MYOGLOBIN ADAPTATION IN TERRESTRIAL AND DIVING BIRDS AND MAMMALS

A Dissertation

by

TRAVER JOSEPH WRIGHT

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Chair of Committee,	Randall W. Davis
Committee Members,	Christopher D. Marshall
	William H. Neill
	Jeremy S. Wasser
Head of Department,	Michael P. Masser

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ABSTRACT

Myoglobin (Mb) is an oxygen binding hemoprotein in vertebrate skeletal muscle that functions in intracellular oxygen storage and transport. Due to the unique oxygen storage demands of diving birds and mammals, these vertebrates can have Mb concentrations ten-fold those found in their terrestrial counterparts making them ideal animal models for studying Mb function. Increased Mb bound muscle oxygen stores are advantageous for diving vertebrates, but Mb concentration optimized to maintain aerobic metabolism while diving or limiting to aerobic dive duration? A numeric model simulating a diving Weddell seal was created to examine physiological factors that influence dive duration and optimal Mb concentration. Mb concentration was limiting to dive duration in postabsorptive dives. However, Mb concentration was optimized for postprandial dives which were limited by blood-bound oxygen stores due to the additional metabolic costs of digestion.

While Mb concentration is adaptive in diving vertebrates, less is known about molecular adaptation of Mb functional properties. Novel methods were developed to extract Mb from frozen muscle and determine Mb oxygen affinity (P_{50}) by generating a high resolution oxygen dissociation curve at 37°C. For comparison, Mb P_{50} was determined for 25 species of diving and terrestrial birds and mammals. Myoglobin P_{50} was conserved among terrestrial vertebrates and most cetaceans at approximately 3.7 mmHg with the exception of the melon-headed whale that had a significantly higher P_{50} (lower oxygen affinity) of 4.85 mmHg. Among pinnipeds (seals and sea lions) the P_{50} ranged from 3.23-3.81 mmHg and showed a trend for higher oxygen affinity in species with longer dive durations. Among diving birds the P_{50} ranged from 2.40-3.36 mmHg and also showed a trend of higher affinities in species with longer dive durations. Both myoglobin concentration and oxygen affinity appear adaptive in diving vertebrates to maintain aerobic metabolism and minimize hypoxic cellular damage in ischemic muscle.

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NOMENCLATURE

ADL	Aerobic dive limit
β_{BO_2}	Capacitance coefficient of oxygen in blood (ml O_2 l^{-1} blood)
cADL	Aerobic dive limit calculated from useable oxygen stores and diving metabolic rate
Ca_{O_2}	Arterial blood oxygen concentration (ml O_2 l^{-1} blood)
CBv_{O_2}	Cerebral venous blood oxygen concentration (ml O_2 l^{-1} blood)
CHv_{O_2}	Coronary venous blood oxygen concentration (ml O_2 l^{-1} blood)
CMv_{O_2}	Skeletal muscle venous blood oxygen concentration (ml O_2 l^{-1} blood)
CNV	Copy-number variant
$CSRCv_{O_2}$	Splanchnic, renal, cutaneous and other peripheral tissue venous blood oxygen concentration (ml O_2 l^{-1} blood)
Cv_{O_2}	Venous blood oxygen concentration (ml O_2 l^{-1} blood)
$C\bar{v}_{O_2}$	Mixed venous blood oxygen concentration (ml O_2 l^{-1} blood)
Cygb	Cytoglobin
DP	Globin distal pocket
E_{BO_2}	Extraction coefficient of oxygen from blood $[(Ca_{O_2} - Cv_{O_2})/ Ca_{O_2}]$
f_H	Heart frequency (beats min^{-1})
Hb	Hemoglobin
Hct	Blood hematocrit
Mb	Myoglobin

Ngb	Neuroglobin
ODC	Oxygen dissociation curve
P_{50}	Globin oxygen affinity defined as partial pressure of oxygen at globin half saturation (mmHg)
Pa_{O_2}	Arterial blood oxygen partial pressure (mmHg)
P_{O_2}	Oxygen partial pressure (mmHg)
Pv_{O_2}	Venous blood oxygen partial pressure (mmHg)
$\dot{Q}$	Blood flow rate (l min^{-1})
$\dot{Q}_B$	Brain blood flow (l min^{-1})
$\dot{Q}_H$	Heart blood flow (l min^{-1})
$\dot{Q}_M$	Skeletal muscle blood flow (l min^{-1})
$\dot{Q}_{O_2}$	Convective oxygen transport in the blood (ml O_2 min^{-1})
$\dot{Q}_{SRC}$	Splanchnic, renal, cutaneous and other peripheral tissue blood flow (l min^{-1})
ROS	Reactive oxygen species
Sa_{O_2}	Arterial blood oxygen saturation (%)
Sv_{O_2}	Venous blood oxygen saturation (%)
$S\bar{v}_{O_2}$	Mixed Venous blood oxygen saturation (%)
$\dot{V}b$	Cardiac output (l min^{-1})
$\dot{V}_{B_{O_2}}$	Brain oxygen consumption rate (ml O_2 min^{-1})
$\dot{V}_{H_{O_2}}$	Heart oxygen consumption rate (ml O_2 min^{-1})
$\dot{V}_{M_{O_2}}$	Skeletal muscle oxygen consumption rate (ml O_2 min^{-1})

$\dot{V}_{O_2}$	Rate of oxygen consumption (ml O_2 min^{-1})
V_S	Stroke volume (l)
$\dot{V}_{SRC_{O_2}}$	Splanchnic, renal, cutaneous and other peripheral tissue oxygen consumption rate (ml O_2 min^{-1})

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CHAPTER I
INTRODUCTION:
MYOGLOBIN FORM AND FUNCTION

Myoglobin (Mb) is a member of a family of oxygen binding globin proteins that share a common globin ancestor but have diversified throughout the vertebrate lineage. Evolution has shaped these proteins to optimize their function in distinct roles and their level of expression in their various tissues. Myoglobin is expressed in vertebrate cardiac and skeletal muscle, and its primary role is to facilitate intracellular diffusion and storage of oxygen. Mutations in Mb structure can affect its functional properties including oxygen affinity, structural stability, and solubility in solution. Although Mb structure is known to vary among species, it is not known how this structural variation affects Mb oxygen affinity. In the muscles of air-breathing diving vertebrates, Mb concentration can be ten-fold greater than in their terrestrial counterparts, making the divers excellent animal models to study the function and expression of Mb. While diving vertebrates benefit from increased Mb expression, questions remain about what might limit the maximum concentration of Mb and whether its functional properties (e.g. oxygen affinity) differ from those of terrestrial species.

Globin Structure and Molecular Evolution

For single-celled and very small multicellular organisms, simple diffusion is adequate for gas exchange. However, oxygen binding proteins and a circulatory system were prerequisites for the evolution of larger, multicellular animals (Hoffmann et al., 2012). Myoglobin (Mb) is a member of the family of oxygen binding globin proteins which also includes hemoglobin (Hb), neuroglobin (Ngb) and cytoglobin (Cygb). These proteins share a common globin ancestor, and their divergence originated from multiple duplication events of an ancestral globin gene early in vertebrate evolution (Storz et al., 2011). Because gene duplicates provide opportunities for genetic mutations without negative consequences, there is a greater rate of mutation within these copy-number

variants (CNV) than in other portions of the genome (Zhang et al., 2009). The duplication and specialization of the globins established separate physiological roles and regulation of expression in these distinct proteins (Terwilliger, 1998).

Despite the diversification of the globin genes, certain functional characteristics have been highly conserved. The globins share a common 'globin fold' structure that nests a heme prosthetic group capable of reversibly binding oxygen. While the amino acid sequence among these homologous genes can vary by as much as 75%, the overall tertiary structure and ligand binding regions are highly conserved (Bashford et al., 1987; Pesce et al., 2003). The functional properties (e.g., oxygen affinity) of these proteins can be altered by mutational changes in structure, and site mutation studies have demonstrated that considerable differences in Mb oxygen affinity can be produced by single amino acid substitutions (Carver et al., 1992; Scott et al., 2001; Dasmeh and Kepp, 2012). Structural variants that increase the stability of the oxygen-bound form result in an increase in globin oxygen affinity (Ajloo et al., 2002). Mb amino acid sequences from a variety of diving and terrestrial air breathing vertebrates show considerable variability in primary protein structure (UniProt Consortium, 2013; www.uniprot.org), and these structural variants produce functional phenotypes that are subject to natural selection (Naylor and Gerstein, 2000; Wittenberg and Wittenberg, 2003). However, little is known about changes in the functional properties of Mb resulting from structural variability among vertebrates. While Mb oxygen affinities are published for various species, they have been measured at a variety of temperatures using differing experimental techniques and are therefore difficult to compare.

Mb is one of the most studied and best known of all proteins. Over half a century ago, Mb became the first protein to have its structure characterized (Kendrew et al., 1958; Kendrew et al., 1960) for which John Kendrew shared the Nobel Prize in chemistry in 1962. Our early understanding of Mb was of a simple muscle heme protein with the function of temporarily storing oxygen, but subsequent research revealed previously unknown roles and unimagined complexities in protein dynamics (Frauenfelder, 2010).

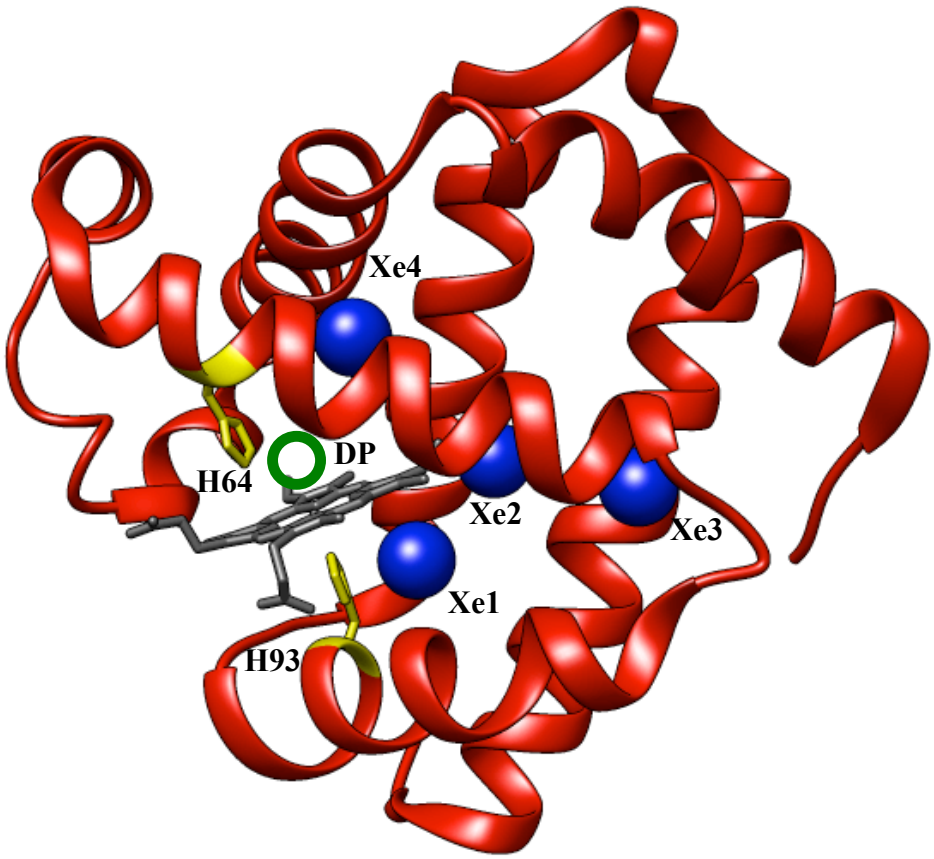

Figure 1.1 Image of sperm whale Mb (PDB accession # 1J52) with bound heme (gray) created using UCSF Chimera (Petterson et al., 2004). On either side of the heme in yellow are the proximal (H93) and distal (H64) histidines. The four Xe pockets are blue, and the distal pocket (DP) is denoted by the green circle.

The structure of vertebrate Mb typifies the globin family. It is a small protein (typically 153 amino acids) arranged in 8 α helices surrounding a heme porphyrin ring with a pentacoordinate central iron (Figure 1.1). Conservation of Mb structure includes a preservation of hydrophilic amino acids on the outward facing regions which enhance solubility and mobility within the cytoplasm, as well as internal hydrophobic amino acids that stabilize the tertiary structure (Bashford et al., 1987). The conserved, internal hydrophobic amino acids form a series of cavities including the heme pocket, the distal pocket, and four additional xenon (Xe) binding pockets (Xe1-Xe4) (Harada et al., 2007). The internally bound heme is stabilized by hydrophobic interactions with the nonpolar

amino acids lining the heme pocket. The distal pocket is an outcropping of the heme pocket adjacent to the heme central iron atom which provides a space for ligand-heme interactions (Harada et al., 2007). Although their contribution to ligand binding kinetics is not well understood, the Xe pockets are thought to transiently harbor ligands such as O_2, CO, and nitric oxide (NO) as well (Tomita et al., 2010).

Myoglobin Function

Mb facilitates the transport of oxygen in muscle, although the functional significance of this is debated and the mechanism is poorly understood (Conley et al., 2000; Jürgens et al., 2000). The compensatory mechanisms exhibited in Mb knockout mice (Gödecke et al., 1999) along with earlier studies (Wittenberg, 1970; Wittenberg, 2007) indicate some role for Mb in maintaining oxygen flux through facilitated diffusion. The contribution of Mb to net oxygen flux is increased under conditions of low P_{O_2} or high Mb concentration (Lin et al., 2007b).

In addition to facilitating oxygen diffusion, Mb also stores intracellular oxygen (Dasmeh and Kepp, 2012) and buffers oxygen availability to mitochondria at the onset of muscle activity. In human subjects performing isolated leg extensions at moderate intensity, Mb oxygen saturation decreases from 100% to ~60% within 20 sec where it is maintained despite increased work load to maximum effort (Richardson et al., 1995). A more deoxygenated steady state level of ~50% saturation was achieved under hypoxic conditions while the subjects breathed 12% oxygen. A steady-state level of saturation up to maximum aerobic capacity supports the hypothesis that partially deoxygenated Mb produces a gradient to facilitate oxygen transport. Although the oxygen storage role of Mb is limited to short-duration buffering of mitochondrial oxygen available at the low concentrations found in terrestrial vertebrates, oxy-Mb can be a significant oxygen reserve in animals with higher Mb concentrations of Mb (Kooyman and Ponganis, 1998; Dasmeh and Kepp, 2012).

In addition to binding oxygen, deoxy-Mb has nitrite reductase activity, making it a significant generator of NO from endogenous nitrite during periods of hypoxia

(Hedgen-Cotta et al., 2014). Nitric oxide not only acts as a potent vasodilator in response to regional muscle hypoxia, but also may play a role in mediating muscle metabolism by inhibiting mitochondrial cytochrome oxidase activity (Wittenberg and Wittenberg, 2003; Shiva et al., 2007). When endogenous oxygen stores and convective oxygen transport to muscles are inadequate, hypoxic conditions can lead to an increase in the production of reactive oxygen species (ROS), resulting in the oxidation of intracellular proteins and lipids and causing cellular damage. Myoglobin can mitigate ROS by two mechanisms: by reducing ROS production in mitochondria and by acting as a peroxidase to scavenge ROS.

Although much is known about Mb, surprisingly little is understood about its functional performance among different species. Studies involving Mb knockout mice showed that these animals were able to live, exercise, and reproduce normally without Mb, thereby raising questions about its physiological significance (Garry et al., 1998). However, the ability of these animals to function normally was due to compensatory mechanisms that increased convective oxygen transport and reduced the diffusive distance for oxygen in muscle (Gödecke et al., 1999; Garry, 2007). While Mb knockout mice were able to perform normally under normoxic conditions, chronic hypoxia resulted in a decrease in left ventricular function compared to wild type mice (Mammen et al., 2003). The various functional roles of Mb including ROS scavenging, NO scavenging, oxygen transport, and oxygen storage appear to be more significant under hypoxic conditions.

Myoglobin in Air-breathing, Diving Vertebrates

Diving vertebrates spend considerable time submerged, and exhibit many adaptations at the behavioral, organ, cellular and biochemical level that maximize aerobic dive duration (Davis, 2014; Yim et al., 2014). Many marine mammals use efficient stroke and glide swimming to exploit changes in buoyancy during descent that minimize energy expenditure (Williams et al., 2000; Davis et al., 2001) and they adjust their dive to remain within their aerobic dive limit (ADL) which minimizes recovery

time at the surface and maximizes the time spent submerged (Davis 2014). Among the physiological adaptations for diving are elevated levels of oxygen binding globin proteins. Blood bound oxygen is enhanced by the combined effects of increased blood volume and Hb concentration. Increased Mb concentration enhances muscle oxygen and represents approximately 20% to over 50% of total body oxygen stores in many cetaceans, seals, and penguins (Ponganis, 2011; Kooyman and Ponganis, 1998).

The level of expression of Mb in muscle is regulated by both genetic and environmental cues (Ordway and Garry, 2004). Since maintaining oxygen delivery to mitochondria in aerobic muscle appears to be the primary function of Mb, it is not surprising its expression is up-regulated in response to hypoxia. Hypoxia alone is sufficient to increase Mb expression in cultured Weddell seal muscle (DeMiranda et al., 2012), but an additional calcium stimulus is needed in terrestrial animals (Kanatous et al., 2009). The unusually high expression of Mb in air-breathing diving vertebrates appears to be regulated by the combined physiological effects of exercise with muscle hypoxia (Kanatous and Mammen, 2010).

Elevated Mb concentrations in diving mammals and birds is positively correlated with breath-hold ability. Cetaceans (whales and dolphns) that make longer dives have a higher Mb concentration than their shorter duration counterparts (Dolar et al., 1999; Kielhorn et al., 2013; Helbo and Fago, 2012). Myoglobin concentration also increases with the ontogenetic development of diving ability in seals (Kanatous et al., 2008; Geiseler et al., 2013) and penguins (Ponganis et al., 1999; Ponganis et al., 2010). Myoglobin is not homogeneously distributed in the locomotory muscles and is highest in areas that produce greater force and consume more oxygen during aerobic swimming in penguins (Ponganis et al., 1997b), cetaceans (Dolar et al., 1999; Polasek and Davis, 2001), and seals (Polasek et al., 2006).

In addition to elevated Mb concentration, diving birds and mammals have a variety of other muscle adaptations for improving diving ability. Oxidative fiber types dominate in the muscles of long-duration diving species to a greater extent than their terrestrial or short-duration counterparts (Kanatous et al., 2002; Kielhorn et al., 2013,

Watson et al., 2003) which further suggests that these animals routinely dive within their aerobic dive limit. Reduced mitochondrial volume density and greater muscle fiber diameter are found in cetaceans (Kielhorn et al., 2013) and seals (Kanatous et al., 2002) that make very long dives which could be adaptive for minimizing muscle energy expenditure (Kielhorn et al., 2013).

During voluntary aerobic dives, marine mammals exhibit a dive response that includes apnea, bradycardia, and peripheral vasoconstriction. The decrease in cardiac output due to bradycardia results in peripheral vasoconstriction to maintain central arterial blood pressure, but it is exercise modulated to maximize the aerobic dive limit at different levels of exertion (Davis and Williams, 2012). The dive response regulates skeletal muscle hypoxia so that blood and muscle oxygen stores are used efficiently at different levels of exercise to maximize aerobic dive duration (Davis, 2014). This is achieved by adjusting cardiac output and convective oxygen transport to muscle according to the rate of muscle oxygen consumption so that the muscle becomes hypoxic (but not anoxic) which allows Mb-bound oxygen to be released so that it can diffuse into the mitochondria. With increasing levels of exercise, convective oxygen transport to active muscle must be increased to maintain aerobic muscle metabolism and simultaneously deplete blood and muscle oxygen stores.

Conclusions

Although Mb has been extensively studied, much of this research has focused on protein chemistry and not on its biological role. Tomita et al. (2010) emphasized the importance of considering recent Mb research from the perspective of comparative physiology. In this dissertation, I will use the unique physiology of diving vertebrates as a model to study the variance in expression, form, and function of Mb to gain insight into adaptive molecular evolution and the physiological role of Mb.

Diving vertebrates have dramatically higher Mb concentrations than their terrestrial counterparts, but it is unclear what limits the maximum concentration and if molecular adaptations influence its role as an intramuscular oxygen store. The evolution

of prolonged breath-hold diving has influenced the expression, structure and function of globin proteins in diving vertebrates (Mircetta et al., 2013; Helbo and Fago, 2012; Dasmeh et al., 2013). The following chapters will explore the role of Mb in diving marine vertebrates including how levels of Mb expression optimize dive duration and the functional properties of Mb oxygen affinity that may be adaptive.

CHAPTER II
THE EFFECT OF MYOGLOBIN CONCENTRATION ON AEROBIC DIVE LIMIT IN A WEDDELL SEAL*

One physiological adaptation for prolonged dive duration in marine mammals is an elevated myoglobin (Mb) concentration in skeletal muscle. To determine the influence of Mb concentration on the aerobic dive limit (ADL), we modified a previously published model that simulated aerobic dives in a Weddell seal (*Leptonychotes weddellii*) and ran it for four Mb concentrations: 5, 27, 54 and 108 g Mb kg^{-1} muscle representing 7%, 50%, 100% and 200%, respectively, of the normal Mb concentration in Weddell seal skeletal muscle. The model was run at increasing levels of muscular exertion and under postabsorptive and postprandial conditions to determine their effect on ADL. For each set of conditions, the model was also run at different levels of cardiac output (i.e., the dive response was varied) to determine the level of convective oxygen transport that optimized the ADL. In a postabsorptive state at a routine level of muscular exertion for a diving Weddell seal, a decrease in Mb concentration to 7% of normal caused a 39% decrease in the ADL (18 min to 11 min), while doubling the Mb concentration increased the ADL by 30% (18 min to 24 min). Under postprandial conditions at a routine level of muscular exertion, doubling the Mb concentration did not increase the ADL (12 min). The convective oxygen transport needed to meet the metabolic demands (Heat Increment of Feeding, HIF) of the splanchnic organs during digestion and assimilation required a cardiac output that was not optimal for the efficient use of muscle oxygen stores. This resulted in an over perfusion of the muscles and incomplete use of myoglobin-bound oxygen. As a result, the postprandial ADL was limited by the amount of oxygen stored in the blood, and increasing the Mb concentration had no effect on the ADL. We hypothesize that myoglobin concentration is optimized for the type and duration of dives routinely made

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Wright, T. J. and Davis, R. W. (2006). The effect of myoglobin concentration on aerobic dive limit in a Weddell seal. J. Exp. Biol. 209 (13), 2576-2585.

by Weddell seals, and that a further increase may not increase the ADL for most free-ranging dives.

Introduction

Weddell seals and other marine mammals exhibit physiological adaptations and behavioral strategies that increase dive duration. These include an elevation in total body oxygen stores through increases in blood volume, hematocrit (Hct, the percentage of blood volume occupied by red blood cells) and muscle myoglobin (Mb) concentration. In addition, marine mammals use efficient modes of locomotion (e.g., gliding during descent, stroke-and-glide swimming) that keep oxygen consumption low during diving (Williams et al., 2000; Williams, 2001). For Weddell seals, these physiological adaptations and behavioral strategies result in an aerobic dive limit (ADL) of about 20 min (Davis and Kanatous, 1999; Ponganis et al., 1993a; Kooyman et al., 1980).

If body oxygen stores are the primary physiological limit to dive duration, why are they not larger? During the evolution of marine mammals, what physiological factors may have set the upper limit to blood volume, Hct and muscle Mb concentration? Weddell seals have a blood volume as high as 21% of their body mass (Ponganis et al., 1993a), almost three times larger than predicted for a terrestrial mammal of the same size (Stahl, 1967). The upper limit to blood volume may be a compromise between increasing oxygen stores and the resultant increase in body mass or abdominal volume.

The Hct of Weddell seals (ca. 60%), which is 1.5-times higher than in most terrestrial mammals, increases blood oxygen stores and maintains convective oxygen transport to organs and tissues as the partial pressure of oxygen in the blood decreases during diving. However, the increased Hct also increases blood viscosity, circulatory resistance and heart work (Elsner and Meiselman, 1995). As a result, the large spleen of Weddell seals sequesters red blood cells, lowers the hematocrit, and decreases blood viscosity when they are at the surface. Only when they begin diving does the spleen contract and release additional red blood cells into the circulation, which increases the

hematocrit while heart rate is reduced due to the dive response (Hurford et al., 1996). A hematocrit greater than 60% would further increase blood viscosity, increase heart work, and could decrease rather than increase convective oxygen transport.(Hedrick and Duffield, 1986). Consequently, the elevated Hct of Weddell seals and other marine mammals may be at its physiological maximum for optimizing blood oxygen storage and convective oxygen transport.

The concentration of Mb in the skeletal muscles of Weddell seals is about 10-times greater than in most terrestrial mammals (Snyder, 1983). Oxygen bound to Mb represents one-third of the total oxygen store in Weddell seals, so it is a major factor in setting the ADL (Davis and Kanatous, 1999; Kooyman and Ponganis, 1998). However, it is not clear what physical or physiological factors may have set the maximum concentration of muscle Mb. The objective of this study was to model the effects of different muscle Mb concentrations on the ADL of Weddell seals. Specifically, we wanted to know how increasing or decreasing Mb concentration beyond normal levels would affect the ADL. Although lowering the Mb concentration would obviously decrease the ADL, would increasing the concentration automatically increase it? To answer this question, we used a previously published model of convective oxygen transport and tissue oxygen consumption (Davis and Kanatous, 1999). We ran the model at different myoglobin concentrations for various levels of muscular exertion under postabsorptive and postprandial conditions to determine their effect on ADL.

Materials and Methods

Theoretical Basis for the Model: Fick's Principal

A numerical integration technique was used to model the relationship between regional convective oxygen transport ($\dot{Q}_{O_2}$) and the rate of oxygen consumption ($\dot{V}_{O_2}$) in a hypothetical Weddell seal during aerobic dives at different levels of muscle oxygen consumption ($\dot{V}_{M_{O_2}}$) (see Nomenclature for a list of symbols). A detailed description of the model and an explanation of the assumptions and equations under postabsorptive

conditions has been published (Davis and Kanatous 1999). This study differed from that of Davis and Kanatous (1999) in that we ran the model with four different muscle Mb concentrations during aerobic dives under both postabsorptive and postprandial conditions. The numerical process iteratively determined arterial blood oxygen concentration (Ca_{O_2}) and venous blood oxygen concentration (Cv_{O_2}) for various tissues and organs based on the circulatory diagram shown in Figure 2.1 and equation 1 (Fick's principle):

$$\dot{V}_{O_2} = \dot{Q}(Ca_{O_2} - Cv_{O_2}) \quad (1)$$

where $\dot{Q}$ is blood flow rate. Cerebral, coronary, and skeletal muscle regional circulations were incorporated into the model individually, while splanchnic, renal and cutaneous circulations were grouped together with all other organs and tissues (e.g., bone and fat). The average temporal resolution (i.e., the period between consecutive computations) was 0.22 min.

This model considers only dives that are within the seal's ADL (Kooyman et al*.,* 1980; Ponganis et al*.,* 1993a). The term ADL was used in this model to describe the maximum duration of an aerobic dive under specific conditions. The basal contribution of anaerobic metabolism in harbor seals has been shown to constitute approximately 2% of ATP production in a resting state and 1% during active swimming (Davis et al., 1991). For this model, this small basal contribution of anaerobic metabolism is ignored, and tissues are considered aerobic as long as there is no increased reliance on anaerobic metabolism resulting in an increase in blood lactate over resting levels. While terms such as diving lactate threshold (DLT) and calculated aerobic dive limit (cADL) are useful for certain applications (Butler and Jones, 1997), they were not applicable to all conditions used to terminate a dive in this model. DLT was not used because increased blood lactate resulting from anaerobic metabolism was not necessary to terminate a dive

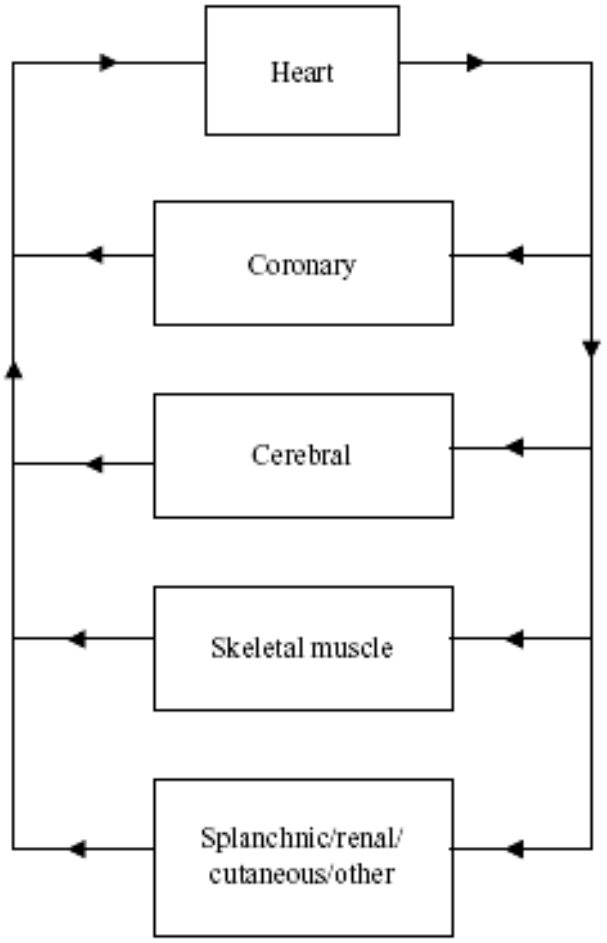

Figure 2.1 Simplified circulatory system used in the model. The cardiovascular system was divided into four regional circulations: coronary, cerebral, skeletal muscle and a combined category that included the splanchnic, renal, cutaneous and other circulatory beds.

in this model. The term cADL is historically used to denote a calculation of aerobic dive limit based on total useable oxygen stores divided by whole body metabolism. While this model does calculate an ADL, it does so through modeling of blood flow and metabolism in individual tissues which can produce vastly different results than whole body calculations in some metabolic states. The rate of oxygen consumption in the tissues is maintained until convective oxygen delivery falls below a critical level and endogenous oxygen stores (skeletal muscle only) are depleted resulting from a

combination of ischemic and hypoxic hypoxia. When any organ (e.g., splanchnic organs) or tissue (e.g., skeletal muscle) no longer has sufficient oxygen to support aerobic metabolism (i.e., the point at which anaerobic energy metabolism commences), then the ADL has been reached and the dive is terminated.

Assumptions and Equations

Organ and tissue masses were based on published values for a 450 kg adult Weddell seal (Fujise et al., 1985; Zapol et al., 1979) as described in Davis and Kanatous in Table 1 (1999). The resting $\dot{V}_{O_2}$ for Weddell seal organs and tissues were estimated from the metabolic mass adjusted $\dot{V}_{O_2}$ for the equivalent organs of a human or rat (Diem and Lentner, 1970; Field et al., 1939; Kety, 1957). The basal, whole body $\dot{V}_{O_2}$ (897 ml O_2 min^{-1} or 2.0 ml O_2 min^{-1} kg^{-1}) was calculated by combining individual organ and tissue metabolic rates. The calculated basal metabolic rate was similar to the minimum metabolic rates measured for adult Weddell seals during rest or sleep (Castellini et al., 1992b; Ponganis et al., 1993a; Williams et al., 2004).

Resting heart rate (f_H) (51.5 beats min^{-1}), cardiac output ($\dot{V}b$) (42.7 l min^{-1}) and stroke volume (V_S) (0.83 l) were based on measured values for Weddell seals (Zapol et al., 1979). During a simulated dive, $\dot{V}b$ was varied from 19-131% of resting levels (see Davis and Kanatous, 1999 Table 2). For brevity, we hereafter refer to these percentages of resting, predive $\dot{V}b$ as percent $\dot{V}b$ (e.g., 19% $\dot{V}b$). When $\dot{V}b$ was below resting levels, most of the reduction resulted from a decrease in f_H (i.e., bradycardia). However, based on studies of seals during forced submergence and voluntary dives (Blix and Folkow, 1983; Kjekshus et al., 1982; Ponganis et al., 1990; Sinnet et al., 1978; Zapol et al., 1979), V_S was also reduced as f_H declined. The maximum reduction in V_S in the model was 25% of the resting value and was proportionate to the reduction in f_H. The reduction in cardiac output (i.e., the severity of the dive response) was immediate and remained

constant throughout a dive. An "anticipatory" increase in $\dot{V}b$ toward the end of a dive was not included in the model. Except for the brain, where circulation was always maintained at resting levels, we assumed that blood flow to the rest of the body decreased proportionately with $\dot{V}b$ during a dive due to reduced $\dot{V}b$ and peripheral vasoconstriction (Blix et al., 1976; Elsner et al., 1964). Peripheral vasoconstriction was assumed to occur in the large arteries (e.g., the renal artery) making it independent of tissue level metabolic dilators that affect arterioles (White et al., 1973). Because vasoconstriction was assumed to occur high in the vascular tree, blood flow was not adjusted independently to individual tissue beds.

Body oxygen stores were confined to the blood and skeletal muscle in this model, since no oxygen storage capability exists in the splanchnic organs (Dodd et al., 1987) and the heart represents less than 2% of the total muscle mass. We assumed that lung oxygen was not available during a dive due to the complete functional pulmonary shunt that occurs in Weddell seals at pressures greater than 3-5 atmospheres (2280-3800 mm Hg; 1 mm Hg = 0,133 kPa; approximately 30-50 m deep) (Falke et al., 1985; Reed et al., 1994b). Even if lung oxygen were available during a dive, it represents only 5% of the total body oxygen store in Weddell seals (Kooyman and Ponganis, 1998).

To calculate total oxygen stores in the blood, we assumed that the blood volume for a 450 kg Weddell seal was 96 liters (Ponganis et al., 1993a) and that 33% of this volume was arterial blood and 67% was venous blood (i.e., venules, small and large veins, hepatic sinus and spleen) (Hurford et al., 1996; Rowell, 1986). The blood hemoglobin (Hb) concentration (assuming complete splenic contraction) was 260 g l^{-1}, and the oxygen binding capacity of Hb was 1.34 ml O_2 g^{-1} Hb (Kooyman et al., 1980; Ponganis et al., 1993a; Qvist et al., 1986). This gave a capacitance coefficient of oxygen in blood (β_{BO_2}) of 348 ml O_2 l^{-1} (260 g Hb l^{-1} blood x 1.34 ml O_2 g^{-1} Hb). At the beginning of a dive, we assumed that the arterial blood was 100% saturated with oxygen as a result of predive hyperventilation (Kooyman et al., 1980; Qvist et al., 1986; Ponganis et al., 1993a). Mixed venous blood was calculated from equation 2 to be 86% saturated at the

beginning of a dive assuming an oxygen content that was 5% by volume less (Ponganis et al., 1993a) than an initial Ca_{O_2} of 348 ml O_2 l^{-1} blood.

$$S\bar{v}_{O_2} = [(348 - 50)/ 348] \times 100 = 86\% \quad (2)$$

where $S\bar{v}_{O_2}$ is the oxygen saturation of mixed venous blood. Arterial and venous blood oxygen stores were calculated as:

$$\text{Arterial blood oxygen (ml)} = 96 \times 0.33 \times 348 = 11{,}025 \quad (3)$$

$$\text{Venous blood oxygen (ml)} = 96 \times 0.67 \times 348 \times 0.86 = 19{,}250 \quad (4)$$

We assumed that 35% of the seal's body mass was skeletal muscle. For this study, we ran the model with four Mb concentrations: 5, 27, 54 and 108 g Mb kg^{-1} muscle representing 7%, 50%, 100% and 200%, respectively, of the normal Mb concentration in Weddell seal skeletal muscle (Ponganis et al., 1993a). The Mb concentration of 5 g kg^{-1} muscle is typical of terrestrial mammals such as a dog, human, or rat (Snyder, 1983). We assumed an oxygen binding capacity of 1.34 ml O_2 g^{-1} Mb, and complete saturation at the beginning of a dive (Gayeski et al., 1987; Schenkman et al., 1997).
Muscle oxygen stores were calculated as:

$$\text{Skeletal muscle oxygen (ml)} = 450 \times 0.35 \times 1.34 \times [\text{Mb}] \quad (5)$$

The total oxygen store (sum of arterial, venous and muscle oxygen) was therefore 31,330 ml O_2 (69.6 ml O_2/kg), 35,973 ml O_2 (79.9 ml O_2/kg), 41,672 ml O_2 (92.6 ml O_2/kg) or 53,068 ml O_2 (117.9 ml O_2/kg) based on the four Mb concentrations, respectively. However, not all of this oxygen is available for metabolism during a dive (Davis and Kanatous, 1999).

As blood circulates through the four vascular beds (Figure 2.1), the organs and tissues extract oxygen from the blood to meet their respective $\dot{V}_{O_2}$ requirements. Cv_{O_2} was calculated for each circulatory bed according to Fick's Principle:

$$CBv_{O_2} = Ca_{O_2} - (\dot{V}_{B_{O_2}} / \dot{Q}_B) \quad (6)$$

$$CHv_{O_2} = Ca_{O_2} - (\dot{V}_{H_{O_2}} / \dot{Q}_H) \quad (7)$$

$$CMv_{O_2} = Ca_{O_2} - (\dot{V}_{M_{O_2}} / \dot{Q}_M) \quad (8)$$

$$CSRCv_{O_2} = Ca_{O_2} - (\dot{V}_{SRC_{O_2}} / \dot{Q}_{SRC}) \quad (9)$$

where $\dot{Q}$ is blood flow rate, $\dot{V}$ is the rate of oxygen consumption, the letters B, H, M indicate brain, heart, and skeletal muscle respectively, and SRC indicates splanchnic, renal, and cutaneous organs and other peripheral tissues. However, the extraction coefficient of oxygen from the blood (E_{BO_2}), where $E_{BO_2} = (Ca_{O_2} - Cv_{O_2}) / Ca_{O_2}$, could never exceed 0.8 (i.e., maximum E_{BO_2} at critical oxygen delivery) during a single pass of the blood through an organ or tissue (Samsel and Schumacker, 1994; Torrance and Wittnich, 1994; Nelson et al., 1988). The mixed venous blood oxygen concentration ($C\bar{v}_{O_2}$) was calculated for the four vascular beds as the difference between the Ca_{O_2} and the total oxygen extracted per ml of blood:

$$C\bar{v}_{O_2} = Ca_{O_2} - [(\dot{V}_{B_{O_2}} + \dot{V}_{H_{O_2}} + \dot{V}_{M_{O_2}} + \dot{V}_{SRC_{O_2}}) / (\dot{Q}_B + \dot{Q}_H + \dot{Q}_M + \dot{Q}_{SRC})] \quad (10)$$

The arterial blood oxygen saturation (Sa_{O_2}) and venous blood oxygen saturation (Sv_{O_2}) were calculated for the blood of each vascular bed as the quotient of their respective oxygen concentrations (Equations 6 to 9) and a β_{BO_2} of 348 ml O_2 l^{-1} blood. The arterial (Pa_{O_2}) and venous (Pv_{O_2}) blood oxygen partial pressures were calculated from their respective Sa_{O_2} and Sv_{O_2} using two polynomial equations fitted to the oxy-hemoglobin dissociation curve (P_{50} = 26.9 mm Hg = 0.133 kPa) for adult Weddell seals (Qvist et al., 1981).

Evidence obtained during the forced submergence of harbor seals and Weddell seals indicates that $\dot{Q}_B$ is generally maintained and $\dot{V}_{B_{O_2}}$ does not decline (Blix and Folkow, 1983; Kerem and Elsner, 1973; Zapol et al., 1979). In this model, we assumed that $\dot{Q}_B$

and $\dot{V}_{B_{O_2}}$ remained at resting levels during a dive and were independent of $\dot{V}b$. We also assumed that the minimum Pa_{O_2} and $P\bar{v}_{O_2}$ for normal cerebral metabolism and function were 22 mm Hg (Sa_{O_2} = 38%) and 18 mm Hg ($S\bar{v}_{O_2}$ = 27%), respectively. This is comparable to the average Pa_{O_2} (24.5 $\pm$ 2.86 mm Hg; mean $\pm$ S.D., N =7) in Weddell seals two minutes before surfacing and to the end tidal P_{O_2} (24 mm Hg) of the first exhalation (assuming that this approximates arterial P_{O_2}) after 17 min aerobic dives (Ponganis et al., 1993a; Qvist et al., 1986). As a result, the model terminated a dive if Pa_{O_2} decreased below 22 mm Hg in the model. However, the Pa_{O_2} of blood perfusing the brain was generally not a consideration in determining ADL.

We assumed that $\dot{Q}_H$ and $\dot{V}_{H_{O_2}}$ changed proportionately with $\dot{V}b$ (Blix and Folkow, 1983; Blix et al., 1976; Kjekshus et al., 1982). When convective oxygen transport to the myocardium changed during a dive, it was proportional to the change in heart work, and the myocardium always received sufficient blood oxygen to maintain aerobic metabolism.

$\dot{Q}_M$ was also assumed to change proportionately with $\dot{V}b$. Oxygen transported to the muscles in the blood was always used (up to a maximum E_{BO_2} of 0.8) before oxygen bound to Mb because of the lower affinity of Hb for oxygen (Schenkman et al., 1997). Oxygen not provided by the blood was obtained from oxymyoglobin stores to meet $\dot{V}_{M_{O_2}}$ requirements. $\dot{V}_{M_{O_2}}$ was assumed to be independent of $\dot{Q}_M$ as long as the combination of convective oxygen transport and oxymyoglobin stores was sufficient to meet metabolic demand. If at any time the combination of these two were no longer sufficient to maintain aerobic muscle metabolism, the dive was terminated.

Postabsorptive $\dot{V}_{O_2}$ (3.73 $\pm$ 0.88 ml O_2 min^{-1} kg^{-1}) and postprandial $\dot{V}_{O_2}$ (5.24 $\pm$ 0.88 ml O_2 min^{-1} kg^{-1}) during aerobic dives were based on indirect calorimetry measurements made by Williams et al. (2004) for foraging and non-foraging Weddell seals. We assumed that the average difference in $\dot{V}_{O_2}$ (1.51 ml O_2 min^{-1} kg^{-1} or 680 ml O_2 min^{-1} for a 450 Kg seal) between postabsorptive and postprandial dives of 7 to 23 min in duration resulted from the metabolic cost of prey warming, digestion, absorption and assimilation, which we refer to as the Heat Increment of Feeding (HIF). This increase in $\dot{V}_{O_2}$ was added to the postabsorptive $\dot{V}_{SRC_{O_2}}$ to give a postprandial $\dot{V}_{SRC_{O_2}}$ of 1,234 ml O_2 min^{-1} (a 2.2-fold increase). We assumed that the $\dot{V}_{SRC_{O_2}}$ was maintained as long as: 1) convective oxygen transport was sufficient to support oxygen demand, 2) E_{BO_2} did not exceed 0.8 and 3) Pa_{O_2} was greater than 22 mm Hg (Kvietys and Granger, 1982; Schlichtig et al., 1992).

Computations

The model was run on a standard spreadsheet program (Quattro Pro for Windows Version 6.0, Novell Applications Group, Orem, Utah) for eight levels of $\dot{V}b$, sixteen levels of $\dot{V}_{M_{O_2}}$ up to a maximum, whole body $\dot{V}_{O_2}$ of 10.7 ml O_2 min^{-1} kg^{-1} and four different Mb concentrations under postabsorptive conditions, which produced 512 combinations. These were then compared to postprandial conditions for the normal and elevated Mb concentration adding an additional 256 combinations. The general procedure was to select a Mb concentration, set the $\dot{V}b$ at a particular level (e.g., 37% of the resting level) and then vary the $\dot{V}_{M_{O_2}}$ from 1 to 16 times the resting level. This process was then repeated for each Mb concentration and $\dot{V}b$. $\dot{V}_{O_2}$ for the four vascular

beds and the entire body were calculated for each combination. The ADL was reached and the dive terminated when: 1) any non-muscle organ or tissue did not receive sufficient oxygen through convective oxygen transport to maintain aerobic metabolism 2) convective oxygen transport and oxymyoglobin stores were no longer sufficient to maintain aerobic muscle metabolism, or 3) when the Pa_{O_2} fell below 22 mm Hg.

Results

The Role of $\dot{V}$b in Optimizing the ADL at Different Levels of Muscle Metabolism

The role of $\dot{V}$b in optimizing the ADL at different levels of muscle metabolism has been described (Davis and Kanatous, 1999). Briefly, the ADL decreases in a non-linear fashion with increasing $\dot{V}_{M_{O_2}}$ for different levels of $\dot{V}$b (range = 19-131% of resting levels) (Davis and Kanatous 1999; Davis et al., 2004) (Figures 2.2 and 2.3). For each level of $\dot{V}_{M_{O_2}}$, there is an optimal $\dot{V}$b that gives a maximum ADL, and this optimal $\dot{V}$b increases (i.e., the dive response is less pronounced) as $\dot{V}_{M_{O_2}}$ increases (see Fig. 4 and Table 4 in Davis and Kanatous, 1999). Since the ADL is inversely proportional to $\dot{V}_{M_{O_2}}$ (assuming a constant level of blood and muscle oxygen depletion), the optimal $\dot{V}$b decreases as the ADL increases (see Fig. 5 in Davis and Kanatous 1999).

The Effect of Myoglobin Concentration on the Postabsorptive ADL

In the postabsorptive state, the resting ADL (28 min) was independent of Mb concentration (Figure 2.2). At a resting level of $\dot{V}_{M_{O_2}}$, the lowest level of convective oxygen transport ($\dot{V}$b = 19%) was still sufficient to supply 97% of the oxygen needed by

the skeletal muscle. As a result, very little Mb oxygen (ranging from 21% to 1% of endogenous oxymyoglobin for concentrations from 5 to 108 mg g^{-1}, respectively) was used while resting submerged, and it was not a factor that limited the ADL (Tables 2.1-2.4).

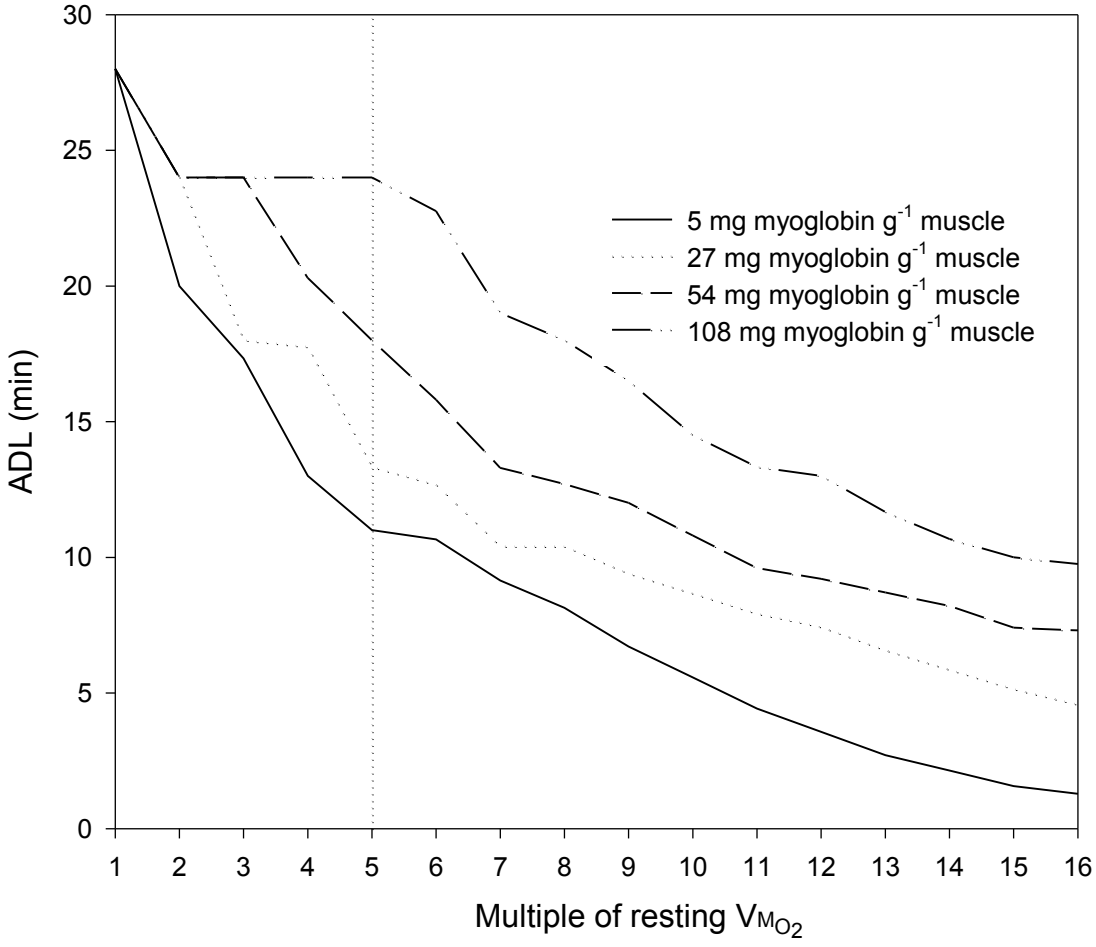

Figure 2.2 Calculated postabsorptive aerobic dive limit (ADL) for four myoglobin concentrations as a function of skeletal muscle oxygen consumption ($\dot{V}_{M_{O_2}}$). Vertical dotted line marks the estimated routine level of diving $\dot{V}_{M_{O_2}}$ for a Weddell seal.

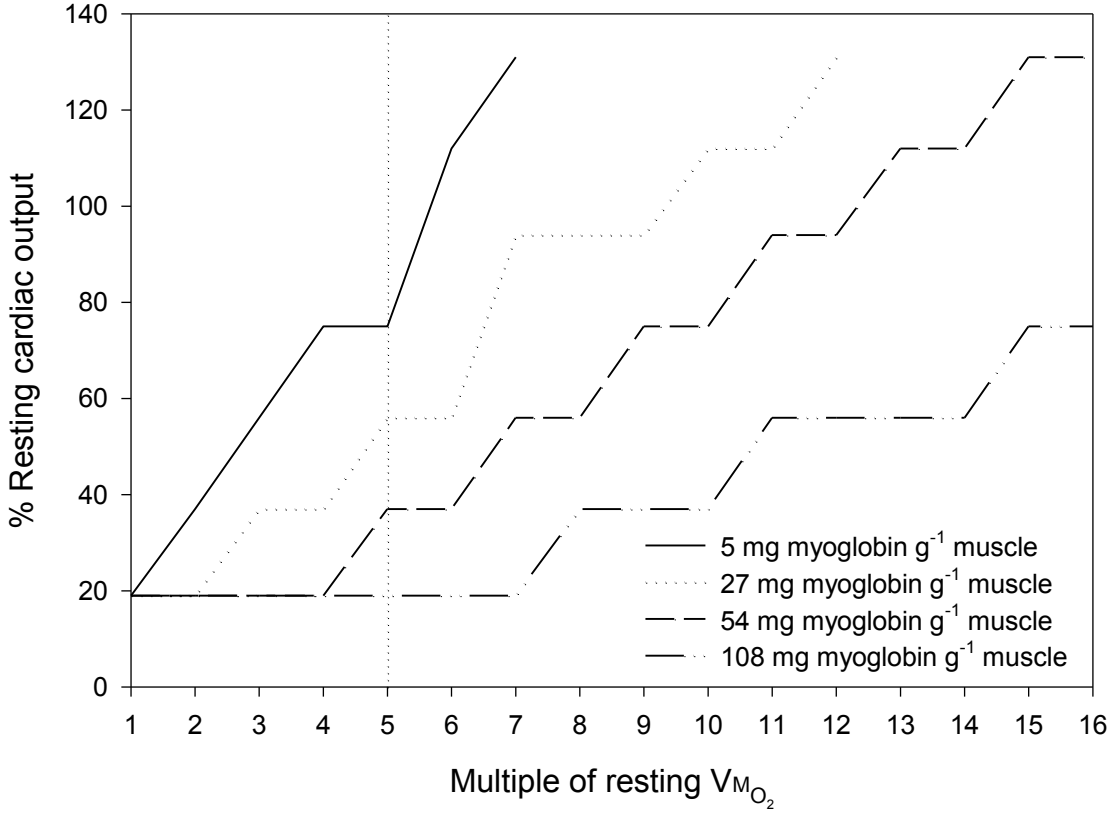

Figure 2.3 Optimal cardiac output as a function of skeletal muscle metabolism ($\dot{V}_{M_{O_2}}$) for four myoglobin concentrations. Vertical dotted line marks the estimated routine level of diving $\dot{V}_{M_{O_2}}$ for a Weddell seal.

Table 2.1 Aerobic dive limit, whole body oxygen consumption, and muscle oxygen consumption for varying multiples of muscular metabolic rate at a reduced [Mb] of 5 mg/g in a postabsorptive state.

$\dot{V}_{M_{O_2}}$	Whole body $\dot{V}_{O_2}$ (ml O_2/min*kg)	ADL (min)	Myoglobin O_2 consumed by muscle during dive (ml O_2)	% Myoglobin O_2 consumed by muscle during dive
1	1.8	28.0	221	21%
2	2.3	20.0	353	33%
3	2.8	17.3	644	61%
4	3.4	13.0	407	39%
5	3.9	11.0	1026	97%
6	4.4	10.7	687	65%
7	4.9	9.1	575	54%
8	5.4	8.1	1008	95%
9	5.9	6.7	1030	98%
10	6.4	5.6	1013	96%
11	6.9	4.4	960	91%
12	7.3	3.6	1001	95%
13	7.8	2.7	974	92%
14	8.3	2.1	984	93%
15	8.8	1.6	964	91%
16	9.3	1.3	1020	97%

Table 2.2 Aerobic dive limit, whole body oxygen consumption, and muscle oxygen consumption for varying multiples of muscular metabolic rate at a reduced [Mb] of 27 mg/g in a postabsorptive state.

$\dot{V}_{M_{O_2}}$	Whole body $\dot{V}_{O_2}$ (ml O_2/min*kg)	ADL (min)	Myoglobin O_2 consumed by muscle during dive (ml O_2)	% Myoglobin O_2 consumed by muscle during dive
1	1.8	28.0	221	4%
2	2.3	24.0	3390	59%
3	2.8	18.0	2232	39%
4	3.3	17.8	5607	98%
5	3.8	13.3	3372	59%
6	4.3	12.7	5609	98%
7	4.9	10.4	2775	49%
8	5.4	10.4	4609	81%
9	5.8	9.4	5577	98%
10	6.3	8.7	4903	86%
11	6.8	7.9	5671	100%
12	7.3	7.4	5300	93%
13	7.8	6.6	5488	96%
14	8.3	5.9	5609	98%
15	8.8	5.1	5617	99%
16	9.3	4.6	5662	99%

Table 2.3 Aerobic dive limit, whole body oxygen consumption, and muscle oxygen consumption for varying multiples of muscular metabolic rate at a normal [Mb] of 54 mg/g in a postabsorptive state.

$\dot{V}_{M_{O_2}}$	Whole body $\dot{V}_{O_2}$ (ml O_2/min*kg)	ADL (min)	Myoglobin O_2 consumed by muscle during dive (ml O_2)	% Myoglobin O_2 consumed by muscle during dive
1	1.8	28.0	221	2%
2	2.3	24.0	3390	30%
3	2.8	24.0	8574	75%
4	3.3	20.3	11279	99%
5	3.8	18.0	9627	84%
6	4.3	15.8	11355	100%
7	4.8	13.3	9003	79%
8	5.2	12.7	11081	97%
9	5.8	12.0	10729	94%
10	6.3	10.8	11389	100%
11	6.8	9.6	9894	87%
12	7.3	9.2	11262	99%
13	7.8	8.7	10404	91%
14	8.3	8.2	11258	99%
15	8.8	7.4	10030	88%
16	9.3	7.3	11298	99%

Table 2.4 Aerobic dive limit, whole body oxygen consumption, and muscle oxygen consumption for varying multiples of muscular metabolic rate at an increased [Mb] of 108 mg/g in a postabsorptive state.

$\dot{V}_{M_{O_2}}$	Whole body $\dot{V}_{O_2}$ (ml O_2/min*kg)	ADL (min)	Myoglobin O_2 consumed by muscle during dive (ml O_2)	% Myoglobin O_2 consumed by muscle during dive
1	1.8	28.0	221	1%
2	2.3	24.0	3390	15%
3	2.8	24.0	8574	38%
4	3.3	24.0	13758	60%
5	3.7	24.0	18942	83%
6	4.2	22.8	22759	100%
7	4.7	19.0	18660	82%
8	5.2	18.0	21291	93%
9	5.7	16.5	22767	100%
10	6.2	14.5	22684	100%
11	6.7	13.3	20523	90%
12	7.2	13.0	22714	100%
13	7.6	11.7	22502	99%
14	8.1	10.7	22593	99%
15	8.7	10.0	21030	92%
16	9.2	9.8	22517	99%

The only way to increase the use of Mb oxygen at rest was to decrease convective oxygen transport even further (i.e., $\dot{V}b$ less than 19%). However, when we ran the model at a $\dot{V}b$ of 9%, the ADL decreased because convective oxygen transport to the splanchnic organs and kidneys was insufficient. Hence, at rest there was no optimal $\dot{V}b$ that provided sufficient oxygen delivery for the kidneys and splanchnic organs while utilizing more Mb-bound oxygen, regardless of the Mb concentration. As a result, there was no difference in ADL for Mb concentrations of 54 and 108 mg g^{-1} until $\dot{V}_{M_{O_2}}$ exceeded 3-times resting.

At muscle Mb concentrations of 5 and 27 mg g^{-1}, the postabsorptive ADL decreased in a curvilinear fashion with increasing $\dot{V}_{M_{O_2}}$ and whole body $\dot{V}_{O_2}$. At normal and elevated Mb concentrations, the ADL decreased in a curvilinear fashion with the exception of a common plateau at 24 min for $\dot{V}_{M_{O_2}}$ of 2 to 3-times resting and 2 to 5-times resting for these two Mb concentrations, respectively (Figure 2.2). At these low levels of $\dot{V}_{M_{O_2}}$, the ADL was limited by blood oxygen stores, and Mb oxygen was not a limiting factor (Tables 2.3 and 2.4). These two curves diverge at higher levels of exertion as muscle oxygen stores are consumed and contribute significantly to setting the ADL. Only when $\dot{V}_{M_{O_2}}$ exceeded 3-times resting did an increase in the Mb concentration above 54 mg g^{-1} increase the ADL.

Based on Williams et al. (2004), we assumed an average postabsorptive diving $\dot{V}_{O_2}$ of 3.8 ml O_2 min^{-1} kg^{-1}, which was equivalent to a $\dot{V}_{M_{O_2}}$ of 5-times resting in our model. At this routine level of diving metabolism, a reduction of Mb concentration from 54 mg g^{-1} to 27 mg g^{-1} and 5 mg g^{-1} reduced the ADL from 18 min to 12.7 min (29%

reduction) and 11.0 min (39% reduction), respectively. Doubling the normal Mb concentration increased the ADL 33% from 18 to 24 min (Figure 2.2).

For all four Mb concentrations, the optimal $\dot{V}b$ (i.e., the $\dot{V}b$ that gave the maximum ADL) increased as muscular exertion increased (Figure 2.3). The optimal $\dot{V}b$ increased more quickly with increasing levels of exertion (i.e. the slope of the trend line was greater) for low muscle Mb concentrations compared to normal and elevated Mb concentrations. As Mb increased, the optimum $\dot{V}b$ for each level of $\dot{V}_{M_{O_2}}$ decreased. For example, at the average diving $\dot{V}_{M_{O_2}}$ of 5-times resting, the optimal $\dot{V}b$ at Mb concentrations of 5, 27, 54 and 108 g Mb kg^{-1} were 75%, 56%, 37% and 19% of resting levels, respectively. As Mb increases, $\dot{V}b$ and muscle blood flow must decrease (i.e., more pronounced dive response) for the muscle to fully use this Mb bound oxygen.

At a $\dot{V}_{M_{O_2}}$ of 5-times resting for normal and elevated Mb concentrations, convective oxygen transport at the optimal $\dot{V}b$ was insufficient to support the aerobic metabolic needs of the muscle. As a result, muscle Mb oxygen stores were used from the beginning and throughout the dive (Figure 2.4). In contrast, the optimal $\dot{V}b$ for reduced Mb concentrations was greater (i.e., less pronounced dive response) resulting in increased convective oxygen transport to the muscles and a delay in the use of Mb oxygen until well into the dive (7 min and 1.33 min for Mb concentrations of 5 and 27 mg g^{-1} respectively). With optimal matching of $\dot{V}b$ to $\dot{V}_{M_{O_2}}$, almost all myoglobin oxygen was consumed at this routine level of exertion regardless of myoglobin concentration.

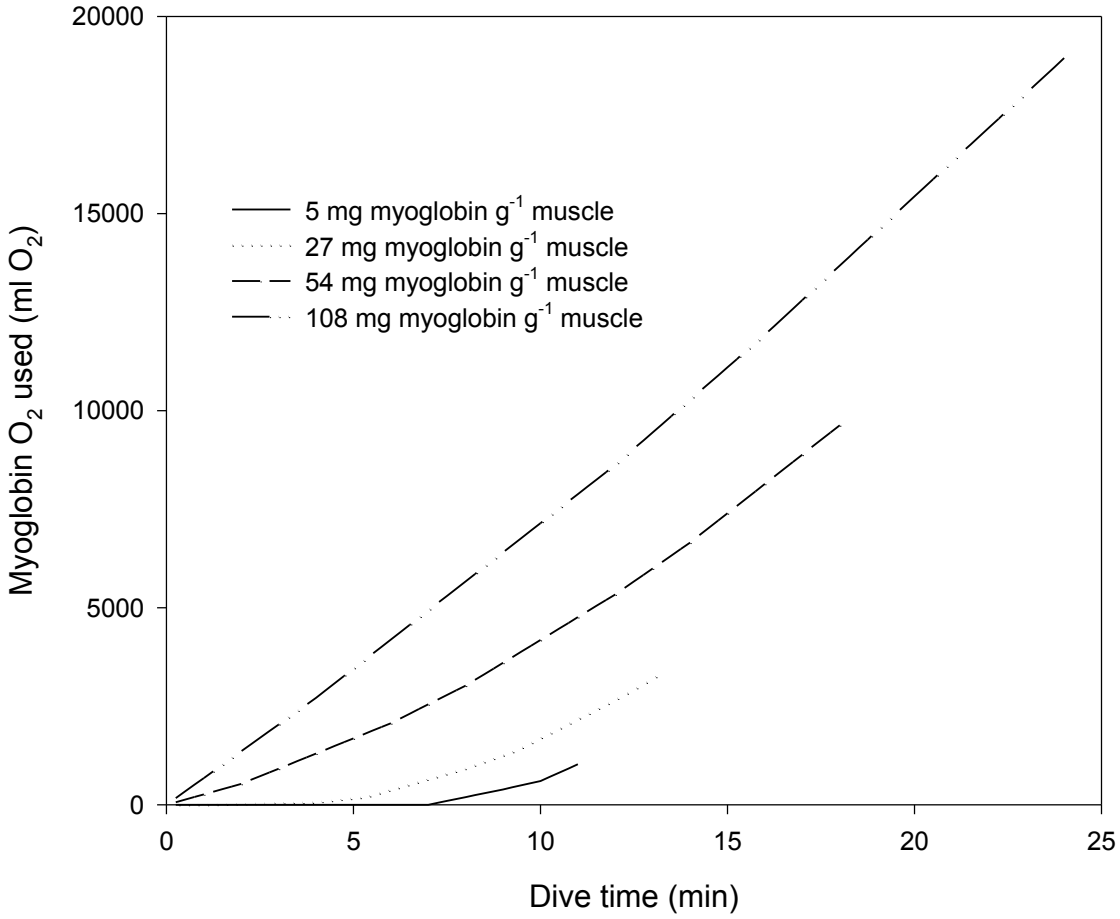

Figure 2.4 Myoglobin oxygen used during diving at a muscular exertion of 5-times resting $\dot{V}_{M_{O_2}}$ for four Mb concentrations.

The Effect of Mb Concentration on the Postprandial ADL

Under postprandial conditions, the ADL decreased at all levels of exertion because of the increased oxygen consumption of the splanchnic organs associated with prey warming, digestion and assimilation. At a routine $\dot{V}_{M_{O_2}}$ of 5-times resting and normal Mb concentration, the postprandial ADL (12 min) was 33% less than under postabsorptive conditions (Figure 2.5). The convective oxygen transport needed by the splanchnic organs required a $\dot{V}$b that was not optimal for the complete use of muscle oxygen at a routine diving $\dot{V}_{M_{O_2}}$ of 5-times resting. Not until $\dot{V}_{M_{O_2}}$ exceeded 7-times resting did this level of perfusion allow for complete utilization of muscle oxygen stores, and Mb oxygen became limiting to the ADL (Figure 2.5 and Table 2.5). As a result, doubling the Mb concentration did not increase the ADL until the level of muscular exertion exceeded 7-times resting. Diving at routine levels of muscular exertion in a postprandial state resulted in convective oxygen transport and not oxy-myoglobin limiting the ADL. Based on the results from our model, digesting and assimilating food while diving decreased the ADL for two reasons: 1) increased splanchnic consumption of blood oxygen and 2) the increased convective oxygen transport needed by the splanchnic organs resulted in a $\dot{V}$b that was not optimal for the complete use of muscle oxygen. As a result, the model indicated that there was no advantage in having a higher than normal myoglobin concentration during postprandial dives at routine levels of $\dot{V}_{M_{O_2}}$.

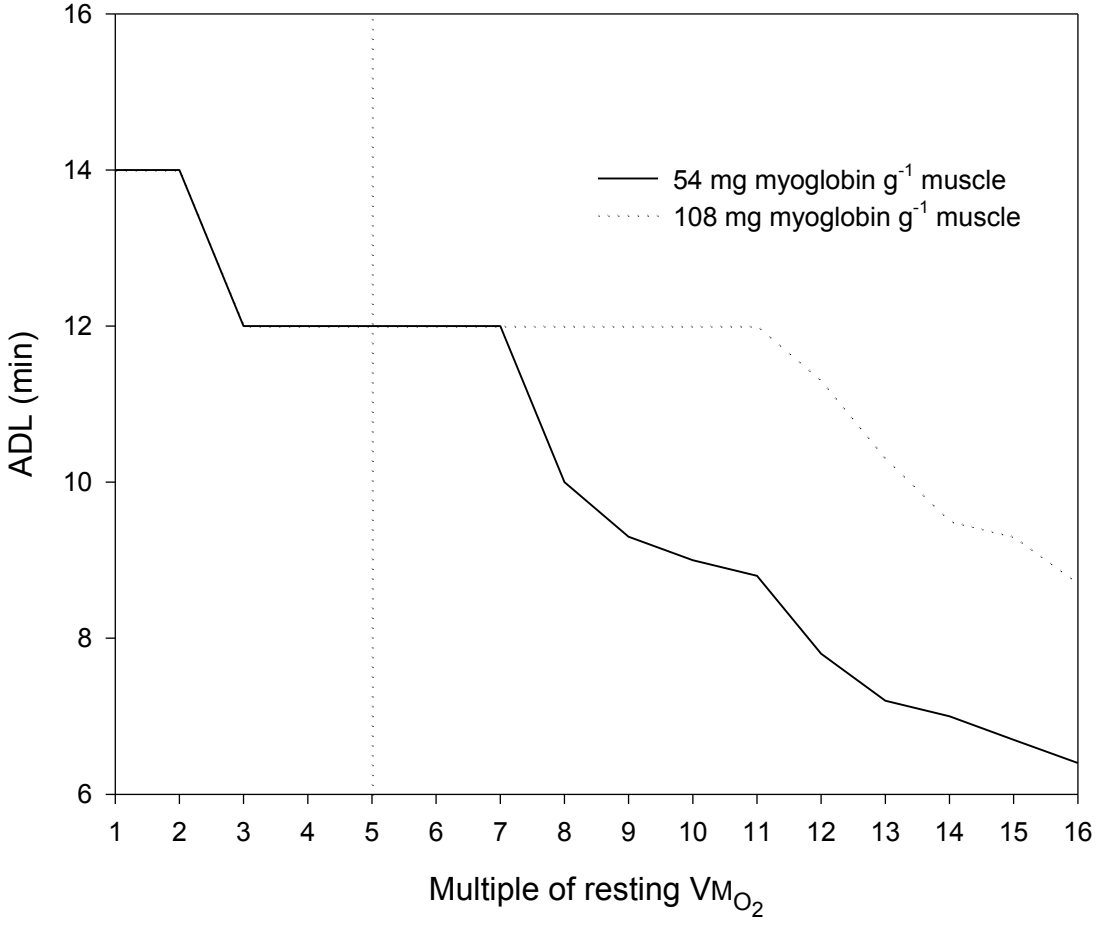

Figure 2.5 Aerobic dive limit (ADL) as a function of muscle oxygen consumption ($\dot{V}_{M_{O_2}}$) for a postprandial Weddell seal with normal and elevated Mb concentrations. Vertical dotted line marks the estimated routine level of diving $\dot{V}_{M_{O_2}}$ for a Weddell seal.

Table 2.5 Aerobic dive limit, whole body oxygen consumption, and muscle oxygen consumption for varying multiples of muscular metabolic rate at a normal [Mb] of 54 mg/g in a postprandial state.

$\dot{V}_{M_{O_2}}$	Whole body $\dot{V}_{O_2}$ (ml O_2/min*kg)	ADL (min)	Myoglobin O_2 consumed by muscle during dive (ml O_2)	% Myoglobin O_2 consumed by muscle during dive
1	3.4	14.0	0	0%
2	3.8	14.0	258	2%
3	4.3	12.0	1206	11%
4	4.8	12.0	3356	29%
5	5.3	12.0	5948	52%
6	5.8	12.0	8540	75%
7	6.2	12.0	11132	98%
8	6.7	10.0	11069	97%
9	7.2	9.3	9930	87%
10	7.8	9.0	9682	85%
11	8.3	8.8	11194	98%
12	8.7	7.8	11143	98%
13	9.3	7.2	10120	89%
14	9.7	7.0	11251	99%
15	10.2	6.7	10487	92%
16	10.7	6.4	11317	99%

Discussion

The Role of Myoglobin in Diving Marine Mammals

Due to myoglobin's high affinity for oxygen (P_{50} = 2-3 mm Hg; Schenkman et al., 1997) compared to Hb (P_{50} = 27 mm Hg; Qvist et al., 1981), it is an endogenous oxygen store for muscle only. To use this source of oxygen, the muscle must become hypoxic by reducing convective oxygen transport. This not only decreases the muscle P_{O_2} so that the oxygen dissociates from myoglobin, but also reserves more blood oxygen for other tissues. In the first publication using this model, Davis and Kanatous (1999) showed the importance of adjusting cardiac output and convective oxygen transport to muscle according to the level of exertion so that the total oxygen available in the muscle and blood was used by the end of a dive. As the level of muscular exertion increased, the dive response was less pronounced and convective oxygen transport to skeletal muscle (and other peripheral organs and tissues) increased. To maximize the ADL, both

oxygen stores had to be depleted simultaneously so that neither was singly responsible for limiting aerobic dive duration.

In a postabsorptive resting state, the ADL was independent of Mb concentration from 5-108 mg myoglobin g^{-1} muscle (Figure 2.2). The model showed that the $\dot{V}b$ needed to maintain resting metabolism in the splanchnic organs (19%) resulted in an over perfusion of the skeletal muscle so that almost all (97%) of the oxygen used by the muscles at rest was supplied by convective oxygen transport in the blood. Greater utilization of Mb oxygen would require less convective oxygen transport to skeletal muscle. However, further reduction in $\dot{V}b$ (9%) resulted in insufficient convective oxygen transport to the splanchnic organs and reduced the ADL.

At a routine diving $\dot{V}_{M_{O_2}}$ of 5-times resting, the postabsorptive ADL increased with higher Mb concentrations (Figure 2.2). In addition, Mb concentration was negatively correlated with optimal $\dot{V}b$ for a dive (Figure 2.3). Higher Mb concentrations (54 and 108 mg g-1) required a greater reduction in cardiac output (more profound dive response). The resultant reduction in convective oxygen transport to muscles decreased the muscle P_{O_2} (i.e., made the muscle hypoxic) so that myoglobin oxygen was used throughout the dive (Figure 2.4).

At a $\dot{V}_{M_{O_2}}$ of 1 to 7-times resting in a postprandial state, the $\dot{V}b$ required to maintain the elevated aerobic metabolism in the splanchnic organs resulted in an over perfusion of the skeletal muscle, which caused the incomplete use of Mb oxygen stores (Table 2.5). Inefficient use of muscle oxygen stores as well as increased use of blood oxygen for digestion and assimilation resulted in blood oxygen limiting the ADL in the postprandial state until $\dot{V}_{M_{O_2}}$ exceeded 7-times resting (Figure 2.5 and Table 2.5). As a result, the doubling of Mb concentration did not increase the ADL under postprandial

conditions until the level of $\dot{V}_{M_{O_2}}$ exceeded 7-times resting, which is 40% higher than the routine level of exertion.

Behavioral Considerations

The results of this model showed that an increase in the Mb concentration increased the ADL at a routine diving $\dot{V}_{M_{O_2}}$ under postabsorptive conditions (Figure 2.2). However, for the same $\dot{V}_{M_{O_2}}$ under postprandial conditions, the convective oxygen transport needed for digestion and assimilation required a $\dot{V}$b which resulted in an over perfusion of the muscle and incomplete use of muscle oxygen stores at routine levels of exertion (i.e., < 7 times resting $\dot{V}_{M_{O_2}}$) (Figure 2.5 and Tables 2.5-2.6). Castellini et al. (1992a) stressed the importance of integrating physiology and behavior in considering the biology of diving. To determine what selective pressures might affect myoglobin concentration, it is important to consider the way Weddell seals routinely dive.

Davis et al. (2003), classified Weddell seal dives into four types. Type 1 were feeding dives with a mean duration of 15.0 min, and these accounted for 14% of all dives made and 29% of total time submerged. Given the assumptions regarding HIF, the postprandial ADL (12 min) at a routine level of exertion calculated by our model agrees well with average duration of feeding dives reported by Davis et al. (2003). Types 2 and 3 dives were relatively short in duration (mean = 3.6 min and 7.9 min respectively) and were rarely associated with feeding. Together these dives accounted for 72% of dives being made. The average duration of these dive types are well below our estimated postabsorptive ADL of 18 min and are not limited by the physiological constraints of the oxygen stores, but by behavior.

Type 4 dives were long in duration (average = 24.7 min), appeared to be exploratory (non-feeding) dives, and accounted for 14% of all dives. This dive type exceeds our estimated postabsorptive ADL of 18 min and relies significantly on

anaerobic metabolism. Our model indicates that an increased myoglobin concentration would prolong aerobic metabolism for this type of dive. However, these long duration dives rarely occur in free diving Weddell seals (Kooyman, 1980).

Table 2.6 Aerobic dive limit, whole body oxygen consumption, and muscle oxygen consumption for varying multiples of muscular metabolic rate at an elevated [Mb] of 108 mg/g in a postprandial state.

$\dot{V}_{M_{O_2}}$	Whole body $\dot{V}_{O_2}$ (ml O_2/min*kg)	ADL (min)	Myoglobin O_2 consumed by muscle during dive (ml O_2)	% Myoglobin O_2 consumed by muscle during dive
1	3.4	14.0	0	0%
2	3.8	14.0	258	1%
3	4.3	12.0	1206	5%
4	4.8	12.0	3356	15%
5	5.3	12.0	5948	26%
6	5.8	12.0	8540	37%
7	6.2	12.0	11132	49%
8	6.7	12.0	13724	60%
9	7.2	12.0	16316	72%
10	7.7	12.0	18908	83%
11	8.2	12.0	21500	94%
12	8.6	11.3	22448	98%
13	9.1	10.3	22471	99%
14	9.6	9.5	22717	100%
15	10.1	9.3	22026	97%
16	10.6	8.7	22097	97%

Factors Determining Myoglobin Concentration

It appears that dives of the type and duration in which an increase in myoglobin concentration would increase the ADL are rare under normal diving behavior. While an increase in myoglobin would prolong aerobic metabolism during some long duration, postabsorptive dives, it does not appear to limit the ADL in the majority of natural dives (i.e., Types 1, 2 and 3). Weddell seals make the majority of their feeding dives in bouts of many dives with short recovery periods on the surface (Castellini et al., 1992a; Kooyman et al., 1980). As a result, many of these feeding dives probably occur in the postprandial condition. Davis et al. (1983) observed that the plasma of Weddell seals became very lipemic during deep foraging dives, indicating that the digestion and intestinal absorption of fat was occurring during the 5 to 6 h foraging session. Increased energy expenditure for digestion during diving is added to the metabolic costs for locomotion and basal metabolism (Williams et al., 2004). This increased metabolism for digestion and assimilation is also thought to reduce the ADL of southern elephant seals during foraging bouts (McConnell et al., 1992). Digestion not only increases oxygen consumption, but also influences the optimal management of the muscle and blood oxygen stores. Our model indicated that diving with the additional metabolic cost of HIF causes blood oxygen to limit the ADL rather than myoglobin oxygen (i.e., myoglobin stores may not be completely used). We hypothesize that myoglobin concentration is optimized for the type and duration of dives routinely made by Weddell seals, and that a further increase may not increase the ADL of most free-ranging dives. Whether physiological constraints associated with the dive response and convective oxygen transport have limited the concentration of myoglobin in muscles remains uncertain, but our model does suggest a possible influence during the evolution of Weddell seals and other long duration divers. In addition, the model indicates that the calculated ADL is more complex than simply the quotient of the available oxygen stores and estimated metabolic rate.

CHAPTER III

MYOGLOBIN EXTRACTION FROM MAMMALIAN MUSCLE TISSUE AND OXYGEN AFFINITY DETERMINATION UNDER PHYSIOLOGICAL CONDITIONS

An accurate determination of myoglobin (Mb) oxygen affinity (P_{50}) can be difficult due to hemoglobin (Hb) contamination in muscle homogenates and the autoxidation of Mb to metMb which is incapable of oxygen binding. If not removed, Hb from residual blood in the homogenate can alter the measured P_{50} for a Mb solution because Hb has a much lower oxygen affinity. To reduce Mb autoxidation, P_{50} is often measured at refrigerated temperatures. However, because Mb oxygen affinity is temperature dependent, these measurements give a greater oxygen affinity (lower P_{50}) than would result at physiological temperature (ca. 37-39° C) for birds and mammals. To avoid these problems, we developed new methods to extract Mb from vertebrate muscle tissue and remove Hb contamination while minimizing globin autoxidation. Cow (*Bos taurus*) muscle tissue (n=5) was homogenized in buffer to form a Mb solution, and Hb contamination was removed using affinity chromatography. A TCS Hemox Blood Analyzer was then used to quickly generate an oxygen dissociation curve for the extracted Mb. The oxygen affinity of extracted bovine Mb was compared to commercially available horse heart Mb. The oxygen affinity of cow Mb (P_{50} = 3.72 ± 0.16 mmHg) was not statistically different from horse Mb (P_{50} = 3.71 ± 0.10 mmHg). With high yield Mb extraction and fast generation of an oxygen dissociation curve, it was possible to consistently determine Mb P_{50} under physiologically relevant conditions for endothermic vertebrates.

Introduction

Myoglobin (Mb) is a globular hemoprotein found in vertebrate cardiac and skeletal muscle that reversibly binds oxygen. In muscle cells, Mb buffers mitochondrial oxygen availability, facilitates oxygen diffusion, and at high concentrations such as those

found in the muscle of many diving birds and mammals can also act as a significant oxygen store to maintain aerobic metabolism during hypoxia (For review see: Wittenberg and Wittenberg, 2003; Ordway and Garry, 2004; Gros et al., 2010; Davis 2014). Although research on Mb has a long history, much of it has focused on Mb as a model for protein chemistry with little regard to its biological role. It was the first protein whose three dimensional structure was determined (Kendrew et al., 1958; Kendrew et al., 1960), and it remains a model for studying the relationship between protein structure and function (Frauenfelder et al., 2003, Brunori, 2010, Storz et al., 2011).

Myoglobin mutational studies have examined the effects of amino acid substitutions on Mb affinity for oxygen (Carver et al., 1992; Dasmeh and Kepp, 2012; Dasmeh et al., 2012; Scott et al., 2001) and other ligands (Olson et al., 2008). However, there has been little work comparing the oxygen affinity of a variety of naturally occurring Mb structural variants for different species. We set out to develop simplified, uniform methods to extract Mb from vertebrate muscle tissue for the purposes of comparing Mb oxygen affinity.

There are two challenges when extracting Mb for measuring oxygen affinity at physiological temperatures (ca. 37-39^{o} C) for birds and mammals: (1) endogenous Hb is an unavoidable contaminant when homogenizing muscle samples, and (2) Mb in solution quickly autoxidizes to non-functioning metMb at physiological temperatures. To minimize the autoxidation of Mb in solution, P_{50} measurements are often made at temperatures below those found in the muscles of birds and mammals. However, because Mb oxygen affinity increases dramatically with decreasing temperature, affinity measurements at low temperature are not physiologically relevant (Schenkman et al., 1997). In living tissue, oxidized metMb is reduced to its active form by the enzyme metMb reductase. This enzyme is present and active in muscle tissue homogenates, but its concentration is dramatically reduced during Mb purification (Hagler et al., 1979). As part of a larger study comparing the oxygen affinity of Mb among a variety of endothermic vertebrates, we developed a reliable method for extracting Mb from

vertebrate muscle. Using purification methods that remove Hb contamination while minimizing Mb oxidation, we eliminated the need for reducing agents (e.g. dithionite) that must be removed by buffer exchange before measuring oxygen affinity.

Materials and Methods

Buffer Selection

The autoxidation rate of Mb to inactive metMb is slowed by conditions of low temperature and alkaline pH (Tomoda et al., 1981). To maximize the yield of functional Mb during extraction and purification, we chose procedures to minimize working time, maintain temperature at 0-4°C, and maintain basic pH (for stepwise methods see Appendix A). A single stock buffer solution of 50 mM TRIZMA® tris buffer (Sigma-Aldrich, T 0694) with 50 mg l^{-1} gentamicin sulfate (Sigma-Aldrich, G-1264) was used throughout tissue homogenization and purification. The temperature dependent shift in pH of this buffer made it possible to chromatographically remove Hb from muscle homogenates and subsequently generate an oxygen dissociation curve (ODC) at a physiologically relevant temperature and pH using one buffer (pH = 7.4 at 37°C; pH = 8.26 at 5°C). This eliminated the need for buffer exchange during purification, thereby reducing preparation time and Mb autoxidation.

Oxygen Affinity

As with Mb, Hb is a globular hemoprotein capable of reversibly binding oxygen. These orthologous globins share much of their overall structure (characteristic "globin fold"), and many key regions are highly conserved (Storz et al., 2013). With similar structure and heme binding properties of these proteins, their optical characteristics are similar (Kelner and Alexander, 1985; Masuda et al., 2008) (Figure 3.1 and 3.2). At wavelengths of 500 – 700 nm, the absorption spectra of HbO_2 and MbO_2 both show twin absorption peaks: 544 and 582 nm for MbO_2 and 542 and 578 nm for HbO_2. In the deoxygenated state, Mb has a single peak at 557 nm and Hb at 554 nm. (For Mb see: Bowen, 1949; Boulton and Huntsman, 1971; Millar et al., 1996. For Hb see: Horecker,

1943; Zijlstra et al., 1991; Zijlstra and Buursma, 1997). Because of the similar optical properties in the oxygenated and deoxygenated state, these globins express nearly identical spectral shifts during oxygen binding and dissociation from 500-700 nm. This shift can be used to monitor oxygen binding state of these pigments in solution, and due to their nearly identical optical responses, identical instrumentation can be used to monitor these pigments.

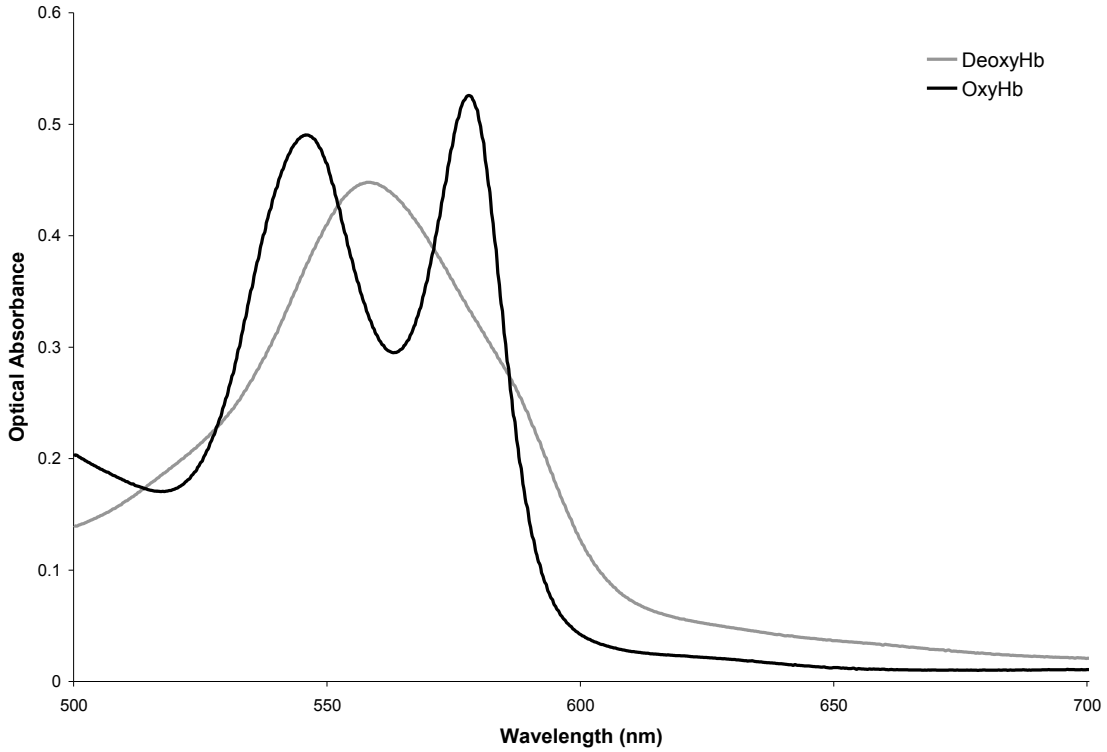

Figure 3.1 Spectral scan of oxyhemoglobin and deoxyhemoglobin at 500 to 700 nm wavelength.

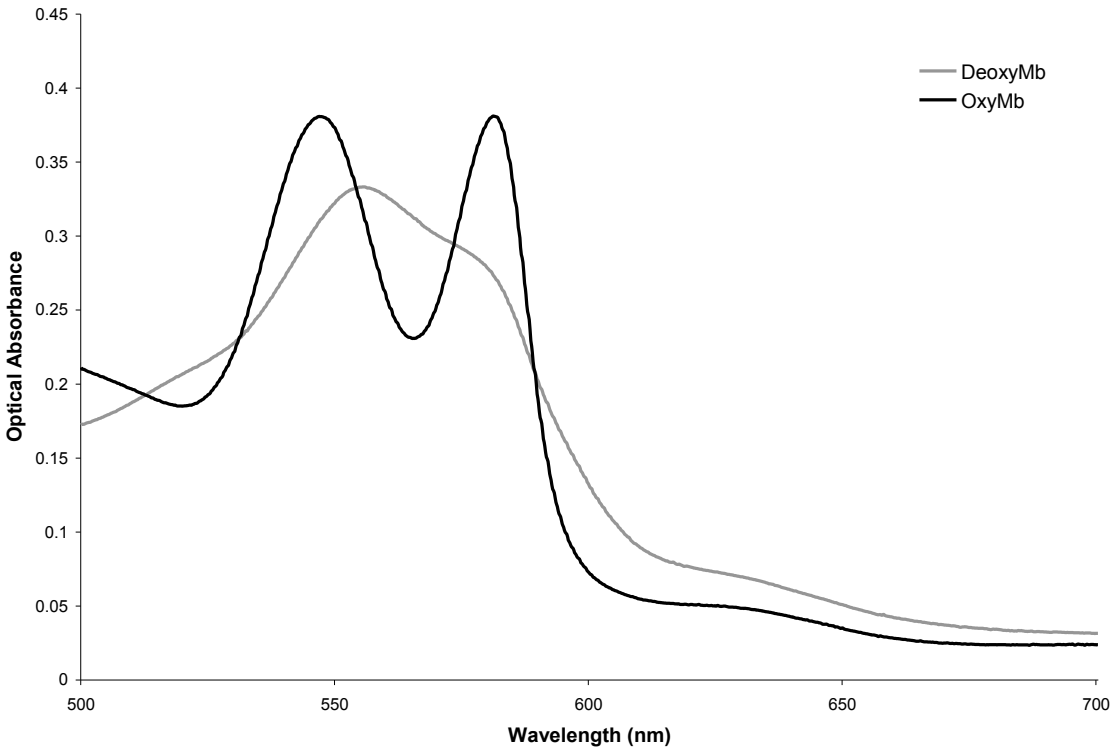

Figure 3.2 Spectral scan of oxymyoglobin and deoxymyoglobin at 500 to 700 nm wavelength.

Oxygen binding respiratory pigments such as Mb and Hb are characterized by the affinity with which they bind oxygen. This oxygen affinity is quantified as the partial pressure of oxygen at which 50% of pigments in solution are bound with oxygen (P_{50}). The P_{50} of these respiratory pigments can be determined by generating an ODC (Figure 3.3). Mb and Hb are optically similar and cannot be distinguished by spectroscopy when in solution together (Kelner and Alexander, 1985). For this study, the P_{50} of Mb was determined by generating an ODC using a TCS Hemox Blood Analyzer (TCS Scientific, New Hope, PA). This instrument was designed for generating oxygen dissociation curves for Hb (Guarnone et al., 1995) using multiwavelength spectroscopy, and we evaluated its performance in ODC generation for Mb.

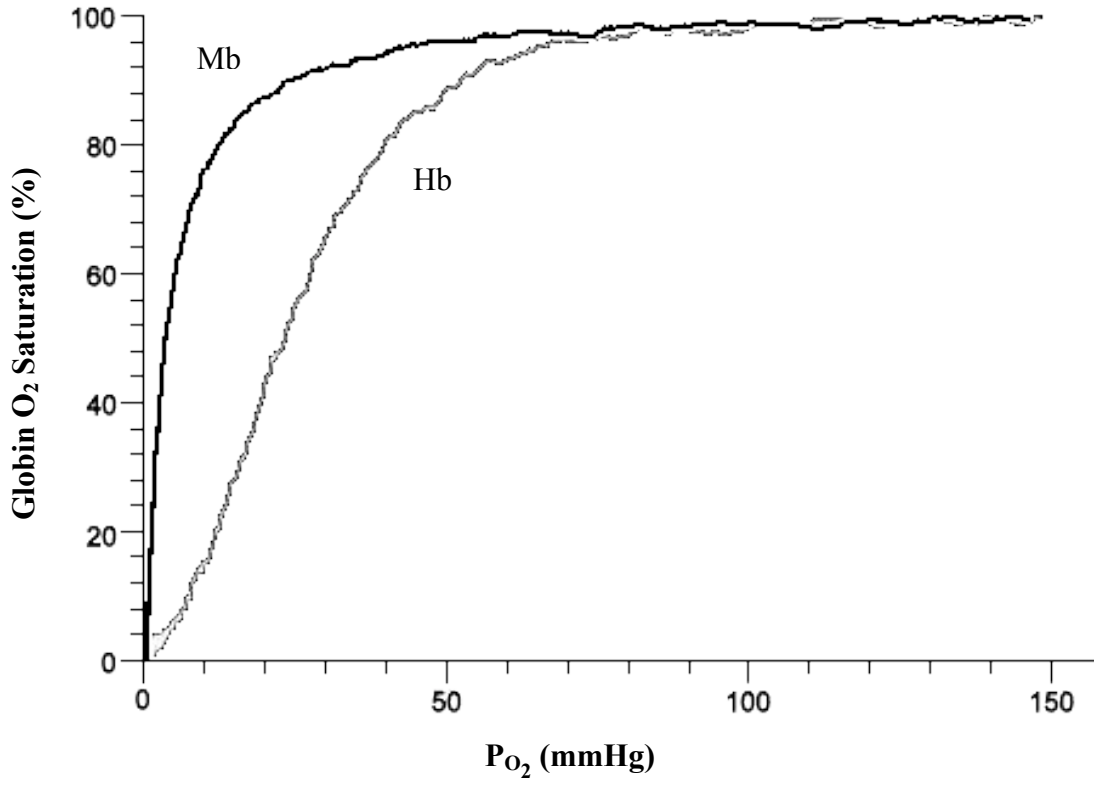

Figure 3.3 Oxygen dissociation curve of horse heart myoglobin (Mb) (P_{50} = 3.70) and human hemoglobin (Hb) (P_{50} = 23.64) determined using TCS Hemox Blood Analyzer.

Lyopholyzed Horse Heart Myoglobin Preparation

Commercially available lyopholyzed horse heart Mb was used as a control for validating myoglobin isolation and standardizing oxygen affinity measurements. To prepare a Mb solution, 9 ml of stock buffer solution was bubbled with nitrogen for ten minutes on ice in a 50 ml flask. The deoxygenated buffer was reduced with the addition of 0.11 g sodium dithionite (Sigma-Aldrich, 157953) to make a 70 mM dithionite solution. The reduced buffer was then bubbled with nitrogen for an additional 5 minutes. After the addition of 0.016 g of lyopholyzed horse heart Mb (Sigma-Aldrich, M1882), the solution was bubbled with nitrogen for one minute which produced a bright red 0.1 mM solution of reduced Mb. Adding lyopholyzed Mb to a pre-reduced hypoxic buffer solution yielded a reduced Mb solution using less dithionite than was required to

reduce Mb solutions after reconstitution. Dithionite was then removed by buffer exchange on a column of G-25 Sephadex® (Sigma-Aldrich, G25150) at 4°C by eluting the sample with chilled stock buffer. The Mb solution was diluted with buffer to the desired concentration (0.02 mM Mb being the minimum [Mb] for our purposes), and frozen in 3.5 ml aliquots at -80°C until needed.

Muscle Myoglobin Extraction

Because Mb and Hb have similar optical properties but very different oxygen affinities, Hb is a significant contaminant that can alter the photometric Mb P_{50} and may constitute as much as 30% of the total globin content of excised muscle tissue in beef cattle (Rickansrud and Henrickson, 1967; Han et al., 1994). The objective of Mb extraction for oxygen affinity was to obtain a solution free of Hb contamination with an adequate concentration of reduced Mb. Affinity chromatography was chosen over other methods for removing Hb contamination. Size exclusion membrane separation under centrifugation has been used to separate Mb from Hb successfully in urine (Kelner and Alexander, 1985), but higher protein concentrations such as those found in homogenized muscle tissue blocked membrane pores which resulted in low Mb recovery. Salt precipitation ("salting out") Hb and Mb in solution does not result in complete globin separation (Kelner and Alexander, 1985) and would require the additional preparatory step of buffer exchange.

Cow muscle samples (n=5) were obtained from a local animal processing facility and stored at -80°C until use. To prepare a Mb solution, frozen muscle tissue was cleared of visible fat and connective tissue and sectioned into cubes roughly 3 mm on each side. Approximately 0.5 g of sectioned lean muscle was homogenized with a glass tissue grinder (Fisher Scientific, 7727-15) using 10 ml of chilled buffer g^{-1} tissue. Samples were chilled on ice throughout the homogenization and purification process. The homogenized solution was centrifuged for 20 min at 1,200 g and 4°C. Hb contamination was removed from the supernatant using low pressure affinity chromatography on a column of DEAE-Sephadex® A-50 (Sigma-Aldrich, A50120).

DEAE affinity chromatography has been used previously to separate hemoglobin and myoglobin in muscle homogenate while eluting with Tris buffer at concentrations of 5 to 50 mM and pH of 8.2-8.6 (Brown, 1961; Yamazaki et al., 1964; Wittenberg and Wittenberg, 1981). Mb was eluted at 4°C using stock buffer solution (pH 8.3 at 4°C) while DEAE preferentially retained Hb, retarding its movement and trapping it in the column. The eluted Mb fraction was collected in 3.5 ml aliquots and frozen at -80°C until analysis. Globin solutions that were too dilute for analysis were concentrated by incomplete thawing under centrifugation using 5 ml cryovials. Freeze centrifugation has proven effective at concentrating proteins including Mb while maintaining enzymatic activity (Virgen-Ortíz et al., 2012, 2013)

Verifying Hemoglobin Removal

To verify that Hb was successfully removed during purification, a test solution was prepared from homogenized cow muscle with additional cow Hb to ensure contamination. A Hb solution was prepared from cow blood obtained from a local meat processing facility. Whole blood was centrifuged for 15 min at 1,200 g and 4°C. Plasma was decanted and the remaining cellular pellet was resuspended and lysed with deionized water. This Hb solution was centrifuged for an additional 15 min at 1,200 g and 4°C to remove cellular debris. The resulting Hb solution was decanted on a column of G 25 Sephadex and eluted with 25mM Tris HCl buffer (pH 8.8 at 4°C).

A Mb solution was prepared using the homogenization methods described above with the addition of 0.1 ml of cow whole blood added g^{-1} of muscle tissue during homogenization to further contaminate the sample with Hb. After homogenization and centrifugation, a subsample was collected as a rough tissue homogenate containing both Hb and Mb. The remaining sample was further purified according to our procedures using DEAE Sephadex chromatography to remove Hb contamination. The Mb solutions were concentrated by incomplete thawing under centrifugation and eluted on a column of G-25 Sephadex to transfer to a buffer of 25mM Tris HCl (pH 8.8 at 4°C) for electrophoresis.

Glycerol was added to the globin solutions to total 10% of solution before loading for electrophoresis. Protein bands were separated using native PAGE on a 13% gel at 200 V for 1.5 hours in TRIS glycine buffer (pH 8.3) (Boulton and Huntsman, 1971). The centrifuged muscle tissue homogenate was found to have pigmented bands corresponding to the Hb standard as well as a smaller more electrophoretically mobile pigmented Mb band. The solution of extracted Mb with Hb contamination that was purified with affinity chromatography had a single, distinct pigmented band for Mb and no Hb band (Figure 3.4).

Determination of Myoglobin P_{50}

The TCS Hemox Analyzer measures the oxygen saturation of respiratory pigments in solution by multiwavelength spectroscopy as a function of the partial pressure of oxygen in the sample cell using a Clark oxygen electrode. The ratio of oxy/deoxy hemoproteins are determined by the ratio of optical absorption at 560 nm (Channel S2) and 570 nm (Channel S1) wavelength. While the optical absorption at 570 nm remains virtually unchanged between the oxy and deoxy states of the hemoprotein (Mb or Hb), the absorption at 560 nm undergoes a significant shift between these two states (Figures 3.1 and 3.2). Because the change in the ratio of absorption of these two spectra is used to determine the percent saturation instead of direct absorbance at a specific wavelength, the effect of Mb oxidation during ODC generation is reduced. The continuous ODC generated by the TCS Hemox Analyzer is of higher resolution than tonometric methods.

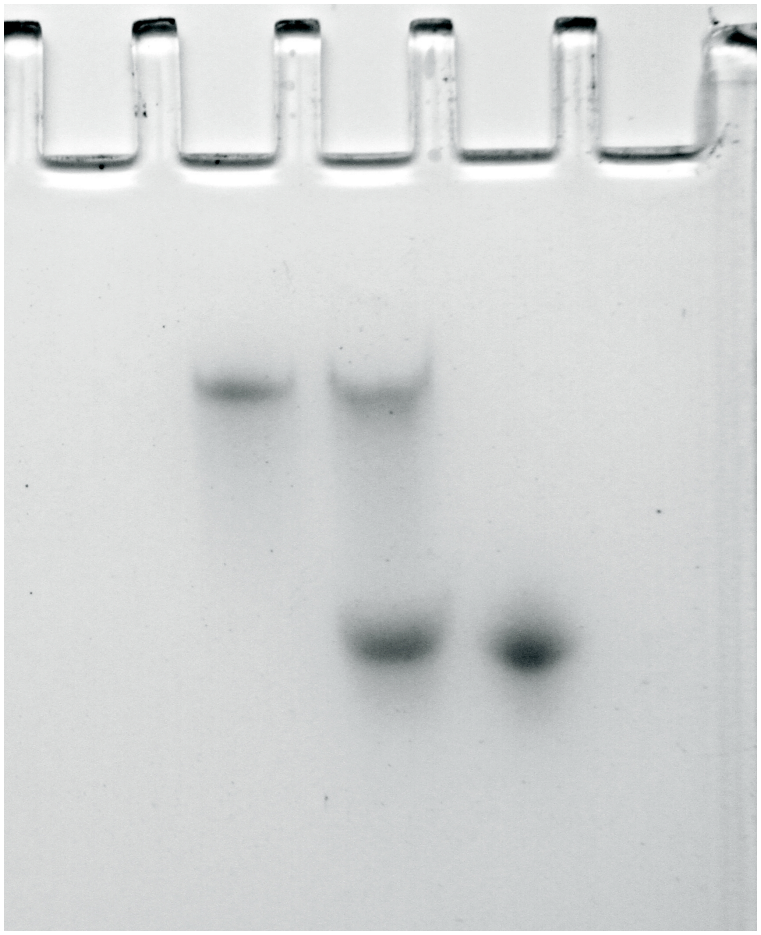

Figure 3.4 Pigmented globin bands from electrophoresis of prepared solutions of (a) prepared cow Hb, (b) homogenized cow muscle with addition of 0.1 ml whole blood g^{-1} muscle to ensure Hb contamination, and (c) solution b after removing Hb contamination using affinity chromatography.

A minimal [Mb] was required to achieve adequate signal amplitude (Channel signal of S1/S2 on TCS Hemox Analyzer), and only samples with adequate concentration to achieve final signal strength of at least 0.025 were used for ODC determination (equivalent to [Mb] of approximately 0.02 mM). Dilute samples with insufficient [Mb] were concentrated by partial thawing under centrifugation. Individual 3.5 ml samples were thawed and 20 µl of antifoam solution was added. Samples were then warmed for 8 min in a water bath of 37°C before transfer to the sample chamber. The sample was bubbled while stirring with compressed air in the chamber and allowed

to equilibrate at 37°C and ambient O_2 partial pressure for an additional 8 min. Saturated oxygen partial pressure was calibrated before each reading using the following equation:

$$(P_{ATM} - 47) * 0.2095 = \text{Oxygen Partial Pressure at Saturation (mmHg)}$$

where P_{ATM} is the atmospheric pressure in mmHg, 47 mmHg is the water vapor pressure at sea level and 37°C, and 20.95% is the percentage of oxygen compressed air. After the sample was equilibrated, an ODC was generated by monitoring the globin saturation and partial pressure of oxygen in solution while deoxygenating with compressed nitrogen. Monitoring of deoxygenation and P_{50} determination was performed using TCS Hemox Data Acquisition System software (V 2.00.13, TCS Scientific). Software monitoring was set to start at a P_{O_2} of 146 mmHg and stop after deoxygenating to 0.5 mmHg. Nitrogen flow rate and stir speed were maintained at a rate that would deoxygenate the sample in 7-10 min. After storing and plotting approximately 1,400 individual data points (ca. 8 min with a sampling rate of 175 min^{-1}), the data acquisition software was used to apply a best fit line for P_{50} calculation.

Results and Discussion

The procedures used in this study produced a Mb solution from homogenized vertebrate muscle tissue that was free of Hb contamination. Autoxidation of Mb during extraction and purification was minimized by maintaining temperature below 4°C, maintaining alkaline pH, and minimizing processing time. The resulting Mb solution could be concentrated or diluted to a desired concentration. Using the TCS Hemox blood analyzer, reproducible high resolution Mb oxygen dissociation curves could be generated with solutions as dilute as 0.02mM Mb.

Mb from homogenized cow muscle had a mean P_{50} that was statistically indistinguishable from commercially available horse heart Mb (cow n = 40, horse n = 67; SPSS 15.0). Due to unequal variance (Levene's test, $p < 0.001$), an unequal variance t-test was used to compare oxygen affinity. There was no statistical difference between

the oxygen affinity of horse heart Mb (P_{50} = 3.70 ± 0.09 mmHg) and Mb extracted from cow muscle (P_{50} = 3.72 ± 0.16 mmHg) (p = 0.415).

Amino acid sequences were available for cow (P02192) and horse (P68082) Mb via the UniProt protein database (www.uniprot.org) (UniProt Consortium, 2013). Sequence alignment comparison of these samples was determined using Jalview 2.8 (Waterhouse et al., 2009) (Figure 3.5). The Mb O_2 affinity of these ungulates appears to be conserved under the conditions tested despite approximately 12% heterogeneity in Mb primary structure. Of the 153 amino acids in these proteins, there are 18 points of variance with mostly conservative substitutions. This is consistent with previous research which showed that Mb structure is highly conserved in critical regions that influence oxygen binding while other regions undergo a relatively greater rate of neutral substitutions (Bogardt et al., 1980; Romero-Herrera et al., 1978; Ma et al., 2013). While there appears to be no significant difference in Mb oxygen affinity for these two species, further research is needed on the Mb oxygen affinities of other species which may reveal differences that are adaptive for their physiological ecology.

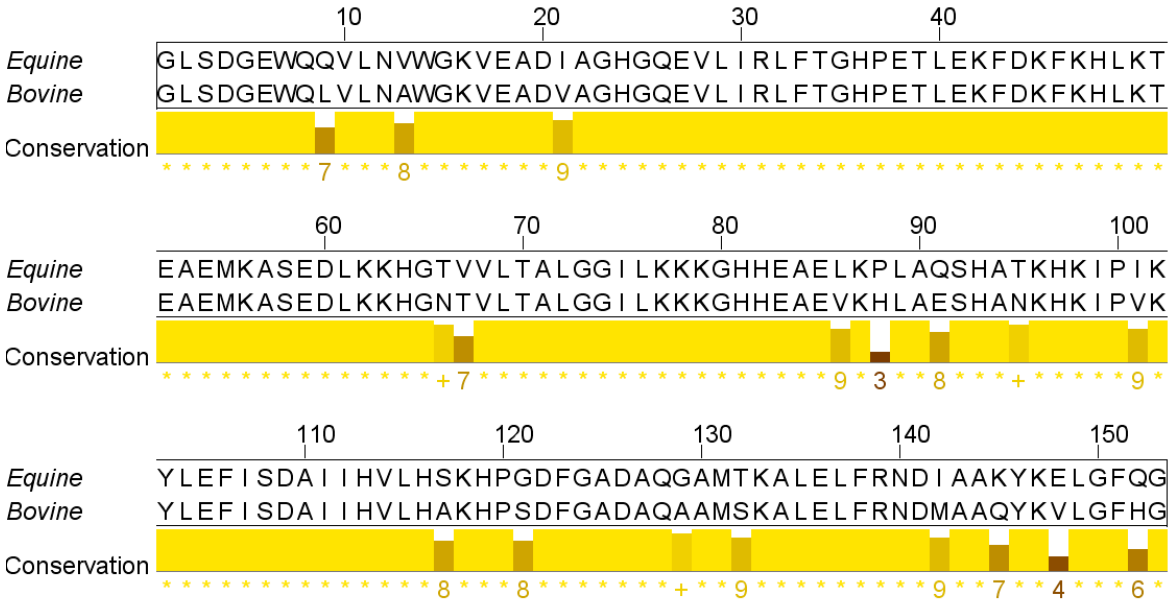

Figure 3.5 Alignment of horse and cow myoglobin sequences using Jalview 2.8. Amino acid residue variants are ranked by conservation of physicochemical properties.

CHAPTER IV
MYOGLOBIN OXYGEN AFFINITY IN DIVING AND TERRESTRIAL BIRDS AND MAMMALS

Myoglobin (Mb) is a member of the family of oxygen binding globin proteins that serves multiple functions in vertebrate skeletal and cardiac muscle, including the transport and storage of oxygen. The extent to which myoglobin functions in each of these roles varies with the physiology and ecology of different species, and a growing body of research suggests selection has shaped the functional properties of Mb to meet unique physiological needs. Diving vertebrates have adaptations that maximize dive duration, which include increased oxygen stores bound to hemoglobin (Hb) in blood and Mb in muscle. Myoglobin concentration is positively correlated with dive duration in diving species, but it is not known if Mb oxygen affinity is adaptive in these animals. In this study, we compared Mb oxygen affinity (P_{50}) of diving and terrestrial birds and mammals to examine whether it is associated with the unique oxygen demands in diving vertebrates. Myoglobin P_{50} was conserved within the narrow range of 2.40-4.85 mmHg across all species examined. The Mb P_{50} of terrestrial ungulates was highly conserved with a mean of 3.72 ± 0.15 mmHg and a narrow range among species (3.70 - 3.74 mmHg). The P_{50} of most cetaceans was similar to terrestrial ungulates but showed greater variability ranging from 3.54-3.82 mmHg with the exception of the melon-headed whale that had a significantly higher P_{50} (lower oxygen affinity) of 4.85 mmHg. Among pinnipeds (seals and sea lions) the P_{50} ranged from 3.23-3.81 mmHg and showed a trend for higher oxygen affinity in species with longer dive durations. Among diving birds the P_{50} ranged from 2.40-3.36 mmHg and also showed a trend of higher affinities in species with longer dive durations. Although the ranges overlapped, the diving birds tended to have a greater oxygen affinity than the diving mammals. Low Mb P_{50} (high oxygen affinity) is associated with avian and mammalian species whose muscles are routinely metabolically active under hypoxic conditions associated with aerobic dives.

Introduction

Globin Evolution

The globins are a group of paralogue globular proteins with an ancient common ancestor (Storz et al., 2011). These related proteins share a common tertiary structure (stereotypic globin fold) with a hydrophobic pocket that contains a heme prosthetic group capable of reversibly binding oxygen. The tertiary structure and ligand binding regions are generally highly conserved, while less critical regions are more variable (Naylor and Gerstein, 2000). Duplication of the ancestral globin gene through whole genome and isolated gene duplication enabled the divergence in globin structure, function, and regulation of expression that produced the current diversity of vertebrate globins observed today (Storz et al., 2011; Hoffmann et al., 2012; Goodman et al., 1975; Wittenberg, 2007; Wystub et al., 2004; Schwarze and Burmester, 2013).

Eight globin proteins are expressed in vertebrates including myoglobin, hemoglobin, cytoglobin, neuroglobin, androglobin, globin E, globin X, and globin Y. However, not all of these globins are present in every species as some have been lost at various divergences during vertebrate evolution (Hoffmann et al., 2011). Myoglobin (Mb) is expressed almost exclusively in vertebrate skeletal and cardiac muscle where it buffers mitochondrial oxygen availability and facilitates oxygen diffusion (Salathe and Chen, 1992; Gödecke et al., 1999; Wittenberg and Wittenberg, 2003; Kanatous and Garry, 2006).

Myoglobin Form and Function

Within the Mb protein are several highly conserved hydrophobic cavities (Figure 1.1) including the heme pocket, the distal pocket (DP), and four additional pockets (Xe1-Xe4) named for their ability to bind xenon, although they also bind O_2, CO and NO (Tomita et al., 2010). A porphyrin ring is present within the heme pocket and is stabilized by hydrophobic interactions with nonpolar amino acids. Additional stability is provided by salt bridges between the heme propionic side chains and polar amino acids near the opening including H97, R45, and S92 (Harada et al., 2007; The standard single

letter abbreviations for amino acids will be used to remain consistent throughout the text).

Notable among the highly conserved amino acids are the proximal (H93) and distal (H64) histidines. The proximal histidine covalently binds the iron at the center of the heme, while the distal histidine has several roles that include stabilizing the oxygen-heme bond. The distal pocket is the gap adjacent to the heme iron and the distal histidine that allows space for the heme to bind oxygen and other ligands (Figure 1.1). Although the backbone of the protein is stable, the conformational states of the amino acid side chains are dynamic. In the lowest energy state, Mb crystallography reveals an enclosed protein with no direct pathway for ligands to enter and bind to the internal heme. Because of this, ligand entry must rely on amino acid side chain fluctuations to open transient channels leading from the protein surface to the distal pocket. There are several proposed transient ligand channels involving the Xe pockets, but the majority (> 75%) of ligand movement in and out of Mb appears to be through a rotation of the distal histidine which serves as a gate to open a direct channel from the protein surface to the distal pocket where heme binding can occur (Scott et al., 2001; Salter et al., 2012). Once oxygen has bound to the heme, the interaction between the heme 6-propionate side chain, R45, and the distal histidine stabilizes the oxy-Mb and decreases heme oxidation (Harada et al., 2007). Although amino acid 45 shows some variability, the R45 and K45 variants appear to be conservative and exhibit similar stabilizing effects in horse and sperm whale Mb, respectively (Harada et al., 2007). Any variation in globin structure that affects the kinetics of binding or releasing oxygen will alter its oxygen affinity (Harada et al., 2007; Dasmeh and Kepp, 2012), and mutations that selectively stabilize the oxygen bound form will increase oxygen affinity (Ajloo et al., 2002).

Diversity of Myoglobin Expression

Myoglobin concentration in the skeletal muscle of birds and mammals varies greatly among species. In air-breathing diving vertebrates, high concentrations of Mb serve as an oxygen store for periods of regional muscle hypoxia during prolonged apnea

(Guyton et al., 1995; Ponganis et al., 1997b; Wright and Davis, 2006; Davis, 2014) and may represent as much as 50% of total oxygen store (Butler and Jones, 1997). While many terrestrial mammals have Mb concentrations < 5 mg g^{-1} muscle tissue (Newcom et al., 2004, Masuda et al., 2008), Mb concentration in diving marine mammals is often 10-fold greater than the levels found in terrestrial animals (Kanatous and Mammen, 2010) with some concentrations exceeding 78 mg g^{-1} (Noren and Williams, 2000). Sedentary birds such as galliforms may have Mb concentrations < 1 mg g^{-1} (Kranen et al., 1999), while long-duration divers such as emperor penguins have concentrations of ca. 64 mg g^{-1} (Kooyman and Ponganis, 1998; Ponganis et al., 1999).

Among diving birds and mammals, Mb concentration increases with increased capacity for dive duration (Reed et al., 1994a; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Dolar et al., 1999; Helbo and Fago, 2012). In addition, Mb concentrations vary within the muscles of vertebrates with the highest concentrations found in those associated with maximum exertion and aerobic metabolism (Polasek and Davis, 2001). The level of expression of Mb within muscle is influenced by intracellular partial pressures of oxygen and enhanced by local hypoxia, which stimulates Mb synthesis (Terrados et al., 1990; Hoppeler and Vogt, 2001).

Myoglobin Structural Variants

Among birds and mammals, Mb varies in primary structure in addition to concentration. Despite variation in amino acid sequence, the overall globin tertiary structure and heme binding regions are largely conserved (Bogardt et al., 1980; Evans and Brayer, 1988; Tamburrini et al., 1999). The oxygen binding properties of respiratory pigments are defined by the P_{50} or the partial pressure (mmHg) of oxygen at which 50% of pigments in solution are bound with oxygen, which is determined from an oxygen dissociation curve (ODC) (Figure 3.3). A lower P_{50} indicates a lower P_{O_2} for half saturation and, therefore, a higher oxygen binding affinity.

Site directed mutational studies of Mb produce variability in oxygen affinity with some amino acid substitutions having a greater influence than others (Carver et al., 1992;

Scott et al., 2001; Dasmeh and Kepp, 2012). Myoglobin has distinct roles of intramuscular storage and transport of oxygen, and the relative importance of these roles varies among animals with different intramuscular oxygen demands (Dasmeh and Kepp, 2012). Diving vertebrates with unique physiological adaptations for storing and transporting oxygen may experience selection pressure that influences the molecular evolution of Mb (Naylor and Gerstein, 2000). Recent studies showed that marine mammal Mb has experienced an increase in the rate of evolution (Dasmeh et al., 2013; Nery et al., 2013b) that has resulted in increased stability (Dasmeh et al., 2013) and net surface charge (Mirceta et al., 2013) compared with terrestrial mammals. Although Mb concentration in cetaceans is high and increases directly with average dive duration, there does not appear to be any evolutionary modification of oxygen affinity in this branch of diving vertebrates (Helbo and Fago, 2012).

Although the structure, concentration, and functional role of Mb are known to vary among species of birds and mammals, no study has comprehensively examined the interspecfic differences in oxygen affinity under identical physiological conditions. Although a range of Mb oxygen affinities has been reported in the literature, the studies used various experimental techniques, instrumentation, and temperatures, which make interpretation of the data for comparative analyses difficult. Additionally, due to the effect of temperature on Mb oxygen affinity, previous experimental results at non-physiological temperatures are not relevant *in vivo* (Schenkman et al., 1997).

The purpose of this study was to compare the oxygen affinity of Mb from a variety of terrestrial and diving birds and mammals to determine if Mb oxygen affinity is adaptive in diving vertebrates. In addition, species with amino acid sequences available in the Uniprot protein database (UniProt Consortium, 2013; www.uniprot.org) were compared to identify structural differences that may account for observed variation in oxygen binding affinity. Among the diving animals, we tested for correlations between Mb P_{50} and routine dive duration that might indicate molecular adaptation for oxygen affinity and storage under hypoxic conditions.

Materials and Methods

Myoglobin Samples

Commercially available horse (*Equus ferus*) Mb (Sigma-Aldrich, M1882) was used as a terrestrial standard of known purity for oxygen affinity comparisons. Muscle samples from oryx (*Oryx dammah*), cow (*Bos taurus*), and sheep (*Ovis aries*) were obtained from local (Houston, TX, USA) animal processing facilities. White-tailed deer (*Odocoileus virginianus*) and redhead duck (*Aythya americana*) samples were donated by licensed local hunters. Penguin muscle samples including macaroni (*Eudyptes chrysolophus*), chinstrap (*Pygoscelis antarctica*), Adélie (*Pygoscelis adeliae*), emperor (*Aptenodytes forsteri*), and king (*Aptenodytes patagonicus*) penguins were collected during necropsy of deceased captive animals and donated by the holding facilities. Bowhead whale (*Balaena mysticetus*) samples were collected during the annual native hunt in Barrow, Alaska. Weddell seal (*Leptonychotes weddellii*) samples were collected by biopsy from live animals in the field as part of a separate research project. The remaining cetacean and seal samples including common dolphin (*Delphinus delphis*), Risso's dolphin (*Grampus griseus*), spinner dolphin (*Stenella longirostris*), bottlenose dolphin (*Tursiops truncatus*), melon-headed whale (*Peponocephala electra*), pygmy sperm whale (*Kogia breviceps*), dwarf sperm whale (*Kogia sima*), sperm whale (*Physeter macrocephalus*), Steller sea lion (*Eumetopias jubatus*), California sea lion (*Zalophus californianus*), harp seal (*Pagophilus groenlandicus*), harbor seal (*Phoca vitulina*), and northern elephant seal (*Mirounga angustirostris*) were collected during necropsies of wild stranded animals by regional marine mammal stranding networks.

Sample preparation and measurement of Mb oxygen affinity have been previously described (Wright and Davis, in review; for stepwise methods see Appendix A). Briefly, a single stock buffer solution of 50 mM TRIZMA® tris buffer (Sigma-Aldrich, T 0694) with 50 mg l^{-1} gentamicin sulfate (Sigma-Aldrich, G-1264) was used throughout all tissue homogenization, purification, and oxygen dissociation curve (ODC) determination. Horse heart Mb solutions were prepared by reconstitution in deoxygenated stock buffer solution and reduced with sodium dithionite (Sigma-Aldrich,

157953). Dithionite was removed by buffer exchange on a column of G25 Sephadex® (Sigma-Aldrich, G25150) at 4°C by eluting with chilled stock buffer.

For preparation of Mb solutions from vertebrate muscle, small sections of tissue (0.5 g or less) were dissected free of visible fat and connective tissue and homogenized with a glass tissue grinder (Fisher Scientific, 7727-15) in 10 ml chilled buffer g^{-1} tissue. Mb solutions were centrifuged to remove cellular debris and eluted on a column of DEAE-Sephadex® A-50 (Sigma-Aldrich, A50120) using stock buffer to remove hemoglobin contamination. Mb solutions that were too dilute to generate an ODC (minimal Mb concentration of 0.02 mM) were concentrated by freeze centrifugation (Virgen-Ortíz et al., 2012, 2013). Samples were frozen in 3.5 ml aliquots and stored at -80°C until analysis.

Determination of Myoglobin P_{50}

Once Mb solutions were prepared, oxygen dissociation curves were generated using a TCS Hemox Blood Analyzer (TCS Scientific, New Hope, PA). Twenty µl antifoam solution was added to thawed 3.5 ml aliquot samples before being transferred to a sample chamber where they were warmed to 37°C and equilibrated to oxygen saturation by bubbling with air. Once temperature and oxygen saturation were stable, an ODC was generated by optically monitoring the solution while deoxygenating by bubbling with compressed nitrogen to a final partial pressure of oxygen of 0.5 mmHg. The TCS Hemox Data Acquisition System software (V 2.00.13) was used to generate the oxygen dissociation curve in real time and calculate P_{50} values. When possible, samples from five individuals were collected for each species with a sample size large enough for eight replicates from each sample. However, due to the opportunistic nature of sample collection the number and size of samples was inconsistent. Despite this, no species was represented by fewer than 14 replicates (Table 4.1).

Table 4.1 Sample size and mean oxygen affinity (P_{50}) from diving and terrestrial vertebrates.

	n	N	P_{50} ± SD (mmHg)
Terrestrial			
Sheep	3	25	3.74 ± 0.18
Cow	5	40	3.72 ± 0.16
Oryx	4	22	3.72 ± 0.19
White-tailed deer	3	14	3.72 ± 0.17
Horse	* 1	67	3.70 ± 0.09
Cetaceans			
Melon-headed whale	4	48	4.85 ± 0.18
Bowhead whale	5	52	3.82 ± 0.10
Sperm whale	1	21	3.76 ± 0.13
Bottlenose dolphin	6	55	3.75 ± 0.17
Kogia sp.	5	54	3.74 ± 0.14
Common dolphin	2	21	3.73 ± 0.15
Spinner dolphin	2	18	3.62 ± 0.14
Risso's dolphin	1	21	3.54 ± 0.09
Pinnipeds			
Steller sea lion	4	29	3.81 ± 0.14
California sea lion	5	45	3.65 ± 0.10
Harbor seal	5	55	3.52 ± 0.18
Harp seal	5	60	3.51 ± 0.16
N. elephant seal	5	47	3.24 ± 0.10
Weddell seal	5	70	3.23 ± 0.22
Birds			
Redhead duck	5	42	3.36 ± 0.21
Macaroni penguin	2	18	3.34 ± 0.14
Chinstrap penguin	2	20	2.94 ± 0.22
Adelie penguin	2	17	2.86 ± 0.14
Emperor penguin	5	46	2.47 ± 0.17
King penguin	4	43	2.40 ± 0.23
Total	91	950	

* Horse heart Mb was purchased from commercially purified and lyophilized Mb.

Aerobic Dive Limit

The standard for quantifying routine vertebrate diving ability is the aerobic dive limit (ADL), which is the maximal duration an animal can remain submerged without appreciable increase in lactic acid resulting from anaerobic metabolism. It is not feasible to measure an ADL in most diving vertebrates (Kooyman et al., 1983), so a calculated ADL (cADL) can be estimated based on diving metabolic rate and useable oxygen stores in animals for which these physiological measures are known or can be reasonably estimated (Butler, 2006). An estimate of ADL based on behavioral diving information is necessary to compare the diving ability of species that have not had an ADL determined experimentally or estimated based on oxygen stores and diving metabolism (cADL). To maximize underwater foraging time, diving vertebrates typically dive within their ADL (Kooyman et al., 1980; Butler, 2004). Weddell seals (Kooyman et al., 1983), elephant seals (Hindell et al., 1992), bottlenose dolphins (Williams et al., 1999), macaroni penguins, and emperor penguins (Green et al., 2003) all make more than 90% of dives within their ADL. Comparison of diving ability based on average dive duration may dramatically underestimate an animal's aerobic diving ability (Noren and Williams, 2000), and a comparison of maximum dive duration can be heavily skewed by extreme anaerobic dive events, which are rare and may be three times the ADL (Kooyman et al., 1980). For species without a published ADL or cADL, we estimated aerobic dive duration using published average dive durations plus one SD, which is a reasonable approximation for a behaviorally determined ADL and results in the maximal dive duration that includes approximately 85% of recorded dives. These estimates were combined with published ADL and cADL estimates to test for correlations between ADL and P_{50}.

Comparison of Myoglobin Structure

Of the 25 species in our study, the complete Mb amino acid sequences of 17 were available in the Uniprot protein database (UniProt Consortium, 2013; www.uniprot.org; see Appendix B for accession numbers by species). Sequences for

three terrestrial ungulates, seven cetaceans, six pinnipeds, and one bird were aligned for comparison using Jalview 2.8 (Waterhouse et al., 2009). A consensus sequence representing the amino acids most common at each site of the multiple alignment was generated for comparison. Conservation of individual amino acids was ranked based on retention of physicochemical properties with a score of 8 or greater being considered a conservative substitution (Livingstone and Barton, 1993). RCSB PDB Ligand Explorer (http://www.rcsb.org) (Bernstein et al., 1977) was used to determine amino acids within interactive distance (taken at 5Å) to the heme, distal pocket (DP) and Xe binding pockets using sperm whale Mb (PDB accession # 1J52 and 1MBO).

Data Analysis

Because *Kogia breviceps* (n=3) and *Kogia sima* (n=2) are phylogenetically similar and share 100% Mb sequence identity, samples from these two species were pooled to form a single *Kogia* group. All statistical analysis was performed with SPSS 15.0 software (IBM Corporation, Somers, NY, USA). Due to unequal variance (Levene test, $p < 0.001$), a Welch test was used to compare Mb oxygen affinity among species. Games-Howell pairwise comparisons were used to form groupings of statistically indistinguishable Mb oxygen affinity.

Results

Myoglobin Oxygen Affinity

Mb P_{50} varied significantly among species (Welch test, $p < 0.001$) and ranged from 2.40 mmHg for emperor penguins to 4.85 mmHg for melon-headed whales (Table 4.1 and Figure 4.1). Pairwise comparison of interspecies P_{50} showed that the terrestrial ungulates and the majority of the cetaceans formed a series of non-exclusive overlapping groups (Figure 4.2). The P_{50} among terrestrial ungulates (horse, deer, oryx, cow, and lamb) were not significantly different (mean of 3.72 ± 0.15 mmHg).

In addition to the overlapping groups composed primarily of terrestrial ungulates and cetaceans, one cetacean and three additional groups varied significantly from all

others (Figure 4.2). Melon-headed whales had a significantly lower P_{50} (4.85 mmHg) than all other species. The closely related king and emperor penguins comprised a group with the lowest P_{50} (mean of 2.44 ± 0.20 mmHg). Closely related Adélie and chinstrap penguins comprised a group with the next lowest P_{50} (mean of 2.90 ± 0.19 mmHg). The Weddell and northern elephant seals had the lowest P_{50} among the mammals although they grouped with the redhead duck and the macaroni penguin, which had the highest P_{50} among the birds (mean of 3.27 ± 0.19 mmHg).

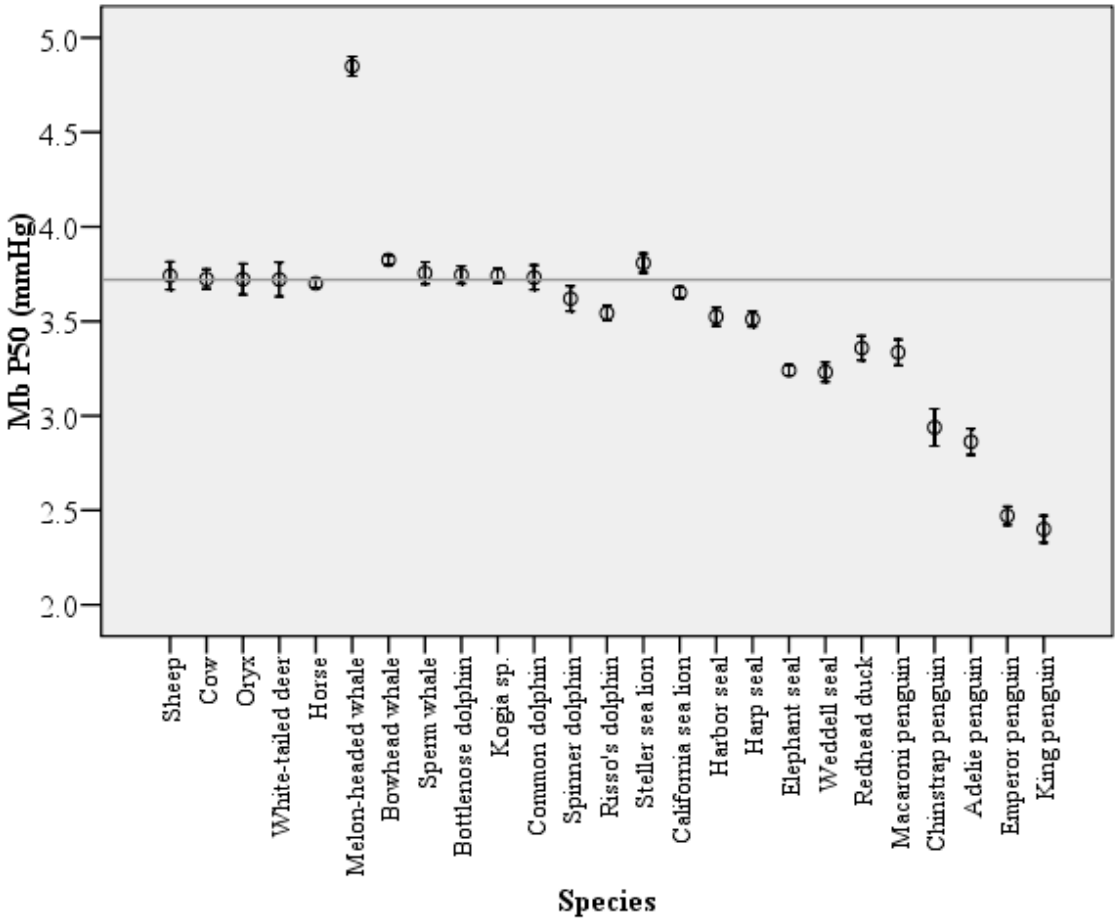

Figure 4.1 Mean myoglobin (Mb) oxygen affinity (P_{50} ± 2 SEM) for a variety of species. Horizontal reference line is at 3.72 corresponding to the P_{50} of commercially available terrestrial horse heart Mb.

King penguin	2.40					
Emperor penguin	2.47					
Adelie penguin		2.86				
Chinstrap penguin		2.94				
Weddell seal			3.23			
Elephant seal			3.24			
Macaroni penguin			3.34			
Redhead duck			3.36			
Harp seal				3.51		
Harbor seal				3.52	3.52	
Risso's dolphin				3.54	3.54	
Spinner dolphin				3.62	3.62	
California sea lion				3.65	3.65	
Horse				3.70	3.70	
White-tailed deer				3.72	3.72	
Oryx				3.72	3.72	
Cow				3.72	3.72	
Common dolphin				3.73	3.73	
Sheep				3.74	3.74	
Kogia sp.				3.74	3.74	
Bottlenose dolphin				3.75	3.75	
Sperm whale				3.76	3.76	
Steller sea lion					3.81	
Bowhead whale					3.82	
Melon-headed whale						4.85

Figure 4.2 Mb oxygen affinity of diving and terrestrial birds and mammals. Columns represent grouping based on Games-Howell pairwise comparison of species with statistically similar Mb oxygen affinity ($p < 0.05$).

Among the diving species, there was a broad range of aerobic diving abilities among cetaceans (3.7 – 51 min), pinnipeds (2.3 – 30 min), and birds (0.5 – 5.7 min) (Table 4.2). For cetaceans, there was no significant correlation between the ADL and Mb oxygen affinity (r^2=0.0113, P=0.894) (Figure 4.3). Among pinnipeds and among birds, there was a significant negative correlation between ADL and Mb P_{50} (i.e., a positive correlation between ADL and Mb oxygen affinity) (r^2=0.8047, P=0.015 and r^2=0.8177, P=0.013, respectively).

Comparison of Myoglobin Structure

Available Mb sequences for this study consisted of 153 amino acids (Figure 4.4). Among these, 81 of the 153 amino acid sites (53%) were completely conserved, and 115 (75%) were highly conserved with a conservation ranking (based on physicochemical properties) of 8 or greater. Of the 30 amino acids lining the conserved pocket regions (heme pocket, DP, and Xe 1-4) (Table 4.3), 21 (70%) were completely conserved and 27 (90%) were highly conserved (Figure 4.4). Much of the variation in alignment was due to the emperor penguin, which was the only bird in our study for which an amino acid sequence was available. Among the mammals, 104 amino acids (68%) were completely conserved and 130 (85%) were highly conserved. Of the 30 amino acids lining the pocket regions, 26 (87%) were completely conserved and 29 (97%) were highly conserved with the one seemingly inconsequential non-conservative T67V horse substitution. There were 39 unique variants in emperor penguin Mb not seen in any other more distantly related mammalian species in the comparison. Of these variants, three conservative substitutions were located in the lining of the heme pocket including the K42R, S92T, and I99V in addition to the less conservative A71Q variant.

Table 4.2 Aerobic dive limits for species in this study determined experimentally (ADL), as calculated estimates (cADL), or estimated based on behavioral data as described in the text.

	ADL (min)	Reference
Cetaceans		
Bowhead whale	16.7 [c]	Simon et al., 2009 *
Sperm whale	51.4 [c]	Watwood et al., 2006
Bottlenose dolphin	3.7 [b]	Williams et al., 1999
Kogia sp.	23.9 [c]	Barlow et al., 1997
Pinnipeds		
Steller sea lion	2.5 [d]	Gerlinsky et al., 2013
California sea lion	2.3 [b]	Ponganis et al., 1997c
Harbor seal	4.5 [c]	Stewart et al., 1989
Harp seal	10.5 [c]	Folkow et al., 2004 [+]
N. elephant seal	30 [c]	Hassrick et al., 2010 [‡]
Weddell seal	20 [a]	Kooyman et al., 1980
Birds		
Redhead duck	0.5 [e]	Furilla and Jones, 1986; Stephenson et al., 1986
Macaroni penguin	2.1 [d]	Green et al., 2003
Chinstrap penguin	2.2 [d]	Culik et al., 1994
Adelie penguin	1.8 [d]	Culik et al., 1994
Emperor penguin	5.6 [a]	Ponganis et al., 1997a
King penguin	5.7 [c]	Kooyman et al., 1992; Le Vaillant et al., 2012

[a] ADL determined experimentally in free diving wild animals.
[b] ADL determined experimentally in trained diving animals.
[c] ADL as mean dive duration of free diving wild animals plus 1 SD as described in text.
[d] ADL calculated as useable oxygen stores divided by diving metabolic rate (cADL).
[e] ADL estimated based on mean dive duration of redhead duck and cADL of tufted duck.
* Averages calculated from U and V shaped mean dive durations plus 1 SD.
[+] Averages calculated from DU index 1993 data.
[‡] Averages calculated from conditioned “recovery” week dives.

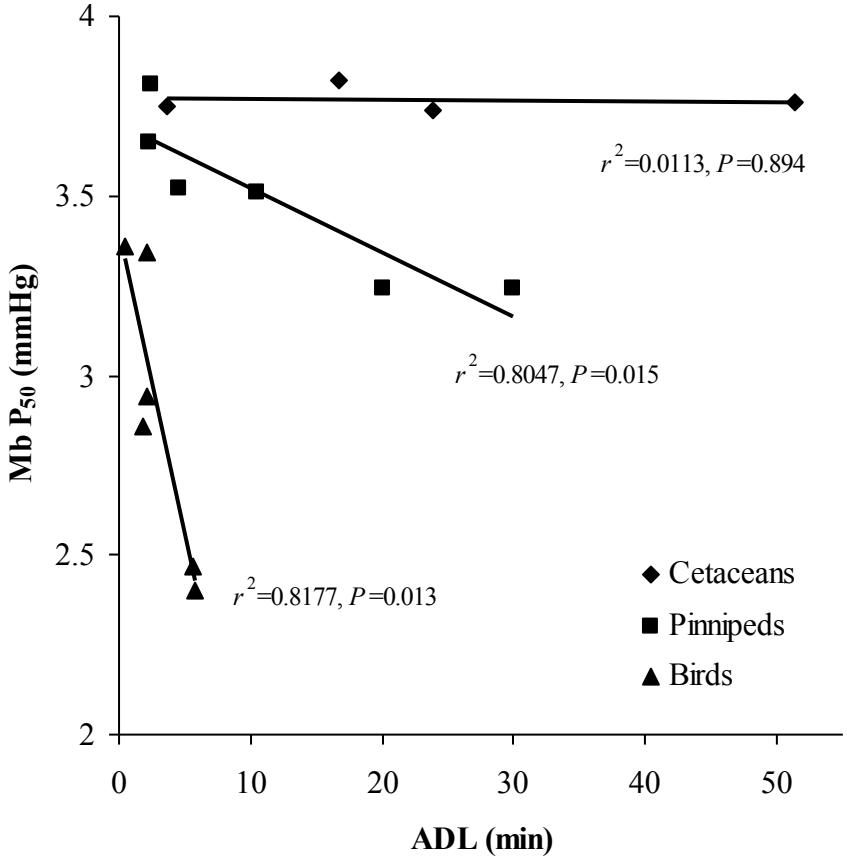

Figure 4.3 Mean Mb oxygen affinity (P_{50}) as a function of dive duration (ADL) for cetaceans, pinnipeds, and birds.

Figure 4.4 Multiple alignment of Mb sequences available from the Uniprot protein database using Jalview 2.8. Amino acid site conservation was ranked based on conservation of physiochemical properties. Amino acids lining the highly conserved heme, distal and Xe pockets are boxed for identification.

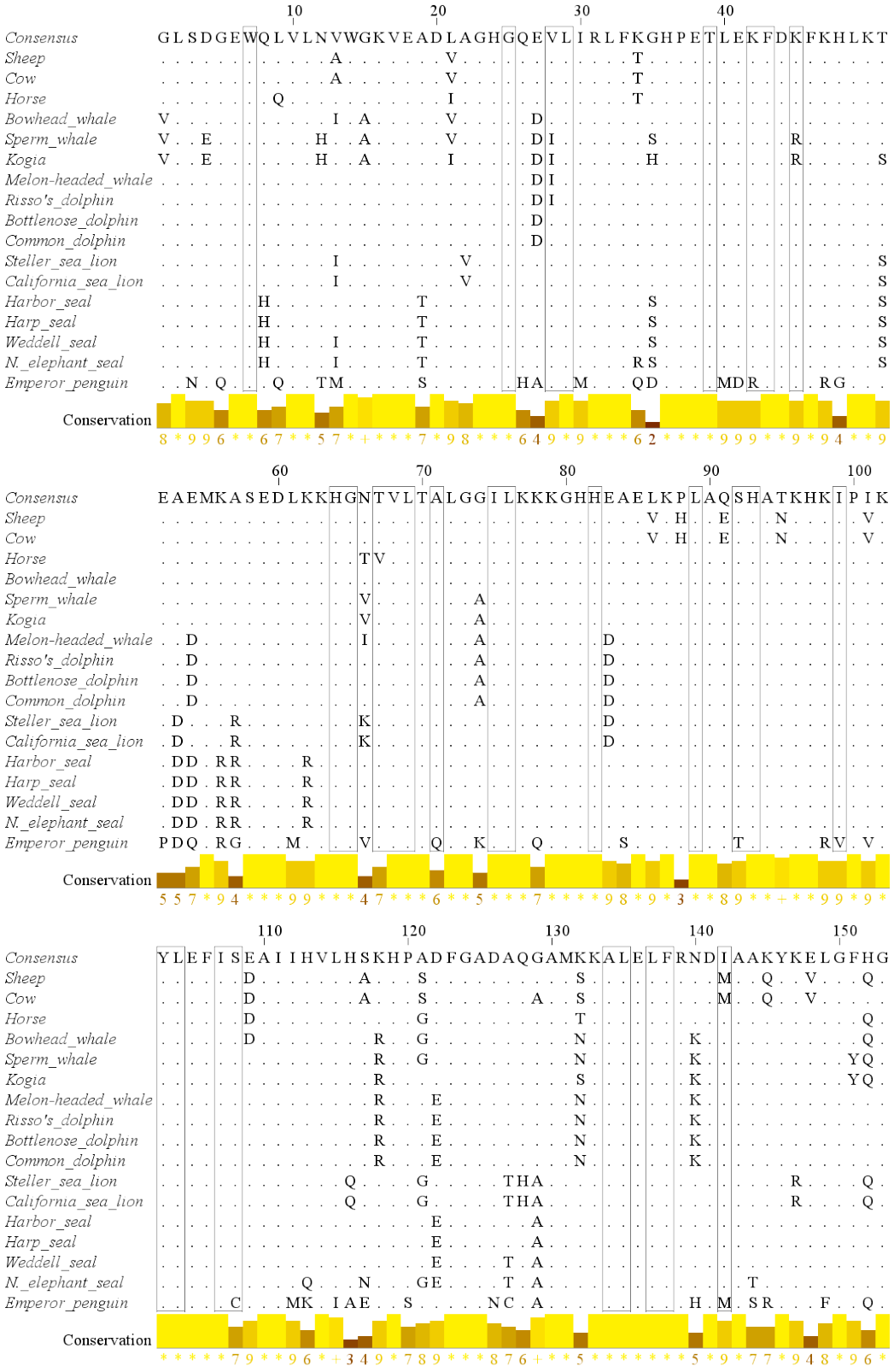
10
20
30
40
Consensus G L S D G E W Q L V L N V W G K V E A D L A G H G Q E V L I R L F K G H P E T L E K F D K F K H L K T
Sheep . . . . . A . . . . . V . . . . . T . . . . . .
Cow . . . . . A . . . . . V . . . . . T . . . . . .
Horse . . . . . Q . . . . . I . . . . . T . . . . . .
Bowhead_whale V . . . . . I . A . . . . V . . . . . D . . . . . .
Sperm_whale V . . E . . . . . H . . A . . . . . V . . . . . D I . . . . . S . . . . . R . . . . .
Kogia V . . E . . . . . H . . A . . . . . I . . . . . D I . . . . . H . . . . . R . . . . . S
Melon-headed_whale . . . . . D I . . . . . .
Risso's_dolphin . . . . . D I . . . . . .
Bottlenose_dolphin . . . . . D . . . . . .
Common_dolphin . . . . . D . . . . . .
Steller_sea_lion . . . . . I . . . . . V . . . . . S
California_sea_lion . . . . . I . . . . . V . . . . . S
Harbor_seal . . . . . H . . . . . T . . . . . S . . . . . S
Harp_seal . . . . . H . . . . . T . . . . . S . . . . . S
Weddell_seal . . . . . H . . . . I . . . . T . . . . . S . . . . . S
N._elephant_seal . . . . . H . . . . I . . . . T . . . . . R S . . . . . S
Emperor_penguin . . N . Q . . . Q . . TM . . . . S . . . . . HA . . M . . . QD . . . MD R . . . . RG . . .
Conservation
8 * 9 9 6 * * 6 7 * * 5 7 * + * * * 7 * 9 8 * * * 6 4 9 * 9 * * * 6 2 * * * * 9 9 9 * * 9 * 9 4 * * 9
60
70
80
90
100
Consensus E A E M K A S E D L K K H G N T V L T A L G G I L K K K G H H E A E L K P L A Q S H A T K H K I P I K
Sheep . . . . . V . H . . E . . . N . . . . . V .
Cow . . . . . V . H . . E . . . N . . . . . V .
Horse . . . . . T V . . . . . .
Bowhead_whale . . . . . .
Sperm_whale . . . . . V . . . . . A . . . . . .
Kogia . . . . . V . . . . . A . . . . . .
Melon-headed_whale . . D . . . . . I . . . . . A . . . . . D . . . . . .
Risso's_dolphin . . D . . . . . A . . . . . D . . . . . .
Bottlenose_dolphin . . D . . . . . A . . . . . D . . . . . .
Common_dolphin . . D . . . . . A . . . . . D . . . . . .
Steller_sea_lion . D . . . R . . . . . K . . . . . D . . . . . .
California_sea_lion . D . . . R . . . . . K . . . . . D . . . . . .
Harbor_seal . D D . R R . . . . R . . . . . .
Harp_seal . D D . R R . . . . R . . . . . .
Weddell_seal . D D . R R . . . . R . . . . . .
N._elephant_seal . D D . R R . . . . R . . . . . .
Emperor_penguin P D Q . R G . . . M . . . . V . . . . Q . . K . . . Q . . . . S . . . . . T . . . . R V . V .
Conservation
5 5 7 * 9 4 * * * 9 9 * * * 4 7 * * * 6 * * 5 * * * 7 * * * * 9 8 * 9 * 3 * * 8 9 * * + * * 9 9 * 9 *
110
120
130
140
150
Consensus Y L E F I S E A I I H V L H S K H P A D F G A D A Q G A M K K A L E L F R N D I A A K Y K E L G F H G
Sheep . . . . . D . . . . . A . . . S . . . . . S . . . . . M . . Q . . V . . . Q .
Cow . . . . . D . . . . . A . . . S . . . . . A . S . . . . . M . . Q . . V . . . . .
Horse . . . . . D . . . . . G . . . . . T . . . . . Q .
Bowhead_whale . . . . . D . . . . . R . . G . . . . . N . . . . . K . . . . . Q .
Sperm_whale . . . . . R . . G . . . . . N . . . . . K . . . . . Y Q .
Kogia . . . . . R . . . . . S . . . . . K . . . . . Y Q .
Melon-headed_whale . . . . . R . . E . . . . . N . . . . . K . . . . . .
Risso's_dolphin . . . . . R . . E . . . . . N . . . . . K . . . . . .
Bottlenose_dolphin . . . . . R . . E . . . . . N . . . . . K . . . . . .
Common_dolphin . . . . . R . . E . . . . . N . . . . . K . . . . . .
Steller_sea_lion . . . . . Q . . . G . . . . . T H A . . . . . R . . . Q .
California_sea_lion . . . . . Q . . . G . . . . . T H A . . . . . R . . . Q .
Harbor_seal . . . . . E . . . . . A . . . . . .
Harp_seal . . . . . E . . . . . A . . . . . .
Weddell_seal . . . . . E . . . T . A . . . . . .
N._elephant_seal . . . . . Q . . N . . . G E . . . T . A . . . . . T . . . . . .
Emperor_penguin . . . . C . . . M K . I A E . . S . . . . . N C . A . . . . . H . M . S R . . . F . . Q .
Conservation
* * * * * 7 9 * * 9 6 * + 3 4 9 * 7 8 9 * * * 8 7 6 + * * 5 * * * * * * * 5 * 9 * 7 7 * 9 4 8 * 9 6 *

Table 4.3 Amino acid sites forming hydrophobic pockets that come within interactive distance of ligands bound by sperm whale myoglobin. RCSB PDB Ligand Explorer was used to select amino acids within 5Å of bound O_2 (PDB accession # 1MBO) for the distal pocket (DP), and Xe and heme (PDB accession # 1J52) for the corresponding pockets.

Pocket	Amino acids
DP	L29, F43, H64, V68
Heme	T39, K42, F43, R45, H64, T67, V68, A71, L89, S92, H93, I99, Y103, L104
Xe1	L89, H93, L104, F138, I142
Xe2	L104, I107, S108, L135, F138
Xe3	W7, I75, L76, H82, A134, L137, F138
Xe4	G25, I28, L29, G65, V68, L69, I107

Discussion

Mutations in Mb structure can affect its oxygen binding properties and stability (Scott et al., 2001; Ochiai et al., 2009; Dasmeh et al., 2013), and interspecies variability generates different phenotypes that are subject to natural selection (Naylor et al., 2000; Wittenberg 2007). Comparing interspecies differences in Mb oxygen affinity from a variety of endothermic vertebrates revealed conservation of P_{50} within a narrow range of 2.45 mmHg despite considerable variability in amino acid sequence. However, among species in this study with available Mb sequences, 47% of amino acid sites showed some level of substitution.

Given the broad range of oxygen affinities that are possible by even a single amino acid substitution (Dasmeh et al., 2012), it appears that the Mb oxygen affinity of endothermic vertebrates is conserved within a narrow range to maintain optimal muscle performance. Marcinek et al. (2001) found evidence for conservation of Mb oxygen affinity in fish with different body temperatures. Among closely related fish species, Mb oxygen affinity measured at 20^{o}C was higher for species that maintain a higher body temperature and lower for lower body temperature fish. When adjusted for physiological body temperature, these oxygen affinities converge. This apparent adaptive conservation of Mb oxygen affinity suggests there may be an optimal value for supporting aerobic respiration under specific physiological conditions. While available

evidence suggests that Mb oxygen affinity is conserved for animals with similar physiology, it is less clear if interspecies variation within the narrow range of oxygen affinity provides an adaptive advantage for species that routinely experience hypoxia during breath-hold diving.

Diving species have an array of multi-level adaptations to cope with recurrent breath-holding and the subsequent hypoxia and muscular ischemia (Davis, 2014). Elevated concentrations of respiratory pigments have obvious adaptive advantages for maintaining aerobic metabolism in diving birds and mammals, and recent studies have examined the potential adaptive molecular evolution of the functional properties of these pigments (Meir and Ponganis, 2009; Soegaard et al., 2012; Helbo and Fago, 2012; Schneuer et al., 2012; Mirceta et al., 2013).

The oxygen affinity of terrestrial ungulates in this study was highly conserved with a mean P_{50} of 3.7 ± 0.15 mmHg. With the exception of melon-headed whales, there was no significant difference in the P_{50} of cetaceans and terrestrial ungulates. Helbo and Fago (2012) also found the P_{50} of toothed whales to be similar to that in horse and concluded that in these animals, Mb's contribution to diving ability is achieved primarily by increasing the concentration rather than altering oxygen affinity. They also noted slightly higher P_{50} in mysticete whales compared to odontocetes which, with the exception of melon-headed whales, was also supported in our study. Our results agree with Helbo and Fago (2012) in that no significant correlation between average dive duration and P_{50} was observed in cetaceans. However, there was a significant trend for diving seals and penguins with a longer ADL to have a lower P_{50}. These correlations do not consider all physiological and behavioral factors that influence ADL such as body mass, diving metabolic rate, total Mb oxygen store, and total Hb oxygen store, but they indicate that within these groups there is a trend for longer duration divers to exhibit a greater Mb oxygen affinity.

Genetic variation in globin function produces phenotypes that are subject to natural selection (Wittenberg, 2007). Myoglobin has experienced an increased rate of evolution in cetactans, suggesting selection pressure in diving vertebrates (Nery et al.,

2013b). Dasmeh et al., (2013) found that mutations that increase Mb protein fold stability are positively selected for in cetaceans, and this increase in protein stability is positively correlated with Mb concentration in these species. In addition, Mirceta et al. (2013) found that Mb concentration correlates positively with protein surface charge in a variety of mammalian divers suggesting a convergent adaptation for maintaining Mb solubility when expressed in high concentrations.

Among the cetaceans, P_{50} ranged from 3.54 mmHg in the Risso's dolphin to 4.85 mmHg in melon-headed whales. Although these P_{50} were significantly different, melon-headed whale Mb only varied from that of Risso's dolphin by a single N66I substitution which, like the N66V substitution found in *Kogia* and sperm whales, increases protein stability (Dasmeh et al., 2013). Mutants of sperm whale Mb at this site have shown modest changes in oxygen affinity (Scott et al., 2001). Due to its location, variation at amino acid 66 has also been hypothesized to shift the oxygen affinity of beluga whale Mb (Stewart et al., 2004). Although amino acid 66 is not located in the heme pocket, it is in close structural proximity and adjacent to amino acids 67 and 68, which line the heme and distal pockets, respectively. It is unclear if this shift in Mb oxygen affinity indicates unique selective adaptation in melon-headed whales or is the result of less directed genetic variation.

The group formed by Adélie and chinstrap penguins had a mean P_{50} of 2.9 mmHg. This is very similar to the previously reported value of 3 mmHg for these species at 40°C (Weber et al., 1974). The slight difference in oxygen affinity between these two studies is diminished if the temperature shift in Mb oxygen affinity is considered.

It is difficult to identify individual amino acid variants that may be responsible for the increased oxygen affinity in emperor penguin Mb due to the large amount of variation from the consensus sequence (Figure 4.4). This is also partly due to the fact that emperor penguins were the only birds in our study whose Mb structure was available in the Uniprot database. The close proximity of the S92T variant to the proximal histidine (H93) may contribute to the high oxygen affinity of emperor penguin

Mb and provides an interesting subject for future point mutation studies. Interestingly, the Mb of emperor penguins also have the identical N66V substitution which appears selected for to increase Mb stability in the deep diving *Kogia* and sperm whale (Dasmeh et al., 2013). The L61 amino acid is in close proximity to the distal histidine and was found to influence oxygen binding in mutational studies (Dasmeh et al., 2012). Although it is a conservative substitution, the L61M emperor penguin substitution may impart some variance to oxygen affinity.

Globin Adaptation to Hypoxia

Oxygen binding globin proteins are obvious candidates for molecular adaptation in diving animals that experience regular hypoxia (Nery et al., 2013 a; Nery et al., 2013 b), and a growing body of evidence supports this hypothesis. Myoglobin oxygen affinity is variable among species, but could the modest variability observed in this study impart an advantage in managing intramuscular oxygen stores under certain physiological conditions? A compensatory shift to account for variability in muscle temperature as observed in fish (Marcinek et al., 2001) could be possible. However, even during prolonged dives, active muscle temperature is maintained at near 37°C in emperor penguins (Ponganis et al., 2003) and Weddell seals (Ponganis et al., 1993b). The absence of an elevated muscle temperature during diving would eliminate the increased oxygen affinity of these species as an adaptation for temperature compensation, and any chilling of muscle temperature would exacerbate the elevated oxygen affinity. Therefore it is unlikely that the shift in oxygen affinity of these species is driven by temperature.

There is considerable evidence that increased oxygen affinity of respiratory globins is advantageous for animals in hypoxic environments. Sprague-Dawley rats that had their Hb oxygen affinity artificially elevated with sodium cyanate had better survivability and lower experimental heart rates in a hypoxic environment (Eaton et al., 1974), and molecular adaptation favoring high affinity Hb is typical in animals that routinely endure hypoxia (Bunn, 1980; Storz, 2007). Burrowing rodents that experience hypoxic environments in underground burrows exhibit an increased Hb oxygen affinity

(Revsbech et al., 2013), and high altitude adapted animals such as bar-headed geese (*Anser indicus*) (Jessen et al., 1991), vicuña (*Vicugna vicugna*) (Hall et al., 1936), and deer mice (*Peromyscus maniculatus*) (Storz et al., 2009) also possess high oxygen affinity Hb compared to their low altitude counterparts.

Hemoglobin appears left shifted in diving harbor porpoises (Soegaard et al., 2012) and emperor penguins (Meir and Ponganis, 2009) compared to terrestrial animals, but there is no clear trend for an adaptive shift in Hb oxygen affinity in diving vertebrates (Davis, 2014). While high affinity Hb may be an advantage when oxygenating blood in a hypoxic environment (e.g., high altitude), this subsequently reduces the gradient for offloading oxygen at the blood-tissue interface (Storz, 2007; Revsbech et al., 2013). An increase in Mb oxygen affinity could increase the diffusion gradient into muscle cells, but potentially reduce the intracellular diffusion gradient for oxygen transport from the sarcolemma to the mitochondria. To effectively facilitate oxygen diffusion, it is critical that the Mb P_{50} be near the partial pressure of oxygen in active muscle to ensure partial saturation of Mb and maintain a diffusive gradient (Marcinek et al., 2001; Wittenberg, 2007). If Mb is acting to facilitate oxygen diffusion to active muscle under conditions of reduced convective oxygen transport, a greater Mb oxygen affinity could have an adaptive advantage for maintaining an intramuscular oxygen diffusion gradient during periods of reduced arterial P_{O_2}.

Models simulating the intramuscular transport of oxygen from the sarcolemma to the mitochondria typically assume an oxygen sink with a P_{O_2} of 0 mmHg at the mitochondria. However, actual mitochondrial P_{O_2} must be some value greater than 0, which would reduce the intracellular O_2 diffusion gradient (Cano et al., 2013). While the significance of this discrepancy may be insignificant in a respiring animal when arterial P_{O_2} and the diffusive gradient are high, it would be more significant as convective oxygen transport, arterial P_{O_2}, and the oxygen diffusion gradient are reduced.

Some long duration divers including elephant seals and emperor penguins tolerate extreme hypoxia by depleting blood oxygen to very low levels by the end of dives (Ponganis et al., 2007; Meir et al., 2009). Despite low arterial oxygen content,

oxygen supply to muscle mitochondria must be maintained by combined contributions of endogenous oxygen stores (MbO_2) and convective oxygen transport to maintain aerobic metabolism (Wright and Davis, 2006). Vasoconstriction in diving vertebrates reduces convective oxygen transport to muscle tissues, and oxygen supply is further reduced as arterial P_{O_2} drops throughout the dive. As a result, unique muscle conditions in diving vertebrates may shape the functional role of Mb.

Recently, attempts have been made to model the functional role of Mb with various oxygen affinities under different conditions (Lin et al., 2007a; Lin et al., 2007b; Dasmeh and Kepp, 2012). Myoglobin has distinct roles of transport and storage of oxygen and they are affected differently by mutations that alter Mb oxygen affinity. As an oxygen store, Mb with a greater oxygen affinity functions better in both normoxic and hypoxic conditions, but this advantage is amplified under hypoxic conditions (Dasmeh and Kepp, 2012).

The contribution of Mb facilitated oxygen diffusion to total oxygen flux increases with Mb concentration, and at the high concentrations found in marine mammals, Mb facilitated oxygen transport dominates over free oxygen diffusion (Lin et al., 2007a, 2007b). Variants in Mb oxygen affinity also affect the oxygen transport role of Mb. Under normoxic conditions, Mb mutants with a lower oxygen affinity function better at oxygen transport, but under hypoxic conditions, high affinity Mb mutants are advantageous (Lin et al., 2007b; Dasmeh and Kepp, 2012). An increased oxygen affinity could maintain oxygen transport in hypoxic muscle when convective oxygen transport is low providing greater efficiency (greater fractional extraction) in extracting oxygen from low P_{O_2} arterial blood.

Variations in Mb oxygen affinity have a greater effect on oxygen storage and transport under hypoxic conditions. Therefore, Mb function under hypoxic conditions provides the primary selective pressure to preserve Mb function (Dasmeh and Kepp, 2012). Recent models of Mb oxygen storage and transport (Lin et al., 2007b; Dasmeh and Kepp 2012) suggest that under conditions of muscular hypoxia, high Mb

concentrations and Mb with high oxygen affinity would be an advantage for both storage and transport of oxygen.

Conclusions

Functional properties of oxygen binding globin proteins such as Mb are mutable and subject to natural selection, and the oxygen affinity of Mb appears to be conserved within a narrow range for terrestrial and aquatic birds and mammals. Small variation within this range may be significant and adaptive for animals that routinely exercise during hypoxia and manage intramuscular transport and storage of oxygen differently. Diving birds and long duration diving seals have Mb oxygen affinities that are significantly greater than terrestrial ungulates, and within these groups there is a trend for greater Mb oxygen affinity in animals with greater diving ability. This increase in Mb oxygen affinity may be adaptive for enhanced oxygen flux during muscular ischemia and hypoxia or could be secondarily adaptive for other roles that protect hypoxic muscle tissue. An adaptive increase in Mb oxygen affinity in deep diving mammals and birds is consistent with other studies demonstrating adaptive increase in globin oxygen affinity in animals that routinely endure hypoxia. Previous studies have demonstrated adaptation of cetacean Mb for greater structural stability and surface charge, but Mb oxygen affinity does not appear to be directly affected in this group of divers.

CHAPTER V
SUMMARY:
ADAPTIVE EXPRESSION AND MOLECULAR EVOLUTION OF MYOGLOBIN IN DIVING BIRDS AND MAMMALS

Myoglobin (Mb) is an oxygen binding hemoprotein in vertebrate cardiac and skeletal muscle. The expression of Mb is up-regulated in response to tissue hypoxia, and diving birds and mammals can have concentrations ten-fold those of terrestrial species. A physiological model simulating Weddell seal diving indicated that Mb concentration is optimized for the type and duration of dives that are typically made (Wright and Davis, 2006). Diving mammals such as cetaceans have an increased rate of Mb evolution (Nery et al., 2013b) and adaptive shifts in its functional properties such as increased surface charge (Mirceta et al., 2013) and protein stability (Dasmeh et al., 2013). My research showed that Mb P_{50} also varies among species of diving birds and seals (but not cetaceans), with oxygen affinity correlating positively with dive duration. Myoglobin has additional enzymatic functions that influence intracellular nitric oxide concentration and reduction of reactive oxygen species that maintain cellular homeostasis and redox stability in hypoxic muscle. As a result, the increased concentration and functional properties of Mb maintain aerobic metabolism and minimize hypoxic cellular damage in ischemic muscle.

Myoglobin Concentration

Diving birds and mammals have increased oxygen stores in the blood (increased blood volume and hemoglobin concentration) and muscle (increased myoglobin concentration) to enhance aerobic dive duration. A dive response (apena, bradycardia and peripheral vasoconstriction) enables the efficient use of blood and muscle oxygen stores, but it is exercise modulated to maximize the aerobic dive limit at various levels of exertion (Davis and Williams, 2012). For Mb-bound oxygen to become available for aerobic metabolism, the intracellular partial pressure of oxygen in the muscle must be

less than 10 mmHg (1.3 kPa); in other words, active muscles must become hypoxic (but not anaerobic) (Davis and Kanatous, 1999; Davis, 2014). However, cardiac output must remain sufficient to maintain convective oxygen transport for aerobic respiration in the heart, brain, and splanchnic organs. A balanced dive response is therefore critical for the animal to efficiently manage blood and muscle oxygen stores, and the aerobic dive limit (ADL) is maximized when blood and muscle oxygen stores are depleted simultaneously (Davis and Kanatous, 1999; Davis and Williams, 2012).

The concept of symmorphosis proposes that within an organism, the capacity of individual components of a biological system are matched to demand so that no single component is either excessive or exclusively limiting to system function (Weibel et al., 1991). Weibel and Hoppeler (2004) proposed that compensatory adaptations in the muscle of Mb knockout mice demonstrate this symmorphosis in the cardiorespiratory system where components of oxygen transport from the environment to the mitochondria are optimized. According to this principle, Mb concentration within the muscle of an animal would be neither excessive nor limiting to oxygen demand. To determine if Mb concentration is optimized for the metabolic needs in the muscle of diving vertebrates, I designed a numerical model based on Fick's principle that integrated cardiac output, regional blood flow, convective oxygen transport, muscle oxymyoglobin desaturation and regional rates of oxygen consumption in a Weddell seal. I then modeled the effect of increasing and decreasing Mb concentration in both postabsorptive and postprandial metabolic states on the ADL.

Myoglobin bound oxygen stores limited the ADL of long duration postabsorptive dives to 18 min, and doubling Mb concentration extended dive duration to 24 min. However, dives of this type are rare in free ranging Weddell seals. Most dives are either short duration dives terminated by behavior and not physiological limits, or occur in extended bouts of repetitive aerobic feeding dives with the added metabolic cost of digestion. The model showed that Mb concentration is optimized for physiological conditions that occur in postprandial feeding dives in which the added metabolic cost of digestion and absorption in splanchnic organs caused blood oxygen stores and *not*

muscle oxy-Mb to be limiting. Under these conditions, increased Mb concentration above levels found in the muscle of adult Weddell seals did not increase the ADL. Without a concomitant increase in blood oxygen stores, increased Mb concentration did not prolong the ADL for most routine dives. Because blood-bound oxygen was typically the limiting physiological factor of ADL, constraints that limit blood oxygen (i.e., diminishing returns of increased blood volume, and viscosity limits to hematocrit) also limit the maximum effective Mb concentration. The principles of symmorphosis apply to Weddell seal Mb concentration which is optimized to the ADL of most physiologically constrained dives.

Kanatous et al. (2008) studied the ontogenetic development of Weddell seal muscle as they matured from non-diving pups to adults. As pups are weaned and begin foraging, dive-induced muscle hypoxia stimulates Mb expression. A rapid ontogenetic increase in Mb concentration in juvenile Weddell seals peaks early in the development of diving ability, but decreases to lower adult levels as the seals develop more efficient modes swimming. The inefficient diving of juvenile seals increases the metabolic cost in the muscle and may induce hypoxic stimuli which would not occur in a more mature diver (Kanatous et al., 2008). It appears the upper limit of Mb concentration seen in adult divers is not limited by intracellular constraints of protein concentration, but by a lack of hypoxic stimuli if Mb oxygen is not limiting to most dives.

Myoglobin Adaptation in Diving Vertebrates

Diving vertebrates have molecular adaptations to cope with physiological stress associated with hypoxia including increased ability to remove reactive oxygen species (Yim et al., 2014). Among these molecular adaptations are selective mutations of Mb. The rate of evolutionary change in Mb structure is accelerated in cetaceans, suggesting selective pressure on this oxygen binding protein in diving vertebrates (Nery et al., 2013b). Dasmeh et al. (2013) found that as Mb concentration increased early in cetacean evolution, there was selection for mutations that increased Mb stability by increasing hydrophobic interactions within the globin molecule. Following the ancestral

increase in Mb concentration and stability, cetaceans that secondarily adapted to shorter duration diving had subsequent reductions in Mb concentration and protein stability. They theorized that the positive correlation between Mb concentration and protein stability selects to minimize the intracellular concentration of unfolded protein. Mirceta et al. (2013) found that in several clades of diving mammals, increased Mb concentration was positively correlated with an adaptive increase in Mb surface charge. This increased surface charge was theorized to increase the repulsive effects of protein at high concentration and prevent protein aggregation.

Molecular adaptation of Mb for increased stability and surface charge are suggested mechanisms for coping with elevated levels of expression in vertebrate divers. Our results also suggest an adaptive modification of the functional properties of Mb in some diving vertebrates. Mb oxygen affinity varied significantly among various diving and terrestrial vertebrates. There was a positive correlation between aerobic dive limit and Mb oxygen affinity in diving birds and seals, although this correlation was not apparent in cetaceans. Recent modeling studies indicate that Mb with an increased oxygen affinity may be advantageous for intracellular transport and storage of oxygen in diving vertebrates that endure prolonged ischemic hypoxia in muscle.

Dasmeh and Kepp (2012) modeled the effect of Mb mutants with varied oxygen affinity on the ability of Mb to store and transport oxygen in muscle. Myoglobin with a high oxygen affinity functions better as an oxygen store than low affinity Mb, and the significance of the oxy-Mb loading of oxygen into the cell increases as P_{O_2} decreases. While low affinity Mb functions better as an oxygen transporter when the partial pressure of oxygen is high, high affinity Mb better facilitates oxygen transport at low P_{O_2}. At the high concentrations found in diving vertebrates, Mb makes an increasingly significant contribution to oxygen flux, and this Mb facilitated oxygen diffusion increases as P_{O_2} decreases (Lin et al., 2007b). Dasmeh and Kepp (2012) concluded that the functional role of Mb is most significant under hypoxic conditions, and therefore the strongest selective pressure influencing Mb oxygen affinity is its functional role at low P_{O_2} (Dasmeh and Kepp, 2012). It is therefore unsurprising that high affinity Mb may be

selected for in the muscle of diving vertebrates where ischemia results in prolonged hypoxic conditions.

Speculations and Future Research

Traditionally, Mb was thought to function exclusively in the intracellular management of oxygen. More recent research shows that Mb has additional roles including nitric oxide (NO) scavenging, nitrite reductase activity, and peroxidase activity. Increased concentration of NO reduces mitochondrial respiration by modulating cytochrome c oxidase activity (Taylor and Moncado, 2010). Mb mediated regulation of cellular metabolism and reduction of cellular damage associated with ischemia and reperfusion may be significant in diving vertebrate muscle.

Nitric Oxide

Under normoxic conditions where the oxygen bound form of Mb dominates, ferrous oxy-Mb acts as a NO scavenger producing ferric met-Mb and nitrate (reaction 1).

$$Mb^{(Fe2+)}O_2 + NO \rightarrow Mb^{(Fe3+)} + NO_3^- \quad (1)$$

Under hypoxic conditions where Mb becomes increasingly deoxygenated, Mb becomes a net NO producer as deoxy-Mb reduces nitrite (reaction 2).

$$Mb^{(Fe2+)} + NO_2^- + H^+ \rightarrow Mb^{(Fe3+)} + NO + OH^- \quad (2)$$

With decreasing P_{O_2}, the point at which Mb transitions from being a scavenger to a net producer of NO is dependent on the ratio of oxy-Mb to deoxy-Mb and is therefore Mb P_{50} dependent (Kamga et al., 2012). Species with high Mb oxygen affinity may maintain normal mitochondrial respiration rate and muscular activity under hypoxic conditions which may be adaptive for maintaining aerobic muscular activity. In both of the above reactions, Mb is oxidized to met-Mb which is incapable of binding oxygen or performing other known Mb functions. The enzyme met-Mb reductase reduces oxidized met-Mb to deoxy-Mb where it can once again function to bind oxygen (Hagler et al., 1979). In diving vertebrates, increased met-Mb reductase activity may be needed due to increased overall Mb concentration, as well as potential increased met-Mb production

due to high rates of NO cycling. Future research should examine the concentration and enzymatic function of met-Mb reductase in diving vertebrates.

An increased concentration of NO reduces mitochondrial respiration by modulating cytochrome c oxidase activity (Taylor and Moncado, 2010). Under very hypoxic conditions where the P_{O_2} is below the P_{50} of Mb, elevated Mb concentrations in marine mammal muscle may increase this nitrite reductase activity providing increased protection from ischemia reperfusion injury (Hendgen-Cotta et al., 2008; Jensen, 2009) and conserving limited oxygen in ischemic tissues by reducing tissue metabolism (Shiva et al., 2007; Lundberg et al., 2008; Jensen, 2009). Elevated Mb oxygen affinity is also associated with increased nitrite reductase activity. Marine mammals that express high oxygen affinity Mb at high concentration may have a compounded effect resulting in an increased capacity for NO generation (Helbo and Fago, 2012). Intriguingly, plasma nitrate and nitrite levels were found to be elevated in harbor porpoise above the levels found in similar sized terrestrial mammals which suggests a unique role of nitrogen cycling in marine mammals (Soegaard et al., 2012).

Myoglobin and ROS

Although the dominant pathways for production are debated, ischemic hypoxia in muscle tissue increases production of reactive oxygen species (ROS) leading to potential cellular damage (Clanton, 2007). The biochemistry in the muscle of diving vertebrates results in an increased rate of ROS production (Zentino-Savin et al., 2010), although this is compensated by an increase in antioxidant activity and increased rate of ROS removal (Wilhelm Filho et al., 2002; Zentino-Savin et al., 2010). In addition to reducing oxidative stress by limiting mitochondrial metabolism during hypoxia via NO generation, Mb also has a peroxidase role to directly modulate ROS (Hendgen-Cotta et al., 2010). Mb scavenging of ROS can produce ferryl-Mb which is capable of oxidizing protein and lipid resulting in cellular damage (Kamga et al., 2012). Despite any contribution to oxidation, Mb has a net effect of maintaining intramuscular redox status and mediating ischemia reperfusion injury (Flögel et al., 2004; Kamga et al., 2012).

Unlike nitrite reductase activity, Mb peroxidase activity does not appear to scale with oxygen affinity (Helbo and Fago, 2012) and, therefore, any increase in Mb ROS scavenging capacity in diving species would be due to increased concentration.

Effects of Pressure

Marine mammals and birds are subject to considerable hydrostatic pressure when diving to depth, and pressure experienced in the deep sea can alter the structural stability and rate of reaction of proteins including globins (Mozhaev et al., 1996; Urayama et al., 2002). As hydrostatic pressure is increased, globin proteins experience a compression that increasingly favors a hexacoordinate structure in which the distal histidine binds directly to the heme iron and restricts ligand binding effectively reducing oxygen affinity (Hamdane et al., 2005; Capece et al., 2009). In cetaceans, there appears to be positive selection for mutations that stabilize Mb structure (Scott et al., 2000; Dasmeh et al., 2013), and selection for stability is positively correlated with Mb concentration and subsequently diving duration and depth (Dasmeh et al., 2013). Increased protein stability may reduce hydrostatic effects and conserve Mb function at depth.

Fatty Acid Transport

Recent research by Shih et al., (2014) has demonstrated that oxy-Mb is capable of reversibly binding palmitate (PA), suggesting potential Mb facilitated fatty acid transport in active muscle. The relative PA dissociation constants and diffusion coefficients of Mb and fatty acid binding protein (FABP) suggest that Mb could rival or even surpass FABP in capacity for fatty acid transport, particularly with the elevated Mb concentration found in diving vertebrates. Additionally, deoxy-Mb does not appear to bind PA which suggests a possible oxygen/fatty acid co-transport mechanism where oxygen and fatty acid are bound to Mb at relatively high P_{O_2} near the sarcolemma and simultaneously offloaded at low P_{O_2} near the mitochondria. Because seals and likely other carnivorous diving vertebrates metabolize fatty acids as their primary source of fuel (Davis, 2014), Mb facilitated transport may be significant. The rate of intracellular

fatty acid diffusion may not be limiting in long duration divers such as Weddell seals where metabolic rate is low, but may be more significant in short duration, higher energy divers such as otariids, penguins, and some cetaceans.

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APPENDIX A

PREPARATION OF MYOGLOBIN FOR MEASURING OXYGEN AFFINITY (P_{50})

Lyophilized Horse Heart Myoglobin Standard

1. Prepare a stock solution of 50 mM tris buffer (Sigma-Aldrich, T 0694) with the addition of 50 mg/l gentamicin sulfate (Sigma-Aldrich, G-1264). The pH of the buffer should be 7.4 at 37°C and 8.26 at 5°C.

2. In a 50 ml flask, bubble 9 ml of stock buffer solution with 100% nitrogen for 10 min while stirring on ice. Add 110 mg sodium dithionite (Sigma-Aldrich, 157953) and continue bubbling for an additional 5 min to complete deoxygenation.

3. Add 16 mg lyophilized horse heart Mb (Sigma-Aldrich, M1882) and continue bubbling with nitrogen for one additional min to produce a reduced and deoxygenated Mb solution that will be dark red in color.

4. Remove dithionite from the solution using buffer exchange chromatography on a bed of G-25 Sephadex (Sigma-Aldrich, G25150) with a bed volume of 5 ml packed media per ml of Mb solution. Elute with stock buffer solution and collect the red Mb fraction. Incomplete removal of dithionite will result in Mb oxidation when frozen.

5. Freeze 1 ml aliquots of the Mb solution for later use.

Myoglobin Extraction from Frozen Muscle

1. Cut approximately 0.5 g of muscle into small pieces (approximately 3 mm on a side) removing visible fat and connective tissue (easiest while tissue is still partially frozen).

2. Homogenize the tissue in 10 ml chilled stock buffer g^{-1} muscle using a glass tissue homogenizer chilled on ice.

3. Decant the homogenized Mb solution into a centrifuge tube and centrifuge at 1,200 g for 20 minutes at 4°C.

4. The following solutions and procedures should be performed at 4°C. Prepare a column of DEAE-Sephadex A-50 (Sigma-Aldrich, A50120) with a bed volume of two times the sample volume according to manufacturer directions. Pour the Mb solution

into the column and elute with stock buffer. (While eluting the Mb solution, Hb movement is slowed in the column due to difference in size and isoelectric points of Hb and Mb.)

5. Samples that are too dilute can be concentrated by freeze centrifugation as described by Virgen-Ortíz et al., (2012, 2013). Pour 3.5 ml of Mb solution into a 5 ml plastic cryovial with a conical bottom. Cap the vial, and freeze upside down sitting on the cap in -80°C freezer. Remove the cryovial from the freezer and burn or cut a small hole at the conical end. Place the cryovial in a centrifuge tube that is slightly larger than the cryovial. This should form a sleeve around the cryovial that keeps it off the bottom of the tube and collects fluid. Centrifuge the frozen sample to elute the concentrated protein from the ice as it thaws. Centrifuge speed and duration can be adjusted to achieve the desired concentration.

Myoglobin Oxygen Dissociation Curve Using TCS Hemox Analyzer

Myoglobin solutions of 3.5 ml and at least 0.02 mM concentration are needed to accurately determine Mb oxygen affinity using the TCS Hemox Analyzer.

An increase in gas flow rate and stir speed greater than the recommended factory settings are needed to achieve a deoxygenation to 0.5 mmHg P_{O_2} in approximately 8 minutes. In addition, high sensitivity membranes (TCS Scientific, HSM-50) are needed for the oxygen electrode.

1. Thaw or prepare a 3.5 ml aliquot of Mb solution at 4°C.

2. Add 20 µl of antifoam solution (TCS Scientific, AFA-25) and heat in a 37°C water bath for 8 minutes.

3. Add the warmed Mb solution to the Hemox chamber and bubble with compressed air to oxygenate and equilibrate to 37°C for 8 minutes.

4. Calibrate the oxygen electrode based on barometric pressure and 20.95% oxygen in compressed air (ca. 150 mmHg P_{O_2}) according to manufacturer's directions.

5. Set the software to begin recording at 146 mmHg P_{O_2} and stop at 0.5 mmHg.

6. Deoxygenate by bubbling with compressed nitrogen to generate the oxygen dissociation curve. Adjust the stir speed and bubble rate so that the oxygen dissociation curve is complete in 7 to 10 minutes. Final signal strength (S1/S2) should be at least 0.025.

APPENDIX B

UNIPROT MYOGLOBIN PROTEIN ACCESSION NUMBERS

Species	Accession number
Sheep	P68251
Cow	P02192
Horse	P68082
Melon-headed whale	Q0KIY3
Bowhead whale	R9RZK8
Bottlenose dolphin	P68279
Kogia s.	P02184
Kogia b.	Q0KIY5
Common dolphin	P68276
Risso's dolphin	R9RY97
Steller sea lion	R9RZ98
California sea lion	P02161
Harbor seal	P68080
Harp seal	R9S078
N. elephant seal	R9S002
Weddell seal	R9RY82
Emperor penguin	D5L2Y3