



Contribution to Special Issue: 'Towards a Broader Perspective on Ocean Acidification Research Part 2'

Original Article

Combined impacts of elevated CO₂ and anthropogenic noise on European sea bass (*Dicentrarchus labrax*)

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Ocean acidification (OA) and anthropogenic noise are both known to cause stress and induce physiological and behavioural changes in fish, with consequences for fitness. OA is also predicted to reduce the ocean's capacity to absorb low-frequency sounds produced by human activity. Consequently, anthropogenic noise could propagate further under an increasingly acidic ocean. For the first time, this study investigated the independent and combined impacts of elevated carbon dioxide (CO₂) and anthropogenic noise on the behaviour of a marine fish, the European sea bass (*Dicentrarchus labrax*). In a fully factorial experiment crossing two CO₂ levels (current day and elevated) with two noise conditions (ambient and pile driving), *D. labrax* were exposed to four CO₂/noise treatment combinations: 400 μatm/ambient, 1000 μatm/ambient, 400 μatm/pile-driving, and 1000 μatm/pile-driving. Pile-driving noise increased ventilation rate (indicating stress) compared with ambient noise conditions. Elevated CO₂ did not alter the ventilation rate response to noise. Furthermore, there was no interaction effect between elevated CO₂ and pile-driving noise, suggesting that OA is unlikely to influence startle or ventilatory responses of fish to anthropogenic noise. However, effective management of anthropogenic noise could reduce fish stress, which may improve resilience to future stressors.

Keywords: elevated CO₂, pile-driving, sensory behaviour, ventilation.

Introduction

Humans depend on the oceans for the goods and ecosystem services they provide, but with global population rise humans are increasingly altering the oceans, with impacts on marine organisms and ecosystems (Vitousek *et al.*, 1997; Halpern *et al.*, 2008). To date, most studies have focused on the effects of a single stressor, yet ecosystems are usually affected by multiple stressors (Breitburg *et al.*, 1998; Crain *et al.*, 2008; Halpern *et al.*, 2008; Ling *et al.*, 2009). This study investigated the combined impacts of two recently acknowledged environmental changes: ocean acidification (OA) and anthropogenic noise (IPCC, 2007; Slabbekoorn *et al.*, 2010; IGBP, IOC, SCOR, 2013).

OA results from atmospheric carbon dioxide (CO₂), primarily released during combustion of fossil fuels, dissolving into the ocean (Orr *et al.*, 2005; Denman *et al.*, 2011; IPCC, 2014). High CO₂ levels and reduced pH affect behaviour and physiology in a range of marine taxa including fish (Fabry *et al.*, 2008; Kroeker

et al., 2010; Hurst *et al.*, 2013). For example, clownfish larvae become highly attracted to predator odours (*Amphiprion percula*; Dixon *et al.*, 2010), while damselfish exhibit bolder behaviour (*Pomacentrus wardi*; Munday *et al.*, 2010) and lose typical lateralization behaviour (*Neopomacentrus azysron*; Domenici *et al.*, 2012). OA-induced disruptions to neurotransmitter function appear to underpin these behavioural changes (Nilsson *et al.*, 2012).

Anthropogenic noise in aquatic environments, including that produced by shipping, seismic surveys, and pile-driving, is also increasing (Bailey *et al.*, 2010; Slabbekoorn *et al.*, 2010; Normandeau Associates, Inc., 2012; Simpson *et al.*, 2015). Since this noise generally overlaps with the hearing ranges of marine taxa, a variety of effects have been documented (Slabbekoorn *et al.*, 2010; Simpson *et al.*, 2015). Increasing interest in offshore renewable energy has stimulated the rapid growth of offshore windfarms, with pile-driving noise being a dominant feature during construction (Gill, 2005; Bailey *et al.*, 2010; Normandeau Associates, Inc., 2012; Bruintjes

et al., 2014). Pile-driving sounds cause freezing reactions in cod (*Gadus morhua*), increases in swimming speed in sole (*Solea solea*; Mueller-Blenkle *et al.*, 2010) and greater oxygen consumption in European sea bass (*Dicentrarchus labrax*; Bruintjes *et al.*, 2014). However, research into its effects on fish remains limited and presents a current knowledge gap to policy-makers and marine managers (Mueller-Blenkle *et al.*, 2010).

OA is predicted to reduce the ocean's capacity to absorb low-frequency sounds produced from human activities (Ilyina *et al.*, 2010). Consequently, anthropogenic noise could propagate further under an increasingly acidic ocean. Additionally, fish otoliths, free-floating earbones in the back of the head that act as accelerometers and enable hearing, were considerably larger in white sea bass (*Atractoscion nobilis*) reared under elevated CO₂ conditions (Checkley *et al.*, 2009). Furthermore, clownfish (*Amphiprion percula*) that normally avoid daytime reef noise during the pelagic larval stage, did not respond to such noise if reared in elevated CO₂ conditions (Simpson *et al.*, 2011). An important question arising from these studies is whether OA influences fish responses to anthropogenic noise. No study has yet investigated the combined impact of OA and anthropogenic noise.

To address this knowledge gap, we investigated the independent and combined impacts of elevated CO₂ and pile-driving noise on the behaviour and physiology of the economically important European sea bass (*D. labrax*). In a crossed design, *D. labrax* were reared in control (~400 µatm) or elevated (~1000 µatm) CO₂ conditions then acutely exposed to ambient or pile-driving noise. Experimental CO₂ levels matched current ocean conditions and predictions made for the end of the century (IPCC, 2014). To establish whether impacts may carry direct fitness consequences, we assessed anti-predator responses using an established looming stimulus assay (Simpson *et al.*, 2015), and measured ventilation rate during acoustic exposure, providing an indication of stress (Simpson *et al.*, 2015).

Previous work that has found ventilation rate in *D. labrax* is considerably higher during playback of pile-driving noise (Bruintjes *et al.*, 2016), a result also seen in the European eel (*Anguilla anguilla*) during playback of ship noise (Simpson *et al.*, 2015), whereas chronic elevated CO₂ exposure previously had no effect on ventilation rate in eels (McKenzie *et al.*, 2003). Therefore, we hypothesized that pile-driving noise would increase ventilation rate, while elevated CO₂ would not. Exposure to high CO₂ levels can impair *D. labrax* sensory response to various smells (Porteus *et al.*, unpublished data), and pile-driving noise can impair *D. labrax* startle response to simulated predator strikes (Everley *et al.*, 2015). Therefore, we further hypothesized that elevated CO₂ or pile-driving noise in isolation, would decrease the number of fish that startle. Previous work investigating multi-stressor impacts have reported interacting effects (Rosa and Seibel, 2008; Munday *et al.*, 2009; Nowicki *et al.*, 2012). Thus, we also tested for an interaction between pile-driving noise and OA to examine whether responses differ when stressors are combined.

Material and methods

Ethics statement

All experimental procedures were approved by the University of Exeter Ethics Committee (2013/247), and were deemed by the Home Office as being below the level of severity that would require licensing, although appropriate licences were in place (PPL 30/2860).

Table 1. Water chemistry parameters for control and elevated CO₂ treatments.

Parameter	Control CO ₂	Elevated CO ₂
pH _{NBS}	8.12 ± 0.004	7.82 ± 0.003
Temperature (°C)	15.3 ± 0.02	15.3 ± 0.02
Salinity (ppt)	35.8 ± 0.04	35.9 ± 0.03
TA (µmol/kgSW)	2407.6 ± 9.1	2377.8 ± 10.2
pCO ₂ (µatm)	471.7 ± 7.4	1032.0 ± 12.8

pH_{NBS}, temperature and salinity data are direct measurements. pCO₂ and TA were calculated from these parameters in CO2SYS. Data are presented as mean ± SE.

CO₂ treatments

Juvenile European sea bass (*D. labrax*; 4–8 g, 7 cm total length TL) were obtained from Ecloserie Marines de Gravelines, France and initially held within a 130 l plastic stock tank (76 × 56 × 30 cm) containing recirculating artificial seawater (15°C) in the University of Exeter Aquatic Resource Centre. Before testing, 48 fish were exposed to seawater at current day CO₂ (~400 µatm) and a further 48 to elevated CO₂ (~1000 µatm) levels for 20–21 d. Each CO₂ treatment consisted of recirculated artificial seawater held in 20 l containers (six fish per container) supplied with seawater at 15.3°C ± 0.02 (SE) at a flow of 1.7 l/min from one of two 100 l header tanks. Photoperiod was controlled (12:12 light:dark) and fish were fed once daily with 1% body mass of Perla MP pellets (Skretting, Shay Lane, Longridge, Preston, UK).

In a recirculating system (450 l), seawater diffused with ambient air scrubbed of CO₂ using Soda lime (Sigma; control), thus offsetting CO₂ produced by fish, or air enriched with CO₂ (elevated) was bubbled into containers. A pH Computer (Aqua Medic, Bissendorf, Germany), calibrated to a set pH and linked to a solenoid valve, controlled the delivery of CO₂ gas (BOC, England, UK) to the elevated CO₂ header tank to maintain a pH of 7.82 ± 0.02. Temperature, salinity, and pH_{NBS} were measured daily using a salinity, conductivity, and temperature system (YSI Model 30, YSI incorporated, Yellow Springs, Ohio, USA), pH meter (Model HI 8314, Hanna Instruments, Leighton Buzzard, UK) and pH probe (Model pHC2401, Radiometer Analytical, Lyon, France) calibrated with National Bureau of Standards (NBS) buffers. Ammonia was monitored weekly using a test kit (TetraTest Ammonia kit, Ark Pets, UK), with water changes every 7–10 d to avoid build up. Seawater was sampled from each container 2–3 times per week, poisoned with mercuric chloride (HgCl₂) according to standard methods and stored at 4°C (following Hurst *et al.*, 2013). Seawater samples were analysed for dissolved inorganic carbon (DIC) using a custom built system (described in Lewis *et al.*, 2013). This allowed a ± 3 µmol/kg precision for DIC measurements. Measured temperature, salinity, pH, and DIC values were used to calculate average seawater pCO₂ and total alkalinity (TA) using the CO2SYS software (<http://cdiac.ornl.gov/oceans/co2rprt.html>) using the GEOSECS constants (NBS scale; Takahashi *et al.*, 1982; Table 1). In preliminary experiments, seawater temperature, oxygen, and CO₂ levels in experimental tanks (see below) were measured over a 35-min period, ensuring no significant deviations in any of these parameters.

General experimental design

The experimental set up comprised two 3 l plastic experimental tanks (20 × 12 × 13 cm, >90% acoustically transparent) filled with water from the CO₂ exposure set up appropriate to each

test fish. Each 3 l tank floated within two larger 90 l glass tanks (54 × 44 × 38 cm) filled with standard seawater in a temperature controlled room (15.6 ± 1°C). Each large tank rested on 5 cm expanded polystyrene and six pieces of evenly distributed plastic pipe insulation to minimize transfer of vibrations from the building. An opaque divider was placed between the two tanks to avoid disruption of ongoing trials or visual interaction between fish during experiments. Recordings were initially made in both tanks to ensure there was no noise transfer between tanks (details of recording equipment below). Playback tracks were MP3 files played through a sound system (identical for both tanks) consisting of a battery (12 V 7.2 Ah sealed lead-acid), MP3 player (PCM-M10, Sony Corp., Japan), amplifier (M033N, 18W, frequency response 0.04–20 kHz; Kemo Electronic, Germany), and underwater speaker (University Sound UW-30; maximal output 156 dB re 1 µPa at 1 m, frequency response 0.1–10 kHz; Lubell Labs, USA). Speakers were positioned facing upwards to the floating experimental tanks and hidden under a false bottom of drilled Perspex.

A fully factorial experiment crossing two CO₂ levels (~400 and ~1000 µatm) with two acoustic conditions (playback of ambient and pile-driving recordings) was used (2 × 2 design). Twenty-four fish were tested in each of the four different CO₂/noise treatment combinations: 400 µatm/ambient (hereafter 400/amb), 1000 µatm/ambient (1000/amb), 400 µatm/pile-driving (400/pile), and 1000 µatm/pile-driving (1000/pile). Using an independent samples design to avoid carry over effects, each fish was tested once in each experiment. For each trial, an individual fish was transferred by scoop into an experimental tank with the relevant CO₂ treatment water. Similar to Simpson *et al.* (2015), each trial included 30 min ambient playback allowing fish to acclimatize, using one of three different ambient tracks, followed by a change to 5 min of another ambient or pile-driving track (see Playback tracks). The CO₂/noise treatment combinations were randomly allocated to each tank, controlling for any tank effect or bias due to the room layout. Treatment order was randomized and counterbalanced between days and in three blocks of four on each day, controlling for any daily variation and time of day effects. After each trial, the test fish was placed in a different holding tank and the water in experimental tanks was changed.

Playback tracks

Thirty-five minutes playback tracks were produced in Audacity 2.0.6 (<http://audacity.sourceforge.net/>) using sounds recorded at four UK ports: Gravesend (51°26'42"N, 0°22'37"E), Plymouth (50°21'33"N, 4°7'26"W), Portsmouth (50°47'21"N, 1°6'25"W), and Newcastle (55°7'46"N, 1°30'24"W) (further details in Wale *et al.*, 2013a, b; Voellmy *et al.*, 2014a, b; Simpson *et al.*, 2015). Field recordings of pile-driving noise, at ca. 120 m distance from the source (55°8'46"N, 1°25'15"W; Narec, Blyth, Newcastle), and ambient harbour noise were taken. Six different ambient noise files (3 × 30 min and 3 × 5 min) and three different pile-driving noise files (all 5 min) were made by looping the sound tracks together (following Voellmy *et al.*, 2014b). Recordings were taken in the centre of the 3 l experimental tanks, in the experimental set up described above, using a hydrophone (HiTech HTI-96-MIN with inbuilt preamplifier, sensitivity -165 dB re 1 V/µPa, frequency range 0.02–30 kHz; High Tech Inc., USA) and calibrated recorder (PCM-M10, 44.1 kHz sampling rate; Sony Corp., Japan).

Before the experiments, Avisoft SASLab Pro (v5.2.07; Avisoft Bioacoustics) was used to analyse the sound pressure level [SPL, root mean square (RMS), full spectra] and power spectra of

recordings when played back in experimental tanks (Figure 1a; following Simpson *et al.*, 2015). The SPL of the ambient tracks was averaged over 30 s recordings (similar to Bailey *et al.*, 2010) and SPL of pile-driving tracks calculated as the average peak pulse level for 21 piling strikes (pulse length = 100 ms, averaging window 10 ms) in a 30 s recording (similar to Everley *et al.*, 2015). The three ambient tracks were played at 127.2 ± 0.03 dB RMS re 1 µPa, while the peak pulse level of the three pile-driving tracks was 161.3 ± 0.05 dB RMS re 1 µPa in experimental tanks. A spherical model of sound propagation predicts that the pile-driving sound levels in the experimental tank were similar in the pressure domain to field conditions at 200 m from the source, while a cylindrical model predicts a distance of several kilometres, although local reflections and scattering would become a factor (Au and Hastings, 2008). These levels could affect a large number of fish in the wild, thus it is important to monitor impacts of noise levels corresponding to these larger spatial scales (Simpson *et al.*, 2015).

Ventilation rate

Fish were tested one at a time. Following Simpson *et al.* (2015), an observer hidden from the fish by a screen measured ventilation rate (opercular beat rate) through a small hole using a counter and stopwatch. The number of opercular beats in 1 min was counted at 27 min from the start of the trial (during ambient noise) then following the change to either another ambient or a pile-driving track. These measurements were used to calculate change in ventilation rate. If opercular beats were not visible, counting was stopped and resumed when they could be seen again, ensuring 1 min of beats was measured for each individual in each test period. Some ventilation measurements were double checked against the video recordings to make sure there was no observer bias and no discrepancies were found.

Startle response

A predator attack was simulated by a looming stimulus (following Simpson *et al.*, 2015). The stimulus was a black squash ball fixed to the end of a swinging transparent Perspex pendulum arm. Fishing line, attached to the pendulum arm, positioned the stimulus at a 45° angle beyond one end of each tank. Once released the squash ball ended up next to the glass but without making contact, due to a foam stopper positioned near to the hinge of the swinging arm on the ceiling. The stimulus was manually released by an observer (through releasing the taut fishing line hooked around a clamp) at 3 min after the track change, thereby standardizing trials. Behaviour was recorded from 27 min until the trial ended using a video camera (Sony HDR-XR155, 25 frames per second) supported by a tripod and pointing through a hole in the screen. A startle was only scored if it was elicited during the first advance of the ball towards the tank. The trial number was visible in the film allowing subsequent subject identification. Videos were analysed without sound (avoiding unintentional observer effects) fish were scored (one per trial) on whether they startled or not in response to the looming stimulus (startle response).

A few trials (1 or 2 per treatment) were omitted from statistical analysis due to problems during the trial, such as accidental stimulus release. Consequently, sample sizes differed slightly between treatment combinations and variables measured.

Data analysis

Statistical analyses were conducted in SigmaPlot (version 10.0) and SPSS (IBM SPSS Statistics, IBM Corporation, Version 22).

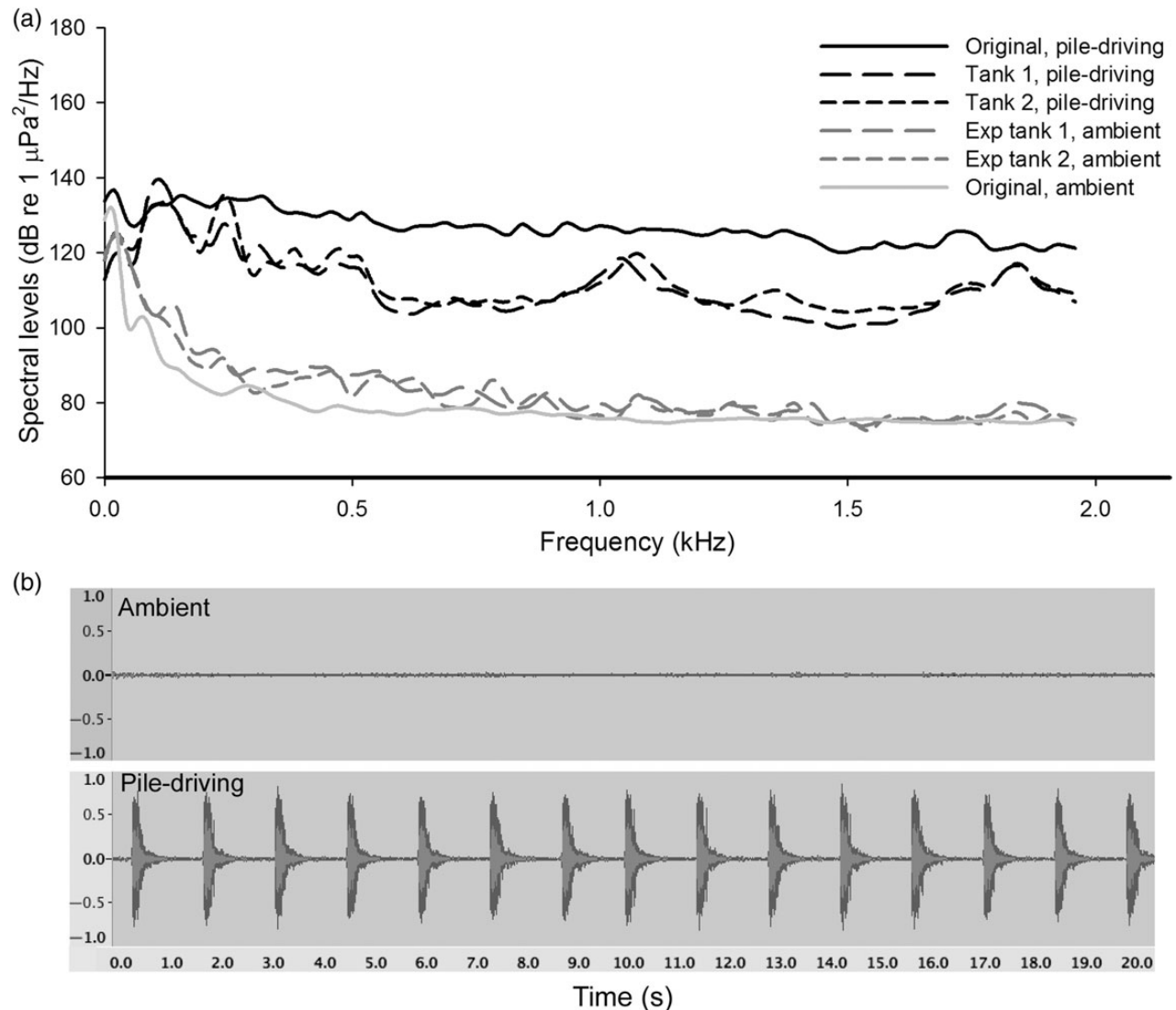


Figure 1. (a) Power spectra for recordings taken in the field and experimental tanks. Analysis conducted in Avisoft SASLab Pro version 5.2.08 (Avisoft Bioacoustics, Berlin, Germany): Hann evaluation window, Fast Fourier Transform size 2048, averaged from a 30 s section from each recording. One recording from each noise treatment is shown for comparison. (b) Example of waveforms of ambient and pile-driving playbacks, 20 s long.

A two-way ANOVA was used to test for differences between CO₂ and noise treatments on the change in ventilation rate before and after the track change. A χ^2 test was used to identify differences in the number of fish that startled across treatment combinations.

Results

Ventilation rate

There was a significant difference in the change in ventilation rate after the track change between noise treatments (two-way repeated measures ANOVA, $F_{1, 88} = 54.854$, n_{amb} , $n = 46$, $p < 0.001$; Figure 2a) but not between CO₂ conditions (two-way repeated measures ANOVA, $F_{1, 88} = 1.342$, $n = 46$, $p = 0.250$; Figure 2a). Thus, there was no interaction. Specifically, ventilation rate increased by 9 beats min⁻¹ (6%) when an ambient track was switched for a pile-driving track in control CO₂ conditions (Figure 2a).

Startle response

The number of fish producing a startle response was not significantly different between the CO₂/noise treatment combinations (χ^2 test, $\chi^2 = 3.892$, $n = 23$, $p = 0.273$), despite 67% more fish startling when exposed to 1000 μatm/pile-driving noise compared with 400 μatm/ambient noise (Figure 2b).

Discussion

In this study, fish ventilation rate significantly increased during playback of pile-driving noise when compared with ventilation rate during ambient noise, supporting the hypothesis that pile-driving noise increases stress. This is consistent with elevated ventilation rate in European eels (Simpson *et al.*, 2015) and greater cortisol levels in three fish species during exposure to playback of ship noise (Wysocki *et al.*, 2006). Furthermore, the ventilation rate of fish exposed to elevated CO₂ was not significantly different to that of control fish, indicating that elevated CO₂ does not have an

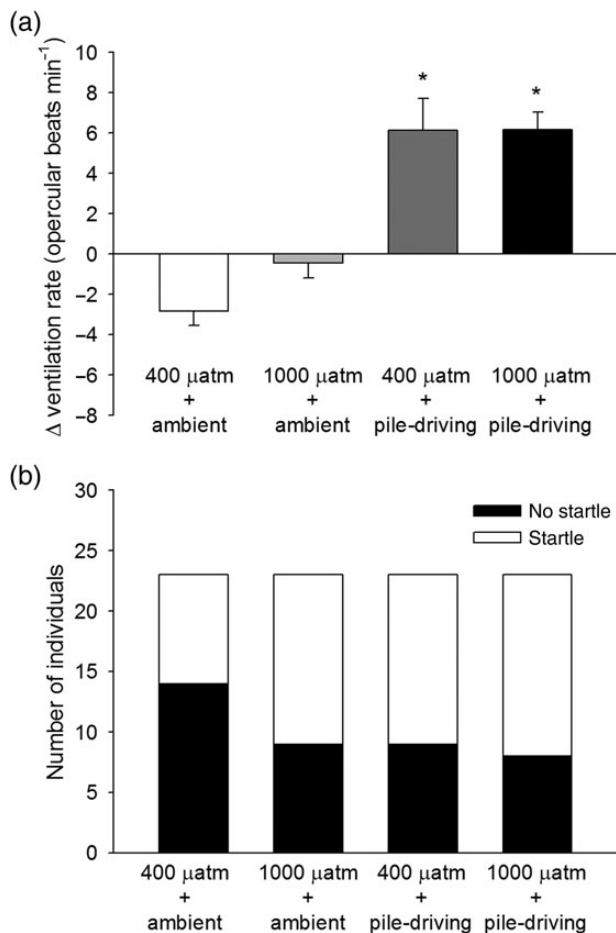


Figure 2. The effect of CO₂ and noise treatments on ventilation rate, and startle response of European sea bass (*D. labrax*). (a) Change in ventilation rate before and after the track change; $n = 23$ for each treatment combination. (b) Number of fish startling or not startling in response to a looming stimulus; $n = 23$ for each treatment combination. Data are presented as mean \pm SE and asterisks above the bars denote significant differences from controls.

additional impact on the ventilatory response of sea bass. This study used acute noise exposures, but long-term stress can negatively impact reproduction and growth (Kight and Swaddle, 2011; Nedelec et al., 2014). Furthermore, behavioural habituation of European sea bass can happen within an hour of exposure to pile-driving noise (Neo et al., 2014). Further work should examine whether elevated CO₂ can affect the habituation of marine fish to noise and how repetitive or chronic exposure to anthropogenic noise affect fish behaviour.

Some fish respond to a predator by freezing; however, with our low-resolution video frame rates (25 fps), we were not able to quantify this response and thus a potential freeze response could not be analysed. Despite this, more fish tended to startle in elevated CO₂ than control CO₂ but this was also not statistically significant. Previous work reported that high CO₂ exposure caused changes to the functioning of gamma-aminobutyric acid type A neurotransmitter receptors, leading to altered behaviours (Nilsson et al., 2012; Chivers et al., 2014; Hamilton et al., 2014; Spady et al., 2014), such as the reversal of olfactory preferences in clownfish (*Amphiprion percula*) larvae (Nilsson et al., 2012). Our initial findings, that warrant additional

investigation, suggest that elevated CO₂ has the potential to increase fish responsiveness to predators, potentially improving their chances of escape through reacting sooner to a threat stimulus. Guppies that reacted earlier to a predator model have a reduced predation risk (Krause and Godin, 1996; Voellmy et al., 2014b). The results suggest that OA could have positive effects on the anti-predator behaviour of *D. labrax*. Future research could determine whether the observed effects are temporary or sustained in the long term (i.e. whole lifespan) and if other processes, such as growth, are compromised. These findings contrast with previous OA studies, demonstrating negative impacts on anti-predator behaviour in damselfish (*Pomacentrus chrysurus*; Ferrari et al., 2011) and conch snails (*Gibberulus gibberulus gibbosus*; Watson et al., 2014).

There was no interaction effect between elevated CO₂ and pile-driving noise on anti-predator behaviour or ventilation rate. This may be because fish that had been exposed to noise had potentially reached a ceiling effect, as there were no significant differences in anti-predator responses or ventilation rate between CO₂ treatments following the addition of pile-driving noise (Hamilton et al., 2014). This is the first study to show that OA is unlikely to influence the anti-predator behaviour and physiological responses of fish to anthropogenic noise. Pile-driving noise increased fish stress, with possible fitness consequences, indicating that effective management of anthropogenic noise will likely reduce stress, especially as oceans further acidify (Denman et al., 2011; Brown et al., 2013; IPCC, 2014; Simpson et al., 2015). However, the value of reducing a present-day stressor to improve the resilience of animals to future stressors should be explored (Pandolfi et al., 2005; Barber et al., 2010; Brown et al., 2013; Simpson et al., 2015). As a recommendation, this question should be considered in policy and management decisions moving further into the 21st century.

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