UNIVERSITY OF CALIFORNIA, SAN DIEGO

Understanding the Aerobic Dive Limit and Dive Performance of Emperor Penguins:

Muscle Oxygen Depletion Patterns and Anaerobic Energy Reserves

A dissertation submitted in partial satisfaction of the requirements for the degree of Doctor of Philosophy

in

Marine Biology

by

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2011

The Dissertation of Cassondra Lee Williams is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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Chair

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2011

DEDICATION

This thesis is dedicated to

my extraordinarily supportive parents.

EPIGRAPH

The seals and birds of Antarctica are the only great populations of large animals that do not live in national parks or special reserves, and are unmanaged. The limits of their populations and distributions are set by natural laws, not by man. In essence, they are our last chance of retaining a fauna that can be studied in a wholly natural state and self-contained, relatively simple ecosystem. There is still much of great value to be learned from such animal populations.

Gerald L. Kooyman
Weddell Seal: consummate diver

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ACKNOWLEDGEMENTS

I may never know what prompted Paul to take on a former lawyer with a degree in business finance as a graduate student to study diving physiology. He is a brave man. Words cannot express how grateful I am to have had the opportunity to work with such an amazing scientist. I have never met such an inspiring, patient and brilliant researcher. It is no wonder that he is breaking new ground in diving physiology with every project. The work I have done with Paul has been some of the most challenging of my life. There were many moments of frustration and exasperation at the difficulties of getting a new instrument to work in the field. I will always remember the words of encouragement when things were going wrong (which they often do in field work), "If it were easy, everyone would be doing it." I have tried to adopt Paul's perseverance and pioneering spirit, which are invaluable attributes in the face of the many challenges of studying freely diving animals. The belief in my ability to develop a new instrument to solve a physiological question that he has instilled in me may be the most valuable thing I take with me from Scripps.

Part of the impetus for pursuing a career in marine biology was a book I read over 10 years ago, "Weddell Seal: consummate diver", by Gerald L. Kooyman. It was an inspiring rendition of research life and work with Weddell seals in Antarctica. It still amazes me to think that, just a few years later; I was working with Jerry at Scripps, hearing firsthand stories of the early days of Antarctic research and then visiting him at Cape Washington, Antarctica while doing my own research. He has been a constant source of inspiration, encouragement and remarkable knowledge. It

has been a true joy to spend time with him and his wonderful wife Mel, both of whom I am happy to call friends. I will take with me many fond memories of time spent with Jerry snorkeling and kayaking at La Jolla Shores, flying to Mexico to see gray whales up close and personal, and the many discussions we had over lunch on the gazebo by the Scripps pier.

Members of the Ponganis lab have been instrumental in my accomplishments at Scripps and in the field. When I first arrived at Scripps, Torre Stockard was there to help me with all the protocols and instruments used in the Ponganis lab. She was incredibly patient in showing me the ropes and quickly became a good friend. Jessica Meir, a fellow graduate student, lab mate, officemate, Antarctic roommate and, most of all, a wonderful friend, made Scripps soon feel like home. From the hard, yet fun and rewarding, work at Penguin Ranch, to sharing laughs and obsessing over everyday things in the office, to stealing away for an afternoon matinee when things just weren't going well, becoming friends with Jessica was one of the highlights of my time at Scripps. Although only at Scripps a short time and technically in the Kooyman lab, Shannon Barber-Meyer's bright and positive personality always made our work feel exciting and rewarding. I will always be grateful to her for getting me involved in outreach activities to encourage young female junior and high school students to explore science as a career, which has become a passion of mine. Finally, our latest member of the Ponganis lab, Gitte McDonald, is a wonderful friend and is always available to listen to me stress about my writing, to read chapters and manuscripts, to meet up at various coffee shops to write and to offer words of encouragement before

talks or interviews. The other members of Penguin Ranch teams in 2005, 2007 and 2008 (Kathi Ponganis, Ed Stockard, Red Howard, Matt Tulis, Cory Champagne, Kozue Shiomi, and Brendon Tribble) provided not only essential help in the field, but also friendship and much needed distractions while on the ice.

It is with great sadness that I leave the Ponganis lab, but I will take with me lots of wonderful memories and plans to make more in the future.

This dissertation would not have been possible without the funding support from the National Science Foundation, including grants OPP 0229638, 0538594, and 0944220. In addition, stroke frequency research in Chapter 2 was supported by a grant from the Japanese Society for Promotion of Science (A19255001). I am thankful for the support I received through a National Institute of Health Marine Biotechnology Training Program Fellowship and a University of California Regents Fellowship. I was also supported by the Scripps Graduate Department for travel to Japan for data analysis and manuscript preparation.

I am grateful to Denise Darling, Josh Reeves, Krystle Shertz, Ron Burton, Douglas Bartlett and the Scripps Graduate Department for financial and other support during my graduate school career. Others in the SIO and UCSD community have also helped me in my graduate studies. In particular, Dale Baker and Vic Vacquier have provided me with insight and assistance in various parts of my research. Douglas Alden, the SIO Hydraulics lab, Ken Duff and the SIO Machine Shop were extremely helpful in designing and building underwater housing units for the NIRS instrument.

I am also indebted to my many friends who have inspired, encouraged and motivated me along the way. From my oldest and dearest friend, Jennifer Lang, to my favorite roommate and fellow law school survivor, Lila Swenson, to the new friends I have made while at Scripps, Jill Leonard-Pingel, Jesse Powell, Megan McKenna, Melania Guerra, Ally Pasulka, and Mary Lynn Price, I have been blessed by these friendships.

I am lucky to have fallen for someone who is understanding and patient in all the field work, lab work and writing activities that take up so many weekends and holidays, in addition to frequent 12-hour weekdays. Eric Dawson has been an amazing man with whom to share my accomplishments, as well as disappointments and frustrations. These last years of graduate school would have been much harder and lonelier without him in my life. His support and heartfelt interest in my research and career is always apparent and his patience in my often long days, nights, weekends and holidays at work seems endless.

The transition from a former Los Angeles attorney to a marine biologist has been challenging and often daunting. This change would not have been possible without the enduring support and love of my family, in particular my wonderful parents, Wenzel and Janice Williams. Many parents might have paused at the thought of their child leaving a successful and stable career as a lawyer in Los Angeles to pursue a career in marine biology. Throughout my life, they have encouraged me to follow my dreams and without their unceasing belief in my abilities, I could not have accomplished half of what I have. Their support of my unusual career path and sincere

interest in my research seems to be without limit. I believe they would have gone to Antarctica to help out on my project if they could have.

MATERIAL PUBLISHED/SUBMITTED FOR PUBLICATION IN THE DISSERTATION

Chapter 1, in full, was published as:

Williams, C. L., Meir, J. U. and Ponganis, P. J. (2011). What Triggers the Aerobic Dive Limit? Patterns of Muscle Oxygen Depletion during Dives of Emperor Penguins *Journal of Experimental Biology* 214: 1802-1812. The dissertation author was the primary investigator and author of this paper.

Chapter 2, in full, is currently being prepared for publication as:

Williams, C. L., Sato, K., Shiomi, K., and Ponganis, P.J. In preparation for *Physiological and Biochemical Zoology*. The dissertation author was the primary investigator and author of this paper.

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ABSTRACT OF THE DISSERTATION

Understanding the Aerobic Dive Limit and Dive Performance of Emperor Penguins: Muscle Oxygen Depletion Patterns and Anaerobic Energy Reserves

by

Cassondra Lee Williams

Doctor of Philosophy in Marine Biology

University of California, San Diego, 2011

Paul J. Ponganis, Chair

Isolation of muscle from the circulation, depletion of the muscle oxygen store and subsequent anaerobic metabolism has been postulated as the physiological basis for the aerobic dive limit (ADL). During complete ischemia, energy production is dependent on the size of the myoglobin oxygen (Mb-O_2) store and phosphocreatine (PCr) and glycogen (Gly) concentrations. 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*). PCr and Gly concentrations in emperor penguin muscle were measured and the depletion of muscle O_2 and those energy stores were modeled under conditions of

complete ischemia. Stroke frequency was also analyzed to assess muscle workload during dives and potential effects on the model.

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 muscle O_2 depletion triggers the ADL. A mean muscle O_2 consumption of 12.4 ml O_2 kg^{-1} muscle min^{-1} and a diving O_2 consumption of 6.8 ml O_2 kg^{-1} muscle min^{-1} were calculated. Type B desaturation patterns demonstrated a more gradual decline, often reaching a mid-dive plateau in Mb desaturation. This pattern suggests maintenance of some muscle perfusion during these dives.

Measured PCr and Gly concentrations, 20.8 and 54.6 mmol kg^{-1} muscle, respectively, were similar to those in non-diving animals. Although stroke frequency was not constant during dives, as was assumed in the model, the model demonstrated that PCr and Gly provide a large anaerobic energy store that can contribute significantly to the penguin's ability to perform long dives.

These results demonstrate that emperor penguins (1) can either isolate muscle from the circulation during dives or perfuse muscle to supplement the muscle O_2 store, (2) have low muscle and total body diving oxygen consumption rates, and (3) have normal PCr and Gly concentrations, which provide significant anaerobic energy stores.

INTRODUCTION

Diving animals are limited in their ability to forage and explore their habitat by their breath hold capacity. Unlike terrestrial animals, which experience tachycardia and increased oxygen consumption while foraging or traveling, diving mammals and birds must maximize time spent underwater by conserving oxygen (O_2). In the extreme, as during forced submersions, O_2 consumption is reduced by severe bradycardia and peripheral vasoconstriction, which reduces perfusion-dependent metabolic rates of organs, isolates muscle from the circulation, and preserves O_2 for vital organs such as the brain and heart (Blix et al., 1983; Scholander, 1940; Scholander et al., 1942; Zapol et al., 1979). In addition, diving animals have increased O_2 storage capacity. One of the hallmarks of diving animals is a high myoglobin (Mb) concentration (Kooyman and Ponganis, 1998). The Mb-O_2 store is often the largest of the three O_2 stores (respiratory system, blood and muscle) in diving animals (Kooyman and Ponganis, 1998). Despite adaptations to regulate O_2 consumption and enhanced O_2 stores, diving animals appear, on occasion, to exceed their aerobic capacities.

In diving mammals and birds, the aerobic dive limit (ADL, dive duration associated with the onset of post-dive blood lactate accumulation) has become an essential criterion in the interpretation of diving behavior and foraging ecology. The elevation in lactate concentration is believed to be related to significant depletion of one or more of the three O_2 stores (Kooyman and Ponganis, 1998; Ponganis et al., 1997). When lactate does accumulate, subsequent surface intervals increase in order to recycle accumulated lactate (Kooyman et al., 1980). Isolation of muscle from the

circulation, depletion of the muscle O_2 store and subsequent anaerobic metabolism in muscle has been postulated as the physiological basis for the aerobic dive limit (Kooyman and Ponganis, 1998).

While much is known about the magnitude of O_2 stores in diving animals and recent advances have shed light on the role of heart rate and the blood O_2 store during diving (Andrews et al., 1997; Kooyman et al., 1983; Meir et al., 2009; Meir et al., 2008; Noren et al., 2005; Noren et al., 2001; Ponganis et al., 2007), very little research has been undertaken on the role of muscle during dives. Locomotory muscles continue to work throughout dives and thus play a key role in understanding what triggers the aerobic dive limit and how marine animals dive beyond their aerobic dive limit. The dearth of research on the physiology of working muscle during dives is due in large part to the lack of commercially available instruments to measure O_2 consumption in muscles. Thus, in order to understand the role of muscle metabolism in the aerobic dive limit and dive performance, innovative approaches must be developed for diving animals.

If locomotory muscles are isolated from the circulation during dives, local Mb provides the O_2 necessary for aerobic metabolism. In dives beyond the aerobic dive limit, muscle metabolism is dependent on anaerobic stores, phosphocreatine and glycogen. Despite the critical role of these anaerobic energy stores, few studies have examined the magnitude of phosphocreatine or glycogen in diving animals. In particular, phosphocreatine concentrations have never been directly measured in any diving animal. In addition, the accumulation of lactate after some dives in marine birds

and mammals indicates the glycogen store also plays an important role in long dives (Kooyman et al., 1983; Kooyman et al., 1980; Ponganis et al., 1997; Shaffer et al., 1997; Williams et al., 1999).

The rate of depletion of muscle O_2 and anaerobic stores will be affected by muscle workload. Muscle workload in freely diving animals is partially a function of stroke rate (Williams et al., 2004). Stroke and glide patterns during a dive will affect muscle metabolic rate and the depletion pattern of the muscle O_2, phosphocreatine and glycogen stores. Thus, an examination of stroke frequency during diving is essential for a more complete understanding of muscle workload, muscle metabolism and depletion of the muscle O_2 and anaerobic energy stores.

Model animal

The emperor penguin provides the ideal model in which to investigate the role of locomotory muscle and muscle metabolism in diving physiology and dive performance. Emperor penguins (*Aptenodytes forsteri*) are the most accomplished of the avian divers, with mean dive durations at sea between 4 and 5 minutes and mean depths to 100 m (Kooyman and Kooyman, 1995). However, these birds are also capable of extreme dives, with maximum depths below 500 m (Kooyman and Kooyman, 1995) and a recent record maximum dive duration of 27.6 min (Sato et al., *in press*). Although extremely long dives (> 12 min) are rare, they comprise about 0.1% of dives (Kooyman and Kooyman, 1995; Wienecke et al., 2007). Some of these extreme dives may be a consequence of the penguin's natural habitat in Antarctica.

While diving in heavy pack ice, where openings in the ice may close at any time, a submerged bird may need to extend its dive in order to find a new breathing hole (Kooyman and Kooyman, 1995). How these birds are able to remain submerged for such extraordinarily long durations is unknown.

Its unique habitat and ability to dive beneath ice also makes the emperor penguin an ideal model animal for diving physiology studies. The development of the isolated dive hole model allows researchers to study physiological phenomena during dives by attaching recorders to penguins and allowing them to dive freely (Kooyman, 1968; Kooyman, 1981; Kooyman et al., 1992). In this model, a sea ice camp (Penguin Ranch) is set up on the fast ice in an area of McMurdo Sound that is free from cracks or holes in the ice for at least 1 km. A dive hole is drilled through the sea ice and a corral is set up around the hole. Penguins are captured and transported to Penguin Ranch where they can dive freely through the isolated hole with physiological instruments attached. However, they must return to the same hole to exit or breathe (Kooyman, 1968; Kooyman, 1981). Using this approach, researchers are able to observe diving behavior of the birds and retrieve instruments with ease.

In addition, significant physiological research on the emperor penguin has been completed in recent years. The aerobic dive limit of emperor penguins has been measured at 5.6 min (Ponganis et al., 1997). Previous research has established that neither O_2 in the air sacs nor O_2 in the blood is depleted at the aerobic dive limit in the emperor penguin (Ponganis et al., 2007; Stockard et al., 2005).

Objectives

This dissertation examines three aspects of the role of locomotory muscles during dives of emperor penguins. First, a new instrument, a near infrared spectrophotometer, was developed in order to measure muscle O_2 depletion in freely diving penguins. The instrument was deployed on emperor penguins diving at an isolated dive hole in Antarctica. Data from these deployments were used to determine muscle O_2 depletion and diving muscle metabolic rate of emperor penguins. Second, the magnitude of the Mb-O_2 store and anaerobic energy stores (phosphocreatine and glycogen) were measured in emperor penguins. Using these data and the diving muscle metabolic rate, the intramuscular depletion of the Mb-O_2, phosphocreatine and glycogen stores and the accumulation of lactate were modeled for different dive durations. Lastly, stroke frequency of emperor penguins diving at sea was analyzed to assess variations in muscle workload and potential implication on the model and metabolic rate.

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CHAPTER 1: What Triggers the Aerobic Dive Limit?

Patterns of Muscle Oxygen Depletion during Dives of Emperor Penguins

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.

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 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.

MATERIAL 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 7.2V battery pack (7.2V 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 artifact from strokes. Reflectance data were stored on an SD 256MB 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 x 7.5 mm, 450 g, SIO Hydraulics Lab & SIO Marine Development Shop) (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 a 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).

Probes were constructed using a small circuit board (19 x 5 x 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 to 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 springs 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 x 1.7 x 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 x 6 x 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 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 mmHg, and are expressed so in the text; pressure axes in graphs are labeled in both mmHg and kPA, using the conversion 0.133 kPA = 1 mmHg. 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 inspiratory 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.

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 mean ± 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, 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 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_IO_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_IO_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 about 4 min at the 20% F_IO_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 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 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, while stroke frequency was high (1-2 Hz), Mb desaturation rate was sometimes fast, as in type A dives, and sometimes moderate (Figs 7B, 8). During the middle phase of the dive, Mb desaturation rate was slow and, sometimes, nearly 0 in the middle of the dive (Figs 7B, 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, 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, Z = -0.259, P = 0.8).

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., 2009; Ponganis et al., 2007; Ponganis et al., 2001; Ponganis et al., 2003; 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 since 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 studies 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 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.

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; but 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 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., 2009; Ponganis et al., 2007). 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 are 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 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 is 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., 1996; Hogan et al., 1992; 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 about one-fifth that of canine gastrocnemius stimulated at 0.25 Hz (see 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 (Figs 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.

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 average 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 duration 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 store 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 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 average value is about 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, 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 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 a 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.

ACKNOWLEDGEMENTS

This work was supported by NSF grants OPP 0229638, 0538594, 0944220. C.L.W. was supported by an NIH Marine Biotechnology Training Program Fellowship

and a UC Regents Fellowship. J.U.M. was supported by a Los Angeles Achievement Rewards for College Scientists fellowship and a Philanthropic Educational Organization Scholar Award. We are grateful for help from T. K. Stockard, K. Ponganis, M. Tulis, C. Champagne, K. Shiomi and B. Tribble for support and assistance in the field, J. Powell for assistance with data processing and filtering, P. Jobsis for support and advice on the development of the NIRS recorder, H. Hanish, T. Wright and M. Loughry of UFI for design and construction of the NIRS recorder, D. Alden of the SIO Hydraulics lab for design of the underwater housing unit and the SIO Machine Shop for construction of underwater housing units, and G.L. Kooyman and B. McDonald for support and manuscript review.

Chapter 1, in full, was published as:

Williams, C. L., Meir, J. U. and Ponganis, P. J. (2011). What Triggers the Aerobic Dive Limit? Patterns of Muscle Oxygen Depletion during Dives of Emperor Penguins *Journal of Experimental Biology* 214: 1802-1812. The dissertation author was the primary investigator and author of this paper.

Table 1-1. 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

Table 1-2. Dive characteristics, Mb desaturation rate and recovery time of type A and type B desaturation profiles

Desaturation pattern	A	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*
75% Recovery time (min)	0.76 ± 0.31	1.16 ± 0.97

*, statistically significant difference between type A and type B

Mb, myoglobin.

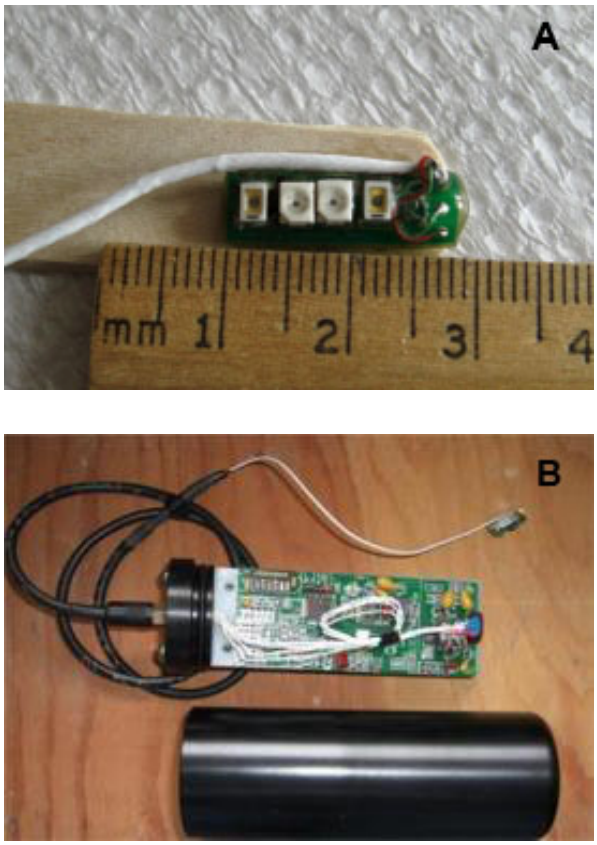

Figure 1-1. (A) Close-up of implantable near-infrared spectrophotometer (NIRS) probe connected to medical cable. (B) The NIRS instrument with microprocessor-based recorder board attached to underwater cable and implantable probe; underwater housing case shown in black.

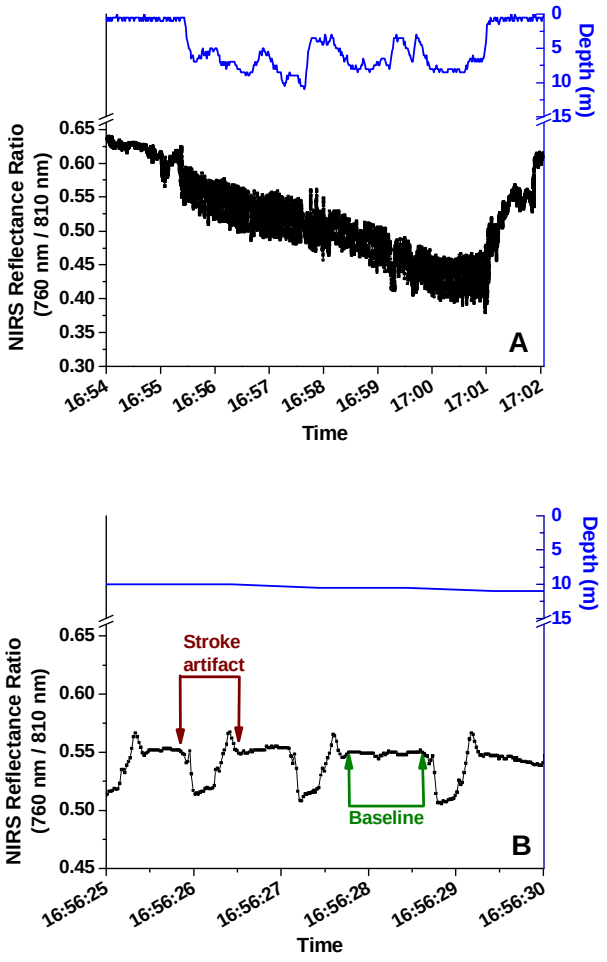

Figure 1-2. (A) Example of data recorded by NIRS instrument and time-depth recorder during a dive. (B) A 5s excerpt from the same dive showing stroke movement artifact and NIRS baseline between strokes. Depth is shown in blue and NIRS reflectance signal is shown in black.

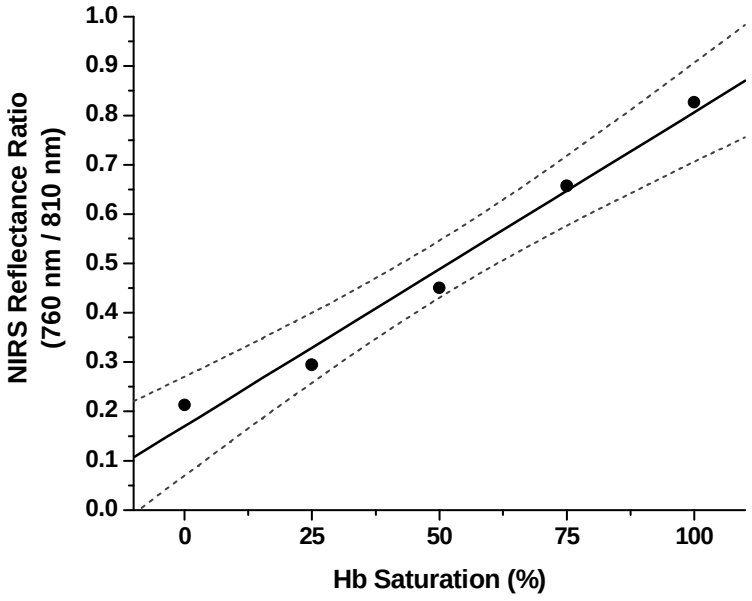

Figure 1-3. Reflectance ratio from the NIRS instrument *vs* hemoglobin (Hb) saturation values. Results confirm a linear relationship ($y = 0.0064x + 0.1702$, $r^2 = 0.98$, $P = 0.0012$; dashed lines reflect 95% confidence interval).

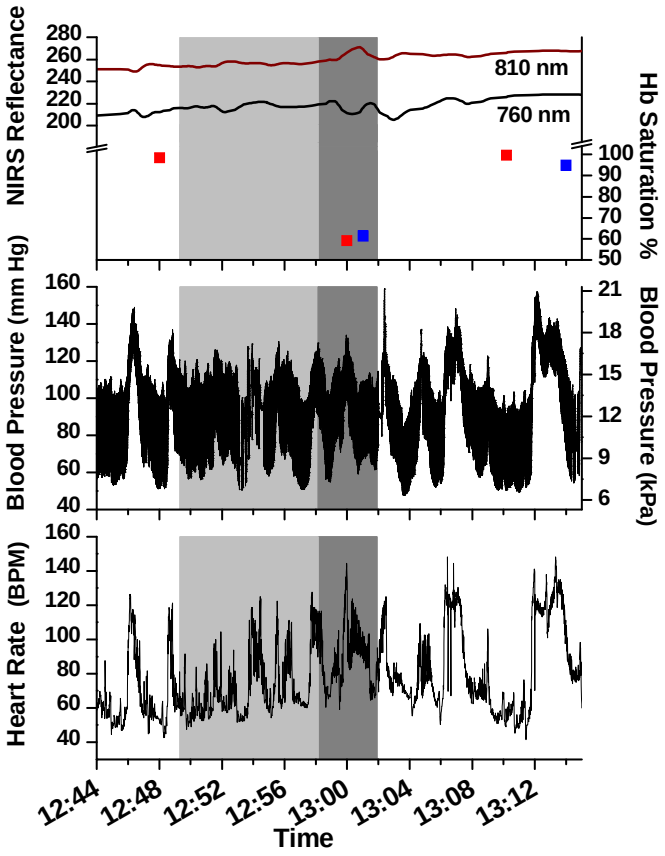

Figure 1-4. Profiles of NIRS reflectance, Hb saturation, blood pressure and heart rate from experiment with a penguin under anesthesia and in which inspiratory oxygen fraction (F_IO_2) was altered. Maroon line, 810 nm reflectance signal; black line, 760 nm reflectance signal; red squares, arterial Hb saturation; blue squares, venous Hb saturation; light gray shaded area, F_IO_2 = 50%,; gray shaded area, F_IO_2 = 20%.

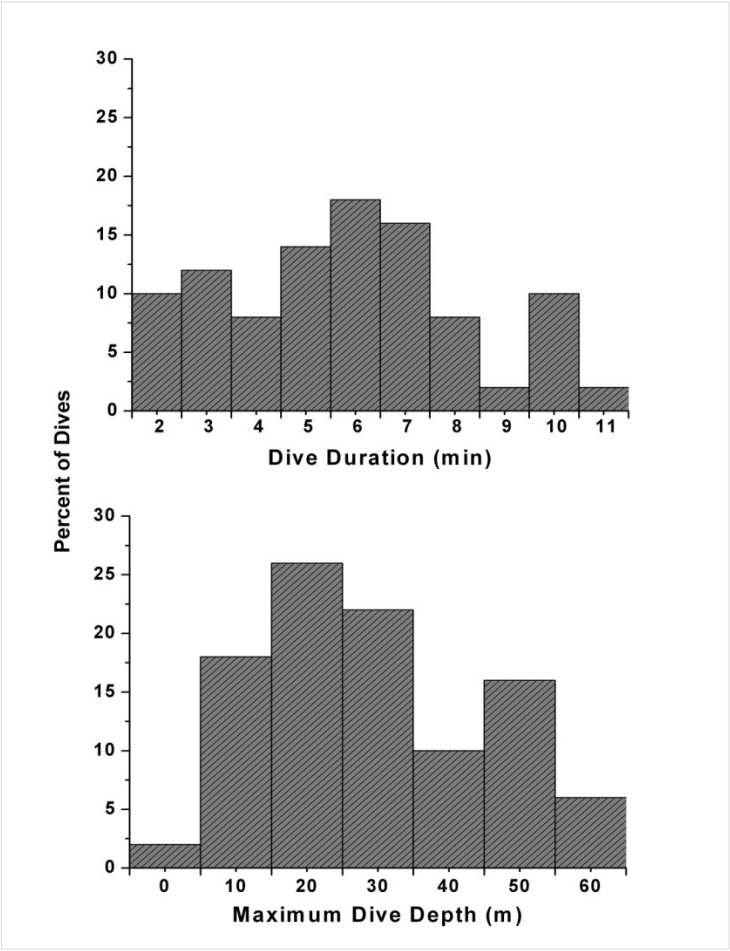

Figure 1-5. Distribution of dive duration (top) and maximum dive depth (bottom) of dives from three emperor penguins outfitted with an NIRS instrument and a time-depth recorder (N=50 dives).

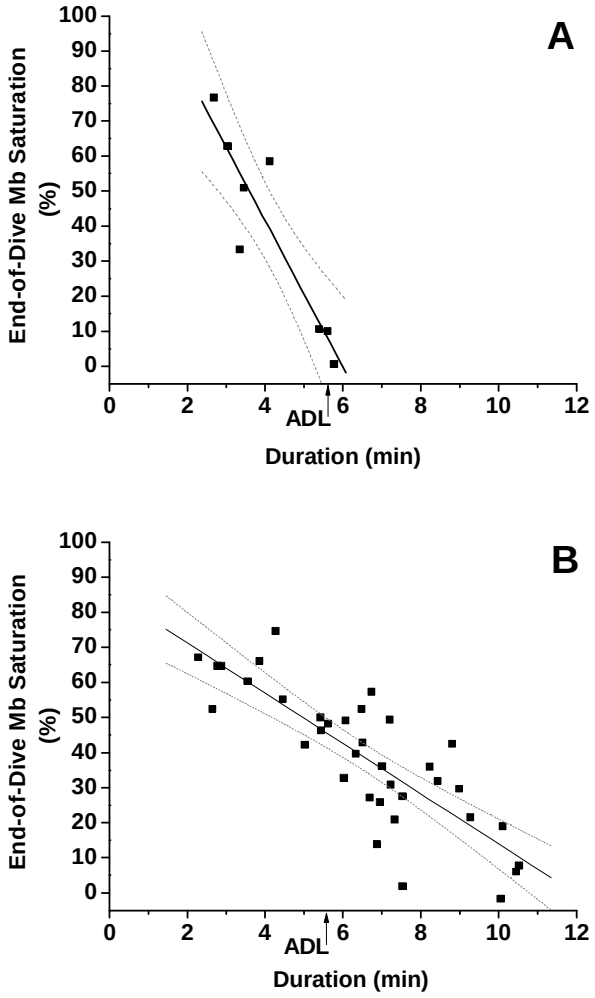

Figure 1-6. Relationship between end-of-dive myoglobin (Mb) saturation values and dive duration from (A) type A and (B) type B dives (linear regression, type A, $y = 125.28 – 20.92x$, $r^2 = 0.74$, $P = 0.0007$; type B, $y = 85.56 – 7.17x$, $r^2 = 0.68$, $P < 0.0001$). Dashed lines reflect 95% confidence intervals. ADL, aerobic dive limit.

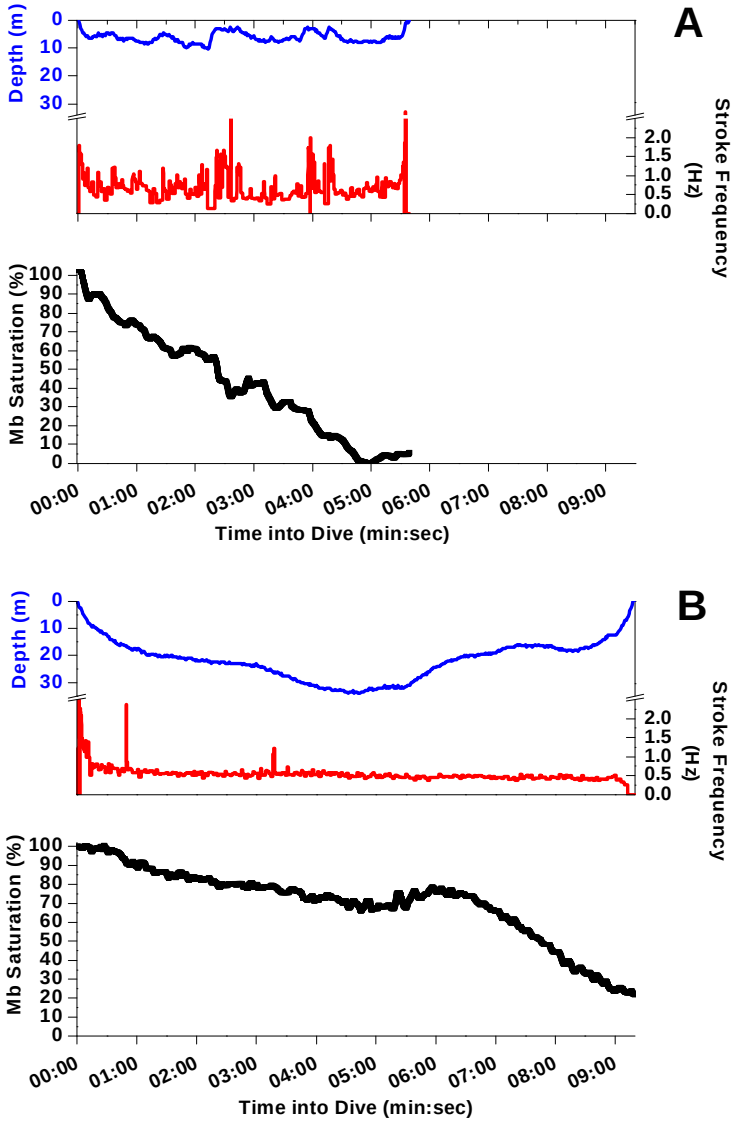

Figure 1-7. Example of Mb desaturation, instantaneous stroke frequency and dive depth profiles for (A) a 5.6 min, type A dive, and (B) a 9.3 min, type B dive.

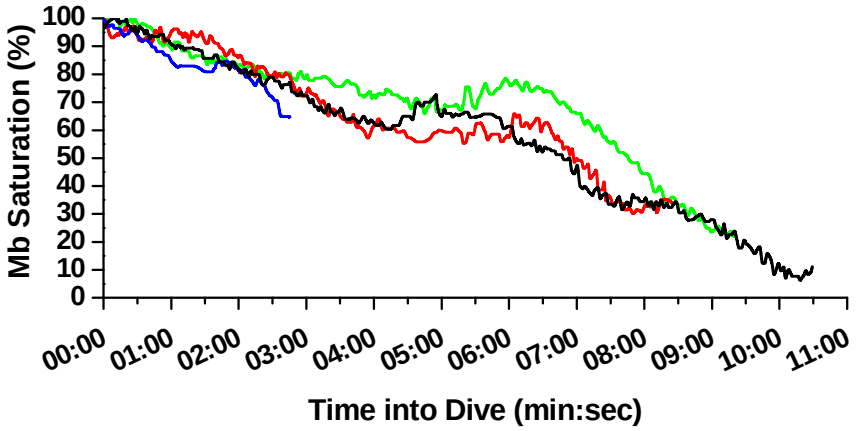

Figure 1-8. Profiles of type B Mb desaturation profiles from four dives (blue, 2.8 min dive; red, 8.4 min dive; green, 9.3 min dive; black, 10.5 min dive).

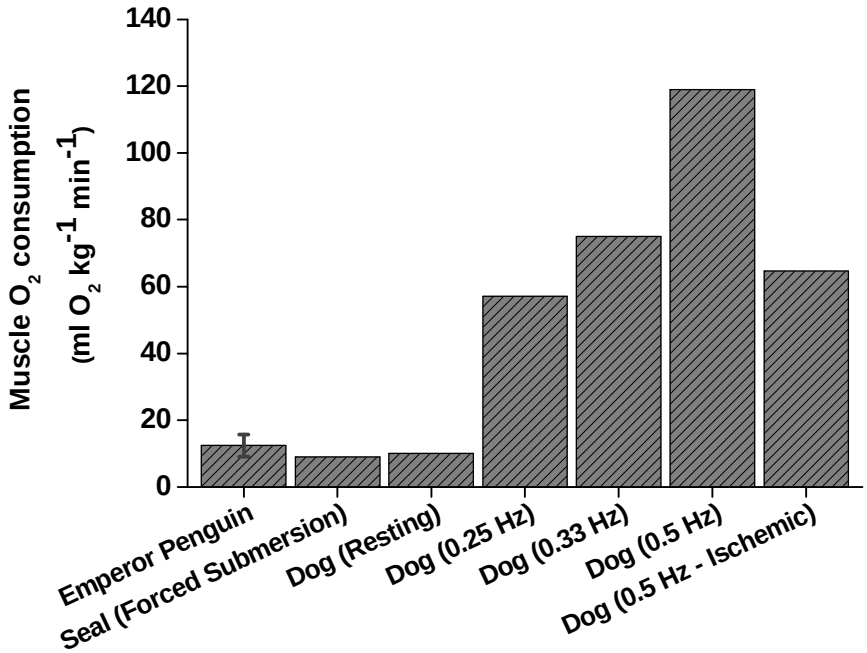

Figure 1-9. Comparison of locomotory muscle O_2 consumption for diving emperor penguins (present study, pectoral muscle), back muscle of forcibly submerged harbor seals (Scholander et al., 1942) and canine gastrocnemius muscle stimulated at different rates (Hogan et al., 1998). Muscle O_2 consumption 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.

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CHAPTER 2: Muscle Energy Stores and Stroke Frequencies of Emperor Penguins: Implications for Muscle Metabolism and Dive Performance

ABSTRACT

In diving animals, bradycardia and peripheral vasoconstriction potentially isolate muscle from the circulation. During complete ischemia, ATP production is dependent on the size of the myoglobin oxygen ($Mb\text{-}O_2$) store and the concentrations of phosphocreatine (PCr) and glycogen (Gly). Therefore, we measured PCr and Gly concentrations in emperor penguin muscle and modeled the depletion of muscle O_2 and those energy stores under conditions of complete ischemia and a previously determined muscle metabolic rate. We also analyzed stroke frequency to assess muscle workload variation during dives and evaluate potential limitations on the model. Measured PCr and Gly concentrations, 20.8 and 54.6 mmol kg^{-1} muscle, respectively, were similar to published values for non-diving animals. The model demonstrated that PCr and Gly provide a large anaerobic energy store, even for dives longer than 20 min. Stroke frequency varied throughout the dive profile indicating muscle workload was not constant during dives as was assumed in the model. The stroke frequency during the first 30 seconds of dives increased with increased dive depth. In extremely long dives, lower overall stroke frequencies were observed. Although O_2 consumption and energy store depletion may vary during dives, the model demonstrated that PCr and Gly, even at concentrations typical of terrestrial birds and mammals, are a significant anaerobic energy store and can contribute significantly to the emperor penguin's ability to perform long dives.

INTRODUCTION

In diving animals, bradycardia and peripheral vasoconstriction may reduce perfusion-dependent metabolic rates of organs, potentially isolate muscle from the circulation, and preserve oxygen for vital organs such as the brain and heart (Blix et al., 1983; Scholander, 1940; Scholander et al., 1942; Zapol et al., 1979). If working muscle is isolated from the circulation during the dive due to extreme vasoconstriction, a continuous supply of ATP must be generated locally as locomotory muscles contract to propel the animal through the water. During aerobic metabolism under these conditions, the oxygen (O_2) necessary to generate ATP is provided by the myoglobin (Mb)-bound O_2 store. High muscle Mb concentration is a hallmark of diving animals and Mb-O_2 stores are large, often the largest of the three O_2 stores (Kooyman and Ponganis, 1998). It has been suggested that the depletion of the Mb-O_2 store is the basis for the aerobic dive limit, (ADL, the dive duration beyond which post-dive blood lactate begins to accumulate), (Kooyman et al., 1983; Williams et al., 2011). In dives beyond the ADL, ATP levels can be maintained anaerobically through phosphocreatine (PCr) hydrolysis and glycolysis. If muscle is completely ischemic for an entire dive, ATP production for muscular work is dependent on the size of the Mb-O_2 store and the concentrations of PCr and glycogen (Gly).

Despite the potentially critical role of anaerobic energy stores in diving animals, few studies have examined the magnitude of these stores. In particular, PCr concentrations have not yet been directly measured in any diving animal. However, based on a study of total creatine (Cr) concentrations, it has been proposed that PCr

concentrations in diving animals are likely similar to values observed in terrestrial species (Blix, 1971). More recently, calibrated nuclear magnetic resonance (NMR) measurements indicated that PCr concentrations in Pekin ducks (*Anas platyrhynchos*) were also comparable to those in terrestrial animals (Stephenson and Jones, 1992). However, even with PCr concentrations assumed to be equivalent to those of terrestrial species, Butler and Jones calculated that, in diving Weddell seals (*Leptonychotes weddelli*), the PCr store would provide up to one-third more ATP than the Mb-O_2 store (Butler and Jones, 1997). Thus, although never directly measured in a diving animal, the PCr store may be a significant anaerobic energy source, even at concentrations found in terrestrial animals.

The accumulation of lactate after some dives in marine birds and mammals indicates the Gly store also plays an important role in dives beyond the ADL (Kooyman et al., 1983; Kooyman et al., 1980; Ponganis et al., 1997b; Shaffer et al., 1997; Williams et al., 1999). In prior studies, skeletal muscle Gly concentrations in diving animals, including fasting penguins, were not elevated compared to terrestrial animals (Groscolas and Rodriguez, 1982; Kerem and Elsner, 1973; Scholander et al., 1942).

The potential contribution of PCr to muscle metabolism in diving animals was first noted by Scholander et al. in their studies of forcibly submerged seals (1942). In those experiments, the Mb-O_2 store was depleted in 5 to 10 min and lactate accumulation (and presumably significant anaerobic glycolysis) began when the Mb-O_2 store reached 15 - 20% saturation. Since those experiments, very few studies have

examined the role of PCr in diving animals. In one such study, only 22% of PCr was consumed during a 6-min forced submersion of a Pekin duck, and both PCr hydrolysis and glycolysis had begun within the first two minutes (Stephenson and Jones, 1992). Although these two studies have provided some important data on rates and timing of Mb desaturation, PCr hydrolysis and glycogen breakdown in forcibly submerged animals (Scholander et al., 1942; Stephenson and Jones, 1992), muscle metabolism in free-diving animals is more complex due to variation both in the degree of muscle ischemia (Williams et al., 2011) and in the work rate of muscle during dives (Williams et al., 2000).

Muscle workload in freely diving animals is partially a function of stroke frequency (Williams et al., 2004). Stroke and glide patterns during a dive will affect muscle metabolic rate and the depletion pattern of the Mb-O_2, PCr and Gly stores. Thus, an examination of stroke frequency during diving is essential for a more complete understanding of muscle workload, muscle metabolism and depletion of the Mb-O_2 and anaerobic energy stores.

The emperor penguin (*Aptenodytes forsteri* Gray 1844) is an accomplished avian diver and makes an ideal model to study anaerobic energy stores and stroke frequency. Emperor penguin dives generally range between 2 and 12 min, with most dives at sea below the measured ADL of 5.6 min (Kooyman and Kooyman 1995; Ponganis et al. 1997b). However, dives longer than 20 min have been observed on rare occasions (Kooyman and Kooyman, 1995; Wienecke et al., 2007). The muscle O_2

depletion rate in diving emperor penguins has been measured at an isolated dive hole in McMurdo Sound, Antarctica (Williams et al. 2011).

The goals of this research were to: (1) quantify the magnitude of the Mb-O_2 and anaerobic energy stores in emperor penguin muscle, (2) model the intramuscular depletion of Mb-O_2, PCr and Gly and the accumulation of lactate during dives of different durations based on the anaerobic energy stores determined in the present study and the previously determined mean diving muscle metabolic rate (Williams et al., 2011) and (3) analyze stroke frequency during dives at sea in order to assess the limitations of the model due to potential variation of muscle workload and metabolic rate throughout a dive. We hypothesized that: (1) PCr and Gly concentrations in the emperor penguin would not be elevated above values in terrestrial species, (2) the estimated intracellular Gly depletion and lactate accumulation during routine dives beyond the ADL would be minimal, consistent with the short surface intervals observed after such dives (Kooyman and Kooyman, 1995; Wienecke et al., 2007) and (3) stroke frequency would vary throughout the dive (Sato et al., 2002; van Dam et al., 2002).

MATERIALS AND METHODS

Mb-O_2 and metabolite concentrations

Experimental approach

Non-breeding emperor penguins (8-15 per season, N = 10 for this experiment) were captured on the sea ice of McMurdo Sound, Antarctica in the austral springs of

2007 and 2008. Penguins were transported to a sea ice camp (Penguin Ranch) with a corral and two isolated dive holes on McMurdo Sound (77° 41', 165° 59'). Penguins dived freely through the isolated dive holes as previously described (Kooyman et al., 1992). They foraged on sub-ice fish, squid and other prey 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.

Muscle biopsy sampling

Muscle biopsies were obtained from ten penguins (mass: 23.6 ± 1.9 kg; range: 21-26 kg) under isoflurane anesthesia (Kooyman et al., 1992) using a Bergstrom muscle biopsy needle (Bergstrom, 1962). All muscle biopsies were taken from the pectoralis-supracoracoideus complex, 3-4 cm lateral to midline at the level of the axilla. Biopsy samples for Mb, PCr, creatine (Cr), Gly, lactate and water content analysis were obtained from a muscle depth of 6 - 8 cm and frozen immediately in liquid nitrogen (N_2). Samples for water content analysis were weighed prior to being frozen. Samples were stored at (-)80° C until analysis, which was within 30 days. To verify the biopsy method did not alter resting muscle condition, lactate values were determined in separate control samples in 4 penguins (Lowry and Passoneau, 1972).

Myoglobin Assay

Frozen muscle samples were thawed, cleaned of fat or connective tissue and weighed. Muscle Mb concentration was determined spectrophotometrically using a modified Reynafarje method (Reynafarje, 1963). After preliminary results showed a high Mb concentration for emperor penguin samples, the dilution factor was changed from 19.25 ml $gram^{-1}$ tissue to 60 ml $gram^{-1}$ tissue. The homogenate was centrifuged at 13,000 g at 0° C for 90 min (Eppendorf Centrifuge 5402 w/ rotor F-45-18-11, Hamburg, Germany). Spectrophotometric measurements were taken at 538 nm and 568 nm on a Shimadzu UV 2501PC spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Equine Mb standard (Sigma-Aldrich, St. Louis, MO, USA) and blanks were run on all assays. All Mb assays (n = 6) were run in duplicate.

Sample preparation for metabolite assays and water content analysis

Muscle tissue samples were lyophilized for 24 hours (bench-top vacuum freeze-dryer, Virtis, Gardiner, NY, USA) and then reweighed. Water content was determined by the difference between the original biopsy weight and the freeze-dried weight. For PCr, Cr and Gly assays, non-muscle material (e.g., blood, connective tissue, feather particles) was removed from samples under a dissecting microscope. Samples were minced and then divided for analysis (2-4 mg for Gly, 4-10 mg for PCr and Cr).

Extraction and neutralization for metabolite assays

For PCr and Cr assays, extraction followed procedures outlined in Lowry and Passoneau (1972). Briefly, 0.05 ml of 2M perchloric acid (PCA) (pre-cooled in ice) per 1 mg tissue sample was added and then the sample mixed at 0° C for 10 min using a vortex mixer. Samples were spun at 15,000 g at 5° C for 10 min and supernatant was removed. The supernatant was cooled in dry ice until a slurry-type consistency was obtained. Then, 0.05 ml of 2M $KHCO_3$ per 1 mg tissue was added to the tube. The sample was spun again at 15,000 g at 5° C for 10 min (Lowry and Passoneau, 1972).

For Gly assays, 0.1 ml of 2N HCl was added per 1 mg tissue sample. The sample was weighed and then incubated at 100° C for 2 hours. Samples were reweighed and deionized H_2O added to obtain the original weight. An equal volume of 2N NaOH was added and the samples spun at 15,000 g for 10 min.

Metabolite assays

PCr, Cr and Gly concentrations were determined using the Lowry and Passoneau (1972) method, with the following modifications. The PCr assay was particularly sensitive to pH. Each individual sample pH was checked after extraction and adjusted to a pH of 8.1 with additional $KHCO_3$ or PCA as necessary. Any resulting changes in volume were accounted for in calculations. To stabilize the creatine kinase enzyme, the enzyme was mixed and stored in a 0.25M glycyl-glycine solution at 5 mg/ml. In addition, 0.1% albumin was added to the reaction buffer to prevent inactivation of creatine kinase due to dilution. Standards and blanks were run

on all assays. All assays were run in duplicate. PCr and Cr were assayed in 7 penguins and Gly in 5 penguins. Sample sizes for PCr and Cr (N = 7), Gly (N = 5) and Mb (N = 6) differed due to size of muscle samples and available extraction volumes for metabolite assays.

Results for lactate, PCr, Cr and Gly assays are reported in mmol kg^{-1} muscle wet weight (w.w.), using the conversion factor obtained from the water content analysis. Myoglobin concentration is reported as g 100 g^{-1}.

Metabolite assay validation

In order to validate sample preparation, extraction and assay methods, all metabolite assays were run on muscle samples from Holtzman rats (*Rattus norvegicus*) supplied by another lab. Muscle biopsies were obtained from the biceps femoris or gluteus maximus muscle after injection of a lethal dose of pentobarbital or, for PCr samples, while under isoflurane anesthesia.

Modeling Mb-O_2 store and anaerobic energy store depletion during dives

Using the PCr and Gly concentrations determined in this study, the modeling of depletion of the Mb-O_2, PCr and Gly stores during dives was based on the following assumptions: (1) no muscle blood flow occurred during the dives (Meir et al., 2008; Ponganis et al., 2009; Ponganis et al., 2007; Williams et al., 2011); (2) muscle metabolic rate was constant after the first minute at a rate of 12.4 ml O_2 kg^{-1} muscle min^{-1} (Williams et al., 2011); (3) in addition to a muscle metabolic rate of 12.4

ml O_2 kg^{-1} muscle min^{-1} during the first min, PCr declined by 30% during the first minute based on the work-rest transition phase requirements demonstrated in exercise physiology studies (Barstow et al., 1994; Haseler et al., 2004; Marsh et al., 1993; McCann et al., 1995); (4) Mb-O_2 depletion continued to provide the required ATP until 6 min into a dive; during that time, aerobic metabolism of glucose from the Gly store provided 33% of the metabolic substrate; it is assumed that intracellular fat provided the remainder (Hochachka and Somero, 1984); (5) once Mb-O_2 declined to 15-20% saturation, PCr and Gly depletion, at a 1:2 ratio (Kemp et al., 2001), provided 80% of ATP requirements. This assumption was based on a previously shown rise in lactate prior to complete Mb-O_2 depletion (Scholander et al., 1942); (6) after Mb-O_2 was fully desaturated, PCr hydrolysis and glycolysis provided 100% of ATP requirements, with Gly accounting for twice the metabolic fuel compared to PCr (Kemp et al., 2001); (7) it was assumed that PCr would not decline below 15% of resting value (Harris et al., 1986) and, thereafter, Gly contributed 100% of ATP requirements; and, (8) lactate levels in the muscle were assumed to be 2.3 mmol kg^{-1} muscle prior to dives, based on results from control values obtained in this study; any accumulation of lactate was assumed to be directly related to anaerobic glycolysis.

Mb-O_2 and metabolites were converted to ATP equivalents in order to calculate depletion of those stores under the model. ATP equivalents, in mmol kg^{-1} muscle, were calculated to be: (1) 0.268 ATP per1 ml O_2 kg^{-1} muscle; (2) 37 ATP per 1 mmol Gly kg^{-1} muscle (glucosyl unit) used as a substrate during aerobic respiration; (3) 1 ATP per 1 mmol PCr kg^{-1} muscle; and (4) 3 ATP per 1 mmol Gly kg^{-1} muscle

(glucosyl unit) used during anaerobic respiration (Hochachka and Somero, 1984). The ATP equivalent for the muscle metabolic rate, 12.4 ml O_2 kg^{-1} muscle min^{-1}, was 3.32 ATP kg^{-1} muscle min^{-1}.

Using the above assumptions, depletion of the Mb-O_2, PCr and Gly stores were calculated for 2, 4, 6, 8, 10, 12 and 23.1 min dives. Dives up to 12 min represent regular dive durations of emperor penguins and the 23.1 min dive represents the longest dive observed at the isolated dive hole (Ponganis et al., 2007).

Using these assumptions, depletion of the Mb-O_2, PCr and Gly stores were calculated for 2, 4, 6, 8, 10, 12 and 23.1 min dives.

Stroke frequency during dives at sea

Penguin capture and instrument attachment

Emperor penguins were captured as they departed on foraging trips from the Cape Washington colony in Antarctica (74°39′ S, 165°24′ E), in the austral spring of 2005. Multi-sensor data loggers (W1000-PD2GT, Little Leonardo Ltd, Tokyo) were attached to seven penguins (mass: 24.6 ± 2.6 kg; range: 21.5 – 29.0 kg) with waterproof tape (Tesa tape 4651, Tesa) and cable ties. The W1000-PD2GT loggers were 22 mm in diameter, 122 mm in length, with a mass of 73 g in air. They recorded depth and temperature at a sampling rate of 1 Hz, and two-dimensional acceleration at a sampling rate of 16 Hz. VHF transmitters (Model MM130, ATS, Isanti, MN, USA) were also attached using cable ties in order to relocate penguins after returning from

foraging trips. Returning instrumented penguins were recaptured and instruments removed.

Data processing

Dive data were processed, graphed and statistically analyzed using IGOR Pro (WaveMetrics, Inc., Lake Oswego, OR, USA), Origin (version 7.5, OriginLab Corp., Northampton, MA, USA), SPSS (version 11.5, SPSS, Inc. Chicago, IL, USA) and Microsoft Excel (Microsoft, Redmond, WA, USA). Several custom-developed macros were used in IGOR Pro to analyze dive and stroke frequency data. Dives were defined as submergences two m or deeper and two min or longer in duration. Dive durations of two min or longer were necessary in order to examine stroke frequency profiles throughout dives and model usage of Mb-O_2 and anaerobic energy stores during dives. Dives were divided into 7 categories of dive depth (0-25, 25-50, 50-100, 100-200, 200-300, 300-400 and >400m). Post-dive surface intervals were calculated from the end of the previous dive to the beginning of the next dive and, as a result, may include short (< 2 min) and shallow submergences (<2 m). Post-dive surface intervals greater than 60 min (N = 42) were excluded from the analysis as it appeared the penguin may have emerged from the water during these long surface intervals (range: 67 – 653 min).

Strokes were marked and counted in IGOR Pro using a custom-made peak detection script and then visually confirmed. Dive stroke frequency for each dive was calculated from the total number of strokes for each dive divided by dive duration.

Instantaneous stroke frequency for 30-sec periods was determined using a custom-made macro and reported as a mean of all beat-to-beat intervals within a 30-sec period. The depth and time of the final glide during ascent to the surface were identified by marking the last stroke during each dive. Start-of-glide depth was expressed as a percentage calculated by dividing the depth of the last stroke by the maximum dive depth. The percentage of time gliding was determined by dividing the time between the last stroke and end of dive by the dive duration.

Statistics

Values are expressed as mean ± s.d., unless otherwise noted. Simple linear regression was used to describe the relationship for each penguin between (1) stroke frequency and maximum dive depth; (2) stroke frequency and dive duration; and, (3) ascent gliding time and maximum dive depth. To examine stroke frequency within dives of different depths, the mean instantaneous stroke frequency for all dives in each dive depth category at 30-sec intervals was calculated. A power regression analysis was performed to assess the relationship between stroke frequency during the first 30-sec period and maximum dive depth. Statistical significance was set at $P < 0.05$.

RESULTS

Mb-O_2 and Energy Store Concentrations

Water content

Water content of emperor penguin muscle tissue was 68.9 ± 1.5%, N = 9, which agrees with previously published data on emperor penguin pectoralis muscle (68.7%) (Groscolas & Rodriguez, 1982). Therefore, in converting dry weight results of metabolite assays to wet weight, a conversion factor of 3.2 was used.

Lactate and myoglobin

Emperor penguin muscle lactate values from control samples were 2.3 ± 0.6 mmol kg^{-1} muscle (N = 4), which are in the same range (2 - 4 mmol kg^{-1} muscle) as resting muscle lactate values in humans, seals and dogs (Eklof et al., 1981; Harris et al., 1986; Scholander et al., 1942; Tesch, 1980). A resting muscle lactate concentration of 2.3 mmol kg^{-1} muscle was used in the model. Myoglobin concentration was 6.5 ± 0.2 g $100g^{-1}$ (N = 6).

Metabolites

Values for concentration of PCr, Cr and Gly in emperor penguins are reported in Table 1. PCr and Gly control values in rats (13.7 ± 1.8 mmol kg^{-1} muscle w.w. and 33.7 ± 0.8 mmol kg^{-1} muscle w.w., respectively, N = 3) were comparable to published values in rat muscle (Brault et al., 2003; Saltin and Gollnick, 1983) (Table 2).

Fuel store depletion during dives

End of dive values obtained from modeling Mb-O_2, PCr and Gly depletion in dives of 0, 2, 4, 6, 8, 10, 12 and 23.1 min durations are reported in Table 3. A theoretical time-course of depletion of the Mb-O_2, PCr and Gly stores and accumulation of lactate during a 23.1 min dive is shown in Fig. 1.

Stroke frequency during dives at sea

General dive data

Stroke frequency and dive data were obtained from seven birds for a total of 3250 dives > 2 min duration (Table 4). Dive durations from all dives ranged from 2.0 to 27.6 min (Fig. 2). Twenty-seven percent of dives were longer than the 5.6 min ADL, and 1% of dives were greater than 10 min. The grand mean dive duration was 4.7 ± 0.5 min. Maximum dive depth for all dives ranged from 7.8 to 513.5 m (Fig. 2), with a grand mean dive depth of 102.9 ± 28.6 m. Forty percent of dives had a maximum depth of 100 m or deeper, and 9% of dives were deeper than 200 m (Fig. 2). Grand mean stroke frequency was 0.67 ± 0.05 Hz, with a range of 0.32 to 1.04 Hz for all dives. Mean post-dive surface intervals ranged from 1.7 to 3.3 min, with a grand mean of 2.6 ± 0.6 min (Table 4).

Stroke frequency, dive depth and dive duration

In six out of seven penguins, there was an inverse relationship between dive stroke frequency and dive duration, which, although significant, was not strong (Fig.

3a) (r^2 = 0.04 to 0.25, $P < 0.001$). Dive stroke frequency was very weakly related to dive depth in four of seven penguins (Fig. 3b) (r^2 = 0.01 to 0.06, $P \leq 0.02$) and not consistent in those penguins. The relationship was inverse in two and positive in the other two birds.

Stroke frequency within dives

Stroke frequency for the first 30-sec of dives increased with increasing maximum dive depth (Fig. 4), up to 200 m where stroke frequency leveled off and remained high, near 1.7 Hz (Fig. 4). A power regression relationship between initial stroke frequency and dive depth was true for all seven penguins (r^2 = 0.59 to 0.76, $P < 0.0001$). Mean stroke frequency at 30-sec intervals of dives representing seven depth categories are shown in Fig. 5. Instantaneous stroke frequency profiles in three dives of varying depth and in the 27.6 min dive, the longest reported dive for an emperor penguin (Sato et al., *in press*) are shown in Fig. 6.

Mean depth of the last stroke before surfacing occurred between 12 and 28 m, or at 21 to 30% of maximum dive depth for all penguins. Grand mean last stroke depth occurred at 22 ± 6 m and at 24.5 ± 2.9% of maximum depth. Mean gliding time during the ascent ranged from 12 to 23 seconds, with a mean of 19 ± 4 seconds for all penguins. This accounted for an average of 5.0 to 8.2% of dive duration, with a grand mean of 6.7 ± 1.0% of dive duration. Gliding time was significantly and positively related to maximum dive depth for each penguin (r^2 = 0.33 to 0.52, $P < 0.0001$) (Fig. 7).

DISCUSSION

Mb-O_2 and energy store concentrations

Myoglobin

Elevated Mb concentrations in locomotory muscle is one of the most striking adaptations of diving birds and mammals, with Mb concentrations in diving animals often tenfold higher than their terrestrial counterparts (Burns et al., 2010). High Mb concentrations provide a significant O_2 store for diving. Previous studies demonstrated an extremely high Mb concentration in emperor penguin locomotory muscle, which was confirmed in the present study (Ponganis et al., 1997a). With an Mb concentration of 6.5 g $100g^{-1}$ and an O_2-binding capacity of 1.34 ml O_2 g^{-1} Mb, the Mb-O_2 store for the model was calculated to be 86 ml O_2 kg^{-1} muscle.

Phosphocreatine and creatine

Although muscle O_2 stores have been measured in many diving species, few studies have examined concentrations of the anaerobic muscle energy sources, particularly PCr concentrations. We report the first PCr concentration directly measured for a freely diving animal (Table 1); it is comparable to most terrestrial animals and ducks (Table 2). In examining total Cr in seals and terrestrial animals, it was initially proposed that an elevated PCr concentration in seals might be beneficial for long dives because it would provide not only additional fuel for extending dives, but would also reduce acidification of muscle resulting from anaerobic metabolism (Blix, 1971). However, as Blix found for total Cr in seals and eiders, emperor

penguins do not have elevated PCr or Cr concentrations in locomotory muscle (Table 2).

It has been reported that PCr concentrations determined from muscle biopsy samples may underestimate the true PCr concentration. When determined in vivo by ^{31}P mass spectrometry, PCr concentrations are greater than those determined from muscle biopsies (Brault et al., 2003). This is likely due to breakdown of PCr to Cr during the biopsy procedure despite rapid freezing of the sample (Brault et al., 2003). Analysis of biopsy samples taken after inhibition of creatine kinase with iodoacetamide resulted in a 6 mmol kg^{-1} (20 – 30%) increase in measured PCr concentrations in resting muscle (Brault et al., 2003). Correcting the assayed PCr concentration in emperor penguin muscle by this amount would result in a concentration of 27 mmol kg^{-1} muscle We believe this value, which is less than total Cr concentration (33 mmol kg^{-1} muscle), is an appropriate value since not all Cr is available to form PCr (Brault et al., 2003). Accordingly, a PCr concentration of 27 mmol kg^{-1} muscle was used in the modeling experiment.

Glycogen

Muscle Gly concentrations vary significantly within and between species. In general, skeletal muscle Gly concentrations in diving animals (31 - 80 mmol kg^{-1} muscle) are highly variable and similar to concentrations in terrestrial animals (26 - 71 mmol kg^{-1} muscle) (Edwards et al., 1999; Goforth, 1986; Groscolas and Rodriguez, 1982; Kerem et al., 1973; Saltin and Gollnick, 1983). Horses, both standardbred and

thoroughbred horses, have appreciably higher Gly concentrations (95-138 mmol kg^{-1} muscle) than most animals (Lindholm and Piehl, 1974; Snow et al., 1985). Gly concentrations determined in this study are not notably different from fasting emperor penguins, other birds or marine mammals (Table 2). This Gly concentration (54.6 mmol kg^{-1} muscle) was used as the initial value for the Gly store in the model.

Mb-O_2, PCr and Gly depletion during dives

Large anaerobic energy stores

The model results illustrate a potential depletion profile of anaerobic energy stores and highlight the role these stores play in emperor penguin diving (Fig. 1, Table 3). Although PCr and Gly concentrations are not elevated above values in terrestrial species, these stores provide a large anaerobic energy reservoir in muscle of emperor penguins during dives (Tables 2, 3). As illustrated in Fig. 1, under the model, the PCr and Gly stores produce almost all of the required ATP after the first 6 min of long dives. At the end of 8-min dives, when the Mb-O_2 store is substantially depleted, PCr concentration is still above 50% of its pre-dive level and the Gly store is less than 2% depleted (Table 3). Even after dives twice as long as the 5.6 min ADL, 44% of the PCr store remains and the Gly store is over 90% of its pre-dive level (Table 3). However, despite the availability of these two anaerobic energy sources in longer dives, most emperor penguin dives at sea are less than the ADL (Table 4) (Kooyman and Kooyman, 1995; Wienecke et al., 2007).

Extreme dives of emperor penguins

Penguins do, on occasion, dive for extraordinarily long durations and, during these dives, the large anaerobic stores become essential (Table 3). In more extreme dives, such as the 23.1 min dive recorded at the isolated dive hole, (Ponganis et al., 2007), both the Mb-O_2 and PCr stores would be nearly depleted under the model, but the Gly store would remain more than 70% of pre-dive level (Table 3). The depletion of muscle Mb-O_2 and anaerobic energy stores, as well as lactate accumulation, for a hypothetical 23.1 min dive is illustrated in Fig. 1. At the end of this dive, muscle lactate concentration would be 30.6 mmol kg^{-1} muscle. Although quite high, this value is much less than the muscle lactate concentration observed in a seal forcibly submerged for 15 min, 42 mmol kg^{-1} muscle (Scholander et al., 1942) and in a thoroughbred horse at a full gallop, 50 mmol kg^{-1} muscle (Snow et al., 1985).

A dive this long is clearly a rare event. In over 150,000 dives at sea, including those discussed below, less than 0.1% of dives were longer than 12 min in duration and only three were longer than 20 min (Kooyman and Kooyman, 1995; Wienecke et al., 2007). Some of these extreme dives may be a consequence of diving in heavy pack ice, where openings in the ice may close at any time, requiring a submerged bird to extend its dive to find a new breathing hole (Kooyman and Kooyman, 1995). Thus, the ability to endure long duration dives may be critical for the habitat in which emperor penguins dive; and, from the model, it is apparent that these extraordinarily long dives are well-within the anaerobic capacities of emperor penguin muscle.

Recovery rates and surface intervals

After the 23.1 min dive, as well as after the now longest dive recorded for an emperor penguin (27.6 min), no diving occurred for at least several hours (Ponganis et al., 2009; Sato et al., *in press*). However, in other extreme dives, surface intervals were less than an hour (Wienecke et al., 2007). Most post-dive surface intervals only last a few minutes (Table 4) (Kooyman and Kooyman, 1995). In emperor penguins, the Mb-O_2 store resaturates within several minutes after dives (Williams et al., 2011). PCr recovery is similarly rapid in exercise studies, where PCr concentrations recovered from 25 to 70% depletion to near resting levels within one to two min (Blei et al., 1993; Forbes et al., 2009; McCully et al., 1994). Thus, Mb-O_2 and PCr recovery should occur within most post-dive surface intervals. Although it is not known how washout of muscle lactate translates to blood lactate concentrations, the decline in blood lactate concentration is also fairly rapid in emperor penguins, near 0.6 mM min^{-1} (Ponganis et al., 1997b).

However, Gly resynthesis can be much slower and resynthesis times vary widely between and within species. While recovery from Gly depletion in penguins has not been studied, Gly recovery in humans is biphasic and dependent several factors, including post-exercise food consumption and Gly depletion level (Jentjens and Jeukendrup, 2003). After strenuous exercise, Gly resynthesis in humans varied between 2 and 8 mmol kg^{-1} muscle hr^{-1} depending on the type and timing of food consumption (Blom et al., 1987; Ivy et al., 1988). Gly recovery in horses can be two to three times longer than in humans (Waller and Lindinger, 2010). In contrast, full

recovery from 50% Gly depletion in rats after moderate exercise occurred within 30 min (Garetto et al., 1984). Since 99% of emperor penguin dives at sea are less than 12 min, Gly depletion is minor after most dives (Table 3). However, repeated Gly-depleting dives may be significant and, depending on recovery rates in penguins, extended surface intervals may be required to replenish the Gly store.

Model provides a reasonable framework for depletion of the Mb-O_2 and anaerobic energy stores

The assumptions in the model are based on diving or exercise physiology studies and the model results are in accord with findings from past diving physiology studies of emperor penguins. For example, the rate of Mb-O_2 depletion, based on a recent study (Williams et al., 2011), and rates of anaerobic energy stores depletion used in the model (Table 3) are consistent with onset of post-dive blood lactate accumulation only in dives beyond the ADL (Ponganis et al., 1997b). The concurrence of PCr and Gly depletion, after the Mb-O_2 store is substantially depleted, has been demonstrated in exercise studies (Kemp et al., 2001), and, in forcibly submerged ducks, where both glycolysis and PCr hydrolysis began within the first two min of the submergence (Stephenson and Jones, 1992). If the entire PCr store was consumed before glycolysis started, lactate accumulation would not begin until dives 11 min or longer, which is inconsistent with the ADL concept, or the rise in blood lactate concentration observed in dives longer than 5-6 min (Ponganis et al., 1997b). Further, although depletion of PCr before glycolysis began would slow the rate of pH decline

since PCr hydrolysis consumes H^+ as part of its reaction, this is likely not necessary due to the high buffering capacity of diving animals (Castellini and Somero, 1981; Mill and Baldwin, 1983).

The model provides a general framework for analyzing the depletion of Mb-O_2, PCr and Gly stores, but necessarily does not account for all variation within dives. For example, the model assumes no muscle blood flow during dives. However, muscle blood flow does appear to occur in the middle of some dives (Williams et al., 2011). If locomotory muscle is perfused during a dive, depletion of the three stores (Mb-O_2, PCr and Gly) and lactate accumulation would be reduced depending on the amount of muscle blood flow. Similarly, the model uses a muscle metabolic rate based on a constant, relatively high stroke frequency (Williams et al., 2011). As discussed below, stroke frequency may vary between and within dives.

In conclusion, from this model, most dives at sea are aerobic in nature and do not significantly deplete anaerobic energy stores. When penguins do dive longer they have substantial anaerobic energy stores for extremely long dives, such as the 27.6 min dive discussed below.

Dive and Stroke frequency patterns at sea

Dive behavior

Dives in this study were typical of dives in earlier studies of penguins at sea (Kooyman and Kooyman, 1995; Wienecke et al., 2007). Despite the exclusion of dives less than 2 min in duration, grand mean dive duration was 4.7 min (Table 4), well

below the ADL of 5.6 min. These results reinforce the conclusion that while at sea, most dives are less than the ADL. However, included in this analysis is a 27.6 min dive, the longest reported dive for an emperor penguin (Sato et al., *in press*).

Stroke frequency and muscle workload

Although a significant relationship between dive stroke frequency and dive duration was present in six penguins; dive duration was only weakly predictive of dive stroke frequency (Fig. 3a). However, in examining dives greater than 12 min, stroke frequencies were less than 0.5 Hz (Fig 3a), well below the grand mean of 0.67 Hz. The relationship between dive depth and dive stroke frequency, although statistically significant in three penguins, was not biologically significant (Fig. 3b). These results suggest that, rather than examining stroke frequency of individual dives, stroke frequency changes within dives may be more indicative of variations in muscle workload during diving. In addition to stroke frequency, stroke amplitude and stroke thrust are variables that also probably contribute to muscle workload. However, these have not been measured in any freely diving penguins.

Within dives, stroke frequency was highest at the beginning of dives (Figs. 4, 5), as penguins worked to overcome their positive buoyancy (van Dam et al., 2002). This high initial stroke frequency increased with deeper maximum dive depths (Fig. 4). For example, in dives less than 25 m, nearly all dives had an initial stroke frequency less than or equal to 1.4 Hz. In contrast, in dives > 200 m, there was only one dive with an initial stroke frequency of less than or equal to 1.4 Hz (Fig. 4). These

results indicate that, not only is muscle workload highest at the beginning of dives, but also that it increases as maximum dive depth increases. In addition, it suggests that penguins anticipate the depth of dives at the very beginning of the dive, stroking significantly faster for dives that will go to deeper depths up to approximately 300 m. These increased stroke frequencies may be secondary to large diving air volumes and greater buoyancy at the start of deeper dives (Sato et al., 2002; Sato et al., *in press*).

Stroke frequency also increased in the middle of deep dives. In dives deeper than 400 m, stroke frequency was above 1.0 Hz at 4 to 5 min into dives (Fig 5). Figure 6D shows a characteristic example of these deep dives with increased instantaneous stroke frequency when the bird reached near maximum depth. Depth profiles revealed small variations at the bottom of these dives coincident with higher instantaneous stroke frequency, suggesting foraging on a mid-water fish, the Antarctic silverfish *Pleuragramma antarcticum* (Kooyman and Kooyman, 1995). Similar increases in stroke frequency also occurred in the middle of 300 to 400 m dives, although the increases were not as great (Fig. 5). These high stroke frequencies did not usually occur in the middle of shallower dives (Figs 5, 6).

Finally, stroke frequency, and thus muscle workload, declined to low levels at the end of most dives (Fig. 5). In all depth categories, stroke frequency at the end of dives was near or less than 0.4 Hz (Fig. 5). Unlike penguins diving at an isolated dive hole (van Dam et al., 2002), penguins at sea glide during ascents (Fig. 7) (Sato et al., 2002). In emperor penguins diving at sea, gliding time increased with maximum depth (Fig. 7). Gliding during the ascent began at about 25% of maximum dive depth and

accounted for 5 to 8% of the dive duration, a cost-saving strategy during ascents (Williams et al., 2000).

The longest recorded dive of an emperor penguin, 27.6 min, provides an informative example of the variation in stroke frequency throughout a dive (Fig. 6C). Instantaneous stroke frequency started out near 2 Hz, decreased rapidly until the penguin approached 100 m, when a secondary increase in stroke frequency to above 1 Hz occurred but then quickly declined to less than 0.5 Hz. After the penguin ascended to about 60 m, instantaneous stroke frequency remained near or less than 0.3 Hz throughout the remainder of the dive, approximately 20 min. The overall stroke frequency for this dive was 0.32 Hz, less than half the mean stroke frequency of dives from which the muscle metabolic rate was measured (0.74 Hz) (Williams et al., 2011).

Stroke frequency variation: implications for the model

The modeling of depletion of Mb-O_2 and anaerobic energy stores provides only a basic framework to examine what occurs in locomotory muscle during dives. After the first minute of diving, the model assumed a constant muscle metabolic rate. However, stroke frequency profiles demonstrated that for all dive depths, stroke frequency, and thus muscle workload, is not constant throughout a dive. Initial stroke frequency increased with increased maximum dive depth, and stroke frequency often changed in the middle of dives (Figs 3, 4). In addition, stroke frequency decreased at the end of almost all dives (Fig. 5). During these changes in stroke frequency, muscle workload would also change. Thus, while the model provides a general framework of

muscle metabolism during dives, muscle metabolic rate is likely not constant during diving and, as a result, consumption of the Mb-O_2 store and depletion of the PCr and Gly stores will vary from the general model depending on, among other factors, stroke frequency within and among dives.

CONCLUSION

The results of this study indicate that the measured PCr and Gly concentrations are typical of non-diving animals, and yet they provide significant anaerobic energy stores. Our simple model of muscle metabolism during dives demonstrates that most dives at sea are aerobic in nature and do not significantly deplete anaerobic energy stores. Even after 12-min dives, the PCr and Gly stores are only 66% and less than 20% depleted, respectively. Serial long dives and extremely long dives may require extended surface intervals for complete Gly resynthesis. Our analysis of stroke frequency demonstrates the limitations of this model in that muscle metabolic rate is likely not constant during dives at sea. Stroke frequency varies both within dives and among dive types.

ACKNOWLEDGEMENTS

This work was supported by NSF grants OPP 0229638, 0538594, 0944220 and a grant from the Japanese Society for Promotion of Science (A19255001). C. Williams was supported by an NIH Marine Biotechnology Training Program Fellowship and a UC Regents Fellowship. C. Williams was also supported by the Scripps Institution of

Oceanography Graduate Department for travel to Japan for data analysis and manuscript preparation. We are grateful for help in the field at Cape Washington from G. Kooyman and G. Marshall, for help in the laboratory at McMurdo Station and field support at Penguin Ranch from C. Champagne and K. Ponganis, J. Meir, M. Tulis, and B. Tribble. We thank the staff of McMurdo Station, especially the Crary Lab and Fixed Wing Operations, for their support. We also thank V. Vacquier for assistance in assay method development, T. Yaksh for supplying rat muscle tissue, M. Woolery and the Moore lab for assistance with and use of laboratory equipment.

Chapter 2, in full, is currently being prepared for publication as: Williams, C. L., Sato, K., Shiomi, K., and Ponganis, P.J. In preparation for *Physiological and Biochemical Zoology*. The dissertation author was the primary investigator and author of this paper.

Table 2-1. Phosphocreatine, creatine and glycogen concentrations (w.w.) of the pectoralis-supracoracoideus muscle complex of emperor penguins (Mean ± s.d.).

Metabolite	**N**	**Concentration (mmol kg^{-1} muscle)**
Phosphocreatine	7	20.8 ± 1.3
Creatine	7	12.0 ± 2.6
Glycogen	5	54.6 ± 8.1

Table 2-2. Phosphocreatine (PCr), creatine (Cr) and total Cr concentrations in muscles of mammals and birds.

Species	PCr (mmol kg^{-1} muscle)	Cr (mmol kg^{-1} muscle)	Total Cr (mmol kg^{-1} muscle)	Glycogen (mmol kg^{-1} muscle)
Emperor Penguin[1]	21	12	33	55
Pekin duck[2,3]	25-32			
Starling[4]	16	13	29	
Pigeon[4]	14	18	32	
Eider[5]			35	
Rat[1,4,6]	14 -23	9	23-31	34
Cheetah[7]	26			40
Horses[8,9]	14 - 19			95-138
Dogs[10]	18 - 24			33-105
Seal[5,11]			31	33-61
Fasting Emperor Penguin[12]				30-60
Chicken[13]				62
Marine mammals[14]				31-80

[1]This study; [2]Stephenson & Jones, 1992; [3]Stephenson et al., 1997; [4]Beis & Newholme, 1975; [5]Blix, 1971; [6]Brault et al, 2003; [7]Williams et al., 1997; [8]Lindholm & Piehl, 1974; [9]Snow et al., 1985; [10]Harris et al., 1986; [11]Kerem et al., 1973; [12]Groscolas & Rodriguez; [13]Edwards et al., 1999; [14]Goforth, 1986.

Table 2-3. End-of-dive myoglobin (Mb) saturation, phosphocreatine (PCr), glycogen (Gly) and lactate concentrations (w.w.) for different dive durations under model assumptions.

Dive Duration (min)	Mb Saturation (%)	PCr (mmol kg^{-1} muscle)	Gly (mmol kg^{-1} muscle)	Lactate mmol kg^{-1} muscle)
0	100	27.0	54.6	2.3
2	71	18.9	54.5	2.3
4	42	18.9	54.4	2.3
6	21	18.3	54.0	3.0
8	6	17.2	53.3	4.5
10	0	15	51.8	7.5
12	0	12	50.3	10.4
23.1	0	4.8*	40.2	30.6

*PCr is assumed to deplete only to approximately 15-20% of resting value, which occurs at 18 min, so PCr remains at 4.8 mmol kg^{-1} muscle in dives beyond 18 min.

Table 2-4. Body mass, number of dives, dive depth, dive duration, stroke frequency and post-dive surface interval of emperor penguins diving at Cape Washington. Surface interval after a 27.6 min dive in Penguin 8 was 8.4 hours. Surface intervals greater than 60 min were excluded from calculation of mean post-dive surface interval. Mean ± s.d. (range).

Penguin	Body mass (kg)	Number of dives	Mean maximum depth (m)	Mean dive duration (min)	Mean stroke frequency (strokes sec^{-1})	Mean post-dive surface interval (min)
1	29	424	54.5 ± 61.2 *(9 - 357.5)*	3.7 ± 1.4 *(2.0 - 8.4)*	0.59 ± 0.10 *(0.35 - 1.0)*	2.0 ± 3.8 *(0.18 - 46.13)*
2	23	448	132.2 ± 94.5 *(15.3 - 418.0)*	5.1 ± 1.8 *(2.0 - 12.2)*	0.67 ± 0.10 *(0.42 - 1.01)*	2.9 ± 3.7 *(0.45 - 29.70)*
3	21.5	452	91.8 ± 56.9 *(10.0 - 423)*	4.6 ± 1.8 *(2.0 - 12.7)*	0.73 ± 0.08 *(0.44 - 0.96)*	1.7 ± 1.8 *(0.37 - 28.85)*
4	23.5	502	91.2 ± 97.0 *(11.5 - 513.5)*	4.7 ± 1.9 *(2.0 - 11.5)*	0.66 ± 0.06 *(0.4 - 0.87)*	2.8 ± 3.9 *(0.30 - 29.93)*
7	24	498	127.1 ± 124.4 *(11.3 - 475.8)*	4.9 ± 2.1 *(2.0 - 10.2)*	0.72 ± 0.08 *(0.41 - 1.04)*	3.3 ± 5.0 *(0.08 - 48.92)*
8	27.5	447	92.7 ± 105.9 *(9.5 - 501.8)*	4.6 ± 2.2 *(2.0 - 27.7)*	0.65 ± 0.09 *(0.32 - 0.92)*	2.5 ± 4.4 *(0.02 - 34.38)*
9	24	479	130.6 ± 112.9 *(7.8 - 499.5)*	5.1 ± 1.9 *(2.0 - 11.0)*	0.66 ± 0.10 *(0.36 - 1.02)*	3.2 ± 4.3 *(0.22- 34.38)*
Grand Mean	**24.6 ± 2.6**	**464 ± 29**	**102.9 ± 28.6**	**4.7 ± 0.5**	**0.67 ± 0.05**	**2.6 ± 0.6**

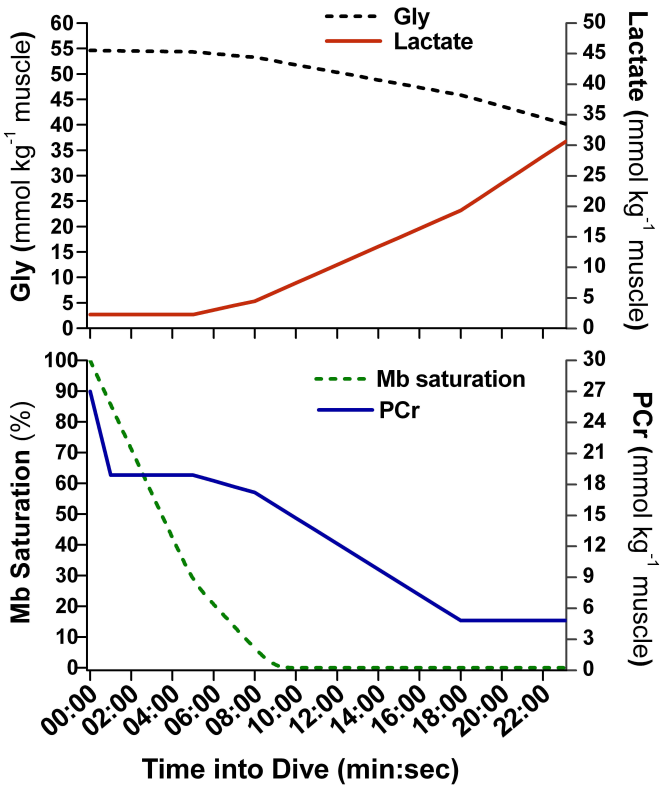

Figure 2-1. Model results showing changes in myoglobin (Mb) saturation, phosphocreatine (PCr) store, glycogen (Gly) store and muscle lactate in a hypothetical 23.1 min dive. Under the model, the PCr and Gly stores produce almost all of the required ATP after the first 6 min of long dives (green dashed line, Mb saturation; blue solid line, PCr; black dashed line, Gly; red solid line, lactate).

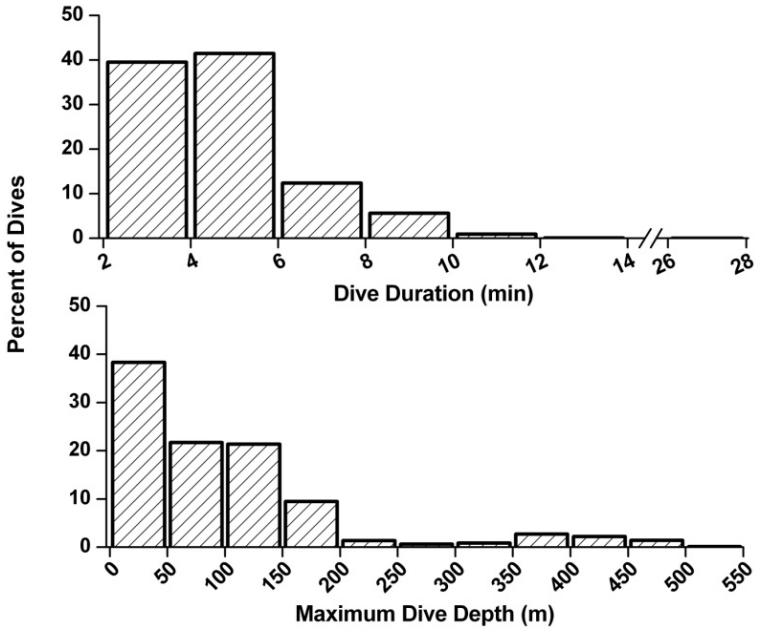

Figure 2-2. Distribution of dive duration (top) and maximum dive depth (bottom) of dives from 7 emperor penguins at sea (N=3250 dives).

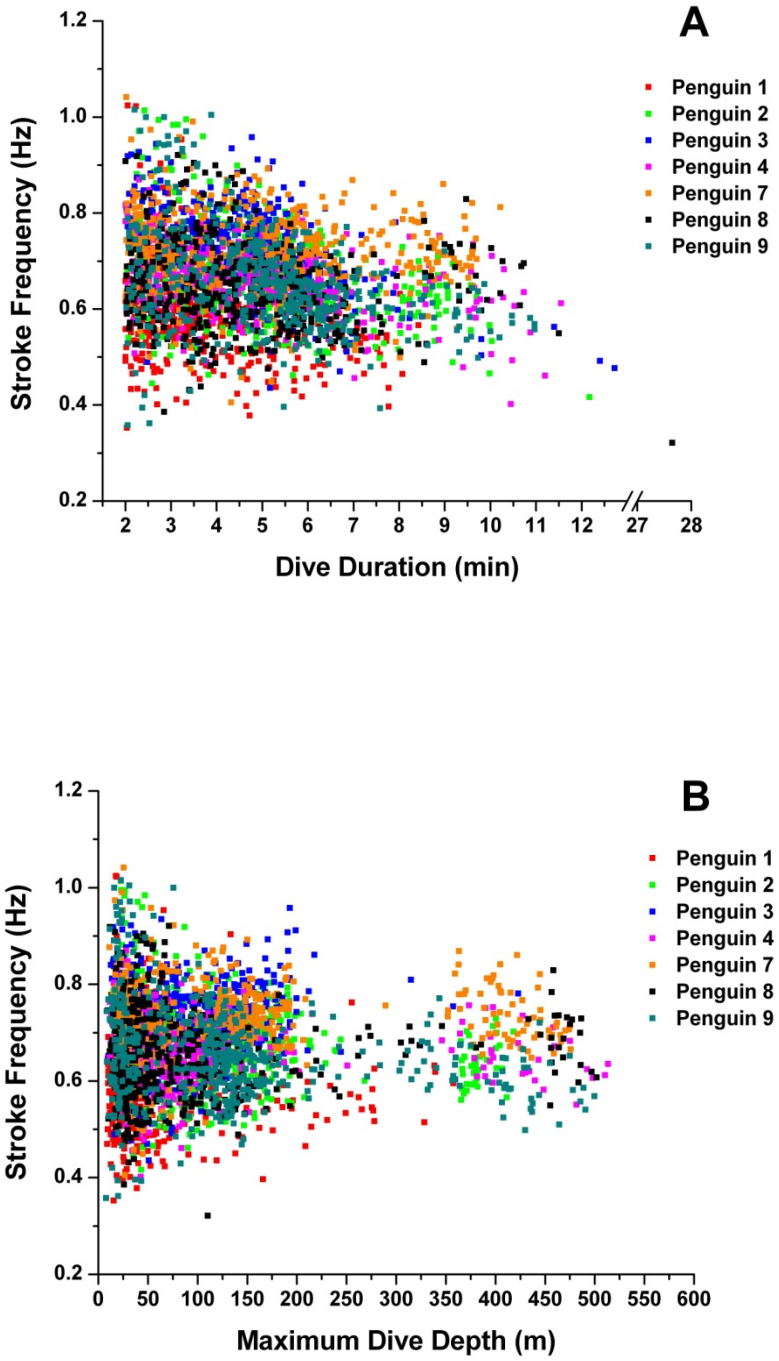

Figure 2-3. Relationship between (A) dive stroke frequency and dive duration and (B) dive stroke frequency and maximum dive depth in 7 penguins.

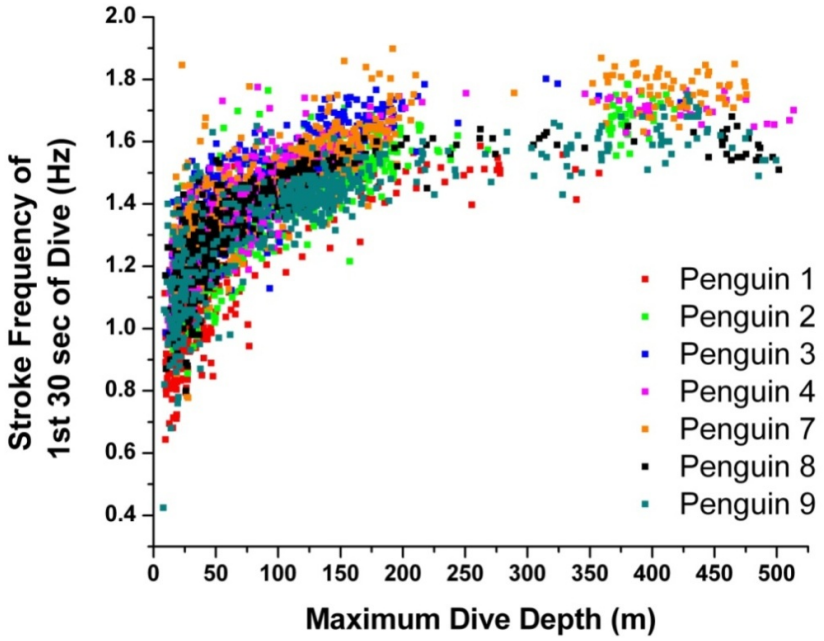

Figure 2-4. Relationship between stroke frequency for the first 30-sec of dives and maximum dive depth. Note first 30-sec stroke frequency increases with increasing dive depth up to 200 m where stroke frequency remains high, near 1.7 Hz

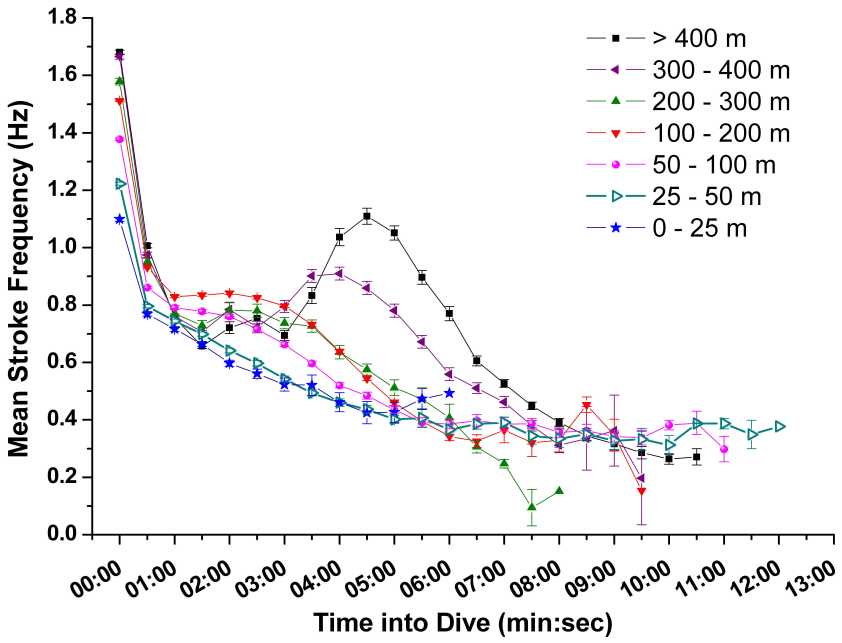

Figure 2-5. Profiles of mean stroke frequency at 30-sec intervals of dives for seven depth categories (0 - 25 m, 25 - 50 m, 50 - 100 m, 100 – 200 m, 200 – 300 m, 300 – 400 m and > 400 m). The 27.6 min dive is excluded from this analysis because it was the only dive beyond 13 min. Instantaneous stroke frequency for the 27.6 min dive is shown separately in Fig 6C. Standard error bars shown.

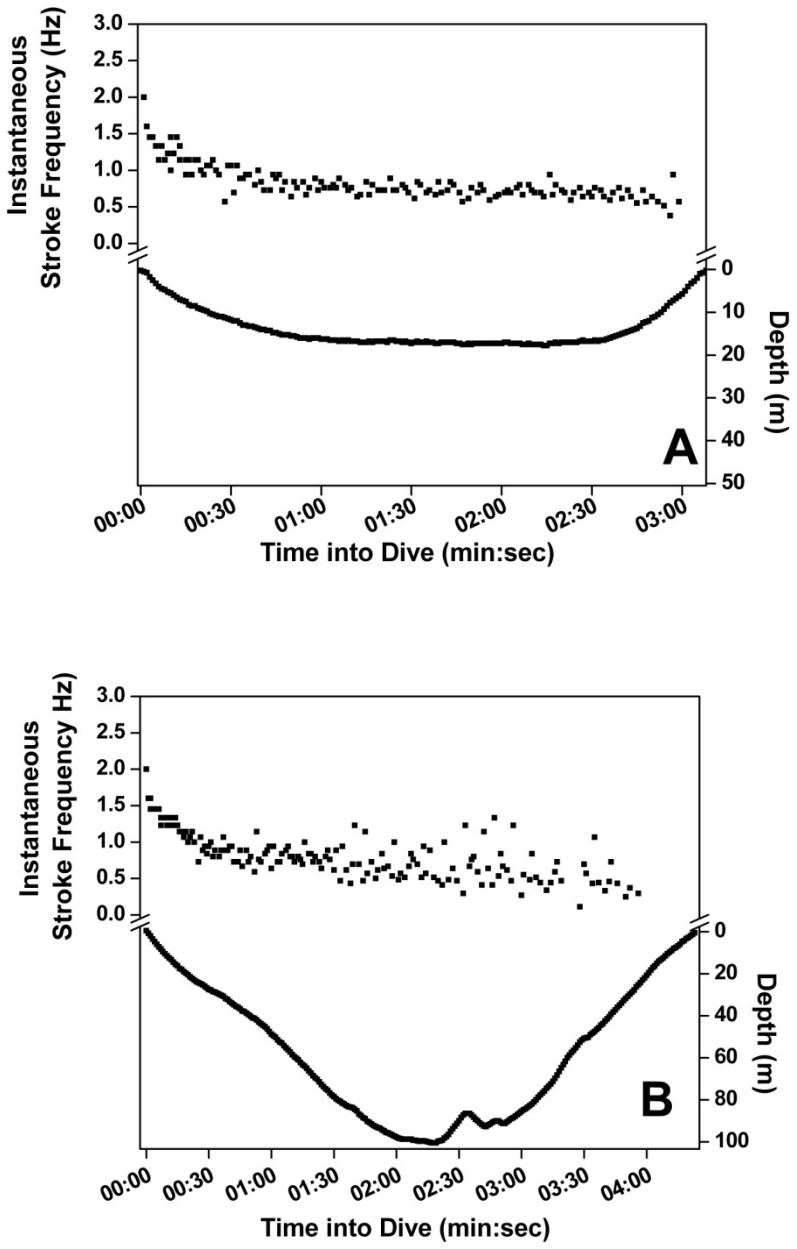

Figure 2-6. Instantaneous stroke frequency profiles of four dives: (A) 18 m, 3.1 min dive; (B) 101 m, 4.6 min dive; (C) 110 m, 27.6 min dive; and (D) 465 m, 9.3 min dive.

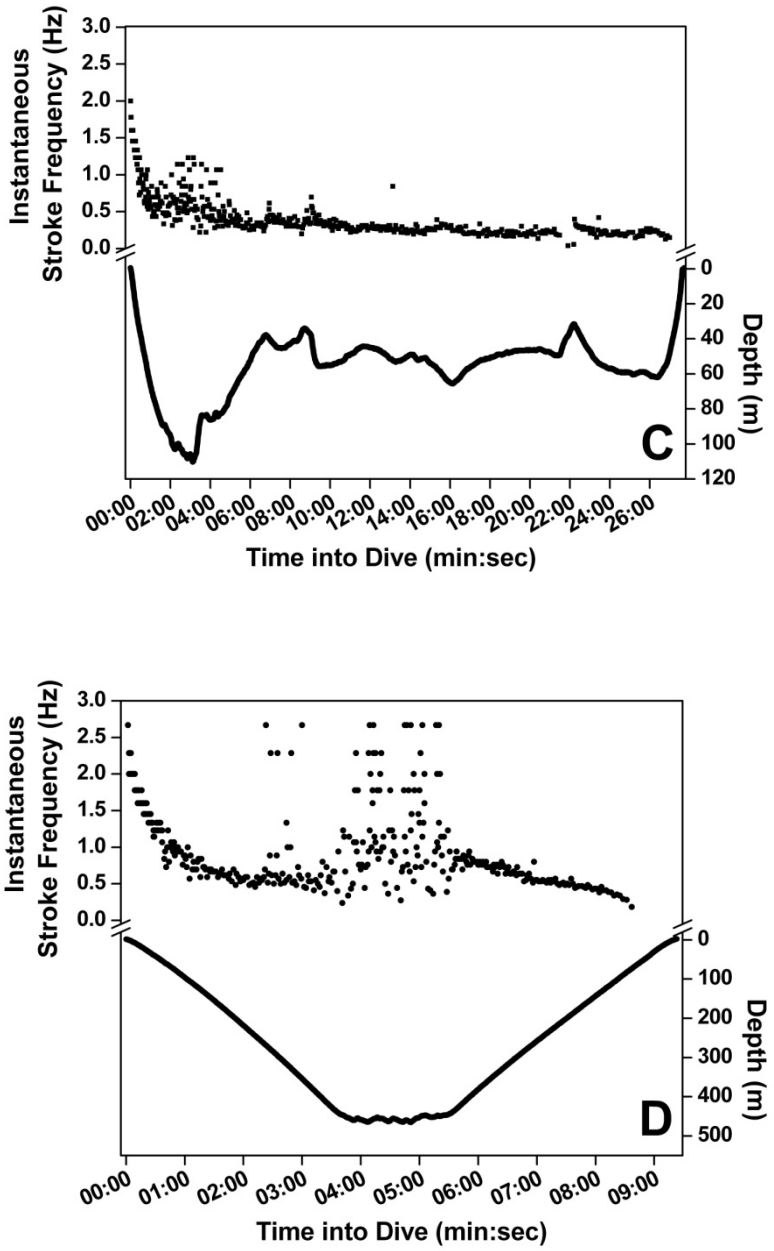

Figure 2-6, continued. Instantaneous stroke frequency profiles of four dives: (A) 18 m, 3.1 min dive; (B) 101 m, 4.6 min dive; (C) 110 m, 27.6 min dive; and (D) 465 m, 9.3 min dive.

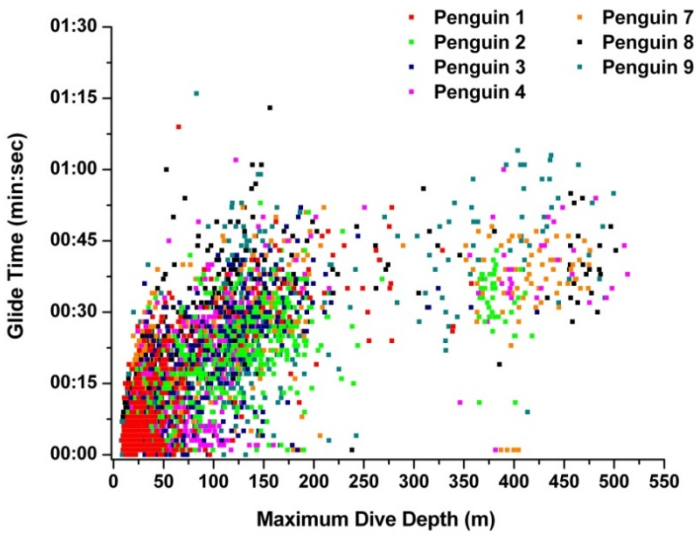

Figure 2-7. Relationship between ascent glide time and maximum dive depth. Ascent glide time was significantly and positively related to maximum dive depth for all penguins (r^2 = 0.33 to 0.52, P < 0.0001).

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