Functional properties of myoglobins from five whale species with different diving capacities

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SUMMARY

Whales show an exceptionally wide range of diving capabilities and many express high amounts of the O2 carrier protein myoglobin (Mb) in their muscle tissues that prolongs their aerobic diving capacity. Although previous studies have mainly focused on the muscle Mb concentration and O2 carrying capacity as markers of diving behavior in whales, it still remains unexplored whether whale Mbs differ in their O2 affinities and in their enzymatic activities as nitrite reductase and peroxidase enzymes, all functions that could contribute to differences in diving capacities. In this study, we have measured the functional properties of purified Mbs from five toothed whales and two baleen whales and have examined their correlation with the average dive duration. Results showed that some variation in functional properties exists among whale Mbs with the toothed whale Mbs having higher O2 affinities and nitrite reductase activities (similar to those of horse) compared with baleen whale Mbs. However, these differences did not correlate with average dive duration. Instead, a significant correlation was found between whale Mb concentration and average duration and depth of dives and between O2 affinity and nitrite reductase activity when including other mammalian Mbs. Despite the fact that the functional properties showed little species-specific differences in vitro, they may still contribute to enhancing diving capacity as a result of the increased muscle Mb concentration found in extreme divers. In conclusion, Mb concentration rather than specific functional reactivities may support whale diving performance.
INTRODUCTION

In contrast to most terrestrial mammals with very limited breath-hold capabilities, diving marine mammals, such as whales (cetaceans), are able to remain submerged for long periods of time. The most extreme divers are found within the toothed whales (odontocetes), with the sperm whale being able to dive to depths of more than 2000 meters and stay submerged for more than two hours (reviewed by Ponganis, 2011). Key adaptations that make these animals capable of such prolonged dives include 1) an increased O2 storage capacity in blood and muscle that makes it possible to rely largely on aerobic metabolism during normal diving (i.e. within the aerobic dive limit, defined as the dive duration beyond which lactate is produced) (Butler and Jones, 1997; Kooyman and Ponganis, 1998), and 2) a greater defense against oxidative stress due to increased mitochondrial production of reactive oxygen species (ROS) at reoxygenation (Cantú-Medellín et al., 2011; Bickler and Buck, 2007). However, knowledge on the molecular mechanisms basic to the diving physiology of these elusive animals is still limited due to the difficulty of obtaining biological samples.

Diving mammals typically have a high concentration of the O2 carrier myoglobin (Mb) in their skeletal muscle (~10-95 mg/g, Ponganis, 2011) compared with non-diving mammals (~3-10 mg/g, Snyder, 1983; Kayar et al., 1988), an adaptation that allows to maintain a prolonged aerobic metabolic activity of swimming muscle during dives by increasing O2 reserves (Scholander, 1940; Butler and Jones, 1997) and by facilitating O2 diffusion within myocytes (Ponganis et al., 2008). In toothed whales (but not in baleen whales or mysticetes) the Mb concentration in muscle has been shown to be positively and significantly correlated with maximum dive duration and with body size (Noren and Williams, 2000). However, for some extreme whale divers, the beaked whales, the duration of a deep foraging dive can be approximately 2-fold longer than the estimated total O2 carrying capacity, even when corrected for the lower metabolic rate due to increased animal size (Tyack et al., 2006). This suggests that these whales may be able to decrease their O2 consumption rate, which would prolong O2 reserves and the aerobic dive limit even further during diving. Additionally, whales in general are also expected to possess an enhanced protection against the generation of ROS that may occur upon resurfacing/reoxygenation especially after long dives, when tissue O2 reserves have been depleted. As described below, the progressive formation of deoxygenated Mb in the muscle due to the release of O2 during diving and the reoxygenation of Mb at resurfacing may both play a central adaptive role in such metabolic and protective mechanisms.
Besides O₂ storage and diffusion (Gros et al., 2010; Wittenberg and Wittenberg, 2003; Dasmeh and Kepp, 2012), other functions of Mb, associated to the protection against damaging effects of hypoxia and reoxygenation, have been discovered in recent years (Shiva et al., 2007; Hendgen-Cotta et al., 2008; Flögel et al., 2004). Specifically, deoxygenated Mb is able to reduce nitrite, an endogenous product of nitric oxide (NO) metabolism, to NO (Shiva et al., 2007). The NO generated downregulates mitochondrial respiration and reduces O₂ consumption during hypoxia (Shiva et al., 2007) by reversible inhibition of cytochrome c oxidase (Brown and Cooper, 1994). The Mb-mediated reduction of nitrite to NO also protects against ischemia/reperfusion injury, as shown in the heart (Hendgen-Cotta et al., 2008). In hemoglobins (Hbs) this nitrite reductase activity has been shown to be positively correlated with O₂ affinity (Huang et al., 2005a), a correlation that may be of importance in the adaptive tolerance to hypoxia given that high-O₂ affinity Hbs are typical of hypoxia-tolerant animals (Huang et al., 2005a; Jensen, 2009). It is not known, however, whether for Mbs a high-O₂ affinity correlates with hypoxia tolerance and/or with a high nitrite reductase activity. Additionally, Mb can act as a peroxidase and scavenge hydrogen peroxide (H₂O₂) (Yusa and Shikama, 1987; Flögel et al., 2004; Helbo et al., 2012), a major ROS produced endogenously when tissues are reoxygenated after hypoxia, thereby protecting against oxidative damage (Bickler and Buck, 2007; Flögel et al., 2004) as it would occur when whales reemerge from diving. Taken together, these functional properties of Mb could contribute to the extreme tolerance to internal hypoxia displayed by whales during diving. In addition, although Mb concentration is known to be correlated with maximal dive durations (Noren and Williams, 2000), it is not known whether Mbs from long-duration divers and short-duration divers differ in any of these functional properties, which would indicate fine-tuning of Mb function according to dive performance.

In this study, we have investigated whether Mb may contribute to whale dive performance by having different functional properties (O₂ affinities and kinetics, nitrite reductase and peroxidase activities) and/or by having different muscle concentrations. To this end, we have purified and characterized Mbs from five cetacean species (three toothed whales and two baleen whales) that are known to differ in their diving performance. We have measured values for O₂ affinities and kinetics, nitrite reductase and peroxidase activities of the purified Mbs from these species and tested for their correlation with the average dive duration known for the individual whale species. Additionally, we have tested whether Mb enzymatic reactivities (nitrite reductase and peroxidase activities) and O₂ affinity are correlated in the mammalian Mbs (whales and horse) included in this study. Furthermore,
we have measured muscle Mb concentration (for minke whale and humpback whale) and examined whether Mb concentration is correlated with average dive duration and dive depth in a range of whale species.

MATERIALS AND METHODS

A muscle sample from Northern bottlenose whale (BNW) (*Hyperoodon ampullatus*, Forster 1770) was obtained from a frozen specimen stranded in the Faroe Islands in 2010. Humpback whale (HW) (*Megaptera novaeangliae*, Borowski, 1781) and arctic minke whale (MW) (*Balaenoptera acutorostrata*, Lacépède 1804) muscle samples from one specimen each were obtained from whales caught by hunters in Nuuk, Greenland (CITES permission 11GL0806453 and 11GL0806452, respectively). Harbor porpoise (HP) (*Phocoena phocoena*, Linnaeus 1758) muscle was obtained from one animal euthanized due to other causes at the Dolfinarium Harderwijk, Holland. All muscle samples were frozen as fast as possible after being cut out of the whales and were kept at -80°C until purification of the Mbs. A frozen (-80°C) sample of purified sperm whale (SW) (*Physeter macrocephalus*, Linnaeus 1758) Mb obtained from a dead specimen stranded at Rømø, Denmark, in 1996 was kindly provided by Prof. Roy E. Weber (Aarhus University). For functional comparisons, purified horse heart Mb (Sigma-Aldrich) was used as representative for a Mb from a non-diving mammal. Chemicals were from Sigma-Aldrich unless otherwise stated and water was milli-Q grade.

*Purification of myoglobins*

Mb from BNW, HW, MW and HP was purified from muscle as previously described (Helbo and Fago, 2011). In brief, Mb was precipitated from muscle homogenates by ammonium sulphate fractionation (40 and 100%) followed by fast protein liquid chromatography (FPLC) gel filtration using a Tricorn Superdex 75 10/300 GL column (GE-Healthcare) equilibrated with 50 mM Tris, 0.5 mM EDTA, 3 mM dithiothreitol (DTT), 0.15 M NaCl, pH 8.3 to separate Mb from contaminating Hb. Horse Mb was converted from the ferric (met) to the ferrous (oxy) form by standard procedures after adding solid dithionite and desalting on a PD10 column (GE-Healthcare) equilibrated with the gel filtration buffer described above. Mb purity was assessed by SDS and isoelectric focusing (IEF) on polyacrylamide gels (Phast System, GE Healthcare). Isoelectric points (pIs) of the native purified Mbs were obtained by IEF in the 3-9 pH range, using a broad pI marker 3-10 (GE Healthcare). Heme oxygenation/oxidation state
was assessed by UV-vis absorption spectroscopy in the range 400-700 nm by using absorption peaks known for Mb (Antonini and Brunori, 1971). All whale Mbs were purified as oxy derivatives, without detectable met heme. This shows that all whale Mbs were in good condition despite muscle samples and purified Mbs being of varying age.

Myoglobin concentration

Muscle Mb concentrations for the toothed whales HP, BNW, SW and for horse were obtained from previously published values (Noren and Williams, 2000; Scholander, 1940; Kayar et al., 1988). The muscle concentrations of the baleen whale (HW and MW) Mbs were measured spectrophotometrically using a modified version of the method by Reynafarje, which takes advantage of characteristic spectral differences to differentiate between Mb and Hb in contaminating blood (Reynafarje, 1963). In this procedure, thawed muscle samples (~0.2 g) were homogenized on ice using an Ultra-Turrax T25 homogenizer in 40 mM potassium phosphate buffer (pH 6.6 for HW and pH 7.7, 3 mM DTT, 0.5 mM EDTA for MW) at a buffer to tissue ratio of 19.25 mL buffer/g wet tissue (Reynafarje, 1963). Samples were centrifuged for 50 min at 15.000 g at 4 °C, and the supernatant was equilibrated with pure CO gas for 3 min. Dithionite (~0.001 g) was added and the sample was equilibrated with CO for 1 more min. Absorbance was recorded at 538 and 568 nm using a HP 8543 UV-vis diode array spectrophotometer and the concentration of Mb (in mg/g wet weight) was calculated using the formula: [Mb] = (A_{538} - A_{568}) \times 117.3, where the difference in absorbance at 538 and 568 nm is multiplied by a constant containing the molar extinction coefficients of carbonyl Hb and Mb, the molecular weights of Hb and Mb and the volume of buffer to weight of tissue (Reynafarje, 1963). No changes in absorbance were found after longer CO equilibration.

Oxygen equilibria

O2 binding curves were determined using a modified diffusion chamber technique previously described (Sick and Gersonde, 1969; Weber, 1992; Weber et al., 2000). Briefly, water-saturated gas mixtures of O2 or air and ultrapure (>99.998%) N2 created by Wösthoff gas mixing pumps were used to equilibrate a thin smear (4 µL, ~100 µM heme) of Mb solution with stepwise increases in oxygen tension (PO2). Changes in absorbance upon oxygenation were recorded continuously at 436 nm by a photomultiplier (model RCA 931-A) and an Eppendorf model 1100M photometer. The absorbance signal was digitalized and measured using a computer with the in-house made data acquisition software,
Spectrosampler. $P_{50}$ (PO$_2$ at half-saturation) and $n_{50}$ (cooperativity) values were calculated from the zero intercept and slope of Hill plots, respectively: log(Y)/(1 − Y) vs. logPO$_2$ where Y is the fractional saturation of Mb. Each curve consists of 4-5 saturation steps. Experiments were carried out in duplicate in 50 mM Tris, 0.5 mM EDTA, 3 mM DTT, 0.15 M NaCl, pH 8.3 at 37°C.

**O$_2$ dissociation kinetics**

O$_2$ dissociation rates ($k_{off}$, s$^{-1}$) were measured using an OLIS RSM 1000 UV/Vis rapid-scanning stopped-flow spectrophotometer (OLIS, Bogart, GA) coupled to a Dell computer with OLIS data collection software. In the stopped flow, oxygenated Mb (~10 μM heme in 200 mM Tris, pH 7.4) was mixed at a 1:1 ratio with 40 mM dithionite prepared in deoxygenated buffer (200 mM Tris, pH 7.4) and spectra (~390-600 nm, 1000/s) were collected over time (~0.2 s). $k_{off}$ rates were calculated from the monoexponential changes of the absorbance spectra using OLIS multiple wavelength data analysis software (SVD). The O$_2$ equilibrium constant ($K$, μM) was calculated by multiplying the measured $P_{50}$ values by the solubility of O$_2$ in water at 37°C (1.407 mM$^{-1}$ torr$^{-1}$) (Boutilier et al., 1984). O$_2$ association rates ($k_{on}$, μM$^{-1}$ s$^{-1}$) were derived from the relation $k_{on} = k_{off}/K$, assuming $P_{50}$ independent of pH for Mbs. Rates were measured at 25 and 37°C.

**Nitrite reduction**

The reaction of nitrite with the Mbs (~10 μM heme in deoxygenated 200 mM Tris, 0.5 mM EDTA 0.1 M KCl, pH 7.4) was measured under pseudo-first order conditions in the presence of dithionite (~200-300 μM) and under anaerobic conditions as previously described (Shiva et al., 2007; Pedersen et al., 2010; Helbo et al., 2012). The nitrite concentration in the stock solution (~20 mM) was verified by the Griess reaction (Giustarini et al., 2008) every day prior to experiments. Varying amounts of nitrite was added anaerobically to the Mb solution via a Hamilton gastight syringe and spectral changes were measured over time in the range 390-650 nm using a HP 8543 UV-vis diode array spectrophotometer. At the end of the reaction, deoxy Mb (peak at 555 nm) was fully converted to the Fe-NO form (peaks at 545 and 572 nm) as predicted by the reaction stoichiometry (Shiva et al., 2007; Pedersen et al., 2010; Helbo et al., 2012). Observed rates ($k_{obs}$, s$^{-1}$) were obtained by fitting absorbance traces at 555 nm to single exponential decay functions. The pH of the reaction mixture was checked after each experiment. Second-order rate constants (μM$^{-1}$ s$^{-1}$) were obtained from the slopes of linear plots of observed rates as a function of nitrite concentrations. Experiments were performed at 25°C to allow for comparison with rate constants determined in previous studies.
Reaction with hydrogen peroxide (peroxidase activity)

To investigate the role of whale Mbs in the overall protection against ROS, H$_2$O$_2$ (~1 mM in water) was added to oxygenated Mb solutions (~10 µM heme, 20 mM Tris pH 7.5) at a 2:1 H$_2$O$_2$/heme molar ratio, as previously described (Helbo et al., 2012). The changes in absorbance spectra were in agreement with those of carp Mbs (Helbo et al., 2012) and with the reaction scheme described for the reaction between oxy Mb and H$_2$O$_2$ (Yusa and Shikama, 1987). The decrease in absorbance at 416 nm over time was monitored at 25°C using a HP 8543 UV-vis diode array spectrophotometer. To correct for autoxidation of the Mbs, absorbance of samples with water added instead of H$_2$O$_2$ was measured in parallel over time. Experiments were performed at 25°C to allow for comparisons with other studies. Observed rates were obtained by fitting a single exponential decay function to the absorbance measured at 420 nm. Rates were measured in duplicate.

Dive duration

Functional properties were correlated to average dive duration values of long, foraging dives reported for the five whale species (Table 1). To investigate whether Mb concentration was dependent on whale dive performance Mb concentration from the investigated species and from additional whale species was correlated with available data on average dive duration and on average dive depth. Species, values and references are listed in Table 1.

Statistics

Least square linear regression analyses were used to test for correlations of O$_2$ affinity, nitrite reductase, peroxidase activities and Mb concentration with average dive duration, to test for correlation of Mb concentration with average dive depth and to test for correlations of O$_2$ affinity with peroxidase and nitrite reductase activities. Results were taken as significant when P ≤ 0.05. Values are presented as means ± SD when n ≥ 3. Statistical tests were performed using the software Sigma Plot 11.

RESULTS

Whale Mbs were successfully purified from skeletal muscles as judged by SDS-PAGE and from ratios of Soret (~416 nm) to protein peak (260 nm) absorbance > 5 (data not shown). An unstained IEF gel of the purified Mb samples (Fig. 1) showed only one red band, which indicates that whale muscles contain
one Mb isoform, having higher pI values (SW: 7.89, BNW: 7.91, HW: 7.77, MW: 7.78, HP: 8.00) than horse Mb (7.36).

The concentrations of Mb in muscle tissues from the baleen whales (HW and MW) were 15.9±0.8 mg/g wet weight (n = 8, from 1 individual) and 7.3 ± 1.0 mg/g wet weight (n = 4, from 1 individual), respectively. The value for the MW Mb concentration may however be a low estimate because of some precipitation of the homogenized sample. In Table 1, showing the data for Mb concentration, we therefore report the average between our value for MW Mb and a value (37 mg/g wet weight) calculated from the measured muscle O2 capacity (Scholander 1940, Dolar et al., 1999). Additional published whale Mb muscle concentrations are also listed in Table 1 together with available data on average dive duration and on average dive depth. When plotting Mb concentration as a function of average dive duration (Fig. 2A), the regression analysis shows that Mb concentration is positively and significantly correlated with average dive duration of the included whale species (r² = 0.522, P = 0.012, n = 11, 7 toothed and 4 baleen whales). When plotting Mb concentration as a function of average dive depth (Fig. 2B), the regression analysis shows that Mb concentration is also positively and significantly correlated with average dive depth of the included species (r² = 0.610, P = 0.013, n = 9, 6 toothed and 3 baleen whales). As data on average dive depth is not available for minke whale, average dive duration was used to investigate the correlations with functional reactivities of the purified Mbs.

Whale Mb O2 binding curves were hyperbolic (Fig. 3) and P50 values for the toothed whale (BNW, SW and HP) Mbs were similar to that for horse Mb (Table 2). In contrast, baleen whales (HW and MW) Mbs had slightly higher P50 values and thus lower O2 affinities (Table 2). Mbs from all species showed cooperativity values (n50) close to 1 (range: 0.9-1.1) (Fig. 3), which is consistent with the monomeric structure of the Mbs and further confirms that Hb was not present as a contaminant.

O2 dissociation stopped-flow kinetics showed that the low-O2 affinity baleen whale Mbs had slightly higher koff rates and slightly lower kon rates than Mbs from toothed whales. Measured (koff) and calculated (kon) values of Mb O2 kinetics are listed in Table 2.

Apparent second order rate constants for the reduction of nitrite to NO by whale and horse deoxy Mbs (at 25°C) were in the range 3-6.6 M⁻¹s⁻¹ (Fig. 4). These results show that toothed whales (HP: 6.5 ± 0.2 M⁻¹s⁻¹, BNW: 6.6 ± 0.3 M⁻¹s⁻¹) deoxy Mbs are >2-fold faster than baleen whales (HW: 3.0 ± 0.1 M⁻¹s⁻¹, MW: 2.6 ± 0.2 M⁻¹s⁻¹) deoxy Mbs at reducing nitrite to NO. The nitrite reductase rates of horse (5.4 ± 0.4 M⁻¹s⁻¹) and SW (4.1 ± 0.4 M⁻¹s⁻¹) Mbs are intermediate.
When reacted with a 2-fold molar excess of H$_2$O$_2$, the whale Mbs investigated here showed similar rates (BNW: 0.51 s$^{-1}$, SW: 0.56 s$^{-1}$, HW: 0.55 s$^{-1}$, MW: 0.41 s$^{-1}$, HP: 0.39 s$^{-1}$), whereas horse Mb was slightly faster (0.70 s$^{-1}$) at removing H$_2$O$_2$.

Mb concentrations, O$_2$ affinities, nitrite reductase activities and peroxidase activities are plotted as a function of the average dive duration of the whale species in Fig. 5A-C. The regression analyses show that the correlations are non-significant (Mb concentration: $r^2 = 0.638$, $P = 0.105$, $n = 5$. O$_2$ affinity: $r^2 = 0.405$, $P = 0.248$, $n = 5$. Nitrite reductase activity: $r^2 = 0.051$, $P = 0.716$, $n = 5$. Peroxidase activity: $r^2 = 0.487$, $P = 0.190$, $n = 5$). Conversely, the functional properties, nitrite reductase activities and $P_{50}$, were significantly correlated (Fig. 6A, $r^2 = 0.719$, $P = 0.033$, $n = 6$), meaning that a high nitrite reductase activity is associated with a high O$_2$ affinity (i.e. a lower $P_{50}$) in the mammalian Mbs (including horse) of this study. No significant correlation was found between peroxidase activity and $P_{50}$ (Fig. 6B, $r^2 = 0.244$, $P = 0.319$, $n = 6$).

**DISCUSSION**

Earlier studies have mainly focused on the Mb concentration and on the muscle O$_2$ carrying capacity as a marker of diving behavior in marine mammals while it has remained unexplored whether Mbs from whales with different diving capacities also differ in their functions as O$_2$ carriers, nitrite reductase and peroxidase enzymes. These are all properties that are expected to be of importance in matching O$_2$ supply with consumption during diving and for limiting oxidative stress after diving. As discussed below, even though these properties show little species-specific difference in vitro for the isolated Mbs, they may be amplified in vivo as a result of the massive increase in muscle Mb concentration present in extreme divers.

Mbs from the three toothed whales included in this study (SW, BNW and HP) are similar to horse Mb in having relatively high O$_2$ affinities and high nitrite reductase activities (Fig. 5A+B). The $P_{50}$ values are all below 3 torr at 37°C, which is typical for mammalian Mbs (Antonini and Brunori, 1971; Nichols and Weber, 1989). Also the nitrite reductase activities of toothed whale and horse Mbs (4.1-6.6 s$^{-1}$ × M$^{-1}$) are similar to reported values for sperm whale (5.6 s$^{-1}$ × M$^{-1}$ Tiso et al., 2011) and horse Mb (6 s$^{-1}$ × M$^{-1}$ Huang et al., 2005b), except from one study on horse Mb, reporting a lower reactivity (2.9 s$^{-1}$ × M$^{-1}$ Tiso et al., 2011). Compared with toothed whales and other mammals, baleen...
whales (MW, HW) have Mbs with slightly lower O2 affinities and lower nitrite reductase rates (Fig. 5A+B). The low O2 affinities of baleen whales Mbs are explained by slight increases and decreases in O2 dissociation and association rates, respectively. Together with the similar peroxidase activity found for whale Mbs (Fig. 5C), our data show that these minor differences in Mb reactivities are not sufficient to contribute significantly to the average dive duration. Instead, genetically-determined functional properties of the Mbs appear overall similar in phylogenetically related whales, such as HP, SW and BNW that differ widely in diving behavior and capability. The data also demonstrate that a correlation exists between the high O2 affinity (low P50) and the nitrite reductase activity of mammalian Mbs (Fig 6), as intrinsic specific properties of the Mbs. Such correlation may be due to a lower reducing potential of the heme, as proposed for Hbs (Huang et al., 2005a), or to a more accessible heme pocket, which would suggest some common molecular mechanism in the regulation of heme reactivity towards O2 or nitrite in Mbs. In contrast, the peroxidase activity does not correlate with either average diving duration or O2 affinity in mammalian Mbs, at least not under the conditions investigated here. This finding suggests that in mammals other enzymes with a more specific peroxidase and catalase activity than Mb may be of greater importance in the detoxification against H2O2 and other ROS.

In general, that Mb O2 affinity and nitrite reductase activity are correlated may be adaptive in hypoxia tolerant animals to 1) improve facilitated diffusion at low PO2 gradients at the blood-muscle interface and 2) increase the rate of NO generated from nitrite per unit time as Mb becomes progressively deoxygenated. In whales, an increased production of NO could be beneficial during long dives by slowing down the mitochondrial respiration, which would prolong O2 reserves (Shiva et al., 2007) and increase cellular protection against the damaging effects of reoxygenation after hypoxic episodes (Hendgen-Cotta et al., 2008). Although this could be of particular importance in extreme divers like SW and BNW, these intrinsic functional properties of the Mbs alone cannot account for the Mb contribution to average dive duration unless Mb concentration is taken into account, as explained below.

When including literature data for additional whale species, we found a significant and positive correlation between average dive duration and Mb concentration for whales, with toothed whales showing the highest durations and the highest Mb concentrations (Fig. 2 and Table 1). This extends the results of a previous study where maximum dive duration (that may far exceed the aerobic dive limit)
was found to be correlated with Mb concentration in toothed whales but not in baleen whales (Noren and Williams, 2000).

The Mb concentrations of some of the whale species differ from what would be predicted from their average dive durations, and in particular the harbor porpoise has a very high Mb concentration despite a modest dive performance. Several reasons may underlie these deviations. First, body size influences the specific metabolism and it may therefore affect O₂ demand and the relationship between dive performance and Mb concentration. This may explain at least in part why the small harbor porpoise expresses comparably high amounts of Mb. Second, whales show great differences in behavior and foraging strategies with the toothed whales spending more energy on capturing prey than the filter-feeding baleen whales. This in turn may also affect O₂ consumption and thus the need for Mb. Data of Fig. 2 suggests that the Mb concentration tends to be higher in toothed whales than in baleen whales showing the same diving abilities. Future investigations on other whale species, especially those with intermediate diving abilities, and on variations in Mb concentration among individuals of the same species, studies that were not feasible here, would represent a useful addition to this study.

Besides increasing the storage capacity for O₂ in muscle and O₂ supplied to the mitochondria per unit time (Ponganis et al., 2008), a higher concentration of Mb will also effectively increase the concentration of enzyme available and thus the rate of nitrite reduction and hydrogen peroxide removal of the muscle tissue. Thus, despite the lack of correlation between the species-specific functional properties of whale Mbs and the average dive duration, simply because of a higher protein concentration, all Mb functions will be enhanced in vivo in long-duration divers compared with short-duration divers. As a case in point, for a given nitrite concentration, the nitrite reductase activity coupled with the high Mb concentration would increase NO production per unit time in the skeletal muscle of BNW ~8-fold compared to HW and horse muscle when O₂ reserves have been depleted. Furthermore, a recent study shows that plasma nitrite levels are elevated in harbor porpoise in comparison with other mammals (Soegaard et al., 2012) indicating that relatively high nitrite levels may also be present in whale skeletal muscle.

In conclusion, we found that some variation exists among whale Mbs in O₂ binding, nitrite reductase activity and in rate of reaction with H₂O₂ but that this variation is not correlated with average dive duration. Instead, the variation may reflect phylogeny and differences in foraging strategies, as the greatest differences in Mb functional properties are found between the toothed and baleen whale.
suborders with toothed whales having comparably higher O₂ affinities and higher nitrite reductase activities. Furthermore, an increased Mb O₂ affinity is at variance with an increased nitrite reductase activity in mammals in general, which may be adaptive in hypoxia-tolerant animals. Average dive duration and depth significantly correlate with Mb muscle concentration in whales, and we conclude that such correlation will entail a consequent enhancement of Mb functions in vivo, including capability for O₂ storage and for nitrite reduction to NO that may considerably prolong dive performance in extreme divers. It appears therefore that differences in Mb concentration rather than differences in specific functional reactivities may contribute to dive performance in whales.
LIST OF ABBREVIATIONS

- ROS: reactive oxygen species
- Mb: myoglobin
- NO: nitric oxide
- Hb: hemoglobin
- H₂O₂: hydrogen peroxide
- BNW: bottlenose whale
- HW: humpback whale
- MW: minke whale
- HP: harbor porpoise
- SW: sperm whale
- pI: isoelectric point
- CO: carbon monoxide
- PO₂: O₂ tension
- \( P_{50} \): PO₂ at half-saturation
- \( n_{50} \): cooperativity
- \( k_{off} \): O₂ dissociation rate
- \( K \): equilibrium constant
- \( k_{on} \): O₂ association rate

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REFERENCE LIST


of hemoglobin as a nitrite reductase that produces NO under allosteric control. J. Clin. Invest. 115, 2099-2107.


**Figure 1.** Unstained IEF polyacrylamide gel (3-9) of purified whale (harbor porpoise (HP), sperm whale (SW), bottlenose whale (BNW), minke whale (MW), humpback whale (HW)) and horse Mbs.

**Figure 2.** Myoglobin concentration as a function of (A) average dive duration and (B) average dive depth of whales. Species abbreviations are: harbor porpoise (HP), sperm whale (SW), bottlenose whale (BNW), minke whale (MW), humpback whale (HW), Cuvier’s beaked whale (CBW), fin whale (FW), bowhead whale (BHW), bottlenose dolphin (BD), spotted dolphin (SD). Data from this study are the Mb concentrations of humpback whale and minke whale. Values and references are reported in Table 1. Least square linear regressions and levels of significance ($r^2$ and $P$ values) are indicated.

**Figure 3.** Oxygen binding equilibria and kinetics of whale Mbs. Representative $O_2$ binding curves for minke whale (open symbols) and bottlenose whale (closed symbols) Mbs measured in 50 mM Tris, 0.5 mM EDTA, 3 mM DTT, 0.15 M NaCl, pH 8.3 at 37°C. Hyperbolic fitting of the data is indicated. Inset shows Hill plots of the same data with the dotted line indicating 50% saturation.

**Figure 4.** Nitrite reductase activity of Mbs from toothed whales (closed symbols) and baleen whales (open symbols) compared with horse Mb (grey symbols). Observed rates of the reaction of deoxy Mbs with nitrite (conditions: 200 mM Tris, 0.5 mM EDTA, 0.1 M KCl, pH 7.4 at 25°C) plotted as a function of nitrite concentration. Second-order rate constants for the nitrite reductase activities are given by the slopes of the linear regression of the data.

**Figure 5.** (A) $P_{50}$, (B) nitrite reductase activity, (C) peroxidase activity as a function of average dive duration for toothed whales (bottlenose whale (BNW), sperm whale (SW) and harbor porpoise (HP)) and for baleen whales (humpback whale (HW) and minke whale (MW)). Least square linear regressions and levels of significance ($r^2$ and $P$ values) are indicated. Horse (H, white circle) is shown as an example of a non-diving mammal but is not included in the regression analyses. Values are means ± S.D.
Figure 6. $P_{50}$ as a function of nitrite reductase activity for bottlenose whale (BNW), sperm whale (SW), harbor porpoise (HP), humpback whale (HW), minke whale (MW) and horse (H). Linear regression analyses and levels of significance ($r^2$ and $P$ values) are indicated. Values are means ± S.D.
Table 1. Myoglobin concentration, average dive duration and average dive depth for some whales

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<th>Average dive duration (min)</th>
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<td><strong>Toothed whales</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bottlenose whale</td>
<td>63</td>
<td>37</td>
<td>1060</td>
<td>Scholander, 1940; Hooker and Baird, 1999</td>
</tr>
<tr>
<td>Sperm whale</td>
<td>54</td>
<td>45</td>
<td>666</td>
<td>Scholander, 1940; Watwood et al., 2006,</td>
</tr>
<tr>
<td>Cuvier’s beaked whale</td>
<td>43</td>
<td>58</td>
<td>1070</td>
<td>Noren and Williams, 2000; Tyack et al., 2006</td>
</tr>
<tr>
<td>Beluga whale</td>
<td>34</td>
<td>13</td>
<td>nd</td>
<td>Noren and Williams, 2000; Martin and Smith, 1999</td>
</tr>
<tr>
<td>Spotted dolphin</td>
<td>25</td>
<td>1.5</td>
<td>23</td>
<td>Castellini and Somero, 1981; Scott and Chivers, 2009</td>
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<tr>
<td>Bottlenose dolphin</td>
<td>27</td>
<td>0.5</td>
<td>20</td>
<td>Noren and Williams, 2000; Mate et al., 1995, Ponganis, 2011</td>
</tr>
<tr>
<td>Harbor porpoise</td>
<td>40</td>
<td>1</td>
<td>25</td>
<td>Noren and Williams, 2000; Westgate et al., 1995</td>
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<tr>
<td><strong>Baleen whales</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bowhead whale</td>
<td>35</td>
<td>10.5</td>
<td>67</td>
<td>Noren and Williams, 2000; Krutzikowsky and Mate, 2000</td>
</tr>
<tr>
<td>Fin whale</td>
<td>24</td>
<td>6.5</td>
<td>98</td>
<td>Noren and Williams, 2000; Croll et al., 2001</td>
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<tr>
<td>Humpback whale</td>
<td>16</td>
<td>8</td>
<td>143</td>
<td>This study; Goldbogen et al., 2008</td>
</tr>
<tr>
<td>Minke whale</td>
<td>22(^b)</td>
<td>4.5</td>
<td>nd</td>
<td>This study and Scholander, 1940; Stern, 1992</td>
</tr>
<tr>
<td>Horse</td>
<td>10</td>
<td>nd</td>
<td>nd</td>
<td>Kayar et al., 1988</td>
</tr>
</tbody>
</table>

\(^a\) Average of duration of feeding dives (long, deep dives).
\(^b\) Value is an average of the concentration measured in this study (7 mg/g) and the concentration calculated from Scholander, 1940 by Dolar et al., 1999 (37 mg/g).
\(^c\) [Mb] reference first, then reference on average dive duration and, lastly, reference on average dive depth if different from that on average dive duration. nd, not determined.
### Table 2. Mb O₂ affinity equilibrium constants ($P_{50}$ and $K$), dissociation ($k_{off}$) and association ($k_{on}$) rates

<table>
<thead>
<tr>
<th></th>
<th>$P_{50}$ (torr)</th>
<th>$K$ (µM)</th>
<th>$k_{off}$ (s⁻¹)</th>
<th>$k_{on}$ (µM⁻¹s⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bottlenose whale</td>
<td>2.2</td>
<td>3.1</td>
<td>79.0 (19.7)</td>
<td>25.5</td>
</tr>
<tr>
<td>Sperm whale</td>
<td>2.7</td>
<td>3.8</td>
<td>88.2 (20.1)</td>
<td>23.2</td>
</tr>
<tr>
<td>Harbor porpoise</td>
<td>2.7</td>
<td>3.8</td>
<td>88.0 (21.5)</td>
<td>23.2</td>
</tr>
<tr>
<td>Humpback whale</td>
<td>3.9</td>
<td>5.5</td>
<td>97.4 (27.7)</td>
<td>17.8</td>
</tr>
<tr>
<td>Minke whale</td>
<td>4.6</td>
<td>6.5</td>
<td>92.7 (25.0)</td>
<td>14.3</td>
</tr>
<tr>
<td>Horse</td>
<td>2.1</td>
<td>3.0</td>
<td>85.8 (20.9)</td>
<td>29.0</td>
</tr>
</tbody>
</table>

Values were measured at 37°C (values at 25°C in parentheses). $n = 3-5$ in all experiments.