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1 **Muscle bioenergetics of two emblematic Mediterranean fish species: *Sardina pilchardus***
2 **and *Sparus aurata*.**

3

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19 **KEY WORDS:** red muscle, bioenergetics, marine fishes, lipids

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21 **RUNNING TITLE:** Cellular fuels in fish muscle

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25 **SUMMARY**

26 We investigated links between swimming behavior and muscle bioenergetics in two
27 emblematic Mediterranean fish species that have very different ecologies and activity levels.
28 European sardines *Sardina pilchardus* are pelagic, they swim aerobically, school constantly
29 and have high muscle fat content. Gilthead seabream *Sparus aurata* are benthopelagic, they
30 show discontinuous spontaneous swimming patterns and store less fat in their muscle.
31 Estimating the proportion of red and white muscle phenotypes, sardine exhibited a larger
32 proportion of red muscle (~10% of the body mass) compared to gilthead seabream (~5% of
33 the body mass). We firstly studied red and white muscle fiber bioenergetics, using high-
34 resolution respirometers, showing a 4-fold higher oxidation capacity for red compared to
35 white muscle. Secondly, we aimed to compare the red muscle ability to oxidize either lipids or
36 carbohydrates. Sardine red muscle had a 3-fold higher oxidative capacity than gilthead
37 seabream and a greater capacity to oxidize lipids. This study provides novel insights into
38 physiological mechanisms underlying the different lifestyles of these highly-prized species.

39

40

41 INTRODUCTION

42 Fishes have a long history as models for energetics and exercise physiology (Fry,
43 1957; Brett, 1971). Currently, they are used to investigate muscle metabolism and pathologies
44 at various levels of integration (Li et al., 2017; Bergen et al., 2019; Krishnan and Rohner,
45 2019). At the *in vivo* level, fish swimming gaits have been well-studied, to define various
46 measures of performance (Brett, 1964; Beamish, 1978; Drucker, 1996; Peake and Farrell,
47 2004). Gaits comprise steady sustained aerobic swimming, for relatively low speed activities
48 such as migration or holding station, and unsteady burst or sprint anaerobic swimming, for
49 high speed swimming such as predator-prey encounters (Webb, 1984). Aerobic and anaerobic
50 swimming relies on structurally separate muscles in fishes (Bone, 1978; Webb, 1984). Slow-
51 twitch oxidative ‘red’ muscle is used for steady aerobic swimming, it is found in strips along
52 the midline and typically represents about 10% of muscle mass (Bone, 1978; McKenzie,
53 2011). Fast-twitch glycolytic ‘white’ muscle comprises the large myotomal blocks that form
54 the majority of the muscle mass (Bone, 1978; Johnston, 1983; Jayne and Lauder, 1994).
55 Moreover, red and white muscle are fueled by different energetic substrates and metabolic
56 pathways relying on carbohydrate or lipid fuels are gradually involved in these different
57 locomotion modes among fish species, depending on their swimming needs (Moyes and West,
58 1995; Weber and Haman, 1996).

59 The European sardine *Sardina pilchardus* and the gilthead seabream *Sparus aurata*
60 have very different ecologies, in particular in terms of their swimming activity. Sardines are
61 pelagic, they swim constantly in schools, covering great distances (Webb, 1984). Gilthead
62 seabream are coastal benthopelagic, usually foraging in a discontinuous pattern for limited
63 distances, although they perform quite extensive seasonal migrations to and from breeding
64 grounds (Lasserre 1976; McClelland et al., 1995; Grigorakis et al., 2002; Steinhausen et al.,
65 2010; Mercier et al., 2012). Gilthead seabream and sardine also present a different lipid

66 storage capacity, which can be related to their lifestyle and locomotor behavior. The sardine
67 stores lipids in its muscle, as the primary site of rapidly available substrate (Venugopal and
68 Shahidi, 1996). By contrast, gilthead seabream mainly stores lipids in the liver (McClelland et
69 al., 1995). Beyond the fundamental ecophysiological interest of comparing these two species,
70 our study has broader ecological and socio-economical implications. *Sparus aurata* is highly
71 prized in inshore fisheries, representing the second most captured species (Weiss et al., 2018,
72 <https://archimer.ifremer.fr/doc/00478/58970/>) and is also the main aquaculture species in the
73 Mediterranean Sea, accounting for 44% of the farmed fish production. *Sardinus pilchardus* is
74 the second most fished species in the Mediterranean as a whole, representing 17% of landings
75 (FAO 2018. Global Capture Production 1950-2016 (online query), www.fao.org). Further, the
76 sardine stock in the Gulf of Lions is currently considered to be ecologically unbalanced, with
77 a major decline in final adult size and body condition (GFCM 2018), possibly mediated by
78 differences in prey composition and feeding behaviour (Saraux et al., 2019; Queiros et al.,
79 submitted). While significant information is available on the seabream, due to its status as a
80 farmed species (Pavlidis and Mylonas, 2011), much less is known about the physiology of
81 sardines, due to the difficulties of maintaining them in captivity.

82 The aim of this study was to characterize aerobic (slow-twitch oxidative) muscle
83 bioenergetics in the sardine and gilthead seabream. We hypothesized that the lifestyle of the
84 sardine would be associated with adaptations for constant aerobic exercise, notably a high
85 capacity for lipid-oxidative metabolism in their muscles compared with those of the more
86 sedentary gilthead seabream.

87

88 **MATERIAL AND METHODS**

89 *Fishes*

90 The *S. aurata* were bought in April 2016 from Cannes Aquaculture (Cannes, France),
91 and transported to the Ifremer research station at Palavas-les-Flots (France). They were then
92 held at a density of 4 kg.m⁻³ in outdoor 4 m³ tanks supplied with well-aerated seawater at
93 prevailing seasonal temperatures (8–25°C). Fish were fed once a day in the morning with 1%
94 body weight of commercial feed. The *S. pilchardus* were captured in March 2016 off Sète
95 (South of France) by a commercial purse-seiner adapted for this purpose. They were
96 transported to the same site as seabream and held in quarantine tanks for health assessment.

97 After confirmation of an absence of pathogens, sardines were moved into indoor 3 m³
98 tanks supplied with water pumped directly from the sea (more details in Queiros et al.,
99 submitted). The photoperiod was adjusted each week to follow the natural cycle and sea water
100 temperature was not controlled (averaging 12°C at this period of time) except to maintain a
101 minimum of 10°C or a maximum of 25°C. Sardines were fed every day with 0.6% body mass
102 of aquaculture pellets. Experiments were performed in February 2017 and approved by the
103 Ministère Français de l'Enseignement Supérieur, de la Recherche et de l'Innovation (APAFIS
104 # 4000-2016020415387815 v.3; APAFIS# 7097-2016093008412692).

105

106 ***Tissue sampling and Fiber bioenergetics***

107 Fishes were fasted for one day before being euthanized with an overdose of benzocaine (1000
108 ppm) and small muscle samples were immediately withdrawn and stored at 4°C in MIR05
109 buffer containing 0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM Taurine, 10
110 mM KH₂PO₄, 20 mM HEPES, 110 mM sucrose, 1g/L free-fatty-acid bovine serum albumin,
111 pH = 7.1 (Kuznetsov et al., 1998). Thereafter, the entire red and white muscles, liver and
112 visceral tissues were dissected and weighed.

113 Respiration rates of both red and white muscle fibers were measured using high resolution
114 respirometers (O2K, Oroboros Instruments; www.oroobros.at) in 2 ml of MIR05 solution at

115 12°C, following a protocol of sequential injections of substrates and inhibitors of the
116 mitochondrial electron transport system (ETS) adapted from a previous study on *Danio rerio*
117 (Teulier et al., 2018). According to this protocol, instead of using chemical permeabilization,
118 fibers were gently mechanically disrupted to measure the cellular respiration rates. Basal non-
119 phosphorylating respiration rate was obtained by addition of either a mixture of respiratory
120 substrates (5 mM pyruvate/2.5 mM malate/5 mM succinate; PMS), which allow a full
121 activation of the ETS by generating a convergent electron flow at the coenzyme-Q junction,
122 or a lipid-derived substrate (40 µM palmitoyl-carnitine/2.5 mM malate; PCM). The
123 phosphorylating rate of respiration was initiated by the injection of ADP (1 mM). The
124 integrity of mitochondrial membranes was systematically checked by adding cytochrome c
125 (10 µM). Maximal respiration rate was obtained using carbonyl cyanide-p-trifluoro-
126 methoxyphenyl hydrazine (FCCP; 1 µM). Finally, the complex III (ubiquinol-cytochrome c
127 oxydoreductase) of the ETS was inhibited by an injection of antimycin A (2.5 µM). The
128 factorial cellular aerobic scope refers to the ratio of FCCP-induced maximal uncoupling
129 respiration rate to basal non-phosphorylating respiration rate (Roussel et al., 2015).

130

131 *Statistical analysis*

132 All data are expressed as means ± SEM. Species (sardine vs. seabream) and substrate (PMS
133 vs PCM) effects on bioenergetics parameters were tested using 2-way ANOVA, or non-
134 parametric 2-way ANOVA on ranks, depending on normality (Shapiro-Wilk test) and
135 homoscedasticity (Levene test). When apposite, pairwise comparisons with adjustments for
136 multiple comparisons (Holm–Sidak method) were conducted to detect further differences
137 between substrates or species. Statistical analysis was performed using SIGMAPLOT 12
138 (Systat Software, Inc.; www.systatsoftware.com).

139

140 **RESULTS**

141 *Body and tissue masses*

142 Because of the much lower body mass of sardines, wet tissue masses were all significantly
143 lower in sardines than in seabream (Table 1). However, relative to their body mass, the
144 proportion of the different tissues varied between species. Sardines had a 2.5-fold higher
145 proportion of red muscle to body mass than seabream, while visceral and white muscles were
146 in similar proportions in both species (Table 1). The wet mass-to-body mass ratios (g/100 g
147 BM) of liver were significantly lower in sardines than in seabream (Table 1).

148

149 *Muscle fibers bioenergetics*

150 Red and white muscle phosphorylating respiration rate (i.e. ADP-induced respiration rates
151 with pyruvate/malate/succinate as respiratory substrates) were markedly different; white
152 muscle fibers exhibited 6 to 12-fold lower respiration rates than red muscle fibers from
153 sardine and seabream, respectively (Fig. 1A). Phosphorylating respiration rate of white
154 muscle was higher in sardine than in gilthead seabream, while red muscle respiration rate was
155 not statistically different between species (Fig. 1A). Using masses of white and red muscles,
156 we then calculated the total muscle oxidative metabolism per gram of fish (Fig. 1B) and the
157 relative contribution of white and red muscle to this total muscle oxidative metabolism (Fig.
158 1C). Sardines exhibited a significantly higher body mass specific-muscle oxidative capacity
159 than gilthead seabream (Fig. 1B), and the contribution of its red muscles to whole body
160 muscle oxidative capacity was slightly but significantly greater than gilthead seabream
161 ($67\pm 1\%$ versus $58\pm 3\%$; Fig. 1C).

162 Because of the very low level of aerobic capacity in white muscles, the study
163 regarding energy substrates oxidation was conducted on red muscles only. On the whole, red
164 muscle fibers of sardines exhibited higher oxidative capacities than gilthead seabream,

165 regardless of respiratory substrates (Table 2). In details, the differences in ADP-induced
166 respiration rate observed between species (2 and 2.5-fold higher in sardines than in gilthead
167 seabream fibers respiring on PMS ($p=0.11$) and PCM ($p=0.07$)) were, however, not
168 statistically significant. (Table 2). The rates of FCCP-induced maximal oxygen consumption
169 were significantly higher in sardine than in gilthead seabream fibers, regardless of respiratory
170 substrates (Table 2). The factorial cellular aerobic scope was not significantly different
171 between fishes when fibers respired on PMS, but higher in sardine for a lipid-derived
172 substrate, such as PCM (Table 2).

173 The higher muscle aerobic metabolism is even more pronounced when the rates of
174 muscle oxygen consumption are expressed per gram of body mass when taking into account
175 the total mass of red skeletal muscle in these two species (Fig. 2A). Despite huge differences
176 in body mass-specific oxidative activities (Fig. 2A), the part of oxygen consumption
177 associated with mitochondrial ATP synthesis was similar in the two species, accounting for
178 about 75% and 87% of phosphorylating respiration rate with PMS and PCM, respectively
179 (Fig. 2B). The remaining 13-25% of oxygen consumption was devoted to counteract proton
180 leakage across the mitochondrial inner membrane (Fig. 2B). Hence, muscle mitochondria of
181 both species allocated the same proportion of oxygen consumed to synthesize ATP. When
182 fibers oxidized PMS, both species exhibited a large mitochondrial extra respiratory capacity
183 (FCCP-induced maximal respiration rate minus ADP-induced phosphorylating respiration
184 rate; Fig. 2B). By contrast, the extra respiratory capacity was reduced in the muscle fibers of
185 gilthead seabream oxidizing a lipid-derived substrate (Fig. 2B). By comparison, sardine
186 muscle fibers retained a large extra respiratory capacity with PCM, which was as high as that
187 calculated with PMS (Fig. 2B).

188

189 **DISCUSSION**

190 The results obtained at the cellular level show that sardines have muscle biochemical
191 adaptations for a lifestyle of constant sustained aerobic exercise, with a better ability to
192 oxidize lipids than the sedentary gilthead seabream.

193 Muscles represent a large proportion of the body mass of both gilthead seabream and
194 sardine (approximately 45%), which is in accordance with the classically described range of
195 40% to 60% (Bone, 1978). Even if the proportion of white muscle is roughly the same
196 between species (~40%), the ratio between red muscle and body mass was significantly
197 higher in sardines (above 10%) than in gilthead seabream (below 5%). Hence, red muscle
198 represents almost 25% of the total muscle mass in sardines and only 10% in gilthead
199 seabream. Our results are in line with the 30% obtained for *Sardina pilchardus* and the 15% in
200 *Pagellus bogaraveo*, a fish closely related to *Sparus aurata* (Greer-Walker and Pull, 1975).
201 Overall, fishes with a more active mode of life have a higher proportion of red fibers (Webb,
202 1984; Dwyer et al., 2014). Red and white muscle also show different aerobic capacity, with
203 respiration rates 6-fold to 12-fold higher in red than in white muscle of sardine and gilthead
204 seabream, respectively. Even though red muscles contribute to a small proportion of total
205 skeletal muscle masses, its high respiratory capacity explains why its oxidative activity
206 contributes to a major part of total muscle respiration rate (Fig. 1). The high metabolic
207 capacity found in the red muscle of the two species is coherent with the extensive evidence of
208 differences in histochemistry, enzyme activity and respiratory capacity between red and white
209 muscles of many fish species (Bone, 1978, Moyes et al., 1992; Martinez et al., 2003; Morash
210 et al., 2008; Strobel et al., 2013; Zak et al., 2017; Teulier et al., 2018).

211 The body-mass specific aerobic metabolism of red muscle is markedly higher in *S.*
212 *pilchardus* than in *S. aurata*. Interestingly, the relative increase in respiration induced by ADP
213 phosphorylation, i.e. the oxygen consumption dedicated to drive ATP synthesis, was the same
214 for both species. This indicates that red muscle fibers of both species allocate the same energy

215 to synthesize ATP and thus to sustain aerobic locomotor performance. However, the FCCP-
216 induced extra respiratory capacity of red muscle fibers differed between species depending on
217 the respiratory substrates. Hence, it was not different when fibers respired on PMS, a mixture
218 of substrates that fully activate the ETS by generating a convergent electron flow at the
219 coenzyme-Q junction. It was, however, significantly lower in gilthead seabream than in
220 sardines when fibers oxidized PCM, a lipid-derived substrate. The FCCP-induced extra
221 respiratory capacity is controlled exclusively by mitochondrial oxidation of substrates, which
222 include the electron transport system, dehydrogenases and translocases. Hence, the results
223 indicate that the capacity to oxidize lipids is significantly higher in the red skeletal muscle of
224 sardine than in gilthead seabream. Indeed, sardine red muscle fibers oxidize PCM at 79% the
225 rate of fully activated ETS, whereas red muscle fibers of seabream oxidize lipids at 47%. This
226 metabolic “specialization” toward lipids oxidation is also supported by a very high value of
227 factorial aerobic scope in sardine red muscle fibers oxidizing a lipid-derived substrate. These
228 results are in accordance with other studies showing that, for instance, red muscles of the
229 pelagic coalfish (Johnston and Moon, 1980) or the endurance swimming rainbow trout
230 (Kiessling and Kiessling, 1993) exhibit a high lipid oxidation ability. And by directly
231 comparing the red muscles of skipjack tuna with the more sedentary freshwater carp, Moyes
232 and collaborators showed that the former contained more active mitochondria with also a
233 greater ability to oxidize lipids than the latter (Moyes et al., 1992).

234 Such difference of lipid aerobic metabolism results from a combination of both a
235 higher respiratory capacity and proportion of red muscle in *S. pilchardus* than in *S. aurata*.
236 However, we cannot completely rule out that part of the muscle metabolism difference
237 between the two species might also be explain by large difference in size, as *S. aurata*
238 weighting 8-fold more than *S. pilchardus*. Indeed, it has been reported that mitochondrial
239 oxidative enzyme activities in both red and white muscles negatively correlate with body

240 mass of fishes (Childress and Somero, 1990; Pelletier et al., 1993; Burness et al., 1999;
241 Almeida-Val et al., 2000; Davies and Moyes, 2007; Young and Egginton, 2009). For instance,
242 the activity of cytochrome-c oxidase, which measure the maximal oxidative capacity of a
243 tissue, correlate negatively with body mass with a scaling coefficient of -0.014 and -0.31 in
244 red muscle of striped bass and white muscle of Atlantic cod, respectively (Pelletier et al.,
245 1993; Young and Egginton, 2009). Based on these equations, body mass would explain
246 between 30% and 60% of the differences in the lipid-supported maximal respiratory capacity
247 between *S. aurata* and *S. pilchardus*. The rest would thus relate to difference in lipid energy
248 metabolic pathway (β -oxidation process and/or mitochondrial translocation system), which
249 might ultimately fit with the lifestyles of the two species. Lipids are well known to be a major
250 fuel for long distance locomotion in animals (Weber, 2011) and to play an important role as
251 energy substrates for sustained aerobic swimming in fishes (Johnston and Moon, 1980; Moyes
252 and West, 1995; McClelland et al., 1995). It is therefore not surprising that pelagic sardines,
253 that swim constantly, exhibit a higher ability to oxidize fat than benthopelagic and less active
254 gilthead seabream. Finally, pelagic fishes are characterized by abundant fat deposits under
255 skin and within red muscle, which are associated with their enhanced capacity to consume
256 lipids as fuel, whereas less active demersal fish typically stock fat in their liver (McClelland et
257 al., 1995; Venugopal and Shahidi, 1996). In this context, the very different lipid stores
258 between *Sardina pilchardus* and *Sparus aurata* would also participate in the differences in the
259 availability of fatty acids to fuel aerobic muscle function (McClelland et al., 1995; Venugopal
260 and Shahidi, 1996).

261 In conclusion, our results show that pelagic sardine exhibits a higher percentage of red
262 muscle than benthopelagic gilthead seabream, with a higher oxidative capacity at the cellular
263 level. Sardine are also able to oxidize lipids at nearly 80% the rate of fully ETS activity,
264 whereas gilthead seabream reach only 47% of the maximum respiratory capacity. However

265 even if muscle bioenergetics clearly show different patterns between these two species,
266 directly linking the locomotion performances to the cellular energetics needs further
267 investigations at the individual scale.

268

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276

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409

410 **Figure legends**

411 **Figure 1: Aerobic metabolism of red and white muscles in sardine and gilthead**

412 **seabream.** A) Phosphorylating respiration rate of white (white bars, n=2-4) and red (red bars,
413 n=6) muscle fibers. B) Contribution of white and red muscles to total body mass-specific
414 respiration rate. C) Relative contribution of white and red muscles to whole body muscle
415 oxidative activity. Values are means \pm sem. † $p < 0.05$, significantly different from white muscle
416 within the same fish species; * $p < 0.05$, significantly different from gilthead seabream.

417

418 **Figure 2: Bioenergetics status of red muscle fibers.** Fibers were respiring either on

419 pyruvate/malate/succinate to fully activate the electron transport system (ETS) or palmitoyl-
420 carnitine/malate (a lipid-derived substrate). A) Contribution of mitochondrial proton leak
421 (basal non-phosphorylating respiration), mitochondrial ATP synthesis (ADP-induced
422 phosphorylating respiration minus basal non-phosphorylating respiration), and mitochondrial
423 respiratory reserve (FCCP-induced maximal respiration minus ADP-induced phosphorylating
424 respiration) to body mass-specific respiration rates of fibers. B) Contribution of mitochondrial
425 proton leak, ATP synthesis and respiratory reserve expressed as percentage of phosphorylating
426 respiratory capacity. Values are means \pm SEM for n=6 independent fiber preparations.
427 * $p < 0.05$, significantly different from seabream.

428

429

430

431 **Table 1: Fish biometrics and tissue masses**

Tissues	Parameters	Gilthead Seabream	Sardine
	Body mass, g	340 ± 20	40 ± 5*
	Body length (mm)	270 ± 6	162 ± 5*
Red muscle	Fresh mass, g	15.5 ± 1.2	4.5 ± 0.5*
	Relative mass, g/100g BM	4.6 ± 0.5	11.3 ± 0.4*
White muscle	Fresh mass, g	137 ± 14	14 ± 2*
	Relative mass, g/100g BM	40 ± 2	36 ± 2
Liver	Fresh mass, g	9.5 ± 0.8	0.4 ± 0.1*
	Relative mass, g/100g BM	2.8 ± 0.2	0.8 ± 0.1*
Visceral tissues	Fresh mass, g	25.3 ± 2.7	3.3 ± 0.8*
	Relative mass, g/100g BM	7.4 ± 0.5	7.7 ± 0.9

432 Values are means ± sem for n=6. * $p < 0.05$, significantly different from gilthead seabream.

433

434 **Table 2: Respiratory variables of red muscle fibers from gilthead seabream and sardines.**

Substrates	Respiratory parameters	Gilthead seabream	Sardine
Pyruvate/Malate/Succinate	Basal non-phosphorylating rate	3.3 ± 0.8	6.1 ± 1.4
	ADP-induced phosphorylating rate	14.1 ± 3.5	27.4 ± 6.8
	FCCP-induced maximal oxidative rate	26.4 ± 4.1	47.6 ± 7.8*
	Factorial cellular aerobic scope	10.2 ± 2.5	8.5 ± 0.9
Palmitoyl-carnitine/Malate	Basal non-phosphorylating rate	1.5 ± 0.1	2.7 ± 0.5 †
	ADP-induced phosphorylating rate	9.4 ± 3.1	23.2 ± 6.1
	FCCP-induced maximal oxidative rate	12.5 ± 3.3 †	37.8 ± 5.9*
	Factorial cellular aerobic scope	7.0 ± 0.9	15.4 ± 1.7*†

435 Oxygen consumption rates are expressed in pmol O₂ s⁻¹ mg of tissue⁻¹ and were determined at 12°C. Basal state, basal non-phosphorylating
 436 respiration measured in the presence of respiratory substrate alone; Phosphorylation state, ADP-induced phosphorylating respiration determined
 437 after addition of 1 mM ADP; Maximal respiratory state, FCCP-induced maximal uncoupling respiration determined after addition of 1 μM
 438 FCCP; Factorial cellular aerobic scope is the ratio of FCCP-induced maximal uncoupling respiration rate to basal non-phosphorylating
 439 respiration rate. Values are means ± sem for n=6 independent fibers preparations. **p*<0.05, significantly different from seabream; † *p*<0.05,
 440 significantly different from pyruvate/malate/succinate within the same fish species.





