

1 **Elevated CO₂ does not alter behavioural lateralization in free-swimming**
2 **juvenile European sea bass (*Dicentrarchus labrax*) tested in groups**

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22 **ABSTRACT**

23 We investigated left-right turning preferences of N=260 juvenile European sea bass
24 (*Dicentrarchus labrax*) reared in either: ambient conditions; ocean acidification (OA)
25 conditions; or reared in ambient conditions but tested in OA water. Groups of 10 individuals
26 were observed alone in a circular tank and individuals' left and right turning during free-
27 swimming were quantified using trajectory data from video. We show that near future OA
28 levels does not affect the number of turns made, or behavioural lateralization (turning
29 preference), in juvenile *D. labrax* tested in groups.

30

31 **KEYWORDS:** *Dicentrarchus labrax*, environmental change, fisheries, laterality, motion
32 tracking, turning

33 Atmospheric CO₂ concentration is predicted to reach between 750 and 1000 ppm by the end of
34 this century, driving ocean acidification (OA) and creating concern for many marine organisms
35 and ecosystems (Pachauri & Meyer, 2014). Experimental work in the laboratory has shown
36 future OA conditions can severely effect fish behaviour, causing attraction to sound or
37 olfactory cues that would normally be avoided (e.g. Dixon *et al.*, 2010; Munday *et al.*, 2009;
38 Simpson *et al.*, 2011) and a loss of lateralisation (a left or right side preference) during
39 behavioural activities (e.g. Domenici *et al.*, 2012). Other work has shown no effect of OA on
40 fish behaviour (e.g. Clark *et al.*, 2020). Differences in species and context studied, and
41 methodology used, may explain these different findings (Munday *et al.*, 2020; Williamson *et*
42 *al.*, 2021).

43

44 The most frequently used method for investigating behavioural lateralisation is a “detour test”
45 where individual fish are presented with right or left travel routes in T-shaped mazes or choice
46 chambers, providing data on individuals’ eye preferences and/or exploration of cues in their
47 environment (e.g. Clark *et al.*, 2020; Domenici *et al.*, 2012; Jutfelt *et al.*, 2013; Lopes *et al.*,
48 2016; Vossen *et al.*, 2016). However, lateralization as measured by detour tests are not always
49 consistent within individuals across time (Roche *et al.*, 2020), and can differ by sex and
50 behavioural context (McLean & Morrell, 2020, 2021). Studies have also measured laterality of
51 individuals’ during free-swimming by recording “spontaneous turns”, normally by viewing
52 video recordings of fish and scoring the proportion of right turns made (e.g. Dadda *et al.* 2012).
53 Here, we test potential effects of future OA on juvenile European sea bass (*Dicentrarchus*
54 *labrax*) left-right turning preferences whilst free-swimming (“spontaneous turns”) in a group,
55 using tracking from video. Using video recordings to explore turning behaviour reduces the
56 risks of observer bias and observer presence affecting behaviour (Rossi *et al.*, 2015); allows
57 for testing in more ecologically relevant contexts (Duteil *et al.*, 2016; Queirós *et al.*, 2015), and

58 has been used to test for potential effects of OA on other aspects of fish behaviour (Duteil *et*
59 *al.*, 2016; Zakroff *et al.*, 2018; Hamilton *et al.*, 2014).

60

61 Juvenile European Sea bass (*Dicentrarchus labrax*) (n=260) were raised in water at 585 μ atm
62 p CO₂ (simulating ambient conditions) or at 1000 μ atm p CO₂ (simulating near-future OA
63 conditions). Fish were tested in groups of 10 fish that were either: reared under ambient
64 conditions (n=9 groups, n=90 fish), reared in OA conditions (n=9 groups, n=90 fish), or reared
65 in ambient conditions but tested in OA water (n=8 groups, n=80 fish). Full details of rearing
66 conditions and experiments are provided in Pope *et al.*, (2014), Duteil *et al.*, (2016) and the
67 supplementary material. Each group was filmed for 1 h in a circular test arena by a Panasonic
68 HDC-SD60 HD video camera positioned directly above the arena (lit by four photographer's
69 lights to dispense light evenly). Fish position was tracked from video recordings at 25 Hz using
70 idTracker (Digital Wave LTD, 2014; Pérez-Escudero *et al.*, 2014) and processed in Matlab
71 (MATLAB, 2014) (Figure 1a). Post-processing eliminated 0.1% of data points that were
72 considered as erroneous (see Duteil *et al.*, (2016) for full details). Using these fish trajectory
73 data, we considered times when fish were moving (>0.7 cm/s: Duteil *et al.*, 2016) and
74 calculated turns (change in directional heading each second) using the 'atan2d' function in
75 Matlab (MATLAB, 2014). Because the atan2d function calculates the directional heading
76 change between two points in an anti-clockwise direction first, positive angles would
77 correspond to a left turn, and negative angles a right turn. Since we wanted to capture
78 "meaningful" turns, we used all turns greater than 76 degrees which is 1 standard deviation
79 from the mean turn angle of 0, representing 30% of all turns recorded (Figure 1b). All turns
80 considered in our analyses and their direction, for each fish in each trial, are presented as
81 supplementary text files.

82

83 We calculated the relative lateralization index for each fish (n=260) based on the total number
84 of left (L) and right turns (R), as: $(R - L)/(R + L) \times 100$ (Bisazza *et al.*, 1998). To test for an
85 overall left or right bias in turning preferences (i.e., population level lateralization), t-tests (for
86 normal data) and one sample Wilcoxon tests (for non-normal data) were performed. Relative
87 lateralization scores were not significantly different from zero for fish raised and tested in
88 ambient conditions ($W = 49$, $p = 0.920$), for fish raised under ambient conditions and tested in
89 OA water ($t_{79} = 1.158$, $p = 0.250$), and for fish raised and tested in OA conditions ($t_{89} = 0.055$,
90 $p = 0.956$). However, these univariate tests do not control for individual identity, and so we
91 used a chi-square test provided by Roche *et al.* (2020) that compares observed variance in turns
92 compared to the expected variance, assuming a normal approximation to the binomial
93 distribution. This is analogous to testing for overdispersion (i.e. are there more observations in
94 the tail ends of the distribution than expected by chance). Because fish made a different number
95 of turns, we tested for lateralisation over 10, 20, and 37 turns (Vinogradov *et al.* 2021). The
96 latter number (37) is the minimum number of turns taken by a fish within a trial, allowing for
97 all fish to be considered. We found no evidence that some of the fish were lateralized across
98 the whole dataset (n=10 turns: $P=0.298$; n=20 turns: $P=0.413$; n=37 turns: $P=0.582$), or within
99 each treatment (n=10 turns: ambient conditions, $P = 0.229$; ambient conditions tested in OA
100 water, $P = 0.660$; OA conditions, $P = 0.303$; n=20 turns: ambient conditions, $P = 0.712$; ambient
101 conditions tested in OA water, $P = 0.520$; OA conditions, $P=0.137$; n=37 turns: ambient
102 conditions, $P=0.523$; ambient conditions tested in OA water, $P=0.653$; OA conditions,
103 $P=0.498$).

104

105 Linear Mixed Models (LMMs) conducted in R with the packages “lme4” and “lmerTest”
106 (Bates *et al.*, 2015; Kuznetsova *et al.*, 2017) were used to test for differences in the total number
107 of turns (LMM1) or lateralisation index (LMM2) for fish in each test condition. We fitted group

108 identity as a random effect in both models to allow for between group differences and
109 “DHARMA” package (Hartig, 2020) was used to check model residuals. Total number of turns
110 by fish raised and tested in ambient conditions was not different to the number of turns made
111 by fish raised under ambient conditions and tested in OA water (LMM1: Effect = -85.23,
112 Standard Error (SE) = 68.35, t-value = -1.25, P = 0.225; Figure 1c; Table S1) or fish raised and
113 tested in OA water (LMM1: Effect = -15.27, SE = 66.31, t-value = -0.23, P = 0.820; Figure 1c;
114 Table S1). The relative lateralization score of fish raised and tested in ambient conditions was
115 not different to the relative lateralization score for fish raised under ambient conditions and
116 tested in OA water (LMM2: Effect = 1.12, SE = 1.28, t-value = 0.87, P = 0.383; Figure 1d;
117 Table S1) or fish raised and tested in OA water (LMM2: Effect = -0.03, SE = 1.24, t-value = -
118 0.02, P = 0.982; Figure 1d; Table S1). We also repeated the above analyses for turns above 130
119 degrees (2 standard deviations) and results are unchanged (supplemental material; Figure S1).

120

121 Our analyses show that near future OA has little effect on left–right turning preference in
122 juvenile European sea bass, free-swimming in a group. Future work should now investigate the
123 applicability of free-swimming in a group as a paradigm for investigating behavioural effects,
124 such as those attributed to high pCO₂. We discuss three issues regarding applicability, below.

125

126 First, fish in our study were hatched and raised from eggs in controlled laboratory conditions
127 with good welfare and survival (Pope *et al.*, 2014); this can be assumed to represent a ‘low
128 risk’ environment. Because laterality measured by detour tests can be impacted by how ‘risky’
129 the test subjects natural environment is (Brown *et al.*, 2004; Ferrari *et al.*, 2015) and ontogeny
130 (Roche *et al.*, 2020; Sundin & Jutfelt, 2016), our set-up may dampen ontogeny or selection for
131 behavioural lateralization (Brown *et al.*, 2004; Ferrari *et al.*, 2015). For example, Brown *et al.*
132 (Brown *et al.*, 2004) found that poeciliid fish (*Brachyrhaphis episcopi*) from high predation

133 regions, but not those from low predation regions, exhibited laterality when inspecting a
134 predator (Brown *et al.*, 2004). Similarly, wild-caught juvenile whitetail damselfish
135 (*Pomacentrus chrysurus*) from high-risk environments were found to be more strongly
136 lateralized than those caught in low-risk environments (Ferrari *et al.*, 2015). Whilst measures
137 of sensory laterality (detour tests) and motor laterality during free swimming (tested here) may
138 not be linked (Dadda *et al.* 2012), it will still be instructive to studying laterality in free-
139 swimming fish reared under different levels of environmental risk and throughout
140 development/lifespans.

141

142 A second and related point is the ‘lack’ of context when studying laterality during free-
143 swimming (within the confines of a simple test environment). Whilst laterality in free-
144 swimming is important for group coordination and predator escape, fish may be unlikely to
145 express turning bias/preferences in ‘safe’ contexts. In contrast, the detour test is designed to
146 elicit a turn response, where fish choose between a control and cue, e.g. a conspecific behind a
147 barrier (Santacà *et al.*, 2019), or a novel object resembling a predator (Roche *et al.*, 2020). In
148 some studies fish are additionally ‘coaxed’ using a small plastic rod to make a choice in such
149 designs (Jarrold & Munday, 2018). Further work is therefore required to understand the causes
150 and consequences laterality in free-swimming and the predicted impact of OA on this.
151 Nevertheless, the method we used here allowed us to assess hundreds of turns for each fish,
152 using different turn criteria, limiting the chance of spurious results (Halsey *et al.*, 2015; Roche
153 *et al.*, 2020).

154

155 Finally, whilst we tested a sociable fish species in an ecologically relevant context – free-
156 swimming in groups – this may have masked any potential OA effects on individual behaviour.
157 For example, fish show decreased variability in their speed when in groups compared to when

158 alone (Herbert-Read *et al.*, 2013), and group-level conformity effects can reduce individual
159 variation in movement metrics because of increased group cohesion (Jolles *et al.*, 2020).
160 Repeated tests of lateralisation for individuals and groups in different contexts (e.g. social
161 conditions and treatments) is therefore surely the way to go (Vinogradov *et al.* 2021), and will
162 allow researchers to explore this alternative method (following further assessment) – extracting
163 turning data from fish trajectories – to investigate changes in laterality (or any movement
164 metric) with changing levels of $p\text{CO}_2$ (or any other potential stressor).

165

166 Overall, our findings support our earlier analyses of the same set of experiments showing
167 elevated $p\text{CO}_2$ does not alter fish movements or interaction with each other and their tank
168 (Duteil *et al.*, 2016), suggesting that behavioural perturbations will not be a major consequence
169 for *D.labrax* in high CO_2 oceans. Furthermore, using video recording and tracking technology
170 provides another way to measure behavioural (relative) lateralization that can be applied across
171 different species and contexts, which can be adapted to investigate a range of questions (e.g.,
172 measuring turning in the light and in the dark and/or making direct comparisons of individuals
173 turning in detour tests: Dadda *et al.* 2012).

174

175 **Ethics**

176 This work was approved by Department of Biosciences Ethics Committee, Swansea
177 University.

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179 **Data accessibility**

180 Fish positional data: <http://dx.doi.org/10.5061/dryad.2dc8k>

181 Turning data: <https://doi.org/10.5061/dryad.cjsxksn5q>

182

183 **Author contributions**

184 A.J.K. and E.C.P. conceived and performed the experiments and collected the video data. M.D.
185 tracked fish trajectories from video and processed fish positional information with input from
186 M.R.B., I.F., and A.J.K. D.M.J calculated turning angles and conducted statistical analyses
187 with input from R.J.D., E.C.P and A.J.K. D.M.J. led writing of the manuscript with input from
188 all authors, who read and approved the final manuscript.

189

190 **Competing interests**

191 We declare we have no competing interests.

192

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