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RESEARCH ARTICLE

What triggers the aerobic dive limit? Patterns of muscle oxygen depletion during dives of emperor penguins

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SUMMARY

The physiological basis of the aerobic dive limit (ADL), the dive duration associated with the onset of post-dive blood lactate elevation, is hypothesized to be depletion of the muscle oxygen (O_2) store. A dual wavelength near-infrared spectrophotometer was developed and used to measure myoglobin (Mb) O_2 saturation levels in the locomotory muscle during dives of emperor penguins (*Aptenodytes forsteri*). Two distinct patterns of muscle O_2 depletion were observed. Type A dives had a monotonic decline, and, in dives near the ADL, the muscle O_2 store was almost completely depleted. This pattern of Mb desaturation was consistent with lack of muscle blood flow and supports the hypothesis that the onset of post-dive blood lactate accumulation is secondary to muscle O_2 depletion during dives. The mean type A Mb desaturation rate allowed for calculation of a mean muscle O_2 consumption of 12.4 ml O_2 kg^{-1} muscle min^{-1}, based on a Mb concentration of 6.4 g 100 g^{-1} muscle. Type B desaturation patterns demonstrated a more gradual decline, often reaching a mid-dive plateau in Mb desaturation. This mid-dive plateau suggests maintenance of some muscle perfusion during these dives. At the end of type B dives, Mb desaturation rate increased and, in dives beyond the ADL, Mb saturation often reached near 0%. Thus, although different physiological strategies may be used during emperor penguin diving, both Mb desaturation patterns support the hypothesis that the onset of post-dive lactate accumulation is secondary to muscle O_2 store depletion.

Key words: diving, myoglobin, emperor penguin, muscle, oxygen store, NIR, aerobic dive limit, ADL, metabolic rate, O_2 consumption.

INTRODUCTION

The aerobic dive limit (ADL) has become an essential tool in the modeling of efficient dive metabolism and the interpretation of diving behavior and foraging ecology of air-breathing marine vertebrates (Butler and Jones, 1997; Kooyman and Ponganis, 1998; Kooyman et al., 1980). The ADL is defined as the dive duration beyond which post-dive blood lactate begins to accumulate (Kooyman et al., 1980). The elevation in lactate concentration is believed to be related to significant depletion of one or more of the three oxygen (O_2) stores (respiratory, blood and muscle) (Kooyman and Ponganis, 1998; Ponganis et al., 1997b). Depletion of the muscle O_2 store and subsequent anaerobic metabolism in muscle has been postulated as the physiological basis for the ADL (Kooyman and Ponganis, 1998).

Early studies on forcibly submerged seals revealed a series of physiological responses, referred to as the dive response (Scholander, 1940; Scholander et al., 1942), that support the hypothesis that muscle is the primary source of post-dive blood lactate accumulation. During forced submersions, muscle was isolated from circulation because of severe bradycardia and peripheral vasoconstriction (Bron et al., 1966; Scholander, 1940; Scholander et al., 1942). Muscle metabolism relied on the intrinsic myoglobin (Mb)-O_2 store and once that store was consumed, lactate began to accumulate in the muscle (Scholander, 1940; Scholander et al., 1942). During the post-submersion tachycardia, washout of lactate from the muscle elevated blood lactate concentration, reinforcing the concept that muscle was isolated from the circulation and was the primary source of post-dive blood lactate (Scholander, 1940; Scholander et al., 1942).

However, other studies suggest that muscle may not be isolated from the blood O_2 store during natural breath holds. Bradycardia and peripheral vasoconstriction are often not as severe during free-ranging dives and spontaneous apneas as during forced submersions (Andrews et al., 1997; Elsner, 1965; Jobsis et al., 2001; Kooyman and Campbell, 1973; Thompson and Fedak, 1993). For example, during sleep apnea in elephant seals (*Mirounga angustirostris*), the bradycardia is mild, muscle blood flow (MBF), although reduced, is maintained during the breath hold and Mb is only partially desaturated (Castellini et al., 1994; Ponganis et al., 2008). Even during a 10 min apnea, Mb remains 80% saturated, phosphocreatine does not break down and lactate does not accumulate (Castellini et al., 1986; Ponganis et al., 2002; Ponganis et al., 2008; Stockard et al., 2007). Similarly, incomplete Mb desaturation during long dives of Weddell seals (*Leptonychotes weddellii*) suggests that muscle metabolism is supported by both the Mb-O_2 store and blood-to-muscle O_2 transfer from the hemoglobin (Hb)-O_2 store (Guyton et al., 1995). In addition, it has been postulated that maintenance of some MBF during dives is necessary to delay the accumulation of lactate, thereby maximizing the ADL (Davis and Kanatous, 1999).

Recent studies at an isolated dive hole in McMurdo Sound, Antarctica, suggest that freely diving emperor penguins (*Aptenodytes forsteri* Gray 1844) may have a dive response similar to the classic dive response demonstrated in forced submersion studies. During the initial descent phase of dives, when stroke frequency (muscle

workload) is highest, there is an immediate reduction in heart rate, which is consistent with reduced muscle perfusion (Meir et al., 2008; van Dam et al., 2002). Similarly, venous partial pressure of O_2 (P_{O_2}) values often increase during the descent phase of emperor penguin dives, at a time when significant O_2 extraction by working muscle would be expected to lower venous P_{O_2} if MBF were maintained (Ponganis et al., 2007).

Accordingly, we hypothesized that muscle is ischemic during diving and that depletion of the muscle O_2 store with subsequent muscle lactate accumulation is the primary mechanism responsible for post-dive blood lactate accumulation in dives beyond the previously measured ADL of 5.6 min (Ponganis et al., 1997b). We predicted that Mb desaturation would follow a monotonic decline until almost complete desaturation by 5.6 min, after which it would plateau near 0% saturation until the end of the dive. To test our hypothesis, we attached a custom-built, near-infrared spectrophotometer (NIRS) instrument to emperor penguins diving at an isolated dive hole. Our goals were to: (1) assess the magnitude and rate of muscle O_2 depletion during dives, (2) describe patterns of Mb desaturation, (3) examine Mb desaturation profiles for indications of muscle perfusion or hypoperfusion during dives and (4) determine muscle O_2 consumption during dives.

MATERIALS AND METHODS

Instrument design

In order to measure Mb desaturation in the emperor penguin muscle during diving, we developed a small, dual wavelength NIRS instrument. NIRS instruments have frequently been used to measure Mb or Hb saturation (Delpy and Cope, 1997; Guyton et al., 1995; Jobsis, 1977; Jobsis et al., 2001; Sako et al., 2001). The absorption of transmitted NIR radiation by specific chromophores, such as Mb, is measured from the reduction in intensity of reflected radiation due to absorption by Mb. The attenuation in radiation intensity is proportional to the absorption by Mb, which can then be related to the NIR absorption spectra of deoxy-Mb and oxygenated Mb (oxy-Mb) to provide a relative degree of oxygenation. The ratio of two wavelengths, 760 nm (where absorption by deoxy-Mb is significantly higher than absorption by oxy-Mb) and 800 nm (an isosbestic point, where absorption coefficients of both deoxy-Mb and oxy-Mb are equal), is linearly correlated to the relative saturation level of Mb (Guyton et al., 1995; Jobsis, 1977; Mancini et al., 1994).

The NIRS instrument consisted of a microprocessor-based recorder, an underwater housing unit and a small implantable probe (Fig. 1). A custom-built microprocessor recorder (UFI, Morro Bay, CA, USA), powered by a 7.2 V battery pack (7.2 V Li-$SOCL_2$, Rose Electronics, Houston, TX, USA), provided an 80 mA current to light emitting diodes (LEDs) on the probe. The probe was implanted on the pectoral muscle, the primary locomotory muscle for diving penguins (Ponganis et al., 1997a). Penguins stroke at approximately 0.75 Hz (van Dam et al., 2002). A high sampling rate of 50 Hz was used in order to compensate for anticipated movement artifacts from strokes. Reflectance data were stored on an SD 256 MB flash card (SanDisk, Milpitas, CA, USA). The NIRS recorder was housed in a custom-designed, aluminum underwater case, which was rated to 350 m (16.2×7.5 mm, 450 g, SIO Hydraulics Lab & SIO Marine Development Shop, La Jolla, CA, USA) (Fig. 1B). The recorder connected to a pluggable underwater cable (HUMG5-BCR & CCP, Sea-Con Brantner & Associates, Inc., El Cajon, CA, USA) (Fig. 1B), which was connected and waterproofed to an FEP-coated ultra-miniature bare copper multi-conductor medical cable (1.1 mm diameter, Cooner Wire, Chatsworth, CA, USA), which was, in turn, attached to the probe (Fig. 1).

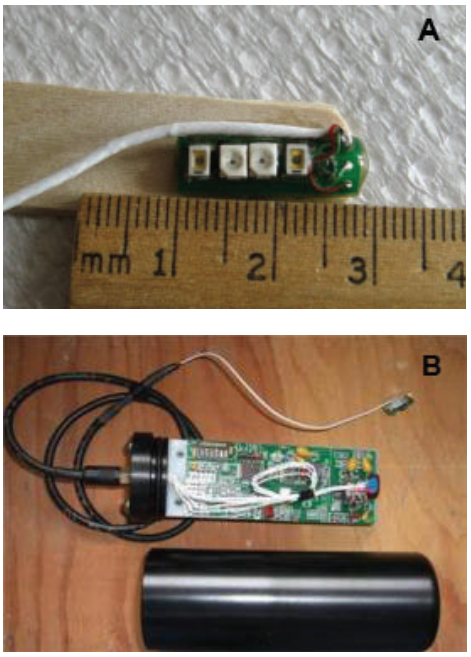

Fig. 1. (A) Close-up of implantable near-infrared spectrophotometer (NIRS) probe connected to medical cable. (B) The NIRS instrument with microprocessor-based recorder board attached to underwater cable and implantable probe; underwater housing case shown in black.

Probes were constructed using a small circuit board (19×5×3 mm, <1 g) with two surface-mount LEDs (SMT760 & SMT810, Epitex, Inc., Kyoto, Japan) positioned in the middle of the board 3 mm apart (Fig. 1A). The 50 Hz sampling rate, alternately powering the two LEDs, resulted in a 2.8 ms pulse width with 17.2 ms between pulses. With a rise and fall time of less than 100 ns for each LED, it was predicted that the sampling cycle would work well with no overlap of pulses. Two surface-mount photodiodes (PD006-SMT, Epitex, Inc.), for receiving reflectance data, were soldered on each end of the board, 3 mm from each LED (Fig. 1A). Rise and fall times for the photodiodes were 6 ns. Probes were sealed using a two-component epoxy with a spectral transmission of >95% in the NIR range (Epo-Tek 302-3M, Epoxy Technology, Inc., Billerica, MA, USA).

Instrument validation

Prior to using the NIRS instrument in the field, the linearity of the NIRS reflectance readings at different saturation values was verified. In the NIR range, absorption spectra for both Hb and Mb are essentially identical; therefore, Hb was used as a proxy for Mb in the linearity verification experiment. Rat (*Rattus norvegicus*) whole blood was mixed in a tonometer attached to a Wösthoff gas-mixing pump to obtain 0 and 100% Hb saturation (Tonometer 237, Instrumentation Laboratories, Bornheim, Germany; H. Wösthoff KG, Bochum, Germany). Blood samples for Hb saturation values of 25, 50 and 75% were obtained using a volumetric mixing technique (Scheid and Meyer, 1978). Blood gas analysis (i-STAT blood gas analyzer, Abbott Point of Care, Princeton, NJ, USA) was used to determine P_{O_2} values for samples at each saturation percentage. Accuracy of the mixing technique was confirmed by comparing P_{O_2} values for each sample with P_{O_2} values for each saturation percentage predicted by the rat O_2–Hb dissociation curve (Schmidt-Nielsen, 1997). A 2 ml

blood sample at each Hb saturation value was transferred anaerobically from the tonometer to a spectrophotometric cuvette. The probe was attached to the side of the cuvette and then covered to prevent light penetration. Readings were immediately taken from the NIRS instrument for each sample.

Field study approach

Non-breeding emperor penguins (8–15 birds per season, N=16 for this experiment, 20–26 kg) were captured on the sea ice of McMurdo Sound, Antarctica, in the austral spring of 2007 and 2008. Penguins were transported to a sea ice camp (Penguin Ranch) set up on McMurdo Sound with two isolated dive holes and a penguin corral as previously described (Kooyman et al., 1992). Penguins dived freely at the isolated dive holes and foraged on sub-ice fish and squid as verified by underwater visual observations and guano deposits. Once experiments were completed, and within 6 weeks of capture, all penguins were released at the sea ice edge. All procedures were approved under a UCSD Animal Subjects Committee protocol and a US Antarctic Treaty permit.

Instrument attachment

The NIRS probe was implanted on the surface of the right pectoral muscle of penguins under general anesthesia (Kooyman and Ponganis, 1994; Kooyman et al., 1992). A small incision (5 cm), located mid-wing level and 10 cm medial from the black–white feather margin, was made in the skin. Muscle was exposed by careful blunt dissection to avoid trauma and bleeding. The probe was sutured to the muscle with three silk sutures previously attached to the back of the probe. Probes were implanted in areas free of major blood vessels. After attachment to the muscle surface and verification of probe function with the recorder, the incision was closed in two layers (subcutaneous and skin) with 2–0 prolene suture. The cable exiting the skin was secured to the feathers with Tesa™ tape and Loctite™ glue. The NIRS recorder was attached to the feathers of the mid-back with a 5 min epoxy glue (Devcon, Danvers, MA, USA), a Velcro™ strip and cable ties as previously described (Stockard et al., 2005). A time–depth recorder (TDR) (MK9, Wildlife Computers, Redmond, WA, USA) (sensitive to 0.5 m, 30 g, 6.5×1.7×1.7 cm, sample rate 1 Hz) was attached above the NIRS instrument midline between the wings as previously described (Stockard et al., 2005). After recovery overnight, instrumented birds were allowed to dive freely for 1 to 2 days. The NIRS instrument, probe and TDR were then removed under anesthesia and all study penguins, observed carefully post-procedure, resumed regular daily diving shortly thereafter.

Zero calibration of probe

During the probe removal procedure, in order to obtain a zero calibration, a portion (~15×6×4 mm, 200 mg) of the pectoral muscle with the probe still attached to it was excised. The excised muscle sample and probe were immediately placed in a watertight reclosable plastic bag wrapped in black electric tape, which was then kept in a 38°C water bath. An opened corner of the bag allowed exit of the probe cable, which was still connected to the NIRS recorder. *Via* small Tygon® tubing, 100% nitrogen gas (N_2, ultrahigh purity, >99.999%) was bubbled through a saline-filled test tube (38°C) and into the open corner of the bag at a rate of 0.1 l min^{-1}. The NIRS instrument continued to record data until there were no further changes in the reflectance signals in order to ensure a 0% Mb saturation value for each penguin muscle sample. NIRS reflectance ratio data were converted to Mb saturation using the 0% saturation value and an assumed 100% Mb saturation value prior to diving.

NIRS probe evaluation

Effects of blood flow and Hb oxygenation on NIRS probe output from muscle were evaluated on two penguins under general isoflurane-O_2 endotracheal anesthesia (Kooyman et al., 1992). The NIRS probe was attached to the pectoral muscle as described above. The birds were also catheterized percutaneously in the wings with a 20 g, 4.5 cm radial artery catheter (RA-04020, Arrow International, Reading, PA, USA) and a 16 g, 4.5 cm brachial vein catheter (BD Insyte™ 381257, Becton Dickinson, Sandy, UT, USA). Arterial blood pressure was transduced by a Hewlett-Packard blood pressure transducer system (78304A/78205D) calibrated by a manometer with the transducer at the level of the penguin's heart. Data were recorded and analyzed in mm Hg, and are expressed so in the text; pressure axes in graphs are labeled in both mm Hg and kPA, using the conversion 0.133 kPA=1 mm Hg. The electrocardiogram (ECG) was recorded with an ECG recorder (RespI/ECG, UFI, Morro Bay, CA, USA) attached to three subcutaneous electrodes placed midline in the back above and below the heart, and laterally in the chest below the heart. Blood pressure and ECG outputs were recorded on a personal computer with Acqknowledge software and a Biopac MP100 interface system (Biopac, Goleta, CA, USA).

Effects of presumed changes in MBF on the NIRS probe output were evaluated with intravenous injections of alpha and beta sympathomimetic agents (phenylephrine and isoproterenol, respectively).

In order to evaluate the effect of Hb saturation on the NIRS probe output, ventilation was maintained with both spontaneous respirations and manually assisted ventilation in order to maintain blood pH in the 7.4 to 7.5 range. The inspired O_2 fraction (F_{IO_2}) was changed with addition of N_2 *via* a second flow meter to the anesthesia circuit and was monitored with an O_2 analyzer (Vascular Technology, Inc., Nashua, NH, USA). Arterial and venous blood P_{O_2} and pH were analyzed with an i-STAT blood gas analyzer. Hb saturation was calculated from the pH 7.5 O_2–Hb dissociation curve of the emperor penguin (Meir and Ponganis, 2009).

Probes and catheters were removed at completion of the studies, and the birds were released back into the penguin corral after recovery from anesthesia.

Data processing and statistics

Data were processed, graphed and statistically analyzed using Origin (OriginLab Corp., Northampton, MA, USA), SPSS (version 11.5, SPSS, Inc. Chicago, IL, USA) and a custom-developed MATLAB program (The MathWorks, Inc., Natick, MA, USA). NIRS data were processed using several custom MATLAB scripts in which the baseline for each LED was determined and stroke artifact was filtered out (Fig. 2). The 760 nm and 810 nm data were smoothed with a 20-point moving average and the ratio 760 nm/810 nm was calculated. Confirmation that movement artifact from the NIRS signal was an accurate record of strokes was made in several ways. First, NIRS movement artifact data were compared with stroke movements recorded by a two-dimensional accelerometer (UME-D2GT, Little Leonardo Co., Tokyo, Japan) during dives in one penguin, which confirmed that artifact was consistent with stroking. Second, manual movement of the wing while the penguin was under anesthesia was confirmed in the NIRS signal. Third, the pattern and rate of the movement artifact during dives were similar to the stroke frequency profiles observed in a prior study (van Dam et al., 2002). Strokes were then determined from a custom-made peak detection script and visually confirmed. Stroke frequency for each dive was calculated from the total number of strokes for each dive divided by dive duration.

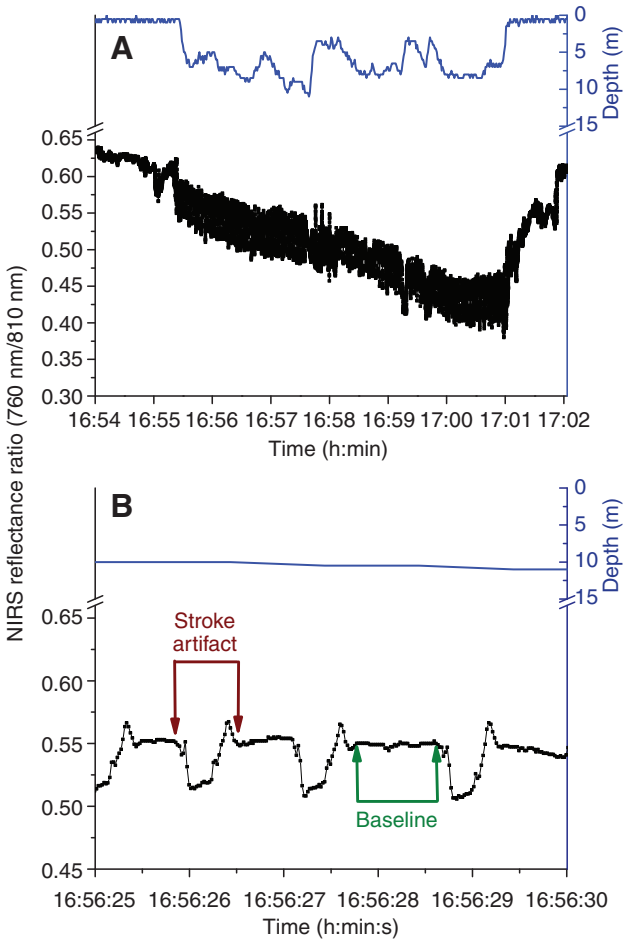

Fig. 2. (A) Example of data recorded by the NIRS instrument and time–depth recorder during a dive. (B) A 5 s excerpt from the same dive showing stroke movement artifact and NIRS baseline between strokes. Depth is shown in blue and NIRS reflectance signal is shown in black.

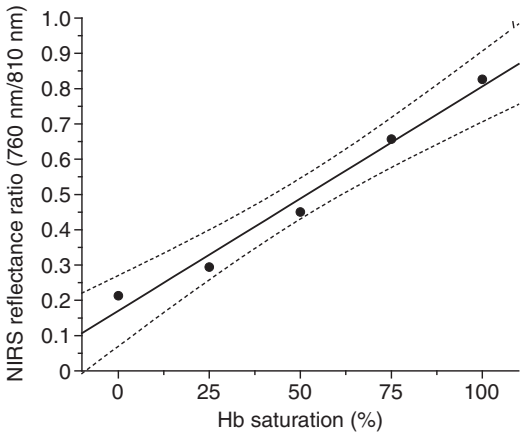

Fig. 3. Reflectance ratio from the NIRS instrument *vs* hemoglobin (Hb) saturation values. Results confirm a linear relationship (y=0.0064x+0.1702, r^2=0.98, P=0.0012; dashed lines reflect 95% confidence intervals).

The relationship between NIRS reflectance ratio from the NIRS instrument and Hb saturation was assessed with simple linear regression in the validation study. End-of-dive Mb saturation values and Mb desaturation rate were determined from converted NIRS data. Regression analysis was used to assess the relationship between end-of-dive saturation and dive duration and Mb desaturation rate and stroke frequency. Statistical significance was assumed at $P \leq 0.05$. Values are expressed as means ± s.d. unless otherwise noted.

RESULTS

Instrument validation

The validation experiment confirmed a significant linear relationship between NIRS reflectance ratio readings at different Hb saturation values (r^2=0.98, P=0.0012; Fig. 3). All probes showed similar statistically significant linear relationships.

NIRS probe evaluation

In the first experiment, to examine the effects of blood flow on the NIRS signals, the baseline systolic blood pressure was 100 to 120 mm Hg and heart rate was approximately 80 beats min^{-1}. After a 0.6 µg intravenous injection of isoproterenol, systolic blood pressure increased to 160 mm Hg and heart rate increased to 140 beats min^{-1}, but no distinct changes in either the 760 nm or the 810 nm NIRS signals occurred. After return of heart rate and blood pressure to baseline, 100 µg of phenylephrine was injected intravenously. Systolic blood pressure rose dramatically to above 220 mm Hg and heart rate decreased transiently to below 40 beats min^{-1}. Again, no significant changes in NIRS signals were observed.

In the second experiment, to examine the effects of changes in Hb oxygenation on the NIRS signals, $F_{I_{O_2}}$ was reduced from 100 to 50% for 10 min and then to 20% for 4 min while monitoring the ECG record and taking periodic blood gas samples. Heart rate rose slightly during the reduction in $F_{I_{O_2}}$, but was quite variable during the entire procedure (Fig. 4). However, there were no associated changes or trends in either NIRS reflectance signal (Fig. 4). After approximately 4 min at the 20% $F_{I_{O_2}}$, arterial and venous Hb saturation had dropped approximately 40% (from 99 and 97% to 59 and 61%, respectively). During this period, there were minimal, inconsistent changes in the NIRS signals (Fig. 4).

Dive behavior

NIRS data were obtained from three birds for a total of 50 dives >2 min duration (Table 1). Muscle biopsies and subsequent zero calibrations were successfully performed in all three birds. The difficulty of obtaining Mb desaturation profiles on a primary locomotory muscle is demonstrated by the number of birds from which we were unable to recover usable NIRS data. Mb desaturation data were not obtained from 13 birds equipped with the NIRS instrument due to: (1) breakage of the medical cable connecting the probe to the recorder during diving, (2) unsuccessful zero calibration experiments and (3) excessive movement artifact in at least one of the LED's reflectance data, which could not be resolved with filtering.

Dive duration and maximum depth data of the three birds are reported in Table 1. Dive durations from all dives ranged from 2.3 to 11.4 min (Fig. 5). Thirty-one dives were equal to or longer than the 5.6 min ADL. Maximum dive depth for all dives ranged from 7 to 64 m (Fig. 5).

Desaturation rates, stroke frequency and recovery times to 75% resaturation are reported in Table 1. Grand mean stroke frequency for all three birds was 0.66±0.08 Hz. Grand mean Mb desaturation

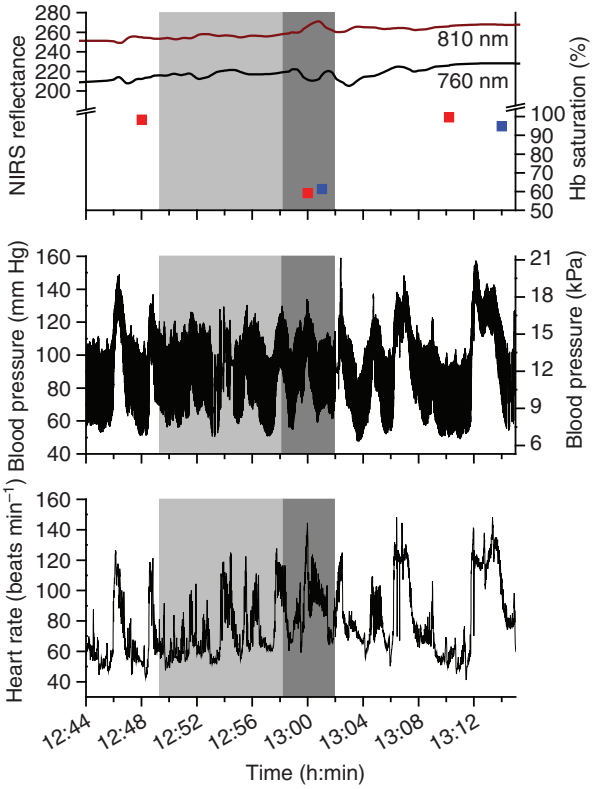

Fig. 4. Profiles of NIRS reflectance, Hb saturation, blood pressure and heart rate from experiment with a penguin under anesthesia and in which the inspired oxygen fraction ($F_{I_{O_2}}$) was altered. Maroon line, 810 nm reflectance signal; black line, 760 nm reflectance signal; red squares, arterial Hb saturation; blue squares, venous Hb saturation; light gray shaded area, $F_{I_{O_2}}$=50%; dark gray shaded area, $F_{I_{O_2}}$=20%.

rate during dives was 11.03±2.4% min^{-1}. Mean recovery times ranged from 0.5 min to almost 2 min, with a grand mean of 1.03±0.76 min.

Mb desaturation patterns

Two distinct Mb desaturation patterns were observed in the birds, a monotonic decline (type A) and a mid-dive plateau pattern (type B). Dives were classified into either type A or type B based on visual verification of the Mb desaturation pattern. Six dives could not be classified as either type A or type B. Bird 2 had primarily type A dives, whereas birds 7 and 11 had predominantly type B dives. Dive characteristics, Mb desaturation rates and recovery times of type A and B dives are summarized in Table 2.

End-of-dive Mb saturation was linearly related to dive duration in type A and type B dives (Fig. 6). In type A dives, end-of-dive Mb saturation was close to 0% in dives near the ADL (Fig. 6A). However, in type B dives, end-of-dive Mb saturation was not significantly depleted until dive durations of 8 to 10 min (Fig. 6B). At 5.6 min into type B dives, Mb saturation was close to 50% (Fig. 6B).

Type A dives

In type A dives, Mb saturation generally followed a monotonic decline throughout the dive (Fig. 7A). Eight dives were classified as type A. The desaturation rate was often slightly higher during the initial descent, but the slope then remained fairly constant throughout the rest of the dive. There were only minor changes in slope or desaturation rate during type A dives (Fig. 7A). In a few longer type A dives, the slope of the desaturation rate leveled off at the end (Fig. 7A). Mean desaturation rate in type A dives was 14.4±3.8% min^{-1}. Mean stroke frequency in type A dives was 0.74±0.04 Hz (Table 2). Instantaneous stroke frequency ranged from 0.4 to 2.0 Hz, with higher stroke frequencies associated with descents and ascents during the dive (Fig. 7A). For dives with 5 to 6 min durations (near the ADL), mean end-of-dive Mb saturation was 7.1±5.6% (N=3, range=0.6–10.7%). Stroke frequency was not a significant predictor of Mb desaturation rate (r^2=0.26, P=0.2).

Type B dives

Thirty-six dives were classified as having a type B desaturation pattern. During initial descent in type B dives, although stroke frequency was high (1–2 Hz), Mb desaturation rate was sometimes fast, as in type A dives, and sometimes moderate (Fig. 7B, Fig. 8). During the middle phase of the dive, Mb desaturation rate was slow and, sometimes, nearly 0 in the middle of the dive (Fig. 7B, Fig. 8) while stroke frequency was 0.4 to 0.6 Hz and fairly constant (Fig. 7B). Finally, during the ascent phase, the desaturation rate increased but stroke frequency remained low, 0.4–0.6 Hz (Fig. 7B), with higher stroke frequencies only observed in some dives at the end of the ascent. Mean desaturation rate in type B dives was 9.8±2.4% min^{-1}. Mean stroke frequency in type B dives was 0.64±0.09 Hz. For dives with 5 to 6 min durations (near the ADL), mean end-of-dive saturation was 44.8±6.5% (N=6, range=32.8–50.1%). Stroke frequency was a weak but statistically significant predictor of Mb desaturation rate (r^2=0.18, P=0.01).

Type A *versus* type B dives

The differences between type A and type B Mb desaturation rates, stroke frequencies, dive duration and maximum dive depth were statistically significant (Mann–Whitney U-test, Z=–2.891, P=0.004; Z=–3.228, P=0.001; Z=–2.800, P=0.005; Z=–4.202, P=0.000, respectively; Table 2). There was no characteristic dive profile for either type A or type B dives. Dives with intra-dive ascents to 2–4 m depth followed by descents to near maximum dive depth occurred in both dive types. The difference in recovery to 75% Mb saturation was not statistically different between type A and type B dives (Mann–Whitney U-test, Z=–0.259, P=0.8).

Table 1. Body mass, dive characteristics, Mb desaturation rate and recovery time of emperor penguins

Penguin	Body mass (kg)	Number of dives	Maximum depth (m)	Dive duration (min)	Stroke frequency (Hz)	Mb desaturation rate (% min^{-1})	Recovery time to 75% Mb saturation (min)
2	22.1	10	12.6±4.7 (7–24.5)	4.1±1.2 (2.7–5.8)	0.74±0.04 (0.67–0.8)	13.7±3.7 (8.7–19.9)	0.68±0.3 (0.2–1.3)
7	22.5	21	48.0±12.0 (22.5–63.5)	6.7±1.8 (2.3–10.1)	0.67±0.1 (0.54–0.91)	9.2±2.8 (5.9–17.9)	0.51±0.5 (0.1–2.2)
11	25.1	19	27.6±5.0 (15.5–33.5)	6.9±2.9 (2.8–11.4)	0.58±0.06 (0.46–0.66)	10.2±1.7 (8.1–14.4)	1.9±0.8 (0.5–3.2)

Values are means ± s.d. (range).
Mb, myoglobin.

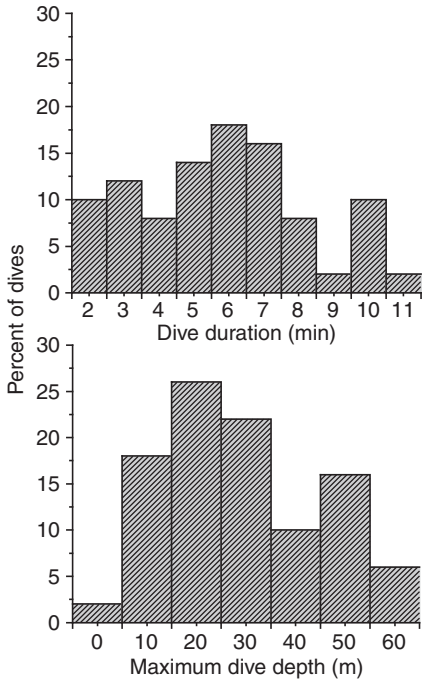

Fig. 5. Distribution of dive duration (top) and maximum dive depth (bottom) of dives from three emperor penguins outfitted with an NIRS instrument and a time–depth recorder (*N*=50 dives).

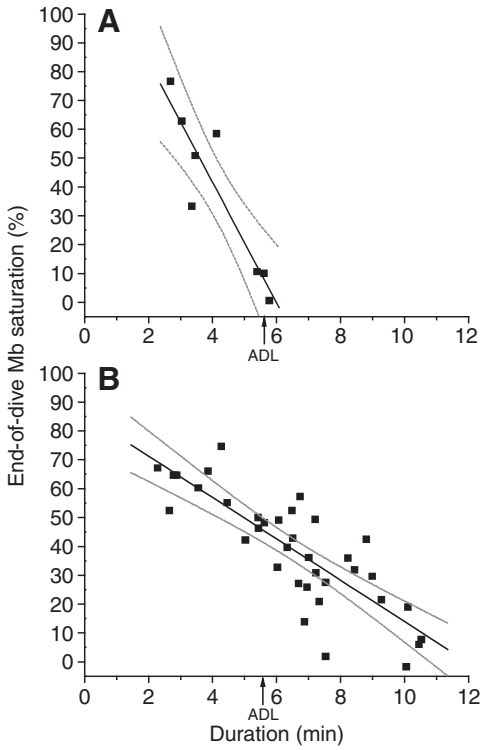

Fig. 6. Relationship between end-of-dive myoglobin (Mb) saturation values and dive duration from (A) type A and (B) type B dives (linear regression, type A, y=125.28–20.92x, r^2=0.74, P=0.0007; type B, y=85.56–7.17x, r^2=0.68, P<0.0001). Dashed lines reflect 95% confidence intervals. ADL, aerobic dive limit.

DISCUSSION

Dive behavior

The NIRS recorder did not appear to have a significant effect on the dive behavior of penguins as dive durations and maximum dive depths were similar to those of birds in past studies (Ponganis et al., 2001; Ponganis et al., 2003; Ponganis et al., 2007; Ponganis et al., 2009; Stockard et al., 2005). Stroke frequencies and stroking patterns were also consistent with results from previous studies (Meir et al., 2008; van Dam et al., 2002). Dive profiles revealed intra-dive ascents to the undersurface of the ice, typical of penguins foraging on sub-ice fish (*Pagothenia borchgrevinki*) (Ponganis et al., 2000), in some but not all of both type A and type B dives.

NIRS probe evaluation studies

NIRS signals may include reflectance data from both Hb and Mb saturation because the absorption spectra of Hb and Mb are essentially the same. Thus, the relative contribution of blood flow, Hb oxygenation and Mb oxygenation to NIRS signals may vary (Mancini et al., 1994; Tran et al., 1999; Wilson et al., 1989). Although the NIRS probe in the present study was attached in areas free of visible blood vessels, the potential contributions of Mb and Hb saturation to the NIRS signals were unknown. Two NIRS instrument evaluation experiments were undertaken on anesthetized birds to address this question. During isoflurane anesthesia, MBF is preserved near levels at rest in pigs and horses, and is even elevated in humans (Lundeen et al., 1983; Raisis et al., 2000; Stevens et al., 1971). The first evaluation experiment was designed to examine the effect of different concentrations of Hb on the NIRS signals by changing the blood flow. After injection of 0.6 μg of isoproterenol, a beta-sympathomimetic agent that increases heart rate and cardiac output and causes peripheral arterial vasodilation, no significant changes in the NIRS signals were observed. If Hb saturation were a significant component of the reflectance signals, the NIRS signals would have been expected to change concomitantly with the observed increase in heart rate and systolic blood pressure. Phenylephrine, an alpha-adrenergic agent, vasoconstricts peripheral vessels and raises blood pressure. The lack of any consistent trend in the NIRS reflectance data, despite systolic blood pressure rising to above 220 mm Hg and heart rate falling to 40 beats min^{-1} immediately after a 100 μg phenylephrine injection, also confirmed that the NIRS data reflected primarily Mb saturation and not changes in the quantity of Hb present due to changes in MBF. Furthermore, because of the transient effect of phenylephrine, the low metabolic rate of muscle at rest and the high O_2 content of penguin muscle, one would not expect to see a large decline in Mb saturation during the transient hemodynamic changes after the phenylephrine injection.

Table 2. Dive characteristics, Mb desaturation rate and recovery time of type A and type B desaturation profiles

Desaturation pattern	Type A	Type B
Number of dives	8	36
Mean maximum depth (m)	12.8±5.2	38.6±13.9*
Mean dive duration (min)	4.2±1.2	6.5±2.3*
Mean stroke frequency (Hz)	0.74±0.04	0.64±0.09*
Mean Mb desaturation rate (% min^{-1})	14.4±3.8	9.8±2.4*
Recovery time to 75% Mb saturation (min)	0.76±0.31	1.16±0.97

*, statistically significant difference between type A and type B.
Mb, myoglobin.

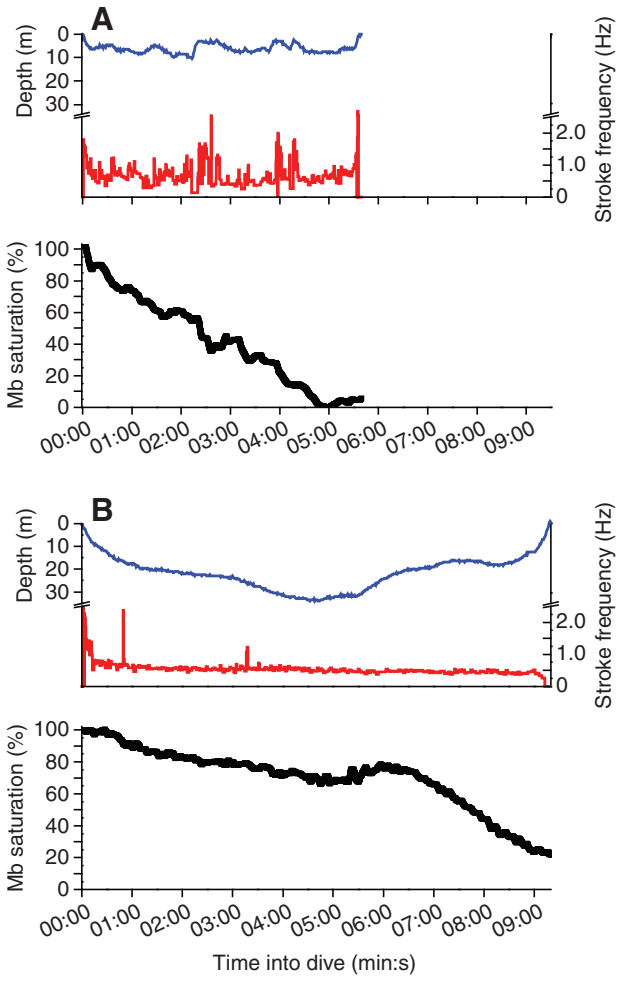

Fig. 7. Example of Mb desaturation, instantaneous stroke frequency and dive depth profiles for (A) a 5.6 min type A dive and (B) a 9.3 min type B dive.

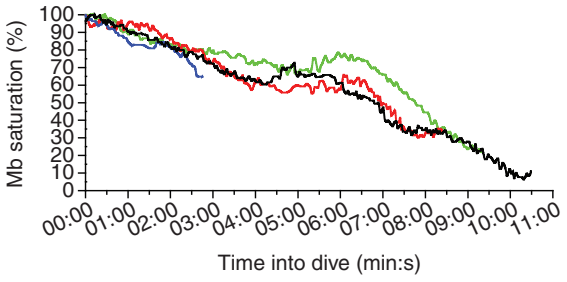

Fig. 8. Profiles of type B Mb desaturation profiles from four dives (blue, 2.8 min dive; red, 8.4 min dive; green, 9.3 min dive; black, 10.5 min dive).

The second evaluation experiment demonstrated that changes in Hb oxygenation due to changes in F_{IO_2} had minimal effect on the NIRS reflectance signals. If the NIRS signals were strongly affected by Hb saturation, the 760 nm reflectance signal would decrease as Hb saturation decreased. However, the 760 nm signal did not show a decreased reflectance; rather, the signal varied slightly in both directions despite a 40% drop in arterial and venous Hb saturation (Fig. 4). As a result of these findings, we concluded that the NIRS reflectance signals predominantly represent Mb saturation during dives.

Type A Mb desaturation profiles

In type A Mb desaturation profiles, the initial descent period was often characterized by a sharp decrease in saturation (Fig. 7A). This sharp decrease generally coincided with high stroke frequency and, thus, high muscle workload (van Dam et al., 2002). After the initial descent, there was a consistent monotonic decline of Mb saturation with only minor changes in desaturation rate throughout the rest of the dive. Small changes in desaturation rate during the dive may be related to variations not just in stroke frequency, but also in stroke amplitude and stroke thrust throughout the dive. However, the overall rate of decline in saturation during a dive appeared constant (Fig. 7A). Individual dive Mb desaturation rates did not correlate with stroke frequency during a dive. We suspect that, in addition to stroke frequency, stroke amplitude and thrust will also contribute to muscle metabolic demand during dives.

Several aspects of type A dives are consistent with isolation of muscle from the circulation during dives. First, the overall Mb desaturation rate (14.4% min^{-1}) is consistent with rapid Mb desaturation observed in early forced submersion studies (Scholander et al., 1942). Second, the Mb desaturation rate is considerably higher than median muscle desaturation rates in Weddell seals (5.1% min^{-1} for short dives and 2.5% min^{-1} for dives >17 min) (Guyton et al., 1995). In that study, it was proposed that those desaturation rates were consistent with maintenance of MBF during dives (Guyton et al., 1995). Third, in type A profiles, no detectable increases in Mb saturation were observed during dives, in contrast to observations in the Weddell seal study (Guyton et al., 1995). Further, the muscle O_2 consumption (discussed below) based on the Mb desaturation rate is consistent with the muscle metabolic rate (12–17 ml O_2 kg^{-1} min^{-1}) calculated based on the O_2 capacity of an isolated pectoralis–supracoracoideus muscle complex and the ADL range of 5–7 min (Ponganis et al., 1997a). Finally, Mb saturation reached low values and plateaued near the ADL in longer dives (5–6 min). Therefore, we conclude that type A Mb desaturation patterns are consistent with our original hypothesis that the muscle O_2 store is isolated from the circulation during these dives and is significantly or completely depleted at the ADL.

Prior research on emperor penguins has also suggested that muscle is isolated from the circulation during dives. During the initial descent phase of the dive, when stroke frequency and, therefore, muscle workload is at its peak (Sato et al., 2002; van Dam et al., 2002), blood O_2 extraction by muscle should be high if there is MBF. However, venous P_{O_2} values often increased rather than decreased during the descent phase of dives (Ponganis et al., 2007; Ponganis et al., 2009). Similarly, penguins often experienced a striking decline in heart rate during early descent (Meir et al., 2008). Again, this is not consistent with maintenance of MBF at that time. Further, the lack of correlation between stroke frequency and heart rate during dives and the rate and pattern of pectoral muscle temperature changes during dives are also consistent with a severe reduction or cessation of MBF (Meir et al., 2008; Ponganis et al., 2003). Finally, although post-dive blood lactate concentrations were elevated in dives 5.6 min or longer (Ponganis et al., 1997b), intra-dive blood samples demonstrated no significant lactate accumulation as far as 10.5 min into a 12.8 min dive (Ponganis et al., 2009). The lack of elevated lactate as late as 10.5 min into a dive suggests that muscle is isolated from the circulation and any accumulated lactate does

not wash out into blood until after a dive, when circulation presumably returns to muscle. In the post-dive period, it is also notable that Mb resaturation is extremely rapid, with mean time to return to 75% saturation near 1 min for both type A and type B dives. Rapid resaturation of Mb also occurs in the post-apneic period of sleeping elephant seals (Ponganis et al., 2008). Such a quick return in Mb saturation is consistent with the post-dive tachycardia and rapid replenishment of air sac and blood O_2 stores in emperor penguins (Meir et al., 2008; Ponganis et al., 2009; Stockard et al., 2005).

Muscle O_2 consumption during dives

If muscle is isolated from the circulation during type A dives and lactate does not accumulate in dives <ADL, then the desaturation rate of the Mb-O_2 store can be used to calculate mean muscle O_2 consumption during dives. The mean Mb desaturation rate for type A dives was 14.4% min^{-1}. Assuming a Mb concentration of 6.4 g $100\,g^{-1}$ muscle (Ponganis et al., 1997a) and an O_2-binding capacity of 1.34 ml $O_2\,g^{-1}$ Mb, mean muscle O_2 consumption is 12.4± 3.3 ml $O_2\,kg^{-1}$ muscle min^{-1}. It should be noted that this value of 12.4 ml $O_2\,kg^{-1}$ muscle min^{-1} was estimated from dives of less than 6 min duration with a mean stroke frequency near 0.74 Hz. Based on this stroke frequency, the cost per stroke is 1.7 ml O_2. Differences in stroke frequency, stroke amplitude and stroke thrust will all contribute to variation in muscle O_2 consumption and stroke cost.

This rate of muscle O_2 consumption during diving is rather low, only two to four times resting muscle metabolic rates in dogs (2–7 ml $O_2\,kg^{-1}$ muscle min^{-1}) (Duran and Renkin, 1974; Hogan et al., 1992; Hogan et al., 1996; Piiper et al., 1985), seals (Ponganis et al., 2008) and humans (2–3 ml $O_2\,kg^{-1}$ muscle min^{-1}) (Mizuno et al., 2003), and near the resting muscle metabolic rate of the Pekin duck (*Anas platyrhynchos*, 11 ml $O_2\,kg^{-1}$ muscle min^{-1}) (Grubb, 1981). The muscle O_2 consumption of the diving emperor penguin is only approximately one-fifth that of canine gastrocnemius stimulated at 0.25 Hz (Fig. 9) (Hogan et al., 1998), less than one-tenth the pectoralis–supracoracoideus muscle O_2 consumption calculated from emperor penguins swimming maximally in a flume (160 ml $O_2\,kg^{-1}$ muscle min^{-1}) (Kooyman and Ponganis, 1994; Ponganis et al., 1997a) and far less than maximal O_2 consumption of human quadriceps femoris muscle (520 ml $O_2\,kg^{-1}$ muscle min^{-1}) (Richardson et al., 1995b). Although muscle ischemia may contribute to decreased muscle O_2 consumption (see example in Fig. 9) (Duran and Renkin, 1974; Hogan et al., 1998; Ponganis et al., 2008), we hypothesize that biomechanical efficiency and hydrodynamics are primarily responsible for this low muscle O_2 consumption during diving of the emperor penguin.

Type B Mb desaturation profiles

In type B dives, Mb desaturation rate during the initial descent was either rapid, as in type A dives, or moderate (Fig. 7B, Fig. 8). However, as descent continued, desaturation rates slowed significantly, often leveling off completely until the ascent phase, when desaturation rates increased. We suggest that the mid-dive plateaus in type B Mb desaturation profiles are secondary to maintenance of muscle perfusion during these segments of type B dives. During the plateau periods, which frequently lasted several minutes or more, stroke frequency was constant (0.4–0.6 Hz), indicating that muscle work continued. The lack of change in Mb saturation implies the muscle O_2 store was supplemented by the circulation during these periods. During the ascent phase, despite continued stroking at the same frequency, Mb desaturation rate again increased, implying that MBF was reduced during this portion of the dive.

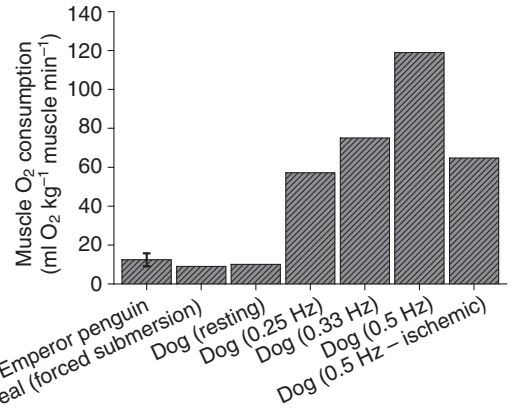

Fig. 9. Comparison of locomotory muscle O_2 consumption for diving emperor penguins (present study, pectoral muscle), back muscle of forcibly submerged harbor seals (Scholander et al., 1942) and canine gastrocnemius muscle stimulated at different rates (Hogan et al., 1998). Muscle O_2 consumption from harbor seal represents only the first 5 min of a 20 min dive, prior to the accumulation of lactate. The last bar represents canine muscle O_2 consumption during stimulation with muscle blood flow reduced to 46% (Hogan et al., 1998). Error bars for emperor penguin data are ±s.d.

A plateau phase of Mb desaturation, as occurred in type B dives, is similar to that during sleep apnea in elephant seals and during exercise of humans to maximum O_2 consumption (Ponganis et al., 2008; Richardson et al., 1995b). In both situations, the decrease in Mb saturation should support blood-to-muscle O_2 flux by maintaining or enhancing the blood-to-muscle P_{O_2} gradient. These type B dive profiles were also similar to Mb desaturation profiles in two Weddell seals, which demonstrated a slow decline at the beginning of some dives and then a flat slope for over half of the dive, which suggested blood-to-muscle O_2 transfer from the Hb-O_2 store (Guyton et al., 1995). However, type B desaturation patterns in emperor penguins are not entirely analogous to results from the prior study on Weddell seals. The increased Mb desaturation rate at the end of type B dives in penguins was not observed in Weddell seals (Guyton et al., 1995).

In addition, the Mb-O_2 store in Weddell seals never approached complete depletion. Even in dives longer than the Weddell seal ADL, including a dive of 27 min, Mb was typically still 40–60% saturated (Guyton et al., 1995). In contrast, saturation values in long type B dives often declined to below 10% (Fig. 8). Thus, type B dives are still supportive of the concept that the onset of post-dive lactate accumulation is secondary to muscle O_2 depletion.

Muscle blood flow during type B dives

If muscle is perfused during these plateau periods of type B dives, MBF can be estimated based on mean muscle O_2 consumption (12.4 ml $O_2\,kg^{-1}$ muscle min^{-1}) previously calculated from type A dives. Because muscle continues to work during this portion of the dive, but Mb saturation does not significantly change, the blood O_2 store likely supplies the required 12.4 ml $O_2\,kg^{-1}$ muscle min^{-1}. Assuming a pectoralis–supracoracoideus muscle mass of 6.25 kg for a 25 kg penguin, 77.5 ml $O_2\,min^{-1}$ is required. With a typical arterio–venous (A–V) O_2 content difference of 5 ml $O_2\,dl^{-1}$ (Ponganis et al., 2007), blood flow to muscle would be approximately 1550 ml min^{-1} or 250 ml kg^{-1} muscle min^{-1}. Given a stroke volume

of 50 ml (Kooyman et al., 1992), 1500 ml min^{-1} of blood flow would require 30 beats min^{-1} in heart rate. This is easily achieved during the transient increases in heart rate frequently observed during the early to mid portions of dives of emperor penguins (Meir et al., 2008). Besides shunting oxygenated blood through peripheral A–V shunts as has been hypothesized during this time period (Ponganis et al., 2009), we propose that emperor penguins also have the option to perfuse muscle instead. Such plasticity in vascular responses could account for the wide variation in previously reported venous P_{O_2} profiles in diving emperor penguins (Ponganis et al., 2009).

A muscle perfusion rate of 250 ml kg^{-1} muscle min^{-1} in a diving emperor penguin is not an exceptional value. MBF values at rest in dogs (Hutter et al., 1999; Pendergast et al., 1985), seals (Blix et al., 1983; Zapol et al., 1979), pigs (Armstrong et al., 1987), sheep (Hales, 1973), horses (Armstrong et al., 1992) and humans (Stevens et al., 1971) are reported between 25 and 250 ml kg^{-1} muscle min^{-1}. In small birds such as ducks and guinea fowl (*Numida meleagris*), MBF at rest is greater, 200–450 ml kg^{-1} muscle min^{-1} (Ellerby and Marsh, 2006; Grubb, 1981; Jones et al., 1979). By comparison, MBF at maximal O_2 consumption can be as high as 1500 to 3000 ml kg^{-1} muscle min^{-1} in dogs (Pendergast et al., 1985), swine (Armstrong et al., 1987) and horses (Armstrong et al., 1992), and up to 3000 to 4000 ml kg^{-1} muscle min^{-1} in humans (Andersen and Saltin, 1985; Richardson et al., 1995a). During the plateau period of type B dives, the estimated MBF is greater than during forced submersions (0–5 ml kg^{-1} muscle min^{-1}) and less than the highest values during recovery periods from forced submersions (1050 ml kg^{-1} muscle min^{-1}) (Blix et al., 1983; Zapol et al., 1979). Therefore, although based on several assumptions, we conclude that this muscle perfusion rate provides a plausible explanation of the type B muscle desaturation pattern.

O_2 consumption during dives: 6.8 ml O_2 kg^{-1} min^{-1}

Mean O_2 store depletion rates have now been calculated in the three O_2 stores of the emperor penguin. The sum of these mean O_2 store depletion rates provides the first calculation of total body O_2 consumption during a dive: 6.8 ml O_2 kg^{-1} min^{-1}. It must be emphasized that depletion rates of O_2 stores are highly variable among individual dives, and that this value of 6.8 ml O_2 kg^{-1} min^{-1} is an overall mean value. Furthermore, longer dives will have lower total body O_2 consumption. For example, as previously estimated on the basis of complete depletion of all O_2 stores (Ponganis et al., 2010), the O_2 consumption during a 23.1 min dive was 2.5 ml O_2 kg^{-1} min^{-1}. In addition, even for dives of similar duration, there is a large range of end-of-dive air sac O_2 fractions, blood Hb saturations and muscle Mb saturations. Such variability may be related to differences in dive depth and buoyancy, stroke frequency, stroke amplitude, stroke thrust and diving respiratory air volume of deep *versus* shallow dives.

This mean value of 6.8 ml O_2 kg^{-1} min^{-1} now allows an estimation of the relative contributions of the individual O_2 stores to metabolic rates during dives predominantly under 10 min duration (based on dive durations in studies on air sac P_{O_2} and blood P_{O_2} and the present study) (Meir and Ponganis, 2009; Stockard et al., 2005). The depletion of the primary locomotory muscle O_2 store provides 3.1 ml O_2 kg^{-1} min^{-1} to the total body O_2 consumption. The depletion of the non-locomotory muscle O_2 store (muscle mass 13% of body mass) (Ponganis et al., 1997a) at a rate of 4 ml O_2 kg^{-1} muscle min^{-1} (i.e. near a resting rate) would contribute another 0.5 ml O_2 kg^{-1} min^{-1} to the total body O_2 consumption. Thus, the entire muscle mass would contribute 3.6 ml O_2 kg^{-1} min^{-1} to total body metabolic rate during a dive. This accounts for over half the average total body O_2 consumption during diving. The respiratory and blood O_2 stores account for 31% (2.1 ml O_2 kg^{-1} min^{-1}) and 16% (1.1 ml O_2 kg^{-1} min^{-1}), respectively, of the total body diving O_2 consumption (Meir and Ponganis, 2009; Stockard et al., 2005).

An O_2 depletion rate of 6.8 ml O_2 kg^{-1} min^{-1} supports frequent suggestions that diving O_2 consumption is extremely low in emperor penguins (Kooyman and Ponganis, 1994; Nagy et al., 2001). This mean value is approximately one-third of both the field metabolic rate measured at the isolated dive hole with doubly labeled water and the lowest rate measured in penguins swimming in a flume (20 and 20.7 ml O_2 kg^{-1} min^{-1}, respectively) (Kooyman and Ponganis, 1994; Nagy et al., 2001). It is equivalent to resting metabolic rate measured in emperor penguins floating in a flume (6.2–6.7 ml O_2 kg^{-1} min^{-1}) (Kooyman and Ponganis, 1994) or standing in their thermoneutral zone (Dewasmes et al., 1980; LeMaho et al., 1976; Pinshow et al., 1976). This mean O_2 consumption is also slightly greater than the resting rate predicted by allometric equations relating metabolic rate to body mass (Aschoff and Pohl, 1970). From this analysis, the actual O_2 depletion rate is close to resting values, confirming that diving is not costly in emperor penguins.

CONCLUSIONS

Two different Mb desaturation patterns in the locomotory muscle of diving emperor penguins were revealed in this study. These patterns are consistent with the high level of variability observed in physiological parameters previously measured in freely diving penguins, including air sac P_{O_2} profiles, venous and arterial P_{O_2} profiles, heart rate, stroke frequency and body temperatures.

The type A pattern, a monotonic decline in Mb desaturation, is reminiscent of results from early forced submersion studies and suggests that no MBF occurred during the dives. Complete or near-complete depletion of the primary locomotory muscle O_2 store at the previously measured ADL supports the concept that muscle is the primary source of the post-dive blood lactate elevation in dives beyond the ADL. Using the Mb desaturation rate determined in these dives, a mean muscle O_2 consumption was calculated at 12.4 ml O_2 kg^{-1} muscle min^{-1}. This low value demonstrates the highly efficient locomotory cost of diving in emperor penguins. We also calculated the first estimate of diving O_2 consumption in a freely diving higher vertebrate from O_2 store depletion rates measured in this study and previous studies. This value, 6.8 ml O_2 kg^{-1} min^{-1}, is near measured rates of resting O_2 consumption in emperor penguins and supports the concept that the bradycardia of diving and efficient hydrodynamics primarily contribute to the slow depletion of O_2 stores and to the dive capacity of emperor penguins.

The type B pattern is a more complex Mb desaturation pattern with either a mid-dive plateau or a slow Mb desaturation rate. This suggests maintenance of MBF for at least a segment of type B dives. It was estimated that an MBF of 250 ml kg^{-1} muscle min^{-1} would maintain a constant Mb saturation during the mid-dive plateau period. This amount of blood flow would require ~30 beats min^{-1} in heart rate, a value consistent with the elevation in heart rate during the first few minutes of dives (Meir et al., 2008). Despite blood O_2 supplementation of the muscle O_2 store, extensive depletion of the muscle O_2 store in dives beyond the ADL still supports the concept that the onset of post-dive lactate accumulation is secondary to muscle O_2 depletion.

The two Mb desaturation patterns revealed in this study, and previously observed large variations in venous P_{O_2} profiles, also reinforce the idea that peripheral vascular responses and blood flow patterns are plastic during the early to middle portions of emperor

penguin dives (Ponganis et al., 2009). These results also suggest emperor penguins have at least two distinct physiological strategies while diving, to either perfuse muscle to supplement the muscle O_2 store, or to not perfuse muscle but rather utilize peripheral A–V shunts to optimize the blood O_2 store.

LIST OF SYMBOLS AND ABBREVIATIONS

ADL	aerobic dive limit
$F_{I_{O_2}}$	inspired O_2 fraction
Hb	hemoglobin
LED	light emitting diode
Mb	myoglobin
MBF	muscle blood flow
NIRS	near-infrared spectrophotometer
P_{O_2}	partial pressure of oxygen
TDR	time–depth recorder

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