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Abstract

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

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

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

Résumé

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

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

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

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

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

1. Introduction

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

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

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

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

Foraging behaviour in fishes is sustained by skeletal muscle activity, with the muscles being structurally separated into discrete slow-twitch oxidative 'red' muscle, used for steady aerobic swimming, and fast-twitch glycolytic 'white' muscle, used for short bursts of high-speed swimming (Bone, 1978; Webb, 1998). Muscular contraction is powered by ATP but, its production depends on muscle type: while red muscle generates this aerobically by mitochondrial oxidative phosphorylation using substrates and oxygen delivered in the 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; Thoral *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

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

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

2. Material and methods

2.1 Experimental conditions

All details of the experiment can be found in Queiros *et al.*, 2019. Briefly, wild sardines were captured off Sète (South of France) in October 2016 and brought back to the Ifremer Palavas-les-Flots research station. The mean age of sardines was 1.24 years. After acclimation and weaning onto commercial pellets, sardines were weighed, measured and marked individually using RFID tags (Biolog-id, Bernay, France). Thereafter, they were distributed among 8 tanks (vol. 300L, 56-57 sardines per tank) with similar mean (± SD) of mass and length in each (mass: 14.1 ± 3.2g; length: 11.9 ± 0.7 cm). A 7-month feeding experiment was performed, from 14/11/2016 to 15/6/2017, with natural photoperiod and water temperature (water was pumped directly from the sea). Animals were fed four times a day with commercial 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 $$Relative\ growth = \frac{Final\ size - Initial\ size}{Initial\ size}$$
216 Where initial and final sizes are expressed in mm.

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

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

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

2.3 Mitochondrial respiration

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

2.3.a Red muscle fibre preparation and oxidative activity

After dissection, the total mass of red muscle was measured and a ~5 mg sample of fibres was withdrawn (Fig. 1). Fibre respiration was monitored at 20°C using high resolution respirometers (Oxygraph-2K, Oroboros® Instruments – WGT Austria) in air-saturated respiration buffer (Mir05: 110 mM sucrose, 0.5 mM EGTA, 3 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH2PO4, 1 g/L fatty acid-free bovine serum albumin and 20 mM Hepes, pH 7.1) using different combination of respiratory substrates (5 mM glutamate/2.5 mM malate; 40 µM palmitoyl-carnitine/2.5 mM malate; 5 mM pyruvate/2.5 mM malate/5 mM succinate) with a protocol adapted from Teulier *et al.,* 2019. These different

combinations of substrates enabled us to assay mitochondrial respiration using substrates derived from amino acid (glutamate), carbohydrate (pyruvate) and lipid (palmitoyl-carnitine) metabolic pathways. In addition, the mixture of NADH- and $FADH_2$-linked substrates (pyruvate/malate and succinate) allowed a full activation of mitochondrial oxidative activity by providing electrons to complex I and complex II of the electron transport system (ETS). Phosphorylating respiration was then initiated by adding 1mM ADP, and the integrity of mitochondria within muscle fibres was subsequently tested by adding 10µM cytochrome c. Finally, a sequential addition of 1 µM p-trifluoromethoxy-carbonyl-cyanide-phenyl hydrazine (FCCP) was performed to measure maximal respiratory activity of the fibres.

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

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

Mitochondrial oxidative phosphorylation efficiency was assessed at 20°C by measuring the rates of ATP synthesis and oxygen consumption in respiratory buffer (120 mM KCl, 5 mM KH_2PO_4, 1 mM EGTA, 2 mM $MgCl_2$, 0.3% fatty acid-free bovine serum albumin (w/v), and 3mM HEPES, pH 7.4) supplemented with 20 mM glucose, 1.5 U/mL hexokinase (Colinet, Renault, & Roussel, 2017; Teulier *et al.*, 2010). Mitochondria were energized with a mixture

of 5 mM pyruvate, 2.5 mM malate, 5 mM succinate to fully activate the ETS. ADP (500 µM) was added to initiate ATP synthesis. After recording the phosphorylating respiration rate using a Clark electrode (Rank Brother Ltd, Cambridge, UK), four 100 µL samples of mitochondrial suspension were withdrawn from the respiratory chamber every 2 min and immediately quenched in 100µL of ice-cold perchloric acid solution (10% $HCLO_4$ and 25mM EDTA). Production of ATP was determined from the slope of the linear accumulation of glucose-6-phosphate content over the sampling time interval (6 min) as described previously (Colinet *et al.*, 2017; Teulier *et al.*, 2010). To make sure that ATP synthesis rate was specific to mitochondrial ATP synthase activity, we determined oxygen consumption and ATP synthesis rates in the presence of oligomycin (1µg/mL) that inhibits mitochondrial ATP synthase. These values were taken into account (*i.e.* subtracted from the raw ATP synthesis) to calculate the rate of mitochondrial ATP synthesis (Teulier *et al.*, 2010). Of note, the basal non-phosphorylating oxygen consumption recorded in the presence of oligomycin is a measure of the maximal rate of proton leak across the inner membrane of mitochondria. Due to the linear relationship between ATP synthesis and oxygen consumption in isolated mitochondria from different tissues and organisms (Beavis & Lehninger, 1986; Fontaine *et al.*, 1996; Nogueira *et al.*, 2002; Roussel *et al.*, 2015; Teulier *et al.*, 2010), we presented these data on a single graph by drawing a linear relationship between the basal non-phosphorylating oxygen consumption activity and the maximal oxidative phosphorylation activity, which is related to maximal rates of oxygen consumption and corresponding ATP synthesis (Fig. 1B).

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

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

2.5. Statistical analyses

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

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

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

3. Results

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

For the effect of food size and quantity on body condition, fat content, growth and final 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,

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

3.3. Mitochondrial oxidative phosphorylation efficiency

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

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

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

3.4. Basal respiration in relation to body condition

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

4. Discussion

The results demonstrate clear links between sardine red muscle energetics and foraging modes, indicating effects of both food size and ration. Reducing the size and/or quantity of food led to a series of mitochondrial energy sparing mechanisms (decrease in maximum oxygen consumption and ATP production, enhanced ATP production efficiency and reduced waste of oxygen linked to proton leak), presumably to maintain energy homeostasis. Such energy saving processes suggest that sardines feeding on small food items or small food quantities faced an energetic challenge. These metabolic processes were linked across different levels, from macroscopic integrative traits such as body mass and body condition to intracellular mechanisms such as mitochondrial efficiency. The effects of food ration were stronger at the whole animal level than at the tissue or mitochondrial level, whereas food size also induced marked mitochondrial adjustments beyond the effects at tissue or whole animal scale (Fig. 4). 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

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

4.2. Food quantity mainly influenced the whole organism.

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

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

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

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

4.3. Body condition index and skeletal muscle mitochondria.

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

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

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

4.4. Conclusions

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

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

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

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FIGURES

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

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

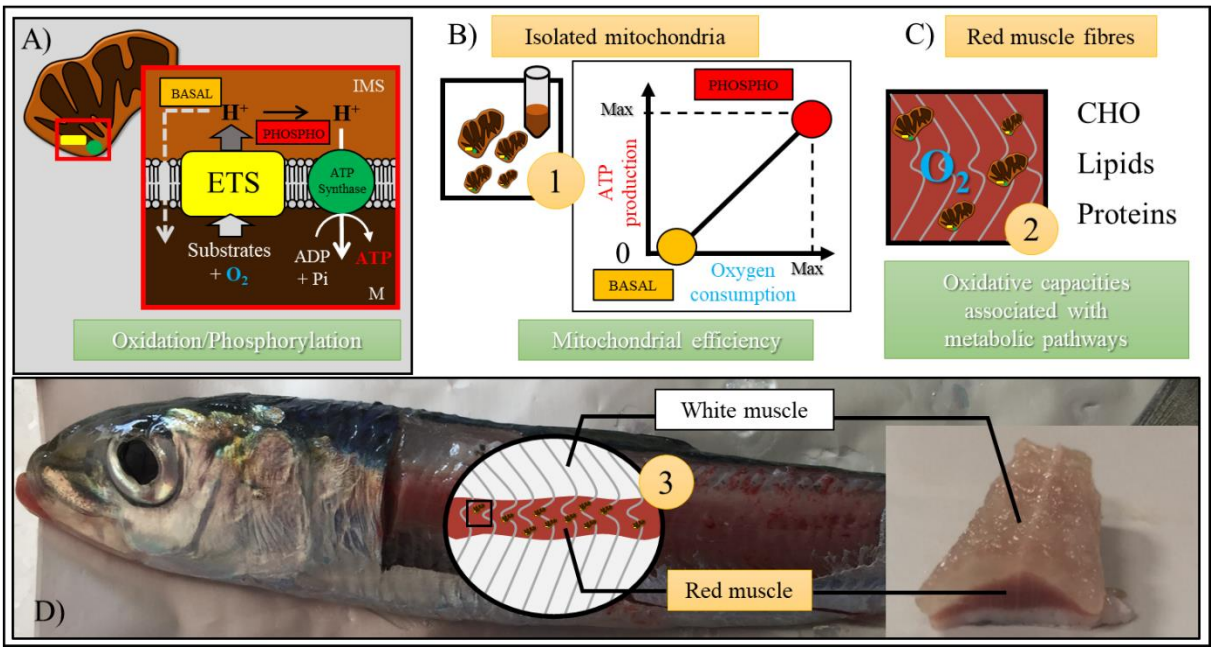

Figure 2. Mitochondrial efficiency of sardine that received different quantities and sizes of food items, at the mitochondrial level (Panel A; LI-LQ: large food items in large quantities, n = 11; LI-SQ: large food items in small quantities, n = 12; SI-LQ: small food items in large quantities, n = 11; SI-SQ: small food items in small quantities, n = 9) **and red muscle level** (Panel B, LI-LQ: n = 11, LI-SQ: n = 11; SI-LQ: n = 10; SI-SQ: n = 9). In panel A, the two trend curves drawn are those of the "large food items" (LI: $y = 3.03x - 44.40$) and "small food items (SI: $y = 3.19x - 37.96$) groups, and in panel B, the trend curves are those of LI-LQ group ($y = 3.17x - 4.52$) and of the other groups together ($y = 2.97x - 1.77$). These data were analysed using a linear-mixed effects model with size and quantity of items as fixed-effects and sex as random effect. The values shown are means ± s.e.m. (the error bars of the basal oxygen consumption are small enough that they are hidden by the points, see zoom boxes). An asterisk represents a significantly difference in maximal oxygen consumption, ATP production and basal oxygen consumption (see zoom boxes) between diets composed of different size food items (Panel A, difference between LI and SI) and between LI-LQ sardines and other groups (Panel B; $*P < 0.05$; $**P < 0.01$).

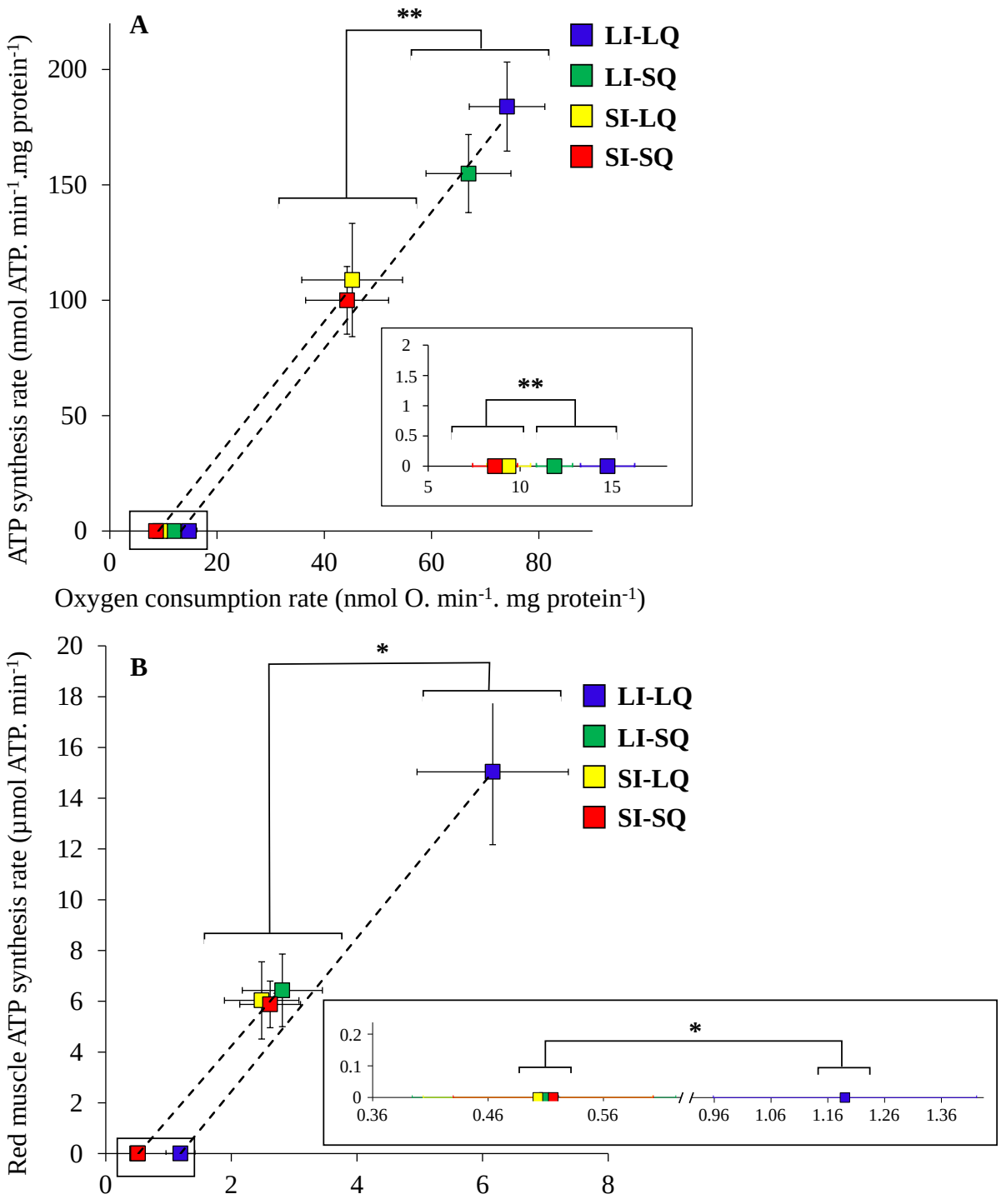
A
**
LI-LQ
LI-SQ
SI-LQ
SI-SQ
ATP synthesis rate (nmol ATP. min-1.mg protein-1)
Oxygen consumption rate (nmol O. min-1. mg protein-1)
B
*
Red muscle ATP synthesis rate (µmol ATP. min-1)
Red muscle oxygen consumption rate (µmol O. min-1)

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Figure 3. Basal oxygen consumption at the mitochondrial level as a function of sardine body condition index. Fish of each treatment are 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 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) quantities, respectively. Basal mitochondrial respiration in nmol $O.min^{-1}.mg\ protein^{-1}$ is positively correlated to the body condition index when the BCI is less than 1.07 ($P < 0.001$). Above this threshold, there is no longer a regression between these two parameters.

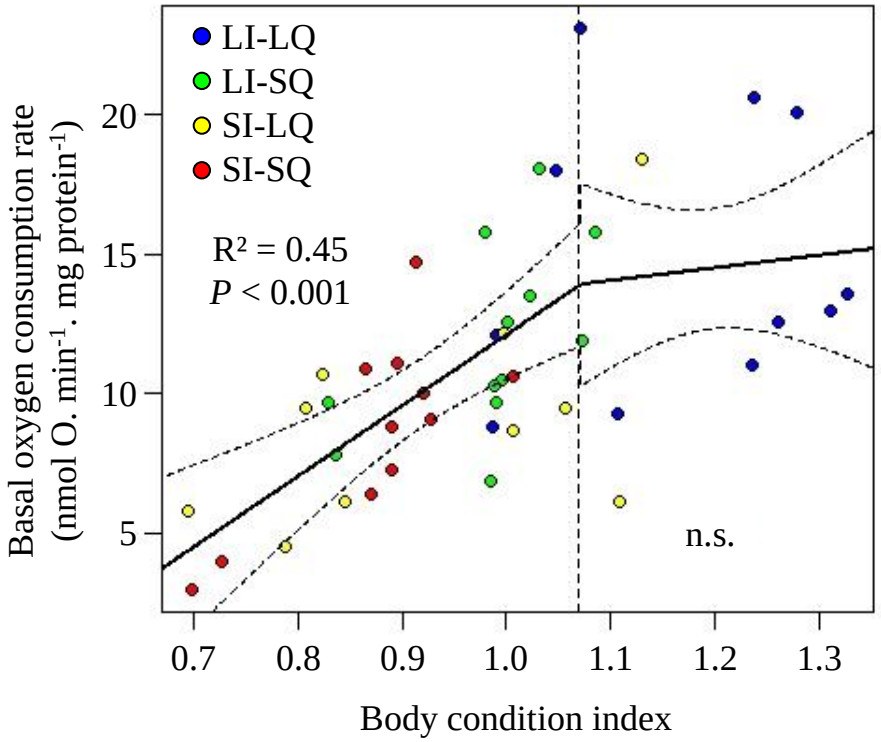

Figure 4. Whole-organism, muscle, and subcellular consequences of the quantity and/or size of food. In order to investigate the effect of size and/or quantity of food, a fully factorial protocol has been set up. Size and quantity impacted the whole-organism and muscle parameters, whereas subcellular bioenergetics have been modified only by size of food items. Significant effects are illustrated by an asterisk. As foraging mode depends 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 food by 50% induces a severe "caloric restriction" (CR). [1] Queiros *et al.*, 2019

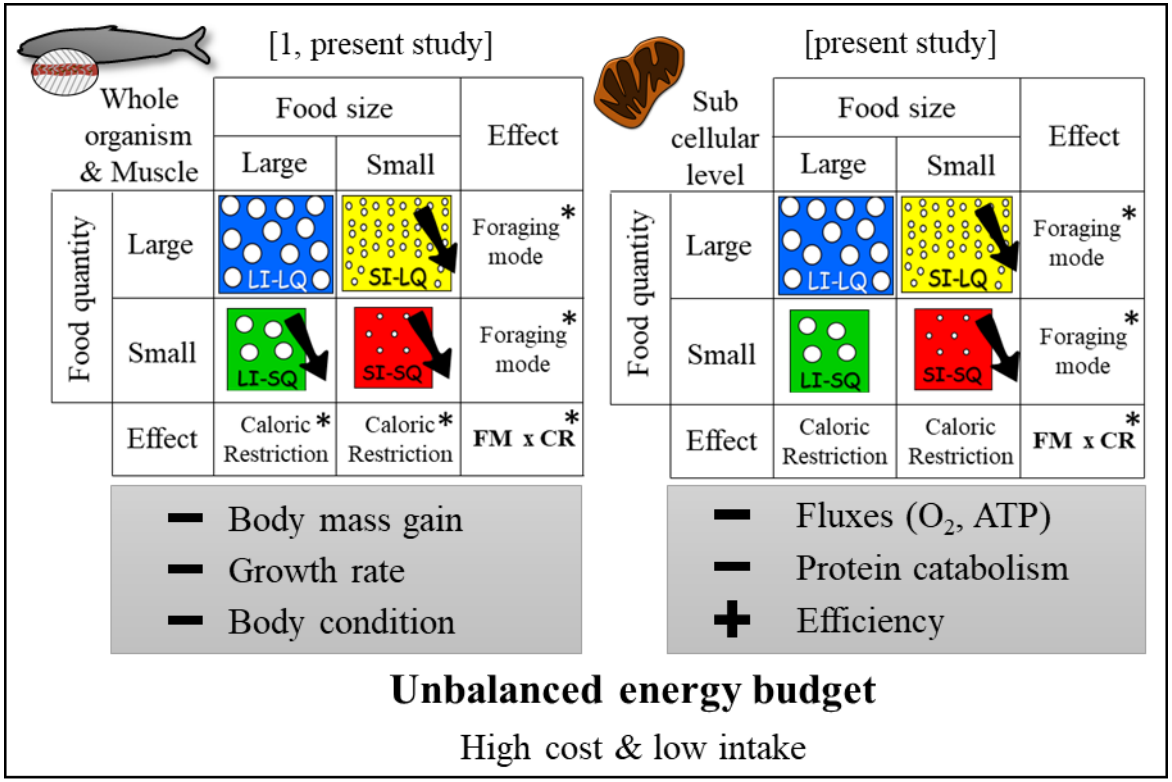

Table 1. Characteristics of sardines used to measure the respiration of red muscle fibres. These data correspond to the measurements made on the day sardines were euthanised. Values are mean ± s.e.m. N corresponds to the number of individuals randomly split into 4 treatments, sorted in three sex categories (male, female and indeterminate). Cumulative growth represents the total length gain in mm, over the entire experiment. 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 with a linear mixed-effect model with size and quantity as fixed-effects and sex as random effect. Data with different superscripts are significantly 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** **Quantity: *P* = 0.001** Size*Quantity: *P* = 0.061
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** **Quantity: *P* = 0.002** Size*Quantity: *P* = 0.055
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** **Quantity: *P* < 0.001** **Size*Quantity: *P* = 0.025**
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** **Quantity: P < 0.001** **Size*Quantity: P = 0.029**
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** **Quantity: *P* = 0.001** Size*Quantity: *P* = 0.058
Red muscle mass (g)	1.59 ± 0.17^{a}	$1.18 \pm 0.06^{a,b}$	0.98 ± 0.13^{b}	0.91 ± 0.07^{b}	**Size: *P* < 0.001** **Quantity: *P* = 0.039**
RM/BM (%)	8.11 ± 0.39	8.46 ± 0.20	** 6.70 ± 0.45	7.09 ± 0.30	**Size: *P* < 0.001**

Table 2. Maximal respiration ETS activity and mitochondrial content of sardine red muscle for each feeding treatment. The maximal respiration rate of ETS induced by FCCP is represented at three different levels of organisation: at the subcellular level (nmol $O.min^{-1}.mg\ proteins^{-1}$) obtained through respiration measurements of isolated mitochondria; at the red muscle fibre level (pmol $O_2.s^{-1}.mg\ muscle^{-1}$) obtained through respiration measurements of a red muscle sample, and at the red muscle level estimated by multiplying maximal respiration rate at the fibre level by red muscle mass or relative red muscle mass (total: pmol $O_2.s^{-1}$; specific: pmol $O_2.s^{-1}.g\ fish^{-1}$). Values are mean ± s.e.m. The mitochondrial content of red muscle is expressed in milligram of mitochondrial proteins per gram of red muscle and was estimated as the ratio between respiration rates at the fibre level and mitochondrial level. Data were analysed with a linear mixed-effects model with size and quantity 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 on the measurements indicates a global effect of item size (*$P < 0.05$; **$P < 0.01$).

		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.*
	Red muscle level — 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: $P = 0.040$