

RESEARCH ARTICLE

Experimental manipulations of tissue oxygen supply do not affect warming tolerance of European perch

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ABSTRACT

A progressive inability of the cardiorespiratory system to maintain systemic oxygen supply at elevated temperatures has been suggested to reduce aerobic scope and the upper thermal limit of aquatic ectotherms. However, few studies have directly investigated the dependence of thermal limits on oxygen transport capacity. By manipulating oxygen availability (via environmental hyperoxia) and blood oxygen carrying capacity (via experimentally induced anaemia) in European perch (*Perca fluviatilis* Linnaeus), we investigated the effects of oxygen transport capacity on aerobic scope and the critical thermal maximum (CT_{max}). Hyperoxia resulted in a twofold increase in aerobic scope at the control temperature of 23°C, but this did not translate to an elevated CT_{max} in comparison with control fish (34.6±0.1 versus 34.0±0.5°C, respectively). Anaemia (~43% reduction in haemoglobin concentration) did not cause a reduction in aerobic scope or CT_{max} (33.8±0.3°C) compared with control fish. Additionally, oxygen consumption rates of anaemic perch during thermal ramping increased in a similar exponential manner to that in control fish, highlighting that perch have an impressive capacity to compensate for a substantial reduction in blood oxygen carrying capacity. Taken together, these results indicate that oxygen limitation is not a universal mechanism determining the CT_{max} of fishes.

KEY WORDS: Metabolism, Oxygen consumption, Aerobic scope, Hyperoxia, Anaemia, Temperature

INTRODUCTION

Anthropogenic climate change is a major determinant of biological change in the world's oceans (Caldeira and Wickett, 2003; Levitus et al., 2000). Given that ambient temperature is a critical determinant of performance in aquatic ectotherms like fish, ocean warming may soon expose many species to temperatures outside their range of tolerance (Huey et al., 2012; Huey and Kingsolver, 2011; Parmesan, 2006). In fact, recent studies demonstrate that the distribution and performance of some fish species have already been affected by the ongoing warming of the oceans (Perry et al., 2005; Seth et al., 2013).

Body temperature is regarded as a key determinant of organismal performance because of the temperature dependence of biochemical reactions and the consequent effects on the physiological function of organisms (Huey and Kingsolver, 2011). According to the metabolic theory of ecology, metabolic rate is a key physiological

factor controlling ecological processes at all levels of organization (Brown et al., 2004). Although the generality of this claim has rightly been questioned (Harte, 2004), the predicted exponential increase in standard metabolic rate (SMR) of ectotherms as temperature increases (Brown et al., 2004; Gillooly et al., 2001) may play a pivotal role in determining an organism's thermal tolerance and future geographical distribution. Moreover, aerobic scope (AS), defined as the difference between SMR and maximum metabolic rate (MMR), has been reported to gradually decline at temperatures exceeding the optimal thermal range in several fishes (Brett, 1971; Fry, 1947). The loss of AS at the upper critical temperature has been suggested to be the fundamental factor in determining the upper thermal limits of fish, as survival is proposed to be time limited beyond this point as a result of a transition to anaerobic metabolism (Lannig et al., 2004; Pörtner, 2002; Sartoris et al., 2003; Zakhartsev et al., 2003). One suggested explanation for the reported loss of AS at high temperatures, formalized as the 'oxygen- and capacity-limited thermal tolerance (OCLTT) hypothesis', is that the cardiorespiratory system progressively fails to maintain the necessary oxygen supply to active tissues (Pörtner and Farrell, 2008).

The generality of this hypothesis has recently been under considerable debate (Clark et al., 2013a,b; Farrell, 2013; Jutfelt et al., 2014; Pörtner, 2014; Pörtner and Giomi, 2013). A growing number of studies show that the exponential rise in SMR can be sustained until lethal temperatures are reached and that AS does not decrease progressively as temperature exceeds an optimal range, but rather crashes immediately below the critical thermal maximum (CT_{max}) (Clark et al., 2011; Fry, 1971; Fry and Hart, 1948; Gräns et al., 2014; Norin et al., 2014). This suggests that mechanisms other than a progressive decline in oxygen transport capacity may underlie upper thermal limits in fish. While different thermal effects on AS may be in part explained by species-specific differences and/or differences in experimental protocols, a fundamental question remains whether a limitation in oxygen supply is the direct causal mechanism determining the upper thermal limit for aquatic ectotherms (Clark et al., 2013a).

Studies on numerous fish species show that acute thermal tolerance is reduced when oxygen tension in the water is decreased below normal air saturation values (i.e. hypoxia; Healy and Schulte, 2012; Rutledge and Beiting, 1989; Weatherley, 1970). While these results lend support to the idea that oxygen supply limits thermal tolerance, one would expect hyperoxia (i.e. an increase in environmental oxygen availability) to increase the upper limit of thermal tolerance if oxygen supply is indeed the limiting factor. To date, few studies have investigated the effects of hyperoxia on the thermal tolerance of fishes, and results are conflicting, with some species displaying an increased thermal tolerance (Weatherley, 1970), while others remain unaffected (Healy and Schulte, 2012; Mark et al., 2002; Rutledge and Beiting, 1989).

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List of symbols and abbreviations

AS	aerobic scope
CT _{max}	upper critical temperature
MMR	maximum metabolic rate
\dot{M}_{O_2}	oxygen consumption rate
SMR	standard metabolic rate

Furthermore, if the CT_{max} of fishes depends on the capacity of the cardiorespiratory system to supply sufficient oxygen to the tissues at increasing temperatures, then individual variability in traits determining oxygen transport should translate into differences in upper thermal limits. Whilst some studies have reported that relative ventricle mass, cardiac myoglobin levels and haematocrit positively correlate with CT_{max} (Anttila et al., 2013; Beers and Sidell, 2011), other studies have not found such correlations (Ekström et al., 2014; Wang et al., 2014). To our knowledge there is only one study that has experimentally reduced blood oxygen carrying capacity in a fish species in order to investigate the dependence of CT_{max} on oxygen transport capacity (Wang et al., 2014). That study suggested that acute thermal tolerance might be determined by factors beyond an inability of the cardiorespiratory system to meet the rise in metabolic demand as temperature increases (Wang et al., 2014).

In the light of the conflicting literature regarding the relationships between environmental oxygen availability, oxygen transport capacity and upper thermal limits, the present study investigated whether increased oxygen availability (via environmental hyperoxia) or experimentally reduced blood oxygen carrying capacity (induced through anaemia) affected oxygen usage capacity (measured through AS) of a temperate eurythermal fish, the European perch (*Perca fluviatilis* Linnaeus). Subsequently, we investigated whether experimentally induced differences in blood oxygen carrying capacity and AS translated into differences in acute upper thermal limits by measuring CT_{max}. We hypothesized that AS and CT_{max} would change in parallel with tissue oxygen supply, leading to higher values in fish exposed to hyperoxia and lower values in anaemic fish.

RESULTS**Haematology**

The haemoglobin concentration, haematocrit and mean corpuscular haemoglobin concentration (MCHC) of all fish measured prior to any experimental manipulations were $80 \pm 2.0 \text{ g l}^{-1}$, $38.6 \pm 1.3\%$ and $211 \pm 6 \text{ g l}^{-1}$, respectively. At the end of the thermal challenge, the haemoglobin concentration of the control group ($75 \pm 2 \text{ g l}^{-1}$) did not significantly differ from that of the hyperoxic group ($68 \pm 3 \text{ g l}^{-1}$, $P=0.097$; Fig. 1A), yet there was a significantly higher haematocrit in the former ($39.6 \pm 1.0\%$) compared with the latter ($33.1 \pm 2.2\%$, $P=0.015$; Fig. 1B). As expected, withdrawing a substantial amount (~40%) of the total blood volume in the anaemic treatment group significantly ($P<0.001$) reduced both the haemoglobin concentration ($43 \pm 5 \text{ g l}^{-1}$) and haematocrit ($19.6 \pm 1.4\%$) when compared with the control group (Fig. 1A,B). No significant differences in MCHC were found between treatment groups and the control group ($P>0.4$ in both cases; Fig. 1C).

Metabolic rate and aerobic scope

Oxygen consumption rates (\dot{M}_{O_2}) of fish from all three treatments were relatively stable for approximately 10 h prior to the fish being subjected to the exhaustive exercise protocol (Fig. 2A). The SMR of

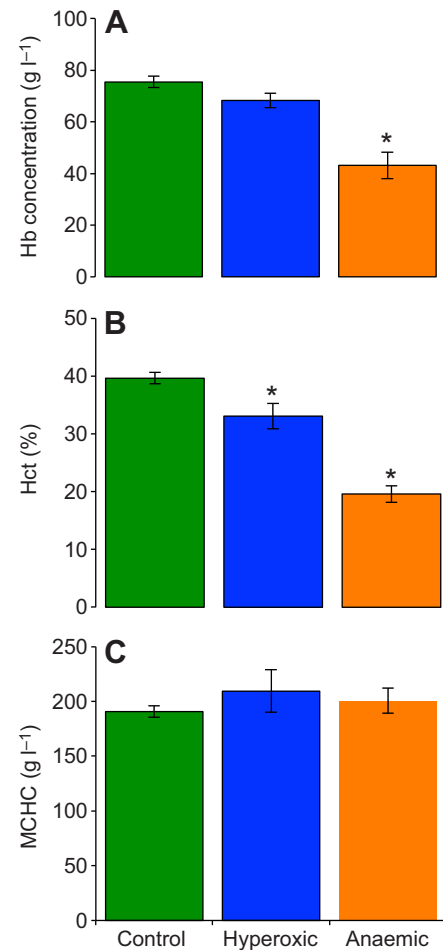


Fig. 1. The effects of increased environmental oxygen availability (hyperoxia) and experimentally reduced blood oxygen carrying capacity (anaemia) on haematological parameters of European perch (*Perca fluviatilis*) after an acute thermal challenge. (A) Haemoglobin (Hb) concentration, (B) haematocrit (Hct) and (C) mean corpuscular haemoglobin concentration (MCHC) of control ($N=8$), hyperoxic ($N=8$) and anaemic ($N=6$) perch after an acute thermal challenge. Data are presented as means \pm s.e.m. Asterisks indicate a significant difference ($P<0.05$) from the control group.

fish from the hyperoxic treatment group at 23°C was $80 \pm 5 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$ and did not differ significantly from that of control fish ($78 \pm 6 \text{ mg O}_2 \text{ kg}^{-1} \text{ h}^{-1}$, $P=0.958$; Fig. 2B). Fish exposed to ambient hyperoxia exhibited an AS that was approximately twofold higher than the control group at 23°C ($P<0.001$), which was due to a significantly higher MMR (calculated as post-exercise \dot{M}_{O_2} ; see Materials and methods) of hyperoxic fish in response to the exhaustive exercise protocol (477 ± 34 versus $286 \pm 24 \text{ mg kg}^{-1} \text{ h}^{-1}$, $P<0.001$; Fig. 2B). Despite a significant reduction in blood oxygen carrying capacity in anaemic fish (~43% reduction in haemoglobin concentration), SMR, MMR and AS were not significantly different from the control group ($P=0.897$, $P=0.468$ and $P=0.387$, respectively; Fig. 2B).

Temperature tolerance

The significantly higher AS of hyperoxic fish compared with the control group did not translate into a significantly higher CT_{max} ($34.6 \pm 0.1^\circ\text{C}$ versus $34.0 \pm 0.5^\circ\text{C}$, $P=0.364$; Fig. 3A). Additionally, the significantly reduced blood oxygen carrying capacity of the anaemic group did not significantly affect CT_{max} ($33.8 \pm 0.3^\circ\text{C}$) in comparison with control fish ($P=0.745$; Fig. 3A).

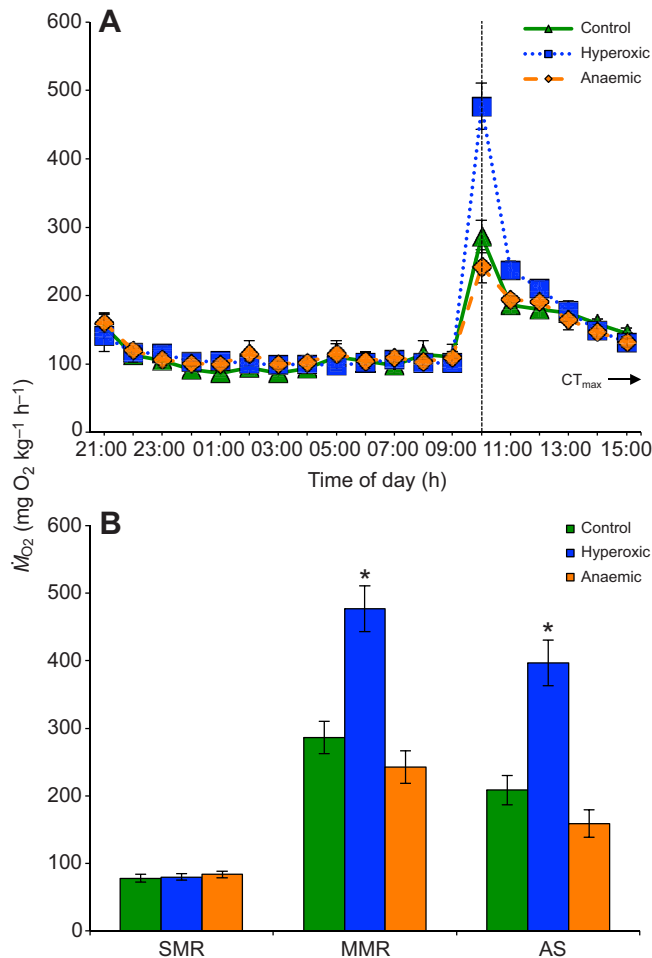


Fig. 2. Metabolic responses of *P. fluviatilis* during environmental hyperoxia or with anaemia. (A) Oxygen consumption rate (\dot{M}_{O_2}) of undisturbed control ($N=8$), hyperoxic ($N=8$) and anaemic ($N=6$) perch before an exhaustive exercise protocol (vertical hatched line). This was followed by a period of exercise recovery (>6 h) before fish were subjected to an acute thermal challenge to assess the critical thermal maximum (CT_{max}). (B) Standard metabolic rate (SMR), maximum metabolic rate (MMR) and aerobic scope (AS) of control, hyperoxic and anaemic perch at 23°C. Data are presented as means \pm s.e.m. Asterisks in B indicate a significant difference ($P<0.05$) from the control group.

During the thermal ramping protocol to determine CT_{max} , \dot{M}_{O_2} was significantly affected by treatment ($P<0.001$), temperature ($P=0.008$) and their interaction ($P=0.001$; Fig. 3B). *Post hoc* tests revealed overall significant differences in \dot{M}_{O_2} between the hyperoxic and control groups ($P<0.001$), but no overall significant differences between the anaemic and control groups ($P=0.993$). These statistical differences were primarily associated with the patterns in \dot{M}_{O_2} at temperatures above 32°C, for which the \dot{M}_{O_2} of control and anaemic fish continued a similar steady exponential increase with temperature while the hyperoxic perch dramatically increased \dot{M}_{O_2} approximately twofold over the 1°C temperature increment between 32 and 33°C (Fig. 3B).

DISCUSSION

Upper thermal limits in aquatic ectotherms have been suggested to be constrained primarily by the progressive inability of the cardiorespiratory system to supply oxygen to tissues at temperatures approaching the upper boundary of the organism's thermal window (Pörtner, 2002; Pörtner and Farrell, 2008). However, surprisingly few

studies have experimentally manipulated oxygen availability and delivery in order to investigate the consequent effects on AS and acute upper thermal limits in fish. We show that a twofold increase in AS induced by environmental hyperoxia, or a substantial reduction in blood oxygen carrying capacity via experimentally induced anaemia did not significantly affect the CT_{max} of European perch. Taken together, these results indicate that it is unlikely that oxygen limitation is a universal mechanism determining the acute upper thermal limit of fishes.

Hyperoxia increases aerobic scope but not acute upper thermal limits

Increased oxygen availability from the surrounding environment has been shown to increase the partial pressure of oxygen in the blood of a range of fish species via increased diffusion of oxygen across the gills and skin (Barrett and Taylor, 1984; Soncini and Glass, 1997; Takeda, 1990). This has been suggested to alleviate the workload of the cardiorespiratory system when supplying oxygen to tissues under increasing temperatures, whilst simultaneously increasing the scope for active oxygen uptake (Mark et al., 2002). Temperature-dependent increases in arterial blood flow (measured via flow-weighted magnetic resonance imaging) and SMR in a species of Antarctic teleost (*Pachycara brachycephalum*) were strongly reduced in individuals exposed to hyperoxia, suggesting that hyperoxia may increase AS (Mark et al., 2002). The present study is the first to directly measure changes in AS of fish under environmental hyperoxia, revealing an approximate doubling of AS in perch subjected to hyperoxic conditions. However, this substantial increase in AS under hyperoxia did not enhance the acute upper thermal limits of perch, as CT_{max} was not significantly elevated when compared with control fish. Although these findings conflict with a study investigating the effects of increased environmental oxygen tension on acute thermal tolerance of goldfish (*Carassius auratus*) at hyperbaric pressures (Weatherley, 1970), they are consistent with findings from a range of other fish species exposed to hyperoxic conditions under ambient pressure (Healy and Schulte, 2012; Mark et al., 2002; Rutledge and Beiting, 1989). Weatherley (1970) also observed that even in the presence of a superabundance of oxygen, enhanced thermal tolerance ended abruptly at a definite 'breakpoint' and thus was indicative of a physiological failure independent of oxygen limitation. Taken together, previous and current findings suggest that mechanisms other than oxygen limitation must be explored in order to decipher the factors governing acute upper thermal limits in fish.

Interestingly, \dot{M}_{O_2} of hyperoxic fish during thermal ramping increased in a similar manner to that of control fish up to 32°C before doubling over a 1°C temperature increment. As all fish in all treatment groups typically remained quiescent throughout the thermal challenge until 1–2°C before CT_{max} , the pronounced increase in \dot{M}_{O_2} is unlikely to be associated with disproportionately higher skeletal muscle activity in the hyperoxic treatment group. As the majority of oxygen transported in the blood of fishes is bound to haemoglobin, one would expect that hyperoxia can only provide a modest increase in the capacity for systemic oxygen delivery because of the relatively small increase in dissolved oxygen levels resulting from the elevated partial pressure of oxygen under hyperoxic conditions (Takeda, 1990). However, our results demonstrate that hyperoxic fish were able to approximately double \dot{M}_{O_2} during both an exhaustive exercise protocol and at temperatures directly preceding CT_{max} . The exact mechanisms underlying these metabolic responses in hyperoxic fish require further investigation but may relate to factors such as significant increases in cutaneous oxygen uptake

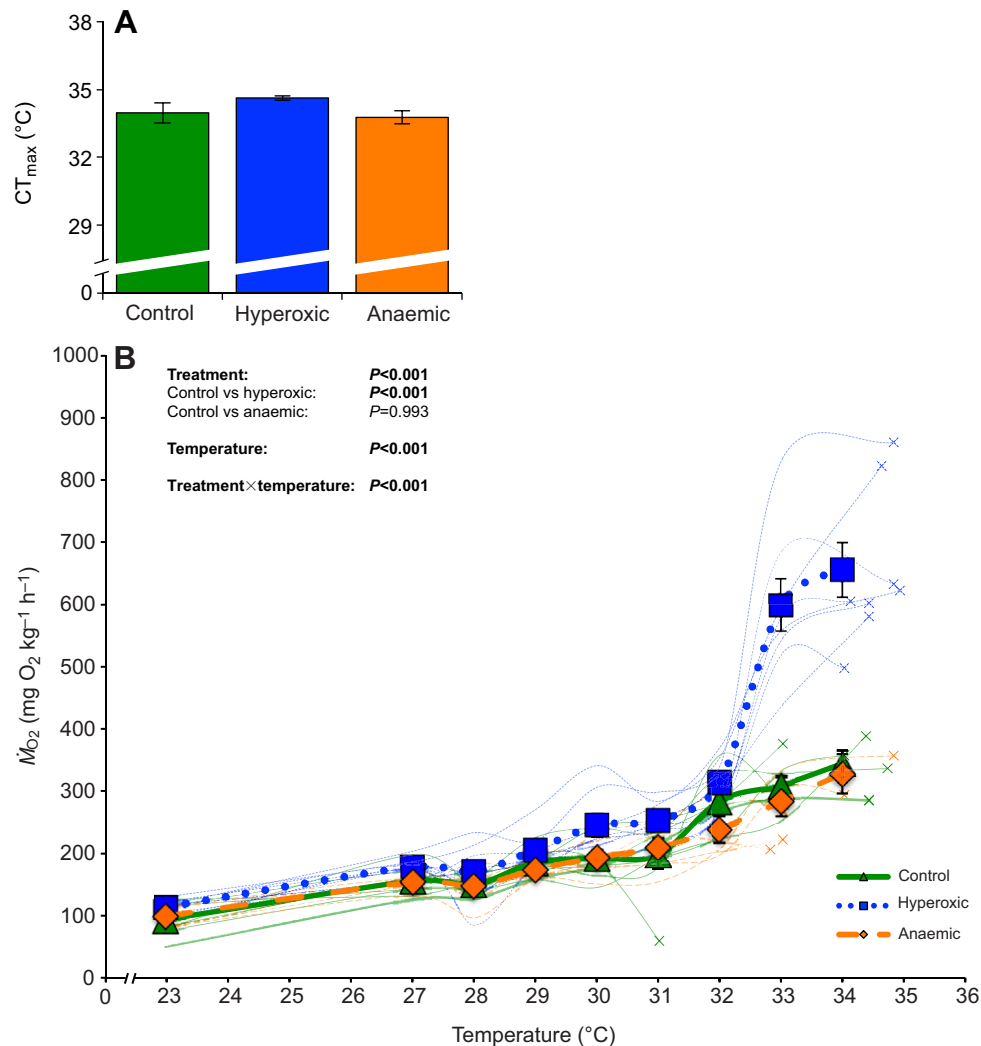


Fig. 3. Acute upper thermal limit and metabolic response to thermal ramping in *P. fluviatilis* during environmental hyperoxia or with anaemia. (A) CT_{max} of control ($N=8$), hyperoxic ($N=8$) and anaemic perch ($N=6$). (B) \dot{M}_{O_2} during an acute thermal challenge of control, hyperoxic and anaemic perch. Data are presented as means \pm s.e.m. Thin variants of the respective lines represent the metabolic response of individual fish within the different treatment groups. The cross at the end of each line indicates the acute CT_{max} for that particular individual. The inset displays the results from the linear mixed model on the metabolic responses of perch during the acute thermal challenge. A significant difference was deemed to be $P < 0.05$.

(Glover et al., 2013), increases in the utilization of the secondary circulatory system (Farrell et al., 2014) and/or maintaining a large diffusion gradient between the blood and tissues (due to an elevated partial pressure of oxygen in the venous system). Nevertheless, it is clear that the greatly increased \dot{M}_{O_2} of hyperoxic fish at temperatures directly preceding CT_{max} did not result in a significantly elevated acute upper thermal limit.

Environmental hyperoxia in the present study was associated with a slightly lower haematocrit but not haemoglobin concentration when compared with control fish following the CT_{max} protocol. Interestingly, a recent study on rainbow trout reported that humoral catecholamine release is reduced during thermal stress in hyperoxia (Currie et al., 2013), suggesting that the difference observed between the hyperoxic and control fish could be associated with a lower degree of adrenergic stimulation and thus red cell swelling in hyperoxic fish (Perry et al., 1996).

Reduced blood oxygen carrying capacity does not affect acute upper thermal limits

The withdrawal of ~40% of the total blood volume from the perch significantly reduced haematocrit (by ~50%) and haemoglobin concentration (by ~43%) when measured after the CT_{max} tests. While haemoglobin concentration is the primary determinant of blood oxygen carrying capacity (Gallaughan and Farrell, 1998), the present study revealed that a ~43% decrease in haemoglobin

concentration did not significantly affect metabolic attributes (SMR, MMR or AS) when compared with control fish. Previous studies on anaemic fish, with anaemia induced by experimental bleeding or intra-peritoneal injection of the haemolytic agent phenylhydrazine, have shown that they can greatly compensate for a reduced blood oxygen carrying capacity by increasing cardiac output and tissue oxygen extraction to maintain overall oxygen transport (Cameron and Davis, 1970; Simonot and Farrell, 2007). Indeed, \dot{M}_{O_2} of anaemic fish during thermal ramping increased in a similar exponential manner to that in control fish and no significant differences in \dot{M}_{O_2} were observed between the groups throughout the entire thermal ramping experiment. These findings are consistent with a recent study on European sea bass (*Dicentrarchus labrax*) where phenylhydrazine-induced anaemia (~50% lower haematocrit) did not affect tissue oxygen delivery across temperatures, as the reduced blood oxygen carrying capacity was compensated for by a significantly higher cardiac output (Wang et al., 2014). Even so, the authors reported a reduction in CT_{max} (by 0.7°C) in anaemic sea bass, which was not the case with the anaemic perch examined in the present study.

Conclusions

This study shows that experimental manipulation of AS and blood oxygen carrying capacity does not translate into altered CT_{max} of European perch. Consequently, it is unlikely that a limitation in

oxygen supply is the direct causal mechanism determining the acute upper thermal limit in fishes. This highlights the need to identify other performance metrics and physiological mechanisms that may operate independently or synergistically to determine the acute thermal tolerance of ectotherms, such as temperature effects on membrane fluidity, ion channel and neural function, as well as thermal inactivation of enzymes and mitochondrial function (Cossins and Bowler, 1987; Iftikar and Hickey, 2013; Overgaard et al., 2012; Vornanen et al., 2014).

As extreme seasonal temperatures are predicted to increase in magnitude and frequency with global climate change (Meehl and Tebaldi, 2004; Seneviratne et al., 2014), the challenge remains for ecophysiologicalists to determine the mechanisms that underlie thermal tolerance. Ultimately, a primary objective must be to formulate predictive models capable of forecasting the effects of climate change on the survival and geographical distribution of ectothermic animals.

MATERIALS AND METHODS

Experimental animals and holding conditions

Wild European perch ($N=22$) were caught by hook and line in the Biotest enclosure ($\sim 1 \text{ km}^2$), a man-made enclosure in the Baltic Sea receiving heated water from the cooling system of the nuclear power plant in Forsmark, Sweden (Hillebrand et al., 2010; Sandstrom et al., 1995). Fish were transported by road ($< 1 \text{ km}$) to a field laboratory and separated randomly between two 1000 l tanks containing air diffusers and flow-through seawater pumped from the Biotest enclosure ($23 \pm 1^\circ\text{C}$; salinity 5 ppt). The fish were held for 2–6 days to allow gut evacuation prior to experiments. Body mass and fork length of the fish (means \pm s.e.m.) were $223 \pm 15 \text{ g}$ and $26.0 \pm 0.5 \text{ cm}$, respectively, and did not differ significantly between treatment groups. Animal care and experimental procedures were approved by the ethical committee of Gothenburg, Sweden (ethical permit 65-2012).

Experimental manipulations and treatment groups

The study consisted of three independent treatments: (1) unmanipulated control fish in normoxic water ($N=8$), (2) experimentally manipulated anaemic fish in normoxic water ($N=6$) and (3) unmanipulated fish in hyperoxic water ($N=8$).

Control fish were anaesthetized in water containing 150 mg l^{-1} MS222 (ethyl-3-aminobenzoate methanesulphonic acid; Sigma-Aldrich Inc., St Louis, MO, USA). Upon loss of equilibrium, a small blood sample ($0.15 \pm 0.01 \text{ ml}$) was taken by puncture of the caudal vasculature with a heparinized syringe to quantify haemoglobin concentration (analytical methods are outlined below). The anaemic fish were handled the same way, except that haemoglobin concentration was experimentally reduced by withdrawing $\sim 40\%$ of the total blood volume (assuming a total blood volume of 3% of body mass in perch; Olson, 1992). The hyperoxic group was exposed to the same anaesthetic protocol but no blood was withdrawn.

While recovering from anaesthesia, fish were individually placed into cylindrical, intermittent flow-through respirometers (volume of 2.8 or 4.0 l depending on fish size) submerged in reservoir baths containing flow-through seawater from the Biotest enclosure at 23°C and with thorough aeration (i.e. 100% air saturation). For the hyperoxic treatment group, the fish were given 3 h to settle after anaesthesia before the reservoir water was bubbled with pure oxygen to maintain the partial pressure of oxygen in the water at $\sim 42 \text{ kPa}$ (200% air saturation). The fish were left undisturbed in the respirometers for at least 16 h while \dot{M}_{O_2} was measured as outlined below.

Respirometry protocol and exhaustive exercise protocol

Measurements of \dot{M}_{O_2} commenced immediately after fish entered the respirometers and were determined using best practices in intermittent flow-through respirometry (see fig. 3B of Clark et al., 2013a; Steffensen, 1989). Briefly, water was continuously circulated through each respirometer using an in-line submersible pump within a recirculation loop, and the oxygen concentration of the water in the respirometer was measured continuously at

0.5 Hz using a FireSting O_2 system (PyroScience, Aachen, Germany) calibrated in accordance with the supplier's manual. Automated flush pumps refreshed the water in the respirometers for 10 min in every 15 min period, ensuring that oxygen levels in the respirometers always remained above 80% air saturation. \dot{M}_{O_2} was calculated from the decline in oxygen concentration in the respirometers during each 5 min period between flush cycles. SMR was calculated for each fish as the mean of the lowest 10% of \dot{M}_{O_2} measurements taken during the $> 16 \text{ h}$ period where the fish were left undisturbed after respirometer entry (excluding outliers, which were considered to be $> 2 \text{ s.d.}$ below the mean of the lowest 10% of values).

Following this period, fish from each group were individually removed from their respirometers and subjected to an exhaustive exercise protocol consisting of a 3 min period of manual chasing around a circular tank (diameter 1.2 m, water depth 20 cm) containing Biotest seawater (23°C) with a partial pressure of oxygen matching that of the treatment group (i.e. $\sim 21 \text{ kPa}$ for control and anaemic groups, $\sim 42 \text{ kPa}$ for the hyperoxic group). This exercise protocol for eliciting MMR was chosen after preliminary tests revealed that this species would not swim continuously in a commercial swim tunnel respirometer. Moreover, this protocol elicits the highest attainable \dot{M}_{O_2} in species with similar ecotypes to the perch used in the present study (see Clark et al., 2013a, and references within). All individuals were visibly exhausted by the end of the 3 min exercise period as highlighted by a lack of response to an experimenter tapping the caudal fin. Immediately following the exercise protocol, fish were returned to their individual respirometers (within 20 s), whereupon respirometers were sealed for 10 min and MMR was taken as the steepest 3 min slope during this time. Once MMR had been determined for each fish, the respirometers were set to the automated flush cycles outlined above and \dot{M}_{O_2} was measured for at least 6 h while the fish recovered.

CT_{max} protocol

Following the post-exercise recovery period, each treatment group was exposed to an acute thermal challenge to determine CT_{max}. Initially, the temperature of the water was gradually increased to 27°C over a 1.5 h period using a thermostatically controlled water heater. From 27°C onwards, temperature was elevated in 1°C increments ($\sim 0.1^\circ\text{C min}^{-1}$) and the fish were held at the new temperature for 20 min prior to the next increment. \dot{M}_{O_2} for each fish was measured during the final 10 min of each temperature plateau. CT_{max} was determined as the temperature (0.1°C precision) where individual fish lost equilibrium for 10 s (Beitinger et al., 2000). When a fish reached CT_{max} it was immediately removed from the respirometer and killed with a sharp cranial blow. A blood sample was promptly taken by caudal puncture with a heparinized syringe and analysed for haematocrit and haemoglobin concentration. Haematocrit was determined as the fractional red cell volume upon centrifugation of a subsample of blood in $80 \mu\text{l}$ microcapillary tubes at 10,000 rpm for 5 min. Haemoglobin concentration was determined using a handheld Hb 201⁺ meter (Hemocue[®] AB, Ängelholm, Sweden) and values were corrected for fish blood (Clark et al., 2008). MCHC was subsequently calculated as haemoglobin concentration/haematocrit $\times 100$.

Statistical analyses

All measured data were assessed for normality (Shapiro–Wilk's test, $P > 0.05$) and homogeneity of variance (Levene's test, $P > 0.05$). To meet these assumptions, the CT_{max} data were transformed using a 'reflect and square root' transformation, whereas the \dot{M}_{O_2} data during temperature ramping were transformed with the natural logarithm. Body mass, fork length, haematological data, SMR, MMR, AS and CT_{max} were analysed using a one-way ANOVA followed by Dunnett's t -tests. No differences in body mass were found between treatments so this factor was excluded from further statistical analyses. For \dot{M}_{O_2} data during the temperature ramping, we used a linear mixed model for comparisons among treatment groups. Individuals were set as subjects and temperatures as repeated measures. AR (1) was used as the type of repeated covariance because recordings that were close in temperature were also more dependent than more temporally distant recordings. \dot{M}_{O_2} was set as the dependent variable. The three experimental groups (i.e. control, hyperoxic and anaemic) and temperature were included in the model as explanatory factors. The explanatory factors and their

interactions were compared using a Bonferroni confidence-interval adjustment. All data are presented as means±s.e.m. unless otherwise stated. Statistical analyses were conducted in SPSS Statistics 21 (IBM Corp., Armonk, NY, USA). Differences where $P < 0.05$ were regarded as statistically significant.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

All authors were involved in the conception and design of the experiment, performing the experiment, analysing the data and completing the manuscript.

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