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BRIEF COMMUNICATION

Chronic experimental hyperoxia elevates aerobic scope: a valid method to test for physiological oxygen limitations in fish

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Abstract

Experimental hyperoxia has been shown to enhance the maximum oxygen uptake capacity of fishes under acute conditions, potentially offering an avenue to test prominent physiological hypotheses attempting to explain impacts of climate warming on fish populations (*e.g.*, gill-oxygen limitation driving declines in fish size). Such benefits of experimental hyperoxia must persist under chronic conditions if it is to provide a valid manipulation to test the relevant hypotheses, yet the long-term benefits of experimental hyperoxia to oxygen uptake capacity have not been examined. Here, the authors measured aerobic metabolic performance of *Galaxias maculatus* upon acute exposure to hyperoxia (150% air saturation) and after 5 months of acclimation, at both 15°C and 20°C. Acute hyperoxia elevated aerobic scope by 74%–94% relative to normoxic controls, and an elevation of 58%–73% persisted after 5 months of hyperoxia acclimation. When hyperoxia-acclimated fish were acutely transitioned back to normoxia, they maintained superior aerobic performance compared with normoxic controls, suggesting an acclimation of the underlying metabolic structures/processes. In demonstrating the long-term benefits of experimental hyperoxia on the aerobic performance of a fish, the authors encourage the use of such approaches to disentangle the role of oxygen in driving the responses of fish populations to climate warming.

KEYWORDS

acclimation effect, gill-oxygen limitation theory, metabolic rate, oxygen- and capacity-limited thermal tolerance, oxygen supersaturation

1 | INTRODUCTION

Current warming of aquatic environments presents a major challenge to ectotherms like fishes, due largely to the thermal-dependency of their physiological processes (Fry 1947). An increase in temperature drives an increase in standard (resting) oxygen uptake rate (≈ aerobic metabolic rate), which has been implicated in the "temperature-size rule" (TSR) – the observed decline in maximum fish size with increasing temperature (Atkinson 1994). Two prominent hypotheses have attempted to describe universal mechanisms underpinning the TSR: (a) the gill-oxygen limitation (GOL) hypothesis proposes that the reduced asymptotic size of fish in warm conditions results from a mismatch between oxygen supply at the gills (gill surface area) and oxygen demand by tissues (standard oxygen uptake rate) (Pauly 1981); whereas (b) the oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis proposes that compromised growth of fish at high temperatures stems from a reduction in aerobic scope (AS, the difference between standard and maximum oxygen uptake rates) (Pörtner 2010). Thus, both of the leading hypotheses to explain the

TSR phenomenon place emphasis on oxygen as the limiting factor, yet this idea remains hotly debated (Clark, Sandblom & Jutfelt 2013; Lefevre, McKenzie & Nilsson 2017).

The debate continues in large part due to the lack of controlled, experimental approaches aimed at disentangling the effects of temperature and oxygen on fish metabolism and growth. In the relatively few available studies, it has been shown that acute hyperoxia [oxygen partial pressure (PO_2) above *c*. 21 kPa or 100% air saturation] can elevate the maximum oxygen uptake rate of fishes [see McArley, Sandblom and Herbert (2021)], increasing AS by as much as twofold in European perch (*Perca fluviatilis*) (Brijs *et al.* 2015). Although such studies in acute hyperoxia can provide some insight into the validity of the GOL and OCLTT hypotheses in driving the TSR, their usefulness is limited by their short temporal scale in comparison with the time span required for fish to reach sexual maturation and asymptote in body mass.

The potential to use experimental hyperoxia to test the validity of the GOL and OCLTT hypotheses in underlying the TSR relies on the physiological responses to hyperoxia enduring through time. Indeed, fish often exhibit a heightened physiological response to acute exposure to novel environmental conditions, but then acclimate over days or weeks of continued exposure such that the physiological parameters approach pre-exposure levels (Angilletta Jr. & Angilletta 2009). This is particularly well studied in relation to temperature, whereby physiological rates like standard oxygen uptake can increase abruptly with acute exposure to high temperature and subsequently decline towards pre-exposure levels after acclimation (Norin, Malte & Clark 2014; Sandblom *et al.* 2014). As far as the authors are aware, however, the observed benefits of hyperoxia in improving oxygen uptake capacity have not been shown to persist beyond acute exposure periods.

Here, to the authors' knowledge, they provide the first long-term assessment of hyperoxia on the aerobic metabolism of a fish. They reared the common galaxias *Galaxias maculatus* for 5 months in an orthogonal design of cold (15°C), warm (20°C), normoxic (PO_2 100%) and hyperoxic (PO_2 150%) conditions, and subsequently evaluated the standard and maximum oxygen uptake rates (herein referred to as standard and maximum aerobic metabolic rates; SMR and MMR, respectively) and aerobic scope (AS) of the fish. Their main aim was to determine whether hyperoxia provided an acute improvement in oxygen uptake capacity and whether this improvement was retained for the duration of the 5-month experiment. In addition, if growth is limited by oxygen and/or AS in normoxia at warm temperatures, as proposed within the GOL and OCLTT hypotheses (Pauly 1981; Pörtner 2010), they expected to observe improved growth rates in the 20°C hyperoxia treatment group if AS was chronically enhanced.

2 | MATERIALS AND METHODS

2.1 | Experimental animals and acclimation treatments

In early November 2020, *G. maculatus* were caught at *c*. 15°C from the Cumberland River mouth, Lorne, Victoria, Australia, using box traps and were subsequently kept in holding tanks for 2 months to acclimate to captivity at Deakin University's Queenscliff Marine Science Centre [see electronic supplementary material (ESM) "Animal capture and rearing"]. Fish were then weighed and evenly distributed across four independent recirculating rack systems at 16.5°C, each housing 10 rectangular tanks (25 L; 22 fish $tank^{-1}$) with salinity of 6–7 ppt. Over a week, the oxygen saturation and temperature of each rack was slowly manipulated as follows: (a) 20°C-hyperoxia (PO_2 150%), (b) 20°C-normoxia, (c) 15°C-hyperoxia (PO_2 150%) and (d) 15°C-normoxia. The warm temperature was chosen to approximate the highest temperature these fish experience for prolonged durations in the wild. Fish were reared in these treatments for at least 5 months (see ESM "Figure S1") and were fed to satiation 5 days a week (Otohime feed; BMAQUA, Frederickton, New South Wales, Australia). Photoperiod started as 12 h:12 h light:dark and was changed to 10 h:14 h light:dark halfway through the study to replicate the fish's natural environment. For specific details see ESM "Experimental acclimation."

2.2 | Respirometry protocol

After 5 months of acclimation and 48 h of fasting (June–July 2021), the authors assessed the MMR and SMR of the reared fish ($n = 14$ per treatment, sourced from the majority of replicate tanks and across the entire available mass range) at their acclimated temperature and at both their acclimated and acute oxygen conditions. To do so, they used an intermittent-flow respirometry system with the capacity to measure whole animal oxygen uptake rates ($\dot{M}_{O_2}$; Firesting, PyroScience, Aachen, Germany) in normoxic and hyperoxic conditions (Oxyguard Pacific oxygen dosing system) and at different temperatures (TECO chiller-heater, model TK-2000, Ravenna, Italy; see ESM "Respirometry system"). In short, pre-selected individuals were removed from their rearing tank and subjected to an MMR-stimulating exhaustive chase protocol (Norin, Malte & Clark 2014) in water identical to their acclimation treatment. Fish were immediately placed into the respirometers (0.7–1.35 L depending on animal size) for recordings of MMR and were then left overnight (for *c*. 18 h) to capture their SMR at equivalent conditions. Subsequently, each fish was acutely exposed within the respirometers to the alternate oxygen condition to which they had been acclimated by turning on or off the oxygen dosing system. After 4 h of exposure to these acute oxygen conditions, the same MMR and SMR protocols were repeated. Background respiration was measured in each respirometer before each trial, and one respirometer in each trial was always left without a fish to quantify dynamic changes in background respiration (see ESM "Respirometry protocol" for details).

2.3 | Tissue sampling

In parallel with respirometry trials, a sub-set of fish ($\sim n = 10$ per treatment taken from the majority of replicate tanks) were randomly

sampled directly from the 20°C holding tanks. The authors did not sample the 15°C treatment group, as the higher temperature group was of most interest because of the heightened potential for oxygen limitation to impact anatomical development. Individuals were euthanized in an ice slurry (*c.* 5 min), weighed to calculate specific growth rate (SGR in % d^{-1}, see ESM "Tissue sampling" for equation) and dissected to assess liver, spleen and ventricle mass (A&D scale, model HR200, Adelaide, Australia). The same cohort of fish was reared until December 2021, at which point an opportunity arose to sample a sub-set of fish ($n = 6$ per treatment) from the 20°C holding tanks for blood measures of haematocrit (Hct), haemoglobin concentration (Hb) and mean corpuscular haemoglobin concentration [MCHC; (Hb)/Hct × 100]. See ESM "Tissue sampling" for details. All experiments were conducted in accordance with the guidelines set by the Deakin University Animal Ethics Committee (no. B27-2018), which complies with the Australian Code for the Care and Use of Animals for Scientific Purposes set out by the Australian federal government.

2.4 | Data analysis and statistics

Metabolic data were imported into LabChart (ADInstruments Pty Ltd., New South Wales, Australia) for analysis. The slope of the linear regression between oxygen and time during closed cycles and the volume of the respective respirometer were used to calculate $\dot{M}_{O_2}$ (mg O_2 h^{-1}) for each individual. MMR for each fish was considered as the steepest slope during any consecutive 2 min period following the exhaustive-chase protocol. The authors assigned a single SMR value to each fish, calculated as the average of the lowest three measurements (out of *c.* 25 measurements). Background (microbial) respiration was measured before each trial and in a parallel fish-free respirometer during each trial; this was usually negligible, but nevertheless was subtracted from all MMR and SMR values (see ESM "Respirometry protocol").

Power regression analyses were used (R version 4.1.2) to extract the best-fit relationship between body mass (g) and $\dot{M}_{O_2}$ (MMR and SMR) for all treatment combinations. To compare MMR, SMR and AS between treatments, raw $\dot{M}_{O_2}$ data were standardized to the overall average mass of 5.27 g. This was achieved by adding the residuals of each individual's measurement to the expected value for a 5.27 g fish, calculated from the power regression equations, resulting in a mass-standardized $\dot{M}_{O_2}$ unit of mg O_2 h^{-1} 5.27 g^{-1}. The authors calculated AS for each individual as the difference between their mass-standardized MMR and SMR. One individual fish from the 20°C-hyperoxia treatment was excluded from analyses due to visible skin damage and irregular MMR and SMR measurements.

Statistical analyses were performed on the mass-standardized $\dot{M}_{O_2}$ data and were conducted using R version 4.1.2. Independent and paired *t*-tests with Bonferroni adjusted *P*-values were used to determine between- and within-subject differences, respectively, and in the single case of abnormal data (see ESM), Yuen's trimmed-mean *t*-tests were used. Normality of data and homogeneity of variance assumptions were checked with the Shapiro–Wilk's test and Levene's test, respectively. Animal sizes were similar between the 20°C treatments; thus all relative organ masses, SGR and blood parameters were compared using independent *t*-tests. SGR data for fish used in respirometry were not included because of the non-random selection of these individuals. See ESM "Data analysis and statistics." All *t*- and *P*-values from *t*-tests can be found in the ESM "Table S1."

3 | RESULTS

At both acclimation temperatures, fish acclimated to normoxia exhibited 17%–19% decreases in SMR, 42%–48% increases in MMR and consequently 74%–95% increases in AS when acutely exposed to hyperoxia during respirometry (paired *t*-tests, $P < 0.05$ in all cases; Figure 1). Many of these metabolic adjustments remained after 5 months of acclimation to hyperoxia. For example, fish acclimated to hyperoxia at 15°C maintained an elevated MMR when measured under hyperoxia, such that MMR was similar between fish acutely exposed to hyperoxia and those which had undergone 5 months of hyperoxia acclimation (independent *t*-test, $P > 0.05$; Figure 1a). Although the SMR of 15°C-acclimated fish increased back up to normoxia control levels after 5 months of hyperoxia acclimation (independent *t*-test, $P < 0.05$; Figure 1b), this increase was not sufficient to compensate for the continued elevation in MMR, and thus AS remained 73% higher in hyperoxia-acclimated fish measured in hyperoxia compared with normoxia-acclimated fish measured in normoxia (independent *t*-test, $P < 0.001$; Figure 1c).

The qualitative patterns were similar but not as pronounced in the 20°C acclimation group. In particular, acute hyperoxia still elicited a decrease in SMR and an increase in MMR (Figure 1d,e), but a decline in MMR throughout the hyperoxia-acclimation period (independent *t*-test, $P < 0.01$) caused a decrease in AS of hyperoxia-acclimated fish compared with those which had been acutely exposed (independent *t*-test, $P < 0.05$; Figure 1f). Nevertheless, AS remained 58% greater in hyperoxia-acclimated fish measured in hyperoxia compared with normoxia-acclimated fish measured in normoxia (independent *t*-test, $P < 0.001$; Figure 1f).

Interestingly, in both the 15°C and 20°C thermal acclimation groups, when hyperoxia-acclimated fish were acutely taken back to normoxia test conditions, MMR and AS decreased, but not to the levels observed in normoxia-acclimated fish under normoxia (Figure 1). Indeed, AS remained 36% (15°C; independent *t*-test, $P < 0.0001$) and 47% (20°C; independent *t*-test, $P < 0.01$) higher in hyperoxia-acclimated fish tested in normoxia than normoxia-acclimated fish tested in normoxia (Figure 1c,f, respectively).

Long-term hyperoxia acclimation at 20°C did not alter SGR or the relative mass of the ventricle, spleen or liver, nor did it change Hct, (Hb) or MCHC levels of the blood compared with fish acclimated to normoxia at 20°C (Table 1).

4 | DISCUSSION

The metabolic measurements made in this study for normoxia-acclimated *G. maculatus* in normoxia align well with values reported for other members of the Galaxiidae family (White *et al.* 2017). Acute exposure to hyperoxia significantly elevated the MMR of *G. maculatus* at both

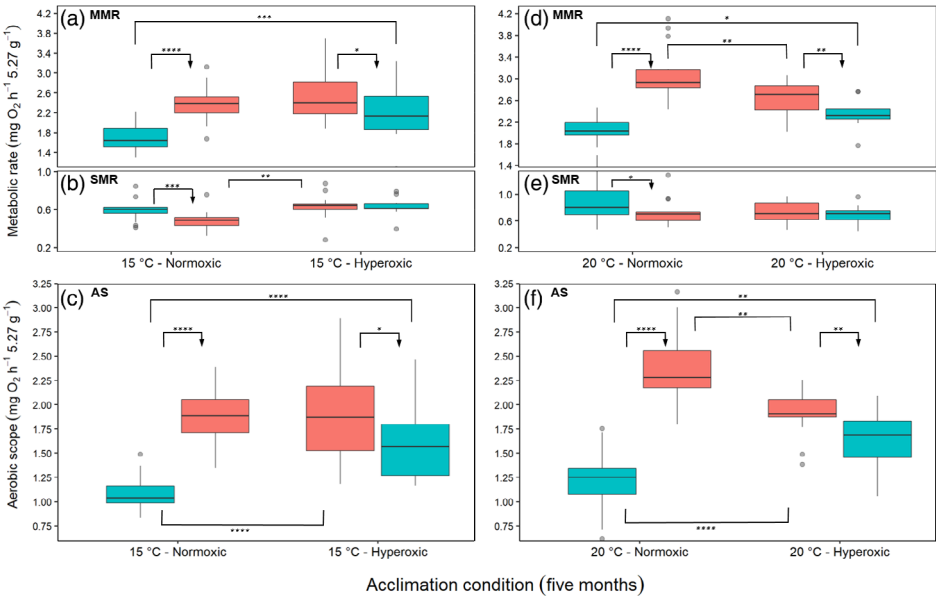

FIGURE 1 Aerobic metabolic performance of *Galaxias maculatus* acclimated to 15°C–normoxia, 15°C–hyperoxia, 20°C–normoxia and 20°C–hyperoxia for 5 months and subsequently tested in both normoxia ( ) and hyperoxia ( ) respirometry conditions at respective acclimation temperatures. (a–c) represent the maximum metabolic rate (MMR), standard metabolic rate (SMR) and absolute aerobic scope (AS), respectively, of fish from the 15°C–normoxia ($n = 14$) and 15°C–hyperoxia ($n = 14$) acclimation treatments, whereas (d–f) depict the respective equivalent for 20°C–normoxia ($n = 14$) and 20°C–hyperoxia ($n = 13$) acclimated fish. Arrows show the direction of repeated measures for fish from their acclimated condition to the acute respirometry test condition. * Statistical significance of *t*-tests, paired and independent as appropriate (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$; see ESM "Table S1")

temperatures, as has been observed in other species including European perch (Brijs *et al.* 2015) and rainbow trout (*Oncorhynchus mykiss*) (McArley *et al.* 2021a). Thus, an elevation in maximum oxygen transport capacity under acute hyperoxia appears to be a common response among fishes, although exceptions have been reported (Duthie & Hughes 1987; Lefrançois & Claireaux 2003). Notably, the persistent improvement in MMR with hyperoxia did not translate to higher SGR in hyperoxia-acclimated fish (Table 1), which contradicts the idea of oxygen- or AS-limited growth in warm environments (Pauly 1981; Pörtner 2010).

The observed decline in SMR under acute hyperoxia – and the consistency of this response across temperatures – is unique, as others have either reported an increase in SMR (McArley, Hickey & Herbert 2018) or no change (Brijs *et al.* 2015; McArley *et al.* 2021b). This hyperoxia-induced, transient decline in SMR aligns with observations that acute hyperoxia causes a decline in ventilation effort, and presumably a decline in the energetic cost of ventilation (McArley, Hickey & Herbert 2018).

For the first time, the authors show that the increase in MMR in response to hyperoxia is largely retained over time, as MMR remained elevated after 5 months of hyperoxia acclimation relative to control fish in normoxia. In trying to understand the underlying mechanisms, they found no evidence of hyperoxia-acclimated fish possessing (a) enlarged ventricles (which could be indicative of higher cardiac stroke volume), (b) enlarged spleens under resting conditions (which could be indicative of an enhanced splenic pooling of erythrocytes) or (c) an increase in blood oxygen-carrying capacity (Table 1). Notably, we cannot discount a possible increase in the oxygen partial pressure (PO_2) of venous blood returning to the heart of hyperoxia-acclimated fish [as found in McArley *et al.* (2021a) and Ekström *et al.* (2016) after acute hyperoxia exposure], which has been proposed to enhance cardiac contractility and thus stroke volume in a way that would be undetectable through ventricle mass measurements. Equally, hyperoxia may promote higher arterial PO_2 after exhaustive exercise [as discussed in McArley *et al.* (2021b)], but this study's data do not provide insight into that possibility.

By acutely exposing hyperoxia-acclimated fish to normoxia and comparing against normoxic controls, the authors found evidence that hyperoxia acclimation may drive changes to the metabolic "machinery." That is, the oxygen transport capacity of hyperoxia-acclimated fish remained higher than normoxia-acclimated fish when both groups were tested in normoxia. The aforementioned cardiac contractility hypothesis could explain this finding only if hyperoxia-acclimated fish retained elevated venous PO_2 after 4 h of normoxia exposure (*e.g.*, by drawing on oxygen stores from myoglobin). There is also the possibility that hyperoxia-acclimated fish possessed modifications to mitochondrial density [as in Mustafa *et al.* (2011)] or morphology (Cook *et al.* 2013), or to haemoglobin-oxygen binding characteristics (Andersen *et al.* 2009; Rasmussen *et al.* 2009), but little is known of how these parameters change in response to chronic hyperoxia.

In contrast to the complete compensation that can be observed in AS after fish are acclimated to high temperatures (Norin, Malte & Clark 2014; Scheuffele, Rubio-Gracia & Clark 2021), the authors of this study reveal that the benefits of hyperoxia in improving the oxygen uptake capacity of fish persist over time. As such, they argue that studies employing experimental hyperoxia will be critical in helping to test the assumptions underlying the GOL and OCLTT hypotheses, and

TABLE 1 Relative organ masses, growth rates and blood oxygen-carrying parameters (mean ± S.D.) for *Galaxias maculatus* acclimated to 20°C–normoxia and 20°C–hyperoxia (body analysis after 6 months of acclimation, blood analysis after 11 months of acclimation)

	Normoxia-acclimated	Hyperoxia-acclimated
Body analysis	$n = 11$	$n = 10$
Whole animal mass (g)	5.83 ± 1.31	5.51 ± 1.76
RVM (%)	0.048 ± 0.013	0.045 ± 0.011
RSM (%)	0.13 ± 0.07	0.16 ± 0.06
RLM (%)	0.68 ± 0.20	0.68 ± 0.22
SGR (% d^{-1})	2.43 ± 0.66	2.40 ± 0.93
Blood analysis	$n = 6$	$n = 6$
Whole animal mass (g)	5.25 ± 1.18	6.76 ± 3.7
Haematocrit (%)	25.5 ± 7.9	26.7 ± 5.7
Haemoglobin (g L^{-1})	44.2 ± 12.2	44.8 ± 7.1
MCHC (g L^{-1})	176.5 ± 18.9	171.5 ± 26.6
SGR (% d^{-1})	1.19 ± 0.36	1.65 ± 1.12

Note. There were no significant differences between the two acclimation groups for any of the measured parameters ($P > 0.05$; see ESM "Table S1").
Abbreviations: MCHC, mean corpuscular haemoglobin concentration; RLM, relative liver mass; RSM, relative spleen mass; RVM, relative ventricle mass; SGR, specific growth rate.

therefore will play a large role in deciphering whether oxygen limitation is a proximate driver of the temperature-size rule in fishes.

AUTHOR CONTRIBUTIONS

M.R.S. was involved in the conceptualization and methodology and led the data collection, analyses and manuscript preparation. H.S. assisted with data collection, review and editing. T.D.C. aided the conceptualization, methodology, data collection, analyses, funding, supervision, review and editing. All authors contributed to revising the manuscript and approved its submission.

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DATA AVAILABILITY STATEMENT

Data that support the findings of this study are openly available in figshare at 10.6084/m9.figshare.20442783

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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