

1 **Reduced n-3 highly unsaturated fatty acids dietary content expected with global change**  
2 **reduces the metabolic capacity of the golden grey mullet**

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8 Key-words: active metabolic rate, aerobic scope, anaerobic capacity, cardiomyocyte  
9 performances, *Liza aurata*, n-3 HUFA, standard metabolic rate,  $U_{crit}$ .

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24 **Abstract:** In this study, we hypothesised that a reduction in n-3 HUFA availability for higher  
25 consumers, as expected with global change, would negatively impact the physiological  
26 performances of fish. The aim was to experimentally evaluate the effect of n-3 HUFA dietary  
27 content on cardio-respiratory performances of the golden grey mullet (*Liza aurata*), a  
28 microalgae grazer of high ecological importance in European coastal areas. These  
29 performances were evaluated in terms of critical swimming speed  $U_{crit}$ , associated oxygen  
30 consumption  $MO_2$ , post-exercise oxygen consumption, and calcium fluxes in cardiomyocytes.  
31 Two replicated groups of fish were fed on a rich (Standard Diet SD diet: 1.2% n-3 HUFA on  
32 dry matter basis, DMB) or a poor n-3 HUFA (Low n-3 HUFA Diet LD diet: 0.2% n-3 HUFA  
33 on DMB) diet during five months, and were called SD and LD groups, respectively. The  
34 results showed that the LD diet reduced growth rate as well as the aerobic capacity of *Liza*  
35 *aurata* at 20°C, suggesting that fish may have to save energy by modifying the proportion of  
36 energy allocated to energy-demanding activities, such as digestion or feeding. In addition, this  
37 LD diet induced higher levels of haematocrit and plasma osmolality, indicating a stress  
38 response at the second and third levels in that group. However, the LD diet caused a massive  
39 increase in swimming efficiency. This should improve the capacity of *L. aurata* to migrate  
40 and to forage over a wide area. In turn, these could then compensate for the reduction of  
41 growth rate and aerobic metabolism.

42

## 43 **Introduction**

44

45 In coastal environments, highly polyunsaturated fatty acids from the n-3 series, otherwise  
46 known as n-3 HUFA, are mainly supplied by the dominating diatoms, which represent an  
47 important lipid source for secondary producers (Kates and Volcani 1966; Roessler 1988; Pahl  
48 et al. 2010; Crawford and Broadhurst 2012). Indeed, n-3 HUFA are weakly synthesised *de*  
49 *novo* by higher organisms, while they function as critical structural and physiological

50 components of the cell membranes of most tissues (Sargent et al. 2002). In marine fish,  
51 membranous n-3 HUFA are dominated by ecosapentaenoic acid (EPA, 20:5n-3) and  
52 docosahexaenoic acid (DHA, 22:6n-3; for reviews see Sargent et al. 2002; Glencross 2009;  
53 Tocher 2010). They are essential for growth, survival, pigmentation, development and  
54 functionality of the brain, vision, and the nervous system, as well as in resistance to stress and  
55 disease, as they are precursors of eicosanoids (for reviews see Sargent et al. 2002; Glencross  
56 2009; Tocher 2010).

57 Over the last twenty years, several studies have shown that diatoms growth rate and n-  
58 3 HUFA profile largely decreased because of environmental variability related to global  
59 change (*i.e.* increasing temperature, pH, and UV; decreasing salinity and oxygen saturation  
60 (Béthoux et al. 1998; Gomez and Souissi 2008; Guschina and Harwood 2009; Pahl et al.  
61 2010; Chen 2012). In addition, the changes in physicochemical parameters, hydrological  
62 regimes and precipitation patterns expected with global change would lead to an over-  
63 enrichment of nitrogen and phosphate with respect to silica and would therefore be  
64 responsible for a shift from diatoms towards non-siliceous species (*e.g.* *Phaeocystis*)  
65 dominance, which are poorer in n-3 HUFA than diatoms (Owens et al. 1989 ; Goffart et al.  
66 2002; Gypens et al. 2007; Gomez and Souissi 2008; Guschina and Harwood 2009).

67 In the context of global change, the expected variability of n-3 HUFA content at the  
68 base of the food web may propagate to higher trophic levels, because, in all animals, the fatty  
69 acid composition of the tissue partly depends on that of the diet (Bell et al. 1996; Leu et al.  
70 2006). As mentioned above, n-3 HUFA are the main components of the membrane bilayer.  
71 Therefore, variability in n-3 HUFA dietary content may lead to variability in the membranous  
72 n-3 HUFA of higher consumers, despite the fact that membrane lipids are regulated to large  
73 extent. This would consequently induce changes in their membrane structure and function.

74           Due to its role in membrane functionality, dietary n-3 HUFA content has been shown  
75 to further impact individual physiological performances, and in particular swimming and  
76 cardio-respiratory performances (McKenzie 2001; Wagner et al. 2004; Chatelier et al. 2006a;  
77 Chatelier 2006b). Swimming performance is essential because it determines food intake,  
78 predator avoidance, migratory capability and consequently the selection of favourable  
79 environmental conditions (Brett 1964; Domenici et al. 2013). For many researchers,  
80 swimming performance is therefore considered determinant of the Darwinian fitness of fish  
81 (Martinez 2003; Yan et al. 2013). Swimming performances are widely evaluated through the  
82 critical swimming speed  $U_{crit}$ , *i.e.*, the water speed at which a fish can no longer maintain its  
83 position when exposed to a swim challenge through a step protocol (Brett 1964; Reidy et al.  
84 2000; Lee 2003). Swimming performances are partly limited by the capacity of heart, which  
85 provides and regulates the internal energy of fish (Paige et al. 1996; McKenzie 2001;  
86 Chatelier et al. 2006a; Guderley et al. 2008). Heart capacity thereby determines aerobic  
87 metabolic scope (AS, Fry 1947), which is the difference between the active metabolic rate  
88 (AMR) and the standard metabolic rate (SMR), *i.e.* the maximal metabolic rate of an  
89 organism in a highly active state minus its metabolic rate when at rest (Fry 1971). AS  
90 estimates the capacity of oxygen allocation, *i.e.* the energy that an organism may allocate to  
91 its different energy-demanding activities (*e.g.* locomotion, digestion, feeding), and is often  
92 monitored because it is claimed to be a relevant proxy of fitness (Claireaux and Lefrancois  
93 2007).

94           In the context of the environmental adaptation of organisms, it is essential to  
95 understand the influence of fatty acids on the swimming and cardio-respiratory performances  
96 of fish. However, only a few studies have reported this effect of fatty acids on fish, and they  
97 are conflicting. More than ten years ago, McKenzie (2001) showed that an enriched n-3  
98 HUFA diet resulted in a decrease in SMR and AMR in the Adriatic sturgeon *Acipenser*

99 *naccarii* and in the eel *Anguilla anguilla*. A reduced dietary n-3 HUFA content has also been  
100 shown to alter the mitochondrial respiratory capacity of rainbow trout (*Onchorynchus mykiss*)  
101 by changing the n-3 HUFA content in membranes, thus influencing the energy status of the  
102 animal (Guderley et al. 2008). Moreover, Wagner et al. (2004) suggested that low dietary n-3  
103 HUFA/saturated fatty acids SFA and n-3 HUFA/arachidonic acid (AA; 20:4n-6) ratios may  
104 negatively affect the swimming performance of Atlantic salmon, as a negative correlation was  
105 found between these ratios and  $U_{crit}$ . However, the opposite was reported in the same species  
106 by Dosanjh et al. (1998) and McKenzie et al. (1998), as well as in sea bass by Chatelier et al.  
107 (2006b). The latter studies reported that low n-3 HUFA dietary levels (replaced by high  
108 monounsaturated fatty acids MUFA content) in the diet always led to higher  $U_{crit}$  in sea bass,  
109 suggesting a preferential use of MUFA than HUFA to provide the necessary energy to the  
110 animal. These inconsistent results reveal the importance of a better evaluation of the effect of  
111 n-3 HUFA dietary content on the swimming and cardio-respiratory performances of fish,  
112 particularly in the context of global change.

113 Therefore, the aim of this study was to test the effect of a reduction in n-3 HUFA  
114 dietary content on critical swimming speed,  $U_{crit}$ , and associated oxygen consumption,  $MO_2$ ,  
115 post-exercise oxygen consumption, as well as on calcium fluxes in cardiomyocytes of the  
116 golden grey mullet (*Liza aurata*). *L. aurata* is of high ecological importance in European  
117 coastal areas because it is a microalgae grazer and a trophic vector of organic matter from  
118 intertidal to subtidal area. Because of its diet, the mullet may be directly subject to the  
119 variability of n-3 HUFA availability in primary producers, as no intermediate trophic steps  
120 will buffer their biochemical variability. To perform this study, two experimental diets were  
121 used: a Standard diet (SD) used as a reference, and a Low-n-3 HUFA diet (LD), which was a  
122 standard diet with a significantly lower content of n-3 HUFA.

123

124 **Materials and methods**

125

126 Fish maintenance

127

128 All fish manipulations were performed according to the French ethics committee.

129 Juvenile golden grey mullets [initial mean weight  $\pm$  standard error (SE):  $26.1 \pm 0.4$  g; initial  
130 mean standard length  $\pm$  SE:  $13.0 \pm 0.1$  cm] were caught in an Italian lagoon (Cabras, Sardinia,  
131 Italy) in 2011 and transported in aerated plastic bags (n = 2 fish per plastic bag) to our  
132 laboratory (Institut du Littoral et de l'Environnement, UMR 7266 CNRS-Université de La  
133 Rochelle, France) where all experiments were conducted. Upon arrival, fish were transferred  
134 into four indoor tanks (volume: 400 L; n = 20 fish per tank) that were individually supplied  
135 with aerated recirculated sand-filtered natural seawater and equipped with an external  
136 biological filter (Eheim professionnel 3 2080, Eheim, Deizisau, Germany). They were  
137 progressively acclimated to the water tank temperature, which was kept constant by a  
138 recirculating water system (TECO TR20, Conselice, Italy), and maintained in a temperature-  
139 controlled room (20°C) exposed to a 12L:12D photoperiod cycle. Temperature ( $19.9 \pm 0.5^\circ\text{C}$ ),  
140 salinity ( $33.4 \pm 0.1$ ) and oxygen ( $87.9 \pm 2.6\%$  air saturation) were monitored daily using a  
141 conductimeter (WTW model oxi 340i, WeilDeim, Germany). After a few days of acclimation  
142 to the experimental structure, fish were fed with a commercial diet (Le Gouessant®  
143 aquaculture, Lamballe, France) once a day for three weeks. Then, 24h-starved fish were  
144 anaesthetised (tricaine methane sulphonate MS-222;  $0.1 \text{ g L}^{-1}$ , Sigma-Aldrich, St Quentin-  
145 Fallavier, France), weighed, and pit-tagged (M120; biolog-id, Réseaumatique, Bernay,  
146 France).

147 Two experimental isolipidic and isoproteic diets differing by their n-3 HUFA content, were  
148 made at the PFOM unit, UMR 6539 LEMAR (Plouzané, France): A standard diet SD (1.2%

149 EPA + DHA on dry matter basis), and a low-n-3 HUFA diet LD (LD: 0.2% EPA + DHA on  
150 dry matter basis DMB) which was obtained by replacing the fish oil present in the standard  
151 diet with soybean oil. For each of these two diets, two groups of fish (n = 2 tanks, *i.e.* 40 fish  
152 per dietary condition) were fed for 5 months (2% of biomass day<sup>-1</sup>). As the HUFA n-3 needs  
153 were not known for *L. aurata*, SD was formulated according to the needs known for other  
154 species. The n-3 HUFA level of that diet ranged between the optimum recommended in  
155 yellow croaker (0.6-0.98%; Zuo et al. 2012), gilthead seabream (0.9%; Kalogeropoulos et al.  
156 1992), juvenile flounder (0.8-1.0%; Kim and Lee 2004) and juvenile starry flounder (at least  
157 0.9% Lee et al. 2003), and that recommended in juvenile cobia *Rachycentron canadum*  
158 (1.49%; Liu et al. 2007) and juvenile cod *Hexagrammo sotakii* (1.2-1.7%, Lee and Cho 2009).  
159 The composition and fatty acid content of the two diets are summarised in Table 1.

160

#### 161 Lipid analysis

162

163 Lipid analysis was performed on the muscle of 12 individuals for each experimental  
164 condition. For each fish, whole frozen muscle was homogenised rapidly with a Hobart®  
165 mixer in order to maintain a low temperature and then more accurately using a Polytron® (PT  
166 2100 Bioblock®, Illkirch, France). A representative portion (~5g) was taken for lipid analysis  
167 and ~3g was taken for dry weight measurements (105°C in an oven for 24h). Lipid analysis  
168 was conducted on duplicates. Extraction of total lipids was done according to Folch et al.  
169 (1957), with chloroform being replaced by dichloro-methane. The separation of neutral NL  
170 and polar lipids PL was performed on fish samples according to the procedure described by  
171 Juaneda and Roquelin (1985). The total lipids TL extracts were fractionated on silica  
172 cartridges (Sep-Pack, Waters®, Guyancourt, France), NL were eluted by chloroform and PL  
173 by methanol. Fatty acid methyl esters (FAME) of TL were prepared by saponification and

174 then methylation while PL FAMES were prepared by transmethylation with sulphuric acid in  
175 methanol. All FAMES were separated by gas chromatography (Auto-system Perkin-Elmer®  
176 with a flame ionisation detector, BPX 70 capillary column: 25 m x 0.22 mm i.d. x 0.25µm  
177 film thickness; split-splitless injector, with helium as a carrier gas). The injector and detector  
178 temperatures were 220 and 260°C, respectively. The temperature of the oven was initially  
179 50°C, and then increased to 180°C in increments of 15°C min<sup>-1</sup>; here, it was maintained for 5  
180 min, and then finally increased to 220°C in increments of 3°C min<sup>-1</sup>. Data acquisition and  
181 handling were carried out by connecting the GLC to a PE Nelson computer. Individual  
182 FAMES were identified by comparing the retention times of authentic standard mixtures. The  
183 results of individual fatty acid composition were expressed as percent of total identified  
184 FAME.

185 Chemical analyses of feed were performed in triplicate for each sample according to AOAC  
186 (Association of Official Analytical Chemists 1984) methods: ash (7 h at 550°C), crude fat  
187 (Folch et al. 1957), and crude protein (Dumas method with an Elementary NA 2000®,  
188 N × 6.25). FAME were extracted and analysed on total lipid fraction using the previously  
189 described method.

190

191 Growth performance

192

193 Each month, 24h-starved fish (n = 40 per experimental condition) were anaesthetised (tricaine  
194 methane sulphonate MS-222; 0.1 g L<sup>-1</sup>, Sigma-Aldrich, St Quentin-Fallavier, France) and  
195 individually identified using a pit-tag reader (MS-120, biolog-id, Réseumatique, Bernay,  
196 France) before measuring fresh weight (± 0.1g), total length, standard length (*i.e.* notochord  
197 length), height and width (± 0.01 cm).



198 Specific growth rate SGR (% day<sup>-1</sup>) was calculated for each experimental condition using  
199 Equation 1:

$$200 \quad \text{SGR} = 100 \times \frac{(\text{Ln final body weight} - \text{Ln initial body weight})}{\text{number of days}} \quad (1)$$

201

202 Swimming performance ( $U_{\text{crit}}$  test)

203

204 *Experimental set-up*

205

206 Fish swimming and metabolic performances were assessed using a swim-tunnel respirometer  
207 (Loligo Systems, Tjele, Denmark), which was made of a respirometer and an external bath.

208 The respirometer (volume: 10 L) was composed of (i) a swim chamber with a square working

209 section (40 cm of length, 10 cm height, 10 cm of width) and (ii) a hydraulic system placed

210 upstream to promote a laminar flow in the swim chamber. No correction for solid blocking

211 effects of the fish in the working section was made, since the calculated fractional error was <

212 5% of the working section area (Webb 1975). The flow in the respirometer was generated by

213 an electric motor with a propeller. It was calibrated before the start of experiments and the

214 speed ranged between 0 and 150 cm s<sup>-1</sup>. Temperature was kept constant by a recirculating

215 water system from the external bath (TECO TR20, Conselice, Italy). A flush pump allowed

216 water exchange between the respirometer and the external bath, in which water temperature

217 and oxygenation were controlled.

218

219 *Oxygen consumption measurements*

220

221 Oxygen concentration in the respirometer was continuously measured during the experiments

222 with an oxygen probe (PreSens, GmbH, Regensburg, Germany) connected to an oxymeter

223 (Oxy-4, PreSens, GmbH, Regensburg, Germany) transferring oxygen data every 10 s to a  
224 storage computer. The oxygen concentration was automatically adjusted according to the real-  
225 time temperature recorded in the respirometer.  $MO_2$  was measured by intermittent-flow  
226 respirometry, based on an alternation between (i) a flushing phase (5 min) and (ii) a  
227 measuring phase (20 min), during which the flush pump was turned off, preventing the inflow  
228 of water from the external bath into the respirometer. The  $MO_2$  ( $mgO_2 kg^{-1} h^{-1}$ ) was calculated  
229 as in Vagner et al. (2008; Equation 2):

$$230 \quad MO_{2meas} = \left( \frac{\Delta [O_2]}{\Delta t} \right) \times \left( \frac{V}{m} \right) \quad (2)$$

231 where  $\Delta [O_2]$  is the oxygen concentration decrease ( $mgO_2 L^{-1}$ ) relative to the fish oxygen  
232 consumption with respect to time  $\Delta t$  (hours),  $V$  the swim tunnel water volume (10 L) minus  
233 the volume of the fish, and  $m$  the fish weight (kg).

234 For each  $MO_2$  measurement, a linear regression was adjusted (Graphical Analysis 3.4,  
235 Beaverton, OR, USA) in order to determine  $\Delta [O_2]/\Delta t$  from the graph plotting  $[O_2]$  versus  
236 time. The regression coefficient of the linear relationship determined  $MO_2$  measurement  
237 accuracy. Only those measurements with a regression coefficient above 0.9 were considered.  
238 The bacterial  $MO_2$  was measured for half an hour before and after each experiment, and the  
239 mean of both was subtracted from the  $MO_2$  measured.

240 As respiratory metabolism depends on the animal weight,  $MO_2$  was standardised for a 100 g  
241 fish et al. (Vagner et al. 2008; Equation 3):

$$242 \quad MO_{2cor} = MO_{2meas} \times \left( \frac{m_{meas}}{m_{cor}} \right)^{1-A} \quad (3)$$

243 where  $MO_{2cor}$  ( $mgO_2 kg^{-1} h^{-1}$ ) is the oxygen consumption for a corrected weight ( $m_{cor} = 100$  g),  
244  $MO_{2meas}$  is the measured  $MO_2$  ( $mgO_2 kg^{-1} h^{-1}$ ) and  $m_{meas}$  is the fish weight (kg).  $A$  is the  
245 allometric exponent describing the relation between the metabolic rate and the fish weight.  $A$   
246 has never been determined for *Liza aurata*. Therefore, we used a value of 0.8 which was  
247 previously estimated in teleosts (Schurmann and Steffensen 1994), and employed in the study

248 by Vagner et al. (2008) for the flathead grey mullet *Mugil cephalus*, a species from the same  
249 family as *L. aurata* (mugilidae).

250

251 *Experimental protocol of the  $U_{crit}$  test*

252 Next, 96h-starved fish [SD group: mean weight  $\pm$  SE:  $47.5 \pm 3.1$  g; mean standard length  $\pm$   
253 SE:  $15 \pm 0.3$ cm, n = 12; LD group: mean weight  $\pm$  SE:  $45.1 \pm 2.1$ ; mean standard length  $\pm$  SE:  
254  $14.6 \pm 0.2$  cm; n = 12] were randomly sampled in tanks using a net, and individually tested in  
255 the swim-respirometer. This long starvation period ensured that  $U_{crit}$  and oxygen consumption  
256 measurements were not influenced by digestion (Beamish 1964). The day before the test, they  
257 were anaesthetised (MS-222;  $0.1 \text{ g L}^{-1}$ ) and transferred into a plastic bag (without exposing  
258 gills to the air) from the indoor acclimatising tank to the swim chamber of the swimming  
259 respirometer. After a short recovery period (about 5 min), the water flow was stabilised at a  
260 very low speed ( $0.5 \text{ Body Length BL s}^{-1}$ ) and a screen darkening the upstream part of the  
261 swim chamber was placed in order to motivate the fish to occupy upstream positions. The fish  
262 were allowed to recover at this low speed for the entire following night.

263 The day after, fish were submitted to a step-protocol involving progressive swimming speed  
264 increments of  $1.5 \text{ BL s}^{-1}$  over 5 min from  $0.5$  to  $3.5 \text{ BL s}^{-1}$ , and increments of  $0.75 \text{ BL s}^{-1}$  for  
265 further increases until fish exhaustion (Brett 1964). At each swimming step, the velocity was  
266 maintained for 20 min and  $\text{MO}_2$  was measured. Oxygen saturation never fell below 75% of air  
267 saturation during  $\text{MO}_2$  measurements. During the phase of swimming speed increment, *i.e.*  
268 between two consecutive swimming steps, water of the respirometer was renewed through the  
269 flush pump reactivation. It allowed the oxygen saturation to return over 85% of air saturation.  
270 The speed increments were repeated until fish exhaustion. Fish were considered exhausted (1)  
271 when they were stuck in a C-shape on the grid placed at the rear of the swim chamber, or (2)

272 when they were unable to swim away from this grid for more than 10 s of lighting  
273 stimulations through the tunnel window at the rear of the swim chamber.

274

275 Excess post-exercise oxygen consumption

276

277 At the end of the  $U_{crit}$  test, the speed was progressively decreased to  $0.5 \text{ BL s}^{-1}$  and fish were  
278 allowed to recover for 70 min according to a cycle of 20 min of measuring and 5 min of  
279 recirculating water, *i.e.* 3  $\text{MO}_2$  measurements were recorded (0.33, 0.75, and 1.17 h after the  
280  $U_{crit}$  test). For each of these three measurement periods and for each fish, the excess  $\text{MO}_2$   
281 ( $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) during the 70 min-recovery period following the test was determined by  
282 integrating (Graphical Analysis vs. 3.4; Beaverton, OR, USA) the area bounded between the  
283 post-test recovery  $\text{MO}_2$  curve and the pre-test  $\text{MO}_2$  ( $0.5 \text{ BL s}^{-1}$ ) measured over the entire recovery  
284 period before the swimming test (Lee et al. 2003; Luna-Acosta et al. 2011). The 6 first hours  
285 of the night recovery period were removed from the  $\text{MO}_2$  ( $0.5 \text{ BL s}^{-1}$ ) calculation, as fish  $\text{MO}_2$   
286 was high due to the stress linked to the fish transfer (personal observation). Routine  $\text{MO}_2$   
287 while swimming at  $0.5 \text{ BL s}^{-1}$  was employed rather than SMR, since it refers to the same and  
288 constant level of swimming activity before and after the test (Lee et al. 2003; Luna-Acosta et  
289 al. 2011).

290

291 Fish sampling after  $U_{crit}$  test

292

293 After the recovery period following the  $U_{crit}$  test, fish were anaesthetised (MS-222;  $0.1 \text{ g L}^{-1}$ ),  
294 identified using a pit-tag reader, weighed ( $\pm 0.1 \text{ g}$ ), and measured ( $\pm 0.1 \text{ cm}$ ). The Fulton  
295 index (FI) was determined using Equation 4 (Fulton 1904):

296 
$$\text{FI} = \frac{W}{L^3} \quad (4)$$

297 where  $W$  is the fish weight in g and  $L$  the total fish length in cm.  
 298 Blood was sampled (1 mL) by caudal puncture using chilled heparinised syringes. All  
 299 manipulations were performed quickly so that blood was obtained within 2 to 3 min following  
 300 transfer into the anaesthetic solution. Plasma was obtained by centrifugation (5 min; 5000  
 301 rpm; 4°C) and stored at -80°C until further analyses of plasma osmolality (mmol kg<sup>-1</sup>;  
 302 Vapro® Vapour Pressure Osmometer 5520, Wescor, UT, USA;  $n = 12$  per experimental  
 303 condition, *i.e.* 6 for each replicate tank per condition) in duplicate for all plasma samples.  
 304 Haematocrit (percentage of red blood cells in the centrifuged blood volume) was measured in  
 305 duplicate in capillary tubes centrifuged for 3 min at 4000 rpm at 4°C. Fish were dissected on  
 306 ice (4°C) to collect liver for assessment of the hepato-somatic index HSI using Equation 5:

$$307 \quad \text{HSI} = \frac{(W_{\text{liver}} \times 100)}{(W_{\text{fish}} - W_{\text{liver}})} \quad (5)$$

308 where  $W_{\text{liver}}$  is the liver weight and  $W_{\text{fish}}$  is the fish weight.

309 Moreover, muscle was collected for lipid analysis above the lateral line in the caudal part in  
 310 order to obtain white muscle (Martinez 2003). It was stored in -80°C under nitrogen until  
 311 further analysis.

312

### 313 Determination of $U_{\text{crit}}$ , SMR, AMR and AS

314

315 For each fish, the following parameters were calculated: critical swimming speed  $U_{\text{crit}}$ , SMR,  
 316 AMR and AS.  $U_{\text{crit}}$  (in BL s<sup>-1</sup>) was calculated using Equation 6 (Brett 1964):

$$317 \quad U_{\text{crit}} = U_t \times \frac{t_1}{t} \times U_1 \quad (6)$$

318 where  $U_t$  (BL s<sup>-1</sup>) is the highest velocity maintained for an entire swimming step,  $t_1$  (min) the  
 319 amount of time spent at the fatigue velocity,  $t$  (min) the prescribed swimming period (20 min),  
 320 and  $U_1$  the last increment velocity (1.5 or 0.75 BL s<sup>-1</sup>).

321 SMR ( $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) was extrapolated as the intercept (*i.e.*  $\text{MO}_2$  when  $U = 0 \text{ BL s}^{-1}$ ; Brett  
322 1964) of Equation 7:

$$323 \quad \text{MO}_2 = \text{SMR} e^{bU} \quad (7)$$

324

325 where  $\text{MO}_2$  is the oxygen consumption ( $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ),  $b$  a constant, and  $U$  the swimming  
326 speed ( $\text{BL s}^{-1}$ ).

327 AMR ( $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) was considered the highest  $\text{MO}_2$  recorded during the  $U_{\text{crit}}$  test.

328 AS ( $\text{mgO}_2 \text{ kg}^{-1} \text{ h}^{-1}$ ) was determined for each fish as the difference between AMR and SMR.

329

330 *Cardiomyocyte performance*

331

332 *Solutions*

333

334 All chemicals were purchased from Sigma-Aldrich (St Quentin-Fallavier, France).

335 The isolation  $\text{Ca}^{2+}$ -free solution: in mM: NaCl, 100; KCl, 10;  $\text{KH}_2\text{PO}_4$ , 1;  $\text{MgSO}_4$ , 5.4;  
336 taurine, 50;  $\alpha\text{D}$ -glucose, 20; HEPES, 10; EGTA, 0.1 (adjusted to pH 7.1 using KOH,  
337 osmolality:  $300 \text{ mOsmol L}^{-1}$ ). The enzymatic digestion solution comprised collagenase (Type  
338 IA,  $0.36 \text{ mg mL}^{-1}$ ), trypsin (Type III,  $0.24 \text{ mg mL}^{-1}$ ) and BSA ( $0.5 \text{ mg mL}^{-1}$ ) added to the  
339 isolation  $\text{Ca}^{2+}$ -free solution.

340 The incubation medium  $\text{Ca}^{2+}$  solution: in mM: Glucose, 10; NaCl, 130; KCl, 5;  $\text{MgSO}_4$ , 3;  
341  $\text{NaH}_2\text{PO}_4$ , 0.5;  $\text{CaCl}_2$ , 2; HEPES, 10 (adjusted to pH 7.35 using KOH).

342 The hyperpotassic  $100 \text{ mM K}^+$  solution (100 K): in mM: glucose, 1; NaCl, 35; KCl, 100;  
343  $\text{MgSO}_4$ , 1.5;  $\text{NaH}_2\text{PO}_4$ , 0.4;  $\text{CaCl}_2$ , 8; HEPES, 10 (adjusted to pH 7.3 using KOH).

344

345 *Ventricular cardiomyocytes isolation*

346

347 Here, 24h-starved fish [SD group: mean weight  $\pm$  SE:  $54.6 \pm 5.3$  g; mean standard length  $\pm$   
348 SE:  $15.0 \pm 0.4$  cm; LD group: mean weight  $\pm$  SE:  $40.8 \pm 5.3$ ; mean standard length  $\pm$  SE:  $14.1$   
349  $\pm 0.5$  cm] were randomly sampled in tanks using a net, and individually anaesthetised (MS-  
350 222;  $0.1 \text{ g L}^{-1}$ ). The heart was rapidly excised and immersed in a  $\text{Ca}^{2+}$ -free solution in order to  
351 disrupt  $\text{Ca}^{2+}$ -dependent cellular bonds. The atrium and bulbus arteriosus were removed.  
352 Single ventricular cells were obtained by enzymatic dissociation using a protocol modified  
353 from that described by Vornanen (1997) and Chatelier et al. (2006a). The ventricle was cut  
354 into small pieces with scissors and immersed in 2 ml of  $\text{Ca}^{2+}$ -free solution containing  
355 proteolytic enzymes. They were gently homogenised for 10 min at  $20^\circ\text{C}$ . Following  
356 enzymatic treatment, (1) the ventricle was transferred in a Petri dish containing 2 mL of fresh  
357  $\text{Ca}^{2+}$  solution with enzymes. This was then (2) mechanically triturated for 5 min through the  
358 opening of a Pasteur pipette; (3) the small pieces of ventricle not yet dissociated were  
359 removed and placed in a new Petri dish containing 2 ml of fresh  $\text{Ca}^{2+}$ -free solution with  
360 enzymes, while the supernatant was centrifuged (5 min, 2500 rpm,  $4^\circ\text{C}$ ); and (4) the pellet  
361 was gently suspended in  $500 \mu\text{L}$  of  $\text{Ca}^{2+}$ -free solution without enzymes. Steps 1 to 4 were  
362 repeated until all of the pieces of ventricle were dissociated (in general 3-4 times). Isolated  
363 cardiomyocytes were then transferred in a new dish equipped with a glass bottom to allow  
364 their fixation. Cells were progressively dropwise immersed in an incubation medium  $\text{Ca}^{2+}$   
365 solution containing  $2 \mu\text{M}$  of blebbistatin (Sigma-Aldrich, St Quentin-Fallavier, France)  
366 dissolved in dimethyl sulphoxide DMSO in order to avoid cell contracture, and maintained in  
367 a dark room at  $20^\circ\text{C}$  for at least 1h15.

368

369 *Measure of  $\text{Ca}^{2+}$  fluxes*

370

371  $\text{Ca}^{2+}$  fluxes were measured using the permeant form of the fluorescent dye FLUO-4 (FLUO-4  
372 AM acetoxy-methyl-ester, Invitrogen, Saint-Aubin, France), dissolved in DMSO. The probe  
373 was added ( $2\ \mu\text{M}$ ) to the physiological medium in the dish in which cells were fixed. After 1h  
374 of loading in a dark room, cells were carefully washed with the incubation medium.  
375 Fluorescence was recorded using a microscope LEICA DMI6000B (Saint-Jorioz, France)  
376 equipped with a FLUO Oligochrome lamp and a camera ORCA-R2. The acquisition software  
377 was Morpho Strider (Explora-Nova, La Rochelle, France). Excitation of FLUO-4 was set at  
378 494 nm, and the fluorescence emission was collected through a dichroic filter at 505 nm.

379 Cells were individually stimulated with 100 K solution in order to change the potassium  
380 equilibrium potential, resulting in a decrease of the inward rectifier potassium current which  
381 is known to stabilise the resting membrane potential (Galli et al. 2009and). Thus,  
382 cardiomyocytes exposed to 100 K solution were depolarised, inducing global  $\text{Ca}^{2+}$  fluxes,  
383 possibly via the activation of L-type  $\text{Ca}^{2+}$  channels and  $\text{Na}^+/\text{Ca}^{2+}$  exchangers. Cells ( $n = 14$  for  
384 SD group and  $n = 16$  for LD group) were locally perfused with incubation medium via a small  
385 delivery tube positioned near the cell (less than 1 mm). This solution was rapidly exchanged  
386 for 100K (one drop,  $20\ \mu\text{L}$ ). Then, stimulation of the cardiomyocytes was stopped by the fast  
387 change of the 100 K solution by the incubation medium.

388 Ryanodin solution (RYA; 1 mM; Ascent Scientific, Bristol, UK) was used to evaluate calcium  
389 mobilization from the sarcoplasmic reticulum (SR) when stimulation occurs. Ryanodin ( $10$   
390  $\mu\text{M}$ ) binds to the ryanodin receptor located on the SR, and locks the SR-  $\text{Ca}^{2+}$  release channel  
391 in the open state, rendering it unable to contribute to  $\text{Ca}^{2+}$  transient (Rousseau et al. 1987). As  
392 a consequence, by subtracting the concentration of  $\text{Ca}^{2+}$  measured in cells stimulated with  
393 RYA to that measured with 100 K, the  $\text{Ca}^{2+}$  concentration released by SR can be estimated.

394 Adrenaline solution (AD;  $10\ \mu\text{M}$ , Sigma-Aldrich; St Quentin-Fallavier, France) was used to  
395 investigate the effects of  $\beta$ -adrenergic receptors upon the cellular calcium responses. As a  $\beta_2$ -



396 type adrenergic receptor agonist, AD increased the density of the L-type calcium current by  
397 elevating the open-probability of the L-type calcium channels (Vornanen 1997; 1998; Hove-  
398 Madsen et al. 1998) after phosphorylation (Shiels et al. 1998). This is supposed to increase the  
399 force of contraction. AD also stimulates the  $\text{Ca}^{2+}$  ATPase pump of the RS, inducing the  $\text{Ca}^{2+}$   
400 re-pumping after the contraction phase, and then accelerating the relaxation. To perform these  
401 experiments, fish cells (RYA:  $n = 16$  for SD group and  $n = 13$  for LD group; AD:  $n = 13$  for  
402 SD group and  $n = 10$  for LD group) were bathed in the stimulating solution (RYA or AD)  
403 diluted in incubation medium for 30 s (one drop, 20  $\mu\text{L}$ ). This allowed the fixation of RYA  
404 and AD to the SR ryanodin and beta-adrenergic receptors respectively. Cells were then briefly  
405 perfused with the stimulating solution (RYA or AD) diluted in 100 K in order to stimulate  
406 intracellular  $\text{Ca}^{2+}$  release.

407 All perfusions were rapid, in order to prevent excessive stimulation and possible contracture  
408 followed by cell death, and a maximum of two cells were stimulated in each dish.

409 For each stimulated cell, the fluorescence was normalised ( $F/F_0$ ), where  $F_0$  was the mean of  
410 the data points recorded during the first 30 s in which cells were perfused with incubation  
411 medium. Then, for each cell, the maximal amplitude of calcium mobilised, the time to reach  
412 the peak ( $tp$ ), and the time to reach 50% relaxation (decay 50%,  $tr$ ) were measured (Imbert-  
413 Auvray et al. 2013).

414 All the analyses performed in this study are summarized in Table 2.

415

416 Statistical analysis

417

418 The data are presented as mean  $\pm$  standard error (SE). All statistical analyses were performed  
419 with Statistica® software vs.7 (StatSoft, Maison-Alfort, France). Concerning growth during  
420 the experimental period, slopes of regression curves were determined for each fish. The effect

421 of diet on these slopes, as well as on SGR,  $U_{crit}$ , SMR, AMR, plasma haematocrit, plasma  
422 osmolality, HSI, FI, and the  $MO_2$  excess during the recovery period integral was tested using  
423 a one-way ANOVA with  $n$  = the number of fish replicates for each dietary condition.  
424 Moreover, the effect of diet on each of the three successive measurements of  $MO_2$   $r(t)$  was  
425 tested using a repeated-measure ANOVA with the three successive measurements used as the  
426 within effect. Concerning cardiomyocyte performance, the effect of diet on the amplitude,  $tp$ ,  
427 and  $tr$  recorded when cells were stimulated with 100K, RYA, or AD was tested using a one-  
428 way ANOVA with  $n$  = the number of cell replicates for each dietary condition. Prior to  
429 ANOVA analyses, normality distribution and homeodasticity were controlled using  
430 Kolmogorov-Smirnov and the Levene test, respectively. Differences were considered  
431 significant when  $\alpha < 0.05$ .

432

## 433 **Results**

434

435 All groups of fish appeared healthy and survival was close to 100% at the end of the  
436 experiment.

437

### 438 Fatty acid composition in fish

439

440 No significant difference of TL, NL or PL content was found between the two dietary groups  
441 of fish (Table 3). As expected, fish fatty acid composition reflected that of the diet, and EPA  
442 and DHA were preferentially incorporated in phospholipids (PL) for both groups (One-way  
443 ANOVA: EPA:  $P < 0.001$ ;  $F_{1,86} = 86.75$ ; DHA:  $P < 0.001$ ;  $F_{1,86} = 20.8$ ) (Table 3).

444 In both lipid classes (PL and NL), the sum of saturated fatty acids ( $\sum$  SFA) was 15%  
445 higher in the group fed the standard n-3 HUFA content diet (SD group) than in that fed the

446 low n-3 HUFA content diet (LD group; Table 3). In NL, this difference reflected that of all  
447 SFA tested (14:0, 16:0, 18:0, and 20:0), while in PL, this mostly reflected the difference in  
448 14:0 and 16:0 FA content, as the 18:0 and 20:0 FA contents were similarly incorporated in  
449 both groups.  $\Sigma$  monounsaturated fatty acids MUFA levels were also 15% higher in SD than in  
450 LD fish, and reflected a difference in both 16:1 and 18:1 in the two dietary groups (Table 3).

451 The  $\Sigma$  n-6 FA content, as well as the 18:2n-6 FA content, were more than two-fold  
452 higher in the LD group than in the SD group, for both PL and NL. However, arachidonic acid  
453 (ARA; 20:4n-6) was similarly present in the PL of both groups ( $P > 0.05$ ), but was  
454 significantly more incorporated (30%) in the NL of SD than in that of LD groups (Table 3).

455 The  $\Sigma$  n-3, as well as n-3 HUFA including EPA and DHA were 1.5- to 2-fold more  
456 present in the NL and PL of the SD group than in the LD group ( $P < 0.05$ ; Table 3). Only the  
457 18:3n-3 FA content was almost 2-fold lower in the SD than LD group. The n-3/n-6 ratio was  
458 only above one in the PL of the SD group, whereas it was below one in the PL of the LD  
459 group, as well as in the NL of the two dietary groups (Table 3).

460 The DHA/EPA ratio was significantly higher in the NL of SD groups, while it was  
461 similar in the PL of fish fed the SD and LD diet. Finally, the ARA/EPA ratio was  
462 significantly higher in the NL of the LD group, but was not different in the PL, regardless of  
463 the dietary group investigated (Table 3).

464

465 Growth performances and body condition

466

467 The SGR of the SD group was significantly higher (25%) than that of the LD group (Table 4).  
468 In addition, fish from the SD group exhibited a significantly higher increase in standard length  
469 throughout the entire experiment than fish from the LD group ( $P < 0.001$ ;  $F_{2,72} = 24.01$ ).  
470 However, the hepatosomatic index (HSI) and FI were not affected by diet ( $P > 0.05$ ; Table 4).

471

472 Swimming performance, energetics and blood parameters

473

474 As expected,  $MO_2$  increased exponentially with swimming speed for both the SD and LD  
475 groups (Fig. 1; *e.g.* Brett 1964; Webb 1975; Vagner et al. 2008; Milinkovitch et al. 2012).

476 Results concerning the effect of HUFA dietary content on  $U_{crit}$ , SMR, AMR, AS, haematocrit,  
477 and plasma osmolality, are summarised in the Table 3.  $U_{crit}$  was not affected by diet ( $P >$   
478 0.05). On the contrary, both AMR and AS measured in the LD group were more than two-fold  
479 lower than in the SD group ( $P < 0.001$ ). Moreover, this was associated with an increase of  
480 more than 20% of haematocrit and 7% of plasma osmolality ( $P < 0.05$ ).

481

482 Excess Post-exercise Oxygen Consumption

483

484 The excess  $MO_2$  measured during the post-exercise period at low speeds was found to be  
485 dependent on diet ( $P < 0.05$ ; Fig. 2), and was more than 45% higher in the SD than in the LD  
486 group.

487

488 Cardiomyocytes performances

489

490 Variables representative of the kinetics of calcium fluxes (peak amplitude, the time to reach  
491 the peak,  $tp$ , and the time to reach 50% relaxation,  $tr$ ), in response to stimulation  
492 (hyperpotassic solution 100K, ryanodin RYA, or adrenaline AD) are presented in Figs 3a, b,  
493 and c for each dietary group. Whatever stimulator was used, there was no effect of diet  
494 reported for these variables ( $P > 0.05$ ). Moreover, no significant effect of the stimulator,  
495 neither on peak amplitude nor on  $tr$ , was observed ( $P > 0.05$ ). However,  $tp$  was significantly

496 reduced by more than 50% in cells stimulated with RYA compared to those stimulated with  
497 100K and AD ( $P < 0.01$ ).

498

## 499 **Discussion**

500

501 This study is the first to measure the effect of a reduction in n-3 HUFA dietary content, as  
502 expected with global change, on physiological performances of the golden grey mullet, a  
503 species of high ecological importance in European coastal areas. The results showed that the  
504 low n-3 HUFA dietary content reduced growth rate and the aerobic capacity of *Liza aurata* at  
505 20°C. This diet also induced higher levels of haematocrit and plasma osmolality. However,  
506 this diet did not impact SMR or  $U_{crit}$ .

507         The n-3 HUFA content of the LD diet was about six-fold lower than that of the SD  
508 diet, suggesting a deficiency of n-3 HUFA in that diet. The immediate consequence of feeding  
509 with both of these diets was a modification of the n-3 HUFA membranous content in fish. As  
510 expected, the cell membrane bilayer reflected the HUFA dietary content in both groups  
511 (Linares and Henderson 1991; Jobling 2001), as (1) fish fed the LD diet presented a  
512 significantly lower n-3 HUFA, including EPA and DHA, content than those fed the SD diet,  
513 and (2) both of these fatty acids were preferentially incorporated in PL rather than NL in both  
514 dietary groups, indicating their integration in cell membranes (Linares and Henderson 1991).

515         As said earlier, the LD diet induced a significantly lower growth rate in *L. aurata* over  
516 the five month experimental period. This result confirms the importance of dietary HUFA,  
517 and especially EPA and DHA, for normal growth, as observed in other species such as  
518 juvenile flounder (Kim and Lee 2004), cod (Lee and Cho 2009), yellow croaker (Zuo et al.  
519 2012), or juvenile cobia (Liu et al. 2007). This lower growth rate did not appear to be due to a  
520 lower food intake in the LD group, as even though the feed intake was not accurately

521 monitored during our experiment for technical reasons, visual daily observations performed  
522 during the meal time allowed us to hypothesise that SD and LD fish ate similarly. In addition  
523 to their lower growth rate, fish from the LD group exhibited a lower increase in standard  
524 length from the beginning to the end of the experiment (results not shown). This resulted in a  
525 similar body condition as shown by FI and HSI measured in both dietary groups (Equation 4).

526 While it reduced growth rate, the low n-3 HUFA diet did not impair the ability of fish  
527 to maintain vital functions such as ventilation and osmoregulation. This was shown by the  
528 similar SMR values measured in both dietary groups (Fry 1971), which were close to that  
529 previously reported in another mugilid species (Vagner et al. 2008). Moreover, the n-3 HUFA  
530 dietary content did not significantly affect  $U_{crit}$  at 20°C, as the LD group achieved the same  
531  $U_{crit}$  as the SD group. As suggested by previous authors, sustained swimming performances  
532 may not only depend on the n-3 HUFA metabolism, but also on other FA metabolism as an  
533 energy source, such as MUFA or SFA (Chatelier et al. 2006b; Regan et al. 2010). Despite this  
534 similar  $U_{crit}$  measurement in both groups, the lower n-3 HUFA diet significantly reduced  
535 AMR and AMS. This indicates that LD fish achieved the same  $U_{crit}$  as SD fish but with a  
536 significantly lower metabolic cost. This is in contrast with results obtained in previous studies,  
537 which showed that lower AMR and AMS were both linked to lower swimming performances  
538 (Chatelier et al. 2006b). Our original results could be partially explained by the marked  
539 accumulation of n-6 FA in both the NL and PL of fish fed the LD diet, which may have  
540 compensated for the lower levels of n-3 FA in that group. In particular, the high levels of  
541 linoleic and oleic acids have been shown to be positively correlated with  $U_{crit}$  performance in  
542 Atlantic salmon (McKenzie et al. 1998) and sea bass (Chatelier et al. 2006b). Linoleic acid  
543 (18:2n-6) has also been shown to increase carnitine palmitoyl transferase activity, which may  
544 improve the aerobic metabolism of fatty acids in red muscle (Sidell and Driedzic  
545 1985 ; Egginton 1996).

546 While LD fish performed the same  $U_{crit}$  as SD fish, the decrease in AMS measured in  
547 that group suggests that the low n-3 HUFA diet could reduce the capacity for oxygen  
548 allocation toward energy-demanding activities such as foraging, growth and digestion (Fry  
549 1971). This result is in accordance with the lower growth rate found in that group.

550 The lower AMS caused by the low n-3 HUFA dietary content could reflect a lower capacity  
551 of oxygen transport in that group. The n-3 HUFA dietary content may have modified the  
552 membrane lipid composition of mitochondria, and consequently reduced the respiratory rate  
553 of mitochondria, as suggested in rainbow trout by Guderley et al. (2008). These authors  
554 reported a higher respiratory rate in the mitochondria of rainbow trout fed a diet enriched in  
555 EPA and DHA compared to fish fed a diet poorer in those FA, and suggested that the level of  
556 these specific FA would be statistically correlated with mitochondria capacity. Another  
557 hypothesis to explain the higher aerobic metabolism in fish fed the enriched n-3 HUFA diet  
558 would be an increasing number of mitochondria as the n-3 HUFA dietary content increased,  
559 as previously demonstrated by Kjaer et al. (2008) in the liver of Atlantic salmon fed a diet that  
560 was rich in EPA. This would induce the higher production of ATP. In other vertebrates such  
561 as several mammals and bird species, a high positive correlation between the molecular  
562 activity ( $ATP\ min^{-1}$ ) of individual  $Na^+K^+ATPase$  units and the content of DHA in the  
563 surrounding membranes bilayer was found, suggesting a higher metabolic activity in tissue  
564 (Turner et al. 2003). However, one consequence of the higher aerobic metabolism measured  
565 in fish fed the SD diet would be an increasing lipid peroxidation in membranes, as  
566 phospholipids are particularly vulnerable to oxidation (Crockett 2008). Lipid peroxidation  
567 represents a distinct set of oxidations initiated by reactive oxygen species that possess  
568 sufficient energy to remove a hydrogen atom from a methylene group within the parent lipid  
569 (Girotti 1985). Lipid peroxidation in biological membranes may be deleterious because of  
570 influences on membrane physical properties likely to affect protein function (Crockett 2008).

571 Contrary to the present study, previous works have reported that a high n-3 HUFA dietary  
572 content reduced AMR and AMS, suggesting that aerobic metabolism in fish is primarily  
573 fuelled by FA oxidation, with MUFA being preferred over SFAs which, in turn, are preferred  
574 over HUFA as substrates (Henderson and Sargent 1985 ; Egginton 1996 ; McKenzie 2001;  
575 Chatelier et al. 2006b). Indeed, MUFA and SFA are mostly incorporated in triacylglycerols  
576 which constitute lipid reserve used as fuels by tissues, while HUFA are mainly used as  
577 structural lipids incorporated in biological membranes or as precursors of eicosanoids (for  
578 review, see Sargent et al. 2002). However, while there is no doubt that dietary and tissue FA  
579 exert profound effects on metabolic and cardio-respiratory physiology of vertebrates, the  
580 explanation of such contrasting results is not known, and further studies are required to  
581 understand the mechanisms responsible for these effects.

582         The lower aerobic metabolic rate induced by the low n-3 HUFA dietary content does  
583 not appear to be due to a lower capacity of oxygen transport by blood. Indeed, the lower AMS  
584 measured in the LD group was associated with a higher haematocrit at the end of the  $U_{crit}$  test,  
585 indicating higher red cells production, and a consequently higher capacity of oxygen transport  
586 in that group. This could have contributed to maintaining swimming at the same velocity, as  
587 in the SD group (Thorarensen et al. 1993). This higher haematocrit could reflect the need for  
588 those fish to increase their oxygen supply in order to survive in a stressful environment, since  
589 a higher haematocrit is one of the indicators of fish health, and may reflect a stress response in  
590 fish. This has already been reported in fish submitted to crowding stress (Trenzado, Morales  
591 and de la Higuera 2006; 2009) or hypoxic conditions (Zambonino et al. 2013). This stress  
592 response is corroborated by (1) the higher plasma osmolality, (2) the lower AMS and AMR,  
593 and (3) the lower growth rate measured in the LD group. Indeed, after a primary response  
594 during which activation of the brain-sympathetic-chromaffin cell axis and the brain-pituitary  
595 inter-renal axis, resulting in the release of stress hormones such as cortisol (not measured in



596 the present study), stress response is characterised by a secondary response during which  
597 these hormones have an effect at blood and tissues levels, including a disturbance of ionic and  
598 osmotic balance, and by a third response characterised by the inhibition of growth and  
599 changes in metabolic rate (Schreck 1982; Barton and Iwama 1991; Wendelaar Bonga 1997;  
600 Mommsen et al. 1999).

601 The higher haematocrit measured in the LD group could also be due to alterations of  
602 the mitochondrial membranes by the low n-3 HUFA dietary content, as suggested in rainbow  
603 trout by Guderley et al. (2008). This would have led to an alteration of the conformation and  
604 activity of the embedded protein, reducing the capacity of oxygen transport across the  
605 membrane and toward mitochondria. This would have resulted in a higher oxygen demand  
606 from mitochondria, and consequently, to increasing red cell production as a strategy to cope  
607 with elevated energy demand.

608 In addition to indicating a secondary stress response, the higher osmolality measured  
609 at the end of the  $U_{crit}$  test in the LD group could also suggest a higher ventilation rate (not  
610 measured) developed in order to increase oxygen supply during the effort. It is well described  
611 that swimming leads to an improving gas exchange at the gills through ventilation rate, and  
612 that the consequence is passive ion movement across the gills. In seawater fish, the ion  
613 movement is from the water to the animal, and the consequence is dehydration. Through an  
614 increasing ventilation rate, fish would have an increased passive ion movement from water to  
615 the gills, increasing osmolality in their system. This ion movement is normally compensated  
616 by water absorption through the intestinal membrane, as well as by ion excretion through the  
617 sodium-chloride channels of the gill membrane in order to maintain the osmotic balance.  
618 However, as this ion excretion represents an energetic cost because it is against the  
619 concentration gradient, fish fed the low n-3 HUFA content diet may have spared that cost in  
620 order to save energy, as their aerobic capacity was reduced.

621 While fish fed the low n-3 HUFA diet displayed a lower capacity for oxygen transport,  
622 they did not show any lower cardiomyocyte performance, measured by the  $\text{Ca}^{2+}$  fluxes in  
623 response to different chemical stimulations, compared to the group fed the standard n-3  
624 HUFA diet. The measure of similar cardiomyocyte performances is in accordance with the  
625 measurements of similar  $U_{\text{crit}}$  values in both dietary groups, as several studies have argued  
626 that  $U_{\text{crit}}$  in active teleosts may be primarily limited by aerobic myocardial performance  
627 (Farrell 2002; Claireaux et al. 2005). These results could suggest that modification of the  
628 membranous n-3 HUFA content in response to diet did not impact the functionality of those  
629 calcium channels embedded in cardiomyocyte membranes or the SR membranes of  
630 cardiomyocytes. However, the cardiomyocyte lipid composition was not measured in the  
631 present study. While our results showed that the white muscle FA composition reflected that  
632 of the diet, we do not know how it varied in heart tissue. Indeed, recent studies showed that  
633 the FA composition of the different fish tissues (liver, heart, kidney, intestine, liver, eyes...)  
634 does not respond in the same way to diet (Benedito-Palos et al. 2010; Böhm et al. 2014). The  
635 absence of any significant differences between dietary groups could also be due to the  
636 experimental temperature used (20°C), as many previous studies have shown differences in  
637 the thermal sensitivity of the mechanisms involved in calcium regulation in fish  
638 cardiomyocytes. For example, the effects of adrenaline were mainly observed in cold-  
639 acclimated teleosts (perch: Tirri and Ripatti, 1982; Tirri and Lehto, 1984; rainbow trout:  
640 Graham and Farrell 1989; Keen et al. 1994). Moreover, SR-  $\text{Ca}^{2+}$  loading and mobilisation  
641 has been shown to depend on species and thermal acclimation (Imbert-Auvray et al. 2013).

642 At 20°C,  $\text{Ca}^{2+}$  release did not seem to depend on the SR in *Liza aurata*, but mainly on  
643 the calcium L-type channel located on the cytoplasmic membrane, as similar amplitudes of  
644  $\text{Ca}^{2+}$  release in response to the stimulation with ryanodine added to 100 K or 100 K alone  
645 were measured. This is in accordance with other authors who showed that  $\text{Ca}^{2+}$  release from

646 the SR was rather limited in the ectothermic vertebrate heart, such as those of fish (Shiels et  
647 al. 2004) and amphibians (Fischmeister and Horackova 1983; Nabauer et al. 1989).

648 The lower anaerobic metabolism measured in fish fed the low n-3 HUFA diet is  
649 coincident with their lower swimming cost measured during the  $U_{crit}$  test, and indicated that  
650 these fish recovered very quickly. Their lower anaerobic capacity was confirmed by their  
651 lower excess  $MO_2$  measured during the 70 min recovery period (Lee et al. 2003; Luna-Acosta  
652 et al. 2011; Zhao et al. 2012). This lower excess  $MO_2$  reflects a lower quantity of oxygen  
653 required to restore tissue and cellular stores of oxygen and high-energy phosphates,  
654 biochemical imbalances in metabolites such as lactate and glycogen, and other functions such  
655 as ionic and osmotic balance in this group (Lee et al. 2003). The higher haematocrit content  
656 measured in that group could have contributed to this better recovery.

657 To conclude, the results of the present study show that a reduction in n-3 HUFA  
658 availability in diet until 0.2% EPA+DHA (on dry matter basis) would reduce the growth rate  
659 as well as the aerobic capacity of *Liza aurata* at 20°C. As stated earlier, aerobic capacity  
660 represents the energy available to perform energy-demanding activities, and the excess energy  
661 will be allocated to growth once other activities have been allocated (Fry 1971). The reduction  
662 of aerobic metabolism by the low n-3 HUFA content diet suggests that fish may have to save  
663 energy by modifying the proportion of energy allocated to energy-demanding activities, such  
664 as digestion or feeding. This also suggests that the growth rate will consequently be reduced.  
665 This long-term strategy of energy allocation could explain the decreasing growth rate  
666 measured when fish were given the deficient diet over the 5 month experimental period. In  
667 addition to a lower growth rate and aerobic metabolism, the lower n-3 HUFA diet induced  
668 higher levels of haematocrit and plasma osmolality, which are all indicators of a stress  
669 response at the second and third levels in that group.

670 However, our results showed that the lower n-3 HUFA diet (or higher MUFA and linoleic  
671 acids) caused a massive change in swimming efficiency and recovery capacity that should  
672 bring ecological advantages in the capacity of fish to migrate or avoid predators. This is of  
673 particular importance to *L. aurata* which is a migratory species that reproduces at sea and uses  
674 coastal areas to grow and to feed.

675 The higher swimming efficiency caused by a deficient n-3 HUFA dietary content would allow  
676 fish to increase their mobility and distribution, thus increasing the range over which feeding  
677 and reproduction occurs. These changes in turn would compensate for the reduction of growth  
678 and aerobic metabolism induced by diet.

679

## 680 **Acknowledgments**

681

682 This work was supported by the Action Initiative of the University of La Rochelle to Marie  
683 Vagner (post-doctoral fellowship). The authors are very grateful to Andrea Satta and Fabio  
684 Antagnarelli for supplying fish; to Michel Prineau for his technical help; and to David Akbar  
685 and Marie Durollet for their help with cellular analysis and microscopy.

686

## 687 **Conflict of interest**

688

689 The authors have no conflict of interest to declare and note that the sponsors of the issue had  
690 no role in the study design, data collection and analysis, decision to publish, or preparation of  
691 the manuscript

692

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916

917 **Table 1** Formulation (ingredient proportion in g 100 g<sup>-1</sup>), chemical composition (% dry matter  
918 DM ± standard error SE; n = 3) and fatty acid composition in total lipids TL (% fatty acid  
919 methyl ester FAME ± SE; n = 3) of the two experimental diets (standard n-3 HUFA diet SD  
920 and low-n-3 HUFA LD diet). Fatty acids for which percentage was lower than 0.2% FAME  
921 are not represented. Each MUFA is represented as a sum of n-7, n-9 and n-11 FA

<i>Ingredients</i> <sup>a</sup>	SD diet	LD diet
Fish meal LT 94	17	17
Casein	30	30
Soy oil	2	10
Fish oil	8	0
Precooked starch	30	30
Vitamin mixture <sup>b</sup>	8	8
Mineral mixture <sup>c</sup>	4	4
Betaine	1	1
<i>Chemical composition</i>		
Dry matter (%)	91.0 ± 0.1	92.0 ± 0.1
Crude protein (% DM)	34.7 ± 0.0	41.3 ± 0.2
Crude fat (% DM)	11.7 ± 0.3	10.7 ± 0.1
Ash (% DM)	7.8 ± 0.2	6.6 ± 0.4
HUFA n-3 (% DM)	1.2 ± 0.0	0.2 ± 0.0
<i>FA composition in TL</i>		
14:0	3.59 ± 0.04	0.59 ± 0.02
16:0	11.84 ± 0.04	11.32 ± 0.02
18:0	2.94 ± 0.01	4.62 ± 0.01
20:0	0.27 ± 0.00	0.46 ± 0.01
Σ SFA	19 ± 0.09	17.63 ± 0.04
16:1	4.03 ± 0.04	0.80 ± 0.02
18:1	26.80 ± 0.05	23.09 ± 0.02
20:1	4.25 ± 0.12	1.20 ± 0.00
22:1	4.69 ± 0.05	1.17 ± 0.01
24:1	0.43 ± 0.01	0.12 ± 0.00
Σ MUFA	40.58 ± 0.14	26.42 ± 0.03
18:2n-6	15.3 ± 0.03	3.63 ± 0.09
20:4n-6	0.38 ± 0.00	0.09 ± 0.00
Σ n-6	16.54 ± 0.02	45.84 ± 0.01
18:3n-3	3.73 ± 0.01	6.38 ± 0.01
18:4n-3	1.05 ± 0.00	0.18 ± 0.00
20:4n-3	0.91 ± 0.00	0.08 ± 0.00
20:5n-3	4.49 ± 0.01	0.79 ± 0.01
22:5n-3	1.62 ± 0.01	0.11 ± 0.00
22:6n-3	6.24 ± 0.06	1.32 ± 0.01
Σ n-3	18.28 ± 0.07	8.87 ± 0.03

$\Sigma$ HUFA n-3	13.51 $\pm$ 0.08	2.31 $\pm$ 0.02
n-3 / n-6	1.11 $\pm$ 0.01	0.19 $\pm$ 0.00
DHA / EPA	1.39 $\pm$ 0.01	1.68 $\pm$ 0.02
ARA / EPA	0.08 $\pm$ 0.00	0.12 $\pm$ 0.00

922 <sup>a</sup> Sources: fish meal LT 94: Norse (Fyllingsdalen, Norway); casein: Sigma-Aldrich  
923 (Germany); soy oil: Système U (Créteil, France); fish oil: pure cod oil Cooper (Melun,  
924 France); precooked starch: Prégéflo Roquette frères (Lestrem, France); vitamin mixture  
925 (INRA Jouy-en-Josas, France)

926 <sup>b</sup> Vitamin mixture (g kg<sup>-1</sup> vitamin mix): retinyl acetate, 1; cholecalciferol, 2.5; DL- $\alpha$ -  
927 tocopheryl acetate, 5; menadione, 1; thiamine-HCL, 0.1; riboflavin, 0.4; D-calcium  
928 panththenate, 2; pyridoxine-HCL, 0.3; cyanocobalamin, 1; niacin, 1; choline, 200; ascorbic  
929 acid (ascorbyl polyphosphate), 5; folic acid, 0.1; D-biotin, 1; meso-inositol, 30

930 <sup>c</sup> Mineral mixture (g kg<sup>-1</sup> mineral mix): KCl, 90; KI, 0.04; CaHPO<sub>4</sub> 2H<sub>2</sub>O, 500; NaCl,  
931 40; CuSO<sub>4</sub> 5H<sub>2</sub>O, 3; ZnSO<sub>4</sub> 7H<sub>2</sub>O, 4; CoSO<sub>4</sub>, 0.02; FeSO<sub>4</sub> 7H<sub>2</sub>O, 20; MnSO<sub>4</sub> H<sub>2</sub>O, 3; CaCO<sub>3</sub>,  
932 215; MgOH, 124; Na<sub>2</sub>SeO<sub>3</sub>, 0.03; NaF, 1

933 Abbreviations: ARA: arachidonic acid; DHA docosahexaenoic acid; EPA: ecosapentaenoic  
934 acid; HUFA: highly unsaturated fatty acids; MUFA: mono-unsaturated fatty acids; SFA:  
935 saturated fatty acids

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942 **Table 2** Summary of all physiological and biochemical analyses performed in this study. For  
 943 each analyse, the number of fish sampled (N) for each experimental condition (standard n-3  
 944 HUFA dietary group and low-n-3 HUFA LD dietary group), and the sampling period are  
 945 indicated. † Indicates paired data points, *i.e.* measurements performed on the same fish

<i>Analyses</i>	<i>Fish replication for each experimental condition</i>	<i>Sampling period</i>
Growth performances (fresh weight, total and standard length, height, width, SGR, Fulton index)	N = 40	Each month during 5 months
Lipid analyses in white muscle†	N = 12	After the 5-month experimental period
Swimming performance ( $U_{crit}$ , SMR, AMR, AS) †	N = 12	After the 5-month experimental period
Anaerobic metabolism †	N = 12	After the 5-month experimental period
Haematocrit †	N = 12	After the 5-month experimental period
Osmolality †	N = 12	After the 5-month experimental period
Hepato-somatic index †	N = 12	After the 5-month experimental period
Cardiac performances	N = 8	After the 5-month experimental period

958 Abbreviations: AMR: active metabolic rate; AS: aerobic scope; SGR: specific growth  
 959 rate; SMR: standard metabolic rate;  $U_{crit}$ : critical swimming speed.

960



961 **Table 3** Total lipid TL content, neutral lipid NL content, polar lipid PL content (mg g<sup>-1</sup> of dry  
 962 weight), fatty acid profile of NL and PL (% of fatty acids methyl esters FAME) in *Liza aurata*  
 963 white muscle according to rearing conditions (LD: fish fed the low-n-3 HUFA diet; and SD:  
 964 fish fed the standard n-3 HUFA diet) Values are mean ± standard error (n = 28 for SD group  
 965 and n = 16 for LD group) Statistical significance of diet is indicated through the *P*, *F* and *df*  
 966 values (one-way ANOVA)

	Fish composition		<i>P</i>	Statistical analysis		
	SD group	LD group		<i>F</i>	<i>df</i> <sub>1</sub>	<i>df</i> <sub>2</sub>
TL	29.34 ± 2.33	30.65 ± 3.46	0.747	0.1	1	43
NL	23.45 ± 2.41	22.34 ± 3.03	0.77	0.08	1	43
PL	5.79 ± 0.46	7.87 ± 1.53	0.12	2.47	1	43
<b>NL</b>						
14:0	4.06 ± 0.10	2.71 ± 0.12	< 0.001	70.8	1	43
16:0	19.08 ± 0.25	17.44 ± 0.42	< 0.001	12.9	1	43
18:0	2.27 ± 0.04	2.55 ± 0.04	< 0.001	23.5	1	43
20:0	0.23 ± 0.01	0.27 ± 0.01	< 0.001	16.7	1	43
Σ SFA	25.86 ± 0.30	23.17 ± 0.49	< 0.001	24.5	1	43
16:1	7.92 ± 0.21	5.52 ± 0.22	< 0.001	56.0	1	43
18:1	26.10 ± 0.34	23.98 ± 0.35	< 0.001	16.1	1	43
Σ MUFA	44.22 ± 0.49	35.85 ± 0.61	< 0.001	109.2	1	43
18:2n-6	11.85 ± 0.75	28.05 ± 1.26	< 0.001	139.9	1	43
20:4n-6	0.37 ± 0.03	0.26 ± 0.02	< 0.001	7.6	1	43
Σ n-6	13.03 ± 0.75	29.14 ± 1.26	< 0.001	137.8	1	43
18:3n-3	2.23 ± 0.06	3.20 ± 0.09	< 0.001	89.3	1	43
20:5n-3	2.38 ± 0.09	1.35 ± 0.15	< 0.001	40.4	1	43
22:6n-3	3.78 ± 0.11	1.62 ± 0.24	< 0.001	86.8	1	43
Σ n-3	11.47 ± 0.22	7.62 ± 0.49	< 0.001	66.3	1	43
Σ HUFA n-3	8.55 ± 0.22	3.98 ± 0.50	< 0.001	94.7	1	43
n-3 / n-6	0.92 ± 0.03	0.29 ± 0.05	< 0.001	144.4	1	43
DHA / EPA	1.62 ± 0.06	1.22 ± 0.07	< 0.001	19.2	1	43
ARA / EPA	0.15 ± 0.01	0.21 ± 0.01	< 0.001	18.7	1	43
<b>PL</b>						
14:0	5.02 ± 0.29	4.00 ± 0.24	0.02	5.7	1	42
16:0	19.40 ± 0.49	17.77 ± 0.55	0.03	4.5	1	42
18:0	5.46 ± 0.54	4.73 ± 0.48	0.37	0.8	1	42
20:0	0.18 ± 0.01	0.17 ± 0.01	0.40	0.7	1	42
ΣSFA	30.41 ± 0.55	26.96 ± 0.52	< 0.001	17.3	1	42
16:1	7.64 ± 0.40	6.18 ± 0.38	0.02	5.8	1	42
18:1	18.01 ± 0.49	16.47 ± 0.44	0.04	4.4	1	42
ΣMUFA	31.85 ± 0.86	26.59 ± 0.76	< 0.001	17.0	1	42

18:2n-6	10.06 ± 0.72	24.79 ± 1.19	< 0.001	127.3	1	42
20:4n-6	1.42 ± 0.17	1.13 ± 0.15	0.25	1.4	1	42
Σ n-6	12.28 ± 0.69	26.81 ± 1.16	< 0.001	132.6	1	42
18:3n-3	2.62 ± 0.15	4.22 ± 0.17	< 0.001	44.1	1	42
20:5n-3	4.85 ± 0.22	3.24 ± 0.26	< 0.001	20.6	1	42
22:6n-3	6.69 ± 0.76	3.80 ± 0.66	< 0.05	6.5	1	42
Σ n-3	18.74 ± 0.98	13.94 ± 0.92	< 0.01	10.5	1	42
Σ HUFA n-3	15.06 ± 1.11	8.88 ± 1.04	< 0.001	13.8	1	42
n-3 / n-6	1.60 ± 0.10	0.56 ± 0.07	< 0.001	51.4	1	42
DHA / EPA	1.34 ± 0.11	1.16 ± 0.14	0.35	0.9	1	42
ARA / EPA	0.28 ± 0.02	0.35 ± 0.03	0.07	3.5	1	42

967 Abbreviations: ARA: arachidonic acid;  $df_1$ : degree of freedom of numerator;  $df_2$ :

968 degree of freedom of denominator; DHA docosahexaenoic acid; EPA: ecosapentaenoic acid;

969 HUFA: highly unsaturated fatty acids; MUFA: mono-unsaturated fatty acids; SFA: saturated

970 fatty acids

971 **Table 4** Critical swimming speed ( $U_{crit}$ ), standard metabolic rate (SMR), active metabolic rate (AMR), aerobic scope (AS), haematocrit, plasma  
 972 osmolality, Fulton index, and hepatosomatic index (HSI) in SD (standard n-3 HUFA fed fish) and LD (low-n-3 HUFA fed fish) groups (mean  $\pm$   
 973 standard error). N (number of replicates) is indicated below each value. The statistical difference between both groups is indicated ( $F$ ,  $P$ ,  $df_1$ :  
 974 degree of freedom of numerator;  $df_2$ : degree of freedom of denominator) for each parameter measured (one-way ANOVA)  
 975

	SGR (% day <sup>-1</sup> )	$U_{crit}$ (BL s <sup>-1</sup> )	SMR (mgO <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	AMR (mgO <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	AS (mgO <sub>2</sub> kg <sup>-1</sup> h <sup>-1</sup> )	Haematocrit (%)	Osmolality (mosmol L <sup>-1</sup> )	Fulton index	HSI
SD group	0.4 $\pm$ 0.02 (n = 40)	6.4 $\pm$ 0.2 (n=11)	66.5 $\pm$ 11.8 (n=10)	590.2 $\pm$ 72.0 (n=10)	523.7 $\pm$ 67.1 (n=10)	35.3 $\pm$ 2.4 (n =12)	374.1 $\pm$ 6.3 (n=11)	0.96 $\pm$ 0.02 (n=12)	0.22 $\pm$ 0.05 (n=12)
LD group	0.3 $\pm$ 0.01 (n = 40)	6.9 $\pm$ 0.4 (n=10)	38.5 $\pm$ 8.7 (n=10)	266.0 $\pm$ 24.8 (n=10)	227.5 $\pm$ 23.3 (n=10)	44.1 $\pm$ 2.9 (n=12)	400.2 $\pm$ 10.5 (n=10)	0.99 $\pm$ 0.02 (n=12)	0.56 $\pm$ 0.32 (n=12)
Statistical analysis									
$P$	0.009	0.276	0.071	0.001	0.001	0.041	0.048	0.305	0.304
$F$	7.26	1.26	3.67	18.12	17.36	4.72	4.43	1.10	1.10
$df_1$	1	1	1	1	1	1	1	1	1
$df_2$	72	19	18	18	18	22	19	22	22

976 **Figure Legends**

977

978 **Fig 1** Oxygen consumption ( $\text{MO}_2$ ;  $\text{mg O}_2 \text{ Kg}^{-1} \text{ h}^{-1}$ ; mean  $\pm$  standard error SE) as a function of  
979 swimming speed ( $\text{BL s}^{-1}$ ) for each fish dietary group: Standard-n-3 HUFA fed fish (SD) are  
980 represented by squares, and Low-n-3 HUFA fed fish (LD) are represented by triangles ( $n = 10$   
981 per experimental condition). Solid line and dotted line indicate exponential curves fitted on  
982  $\text{MO}_2$  as a function of swimming speed before a plateau was attained for the SD and LD  
983 groups, respectively (SD group curve:  $y = 74.003 e^{0.3061x}$ ;  $r^2 = 0.98$ ; LD group curve:  $y =$   
984  $45.051 e^{0.2609x}$ ;  $r^2 = 0.98$ ).

985

986 **Fig 2** Recovery status of *L aurata* after the  $U_{\text{crit}}$  test for each fish dietary group:  
987 Mean excess of post-exercise oxygen consumption ( $\pm$  standard error SE, EPOC in  $\text{mgO}_2 \text{ kg}^{-1}$ )  
988 for each dietary group assessed over the whole recovery period (70 min). A significant  
989 difference was observed between the groups ( $P = 0.0037$ ;  $F_{1, 18} = 4.98$ )

990

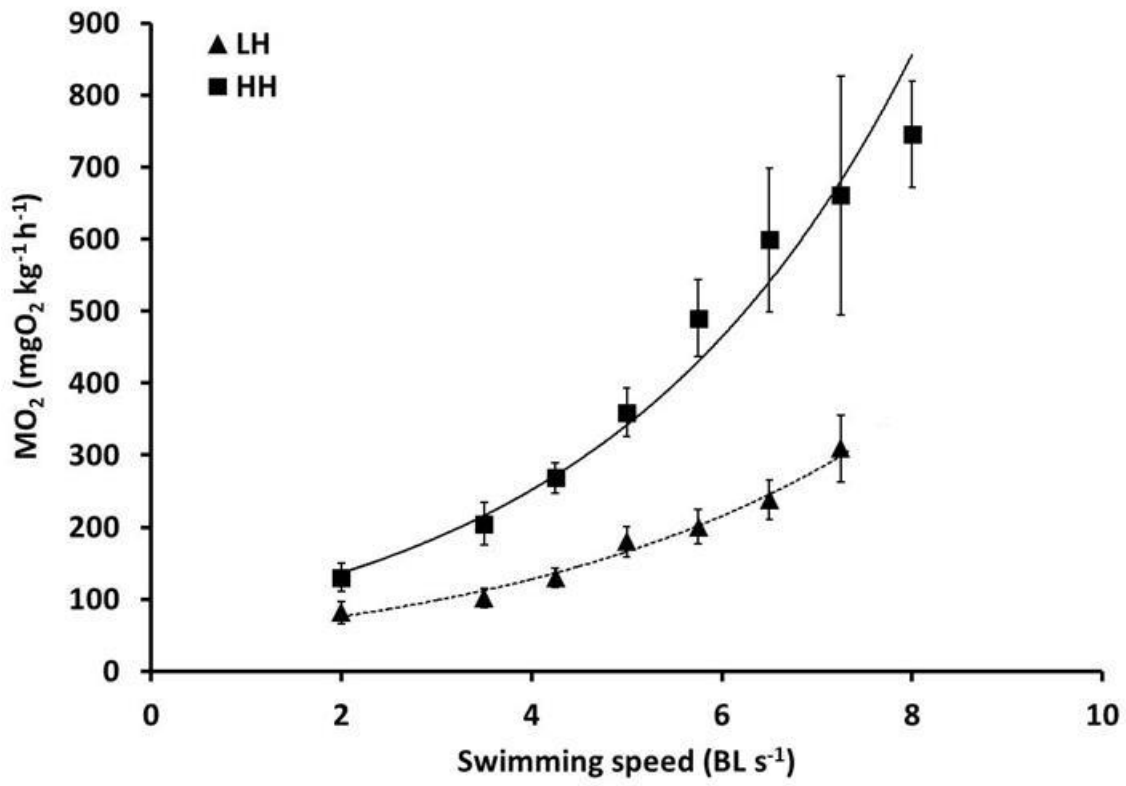
991 **Fig 3** Kinetics of calcium fluxes as a function of chemical stimulation for each fish dietary  
992 group (standard-n-3 HUFA SD or low-n-3 HUFA LD diet): (a) time to rise (s), (b) time to  
993 decay at 50% (s), and (c) amplitude (V) of calcium concentration measured in fish ventricular  
994 myocytes following stimulation by hyperpotassic solution (100 k at 100 mM), ryanodine  
995 (RYA at 1 $\mu\text{M}$ ), or adrenaline (AD at 1 mM). Results are mean  $\pm$  standard error SE with the  
996 number of cells tested indicated on each mean. One-way ANOVA revealed no significant  
997 effect of diet ( $P > 0.05$ ); \*\* indicates that RYA had a significant effect on time to rise at  $P <$   
998 0.01 ( $F_{2, 41} = 10.29$ ).

999

1000

1001 Fig 1

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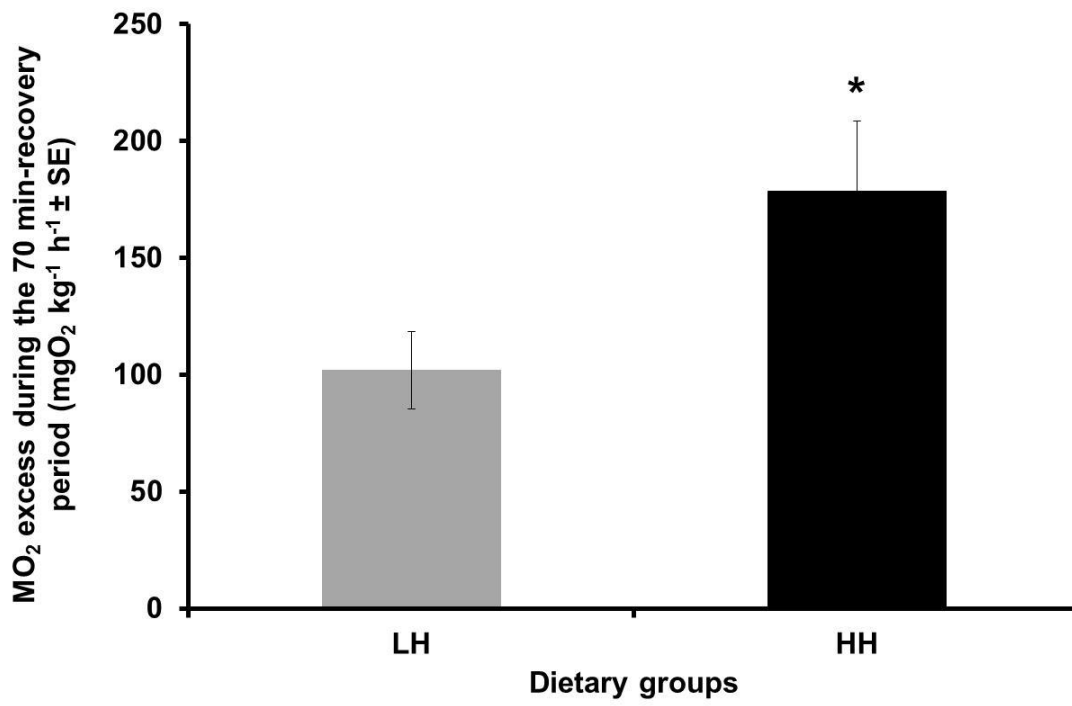


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1006 **Fig 2**



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