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 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 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.
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 (CTmax) 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 focussed 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 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 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 & 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, causing the arterial-venous oxygen concentration difference to widen, due to increased extraction (e.g. Heath and Hughes, 1973; Eliasson et al., 2013). Studies of acute warming of fishes, crustaceans and amphibians have not, however, found that aerobic scope declined until temperatures immediately below CTmax. 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,b; 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 of ectotherms.

One approach to assess this is to manipulate oxygen availability and transport capacity. Several studies have demonstrated that aquatic hyperoxia increases CTmax in fishes (Alabaster and Welcome, 1962; Weatherley, 1970; Rutledge and Beintinger, 1989), although not in the Antarctic
teleost *Pachycara brachycephalum* (Mark et al., 2002), and severe hypoxia caused a reduction in CTmax in three teleosts (Rutledge and Beitinger, 1989). Most of the oxygen transported in the blood of fishes is, however, bound to haemoglobin, so hyperoxia only provides modest increases in capacity for systemic oxygen delivery (cardiac output x arterial O2 concentration; \( Q \times [O_2]_a \)). While the increase in dissolved oxygen levels in the 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 dependence of CTmax on oxygen transport capacity is to reduce haemoglobin concentration. We therefore created anaemia by intra-peritoneal injection of the haemolytic agent phenylhydrazine (PHZ) in European sea bass (*Dicentrarchus labrax*, L. 1758). In a parallel series of experiments, we characterized how an acute temperature rise affected cardiac output \((Q)\) and the rate of oxygen consumption \((\dot{M}O_2)\) in fish with normal or low haematocrit (hct).

**RESULTS AND DISCUSSION**

CTmax 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 CTmax of 35.1 ± 0.2°C (Fig. 1A). Furthermore, the individual values for CTmax 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 CTmax of 0.7°C. The parallel studies revealed that anaemic sea bass maintained \(\dot{M}O_2\) up to temperatures immediately below CTmax by increasing \(Q\) 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 CTmax, despite the reduced hct. Thus, the CTmax 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 CTmax.

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 reported here are therefore likely to be overestimates compared to undisturbed fish. Nevertheless, any such handling effects should have been similar in both 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 haemoglobin concentration of 20 mmol Hb per litre red blood cells, we estimate total arterial oxygen concentration of around 8.15 mmol O$_2$ per litre blood (with 8 mmol bound to haemoglobin and 0.15 mmol dissolved in plasma at a PO$_2$ of 100 mmHg). By these assumptions, a reduction of hct to 20% would cause a decline of 4 mmol per litre in the amount of oxygen carried by haemoglobin, with the minor amount of dissolved oxygen being unaffected.

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 hcts of fish used to determine CTmax. $\dot{M}$O$_2$ increased significantly as temperature rose from 25 to 33°C, with $Q_{10}$’s 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 maximum aerobic swimming speed of sea bass seasonally acclimatised to 30°C (Claireaux et al. 2006). The fact that the anaemic sea bass could meet their oxygen demands at temperatures immediately below CTmax is strong evidence that that there was no oxygen or capacity limitation.

The $f_{hi}$ increased with temperature ($Q_{10}$’s 2.1 and 1.8 in control and PHZ-treated fish, respectively) and PHZ-treated fish had significantly higher $f_{hi}$ at 25°C and 31°C (Fig 2B). Stroke volume ($V_s$) was not affected by either hct or temperature (Fig. 2C), but $Q$ tended to increase with temperature ($Q_{10}$ of 1.6 in both groups) and was consistently greater in the PHZ group (Fig. 2D). $Q$ reached a maximum at 31°C and did not increase further at 33°C. There are no other measures of $Q$ and $f_{hi}$ at similar temperatures in this species. 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 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 within 48h after PHZ-treatment in the sea bass. Although reliance on tachycardia to increase $Q$ is consistent with previous exercise studies in sea bass (Chatelier et al. 2005, 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 $PO_2$ 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 $Q$ of the anaemic fish persisted over the entire temperature range, to compensate fully for the reduced oxygen carrying capacity and maintain delivery, i.e. $Q \times [O_2]_a$, 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 $PO_2$. In any event, the sea bass did not appear to be limited by their capacity for oxygen delivery as they approached CT$_{max}$.

To evaluate the compensatory rise in $Q$ in anaemic fish, we expressed $\dot{M}O_2$ relative to $Q$ ($\dot{M}O_2/Q$) in Figure 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 $PO_2$ was presumably reduced in anaemic fish, particularly at higher temperatures, this did not translate into impaired cardiac function or 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,b; Farrell, 2013 and Pörtner and Giomi, 2013). Few studies have manipulated oxygen delivery to investigate 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 cardiac output, 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 tolerance of acute warming in sea bass. These could include an effect of temperature on nervous function, membrane stability and enzymatic and mitochondrial functions (Prosser, 1973).
MATERIALS AND METHODS

Experimental Animals

European sea bass (*Dicentrarchus 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 three 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 upper critical temperature and cardiovascular responses to increased temperature were performed on separate groups. In all cases, hct was lowered by intra-peritoneal injection of phenyl hydrazine hydrochloride (PHZ; 10 mg kg⁻¹ dissolved in 1 ml saline kg⁻¹) no less than 24 h prior to temperature challenges.

**Haematocrit effects on upper critical temperature.** Twelve fish were lightly anesthetized in MS-222 (200 mg l⁻¹) and injected with either PHZ (N=5) or saline (N=7), then recovered for 24 h in their holding tank. The following day, temperature was increased by 1°C every 30 min and behaviour 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 80 μL capillary tubes at 8000 rpm for 3 min.

**Cardio-respiratory responses to increased temperature.** Fish were anesthetized 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 measurements of cardiac output (*Q*), and an intra-peritoneal polyethylene catheter (PE50) inserted. Six fish were given PHZ immediately after surgery, whereas 7 control fish received a sham injection of a similar volume of saline. All fish were allowed to recover for 48 h before experiments.
For simultaneous measures of $\dot{M}O_2$ and $Q$, fish were placed in a 10 litre respirometer, submerged in sea water at 25°C, approximately 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 $Q$ were measured for 40 min at each new temperature. This gave an overall heating rate similar to the CTmax experiment (2°C per hour). $Q$ was measured using the Transonic flow probe and recorded by a MP100 (BIOPAC systems Inc., CA, USA) using AcqKnowledge 3.9.1. $f_{HI}$ was derived from the pulsatile flow signal and $V_s$ was calculated as $Q/f_{HI}$. As CTmax of sea bass was approximately 35°C, we did not raise temperature above 33°C. When the experiment terminated, fish were anaesthetized (200mg/L MS-222) and a blood sample 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 (Neligo Systems, Denmark). Blank measurements were performed a few times to assess background bacterial $M_2$, but never exceeded 10% of fish $\dot{M}O_2$.

Statistics

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-Sidak method), this being no PHZ and 25°C. A $P < 0.05$ was considered significant, all data is presented as mean ± s.e.m. unless indicated.

Acknowledgements

We thank Erik Sandblom for stimulating discussions.

Author contributions

All authors contributed to the design and execution of the experiments, and all authors contributed to data analysis and writing of the manuscript.

Competing interests

No competing interests.

Funding

This research was supported by the Centre National de la Recherche Scientifique (CNRS), the Danish Research Council, The Ambassade de France in Copenhagen and Université Montpellier 2. TW was supported by a fellowship from Région Languedoc-Roussillon (RLR) as a visiting
professor at Université Montpellier 2. IF and NKI was supported by a student grant from The
Ambassade de France in Copenhagen.
List of abbreviations

$P_{O_2}$ oxygen partial pressure

$Q$ cardiac output

$[O_2]_a$ arterial $O_2$ concentration

$CT_{max}$ upper critical temperature

$\dot{M}O_2$ rate of oxygen consumption

$\text{PHZ}$ phenylhydrazine

$hct$ haematocrit

$f_H$ heart rate

$V_s$ stroke volume

OCLTT oxygen and capacity limited thermal tolerance model
References


FIGURE LEGENDS

**Figure 1.** The upper critical temperature (CTmax) as determined from the temperature where the sea bass were no longer able to maintain body position (Knockout) (A). The haematocrit (hct) for the two groups of fish, PHZ-treated and control fish, respectively, are presented as an insert in this figure. Figure 1B shows the correlation between hct and CTmax of each individual fish. Regression: CTmax = 34.8°C + 0.02hct; R²=0.2325, p=0.1124. N=5 for phenylhydrazine and N=7 for saline.

**Figure 2.** Oxygen uptake (ṀO₂) (A), heart rate (fH) (B), cardiac output (Q) (C), stroke volume (Vs) (D), and oxygen consumption relative to blood flow (ṀO₂/Q) (E) for fish treated with PHZ or saline (control). N=6 for the PHZ-treated group and N=7 for the control group. Hashes and asterisks indicate significant difference from 25°C in control and PHZ-treated fish, respectively. Daggers indicate significant difference between control fish and PHZ-treated fish at a given temperature.
Figure 1
Figure 2

A

\( \dot{V}O_2 \) (mg O₂ kg⁻¹ h⁻¹)

- control
- PHZ

B

\( \dot{f}_H \) (min⁻¹)

C

\( V_s \) (mL kg⁻¹)

D

\( Q \) (mL min kg⁻¹)

E

\( \dot{V}O_2/Q \) (mg O₂ / mL blood)

Temperature (°C)

Temperature (°C)