280 Figure 4 [next page]

(a) Baseline behaviour



(b) Behavioural changes

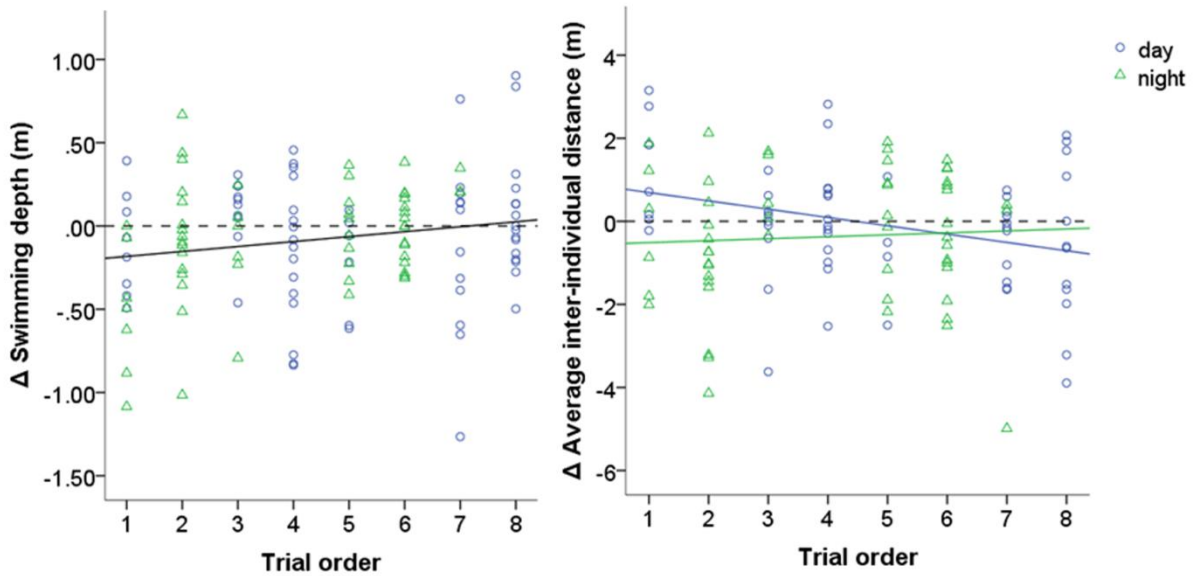


281

282 Fig. 4. (a) Baseline behaviour (mean  $\pm$  SE) during the day and during the night for swimming  
283 speed, swimming depth (from bottom), average inter-individual distance and distance from  
284 the speaker. (b) Behavioural changes from before to the start of sound exposure during the  
285 day and during the night. An asterisk (\*) denotes a significant difference ( $P \leq 0.05$ ) and a  
286 plus (+) denotes a non-significant trend ( $0.05 < P \leq 0.1$ ). The symbol between the bars  
287 indicates a difference between day and night, and the symbol above the bars indicates a  
288 difference from zero.

289

290 Figure 5 [next page]



291

292 Fig. 5. Change in swimming depth (left) and average inter-individual distance (right)  
 293 throughout the series of eight trials. The change in swimming depth diminishes with  
 294 subsequent trials, indicating inter-trial habituation. The influence of trial order on the change  
 295 in group cohesion is different between day and night.

296

## 297 Discussion

298 We showed significant variation in swimming patterns throughout the diurnal cycle of  
 299 European seabass in semi-captive conditions in an outdoor floating pen. Comparing baseline  
 300 behaviour at night to during the day, the fish swim significantly slower and in a looser shoal,  
 301 and also tended to stay nearer to the surface (non-significant trend). When exposed to sound,  
 302 the fish increased their swimming speed, swimming depth and group cohesion. These  
 303 changes were stronger at night (significant for speed and depth and a non-significant trend for  
 304 group cohesion). Additionally, the observed changes in swimming depth gradually reduced  
 305 for subsequent sound exposures, indicating inter-trial habituation.

306

307 STRONGER RESPONSE AT NIGHT

308 The European seabass in our study were spatially restricted by the floating pen and relatively  
309 shallow water but showed clear diurnal swimming patterns. Such daily behavioural rhythms  
310 have also been shown in free-ranging dusky grouper (*Epinephelus marginatus*) and yellow  
311 fin tuna (*Thunnus albacares*), where the fish swam closer to the surface at night (Mitsunaga  
312 *et al.*, 2013; Koeck *et al.*, 2014) or in sprat (*Sprattus sprattus*), who form dense schools during  
313 the day and disperse during the night (Hawkins *et al.*, 2012). This daily rhythmicity in  
314 movement is possibly driven by diel cycles in hormones and metabolites (Kühn *et al.*, 1986;  
315 Pavlidis *et al.*, 1999; De Pedro *et al.*, 2005; Polakof *et al.*, 2007). For example, our study  
316 species, the European seabass, has been shown to have significant daily variation in plasma  
317 glucose, insulin and cortisol (Planas *et al.*, 1990; Cerdá-Reverter *et al.*, 1998). The daily  
318 peaks of these parameters depend on whether the species is diurnal or nocturnal. Diurnal  
319 species typically produce most cortisol at the start of the day, while nocturnal species at the  
320 start of the night (Montoya *et al.*, 2010; Oliveira *et al.*, 2013; Vera *et al.*, 2014).

321         Upon sound exposure, European seabass in our study showed stronger behavioural  
322 changes at night compared to during the day. The influence of the time of the day on stress  
323 response during exposure to some external stimulus has been shown in three nocturnal fishes  
324 (Lankford *et al.*, 2003; López-Olmeda *et al.*, 2013; Vera *et al.*, 2014). Two of the species  
325 showed stronger cortisol increase at night and one during the day in response to experimental  
326 exposure to air (taking fish out of the water), suggesting that daily variation in sensitivity to  
327 stressors is species-specific. The mechanism of such differential sensitivity is still unknown,  
328 although it may be related to potential daily rhythms in the sensitivity of the associated  
329 endocrine glands (Engeland & Arnhold, 2005; Dickmeis, 2009). The response to sound  
330 exposure during the day was particularly small compared to a previous experiment conducted  
331 before the current experiment using the same setup on the same animals. In the previous



332 experiment, the fish were exposed to a series of four sound treatments varying in their  
333 temporal structure (one of the sound treatments was re-used in the current study), which took  
334 place during the day over a two-day period (Neo *et al.*, 2016). This prior experience may  
335 have induced anticipation in the fish to the ensuing sound exposure in the current study,  
336 yielding lower response levels, especially during the day. Nevertheless, the fish still  
337 responded strongly to sound exposure at night, potentially because they were woken up from  
338 their resting or sleep-like state (Zhdanova, 2006, 2011). Such disruption can be particularly  
339 harmful to the fish as it may affect their daily activities. For example, when subjected to  
340 unpredictable and chronic exposure to stressors at night compared to during the day,  
341 zebrafish (*Danio rerio*) learned less well in an inhibitory avoidance task (Manuel *et al.*,  
342 2014).

343         Despite low response levels during the day, our observations suggest that sound  
344 exposure at night may have more impact on European seabass than during daytime. However,  
345 application of these findings with regard to managing anthropogenic marine activities  
346 requires careful consideration, as some species within an affected area may actually be more  
347 sensitive to stress during the day (López-Olmeda *et al.*, 2013). Also, care should be taken  
348 when extrapolating results from hatchery-reared fish in a constrained set-up to wild free-  
349 ranging fish. Nonetheless, our findings suggest that the responsiveness of fish to sound  
350 exposure may be affected by the natural rhythms in physiology as well as the environmental  
351 contexts. Consequently, such factors should also be considered when evaluating potential  
352 impacts of noisy offshore activities.

353

354 INTER-SESSION HABITUATION

355 European seabass not only habituate to sound exposure within a session, as shown in  
356 previous experiments (Neo et al., 2014, 2015, 2016), they also habituated over subsequent  
357 exposures, as shown in the current study. Such inter-trial reduction in behavioural response  
358 has also been reported for the coral reef fish, threespot dascyllus. Its hiding behaviour during  
359 boat noise diminished during a two-week period with repeated playback of boat noise. This  
360 reduced behavioural response was in line with diminished elevated ventilation rates  
361 (opercular beat) after one and two weeks (Nedelec *et al.*, 2016). Other relatively long-term  
362 studies that looked into physiological measures showed similar results. Post-larval European  
363 seabass, that had been exposed to impulsive sound for 12 weeks, no longer showed elevated  
364 ventilation rates upon exposure of the same noise type (Radford *et al.*, 2016). In a split-brood  
365 experiment using larval Atlantic cod, two days of noise treatment reduced growth whereas  
366 the growth had converged again at the end of the experiment which lasted for 16 days  
367 (Nedelec *et al.*, 2015).

368         In the current study, the European seabass reduced the change in swimming depth at  
369 the onset of sound exposure. Compared to the intra-trial habituation of earlier studies (Neo et  
370 al. 2014, 2015, 2016), the inter-trial habituation was less prominent. For example, inter-trial  
371 habituation only occurred with swimming depth, but not for the other test parameters. The  
372 lack of inter-trial habituation in other parameters suggests that the fish may not have  
373 completely habituated to repeated exposures. However, it can also be explained by the more  
374 variable nature of these responses. Furthermore, the behaviour of the fish was constrained by  
375 the floating pen set-up and absolute levels or the nature of behavioural changes in our study  
376 should not be taken to extrapolate to the outside world. Nevertheless, relative differences  
377 with context (day and night) or variation among subsequent exposures provide conceptual  
378 insights and can be considered a proof of principle.

379           It is debatable whether habituation is necessarily beneficial to the fish under sound  
380 exposure (Bejder *et al.*, 2009). On the one hand, habituation may reduce spatial and  
381 distributional changes, which is critical when a site is crucial for foraging or spawning. On  
382 the other hand, habituation may also cause fish to stay within an affected area, while still  
383 causing physiological stress (Anderson *et al.*, 2011; Filiciotto *et al.*, 2013), auditory masking  
384 (Vasconcelos *et al.*, 2007) and attentional shifts (Purser & Radford, 2011; Simpson *et al.*,  
385 2014; Shafiei Sabet *et al.*, 2015). Hence, more insights into the consequences of fish  
386 habituation to repeated sound exposures (Davis, 1970; Chanin *et al.*, 2012; Neo *et al.*, 2015)  
387 and specific features such as interval regularity of repeated trials (Nedelec *et al.*, 2015;  
388 Shafiei Sabet *et al.*, 2015; current study), are critical for valid impact assessments.

389

## 390 CONCLUSION

391 Our study showed that European seabass responded more strongly to sound exposure at night  
392 and that they habituated to repeated exposures. These findings demonstrate that  
393 environmental context and exposure experience may modulate sound impact on fish due to  
394 noisy human activities. Consequently, mitigation efforts aiming at minimising sound impact  
395 should take these factors into account when devising pile-driving or seismic survey  
396 operations. Our study did not aim at assessing absolute thresholds to extrapolate to real-world  
397 conditions, but the natural water body conditions and the relatively large swimming area in  
398 the floating pen provide fundamental insights and may help in predicting variation in  
399 potential for sound impact between day and night and between brief and long-term or  
400 repeated exposure conditions. However, studies on free-ranging fish and exposure conditions  
401 in deeper water are needed to gain critical knowledge for impact assessments and potential  
402 for mitigation.

403

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