**Low oxygen levels can help to prevent the detrimental effect of acute warming on mitochondrial efficiency in fish**

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**Journal name:** Biology Letters

**Electronic supplementary material**

**Animal cares**

In April 2017, fish were moved from the hatchery (Les Poissons du Soleil, Balaruc-les-Bains, France) to fish facilities in Ifremer (Plouzané, France). The fish were kept in a communal tank of 1m3, fed once a day with pellets (Neo grower extra marin, Le Gouessant, Lamballe, France), with a photoperiod of 12:12. The seawater was directly pumped in the harbour. Because of the high capacity of the stock tank (350 m3), the seawater temperature matched the mean temperature of the harbour [1] but with virtually no daily fluctuation.

In January 2019, fish (n = 8) were moved into two replicated 500L-tanks which were supplied with filtered seawater at a flow rate of 550L/hour. Extra fish (n = 24) were added to obtain an optimal fish density in the tanks. Fish were then left 3 months in this new condition, fed to satiation every second day. In April 2019, water temperature was monitored twice daily (CellOx® 325, WTW) over the two weeks before the mitochondrial assay to match the assay temperature of the mitochondria with the water temperature in fish tanks. Fish were fasted for 24h before sacrifice. Because of the logistics constraint associated with assaying fresh mitochondria, fish sampling and mitochondrial assay were staggered over four days. Each day, one fish was processed the morning and the afternoon. Preliminary analyses show that mitochondrial properties in our study were not affected by the day of measurement and the time of the day.

**Red muscle homogenate preparation**

The fish was anesthetized with eugenol [0.5 mg mL-1 eugenol and 0.5 mg mL-1 ethanol] diluted in seawater [final concentration = 1 mg L-1]. After the fish showed signs of profound anaesthesia (total loss of equilibrium, slow and irregular opercular movement, see [2]), the fish was immediately taken out of the water, weighed and culled by decapitation [3,4]. Then a piece of red muscle was taken from the left flank below the dorsal fin [muscle sample mass: mean ± s.e.m. = 13.4 ± 0.8 mg] and placed in 1 mL of respirometry buffer (20 mmol L-1 Taurine, 10 mmol L-1 KH2PO4, 20 mmol L-1 HEPES, 110 mmol L-1 D-sucrose, 60 mmol L-1 K-lactobionate, 1 g L-1 BSA fatty acid free, and pH 7.2 at 12°C). The piece of red muscle was then cut with microdissecting scissors to obtain a homogenous solution (see the Appendix of [5]). Then this shredded solution was diluted in respirometry buffer to a concentration of 1 mg mL-1 [concentration: mean ± s.e.m. = 1.00 ± 0.00 mg mL-1). This homogenization technique permeabilized the plasma membranes of muscle myocytes. This can be shown with the very high stimulation of respiratory fluxes after addition of ADP molecules (mean ± s.e.m. = 6.3 ± 0.1-fold increase, n = 32) as ADP molecule only crosses a permeable plasma membrane. This homogenization procedure is as efficient as procedures that used detergents for the permeabilization of the plasma membrane, as validated in Appendix of [5].

**Mitochondrial respiration and phosphorylation**

*Temperature and oxygen conditions*

Mitochondria properties of red muscle were determined in four *in vitro* conditions: (i) 13.00°C – High [O2], (ii) 13.00°C – Low [O2], (iii) 16.00°C – High [O2] and (iv) 16.00°C – Low [O2]. Oxygen levels were determined for practical reasons. Oxygen levels have been manipulated in the respirometry chambers with addition of gas (e.g. pure oxygen). For the condition “Low [O2]”, we have reoxygenated the respirometry chambers once during the protocol to stay above a sufficient oxygen level to prevent limitation of oxygen diffusion [6]. See minimum and maximum oxygen levels over the course of the assay in Table S1.

The 3°C warming of the mitochondrial assay simulated the acute warming conditions seabass would naturally experience at the season of the study. Temperature was monitored at least every 60 minutes over eleven years (2007 – 2017) in the Brest Harbour [1], where juvenile seabass naturally occurs. Temperature data from 1st April until 31st May was used to calculate the daily thermal range in Brest Harbour. Maximal daily thermal ranges between 1st April and 31st May for each year were averaged (3.06 ± 0.09°C). A warming of 3°C is considered as representative of extreme acute warming that juvenile seabass can naturally meet in their environment from April to May. The 13°C experimental condition corresponded to the water temperature of fish tanks at the time of the tissue sampling (12.8°C ± 0.3°C) and to which mitochondria were acclimatized. This temperature was also representative of the temperature in Brest Harbour at this time of the year, when a daily +3°C warming can be considered extreme.

*Equipment preparation*

We used two respirometers Oroboros® (Oroboros Instruments, Innsbruck, Austria) to measure the mitochondrial respiration. ATP flux was estimated with variation in concentration of free magnesium with the fluorescence of the Magnesium Green probe (MgGreen). Each had two respirometry chambers and was equipped with fluorescent sensors. One respirometer was set at 13.00 ± 0.00°C and the other at 16.00 ± 0.00°C. For each respirometer, one chamber was at Low [O2] and the other chamber was at High [O2]. Respirometers have been calibrated for instrumental background over a large range of [O2] (100 to 600 µM), zero oxygen and air saturation oxygen level at 13°C and 16°C. The simultaneous measurement of oxygen consumption and ATP production allow us to determine the mitochondrial efficiency to produce ATP (ATP/O ratio).

*Measurement of mitochondrial metabolic traits*

Immediately after the red muscle homogenate preparation, 2.1 mL of the homogenous solution were pipetted and placed in each respirometry chambers of respirometers. The mitochondrial efficiency measurement protocol is based on [7]. We started with an injection of an ATPase inhibitor, Ap5A (100µM). The respiration of red muscle fibres was then stimulated by the addition of pyruvate (5 mM) and malate (0.5 mM). After the addition of the probe Magnesium Green (MgGreen) (2.2µM), EGTA (0.1mM) and EDTA (15µM) were subsequently added. Then, stepwise additions of MgCl2 were performed. Succinate (2 mM) was then added, and the phosphorylation respiration rate (i.e. OXPHOS respiration) was reached by adding a saturating concentration of ADP (2 mM). Carboxylatractyloside (4 µM) was added to inhibit adenylate nucleotide translocases and to obtain the respiration rate that offsets proton leak (i.e. LEAK respiration). Antimycine A (2.5 µM) was added to inhibit complex III and then obtained a residual oxygen consumption, which was removed from other oxygen consumption measurements. Finally, cytochrome c oxidase (COX) activity was measured in presence of ascorbate (8 mM) and N, N, N’, N’ -tetramethyl-p-phenylenediamine dihydrochloride (500 µM). In a separate run, auto-oxidation of ascorbate and TMPD was measured under each oxygen and temperature conditions. The chemical auto-oxidation was 13.41 pmol O2 s-1 mL-1 at 13°C/High [O2], 9.28 pmol O2 s-1 mL-1 at 13°C/Low [O2], 16.34 pmol O2 s-1 mL-1 at 16°C/High [O2], and 9.82pmol O2 s-1 mL-1 at 16°C/Low [O2]. This chemical auto-oxidation was removed from the respiratory fluxes after adding ascorbate and TMPD in presence of red muscle homogenate.

*Determination of the affinity (Kd) of free magnesium for ADP and ATP*

The ATP concentration in the respirometry chamber was estimated from measurement of changes in free magnesium concentration, [Mg2+] and is based on the unequal affinities of ATP and ADP for [Mg2+] [8]. The method for determining the affinity of the magnesium with nucleotides was adapted from [9]. Determination of *Kd* was performed at 1 mg red muscle homogenate per mL of respiration buffer, in presence of carboxylatractyloside (4 µM), oligomycin (2 µg mL-1) and P1,P5 - Di(adenosine-5') pentaphosphate (Ap5A, 25 µM) to inhibit the adenylate nucleotide translocases, the ATP synthase and adenylate kinases, respectively. Pyruvate (5 mM) and malate (0.5 mM) were then injected, followed by the fluorescent probe MgGreen (2.2 µM). The calcium and magnesium ions were chelated by EGTA (0.1 mM) and EDTA (15 µM). MgCl2 was then added per 10-step addition (final concentration, Cf = 0.1 mM) for the calibration of the fluorescent signal into Mg2+. After addition of succinate (10 mM), the *Kd* of Mg2+ for ATP and ADP was estimated with a 32-step addition of ATP (Cf = 6.4 mM) and ADP (Cf = 8 mM), respectively. The *Kd* of free magnesium for ATP (13°C= 0.266 mmol L-1, 16°C= 0.221 mmol L-1) and for ADP (13°C= 1.803 mmol L-1, 16°C= 1.886 mmol L-1) was estimated to convert Mg2+ signal into ATP flux (adapted from [9]).

**Table S1. Minimum and maximum oxygen levels reached in the respirometry chamber, for each temperature and oxygen conditions.** The oxygen levels are expressed in mean ± SD µmol O2 L-1.

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| --- | --- | --- | --- |
| Temperature | Oxygen conditions | Minimum [O2] | Maximum [O2] |
| 13°C | High [O2] | 287.9 ± 45.0 | 552.4 ± 51.3 |
| 13°C | Low [O2] | 177.1 ± 28.0 | 288.3 ± 5.4 |
| 16°C | High [O2] | 243.4 ± 63.0 | 557.7 ± 33.2 |
| 16°C | Low [O2] | 117.8 ± 54.4 | 263.4 ± 7.6 |

**References**

1. Petton S, Le Roy V, Bellec G, Queau I, Le Souchu P, Pouvreau S. 2018 Marine environmental station database of Daoulas bay. (doi:10.17882/42493)

2. Hoseini SM, Rajabiesterabadi H, Tarkhani R. 2015 Anaesthetic efficacy of eugenol on iridescent shark, *Pangasius hypophthalmus* (Sauvage, 1878) in different size classes. *Aquac. Res.* **46**, 405–412. (doi:10.1111/are.12188)

3. AVMA. 2020 Guidelines for the Euthanasia of Animals. (doi:10.1163/9789004294530\_006)

4. Hilton Z, Clements KD, Hickey AJR. 2010 Temperature sensitivity of cardiac mitochondria in intertidal and subtidal triplefin fishes. *J. Comp. Physiol. B Biochem. Syst. Environ. Physiol.* **180**, 979–990. (doi:10.1007/s00360-010-0477-7)

5. Salin K, Auer SK, Rudolf AM, Anderson GJ, Selman C, Metcalfe NB. 2016 Variation in metabolic rate among individuals is related to tissue-specific differences in mitochondrial leak respiration. *Physiol. Biochem. Zool.* **89**, 511–523. (doi:10.1086/688769)

6. E Gnaiger, B Lassnig, A Kuznetsov GR and RM. 1998 Mitochondrial oxygen affinity, respiratory flux control and excess capacity of cytochrome c oxidase. *J. Exp. Biol.* **1139**, 1129–1139.

7. Salin K, Villasevil EM, Auer SK, Anderson GJ, Selman C, Metcalfe NB, Chinopoulos C. 2016 Simultaneous measurement of mitochondrial respiration and ATP production in tissue homogenates and calculation of effective P/O ratios. *Physiol. Rep.* **4**, 1–9. (doi:10.14814/phy2.13007)

8. Leyssens A, Nowicky A V, Patterson L, Crompton M, Duchen MR. 1996 The relationship between mitochondrial state, ATP hydrolysis, [Mg2+]i and [Ca2+]i studied in isolated rat cadiomyocytes. *J. Physiol.* **496.1**, 111–128.

9. Chinopoulos C, Kiss G, Kawamata H, Starkov AA. 2014 Measurement of ADP-ATP exchange in relation to mitochondrial transmembrane potential and oxygen consumption. *Methods Enzymol.* **542**, 333–348. (doi:10.1016/B978-0-12-416618-9.00017-0)