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SHORT COMMUNICATION

Anaemia only causes a small reduction in the upper critical temperature of sea bass: is oxygen delivery the limiting factor for tolerance of acute warming in fishes?

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ABSTRACT

To address how the capacity for oxygen transport influences tolerance of acute warming in fishes, we investigated whether a reduction in haematocrit, by means of intra-peritoneal injection of the haemolytic agent phenylhydrazine, lowered the upper critical temperature of sea bass. A reduction in haematocrit from 42±2% to 20±3% (mean ± s.e.m.) caused a significant but minor reduction in upper critical temperature, from 35.8±0.1 to 35.1±0.2°C, with no correlation between individual values for haematocrit and upper thermal limit. Anaemia did not influence the rise in oxygen uptake between 25 and 33°C, because the anaemic fish were able to compensate for reduced blood oxygen carrying capacity with a significant increase in cardiac output. Therefore, in sea bass the upper critical temperature, at which they lost equilibrium, was not determined by an inability of the cardio-respiratory system to meet the thermal acceleration of metabolic demands.

KEY WORDS: Haematocrit, Cardiovascular, Oxygen transport, Fish, Thermal tolerance

INTRODUCTION

Temperature affects the rate of virtually all bodily functions. Global warming is altering the distribution of natural populations, so a major challenge for experimental biology is to provide a mechanistic model that relates physiological capacity to temperature tolerance (e.g. Helmuth et al., 2005). It has long been appreciated that thermal sensitivity decreases as the level of biological organization increases, such that thermal tolerance of the whole living organism is lower than that of the organs, which again is lower than thermal sensitivity of cells and enzymes (e.g. Orr, 1955; Ushakov, 1964; Prosser, 1973; Cossins and Bowler, 1987; Pörtner, 2002; Pörtner and Peck, 2010). Based on this hierarchy of thermal sensitivity, it has been argued that the upper critical temperature (CT_{max}) of the organism reflects loss of integration of physiological functions rather than denaturation of enzymes or increased fluidity of biological membranes (Cossins and Bowler, 1987).

In ectotherms, much research has focused on the hypothesis that, as temperature increases, the cardio-respiratory system eventually fails to meet the inexorable rise in tissue oxygen demands. Therefore, the upper boundary of the thermal window would be defined by the capacity for oxygen transport relative to metabolic demand (e.g. Pörtner and Knust, 2007; Wang and Overgaard, 2007; Pörtner and Farrell, 2008). This apparent dependence of temperature tolerance on the capacity for oxygen delivery is supported by a decline in aerobic scope (the difference between standard and maximal rates of oxygen consumption) reported for several fishes as temperature rises (e.g. Fry and Hart, 1948; Fry, 1971; Brett, 1971; Claireaux and Lagardère, 1999; Nilsson et al., 2009; Eliason et al., 2013). Furthermore, in salmonids, venous oxygen concentration decreases with increased temperature, indicating that increased metabolic demand is not matched proportionally by increased cardiac output ($\dot{Q}$), causing the arterial–venous oxygen concentration difference to widen, as a result of increased extraction (e.g. Heath and Hughes, 1973; Eliason et al., 2013). Studies of acute warming of fishes, crustaceans and amphibians, however, have found that aerobic scope does not decline until temperatures immediately below CT_{max}. This indicates that mechanisms other than limited oxygen transport may underlie tolerance of acute warming (e.g. Overgaard et al., 2012; Clark et al., 2013a; Clark et al., 2013b; Norin et al., 2014; Ern et al., 2014). This is important to investigate because extreme seasonal temperatures are predicted to increase in frequency with global climate change, with potential acute effects on ectotherms.

One approach to assess this is to manipulate oxygen availability and transport capacity. Several studies have demonstrated that aquatic hyperoxia increases CT_{max} in fishes (Alabaster and Welcomme, 1962; Weatherley, 1970; Rutledge and Beitinger, 1989), although not in the Antarctic teleost *Pachycara brachycephalum* (Mark et al., 2002), and severe hypoxia caused a reduction in CT_{max} in three teleosts (Rutledge and Beitinger, 1989). Most of the oxygen transported in the blood of fishes is, however, bound to haemoglobin (Hb), so hyperoxia only provides modest increases in the capacity for systemic oxygen delivery ($\dot{Q} \times [O_2]_a$, where $[O_2]_a$ is arterial O_2 concentration). While the increase in dissolved oxygen levels in arterial blood would be of little quantitative value, hyperoxia may have confounding effects if it elevates venous levels by increasing cutaneous oxygen uptake.

An alternative method to investigate the dependence of CT_{max} on oxygen transport capacity is to reduce Hb concentration. We therefore created anaemia by intra-peritoneal injection of the haemolytic agent phenylhydrazine (PHZ) in European sea bass, *Dicentrarchus labrax* (Linnaeus 1758). In a parallel series of experiments, we characterized how an acute temperature rise affected $\dot{Q}$ and the rate of oxygen consumption ($\dot{M}_{O_2}$) in fish with normal or low haematocrit (Hct).

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List of symbols and abbreviations

$[O_2]_a$	arterial O_2 concentration
CT_{max}	upper critical temperature
f_H	heart rate
Hct	haematocrit
$\dot{M}_{O_2}$	rate of oxygen consumption
OCLTT	oxygen and capacity limited thermal tolerance model
PHZ	phenylhydrazine
P_{O_2}	oxygen partial pressure
$\dot{Q}$	cardiac output
V_s	stroke volume

RESULTS AND DISCUSSION

CT_{max} of sea bass with a normal Hct of 42±2% was 35.8±0.1°C, whereas fish with a Hct that had been lowered significantly to 20±3% by PHZ had an only slightly, albeit significantly, lower CT_{max} of 35.1±0.2°C (Fig. 1A). Furthermore, the individual values for CT_{max} were not correlated significantly to Hct (linear regression in Fig. 1B, R^2=0.23, P=0.11). Therefore, our main finding is that a 50% reduction in Hct, and hence blood oxygen carrying capacity, only caused a small reduction in CT_{max} of 0.7°C. The parallel studies revealed that anaemic sea bass maintained $\dot{M}_{O_2}$ up to temperatures immediately below CT_{max} by increasing $\dot{Q}$ and elevating oxygen extraction relative to controls (Fig. 2). This clearly indicates that the capacity of the sea bass to meet their oxygen demands was not limited at the temperatures immediately preceding CT_{max}, despite the reduced Hct. Thus, the CT_{max} was clearly not only determined by an inability of the cardio-respiratory system to meet the rise in metabolic demands as temperature increased, unless this manifested itself with only a 0.7°C increase in CT_{max}.

The blood samples to determine Hct were taken by venous puncture and the associated stress undoubtedly caused catecholamine release and red cell swelling in response to adrenergic stimulation (Perry et al., 1996). The Hct values reported here are therefore likely to be overestimates compared with those of undisturbed fish. Nevertheless, any such handling effects should have been similar in control and PHZ-treated fish, so the relative differences were genuine and confirm that PHZ did rupture erythrocytes (Smith et al., 1971) to reduce oxygen carrying capacity. With a Hct of 40% and a typical piscine mean cellular Hb concentration of 20 mmol Hb l^{-1} red blood cells, we estimate a total arterial oxygen concentration of around 8.15 mmol O_2 l^{-1} blood [with 8 mmol bound to Hb and 0.15 mmol dissolved in plasma at an oxygen partial pressure (P_{O_2}) of 100 mmHg]. By these assumptions, a reduction of Hct to 20% would cause a decline of 4 mmol l^{-1} in the amount of oxygen carried by Hb, with the minor amount of dissolved oxygen being unaffected.

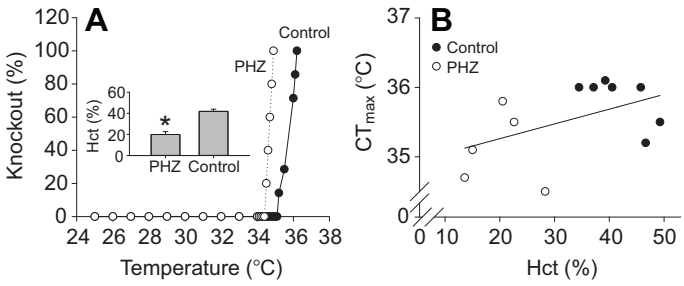

Fig. 1. Upper critical temperature and haematocrit for phenylhydrazine (PHZ)-treated and control fish. (A) The upper critical temperature (CT_{max}) was determined from the temperature where the sea bass were no longer able to maintain body position (knockout). The haematocrit (Hct) for the two groups of fish (PHZ-treated and control fish) is presented as an inset. (B) The correlation between Hct and CT_{max} of each individual fish. Regression: CT_{max}=34.8°C+0.02Hct; R^2=0.2325, P=0.1124, N=5 for PHZ and N=7 for saline.

In the parallel experiments where we measured cardiovascular and respiratory responses to increased temperature, PHZ significantly reduced Hct from 41±2% to 18±4%; values that did not differ significantly from those of fish used to determine CT_{max}. $\dot{M}_{O_2}$ increased significantly as temperature rose from 25 to 33°C, with Q_{10} values of 2.1 and 2.3 for normal and reduced Hct, respectively. The reduction in Hct had no effect on $\dot{M}_{O_2}$ at any temperature (Fig. 2A). The $\dot{M}_{O_2}$ measured in resting fish at 25°C was similar to previous reports for sea bass at similar temperatures (Iversen et al., 2010; Claireaux et al., 2006), and the rise in $\dot{M}_{O_2}$ as temperature was acutely increased to 33°C resembled measurements on seasonally acclimatized sea bass (Claireaux et al., 2006). The acute Q_{10} of ~2 is within the normal range for fishes (Clarke and Johnston, 1999). The maximum $\dot{M}_{O_2}$ measured here at 33°C was about 60% of that measured at the maximum aerobic swimming speed of sea bass seasonally acclimatized to 30°C (Claireaux et al., 2006). The fact that the anaemic sea bass could meet their oxygen demands at temperatures immediately below CT_{max} is strong evidence that there was no oxygen or capacity limitation.

Heart rate (f_H) increased with temperature (Q_{10} values of 2.1 and 1.8 in control and PHZ-treated fish, respectively) and PHZ-treated fish had significantly higher f_H at 25 and 31°C (Fig. 2B). Stroke volume (V_s) was not affected by either Hct or temperature (Fig. 2C), but $\dot{Q}$ tended to increase with temperature (Q_{10} of 1.6 in both groups) and was consistently greater in the PHZ group (Fig. 2D). $\dot{Q}$ reached a maximum at 31°C and did not increase further at 33°C. There are no other measures of $\dot{Q}$ and f_H at similar temperatures in this species. Farrell et al. (Farrell et al., 2007) found that maximum *in vitro* performance of the sea bass heart was very sensitive to increases from 18 to 22°C, with *in vitro* values at 22°C being higher than we observed at 33°C in both control and anaemic fish. The rise in f_H and $\dot{Q}$ are similar to previous studies on fishes (Cameron and Wohlsclag, 1969; Wood et al., 1979; Simonot and Farrell, 2007). In some species, V_s also increases in anaemia, which has been linked to a prominent cardiac hypertrophy (Sun et al., 2009; Simonot and Farrell, 2007). It seems unlikely, however, that cardiac growth would have manifested itself within 48 h of PHZ treatment in the sea bass. Although reliance on tachycardia to increase $\dot{Q}$ is consistent with previous exercise studies in sea bass (Chatelier et al., 2005; Chatelier et al., 2006; Dupont-Prinet et al., 2009; Sandblom et al., 2005), the underlying regulatory pathways for tachycardia in the anaemic fish are not easy to resolve without blood pressure measurements. They may involve reflex responses to P_{O_2}-sensitive chemoreceptors perfused by venous blood, for example on the venous side of afferent branchial arteries (e.g. Milsom, 2012). It is also likely that tachycardia reflected barostatic responses (Sandblom and Axelsson, 2005) to maintain blood pressure, to compensate for reduced blood viscosity and a general vasodilation, and the associated reduction in total peripheral resistance to maintain tissue oxygenation. In any event, the increased $\dot{Q}$ of the anaemic fish persisted over the entire temperature range, to compensate fully for the reduced oxygen carrying capacity and maintain delivery, i.e. $\dot{Q}\times[O_2]_a$, where the arterial oxygen concentration ($[O_2]_a$) is directly proportional to Hct. Thus, the reduction in Hct did compromise systemic oxygen delivery but must, presumably, have resulted in a considerable reduction in venous P_{O_2}. In any event, the sea bass did not appear to be limited by their capacity for oxygen delivery as they approached CT_{max}.

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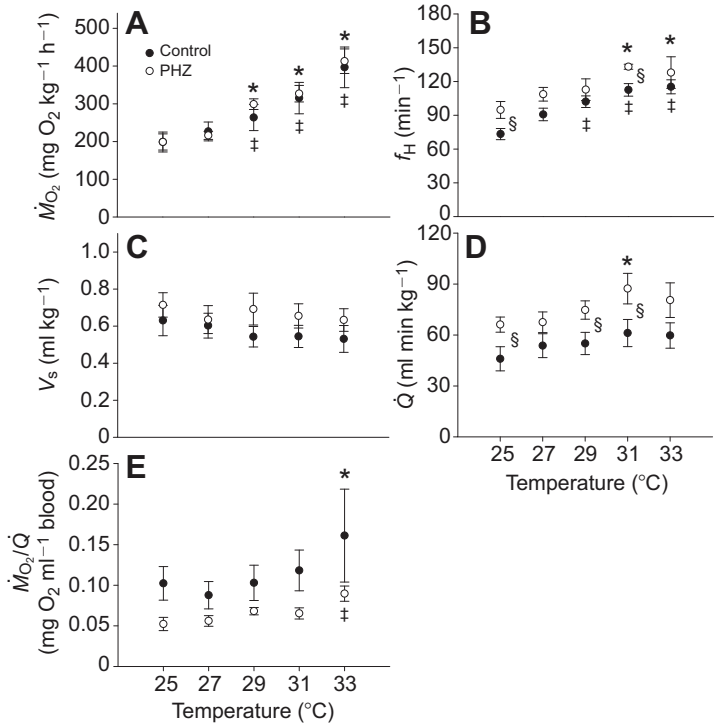

Fig. 2. Metabolic and cardiovascular responses to acute temperature increases in phenylhydrazine (PHZ)-treated and control fish. Oxygen uptake ($\dot{M}_{O_2}$) (A), heart rate (f_H) (B), cardiac output ($\dot{Q}$) (C), stroke volume (V_s) (D) and oxygen consumption relative to blood flow ($\dot{M}_{O_2}/\dot{Q}$) (E) for fish treated with PHZ or saline (control). *N*=6 for the PHZ-treated group and *N*=7 for the control group. Double daggers and asterisks indicate a significant difference from 25°C in control and PHZ-treated fish, respectively. Daggers indicate a significant difference between control fish and PHZ-treated fish at a given temperature.

To evaluate the compensatory rise in $\dot{Q}$ in anaemic fish, we expressed $\dot{M}_{O_2}$ relative to $\dot{Q}$ ($\dot{M}_{O_2}/\dot{Q}$) in Fig. 2E. This measure of oxygen extracted by the tissues relative to the volume of blood pumped by the heart tended to be lower in the anaemic fish, although not significantly. Nevertheless, because of the 50% reduction in Hct, PHZ caused the expected reduction in convective oxygen transport by the cardiovascular system when expressed relative to $\dot{M}_{O_2}$. Overall, although venous P_{O_2} was presumably reduced in anaemic fish, particularly at higher temperatures, this did not translate into impaired cardiac function or an impaired ability to increase cardiac output and meet oxygen demands. Taken together, all of these measurements indicate that the cardio-respiratory system was able to cope with the metabolic demands imposed by the increased temperature, despite reductions in oxygen carrying capacity.

The hypothesis that oxygen delivery by the cardio-respiratory system is the primary factor limiting upper temperature tolerance is widely assumed, but is primarily based on associations between aerobic scope and temperature (Clark et al., 2013a; Clark et al., 2013b; Farrell, 2013; Pörtner and Giomi, 2013). Few studies have manipulated oxygen delivery to investigate the effects on tolerance of acute warming. Our results reveal that sea bass had the capacity to compensate for a profound reduction in Hct by increasing $\dot{Q}$, even at temperatures only 2°C below CT_{max}. Cardiovascular function did not, therefore, appear to be compromised at the highest temperatures. This indicates that additional explanations beyond limitations to oxygen delivery should be considered to explain the tolerance of acute warming in sea bass. These could include an effect of temperature on nervous function, membrane stability and enzymatic and mitochondrial function (Prosser, 1973).

MATERIALS AND METHODS

Experimental animals

European sea bass (*D. labrax*) with a body mass of 453±25 g were obtained from Extramer SrL (Salses le Chateau, Roussillon, France) and transported to the Station Méditerranéenne de l'Environnement Littoral in Séte (Languedoc, France). They were maintained for at least 3 weeks in large tanks with recirculating, aerated and bio-filtered seawater at 25°C (similar to prevailing seasonal water temperatures in their previous aquaculture facility), salinity of 35‰, and natural photoperiod. Animals were fed with commercial pelleted feed daily; food was withheld for at least 24 h before surgery.

Experimental protocols and surgeries

Studies on CT_{max} and cardiovascular responses to increased temperature were performed on separate groups. In all cases, Hct was lowered by intra-peritoneal injection of PHZ (10 mg kg^{-1} dissolved in 1 ml saline kg^{-1}) no less than 24 h prior to temperature challenges.

Hct effects on CT_{max}

Twelve fish were lightly anaesthetized in MS-222 (200 mg l^{-1}) and injected with either PHZ (*N*=5) or saline (*N*=7), then allowed to recover for 24 h in their holding tank. The following day, temperature was increased by 1°C every 30 min and behaviour was observed. When fish lost equilibrium, they were immediately removed and a blood sample taken by caudal puncture, to determine Hct. Fish were returned to water at 25°C and allowed to recover. Hct was determined as fractional red cell volume upon centrifugation of blood samples in 8 µl capillary tubes at 8000 rpm for 3 min.

Cardio-respiratory responses to increased temperature

Fish were anaesthetized in MS-222 (200 mg l^{-1}) until spontaneous ventilation and reflexes subsided, then transferred to an operating table where the gills were irrigated with aerated water containing MS-222 (100 mg l^{-1}). A Transonic flow probe was placed around the ventral aorta for measurement of $\dot{Q}$, and an intra-peritoneal polyethylene catheter (PE50) inserted. Six fish were given PHZ immediately after surgery, whereas seven control fish received a sham injection of a similar volume of saline. All fish were allowed to recover for 48 h before experiments began.

For simultaneous measurement of $\dot{M}_{O_2}$ and $\dot{Q}$, fish were placed in a 10 l respirometer, submerged in seawater at 25°C, ~24 h after surgery and allowed to habituate overnight. The following morning, temperature was increased in 2°C steps, each increment taking 20 min to complete, whereupon $\dot{M}_{O_2}$ and $\dot{Q}$ were measured for 40 min at each new temperature. This gave an overall heating rate similar to that of the CT_{max} experiment (2°C h^{-1}). $\dot{Q}$ was measured using the Transonic flow probe and recorded by a MP100 (BIOPAC Systems Inc., CA, USA) using AcqKnowledge 3.9.1. f_H

was derived from the pulsatile flow signal and V_s was calculated as $\dot{Q}/f_H$. As CT_{max} of sea bass was ~35°C, we did not raise the temperature above 33°C. When the experiment terminated, fish were anaesthetized (200 mg l^{-1} MS-222) and a blood sample was taken to determine Hct. $\dot{M}_{O_2}$ was measured by intermittent closed respirometry with 5 min closed periods and 10 min flush periods, where $\dot{M}_{O_2}$ was determined from the rate at which water P_{O_2} declined during the closed phase. Water P_{O_2} was measured by a fibre optic electrode using Oxy-4 (Loligo Systems, Denmark). Blank measurements were performed a few times to assess background bacterial $\dot{M}_{O_2}$, but never exceeded 10% of fish $\dot{M}_{O_2}$.

Statistics

The effects of PHZ and temperature were analysed by two-way repeated measures ANOVA (Sigmaplot 12.5, Systat Software, Inc.). Differences were identified by multiple comparison versus control (Holm–Šidák method), this being no PHZ and a temperature of 25°C. $P<0.05$ was considered significant; all data are presented as means ± s.e.m. unless otherwise indicated.

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Competing interests

The authors declare no competing financial interests.

Author contributions

T.W., S.L., I.F., N.K.I. and D.J.M. designed the experiment, all authors contributed to the execution of the experiments as well as the analysis and interpretation of the data. T.W., S.L., I.F. and D.J.M. drafted the manuscript, which was subsequently edited and approved by all authors.

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