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RESEARCH ARTICLE

Normoxic limitation of maximal oxygen consumption rate, aerobic scope and cardiac performance in exhaustively exercised rainbow trout (*Oncorhynchus mykiss*)

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ABSTRACT

In fish, maximum O_2 consumption rate ($\dot{M}_{O_2,max}$) and aerobic scope can be expanded following exhaustive exercise in hyperoxia; however, the mechanisms explaining this are yet to be identified. Here, in exhaustively exercised rainbow trout (*Oncorhynchus mykiss*), we assessed the influence of hyperoxia on $\dot{M}_{O_2,max}$, aerobic scope, cardiac function and blood parameters to address this knowledge gap. Relative to normoxia, $\dot{M}_{O_2,max}$ was 33% higher under hyperoxia, and this drove a similar increase in aerobic scope. Cardiac output was significantly elevated under hyperoxia at $\dot{M}_{O_2,max}$ because of increased stroke volume, indicating that hyperoxia released a constraint on cardiac contractility apparent with normoxia. Thus, hyperoxia improved maximal cardiac performance, thereby enhancing tissue O_2 delivery and allowing a higher $\dot{M}_{O_2,max}$. Venous blood O_2 partial pressure (P_{vO_2}) was elevated in hyperoxia at $\dot{M}_{O_2,max}$, suggesting a contribution of improved luminal O_2 supply in enhanced cardiac contractility. Additionally, despite reduced haemoglobin and higher P_{vO_2}, hyperoxia treated fish retained a higher arterio-venous O_2 content difference at $\dot{M}_{O_2,max}$. This may have been possible because of hyperoxia offsetting declines in arterial oxygenation that are known to occur following exhaustive exercise in normoxia. If this occurs, increased contractility at $\dot{M}_{O_2,max}$ with hyperoxia may also relate to an improved O_2 supply to the compact myocardium via the coronary artery. Our findings show $\dot{M}_{O_2,max}$ and aerobic scope may be limited in normoxia following exhaustive exercise as a result of constrained maximal cardiac performance and highlight the need to further examine whether or not exhaustive exercise protocols are suitable for eliciting $\dot{M}_{O_2,max}$ and estimating aerobic scope in rainbow trout.

KEY WORDS: Aerobic scope, Hyperoxia, Exhaustive exercise, Cardiac output, Oxygen consumption, Cardiorespiratory performance

INTRODUCTION

In fish, exhaustive exercise (also referred to as strenuous exercise, burst swimming or chase stress) is a form of swimming powered mostly by anaerobically derived adenosine triphosphate (ATP) production within the highly differentiated white musculature (Kieffer, 2000). Before it becomes 'exhaustive', the powerful, swift bursts of movement produced by this form of swimming are undoubtedly ecologically and evolutionarily important, for example, in predator–prey interactions (Harper and Blake, 1990; Webb, 1976) or in overcoming strong river flows in upstream migrations (Burnett et al., 2014; Hinch and Bratty, 2000). However, if anaerobically powered swimming is performed for too long, fish become exhausted and display a suite of severe physiological disturbances including, but not limited to, depletion of fermentable fuels, blood and tissue acidosis, lactate accumulation in the blood and white muscle, and ionic and osmotic imbalances (see reviews by Kieffer, 2000; Milligan, 1996; Wood, 1991a). Collectively, these disturbances generate an O_2 debt – the so called excess post-exercise O_2 consumption (EPOC) – that must be repaid following exhaustive exercise as a consequence of homeostasis being restored (Brett, 1964; Scarabello et al., 1991; Zhang et al., 2018).

EPOC is typically characterised by whole animal mass-specific O_2 consumption rate ($\dot{M}_{O_2}$) that follows a pattern of exponential decay: a peak in $\dot{M}_{O_2}$ is observed immediately or soon after exhaustive exercise ceases, before it falls rapidly and then gradually returns to a resting rate over several hours. For example, after an immediate peak following exhaustive exercise, $\dot{M}_{O_2}$ returns to a resting rate (indicating EPOC has been repaid) in 3.5–4.5 h in juvenile rainbow trout (*Oncorhynchus mykiss*) (Scarabello et al., 1991). Longer duration EPOC (12–15 h), however, may be apparent when defining its endpoint as a return to a basal $\dot{M}_{O_2}$ (in fish termed standard metabolic rate; SMR) rather than one-off measures of routine $\dot{M}_{O_2}$ (Zhang et al., 2018). The $\dot{M}_{O_2}$ peak observed during EPOC has attracted much interest as a proxy measure of maximum metabolic rate in fish (Norin and Clark, 2016). This is because chasing a fish to exhaustion is a convenient, time efficient method of eliciting maximal O_2 consumption rate ($\dot{M}_{O_2,max}$) in fishes in general, and also because it is often the only method suitable for doing so in sluggish, benthic species that will not swim in a flume (Norin and Clark, 2016). These estimates of $\dot{M}_{O_2,max}$ are important because together with estimates of SMR they are used to derive a measure of physiological performance known as aerobic scope (i.e. the difference between $\dot{M}_{O_2,max}$ and SMR) (Clark et al., 2013). Although aerobic scope has long been used as a measure of physiological performance (Fry, 1947), it has risen to prominence in the past 15 years as a means of assessing the vulnerability of fish to climate warming (Claireaux and Lefrançois, 2007; Clark et al., 2013; Gräns et al., 2014; Lefevre, 2016; McArley et al., 2017; Norin et al., 2014; Pörtner and Farrell, 2008).

It is well known that various environmental factors modulate aerobic scope. Indeed, the consistency, constraint or expansion of aerobic scope under different conditions (e.g. at different temperatures) is a major reason it has been promoted as a metric of performance (Claireaux and Lefrançois, 2007; Clark et al., 2013; Pörtner and Farrell, 2008). Water oxygenation is one such environmental factor modulating aerobic scope. While there is a consensus that as O_2 levels fall below normoxia (i.e. become

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hypoxic) aerobic scope will eventually be constrained because of a limitation of $\dot{M}_{O_2,max}$ (Claireaux and Lefrançois, 2007; Claireaux and Chabot, 2016; Mandic and Regan, 2018), it is unclear whether $\dot{M}_{O_2,max}$ and aerobic scope are constrained by O_2 availability under normoxia, because few studies have examined the influence of hyperoxia (O_2 levels above normoxia) on these parameters (reviewed in McArley et al., 2021). Two recent studies, however, provide evidence $\dot{M}_{O_2,max}$ and aerobic scope can be constrained under normoxia in fish. In European perch (*Perca fluvialitis*), $\dot{M}_{O_2,max}$ was ∼92% higher under hyperoxia (200% air saturation) compared with normoxia (Brijs et al., 2015). Also, in two species of triplefin fishes, the twister (*Belapiscis medius*) and common triplefin (*Forstyregion lapillum*), $\dot{M}_{O_2,max}$ was ∼25% higher under hyperoxia (200% air saturation) than it was in normoxia (McArley et al., 2018). In both these studies, elevated $\dot{M}_{O_2,max}$ under hyperoxia drove a corresponding expansion of aerobic scope, because with hyperoxia SMR was unchanged in perch and only slightly elevated in both triplefin species. Additionally, in the perch study, maximal routine $\dot{M}_{O_2}$ approximately doubled relative to normoxia at temperatures approaching thermal limits during acute thermal ramping (Brijs et al., 2015), and in the triplefin study, $\dot{M}_{O_2,max}$ was ∼50% higher under hyperoxia when fish were exhaustively exercised after an acute temperature increase from 21°C to 29°C (McArley et al., 2018). While these studies clearly indicate an expansion of $\dot{M}_{O_2,max}$ with hyperoxia, as yet, the mechanisms that drive this have not been identified. Furthermore, whether an expansion of $\dot{M}_{O_2,max}$ following exhaustive exercise under hyperoxia is a common response in fish is unclear. This possibility has only been considered in one other study, which found $\dot{M}_{O_2,max}$ and aerobic scope unchanged with hyperoxia in common sole (*Solea solea*) (Lefrançois and Claireaux, 2003).

In this study, we added rainbow trout to the short list of fishes in which the influence of hyperoxia on $\dot{M}_{O_2,max}$ and aerobic scope following exhaustive exercise has been examined. The focus was to determine whether any changes (presumably an increase) in $\dot{M}_{O_2,max}$ and aerobic scope following exhaustive exercise under hyperoxia could be linked to changes in cardiac function. Some evidence that cardiac performance is improved under hyperoxia at times of high O_2 demand already exists. In European perch, Ekström et al. (2016) found cardiac output was higher under hyperoxia (200% air saturation) relative to normoxia at temperatures approaching thermal limits during acute thermal ramping. Although these authors did not measure $\dot{M}_{O_2}$, the elevated cardiac output seen in their study corresponds well with the increased routine $\dot{M}_{O_2}$ at high temperatures observed in perch under hyperoxia by Brijs et al. (2015). As such, it was hypothesised that if $\dot{M}_{O_2,max}$ was expanded following exhaustive exercise with hyperoxia in rainbow trout, it would correspond with elevated cardiac output. Ekström et al. (2016) also observed that the partial pressure of oxygen in venous blood (P_{vO_2}) was elevated in hyperoxia across acute test temperatures, suggesting the possibility that hyperoxia improves luminal O_2 supply to the myocardium. To see if this was also the case in rainbow trout following exhaustive exercise, we assessed P_{vO_2} by sampling venous blood from an implanted cannula. In addition to $\dot{M}_{O_2}$, cardiac and P_{vO_2} assessment, the influence of hyperoxia on the magnitude and recovery dynamics of physiological disturbances resulting from exhaustive exercise was examined. This included haematological variables (haemoglobin and haematocrit), venous blood pH, plasma lactate and plasma osmolality. We also determined EPOC to assess whether hyperoxia affected the magnitude or the rate of repayment of the O_2 debt associated with exhaustive exercise.

MATERIALS AND METHODS

Animals, holding conditions and experimental set-up

The rainbow trout [*Oncorhynchus mykiss* (Walbaum 1792)] used in this study were of mixed sex and obtained from a commercial hatchery (Vänneåns Fiskodling AB, Halland, Sweden). At the time of experimentation, there was no difference in body mass between fish in the normoxic (896.5±47.5 g; mean±s.e.m.) and hyperoxic (883.5±68.57 g) treatment groups (t=0.1518, d.f.=17, P=0.88). Prior to experimentation, the fish were held in 600 litre tanks for a period of at least 4 weeks for laboratory acclimation. These tanks were supplied with air saturated, recirculated fresh water at ∼10°C and maintained under a 12 h:12 h light:dark photoperiod. Fish were fed pellets (7 mm, Protec Trout pellets, Skretting, Norway) twice a week until the start of the experiments, but food was always withheld for a period of 3 days prior to experimentation. All experimental procedures were covered by ethical permit 165-2015, approved by the regional ethical committee in Gothenburg.

All measurements outlined in the following sections were completed with the fish housed in a respirometer (see 'Respirometry and data acquisition for cardiac variables' below) held in a 120 litre experimental tank. Two of these identical tanks, which each held an individual respirometer, were used to perform the experiments. The experimental tanks received temperature-controlled freshwater (mean: 9.9±0.02°C, range: 9.7–10.8°C) from the main recirculated supply feeding the fish holding tanks. The normoxia treatment condition was maintained by bubbling the water supply to respirometers with air, while the hyperoxia treatment condition was maintained by bubbling pure O_2 at a set rate from a manually adjusted gas bottle (see section 'Experimental protocol' for details).

Surgery and instrumentation

Surgery began by anaesthetising fish in 10°C freshwater containing 150 mg l^{-1} MS-222 (Tricaine methanesulfonate, Scan Aqua AS, Årnes, Norway) buffered with 300 mg l^{-1} $NaHCO_3$. Once ventilatory strokes ceased, mass was taken before placing fish right side up on a surgery table lined with foam. Throughout surgery, the gills were perfused with a continuous flow of aerated fresh water (10°C) containing 75 mg l^{-1} MS-222 and 150 mg l^{-1} $NaHCO_3$. Two procedures, which were completed in approximately 90 min, were carried out during surgery. Firstly, the ventral aorta was accessed via a small incision made in the isthmus within the right opercular cavity and freed from surrounding tissue by blunt dissection. A 2.5 mm Transonic transit-time blood flow probe (L type; Transonic Systems, Ithaca, NY) was then placed around the ventral aorta to allow recordings of ventral aortic blood flow (cardiac output). The flow probe was secured in place with two 3-0 silk sutures placed around the probe lead within the opercular cavity and a suture placed around the probe lead where it exited the opercular cavity. Additional silk sutures (2-0) positioned below the pectoral fin, below the lateral line ∼5 cm posterior to the operculum, and adjacent to the dorsal fin fixed the probe lead to the fish's body. In the second part of surgery, the ductus of Cuvier was cannulated with a PE50 catheter to allow venous blood sampling as previously described by Sandblom et al. (2006). The catheter was filled with heparinised saline (25 IU ml^{-1}) and attached to the fish's body with silk sutures (3-0) positioned adjacent to where the catheter exited the opercula cavity and adjacent to the dorsal fin.

Experimental protocol

Following surgery, fish were transferred to the respirometers and immediately placed under treatment conditions of either normoxia

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or hyperoxia. The mean water O_2 level inside the respirometers throughout the duration of the experimental protocol was 95.9±0.3% air saturation (∼20.1 kPa) and 210.2±2.3% air saturation (∼44 kPa) in the normoxia and hyperoxia treatments, respectively. In the hyperoxic treatment, the maximum value inside the respirometers ranged from 207 to 230% air saturation (∼43–48 kPa) among the ten replicates. The treatment groups are referred to as ∼100% air saturation and ∼200% air saturation throughout the remainder of this paper. After entering the respirometer, fish were left undisturbed for a post-surgery recovery period of ∼22 h. $\dot{M}_{O_2}$ and cardiac function (cardiac output and heart rate) were recorded continuously and a venous blood sample was drawn at the end of the post-surgery recovery period for assessment of pre-exhaustive exercise (from here referred to as pre-chase) baseline blood variables (see 'Blood analysis' below). The fish was then disconnected from the recording equipment, removed from the respirometer and placed into a circular 50 litre tank where it was exhaustively exercised by manual chasing (i.e. repeated tail grabbing) for a period of 5 min. The transfer process to the chasing tank was completed in approximately 15 s. During the exhaustive exercise protocol, the water O_2 level in the tank was maintained at the level assigned to the respective treatment groups (i.e. the normoxia group was chased in ∼100% air saturation, and the hyperoxia group was chased in ∼200% air saturation) and a temperature of ∼10°C. All fish reached an exhausted state (i.e. they no longer performed burst movements when the tail was grabbed) after 5 min chasing, and there were no obvious differences between treatment groups as to the speed at which fish became exhausted during chasing. After chasing, the fish was transferred back into the respirometer, reconnected to the recording equipment and post-exhaustive exercise (from here referred to as post-chase) respirometry cycles were initiated. This transfer process was always completed within 45 s of the completion of chasing. At this point, a venous blood sample was also drawn to assess blood variables immediately post-chase (0 h post-chase). $\dot{M}_{O_2}$ and cardiac function were then again measured continuously for a period of ∼20 h, and further venous blood samples were taken at 1, 2, 4 and 20 h post-chase.

Respirometry and data acquisition for cardiac variables

$\dot{M}_{O_2}$ was measured using intermittent stop-flow respirometry (Steffensen, 1989). Respirometers (10 litre volume) were constructed of a section of cylindrical PVC tube that was sealed with end caps fitted with O-rings. A spout placed in the middle of the respirometer, which extended above the water level of the experimental set-up, provided an exit for the catheter and flow probe lead. To flush the respirometer after each measurement cycle (see 'Calculation of cardiorespiratory variables'), a submersible flush-pump (Eheim Universal 1200, Eheim, Deizisau, Germany) was connected to a fitting in the end cap via silicon tubing. The flush-pump was connected to a control switch coupled to a PowerLab system (ADInstruments, Castle Hill, Australia), which allowed the flush pump to be switched on and off automatically using LabChart pro data acquisition software (version 7.3.2, ADInstruments, Castle Hill, Australia). To continually mix water within the respirometer, an inline pump (Eheim Universal 1200) was connected to a fitting in each end cap via a silicon tubing recirculation loop. A fibre optic O_2 probe (OXROB10, PyroScience, Aachen, Germany), sealed in a fitting placed in the recirculation loop, continually measured the water O_2 level (% air saturation) within the respirometer, and O_2 values were recorded using LabChart Pro data acquisition software via a Firesting O_2 meter (PyroScience, Aachen, Germany) connected to a PowerLab. To assess cardiac function, the flow probe (individually bench calibrated at 10°C according to the manufacturer's instructions) placed around the ventral aorta was connected to a Transonic blood flow meter (model T206; Transonic Systems, Ithaca, NY), and the signals were recorded at a sampling rate of 10 Hz using a PowerLab system and LabChart pro data acquisition software.

Calculation of cardiorespiratory variables

The slope of the linear decline in O_2 within the respirometer during measurement cycles (i.e. when only the mixing pump was on) was used to determine $\dot{M}_{O_2}$ using the following formula:

$$\dot{M}_{O_2} = [(V_r - V_f) \times (\Delta\%\mathrm{Sat}/t) \times \alpha]/(M_b), \quad (1)$$

where V_r is the volume of the respirometer, V_f is the volume of the fish assuming that 1 g of fish equals 1 ml water, $\Delta\%\mathrm{Sat}/t$ is the change in O_2 (% air saturation) per unit time, α is the temperature specific solubility coefficient of O_2 in fresh water and M_b is the body mass of the fish (Clark et al., 2013). The first 30–60 s of each measurement cycle was excluded to ensure only the linear section of the decline in O_2 was included in slope determinations (R^2 values for these slopes always remained above 0.98). Measurement cycles were interspersed with flush cycles to replace respirometer water. The first five cycles following exhaustive exercise always consisted of 90 s measurement periods and 3 min flush periods; however, as the $\dot{M}_{O_2}$ of the fish recovered, the length of measurement and flush cycles were increased to allow a sufficient decline in O_2 during each cycle. This occurred in stages for approximately 4 h following exhaustive exercise, and then overnight measurement (∼8–10 min) and flush (∼5–7 min) cycles were initiated. The O_2 level in the respirometer remained above 85% air saturation in the normoxic treatment for >98% of the protocol and above 185% air saturation in the hyperoxic treatment for >99% of the protocol. In background O_2 consumption checks, a positive slope was detected, which was likely related to a small temperature increase (∼0.10°C) inside the respirometer during measurement cycles. To account for this in estimates of fish $\dot{M}_{O_2}$, the positive background slope determined at the start of the protocol was added to the slope value of each measurement cycle.

SMR was estimated as the mean of the lowest 10 $\dot{M}_{O_2}$ values recorded during the ∼20 h post-chase recovery period (Brijs et al., 2015; Norin et al., 2014). $\dot{M}_{O_2}$ values more than two standard deviations (i.e. the standard deviation of the lowest 10 $\dot{M}_{O_2}$ values for an individual fish) below a fish's SMR were removed. In total, 9 $\dot{M}_{O_2}$ values were removed, and these were on average 0.25 mg O_2 kg^{-1} h^{-1} (range: 0.05–0.65 mg O_2 kg^{-1} h^{-1}) below two standard deviations of SMR. $\dot{M}_{O_2,max}$ was defined as the highest $\dot{M}_{O_2}$ value measured at any point post-chase and was seen within the first 5 post-chase measurement cycles in all fish. Aerobic scope was calculated as the difference between a fish's SMR and $\dot{M}_{O_2,max}$. EPOC was calculated as the area under the curve bounded by post-chase $\dot{M}_{O_2}$ and SMR as a baseline. This was done using the area under the curve function in Graph Pad Prism (version 8.4.2). The baseline value indicating when EPOC was completed was set as the highest $\dot{M}_{O_2}$ value used in the estimation of SMR for an individual fish (i.e. the tenth lowest $\dot{M}_{O_2}$ value recorded post-chase). The $\dot{M}_{O_2}$ curves were smoothed to prevent periods of elevated $\dot{M}_{O_2}$ that likely resulted from increased activity from being included in EPOC calculation. Smoothing involved removing $\dot{M}_{O_2}$ values that increased by more than 5% relative to the previous $\dot{M}_{O_2}$ value until the baseline $\dot{M}_{O_2}$ threshold was reached (Zhang et al., 2018). In

these instances, $\dot{M}_{O_2}$ values were removed until $\dot{M}_{O_2}$ returned to within 5% of the $\dot{M}_{O_2}$ value recorded immediately prior to the first removed value. The EPOC duration was defined as the time after exhaustive exercise at which $\dot{M}_{O_2}$ became equal to the tenth lowest $\dot{M}_{O_2}$ value used to estimate an individual fish's SMR. The rate of EPOC repayment was calculated as total EPOC divided by the EPOC duration.

Cardiac output was determined from blood flow data and normalised to body mass (ml min^{-1} kg^{-1}), and heart rate was determined from the pulsatile blood flow measurements. Cardiac stroke volume (ml $heart\ beat^{-1}$) was calculated by dividing cardiac output by heart rate. The main purpose of the post-chase cardiac measurements was to identify whether improved heart performance was a driver of higher $\dot{M}_{O_2,max}$ under hyperoxia. As such, the maximum values for cardiac output, heart rate and stroke volume presented here are taken from the same time when $\dot{M}_{O_2,max}$ was measured. Resting values for cardiac output, heart rate and stroke volume were defined as the mean value for each variable over the ten lowest $\dot{M}_{O_2}$ measurement cycles (i.e. they were tied to SMR). Scope for each of these cardiac variables was calculated as the difference between the variable at $\dot{M}_{O_2,max}$ and the variable at SMR. Subsequently, coupled measurements of $\dot{M}_{O_2}$ and cardiac output were used to approximate the arterio-venous oxygen content difference (A–V O_2 content difference) according to rearrangement of the Fick equation:

$$\text{A}-\text{V}\,O_2\,\text{content difference} = \dot{M}_{O_2}/\text{cardiac output}. \quad (2)$$

Maximum, resting and scope for A–V O_2 content difference were defined as for cardiac variables (i.e. tied to SMR and $\dot{M}_{O_2,max}$).

To assess changes in $\dot{M}_{O_2}$, cardiac variables and A–V O_2 content difference, pre-chase and post-chase routine values for each of these variables were determined for multiple time points throughout the protocol. The sampling time points were pre-chase (treated as a resting control value as described above), immediately post-chase (0 h) and 1, 2, 4, 8, 14 and 20 h post-chase. Routine values for each cardiorespiratory variable were defined as the mean value across two $\dot{M}_{O_2}$ measurement cycles at each of the predefined sampling time points. The exception to this was the 0 h cardiorespiratory measurements, which were always taken from the first $\dot{M}_{O_2}$ measurement cycle following exhaustive exercise.

Blood analysis

At most sampling time points (pre-chase and 0, 1, 2, 4 and 20 h post-chase), ∼250 µl of blood was drawn into a heparinised 1 ml syringe via the venous cannula. $P_{V_{O_2}}$ was measured using a fibre optic O_2 probe (PyroScience, Aachen, Germany) calibrated at 10°C. To minimise exposure of the blood sample to air, the O_2 probe was pushed through the syringe's rubber stopper and sealed in place with silicone. Thus, when a sample was taken, the blood came into contact with the O_2 probe tip sealed within the syringe. Once the blood sample was drawn, the catheter was removed from the syringe and the tip of the syringe was sealed. The syringe was then placed in 10°C water until the $P_{V_{O_2}}$ signal plateaued, which typically occurred within ∼3 min. The $P_{V_{O_2}}$ signal was recorded via a Firesting O_2 meter connected to a PowerLab. After $P_{V_{O_2}}$ was taken, the blood sample was transferred to a 1 ml centrifuge tube and placed in a 10°C water bath. Extracellular blood pH (pHe) was then measured using a handheld pH meter (Sentron SI400, Sentron Europe, Leek, The Netherlands) calibrated at 10°C. Next, haematocrit (Hct) and haemoglobin concentration ([Hb]) were measured in duplicate sub-samples. Hct was determined as the fractional red cell volume after centrifugation of blood in 80 µl microcapillary tubes at 10,000 rpm for 5 min, and [Hb] was determined using a handheld Hb 201+ meter (Hemocue AB, Ängelholm, Sweden). The Hb values were corrected for fish blood following Clark et al. (2008). Mean corpuscular haemoglobin concentration (MCHC) was subsequently calculated as MCHC=[Hb]/Hct×100. The remaining blood was centrifuged at 10,000 rpm for 5 min, and the plasma was immediately frozen and stored at −80°C. The plasma samples were subsequently used to determine plasma lactate using a commercially available lactate assay kit (Lactate Colorimetric Assay Kit II, Biovision, CA, USA). Osmolality was determined on thawed plasma samples using an osmometer (Model 3320 Osmometer, Advanced Instruments INC, MA, USA).

Statistics

All statistical analyses were performed using the IBM SPSS Statistics 26 software package, and significance was set at $P<0.05$. SMR, $\dot{M}_{O_2,max}$, and EPOC were compared between normoxia and hyperoxia using independent samples *t*-tests, and aerobic scope was compared between normoxia and hyperoxia using Welch's *t*-test. Cardiac output, heart rate, stroke volume and A–V O_2 content difference at $\dot{M}_{O_2,max}$ and SMR, as well as the scope for each of these variables, were compared between normoxia and hyperoxia with independent sample *t*-tests. For stroke volume scope, means were compared using Welch's *t*-test.

Mixed two-way analysis of variance (ANOVA) was used to compare routine $\dot{M}_{O_2}$, routine heart function, routine A–V O_2 content difference and blood parameters between normoxia and hyperoxia pre- and post-chase. Water O_2 level (normoxia or hyperoxia) was set as the between-subjects variable, and sampling time point was set as the within-subjects variable. Sampling time points included in the analysis of routine $\dot{M}_{O_2}$, routine heart function and routine A–V O_2 content difference were pre-chase and 0, 1, 2, 4, 8, 14 and 20 h post-chase. Blood parameters were analysed using samples taken pre-chase and 0, 1, 2, 4 and 20 h post-chase. For the two-way mixed ANOVA of $\dot{M}_{O_2}$, there was a violation of equality of variances among treatments at the 1 h (Levene's test: $P=0.012$) post-chase time point. Equality of variances could not be satisfied through data transformations, so the analysis was run with and without the 1 h time point included to determine if the outcome of the analysis was impacted. There was no difference in the conclusion drawn from the analysis when the 1 h time point was excluded, so the analysis including all time points is reported in the Results section. The same approach was taken for the two-way mixed ANOVA of stroke volume and plasma lactate, where the exclusion of one time point (14 h for stroke volume and 2 h for plasma lactate) with unequal variances (Levene's test: $P=0.017$ and $P=0.018$, respectively) among treatment groups had no influence on the conclusion drawn from the analysis. For the two-way mixed ANOVA of A–V O_2 content difference, the analysis was performed with the 20 h time point excluded to satisfy the assumption of equality of covariance matrices as assessed by a Box's *M*-test. $P_{V_{O_2}}$ was analysed using natural log transformed data. Owing to violations of equality of variance and equality of covariance matrices, which could not be corrected with transformations, the two-way mixed ANOVA for plasma osmolality was performed with the 1 h, 2 h and 20 h post-chase time points excluded. A second two-way mixed ANOVA was performed with the pre-chase and 20 h post-chase time points to confirm if recovery of osmolality was completed by the end of the protocol. In cases where there was a violation of sphericity (Mauchly's test of sphericity $P<0.05$), Greenhouse–Geisser adjusted *F*-tests and *P*-values were interpreted

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in the analysis. Where an interaction was significant, simple main effects were assessed at each level of the within-subjects variable to determine if there were significant differences between normoxia and hyperoxia at a particular sampling time point. Additionally, in the case of a significant interaction, a repeated measures ANOVA with Bonferroni adjusted *post hoc* comparisons was performed within each level of the between-subjects variable to determine if there were differences in post-chase recovery dynamics between normoxia and hyperoxia. As the main focus was to determine if there were any differences between normoxia and hyperoxia in the rate of recovery to a resting condition post-chase, only the *post hoc* comparisons between pre-chase values (treated as a resting control value) and post-chase values are reported in the Results. In the case of significant main effects without an interaction, Bonferroni adjusted *post hoc* comparisons among sampling time points were made across the normoxia and hyperoxia treatments. Again, only the *post hoc* comparisons between the pre-chase and post-chase values are reported in the Results. No *post hoc* comparisons were required in the case where there was a significant main effect of water O_2 level because there were only two levels of this variable (i.e. normoxia versus hyperoxia).

RESULTS

Maximum cardiorespiratory responses and physiological status immediately following exhaustive exercise in normoxia and hyperoxia

$\dot{M}_{O_2,max}$, which was observed within the first 5 post-chase respirometry cycles in all fish (i.e. no more than 14 min post-chase), was 33% higher under hyperoxia than normoxia (hyperoxia: 391.6±24.8 mg O_2 kg^{-1} h^{-1}; normoxia: 294.1±12.2 mg O_2 kg^{-1} h^{-1}; t=−3.40, d.f.=17, P=0.003; Fig. 1F). A significantly higher $\dot{M}_{O_2}$ (P<0.05) was also seen with hyperoxia when only the 0 h post-chase time point (i.e. the first post-chase $\dot{M}_{O_2}$ measurement) was considered (Fig. 2A). Since SMR was similar between treatments (normoxia: 52.4±2.4 mg O_2 kg^{-1} h^{-1}; hyperoxia: 49.3±1.2 mg O_2 kg^{-1} h^{-1}; t=1.01, d.f.=17, P=0.33, Fig. 1A), the higher $\dot{M}_{O_2,max}$ seen under hyperoxia translated to a significant elevation of aerobic scope under hyperoxia relative to normoxia (hyperoxia: 342.3±25.1 mg O_2 kg^{-1} h^{-1}; normoxia: 241.8±12.3 mg O_2 kg^{-1} h^{-1}; t=−3.59, d.f.=13.10, P=0.003; Fig. 1K). The higher $\dot{M}_{O_2,max}$ under hyperoxia was mainly explained by improved cardiac function, because at $\dot{M}_{O_2,max}$ cardiac output was also significantly elevated under hyperoxia (hyperoxia: 32.6±1.6 ml min^{-1} kg^{-1}; normoxia: 27.1±2 ml min^{-1} kg^{-1}; t=−2.17, d.f.=17, P=0.045, Fig. 1G). Higher cardiac output under hyperoxia at $\dot{M}_{O_2,max}$ was mainly due to a trend for increased stroke volume (hyperoxia: 0.52±0.02 ml kg^{-1}; normoxia: 0.45±0.03 ml kg^{-1}; t=−1.84, d.f.=17, P=0.084, Fig. 1I), as heart rate was nearly identical at $\dot{M}_{O_2,max}$ between treatments (normoxia: 60.7±1.5 beats min^{-1}; hyperoxia: 62.4±1.1 beats min^{-1}; t=−0.95, d.f.=17, P=0.36, Fig. 1H). An identical pattern was observed for these cardiac variables when only the 0 h post-chase time point was considered (Fig. 2B–D). There was no difference in A–V O_2 content difference at $\dot{M}_{O_2,max}$ between hyperoxia and normoxia (hyperoxia: 0.20±0.01 mg O_2 ml^{-1}; normoxia: 0.19±0.01 mg O_2 ml^{-1}; t=−0.55, d.f.=17, P=0.59; Fig. 1J), and this was also the case at the 0 h post-chase time point (Fig. 2E). Moreover, at 0 h post-chase, close to the point when $\dot{M}_{O_2,max}$ was observed, [Hb] and Hct were significantly lower (P>0.05) under hyperoxia than normoxia, but there was no difference in MCHC between treatments (Fig. 3). Additionally, Pv_{O_2} was significantly (P<0.05) elevated under hyperoxia relative to normoxia at 0 h post-chase (Fig. 4A). No differences between treatments were observed for venous pH_e, plasma lactate and plasma osmolality at the 0 h post chase time point (Fig. 4B–D).

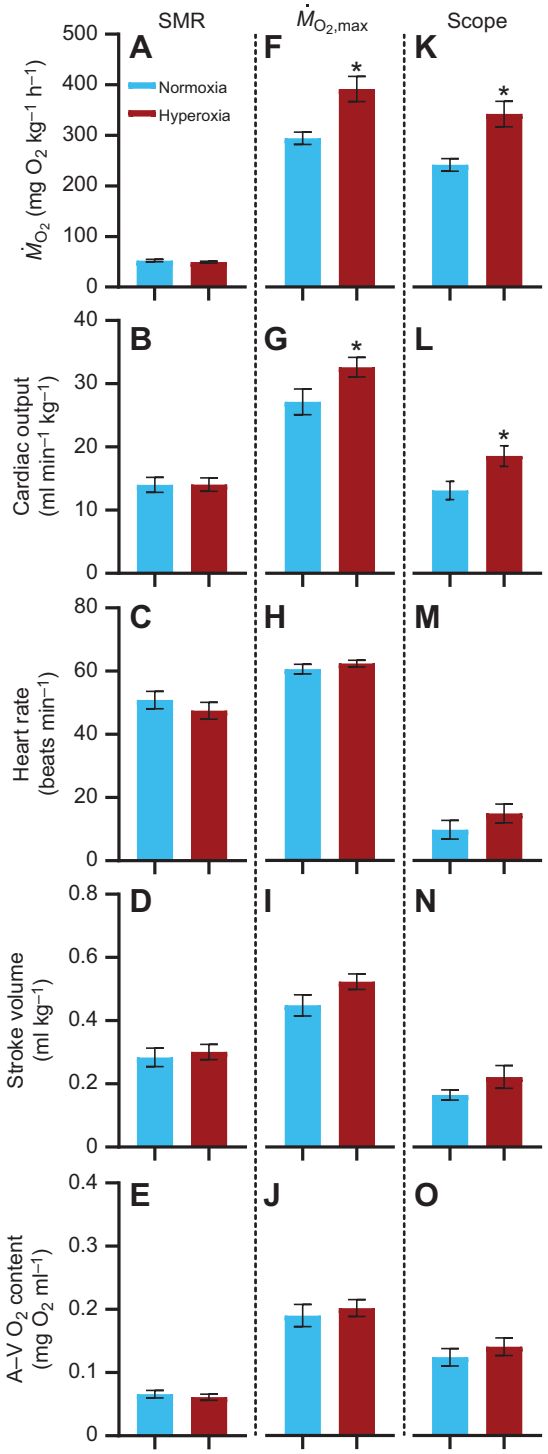

Fig. 1. Cardiorespiratory function in rainbow trout (*Oncorhynchus mykiss*) exhaustively exercised under normoxia (~100% air saturation) or hyperoxia (~200% air saturation). All values are means±s.e.m. (normoxia, N=9; hyperoxia, N=10). Cardiorespiratory responses are shown at standard metabolic rate (SMR; A–E) and maximum O_2 consumption rate ($\dot{M}_{O_2,max}$; F–J). Scope=variable at $\dot{M}_{O_2,max}$–variable at SMR (K–O). $\dot{M}_{O_2}$=mass-specific O_2 consumption rate; A–V O_2 content=arterial–venous O_2 content difference (estimated by the Fick equation). Asterisks indicate a significant difference (P<0.05) between normoxia and hyperoxia.

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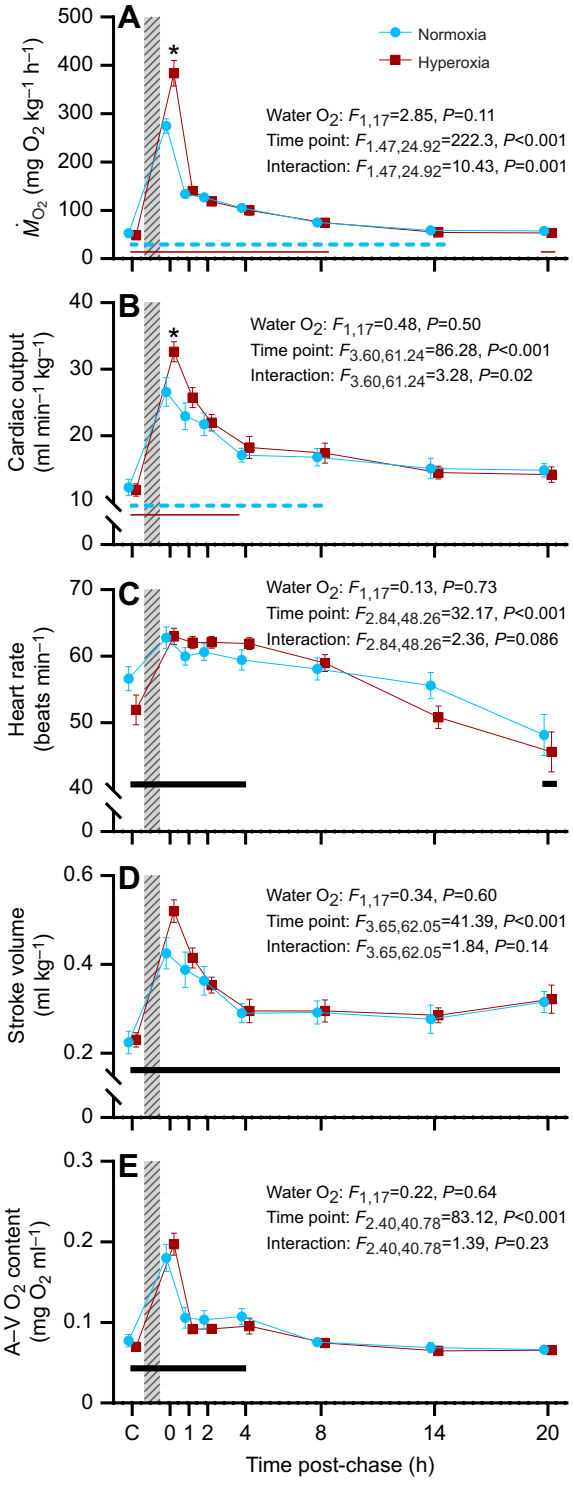

Fig. 2. Routine cardiorespiratory function in rainbow trout exhaustively exercised under normoxia (~100% air saturation) or hyperoxia (~200% air saturation). All values are means±s.e.m. (normoxia, N=9; hyperoxia, N=10). (A) $\dot{M}_{O_2}$ (mass-specific O_2 consumption rate). (B) Cardiac output. (C) Heart rate. (D) Cardiac stroke volume. (E) Arterial–venous O_2 content difference (A–V O_2 content; estimated by the Fick equation). The hatched, grey bar represents 5 min exhaustive exercise (chasing) separating pre-chase control values (C on the x-axis) from post-chase values (0–20 h). Note, data points are intentionally staggered on the x-axis for ease of interpretation. Text insets show the results of a two-way mixed analysis of variance between water O_2 level and sample time point for each variable. Asterisks indicate significant differences (P<0.05) between normoxia and hyperoxia within a sampling time point. Dashed, blue and thin, maroon horizontal lines show significant differences (P<0.05) between pre-chase control values and post-chase values within the normoxia and hyperoxia treatments, respectively. Thick, black horizontal lines indicate where post-chase values were significantly different (P<0.05) from pre-chase control values across water O_2 levels (i.e. where there was only a significant main effect of sampling time point).

Cardiorespiratory function and physiological status at rest in normoxia and hyperoxia

At SMR, which was defined as the mean of the 10 lowest $\dot{M}_{O_2}$ values measured post-chase, no differences existed between treatments in cardiac output (normoxia: 14±1.2 ml min^{-1} kg^{-1}; hyperoxia: 14.1±1.1 ml min^{-1} kg^{-1}; t=−0.03, d.f.=17, P=0.98; Fig. 1B), heart rate (normoxia: 50.9±2.8 beats min^{-1}; hyperoxia: 47.5±2.6 beats min^{-1}; t=−0.03, d.f.=17, P=0.98; Fig. 1C), stroke volume (normoxia: 0.28±0.03 ml kg^{-1}; hyperoxia: 0.30±0.02 ml kg^{-1}; t=−0.46, d.f.=17, P=0.65; Fig. 1D) and A–V O_2 content difference (normoxia: 0.066±0.006 mg O_2 ml^{-1}; hyperoxia: 0.061±0.005 mg O_2 ml^{-1}; t=0.62, d.f.=17, P=0.54; Fig. 1E). Similarly, there were no differences in routine $\dot{M}_{O_2}$, cardiac variables and A–V O_2 content difference between treatments when fish were in a rested state pre-chase and 20 h post-chase (Fig. 2). Pre-chase and 20 h post-chase [Hb] and Hct, however, were significantly (P<0.05) depressed under hyperoxia relative to normoxia, while MCHC was similar between treatments at both times (Fig. 3). Alongside depressed [Hb] and Hct, Pv_{O_2} was significantly (P<0.05) elevated under hyperoxia relative to normoxia at both the pre-chase and 20 h post-chase time points (Fig. 4A). Across treatments, however, no differences (P>0.05) in venous pH_e, plasma lactate and plasma osmolality existed at these times (Fig. 4B–D). Thus, at rest, both pre-chase and 20 h post-chase, the only differences observed between treatments were depressed [Hb] and Hct, and elevated Pv_{O_2} with hyperoxia.

Excess post-exercise oxygen consumption and recovery of cardiorespiratory function following exhaustive exercise in normoxia and hyperoxia

Despite higher $\dot{M}_{O_2,max}$ with hyperoxia, there were no differences in total EPOC (normoxia: 530.4±26 mg O_2 kg^{-1}; hyperoxia: 533.9±37.1 mg O_2 kg^{-1}, t=−0.07, d.f.=17, P=0.94; Fig. 5A), EPOC duration (normoxia: 13.4±0.9 h; hyperoxia: 12.4±1.2 h, t=0.72, d.f.=17, P=0.48; Fig. 5B) and the rate of EPOC repayment (normoxia: 40.7±2.9 mg O_2 kg^{-1} h^{-1}; hyperoxia: 46.6±5.1 mg O_2 kg^{-1} h^{-1}, t=−0.97, d.f.=17, P=0.33; Fig. 5C) between treatments. When considering routine $\dot{M}_{O_2}$, however, fish in hyperoxia recovered to a pre-chase level faster than did fish in normoxia; this occurred by 8 h post-chase in hyperoxia and by 14 h post-chase in normoxia (Fig. 2A). Matching the pattern for routine $\dot{M}_{O_2}$, post-chase routine cardiac output recovered to a pre-chase level faster under hyperoxia (by 4 h post-chase) than under normoxia (by 8 h post-chase) (Fig. 2B). There were no differences between treatments in the post-chase recovery dynamics of heart rate, stroke volume and A–V O_2 content difference (Fig. 2C–E). Exhaustive exercise impacted [Hb], Hct, MCHC, Pv_{O_2}, venous pH_e, plasma lactate and plasma osmolality similarly in hyperoxia and normoxia, such that there were no differences in recovery dynamics between treatments for any of these variables (Figs 3 and 4).

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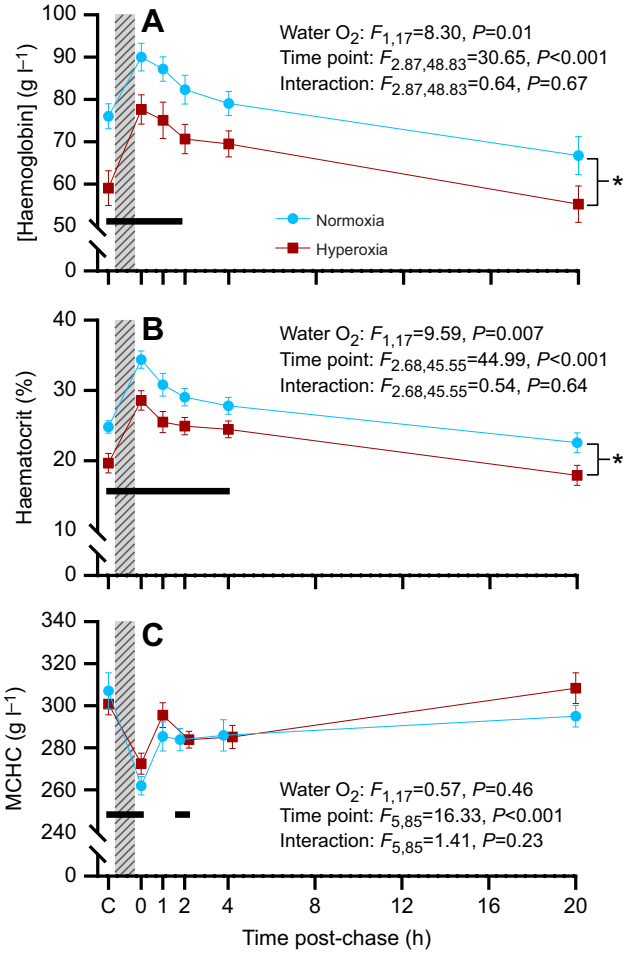

Fig. 3. Haematological responses in rainbow trout exhaustively exercised under normoxia (~100% air saturation) or hyperoxia (~200% air saturation). All values are means±s.e.m. (normoxia, N=9; hyperoxia, N=10). (A) Haemoglobin. (B) Haematocrit. (C) Mean corpuscular haemoglobin concentration (MCHC). Note, data points at 2 h and 4 h in C are intentionally staggered on the x-axis for ease of interpretation. The hatched, grey bar represents 5 min exhaustive exercise (chasing) separating pre-chase control values (C on x-axis) from post-chase values (0–20 h). Text insets show the results of a two-way mixed analysis of variance between water O_2 level and sampling time point for each variable. Asterisks show a significant difference (P<0.05) between normoxia and hyperoxia across sampling time points (i.e. a significant main effect of water O_2 level). Black horizontal lines indicate where post-chase values were significantly different (P<0.05) from pre-chase control values across water O_2 levels.

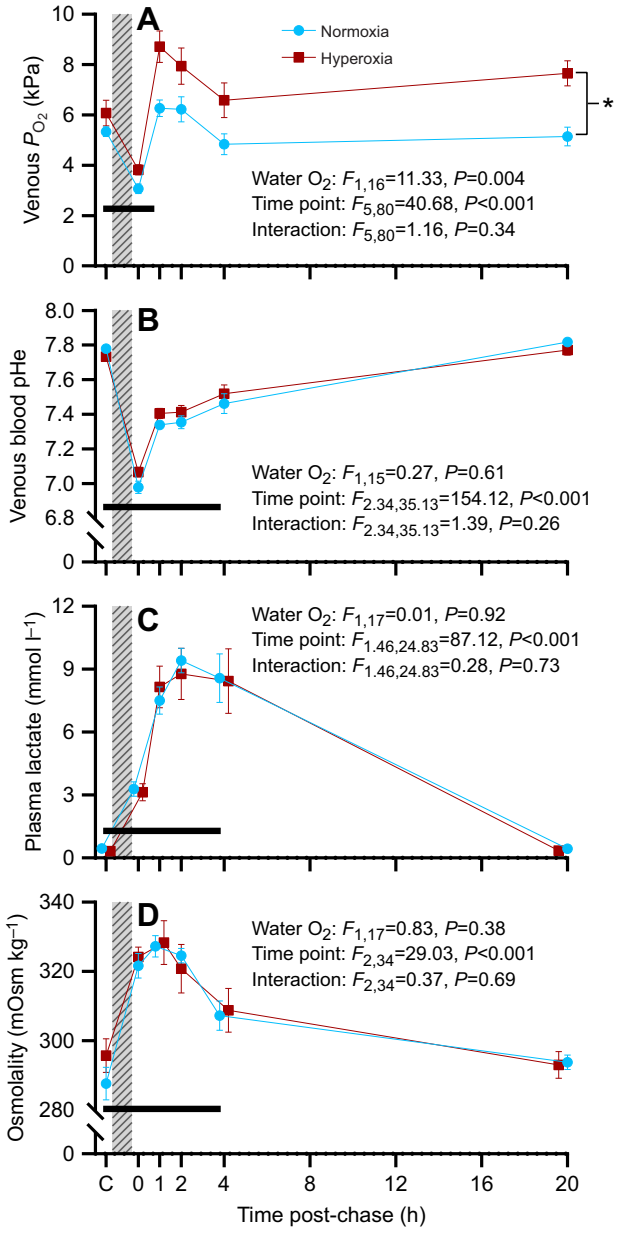

Fig. 4. Venous blood O_2 partial pressure, venous acid–base status, plasma lactate and plasma osmolality in rainbow trout exhaustively exercised under normoxia (~100% air saturation) or hyperoxia (~200% air saturation). All values are means±s.e.m. (normoxia, N=9; hyperoxia, N=10). (A) Venous blood O_2 partial pressure (P_{O_2}). (B) Venous blood extracellular pH (pH$_e$). (C) Plasma lactate. (D) Plasma osmolality. Note, some data points are intentionally staggered on the x-axis for ease of interpretation. The hatched grey bar represents 5 min exhaustive exercise (chasing) separating pre-chase control values (C on the x-axis) from post-chase values (0–20 h). Text insets show the results of a two-way mixed analysis of variance between water O_2 level and sample time point for each variable. Asterisks indicate a significant difference (P<0.05) between normoxia and hyperoxia across sampling time points (i.e. a significant main effect of water O_2 level). Black horizontal lines indicate where post-chase values were significantly different (P<0.05) from pre-chase control values across water O_2 levels.

DISCUSSION

Hyperoxia increases maximum cardiorespiratory performance in rainbow trout following exhaustive exercise but does not influence repayment of O_2 debt

Here, in rainbow trout, $\dot{M}_{O_2,max}$ was 33% higher under hyperoxia than normoxia, and since SMR was essentially the same between treatments, this also drove a corresponding 41% increase of aerobic scope. At the same hyperoxic water O_2 level (~200% air saturation), the magnitude of increase in $\dot{M}_{O_2,max}$ and aerobic scope seen here was comparable to that seen in two triplefin fishes, ~25% and ~30% for $\dot{M}_{O_2,max}$ and aerobic scope, respectively (McArley et al., 2018), whereas it was less than the ~92% increase in $\dot{M}_{O_2,max}$ and aerobic scope seen in European perch (Brijs et al., 2015). Although $\dot{M}_{O_2,max}$ increased with hyperoxia, the total O_2 debt (EPOC) generated by exhaustive exercise – a highly anaerobic form of swimming (Milligan and Girard, 1993; Milligan, 1996) – was identical between normoxia and hyperoxia. The similarity of EPOC between oxygenation treatments, near identical profiles of lactate accumulation in the plasma and similar changes in venous blood pH, suggests the anaerobic requirements of exhaustive exercise were

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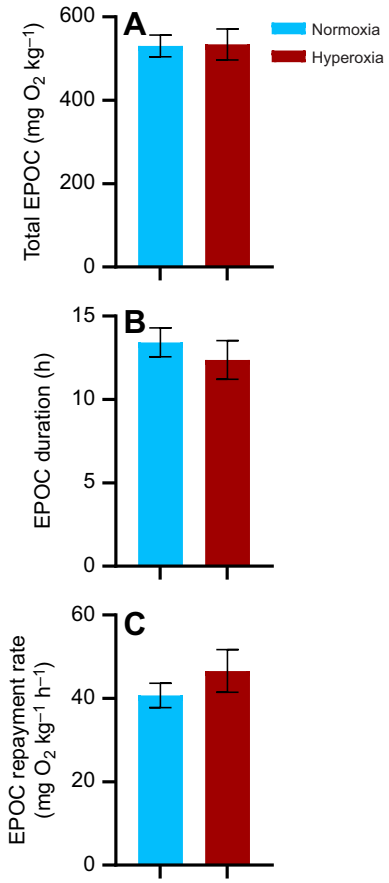

Fig. 5. Excess post-exercise oxygen consumption (EPOC) in rainbow trout exhaustively exercised under normoxia (~100% air saturation) or hyperoxia (~200% air saturation). All values are means±s.e.m. (normoxia, *N*=9; hyperoxia, *N*=10). (A) Total EPOC. (B) EPOC duration. (C) Rate of EPOC repayment (i.e. total EPOC/EPOC duration).

unmodified by hyperoxia. The energetic requirements of correcting osmotic imbalances caused by fluid and ion shifts between intracellular and extracellular compartments also make up a significant proportion of EPOC in fish (Wood, 1991a), but as indicated by near identical changes in plasma osmolality following exhaustive exercise, these, too, appeared unaffected by hyperoxia. Overall, it appears hyperoxia, despite increasing $\dot{M}_{O_2,max}$, does not influence either the magnitude of O_2 debt generated from exhaustive exercise or the rate at which this O_2 debt is repaid in any meaningful way.

The elevated $\dot{M}_{O_2,max}$ and aerobic scope seen with hyperoxia in the current study was mainly the result of a 20% higher cardiac output. This was due to higher cardiac stroke volume, as heart rate was essentially identical at $\dot{M}_{O_2,max}$ between treatments. Thus, immediately after exhaustive exercise, hyperoxia released a constraint on cardiac contractility apparent with normoxia, and this allowed, at least in part, for $\dot{M}_{O_2}$ to proceed at a higher rate. While it was confirmed that improved cardiac contractility following exhaustive exercise with hyperoxia was associated with elevated Pv_{O_2}, the magnitude of difference in Pv_{O_2} was relatively small (+0.8 kPa with hyperoxia), and it is questionable whether this would have driven the observed differences in contractility. In European perch subjected to thermal ramping, hyperoxia (200% air saturation) treated fish maintained higher routine cardiac output at temperatures approaching upper critical thermal limits (29–31°C) than did perch in normoxia (Ekström et al., 2016). In the same study, Pv_{O_2} was elevated with hyperoxia, which the authors suggested may have led to improved cardiac performance through an enhanced luminal O_2 supply to the myocardium. It should be noted, however, that at the temperatures where differences in cardiac output emerged under hyperoxia in perch (29−30°C), a greater difference in Pv_{O_2} between hyperoxia and normoxia was observed than that seen in rainbow trout in the current study (in perch, +3.9 kPa and +1.7 kPa with hyperoxia at 29°C and 30°C, respectively, and in the current study, +0.8 kPa with hyperoxia following exhaustive exercise).

In salmonids, the outer compact layer of the heart (compact myocardium) is supplied directly with oxygenated blood from the gills via the coronary artery. This contrasts with approximately 2/3 of all teleost species, including European perch, in which the heart only receives O_2 via the venous blood returning to the heart (Ekström et al., 2016, 2017; Farrell et al., 2012). Thus, here, in addition to the slightly elevated Pv_{O_2} with hyperoxia, it is also possible that hyperoxia enhanced the coronary arterial O_2 supply to the compact myocardium, which in turn may have contributed to improved contractility. All but one study assessing the influence of hyperoxia on blood oxygenation in fish, including several in rainbow trout, show arterial blood O_2 partial pressure (Pa_{O_2}) increases during environmental hyperoxia exposure (McArley et al., 2021). It is likely, therefore, that Pa_{O_2} was increased with hyperoxia in the current study. At rest under normoxia, Pa_{O_2} is normally around 14.5–18.0 kPa in rainbow trout (e.g. Kiceniuk and Jones, 1977; Morgenroth et al., 2019; Wang et al., 1994; Wood and Jackson, 1980). If such levels of Pa_{O_2} remained after exhaustive exercise, then any increase in Pa_{O_2} with hyperoxia leading to improved cardiac output might suggest an *in vivo* limitation of maximal contractility, presumably within the compact myocardium, at normoxic Pa_{O_2}. It is possible, however, that hyperoxia does not increase Pa_{O_2} above a typical resting normoxic level following exhaustive exercise. Indeed, several studies in rainbow trout have demonstrated Pa_{O_2} can fall to around ~8 kPa immediately following exhaustive exercise (Ferguson and Tufts, 1992; Milligan and Wood, 1987; Primmett et al., 1986; Wang et al., 1994). Therefore, a window may exist after exhaustive exercise where hyperoxia would not simply elevate Pa_{O_2} beyond the typical normoxic range (~16 kPa), but instead prevent it from collapsing and becoming mildly hypoxic, as seems to be the case in normoxia. Further investigation, however, is required to evaluate whether there are differences in arterial blood oxygenation in fish undergoing exhaustive exercise under hyperoxia or normoxia.

Hyperoxia may safeguard arterial haemoglobin oxygen saturation following exhaustive exercise

The A–V O_2 content difference (estimated by the Fick equation) at $\dot{M}_{O_2,max}$ was 6.3% higher under hyperoxia than normoxia. Although this difference was not significant, combined with higher cardiac output, it may have contributed to the higher $\dot{M}_{O_2,max}$ in hyperoxia. The increased A–V O_2 content difference was apparent despite [Hb] being, on average, 12.5 g l^{-1} lower under hyperoxia immediately post-chase, which would tend to reduce arterial O_2 content, and Pv_{O_2} being elevated under hyperoxia immediately post-chase, which would tend to increase venous O_2 content (i.e. through increasing Hb O_2 saturation). Together, in a situation where Hb was fully saturated in arterial blood and assuming the Hb O_2 dissociation curve was similarly shaped with hyperoxia and normoxia, this should have caused a reduced A–V O_2 content difference under hyperoxia relative to normoxia. There are two possibilities that could explain this seemingly impossible outcome. Firstly, following

exhaustive exercise, it may be that Hb O_2 saturation in arterial blood is higher under hyperoxia than normoxia. As noted above, it is known that Pa_{O_2} can fall to around ∼8 kPa following exhaustive exercise in normoxia, and this, along with possible contributions of Bohr/Root effects associated with blood acidosis, can cause reductions in Hb O_2 saturation in arterial blood (Ferguson and Tufts, 1992; Milligan and Wood, 1987). As hyperoxia increases Pa_{O_2} in rainbow trout (Wood and Jackson, 1980), it is possible hyperoxia offsets declines in Pa_{O_2} following exhaustive exercise in normoxia, thereby promoting higher Hb O_2 saturation and a higher arterial O_2 content. Duthie and Hughes (1987) demonstrated there is no difference in $\dot{M}_{O_2,max}$ between hyperoxia and normoxia treated rainbow trout swum at critical swimming speed (U_{crit}). While this may seem paradoxical in relation to the elevated $\dot{M}_{O_2,max}$ observed in hyperoxia following exhaustive exercise in the present study, one reason to explain the lack of an effect of hyperoxia in Duthie and Hughes (1987) could be that a reduction in Pa_{O_2} and Hb O_2 saturation is less likely to occur at U_{crit} under normoxia when swimming fish are able to ram ventilate. Indeed, Kiceniuk and Jones (1977) showed that Pa_{O_2}, HbO_2 saturation and arterial O_2 content all remained similar to resting levels in rainbow trout swimming at U_{crit} under normoxia. In contrast, also in rainbow trout, Neumann et al. (1983) showed a significant decline in arterial O_2 content relative to resting levels – presumably linked to lower Pa_{O_2} and Hb O_2 saturation – following exhaustive exercise. If a reduction in arterial Hb O_2 saturation following exhaustive exercise is common in fish and does not occur when fish are swimming (and ram ventilating), this could offer an explanation as to why, at least in some cases, higher $\dot{M}_{O_2,max}$ values are seen in fish swum at U_{crit} than in fish following exhaustive exercise (Raby et al., 2020).

The second possible reason explaining a higher A–V O_2 content difference with hyperoxia relates to a limitation with using the Fick equation (i.e. dividing $\dot{M}_{O_2}$ by cardiac output) to estimate this parameter. In doing so, it is assumed that the total $\dot{M}_{O_2}$ of the organism is only the product of the O_2 delivered to the tissues via arterial blood multiplied by the rate of arterial blood flow (i.e. cardiac output). In fish, however, this assumption is erroneous because O_2 can be consumed directly from the water by the skin and gills (Farrell et al., 2014; Feder and Burggren, 1985; Zena et al., 2017). The proportion of total $\dot{M}_{O_2}$ consumed directly by the skin in rainbow trout, for example, is estimated to be 15% under normoxia (Kirsch and Nonnotte, 1977). While it has not been evaluated in rainbow trout, the proportion of total $\dot{M}_{O_2}$ consumed directly by the skin has been shown to increase under hyperoxia in other fishes, for example, in European eels (*Anguilla anguilla*; Kirsch and Nonnotte, 1977; Le Moigne et al., 1986) and common carp (*Cyprinus carpio*; Takeda, 1989). An important distinction to make, however, is that, although the proportion of O_2 consumed directly by the skin and gills increases with hyperoxia – presumably because of increased O_2 diffusion gradients between hyperoxic water and cutaneous tissues – total $\dot{M}_{O_2}$ remains unchanged (Kirsch and Nonnotte, 1977; Le Moigne et al., 1986; Takeda, 1989). Thus, in itself, hyperoxia does not appear to increase total O_2 demand. If the proportion of total $\dot{M}_{O_2}$ attributed to direct O_2 uptake by the skin and gills was higher under hyperoxia than normoxia in the current study, then the estimates of A–V O_2 content difference derived from rearrangement of the Fick equation would be overestimated for the hyperoxic treatment, thereby inflating estimates relative to normoxia. In reality, the estimate of A–V O_2 content difference calculated here is actually an estimate of the total amount of O_2 removed from the water via any route per unit of blood volume pumped by the heart, and direct measures of arterial and venous O_2 content alongside cardiac output and $\dot{M}_{O_2}$ are required to pinpoint where the O_2 consumed by the organism is being taken up and in what proportions. An analysis of this type has been completed in sockeye salmon (*Oncorhynchus nerka*), although not in relation to hyperoxia (Farrell et al., 2014). It revealed the proportion of total $\dot{M}_{O_2}$ attributed to direct uptake by cutaneous tissues ranges from 12 to 48% and does not necessarily decline when metabolic rate increases, as has been previously assumed (Farrell et al., 2014). These findings show the potential for substantial and variable direct O_2 uptake by cutaneous tissues in salmonids, which in turn highlights the need for caution when interpreting parameters, such as the A–V O_2 content difference, derived from the Fick equation.

Hyperoxia induces reduced haemoglobin and haematocrit

The Hb concentration and Hct of blood were reduced in hyperoxia relative to normoxia under resting conditions, and this was apparent both before and following recovery from exhaustive exercise. In both normoxia and hyperoxia, [Hb] and Hct increased after exhaustive exercise, but here, too, the haematological differences seen between treatments remained. MCHC was also similar among treatments at rest and following exhaustive exercise. The observed pattern of a reduction in MCHC after exhaustive exercise accompanied by increases in [Hb] and Hct indicate a combination of splenic release of stored red blood cells, which boosts blood O_2-carrying capacity (Brijs et al., 2020; Kita and Itazawa, 1989; Pearson and Stevens, 1991; Yamamoto et al., 1980), and red blood cell swelling, which serves to protect blood O_2 transport through regulation of intracellular red blood cell pH and dilution of allosteric modifiers of Hb (Milligan and Wood, 1987; Nikinmaa, 1986). Both these responses (i.e. splenic release and red blood cell swelling) are linked to the action of neurally and hormonally released catecholamines (i.e. adrenaline and noradrenaline) (Kita and Itazawa, 1989; Nikinmaa, 1986), the latter which increase substantially in the plasma of exhaustively exercised rainbow trout (Milligan and Wood, 1987; Wood et al., 1990). It is also likely that part of the observed haemoconcentration following exhaustive exercise resulted from a reduction in plasma volume associated with fluid shifts from extracellular to intracellular fluid compartments (Pearson and Stevens, 1991; Wood, 1991a). The sharp increase in plasma osmolality following exhaustive exercise indicates such a fluid shift occurred.

The general reduction of [Hb] and Hct with hyperoxia suggests that after only ∼22 h exposure to elevated water O_2 levels, when the first blood sample was taken, red blood cells had been removed from the circulation. A dilution of the blood, for example, due to possible fluid shifts associated with respiratory acidosis and bicarbonate accumulation in plasma during hyperoxia (Wood, 1991b), could also have caused [Hb] and Hct to decrease, but this seems unlikely as there were no differences in resting plasma osmolality or overall cardiovascular status between treatments. Similarly, in Senegalese sole (*Solea senegalensis*), lower [Hb] accompanied by lower red blood cell numbers were observed after 24 h exposure to 200% air saturation (Machado et al., 2018). Other studies involving short duration (1−4 days) hyperoxia, however, have not reported similar haematological changes (Brauner et al., 2000; Ekström et al., 2016; Karlsson et al., 2011; Mustafa et al., 2011; Takeda, 1990; Vanlandeghem et al., 2010). More often, the haematological responses to long term (weeks to months) hyperoxia have been assessed in fish (reviewed in McArley et al., 2021), and several studies in rainbow trout, specifically, have demonstrated either reduced [Hb] or Hct, or both in such circumstances (Dabrowski et al., 2004; Edsall and Smith, 1990; Jewett et al., 1991; Ritola et al.,

2002). Thus, although the reduction in [Hb] and Hct with hyperoxia observed in the current study is not unique in rainbow trout, it is, to the best of our knowledge, the first to show these haematological changes can occur so rapidly. Assessing the role of the spleen, the known storage organ for red blood cells in fish (Fänge and Nilsson, 1985), in mediating changes in [Hb] and Hct during hyperoxia exposure may provide a useful avenue to begin understanding exactly how these acute haematological responses occur.

Conclusion

Our findings show, at least in rainbow trout, that one way hyperoxia can increase $\dot{M}_{O_2,max}$ and aerobic scope following exhaustive exercise is to improve cardiac contractility, thereby allowing higher cardiac output. An improvement of maximal cardiac performance alone, however, did not fully explain the higher $\dot{M}_{O_2,max}$ seen with hyperoxia. Somehow, despite a significantly reduced [Hb] and higher Pv_{O_2}, fish in hyperoxia maintained a higher A–V O_2 content difference as estimated by the Fick equation. One way this might have been possible is that hyperoxia offset declines in arterial Hb O_2 saturation following exhaustive exercise that are known to occur under normoxia. Although somewhat speculative, this could raise arterial blood O_2 content higher under hyperoxia relative to normoxia, thus driving a higher A–V O_2 content difference. Future studies assessing arterial Pa_{O_2}, arterial Hb O_2 saturation and arterial O_2 content would further elucidate exactly how hyperoxia expands $\dot{M}_{O_2,max}$ following exhaustive exercise and could have important implications for the utility of using exhaustive exercise to elicit $\dot{M}_{O_2,max}$ and estimate aerobic scope in fish.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: T.J.M., E.S.; Methodology: T.J.M., D.M., A.T.E., E.S.; Formal analysis: T.J.M.; Investigation: T.J.M., D.M., L.A.Z., A.T.E.; Resources: E.S.; Data curation: T.J.M.; Writing - original draft: T.J.M.; Writing - review & editing: T.J.M., D.M., L.A.Z., A.T.E., E.S.; Visualization: T.J.M.; Supervision: E.S.; Project administration: T.J.M., E.S.; Funding acquisition: T.J.M., E.S.

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Data availability

Data are publicly available on Figshare at https://doi.org/10.6084/m9.figshare.14815818.v1.

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