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

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Abstract :

In this study, we hypothesised that a reduction in n-3 HUFA availability for higher consumers, as expected with global change, would negatively impact the physiological performances of fish. The aim was to experimentally evaluate the effect of n-3 HUFA dietary content on cardio-respiratory performances of the golden grey mullet (Liza aurata), a microalgae grazer of high ecological importance in European coastal areas. These performances were evaluated in terms of critical swimming speed U (crit), associated oxygen consumption MO2, post-exercise oxygen consumption and calcium fluxes in cardiomyocytes. Two replicated groups of fish were fed on a rich (standard diet, SD diet: 1.2 % n-3 HUFA on dry matter basis, DMB) or a poor n-3 HUFA (low n-3 HUFA diet, LD diet: 0.2 % n-3 HUFA on DMB) diet during 5 months and were called SD and LD groups, respectively. The results showed that the LD diet reduced growth rate as well as the aerobic capacity of L. aurata at 20 A degrees C, suggesting that fish may have to save energy by modifying the proportion of energy allocated to energy-demanding activities, such as digestion or feeding. In addition, this LD diet induced higher levels of haematocrit and plasma osmolality, indicating a stress response at the second and third levels in that group. However, the LD diet caused a massive increase in swimming efficiency. This should improve the capacity of L. aurata to migrate and to forage over a wide area. In turn, these could then compensate for the reduction in growth rate and aerobic metabolism.

1. Introduction

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

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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 ± standard error (SE): 26.1 ± 0.4 g; initial
130 mean standard length ± SE: 13.0 ± 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 (20ºC) exposed to a 12L:12D photoperiod cycle. Temperature (19.9 ± 0.5°C),
140 salinity (33.4 ± 0.1) and oxygen (87.9 ± 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 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^{-1}). 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 were220 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^{-1}; here, it was maintained for 5
180 min, and then finally increased to 220°C in increments of 3°C min^{-1}. 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^{-1}, Sigma-Aldrich, St Quentin-Fallavier, France) and
195 individually identified using a pit-tag reader (MS-120, biolog-id, Réseaumatique, 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 $^{-1}$) was calculated for each experimental condition using
199 Equation 1:

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

201

202 Swimming performance (U_{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^{-1}. 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 $$MO_{2meas} = \left(\frac{\Delta\,[O2]}{\Delta\,t}\right) \times \left(\frac{V}{m}\right) \qquad (2)$$

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

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 ± SE: 47.5 ± 3.1 g; mean standard length ±
253 SE: 15 ± 0.3cm, n = 12; LD group: mean weight ± SE: 45.1± 2.1; mean standard length ± SE:
254 14.6 ± 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 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 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 BL s^{-1} over 5 min from 0.5 to 3.5 BL s^{-1}, and increments of 0.75 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 MO_2 was measured. Oxygen saturation never fell below 75% of air
267 saturation during 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 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 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 MO_2
281 (mgO_2 kg^{-1} 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 MO_2 curve and the pre-test $MO_{2\ (0.5\ BL\ s^{-1})}$ 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 $MO_{2\ (0.5\ BL\ s^{-1})}$ calculation, as fish MO_2
286 was high due to the stress linked to the fish transfer (personal observation). Routine MO_2
287 while swimming at 0.5 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 g L^{-1}),
294 identified using a pit-tag reader, weighed (± 0.1 g), and measured (± 0.1 cm). The Fulton
295 index (FI) was determined using Equation 4 (Fulton 1904):
296 $$FI = \frac{W}{L^3} \quad (4)$$

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^{-1};
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 $$\mathrm{HSI} = \frac{(\mathrm{W\ liver} \times 100)}{(\mathrm{W\ fish} - \mathrm{W\ liver})} \qquad (5)$$

308 where W_{liver} is the liver weight and W_{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_{crit}, SMR, AMR and AS

314

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

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

318 where U_t (BL s^{-1}) 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^{-1}).

321 SMR ($mgO_2\ kg^{-1}\ h^{-1}$) was extrapolated as the intercept (*i.e.* MO_2 when U = 0 BL s^{-1}; Brett
322 1964) of Equation 7:

323 $$MO_2 = SMR\ e^{bU} \qquad (7)$$

324

325 where MO_2 is the oxygen consumption ($mgO_2\ kg^{-1}\ h^{-1}$), b a constant, and U the swimming
326 speed (BL s^{-1}).

327 AMR ($mgO_2\ kg^{-1}\ h^{-1}$) was considered the highest MO_2 recorded during the U_{crit} test.

328 AS ($mgO_2\ kg^{-1}\ h^{-1}$) 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 Ca^{2+}-free solution: in mM: NaCl, 100; KCl, 10; KH_2PO_4, 1; $MgSO_4$, 5.4;
336 taurine, 50; αD-glucose, 20; HEPES, 10; EGTA, 0.1 (adjusted to pH 7.1 using KOH,
337 osmolality: 300 mOsmol L^{-1}). The enzymatic digestion solution comprised collagenase (Type
338 IA, 0.36 mg mL^{-1}), trypsin (Type III, 0.24 mg mL^{-1}) and BSA (0.5 $mgmL^{-1}$) added to the
339 isolation Ca^{2+}-free solution.

340 The incubation medium Ca^{2+} solution: in mM: Glucose, 10; NaCl, 130; KCl, 5; $MgSO_4$, 3;
341 NaH_2PO_4, 0.5; $CaCl_2$, 2; HEPES, 10 (adjusted to pH 7.35 using KOH).

342 The hyperpotassic 100 mM K^+ solution (100 K): in mM: glucose, 1; NaCl, 35; KCl, 100;
343 $MgSO_4$, 1.5; NaH_2PO_4, 0.4; $CaCl_2$, 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 ± SE: 54.6 ± 5.3 g; mean standard length ±

348 SE: 15.0 ± 0.4 cm; LD group: mean weight ± SE: 40.8 ± 5.3; mean standard length ± SE: 14.1

349 ± 0.5 cm] were randomly sampled in tanks using a net, and individually anaesthetised (MS-

350 222; 0.1 g L^{-1}). The heart was rapidly excised and immersed in a Ca^{2+}-free solution in order to

351 disrupt 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 Ca^{2+}-free solution containing

355 proteolytic enzymes. They were gently homogenised for 10 min at 20°C. Following

356 enzymatic treatment, (1) the ventricle was transferred in a Petri dish containing 2 mL of fresh

357 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 Ca^{2+}-free solution with

360 enzymes, while the supernatant was centrifuged (5 min, 2500 rpm, 4°C); and (4) the pellet

361 was gently suspended in 500 µL of 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 Ca^{2+}

365 solution containing 2 µM 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°C for at least 1h15.

368

369 *Measure of Ca^{2+} fluxes*

370

371 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 µM) 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 Ca^{2+} fluxes,
383 possibly via the activation of L-type Ca^{2+} channels and Na^{+}/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 µL). 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 µM) binds to the ryanodin receptor located on the SR, and locks the SR- Ca^{2+} release channel
391 in the open state, rendering it unable to contribute to Ca^{2+} transient (Rousseau et al. 1987). As
392 a consequence, by subtracting the concentration of Ca^{2+} measured in cells stimulated with
393 RYA to that measured with 100 K, the Ca^{2+} concentration released by SR can be estimated.
394 Adrenaline solution (AD; 10 µM, Sigma-Aldrich; St Quentin-Fallavier, France) was used to
395 investigate the effects of β-adrenergic receptors upon the cellular calcium responses. As a β_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 Ca^{2+} ATPase pump of the RS, inducing the 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 µL). 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 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/F0), where F0 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 ± 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 (∑ 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. ∑ 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 ∑ 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 ∑ 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-pituary
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 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_{crit} values in both dietary groups, as several studies have argued
626 that U_{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- 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, 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 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 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

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681

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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
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692

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694

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917 **Table 1** Formulation (ingredient proportion in g 100 g^{-1}), 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

Ingredients [a]	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 [b]	8	8
Mineral mixture [c]	4	4
Betaine	1	1
Chemical composition		
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
FA composition in TL		
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
ΣMUFA	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

Σ HUFA n-3	13.51 ± 0.08	2.31 ± 0.02
n-3 / n-6	1.11 ± 0.01	0.19 ± 0.00
DHA / EPA	1.39 ± 0.01	1.68 ± 0.02
ARA / EPA	0.08 ± 0.00	0.12 ± 0.00

922 [a] 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 [b] Vitamin mixture (g kg^{-1} vitamin mix): retinyl acetate, 1; cholecalciferol, 2 5; DL-α-
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 [c] Mineral mixture (g kg^{-1} mineral mix): KCl, 90; KI, 0 04; $CaHPO_4$ $2H_2O$, 500; NaCl,
931 40; $CuSO_4$ $5H_2O$, 3; $ZnSO_4$ $7H_2O$, 4; $CoSO_4$, 0 02; $FeSO_4$ $7H_2O$, 20; $MnSO_4$ H_2O, 3; $CaCo_3$,
932 215; MgOH, 124; Na_2SeO_3, 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

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Analyses	*Fish replication for each experimental condition*	*Sampling period*
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

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

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961 **Table 3** Total lipid TL content, neutral lipid NL content, polar lipid PL content (mg g^{-1} 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		Statistical analysis			
	SD group	LD group	*P*	*F*	df_1	df_2
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
NL						
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
PL						
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 ±
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^{-1})	U_{crit} (BL s^{-1})	SMR (mgO_2 kg^{-1} h^{-1})	AMR (mgO_2 kg^{-1} h^{-1})	AS (mgO_2 kg^{-1} h^{-1})	Haematocrit (%)	Osmolality (mosmol L^{-1})	Fulton index	HSI
SD group	0.4 ± 0.02 (n = 40)	6.4 ± 0.2 (n=11)	66.5 ± 11.8 (n=10)	590.2 ± 72.0 (n=10)	523.7 ± 67.1 (n=10)	35.3 ± 2.4 (n =12)	374.1 ± 6.3 (n=11)	0.96 ± 0.02 (n=12)	0.22 ± 0.05 (n=12)
LD group	0.3 ± 0.01 (n = 40)	6.9 ± 0.4 (n=10)	38.5 ± 8.7 (n=10)	266.0 ± 24.8 (n=10)	227.5 ± 23.3 (n=10)	44.1 ± 2.9 (n=12)	400.2 ± 10.5 (n=10)	0.99 ± 0.02 (n=12)	0.56 ± 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
df1	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 (MO_2; mg O_2 Kg^{-1} h^{-1}; mean ± standard error SE) as a function of
979 swimming speed (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 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_{crit} test for each fish dietary group:
987 Mean excess of post-exercise oxygen consumption (± standard error SE, EPOC in $mgO_2\ kg^{-1}$)
988 for each dietary group assessed over the whole recovery period (70 min). A significant
989 difference was observed between the groups ($P = 0.0037$; $F_{1, 18} = 4.98$)

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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 1µM), or adrenaline (AD at1 mM). Results are mean ± 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 ($F_{2, 41} = 10.29$).

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1001 **Fig 1**

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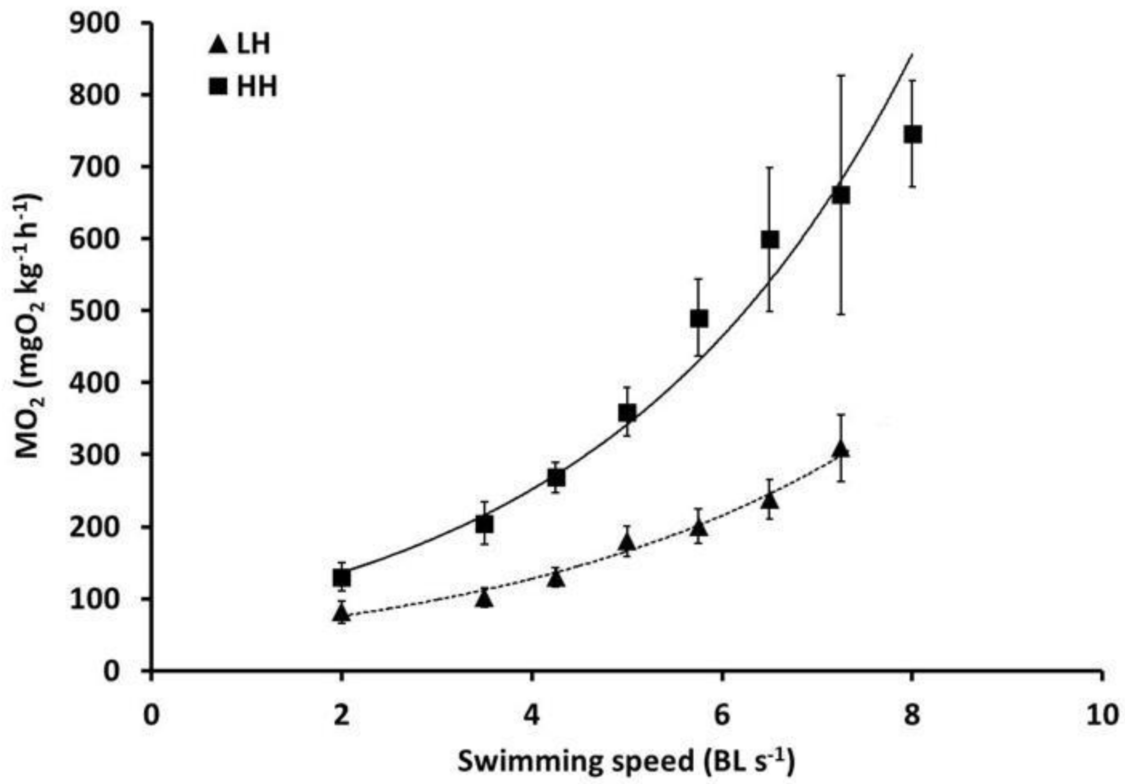

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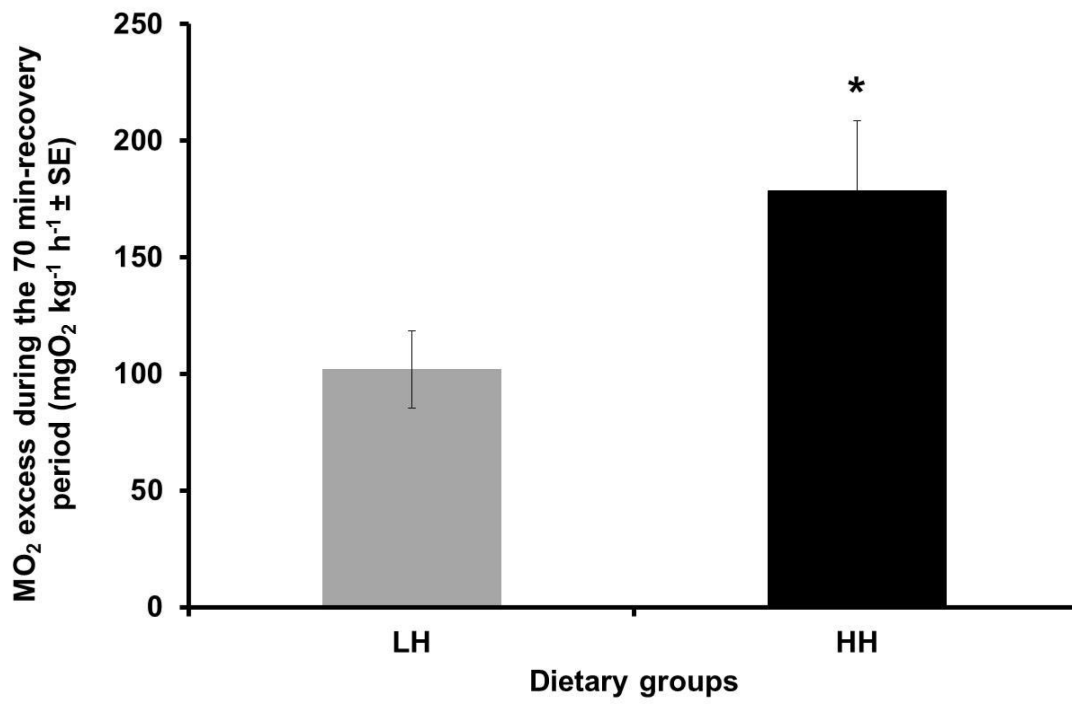
1006 **Fig 2**



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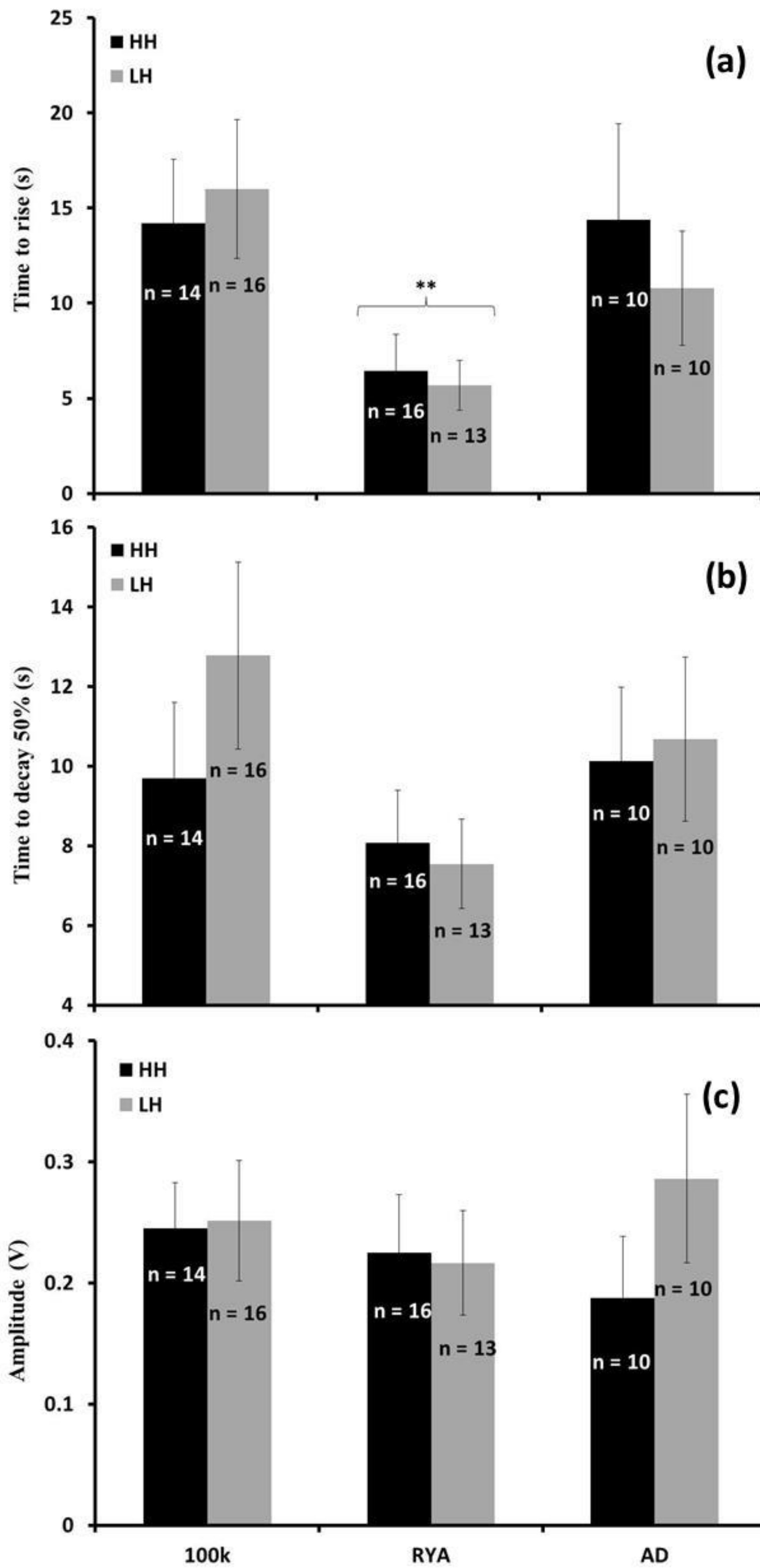
1022 **Fig 3**



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