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RESEARCH ARTICLE

Temperature independence of haemoglobin–oxygen affinity in smalleye Pacific opah (*Lampris incognitus*) and swordfish (*Xiphias gladius*)

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ABSTRACT

Smalleye Pacific opah and swordfish can conserve metabolic heat and maintain specific body regions warmer than ambient water temperature (i.e. regional heterothermy). Consequently, blood O_2 uptake at the gills occurs at the environmental temperature at which the individual is found, but O_2 offloading will occur at different temperatures in different tissues. While several regionally heterothermic fishes (e.g. billfishes, tunas and sharks) show a reduced temperature effect on haemoglobin (Hb)–O_2 affinity, the temperature dependence of Hb–O_2 affinity in opah and swordfish is unknown. We hypothesized that the Hb of opah and swordfish would also show a reduced temperature dependence. Opah whole-blood–O_2 affinity exhibited a reverse temperature dependence above 50% Hb–O_2 saturation (10–20°C, pH 7.2–8.0), while the temperature dependence of swordfish blood–O_2 affinity (10–25°C) was saturation and pH dependent, becoming temperature independent below 50% Hb–O_2 saturation and pH 7.4. Experiments on stripped haemolysates showed that adding ATP ([ATP]/[Hb]=30) decreased the temperature sensitivity of Hb–O_2 affinity, changing the overall oxygenation enthalpy ($\Delta H'$) values of opah (10–20°C) and swordfish (10–25°C) Hbs at pH 7.4 from −15 and −42 kJ mol^{-1} O_2, respectively, to +84 and −9 kJ mol^{-1} O_2. Swordfish blood–O_2 affinity was high compared with that of other large, pelagic, marine teleosts, which may be the result of unusually low ATP/Hb levels, but might also enable swordfish to forage in the potentially low-oxygenated water of the upper reaches of the oxygen minimum layer. The existence of Hbs with reduced temperature sensitivity in regionally heterothermic fishes may prevent marked changes in Hb–O_2 affinity between the cold and warm tissues.

KEY WORDS: Haemoglobin, Blood, Oxygen, Temperature, Regional heterothermy

INTRODUCTION

The swordfish (*Xiphias gladius*) and the smalleye Pacific opah (*Lampris incognitus*) are both large, mesopelagic species that can conserve metabolic heat and maintain the temperature of select tissues or organs warmer than the surrounding water (i.e. regional heterothermy). Opah can maintain the cranial region and entire body core, including the heart, warmer than ambient water, which is unique among fishes because the hearts of all other fishes remain near ambient temperature (Runcie et al., 2009; Wegner et al., 2015). In swordfish, thermogenic extraocular muscles heat the eyes and brain (Carey, 1982b), and recent work shows that their red slow-twitch swimming muscles can maintain some degree of temperature elevation relative to the environment (Carey, 1990; Carey and Gibson, 1987; Stoehr et al., 2018, 2020). Only a few other fishes are also capable of heating their eyes and brain [i.e. billfishes (family Istiophoridae), tunas (family Scombridae), lamnid sharks (family Lamnidae) and the butterfly mackerel (*Gasterochisma melampus*)] or the red swimming muscles [i.e. tunas, lamnids and the common thresher shark (*Alopias vulpinus*)] warmer than ambient water (Bernal and Sepulveda, 2005; Block, 1986; Carey and Teal, 1966, 1969a,b; Carey et al., 1985; Sepulveda et al., 2008). The ability to maintain a warm tissue temperature while residing in cold water provides regionally heterothermic fishes with certain physiological advantages over their ectothermic competitors and ectothermic prey, such as greater power output by the warm swimming muscles, and superior vision as a result of enhanced temporal resolution in the heated retinas (Altringham and Block, 1997; Fritsches et al., 2005; Stoehr et al., 2020).

Regionally heterothermic fishes are able to conserve metabolic heat in select body regions via vascular specializations (retia mirabilia) that form countercurrent heat exchangers, enabling cold arterial blood flowing from the gills to be warmed by venous blood (except in the opah) that is returning to the gills (Bernal et al., 2001; Block, 1986; Carey, 1982a; Carey and Teal, 1966,a; Carey et al., 1985; Fudge and Stevens, 1996; Patterson et al., 2011; Runcie et al., 2009; Wegner et al., 2015). This heat transfer diminishes convective heat loss, as the venous blood cools to near ambient temperature before reaching the gill lamellae, where blood and water reach thermal equilibrium (Brill et al., 1994; Stevens and Sutterlin, 1976). Opah and swordfish have heat exchanging retia in the orbital circulation (Carey, 1982b; Runcie et al., 2009), and warmed blood perfuses the opah's entire body, including the heart, as a result of a series of unique afferent–efferent arterial heat exchanging retia within the gill arches, each of which is relatively thick and insulated with fat (Wegner et al., 2015). The swordfish also has putative heat exchanging retia that supply blood to the medially located red muscle; however, little is known about the degree of control swordfish have over red muscle temperature fluctuation during extended exposure to cold environmental temperatures (Carey, 1990; Stoehr et al., 2018). A consequence of efficient vascular heat exchange is that blood O_2 uptake at the gills occurs at quite different temperatures to O_2 offloading in the tissues that are thermally

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isolated by a rete, and these internal temperature gradients are exacerbated when opah and swordfish dive into cold water below the thermocline (Carey, 1990; Wegner et al., 2015).

During daily sojourns between the warm upper mixed layer above the thermocline (e.g. >20°C) and deeper colder waters below the thermocline (e.g. <5°C), swordfish may experience large and rapid changes in water temperature in addition to possibly low environmental oxygen levels near the oxygen minimum layer (Carey, 1990; Carey and Robinson, 1981; Dewar et al., 2011; Sepulveda et al., 2010), whereas below the thermocline, swordfish cranial temperature is relatively constant and can be elevated as much as 12°C above ambient water temperature (Carey, 1990). Although opah occasionally swim into the warm upper mixed layer, they tend to remain in cooler water below the thermocline where cranial temperatures are relatively constant and elevated at least 6°C above the surrounding water, while body and heart temperature are elevated at least 3–5°C above the surrounding water (Wegner et al., 2015). Therefore, blood O_2 uptake at the gills must occur over the range of environmental temperatures encountered by opah and swordfish, but the blood must also transport O_2 over steep internal temperature gradients, and O_2 offloading must occur from the lowest temperature (e.g. at the gills) to the highest body temperatures (e.g. at the ocular muscles). Moreover, as blood flows through a heat exchanging rete, it is subjected to what has been described as 'closed-system' temperature changes as the blood P_{O_2} and P_{CO_2} can vary with temperature, but the content of blood gases remains essentially constant as a result of the size and thickness of rete vessels, diminishing diffusion of gases out of the blood (Brill and Bushnell, 1991; Cech et al., 1984; Stevens et al., 1974). The O_2 affinity of the blood can, thus, be directly affected by closed-system temperature changes as well as by variable environmental and body temperatures.

The O_2 affinity of most jawed vertebrate haemoglobins (Hbs) typically decreases with increasing temperature. This is because the heat of O_2 binding to the haem groups (ΔH^{O_2}) is intrinsically exothermic, so the overall enthalpy of oxygenation ($\Delta H'$) is usually also exothermic (i.e. numerically negative). However, temperature-independent Hb–O_2 affinity and even reverse temperature dependence (i.e. increasing temperature increases Hb–O_2 affinity) have been reported in several species of ectothermic and regionally heterothermic fishes, including tuna, billfish and lamnid sharks (Andersen et al., 1973; Barlow et al., 2017; Bernal et al., 2018; Brill and Bushnell, 1991, 2006; Carey and Gibson, 1977, 1983; Cech et al., 1984; Clark et al., 2008, 2010; Graham, 1973; Larsen et al., 2003; Lilly et al., 2015; Lowe et al., 2000; Sharp, 1975; Weber et al., 2010). Among fishes, reductions and reversals in the temperature dependence of Hb–O_2 affinity appear to stem predominantly from oxygenation-linked dissociation of allosteric effectors, such as hydrogen ions (i.e. Bohr protons) and organic phosphates (e.g. adenosine triphosphate, ATP), which contribute endothermically to $\Delta H'$, reducing the overall effect of temperature on Hb–O_2 affinity (Carey and Gibson, 1977; Dickinson and Gibson, 1981; Ikeda-Saito et al., 1983; Larsen et al., 2003; Morris and Gibson, 1982). The main effector of tuna Hb is protons, whereas ATP is the primary effector of lamnid shark Hb, and in billfishes it is pH-dependent binding of ATP (Ikeda-Saito et al., 1983; Larsen et al., 2003; Weber et al., 2010). In previous experiments on swordfish Hb, increasing temperature considerably decreased Hb–O_2 affinity (i.e. a normal temperature dependence), although it is not clear whether those experiments were conducted on whole blood, erythrolysates or stripped haemolysates (Andersen et al., 1973). Therefore, it is not known whether swordfish Hb exhibits an ATP-induced temperature independence in a manner like that of the closely related istiophorid billfishes. We are not aware of any O_2 equilibria studies on Hb or blood from any of the opah species.

Several hypotheses have been proposed for the functional significance of reduced and reverse temperature-dependent Hb–O_2 affinity in regionally heterothermic fishes. In most, the importance is attributed to either a decreased influence of temperature on blood O_2 transport across large internal temperature gradients or the energetic savings of an increased $\Delta H'$ as less energy would be required to bind and unload O_2 (Carey and Gibson, 1977; Clark et al., 2008; Giardina et al., 1989; Graham, 1973; Weber and Campbell, 2011; Weber et al., 2010). Information on the effect of temperature on Hb–O_2 affinity in understudied regionally heterothermic fishes, such as swordfish and opah, can further broaden our understanding of the potential functional significance of reduced and reverse temperature-dependent Hb–O_2 affinity in regionally heterothermic fishes. To that end, we investigated the effect of temperature on Hb–O_2 affinity in blood and haemolysates from the swordfish and the smalleye Pacific opah.

As reductions in the temperature sensitivity of Hb have been reported in other regional heterotherms, including billfishes, we hypothesized that both swordfish and opah Hb would have a similar reduced temperature sensitivity. Furthermore, as swordfish and istiophorid billfishes are closely related and their specialized extraocular muscles and heat-exchanging retia are very similar (Block, 1986, 1991), we expected that swordfish Hb would exhibit an ATP-induced temperature independence like previously studied billfish Hbs. We assessed the temperature sensitivity of whole blood from these species by constructing oxygen equilibrium curves (OECs) and quantifying P_{50} (the P_{O_2} at 50% Hb–O_2 saturation) at different temperatures, as well as by measuring blood P_{O_2} during closed-system temperature changes in an experimental system meant to mimic the temperature changes that the blood experiences in a heat exchanging rete. We also constructed OECs and determined P_{50} in stripped haemolysates buffered at different pH levels, and in the absence and presence of ATP. Experiments were also conducted on haemolysates of Atlantic bluefin tuna that were opportunistically sampled, which allowed us to evaluate the enthalpic contributions of oxygenation-linked effector dissociation among the different lineages of regionally heterothermic teleosts. Collectively, this information gives insight into the evolution and functional significance of reduced and reverse temperature-dependent Hb–O_2 affinity among disparate lineages of regionally heterothermic teleosts.

MATERIALS AND METHODS

All capture, handling and experimental procedures followed guidelines approved by the University of Massachusetts (animal care protocol no. 13-06), the California Department of Fish and Wildlife (Scientific Collection permit nos SC-2471, SC-12372) and the University of British Columbia (UBC) Animal Care Committee (animal care no. A11-0235 and A15-0266). All partial pressures and P_{50} values are reported in mmHg (1 mmHg=0.133 kPa).

Blood collection

Swordfish, *Xiphias gladius* Linnaeus 1758 (n=7), were captured by deep-set buoy gear (Sepulveda et al., 2014), and opah, *Lampris incognitus* Underkoffler, Luers, Hyde and Craig 2018 (n=4), were captured by deep-set buoy gear or short set pelagic long-line in the coastal waters off Southern California (i.e. the Southern California Bight). Atlantic bluefin tuna, *Thunnus thynnus* Linnaeus 1758 (n=2), were captured by hook and line off Massachusetts (fork lengths are reported in Table 1). Blood was drawn by caudal

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Table 1. Fork length and blood variables for smalleye Pacific opah, swordfish and Atlantic bluefin tuna

	Opah	Swordfish	Bluefin tuna
FL (cm)	113±4 (4)	156±12 (5)*	107, 109
Hct (%)	59.1±2.5 (4)	46.3±4.7 (7)	57.8, 53.5
[Hb_4] (mmol l^{-1})	2.21±0.12 (4)	1.81±0.15 (7)	2.53, 2.36
MCHC (mmol l^{-1})	3.74±0.14 (4)	3.96±0.20 (7)	4.37, 4.41
ATP:Hb_4 (mol mol^{-1})	1.32, 0.68	0.67±0.16 (7)	0.40, 0.54
Plasma osmolality (mOsm kg^{-1})	565.2±6.4 (3)	437.5±19.9 (7)	410, 379
Plasma lactate (mmol l^{-1})	14.7±0.79 (3)	12.0±1.53 (6)	18.7, 18.6

Values are means±s.e.m. with samples sizes in parentheses. If values were measured in only two individuals, then the individual measurements are reported. FL, fork length; Hct, haematocrit; Hb, haemoglobin; MCHC, mean corpuscular haemoglobin concentration. *Swordfish fork length was measured from the lower jaw.

puncture into heparinized syringes. Blood samples were kept on ice and shipped by courier to the Department of Zoology, UBC Vancouver campus, where experiments on whole blood were conducted within 1–4 days after the blood was collected. Preliminary experiments with swordfish blood (n=3) that were kept under refrigeration (4°C) for up to 6 days post-collection showed no changes in Hb concentration, haematocrit (Hct; the proportion of red blood cells in blood) and plasma pH, and there was no evidence of red blood cell (RBC) lysis (Morrison, 2020). However, during this time period, RBC intracellular nucleoside triphosphate (NTP) levels likely changed from levels typical of freshly sampled blood, possibly causing Hb–O_2 affinity to increase over time. Although we were not able to construct OECs on freshly drawn blood (i.e. <1 day post-collection), we found that whole-blood P_{50} at 15°C and 3.8 mmHg CO_2 was relatively unchanged from 4 to 8 days post-collection in two swordfish (P_{50} ranged between 24 and 30 mmHg), and blood P_{50} of a third swordfish was unchanged from 5 to 8 days post-collection (23–24 mmHg). Moreover, in a previous study of chub mackerel (*Scomber japonicus*) blood–O_2 affinity, experiments were also conducted at the UBC Vancouver campus on blood collected from mackerel captured off Southern California, and it was concluded that mackerel blood was viable for up to 6 days when stored at 4°C (Clark et al., 2010).

Experimental protocol

Immediately after blood samples arrived at UBC, Hb concentration and Hct were measured, and subsamples of blood were centrifuged to separate the plasma from the RBCs for measurement of plasma osmolality. The packed RBCs and remaining plasma were frozen at −80°C for determination of RBC intracellular ATP concentration and plasma lactate concentration. Whole-blood OECs were constructed by quantifying the relative Hb–O_2 saturation at a range of equilibration P_{O_2} values at two CO_2 levels (1.9 and 7.6 mmHg), and at 10 and 25°C for swordfish, and 10, 15 and 20°C for opah. Blood pH and P_{O_2} were measured in subsamples of blood equilibrated with gas mixes at each of the OEC temperature treatments. After completion of the whole-blood experiments, RBCs were separated from blood plasma by centrifugation. The RBCs were then thrice rinsed in ice-cold marine teleost saline (Hoar and Hickman, 1983) and frozen at −80°C for experiments on stripped haemolysates. OECs were constructed for stripped haemolysates in the presence and absence of effector ions at 10 and 25°C for swordfish, 10 and 20°C for opah, and 15 and 25°C for bluefin tuna.

Haematological parameters

Haemoglobin concentration, expressed as tetrameric Hb ([Hb_4], in mmol l^{-1}), was measured by the cyanmethaemoglobin method using Drabkin's reagent and a haem-based extinction coefficient of 11 $mmol^{-1}$ cm^{-1} at a wavelength of 540 nm (Völkel and Berenbrink, 2000). Hct was measured as the percentage of packed RBCs relative to total blood volume after centrifuging samples in glass microcapillary tubes at approximately 13,000 RCF for 5 min. Mean corpuscular haemoglobin concentration (MCHC, in mmol l^{-1}) was calculated by dividing [Hb] by Hct. Plasma osmolality (mOsm kg^{-1}) was measured in 10 µl of undiluted plasma with a vapour pressure osmometer (VAPRO 5520, Wescor, Logan, UT, USA). ATP was assayed with an ATP colorimetric assay kit (MAK190, Sigma-Aldrich Co. LLC, St Louis, MO, USA), and plasma lactate was measured spectrophotometrically using the LDH-catalysed reaction converting lactate to pyruvate, where the reduction of NAD^+ to NADH was measured at 340 nm (Bergmeyer et al., 1983).

Whole-blood oxygen equilibria, pH and P_{O_2}

Oxygen equilibria experiments were conducted at 10 and 25°C for swordfish, and 10, 15 and 20°C for opah. The coldest experimental temperature was 10°C for both species as this is close to the coldest water temperature regularly encountered by both swordfish and opah (Sepulveda et al., 2010; Wegner et al., 2015). The warmer experimental temperatures were chosen because 25°C is near to the warmest cranial and water temperatures for swordfish, and 15 and 20°C are near the warmest body and cranial/water temperatures, respectively, for opah (Carey, 1990; Sepulveda et al., 2010; Wegner et al., 2015). At each temperature treatment, experiments were conducted at two physiologically relevant CO_2 levels, low (1.9 mmHg) and high (7.6 mmHg), to quantify the Bohr coefficient (i.e. $\Delta P_{50}/\Delta$pH).

The relationship between Hb–O_2 saturation and P_{O_2} (i.e. an OEC) was assessed on 2–3 replicate samples using a custom microplate-based, parallel assay, multi-cuvette tonometry cell as described by Lilly et al. (2013). Cuvettes were formed by sandwiching blood samples (~3 µl) between two sheets of low-density polyethylene (Glad® ClingWrap) that were secured on an aluminium ring with two plastic O-rings, which were then placed in a gas-tight tonometry cell designed to fit into a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA, USA). Optical density (OD) was measured every 20–30 s at 390 nm (near an isosbestic point between oxygenated and deoxygenated Hb, where OD is independent of Hb–O_2 saturation), and at 430 and 436 nm (near the peak absorption for deoxygenated Hb), wavelengths commonly used in thin-film optical methods for measuring Hb–O_2 binding (e.g. Clark et al., 2008; Reeves, 1980; Weber et al., 2010). Initially, blood was equilibrated with pure N_2 for a minimum of 30 min until OD at 430 and 436 nm was stable, which was assumed to indicate full Hb deoxygenation. After deoxygenation, the Hb–O_2 saturation was increased with at least nine stepwise increments of the O_2 tension, balanced with N_2, up to a P_{O_2} of 159.6 mmHg (i.e. approximate atmospheric P_{O_2} at sea level). In a tenth and final increment, the P_{O_2} was increased to 228 mmHg in the absence of CO_2 to achieve full Hb–O_2 saturation. OEC experiments lasted between 1 and 2 h. In preliminary experiments, absorption spectra were recorded following the last oxygenation step (i.e. full Hb–O_2 saturation), and these spectra showed no evident peak at 630 nm, an absorption maximum for metHb (e.g. Völkel and Berenbrink, 2000). Moreover, the OD at 630 nm was not unusually high compared with an absorption maxima for oxygenated Hb

(OD630 nm/OD575 nm<0.04), and α/β peak OD ratios (OD575 nm/OD540 nm) were around 1.06, which are both typical of oxygenated Hb with no significant metHb fraction (Völkel and Berenbrink, 2000; Weber et al., 2010; Zijlstra and Buursma, 1997). Therefore, in the remainder of the experiments, we did not consider metHb formation to have significantly affected our Hb–O_2 saturation measurements, and full Hb–O_2 saturation was assumed at the final oxygenation step. Gas mixtures of O_2, CO_2 and N_2 were obtained using a Wösthoff DIGAMIX® gas mixing pump (H. Wösthoff Messtechnik, Bochum, Germany). At each equilibration step, the difference in OD (ΔOD) between 390 nm and 430 nm or 436 nm (ΔOD=430 nm or 436 nm–390 nm) was used to calculate the fractional Hb–O_2 saturation ([Hb–O_2]/[Hb]) from the change in ΔOD from full deoxygenation, relative to that between full deoxygenation (pure N_2) and full oxygenation (P_{O_2} of 228 mmHg and no CO_2). OECs constructed using 430 nm were identical to those constructed using 436 nm.

Whole-blood pH was measured in approximately 500 µl of blood equilibrated for 1 h with either 1.9 mmHg or 7.6 mmHg CO_2 and a range of O_2 tensions between 7.6 mmHg and 159.6 mmHg (balanced with N_2), in rotating glass tonometers thermostatically set to 10, 15, 20, or 25°C. The gas mixtures were humidified at the experimental temperature prior to entering the tonometers. Blood was drawn into a gas-tight syringe pre-flushed with the gas mixture, and pH was measured by drawing the blood through a Microelectrodes 16-705 flow-thru pH electrode in combination with a 16-702 flow-thru reference electrode (Microelectrodes Inc., Bedford, NH, USA) thermostatically set to the experimental temperature.

To mimic the closed-system temperature changes that blood may experience in the arterioles and venules of a heat exchanging rete, approximately 500 µl blood samples equilibrated at 10, 15, 20 or 25°C were injected into a Radiometer E5046 P_{O_2} electrode thermostatically set at the equilibration temperature as well as another P_{O_2} electrode thermostatically set to a warmer or cooler experimental temperature according to Cech et al. (1984) and Brill and Bushnell (1991). Swordfish blood temperature was changed between 10 and 25°C, whereas opah blood temperature was changed either between 10 and 15°C or between 15 and 20°C. Although the blood was static within the electrode chamber, it was rapidly heated or cooled in a system where there is minimal exchange of gases and ions between the blood and another medium. Prior to injecting the blood, each P_{O_2} electrode was flushed with the experimental gas mixture to minimize electrode response time to the respective P_{O_2}. Temperature-induced changes in P_{O_2} were monitored using Acqknowledge® Data Acquisition Software (v.3.7.3, BIOPAC Systems, Inc.) by viewing traces of P_{O_2}, and when it appeared that the traces had stabilized, the respective values of each were recorded.

Haemolysates

Frozen and packed RBCs were placed on ice and put in a refrigerator (4°C) where they thawed over 24 h. Thawed RBCs were then mixed with an equal volume of cold 0.1 mmol l^{-1} Hepes buffer (pH 8.0) and centrifuged at 10,000 RCF to remove cell debris. The resulting erythrolysates were stripped of endogenous ionic effectors by passage through mixed-bed ion exchange resin (Amberlite® MB-20). MetHb (Hb^+) levels were assessed by oxygenating 10–20 µl of the haemolysates in 1000 µl of 100 mmol l^{-1} Hepes buffer (pH 7.4) that was bubbled with 100% O_2, and a spectral scan was made from 500 to 700 nm (i.e. an oxyHb spectrum). If there was an evident peak or unusually high absorbance at 630 nm, an absorption maximum for metHb (Völkel and Berenbrink, 2000; Zijlstra and Buursma, 1997), then the Hb^+ was reduced by adding a molar excess of sodium dithionite to the haemolysate, followed by passage through mixed-bed ion exchange resin. If spectral scans of reduced oxyHb showed α/β peak OD ratios (OD575 nm/OD540 nm) around 1.06 (see above: Weber et al., 2010; Zijlstra and Buursma, 1997), and no evident peak at 630 nm relative to the OD at wavelengths either side of 630 nm (~600–660 nm), then the haemolysates were concentrated with centrifugal filters (30 kDa exclusion size limit) and used for OEC experiments. Oxygen equilibria were determined in 100 mmol l^{-1} Hepes buffer (pH 6.9–7.8 and no Cl^-) at a Hb concentration of 0.6 mmol l^{-1} in the absence and presence of saturating levels of ATP (ratio of the concentration of ATP/Hb=30). Although guanosine triphosphate (GTP), a potent allosteric effector of teleost Hbs, is also present in fish RBCs, ATP was used in these experiments as ATP concentrations are around sixfold higher than GTP concentrations in billfish RBCs (Weber et al., 2010). ATP wasadded from a 500 mmol l^{-1} stock solution of ATP di-sodium salt (Sigma-Aldrich A7699) in water. OECs were generated at 10 and 20°C for opah haemolysates, 10 and 25°C for swordfish haemolysates, and 15 and 25° bluefin tuna haemolysates following the procedures described above, except without CO_2, and the final O_2 equilibration step (i.e. full saturation) was with 100% O_2. Experiments on bluefin tuna haemolysates were not conducted in the presence of ATP as others have reported that organic phosphates negligibly affect tuna Hb–O_2 affinity (Andersen et al., 1973; Ikeda-Saito et al., 1983). The pH of the haemolysate solutions was measured at the experimental temperature with a thermostatically controlled glass pH electrode (InLab® Micro, Mettler-Toledo LLC, Columbus, OH, USA).

Data analysis

All statistical analyses and curve fitting were performed in R v.3.5.2 (http://www.R-project.org/). For each blood or haemolysate paired dataset of fractional Hb–O_2 saturation (response variable) and P_{O_2} (explanatory variable), non-linear regression was used to fit a three-parameter form of the Hill equation:

$$y = \frac{d}{1 + \left(\frac{a}{x}\right)^b}, \quad (1)$$

where y is the fractional Hb–O_2 saturation, x is the P_{O_2} (i.e. dosage), d is the maximum asymptote (i.e. the response value for infinite dosage), a is the point of inflection (i.e. where $y=d/2$) and b is the slope of the steepest portion of the curve (i.e. the Hill coefficient, n_H). The best-fit parameter values (a, b and d) were used to calculate the P_{O_2} values corresponding to specific Hb–O_2 saturations (P_S; i.e. P_{10}, P_{20}, P_{30}, P_{40}, P_{50}, P_{60}, P_{70}, P_{80}, P_{90} and P_{95}). Non-linear least-squares curve fitting by the Levenberg–Marquardt algorithm was performed using the nlsLM function from the 'minpack.lm' package for R (https://CRAN.R-project.org/package=minpack.lm). Because teleost blood pH is typically dependent on Hb–O_2 saturation (Brauner et al., 1996; Jensen, 1986; Lowe et al., 1998), OEC parameters for each individual were used to calculate Hb–O_2 saturation at the equilibration O_2 tensions, and the pH at a specific Hb–O_2 saturation (pH_S) was then estimated from plots of %Hb–O_2 versus pH. The effects of pH and temperature on whole-blood Hb–O_2 affinity were assessed with linear mixed models, where the response variable was $\log_{10}P_S$ (e.g. $\log_{10}P_{50}$) and the explanatory variables were pH_S (continuous), assay temperature (as a factor), the interaction term between pH_S and assay temperature, and

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individual (id) as a random effect (R-language formula, 'log10(P_S)~pH$_S$*temperature+(1|id)'). Linear mixed models were fitted using the lmer function from the 'lme4' package with the 'lmerTest' package (Bates et al., 2015; Kuznetsova et al., 2017). Mixed models were fitted at each saturation from P_{10} to P_{95}, and for each model a likelihood ratio test (LRT) of fixed effects, fitted with maximum likelihood estimation using a χ^2 distribution, was used to assess the relative importance of temperature in the model (i.e. to test the null hypothesis that temperature is a significant effector of Hb–O_2 affinity). The effects of pH$_{50}$ and temperature on n_H values were also assessed with linear mixed models. For stripped haemolysate data, the effect of temperature on Hb–O_2 affinity (P_{50}), and the pH dependency of P_{50} were analysed with linear models, where the response variable was $\log_{10}P_{50}$ and the explanatory variables were pH (continuous) and assay temperature (as a factor). As the number of OECs generated from a stripped haemolysate differed among individuals, and pH was experimentally controlled, id was not included as a random effect and an interaction between temperature and pH was also not included in the model.

The mixed model fits (Bohr plots) were used to predict $\log_{10}P_S$ values with bootstrap estimated standard errors (500 replications), and these were used to construct whole-blood OECs at constant pH (pH 7.4, 7.5, 7.6 and 7.7) for each species' temperature treatments. Predicted P_{50} values (i.e. $10^{\log_{10}P_{50}}$) were used as a proxy for whole-blood O_2 affinity. Haemolysate $\log_{10}P_S$ values were calculated at specific pH values from linear models fitted to data for each temperature and effector treatment. The temperature dependence of whole-blood and haemolysate O_2 affinity was quantified by calculating $\Delta H'$ values using the van't Hoff equation (Wyman, 1964):

$$\Delta H' = 2.303R\,\frac{\Delta \log P_S}{\Delta \frac{1}{T}}, \qquad (2)$$

where R is the universal gas constant (0.008314 kJ K^{-1} mol^{-1}) and T is the temperature in Kelvin. Whole-blood $\Delta H'$ calculations may not correctly quantify the heat of Hb oxygenation as the contribution of other reactions to $\Delta H'$ was not known for both the plasma and RBC intracellular compartments, and the RBC intracellular concentrations of allosteric effectors were not known or controlled. Nevertheless, the temperature sensitivity of the blood from different species, both within and across studies, can be conveniently compared with whole-blood $\Delta H'$ values. Whole-blood calculations are denoted as $\Delta H'_{WB}$, and for their calculation $\log_{10}P_S$ values were determined at constant extracellular or plasma pH (pH 7.4, 7.5, 7.6 and 7.7), which is typically alkaline relative to RBC intracellular pH. The heat of solution of O_2 (−14 to −12 kJ mol^{-1} between 15 and 25°C; Olofsson et al., 1984) is included in both $\Delta H'_{WB}$ and haemolysate $\Delta H'$ values. The pH dependency of Hb–O_2 affinity was determined by calculating Bohr coefficients at different %Hb–O_2 saturations (P_S):

$$\varphi = \frac{\Delta \log_{10} P_S}{\Delta\,\mathrm{pH}}, \qquad (3)$$

where φ values are the slopes (±95% confidence intervals) from the fitted models of $\log_{10}P_S$ versus pH values.

RESULTS

Whole-blood experiments

Blood parameters and fork length are summarized in Table 1. Whole-blood OECs (Figs 1 and 2) were successfully constructed for four opah and five swordfish, and the blood pH levels measured in the OEC experiments ranged from pH 7.23 to 8.04 for opah, and pH 7.33 to 7.94 for swordfish (Fig. 3). Whole-blood P_{50} values at pH 7.7 (an approximation of arterial blood pH at 25°C from measurements reported from swimming yellowfin tuna; Korsmeyer et al., 1997), n_H values and Bohr coefficients are reported in Table 2. Measured whole-blood OECs are shown in Figs 1 and 2, the pH dependence of blood–O_2 affinity (P_{50}) is shown in Fig. 3, and modelled OECs at different temperatures and blood pH are presented in Fig. 4.

The aim of our analysis was to assess the effects of temperature and pH on P_S, the blood P_{O_2} corresponding to specific Hb–O_2 saturations (e.g. P_{50}). In opah blood, temperature was an important model factor for only the P_{90} regression (χ^2=10.101 d.f.=4, P=0.039), where a reverse temperature dependence was evident in the corresponding Bohr plot (additional Bohr plots are available in Fig. S1). At lower Hb–O_2 saturations, temperature did not

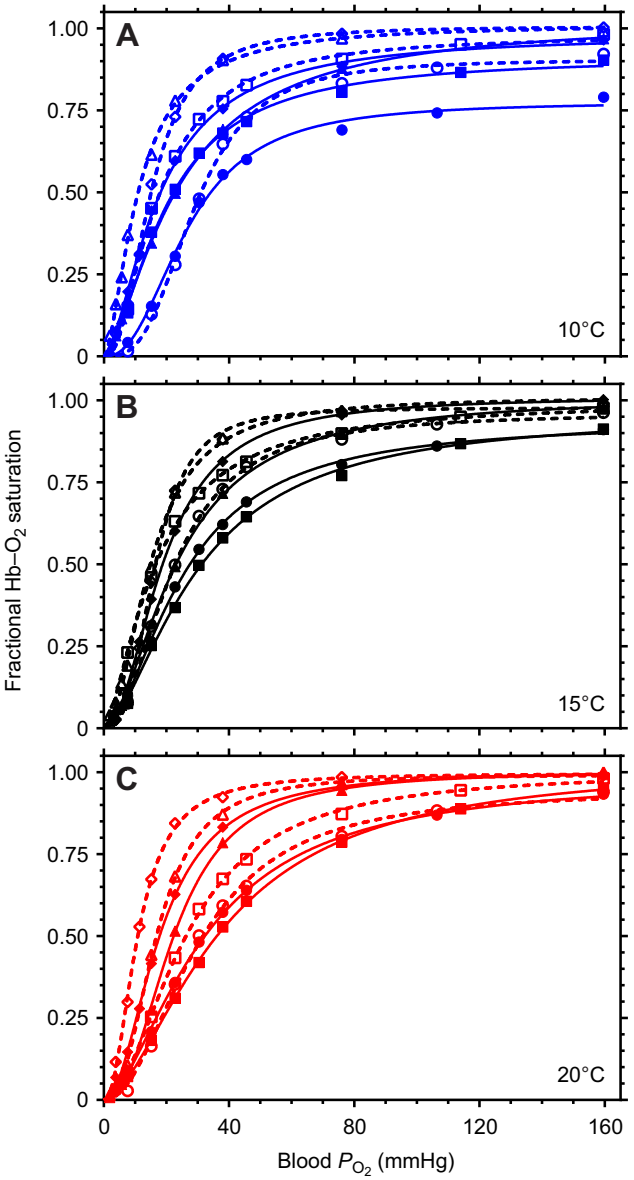

Fig. 1. Oxygen equilibrium curves (OECs) of smalleye Pacific opah (*Lampris incognitus*) blood. Symbols indicate measured values for 4 opah: circles, fork length (FL)=105 cm; squares, FL=116 cm; triangles, FL=123 cm; diamonds, FL=110 cm. OECs were constructed at a low CO_2 (P_{CO_2}=1.9 mmHg; open symbols and dashed curves) and a high CO_2 (P_{CO_2}=7.6 mmHg; filled symbols and solid curves), at 10°C (A), 15°C (B) and 20°C (C).

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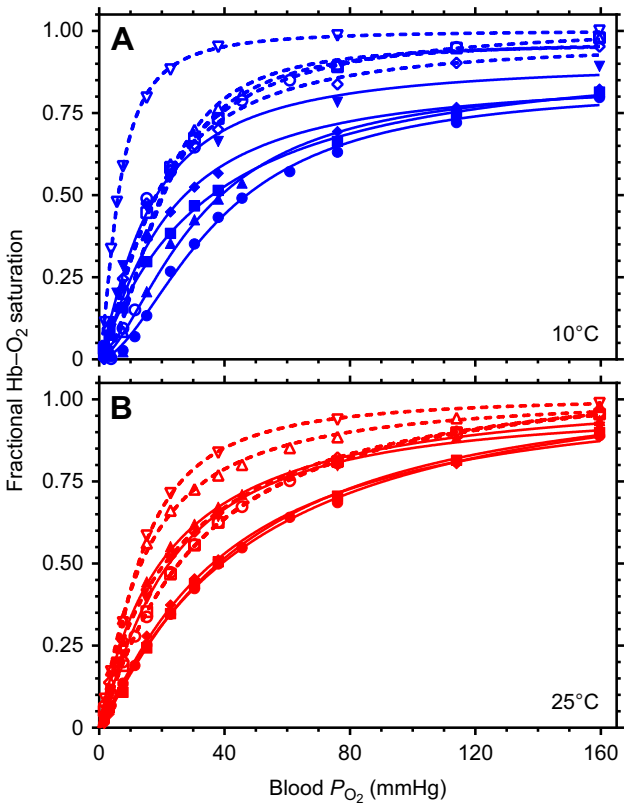

Fig. 2. OECs of swordfish (*Xiphias gladius*). Symbols indicate measured values for 5 swordfish: circles, 182 cm; squares, 122 cm; triangles, 150 cm; diamonds, 144 cm; down-pointing triangles, 183 cm. OECs were constructed at a low CO_2 (P_{CO_2}=1.9 mmHg; open symbols and dashed curves) and a high CO_2 (P_{CO_2}=7.6 mmHg; filled symbols and solid curves), at 10°C (A) and 25°C (B).

significantly predict blood–O_2 affinity, which is evident by the overlapping data and regression lines presented in the Bohr plots (Fig. 3A). In swordfish blood, temperature was an important model factor from P_{50} to P_{95} (χ^2=7.284–18.790, d.f.=2, $P\leq$0.026), where increasing temperature decreased blood–O_2 affinity (Fig. 4E–H). The effect of temperature on swordfish blood–O_2 affinity showed a pH dependence, with temperature having a reduced effect on blood–O_2 affinity with declining pH (Fig. 4E–H), as is evident by the merging or crossing of the Bohr plot regression lines (Fig. 3C). The random intercept, id, accounted for 27–58% of the total variance for the swordfish regression models, and 28–80% of the total variance for the opah regression models.

OECs of both opah and swordfish blood exhibited considerable Bohr effects, evident as a right-shift (i.e. decreased blood–O_2 affinity) with declining pH (Fig. 4; additional modelled OECs are shown in Fig. S2). Opah blood pH was a significant predictor of logP_S from 40% to 95% saturation (i.e. P_{40}–P_{95}) at all temperatures ($\beta\leq-0.38$, $P\leq$0.025). From 10% to 30% saturation (i.e. P_{10}–P_{30}), blood pH was not a significant predictor of logP_S, and an interaction between pH and temperature was not a significant predictor of logP_S at any saturation. Swordfish blood pH was a significant predictor of logP_S at all Hb–O_2 saturation levels and at both temperatures ($\beta\leq-1.11$, $P\leq$0.036), but blood–O_2 affinity was more sensitive to pH changes at 10°C, as is evident by larger Bohr coefficients at 10°C than at 25°C (Table 2 and Fig. 3C). An interaction between pH and temperature was a significant predictor of logP_S for swordfish logP_{50}–logP_{90} ($\beta\geq$0.74, $P\leq$0.049). The pH dependency of the effect of temperature on whole blood-O_2 affinity is evident in plots of $\Delta H'_{WB}$ (Fig. 5).

Opah n_H values ranged from 1.49 to 3.00 at 10°C, from 1.51 to 2.97 at 15°C, and from 1.69 to 2.49 at 20°C (Fig. 3B), and swordfish n_H values ranged from 1.06 to 2.11 at 10°C, and from 0.93 to 1.46 at 25°C (Fig. 3D). Model-predicted n_H values are presented in Table 2. Blood pH was not a significant predictor of n_H for either species, temperature was also not an important predictor of opah n_H values, but temperature was an important predictor of swordfish n_H values ($\beta=-0.44$, $t_{13.47}=-4.21$, P=0.0009), with higher values at 10°C than at 25°C.

The effects of closed-system temperature changes on blood P_{O_2} are shown in Fig. 6, with modelled temperature-induced changes in plasma P_{O_2} predicted by Henry's law (i.e. increasing temperature will increase P_{O_2} in a closed-system as a result of a reduction in plasma O_2 solubility and vice versa) using O_2 solubilities at different temperatures from Boutilier et al. (1984). Closed-system warming of opah blood generally decreased blood P_{O_2}, presumably as a result of increased Hb–O_2 affinity with increasing temperature (i.e. a reverse temperature dependence). Closed-system cooling of opah blood tended to increase blood P_{O_2}, but this effect was more variable. In contrast, closed-system temperature changes of swordfish blood changed blood P_{O_2} beyond the predicted temperature-induced change in plasma P_{O_2}.

Haemolysate experiments

Haemolysate experiments were conducted on samples from three opah, six swordfish and the two bluefin tuna. Stripped haemolysate P_{50} values at pH 7.4 (an approximation of RBC intracellular pH; Weber et al., 2010), Bohr coefficients and $\Delta H'$ values are summarized in Table 3. At 10°C, stripped haemolysate P_{50} of swordfish (1.8 mmHg) was lower than that of opah (5.9 mmHg), and at 25°C, swordfish P_{50} (4.5 mmHg) was lower than that of bluefin tuna (13.7 mmHg). The addition of ATP increased haemolysate P_{50} (i.e. decreased Hb–O_2 affinity) for both opah and swordfish (Fig. 7 and Table 3). The relationships between haemolysate pH and logP_{50}, with associated n_H values, are presented in Fig. 7.

Opah stripped haemolysates in the absence of ATP showed a significant effect of pH on logP_{50} ($\beta=-0.527$, P=0.005), and P_{50}

Table 2. Whole-blood oxygen equilibria parameters for smalleye Pacific opah and swordfish at different temperatures

	Opah (4)			Swordfish (5)	
	10°C (*n*=8)	15°C (*n*=8)	20°C (*n*=8)	10°C (*n*=10)	25°C (*n*=10)
logP_{50} at pH 7.7	1.25±0.11	1.25±0.11	1.25±0.12	1.08±0.14	1.29±0.12
P_{50} (mmHg) at pH 7.7	17.9	17.7	17.9	12.1	19.6
n_H at pH 7.7	2.03±0.15	2.14±0.15	2.26±0.15	1.67±0.14	1.20±0.10
Bohr coefficient	−0.49±0.28	−0.46±0.32	−0.64±0.30	−1.29±0.50	−0.65±0.60

logP_{50} and n_H values are reported ±bootstrap estimated s.e., and Bohr coefficients ($\Delta\log P_{50}/\Delta$pH) are reported with 95% confidence intervals. Numbers in parentheses beside each species name indicate the number of individuals sampled, and the sample sizes beside each temperature indicate the number of oxygen equilibrium curves (OECs) generated for each temperature treatment (i.e. two per individual).

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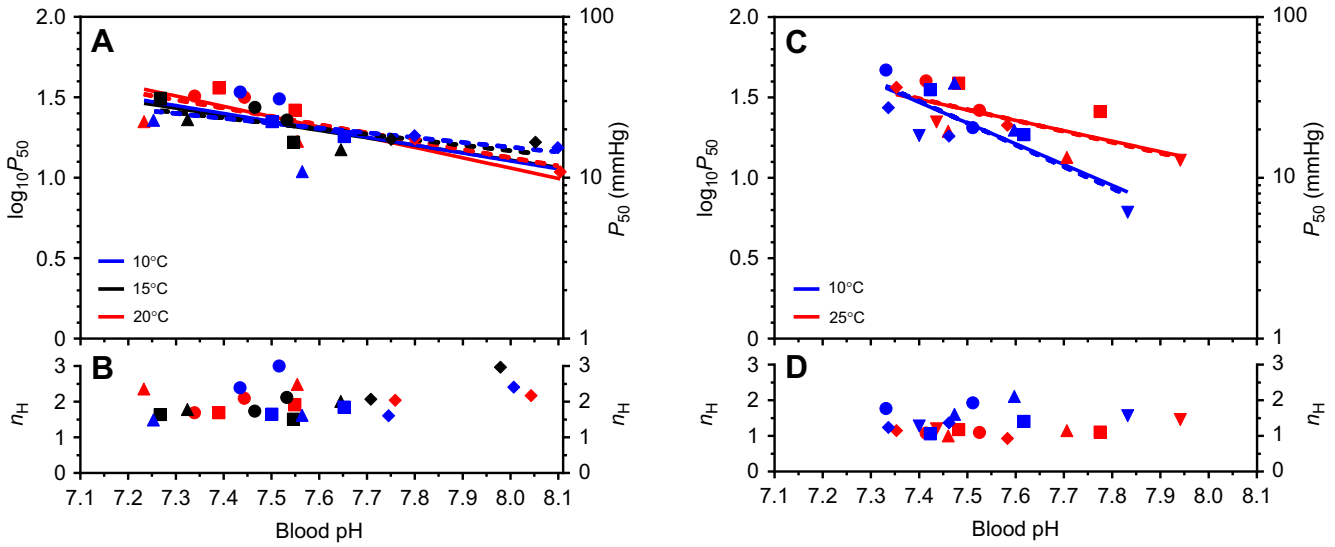

Fig. 3. Dependence of blood oxygen affinity (P_{50}) and the Hill coefficient (n_H) on whole-blood pH for smalleye Pacific opah and swordfish. Opah (A,B) and swordfish (C,D) P_{50} (mmHg; A,C) and n_H (B,D) values correspond to the OECs shown in Figs 1 and 2, with different symbols indicating different individuals (see legends of Figs 1 and 2). Opah data are at 10, 15 and 20°C, and swordfish data are at 10 and 25°C. Solid lines are the best-fit lines from mixed models at each temperature, and dashed lines are the best-fit lines from ordinary least squares regression (presented for comparison).

increased with increasing temperature ($\Delta H'$=−15 kJ mol^{-1}), but logP_{50} values at pH 7.4 were not significantly different between 10 and 25°C (overlapping 95% confidence intervals; Table 3) and temperature was not a significant predictor of logP_{50} (β=0.083, P=0.211). In the presence of ATP, temperature was a significant predictor of logP_{50} (β=−0.506, P<<0.001), but opah haemolysate P_{50} exhibited a reverse temperature dependence ($\Delta H'$=+84 kJ mol^{-1}) with significantly lower haemolysate P_{50} (pH 7.4) values at 25°C than at 10°C (Table 3 and Fig. 7A). Opah Bohr coefficients were increased with the addition of ATP (Table 3). For opah stripped haemolysates, temperature and pH were not significant predictors of n_H, both in the absence and presence of ATP.

Swordfish stripped haemolysates in the absence of ATP showed no significant effect of pH on logP_{50} (β=−0.381, P=0.700), as indicated by Bohr coefficients that were not significantly different from zero (Table 3). However, temperature was a significant predictor of logP_{50} (β=0.389, P<<0.001), with haemolysate P_{50} increasing with increasing temperature ($\Delta H'$=−42 kJ mol^{-1}). In the presence of ATP, pH was a significant predictor of swordfish logP_{50} (β=−1.180, P<<0.001), but temperature was not (β=0.073, P=0.061). The addition of ATP to swordfish stripped haemolysates significantly increased the Bohr coefficients at both 10 and 25°C (−1.32 at 10°C, and −1.06 at 25°C), and the effect of temperature on swordfish P_{50} was reduced ($\Delta H'$=−9 kJ mol^{-1}) compared with when ATP was absent ($\Delta H'$=−42 kJ mol^{-1}; Table 3). For swordfish stripped haemolysates, pH was not a significant predictor of n_H, but temperature was a significant predictor of n_H, in both the absence (β=−0.804, P=0.012) and presence of ATP (β=0.339, P<<0.001).

For bluefin tuna stripped haemolysate (no ATP), pH was a significant predictor of logP_{50} (β=−0.670, P<<0.001), as was temperature (β=−0.169, P=0.004). However, tuna haemolysate P_{50} exhibited a reverse temperature dependence ($\Delta H'$=+29 kJ mol^{-1}; Fig. 7E), with significantly lower P_{50} (pH 7.4) values at 25°C than at 15°C (Table 3). Temperature was not a significant predictor of tuna stripped haemolysate n_H values, and although pH did not influence n_H at 15°C, pH was a significant predictor of n_H at 25°C (β=0.989, P=0.039).

DISCUSSION

The purpose of this study was to investigate the temperature dependence of Hb–O_2 affinity in two regionally heterothermic teleosts, the smalleye Pacific opah and the swordfish. As reductions in the temperature sensitivity of Hb seem to be associated with regional heterothermy in vertebrates (e.g. Weber and Campbell, 2011), and reduced and reverse temperature effects have been reported from regionally heterothermic fishes, including istiophorid billfishes, we expected both swordfish and opah Hb to have a similar reduced temperature sensitivity. The results show temperature-independent and reverse temperature dependence of Hb–O_2 affinity in blood and stripped haemolysates of opah, and a pH- and saturation-dependent effect of temperature on Hb–O_2 affinity in blood and stripped haemolysates of swordfish (Figs 3 and 4, 5 and 7).

Opah and swordfish both have elevated Hb concentrations, suggesting high blood O_2 carrying capacity when compared with other less active teleosts (Bernal et al., 2001; Brill and Bushnell, 2006; Gallaugher and Farrell, 1998). The Hct values (mean±s.e.m.) for swordfish (46.3±4.7%), opah (59.1±2.5%) and the two bluefin tuna (54% and 58%) were higher than values reported for resting tuna (35–44%) (Brill and Bushnell, 1991, 2006; Lowe et al., 2000). These relatively high Hct values are, however, within the range of values reported for capture-stressed marlins (43–55%) and tunas (75–83%) (Dobson et al., 1986; Wells et al., 1986), and our opah Hct was similar to a published value (53.5±14.1% mean±s.d.) (Wegner et al., 2015). Capture stress can cause high Hct in fish, usually as a result of adrenergic release of RBCs stored in the spleen (i.e. splenic contraction), β-adrenergic receptor stimulated RBC swelling, or a combination of the two (e.g. Wells et al., 1986; Wendelaar Bonga, 1997). The Hb concentrations reported in this study (1.8–2.1 mmol l^{-1}; Table 1) are within the range of values reported for resting tunas (1.9–2.3 mmol l^{-1}), while MCHCs (3.7–4.4 mmol l^{-1}; Table 1) are lower than those of resting tunas (5.1–5.7 mmol l^{-1}) but are within the range of values reported for capture-stressed tunas and marlins (3.8–4.8 mmol l^{-1}) (Brill and Bushnell, 1991, 2006; Lowe et al., 2000; Wells et al., 1986). Therefore, RBC swelling in response to capture stress likely contributed to the high Hct that we measured (Wells et al., 1986).

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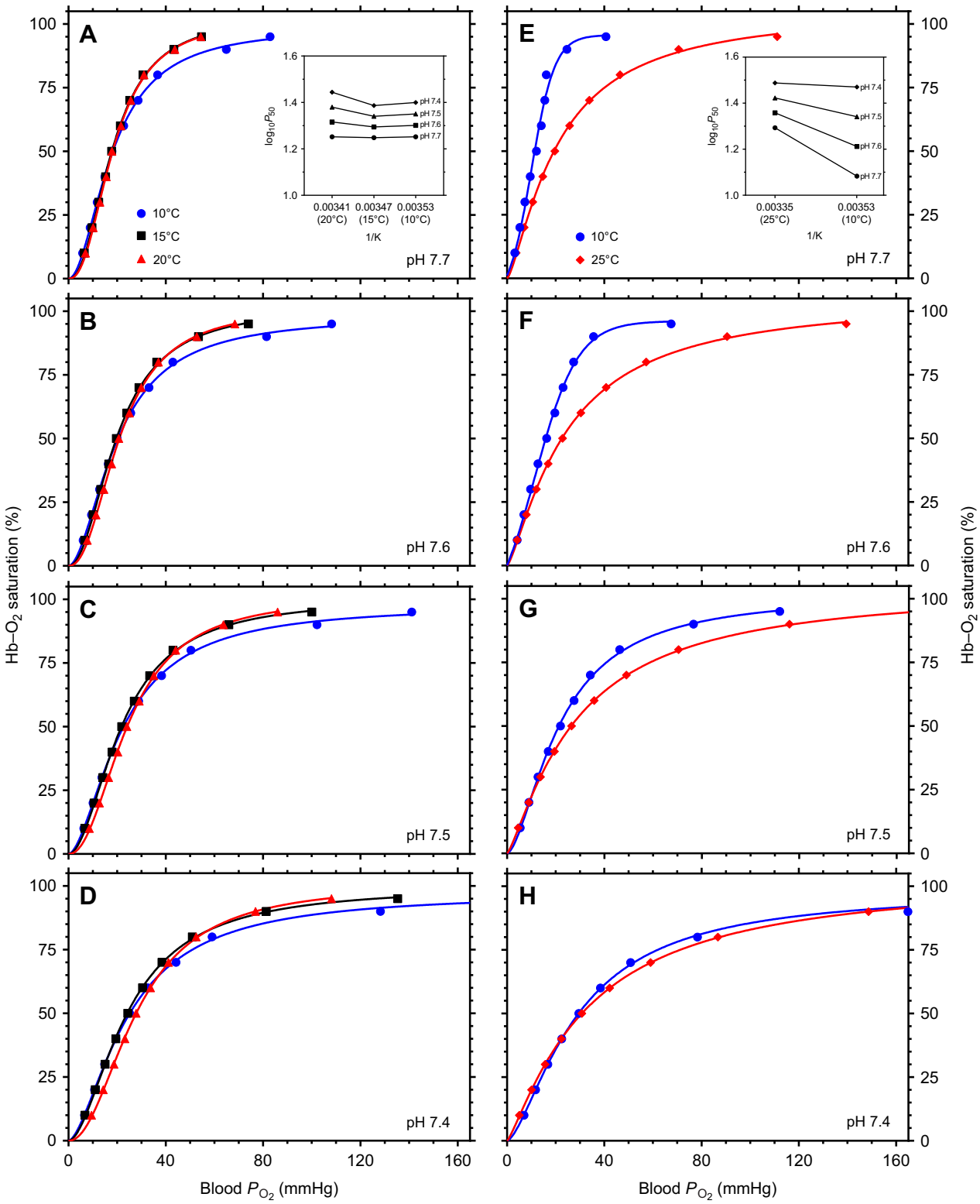

Fig. 4. Modelled whole-blood OECs of smalleye Pacific opah and swordfish at different pH and temperatures. OECs were constructed at standardized pH levels by interpolating blood P_{O_2} values from linear mixed models of logP_{O_2} versus pH for specific Hb–O_2 saturation levels at each experimental temperature. Opah OECs (A–D) were modelled at 10, 15 and 20°C. Swordfish OECs (E–H) were modelled at 10 and 25°C. OECs were constructed at four pH levels: pH 7.7 (A,E), pH 7.6 (B,F), pH 7.5 (C,G) and pH 7.4 (D,H). Insets in A and E show van't Hoff plots of logP_{50} (where P_{50} is in mmHg) versus 1/K, where K is the blood temperature in kelvin.

In this study, blood was withdrawn from swordfish and opah shortly after capture at sea, because it was unrealistic to sample resting and cannulated fish. Consequently, the fish probably experienced varying levels of respiratory and metabolic acidosis concomitant with burst swimming and capture-induced fatigue, which is indicated by relatively high plasma lactate and osmolality

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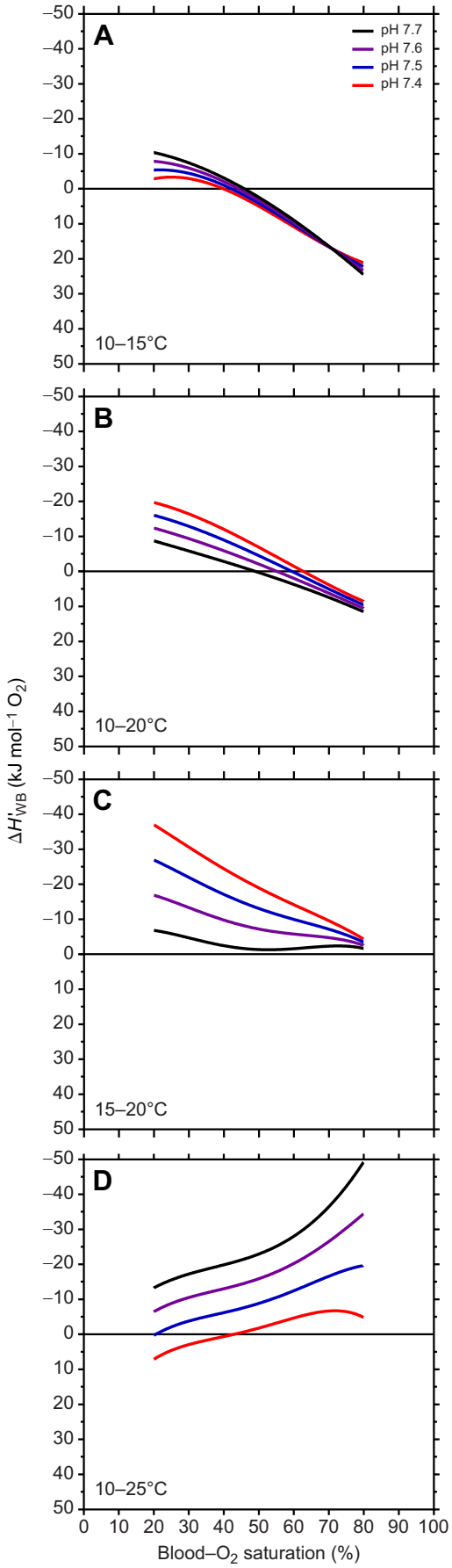

Fig. 5. Predicted enthalpy of oxygenation ($\Delta H'_{WB}$) as a function of whole-blood pH and Hb–O_2 saturation for the smalleye Pacific opah and the swordfish. $\Delta H'_{WB}$ values were calculated with the van't Hoff isochore at constant pH between 10 and 15°C (A), 10 and 20°C (B) and 15 and 20°C (C) for opah blood, and between 10 and 25°C (D) for swordfish blood. The blood O_2 tension (P_{O_2}) at specific blood O_2 saturation levels at a given pH and temperature are presented in 4.

levels (Table 1) that are comparable to values reported for capture-stressed tunas and marlins (Dobson et al., 1986; Wells et al., 1986). Even so, we were able to model OECs over a range of pH levels that are comparable to published studies on marlins and tunas, because the blood pH levels that we achieved with the CO_2 exposures (pH 7.23–8.04 for opah, and pH 7.33–7.94 for swordfish) overlapped with the range of pH levels reported in O_2 equilibria studies of blood from both capture-stressed marlins (25°C) and resting tunas (15–30°C) (Brill and Bushnell, 1991, 2006; Dobson et al., 1986; Lowe et al., 2000; Wells and Davie, 1985). However, the RBC ATP levels were low relative to the RBC Hb concentrations (Table 1), and although they are lower than ATP levels reported for most teleosts under non-stressed conditions, they are within the range of values reported for capture-stressed tunas and marlins (Filho et al., 1992; Wells and Davie, 1985; Wells et al., 1986). Given the findings of our haemolysate experiments and those of previous studies, the amount of ATP present in the RBCs will undoubtably affect Hb–O_2 affinity and its temperature dependence (e.g. Larsen et al., 2003; Nelson et al., 2019; Weber et al., 2010). Therefore, fresh blood from unstressed opah and swordfish may have higher ATP levels, potentially causing different temperature dependencies and lower O_2 binding affinities than we measured (i.e. higher P_{50} values than reported here). The effects of temperature that we observed in our experiments on whole blood were qualitatively like those for our experiments on stripped haemolysates, in that swordfish Hb showed a reduced temperature dependence that was dependent on pH in both blood and stripped haemolysates (in the presence of ATP), and the reduced and reverse temperature dependencies evident in opah blood were also observed in stripped haemolysates either in the absence of ATP (reduced temperature dependence) or in the presence of ATP (reverse temperature dependence) (Figs 3 and 7).

Temperature dependence of Hb–O_2 affinity

Opah whole blood–O_2 affinity showed both reduced and reversed temperature dependencies that were dependent on O_2 saturation, while the temperature dependence of swordfish blood–O_2 affinity was dependent on both O_2 saturation and blood pH (Figs 4 and 5). Saturation- and pH-dependent effects of temperature on Hb–O_2 affinity have also been reported in studies of blood, stripped haemolysates or isolated Hb components from some other regionally heterothermic fishes (Andersen et al., 1973; Carey and Gibson, 1977, 1983; Ikeda-Saito et al., 1983; Sharp, 1975; Weber et al., 2010). In teleosts, the saturation dependence of the effect of temperature is largely due to the non-linear release of Bohr protons, with most proton dissociation occurring between 50% and 100% Hb–O_2 saturation (Brauner et al., 1996; Ikeda-Saito et al., 1983; Lowe et al., 1998). Therefore, the results of this study exemplify the importance of evaluating how temperature influences the shape and position of the entire OEC, and that generalized conclusions can be misleading if only P_{50} values are evaluated (Sharp, 1975).

Among regionally heterothermic fishes, reductions in the thermal sensitivity of Hb–O_2 affinity have been attributed to oxygenation-linked effector dissociation that contributes endothermically to $\Delta H'$ (e.g. Ikeda-Saito et al., 1983; Larsen et al., 2003; Weber and

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Table 3. Haemolysate oxygen equilibria parameters of smalleye Pacific opah, swordfish and Atlantic bluefin tuna at two experimental temperatures and in the absence or presence of ATP

	Opah (2)		Swordfish (6)		Bluefin tuna (2)	
	10°C	20°C	10°C	25°C	15°C	25°C
No. of OECs						
Stripped Hb	6	6	25	22	7	9
Hb+ATP	6	12	16	22		
logP_{50}, pH 7.4						
Stripped Hb	0.77±0.15	0.87±0.11	0.26±0.08	0.65±0.10	1.31±0.11	1.14±0.09
Hb+ATP	2.01±0.06	1.48±0.14	1.46±0.05	1.54±0.06		
P_{50} (mmHg), pH 7.4						
Stripped Hb	5.9	7.3	1.8	4.5	20.6	13.7
Hb+ATP	102.4	30.5	29.0	34.8		
Bohr coefficients						
Stripped Hb	−0.60±0.52	−0.47±0.70	−0.05±0.28	0.00±0.41	−0.71±0.21	−0.68±0.32
Hb+ATP	−2.78±1.48	−2.10±1.65	−1.32±0.23	−1.06±0.31		
$\Delta H'$ (kJ mol^{-1}), pH 7.4						
Stripped Hb	−15		−42		+29	
Hb+ATP	+84		−9			

$\log_{10}P_{50}$ values ±bootstrap s.e. were predicted at pH 7.4, and Bohr coefficients ($\Delta\log P_{50}/\Delta$pH) are reported ±95% confidence intervals. $\Delta H'$ values include the heat O_2 solubilization (−14 kJ mol^{-1} at 15°C; Olofsson et al., 1984). Numbers in parentheses beside each species name indicate the number of individuals sampled.

Campbell, 2011; Weber et al., 2010). In stripped haemolysate from three species of istiophorid billfishes, oxygenation-linked dissociation of ATP with secondary contributions from additional Bohr proton dissociation greatly reduced or reversed the temperature dependence of Hb–O_2 affinity (Weber et al., 2010). We observed similar effects of ATP and Bohr protons on the temperature dependence of Hb–O_2 affinity in stripped haemolysates of swordfish, where the addition of ATP induced a Bohr effect and increased $\Delta H'$ (pH 7.4) from −42 to −9 kJ mol^{-1} O_2 in the absence of ATP (Fig. 7C and Table 3). In opah stripped haemolysates in the absence of ATP, temperature had a relatively reduced effect on Hb–O_2 affinity with a $\Delta H'$ of −15 kJ mol^{-1} O_2 at pH 7.4, but the

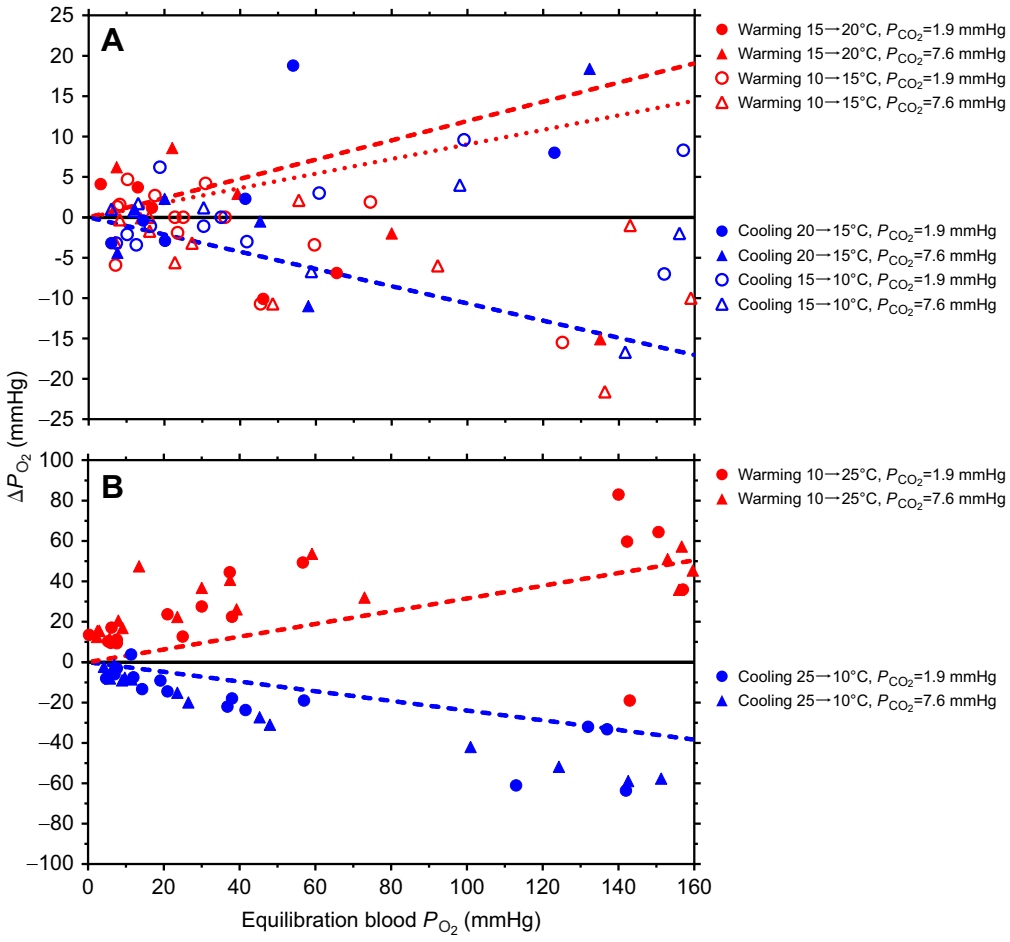

Fig. 6. Effects of closed-system temperature changes on the measured change in blood P_{O_2} (ΔP_{O_2}) of smalleye Pacific opah and swordfish. Blood from four opah (A) and five swordfish (B) was equilibrated at a range of O_2 tensions, at a P_{CO_2} of either 1.9 mmHg (circles) or 7.6 mmHg (triangles) and then warmed (red) or cooled (blue). Opah blood temperature was changed between either 10 and 15°C (open symbols) or 15 and 20°C (filled symbols), and swordfish blood was changed between 10 and 25°C. Coloured lines indicate the theoretical temperature-induced ΔP_{O_2} expected as a result of changes in the solubility of blood plasma at a given equilibration P_{O_2} (i.e. Henry's law) with warming (red) or cooling (blue) between 10 and 15°C (dashed line), and 15 and 20°C (dotted line) for opah blood, and between 10 and 25°C (dashed line) for swordfish blood. Oxygen solubilities for plasma at the different temperatures were taken from Boutilier et al. (1984). Note the different scales on the *y*-axes.

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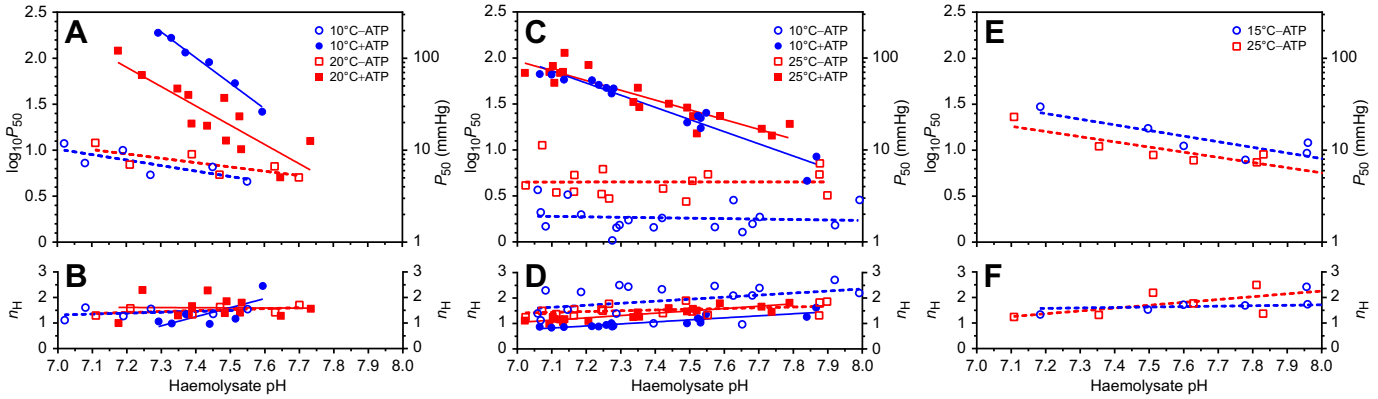

Fig. 7. Temperature and pH dependence of P_{50} and n_H values of stripped haemolysates of smalleye Pacific opah, swordfish and Atlantic bluefin tuna (*Thunnus thynnus*). Experiments were conducted in 100 mmol l^{-1} Hepes buffer at tetrameric Hb concentrations of 0.6 mmol l^{-1}, in the absence of ATP (open symbols) and presence of ATP (filled symbols; [ATP]=18 mmol l^{-1}; [ATP]/[Hb_4]=30). Low temperature data (circles) are at 10°C for opah (A,B) and swordfish (C,D), and 15°C for bluefin tuna (E,F). Warm temperature data (squares) are at 20°C for opah and 25°C for swordfish and bluefin tuna. (A,C,E) Bohr plots of $\log_{10}P_{50}$ (where P_{50} is in mmHg) and haemolysate pH. (B,D,F) Hill coefficients (n_H) plotted against haemolysate pH. Dashed lines (absence of ATP) and solid lines (presence of ATP) are the best-fit lines from ordinary least squares regression models across the range of experimental pH values.

addition of ATP caused a reverse temperature dependence, increasing $\Delta H'$ to +84 kJ mol^{-1} O_2 (Fig. 7A and Table 3). In the absence of ATP, opah stripped haemolysate–O_2 affinity may decrease with increasing temperature at high pH (extrapolated from regression lines in Fig. 7A), indicating that Bohr proton dissociation potentially contributes to the low $\Delta H'$, although other processes such as Hb conformational changes (i.e. T→R state transitions) may also have significant enthalpic contributions. We observed reverse temperature-dependent Hb–O_2 affinity in haemolysates (no ATP) of Atlantic bluefin tuna (Fig. 7E and Table 3). This finding is similar to previous work on bluefin tuna Hb showing that proton dissociation from many Bohr groups, concomitant with cooperative O_2 binding, underlies the reversed effect of temperature on Hb–O_2 affinity (Carey and Gibson, 1977; Ikeda-Saito et al., 1983; Morris and Gibson, 1982).

Closed-system temperature changes

The results of the effect of temperature on whole-blood–O_2 affinity were paralleled during closed-system temperature changes. Opah whole-blood–O_2 affinity showed evidence of a reverse temperature dependence during the closed-system experiments, in which warming tended to cause a reduction in blood P_{O_2} and cooling tended to increase blood P_{O_2} (Fig. 6A). In contrast, closed-system warming and cooling of swordfish blood caused blood P_{O_2} to increase and decrease, respectively (Fig. 6B). The changes to swordfish blood P_{O_2} were greater than predicted from the temperature dependence of the O_2 solubility of blood plasma, which indicates that closed-system warming probably caused Hb–O_2 offloading as a result of decreased Hb–O_2 affinity and, vice versa, cooling probably caused Hb–O_2 binding as a result of an increased Hb–O_2 affinity.

Early experiments on closed-system temperature changes on blood P_{O_2} from regionally heterothermic fishes tested the hypothesis that temperature-independent Hb–O_2 affinity prevents premature Hb–O_2 offloading during closed-system warming in a heat exchanging rete and the surrounding muscle (Carey and Gibson, 1977; Cech et al., 1984; Graham, 1973). It was thought that if temperature-induced Hb–O_2 offloading occurred as the blood was warmed in an artery or arteriole, then O_2 may be lost from the arterial blood, potentially by arterio-venous O_2 diffusion in a heat exchanging rete, which could reduce arterial blood O_2 levels prior to perfusing the muscle capillaries (Carey and Gibson, 1977, 1983; Graham, 1973). In addition to our results for opah, temperature-independent and reverse temperature-dependent blood–O_2 affinity during closed-system warming has been reported for several species of tuna and regionally heterothermic sharks (Bernal et al., 2018; Brill and Bushnell, 1991, 2006; Cech et al., 1984; P.R.M., D.B., C.A.S. and C.J.B., unpublished data). However, if warming of arterial blood in a heat exchanging rete causes premature Hb–O_2 offloading that is detrimental to circulatory O_2 delivery, then it would be expected that temperature-independent blood–O_2 affinity would be evident during closed-system temperature changes for all regionally heterothermic fishes. This is not the case as closed-system warming greatly increased the P_{O_2} of swordfish blood (Fig. 6B), and similar results have been reported for bigeye tuna (*Thunnus obesus*) and kawakawa (*Euthynnus affinis*) (Jones et al., 1986; Lowe et al., 2000). It seems unlikely that O_2 would be lost from the arterial vessels upstream of the capillaries, especially given the arterioles and venules of vertebrate heat-exchanging retia, as well as the diffusion distance between them, are about an order of magnitude greater than the vessels of a rete specialized for gas exchange, the swim bladder rete (Carey et al., 1985; Clark et al., 2008; Graham and Dickson, 2001; Lemons et al., 1987; Stevens et al., 1974). As the anatomy of heat-exchanging retia likely precludes significant arterio-venous O_2 diffusion, reduced and reverse temperature-dependent Hb–O_2 affinity is probably not needed to prevent premature Hb–O_2 offloading. It is probably more important that Hb–O_2 affinity is not too different in the capillaries of warm and cold tissues.

The functional significance of reduced and reversed temperature dependence of Hb–O_2 affinity

In most animals, endothermic Hb O_2 offloading in the tissues and exothermic Hb–O_2 binding at the gas-exchange surface causes the outward transport and loss of metabolic heat to the environment, which contributes to ectothermy in fishes (Jensen et al., 1998; Stevens and Sutterlin, 1976; Weber and Fago, 2004). However, in the opah, most tunas and regionally heterothermic sharks, $\Delta H'_{WB}$ values are near zero or positive (i.e. temperature-independent and reverse temperature-dependent Hb–O_2 affinity), potentially eliminating any outward heat transport linked to Hb O_2 offloading and Hb–O_2 binding at the tissues and gills, respectively, which could

potentially save up to 13% of the heat produced during glucose metabolism, assuming an intrinsic enthalpy of haem oxygenation of −62 kJ mol^{-1} and an oxycalorific equivalent for glucose metabolism of 473 kJ mol^{-1} (Weber and Wells, 1989; Weber et al., 2010). This may increase the efficiency of heat retention in the warm red muscles of the opah and species that inhabit cold temperate or polar waters (e.g. some tunas and lamnid sharks). However, it is not clear whether Hb oxygenation enthalpy significantly contributes to thermoconservation, as both swordfish and bigeye tuna $\Delta H'_{WB}$ values were negative (i.e. exothermic), yet swordfish undertake prolonged (up to 12 h) foraging excursions below the thermocline, where they remain within cold water (6–12°C) while maintaining cranial and red muscle temperatures elevated up to 10°C or more above the surrounding water (Carey, 1990; Lowe et al., 2000; Sepulveda et al., 2010; D.B. and C.A.S., unpublished data).

Reduced and reverse temperature-dependent Hb–O_2 affinity should prevent excessive temperature-induced shifts to the physiological OEC (i.e. *in vivo* Hb–O_2 affinity), and thus blood P_{O_2}, as peripheral tissue temperature changes with environmental temperature, and as blood flows from the gills to warmer tissues. In other words, Hb–O_2 affinity would not be greatly affected by temperature changes in the body or environment of a regionally heterothermic fish such as the opah. A rightward shifted OEC at colder temperatures should ensure that Hb–O_2 affinity is not high enough to impair Hb O_2 offloading to the cold peripheral tissues, or possibly even enhance O_2 offloading compared with Hb with a 'normal' temperature dependence (Clark et al., 2008; Giardina et al., 1989; Weber and Campbell, 2011). If the physiological OEC is not too different in the capillaries of the warm and cold tissues, then metabolically produced CO_2 and protons will predominantly promote Hb–O_2 offloading and maintain matching between O_2 supply and O_2 demand to all the tissues and organs even though they vary in temperature.

Swordfish Hb, however, has a 'normal' temperature dependence at high O_2 saturations and high blood pH, but at low pH, Hb–O_2 affinity becomes temperature independent. The possible benefits of temperature-independent Hb–O_2 affinity in billfishes have been generally attributed to a relatively left-shifted OEC at high temperatures, potentially preventing large decreases to Hb–O_2 affinity as the blood is rapidly warmed (Weber et al., 2010). However, rather than preventing a right-shifted OEC in warm tissues (i.e. decreased Hb–O_2 affinity), the pH dependency of the effect of temperature on swordfish and billfish Hb may ensure a right-shifted OEC (i.e. decreased Hb–O_2 affinity), in cold tissues, which combined with a larger Bohr coefficient at low temperatures (Table 2), may promote Hb O_2 offloading in the capillaries of cold tissues and organs with a high metabolic demand (Clark et al., 2008). This may have the greatest physiological relevance to swordfish, in which organs such as the heart operate at cold ambient water temperatures (6–12°C) during foraging excursions below the thermocline, but the cardiac and swimming muscles of swordfish exhibit relatively greater tolerance to functioning in cold water, potentially elevating the O_2 demand of these muscles compared with those of other teleosts at cold temperatures (Galli et al., 2009; Stoehr et al., 2018, 2020).

Reductions in the thermal sensitivity of Hb may also be influenced by the species' thermal behaviour as reduced temperature-dependent Hb–O_2 affinity is not a trait that is exclusive to regional heterotherms, and has been reported for several ectothermic elasmobranchs and teleosts (e.g. Barlow et al., 2017; Bernal et al., 2018; Cech et al., 1994; Clark et al., 2010; Hopkins and Cech, 1994; Weber et al., 1976). Temperature-independent Hb–O_2 affinity was originally proposed to potentially enable O_2 uptake as Atlantic bluefin tuna rapidly swim through waters of varying temperatures (Rossi Fanelli and Antonini, 1960). Ectothermic fishes could plausibly gain the same advantage from Hb with a reduced thermal sensitivity; however, it is not clear whether temperature-independent Hb–O_2 affinity in ectothermic fishes is a consequence of effector binding that was selected for reasons unrelated to the temperature sensitivity of Hb.

Hb–O_2 affinity and low environmental oxygen

Opah and swordfish whole blood P_{50} values at pH 7.7 and 10°C were around 18 and 12 mmHg, respectively (Table 2), suggestive of relatively high blood–O_2 affinity in these species compared with other marine teleosts, including some tunas (e.g. Brill and Bushnell, 1991; Brill and Bushnell, 2006; Clark et al., 2008; Harter et al., 2022; Lowe et al., 2000). Low blood P_{50} values in this study were possibly linked to the low RBC ATP levels that may have been caused by capture stress and the blood storage duration (1–4 days), although the stripped haemolysate experiments revealed high intrinsic Hb–O_2 affinity in both opah and swordfish (Table 3), with P_{50} values (pH 7.4) for swordfish (1.8 and 4.5 mmHg at 10 and 25°C, respectively) being lower than those of opah (5.9 and 7.3 mmHg at 10 and 20°C, respectively) and other istiophorid billfishes (3.5–4.0 mmHg at 10°C, and 10.5–14.8 mmHg at 25°C; Weber et al., 2010). Low swordfish P_{50} values were also reported by Andersen et al. (1973), around 4 and 12 mmHg at 5 and 25°C, respectively, although they did not report the pH and it is unclear whether their experiments were conducted on blood or haemolysates. Furthermore, in two capture-stressed istiophorid billfishes, blue marlin (*Makaira nigricans*) and striped marlin (*Kajikia audax*), blood P_{50} values at pH 7.6 and 25°C were around 13 and 16 mmHg, respectively, and the striped marlin ATP:Hb ratio was around 0.22, lower than the mean for the swordfish in this study (Dobson et al., 1986; Wells and Davie, 1985). Although a high blood–O_2 affinity in the swordfish may be due to the relatively low RBC ATP levels, we suspect that the high intrinsic Hb–O_2 affinity of the swordfish causes a high whole-blood–O_2 affinity that might help swordfish to exploit relatively deep and potentially low oxygenated waters (Carey and Robinson, 1981; Dewar et al., 2011; Sepulveda et al., 2010).

Swordfish diurnal movements follow the vertical migrations of the organisms that form the deep scattering layer, thus exposing swordfish to depths (during the daytime) that are typically proximal to the upper reaches of the oxygen minimum layer (e.g. Sepulveda et al., 2010). Consequently, swordfish likely encounter low environmental oxygen levels during the day. In addition to a potentially high blood–O_2 affinity, swordfish also have a relatively large gill surface area that is comparable to tunas, but above that of istiophorid billfishes as well as most other marine teleosts (Wegner et al., 2010). A large gill surface area combined with a high blood-O_2 affinity should enable adequate O_2 extraction from oxygen poor water and allow swordfish to exploit the upper reaches of the oxygen minimum layer during the day.

Summary of findings

The results presented here show temperature-independent and reversed temperature-dependent Hb–O_2 affinity in blood and haemolysates of opah, and temperature-independence at low pH in blood and haemolysates of swordfish. In regionally heterothermic fishes such as opah and swordfish, an increased $\Delta H'$ might conserve heat-energy and possibly prevents Hb–O_2 affinity from being too different from the cold to the warm tissues and organs. The latter effect should promote O_2 unloading uniformly to all tissues despite differences in tissue temperature. Whole blood-O_2 affinities were

relatively low for both opah and swordfish, which may have resulted from RBC ATP/Hb ratios that were relatively low for teleosts. RBC ATP/Hb ratios were, however, similar to those of capture-stressed tunas and marlins, and swordfish stripped haemolysates had high intrinsic O_2 affinities. A potentially high blood-O_2 affinity in the swordfish, may enable oxygen uptake from the water of the upper reaches of the oxygen minimum layer where swordfish tend to forage during daylight hours. Among regionally heterothermic teleosts, the relative contributions of the different effector ions that underlie the molecular mechanism of modulations to the enthalpy of Hb–O_2 binding differ among the opah, billfishes, and tunas, which all differ from lamnid sharks (Larsen et al., 2003; Weber and Campbell, 2011). It does not seem to be a coincidence that Hbs with reduced temperature sensitivity are present in all lineages of regionally heterothermic fishes investigated to date, yet this trait is shared with some ectothermic fishes (e.g. Clark et al., 2010; Nelson et al., 2019). Further studies on the temperature-dependence of Hb–O_2 affinity in regionally heterothermic fishes, including other opah and billfish species, and closely related ectothermic species are warranted to provide further insight into the evolution and functional significance of reduced and reverse temperature-dependent Hb–O_2 affinity.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: P.R.M., D.B., C.A.S., N.C.W., C.J.B.; Methodology: P.R.M., C.J.B.; Formal analysis: P.R.M.; Investigation: P.R.M., D.B., C.A.S., N.C.W.; Resources: D.B., C.A.S., N.C.W., C.J.B.; Writing - original draft: P.R.M.; Writing - review & editing: P.R.M., D.B., C.A.S., N.C.W., C.J.B.; Supervision: C.J.B.; Funding acquisition: D.B., C.A.S., C.J.B.

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