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ORIGINAL PAPER

Reduced and reversed temperature dependence of blood oxygenation in an ectothermic scombrid fish: implications for the evolution of regional heterothermy?

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Abstract Tunas (family Scombridae) are exceptional among most teleost fishes in that they possess vascular heat exchangers which allow heat retention in specific regions of the body (termed 'regional heterothermy'). Seemingly exclusive to heterothermic fishes is a markedly reduced temperature dependence of blood–oxygen (blood–O_2) binding, or even a reversed temperature dependence where increasing temperature increases blood–O_2 affinity. These unusual binding properties have been documented in whole blood and in haemoglobin (Hb) solutions, and they are hypothesised to prevent oxygen loss from arteries to veins within the vascular heat exchangers and/or to prevent excessive oxygen unloading to the warm tissues and ensure an adequate supply of oxygen to tissues positioned efferent to the heat exchangers. The temperature sensitivity of blood–O_2 binding has not been characterised in an ectothermic scombrid (mackerels and bonitos), but the existence of the unusual binding properties in these fishes would have clear implications for their proposed association with regional heterothermy. Accordingly, the present study examined oxygenation of whole blood of the chub mackerel (*Scomber japonicus*) at 10, 20 and 30°C and at 0.5, 1 and 2% CO_2. Oxygen affinity was generally highest at 20°C for all levels of CO_2. Temperature-independent binding was observed at low (0.5%) CO_2, where the PO_2 at 50% blood–O_2 saturation (P_{50}) was not statistically different at 10 and 30°C (2.58 vs. 2.78 kPa, respectively) with an apparent heat of oxygenation ($\Delta H°$) close to zero (-6 kJ mol^{-1}). The most significant temperature-mediated difference occurred at high (2%) CO_2, where the P_{50} at 10°C was twofold higher than that at 20°C with a corresponding $\Delta H°$ of $+43$ kJ mol^{-1}. These results provide clear evidence of independent and reversed open-system temperature effects on blood oxygenation in *S. japonicus*, and it is therefore speculated that these unusual blood–O_2 binding characteristics may have preceded the evolution of vascular heat exchangers and regional heterothermy in fishes.

Keywords Apparent heat of oxygenation · Blood respiratory properties · Bohr effect · Carbon dioxide · Chub mackerel · Evolution of endothermy · Haemoglobin–oxygen equilibrium curves · pH · Root effect · *Scomber japonicus* · Tunas

Introduction

The development of regional heterothermy (often termed 'regional endothermy') in the tunas is arguably one of the most significant events in the evolution of modern teleost fishes. Regional heterothermy likely allowed thermal niche expansion in tunas and has been documented in only a few other fish groups, including some sharks, some billfishes,

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and recently, the opah (*Lampris guttatus*) (Dickson and Graham 2004; Runcie et al. 2009). Regional heterothermy is made possible by countercurrent vascular heat exchangers (*retia mirabilia*), which facilitate heat conservation in one or more specific areas of the body including the swimming muscles (tunas and some sharks), cranium (tunas, billfishes, butterfly mackerel, some sharks, and the opah), and viscera (some tunas and some sharks) (Carey 1973; Fudge and Stevens 1996; Bernal et al. 2001; Graham and Dickson 2004; Sepulveda et al. 2005; Shadwick 2005; Runcie et al. 2009). Consequently, the blood of tunas may experience abrupt temperature changes (as much as 20°C) during its transit from the gills to those tissues served by heat exchangers (Carey and Lawson 1973). Additionally, some tunas typically dive rapidly from warm surface waters (which may be up to 33°C) to depths below the thermocline, where temperatures may be less than 5°C (Gunn and Young 1999; Block et al. 2001). Therefore, the binding properties of tuna haemoglobin (Hb) must be adapted to accommodate sufficient oxygen loading at the gills and delivery to the tissues while faced with extreme and abrupt changes in temperature.

Previous studies have demonstrated that temperature affects the haemoglobin–oxygen (Hb–O_2) binding properties of tuna in an unusual way in comparison with other vertebrates. In nearly all other vertebrates, the exothermic nature of Hb–O_2 binding facilitates oxygen dissociation with increasing temperature (i.e. decreases Hb–O_2 affinity). In contrast, Rossi-Fanelli and Antonini (1960) reported that Hb–O_2 affinity was essentially independent of temperature in purified Atlantic bluefin tuna (*Thunnus thynnus*) haemolysates. This phenomenon has since been documented in whole blood samples from several other tuna species, and a reversed temperature dependence (where an increase in temperature increases Hb–O_2 affinity) has also been discovered (Cech et al. 1984; Jones et al. 1986; Brill and Bushnell 1991; Lowe et al. 2000; Brill and Bushnell 2006; Clark et al. 2008b). The molecular basis for the reduced and reversed temperature dependence of Hb–O_2 binding is likely attributed, at least in part, to the counteractive effects of the endothermic dissociation of organic phosphates, chloride ions and Bohr protons upon oxygen binding (Wood 1980; Weber and Jensen 1988; Larsen et al. 2003; Weber and Fago 2008; Rasmussen et al. 2009).

Three distinct hypotheses exist to explain the functional significance of the unusual blood–O_2 binding properties of tunas. It was originally proposed that the atypical Hb characteristics evolved to enable exploitation of waters of greatly differing temperature without compromising oxygen uptake at the gills (Rossi-Fanelli and Antonini 1960). An alternative hypothesis proposed that such binding properties exist to prevent any potential oxygen transfer between the neighbouring arteries and veins in the vascular countercurrent heat exchangers (Graham 1973). A third hypothesis stated that reduced and reversed temperature dependence of blood–O_2 binding may exist to prevent excessive oxygen unloading to the warmer tissues and ensure an adequate supply of oxygen to the tissues efferent to the heat exchangers (Graham 1973; Clark et al. 2008b). Studies on the porbeagle shark (*Lamna nasus*), a species which belongs to a family of sharks possessing vascular heat exchangers (family Lamnidae), support the suggestion of an intimate association between vascular heat exchangers and atypical Hb–O_2 binding properties (Andersen et al. 1973; Larsen et al. 2003; Dickson and Graham 2004). Nevertheless, the functional significance of reduced and reversed temperature dependence of blood–O_2 binding, and their link with regional heterothermy in fishes, remains a matter of speculation. To determine if there exists an exclusive association between vascular heat exchangers and the atypical binding properties of tuna haemoglobin, it is necessary to examine haemoglobin enthalpy in equivalent ecotypes that are less derived than tunas and do not possess heat exchangers, such as the ectothermic scombrids (mackerels and bonitos).

Accordingly, the present study examined, for the first time, the effect of temperature on the blood–O_2 binding properties of an ectothermic scombrid, the chub mackerel (*Scomber japonicus* Houttuyn). *S. japonicus* lacks vascular heat exchangers and cannot achieve tissue temperatures greater than 1°C above ambient (Roberts and Graham 1979; Sepulveda and Dickson 2000; Dickson and Graham 2004) but, like tunas, it is tolerant of a broad range of environmental temperatures and may dive to depths below the thermocline (Schaefer 1986; Hernandez and Ortega 2000). *S. japonicus* shares further similarities with tunas, including a fusiform body shape to reduce drag (Roberts and Graham 1979), high Hb concentration (Klawe et al. 1963; Greer-Walker and Pull 1975), large gill surface area with short diffusion distance (Hughes 1966; Steen and Berg 1966), high sustainable swimming speeds and a large dependence on ram gill ventilation (Roberts 1975; Roberts and Graham 1979; Boutilier et al. 1984; Sepulveda and Dickson 2000). The thermal tolerance of *S. japonicus* is emphasised by its broad distribution throughout the temperate and subtropical waters of both hemispheres (Kishinouye 1923; Matsui 1967; Schaefer 1986). Temperatures of 10, 20 and 30°C were chosen for the present study to approximate, respectively the minimum, preferred, and maximum temperatures of this species (Schaefer 1986). This study is the first to test the hypothesis that reduced and reversed temperature dependence of blood–O_2 binding are exclusive to scombrids that possess vascular heat exchangers.

Materials and methods

Animals and blood sampling

Chub mackerel (*Scomber japonicus*) were caught by hook and line off the coast of southern California, USA, in May 2008, at sea surface temperatures of 18 ± 2°C. Five mackerel (fork length 23–39 cm) were placed within an aerated 100-l holding tank for 4 h to recover from capture. Fish were individually netted and a 2–5 ml heparinised blood sample was rapidly obtained by caudal venepuncture. Blood sampling was completed within 30 s of a fish being netted, and the sample was immediately placed on ice. Blood was transported overnight to the University of British Columbia, Vancouver, Canada, where experiments were performed between 1 and 6 days post-capture. Using these sampling techniques, it was not expected that a significant methaemoglobin concentration would exist (Wells et al. 1997), and it is likely that any catecholamines released during capture were degraded within a few hours of blood storage (Tetens et al. 1988; Randall and Perry 1992). Measurements were conducted on individual blood samples from all five fish. Blood was stored at 3°C when not in use, and regularly mixed. Blood was not stored for more than 6 days prior to use, as it has been demonstrated for rainbow trout whole blood that erythrocyte function, as indicated by activation of membrane Na^+/H^+ exchangers following addition of isoproterenol, remains viable for 6 days at 4°C (Caldwell et al. 2006).

Oxygen equilibrium curves

Oxygen equilibrium curves (OECs) were determined using similar techniques and a modified version of the custom-built spectrophotometer ($P_{wee}50$, La Trobe University, Bundoora, Australia) described previously (Clark et al. 2008b). Briefly, in the presence of light, 1 µl of well-mixed whole blood was smeared between two 6-µm Teflon membranes held taut with a neoprene O-ring on a ring-shaped sample holder. Any residual catecholamines that might influence oxygen equilibrium parameters were expected to have been rapidly degraded in the thin blood film using these techniques (Wells et al. 2003) if they had not already been degraded from storage (Tetens et al. 1988). The sample holder was positioned into a 5-ml air-tight sample chamber, with the center of the ring, and hence the blood sample, located directly above a focused bank of light-emitting diodes (LEDs). The equipment was housed within a temperature-controlled room such that the temperature within the sample chamber could be regulated (±0.3°C) to expose the blood to open-system temperature changes. Humidified, medical-grade gas mixtures were introduced into the sample chamber at a flow rate of 50 ml min^{-1} using a gas mixing pump (Corning 192 Precision Gas Mixer; Corning Medical and Scientific, Medfield, Massachusetts, USA). The blood film equilibrated with each new gas mixture within 3–10 min, as indicated by a steady output from the spectrophotometer. The wavelength of light emitted by the LEDs sequentially switched between 435 nm (near the peak absorption for deoxygenated Hb) and 390 nm (near the isosbestic point between oxy- and deoxy-haemoglobin; i.e. the wavelength at which absorption is independent of oxygen saturation) and the values obtained from these wavelengths were used to calculate the level of blood–O_2 saturation for each gas mixture.

OECs were constructed using the $P_{wee}50$ at 10, 20 and 30°C and at three levels of CO_2 (0.5, 1.0 and 2.0%), using stepwise increments in O_2 (typically 1% increments in O_2 until exceeding 50% blood–O_2 saturation; balance N_2) up to a maximum of 21% O_2. The levels of CO_2 were chosen to encompass in vivo values measured previously from arterial and venous systems of athletic fishes (e.g. Korsmeyer et al. 1997), with a goal to encompass the maximum value that might be attainable in an intensely exercising mackerel. At each temperature, a given blood sample was subjected to all three CO_2 tensions. One hundred percent blood–O_2 saturation for each blood sample was defined as the peak absorbance value obtained when the blood sample was exposed to a CO_2–free gas mixture of 21% O_2 and 79% N_2 [pilot experiments confirmed full saturation of *S. japonicus* blood under these conditions, and this is in agreement with previous reports for *S. scombrus* blood at 10°C (Herbert et al. 2006)]. This allowed calculation of 100% blood–O_2 saturation at the different CO_2 tensions within a given temperature, which enabled calculation of the Root effect at each CO_2 tension. Fresh subsamples of blood were used for each curve, and the order of performing the curves was randomised. At the conclusion of constructing an OEC, absorbances at 0 and 100% blood–O_2 saturation were rechecked, as well as at two oxygen tensions either side of 50% blood–O_2 saturation (i.e. either side of the P_{50}). Changes in absorbance at a given oxygen tension were negligible between the two tests at all temperatures, and the calculated P_{50}s from both tests were always within 20% of each other, suggesting that there was not an appreciable change in organic phosphates or methaemoglobin concentration throughout the period required to construct an OEC. Furthermore, to examine for changes in blood–O_2 affinity during the days of blood storage (e.g. due to a gradual depletion of organic phosphates which may increase blood–O_2 affinity over time), values of P_{50} under each treatment were plotted against 'time after blood arrival' to inspect for significance of linear regressions. Under no instances did P_{50} significantly correlate with blood storage time ($P > 0.143$ in all cases).

Tonometry, pH and haematology

Subsamples of whole blood (0.3 ml) from all five fish were incubated in glass Eschweiler tonometers (Kiel, Germany) in the presence of light, with a gas mixture of 0.25% CO_2, 50% air and 49.75% N_2 (=0.27% CO_2, 10.50% O_2 and 89.23% N_2; using a Wösthoff DIGAMIX 6KM 422 gas mixing pump, Bochum, Germany) at 10°C for subsequent measurement of extracellular pH (pHe) using a thermostatted capillary pH electrode (BMS 3 MK 2, Radiometer, Denmark) (see Table 1). Further subsamples were incubated in the tonometers with gas mixtures containing varying levels of CO_2 (0.25–2.0% CO_2 with approximately 0.3% increments) in order to quantify the relationship between $\%CO_2$ and pHe at 10°C. The slope of this relationship (−0.236 pHe units $\%CO_2^{-1}$) was used to assign pHe values to the OECs at different CO_2 tensions at 10°C. Additionally, the pHe values at 10°C were temperature-corrected to 20 and 30°C using an established relationship determined for vertebrates including tunas (ΔpHe $°C^{-1} = -0.016$; (Reeves 1972; Cech et al. 1984)).

Haemoglobin concentration ([Hb]) was determined using a HemoCue analyser (Angelholm, Sweden) calibrated for fish blood (see Clark et al. 2008a). Haematocrit (Hct) was measured following centrifugation of whole blood in micro-haematocrit tubes at 7,000×g for 10 min, and mean corpuscular haemoglobin concentration (MCHC) was calculated as [Hb]/(Hct/100). Whole blood pHe, [Hb] and Hct were regularly measured following tonometry at 10°C and 1.0% CO_2 throughout the duration of 3°C storage and prior to OEC determinations to ensure samples were viable. No significant differences were observed over the storage duration of 6 days (e.g. pHe = −0.0013 (day) + 7.841 [$r^2 = 0.000$, $P = 0.966$]), and consistently clear plasma colour during Hct measurements confirmed negligible haemolysis.

Table 1 Haematological variables for whole blood of chub mackerel, *Scomber japonicus* ($N = 5$)

Fish fork length (cm)	28.3 ± 3.4
pHe at 10°C[a]	8.033 ± 0.093
Haematocrit (%)	35.4 ± 3.7
Haemoglobin concentration	
(g l^{-1})	99.8 ± 9.5
(mM)	1.47 ± 0.14
MCHC	
(g l^{-1})	282.8 ± 7.5
(mM)	4.16 ± 0.11

[a] pHe measured after tonometry with a gas mixture of 0.25% CO_2, 50% air and 49.75% N_2 (=0.27% CO_2, 10.50% O_2 and 89.23% N_2)

Data analysis and statistics

OECs were constructed by fitting oxygen partial pressure (PO_2) and percent blood–O_2 saturation data to a logistic function by least-squares regression (SigmaPlot, Systat Software Inc.). The effect of carbon dioxide partial pressure (PCO_2) on the blood–O_2 affinity constant, P_{50} (the PO_2 at which blood–O_2 saturation is 50%), was calculated for each temperature by the CO_2 coefficient ($\Delta\log P_{50}/\Delta\log PCO_2$), and the effect of pHe on P_{50} was calculated as the Bohr coefficient ($\Delta\log P_{50}/\Delta$pHe). The temperature sensitivity of the OEC was expressed by the van't Hoff integration by the apparent heat of oxygenation for the Hb–O_2 reaction, $\Delta H° = 2.303R((\Delta\log P_{50})/(\Delta 1/T))$ kJ mol^{-1}, where R = universal gas constant (0.008314 kJ K^{-1} mol^{-1}), and T = measurement temperature in K. A positive $\Delta H°$ indicates a left-shift in the OEC with increasing temperature. Hill plots were constructed on mid-range data (~20–80% saturation) to give a linear regression of log PO_2 versus log ($S/(100 - S)$), where S is the percent blood–O_2 saturation, and the slope through the P_{50} defines the Hill coefficient (n_H).

Comparisons between data were conducted using paired t tests or repeated measures ANOVA where appropriate. Statistical significance was considered as $P < 0.05$. All results are given as means ± standard error of the mean (SEM). Pressures are given in kPa. For conversion of units, 1 mm Hg = 1 Torr = 0.1333 kPa.

Results

Haematological variables of stored blood following tonometry at 10°C are given in Table 1. At 10°C, and with a gas mixture of 0.27% CO_2, 10.50% O_2 and 89.23% N_2, pHe was 8.033 ± 0.093. The measured relationship between $\%CO_2$ (range 0.25 – 2.0% CO_2) and pHe at 10°C yielded a slope of -0.236 pHe units $\%CO_2^{-1}$ (Tables 1, 2). On the basis that 1 g Hb binds 1.39 ml O_2, the estimated oxygen carrying capacity of *S. japonicus* blood was 139 ml O_2 l^{-1}.

Increasing PCO_2 (and therefore decreasing pHe) typically shifted the OEC to the right at all temperatures and at all levels of PCO_2, significantly increasing P_{50} values ($P < 0.05$; Fig. 1; Table 2). The Bohr coefficient between 0.5 and 1.0% CO_2 tended to increase in magnitude with increasing temperature and ranged from −1.04 at 10°C to −1.52 at 30°C. The Bohr coefficient between 1.0 and 2.0% CO_2 was around −0.40 at 20 and 30°C, but was of greater magnitude at 10°C due to a large Root effect (Table 2; Fig. 1). Indeed, increasing CO_2 from 0 to 2.0% at 10°C and 21% O_2 initiated the most dramatic Root effect of all treatments (31.9 ± 6.0% decrease in blood–O_2 saturation;

Table 2 Effects of temperature and carbon dioxide on whole blood oxygen equilibrium parameters from chub mackerel, *Scomber japonicus*

	10°C			20°C			30°C		
CO_2 (%)	0.5%	1.0%	2.0%	0.5%	1.0%	2.0%	0.5%	1.0%	2.0%
PCO_2 (kPa)	0.50	1.00	2.00	0.49	0.99	1.98	0.49	0.97	1.94
pHe	7.947	7.830	7.594	7.787	7.670	7.434	7.627	7.510	7.274
P_{50} (kPa)	2.58±0.19^{ACD}	3.49±0.43^{C}	7.37±1.87^{BD}	1.97±0.12^{AE}	2.93±0.40^{F}	3.66±0.50^{BEF}	2.78±0.28^{GH}	4.19±0.40^{G}	5.45±1.22^{H}
Root (%)	-11.1±1.5	-12.7±3.9	-31.9±6.0^{IJ}	-6.9±1.4^{I}	-11.4±4.4	-15.7±6.4	-5.4±1.6^{J}	-13.0±4.7	-18.7±6.9
n_H	0.92±0.07	1.04±0.13	0.80±0.06	1.02±0.06	1.04±0.09	1.08±0.15	1.17±0.09	1.07±0.09	1.17±0.27
CO_2 coeff.	0.41±0.10	0.97±0.34		0.54±0.19	0.32±0.11		0.60±0.01	0.32±0.23	
Bohr coeff.	-1.04±0.24	-1.23±0.43		-1.38±0.48	-0.41±0.14		-1.52±0.03^{K}	-0.40±0.29^{K}	

Data are means ± SEM. Calculations: n_H ($\log PO_2/\log(S/(100 - S))$), CO_2 coefficient ($\Delta\log P_{50}/\Delta\log PCO_2$), Bohr coefficient ($\Delta\log P_{50}/\Delta$pHe). pHe at 10°C calculated using the measured slope relating pHe and $\%CO_2$ (-0.236 pHe units $\%CO_2^{-1}$) following the initial pHe measurement of 8.033 ± 0.093 at 10°C (see Table 1). pHe values were temperature-corrected to 20 and 30°C using -0.016 pHe units $°C^{-1}$ (see "Materials and methods"). Root effect is given as a percentage decrease in blood-oxygen saturation, assuming 100% saturation in a gas mixture of 0% CO_2, 21% O_2 and 79% N_2 at each respective temperature. Same superscript letters indicate significant differences

Fig. 1). Values of n_H tended to increase with temperature but never exceeded 1.2, resulting in an essentially hyperbolic relationship between blood–O_2 saturation and PO_2.

The effect of temperature on the OEC at each PCO_2 is illustrated in Fig. 2, and P_{50} values are presented in Table 2. Oxygen affinity was generally highest at 20°C for all levels of CO_2 (Table 2), which is best illustrated by Arrhenius plots (Fig. 2, inset). Temperature-independent binding was observed at 0.5% CO_2, where P_{50} was not statistically different at 10 and 30°C (2.58 vs. 2.78 kPa, respectively; $P > 0.05$) with a $\Delta H°$ close to zero (-6.1 ± 1.2 kJ mol^{-1}; Table 3). Representative OECs for sockeye salmon (*Oncorhynchus nerka*) at 12 and 24°C, constructed using the same techniques and equipment used for *S. japonicus* OECs, are provided in Fig. 2 (top panel) to illustrate the classical response of the vertebrate OEC to temperature. The most significant temperature-mediated difference for *S. japonicus* occurred at 2.0% CO_2, where the P_{50} at 10°C was twofold higher than that at 20°C ($P < 0.05$) with a corresponding $\Delta H°$ of $+43.2 \pm 22.2$ kJ mol^{-1} (Tables 2, 3). These results provide clear evidence of reduced and reversed temperature dependence of blood oxygenation in *S. japonicus*.

Discussion

Haemoglobin cooperativity, Root effect and Bohr effect

The [Hb] and Hct measured for *S. japonicus* compare favourably with values previously reported for various mackerel species, which are known to be higher than most other fishes with the exception of tunas (Hall and Gray 1929; Root 1931; Klawe et al. 1963; Wells et al. 1986; Brill and Bushnell 1991; Dickson 1996; Lowe et al. 2000; Brill and Bushnell 2006; Clark et al. 2008b). Furthermore, the pHe values of *S. japonicus* blood after tonometry are consistent with in vivo values reported for mackerels and tunas (Boutilier et al. 1984; Jones et al. 1986; Korsmeyer et al. 1997). The P_{50} values of *S. japonicus* may suggest a similar level of hypoxia tolerance as the tunas, perhaps with the exception of bigeye tuna (Lowe et al. 2000), and the Hb subunits of *S. japonicus* have a low cooperativity ($n_H < 1.2$) that falls within the range reported for other scombrids (Cech et al. 1984; Jones et al. 1986; Brill and Bushnell 1991; Lowe et al. 2000; Brill and Bushnell 2006; Herbert et al. 2006).

The magnitude of the Root effect in *S. japonicus* increased with decreasing temperature (Table 2). Such thermal dependence of the Root effect is consistent with studies on tunas (Brill and Bushnell 1991, 2006). In fact, while Atlantic bluefin tuna (*Thunnus thynnus*) blood displayed a significant Root effect between 0.5 and 1.5% CO_2 at 15°C, no Root effect was detected at 25°C over the same CO_2 range (Brill and Bushnell 2006). It is questionable whether this in vitro thermal dependence of the Root effect in scombrids has functional significance in vivo, as this would potentially result in insufficient oxygen supply to the eye and visual impairment of the fish at warm temperatures (Berenbrink et al. 2005). It may be that a stronger acidification is required at high temperatures to induce the same level of oxygen unloading. Nevertheless, the greatest Root effect in the present study occurred at 10°C (32% decrease in blood–O_2 saturation from 0 to 2 kPa PCO_2) and at all temperatures was within the range previously documented for *S. scombrus* at 10 and 20°C (Root 1931; Herbert et al.

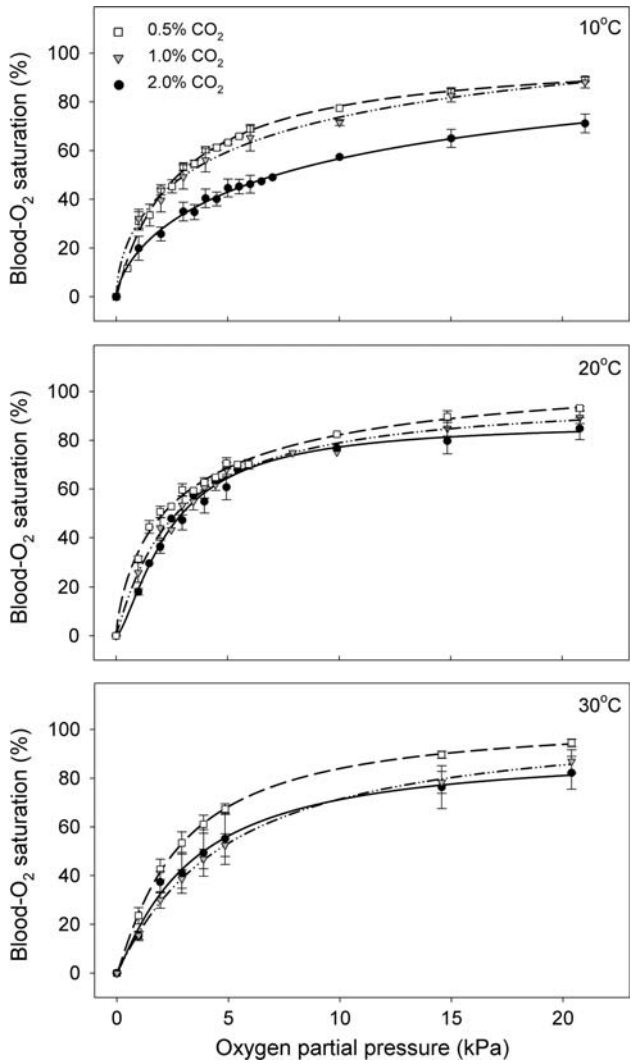

Fig. 1 Oxygen equilibrium curves (OECs) for chub mackerel (*Scomber japonicus*) at 0.5% CO_2 (*squares* and *dashed line*), 1.0% CO_2 (*diamonds* and *dash-dot line*) and 2.0% CO_2 (*circles* and *solid line*), and at 10, 20 and 30°C (data are means ± SEM). 100% haemoglobin saturation was determined at each temperature using a CO_2–free gas mixture of 21% O_2 and 79% N_2

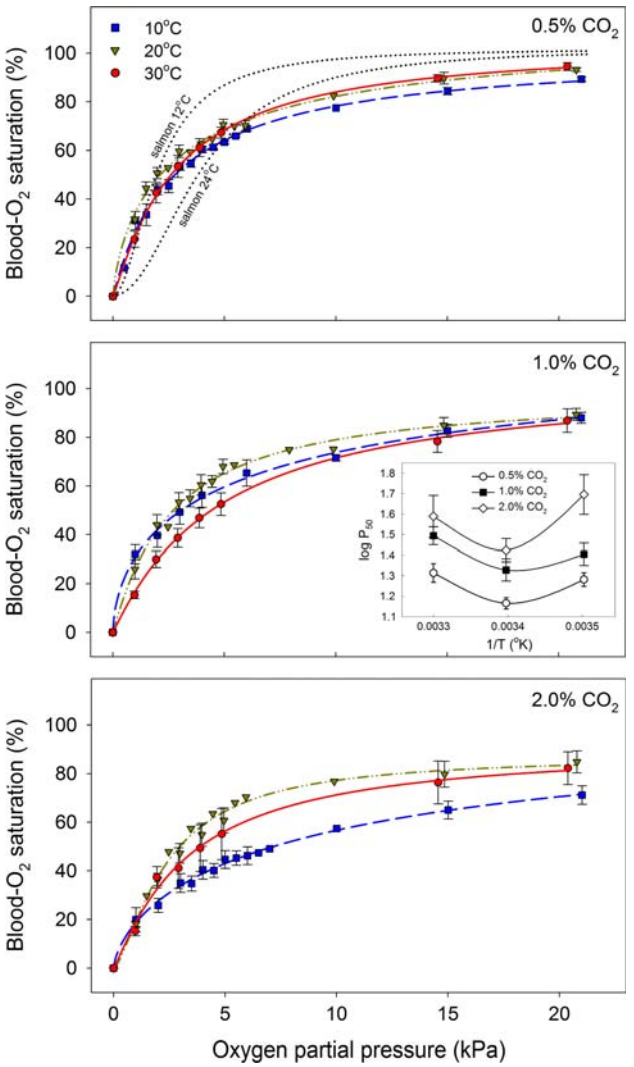

Fig. 2 Oxygen equilibrium curves (OECs) for chub mackerel (*Scomber japonicus*) at 10°C (*squares* and *dashed line*), 20°C (*diamonds* and *dash-dot line*) and 30°C (*circles* and *solid line*), and at 0.5, 1.0 and 2.0% CO_2 (data are means ± SEM). 100% haemoglobin saturation was determined at each temperature using a CO_2–free gas mixture of 21% O_2 and 79% N_2. *Inset* Arrhenius plot of log P_{50} versus $1/T$ at three CO_2 tensions, where T is blood temperature in K. In order to illustrate the classical response of the vertebrate OEC to temperature, included on the *top panel* are OECs for sockeye salmon (*Oncorhynchus nerka*) at 12 and 24°C (0.03% CO_2) constructed using the same techniques and the same equipment (i.e. the $P_{wee}50$) used in the present study (data courtesy of Linda Hanson, University of British Columbia, Canada)

2006). Over a similar temperature range of 20–30°C and over a comparable PCO_2 increment of 1 kPa, the Root effect of *S. japonicus* (~12% decrease in blood–O_2 saturation from 0 to 1 kPa PCO_2; Table 2) was similar to that reported for species of tuna [~10% decrease in blood–O_2 saturation from 0.5 to 1.5 kPa PCO_2; (Brill and Bushnell 1991; Lowe et al. 2000)].

The Bohr coefficient of *S. japonicus* was greatly negative at most temperatures and CO_2 tensions (Table 2). Large Bohr coefficients are common among fishes that possess a large Root effect (Lowe et al. 2000; Berenbrink et al. 2005), as well as those that possess $\Delta H°$ values that are positive or near zero (Cech et al. 1984; Brill and Bushnell 1991; Lowe et al. 2000; Clark et al. 2008b). The details of the latter are not well understood, but a large Bohr coefficient may compensate for the lack of temperature influence on oxygen unloading at the tissues. It is possible that the large Bohr coefficient actually confers the lack of temperature influence on blood–O_2 binding based on the exothermic nature of proton–haemoglobin binding (Weber et al. 2008).

Table 3 The effect of temperature on the apparent heat of oxygenation ($\Delta H°$) for haemoglobin of chub mackerel, *Scomber japonicus*, at three CO_2 tensions

	$\Delta H°$ (kJ mol^{-1})		
	10–20°C	20–30°C	10–30°C
0.5% CO_2	+18.5 ± 6.9	−24.4 ± 12.3[a]	−6.1 ± 1.2
1.0% CO_2	+12.2 ± 10.1	−27.9 ± 14.4[b]	−13.9 ± 1.4
2.0% CO_2	+43.2 ± 22.2[abc]	−23.1 ± 16.4[c]	+8.5 ± 14.4

Same superscript letters indicate significant differences

Functional role of reduced and reversed thermal dependence of haemoglobin oxygenation

This study presents the first evidence that an ectothermic scombrid possesses reduced and reversed thermal effects on blood–O_2 binding. However, an examination of previous reports for *S. scombrus* may have provided some insight in this regard, as P_{50} values of 3.0 kPa at 10°C (Herbert et al. 2006) and 2.3 kPa at 20°C (Root 1931) (each at pH ~7.9) have been reported in studies conducted more than 70 years apart. While blood oxygenation was essentially independent of temperature in *S. japonicus* at 10, 20 and 30°C at 0.5% CO_2, OECs separated at higher CO_2 tensions such that blood–O_2 affinity was markedly lower at 10°C and 2.0% CO_2 than at either of the higher temperatures at the same CO_2 tension (Fig. 2). These results indicate that the OEC in vivo may be less influenced by temperature in arterial blood (where CO_2 tensions are the lowest) than in venous blood (where CO_2 tensions are higher). It may be that maintaining temperature-independent blood–O_2 binding in arterial blood at the gills ensures a consistent supply of oxygen from the ambient water over the thermal range experienced by *S. japonicus* (Rossi-Fanelli and Antonini 1960), whereas there may be a requirement for blood–O_2 affinity to be differentially affected by temperature in the venous system where blood CO_2 levels can be substantially elevated during high levels of sustained exercise (Brauner et al. 2000). The functional significance of these findings is not understood, although a similar CO_2-linked (and pH-linked) temperature dependence of blood–O_2 affinity has been reported for southern bluefin tuna [*Thunnus maccoyii*; (Clark et al. 2008b)] and the porbeagle shark [*Lamna nasus*; (Larsen et al. 2003)], indicating the prevalence of this phenomenon among regionally heterothermic fishes.

Our findings for an ectothermic scombrid have important implications for the hypothesised link between unusual thermal dependence of blood–O_2 binding and the capacity to maintain elevated tissue temperatures (Rossi-Fanelli and Antonini 1960; Cech et al. 1984; Clark et al. 2008b). Despite the limited available information on reduced and reversed thermal effects on blood–O_2 binding, there are sufficient data to allow some conclusions to be drawn. First, regional heterothermy has been reported in some species of elasmobranchs from the family Lamnidae (Dickson and Graham 2004; Bernal and Sepulveda 2005; Shadwick 2005), and independent and reversed temperature dependence of Hb–O_2 binding has been reported in a heterothermic member of the family Lamnidae, the porbeagle shark (Andersen et al. 1973; Larsen et al. 2003) (Fig. 3). This provides some evidence that heterothermy and the atypical temperature effects on blood–O_2 binding evolved independently in the elasmobranchs and teleosts (Bernal et al. 2001; Dickson and Graham 2004). Previous studies have also reported regional heterothermy in the elasmobranch family Alopiidae (Bernal and Sepulveda 2005; Sepulveda et al. 2005), although nothing is currently known of the effects of temperature on blood respiratory properties in this lineage. Second, heterothermy and atypical temperature dependence of Hb–O_2 binding have been reported in the blue marlin (*Makaira nigricans*) and the striped marlin (*Tetrapturus audax*), members of the billfish family Istiophoridae (Weber and Jensen 1988; Weber and Fago 2008), indicating that these traits exist in regionally heterothermic teleost lineages outside of the family Scombridae (Fig. 3). Finally, the present study on *S. japonicus* confirms that thermally independent and reversed thermal dependence of blood–O_2 binding exist in ectothermic members of the suborder Scombroidei, which suggests that vascular heat exchangers and regional heat conservation were not prerequisites for the development of reduced or reversed thermal effects on blood–O_2 binding (Fig. 3).

In fact, it seems more likely that the series of evolutionary events was reversed, at least within the Scombroidei. Based on the available data, it may be speculated that (1) independent and reversed thermal effects on blood–O_2 binding evolved early in the Scombroidei lineage, which may have allowed enhanced gill oxygen uptake over a broad range of ambient temperatures and therefore some level of thermal niche expansion; and (2) heterothermy evolved following the evolution of the unusual blood–O_2 binding characteristics, the latter of which were beneficial in preventing excessive oxygen unloading to the warmer tissues and ensuring oxygen delivery to tissues efferent to the vascular heat exchangers (Clark et al. 2008b). Regional heterothermy may have enhanced various physiological processes, such as digestion, visual acuity, neural processing, and skeletal muscle contraction frequencies (reviewed in Dickson and Graham 2004), and therefore allowed further thermal niche expansion in those species which evolved this trait. In contrast, it may be argued that regional heterothermy evolved first and was a prerequisite for the atypical blood–O_2 binding characteristics. If this were true, *S. japonicus* must have lost the ability to

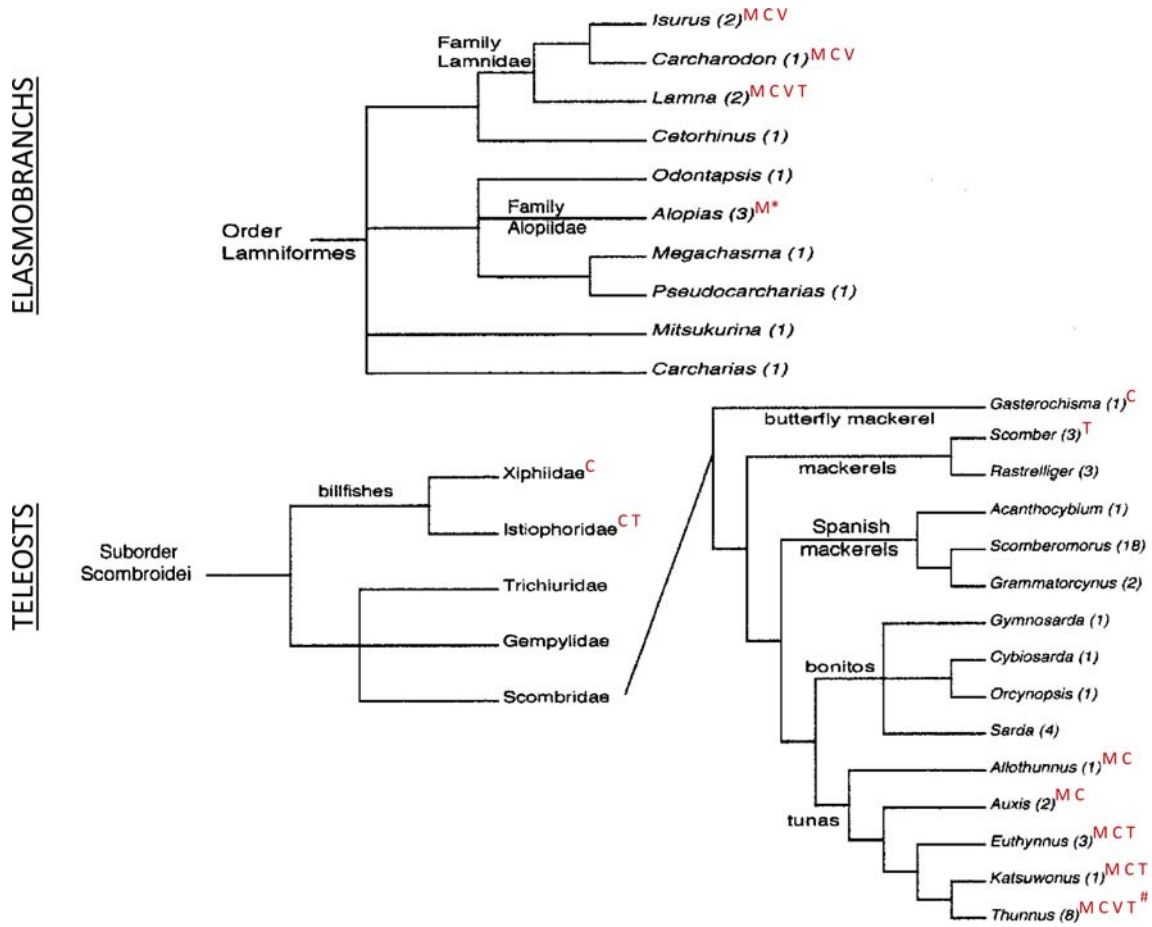

Fig. 3 Cladograms showing phylogenetic relationships among the main fish groups with heterothermic (=endothermic) species (sharks of the families Lamnidae and Alopiidae and Scombroid fishes of the families Xiphiidae, Istiophoridae, and Scombridae). Reproduced and modified from Dickson and Graham (2004), where cladograms were derived from morphological and gene sequence data (from Carpenter et al. 1995; Lydeard and Roe 1997; Naylor et al. 1997; Graham and Dickson 2000; Bernal et al. 2001; Collette et al. 2001). *Numbers in parentheses* represent the number of species in the genus. *Superscript letters* are given for the order Lamniformes and the suborder Scombroidei to indicate empirically confirmed heterothermy in skeletal red muscle (*M*), cranium (*C*) or viscera (*V*). Those groups in which there exists evidence of reduced or reversed thermal effects on blood–O_2 binding are also indicated (*T*). *Only *Alopias vulpinus* (not *A. superciliosus* or *A. pelagicus*) has red muscle heterothermy. #Not all members have visceral heterothermy. Data associated with superscript letters were obtained from: Dickson and Graham 2004 and references within; present study; Brill and Bushnell 1991; Larsen et al. 2003; Bernal and Sepulveda 2005; Sepulveda et al. 2005, 2007, 2008; Clark et al. 2008b; Weber and Fago 2008

conserve heat (i.e. lost heat exchangers and/or cranial heater tissue; Dickson and Graham 2004) throughout evolution but retained the atypical blood–O_2 binding properties. This possibility does not agree with current views on the evolution of regional heterothermy in fishes (Block and Finnerty 1994; Dickson and Graham 2004). Additionally, there appears to be a body size component to regional heterothermy in fishes (Dickson 1994). Regional heterothermy is typically associated with fish species that spend the majority of their lives at relatively large body size (Dickson 1994; Sepulveda et al. 2007, 2008), and to our knowledge there is no evidence of ancestral mackerel being substantially larger than extant species (Collette and Nauen 1983; Monsch 2006). Thus, the evolution and subsequent loss of heterothermic capacity in *S. japonicus* is not considered likely.

In summary, this study examined the blood respiratory properties of an ectothermic scombrid, *S. japonicus*, to test the hypothesis that there exists an exclusive relationship between regional heterothermy and reduced or reversed thermal dependence of blood oxygenation. The findings confirm the presence of atypical blood–O_2 binding properties in *S. japonicus* and therefore reject the proposed hypothesis. We speculate that reduced and reversed thermal dependence of blood oxygenation may have preceded the evolution of regional heterothermy in fishes, functioning initially to enable some level of thermal niche expansion and secondarily to ensure a sufficient oxygen

supply to all tissues following the evolution of vascular countercurrent heat exchangers. In addition to further quantifying phylogenetic relationships and examining for regional heat conservation, future work must determine the effects of temperature on blood oxygenation in ectothermic and regionally heterothermic sharks and teleosts, particularly those of the order Lamniformes [e.g. goblin shark (*Mitsukurina owstoni*) and family Alopiidae] and the suborder Scombroidei (e.g. billfishes, butterfly mackerel, bonitos and primitive tunas; Fig. 3). Indeed, of the members of these two groups that have been studied for thermal effects on blood–O_2 binding, all have displayed reduced and/or reversed temperature dependence (Fig. 3). Further insight will come from comparative examinations of the molecular structure and function of haemoglobins within these groups, to better characterise the role of allosteric modulation in the temperature dependence of oxygen affinity.

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