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Physiology

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

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Aerobic metabolism of aquatic ectotherms is highly sensitive to fluctuating climates. Many mitochondrial traits exhibit phenotypic plasticity in response to acute variations in temperature and oxygen availability. These responses are critical for understanding the effects of environmental variations on aquatic ectotherms' performance. Using the European seabass, *Dicentrarchus labrax*, we determined the effects of acute warming and deoxygenation *in vitro* on mitochondrial respiratory capacities and mitochondrial efficiency to produce ATP (ATP/O ratio). We show that acute warming reduced ATP/O ratio but deoxygenation marginally raised ATP/O ratio, leading to a compensatory effect of low oxygen availability on mitochondrial ATP/O ratio at high temperature. The acute effect of warming and deoxygenation on mitochondrial efficiency might be related to the leak of protons across the mitochondrial inner membrane, as the mitochondrial respiration required to counteract the proton leak increased with warming and decreased with deoxygenation. Our study underlines the importance of integrating the combined effects of temperature and oxygen availability on mitochondrial metabolism. Predictions on decline in performance of aquatic ectotherms owing to climate change may not be accurate, since these predictions typically look at respiratory capacity and ignore efficiency of ATP production.

1. Introduction

Temperature exerts profound effects on the performance of ectothermic animals, partly through its effect on metabolism. The issue is particularly severe for aquatic ectotherms since water warming also results in a decrease in dissolved oxygen concentration [1]. Oxygen depletion is often more dramatic for marine organisms that face exacerbated eutrophication-induced deoxygenation in most estuarine and coastal habitats [2]. Rising temperature causes an increase in aerobic metabolism but the lower oxygen availability might lead to a point where the organism has insufficient oxygen to supply to mitochondria and meet metabolic demands [3–6]. In the long term, many species can remodel their mitochondrial metabolism, and this acclimation response can help to maintain cell energy homeostasis across environmental conditions [7,8]. Generally, capacity for acclimation to mean warming and oxygen depletion is considered as a key feature determining resilience to climate change [9]. However, an increase in the frequency and magnitude of extreme temperature events, such as heat waves [10], is likely to be biologically more significant [11]. Because chemical reactions are inherently sensitive to temperature and substrate availability (e.g. oxygen) on an acute time scale, we contend

that acute metabolic response on a short time scale is critical for understanding the effects of climate extremes on the performance of aquatic ectotherms [12].

Changes in mitochondrial metabolism may have important consequences for an organism's ability to meet metabolic demands during acute warming and a decline in oxygen availability. This is because mitochondrial metabolism not only defines the cellular oxygen needs but also determines cells' ability to produce ATP molecules that fuel nearly all the important functions such as muscle contraction, growth and reproduction. Oxygen consumption related to ATP production (OXPHOS respiration) by mitochondria exposed to acute warming *in vitro* increases exponentially [13] (until mitochondria are no longer functional [14]). The activities of enzymes associated with respiratory processes are typically higher under acute warming conditions owing to the thermodynamics of molecular movements [8]. For example, cytochrome c oxidase (COX) activity, a measure of the maximal oxidative capacities of the final electron acceptor, increases of 1.56-fold and 2-fold with a 7°C and a 10°C temperature increase in the skeletal muscle mitochondria of rat [15] and crocodile [16], respectively. Warming conditions also lead to changes in other mitochondrial traits affecting ATP production, including the proportion of oxygen consumed to counteract the proton leakage across the mitochondrial inner membrane (LEAK respiration) and the proportion of oxygen consumed for ATP production. During a transient temperature increase, such as episodic heat waves, the increase in oxygen dissipated in proton leak may lead to a decrease in ATP production, unless the oxygen consumption related to ATP production increases by an equivalent or greater amount [17]. Importantly, in warming aquatic environments the ability to produce ATP might no longer be compensated by the capacity to take up higher amounts of oxygen, since there is likely not enough dissolved oxygen available.

Shifts in the ability of mitochondria to make ATP are another important mechanism for organisms to cover their ATP needs. The number of ATP molecules produced for each oxygen consumed by the mitochondria (termed the ATP/O ratio), in addition to the proportion of oxygen consumed for ATP production, can acutely vary [18,19]. Previous studies have demonstrated that the ATP/O ratio *in vitro* decreases when the temperature is acutely raised [20] and increases when oxygen levels decline [21]. While these studies suggest that ATP/O ratio can be drastically modulated with acute warming and oxygen depletion in the opposite direction, combined effects of temperature and oxygen remain largely unexplored although this would have important implications for aquatic ectotherms in a more thermally variable future.

Our aim was to investigate the acute effects of warming and oxygen depletion on fish mitochondria *in vitro*. We use a fully factorial approach to examine acute effects of temperature and oxygen availability on the mitochondrial respiratory capacities (OXPHOS and LEAK respirations, and COX activity), ATP production and efficiency to produce ATP (ATP/O ratio) of the red muscle of European seabass *Dicentrarchus labrax*. We predict that low dissolved oxygen concentration may have a compensatory effect on mitochondrial efficiency at high temperature, whereby the changes in ATP/O ratio with warming are counteracted by changes in ATP/O ratio with deoxygenation.

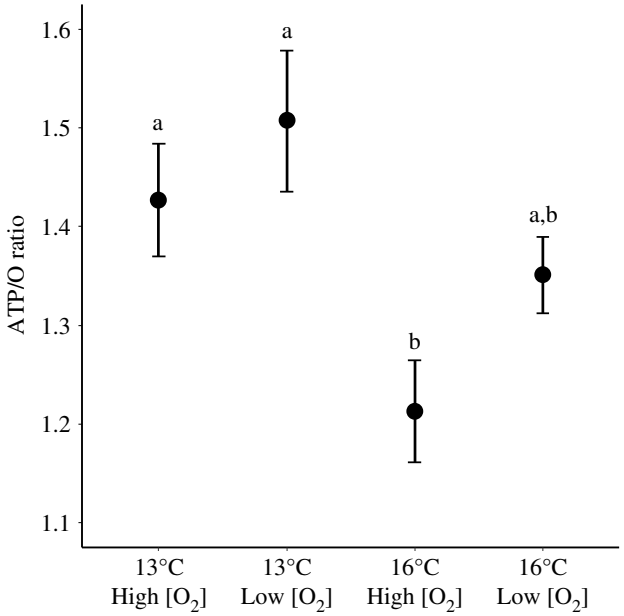

Figure 1. Red muscle mitochondrial efficiency (ATP/O ratio) of European seabass (*Dicentrarchus labrax*) as a function of temperature and oxygen availability. Means ± s.e.m. are shown. $N = 8$ fish per condition. Letters represent a significant difference between testing conditions ($p < 0.05$).

2. Material and methods

(a) Animals

Juvenile seabass were moved from the hatchery Les Poissons du Soleil (Balaruc-les-Bains, France) to Ifremer, Plouzané, in April 2017. The fish were kept in a communal tank, fed with commercial pellets (Le Gouessant, Lamballe), with a photoperiod of 12 : 12 and a water temperature following the daily mean natural temperature in Brest harbour. In April 2019, juvenile fish were culled ($n = 8$, body mass: mean ± s.e.m. = 163.3 ± 11.3 g) and samples of red muscle (13.4 ± 0.8 mg) were immediately collected and kept in ice-cold respirometry buffer for analysis of mitochondrial properties (see the electronic supplementary material for details on animal maintenance, culling of the fish and buffer composition).

(b) Mitochondrial respiratory capacities and efficiency

Muscle tissue was homogenized and replicate samples of homogenate were then added to four chambers of high-resolution respirometers equipped with fluorescent sensors (Oroboros Instruments, Innsbruck, Austria). Oxygen and magnesium green fluorescence were detected simultaneously as in [22,23]. Mitochondria were measured simultaneously for each fish in a fully factorial acute test temperature and oxygen availability design: 13°C/High [O_2] (552.4 ± 51.3 µM), 13°C/Low [O_2] (288.3 ± 5.4 µM), 16°C/High [O_2] (557.7 ± 33.2 µM) and 16°C/Low [O_2] (263.4 ± 7.6 µM). After addition of homogenate to respirometers at 13°C or 16°C, pure oxygen gas was added into two High [O_2] chambers to double the concentration compared to Low [O_2] chambers. The 3°C warming of the mitochondrial assay simulated the acute warming conditions juvenile seabass would naturally experience at this life stage (3.06 ± 0.09°C [24]). 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. See electronic supplementary material for full details of the experimental conditions of the mitochondrial assay.

The rates of OXPHOS and ATP production were assessed by adding ADP to the chamber containing energy substrates and magnesium green (see electronic supplementary material).

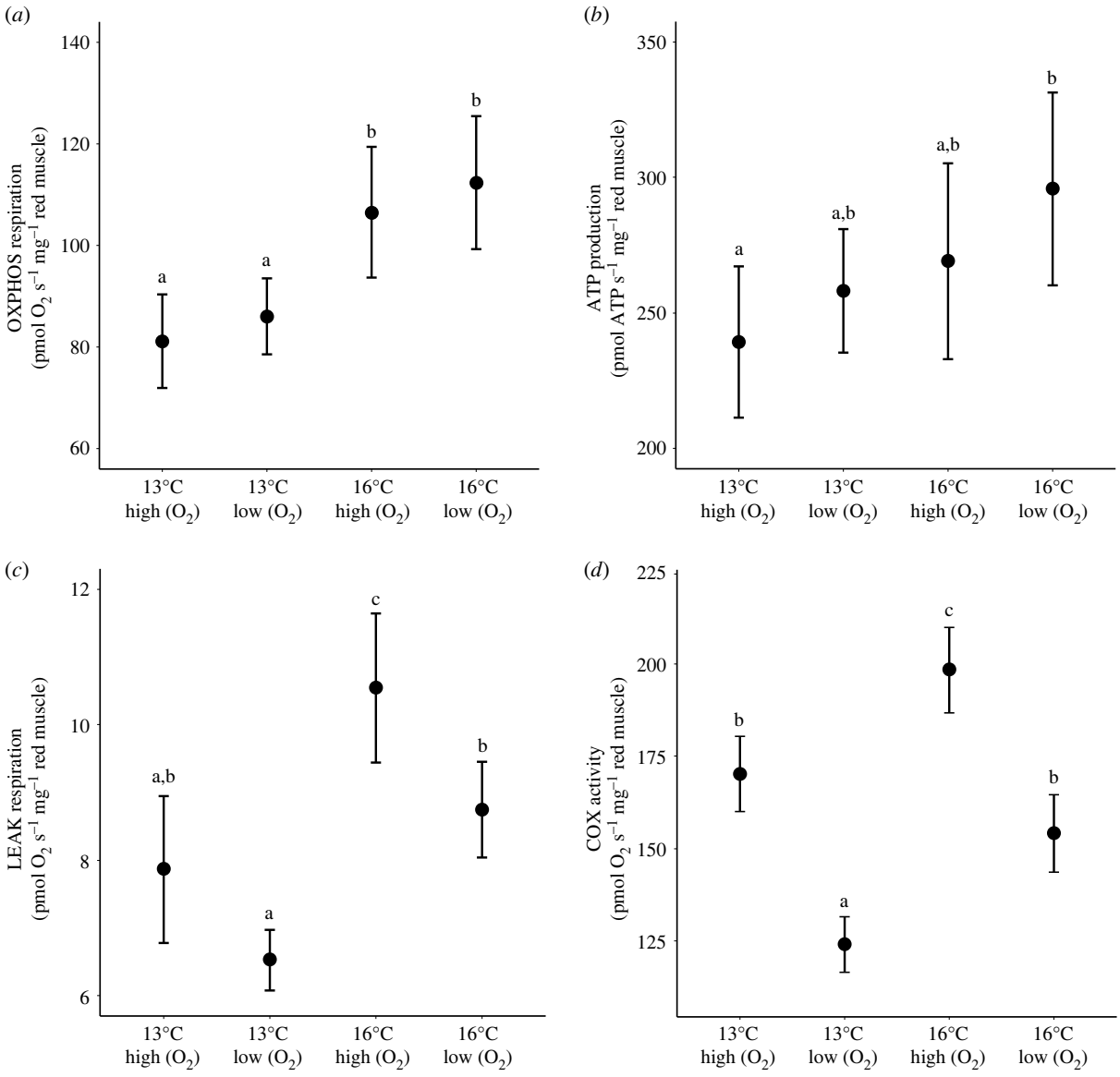

Figure 2. Effect of temperature and oxygen availability on red muscle mitochondrial properties of European seabass (*Dicentrarchus labrax*): (*a*) rate of oxygen consumption associated with ATP synthesis (OXPHOS respiration), (*b*) rate of ATP production, (*c*) rate of oxygen consumption to offset the proton leak (LEAK respiration) and (*d*) cytochrome c oxidase (COX) activity. Means ± s.e.m. are shown. $N = 8$ fish per condition. Letters represent a significant difference between testing conditions ($p < 0.05$).

The rate of LEAK respiration was then measured by inhibiting ATP synthesis through the addition of carboxyatractyloside and COX activity was measured after addition of ascorbate and N,N,N′,N′-tetramethyl-p-phenylenediamine. Finally, the ATP/O ratio was calculated as the ratio of ATP production to OXPHOS. See electronic supplementary material for full details of the protocol.

(c) Statistical analysis

We analysed the effects of temperature and oxygen conditions on mitochondrial function using repeated-measures two-way ANOVAs and Tukey adjusted post hoc tests. The ANOVAs included mitochondrial properties (i.e. ATP/O ratio, OXPHOS respiration, ATP production, LEAK respiration and COX activity) as dependant variables, and temperature (13°C and 16°C) and oxygen availability (Low and High) as categorical predictors, and two-way interactions between temperature and oxygen levels. The level of significance was $p < 0.05$. All data are presented as means ± s.e.m. Statistical analyses were performed in *R* v. 3.6.1 with the packages *lme4* and *lsmeans*.

3. Results

The ATP/O ratio significantly decreased with acute warming ($F_{1,21} = 11.7$, $p = 0.003$) and increased slightly—but not significantly—with acute deoxygenation ($F_{1,21} = 4.1$, $p = 0.057$). As a result, mitochondria tested at 13°C/High [O_2] and 16°C/Low [O_2] did not differ with respect to ATP/O ratio (Tukey adjusted post hoc test: $p = 0.759$, figure 1).

OXPHOS respiration ($F_{1,21} = 40.1$, $p < 0.001$), ATP production ($F_{1,21} = 8.6$, $p = 0.008$), LEAK respiration ($F_{1,21} = 29.1$, $p < 0.001$) and COX activity ($F_{1,21} = 35.5$, $p < 0.001$, figure 2) increased with temperature. However, the effects of deoxygenation and combined warming and deoxygenation on mitochondrial responses differed among traits. OXPHOS respiration did not change with oxygen levels ($F_{1,21} = 1.7$, $p = 0.205$), ATP production tended to increase with deoxygenation, but this difference was marginally not significant ($F_{1,21} = 3.9$, $p = 0.061$, figure 2*b*), LEAK respiration and COX activity decreased with oxygen depletion (LEAK: $F_{1,21} = 11.9$, $p = 0.002$; COX activity: $F_{1,21} = 85.9$, $p < 0.001$). Finally,

OXPHOS respiration and ATP production in mitochondria tested at 16°C/Low [O_2] were significantly higher than those in mitochondria tested at 13°C/High [O_2] (Tukey adjusted post hoc test: OXPHOS respiration: $p < 0.001$; ATP production: $p = 0.011$, figure 2*a*,*b*), while there was no difference in LEAK respiration and COX activity between mitochondria tested at 16°C/Low [O_2] and 13°C/High [O_2] (Tukey adjusted post hoc test: LEAK: $p = 0.53$; COX activity: $p = 0.12$, figure 2*c*,*d*).

4. Discussion

The key result from our study is that plasticity of the mitochondrial metabolism has the capacity to compensate for the acute warming effect on ATP/O ratio when oxygen availability declines. Acute warming decreased ATP/O ratio but deoxygenation marginally raised ATP/O ratio. As a result, mitochondria under high temperature and low oxygen availability operate as efficiently as mitochondria under low temperature and high oxygen availability. The predicted decline in the performance of aquatic ectotherms owing to acute warming and oxygen depletion may be not as severe, since the predictions typically look at respiratory capacity and ignore ATP synthesis capacities and efficiencies ([7,25] but see [17]).

The vast majority of the ATP present in organisms is generated by mitochondria and can only be measured through *in vitro* assays of the rate of ATP production ([22] but see [26]). However, these assays measure mitochondrial ATP generated under highly controlled conditions. Thus, there are benefits and limitations to phenotype mitochondrial metabolism *in vitro*. On the one hand, while previous studies generally describe the response of mitochondrial properties to very high change in assay temperature, the 3°C warming used in our study might be more biologically relevant acute warming in relation to the seabass environment. This warming was defined based on recorded extremes of daily thermal range over the past decade in Brest Harbour [24], where juvenile seabass naturally live. Because variation in the daily thermal ranges is predicted to increase in both frequency and intensity as a result of climate change [10], quantifying mitochondrial responses at extended thermal range may also be warranted. On the other hand, assessing mitochondrial properties in response to the warming-induced decline in oxygen availability would require partial pressures of oxygen set to *in vivo* levels at 13°C and 16°C during measurement of mitochondrial properties. These considerations can quickly become logistically challenging as the difference in cellular oxygen levels *in vivo* for a 3°C warming is difficult to reach with the equipment suitable for ATP/O ratio assay in fish mitochondria. There is a need to develop more sophisticated techniques for measuring mitochondrial metabolism to extrapolate *in vitro* results to the *in vivo* situation.

It has recently become apparent that mitochondrial efficiency has a strong positive effect on organism performance, such as locomotor ability [27], feeding efficiency [28] and growth performance [29,30]. Understanding the scope for mitochondrial plasticity has then become critical for predicting how populations can respond to environmental change [31,32]. Our study is the first to indicate compensation of the combined effects of deoxygenation and warming on mitochondrial ATP/O ratio. The decrease in the ATP/O ratio in response to warming and the increase in ATP/O ratio in response to deoxygenation have previously been observed independently in mitochondria from birds [33] and rats [21]. Our study therefore underlines the importance of integrating the combined effects of temperature and oxygen availability on mitochondrial metabolism. Interestingly, trends for increase in ATP/O ratio in response to deoxygenation suggest that mitochondrial efficiency in the red muscle of seabass is controlled by a corresponding decline in LEAK respiration. An increase in ATP/O ratio with decreasing LEAK respiration may therefore represent a decrease in the proportion on energy dissipated through proton leakage across the inner mitochondrial membrane, and in turn increasing that energy in ATP synthesis [18]. These *in vitro* measurements nonetheless are likely to indicate the direction of the mitochondrial responses in the live animal [17]. This is particularly relevant in ectotherms where the temperature and oxygen conditions that the mitochondria are exposed to can vary within the animal on a short time scale. Our results suggest that animal performance under ongoing climate change will likely depend on their plasticity in the aerobic metabolism, especially the change in the efficiency of their mitochondria in producing ATP.

Ethics. Use of animals was in accordance with directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes. All procedures were carried out under the jurisdiction of the French Department of Veterinary Services (license no. B 29-212-05).

Data accessibility. Data are available via Dryad Digital Repository: https://doi.org/10.5061/dryad.dfn2z34zk. [34]. Thoral E, Roussel D, Chinopoulos C, Teulier L, Salin K. 2020 Data from: Low oxygen levels can help to prevent the detrimental effect of acute warming on mitochondrial efficiency in fish. Dryad Digital Repository. (doi:10.5061/dryad.dfn2z34zk)

Authors' contributions. E.T. and K.S. conceived the study and conducted the experiment; all authors analysed and interpreted the data. E.T. and K.S. led the writing of the manuscript. L.T., D.R. and C.C. revised the manuscript and added comments; all authors approved the final version of the manuscript and agree to be held accountable for the work performed.

Competing interests. We declare we have no competing interests.

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