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RESEARCH ARTICLE

The effect of temperature on haemoglobin–oxygen binding affinity in regionally endothermic and ectothermic sharks

Phillip R. Morrison[1,*,‡], Diego Bernal[2], Chugey A. Sepulveda[3] and Colin J. Brauner[1]

ABSTRACT

Haemoglobin (Hb)–O_2 binding affinity typically decreases with increasing temperature, but several species of ectothermic and regionally endothermic fishes exhibit reduced Hb thermal sensitivity. Regionally endothermic sharks, including the common thresher shark (*Alopias vulpinus*) and lamnid sharks such as the shortfin mako shark (*Isurus oxyrinchus*), can maintain select tissues and organs warmer than ambient temperature by retaining metabolic heat with vascular heat exchangers. In the ectothermic bigeye thresher shark (*Alopias superciliosus*), diurnal movements above and below the thermocline subject the tissues, including the blood, to a wide range of operating temperatures. Therefore, blood–O_2 transport must occur across internal temperature gradients in regionally endothermic species, and over the range of environmental temperatures encountered by the ectothermic bigeye thresher shark. While previous studies have shown temperature-independent Hb–O_2 affinity in lamnid sharks, including shortfin mako, the Hb–O_2 affinity of the common and bigeye thresher sharks is unknown. Therefore, we examined the effect of temperature on whole-blood Hb–O_2 affinity in common thresher shark and bigeye thresher shark. For comparison, analyses were also conducted on the shortfin mako shark and two ectothermic species, blue shark (*Prionace glauca*) and spiny dogfish (*Squalus acanthias*). Blood–O_2 binding affinity was temperature independent for common thresher shark and shortfin mako shark, which should prevent internal temperature gradients from negatively affecting blood–O_2 transport. Blue shark and spiny dogfish blood–O_2 affinity decreased with increasing temperature, as expected, but bigeye thresher shark blood exhibited both a reduced temperature dependence and a high Hb–O_2 affinity, which likely prevents large changes in environment temperature and low environmental oxygen from affecting O_2 uptake.

KEY WORDS: Hb thermal sensitivity, Blood-O_2 transport, Lamnidae, Alopiidae, Blood, Regional endothermy

INTRODUCTION

Like many highly migratory marine species, sharks in the families Lamnidae (i.e. mackerel sharks) and Alopiidae (i.e. thresher sharks) encounter a wide range of environmental temperatures during vertical movements through the water column or over long latitudinal migrations (e.g. Bernal et al., 2009; Sepulveda et al., 2019; Weng et al., 2005). For example, shortfin mako sharks (*Isurus oxyrinchus*) and common thresher sharks (*Alopias vulpinus*) periodically descend below the thermocline into water that may be more than 10°C cooler than surface waters (Cartamil et al., 2011, 2016; Holts and Bedford, 1993; Sepulveda et al., 2004). Similarly, bigeye thresher sharks (*Alopias superciliosus*) also experience particularly large and rapid changes in environmental temperature as they spend most of the day in deep cold water (<10°C) proximal to the upper reaches of the oxygen minimum layer, but during the night they ascend into the warmer mixed layer (>20°C) (Coelho et al., 2015; Nakano et al., 2003; Sepulveda et al., 2019; Weng and Block, 2004). During these diurnal movements above and below the thermocline, bigeye thresher shark body temperature closely tracks environmental temperature, which subjects the muscles and organs, including the blood, to a wide range of operating temperatures (e.g. 6–25°C; Aalbers et al., 2021; Sepulveda et al., 2019). Because the body temperature of most fishes mirrors that of the surrounding water temperature (Carey et al., 1971), and temperature affects the O_2 affinity of blood (reviewed by Weber and Campbell, 2011), moving between disparate thermal environments causes temporal changes in body temperature that have important implications for the transport of O_2 from the water to the tissues (e.g. Carey, 1982).

Among jawed vertebrates, haemoglobin (Hb)–O_2 binding affinity generally decreases with increasing blood temperature because O_2 binding to the haem groups is exothermic (Barcroft and Hill, 1910). This effect of temperature on Hb–O_2 affinity is indicated by overall values for the enthalpy change of oxygenation that are negative (i.e. exothermic) (reviewed by Weber and Campbell, 2011). However, Atlantic bluefin tuna (*Thunnus thynnus*) Hb–O_2 binding affinity was shown to be insensitive to temperature (Rossi-Fanelli and Antonini, 1960), even exhibiting a reverse temperature dependence (i.e. $\Delta H'$ is positive and Hb–O_2 affinity increases with increasing temperature) (Carey and Gibson, 1977; but also see Brill and Bushnell, 2006), and reduced and reversed temperature dependence has been reported in several other tuna (Scombridae) (Brill and Bushnell, 1991; Cech et al., 1984; Clark et al., 2008; Lilly et al., 2015; Sharp, 1975). Lamnid sharks also have Hbs with reduced or reversed temperature dependence (Andersen et al., 1973) and, like tunas, they have evolved the ability to maintain distinct regions of their bodies warmer than the surrounding water (regional endothermy) (Bernal et al., 2001; Carey and Teal, 1966, 1969; Carey et al., 1971, 1985). The common thresher shark is also regionally endothermic (Bernal and Sepulveda, 2005), and although the thermal sensitivity of common thresher shark Hb is unknown, reduced and reverse temperature-dependent Hb–O_2 affinity in tunas and lamnid sharks has been linked to the oxygenation-dependent dissociation of allosteric effectors (e.g. protons and organic phosphates), which contribute endothermically to $\Delta H'$ (Ikeda-Saito et al., 1983; Larsen et al., 2003; Weber and Campbell, 2011).

[1]Department of Zoology, University of British Columbia, Vancouver, BC, Canada, V6T 1Z4. [2]Department of Biology, University of Massachusetts, Dartmouth, MA 02747, USA. [3]Pfleger Institute of Environmental Research, Oceanside, CA 92054, USA.
*Present address: Biology Department and Department of Resource Management and Protection, Vancouver Island University, Nanaimo, BC, Canada, V9R 5S5.

‡Author for correspondence (phillip.r.morrison@gmail.com)

P.R.M., 0000-0001-9470-4540; D.B., 0000-0002-4192-9559; C.A.S., 0000-0002-2987-7880; C.J.B., 0000-0002-3695-7707

Received 7 September 2022; Accepted 20 December 2022

Journal of Experimental Biology (2023) 226, jeb244979. doi:10.1242/jeb.244979

Regionally endothermic sharks can retain the metabolic heat produced by their red, slow-twitch, swimming muscles (RM) with vascular heat exchangers (i.e. *retia mirabilia)*, which enable RM temperatures to be elevated by 4–10°C above ambient temperature in the shortfin mako shark and the common thresher shark (Bernal and Sepulveda, 2005; Carey and Teal, 1969; Carey et al., 1985; Patterson et al., 2011). In contrast, ectothermic fishes thermoconform with their environment, in large part as a result of the convective transfer of metabolic heat in the blood to the gill circulation where heat is lost to the inspired water (Stevens and Sutterlin, 1976). Like tunas, the RM of regionally endothermic sharks is deeply set in the body compared with the superficial position of the RM in most other fishes (Bernal et al., 2001; Carey and Teal, 1966, 1985; Sepulveda et al., 2005). The bulk of the arterial blood flow entering the systemic circulation is directed laterally through subcutaneous arteries and veins, which redirects the blood supply to the medially situated RM through heat exchanging retia, where the cold arterial blood is warmed before it perfuses the RM (Carey and Teal, 1969; Carey et al., 1985; Patterson et al., 2011). The retia enable heat transfer to the arterial blood from the warmer venous blood exiting the metabolically active (i.e. contracting) RM, efficiently conserving heat and cooling the outflowing venous blood near to ambient temperature before it reaches the gills. Consequently, blood flowing through a heat exchanging rete is subjected to what has been described as 'closed-system' warming and cooling, as the change in blood temperature will directly affect the partial pressures of oxygen (P_{O_2}) and carbon dioxide (P_{CO_2}), but the content of blood gases will remain relatively constant as the rete vessels (i.e. arterioles and venules) should preclude diffusion of gases out of the blood (Brill and Bushnell, 1991; Cech et al., 1984; Stevens et al., 1974). Accordingly, it has also been proposed that temperature-independent Hb–O_2 affinity may avert premature Hb–O_2 unloading as the blood is warmed in transit from the gills to the warmer tissues of regionally endothermic fishes (Carey and Gibson, 1977; Graham, 1973).

While there are three extant species of thresher shark – the common thresher shark, the bigeye thresher shark and the pelagic thresher shark (*Alopias pelagicus*) – only the common thresher shark is a known regional endotherm (Bernal and Sepulveda, 2005; Carey et al., 1971; Patterson et al., 2011). As in other ectothermic sharks, the RM is positioned superficially in the bigeye and pelagic thresher sharks, and RM temperature does not differ from that of the surrounding water (Patterson et al., 2011; Sepulveda et al., 2005). While it has been speculated that the bigeye thresher shark has warm eyes (cranial endothermy) because the orbital circulation is enlarged, suggestive of cranial endothermy (Block and Carey, 1985; Weng and Block, 2004), preliminary data on cranial temperature measurements obtained for bigeye thresher sharks suggest that the temperature of these tissues does not differ significantly from that of the white muscle (D.B. and C.A.S., unpublished). However, bigeye thresher sharks do exhibit extreme temperature tolerance, transitioning between warm surface waters (~18–25°C) and colder water below the thermocline (<10°C), and remain in these disparate thermal environments for extended periods (10–12 h) (Aalbers et al., 2021; Nakano et al., 2003; Sepulveda et al., 2019; Weng and Block, 2004). Thus, Hb–O_2 transport must occur over the range of environmental temperatures encountered by the ectothermic bigeye thresher shark, and these daily temperature changes may potentially impair O_2 transport unless the temperature dependence of Hb–O_2 affinity is reduced, which we propose here.

We are not aware of any published studies on Hb–O_2 binding affinity in any thresher shark species, so this was the drive behind our own investigation. Previous studies have shown reduced Hb thermal sensitivity in at least three lamnid sharks (regional endotherms): the shortfin mako shark, the porbeagle shark (*Lamna nasus*) and the salmon shark (*Lamna ditropis*) (Andersen et al., 1973; Bernal et al., 2018; Dickinson and Gibson, 1981). We therefore hypothesized analogous reduced Hb thermal sensitivity in common thresher shark. Furthermore, as the bigeye thresher shark remains poorly studied and exhibits extreme fluctuations in ambient daily temperatures, we also hypothesized that bigeye thresher shark Hb has a reduced temperature dependence. Bigeye thresher sharks spend most of the day proximal to the upper reaches of the oxygen minimum layer (Aalbers et al., 2021; Sepulveda et al., 2019), so we also hypothesized that this species has a high Hb–O_2 affinity to extract oxygen from the relatively hypoxic water. We also conducted experiments on blood from shortfin mako sharks to corroborate and increase the sample size from Bernal et al. (2018). Finally, to corroborate our findings with a comparative approach, analyses were also conducted on blood from two previously studied ectothermic sharks, the blue shark (*Prionace glauca*) and the spiny dogfish (*Squalus acanthias*) (Bernal et al., 2018; Wells and Weber, 1983). The blue shark is pelagic and occupies an ecological niche that overlaps with those of the shortfin mako, common thresher and bigeye thresher sharks (i.e. sympatric), while the spiny dogfish is generally a temperate-coastal species but sometimes encounters variable depths (generally <600 m) and water temperatures (~5–15°C) (Bernal et al., 2009; Sulikowski et al., 2010). We assessed the temperature sensitivity of whole blood from these sharks by constructing oxygen equilibrium curves (OECs; the relationship between P_{O_2} and Hb–O_2 saturation) and quantifying P_{50} (the P_{O_2} at 50% Hb–O_2 saturation) at different temperatures, as well as by measuring the P_{O_2} while the blood was warmed or cooled in an experimental closed-system meant to mimic the temperature changes that the blood experiences in the arterioles and venules of a heat exchanging rete.

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 pressure and P_{50} values are reported in mmHg (1 mmHg=0.133 kPa).

Blood collection

Bigeye thresher sharks, *Alopias superciliosus* Lowe 1841 (n=9), were captured by deep-set buoy gear (Sepulveda et al., 2014, 2019) in the coastal waters off Southern California (i.e. the Southern California Bight). Shortfin mako, *Isurus oxyrinchus* Rafinesque 1810 (n=7), and blue sharks, *Prionace glauca* (Linnaeus 1758) (n=5), were captured by hook and line off the coast of Southern California, and common thresher sharks, *Alopias vulpinus* (Bonnaterre 1788) (n=2) and spiny dogfish, *Squalus acanthias* Linnaeus 1758 (n=8), were captured by hook and line off the coast of Massachusetts (see Table 1 for fork lengths). Individuals were either restrained alongside the research vessel or quickly brought aboard and restrained. Blood was then withdrawn by caudal puncture using heparinized syringes, a method previously used for obtaining shark blood for oxygen equilibria experiments (Bernal et al., 2018; Brill et al., 2008; Cooper and Morris, 1998). All sharks were released after sampling. Blood samples were kept on ice and

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Table 1. Fork length and blood variables for shortfin mako sharks, common thresher sharks, bigeye thresher sharks, blue sharks and spiny dogfish

	Shortfin mako shark	Common thresher shark	Bigeye thresher shark	Blue shark	Spiny dogfish
Fork length (cm)	118±7 (7)	135, 175	166±4 (8)	93±15 (6)	52±3 (8)
Haematocrit (%)	34.1±2.0 (7)	29.7, 36.8	25.5±1.0 (9)	19.1±1.6 (6)	17.9±1.5 (8)
Haemoglobin (mmol l^{-1})	1.60±0.12 (7)	1.25, 1.65	1.13±0.04 (9)	0.69±0.04 (6)	0.63±0.06 (8)
MCHC (mmol l^{-1})	4.68±0.24 (7)	4.20, 4.49	4.45±0.08 (9)	3.64±0.16 (6)	3.52±0.08 (8)
Plasma osmolality (mOsm kg^{-1})	959±6 (7)	1004	931±27 (9)	914±32 (6)	937
Plasma lactate (mmol l^{-1})	8.8±4.2 (4)	7.14	3.4±0.35 (7)	7.2±2.0 (4)	1.4±0.2 (6)
Blood pH (range)	15°C: 7.73 (7.35–8.13) 25°C: 7.60 (7.34–8.04)	15°C: 7.30, 7.38 22°C: 7.36, 7.27	10°C: 7.51 (7.42–7.70) 25°C: 7.54 (7.43–7.70)	15°C: 7.70 (7.52–7.86) 25°C: 7.54 (7.27–7.69)	15°C: 7.72 (7.64–7.88) 25°C: 7.69 (7.59–7.83)

MCHC, mean corpuscular haemoglobin concentration. Values are means±s.e.m. with samples sizes in parentheses. Blood pH was measured in blood equilibrated to 0.25% CO_2 and saturating levels of O_2, and is reported as the mean with the range of pH values in parentheses. If values were measured in only one or two individuals, then the individual measurements are reported.

overnight shipped by courier to the University of British Columbia (UBC), Vancouver, Canada, where the blood was stored at 4°C. All experiments on whole blood were conducted within 1–4 days post-capture. This sampling technique has been successfully employed for previous studies on both teleosts and sharks (Bouyoucos et al., 2020; Clark et al., 2010; Morrison et al., 2022; Polinski et al., 2021; Zhang et al., 2019). However, during this time, red blood cell (RBC) intracellular nucleoside triphosphate (NTP) levels likely decreased relative to *in vivo* levels, possibly causing higher blood–O_2 affinity with altered temperature sensitivity versus that in freshly sampled blood. Although we were not able to construct OECs on freshly drawn blood, we have shown that swordfish (*Xiphias gladius*) blood P_{50} was relatively stable from 4 to 8 days post-collection (Morrison et al., 2022). In another previous study conducted at the UBC Vancouver campus using blood collected from chub mackerel (*Scomber japonicus*) off the coast of Southern California, it was concluded that blood was viable for up to 6 days when stored at 4°C (Clark et al., 2010). Possible shortfalls of this study are further discussed below.

Haematological parameters

Immediately after blood samples arrived at UBC, Hb concentration and haematocrit (Hct) were measured, and subsamples of blood were centrifuged to separate the plasma from the RBCs for measurement of plasma osmolality. The remaining plasma was frozen at −80°C for determination of plasma lactate concentration. Hb 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 (e.g. 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 as [Hb_4]/(Hct/100). Plasma osmolality (mOsm kg^{-1}) was measured in 10 µl of undiluted plasma with a vapour pressure osmometer (VAPRO 5520, Wescor, Logan, UT, USA). 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}

Whole-blood OECs were constructed by quantifying the relative Hb–O_2 saturation over a range of equilibration P_{O_2} values at two physiologically relevant carbon dioxide levels, low (1.9 mmHg/0.25%) and high (3.8 mmHg/0.50% for spiny dogfish and 7.6 mmHg/1.00% for all others), and at two temperatures, 10 and 25°C for bigeye thresher shark, 15 and 22°C for common thresher shark, and 15 and 25°C for shortfin mako shark, blue shark and spiny dogfish. The colder experimental temperatures (10 or 15°C) are within the range of the colder water temperatures regularly encountered by these species (Carey et al., 1990; Cartamil et al., 2016; Sepulveda et al., 2004, 2019; Sulikowski et al., 2010). The warmest experimental temperatures corresponded to the warmest water temperatures encountered by bigeye thresher sharks and blue sharks (25°C), and the warmest RM temperatures in the mako (25°C) and the common thresher sharks (22°C) (Bernal and Sepulveda, 2005; Carey et al., 1990; Carey and Teal, 1969; Patterson et al., 2011; Sepulveda et al., 2019). Spiny dogfish rarely encounter water as warm as 25°C, but constructing OECs at 25°C allowed direct comparison with the other species included in this study.

The relationship between Hb–O_2 saturation and P_{O_2} (i.e. an OEC) was assessed on two to three replicate samples using a custom microplate-based, parallel assay, multi-cuvette tonometry cell as described by Lilly et al. (2013), and following the procedure outlined in Morrison et al. (2022). Cuvettes were formed by sandwiching blood samples (~3–5 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). Experimental temperatures were achieved by placing the microplate reader in a temperature-controlled environmental chamber. 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 nm and 436 nm (near the peak absorption for deoxygenated Hb), wavelengths commonly used in thin-film optical methods for measuring Hb–O_2 saturation (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. 21% O_2, an approximation of the P_{O_2} in dry atmospheric air at sea level). Full Hb–O_2 saturation was assumed after a final increment to a P_{O_2} of 228 mmHg in the absence of CO_2. Dry gas mixtures of O_2, CO_2 and N_2 (>99.5% pure medical gases; Linde Canada Inc.) were obtained using a DIGAMIX® gas mixing pump (H. Wösthoff Messtechnik, Bochum, Germany), and gases were not humidified as the blood samples were sealed in plastic wrap. OEC experiments

lasted between 1 and 2 h, and in preliminary experiments no significant methaemoglobin (MetHb) formation occurred during the experiments. MetHb was qualitatively assessed by recording absorption spectra following the final oxygenation step, and the spectra showed no unusually high absorbance at 630 nm, an absorption maximum for metHb (e.g. Völkel and Berenbrink, 2000; Zijlstra and Buursma, 1997). Therefore, we conclude that metHb formation did not significantly affect our Hb–O_2 saturation measurements in the remainder of the experiments. At each equilibration step, the difference in OD (ΔOD) between 390 nm and 430 nm or between 390 nm and 436 nm (ΔOD=OD 430 nm−OD 390 nm or OD 436 nm−OD 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 OD at 430 nm were identical to those constructed using OD at 436 nm.

Blood pH was measured in approximately 500 µl subsamples of blood equilibrated for 1 h with either the low or high CO_2 tension, and a range of O_2 tensions between 7.6 and 159.6 mmHg (balanced with N_2) in rotating glass tonometers thermostatically set to 10, 15, 22 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 pH electrode (16-705 flow-thru pH electrode, Microelectrodes Inc., Bedford, NH, USA) in combination with a 16-702 flow-thru reference electrode (Microelectrodes Inc.) thermostatically set to the experimental temperature.

Closed-system temperature changes

To mimic the closed-system temperature changes that blood experiences in the arterioles and venules of a heat exchanging rete, approximately 500 µl blood samples equilibrated at 10, 15, 22 or 25°C were injected into a pH electrode (as described above) and a Radiometer E5046 P_{O_2} electrode thermostatically set to the equilibration temperature as well as another pair of electrodes thermostatically set to a warmer or cooler experimental temperature according to Cech et al. (1984), Brill and Bushnell (1991) and Bernal et al. (2018). Bigeye thresher shark blood temperature was changed between 10 and 25°C, common thresher shark blood temperature was changed between 10 and 22°C, and shortfin mako shark, blue shark and spiny dogfish blood temperature was changed between 15 and 25°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 blood injection, 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 pH and P_{O_2} were monitored using Acqknowledge® Data Acquisition Software (version 3.7.3, BIOPAC Systems, Inc.) by viewing traces of pH and P_{O_2}, and when it appeared that the traces had stabilized, the respective values of each were recorded.

Data analysis

[Hb], Hct and MCHC were compared among species by one-way ANOVA, and differences among the species means were assessed by Tukey's multiple comparison test using GraphPad Prism version 6.01 (GraphPad Software, La Jolla, CA, USA). All other statistical analyses, curve fitting and linear mixed model fitting were performed in R v 4.1.3 (http://www.R-project.org/).

An OEC was constructed for each paired dataset of fractional Hb–O_2 saturation (response variable) and blood P_{O_2} (explanatory variable), by fitting 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 where $y=d/2$, and b is the slope of the steepest part of the curve (i.e. the Hill coefficient, n_H). 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). The best-fit parameter values (a, b and d) were used to calculate the P_{O_2} values corresponding to specific % Hb–O_2 saturation (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}). The effects of pH and temperature on blood–O_2 affinity and n_H values were assessed with linear mixed models, where the response variable was either $\log_{10}P_S$ (e.g. $\log_{10}P_{50}$) or n_H, and the explanatory variables were pH (continuous), assay temperature (as a factor), the interaction term between pH and assay temperature, and individual (i.d.) as a random effect [R-language formula, 'log10(P_S)~pH*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 mixed model fits (i.e. Bohr plots) were used to predict P_S values with bootstrap estimated standard errors (500 replications), and these were used to construct OECs at constant pH (i.e. isohydric OECs) for each species' temperature treatment. The temperature dependence of blood–O_2 affinity was quantified by calculating $\Delta H'$ values using the van't Hoff equation (Wyman, 1964):

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

where R is the gas constant (0.008314 kJ K^{-1} mol^{-1}) and T is the absolute temperature (Kelvin). $\Delta H'$ values determined from whole-blood experiments may not accurately quantify the heat of Hb-oxygenation in whole blood because the enthalpic contribution of other reactions to $\Delta H'$ was unknown, and the concentrations of Hb allosteric effectors were not known or controlled. The heat of solution of O_2 is included in $\Delta H'$ values, and values of $\Delta H'$ were calculated with P_S values that were determined at constant extracellular plasma pH, which is usually alkaline relative to RBC intracellular pH. The pH dependency of Hb–O_2 affinity was determined by calculating Bohr coefficients at different %Hb–O_2 saturations (P_S):

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

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

RESULTS

Blood parameters and species lengths are summarized in Table 1. Mean [Hb] and Hct values for shortfin mako sharks were significantly greater than those of bigeye thresher sharks, and while [Hb] and Hct were not different between blue sharks and spiny dogfish, they were lower than those of shortfin mako sharks and bigeye thresher sharks (Hb: $F_{3,26}$=40.60, P<0.0001; Hct: $F_{3,26}$=26.74, P<0.0001). MCHC was not different between shortfin makos and bigeye threshers, which both had higher MCHC than blue sharks and spiny dogfish ($F_{3,26}$=16.30, P<0.0001). Common thresher shark haematological values were not included in the analyses as only two individuals were sampled, but [Hb], Hct and MCHC for the two common thresher sharks were within the range of values measured in shortfin makos (Table 1).

Whole-blood experiments

Whole-blood OECs were successfully constructed for seven shortfin mako sharks, two common thresher sharks, five bigeye thresher sharks, four blue sharks and seven spiny dogfish. Measured whole-blood OECs are shown in Fig. 1, the effect of temperature and pH, and blood–O_2 affinity (P_{20}, P_{50} and P_{80}) are shown in Bohr plots in Fig. 2, and modelled OECs at different temperatures and pH are shown in Fig. 3. The range of blood pH levels measured in the whole-blood OEC experiments is reported in Table 2 for each species, along with P_{50} values, n_H values and Bohr coefficients. Blood P_{50} values for the shortfin mako shark, the bigeye thresher shark and the blue shark were predicted at pH 7.7 and pH 7.5, which are approximated arterial pH values for the blue shark and the mako shark, respectively (Lai et al., 1997). Spiny dogfish P_{50} values are reported at an arterial pH of 7.85 (Swenson and Maren, 1987; Wells and Weber, 1983), as well as pH 7.7 for comparison among species. Common thresher shark P_{50} values are reported at pH 7.3 because of the relatively acidotic state of the blood from the two individuals that were sampled (Fig. 3C and Table 2). Blood pH and P_{CO_2} had little influence on P_{50} for all species, as indicated by low Bohr coefficients and 95% CIs that included zero (Table 2).

Temperature was not a significant predictor of blood P_{O_2} at any saturation level in blood of both shortfin mako shark and common thresher shark. In bigeye thresher shark blood, temperature was not a significant predictor of blood P_{O_2} at or below 60% saturation, but from 70% to 95% saturation, temperature was an important model factor (χ^2=8.052–26.321, d.f.=2, P≤0.018), where increasing temperature decreased blood–O_2 affinity. Blood–O_2 affinity was significantly decreased by increasing temperature for both blue shark and spiny dogfish, and temperature was an important predictor of blood P_{O_2} at all saturations (blue shark: χ^2=8.481–41.751, d.f.=2, P≤0.014; spiny dogfish: χ^2=39.488–54.670, d.f.=2, P<0.001).

Hill coefficients were not significantly influenced by blood pH or temperature for any species except the bigeye thresher shark (Fig. 2). Temperature was an important predictor of bigeye thresher n_H values (χ^2=19.847, d.f.=2, P=0.000049), with lower values at 25°C than at 10°C, and pH was an important predictor of bigeye thresher n_H values at 10°C (β=1.034, P=0.017) but not at 25°C. Shortfin mako shark n_H values ranged from 1.25 to 2.91 at 15°C and 1.44 to 2.20 at 25°C, common thresher shark n_H values ranged from 1.12 to 1.63 at 15°C and 1.10 to 1.73 at 22°C, bigeye thresher shark n_H values ranged from 1.52 to 2.28 at 10°C and 1.07 to 1.77 at 25°C, spiny dogfish n_H values ranged from 0.91 to 1.17 at 15°C and 1.03 to 1.67 at 25°C, and blue shark n_H values ranged from 1.27 to 1.47 at 10°C, 1.13 to 1.79 at 15°C, and 0.89 to 1.39 at 25°C (Fig. 2).

The effects of closed-system temperature changes on blood P_{O_2} are shown in Fig. 4, with predicted temperature-induced changes in plasma P_{O_2} following Henry's law (i.e. the temperature dependence of plasma O_2 solubility will cause P_{O_2} in a closed system to increase or decrease by increasing or decreasing temperature, respectively) using O_2 solubilities from Boutilier et al. (1984). Closed-system warming and cooling of shortfin mako and common thresher shark blood generally increased and decreased blood P_{O_2}, respectively, but the change in P_{O_2} (ΔP_{O_2}) was less than the predicted change. Closed-system warming and cooling of blood from bigeye thresher sharks, blue sharks and spiny dogfish changed P_{O_2} beyond that predicted by the change in solubility of O_2, presumably as a result of temperature induced Hb–O_2 offloading and binding with warming and cooling, respectively. The greatest ΔP_{O_2} occurred in bigeye thresher shark blood (Fig. 4D).

DISCUSSION

Our primary objective was to compare the effect of temperature on whole-blood Hb–O_2 binding affinity in two closely related sharks that experience either frequent and large temporal changes in body temperature (bigeye thresher shark) or are capable of regional endothermy (common thresher shark). We also measured the effect of temperature on blood–O_2 affinity in shortfin mako shark, blue shark and spiny dogfish using comparable methods to those used for thresher sharks. This study tested the hypothesis that blood–O_2 affinity in both the common thresher shark and the bigeye thresher shark is less affected by temperature than in most ectothermic species, similar to the blood and Hbs of regionally endothermic lamnid sharks (i.e. the shortfin mako shark, the porbeagle and the salmon shark) (Andersen et al., 1973; Bernal et al., 2018; Dickinson and Gibson, 1981; Larsen et al., 2003). Additionally, this study also tested the hypothesis that the bigeye thresher shark has a relatively high blood–O_2 affinity as this species encounters low environmental oxygen levels, daily. We observed that whole-blood Hb–O_2 affinity was independent of temperature for the regionally endothermic common thresher shark (Figs 2C and 3B) and shortfin mako shark (Figs 2A and 3A), whereas bigeye thresher shark blood exhibited a temperature dependence that was dependent on Hb–O_2 saturation (Figs 2G and 3D). Bigeye thresher shark blood also had a high O_2 affinity with a P_{50} around 8 mmHg at 10 and 25°C, which is less than half the P_{50} for the common thresher shark, blue shark and spiny dogfish at 25°C (Table 2).

Justification of blood sampling methodology

To obtain blood samples for this study, blood was taken from sharks shortly after capture at sea, as it is impracticable and unsafe to obtain blood samples from resting and cannulated large sharks held in laboratory aquaria. The individuals included in this study had likely experienced varying levels of fatigue and consequent respiratory and metabolic acidosis. This was indicated by relatively high plasma lactate levels (Table 1; mean±s.e.m.) in shortfin mako sharks (8.8±4.2 mmol l^{-1}), common thresher sharks (7.1 mmol l^{-1}) and blue sharks (7.2±2.0 mmol l^{-1}); however, these mean values are lower than mean lactate levels reported from capture-stressed shortfin mako sharks (13–16 mmol l^{-1}) and blue sharks (9 mmol l^{-1}) (Wells and Davie, 1985; Wells et al., 1986). Spiny dogfish plasma lactate levels (1.4±0.2 mmol l^{-1}) were similar to resting levels of 1 mmol l^{-1} (Richards et al., 2003), while bigeye thresher shark plasma lactate levels (3.4±0.35 mmol l^{-1}) were similar to levels measured in capture-stressed sandbar sharks (*Carcharhinus plumbeus*; ∼4 mmol l^{-1}; Brill et al., 2008), and were intermediate between those for spiny dogfish and those for shortfin

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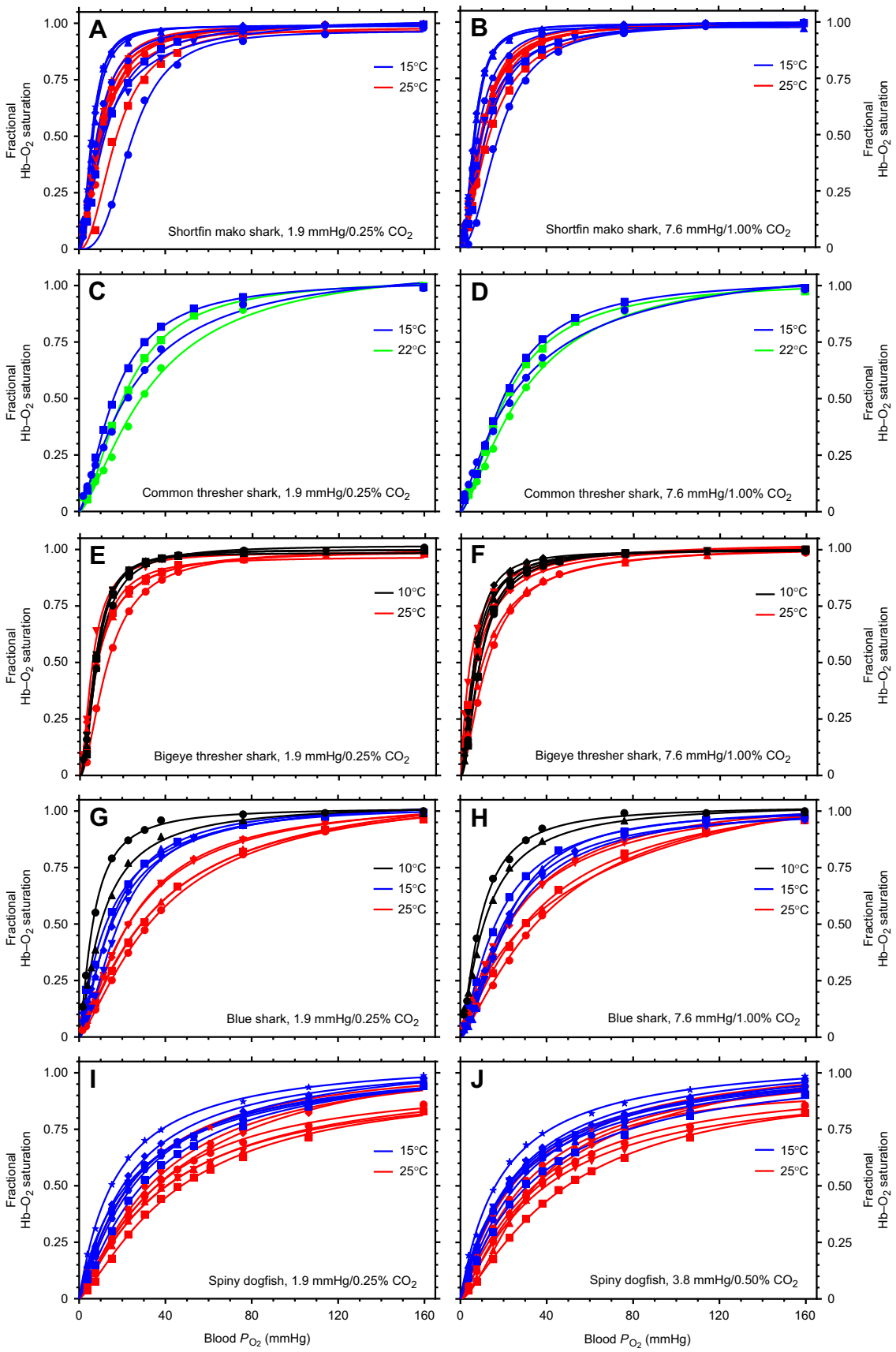

Fig. 1. See next page for legend.

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Fig. 1. Whole-blood oxygen equilibrium curves (OECs) of shortfin mako shark (*Isurus oxyrinchus*), common thresher shark (*Alopias vulpinus*), bigeye thresher shark (*Alopias superciliosus*), blue shark (*Prionace glauca*) and spiny dogfish (*Squalus acanthias*). Data are for individual sharks, as follows (symbol shape, fork length). (A,B) Shortfin mako sharks: circles, 125 cm; squares, 141 cm; upward triangles, 100 cm; downward triangles, 140 cm; diamonds, 100 cm; hexagons, 105 cm; stars, 114 cm. (C,D) Common thresher sharks: circles, 135 cm; squares, 175 cm. (E,F) Bigeye thresher sharks: circles, 172 cm; squares, no length; upward triangles, 159 cm; downward triangles, 160 cm; diamonds, 152 cm. (G,H) Blue shark: circles, 60 cm; squares, 120 cm; upward triangles, 65 cm; downward triangles, 95 cm; diamonds, 150 cm. (I,J) Spiny dogfish: circles, 48 cm; squares, 50 cm; upward triangles, 55 cm; downward triangles, 50 cm; diamonds, 50 cm; hexagons, 65 cm; stars, 40 cm. OECs were constructed at a low P_{CO_2} of 1.9 mmHg/0.25% CO_2 (A,C,E,G,I) and a high P_{CO_2} of 7.6 mmHg/1.00% CO_2 (B,D,F,H) or 3.8 mmHg/0.50% CO_2 (J). Symbols indicate measured values, and curves were generated by fitting the Hill equation to the data (see Materials and Methods) at 10°C (black), 15°C (blue), 22°C (green) or 25°C (red).

mako, common thresher and blue sharks. Except for the two common thresher sharks, the blood pH levels that we achieved with the CO_2 exposures (Tables 1 and 2) were within the range of arterial and venous blood pH levels measured in resting or slowly swimming sharks (~pH 7.9–7.3; reviewed by Morrison et al., 2015), which allowed us to construct OECs within a physiologically relevant pH range (pH 7.7–7.3; Fig. 3). Although pH was low in common thresher shark blood, the blood showed no signs of RBC lysis and Hct was close to previously published values (Emery, 1986; Filho et al., 1992a). Hct, [Hb] and MCHC values for the other species included in this study (Table 1) were also within the range of previously published values for shortfin mako, blue shark and spiny dogfish (reviewed by Morrison et al., 2015).

In this study, we did not measure the RBC intracellular concentrations of NTPs such as adenosine triphosphate (ATP) and guanosine triphosphate (GTP), which can affect Hb–O_2 affinity and its dependence on temperature and pH (Larsen et al., 2003; Morrison et al., 2015). Strenuous swimming associated with capture stress can cause RBC NTP levels to decrease (Brill et al., 2008), and NTP levels can also decline over time after blood withdrawal, which both will cause the ratio of RBC NTP concentration to MCHC (NTP/Hb) to decline and Hb–O_2 affinity to potentially increase (i.e. lower P_{50} at lower NTP levels). Therefore, RBC NTP levels may be higher in fresh blood from resting sharks than in blood withdrawn from capture-stressed sharks and stored in a refrigerator for several days, potentially causing lower binding affinities with different temperature dependencies from those that we measured. However, NTP/Hb ratios for sharks are generally lower and relatively stable compared with those of teleosts, and mean NTP/Hb ratios of capture-stressed shortfin mako (0.3–0.7) and blue sharks (0.5) were within the range of ratios reported for unstressed elasmobranchs (0.3–1.6) (Filho et al., 1992b; Morrison et al., 2015; Wells and Davie, 1985; Wells et al., 1986).

Lastly, although only two common thresher sharks were included in this study, the results of the whole-blood experiments were consistent between the two individuals. Furthermore, our results on the effect of temperature on shortfin mako shark blood–O_2 affinity (n=7) are consistent with the limited sample size (n=3) of Bernal et al. (2018). This evidence is suggestive that the common thresher shark data are representative of the species.

Temperature dependence of Hb–O_2 affinity

Temperature had little to no effect on blood P_{50} of the two regionally endothermic species, shortfin mako shark and common thresher shark, and the blood P_{50} of the ectothermic bigeye thresher shark was also not affected by temperature. In contrast, increasing temperature increased P_{50} (i.e. decreased blood–O_2 affinity) in blue shark and spiny dogfish blood (Table 2), and as expected for ectotherms, the temperature dependence of blood–O_2 affinity was consistent across most of the OEC (i.e. at all saturation levels; Fig. 3). However, temperature did not uniformly affect the OEC of the bigeye thresher shark, which is also an ectotherm. At low Hb–O_2 saturation, bigeye thresher shark blood–O_2 affinity was independent of temperature, but above 60% saturation, O_2 affinity decreased with increasing temperature (Fig. 3D).

Temperature-dependent binding of allosteric effectors such as ATP and H^+ ions (Bohr protons) has been proposed to underlie reduced and reverse temperature-dependent Hb–O_2 affinity among regionally endothermic fishes (Ikeda-Saito et al., 1983; Larsen et al., 2003; Morrison et al., 2022; Weber and Campbell, 2011; Weber et al., 2010). The major Hb components from the porbeagle have high intrinsic O_2 affinities with a normal temperature dependence, but the addition of ATP to stripped Hb reduces O_2 affinity and reverses the effect of temperature, as a result of oxygenation-linked dissociation of ATP and protons that contribute endothermically to $\Delta H'$, causing it to become positive (Larsen et al., 2003). It is not yet clear what underlies temperature-independent Hb–O_2 affinity in other lamnid sharks and the thresher sharks, but the molecular and structural underpinnings of reduced thermally sensitive Hbs in lamnid and alopiid is an area worthy of additional research.

Closed-system temperature changes

Temperature-independent Hb–O_2 affinity in blood from the shortfin mako shark and the common thresher shark was reflected in closed-system changes to blood temperature, when blood P_{O_2} changed less than would be expected due to the temperature dependence of plasma O_2 solubility (Fig. 4A,B). Similar results have been previously reported for the shortfin mako shark, and tunas with Hbs that exhibit temperature-independent or reverse temperature-dependent O_2 affinity (Bernal et al., 2018; Brill and Bushnell, 1991, 2006; Cech et al., 1984). In comparison, closed-system temperature changes caused large changes to blood P_{O_2} for the ectothermic species included in this study – bigeye thresher shark, blue shark and spiny dogfish (Fig. 4C–E) – presumably indicating that warming and cooling of blood caused Hb–O_2 unloading and binding, respectively. In bigeye thresher shark blood, the magnitude of temperature-induced changes to blood P_{O_2} exceeded those measured in blue shark and spiny dogfish blood. Most of the equilibration O_2 tensions used in this study greatly exceeded the bigeye thresher shark P_{50} of 8 mmHg, so the closed-system temperature changes likely occurred at high Hb–O_2 saturation levels where temperature has the greatest influence on the bigeye thresher shark OEC (Fig. 3D), probably causing excessive Hb–O_2 dissociation and binding as the blood is warmed and cooled, respectively.

Blood–O_2 carrying capacity, blood–O_2 affinity and low environmental O_2

Shortfin mako shark and common thresher shark Hct and [Hb] are higher than typical values for ectothermic sharks, indicating a higher blood–O_2 carrying capacity that is consistent with previous studies of sharks capable of red muscle endothermy (Bernal et al., 2001; Emery, 1986). The Hct and [Hb] for bigeye thresher shark are similar to those of hammerhead sharks

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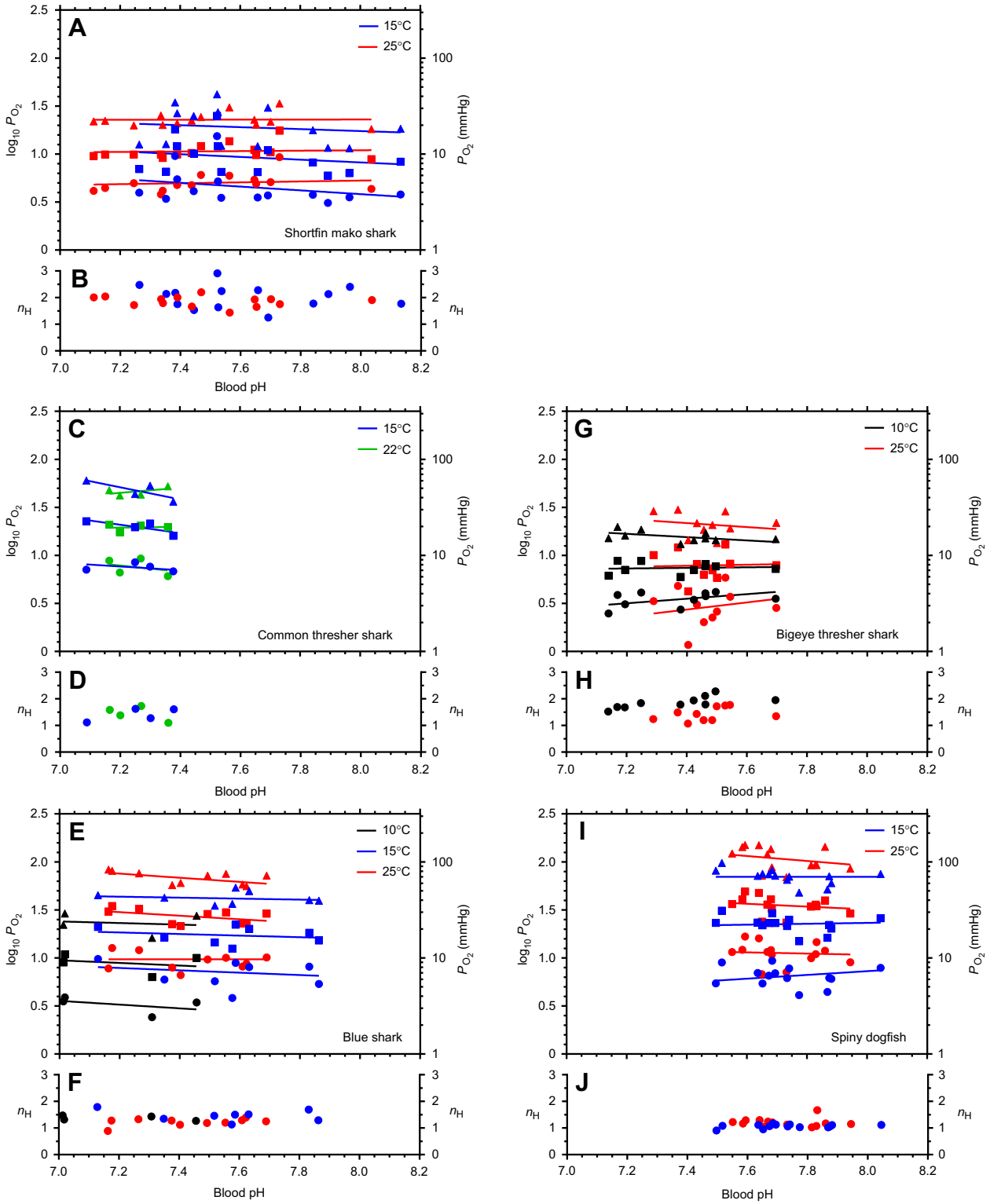

Fig. 2. Dependence of blood-oxygen affinity (P_{20}, P_{50} and P_{80}) and the Hill coefficient (n_H) on blood pH at different temperatures in shortfin mako shark, common thresher shark, blue shark, bigeye thresher shark and spiny dogfish. P_{O_2} values (A,C,E,G,I) correspond to P_{20} (circles), P_{50} (squares) and P_{80} (triangles) values that were interpolated from the fitted curves shown in Fig. 1, and n_H values (B,D,F,H,J) are the slopes of the curves (i.e. the parameter *b* in the Hill equation; see Materials and Methods). Data are shown at 10°C (black), 15°C (blue), 22°C (green) or 25°C (red). Lines are the best-fit lines from mixed models at each temperature.

in the genus *Sphyrna*, which are intermediate between typical values for other ectothermic sharks and those for regionally endothermic sharks (reviewed by Morrison et al., 2015). This may indicate that bigeye thresher sharks as well as some other large pelagic sharks have higher oxygen demands (i.e. higher active metabolic rates) than most other ectothermic sharks, so they potentially have relatively higher blood-O_2 carrying capacities to match.

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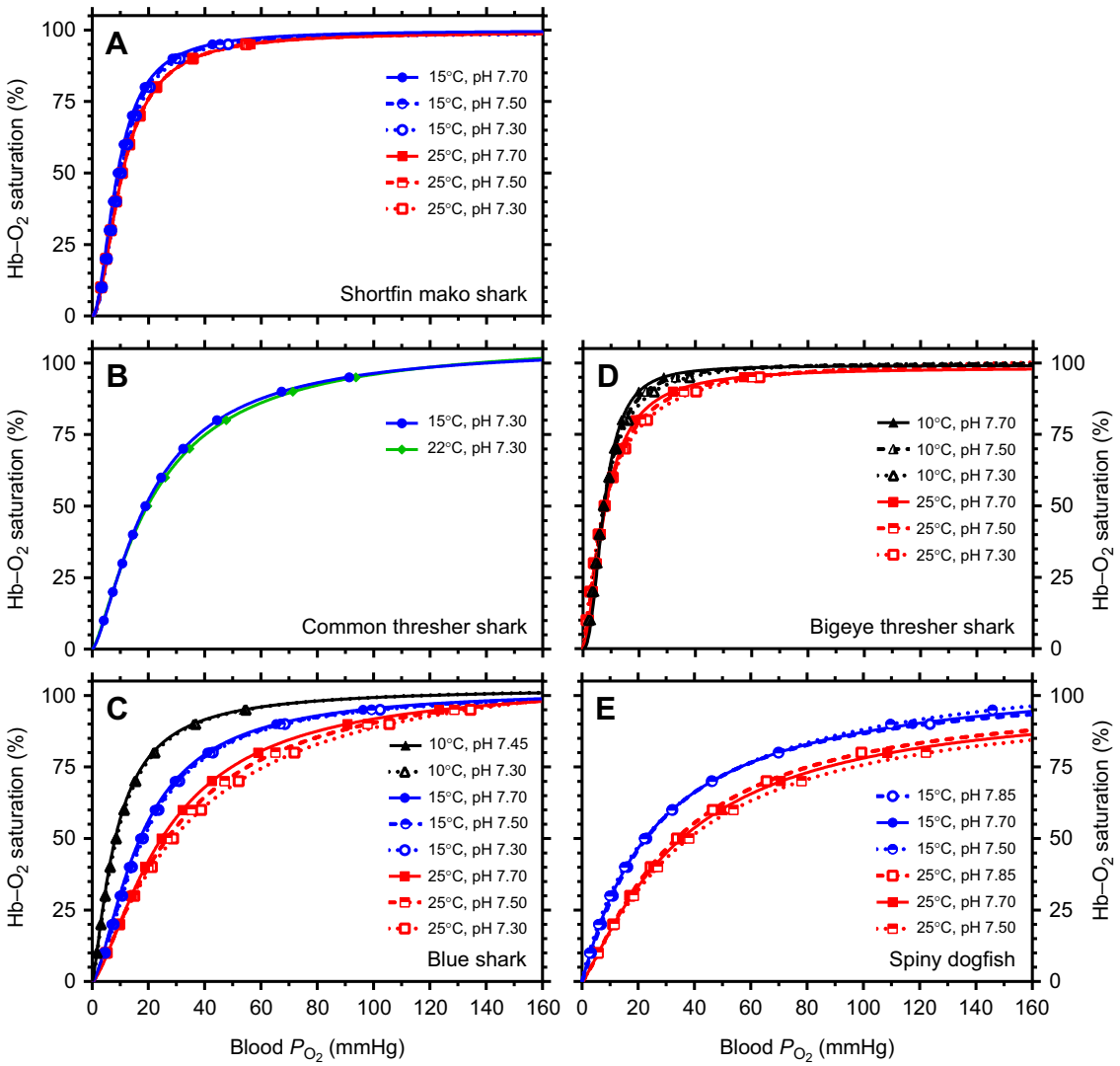

Fig. 3. Modelled whole-blood OECs of shortfin mako shark, common thresher shark, blue shark, bigeye thresher shark and spiny dogfish at different pH and temperature. OECs were constructed at standardized pH levels by interpolating blood P_{O_2} values from linear mixed models of logP_{O_2} versus pH for specific Hb–O_2 saturation levels at each experimental temperature (shown in Fig. 2 for P_{20}, P_{50} and P_{80}). OECs were modelled at 10°C (black curves and triangles), 15°C (blue curves and circles), 22°C (green curves and diamonds) or 25°C (red curves and squares). Species names, temperatures and pH levels are given in each panel.

This study's whole-blood–O_2 equilibria results for shortfin mako shark and blue shark blood are qualitatively like those reported by Bernal et al. (2018). As in this study, Bernal et al. (2018) also observed a reduced effect of temperature on shortfin mako shark blood–O_2 affinity, and a normal decrease in blood–O_2 affinity with increasing temperature in blue shark blood. However, their shortfin mako shark P_{50} values (14.5–22.3 mmHg) and Bohr coefficients (ϕ=−0.11 to −0.74) are larger than those reported here (Table 3). These discrepancies may be due to potentially low RBC NTP levels in this study compared with the relatively fresher blood samples used by Bernal et al. (2018), although the lower P_{50} value (10.6 mmHg at pH 7.6) and small Bohr coefficient (ϕ=0.16) reported by Wells and Davie (1985) for shortfin mako shark blood at 25°C (Table 3) are almost identical to those of this study at the same temperature and pH (P_{50}=10.8 mmHg and φ=0.02; Table 2). Blue shark P_{50} values at 25°C are about 3–5 mmHg lower in this study than in that of Bernal et al. (2018) (Tables 2 and 3), which may be due to potentially lower RBC NTP levels in this study, but at 15°C P_{50} values were similar between the studies, and the slightly larger Bohr coefficients that Bernal et al. (2018) reported at both 15 and 25°C (Table 3) fall within the 95% CIs for the Bohr coefficients reported here (Table 2). The spiny dogfish P_{50} value reported by Wells and Weber (1983) at 15°C and pH 7.85 (17.9 mmHg) is slightly lower than the value that we determined (22.8 mmHg), and they reported a Bohr coefficient of −0.28 (Table 3), whereas we observed no significant effect of pH on P_{50} at 15°C (Table 2).

The bigeye thresher shark whole-blood P_{50} values are among the lowest reported for any elasmobranch, and they are similar to those of some hypoxia-tolerant freshwater teleosts (Harter et al., 2022; Morrison et al., 2015). Although it is possible that the low P_{50} values may be partly due to low RBC NTP levels associated with capture stress and blood storage duration (as discussed above), we suspect that such a low P_{50} that is temperature independent likely benefits O_2 uptake over the range of environmental conditions that bigeye thresher sharks encounter, as a consequence of the vastly different daytime and nocturnal distributions. Bigeye thresher

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Table 2. Whole-blood oxygen equilibria parameters of sharks at different temperatures

Species	T (°C)	n	pH	P_{50} (mmHg)	logP_{50}	n_H	Bohr coefficient	pH range
Shortfin mako shark (7)	15	14	7.70	9.0	0.96±0.05	2.01±0.11	−0.14±0.24	7.264–8.134
			7.50	9.7	0.98±0.05	2.07±0.11		
	25	14	7.70	10.8	1.03±0.06	1.91±0.13	0.02±0.33	7.112–8.037
			7.50	10.7	1.03±0.05	1.86±0.10		
Common thresher shark (2)	15	4	7.30	18.9	1.28±0.02	1.43±0.19	−0.43±0.60	7.089–7.378
	22	4	7.30	19.6	1.29±0.03	1.40±0.19	0.03±1.04	7.165–7.360
Bigeye thresher shark (5)	10	10	7.70	7.6	0.88±0.07	2.20±0.15	0.03±0.32	7.139–7.695
			7.50	7.5	0.87±0.05	2.00±0.09		
	25	10	7.70	8.1	0.91±0.08	1.67±0.16	0.05±0.58	7.289–7.697
			7.50	7.9	0.90±0.05	1.45±0.08		
Blue shark (5)	10	4	7.45	8.2	0.91±0.07	1.30±0.13	−0.14±0.40	7.013–7.456
			7.30	8.6	0.93±0.05	1.30±0.11		
	15	8	7.70	16.8	1.22±0.04	1.44±0.09	−0.08±0.46	7.128–7.863
			7.50	17.4	1.24±0.03	1.53±0.08		
	25	10	7.70	24.2	1.38±0.05	1.40±0.11	−0.19±0.48	7.163–7.689
			7.50	26.4	1.42±0.03	1.27±0.08		
Spiny dogfish (7)	15	14	7.85	22.8	1.36±0.04	1.09±0.04	0.05±0.14	7.497–8.045
			7.70	22.4	1.35±0.03	1.06±0.04		
	25	14	7.85	33.7	1.52±0.04	1.20±0.05	−0.15±0.20	7.551–7.944
			7.70	35.4	1.55±0.03	1.20±0.03		

Blood-oxygen affinity (logP_{50}) and Hill coefficient (n_H) values are reported with bootstrap estimated standard errors, and Bohr coefficients are reported with the 95% confidence intervals for the slopes from linear models of logP_{50} versus pH (see Materials and Methods). Numbers in parentheses beside each species name indicate the number of individuals sampled, and the sample sizes (n) beside each temperature indicate the number of OECs generated for each temperature treatment (i.e. two per individual).

sharks spend most of the night in the warmer upper mixed layer (~7 mg O_2 l^{-1}; P_{O_2}>150 mmHg), but during the day they descend well below the thermocline, proximal to the upper reaches of the oxygen minimum layer (<2 mg O_2 l^{-1}; P_{O_2}<34 mmHg) (Aalbers et al., 2021; Sepulveda et al., 2019; oxygen tensions determined with depth, temperature and dissolved oxygen concentrations reported by Sepulveda et al., 2019). While near the oxygen minimum layer, a low P_{50} combined with relatively thin lamellar diffusion distances and a gill surface area (relative to body mass) that is larger than that of any other studied elasmobranch (Wootton et al., 2015) should facilitate better O_2 diffusion into the blood from the relatively cold hypoxic water. The P_{50} values of the shortfin mako shark (15 and 25°C) and the blue shark (10°C) are also relatively low for elasmobranchs, only about 1–3 mmHg greater than those of the bigeye thresher shark, but neither shortfin mako sharks nor blue sharks routinely spend prolonged periods in hypoxic waters (Bernal et al., 2009). However, a low P_{50} likely only confers a significant physiological advantage in low environmental oxygen when it occurs in combination with other traits, such as the relatively large gill surface and thin lamellae of the bigeye thresher shark.

The ecophysiological significance of reduced and reverse temperature-dependent Hb–O_2 affinity

Temperature-independent Hb–O_2 binding affinity was first reported in Hb from the Atlantic bluefin tuna (*Thunnus thynnus*), and it was proposed that this trait may prevent thermally induced changes to Hb–O_2 affinity, and thus abate disturbances to O_2 uptake, in the face of large and sometimes rapid changes in environmental temperature associated with their large latitudinal and vertical (depth) movements (Rossi-Fanelli and Antonini, 1960). It seems reasonable that temperature-independent Hb–O_2 affinity enables O_2 uptake over a broad range of ambient temperatures, as several fishes that are tolerant of a wide range of temperatures (i.e. eurythermal), including both ectothermic and regionally endothermic fishes that have Hbs with reduced or reverse temperature dependence (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). Although Hb with a reduced temperature sensitivity is not exclusive to regionally endothermic fishes, it does not seem to be a coincidence that this trait is present in all lineages of regionally endothermic fishes investigated to date, nor that the temperature dependence of Hb–O_2 affinity is considerably reduced in regionally endothermic fishes compared with most other vertebrates, including mammals with heat exchanging retia in their limbs or appendages (Weber and Campbell, 2011).

In regionally endothermic sharks such as the shortfin mako and common thresher shark, temperature-independent Hb–O_2 affinity likely prevents body temperature from affecting the *in vivo* blood–O_2 affinity, which should maintain a relatively constant blood–O_2 affinity from the coldest to the warmest tissues. This may protect Hb–O_2 unloading in the colder tissues, matching O_2 supply to O_2 demand irrespective of tissue temperature (Clark et al., 2008). In bigeye thresher sharks, Hb with a reduced temperature dependence may be linked to their thermal ecology and diurnal dive patterns, in which they spend the day in cold water (<10°C) deep below the thermocline, but ascend into the warmer upper mixed layer at night (Aalbers et al., 2021; Nakano et al., 2003; Sepulveda et al., 2019; Weng and Block, 2004). These diel migrations subject bigeye thresher sharks to abrupt changes in water temperatures ranging between 5 and 25°C (Aalbers et al., 2021; Sepulveda et al., 2019). This requires that O_2 uptake is effective at the extreme temperatures, so a reduced effect of temperature on Hb–O_2 affinity may ensure that environmental temperature does not excessively shift blood P_{50} as bigeye thresher sharks move between warm and cold water. At high Hb–O_2 saturation levels, the bigeye thresher shark OEC right shifts with increasing temperature (Fig. 3D), although Hb–O_2 affinity remains relatively high compared with that of other species such as the blue shark (Fig. 3C). The absence of a substantial right shift of the OEC at high O_2 saturations and high temperatures may ensure that large changes to internal temperatures do not impair Hb–O_2 unloading to muscles, which are specialized to function over a broader temperature range than in sympatric species (Stoehr et al., 2020). Swordfish exhibit similar diurnal distributions to bigeye

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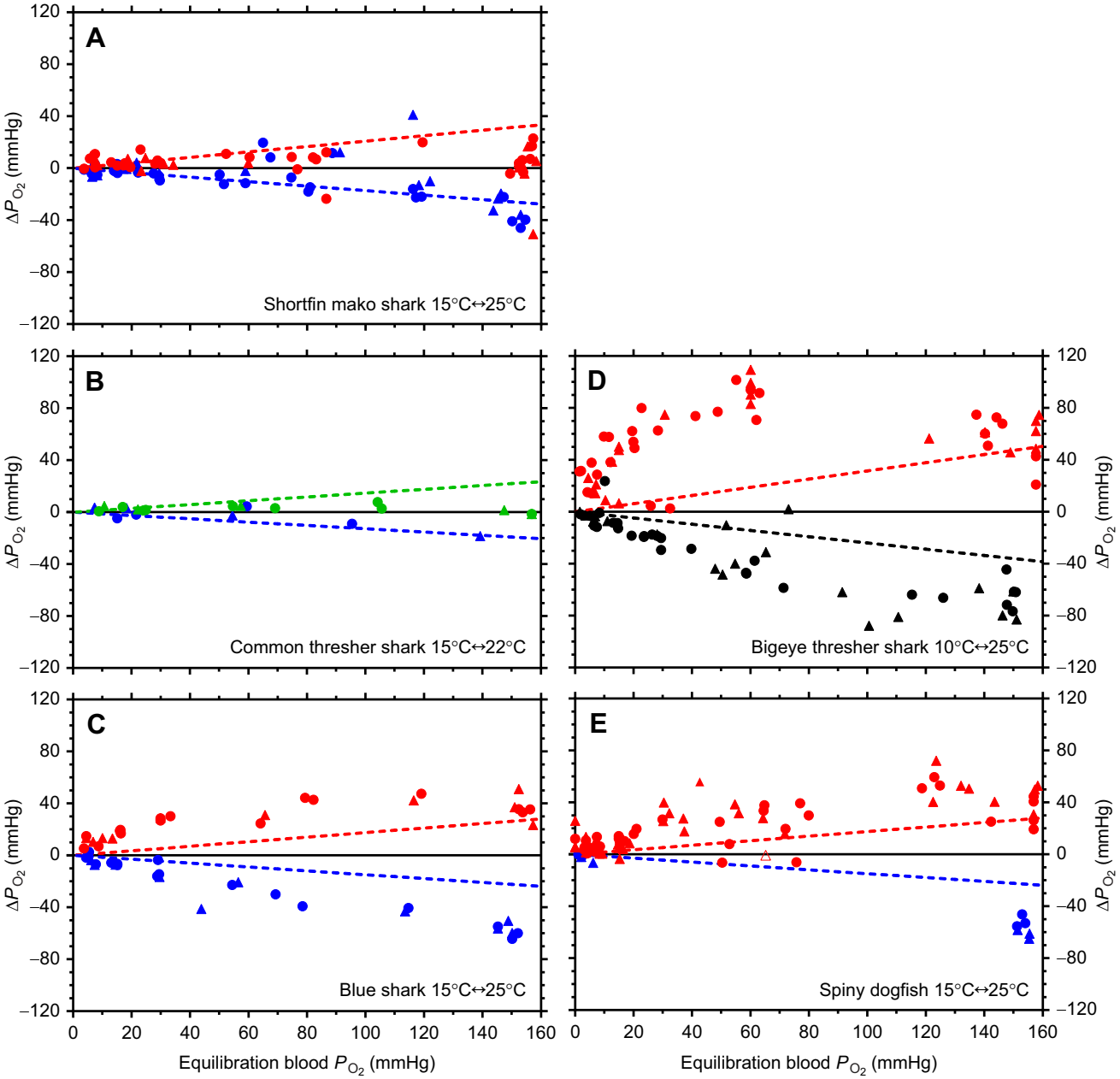

Fig. 4. Effects of closed-system temperature changes on the measured change in blood P_{O_2} (ΔP_{O_2}) of shortfin mako shark, common thresher shark, blue shark, bigeye thresher shark and spiny dogfish. Blood from 7 shortfin mako sharks (A), 2 common thresher sharks (B), 4 blue sharks (C), 7 bigeye thresher sharks (D) and 8 spiny dogfish (E) was equilibrated at a range of O_2 tensions (equilibration blood P_{O_2}), at a low P_{CO_2} (circles) of 1.9 mmHg and a high P_{CO_2} (triangles) of 3.8 mmHg (spiny dogfish) or 7.6 mmHg (all other sharks), and then warmed (red and green) or cooled (blue and black). Shortfin mako shark, blue shark and spiny dogfish blood temperature was changed between 15 and 25°C, common thresher shark blood was changed between 15 and 22°C, and bigeye thresher shark blood was changed between 10 and 25°C. Dotted lines indicate the theoretical temperature-induced ΔP_{O_2} expected as a result of changes in solubility of blood plasma at a given equilibration P_{O_2} (i.e. Henry's law) with warming (red and green) or cooling (blue and black). Oxygen solubilities for plasma at the different temperatures were taken from Boutilier et al. (1984).

thresher sharks, and the temperature dependence of Hb–O_2 affinity is comparable between the two species, as are some other aspects of their physiology (Morrison et al., 2022; Sepulveda et al., 2010; Stoehr et al., 2020).

Summary

Here, we show that whole-blood Hb–O_2 affinity is temperature independent for the regionally endothermic common thresher shark. Temperature-independent Hb–O_2 affinity was previously shown in blood from the shortfin mako shark and was corroborated in this study. We also show that the bigeye thresher shark P_{50} is insensitive to temperature, and is relatively low compared with that of most sharks and marine teleosts (Harter et al., 2022; Morrison et al., 2015). This potentially indicates a tolerance to hypoxia and may allow bigeye thresher sharks to exploit depths proximal to the upper reaches of the oxygen minimum layer. Blue shark and spiny dogfish blood–O_2 affinity decreased with increasing temperature, as expected for these species. In regionally endothermic sharks such as the shortfin mako shark and the common thresher shark, temperature-independent Hb–O_2 affinity may avert excessive

Table 3. Whole-blood P_{50} values (mmHg) and Bohr coefficients (ϕ) reported in the literature and this study for shortfin mako sharks, blue sharks and spiny dogfish

Species	Reference	T (°C)	pH	ϕ	This study: ϕ	P_{50} (mmHg)	This study: P_{50} (mmHg)
Shortfin mako shark	Bernal et al. (2018)	15	7.93	−0.74	−0.14	14.5	8.4
		15	7.68			22.3	9.1
		25	8.13	−0.11	0.02	18.6	11.0
		25	7.64			20.9	10.8
	Wells and Davie (1985)	25	7.6	0.16	0.02	10.6	10.8
Blue shark	Bernal et al. (2018)	15	8.05	−0.33	−0.08	12.1	15.8
		15	7.45			19.1	17.6
		25	7.95	−0.22	−0.19	25.0	21.7
		25	7.48			31.7	26.6
Spiny dogfish	Wells and Weber (1983)	15	7.85	−0.28	0.05	17.9	22.8

This study's P_{50} values were interpolated at the same pH as the literature values using the Bohr coefficients reported in Table 2.

decreases in Hb–O_2 affinity in warm tissues, while also preventing Hb–O_2 affinity from being too high to unload sufficient O_2 to the cold tissues.

Haemoglobins with a low temperature dependence of O_2 binding have been reported in all studied groups of regionally endothermic fishes, although $\Delta H'$ varies among species and not all species exhibit reductions in $\Delta H'$ (e.g. bigeye tuna, *Thunnus obesus*; Lowe et al., 2000). The oxygenation-dependent release of allosteric effectors such as ATP and Bohr protons contributes endothermically to $\Delta H'$, causing reductions or reversals in the temperature sensitivity of Hb from regionally endothermic as well ectothermic fishes (Ikeda-Saito et al., 1983; Larsen et al., 2003; Morrison et al., 2022; Nelson et al., 2019; Weber et al., 2010). ATP has been implicated as an important effector of Hb from the regionally endothermic porbeagle shark (Larsen et al., 2003), although further functional, structural and molecular studies of Hbs from regionally endothermic sharks, as well as closely related ectothermic sharks, should provide insight into the evolution of this trait and its physiological and ecological significance.

Acknowledgements
The authors thank Anne Kim (Department of Zoology, UBC) for help with the OEC experiments, as well as Scott Aalbers and Jennifer Thirkell (Pfleger Institute of Environmental Research) for field and logistical assistance. We also thank Dr Richard Brill and Dr Peter Bushnell for their assistance developing parts of this work.

Competing interests
The authors declare no competing or financial interests.

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

Funding
This work was supported by the National Science Foundation [1354593 to D.B., 1354772 to C.A.S.]. Funding for C.A.S. was also provided by the NOAA Saltonstall Kennedy Program [NA17NMF4270216] as well as the California Ocean Protection Council, under Grant Agreement no. C0303000, Project #R/OPCSFAQ-07 through the Sea Grant College Program. Funding was also provided by Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery grants to C.J.B. (261924-13; 2018-04172), and a Natural Sciences and Engineering Research Council of Canada Postgraduate Scholarship to P.R.M.

Data availability
All relevant data can be found within the article and its supplementary information.

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