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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 O₂ 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 PO₂ 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 (O₂) 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 O₂ 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⁺ 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 O₂ 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-O₂ 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 O₂ 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 O₂ [50 % saturation (% sat.) at 20 °C] displayed a full

100 postprandial peak O₂ 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 O₂
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⁻¹ 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⁻¹) 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 O₂ level of ~20.4 kPa (dissolved O₂ 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⁻¹ 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 ± 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 ≥ 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 $\dot{M}O_{2max}$ (~ 400 mg O_2 h⁻¹ kg⁻¹; 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 ~ 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⁻¹ or ~1.5% sat. min⁻¹) and then at a slower rate of deoxygenation (0.0313 kPa min⁻¹ or
206 ~0.15% sat. min⁻¹) 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 μ 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 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 $\dot{M}O_{2\text{max}}$ (503.4 ± 27.2 vs. 427.3 ± 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 ± 23.3 vs. 318.0 ± 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 ± 0.2 vs. 4.0 ± 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 ≥ 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 ± 2.1 vs. 109.3 ± 3.5 mg O₂ h⁻¹ kg⁻¹, $F_{2,53} = 6.0$, $p = 0.003$;
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 ± 3.7 vs. 138.1 ± 4.4 mg O₂ h⁻¹ kg⁻¹, $F_{2,53} = 3.6$, $p = 0.033$; 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_{crit} by 28% (2.69 ± 0.10 vs. 3.75 ± 0.14 kPa, $F_{2, 53} = 18.1; p < 0.001$), ILOP by 22%
341 (1.02 ± 0.084 vs. 1.31 ± 0.063 kPa, $F_{2, 52} = 4.2; p = 0.03$) and SOD by 34% (1.65 ± 0.09 vs. 2.39
342 ± 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 ± 15.3 vs. 334.6 ± 13.3 O₂ h⁻¹ kg⁻¹, 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_{AAS-50} [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 O₂ uptake in
363 hypoxia, a lower P_{crit}, and a left-shifted HPC (a higher $\dot{M}O_{2peak}$ under moderate hypoxia). While
364 the lower P_{crit} 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_{crit} 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; Thorat *et al.*, 2022). While P_{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 O₂ 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 O₂ cost of ventilation. Improvements to O₂ 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-O₂ affinity are all possible contributing
453 mechanisms to improve O₂ transfer at the gills, but ventilation volume would still have to
454 increase for the halving of water O₂ 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 O₂ 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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505 experiments and respirometry data acquisition. L.P., K.A. analyzed molecular samples. H.O., F.L., FM.
506 greatly contribute to all aspects of the project. A.P.F., G.C., and K.A. collaborated in editing the
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- 833

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 O₂ (PO₂, 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 O₂ 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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