

## MYOGLOBIN OXYGEN AFFINITY IN AQUATIC AND TERRESTRIAL BIRDS AND MAMMALS

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### Abstract

Myoglobin (Mb) is an oxygen binding protein found in vertebrate skeletal muscle where it facilitates intracellular transport and storage of oxygen. This protein has evolved to suit unique physiological needs in the muscle of diving vertebrates that express Mb at much greater concentrations than their terrestrial counterparts. In this study, we characterized Mb oxygen affinity ( $P_{50}$ ) from 25 species of aquatic and terrestrial birds and mammals. Among diving species we tested for correlations between Mb  $P_{50}$  and routine dive duration. Across all species examined, Mb  $P_{50}$  ranged from 2.40-4.85 mmHg. The mean  $P_{50}$  of Mb from terrestrial ungulates was  $3.72 \pm 0.15$  mmHg (range 3.70 - 3.74 mmHg). The  $P_{50}$  of cetaceans was similar to terrestrial ungulates ranging from 3.54-3.82 mmHg with the exception of the melon-headed whale that had a significantly higher  $P_{50}$  of 4.85 mmHg. Among pinnipeds, 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. In pinnipeds and birds, low Mb  $P_{50}$  was associated with species whose muscles are metabolically active under hypoxic conditions associated with aerobic dives. Given the broad range of potential globin oxygen affinities, Mb

P<sub>50</sub> from diverse vertebrate species appears constrained within a relatively narrow range. High Mb oxygen affinity within this range may be adaptive for some vertebrates that make prolonged dives.

### **Key Terms**

myoglobin, oxygen affinity, aerobic dive limit, marine mammal, hypoxia, molecular evolution, nitric oxide

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### **Symbols and abbreviations**

|      |  |
|------|--|
| ADL  | Aerobic dive limit   |
| cADL | Aerobic dive limit calculated from useable oxygen stores and diving metabolic rate |
| DP   | Globin distal pocket   |
| Hb   | Hemoglobin   |
| Mb   | Myoglobin  |
| NO   | Nitric oxide   |
| ODC  | Oxygen dissociation curve  |

- $P_{50}$  Globin oxygen affinity defined as partial pressure of oxygen at globin half saturation (mmHg)
- $P_{O_2}$  Oxygen partial pressure (mmHg)

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TW contributed to the research conceptualization, sample preparation, data collection, data analysis, and manuscript preparation. RD contributed to the research conceptualization, data analysis, and manuscript preparation.

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## Introduction

Myoglobin (Mb) is an oxygen binding heme protein expressed primarily 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). The concentration of Mb 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  $< 5 \text{ mg g}^{-1}$  muscle tissue (Newcom et al., 2004, Masuda et al., 2008), Mb concentration in diving mammals is often 10-fold greater (Kanatous and Mammen, 2010) with some concentrations exceeding  $78 \text{ mg g}^{-1}$  (Noren and Williams, 2000). Sedentary birds such as galliforms may have Mb concentrations  $< 1 \text{ mg g}^{-1}$  (Kranen et al., 1999), while long-duration divers such as emperor penguins have concentrations of ca.  $64 \text{ mg g}^{-1}$  (Kooyman and Ponganis, 1998; Ponganis et al., 1999).

Among species of diving birds and mammals, Mb concentration is positively correlated with increased routine dive duration (Reed et al., 1994; Butler and Jones, 1997; Kooyman and Ponganis, 1998; Dolar et al., 1999; Helbo and Fago, 2012). In addition, Mb concentrations can vary within the musculature of an individual with the highest concentrations found in muscles associated with routine exertion and aerobic metabolism (Polasek and Davis, 2001). Although multiple factors influence Mb transcription (Kanatous and Mammen, 2010), localized muscle hypoxia generally acts as a stimulus for Mb synthesis (Terrados et al., 1990; Hoppeler and Vogt, 2001).

In addition to varied Mb concentration, the primary structure of Mb is also mutable and varies among vertebrate species. Despite variations 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. This  $P_{50}$  can be determined by generating an oxygen dissociation curve (ODC) from a globin solution. When interpreting an ODC, 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 selective pressure that influences the molecular evolution of Mb (Naylor and Gerstein, 2000). Recent studies have shown that marine mammal Mb experienced an increase in the rate of evolution (Dasmeh et al., 2013; Nery et al., 2013b) that resulted in increased stability (Dasmeh et al., 2013) and net surface charge (Mirceta et al., 2013) compared with terrestrial mammals.

Although the structure, concentration, and functional role of Mb are known to vary among birds and mammals, few studies (Nichols and Weber, 1989; Marcinek et al., 2001; Helbo and Fago, 2012) have examined the interspecific differences in oxygen affinity using *identical* experimental methods. A range of Mb oxygen affinities have been reported in the literature, however past studies used a variety of experimental techniques, instrumentation and temperatures, making comparative analysis among studies difficult. Additionally, many previous studies measured Mb oxygen affinity at refrigerated temperatures to minimize autoxidation of Mb. However, due to the effect of temperature on Mb oxygen affinity, previous experimental results at non-physiological temperatures are not directly relevant *in vivo* (Schenkman et al., 1997).

In this study, we measured the oxygen affinity of Mb for a variety of terrestrial and diving mammals and aquatic birds of varied diving ability at a physiologically significant temperature (37° C) using uniform methods for comparative analysis. 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 could account for observed variation in oxygen binding affinity and provide insight for future Mb mutational studies. Among the diving animals, we tested for correlations between routine dive duration and Mb P<sub>50</sub> that might indicate molecular adaptation of Mb to meet the unique oxygen demands of diving vertebrate muscle.

## Results

### *Myoglobin Oxygen Affinity*

Mb P<sub>50</sub> 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 1 and Fig. 2). Pairwise comparison of interspecies P<sub>50</sub> showed that the terrestrial ungulates and the majority of the cetaceans formed a series of non-exclusive overlapping groups (Fig. 3). The P<sub>50</sub> among terrestrial ungulates (horse, deer, oryx, cow, and lamb) were highly conserved and not significantly different (mean of  $3.72 \pm 0.15$  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 (Fig. 3). Melon-headed whales had a significantly higher P<sub>50</sub> (4.85 mmHg) than all other species. The closely related king and emperor penguins comprised a group with the lowest P<sub>50</sub> (mean of  $2.44 \pm 0.20$  mmHg). Closely related Adélie and chinstrap penguins comprised a group with the next lowest P<sub>50</sub> (mean of  $2.90 \pm 0.19$  mmHg). Weddell and northern elephant seals had the lowest P<sub>50</sub> among the mammals although they grouped with the redhead duck and the macaroni penguin, which had the highest P<sub>50</sub> among the birds (mean of  $3.27 \pm 0.19$  mmHg).

The maximal duration an air-breathing vertebrate can remain submerged without appreciable increase in lactic acid from anaerobic metabolism is the aerobic dive limit (ADL). For diving species, we tested for correlations between Mb P<sub>50</sub> and ADL. There was a broad range of ADLs among cetaceans (3.7 – 51 min), pinnipeds (2.3 – 30 min), and birds (0.5 – 5.7 min) (Table 2). For cetaceans, there was no significant correlation between ADL and Mb P<sub>50</sub> ( $r^2=0.0113$ ,  $P=0.894$ ) (Fig. 4). For both pinnipeds and birds, there were significant negative correlations between ADL and Mb P<sub>50</sub> (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*

All available Mb amino acid sequences for species in this study were composed of 153 amino acids (Fig. 5). Among these, 81(53%) were completely conserved, and 115 (75%) were highly conserved with a conservation ranking (based on physicochemical properties) of 8 or greater (Livingstone and Barton, 1993). Of the 30 residues lining the conserved pocket regions (heme pocket, DP, and Xe 1-4) (Table 3), 21 (70%) were completely conserved and 27 (90%)

were highly conserved (Fig. 5). 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 residues (68%) were completely conserved and 130 (85%) were highly conserved. Of the 30 residues 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 (single letter abbreviations for amino acids will be used to remain consistent throughout the text, tables, and figures). 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.

## Discussion

### *Globin Form and Function*

The tertiary structure and ligand binding regions of Mb and other globin proteins are generally highly conserved, while less critical regions are more variable (Naylor and Gerstein, 2000). Within the Mb protein are several highly conserved hydrophobic cavities (Fig. 1) including the heme pocket, the distal pocket (DP), and four additional pockets (Xe1-Xe4) (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).

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 (Fig. 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 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). 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).

### ***Mb Structural Variants and Oxygen Affinity***

Interspecies variability in Mb structure produces phenotypes that are subject to natural selection (Naylor et al., 2000; Wittenberg, 2007), and these variances can affect Mb oxygen binding properties and stability (Scott et al., 2001; Ochiai et al., 2009; Dasmeh et al., 2013). Among the species in this study with available Mb sequences, 47% of amino acid residues showed some level of substitution. Mutations of sperm whale Mb causing even single amino acid substitutions can produce a range of Mb P<sub>50</sub> from less than 1 mmHg to more than 100 mmHg (Scott et al., 2001; Dasmeh et al., 2012). Comparison of interspecies differences in Mb oxygen affinity from a variety of endothermic vertebrates in this study revealed conservation of P<sub>50</sub> within a relatively narrow range (2.40 mmHg – 4.85 mmHg) despite considerable variability in primary protein structure. Given the broad range of oxygen affinities that are possible by even single amino acid substitutions, Mb oxygen affinity of vertebrates is conserved within a narrow range to maintain optimal muscle performance. With the exception of the melon-headed whale, the Mb P<sub>50</sub> of the species in this study fell within the range previously reported for birds and mammals (Weber et al., 1974; Nichols and Weber, 1989; Helbo and Fago, 2012).

While the Mb oxygen affinity of birds and mammals in this study are conserved within a relatively narrow range, it is less clear if interspecies variation within this range 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).

Oxygen binding globin proteins are obvious candidates for molecular adaptation in diving animals that experience regular hypoxia (Nery et al., 2013 a), and Mb has experienced an increased rate of evolution in cetaceans (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 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. Together, these studies indicate that Mb is under selective pressure and adapted to the unique physiological demands in the muscle of diving vertebrates. Our study indicates that in addition to stability and surface charge, there may be an adaptive increase in Mb oxygen affinity in some diving vertebrates with high ADLs.

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 increased concentration rather than altered 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) that no significant correlation between average dive duration and  $P_{50}$  was observed in cetaceans.

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 sp.* 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 residue 66 has also been hypothesized to shift the oxygen affinity of beluga whale Mb (Stewart et al., 2004). Although residue 66 is not located in the heme pocket, it is in close structural proximity and adjacent to residues 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.

Although there was no correlation between ADL and Mb oxygen affinity in cetaceans, there was a significant trend for seals and penguins with longer ADLs 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. 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). 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 (Fig. 5). 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 has the identical N66V substitution which increases Mb stability in 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 affect oxygen affinity.

### ***Globin Adaptation to Temperature***

Because the oxygen affinity of Mb increases with decreasing temperature, Mb adaptation would be required to maintain a theoretically optimal oxygen affinity for species with different muscle temperatures (*e.g.* ectotherms in different temperature environments). Marcinek et al. (2001) found evidence for conservation of Mb oxygen affinity among fish with different body temperatures. Among closely related fish species, Mb oxygen affinity at 20°C was higher for species with a higher body temperature than their lower body temperature counterparts. When the Mb  $P_{50}$  for these species was adjusted for the temperature typically experienced in the muscle tissue of these species, the oxygen affinities converged. Nichols and Weber (1989) also found differences in Mb oxygen affinity of fish and mammal species that they attributed to adaptive

maintenance of Mb oxygen affinity at a biologically useful level despite different body temperatures. This apparent adaptive conservation of Mb oxygen affinity suggests there may be an optimal value for supporting aerobic respiration under specific 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., 1993). 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 diving species is driven by temperature. Despite overlapping ranges of Mb oxygen affinity, aquatic birds tended to have greater oxygen affinities than mammals. However, Mb P<sub>50</sub> is positively correlated with temperature, and birds tend to have a higher body temperature than mammals (Clarke and O'Connor, 2014). If corrected for body temperature, the discrepancy between Mb oxygen affinity in aquatic birds and mammals seen in this study would be reduced. Without the inclusion of terrestrial birds in this study it is unclear if they exhibit a similar trend and also have a greater Mb oxygen affinity than their terrestrial mammalian counterparts.

### ***Globin Adaptation to Hypoxia***

There is considerable evidence that increased oxygen affinity of respiratory globins is advantageous for animals in hypoxic environments. Mammalian Hb oxygen affinity varies more than two fold with P<sub>50</sub> values ranging from less than 20 mmHg to over 40 mmHg, and molecular adaptation favoring high affinity Hb is typical in animals that routinely endure hypoxia (Bunn, 1980; Storz, 2007). 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). 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 is also left shifted (higher affinity) 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). To effectively facilitate oxygen diffusion in active muscle, it is critical that the  $P_{50}$  of Mb be near the partial pressure of oxygen in active tissues 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 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 reduces the intracellular  $O_2$  diffusion gradient (Cano et al., 2013). While this discrepancy may be insignificant when the diffusive gradient is high (high arterial  $P_{O_2}$ ), it would be more significant as convective oxygen transport, arterial  $P_{O_2}$ , and the oxygen diffusion gradient are reduced (e.g., hypoxia during breath-hold diving). However, it is presently unclear what the effect of higher oxygen affinity Mb may have on the final stage of oxygen delivery from Mb to mitochondria.

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 is normally maintained by 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 in 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 enhanced 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). 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.

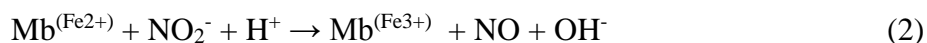
Because variations in Mb oxygen affinity have a greater effect on oxygen storage and transport when  $P_{O_2}$  is low, hypoxic conditions provide the primary selective pressure to preserve Mb function (Dasmeh and Kepp, 2012). Recent models of Mb oxygen storage and transport (Lin et al., 2007b; Dasmeh and Kepp 2012) suggest that under conditions of muscular hypoxia, high Mb concentrations and Mb with high oxygen affinity are advantageous for both storage and transport of oxygen.

### ***Additional Roles of Mb***

Traditionally, Mb was thought to function exclusively in the intracellular management of oxygen, but recent work has demonstrated that Mb also contributes to regulation of intramuscular bioavailability of nitric oxide (NO), thereby influencing mitochondrial respiration (Ordway and Garry, 2004; Hendgen-Cotta et al., 2008). Mb acts as an oxygen sensor and transitions from being a NO scavenger to a NO producer as  $P_{O_2}$  decreases (Hendgen-Cotta et al., 2015). Under normoxic conditions where the oxygen bound form of Mb dominates, ferrous oxy-Mb scavenges NO producing ferric met-Mb and nitrate (reaction 1).



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



Increased concentration of NO reduces mitochondrial respiration by modulating cytochrome c oxidase activity (Taylor and Moncado, 2010). With decreasing  $\text{P}_{\text{O}_2}$ , 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  $\text{P}_{50}$  dependent (Kamga et al., 2012). Under very hypoxic conditions where the  $\text{P}_{\text{O}_2}$  is below the  $\text{P}_{50}$  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). In addition, elevated Mb oxygen affinity is associated with increased nitrite reductase activity. Marine mammals that express high oxygen affinity Mb at high concentration may have a compounding effect resulting in an increased capacity for NO generation (Helbo and Fago, 2012). Given conditions of decreasing muscle  $\text{P}_{\text{O}_2}$ , higher affinity Mb would transition from being a net NO scavenger to a producer at a lower  $\text{P}_{\text{O}_2}$ . High Mb oxygen affinity may therefore be adaptive in diving species to maintain mitochondrial respiration and muscular activity at lower  $\text{P}_{\text{O}_2}$  during ischemic muscle hypoxia.

In both the scavenging and generation of NO (equations 1 and 2), 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.

### ***Conclusions***

Functional properties of oxygen binding globin proteins such as Mb are mutable and subject to natural selection. Given the more than 100 fold increase in Mb  $\text{P}_{50}$  that is achievable by Mb mutation, the oxygen affinity of Mb appears to be conserved within a relatively narrow range for terrestrial and aquatic birds and mammals. Variations within this range may be significant and adaptive for animals that routinely exercise during diving 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 longer routine dive durations. 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 including metabolic regulation by modulating intramuscular nitric oxide. An adaptive increase in Mb oxygen affinity in long-duration diving mammals and birds is consistent with other studies demonstrating adaptive increase in globin oxygen affinity in animals that routinely experience severe hypoxia.

## 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 as part of a separate research project 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 described in detail (Wright and Davis, 2015). Briefly, a single stock buffer solution of 50 mM tris buffer (Sigma-Aldrich, T 0694) with 50 mg l<sup>-1</sup> gentamicin sulfate (Sigma-Aldrich, G-1264) was used throughout all tissue homogenization, purification, and 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<sup>-1</sup> 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; Virgen-Ortiz et al., 2013). Samples were frozen in 3.5 ml aliquots and stored at -80°C until analysis.

### ***Determination of Myoglobin P<sub>50</sub>***

Once Mb solutions were prepared, oxygen dissociation curves were generated using a TCS Hemox Blood Analyzer (TCS Scientific, New Hope, PA). Twenty µl of 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 dual wavelength optical monitoring of the solution while deoxygenating by bubbling with compressed nitrogen to a final oxygen partial pressure of 0.5 mmHg. The TCS Hemox Data Acquisition System software (V 2.00.13) was used to generate an oxygen dissociation curve in real time and calculate P<sub>50</sub> values. When possible, samples from five individuals were collected for each species with a sample size large enough for eight replicates from each sample. Due to the opportunistic nature of sample collection the number and size of samples varied; however, no species was represented by fewer than 14 replicates (Table 1).



### ***Aerobic Dive Limit***

While it is not feasible to measure an ADL in most diving vertebrates (Kooyman et al., 1983), 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). In order to compare the diving ability of species that have not had an ADL or cADL determined, an estimate of ADL based on behavioral diving information is necessary. Using only average dive duration as an estimate for ADL may dramatically underestimate an animal's aerobic diving ability (Noren and Williams, 2000). Similarly, a comparison of maximum dive duration can be heavily skewed by extreme anaerobic dive events, which are rare and may be three times the actual ADL (Kooyman et al., 1980). An understanding of vertebrate dive behavior allows us to better estimate diving ability based on behavioral data. In order 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. For species in this study without a published ADL or cADL, an ADL was estimated based on behavioral information using published mean dive durations plus one s.d. This 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<sub>50</sub>.

### ***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](http://www.uniprot.org); see Appendix 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.

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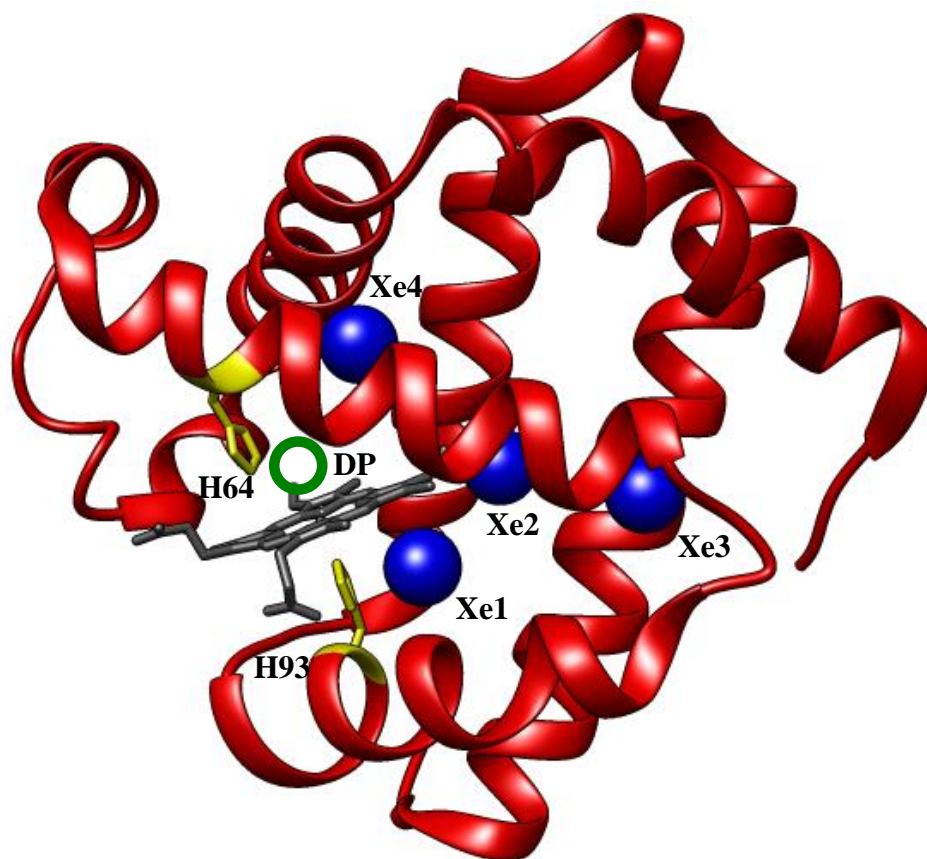
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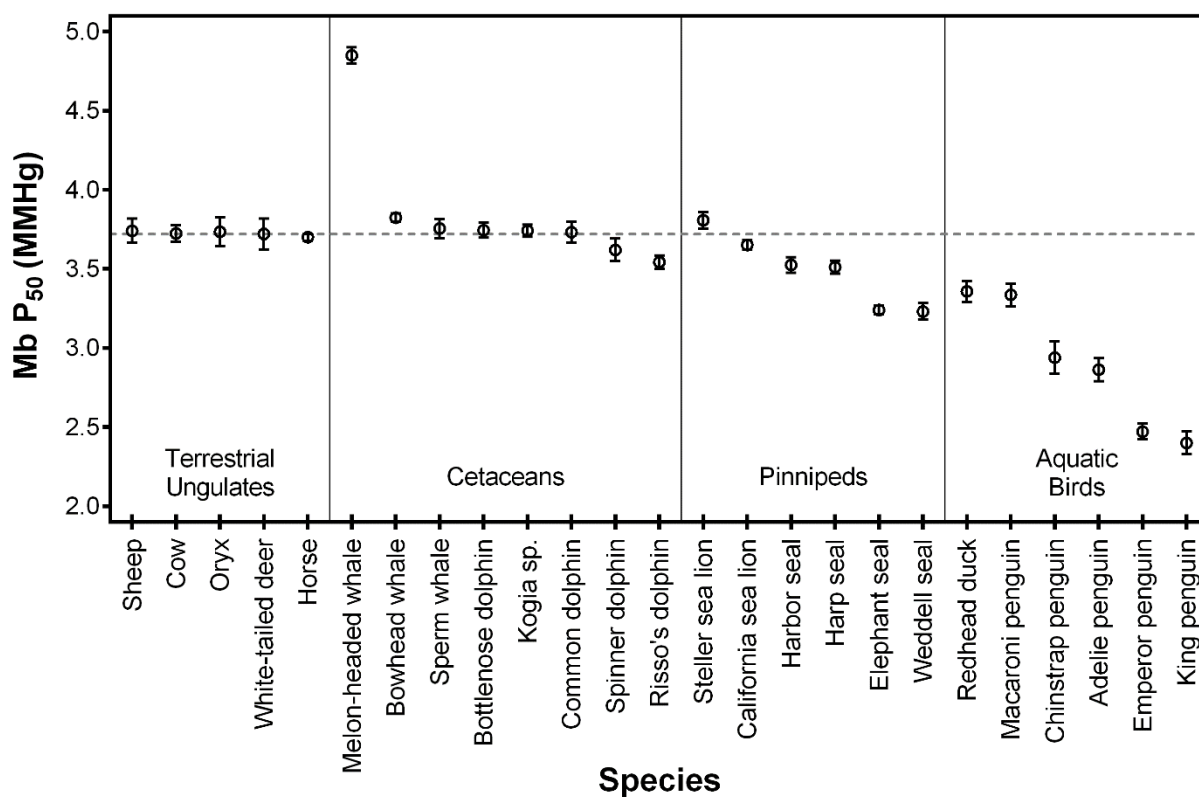
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## Figures



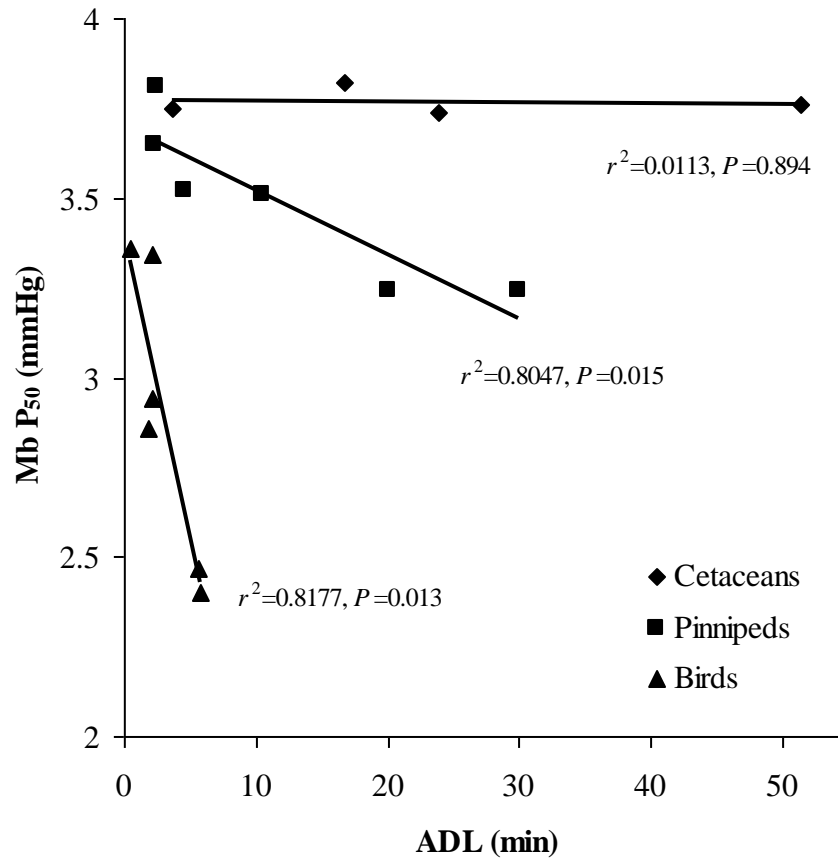
**Fig. 1** Image of sperm whale myoglobin (PDB accession # 1J52) with bound heme (gray), proximal (H93) and distal (H64) histidines (yellow), four Xe pockets (blue), and the distal pocket (green circle). Image was created using UCSF Chimera (Pettersen et al., 2004).



**Fig. 2 Mean myoglobin oxygen affinity ( $P_{50} \pm 2$  s.e.m.) for a variety of species.** Horizontal reference line is at 3.72 corresponding to the  $P_{50}$  of commercially available terrestrial horse heart myoglobin.

|                     |      |      |      |      |      |      |
|---------------------|------|------|------|------|------|------|
| 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 |      |
| <i>Kogia</i> 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 |

**Fig. 3 Statistical grouping of myoglobin oxygen affinity of diving and terrestrial birds and mammals.** Columns represent grouping based on Games-Howell pairwise comparison of species with statistically similar myoglobin oxygen affinity ( $p < 0.05$ ).



**Fig. 4** Mean myoglobin oxygen affinity ( $P_{50}$ ) as a function of aerobic dive limit for cetaceans, pinnipeds, and birds.





## Tables

**Table 1** Sample size and mean oxygen affinity ( $P_{50}$ ) from diving and terrestrial vertebrates.

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

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

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

<sup>a</sup> ADL determined experimentally in free diving wild animals.

<sup>b</sup> ADL determined experimentally in trained diving animals.

<sup>c</sup> ADL as mean dive duration of free diving wild animals plus 1 s.d. as described in text.

<sup>d</sup> ADL calculated as useable oxygen stores divided by diving metabolic rate (cADL).

<sup>e</sup> 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 s.d.

<sup>+</sup> Averages calculated from DU index 1993 data.

<sup>‡</sup> Averages calculated from conditioned “recovery” week dives.

**Table 3** Amino acid residues 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<sub>2</sub> (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                                     |

## APPENDIX

### UNIPROT MYOGLOBIN PROTEIN ACCESSION NUMBERS

| Species             | Accession number |
|---------------------|------------------|
| Sheep               | P68251           |
| Cow                 | P02192           |
| Horse               | P68082           |
| Melon-headed whale  | Q0KIY3           |
| Bowhead whale       | R9RZK8           |
| Bottlenose dolphin  | P68279           |
| <i>Kogia s.</i>     | P02184           |
| <i>Kogia b.</i>     | 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           |