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Multigenerational exposure to temperature influences mitochondrial oxygen fluxes in the Medaka fish (Oryzias latipes)

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Abstract :

Aim

Thermal sensitivity of cellular metabolism is crucial for animal physiology and survival under climate change. Despite recent efforts, effects of multigenerational exposure to temperature on the metabolic functioning remain poorly understood. We aimed at determining whether multigenerational exposure to temperature modulate the mitochondrial respiratory response of Medaka fish.

Methods

We conducted a multigenerational exposure with Medaka fish reared multiple generations at 20 and 30°C (COLD and WARM fish, respectively). We then measured the oxygen consumption of tail muscle at two assay temperatures (20 and 30°C). Mitochondrial function was determined as the respiration supporting ATP synthesis (OXPHOS) and the respiration required to offset proton leak (LEAK(Omy)) in a full factorial design (COLD-20°C; COLD-30°C; WARM-20°C; WARM-30°C).

Results

We found that higher OXPHOS and LEAK fluxes at 30°C compared to 20°C assay temperature. At each assay temperature, WARM fish had lower tissue oxygen fluxes than COLD fish. Interestingly, we did not find significant differences in respiratory flux when mitochondria were assessed at the rearing temperature of the fish (i.e., COLD-20°C vs. WARM −30°C).

Conclusion

The lower OXPHOS and LEAK capacities in warm fish are likely the result of the multigenerational exposure to warm temperature. This is consistent with a modulatory response of mitochondrial capacity to compensate for potential detrimental effects of warming on metabolism. Finally, the absence of

significant differences in respiratory fluxes between COLD-20°C and WARM-30°C fish likely reflects an optimal respiration flux when organisms adapt to their thermal conditions.

Keywords : cellular respiratory, ectotherms, global warming, long-term exposure, tail muscle

1. INTRODUCTION

Temperature is one of the most important drivers of climate change. In particular, increasing temperature can result in (a) higher individual growth rates [1,2], (b) earlier maturity [1,3], (c) smaller adult body size [1,4,5] and (d) shorter lifespan [6]. These responses are at least partly driven by processes at the metabolic scale [4,7,8] which suggests that better understanding how temperature affects metabolism is key to determine ecological consequences of climate change. It was early demonstrated that the metabolic rate of ectotherms increases with warming, accelerating cellular respiratory and oxygen demand following the Arrhenius law [9–11]. Conversely, decreasing temperature reduces the production of adenosine triphosphate (ATP) in ectotherms mitochondria [12–16]. These thermal effects may be particularly important for aquatic freshwater ectotherms, especially threatened by global warming [17,18] and that have fewer possibilities for behavioural thermoregulation or dispersal compared to marine or terrestrial species [19]. Warming also decreases oxygen concentration in water [20] inducing a mismatch between the oxygen demand and oxygen concentrations [2,21,22].

Mitochondria are the central organelle of metabolism and are responsible for almost 90% of the cellular oxygen consumption of an organism [23]. They contain the enzymatic systems generating cellular energy in the form of ATP by oxidative phosphorylation (OXPHOS), i.e. the coupling between substrate oxidation requiring oxygen, and the phosphorylation of ADP (Adenosine diphosphate) into ATP [24]. Mitochondrial responses to elevated temperatures are multiple [25]. According to previous studies, temperature leads to (*a*) an exponential increase in the rate of consumed oxygen molecules related to ATP production [11,26,27], (*b*) the production of cellular damages [26–28] and (*c*) a modification of the activities and functionalities of several enzymes, membrane proteins and gene regulatory mechanisms [29,30].

Climate is changing gradually, acclimation and evolutionary responses can be fast [31,32], it is therefore essential to better understand the long term mitochondrial responses induced by the environmental temperature on aquatic freshwater organisms. Previous studies highlighted that warm and cold acclimation in ectotherms can lead to compensation in mitochondrial functions [33–36]. In particular, long-term exposure to higher temperature can increase mitochondrial efficiency and decrease mitochondrial metabolism through: (*a*) a decrease in mitochondrial respiration rates, (*b*) a decrease in mitochondrial density and (*c*) modifications of enzyme structure or compositional changes within mitochondrial membranes (i.e. phospholipid proportions and fatty acid composition) [25,33,35–45]. Studies have reported that ectotherms from colder environments (higher latitudes or altitudes) have elevated metabolic rates compared to those from warmer climates [46,47]. This has been reported for fish where northern populations are increasing their mitochondrial oxidative capacities and mitochondrial density as a compensatory mechanism [12,15,48]. In addition, seasonal exposure to cold temperature can enhance aerobic metabolism by increasing mitochondrial densities and/or capacities of mitochondrial enzymes [13,16].

These adjustments may result both from genetic adaptation over generations or reversible plastic modifications in mitochondrial structure and function. Although there is a growing interest for the study of mitochondrial adjustments in response to temperature, most studies focused on populations from thermally contrasted environment [33,38,39,49] or on seasonal acclimatization [41,45,50,51]. However, in these empirical studies, other environmental factors such as luminosity, photoperiod, pH and nutrients can covariate with temperature which makes it difficult to quantify the influence of temperature on mitochondrial response. In addition, the time required for thermal acclimation to be completed is often assumed rather than measured, which can biased conclusions regarding the acclimation status of the studied processes [52]. In a rare study, Shama *et al.* (2016) [44] showed that, in marine sticklebacks, acclimation of

grandmother to warming lead to lower and optimized offspring mitochondrial respiratory. However, there is no consensus on how acclimation within or across generations modulates mitochondrial oxygen flux in response to temperature. In addition, to our knowledge, there is no multigenerational exposure study on mitochondrial responses to temperature in freshwater aquatic organisms, despite their lower possibilities for behavioural thermoregulation or dispersal in comparison to marine or terrestrial species [19]. This investigation is thus important to improve our understanding of environmentally driven variation in mitochondrial responses [25,53,54] and by consequence, to understand how freshwater organisms can cope with climate change.

To determine whether multigenerational exposure to temperature can modulate mitochondrial thermal response, we investigated mitochondrial oxygen fluxes in the Medaka fish *Oryzias Latipes* (Adrianichthyidae) after rearing them for two years at two contrasted temperatures (20 and 30°C). These represent non extreme temperatures falling within the natural range experienced by the species [55,56]. Using a full factorial design with fish from the same parental group (F_0) reared over several generations at 20 °C (F_3) or 30 °C (F_4) crossed with two assay temperatures (20 °C or 30 °C). We expected (H1) higher oxygen fluxes for mitochondria assessed at 30 °C compared to 20 °C. We also hypothesized that (H2) long-term exposure to temperature would lead to a modulation of mitochondrial response through modifications in (H2.i) mitochondrial efficiency, (H2.ii) mitochondrial density or (H2.iii) mitochondrial capacities. H1 is supported by previous studies showing that mitochondrial activities is higher at warmer assay temperatures compared to cooler ones [10,13,16,26,57,58] whereas H2 is motivated by the hypothesis that thermal acclimations in ectotherms can results in compensation of mitochondrial functions [33–36].

2. RESULTS

Tissue oxygen consumption:

After two years of multigenerational exposure to 20 and 30°C, we measured oxygen consumption from a wet weight-controlled skeletal tail muscle minced of Medaka fish using two Oxygraph-2k high-resolution respirometers (Oroboros Instruments, Innsbruck Austria). Oxygen consumption was measured for two mitochondrial states, corresponding to the rate of respiration supporting ATP synthesis (OXPHOS) and the respiration required to drive proton leak (LEAK), obtained using oligomycin (i.e.LEAK(Omy)). Finally, we estimated the COX capacity as a proxy of maximal mitochondrial oxidative capacity, using Ascorbate + TMPD as substrates. This choice is motivated by results from Larsen et al, 2012 who reported a strong correlation (r = 0.94, p < 0.001; Figure 3.H) between permeabilized fiber respiration rate using Ascorbate + TMPD as substrates, and the mitochondrial content (total mitochondrial volume) for skeletal muscle of human subject [59].

For each respiratory state and the COX capacity, we used a linear mixed model (LMM) to test effects of rearing temperature, assay temperature, and their interaction on the tissue oxygen fluxes (in picomol.s^{-1}.mg^{-1}). The statistical interaction between fish rearing temperature and assay temperature was non-significant for both OXPHOS *(Chisq = 0.97, df = 1, p = 0.32*) and LEAK(Omy) (*Chisq = 0.80, df=1, p = 0.37*) indicating that effects of assay and rearing temperatures on oxygen fluxes were additive. Assay temperature significantly affected OXPHOS oxygen flux (*Chisq =7.42, df =1, p = 0.005****) (Fig. 1A) and LEAK(Omy) oxygen flux *(Chisq = 10.05, df = 1, p = 0.002 **)* (Fig. 1B). For both WARM and COLD fish, we found that tissue assessed at 30°C have higher oxygen fluxes than those assessed at 20°C (Fig. 1A, Fig. 1B). Moreover, we found a significant effect of fish rearing temperature on oxygen fluxes at OXPHOS (*Chisq = 20.76, df = 1, p =* <0.0001***) (fig.1A) and LEAK(Omy) states *(Chisq = 17.62, df= 1, p=* <0.0001***) (Fig. 1B). At each assay temperature, the tissue of WARM fish had lower oxygen fluxes compared to the tissue of COLD fish (Fig. 1A, Fig. 1B).

Finally, we compared oxygen fluxes between COLD fish assessed at 20°C and WARM fish assessed at 30°C to test if oxygen fluxes can converge to similar levels after multigenerational exposure. We found that no significant differences in their oxygen fluxes for both OXPHOS *(Chisq = 2.05, df = 1, p = 0.15)* and LEAK(Omy) (*Chisq = 2.006, df = 1, p = 0.16*) despite a tendency towards lower flux for WARM fish.

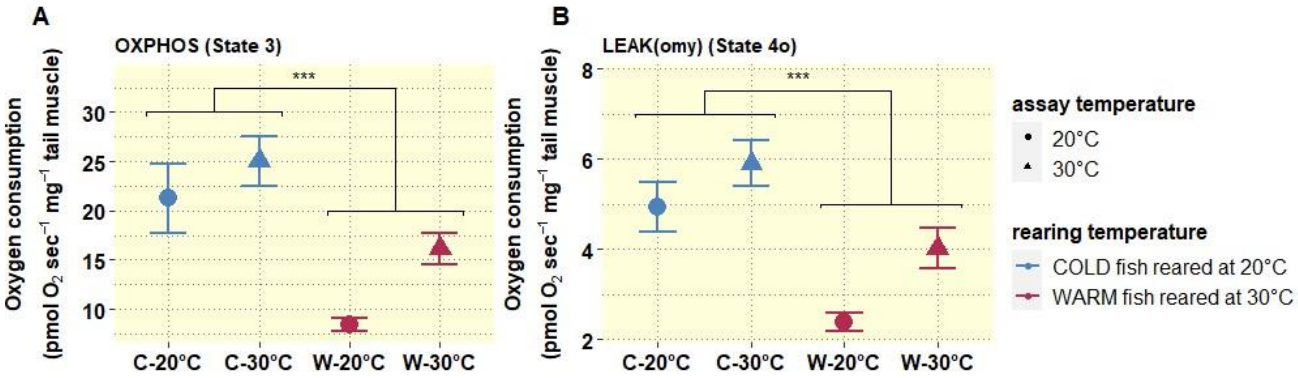

Figure 1 *: (A) OXPHOS respiration mean (± SE) O_2 fluxes in picomol.s^{-1}.mg^{-1} and (B) LEAK(Omy) respiration mean (± SE) O_2 fluxes in picomol.s^{-1}.mg^{-1} according to fish rearing temperature (COLD in blue or WARM in red) and assay temperature (20°C, dots or 30°C, triangles).*

Respiratory Control Ratio (RCR):

For each sample, we next computed the Respiratory Control Ratio (RCR) as the ratio of ATP-synthesizing tissue respiration (OXPHOS respiration) over tissue respiration in the absence of ATP-synthesizing (LEAK(Omy) respiration) [60,61]. The RCR thus reflects the coupling of oxidative phosphorylation through the Electron Transport System flux and is thereby a measure of the efficiency of converting substrates to ATP (respiratory efficiency). We tested the effects of fish rearing temperature (20 °C or 30 °C), assay temperature (20 °C or 30 °C) and their interactions on the respiratory control ratio (RCR) using a LMM.

We found no significant effect of fish rearing temperature (*Chisq = 0.21, df = 1, p = 0.65*), assay temperature (*Chisq* = 2.78, *df* = 1, *p* = 0.09) or their interaction (*Chisq = 0.87, df = 1, p = 0.35*) on the RCR (Fig. 2).

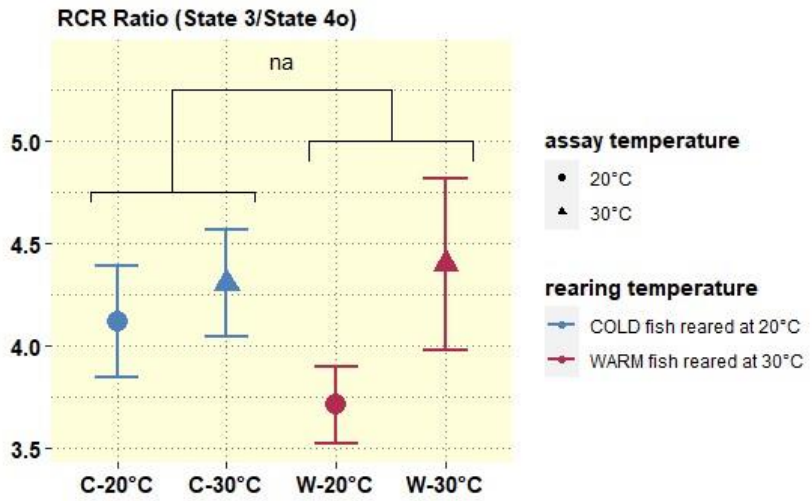

Figure 2: *Mean (± SE) of RCR according to fish rearing temperature (COLD in blue or WARM in red) and assay temperature (20 °C, dots or 30 °C, triangles).*

OXPHOS and LEAK fluxes across COX capacity gradients:

To test whether the OXPHOS (state 3) and LEAK(Omy) (state 4o) oxygen fluxes remain similar across fish rearing temperature along the COX capacity flux gradient, we used a mixed ANCOVA model with OXPHOS or LEAK(Omy) fluxes as response variables and COX capacity flux and fish rearing temperature as fixed independent variables.

We found no significant effect of the interaction between fish rearing temperature and COX capacity for both OXPHOS *(Chisq = 0.99, df = 1, p = 0.32*) and LEAK(Omy) fluxes (*Chisq = 3.79, df = 1, p = 0.052*). For both OXPHOS and LEAK(Omy) states, we found that WARM fish had a lower intercept than COLD fish but their slopes were not significantly different. (Fig. 3A, Fig. 3B). We found no significant impact of fish rearing temperature on oxygen fluxes for the COX capacity state (*Chisq* = 0.01, *df* = 1, *p* = 0.9).

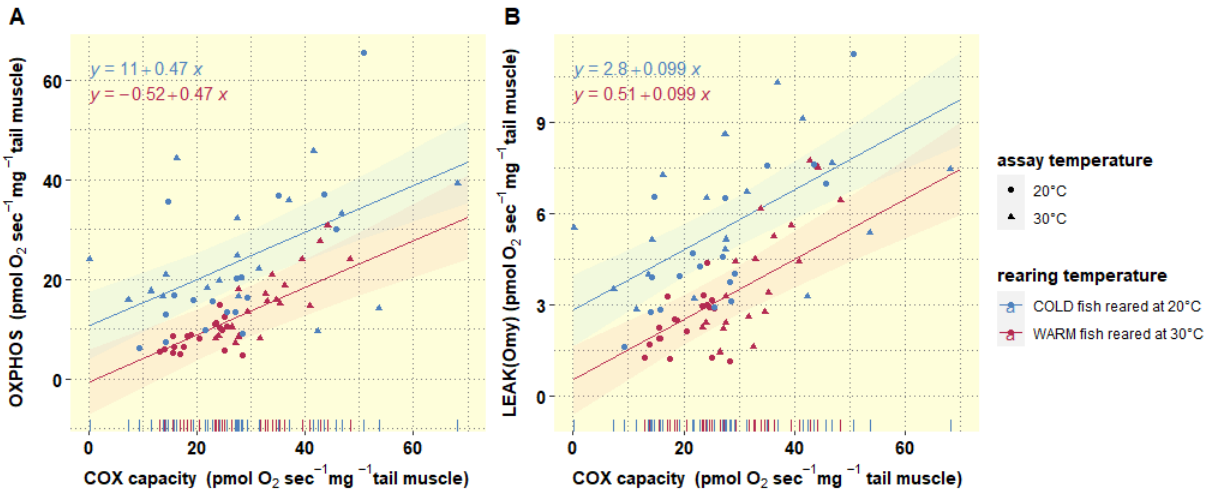

Figure 3: *(A) OXPHOS O_2 fluxes in picomol.s^{-1}.mg^{-1} and (B) LEAK(Omy) O_2 fluxes in picomol.s^{-1}.mg^{-1} as a function of the COX* capacity *oxygen flux according to fish rearing temperature (COLD in blue or WARM in red) and Assay temperature (20°C, dots or 30°C, triangles).*

However, we found a significant impact of fish assay temperature on oxygen fluxes for the COX capacity (*Chisq* = 8.6, *df* = 1, *p* = 0.003**) reflecting the dependency of COX capacity to temperature (Fig. 4).

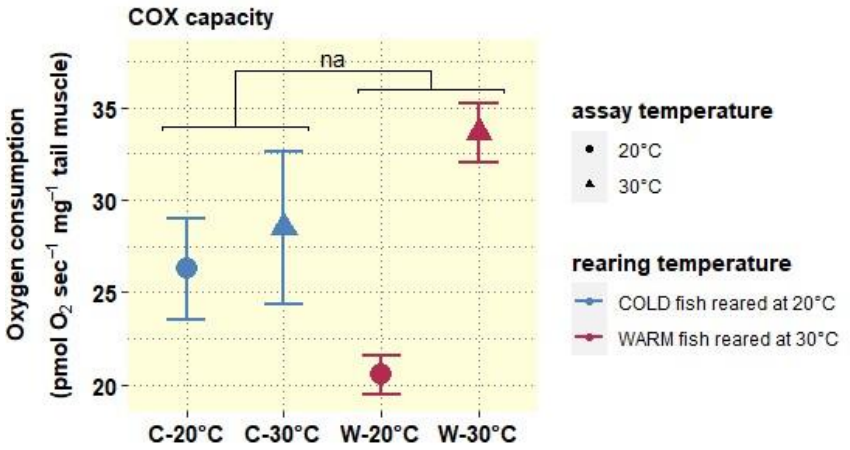

Figure 4: *COX* capacity *mean (± SE) O_2 fluxes in picomol O_2.s^{-1}.mg^{-1} according to fish rearing temperature (COLD in blue or WARM in red) and assay temperature (20°C, dots or 30°C, triangles).*

3. DISCUSSION

The long-term impact of temperature on mitochondrial respiration is crucial for understanding how organisms can cope with temperature changes. It is well established that elevated temperature increases cellular oxygen demand which, in turn, increases mitochondrial oxygen consumption rates and ATP production [9,4,10,27,13,16]. Some studies highlight that warm and cold acclimations in ectotherms can result in compensation of mitochondrial functions through modifications in mitochondrial respiration rates, density or compositional changes within membranes [33–36]. However, these responses can vary across studies depending on exposure duration and species studied. As a result, there is no consistent general pattern in the way within or across generation, acclimation to temperature modulates mitochondrial oxygen fluxes. Following these previous studies, our objective was to determine whether ectotherms could modulate their mitochondrial oxygen fluxes after a multigenerational (2 years) exposure to temperature.

We found that, for both COLD and WARM fish, OXPHOS and LEAK(Omy) respiration fluxes were higher for mitochondria assessed at 30°C than at 20°C. Our findings are in line with previous studies showing that temperature increases the rate of consumed oxygen molecules related to ATP production and accelerates the speed of biochemical reactions [10,13,16,26,27,37,57,58]. This thermal impact can lead to a live fast-die young strategy where organisms live fast with higher reproduction and growth (maximizing fitness), which may counteract cellular damages accumulation that reduces performance with age [4,62].

Interestingly, we found that, at each assay temperature, mitochondria from WARM fish had lower oxygen fluxes than those of COLD fish. These results are in line with our second hypothesis, suggesting a modulation of the mitochondrial response over the long-term which

could be a mechanism to counteract short-term temperature exposure effects. Portner (2002) [22] highlights that cold adaptation may lead to an increasing temperature dependence of mitochondrial oxygen demand. This effect is at least partially explained by compensatory mechanism leading to an increase in mitochondrial oxidative capacities and/or mitochondrial density, as reported in previous studies on cold long-term exposure [12,13,15,48]. According to Skulachev (1998) [63], mitochondrial uncoupling can decrease the production of cellular damage, and thus potentially counteract effects of high temperatures in the long term (mechanism H2i). This phenomenon can reduce damage formation via phospholipids membrane composition and uncoupling proteins (UCPs) [58,61,64,65], leading to lower ATP production and mitochondrial efficiency [63]. Studies suggest that UCPs may be differentially regulated in response to thermal long-term exposure in ectotherms and thus can modulate the mitochondrial response [66,67]. Under the uncoupling hypothesis, we expected a low RCR for warm fish indicating low phosphorylating oxidation capacity relative to the oxidation required to compensate for proton leakage (mitochondrial coupling efficiency). However, we found no significant difference in RCR between treatments, which implies that fish maintained their respiratory efficiency regardless of the temperature treatments. To deepen our results understanding, it would be interesting to quantify uncoupling proteins (UCPs) even if their physiological functions are not yet clearly established in fish [68,69], as well as phospholipids in the membrane and the ATP production/O_2 consumption ratio.

A second mechanism that may explain a modulation of the mitochondrial response is related to mitochondrial density (H2.ii). Previous studies suggest that the extent of thermal tolerance results from limitations imposed by mitochondrial function and density [22,70]. For instance, fish seasonal cold acclimatization can increase mitochondrial density or mitochondrial aerobic capacity [50,71], while fish populations living in warm environments often have lower mitochondrial densities [72]. Thus, a lower mitochondrial density would decrease oxygen fluxes

and could explain an overall decrease in oxygen consumption in warm exposed fish. Conversely, in cold acclimated fish, an increase in mitochondrial density would increase the amount of mitochondrial enzyme needed to maintain high reaction rates [73] which may also contribute to explain the differences between WARM and COLD exposed fish in our study. Using COX capacity fluxes, we investigated if the differences between WARM and COLD fish are associated to difference in mitochondrial densities. We found no significant effects of fish rearing temperature on oxygen fluxes for COX capacity state. This result could suggest that mitochondrial density was not influenced by the multigenerational exposure to temperature in our study. We also found that, for a similar COX capacity flux, WARM fish have lower oxygen fluxes that COLD fish for OXPHOS and LEAK(Omy) states. However, we need to nuance this results. First, the rate of auto-oxidation of Asc and TMPD in presence of sodium-azide was not evaluated in our study. Second, strong correlation between fiber respiration and Asc+TMPD and mitochondrial content found in Larsen et al. (2012) might be different in minced tissue. We therefore cautiously conclude that our results are probably not explained by changes in mitochondria density.

Overall, our findings suggest that uncoupling or mitochondrial density changes are not the main mechanisms that could explain the observed modulation in mitochondrial respiration. A third mechanism could be a modulation of mitochondrial metabolic capacity in response to multigenerational exposure to high and low temperatures (H2.iii). This suggests internal changes in mitochondrial function through the implementation of regulatory mechanisms over generations through physiological changes, enzyme isoform, protein structure and membrane fluidity modifications or in the degree of unsaturation of the fatty acids [37,42,74]. However, it is difficult to determine when and where (internal or external to the mitochondria) this regulatory mechanisms occur. Another possibility could be (ii) a maintenance of the ATP production/O_2 consumption ratio. After several generation, WARM fish may produce less ATP and thus have

lower oxygen requirements while still having an efficient ATP/O_2 ratio. Conversely, COLD fish may produce more ATP and have higher oxygen requirements.

Understanding whether modifications in mitochondrial oxygen fluxes translate to changes in individual respiration rate is important for the integration of thermal effects across scales. Interestingly, a recent study showed that nine years of experimental warming did not influence the thermal sensitivity of individual respiration rate in medaka fish [75] which contrasts with previous studies [71,76,77]. This phenomenon of maintaining respiratory efficiency could also be related to the absence of significant differences in respiratory flux when fish were assessed at their rearing temperature (i.e. COLD fish assessed at 20°C vs WARM fish assessed at 30°C). This could reflect the existence of an optimal respiration flux when organisms have enough time to acclimate or adapt to local conditions. A next step would be to investigate whether these processes are linked to selection over several generations or to plasticity of individuals or of the mitochondria itself. In a global change context, our results indicate that exposure to temperature during two years over several generations can modulate the thermal responses at the mitochondria level. Interestingly, this lead to similar mitochondrial respiratory rates when comparing the mitochondrial respiration of fish assessed at their rearing temperature (i.e. COLD-20°C vs. WARM-30°C). This result suggests that multigenerational exposure to temperature should be taken into account to predict the ecological impacts of global warming that are assessed using the metabolic theory of ecology under which metabolic rate increases exponentially with temperature [8,78,79]. It would thus be interesting to investigate potential trade-offs between increasing energy allocation in life history traits and reducing the production of pro-oxydant, and, in turn, cell senescence. We also need to determine the origin of changes in mitochondrial phenotypes because, after several generations, plasticity, genetic and/or epigenetic inheritance could have influenced the frequency of the phenotype in our populations [80,81]. Especially, epigenetic mechanisms such as histone acetylation, DNA and RNA

methylation, can be passed from one generation to another and induce changes across generations [82–86]. It is possible that differences in the number of generations in our study can lead to a higher accumulation of epigenetic modifications for WARM fish compared to COLD fish and affect how mitochondrial traits are responding to assay temperature. However, in a previous study on Médaka (*Orizias Latipes*) testing the impact of warm multigenerational exposure on growth pattern [49], we found that the effects of growth and reproduction were mainly plastic responses. We thus attribute our result to plastic acclimation effect although molecular analyses would be need to rule out the influence of genetic or epigenetic variations.

Another potential factor of metabolic stress is a lower oxygen concentration in warmer waters, while metabolic demand increases with temperature. In our experiment, oxygen concentration was lower at 30°C but was not limiting (88,4%) and non-stressful. Furthermore, the impacts of low oxygen concentration on fish are complex and the question of whether partial pressure or solubility limits oxygen supply is still poorly understood [87]. This suggests that our results are probably not explained by limitations in oxygen availability.

To conclude, our results indicate that multigenerational exposure leads to modifications in mitochondrial respiration without modifications of the oxidative phosphorylation process efficiency or mitochondrial density. Our study highlights that multigenerational exposure can leads to a modulatory response to compensate for potential detrimental consequences of temperature and highlights that both responses (i.e. rearing temperature versus assay temperature) should be taken into account to better understand how organisms can cope with temperature changes. Finally, the absence of significant differences in respiratory flux between COLD fish assessed at 20°C and WARM fish assessed at 30°C in our experiment could reflect the existence of an optimal respiration flux when organisms have sufficient time to acclimate or adapt to local conditions. Future studies are required to better understand the diversity of mechanisms by which organisms can modulate their mitochondrial capacity under temperature changes.

4. MATERIALS AND METHODS

Biological model and rearing conditions:

The medaka fish *Oryzias Latipes* is a small (20-40 mm) amphidromous freshwater fish from Southeast Asia. It is an eurythermal fish that can live in temperatures ranging from 0 to 40°C [55,56] with an optimum individual growth temperature at 25°C [88] and can reach sexual maturity within only 10–12 weeks at 27°C [89], making it a good biological model for multigenerational thermal experimentation. It has an average lifespan of 1 year in the wild and up to 5 years in captivity [56]. Medaka used in this study were reared at 20 °C or 30 °C (non-stressful temperatures) (COLD and WARM fish, respectively) during two years over several generations under controlled experimental conditions in climatic chambers. WARM and COLD fish used in the experiments present differences in generation numbers (4 and 3, respectively) due to a shorter generation time at 30°C compared to 20°C. Difference in the number of generation is due to warming condition applied in this study, as fish grow faster and have an early sexual maturity when temperature increase [1,3,75]. To get F_4 COLD fish, the experiment should have been run for an additional year. As a consequence, we preferred comparing mitochondrial respiration of fish at the same date rather than at the same generation to reduce the risk of changes in experimental conditions experienced by WARM and COLD fish (e.g. water quality or potential diseases) over a year, as well as potential changes in conditions of oxygen flux measurements.

The climatic chambers are equipped with temperature and humidity sensors to control room conditions. Water is controlled by mechanical, biological and UV filters and is supplied from a buffer tank via an open-circuit system, with drip feed (1 L. h^{-1}). Photoperiod is controlled by timers and set at 12:12 (L:D). The two climate chambers are composed of two rows of five

aquariums. The two lineages were obtained from a starting F_0 population of 320 Medakas (160 females & 160 males) belonging to the CAB strain from Carolina Biological Supply Company (USA) and provided by AMAGEN© (France). F_0 generation fish were maintained at 25°C (i.e. the temperature at which AMAGEN reared their strain) and were then progressively acclimatized to 20 or 30 °C at a rate of one degree every 2 days. To start a new fish generation, we collected egg clutches during the optimal fecundity period (i.e. when the number of laying females and the average number of eggs per female per day were at their maximum) and placed them in hatcheries at the parental temperature (20 °C or 30 °C). We maintained the newborn fish in climatic chambers at the same temperature as their parents (20 °C or 30 °C) and then placed them in five replicates aquariums (25 × 40 × 20 cm) until maturity, with a density of ~30 adult fish per aquarium. Fish were fed "ad libitum" with dry flakes food TétraMin© three times a day (at 9h, 13h and 18h). Oxygen concentration during the experimental period in our climatic chamber tanks were 8.32 ± 0.33 O2_mg/L (91,5%) at 20°C and 6.62 ± 0.59 O2_mg/L (88,4%) at 30°C. The oxygen concentration is maintained by means of an air pump feeding bubbler-filters in each aquarium. In our experiment, oxygen concentration was lower at 30°C but was not limiting and non-stressful.

Experimental design:

After two years of multigenerational exposure to 20°C and 30°C, we aimed at measuring oxygen consumption from minced skeletal tail muscle of Medaka fish using full factorial experimental design with two rearing temperatures: [20 °C (COLD; C) and 30 °C (WARM; W)] crossed with two assay temperatures (20 and 30 °C). To measure oxygen consumption we used 18 COLD fish from F_3: 10 adult females (mean age ± s.e: 326.8 ± 4.5 days) and 8 adult

males (325. 5 ± 6.5 days) from F_3. We used 19 WARM fish: 9 adult females (230.3 ± 6.6 days) and 10 adult males (227.4 ± 6.6 days) from F_4.

We used two muscle samples of each fish for the two assay temperatures (one sample per temperature) in the oxygraph (i.e. 36 muscular samples for COLD fish and 38 muscular samples for WARM fish).

Tissue respiratory measurement:

Medakas were euthanized by percussion of the cranial cavity and then measured (mm), weighed (mg) and dissected to sample muscle from the two flanks of each fish. Dissections were conducted within five minutes following euthanasia on a freezer block to slow down cellular destruction. For each fish, 4 mg (Mean ± SEM = 4.02 ± 0.50 mg) of skeletal tail muscle from each of the two flanks were collected with a scalpel and fine forceps under binocular magnifying glass, then they were cut using micro-dissecting scissors and minced to obtain a minced solution with particle size lower than 0.5 mm (tested by pipetting through 1mL tip) buffered in 1 mL of 0.5M Respiratory Medium (MIR05) [90] (EGTA 0.5mM, MgCl2 3 mM, Lactobionic acid 60mM, Taurine 20mM, KH2PO4 10 mM, HEPES 20 mM, D-Sucrose 110 mM). Tissue minced were then diluted in 1 mL MIR05 to obtain a final concentration of approximately (2mg muscle/mL of MIR). Conventional homogenization and mitochondrial isolation (e.g. using homogenisers) procedures were not feasible for the small tissue samples of this study. The technique for preparation of the muscle shredding was adapted from [91–93] and has already been validated in fish tissue and published in full detail elsewhere [94,95].

Oxygen consumption (in picomol.s^{-1}.mg^{-1}) from a wet weight-controlled skeletal tail muscle minced of Medaka fish were measured using two Oxygraph-2k high-resolution respirometers (*Oroboros Instruments, Innsbruck Austria*), each equipped with two independent measuring

chambers (2.1 mL). Immediately following preparation, minced tissue from each fish were added to one of the two measurement chambers of the oxygraph. For each individual fish, we tested simultaneously one flank at 20 °C and the other at 30 °C. We established oxygen flux curves at different states of oxidative phosphorylation, corresponding to different functions of the respiratory chain (OXPHOS, LEAK(Omy), COX-capacity and ROX state, described hereafter). To fulfill this purpose we followed an injection protocol of different substrates enabling the activation or inhibition of ATP-producing enzyme complexes (following [94,96,97]). The oxygen electrodes were calibrated at air-saturated MiR05 and zero oxygen after sodium dithionite addition. The minced muscle is added to the measurement chamber at 20 °C or 30 °C and a sequential substrate/inhibitor protocol is initiated. Several minutes after each substrate injection, a plateau is reached for the oxygen flux. The average flux value of the plateau is retrieved using Datlab software (*Oroboros Instruments*) in picomol.s^{-1}.mg^{-1}. First, the Krebs cycle and glycolysis are simulated by adding 5 µL of 2 M (5 mM) Pyruvate, 2.5 µL of 400 mM (0.5 mM) Malate and 20 µL of 1 M succinate (10 mM). These substrates promote the electrons entry. Then, the OXPHOS state (state 3) is reached by adding a saturating concentration of ADP of 16 µL at 500 mM (4 mM). This state corresponds to the maximum oxidative phosphorylation capacity of the membrane (state 3) that comprises both proton leak rate and phosphorylating rate. The LEAK(Omy) state (state 4o) is then induced by the addition of 1 µL of Oligomycin at 0.01 mM (5 nM), a complex V inhibitor thereby prevent phosphorylation so that proton leak rate can be isolated from state 3. The residual oxygen consumption, ROX state, is obtained by the addition of 1 µL of 5 mM (2.5 µM) Antimycin A, an inhibitor stopping the functioning of the respiratory chain, in order to determine residual oxygen consumption. Finally, the COX capacity state is reached by adding 20 µL of of 800 mM Ascorbate (8 mM), and a 5µL of 200mM TMPD (0.5 mM) to obtain the Cytochrome C oxidase (COX) associated flux that is a proxy of mitochondrial density [59]. The auto-oxidation of Asc and TMPD was not evaluated in

this protocol. The residual oxygen flux (ROX) is subtracted from each state (OXPHOS, LEAK(Omy) and COX capacity) to correct fluxes values.

Respiratory Control Ratio:

The Respiratory Control Ratio (RCR) reflects the coupling of oxidative phosphorylation through the Electron Transport System flux and is thereby a measure of the efficiency of converting substrates to ATP (respiratory efficiency). It is calculated as the ratio of ATP-synthesizing mitochondrial respiration (OXPHOS state) over mitochondrial respiration in the absence of ATP-synthesizing (LEAK(Omy) state) [60,61].

Statistical analyses:

We tested the effects of fish rearing temperature, assay temperature and their interactions on oxygen fluxes at the OXPHOS, LEAK(Omy), COX capacity state and on the RCR using linear mixed models (LMMs). We initially included the time from dissection to the start of the measurement, and the identification number of the measurement chamber as fixed effects in the LMMs but, in the absence of significant effects, they were finally excluded from the analyses. The measurement date was included as a random effect, as well as the identity of individual fish and their sex, to account for the repeated measurements (two measurements for each fish). Application conditions for the LMMs (homoscedasticity, normality, independence of residuals) were tested and the significance of the fixed model terms was assessed using analyses of deviance (Anova function from the car package). LMMs were computed using the lme4 package [98]. To test for differences in mitochondrial density between the two fish rearing temperature, we used a linear mixed model with COX capacity flux as a response variable and rearing temperature as fixed independent variable. Assay temperature was included in the

model as random effect to account for the thermal sensitivity of the COX capacity. The measurement date, the identity of individual fish and their sex were also included as random effects. To test whether the OXPHOS and LEAK(Omy) fluxes remain similar across rearing temperature for the correlated COX capacity flux, we used a mixed ANCOVA model with OXPHOS or LEAK(Omy) fluxes as response variables and COX capacity flux, rearing temperature and their interactions as fixed independent variables. Assay temperature, the measurement date, the identity of individual fish and their sex were also included as random effects. This model allowed to standardize fluxes by COX capacity and thus removing the potential influence of changes in mitochondrial density on oxygen fluxes.

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DATA AVAILABILITY STATEMENT:

Data are available online: https://doi.org/10.5281/zenodo.7904757

AUTHORS' CONTRIBUTIONS:

Julie Morla: Conceptualization-Equal, Data curation-Equal, Formal analysis-Lead, Investigation-Lead, Methodology-Equal, Writing – original draft-Lead, Writing – review & editing-Lead

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ANIMAL ETHICAL DECLARATION: No ethical permit was required under French legal requirements to conduct this experiment because experimental temperatures are within the range of non-stressful temperature for this species (Leaf et al. 2011; Shima and Mitani 2004). Fish were maintained in the laboratory under permit number A1300101 by the French authority of the "Direction départementale de la protection des populations des Bouches-du-Rhones" under the articles R 214-87, R 214-122, and R 215-10 of the French law.

Figures:

Figure 1 *: (A) OXPHOS respiration mean (± SE) O_2 fluxes in picomol.s^{-1}.mg^{-1} and (B) LEAK(Omy) respiration mean (± SE) O_2 fluxes in picomol.s^{-1}.mg^{-1} according to fish rearing temperature (COLD in blue or WARM in red) and assay temperature (20°C, dots or 30°C, triangles).*

Figure 2: *Mean (± SE) of RCR according to fish rearing temperature (COLD in blue or WARM in red) and assay temperature (20 °C, dots or 30 °C, triangles).*

Figure 3: *(A) OXPHOS O_2 fluxes in picomol.s^{-1}.mg^{-1} and (B) LEAK(Omy) O_2 fluxes in picomol.s^{-1}.mg^{-1} as a function of the COX* capacity *oxygen flux according to fish rearing temperature (COLD in blue or WARM in red) and Assay temperature (20°C, dots or 30°C, triangles).*

Figure 4: *COX* capacity *mean (± SE) O_2 fluxes in picomol $O_2.s^{-1}.mg^{-1}$ according to fish rearing temperature (COLD in blue or WARM in red) and assay temperature (20°C, dots or 30°C, triangles).*

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