

1 **Title: Temperature and O₂, but not CO₂, interact to affect aerobic performance**
2 **of European sea bass (*Dicentrarchus labrax*)**

3 **Running title: Temperature & O₂, not CO₂, affect bass**

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22 **Abstract**

23 Climate change causes warming, decreased O₂, and increased CO₂ in marine
24 systems and responses of organisms will depend on interactive effects between
25 these factors. We provide the first experimental assessment of the interactive effects
26 of warming (14 to 22°C), reduced O₂ (~3 – 21 kPa O₂), and increased CO₂ (~400 or
27 ~1000 µatm ambient CO₂) on four indicators of aerobic performance (standard
28 metabolic rate, SMR, maximum metabolic rate, MMR, aerobic scope, and hypoxia
29 tolerance, O_{2crit}), blood chemistry, and O₂ transport (P₅₀) of a marine fish, the
30 European sea bass (*Dicentrarchus labrax*). Warming increased SMR and O_{2crit} (i.e.
31 reduced hypoxia tolerance) as well as MMR in normoxia but there was an interactive
32 effect with O₂ so that hypoxia caused larger reductions in MMR and aerobic scope at
33 higher temperatures. Increasing CO₂ had minimal effects on SMR, MMR and O_{2crit}
34 and did not show interactive effects with temperature or O₂ for any measured
35 variables. Aerobic performance was not linked to changes in blood chemistry or P₅₀.
36 Despite lack of effects of CO₂ on aerobic performance, increased CO₂ induced 30%
37 mortality of fish exercised in low O₂ at 22°C indicating important threshold effects
38 independent of aerobic performance. Overall, our results show temperature and O₂,
39 but not CO₂, interact to affect aerobic performance of sea bass, disagreeing with
40 predictions of the oxygen- and capacity-limited thermal tolerance hypothesis.

41 1. Introduction

42 Atmospheric CO₂ levels are increasing and could reach ~1000 µatm by the end
43 of the century (IPCC, 2014). Rising atmospheric greenhouse gases increase ocean
44 temperatures (Bopp et al., 2013), which reduces oceanic O₂ content and
45 exacerbates the frequency and severity of hypoxic (low O₂) events (Breitburg et al.,

46 2018; Diaz & Rosenberg, 2008). As atmospheric CO₂ levels continue to rise so too
47 does the concentration of CO₂ in marine systems (Caldeira & Wickett, 2003).
48 Therefore, responses of marine organisms to climate change will be a result of
49 simultaneous changes in temperature, O₂, and CO₂.

50 Changes in temperature, O₂, and CO₂ can individually impact physiological
51 performance of fish. Concern has been raised that interactions between these three
52 may occur in a non-linear manner, so that their combined impact cannot be predicted
53 from responses to an individual variable (Côté et al., 2016; Crain et al., 2008;
54 McBryan et al., 2013; Todgham & Stillman, 2013). As such, we need to understand
55 how temperature, O₂, and CO₂ interact to affect the physiology of fish to enable
56 accurate predictions of how climate change will influence fish species (Hollowed et
57 al., 2013; Pörtner & Peck, 2010; Wernberg et al., 2012). One approach is to examine
58 how these environmental factors affect the fish's range of aerobic metabolism
59 (aerobic scope), defined as the difference between an animal's maximum rate of O₂
60 consumption (maximum metabolic rate, MMR) and the minimum rate needed to
61 meet basal energy demands (standard metabolic rate, SMR) (Fry, 1971).

62 It has been proposed that aerobic scope provides a single metric of whole-animal
63 performance in a particular environment. Therefore, aerobic scope can be directly
64 linked to processes such as growth and reproduction and in turn overall organism
65 fitness, through the concept of oxygen- and capacity- limited thermal tolerance,
66 OCLTT (Pörtner, 2012; Pörtner et al., 2017). The OCLTT hypothesis assumes that
67 an organism's overall fitness is maximised at an optimal temperature at which
68 aerobic scope peaks. As such, anything that reduces aerobic scope will also
69 diminish fitness, potentially changing the distribution of populations (Pörtner &
70 Farrell, 2008). Hypoxia affects aerobic scope by limiting environmental O₂ availability

71 and, therefore, the maximum O₂ uptake rate possible by fish. Increased CO₂ has
72 been proposed to affect aerobic scope both by increasing the SMR of fish (e.g.
73 through increased cost of acid-base regulation) and by decreasing MMR (potentially
74 because subsequent changes in internal acid-base chemistry can reduce O₂
75 transport capacity of the blood or impair tissue functioning). The OCLTT hypothesis
76 therefore predicts that reduced O₂ and increased CO₂ will interact to reduce aerobic
77 scope across the thermal performance curve of a species. This would result in a
78 lower optimal temperature (where peak aerobic scope occurs) and reduced thermal
79 tolerance. While this concept has been successfully used to explain changes in
80 habitat suitability and population distributions of some fish species (Cucco et al.,
81 2012; Del Raye & Weng, 2015), the assertion that it represents a universal
82 framework to predict effects of climate change on all fish populations (Farrell, 2016)
83 has been challenged (Jutfelt et al., 2018; Lefevre, 2016).

84 The proposal of the OCLTT hypothesis has led to numerous studies examining
85 how O₂ or CO₂ interact with temperature to affect aerobic performance (for examples
86 see Chabot and Claireaux, 2008; Rummer et al., 2013; Grans et al., 2014). However,
87 to date no experimental work has sought to investigate how combined changes in all
88 three factors (temperature, O₂, and CO₂) interact to affect aerobic performance in
89 fish. Combining all three environmental variables is vital to accurately assess
90 potential interactive effects for three reasons. Firstly, meta-analysis of multi-factor
91 studies indicates that the prevalence of non-linear effects doubles when moving from
92 studies that combine two factors to three factors (Crain et al., 2008). Secondly, the
93 role of CO₂ as a limiting factor of aerobic scope was originally suggested to occur
94 primarily in combination with hypoxia (Fry, 1971). Thirdly, low O₂ conditions in the
95 environment always co-occur with increased CO₂ (Melzner et al., 2013). As such,

96 experiments investigating effects of O₂ or CO₂ and temperature on aerobic
97 performance may not accurately reflect interactive effects caused by all three
98 environmental factors.

99 In this study we investigated how temperature, O₂, and CO₂ interact to affect
100 aerobic performance of European sea bass (*Dicentrarchus labrax*), a species
101 showing recent northward range expansions thought to be related to warming
102 (Pawson et al., 2007). Two separate populations exist (Souche et al., 2015) and,
103 although the physiological responses of this species to environmental change have
104 been regularly examined, to date only one study has used fish from the Atlantic
105 population. As such, our experiment had three aims:

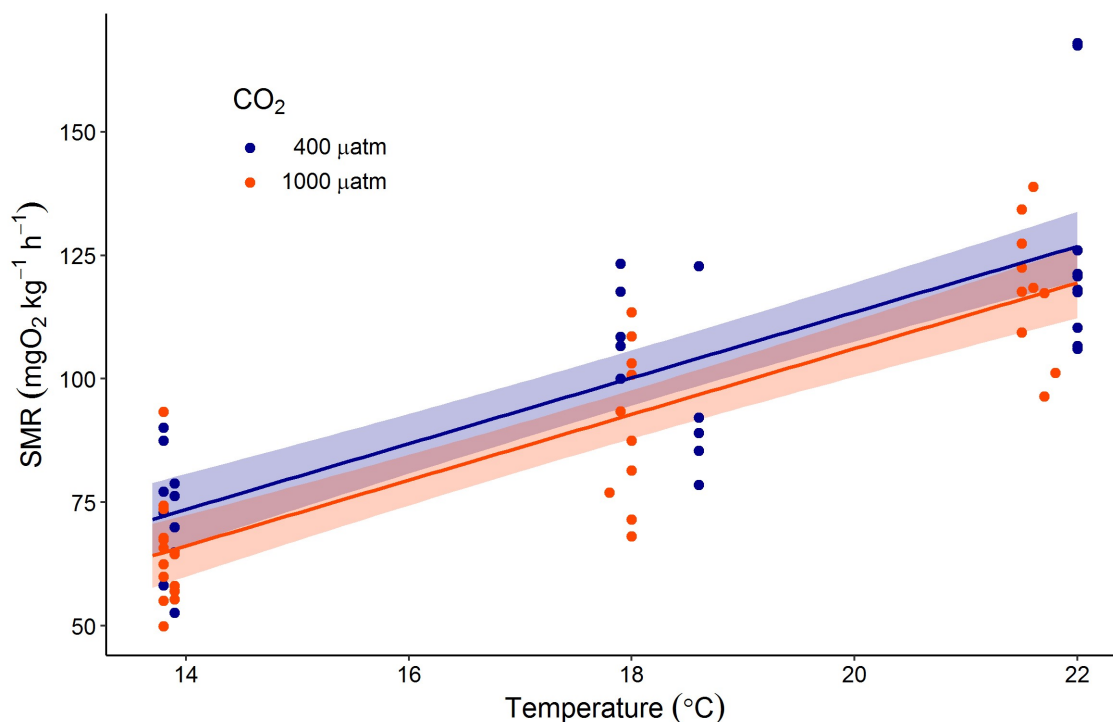
- 106 i. to assess how aerobic performance of sea bass from the Atlantic population
107 will respond to predicted future environmental changes;
- 108 ii. to determine whether combinations of hypoxia and increased CO₂ interact
109 with temperature to affect aerobic scope, as predicted by the OCLTT
110 hypothesis;
- 111 iii. to determine whether changes in aerobic performance are linked to blood
112 chemistry and O₂ transport capacity.

113 2. Results

114 2.1. Standard metabolic rate and hypoxia tolerance

115 The best supported model for SMR included both temperature and CO₂ as fixed
116 effects (Linear Mixed-Effects Model, marginal R² = 0.70, conditional R² = 0.70, Table
117 S6). Standard metabolic rate approximately doubled between 14 and 22°C,
118 exhibiting a Q₁₀ temperature coefficient of 2.09 (Figure 1). The best model indicated

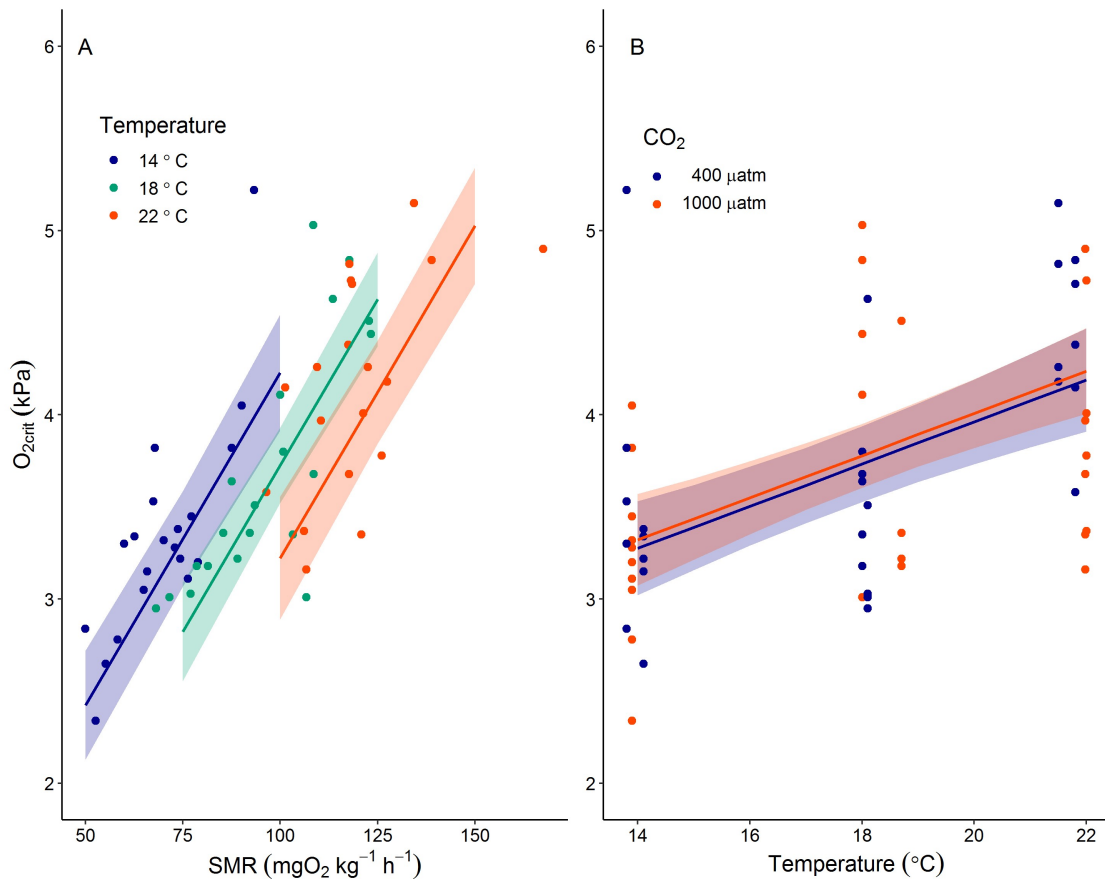
119 that CO₂ had a small effect on SMR - reducing SMR by 7.4 mgO₂ kg⁻¹ h⁻¹, ~10 % of
120 the smallest SMR, (95 % CI = -1.65 to 16.43 mgO₂ kg⁻¹ h⁻¹) across all temperatures at
121 ~1000 μatm CO₂ (Figure 1). However, the model including temperature but not CO₂
122 had a ΔAICc <2 indicating that including the effect of CO₂ in the best model did not
123 lead to a large improvement in model fit (Table S5). There was no evidence of an
124 interactive effect between increasing temperatures and increasing CO₂.



125

126 **Figure 1:** Impact of temperature and CO₂ on standard metabolic rates (SMR) of
127 juvenile sea bass. The best supported model (marginal R² = 0.70, conditional R² =
128 0.70) to explain variation in SMR included temperature and CO₂ as explanatory
129 variables but not their interaction. Points represent calculated SMR for individual fish,
130 lines represented predicted SMR at two CO₂ levels (blue = present day ~400 μatm
131 CO₂, orange = end of century ~ 1000 μatm CO₂) from the best supported model, and
132 shaded areas represent bootstrapped 95 % CI of predictions (n = 1000).

133 The best supported model of O_{2crit} included the effects of temperature, CO_2 and
134 SMR but no interactions (Linear Mixed-Effects Model, marginal $R^2 = 0.72$, conditional
135 $R^2 = 0.77$, Table S7). A doubling in SMR from 60 to 120 $mgO_2 kg^{-1} h^{-1}$ is predicted to
136 increase O_{2crit} by 2.16 kPa O_2 (95 % CI = 1.66 to 2.66 kPa O_2). Independent of their
137 effects on SMR, both temperature and CO_2 were included in the best model of O_{2crit} .
138 The effect of temperature meant that for a given value of SMR O_{2crit} would reduce as
139 temperature increased. For instance, a fish at 14 °C is predicted to have an O_{2crit}
140 0.50 kPa O_2 (95 % CI = 0.13 to 0.87 kPa O_2) higher than a fish with the same SMR
141 at 18 °C (Figure 2A). Combined the effects of SMR and temperature mean warming
142 from 14 to 22 °C will increase O_{2crit} by 0.91 kPa O_2 (95 % CI = 0.53 to 1.29 kPa O_2 ,
143 Figure 2B). The additional effect of CO_2 is predicted to increase O_{2crit} . Despite this,
144 when taking into account the effect of CO_2 on SMR from the best model of SMR
145 (Figure 1), and the effect of SMR and temperature on O_{2crit} , the resulting effect of
146 CO_2 causes an increase in O_{2crit} of only 0.04 kPa O_2 (95 % CI = -0.32 to 0.40 kPa
147 O_2 , Figure 2B).



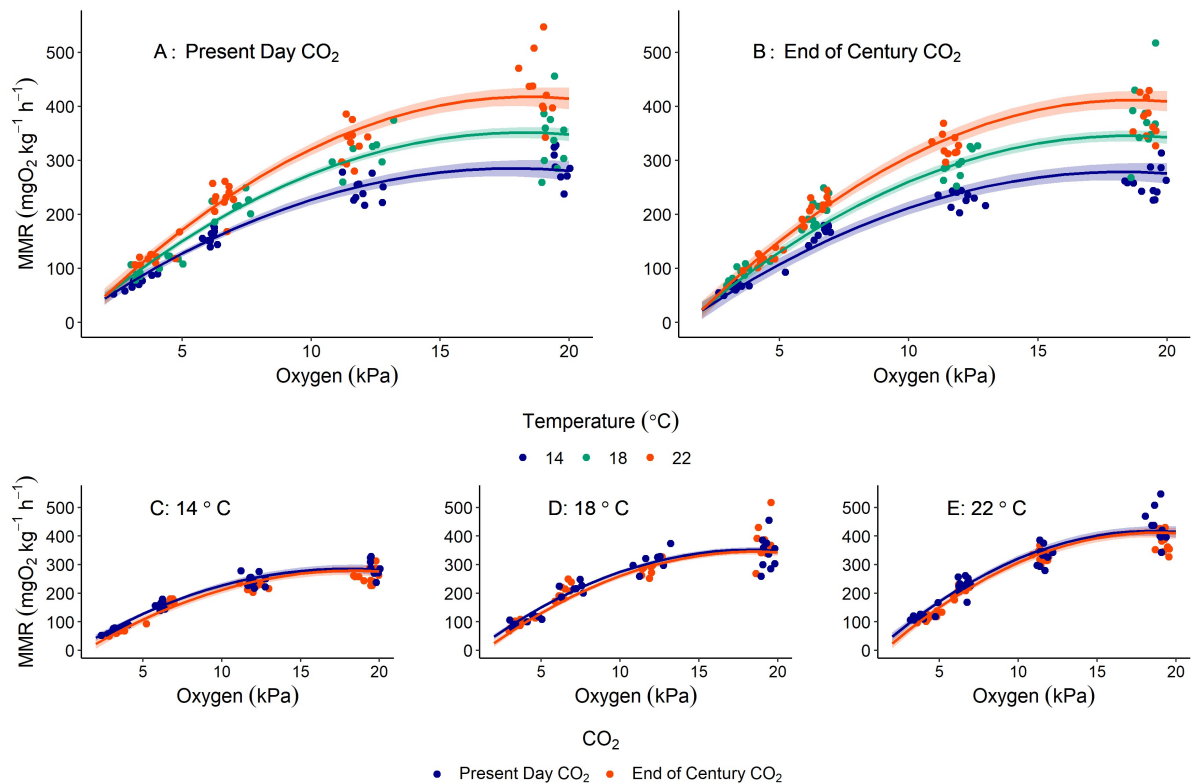
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149 **Figure 2:** Combined impacts of SMR, temperature and CO_2 on hypoxia tolerance
150 (O_{2crit}) of juvenile European sea bass. **A.** The best supported model (marginal $R^2 =$
151 0.72, conditional $R^2 = 0.77$) predicted a positive effect of increasing SMR on O_{2crit}
152 with the effect of increased temperature resulting in lower O_{2crit} for a given value of
153 SMR. **B.** Combined effects of SMR and temperature result in an increase in O_{2crit}
154 between 14 °C and 22 °C. The predicted positive effect of increased CO_2 on O_{2crit} is
155 small compared to changes in SMR and temperature. Points represent calculated
156 O_{2crit} for individual fish, lines represented predicted O_{2crit} from the best supported
157 model, and shaded areas represent bootstrapped 95 % CI of predictions ($n = 1000$).

158 2.2. Maximum metabolic rate and aerobic scope

159 Maximum metabolic rate of sea bass was affected by temperature, O_2 , and CO_2
160 level. Warming from 14 to 22 °C, in normoxia combined with normocapnia, caused a

161 50 % increase in MMR from 293.5 ± 10.4 to 435.9 ± 18.9 $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$, with a Q_{10} of
162 1.64. In high CO_2 the temperature effect on MMR was very similar ($Q_{10} = 1.60$). The
163 best supported model predicted that O_2 had a non-linear quadratic effect on MMR,
164 so that a given reduction in O_2 caused a larger reduction in MMR at lower O_2 levels,
165 as well as interactive effects between O_2 and temperature (Figure 3; Linear Mixed
166 Model, marginal $R^2 = 0.91$, conditional $R^2 = 0.96$, Table S8). For example a 5 kPa
167 reduction in O_2 at 18 °C (in normal CO_2) from air saturated levels (~ 20 to ~15 kPa
168 O_2) results in a predicted decrease in MMR of $8.0 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ (95 % CI = -7.2 to
169 $23.2 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) whereas the same reduction in O_2 from 10 to 5 kPa resulted in a
170 15-fold larger predicted decrease in MMR of $123.8 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ (95 % CI = 115.1 to
171 $132.5 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$). In addition, the non-linear O_2 effect interacted with the
172 temperature effect so that the same reduction in O_2 caused a larger reduction in
173 MMR as temperature increased (Figure 3A & B). This was particularly noticeable
174 between 15 and 10 kPa O_2 where fish at 22 °C (in normal CO_2) exhibited a predicted
175 decline in MMR that was 60 % larger than fish at 14 °C, $80.9 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ (95 % CI
176 = 63.2 to $98.6 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$) versus $50.8 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ (95 % CI = 35.9 to 65.7 mgO_2
177 $\text{kg}^{-1} \text{ h}^{-1}$). Finally, environmental CO_2 level had a small, negative effect independent of
178 interactions between temperature and O_2 . As a result, an increase in CO_2 of 1000
179 μatm is predicted to reduce MMR by $8.5 \text{ mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ (95 % CI = -18.2 to 35.3 mgO_2
180 $\text{kg}^{-1} \text{ h}^{-1}$) irrespective of temperature and O_2 (Figure 3C, D, & E).

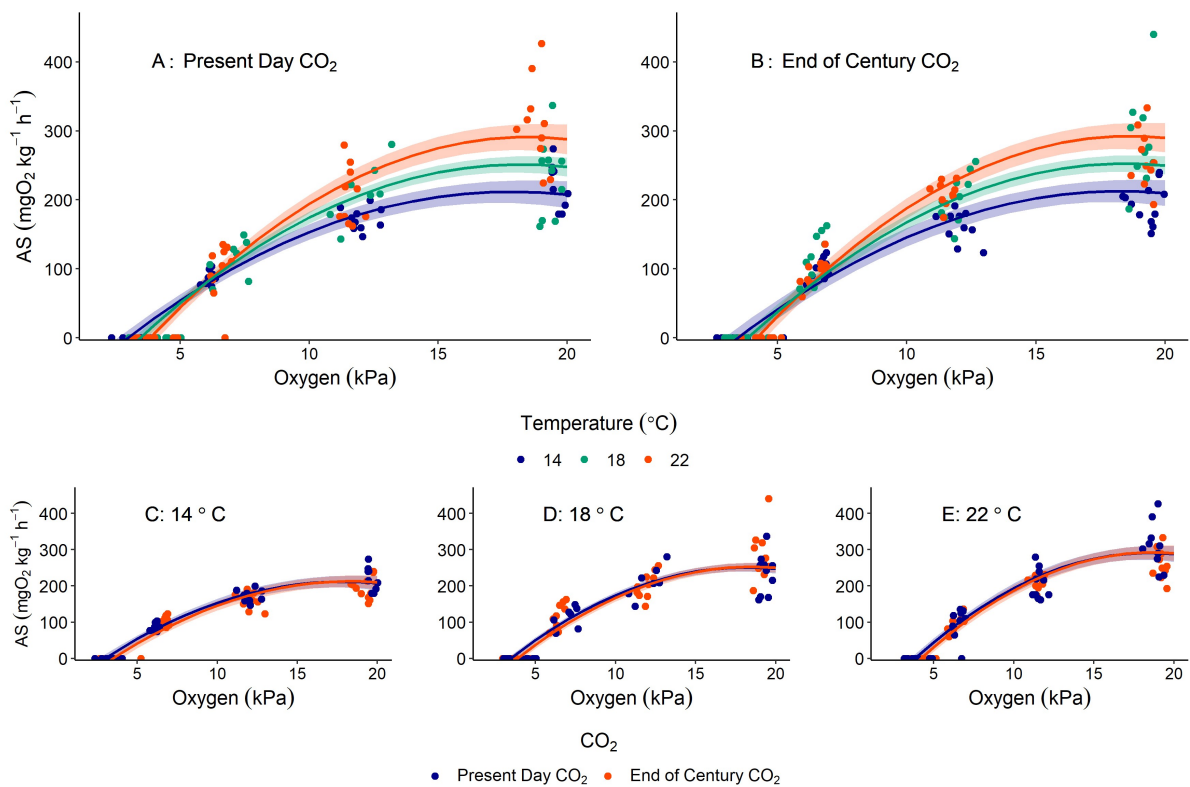


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182 **Figure 3:** Effects of combinations of temperature, O₂, and CO₂ on the maximum
183 metabolic rate (MMR) of European sea bass. There was a synergistic interactive
184 effect of temperature and O₂ on MMR for sea bass exposed to both **A.** present day
185 CO₂ conditions (present day CO₂ = 400 μatm at ~20 kPa O₂) and **B.** end of century
186 CO₂ conditions (end of century CO₂ = 1000 μatm at ~ 20 kPa O₂). The additive
187 effects of reduced O₂ and increased CO₂ levels are displayed for sea bass at **A.** 14
188 °C **B.** 18 °C and **C.** 22 °C. Points represent calculated MMR for individual fish, lines
189 represent predicted MMR from the best supported model, and shaded areas
190 represent bootstrapped 95 % confidence intervals (n = 1000).

191 We predicted the impacts of temperature, O₂, and CO₂ on aerobic scope from the
192 best supported models fitted to measurements of SMR and MMR (Figure 4). At
193 normoxia (~20 kPa O₂) aerobic scope is predicted to increase by 80.2 mgO₂ kg⁻¹ h⁻¹
194 (95 % CI = 24.8 to 135.7 mgO₂ kg⁻¹ h⁻¹) as temperature increases from 14 to 22 °C

195 independent of changes in CO₂ level (Figure 4A & B). Interactive effects between
196 temperature and O₂ on MMR are reflected in predictions of aerobic scope (Figure 4A
197 &B). As increasing CO₂ has the same direction of effect on both SMR and MMR the
198 impact of CO₂ on aerobic scope is minimal (Figure 4C, D, & E).



199

200 **Figure 4:** Effects of combinations of temperature, O₂, and CO₂ on the aerobic scope
201 (AS) of European sea bass. There was a non-linear interactive effect of temperature
202 and O₂ on aerobic scope for sea bass exposed to both **A.** present day CO₂
203 conditions (present day CO₂ = 400 μatm at ~20 kPa O₂) and **B.** end of century CO₂
204 conditions (end of century CO₂ = 1000 μatm at ~ 20 kPa O₂). The additive effects of
205 reduced O₂ and increased CO₂ levels are displayed for sea bass at **A.** 14 °C **B.** 18
206 °C and **C.** 22 °C. Points represent aerobic scope of individual fish derived from
207 calculated RMR and MMR of that individual, lines represent predicted aerobic scope
208 calculated by subtracting model predictions of RMR and MMR and shaded areas

209 represent 95 % confidence intervals calculated from bootstrapped standard errors of
210 predicted RMR and MMR (n = 1000).

211 2.3. Blood chemistry & Hb-O₂ affinity

212 Increasing ambient CO₂ from ~400 to ~1000 μ atm increased plasma pCO₂ (Two-
213 way ANOVA, F = 15.84, df = 1, p < 0.001) whereas warming from 14 to 22 °C did not
214 (F = 0.772, df = 2, p = 0.468), and there was no interactive effect noted (F = 2.067, df
215 = 2, p = 0.138). Despite the significant overall effect of increased CO₂ on plasma
216 pCO₂ pairwise comparisons (Figure 5) only revealed a significant increase in plasma
217 pCO₂ in bass at 18 °C exposed to ~1000 μ atm (0.53 ± 0.05 kPa pCO₂) compared to
218 ambient conditions (0.29 ± 0.05 kPa pCO₂) (Pairwise comparisons of least square
219 means, t = 3.688, df = 1, p = 0.008).

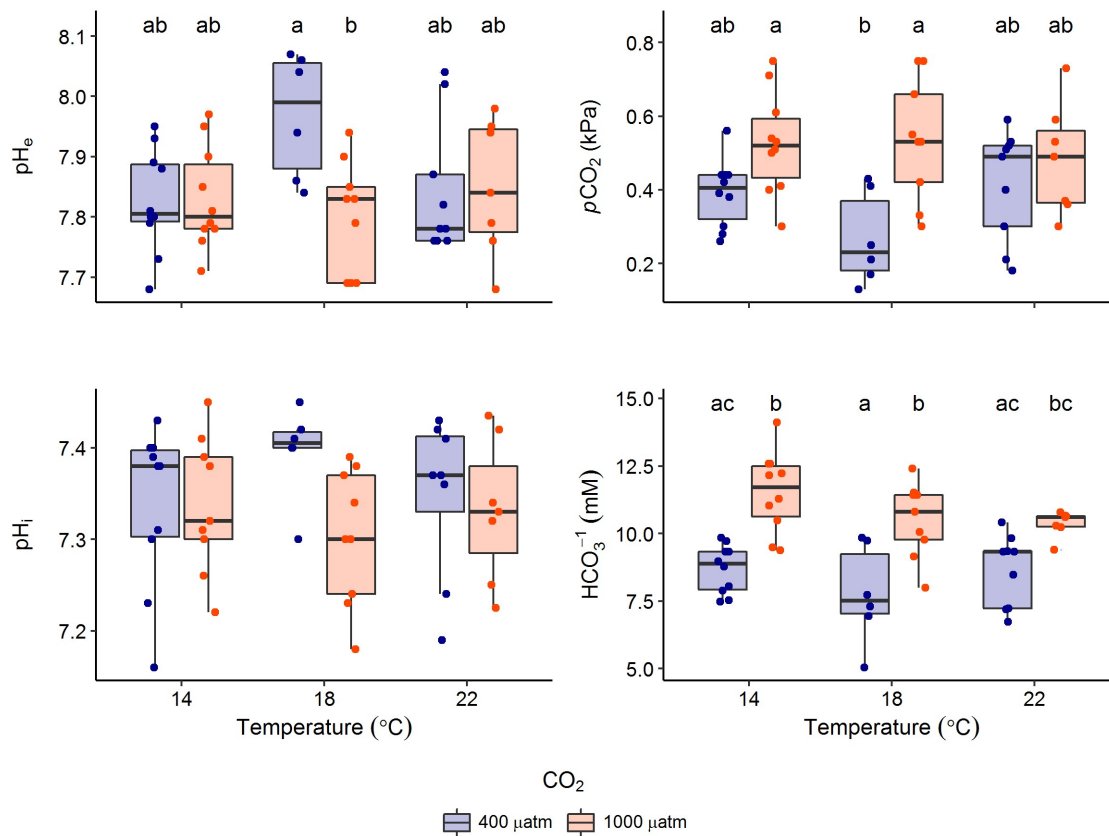
220 To compensate for increased plasma pCO₂ in ~1000 μ atm CO₂ treatments sea
221 bass accumulated ~3 mM extra HCO₃⁻ (Two-way ANOVA, F = 44.34, df = 1, p <<
222 0.001) (Figure 5), compared to fish in ambient CO₂ conditions at 14 and 18 °C. Fish
223 exposed to ~1000 μ atm CO₂ at 22 °C showed a non-significant increase in HCO₃⁻ of
224 just under 2 mM (95 % CI = 0.60 to 2.84 mM) when compared to fish at ~400 μ atm
225 CO₂ (t = 2.66, df = 1, p = 0.103). There was no temperature effect on plasma HCO₃⁻
226 (F = 2.538, df = 2, p = 0.0903) and no interactive effect between temperature and
227 CO₂ (F = 0.969, df = 2, p = 0.387).

228 As a result of compensatory accumulation of HCO₃⁻, blood extracellular pH (pH_e)
229 was regulated in response to high CO₂ (F = 3.56, df = 1, p = 0.066) and sea bass did
230 not show significant effects of temperature on pH_e (Two way ANOVA, F = 1.425, df =
231 2, p = 0.251). However, there was a significant interactive effect between
232 temperature and CO₂ (F = 3.952, df = 2, p = 0.026). This interactive effect was

233 caused by a significant reduction in pH_e in sea bass exposed to $\sim 1000 \mu\text{atm CO}_2$ at
234 $18 \text{ }^\circ\text{C}$ (7.80 ± 0.03) when compared to fish at ambient CO_2 levels (7.97 ± 0.04)
235 (pairwise comparisons of least square means, $t = 3.242$, $\text{df} = 1$, $p = 0.026$). We did
236 not find significant differences between pH_e across all other treatment groups.
237 Additionally, intracellular pH of red blood cells (pH_i) showed no significant differences
238 between all treatments (Kruskal-Wallis test, $\chi^2 = 6.79$, $\text{df} = 5$, $p = 0.237$) (Figure 5).

239 There were no significant differences in plasma lactate levels across all
240 treatments (Kruskal-Wallis, $\chi^2 = 6.40$, $\text{df} = 5$, $p = 0.269$), with mean lactate for all fish
241 of $0.42 \pm 0.06 \text{ mM}$ ($\pm \text{S.E.}$). Plasma glucose levels (mean for all fish of 4.30 ± 0.14
242 mM , $\pm \text{S.E.}$) were not significantly affected by temperature (Two-way ANOVA, $F =$
243 0.864 , $\text{df} = 2$, $p = 0.429$), CO_2 ($F = 0.0138$, $\text{df} = 1$, $p = 0.907$) or the interaction
244 between temperature and CO_2 ($F = 1.052$, $\text{df} = 2$, $p = 0.358$).

245



246

247 **Figure 5:** Impact of temperature and CO₂ on blood acid-base characteristics of
 248 European sea bass. Significant differences in pairwise comparisons of least square
 249 means between treatments are indicated by different letters (a,b,c). No significant
 250 differences between any treatments were noted for measurements of pH_i.

251 Our measurements of blood O₂ transport showed no consistent impacts of
 252 temperature or CO₂. Haemoglobin-O₂ binding affinity (P₅₀) (Kruskal-Wallis, $\chi^2 =$
 253 6.503, df = 4, p = 0.165) and Hills' number (One-way ANOVA, F = 0.878, df = 4, p =
 254 0.487) were not significantly different between any treatments (Figure S1).
 255 Haematocrit (Hct) was not affected by temperature (Two-way ANOVA, F = 0.832, df
 256 = 2, p = 0.442) or CO₂ (F = 2.945, df = 1, p = 0.093) and no interactive effects were
 257 evident (F = 0.069, df = 2, p = 0.934). Haemoglobin (Hb) levels were affected by
 258 temperature (Two-way ANOVA, F = 7.094, df = 2, p = 0.002) with fish sampled at 22
 259 °C having Hb levels 0.20 and 0.24 mM higher than fish sampled at 18 and 14 °C

260 respectively. Haemoglobin levels were not affected by CO₂ ($F = 0.690$, $df = 1$, $p =$
261 0.411) and there were no interactive effects ($F = 3.067$, $df = 2$, $p = 0.057$) (Figure
262 S2).

263 2.4. Mortality

264 Three fish (30 %) died ~1 hour post-chase after exposure to ~6 kPa O₂ at 22 °C
265 in the high CO₂ treatment. No fish died post-chase in any other treatment
266 combinations.

267 3. Discussion

268 Our results demonstrate, for the first time, the interactive effects of temperature,
269 O₂, and CO₂ on aerobic performance of an active predatory marine fish. We show
270 that temperature and O₂ have a non-linear interactive effect on aerobic performance
271 of European sea bass but CO₂'s impact is minor and independent. Both SMR and
272 MMR increased with temperature from 14 to 22 °C, but changes in MMR were
273 greater leading to positive effects on absolute aerobic scope. These results suggest
274 that European sea bass populations in the North-East Atlantic (typical temperatures
275 are < 22 °C) could physiologically benefit from global warming. However, hypoxia
276 tolerance reduced at higher temperatures, and hypoxia reduced MMR and aerobic
277 scope by a greater amount at high temperatures, indicating that in warmer waters
278 European sea bass will be more susceptible to hypoxia.

279 We confirm similar findings from previous research investigating effects of
280 temperature and O₂ on European sea bass. For example, the increase in SMR with
281 temperature we observed closely follows results from aquaculture sourced European
282 sea bass (Claireaux & Lagardère, 1999). That is the Q₁₀ temperature coefficient
283 dropped from 2.33 between 14 and 18 °C to 1.82 between 18 and 22 °C. This drop in

284 Q₁₀ as temperature increases provides further evidence that SMR does not increase
285 exponentially after chronic vs acute exposure (Sandblom et al., 2014; Schulte et al.,
286 2011). Responses of MMR to temperature showed similar Q₁₀'s indicating a linear
287 increase in MMR between 14 and 22 °C. This also closely corresponds with
288 observations from Claireaux and Lagardère (1999) who found that MMR increased
289 approximately linearly between 14 and 22 °C, before peaking between 22 and 24 °C
290 and declining at higher temperatures. Further work with sea bass from
291 Mediterranean stock has shown that specific growth rate and feed conversion
292 efficiency peak at ~25 °C and decline at higher temperatures (Person-Le Ruyet et
293 al., 2004), similar to changes in metabolic scope shown by Claireaux and Lagardère
294 (1999). Finally, aquaculture-produced sea bass display an Arrhenius break point of
295 heart rate at ~21.5 °C and developed arrhythmia at ~26 °C (Crespel et al., 2019).
296 The consistency in temperature of peak performance in sea bass from distinct sub-
297 populations with vastly different environmental experiences supports the idea that
298 fish face ceilings to physiological performance in the face of environmental change
299 (Sandblom et al., 2016). Despite these similarities with previous research, SMR of
300 fish in our study was higher at a given temperature than for fish from Mediterranean
301 stocks (Claireaux & Lagardère, 1999). This may support the theory of metabolic cold
302 adaptation, that basal energy demand in fish from warmer environments will be lower
303 than in fish from cold environments when measured at the same temperature
304 (Krogh, 1916). This has recently been supported by evidence from wild populations
305 of three-spine stickleback (*Gasterosteus aculeatus*) (Pilakouta et al., 2020).

306 Declining O₂ caused a decrease in MMR along a limiting oxygen curve, similar to
307 that seen previously in sea bass (Claireaux & Lagardère, 1999; Lagardère et al.,
308 1998) and other fish species (Chabot & Claireaux, 2008; Claireaux et al., 2000;

309 Lefrancois & Claireaux, 2003; Mallekh & Lagardère, 2002). This result questions
310 recent predictions made by Seibel and Deutsch (2020) that MMR of fish should
311 decrease linearly from ~21 kPa O₂ to O_{2crit} values. The curved, rather than linear,
312 response of MMR we observed may occur as a result of compensatory mechanisms
313 (e.g. increased ventilation, cardiac output, gill lamellar perfusion and surface area,
314 and haematocrit) to maintain O₂ delivery (Farrell & Richards, 2009). While these
315 adjustments may limit reductions in MMR during mild to moderate hypoxia they may
316 reach their performance limits as O₂ approaches critical levels, resulting in a steeper
317 decline in MMR. For example, in moderate hypoxia rainbow trout (*Oncorhynchus*
318 *mykiss*) increase cardiac output via increased stroke volume but in severe hypoxia
319 cardiac output cannot be increased further leading to bradycardia (Sandblom &
320 Axelsson, 2005).

321 Temperature and O₂ interacted to affect metabolism of sea bass so that impacts
322 of hypoxia on MMR increased with temperature (Figure 3). This result supports
323 previous research by Claireaux & Lagardère (1999). However, sea bass in our study
324 displayed higher MMR at similar temperature and pO₂ when compared to bass from
325 Claireaux and Lagardère (1999). In addition, the O_{2crit} (the point at which SMR =
326 MMR) of our sea bass increased between 14 °C and 22 °C (Figure 2) whereas
327 results from previous work suggested that O_{2crit} increases or remains constant
328 across this temperature range (Claireaux & Lagardère, 1999). The reduction in
329 hypoxia tolerance of sea bass with warming was primarily a result of strong positive
330 correlation between O_{2crit} and SMR (Figure 1). This relationship has been shown for
331 numerous fish species, and most recently in work with black sea bass (*Centropristus*
332 *striata*) (Slesinger et al., 2019). However, our results also indicate that temperature
333 had a secondary effect which resulted in lower O_{2crit} at higher temperatures for a

334 given SMR. This suggests that temperature affects O_{2crit} via another mechanism (or
335 mechanisms) independent of SMR. This is unlikely to be related to O_2 transport
336 capacity of the blood as there were few consistent effects of temperature on Hct, Hb
337 or P_{50} of sea bass (Figure 5, S1 and S2). It has previously been observed that
338 improved hypoxia tolerance of fish after acclimation to increased temperature
339 correlates to increased gill lamellar surface area (McBryan et al., 2016) or changes
340 to heart structure (Anttila et al., 2015). Gomez Isaza *et al.* (2021) demonstrated that
341 both of these cardiorespiratory responses can improve O_2 supply capacity.
342 Combined, these results suggest that thermal acclimation can cause structural
343 changes to the gills and the heart, improving performance in low O_2 conditions and
344 mitigating the negative effect of temperature induced increases in SMR on hypoxia
345 tolerance.

346 The independent effect of rising CO_2 reduced MMR. Previous research has not
347 shown consistent effects of CO_2 on MMR or SMR of fish species, with the majority
348 showing no effects of CO_2 (Lefevre, 2016, 2019). Interestingly the most recent
349 research with European sea bass found that long term exposure to elevated CO_2
350 increased MMR. This may indicate that negative effects of short term exposures
351 used in our study (i.e. weeks) can be overcome in the long term (i.e. years) (Crespel
352 et al., 2019). As such, future work needs to determine whether interactions between
353 CO_2 and temperature or O_2 occur over longer time scales. The effect of CO_2 on
354 metabolic rate in our study was only identifiable by including the rise in CO_2 which
355 co-occurs when environmental O_2 declines. Without the additional data from
356 increased CO_2 exposures at lower O_2 levels the effect of ambient CO_2 on MMR at
357 normoxia was not significant. Although the best model of SMR also included a
358 negative effect of CO_2 this was predicted to be small with confidence intervals

359 overlapping zero (Figure 1). Additionally, removal of CO₂ as an explanatory variable
360 from the model of SMR did not greatly impair model fit ($\Delta AICc < 2$) which indicates
361 that the effect of CO₂ on SMR was not critically important for overall model
362 performance.

363 As CO₂ increases in the environment when O₂ declines the negative effect of
364 CO₂ has a greater impact on MMR at lower O₂ levels (Figure 3 C, D, and E).
365 Decreased MMR when fish are exposed to acutely increased CO₂ is usually thought
366 to be a result of an internal acidosis causing a decrease in Hb-O₂ binding affinity,
367 reducing the capacity of O₂ transport in the blood (Heuer & Grosell, 2016). However,
368 fish have well developed acid-base regulatory mechanisms and blood sampling
369 showed that sea bass in normoxia had fully compensated for the effects of increased
370 environmental CO₂ on blood pH by compensatory accumulation of extra HCO₃⁻,
371 resulting in no changes in Hb-O₂ binding affinity (Figure 5 and S1). Additionally, we
372 have recently shown that sea bass are able regulate blood pH when exposed to
373 concurrent progressive hypercapnia during progressive hypoxia over the course of
374 several hours and have higher Hb-O₂ binding affinity in these conditions when
375 compared to fish exposed to progressive hypoxia with no concurrent hypercapnia
376 (Montgomery et al., 2019). In addition, we did not see significant changes in Hb
377 levels or Hct between CO₂ treatments (Figure 7). We conclude that the negative
378 effect of increased CO₂ on MMR is unlikely to be related to changes in O₂ transport
379 capacity in the blood. Whilst beyond the scope of the present study we can
380 speculate that instead CO₂ may affect MMR via changes in mitochondrial
381 metabolism (Leo et al., 2017; Strobel et al., 2012, 2013), cardiac performance
382 (Crespel et al., 2019; Perry & Abdallah, 2012) or via shifts from aerobic to anaerobic
383 metabolic pathways (Michaelidis et al., 2007). Indeed, recent results show that CO₂

384 impacts mitochondrial function of sea bass from the Atlantic population in
385 combination with acute warming (Howald et al., 2019)

386 While the effect of CO₂ on MMR was part of the best supported model it is
387 uncertain whether it would cause biologically relevant impacts. Previous research
388 has linked declines in MMR caused by increased CO₂ with decreased swimming
389 performance (Lefevre, 2019) but it is unknown if the relatively small changes in MMR
390 shown in the present study would translate to other aspects of whole animal
391 performance. This is especially the case as predictions of aerobic scope from the
392 combined best supported models of MMR and SMR essentially show no effect of
393 CO₂ on aerobic scope at O₂ levels >10 kPa (Figure 4 C, D, and E). This occurs
394 because predicted effects of CO₂ act in the same direction for both SMR and MMR.
395 As changes in aerobic scope typically predict environmental impacts on processes
396 such as growth and reproduction (Clark et al., 2013; Pörtner et al., 2017) we would
397 predict that climate change relevant CO₂ increases have negligible effects on these
398 endpoints. However, the effect of CO₂ may have important consequences not
399 reflected in changes in aerobic scope. In our most extreme treatment (22 °C, ~30 %
400 air saturation, end of century CO₂) we observed 30 % mortality of sea bass after
401 exhaustive exercise. Fish exercised at the same temperature and O₂ levels in
402 ambient CO₂ conditions showed no mortality (and no mortality was observed in any
403 other treatment combinations) – consequently it appears the elevated CO₂ during
404 hypoxia in a future ocean scenario (which was approximately 1100 µatm higher than
405 in the present day CO₂ scenario) may impair recovery from exercise when O₂ is
406 limiting. Mortality in fish post-exercise has been theorised to result from intracellular
407 acidosis generated during anaerobic respiration (Wood et al., 1983). Therefore, the
408 greater increase in CO₂ during hypoxia in the future ocean CO₂ scenario may either

409 exacerbate the intracellular acidosis caused by anaerobic activity or impair the ability
410 of fish to process anaerobic end products.

411 3.1. Evidence to support OCLTT?

412 The OCLTT hypothesis suggests climate change will affect fish because
413 combined effects of reduced O₂ and increasing CO₂ will synergistically interact,
414 lowering aerobic scope across its thermal performance curve (Pörtner & Peck,
415 2010), and that changes in aerobic scope can be used as a single metric for
416 predicting whole animal performance (Pörtner, 2012). Our data provides some
417 support for the OCLTT hypothesis as the observed interactions between increased
418 temperature and reduced O₂ on aerobic scope would be expected to result in
419 changes to the thermal performance curve as predicted by Pörtner and Farrell
420 (2008). However, our highest temperature treatment did not decrease MMR or
421 aerobic scope and so we cannot confirm whether interactive effects between
422 temperature and hypoxia would follow predictions of the OCLTT above the optimum
423 temperature of aerobic scope. In contrast to hypoxia, the effects of CO₂ did not
424 follow predictions from the OCLTT as CO₂ did not interact with either temperature or
425 hypoxia and had minimal impacts on aerobic scope.

426 The interactive effects of O₂ and temperature on the MMR of sea bass in this
427 study closely resemble predictions of the metabolic niche framework which Ern
428 (2019) proposed as an update to the OCLTT hypothesis. In particular the concept of
429 aerobic scope isopleths (where aerobic scope remains constant across a range of
430 temperatures as a result of changes in O₂ or vice versa) is supported by our data,
431 showing that aerobic scope of sea bass would be expected to remain constant
432 across an 8 °C temperature range at an O₂ level of ~6 kPa (Figure 3 A and B). As

433 such, we would support Ern's suggestion to experimentally assess how aerobic
434 scope affects important processes, such as growth, independently of changes in
435 temperature and O₂ by utilising these isopleths. Although increased aerobic scope
436 has long been linked to improved individual fitness there is a still a lack of evidence
437 to confirm this relationship occurs – as such the assumption that peak physiological
438 fitness occurs when aerobic scope peaks (central to the OCLTT) may be erroneous.

439 Alternatively, Deutsch *et al.* (2015) have suggested a framework which aims to
440 predict impacts of climate change on the physiological suitability of a habitat for a
441 species via a metabolic index, relating the ratio of O₂ supply to resting metabolic
442 demand (i.e. SMR), rather than aerobic scope. This metabolic index appears to
443 provide a tool for predicting the biogeographical distributions of species with the
444 biogeographical distribution limits of many marine species corresponding to a
445 metabolic index of ~2-5 (Deutsch *et al.*, 2015). This approach has been supported by
446 experimental work with black sea bass, *Centropristis striata*, (Slesinger *et al.*, 2019)
447 and Roman sea bream, *Chrysolephus laticeps*, (Duncan *et al.*, 2020) which showed
448 that the metabolic index can accurately predict changes in population distributions of
449 these species . Applying the principle of the metabolic index to our data suggests
450 that temperatures of 22 °C are close to the upper temperature limits of European sea
451 bass from the North-East Atlantic sub-population (metabolic index at 22 °C and 20
452 kPa O₂ was ~5).

453 4. Conclusion

454 In summary, our research shows that aerobic scope of European sea bass will
455 increase with expected warming in the North-East Atlantic, and that even extreme
456 summer temperatures (~22 °C) at the end of the century will positively impact on the

457 absolute aerobic performance of sea bass. However, synergistic interactions
458 between warming and reduced O₂ indicate that hypoxic conditions will have greater
459 impacts on sea bass in future oceans. Increased CO₂ levels showed no interactions
460 with either temperature or O₂ changes but were predicted to cause a small decline in
461 MMR – although this had little impact on aerobic scope because increased CO₂
462 caused a trend for decreased SMR. Sea bass fully compensated blood pH for
463 increased CO₂ levels and increases in SMR and reductions in MMR with
464 temperature were not linked to changes in O₂ transport. Despite end of century CO₂
465 levels having minimal effects on aerobic scope, they did cause increased mortality of
466 fish recovering from exercise in the more extreme hypoxic scenario (~30 % air
467 saturation) at 22 °C. This effect would not have been observed without including
468 expected increases in CO₂ as O₂ declines in hypoxia treatments. Thus,
469 environmentally relevant changes in CO₂ during hypoxia may lead to important
470 threshold effects which could be missed if experiments only consider changes in
471 CO₂ related to atmospheric concentrations. Interactive effects of temperature and O₂
472 support predictions from the oxygen-and temperature-limited metabolic niche
473 framework proposed as an update to the OCLTT hypothesis by Ern (2019), however
474 the effect of CO₂ did not support predictions of the OCLTT (Pörtner & Farrell, 2008).
475 Changes in the metabolic index proposed as a physiological constraint by Deutsch *et*
476 *al.* (2015) suggest that despite increases in MMR and aerobic scope future climate
477 change may result in conditions which will begin to constrain growth and
478 reproduction of sea bass in areas where temperatures increase above 22 °C.
479 However, there is a vital need for increased research to link changes in aerobic
480 scope to population relevant metrics such as growth and reproduction to better

481 assess what environmental impacts on aerobic performance may mean for wider
482 populations.

483 5. Materials & Methods

484 5.1. Animal Collection and Husbandry

485 We collected juvenile sea bass from estuaries and coastal lagoons on the south
486 Dorset coast and Isle of Wight in June 2017. Fish were held in a marine recirculating
487 aquaculture system (RAS) at the University of Exeter for 332 days before
488 experimental work began (see water chemistry data in Table 1). Sea bass were fed a
489 diet of commercial pellet (Horizon 80, Skretting) at a ration of ~1-2 % body mass
490 three times per week and supplemented with ~1 % body mass of chopped mussel
491 (*Mytilus edulis*) once per week. All experimental procedures were carried out under a
492 UK Home Office licence (P88687E07) and approved by the University of Exeter's
493 Animal Welfare and Ethical Review Board.

494 **Table 1:** Water chemistry parameters of the recirculating aquaculture system in
495 which sea bass were held prior to experimental work beginning (means \pm S.D.
496 shown). Fish were initially held at 15 °C before stock systems were raised to 18 °C
497 approximately 6 months prior to experimental work beginning. The temperature
498 shown in the table represents the mean temperature for the entire time fish were
499 held in the RAS prior to experimental trials beginning.

Time in system (days)	Temperature (°C)	pH (NBS scale)	Salinity	Total Alkalinity (μM)	pCO₂ (μatm)
332	17.1 \pm 1.4	8.02 \pm 0.05	32.98 \pm	2072.0 \pm	534.0 \pm

0.65 130.2 60.2

500

501 5.2. Treatment conditions

502 Sea bass were transferred to an experimental RAS, in a temperature-controlled
503 room for a minimum of 14 days acclimation to treatment conditions (Figure 6). Six
504 treatment conditions were used combining three temperatures and two CO₂ levels in
505 a three x two factorial design (Table 2). Temperature treatments (14, 18 and 22 °C)
506 were chosen to reflect temperature ranges in coastal UK waters from spring to
507 autumn as well as potential future summer temperatures at the end of the century
508 (IPCC, 2014; Tinker et al., 2020). CO₂ treatments (~400 & ~1000 µatm) were chosen
509 to reflect annual average ambient atmospheric CO₂ levels currently and possible
510 end-of-century ambient atmospheric levels according to an RCP 8.5 scenario (IPCC,
511 2014). Sea bass were transferred to the experimental RAS at 18 °C before
512 temperatures were adjusted at a rate of 2 °C per day to reach treatment conditions
513 (i.e. 14 or 22 °C). A header tank (~500 L) in the experimental RAS was used to
514 adjust CO₂ to the desired level for each treatment before entering the treatment tank
515 which contained the sea bass. For present day CO₂ treatments (~400 µatm) the
516 header tank was aerated using CO₂ scrubbed air to remove excess CO₂ added to
517 the RAS by the biological filters. For end of century CO₂ treatments (~1000 µatm) an
518 Aqua Medic pH computer was used to adjust RAS water to an appropriate pH (7.8).
519 Additionally, treatment tanks were aerated with a gas mix with the appropriate CO₂
520 content for each treatment.

521 Measurements of treatment tank pH (NBS scale), salinity, temperature, and a 12
522 mL water sample to measure Dissolved Inorganic Carbon (DIC) were taken every 2-

523 3 days. Seawater DIC analysis was conducted using a custom built system
524 described in detail by Lewis *et al.* (2013). Data for pH, salinity, temperature and DIC
525 were then input into the seawater carbon calculator programme, CO2SYS (Pierrot *et*
526 *al.*, 2006) to calculate $p\text{CO}_2$ based on the equilibration constants refitted by Dickson
527 and Millero (1987), and KSO_4 dissociation constants from Dickson (1990) (Table 2).

528 **Table 2:** Mean \pm S.D. of water chemistry parameters in treatment tanks during
529 treatments at 3 temperature (14, 18, and 22 °C) and 2 CO_2 levels (~400 and ~1000
530 μatm). Treatment order represents time course of treatments (i.e. treatment 1 was
531 conducted first and treatment 6 last). Total Alkalinity (TA) varied somewhat over time
532 as a result of biological activity and so was adjusted periodically by addition of 1.0 M
533 NaHCO_3 to restore TA levels back to $>2000 \mu\text{M}$.

Parameter	Treatment					
	1	2	3	4	5	6
Temperature (°C)	17.9 \pm 0.0	22.0 \pm 0.0	13.9 \pm 0.1	21.7 \pm 0.5	13.9 \pm 0.1	18.0 \pm 0.1
$p\text{CO}_2$ (μatm)	460 \pm 34	375 \pm 28	333 \pm 38	1065 \pm 172	1057 \pm 65	973 \pm 114
pH (NBS)	8.06 \pm 0.03	8.12 \pm 0.02	8.14 \pm 0.04	7.79 \pm 0.03	7.81 \pm 0.04	7.83 \pm 0.05
Salinity	32.9 \pm 0.6	32.5 \pm 2.9	33.3 \pm 0.3	32.6 \pm 0.3	34.6 \pm 0.5	33.8 \pm 0.6
TA (μM)	1935 \pm 93	1745 \pm 26	1861 \pm 88	2147 \pm 292	2393 \pm 188	2170 \pm 20

535 5.3. Respirometry measurements (SMR, MMR, and O_{2crit})

536 Rates of oxygen consumption ($\dot{M}O_2$) were made as a proxy of metabolic rate
537 using an intermittent-flow respirometer system, details of which can be found in
538 Montgomery *et al.* (2019), set-up following recommendations by Svendsen *et al.*
539 (2016). Sea bass were starved for 72 hours prior to the start of measurements to
540 ensure that metabolism was not affected by the specific dynamic action of digestion
541 (Chabot *et al.*, 2016). Individual sea bass were then transferred to respirometer
542 chambers and left to acclimate for a minimum of 12 hours overnight before
543 measurements of $\dot{M}O_2$ began. For each treatment all respirometry measurements
544 were conducted in two groups (hereafter referred to as respirometry group), with five
545 fish being measured simultaneously for each group. Following the 12 hour
546 acclimation period we measured $\dot{M}O_2$ of each sea bass for ~3-4 hours (from ~6 am
547 to ~10 am) before hypoxia tolerance was assessed using a critical O_2 tension (O_{2crit})
548 trial (Figure 6), following protocols set out in Montgomery *et al.* (2019). Carbon
549 dioxide levels in the water were simultaneously increased as O_2 declined during O_{2crit}
550 trials to reflect the natural rise in CO_2 during hypoxic events in aquatic systems
551 (Melzner *et al.*, 2013; Montgomery *et al.*, 2019). During O_{2crit} trials water pH,
552 temperature, salinity, and DIC were measured every hour to calculate water
553 carbonate chemistry. Changes in system pCO_2 and pH during O_{2crit} trials for each
554 treatment are given in supplementary materials (Table S1).

555 O_{2crit} trials were stopped once a minimum of three consecutive $\dot{M}O_2$
556 measurements showed a transition from an oxy-regulating to oxy-conforming state
557 for each fish. Following completion of O_{2crit} trials the respirometer system was
558 aerated with ambient air (CO_2 ~400 μatm) or a 0.1 % CO_2 in air gas mix (CO_2 ~1000
559 μatm) to rapidly restore O_2 levels to normoxia and CO_2 to the appropriate treatment

560 level. Sea bass were left to recover in respirometers, for a minimum of one hour
561 post-trial, until O_2 levels reached ~ 21 kPa O_2 (~ 100 % air saturation) before
562 removing the fish and measuring background respiration for a minimum of one hour
563 (six measurement cycles) for all respirometers immediately post trial. Each sea bass
564 was then placed in an individual ~ 10 L isolation tank which was subsequently fed by
565 the respirometry system sump (at a rate of ~ 4 L min^{-1}) to maintain treatment
566 conditions (with overflowing water from the isolation tanks recirculating back to the
567 sump).

568 After fish had rested overnight in isolation tanks, MMR was measured for each
569 fish (using an exhaustive chase protocol; Norin and Clark, 2016) on three
570 consecutive days (with overnight recovery in between) at three different levels of O_2
571 (100, 60 and 30 % air saturation) with increasing CO_2 levels for each O_2 level as
572 detailed for $O_{2\text{crit}}$ trials (Figure 6). The appropriate O_2 and CO_2 level was achieved by
573 aerating isolation boxes with a mix of N_2 , O_2 and CO_2 (G400 Gas mixing system,
574 Qubit Biology Inc.) at a rate of 5 L min^{-1} . Fish were exposed to the new O_2 and CO_2
575 conditions for ~ 2 hours before chase protocols were conducted. Chase protocols and
576 subsequent respirometry measurements were conducted at the appropriate
577 temperature, O_2 , and CO_2 conditions for each treatment. Measurements of water pH,
578 temperature, salinity, and DIC were taken for each isolation tank, the chase tank
579 (after all fish were chased) and the respirometer system (during $\dot{M}O_2$ measurements)
580 to calculate water carbonate chemistry. Temperature, O_2 , and CO_2 conditions for all
581 MMR trials are given in Table S2.

582 For all $\dot{M}O_2$ measurements dissolved O_2 concentration (% air saturation) was
583 measured continuously (frequency ~ 1 Hz) in respirometer chambers using a fibre
584 optic O_2 optode mounted in the recirculation loop of the respirometer chamber.

585 These optodes were linked to two Firesting Optical Oxygen Meters (Pyro Science,
586 Aachen, Germany) which were connected to a PC running AquaResp 3 software
587 which automatically logged all measurements.

588 $\dot{M}O_2$ was automatically calculated by the AquaResp3 software by fitting a linear
589 regression to the O_2 versus time data for each measurement period. The slope (s) of
590 this regression ($kPa O_2 h^{-1}$) was then used to calculate $\dot{M}O_2$ ($mg O_2 kg^{-1} h^{-1}$) using
591 the equation outlined by Svendsen *et al.*(2016):

592

$$593 \quad \dot{M}O_2 = sV_{resp}\alpha m^{-1}$$

594

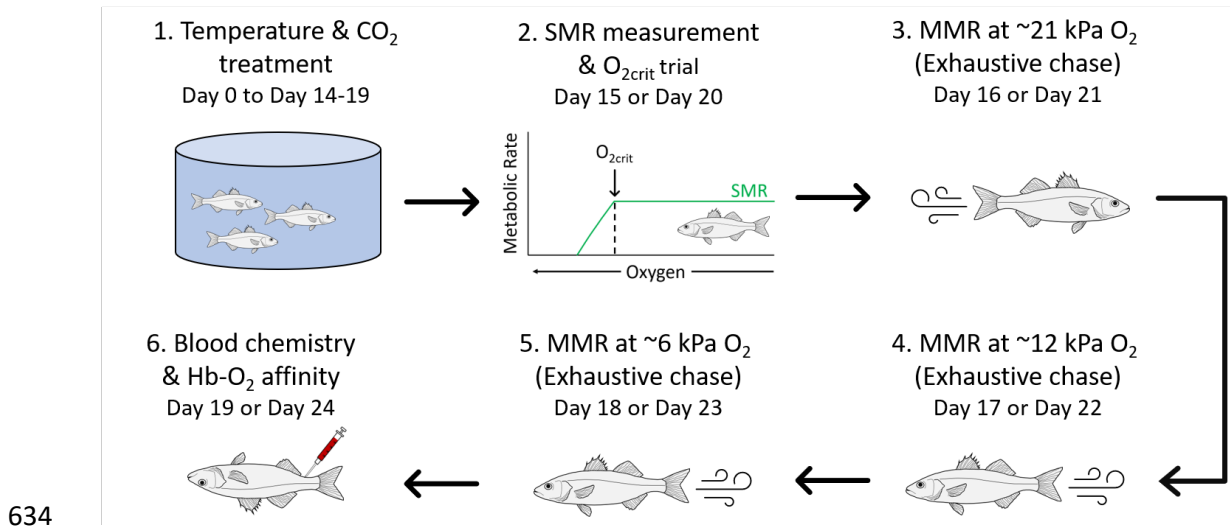
595 where V_{resp} is the respirometer volume minus the volume of the fish (L), α is the
596 solubility of O_2 in water ($mgO_2 L^{-1} kPa^{-1}$) for the relevant salinity and temperature,
597 and m is the mass of the fish (kg). For the purpose of establishing the impacts of
598 reduced O_2 on MMR and determining O_{2crit} values, the O_2 level of each
599 measurement period was defined as the mean dissolved O_2 measurement over the
600 measurement period. The mean background respiration for each respirometer over
601 the 1 hour post-trial measuring period (average was $< 2\%$ of fish $\dot{M}O_2$) was
602 subtracted from $\dot{M}O_2$ measurements. Background corrected $\dot{M}O_2$ was then scaled to
603 an average individual mass of 120 g using a mass exponent of 0.89 prior to
604 subsequent analysis (Jerde *et al.*, 2019).

605 We calculated SMR of each fish as the mean of the lowest 10 $\dot{M}O_2$
606 measurements from the ~3-4 hour period prior to O_{2crit} trials in which mean dissolved
607 O_2 saturation was $>80\%$ air saturation. The critical O_2 tension (O_{2crit}) of each
608 individual fish was then calculated using $\dot{M}O_2$ measurements from O_{2crit} trials with

609 function 'calcO2crit' from package 'fishMO2' (Chabot *et al.* 2016) in R v.3.6.3 (R
610 Core Team, 2020), using the estimated SMR of each individual, as detailed in the
611 supplementary material of Claireaux & Chabot (2016). Finally, we defined MMR as
612 the single highest measurement of $\dot{M}O_2$ in the one hour period immediately following
613 exercise to exhaustion (Norin & Clark, 2016). This point usually occurred during the
614 first measurement period immediately after each fish was moved to the respirometer
615 chamber i.e. ~ 2-5 minutes after the cessation of the chase protocol. However, for
616 some fish in normoxia spontaneous activity inside the respirometer chamber during
617 SMR or O_{2crit} trials resulted in instantaneous measurements of $\dot{M}O_2$ higher than
618 those noted following chase protocols. In these occasions this higher value of $\dot{M}O_2$
619 was used as the estimated MMR for that fish (n = 2 out of 65 fish).

620 5.4. Blood chemistry and Hb-O₂ affinity measurements

621 Following MMR measurements, sea bass were left overnight in the isolation
622 boxes before blood samples were taken (Figure 6), following methods outlined in
623 Montgomery *et al.* (2019), from each fish in normoxic conditions and at the relevant
624 treatment temperature and CO₂ level (Table S3). We then measured extracellular pH
625 (pH_e), haematocrit (Hct), TCO₂, haemoglobin content (Hb), plasma glucose, and
626 plasma lactate and calculated pCO_2 and HCO₃⁻ following methods detailed in
627 Montgomery *et al.* (2019). We also followed the freeze-and-thaw method to measure
628 intracellular pH of RBCs (pH_i) as described by Zeidler and Kim (1977), and validated
629 by Baker *et al.* (2009). All measurements or storage of blood for subsequent analysis
630 occurred within 10 minutes of blood sampling. Finally, we measured Hb-O₂ affinity
631 using a Blood Oxygen Binding System (BOBS, Loligo systems), detailed in general
632 in Oellermann *et al.* (2014) and specifically for fish blood in Montgomery *et al.*
633 (2019).



635 Figure 6: Summary of the timeline over which experimental end points were
636 measured. Sea bass were acclimated for a minimum of 14 days to a temperature
637 (14, 18, 22 °C) and CO₂ (~400 µatm or ~1000 µatm ambient CO₂) treatment before
638 measurements of SMR/O_{2crit}, MMR (at ~21 kPa, ~12 kPa, and ~6 kPa O₂), and blood
639 chemistry/Hb-O₂ affinity were obtained on consecutive days for each individual.

640 5.5. Statistical Analysis

641 5.5.1. Respirometry data analysis

642 We conducted all statistical analysis in R v3.6.3 (R Core Team, 2020). Results
643 are reported as mean ± S.E unless otherwise stated. Sample sizes for respirometry
644 data can be seen in Table S4. The effects of temperature, O₂, and CO₂ on individual
645 physiological performance metrics (SMR, MMR, and O_{2crit}) were analysed using
646 separate general linear mixed-effects models (GLMM) in package 'lme4' (Bates et
647 al., 2015; Pinheiro et al., 2018). All models included respirometry group as a random
648 intercept term to account for potential tank effects introduced during respirometry
649 measurements. For each physiological metric the best supported model was
650 determined as the model with the lowest corrected Akaike's information criterion,
651 AICc (Burnham & Anderson, 1998; Hurvich & Tsai, 1989). Residual diagnostic plots

652 of each GLMM were then assessed using package ‘DHARMA’ to confirm validity of
653 model fit (Hartig, 2020). Once the best supported model for each physiological
654 parameter was identified (see Table S5 for model comparisons) predictions were
655 made across a range of temperatures, O₂ levels, and CO₂ levels to visualise
656 combined effects of these variables on the physiology of seabass. We then used
657 function bootMer from lme4 (Pinheiro et al., 2018) to calculate 95 % confidence
658 intervals of model predictions.

659 5.5.2. Blood chemistry and Hb-O₂ affinity data analysis

660 Measurements of blood chemistry parameters (pH_e, pH_i, pCO₂, HCO₃⁻, P₅₀, Hills’
661 number, Hct, Hb, lactate, and glucose) were analysed using the ambient water
662 temperature of the treatment and using a categorical CO₂ level of low (i.e. ~400 µatm
663 treatment) or high (i.e. ~1000 µatm treatment). Measurements were analysed using
664 a type III sum of squares two-way ANOVA (to account for unequal sample sizes).
665 Post hoc-tests were then conducted on least-square means generated by package
666 ‘emmeans’ (Lenth, 2020), with Tukey adjusted p-values for multiple comparisons.
667 Model residuals from analysis of plasma lactate and glucose measurements did not
668 meet the assumptions of normality or equal variances required by two-way ANOVA,
669 as such this data was analysed using the alternative non-parametric Kruskal-wallis
670 test.

671 Blood-oxygen binding parameters (P₅₀ and Hills’ number) could not be obtained
672 for fish in the 14 °C and high CO₂ treatment as a result of an equipment failure. As
673 such these data were analysed using a one-way ANOVA. If statistical assumptions
674 of one-way ANOVA were not met then data were analysed using the non-parametric
675 Kruskal-Wallis test. Sample sizes for blood chemistry data can be seen in Table S4.

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690 assistance with blood chemistry measurements.

691 Competing Interests

692 The authors declare no competing interests.

693 Data availability

694 Data will be made publicly available via the University of Exeter's online repository if
695 the manuscript is accepted: <https://ore.exeter.ac.uk/repository/>

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995 **Supplementary materials**

996 **Table S1:** Measurements of $p\text{CO}_2$ (μatm , top row) and pH (NBS scale, bottom row)
 997 as O_2 declined during $\text{O}_{2\text{crit}}$ trials for each treatment. All values are mean \pm S.D. apart
 998 from for Treatment 3 at $\sim 30\%$ dissolved O_2 where only one measurement of $p\text{CO}_2$
 999 and pH was made and calculations of mean and S.D. were not possible

~Dissolved O_2 (% air saturation)	Treatment					
	14 °C		18 °C		22 °C	
	Present CO_2	Present CO_2	Present CO_2	Future CO_2	Future CO_2	Future CO_2
100	377 \pm 23	483 \pm 20	455 \pm 35	1019 \pm 50	1070 \pm 10	1172 \pm 89
	8.14 \pm 0.02	8.09 \pm 0.02	8.10 \pm 0.04	7.79 \pm 0.01	7.78 \pm 0.00	7.80 \pm 0.03
80	418 \pm 9	508 \pm 10	486 \pm 47	1149 \pm 2	1156 \pm 86	1209 \pm 18
	8.10 \pm 0.01	8.07 \pm 0.01	8.07 \pm 0.04	7.74 \pm 0.01	7.75 \pm 0.02	7.79 \pm 0.02
60	477 \pm 25	613 \pm 90	497 \pm 23	1383 \pm 125	1315 \pm 73	1335 \pm 92
	8.05 \pm 0.02	7.99 \pm 0.01	8.07 \pm 0.02	7.67 \pm 0.02	7.69 \pm 0.02	7.75 \pm 0.03
40	625 \pm 44	746 \pm 40	577 \pm 46	1637 \pm 83	1488 \pm 24	1658 \pm 237
	7.94 \pm 0.03	7.92 \pm 0.02	8.01 \pm 0.04	7.59 \pm 0.01	7.64 \pm 0.00	7.66 \pm 0.05
30	822	1104 \pm 155	647 \pm 18	1973 \pm 159	1964 \pm 134	2179 \pm 63
	7.83	7.76 \pm 0.06	7.96 \pm 0.01	7.51 \pm 0.04	7.53 \pm 0.02	7.55 \pm 0.01
20	1162 \pm 2	2103 \pm 79	808 \pm 35	2491 \pm 238	2416 \pm 265	2547 \pm 266
	7.69 \pm 0.00	7.50 \pm 0.02	7.88 \pm 0.02	7.42 \pm 0.05	7.45 \pm 0.03	7.48 \pm 0.06
15	1734 \pm 92	2431 \pm 663	1318 \pm 108	3099 \pm 448	2579 \pm 4	3380 \pm 354
	7.53 \pm 0.02	7.45 \pm 0.11	7.68 \pm 0.04	7.32 \pm 0.07	7.41 \pm 0.00	7.36 \pm 0.06

1000 **Table S2:** Temperature (°C), pCO₂ (µatm), and O₂ (kPa) conditions sea bass were exposed to pre-chase, during chase, and when recovering from exercise during measurements of MMR. All
 1001 values are presented as mean ± S.E. - indicates measurements for which data was not collected. For the 14 °C present day CO₂ treatment missing pre-chase values would be expected to be
 1002 extremely similar to values recorded during chase as shown by values for all other treatments.

		Treatment																	
		14 °C Present CO ₂			18 °C Present CO ₂			22 °C Present CO ₂			14 °C Future CO ₂			18 °C Future CO ₂			22 °C Future CO ₂		
		Pre- chase	During chase	Recovery	Pre- chase	During chase	Recovery	Pre- chase	During chase	Recovery	Pre- chase	During chase	Recovery	Pre- chase	During chase	Recovery	Pre- chase	During chase	Recovery
~100 % air saturation	Temp (°C)	13.9 ± 0.1	13.9 ± 0.1	13.9 ± 0.1	-	18.3 ± 0.5	18.3 ± 0.5	22.0 ± 0.0	21.8 ± 0.1	22.0 ± 0.0	13.9 ± 0.0	14.2 ± 0.5	13.9 ± 0.1	18.0 ± 0.1	18.5 ± 0.0	18.0 ± 0.2	21.6 ± 0.1	22.3 ± 1.1	21.9 ± 0.1
	pCO ₂ (µatm)	-	-	364 ± 12	-	522 ± 105	522 ± 105	-	-	404 ± 0	1056 ± 27	1148 ± 171	1040 ± 4	1085 ± 57	1129 ± 44	1195 ± 44	1092 ± 27	1168 ± 177	1100 ± 52
	O ₂ (kPa)	-	-	19.6 ± 0.1	20.8	20.7	19.3 ± 0.2	-	-	18.8 ± 0.2	20.0 ± 0.3	20.7 ± 0.2	19.2 ± 0.6	20.8 ± 0.4	20.4 ± 0.6	19.1 ± 0.3	20.5 ± 0.2	20.3 ± 0.1	19.2 ± 0.9
~60 % air saturation	Temp (°C)	13.3 ± 0.1	13.6 ± 0.1	13.9 ± 0.1	-	17.7 ± 0.1	18.7 ± 0.1	20.5 ± 0.1	21.4 ± 0.2	22.0 ± 0.0	13.7 ± 0.2	13.8 ± 0.4	14.0 ± 0.2	17.5 ± 0.2	17.7 ± 0.2	18.0 ± 0.1	21.2 ± 0.4	21.2 ± 0.3	21.9 ± 0.1
	pCO ₂ (µatm)	542 ± 67	-	501 ± 69	-	619 ± 98	674 ± 37	554 ± 25	-	579 ± 93	1533 ± 62	1577 ± 67	1528 ± 44	1457 ± 137	1486 ± 168	1540 ± 38	1468 ± 67	1436 ± 58	1468 ± 60
	O ₂ (kPa)	13.9 ± 0.3	14.5 ± 0.2	12.0 ± 0.1	14.3 ± 0.3	14.3 ± 0.6	11.8 ± 1.0	14.3 ± 0.2	14.8 ± 0.0	11.6 ± 0.2	14.5 ± 0.2	14.4 ± 0.0	12.0 ± 0.1	14.4 ± 0.3	14.4 ± 0.2	11.9 ± 0.2	14.5 ± 0.2	14.5 ± 0.1	11.5 ± 0.1
~30 % air saturation	Temp (°C)	13.1 ± 0.2	13.4 ± 0.0	13.8 ± 0.1	-	17.6 ± 0.5	18.7 ± 0.1	20.5 ± 0.1	21.4 ± 0.1	22.0 ± 0.0	12.9 ± 0.2	13.1 ± 0.6	13.9 ± 0.1	17.6 ± 0.0	17.6 ± 0.1	18.3 ± 0.2	20.9 ± 0.1	20.9 ± 0.1	21.9 ± 0.1
	pCO ₂ (µatm)	1173 ± 95	-	839 ± 53	-	854 ± 40	793 ± 118	929 ± 85	-	898 ± 101	2575 ± 243	2467 ± 110	2219 ± 216	2213 ± 135	2278 ± 179	2123 ± 272	2400 ± 126	2399 ± 34	2027 ± 100
	O ₂ (kPa)	8.3 ± 0.3	8.8 ± 0.0	6.1 ± 0.1	8.4 ± 0.5	8.6 ± 0.3	6.8 ± 0.8	8.5 ± 0.3	8.8 ± 0.2	6.6 ± 0.2	9.1 ± 0.3	8.9 ± 0.0	6.7 ± 0.1	8.8 ± 0.4	9.1 ± 0.1	6.4 ± 0.3	8.7 ± 0.5	8.7 ± 0.3	6.4 ± 0.5

1003 **Table S3:** Temperature and $p\text{CO}_2$ levels fish were exposed to when they were
 1004 anaesthetised immediately prior to blood sampling. All measures are given as
 1005 mean \pm S.D.

Parameter	Treatment					
	14 °C	18 °C	22 °C	14 °C	18 °C	22 °C
	Present CO ₂	Present CO ₂	Present CO ₂	Future CO ₂	Future CO ₂	Future CO ₂
Temperature (°C)	13.1 \pm 0.1	17.4 \pm 0.1	21.2 \pm 0.3	13.3 \pm 0.3	17.8 \pm 0.1	21.3 \pm 0.2
$p\text{CO}_2$ (μatm)	350 \pm 13	445 \pm 52	398 \pm 28	1130 \pm 33	1103 \pm 47	1221 \pm 35

1006

1007 **Table S4:** Samples sizes for each measurement in all treatments. We were
 1008 unable to make measurements of P₅₀ or Hills number for the 14 °C future CO₂
 1009 scenario due to an equipment failure.

Measurement	Treatment					
	14 °C	18 °C	22 °C	14 °C	18 °C	22 °C
	Present CO ₂	Present CO ₂	Present CO ₂	Future CO ₂	Future CO ₂	Future CO ₂
SMR	10	10	10	10	10	10
O _{2crit}	10	10	10	10	10	10
MMR at ~21 kPa O ₂	9	10	10	12	10	10
MMR at ~12 kPa O ₂	10	7	10	10	10	10
MMR at ~6 kPa O ₂	10	7	10	10	10	10
Blood pH _e	10	6	9	10	9	7
Plasma $p\text{CO}_2$	10	6	9	10	9	7
Plasma HCO ₃ ⁻	10	6	9	10	9	7
RBC pH _i	10	6	8	9	9	7
P ₅₀	10	6	9	NA	8	7
Hills number	10	6	9	NA	8	7
Haematocrit	10	6	9	10	9	7
Haemoglobin	7	6	9	10	9	7
Plasma lactate	9	6	9	7	8	7
Plasma glucose	9	6	10	7	8	7

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1013 **Table S5:** Structure of models and model comparison results explaining
 1014 variation in SMR, O_{2crit} , and MMR of sea bass exposed to differing combinations
 1015 of temperature, O_2 , and CO_2 . The best supported model for each response
 1016 variable is highlighted in bold.

Response Variable	Explanatory Variables Structure	AICc	Δ AICc	d.f.	
SMR	Temperature + CO_2	546.3	0	5	
	Temperature	548.2	1.9	4	
	Temperature* CO_2	548.7	2.4	6	
	CO_2	578.4	32.1	4	
O_{2crit}	Temperature + CO_2 + SMR	72.7	0	6	
	Temperature * SMR + CO_2	75.2	2.5	7	
	Temperature * CO_2 + SMR	75.2	2.5	7	
	Temperature + CO_2 * SMR	75.3	2.6	7	
	Temperature + SMR	76.2	3.5	5	
	Temperature*SMR	78.6	5.9	6	
	Temperature * CO_2 * SMR	83.0	10.3	10	
	SMR	84.0	11.3	4	
	SMR + CO_2	84.8	12.1	5	
	SMR* CO_2	87.2	14.5	6	
	Temperature	134.2	61.5	4	
	Temperature + CO_2	136.4	63.7	5	
	Temperature* CO_2	138.8	66.1	6	
	CO_2	144.0	71.3	4	
	MMR	CO_2 + Temperature*O_2 + Temperature*(O_2)²	2240.8	0	12
		CO_2 *Temperature* O_2 + Temperature*(O_2) ²	2243.0	2.2	15
CO_2 + Temperature* O_2 + (O_2) ²		2247.5	6.7	11	
CO_2 *Temperature* O_2 + (O_2) ²		2248.8	8.0	14	
Temperature + CO_2 * O_2 + CO_2 *(O_2) ²		2280.1	39.3	12	
CO_2 *Temperature* O_2		2349.8	109.0	13	
Temperature + CO_2 * O_2		2360.7	119.9	10	
CO_2 + Temperature* O_2		2362.8	122.0	10	
CO_2 * O_2		2365.1	124.3	9	
Temperature* CO_2 + O_2		2371.4	130.6	10	
Temperature + CO_2 + O_2		2379.7	138.9	9	
Temperature + CO_2		2464.3	223.5	8	
Temperature + O_2		2464.7	223.9	8	
Temperature * CO_2		2465.4	224.6	9	
Temperature* O_2		2467.0	226.2	9	

CO ₂	2478.3	237.5	7
O ₂	2488.8	248.0	7
Temperature	2638.1	397.3	7

1017

1018 **Table S6:** General linear mixed-effects model outputs for analysis of standard
 1019 metabolic rate. The best supported model was fitted using a Gaussian
 1020 distribution and included the parameters temperature and CO₂ as explanatory
 1021 variables and group ID as a random intercept term. Parameter effects are
 1022 compared against a reference level where temperature and CO₂ are 0. Marginal
 1023 $R^2 = 0.70$, Condition $R^2 = 0.70$. Confidence intervals for each parameter were
 1024 determined from function confint in package lme4. Marginal and conditional R^2
 1025 of the model were determined using function r.squaredGLMM from package
 1026 MuMIn.

Parameter	Estimate	Standard Error	Confidence Interval (95%)	t-value	Variance	Standard Deviation
Best supported model <- lmer(SMR ~Temperature + CO ₂ + (1 Group))						
Intercept	-14.77	11.62	-37.35 – 7.81	-1.27		
Temperature	6.66	0.57	5.55 – 7.78	11.61		
CO ₂	-0.012	0.006	-0.024 – -0.001	-2.04		
Group					0.0	0.0

1027

1028 **Table S7:** General linear mixed-effects model outputs for analysis of O_{2crit}. The
 1029 best supported model was fitted using a Gaussian distribution and included the
 1030 parameters temperature, CO₂, and SMR as explanatory variables and group ID
 1031 as a random intercept term. Parameter effects are compared against a
 1032 reference level where temperature, CO₂, and SMR are 0. Marginal $R^2 = 0.72$,
 1033 Condition $R^2 = 0.77$. Confidence intervals for each parameter were determined
 1034 from function confint in package lme4. Marginal and conditional R^2 of the model
 1035 were determined using function r.squaredGLMM from package MuMIn.

Parameter	Estimate	Standard Error	Confidence Interval (95%)	t-value	Variance	Standard Deviation
Best supported model <- lmer(O2crit~ Temperature+CO ₂ +SMR) + (1 Group)						
Intercept	2.174	0.421	1.401 – 2.945	5.16		
Temperature	-0.126	0.031	-0.185 – -0.068	-4.00		

CO ₂	0.0005	0.0002	0.0001 – 0.0009	10.75		
SMR	0.036	0.003	0.030 – 0.043	2.37		
Group					0.03	0.18

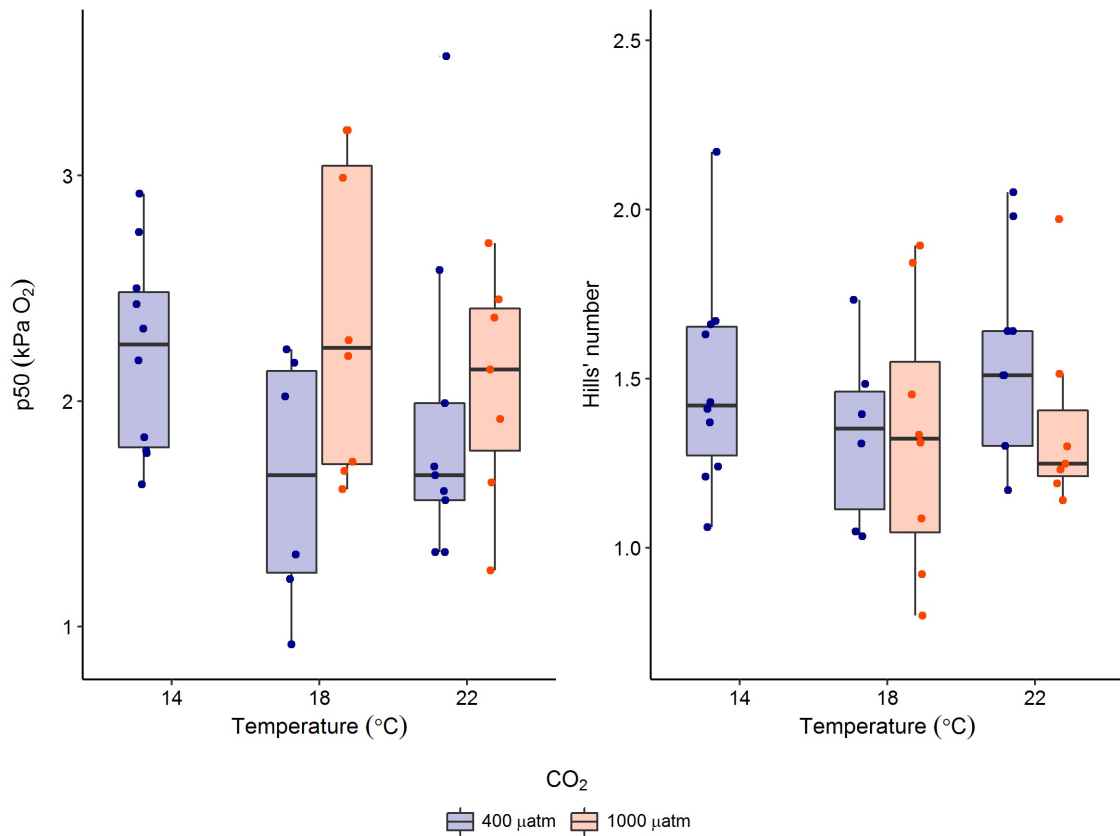
1036

1037 **Table S8:** General linear mixed model outputs for analysis of maximum
 1038 metabolic rate. The best supported model was fitted using a Gaussian
 1039 distribution and included the parameters temperature and CO₂ as explanatory
 1040 variables and group ID as a random intercept term. Parameter effects are
 1041 compared against a reference level where temperature and CO₂ are 0. Marginal
 1042 $R^2 = 0.91$, Condition $R^2 = 0.96$. Confidence intervals for each parameter were
 1043 determined from function confint in package lme4. Marginal and conditional R^2
 1044 of the model were determined using function r.squaredGLMM from package
 1045 MuMIn.

1046

Parameter	Estimate	Standard Error	Confidence Interval (95%)	t-value	Variance	Standard Deviation
Best supported model < - lmer(MMR ~ scale(Temperature)*scale(O ₂) + scale(Temperature)*scale(O ₂ ²) + scale(CO ₂) + (O ₂ Fish ID) + (1 Group)						
Intercept	231.99	2.58	227.01 – 236.98	89.96		
scale(Temperature)	32.53	2.55	27.60 – 37.46	12.76		
scale(O ₂)	240.35	9.50	221.92 – 258.74	25.31		
scale(Temperature*O ₂)	41.95	8.60	25.26 – 58.62	4.88		
scale(O ₂ ²)	-157.69	8.68	-174.50 – -140.85	-18.17		
scale(Temperature*O ₂ ²)	-25.10	8.36	-41.31 – -8.86	-3.00		
scale(CO ₂)	-7.52	2.58	-12.08 – -2.97	-3.20		
Fish ID (Intercept)					90.02	9.49
Fish ID (O ₂)					6.53	2.56
Group					4.78 x 10 ⁻⁶	0.00

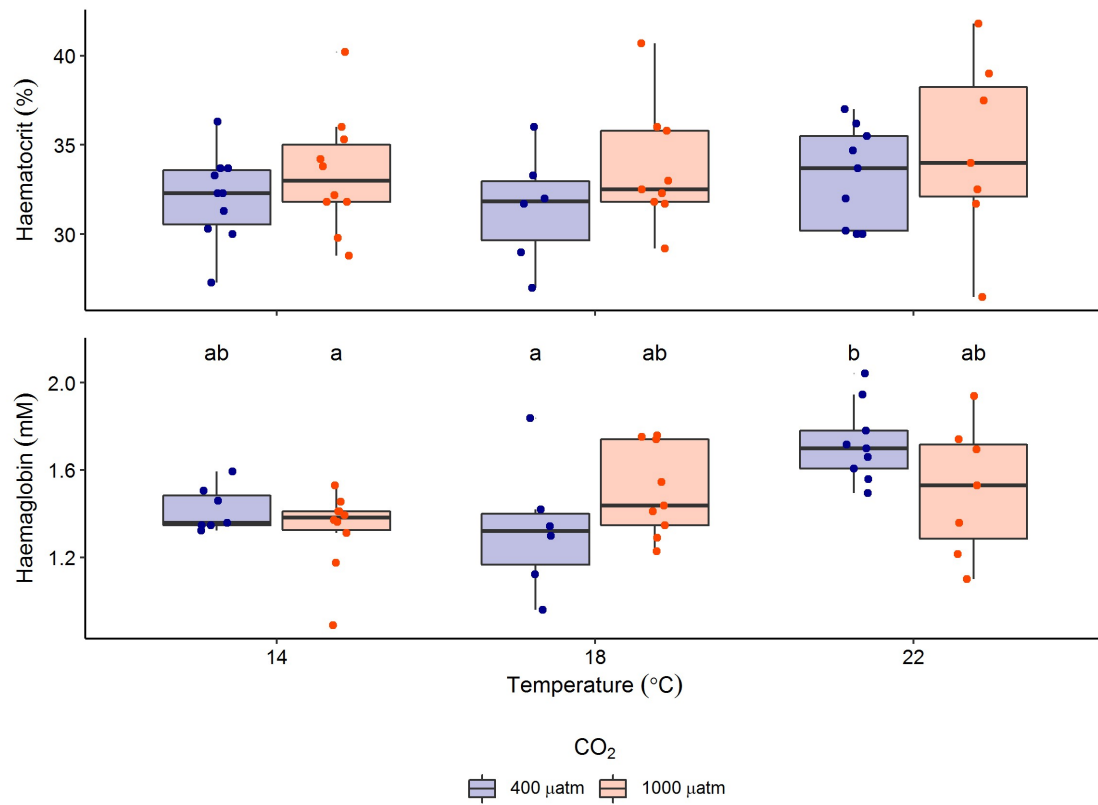
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1048

1049 **Figure S1:** No impacts of temperature and CO₂ were observed for
1050 measurements of haemoglobin-O₂ binding affinity (measured using P₅₀) and
1051 Hill's' number. Due to an equipment failure no measurements were possible
1052 during the original experimental period for fish at 14 °C exposed to ~1000 μatm
1053 CO₂.

1054



1055

1056 **Figure S2:** Impact of temperature and CO₂ on haematological parameters of
1057 sea bass. No significant difference in haematocrit were observed between any
1058 treatments. Significant difference in haemoglobin content were noted between
1059 fish sampled at different temperature and CO₂ treatments and are represented
1060 by different lower case letters.

1061