The Journal of Experimental Biology 215, 1938-1943

doi:10.1242/jeb.069872

RESEARCH ARTICLE

Respiratory properties of blood in the harbor porpoise, *Phocoena phocoena*

Lisette B. Soegaard[1,2], Marie N. Hansen[1], Cornelis van Elk[3], Jesper Brahm[4] and Frank B. Jensen[1,*]

[1]Institute of Biology, University of Southern Denmark, DK-5230 Odense M, Denmark, [2]Fjord and Belt, DK-5300 Kerteminde, Denmark, [3]Dolfinarium Harderwijk, NL-3841 Harderwijk, The Netherlands and [4]Department of Cellular and Molecular Medicine, University of Copenhagen, Denmark

*Author for correspondence (fbj@biology.sdu.dk)

Accepted 16 February 2012

SUMMARY

Harbor porpoises are active divers that exchange O_2 and CO_2 with the environment during a fast single breath upon surfacing. We investigated blood O_2-transporting properties, buffer characteristics, Cl^- transport *via* the erythrocyte anion exchanger (AE1), circulating nitric oxide metabolites and hemoglobin nitrite reduction in harbor porpoises with the aim to evaluate traits that are adaptive for diving behavior. Blood O_2 affinity was higher in harbor porpoises than in similar sized terrestrial mammals, as supported by our parallel recordings of O_2 equilibria in sheep and pig blood. Further, O_2 affinity tended to increase with increasing body mass. A high O_2 affinity favors O_2 extraction from the lungs, but a normal Bohr effect ($\Delta\log P_{50}/\Delta pH$=–0.46) gradually lowers O_2 affinity during dives (where CO_2 accumulates) to assist O_2 off-loading to perfused tissues. The true plasma non-bicarbonate buffer value was moderately higher than in terrestrial mammals and increased upon deoxygenation. Plasma bicarbonate was also relatively high, contributing to increase the overall buffer capacity. The apparent Cl^- permeability of harbor porpoise erythrocytes was similar to the human value at 37°C, showing absence of a comparative increase in the velocity of erythrocyte HCO_3^-/Cl^- exchange to aid CO_2 excretion. The Q_{10} for AE1-mediated Cl^- transport in harbor porpoises was lower than in humans and seemed to match the Q_{10} for metabolism (Q_{10}≈2). Plasma nitrite, plasma nitrate and hemoglobin-mediated nitrite reduction were elevated compared with mammalian standards, suggesting that increased nitric oxide bioavailability and nitrite-derived nitric oxide could play important roles in diving physiology.

Key words: diving mammal, oxygen affinity, buffer value, erythrocyte anion exchange, hemoglobin nitrite reduction.

INTRODUCTION

The harbor porpoise is a relatively abundant species in the waters surrounding Denmark (Hammond et al., 2002). It is one of the smallest whales, and as such has a relatively large mass-specific metabolic rate (Reed et al., 2000). In addition, it is an active swimmer that forages the costal waters for long distances. Harbor porpoises spend most of the time submerged but have to surface occasionally to breathe. A surface event typically involves only one ventilatory cycle that is completed in less than 1 s. The breath is characterized by a large tidal volume and high gas flow rates during both expiration and inspiration (Reed et al., 2000). The aerobic dive limit is close to 5 min (Reed et al., 2000), which corresponds with maximum dive duration observed in the field, but most dives are considerably shorter (Westgate et al., 1995).

Whales rely on O_2 stores in lung, blood and muscles to sustain aerobic metabolism during dives (Butler and Jones, 1997; Ponganis, 2011). O_2 stores are therefore slowly depleted, while the metabolic CO_2 production leads to an increase in internal CO_2 tensions (Ponganis, 2011). Ideally, in the harbor porpoise, O_2 stores should be refilled and CO_2 fully eliminated as result of the single breath while surfacing. This also seems the case during short dives, whereas long breath-hold periods call for several surfacing events in close succession, mainly because it is slower to eliminate CO_2 built up in blood and tissues than to replenish O_2 (Boutilier et al., 2001).

The respiratory properties of blood are an important component in the adaptation of marine mammals to their diving behavior. The concentration of hemoglobin (Hb) and the blood volume set the capacity of the blood O_2 reservoir; and the blood O_2 affinity and its pH sensitivity are instrumental in setting blood values of O_2 tension (P_{O2}) and thus diffusion conditions in the microvasculature of lungs and tissues. The buffering capacity of the blood is important in limiting acid–base disturbances as result of CO_2 accumulation (and lactic acid formation during long dives). Some information on blood O_2 affinity and buffering properties in the harbor porpoise is available (Reed et al., 2000), but this information is based on two juvenile individuals, and more data are needed to assess the generality of the findings. The transport of CO_2 from tissues to lungs involves a series of steps, but among these the Cl^-/HCO_3^- exchange across the red blood cell membrane *via* anion exchanger-1 (AE1) is considered to be the rate-limiting step for CO_2 excretion (Wieth et al., 1982; Jensen, 2004). Given the fast ventilatory cycle and short time period for CO_2 excretion during surfacing, the kinetics of anion exchange in the harbor porpoise attracts attention, because a fast erythrocyte anion exchanger would aid CO_2 elimination. At present, no information is available on the rate of erythrocyte anion exchange in diving mammals. The AE1-mediated anion transport shows very large temperature sensitivity in humans (Brahm, 1977), thus the temperature sensitivity of AE1-mediated anion transport in the harbor porpoise also deserves investigation. Diving marine mammals can experience large decreases in local temperature in the peripheral circulation and may also reduce core temperature during dives. A large temperature sensitivity of Cl^-/HCO_3^- exchange (as in humans) could therefore lead to an inappropriate slow-down of anion exchange upon cooling.

In addition to the well-documented functions of Hb in blood O_2 and CO_2 transport and in H^+ buffering, it has been suggested that Hb also functions as a nitrite reductase. Thus, deoxygenated Hb reduces nitrite to nitric oxide (NO), which has been implicated in blood flow regulation (Cosby et al., 2003). Indeed, it appears that nitrite, which is a natural end-product of NO generated by nitric oxide synthases (NOS), functions as an alternative NO source that can be activated by deoxygenated Hb and other cellular proteins under hypoxic conditions (Lundberg et al., 2008). Additionally, in mammals, nitrate (another main NO metabolite) can be reduced to nitrite and then subsequently to NO under hypoxia (Lundberg et al., 2008). Apart from participating in blood flow regulation, nitrite and nitrite-derived NO has been shown to protect tissue cells from ischemia(anoxia)/reperfusion injury (Dezfulian et al., 2007; Shiva and Gladwin, 2009). Because diving mammals naturally experience hypoxia during long dives, and because peripheral vasoconstriction during dives leads to natural cycles of ischemia and reperfusion in several tissues of diving mammals (Butler and Jones, 1997), it seems plausible that nitrite may play important roles in the physiology of diving mammals (Jensen, 2009).

The aims of the present study were to evaluate the respiratory properties of harbor porpoise blood and to characterize the kinetics of AE1-mediated Cl^- transport across the erythrocyte membrane. Furthermore, to initiate research on the role of NO and its metabolites in the physiology of diving mammals, we measured nitrite and nitrate levels in blood plasma and studied the nitrite reductase properties of harbor porpoise Hb at different O_2 saturations.

MATERIALS AND METHODS

Animals and blood sampling

Blood was obtained from individual harbor porpoises [*Phocoena phocoena* (Linnaeus 1758)] during routine veterinary blood sampling at Fjord and Belt in Kerteminde (Denmark) and Harderwijk Dolfinarium (The Netherlands). Both of these facilities have permission to house harbor porpoises. The animals had a body mass of 44.4±14.2 kg (mean ± s.d., N=8). Blood was sampled from a vein in the tail fluke, using heparin as anticoagulant, and immediately placed on ice. Experiments on individual blood samples were completed within 48 h. For comparative purposes, blood from pig (*Sus scofa domesticus*) was obtained from a local slaughterhouse (Danish Crown, Odense) and blood from sheep (*Ovis aries*) was acquired from the Biomedical Laboratory, University of Southern Denmark.

Blood O_2 equilibria and acid–base properties

Sub-samples of blood were pipetted into two Eschweiler (Kiel, Germany) tonometers and equilibrated at 37°C for 45 min to gas mixtures with constant CO_2 tension (P_{CO_2}) and either air (oxygenated blood, tonometer 1) or N_2 (deoxygenated blood, tonometer 2) as balance. Gas mixtures were delivered *via* Wösthoff (Bochum, Germany) gas mixing pumps, and the gases were humidified by bubbling in humidifiers before they reached the tonometers. Separate equilibrations were performed with CO_2 levels of 1, 5 and 9% CO_2 in the gas mixtures. Plasma for studies on separated plasma was obtained by centrifugation of whole blood, and the plasma was subsequently equilibrated to 1, 5 and 9% CO_2.

Oxygen equilibrium curves were constructed by the mixing method (Scheid and Meyer, 1978). Known amounts (mass determination) of deoxygenated blood and oxygenated blood (providing oxygen saturation, S_{O_2}) were mixed in a syringe containing a stainless steel ball, and the P_{O_2} of the mixture was measured at 37°C with a Radiometer (Copenhagen, Denmark) E5046 oxygen electrode connected to a PHM 73 monitor. At least three different mixtures (S_{O_2} values) were analyzed in each experiment, and the results were plotted in a Hill diagram [$\log(S_{O_2}/(100–S_{O_2}))$ *versus* $\log P_{O_2}$] from which P_{50} (O_2 tension at 50% saturation) and Hill's n (slope of the linear Hill plot, which expresses cooperativity) were calculated.

Blood pH was measured with the capillary pH electrode of a Radiometer BMS 3 electrode setup. Total CO_2 contents in true plasma (obtained by centrifugation of equilibrated blood) and separated plasma were measured with the Cameron method (Cameron, 1971). Bicarbonate was evaluated as the difference between total CO_2 and dissolved CO_2, using the human value of 0.0306 mmol l^{-1} $mmHg^{-1}$ for CO_2 solubility (Siggaard-Andersen, 1976).

Hematocrit (Hct) was determined by centrifugation (3 min at 14,980 ***g***), and hemoglobin (Hb) was measured spectrophotometrically with the cyanmethemoglobin method, using an extinction coefficient of 11 $mmol^{-1}$ l^{-1} cm^{-1} at 540 nm.

Unidirectional $^{36}Cl^-$ efflux *via* the red blood cell anion exchanger

Whole blood was centrifuged and the red blood cells (RBCs) were washed three times in an iso-osmotic saline consisting of: 128 mmol l^{-1} NaCl, 25 mmol l^{-1} $NaHCO_3$, 2.5 mmol l^{-1} KH_2PO_4, 2 mmol l^{-1} $CaCl_2$, 1 mmol l^{-1} $MgSO_4$, 3.9 mmol l^{-1} D-glucose and 10 mmol l^{-1} Hepes buffer. The RBCs were suspended in the saline at a Hct of 50% and equilibrated for 45 min with humidified air (oxygenating the cells) in Eschweiler tonometers. Equilibrations were performed at 15, 25 and 37°C. Extracellular pH was 8.01±0.08 (mean ± s.e.m., N=21). From human RBCs it is known that Cl^- exchange fluxes are maximal and constant when extracellular pH is between pH 7 and 9 (Wieth et al., 1982). The isotope $^{36}Cl^-$ (as NaCl) was added 10 min prior to the end of equilibration (final radioactivity 15 kBq ml^{-1}). After equilibration, the RBC suspension was centrifuged (10 min at 3850 ***g***) to obtain packed RBCs for $^{36}Cl^-$ efflux measurements. Unidirectional $^{36}Cl^-$ efflux *via* the RBC anion exchanger (which greatly exceeds and is much faster than Cl^- transport *via* other transporters) was measured under Cl^- self-exchange conditions at the three different temperatures, using the continuous flow tube method, as previously described (Brahm, 1977; Jensen et al., 2001). The rate coefficients (k; s^{-1}) for unidirectional Cl^- efflux determined in these experiments were converted into apparent Cl^- permeability coefficients (P_{Cl}; µm s^{-1}) by multiplying k with the ratio between cell water volume and membrane surface area (V_W/A_m=0.447 µm). The RBC volume, area and water content were assessed as previously described (Jensen and Brahm, 1995).

Nitrite and nitrate in plasma, and nitrite reactions with hemoglobin

Freshly drawn blood was centrifuged and the plasma was frozen and kept at –80°C until measurements. Plasma nitrite and nitrate were measured by reductive chemiluminescence using a Sievers (Boulder, CO, USA) Nitric Oxide Analyzer model 280i (Hansen and Jensen, 2010).

Purified Hb solutions (in 0.05 mol l^{-1} Tris buffer, pH 7.3 and 0.1 mol l^{-1} KCl) were prepared as previously described (Jensen, 2008). The reaction between nitrite and Hb (at 155 µmol l^{-1} heme and a $[NO_2^-]$/[heme] ratio of 2.7) was recorded spectrophotometrically at 25°C, using a specially constructed glass tonometer with a built-in cuvette (Jensen, 2008). Experiments were performed at different constant P_{O_2} values, producing S_{O_2} values ranging from 0% (fully deoxygenated Hb) to 99% S_{O_2} (oxygenated

Hb). Gas with constant P_{O_2} was supplied to the tonometer from a Wösthoff (Bochum, Germany) gas mixing pump (mixing air with N_2). The concentrations of deoxygenated Hb (deoxyHb), oxygenated Hb (oxyHb), nitrosyl-hemoglobin (HbNO) and methemoglobin (metHb) in the course of the reaction were determined by spectral deconvolution of absorbance spectra recorded at different time points during the reaction (Jensen, 2008).

RESULTS AND DISCUSSION

Blood oxygen transporting properties

Oxygenated blood equilibrated to 5% CO_2 had a Hct of 46.9±6.5% and a tetrameric Hb concentration of 2.7±0.5 mmol l^{-1} (means ± s.d., N=8), the latter corresponding to a Hb-bound O_2 capacity of 10.8 mmol l^{-1} or 24.2 vol.% (ml O_2 100 ml^{-1}). These values are similar to earlier reported values for harbor porpoise (Reed et al., 2000) and only marginally higher than values for terrestrial mammals (Snyder, 1983). Although a higher Hct and [Hb] would have increased O_2 capacity, as observed in long- and deep-diving species, this would come at the expense of an increased blood viscosity (Hedrick and Duffield, 1991). The observed Hct in harbor porpoise is close to the optimal Hct for oxygen transport (Hedrick and Duffield, 1991).

The mean P_{50} value of harbor porpoise blood at 5% CO_2 (P_{CO_2}≈35.7 mmHg) was 29.8 mmHg, which increased to 36.2 mmHg at 9% CO_2 and decreased to 19.3 mmHg at 1% CO_2. On a logarithmic scale, P_{50} values were linearly correlated to pH, revealing a Bohr coefficient ($\Delta\log P_{50}/\Delta$pH) of –0.46 (Fig. 1). The O_2 affinity of harbor porpoise blood is somewhat higher (i.e. P_{50} is somewhat smaller) than in terrestrial mammals of similar size (Schmidt-Nielsen, 1997). This conclusion is supported by our findings of larger P_{50} values in pig and sheep than in harbor porpoise, using the same experimental method on all species (Fig. 1). The O_2 affinity of pig and sheep blood resembles earlier reports (Willford and Hill, 1986; Moraga et al., 1996), whereas the oxygen affinity of harbor porpoise blood is comparable to that in bottlenose dolphin and common dolphin (Kooyman, 1989). A relatively high blood O_2 affinity in the harbor porpoise is advantageous for effective O_2 extraction from the pulmonary O_2 store during dives (Snyder, 1983), as recently documented in diving emperor penguins (Meir and Ponganis, 2009). This mechanism is particularly applicable in divers that inhale before diving and perform relative shallow dives, where the alveoli do not collapse (e.g. harbor porpoise). The mechanism is not applicable in deep-diving seals that exhale before diving, and where the alveoli collapse. Deep- and long-diving seals instead have relatively low O_2 affinity that favors O_2 off-loading in tissues (Meir et al., 2009).

Although the O_2 affinity of harbor porpoise blood is increased compared with similarly sized terrestrial mammals, it is not inappropriately high. O_2 off-loading to tissues can therefore still occur at a relatively high off-loading P_{O_2}. This is aided by the normal-sized Bohr effect ($\Delta\log P_{50}/\Delta$pH=–0.46), which provides a significant rightward shift of the O_2 equilibrium curve when CO_2 is added during blood passage of tissue capillaries. Also, the Bohr effect contributes a gradual increase in P_{50} in the course of a dive, as P_{CO_2} increases and pH decreases with dive duration, helping to off-load O_2 when O_2 stores gradually decline. Additionally, the O_2 cooperativity of harbor porpoise blood is comparatively high: Hill's n was approximately 3 throughout the examined pH interval (not illustrated), which compares with a value of 2.7 in human blood (Siggaard-Andersen, 1976). The high Hill's n reflects a steep O_2 equilibrium curve that favors O_2 off-loading in tissues and O_2 uptake in lungs for only small changes in P_{O_2}.

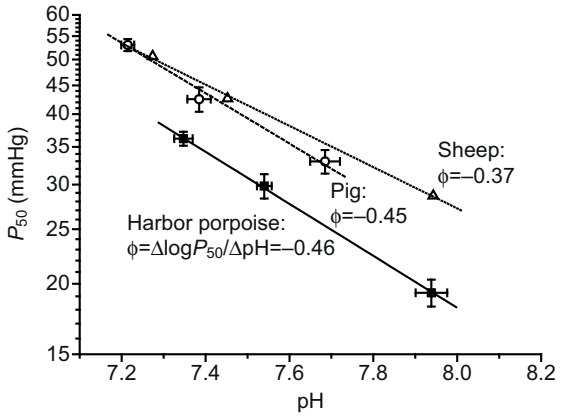

Fig. 1. Oxygen tension at 50% saturation (P_{50}; logarithmic scale) as a function of pH in whole blood from harbor porpoise, pig and sheep at 37°C. Values are means ± s.e.m. (N=8 for harbor porpoise; N=6 for pig; N=2 for sheep). The Bohr factors ($\phi=\Delta\log P_{50}/\Delta$pH) were determined from the slopes of the linear relationships and are indicated for each species.

Interspecific comparisons have revealed that mammals with low body mass have lower O_2 affinity than mammals with high body mass (Schmidt-Nielsen, 1997). Because body mass of the present harbor porpoises ranged from 29 to 68 kg, we tested for the presence of an intraspecific correlation between O_2 affinity and body mass. Indeed, there was a tendency for a decrease in P_{50} with increasing body mass (Fig. 2), but the correlation failed to be significant at the P<0.05 level (P=0.087).

Blood acid–base properties

Equilibration of oxygenated blood to different P_{CO_2} values resulted in a practically linear relationship between plasma [HCO_3^-] and extracellular pH from which the non-bicarbonate true plasma buffer value ($\beta_{oxy}=-\Delta[HCO_3^-]/\Delta$pH) of 27 Slykes (mmol l^{-1} pH $unit^{-1}$) was calculated (Fig. 3). This value compares with the value of 29.2 Slykes previously reported for the harbor porpoise (Reed et al., 2000). In deoxygenated blood, the non-bicarbonate buffer value was higher (β_{deoxy}=34.3 Slykes) and [HCO_3^-] was increased compared with oxygenated blood (Fig. 3), showing the presence of a Haldane effect, which follows naturally from the presence of a Bohr effect (Jensen, 2004). Upon deoxygenation, the conformational change from oxyHb to deoxyHb is associated with increased pK_a values of the Bohr groups (i.e. the specific amino acid residues responsible for the Bohr effect), which increases H^+ binding to the Hb inside the RBCs. At any given P_{CO_2}, this extra H^+ binding drives the CO_2 hydration equilibrium reaction ($CO_2 + H_2O \leftrightarrow HCO_3^- + H^+$) further towards HCO_3^- formation, which subsequently is shifted to plasma *via* the RBC anion exchanger, elevating plasma [HCO_3^-] in deoxygenated blood.

The true plasma buffer value primarily reflects the buffering capacity of Hb. The β-values in the harbor porpoise are slightly higher than in terrestrial mammals [e.g. 22 Slykes in pigs (L.B.S., unpublished), 28 Slykes in humans (Siggaard-Andersen, 1976) and 16 Slykes in mice (Iversen et al., 2012)] and similar to the value of 32 Slykes reported for the gray seal (Boutilier et al., 1993). Large elevations in β-values compared with terrestrial mammals are mainly found in diving species with high blood Hb concentrations (Lapennas and Reeves, 1982), which may be essential for buffering acid loads during long dives.

The buffer value of separated plasma reflects the buffering properties of plasma proteins. The value in the harbor porpoise was

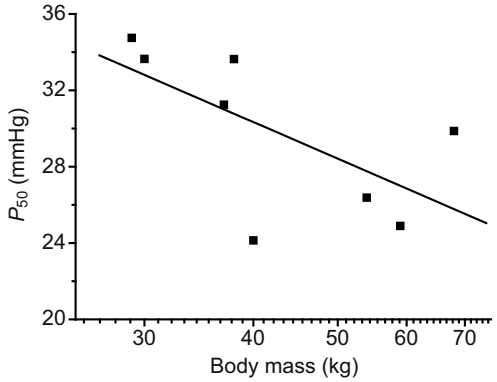

Fig. 2. P_{50} of harbor porpoise blood equilibrated to 5% CO_2 as a function of body mass (logarithmic scale). Values are from eight individual harbor porpoises. The line depicts the linear regression of the data (R=0.64, P=0.087, N=8).

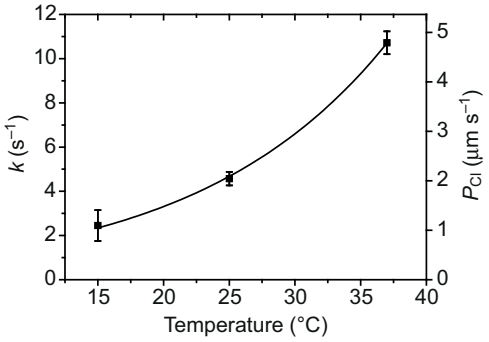

Fig. 4. Temperature dependency of rate coefficients (k; left y-axis) for unidirectional Cl^- efflux from harbor porpoise red blood cells, with the corresponding apparent Cl^- permeability coefficient (P_{Cl}) depicted on the right y-axis. Experiments were performed on red blood cells from two individuals with three to four replicates per individual at each temperature. There were no difference between individuals, and the pooled results are shown (N=6–8 at each point). The drawn curve is an exponential fit to the data (R^2=0.9994). The three mean values are all significantly different (one-way ANOVA followed by Bonferroni test).

8 Slykes (Fig. 3), which is not significantly elevated compared with mammalian standards. Killer whales, and particularly gray seals, have been reported to have high non-bicarbonate buffer values in separated plasma, presumably as result of elevated levels of plasma proteins (Boutilier et al., 1993), but the precise mechanism has not been resolved.

Plasma bicarbonate concentrations were somewhat higher in the harbor porpoise than in humans under standard conditions [i.e. 30 mmol l^{-1} (Fig. 3) *versus* 25 mmol l^{-1}, respectively, at pH 7.4]. This provides the harbor porpoise with a high bicarbonate buffer capacity that assists the buffering of metabolic acidosis during and after long dives or extensive exercise.

RBC anion exchange

The rate coefficient k (s^{-1}) for unidirectional Cl^- efflux *via* the RBC anion exchanger increased exponentially with temperature (Fig. 4). The apparent Cl^- permeability coefficient P_{Cl} (depicted on the right y-axis of Fig. 4) reached a value close to 5 μm s^{-1} at 37°C. This value is similar to the P_{Cl} value in human RBCs at 37°C (Jensen and Brahm, 1995) and to P_{Cl} values observed in a number of ectothermic animals at their respective preferred temperatures (Jensen et al., 2001). Thus, the velocity of anion exchange across the RBC membrane in the harbor porpoise is not increased compared with other animals at normal body temperature, suggesting that the RBC HCO_2/Cl^- exchange capacity is adequate to secure sufficient CO_2 excretion during single breaths at surfacing events.

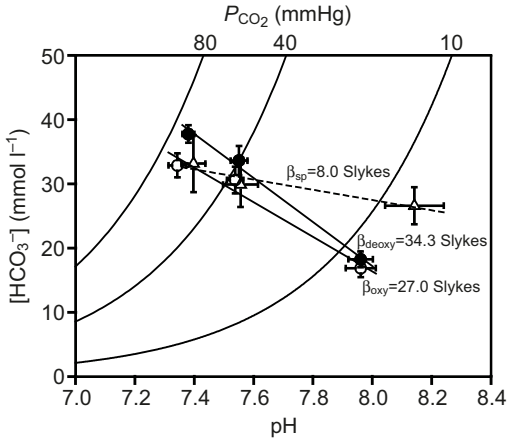

Fig. 3. Relationship between plasma [HCO_3^-] and pH in true plasma of oxygenated (open circles) and deoxygenated (filled circles) harbor porpoise blood, and in separated plasma (open triangles). Non-bicarbonate buffer values (β=–Δ[HCO_3^-]/ΔpH; in Slykes=mmol l^{-1} pH $unit^{-1}$) were obtained from linear regressions of the data. Values are means ± s.e.m. (N=8).

The temperature dependency of k and P_{Cl} (Fig. 4) corresponded to an overall Q_{10} value of 2.0 for anion exchange in the considered temperature interval. This temperature sensitivity is lower than that reported for RBC anion exchange in other endotherms, specifically chickens (Brahm and Wieth, 1977) and humans (Brahm, 1977), where Q_{10} is 3.4 between 15 and 38°C. The temperature sensitivity in the harbor porpoise is, however, similar to that observed in ectothermic animals that normally experience changes in body temperature (Jensen et al., 2001). This suggests that the Q_{10} for anion exchange and the Q_{10} for metabolism and gas transport (often close to 2) match each other. Given that RBC anion exchange is the rate-limiting step in CO_2 transport, this means that the degree of rate limitation offered by RBC anion exchange does not change significantly as temperature changes (Jensen et al., 2001). The matching of Q_{10} values for RBC anion exchange and CO_2 transport is clearly adaptive in ectotherms, whereas its importance in an endotherm seems less obvious. We speculate that a minor decrease in core temperature during long dives as well as peripheral cooling (e.g. in flippers) could make a match of Q_{10} values advantageous in diving mammals. Information on body temperature in diving mammals is limited, but core temperature can decrease by 2–3°C (Kooyman et al., 1981) and occasionally even up to 7°C (Meir and Ponganis, 2010) in diving seals. A decrease in body temperature would help to reduce metabolic rate (and thus O_2 demand), but it appears that core temperature is relatively tightly controlled during routine dives and is only allowed to decrease appreciably during exceptional long dives (Meir and Ponganis, 2010).

Circulating nitrite and nitrate

The plasma concentration of nitrite measured by reductive chemiluminescence was 3.3±0.7 μmol l^{-1} (mean ± s.e.m., N=6) and the concentration of nitrate was 109±23 μmol l^{-1} (mean ± s.e.m., N=6). Both mean values are appreciably higher than corresponding

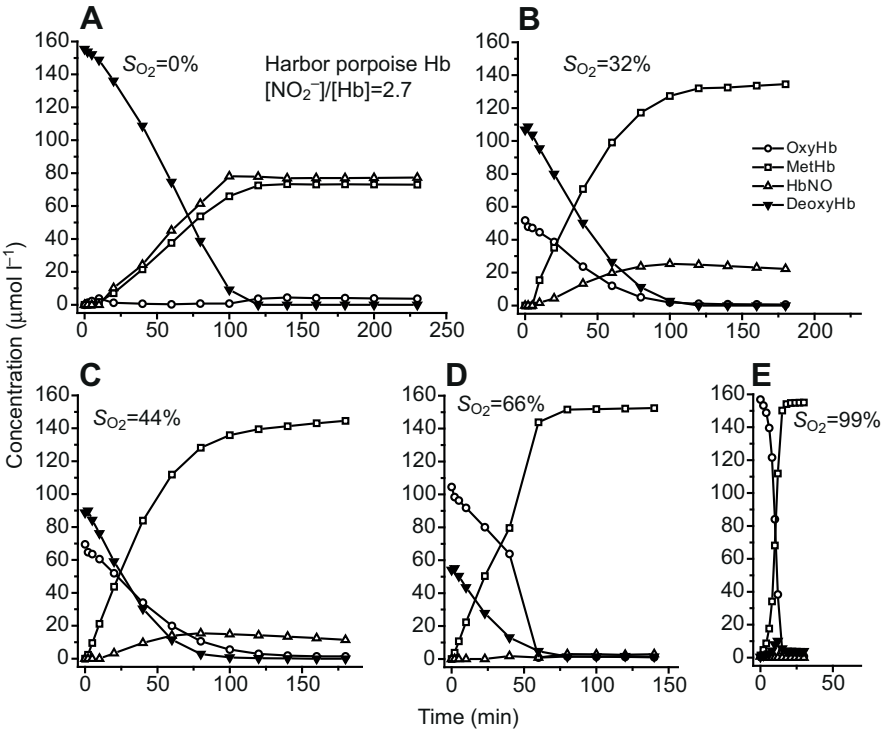

Fig. 5. Time-dependent changes in the concentrations of oxygenated hemoglobin (oxyHb), methemoglobin (metHb), nitrosyl-hemoglobin (HbNO) and deoxygenated hemoglobin (deoxyHb) during the reaction of nitrite with harbor porpoise Hb at different oxygen saturations (S_{O_2}). Initial S_{O_2} values are depicted in the individual panels. All panels are drawn to the same scale. The Hb concentration was 155 μmol l^{-1} and the nitrite/heme concentration ratio was 2.7. The temperature was 25°C. Measurements were made in 0.05 mol l^{-1} Tris buffer, with 0.1 mol l^{-1} KCl, at a pH of 7.3.

values in terrestrial mammals that do not normally encounter hypoxia (nitrite <1 μmol l^{-1}; nitrate ~20–40 μmol l^{-1}) (Kleinbongaard et al., 2003; Lundberg et al., 2008), suggesting that circulating nitrite and nitrate levels are elevated in diving mammals, possibly as result of elevated NOS activity. High nitrite and nitrate levels have also been reported in Tibetan highlanders (Erzurum et al., 2007) and in some hypoxia-tolerant fish (Sandvik et al., 2012) and turtles (Jacobsen et al., 2012). Collectively, this suggests that elevated circulating nitrite could be part of the adaptation to hypoxia. In harbor porpoises, high nitrite and nitrate levels may provide protection against ischemia/reperfusion injury (Shiva and Gladwin, 2009) in organs that are cut-off from blood flow during long dives. High nitrate/nitrite levels can also reduce whole-body oxygen cost during exercise by increasing the metabolic efficiency of mitochondria, as recently described in humans (Larsen et al., 2007; Bailey et al., 2009; Larsen et al., 2011). Indeed, an improved efficiency of O_2 utilization during exercise would help active divers such as the harbor porpoise to economize with O_2 reserves during dives.

It is noteworthy that the measured values of nitrite and nitrate were variable, as they were in Tibetan highlanders (Erzurum et al., 2007). Endogenous nitrite and nitrate are derived both from oxidation of NO and from dietary sources (Lundberg et al., 2008). The variability can therefore originate from a difference in NOS activity as well as variable dietary contributions among individuals. More research is needed to verify whether nitrite and nitrate are generally elevated in diving mammals.

Nitrite reactions with Hb

Addition of nitrite to fully deoxygenated Hb in solution resulted in a progressive decrease in the concentration of deoxyHb to zero and a concurrent 1:1 increase in the concentrations of metHb and HbNO to half the initial concentration of deoxyHb (Fig. 5A). This outcome shows that nitrite reacts with deoxygenated ferrous (Fe^{2+}) heme to form met(Fe^{3+})Hb and NO, with subsequent rapid binding of the formed NO to vacant ferrous heme (Gladwin and Kim-Shapiro, 2008):

$$Hb(Fe^{2+}) + NO_2^- + H^+ \rightarrow Hb(Fe^{3+}) + NO + OH^-, \quad (1)$$

$$Hb(Fe^{2+}) + NO \rightarrow Hb(Fe^{2+})NO. \quad (2)$$

The reaction of nitrite with fully oxygenated Hb was faster and resulted in conversion of all oxyHb to metHb (Fig. 5E), in agreement with the stoichiometry for the complex autocatalytic oxidation of oxyHb by nitrite (Kosaka and Tyuma, 1987):

$$4Hb(Fe^{2+})O_2 + 4\ NO_2^- + 4H^+ \rightarrow 4Hb(Fe^{3+}) + 4\ NO_3^- + O_2 + 2\ H_2O. \quad (3)$$

At intermediate S_{O_2} values (as occurs *in vivo*), nitrite can react with both deoxyHb and oxyHb, but the deoxyHb reaction producing NO (reaction 1) was clearly favored, as seen from the steeper decline in [deoxyHb] with time (i.e. higher reaction rate) compared with [oxyHb] at 32% S_{O_2} (Fig. 5B) and 44% S_{O_2} (Fig. 5C). At 66% S_{O_2}, the same applied, but the oxyHb reaction became autocatalytic when [deoxyHb] approached zero, quickly converting remaining oxyHb to metHb (Fig. 5D). These findings match observations made in two other mammalian Hbs: human Hb (Grubina et al., 2007) and rabbit Hb (Jensen, 2008). A Hill plot, using the initial S_{O_2} values and corresponding P_{O_2} values, revealed a P_{50} of 6.1 mmHg and an *n*-value of 2.7 for harbor porpoise Hb under the conditions used in the experiments (data not shown).

The reactions between nitrite and Hb were studied under the same conditions as previously used, e.g. with rabbit Hb (Jensen, 2008), allowing for a direct comparison. The reaction of nitrite with fully deoxygenated Hb was considerably faster in harbor porpoise than in rabbit [completed in 120 min (Fig. 1A) *versus* 370 min (Jensen, 2008), respectively], and reaction rates were also higher in harbor porpoise than in rabbit at intermediate S_{O_2}. Furthermore, the formation of NO and HbNO was higher at intermediate S_{O_2} in harbor porpoise. Collectively, these results reveal a higher nitrite reductase activity in harbor porpoise Hb than in rabbit Hb. An increase in O_2 affinity can increase the reaction rate by favoring the R conformation of Hb, which lowers the heme redox potential and increases the reactivity of deoxygenated hemes (Crawford et al., 2006; Jensen,

2008). However, this does not explain the difference between harbor porpoise and rabbit, because P_{50} of the Hb was similar in the two species. The difference must therefore mainly result from differences in redox potential that are unrelated to O_2 affinity.

The deoxyHb-mediated production of NO has been suggested to contribute to hypoxic vasodilation (Cosby et al., 2003; Crawford et al., 2006), but whether this has a bearing on blood flow in marine mammals remains to be determined. The situation is special in diving mammals, because blood flow to muscles and some internal organs is curtailed by vasoconstriction during long dives. Release of NO activity from deoxygenating RBCs during a dive may, however, help O_2 delivery to tissues such as the heart and the brain that remain perfused. Nitrite-derived NO may also be important by inducing vasodilation in the pulmonary vasculature (Ingram et al., 2010). Global hypoxia (as develops in mammals during apnea or at high altitude) routinely constricts pulmonary arteries throughout the lungs, which increases pulmonary vascular resistance, pulmonary artery blood pressure and right ventricular work (Moudgil et al., 2005). This response is maladaptive in global hypoxia (Storz et al., 2010), but would be countered by vasodilation from nitrite reduction to NO in RBCs and/or in the vessel wall. The elevated nitrite levels in the harbor porpoise may therefore prevent hypoxic pulmonary vasoconstriction. The pulmonary artery pressure does not increase during sleep-apnea-induced hypoxia in elephant seals (Ponganis et al., 2006), supporting that hypoxic pulmonary vasoconstriction may be weak in diving mammals.

ACKNOWLEDGEMENTS

We thank Genevieve Desportes and Angela Fago for useful discussions, and the staff at Fjord and Belt and Dolfinarium Harderwijk for their help during blood sampling.

FUNDING

The work was supported by the Danish Natural Science Research Council (21-04-0326 and 10-084565).

REFERENCES

Bailey, S. J., Winyard, P., Vanhatalo, A., Blackwell, J. R., DiMenna, F. J., Wilkerson, D. P., Tarr, J., Benjamin, N. and Jones, A. M. (2009). Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans. *J. Appl. Physiol.* **107**, 1144-1155.

Boutilier, R. G., Nikinmaa, M. and Tufts, B. L. (1993). Relationship between blood buffering properties, erythrocyte pH and water content, in gray seals (*Halichoerus grypus*). *Acta Physiol. Scand.* **147**, 241-247.

Boutilier, R. G., Reed, J. Z. and Fedak, M. A. (2001). Unsteady-state gas exchange and storage in diving marine mammals: the harbor porpoise and grey seal. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **281**, R490-R494.

Brahm, J. (1977). Temperature-dependent changes of chloride transport kinetics in human red cells. *J. Gen. Physiol.* **70**, 283-306.

Brahm, J. and Wieth, J. O. (1977). Separate pathways for urea and water and for chloride in chicken erythrocytes. *J. Physiol.* **266**, 727-749.

Butler, P. J. and Jones, D. R. (1997). The physiology of diving of birds and mammals. *Physiol. Rev.* **77**, 837-899.

Cameron, J. N. (1971). Rapid method for determination of total carbon dioxide in small blood samples. *J. Appl. Physiol.* **31**, 632-634.

Cosby, K., Partovi, K. S., Crawford, J. H., Patel, R. P., Reiter, C. D., Martyr, S., Yang, B. K., Waclawiw, M. A., Zalos, G., Xu, X. et al. (2003). Nitrite reduction to nitric oxide by deoxyhemoglobin vasodilates the human circulation. *Nat. Med.* **9**, 1498-1505.

Crawford, J. H., Isbell, T. S., Huang, Z., Shiva, S., Chacko, B. K., Schechter, A. N., Darley-Usmar, V. M., Kerby, J. D., Lang, J. D., Jr, Kraus, D. et al. (2006). Hypoxia, red blood cells, and nitrite regulate NO-dependent hypoxic vasodilation. *Blood* **107**, 566-574.

Dezfulian, C., Raat, N., Shiva, S. and Gladwin, M. T. (2007). Role of the anion nitrite in ischemia-reperfusion cytoprotection and therapeutics. *Cardiovasc. Res.* **75**, 327-338.

Erzurum, S. C., Ghosh, S., Janocha, A. J., Xu, W., Bauer, S., Bryan, N. S., Tejero, J., Hemann, C., Hille, R., Struehr, D. J. et al. (2007). Higher blood flow and circulating NO products offset high-altitude hypoxia among Tibetans. *Proc. Natl. Acad. Sci. USA* **104**, 17593-17598.

Gladwin, M. T. and Kim-Shapiro, D. B. (2008). The functional nitrite reductase activity of the heme-globins. *Blood* **112**, 2636-2647.

Grubina, R., Huang, Z., Shiva, S., Joshi, M. S., Azarov, I., Basu, S., Ringwood, L. A., Jiang, A., Hogg, N., Kim-Shapiro, D. B. et al. (2007). Concerted nitric oxide formation and release from the simultaneous reactions of nitrite with deoxy- and oxyhemoglobin. *J. Biol. Chem.* **282**, 12916-12927.

Hammond, P. S., Berggren, P., Benke, H., Borchers, D. L., Collet, A., Heide-Jorgensen, M. P., Heimlich, S., Hiby, A. R., Leopold, M. F. and Oien, N. (2002). Abundance of harbour porpoise and other cetaceans in the North Sea and adjacent waters. *J. Appl. Ecol.* **39**, 361-376.

Hansen, M. N. and Jensen, F. B. (2010). Nitric oxide metabolites in goldfish under normoxic and hypoxic conditions. *J. Exp. Biol.* **213**, 3593-3602.

Hedrick, M. S. and Duffield, D. A. (1991). Haematological and rheological characteristics of blood in seven marine mammal species: physiological implications for diving behavior. *J. Zool.* **225**, 273-283.

Ingram, T. E., Pinder, A. G., Bailey, D. M., Fraser, A. G. and James, P. E. (2010). Low-dose sodium nitrite vasodilates hypoxic human pulmonary vasculature by a means that is not dependent on a simultaneous elevation in plasma nitrite. *Am. J. Physiol. Heart Circ. Physiol.* **298**, H331-H339.

Iversen, N. K., Malte, H., Baatrup, E. and Wang, T. (2012). The normal acid-base status of mice. *Respir. Physiol. Neurobiol.* **180**, 252-257.

Jacobsen, S. B., Hansen, M. N., Jensen, F. B., Skovgaard, N., Wang, T. and Fago, A. (2012). Circulating nitric oxide metabolites and cardiovascular changes in the turtle *Trachemys scripta* during normoxia, anoxia and reoxygenation. *J. Exp. Biol.* **215** (in press).

Jensen, F. B. (2004). Red blood cell pH, the Bohr effect, and other oxygenation-linked phenomena in blood O_2 and CO_2 transport. *Acta Physiol. Scand.* **182**, 215-227.

Jensen, F. B. (2008). Nitric oxide formation from the reaction of nitrite with carp and rabbit hemoglobin at intermediate oxygen saturations. *FEBS J.* **275**, 3375-3387.

Jensen, F. B. (2009). The role of nitrite in nitric oxide homeostasis: a comparative perspective. *Biochim. Biophys. Acta* **1787**, 841-848.

Jensen, F. B. and Brahm, J. (1995). Kinetics of chloride transport across fish red blood cell membranes. *J. Exp. Biol.* **198**, 2237-2244.

Jensen, F. B., Wang, T. and Brahm, J. (2001). Acute and chronic influence of temperature on red blood cell anion exchange. *J. Exp. Biol.* **204**, 39-45.

Kleinbongard, P., Dejam, A., Lauer, T., Rassaf, T., Schindler, A., Picker, O., Scheeren, T., Godecke, A., Schrader, J., Schulz, R. et al. (2003). Plasma nitrite reflects constitutive nitric oxide synthase activity in mammals. *Free Radic. Biol. Med.* **35**, 790-796.

Kooyman, G. L. (1989). *Diverse Divers. Physiology and Behaviour.* Zoophysiology, Vol. 23. Berlin: Springer.

Kooyman, G. L., Castellini, M. A. and Davis, R. W. (1981). Physiology of diving in marine mammals. *Annu. Rev. Physiol.* **43**, 343-356.

Kosaka, H. and Tyuma, I. (1987). Mechanism of autocatalytic oxidation of oxyhemoglobin by nitrite. *Environ. Health Perspect.* **73**, 147-151.

Lapennas, G. N. and Reeves, R. B. (1982). Respiratory properties of blood of the gray seal, *Halichoerus grypus*. *J. Comp. Physiol.* **149**, 49-56.

Larsen, F. J., Weitzberg, E., Lundberg, J. O. and Ekblom, B. (2007). Effects of dietary nitrate on oxygen cost during exercise. *Acta Physiol.* **191**, 59-66.

Larsen, F. J., Schiffer, T. A., Borniquel, S., Sahlin, K., Ekblom, B., Lundberg, J. O. and Weitzberg, E. (2011). Dietary inorganic nitrate improves mitochondrial efficiency in humans. *Cell Metab.* **13**, 149-159.

Lundberg, J. O., Weitzberg, E. and Gladwin, M. T. (2008). The nitrate-nitrite-nitric oxide pathway in physiology and therapeutics. *Nat. Rev. Drug Dis.* **7**, 156-167.

Meir, J. U. and Ponganis, P. J. (2009). High-affinity hemoglobin and blood oxygen saturation in diving emperor penguins. *J. Exp. Biol.* **212**, 3330-3338.

Meir, J. U. and Ponganis, P. J. (2010). Blood temperature profiles of diving elephant seals. *Physiol. Biochem. Zool.* **83**, 531-540.

Meir, J. U., Champagne, C. D., Costa, D. P., Williams, C. L. and Ponganis, P. J. (2009). Extreme hypoxemic tolerance and blood oxygen depletion in diving elephant seals. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, R927-R939.

Moraga, F., Monge, C., Riquelme, R. and Llanos, A. (1996). Fetal and maternal blood oxygen affinity: a comparative study in llamas and sheep. *Comp. Biochem. Physiol.* **115A**, 111-115.

Moudgil, R., Michelakis, E. D. and Archer, S. L. (2005). Hypoxic pulmonary vasoconstriction. *J. Appl. Physiol.* **98**, 390-403.

Ponganis, P. J. (2011). Diving mammals. *Compr. Physiol.* **1**, 447-465.

Ponganis, P. J., Stockard, T. K., Levenson, D. H., Berg, L. and Baranov, E. A. (2006). Intravascular pressure profiles in elephant seals: Hypotheses on the caval sphincter, extradural vein and venous return to the heart. *Comp. Biochem. Physiol.* **145A**, 123-130.

Reed, J. Z., Chambers, C., Hunter, C. J., Lockyer, C., Kastelein, R., Fedak, M. A. and Boutilier, R. G. (2000). Gas exchange and heart rate in the harbor porpoise, *Phocoena phocoena*. *J. Comp. Physiol. B* **170**, 1-10.

Sandvik, G. K., Nilsson, G. E. and Jensen, F. B. (2012). Dramatic increase of nitrite levels in hearts of anoxia-exposed crucian carp supporting a role in cardioprotection. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **302**, R468-R477.

Scheid, P. and Meyer, M. (1978). Mixing technique for study of oxygen-hemoglobin equilibrium: a critical evaluation. *J. Appl. Physiol.* **45**, 818-822.

Schmidt-Nielsen, K. (1997). *Animal Physiology. Adaptation and Environment.* Cambridge: Cambridge University Press.

Shiva, S. and Gladwin, M. T. (2009). Nitrite mediates cytoprotection after ischemia/reperfusion by modulating mitochondrial function. *Basic Res. Cardiol.* **104**, 113-119.

Siggaard-Andersen, O. (1976). *The Acid-Base Status of the Blood.* Munksgaard, Copenhagen.

Snyder, G. K. (1983). Respiratory adaptations in diving mammals. *Respir. Physiol.* **54**, 269-294.

Storz, J. F., Scott, G. R. and Cheviron, Z. A. (2010). Phenotypic plasticity and genetic adaptation to high-altitude hypoxia in vertebrates. *J. Exp. Biol.* **213**, 4125-4136.

Westgate, A. J., Read, A. J., Berggren, P., Koopman, H. N. and Gaskin, D. E. (1995). Diving behavior of harbor porpoises, *Phocoena phocoena*. *Can. J. Fish. Aquat. Sci.* **52**, 1064-1073.

Wieth, J. O., Andersen, O. S., Brahm, J., Bjerrum, P. J. and Borders, C. L. (1982). Chloride-bicarbonate exchange in red blood cells: physiology of transport and chemical modification of binding sites. *Philos. Trans. R. Soc. Lond. B* **299**, 383-399.

Willford, D. C. and Hill, E. P. (1986). Modest effect of temperature on the porcine oxygen dissociation curve. *Respir. Physiol.* **64**, 113-123.