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► To cite this version:

Y. Voituron, D. Roussel, L. Teulier, Marie Vagner, Q. Ternon, et al.. Warm Acclimation Increases Mitochondrial Efficiency in Fish: A Compensatory Mechanism to Reduce the Demand for Oxygen. *Physiological and Biochemical Zoology*, 2022, 95 (1), pp.15-21. 10.1086/716904 . hal-03510408

HAL Id: hal-03510408

<https://hal.science/hal-03510408v1>

Submitted on 4 Jan 2022

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

Journal:	<i>Biology Letters</i>
Manuscript ID	RSBL-2021-0122
Article Type:	Research
Date Submitted by the Author:	25-Feb-2021
Complete List of Authors:	Voituron, Yann; UMR 5023 Ecology of Natural and Man-impacted Hydrosystems, Biology Roussel, Damien; UMR 5023 Ecology of Natural and Man-impacted Hydrosystems, Biology Teulier, Loic; UMR 5023 Ecology of Natural and Man-impacted Hydrosystems, Biology Vagner, Marie; Université de Bretagne Occidentale Ternon, Quentin; Université de Bretagne Occidentale Romestaing, Caroline; Université Claude Bernard Lyon 1, UMR5023 Ecology of Natural and Man-Impacted Hydrosystems Dubillot, Emmanuel; UMR 7266 (La Rochelle University-CNRS) Lefrançois, C; University La Rochelle/CNRS,
Subject:	Cellular Biology < BIOLOGY, Ecology < BIOLOGY
Categories:	Physiology
Keywords:	Mitochondria, Thermal acclimation, Skeletal muscle, ATP/O, oxidative phosphorylation, <i>Dicentrarchus labrax</i>

Author-supplied statements

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

Statement (if applicable):

Conception of the study and design of the work: D.R., Y.V., C.L.; Acquisition and analysis: L.T., C.R., E.D., DR. Q.T., MV.; Interpretation of data and validation: Y.V., D.R., C.L. L.T.

Authors' contributions

This paper has multiple authors and our individual contributions were as below

Statement (if applicable):

CUST_AUTHOR_CONTRIBUTIONS_TEXT :No data available.

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

42 **1. Introduction**

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

52 An acute temperature variation results in membrane destabilization and a compensatory
53 increase in respiratory activity to counteract increased proton leakage across the mitochondrial
54 membrane, in order to maintain the driving force (proton motive force) and the ATP synthesis
55 [5-7]. In addition, most of the studies have also reported in many different ectothermic species,
56 such as bivalves, lugworms, crabs, insects, toads and fishes, that an acute elevation of
57 temperature triggers oxidative and energy costs of mitochondrial functioning [6,7,8-15]. The
58 oxidative cost is associated with an increased mitochondrial reactive oxygen species
59 production, and the energy cost is associated with a decreased mitochondrial coupling
60 efficiency due to an increased proton leakage. All together the heat stress would force
61 ectotherms to allocate extra oxygen and energy resources to maintain ATP production. In
62 contrast, a thermal acclimation can profoundly modify mitochondrial properties [16], like
63 mitochondrial oxidative and enzyme activities [3,6,17-19], inner mitochondrial membrane
64 composition [19-22], and mitochondrial volume density [23]. The functional results of such
65 compensatory adjustments during acclimation lead to a “mitochondrial homeostasis”, that is a
66 profound ability to maintain constant mitochondrial oxidative rates and enzyme activities
67 despite pronounced body temperature variations [1,11,24]. However, the impact of thermal
68 acclimation on the energy cost of mitochondrial functioning remains puzzling. Few studies have
69 reported that the ADP/O ratio, an oximetry measure of mitochondrial efficiency, could be either
70 changed [25,26] or not by thermal acclimation [27,28]. All these studies aiming at linking
71 variations of environmental temperatures to animal performances have been based on fluxes of
72 oxygen as proxy of energy output without knowing, whether the mitochondrial ATP synthesis
73 rate and the efficiency of mitochondrial energy transduction system (i.e. ATP/O ratio), vary
74 with acclimation.

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

80

81

82 **2. Materials and Methods**

83 **(a) Fish acclimation and Ethics statement**

84 The juvenile European sea bass, initially produced in a farm (Graveline hatchery, Pas-de-
85 Calais), were supplied (initial fish weight: 78.7 ± 3.5 g) by the CEDRE (Brest, France), and
86 acclimated at the LIENSs facilities (La Rochelle, France) in January 2018. Upon arrival, fishes
87 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 =
88 35.3 ± 0.3). Each tank was individually equipped with an external biological filter (Eheim
89 professional 3 2080, Eheim, Deizisau, Germany) and supplied with aerated natural seawater.
90 After 6 weeks of acclimation to these laboratory conditions, fish were progressively acclimated
91 (1°C changing every 3 days) to the one of the three experimental temperature (18, 22, 26°C, n
92 = 2 tanks per acclimation temperature tested). These temperatures were chosen in regards to the
93 temperature considered as optimal in term of aerobic scope for this species, when located in
94 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
95 $\pm 0.2^{\circ}\text{C}$) were kept constant (TECO TR20, Conselice, Italy), as well as salinity (33.7 ± 0.3 ;
96 34.1 ± 0.4 ; 34.3 ± 1.0 respectively); both were monitored daily using a conductimeter (WTW
97 model 3110 Weilheim, Germany). Oxygen content was never below 90 % air saturation. Fishes
98 were maintained in their respective thermal condition during 2 months with a 12L:12D
99 photoperiod cycle. Fishes were fed with commercial pellets (Le Gouessant aquaculture,
100 Lamballe, France) once a day, three times a week, during the whole experimental period. Then,
101 fishes were randomly caught and sacrificed by long lasting anesthesia (tricaine methane
102 sulphonate MS-222; Sigma-Aldrich, St Quentin-Fallavier, France). All fish manipulations were
103 performed according to the Animal Care Committee of France under authorization n°17-300-
104 2.

105

106 **(b) Mitochondrial isolation**

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

109 Briefly, skeletal muscles were homogenized with a Potter-Elvehjem homogenizer and treated
110 with subtilisin (1 mg g⁻¹ muscle wet mass) for 5 min in an ice bath. The mixture was diluted
111 1:2, homogenized and centrifuged at 1000g for 10 min. The resulting supernatant was
112 centrifuged at 9000g for 10 min, and the pellet was re-suspended in 10 mL of isolation buffer,
113 and centrifuged at 1000g for 10 min to pellet any remaining cell debris contamination. The
114 resulting supernatant was filtered through cheesecloth and centrifuged at 9000g for 10 min. The
115 pellet containing mitochondria was washed once by suspension in the isolation buffer and
116 centrifuged at 9000g for 10 min. All steps were carried out at 4°C. Protein content of the
117 mitochondrial preparation was assayed in duplicate at 540 nm using the biuret method, with
118 bovine serum albumin as a standard.

119

120 **(c) Mitochondrial oxidative phosphorylation activity and efficiency**

121 Whatever the acclimation temperature experienced by sea bass, mitochondrial oxygen
122 consumption and ATP synthesis rates were measured at 18, 22, and 26°C in respiratory buffer
123 (120mM KCl, 5mM KH₂PO₄, 1mM EGTA, 2mM MgCl₂, 0.3% fatty acid-free bovine serum
124 albumin (w/v), and 3mM HEPES, pH 7.4) supplemented with 20 mM glucose and 1.5 U mL⁻¹
125 hexokinase [32]. Oxygen consumption was measured using a Clark electrode (Rank Brother
126 Ltd, Cambridge, UK), assuming an oxygen concentration of 544, 501, and 468 nmol O mL⁻¹,
127 at 18, 22, and 26°C, respectively. Muscle mitochondria were energized with a mixture of
128 substrates (5mM pyruvate, 2.5mM malate, 5mM succinate). ADP (500µM) was added to
129 initiate ATP synthesis. After recording the phosphorylating respiration rate, four aliquots of
130 mitochondrial suspension were withdrawn from the respiratory chamber every 2 min and
131 immediately quenched in ice-cold perchloric acid solution (10% HClO₄ and 25mM EDTA).
132 After centrifugation of the denatured protein (20000g for 5 min), supernatants were neutralized
133 with a KOH solution (0.2M KOH and 0.3M MOPS). The ATP production was determined from
134 the slope of the linear accumulation of glucose-6-phosphate content over the sampling time
135 interval (6 min). Glucose-6-phosphate was assayed spectrophotometrically at 340nm by
136 monitoring the production of NADH in an assay medium (50 mM triethanolamine-HCl, 7.5mM
137 MgCl₂, 3.75mM EDTA, pH 7.4), supplemented with 0.5 mM NAD and 0.5 U glucose-6-
138 phosphate dehydrogenase from *Leuconostoc mesenteroides* [32].

139

140 **(d) Statistical analyses**

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

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

150

151

152 3. Results

153 Acute increase in assay temperature increased significantly the rates of phosphorylating oxygen
154 ($F_{(2,75)}=47.89$; $P<0.0001$; Fig. 1A) and corresponding ATP synthesis ($F_{(2,75)}=42.64$; $P<0.0001$;
155 Fig. 1B). Both were also significantly affected by thermal acclimation (Fig. 1A; $F_{(2,75)}=36.84$;
156 $P<0.0001$ and Fig. 1B; $F_{(2,75)}=20.99$; $P<0.0001$, respectively), being higher in 18°C acclimated
157 fish than in the two other groups. When measured at the acclimation temperature, the rates of
158 phosphorylating oxygen consumption (Fig. 1D) and ATP synthesis (Fig. 1E) were not
159 significantly different between experimental groups. The mitochondrial efficiency (ATP/O)
160 was significantly affected by assay temperatures ($F_{(2,75)}=4.22$; $P<0.05$) and acclimation
161 temperature ($F_{(2,75)}=8.70$; $P<0.001$); the mitochondrial efficiency being significantly higher in
162 26°C acclimated fish than in the two other thermal acclimation groups (Fig. 1C). However, the
163 effect of assay temperatures was not statistically significant when tested within each of the three
164 acclimation groups. When measured at the acclimation temperature, the mitochondrial
165 efficiency was significantly higher in 26°C acclimated fish than that in the two other groups
166 (Fig. 1F).

167 The basal-non phosphorylating respiration, which measures the maximal rate of proton leak,
168 was significantly affected by assay temperatures and acclimation temperature ($F_{(2,72)}=19.1$;
169 $P<0.0001$ and $F_{(2,72)}=4.1$; $P<0.05$, respectively; Fig. 2). The basal respiration of 18 and 22°C
170 acclimated fish was more sensitive to acute changes in assay temperature than warm acclimated
171 fish (Fig. 2A). At the acclimation temperature, the leak respiration was not significantly
172 different between groups (Fig. 2B).

173

174

175 4. Discussion

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

191 An acute rise in temperature is well known to increase oxidative phosphorylation fluxes
192 and to decrease mitochondrial efficiency (i.e. ATP/O or ADP/O ratios) [7-9,15]. The loss of
193 efficiency indicates that more oxygen and fuels are needed to produce a given amount of ATP
194 and sustain performances. The issue can be very challenging for aquatic ectotherms since water
195 warming results in a decrease in dissolved oxygen concentration. In warm-acclimated fishes,
196 the increased mitochondrial efficiency reported alleviates the need for oxygen and therefore can
197 be view as a compensatory mechanism allowing to maintain enough cellular energy under
198 challenging thermal conditions. Several biochemical adjustments might explain why
199 mitochondrial efficiency increased in warm-acclimated fish. The reduction of mitochondrial
200 respiration rates during warm acclimation (Fig. 1A; [3,6,19,33]) is able to improve
201 mitochondrial efficiency by limiting the rise in oxygen dissipated in the proton leak (Fig. 2A;
202 [34]). The alteration of mitochondrial phospholipids, such as a decreased in cardiolipin content
203 [24] and/or changes in the fatty acids composition of mitochondrial membrane (like a decrease
204 in 22:6n3, 20:5n-3 fatty acid and/or a low 20:4n6/18:2n6 ratio) [19,22], can also improve
205 mitochondrial efficiency by triggering a strong decrease in mitochondrial proton leak activity
206 [35, 36]. This last hypothesis is supported by a recent study showing that fish fed with low n-3
207 highly unsaturated fatty acids diet exhibited higher mitochondrial ATP/O ratio [37].

208 Notwithstanding the underlying mechanisms, which may also depend upon the species
209 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].

223

224

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- 340

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

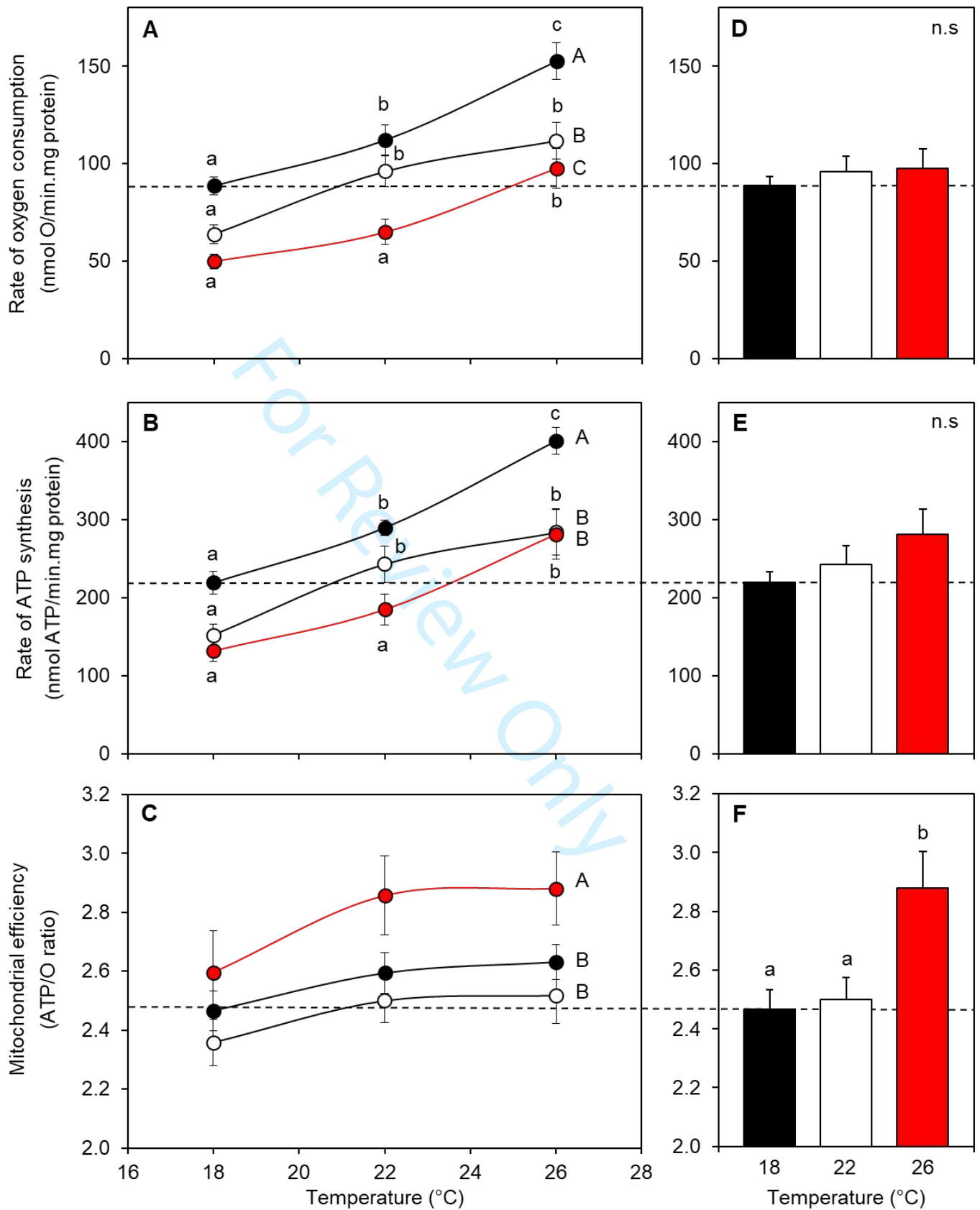


Figure 2

