ON THE TEMPERATURE-DEPENDENCE OF HÆMOGLOBIN-OXYGENATION AND BLOOD-OXYGEN TRANSPORT IN REGIONALLY HETEROTHERMIC TELEOSTS AND SHARKS

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Abstract

Hæmoglobin (Hb)-O_2 affinity in most vertebrates typically decreases with increasing blood temperature because the oxygenation enthalpy ($\Delta H'$) is usually exothermic. However, in regionally heterothermic fishes, such as tunas and some sharks, $\Delta H'$ is commonly low or even endothermic, causing a very reduced or reversed effect of temperature on Hb-O_2 affinity. Regionally heterothermic fishes conserve metabolic heat with vascular heat exchangers that prevent circulatory heat loss and establish internal temperature gradients. My objective was to investigate the functional significance of reduced and reverse temperature-dependent Hb-O_2 affinity in regionally heterothermic fishes. I hypothesized that temperature-independent Hb-O_2 affinity conserves heat energy and matches O_2 supply to O_2 demand despite large internal temperature gradients, so I expected this trait to be shared by all regionally heterothermic fishes. I investigated this by (1) examining the effect of temperature on Hb-O_2 affinity in three regionally heterothermic species whose Hb has not been well studied (swordfish, opah, and common thresher shark), and (2) mathematical modelling of O_2 transport to quantitatively assess the relative contributions of Hb concentration and Hb-O_2 affinity to O_2 and heat transport.

I found that opah Hb-O_2 affinity is temperature-independent, the temperature-dependence of swordfish Hb-O_2 affinity is pH dependent, becoming temperature-independent at low pH, and common thresher shark Hb-O_2 affinity is temperature-independent. I also found that Hb from bigeye thresher shark, a suspected regional heterotherm, is temperature-independent below 50% Hb-O_2 saturation. Using a mathematical model of O_2 transport I demonstrated that Hb concentration and Hb-O_2 affinity are relatively more important than other factors of O_2 transport in determining maximum O_2 consumption in yellowfin tuna, a regional heterotherm with a

"high-energy demand." I also showed that Hb with a reversed temperature-dependence diminishes temperature induced changes to blood O_2 tension and prevents Hb-heat loss, as much as 13% of metabolic heat production.

These results provide insight into the functional significance of reduced and reversed temperature-dependent Hb-O_2 affinity in regionally heterothermic fishes. All known lineages of regionally heterothermic fishes have Hb with a low $\Delta H'$, and increases to $\Delta H'$ have convergently evolved by different molecular mechanisms with underlying dependence on different allosteric effectors.

Lay Summary

Hæmoglobin is a protein found inside red blood cells of vertebrates. Inspired oxygen binds to hæmoglobin and is transported in the blood throughout the body where oxygen is unloaded to fuel metabolism. In most animals, increasing blood temperature decreases hæmoglobins binding affinity for oxygen. However, the temperature-sensitivity of hæmoglobin is unusually low in some animals, including "warm-bodied" fishes such as tunas, marlins, and some sharks that are able to keep regions of their bodies warmer than the surrounding water (i.e., regional heterothermy). In this thesis, I show that the warm-bodied opah, common thresher shark, and swordfish also have hæmoglobin with a low temperature-sensitivity. I also examined how temperature-insensitive hæmoglobin may be useful in warm-bodied fish, which I conclude is a remarkable example of a trait that has repeatedly evolved to fulfill a similar function: to transport and uniformly unload oxygen to regions of the body that are maintained at very different temperatures.

Preface

Chapter 2 is based on analyses that I conceived. I performed all of the *in silico* experiments, and analyzed the data.

Chapter 3 was a collaborative project with Diego Bernal (University of Massachusetts, Dartmouth), Chugey A. Sepulveda (Pfleger Institute of Environmental Research), and Nicholas C. Wegner (National Oceanic and Atmospheric Administration), and Colin J. Brauner (University of British Columbia). Diego Bernal, Chugey Sepulveda, and Nick Wegner collected the blood samples that I used. Diego Bernal, Chugey Sepulveda, and I designed the experiments. I conducted the experiments and analyzed the data under supervision from Colin Brauner.

Chapter 4 was a collaborative project with Diego Bernal, Chugey A. Sepulveda, and Colin J. Brauner. Diego Bernal and Chugey Sepulveda collected the blood samples that I used. Diego Bernal, Chugey Sepulveda, and I designed the experiments. I conducted the experiments and analyzed the data under supervision from Colin Brauner.

The experiments in this thesis followed protocols that were approved by the UBC animal care committee (animal care no: A11-0235).

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List of Symbols and Abbreviations

ATP	Adenosine triphosphate
βbO_2	Blood-O_2 capacitance coefficient
CaO_2	Arterial blood-O_2 content
CvO_2	Venous blood-O_2 content
Hb	Hæmoglobin
[Hb]	Hæmoglobin concentration
Hct	Hæmatocrit (proportion of red blood cells in blood)
ΔH^{O2}	Enthalpy of hæme oxygenation
$\Delta H'$	Overall enthalpy of hæmoglobin oxygenation
$\Delta H'_{WB}$	Overall enthalpy of hæmoglobin oxygenation in whole blood
L	Allosteric equilibrium constant
MCHC	Mean corpuscular hæmoglobin concentration
$\dot{M}O_2$	Moles or mass of O_2 consumed (i.e., flow rate of O_2)
NTP	Nucleoside triphosphate
n_{50}	Hill coefficient determined at P_{50}
OEC	Oxygen equilibrium curve
OML	Oxygen minimum layer
PCO_2	Partial pressure of CO_2
PO_2	Partial pressure of O_2
PaO_2	Arterial blood PO_2
PvO_2	Venous blood PO_2
P_S	The PO_2 that corresponds to a given Hb-O_2 saturation (S)

P_{50}	The PO_2 where 50% of Hb is saturated with O_2
P_{95}	The PO_2 where 95% of Hb is saturated with O_2
$\dot{Q}$	Cardiac output
RBC	Red blood cell
RM	Red muscle
R-state	High affinity hæmoglobin conformation (i.e., the relaxed state)
T-state	Low affinity hæmoglobin conformation (i.e., the tense state)
$\dot{V}O_2$	Volume of O_2 consumed (i.e., flow rate of O_2)
φ	Bohr coefficient

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I also received help, advice, and guidance from Richard Brill (National Oceanic and Atmospheric Administration), Hans Malte (Aarhus University), Sally Otto (UBC), as well as many graduate students and postdocs in the Department of Zoology at UBC.

Chapter 1 General Introduction

1.1 Hæmoglobin

"[T]he hæmoglobin of different animals does not necessarily possess the same relative affinity for oxygen." (August Krogh, 1910, p. 220)

Hæmoglobin (Hb) is a protein that is encapsulated in the red blood cells of jawed vertebrates (gnathostomes) where it plays an essential role in gas transport between the respiratory exchange surface (e.g., gills, lungs, or skin) and the respiring cells of an animal. The steady consumption of oxygen (O_2) during mitochondrial respiration maintains a low partial pressure of O_2 (PO_2) in the cells relative to the inspired water or air, and metabolically produced carbon dioxide (CO_2) elevates the partial pressure of cellular CO_2 (PCO_2). In all but a few vertebrates, Hb is pivotal to sustaining aerobic metabolism by expediting the transport of O_2 down its PO_2 gradient in the circulatory system from the respiratory surface to the respiring tissues (the oxygen transport cascade), while also facilitating blood-CO_2 transport to the respiratory surface where CO_2 is eliminated in the expired water or air. Because Hb forms a physiological link between aerobic metabolism and the environment, Hb function has been shaped by internal constraints as well as by external selective forces during vertebrate evolution, resulting in numerous examples of adaptive evolution and convergence of Hb function (e.g., Natarajan et al., 2016; Storz, 2016; Tufts et al., 2015; Weber and Campbell, 2011; Weber and Fago, 2004).

Oxygen binding to Hb is an exothermic process (i.e., the overall enthalpy, $\Delta H'$, is negative) in most vertebrates, so increasing temperature typically decreases Hbs affinity for O_2; a so-called "normal" temperature dependence (Figure 1.1). However, an excellent example of

convergent Hb function is the multiple evolutionary origins of Hbs with a reduced sensitivity to temperature among regionally heterothermic vertebrates (Weber and Campbell, 2011). Regional heterotherms are endothermic animals since they have the physiological capacity to regulate body temperature with internally generated heat, but they maintain distinct regions of their bodies at different temperatures. This is often made possible by heat exchanging *retia mirabilia* that facilitate heat transfer from blood leaving to blood entering a warm body region. For example, some regionally heterothermic mammals and birds have heat exchanging *retia* in their limbs or appendages that reduce heat loss to the environment (Scholander, 1955). Some teleosts and sharks have independently evolved endothermy, which is partially facilitated by heat exchanging *retia* that prevent internally generated heat being lost via the blood flow to the surrounding water. Because the heat exchanging *retia* elevate the temperatures of only select body regions warmer than ambient water temperature, and some tissues remain in thermal equilibrium with ambient water, endothermic fishes and sharks are functionally regional heterotherms. For the remainder of this thesis I will refer to these fishes as regionally heterothermic. In all but a couple of regionally heterothermic fishes investigated so far, Hb-O_2 affinity is independent of temperature or even exhibits a reverse temperature-dependence (i.e., Hb-O_2 affinity increases with increasing temperature, evident as a leftward shift of the black curves in Figure 1.1) (Morrison et al., 2015; Weber and Campbell, 2011). Furthermore, regional heterothermy is energetically costly, as is apparent by the high metabolic rates of those species for which it has been measured (Korsmeyer and Dewar, 2001; Sepulveda et al., 2007b). Thus, to fuel heat production and support their high metabolic rates, regionally heterothermic fishes tend to have high blood-O_2 transport capacities, due in part to high Hb concentrations (e.g., Bernal et al., 2001b; Morrison et al., 2015).

I further review Hb function and the Hb of regionally heterothermic fishes in this chapter. Based on this information, I have developed two general hypothesis that unify this thesis: 1) temperature-independent Hb-O_2 affinity conserves heat-energy, which may help maintain stably elevated body temperatures in regionally heterothermic fishes. Thus, I expected this trait to be shared by all regionally heterothermic teleosts and sharks; and 2) the Hb-oxygen-binding properties and high Hb concentrations of regionally heterothermic fishes maintain a match between O_2 supply and O_2 demand despite large internal temperature gradients. At the end of this chapter I outline my objectives and the research questions that I address in the subsequent chapters.

1.1.1 Hæmoglobin structure and function

Genome duplication events early in vertebrate evolution produced a functional diversification of globin genes that gave rise to the modern jawed vertebrate Hb, which is a heterotetramer composed of two α-chain subunits and two β-chain subunits (Pillai et al., 2020; Storz, 2016). Each subunit contains a hæme group with a porphyrin ring that holds an iron atom, which reversibly binds one O_2 molecule when the iron atom is in the ferrous state (Fe^{2+}); thus, one Hb tetramer can bind up to four O_2 molecules. The quaternary structure of the Hb tetramer comprises an oxygenation-linked equilibrium between two distinct conformations: a high-affinity (oxygenated) R-state and a low-affinity (deoxygenated) T-state (Monod et al., 1965). Homotropic and heterotropic allostery regulate oxygenation-linked shifts in the T↔R conformational equilibrium, in such a way that interactions among the hæme binding sites instigate cooperative O_2-binding (homotropic), and ligand binding to non-hæme sites regulates Hb-O_2 affinity (heterotropic) (Baldwin and Chothia, 1979; Perutz, 1970).

The oxygen equilibrium curve (OEC) characterises the relationship between Hb-O_2 saturation and PO_2, and the shape and position of the OEC reflect homotropic allostery and Hb-O_2 binding affinity, respectively (Figure 1.1) (Bohr et al., 1904; Wyman, 1964). August Krogh and Isabella Leitch introduced the term "tension of unloading" (t_u) to denote the "O_2 pressure at which the Hb is just half saturated with oxygen" and the "tension of loading (t_l) as corresponding to 95 per cent. saturation" (Krogh and Leitch, 1919). Their t_u term is the equilibrium constant, K, in Archibald V. Hill's mathematical description of the OEC (i.e., Hill's equation) (Barcroft and Hill, 1910; Hill, 1910) and is now typically referred to as P_{50} (i.e., the PO_2 at 50% Hb-O_2 saturation), which is "used as a single though incomplete characteristic" of the OEC that quantifies Hb-O_2 affinity (Krogh and Leitch, 1919). Non-hæme binding of heterotropic effectors (i.e., allosteric effectors) typically increases P_{50} and right shifts the OEC of whole blood relative to that of stripped Hb (i.e., a Hb solution purified of endogenous allosteric effectors), and oxygenation-linked binding and dissociation of allosteric effectors shifts the T↔R conformational equilibrium, modulating blood-O_2 affinity between the respiratory exchange surface and the peripheral tissues (i.e., the sites of O_2 binding and offloading, respectively) (Figure 1.1). Therefore, Hb-O_2 affinity is an important factor in the O_2 transport cascade because it is a link between the PO_2 of the inspired water or alveolar air and the tissue O_2 supply.

1.1.2 Blood-oxygen transport

"In the respiratory organs proper the gases are transported from one medium to another by diffusion and, for all we know, diffusion is also the only mechanism for the transport into and out of the ultimately respiring cells, but in between we have a transport by convection in the flowing blood" (Krogh, 1941, p. 88). The oxygen capacity and the OEC are important respiratory characteristics of the blood that are central to blood-O_2 transport from the respiratory organ to

the tissues. The diffusive steps of the O_2 transport cascade can be quantified by Fick's law of diffusion, while convection can be quantified by the Fick Principle, which is given by

$$\dot{V}O_2 = \dot{Q} \cdot (CaO_2 - CaO_2) \quad (1)$$

where $\dot{V}O_2$ is the flow rate of O_2 (i.e., O_2 consumption rate), $\dot{Q}$ is the cardiac output, and (CaO_2-CvO_2) is the arteriovenous O_2 content difference. In equation 1, circulatory O_2 delivery is equal to the product of $\dot{Q}$ and CaO_2, and the O_2 extraction is equal to percentage of O_2 removed from the blood [i.e., (CaO_2-CvO_2)/CaO_2 · 100%]. The arteriovenous O_2 content difference can be expressed by the following

$$CaO_2 - CaO_2 = \beta bO_2 \cdot (PaO_2 - PvO_2) \quad (2)$$

where (PaO_2-PvO_2) is the arteriovenous PO_2 difference and βbO_2 is the O_2 capacitance coefficient of the blood. βbO_2 defines the gradient of the functional portion of the OEC (Figure 1.2), which is the slope of the relation between O_2 content and PO_2:

$$\beta bO_2 = \frac{CaO_2 - CaO_2}{PaO_2 - PvO_2} \quad (3)$$

It follows that for any given cardiac output, circulatory O_2 delivery and O_2 extraction depend on the arterial O_2 content and left or right shifts in the OEC that change βbO_2 (Figure 1.2). Thus, blood-O_2 transport is largely determined by Hb concentration and shifts in Hb-O_2 affinity between the respiratory organ and the tissues.

1.1.3 Allosteric regulation of Hb-O_2 affinity

"The affinity of blood or pure hæmoglobins for oxygen is a complex phenomenon, depending upon a number of conditions, the most important of which are temperature and hydrogen ion concentration" (Krogh, 1941, p. 95). Hb-O_2 affinity is also affected by binding of chloride ions (Cl^+) and organic phosphates to non-hæme sites, but rapid left and right shifts to

the OEC *in vivo* (i.e., the physiological OEC) are induced primarily by non-hæme binding of hydrogen ions (protons) and the thermodynamics of Hb-O_2 binding (Figure 1.1).

1.1.3.1 The effects of protons and *P*CO_2: The Bohr, Haldane, and Root effects

As metabolic CO_2 enters the blood from the tissues there is a simultaneous and associated decline in blood pH that decreases blood-O_2 affinity via negative, heterotropic interactions linked to proton binding sites. The influence of CO_2 on blood-O_2 equilibria was first described in the seminal paper by Bohr et al. (1904), in which Christian Bohr, Karl Hasselbalch, and August Krogh showed that increasing *P*CO_2 right shifted the OEC. A few years later, Joseph Barcroft and Leon Orbeli (1910) reported a similar effect by adding acid to blood. The influence of CO_2 and pH on Hb-O_2 affinity has since become known as the "Bohr effect" (given by John Scott Haldane) and is quantified as the Bohr factor or Bohr coefficient (φ):

$$\varphi = \frac{\Delta \log_{10} P_{50}}{\Delta \mathrm{pH}} \qquad (4)$$

The magnitude of Bohr effect directly influences Hb-O_2 affinity, and the protons bound during oxygen unloading in the tissues profoundly right shifts the physiological OEC (Malte and Lykkeboe, 2018). This deoxygenation linked proton binding facilitates CO_2 transport by increasing the CO_2 content of the deoxygenated venous blood relative to oxygenated arterial blood, a phenomenon established by the work of Haldane and co-workers (Christiansen et al., 1914) and is now known as the "Haldane effect". The Bohr and Haldane effects are reciprocal to one another and are considered "two sides of the same coin" because they are thermodynamically linked at the molecular level (Wyman, 1964). Consequently, CO_2 that diffuses into the blood in the tissue capillaries boosts O_2 offloading by decreasing Hb-O_2 affinity, but elimination of CO_2 at the respiratory organ promotes O_2 binding by increasing Hb-O_2

affinity. Thus, the Bohr effect plays an essential role in shifting the OEC *in vivo* from high to low affinity at the respiratory organ and tissues, respectively. Furthermore, because P_{50} and the Bohr coefficient are not independent of one another, over the course of Hb evolution structural changes that increased the magnitude of the Bohr coefficient and/or the effectiveness of a given acid load to promote Hb-O_2 unloading, would have also increased whole blood P_{50}, and vice versa (Berenbrink, 2006; Malte and Lykkeboe, 2018).

Teleosts express multiple, functionally distinct Hb isoforms, which is at least partly due to a teleost-specific genome duplication event (Opazo et al., 2013; Storz, 2016), and some teleost Hbs exhibit what has been described as an exaggerated Bohr effect (Root, 1931). Krogh and Leitch (1919) were the first to note that the blood of fishes "is very sensitive to small increases in CO_2 tension which reduce the affinity for oxygen very considerably" (Berenbrink et al., 2011). The pH sensitivity of teleost Hb is known as the Root effect, named in recognition of Raymond Root (Scholander and Van Dam, 1954), and it is characterised by an exaggerated right shift of the OEC with declining pH, and a loss of cooperative Hb-O_2 binding associated with the inability to fully saturate Hb below a certain pH threshold (Green and Root, 1933; Root, 1931). Root effect Hbs are part of a complex physiological system that is specialized for O_2 secretion in the eye and swim bladder of teleost fishes by inducing large decreases in Hb-O_2 affinity and O_2 carrying capacity, which are corrected by the time the blood circulates back to the respiratory surface to replenish blood-O_2 levels (e.g., Berenbrink, 2007; Berenbrink et al., 2005). Although teleost Bohr coefficients are generally numerically higher than in other vertebrates, a Bohr effect is present in all lineages of jawed vertebrate (e.g., Berenbrink, 2006; Giardina et al., 2004; Pough, 1980), but not necessarily in all species on a particular branch of the vertebrate tree (e.g., chondrichthyans; Morrison et al., 2015).

1.1.3.2 The effect of temperature and thermodynamics of O_2 binding

Sir Joseph Barcroft and collaborators were the first to quantify the kinetics and thermodynamics of Hb-O_2 equilibria, and they determined that the overall enthalpy of Hb-oxygenation ($\Delta H'$) is numerically negative (Barcroft, 1914; Barcroft and Hill, 1910). Thus, Hb-O_2 binding is an exothermic process ($Hb + O_2 \leftrightharpoons HbO_2$ + heat), so increasing temperature will shift the equilibrium towards the deoxygenated form of Hb, consequently decreasing Hb-O_2 affinity (Barcroft and Hill, 1910; Barcroft and King, 1909). Because the heat of O_2 binding to the haeme groups (ΔH^{O2}) is intrinsically exothermic, the overall ΔH is usually also exothermic. Values for ΔH^{O2} are very similar among the hæmolysates of jawed vertebrates [e.g., ~ -62 kJ mol^{-1} for billfish hemolysates at pH 8.0 (Weber et al., 2010)] (Morrison et al., 2015; Powers et al., 1979). However, $\Delta H'$ is typically numerically less negative than ΔH^{O2}. This is because $\Delta H'$ comprises the sum of contributions from ΔH^{O2}, the heat of solution of O_2 (~ -12.6 kJ mol^{-1}), the heat of conformational changes (T↔R transitions), and the heats of ionization and dissociation of allosteric effectors (e.g., protons, organic phosphates, and Cl^- ions). Oxygenation linked dissociation of allosteric effectors contribute endothermically to $\Delta H'$, reducing the overall effect of temperature on Hb-O_2 affinity. Values of $\Delta H'$ are typically negative in jawed vertebrates, and are quantified using the van't Hoff isochore (Wyman, 1964):

$$\Delta H' = \ln 10 \cdot R \cdot \frac{\Delta \log_{10} P_{50}}{\Delta \frac{1}{T}} \quad (5)$$

where R is the gas constant and T is the absolute temperature.

Krogh and Leitch (1919) noted the significance of the temperature sensitivity of Hb-O_2 affinity for O_2 uptake and transport in fishes, recognizing that fishes thermoconform to their environment (i.e., most fishes are poikilotherms) so the surrounding water temperature will

directly influence Hb-O_2 uptake and transport. Furthermore, endothermic Hb-O_2 unloading in the tissues and exothermic Hb-O_2 binding at the gills cause the outward transport and loss of metabolic heat to the environment, contributing to poikilothermy in fishes (Jensen et al., 1998; Stevens and Sutterlin, 1976; Weber and Fago, 2004). Over 40 years after Krogh and Leitch reported their blood-O_2 equilibrium experiments at the environmental temperatures of fishes, Alessandro Rossi Fanelli and Eraldo Antonini reported that temperature had very little effect on the O_2 affinity of crystalline Hb from Atlantic bluefin tuna (*Thunnus thynnus*) (Rossi Fanelli and Antonini, 1960). Bluefin tuna Hb exhibited an obvious pH sensitivity, but over a wide pH range (pH 6.45 to 8.7) changing temperature between 5°C and 35°C (~5, 10, 20, 30, and 35°C) had no appreciable effect on the shape or position of the OEC. At that time, the discovery that bluefin tuna Hb was "practically unaffected by temperature" was considered novel enough that the findings were published in *Nature* along with a companion paper on tuna myoglobin, which was shown to be functionally similar to mammalian myoglobin (Rossi Fanelli and Antonini, 1960; Rossi Fanelli et al., 1960).

Rossi Fanelli and Antonini proposed that "the insensitivity of the oxygen equilibrium of tuna hæmoglobin to changes in temperature could represent a sort of molecular adaptation which would enable the animal to live in waters of very different temperatures without modification of the functional properties of its respiratory pigment" (Rossi Fanelli and Antonini, 1960, p. 896). In other words, they concluded that bluefin tuna should have no problem binding O_2 as they move between warm and cold water above and below the thermocline, respectively. This may indeed be a benefit of temperature independent Hb-O_2 affinity, but what Rossi Fanelli and Antonini may not have known, or at least did not mention, is that bluefin tuna are regionally heterothermic and can maintain the core of their body much warmer than their peripheral tissues

and the ambient water. It was another four years before the body temperatures of yellowfin tuna (*Thunnus albacares*) and skipjack tuna (*Katsuwonus pelamis*) were reported (Barrett and Hester, 1964), and another two more years before Francis "Frank" Carey and John Teal reported the body temperatures of bluefin tuna and described the anatomical specialisations that facilitate regional heterothermy (Carey and Teal, 1966). Reports of warm bodied tuna and descriptions of their anatomy date back to at least 1835 (reviewed by Fudge and Stevens, 1996), and although Rossi Fanelli and Antonini may not have known of these earlier publications, their discovery that bluefin tuna Hb-O_2 affinity is unaffected by temperature led others to hypothesize that this trait may prevent unnecessary temperature induced shifts in the OEC between the cold and warm tissues (e.g., Carey and Gibson, 1977; Graham, 1973). Investigations into the effect of temperature on Hb-O_2 affinity in other species of regionally heterothermic vertebrates has spanned the 60 years since the formative publication by Rossi Fanelli and Antonini (Weber and Campbell, 2011), and this line of research is the basis for this PhD thesis. Before reviewing the literature on the effect of temperature on Hb-O_2 affinity in regionally heterothermic teleosts and sharks, I will briefly review the anatomical and physiological specializations that conserve body heat in these disparate species.

1.2 Regionally heterothermic fishes and sharks

From the late 1960's until his death in 1994, Frank Carey and his collaborators established that tuna and lamnid sharks are "warm-bodied," and they began to describe the anatomical and physiological specialisations for endothermy as well as the physiological ecology of these fishes (e.g., Block and Carey, 1985; Carey, 1973; Carey and Lawson, 1973; Carey and Teal, 1966; Carey and Teal, 1969a; Carey and Teal, 1969b; Carey et al., 1971; Carey et al., 1981; Carey et al., 1981; Carey et al., 1982; Carey et al., 1984; Carey et al., 1985; Linthicum and

Carey, 1972; Wolf et al., 1988). In the 1980's and early 1990's, Frank Carey and Barbara Block would also describe the "brain heaters" and aspects of the ecology of billfishes (e.g., Block, 1986; Block, 1991b; Block et al., 1992a; Block et al., 1992b; Carey, 1982b; Carey, 1990). Carey's pioneering research on tuna has been reviewed by Graham and Dickson (2001), and research on fish endothermy and the physiological ecology of large pelagic fishes is carried on by others to this day. Since Carey and Teal (1966) described the endothermic capacity of the Atlantic bluefin tuna, it has been well established that endothermy has evolved independently in several teleost and elasmobranch lineages, including tunas (Scombridae: *Euthynnus*, *Auxis*, *Katsuwonus*, *Thunnus*), billfishes (Istiophoridae and Xiphiidae), the smalleye Pacific opah (*Lampris incognitus*), the common thresher shark (*Alopias vulpinus*), and sharks in the family Lamnidae (salmon shark, *Lamna ditropis*, porbeagle shark, *Lamna nasus*, shortfin mako, *Isurus oxyrinchus*, longfin mako, *Isurus paucus*, white shark, *Carcharodon Carcharias*) (Figure 1.3) (Bernal and Sepulveda, 2005; Block, 1986; Block and Carey, 1985; Carey, 1982b; Carey and Teal, 1966; Carey and Teal, 1969a; Carey and Teal, 1969b; Carey et al., 1971; Carey et al., 1985; Runcie et al., 2009; Sepulveda et al., 2008; Wegner et al., 2015).

Endothermic fishes are functionally regional heterotherms (sometimes referred to as regional endotherms) since they conserve metabolic heat to maintain only select tissues or regions of their bodies warmer than the surrounding water. In contrast, most other fishes are poikilothermic and thermoconform with their environment because metabolic heat is transferred to the environment through conduction across the body wall, as well as by convective transfer in the blood to the gill circulation where heat is lost to the inspired water (Brill et al., 1994; Stevens and Sutterlin, 1976). Thus, for regionally heterothermic fishes to maintain elevated body temperatures in their aquatic environments they need a source of metabolic heat and a

mechanism to retain that heat (Graham and Dickson, 2001). Although the source of heat varies among species, all regionally heterothermic fishes have *retia mirabilia* that function as countercurrent heat exchangers, like those of mammals (Scholander and Krog, 1957), and these *retia* are situated between the warm tissues and the gills to mitigate circulatory heat loss. The anatomical and physiological specialisations that facilitate thermoconservation in fishes are well reviewed (e.g., Bernal and Lowe, 2015; Bernal et al., 2001b; Bernal et al., 2012; Block, 1991a; Block, 1991b; Carey, 1973; Dickson and Graham, 2004; Fudge and Stevens, 1996; Graham and Dickson, 2000; Graham and Dickson, 2001; Graham and Dickson, 2004), and another in depth analysis could easily take up too much of this thesis. So here I will provide a brief overview of the features that are most pertinent to Hb function and blood-O_2 transport.

1.2.1 Sources of heat in regionally heterothermic fishes

Tunas, sharks in the family Lamnidae, and the common thresher shark are able to maintain a warm body core (Figure 1.4) by retaining the heat generated by the continuous contraction of their red myotomal swimming muscles (RM) (Bernal and Sepulveda, 2005; Carey and Teal, 1966; Carey and Teal, 1969a; Carey and Teal, 1969b; Carey et al., 1985; Sepulveda et al., 2008). Unlike most other fishes in which the RM is positioned subcutaneously, the warm RM of regional heterothermic tunas and sharks is located medially and more anteriorly in the body, close to the vertebral column (Carey and Teal, 1966; Carey and Teal, 1969a; Carey et al., 1985; Sepulveda et al., 2005; Sepulveda et al., 2008). Because these species must swim to ram ventilate their gills, the continuous contraction of their internalized RM provides a constant source of heat that is somewhat insulated from the environment by the surrounding white muscle. Additionally, tunas and lamnid sharks are also capable of elevating cranial (brain and eyes) and visceral temperatures (Figure 1.4) (Anderson and Goldman, 2001; Bernal et al., 2001b; Block and Carey,

1985; Carey et al., 1981; Carey et al., 1984; Goldman, 1997; Goldman et al., 2004; Sepulveda et al., 2004; Sepulveda et al., 2007a; Wolf et al., 1988).

Billfishes (Istiophoridae) and the swordfish (*Xiphias gladius*) are also capable of cranial endothermy and warm their heads with heat generating organs that evolved from the superior rectus extraocular muscles (Figure 1.4) (Block, 1986; Block, 1991b; Carey, 1982b). A putative heater organ also independently evolved in the butterfly kingfish (also known as the butterfly mackerel, *Gasterochisma melampus*), a scombrid fish closely related to tunas, but cranial endothermy has yet to be confirmed in butterfly kingfish (Tullis et al., 1991). Although the ocular muscles of some other regionally heterothermic fishes likely also contribute to cranial endothermy (Bernal et al., 2001b; Block and Finnerty, 1994; Runcie et al., 2009; Tubbesing and Block, 2000; Wolf et al., 1988), the heater organs of billfishes and the butterfly kingfish are unique because they are the only examples in fishes of tissues that are specialised for heat production (Block, 1994; Tullis et al., 1991).

The smalleye Pacific opah stands out among regionally heterothermic fishes because it has evolved what has been referred to as a form of whole-body endothermy (Wegner et al., 2015). The opah heats its cranium and most of its body core including the heart (Wegner et al., 2015), which is unique among fishes because the hearts of other fishes, including regional heterotherms, remain near ambient water temperature (e.g., Bernal et al., 2001b; Brill and Bushnell, 2001; Brill and Lai, 2015). The opahs heart receives warm blood from both the arterial and venous circulations, and is insulated from the opercular cavity by a fat layer. The lateral rectus extraocular muscle appears to be involved in heating the opahs cranium (Runcie et al., 2009), but most of the metabolic heat that warms the body is produced by the dark red, aerobic pectoral muscles that power swimming by the continuous “flapping” of their pectoral fins. A fat

layer also insulates the body core from the surrounding water, preventing heat loss from the peripherally situated pectoral muscles (Wegner et al., 2015). The system of heat exchanging *retia* is also quite different in the opah because the *retia* are associated with the gill vasculature and send warm blood to the entire body (Wegner et al., 2015), whereas the *retia* of other regional heterotherms are associated with the blood supply to the warm tissues only (see next section).

1.2.2 Retia mirabilia and circulatory heat exchange

The heat exchanging *rete mirabile*, or "wonderful net," is a tissue of closely intermingled and countercurrent running arterioles and venules (e.g., Carey et al., 1985; Lemons et al., 1987; Stevens et al., 1974), which conserves heat in a warm tissue by enabling the inflowing arterial blood to be warmed by the outflowing venous blood that is returning to the gills (Bernal et al., 2001b; Block, 1986; Carey, 1982b; Carey and Teal, 1966; Carey and Teal, 1969a; Fudge and Stevens, 1996; Graham and Dickson, 2001; Runcie et al., 2009; Wegner et al., 2015). Because thermal diffusion occurs about ten times faster than molecular diffusion, the blood of fishes thermally equilibrates with water in the time it takes for the blood to transit through the gill lamellae and exchange gases with the inspired water (Brill et al., 1994; Graham and Dickson, 2001; Stevens and Sutterlin, 1976). Thus, heat exchanging *retia* mitigate excessive metabolic heat loss from the warm tissues by cooling the outflowing venous blood near to ambient temperature before it reaches the gills, which creates a stable thermal gradient or thermal excess between the warm tissues and the ambient water (Figure 1.4) (Graham and Dickson, 2001).

The systemic circulation of regionally heterothermic sharks and most tunas are highly modified to route the bulk of the systemic blood flow through subcutaneous arteries and veins, which direct the RM blood supply through lateral heat exchanging *retia* (Bernal et al., 2001b; Burne, 1924; Carey and Teal, 1966; Carey and Teal, 1969a; Carey et al., 1985; Patterson et al.,

2011). This contrasts with most other fishes, in which the major conduits of the systemic circulation are situated medially, just ventral to the vertebral column. However, some tunas have only a central circulation that supplies blood to the RM via a central heat exchanging *retia*, and some *Thunnus* species lack a central *rete* and appear capable of adjusting the relative amount of systemic blood flow between the central circulation and the lateral heat exchanging *retia*, which should increase their capacity to physiologically thermoregulate (Bernal et al., 2001b; Bernal et al., 2017; Graham and Dickson, 2001; Sepulveda et al., 2008). Lamnid sharks may also be able to physiologically thermoregulate by redistributing the systemic blood flow in a similar way (Bernal and Lowe, 2015; Bernal et al., 2001a; Bernal et al., 2009). The RM heat exchanging *retia* establish elevated and relatively stable operating temperatures for the RM, which in some species creates a thermal excess of 10 to 20°C even in frigid, sub-polar water (Bernal and Lowe, 2015; Bernal et al., 2001b; Bernal et al., 2005; Carey and Teal, 1969b; Carey et al., 1971; Patterson et al., 2011; Sepulveda et al., 2008). The swordfish also has putative heat exchanging *retia* that supply blood to the medially located red aerobic swimming muscles, but these *retia* are not as efficient at heat retention as the red muscle *retia* of tunas and sharks and likely serve to just slow down cooling of the red muscle (Carey, 1990; Stoehr et al., 2018).

Tunas and lamnid sharks also possess heat exchanging *retia* in the orbital and visceral circulations, and orbital *retia* also conserve heat generated by the thermogenic organs of billfishes (Bernal et al., 2001b; Block, 1986; Block and Carey, 1985; Burne, 1924; Carey, 1982b; Carey et al., 1981; Fudge and Stevens, 1996; Linthicum and Carey, 1972; Wolf et al., 1988). In the case of the swordfish, the orbital *rete* maintains cranial temperature within a narrow range that is elevated as much as 12°C above ambient water temperature when swordfish are in deep, cool water during daylight hours (< 10°C) (Carey, 1982b; Carey, 1990). Likewise, the orbital

retia of tuna and lamnid sharks efficiently elevate eye and brain temperatures, as much as 18°C above ambient in Atlantic bluefin tuna, and 10 to 13°C above ambient in lamnid sharks (Figure 1.4) (Bernal and Lowe, 2015; Bernal et al., 2001b; Block and Carey, 1985; Linthicum and Carey, 1972). The ability to maintain warm brain and eyes should increase integration and processing of sensory information, and previous work on swordfish eyes has shown that the elevated temperature increases temporal resolution (Fritsches et al., 2005), which should provide regionally heterothermic fishes with crucial advantages over their thermoconforming prey (Block, 1986).

The smalleye Pacific opah is quite different from other regionally heterothermic fishes because it has a heat exchanging *rete mirabile* located inside each gill arch, each of which is relatively thick and insulated with fat (Wegner et al., 2015). These gill *retia* warm the arterial blood immediately after being oxygenated at the gills, warming the blood that then perfuses the entire body, including the heart (Wegner et al., 2015). Furthermore, an orbital *rete* enables further heat localization in the cranial region, causing it to be even warmer than the rest of the body (Runcie et al., 2009; Wegner et al., 2015). Cranial temperatures in the opah are also relatively constant and elevated at least 6°C above the ambient environment, while body and heart temperature are elevated at least 3 to 5°C (Figure 1.4) (Wegner et al., 2015).

The bigeye thresher shark (*Alopias* superciliosus) has been suspected to be capable of cranial endothermy because it has unusually large eyes and a putative orbital *rete* (Block and Carey, 1985; Block and Finnerty, 1994; Dickson and Graham, 2004; Weng and Block, 2004). However, two of my collaborators that were involved in the work included in Chapters 3 and 4 of this thesis, have made opportunist measurements of the eye temperature of bigeye threshers and found them to be no warmer than ambient sea surface temperature (Diego Bernal and

Chugey A. Sepulveda, personal communication). Furthermore, the pelagic thresher shark (*Alopias pelagicus*) also has an orbital *rete* (Block and Carey, 1985), the common thresher shark has a *rete* associated with the viscera, there are RM associated *retia* in the pectoral fins of the Chilean devil ray (*Mobula tarapacana*), and orbital *retia* are present in some species of mobulid rays (Alexander, 1995; Alexander, 1996; Fudge and Stevens, 1996), but it has yet to be confirmed if these *retia* have a thermoconserving function (Bernal and Lowe, 2015).

The heat exchanging *retia* of regionally heterothermic fishes as well as other regionally heterothermic vertebrates (e.g., some mammals and birds) cause blood-O_2 uptake to occur at a very different temperature to O_2 unloading in the tissues that are thermally isolated by a *rete*. In regionally heterothermic fishes, when blood flows through a *rete* into or out of the warmer tissues it is subjected to what has been described as "closed-system" temperature changes (Brill and Bushnell, 1991a). This is because the change in blood temperature can potentially affect blood PO_2 and PCO_2, but the content of blood gases should remain constant due to the size and thickness of *rete* vessels (i.e., arterioles and venules) that ought to preclude diffusion of gases out of the blood (Carey et al., 1985; Graham and Dickson, 2001; Stevens et al., 1974). Furthermore, most regionally heterothermic fishes are pelagic predators that subject themselves to varying water temperatures while moving above and below the thermocline or during latitudinal migrations (e.g., Bernal et al., 2009; Bernal et al., 2017; Block et al., 1992b; Block et al., 2001; Carey, 1990; Dewar et al., 2011; Sepulveda et al., 2010; Wegner et al., 2015; Weng et al., 2005). Thus, blood-O_2 uptake must occur over the range of temperatures encountered by a species, but the blood must also transport O_2 over steep internal temperature gradients, and O_2 offloading must occur from the lowest (usually at the gills) to the highest body temperatures (usually at the muscle).

1.2.3 Elevated metabolic rates and oxygen transport

Tunas and lamnid sharks exhibit elevated metabolic rates and a remarkable evolutionary convergence for "thunniform" swimming, which is due to a number of shared morphological and physiological traits, including internalized red muscle (Bernal et al., 2001b; Donley et al., 2004). Maintaining elevated red muscle temperatures also elevates metabolic rates, and tuna are often referred to as "high-energy-demand" or "high-performance" fishes because of their high metabolic rates and large aerobic scopes (Brill and Bushnell, 1991b; Korsmeyer and Dewar, 2001). Lamnid sharks also appear to have elevated metabolic rates compared to other sharks (Bernal et al., 2012; Ezcurra et al., 2012; Sepulveda et al., 2007b), and in both tunas and lamnids, the increased metabolic demand for O_2 has been matched by parallel increases in the structural and physiological factors of the O_2 transport cascade (Bernal et al., 2001b; Brill and Lai, 2015; Wegner, 2015; Wegner et al., 2010). This is especially apparent in the cardiovascular system of tunas (Brill and Bushnell, 2001).

Tunas have very high blood-O_2 carrying capacities due to high Hb concentrations that are comparable to mammalian values (Bernal et al., 2001b; Brill and Bushnell, 2001; Gallaugher and Farrell, 1998). Tunas also have gills with a large surface area and thin diffusion distances to increase O_2 extraction from the water, and large powerful hearts to circulate blood at high rates (Brill and Bushnell, 2001; Muir and Hughes, 1969; Wegner et al., 2006; Wegner et al., 2010). Thus, rates of circulatory O_2 delivery are exceptionally high in tuna. High Hb concentration in tunas and other regionally heterothermic fishes has been proposed to be necessary to supply O_2 at high rates while minimizing blood flow, which should match the high O_2 demands of heat production and help maintain elevated tissue temperatures by reducing circulatory heat loss (Carey and Gibson, 1983; Gibson and Carey, 1982). However, heat production and heat loss may

be independent of blood flow (Brill et al., 1994; Graham and Dickson, 2004), and Brill and Bushnell have proposed that the high Hb concentration facilitates high rates of circulatory O_2 delivery without unnecessarily increasing cardiac output when aerobic metabolism is elevated (Brill and Bushnell, 1991b; Brill and Bushnell, 2001). I tend to agree with Brill and Bushnell because tuna hearts appear to be extremely "specialised" for pumping large volumes of blood, and tuna Hb concentrations may have reached a level that cannot be surpassed without greatly increasing blood viscosity and causing cardiovascular impairment. Therefore, high blood-O_2 carrying capacities should match O_2 supply to O_2 demand while also maintaining sufficient O_2 stores to recover from strenuous swimming without imposing exhaustive demands on cardiac function. Furthermore, tuna have evolved certain traits to facilitate high rates of O_2 diffusion into the muscles (Korsmeyer and Dewar, 2001), and all studied species of tuna have a large Bohr coefficient (Brill and Bushnell, 1991a; Cech et al., 1984; Jones et al., 1986). The latter is very likely necessary to right-shift the OEC in the tissue capillaries to unload O_2 at high enough rates to meet the demand for O_2 without increased blood flow.

1.3 Review and critique of Hb-O_2 equilibrium studies of regionally heterothermic fishes

"Bluefin tuna hemoglobin, in contrast to all other vertebrate hemoglobins has an affinity for oxygen which is almost independent of temperature . . . Such low thermal coefficients make it seem possible that in the warm fishes part of the integration of life processes has been achieved by evolving systems with low sensitivity to temperature with the bluefin having in addition a fairly well-controlled temperature." (Carey and Lawson, 1973, p. 390)

It was not until 1973 that further research was published on the hæmoglobins of regionally heterothermic fishes (Andersen et al., 1973), 13 years after Rossi Fanelli and Antonini reported their findings on bluefin tuna hæmoglobin. This research was led by Quentin Gibson

and over the next decade Gibson and co-workers, often in collaboration with Frank Carey, further investigated the effect of temperature on hæmoglobin function in tunas and lamnid sharks. Since then, hæmoglobins with reduced thermal sensitivity have been reported from many species of regionally heterothermic vertebrates with heat-exchanging *retia*, including fishes and mammals (Weber and Campbell, 2011). Among these animals, relative increases in the enthalpy of hæmoglobin-oxygenation have convergently evolved by different molecular mechanisms. Here I will review the literature on the effect of temperature on Hb-O_2 affinity in tunas, billfishes, and lamnid sharks. I am not aware of any published studies on the temperature sensitivity of Hb from opah or thresher sharks.

1.3.1 Tuna

Atlantic bluefin tuna Hb is one of the most well studied fish Hbs, including structural and functional studies of ligand binding, as well as studies on the effect of temperature on Hb-O_2 affinity and the mechanism of the Root effect (Brunori, 1966; Carey and Gibson, 1977; Ikeda-Saito et al., 1983; Jensen, 2001; Morris and Gibson, 1982; Morris et al., 1981; Rodewald et al., 1987; Yokoyama et al., 2004). Seventeen years after the first report that Atlantic bluefin tuna Hb-O_2 affinity was unaffected by temperature, Carey and Gibson (1977) reported that the shape of the OEC for bluefin tuna Hb was unusually temperature dependent (Carey and Gibson, 1977; Rossi Fanelli et al., 1960). Carey and Gibson found that below 20% Hb-O_2 saturation, bluefin tuna hæmolysate had a higher O_2 affinity at 14°C than at 22°C, but above 20% saturation O_2 affinity was higher at 22°C causing the OECs for the two temperatures to crossover (i.e., switching from a normal to a reverse temperature-dependence). Similar reverse temperature effects were later reported for whole blood from Atlantic bluefin tuna (Carey and Gibson, 1983). In a study on the primary Hb isoform of Atlantic bluefin tuna (Hb I) at various pH levels and

temperatures, a saturation dependent reverse temperature effect (i.e., positive $\Delta H'$) was also observed, but the OECs at different temperatures crossed over around 50% saturation for Hb I (Figure 1.5) (Ikeda-Saito et al., 1983). The positive $\Delta H'$ is due to endothermic contributions from oxygenation-dependent Bohr protons released at high Hb-O_2 saturation (Ikeda-Saito et al., 1983). Organic phosphates (i.e., adenosine triphosphate, ATP) probably do not have an appreciable endothermic contribution to $\Delta H'$ since organic phosphates do not appear to influence Hb from both bluefin tuna and bigeye tuna (*Thunnus obesus*) (Andersen et al., 1973; Ikeda-Saito et al., 1983). The increased Hb-O_2 affinity at high saturation with increasing temperature is linked to an unusually high temperature dependency of the allosteric equilibrium constant, *L*, which defines the proportions of the T- and R-states ($L \propto \frac{T}{R}$) (Carey and Gibson, 1977; Carey and Gibson, 1983; Ikeda-Saito et al., 1983; Morris and Gibson, 1982). Low temperatures stabilize the low affinity T-state conformation (high *L*), preventing the T→R transition even at high O_2 saturation, owing to shifts between multiple T-state conformations that can be explained by an extended form of the MWC two-state model (Ikeda-Saito et al., 1983; Morris and Gibson, 1982; Morris et al., 1981). The large number of Bohr groups stabilize the T-state, but increasing temperature causes the release of Bohr protons that are bound at high O_2 saturation, decreasing *L* and increasing Hb-O_2 affinity (Ikeda-Saito et al., 1983).

Prior to most of this research on Atlantic bluefin tuna Hb, Andersen et al. (1973) investigated the temperature dependence of Hb-O_2 affinity in several regionally heterothermic teleosts and elasmobranchs, including the bigeye tuna. However, it is not clear if Andersen and coworkers experimented on whole blood or hæmolysates since they used both terms to describe the same set of experiments, and they did not report the sample pH for their OEC experiments. Nonetheless, they reported that the shape of bigeye tuna OECs were affected by temperature

(5°C, 15°C, 25°C, and 35°C), where the upper portion of the OECs at 5 and 15°C were relatively close together. This reduced effect of temperature at high O_2 saturation between 5 and 15°C was likely due to endothermic contributions to $\Delta H'$ from Bohr proton release and a decrease in L with increasing temperature, but temperature does not have as large an effect on L as it does on bluefin tuna Hb. Furthermore, the effect of temperature on L appears to be temperature dependent because the OECs at 25 and 35°C are quite right shifted compared to the 15°C OEC. Like bluefin tuna Hb, organic phosphate (ATP) did not influence bigeye tuna Hb (Andersen et al., 1973).

Also in 1973, Jeffrey Graham reported that preliminary experiments showed no pronounced effect of temperature on the O_2 affinity of whole blood from black skipjack tuna (*Euthynnus lineatus*), although he provided no data or description of the experiments (Graham, 1973). A couple of years later Gary Sharp reported O_2 equilibrium experiments for red blood cell (RBC) suspensions from yellowfin tuna (*Thunnus albacares*), albacore tuna (*Thunnus alalunga*), bigeye tuna, and a closely related ectothermic species, wahoo (*Acanthocybium solandri*) (Sharp, 1975). Sharp used relatively fresh blood samples from yellowfin and albacore tuna, but the bigeye tuna and wahoo samples were taken from deceased fish obtained at a fish market. The experiments were conducted on RBCs removed from blood plasma, rinsed, and resuspended in buffered glycerol solutions (i.e., not whole blood). Sharp provided complete OECs for a range of temperatures and pH levels, as well as P_{50} values and precise pH measurements made at 50% Hb-O_2 saturation (pH_{50}). However, the experimental pH treatments were very low, with pH_{50} values ranging from 7.44 to 6.69, which are now known to be well below physiologically routine arterial and venous levels for tuna (~7.7-7.8) (Bushnell and Brill, 1992; Jones et al., 1986; Korsmeyer et al., 1997a). The low pH levels for bigeye tuna and wahoo

RBC suspensions were attributed to the poor quality and relatively acidotic state of the blood (pH 6.7-7.0) sampled from fish caught at least 24 hours prior to blood withdrawal. The results of the O_2 equilibrium experiments showed that P_{50} was temperature independent for RBCs from yellowfin and albacore tuna, whereas increasing temperature increased P_{50} (i.e., decreased Hb-O_2 affinity) for bigeye tuna and wahoo. The yellowfin tuna OECs also crossed over around 50% Hb-O_2 saturation, whereby Hb-O_2 affinity increased with increased temperature (i.e., a reverse temperature-dependency) above 50% saturation, similar to what would be observed a few years later for bluefin tuna Hb. This observation prompted Sharp to note that "the effects of changes of parameters such as temperature on the dissociation properties of yellowfin hemoglobin could be misleading if only P_{50} values are evaluated" (Sharp, 1975, p. 684). The effect of temperature on the position of the OEC at O_2 tensions above and below P_{50} was an important observation that has been overlooked in more recent studies. Furthermore, although Sharp did not quantitatively assess the effect of temperature on the entire OEC he did provide enough figures and information that the appropriate data can be extracted from his study for further analysis.

Reduced and reversed effects of temperature on whole blood-O_2 affinity have been reported for quite a few species of tuna. A lot of the work on the blood respiratory properties of tuna has been led by Richard Brill and Peter Bushnell who, along with David Jones, were able to develop techniques to sample blood from resting and cannulated tuna, which is the best procedure for O_2 equilibrium experiments on whole blood from fish. In the following studies the effect of temperature on blood-O_2 affinity was determined by comparing only P_{50} values from OECs constructed with whole blood at different temperatures. Cech et al. (1984) reported a reverse temperature-dependency for albacore P_{50}, which appeared to be saturation dependent (*Thunnus alalonga*; 5, 10, 15, 20, and 25°C); Brill and Bushnell (1991a) reported that P_{50} was

independent of temperature for skipjack and yellowfin tuna (20 and 30°C); Lowe et al. (Lowe et al., 2000) reported that bigeye tuna P_{50} was "essentially unaffected" by temperature (15 and 25°C); Brill and Bushnell (2006) reported a slight reverse effect of temperature on P_{50} for Atlantic bluefin tuna (15 and 25°C); Clark et al. (2008a) studied blood from southern bluefin tuna (*Thunnus maccoyii*), and they found that temperature had a reverse effect on P_{50} between 10 and 23°C, and P_{50} was independent of temperature between 23 and 36°C; Lilly et al. (2015) reported temperature-independence and reverse temperature-dependence of P_{50} for Pacific bluefin tuna (*Thunnus orientalis*), and variable effect of temperature on yellowfin tuna P_{50}, including normal temperature-dependence, temperature-independence, and reverse temperature-dependence (RBCs rinsed and resuspended in buffered saline; 15, 20, 25, and 30°C).

The studies described in the previous paragraph provide good evidence (qualitative) that all tunas probably have Hbs with a reduced thermal sensitivity, albeit to varying degrees among species. However, the effect of temperature on blood-O_2 affinity was not quantitatively assessed in an appropriate way in any of those studies. Furthermore, except for the study by Brill and Bushnell (1991), the data presented by the authors offer limited opportunities for further analysis. I will outline the major reasons for these shortcomings because they are relevant to the results that I present in Chapters 3 and 4:

1. The authors of each study did not assess the effect of temperature on the shape of the OEC (i.e., above and below 50% Hb-O_2 saturation), even though previous studies have shown that the effect of temperature on Hb-O_2 affinity is dependent on Hb-O_2 saturation (Andersen et al., 1973; Carey and Gibson, 1977; Carey and Gibson, 1983; Ikeda-Saito et al., 1983; Sharp, 1975). 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 in teleosts (Brauner et al., 1996; Ikeda-Saito et al., 1983). Some of the authors listed above have even shown that in yellowfin tuna blood most of the oxygenation-dependent release of Bohr protons occurs between 40 and 100% O_2 saturation (Lowe et al., 1998).

2. Exemplary or representative OECs are presented in each study, but in some it is obviously apparent that the OEC is not a good fit to the data. This is likely due to imprecise measurements of Hb-O_2 saturation and/or blood PO_2, and not a poor choice of OEC model. Precise O_2 equilibrium measurements should yield data that take the form of an OEC, so an appropriate OEC model should exactly fit to the data, or at least fit reasonably well enough that most of the data points lie on the curve. However, in some of the representative OECs very few, and in some cases none, of the data points lie on the fitted OEC (e.g., Lowe et al., 2000).
3. The analyses were not appropriate for comparing P_{50} values at different temperatures. To compare among temperature treatments, the influence of blood pH on P_{50} must be controlled somehow. In all but one of the studies the authors reported that pH greatly affected P_{50}, as to be expected in teleosts. However, the authors reported a mean P_{50} for all blood samples included in each temperature and PCO_2 treatment (OECs were constructed at different PCO_2 treatments to manipulate blood pH), but the P_{50} values for all individuals in a treatment had different blood pH values. Cech and co-workers even stated that "[t]he variation in P_{50} seemed to stem primarily from small differences in blood pH measured for individual fish" (Cech et al., 1984, p. 25), yet they and others did not determine P_{50} at specific pH values from plots of logP_{50} vs pH (i.e., Bohr plots) as is standard practice (e.g., Fago et al., 1997; Hall, 1966; Lahiri, 1975; Larsen et al., 2003;

Reeves, 1980; Riegel et al., 1966; Weber et al., 1976; Weber et al., 2010). The "small differences in blood pH" that Cech et al. referred to were actually large differences, with blood pH ranging from 7.61 to 7.76, which is typical of most studies on fish blood. Lilly et al. (2015) did not measure the pH of their samples.

Because of these shortcomings, the summary data presented in these studies probably do not accurately describe the blood respiratory properties of each species, and additional information is needed to use the data for further analysis of the effect of temperature on blood P_{50}. The exception is Brill and Bushnell (1991a) who included a table of all P_{50} and pH values determined for each individual yellowfin and skipjack tuna that they studied. I have further analyzed these data in Chapter 2.

The effect of closed-system warming and cooling on blood PO_2 were assessed in some of the tuna whole blood studies, and these data are interesting and are worth brief discussion here because I present the same type of measurements in Chapters 3 and 4. In the absence of Hb, increasing temperature will considerably increase PO_2 in a closed-system due to a reduction in plasma O_2 solubility, and *vice versa* (i.e., Henry's law). If the effect of temperature on plasma O_2 solubility is removed from the total change in blood PO_2 (ΔPO_2), then it can be reasonably assumed that any change to blood PO_2 beyond what would be expected solely in plasma is due to temperature induced Hb-O_2 unloading or binding. In blood with a normal temperature-dependence, warming will decrease Hb-O_2 affinity and cause O_2 to dissociate from Hb and diffuse across the RBC membrane into the plasma, which will increase blood PO_2 beyond what would be expected due to the temperature dependence of plasma O_2 solubility. Cooling will have the opposite effect; Hb-O_2 affinity will increase and promote Hb-O_2 binding, which will decrease blood PO_2 because O_2 will diffuse form plasma into the RBCs. Temperature-independent Hb-O_2

affinity would cause $\Delta P O_2$ to be dependent only on the temperature-dependence of plasma O_2 solubility, while reverse-temperature dependent Hb-O_2 affinity would cause blood PO_2 to decrease with warming, but increase with cooling. In species where the temperature-dependence of Hb-O_2 affinity is saturation dependent (i.e., when the OECs crossover), $\Delta P O_2$ would also be expected to be saturation dependent.

Joseph Cech and coworkers initiated these closed-system experiments to test Jeffrey Graham's hypothesis that "temperature-insensitive hemoglobin has evolved in tuna to eliminate the problem of premature oxygen dissociation as blood crosses a thermal gradient [in a heat exchanging *rete mirabile*] en route to the muscles" (Graham, 1973, p. 1967). They reported that albacore blood equilibrated at different PO_2's and at 10 or 25°C then rapidly warmed to 30°C in a closed-system caused a reduction of blood PO_2, the opposite effect for blood equilibrated at 30°C then cooled to 10°C. These results are not unexpected given that Cech and coworkers observed a reverse temperature-dependence in their whole blood OECs. David Jones and coworkers did similar experiments with blood from kawakawa (also known as mackerel tuna; *Euthynnus affinis*), but they used dorsal aortic blood from fish maintained at 25°C and then warmed the blood to 35°C (Jones et al., 1986). They reported that warming dorsal aortic blood caused PO_2 to increase by about 25%, so Jones et al. concluded that "our tuna can live quite happily in the absence of any advantage conferred by reversed thermal effects" (Jones et al., 1986, p. 210). Brill and Bushnell (1991a) reported that skipjack tuna blood had a low sensitivity to closed-system temperature changes, but yellowfin tuna blood had a normal temperature-dependence. Bigeye tuna blood also exhibited a normal temperature-dependence during closed-system temperature changes, whereas Atlantic bluefin tuna blood exhibited a reverse temperature-dependence like albacore blood (Brill and Bushnell, 2006; Lowe et al., 2000). The

different closed-system results for skipjack, yellowfin, and bigeye tuna seem to have perplexed the researchers that made those observations (Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Lowe et al., 2000). However, in those studies the effect of temperature on plasma O_2 solubility and thus, plasma PO_2 was not considered.

In Figure 1.6, I have replotted curves that were fit to the closed-system PO_2 data for skipjack tuna, yellowfin tuna, and bigeye tuna, as reported by Brill and Bushnell (2006), but I have also included the expected change to plasma PO_2 due to the temperature dependence of plasma O_2 solubility. Skipjack blood PO_2 changed less than would be expected due to the temperature dependence of plasma O_2 solubility, yellowfin tuna plasma PO_2 closely follows the expected change, and bigeye tuna PO_2 exceeds the expected change. This indicates a reverse temperature-dependence of O_2 affinity in skipjack blood, temperature-independence in yellowfin tuna blood, and normal temperature-dependence in bigeye tuna blood. Whole blood-O_2 affinity (i.e., P_{50}) exhibits a reverse temperature-dependence in skipjack tuna, and is temperature-independent in yellowfin tuna (Brill and Bushnell, 1991a). Therefore, closed-system temperature changes to blood PO_2 in skipjack and yellowfin tuna are qualitatively like the temperature-dependence of blood P_{50}. The same is true of bigeye tuna blood, although further explanation is justified here.

Bigeye tuna blood P_{50} was reported to be almost temperature-independent and quite low (i.e., high Hb-O_2 affinity), which was interpreted to benefit O_2 loading during dives to deep, cold and hypoxic water (Lowe et al., 2000). However, the large change to blood PO_2 with closed-system warming was interpreted to benefit O_2 unloading to the red muscle, despite the finding of temperature-independent Hb (Lowe et al., 2000). This interpretation would require large changes of $\Delta H'$ between the gills and the systemic circulation, although this is not consistent with the data

and the physiological mechanisms have never been proposed. Lowe et al. (2000) presumed that bigeye tuna has a high blood-O_2 affinity based on their reported P_{50} values of 12.5 mmHg (pH 8.05) at 15°C, and 15.9 mmHg (pH 8.08) at 25°C. However, these P_{50} values were determined at a high blood pH. The authors also reported values at more physiologically relevant pH values: 26.7 mmHg (pH 7.710) at 15°C, and 29.7 (pH 7.780) mmHg at 25°C. If these values are adjusted with their reported Bohr coefficients to a common pH of 7.71, then the P_{50} at 25°C is 34.2 mmHg. The $\Delta H'$ value for this temperature change is -18 kJ mol^{-1}, which is relatively reduced compared to many other vertebrates, but is normal relative to other tuna (i.e., increasing temperature increases P_{50}). Therefore, the effect of closed-system temperature changes on bigeye tuna blood PO_2 is similar to the effect of temperature on whole blood-O_2 equilibria.

1.3.2 Billfishes

Andersen et al. (1973) reported that increased temperature considerably right-shifted swordfish OECs (5, 25, and 35°C), and ATP had a marked effect on swordfish hæmolysates. The major Hb component from striped marlin (*Kajikia audax*) also exhibited a normal temperature-dependence (Brittain, 1986), but Roy Weber and coworkers found that adding ATP to hæmolysates of striped marlin caused temperature-independence of P_{50} (10 and 25°C) within a physiologically relevant pH range. The presence of ATP had a similar effect on the temperature-dependence of shortbill spearfish (*Tetrapturus angustirostris*) hæmolysate P_{50}, but reversed the temperature-dependence of blue marlin (*Makaira nigricans*) hæmolysate P_{50} (10 and 25°C). However, Weber et al. did not assess the effect of temperature on the shape of the OEC. Like tuna, the temperature-dependence of billfish Hb is reduced by oxygenation-linked dissociation of allosteric effectors that contribute endothermically to $\Delta H'$. Unlike tuna, however, the primary

effector of billfish Hb is ATP, with minor enthalpic contributions from other allosteric effectors (likely protons) (Weber et al., 2010).

1.3.3 Sharks

Hæmoglobins from the shortfin mako shark and the porbeagle shark were also included in the study by Andersen et al (1973). Mako OECs showed a reduced temperature-dependence with no effect of temperature on the shape of the OEC (5, 15, 25, and 35°C), whereas porbeagle OECs showed a reduced temperature-dependence at low O_2 saturations, but a reverse temperature-dependence at high O_2 saturations causing the OECs to crossover around 50% O_2 saturation, like those of Atlantic bluefin tuna. Dickinson and Gibson (1981) reported similar temperature-independence for Hb from the salmon shark (*Lamna ditropus*; described as the Pacific porbeagle shark, *Lamna ditrotus*), and for both porbeagle and salmon shark Hb temperature-independence is due to a decrease in the allosteric equilibrium constant, *L*, with increasing temperature (Andersen et al., 1973; Dickinson and Gibson, 1981). Further work in Roy Weber's lab by Larsen et al. (2003) showed that ATP induced the reverse temperature-dependence of porbeagle Hb (Figure 1.5). Porbeagle Hb has a high intrinsic O_2-affinity with a normal temperature-dependence, as appears to be the case for most elasmobranch Hbs (Morrison et al., 2015), but the addition of ATP to stripped Hb reduces the O_2-affinity and reverses the effect of temperature. Like bluefin tuna Hb, the reverse temperature-dependence of porbeagle Hb stems from a reduction in *L* with increasing temperature and a stabilization of the T-state at low temperature. In porbeagle Hb, the presence of ATP decreases both the T-state association constant (K_T) and the R-state association constant (K_R), but causes K_T to become temperature invariant while K_R decreases with increasing temperature. Stabilization of the T-state at low temperature is due to preferential binding of ATP to the T-state relative to the R-state, but as

temperature increases the T-state becomes less stable and a T→R transition occurs, promoting the release of ATP and protons. The release of these allosteric effectors contributes endothermically to $\Delta H'$, causing it to become positive.

I am aware of only one study that has investigated the effect of temperature on whole blood-O_2 affinity of a regionally heterothermic shark. Bernal and coworkers reported a very reduced effect of temperature on the blood-O_2 affinity, almost temperature-independent, for the shortfin mako shark (15, 20, and 25°C). The effect of temperature on blood-O_2 affinity appeared to be saturation dependent, but not to the same extent as previously reported for the porbeagle shark and the bluefin tuna, and closed-system warming of mako blood caused a slight reduction in blood PO_2 (Bernal et al., 2018). Bernal et al. also reported sizeable Bohr coefficients from mako blood, which contrasts with the pH-independence of blood-O_2 affinity reported by Wells and Davie (1985) for mako blood at 25°C..

1.3.4 Hypotheses for the functional significance of reduced- and reversed-temperature dependence of Hb-O_2 affinity in regionally heterothermic fishes

Reductions or reversals in the temperature-dependence of Hb-O_2 affinity have been reported for many regionally heterothermic vertebrates with heat-exchanging *retia*, including fishes, mammals, and birds (Weber and Campbell, 2011). Among these animals, relative increases in the enthalpy of hæmoglobin-oxygenation have convergently evolved by different molecular mechanisms. However, some non-heterothermic vertebrates also have Hbs with a reduced-temperature dependency, including some poikilothermic teleosts and sharks (e.g., Bernal et al., 2018; Clark et al., 2010; Hopkins and Cech, 1994). Although this trait is not exclusive to regionally heterothermic vertebrates, it does seem to be shared by them. Several

hypotheses have been proposed for the functional significance of reduced- and reversed-temperature dependent Hb-O_2 affinity, which I will review.

Environment temperature and O_2 loading

As I explained in section 1.1.3.2, the first report of temperature-independent Hb-O_2 affinity was made by Rossi Fanelli and Antonini (1960), and they proposed that this trait may enable O_2 uptake as tuna swim through varying water temperatures. This trait is not exclusive to regionally heterothermic vertebrates as some non-heterothermic vertebrates also have Hbs with a reduced-temperature dependence, including some pelagic teleosts and sharks (e.g., Bernal et al., 2018; Clark et al., 2010; Hopkins and Cech, 1994). This trait may therefore benefit O_2 loading at different environmental temperatures, but this does not explain why this trait is possibly shared by all regionally heterothermic vertebrates, including mammals and birds. Furthermore, some species such as the circumtropical yellowfin tuna infrequently descend below the thermocline, so their realized thermal niche is warmer and narrower (~ 17-24°C) relative to that of the Atlantic bluefin tuna, which subjects itself to a wide range of water temperatures during migrations and descents below the thermocline (Bernal et al., 2009; Bernal et al., 2017; Block et al., 2001). Thus, the functional convergence for reduced temperature-dependent Hb-O_2 affinity among regional heterotherms is likely related to the internal temperature gradients caused by heat-exchanging *retia*.

Premature O_2 unloading in the retia causing an arterio-venous PO_2 gradient

Jeffrey Graham "proposed that the function of temperature-independent hemoglobin is to ensure the efficient delivery of oxygen to muscle tissue by preventing the premature unloading of this gas in the rete or outer layers of the muscle mass" (Graham, 1973, p. 1964). He hypothesized

that Hb with a normal temperature-dependence would progressively unload O_2 as the blood is warmed in a *rete*, possibly lowering the amount of O_2 in blood before its reaches the muscles (Graham, 1973). Carey and Gibson further explained that if tuna Hb had a normal temperature dependence, then "as the cold arterial blood coming from the gills was warmed in the heat exchanger the partial pressure of oxygen would rise while at the same time the partial pressure in the venous blood would fall as it cooled, both effects operating to increase the head of oxygen pressure transferring oxygen from the arterial to the venous blood and so to decrease respiratory efficiency" (Carey and Gibson, 1977, p. 1377). It seems unlikely that arterio-venous O_2 diffusion would occur because the heat-exchanging *retia* are composed of vessels that resemble arterioles and venules. The size of the arterioles and venules and the diffusion distance between them are about an order of magnitude greater than the vessels of a *rete* specialized for gas exchange (e.g., the swim bladder *rete*) (Carey et al., 1985; Graham and Dickson, 2001; Lemons et al., 1987; Stevens et al., 1974). However, even though arterio-venous O_2 diffusion likely does not occur, reverse temperature-dependence may be important for preventing premature O_2 unloading in species that maintain a large thermal excess between the gills and warm muscles (~10-20°C); species such as the bluefin tunas, the porbeagle sharks, and the salmon shark. However, it should only take seconds for the blood to move from a *rete* to the tissue capillaries (Brill and Bushnell, 2001), and the amount of O_2 that would be unloaded from Hb with a normal temperature-dependence and warmed by 10°C corresponds to a decreases in Hb-O_2 saturation of less than 0.3% [calculated from closed-system warming of big-eye tuna blood from 15 to 25°C (Lowe et al., 2000)], which seems unlikely to negatively affect circulatory O_2 delivery.

Energy conservation and maintenance of O_2-supply to tissues of different temperatures

Reductions in the temperature-dependence of Hb-O_2 affinity have also been proposed to save energy and prevent excessive leftward shifts of the OEC, and thus maintain O_2 unloading, as blood flows from the warm body to the colder legs or appendages of regionally heterothermic mammals (e.g., Brix et al., 1989a; Brix et al., 1989b; Giardina et al., 1989a; Giardina et al., 1989b). An increased $\Delta H'$ would require less energy to bind and unload O_2, which has been regarded as a potential energy saving mechanism in arctic mammals (Brix et al., 1989a).

It may seem intuitive that a normal temperature-dependence would enhance O_2 transport since a left shifted OEC at the cold gills should benefit O_2 uptake, and a right shifted OEC in the warm tissues should benefit O_2 unloading. However, a normal temperature-dependence could impair O_2 unloading to the colder tissues in a regional heterotherm. Temperature-independence of Hb-O_2 affinity would prevent temperature from directly influencing P_{50} in the warm and cold tissues of regionally heterothermic fishes, and a reverse temperature-dependence may actually enhance O_2 unloading from the venous return to the myocardium in the cold hearts of tunas, billfishes, and sharks (Clark et al., 2008a). An increased $\Delta H'$ would also reduce the energy required for O_2 unloading in the cold peripheral tissues of regionally heterothermic fishes, and temperature independence of P_{50} should prevent liberation of heat upon O_2 binding at the gills (Weber and Fago, 2004; Weber and Wells, 1989). These ideas are further explored in the following chapters.

1.4 Thesis objectives and organisation

I have developed two hypotheses that underlie the objectives of this thesis: 1) temperature-independent Hb-O_2 affinity conserves heat-energy, which may help maintain stably elevated body temperatures in regionally heterothermic fishes. Thus, I expected this trait to be shared by all regionally heterothermic teleosts and sharks; and 2) the Hb-oxygen-binding

properties and high Hb concentrations of regionally heterothermic fishes maintain matching between O_2 supply and O_2 demand despite large internal temperature gradients. I will briefly explain the justification for these hypotheses before stating my research objectives and questions.

Hæmoglobin of regionally heterothermic fishes must transport O_2 despite internal temperature gradients. Heat is also transported by Hb, and because heat absorbed during O_2 unloading is liberated upon O_2 binding at the gills, Hb contributes to heat loss to the environment. However, temperature-independent Hb-O_2 affinity would eliminate heat absorption by Hb upon O_2 unloading, preventing Hb-heat transport away from the warm tissues. Moreover, reductions and reversals in the temperature dependence of Hb-O_2 affinity may prevent unwanted temperature-induced shifts of the OEC, maintaining O_2 unloading to cold tissues with a high metabolic demand such as the heart. Therefore, it would be expected that all regionally heterothermic fishes would have Hbs with a reduced or reverse temperature-dependence to conserve metabolic heat in the warm tissues (hypothesis 1), and match O_2 supply with O_2 demand in their warm and cold tissues (hypothesis 2). Furthermore, blood-O_2 transport depends on Hb concentration and Hb-O_2 affinity, both of which influence the O_2 capacitance of the blood (βbO_2). The evolution of endothermy is associated with increased metabolic rates (Block and Finnerty, 1994; Brill, 1996; Ciezarek et al., 2019; Sepulveda et al., 2007b), and regionally heterothermic fishes have high Hb concentrations that are necessary to achieve high rates of circulatory O_2 delivery without unnecessarily increasing cardiac output (Brill and Bushnell, 1991b; Gibson and Carey, 1982). Tunas exhibit exceptionally high metabolic rates (Korsmeyer and Dewar, 2001), and I hypothesize that during aerobically sustained exercise they depend on a high βbO_2 to unload O_2 to the swimming muscles (hypothesis 2). In this thesis, the general objectives that are addressed in the subsequent four chapters are outlined by the following

questions: 1) How do Hb concentration and Hb-O_2 affinity contribute to the determinants of maximal O_2 consumption, circulatory O_2 delivery and tissue O_2 extraction, in the yellowfin tuna? 2) How does the enthalpy of Hb-oxygenation influence O_2 and heat transport? 3) How does temperature affect Hb-O_2 affinity in the swordfish and the smalleye Pacific opah? And 4) How does temperature affect Hb-O_2 affinity in the common thresher shark and the bigeye thresher shark?

1.4.1 How do Hb concentration and Hb-O_2 affinity contribute to the determinants of maximal O_2 transport in the yellowfin tuna?

Sensitivity analyses of the structural and physiological factors that determine maximal O_2 transport ($\dot{M}O_2max$) have been conducted for species representing all the major extant tetrapod clades, but no analysis of the O_2 transport system of fishes has been published. The yellowfin tuna is an excellent model to test for O_2 transport limitations to maximal O_2 flux, because the yellowfin tuna has a relatively high $\dot{M}O_2max$ and all components of the O_2 transport cascade appear to have evolved in parallel to maintain matching between O_2 supply and demand (Brill and Bushnell, 2001; Korsmeyer and Dewar, 2001; Wegner et al., 2010). Furthermore, the yellowfin tuna has high Hb concentration and a relatively large Bohr effect (Brill and Bushnell, 1991a), both of which will contribute to a high βbO_2 and are likely important for enhancing circulatory O_2 delivery and tissue O_2 extraction.

In Chapter 2 I have used a mathematical model of the fish O_2 transport cascade to quantitatively assess the possible contributing factors to O_2 supply limitation at $\dot{M}O_2max$ in yellowfin tuna. In doing this, I have also quantified how Hb concentration and Hb-O_2 affinity influence circulatory O_2 delivery and tissue O_2 extraction. Because the yellowfin tuna has a relatively large mixed venous O_2 reserve at $\dot{M}O_2max$, I hypothesized that tissue O_2 extraction

may be constrained by βbO_2, which will cause an apparent tissue O_2-diffusion limitation in the model.

1.4.2 How does the enthalpy of Hb-oxygenation influence O_2 and heat transport?

The thermodynamics of Hb-oxygenation mandate that heat energy is absorbed and released by the circulating Hb. Reductions and reversals of $\Delta H'$ should reduce or eliminate the heat absorbed during Hb-O_2 unloading in the warm tissues of regionally heterothermic fishes (Weber and Wells, 1989). Furthermore, temperature-independence and reverse temperature-dependence of Hb-O_2 affinity should prevent excessive temperature induced shifts to the physiological OEC between tissues maintained at very different temperatures (e.g., >10°C difference), possibly enhancing O_2 unloading to cold organs such as the heart. Although these concepts are intuitively obvious, the effect of the temperature-dependence of Hb-O_2 affinity on heat-conservation and blood PO_2 over a large internal temperature gradient have not been quantitatively evaluated.

In the second part of Chapter 2, I address the question of how does the enthalpy of Hb-oxygenation influence O_2 and heat transport? This was investigated using previously published data on tuna blood-O_2 affinity to calculate the heat-energy transported by Hb, and to calculate blood PO_2 (~P_{50}) over a range of temperatures and $\Delta H'$ values. I hypothesized that since temperature-independent Hb-O_2 affinity will prevent a left-ward shift of the OEC with decreasing temperature, then blood PO_2 at approximately 50% Hb-O_2 saturation, and thus Hb-O_2 unloading, will remain relatively constant over a wide range of body temperatures (15-30°C).

1.4.3 How does temperature affect Hb-O_2 affinity in the swordfish and the smalleye Pacific opah?

Hæmoglobins from regionally heterothermic tunas and billfishes (Istiophoridae) exhibit reduced temperature-dependence, temperature-independence, or reverse temperature-dependence. The swordfish is closely related to billfishes in the family Istiophoridae, but the limited available evidence indicates that swordfish Hb may have a normal temperature-dependence (Andersen et al., 1973). However, since Weber et al. (Weber et al., 2010) found that ATP induced temperature-independence of Hb-O_2 affinity in billfishes, further investigations of swordfish Hb are warranted. The smalleye Pacific opah is another regionally heterothermic teleost, but I am not aware of any published studies on the effect of temperature on Hb-O_2 affinity in the opah.

In Chapter 3 I address the following question: How does temperature affect Hb-O_2 affinity in the swordfish and the smalleye Pacific opah? If temperature-independent Hb-O_2 affinity is necessary to maintain regional heterothermy, then I expect that both the swordfish and the opah should have Hbs that are insensitive to temperature. I address this by constructing OECs at different temperatures in whole blood and hæmolysates, and by measuring the effect of closed-system temperature changes on blood PO_2.

1.4.4 How does temperature affect Hb-O_2 affinity in the common thresher shark and the bigeye thresher shark?

At least three species of regionally heterothermic sharks in the family Lamnidae have Hbs with a reduced or reversed temperature-dependence. However, the effect of temperature on Hb-O_2 affinity has not been investigated in the common thresher shark or the bigeye thresher

shark. Moreover, the temperature sensitivity of shortfin mako Hb has been previously studied, but a more thorough investigation is warranted to be able to quantify the effect of temperature on mako Hb-O_2 affinity.

In Chapter 4, I address the question of how does temperature affect Hb-O_2 affinity in the common thresher shark and the bigeye thresher shark? If temperature-independent Hb-O_2 affinity is necessary for sharks to maintain regional heterothermy, then like my expectations for the regionally heterothermic teleosts in Chapter 3, I also expect that the common thresher shark will have Hbs that are insensitive to temperature. The ecology and vascular anatomy of the bigeye thresher shark make for a curious case. The bigeye thresher makes diel movements from warm water above the thermocline to cold water deep below thermocline, and it also has an orbital *rete*, although its function is unclear. Therefore, the temperature-dependence of Hb from the bigeye thresher may provide some insight into the functional significance of temperature-independent Hb-O_2 affinity in regionally heterothermic fishes.

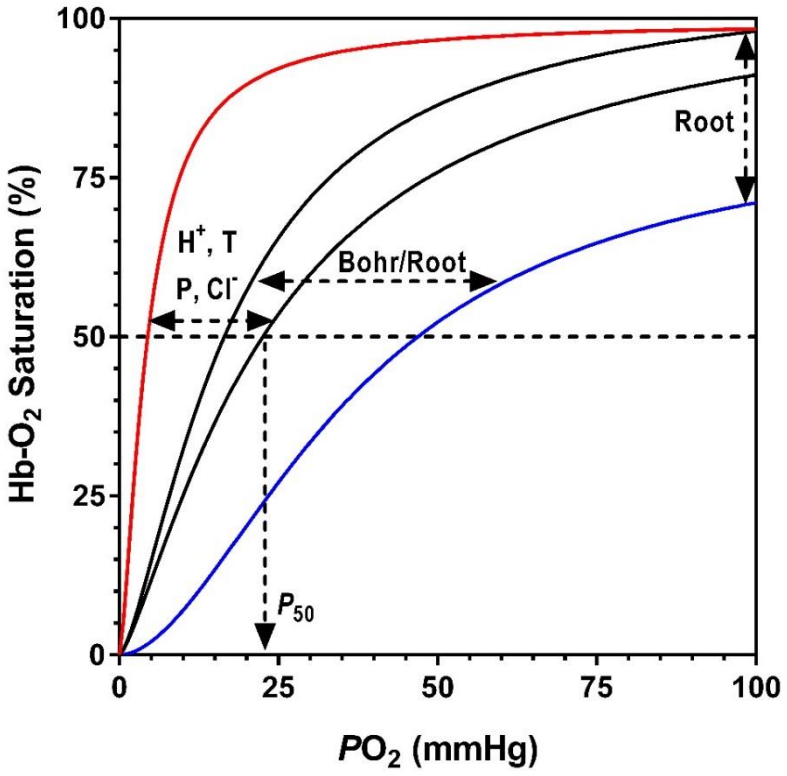

Figure 1.1 Oxygen equilibrium curves (OECs) showing that allosteric effectors and increased temperature decrease hæmoglobin (Hb)-O_2 affinity and right shift the OEC.

Hb-O_2 affinity is quantified as P_{50}, the partial pressure of O_2 at 50% Hb-O_2 saturation. The red OEC farthest to the left represents the intrinsic O_2 affinity (pH 7.4) of a stripped hæmolysate (i.e., purified of endogenous allosteric effectors). The two black OECs in the middle are for whole blood, and are right shifted relative to stripped Hb due to binding of allosteric effectors (heterotropic interactions), which include hydrogen ions (H^+), chloride ions (Cl^-), and organic phosphates (P). Left and right shifts of the physiological OEC are primarily due to the Bohr effect (i.e., oxygenation linked binding of H^+ ions) and changes in temperature (T). Some teleost Hbs exhibit a Root effect, which is represented by the blue OEC to the far right. Below a threshold pH level, the Root effect is generally characterized by a very right-shifted OEC, a loss of cooperative O_2-binding (homotropic interactions), and the inability to fully saturate Hb.

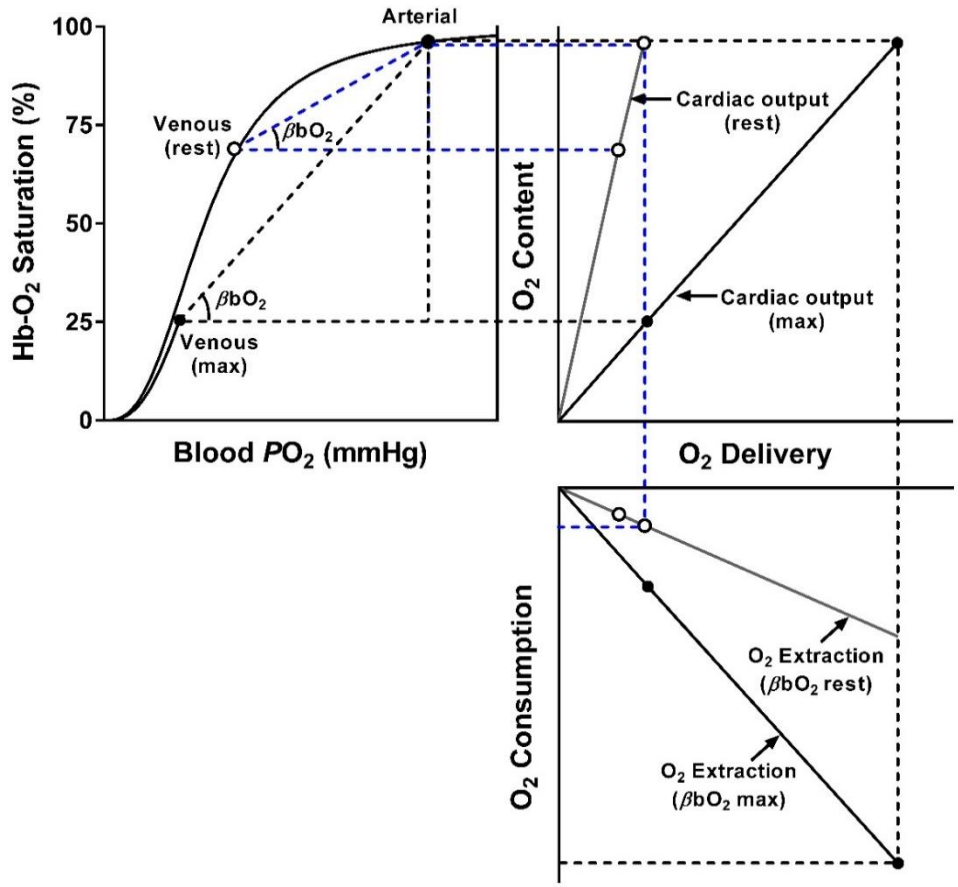

Figure 1.2 The interrelationships of the determinants of oxygen transport.

Circulatory O_2 transport and tissue O_2 extraction govern rates of O_2 consumption. Circulatory O_2 delivery is the product of the arterial O_2 content and cardiac output. The O_2 capacitance of the blood (βbO_2) is the slope of the functional portion of the oxygen equilibrium curve, and is an important determinant of tissue O_2 extraction (the O_2 content removed from the blood expressed as a percentage of arterial O_2 content). Hæmoglobin is an important determinant of both circulatory O_2 delivery and tissue O_2 extraction because blood Hb concentration determines arterial O_2 content, and left and right shifts of the OEC influence βbO_2. For a given cardiac output, O_2 transport depends on arterial O_2 content and βbO_2. The plots were constructed with data from humans at rest and exercising at their maximum rates of O_2 consumption (Sun et al., 2000).

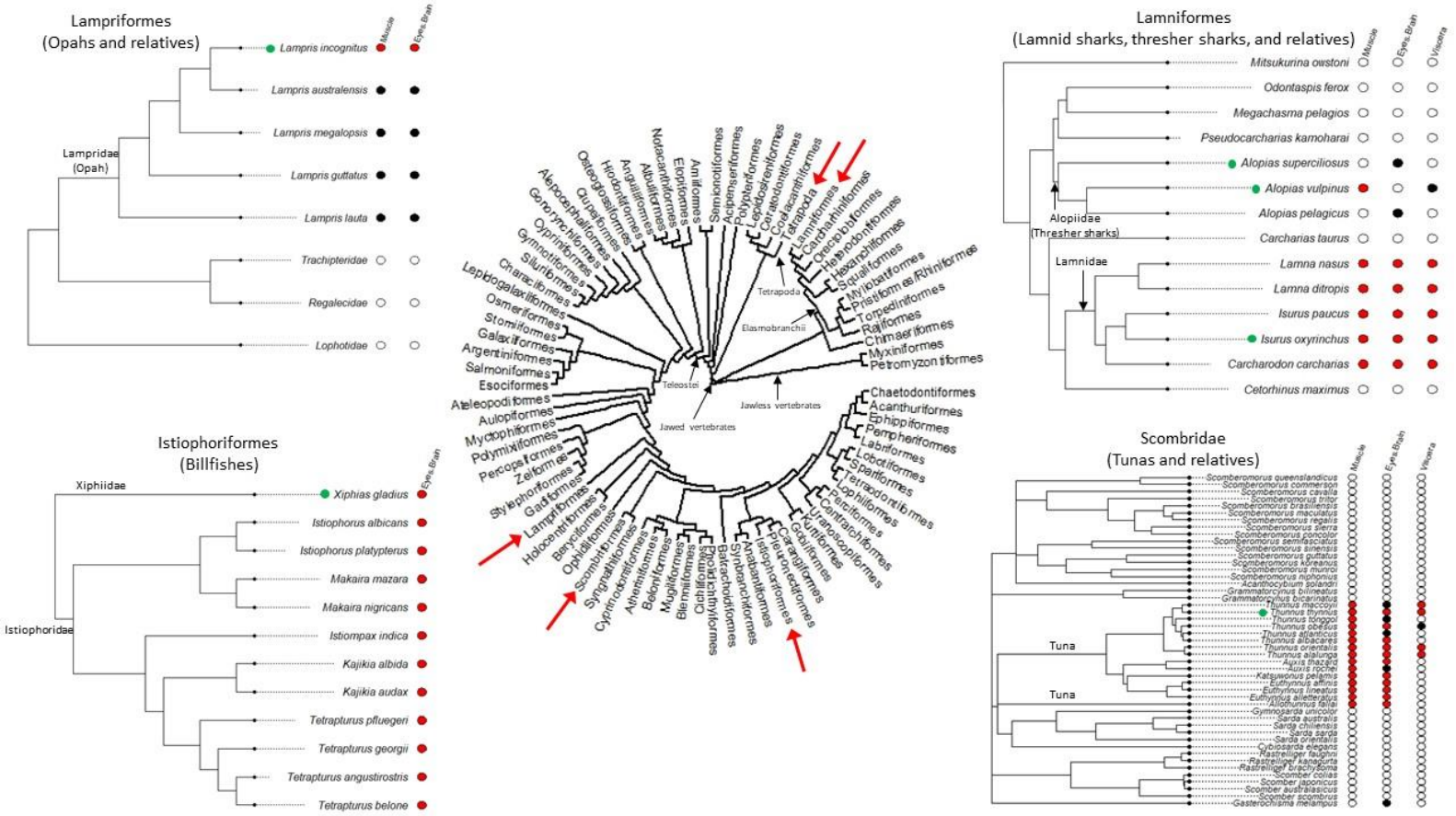

Figure 1.3 Proposed phylogenetic relationships among regionally heterothermic fishes.

Red arrows indicate orders (plus tetrapod) that have evolved endothermy. Red circles indicate the ability of a species to elevate the temperature of the specified tissue; black circles indicate a species suspected or assumed to be able to elevate tissue temperature; white circles indicate ectotherms. Green circles indicate species studied in this thesis. The phylogenetic relationships are based on those of Kumar et al. (2017), Santini and Sorenson (2013), Santini et al. (2013), Sorenson et al. (2014), and Underkoffler et al. (2018).

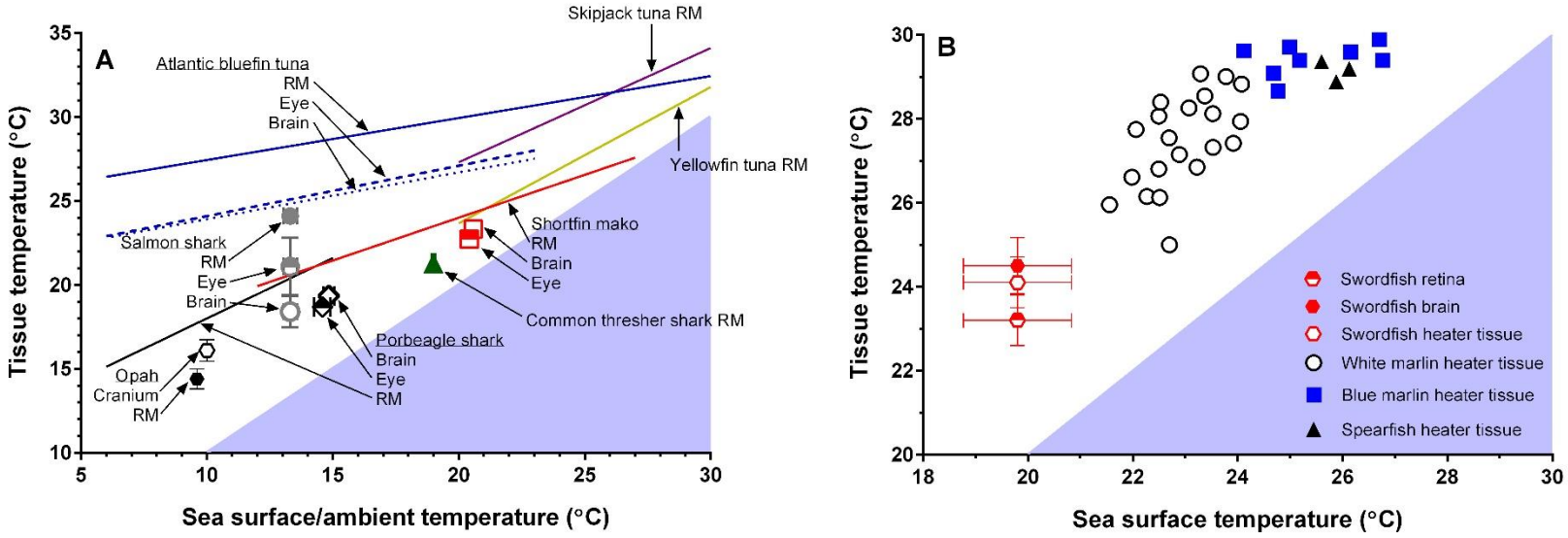

Figure 1.4 Endothermic tissue temperatures in regionally heterothermic teleosts and sharks.

(A) Red muscle (RM) and cranial temperatures of representative regionally heterothermic fishes described in Chapter 1. Tissue temperatures are plotted as function of sea surface temperature (tunas and sharks) or ambient temperature (opah). (B) Heater organ temperature relative to sea surface temperature in billfishes. The white regions represent temperatures above ambient water temperature. Figures A and B recreated from Bernal et al. (2009) and Block (1990), respectively, with data from the following: Barrett and Hester (1964) (yellowfin tuna, *Thunnus albacares*, RM); Carey and Teal (1969b) (Atlantic bluefin tuna, *Thunnus thynnus*, and skipjack tuna, *Katsuwonus pelamis*, RM); Linthicum and Carey (1972) (Bluefin tuna eye and brain); Block and Carey (1985) and

Bernal et al. (2001b) (porbeagle shark, *Lamna nasus*, and shortfin mako shark, *Isurus oxyrinchus*); Bernal and Sepulveda (2005) and Patterson et al. (2011) (common thresher shark, *Alopias vulpinus*); Anderson and Goldman (2001) (salmon shark, *Isurus oxyrinchus*); Wegner et al. (2015) (smalleye Pacific opah, *Lampris incognitus*); Carey (1982) (swordfish, *Xiphias gladius*); Block (1990) (white marlin, *Kajikia albida*, blue marlin, *Makaira nigricans*, spearfish, *Tetrapturus angustirostris*).

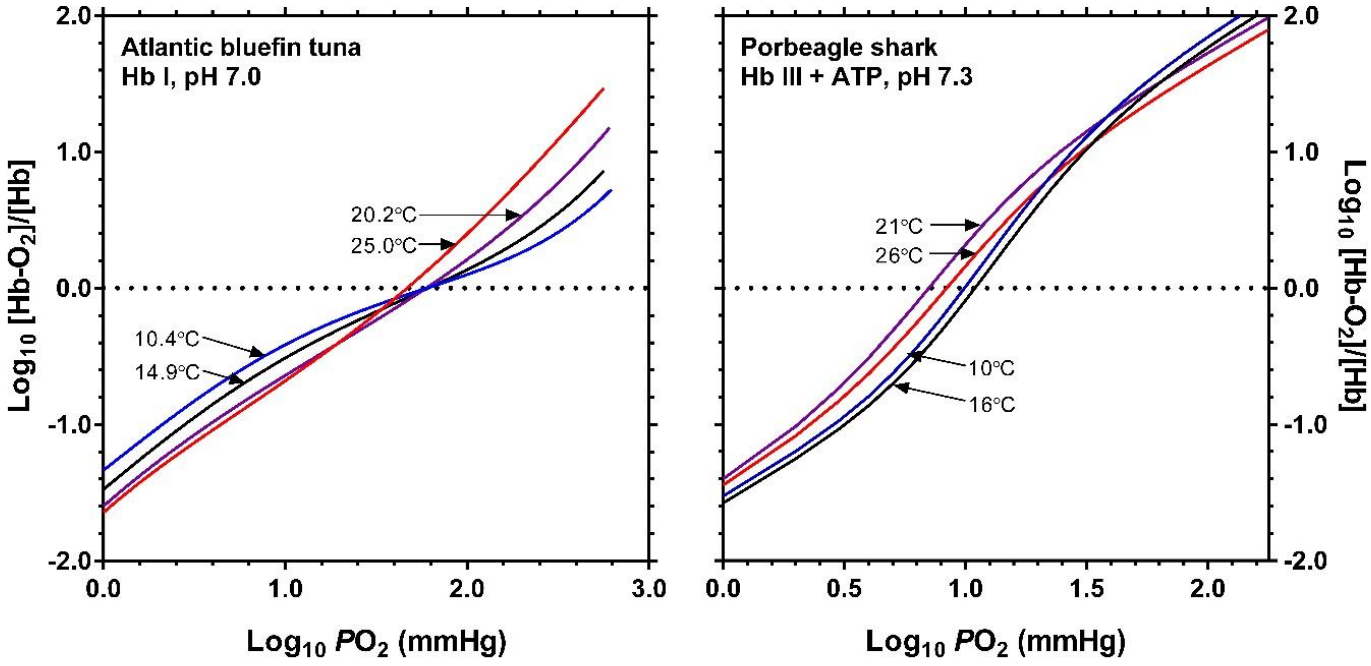

Figure 1.5 Hill plots of oxygen equilibria of Hb from the Atlantic bluefin tuna and the porbeagle shark

Atlantic bluefin-tuna O_2 equilibria for Hb component I were at pH 7.0, and show the temperature-independence at 50% Hb-O_2 saturation and a reverse temperature-dependence above 50% saturation (modified after Ikeda-Saito et al., 1983). Porbeagle shark O_2 equilibria for Hb component III were at pH 7.4 in the presence of ATP, and show a reverse temperature-dependence below ~75% Hb-O_2 saturation and the crossing over of the OECs and almost temperature independence above ~75% saturation (constructed with parameters reported by Larsen et al., 2003).

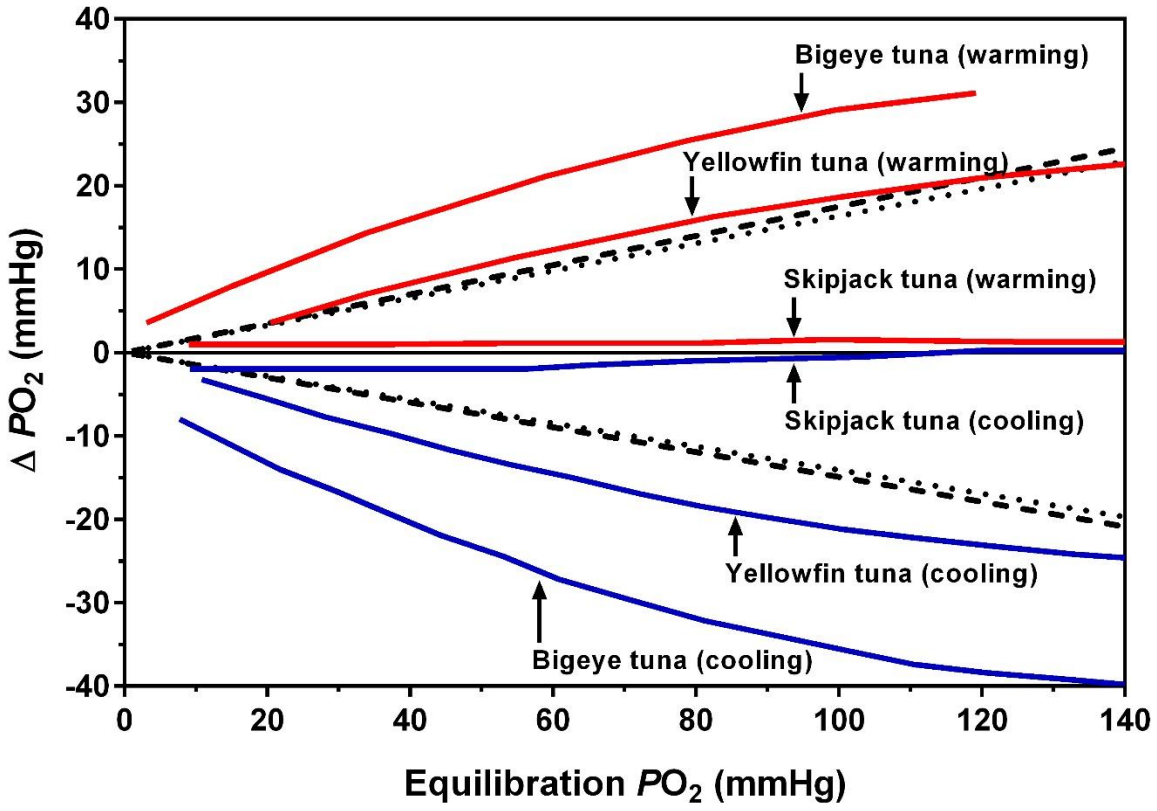

Figure 1.6 Effects of closed-system temperature changes on the measured change in blood PO_2 (ΔPO_2) in bigeye tuna, yellowfin tuna, and skipjack tuna.

This figure was created from a figure in Brill and Bushnell (Brill and Bushnell, 2006), with data from Brill and Bushnell (1991a) and Lowe at al. (2000). Tuna blood was equilibrated at a range of O_2 tensions (Equilibration PO_2) and then heated (red curves) or cooled (blue curves). Bigeye tuna blood temperature was changed between either 15 and 25°C, and skipjack and yellowfin tuna blood temperatures were changed between 20 and 30°C (circles). Black lines indicate the temperature induced ΔPO_2 expected due to changes in solubility of blood plasma alone at a given equilibration PO_2 (i.e., Henry's Law) between 15 and 25°C (dashed), and 20 and 30°C (dotted).

Chapter 2 Determinants of Maximal Oxygen Transport in Yellowfin Tuna (*Thunnus albacares*) and Rainbow Trout (*Oncorhynchus mykiss*), and the Functional Significance of the Enthalpy of Hb-Oxygenation on Heat and Oxygen Transport

"The circulatory system of man and the vertebrate animals can be considered as made up of a small number of organs or subordinate systems, which are easy to recognize anatomically, and the functions of which are on the whole quite distinct. We have a propulsive organ: the heart; a distributing organ: the system of arteries; an organ for interchanges of substances between the blood and the tissues: the capillaries; an organ for collecting the blood and carrying it back to the heart: the venous system." (August Krogh, 1922, p. 1)

2.1 Introduction

As August Krogh so eloquently explained, "diffusion and convection are the only processes responsible for the oxygen transport into respiratory organs and within organisms" (Krogh, 1941, p. 20). These diffusive and convective processes that form the O_2 transport cascade can be simplified into four steps: 1) convective flow of water or air to the respiratory organ by ventilation; 2) diffusion of O_2 across the respiratory barrier into the blood; 3) convective O_2 transport in the blood to the tissue capillaries; 4) diffusion of O_2 from the blood into the cells where O_2 is consumed by the mitochondria during oxidative phosphorylation of adenosine triphosphate (ATP). Because each O_2 transport step can be described by relatively simple and well-known equations (e.g., Rahn and Fenn, 1955; Taylor and Weibel, 1981), O_2 transport can be quite easily mathematically modelled. At each step, the flow rate of O_2 (expressed in mols, $\dot{M}O_2$, or as a volume, $\dot{V}O_2$, and from herein referred to as $\dot{M}O_2$) can be

expressed as the product of an O_2 partial pressure gradient (ΔPO_2) and a conductance (G) that is defined by structural and physiological factors (Taylor and Weibel, 1981; Taylor et al., 1987; Wang and Malte, 2011). Therefore, if the O_2 transport cascade is modelled as an integrated system with the conductances arranged in series (Figure 2.1), the relative contribution of each structural or physiological factor in determining the maximum rate of O_2 transport ($\dot{M}O_2max$) can be quantitatively evaluated (di Prampero, 1985; Jones and Karas, 1988; Shephard, 1969; Wagner, 1988; Wang and Malte, 2011).

During exercise, $\dot{M}O_2$ increases linearly with exercise intensity to a point where $\dot{M}O_2max$ can be experimentally defined (Hillman et al., 1979; Norin and Clark, 2016; Robergs et al., 2010; Seeherman et al., 1981; Zhang et al., 2019). In healthy individuals, it is typically only when exercising to $\dot{M}O_2max$ that circulatory O_2 transport may be maximized and O_2 supply may limit ATP synthesis (Robergs, 2001; Wagner, 2000). The factors that contribute to circulatory O_2 transport include ventilation ($\dot{V}$), respiratory diffusion conductance (Gd), hæmoglobin (Hb) concentration, Hb-O_2 affinity, cardiac output ($\dot{Q}$), and tissue O_2 conductance (Gdt). Importantly, these factors determine arterial-O_2 saturation, arterial-O_2 concentration, circulatory O_2 delivery (the product of $\dot{Q}$ and arterial-O_2 concentration), and tissue O_2 extraction (i.e., all processes included in the diffusion of O_2 from the blood to the mitochondria) (Wagner, 2000). $\dot{M}O_2max$ is determined by the interactions of all the factors mentioned above, and experimental or evolutionary increases in the relative magnitude of any one factor must generally be matched with an increase in some or all of the other factors to result in an increase in $\dot{M}O_2max$ (di Prampero, 1985; Jones and Karas, 1988; Jones and Lindstedt, 1993; Taylor and Weibel, 1981; Taylor et al., 1987; Wagner, 1996a; Wagner, 2011). Mathematical models of O_2 transport and the relative importance of each physiological factor in determining $\dot{M}O_2max$ have been

investigated in a limited range of vertebrates representing mammals, birds, reptiles, and amphibians (di Prampero and Ferretti, 1990; Jones, 1998; Jones and Karas, 1988; Scott and Milsom, 2006; Wagner, 1993; Wagner, 1996b; Wagner, 2011; Wang and Hicks, 2002; Wang and Hicks, 2004; Withers and Hillman, 1988).

Comparative biologists are interested in $\dot{M}O_2max$ because it determines an animal's maximal aerobic performance, and the traits that determine $\dot{M}O_2max$ are heritable and thus are very likely subject to natural and sexual selection (e.g. Garland and Bennett, 1990; Hayes and O'Connor, 1999; Nespolo et al., 2016; Storz et al., 2019). Across species, $\dot{M}O_2max$ is generally correlated with the oxidative capacity of the active muscles (Hoppeler, 1990), and matching in the O_2 supply-demand relationship is heavily dependent on circulatory O_2 delivery, making the heart and Hb important targets for evolutionary increases to $\dot{M}O_2max$ (e.g., Dohm et al., 1994; Gallaugher et al., 2001; Garland and Bennett, 1990; Gonzalez et al., 2006). In support of this, arterial O_2 delivery is strongly correlated with $\dot{M}O_2max$ in a variety of vertebrates (Gallaugher et al., 2001; Hillman et al., 2013; Kayar et al., 1994), and integrative models of the O_2 transport cascade have consistently shown $\dot{Q}$ to be one of the more important factors determining $\dot{M}O_2max$ (di Prampero and Ferretti, 1990; Jones, 1998; Scott and Milsom, 2006; Wagner, 2011; Wang and Hicks, 2002; Withers and Hillman, 1988). However, an increased capacity for circulatory O_2 delivery requires corresponding increases in respiratory and muscle diffusive conductance. Accordingly, animals with a relatively high $\dot{M}O_2max$ have so-called adaptive changes to increase O_2 conductance at each step of the O_2 transport cascade (Bennett et al., 1984; Bernal et al., 2001b; Bishop, 1999; Brill and Bushnell, 2001; Bushnell and Jones, 1994; Henderson et al., 2002; Jones et al., 1989; Lindstedt et al., 1991; Longworth et al., 1989; Taylor et al., 1987).

The fish O_2 transport cascade has not yet been modeled as an integrated system to assess the relative contribution of each factor to determining $\dot{M}O_2max$, even though extensive studies indicate that circulatory O_2 delivery is very likely maximized during maximal aerobic exercise, at least in Pacific salmonids (*Oncorhynchus spp.*). A three-fold increase in $\dot{Q}$ supports increased circulatory O_2 delivery in salmonids swimming at their $\dot{M}O_2max$, but there is good experimental evidence to suggest that $\dot{Q}$ and $\dot{M}O_2$ reach a maximum and plateau prior to a fish achieving its maximum aerobically sustained swimming speed (Eliason et al., 2013; Gallaugher et al., 2001; Kiceniuk and Jones, 1977; Lee et al., 2003; Thorarensen et al., 1996). Furthermore, hæmatocrit and intraspecific variability in maximum $\dot{Q}$ are both correlated with $\dot{M}O_2max$ in rainbow trout (*Oncorhynchus mykiss*) (Claireaux et al., 2005; Gallaugher et al., 1995). Although, when trout were made anemic there was no compensatory increase in $\dot{Q}$ to maintain circulatory O_2 delivery (Gallaugher et al., 1995). Thus, during maximal aerobic exercise, $\dot{Q}$ may reach a maximum causing arterial-O_2 content to predominantly determine maximum circulatory O_2 delivery. Then again, reduced arterial-O_2 content may prevent a compensatory increase in $\dot{Q}$ by causing myocardial ischemia, and it has been suggested that peripheral diffusion limitations may maintain a venous O_2 threshold that protects cardiac O_2 supply (Farrell, 2002; Farrell and Clutterham, 2003; Steffensen and Farrell, 1998). However, rainbow trout $\dot{M}O_2max$ was not increased with hyperoxia (Duthie and Hughes, 1987), which contrasts results in mammals (Knight et al., 1993; Wagner et al., 1996) and may indicate that in rainbow trout, both O_2 and substrate supply are maximized to meet the demand of oxidative phosphorylation at $\dot{M}O_2max$. This may be the case because as rainbow trout approach $\dot{M}O_2max$, oxidative substrates are depleted in the red-oxidative muscle fibers concomitant with an increased reliance on white-

glycolytic muscle fibers to power swimming and potentially supply substrate, as lactate, to the red muscle (Richards et al., 2002).

The high performance tunas have some of the highest $\dot{M}O_2$ values measured among fishes, which is at least in part related to the high metabolic demands of endothermy and their exceptional swimming performance (Korsmeyer and Dewar, 2001). To sustain high rates of circulatory O_2 delivery and tissue O_2 extraction, all factors of the tuna O_2 transport cascade seem to have evolved in conjunction with their high metabolic rates (Bernal et al., 2001b; Brill and Bushnell, 2001; Bushnell and Jones, 1994). A likely hypothesis proposes that the high-performance physiology of tunas, including exceptionally large hearts and high cardiac output, evolved to supply O_2 at high enough rates to fuel multiple metabolically demanding activities, not exclusively for exceptionally high swimming performance (Brill, 1996; Korsmeyer et al., 1996a). Nevertheless, the cardiovascular systems of tunas are relatively well studied and seem adequately equipped to support high levels of aerobic exercise (Brill and Bushnell, 1991b; Brill and Bushnell, 2001; Bushnell and Jones, 1994). Although there are limited data on the swimming $\dot{M}O_2max$ of tunas (Blank et al., 2007; Korsmeyer and Dewar, 2001), Korsmeyer et al. (1997a) were able to measure cardiac performance and arterial and venous blood gases in yellowfin tuna swum to fatigue. Thus, the yellowfin tuna is the only regionally heterothermic or high-energy demand fish that has been studied to a reasonable degree to quantify how Hb concentration and Hb-O_2 affinity affect βbO_2, circulatory O_2 delivery, and tissue O_2 extraction at $\dot{M}O_2max$.

Tuna are regionally heterothermic, so blood must transport and unload O_2 to tissues of widely different temperatures. Yellowfin tuna blood-O_2 affinity is temperature-independent, but among other species of tuna, the temperature-dependence of blood-O_2 affinity ranges from

slightly reduced to greatly reversed, so hypotheses for the functional significance of this trait in tuna are equivocal (Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Cech et al., 1984; Clark et al., 2008a; Jones et al., 1986; Lowe et al., 2000). However, this trait likely maintains a relatively stable blood PO_2 in tissues of different temperatures, and probably also conserves metabolic heat energy in the warm tissues (Weber and Wells, 1989).

In this study I have assessed the possible contributing factors to O_2 supply limitation at $\dot{M}O_2max$ in two fish models: the rainbow trout and the yellowfin tuna. For each model, I quantitively evaluated the relative importance of each factor to determining $\dot{M}O_2max$. To do this, I have used published data for each species (Kiceniuk and Jones, 1977; Korsmeyer et al., 1997a), and a mathematical model of the O_2 transport cascade as described by Wang and Malte (2011), which includes a model of countercurrent exchange of O_2 in the gill (Malte and Weber, 1985). In doing this, I have also quantified how Hb concentration and Hb-O_2 affinity influence circulatory O_2 delivery and tissue O_2 extraction. Because the yellowfin tuna has a relatively large mixed venous O_2 reserve at $\dot{M}O_2max$, I hypothesized that tissue O_2 extraction is dependent on βbO_2 (i.e., the magnitude of the Bohr shift), which will cause an apparent tissue O_2-diffusion limitation in the model. I have also evaluated how the temperature-dependence of Hb-O_2 affinity influences venous PO_2 in tuna. I hypothesized that since temperature-independent Hb-O_2 affinity will prevent a left-ward shift of the OEC with decreasing temperature, then blood PO_2 will remain relatively constant over a wide range of body temperatures (15-30°C). Lastly, I have calculated what the energetic savings are relative to the oxycalorific equivalent of glucose metabolism over a range of $\Delta H'$ values.

2.2 Methods

Mathematical model of fish O_2 transport

The following system of equations conserve O_2 mass at each step as O_2 molecules are passed from water to the blood, and from the blood to the tissues. The equations used to model O_2 transport, and the procedure to simultaneously solve the system of equations was described by Wang and Malte (2011). The countercurrent exchange of O_2 at the respiratory surface of the gill, as formulated by Malte and Weber (1985), is described by two coupled differential equations, one for water and one for blood:

$$\frac{dPw}{dl} = \frac{Gd}{Gv} \cdot (Pw - Pb) \tag{1a}$$

$$\frac{dPb}{dl} = \frac{Gd}{Gp} \cdot (Pw - Pb) \tag{1b}$$

In these equations *Pw* and *Pb* are the PO_2 in water and blood, respectively, as a function of length (*l*) along the exchange surface (i.e., *Pw* = *Pw*(*l*), and *Pb* = *Pb*(*l*)). *Gd*, *Gv*, and *Gp*, are the diffusion, ventilation, and perfusion conductances, respectively. *Gd* includes conductances for the gill membrane (*Gdm*) and the erythrocytes (*Gde*) (Weibel et al., 1993), and these are given by

$$Gd^{-1} = Gdm^{-1} + Gde^{-1} \tag{2}$$

$$Gdm = K \cdot \frac{SA}{t} \tag{3}$$

$$Gde = \theta_{O_2} \cdot Vc \tag{4}$$

where *K* is the Krogh diffusion constant, *SA* is the area of the exchange surface, *t* is its thickness, θ_{O_2} is the rate of O_2 binding by whole blood (i.e., Hb), and *Vc* is the volume of the capillaries in

the gill lamellae. In this model, *Gd* is fixed at a constant value that is calculated as the conductance required to match O_2 exchange in the gill and tissues, respectively (i.e., an assumed maximal value). The ventilation conductance is calculated by

$$Gv = \dot{V}w \cdot \alpha w_{O_2} \tag{5}$$

where $\dot{V}w$ is the ventilation volume and αw_{O_2} is the solubility of O_2 in water (i.e., the capacitance coefficient of O_2 in water). The perfusion conductance is calculated by

$$Gp = \dot{Q} \cdot \beta b_{O_2} \tag{6}$$

where $\dot{Q}$ is cardiac output and βb_{O_2} is the O_2 capacitance coefficient in blood. Due to the nonlinearity of the OEC, which describes the relationship between Hb-O_2 saturation and PO_2, βb_{O_2} is not a constant and depends on blood PO_2 (i.e., *Pb*). βb_{O_2} is defined from the slope of the relationship between blood-O_2 content (*Cb*) and *Pb*

$$\beta b_{O_2} = \frac{dCb}{dPb} \tag{7}$$

In this model the OEC is described by the Hill equation, which requires only two parameters and gives a reasonable approximation of the relationship between fractional Hb-O_2 saturation (S_{Hb}) and *Pb* if the parameters for other OEC models are not available. The Hill equation is given by

$$S_{Hb} = \frac{Pb^n}{P_{50}^n + Pb^n} \tag{8}$$

where P_{50} is the PO_2 at which 50% of Hb is saturated, and *n* is the Hill coefficient (i.e., a parameter that describes the sigmoidicity of the OEC, and quantifies cooperativity among the ligand binding sites). The blood-O_2 content (i.e., *Cb*) is determined from S_{Hb}, the tetrameric Hb concentration (C_{Hb}), and the O_2 that is physically dissolved in the blood plasma. The relationship between *Cb* and *P*b then becomes

$$Cb = Pb \cdot \alpha b_{O_2} + 4 \cdot C_{Hb} \cdot \frac{Pb^n}{P_{50}^n + Pb^n} \quad (9)$$

where αb_{O_2} is the solubility of O_2 in plasma.

Gas exchange in the tissues is described by a third differential equation

$$\frac{dPb}{dl} = \frac{Gdt}{Gp} \cdot (Pb - Pt) \quad (10)$$

where *Gdt* is the diffusion conductance of the tissues, and *Pt* is the tissue PO_2. *Gdt* is fixed at a constant value that is calculated as the conductance required to match O_2 exchange to the reported $\dot{M}O_2max$ value (Table 2.1). Here, *Pt* is treated as the mitochondrial PO_2 ($PmitO_2$) and is assumed to be zero at $\dot{M}O_2max$, as justified by Cano et al. (Cano et al., 2013). Finally, $\dot{M}O_2$ is determined by the Fick principle

$$\dot{M}O_2 = \dot{Q} \cdot (Ca - Cv) \quad (11)$$

where *Ca – Cv* is the arteriovenous O_2 content difference.

Solution of the equations

The system of equations (Eqns. 1a, 1b, and 10) are simultaneously solved numerically by an iterative method. The equations describing countercurrent O_2 exchange at the gill (Eqns. 1a and 1b) form a boundary value problem that requires initial inputs of *Pw* and *Pb* at the same point on the exchange surface [i.e., *Pb*(*l* = 0) and *Pw*(*l* = 0)]. The mixed venous PO_2 (*Pv*) and the inspired PO_2 (*PI*) are the independent values and are initial inputs of the model at *Pb*(*l* = 0) and *Pw*(*l* = *l*), respectively. The dependent values are the arterial PO_2 (*Pa*) and expired PO_2 (*PE*), which are the model outputs at *Pb*(*l* = *l*) and *Pw*(*l* = 0), respectively, but because *Pw*(*l* = 0) needs to be known initially, an estimate of *PE* is input. This results in values for *Pa* and *PE*, and the *Pa* value is then input into the equation for O_2 exchange in the tissues (Eqn. 10), which results in a

new *Pv* value that is used to initiate a new cycle of the model. This is repeated until the values for *Pa*, *Pv* (and *PE*) no longer change. The parameters required to determine *Gv* and *Gp* are taken from the literature (see below).

Data

The experimental data used in this study are summarized in Table 2.1. Rainbow trout data are from Kinceniuk and Jones (1977), and yellowfin tuna data are from Korsmeyer et al. (1997). As described by Korsmeyer et al (1997), the yellowfin tuna $\dot{M}O_2max$ was estimated from the relationship between swim speed and $\dot{M}O_2$ from another study on similar sized yellowfin tuna (Dewar and Graham, 1994). The corresponding cardiac output was calculated with the Fick principle and is in line with the heart rate measurements made by Korsmeyer et al. (1997) and previous measurements of stroke volume (Brill and Bushnell, 2001; Korsmeyer et al., 1997a). Tuna ventilation volume was determined as

$$\dot{V}w = \frac{\dot{M}O_2}{PI \cdot \alpha w_{O_2} \cdot U} \tag{12}$$

where *U* is the fraction of O_2 extracted from the incurrent/inspired water and is often referred to as O_2 utilization. Bushnell and Brill (Bushnell and Brill, 1991) determined a *U* of 0.553 in swimming yellowfin tuna, which was used to estimate a $\dot{V}w$ of 3.3 L min^{-1} kg^{-1} for the tuna studied by Korsmeyer et al. (1997), close to previous determinations of $\dot{V}w$ in yellowfin tuna (Bushnell and Brill, 1991; Jones et al., 1990).

The P_{50} values at the gill (P_{50gill}) were calculated using published studies on blood from rainbow trout and yellowfin tuna (Brill and Bushnell, 1991a; Weber et al., 1976). For yellowfin tuna, Bohr plots (i.e., plots of $\log_{10}P_{50}$ vs pH) were constructed with published P_{50} values and accompanying pH values at 20 and 30°C (Brill and Bushnell, 1991a). The slope of the

relationship between $\log_{10}P_{50}$ and pH is the Bohr coefficient, which was used to determine P_{50} at the reported blood pH of swimming yellowfin tuna. The rainbow trout Bohr coefficient reported by Weber et al. (1976) was used to adjust rainbow trout P_{50gill} to in vivo blood pH. Estimates of P_{50gill} are likely physiologically applicable because *in vitro* O_2 equilibria experiments can be designed to closely match *in vivo* conditions in the gill or lung. However, they do not necessarily match condition in the tissue capillaries, where metabolic CO_2 diffuses into the blood (Figure 2.1) and acidifies the blood relative to arterial pH, which decreases Hb-O_2 affinity and right shifts the OEC via the Bohr effect (i.e., Hb-O_2 dissociation linked binding of hydrogen ions, commonly referred to as Bohr protons). Although the concentration of hydrogen ions is greater in venous blood than in arterial blood, mixed venous pH is often close to or even relatively more alkaline than arterial pH (e.g., Korsmeyer et al., 1997a) because Hb acts as a pH buffer. Hb-proton binding prevents large changes to blood pH while also decreasing venous blood-O_2 affinity by stabilising the low affinity T-state Hb confirmation, so it is Bohr proton saturation and not pH that is relevant to venous blood-O_2 affinity. Consequently, mixed venous blood pH cannot be treated as an independent variable because it is dependent on blood-tissue gas exchange and non-bicarbonate buffering (e.g., Hb-H^+ binding). Thus, measurements of venous blood pH are not useful to estimate P_{50} in the tissue capillaries ($P_{50tissue}$). Here, $P_{50tissue}$ was estimated by fitting an OEC to the in vivo *Pv* and *Cv* values, and the P_{50} of that OEC was used as an approximation of $P_{50tissue}$. In this model, P_{50gill} was used to determine *Gp* in equations 1a and 1b, and $P_{50tissue}$ was used to determine *Gp* in equation 10.

Tetrameric Hb concentrations (i.e., C_{Hb}) were calculated to match the reported values for arterial O_2 content, and the venous O_2 contents (i.e., *Cv*) were calculated from the reported

values for *Ca* and *Ca-Cv*. Values for the solubilities of O_2 in plasma and water were taken from Boutilier et al. (1984).

Sensitivity analysis to assess the possible contributing factors to O_2 supply limitation at $\dot{M}O_2max$

After initially determining values of *Gd* and *Gdt* that resulted in model outputs of *Pa*, *Pv*, *Ca*, *Cv*, and $\dot{M}O_2max$ that closely matched *in vivo* values (Table 1), the model was used to test the sensitivity of the system for each species data set. Following a similar procedure to Jones (1998), each of the four conductances was individually changed by increasing or decreasing by 1% the values of the diffusion conductances, *Gd* and *Gdt*, and the factors that determine *Gv* and *Gp* ($\dot{V}w$, $\dot{Q}$, C_{Hb}, and $P_{50tissue}$). For each change of a variable (i.e., *Gd*, *Gdt*, $\dot{V}w$, $\dot{Q}$, C_{Hb}, and $P_{50tissue}$), the corresponding change in $\dot{M}O_2max$ was expressed as a fractional change relative to the reported $\dot{M}O_2max$ value. The sensitivity of flux through the system to changes of each variable (*i*) was quantified as the slope of the line (b_i) fit to the fractional $\dot{M}O_2max$ values calculated for each variable at -1%, control (i.e., no change), and +1% values. The slopes were standardized to fractional flux control coefficients (F_i) for each of the four conductances by dividing the slope for each variable by the sum of the slopes for all the variables in the system (Eqns. 13 and 14).

$$F_i = \frac{b_i}{\sum b_i} \tag{13}$$

$$F_{system} = 1.0 = F_{Gv} + F_{Gd} + F_{Gp} + F_{Gdt} \tag{14}$$

In equation 14, changes in $\dot{V}w$ and $\dot{Q}$ were used to change *Gv* and *Gp*, respectively. By doing this, the sum of the slopes (Eqn. 13) for $\dot{V}w$, $\dot{Q}$, *Gd*, and *Gdt* are equal to one.

To assess the extent to which $P_{50tissue}$ affects O_2 unloading, tissue O_2 extraction, and $\dot{M}O_2max$, $P_{50tissue}$ was decreased stepwise until it was equal to P_{50gill} (i.e., no Bohr shift). At each step, the effect of decreasing $P_{50tissue}$ was determined by comparing model output values to the respective values with no change to the model.

Assumptions for the O_2 transport model

In this model it is assumed that $\dot{M}O_2max$ is O_2 supply limited, that the entire system is in a steady state of O_2 exchange (i.e., $\dot{M}O_2$ transients at the onset and cessation of exercise are not considered), and that the gills can be represented each by a single compartment (i.e., an exchange surface of length *l*). There are also several implicit assumptions made in this analysis, as described by Malte and Weber (1985) and Wagner (1993). At the gill, it is assumed that diffusion limitation is the primary factor affecting O_2 exchange, that there is no ventilation/perfusion inhomogeneity in the gills, that $\dot{V}w$ and $\dot{Q}$ are not cyclic processes, and that there is no gill metabolism. In the tissues it is assumed that tissue-O_2 extraction is diffusion limited, and that there is no perfusion/metabolism inhomogeneity, and that there are no intramuscular limitations to O_2 transport. The tissue diffusion conductance, *Gdt*, is assumed to comprise all components of peripheral diffusion and mitochondrial O_2 uptake, including the kinetics of cellular O_2 diffusion and intracellular O_2 flux. It is assumed that there are no shunts for blood flow or diffusion. The blood is assumed to be a homogenous medium (i.e., no plasma or cells) that O_2 diffuses to and from without any hinderance by Hb-O_2 binding and dissociation or any other chemical reactions.

Calculation of Hb-heat transport

Metabolic heat production (*HP* kJ min^{-1}) is given by

$$HP = O_2kJ \cdot (Ca - Cv) \cdot \dot{Q} \quad (15)$$

where $O_2\ kJ$ is the oxycalorific equivalent for glucose metabolism and is equal to 473 kJ mol^{-1}. The heat transferred by Hb is given by

$$H_{Hb} = \Delta H' \cdot (Ca - Cv) \cdot \dot{Q} \quad (16)$$

where $\Delta H'$ (kJ mol^{-1}) is the overall enthalpy of Hb-oxygenation. If H_{Hb} is quantified as a percentage of *HP*, then the percentage of heat transport by Hb is independent of O_2 extraction and cardiac output, and is a relatively constant ratio of $\Delta H'/O_2kJ$. The heat potentially transported to the environment during Hb-oxygenation at the gills was modeled as a function of $\Delta H'$, assuming that $\Delta H'$ is constant across the entire OEC.

Temperature dependence of blood PO_2

The effect of temperature on yellowfin tuna blood PO_2 was evaluated at four different values of $\Delta H'$ from 15°C to 30°C. The results of this model are meant to predict the blood PO_2 in the peripheral tissues that are not protected by heat exchanging *retia*, and are thus influenced by ambient water temperature. Venous PO_2 values for the tissue capillaries are not known, so mixed venous blood PO_2 of slowly swimming yellowfin tuna was used as a starting reference. Although this may be considered a limitation of the model, it allowed me to estimate the effect of temperature on *in vivo* P_{50} since mixed venous blood is close to 50% saturated, which also validates using $\Delta H'$ values determined at P_{50} (i.e., 50% Hb-O_2). Starting values, as reported by Korsmeyer et al. (1997a), were: PO_2 = 40mmHg; pH = 7.800; temperature = 25°C. Blood PO_2 was predicted at the different temperatures using the van't Hoff isochore (Wyman, 1964)

$$\Delta H' = \ln 10 \cdot R \cdot \frac{\Delta \log_{10} P_{50}}{\Delta \frac{1}{T}} \quad (17)$$

where R is the gas constant, and T is the absolute temperature. Similar to a previous model of O_2 transport in regionally heterothermic mammals (Brix et al., 1990), blood PO_2 was determined as a function of blood pH at each temperature, and at a constant pH of 7.800. Blood pH was adjusted by -0.017 pH units/°C according to the results of Brill and Bushnell (1991a), and the blood PO_2 was then adjusted to that pH using equation 18 and the Bohr coefficient (φ) determined for yellowfin tuna (as described above):

$$PO_{2(pH,T)} = PO_{2(T)} \cdot 10^{(\varphi \cdot \Delta pH)} \quad (18)$$

$\Delta H'$ values were calculated for yellowfin tuna and skipjack tuna using the van't Hoff isochore. As blood temperature changes, the degree of relative alkalinity to pH of neutrality should remain fairly constant (Kim and Milsom, 2019; Rahn, 1967; Reeves, 1972), so blood PO_2 was also modeled as a function of blood temperature only and assuming a constant pH of 7.800. Skipjack tuna Bohr plots were constructed with data from Brill and Bushnell (1991a) as described above for yellowfin tuna. A third ΔH of -19 kJ mol^{-1} was calculated for Bigeye tuna (*Thunnus obesus*) using data reported in Lowe et al. (2000), and a fourth ΔH of -35 kJ mol^{-1} for rainbow trout blood at pH 7.8 was taken from Weber et al. (Weber et al., 1976).

2.3 Results

O_2 transport

For both the tuna and trout models, the mean partial pressure gradient between *PI* and *Pb* (ΔPO_{2gill}), the mean gill capillary *Pb* ($\bar{P}c_{gill}$), and the mean tissue capillary PO_2 ($\bar{P}c_{tissue}$) are reported in Table 2.1 and illustrated in Figure 2.2. The estimates of *Gd* and *Gdt* (Table 2.1) were

calculated to match model predictions of *Pa* and *Pv* to the reported values, which resulted in estimated $\dot{M}O_2max$ values that were within 0.1% of the reported values. The model estimates of $\dot{M}O_2max$ were then used as the "control" values for the sensitivity analyses. The relative changes in $\dot{M}O_2max$ caused by ± 1% changes in model variables (i.e., *Gd*, *Gdt*, $\dot{V}w$, $\dot{Q}$, C_{Hb}, and $P_{50tissue}$) are shown in Figure 2.3. The three points (-1, 0, and +1% change) were collinear ($R^2 > 0.999$) for each variable in both models, and the slopes of linear regressions fit to the three points for each variable are reported in Table 2.2. For rainbow trout, altering $\dot{Q}$ had the greatest affect on $\dot{M}O_2max$ followed by C_{Hb}, $P_{50tissue}$, *Gdt*, *Gd*, and $\dot{V}w$. For yellowfin tuna, altering $P_{50tissue}$ had the greatest affect on $\dot{M}O_2max$ followed by $\dot{Q}$, C_{Hb}, *Gdt*, *Gd*, and $\dot{V}w$. The resulting fractional flux control coefficients for the four conductances are shown in Figure 2.4.

H-O_2 affinity and Hb-heat transport

Figure 2.5 shows the effect of reducing $P_{50tissue}$ on $\dot{M}O_2$, circulatory O_2 delivery, tissue O_2 extraction and blood O_2 levels. In the absence of a right shifted OEC in the tissue compartment (i.e., $P_{50gill} = P_{50tissue}$), yellowfin tuna $\dot{M}O_2$ was reduced by 44% whereas rainbow trout $\dot{M}O_2$ decreased by 16%. The relatively larger decrease of yellowfin tuna $\dot{M}O_2$ was primarily due to reduced tissue-O_2 extraction with decreasing $P_{50tissue}$ (Figure 2.5).

The relationship between log transformed P_{50} and blood pH for both yellowfin tuna and skipjack tuna are shown in Figure 2.6 (the regression equations are given in the caption of Figure 2.6). The Y-intercept values from least-squares linear regressions were not different between the two temperatures for yellowfin tuna blood [$F = 0.260$, *d.f.* = (1, 19), $P = 0.616$], indicating that P_{50} is independent of temperature, so a single linear regression was fit to the combined 20°C and 30°C data. A reverse temperature effect was evident in skipjack tuna blood, as indicated by a lower Y-intercept at 30°C than at 20°C [$F = 9.439$, *d.f.* = (1, 19), $P = 0.006$]. Both species

exhibited a marked Bohr effect (i.e., an inverse relationship between $\log_{10}P_{50}$ and pH). A Bohr coefficient of -0.625 (Figure 2.6) was determined for yellowfin tuna, which was then used to adjust PO_2 over a range of temperatures and associated pH values shown. The resulting effect of temperature on blood PO_2 is shown in Figure 2.7. Over a temperature gradient from 30°C to 15°C and the concomitant pH change, Hb with a reversed temperature effect (i.e., skipjack tuna; $\Delta H' = 16$ kJ mol^{-1}; Figure 2.5) causes no change to blood PO_2, whereas Hb with an O_2 affinity that is independent of temperature (i.e., yellowfin tuna; $\Delta H' = 0$ kJ mol^{-1}; Figure 5) causes a pH dependent decline in PO_2 with declining temperature ($\Delta PO_2 \approx$ -9 mmHg from 25 to 15°C).

The $\Delta H'$ values calculated for yellowfin tuna and skipjack tuna blood were used to determine the amount of heat transported by Hb. The enthalpy of Hb-O_2 binding expressed as a percentage of the oxycaloric equivalent of glucose catabolism (~473 kJ mol^{-1}) for a range of enthalpy values is shown in Figure 2.7. In rainbow trout blood between pH 7.8 and pH 7.5 ($\Delta H'$ values of -35 and -11 kJ mol^{-1}, respectively), about 7% to 2% of the heat produced is transported by Hb and potentially lost to the environment in conjunction with Hb-O_2 binding at the gills. Yellowfin tuna blood shows no effect of temperature on Hb-O_2 affinity (Figure 2.6) (Bushnell and Brill, 1991), which should avert the absorption and transport of heat away from the warm tissues by Hb (Figure 2.8). A reverse temperature-dependence like that observed by Brill and Bushnell (1991) in skipjack tuna blood, may cause Hb-oxygenation linked retention of heat equivalent of up to 4% or more of the energy produced during glucose catabolism.

2.4 Discussion

Main findings

The main objectives of this study were twofold: 1) to quantitatively evaluate the factors determining the maximal rate of O_2 transport in the rainbow trout and the yellowfin tuna; and 2) to determine the influence of the enthalpy of Hb-O_2 binding on Hb-heat transport and blood PO_2. In both the yellowfin tuna and rainbow trout O_2 transport models, circulatory O_2 delivery and tissue diffusing capacity (i.e., perfusion conductance and tissue diffusion conductance, respectively) were relatively more important determinants of $\dot{M}O_2max$ than were $\dot{V}w$ and gill diffusing capacity (Figures 2.3 and 2.4). Thus, when rainbow trout and yellowfin tuna are forced to swim in controlled and normoxic conditions (Kiceniuk and Jones, 1977; Korsmeyer et al., 1997a), gill O_2 uptake is not a major determinant of O_2-supply limitation. The model results of this study and the results of many others give good reason to suggest that $\dot{M}O_2max$ is governed by the interactions between central and peripheral cardiovascular O_2 transport (i.e., circulatory O_2 delivery and tissue O_2 extraction). Under exercise conditions when O_2 supply limits $\dot{M}O_2max$ in fish, the anatomical separation of muscle fiber types and a need to secure venous O_2 supply to the heart may contribute to circulatory O_2 delivery and muscle O_2 extraction apparently limiting O_2 supply to the muscles. These ideas are further explored below. Temperature independent and endothermic $\Delta H'$ values that are characteristic of most tuna have been hypothesized by others to conserve metabolic heat and avert disruptions to Hb-O_2 unloading over internal temperature gradients, and my model results support these hypotheses.

In mathematical analyses of the O_2 transport systems of air-breathing vertebrates, the sensitivity of the system has often been tested by using large changes in model variables (i.e., 20-100%) (Jones and Karas, 1988; Scott and Milsom, 2006; Wagner, 1996b; Wagner, 2011; Withers

and Hillman, 1988). However, because of the sigmoidal shape of the OEC, large changes to variables can cause large and unrealistic changes to model outputs of blood O_2 content and $\dot{M}O_2max$, which precludes quantitative determination of the sensitivity of the system. To test the sensitivity of the O_2 transport systems modeled in this study, the model variables were perturbed by 1%, *ceteris paribus*. This approach was adopted from the elegant sensitivity analysis of the equine O_2 transport system by Jones (1998), and a similar approach by diPrampero (1985) who analysed the factors determining maximum O_2 transport in humans using the results of experimental perturbations of variables in the O_2 transport system. Limiting the variable perturbations to 1% allows for "fine-tuning" of the system within a range of realistic deviations of the model variables. For the fish O_2 transport models, qualitatively similar results were achieved by perturbing the model variables by 1, 5, 10, and 20%, but the effects on $\dot{M}O_2max$ deviated from linearity at the higher relative perturbations. However, the results of this analysis are likely qualitatively comparable to other analyses that used larger variable perturbations (i.e., the conclusions from analyses that used >20% changes would likely not have changed if smaller model perturbations were used).

Tests of the O_2 transport system have also often tested the hypothesis of symmorphosis (Garland and Huey, 1987; Jones, 1998; Weibel and Taylor, 1981). As originally formulated, symmorphosis proposed that regulated morphogenesis should result in changes to the structural and functional factors of the O_2 transport cascade that are proportional to changes to maximal flux through the system, such that no one factor or component of the system should be more limiting than the others (Taylor and Weibel, 1981). Thus, symmorphosis has been suggested as null hypothesis in empirical tests for rate-matching of O_2 flux through each step of the O_2 transport cascade (Jones et al., 1990; Weibel et al., 1991). However, perfect rate matching at

each step of the O_2 transport cascade should not necessarily be an *a priori* hypothesis because symmorphosis may not be a likely evolutionary outcome (Dudley and Gans, 1991; Garland, 1998; Garland and Huey, 1987). An important reason why it is unlikely that the physiology of all animals has been "optimized" for $\dot{M}O_2max$ (e.g., Jones, 1998; Jones and Lindstedt, 1993) is that biological structures (e.g., proteins, tissues, or organs) may not evolve in parallel (e.g., Dalziel et al., 2012), because each may play important roles in multiple physiological processes (Dudley and Gans, 1991; Garland, 1998). This is exemplified by the fish gill, which is a multifunctional, metabolic organ that has central roles in acid-base regulation, osmotic and ionic regulation, and excretion of nitrogenous wastes (Evans et al., 2005). Nevertheless, aquatic gas exchange is a dominant function of the gill and the gill morphometrics that govern gas diffusion (i.e., lamellar surface area and the thickness of the blood-water barrier) seem to be correlated with metabolic capacity among fishes, with tunas having the highest relative gill surface areas (Bigman et al., 2018; Hughes and Morgan, 1973; Wegner, 2015; Wegner et al., 2006; Wegner et al., 2010).

Gill diffusion conductance was not a relatively important determinant of O_2 supply limitation in either fish model, in contradiction to symmorphosis, at least regarding maximal O_2 transport in normoxia. Comparably, even though the lungs of air-breathing tetrapods are relatively unmalleable (e.g., Henderson et al., 2002; Weibel, 2000) pulmonary diffusing capacity is not a major limiting factor to O_2 flux in normoxia (Scott and Milsom, 2006; Wagner, 1996b; Withers and Hillman, 1988). However, in the Thoroughbred racehorse, ventilation and pulmonary diffusing capacity are the most important factors determining maximum O_2 flux, which has likely resulted from strong selection for aerobic power that has pushed the capacity for O_2 transport to the physiological limits of the equine lung (Jones, 1998). Interestingly, highly aerobic human athletes experience similar pulmonary limitations to exercise (Hopkins, 2005;

Hopkins and Harms, 2004; Hopkins et al., 1996). When swimming at $\dot{M}O_2max$, rainbow trout likely approach the functional limits of gill O_2 uptake, but yellowfin tuna do not seem to use the entire functional capacity of the gill. Experimental manipulations of rainbow trout gill surface area provide good evidence that most of the gill lamellae are perfused to increase O_2 uptake during maximal aerobic swimming (Duthie and Hughes, 1987). Increased blood pressure in the ventral aorta and increased blood flow will decrease the blood-water barrier and increase the functional gill surface area by recruiting distal lamellae, which maintains a high arterial O_2 saturation at $\dot{M}O_2max$ that is comparable to resting values (Kiceniuk and Jones, 1977). In comparison, yellowfin tuna arterial PO_2 and O_2 saturation increase with increasing swimming speed but are still relatively low during exercise (Korsmeyer et al., 1997a). Higher blood pressure and blood flow likely cause recruitment of lamellar surface area (Korsmeyer et al., 1997a), but the high vascular resistance reported in yellowfin tuna (Bushnell and Brill, 1992) may limit or cause heterogeneous lamellar perfusion. In general, when considering total O_2 exchange between the environment and the metabolizing tissues, it is not advantageous to decrease blood flow even if it increases O_2 extraction efficiency (Malte, 2011). In tuna, decreased blood flow would probably not increase O_2 extraction since the functional area for diffusion would also decrease. Even though tuna arterial saturation was low, it did not seem to hinder total O_2 flux. Since tuna ram-ventilate their gills it seems likely that as tuna swim faster, and increase ventilation and perfusion, that arterial blood saturation may increase in proportion to O_2 demand.

A digression concerning the determination of $\dot{M}O_2max$ in fishes is necessary before discussing peripheral cardiovascular O_2 transport limitations. To assess the $\dot{M}O_2max$ of mammals and some other tetrapods (usually reported as $\dot{V}O_2max$ in air breathing animals), researchers

typically use a graded exercise test and determine $\dot{M}O_2max$ as the point where $\dot{M}O_2$ plateaus and does not increase further with increasing exercise intensity (e.g., Seeherman et al., 1981). Testing protocols often include other criteria to assess $\dot{M}O_2max$, including but not limited to measurements of heart rate, blood lactate, the respiratory exchange ratio, and the rate of perceived exertion (humans). Graded exercise protocols have also been developed for fish (Beamish, 1978; Brett, 1964), in which the highest or peak $\dot{M}O_2$ ($\dot{M}O_2peak$) measured during swimming is used to approximate $\dot{M}O_2max$, sometimes also supported with measurements of heart rate or blood lactate (Norin and Clark, 2016; Zhang et al., 2019). However, due to methodological constraints of measuring aquatic respiration, and the typical recruitment of glycolytic muscles that accompany dynamic swimming gait changes when a fish nears fatigue (Peake and Farrell, 2004; Richards et al., 2002), $\dot{M}O_2max$ may not always characterize the same thing in fish as it does in mammals (Zhang et al., 2019). However, in many cases $\dot{M}O_2peak$ is likely a good enough approximation of $\dot{M}O_2max$ in fish (e.g., Lee et al., 2003; Norin and Clark, 2016; Zhang et al., 2020).

Kiceniuk and Jones (1977) and Korsmeyer et al. (1997) used the same graded exercise protocol for their studies on rainbow trout and yellowfin tuna, respectively, which included velocity increments every 60 minutes until fish fatigued. The rainbow trout showed a plateau in heart rate at the penultimate velocity increment, which is good evidence that $\dot{M}O_2max$ was likely achieved at the fatiguing swimming velocity (Kiceniuk and Jones, 1977). Yellowfin tuna were reported to have been swum to fatigue, as characterized by dynamic changes to swimming gait and elevated blood lactate levels, although their maximal swimming velocities, heart rates, and levels of O_2 extraction were lower than previously observed or predicted (Brill and Bushnell, 1991b; Dewar and Graham, 1994; Korsmeyer et al., 1996b; Korsmeyer et al., 1997a; Korsmeyer

et al., 1997b). During exercise, tuna rely almost entirely on changes in heart rate to modulate cardiac output, whereas trout increase cardiac output by increasing both heart rate and stroke volume (Brill and Bushnell, 2001). The lower than expected heart rate reported for yellowfin tuna may indicate that cardiac output could have been increased further, which would increase circulatory O_2 delivery (Korsmeyer et al., 1997a). The arteriovenous O_2 content difference was similar between rainbow trout and yellowfin tuna, but tissue O_2 extraction reached 86% in rainbow trout and only 49% in yellowfin tuna, with resulting mixed venous saturation of around 13% and 44%, respectively (Kiceniuk and Jones, 1977; Korsmeyer et al., 1997a). Thus, it is possible that the large venous O_2 reserve in yellowfin tuna indicates that they did not reach their maximal aerobic swimming limits or $\dot{M}O_2max$, but Korsmeyer et al. (1997) explained that the mixed venous O_2 content may not reflect the O_2 extracted by the swimming muscles (i.e., the loins of red muscle), which are anatomically separated from the other skeletal muscles in fishes and have their own vascular supply (Bone, 1978).

Systemic blood flow is redistributed during swimming exercise, causing a disproportionate increase in blood flow to the red muscle ($\dot{Q}_{RM}$) to support the increased metabolic demand. At rest, rainbow trout $\dot{Q}_{RM}$ is about 9% of cardiac output, but when swimming at 80% of maximum effort $\dot{Q}_{RM}$ increases to 42% of cardiac output, which supports a 12-fold increase in red muscle $\dot{M}O_2$ (Randall and Daxboeck, 1982). Randall and Daxboeck (1982) estimated red muscle $\dot{M}O_2$ to be 87% of total $\dot{M}O_2$, based on a red muscle O_2 extraction of 96%. Thus, the muscles powering swimming can be assumed to account for most of the increased $\dot{M}O_2$, so the results of the rainbow trout model can be argued to reflect limiting conditions at $\dot{M}O_2max$. Yellowfin tuna $\dot{Q}_{RM}$ has not been determined, but based on measurements made in lightly anesthetized albacore tuna (*Thunnus allalunga*) (White et al.,

1988), Korsmeyer et al. (1996b) conservatively estimated that $\dot{Q}_{RM}$ would be 36% of maximum cardiac output, which they used to model red muscle $\dot{M}O_2$ during maximal aerobic swimming. They assumed that if red muscle extracts 90% of the O_2 in the blood, then the red muscle $\dot{M}O_2$ would be 67% of $\dot{M}O_2max$ in a 2.1 kg yellowfin tuna with an estimated $\dot{M}O_2max$ of 1600 mg O_2 kg^{-1} hr^{-1} (0.834 mmol O_2 kg^{-1} min^{-2}). Taking their model a step further, I calculated a total O_2 extraction of 48% for the entire circulation, which is almost identical to their *in vivo* measurements that were used for this study's model (Korsmeyer et al., 1997a). Thus, yellowfin tuna red muscle O_2 extraction likely exceeds 49%, but an O_2 extraction of 49% may be reasonable for the entire cardiac output at $\dot{M}O_2max$. Therefore, the relative importance of *Gdt* in this study's yellowfin tuna model likely reflects whole body O_2 extraction, and not necessarily a limitation for O_2 diffusion across the red muscle capillaries. Tissue specific PO_2 and blood O_2 content values would permit determination of O_2 extraction in each tissue compartment, but to the best of my knowledge those measurements have not been reported.

A major assumption of the model that I used is that the tissue diffusion conductance, *Gdt*, comprises everything that may affect O_2 diffusion and transport from the blood to the mitochondria. In models for humans and birds, *Gdt* has been shown to be a relatively important determinant of $\dot{M}O_2max$, particularly in elite athletes or in hypoxia (di Prampero, 1985; Jones and Karas, 1988; Scott and Milsom, 2006; Wagner, 1996a; Wagner, 1996b). This seems intuitive because if all factors contributed equally to O_2 flux then *F* would equal 0.25 for each of the four conductances. So, if *Gv* and *Gd* are relatively non-limiting, then it would be expected that the resistance to O_2 flux would be shared by the factors determining circulatory O_2 delivery and tissue O_2 extraction (i.e., $\dot{Q}$, [Hb], and *Gdt*). However, it has been argued that tissue diffusion cannot be the primary limitation to $\dot{M}O_2max$ for at least two reasons: 1) because not all

capillaries are perfused during maximal exercise, so if there is unused diffusing capacity then tissue diffusion cannot be limiting to O_2 transport; and 2) because in ectothermic vertebrates that experience different body temperatures, temperature has a greater affect on $\dot{M}O_2max$ than it does on gas diffusion coefficients (Hedrick et al., 2015; Hillman et al., 2013). This argument is from the perspective that there is a single rate-limiting step to O_2 transport, whereas I have adopted the well-established hypothesis that $\dot{M}O_2max$ is set by all steps in the O_2 transport cascade (Jones and Karas, 1988; Wagner, 1988; Wagner, 1993). Put differently, $\dot{M}O_2max$ is governed by the interaction between circulatory O_2 delivery and tissue O_2 extraction, the two components of O_2 transport to the mitochondria. Moreover, there is good experimental evidence showing that raising the PO_2 gradient for O_2 diffusion into exercising muscle without increasing circulatory O_2 delivery increases O_2 extraction and $\dot{M}O_2max$ (Richardson et al., 1998; Wagner et al., 1990), which emphasizes the relative importance of peripheral O_2 diffusion to determining $\dot{M}O_2max$.

In salmonids, a peripheral O_2 diffusion limitation has been proposed as a possible mechanism to prevent venous PO_2 falling below a threshold that protects cardiac O_2 supply, thus preventing myocardial ischemia during exercise (Farrell, 2002; Farrell and Clutterham, 2003; Steffensen and Farrell, 1998). The relative importance of *Gp* and *Gdt* to determining $\dot{M}O_2max$ in the rainbow trout model likely reflects a limited capacity for O_2 extraction and diffusion from the blood to the tissues other than red muscle, so the mixed venous PO_2 is most likely dependent on the remainder of the systemic blood flow. The situation is different in tuna, which have hearts with large ventricles that are well-endowed with a coronary circulation, so tuna hearts may not be as dependent on venous O_2 supply to the myocardium (Brill and Bushnell, 2001).

The intrinsic metabolic capacity of tuna red muscle is similar to that of other active pelagic teleosts, but the elevated temperatures of tuna red muscle increases its aerobic metabolic

capacity and also warms the adjacent white muscle, which has higher anaerobic and aerobic metabolic capacity relative to most other fishes (Dickson, 1995; Dickson, 1996; Korsmeyer and Dewar, 2001). The gills and cardiovascular systems of tuna are capable of high rates of circulatory O_2 delivery (Brill and Bushnell, 2001; Muir and Hughes, 1969; Wegner et al., 2006; Wegner et al., 2010), and the muscles have evolved certain traits that facilitate high rates O_2 diffusion, including: relatively small red muscle fibers with high capillarity, relatively high amounts of mitochondrial protein due to densely packed mitochondrial cristae, and high concentrations of myoglobin in both the red and white muscle (Dickson, 1996; Korsmeyer and Dewar, 2001; Mathieu-Costello et al., 1992; Mathieu-Costello et al., 1996; Moyes et al., 1992). Collectively, these traits plus elevated muscle temperatures will increase O_2 extraction and may ensure that the red muscle is never hypoxic during exhaustive exercise (Brill, 1996). Thus, it seems that muscle O_2 extraction and circulatory O_2 delivery are well matched in tunas (reviewed by: Brill and Bushnell, 2001; Korsmeyer and Dewar, 2001), which is supported by the model results of this study (Figures 2.3 and 2.4). Therefore, it is not unreasonable to propose that at $\dot{M}O_2max$, red muscle O_2 extraction may even exceed the 90% extraction predicted by Korsmeyer et al. (1997a).

Hæmoglobin concentration and shifts in Hb-O_2 affinity had relatively important contributions to determining $\dot{M}O_2max$ in both the rainbow trout and yellowfin tuna models (Figure 2.3). As outlined by Brill and Bushnell (1991b), the high Hb concentrations and blood-O_2 carrying capacity of tunas likely contribute to high circulatory O_2 delivery without excessive increases in cardiac output. A high Hb concentration may also be important for maintaining a high mixed venous O_2 content in the blood flow through the non-red muscle portion of the systemic circulation, which has a lower O_2 extraction than the red muscle. Thus, high Hb

concentrations are likely necessary to match O_2 supply with the high metabolic capacity of the warmed red muscle, while also securing a high O_2 supply to fuel other metabolically demanding processes, as well as to facilitate rapid recovery after exhaustive exercise in the pelagic environment where there is nowhere to hide and rest (Brill, 1996; Korsmeyer et al., 1996a).

In the tissue capillaries, an increased P_{50} due to the Bohr and Root effects appears to be necessary to maintain O_2 supply by enhancing O_2 offloading relative to Hbs that lack a Bohr or Root effect. Reducing $P_{50tissue}$, which would be equivalent to reducing the Bohr coefficient or βbO_2, reduced $\dot{M}O_2$ more so in yellowfin than in rainbow trout. As described above, tuna have evolved several traits that should enhance tissue O_2 diffusion, and it seems likely that natural selection has pushed the cardiovascular system of tunas close to the functional limits for O_2 transport. Thus, the numerically large Bohr coefficients are probably necessary to increase O_2 unloading and tissue O_2 extraction to match O_2 supply to the high O_2 demand of the red muscle. This dependence on large right shifts of the OEC to unload O_2 likely contributed to the relative importance of *Gdt* in the yellowfin tuna model. As is evident in Figure 2.5, decreasing $P_{50tissue}$ appreciably decreased O_2 extraction, which would influence *Gdt* in the model. An emerging model for teleosts with pH sensitive Hbs proposes that plasma accessible carbonic anhydrase in the tissue capillaries accelerates CO_2 hydration, which rapidly acidifies the red blood cells and decreases Hb-O_2 affinity via the Bohr and Root shifts (Brauner and Harter, 2017; Rummer et al., 2013). There is good evidence to suggest that in salmonids this system maintains rates of O_2 unloading high enough that cardiac output is not unnecessarily increased to possibly unsustainable levels to maintain circulatory O_2 transport (e.g., Harter et al., 2019). Although it is not clear if all tuna have Root effect Hbs, tuna blood-O_2 affinity does show a pronounced sensitivity to pH (Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Cech et al., 1984; Clark et

al., 2008a; Jones et al., 1986; Lowe et al., 2000). Therefore, it seems likely that tunas would be highly dependent on such a system during strenuous swimming to unload O_2 to the exercising red muscle without unnecessarily large increase in cardiac output, which is in line with my results (e.g., Figures 2.3 and 2.5) and the extensive research by Brill and Bushnell (Brill and Bushnell, 1991b; Brill and Bushnell, 2001). Moreover, temperature-independent Hb-O_2 affinity could be interpreted to impair O_2 unloading to the warm tissues such as the RM during exercise, but plasma accessible carbonic anhydrase in the tissue capillaries would overcome this by promoting Hb-O_2 unloading in the absence of temperature induced shift of the OEC.

Hæmoglobin may also serve an important role in thermoregulation, because Hb-O_2 transport is linked to heat transport. The intrinsic enthalpy of hæme-oxygenation (i.e., ΔH^{O_2}) is around 13% of the oxycalorific equivalent for glucose metabolism (473 kJ mol^{-1}; Figure 6), but the oxygenation dependent dissociation of allosteric effectors contributes endothermically to $\Delta H'$, so *in vivo* Hb-heat transport will typically be lower than 13% of metabolic heat production. In humans about 9% of the heat produced is transported by Hb, and in rainbow trout about 2-8% may be transported to the gills by Hb and subsequently lost to the environment upon Hb-oxygenation (Figure 2.8) (Coates, 1975; Weber and Wells, 1989; Weber et al., 1976). Temperature-independent Hb-O_2 affinity eliminates Hb-heat transport (yellowfin tuna in Figure 2.8), and a reversed temperature-dependency, where $\Delta H'$ is positive, would cause heat to be released to the tissues upon Hb-O_2 unloading (skipjack tuna in Figure 2.8), which may contribute to maintaining the RM warm.

Reduced and reverse temperature-dependent Hb-O_2 affinity may also avert possible impaired Hb-O_2 unloading to the cold peripheral tissues, which are separated from the warm body regions by heat exchanging *retia* (Brix et al., 1989b; Clark et al., 2008a; Giardina et al.,

1989a). For example, in Figure 2.7 it is evident that temperature-independence stabilizes mixed venous PO_2 over a wide range of temperatures. This is further discussed in Chapter 5 (5.3.3).

Summary and conclusions

Oxygen transport from the environment to the mitochondria is governed by the interactions between circulatory O_2 delivery and tissue O_2 extraction. All factors of the O_2 transport cascade contribute to determining $\dot{M}O_2max$, although their relative importance may vary. In both the yellowfin tuna and the rainbow trout models, perfusion conductance had the greatest relative contribution to determining $\dot{M}O_2max$, with F values of 0.47 and 0.50 for tuna and trout, respectively. Tissue diffusion conductance also had a relatively important contribution to overall O_2 flux, but more so for yellowfin tuna than for rainbow trout (F values of 0.39 and 0.28, respectively). This likely reflects the anatomical separation of skeletal muscle fiber types, and a lower O_2 extraction in the blood flow to the tissues other than the exercising red muscle. Hæmoglobin concentration and cardiac output contributed almost equally to determining Gp in yellowfin tuna. A high P_{50} in the tissues (i.e., a right shift OEC due to proton binding) was an important determinant of $\dot{M}O_2max$ due to its influence on Hb-O_2 unloading and tissue O_2 extraction. Therefore, in the absence of temperature-induced right shift of the OEC in the warm and exercising RM, a large Bohr effect is required to unload O_2. High Hb concentrations and numerically high Bohr coefficients in tuna are very likely important for them to achieve such high rates of O_2 consumption, by increasing circulatory O_2 delivery and tissue O_2 extraction without unnecessarily large increases in cardiac output. Reductions in the temperature sensitivity of tuna hæmoglobin probably has an important heat conserving function, and may avert impaired to O_2 unloading to cold tissues by preventing leftward shifts of the OEC.

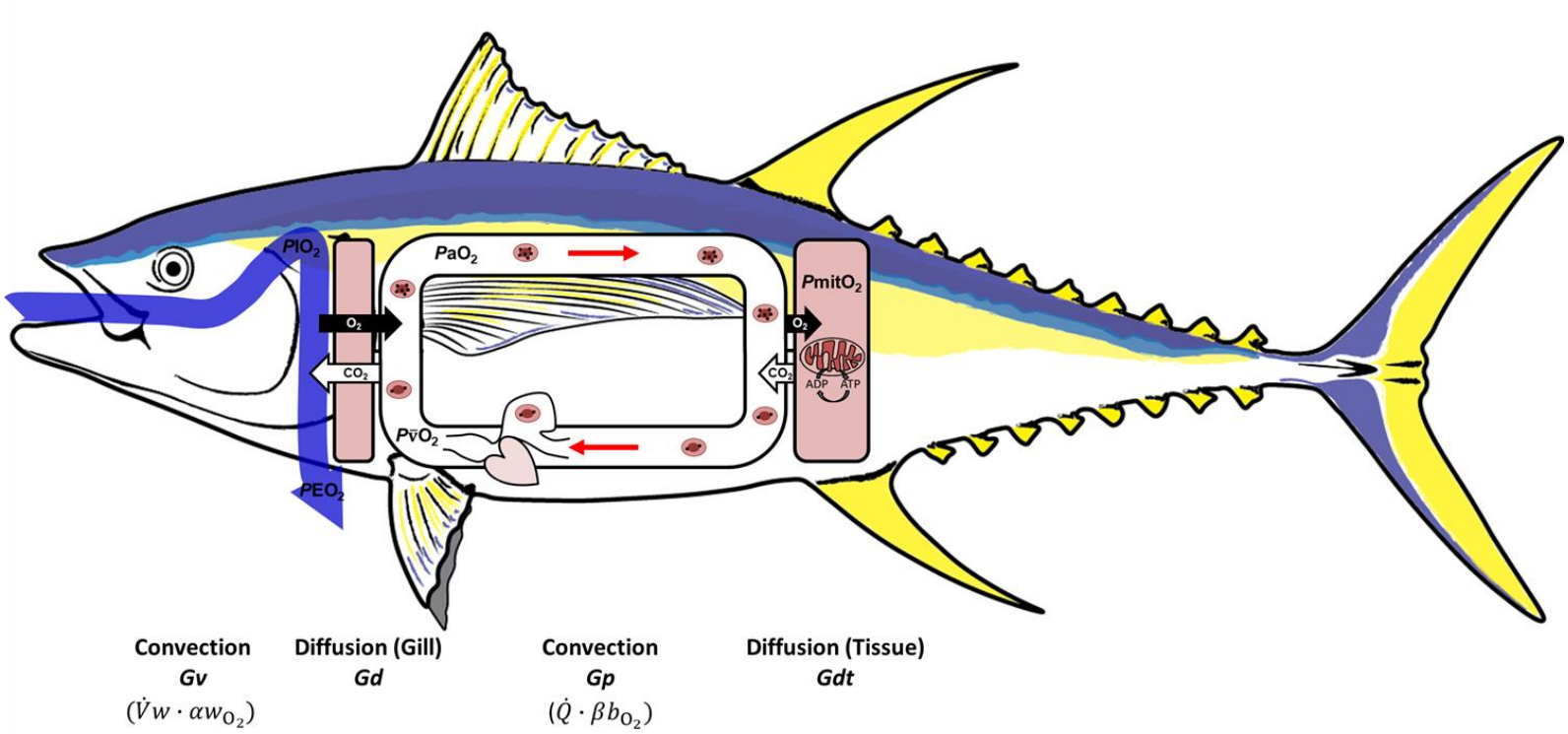

Figure 2.1 Schematic of the fish O_2 transport cascade with O_2 transfer from water to the mitochondria occurring in four steps.

At each step, the flow rate of O_2 ($\dot{M}O_2$) can be expressed as the product of an O_2 partial pressure gradient (ΔPO_2) and a conductance (G). The ventilation conductance (Gv) is a product of the ventilation volume ($\dot{V}w$) and the solubility of O_2 in water (αw_{O_2}), and diffusion from water to blood is characterized by a diffusion conductance (Gd). The perfusion conductance (Gp) is a product of the cardiac output ($\dot{Q}$) and the blood-O_2 capacitance (βb_{O_2}), and diffusion from blood to the tissues is characterized by a diffusion conductance (Gdt). PIO_2, inspired PO_2 (PI); PEO_2, expired PO_2 (PE); PaO_2, arterial PO_2 (Pa); $P\bar{v}O_2$, mixed venous PO_2 (Pv); $PmitO_2$, mitochondrial or tissue PO_2 (Pt).

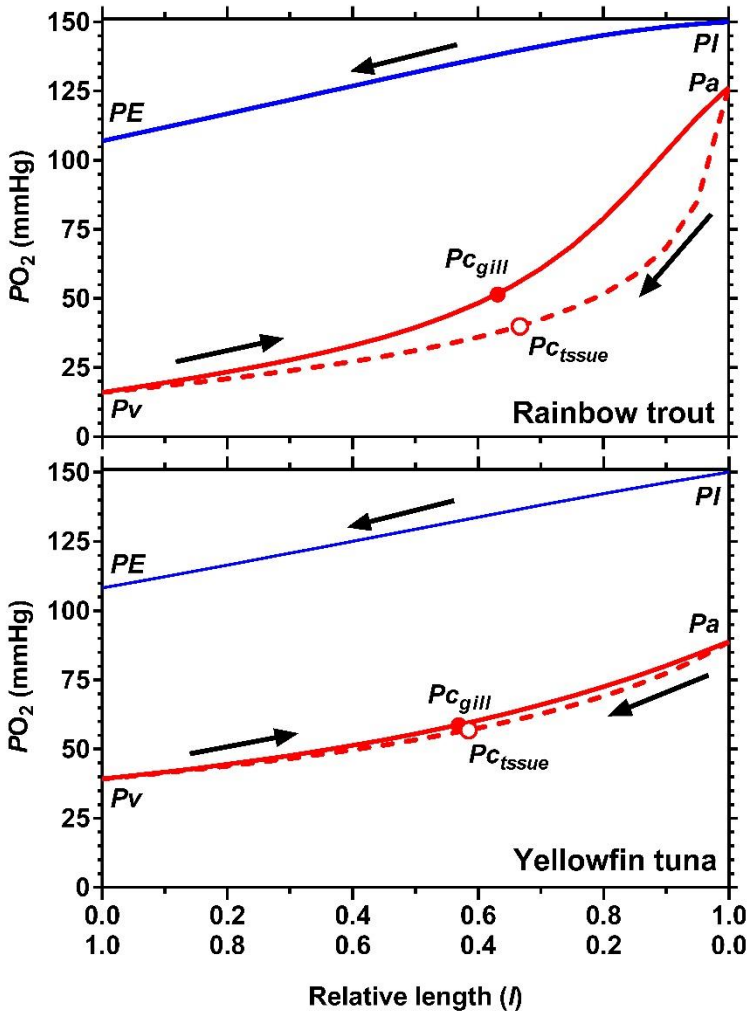

Figure 2.2 The change in water and blood PO_2 (mmHg) along the length (l) of the exchange surface for rainbow trout and yellowfin tuna.

Arrows indicate the direction of flow of water (blue) or blood (red). The solid red curve represents blood along the gill capillary exchange surface, and the dashed red curve is the blood along the tissue capillary exchange surface. The inspired PO_2 (*PI*), expired PO_2 (*PE*), arterial PO_2 (*Pa*), and the mixed venous PO_2 (*Pv*) are indicated at the point where the curves meet the vertical axes. The mean PO_2 in the gill capillary (Pc_{gill}; solid circle) and in the tissue capillary (Pc_{tissue}; open circle) are indicated at their respective positions along the exchange surface.

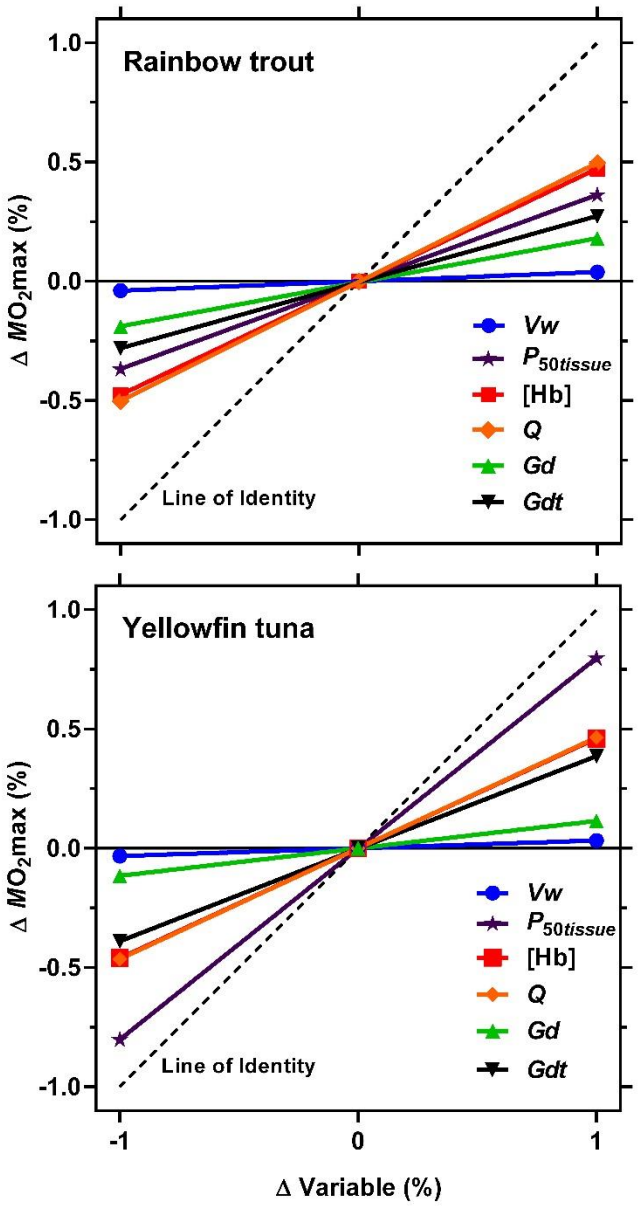

Figure 2.3 Change in $\dot{M}O_2max$ caused by ± 1% change in different components of the O_2 transport cascade for rainbow trout and yellowfin tuna.

The changes in $\dot{M}O_2max$ were determined by solving the mathematical model described in the text. The lines are linear regressions for the three data points for each variable: ventilation ($\dot{V}w$), hæmoglobin concentration (Hb), cardiac output ($\dot{Q}$), P_{50} in the tissue capillaries ($P_{50tissue}$), gill diffusion conductance (Gd), and tissue diffusion conductance (Gdt).

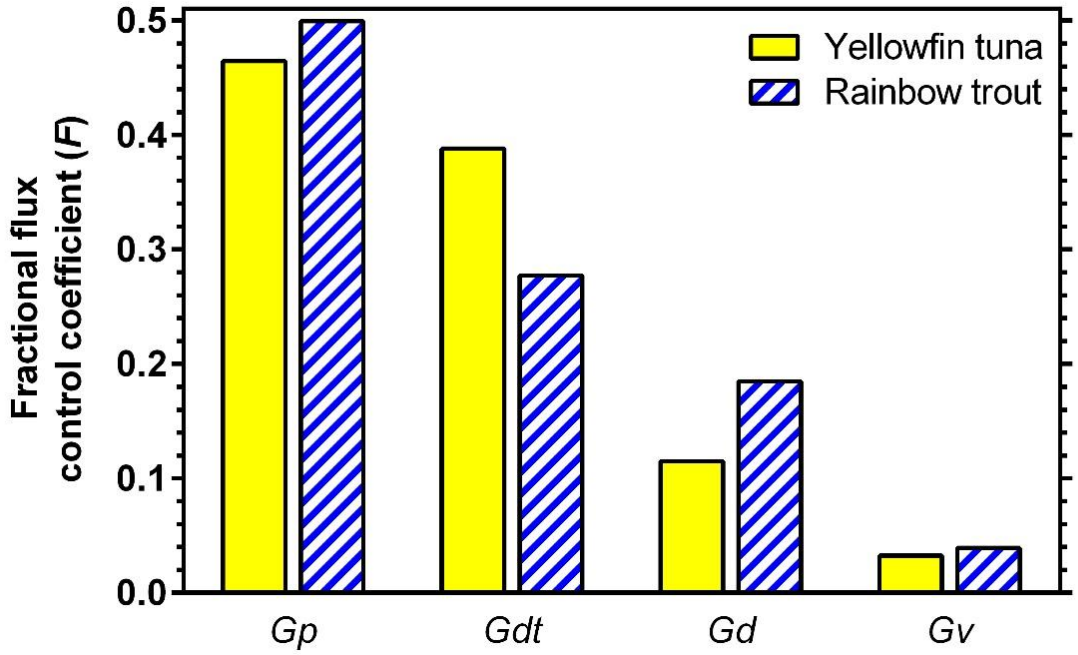

Figure 2.4 Fractional flux control coefficients (*F*) for the four conductances that govern flow through the O_2 transport cascade and contribute to determining $\dot{M}O_2max$.

Solid yellow bars to the left of each pair are those for the yellowfin tuna model, and diagonal striped bars to the right are those for the rainbow trout model. *Gp*, perfusion conductance; *Gdt*, tissue diffusion conductance; *Gd*, gill diffusion conductance; *Gv*, ventilation conductance. The *F* values were calculated as the slopes reported in Table 2.2 divided by the sum of the slopes of the four conductances. Changes to cardiac output ($\dot{Q}$) and ventilation ($\dot{V}w$) were used to determine *F* values for *Gp* and *Gv*, respectively.

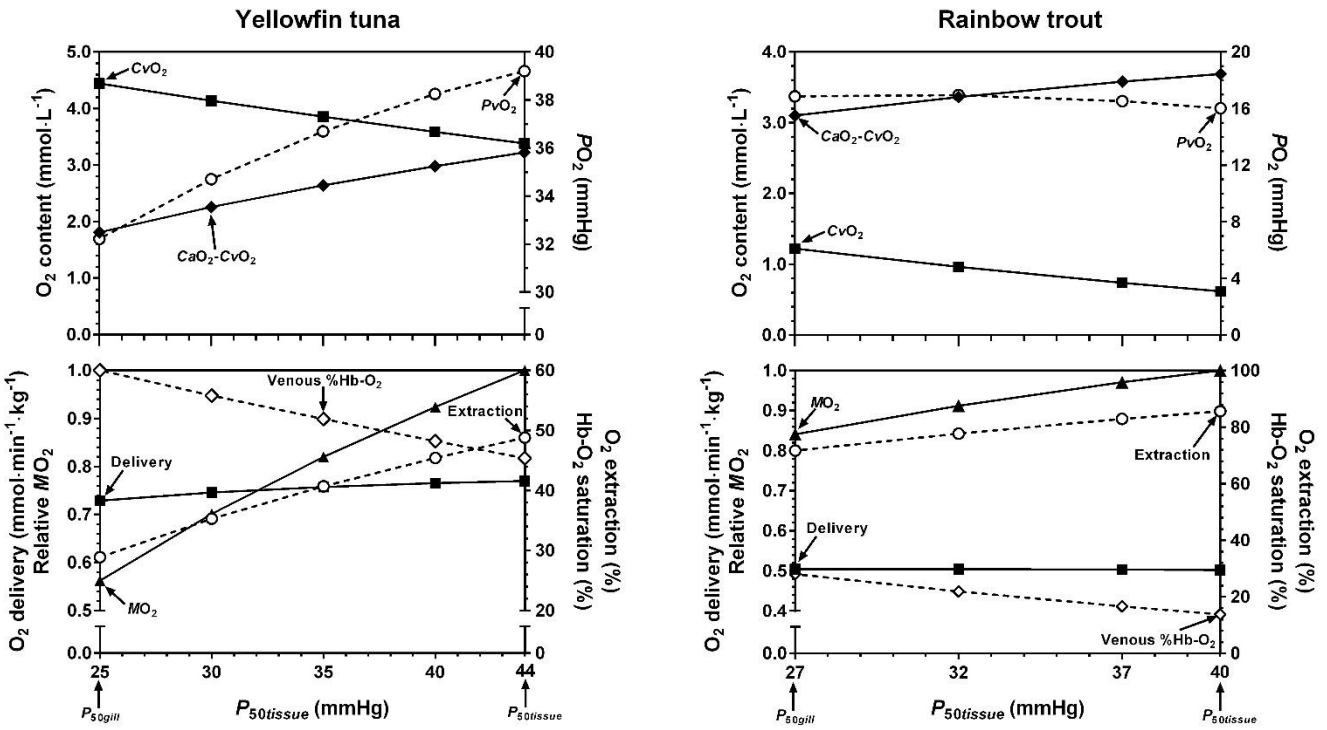

Figure 2.5 The effect of $P_{50tissue}$ on blood-O_2 levels, O_2 delivery, tissue O_2 extraction, and maximum O_2 transport.

$P_{50tissue}$ was decreased stepwise until it was equal to P_{50gill} (i.e., no Bohr shift), and at each step the effect of decreasing $P_{50tissue}$ on O_2 transport was determined. Closed symbols correspond to the left y-axis, and open symbols correspond to the right y-axis. Top panel: venous O_2 content (CvO_2; closed squares), the arteriovenous O_2 content difference (CaO_2-C_vO_2; closed diamonds), venous PO_2 (PvO_2; closed circles). Bottom panel: Circulatory O_2 delivery (closed squares), relative O_2 consumption (MO_2; closed triangles), venous Hb-O_2 saturation (open diamonds), tissues O_2 extraction (open circles).

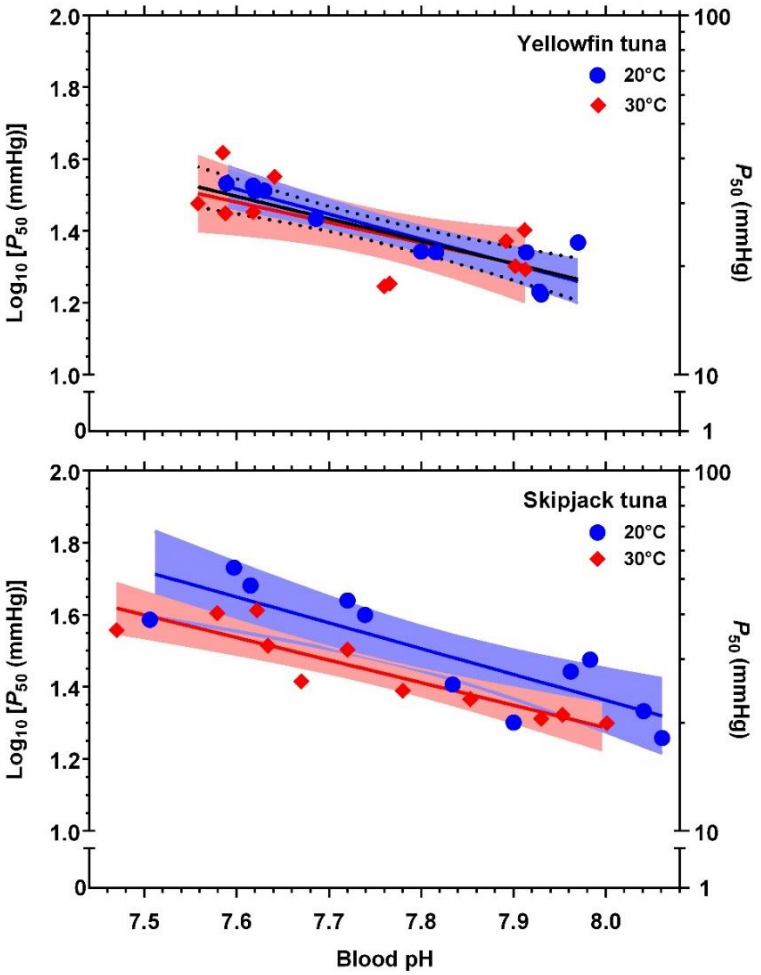

Figure 2.6 Yellowfin tuna and skipjack tuna Bohr plots.

Linear regressions (± 95% CIs) were fit to data reported by Brill and Bushnell (1991a) and measured at 20°C (blue circles) and 30°C (red diamonds). Yellow fin tuna P_{50} was temperature-independent, as indicated by similar regression intercepts [F = 0.260, $d.f.$ = (1, 19), P = 0.616], so a single regression (black line) was fit to the combined data. Skipjack tuna P_{50} showed a reversed temperature-dependence, as indicated by a lower intercept at 30°C than at 20°C [F = 9.439, $d.f.$ = (1, 19), P = 0.006]. Yellowfin tuna 20°C: combined data [$\log_{10}P_{50}$ = -0.625 (± 0.230) · pH + 6.244 (± 1.782)]. Skipjack tuna 20°C: $\log_{10}P_{50}$ = -0.717 (± 0.337) · pH + 7.096 (± 2.638); 30°C: $\log_{10}P_{50}$ = -0.626 (± 0.219) · pH + 6.294 (± 1.693). Parameters ± 95% CIs.

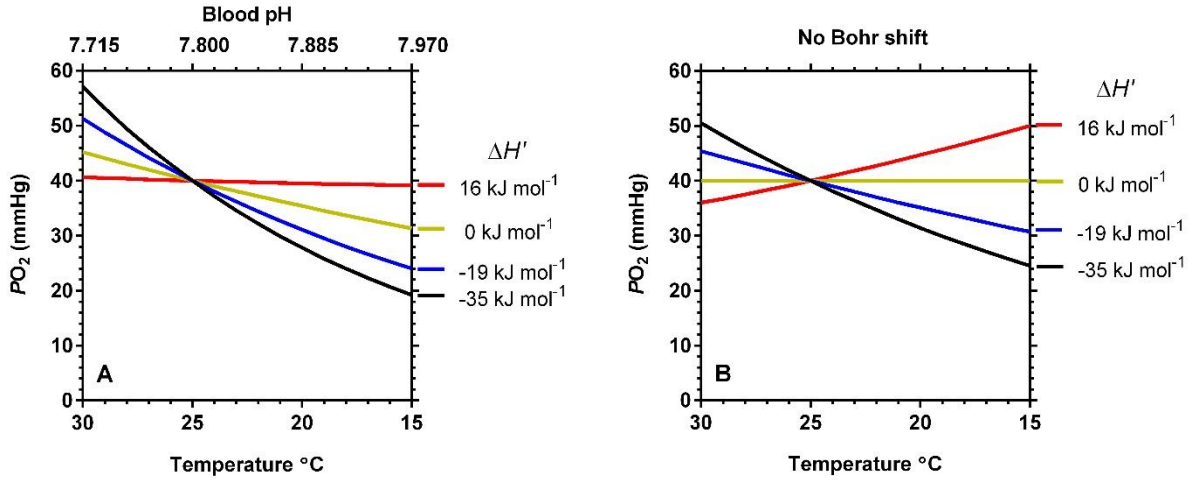

Figure 2.7 Theoretical effect of temperature on mixed venous blood PO_2 with varying enthalpy of Hb-oxygenation ($\Delta H'$).

The venous blood PO_2 of slowly swimming yellowfin tuna was used as a starting reference: PO_2 = 40 mmHg; Hb-O_2 ~ 50%; pH = 7.800; temperature = 25°C (Korsmeyer et al., 1997a). The blood PO_2 was adjusted to each temperature using varying $\Delta H'$ values and the van't Hoff isocore (see methods). It was assumed that yellowfin tuna blood pH changes -0.017 pH units/°C, according to Brill and Bushnell (1991a). (A) The blood PO_2 was then adjusted to the pH at each temperature using a Bohr coefficient of -0.625, or (B) it was assumed that blood pH was constant at pH 7.800 since the degree of relative alkalinity to pH of neutrality should remain constant as temperature changes (e.g., Rahn, 1967). $\Delta H'$ values: 16 kJ mol^{-1}, calculated for skipjack tuna (*Katsuwonus pelamis*) (Figure 2.5); 0 kJ mol^{-1}, calculated for yellowfin tuna (*Thunnus albacares*) (Figure 5); -19 kJ mol^{-1}, estimated for bigeye tuna (*Thunnus obesus*) using P_{50} values and Bohr coefficients presented in Lowe et al. (2000); -35 kJ mol^{-1} for rainbow trout (*Oncorhynchus mykiss*) at pH 7.8 (Weber et al., 1976).

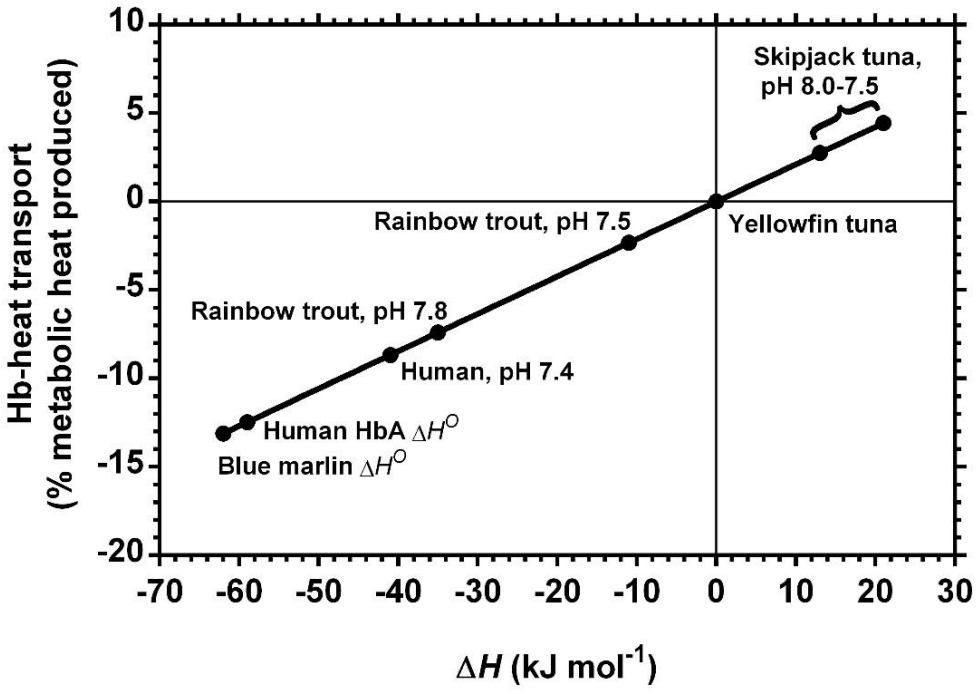

Figure 2.8 The relationship between hæmoglobin (Hb)-heat transport and the enthalpy of Hb-oxygenation ($\Delta H'$ kJ mol^{-1}).

Hb-heat transport is $\Delta H'$ expressed as a percentage of the oxycalorific equivalent for glucose metabolism (473 kJ mol^{-1}). Values for specific species are indicated by solid circles. Rainbow trout values are from Weber et al. (1976), human values at pH 7.4 were determined with data reported by Reeves (1980), and yellowfin and skipjack tuna values were determined with data from Brill and Bushnell (1991; Figure 5). $\Delta H'$ values were determined with P_{50} values, although in some species $\Delta H'$ is saturation dependent. The intrinsic enthalpy of hæme oxygenation (ΔH^{O_2}) for blue marlin and human HbA were determined in purified Hb solutions and were taken from Weber at al. (2010) and Atha and Ackers (1974), respectively. Negative Hb-heat transport values indicate that heat absorbed upon deoxygenation in the tissues is released upon oxygenation in the lungs or gills, whereas positive values indicate no heat loss associated with Hb-oxygenation.

Table 2.1 Input data used to model $\dot{M}O_2max$ in rainbow trout and yellowfin tuna.

Physiological variable	Symbol	Unit	Rainbow trout	Yellowfin tuna
Temperature	T	°C	10	25
[1]Diffusion conductance, gill	Gd	µmol min^{-1} kg^{-1} $mmHg^{-1}$	2.07	2.66
[1]Diffusion conductance, tissue	Gdt	µmol min^{-1} kg^{-1} $mmHg^{-1}$	4.82	4.70
[2]Ventilation	$\dot{V}w$	mL min^{-1} kg^{-1}	1730	3300
[2]Cardiac output	$\dot{Q}$	mL min^{-1} kg^{-1}	52.6	116.6
O_2 solubility, water	αw_{O_2}	µmol L^{-1} $mmHg^{-1}$	2.240	1.368
O_2 solubility, blood plasma	αb_{O_2}	µmol L^{-1} $mmHg^{-1}$	1.986	1.510
Inspired PO_2	PI	mmHg	150	150
[2]Arterial PO_2	Pa	mmHg	126	89
[2]Mixed venous PO_2	Pv	mmHg	16	39
[2]Hb concentration (tetrameric)	C_{Hb}	mmol L^{-1}	1.06	1.83
[2]Arterial O_2 content	Ca	mmol L^{-1}	4.33	6.60
[2]Arteriovenous O_2 content difference	$Ca - Cv$	mmol L^{-1}	3.70	3.21
[3]Blood/Hb-O_2 affinity, gill	P_{50gill}	mmHg	27	25
Blood/Hb-O_2 affinity, tissue	$P_{50tissue}$	mmHg	40	44
[3]Hill coefficient	n		2	1.6
[3]Bohr coefficient	φ		-0.52	-0.62
Enthalpy of Hb-oxygenation	$\Delta H'$	kJ mol^{-1}	-11.3	0
[1]Mean PO_2 gradient, gill	$\Delta(PO_{2gill})$	mmHg	79.4	70.7
[1]Mean capillary PO_2, gill	$\bar{P}c_{gill}$	mmHg	51.4	58.6
[1]Mean capillary PO_2, tissue	$\bar{P}c_{tissue}$	mmHg	39.9	56.8
[2]Maximum rate of O_2 transport	$\dot{M}O_2max$	mmol min^{-1} kg^{-1}	0.194	0.375

[1] Variable determined with the model.
[2] Variable taken from or calculated from literature data: rainbow trout, Kiceniuk and Jones (1977); yellowfin tuna, Korsmeyer et al. (1997a).
[3] Rainbow trout P_{50}, Hill coefficient, and Bohr coefficient are from (Weber et al., 1976).

Table 2.2 Slopes of linear regressions presented in Figure 2.3, representing the magnitude of the effect of changes in each variable on $\dot{M}O_2max$ in rainbow trout and yellowfin tuna.

Variable	Rainbow trout	Yellowfin tuna
$\dot{V}w$	0.039	0.033
Gd	0.185	0.115
Gdt	0.277	0.388
$P_{50tissue}$	0.365	0.800
C_{Hb}	0.474	0.461
$\dot{Q}$	0.499	0.465

Chapter 3 The Effect of Temperature on Hæmoglobin-Oxygen Affinity of Swordfish (*Xiphias gladius*) and Smalleye Pacific Opah (*Lampris incognitus*)

"[T]emperature invariance of oxygen binding is not some second-order effect, but is of primary importance to these fish." (Francis G. Carey, 1982a, p. 228)

3.1 Introduction

The swordfish (*Xiphias gladius*) and the smalleye Pacific opah (*Lampris incognitus*) are both highly active and regionally heterothermic species. While the opah is capable of a whole-body form of endothermy and can heat the cranial region and entire body core including the heart, the swordfish (family Xiphiidae) and other billfishes (family Istiophoridae) are often referred to as "regional endotherms" because they heat the eye and brain region only (i.e., cranial endothermy) (Block, 1986; Block, 1991b; Carey, 1982b; Runcie et al., 2009; Wegner et al., 2015). Endothermy probably provides the opah and the swordfish with crucial advantages over their thermoconforming prey, since both species spend most of the day in cold water deep below the thermocline (Carey, 1990; Carey and Robinson, 1981; Dewar et al., 2011; Sepulveda et al., 2010; Wegner et al., 2015), where warm eyes will enhance temporal resolution (Fritsches et al., 2005) and the opahs warm heart and swimming muscles likely enhance aerobic performance.

In opah and billfishes, metabolic heat generated by the extraocular muscles is conserved with a heat exchanging *rete* that arises from the carotid artery, warming the eyes and brain (Block, 1986; Carey, 1982b; Runcie et al., 2009). Warm blood perfuses the entire body of the opah, including the heart, due to a series of heat exchanging *retia* within the gills as well as insulting fat layers around the gill *retia* and the body core, which conserve the heat produced by the continuous contraction of the swimming muscles (Wegner et al., 2015). The cranial *retia*

effectively maintain swordfish cranial temperatures relatively constant and elevated as much as 12°C above ambient water temperature when swordfish are in deep cold water (Figure 3.1) (Carey, 1990). Cranial temperatures in the opah are also relatively constant and elevated at least 6°C above the surrounding water, while body and heart temperature are elevated at least 3 to 5°C (Figure 3.1) (Wegner et al., 2015).

A consequence of efficient heat exchange within the circulation is the establishment of large internal temperature gradients (i.e., regional heterothermy), which are exacerbated when opah and swordfish dive below the thermocline into cold water (Figure 3.1). During daily sojourns between warm and cold waters above and below the thermocline, respectively, swordfish may experience water temperatures from as low as 4°C to as high as 30°C in less than a few hours, in addition to possibly low environmental oxygen levels in deep water (Carey, 1990; Carey and Robinson, 1981; Dewar et al., 2011; Sepulveda et al., 2010). Therefore, blood-O_2 uptake at the gills must occur over the range of environmental temperatures that are encountered by opah and swordfish, but blood-O_2 transport occurs over the steep internal temperature gradients between the cold gills and warmest tissues.

As reviewed in Chapter 1, the O_2 affinity of most jawed vertebrate Hbs typically decreases with increasing temperature, but regional heterotherms tend to have Hbs with a reduced or even reverse temperature-dependence (Weber and Campbell, 2011). In one of the first comparative studies of Hb from regionally heterothermic fishes, Andersen et al. (1973) reported that increasing temperature greatly decreased the O_2-affinity of swordfish Hb (i.e., a normal temperature-dependence), but the blood pH and detailed methods were not reported in that study. However, Weber et al. (2010) reported an ATP-induced temperature-independence of P_{50} in hæmolysates from three billfishes that are closely related to swordfish: striped marlin (*Kajikia*

audax = *Tetrapturus audax*), blue marlin (*Makaira nigricans*), and shortbill spearfish (*Tetrapturus angustirostris*) (reviewed in Chapter 1, section 1.3.2). Thus, a more thorough investigation of the effect of temperature on blood and Hb-O_2 affinity in swordfish is warranted. I am not aware of any O_2 equilibria studies on Hb or blood from any of the opah species.

In this Chapter I address the following question: How does temperature affect Hb-O_2 affinity in the swordfish and the smalleye Pacific opah? If temperature-independent Hb-O_2 affinity is necessary to maintain regional heterothermy, then I expect that both the swordfish and the opah should have Hbs that are insensitive to temperature. I address this by constructing OECs at different temperatures in whole blood and hæmolysates, and by measuring the effect of closed-system temperature changes on blood PO_2. Furthermore, since swordfish and other billfishes are closely related and share similar anatomy and physiology related to their capacity for endothermy, I expect that swordfish Hb would exhibit an ATP-induced temperature independence like previously studied billfish Hbs. I also present data from Atlantic bluefin tuna hæmolysates to compare the enthalpic contributions of oxygenation linked effector dissociation among the different lineages of regionally heterothermic teleosts.

3.2 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 no. SC-2471), and the University of British Columbia (UBC) Animal Care Committee (animal care no. A11-0235 and A15-0266).

Blood collection

Swordfish (n = 7) were captured by deep-set buoy gear (Sepulveda et al., 2014), opah (n = 4) were captured by hook and line in the coastal waters off Southern California (i.e., the Southern California Bight), and Atlantic bluefin tuna (n = 2) were captured by hook and line off Massachusetts (fork lengths are reported in Table 3.1). Blood was drawn by caudal puncture into heparinized syringes. Blood samples were kept on ice and shipped by courier to the UBC campus in Vancouver, Canada, where experiments were conducted within 1 to 4 days after the blood was collected. Preliminary experiments with swordfish blood showed no changes in Hb concentration, hæmatocrit (Hct; the proportion of red blood cells in blood), plasma pH, or Hb-O_2 affinity (i.e., P_{50}, the PO_2 at 50% Hb-O_2 saturation), and no evidence of red blood cell (RBC) lysis for up to 6 days after blood was collected, provided blood was refrigerated during this time (see Appendix).

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 red blood cells (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 oxygen equilibrium curves (OECs) were constructed by quantifying the relative Hb-O_2 saturation at a range of equilibration PO_2's at two CO_2 levels and two (swordfish) or three (opah) temperatures. Blood pH, and PO_2 were measured in subsamples of blood equilibrated with gas mixes at each of the OEC temperature treatments. After completing the whole blood experiments, RBCs were separated from blood plasma by centrifugation, then the RBCs were thrice rinsed in ice cold saline and frozen at -80°C for

experiments on stripped hæmolysates. OECs were constructed for stripped hæmolysates in the presence and absence of effector ions at two different temperatures for each species (see below).

Hæmatological parameters

Hæmoglobin concentration was measured by the cyanmethæmoglobin method using Drabkin's reagent and a hæm-based extinction coefficient of 11 $mmol^{-1}$ cm^{-1} (Völkel, and Berenbrink, 2000). All Hb concentrations are expressed as tetrameric Hb ([Hb], in mM). Hct was measured as the percentage of packed RBCs relative to total blood volume after centrifuging samples at 11,500 rpm for five minutes. Mean corpuscular hæmoglobin concentration (MCHC, in mM) was calculated by dividing [Hb] by Hct. Plasma osmolality (mOsm kg^{-1}) was measured in 10µL of undiluted plasma with a vapour pressure osmometer (VAPRO 5520, Wescor, Logan, Utah). ATP was assayed with a colourimetric assay kit (SIGMA-ALDRICH MAK190, Sigma-Aldrich Co. LLC, St. Louis, Missouri), and plasma lactate was measured spectrophotometrically using the LDH-catalyzed 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 PO_2

Oxygen equilibria experiments were conducted at temperatures that correspond near to the coldest environmental temperatures and the warmest tissue temperatures in the opah and the swordfish. The coldest experimental temperature was 10°C, which is close to the coldest water temperature regularly encountered by both the swordfish and the opah (Sepulveda et al., 2010; Wegner et al., 2015). The warmest experimental temperature was 25°C for swordfish (i.e., near the warmest cranial and water temperatures), and 15°C and 20°C for the opah (near the warmest body and cranial/water temperatures, respectively) (Carey, 1990; Sepulveda et al., 2010; Wegner

et al., 2015). At each temperature treatment, experiments were conducted at two physiologically relevant CO_2 levels, 0.25% CO_2 and 1.00% CO_2, to manipulate blood pH at a high and low level to quantify the Bohr coefficient (i.e., $\frac{\Delta P_{50}}{\Delta pH}$).

The relationship between Hb-O_2 saturation and PO_2 (i.e., an OEC) was assessed on 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 (~ 3μL) between two sheets of low density polyethylene (Glad® ClingWrap) that were secured on an aluminum ring with two plastic O-rings, which were then placed in a gas tight tonometry cell modified to fit into a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, USA). Optical density (OD) was measured every 20 to 30 seconds at 390nm (an isosbestic point where OD is independent of Hb-O_2 saturation), and at 430 nm and 436 nm (wavelengths that typically correspond to a maximum absorbance for deoxygenated Hb). Initially, blood was equilibrated with pure N_2 for a minimum of 30 minutes until OD at 430/436 nm was stable, which was assumed to indicate full Hb deoxygenation. After deoxygenation, the Hb-O_2 saturation was increased with stepwise increments of the O_2 tension, balanced with N_2, up to 21% O_2. Full Hb-O_2 saturation was assumed after a final increment to 30% O_2 in the absence of CO_2. Gas mixtures of O_2, CO_2, and N_2 were obtained using a Wösthoff DIGAMIX® gas mixing pump (H. Wösthoff Messtechnik, Bochum, Germany). Fractional Hb-O_2 saturations were calculated for each equilibration step as the change in ΔOD (ΔOD = 430 nm or 436 nm - 390 nm) from full deoxygenation, relative to that between full deoxygenation (pure N_2) and full oxygenation (30% O_2).

Whole blood pH was measured in approximately 500 μL of blood equilibrated for 1 h with either 0.25 or 1.00% CO_2 and a range of O_2 tensions (balanced with N_2) in rotating glass

tonometers thermostated to either 10, 15, 20, or 25°C. 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) thermostated to the experimental temperature.

To mimic the closed-system temperature changes that blood experiences in the arterioles and venules of a heat exchanging *rete mirabile*, approximately 500 µL blood samples equilibrated at either 10, 20, or 25°C were injected into a pH electrode (as described above) and a Radiometer E5046 PO_2 electrode thermostated at the equilibration temperature as well as another pair of electrodes thermostated to a warmer or cooler experimental temperature according to Cech et al. (1984) and Brill and Bushnell (1991a). Although the blood was static within the electrode chamber, the blood 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 PO_2 electrode was flushed with the experimental gas mixture to minimise electrode response time to the respective PO_2. Temperature induced changes in pH and PO_2 were monitored using data acquisition software, and when it appeared that pH and PO_2 traces had stabilized, the respective values of each were recorded.

Hæmolysates

Frozen and packed RBCs were slowly thawed on ice (>24 h) then mixed with an equal volume of cold 0.1 mM 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 hæmolysates in 1000 µL of 100 mM Hepes buffer (pH 7.4) that

was bubbled with 100% O_2, and a spectral scan was made from 500-700 nm (i.e., an oxyHb spectrum). If there was an evident peak or unusually high absorbance at 630 nm, an absorption maximum for metHb, then the Hb^+ was reduced by adding a molar excess of sodium hydrosulfite to the hæmolysate, followed by passage through mixed bed ion exchange resin. Hæmolysates were concentrated with centrifugal filters (30 kDa). Oxygen equilibria were determined in 0.1 M Hepes buffer at a Hb concentration of 0.6 mM in the absence and presence of saturating levels of ATP (ratio of the concentration of ATP/Hb = 30). OECs were generated at 10 and 20°C for opah hæmolysates, and 10 and 25°C for swordfish hæmolysates, and 15 and 25° bluefin tuna hæmolysates 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. The pH of the hæmolysate solutions was measured at the experimental temperature with a thermostated Mettler Toledo InLab Micro glass pH electrode (Mettler-Toledo LLC, Columbus, OH, USA).

Data analysis

All statistical analyses and curve fitting were performed in R v 3.5.2 (R Core Team, 2017). An oxygen equilibrium curve (OEC) was constructed for each blood or hæmolysate sample by fitting a three-parameter logistic (3PL) model to paired data of fractional Hb-O_2 saturation (response variable) and PO_2 (explanatory variable). The R-language formula for the OEC model was '$HbO_2 \sim d/(1 + \exp(b*(\log10(PO_2) - \log10(e)))$'. The best-fit parameter values (b, d, and e) were used to calculate the PO_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}). Hill cooperativity coefficients were determined at P_{50} by differentiating the 3PL equation at P_{50}. Because teleost blood pH is typically dependent on Hb-O_2 saturation (Brauner et al., 1996; 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 vs pH. The effects of pH and temperature on 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 individual (id) as a random effect (R-language formula, '$\log10(P_S) \sim pH_S$*temperature + (1|id)'). Mixed models were fit at each saturation from P_{10} to P_{95}, and for each model a Likelihood Ratio Test (LRT) of fixed effects, fit with maximum likelihood estimation using a Chi square 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). Nonlinear least-squares curve fitting by the Levenberg-Marquardt algorithm was performed using the nlsLM function from the 'minpack.lm' package for R (Elzhov et al., 2010), and linear mixed models were fit using the lmer function from the 'lme4' package with the 'lmerTest' package (Bates et al., 2014; Kuznetsova et al., 2017).

The mixed model fits were used to predict P_S values with bootstrap estimated standard errors (500 replications), and these were used to construct whole blood OECs at constant pH for each species temperature treatments. Predicted P_{50} values were used as a proxy for whole blood-O_2 affinity. Hæmolysate P_S values were calculated at specific pH values from mixed linear models fit to data for each temperature and effector treatment. The temperature-dependence of whole blood and hæmolysate O_2 affinities were quantified by calculating $\Delta H'$ values using the van't Hoff equation (Wyman, 1964):

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

where R is the gas constant and T is the absolute temperature (Kelvin). Because $\Delta H'$ calculations requires that the concentration of allosteric effectors be known and the experimental conditions for Hb are carefully controlled (e.g., stripped hæmolysates), I consider the whole blood calculations as estimates at best. I denote the whole blood calculations as $\Delta H'_{WB}$, and for their calculation I determined P_S values at constant pH and I assumed that RBC intracellular concentrations of other allosteric effector were constant. The heat of solution of O_2 (~12.6 kJ mol^{-1}) is included in whole blood $\Delta H'_{WB}$ values, but excluded from hæmolysate $\Delta H'$ values. The pH dependency of Hb-O_2 affinity was determined by calculating Bohr coefficients at different %Hb-O_2 saturations (P_S):

$$\varphi = \frac{\Delta \log_{10} P_S}{\Delta \mathrm{pH}}$$

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

3.3 Results

Species lengths and blood parameters are summarized in Table 3.1. Whole blood OEC's were successfully constructed for four opah and five swordfish. Hæmolysate experiments were conducted on samples from three opah, six swordfish, and the two bluefin tuna.

Whole-blood experiments

Whole blood P_{50} values at pH 7.7 [an approximation of arterial blood pH from measurements reported from swimming yellowfin tuna (Korsmeyer et al., 1997a)], Hill coefficients, and Bohr coefficients are reported in Table 3.2. Both the opah and the swordfish have relatively high blood-O_2 affinities (i.e., low P_{50} values), and large Bohr coefficients. Hill coefficients tended to decline with decreasing pH, and although temperature did not influence n_{50}

values for opah, they were highest at 10°C in swordfish blood (χ^2 = 4.209 df = 1, P = 0.0402). Whole blood OECs at pH 7.7 and lower pH levels that correspond to temperate dependent pH shifts are presented in Figure 3.2.

In opah whole blood, temperature had a negligible effect on blood-O_2 affinity below 90% saturation. At 90% saturation temperature was an important model factor (χ^2 = 10.101 df = 4, P = 0.039), which is evident as a significant increase in blood-O_2 affinity (decreasing P_{90}) as temperature increased from 10 to 15°C, and no evident effect of temperature between 15 and 20°C. In opah blood at pH 7.7 and between 10 and 15°C, $\Delta H'_{WB}$ values were negative (exothermic) at low saturation, but increased with increasing saturation, becoming positive (endothermic) above ~44% saturation. This transition from exothermic to endothermic $\Delta H'_{WB}$ values occurred at lower saturations with lower blood pH (Figure 3.3). Between 15 and 20°C, $\Delta H'_{WB}$ values were negative but near to zero at pH 7.7. At lower blood pH, $\Delta H'_{WB}$ values were negative and quite exothermic below ~80% saturation (Figure 3.3), although below 90% saturation there were no apparent differences between P_S values at 15 and 20°C (Figure 3.2).

In swordfish whole blood temperature had a negligible effect on blood-O_2 affinity below 50% saturation, but above 50% saturation temperature and the interaction between temperature and pH were important model factors ($P_{50:}$ χ^2 = 7.284, df = 2, P = 0.026; $P_{60:}$ χ^2 = 12.532, df = 2, P = 0.002; $P_{70:}$ χ^2 = 18.790, df = 2, P = 0.0000831; $P_{80:}$ χ^2 = 17.621, df =2, P = 0.000149; $P_{90:}$ χ^2 = 9.7449, df = 2, P = 0.007654; $P_{95:}$ χ^2 = 7.605, df = 2, P = 0.02232). The effect of temperature on blood-O_2 affinity was pH dependent in swordfish blood. At high pH, an increase in temperature decreased blood-O_2 affinity above 50% saturation, evident as a right shift of the upper portion of the OECs (pH 7.7) between 10 and 25°C. The effect of temperature was reduced as pH declined, and around pH 7.4 temperature had a negligible effect on blood-O_2 affinity. The

pH dependency of the effect of temperature on whole blood-O_2 affinity is evident in plots of $\Delta H'_{WB}$ as a function of saturation, where $\Delta H'_{WB}$ becomes more endothermic with decreasing saturation and pH (Figure 3.3).

The effects of closed-system temperature changes on blood PO_2 are shown in Figure 3.4. Closed-system warming of opah blood generally decreased blood PO_2, due to increased Hb-O_2 affinity with increasing temperature (i.e., a reverse temperature-dependence). Closed-system cooling of opah blood tended to increase blood PO_2, but this effect was variable. In contrast, closed-system temperature changes of swordfish blood changed blood PO_2 beyond that predicted by Henry's law [i.e., increasing temperature will increase PO_2 in a closed system due to a reduction in plasma O_2 solubility and vice versa]. The change in PO_2 due to the change in solubility between 10 and 25°C was subtracted from the total change in blood PO_2 (ΔPO_2) to estimate the contribution to ΔPO_2 from temperature induced O_2 unloading or binding to Hb (ΔPO_2^{Hb}). Values of ΔPO_2^{Hb} with warming did not show any evident trend with initial pH or equilibration PO_2 (i.e., initial PO_2), and averaged (± 95% CI) 12.2 (± 6.5) mmHg. This increase in PO_2 was assumed to be due to temperature induced Hb-O_2 unloading and would be caused by < 1% decrease in Hb-O_2 saturation. With closed-system cooling of swordfish blood, ΔPO_2^{Hb} ranged from -34 to 6.5 and was correlated with the initial equilibration PO_2 (Pearson's r = -0.603, P = 0.0004) with greater decreases at higher equilibration PO_2.

Hæmolysate experiments

Hill plots of the hæmolysate experiments are presented in Figure 3.5, and P_{50} and enthalpy values are summarized in Table 3.3. Opah stripped hæmolysates showed a very slight reduction in Hb-O_2 affinity by increasing temperature from 10 to 20°C, whereas the O_2 affinity of swordfish hæmolysates was greatly reduced by increasing temperature from 10 to 25°C.

Bluefin tuna hæmolysates showed a reverse temperature effect. The intrinsic O_2 affinity of Swordfish hæmolysate was higher (i.e., lower P_{50}) than those of opah and bluefin tuna. ATP greatly reduced the O_2 affinities and temperature sensitivities of both opah and swordfish hæmolysates. The presence of ATP caused a reverse temperature-dependency of O_2 affinity in Opah hæmolysates, resulting in positive $\Delta H'$ values (i.e., endothermic) (Figure 3.6 and Table 3.3). For swordfish hæmolysates, the effect of temperature on O_2 affinity was both pH and ATP dependent, with $\Delta H'$ becoming increasingly more endothermic with declining pH (Figure 3.6). At pH 7.4 [an approximation of RBC intracellular pH (Weber et al., 2010)] and in the presence of ATP, the effect of temperature on swordfish hæmolysate O_2 affinity was reversed above 30% saturation (Figure 3.6B).

Bohr factors were similar between temperature treatments for stripped hæmolysates of both opah and swordfish. The addition of ATP increased the effect of pH on Hb-O_2 affinity, and caused Bohr factors to be higher at 10°C than at higher temperatures (Table 3.3).

3.4 Discussion

Main findings

The purpose of this study was to investigate the temperature-dependence of Hb-O_2 affinity in blood and stripped hæmolysates of two regionally heterothermic teleosts, the opah and the swordfish. I hypothesized that temperature-independent Hb-O_2 affinity is associated with the evolution of regional heterothermy, so I expected that both the swordfish and the opah should have Hbs that are insensitive to temperature. I also conducted experiments on Atlantic bluefin tuna hæmolysates to compare the enthalpic contributions of oxygenation linked effector dissociation among the different lineages of regionally heterothermic teleosts.

The results show temperature-independence of Hb-O_2 affinity in the opah, and a pH and saturation dependent effect of temperature on Hb-O_2 affinity in the swordfish (Figures 3.2-3.6). Opah whole blood-O_2 affinity showed a reverse temperature-dependence, which caused a reduction in PO_2 when blood was warmed in a closed system (Figure 3.4). These findings were supported by the results of experiments on hæmolysates, which showed that opah Hb-O_2 affinity was temperature-independent within a physiologically relevant pH range, and the addition of ATP caused a reverse temperature-dependence (Figures 3.5 and 3.6). Swordfish blood-O_2 affinity decreased with increasing temperature, but the effect of temperature was dependent on O_2 saturation and blood pH (Figures 3.2 and 3.3). These results were paralleled in closed system warming of swordfish blood, which caused blood PO_2 to increase beyond what would be expected due to the temperature dependence of the O_2 solubility of blood plasma (Figure 3.4). Experiments on swordfish hæmolysates showed an ATP induced temperature independence of Hb-O_2 affinity below pH 7.5, like other billfishes (Figures 3.5 and 3.6) (Weber et al., 2010). Bluefin tuna hæmolysates showed a reverse temperature-dependence (Figures 3.5 and 3.6), in accordance with previous studies (Carey and Gibson, 1977; Ikeda-Saito et al., 1983). 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 "could be misleading if only P_{50} values are evaluated" (Sharp, 1975).

Whole blood and closed-system temperature changes

Opah and swordfish have relatively high Hb-O_2 affinities, with whole blood P_{50} values around 18 mmHg in the opah and 12 mmHg in the swordfish at pH 7.7 and 10°C. These relatively low P_{50} values may ensure adequate O_2 uptake when opah and swordfish are at depths near the oxygen minimum layer (Figure 3.1) (Sepulveda et al., 2010; Wegner et al., 2015). The

$\Delta H'_{WB}$ values for opah and swordfish blood P_{50} at pH 7.7 were around 1 kJ mol^{-1} O_2 and -23 kJ mol^{-1} O_2, respectively.

Temperature induced shifts of the swordfish OEC were observed during closed-system warming and cooling, which caused respective increases and decreases to the blood PO_2 (Figure 3.4) that were similar to previous reports from bigeye tuna blood (Figure 1.6) (Lowe et al., 2000). The closed-system PO_2 changes observed in bigeye tuna and swordfish blood exceed the PO_2 changes expected in plasma, indicating that O_2 is being released or bound to Hb with warming and cooling, respectively. The swordfish $\Delta H'_{WB}$ value is close to that for bigeye tuna (~ -18 kJ mol^{-1} O_2), which likely underlies the similar closed-system results for these species (Lowe et al., 2000). Furthermore, if swordfish blood is returning to the gills around 50% saturated and leaving the gills around 95% saturated, then the average enthalpy of oxygenation might be around -37 kJ mol^{-1}, potentially causing about 8% of the heat produced during glucose metabolism to be liberated and lost to the inspired water during Hb-oxygenation. This heat loss may prevent efficient heat retention in a large tissue mass such as the red muscle, which may contribute to why the red muscle *retia* of swordfish are not as efficient at heat retention as those of tunas and sharks and likely serve to just slow down cooling of the red muscle (Carey, 1990; Stoehr et al., 2018).

The temperature-independence and reverse temperature-dependence exhibited by opah blood during closed system warming and cooling is probably necessary for O_2 transport to the tissues, which may range from cold ambient temperatures as low as 8°C to the warmest tissues that are around 17°C. Over this temperature range, opah blood PO_2 should remain relatively stable throughout the circulation. Furthermore, the enthalpy of oxygenation for opah Hb-O_2

binding from 50% to 95% saturation would be about +22 kJ mol^{-1}, which should eliminate any loss of metabolic heat during Hb-oxygenation.

Molecular mechanisms of reduced temperature dependence of Hb-O_2 affinity

Reductions in the thermal sensitivities of Hb-O_2 affinity results from oxygenation-linked effector dissociation that contribute endothermically to $\Delta H'$, but the relative contributions of different effectors vary among lineages of regional heterotherms. In swordfish, the primary effector is ATP with secondary contributions from protons, which is the same as other billfishes in the family Istiophoridae (Weber et al., 2010). In billfishes, ATP binding reduces Hb-O_2 affinity below pH 8.0 and causes Hb-O_2 affinity to be temperature-independent above 50% O_2 saturation and within the physiological RBC intracellular pH range (~ pH 7.0 – 7.5; Figure 3.6) (Weber et al., 2010). At low temperature ATP is likely preferential bound at high O_2 saturations, but rising temperature causes dissociation of ATP and protons from Hb, stabilizing the high affinity conformation and causing $\Delta H'$ to approach zero or become positive. Although ATP had large enthalpic contributions to $\Delta H'$ in opah hæmolysates, temperature appears to have no effect on stripped hæmolysate O_2 affinity in opah, at least within a physiologically relevant pH range (Figure 3.6). Thus, the decreased effect of temperature on Hb-O_2 affinity is likely due to temperature-dependent dissociation of protons that stabilizes the high-affinity conformation at higher temperatures and increases $\Delta H'$ to 0 $kJmol^{-1}$. Preferential binding of ATP at low temperature probably stabilizes the low affinity conformation, but increasing temperature causes dissociation of ATP, probably decreasing the allosteric constant, *L*, by stabilizing the high affinity R-state conformation. The enthalpic contribution of ATP dissociation will cause $\Delta H'$ to become positive. Protons are the main effector of Atlantic bluefin tuna Hbs, consistent with previous work (Ikeda-Saito et al., 1983). Therefore, although the molecular mechanisms

underlying reductions in the temperature sensitivity of Hb-O_2 affinity are similar among regionally heterothermic teleosts, the varying roles of allosteric effectors on reducing $\Delta H'$ indicate the independent evolution of temperature insensitivity among tunas, billfishes, opah. Elucidation of the effector binding sites and the amino acid arrangements and mutations that gave rise to them among different lineages of regionally heterothermic teleosts is worthy of future research.

Possible limitations

My main objective was to construct OECs at temperatures close to the minimum and maximum temperatures that the blood would experience *in vivo*. Swordfish make diel, vertical movements from the warm upper mixed layer to cold waters below the thermocline, subjecting themselves to ambient temperatures from 4 to 30°C while maintaining relatively stable cranial temperatures from around 19 to 24°C (Carey, 1990; Dewar et al., 2011; Sepulveda et al., 2010). Although opah occasionally swim into warm water of around 20°C in the warm upper mixed layer, they tend to remain in cooler water below the thermocline where they maintain pectoral muscle temperatures around 14 to 17°C, or around 5°C above ambient (Wegner et al., 2015). Cranial temperatures measured in decked opah have been reported to average around 21°C, although this may be warmer than experienced in free swimming fish (Runcie et al., 2009). The experimental temperatures (10, 15 and 20°C for opah, and 10 and 25°C for swordfish) encompass the low and high ambient and body temperatures reported for these two species, and these temperatures are also comparable to previous studies on closely related or ecologically similar species (Andersen et al., 1973; Brill and Bushnell, 2006; Lowe et al., 2000; Weber et al., 2010). The experimental temperatures used for the Atlantic bluefin tuna hæmolysates matched those used for bluefin tuna blood by Brill and Bushnell (2006). A potential limitation is that fish

were captured at-sea because it was unrealistic to sample resting and cannulated swordfish and opah. The individuals included in this study had likely experienced varying levels of exercise induced fatigue and consequent respiratory and metabolic acidosis, which are indicated by relatively high plasma lactate levels (Table 3.1). However, the blood pH levels achieved with the CO_2 exposures were within physiologically relevant ranges, which allowed me to estimate blood P_S at physiologically relevant pH values. The main objective of this chapter was to investigate the effect of temperature on Hb-O_2 affinity, and it is very unlikely that any exercise or stress induced physiologically changes would have compromised the thermodynamics of Hb-O_2 binding in whole blood. However, my measurements of RBC intracellular ATP levels were relatively low, and if *in vivo* ATP:Hb ratios are higher in swordfish blood, then the effect of temperature on Hb-O_2 affinity would likely be reduced at higher pH than I measured *in vitro*. Nonetheless, our results from whole blood experiments were corroborated by the results of experiments on hæmolysates purified of endogenous ionic effectors.

Summary

The results presented here show temperature-independent and reversed temperature-dependence of Hb-O_2 affinity in blood and hæmolysates of opah, and temperature-independence at low pH in swordfish blood and hæmolysates. Reductions and reversals of the temperature sensitivity of Hb are likely associated with the evolution of heat exchanging *retia mirabilia* (Weber and Campbell, 2011) that facilitated the evolution of regional heterothermy among disparate lineages of teleosts and sharks (Block and Finnerty, 1994; Carey, 1982a; Dickson and Graham, 2004). Among regionally heterothermic teleosts, the effector ions that underlie the molecular mechanism of modulations to the enthalpy of Hb-O_2 binding differ among the opah,

billfishes, and tunas, and this striking functional convergence is paralleled in the Hbs of other regionally heterothermic vertebrates with heat exchanging *retia* (Weber and Campbell, 2011).

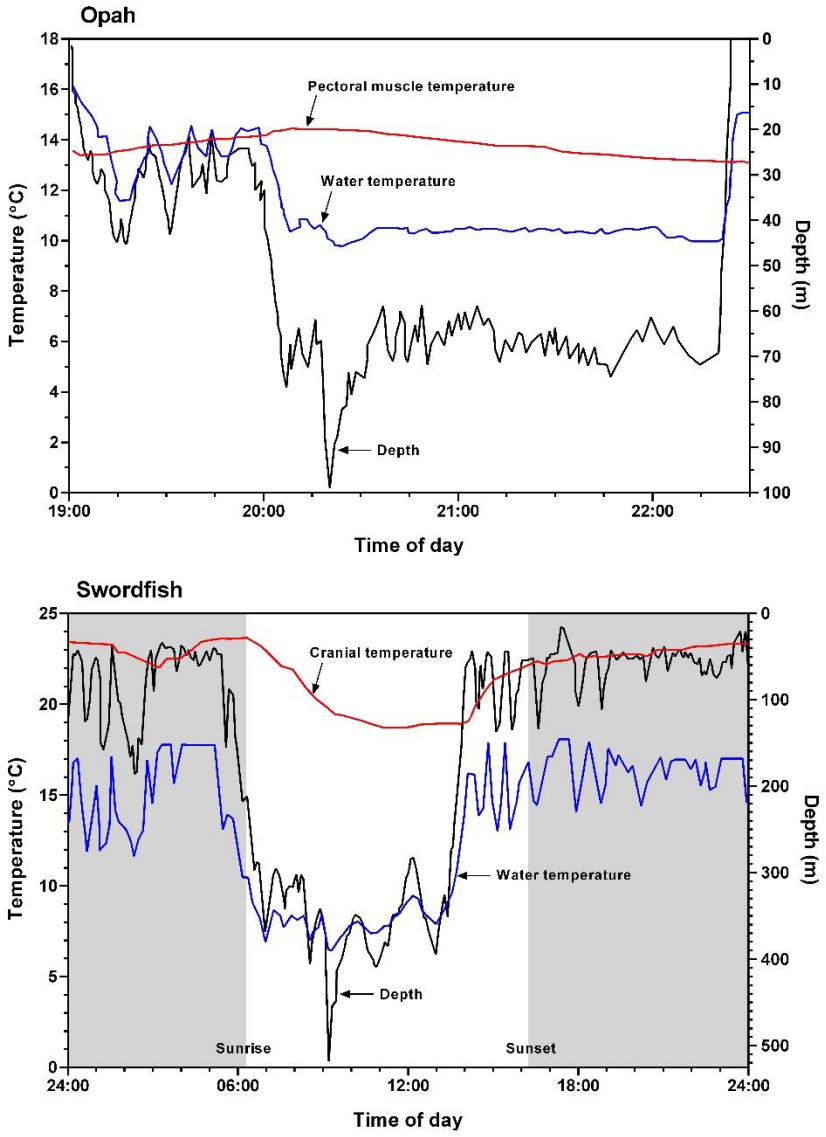

Figure 3.1 Opah i*n vivo* pectoral muscle temperature and swordfish *in vivo* cranial temperature while swimming at depth.

Depth (black), water temperature (blue) and tissue temperature (red) profiles were redrawn with data from Wegner et al. (2015; Opah) and Carey (1990; Swordfish).

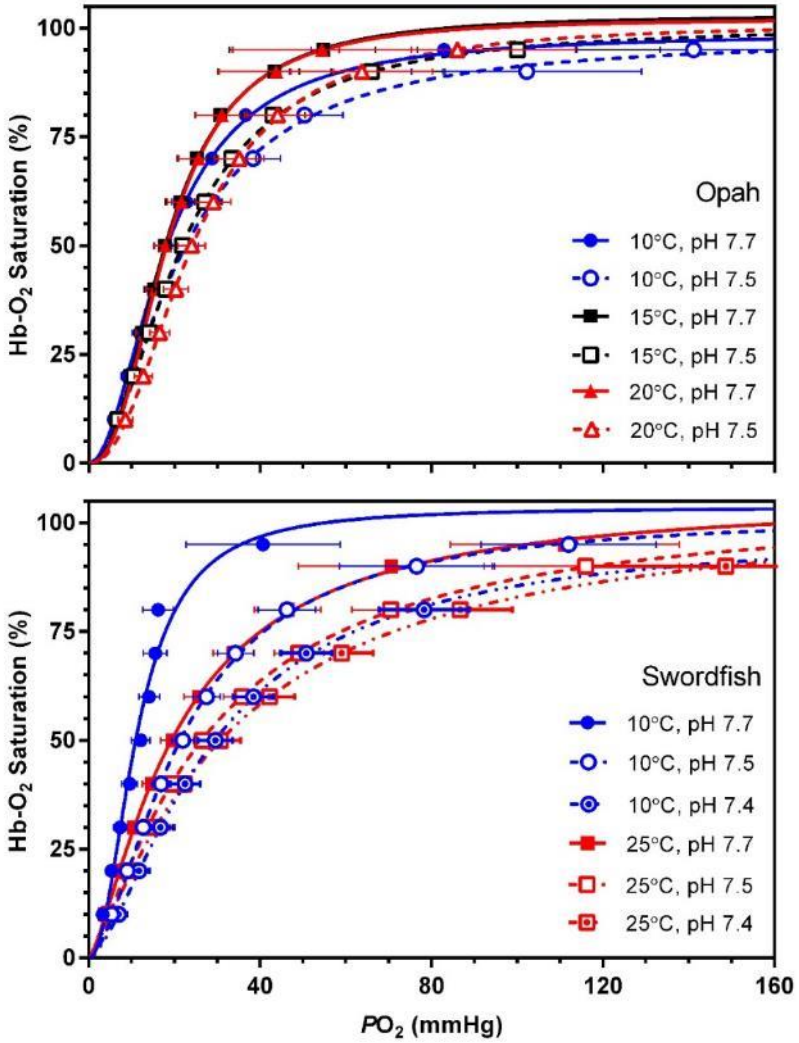

Figure 3.2 Whole blood oxygen equilibrium curves (OECs) for the smalleye Pacific opah and the swordfish at different pH and temperatures.

Blood PO_2 values were interpolated (± bootstrap standard errors) from linear mixed models of logPO_2 vs pH at specific Hb-O_2 saturation levels (see Methods section). Opah OECs were constructed at 10°C (circles), 15°C (squares), and 20°C (triangles) at pH 7.7 (filled symbols and solid curves) and pH 7.5 (open symbols and dashed curves). Swordfish OECs were constructed at 10°C (circles) and 25°C (squares) at pH 7.7 (filled symbols and solid curves), pH 7.5 (open symbols and dashed curves) , and pH 7.4 (open symbols plus a dot, and dash-dotted curves).

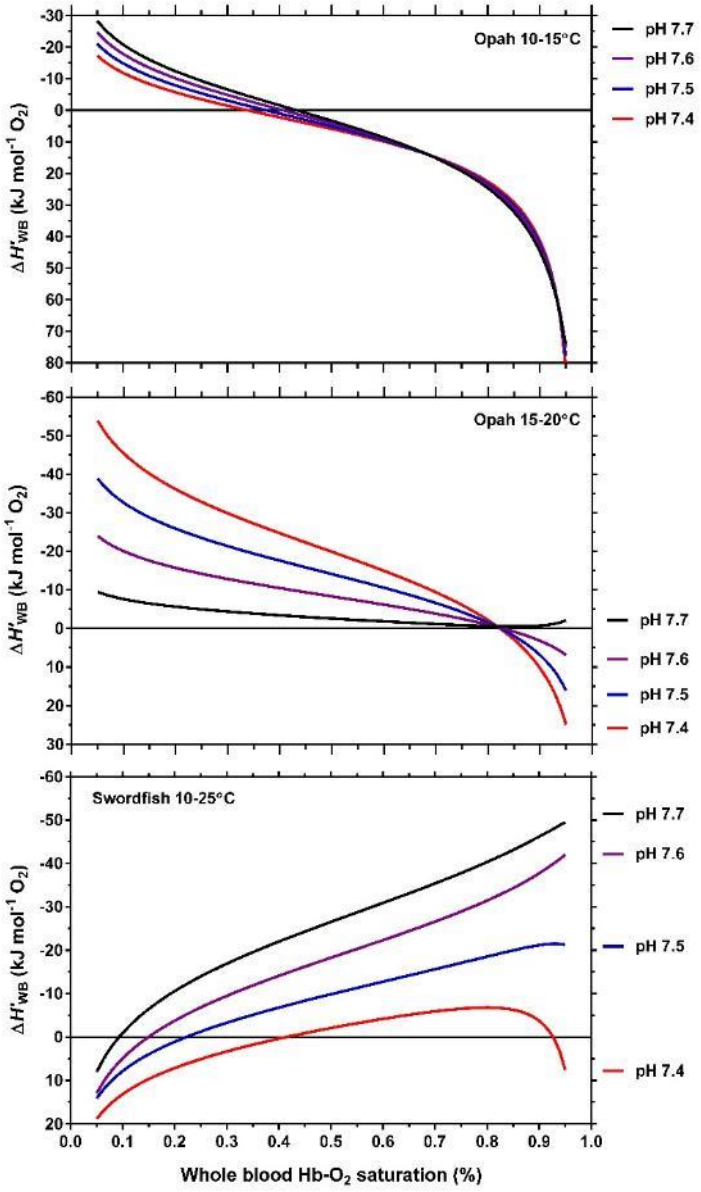

Figure 3.3 Predicted enthalpy of oxygenation ($\Delta H'_{WB}$) as a function of whole blood pH and whole blood Hb-O_2 saturation for the smalleye Pacific opah and the swordfish.

$\Delta H'_{WB}$ values were calculated with the van't Hoff isochore (see methods) at constant pH between 10-15°C and 15-20°C for opah blood, and between 10-25°C for swordfish blood. The blood-O_2 tensions (PO_2) at specific blood-O_2 saturation levels at a given pH and temperature were determined from the data presented in Figure 3.1.

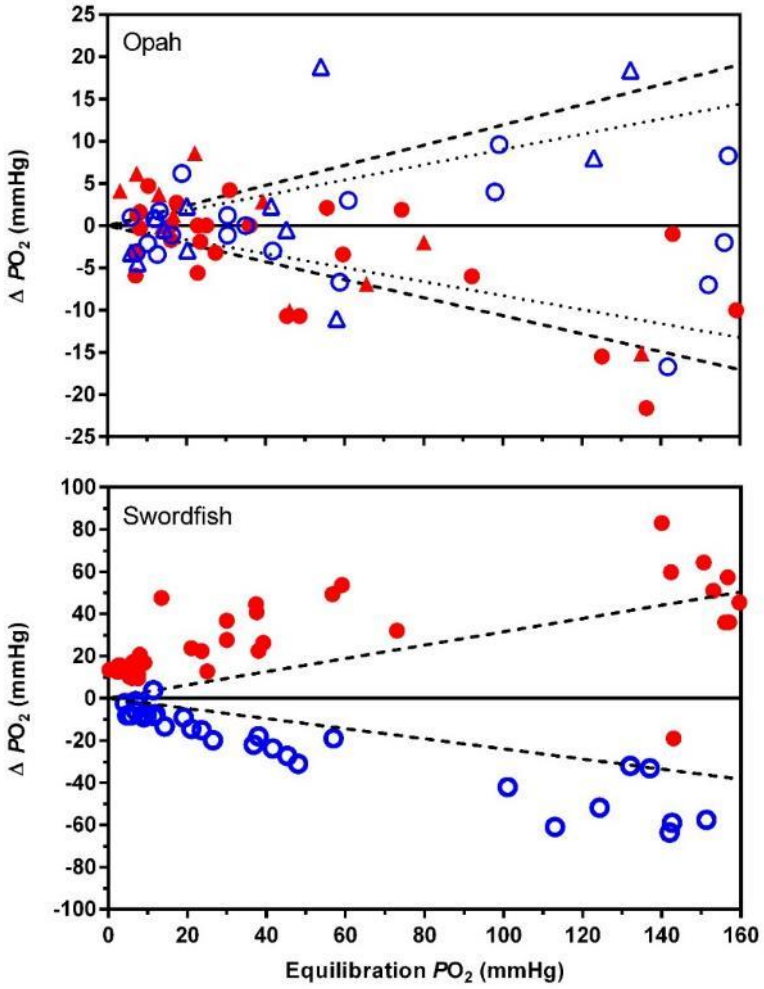

Figure 3.4 Effects of closed-system temperature changes on the measured change in blood PO_2 (ΔPO_2) in blood from the smalleye Pacific opah and the swordfish.

Opah and swordfish blood was equilibrated at a range of O_2 tensions (Equilibration PO_2) and then heated (filled symbols) or cooled (open symbols). Opah blood temperature was changed between either 10 and 15°C (triangles) or 15 and 20°C (circles), and swordfish blood was changed between 10 and 25°C. Dotted lines indicate the temperature induced ΔPO_2 expected due to changes in solubility of blood plasma at a given equilibration PO_2 (i.e., Henry's Law) between 10 and 15°C (dashed), and 15 and 20°C (dotted) for opah blood, and between 10 and 25°C (dashed) for swordfish blood

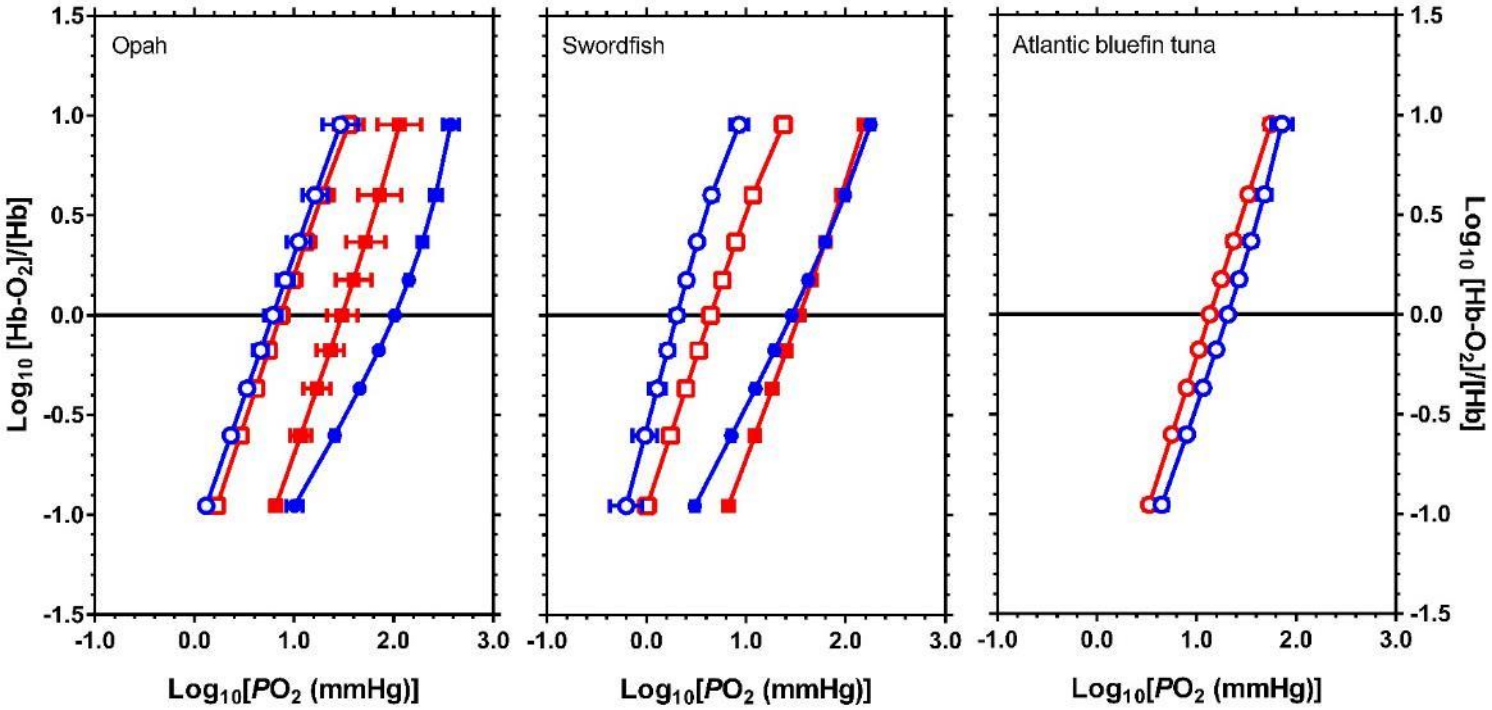

Figure 3.5 Hill plots of Hb-O_2 equilibria of smalleye Pacific opah, swordfish, and Atlantic bluefin tuna at pH 7.4.

Experiments were conducted on stripped hæmolysates in the absence of ATP (open symbols), and in the presence of ATP (filled symbols) at two temperatures. Low temperature data (circles) are at 10°C for opah and swordfish, and 15°C for bluefin tuna. Warm temperature data (squares) are at 20°C for opah, and 25°C for swordfish and bluefin tuna. Data points (± bootstrap standard errors) were predicted at pH 7.4 from linear mixed models of logP_S vs pH (see Methods section). Experiments were conducted in 0.1 M Hepes buffer at Hb concentrations of 0.6 mM, and ATP was added at saturating concentrations (18 mM; ATP/Hb = 30).

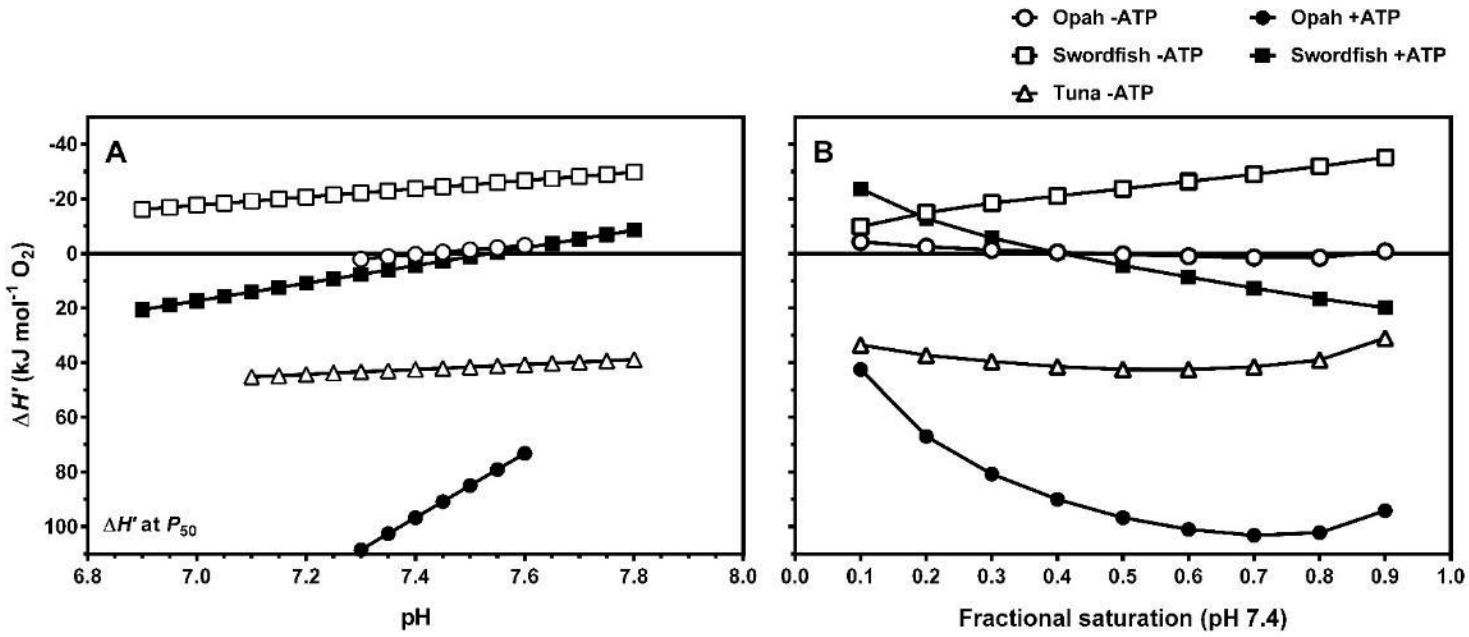

Figure 3.6 Heats of oxygenation (Δ*H'*) of stripped hæmolysates of smalleye Pacific opah, swordfish, and Atlantic bluefin tuna in the absence and presence of ATP.

In panel A, Δ*H'* values were calculated with the van't Hoff equation (see Methods) with logP_{50} values that were interpolated from logP_{50}/pH relationships. Δ*H'* values in panel B were calculated from data reported in Figure 3.4, and plotted as function of Hb-O_2 fractional saturation at pH 7.4. Experiments were conducted in 0.1 M Hepes buffer at Hb concentrations of 0.6 mM, and ATP was added at saturating concentrations (18 mM; ATP/Hb = 30). Δ*H'* values exclude the heat of O_2 in solution (-12.6 kJ mol^{-1}).

Table 3.1 Fork length (FL), and blood variables for smalleye Pacific opah, swordfish, and Atlantic bluefin tuna.

Adenosine triphosphate (ATP) concentration (mM) is reported relative to mean corpuscular hæmoglobin concentration (MCHC). Values are means ± standard error with samples sizes in parentheses. If values were measured in only one or two individuals, then the individual measurements are reported.

	Opah	Swordfish	Bluefin Tuna
FL (cm)	116	149.5 ± 12.4 (4)	107, 109
Hæmatocrit (%)	59.1 ± 2.5 (4)	46.3 ± 4.7 (7)	57.8, 53.5
Hæmoglobin (mM)	2.21 ± 0.12 (4)	1.81 ± 0.15 (7)	2.53, 2.36
MCHC (mM)	3.74 ± 0.14 (4)	3.96 ± 0.20 (7)	4.37, 4.41
ATP:Hb	1.32, 0.68	0.67 ± 0.16 (7)	0.40, 0.54
Plasma osmolality (mOsm/kg)	565.2 ± 6.4 (3)	437.5 ± 19.9 (7)	410, 379
Plasma lactate (mM)	14.7 ± 0.79 (3)	12.0 ± 1.53 (6)	18.7, 18.6

Table 3.2 Whole blood oxygen equilibria parameters for smalleye Pacific opah and swordfish at different temperatures.

Whole blood P_{50} (PO_2 at 50% Hb-O_2 saturation) is reported at pH 7.7, a proxy for arterial blood pH, and was predicted from linear mixed models of logP_{50} vs pH (see Methods section). Bohr coefficients were determined as the the model slopes (i.e., $\Delta \log P_{50}/\Delta$pH). Hill cooperativity coefficients (n_{50}) were determined at P_{50} and estimated at pH 7.7 from mixed linear models of n_{50} vs pH. P_{50} and n_{50} values are reported with bootstrap estimated standard errors, and Bohr coefficients are reported with the 95% confidence intervals for the slopes of the linear mixed models.

	Opah			Swordfish	
	10°C	**15°C**	**20°C**	**10°C**	**25°C**
P_{50} (mmHg) at pH 7.7 (SE)	17.9 (15.7 to 20.4)	17.7 (15.5 to 20.2)	17.9 (15.7 to 20.4)	12.1 (10.3 to 14.3)	19.6 (17.1 to 22.5)
n_{50} at pH 7.7 (SE)	2.03 (0.15)	2.14 (0.15)	2.26 (0.15)	1.57 (0.10)	1.36 (0.09)
Bohr coefficient (95% CI)	-0.49 (-0.77 to -0.20)	-0.46 (-0.75 to -0.11)	-0.64 (-1.09 to -0.49)	-1.29 (-1.80 to -0.80)	-0.65 (-1.23 to -0.01)

Table 3.3 Hæmolysate oxygen equilibria parameters of smalleye Pacific opah, swordfish, and Atlantic bluefin tuna at two experimental temperatures and in the absence or presence of ATP.

P_{50} values (± bootstrap standard errors) were predicted at pH 7.4 from linear mixed models of logP_{50} vs pH (see Methods section), and Bohr coefficients are the model slopes (i.e., $\Delta\log P_{50}/\Delta$pH) with their 95% confidence intervals. $\Delta H'$ values were calculated with logP_{50} values at pH 7.4, and excludes the heat of O_2 in solution (-12.6 kJ mol^{-1} O_2).

	Opah		Swordfish		Bluefin tuna	
	10°C	**20°C**	**10°C**	**25°C**	**15°C**	**25°C**
***P*$_{50}$ (mmHg), pH 7.4**						
Stripped Hb	6.13 (± 1.40)	7.32 (± 1.45)	2.02 (± 0.30)	4.39 (± 0.73)	20.58 (± 2.31)	13.57 (± 1.57)
Hb + ATP	102.45 (± 4.57)	30.31 (± 12.74)	28.71 (± 1.54)	34.26 (± 2.37)		
Bohr coefficients						
Stripped Hb	-0.50 (-0.69 to –0.34)	-0.40 (-0.55 to -0.25)	-0.26 (-0.37 to -0.15)	-0.12 (-0.24 to 0.02)	-0.71 (-0.91 to -0.51)	-0.66 (-0.89 to -0.45)
Hb + ATP	-2.78 (-3.15 to -2.42)	-2.04 (-2.45 to -1.65)	-1.28 (-1.44 to -1.12)	-0.98 (-1.15 to -0.79)		
Δ*H*' (kJ mol^{-1}), pH 7.4						
Stripped Hb	0.28		-23		42	
Hb + ATP	97		4			

Chapter 4 The Effect of Temperature on Hæmoglobin-Oxygen Affinity in Sharks

"The ability to swim swiftly, achieved through the extra power available from warm muscles, must have been a powerful selective advantage in the evolution of tuna and lamnid sharks. Starting with the basic features of a fish vascular system the stringent requirements of speed have resulted in the remarkable parallel evolution of almost identical systems in two completely unrelated groups of fish." (Francis G. Carey and John M. Teal, 1969a, p. 203)

"[A]lthough the lamnid sharks are powerful and vigorous predators, they are also delicate animals." (Francis G. Carey et al., 1985, p. 93)

4.1 Introduction

Sharks in the family Lamnidae (salmon shark, *Lamna ditropis*, porbeagle shark, *Lamna nasus*, shortfin mako, *Isurus oxyrinchus*, longfin mako, *Isurus paucus*, white shark, *Carcharodon Carcharias*), as well as the common thresher shark (*Alopias vulpinus*) have evolved specialised circulatory systems that retain the heat produced by their RM, which are internalized relative to other sharks (Figure 1.3) (Bernal et al., 2001b; Bone and Chubb, 1983; Burne, 1924; Carey and Teal, 1969a; Carey et al., 1985; Patterson et al., 2011). In these regionally heterothermic sharks, the bulk of the systemic circulation is directed laterally through subcutaneous arteries and veins, which redirect the blood supply to the medially situated RM through a heat exchanging *rete mirabile* on either side of the body. Lamnid sharks also have warm brains, eyes, and viscera that are all supplied with blood that passes through additional heat exchanging *retia* in transit to those organs (Alexander, 1998; Bernal et al., 2001b; Block and Carey, 1985; Burne, 1924; Carey et al., 1981; Carey et al., 1985; Tubbesing and Block, 2000). Interestingly, the bigeye thresher shark

(*Alopias superciliosus*), a close relative of the common thresher shark, has unusually large eyes that are supplied with blood through an orbital *rete* suspected of having a heat exchanging function (Block and Carey, 1985; Block and Finnerty, 1994; Dickson and Graham, 2004; Weng and Block, 2004).

In the temperately distributed shortfin mako and common thresher shark, RM temperatures are elevated around 5 to 10°C over ambient in the mako, and around 2 to 5°C in the common thresher shark (Figure 1.4) (Bernal and Sepulveda, 2005; Carey and Teal, 1969a; Carey et al., 1985; Patterson et al., 2011). It is not clear if the bigeye thresher shark has warm eyes, since opportunistic measurements of bigeye thresher eye temperatures have shown them to be no warmer than ambient sea surface temperature (Diego Bernal and Chugey A. Sepulveda, personal communication). However, mako eye, brain, and visceral temperatures are also much warmer than ambient (Anderson and Goldman, 2001; Block and Carey, 1985; Carey et al., 1981). For example, mako stomach temperatures remain warmer than ambient water, even during repeated descents below the thermocline, and are on average about 4°C warmer than the surrounding water (Figure 4.1) (Sepulveda et al., 2004).

Like many other pelagic species, regionally heterothermic sharks encounter a wide range of water temperature during vertical movements through the water column or over long latitudinal migrations into temperate and subpolar waters (Bernal et al., 2009; Weng et al., 2005). The shortfin mako and the common thresher routinely descend below the thermocline into water that may be more than 10°C cooler than surface waters (Figure 4.1) (Cartamil et al., 2011; Cartamil et al., 2016; Holts and Bedford, 1993; Sepulveda et al., 2004), and bigeye thresher sharks subject themselves to an extremely wide range of water temperatures (~ 6 to 25°C) during diel vertical migrations (Figure 4.2) (Coelho et al., 2015; Nakano et al., 2003; Sepulveda et al.,

2019; Weng and Block, 2004). Consequently, blood-O_2 uptake at the gills must occur over the range of environmental temperatures that these sharks encounter, and movements into cold water increase internal temperature gradients. Furthermore, the bigeye thresher shark spends most of the day in deep, cold water near the oxygen minimum layer (OML) where the concentration of dissolved oxygen may be less than 2 mg L^{-1} (Figure 4.2), so a high Hb-O_2 affinity may be necessary to extract O_2 from the hypoxic water.

Since $\Delta H'$ is negative (i.e., exothermic) for most vertebrate Hbs, increasing blood temperature typically decreases Hb-O_2 affinity. This can potentially benefit O_2 unloading to warm active tissues, or O_2 uptake in fishes in cold hypoxic water. However, most studied regionally heterothermic fishes have Hb with a greatly reduced or even reversed temperature-dependence (reviewed in Chapter 1, section 1.3). It may seem unlikely that Hbs from regionally heterothermic sharks would have evolved temperature-independent O_2-affinty linked to enthalpic contributions from allosteric effectors, since sharks in general tend to have high Hb-O_2 affinities, small Bohr effects, and lower concentrations of nucleoside triphosphates (NTPs) in their RBCs relative to teleosts (Morrison et al., 2015). However, ATP does affect the O_2 affinity, the Bohr effect, and temperature sensitivity of shark Hbs (Larsen et al., 2003; Weber et al., 1983a), and Larsen et al. (2003) have shown that ATP is responsible for reversing the temperature-dependence of O_2 affinity in porbeagle shark Hb. There are not many whole blood O_2 equilibria studies of lamnid sharks, although Bernal et al. (2018) reported that shortfin mako whole blood-O_2 affinity was almost temperature-independent. However, Bernal et al. (2018) reported sizable Bohr coefficients, in contrast to a previous report of pH independence in mako blood (Wells and Davie, 1985), and the measures of blood-O_2 affinity were not reported at precise blood pH values, making it difficult to quantify the effect of temperature on blood-O_2 affinity in mako

blood. I am not aware of any published studies on the Hb or blood of the common thresher shark or the bigeye thresher shark.

In this chapter I address the question of how does temperature affect Hb-O_2 affinity in the common thresher shark and the bigeye thresher shark? If temperature-independent Hb-O_2 affinity is necessary for sharks to maintain regional heterothermy, then like my expectations for the regionally heterothermic teleosts in Chapter 3, I also expect that the common thresher shark will have Hbs that are insensitive to temperature. The bigeye thresher makes diel movements from warm water above the thermocline to cold water deep below thermocline, and it also has an orbital *rete*, although its function is unclear. Therefore, I expect the bigeye thresher shark to have a high Hb-O_2 affinity, and the temperature-dependence of bigeye thresher shark Hb may provide some insight into the functional significance of temperature-independent Hb-O_2 affinity in regionally heterothermic fishes. Additionally, I have also conducted experiments of blood from shortfin mako sharks to add to the results of Bernal et al. (2018) on blood from three makos, to corroborate the findings of that study with a larger sample size. For comparison, experiments 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).

4.2 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 no. SC-2471), and the University of British Columbia (UBC) Animal Care Committee (animal care no. A11-0235 and A15-0266).

Blood collection

Bigeye thresher sharks (n = 9) were captured by deep-set buoy gear (Sepulveda et al., 2014) in the coastal waters off Southern California (i.e., the Southern California Bight). Common thresher sharks (n = 2) and spiny dogfish (n = 8) were captured by hook and line off Massachusetts. Shortfin mako (n = 9) and blue sharks (n = 8) were captured by hook and line off Southern California or off Massachusetts. Blood was immediately drawn by caudal puncture into heparinized syringes. Blood samples were kept on ice and shipped by courier to the UBC campus in Vancouver, Canada, where experiments were conducted within 1 to 4 days after the blood was collected. Preliminary experiments with swordfish blood (*Xiphias gladius*) showed no changes in Hb concentration, hæmatocrit (Hct), or blood P_{50}, and no evidence of RBC lysis for up to 6 days after blood was collected, provided blood was refrigerated during this time (see Appendix).

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 measurements 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 was kept in the fridge at 4°C and OECs were constructed by quantifying the relative Hb-O_2 saturation at a range of equilibration PO_2's at two CO_2 levels and two temperatures. Blood pH, and PO_2 were measured in subsamples of blood equilibrated with gas mixes at each of the CO_2 and temperature treatments.

Hæmatological parameters

Hæmoglobin concentration was measured by the cyanmethæmoglobin method using Drabkin's reagent and a hæm-based extinction coefficient of 11 $mmol^{-1}$ cm^{-1}. All Hb concentrations are expressed as tetrameric Hb ([Hb], in mM). Hct was measured as the percentage of packed RBCs relative to total blood volume after centrifuging samples at 11,500 rpm for five minutes. Mean corpuscular hæmoglobin concentration (MCHC, in mM) was calculated by dividing [Hb] by Hct. Plasma osmolality (mOsm kg^{-1}) was measured in 10µL of undiluted plasma with a vapour pressure osmometer (VAPRO 5520, Wescor, Logan, Utah). ATP was assayed with a colourimetric assay kit (SIGMA-ALDRICH MAK190, Sigma-Aldrich Co. LLC, St. Louis, Missouri), and plasma lactate was measured spectrophotometrically using the LDH-catalyzed 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 PO_2

Oxygen equilibria experiments were conducted at temperatures that correspond near to the coldest environmental temperatures and the warmest tissue or environmental temperatures for each species. The coldest experimental temperature was 10°C for the bigeye thresher shark, and 15°C for the common thresher shark and the mako, which are within the range of the colder water temperatures regularly encountered by these species (Cartamil et al., 2016; Sepulveda et al., 2004; Sepulveda et al., 2019). The warmest experimental temperatures corresponded to the warmest water temperatures encountered by bigeye thresher 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 and Teal, 1969a; Patterson et al., 2011; Sepulveda et al., 2019). At each temperature treatment, experiments were conducted at two physiologically relevant CO_2 levels, 0.25% CO_2

and 1.00% CO_2 (0.25% and 0.50% CO_2 for spiny dogfish), to manipulate blood pH at a high and low level to quantify the effect of pH on blood-O_2 affinity (i.e., the Bohr effect).

The relationship between Hb-O_2 saturation and PO_2 (i.e., an OEC) was assessed on 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 (~ 3μL) between two sheets of low density polyethylene (Glad® ClingWrap) that were secured on an aluminum ring with two plastic O-rings, which were then placed in a gas tight tonometry cell modified to fit into a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, USA). Optical density (OD) was measured every 20 to 30 seconds at 390nm (an isosbestic point where OD is independent of Hb-O_2 saturation), and at 430 nm and 436 nm (wavelengths that typically correspond to a maximum absorbance for deoxygenated Hb). Initially, blood was equilibrated with pure N_2 for a minimum of 30 minutes until OD at 430/436 nm was stable, which was assumed to indicate full Hb deoxygenation. After deoxygenation, the Hb-O_2 saturation was increased with stepwise increments of the O_2 tension, balanced with N_2, up to 21% O_2. Full Hb-O_2 saturation was assumed after a final increment to 30% O_2 in the absence of CO_2. Gas mixtures of O_2, CO_2, and N_2 were obtained using a Wösthoff DIGAMIX® gas mixing pump (H. Wösthoff Messtechnik, Bochum, Germany). Fractional Hb-O_2 saturations were calculated for each equilibration step as the change in ΔOD (ΔOD = 430 nm or 436 nm - 390 nm) from full deoxygenation, relative to that between full deoxygenation (pure N_2) and full oxygenation (30% O_2).

Whole blood pH and PO_2 were measured in approximately 100-200 μL of blood equilibrated for 1 h with either 0.25 or 1.00% CO_2 (0.50% for spiny dogfish) and a range of O_2 tensions (balanced with N_2) in rotating glass tonometers thermostatted to either 10, 15, 22, or

25°C. Blood was drawn into a gas tight syringe pre-flushed with the gas mixture, and then the blood was injected into pH and P_{O_2} electrodes thermostatted to the respective experimental temperature. Blood 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), and P_{O_2} was measured with a Radiometer E5046 oxygen electrode.

To mimic the closed-system temperature changes that blood experiences in the arterioles and venules of heat exchanging retia mirabilia, approximately 100-200 μL blood samples equilibrated at either 10, 15, 22 or 25°C were injected into both a pH microelectrode and an oxygen electrode (as described above) thermostatted to a warmer or cooler experimental temperature according to previous studies (Bernal et al., 2018; Brill and Bushnell, 1991a; Cech et al., 1984). Although the blood was static within the electrode chamber, the blood was rapidly heated or cooled in a system where there is minimal exchange of gases and ions between the blood and another medium. Immediately prior to blood injection, each oxygen electrode was flushed and thus pre-equilibrated with the respective experimental gas mixture to minimise electrode equilibration time with the blood. Temperature induced changes in pH and P_{O_2} (ΔP_{O_2}) were monitored using data acquisition software, and when it appeared that pH and P_{O_2} traces had stabilized, the respective values of each were recorded.

Common thresher shark hæmolysate

Frozen and packed RBCs from one common thresher shark were slowly thawed on ice (>24 h) then mixed with an equal volume of cold 0.1 mM Hepes buffer (pH 8.0) and centrifuged at 10,000 RCF to remove cell debris. The resulting erythrolysate was 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 hæmolysates in 1000 µL of 100 mM Hepes buffer (pH 7.4) that was bubbled with 100% O_2, and a spectral scan was made from 500-700 nm (i.e., an oxyHb spectrum). The hæmolysate was concentrated with a centrifugal filter (30 kDa). Oxygen equilibria at 15 and 22°C were determined in 0.1 M Hepes buffer at a Hb concentration of 0.6 mM in the absence and presence of saturating levels of ATP (ratio of the concentration of ATP/Hb = 30). The experiments were also conducted in the absence or presence of 500 mM of urea, because elasmobranch tissues have high urea concentrations, and urea can have an antagonistic effect on the ATP sensitivity of elasmobranch Hbs (reviewed by Morrison et al., 2015). OECs were generated 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. The pH of the hæmolysate solutions was measured at the experimental temperature with a thermostated Mettler Toledo InLab Micro glass pH electrode (Mettler-Toledo LLC, Columbus, OH, USA).

Data analysis

All statistical analyses, curve fitting, and linear mixed model fitting were performed in R v 3.5.2 (R Core Team, 2017). Linear regressions were performed with GraphPad Prism version 6.01 (GraphPad Software, La Jolla California, USA). Nonlinear least-squares curve fitting by the Levenberg-Marquardt algorithm was performed using the nlsLM function from the 'minpack.lm' package for R (Elzhov et al., 2010), linear mixed models were fit using the lmer function from the 'lme4' package with the 'lmerTest' package (Bates et al., 2014; Kuznetsova et al., 2017).

Hæmoglobin concentration, Hct, and MCHC were compared among species by one-way analysis of variance, and differences among the species means were assessed by Tukey's multiple comparison test. An oxygen equilibrium curve (OEC) was constructed for each blood or hæmolysate sample by fitting a three-parameter logistic (3PL) model to paired data of fractional

Hb-O_2 saturation (response variable) and PO_2 (explanatory variable). The R-language formula for the OEC model was 'HbO_2 ~ d/(1 + exp(b*(log10(PO_2) - log10(e)))'.The best-fit parameter values (b, d, and e) were used to calculate the PO_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}). Hill cooperativity coefficients (n_{50}) were determined at P_{50} by differentiating the 3PL equation at P_{50}.

The effects of pH and temperature on whole blood-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 (continuous), assay temperature (as a factor), the interaction term between pH and assay temperature, and individual (id) as a random effect (R-language formula, 'log10(P_S) ~ pH*temperature + (1|id)'). Mixed models were fit at each saturation from P_{10} to P_{95}, and for each model a Likelihood Ratio Test (LRT) of fixed effects, fit with maximum likelihood estimation using a Chi square 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). Linear models were used to assess the relationship between common thresher shark hæmolysate-O_2 affinity (response variable; $\log_{10} P_S$) and pH (explanatory variable) for each experimental treatment.

The mixed model fits (whole blood) were used to predict P_S values with bootstrap estimated standard errors (500 replications), and these were used to construct OECs (whole blood) at constant pH (i.e., isohydric OECs) for each species temperature treatment. Hæmolysate P_S values were calculated at specific pH values from mixed linear models fit to data for each temperature and effector treatment, and the curve fitting function of GraphPad Prism was used to determine the 95% CI's of the interpolated values. The temperature-dependence of whole blood

and hæmolysate O_2 affinities were quantified by calculating $\Delta H'$ values using the van't Hoff equation (Wyman, 1964):

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

where R is the gas constant and T is the absolute temperature (Kelvin). Because $\Delta H'$ calculations requires that the concentration of allosteric effectors be known and the experimental conditions for Hb are carefully controlled (e.g., stripped hæmolysates), I consider the whole blood calculations as estimates at best. However, they are useful for comparisons among species and studies. I denote the whole blood calculations as $\Delta H'_{WB}$, and for their calculation I determined P_S values at constant pH, and I assumed that RBC intracellular concentrations of allosteric effectors were constant. The heat of solution of O_2 (~12.6 kJ mol^{-1}) is included in whole blood $\Delta H'_{WB}$ values, and excluded from hæmolysate $\Delta H'$ values. The pH dependency of Hb-O_2 affinity was determined by calculating Bohr coefficients at different %Hb-O_2 saturations (P_S):

$$\varphi = \frac{\Delta \log_{10} P_S}{\Delta \mathrm{pH}}$$

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

4.3 Results

Species lengths and blood parameters are summarized in Table 4.1. Hb concentration and Hct were not different between blue sharks and spiny dogfish, but were lower in these species compared to bigeye threshers and shortfin makos, and bigeye thresher values were lower than those for shortfin mako (Hb: $F_{(3, 31)} = 27.00$, $P < 0.0001$; Hct: $F_{(3, 31)} = 17.72$, $P < 0.0001$). MCHC was not different between shortfin makos and bigeye threshers, which both had higher

MCHC than blue sharks and spiny dogfish ($F_{(3, 31)} = 18.76$, $P < 0.0001$). Hb concentration, Hct, and MCHC for the two common thresher sharks were within the range of values measured in shortfin makos. Blood pH was low in some individuals (at 0.25% CO_2), and plasma lactate levels were generally high except for dogfish (Table 4.1).

Whole-blood experiments

Whole blood OEC's were successfully constructed for seven shortfin mako sharks, two common thresher sharks, five bigeye thresher sharks, four blue sharks, and seven spiny dogfish. Whole blood OECs and P_{50} values for the shortfin mako, the bigeye thresher, and the blue shark were predicted at pH 7.7 and pH 7.5, approximated arterial pH for the blue shark and the mako shark, respectively (Lai et al., 1997). Spiny dogfish OECs and 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 OECs and P_{50} values are reported at pH 7.3 due to the relatively acidotic state of the blood from the two individuals that we sampled (Table 4.1 and inset of Figure 4.3).

Oxygen equilibria parameters are reported in Table 4.2 and OECs are presented in Figure 4.3. All shark species had relatively low P_{50} values (i.e., high blood-O_2 affinities), but those of the mako shark and the bigeye thresher shark were exceptionally low. Blood pH and PCO_2 had little influence on P_{50}, as indicated by low Bohr coefficients or Bohr coefficients with 95% confidence intervals that included zero (e.g., common thresher shark blood at 22°C). Hill coefficients were relatively low and were generally independent of blood pH (Figure 4.3 and Table 4.3). Temperature was an important predictor of $n_{50,}$ but the response was relatively negligible and inconsistent with lower n_{50} values at higher temperatures for the shortfin mako ($\chi^2 = 7.450$, df = 2, $P = 0.024$) and the bigeye thresher ($\chi^2 = 18.922$, df = 2, $P = 0.00008$), but higher

n_{50} values at higher temperatures for the common thresher (χ^2 = 10.358, df = 2, P = 0.006), the blue shark (χ^2 = 7.118, df = 2, P = 0.028), and the spiny dogfish (χ^2 = 18.922, df = 2, P = 0.00008).

In shortfin mako blood, temperature negligibly affected blood-O_2 affinity below 90% saturation. At 95% saturation blood PO_2 was 9-10 mmHg greater at 25°C than at 15°C, but the P_{95} standard errors overlap (Figure 4.3) and temperature was not an important model factor for P_{95} (χ^2 = 5.520, df = 2, P = 0.063). Mako $\Delta H'_{WB}$ values were positive (endothermic) at low saturation, but decreased to negative values (exothermic) with increasing saturation and at high pH, although $\Delta H'_{WB}$ values were still relatively temperature independent (< 10 kJ mol^{-1} O_2) (Figure 4.4). In common thresher shark blood, a slight reverse effect of temperature on blood-O_2 affinity was evident (Figures 4.3 and 4.4), although temperature was not an important model factor. Bigeye thresher blood-O_2 affinity was unaffected by temperature below 60% O_2 saturation, but from P_{60} to P_{95} temperature was an important factor ($P_{50:}$ χ^2 = 5.258, df = 2, P = 0.073; $P_{60:}$ χ^2 = 10.970, df = 2, P = 0.004; $P_{70:}$ χ^2 = 17.511, df = 2, P = 0.0002; $P_{80:}$ χ^2 = 24.426, df =2, P = 0.000005; $P_{90:}$ χ^2 = 32.580, df = 2, P = 0.00000008; $P_{95:}$ χ^2 = 31.867, df = 2, P = 0.0000001). The saturation dependency of $\Delta H'_{WB}$ in bigeye thresher shark blood is evident as positive $\Delta H'_{WB}$ values below 60% saturation, but decreasingly negative values above 60% saturation (Figure 4.4), as well a right shift of the upper portion of the OEC with increasing temperature (Figure 4.3). Increasing temperature decreased blood-O_2 affinity in both blue shark and spiny dogfish blood across most of the OEC (Figure 4.3) due to exothermic $\Delta H'_{WB}$ at all saturations (Figure 4.4). In blue shark blood, temperature did not significantly affect P_{10} or P_{20}, but temperature was an important model factor from P_{30} to P_{95} (χ^2 = 8.481 to 25.748, df = 2, P = 0.014 to 0.000009).

The effects of closed-system temperature changes on blood P_{O_2} are shown in Figure 4.5. Closed-system warming of mako and common thresher blood generally increased blood P_{O_2}, but less so than that predicted by Henry's law (i.e., increasing temperature will increase P_{O_2} in a closed system due to a reduction in plasma O_2 solubility, and vice versa). Closed-system warming of blood from bigeye threshers, blue sharks, and spiny dogfish increased P_{O_2} beyond that predicted by the change in solubility of O_2, indicating that temperature induced Hb-O_2 offloading likely contributed to ΔP_{O_2}. The greatest ΔP_{O_2} occurred in bigeye thresher blood. Closed-system cooling caused blood P_{O_2} to decrease, but less so in mako and common thresher blood than in the other species.

Common thresher shark hæmolysate experiments

Hill plots of the hæmolysate experiments are presented in Figure 4.6 and Figure 4.7. Summary data at pH 7.3 are presented in Table 4.4. Common thresher shark stripped hæmolysates had a low intrinsic O_2 affinity that was independent of temperature and pH. The addition of ATP reduced O_2 affinity and induced a pH dependency at 15°C that was not evident at 25°C. This pH dependency is evident as large Bohr coefficients with confidence intervals that do not include zero (Table 4.4). At 25°C urea reduced the effect of ATP on Hb-O_2 affinity causing a reverse temperature-dependence (Figure 4.7), although the confidence intervals for the data at each temperature overlap (Figure 4.6). At pH 7.3 and in the presence of ATP and urea, $\Delta H'$ was +35 kJ mol^{-1} O_2 (excluding the heat of O_2 in solution).

4.4 Discussion

Main findings

The main objective of this study was to investigate the effect of temperature on whole blood Hb-O_2 affinity in the regionally heterothermic common thresher shark, and a closely related species that is a suspected of having warm eyes, the bigeye thresher shark. The effect of temperature on blood-O_2 affinity in shortfin mako shark was also investigated, although this species has been studied previously (Bernal et al., 2018). This study tested the hypothesis that the blood-O_2 affinity of the common thresher is less affected by temperature than most non-heterothermic species, similar to the blood and Hbs of the shortfin mako, the porbeagle shark, 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 due to the low environmental oxygen levels that this species encounters daily (Figure 4. 2). Whole blood Hb-O_2 affinity was independent of temperature for both common thresher shark and shortfin mako (Figure 4.3). When blood from these two species was warmed in a closed-system meant to mimic warming of blood in a heat exchanging *rete mirabile*, blood PO_2 changed less than would be expected due to temperature dependence of the solubility of O_2 in plasma (Figure 4.4). Bigeye thresher blood had a high O_2 affinity (i.e., low P_{50}; Table 4.2) that exhibited a temperature-dependence that was dependent on Hb-O_2 saturation. At low saturation, bigeye thresher blood-O_2 affinity was independent of temperature (Figure 4.3), but above ~50% saturation O_2 affinity decreased with increasing temperature due to an increasingly exothermic $\Delta H'_{WB}$ (Figure 4.4). Closed-system warming and cooling of bigeye thresher blood caused large changes to blood PO_2.

Blood-O_2 carrying capacity and O_2 affinity

Hæmatocrits and Hb concentrations reported in this study (Table 4.1) are similar to previous reports for shortfin mako, common thresher, blue shark, and spiny dogfish (reviewed by Morrison et al., 2015). Shortfin mako and common thresher Hct and Hb concentrations 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 RM endothermy (Bernal et al., 2001b; Emery, 1986). Those for bigeye thresher shark are similar to hammerhead sharks in the genus *Sphyrna*, which are intermediate between typical values for other ectothermic sharks and those for regionally heterothermic sharks (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 metabolic rates) than most non-heterothermic sharks, so they have relatively higher blood-O_2 carrying capacities to match. Curiously, the blue shark occupies an ecological niche that overlaps with those of the shortfin mako, the common thresher, and the bigeye thresher (Bernal et al., 2009; Cartamil et al., 2011; Kinney et al., 2020), but unlike these sharks, the blue sharks cardiovascular system (i.e., the relative size and physiology of the heart, and blood-O_2 carrying capacity) does not appear capable of higher rates of circulatory O_2 delivery compared to other ectothermic sharks (Bernal et al., 2001b; Brill and Lai, 2015; Morrison et al., 2015).

This study's results for shortfin mako and blue shark blood are qualitatively like those reported by Bernal et al. (2018), although their shortfin mako Bohr coefficients and P_{50} values are larger than those reported here (Table 4.3). However, the low P_{50} value and small Bohr coefficients reported by Wells and Davie (1985) for shortfin mako blood at 25°C (Table 4.3) are close to those in this study (Table 4.2). Blue shark P_{50} values are similar between this study and that by Bernal et al. (2018), and the slightly larger Bohr coefficients that they reported (Table 4.3) fall within the 95% confidence intervals for the Bohr coefficients reported here (Tables 4.2).

The Spiny dogfish P_{50} value (pH 7.85) reported by Wells and Weber (1983) at 15°C is slightly lower than the value that I determined, and they reported a Bohr coefficient of -0.28 (Table 4.3), whereas I observed no significant effect of pH on P_{50} at 15°C (Table 4.2).

Temperature had little to no effect on blood P_{50} of the shortfin mako, the common thresher shark, or the bigeye thresher shark (Table 4.2). In contrast, increasing temperature increased P_{50} (i.e., decreased blood-O_2 affinity) in blue shark and spiny dogfish blood (Table 4.2), and as expected for these two species, the temperature dependence of blood-O_2 affinity was consistent across most of the OEC (Figure 4.3). Temperature did not uniformly affect the bigeye thresher OEC, which is evident as a right shift of the 25°C OEC above 50% Hb-O_2 saturation (Figure 4.3). At low O_2 saturations, bigeye thresher shark $\Delta H'_{WB}$ values were positive (endothermic) and are comparable to the those of the shortfin mako and the common thresher shark, but $\Delta H'_{WB}$ became decreasingly negative (exothermic) with increasing saturation. Around 90% O_2 saturation, the $\Delta H'_{WB}$ values are comparable to those of the blue shark and the spiny dogfish.

The bigeye thresher shark whole blood P_{50} values are among some of the lowest that I have measured, and one of the lowest reported from elasmobranchs (Morrison et al., 2015). I 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 due to vastly different daytime and nocturnal distributions. Bigeye thresher sharks spend most of the day well below the thermocline near the upper reaches of the oxygen minimum layer (OML), but ascend to the warmer upper mixed layer at night (Figure 4.2) (Sepulveda et al., 2019). While near the OML, a low P_{50} combined with relatively thin lamellar diffusion distances and a gill surface area 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. Swordfish make similar diel vertical migrations (Sepulveda et al., 2010), and also like bigeye thresher sharks, swordfish have relatively large gill surface areas and a low P_{50}. Thus, the respiratory characteristics of the bigeye thresher shark and the swordfish seem to be well adapted for extracting O_2 from the hypoxic water near the OML.

It is notable that at 10°C the blue shark's P_{50} is the same as the bigeye thresher's, which are both only slightly lower than the shortfin mako's P_{50} at 15°C (Table 2). However, blue sharks and shortfin makos do not routinely enter water low in O_2 (Bernal et al., 2009). Elasmobranchs in general tend to have high Hb-O_2 affinities and small Bohr coefficients (Morrison et al., 2015), so a low P_{50} may not be remarkable in the bigeye thresher. Alternatively, if bigeye thresher stripped hæmolysate has a lower intrinsic O_2 affinity than its congeners, the common thresher and the pelagic thresher (*Alopias pelagicus*), then it is reasonable to hypothesize that a low P_{50} is adaptive to low environmental O_2 in the bigeye thresher shark. To that end, I have completed some preliminary experiments on bigeye thresher shark stripped hæmolysates, and at 10°C and 25°C the intrinsic P_{50} values (pH 7.3) are 1.5 mmHg and 3.2 mmHg, respectively. These values are not too dissimilar to the common thresher shark values reported in Table 4.4, or the swordfish values reported in Table 3.3. Furthermore, although the intrinsic Hb-O_2 affinities of both the bigeye and the common thresher sharks are much lower than many other elasmobranchs (Morrison et al., 2015), they are similar to some, including the porbeagle shark and the spiny dogfish (Larsen et al., 2003; Weber et al., 1983a). Therefore, the high whole blood-O_2 affinity of the bigeye thresher shark does not appear to involve evolutionary changes in the intrinsic Hb-O_2 affinity, and is likely due to differences in the

sensitivity of Hb to allosteric effectors (i.e., heterotropic allostery) and/or the physicochemical working environment for Hb within the red blood cells.

The absence of a Bohr shift in shortfin mako blood was also reported by (Wells and Davie, 1985) and is interesting because the Bohr effect is generally considered beneficial for O_2 offloading in muscle capillaries (e.g., Jensen, 2004). The shortfin mako has the highest measured rates of oxygen consumption among sharks (Sepulveda et al., 2007b), so it would be expected that a Bohr shift would benefit O_2 unloading to the swimming muscles. It is not clear if there is an adaptive benefit to a low P_{50} that is insensitive to pH in the shortfin mako, or if these are general traits of elasmobranch Hbs that may potentially contribute to the upper limit of aerobically sustained exercise.

Closed-system temperature changes

Temperature-independent Hb-O_2 affinity in blood from the shortfin mako and the common thresher shark was reflected in closed-system changes to blood temperature, when blood PO_2 changed less than would be expected due to the temperature dependence of plasma O_2 solubility (Figure 4.5). In comparison, closed-system temperature changes caused large changes to blood PO_2 for the bigeye thresher, blue shark, and the spiny dogfish (Figure 4.5), indicating that warming and cooling of blood caused Hb-O_2 unloading and binding, respectively. In bigeye thresher blood, the magnitude of temperature induced changes to blood PO_2 exceeded those measured in blue shark and spiny dogfish blood. Because bigeye thresher blood has such a high O_2 affinity, most of the equilibration O_2 tensions used in this study would have caused high Hb-O_2 saturation levels where increasing temperature greatly right shifts the bigeye thresher OEC (Figure 4.3), causing excessive Hb-O_2 unloading as the blood is warmed. In mako and common thresher blood, temperature-independent Hb-O_2 affinity and possible reverse temperature-

dependence likely caused the relatively decreased effect of closed-system temperature changes on blood PO_2. Similar results have been previously reported for the shortfin mako and some tunas (Bernal et al., 2018; Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Cech et al., 1984).

During closed-system warming, the average change in blood pH of the shortfin mako and the blue shark was around -0.023 U/°C (Table 4.2), which is at the higher end of previous reports from tunas (-0.021 to -0.010 U/°C) (Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Lowe et al., 2000). However, bigeye thresher and common thresher blood pH was unaffected by closed system temperature changes. It is not clear what caused this lack of a shift in blood pH during temperature changes, but it is worthy of future research as are studies on the effect of temperature on acid-base state in sharks in general (Heisler, 1988).

Molecular mechanisms of temperature-independent Hb-O_2 affinity in the common thresher shark

Common thresher shark intrinsic Hb-O_2 affinity is temperature-independent, and in the presence of ATP and urea, temperature has a reversed effect on Hb-O_2 affinity. It is not clear what underlies the temperature-independence since pH had no effect on Hb-O_2 affinity in the absence of urea and ATP. It seems unlikely that I failed to strip all endogenous allosteric effectors from the hæmolysates since the P_{50} was low, 2.5 mmHg. Urea reduces the O_2 affinity of many vertebrate Hbs, including those of some, but not all elasmobranchs (reviewed by Morrison et al., 2015). Urea did not affect common thresher shark Hb-O_2 affinity, but it did decrease the effect of ATP on Hb-O_2 affinity, which has also been reported for Hbs from some sharks (e.g., spiny dogfish) but not others (e.g., sandbar shark, *Carcharhinus plumbeus*) (Brill et al., 2008; Weber et al., 1983a; Weber et al., 1983b). Urea insensitivity may somehow be linked to the intrinsic temperature-independence, but I can offer no mechanistic explanation for this.

Alternatively, the common thresher shark may have multiple Hb components that are differentially affected by proton binding, in which case the combined hæmolysate may appear to be intrinsically temperature-independent due to proton linked increases to $\Delta H'$ for some Hb components but not others. The major Hb components of porbeagle shark show a normal temperature-dependence in the absence of ATP, but a reverse temperature-dependence in the presence of ATP (Larsen et al., 2003). Thus, it seems that ATP is the major effector that underlies reverse-temperature-dependence in lamnid sharks and the common thresher shark, but further research is warranted to elucidate any structural or functional differences and the possible independent evolution of this trait in common thresher shark Hb.

Possible limitations

The primary objective of this study was to construct whole blood OECs at two different but physiologically relevant temperatures for the shortfin mako, common thresher, and bigeye thresher. Our experimental temperatures encompass the low and high ambient and body temperatures reported for these species (Carey and Teal, 1969a; Cartamil et al., 2016; Patterson et al., 2011; Sepulveda et al., 2004; Sepulveda et al., 2019), and these temperatures are also comparable to previous studies (Andersen et al., 1973; Bernal et al., 2018). The individuals included in this study had likely experienced varying levels of exercise induced fatigue and consequent respiratory and metabolic acidosis. This is indicated by relatively high plasma lactate levels in shortfin mako, common thresher, and blue sharks (Table 4.1), which are comparable to lactate levels reported from capture-stressed shortfin mako and blue sharks (Wells et al., 1986). However, bigeye thresher and spiny dogfish plasma lactate levels were relatively low. Furthermore, Hct and Hb concentration values (Table 4.1) were similar to previously published values (Morrison et al., 2015), indicating that capture stress did not cause lasting RBC swelling.

It is not possible or safe to obtain blood samples from resting and cannulated large sharks held in laboratory aquaria, so collecting blood samples by caudal peduncle puncture is an accepted method for obtaining shark blood for oxygen equilibria experiments (Brill et al., 2008; Cooper and Morris, 1998). Except for the two common thresher sharks, the blood pH levels that we achieved with the CO_2 exposures were within physiologically relevant ranges, which allowed us to estimate blood P_S at physiologically relevant pH values. The common thresher shark blood was, however, in good shape and showed no signs of RBC lysis or RBC swelling [i.e., Hcts were close to previously published values (Emery, 1986; Filho et al., 1992)]. The main objective of this study was to investigate the effect of temperature on Hb-O_2 affinity, and it is very unlikely that any exercise or stress induced physiologically changes would have compromised the allosteric effect of temperature on Hb function in whole blood. This is supported by the experiments on common thresher shark stripped hæmolysates that showed temperature effects that are comparable to those observed in whole blood. Lastly, although only two common thresher sharks were included in this study, the results were consistent between the two individuals and with the stripped hæmolysate experiments, and since this studies results on the effect of temperature on shortfin mako (n = 7) are consistent with the limited sample size (n = 3) of Bernal et al. (2018), it is unlikely that the common thresher shark data are not representative of the species.

Conclusion

This study shows for the first time that Hb-O_2 affinity is independent of temperature in blood and hæmolysates from the regionally heterothermic common thresher shark. Temperature independent Hb-O_2 affinity was previously shown in blood from the shortfin mako and was corroborated in this study. It was also shown that the bigeye thresher shark has a low P_{50} that is

insensitive to temperature, which indicates an exceptional tolerance to hypoxia and likely allows this species to exploit the OML and a wide range of water temperatures.

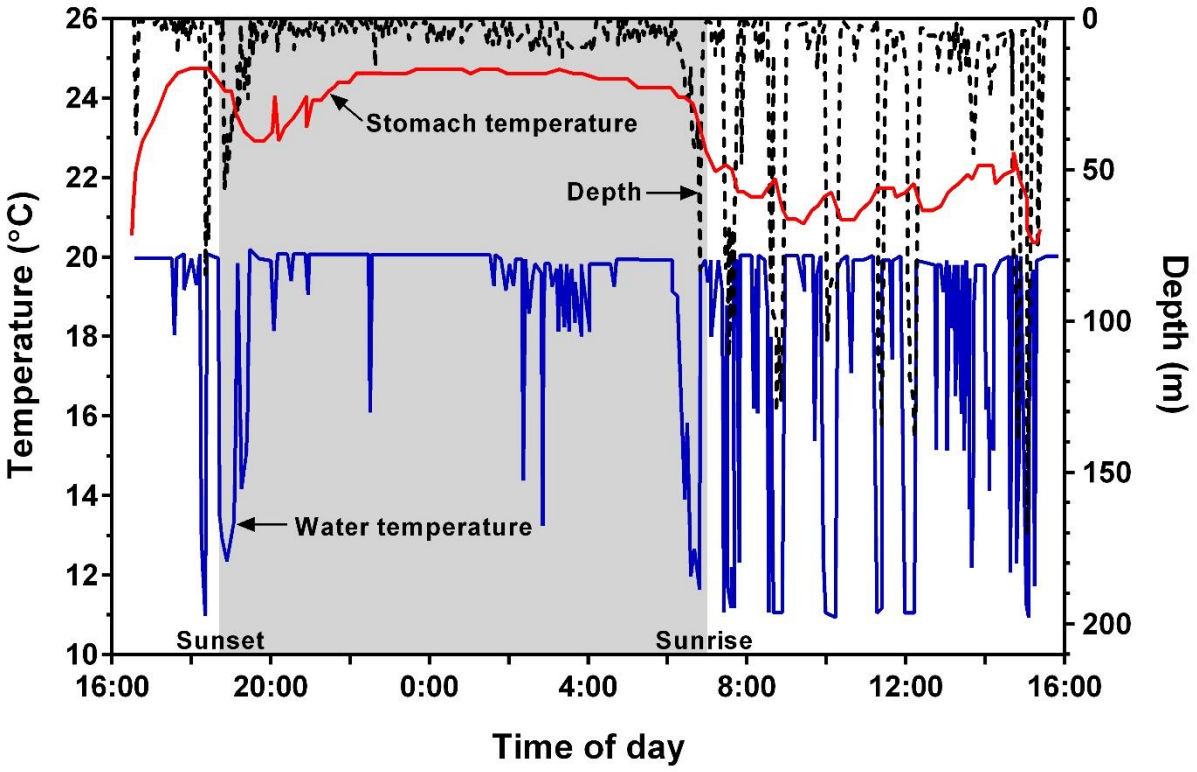

Figure 4.1 Vertical movements, and stomach and ambient temperatures for a free-swimming shortfin mako shark tracked in the Southern California Bight.

The mako was 18.0 kg and 120 cm fork length, and was tracked in 2002 by Chugey Sepulveda (Pfleger Institute of Environmental Research). Shaded area represents night, depth is shown in black, stomach temperature in red, and water temperature in blue. Figure created from data and figures in Sepulveda et al. (2004).

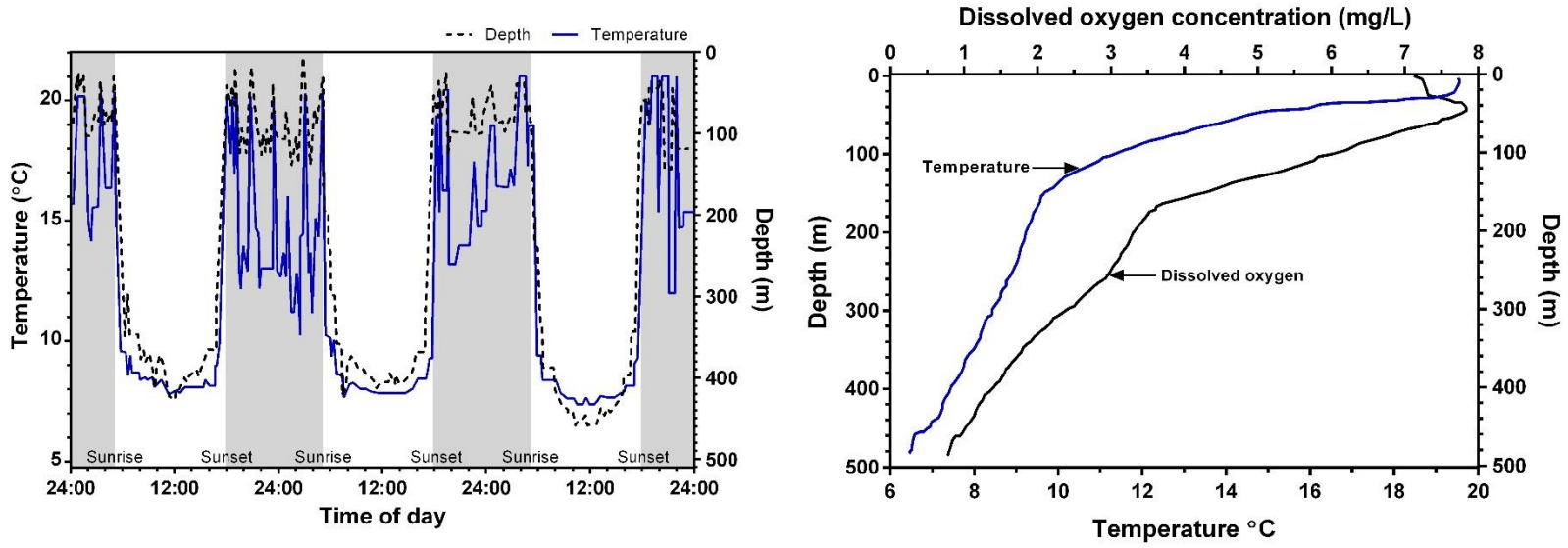

Figure 4.2 Diurnal movements of a bigeye thresher shark captured in the Southern California Bight.

The bigeye thresher shark was tagged with a pop-up satellite archival transmitter and tracked for 30 days by Sepulveda et al. (2019). The panel on the left shows a 3-day portion of the 30-day track. The shaded area represents night, depth is shown in black (dashed line), and water temperature in blue. The panel on the right shows the typical temperature (blue) and dissolved oxygen concentration (black) as a function of depth in the Southern California Bight, where the bigeye thresher sharks were tagged. Daytime depths are near the upper reaches of the oxygen minimum layer (< 2 mg O_2 L^{-1}). Figure created from data and figures in Sepulveda et al. (2019).

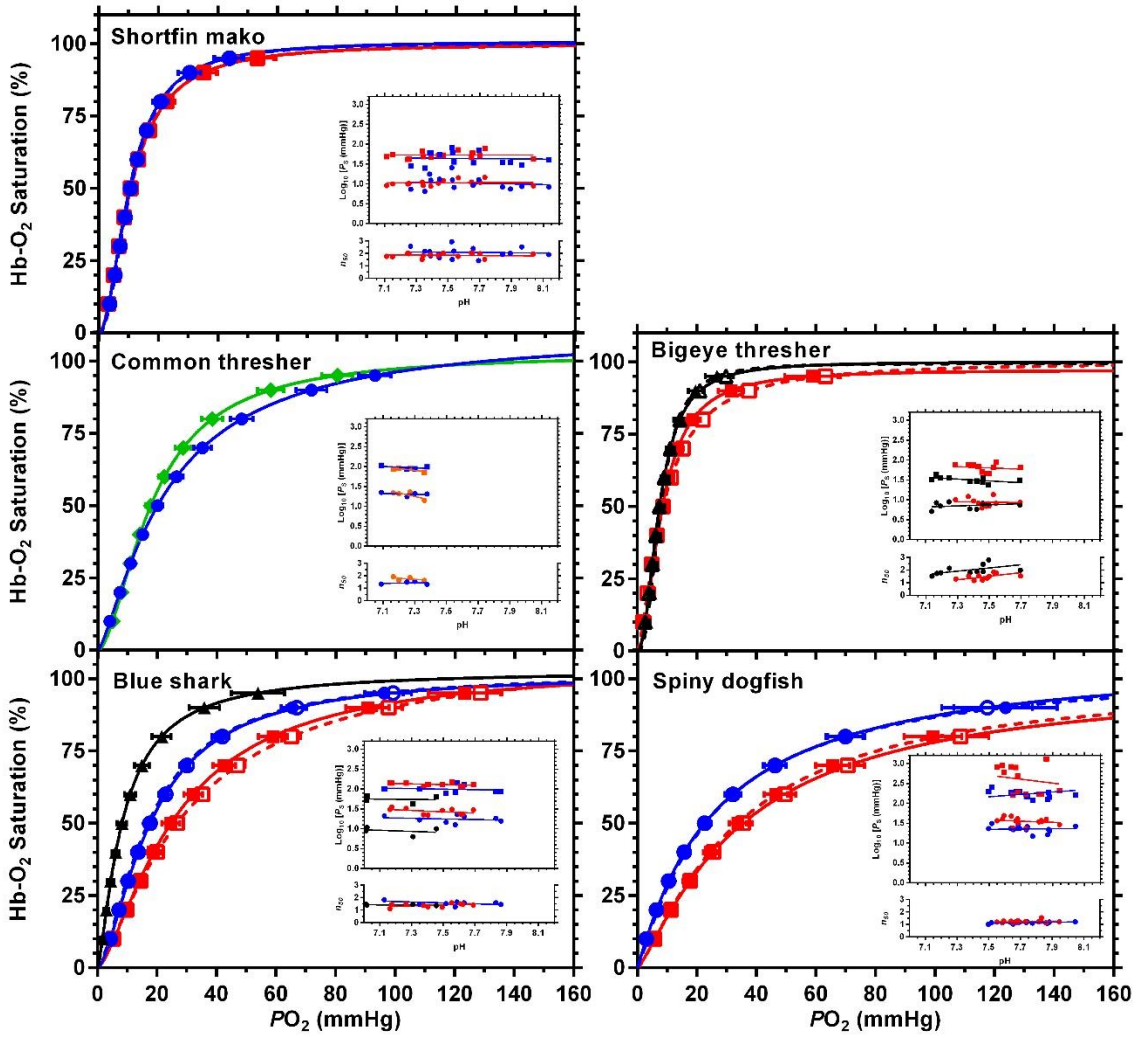

Figure 4.3 Whole blood oxygen equilibrium curves (OECs) at different pH and temperatures for shortfin mako shark, common thresher shark, bigeye thresher shark, blue shark, and spiny dogfish.

OECs were constructed at either 10°C (black triangles), 15°C (blue circles), 22°C (green diamonds), or 25°C (red squares). Blood PO_2 values were interpolated (± bootstrap standard errors) from linear mixed models of logPO_2 vs pH at specific Hb-O_2 saturation levels (see Methods section). Shortfin mako, common thresher, bigeye thresher, and blue shark (15 and 25°C) OECs are at pH 7.7 (filled symbols and solid curves) and pH 7.5 (open symbols and

dashed curves). The blue shark OEC at 10°C is at pH 7.45 (filled triangles and solid curve), and spiny dogfish OECs are at pH 7.85 (filled symbols and solid curves), and pH 7.7 (open symbols and dashed curves). Inset figure show the relationships between $\log_{10}P_S$ vs pH at 50 and 95% Hb-O_2 saturation (P_{50} and P_{95}, respectively), and n_{50} vs pH, plotted with the line from the linear mixed models (see methods).

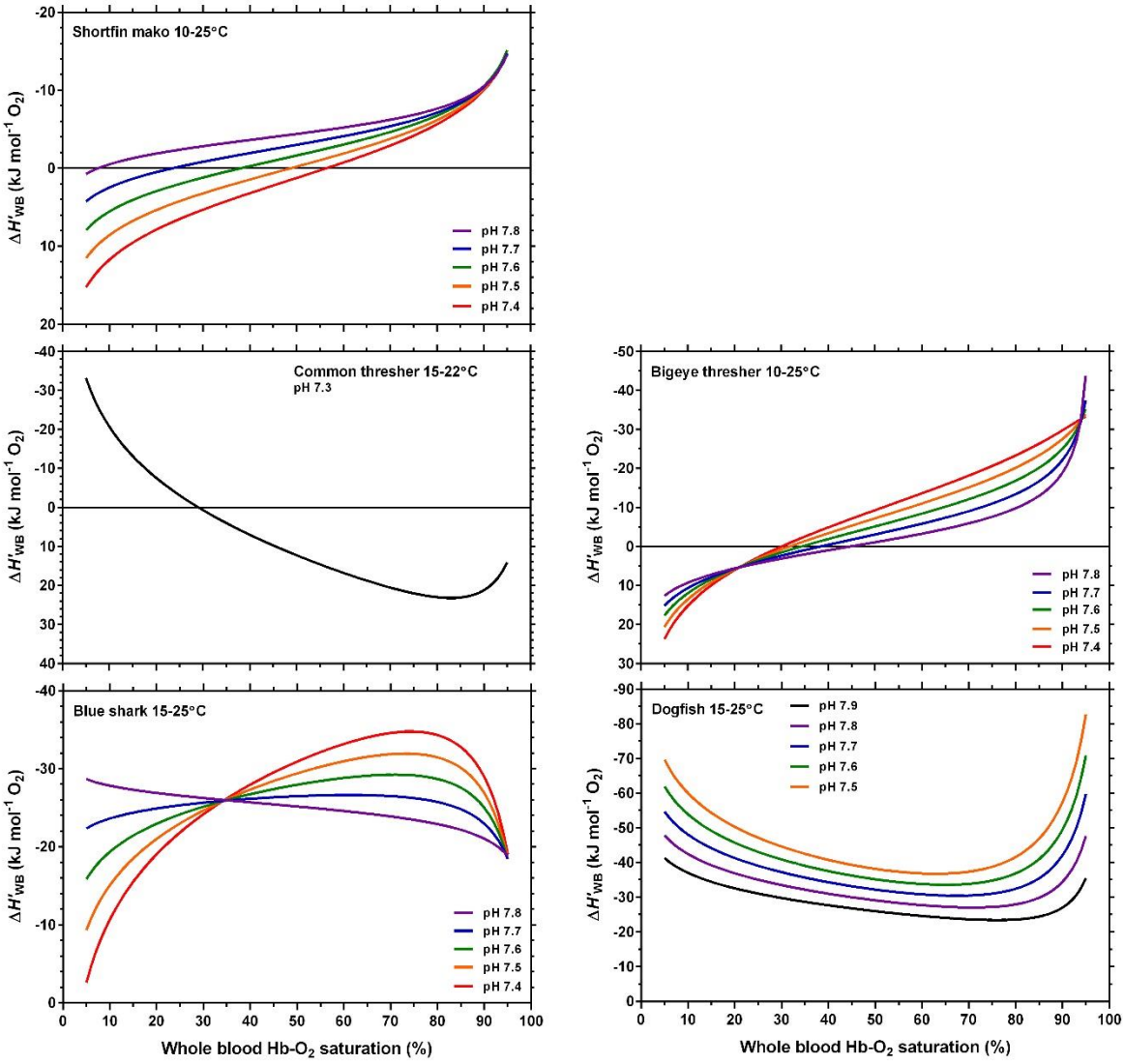

Figure 4.4 Predicted enthalpy of oxygenation ($\Delta H'_{WB}$) as a function of whole blood pH and whole blood Hb-O_2 saturation for shortfin mako shark, common thresher shark, bigeye thresher shark, blue shark, and spiny dogfish.

$\Delta H'_{WB}$ values were calculated with the van't Hoff isochore (see methods) at constant pH between the indicated temperatures. The blood-O_2 tensions (PO_2) at specific blood-O_2 saturation levels at a given pH and temperature were determined from the data presented in Figure 4.3.

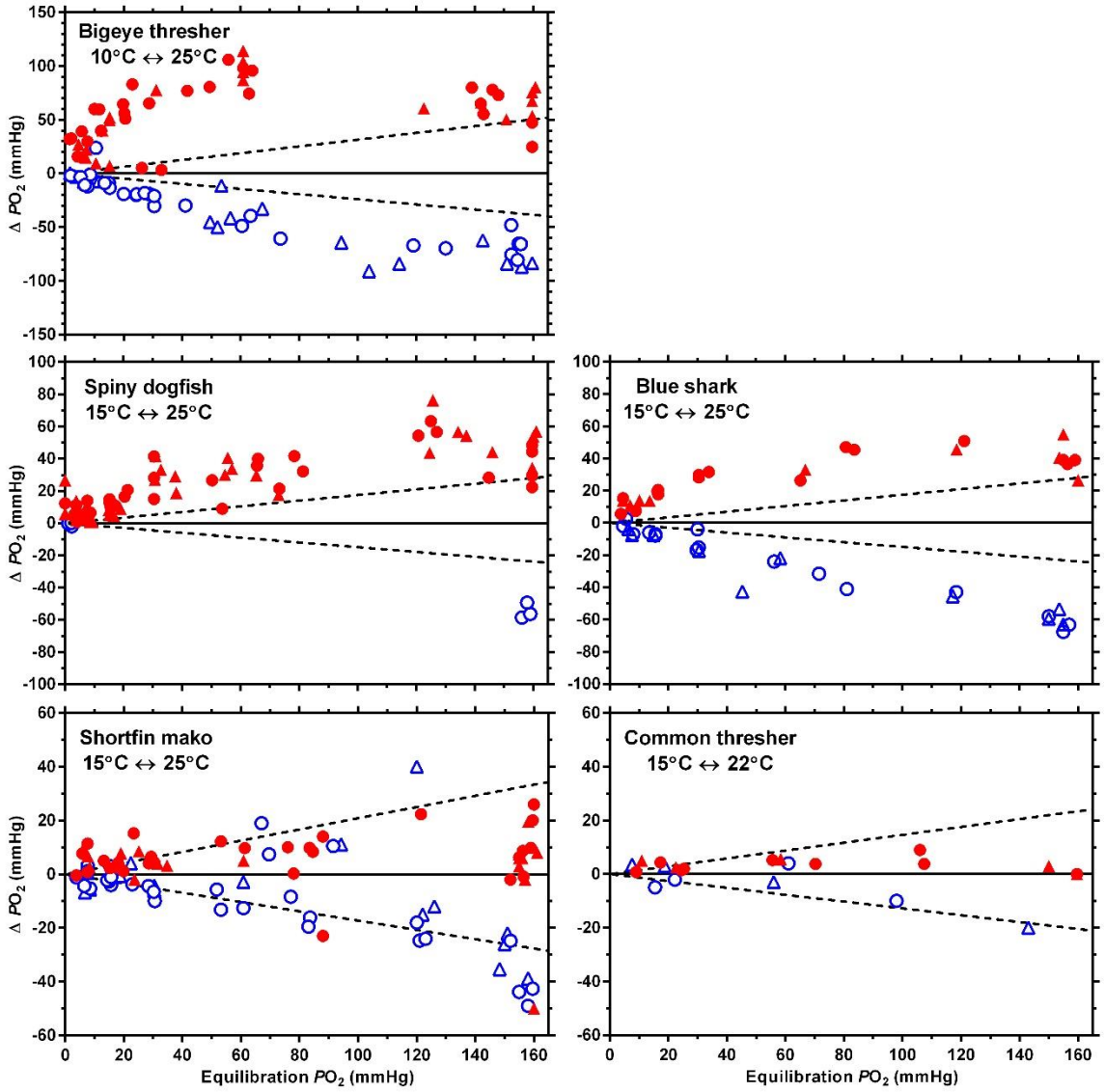

Figure 4.5 Effects of closed-system temperature changes on the measured change in blood P_{O_2} (ΔP_{O_2}).

Shark blood was equilibrated at a range of O_2 tensions and a low CO_2 tension (circles; P_{CO_2} = 1.9 mmHg) or a high CO_2 tension (triangles; P_{CO_2} = 3.8 mmHg in spiny dogfish, and 7.6 mmHg in the other sharks), was heated (filled red symbols) or cooled (open blue symbols) between the temperatures specified above. Dotted lines indicate the temperature induced ΔP_{O_2} expected due to changes in solubility of blood plasma at a given equilibration P_{O_2} (i.e., Henry's Law).

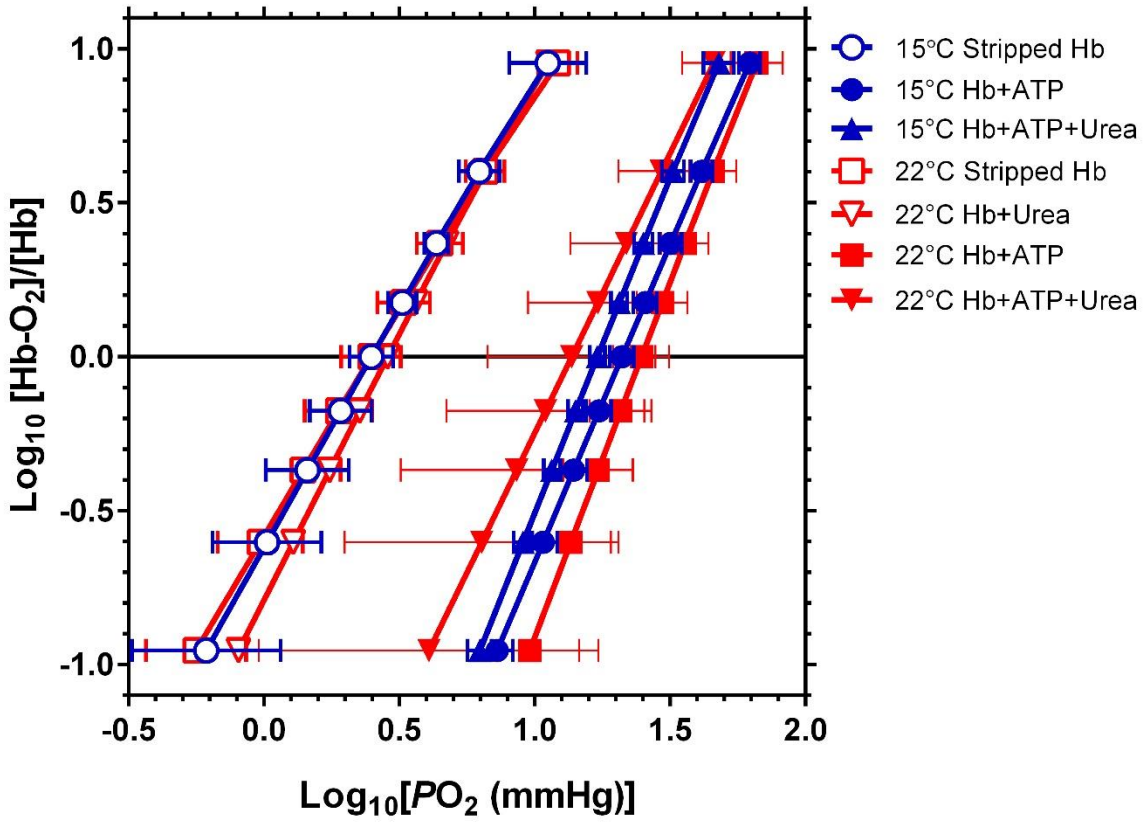

Figure 4.6 Hill plots of Hb-O_2 equilibria of common thresher shark at pH 7.3.

Experiments were conducted on stripped hæmolysates in the absence or presence of ATP and urea at two temperatures (15°C and 22°C). Data points 95% confidence intervals were predicted at pH 7.3 from linear models of logP_S vs pH (see Methods section). Experiments were conducted in 0.1 M Hepes buffer at Hb concentrations of 0.6 mM, ATP was added at saturating concentrations (18 mM; ATP/Hb = 30), and urea was added at a concentraion of 500 mM. The data points for Hb + Urea at 15°C overlapped with those of Stripped Hb, so were omitted for clairty.

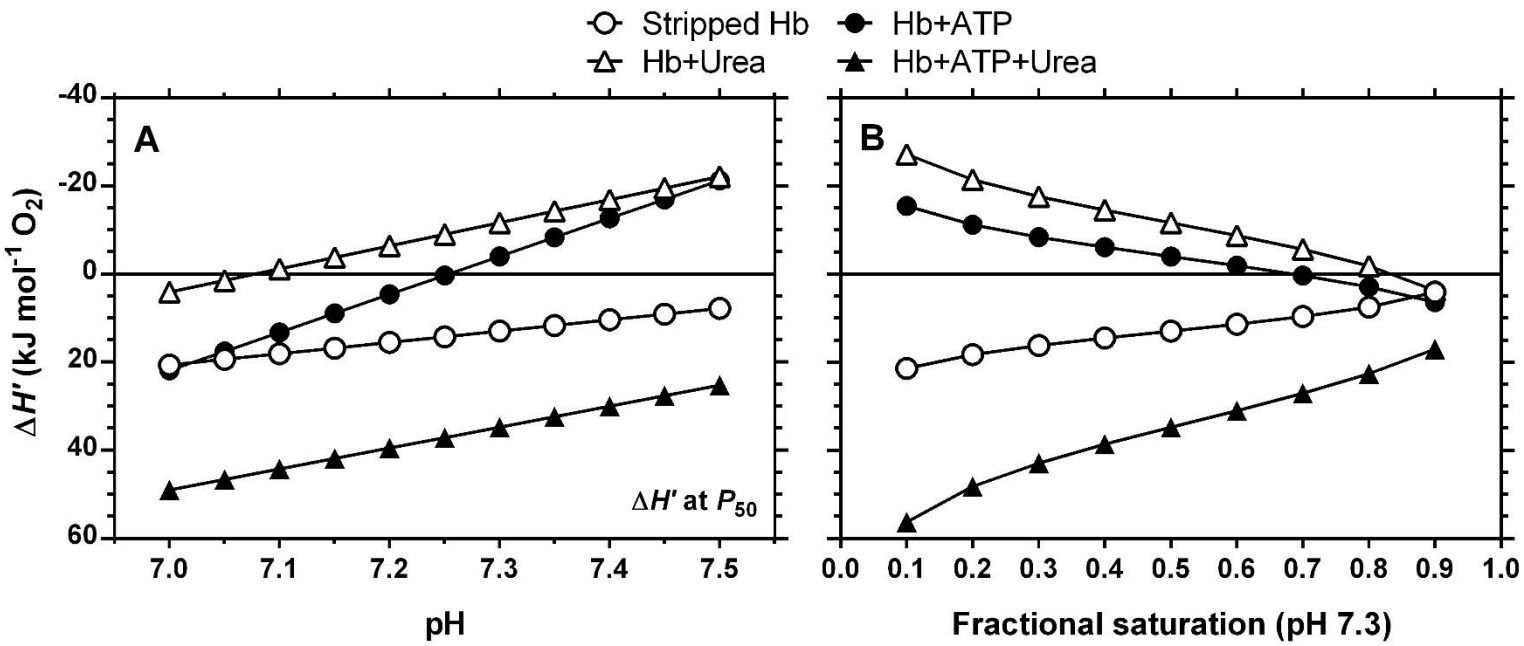

Figure 4.7 Heats of oxygenation (Δ*H'*) of stripped hæmolysates of common thresher shark.

In panel A, Δ*H'* values were calculated with the van't Hoff equation (see Methods) with logP_{50} values that were interpolated from logP_{50}/pH relationships. In panel B, Δ*H'* values were calculated from data reported in Figure 4.6, and plotted as function of Hb-O_2 fractional saturation at pH 7.3. Experiments were conducted at 15°C and 22°C in 0.1 M Hepes buffer at Hb concentrations of 0.6 mM, and ATP was added at saturating concentrations (18 mM; ATP/Hb = 30), and urea was added at a concentration of 500 mM. Δ*H'* values exclude the heat of O_2 in solution (-12.6 kJ mol^{-1}).

Table 4.1 Fork length and blood variables for shortfin mako sharks, common thresher sharks, bigeye thresher sharks, blue sharks, and spiny dogfish.

Values are means ± standard error with samples sizes in parentheses. Mean blood pH is reported with the range of values. If values were measured in only one or two individuals, then the individual measurements are reported.

	Shortfin mako shark	Common thresher shark	Bigeye thresher shark	Blue shark	Spiny dogfish
Fork length (cm)	128 ± 8 (9)	135, 175		135 ± 13 (5)	52 ± 3 (8)
Hæmatocrit (%)	31.6 ± 2.0 (10)	29.7, 36.8	25.5 ± 1.0 (9)	19.2 ± 1.2 (8)	17.9 ± 1.5 (8)
Hæmoglobin (mM)	1.48 ± 0.12 (10)	1.25, 1.65	1.13 ± 0.04 (9)	0.69 ± 0.03 (8)	0.63 ± 0.06 (8)
MCHC (mM)	4.65 ± 0.19 (10)	4.20, 4.49	4.45 ± 0.08 (9)	3.61 ± 0.12 (8)	3.52 ± 0.08 (8)
Plasma osmolality (mOsm/kg)	959 ± 6 (7)	1004	931 ± 27 (9)	914 ± 32 (6)	937
Plasma lactate (mM)	8.8 ± 4.2 (4)	7.14	3.4 ± 0.35 (7)	6.6 ± 1.7 (5)	1.4 ± 0.2 (6)
Blood pH (range)[4]	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)

[4] Measured in blood equilibrated to 0.25% CO_2 and saturating levels of O_2.

Table 4.2 Whole blood oxygen equilibria parameters at different temperatures and pH.

Shortfin mako shark	**15°C (n = 7)**		**25°C (n = 7)**
P_{50} mmHg (SE), pH 7.7	10.3 (9.3 to 11.4)		10.7 (9.5 to 12.1)
pH 7.5	10.7 (9.6 to 11.8)		10.7 (9.7 to 11.8)
n_{50} (± SE), pH 7.7	2.07 (± 0.11)		1.83 (± 0.12)
pH 7.5	2.09 (± 0.11)		1.85 (± 0.11)
Bohr coefficients (95% CI)	-0.08 (-0.30 to 0.14)		0.01 (-0.20 to 0.40)
pH range	pH 7.264 to 8.134		pH 7.112 to 8.037
ΔpH/ΔT°C (± 95% CI)	-0.023 (± 0.011)		0.029 (± 0.013)
CS temperature change	15 → 25°C		25 → 15°C
Common thresher shark	**15°C (n = 2)**		**22°C (n = 2)**
P_{50} mmHg (SE), pH 7.3	20.0 (18.4 to 21.7)		17.7 (16.2 to 19.3)
n_{50} (± SE), pH 7.3	1.43 (± 0.08)		1.72 (± 0.08)
Bohr coefficients (95% CI)	-0.13 (-0.60 to 0.33)		-0.88 (-1.56 to 0.06)
pH range	pH 7.089 to 7.378		pH 7.165 to 7.360
ΔpH/ΔT°C (± 95% CI)	-0.0003 (± 0.007)		-0.002 (± 0.006)
CS temperature change	15 → 22°C		22 → 15°C
Bigeye thresher shark	**10°C (n = 5)**		**25°C (n = 5)**
P_{50} mmHg (SE), pH 7.7	7.9 (6.9 to 9.2)		8.5 (7.2 to 9.9)
pH 7.5	7.5 (6.7 to 8.2)		8.7 (8.0 to 9.5)
n_{50} (± SE), pH 7.7	2.42 (± 0.19)		1.80 (± 0.22)
pH 7.5	2.17 (± 0.13)		1.51 (± 0.11)
Bohr coefficients (95% CI)	0.14 (-0.16 to 0.42)		-0.06 (-0.74 to 0.33)
pH range	pH 7.139 to 7.695		pH 7.289 to 7.697
ΔpH/ΔT°C (± 95% CI)	-0.003 (± 0.003)		-0.0002 (± 0.005)
CS temperature change	10 → 25°C		25 → 10°C
Spiny dogfish	**15°C (n = 7)**		**25°C (n = 7)**
P_{50} mmHg (SE), pH 7.85	22.9 (21.1 to 24.8)		33.8 (31.1 to 36.7)
pH 7.7	22.5 (20.9 to 24.3)		35.6 (32.9 to 38.5)
n_{50} (± SE), pH 7.85	1.17 (± 0.03)		1.26 (± 0.04)
pH 7.7	1.14 (± 0.02)		1.23 (± 0.02)
Bohr coefficients (95% CI)	0.04 (-0.12 to 0.19)		-0.15 (-0.41 to -0.03)
pH range	pH 7.497 to 8.045		pH 7.551 to 7.944
Blue shark	**10°C (n = 3)**	**15°C (n = 4)**	**25°C (n = 4)**
P_{50} mmHg (SE), pH 7.7		17.0 (15.6 to 18.6)	24.7 (21.8 to 27.9)
pH 7.5/7.45	8.2 (6.7 to 9.7), pH 7.45	17.7 (16.4 to 19.0), pH 7.5	26.7 (24.9 to 28.7), pH 7.5
n_{50} (± SE), pH 7.7		1.51 (± 0.07)	1.50 (± 0.09)
pH 7.5/7.45	1.39 (± 0.13), pH 7.45	1.58 (± 0.06), pH 7.5	1.42 (± 0.06), pH 7.5
Bohr coefficients (95% CI)	-0.14 (-0.54 to 0.26)	-0.08 (-0.41 to 0.53)	-0.17 (-0.52 to 0.45)
pH range	pH 7.013 to 7.456	pH 7.128 to 7.863	pH 7.163 to 7.689
ΔpH/ΔT°C (± 95% CI)		-0.022 (± 0.013)	0.023 (± 0.010)
CS temperature change		15→25°C	25→15°C

Table 4.3 Whole blood P_{50} values (mmHg) and Bohr coefficients (φ) reported in the literature for shortfin mako sharks, blue sharks, and spiny dogfish.

Species	T°C	pH	P_{50} (mmHg)	φ	Reference
Shortfin mako shark	15	7.93	14.5	-0.74	Bernal et al. (2018)
	15	7.68	22.3		
	25	8.13	18.6	-0.11	
	25	7.64	20.9		
	25	7.6	10.6	0.16	Wells and Davie (1985)
Blue shark	15	8.05	12.1	-0.33	Bernal et al. (2018)
	15	7.45	19.1		
	25	7.95	25.0	-0.22	
	25	7.48	31.7		
Spiny dogfish	15	7.85	17.9	-0.28	Wells and Weber (1983)

Table 4.4 Hæmolysate oxygen equilibria parameters of common thresher shark at two experimental temperatures and in the absence or presence of ATP and urea.

P_{50} values (± 95% confidence intervals) were predicted at pH 7.3 from linear models of logP_{50} vs pH (see Methods section), and Bohr coefficients are the model slopes (i.e., $\Delta\log P_{50}/\Delta$pH) with their 95% confidence intervals. $\Delta H'$ values were calculated with logP_{50} values at pH 7.3, and excludes the heat of O_2 in solution (-12.6 kJ mol^{-1} O_2).

	15°C		22°C	
P_{50}, pH 7.3	**Log_{10} P_{50} ± 95% CI**	**P_{50} (mmHg)**	**Log_{10} P_{50} ± 95% CI**	**P_{50} (mmHg)**
Stripped Hb	0.397 ± 0.080	2.49	0.395 ± 0.110	2.48
Hb + Urea	0.352 ± 0.322	2.25	0.456 ± 0.786	2.86
Hb + ATP	1.322 ± 0.044	21.01	1.394 ± 0.103	24.76
Hb + ATP + Urea	1.232 ± 0.028	17.04	1.136 ± 0.310	13.69
Bohr coefficients (φ)	**φ ± 95% CI**		**φ ± 95% CI**	
Stripped Hb	-0.006 ± 0.226		0.105 ± 0.346	
Hb + Urea	-0.498 ± 1.263		-0.273 ± 4.132	
Hb + ATP	-0.451 ± 0.198		-0.081 ± 0.302	
Hb + ATP + Urea	-0.388 ± 0.122		-0.184 ± 0.738	
$\Delta H'$ (kJ mol^{-1}), pH 7.3				
Stripped Hb		+13		
Hb + Urea		-12		
Hb + ATP		-4		
Hb + ATP + Urea		+35		

Chapter 5 General Discussion and Conclusions

"I look upon controversy especially as one of the chief ways in which truth is approached. We may fondly imagine that we are impartial seekers after truth, but with a few exceptions, to which I know that I do not belong, we are influenced and sometimes strongly by our personal bias and we give our best thoughts to those ideas which we have to defend. Nevertheless we should of course all do our best to avoid controversy, in the sense that we should take every possible care to verify our facts and substantiate our conclusions before publishing our results." (August Krogh, 1929, p. 248)

5.1 Thesis overview and major contributions

My objective for this thesis was to investigate the functional significance of reduced and reverse temperature-dependent Hb-O_2 affinity in regionally heterothermic teleost and sharks. There are two underlying hypotheses that guided this work: 1) temperature-independent Hb-O_2 affinity conserves heat-energy, which may help maintain stably elevated body temperatures in regionally heterothermic fishes, so I expected this trait to be shared by all regionally heterothermic teleosts and sharks capable of maintaining internal temperature gradients; and 2) the Hb-oxygen binding properties and high Hb concentrations of regionally heterothermic fishes maintain matching between O_2 supply and O_2 demand despite large internal temperature gradients. To address these hypotheses, I developed four specific research questions: 1) how do Hb concentration and Hb-O_2 affinity contribute to the determinants of maximal O_2 consumption, circulatory O_2 delivery and tissue O_2 extraction, in the yellowfin tuna? 2) How does the enthalpy of Hb-oxygenation influence O_2 and heat transport by Hb? 3) How does temperature affect Hb-O_2 affinity in two regionally heterothermic teleosts, the swordfish and the smalleye Pacific opah?

4) How does temperature affect Hb-O_2 affinity in the regionally heterothermic common thresher shark, and a suspected regional heterotherm, the bigeye thresher shark?

Addressing the first question, using a mathematical model of O_2 transport I demonstrated that Hb concentration and Hb-O_2 affinity are relatively important factors determining $\dot{M}O_2max$ in the yellowfin tuna, a regional heterotherm with a "high-energy demand" (Chapter 2). Addressing the second question, I used published data to determine a range of enthalpy values for different species of tuna, which I used to model the potential heat-energy savings and influence of temperature on blood PO_2. I showed that Hb with a reversed temperature dependence should prevent Hb-heat loss, as much as 13% of metabolic heat production, and prevent any changes to blood PO_2 over a wide temperature range (Chapter 2). Addressing the third question, I showed that smalleye Pacific opah Hb-O_2 affinity was independent of temperature, and the temperature-dependence of swordfish Hb-O_2 affinity was pH dependent, becoming temperature-independent at low pH (Chapter 3). Addressing the fourth question, I showed that common thresher shark Hb-O_2 affinity was independent of temperature, and bigeye thresher shark Hb has a high O_2 affinity that is temperature-independent at low saturation, but has a normal temperature-dependence at high saturation (Chapter 4).

These results provide insight into the functional significance of reduced and reversed temperature-dependent Hb-O_2 affinity in regionally heterothermic fishes (Table 5.1). In this chapter I offer my interpretation of these results and those of previous studies to provide some context for the evolution and functional significance of this Hb trait in regionally heterothermic teleosts and sharks.

5.2 The functional significance of an increased enthalpy of hæmoglobin-oxygenation

5.2.1 Temperature gradients in the heat-exchanging *retia mirabilia*: closed-system temperature changes

Hæmoglobin of regionally heterothermic fishes must transport O_2 across internal temperature gradients established by the heat exchanging *retia*. Experiments on the effects of closed-system temperature changes on blood PO_2 were initiated to test the hypothesis that temperature-independent Hb-O_2 affinity prevents premature Hb-O_2 unloading during closed-system warming in the heat exchanging *retia* (Carey and Gibson, 1977; Cech et al., 1984; Graham, 1973). Although the relative importance of closed-system changes to blood PO_2 in the heat exchanging *retia* has likely been overstated, and it is probably more important that P_{50} is not too different in the capillaries of warm and cold tissues, the results of closed-system experiments are good for qualitative comparisons of the temperature-dependence of blood-O_2 affinity among species.

Blood from smalleye Pacific opah, shortfin mako shark, and common thresher shark shows reverse temperature-dependence during closed-system warming, when blood PO_2 changed less than and/or in the opposite direction as would be expected due to the temperature dependence of the solubility of O_2 in plasma (Figure 3.4). These results are similar to previous reports for Atlantic bluefin tuna, albacore tuna, and skipjack tuna (section 1.3.1 and Figure 1.6) (Bernal et al., 2018; Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Cech et al., 1984). In contrast, swordfish and bigeye thresher shark blood PO_2 increased with closed-system warming (Figure 3.4 and Figure 4.5), more than would be expected due to the temperature dependence of the solubility of O_2 in plasma, indicating that increasing temperature caused Hb-O_2 unloading due to decreased Hb-O_2 affinity. The unloaded O_2 diffuses from the RBCs into the plasma,

which increases plasma PO_2. These results are similar to those previously reported for bigeye tuna blood (Figure 1.6) (Lowe et al., 1998). Furthermore, all of the closed-system effects of temperature on blood PO_2 described above correspond to the effect of temperature on whole blood-O_2 affinity for each species, in contrast to previous speculation that the effect of temperature on blood-O_2 affinity may differ between the gills and systemic circulation of some species (Lowe et al., 2000).

If warming of arterial blood in a heat exchanging *rete* causes premature Hb-O_2 unloading that is detrimental to circulatory O_2 delivery, then it would be expected that temperature-independent Hb-O_2 affinity would be a trait common to all regionally heterothermic fishes. Since closed-system warming decreased blood-O_2 affinity and increased blood PO_2 in both bigeye tuna and swordfish, it seems unlikely that premature O_2 unloading in a heat exchanging *rete* adversely affects circulatory O_2 delivery. Moreover, relatively short blood transit times through the heat exchanging *retia* may limit excessive temperature induced O_2 unloading (Brill and Bushnell, 2001), and even if closed-system warming induced rapid Hb-O_2 unloading, the reduction in saturation is probably inconsequential to circulatory O_2 delivery.

5.2.2 Heat-energy conservation in species with warm red muscle

An increased $\Delta H'$ will reduce the energy required for O_2 unloading in the cold peripheral tissues of regionally heterothermic fishes, and prevent liberation of heat upon O_2 binding at the gills (Giardina et al., 1989b; Weber and Fago, 2004; Weber and Wells, 1989). In most tunas and regionally heterothermic sharks, $\Delta H'$ values are near zero or positive (i.e., temperature-independent and reverse-temperature dependent Hb-O_2 affinity, respectively; Table 5.1) eliminating any outward heat transport linked to Hb-O_2 unloading and binding. This could potentially save up to 13% of metabolic heat production compared to the intrinsic $\Delta H'$ (i.e, ΔH^{O2}

≈ -61 kJ mol^{-1} O_2; Figure 2.8). Therefore, it seems reasonable to hypothesize that the heat-energy savings of this trait may be important for preventing Hb-heat transport away from the warm red muscle, increasing the efficiency of heat retention. In contrast to this hypothesis, bigeye tuna and swordfish $\Delta H'$ values are negative, and therefore exothermic, so it is not clear if the energy savings of a numerically low $\Delta H'$ contributes appreciably to thermoconservation in regionally heterothermic fishes. There are, however, important differences between bigeye tuna and other cold tolerant tunas, and between billfishes and other regionally heterothermic fishes, which are worthwhile considering and are briefly discussed below.

Red muscle temperatures are not stable in either bigeye tuna or swordfish, and are influenced by ambient water temperature more so than in cold tolerant tunas and lamnid sharks. During daily sojourns into cold water deep below the thermocline, bigeye tuna routinely return to warmer surface waters before their RM temperature falls below about 17°C, and while swordfish are at depth their RM cools close to ambient temperature (Bernal et al., 2009; Holland et al., 1992; Malte et al., 2007; Stoehr et al., 2018). However, the behavioural thermoregulation exhibited by the bigeye tuna prevents the warm tissues from becoming hypothermic, and warming-up in surface waters is accelerated by redistributing blood flow between the central circulation and lateral heat-exchanging *retia* (i.e., physiological thermoregulation) (Holland and Sibert, 1994; Holland et al., 1992). In comparison, some large tunas and lamnid sharks that inhabit cold temperate or polar waters for months at a time with no warm refuges, maintain RM temperatures stably elevated by as much as 10 to 20°C above ambient (Anderson and Goldman, 2001; Bernal and Lowe, 2015; Bernal et al., 2017; Block et al., 2001; Carey and Teal, 1969b; Goldman et al., 2004; Gunn and Block, 2001). In the blood of these cold-tolerant tunas and sharks the temperature-dependence of Hb-O_2 affinity is reversed (Brill and Bushnell, 2006;

Carey and Gibson, 1977; Cech et al., 1984; Clark et al., 2008a; Dickinson and Gibson, 1981; Larsen et al., 2003; Lilly et al., 2015).

Billfish cranial endothermy is quite remarkable, but the warm tissues make up a relatively small proportion of the entire body compared to regionally heterothermic fishes capable of RM endothermy. Since most of the systemic circulation perfuses tissues and organs that are near ambient water temperature, reversed Hb-oxygenation enthalpy would probably not greatly affect thermoregulation and heat conservation in billfishes, including swordfish. Furthermore, in at least swordfish, the orbital *rete* effectively retains heat generated by the heater organ, keeping cranial temperature elevated well above cold ambient temperatures (Figure 3.1) (Carey, 1990).

Although numerically low or positive $\Delta H'$ values will conserve heat-energy, it is not clear if these savings significantly influence body temperatures in regionally heterothermic fishes. It seems unlikely that reversed Hb-oxygenation enthalpy helps maintain stable cranial temperatures in billfishes, and the absence of temperature-independent Hb-O_2 affinity in bigeye tuna may justify rejecting the hypothesis that this trait has an appreciable contribution to thermoconservation. If a regional heterothermic fish had a $\Delta H'$ equivalent to rainbow trout, -35kJ mol^{-1}, then about 7.4% of the metabolic heat production would be lost from Hb to the environment (Figure 2.8). In a slowly swimming yellowfin tuna, this may increase blood heat-energy loss from 0.105 to 0.112 kJ kg^{-1} min^{-1}. This may be inconsequential over short periods, but tuna are considered “energy speculators” (Brill, 1996; Korsmeyer and Dewar, 2001), so any energy savings may have long term value, but may not be essential for thermoconservation.

5.2.3 Hæmolgobin-O_2 unloading to warm and cold tissues

Reduced and reverse temperature-dependent Hb-O_2 affinity should prevent excessive temperature induced shifts to the physiological OEC, matching O_2 supply to O_2 demand in

tissues maintained at very different temperatures (Giardina et al., 1989a; Weber and Campbell, 2011). Reverse temperature-dependent Hb-O_2 affinity is probably most important in cold-tolerant species like Atlantic bluefin tuna, because blood-O_2 affinity and PO_2 will not drastically change as peripheral tissue temperature changes with environmental temperature, and as blood flows from the gills to warmer tissues. In other words, blood PO_2 would not be greatly affected by temperature changes in the body or environment of a regionally heterothermic fish, which I showed in Chapter 2. Using yellowfin tuna mixed venous blood gas data at 25°C (Korsmeyer et al., 1997a) and applying different $\Delta H'$ values to adjust blood PO_2 to temperatures ranging from 30°C down to 15°C, it becomes evident that temperature-independence stabilizes mixed venous PO_2 over the temperature range, and reverse temperature-dependence may enhance O_2 unloading at cold temperatures (Figure 2.7).

When cold tolerant regional heterotherms enter cold water, a reverse temperature-dependence probably protects Hb-O_2 unloading to the cold peripheral tissues by preventing blood PO_2 from decreasing so low that it may impair Hb-O_2 unloading and tissue O_2 extraction. This may be particularly important for cardiac O_2 delivery (Clark et al., 2008a) because tuna, shark, and billfish hearts remain near ambient water temperature due to their anatomical position and a coronary circulation that supplies arterial blood directly from the gills (Bernal et al., 2001b; Brill, 1987; Brill and Bushnell, 2001; Cox, 2015; Daxboeck and Davie, 1986). Thus, ambient temperature directly influences cardiac function (Clark et al., 2008b; Clark et al., 2013). Although the hearts of most lamnid sharks are not too different from ectothermic sharks (Brill and Lai, 2015), tuna have large powerful hearts compared to other teleosts (Brill and Bushnell, 1991b; Brill and Bushnell, 2001; Farrell et al., 1992). This is exemplified by the relatively large hearts of bigeye tuna, which also appear to have enhanced performance at colder temperatures

relative to some tuna with warmer distributions (Bernal et al., 2017; Brill and Bushnell, 1991b; Galli et al., 2009). The hearts of some other cold-tolerant tuna, swordfish, and salmon shark also appear to have evolved a greater tolerance to functioning in cold water (Bernal et al., 2017; Galli et al., 2009; Weng et al., 2005). Thus, in species such as Atlantic bluefin tuna, a reverse temperature-dependence has been proposed to enhance O_2 unloading to cold organs with a high metabolic demand, such as the heart (Clark et al., 2008a).

At approximate arterial pH values, swordfish $\Delta H'_{WB}$ (-23 mol^{-1} O_2) is more exothermic than other regionally heterothermic fishes, but it is more endothermic (less numerically negative) than values for some regionally heterothermic mammals that have heat exchanging *retia* in their limbs, which can function to retain heat or dissipate heat. For example, $\Delta H'_{WB}$ values (including the heat of O_2 in solution) for both reindeer and musk-ox are about -27 kJ mol^{-1} O_2, which is much less than for humans (~ -52 kJ to -44 kJ mol^{-1} O_2) (Brix et al., 1990; Reeves, 1980). The temperature-dependence of reindeer Hb is also saturation dependent, but unlike swordfish Hb, the effect of temperature on Hb-O_2 affinity decreases with increasing saturation in reindeer blood. At high O_2 saturations that are similar to arterial levels, reindeer Hb-O_2 affinity is almost independent of temperature, which likely prevents temperature induced shifts to Hb-O_2 affinity as the arterial blood is cooled in the heat exchanging *retia* en route to the cold legs (Giardina et al., 1989a). Swordfish Hb, however, has a “normal” temperature dependence at high O_2 saturations and arterial blood pH values, but at low pH, Hb-O_2 affinity becomes temperature-independent. This is due to proton and ATP binding that is more favourable at low than at high temperature, which may also avert temperature induced shifts to Hb-O_2 affinity in the capillaries of the cold tissues.

Smalleye Pacific Opah are quite different than other regionally heterothermic fishes because they have warm hearts and maintain most of their body warmer than ambient (Wegner et al., 2010). However, temperature varies within the body, so temperature-independent Hb-O_2 affinity within the range of body temperatures should maintain matching between O_2 supply and O_2 demand to all the tissues and organs even though they vary in temperature.

5.3 Matching O_2 supply and O_2 demand

Hæmoglobin concentration and O_2 affinity both have important contributions to blood-O_2 transport because they determine blood-O_2 carrying capacity and the O_2 capacitance of the blood (i.e., βbO_2). Thus, Hb is an important determinant of circulatory O_2 delivery and tissue O_2 extraction. Furthermore, tuna cardiac outputs are quite low during routine swimming (Clark et al., 2008b; Clark et al., 2013) but routine metabolic rates are relatively high (Korsmeyer and Dewar, 2001), so Hb likely plays an essential role in sustaining aerobic metabolic rates and heat production. In Chapter 2, I showed that Hb concentration and an increased P_{50} in the tissues (i.e., a Bohr shift) are important determinants of maximum O_2 transport in yellowfin tuna.

Sharks, however, differ. In general, cardiac performance does not appear to be exceptional in regionally heterothermic sharks compared to ectothermic sharks, quite unlike the situation in tuna (Brill and Lai, 2015). Although shortfin mako routine and maximum (i.e., the highest measured) O_2 consumption rates are higher than other sharks (Sepulveda et al., 2007b), the routine rates are about half that of yellowfin tuna (Brill and Lai, 2015). Because mako shark cardiac output does not seem to increase considerably with increased swimming activity, circulatory O_2 delivery also will not greatly increase, so more O_2 will have to be extracted from the blood to match O_2 supply to the elevated O_2 demand. High Hb-O_2 affinity and high Hb

concentration maintain elevated levels of arterial saturation and blood-O_2 carrying capacity. However, mako blood, and that of other large active sharks, does not exhibit a significant Bohr shift. Therefore, the maximum aerobic metabolic rates of regionally heterothermic sharks may be constrained by reduced circulatory O_2 delivery due to low cardiac outputs, and relatively low tissue O_2 extraction due to their inability to alter Hb-O_2 affinity, and thus βbO_2, between the gills and the tissues.

5.4 Is reduced temperature-dependence of Hb-O_2 affinity an example of convergent physiological function among regionally heterothermic vertebrates?

Many cold-tolerant mammals, aquatic mammals, and birds have heat exchanging *retia mirabilia* that supply blood to limbs or appendages (Figure 1.3) (e.g., Cutright and McKean, 1979; Kahl, 1963; Øritsland, 1970; Rommel and Caplan, 2003; Scholander, 1955; Scholander and Krog, 1957; Scholander and Schevill, 1955). In some regionally heterothermic mammals and birds, the limbs are colder than the rest of the body so the heat exchanging *retia* cool and warm the blood flowing into and out of the appendages, respectively. However, the *retia* can also function to dissipate heat and are important for whole animal temperature regulation (e.g., Steen and Steen, 1965). Furthermore, carnivores and artiodactyls (Cetartiodactyla) have a carotid *rete* that cools the brain, which is thought to have contributed to niche expansion and diversification of artiodactyls relative to similar mammals like perissodactyls that lack a carotid *rete* (Baker, 1972; Lust et al., 2007; Mitchell and Lust, 2008).

It is generally regarded that reductions in the temperature dependence of Hb-O_2 affinity averts impaired blood-O_2 transport across internal temperature gradients, which exist between tissues or organs that are much warmer or colder than the respiratory organs of regionally heterothermic vertebrates with heat exchanging *retia* (Weber and Campbell, 2011). I did not

question this hypothesis until I started writing this thesis during the "lockdown" imposed due to the COVID-19 pandemic, even though a few years ago I amassed all of the mammal blood P_{50} data that I could find, but I found few studies on the effect of temperature on Hb-O_2 affinity in mammals. Most of these studies investigated the effect of temperature on Hb-O_2 affinity in regionally heterothermic mammals with limited comparisons to non-heterotherms, so I find the results of these studies equivocal regarding this hypothesis.

Low temperature-dependence of Hb-O_2 affinity may be a trait shared by cetartiodactyls, with good evidence for this from reindeer/caribou (*Rangifer tarandus*), red deer (*Cervus elaphus*), musk-ox (*Ovibos moschatus*), common minke whale (*Balaenoptera acutorostrata*), and pig (*Sus* sp.) (Brix et al., 1989a; Brix et al., 1989b; Brix et al., 1989a; Pellegrini et al., 1999; Willford and Hill, 1986). Among carnivores, cat (*Felis catus*) and harbour seal (*Phoca vitulina*) Hbs also have a low temperature-dependence (Cambier et al., 2004; Willford and Hill, 1986), but there are not many other published studies on the temperature-dependence of carnivore Hbs, including limited data on Hb from the polar bear (*Ursus maritimus*), which has heat exchanging *retia* in its legs (Øritsland, 1970). Furthermore, there is a dearth of information on the temperature-dependence of Hb from heterothermic birds (reviewed by Weber and Campbell, 2011). Lastly, in all of these examples of Hb with a low temperature-dependence, including other examples provided by Weber and Campbell (2011), the conclusion of a reduced temperature-dependence is based on limited comparisons to few other mammalian Hbs aside from human HbA. It seems that the O_2 affinity of HbA may be particularly sensitive to temperature ($\Delta H' \approx$ -45kJ mol^{-1} O_2), so further comparisons among regionally heterothermic and non-heterothermic mammals are warranted to investigate whether reductions in the temperature-dependence of Hb-O_2 affinity are adaptive to animals with heat exchanging *retia*.

Reduced temperature-dependence of Hb-O_2 affinity is not a trait that is exclusive to regional heterotherms. For example, this trait has been reported for several ectothermic fishes, including some 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; Morrison et al., 2015; Weber et al., 1976). However, in most studies of the effect of temperature on Hb-O_2 affinity of fishes, the effect of pH and temperature on Hb-O_2 affinity were not appropriately quantitively assessed (except for studies by Roy Weber or Angela Fago and colleagues), and the reported summary data provide limited opportunities for further analyses. Furthermore, in a couple of studies on Hb of Atlantic Cod (*Gadus morhua*), the authors reported reduced and reverse temperature-dependence, but the figures of their data clearly show a normal temperature dependence (e.g., Barlow et al., 2017; Nelson et al., 2019). Therefore, there is reasonable evidence that reduced temperature-dependent Hb-O_2 affinity may be a trait that is quite widespread among fishes, and is worthy of further research since the apparent existence of this trait in some species may be a result of inadequate data analysis. Furthermore, it is not clear if this trait has any functional significance in ectotherms or if it is a consequence of effector binding that was selected for reasons unrelated to the temperature sensitivity of Hb. Interestingly, reduced and reversed temperature-dependent blood-O_2 affinity has also been reported in chub mackerel (*Scomber japonicus*), an ectothermic scombrid that is closely related to tuna (Clark et al., 2010), so it reasonable to propose this trait was present in the common ancestor to tuna. However, it does not seem to be a coincidence that all regionally heterothermic teleosts and sharks investigated to date have Hb with reduced temperature sensitivities (Table 5.1).

Hæmoglobins with reduced or reversed temperature-dependence are present in blood of common thresher shark, lamnid sharks, smalleye Pacific opah, tunas, swordfish, and istiophorid

billfishes. Among studied tunas, all species exhibit either temperature-independent or reverse temperature-dependent Hb-O_2 affinity, except for bigeye tuna (Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Cech et al., 1984; Clark et al., 2008a; Graham, 1973; Lilly et al., 2015; Lowe et al., 2000). The temperature-dependence of bigeye tuna Hb-O_2 affinity is, however, relatively reduced compared to that of most other vertebrates.

I do not disagree with the proposal that reductions in the temperature-dependence of Hb-O_2 affinity are an example of convergent physiological function among regionally heterothermic vertebrates, but more research and thought to this topic is needed.

5.5 Future research directions

The findings of this thesis add to the list of regional heterotherms that posses Hb with a numerically low $\Delta H'$ (Table 5.1), and provides some insight into the functional significance of this trait. However, there are still a lot of unanswered questions and work to be done to better understand the evolution and functional significance of reduced and reverse temperature-dependent Hb-O_2 affinity.

Does the butterfly kingfish also have Hb with a reduced temperature-dependence? The butterfly kingfish is a close relative of the tunas in the family Scombridae and is likely capable of elevating cranial temperature (Tullis et al., 1991), although endothermy has yet to be confirmed with temperature measurements (Figure 1.3; Table 5.1). Investigations into cranial temperature and the effect of temperature on blood- and Hb-O_2 affinity are needed in the butterfly kingfish.

Hoes does the temperature-dependence of Hb-O_2 affinity differ among the tunas, and how do the tunas compare to ectothermic scombrid fishes? There appears to be differences among tunas that may be related to internal temperature gradients that are increased by the

depths and water temperatures that each species exploits, as well as species specific capacities to maintain stably elevated RM temperatures. Furthermore, at least one ectothermic scombrid fish exhibits reduced and reverse temperature dependence. I think that the family Scombridae are an excellent model for functional, structural, and molecular studies to further investigate the evolution and significance of reduced and reverse temperature-dependent Hb-O_2 affinity. Although the Atlantic bluefin tuna is well studied, no other species in the family Scombridae has been studied in such detail (see Chapter 1, section 1.3.1).

Has temperature-independent Hb-O_2 affinity evolved independently in lamnid sharks and the common thresher shark? There is good evidence to suggest that RM endothermy evolved independently in lamnid sharks and the common thresher shark. Structural and molecular studies of Hbs from these sharks, as well as closely related ectothermic sharks, would provide insight into the evolution of this trait.

Is the temperature-dependence of Hbs from regionally heterothermic mammals and birds reduced compared to non-heterothermic species? Although it is assumed that in regionally heterothermic mammals $\Delta H'$ is relatively low, it is not well known how the temperature-dependence of Hb compares to closely related non-heterothermic mammals. The order Cetartiodactyla (even-toed ungulates and whales) is an excellent group to investigate the evolution and functional significance of this trait among regionally heterothermic mammals. Some species Hbs are already well characterized (e.g., reindeer Brix et al., 1990; Giardina et al., 1989b; Giardina et al., 1989b), phylogenetic relationships are quite well established (Zurano et al., 2019), and the heat exchanging carotid *rete* is thought to have been important to the diversification and niche expansion of cetartiodactyls (Lust et al., 2007; Mitchell and Lust, 2008).

Does the bigeye thresher shark have warm eyes? Although the bigeye thresher shark has an orbital *rete* suspected of having a heat exchanging function (Block and Carey, 1985; Weng and Block, 2004), cranial temperatures are not warmer than sea surface temperature (Diego Bernal and Chugey Sepulveda, personal communication). However, I suspect that ocular muscles are capable of elevating eye temperatures above ambient temperature when bigeye thresher sharks are in deep cold water, but like opah, eye temperatures are probably not warmer than surface waters (Wegner et al., 2015). Temperature measurements of the eyes and ambient water, while bigeye thresher sharks are in cold water below the thermocline, are needed to establish if this shark is regionally heterothermic.

5.6 Summary and final thoughts

When I first started thinking about applying for NSERC funding to start a PhD thesis, my objective was to develop a project where I could study sharks and respiratory physiology. That seems to have worked out. I cannot remember what led me to start searching the literature for papers on Hb function in regionally heterothermic sharks, but my introduction to the topic of the temperature-dependence of Hb-O_2 affinity in regional heterotherms was through papers by Roy Weber and colleagues (Larsen et al., 2003; Weber and Campbell, 2011; Weber et al., 2010). With these papers, and others (Andersen et al., 1973; Brill and Bushnell, 1991a; Brill and Bushnell, 2006; Carey and Gibson, 1977; Clark et al., 2008a; Clark et al., 2010; Lowe et al., 2000; Rossi Fanelli and Antonini, 1960), I adopted the hypothesis that the temperature-dependence of Hb-O_2 affinity will be reduced or reversed in all regionally heterothermic fishes, so as to prevent premature O_2 unloading as blood is warmed in a heat exchanging *retia*. This was at odds with what some others thought, but seemed like a testable hypothesis if I could get blood from enough unstudied species. Since then, I have amended that hypothesis and learned enough

that I am confident that I can critically assess and synthesize the literature on this topic. I hope I have done that reasonably well.

In this thesis I have provided evidence that all lineages of known regionally heterothermic teleosts and sharks have Hb with a low overall $\Delta H'$. Specifically, I have shown temperature-independence in smalleye Pacific opah, common thresher shark, and mako shark, a pH-dependent reduced temperature-dependence in swordfish, and a saturation-dependent reduced temperature-dependence in bigeye thresher shark. The oxygenation-dependent release of allosteric effectors contributes endothermically to $\Delta H'$ and stabilizes the high affinity Hb conformation at high relative to cold temperature. The main effector of tuna Hb is protons (Ikeda-Saito et al., 1983), whereas in Hbs of billfishes it is pH-dependent binding of ATP (Weber et al., 2010), and ATP is the primary effector of lamnid shark Hbs (Larsen et al., 2003). I have shown that ATP and protons are the main effectors of swordfish Hb, like other billfishes. In opah Hb, proton binding and dissociation cause temperature-independence, but ATP reverses it. Common thresher shark Hb seems to have an intrinsic temperature-independence that is reversed in the presence of ATP. Thus, increases to $\Delta H'$ appear to have repeatedly evolved by different molecular mechanisms with underlying dependence on different allosteric effectors.

I have also attempted to explain the functional significance of high Hb concentration in regionally heterothermic fishes, as well as the functional significance of low $\Delta H'$. In all regional heterotherms, high Hb concentration is probably essential to matching O_2 supply to O_2 demand without unnecessarily increasing cardiac output. There are two major effects of an increased $\Delta H'$: it will reduce the energy used during the Hb-O_2 binding and unloading cycle, and it will reduce the effect of temperature on Hb-O_2 affinity. Presumably, the functional significance of reduced and reverse temperature dependence is associated with at least one of these effects.

Swordfish and billfishes heat only the cranial region, and swordfish have a normal temperature dependence at high pH and high saturation, which I suspect is also the case for other billfishes. Any Hb linked energy savings are probably inconsequential to billfishes, but a right shifted OEC at low temperature due to temperature dependent ATP and proton binding should promote O_2 unloading uniformly to all tissues despite the tissue temperature. In species that heat their body core (tuna, lamnid sharks, common thresher shark, and opah), Hb-O_2 affinity is temperature-independent or exhibits a reverse temperature-dependence, except for the bigeye tuna. In these species, other than bigeye tuna, reduced and reverse temperature-dependence probably conserves heat-energy and prevents Hb-O_2 affinity from being too high to unload O_2 to the cold tissues and organs, especially those with a high metabolic demand like the heart. That these disparate lineages of teleosts and sharks have independently evolved regional heterothermy is quite remarkable, but the evolution of temperature insensitive Hbs through different molecular strategies to modulate $\Delta H'$ is an exceptional example of convergent physiological function.

Table 5.1 $\Delta H'$ values for known and suspected regionally heterothermic teleosts and sharks

Taxon	Common name	Endothermy: Swimming muscle	Endothermy: Eye / brain	Endothermy: Viscera	$\Delta H'$ (kJ mol^{-1})	$\Delta H'$ conditions[1]	$\Delta H'$ reference
Teleostei							
Order Istiophoriformes							
Family Xiphiidae							
Xiphias gladius	Swordfish		X		-23	WB; pH 7.7; 10-25°C	1
					+4	Hb+ATP; pH 7.4; 10-25°C	1
Family Istiophoridae							
Istiophorus albicans	Atlantic sailfish		X				
Istiophorus platypterus	Indo-Pacific sailfish		X				
Makaira mazara	Indo-Pacific blue marlin		X		+26	Hb+ATP; pH 7.4; 10-25°C	2
Makaira nigricans	Blue marlin		X				
Istiompax indica	Black marlin		X				
Kajikia albida	Atlantic white marlin		X				
Kajikia audax	Striped marlin		X		+4	Hb+ATP; pH 7.4; 10-25°C	2
Tetrapturus angustirostris	Shortbill spearfish		X		-7	Hb+ATP; pH 7.4; 10-25°C	2
Tetrapturus belone	Mediterranean spearfish		X				
Tetrapturus georgii	Roundscale spearfish		X				
Tetrapturus pfluegeri	Longbill spearfish		X				
Order Lampriformes							
Family Lamdridae							
Lampris australensis	Southern spotted opah	Assumed	Assumed				
Lampris guttatus	North Atlantic opah	Assumed	Assumed				
Lampris immaculatus	Southern opah	Assumed	Assumed				
Lampris incognitus	Smalleye Pacific opah	X	X		+1	WB; pH 7.7; 10-20°C	1
Lampris lauta	East Atlantic opah	Assumed	Assumed				
Lampris megalopsis	Bigeye Pacific opah	Assumed	Assumed				
Order Scombriformes							
Family Scombridae							
Gasterochisma melampus	Butterfly kingfish		Assumed				
Scomber japonicus	Pacific chub mackerel	Ectotherm	Ectotherm	Ectotherm	+42	WB; pH 7.7; 10-20°C	3
Allothunnus fallai	Slender tuna	X	X				
Auxis rochei	Bullet tuna	X	Assumed				
Auxis thazard	Frigate tuna	X	X				
Euthynnus affinis	Kawakawa tuna	X	X				
Euthynnus alletteratus	Little tunny	X	X				
Euthynnus lineatus	Black skipjack tuna	X	X		≈0	WB[2]	4
Katsuwonus pelamis	Skipjack tuna	X	X		+18	WB; pH 7.7; 20-30°C	1, 5
Thunnus alalunga	Albacore tuna	X	X	X	+7	WB; pH not given; 5-25°	6
Thunnus albacares	Yellowfin tuna	X	X		+4	WB; pH 7.7; 20-30°C	1, 5
Thunnus atlanticus	Blackfin tuna	X	Assumed				
Thunnus maccoyii	Southern bluefin tuna	X	Assumed	X	>0	WB[3]	7
Thunnus obesus	Bigeye tuna	X	X	?	-18	WB; pH 7.7; 15-25°C	8
Thunnus orientalis	Pacific bluefin tuna	X	X	X	>0	WB[3]	9
Thunnus thynnus	Atlantic bluefin tuna	X	X	X	+21	WB; pH 7.7; 15-25°C	10
Thunnus tonggol	Longtail tuna	X	Assumed				
Elasmobranchii							
Order Lamniformes							
Family Alopiidae							
Alopias pelagicus	Pelagic thresher shark		?				
Alopias superciliosus	Bigeye thresher shark		?		-3	WB; pH 7.7; 10-25°C	1
Alopias vulpinus	Common thresher shark	X		?	+12	WB; pH 7.3; 15-22°C	1
Family Lamnidae							
Carcharodon carcharias	White shark	X	X	X			
Isurus oxyrinchus	Shortfin mako shark	X	X	X	-3	WB; pH 7.7; 15-25°C	1
Isurus paucus	Longfin mako shark	?	X	X			
Lamna ditropis	Salmon shark	X	X	X	≥0	Hb	11
Lamna nasus	Porbeagle shark	X	X	X	+12	Hb+ATP; pH 7.3; 10-26°C	12

[1] WB = whole blood; Hb = hæmolysate or Hb component

[2] Graham (1973) reported no effect of temperature on blood-O_2 affinity, bot no other details were given.

[3] $\Delta H'$ cannot be calculated with the data provided in the publication.

This table was adapted from Dickson and Graham (2004). X = evidence of endothermy including measurements of elevated tissue temperatures; ? = putative heat exchanging *retia mirabilia* reported, but temperature measurements are lacking; Assumed = endothermy is assumed due to morphological characteristics and/or phylogenetic relationships to known endothermic species. References for the evidence for endothermy: (Bernal and Sepulveda, 2005; Block, 1990; Block, 1991a; Block and Carey, 1985; Dickson and Graham, 2004; Patterson et al., 2011; Runcie et al., 2009; Sepulveda et al., 2007a; Sepulveda et al., 2008; Underkoffler et al., 2018; Wegner et al., 2015). $\Delta H'$ values are from the following: 1 = this thesis; 2 = (Weber and Campbell, 2011); 3 = (Clark et al., 2010); 4 = (Graham, 1973); 5 = (Brill and Bushnell, 1991a); 6 = (Cech et al., 1984); 7 = (Clark et al., 2008a); 8 = (Lowe et al., 2000); 9 = (Lilly et al., 2015); 10 = (Brill and Bushnell, 2006); 11 = (Dickinson and Gibson, 1981); 12 = (Larsen et al., 2003).

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Appendix: A note on the storage duration of fish blood

In Chapter 3 and Chapter 4, oxygen equilibrium curves were constructed on whole blood from fish and sharks captured off Southern California (i.e., the Southern California Bight) or off Massachusetts. Blood samples were drawn by caudal puncture into heparinized syringes, and were then shipped, on ice, by courier to the UBC campus in Vancouver, Canada, where experiments were conducted within 1 to 4 days after the blood was collected. This sampling technique was previously used for a study on chub mackerel blood-O_2 affinity (Clark et al., 2010). In that study, experiments were conducted at the UBC Vancouver campus, but blood was collected from mackerel captured off Southern California by the same researcher (Dr. Chugey Sepulveda) that sampled blood for my thesis research. Clark et al. (2010) concluded that fish blood is viable for up to 6 days if stored at 4°C. To verify this sampling technique, we conducted preliminary experiments on swordfish blood.

Swordfish (n = 3) were captured by deep-set buoy gear (Sepulveda et al., 2014) off Southern California. Blood was drawn by caudal puncture into heparinized syringes. Blood samples were kept on ice and shipped by courier to the UBC campus in Vancouver, Canada, where experiments were conducted over 11 days after the blood was collected. Blood was refrigerated at 4°C during this time. Each day after blood samples arrived at UBC (2 days post-capture) hæmoglobin (Hb) concentration (mM) and hæmatocrit (Hct; the ratio of the volume of red bloods cells to the total volume of blood) were measured (Figure A.1). Whole blood pH, plasma Hb concentration, and whole blood P_{50} (mmHg; the partial pressure of oxygen that corresponds to 50% Hb-O_2 saturation) were also determined, but not every day (see Figure A.1). Hb concentration, Hct, pH, and P_{50} were determined as described in Chapter 3 (section 3.2). To assess if considerable red blood cell lysis occurred during the storage duration, plasma Hb

concentration was measured in triplicate 10 µl samples of blood plasma that were separated from the red blood cells by centrifugation. Mean corpuscular hæmoglobin concentration (MCHC, in mM) was calculated by dividing [Hb] by Hct.

To assess if Hb concentration, plasma Hb concentration, Hct, MCHC, and pH changed over time, linear models were fit to the data for each individual swordfish. For each of these variables, the slopes of the fitted lines were not significantly greater than zero, indicating that there was no biologically relevant change in each variable. The P_{50} data were inspected visually, and based on these data and a previous study by Clark et al. (2010), it was concluded that OECs could be constructed on blood stored for up to 6 days after blood was collected, provided blood was refrigerated at 4°C during this time. For the work reported in Chapters 3 and 4, OECs were constructed within 4 days post-capture, less than the proposed maximum of 6 days.

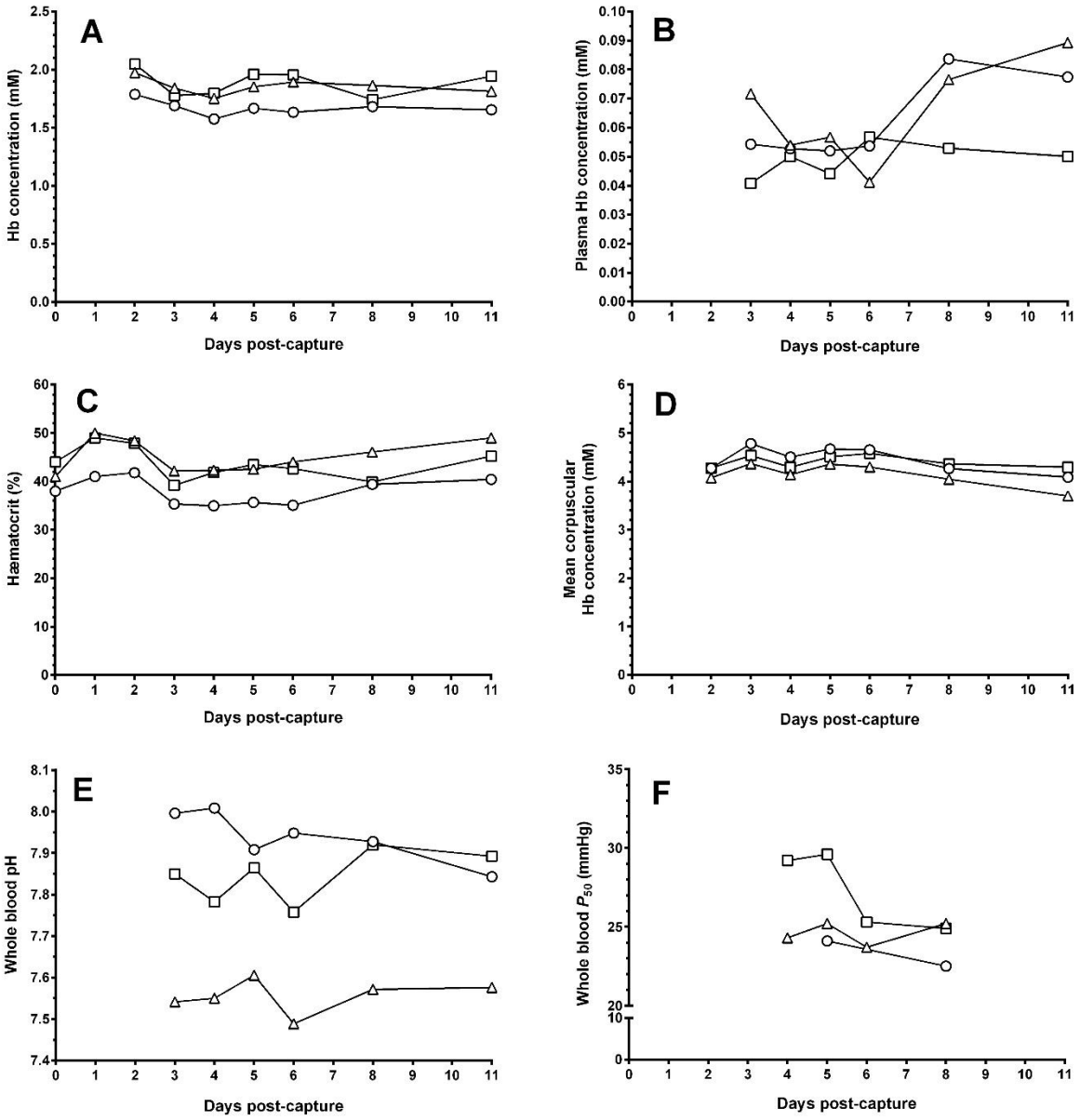

Figure A.1 The effect of storage duration on blood from swordfish (*Xiphias gladius*).

Blood from swordfish (n = 3, represented by different shaped symbols) was stored at 4°C for 11 days post-capture. Hb concentration (A), plasma Hb concentration (B), hæmatocrit (C), mean corpuscular Hb concentration (D), whole blood pH (E), and whole blood P_{50} (F) were not appreciably changed over the respective sampling periods.