**Warm acclimation increases mitochondrial efficiency in fish:
a compensatory mechanism to alleviate the need for oxygen**

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Relevant information will appear here if provided.

Ethics

Does your article include research that required ethical approval or permits?:

Yes

Statement (if applicable):

All fish manipulations were performed according to the Animal Care Committee of France under authorization n°17-300-2.

Data

It is a condition of publication that data, code and materials supporting your paper are made publicly available. Does your paper present new data?:

Yes

Statement (if applicable):

At this stage, the data have been uploaded as supplementary files.
The data will be deposit in DRYAD if the paper is accepted for publication.

Conflict of interest

I/We declare we have no competing interests

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**Warm acclimation increases mitochondrial efficiency in fish: a compensatory
mechanism to alleviate the need for oxygen**

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24 **Abstract:**

25 In ectotherms, it is well described that thermal acclimation induces compensatory adjustments
26 maintaining mitochondrial functions across large shifts in temperature. However, till now,
27 studies mostly focused on fluxes of oxygen without knowing whether mitochondrial efficiency
28 to produce ATP (ATP/O ratio) is also dependent on temperature acclimation. We thus measured
29 thermal reaction norms of mitochondrial oxidative phosphorylation activity and efficiency in
30 skeletal muscle of sea bass *Dicentrarchus labrax* juveniles acclimated at optimal (22°C), low
31 (18°C) and high (26°C) temperatures. The mitochondrial fluxes (oxygen consumption and ATP
32 synthesis) increased with increasing assay temperatures, and were on the whole higher in 18°C
33 acclimated fishes than in the two other groups. However, these mitochondrial rates were not
34 significantly different between experimental groups, when they were compared at the
35 acclimation temperature. In contrast, we show that acclimation to high, and not low,
36 temperature improved mitochondrial efficiency (on average +15%). This higher efficiency in
37 high temperature acclimated fishes is also detected when acclimation and test temperatures are
38 identical and compared. This mitochondrial phenotype would favor an economical management
39 of oxygen in response to harsh energetic constraints associated with warming water, i.e. an
40 increased metabolic rate associated with a decreased dissolved oxygen level.

41

1. Introduction

Living systems are highly dissipative, consuming energy to carry out different vital functions mainly fulfilled by the cellular breakdown of adenosine triphosphate (ATP). In aerobic organisms, mitochondria are the major ATP cell generator, oxidizing reduced coenzymes obtained from the breakdown of food molecules with oxygen to synthesize ATP through the oxidative phosphorylation processes. As such, mitochondria constitute a strong physiological link between energy resources in the environment of an animal and its performances [1,2]. Because every biochemical process is temperature dependent, the thermal impact on mitochondrial functioning has long been explored in ectotherms providing evidence of a time-dependent response to temperature [3,4].

An acute temperature variation results in membrane destabilization and a compensatory increase in respiratory activity to counteract increased proton leakage across the mitochondrial membrane, in order to maintain the driving force (proton motive force) and the ATP synthesis [5-7]. In addition, most of the studies have also reported in many different ectothermic species, such as bivalves, lugworms, crabs, insects, toads and fishes, that an acute elevation of temperature triggers oxidative and energy costs of mitochondrial functioning [6,7,8-15]. The oxidative cost is associated with an increased mitochondrial reactive oxygen species production, and the energy cost is associated with a decreased mitochondrial coupling efficiency due to an increased proton leakage. All together the heat stress would force ectotherms to allocate extra oxygen and energy resources to maintain ATP production. In contrast, a thermal acclimation can profoundly modify mitochondrial properties [16], like mitochondrial oxidative and enzyme activities [3,6,17-19], inner mitochondrial membrane composition [19-22], and mitochondrial volume density [23]. The functional results of such compensatory adjustments during acclimation lead to a “mitochondrial homeostasis”, that is a profound ability to maintain constant mitochondrial oxidative rates and enzyme activities despite pronounced body temperature variations [1,11,24]. However, the impact of thermal acclimation on the energy cost of mitochondrial functioning remains puzzling. Few studies have reported that the ADP/O ratio, an oximetry measure of mitochondrial efficiency, could be either changed [25,26] or not by thermal acclimation [27,28]. All these studies aiming at linking variations of environmental temperatures to animal performances have been based on fluxes of oxygen as proxy of energy output without knowing, whether the mitochondrial ATP synthesis rate and the efficiency of mitochondrial energy transduction system (i.e. ATP/O ratio), vary with acclimation.

The aim of the present study was to provide a more accurate estimate of ATP/O ratio and its sensitivity to thermal acclimation and acute thermal challenge. We thus measured oxygen consumption, ATP synthesis and mitochondria efficiency in skeletal muscle mitochondria at three assay temperatures (18, 22 and 26°C) in sea bass *Dicentrarchus labrax* juveniles that were acclimated during 2 months at 18, 22, 26°C.

2. Materials and Methods

(a) Fish acclimation and Ethics statement

The juvenile European sea bass, initially produced in a farm (Graveline hatchery, Pas-de-Calais), were supplied (initial fish weight: 78.7 ± 3.5 g) by the CEDRE (Brest, France), and acclimated at the LIENSs facilities (La Rochelle, France) in January 2018. Upon arrival, fishes were transferred into six tanks of 600 L ($n = 14$ fish per tank, $T^{\circ}\text{C} = 12.6 \pm 0.6^{\circ}\text{C}$ and salinity = 35.3 ± 0.3). Each tank was individually equipped with an external biological filter (Eheim professional 3 2080, Eheim, Deizisau, Germany) and supplied with aerated natural seawater. After 6 weeks of acclimation to these laboratory conditions, fish were progressively acclimated (1°C changing every 3 days) to the one of the three experimental temperature (18, 22, 26°C, $n = 2$ tanks per acclimation temperature tested). These temperatures were chosen in regards to the temperature considered as optimal in term of aerobic scope for this species, when located in North Atlantic, which is 22°C [29-31]. Rearing temperature ($18.3 \pm 0.2^{\circ}\text{C}$; $22.0 \pm 0.3^{\circ}\text{C}$; $26.0 \pm 0.2^{\circ}\text{C}$) were kept constant (TECO TR20, Conselice, Italy), as well as salinity (33.7 ± 0.3 ; 34.1 ± 0.4 ; 34.3 ± 1.0 respectively); both were monitored daily using a conductimeter (WTW model 3110 Weilheim, Germany). Oxygen content was never below 90 % air saturation. Fishes were maintained in their respective thermal condition during 2 months with a 12L:12D photoperiod cycle. Fishes were fed with commercial pellets (Le Gouessant aquaculture, Lamballe, France) once a day, three times a week, during the whole experimental period. Then, fishes were randomly caught and sacrificed by long lasting anesthesia (tricaine methane sulphonate MS-222; Sigma-Aldrich, St Quentin-Fallavier, France). All fish manipulations were performed according to the Animal Care Committee of France under authorization n°17-300-2.

(b) Mitochondrial isolation

Mitochondria were isolated from whole skeletal muscle of one flank in an ice-cold isolation buffer containing 100mM sucrose, 50mM KCl, 5mM EDTA, 50mM Tris-base, pH 7.4 at 4°C.

Briefly, skeletal muscles were homogenized with a Potter-Elvehjem homogenizer and treated with subtilisin (1 mg g⁻¹ muscle wet mass) for 5 min in an ice bath. The mixture was diluted 1:2, homogenized and centrifuged at 1000g for 10 min. The resulting supernatant was centrifuged at 9000g for 10 min, and the pellet was re-suspended in 10 mL of isolation buffer, and centrifuged at 1000g for 10 min to pellet any remaining cell debris contamination. The resulting supernatant was filtered through cheesecloth and centrifuged at 9000g for 10 min. The pellet containing mitochondria was washed once by suspension in the isolation buffer and centrifuged at 9000g for 10 min. All steps were carried out at 4°C. Protein content of the mitochondrial preparation was assayed in duplicate at 540 nm using the biuret method, with bovine serum albumin as a standard.

(c) Mitochondrial oxidative phosphorylation activity and efficiency

Whatever the acclimation temperature experienced by sea bass, mitochondrial oxygen consumption and ATP synthesis rates were measured at 18, 22, and 26°C in respiratory buffer (120mM KCl, 5mM KH₂PO₄, 1mM EGTA, 2mM MgCl₂, 0.3% fatty acid-free bovine serum albumin (w/v), and 3mM HEPES, pH 7.4) supplemented with 20 mM glucose and 1.5 U mL⁻¹ hexokinase [32]. Oxygen consumption was measured using a Clark electrode (Rank Brother Ltd, Cambridge, UK), assuming an oxygen concentration of 544, 501, and 468 nmol O mL⁻¹, at 18, 22, and 26°C, respectively. Muscle mitochondria were energized with a mixture of substrates (5mM pyruvate, 2.5mM malate, 5mM succinate). ADP (500μM) was added to initiate ATP synthesis. After recording the phosphorylating respiration rate, four aliquots of mitochondrial suspension were withdrawn from the respiratory chamber every 2 min and immediately quenched in ice-cold perchloric acid solution (10% HClO₄ and 25mM EDTA). After centrifugation of the denatured protein (20000g for 5 min), supernatants were neutralized with a KOH solution (0.2M KOH and 0.3M MOPS). The ATP production was determined from the slope of the linear accumulation of glucose-6-phosphate content over the sampling time interval (6 min). Glucose-6-phosphate was assayed spectrophotometrically at 340nm by monitoring the production of NADH in an assay medium (50 mM triethanolamine-HCl, 7.5mM MgCl₂, 3.75mM EDTA, pH 7.4), supplemented with 0.5 mM NAD and 0.5 U glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* [32].

(d) Statistical analyses

All statistical analyses were performed with linear models in JMP 12 (SAS Institute Inc., Cary, NC). The normality and homogeneity of variance of residuals were systematically checked in

the full models and were satisfactory. All models included fixed effects of acclimation treatments, since the same samples were assessed at three assay temperatures, this latter parameter was imbricated into acclimation factor. In addition, values obtained at the temperature at which fishes were acclimated have been compared with One-Way ANOVA. Whenever significant differences were found among acclimation groups, we used the Tukey's procedure to conduct post hoc tests (pairwise comparisons between the acclimated groups). Results are presented as mean $\pm$ s.e.m unless otherwise stated.

3. Results

Acute increase in assay temperature increased significantly the rates of phosphorylating oxygen ($F_{(2,75)}=47.89$; $P<0.0001$; Fig. 1A) and corresponding ATP synthesis ($F_{(2,75)}=42.64$; $P<0.0001$; Fig. 1B). Both were also significantly affected by thermal acclimation (Fig. 1A; $F_{(2,75)}=36.84$; $P<0.0001$ and Fig. 1B; $F_{(2,75)}=20.99$; $P<0.0001$, respectively), being higher in 18°C acclimated fish than in the two other groups. When measured at the acclimation temperature, the rates of phosphorylating oxygen consumption (Fig. 1D) and ATP synthesis (Fig. 1E) were not significantly different between experimental groups. The mitochondrial efficiency (ATP/O) was significantly affected by assay temperatures ($F_{(2,75)}=4.22$; $P<0.05$) and acclimation temperature ($F_{(2,75)}=8.70$; $P<0.001$); the mitochondrial efficiency being significantly higher in 26°C acclimated fish than in the two other thermal acclimation groups (Fig. 1C). However, the effect of assay temperatures was not statistically significant when tested within each of the three acclimation groups. When measured at the acclimation temperature, the mitochondrial efficiency was significantly higher in 26°C acclimated fish than that in the two other groups (Fig. 1F).

The basal-non phosphorylating respiration, which measures the maximal rate of proton leak, was significantly affected by assay temperatures and acclimation temperature ($F_{(2,72)}=19.1$; $P<0.0001$ and $F_{(2,72)}=4.1$; $P<0.05$, respectively; Fig. 2). The basal respiration of 18 and 22°C acclimated fish was more sensitive to acute changes in assay temperature than warm acclimated fish (Fig. 2A). At the acclimation temperature, the leak respiration was not significantly different between groups (Fig. 2B).

4. Discussion

Cold and high temperature acclimation are known to trigger different physiological mechanisms leading to contrasted structure and function in acclimated ectotherms. This is mainly due to differential thermal effects on oxygen demand (being a function of cellular energy needs) and oxygen supply (being a function of oxygen concentration, viscosity and diffusion rates). Ectotherms thus developed different biological mechanisms aiming at alleviate the effect of temperature. As previously reviewed [1,24], the present data confirm the occurrence of mitochondrial adjustments during thermal acclimation to restore mitochondrial fluxes (phosphorylating respiration and ATP synthesis) in skeletal muscle of European sea bass. Our results indicate that thermal acclimation also restores mitochondrial efficiency at temperatures considered as cold (18°C) or optimal (22°C) for the species used in the present study [29-31]. In contrast, warm-acclimated fishes displayed an increased mitochondrial efficiency, both across the range of assay temperatures and when individuals were compared at their own acclimation temperature. This fundamental result reinforces a recent study using an indirect measurement of mitochondrial efficiency and reporting similar increased in mitochondrial efficiency following warm acclimation in fish [26].

An acute rise in temperature is well known to increase oxidative phosphorylation fluxes and to decrease mitochondrial efficiency (i.e. ATP/O or ADP/O ratios) [7-9,15]. The loss of efficiency indicates that more oxygen and fuels are needed to produce a given amount of ATP and sustain performances. The issue can be very challenging for aquatic ectotherms since water warming results in a decrease in dissolved oxygen concentration. In warm-acclimated fishes, the increased mitochondrial efficiency reported alleviates the need for oxygen and therefore can be view as a compensatory mechanism allowing to maintain enough cellular energy under challenging thermal conditions. Several biochemical adjustments might explain why mitochondrial efficiency increased in warm-acclimated fish. The reduction of mitochondrial respiration rates during warm acclimation (Fig. 1A; [3,6,19,33]) is able to improve mitochondrial efficiency by limiting the rise in oxygen dissipated in the proton leak (Fig. 2A; [34]). The alteration of mitochondrial phospholipids, such as a decreased in cardiolipin content [24] and/or changes in the fatty acids composition of mitochondrial membrane (like a decrease in 22:6n3, 20:5n-3 fatty acid and/or a low 20:4n6/18:2n6 ratio) [19,22], can also improve mitochondrial efficiency by triggering a strong decrease in mitochondrial proton leak activity [35, 36]. This last hypothesis is supported by a recent study showing that fish fed with low n-3 highly unsaturated fatty acids diet exhibited higher mitochondrial ATP/O ratio [37].

Notwithstanding the underlying mechanisms, which may also depend upon the species or the tissue investigated, the improvement of mitochondrial efficiency in warm-acclimated

210 fishes indicates that less oxygen is required to synthesize a given amount of ATP. Hence,
211 acclimation of aquatic ectotherms to sub-optimal temperature triggers an adjustment of
212 mitochondrial energy transducing system toward a more economical management of oxygen,
213 which may contribute to compensate a mismatch between increasing energy needs and the
214 ability to supply the consequent oxygen demand of the cells. The model of oxygen- and
215 capacity-limited thermal performance [38] posits that loss of performance at high temperatures
216 is induced by a mismatch between oxygen delivery and demand of aerobic processes [38]. Our
217 results indicate that the higher mitochondrial ATP/O ratio following warm temperature
218 acclimation may compensate from a loss of oxygen delivery, by maintaining ATP synthesis
219 and its allocation to the animal energy-demanding activities. Interestingly, an increased
220 mitochondrial efficiency as a compensatory mechanism to fulfil energy needs of cells has also
221 been described in other organisms that acclimate to harsh environmental or nutritional energy
222 constraints, such as long-term fasting or hypoxia [39, 40].

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341
342 **Figure 1:** Effect of assay temperature and thermal acclimation on (A) oxygen consumption, (B)
343 ATP synthesis and (C) coupling efficiency in skeletal muscle mitochondria of juvenile sea bass.
344 Fishes were acclimated at 18°C (black circles), 22°C (open circle), or 26°C (red circles). Panels
345 D, E and F reports values obtained when mitochondrial parameters were measured at the
346 temperature at which fishes were acclimated. Values are means $\pm$ s.e.m., from $N=7-10$
347 individual mitochondrial preparation per group. Data with different lowercase letters are
348 significantly different at $P<0.05$ within the same acclimation group. Curves with different
349 capital letters indicate significant effect of thermal acclimation ($P<0.05$).

350
351 **Figure 2:** Effect of temperature and thermal acclimation on (A) basal non-phosphorylating
352 oxygen consumption in skeletal muscle mitochondria of juvenile sea bass. Fishes were
353 acclimated at 18°C (black circles), 22°C (open circle), or 26°C (red circles). The rates of basal
354 non-phosphorylating respiration were measured in the presence of $1 \mu\text{g ml}^{-1}$ oligomycin. Panel
355 B reports values obtained when mitochondrial parameters were measured at the temperature at
356 which fishes were acclimated. Values are means $\pm$ s.e.m., from $N=7-10$ individual
357 mitochondrial preparation per group. Data with different lowercase letters are significantly
358 different at $P<0.05$ within the same acclimation group. Curves with different capital letters
359 indicate significant effect of thermal acclimation regardless of the assay temperature ($P<0.05$).

For Review Only

Figure 1

Figure 2