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Long-term effects of ocean acidification upon energetics and oxygen transport in the European sea bass (*Dicentrarchus labrax,* Linnaeus)

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Abstract :

The accumulation of CO2 in the atmosphere and resulting ocean acidification represent a threat to marine ecosystems. While acid–base regulatory capacity is well developed in marine fish, allowing compensation of extra-cellular pH during short-term hypercapnia, the possible energetic costs of such regulation during long-term exposure remain to be established. In this study, juvenile European sea bass (*Dicentrarchus labrax*) were exposed from 2 days post-hatching to three different ocean acidification scenarios: control (present condition, PCO2 = 520 µatm, pH 7.9), moderate acidification (PCO2 = 950 µatm, pH 7.7), and high acidification (PCO2 = 1490 µatm, pH 7.5). After 1.5 years of exposure, fish aerobic metabolic capacities, as well as elements of their oxygen extraction and transport chain, were measured. Compared to control, PCO2 treatments did not affect fish standard metabolic rate (SMR). However, the most severe acidification condition was associated with a significantly elevated maximum metabolic rate (MMR).This was supported by heavier gill system and higher blood haemoglobin concentration. A reduction of maximum cardiac frequency (fHmax) during incremental warming of anaesthetized fish was also observed in both acidification scenarios. On the other hand, the critical oxygen level (O2crit), the minimum oxygen level required to sustain SMR, did not differ among groups. The increased MMR, associated with maintained SMR, suggests that acid–base compensatory processes, although not increasing maintenance costs, may affect components of bass homeostasis, resulting in new internal physico-chemical conditions. The possibility that these alterations influence metabolic pathways and physiological functions involved in fish aptitude to maximally transport oxygen is discussed.

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45 **INTRODUCTION**

46

47 Over the last century, intensification of anthropogenic activities has led to increased carbon dioxide
48 (CO_2) emissions (Intergovernmental Panel on Climate Change 2014) and atmospheric CO_2
49 concentration is now reaching an unprecedented level in the last thousand year, in excess of 400
50 ppm (Lüthi et al. 2008). Oceans, which are representing 70% of the earth surface, absorb a large
51 proportion of atmospheric CO_2. When dissolved in water, CO_2chemically reacts to produce
52 carbonic acid (H_2CO_3) which then dissociates into bicarbonate ions (HCO_3^-) and protons (H^+). The
53 increased concentration of protons in the world oceans is now a widely recognized phenomenon
54 named 'ocean acidification' (OA).Since the beginning of the twentieth century, ocean surface pH
55 has already declined by 0.1 U (Intergovernmental Panel on Climate Change 2014) and projections
56 suggest an additional decrease of 0.3 to 0.5 U by 2100 (Caldeira and Wickett 2005;
57 Intergovernmental Panel on Climate Change 2014).

58

59 Ocean acidification and related changes in marine water's chemistry are recognized to have
60 negative effects on the survival, calcification, growth and reproduction of many calcifying marine
61 organisms such as corals, echinoderms and bivalves (Kroeker et al. 2010; Kroeker et al. 2013).
62 Available information regarding the impact on fish leans toward an absence of effect due to their
63 acid-base regulatory capacity, which is believed to exceed what is required to face the predicted
64 acidification of their environment (Heuer and Grosell 2014). However, whereas the mechanistic
65 bases of acid-base regulation are well described in fish (Pörtner et al. 2004; Heuer and Grosell
66 2014), this knowledge is not yet matched with a full understanding of the implications for fish
67 populations in their natural environment. One missing piece of information relates to the potential
68 long term consequences of ocean acidification and in particular its cumulative effects over life
69 stages on integrated processes such as, for instance, energy metabolism, ontogeny and growth. To

70 our knowledge, only one study has investigated the long term (14-16 weeks) effect of hypercapnia
71 at ecologically relevant level (1000 µatm) (Gräns et al. 2014). Using the Atlantic halibut
72 (*Hippoglossus hippoglossus,* Linnaeus), these authors revealed impaired growth (only at cold
73 temperature, 4°C) but increased aerobic metabolic scope at every tested temperature (ranging from
74 4°C to 18°C), the latter suggesting that fish aerobic performance was not compromised by long-
75 term exposure to elevated ambient CO_2. However, these authors also pointed out that the causal link
76 between oxygen supply and whole-animal performance and fitness under hypercapnic conditions
77 remained unclear and needed further investigation.
78
79 The notion of capacity for aerobic metabolic activities (also named aerobic metabolic scope), put
80 forth by Fry (1971), has been proposed as a useful measure to investigate the influence of the
81 environment upon fish performance (for review see Claireaux and Lefrançois 2007). According to
82 Fry's original definition, the aerobic metabolic scope is the difference between the standard
83 metabolic rate (SMR, the cost of maintenance measured in unstimulated, inactive and fasted fish,
84 Chabot et al., 2016) and the maximal metabolic rate (MMR). The aerobic metabolic scope therefore
85 quantifies the capacity of a given fish, in a given set of environmental conditions, to allocate energy
86 to physiological activities beyond SMR (such as digestion, growth, locomotion and reproduction)
87 and represents an integrative approach to examine the physiological basis of environmental
88 adaptation (Claireaux and Lefrançois 2007). On that basis, it has been predicted that the anticipated
89 increase in marine CO_2 levels will contribute to reduce fish capacity for aerobic activities (Pörtner
90 and Farrell 2008), with expected impacts upon individual's fitness and, ultimately, upon the
91 resilience of populations. Yet, literature reviews shows that reported effects of near-future
92 hypercapnia on fish aerobic capacity are contrasted (Lefevre 2016; Esbaugh 2018; Hannan and
93 Rummer 2018), with an increased aerobic metabolic scope observed in damselfish (*Acanthochromis*

94 *polyacanthus,* Bleeker) (Rummer et al. 2013) or no effect in Atlantic cod (*Gadus morhua,*
95 Linnaeus) (Melzner et al. 2009).
96
97 According to the Fick equation, the aerobic metabolic rate of an animal is a function of its capacity
98 to extract oxygen from the ambient water and to deliver it to the ATP-producing mitochondria
99 (Farrell et al. 2014). The heart therefore represents a key component of the Fick equation as it
100 determines internal oxygen fluxes and allocation among the circulatory beds. Accordingly, using an
101 *in situ* heart preparation, Gräns et al. (2014) observed that the maximum flow-generating capacity
102 of Atlantic halibut heart increased under hypercapnia. The gill is also an important component of
103 the Fick equation as it is the main site of oxygen extraction from the ambient water (Evans et al.
104 2005). It has long been demonstrated that a relationship exists between gill surface area and fish
105 metabolic demand for oxygen and maximum metabolic rate (Schmidt-Nielsen 1997). Blood oxygen
106 carrying capacity is as well a crucial component of the oxygen transport and delivery chain.
107 However, a previous study reported a reduced blood oxygen contentin species maintained under
108 rather severe hypercapnic condition (40 000 µatm ; McKenzie et al. 2003), possibly limiting aerobic
109 capacity. Nevertheless, there exist to date no integrated long term study of the components of the
110 oxygen transport chain under predicted capnic conditions and following long term exposure and
111 compensation.
112
113 Oxygen availability is an important environmental issue for aquatic organisms. Classically, the
114 capacity of these organisms to tolerate an episode of reduced oxygen availability (hypoxia) is
115 assessed by measuring the critical oxygen level (O_{2crit}) i.e., the oxygen threshold below which SMR
116 is no longer sustainable aerobically. Below this threshold, some of the ATP must be produced
117 anaerobically and/or metabolic depression takes place (Nilsson and Renshaw 2004).With the
118 predicted global warming, hypoxic events are expected to become more frequent and more severe

119 (Intergovernmental Panel on Climate Change 2014; Rogers et al. 2016). Yet, very few studies have
120 investigated the potential interaction between projected ocean acidification and deoxygenation in
121 fish. These few studies report, however, no effect on O_{2crit} after both short-term (four days) or
122 following long-term (six weeks) exposure to hypercapnia in two damselfish, *Pomacentrus*
123 *moluccensis* (Bleeker) and *Pomacentrus amboinensis* (Bleeker), (Couturier et al. 2013) and in the
124 European eel, *Anguilla Anguilla* (Linnaeus), (McKenzie et al. 2003).
125
126 In this context, the objective of the present study was to investigate the effect of a long-term
127 exposure to elevated water CO_2 content upon the energetics and oxygen transport capacity of a
128 commercially relevant, temperate fish, the European sea bass (*Dicentrarchus labrax,* Linnaeus). In
129 this experiment, the possibility of carry-over effects of early environmental conditions across life
130 history stages was taken into consideration (Vanderplancke et al. 2015). Accordingly, fish were
131 maintained under hypercapnia from two days post-hatch and untilthey were 1.5-year old. Three
132 P_{CO2} treatments were tested i.e., control (P_{CO2} = 520 µatm), moderate acidification (P_{CO2} = 950
133 µatm) and high acidification (P_{CO2} = 1490 µatm). The specific objectives of our study were (1) to
134 examine the influence of hypercapnia on aerobic performance (SMR and MMR) through
135 respirometry measurements, (2) to evaluate simple determinants of fish capacity for oxygen
136 extraction and transport such as cardiac and gill masses to body mass ratios, maximal heart rate (on
137 anaesthetized fish) as well as blood haematocrit and haemoglobin concentration, and (3) to measure
138 the critical oxygen level as an index of hypoxia tolerance.
139

140 **MATERIALS AND METHODS**

141

142 **Animals**

143

144 Fish were obtained in October 2013 from a local commercial hatchery (Aquastream, Ploemeur,
145 France). At two days post-hatch (dph), they were brought to Ifremer rearing facility (Brest, France)
146 and randomly distributed among nine tanks (38 L; 19 °C; n= 2200 larvae per tank) corresponding to
147 three experimental treatments in triplicates *i.e.*, control (labelled *C;* pH total 7.9; P_{CO2} = 520 µatm),
148 moderate acidification (*MA*; pH total 7.7; P_{CO2} = 950 µatm) and high acidification (*HA;* pH total
149 7.5; P_{CO2} = 1490 µatm). The photoperiod was set at 16 h light: 8 h dark. Larvae were fed *ad libitum*
150 with Artemia until 28 dph and then with commercial pellets according to feeding charts (about 1%
151 ration, w/w, Néo-start and néo-grower, Le Gouessant, France). At 45 dph, some fish within each
152 treatment were pooled and transferred to three larger tanks (450 L, n= 1500 fish per tank) with
153 identical water P_{CO2} and pH as above. At that time, there was no tank replication within the
154 treatments but special care was taken to standardize every rearing conditions. Temperature was set
155 at 15 °C (Table 1) and photoperiod followed the natural day-night cycle. When fish reached
156 approximately 10 g (about 8 months), 700 fish per condition were anaesthetized with tricaine
157 methane sulphonate (MS222, Pharmaq, UK) and a passive integrated transponder (PIT tag;ISO
158 1.4 mm × 9 mm, Biolog-id, France) was inserted subcutaneously behind the dorsal fin. No
159 difference in the mortality was observed among the conditions from the larval (Crespel et al. 2017)
160 to the juvenile stage. Fish were unfed for 24 h before any manipulation or experiment. The protocol
161 was in conformity with current rules and regulations in France (project code: APAFIS 4341.03,
162 #201620211505680.V3).
163
164 **Experimental conditions**
165
166 Experimental conditions were obtained using an automatic CO_2 injection system connected by a pH
167 electrode (pH Control, JBL, Germany). Salinity (WTW LF325, Xylem Analytics Germany,
168 Weilheim, Germany) was measured monthly. A daily control of water temperature and pH

169 (National Bureau of Standards scale, pH_{NBS}) was performed every morning before feeding with a
170 hand held pH meter (330i, WTW, Germany) calibrated daily with fresh certified WTW technical
171 buffers pH 4.01 an pH 7.00 (Xylem Analytics Germany, Weilheim, Germany). In addition, total pH
172 was determined monthly following Dickson et al. (2007) using m-cresol purple as the indicator.
173 Total alkalinity (TA) in each tank was measured monthly by titration (Labocea, France). Phosphate
174 and silicate concentrations were determined by segmented flow analysis following Aminot et
175 al.(2009). CO_2 partial pressure (P_{CO2}) was calculated using the total pH measurements and the
176 Microsoft excel macro CO2SYS software (Lewis and Wallace 1998) and constants from Mehrbach
177 et al. (1973). Water chemistry is summarized in Table 1.
178
179 **Respirometry**
180
181 Experimental set-up
182
183 Fish oxygen uptake (MO_2) was measured using eight static, intermittent flow respirometry
184 chambers (2.1 L). The set-up was immersed in a tank filled with aerated (> 90% air saturation) and
185 thermoregulated (15 ± 0.25 °C) seawater. Water $P_{CO2}$was regulated at the level of the fish original
186 rearing tank using the same automatic CO_2 injection system as described above. The respirometry
187 system was placed behind an opaque curtain and movements in and out of the room were kept to a
188 minimum to prevent fish disturbance. Submersible pumps (Eheim GmbH, Germany) supplied water
189 from the outer tank to the respirometer chambers. These pumps were controlled by Aquaresp
190 software (aquaresp.com) which set the frequency and duration of the open (flush) and closed
191 (measure) modes of the measuring cycle. A second series of pumps (Eheim GmbH, Germany)
192 connected, *via* a closed circuit, an optode (PreSens GmbH, Germany or Firesting Pyro Science,
193 Germany) to the respirometry chambers. This closed circuit allowed the monitoring of the oxygen

194 level in the chambers (% air saturation) and insured good mixing of water in each respirometer.
195 Optodes were calibrated before each respirometry trials using fully aerated water and a 0% oxygen
196 solution (sodium sulfite in excess).
197 The rate of oxygen consumption (MO_2, in mg O_2 h^{-1} kg^{-1}) was calculated by Aquaresp software
198 using the following formula:
199
200 $MO_2 = a \times \beta \times V_{rem} / mf$
201
202 where a is the slope of the decrease in water oxygen level over time (% O_2 saturation h^{-1}), β is the
203 solubility of O_2 (mg O_2 L^{-1} 100% O_2 saturation^{-1}), V_{rem} is the volume of the chamber minus the
204 volume of the fish (L) and mf is the fish mass (kg).
205
206 Respirometry protocol
207
208 Experiments were conducted from February to April 2015. Fish mean mass was 79.6 ± 1.7 g and
209 mean length was 18.1 ± 0.1 cm (n = 24 per acclimation group, no statistical differences between
210 groups, ANOVA, $F(2, 69) = 1.439$, $P = 0.244$, ANOVA, $F(2, 69) = 0.960$, $P = 0.387$, respectively).
211 A typical respirometry trial is presented in Fig.1. Fish were fasted three days prior to measurements
212 to prevent residual specific dynamic action (Jourdan-Pineau et al. 2010). Three sets of eight fish per
213 experimental treatment were tested in three blocks and all treatments were tested once per block in
214 systematic order (C, MA, HA). The three blocks were completed in 30 days. For each run, eight fish
215 were selected at random from the treatment tanks, identified (PIT tag reading) and their mass and
216 length measured. They were then placed in a 10 L tank where they were manually chased, typically
217 less than 10 minutes, until exhaustion i.e., they would not respond to further stimulation. The fish
218 were then rapidly placed in a respirometer chamber and the oxygen consumption measurement

219 immediately started (Zhang et al. 2018). The respirometry cycle included 210 s in closed mode
220 (measurement) followed by 90 s of open mode (chamber flushing). The first 30 s in closed mode
221 (wait period) were not used to calculate fish oxygen consumption to insure that the decrease in O_2
222 with time had become linear. Therefore, only the last 180 s of the slope was used for each
223 measurement of oxygen uptake. The highest oxygen uptake measured during the 2 h post-
224 exhaustion recovery period (obtained during the first 30 minutes) was used to estimate fish
225 maximum metabolic rate (MMR). At 2 h post-exhaustion, as fish had partially recovered from
226 exhaustion and MO_2 was approximately half of the maximal value, the respirometry measuring
227 cycle was modified with 360 s in closed mode (30 s wait and 330 s for measurement) and 240 s of
228 chamber flushing. These conditions were maintained during at least the next 65h, allowing a
229 reliable estimation of fish standard metabolic rate (SMR) (Chabot et al. 2016). Note that during
230 MO_2 measurements, water oxygen level in the respirometers never dropped below 75%. The last
231 phase of each experimental trial was dedicated to estimating fish critical oxygen level (O_{2crit}). To
232 this end, the water in the outer tank was deoxygenated by passing through a gas equilibration
233 column supplied with nitrogen before it was pumped into the respirometers. Water oxygen level in
234 the respirometry chambers was dropped from 100% air saturation (% air sat) to approximately 8%
235 air sat over a period of 4-5 hours during which fish oxygen consumption continued to be monitored
236 using the same measurement cycle as before (Claireaux and Chabot 2016). At the end of the
237 hypoxic trial, fish were removed from the respirometry chambers and returned to their original
238 rearing tank. Background bacterial MO_2 was then recorded in the empty chambers and estimated at
239 every time during the experiment using linear regression, assuming zero background respiration at
240 the beginning of the run as the entire system was disinfected with household bleach between each
241 trial. Each fish MO_2 measurement was then corrected for the calculated background respiration.
242

243 Respirometry data analysis and calculations

244

245 The accuracy of the MO_2 estimation is reflected by the regression coefficient (R^2) between water

246 oxygen level and time during the measurement period (closed mode) of the respirometry cycle.

247 Values of MO_2 associated with R^2 below 0.85 were removed from the analysis (maximum 5% in

248 some fish). MMR was determined using the highest MO_2 values recorded during the 2-h post

249 exhaustion period. Fish SMR was determined using a R script (Chabot et al., 2016) and

250 MO_2measurements obtained after fish full recovery (typically 10 h) and before the beginning of the

251 hypoxia treatment. Briefly, the script analyses the distribution of MO_2 measurements (mclust

252 function in R package) and selects the number of normal distributions that best fit the data (between

253 one and four). The coefficient of variation (CV) of the values assigned to the normal distribution

254 with the lowest mean value among the four distributions is then calculated. When CV is below 7 the

255 mean of the values assigned to the lowest normal distribution is considered to represent SMR.

256 When CV is more than 7, the 0.2 quantile of the values is preferred to represent SMR (Chabot et al.

257 2016).

258

259 The critical oxygen level (O_{2crit}) was determined using a R script from Claireaux and Chabot

260 (2016). This script establishes the linear regression between the ambient oxygen level and fish MO_2

261 as hypoxic conditions develop. The calculated intersection between this regression line and SMR

262 corresponds to O_{2crit} (Fig. 2).

263

264 **Maximum heart rate**

265

266 Measurement of maximum heart rate (f_{Hmax}) were performed during September 2014, following

267 Casselman et al. (2012). At that time fish weighted 30.1±1.0 g and were 13.5±0.1 cm in length (no

268 statistical differences between experimental treatments, ANOVA, $F(2, 39) = 1.643$, $P = 0.207$,
269 ANOVA, $F(2, 39) = 0.114$, $P = 0.738$, respectively).
270
271 The fish (N = 14 per treatment) were anesthetized in 32 ppm seawater containing 5 mgL^{-1} MS222,
272 the pH was adjusted with NaOH to similar pH as in the experimental treatments. After being
273 weighted, fish were placed in an experimental setup that received aerated and temperature
274 controlled water containing a maintenance dose of MS222 (5 mgL^{-1}). At the beginning of the
275 experiment the water temperature was 17°C. The water was partially directed over the fish gills.
276 Fish electrocardiogram (ECG) was detected with silver electrodes positioned on the skin just above
277 and below the heart, a ground electrode was in the water. The ECG was recorded with BioPac
278 MP36R (BIOPAC Systems Inc, Essen, Germany) with build-in amplifiers and filters.
279
280 Fish were allowed to stabilize in the setup for 30 minutes before intraperitoneal injections of
281 atropine sulphate (3 $mgkg^{-1}$) and isoproterenol (8 μgkg^{-1})to increase heart rate to its maximum value
282 (f_{Hmax}) (Casselman et al. 2012). Both drugs were purchased from Sigma-Aldrich Chemie Gmbh
283 (Munich, Germany) and dissolved in saline (0.9% NaCl). The time-interval between injections was
284 15 minutes after which the temperature of water was increased in1°C increments every six minutes
285 (10°Ch^{-1}). At each step, the heart rate was allowed to stabilize for five minutes. f_{Hmax} was recorded
286 at each temperature increment by measuring the duration of 15 heart beats R–R intervals and
287 transforming into a frequency. When cardiac arrhythmias (missing QRS complex in ECG signal i.e.
288 atrioventricular block, see Anttila et al. 2013) were first observed the temperature of the water was
289 recorded (=arrhythmia temperature, T_{ARR}), fish were removed from setup and returned to their
290 rearing tank. No mortalities were observed during the days that followed the trials.
291

292 For each fish the Arrhenius break point temperature (T_{AB}), was calculated using Arrhenius plots
293 according to Yeager and Ultsh (1989). The analyses were done with SigmaPlot (12.3; Systat
294 Software Inc., USA) Regression Wizard program using two segment linear regression formula. In
295 the analyses the temperature was transformed to Kelvins and expressed in x-axis while heart rate
296 was transformed to natural logarithm of f_{Hmax} and expressed in y-axis. The plot included all the
297 testing temperatures and heart rates from 17°C until arrhythmias were observed. The software fitted
298 two linear regression lines to plot and the intersection of the two linear regression lines indicated the
299 Arrhenius break point temperature (T_{AB}).
300
301 **Samplings**
302
303 One month after the respirometry experiments, eight fish from each treatment were selected at
304 random, anesthetized with MS222 and blood samples were quickly drawn by caudal puncture using
305 heparinised syringes. Haematocrit was determined immediately. The remaining blood was kept at
306 4 °C in heparinised tubes for haemoglobin measurements within the hour post-sampling. Fish were
307 then sacrificed with a spinal cut and their mass and length measured. Left gill arches were then
308 excised, rinsed in a physiological solution (Ringer solution) and stored at 4°C for 24h. The ventricle
309 was also excised and immediately wet weighted. Ventriculo-somatic index was obtained dividing
310 the ventricle mass by total body mass.
311
312 Blood haemoglobin concentration was measured with a colorimetric kit (Drabkin, Sigma, France).
313 Mean corpuscular haemoglobin concentration (MCHC) was calculated by dividing values of
314 haemoglobin concentration by the haematocrit. Gill filaments were carefully cut from each gill arch
315 under a binocular and wet weighted. They were then dried for 72h at 60°C and dry weighted for

316 calculation of the gill water content. Gill-somatic index was obtained dividing the gill filaments
317 mass by the total body mass.
318
319 **Statistical analysis**
320
321 Data normality and homogeneity were tested with analysis of the distribution of the residuals and
322 Levene tests respectively. A general linear model was used to analyse MMR, SMR, aerobic scope
323 and O_{2crit}, with experimental CO_2 condition fitted as fixed effect, date of run start fitted as random
324 effect and body mass as a covariate. A two-way repeated measures of ANOVA was used to analyse
325 f_{Hmax} differences between experimental CO_2 conditions and measuring temperatures. Mass, T_{AB},
326 T_{ARR}, gill, heart and blood data (gill-somatic index, gill water content, ventriculo-somatic index,
327 haematocrit, haemoglobin concentration, MCHC) were analyzed using one-way ANOVAs with
328 experimental CO_2 condition as factor. The Bonferonni correction was applied to the haematocrit,
329 haemoglobin concentration and MCHC. *A posteriori* Tukey's tests were performed when variances
330 were homogenous, otherwise, Games & Howell test was preferred.Statistical analyses were
331 performed using Statistica7 (Statsoft, USA) and SigmaPlot 12.3 (Systat Software Inc., USA). A
332 significance level of $\alpha = 0.05$ was used in all statistical tests.
333
334 **RESULTS**
335
336 **Respirometry**
337
338 Experimental CO_2 treatments had no significant effect on SMR (GLM, $F(2,6) = 0.681$, $P = 0.542$)
339 (Fig. 3) but significantly affected MMR (GLM, $F(2,6) = 4.414$, $P = 0.016$). Fish exposed to severe
340 hypercapnia (HA) had a significantly higher MMR than control (C) and moderate hypercapnic

341 (MA) fish (Fig. 3). No significant difference between C and MA fish was observed. Experimental
342 CO_2 treatments had no significant effect on aerobic metabolic scope (GLM, $F(2,6) = 0.664$, P =
343 0.549), which was 231.9 ± 7.4mg$O_2h^{-1}kg^{-1}$, 237.0± 6.4mg$O_2h^{-1}kg^{-1}$ and 254.2± 11.1mg $O_2h^{-1}kg^{-1}$ for
344 the C, MA and HA fish respectively. Body mass covariate was having a significant effect on SMR
345 (GLM, $F(2,6) = 8.62$, P = 0.005) and on MMR (GLM, $F(2,6) = 7.46$, P = 0.008), but not on the
346 aerobic metabolic scope (GLM, $F(2,6) = 3.24$, P = 0.077). The body mass of the fish was ranging
347 from 53.7g to 118.5g, the mean being 79.6 ± 1.7 g.
348
349 **Maximum heart rate measurements**
350
351 During warming, both the experimental CO_2 treatments and water temperature had significant
352 effects on f_{Hmax} (ANOVA, $F(2,344) = 13.6$, P < 0.001 for CO_2 and $F(10,344) = 3.2$, P < 0.001 for
353 temperature) as no interaction between the main factors was found (ANOVA, $F(2,10) = 0.48$, P =
354 0.97). *A posteriori* tests revealed that there were significant differences between C and MA fish and
355 between C and HA fish while the MA and HA fish did not differ significantly from each other. The
356 control group had the highest f_{Hmax} values during warming, whereas exposure to hypercapnic
357 conditions lowered the maximum heart rate significantly (Fig.4).
358
359 Although heart rate measured during warming was lowered in hypercapnic-reared fish, thermal
360 tolerances (T_{AB}: ANOVA, $F(2, 40) = 0.006$, P = 0.99, and T_{ARR}: ANOVA, $F(2, 40) = 0.53$, P =
361 0.59) were not found statistically different among rearing CO_2 treatments (Table 2).
362
363 **Tissues and blood response**
364

365 The experimental CO_2 treatments had no significant effect on the ventriculo-somatic index

366 (ANOVA, $F(2,21) = 0.93$, P = 0.41) (Table 3).

367

368 Rearing CO_2 conditions had a significant influence upon the wet (data not shown) and dry gill

369 mass/body mass ratio (Fig.5A) (ANOVA, $F(2,21) = 3.8$, P = 0.039 and $F(2,21) = 6.6$, P = 0.006,

370 respectively). This ratio was significantly higher in HA fish compared to C fish while MA fish were

371 similar to both C and HA fish. Rearing CO_2 conditions also had a significant influence upon gill

372 water content (ANOVA, $F(2,21) = 4.1$, P = 0.032). Gill water content was significantly lower in

373 HA fish compared to MA fish (Fig. 5B) whereas control fish were similar to both MA and HA fish.

374

375 No significant difference in blood haematocrit was observed among the three experimental CO_2

376 groups (ANOVA, $F(2,21) = 1.0$, P = 0.38, Bonferroni correction P = 1) (Table 3). However,

377 experimental CO_2 treatments had a significant influence upon blood haemoglobin concentration

378 (ANOVA, $F(2,21) = 6.9$, P = 0.005, Bonferroni correction P = 0.015) (Table 3) which was

379 significantly higher in HA fish compared to C and MA fish. No difference between C and MA was

380 observed. The resulting mean corpuscular haemoglobin concentration (MCHC) differed among

381 experimental CO_2 groups (ANOVA, $F(2,21) = 12.1$, $P < 0.001$, Bonferroni correction P = 0.003)

382 (Table 3). Higher MCHC levels were observed in HA and MA fish compared to the C fish. No

383 difference between HA and MA was observed.

384

385 **Critical oxygen level**

386

387 No significant difference among experimental treatments was observed on O_{2crit} (GLM,

388 $F(2, 6) = 0.509$, P = 0.625) (Table 3).

389

DISCUSSION

The objective of this study was to examine the integrated consequences of exposing fish to projected ocean acidification conditions over a 1.5-year period which included larval and juvenile life stages. In this study, fish aerobic metabolic capacity (SMR, MMR, aerobic metabolic scope and critical oxygen level) as well as characteristics of the oxygen extraction and transport chain (namely maximal heart rate, gill and ventricular mass, blood haematocrit and haemoglobin concentration) were measured. Compared to the control treatment (*C*; pH 7.9, P_{CO2}: 520 µatm), moderate acidification conditions (*MA*; pH 7.7, P_{CO2}: 950 µatm) had no effect on the fish aerobic metabolic capacity nor gill mass to body mass ratio. However, the cardiac response to the acute increase in water temperature was altered as values of maximal heart rate were significantly lower. In the high acidification condition (*HA*; pH 7.5, P_{CO2}: 1490 µatm), no difference in fish standard metabolic rates was observed but higher maximal metabolic rates were measured. Despite these results, no significant difference in aerobic metabolic scope was found, likely as a result of high inter-individual variability in both variables. Fish from this condition also presented a heavier gill system, as well as higher haemoglobin concentration and mean corpuscular haemoglobin concentration, suggesting higher oxygen extraction and transport capacity, even if they also presented a reduced maximal heart rate during warming. However, these fish displayed similar critical oxygen level to control fish. Overall, these results suggest that projected high acidification condition can lead to a new aerobic metabolic condition in sea bass (*Dicentrarchus labrax,* Linnaeus) juveniles.

A 1.5-year exposure to P_{CO2} levels above current situation (950 µatm and 1490 µatm) did not affect sea bass standard metabolic rate. Conflicting data exist in the literature regarding the effect of hypercapnia on SMR. A similar lack of effect has been reported in Atlantic Cod (*Gadus morhua,*

415 Linnaeus, acclimated to extreme hypercapnia ,6000 µatm, for 12 months, Melzner et al. 2009) and
416 in damselfish (*Pomacentrus moluccensis*, Bleeker, and *Pomacentrus amboinensis*, Bleeker,
417 acclimated to near-future hypercapnia, 860µatm, for four days, (Couturier et al. 2013). Conversely,
418 spiny damselfish (*Acanthochromis polyacanthus,* Bleeker, exposed to 946 µatm P_{CO2} for 17 days)
419 displayed lower resting metabolic rate (Rummer et al. 2013). Such discrepancies in fish responses
420 to hypercapnia prevents definitive conclusion. However, the diversity in experimental conditions
421 tested in the above studies may explain, at least partially, the variability in the reported effects.
422 Exposure duration is certainly an important element to take into account. Fish exposed to
423 hypercapnic conditions must restore internal acid-base balance by pumping bicarbonate from the
424 surrounding water mostly in exchange for chloride. This entry of bicarbonate compensates CO_2-
425 related acidosis by restoring extra-cellular pH and, depending on the species and experimental
426 conditions tested, these adjustments may take from hours to days to be completed (Esbaugh et al.
427 2012; Heuer and Grosell 2014). Thus, measures of MO_2 made within a few days following
428 exposure may not represent true SMR as they may include the masking effect of this additional
429 regulatory work. In addition, in some of the previous studies, unusual respirometry protocols were
430 used such as recording SMR during daytime, the later potentially leading, in diurnal species, to an
431 overestimation of SMR due to residual activity and vigilance. As also pointed out by Gräns et al.
432 (2014), this may potentially result in increased inter-individual variability in SMR masking the
433 modest cost of acid-base compensation.
434
435 The similar standard metabolic rate observed among experimental treatments suggests that long
436 term acclimation to even the most severe ocean acidification scenario did not affect sea bass
437 maintenance demand for oxygen. It remains to be determined, however, whether this observation
438 implies no additional regulatory costs or rather that the additional cost is compensated through, for
439 instance, a different setting in the trade-off among life sustaining activities. Several authors have

440 reported increased homeostasis-related activities such as ion transports, acid-base regulation and
441 energy metabolism enzymes following long term (>14 weeks) exposure to high P_{CO2} levels (Evans
442 et al. 2005; Esbaugh et al. 2012; Bresolin de Souza et al. 2014). It was then hypothesized that these
443 increased activities should lead to increased SMR (Deigweiher et al. 2010; Bresolin de Souza et al.
444 2014; Esbaugh 2018; Hannan and Rummer 2018) but the few published studies that actually
445 measured the cost of hypercapnia suggested that exposure to hypercapnia was not associated with
446 increased metabolic expenditure (Deigweiher et al. 2008; Melzner et al. 2009; Esbaugh et al. 2016;
447 Lefevre 2016). The absence of difference in growth in the present study additionally supports the
448 lack of energetic cost of future hypercapnia. As mentioned above, revised trade-off among the life
449 sustaining functions may contribute to preserve SMR under hypercapnic conditions. To our
450 knowledge, however, no published information is available to document this possible change in fish
451 prioritisation of physiological functions.
452
453 In the current study, the long-term exposure to high CO_2 level (1490 µatm) resulted in significantly
454 elevated MMR (+10%). Previous studies also reported increased MMR (~20%) in the spiny
455 damselfish (*Acanthochromis polyacanthus*, Bleeker, exposed to 946 µatm P_{CO2}, Rummer et al.
456 2013) and (28-39%) in the damselfish (*Pomacentrus amboinensis,* Bleeker, exposed to 860 µatm
457 P_{CO2}, Couturier et al. 2013). On the contrary, in a different species of damselfish (*Pomacentrus*
458 *moluccensis,* Bleeker), as well as in its predator (*Pseudochromis fuscus,* Muller and Troschel) no
459 change in MMR was observed (exposure to 860 µatm P_{CO2}, Couturier et al. 2013). Lack of effect
460 has also been reported in the European eel (*Anguilla anguilla,* Linnaeus, exposed up to 60000µatm
461 P_{CO2}, McKenzie et al. 2003), in the Atlantic Cod (*Gadus morhua,*Linnaeus, exposed to 6000 µatm
462 P_{CO2}, Melzner et al. 2009), . As for SMR, these differences are likely at least partially the result of
463 differences in experimental conditions, including the duration of exposure. Furthermore, some
464 authors measured MMR in swimming chambers during steady-state swimming while others used

465 static chambers measuring MMR during recovery from an episode of chasing until exhaustion.
466 During steady swimming, all the components of the oxygen transport chain are solicited and in
467 relative steady state to provide oxygen to the working muscles. On the other hand, following
468 exhaustion, oxygen demanding activities are mostly involved in restoring tissue and cellular
469 homeostasis and steady state in oxygen allocation and use is unlikely (Zhang et al. 2018). Although
470 some evidences suggests that steady swimming and exhaustive exercise can generally give
471 comparable measures of MMR (Killen et al. 2017), it has to be noted that this may depend on the
472 species and its lifestyle. Inter-species variation in the response to hypercapnia should also be
473 expected, especially since Couturier et al. (2013) demonstrated that different species can exhibit
474 different MMR responses to increase P_{CO2}.
475
476 Following long term exposure to acidification juvenile sea bass displayed significantly lower
477 maximum heart rate (f_{Hmax}) (7-15%) during acute warming than control individuals. It has been
478 recently shown that combining ocean acidification (1170 µatm) with increased temperature (from 0
479 to 8 or 16 °C in Polar, *Boreogadus saida*, Lepechin, and Atlantic cod, *Gadus morhua*, Linnaeus,
480 respectively for 4 weeks) reduced heart mitochondrial ATP production (Leo et al. 2017). This could
481 be one mechanistic reason for the lower maximal heart rate recorded in the hypercapnic fish.
482 However, the present result contrasts with the only other study on cardiac performance following
483 long-term acclimation (14-16 weeks) to hypercapnia (1000 µatm) (Gräns et al. 2014). These authors
484 indeed reported that hypercapnia acclimated Atlantic halibut (*Hippoglossus hippoglossus,*
485 Linnaeus) displayed higher maximum cardiac output than control fish. Because of regulatory
486 change in stroke volume measurement, heart rate and cardiac output do not necessarily correlate.
487 The contrasting results may be resolved by considering a possible compensatory increase in stroke
488 volume. This, indeed, could be the case since there were no differences in the thermal capacities of
489 cardiac function among groups. However, it remains to be tested which are the compensatory

490 mechanisms (stroke volume or e.g. changes in energy metabolism of cardiac function). In addition,
491 it has to be acknowledged that in the present study heart rates and metabolic rates were measured
492 several months apart, possibly influencing the relation between them.
493
494 Fish exposed to 1490 µatm CO_2 for 1.5 year displayed heavier gills (+15%) and this was not the
495 result of water movements into the gill epithelium as no difference in gill water content with the
496 control group was observed. Although gill surface area was not actually measured, it is tempting to
497 hypothesise that heavier gills indicate increased respiratory surface, as reported in the striped catfish
498 *Pangesianodon hypophthalmus* (Phuong et al. 2018) and, therefore, increased oxygen extraction
499 capacity. This would also require, however, that the oxygen diffusion distance across the gill
500 epithelium is at least maintained in the high CO2 treatment. Accordingly, it has been shown that a
501 14-day exposure of the estuarine red drum (*Sciaenops ocellatus*) to 1000 µatm resulted in a
502 significant reduction in the branchial diffusion distance (Esbaugh et al. 2016). Fish are known for
503 having highly plastic gills, changes having been reported in relation with water oxygenation,
504 temperature, salinity and acidification (Evans et al. 2005; Sollid and Nilsson 2006; Chapman et al.
505 2008; Rummer et al. 2013). This regulatory mechanism could provide the functional basis for the
506 increased MMR observed in hypercapnic acclimated fish and a compensation for the lower
507 maximal heart rate measured during warming. As maximal heart rate is decreasing, less oxygen
508 may be available to organs, potentially resulting in hypoxemia. Increased gill surface area may
509 have occurred to compensate this phenomenon, leading to increased MMR. Rummer et al (2013)
510 also suggested that the increased MMR they observed in damselfish under acidification condition
511 was obtained via increased gill oxygen extraction capacity, through increased blood perfusion and
512 lamellar recruit.
513

514 Along the same line, fish exposed to acidification displayed higher haemoglobin concentration
515 (+ 30%, 1490 µatm P_{CO2}) and MCHC (+15%, 950 and +25%, 1490 µatm P_{CO2}), suggesting higher
516 oxygen carrying capacity than control fish. As no difference in haematocrit was found between
517 treatments, this was obtained without affecting blood viscosity, hence cardiac workload. Similarly,
518 no change in haematocrit has been observed in Gilthead seabream (*Sparus aurata*, Linnaeus)
519 exposed to 5000 µatm P_{CO2} (Michaelidis et al. 2007). In contrast to our results, however, these
520 authors, as well as Rummer et al. (2013), did not find any difference in haemoglobin concentration.
521 Since it can take up to an average of eight months to renew red blood cells stores (Witeska 2013),
522 blood haemoglobin acclimation is a long-term process that may have been missed in short-term
523 acclimation studies.
524
525 When exposed to severe hypoxic conditions, hypercapnic-reared fish displayed similar critical
526 oxygen level (O_{2crit}) than normocapnic fish. The O_{2crit} corresponds to the minimal oxygenation level
527 required to sustain standard metabolic rate (SMR). Below O_{2crit}, aerobic metabolic scope is nil and
528 an increased proportion of fish ATP production shifts from being aerobic to being anaerobic. The
529 only few studies that have investigated the effect of hypercapnia on O_{2crit} were concordant with
530 present results (McKenzie et al. 2003; Couturier et al. 2013; Ern et al. 2017). Other studies have
531 used different indicators to document the transition from aerobic to anaerobic metabolism. Rummer
532 et al. (2013) compared the kinetics of plasma lactate accumulation during an hypoxic episode in the
533 spiny damselfish (*Acanthochromis polyacanthus,* Bleeker). They found that hypercapnia exposed
534 fish (P_{CO2} = 946 µatm) had similar lactate threshold than control fish. It must be noted, however,
535 that plasma lactate is a difficult indicator to handle as its accumulation in the blood stream is
536 generally the result of the mismatch between production and disposal (Omlin and Weber 2010).
537 Nonetheless, the ability of sea bass juveniles to preserve O_{2crit} under hypercapnic conditions
538 suggests that the implemented physiological adjustments to compensate for extra-cellular

539 acidification did not affect fish capacity to meet maintenance oxygen demand under reduced
540 oxygen availability. This is an important result as potential trade-off between hypoxia tolerance and
541 high oxygen transport capacity and aerobic metabolism might arise from conflicting influence of
542 haemoglobin oxygen affinity (Burggren et al. 1991). It is interesting to point out that in the present
543 study such trade-off didn't seem to occur as no difference was observed in fish capacity to maintain
544 aerobic metabolism under hypoxia (O_{2crit}) while increasing aerobic capacity (MMR).
545

546 **CONCLUSION**
547

548 Long-term, across life-stages exposure to acidification levels predicted for 2100 (Caldeira and
549 Wickett 2005; Intergovernmental Panel on Climate Change 2014) resulted in a new aerobic
550 metabolic condition in the sea bass, *Dicentrarchus labrax* (Linnaeus) juveniles. Fish from the high
551 acidification condition (1490 µatm) had higher aerobic capacities (MMR) which correlated with
552 heavier gills and increased blood haemoglobin concentration, suggesting potentially higher oxygen
553 extraction and transport capacity, even if lower maximal heart rate during warming. These results
554 suggest that sea bass juveniles have some metabolic capacities to face projected acidification
555 scenarios. However, further experiments are needed to investigate more deeply the underlying
556 mechanisms involved in the acclimation process. Measurements of extra- and intra-cellular pH and
557 bicarbonate concentration, as well as activities of transporters involved in acid-base regulation,
558 should be conducted to confirm that acid-base balance was fully restored under acidified condition.
559 Moreover, even though the oxygen threshold below which an increased proportion of ATP
560 production shifts from being aerobic to being anaerobic was not affected by hypercapnia, it may
561 have affected fish anaerobic metabolic capacity (Claireaux and Chabot, 2016). In addition, ocean
562 acidification represents just one component of global climate change together with, for instance,
563 ocean warming and deoxygenation. Therefore, there is a pressing need to examine the synergistic

effect of these stressors, as some studies revealed that together they have stronger impacts on marine organisms than when occurring alone (Enzor et al. 2013; Leo et al. 2017). These investigations are essential to provide strong physiological basis and allow a better understanding of the possible adaptation of fish populations in a changing world.

Compliance with Ethical Standards

Conflict of Interest The authors declare no competing or financial interests

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Project code: APAFIS 4341.03, #201620211505680.V3

Author contributions

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Funding acquisition: JLZI, GC

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Figure1

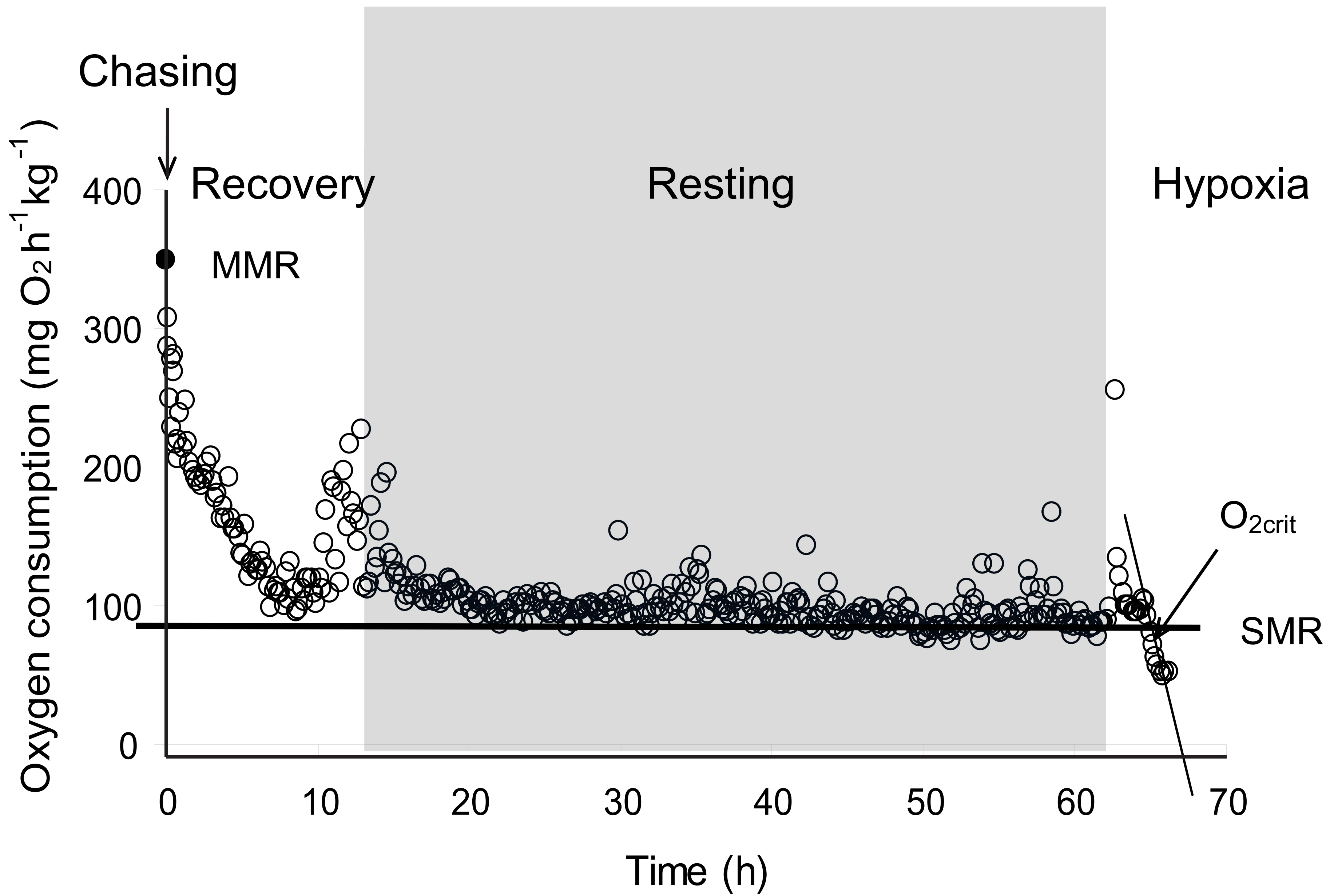


Figure2

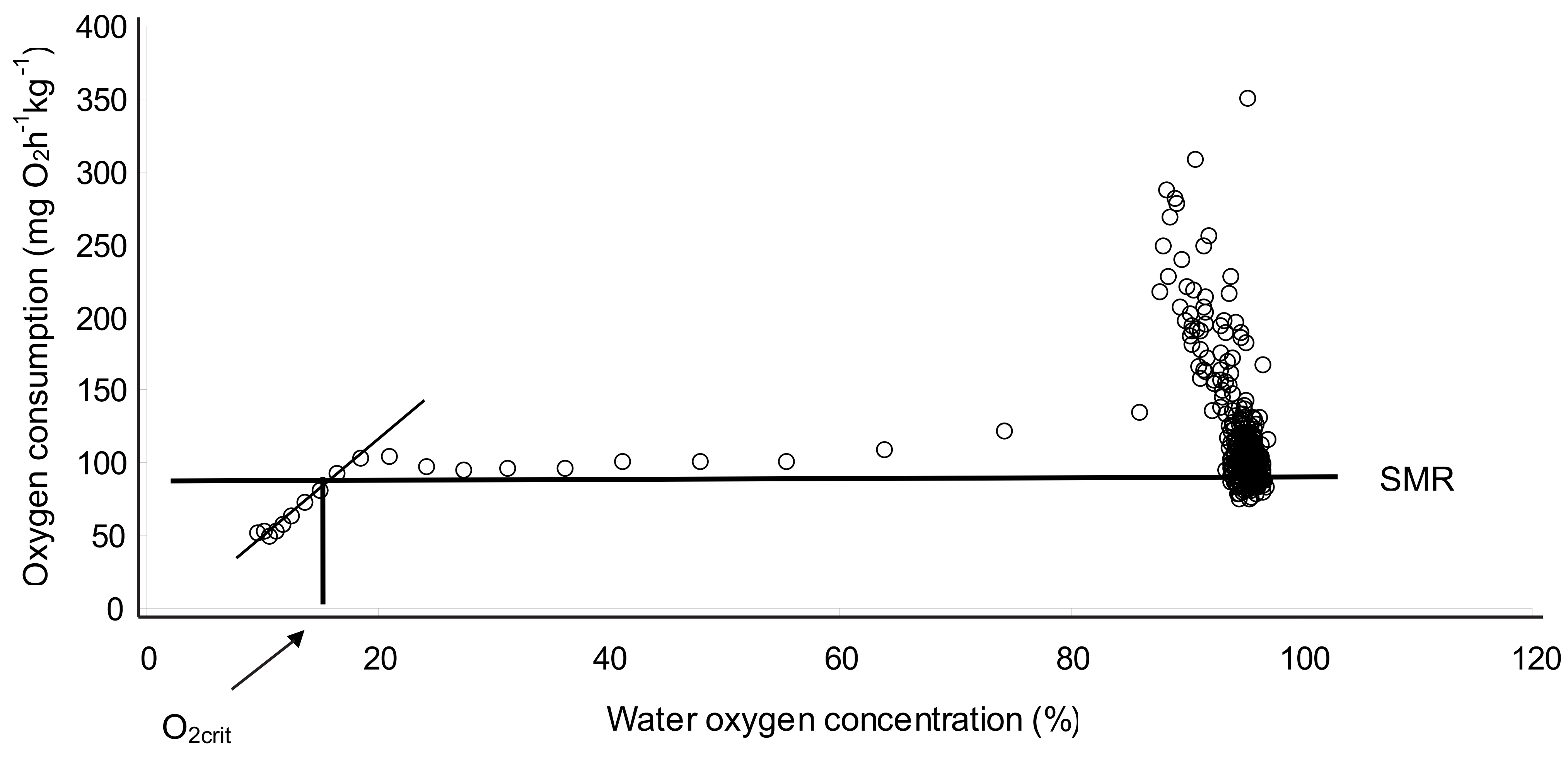
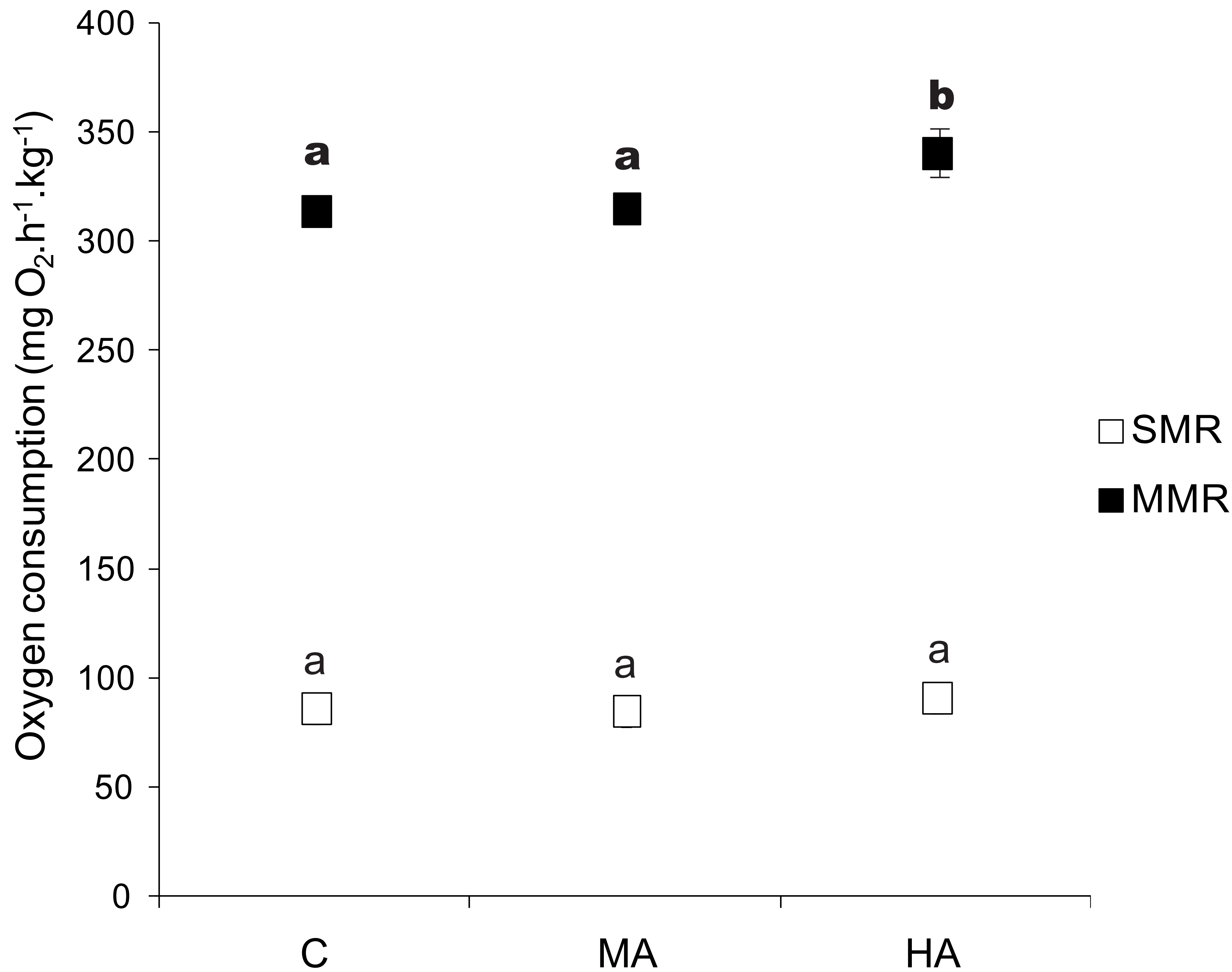


Figure3



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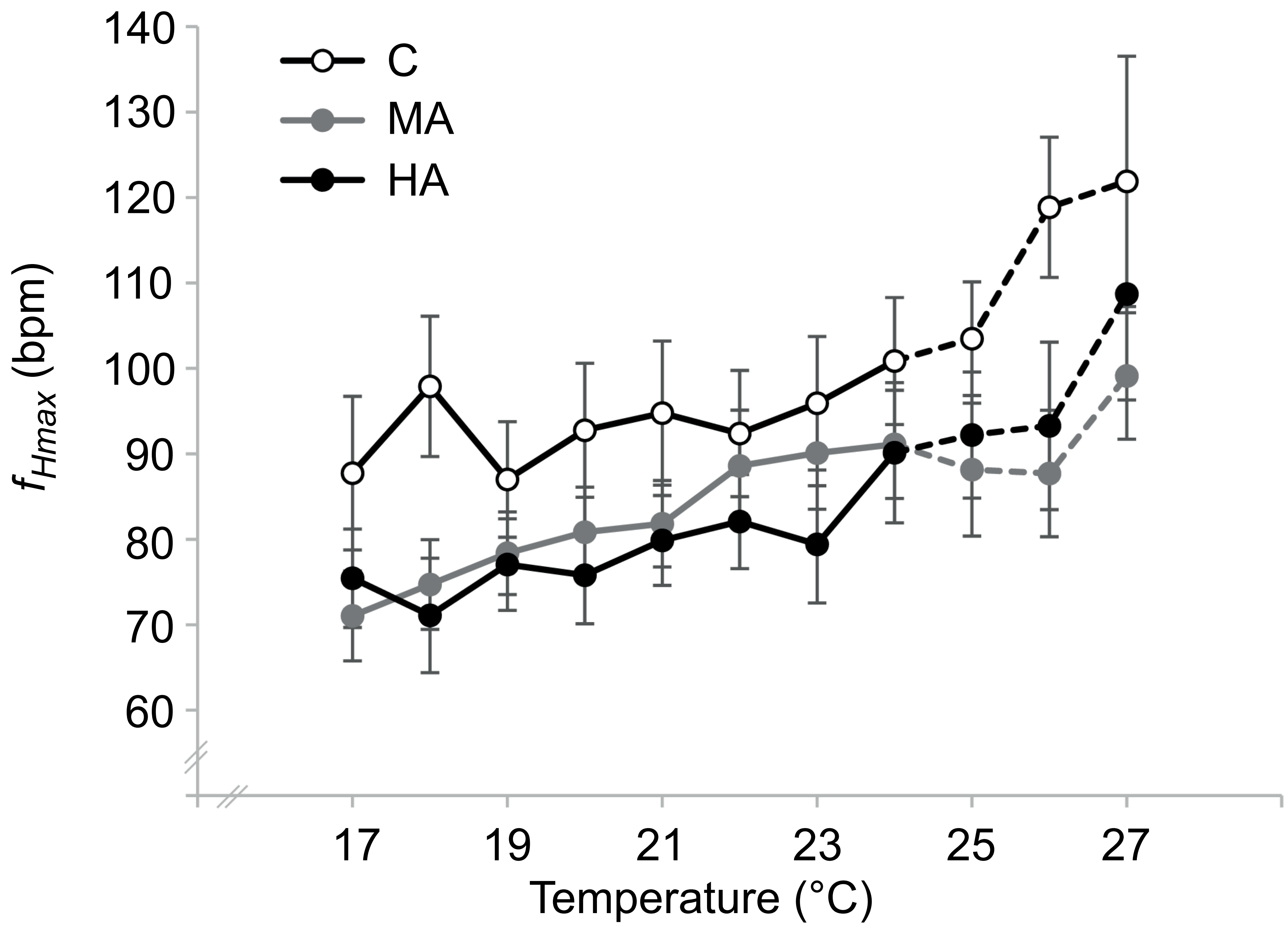
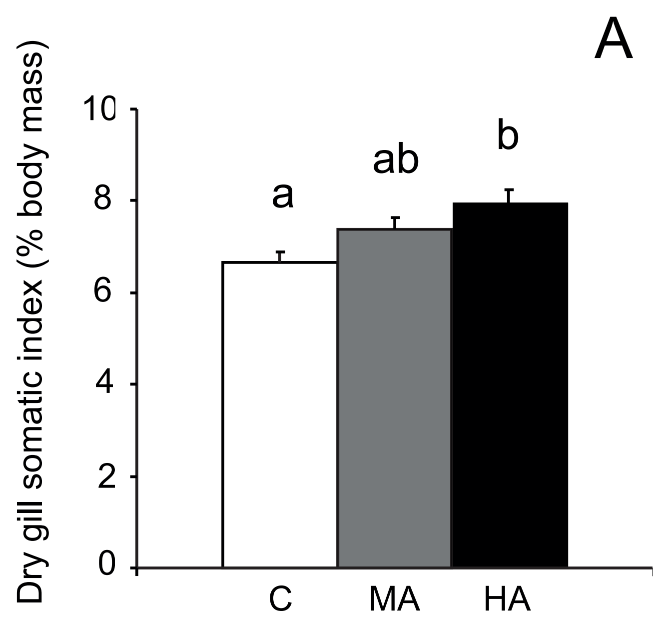
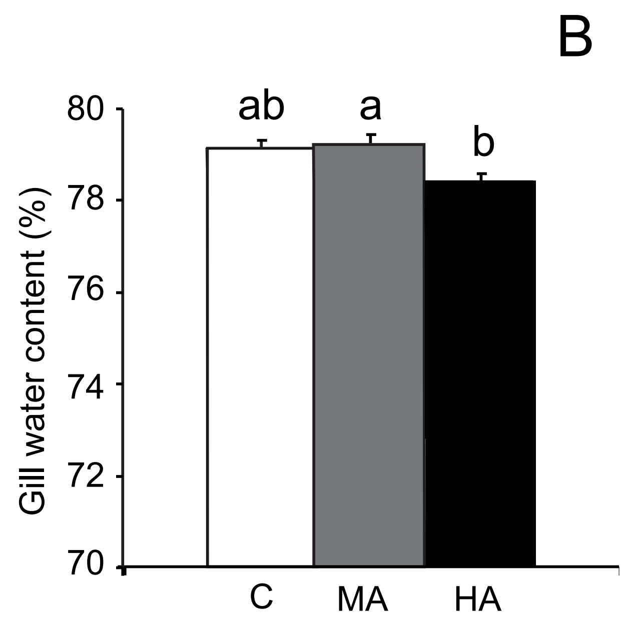


Figure5





1 **Figure captions**

2

3 **Fig. 1** Oxygen consumption (mg $O_2h^{-1}kg^{-1}$) over time (h) of a typical fish. Fish were first chased
4 until exhaustion (Chasing) to determine the maximal metabolic rate (MMR). Then fish were
5 allowed to rest over a period of 65h (Resting) to determine the standard metabolic rate (SMR).
6 Finally, fish were exposed to a progressive hypoxia (Hypoxia) to determine the fish critical oxygen
7 limit (O_2crit).

8

9 **Fig. 2** Oxygen consumption (mg $O_2h^{-1}kg^{-1}$) over water oxygen concentration (%) of a typical fish.
10 Fish were exposed to a progressive hypoxia to determine the fish critical oxygen limit (O_{2crit}). When
11 ambient oxygen drops below O_{2crit}, fish MO_2 decline proportionally and reveal a linear regression
12 (LR) between oxygen level and MO_2 at the end of hypoxia. The intersection between the regression
13 line and the horizontal line corresponding to SMR was O_{2crit}.

14

15 **Fig. 3** The oxygen consumption (mg $O_2h^{-1}kg^{-1}$) in the fish exposed to control P_{CO2} (C; 520 ppm),
16 moderate hypercapnia (MA; 950 ppm) and high hypercapnia (HA; 1490 ppm). Standard (□SMR)
17 and maximal (■MMR) metabolic rates of fish exposed during 1.5-year. Values are mean ± s.e.m., n
18 = 22 to 24 per group. The oxygen consumption was analysed using the absolute values (in mg O_2h^-
19 ^{1}) and body mass as a covariate, the data are presented as mass-specific for clarity. Different letters
20 indicate significant differences ($P < 0.05$).

21

22 **Fig. 4** The maximum heart rate (f_{Hmax}) values of fish exposed to control P_{CO2} (C; 520 ppm),
23 moderate hypercapnia (MA; 950 ppm) and high hypercapnia (HA; 1490 ppm) during incremental
24 warming. The f_{Hmax} was achieved by intraperitoneal injections of atropine sulphate and
25 isoproterenol. The heating rate was 10°C h^{-1}. Values are mean beats per minute (bpm) ± (s.e.m.), n

26 = 14 per group. There is significant differences between *C* and *MA* ($P < 0.001$, $T = 4.0$) and

27 between *C* and *HA* ($P < 0.001$, $T = 4.9$) while the *MA* and *HA* did not differed significantly from

28 each other ($P = 0.68$, $T = 0.4$).The dotted lines indicate temperatures at which arrhythmias were

29 observed in individual fish.

30

31 **Fig. 5** The gill responsein the fish exposed to control P_{CO2} (C; 520 ppm), moderate hypercapnia

32 (MA; 950 ppm) and high hypercapnia (HA; 1490 ppm). (A) Dry gill mass to body mass ratio and

33 (B) gill water content of fish exposed during 1.5-year. Values are mean ± s.e.m., n = 8 per group.

34 Different letters indicates significant difference ($P < 0.05$).

35

1 **Table 1 Water chemistry of the experimental tanks.** Water temperature (T °C), pH $_{NBS}$ (NBS
2 scale), pH tot (total scale), TA (total alkalinity), PO_4^{3-}(phosphate concentration), SiO_4(silicate
3 concentration) were measured in the different conditions. P_{CO2} (the projected partial pressure of
4 CO_2) was calculated using CO2SYS software in the different conditions.
5

	Salinity (‰) n = 5	T°C n = 525	pH $_{NBS}$ n = 525	pH tot n = 5	TA (µML^{-1}) n = 9	PO_4^{3-} (µML^{-1}) n = 6	SiO_4 (µML^{-1}) n = 6	P_{CO2} (µatm) n = 9
C	34.3 (0.2)	15.3 (0.1)	8.05 (0.01)	7.94 (0.03)	2294 (10)	0.71 (0.08)	8.35 (0.26)	516 (31)
MA	34.3 (0.2)	15.3 (0.1)	7.82 (0.01)	7.71 (0.02)	2293 (14)	0.71 (0.08)	8.35 (0.26)	953 (28)
HA	34.3 (0.2)	15.3 (0.1)	7.61 (0.01)	7.53 (0.02)	2280 (16)	0.71 (0.08)	8.35 (0.26)	1489 (42)

6 Values are mean ± (s.e.m.), n is the number of samples
7

8 **Table 2** The Arrhenius break point temperature (T_{AB}) and arrhythmia temperature (T_{ARR}) of fish

9 exposed during 1.5-year to control P_{CO2} (C; 520 ppm), moderate hypercapnia (MA; 950 ppm) and

10 high hypercapnia (HA; 1490 ppm).

11

	T_{AB} (°C)	T_{ARR} (°C)
C	21.5 (0.5)	25.7 (0.8)
MA	21.5 (0.5)	26.6 (0.5)
HA	21.6 (0.5)	25.9 (0.6)

12 Values are mean ± (s.e.m.), n = 14 per group. No significant differences were found between

13 groups.

14

15 **Table 3** Ventriculo-somatic index (VSI), blood haematocrit (Hct), blood haemoglobin

16 concentration (Hb), mean corpuscular haemoglobin concentration (MCHC) and critical oxygen

17 level (O_{2crit}) of fish exposed during 1.5-year to control P_{CO2} (C; 520 ppm), moderate hypercapnia

18 (MA; 950 ppm) and high hypercapnia (HA; 1490 ppm).

19

	VSI (% body mass)	Hct (%)	Hb ($mgdL^{-1}$)	MCHC($mgdL^{-1}$)	O_{2crit} (%)
C	0.053 (0.001)	35.1 (1.6)	7.3 (0.4)[a]	20.9 (1.2)[a]	17.8 (0.5)
MA	0.055 (0.004)	32.6 (1.7)	7.9 (0.4)[a]	24.2 (0.3)[b]	17.1 (0.5)
HA	0.058 (0.001)	35.5 (1.3)	9.4 (0.4)[b]	26.4 (0.6)[b]	16.8 (0.5)

20 Values are mean ± (s.e.m.), n = 24 per group. Different letters indicates significant difference

21 among groups ($P < 0.05$).