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1 **Changes in foraging mode caused by a decline in prey size have major bioenergetic**
2 **consequences for a small pelagic fish**

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35

36 **Abstract**

- 37 1. Global warming is causing profound modifications of aquatic ecosystems and one major
38 outcome appears to be a decline in adult size of many fish species. Over the last decade,
39 sardine populations in the Gulf of Lions (NW Mediterranean Sea) have shown severe
40 declines in body size and condition as well as disappearance of the oldest individuals,
41 which could not be related to overfishing, predation pressure or epizootic diseases.
- 42 2. In this study, we investigated whether this situation reflects a bottom-up phenomenon
43 caused by reduced size and availability of prey that could lead to energetic constraints.
44 We fed captive sardines with food items of two different sizes eliciting a change in
45 feeding mode (filter-feeding on small items and directly capturing larger ones) at two
46 different rations for several months, and then assessed their muscle bioenergetics to test
47 for changes in cellular function.
- 48 3. Feeding on smaller items was associated with a decline in body condition, even at high
49 ration, and almost completely inhibited growth by comparison to sardines fed large
50 items at high ration.
- 51 4. Sardines fed on small items presented specific mitochondrial adjustments for energy
52 sparing, indicating a major bioenergetic challenge. Moreover, mitochondria from
53 sardines in poor condition had low basal oxidative activity but high efficiency of ATP
54 production. Notably, when body condition was below a threshold value of 1.07, close
55 to the mean observed in the wild, it was directly correlated with basal mitochondrial
56 activity in muscle.
- 57 5. The results show a link between whole-animal condition and cellular bioenergetics in

58 the sardine, and reveal physiological consequences of a shift in feeding mode. They
59 demonstrate that filter-feeding on small prey leads to poor growth, even under abundant
60 food and an increase in the efficiency of ATP production. These findings may partially
61 explain the declines in sardine size and condition observed in the wild.

62

63 **Keywords:** Bioenergetics, red muscle, mitochondria, fish shrinking, sardine, foraging
64 behaviour, food restriction

65 **Résumé**

66 Le changement global entraîne de profondes modifications des écosystèmes aquatiques, l'une
67 des principales étant le déclin de la taille des adultes chez de nombreuses espèces de poissons.
68 Au cours de la dernière décennie, les populations de sardines du Golfe du Lion (Nord-Ouest de
69 la Méditerranée) ont montré une importante diminution de leur taille et de leur condition
70 corporelle ainsi qu'une disparition des individus les plus âgés, qui n'ont pas pu être liées à la
71 surpêche, à la pression de prédation ou aux épizooties.

72 Dans cette étude, nous avons cherché à savoir si cette situation reflète un phénomène ascendant
73 causé par la réduction de la taille et de la disponibilité des proies qui pourrait entraîner des
74 contraintes énergétiques chez la sardine. Nous avons ainsi nourri des sardines captives avec des
75 granulés de deux tailles différentes provoquant un changement de mode d'alimentation
76 (filtration des petits granulés et capture directe des plus gros) et à deux rations différentes
77 pendant plusieurs mois, puis nous avons évalué leur bioénergétique musculaire pour tester les
78 changements au niveau de leur fonction cellulaire.

79 L'alimentation à base de petits granulés a été associée à un déclin de la condition corporelle,
80 même à une ration élevée, et à une croissance quasiment inhibée par rapport aux sardines
81 nourries avec des plus gros granulés à une ration élevée.

82 Les sardines nourries avec des petits granulés ont également présenté des ajustements
83 mitochondriaux spécifiques pour économiser de l'énergie, indiquant un défi bioénergétique
84 majeur. De plus, les mitochondries des sardines en mauvaise condition présentaient une faible
85 activité oxydative basale, mais une efficacité élevée de production d'ATP. Notamment, lorsque
86 la condition corporelle était inférieure à une valeur seuil de 1,07, proche de la moyenne observée
87 dans la nature, elle était directement corrélée à l'activité mitochondriale basale dans le muscle.

88 Ces résultats montrent un lien entre la condition de l'animal entier et la bioénergétique cellulaire
89 chez la sardine, et révèlent les conséquences physiologiques d'un changement de mode
90 d'alimentation. Ils démontrent que le nourrissage via la filtration de petites proies entraîne une
91 faible croissance, même en cas de nourriture abondante, et une augmentation de l'efficacité de
92 la production d'ATP. Ces résultats peuvent expliquer en partie le déclin de la taille et de la
93 condition des sardines observé dans la nature.

94 **1. Introduction**

95 Marine ecosystems face strong pressures from a combination of climate forcing,
96 anthropogenic disturbance (pollution, navigation, recreational activities, by-catch, climate
97 change) and anthropogenic competition for resources (e.g. fisheries) (Halpern *et al.*, 2008), all
98 of which can impact organismal phenotypes. While water warming and a relaxation of density-
99 dependence phenomena through fishing should promote an increase in growth rates of
100 ectothermic organisms (Morrongiello, Sweetman, & Thresher, 2019), there is evidence that the
101 final adult size of many aquatic ectotherms is declining globally (Daufresne, Lengfellner, &
102 Sommer, 2009; Forster, Hirst, & Atkinson, 2012). Whereas recently debated (Audzijonyte *et*
103 *al.*, 2020), this size reduction occurs within and amongst species, throughout the food web,
104 from top predators (Sibert *et al.*, 2006) to primary producers (Sommer, Paul, & Moustaka-
105 Gouni, 2015). The decrease in size could result from fishery-induced evolution (as fisheries
106 targeting mostly larger organisms should favour the selection of slow growth and smaller
107 phenotypes, Conover & Munch, 2002; Jørgensen *et al.*, 2007) and/or from within-generation
108 plastic physiological responses. It could also result from direct effects of warming on
109 physiology (e.g. the temperature-size rule; Atkinson, 1994) or global-change-derived
110 modifications of primary production (Bopp *et al.*, 2013).

111 There has been a profound decline in size and body condition of sardines *Sardina*
112 *pilchardus* and anchovies *Engraulis encrasicolus* in the Gulf of Lions (northwestern
113 Mediterranean Sea), due to lower growth and disappearance of larger and older individuals
114 (Brosset *et al.*, 2017; Van Beveren *et al.*, 2014). Extensive research has established that this is
115 not due to top-down effects, such as predation or fishing pressure, nor to pathogens (reviewed
116 in Saraux *et al.*, 2019). Rather, a bottom-up process appears to be at play, linked to a shift in
117 plankton communities that the fishes feed upon, towards smaller zooplankton species (Brosset
118 *et al.*, 2016a; Queiros *et al.*, 2019). When maintained in captivity on artificial feeds, wild

119 sardines exhibited high growth rates and condition, indicating that the decline in size and
120 condition was a plastic rather than an evolutionary response (Saraux *et al.*, 2019). While it is
121 obvious that food quantity can have a significant impact on growth and body condition in fishes
122 (Lee *et al.*, 2018; Sun & Chen, 2009), the potential implications of prey size are less well
123 known. They deserve to be investigated because of climate-change related regime shifts in food
124 webs (Garzke, Ismar, & Sommer, 2015). A reduction in prey size is often accompanied by
125 lower energy content (Zarubin *et al.*, 2014), which may present a major energetic challenge to
126 predators, even for sardines that are opportunistic planktivores (Palomera *et al.*, 2007; Rumolo
127 *et al.*, 2016).

128 It has been demonstrated that, for rations of similar energy content, sardines fed small
129 prey have lower growth and condition than those fed larger prey (Queiros *et al.*, 2019). The
130 sardine modifies its feeding behaviour with prey size, switching from filter feeding on small
131 prey to direct capture of larger prey, regardless of prey availability (Costalago, Garrido, &
132 Palomera, 2015; Garrido *et al.*, 2007). Foraging duration was much higher for sardines fed on
133 small food items than those fed on larger ones (Queiros *et al.*, 2019). Thus, a possible
134 mechanism for the poor growth and condition may relate to high energy requirements for
135 extended swimming activity during filter-feeding, compared to shorter bouts of swimming to
136 capture large items (Costalago & Palomera, 2014; Crowder, 1985).

137 Foraging behaviour in fishes is sustained by skeletal muscle activity, with the muscles
138 being structurally separated into discrete slow-twitch oxidative ‘red’ muscle, used for steady
139 aerobic swimming, and fast-twitch glycolytic ‘white’ muscle, used for short bursts of high-
140 speed swimming (Bone, 1978; Webb, 1998). Muscular contraction is powered by ATP but, its
141 production depends on muscle type: while red muscle generates this aerobically by
142 mitochondrial oxidative phosphorylation using substrates and oxygen delivered in the
143 bloodstream, white muscle generates ATP mostly through anaerobic glycolysis of intracellular

144 substrates (Fig. 1, Bone, 1978; Webb, 1998). In the sardine, swimming muscle represents up to
145 70% of body mass and, although the red muscle mass only represents ~10% of the body mass,
146 its mitochondrial activity accounts for 70% of muscle metabolic activity (Teulier *et al.*, 2019).
147 Mitochondrial oxidative phosphorylation is a series of reactions that couple substrate oxidation
148 (catabolism of carbohydrates, lipids or proteins) and oxygen consumption with the generation
149 of a proton motive force, which is then used by ATP synthase to synthesize ATP (Fig. 1). A
150 significant proportion of mitochondrial oxygen consumption is not, however, coupled to ATP
151 synthesis but is used to compensate for proton leakage across the inner mitochondrial
152 membrane. Mitochondrial proton leak is a ubiquitous waste of potential energy, which reduces
153 the efficiency of ATP production (*i.e.* the ATP/O ratio) (Brand, 2005; Fig. 1). The ATP/O ratio
154 defines how much oxygen and nutrients are needed to meet cell ATP demands and, ultimately,
155 sustain animal performance (Brand, 2005). For instance, mitochondrial ATP/O has been
156 positively related to muscle performance (Conley *et al.*, 2013; Distefano *et al.*, 2018) and
157 growth efficiency (Salin *et al.*, 2019; Toyomizu *et al.*, 2011). Mitochondrial efficiency is also
158 flexible, varying among individuals over time, notably in response to environmental and
159 physiological constraints such as temperature, oxygen or food availability (Roussel & Voituron,
160 2020; Salin *et al.*, 2015; Salin *et al.*, 2012; Thorald *et al.*, 2021b). For example, mitochondrial
161 efficiency can increase to enhance energy conservation during food deprivation (Bourguignon
162 *et al.*, 2017; Monternier *et al.*, 2017) or decrease to promote heat generation, as in brown
163 adipose tissue of cold-adapted mammals (Nedergaard & Cannon, 2018). The maintenance of a
164 high ATP/O ratio and related efficiency may carry significant costs, especially increased
165 production of reactive oxygen species (Brand, 2000), and/or a decreased carbon flux from
166 mitochondria that can compromise aspects of cellular biosynthesis (Rolfe & Brand, 1997).
167 Overall, there is strong evidence that mitochondrial efficiency (ATP/O ratio) can be adjusted

168 plastically, in response to metabolic constraints and energy demands, leading to a dynamic
169 interplay with the biological condition of an organism (Koch *et al.*, 2021).

170 In this study we investigated whether aerobic muscle energy metabolism and, in
171 particular, mitochondrial function, might represent a mechanistic nexus for how sardine
172 foraging mode impacts upon their physical condition (Fig. 1). We thus investigated the
173 oxidative activity of red muscle fibres and the coupling efficiency of mitochondrial oxidative
174 phosphorylation in sardines fed for 7 months with either small or large food items (0.1mm and
175 1.2mm diameter commercial pellets), at either low or high ration (0.3% and 0.6% of fish mass
176 per day). We predicted that fish fed on small items would show specific mitochondrial
177 adjustments for energy sparing, with decreased oxygen consumption but enhanced efficiency
178 (ATP/O ratio), which would indicate that increased metabolic costs of foraging mode
179 represented an energetic challenge.

180

181 **2. Material and methods**

182 **2.1 Experimental conditions**

183 All details of the experiment can be found in Queiros *et al.*, 2019. Briefly, wild sardines
184 were captured off Sète (South of France) in October 2016 and brought back to the Ifremer
185 Palavas-les-Flots research station. The mean age of sardines was 1.24 years. After acclimation
186 and weaning onto commercial pellets, sardines were weighed, measured and marked
187 individually using RFID tags (Biolog-id, Bernay, France). Thereafter, they were distributed
188 among 8 tanks (vol. 300L, 56-57 sardines per tank) with similar mean (\pm SD) of mass and length
189 in each (mass: 14.1 ± 3.2 g; length: 11.9 ± 0.7 cm). A 7-month feeding experiment was
190 performed, from 14/11/2016 to 15/6/2017, with natural photoperiod and water temperature
191 (water was pumped directly from the sea). Animals were fed four times a day with commercial
192 pellets (except on weekends where one daily meal was provided). The experiment included the

193 reproductive season, which occurs once a year between December and March. As detailed in
194 Queiros *et al.*, 2019, the daily weight gain of sardines *in natura* was 0.2% with good condition
195 and 0.1% with bad condition. A preliminary study found that these growth rates were achieved
196 with daily feeding rates of 0.6% and 0.3% of fish mass. Thus, the combination of two pellet
197 sizes (0.1mm and 1.2mm, within the natural range of sardine prey size) (Le Bourg *et al.*, 2015)
198 and two rations (0.3% and 0.6% of fish biomass per day) resulted in four treatments: (1) small
199 items in small quantity (SI-SQ), (2) small items in large quantity (SI-LQ), (3) large items in
200 small quantity (LI-SQ), (4) large items in large quantity (LI-LQ). The two sizes elicited two
201 distinct foraging modes, filtration *vs.* particulate capture (Queiros *et al.*, 2019). Each treatment
202 was randomly assigned to two tanks for replicates. Biometries were performed each month to
203 correct food quantity against increasing biomass, adjusted each week based on linear
204 extrapolation of biomass across successive biometries.

205

206 **2.2 Sardine sacrifices and morphometric measurements**

207 At the end of the experiment, 1 or 2 sardines were randomly removed from each tank
208 every day over a period of 1 week, *i.e.* 11 to 12 fish per group, and killed by an overdose of
209 benzocaine (1000 ppm). Sardines were fasted for 24h before euthanasia. Mass and size of each
210 sardine were recorded. Intramuscular fat was estimated by fatmeter (Distell Fish Fatmeter
211 MFM-992, as described in Brosset *et al.*, 2015a) and sex was determined visually. Experiments
212 were performed in June when sardines were out of the breeding season (Brosset *et al.*, 2016b).
213 Cumulative growth was estimated based on the increase in size divided by the experiment
214 duration (mm/day), and the relative growth (in %) was calculated as follows:

$$215 \quad \text{Relative growth} = \frac{\text{Final size} - \text{Initial size}}{\text{Initial size}}$$

216 Where initial and final sizes are expressed in mm.

217 Body condition was calculated following the Le Cren index K_n as estimated by Brosset
218 *et al.*, 2015b (based on more than 24,000 wild sardines sampled over the last 50 years):

$$219 \quad K_n = \frac{BM}{0.00607 \times TL^{3.057}}$$

220 where TL is the total length (cm) and BM is the wet body mass (g). This index compares the
221 actual wet mass of an individual to the mass predicted by the relationship of mass to length for
222 the entire wild population. As such, $K_n < 1$ (or $K_n > 1$) means that body condition is lower (or
223 higher) than the long-term average observed in the wild population

224

225 **2.3 Mitochondrial respiration**

226 Oxygen consumption measurements were performed in red muscle at two levels: in
227 muscle fibres and in isolated mitochondria. At the mitochondrial level, oxygen consumption
228 was expressed in $\text{nmol O}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of protein, and it was expressed in $\text{nmol O}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$ of
229 red muscle at the fibre level. The oxygen consumption was then estimated at the muscle level
230 by multiplying the oxygen consumption obtained at the fibre level by the red muscle mass
231 ($\mu\text{mol O}\cdot\text{min}^{-1}$) or by the relative red muscle mass ($\text{nmol O}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$ of fish) (see Table 2).

232

233 **2.3.a Red muscle fibre preparation and oxidative activity**

234 After dissection, the total mass of red muscle was measured and a ~5 mg sample of
235 fibres was withdrawn (Fig. 1). Fibre respiration was monitored at 20°C using high resolution
236 respirometers (Oxygraph-2K, Oroboros® Instruments – WGT Austria) in air-saturated
237 respiration buffer (Mir05: 110 mM sucrose, 0.5 mM EGTA, 3 mM MgCl_2 , 60 mM K-
238 lactobionate, 20 mM taurine, 10 mM KH_2PO_4 , 1 g/L fatty acid-free bovine serum albumin and
239 20 mM HEPES, pH 7.1) using different combination of respiratory substrates (5 mM
240 glutamate/2.5 mM malate; 40 μM palmitoyl-carnitine/2.5 mM malate; 5 mM pyruvate/2.5 mM
241 malate/5 mM succinate) with a protocol adapted from Teulier *et al.*, 2019. These different

242 combinations of substrates enabled us to assay mitochondrial respiration using substrates
243 derived from amino acid (glutamate), carbohydrate (pyruvate) and lipid (palmitoyl-carnitine)
244 metabolic pathways. In addition, the mixture of NADH- and FADH₂-linked substrates
245 (pyruvate/malate and succinate) allowed a full activation of mitochondrial oxidative activity by
246 providing electrons to complex I and complex II of the electron transport system (ETS).
247 Phosphorylating respiration was then initiated by adding 1mM ADP, and the integrity of
248 mitochondria within muscle fibres was subsequently tested by adding 10µM cytochrome c.
249 Finally, a sequential addition of 1 µM p-trifluoromethoxy-carbonyl-cyanide-phenyl hydrazine
250 (FCCP) was performed to measure maximal respiratory activity of the fibres.

251

252 ***2.3.b Mitochondrial isolation, coupling efficiency, respiratory capacity and content***

253 Mitochondria were isolated from red skeletal muscle in an ice-cold isolation buffer
254 containing (100 mM sucrose, 50 mM KCl, 5 mM EDTA, 50 mM Tris-base, pH 7.4 at 4°C),
255 according to previously described protocol for small tissue samples (Boël *et al.*, 2019),
256 involving potter homogenizer, partial protease digestion, and differential centrifugation with
257 mitochondria pelleted at 9000 × g, all steps at 4°C. Protein content of the preparation was
258 assayed in duplicate at 540nm using the biuret method, with bovine serum albumin as standard
259 (Gornall *et al.*, 1949). Absorbance of the same volume of mitochondria was also assayed at
260 540nm in a solution containing 0.6% Na-K-tartrate and 3% NaOH and subtracted, to correct
261 for any contamination with pigments absorbing at 540nm.

262 Mitochondrial oxidative phosphorylation efficiency was assessed at 20°C by measuring
263 the rates of ATP synthesis and oxygen consumption in respiratory buffer (120 mM KCl, 5 mM
264 KH₂PO₄, 1 mM EGTA, 2 mM MgCl₂, 0.3% fatty acid-free bovine serum albumin (w/v), and
265 3mM HEPES, pH 7.4) supplemented with 20 mM glucose, 1.5 U/mL hexokinase (Colinet,
266 Renault, & Roussel, 2017; Teulier *et al.*, 2010). Mitochondria were energized with a mixture

267 of 5 mM pyruvate, 2.5 mM malate, 5 mM succinate to fully activate the ETS. ADP (500 μ M)
268 was added to initiate ATP synthesis. After recording the phosphorylating respiration rate using
269 a Clark electrode (Rank Brother Ltd, Cambridge, UK), four 100 μ L samples of mitochondrial
270 suspension were withdrawn from the respiratory chamber every 2 min and immediately
271 quenched in 100 μ L of ice-cold perchloric acid solution (10% HClO₄ and 25mM EDTA).
272 Production of ATP was determined from the slope of the linear accumulation of glucose-6-
273 phosphate content over the sampling time interval (6 min) as described previously (Colinet *et*
274 *al.*, 2017; Teulier *et al.*, 2010). To make sure that ATP synthesis rate was specific to
275 mitochondrial ATP synthase activity, we determined oxygen consumption and ATP synthesis
276 rates in the presence of oligomycin (1 μ g/mL) that inhibits mitochondrial ATP synthase. These
277 values were taken into account (*i.e.* subtracted from the raw ATP synthesis) to calculate the rate
278 of mitochondrial ATP synthesis (Teulier *et al.*, 2010). Of note, the basal non-phosphorylating
279 oxygen consumption recorded in the presence of oligomycin is a measure of the maximal rate
280 of proton leak across the inner membrane of mitochondria. Due to the linear relationship
281 between ATP synthesis and oxygen consumption in isolated mitochondria from different tissues
282 and organisms (Beavis & Lehninger, 1986; Fontaine *et al.*, 1996; Nogueira *et al.*, 2002; Roussel
283 *et al.*, 2015; Teulier *et al.*, 2010), we presented these data on a single graph by drawing a linear
284 relationship between the basal non-phosphorylating oxygen consumption activity and the
285 maximal oxidative phosphorylation activity, which is related to maximal rates of oxygen
286 consumption and corresponding ATP synthesis (Fig. 1B).

287 Maximal activity of the electron transport system (*i.e.* the respiratory capacity) was
288 measured at 20°C by adding 2 μ M FCCP in the respiratory buffer supplemented with 5mM
289 pyruvate, 2.5 mM malate, 5 mM succinate, and 1 μ g/mL oligomycin. Mitochondrial content of
290 skeletal muscle was estimated from the ratio between the maximal oxygen consumption rates

291 of muscle fibres (expressed per gram of muscle) and mitochondria (expressed per milligram of
292 protein).

293

294 **2.5. Statistical analyses**

295 Linear mixed-models (LMM) were used to evaluate the effects of food size and quantity
296 on phenotypic traits, red muscle mitochondrial respiration and efficiency. These models all
297 followed a similar structure, with food size and quantity as well as their interaction as fixed
298 effects, while sex was included as a random effect to remove any potential bias associated to
299 differences between males and females. When an interaction between size and quantity of items
300 was significant ($P < 0.05$) or tended to be significant ($P < 0.07$), post-hoc tests comparing all
301 four treatments (*lsmeans*) were performed. When the interaction was not significant, it was
302 removed from the model and the p- values obtained in the simplified model were reported in
303 the results section.

304 The relationship between individual body condition and basal mitochondrial respiration
305 was analysed by piecewise linear regression (Fig. 3). Once the breakpoint was estimated, linear
306 regressions were evaluated over each segment of condition (*i.e.* before and after the breakpoint).

307 Data are presented as means \pm SE. Sample sizes varied among models because some
308 data were missing for some fish, or at the fibre level, data were removed from analyses if
309 addition of cytochrome c caused oxygen consumption to increase by 30% or more (see 2.3.a).
310 Statistical analyses were performed in *R* v. 3.6.1. with the packages *lme4* and *lsmeans*.

311

312 **3. Results**

313 **3.1. Effect of food size and quantity on body condition and growth**

314 For the effect of food size and quantity on body condition, fat content, growth and final
315 mass, the interaction between size and quantity was always significant or close to significance

316 (LMMs: $P = 0.025-0.061$). LI-LQ sardines showed the highest body condition, fat content, body
317 mass, cumulative and relative growth compared to the other three groups (post-hoc lsmeans: P
318 < 0.010), while LI-SQ, SI-LQ and SI-SQ did not differ significantly (Table 1). By contrast,
319 there was no interaction between food size and quantity for effects on red muscle mass, whether
320 in absolute terms ($F = 2.359$; $P = 0.133$, $n = 9$) or relative to body mass ($F = 0.002$; $P = 0.962$,
321 $n = 9$). Still, LI sardines had greater absolute red muscle mass than SI sardines ($F = 15.152$; P
322 < 0.001), as did LQ compared to SQ sardines ($F = 4.551$; $P = 0.039$). Finally, the size of items
323 also had an effect on relative red muscle mass ($F = 16.515$; $P < 0.001$) with LI sardines being
324 higher than SI (Table 1).

325

326 **3.2. Oxidative metabolism at different levels of organisation**

327 The phosphorylating respiration state of skeletal muscle fibres obtained with different
328 substrates is presented in Supplementary Material (Figure S1). Apart from glutamate/malate,
329 there was no effect of food size, quantity or their interaction on the phosphorylating state of
330 fibres (all $P > 0.05$). Regarding glutamate/malate, the interaction between food size and quantity
331 tended to be significant ($F = 3.614$, $P = 0.066$), and muscle phosphorylating respiration was
332 affected by food size ($F = 4.220$, $P = 0.048$, post-hoc tests: SI-LQ $<$ LI-LQ, $P = 0.040$).

333 Table 2 reports the maximal respiration state following addition of FCCP, as measured
334 in isolated mitochondria and muscle fibres, and further estimated in whole red muscle and fish.
335 Maximal respiration of isolated mitochondria was only affected by food size ($F = 9.334$; $P =$
336 0.004), and was significantly higher in LI than in SI sardines (Table 2). Similarly, mitochondrial
337 content was only affected by food size ($F = 4.604$; $P = 0.040$) but in an opposite direction, being
338 significantly lower in LI groups. The interaction between food size and quantity was close to
339 significance ($F = 3.228$; $P = 0.082$), suggesting that the negative effect of food size on
340 mitochondrial content was mostly observed in sardines fed in small quantity. At the fibre level,

341 while the interaction between food size and quantity was significant for the maximal respiration
342 state measured at this level ($F = 6.697$; $P = 0.014$), no differences in fibre respiration were
343 highlighted among groups. Finally, the food treatments had no effect on maximal respiration
344 rates at the red muscle or whole fish level ($P > 0.05$ for size, quantity, and its interaction).

345

346 **3.3. Mitochondrial oxidative phosphorylation efficiency**

347 At the mitochondrial level, there was no interaction between food size and quantity on
348 oxidative phosphorylation activity (*i.e.* the rates of oxygen consumption and associated ATP
349 synthesis). However, activity was higher in sardines fed large food items (oxygen consumption:
350 $F = 9.807$; $P = 0.003$; ATP production: $F = 10.638$; $P = 0.002$), regardless of quantity ($P > 0.05$,
351 Fig. 2A). Basal non-phosphorylating respiration state measured in the presence of oligomycin
352 (*i.e.* the intercept with the x-axis) was also significantly higher in LI sardines ($F = 11.746$; $P =$
353 0.002 , see box in Fig. 2A). There was no significant interaction of size and quantity, and no
354 effect of size or quantity on the slopes of the relationships between rates of ATP synthesis and
355 oxygen consumption (Size*Quantity: $F = 0.014$; $P = 0.906$; Size: $F = 0.784$; $P = 0.382$;
356 Quantity: $F = 2.017$; $P = 0.164$). Thus, these slopes were similar between SI and LI sardines
357 (Slope_{SI}: 3.19 ± 0.15 ; Slope_{LI}: 3.03 ± 0.10). Altogether, these results indicate that the
358 regression line relating ATP synthesis to oxygen consumption was shifted to the right in LI
359 compared to SI groups, meaning that mitochondria from LI sardines had to consume more
360 oxygen to produce the same amount of ATP, due to a higher basal non-phosphorylating
361 respiration or “leak” respiration (Fig. 2A).

362 When mitochondrial function was quantified for the entire red muscle, oxidative
363 phosphorylation activity did not differ among treatments except for LI-LQ sardines, which
364 exhibited higher rates of ATP synthesis and corresponding phosphorylating respiration, and
365 higher basal non-phosphorylating respiration than the other groups ($P < 0.05$, Fig. 2B).

366 However, as the slopes were similar between LI-LQ and the other groups (Slope_{LI-LQ}: $3.17 \pm$
367 0.19 ; Slope_{other groups}: 2.96 ± 0.18 ; $P > 0.05$), red muscles of LI-LQ sardines needed to consume
368 more oxygen to produce the same amount of ATP compared to the sardines from the other three
369 groups (Fig. 2B).

370

371 **3.4. Basal respiration in relation to body condition**

372 The rate of basal non-phosphorylating respiration at the mitochondrial level was
373 significantly related to Le Cren body condition (Fig. 3). The segmented regression analysis
374 showed a significant increase in basal non-phosphorylating respiration rate with body
375 condition, until a condition threshold of 1.07 ($R^2 = 0.45$; $P < 0.001$) beyond which no significant
376 relation was found ($P > 0.05$, Fig. 3).

377

378 **4. Discussion**

379 The results demonstrate clear links between sardine red muscle energetics and foraging
380 modes, indicating effects of both food size and ration. Reducing the size and/or quantity of food
381 led to a series of mitochondrial energy sparing mechanisms (decrease in maximum oxygen
382 consumption and ATP production, enhanced ATP production efficiency and reduced waste of
383 oxygen linked to proton leak), presumably to maintain energy homeostasis. Such energy saving
384 processes suggest that sardines feeding on small food items or small food quantities faced an
385 energetic challenge. These metabolic processes were linked across different levels, from
386 macroscopic integrative traits such as body mass and body condition to intracellular
387 mechanisms such as mitochondrial efficiency. The effects of food ration were stronger at the
388 whole animal level than at the tissue or mitochondrial level, whereas food size also induced
389 marked mitochondrial adjustments beyond the effects at tissue or whole animal scale (Fig. 4).
390 This shows that energy saving processes were not adequate to counteract detrimental effects of

391 foraging on small particles. Indeed, despite an enhanced mitochondrial efficiency to produce
392 ATP, SI sardines still exhibited lower body condition, body mass and growth performance,
393 regardless of the ration provided (Queiros *et al.*, 2019). Our results therefore support the
394 hypothesis that filter-feeding behaviour is more costly than particulate-feeding in
395 Mediterranean sardines (Queiros *et al.*, 2019), unlike in other small pelagic species (Gibson &
396 Ezzi, 1992; Sanderson & Cech, 1992; Van Der Lingen, 1995), and that this induces plastic
397 responses in cellular energetics.

398

399 **4.1. Food size induces subcellular bioenergetic adjustments.**

400 Why would the size of food items affect traits like body condition, body mass or growth
401 performance? Part of the answer may reside in the most striking result of our study, which is
402 that small food items triggered bioenergetic adjustments in sardine muscle mitochondria. The
403 effects of food size overwhelmed any potential effect of quantity. Small food items may have
404 induced an increased utilisation of lipids, as intramuscular lipids were ~50% lower in SI-SQ
405 compared to LI-LQ sardines. Moreover, a reduction in glutamate oxidation is a known protein-
406 sparing mechanism, which suggests that protein catabolism may have been occurring. This,
407 together, may explain why SI sardine exhibited lower red muscle mass both in absolute and
408 relative terms.

409 Feeding on small food items was also associated with decreased oxidative capacity and
410 reduced energy (ATP) outflow, from mitochondria up to whole muscle. This involved a severe
411 loss of mitochondrial power, *i.e.* a ~50% decrease in both maximal rates of ATP synthesis and
412 oxygen consumption. These reduced maximal rates were balanced by a higher coupling
413 efficiency; mitochondria of SI sardines consumed less oxygen than LI sardines to produce a
414 given amount of ATP. The lower maximal respiratory capacity of skeletal muscle and economic
415 management of cellular resources are energy saving mechanisms, decreasing the quantity of

416 substrates required to produce ATP. Similar adjustments have been reported in skeletal muscle
417 of birds at a critical stage of fasting, when endogenous lipid stores are depleted (Bourguignon
418 *et al.*, 2017; Monternier *et al.*, 2017), and also in skeletal muscle of mammals fed on protein-
419 deficient diets (Zangarelli *et al.*, 2006). Overall, these results indicate that mitochondrial
420 efficiency improves when energy balance is severely threatened in the sardine. Consequently,
421 our results indicate that sardines were unable to cope with the high energy expenditure imposed
422 by filter-feeding on small items, even when these were provided at high ration.

423

424 **4.2. Food quantity mainly influenced the whole organism.**

425 It is well established that food shortage triggers adaptive behavioural, physiological and
426 cellular responses to lower costs of living, reducing metabolic rates, spontaneous activity and
427 somatic growth (Metcalf, Van Leeuwen, & Killen, 2016). For example, the standard metabolic
428 rate of juvenile brown trout *Salmo trutta* changes as a function of food availability, decreasing
429 or increasing when fish are fed on a low or high ration, respectively (Auer *et al.*, 2016), which
430 was closely related to their growth rate (Auer *et al.*, 2015). In our study, a 50% reduction in
431 ration negatively impacted body condition (-26%), body mass (-33%) and dramatically slowed
432 growth rate of LI sardines (-64%, similarly to what Queiros *et al.*, 2019 found). While a similar
433 trend was observed for growth rate in SI sardines when comparing between rations (-68%) the
434 difference was not significant, presumably because the energetic challenge of foraging on small
435 items transcended any effect of ration (Table 1).

436 Energy conservation during food shortage is also enacted at a cellular level, by
437 reductions in energy demanding processes and oxidative activity of metabolically active tissues,
438 such as liver and skeletal muscles (McCue, 2010). For instance, the oxidative activity of brown
439 trout muscle is positively correlated with food intake and maximum metabolic rate (Salin *et al.*,
440 2016a; 2016b). In the sardines, bioenergetics of whole red muscle in the LI group was positively

441 correlated with food availability, which is likely due to muscle mass. Because of lower body
442 mass and red muscle mass in food-restricted fish, maximal oxygen consumption and ATP
443 synthesis rates of the muscle are depressed. Down regulation of aerobic metabolic pathways
444 has been reported in the red muscle of food-restricted gilthead sea bream *Sparus aurata*
445 (Bermejo-Nogales *et al.*, 2011).

446 The lowered respiratory capacity of whole muscle in LI-SQ sardines appears mainly to
447 be due to decreased mitochondrial content rather than to changes in mitochondrial activity or
448 skeletal muscle protein content. Changes in mitochondrial content could be caused by
449 dysregulation of autophagy and biogenesis. The former could be induced by caloric restriction
450 (reviewed in Mehrabani *et al.*, 2020) while the latter, which is extremely costly, is probably
451 inhibited to conserve energy. It is also possible that the decrease in mitochondrial content and
452 associated loss of metabolic power in the sardines is linked to decreased use of slow-twitch
453 aerobic red muscle for a particulate capture feeding mode (Hood, 2009; Novak *et al.*, 2005), as
454 prey capture tends to use fast-twitch anaerobic white muscle (Bone, 1978).

455

456 **4.3. Body condition index and skeletal muscle mitochondria.**

457 The energetic constraints associated with the filtering mode led to very low body
458 condition in the sardines. It is fascinating that, below a threshold value of 1.07, declines in body
459 condition index were strongly correlated with declines in basal non-phosphorylating respiration
460 rate of muscle mitochondria. This threshold is surprisingly close to the mean body condition of
461 sardines observed in the wild, around 1 (Brosset *et al.*, 2015b), which may imply that wild
462 populations are also at a threshold of bioenergetic disequilibrium. Basal non-phosphorylating
463 respiration represents maximal proton leakage across the inner mitochondrial membrane.
464 Limiting proton leak lowers costs of basal maintenance (Boutilier & St-Pierre, 2002) but may
465 carry an oxidative risk, such as an increased reactive oxygen species production (Salin *et al.*,

466 2018). A reduction of maintenance costs could also be achieved by decreasing mitochondrial
467 content (Guderley, 2004), as observed in LI-SQ sardines (Fig 2b). That is, a decrease in
468 mitochondrial content seems less harmful than directly reducing basal respiration rate itself, as
469 LI-SQ body condition stayed closer to 1 than the SI groups.

470 Hence, the decline in mitochondrial energy wastage in sardines with poor condition
471 suggests that modifying mitochondrial function provides an economical phenotype when
472 metabolic costs of foraging are high compared to energy acquired.

473

474 **4.4. Conclusions**

475 Within our experimental conditions, we provide clear evidence that, in sardines, feeding
476 on small prey can present a greater energetic challenge than reduced availability of large prey.
477 Feeding sardines a restricted ration of large food items reduced their body condition, growth
478 rate and body mass, revealing an unbalanced energy budget, but did not stimulate plastic
479 metabolic responses within mitochondria. By contrast, feeding small items, whatever the
480 quantity, induced energy-saving adjustments in mitochondria that are typical of a severe
481 energetic challenge (Bourguignon *et al.*, 2017; Costalago & Palomera, 2014). The food size
482 effect seems to be mediated by feeding mode in the sardines, with the switch from prey capture
483 to filtering. Therefore, impacts of prey size on population condition could be profoundly non-
484 linear. Prey size might only have minor influence on condition as long as sardines use a capture
485 feeding mode, but when prey species are small enough to trigger a change to filtering mode,
486 there are dramatic consequences for body condition.

487 While this study focuses on sardines in the north-western Mediterranean Sea, it may be
488 indicative of a widespread phenomenon linked to climate change. That is, if global warming
489 causes systematic reductions in prey size (Daufresne *et al.*, 2009), the extra time and energy
490 required to forage could cause energy challenges at multiple levels of the food web. Coupled

491 with increased metabolic demands of ectotherms in a warmer environment, this could lead to
492 reduced growth rates and be a major contributor to declining final adult sizes in fishes (Gardner
493 *et al.*, 2011).

494

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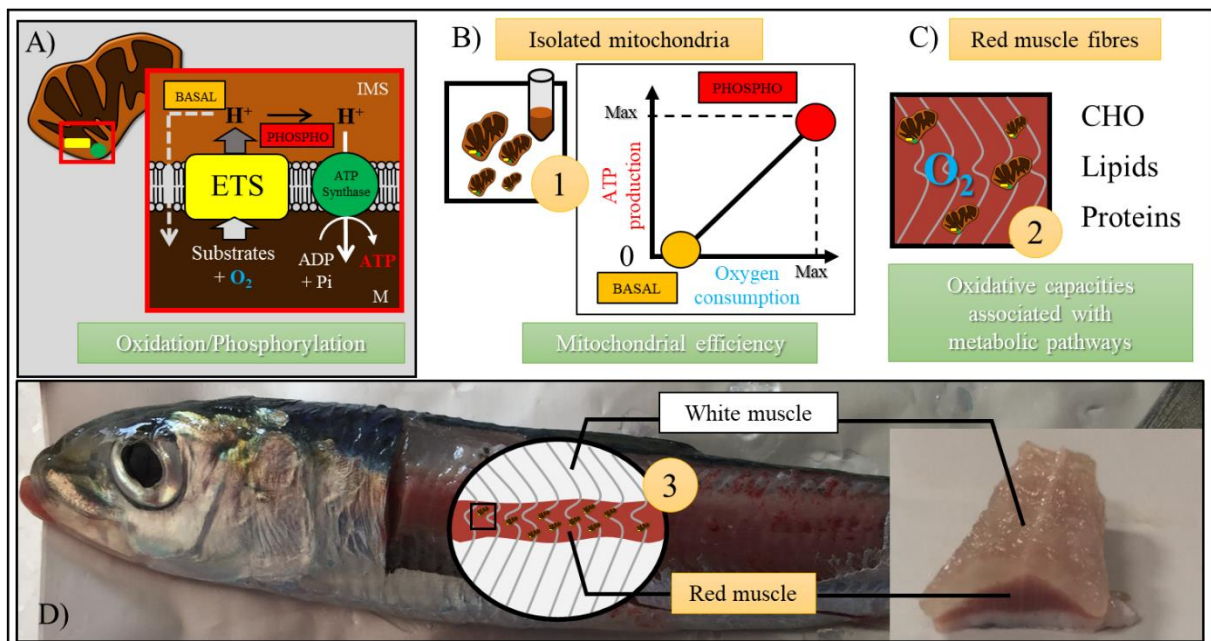
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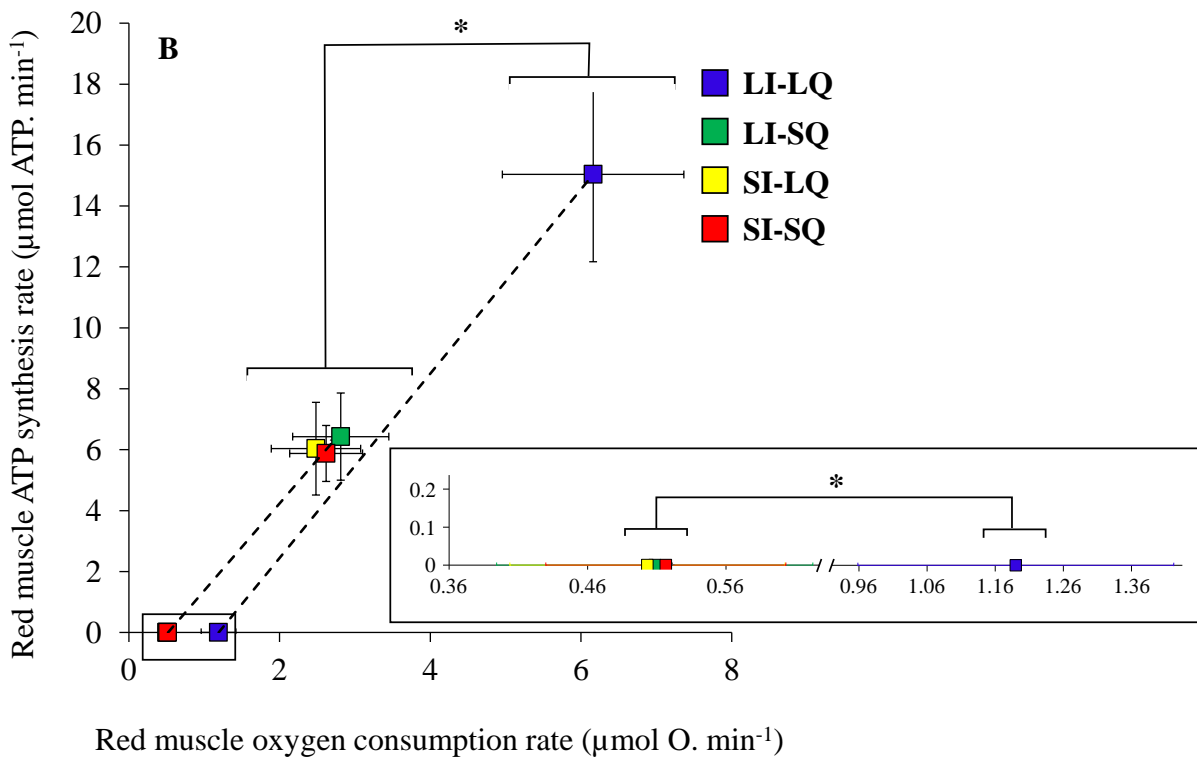
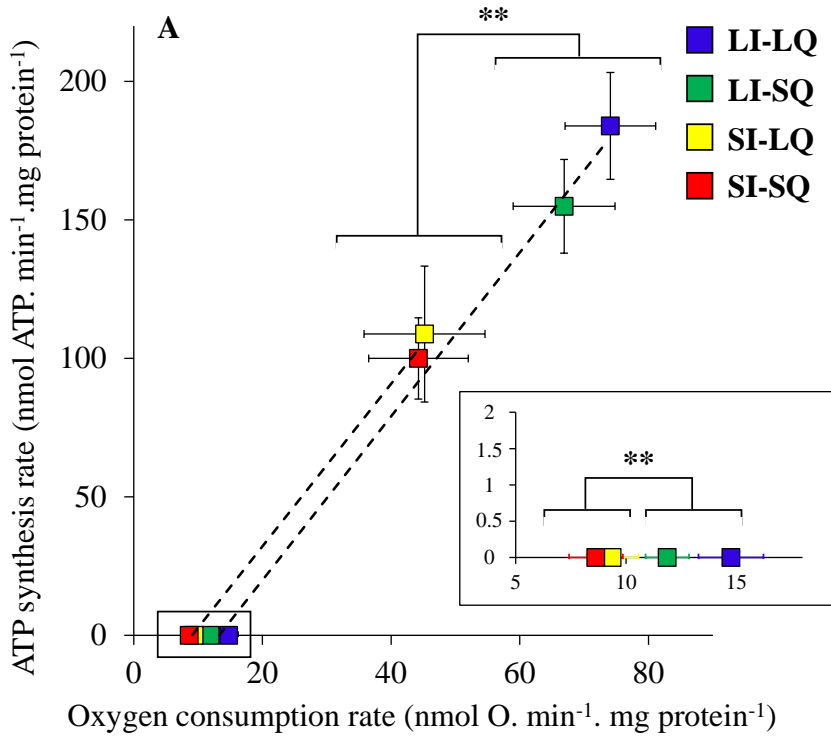
751 **FIGURES**
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753 **Figure 1. Illustration of the integrative levels of the red muscle bioenergetics in sardine,**
 754 **from isolated mitochondria, to the whole tissue.**

755 Substrates are oxidized by the complexes of the electron transport system (ETS) and ATP is
 756 produced through a phosphorylation process by the ATP synthase. The ETS and ATP synthase
 757 are enclosed in the inner membrane of the mitochondria (panel A). Mitochondrial respiration
 758 rates associated to either basal non-phosphorylating state (BASAL) and/or phosphorylating
 759 state (PHOSPHO) were assessed at different levels of integration. 1) Mitochondrial oxygen
 760 consumption was coupled to the amount of ATP produced, to obtain the mitochondrial
 761 efficiency which is the slope of the linear regression between oxygen consumption and ATP
 762 production, called P/O ratio (panel B). 2) The muscle fibre respiration rates were induced by
 763 addition of different substrates to discriminate the metabolic pathways fuelling the red muscle
 764 (panel C). 3) The whole red muscle oxidation phosphorylation activity was calculated to
 765 estimate the relative importance of the whole muscle bioenergetics. The red muscle is
 766 positioned along the lateral line and is clearly separated from the white muscle (panel D). ETS:
 767 Electron transport system; ATP: Adenosine triphosphate; ADP: adenosine diphosphate; Pi:
 768 Phosphate inorganic; IMS: intermembrane space of the mitochondrion; M: matrix of the
 769 mitochondrion. See the “Material and Methods” section for further details.



770 **Figure 2. Mitochondrial efficiency of sardine that received different quantities and sizes**
771 **of food items, at the mitochondrial level** (Panel A; LI-LQ: large food items in large quantities,
772 n = 11; LI-SQ: large food items in small quantities, n = 12; SI-LQ: small food items in large
773 quantities, n = 11; SI-SQ: small food items in small quantities, n = 9) **and red muscle level**
774 (Panel B, LI-LQ: n = 11, LI-SQ: n = 11; SI-LQ: n = 10; SI-SQ: n = 9). In panel A, the two trend
775 curves drawn are those of the “large food items” (LI: $y = 3.03x - 44.40$) and “small food items
776 (SI: $y = 3.19x - 37.96$) groups, and in panel B, the trend curves are those of LI-LQ group ($y =$
777 $3.17x - 4.52$) and of the other groups together ($y = 2.97x - 1.77$). These data were analysed
778 using a linear-mixed effects model with size and quantity of items as fixed-effects and sex as
779 random effect. The values shown are means \pm s.e.m. (the error bars of the basal oxygen
780 consumption are small enough that they are hidden by the points, see zoom boxes). An asterisk
781 represents a significant difference in maximal oxygen consumption, ATP production and
782 basal oxygen consumption (see zoom boxes) between diets composed of different size food
783 items (Panel A, difference between LI and SI) and between LI-LQ sardines and other groups
784 (Panel B; $*P < 0.05$; $**P < 0.01$).
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787 **Figure 3. Basal oxygen consumption at the mitochondrial level as a function of sardine body condition index.** Fish of each treatment are
788 represented by a coloured circle. Sardines fed with large food items are represented in blue for large quantity (LI-LQ, n = 11) and in green for small
789 quantity (LI-SQ, n = 12). Yellow and red circles represent sardines fed with small food items, in large (SI-LQ, n = 10) and small (SI-SQ, n = 11)
790 quantities, respectively. Basal mitochondrial respiration in $\text{nmol O} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ is positively correlated to the body condition index when the
791 BCI is less than 1.07 ($P < 0.001$). Above this threshold, there is no longer a regression between these two parameters.

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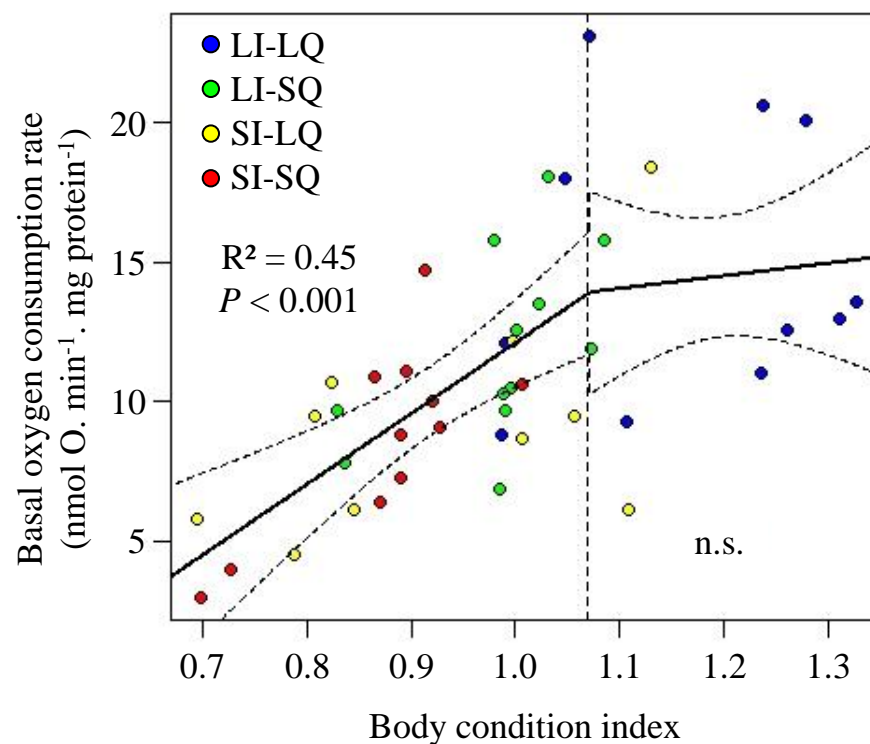
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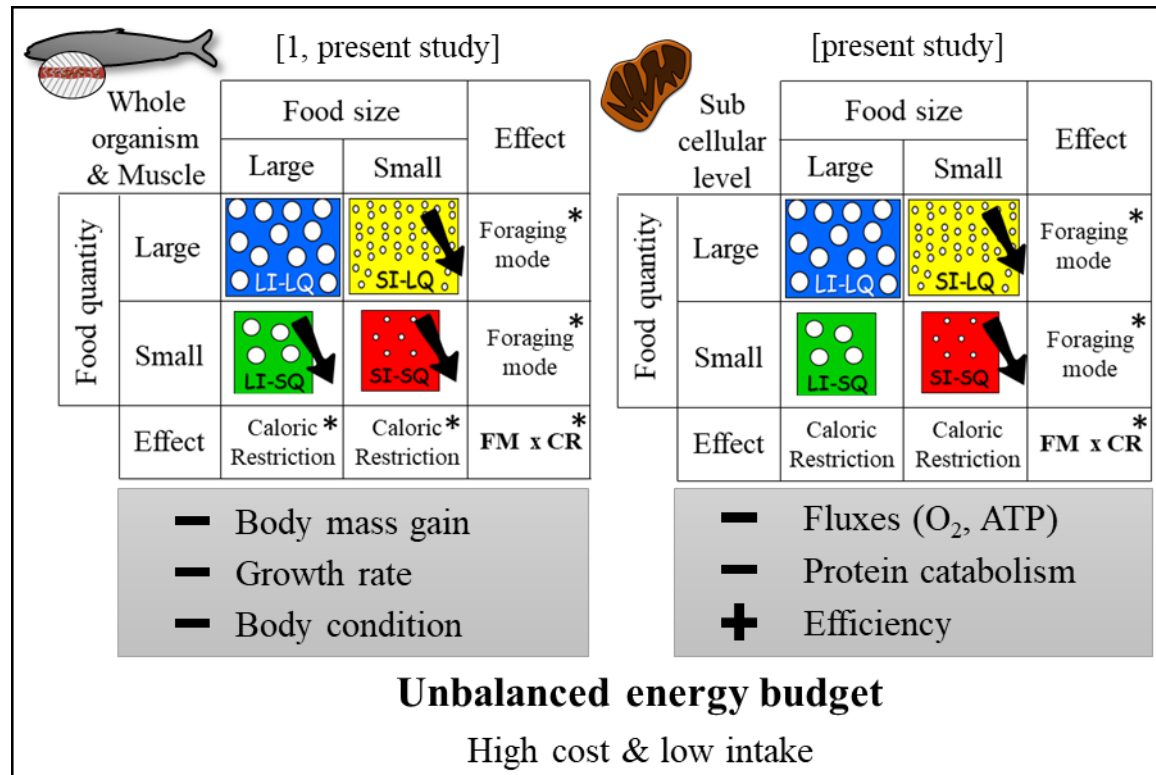
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809 **Figure 4. Whole-organism, muscle, and subcellular consequences of the quantity and/or size of food.** In order to investigate the effect of size
 810 and/or quantity of food, a fully factorial protocol has been set up. Size and quantity impacted the whole-organism and muscle parameters, whereas
 811 subcellular bioenergetics have been modified only by size of food items. Significant effects are illustrated by an asterisk. As foraging mode depends
 812 on food size (Costalago *et al.*, 2015; Garrido *et al.*, 2007), the effect described by size is called “foraging mode” (FM). Lowering the quantity of
 813 food by 50% induces a severe “caloric restriction” (CR). [1] Queiros *et al.*, 2019



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818 **Table 1. Characteristics of sardines used to measure the respiration of red muscle fibres.** These data correspond to the measurements made
819 on the day sardines were euthanised. Values are mean \pm s.e.m. N corresponds to the number of individuals randomly split into 4 treatments, sorted
820 in three sex categories (male, female and indeterminate). Cumulative growth represents the total length gain in mm, over the entire experiment.
821 RM/BM ratio corresponds to the relative red muscle mass, which is the proportion of the red muscle mass over the body mass. Data were analysed
822 with a linear mixed-effect model with size and quantity as fixed-effects and sex as random effect. Data with different superscripts are significantly
823 different at $P < 0.05$. The boxes with the asterisks indicated a global effect of item size (** $P < 0.001$).

	LI-LQ	LI-SQ	SI-LQ	SI-SQ	Statistical analysis		
N	8-11	10-12	9-11	10-12			
(M/F/indeterminate)	(6/3/2)	(5/6/1)	(3/5/2)	(3/8/1)			
Final BCI	1.17 ± 0.04 ^a	0.98 ± 0.02 ^b	0.93 ± 0.05 ^b	0.87 ± 0.02 ^b	Size: P < 0.001	824	
					Quantity: P = 0.001	825	
					Size*Quantity: P = 0.061	826	
Fat content (%)	13.46 ± 1.40 ^a	8.86 ± 0.76 ^b	8.12 ± 0.81 ^b	6.93 ± 0.37 ^b	Size: P < 0.001	827	
					Quantity: P = 0.002	828	
					Size*Quantity: P = 0.055	829	
Cumulative growth (mm)	14.29 ± 1.68 ^a	5.05 ± 1.07 ^b	5.27 ± 1.42 ^b	1.64 ± 0.46 ^b	Size: P < 0.001	830	
					Quantity: P < 0.001	831	
					Size*Quantity: P = 0.025	832	
Relative growth (%)	12.55 ± 1.56 ^a	4.35 ± 0.91 ^b	4.60 ± 1.29 ^b	1.34 ± 0.36 ^b	Size: P < 0.001	833	
					Quantity: P < 0.001	834	
					Size*Quantity: P = 0.029	835	
Body mass (g)	19.23 ± 1.38 ^a	13.98 ± 0.62 ^b	14.22 ± 1.19 ^b	12.81 ± 0.73 ^b	Size: P = 0.005	836	
					Quantity: P = 0.001	837	
					Size*Quantity: P = 0.058	838	
Red muscle mass (g)	1.59 ± 0.17 ^a	1.18 ± 0.06 ^{a,b}	0.98 ± 0.13 ^b	0.91 ± 0.07 ^b	Size: P < 0.001	839	
					Quantity: P = 0.039	840	
RM/BM (%)	8.11 ± 0.39	8.46 ± 0.20	**	6.70 ± 0.45	7.09 ± 0.30	Size: P < 0.001	841

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847 **Table 2. Maximal respiration ETS activity and mitochondrial content of sardine red muscle for each feeding treatment. The maximal**
848 **respiration rate of ETS induced by FCCP is represented at three different levels of organisation: at the subcellular level (nmol O.min⁻¹.mg**
849 **proteins⁻¹) obtained through respiration measurements of isolated mitochondria; at the red muscle fibre level (pmol O₂.s⁻¹.mg muscle⁻¹) obtained**
850 **through respiration measurements of a red muscle sample, and at the red muscle level estimated by multiplying maximal respiration rate at the**
851 **fibre level by red muscle mass or relative red muscle mass (total: pmol O₂.s⁻¹; specific: pmol O₂.s⁻¹.g fish⁻¹). Values are mean ± s.e.m. The**
852 **mitochondrial content of red muscle is expressed in milligram of mitochondrial proteins per gram of red muscle and was estimated as the ratio**
853 **between respiration rates at the fibre level and mitochondrial level. Data were analysed with a linear mixed-effects model with size and quantity**
854 **as fixed-effects and sex as random effect. Different letters indicate a significant difference among groups at P < 0.05. The boxes with the asterisks**
855 **on the measurements indicates a global effect of item size (*P < 0.05; **P < 0.01).**
856

	LI-LQ	LI-SQ	SI-LQ	SI-SQ	Statistical analysis
N	9-11	11-12	9-11	9-12	
Maximal respiration ETS activity					
Mitochondrial level (nmol O.min ⁻¹ .mg protein ⁻¹)	90 ± 9	96 ± 7	59 ± 12	65 ± 12	Size: P = 0.004
Red muscle fibre level (nmol O.min ⁻¹ .mg muscle ⁻¹)	5 ± 0.8 ^a	3.5 ± 0.4 ^a	3.3 ± 0.4 ^a	5.3 ± 0.9 ^a	Size*Quantity: P = 0.014
Red muscle level					
Total (µmol O.min ⁻¹)	6.3 ± 0.9 ^a	4.4 ± 0.7 ^a	3.6 ± 0.7 ^a	4.1 ± 0.8 ^a	n.s.
Specific (nmol O.min ⁻¹ .g fish ⁻¹)	350 ± 44 ^a	306 ± 38 ^a	245 ± 34 ^a	307 ± 58 ^a	n.s.
Mitochondrial content (mg protein. g muscle ⁻¹) ^x	50.9 ± 7.7	34.8 ± 5.4	52.6 ± 8.2	62.2 ± 7.6	Size: P = 0.040

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