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## Respiratory plasticity during acclimation to hypoxia and following a recovery in normoxia

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### Abstract :

Phenotypic plasticity manifested after acclimatization is a very important source of biological variability among fish species. We hypothesized that hypoxic acclimation, besides potentially generating a temporary hypoxic respiratory phenotype, would also manifest as a continued benefit after re-acclimation to normoxia. Hence, we holistically characterized the respiratory phenotype of European sea bass (*Dicentrarchus labrax*) acclimated to normoxia with or without prior acclimation to hypoxia. Compared with the original normoxic phenotype, prior acclimation to hypoxia and return to normoxia produced a 27% higher absolute aerobic scope (AAS), a 24% higher citrate synthase activity in red muscle and a 28% lower excess post-exercise O<sub>2</sub> consumption. Additional testing of hypoxia-acclimated fish under normoxia explored the specific effects of hypoxic acclimation. The hypoxic phenotype, when compared with the original normoxic phenotype, had a lower standard metabolic rate, a better hypoxia performance and a lower minimum PO<sub>2</sub> for supporting 50% AAS. Given this respiratory malleability, general predictions for marine fish exploiting a more hypoxic future should better consider respiratory plasticity and prolonged effects of hypoxic exposures.

48 **Introduction**

49 Animals respond to environmental change either by moving to a more favourable location, by  
50 changing their phenotype (acclimatization), or potentially by evolving over multiple generations  
51 (genetic adaptation). Yet, the drastic decrease in dissolved oxygen (O<sub>2</sub>) availability (*i.e.*,  
52 hypoxia) during the Permian era (299-251 million years ago), which extirpated over 90% of  
53 marine fish species (Graham *et al.*, 1995; Clack, 2007), illustrates potential limits to such coping  
54 strategies. Nonetheless, fish species subsequently radiated into the most specious vertebrate

55 group, suggesting that successful and broad environmental adaptations did occur among the  
56 survivors of the Permian era.

57 Today, the Anthropocene is presenting extant marine fishes with another hypoxic  
58 challenge. Associated with global warming, the increased frequency of hypoxic episodes can be  
59 one of the key driving forces well into the future, reshaping the distribution and evolution of  
60 marine species (Deutsch *et al.*, 2015). The hypoxia-induced redistribution can be affected by the  
61 ability of aquatic ectotherms to obtain O<sub>2</sub> in the hypoxia (Seibel and Deutsch, 2020; Seibel *et al.*,  
62 2021). With this prospect, a key question concerning the biodiversity of marine fishes is how  
63 plastic is the respiratory phenotype of marine fishes? We define respiratory phenotype as a suite  
64 of respiratory performance metrics that characterize whole-animal aerobic and glycolytic  
65 metabolism.

66 In nature, ample opportunities exist for fishes to manifest new respiratory phenotypes that  
67 preserve their capacity to sustain their activities. These opportunities are the daily and seasonal  
68 environmental cycles experienced by fish in freshwater (Morash *et al.*, 2018), intertidal (Somero,  
69 2002) and marine (Drinkwater *et al.*, 2003) ecosystems, as well as diurnal or seasonal migrations  
70 into hypoxic zones of certain marine fishes for foraging (Douglas *et al.*, 1976; Gjøsaeter, 1984;  
71 MacKenzie and Mariani, 2012). In the present study, we were particularly interested in  
72 examining the aerobic performance of fish, the development of a hypoxic phenotypic and its  
73 reversibility, *i.e.*, would a fish restore its original normoxic respiratory phenotype after being  
74 returned to normoxia following hypoxic acclimation, or would a new phenotype emerge?

75 Phenotypic plasticity in response to hypoxia can occur rapidly in fishes and enhances  
76 aerobic performance under hypoxic conditions (*see summaries* by Wang *et al.*, 2009; Gamperl  
77 and Driedzic, 2009; Richards, 2009). The high-latitude minnow (*Rhynchocypris lagowskii*

78 Dybowski, 1869), for example, remodels its entire gill structure after just a 30-min hypoxic  
79 exposure (Yang *et al.*, 2021). Similarly, the mangrove rivulus (*Kryptolebias marmoratus* Poey,  
80 1880) remodels epidermal ionocytes and respiratory traits after just 24 h of air exposure  
81 (Blanchard *et al.*, 2019; Dong *et al.*, 2021). Moreover, a brief ischemic period can precondition  
82 fish cardiac myocytes to help maintain stroke volume and cardiac output, can induce cardiac  
83 hypertrophy and can enhance the sarcolemmal ATP-sensitive K<sup>+</sup> channels, helping fish to  
84 perform better in a subsequent hypoxic episode (Gamperl *et al.*, 2001b; Gillis and Johnston,  
85 2017; Carnevale *et al.*, 2021). Hence, we hypothesized that hypoxic acclimation, besides  
86 generating a hypoxic respiratory phenotype, would also provide respiratory benefits upon re-  
87 acclimation to normoxia. Our broader objective was to contribute to understanding how marine  
88 fishes might respond to seasonal O<sub>2</sub> cycling and thereby better predict future distributions of  
89 marine fish species.

90 Our model species was European sea bass (*Dicentrarchus labrax* Linnaeus, 1758)  
91 because it has an active lifestyle and naturally exploits hypoxic habitats. Adults require a high  
92 aerobic capacity to capture prey in hydraulically dynamic coastal waters (Pickett and Pawson,  
93 1994), while juveniles successfully exploit hypoxic estuaries and coastal lagoons. Indeed, their  
94 hemoglobin-O<sub>2</sub> affinity ( $P_{50} = \sim 1.7$  kPa; Pichavant *et al.*, 2003), which lies between that of the  
95 hypoxia-tolerant common carp ( $P_{50} = 0.9$  kPa; Roy and Lykkeboe, 1978) and that of the active,  
96 hypoxia-sensitive rainbow trout ( $P_{50} = 2.9$  kPa; Weber *et al.*, 1976), suggests a moderate hypoxia  
97 tolerance. Furthermore, when ambient O<sub>2</sub> was cycled between 8.3 and 17.8 kPa (Thetmeyer *et*  
98 *al.*, 1999), growth rate and feed conversion efficiency were preserved over a 4-week period. In  
99 fact, sea bass held at 10.4 kPa ambient O<sub>2</sub> [50 % saturation (% sat.) at 20 °C] displayed a full

100 postprandial peak O<sub>2</sub> uptake (Zambonino-Infante *et al.*, 2017). Thus, hypoxia acclimation of the  
101 present study used 50 % sat. for the maintenance of normal growth during hypoxia.

102 Our hypothesis was tested by returning European sea bass that had been acclimated to a  
103 stable hypoxic environment back into their original normoxic environment. We asked whether  
104 they simply reverted to the original normoxic phenotype, or whether a new normoxic phenotype  
105 would emerge (Fig. 1). Also, to provide clues to the mechanisms underlying any new respiratory  
106 phenotype, we tested hypoxia-acclimated fish under normoxic and hypoxic conditions. Thus, by  
107 holistically characterizing a normoxic and hypoxic phenotype might provide insights into the O<sub>2</sub>  
108 cost of breathing hypoxic water as well as establishing the nature of hypoxic acclimation. We  
109 holistically characterized the individual respiratory phenotype of sea bass using whole-animal  
110 respirometry (Claireaux and Lagardère, 1999; Svendsen *et al.*, 2016; Zhang *et al.*, 2019) and  
111 measured muscle enzymes activities of citrate synthase (CS) and lactate dehydrogenase (LDH)  
112 (Childress and Somero, 1979; Dalziel *et al.*, 2012). In addition, we generated individual hypoxic  
113 performance curves (Zhang *et al.*, 2022).

114

## 115 **Materials and methods**

### 116 **(a) Experimental animals and acclimation procedures**

117 Before experiments started, a stock of juvenile European sea bass (*Dicentrarchus labrax*,  
118 Linnaeus 1758; n = 150; Aqualstream, Lorient, France) was reared for 12 weeks under normoxic  
119 laboratory conditions in a 2000-L indoor tank in Ifremer research facilities (Plouzané, France).  
120 They were fed *ad libitum* twice weekly (Le Gouessant, Lamballe, France). Individual radio-  
121 frequency identification tags were subcutaneously implanted under anaesthesia (100 mg L<sup>-1</sup> MS-  
122 222) at the end of the sixth week of the rearing period. Fish holding and all experimental

123 procedures followed the guidelines of current animal care rules and regulations in France (Apafis  
124 2018040916374437).

125         Acclimation prior to respirometry measurements involved equally redistributing fish into  
126 two acclimation tanks (500-L) that received flow-through (300 L h<sup>-1</sup>) and thermoregulated  
127 seawater (16 °C; the average summer temperature of the species experience in the region).

128 Photoperiod was synchronized to the natural regional cycle with an adjustment to the diurnal  
129 cycle each week. One acclimation tank contained control normoxic fish that remained at a partial  
130 pressure of O<sub>2</sub> level of ~20.4 kPa (dissolved O<sub>2</sub> of ~98 % sat.). Some of these fish were tested  
131 after 4 weeks of acclimation to on-going normoxic conditions (N-N: 61.5 ± 2.0 g, n = 28). The  
132 other acclimation tank had been made progressively hypoxic at a rate of 10 % sat. h<sup>-1</sup> to 50 %  
133 sat. (10.4 kPa) using a custom-built, 50-L gas-equilibration column that was situated upstream of  
134 the aquarium and received the thermoregulated seawater into the top while nitrogen gas was  
135 injected at the bottom. The hypoxic water was maintained for six weeks for the hypoxia-  
136 acclimated group. Thereafter, some fish respirometry tests were performed either under the same  
137 hypoxic conditions (10.4 kPa; H-H; 63.6 ± 5.7 g; n = 16; Suppl. Mat.) or under normoxic  
138 conditions (H-N: 74.7 ± 4.5 g, n = 16). The remainder were returned to normoxia for four weeks  
139 for re-acclimation before being tested under normoxic conditions (HN-N: 63.3 ± 6.1 g, n = 13)  
140 and comparison with the N-N group.

141         A common acclimation period for temperate fish species is 3-4 weeks under normoxic  
142 conditions (*e.g.* Fanguie *et al.*, 2009); a new cardiac phenotype can begin to appear even after 8 h  
143 of 4 °C acclimation (Sutcliffe *et al.*, 2020; Gilbert *et al.*, 2022). Therefore, we assumed that a  
144 steady-state phenotype would emerge after a normoxic acclimation period of four weeks and be  
145 stable thereafter. The respiratory phenotype of a temperate fish can be stable for 9–18 weeks

146 under controlled laboratory conditions (Table S1; Zhang *et al.*, 2019; Polinski *et al.*, 2021;  
147 Zhang, 2021). Hypoxia, however, could slow the acclimation processes. Therefore, as a  
148 precaution, we used a 6-week hypoxic acclimation period in the event of a slower acclimation  
149 process in an oxygen limiting environment. Our reasoning was partly based on a 6-week hypoxic  
150 (~40 % sat., 8.4 kPa) acclimation period being previously used for a hypoxic phenotype of  
151 Atlantic cod (*Gadus morhua*, Linnaeus, 1758) at a colder temperature of 10 °C (Petersen and  
152 Gamperl, 2010; Petersen and Gamperl, 2011).

153

#### 154 **(b) Protocol used to characterize the individual respiratory phenotypes**

155 We followed simultaneously individual  $\dot{M}O_2$  for eight, fasted (for 48 h) fish over a 3-day period  
156 using an automatic respirometry system (Steffensen, 1989), as previously validated and  
157 described for the Integrated Respiratory Assessment Protocol (IRAP; Zhang *et al.*, 2016; 2019).  
158 Phenotyping of each treatment group (n~16) consequently involved two sets of measurements  
159 over a 5-day period using eight 2.25-L Loligo®-type respirometer chambers (water volume: fish  
160 ratio = 36:1) that were immersed in a 500-L seawater bath at an ambient water temperature of 16  
161  $\pm 0.5$  °C. This outer bath was connected via a pump to the gas-equilibrium column used to  
162 control water  $PO_2$ . Water from the outer bath (normoxic or hypoxic depending on the test  
163 conditions) was supplied to each respirometry chamber via a dedicated individual water pump.  
164 Water  $PO_2$  in each of the eight respirometers was continuously measured using an optical  $O_2$   
165 probe (Robust  $O_2$  Probe OXROB2, PyroScience GmbH, Aachen, Germany).  $\dot{M}O_2$  of each  
166 individual fish was reported on-line every 10 min (*see* Fig. 2a) by AquaResp software (Svendsen  
167 *et al.*, 2019), which used a sequential interval regression analysis for a 420-s period when the  
168 respirometer was sealed (*see* Suppl. Mat.). The remainder of the 10-min  $\dot{M}O_2$  measurement cycle

169 was taken up by a 120-s flush period (the respirometer open) and a 60-s stabilization period (the  
170 respirometer closed) prior to the actual 420-s  $\dot{M}O_2$  measurement period.

171 Each respirometry chamber was equipped with a customized chasing device (a 14-cm  
172 soft, flexible plastic strip located at the mid-point of the chamber; Zhang *et al.*, 2020). This  
173 device individually agitated fish after a  $\geq 30$ -min period of habituation to the respirometer.  
174 During the 10-min agitation period each fish become refractory and during this agitation the on-  
175 line monitoring of  $\dot{M}O_2$  revealed peaks and plateaus in  $\dot{M}O_2$  associated with activity and rest  
176 periods. Peaks in  $\dot{M}O_2$  were occasionally seen in the 10-min measurement cycle when the fish  
177 was no longer being agitated and  $\dot{M}O_2$  was in a declining phase. Maximum  $O_2$  uptake ( $\dot{M}O_{2max}$ )  
178 was determined from these peaks in  $\dot{M}O_2$  ( $\dot{M}O_{2peak}$ ) using a more precise, off-line analysis  
179 (Zhang *et al.*, 2019; Zhang and Gilbert, 2017 an iterative algorithm applied to 2-min  
180 measurement windows (Fig. S1); *see* Appendix). This method of generating an  $\dot{M}O_{2max}$  was  
181 previously validated for rainbow trout because  $\dot{M}O_{2max}$  was higher compared with a protocol that  
182 chased rainbow trout outside of the respirometer (Zhang *et al.*, 2020). Indeed, sea bass chased to  
183 exhaustion at 16 °C outside of the respirometer at the Ifremer laboratory (Brest, France) had a  
184 numerically lower  $\dot{M}O_{2max}$  ( $\sim 400$  mg  $O_2$  h<sup>-1</sup> kg<sup>-1</sup>; Zhang *et al.*, 2017) when compared with our  
185  $\dot{M}O_{2max}$  measurements (*see Results*).

186 After the agitation, we followed the decline in  $\dot{M}O_2$  of fish for about 10 h to calculate the  
187 total  $O_2$  consumed during the recovery (Zhang *et al.* 2018) and estimate the excess post-  
188 exhaustion  $O_2$  consumption (EPOC; *see* calculation in Appendix). After this recovery, each fish  
189 remained undisturbed (except for a daily visual check) for the ensuing two-day quiescent period  
190 that yielded  $\sim 240$  measurements of routine  $\dot{M}O_2$  per fish, from which standard metabolic rate  
191 (SMR) and routine metabolic rate (RMR) were estimated using established analytical



192 procedures. SMR was analysed off-line with a quantile algorithm (q0.2) (Chabot *et al.*, 2016)  
193 applied to the ~240  $\dot{M}O_2$  measurements. Absolute aerobic scope (AAS) was derived from the  
194 numerical difference between  $\dot{M}O_{2max}$  and SMR, while factorial aerobic scope (FAS) was  
195 derived from the quotient of  $\dot{M}O_{2max}$  and SMR. RMR was determined as the average of the ~240  
196  $\dot{M}O_2$  measurements and the standard deviation of an individual's RMR was used as an index of  
197 spontaneous activity, *i.e.* the more active a fish, the greater the variability of RMR measurements  
198 for an individual fish. All  $\dot{M}O_2$  values were corrected for the background  $\dot{M}O_2$ , which was  
199 measured for 20 min in each respirometer without a fish, both before and immediately after  
200 every trial. A logarithmic microbial growth model was applied to background measurement over  
201 the entire period of respirometry so that the background  $\dot{M}O_2$  could be subtracted from each  
202 relevant  $\dot{M}O_2$  measurement.

203 IRAP ended with a hypoxia challenge test (HCT) when the gas-equilibration column  
204 reduced  $PO_2$  in the outer bath initially to ~6.25 kPa (DO = ~30 % sat.) within 45 min (*i.e.*, 0.313  
205 kPa min<sup>-1</sup> or ~1.5% sat. min<sup>-1</sup>) and then at a slower rate of deoxygenation (0.0313 kPa min<sup>-1</sup> or  
206 ~0.15% sat. min<sup>-1</sup>) until the fish lost its dorso-ventral equilibrium. The incipient lethal  $O_2$  partial  
207 pressure (ILOP; Claireaux *et al.*, 2013) was assigned to the partial  $O_2$  pressure ( $PO_2$ ) when the  
208 fish first lost equilibrium. At this point, fish were immediately removed from the respirometer  
209 and successfully revived before returning them to their holding aquaria. The off-line analysis of  
210  $\dot{M}O_2$  during the HCT and as a fish became progressively hypoxic yielded the  $PO_2$  level at which  
211 SMR could no longer be maintained, the critical  $O_2$  partial pressure ( $P_{crit}$ ; see calculation in  
212 Appendix). The scope for  $O_2$  deficit (SOD) was assigned to the difference between  $P_{crit}$  and  
213 ILOP.

214 Our holistic respiratory phenotyping was based, therefore, on 10 measured or derived  
215 respiratory indices for individual fish. The respiratory phenotype of the H-N group was  
216 compared with the N-N group to ascertain the nature of the that had emerged for the hypoxic  
217 phenotype. The H-H and H-N groups were also compared (Suppl. Mat.) to understand the  
218 limiting effect of ambient hypoxia (Fry, 1971).

219 Respiratory indices were statistically compared among treatment groups (N-N, H-N and  
220 HN-N) with general linear effect models and body mass as a covariate. Logarithm  
221 transformations were needed for comparisons of the variance of RMR to meet the assumptions of  
222 normality of residuals and homoscedasticity of the variance.

### 224 (c) Protocol used for measuring an individual hypoxic performance curve

225 A hypoxic performance curve (HPC) can quantify the constraint of a progressive decrease of  
226 ambient water  $PO_2$  on  $\dot{M}O_{2max}$ , *i.e.*, the relationship between  $\dot{M}O_{2peak}$  and water  $PO_2$ . Previous  
227 studies have generated and validated an HPC for group activity of fish (Lefrancois and  
228 Claireaux, 2003) and for individual fish (Zhang *et al.*, 2021; Zhang *et al.*, 2022). We generated  
229 HPC on individual fish after normoxic acclimation (N-N; n=8) and after hypoxic acclimation (H-  
230 N; n=8). These sea bass were tested following a 7-day recovery (5 days feeding and 2 days  
231 fasting) from their IRAP test and assumed full recovery from the exhaustive and the HCT would  
232 take < 24 h (Milligan, 1996; Zhang *et al.*, 2018).

233 Fish were placed in individual respirometers, as described above, for the HPC. They  
234 habituated to the respirometer in a flush mode and received normoxic seawater (with DO =  
235 ~95 % sat., 19.8 kPa, 16 °C) for 30 min. The  $\dot{M}O_2$  measurement cycle was 5 min: a 120-s  $\dot{M}O_2$   
236 recording period, a 150-s flush period and a 30-s stabilization period to better capture  $\dot{M}O_{2peak}$ .

237 An initial agitation for 10 min generated a  $\dot{M}O_{2peak}$  under normoxic condition (again using off-  
 238 line analysis; *see* Appendix). During the ensuing 25 min, while the water  $PO_2$  was progressively  
 239 reduced with the respirometer in flush mode (*see* above), a fish would partially recover. The next  
 240  $\dot{M}O_2$  measurement cycle started 25 min after the previous measurement and at a lower  $PO_2$ ,  
 241 which was maintained while the fish was again agitated to generate a new  $\dot{M}O_{2peak}$ . This  
 242 procedure was then repeated every 10 min at a progressively lower  $PO_2$  down to 4.2 kPa (DO =  
 243 20% sat.) (*i.e.*, slightly higher than our measured  $P_{crit}$ ). The total test time of an HPC was ~125  
 244 min and yielded 11  $\dot{M}O_{2peak}$  values at progressively lower levels of water  $PO_2$ . After an HPC test,  
 245 the fish were removed from the respirometer and returned to a well-aerated aquarium where they  
 246 all recovered.

247 An individual-based HPC was based on a one-phase association regression equation  
 248 (Eqn. 1), which best modelled the relationship between the measured  $\dot{M}O_{2peak}$  and the ambient  
 249  $PO_2$  (Mueller and Seymour, 2011). We only used individual regression models that had  $0.65 \leq$   
 250  $R^2 \leq 0.99$  (three fish were rejected). Those satisfying this level of quality assurance were pooled  
 251 for averaged HPCs of the normoxia-acclimated (n=6) and hypoxia-acclimated (n=7) test groups.  
 252 Individual variation among the individual HPCs was accounted for by normalizing  $\dot{M}O_{2peak}$  as a  
 253 percentage of the individual AAS (derived from the individual  $\dot{M}O_{2peak}$  measured at normoxia).  
 254 This normalized HPC was then used to interpolate the minimum  $PO_2$  at which a fish could  
 255 generate 50% of its normoxic aerobic scope,  $P_{AAS-50}$  (Zhang *et al.*, 2022). These individual data  
 256 were used to statistically compare  $P_{AAS-50}$  normoxia-acclimated and hypoxia-acclimated test  
 257 groups with an independent sample t-test.

258

259  $y = I + (Asymptote - I) * [1 - \exp(-K * x)]$  One-phase association equation (Eqn. 1)

260

261 Where  $I$  is the intercept at the y-axis, *Asymptote* is a line that the curve continues to approach at  
262 infinity.  $I$  and *Asymptote* are expressed in the same unit as  $y$ .  $K$  is the rate constant for a  
263 hyperbolic increase.

264

#### 265 **(d) Organ and enzyme activity measurements**

266 Additional fish were directly sampled by removing them directly from the acclimation tanks  
267 (normoxic, hypoxic and re-aerated hypoxic fish) to provide representative measurements of  
268 organ size, hematology and metabolic enzyme activity of each acclimation phenotype. They  
269 were sacrificed with a blow on the head (N:  $n = 23$ ; H:  $n = 13$  and HN:  $n = 12$ ). Blood was  
270 removed immediately by caudal puncture into a heparinized syringe to determine hematocrit  
271 (Sigma 201m microhematocrit centrifuge) and hemoglobin concentration [Hb]. The [Hb] was  
272 calculated as described by Clark *et al.* (2008) from the absorbance measured in triplicates  
273 (PerkinElmer EnSpine™ 2300 Multilabel plate reader, Perkin Elmer, Turku, Finland) at 540  
274 nm for 10  $\mu$ l of blood diluted to 1 ml with a solution containing: 50 mg  $K_3Fe(CN)_6$  (Merck,  
275 Espoo, Finland), 12.5 mg KCN (Pharmakon Inc, NJ, USA), 40 mg  $KH_2PO_4$  (MilliporeSigma,  
276 Darmstadt, Germany) in 175 ml  $H_2O$ . The ventricle and liver were removed and weighed to  
277 calculate relative liver and ventricular masses as a percentage of fish body mass. Samples of red  
278 and white skeletal muscle (7-8 mm thickness) were removed from the cross-section of the second  
279 dorsal fin and caudal fin. They were flash-frozen with liquid nitrogen before storage at  $-80^\circ C$   
280 until analysis. We reasoned that, because skeletal muscle is the largest and most active organ in  
281 fish, citrate synthase (CS, EC 2.3.3.1) and lactate dehydrogenase (LDH, EC 1.1.1.27) activities  
282 from the red and white muscle of fish are useful index of oxidative and substrate-level energy

283 metabolic capacity of the fish. These muscle samples were homogenized in 19 and 6 vol.  
284 homogenization buffer (0.1% Triton, 50 mM Hepes, 1 mM EDTA, pH 7.4) for CS activity, and  
285 in 19 and 40 vol. homogenization buffer for LDH. Both assays were performed in triplicate  
286 (randomized) at room temperature measuring the maximal activity for three minutes with the  
287 EnSpire 2300 Multilabel Reader and subtracting the background reaction rate (Dalziel *et al.*,  
288 2012). The concentration of protein in muscle homogenates was analyzed with a BCA protein  
289 assay kit (ThermoFisher, Waltham, MA, USA) to express enzyme activity as  $\text{g}^{-1}$  protein. Organ,  
290 hematocrit, haemoglobin and enzyme activity metrics were statistically compared among  
291 treatment groups using ANOVA with Tukey-Kramer *post-hoc* tests. Statistical significances for  
292 all analyses were assigned when  $\alpha \leq 0.05$ .

293

## 294 Results

### 295 (a) Re-acclimation to normoxia of hypoxia-acclimated sea bass produced a new normoxic 296 respiratory phenotype, one with an improved aerobic capacity

297 Hypoxia-acclimated sea bass returned to normoxia for 4 weeks did not fully return to their  
298 original normoxic phenotype. Notably, aerobic performance was significantly improved. While  
299 SMR was similar compared with the N-N test group, the HN-N test group had an 18% higher  
300  $\dot{M}\text{O}_{2\text{max}}$  ( $503.4 \pm 27.2$  vs.  $427.3 \pm 11.7$   $\text{mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$ ,  $F_{2,53} = 6.4$ ,  $p = 0.003$ ), which contributed  
301 to a 27% higher AAS ( $402.5 \pm 23.3$  vs.  $318.0 \pm 13.7$   $\text{mg O}_2 \text{ h}^{-1} \text{ kg}^{-1}$ ,  $F_{2,53} = 7.2$ ,  $p = 0.001$ ) and a  
302 28% higher FAS ( $5.1 \pm 0.2$  vs.  $4.0 \pm 0.2$ ,  $F_{2,53} = 7.1$ ,  $p = 0.003$ ; Fig. S6). The HN-N phenotype  
303 also had a 24% higher citrate synthase activity in red muscle ( $F_{2,47} = 3.2$ ,  $p = 0.049$ , Fig. 3a) than  
304 the N-N phenotype.

305 Furthermore, the HN-N group had a 10% lower  $P_{crit}$  ( $F_{2,53} = 18.1$ ,  $p = 0.001$ ; Fig. 4a) than  
306 the N-N phenotype, indicating an improved hypoxia tolerance. The relative liver mass of the  
307 HN-N phenotype was significantly lower ( $F_{2,25} = 4.5$ ,  $p = 0.016$ , Fig. S3d) and their EPOC was  
308 28% lower ( $F_{2,46} = 5.4$ ,  $p = 0.013$ ; Fig. 4d) compared to hypoxia-acclimated fish.

309 While a new normoxic phenotype certainly emerged after the hypoxia-acclimated sea  
310 bass were re-acclimated to normoxia, SMR, ILOP and SOD remained statistically the same as  
311 those of the original N-N group ( $F_{2,53} \leq 7.7$ ,  $p \geq 0.059$ , power  $\geq 0.71$ , Fig. 2c & Fig. 4b, c).

### 313 **(b) Hypoxic acclimation of sea bass produced a new respiratory phenotype with improved** 314 **hypoxia tolerance and hypoxic performance**

315 As anticipated (Zambonino-Infante *et al.*, 2017), hypoxic acclimation at 10.4 kPa (50% sat.) did  
316 not affect body size (Fig. S2). Likewise, the maximum lactate dehydrogenase activities for both  
317 red and white muscles ( $F_{2,46} \leq 0.27$ ,  $p \geq 0.93$ , Fig. S7), hematocrit, [Hb] and relative masses of  
318 the ventricle and liver were similar for the hypoxia-acclimated fish ( $F_{2,25} \leq 3.5$ ,  $p \geq 0.07$ ; Fig.  
319 S3) when compared to the normoxia-acclimated fish.

320 All the same, a new hypoxic phenotype was confirmed by testing the hypoxic and  
321 normoxic phenotypes in normoxia (*i.e.* H-N and N-N test groups) and revealing significant  
322 differences in their respiratory indices. Notably, SMR was 19% lower in the H-N test group  
323 when compared with N-N fish ( $88.0 \pm 2.1$  vs.  $109.3 \pm 3.5$  mg O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>,  $F_{2,53} = 6.0$ ,  $p = 0.003$ ;  
324 Fig. 2c). This hypometabolic state was also reflected in the RMR of the H-N test group. RMR  
325 was similarly 15% lower in the H-N test group over the 2-day quiescent period when compared  
326 with N-N fish ( $117.3 \pm 3.7$  vs.  $138.1 \pm 4.4$  mg O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>,  $F_{2,53} = 3.6$ ,  $p = 0.033$ ; Fig. 2d).  
327 Furthermore, a sustained metabolic depression of the hypoxia-acclimated group was quite

328 evident throughout the quiescent period after inspection of both individual (Fig. 2a) as well as  
329 grouped mean  $\dot{M}O_2$  traces (Fig. 2b). Such a sustained reduction in RMR was likely not due to a  
330 lower spontaneous activity because the individual variance for RMR, our index of spontaneous  
331 activity, was similar for the H-N and N-N test groups ( $F_{2, 53} = 1.7, p = 0.98, \text{power} = 0.334$ ; Fig.  
332 2e).

333 Despite a reduction of SMR in the hypoxia-acclimated phenotype, both  $\dot{M}O_{2\text{max}}$  and AAS  
334 were maintained; they were similar for the H-N and N-N tests groups ( $F_{2, 53} \leq 7.2, p \geq 0.53,$   
335  $\text{power} \geq 0.98$ ; Fig. 2f, g). With maximal aerobic capacity unchanged and SMR lowered, FAS  
336 was significantly higher for the H-N test group compared with the N-N test group ( $F_{2, 53} = 7.1, p$   
337  $= 0.035$ ; Fig. 2h).

338 The phenotype after hypoxic acclimation also had an improved hypoxia tolerance.  
339 Specifically, three indicators were significantly lower for the H-N test group than for the N-N  
340 test group:  $P_{\text{crit}}$  by 28% ( $2.69 \pm 0.10$  vs.  $3.75 \pm 0.14$  kPa,  $F_{2, 53} = 18.1; p < 0.001$ ), ILOP by 22%  
341 ( $1.02 \pm 0.084$  vs.  $1.31 \pm 0.063$  kPa,  $F_{2, 52} = 4.2; p = 0.03$ ) and SOD by 34% ( $1.65 \pm 0.09$  vs.  $2.39$   
342  $\pm 0.14$  % sat.,  $F_{2, 52} = 7.7, p = 0.001$ ) (Fig. 4).

343 Nonetheless, the hypoxic ambient environment clearly constrained peak respiratory  
344 performance, as revealed when the hypoxic phenotype was tested under the ambient hypoxic  
345 condition (*i.e.*, the H-H test group). For example,  $\dot{M}O_{2\text{max}}$ , AAS and FAS of the H-H test group  
346 were all significantly reduced, almost halved ( $F_{3, 69} \geq 14.6, p \leq 0.0007$ ) when compared with  
347 the H-N test group. However, SMR and RMR were the same as the H-N test group ( $F_{3, 69} \leq 7.9,$   
348  $p \geq 0.29$ ; Fig. S4).

349 Given the improved aerobic performance and hypoxia tolerance of the hypoxic  
350 phenotype, the original normoxic phenotype was compared with the hypoxic phenotype using a  
351 hypoxic performance curve (HPC). Their  $\dot{M}O_{2peak}$  values in normoxia were statistically  
352 indistinguishable ( $365.1 \pm 15.3$  vs.  $334.6 \pm 13.3$  O<sub>2</sub> h<sup>-1</sup> kg<sup>-1</sup>, respectively; t-test:  $t = 1.4$ ,  $p = 0.17$ ,  
353 power = 0.296). The hypoxic phenotype, however, had a significantly left-shifted HPC compared  
354 with the normoxic phenotype. This shift produced a 21% lower P<sub>AAS-50</sub> [7.92 vs. 10.0 kPa (38 vs.  
355 48 % sat.);  $t = 1.6$ ,  $p = 0.0031$ ; Fig. 5]. As quality assurance for the HPC of the hypoxic  
356 phenotype, the interpolated  $\dot{M}O_{2peak}$  at 10.4 kPa (50% sat.) was compared to and was similar to  
357 that measured as  $\dot{M}O_{2max}$  at a similar level of ambient hypoxia [*i.e.*, H-H; 10.4 kPa (50% sat.)].  
358

## 359 Discussion

360 As an extension of the cellular metabolic signalling pathways proposed for hypoxic phenotypes  
361 (Hochachka, 1986), we considered the lesser studied whole-animal respiratory phenotype. We  
362 demonstrated that the hypoxia-acclimated phenotype of sea bass had an enhanced O<sub>2</sub> uptake in  
363 hypoxia, a lower P<sub>crit</sub>, and a left-shifted HPC (a higher  $\dot{M}O_{2peak}$  under moderate hypoxia). While  
364 the lower P<sub>crit</sub> is clearly influenced by a lower SMR, the  $\dot{M}O_{2peak}$  under moderate hypoxia is not.  
365 Moreover, the re-acclimation of hypoxia-acclimated sea bass to normoxia produced a new  
366 normoxic phenotype. This new normoxic phenotype also had an enhanced  $\dot{M}O_{2max}$  and AAS, as  
367 well as a reduced P<sub>crit</sub> like the hypoxic phenotype, but not the reduced SMR. Thus, it seems  
368 probable, but not definitely demonstrated, that certain (but not all) respiratory enhancements  
369 shown for the hypoxic phenotype were retained after 4-weeks of re-acclimation to normoxia.  
370 Such potential and sustained (many weeks) effects on the current performance of an animal's  
371 previous experience are more generally termed a carryover effect (O'Connor *et al.*, 2014), but



372 we cannot be certain that our demonstration for sea bass is necessarily a carryover effect.  
373 Nonetheless, and regardless of the exact mechanism, phenotypic plasticity following a hypoxic  
374 acclimation can clearly benefit whole-animal aerobic performance and  $O_2$  handling in normoxia  
375 as well as hypoxia. Moreover, European sea bass could be a 'fence-sitter' when exploiting  
376 hypoxic habitats, taking advantage of both a reduced maintenance metabolic demand and an  
377 improved aerobic performance during the hypoxic experience, two processes that are typically  
378 thought of as mutually exclusive strategies for fish living in an oxygen-limiting environment.

379

### 380 **(a) Sustained effect of improved aerobic performance**

381 A hypoxia-acclimated fish returned to their original normoxic condition can have three general  
382 outcomes (Fig. 1): i) Restoring their original normoxic phenotype, *i.e.*, status quo, ii) Suffering a  
383 compromised normoxic performance, a negative consequence of hypoxic acclimation, or iii)  
384 Acquiring enhanced normoxic performance, a beneficial prolonged consequence of hypoxic  
385 acclimation. We observed increased aerobic performance and capacity ( $\dot{M}O_{2max}$ , AAS & FAS)  
386 after hypoxia-acclimated sea bass were re-acclimated to and tested in normoxia. These whole  
387 animal changes align with the observed increase in CS activity in their red muscles. This new  
388 normoxic phenotype may have maintained some of the same physiological improvements for at  
389 least 4 weeks after hypoxia-acclimated sea bass were returned to normoxia. An enhanced aerobic  
390 capacity, for example, reduced the need for glycolytic capacity (Farrell, 2016; Zhang *et al.*,  
391 2018) because EPOC was reduced in the normoxia re-acclimated group when compared to the  
392 normoxia group (Fig. 4).

393 A novel discovery was a left-shifted HPC after an acclimation of sea bass to hypoxia.

394 This means that hypoxia-acclimated sea bass had improved  $O_2$  handling for  $\dot{M}O_{2peak}$ , as indicated

395 by their 21% lower  $P_{AAS-50}$  compared with hypoxia-acclimated fish. Likewise, a lower  $P_{crit}$  has  
396 been correlated with a higher AAS among eight populations of four fish species (Zhang *et al.*,  
397 2018; Zhang *et al.*, 2021). However, in terms of hypoxic acclimation of sea bass, SMR and  $P_{crit}$   
398 were not reduced when they were tested in hypoxia, but were reduced when tested in normoxia.  
399 Therefore, the observed suppression of SMR is likely not a contributing factor to the lower  $P_{AAS-}$   
400  $_{50}$  because the HPC and IRAP testing was performed at a similar 50% hypoxia. Other beneficial  
401 hypoxia acclimation mechanisms include remodeling of gill secondary lamellae (Brauner and  
402 Rombough, 2012; Anttila *et al.*, 2015; Yang *et al.*, 2021), which would reduce the  $O_2$  diffusion  
403 distance (Randall, 1982), could improve hypoxia tolerance and could defend  $\dot{M}O_{2peak}$  under  
404 ambient hypoxia. Improved cardiovascular  $O_2$  delivery and  $O_2$  utilization by mitochondria are  
405 also possible. For example, hemoglobin- $O_2$  binding affinity could increase (Weber and Jensen,  
406 1988; Montgomery *et al.*, 2019; Wells *et al.*, 1989), venous blood stores could be better  
407 mobilized by increasing venous tone, and capillarity could increase in cardiac (Gillis and  
408 Johnston, 2017) and swimming muscles (McKenzie *et al.*, 2004).

409 Future experiments should test whether or not the beneficial  $P_{AAS-50}$  is retained on re-  
410 acclimation to normoxia because logistical constraints prevented us from doing so in the present  
411 study. Indeed, and more generally, the malleability of the  $O_2$  transport cascade system of sea bass  
412 could be a useful model system to study how a fish might benefit from prior hypoxic exposures.  
413 Beyond hypoxia, other environmental stressors are also known to have sustained effects,  
414 increasing subsequent tolerance to that stressor (Kawabata *et al.*, 1998; Gamperl *et al.*, 2001).  
415 Another uncertainty generated by the present study is the exact time course for developing new  
416 phenotypes and for how long the benefits of a hypoxic exposure might persist. Our acclimation  
417 periods (4 weeks for normoxia and 6 weeks for hypoxia) were based on many previous studies

418 and did not consider any potential modulating effects of seasonality. We do know, however, that  
419 IRAP metrics in normoxic fish can be stable for up to 18 weeks (Zhang *et al.*, 2019; Polinski *et*  
420 *al.*, 2021; Zhang, 2021), but time-series studies with time-matched controls over different  
421 acclimation periods and developmental stages will be needed to resolve this unknown.

#### 422

#### 423 **(b) Reduced SMR as a mechanism for hypoxic acclimation in active marine fish**

424 While a maintained aerobic scope of hypoxia-acclimated sea bass is consistent with previous  
425 hypoxia acclimation studies for rainbow trout (Bushnell *et al.*, 1984), Atlantic cod (*Gadus*  
426 *morhua*) (Petersen and Gamperl, 2010; 2011) and silver seabream (*Pagrus auratus* Forster,  
427 1801) (Cook *et al.*, 2013), all of which maintained  $\dot{M}O_{2\max}$  and aerobic scope when they were  
428 tested in normoxia, none of these previous studies measured SMR. Therefore, a reduced  
429 metabolism (a lower SMR and RMR without any apparent change in locomotory activity, differs  
430 from the inactivity in overwintering fishes, Reeve *et al.*, 2022) in active marine fish after  
431 acclimation to a moderate hypoxic condition is, to the best of our knowledge, a novel finding and  
432 adds to previous reports a much larger suppression seen typically in anoxia for a limited group of  
433 extremely anoxia-tolerant fish species (*see* review by Stecyk, 2017). Nonetheless, a 19% SMR  
434 reduction in sea bass was not nearly as extreme as the up to 90% reduction seen for anoxia-  
435 tolerant fishes in very severe hypoxia (Vornanen *et al.*, 2009; Thoral *et al.*, 2022). While  $P_{\text{crit}}$  was  
436 not improved when measured in hypoxia, this index of hypoxic tolerance was improved after an  
437 acute transfer to (and IRAP testing) in normoxia where SMR reduction was manifested, and was  
438 subsequently retained after 4 weeks in normoxia. Yet, the reduced SMR remains as a meaningful  
439 energy saving in hypoxia because it cascaded through to a 15% reduction in RMR. Notably,  
440 body condition of hypoxia-acclimated sea bass was unaffected by a 50% reduction in oxygen

441 availability in the water, along with a similar liver mass (Fig. S3d) and activity of citrate  
442 synthase in both red and white skeletal muscle (Fig. 3; Fig S7) compared to the normoxia-  
443 acclimated group.

444 Unanswered, however, is why SMR of the hypoxia-acclimated fish tested in hypoxia (H-  
445 H) was similar to that for normoxia-acclimated fish tested in normoxia (N-N) (Fig. S4 & S5) and  
446 the metabolic suppression was only revealed by testing hypoxia-acclimated sea bass after an  
447 acute transfer to normoxia (H-N). A possible explanation, one that would be worth testing,  
448 relates to the fact that in hypoxia O<sub>2</sub> availability was reduced by 50%, and so the hypoxic  
449 phenotype would have had to compensate by increasing ventilation volume, which would present  
450 an increased O<sub>2</sub> cost of ventilation. Improvements to O<sub>2</sub> extraction at the gills through expansion  
451 of gill blood vessels, an increase in cardiac output and gill blood flow, lamellar recruitment,  
452 increased barrier permeability and greater Hb-O<sub>2</sub> affinity are all possible contributing  
453 mechanisms to improve O<sub>2</sub> transfer at the gills, but ventilation volume would still have to  
454 increase for the halving of water O<sub>2</sub> content given the efficiency of oxygen exchange in  
455 normoxia. A review of the ventilatory response of 34 teleost species to acute hypoxia found that  
456 approximately halving the ambient water O<sub>2</sub> content, as in the present study, produced at least a  
457 100% compensatory increase in ventilation volume for the majority of species (*see* Table S2;  
458 Perry *et al.*, 2009). Even the exceptions (*i.e.*, tuna species, dourado and plaice) had a 45–75%  
459 compensatory increase in ventilation volume (Table S2). Consequently, if ventilation costs 10–  
460 15% of RMR for normoxic, resting rainbow trout (Farrell and Steffensen, 1987), a doubling of  
461 this for the hypoxic sea bass phenotype in hypoxia might double the energy cost of ventilation.  
462 In this case, the observed reduction in SMR of the hypoxic phenotype would largely offset this  
463 increase in routine energy expenditure. While this quantitative matching could be an association

464 rather than a causation, we did not observe the well-documented increase in restlessness  
465 associated with an acute hypoxic exposure (*e.g.* Steffensen *et al.*, 1982; van Raaij *et al.*, 1996)  
466 because neither RMR nor its variability increased in hypoxia-acclimated sea bass tested in  
467 hypoxia.

468         How widespread a modest reduction in SMR is a strategy used by active marine fishes to  
469 acclimate to a challenging hypoxic environment is unclear until we have a better understanding  
470 of the specific mechanisms (*see* review by Hochachka *et al.*, 1996). For example, we know that  
471 SMR is stable for up to 18 weeks under normoxic conditions (Zhang *et al.*, 2019; Polinski *et al.*,  
472 2021; Zhang, 2021), we cannot exclude the possibility of the seasonal effects on SMR.  
473 Furthermore, if suppression of protein turnover was the mechanism to reduce SMR, protein  
474 turnover would need to be halved to quantitatively account for our observed 19% decrease in  
475 SMR given that protein turnover accounts for about 30–40% of SMR (Houlihan *et al.*, 1988;  
476 Houlihan *et al.*, 1992; Carter *et al.*, 1993). While suppression of protein synthesis occurs with  
477 acute hypoxia exposure in cichlids (*Astronotus ocellatus* Agassiz, 1831) (Cassidy *et al.*, 2018),  
478 Arctic char (*Salvelinus alpinus* Linnaeus, 1758) (Cassidy and Lamarre; 2019), and jumbo squid  
479 (*Dosidicus gigas* d'Orbigny, 1835) (Seibel *et al.*, 2014), which are all hypoxia-sensitive  
480 organisms, no acclimation studies besides the present study have shown a similar response. What  
481 is also clear from the present study is that a reduction of SMR, while a key response to hypoxic  
482 acclimation in sea bass, was not carried over on their return to normoxia.

483

## 484 **Conclusion**

485 Prolonged encounters with environmental stressors such as hypoxia can substantially change the  
486 respiratory phenotype of fish. Indeed, we characterized how European sea bass, an athletic

487 marine fish that naturally exploits both hypoxic and normoxic habitats, remodeled its respiratory  
488 phenotype during hypoxia acclimation (a reduced minimum maintenance metabolism, better  
489 hypoxia performance, enhanced aerobic performance and capacity). Moreover, after hypoxia-  
490 acclimated sea bass were returned to normoxia, a different normoxic phenotype was still evident  
491 after 4 weeks in normoxia, one that displayed a better hypoxia performance and enhanced  
492 aerobic performance and scope compared with the original normoxic phenotype. Given these  
493 findings for sea bass, greater attention should be given the cyclic nature of the ambient  
494 environment (both short-term and long-term), especially in view of the scarcity of studies on  
495 phenotype reversibility (*see* review by Burggren, 2020).

496  
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834 **Figure Captions**

835  
836 Fig. 1. A theoretical framework for phenotypic plasticity (*i.e.* within a generation) for hypoxic  
837 acclimation and a return to ambient normoxia, *i.e.*, environmental changes. (a) Even with acclimation,  
838 which may take some time, the performance of a hypoxic phenotype may be constrained by the limiting  
839 ambient environmental factor, *i.e.*, hypoxic water at 10.4 kPa (50 % air saturation) (panel b). Also, when  
840 the hypoxia-acclimated animal is returned to normoxia, the normoxic phenotype might revert to the  
841 original normoxic phenotype, or another normoxic phenotype might emerge with either a compromised or  
842 even an enhanced performance in normoxia. The present study investigated the respiratory plasticity of a  
843 marine fish species, the European sea bass (*Dicentrarchus labrax*).

844  
845 Fig. 2. The respiratory phenotype in juvenile European sea bass (*Dicentrarchus labrax*) at 16 °C based on  
846 individual oxygen uptake ( $\dot{M}O_2$ ) measurements. The three test groups were normoxia-acclimated fish  
847 tested in normoxia (N-N; grey), hypoxia-acclimated fish tested in normoxia (H-N; orange), and hypoxia-  
848 acclimated fish re-acclimated to and tested in normoxia (HN-N; green). (a) Continuous  $\dot{M}O_2$  traces from  
849 representative individuals for the three treatment groups over the first 40 h of IRAP. The individual's  
850 standard metabolic rate (SMR; solid horizontal lines) and maximum oxygen uptake ( $\dot{M}O_{2max}$ ; dotted  
851 horizontal lines) are provided for reference. White-&-grey segments indicate average summer diel cycles  
852 in western France (~15 L:9 D). (b) Continuous mean  $\dot{M}O_2$  traces (solid line)  $\pm$  s.e.m (shaded area) for all  
853 individuals in each of the three test groups over the first 40 h of IRAP. Panels (c) to (h) summarize mean  
854 values for five key aerobic respiratory indices derived from  $\dot{M}O_2$ : (c) SMR, (d) routine metabolic rate  
855 (RMR), (e) the variance of RMR, (f)  $\dot{M}O_{2max}$ , and (g) absolute aerobic scope (AAS =  $\dot{M}O_{2max} - \text{SMR}$ ).  
856 Phenotypic plasticity associated with hypoxic acclimation is indicated by statistically significant  
857 differences between N-N (grey) and H-N (orange) test groups. Comparison of N-N (grey) and HN-N  
858 (green) reveals the new normoxic phenotype that results from a prior hypoxic acclimation. The boxplots  
859 indicate the bar as the 25-75 percentile, the whiskers as the 10-90 percentile, the line as the median and

860 '+' as the mean (n = 13–28). Different letters denote a statistical significance (ANCOVA with Holm-  
861 Šídák *post-hoc* tests,  $\alpha < 0.05$ ). No mathematical or statistical transformations are applied to the data  
862 presented.

863  
864  
865 Fig. 3. Effects of a 6-week hypoxic acclimation on the maximal activity of citrate synthase (CS) in red  
866 and white muscles of juvenile European sea bass (*Dicentrarchus labrax*) at 16 °C. Phenotypic plasticity  
867 associated with hypoxic acclimation is indicated by statistically significant differences between  
868 normoxia-acclimated fish (N; grey) with hypoxia-acclimated (H; orange) test groups. A comparison of the  
869 normoxia-acclimated (grey) and the hypoxia-acclimated re-acclimated to normoxia (HN; green) reveals  
870 the new normoxic phenotype that results from a prior hypoxic acclimation. The boxplots indicate the bar  
871 as the 25-75 percentile, the whiskers as the 10-90 percentile, the line as the median and '+' as the mean (n  
872 = 13–23). Different letters denote a statistical significance (one-way ANOVA with Tukey-Kramer *post-*  
873 *hoc* tests,  $\alpha < 0.05$ ). No mathematical or statistical transformations are applied to the data presented.

874  
875 Fig. 4. The respiratory phenotype in juvenile European sea bass (*Dicentrarchus labrax*) at 16 °C based on  
876 individual oxygen uptake ( $\dot{M}O_2$ ) measurements. Panel (a, critical oxygen partial pressure,  $P_{crit}$ ) is the  $PO_2$   
877 level at which SMR could no longer be maintained, (b) incipient lethal oxygen partial pressure (ILOP).  
878 Panels (c) & (d) summarize mean values for four key indices of glycolytic capacity derived from  $\dot{M}O_2$   
879 [(c) scope for oxygen deficit (SOD) and (d) excess post-exercise oxygen consumption (EPOC)]. The three  
880 test groups were normoxia-acclimated fish tested in normoxia (N-N; grey), hypoxia-acclimated fish tested  
881 in normoxia (H-N; orange), and hypoxia-acclimated fish re-acclimated to and tested in normoxia (HN-N;  
882 green). Phenotypic plasticity associated with hypoxic acclimation is indicated by statistically significant  
883 differences between N-N (grey) and H-N (orange) test groups. A comparison of N-N (grey) and HN-N  
884 (green) reveals the new normoxic phenotype that results from a prior hypoxic acclimation. The boxplots  
885 indicate the bar as the 25-75 percentile, the whiskers as the 10-90 percentile, the line as the median and  
886 '+' as the mean (n = 13–28). Different letters denote statistical significance (one-way ANCOVA with

887 Holm-Šídák *post-hoc* tests,  $\alpha < 0.05$ ). No mathematical or statistical transformations are applied to the  
888 data presented.

889

890

891 Fig. 5. Hypoxic performance curves of normalized oxygen uptake ( $\dot{M}O_2$ ) for normoxia- and hypoxia-  
892 acclimated juvenile European sea bass (*Dicentrarchus labrax*) at 16 °C. The data were taken from  
893 individual-based hypoxic performance curves where  $\dot{M}O_2$  were normalized as a percentage of each  
894 individual's absolute aerobic scope (% AAS). (a) Mean % AAS (dots)  $\pm$  s.e.m (error bars) across a range  
895 of partial pressure of O<sub>2</sub> (PO<sub>2</sub>, kPa) were modeled using one-phase association equations [normoxic  
896 phenotype:  $y = -39.5 + (130.0 + 39.5) \times [1 - e^{(-0.005 \times x)}]$ ;  $R^2 = 0.88$ , AIC=339.8; hypoxic phenotype:  $y = -$   
897  $85.8 + (88.3 + 85.8) \times [1 - e^{(-0.057 \times x)}]$ ;  $R^2 = 0.81$ , AIC=412.6]. The solid curves are one-phase association  
898 regression models, and the shaded areas are the 95% confidence intervals of these curves. Blue dash lines  
899 graphically illustrate the comparison of mean values for the minimum O<sub>2</sub> partial pressure that supports  
900 50% of AAS ( $P_{AAS-50}$ ). (b) A statistic comparison of the interpolated  $P_{AAS-50}$  values for the hypoxic and  
901 normoxic phenotype. The mean values were based on the  $P_{AAS-50}$  values interpolated from each individual  
902 hypoxic performance curve. The boxplots indicate the bar as the 25-75 percentile, the whiskers as the 10-  
903 90 percentile, the line as the median and '+' as the mean (n = 6–7). Different letters denote statistical  
904 significance (independent sample t-test,  $\alpha < 0.05$ ). No mathematical or statistical transformations are  
905 applied to the data presented.

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