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High-affinity hemoglobin and blood oxygen saturation in diving emperor penguins

Jessica U. Meir* and Paul J. Ponganis

Center for Marine Biotechnology and Biomedicine, Scripps Institution of Oceanography, University of California San Diego, La Jolla, CA 92093, USA

*Author for correspondence (jmeir@ucsd.edu)

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SUMMARY

The emperor penguin (*Aptenodytes forsteri*) thrives in the Antarctic underwater environment, diving to depths greater than 500 m and for durations longer than 23 min. To examine mechanisms underlying the exceptional diving ability of this species and further describe blood oxygen (O_2) transport and depletion while diving, we characterized the O_2–hemoglobin (Hb) dissociation curve of the emperor penguin in whole blood. This allowed us to (1) investigate the biochemical adaptation of Hb in this species, and (2) address blood O_2 depletion during diving, by applying the dissociation curve to previously collected partial pressure of O_2 (P_{O_2}) profiles to estimate *in vivo* Hb saturation (S_{O_2}) changes during dives. This investigation revealed enhanced Hb–O_2 affinity (P_{50}=28 mmHg, pH 7.5) in the emperor penguin, similar to high-altitude birds and other penguin species. This allows for increased O_2 at low blood P_{O_2} levels during diving and more complete depletion of the respiratory O_2 store. S_{O_2} profiles during diving demonstrated that arterial S_{O_2} levels are maintained near 100% throughout much of the dive, not decreasing significantly until the final ascent phase. End-of-dive venous S_{O_2} values were widely distributed and optimization of the venous blood O_2 store resulted from arterialization and near complete depletion of venous blood O_2 during longer dives. The estimated contribution of the blood O_2 store to diving metabolic rate was low and highly variable. This pattern is due, in part, to the influx of O_2 from the lungs into the blood during diving, and variable rates of tissue O_2 uptake.

Key words: oxygen-hemoglobin dissociation curve, aerobic dive limit, hypoxia, P_{O_2}, oxygen depletion.

INTRODUCTION

Extreme environments, particularly those with limited oxygen (O_2) availability, are especially challenging to most life forms, yet still host diverse and abundant life. Species that flourish in such environments are ideal models in which to examine the physiological, cellular and biochemical mechanisms underlying tolerance to hypoxia and O_2 depletion. The emperor penguin (*Aptenodytes forsteri*), the consummate avian diver, thrives in the extreme Antarctic underwater environment, diving to depths greater than 500 m (Kooyman and Kooyman, 1995; Wienecke et al., 2007) and for durations longer than 23 min (Ponganis et al., 2007). Recent measurements of air sac and blood partial pressure of O_2 (P_{O_2}) in diving emperor penguins have revealed exceptional tolerance to low O_2 in this species (Ponganis et al., 2007; Stockard et al., 2005). It has been hypothesized that this underlying tolerance, well below the limits of many birds and mammals, may necessitate biochemical and molecular adaptations including a shift in the emperor penguin O_2–hemoglobin (Hb) dissociation curve, relative to other birds.

The O_2–Hb dissociation curve of whole blood of emperor penguins has not been determined. In general, the hemoglobin of birds has lower O_2 affinity than that of mammals. This may reflect a shift toward favoring O_2 unloading to the tissues, as the avian respiratory system is inherently more efficient at O_2 uptake (Piiper and Scheid, 1975; Powell, 2000; Powell et al., 1989). Consequently, the P_{50} (P_{O_2} at which Hb is 50% saturated) values of most birds (44–52 mmHg) are much higher than those of mammals (25–31 mmHg) (Christensen and Dill, 1935; Hirsowitz et al., 1977; Lenfant et al., 1970; Lutz, 1980; Wastl and Leiner, 1931). However, Adélie (*Pygoscelis adeliae*), chinstrap (*P. antarctica*), and gentoo penguins (*P. papua*), and the bar-headed goose (*Anser indicus*), a bird adapted to life at high altitudes, have P_{50} values in the mammalian range (Black and Tenney, 1980; Lenfant et al., 1969b; Milsom et al., 1973; Petschow et al., 1977), favoring O_2 uptake from the lungs when P_{O_2} is low. The P_{50} for isolated emperor penguin Hb (36 mmHg) has been measured (Tamburrini et al., 1994). Determination of the P_{50} and dissociation curve in whole blood for this species remains necessary, however, as the P_{50} of isolated Hb of this species was highly sensitive to the presence of various cofactors (Tamburrini et al., 1994).

We characterized the O_2–Hb dissociation curve of the emperor penguin in whole blood in order to (1) investigate the biochemical adaptation of Hb in emperor penguins, and (2) address blood O_2 depletion during diving, by applying the dissociation curve to previously collected P_{O_2} profiles (Ponganis et al., 2009; Ponganis et al., 2007) to estimate *in vivo* Hb saturation (S_{O_2}) changes during dives. It was hypothesized that the P_{50} of the emperor penguin would be similar to that of the bar-headed goose and other penguin species, an adaptation which could contribute to tolerance to low O_2 in the emperor penguin (Black and Tenney, 1980; Lenfant et al., 1969b; Milsom et al., 1973; Ponganis et al., 2007). Hemoglobin with O_2 affinity in this range would also be consistent with blood P_{O_2} and O_2 contents of emperor penguins at rest (Ponganis et al., 2007). The magnitude of the Bohr effect was expected to be similar to those in other birds and penguins (–0.4 to –0.6) (Lenfant et al., 1969b). A similar range of Bohr effects, which should favor O_2 unloading to the tissues, has also been observed in a variety of marine mammals (Lenfant et al., 1970; Qvist et al., 1981; Willford et al., 1990). An accurate determination of the Bohr effect in emperor penguin whole blood also allows refinement of the estimation of S_{O_2} from P_{O_2} profiles since P_{CO_2} and pH data are available at various points in dives of this species (Ponganis et al., 2009; Ponganis et al., 2007).

The extent and rate of respiratory O_2 store depletion have been previously determined for the emperor penguin (Stockard et al., 2005). The addition of blood O_2 store depletion data provides the next component of the investigation of total O_2 consumption in this exceptional diver. It was hypothesized that S_{O_2} profiles would reflect the wide range of P_{O_2} values at the end of dives (Ponganis et al., 2009; Ponganis et al., 2007) and that the rate of decrease of blood O_2 would vary inversely with dive duration since heart rate in this species progressively decreases during longer dives (Meir et al., 2008). An inverse relationship to dive duration has been demonstrated in the respiratory O_2 store depletion rate of the diving emperor penguin (Stockard et al., 2005) and in blood O_2 store depletion rates of seals (Elsner et al., 1964; Stockard et al., 2007).

MATERIALS AND METHODS

As in past studies (Kooyman et al., 1992; Ponganis et al., 2001), non-breeding emperor penguins (*Aptenodytes forsteri* Gray; ~15 birds/season, 20–30 kg) were captured near the McMurdo Sound ice edge or at Terra Nova Bay in October 2003–2005, 2007 and 2008 and were maintained for 6 weeks at an isolated dive hole enclosed within a corral at the Penguin Ranch on the McMurdo Sound sea ice (77°41′, 165°59′). All procedures were approved under a UCSD Animal Subjects Committee protocol and US Antarctic Treaty Permit. All birds were returned to the ice edge and released upon completion of the study.

P_{O_2} electrode deployments

P_{O_2} electrodes (Licox C1.1 Revoxode; Integra LifeSciences, Plainsboro, NJ, USA) and thermistors (model 554, Yellow Springs Instruments, Yellow Springs, OH, USA) were inserted percutaneously into the aorta or vena cava of emperor penguins under general isoflurane anesthesia as described previously (Ponganis et al., 2007; Ponganis et al., 2001; Ponganis et al., 2004; Ponganis et al., 2003). An additional arterial data set was obtained in 2008 from a bird equipped with only an arterial P_{O_2} electrode as described in the previous study (Ponganis et al., 2009). The electrodes were connected to a custom-built P_{O_2}/temperature recorder (UFI, Morro Bay, CA, USA) and an Mk9 time–depth recorder (TDR) was also attached (Wildlife Computers, Redmond, WA, USA) as previously described (Stockard et al., 2005). After overnight recovery from anesthesia, birds were allowed to dive at the isolated dive hole. The instrumented birds dived for 1–2 days, after which catheters, probes and recorders were removed under general anesthesia. All P_{O_2} values were corrected to 38°C for construction of the P_{O_2} profiles, as previously described (Ponganis et al., 2007).

O_2-Hb dissociation curve characterization

O_2–Hb dissociation curves on fresh whole blood were determined with the mixing technique of tonometered blood (Scheid and Meyer, 1978). Blood samples were obtained from the Penguin Ranch emperor penguins during anesthesia, placed on ice, and processed immediately. All dissociation curve analyses were completed within 6 h of blood collection in order to prevent depletion of labile organic phosphates such as inositol pentaphosphate (IPP) which would influence the dissociation curve, and to avoid prolonged metabolism of these nucleated avian red blood cells. The mixing technique consisted of the volumetric mixing of 0% O_2-saturated blood and 100% O_2-saturated blood to achieve the desired S_{O_2} at various points (i.e. 90, 70, 50, 40, 20, 10, 5% S_{O_2}) along the curve with subsequent measurement of the P_{O_2} of the resulting mixture using an i-STAT blood gas analyzer (37°C; Abbott Point of Care, Princeton, NJ, USA) (Black and Tenney, 1980; Johansen et al., 1987; Nörgaard-Pedersen et al., 1972; Qvist et al., 1981). Use of the i-STAT analyzer also allowed verification of pH and P_{CO_2}, and Tucker chamber analyses (Tucker, 1967) provided verification of blood O_2 content. The CO_2 Bohr effect was determined by changing the CO_2 concentration of the gas in the tonometer in order to adjust pH. Dissociation curves were determined at pH values of 7.5, 7.4, 7.3 and 7.2. The $\log[S_{O_2}/(100–S_{O_2})]$ *vs* $\log(P_{O_2})$ was plotted and linear regression analysis performed in order to generate the equation for the O_2–Hb dissociation curve at each pH (Nicol, 1991). As in similar studies (Nicol, 1991), all S_{O_2} points from all birds were combined in order to generate a general O_2–Hb dissociation curve for this species. This is justified as, with the exception of disease, the structure of hemoglobin varies among species, not individuals of a species. The Bohr coefficient was derived from linear regression of the log P_{50} on pH (each point averaged from all data of all birds for pH 7.5, 7.4, 7.3 and 7.2) (Nicol, 1991; Willford et al., 1990). In addition, the fixed acid Bohr effect was determined by titrating with HCl to pH 7.4, 7.3 and 7.2 while P_{CO_2} was maintained at the level required for the standard pH 7.5 curve in that penguin (Willford et al., 1990). The effect of lactic acid on the dissociation curve was also evaluated in whole blood by determining additional dissociation curve points after adding lactic acid (L6661, Sigma, St Louis, MO, USA) to the blood to a final concentration of 5 and 10 mmol l^{-1} (Nicol, 1991).

In order to validate the specific equipment and methods used in this study, dissociation curves were also determined with blood from an avian (chicken, *Gallus gallus domesticus*) and pinniped (*Leptonychotes weddelli*, *Phoca vitulina*, *Zalophus californianus*) species with previously published O_2–Hb binding data (Bartels et al., 1966; Hirsowitz et al., 1977; Lenfant, 1969; Lenfant et al., 1969a). Mixing technique tests were also conducted to verify that no hemolysis occurred during mixing.

% Hb saturation (S_{O_2}) calculations

Percent hemoglobin O_2 saturation (S_{O_2}) values were obtained by applying data from P_{O_2} profiles [from previous studies (Ponganis et al., 2009; Ponganis et al., 2007) and current studies] to the linear regression equation generated by the $\log[S_{O_2}/(100–S_{O_2})]$ *vs* $\log(P_{O_2})$ plot and solving for S_{O_2} (at the appropriate pH, see below).

Blood O_2 store depletion calculations

Blood samples taken for the O_2–Hb dissociation curve were also used for hemoglobin analyses [cyanomethemoglobin technique (Ponganis et al., 1993)] in order to obtain Hb concentration. Oxygen content for initial and final (end-of-dive) time points for each dive were calculated from the corresponding S_{O_2} values, using a Hb concentration of 18.3 g dl^{-1} ([this study, agrees well with previous findings (Kooyman and Ponganis, 1998; Ponganis et al., 1997a)] with the formula: O_2 content (ml O_2 dl^{-1} blood)= (1.34 ml O_2 g^{-1} Hb)×[Hb](g dl^{-1})×S_{O_2}+(0.003×P_{O_2}). Initial S_{O_2} (S_{O_2} at the start of the dive) was estimated with the pH 7.5 dissociation curve, and the final S_{O_2} value (from the final P_{O_2} value, measured within the last 5 or 15 s of the dive depending on the specific recorder) was estimated with the pH 7.4 dissociation curve. The effect of pH is critical in estimation of final S_{O_2}. For example, from data of Weddell seals (Qvist et al., 1981), a P_{O_2} of 10 mmHg corresponds to 8% S_{O_2} at pH 7.4, but only 2% at pH 7.0. Blood lactate concentration does not increase significantly during dives of emperor penguins, even during dives longer in duration than the previously measured aerobic dive limit [ADL; duration beyond which blood lactate concentration increases above resting levels (Kooyman et al., 1983); 5.6 min for the emperor penguin (Ponganis

Table 1. Dive, S_{O_2}, % O_2 content depletion and depletion rate of the arterial and venous blood for the emperor penguins in this study

P_{O_2} electrode location	Duration (min)	Maximum depth (m)	Pre-dive S_{O_2} (%)	Initial S_{O_2} (%)	Maximum S_{O_2} (%)	Final S_{O_2} (%)	Δ S_{O_2} (initial S_{O_2}– final S_{O_2})	% O_2 content depletion	Depletion rate (ml O_2 dl^{-1} min^{-1})
Venous (*N*=9 penguins, 130 dives)	6.1±4.6 (0.8–23.1)	38.6±24.7 (4–155)	74.1±13.7 (29.7–95.8)	75.6±13.0 (38.4–95.3)	80.9±12.7 (49.5–98.8)	46.6±30.2 (0–97.0)	29.0±30.3 (–27.8–+92.3)	37.8±40.0 (–58.0–+100)	0.9±1.4 (–6.2–+4.7)
Arterial (*N*=6 penguins, 71 dives)	5.1±2.1 (1.1–11.9)	29.1±17.3 (11–91.5)	96.2±2.3 (85.7–99.3)	96.5±2.3 (85.7–99.2)	99.1±0.8 (93.5–100)	75.6±13.4 (47.4–98.9)	20.9±14.1 (–5.0–+50.7)	21.6±14.5 (–5.5–+51.7)	0.9±0.6 (–0.4–+2.0)

Values are means ± s.d. and range. Pre-dive, initial and maximum S_{O_2} were determined at pH 7.5, final S_{O_2} at pH 7.4.

et al., 1997b)] for this species (Ponganis et al., 2009). The rise in blood lactate does not occur until the post-dive period in this species (Ponganis et al., 1997b; Ponganis et al., 2009). Based on lactate, P_{CO_2} and pH measurements obtained from emperor penguin blood samples drawn at various time points during diving (Ponganis et al., 2009), a pH of 7.4 was deemed most representative of conditions at the end of the dive.

The percentage of net O_2 content depletion in either the arterial or venous system (dependent on the site of insertion) for each dive of each penguin was calculated [% O_2 content depletion=(initial O_2 content–final O_2 content)/initial O_2 content×100]. The rate of O_2 content depletion [(initial O_2 content–final O_2 content)/dive duration] was also calculated for the arterial and venous systems. This provides an overall depletion rate, not an instantaneous measure of O_2 consumption. Data from penguins with arterial P_{O_2} electrodes provided an estimate of the net depletion of O_2 in the arterial system; those with venous probes provided estimates of net venous O_2 depletion.

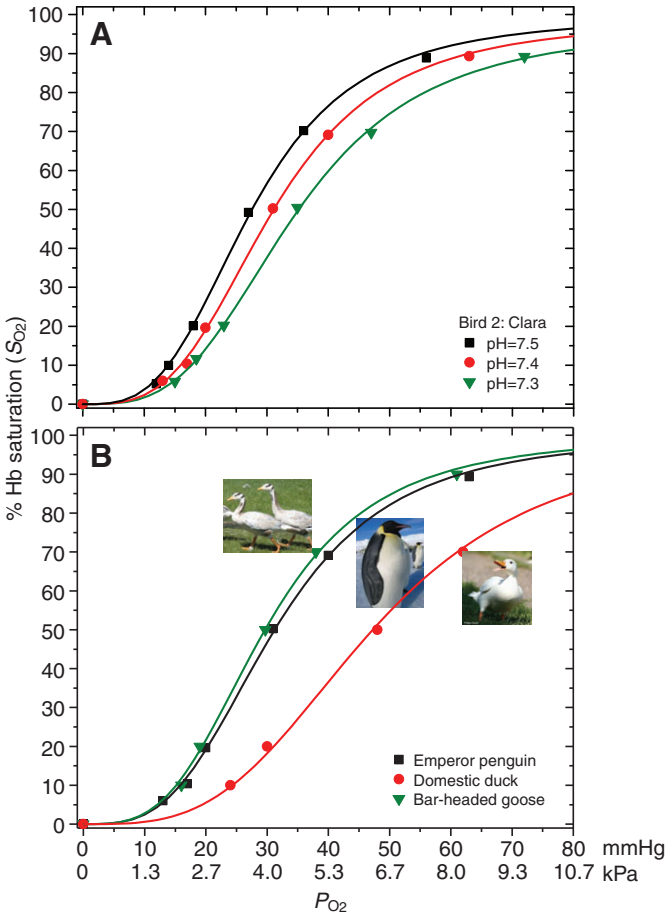

Fig. 1. An example of oxygen–hemoglobin (O_2–Hb) dissociation curves from (A) one penguin at pH 7.5, 7.4 and 7.3, and (B) the emperor penguin, the bar-headed goose (*Anser indicus*) (Black and Tenney, 1980) and the domestic duck *(Anas platyrhynchos,* forma domestica) (Hudson and Jones, 1986) at pH 7.4. Note that as for the bar-headed goose, the O_2–Hb dissociation curve of the emperor penguin is significantly left-shifted as compared with the domestic duck (and most birds). The bar-headed goose photo is courtesy of Graham Scott; the domestic duck photo is by Maren Winter (licensed under the terms of the GNU Free Documentation License, Version 1.2 or any later version); the penguin photo is by J.M.

Data analysis and statistics

As outlined above, the dissociation curve, calculated using linear regression, was applied to P_{O_2} values to yield S_{O_2} values, and calculations of O_2 depletion rate and percentage of O_2 depletion were made. Differences between arterial and venous results were determined with one-way ANOVA. Correlation between dive duration and the variables of final S_{O_2}, pre-dive S_{O_2}, percentage O_2 content depleted and depletion rate was addressed with Spearman rank order correlation tests. Statistical significance was assumed at $P<0.05$ and the significance level is quoted in the text. Values are expressed as means ± s.d. P_{O_2} values are expressed in mmHg (as measured). Figures include the corresponding values in kPa, assuming 1 mmHg=0.133 kPa.

RESULTS

Dive behavior of emperor penguins at the Penguin Ranch for the dives associated with the P_{O_2} profiles in this study has been described previously (Ponganis et al., 2009; Ponganis et al., 2007). Dive duration and depth data are given in Table 1.

O_2–Hb dissociation curve

Complete O_2–Hb dissociation curves were determined with blood from 12 penguins, with additional P_{50} values at specific pH levels and fixed acid Bohr effects obtained in an additional two penguins. The P_{50} was 28±1 mmHg (N=12 penguins) at pH 7.5 (Fig. 1A). The CO_2 Bohr effect (slope of log P_{50} *vs* pH) was –0.45 (y=–0.45x+4.81, r^2=0.98, P=0.008). The fixed acid Bohr effect (addition of HCl or lactic acid) was not significantly different from that of CO_2.

The resulting regression equations from the plots of $\log[S_{O_2}/(100–S_{O_2})]$ *vs* $\log(P_{O_2})$ (all saturation points, all penguins combined) were:

$$\text{pH 7.5: } \log[S_{O_2}/(100–S_{O_2})] = 2.92589 \times \log(P_{O_2}) – 4.24338 \ (N=43, r^2=0.98, P<0.0001),$$

$$\text{pH 7.4: } \log[S_{O_2}/(100–S_{O_2})] = 2.94767 \times \log(P_{O_2}) – 4.39858 \ (N=70, r^2=0.98, P<0.0001),$$

$$\text{pH 7.3: } \log[S_{O_2}/(100–S_{O_2})] = 3.04945 \times \log(P_{O_2}) – 4.72019 \ (N=38, r^2=0.99, P<0.0001),$$

$$\text{pH 7.2: } \log[S_{O_2}/(100–S_{O_2})] = 3.15958 \times \log(P_{O_2}) – 4.97618 \ (N=9, r^2=0.99, P<0.0001).$$

Samples from other species (chicken and pinnipeds) run for dissociation curve method validation agreed with previously published values (Bartels et al., 1966; Hirsowitz et al., 1977; Lenfant, 1969; Lenfant et al., 1969a). Mixing technique verification tests confirmed that no hemolysis occurred while mixing blood samples in the syringes, based on clear plasma color in hematocrit tubes after centrifugation. The mean hemoglobin concentration in this study (18.3±1.1 g dl^{-1}, N=17) was equivalent to that of previous studies (Kooyman and Ponganis, 1998; Ponganis et al., 1997a). Oxygen content analyses performed with 100% O_2-saturated blood (Tucker chamber) agreed within 5% of the maximal O_2 content calculated using the equation in this study [O_2 content (ml O_2 dl^{-1} blood)=(1.34 ml O_2 g^{-1} Hb)×[Hb](g dl^{-1})×S_{O_2}+(0.003×P_{O_2})].

P_{O_2} profiles

In addition to the arterial P_{O_2} data collected in previous studies (Ponganis et al., 2009; Ponganis et al., 2007), an arterial P_{O_2} and dive profile was obtained for one more emperor penguin in the 2008 Antarctic field season (Fig. 2A). This increased the arterial data sample size to six penguins (71 dives in total). The venous data were from nine penguins (130 total dives). These profiles have been adequately described in previous studies (Ponganis et al., 2009; Ponganis et al., 2007).

S_{O_2} and blood O_2 depletion

In vivo arterial Hb saturation (S_{a,O_2}) often remained near 100% for much of the dive, reflecting the large increase in arterial P_{O_2} at the beginning of the dive and the fact that despite a subsequent decrease, P_{O_2} remained relatively high until the later dive phase (Fig. 2A) (Ponganis et al., 2007). A rapid decline in S_{a,O_2} generally began during the final ascent phase of the dive (Fig. 2A). S_{v,O_2} (venous Hb saturation) profiles reflected venous P_{O_2} profiles (Ponganis et al., 2007) in that they were quite variable among dives (Fig. 2B, Fig. 3), with marked fluctuations, transient increases during the dive, and a large range of final S_{v,O_2} values (Fig. 4). Re-oxygenation occurred rapidly upon return to the surface, as demonstrated by the return in both S_{a,O_2} and S_{v,O_2} to values consistent with those at rest within approximately 2 min post-dive (Fig. 2). Pre-dive and initial S_{v,O_2} values (Table 1, Fig. 2B, Fig. 3) were often higher than those corresponding to the mean venous P_{O_2} of emperor penguins at rest (Ponganis et al., 2007).

Final S_{v,O_2} was ≤20% in 28% of dives, ≤5% in 15% of dives and ≤2% in 6% of dives. With the exception of only one dive, S_{v,O_2} decreased below 20% only in dives greater than the previously measured ADL (Ponganis et al., 1997b) (Table 1, Fig. 4).

Max S_{O_2} (the peak S_{O_2} during the dive), pre-dive S_{O_2}, initial S_{O_2}, final S_{O_2}, ΔS_{O_2} (initial S_{O_2} minus final S_{O_2}), percentage O_2 content depletion, and blood O_2 store depletion rate data are given in Table 1. Max S_{O_2}, initial S_{O_2}, final S_{O_2}, ΔS_{O_2} and percentage O_2 content depletion were all significantly different between the arterial and venous compartments [one-way ANOVA: P≤0.001 for all except ΔS_{O_2} (P=0.035); F=144.40, 180.42, 59.14, 4.53, 10.93, respectively]. The blood O_2 store depletion rates between the two compartments, however, were not significantly different (one-way ANOVA: P=0.94; F=0.00627).

Both final S_{a,O_2} and S_{v,O_2} demonstrated a strong and significant negative correlation to dive duration (Spearman rank order correlation, Table 2, Fig. 4). The percentage of O_2 content depleted during the dive showed a strong and significant positive correlation to dive duration for both compartments (Spearman rank order correlation, Table 2). The pre-dive S_{v,O_2} had a significant, but weak positive correlation to dive duration, whereas pre-dive S_{a,O_2} was not significantly related to dive duration (Spearman rank order correlation, Table 2). Blood O_2 store depletion rate had a significant, but weak, positive relationship to dive duration in both the arterial and venous compartments (Spearman rank order correlation, Table 2).

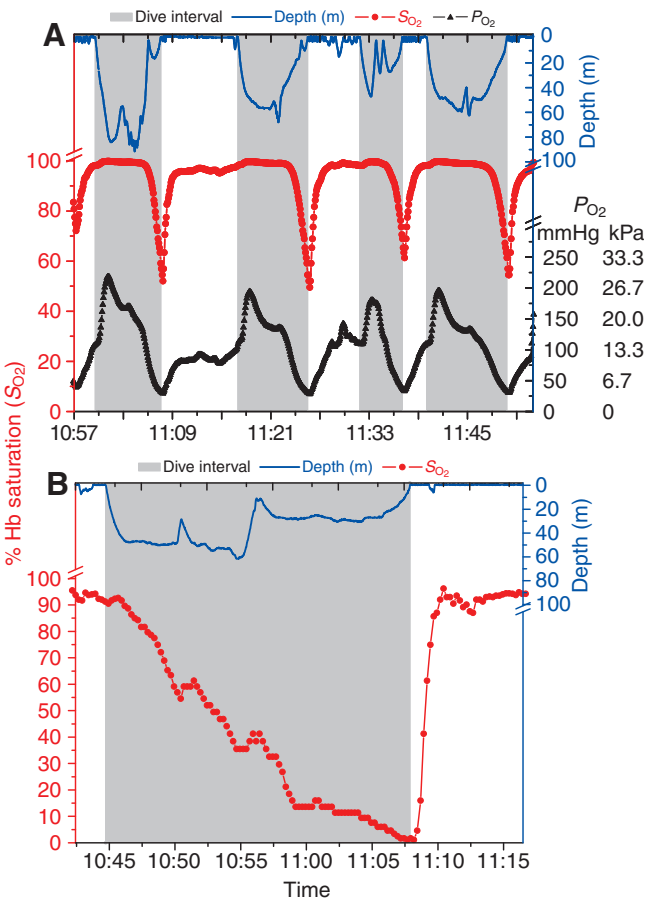

Fig. 2. The S_{O_2} profile during (A) 1 h of diving of bird 1 (2008) (arterial S_{O_2}) with P_{a,O_2} superimposed. Note that $P_{a,O_2}/S_{a,O_2}$ are at low levels at the start of the measurements because a dive had occurred just before the series recorded, and (B) the current record dive (23.1 min) of an emperor penguin [bird 19 (Ponganis et al., 2007), venous S_{O_2}]. Note the arterialization of the venous blood O_2 store in this dive, as S_{v,O_2} before the dive is as high as 95% and the initial S_{v,O_2} of the dive is 91%. S_{v,O_2} decreased to 1% by the end of this long dive. S_{O_2} was determined at pH 7.5 throughout the entire dive to maintain consistency and to provide a conservative estimate of continuous S_{O_2}.

DISCUSSION

O_2–Hb dissociation curve

As hypothesized because of its potential to contribute to tolerance to low O_2 in this species (Ponganis et al., 2007), the O_2–Hb dissociation curve of the emperor penguin is indeed left-shifted relative to most birds, similar to that of other penguin species and the high-flying bar-headed goose (Black and Tenney, 1980; Lenfant et al., 1969b; Milsom et al., 1973; Petschow et al., 1977). Left-shifted hemoglobin (higher Hb–O_2 affinity) is advantageous for the diving emperor penguin, as it implies that more O_2 is available at any given P_{O_2}. This becomes particularly relevant for a breath hold diver that often experiences low P_{O_2} values during diving. For

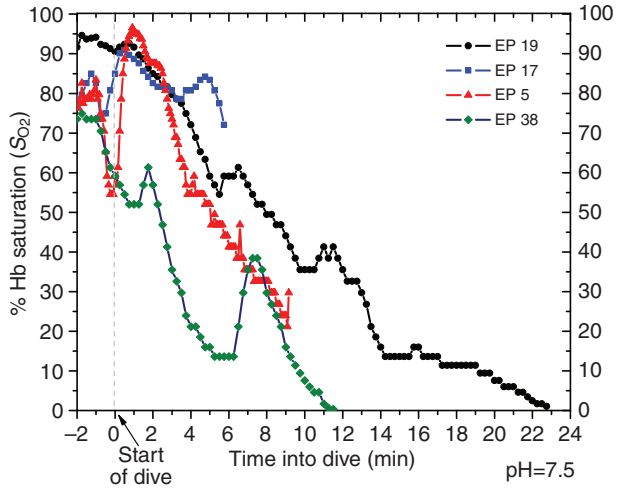

Fig. 3. Comparisons of the $S_{v,O2}$ profile [based on P_{O2} profiles from a prior study (Ponganis et al., 2009)] in dives of four emperor penguins (EP). S_{O2} was determined at pH 7.5 throughout the entire dive to maintain consistency and to provide a conservative estimate of continuous S_{O2}. Note the variability of the $S_{v,O2}$ profile among different dives.

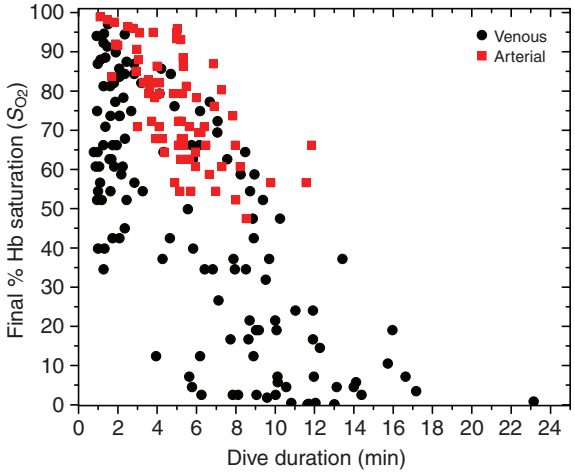

Fig. 4. The final S_{O2} *vs* dive duration from the compiled P_{O2} profiles of all dives [venous and arterial; Ponganis et al. and current study (Ponganis et al., 2009; Ponganis et al., 2007)]. Note the significant amount of overlap between arterial and venous values.

example, at a P_{O2} of 20 mmHg, a pekin duck would be stripped of all its O_2, whereas the hemoglobin of the emperor penguin, with its left-shifted dissociation curve, is still 27% saturated (pH 7.5; 21.5% at pH 7.4) at this same P_{O2} (Fig. 1B). This advantage could also assist in the prevention of deleterious effects such as shallow water blackout if $P_{a,O2}$ drops to low levels during diving.

An increased Hb–O_2 affinity also allows for more complete depletion of the respiratory O_2 store. As opposed to marine mammals whose lungs do not constitute a significant portion of total O_2 stores, the respiratory store of the diving emperor penguin remains a significant contributor to O_2 stores while diving [19% of total O_2 stores (Kooyman and Ponganis, 1998)], and this species dives upon inspiration (Kooyman et al., 1971). The increased O_2 affinity of emperor penguin Hb allows for continued extraction of this O_2 from the respiratory O_2 store for use during diving. By contrast, a pekin duck forcibly submerged to the point of 'imminent cardiovascular collapse' was left with 25% of its respiratory O_2 store unused, as the blood contained no O_2 at an air sac P_{O2} near 30 mmHg (Hudson and Jones, 1986).

Presumably, the biochemical adaptation underlying the increased Hb–O_2 affinity is achieved with specific amino acid substitution(s), similar to that of other species. In two species of high-altitude birds with increased Hb–O_2 affinity (bar-headed and Andean geese), the same $\alpha_1\beta_1$ intradimer contact site is disrupted, accomplished by two different amino acid substitutions (on the α-chain in one of the species, and on the corresponding β-chain site in the other) (Perutz, 1983; Weber and Fago, 2004). This same intradimer contact site is altered in high O_2 affinity fish Hb, and even reconstituted human Hb demonstrates significantly higher affinity for O_2 when this single site is altered (Jessen et al., 1991; Weber and Fago, 2004). Upon examination of the published amino acid sequence of emperor penguin hemoglobin (Tamburrini et al., 1994), it is revealed that this specific site is not altered in emperor or rockhopper (*Eudyptes crestatus*) penguins. However, as compared to human Hb, there are changes in six α-chain and five β-chain $\alpha_1\beta_1$ contact sites, and one α-chain $\alpha_1\beta_2$ contact site. It is possible that one of these modifications accounts for the emperor penguin's increased Hb–O_2 affinity, although other structural features might also be responsible.

We hypothesize that the small Bohr effect (–0.25) found in the previous study of isolated emperor penguin Hb may be secondary to experimental conditions and the sensitivity of the isolated Hb to co-factors (Tamburrini et al., 1994). It was hypothesized in that study that the low Bohr effect prevents 'stripping' of O_2 during the long dives of emperor penguins (Tamburrini et al., 1994). However, such a low Bohr effect for Hb in whole blood was not found in this study, nor is it present in other diving penguins and marine mammals (Lenfant et al., 1970; Lenfant et al., 1969b). A small Bohr effect is a potential disadvantage in terms of unloading O_2 to the tissues in a diving animal, especially in those with relatively high Hb–O_2 affinities. Blood pH data during dives have also demonstrated that emperor penguin blood does not become very acidotic during the dive, indicating that the effects of the Bohr shift may not be particularly relevant during diving in this species (Ponganis et al., 2007).

S_{O2} extremes

Final $S_{v,O2}$ reached very low levels, ≤5% in 15% of dives (corresponding to O_2 contents <1.2 ml O_2 dl^{-1} blood) and approached

Table 2. Spearman rank order correlation results

		Final S_{O2}/duration		Pre-dive S_{O2}/duration		% O_2 content depletion/duration		Depletion rate/duration	
Location	*N*	Spearman *R*	*P*	Spearman *R*	*P*	Spearman *R*	*P*	Spearman *R*	*P*
Venous	130	–0.722*	<0.001	0.216*	0.007	0.804*	<0.001	0.272*	0.001
Arterial	71	–0.677*	<0.001	0.174	0.074	0.667*	<0.001	0.210*	0.040

*Correlation is significant at the 0.05 level.

0% in some dives, particularly in those with durations longer than the previously measured ADL (Ponganis et al., 1997b) (Table 1, Fig. 2B, Figs 3 and 4). This resulted in a large percentage venous O_2 content depletion during the dive (Table 1). The wide range of final $S_{v,O2}$ values for dives of similar duration (Fig. 4) and the fluctuations in the venous P_{O2} and S_{O2} profiles during dives (Fig. 3) suggest significant variability in tissue O_2 extraction and blood flow patterns during dives. As previously suggested (Ponganis et al., 2009), this may reflect differences or changes in the peripheral vascular response including regulation of blood flow to muscle and other organs as well as through arterio-venous (a-v) shunts.

Final $S_{a,O2}$ reached only as low as 47% (Table 1, Fig. 4; corresponding to an O_2 content of 11.5 ml O_2 dl^{-1} blood) in dives as long as 12 min. Such high values contrast with the lower values found in elephant seals (*Mirounga angustirostris*) (Meir et al., 2009) and, again, are considered secondary to the size of the respiratory O_2 store in emperor penguins and to the maintenance and efficiency of respiratory gas exchange during these dives. Such high $S_{a,O2}$ may well minimize the risk of shallow water blackout in these birds (Fig. 2A). It should be noted that many of these final $S_{a,O2}$ values are greater than would have been predicted from final air sac P_{O2} values for dives of similar duration (Ponganis et al., 2009; Ponganis et al., 2007). It is unclear why final $P_{a,O2}$ values are higher than those recorded in the air sacs, especially since air sac P_{O2} prior to dives and during dives is greater than $P_{a,O2}$ (Ponganis et al., 2009). Sample size, type of dive, circulatory lag time, and the location of the sampled air sac may contribute to these differences. Regardless of the explanation, it is apparent that emperor penguins can perform dives as long as 12 min in which final arterial P_{O2} and S_{O2} are relatively well preserved.

It should also be noted that there is considerable overlap between final arterial and many final venous S_{O2} values of the dives in this study (Fig. 4). Such overlap may be secondary to the previously suggested intra-dive a-v shunting (Ponganis et al., 2009) and the close equilibration of air sac, arterial and venous P_{O2} in the latter parts of such dives. Equilibration of arterial and venous P_{O2} and S_{O2} values toward the end of long breath holds has been reported in seals (Elsner et al., 1964; Stockard et al., 2007). As the sample size for arterial records was smaller than that of venous records, and because there were no dives longer than 12 min for arterial data (compared with >23 min for venous data), we believe that final $S_{a,O2}$ values in longer dives may well be lower than those documented in this study.

Both $S_{a,O2}$ and $S_{v,O2}$ returned to values equivalent to those at rest within about 2 min post-dive (Fig. 2), consistent with P_{O2} profiles of this species (Ponganis et al., 2009; Ponganis et al., 2007). In the previous study, no relationship was shown between surface interval duration and the time to return to the P_{O2} value at rest (Ponganis et al., 2009). Thus, re-oxygenation occurred very rapidly upon return to the surface, and does not represent a limitation to the onset of the subsequent dive.

S_{O2} profiles and implications for O_2 store utilization

As previously discussed (Ponganis et al., 2009), the initial transient increase in $P_{a,O2}$ during dives reflected the compression hyperoxia observed during diving in the air sacs of emperor penguins (Stockard et al., 2005) and demonstrated the maintenance of pulmonary gas exchange and transfer of O_2 from the respiratory to the blood O_2 store. Even as $P_{a,O2}$ declined from the early peak value, its value was relatively high for much of the dive (Fig. 2A). As a consequence, $S_{a,O2}$ remained near 100% for much of the dive (Fig. 2A), preserving a high O_2 content in the arterial system for critical organs such as the brain. $S_{a,O2}$ generally did not decrease significantly until the final ascent phase of the dive, consistent with the decline in ambient pressure and decrease in both air sac and arterial P_{O2} during ascent (Ponganis et al., 2007; Stockard et al., 2005) (Fig. 2A). The net transfer of O_2 from the lungs to the blood also resulted in some final $P_{a,O2}$ values greater than the $P_{a,O2}$ of birds at rest and some final $P_{v,O2}$ values not only greater than that at rest, but even greater than $P_{v,O2}$ at the start of the dive (Ponganis et al., 2007). The corresponding S_{O2} values resulted in negative values for ΔS_{O2}, percentage O_2 content depleted and blood O_2 depletion rates (i.e. an overall increase in S_{O2} during the dive) in such dives (Table 1, Fig. 5). These findings are consistent with the significant role of the left-shifted dissociation curve and with the contribution of respiratory O_2 to total O_2 stores in this species.

By contrast, although there was also a transient increase in $P_{a,O2}$ and consequently $S_{a,O2}$ in diving elephant seals at the beginning of dives, max $P_{a,O2}$ in the emperor penguin was nearly twice that in the elephant seal [mean max $P_{a,O2}$=88±20 mmHg for the elephant seal (Meir et al., 2009) and 157±52 mmHg in the emperor penguin]. As in the emperor penguin, $S_{a,O2}$ peaked near 100% in the elephant seal, but this high level of $S_{a,O2}$ was not maintained and instead decreased rapidly after the peak (Meir et al., 2009). These findings reflect the difference in the magnitude of the respiratory O_2 store between these two species. Unlike emperor penguins, elephant seals dive upon expiration and the lungs do not constitute a significant portion of total O_2 stores (Falke et al., 1985; Kooyman and Ponganis, 1998).

It should be noted that maximum S_{O2} values in this study were probably slightly underestimated, because it was not possible to determine a true, critical P_{O2} value at 100% S_{O2}. In order to ensure complete saturation in the tonometry of the 100% O_2-saturated blood for O_2–Hb dissociation curve determination, a high P_{O2} (>200 mmHg) was used for that sample. Thus, the actual inflection point in P_{O2} at which S_{O2} first reaches 100% was not determined (Fig. 1A). The lack of this data point in the regression equations results in a slightly lower calculated S_{O2} for P_{O2} values that approach the inflection point of 100% S_{O2}. In turn, this implies that net depletion values and O_2 depletion rates are slightly underestimated.

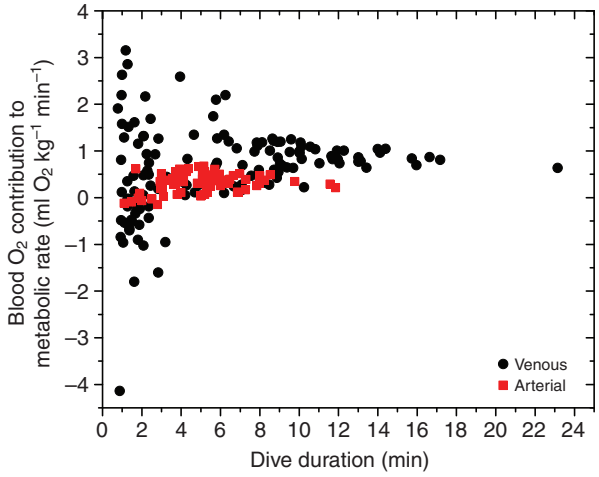

Fig. 5. The blood O_2 contribution to metabolic rate *vs* dive duration for all venous and arterial dives. Note that since S_{O2} was sometimes higher at the end of the dive than at the initial time point, negative values resulted in some cases for this variable.

Arterio-venous shunts

Pre-dive and initial $S_{v,O2}$ values were often higher than those corresponding to $P_{v,O2}$ of emperor penguins at rest (Ponganis et al., 2007), reaching as high as 95% (Table 1, Fig. 2B, Fig. 3). Although maximum $S_{v,O2}$ during a dive was much more variable than $S_{a,O2}$ (Table 1), the mean of maximum $S_{v,O2}$ was also quite high at 81%, and, in some dives, max $S_{v,O2}$ values were almost 100% (Table 1, Fig. 2B, Fig. 3). These 'arterialized' venous values imply some degree of arterio-venous (a-v) shunting or at least a lack of tissue O_2 uptake from the blood. In addition to previous findings in these species, including increases in $P_{v,O2}$ during the descent period, the lack of blood lactate accumulation during the dive, muscle temperature profiles, dramatic bradycardias and a lack of association between heart rate and stroke frequency during diving, these results further support the concept that muscle is isolated from the circulation during dives of emperor penguins (Meir et al., 2008; Ponganis et al., 2009; Ponganis et al., 2007; Ponganis et al., 2003).

Such high values during surface intervals may be secondary to an increased gas exchange rate, enhanced reloading of O_2 stores and improved ventilation perfusion matching due to a more pronounced hyperventilation and tachycardia before such dives. In addition to the maximization of the blood O_2 store by the arterialization of its venous blood, these birds often made full use of this component of their O_2 store by decreasing $S_{v,O2}$ to near 0% in longer dives, as discussed above (Table 1, Fig. 2B, Fig. 3). For example, in the longest recorded dive to date for an emperor penguin (23.1 min), pre-dive S_{O2} was 91–95%, initial S_{O2} was 91%, and final S_{O2} decreased to as low as 1%, demonstrating a near complete utilization of the venous blood O_2 store in this remarkable dive (Fig. 2B).

Based on pre-dive S_{O2} values, the magnitude of a peripheral a–v shunt prior to the dive can be estimated with a shunt equation, as follows, assuming a 5 ml O_2 dl^{-1} a-v O_2 difference at rest (Ponganis et al., 2007): {venous O_2 content=[arterial O_2 content×(% a-v shunt)]–[(arterial O_2 content–5 ml O_2 dl^{-1})×(1–(% a-v shunt))]}. For example, the maximum pre-dive $S_{v,O2}$ in this study was 95.8% (Table 1). Using O_2 contents calculated for this level of $S_{v,O2}$ (23.5 ml O_2 dl^{-1}) and the maximal pre-dive $S_{a,O2}$ (24.4 ml O_2 dl^{-1}) an arterio-venous shunt of 82% would be necessary to reach this venous S_{O2}. For the pre-dive $S_{v,O2}$ of 91.3% (22.4 ml O_2 dl^{-1}) prior to the 23.1 min dive (Fig. 2B), the magnitude of the a-v shunt would be 60%. These calculations should be considered slight overestimates since the $S_{v,O2}$ values are vena caval and not true mixed venous samples (myocardial O_2 extraction would decrease the mixed venous $S_{v,O2}$ further).

Intrapulmonary shunts

As discussed in previous studies, the mean $P_{a,O2}$ of emperor penguins at rest (68±7 mmHg) is less than two-thirds of the mean value in the air sac (Ponganis et al., 2009; Ponganis et al., 2007), probably mainly because of ventilation–perfusion mismatch (Powell, 2000; Powell et al., 1989). Using the O_2–Hb dissociation curve and the classic pulmonary shunt equation [% shunt=(capillary O_2 content–arterial O_2 content)/(capillary O_2 content–venous O_2 content)], calculations can be made to estimate the size of the pulmonary shunt in the emperor penguin both at rest and in the pre-dive period. Calculating S_{O2} from the previously measured mean $P_{a,O2}$ and $P_{v,O2}$ values at rest (Ponganis et al., 2007) and assuming capillary S_{O2}=100%, based on air sac P_{O2} measurements of 120 mmHg from previous studies (Stockard et al., 2005), and a [Hb]=18.3 g dl^{-1} (this study) to calculate O_2 content, the extent of the intrapulmonary shunt in a resting emperor penguin is 28%. Again, this value may be slightly overestimated since mixed venous values were not used in the calculation. Nonetheless, shunting of pulmonary blood flow is usually very small in birds, less than 1–2.7% of cardiac output in anesthetized artificially ventilated geese and ducks [representing the true intrapulmonary shunt (Burger et al., 1979; Powell and Wagner, 1982)], or 6.3–8% in ducks [representing intrapulmonary plus extrapulmonary shunts (Bickler et al., 1986)]. The high percentage shunt value in emperor penguins may also be due in part to an overestimation of the capillary O_2 content because capillary P_{O2} and saturation may be decreased secondarily to the thickened parabronchial capillary blood-to-air barrier reported in emperor penguins (Welsch and Aschauer, 1986). Using pre-dive $S_{a,O2}$ and $S_{v,O2}$ values (mean values for pre-dive $S_{v,O2}$ and $S_{a,O2}$ in this study; Table 1) instead of values at rest, however, the intrapulmonary shunt is reduced to 14.3%. This suggests that the hyperventilation and tachycardia characteristic of the pre-dive period in this species (Kooyman et al., 1971; Kooyman et al., 1992; Meir et al., 2008) improves ventilation–perfusion matching prior to the dive.

Blood O_2 store contribution to metabolic rate

Since the blood volume of an emperor penguin of a given mass can be calculated (based on 100 ml kg^{-1}) (Kooyman and Ponganis, 1998; Ponganis et al., 1997a), a calculation of the contribution of the blood O_2 store to overall metabolic rate was made: blood O_2 store contribution=[(final O_2 content–initial O_2 content)/dive duration×blood volume], with the assumption that one-third of blood volume is arterial, and two-thirds venous in distribution (Kooyman, 1989). Using the mean values for all arterial and all venous dives combined, the blood O_2 store contribution alone to metabolic rate from the arterial compartment is 0.3±0.2 ml O_2 kg^{-1} min^{-1} (range=–0.1–+0.7) and from the venous compartment is 0.6±0.9 ml O_2 kg^{-1} min^{-1} (range=–4.1–+3.1). These values are only approximately 5% (arterial) and 10% (venous) of the resting metabolic rate measured for this species (Kooyman and Ponganis, 1994; LeMaho et al., 1976; Pinshow et al., 1977). If dives in which S_{O2} increased during the dive are excluded from the analysis, the blood O_2 store contribution alone to metabolic rate from the arterial compartment is 0.4±0.2 ml O_2 kg^{-1} min^{-1} (range=0.0–+0.7) and from the venous compartment is 0.9±0.6 ml O_2 kg^{-1} min^{-1} (range=0.1–+3.1). In comparison, respiratory O_2 store depletion rates were 2.3 and 5.3 times that in the venous and arterial blood O_2 store compartments, respectively [mean=2.1±0.8 ml O_2 kg^{-1} min^{-1} (Stockard et al., 2005)].

As previously discussed, since S_{O2} was sometimes higher at the end of the dive than at the initial time point, negative values resulted for ΔS_{O2}, percentage O_2 content depletion, and depletion rates (Table 1, Fig. 5). For example, the venous O_2 store contribution to metabolic rate was –1.8 ml O_2 kg^{-1} min^{-1} for a 1.6 min dive. Assuming a gain in the venous blood O_2 store of 1.8 ml O_2 kg^{-1} min^{-1} in this 1.6 min dive, this indicates a minimum loss of 2.9 ml O_2 kg^{-1} from the respiratory system during the dive. If the respiratory system initially has 20% O_2 at 69 ml kg^{-1} (Ponganis et al., 1999), it contains approximately 14 ml O_2 kg^{-1} at the beginning of the dive. For the 1.6 min dive, a minimum of 21% (2.9/14) of the respiratory O_2 was transferred into the blood. This represents a minimum value, as it does not include respiratory O_2 that entered the blood and replaced any O_2 taken up by tissue from the blood. However, such calculations are useful to illustrate the maintenance of gas exchange during the dive and the potential magnitude of the transfer of respiratory O_2 into the blood.

These calculations illustrate the complexity of estimating the contribution of the net blood O_2 store to metabolic rate as the blood O_2 depletion rate in a penguin (or any diver with a large respiratory O_2 store) is a function of both the transfer of O_2 into the blood and

the uptake of O_2 from blood by the tissues. Simultaneous air sac and blood P_{O_2} data would allow calculation of the net contribution of these O_2 stores to diving metabolic rate, but such measurements are not currently feasible. However, because the separately determined O_2 contributions from the lungs and blood are both low, these data are consistent with (1) a significant contribution from the exceptionally large muscle O_2 store to diving metabolic rate in emperor penguins (Kooyman and Ponganis, 1998; Ponganis et al., 1997a), and (2) the low field metabolic rate and the true diving bradycardia (heart rate while diving significantly lower than that of heart rate at rest) exhibited by emperor penguins at the isolated dive hole (Meir et al., 2008; Nagy et al., 2001).

Conclusions

This investigation has revealed enhanced O_2 affinity of emperor penguin hemoglobin, similar to that of high-altitude geese and other penguin species. These species are distantly related (Sphenisciformes and Anseriformes) (Hackett et al., 2008) and have evolved in different environments under different constraints. The conditions of hypoxia that high-altitude birds and penguin species encounter are also different, ranging from that of extended hypoxia during sustained flight for the bar-headed goose, to transient, though perhaps frequent hypoxia experienced by diving penguins. Despite these differences, both bar-headed geese and emperor penguins have evolved hemoglobins with remarkably similar O_2 affinities. As neither of the two amino acid substitutions responsible for the increased Hb affinity in high-altitude birds is present in emperor penguin hemoglobin, the mechanisms underlying this shared trait are also different. Perhaps this level of O_2 affinity is closer to the physiological limit of maximizing O_2 uptake during hypoxia. As this is also the range of O_2 affinity optimized by mammalian hemoglobins, including those of diving mammals, it demonstrates the compromise between O_2 uptake from the respiratory system and O_2 unloading to the tissues intrinsic to the O_2–Hb dissociation curve.

S_{O_2} profiles during diving, based on the O_2–Hb dissociation curve, demonstrated (1) the maintenance of S_{a,O_2} levels near 100% throughout much of the dive, (2) a wide range of final $S_{v,O2}$ values and optimization of the venous blood O_2 store resulting from arterialization and near complete depletion of venous blood O_2 during longer dives, and (3) that estimated contribution of the blood O_2 store to diving metabolic rate was low and highly variable. This pattern is due, in part, to the influx of O_2 from the lungs into the blood during diving, and variable rates of tissue O_2 uptake.

LIST OF ABBREVIATIONS

ADL	aerobic dive limit
Hb	hemoglobin
P_{CO_2}	partial pressure of carbon dioxide
P_{O_2}	partial pressure of oxygen (P_{a,O_2} or P_{v,O_2}: arterial or venous)
P_{50}	partial pressure of O_2 at which Hb is 50% saturated
S_{O_2}	% hemoglobin saturation (S_{a,O_2} or S_{v,O_2}: arterial or venous)

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