OXYGEN STORES, CARBON DIOXIDE ACCUMULATION AND NUTRITIONAL STATUS AS DETERMINANTS OF DIVING ABILITY OF STELLER SEA LIONS (*EUMETOPIAS JUBATUS*)

by

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Abstract

The diving ability of marine mammals is limited by body oxygen stores (TBO) and rates of oxygen depletion (diving metabolic rate; DMR), which can be expressed as the calculated aerobic dive limit (cADL). Diving ability must also be influenced by CO_2 production and control of ventilation. I investigated the factors that limit the diving ability of Steller sea lions (*Eumetopias jubatus*), including the effect of nutritional stress on the cADL. Specifically, I 1) determined the cADL of Steller sea lions by measuring TBO and DMR, 2) determined whether nutritional stress alters the cADL and 3) examined the post-dive elimination of CO_2, and the sensitivity of Steller sea lions to hypercapnia (high inspired CO_2). TBO was estimated from measured blood oxygen stores and body composition—and metabolic rate, breathing frequency and dive behaviour were recorded prior to and during a period of nutritional stress where animals lost ~10% of their mass. Animals breathed ambient, hypercapnic or hypoxic (low O_2) air to experimentally alter pCO_2 levels and decrease rates of CO_2 elimination and O_2 consumption. I found that the TBO (35.9 ml O_2 kg^{-1}) and cADL (3.0 minutes) in actively diving Steller sea lions were lower than previously reported for other species of sea lions and fur seals. I also found a significant increase in mass-specific DMR and blood volume (resulting in higher TBO) in nutritionally stressed animals that resulted in a longer cADL. Hypercapnia was found to significantly affect ventilation, but had no effect on dive behaviour—and elimination of CO_2 between dives took longer than replenishing O_2 stores. Overall, nutritional stress and hypercapnic conditions did not directly limit the diving ability of the Steller sea lions, but had an indirect effect on foraging efficiency by increasing the time they spent on the surface between dives. Accumulation of CO_2 over several dives in a foraging bout also appeared to reduce foraging efficiency, which likely ultimately limits the time a sea lion spends in apnea and therefore overall foraging duration and net energy intake.

Preface

I was the main designer of the experiments described in this thesis, with suggestions from supervisors David Rosen and Andrew Trites. I performed all data collection and analysis and prepared all manuscripts. Chapters 2, 3 and 4 were written as manuscripts and benefited from comments and edits by co-authors David Rosen and Andrew Trites. A version of Chapter 2 has been published as: Gerlinsky, C.D., Rosen, D.A.S., and Trites. A.W. (2013) High diving metabolism results in a short aerobic dive limit for Steller sea lions. *Journal of Comparative Physiology B* **189** (5):699-708.

All research was conducted under UBC Animal Care Permits A07-0413 and A11-0397 and was approved by the Vancouver Aquarium Animal Care Committee. I completed the ethics training requirements of the Canadian Council on Animal Care (CCAC) / National Institutional Animal User Training program (NIAUT) certificate # 4530 – 11.

Blood samples were analyzed by Metabolic Solutions Inc. (Nashua, NS, USA) for deuterium concentration and by Idexx Laboratories (Delta, BC, CAN) for clinical blood chemistry and hematology.

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List of abbreviations

ADL	Aerobic dive limit
cADL	Calculated aerobic dive limit
TBO	Total body oxygen stores
RMR	Resting metabolic rate
MR_S	Surface (pre-dive) metabolic rate
DMR_{cycle}	Diving metabolic rate, measured over the 'dive event' (dive & recovery)
DMR_{dive}	Diving metabolic rate, all excess O_2 to dive duration only
$\dot{V}_{O_2}$	Rate of oxygen consumption
$\dot{V}_{CO_2}$	Rate of carbon dioxide production
F_iCO_2	Fractional content of inspired air that is carbon dioxide
F_iO_2	Fractional content of inspired air that is oxygen
P_{CO_2}	Partial pressure of carbon dioxide (in blood)
P_{O_2}	Partial pressure of oxygen (in blood)
bpm	Breaths per minute
F_B	Breathing frequency
P_{ACO_2}	Alveolar partial pressure of carbon dioxide
M_b	Body mass
LBM	Lean body mass
BV	Blood volume
PV	Plasma volume

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Chapter 1: Introduction

Marine mammals are capable of diving well beyond the abilities of a terrestrial mammal, enabling them to successfully forage in aquatic environments. They have evolved a suite of physiological and behavioural adaptations to endure the stresses of diving. One of the most fundamental stresses they face is the hypoxemia (low blood O_2 levels; low P_{O_2}) and hypercapnia (high blood CO_2 levels; high P_{CO_2}) that results from the extended durations of breath-hold (apnea; Kooyman et al., 1973; Qvist et al., 1986; Ponganis et al., 1993; Meir et al., 2009). Adaptations to diving serve to significantly increase the duration that marine mammals can stay in apnea and actively exercise while doing so. Determining the physiological and behavioural factors that contribute to the diving patterns and limitations of individuals contributes to understanding what confines their ability to forage.

A significant constraint to diving for air-breathing mammals is the depletion of O_2 stores during apnea (breath-hold). The aerobic dive limit (ADL) represents the theoretical maximum duration an animal can dive if relying solely on O_2 stores for metabolism. It is a commonly used metric to compare diving ability among and within species of marine mammals. To extend breath-hold duration, animals can increase the amount of O_2 available and decrease the rate of O_2 use (hypometabolism). Marine mammals have evolved physiological adaptations to exploit both of these avenues. However, the extent to which they are affected by other physiological changes—such as those related to differences in nutritional status or exercise regime—is unknown. In addition, while considerable research into the diving physiology of marine mammals has concentrated on the role of oxygen depletion, less research has focused on the potential effect of carbon dioxide accumulation in determining diving limits.

The goals of my research were to define the dive limits of Steller sea lions (*Eumetopias jubatus*) in terms of O_2 stores and their rate of depletion (ADL), to determine if changes in body composition and metabolism due to nutritional stress affect these limits (ADL) and oxygen management strategies while foraging. I also sought to examine the role that CO_2 accumulation plays in the physiological control of dive behaviour.

Body oxygen stores & the aerobic dive limit

The mammalian diving reflex evolved to conserve limited onboard O_2 stores, enabling animals to extend breath-hold duration, and is most strongly exhibited in marine mammals. The dive reflex is characterized by apnea, peripheral vasoconstriction and bradycardia, a slowing of the heart rate to maintain blood pressure (Scholander, 1940; Butler and Jones, 1997). This optimizes management of O_2 stores by reducing O_2 delivery to certain parts of the body, allowing them to function anaerobically and maximizing sequestration of O_2 from hemoglobin and myoglobin. This diving reflex also conserves O_2 for critical systems that can only function aerobically, such as the brain and heart.

The aerobic dive limit (ADL) is defined as the dive duration after which lactate levels in the blood begin to increase above normal resting levels due to anaerobic metabolism (Kooyman, 1989). Hence, this has more recently been referred to as the diving lactate threshold (Butler and Jones, 1997). Given the logistical difficulties in directly measuring blood lactate levels in actively diving animals, the ADL can be estimated from measures of total body O_2 stores and diving metabolic rate (Kooyman et al., 1983; Butler, 2006). This calculated ADL (cADL) is often used as a proxy for the measured ADL.

There are three major areas where oxygen is stored in the body: the lungs, muscle and blood. The proportion of oxygen stored in each varies among different groups of marine mammals and corresponds with general dive behaviour (for review see Kooyman, 1989). Species that generally dive for shorter durations at shallow depths, such as the Steller sea lion (Merrick et al., 1994; Merrick and Loughlin, 1997; Loughlin et al., 1998) normally inhale before a dive—hence the lung contributes to TBO as gas exchange continues during the first part of a dive. Deep divers generally exhale before diving to facilitate lung collapse, and therefore rely on muscle and blood oxygen stores. The oxygen carrying capacity of the blood is based on total red blood cell counts (a function of hematocrit and blood volume) and haemoglobin concentration within a RBC—all of which are generally higher in marine mammals compared to terrestrial mammals (for review see Kooyman and Ponganis, 1998). Marine mammals also have greater muscle O_2 stores due to myoglobin concentrations that are 10-30 times higher than in terrestrial mammals (Kooyman et al., 1981). Higher myoglobin concentrations generally correlate with increased diving ability (Kooyman and Ponganis, 1998; Noren and Williams, 2000). In many species, body O_2 stores increase in association

with diving ability in pups and juveniles (Burns et al., 2005; Richmond et al., 2006; Fowler et al., 2007; Weise and Costa, 2007).

Estimates of TBO exist for several species of otariids, including juvenile Steller sea lions (Lenfant et al., 1970; Richmond et al., 2006), but there are no direct measures for adult Steller sea lions. Blood O_2 stores of otariids are often calculated based on measures of blood cell counts and haemoglobin concentration (but rarely incorporate direct measures of blood volume), and muscle O_2 stores have been estimated from some measures of myoglobin concentration (but no direct measures of muscle mass). However, muscle mass and lung O_2 stores are usually estimated from allometric relationships. A single measure of TBO is generally used to estimate diving ability; however this may vary in adult animals due to season, nutritional or physiological status. Very few studies have examined the variation of TBO within individuals, which could impact diving abilities.

Calculation of ADL also requires accurate measures of diving metabolic rate (DMR). Determining the rates of O_2 consumption ($\dot{V}_{O_2}$) and CO_2 production ($\dot{V}_{CO_2}$) via gas respirometry is one of the most accurate and direct measures of metabolism. A number of studies have directly measured DMR in Steller sea lions, but estimates vary widely (Hastie et al., 2007; Fahlman et al., 2008; Young et al., 2010). This is partly because DMR can be calculated from measures of $\dot{V}_{O_2}$ based on two different methods; either all excess O_2 consumed in the post-dive recovery period is attributed to the dive itself (DMR_{dive}), or $\dot{V}_{O_2}$ is calculated as an average over the "dive event", i.e., the total duration of the dive and subsequent recovery period (DMR_{cycle}). Alternate estimates of metabolic rate (although not necessarily DMR per se) have been obtained using the doubly labeled water turnover technique, accelerometry (which estimates activity), heart rate, or projected from allometric relationships (Boyd et al., 1995; Butler et al., 2004; Green, 2011; Halsey et al., 2011). However, accurate simultaneous measures of DMR and body oxygen stores are required to calculate the aerobic diving capacity of specific marine mammals.

Respiratory drive & carbon dioxide

Research into the diving capacity of marine mammals has primarily focused on the role of O_2 in determining dive limits. Although considered inefficient, marine mammals are

capable of diving well beyond their O_2 limits using anaerobic metabolism in tissues that do not rely critically on O_2 to fuel metabolism (primarily in the muscles). Many marine mammals have been observed diving ~4 times longer than their aerobic limits. For example, Weddell seals have an ADL of 19 min (Ponganis et al., 1993) and a maximum recorded dive duration of 82 min (Castellini et al., 1992), while a juvenile California sea lion has an ADL of 2.3 min (Ponganis et al., 1997a) and maximum dive durations of ~8 – 10 minutes (Feldkamp et al., 1989). The extent of this "anaerobic capacity" to dive and the factors that ultimately end the dive (whether behavioural or physiological) are unclear. Factors such as low P_{O_2}, high P_{CO_2} or changes in blood pH due to anaerobic metabolism could all potentially contribute to defining the physiological limits of breath-hold duration.

Regardless of the precise mechanisms, physiological control of diving in air breathers must be related to control of ventilation. As O_2 stores are consumed during breath-hold diving, CO_2 is being produced and must be stored in the body until ventilation resumes following a dive. In mammals, control of ventilation depends on CO_2 as it is more difficult for an air-breathing organism to offload CO_2 than to obtain O_2 from the environment. Rising P_{CO_2} is sensed by peripheral and central chemoreceptors in the arteries and brain respectively, stimulating respiratory drive, which generally occurs long before oxygen is limiting. Adaptations that marine mammals have to decrease the rate of O_2 use and extend the ADL also serve to decrease the rate of CO_2 production and subsequent rise of P_{CO_2}. However, to extend breath hold duration, air breathers will ultimately have to delay the onset of ventilation and cope with the resulting accumulation of CO_2 and associated respiratory acidosis.

Marine mammals appear to have exceptionally high blood buffering capacity that maintains pH to protect against CO_2 build-up and resulting respiratory acidosis (Lenfant et al., 1970; Castellini and Somero, 1981). As with all mammals, CO_2 in the blood combines with water to produce carbonic acid (catalyzed by the enzyme carbonic anhydrase), which then dissociates into protons and bicarbonate ions (HCO_3^-). As P_{CO_2} increases and bicarbonate is produced, $[H^+]$ also increases. Haemoglobin then binds to CO_2 and protons, reducing oxygen affinity (Bohr effect), and facilitating the release of remaining O_2 from blood stores. Hence, during breath hold, P_{O_2} decreases and CO_2 carrying capacity of the blood increases. A similar mechanism is responsible for oxygen release from myoglobin in muscles (Davis et al., 2004).

Previous studies that have examined the sensitivity of mammals to CO_2 have tested the effect of high inspired levels of CO_2 (hypercapnia) on their ventilatory response. A few studies, mainly on phocid seals in laboratory experiments, have shown that marine mammals may have a reduced sensitivity or blunted response to CO_2 compared to terrestrial mammals. However, the role of blood pCO_2 levels in limiting dive behaviour, or terminating a dive in actively diving and foraging animals, is unclear. Although CO_2 must contribute to control of ventilation in marine mammals, there are few studies that relate it to determining dive behaviour (Butler and Jones, 1997; Boutilier et al., 2001). No studies have examined the ventilatory response to high CO_2 or the effect of hypercapnic conditions on dive behaviour in an otariid. Otariids generally have lower diving capacity (TBO and ADL) than phocid seals, and may therefore be more sensitive to CO_2.

Steller sea lions & nutritional stress

Individual variation of TBO and DMR due to physiological or environmental changes in adult marine mammals has received little research attention. Nor has consideration been given to how these parameters might be affected by changes in nutritional status. Such changes may play a significant role in understanding the impact that hypothesized changes in the biotic environment may have on species of concern, such as Steller sea lions. Malnutrition and the resulting changes in body composition (lipid and lean body mass) may affect muscle and blood O_2 stores, and diving metabolic rates may be affected by changes in body mass or other physiological adaptations. Nutritionally stressed animals may also alter their dive behaviour in response to changes in abundance or distribution of prey, or their need to increase food consumption to compensate for nutritional deficits. If animals that are nutritionally stressed are diving close to their physiological (aerobic) limits, they may rely more on energetically expensive anaerobic metabolism, which would reduce foraging efficiency. Nutritional stress can therefore not only result in animals having to dive longer and more frequently to find prey, but may also result in them having to spend more energy doing so, further compounding losses in net energy intake.

Steller sea lions are the largest member of the pinniped family Otariidae (sea lions and fur seals), and occur in the North Pacific Ocean, mainly along the coastal regions from California to northern Japan. When not at sea, Steller sea lions are typically found in

aggregations resting at haul-out sites (non-breeding areas) and reproducing at rookeries (breeding areas). Steller sea lions have declined in the western portion of their range (Gulf of Alaska to Russia) by ~80% since the mid-1970's (Trites and Larkin, 1996), and are classified in central and western Alaska as endangered under U.S. legislation (Sease et al., 2001).

Several theories have been proposed to explain the decline of Steller sea lion populations such as predation, disease, and nutritional stress. The Nutritional Stress hypothesis states that Steller sea lions are not getting enough high quality prey (Rosen, 2009) causing deleterious changes in body composition resulting in reduced body size and productivity, a higher mortality rate among juveniles, and changes in morphology and blood parameters (Trites and Donnelly, 2003). It has been demonstrated that nutritional stress results in decreased body condition in Steller sea lions (for review see Rosen, 2009), but the subsequent effect on physiological foraging capacity through changes in TBO or DMR is unknown.

Research goals

The overall objective of my research was to examine the factors that may limit the diving capacity and behaviour – and hence foraging ability – of Steller sea lions. Studies of diving physiology and the limits to dive behaviour have traditionally focused on oxygen use and storage. However, there is limited information about O_2 stores in adult Steller sea lions and how these stores may vary within adult individuals. Thus, my first goal was to define the diving capacity of adult Steller sea lions by calculating the aerobic dive limit through measures of O_2 stores and diving metabolic rate. I also wanted to determine whether exercise during a dive (in an aquarium vs. open water environment) affected O_2 stores (Chapter 2).

Nutritional stress has been proposed as a factor that may have contributed to the decline of Steller sea lions in the wild, but there are no studies examining the effect of nutritional stress on diving capacity and behaviour of marine mammals. Hence, my second goal was to determine whether changes in body composition due to a controlled episode of nutritional stress affect dive limits (TBO & ADL) and the cost of foraging (DMR; Chapter 3).

While the role that depletion and storage of O_2 play in limiting diving capacity have been well studied, significantly less attention has been given to how CO_2 and its control of ventilation contribute to the limitations and control of dive behaviour. A few studies have

undertaken laboratory experiments to answer this question, but none have involved actively diving animals that may have significantly different responses while diving in a natural environment. Nor has any study looked at the sensitivity of otariids to CO_2. My third goal was therefore to examine the sensitivity of Steller sea lions to CO_2 while at rest (ventilatory response) and to determine whether hypercapnia affects metabolism (at rest and while diving), or dive behaviour (Chapter 4). For this part of my study, I exposed sea lions to high-inspired CO_2 (hypercapnia) and low inspired O_2 (hypoxia).

Concurrent to assessing CO_2 sensitivity while resting, I wanted to determine whether CO_2 production while diving indirectly limited foraging efficiency by examining how animals recovered from diving. I therefore examined the recovery period following diving, in terms of the time it took to refill O_2 stores, versus the time to offload accumulated CO_2 stores. I also examined whether limiting either of these processes (through altered inspired gas concentrations) would limit diving indirectly (by extending recovery time and surface intervals) and reduce overall foraging efficiency.

My thesis includes three chapters that were written to be submitted separately for publication in peer-reviewed journals. The first Chapter focuses on the ADL of Steller sea lions, the second on the impact of nutritional stress on diving, and the third on the role of CO_2 in diving. Although these chapters detail separate aspects of the research, there is some unavoidable contextual overlap between them.

While my research focuses on the physiological controls of diving in an otariid, the results are important to specific species such as Steller sea lions that may be impacted by changes in their biotic environment. Conservation of Steller sea lion populations depends on determining the accessibility and profitability of prey, which depends on specific and accurate estimates of their ability to dive and forage in the wild. The diving ability of Steller sea lions may be overestimated as the study of diving physiology has largely focused on phocid seals that tend to have lower diving metabolic rates and greater oxygen stores.

My research sought to define the normal limitations of diving, and assess how it might be further limited by nutritional stress associated with reduced prey availability. Additionally, I wanted to determine how hypercapnia affects an actively diving and foraging animal, and how they respond behaviourally and physiologically to changes in inspired gas composition. My hope is that my research furthers understanding of the potential impacts of changes in the

biotic environment on Steller sea lion populations, and highlights the importance of physiological limits to diving in constraining the ability of an air-breathing mammal to forage underwater.

Chapter 2: High diving metabolism results in a short aerobic dive limit for Steller sea lions (*Eumetopias jubatus*)

Summary

The diving capacity of marine mammals is typically defined by the aerobic dive limit (ADL) which, in lieu of direct measurements, can be calculated (cADL) from total body oxygen stores (TBO) and diving metabolic rate (DMR). To estimate cADL, I measured blood oxygen stores, and combined this with diving oxygen consumption rates ($\dot{V}_{O_2}$) recorded from 4 trained Steller sea lions diving in the open ocean to depths of 10 or 40 m. I also examined the effect of diving exercise on O_2 stores by comparing blood O_2 stores of the diving animals to non-diving individuals at an aquarium. Mass-specific blood volume of the non-diving individuals was higher in the winter than in summer, but there was no overall difference in blood O_2 stores between the diving and non-diving groups. Estimated TBO (35.9 ml O_2 kg^{-1}) was slightly lower than previously reported for Steller sea lions and other otariids. Calculated ADL was 3.0 minutes (based on an average DMR of 2.24 L O_2 min^{-1}) and was significantly shorter than the average 4.4 minute dives the study animals performed when making single long dives—but was similar to the times recorded during diving bouts (a series of 4 dives followed by a recovery period on the surface), as well as the dive times of wild animals. My study is the first to estimate cADL based on direct measures of $\dot{V}_{O_2}$ and blood oxygen stores for an otariid and indicates they have a much shorter ADL than previously thought.

Introduction

Marine mammals that exploit resources at depth rely on physiological adaptations to extend the time they can remain submerged to increase their foraging efficiency (Houston and Carbone 1992). Foraging ability for a marine mammal inherently depends on overall diving ability, with the most efficient foraging thought to be done aerobically. This aerobic diving capacity is typically defined by the aerobic dive limit (ADL or diving lactate threshold; Butler 2006). The ADL is measured as the maximum dive duration before there is an increase in post-dive blood lactate concentration beyond resting levels (Kooyman 1985). An increase in post-dive lactate indicates anaerobic metabolism is being used to conserve onboard oxygen stores for critical systems that can only function aerobically (Kooyman et al. 1980). It is

thought that marine mammals should preferentially dive within their ADL (Kooyman 1989); the extra time needed to recover from anaerobic dives would result in a lower overall foraging efficiency, as the surface interval needed to recover increases disproportionately with longer dive times (Kooyman et al. 1980).

The ADL has only been measured in five species of marine mammals: two cetaceans, two phocid seals and one otariid, the California sea lion (Ponganis et al. 1993; Ponganis et al. 1997b; Ponganis et al. 1997a; Williams et al. 1993; Shaffer et al. 1997). Due to the challenges of measuring blood lactate in a freely diving animal, the calculated ADL (cADL) is commonly used to compare diving ability among species (Butler 2006). The cADL is a simplified estimate of aerobic diving capacity based on the amount of oxygen stored in the body and the rate at which this oxygen is depleted (Ponganis et al. 1993; Castellini et al. 1992; Kooyman et al. 1980). In general, marine mammals have higher total body oxygen stores (TBO) than their terrestrial counterparts (Lenfant et al. 1970; Kooyman and Ponganis 1998). In addition, marine mammals can extend their cADL by decreasing the rate at which they use oxygen stores, such as by suppressing metabolic rate (Butler and Jones 1997; Kooyman and Ponganis 1998). They also have a high tolerance to very low partial pressures of oxygen in the blood and can control the rate and pattern of oxygen store utilization (Ponganis et al. 2011; Meir et al. 2009).

TBO consist of the oxygen carrying capacity of the blood, muscle and lungs. Unlike phocids (true seals), in otariids (sea lions and fur seals) the lung can make a significant contribution to onboard oxygen stores, since they inhale before a dive. Blood oxygen stores are a function of blood volume, red blood cell concentration (measured as hematocrit) and hemoglobin concentration. Muscle oxygen stores are a function of myoglobin concentration, which has also been shown to be higher in marine than in terrestrial mammals (Castellini and Somero 1981; Kooyman and Ponganis 1998). Blood and muscle oxygen stores (and hence diving ability) have been shown to develop with age (Richmond et al. 2006; Weise and Costa 2007; Fowler et al. 2007), and generally correlate with the time of weaning and the beginning of independent foraging (Horning and Trillmich 1997; Burns et al. 2005). Activity level and exposure to diving may also affect body oxygen stores during development (Noren et al. 2001), but the influence of exercise and diving on TBO of adult marine mammals post-development is unknown.

In addition to knowing TBO, obtaining an accurate estimate of the cADL also depends on calculating the rate at which oxygen stores are used during diving. Much of the large disparity among cADLs within and between species can be attributed to the considerable variation in how diving metabolic rate (DMR) is measured; such as estimated from field metabolic rate (Costa and Gales 2003; Costa and Gales 2000; Fowler et al. 2007) or calculated from allometric scaling (Richmond et al. 2006). The most accurate measure of metabolic rate is made directly from oxygen consumption rates ($\dot{V}_{O_2}$), but even this can vary depending on the method of calculation, the environment in which it is measured (restricted aquarium tanks versus freely diving in open water) and on behavioural or physiological differences between trained and wild animals. This variation makes interspecific comparisons of cADL measurements difficult, as well as those among multiple studies on the same species.

Despite the difficulties of estimating ADL, the assumption that most dives are aerobic appears to be true for most species with an empirically measured ADL (Butler and Jones 1997; Kooyman et al. 1980; Costa et al. 2001). In contrast, many species of otariids appear to be diving beyond their calculated aerobic limits (Costa et al. 2001). The discrepancy might be attributable to the fact that few studies have reported cADL based on direct measures of DMR (via $\dot{V}_{O_2}$) on actively diving animals, and none have simultaneously measured TBO.

The only measured ADL for an otariid is 2.3 minutes for a juvenile California sea lion and is significantly lower than previously estimated cADLs of up to 15 minutes for Steller sea lions (Ponganis et al. 1997; Richmond et al. 2006). This estimated cADL is also high compared to the typical dive behaviour of Steller sea lions in the wild. Studies on adult and juvenile Steller sea lions report dives being normally short, shallow and frequent, with most dives <4 minutes and the longest recorded dive being ~12 minutes (Merrick et al. 1994; Loughlin et al. 1998; Merrick and Loughlin 1997; (Pitcher et al. 2005).

My objective was to calculate the ADL of Steller sea lions by measuring blood oxygen stores (to calculate TBO) and DMR of a group of adult females trained to dive regularly in the open ocean. I compared the cADL to their actual dive durations, as well as to those of Steller sea lions recorded in the wild. I also investigated the effect of previous exercise on blood O_2 stores and compared this between seasons in non-diving animals. I hypothesized that blood O_2 stores would be greater in the diving group as compared to the non-diving group. My study is

the first to calculate the ADL for an otariid based on concurrent measurements of blood O_2 stores and direct measurements of DMR via $\dot{V}_{O_2}$, and results in one of the most accurate estimates of cADL for a marine mammal.

Methods

Study design/data collection

I used eight adult, female Steller sea lions that were collected from breeding rookeries as pups and raised in captivity at the Vancouver Aquarium (British Columbia, Canada). All animals were previously trained to use experimental equipment and performed all trials voluntarily under trainer control. Four of the sea lions (between 12 – 15 years old) were housed at the Open Water Research Laboratory (Port Moody, BC), and have been actively diving in the open ocean for research purposes since 2003 (F00SI, F00BO), 2005 (F00HA) or 2008 (F00YA). Animals were fed a diet of herring (*Clupea pallassi*) and market squid (*Doryteuthis opalescens*) supplemented with vitamins. All experiments were conducted under UBC Animal Care Permit #A07-0413.

The four sea lions at the Open Water facility (OW) each underwent several trials to determine DMR from May – July 2011. Trials were conducted concurrent with a study on the effect of altered inspired gas concentrations on dive behaviour and recovery time, but there was no significant affect of inspired gas on DMR. Briefly, TBO was calculated based on direct measures of blood oxygen stores and estimates from previous studies for muscle and lung oxygen stores (detailed below). This was done concurrently with the DMR trials (July 2011) for the sea lions at the OW facility and on four sea lions (9 years of age) at the Vancouver Aquarium (February and May 2012) for comparison of diving and non-diving individuals, and seasonal differences within the non-diving group. Calculated ADL was estimated by dividing TBO by DMR. These values were then compared to the voluntary dive behaviour of the sea lions.

Diving metabolic rate

Diving metabolic rate was determined from four Steller sea lions diving voluntarily over a series of dive trials. Dive trials were conducted at a depth of either 10 or 40 m (to match behaviour typical of wild sea lions). The experimental set-up consisted of a floating platform

with a square opening in the middle containing a submerged cage (1.52 m x 1.52 m x 2.5 m) and a floating transparent Plexiglas respirometry dome (100 L). Metabolic rate was measured in the dome using flow through respirometry. Air was drawn through the dome at 350 L min^{-1}. Air was sub-sampled and scrubbed of water vapor, then fractional concentrations of oxygen and carbon dioxide were measured using Sable system FC-1B and CA-1B analyzers, coupled to a 500H mass flow generator and controller (Sable Systems Inc., Las Vegas, NV, USA). Gas concentrations in the excurrent air stream were recorded every 0.5 seconds (Sable Data Acquisition system, Sable Systems Inc.). Barometric pressure, relative humidity, and air temperature were also recorded.

Animals were fasted overnight prior to trials and weighed each morning. They were fed less than 0.5 kg during transport to the dive site to minimize any effect of heat increment of feeding on metabolic rate (Rosen and Trites 1997). During trials, the animals wore a harness with a VHF transmitter and time depth recorder (ReefNet, Inc., Mississauga, ON, Canada). Pre-dive metabolic rate was measured at the start of each trial while animals rested inside the metabolic dome, as the last 3 minutes of 5-10 minute period, when $\dot{V}_{O_2}$ was constant. Animals then dove voluntarily to the bottom of 2 feeding tubes that delivered ~20 gram pieces of herring at depth (10 or 40 m) every 5 seconds for the duration of the dive.

The animals were asked to do either a single long dive or a 4-dive bout cycle, during which the animals chose both their dive and inter-dive surface interval durations. Following the single dive or dive bout, the sea lions remained at the surface in the respirometry dome for a post-dive 'recovery' measurement, defined as the time it took for the rate of oxygen consumption ($\dot{V}_{O_2}$) to return to within 5% of pre- or post-dive baseline values, whichever was lower. The four diving animals each completed 5 or 6 dives of each type (single and bout) and at two different depths (10 or 40 m). To facilitate comparisons to other studies, DMR was calculated two different ways: by dividing the total oxygen consumed in excess of post-dive baseline in the recovery period over the dive duration only (DMR_{dive}; as per Hastie et al. 2007; Hurley and Costa 2001) and as the average $\dot{V}_{O_2}$ over the dive and recovery period (DMR_{cycle}; as per Fahlman et al. 2008; Kooyman et al. 1980). For bout dives, DMR_{cycle} was the average $\dot{V}_{O_2}$ for the entire cycle of dives and recovery period.

Metabolic data was analyzed using LabAnalyst X (Warthog systems, Mark Chappell, University of California). Data was corrected for electronic drift by baselining gas concentrations to ambient air at the beginning and end of the trial. The entire gas analysis system was periodically calibrated with gases at known concentrations. Rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were calculated using equations 11.7 and 11.8 in Lighton (2008).

Resting metabolic rate of non-diving animals

Resting metabolic rate (RMR) of non-diving Steller sea lions was measured while animals were resting in water in a small tank at the Vancouver Aquarium using the same metabolic equipment as described above for diving animals. They were fasted overnight prior to measurements of $\dot{V}_{O_2}$ and all trials were completed within their assumed thermo-neutral zone. RMR was calculated as the lowest 20 minute average of a 40 - 45 minute trial period (when $\dot{V}_{O_2}$ had reached a steady state) and was measured once for each animal in each season.

Total body oxygen stores

Estimates of oxygen stores in the lung, blood and muscle were combined to determine TBO. I assumed full use of the lung O_2 stores during the dive. Lung O_2 stores were estimated based on actual measures of body mass (M_b), and assumed diving lung volume of 55 ml kg^{-1} (Lenfant et al. 1970) and 15% O_2 content (Kooyman et al. 1971) such that:

$$Lung\ O_2(mL) = M_b\ (kg) * 55\ mL\ kg^{-1}\ * 0.15$$

Muscle O_2 stores were calculated assuming a muscle mass of 37% of total body mass (Richmond et al. 2006), and a myoglobin concentration [Mb] for adult Steller sea lions of 28.7 mg g^{-1} and 20.0 mg g^{-1} of wet weight muscle, for swimming muscle and non-swimming muscle, respectively (Kanatous et al. 1999). Muscle was assumed to be 52% swimming and 48% non-swimming as measured on one-month old pups (Richmond et al. 2006). Muscle O_2 stores were calculated assuming an oxygen binding capacity of 1.34 ml O_2 g^{-1} Mb (Kooyman and Sinnett 1982) using the equation:

$$Muscle\ O_2(mL) = 0.37 * M_b\ (kg) * 1.34\ mL\ g^{-1} Mb * \%\ muscle\ type\ *\ [Mb]$$

Blood oxygen stores were measured directly from each sea lion. Blood samples were taken from the caudal gluteal vein shortly after animals were anaesthetized using isofluorane gas (maximum 5% isofluorane) under veterinary control. Blood samples were taken as early in the anaesthetic procedure as possible, as soon as animals were induced (10 – 15 minutes). Samples were analyzed by a commercial laboratory (Idexx Laboratories, Delta, BC) for hematocrit and hemoglobin values.

Plasma volume was measured using Evan's blue dilution procedure (Gibson and Evans 1937). After taking background samples, Evan's blue dye (0.5 mg kg^{-1} dose, Sigma-Aldrich; E2129) was injected into a vein in the rear flipper through an intravenous catheter. Serial samples were taken at approximately 8, 16, 24 and 32 minutes after injection (exact time noted per sample). Plasma was analyzed using a simplified technique whereby the relationship between the optical densities at two different wavelengths (624 and 740 nm) can be used to correct for the presence of plasma in dye samples (Nielsen and Nielsen 1962; Foldager and Blomqvist 1991; El-Sayed et al. 1995). A linear regression of the absorption over time was used to determine the concentration of dye at time of injection from the y-intercept, which was then used to calculate instantaneous dilution volume using standard curves created from stock solutions.

Blood volume (BV) was calculated from hematocrit (Hct) and plasma volume (PV) using the equation:

$$BV(L) = PV(L) * \frac{100}{(100 - Hct)}$$

Blood O_2 stores were calculated using BV and hemoglobin concentration [Hb], assuming an oxygen binding capacity of hemoglobin of 1.34 ml O_2 g^{-1} Hb (Kooyman and Sinnett 1982). Blood was assumed to be 1/3 arterial, that it was 95% saturated at the beginning of the dive and reduced to 20% at the end of the dive, and 2/3 venous, assumed to be 5 vol% less than initial arterial saturation (Richmond et al. 2006; Ponganis et al. 1993). Hence:

$$Arterial\ O_2(mL) = 0.33\ * BV\ (mL) * (0.95 - 0.20) * (1.34\ mL\ g^{-1}Hb) * [Hb](g\ mL^{-1})$$

$$Venous\ O_2(mL) = 0.67\ * BV\ (mL) * (arterial\ O_2content - 5\ vol\%)$$

Statistical analysis

All data was analyzed using R software (R Development Core Team, 2011). Data from each animal were treated as repeated measures by including animal ID as a random effect, using linear mixed-effects models (lme) from the nlme package (Pinheiro et al. 2011). Models were run using the maximum likelihood method. Fixed factors for blood and TBO parameters included group (diving or non-diving) and season, and for dive data included dive duration (2 – 7 minutes), depth (10 m or 40 m) and type of dive (single or bout). If multiple fixed factors were significant, nested models (with or without a fixed effect) were compared using a log likelihood ratio test to determine the best overall model to fit the data (Pinheiro and Bates 2000). A repeated measures ANOVA on a single model was performed to determine if the slope and intercept were significant. For significant categorical factors, post-hoc tests (using the Bonferroni method) were performed to compare the means between multiple groups. Values are reported as means (± st. dev.) and significance was set at $\alpha = 0.05$.

Results

Blood oxygen stores of diving and non-diving sea lions

There were no significant differences in blood oxygen stores between the diving and non-diving groups of captive-raised animals or between the seasons within the non-diving group (Table 2.1). Mass-specific blood oxygen stores averaged 15.5 (± 2.6) ml O_2 kg^{-1}. Therefore, estimated TBO averaged 35.9 (± 2.6) ml O_2 kg^{-1} (with lung and muscle O_2 stores comprising 8.25 and 12.2 ml O_2 kg^{-1}, respectively). All blood parameters measured (BV, PV, Hct and [Hb]; Table 2.1) were slightly higher in winter (non-diving group), but only mass-specific blood volume showed a significant difference between seasons (Fig. 2.1; Season vs. BV (ml kg^{-1}), $p = 0.002$; Bonferroni: $p < 0.001$ for winter vs. both spring and summer) resulting in slightly higher blood O_2 stores as well (p 0.051). Blood volume averaged 109 ml kg^{-1} in winter (non-diving) and 99.6 ml kg^{-1} and 97.5 ml kg^{-1} in the spring (non-diving) and summer (diving) groups (overall range 94 – 115 ml kg^{-1}). Plasma volume ranged from 51 – 63 ml kg^{-1}. I did not compare TBO between groups as the lung and muscle components were estimated based on mass scalars.

Metabolic rate

Resting metabolic rate (RMR) in non-diving animals was 1.29 L O_2 min^{-1} (for animals averaging 172 kg), which was lower than pre-diving metabolism in diving animals (1.78 L O_2 min^{-1} for animals averaging 193 kg). However metabolism was measured in different environments for the two groups; the non-diving animals spent up to 40 minutes in a pool compared with the diving animals that rested in a small open ocean pen prior to their first dive. As the latter was measured just prior to their dive it was likely higher than true resting values, and is defined as a "pre-dive" metabolic rate.

Diving metabolic rate was higher than pre-dive metabolism when calculated based on both the "dive event" (dive + recovery, DMR_{cycle}) and when all excess oxygen consumed was attributed to the dive only (DMR_{dive}). Mean DMR_{cycle} for single dives (n = 43 single dives, both depths included, grand mean calculated from average for each individual) was 2.24 L O_2 min^{-1} and for a bout of four consecutive dives (n = 40 bout dives, both depths included, grand mean calculated from average for each individual) was 2.44 L O_2 min^{-1} (Table 2.2). Mean DMR_{dive} for single dives was 2.88 L O_2 min^{-1} but also depended significantly on dive duration (Fig. 2.2; $p < 0.001$). A "minimum" DMR_{dive} was estimated as 2.68 L O_2 min^{-1} for dives >4.5 minutes, as DMR_{dive} for these dives no longer depended on dive duration (Fig. 2.2).

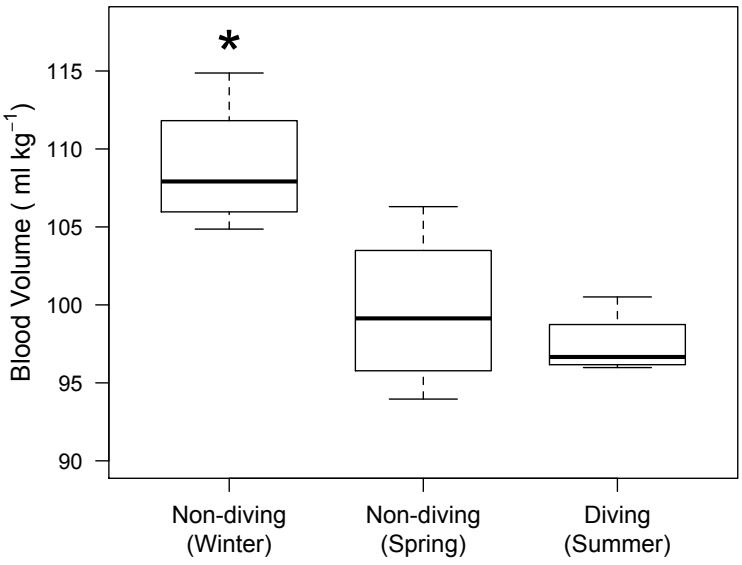

Figure 2.1 Blood volume (ml kg^{-1}) of diving and non-diving Steller sea lions as measured by Evan's blue dilution. Blood volume during winter (non-diving, n = 4) was significantly higher (*) than in the spring (non-diving, n = 4) or summer (diving, n = 4)

Table 2.1 Blood parameters including hematocrit (Hct), plasma volume (PV), blood volume (BV), hemoglobin concentration [Hb], blood O_2 stores, total body oxygen stores (TBO) and mass (day of blood O_2 store measurement) of diving and non-diving captive Steller sea lions

Animal	Group	Season	Mass (kg)	Hct	PV (ml kg^{-1})	BV (ml kg^{-1})	[Hb] (g/l)	Blood O_2 (ml kg^{-1})	TBO (ml kg^{-1})
F97BO	Diving	Summer	157	0.42	55.7	96.0	155	14.4	34.8
F00HA	Diving	Summer	175	0.44	56.3	100.5	160	15.7	36.1
F00YA	Diving	Summer	218	0.43	55.3	97.0	141	13.0	33.4
F97SI	Diving	Summer	225	0.41	56.8	96.3	150	13.9	34.3
Average			**194**	**0.43**	**56.0**	**97.5**	**152**	**14.2**	**34.6**
F03AS	Non-diving	Spring	171	0.40	56.4	94.0	137	12.1	32.5
F03RO	Non-diving	Spring	171	0.49	51.3	100.7	170	16.9	37.3
F03WI	Non-diving	Spring	178	0.50	53.1	106.3	166	17.3	37.8
F03IZ	Non-diving	Spring	180	0.40	58.6	97.6	137	12.6	33.0
Average			**175**	**0.45**	**54.9**	**99.6**	**153**	**14.7**	**35.1**
F03AS	Non-diving	Winter	174	0.49	55.5	108.8	175	18.9	39.3
F03RO	Non-diving	Winter	164	0.48	59.7	114.9	169	19.1	39.6
F03WI	Non-diving	Winter	176	0.49	54.6	107.1	173	18.4	38.8
F03IZ	Non-diving	Winter	169	0.40	62.9	104.9	137	13.5	33.9
Average			**171**	**0.47**	**58.2**	**108.9***	**164**	**17.5**	**37.9**

*Mass specific blood volume is significantly higher in winter than in spring or summer

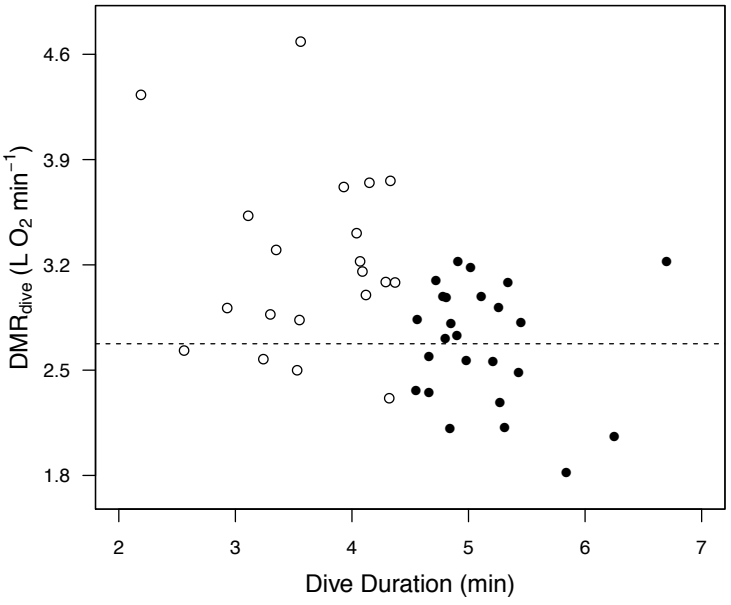

Figure 2.2 Diving metabolic rate as a function of dive duration. DMR is calculated vs. dive time only (DMR_{dive}). DMR_{dive} decreases significantly as dive duration increases. The dashed line represents the average DMR_{dive} for dive durations > 4.5 minutes (filled circles) used to calculate ADL.

Table 2.1 Metabolic rates and aerobic dive limit of Steller sea lions including pre-dive (surface) metabolic rate (MR_S), diving metabolic rate as a function of: the single dive cycle (DMR_{cycle}), dive time only (DMR_{dive}) and a dive bout cycle (DMR_{bout}). Mass (average for the trial period), calculated aerobic dive limit (cADL) based on DMR_{cycle} and on the minimum estimate of DMR_{dive}, mean dive duration of single long dives and mean dive duration to cADL ratio are also listed

Animal	Mass (kg)	MR_S (L O_2 min^{-1})	DMR_{dive} (L O_2 min^{-1})	DMR_{cycle} (L O_2 min^{-1})	DMR_{bout} (L O_2 min^{-1})	TBO (ml O_2 kg^{-1})	cADL (DMR_{cycle})	cADL (minimum DMR_{dive})	Mean dive duration (min)	Dive duration: cADL ratio
F97BO	158	1.42	2.54	1.88	2.17	34.8	2.9	2.5	3.8	1.3
F00HA	170	1.72	2.60	2.11	2.21	36.1	2.9	2.6	5.0	1.7
F00YA	216	1.77	3.05	2.34	2.59	33.4	3.1	2.4	4.2	1.3
F97SI	226	2.20	3.33	2.62	2.80	34.3	3.0	2.5	4.5	1.5
Average	**193**	**1.78**	**2.88**	**2.24**	**2.44**	**34.6**	**3.0**	**2.5**	**4.4**	**1.5**

Aerobic dive limit of Steller sea lions

Aerobic dive limit based on DMR_{cycle} for a single dive was 3.0 minutes and based on DMR_{dive} (for dives >4.5 minutes) was 2.5 minutes. Both of these estimates were shorter than the average single dive duration of the captive animals for my study, which was 4.4 (± 0.5) minutes (Fig. 2.3). However, they were slightly longer than the average dive time for repeated dives in a bout, which was 2.0 (± 1.3) minutes, with an average surface interval of about 18 (± 14) seconds between these dives.

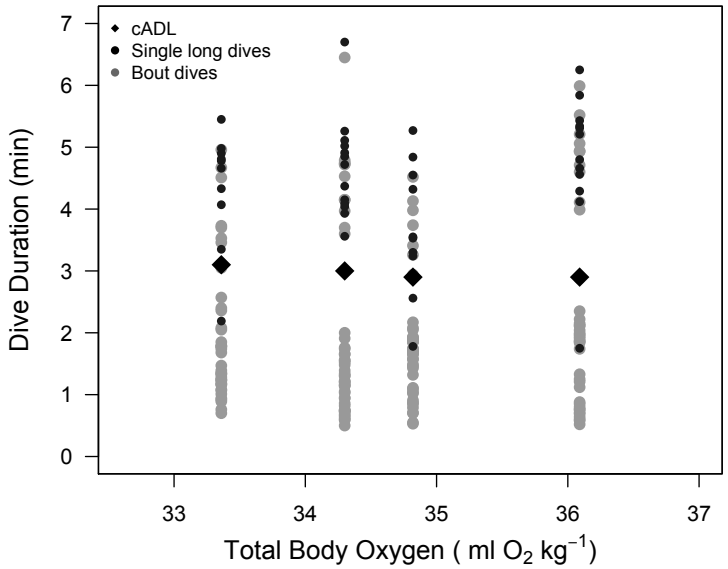

Figure 2.3 Calculated aerobic dive limit (cADL) and dive durations as a function of total body oxygen stores (ml O_2 kg^{-1}) for each of the four diving animals. Single long dives (*dark circles*) are typically longer than the cADL (*diamonds*, based on DMR_{cycle}). Bout dive durations (*grey circles*) for each animal are also shown.

Discussion

Oxygen stores and consumption

The average TBO of adult female Steller sea lions in my study was 35.9 ml O_2 kg^{-1}, which was slightly lower than estimates from previous studies of 38.8 and 40.4 ml O_2 kg^{-1} conducted on younger sea lions (Richmond et al. 2006; Lenfant et al. 1970). The effect of exercise and diving activity on TBO in adult animals is unknown. I predicted actively diving animals may have higher hemoglobin concentrations or higher blood volumes that would result in higher blood oxygen stores. However, blood O_2 stores did not differ significantly between the sea lions that were on an active diving regime and those that were not. Although development of oxygen stores in otariids occurs over the first few years of development (Fowler et al. 2007; Richmond et al. 2006; Weise and Costa 2007), it may be relatively static in later years. Both groups of sea lions had been initially raised in the same captive environment that did not allow them to dive beyond three meters during the developmental period. Thus, the group that was later moved to the Open Water site did not have the opportunity to develop a strong diving ability during this formative period, resulting in the lack of differences between groups that was not overcome by later differences in exercise regimes. Conversely, blood O_2 stores may not be significantly affected by early diving experience (Kodama et al., 1977), and therefore my values are representative of animals in the wild. Surprisingly, I found that blood volume was higher in non-diving animals. However, these differences in blood volume did not translate into significantly greater blood oxygen stores and were only higher in the winter season. Thus I suspect the differences reflected seasonal changes in physiology rather than previous diving activity.

Differences in TBO due to activity or exercise could be manifested as changes in myoglobin concentration or muscle mass, which I was unable to measure. This has not been studied in pinnipeds, but myoglobin concentrations have been shown to be higher in dive-trained tufted ducks and exercising bar-headed geese than inactive controls (Stephenson et al. 1989; Saunders and Fedde 1991). Based on this, it is conceivable that the diving sea lions in my study may also have had elevated myoglobin concentration (that would have resulted in higher TBO) due to their years of diving experience, even though they were not diving at a young age.

In general, otariids have much lower mass-specific TBO than has been found in phocid seals (Lenfant et al. 1970; Burns et al. 2005; Burns et al. 2007; Kooyman et al. 1983; Ponganis et al. 1993; Noren et al. 2005). This is also reflected by shorter than expected dive times relative to their body size (Schreer and Kovacs 1997). Among otariids, Steller sea lions also have a lower mass-specific TBO than most other sea lions and fur seals. This can be attributable to differences in either blood volume or muscle myoglobin concentration. For example, while blood volume of Steller sea lions in my study was similar to northern fur seals, young Steller sea lions and California sea lions (Lenfant et al. 1970; Ponganis et al. 1997b), adult female California sea lions have higher myoglobin concentration resulting in greater TBO (Weise and Costa 2007). Conversely, while the muscle myoglobin concentration in Steller sea lions is similar to that of New Zealand and Australian sea lions, these species have a much higher blood volume (i.e., 152 ml O_2 kg^{-1} in New Zealand sea lions and 178 ml O_2 kg^{-1} in Australian sea lions, vs. 97.5 ml O_2 kg^{-1} in the Steller sea lions in my study) also resulting in greater TBO (Costa et al. 1998; Fowler et al. 2007). Clearly, a better understanding of the selective pressures and ecological consequences of differences in TBO among different otariids would benefit from a more comprehensive study of actual blood and muscle oxygen store measurements across species.

In my study, diving $\dot{V}_{O_2}$ was higher than "pre-dive" metabolism (1.78 L O_2 min^{-1}; 9.44 ml O_2 kg^{-1} min^{-1}), whether measured over the entire dive cycle ($DMR_{cycle,}$ 2.24 L O_2 min^{-1}; 11.6 ml O_2 kg^{-1} min^{-1}) or when all excess oxygen was attributed to the dive only ($DMR_{dive,}$ 2.88 L O_2 min^{-1}; 15.0 ml O_2 kg^{-1} min^{-1}). A previous study on the same group of animals found slightly lower metabolic rates, but a similar pattern — DMR_{cycle} was 9.9 ml O_2 kg^{-1} min^{-1} and was slightly higher than resting rates of 8.2 ml O_2 kg^{-1} min^{-1} (Fahlman et al. 2008). These results are in contrast to an earlier study that found Steller sea lions had a relatively low DMR_{dive} which decreased to ~6 ml O_2 kg^{-1} min^{-1} from pre or post-dive resting rates (Hastie et al. 2007). However, the study by Hastie et al. (2007) only used one feeding tube at depth, so it is likely the animals were much less active while diving than in the previously mentioned studies that employed two feeding tubes to encourage an active dive, representative of foraging behaviour. The apparent decrease in metabolism during diving may also have been because pre-dive MR's (~11 ml O_2 kg^{-1} min^{-1}) were slightly higher and recovery periods were assumed to be shorter, resulting in relatively less consumed oxygen being attributed to the

dive itself. This highlights the importance of ensuring complete metabolic recovery when estimating the true costs of diving. A similar reduction in metabolism during diving from "pre-dive" metabolic rate (10.23 down to ~6 ml O_2 kg^{-1} min^{-1}) was seen in California sea lions (Hurley and Costa 2001). However, this study reported the "submersion" metabolic rate on animals in an aquarium, not from animals that were actively diving. The only other oxygen consumption rates that have been measured on freely diving animals are those of Weddell seals. Kooyman (1973) originally found their DMR to be about twice as high as resting values, whereas Castellini et al. (1992) found that diving $\dot{V}_{O_2}$ was similar to metabolism at rest except for long dives in which there was a significant decrease in DMR that was also correlated with dive duration.

Aerobic Dive Limit

The cADL based on DMR_{cycle} for a single dive was 3.0 minutes, and was 2.5 minutes based on the estimate of DMR_{dive} for long dives. These values are shorter than the 3.7 to 15 minutes (Richmond et al. 2006) and 3.7 to 12.2 minutes (Hastie et al. 2007) that have been previously estimated for Steller sea lions. The 12.2 minute estimate of cADL was based on a DMR that was much lower than I found in my study (and was also lower than the DMR estimated by Fahlman et al. 2008). The 3.7 minute estimate by Richmond et al. (2006) was based on an estimated DMR that was very close to my value (but calculated to account for the cost of transport while diving; Rosen and Trites 2002)—but was longer due to a higher TBO estimate.

Interestingly, my estimate of cADL coincides with the only study to compare the cADL and measured "lactate" ADL in an otariid. In this case, a field metabolic rate corresponding to 4.8 x basal metabolic rate (BMR; Kleiber 1975) was used to calculate an ADL of 1.8 – 2.0 minutes for juvenile California sea lions, which underestimated their measured ADL of 2.3 minutes (Ponganis et al. 1997b). For California sea lions, a DMR of ~4 x BMR would be needed to accurately predict the measured ADL. Coincidentally, the measured DMR over a dive cycle (DMR_{cycle}) in my study was 4 x BMR, suggesting that I determined an accurate estimate of the ADL in Steller sea lions. However, further direct measures of ADL in otariids are needed to provide support for this value, as it contrasts with studies comparing the measured and calculated ADL in phocids and cetaceans that suggest

assuming a DMR of 2 x BMR best approximates the ADL (Williams et al. 1999; Noren et al. 2012).

The cADL of 2.5 to 3.0 minutes for Steller sea lions is also much shorter than the average duration of their single dives of 4.4 minutes. Although this may imply the cADL is underestimated, Steller sea lions in the wild rarely dive this long. Dives of adult female Steller sea lions tend to be brief (averaging 1.9 to 2.4 minutes), shallow (averaging 21 to 53 m), and frequent (Merrick et al. 1994; Loughlin et al. 1998). The longest recorded dives for Steller sea lions have been for juveniles at >12 minutes but such long dives are extremely rare (0.04% of 692,000 recorded dives) and their mean maximum durations were ~6 minutes (Pitcher et al. 2005). In fact, 92% of dives in winter and 98% of dives in summer made by adult females were <4 minutes (Merrick and Loughlin 1997). This suggests that anaerobic processes allowed the longer, single dives I recorded to extend beyond the ADL. Although the animals in my study were diving freely in the open ocean to depths typical of a wild animal, there were also significant behavioural differences in their "motivation" to dive. The captive animals in my study only did one dive trial per day and had several days to rest and recover. Hence, they may not have been as concerned about developing a long term "anaerobic debt" or may simply have been more naïve about the consequences of diving beyond their ADL.

In contrast to the single long dives, dive durations of the sea lions when performing consecutive dives in a bout became progressively shorter and were, on average, ~2.2 minutes each, slightly less than their cADL, and more similar to the behaviour of wild animals. The sea lions also spent short (average 18 second) surface intervals between their dives, which may have been insufficient to fully replenish their oxygen stores—indicating a delayed payoff of oxygen debt. If there is no immediate need to fully balance oxygen stores or process accumulated lactate, single dives may be "uncoupled" from aerobic limits (Horning 2012). Hence a consecutive bout of diving (such as what animals in the wild perform) may be more constrained by aerobic limits than a single dive (Horning 2012) and the average duration of dives in a bout may be more similar to the ADL.

The cADL of Steller sea lions is low compared to other marine mammals, and is much lower than expected given they are the largest species of otariid. A review by Schreer and Kovacs (1997) defining allometric relationships for maximum dive duration and depth with body mass between all groups of diving mammals suggested that the maximum dive duration

of a 200 kg Steller sea lion should be 12.9 minutes. However, maximum dive durations of otariids do not correlate with body mass — unlike for phocids (Schreer and Kovacs 1997). Thus, this high ADL predicted from body allometery seems unreasonable, particularly in light of my direct measurements of metabolism and blood O_2 stores.

Some otariids have a greater tendency to dive beyond their cADL than others. This apparent reliance on anaerobic metabolism is likely more related to different foraging strategies and dive behaviour than on physiological limits (Costa et al. 2001; Costa et al. 2004). For example, benthic hunters are more likely to dive beyond their cADL than those that forage pelagically. It is thought this maximizes bottom time by decreasing transit time, especially when foraging for a more predictable prey source (Costa et al. 2004). Although using anaerobic metabolism may result in inefficient foraging in terms of submergence to surface times, other foraging strategies, or other O_2 management strategies may maximize prey capture by relying on some amount of anaerobic metabolism.

ADL has yet to be directly measured in Steller sea lions. However, I have shown that Steller sea lions have a much shorter cADL than previously thought and that blood oxygen stores are not related to level of physical activity or fitness. Given their relatively low body oxygen stores and higher diving metabolism, it would be interesting to determine whether the measured ADL of Steller sea lions is also lower than expected. My findings agree with the only study that compared measured and calculated ADL, and is the first to estimate cADL based on direct measures of $\dot{V}_{O_2}$ for an otariid. I believe that the relatively short 2.5 – 3 minute dive limit accurately reflects the cADL for Steller sea lions despite the consistently longer single dives performed by the animals in my study. In fact, most marine mammals are capable of diving well beyond their aerobic limits if needed; the maximum recorded dive duration of a Weddell seal is 82 minutes, which is 4 times their ADL of 20 minutes (Ponganis et al. 1993; Kooyman et al. 1980; Castellini et al. 1992), and the ADL of a juvenile California sea lion at 2.3 minutes, is much shorter than their longest recorded dive of 9.9 minutes (Feldkamp et al. 1989; Ponganis et al. 1997b). While limited evidence shows that wild Steller sea lions typically dive within the limits of my cADL, my study on captive animals shows that Steller sea lions can voluntarily dive much longer. However, extending dives beyond 3 minutes likely requires Steller sea lions to use anaerobic metabolism.

Chapter 3: Steller sea lions (*Eumetopias jubatus*) have greater blood volumes, higher diving metabolic rates and a longer aerobic dive limit when nutritionally stressed

Summary

Marine mammal foraging behaviour inherently depends on their diving ability. Declining populations of Steller sea lions in Alaska may be facing nutritional stress that could affect their diving ability through changes in body composition or metabolism. My objective was to determine whether nutritional stress (restricted food intake resulting in a ~10% decrease in mass) altered the calculated aerobic dive limit (cADL) of four captive Steller sea lions diving in the open ocean, and how this related to changes in observed dive behaviour. I measured diving metabolic rate (DMR), blood O_2 stores, body composition and dive behaviour prior to and while under nutritional restriction. I found that, when nutritionally stressed, the sea lions increased the duration of their single long dives, but also increased the proportion of time spent at the surface during a cycle of four dives and had higher mass-specific metabolic rates. While both lipid and lean body mass decreased, resulting in potentially lower muscle O_2 stores, there were greater offsetting increases in blood O_2 stores associated with higher blood volume (due primarily to increases in plasma volume) that increased total body O_2 stores. The greater rise in O_2 stores relative to the increase in DMR resulted in the sea lions having a longer cADL when nutritionally stressed. I concluded that nutritional stress did not negatively affect the diving ability of Steller sea lions, but that it did increase their DMRs and surface recovery intervals, requiring them to spend more time foraging to meet their energy requirements due to the resulting lower foraging efficiency.

Introduction

The foraging ability of marine mammals, such as Steller sea lions (*Eumetopias jubatus* Schreber 1776), is tied to their diving ability—particularly the depth and duration that they can regularly dive in order to obtain food. Limitations in diving capacity will affect both the quantity and type of food that is accessible and hence the amount of nutrition they can obtain. Nutritional stress is hypothesized to have contributed to the decline of Steller sea lions in the

wild (for review see Trites and Donnelly, 2003) through changes in the abundance, distribution or species composition of their prey (Benson and Trites, 2002; Trites et al., 2007).

Nutritional stress can lead to increased time spent foraging and diving to overcome this deficit. However, physiological and anatomical changes resulting from nutritional stress may negatively affect the foraging ability of Steller sea lions. Resulting decreases in total body oxygen stores (TBO) or increases in diving metabolic rate (DMR) could affect overall diving capacity or increase the energetic cost of diving and, therefore, impact subsequent energy intake (Rosen et al., 2007). For example, increases in resting metabolism (hypermetabolism) associated with an animal's "hunger response" (Cornish and Mrosovsky, 1965; Collier, 1969) may increase DMR. Conversely, fasting-induced hypometabolism could reduce the rate of O_2 use (Guppy and Withers, 1999), thereby decreasing foraging costs (although this is unlikely to occur in an actively foraging animal).

Previous studies have shown varying metabolic responses to fasting and food restriction in pinnipeds while on land. However, little is known about these metabolic responses while diving. Among Steller sea lions and harbour seals (*Phoca vitulina* Linnaeus 1758), periods of fasting or restricted diets of low quality prey result in decreased resting metabolic rates, typical of a 'fasting' response to conserve energy (Markussen, 1995; Rosen and Trites, 1999, 2002). However, restricted diets of high quality prey causes the metabolic rate of Steller sea lions to rise, which is indicative of a 'hunger' response that may be related to increased foraging effort (Rosen and Trites, 2002). The only study to have examined changes in DMR in fasted sea lions found that DMR increased in winter and remained unchanged in summer following a 10 day fast (Svärd et al., 2009). It is unclear whether the same response would be seen in an animal nutritionally stressed over a longer time period, or how a change in DMR corresponds to changes in body composition or translate into changes in foraging behaviour.

Net energy gained during foraging depends on the energy consumed (amount and nutritional quality of prey) and the energy needed to dive, and is considered most energetically efficient if done aerobically (i.e. relying only on O_2 stores). This aerobic diving ability is generally expressed as the aerobic dive limit (ADL, or diving lactate threshold; Butler, 2006). This can be calculated (cADL), using measures of O_2 stores and DMR, and used as a proxy for the measured ADL; originally defined by Kooyman et al., (1980) as the dive duration after

which post-dive blood lactate levels rise beyond resting levels due to anaerobic metabolism. Anaerobic metabolism is energetically expensive (Kooyman, 1989) and results in longer recovery times for a given dive duration—hence relying on anaerobic metabolism while diving could reduce net energy gained during a foraging bout depending on the quality, abundance and distribution of prey available. A potential increase in DMR in a nutritionally stressed animal would result in a decrease in the cADL, either limiting foraging time or increasing reliance on anaerobic metabolism, thereby decreasing foraging efficiency through increased metabolic overhead.

However, the aerobic dive limit also depends on total body O_2 stores (TBO). TBO consist of the O_2 stored in the lungs, blood and muscle, and generally scales to body mass (M_b). Marine mammals have adaptations that result in higher O_2 stores through increased mass-specific blood volume, hematocrit (packed cell volume), hemoglobin concentration and myoglobin concentration. Studies with captive animals have shown Steller sea lions experience changes in body composition due to a loss of lipid stores and lean body mass when on restricted or low quality diets (for review see Rosen, 2009). Such nutrition-induced changes in body composition could potentially alter TBO through changes in muscle mass (due to loss of lean body mass) or blood volume (possibly due to changes in body water content). TBO has been shown to change with age in several species of pinnipeds, as young animals develop O_2 stores and diving ability (Burns et al., 2005; Noren et al., 2005; Richmond et al., 2006; Fowler et al., 2007; Weise and Costa, 2007). However, aside from a few studies of seasonal changes in TBO (MacArthur, 1990; Villegas-Amtmann and Costa, 2010; Villegas-Amtmann et al., 2012), plasticity in O_2 stores in adult animals has received relatively little attention, and there are no studies examining how changes in nutritional status or body composition could alter TBO in adult individuals.

In addition to physiological changes, nutritional stress may also alter dive behaviour, whereby animals increase dive depth and durations, or alter O_2 management strategies to compensate for changes in abundance and distribution of prey. For example, animals may have to decrease dive durations to maintain efficient foraging within O_2 store limitations if TBO decreases or if the cost of diving is elevated by a higher DMR. Conversely, animals may be more likely to dive closer to their physiological limits to maximize prey capture (including

relying more heavily upon anaerobic metabolism) if prey abundance or distribution is limited, or in order to overcome past nutritional deficits.

My objective was to determine whether nutritional stress impacts the aerobic diving capacity and behaviour of Steller sea lions. Four captive animals trained to dive in the open ocean were subjected to a period of restricted food intake to simulate a nutritional restriction in the wild. I measured dive behaviour and metabolic rates of animals actively diving in the open ocean prior to and while under nutritional stress, and estimated changes in TBO by measuring changes in body composition (to estimate loss of muscle mass) and blood O_2 stores. I then used TBO and DMR to calculate the ADL for the sea lions before and during nutritional stress, and compared this to observed changes in dive behaviour. I hypothesized that nutritionally stressed animals would have a lower ADL, due to decreases in TBO. The results of my study can be used to assess potential impacts of changes in diving capacity due to changes in body composition and physiology on foraging costs, and infer whether nutritionally stressed sea lions in the wild have to expend more energy to dive and obtain food.

Methods

Study design/data collection

I measured changes in the blood O_2 stores, body composition, metabolism and dive behaviour before and during a period of nutritional stress of four adult, female Steller sea lions in May – August 2011. All sea lions were collected from breeding rookeries as pups and raised in captivity at the Vancouver Aquarium (British Columbia, Canada). The animals (between 12 – 15 years old) were housed at the Open Water Research Station (Port Moody, BC), and have been actively diving in the open ocean for research purposes for 3 to 8 years (since 2003: F00SI, F00BO, 2005: F00HA or 2008: F00YA). All animals were previously trained to use the experimental equipment and performed all trials voluntarily under trainer control. All experiments were conducted under UBC Animal Care Permit #A07-0413.

Animals were fed a diet of herring (*Clupea pallassi*) and market squid (*Doryteuthis opalescens*) supplemented with vitamins. Animals were nutritionally stressed by restricting daily food intake, such that they lost ~10% (9.1 – 10.9%) of their initial M_b over a three week period. I then continued to restrict their daily intake to maintain them at ~10% below their initial mass (to a maximum of 15% as per Animal Care regulations) for an additional three

weeks, while the nutritionally stressed dive trials were conducted. Blood O_2 stores and body composition were measured at the beginning and end of the initial three week period of food restriction. Trials occurred concurrently with a study to assess how inspired gas concentrations affect post-dive recovery of O_2 stores and elimination of CO_2. Inspired gas concentrations were altered prior to the animal entering the metabolic dome, such that I could baseline [O_2] and [CO_2] to altered inspired levels, in order to calculate DMR.

Diving metabolic rate

DMR was determined for four Steller sea lions diving voluntarily over a series of dive trials. The experimental dive set-up and measurement of diving metabolism are described in chapter 2. Briefly, the animals were trained to dive between a (100 L) metabolic dome floating at the surface and feeding tubes that delivered fish pieces at depth. The rates of O_2 consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were measured using flow through respirometry. Specifically, a dried subsample of excurrrent air was analyzed for O_2 and CO_2 concentrations using Sable System FC-1B and CA-1B analyzers, respectively, coupled to a 500H mass flow generator and controller (Sable Systems Inc., Las Vegas, NV, USA) pulling air at 350 L min^{-1}. Metabolic data was analyzed using LabAnalyst X (Warthog systems, University of California) and equations from Lighton (2008).

Animals were fasted overnight prior to trials and weighed each morning. They were fed <0.5 kg during transport to the dive site to minimize any effect of heat increment of feeding on metabolic rate (Rosen and Trites, 1997). Pre-dive (surface) metabolic rate (MR_S) was measured at the start of each trial while animals rested inside the metabolic dome, and was calculated from the last 3 minutes of a 5-10 minute measurement period, when $\dot{V}_{O_2}$ was constant.

The animals were directed to undertake a single long dive, which was then followed by a 4-dive bout cycle. Following completion of either the single dive or dive bout, the sea lions remained at the surface in the respirometry dome until they were physiologically recovered ($\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ returned to pre-dive levels, ~8-12 minutes). During the dives, animals received ~20 gram pieces of herring every 5 seconds from two feeding tubes set at a depth of 40 m. The feeding rate was constant for both dive types and both nutritional states. Single dives were only included if >1.5 min and dive bouts continued regardless of individual dive duration

as long as the animal continued to surface inside the metabolic dome. Animals were previously trained for this experimental protocol (to complete a single dive, then a 4 dive bout separated by a full recovery) so they may have had some prior knowledge as to what dive type was expected of them. They chose both their dive and (in the case of dive bouts) inter-dive surface interval durations.

To facilitate comparing my results with those from other studies, I calculated DMR in two ways: first, by dividing the total O_2 consumed in excess of post-dive baseline in the recovery period over only the dive duration (DMR_{dive}) and, second, as the average $\dot{V}_{O_2}$ over the entire dive and following recovery period (DMR_{cycle}). In the case of bout dives, DMR_{cycle} was calculated as the average $\dot{V}_{O_2}$ for the entire cycle of dives (including inter-dive surface periods) and subsequent recovery period.

Blood oxygen stores

Blood O_2 stores were measured for each of the four diving sea lions at the Open Water Research Station as per chapter 2. Briefly, blood samples were taken from the caudal gluteal vein shortly after animals were anaesthetized using isofluorane gas. Blood O_2 stores were calculated from measures of blood volume (BV) and hemoglobin concentration (as per Ponganis et al., 1993; Richmond et al., 2006), where:

$$BV = PV * \frac{100}{(100 - Hct)} \qquad (3)$$

BV was calculated from hematocrit and plasma volume (PV), which was determined using Evan's blue dilution procedure (Gibson and Evans, 1937). Hematocrit, hemoglobin concentration (using the Sysmex sodium lauryl sulfate method), red blood cell (RBC) count, mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) values were determined by a commercial laboratory using a Sysmex automated hematology analyzer (Idexx Laboratories, Delta, BC).

Blood O_2 stores were calculated using BV and hemoglobin concentration, assuming an O_2 binding capacity of hemoglobin of 1.34 ml O_2 g^{-1} Hb (Kooyman and Sinnett, 1982). Blood was assumed to be 1/3 arterial, that it was 95% saturated at the beginning of the dive and

reduced to 20% at the end of the dive, and 2/3 venous, assumed to be 5 vol% less than initial arterial saturation (Ponganis et al., 1993; Richmond et al., 2006).

Body composition

Body composition was determined by deuterium dilution (Reilly and Fedak, 1990). After obtaining a background blood sample, animals were injected intramuscularly with a measured dose of deuterium oxide (D_2O dose, 0.10 - 0.15 mg kg^{-1}). A second blood sample was taken after a two-hour equilibrium period. Samples were centrifuged at 3500 rpm for 5 min and serum was stored at -70 °C until analysis. Serum and dose samples were analyzed by Metabolic Solutions Inc. (Nashua, NS, USA) for isotope concentrations to determine total body water (TBW). TBW was used to calculate total body lipid (TBL) using the equation from Arnould et al., (1996) derived for Antarctic fur seals (*Arctocephalus gazella* Peters 1875). The remaining mass of the sea lions was assumed to represent lean body mass (LBM) and was calculated by subtracting TBL from M_b (Arnould et al., 1996).

$$TBL\ (kg) = 0.927 * M_b - 1.309 * TBW(kg) + 0.265 \quad (4)$$

Aerobic dive limit

I calculated an ADL for the Steller sea lions before and during the time they were nutritionally stressed, taking into account the changes in TBO and DMR. Lung O_2 stores (based on a diving lung volume of 55 mL kg^{-1} and 15% O_2 content) and muscle O_2 stores were calculated as per previous studies (Richmond et al., 2006; also see Chapter 2) and I assumed no change in lung O_2 stores. I was unable to measure myoglobin concentration [Mb], and there is no published evidence to suggest that it changes when animals are nutritionally stressed. My calculations of ADL therefore assumed that [Mb] was constant and independent of nutritional state. I assumed muscle mass was initially 37% of M_b and, for the calculation of TBO and ADL, that all LBM lost was muscle mass (i.e. maximum muscle mass loss). Lung, muscle and measured blood O_2 stores were then combined to estimate TBO, which was divided by DMR_{cycle} to estimate cADL.

Statistical analysis

All data was analyzed using R software (R Development Core Team, 2011). Data from each animal were treated as repeated measures by including animal ID as a random effect, using linear mixed-effects models (lme) from the nlme package (Pinheiro et al., 2011).

Models were run using the maximum likelihood method. If multiple fixed factors were significant, nested models (with or without a fixed effect) were compared using a log likelihood ratio test to determine the best overall model to fit the data (Pinheiro and Bates, 2000). Conditional R^2 statistics (describing the proportion of variance explained by both the fixed and random effects) were calculated using the package lmmfit, v.1.0 for R (Maj, 2011). Values are reported as means (± s.d.) and significance was set at $\alpha = 0.05$.

For measures of blood parameters, body composition, MR_S, DMR_{cycle} and dive behaviour (dive and surface interval duration), linear mixed models were used to determine if there was a difference between pre- and post-treatment values. A repeated measures ANOVA on a single model was performed to determine if there was a linear relationship between DMR and dive duration (tested as a fixed covariate), with nutritional state tested as a fixed factor to determine if this altered the relationship. Metabolic rates were tested as absolute values, scaled to $M_b^{0.75}$ and scaled to M_b directly.

Results

Oxygen stores & body composition

Total M_b of the four Steller sea lions dropped by an average of 10.1% (9.1 – 10.9%) during the initial three-week period of food restriction, while significant increases occurred in total plasma volume, total blood volume, and mass-specific plasma and blood volume (Table 3.1). There was an increase in hematocrit (due to an increase in RBC count and no change in MCV), and a small but insignificant increase in [Hb] and decrease in MCH; hence hemoglobin production must have increased slightly following the increase in blood volume. Combined, absolute blood O_2 stores increased 32% (range 12 – 55%) from an average of 2.74 (±0.36) to 3.66 (±0.99) L O_2. This translated into a 48% increase on a mass-specific basis, from 14.2 (±1.1) mL O_2 kg^{-1} to 21.0 (±2.5) mL O_2 kg^{-1}. The mean values and range for each measured parameter before and during nutritional stress, as well as the mean, st. dev. and significance of the difference between pre- and post-restriction values are reported in Table 3.1.

I saw a significant decrease in total LBM, total body lipid and total body water (Table 3.1). Taking changes in M_b into account, there was a significant decrease in mass-specific body lipid and an increase in mass-specific LBM and mass-specific body water. In absolute terms, LBM decreased by an average of 9.9 (±0.7) kg, which assumedly resulted in a decrease

in muscle O_2 stores. All LBM lost may not have been skeletal muscle, and it is plausible [Mb] increased in nutritionally stressed animals, but I was unable to measure these parameters. To conservatively estimate TBO given these uncertainties, I assumed that all of the LBM lost was derived from skeletal muscle and that myoglobin concentration remained constant. This means that - at most - muscle O_2 stores would have decreased on average from 2.37 L O_2 (12.3 mL O_2 kg^{-1}) to 2.05 L O_2 (11.8 mL O_2 kg^{-1}).

Lung O_2 stores were assumed to remain constant (8.25 mL O_2 kg^{-1}) during nutritional stress and estimates were based on pre-nutritionally stressed mass. The increase in blood O_2 stores was much greater than the estimated loss of muscle O_2 stores, resulting in a slight increase in absolute TBO and a significant increase in mass-specific TBO (Table 3.1). Given that I assumed the maximum amount of muscle O_2 store loss and that [Mb] may also have been higher in nutritionally stressed animals, the increase in TBO was likely even greater than my calculation indicates.

Table 3.1 Mean, minimum and maximum values for body composition, blood parameters, estimated O_2 stores, diving metabolic rate and calculated aerobic dive limit at normal M_b and while nutritionally stressed in Steller sea lions (n=4). Mean and st. dev. of the difference in each parameter (final – initial value) are presented. P values are from linear mixed effects models accounting for repeated measures between animals.

		Normal			Stressed			Difference		
Parameter	**Units**	**Mean**	**Min**	**Max**	**Mean**	**Min**	**Max**	**Mean**	**St. Dev.**	**p**
Mass	kg	**194**	157	225	**174**	140	201	**-19.6**	3.90	<0.001
Total body water	kg	**116**	93.0	138	**109**	85.9	131	**-6.44**	0.51	<0.001
Total body lipid	kg	**28.1**	24.0	35.2	**18.4**	14.3	23.5	**-9.77**	3.78	0.002
Lean body mass	kg	**166**	133	197	**156**	122	186	**-9.86**	0.68	<0.001
Body water	% body mass	**59.8**	58.6	61.3	**62.7**	61.4	65.5	**2.94**	1.00	0.001
Body lipid	% body mass	**14.6**	12.6	16.2	**10.8**	7.1	12.5	**-3.83**	1.31	0.001
Lean body mass	% body mass	**85.5**	83.8	87.4	**89.4**	87.5	92.9	**3.83**	1.31	0.001
Plasma volume	L	**10.9**	8.7	12.8	**12.5**	10.3	15.8	**1.68**	1.07	0.016
Blood volume	L	**18.9**	15.1	21.7	**23.2**	18.1	29.8	**4.32**	2.77	0.016
Mass-specific PV	ml kg^{-1}	**56.0**	55.3	56.8	**71.7**	64.6	78.9	**15.7**	5.82	<0.001
Mass-specific BV	ml kg^{-1}	**97.5**	96.0	101	**132**	122	149	**34.8**	13.0	<0.001
Hemoglobin Conc.	g ml^{-1}	**152**	141	160	**161**	152	166	**9.00**	11.3	0.065
MCH	pg	**39.5**	38.3	41.0	**38.9**	37.1	39.9	**-0.60**	0.84	0.148
MCV	fl	**111**	105	120	**111**	105	116	**-0.58**	4.89	0.787
Hematocrit	%	**0.43**	0.41	0.44	**0.46**	0.43	0.47	**0.03**	0.02	0.008
RBC count	x 10^{12} L^{-1}	**3.9**	3.6	4.1	**4.2**	4.1	4.3	**0.30**	0.24	0.014
Blood O_2 store	L O_2	**2.74**	2.26	3.13	**3.66**	2.65	4.87	**0.92**	0.68	0.027
TBO	L O_2	**6.71**	5.48	7.74	**7.30**	5.52	9.14	**0.59**	0.68	0.096
Mass-specific Blood O_2	ml O_2 kg^{-1}	**14.2**	13.0	15.7	**20.7**	18.9	24.3	**6.51**	3.08	<0.001
Mass-specific TBO	ml O_2 kg^{-1}	**34.7**	33.5	36.2	**41.6**	39.4	45.6	**6.90**	3.44	<0.001
DMR Cycle (single)	ml O_2 kg^{-1} min^{-1}	**11.5**	10.8	11.9	**12.7**	12.1	13.3	**1.23**	0.73	0.004
DMR Cycle (bout)	ml O_2 kg^{-1} min^{-1}	**12.9**	11.2	14.3	**14.4**	12.0	16.1	**1.44**	0.57	<0.001
cADL	minutes	**3.0**	3.0	3.1	**3.3**	3.0	3.4	**0.25**	0.16	0.008

Dive metabolism and behaviour

There was no change in absolute MR_S or DMR_{cycle} (calculated over the "dive event") for either single or bout dives attributable to the nutritional stress event. However, due to the resultant decreased M_b, there was a significant increase in mass-specific MR_S and DMR_{cycle} for both single dives (Fig. 3.1, $p < 0.001$) and dive bouts (cycle of 4 dives; Fig. 3.2, $p < 0.001$) during the period the animals were nutritionally stressed. DMR_{cycle} for bout dives also depended on bout cycle duration ($p = 0.005$) for both stressed and non-stressed states. The same relationship is found whether mass-specific metabolic rates are scaled to $M_b^{0.75}$ (as is shown in Figs 3.1 & 3.2 as a function of dive duration) or to M_b directly.

The duration of single dives significantly depended on nutritional state, increasing from an average of 4.6 to 5.2 minutes when nutritionally stressed (Fig. 3.3, $p = 0.018$). Time to reach baseline MR post-dive (recovery in terms of $\dot{V}_{O_2}$) for single dives was dependent on dive duration (increased with longer dives) but was not significantly dependent on nutritional state. As a result, total dive cycle duration (dive and recovery) also increased in nutritionally stressed animals ($p = 0.006$).

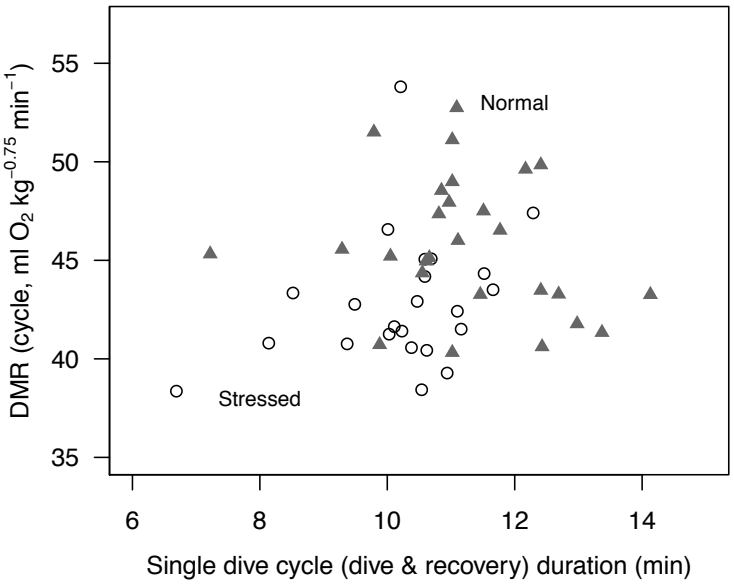

Figure 3.1 Diving metabolic rate as a function of dive cycle (dive and post-dive recovery) duration for single long dives in four adult Steller sea lions. DMR is scaled to $M_b^{0.75}$ and calculated as DMR_{cycle} (excess O_2 averaged over the entire "dive event"). DMR_{cycle} was independent of dive duration, but higher when nutritionally stressed ($p < 0.001$, *triangles*) than at normal mass (*circles*).

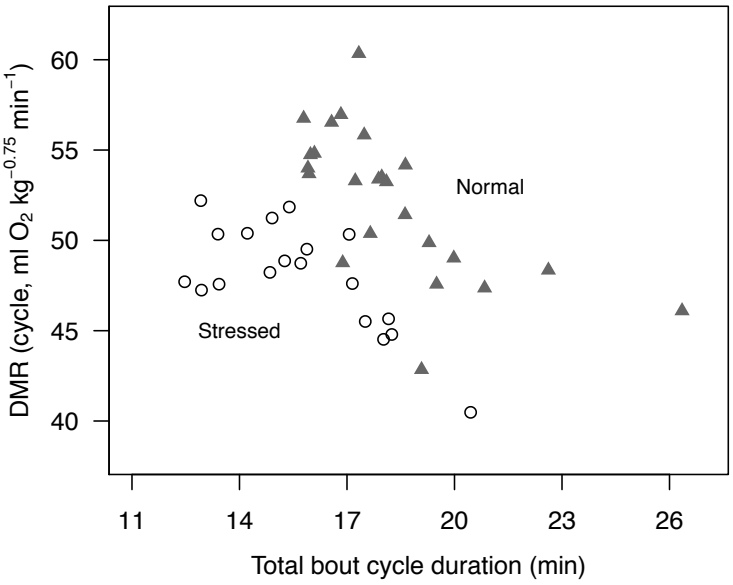

Figure 3.2 Diving metabolic rate as a function of bout (4 dives with surface intervals and post-dive recovery) cycle duration in four adult Steller sea lions (DMR_{cycle} calculated over the dive "event" and scaled to $M_b^{0.75}$). DMR_{cycle} was higher during nutritional stress (*triangles*, $p < 0.001$) than at normal mass (*circles*) and depended on bout duration ($p = 0.005$).

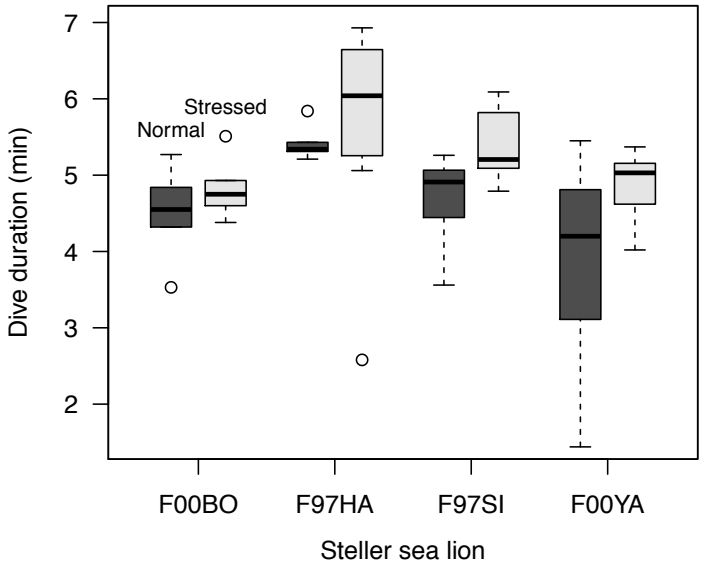

Figure 3.3 Average duration of single long dives for each of four adult Steller sea lions under each nutritional state (5 – 6 single dives per animal per state). Duration increased when nutritionally stressed (lt. grey, $p = 0.018$) as compared to normal mass (dk. grey). Open circles represent outliers.

This effect of nutritional state on single dive duration confounded measures of DMR_{dive} (calculated by dividing all excess O_2 consumed in the post-dive recovery period above baseline levels by dive duration only), which decreased significantly with increasing dive duration in both stressed and non-stressed sea lions. In other words, independent of any changes due to nutritional stress, DMR_{dive} should be lower in nutritionally stressed sea lions partly as a function of the longer dives they undertook. Therefore, to examine whether DMR_{dive} changed independent of dive duration, I compared the relationship between DMR_{dive} and dive duration for stressed and non-stressed animals. The relationship between absolute measures of DMR_{dive} and duration did not change with nutritional state, but nutritional state did significantly affect mass-specific DMR_{dive} such that, for a given duration, mass-specific DMR_{dive} is higher when animals were nutritionally stressed (Fig. 3.4, $p < 0.001$). Hence, as with MR_S and DMR_{cycle}, nutritional state had little affect on absolute values of DMR_{dive} but did result in increases in mass-specific values, seen when scaled to either $M_b^{0.75}$ (as shown in Fig. 3.4) or to M_b directly.

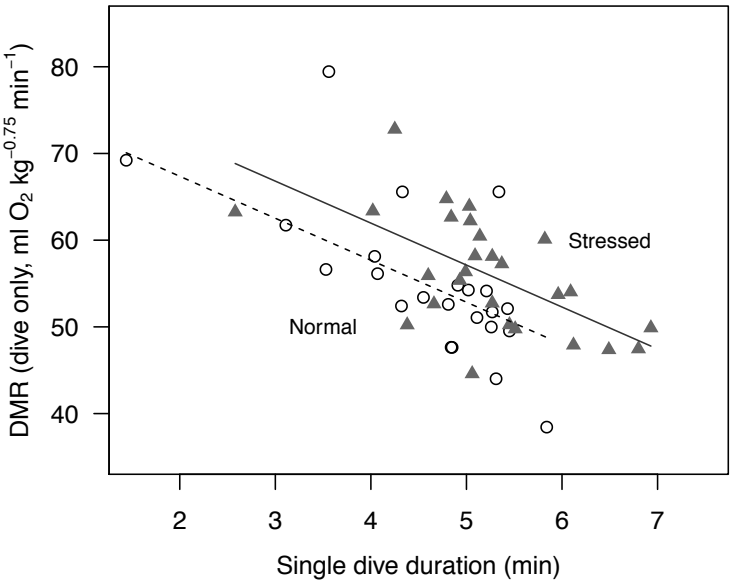

Figure 3.4 Diving metabolic rate as a function of dive duration for single long dives in four adult Steller sea lions ($R^2 = 0.49$). DMR is scaled to $M_b^{0.75}$ and calculated as DMR_{dive} (excess O_2 consumed in the post-dive recovery period divided by dive duration only). DMR_{dive} was higher during nutritional stress ($p = 0.017$, *triangles*) than at normal mass (*circles*) and depended on dive duration ($p < 0.001$).

As with single dive cycle duration, the total duration of a bout dive cycle (4 dive cycles and recovery) increased with nutritional stress ($p < 0.001$); however, this was due to the consistent increase in post-bout recovery time (time to return to baseline MR in terms of $\dot{V}_{O_2}$, $p = 0.002$; Fig. 3.5) rather than dive duration. Of the four animals, only two increased the duration of dives in a bout whereas the other two decreased bout dive duration. Hence, in contrast to single dives, there was no overall increase in the dive duration in the bouts during nutritional stress (Fig. 3.6a; average duration of bout dives was 2.2 ± 1.2 min for unstressed sea lions and 2.5 ± 1.6 min when stressed).

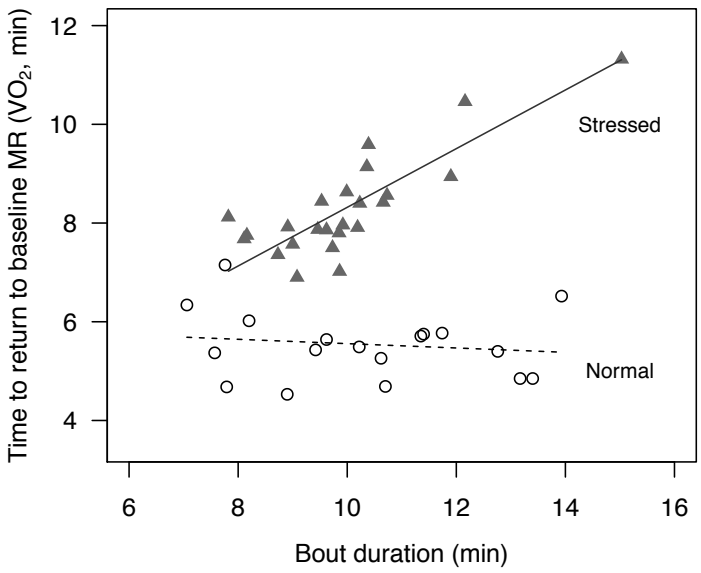

Figure 3.5 Time to reach baseline metabolic rate (recovery, in terms of $\dot{V}_{O_2}$) following a cycle of bout dives as a function of bout duration in four adult Steller sea lions under each nutritional state, normal (*circles*) and stressed (*triangles*). Bouts consisted of 4 dives to 40 m and 3 surface intervals, which comprised a higher proportion of the total bout duration in nutritionally stressed individuals. Significantly greater recovery time when stressed ($p < 0.001$) suggests they ended their bout with a greater depletion of O_2 stores, while the dependence on bout duration ($p < 0.001$ for stressed dives only) suggests they were not recovering O_2 stores between bout dives as shown by the constant recovery time following normal diving bouts.

There was also an overall increase in surface interval duration (from 21 ± 14 to 25 ± 12 secs; $p = 0.031$) during bouts when nutritionally stressed. As a result, all nutritionally stressed sea lions spent a greater proportion of time at the surface breathing in the metabolic dome for a given bout of diving (4 dives, with 3 inter-dive surface intervals; $p = 0.001$, Fig. 3.6b), in addition to the longer post-bout recovery. Spending more time at the surface relative to dive duration makes sense if the sea lions were using O_2 stores and producing CO_2 at a higher rate—which is consistent with the higher DMR_{cycle} for bout dives seen when they were nutritionally stressed (shown in Fig. 3.5 as a function of bout duration). When nutritionally stressed, recovery following a bout of diving also depended significantly on bout duration (Fig. 3.6, $p < 0.001$), indicating that nutritionally stressed animals were depleting O_2 stores to a greater level.

I calculated foraging efficiency as the amount of fish caught (grams) per ml of O_2 expended using mean measures of DMR and dive behaviour for each dive type and nutritional state (Table 3.2). The amount of prey available (fish delivery rate at depth) was kept constant between trials, and I assumed the energy needed to digest prey (digestive efficiency) did not vary with nutritional state. For single dives, the animals were able to consume slightly more fish per minute of a dive cycle (dive and recovery duration), but higher metabolic rates resulted in slightly lower foraging efficiency (~4% lower) when nutritionally stressed. For bout dives, both the amount of fish consumed per minute of dive cycle was lower and DMR was higher when nutritionally stressed, which combined to reduce foraging efficiency by 28%.

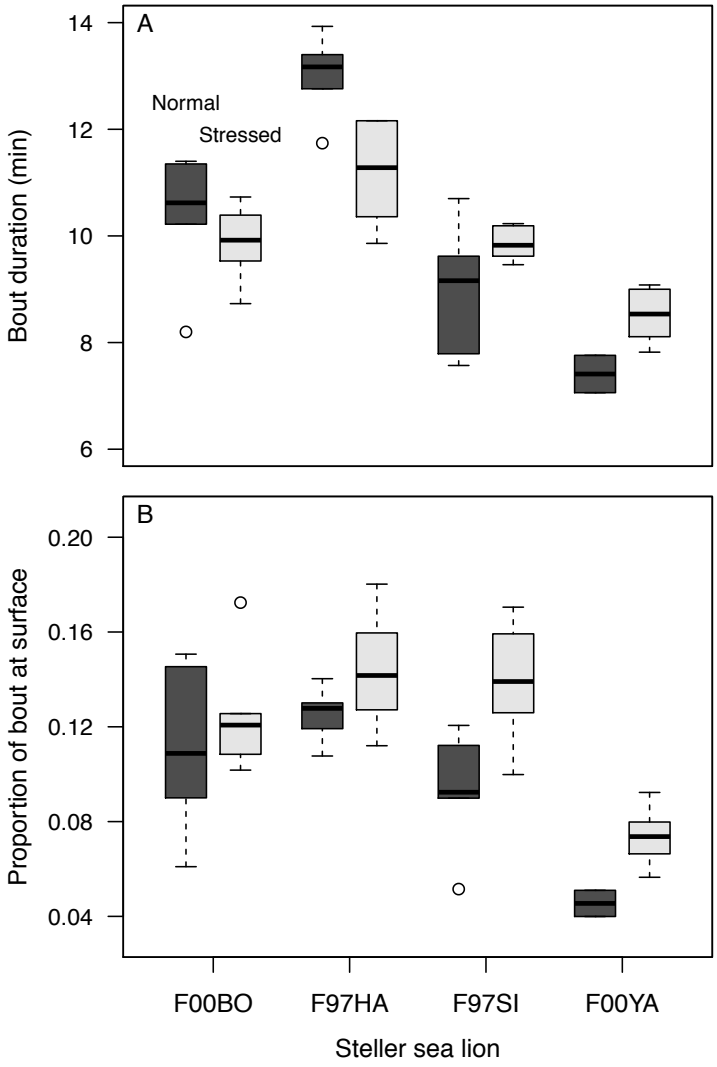

Figure 3.6 Bout dive behaviour for each of four adult Steller sea lions under each nutritional state (normal; lt. grey and stressed; dk. grey, 4 – 6 bouts per animal per state). (A) Total bout duration (4 dives & 3 surface intervals, not including final recovery period). (B) Proportion of the total bout duration spent at the surface. When nutritionally stressed animals either increased surface interval duration resulting in an increase in bout duration (F97SI & F00YA), or decreased dive duration and maintained surface duration (F00BO & F97HA). This resulted in a greater proportion of a 4-dive bout cycle (total surface interval duration/total bout duration) being spent at the surface when nutritionally stressed ($p = 0.001$), indicating they required longer surface intervals to recover O_2 stores. Open circles represent outliers

Table 3.2 Foraging efficiency per dive type and nutritional state (based on an average 194 kg sea lion at normal mass, and 174 kg while stressed). Mean duration is for a single long dive or a bout of 4 dives (total duration of 4 dives). Total dive cycle duration includes mean recovery time and (for bouts) total surface interval duration. Mean bottom time accounts for an average of 0.83 minutes for animals to transit to a depth of 40 m and back. Fish consumed is based on total bottom time per cycle multiplied by ~0.02 kg pieces of fish delivered via feeding tubes every 5 seconds to depth and reported per minute of a total dive cycle. Energy expended is based on DMR_{cycle} (assuming 20.35 kJ per ml O_2) and energy gained assumes an energy content of Herring of 7.72 kJ per gram (Rosen, pers. comm.). Foraging efficiency is the total energy gained divided by the total energy consumed (dive depth and fish delivery rates were kept constant).

Dive type	**Nutritional state**	**Mean single or bout dive duration**	**Mean recovery time**	**Total dive cycle/bout duration**	**Bottom time (% dive cycle)**	**Fish consumed (g min^{-1} of dive cycle)**	**DMR (energy expended, kJ min^{-1})**	**Energy gained (kJ min^{-1})**	**Efficiency (kJ gained/ expended)**
Single	Normal	4.59	5.64	10.23	36.8%	88.2	45.2	681	15.1
Single	*Stressed*	*5.20*	*6.03*	*11.23*	*38.9%*	*93.4*	*44.8*	*721*	*16.1*
Bout	Normal	9.10	5.51	15.71	36.8%	88.3	51.0	681	13.4
Bout	*Stressed*	*8.77*	*8.30*	*18.30*	*29.8%*	*71.5*	*51.1*	*552*	*10.8*

Discussion

Diving capacity is largely influenced by the aerobic dive limit and is dependent on limited O_2 stores and the rate at which these are consumed (Kooyman et al., 1980). Nutritional stress can significantly affect body composition and metabolic rate (for review see Rosen, 2009), and likely affects the aerobic dive limit as a result. Although developmental changes in both O_2 stores and metabolic rate have been demonstrated in pinnipeds, few studies have examined potential variation within individual adult animals. Specifically, it is unknown how changes in body condition or physiology due to nutritional stress will affect the cADL or subsequent dive behaviour and ability to forage.

Given the hypometabolic response generally seen in fasting pinnipeds on land (Markussen, 1995; Rosen and Trites, 2002), it is possible that the nutritionally stressed sea lions could extend their ADL by lowering their metabolism while diving. Steller sea lions tend to have higher DMRs relative to their mass than phocid seals (e.g. Weddell seals; Castellini et al., 1992), and may have a greater capacity to lower their metabolism while diving. However, it is more likely that adaptations to conserve O_2 stores and extend the ADL (such as the dive response) that are employed during diving under normal physiological conditions have already lowered diving metabolism to minimal levels, precluding any further reduction in diving metabolism when stressed. Hence, I expected that the sea lions' DMR would fall due to loss of mass (but remain unchanged on a mass-specific basis), since these individuals are actively foraging and hence likely cannot lower their metabolism as is seen in land-bound animals. However, in my study I saw no reduction in either DMR or MR_S, resulting in an increase in mass-specific metabolic rates.

It is generally accepted that marine mammals subjected to nutritional stress will experience differential losses of lipid and lean mass because of their tendency to conserve LBM and use (largely metabolically inert) lipid from the blubber layer as their primary energy source while fasting (Oritsland, 1990), although this generalization is largely formulated from studies of phocid seals that have greater overall lipid reserves. Hence, mass-specific DMR may increase simply due to the relative increase in the proportion of LBM. In my case, 48.4% (range 38.2% - 57.6%) of the mass loss of the study animals can be attributed to the lipid layer. However, when metabolism is scaled to LBM I still see an increase in mass-specific MR_S and DMR indicating that other factors besides the proportional increase in lean mass

must contribute to the overall increase in metabolic rate. An increase in resting metabolism was previously observed in Steller sea lions while consuming restricted quantities of high quality prey (Rosen and Trites, 2002), and DMR_{cycle} has also been observed to increase following 9-10 day fasts (Svärd et al., 2009).

This increase in MR may reflect a 'hunger' response, as suggested for (non-diving) sea lions by Rosen and Trites (2002), which is congruent with the increased foraging effort (longer dives) seen in the nutritionally stressed animals. Alternately, although mass was kept constant, it is also possible that body composition (% LBM) of the sea lions continued to change over the subsequent three-week period during which dive trials were conducted and MR was measured. However, there was no observed change in MR over the course of the nutritionally stressed trials. There could also be an added thermoregulatory cost due to loss of insulative lipid stores as I saw increases in both pre-dive MR_S and DMR, although summer water temperatures make this unlikely.

It might be suggested that the increased DMR seen in the nutritionally stressed sea lions was a product of increased digestive costs. While diving and digestion are often considered incompatible physiological processes under normal foraging conditions (Rosen 2009; Sparling et al., 2007), Svärd et al. (2009) suggested that the increased metabolism seen during diving in Steller sea lions following fasting might be due to the simultaneous costs of diving and the heat increment of feeding. Although such a strategy would decrease the efficiency of foraging, they argue that the immediate need to gain energy and replace depleted lipid stores from ingested food imposed by their previous mass loss overrides this consideration. Although a previous study found that MR did not increase until 60 min after a 2 or 4 kg meal in resting animals (Rosen and Trites, 1997), and the course of my dive trials typically lasted only about 40 min., the onset of digestion may be earlier in nutritionally stressed animals when diving. However, this is unlikely since the resting rates measured at the end of the dive trial were not elevated as compared to the pre-dive rates, as would likely be seen if digestion was occurring towards the end of my dive trials. Hence, increased digestion does not likely explain the higher DMR seen in the nutritionally stressed sea lions.

Differences in activity levels may also be responsible for the higher DMRs I observed in nutritionally stressed animals. Although the trials were designed to elicit the same activity levels (i.e. same dive depth, two tubes delivering food to create the same prey patch) they may

have been different if animals were more motivated to search for and catch prey while nutritionally stressed.

It is difficult to tease apart the effects of scaling and changes in M_b on metabolism—to know whether the observed increases in mass-specific MR_S and DMR_{cycle} were an artifact of decreases in M_b, or whether they described true physiological changes. The longer surface intervals (but not longer dive times) that occurred between the consecutive dives in a bout by the animals suggests the increase in mass-specific rate was an actual added cost to diving, as the animals spent more time in the surface interval to refill O_2 stores and remove accumulated CO_2 relative to dive durations, and were unable to delay this added surface time to the post-dive recovery period (Fig. 3.5).

Animals can dive past their physiological (aerobic) limits and rely more on anaerobic metabolism, which could explain the significantly longer recovery times of the sea lions following a diving bout of equal length when nutritionally stressed. The longer recovery times following a series of dives occurred despite longer surface intervals between those dives that, all other things being equal, should have decreased the recovery time needed following the bout. In fact, the time to return to baseline MR increased with the duration of the bout of diving indicating the nutritionally stressed sea lions were not recovering their O_2 stores between dives, hence were depleting O_2 stores to a lower level (and were likely accumulating greater amounts of CO_2). This contrasts sharply with the constant recovery times that followed the diving bouts (independent of duration) when the sea lions were not nutritionally stressed (Fig. 3.5). The surface interval duration may be more dependent on CO_2 elimination than on refilling of O_2 stores, as it typically takes longer for CO_2 to be fully removed from the body than for O_2 stores to be refilled (Boutilier et al., 2001). The time to return to baseline $\dot{V}_{CO_2}$ was also slightly higher in nutritionally stressed animals, although, the effect of nutritional stress on CO_2 recovery was not nearly as great as the increase in O_2 recovery time.

Although a higher DMR increased the cost of diving, it did not shorten the cADL of Steller sea lions as they had significantly higher blood O_2 stores as well. I had expected nutritionally stressed sea lions to have lower TBO simply because of mass loss (and related muscle loss). Loss of LBM impacts muscle O_2 stores due to a loss of skeletal muscle mass as protein stores are catabolized. Unfortunately, I was unable to directly measure muscle mass. However, I assumed that substantial loss of muscle O_2 stores must have occurred given the

significantly lower LBM of the sea lions after the episode of nutritional stress. Although it is unclear how much of the LBM lost during periods of food restriction or fasting comes from skeletal muscle, it is likely a significant portion (see Cherel et al., 1994). By my calculation the amount of LMB lost over the initial 21 day period of food restriction would represent ~14% (±2.9%) of initial muscle mass, assuming the 'worse-case scenario' (i.e., that 100% of LBM lost was muscle). The only study to have directly measured muscle mass (by computed tomography) during a mass loss event found grey seal pups (*Halichoerus grypus* Fabricius 1791) lost ~20% of their muscle mass during a 31 day post weaning fast (Nordoy and Blix, 1985).

Resultant muscle O_2 stores will also depend on myoglobin concentration, which could increase as skeletal muscle protein is utilized. Several studies have shown increases in muscle myoglobin concentration concurrent with mass loss due to hibernation or fasting (Galster and Morrison, 1975; MacArthur, 1990; Noren et al., 2005). This potential increase in muscle myoglobin concentration implies that loss of muscle O_2 stores may be significantly less than estimated based on changes in muscle mass alone, and that mass-specific muscle O_2 stores may be maintained or actually higher in nutritionally stressed animals.

Changes in body composition also affected blood O_2 stores through changes in blood volume. Blood volume scales to M_b between species and individuals, but the effects of M_b changes within an individual are less clear. Significant increases in the blood volume of sea lions in my study was accompanied by increases in body water as a percent of M_b, although total body water decreased on an absolute basis. This may indicate that the sea lions were retaining more water during the food restriction as lipid and protein stores were catabolized, which could have contributed to the increase in plasma volume despite the loss of mass. Higher protein concentration in the blood (caused by catabolism of protein stores) may also have an osmotic influence, causing retention of water. Increases in urea and lower BUN:Creatinine ratios generally indicate dehydration in nutritionally stressed animals (Dierauf and Gulland, 2001). However, I saw opposite trends in these parameters, which may be indicative of a high level of hydration that contributed to the increase in plasma volume. Decreases in urea (as opposed to the expected increase) were also seen in previous studies of Steller sea lions on restricted herring diets (Rosen et al., 2004). The changes in blood volume I

observed may also be transient in nature (i.e. more a consequence of losing mass, than being at a lower mass or altered body composition).

Blood O_2 stores were also measured in a group of sea lions (non-diving; i.e. in an aquarium) before and after an episode of nutritional stress during another study where the sea lions were consuming different types of prey (Fig. A.5.1; Table A.5.1). Overall, the effect of nutritional stress on plasma volume was less pronounced in these animals, but the data also indicate the effect could be dependent on prey quality. Of the four animals measured, one sea lion that was fed the same restricted diet (herring) had higher plasma volumes, whereas one individual that was fed a low fat diet (Atka mackerel) had significantly lower plasma volume. In general, it seems animals on a restricted diet of higher quality prey show differing trends in body composition and utilization of lean and lipid mass stores than those on lower quality diets (Rosen and Trites, 2001; Jeanniard du Dot et al., 2008). Hence the effect of nutritional stress on plasma volume may also be dependent on diet quality. However, it is important to note that these animals were also measured in a different season (winter) and were not actively diving, either factor could also explain the differences seen.

Increased blood O_2 stores have also been seen in grey seal pups during the post-weaning fast, although this was due to higher hemoglobin concentrations as opposed to higher blood volumes (Noren et al., 2005) and was attributed only to developmental changes (as opposed to a reaction to mass loss). I saw a small, but insignificant increase in hemoglobin concentration in my study (paralleled with a small decrease in MCH), indicating there was a small increase in hemoglobin production, but not enough to match the increase in red blood cells. However, these changes were all fairly small and probably not biologically significant as they were within the range normally seen in this group of animals (D. A. S. Rosen, unpublished data).

The substantial increase in blood O_2 stores of the sea lions experiencing nutritional stress more than compensated for the potential loss of muscle O_2 stores. This resulted in an increase in both absolute and mass-specific TBO, in spite of the lower mass of the nutritionally stressed animals. Despite a higher DMR this increase in O_2 available for use during the dive increased the cADL (from 3.0 to 3.3 minutes) and was consistent with the significant increase in the average duration of their single long dives (from 4.6 to 5.2 minutes).

The single long dives performed by the animals in this study were significantly longer than their cADL, under both stressed and unstressed nutritional states. As I did not directly measure ADL, it is plausible the cADL is underestimated. However, my estimate of cADL agrees with the behaviour of Steller sea lions in the wild, that typically undertake dives ~2 - 2.4 minutes long (Merrick and Loughlin, 1997; Loughlin et al., 1998). Additionally, the average duration of the 4 dives performed in a cycle of dives (bout), was much shorter (2.3 min when unstressed and 2.5 min when stressed) than the single long dives, and closer to the cADL estimate. As my study sought to determine whether nutritional stress limited dive behaviour or ability, the parameters of the dive trials encouraged the sea lions to dive as long as possible (i.e. food delivery was at a high rate to encourage longer dives, and animals were fed minimally when not participating in trials). Therefore, I believe the animals were diving beyond their aerobic limits for their single long dives, especially when nutritionally stressed. It is conceivable that captive animals may be naïve to the consequences of diving beyond their aerobic limits, or less concerned with developing an O_2 debt. As transit time was constant, the increase in dive duration for these single dives increased their effective foraging (bottom) time (by 17%), thereby allowing them to increase their intake of energy for a single dive cycle. However I saw evidence that this strategy was not feasible when performing several consecutive dives (there was no overall significant increase in bout dive duration).

The drawback to the sea lions undertaking longer dives (with a higher DMR) when nutritionally stressed is that they require longer post-bout recovery (Fig. 3.5) and longer surface intervals between consecutive dives. This extra inter-dive time is a consequence of having a higher DMR when nutritionally stressed, which would deplete onboard O_2 stores faster and cause faster accumulation of CO_2. As a consequence, the sea lions that were nutritionally stressed decreased their foraging efficiency by having to spend more time and energy gaining food—and more time recovering from their dives (possibly because they were diving anaerobically). This was particularly evident during bout dives, when they spent a greater proportion of their bout at the surface (Fig. 3.6b) and the amount of fish caught per minute of a dive cycle decreased by nearly 20% (Table 3.2).

It is interesting to note that the sea lions in my study seemed to compensate for the increased DMR with two distinct foraging strategies. While two animals increased both dive and surface durations, the other two animals chose to decrease dive duration, such that they

did not have to increase surface duration between dives in a bout (Fig. 3.6a), resulting in no overall change in the duration of these bouts of several dives due to nutritional stress.

In summary, my study has shown that changes in nutritional status can lead to significant variation in body O_2 stores in adult sea lions. While diving ability was not directly limited (and actually enhanced) due to the sea lions having greater O_2 stores when they were nutritionally stressed, the cost of diving was significantly higher and lowered their overall foraging efficiency. This decrease in foraging efficiency, combined with an increased need for energy intake, required the sea lions to change their dive behaviour by increasing the duration of their single dives and potentially relying more on energetically expensive anaerobic metabolism.

The extent to which animals may be able to significantly increase foraging time in the wild may be limited. An increase in foraging time means less time for other aspects of life history, and could negatively affect survival by exposing adults to greater risk of predation and increasing fasting times for pups. Combining increased cost of diving with changing prey distributions could significantly impact foraging costs, net energy intake and subsequent pup condition and juvenile survival. Animals faced with unexpected episodes of nutritional stress will have to increase their foraging times to maintain energy intake and acquire the extra energy needed to replace mass and fat stores.

Overall, the capacity (at least aerobically) of Steller sea lions to dive and forage while nutritionally stressed appears to be higher compared to sea lions that are not stressed. However, a consequence of this increased ability to dive is that the greater proportion of time spent foraging will be both energetically expensive and affect other aspects of life history—a tradeoff that could ultimately impact other aspects of population growth.

Chapter 4: Sensitivity to hypercapnia and elimination of CO_2 following diving

Summary

The diving ability of marine mammals is not only a function of how they use and store oxygen, but also the physiological control of ventilation, which is dependent on the accumulation of carbon dioxide (CO_2). I assessed the influence of CO_2 on physiological control of dive behaviour, by testing how increasing inspired CO_2 (hypercapnia) and decreasing inspired O_2 (hypoxia) affect the diving metabolic rate, submergence times, and dive recovery times (time to replenish O_2 stores and eliminate CO_2) of freely diving Steller sea lions. I also measured changes in breathing frequency of non-diving individuals. My findings show that breathing frequency is affected by hypercapnia (at levels as low as 2% inspired CO_2), but not by hypoxia (down to 17% inspired O_2). Although hypercapnia affected breathing rates, it did not affect the duration of dives or surface intervals. Changes in breathing rates indicate respiratory drive was altered by hypercapnia at rest, but the increase in ventilation may be enough to mediate increases in blood CO_2 levels due to changes in inspired CO_2, such that animals can maintain normal dive behaviour. It took the sea lions longer to remove accumulated CO_2 than it did for them to replenish their O_2 stores following dives. This difference in recovery time between O_2 and CO_2 grew with increasing dive durations, hypercapnic conditions, and was greater for bout dives, suggesting there could be a buildup of CO_2 load with repeated dives. I predict the increasingly longer time required for the sea lions to remove CO_2 would eventually exhibit control over the overall time they can spend in apnea and therefore overall foraging duration.

Introduction

Exhaustion of on-board oxygen stores is generally assumed to limit the time that diving vertebrates can remain submerged and has been the basis for calculating commonly used metrics, such as the aerobic dive limit, to compare the diving abilities of different species (reviewed in Kooyman and Ponganis, 1998). However physiological control of dive behaviour is inherently related to the control of ventilation. The observation that many marine mammals routinely end their dives long before they have depleted their O_2 stores and are also

capable of diving well beyond their aerobic limits, suggests that other factors besides O_2 limitations must be involved in terminating a dive, such as a build-up of CO_2 (Butler, 1982).

CO_2 plays a central role in controlling ventilation in terrestrial mammals (Phillipson et al., 1981). However, its contribution to the control of diving in marine mammals is unclear (for review see Butler, 1982). CO_2 is produced as O_2 stores are depleted during breath holding, and results in an increase in the partial pressure of CO_2 (P_{CO_2}) in tissues and blood (Kooyman et al., 1980; Qvist et al., 1986). Small increases in blood P_{CO_2} in mammals stimulates respiratory drive, perfusing tissues with O_2 and preventing the further rise of CO_2 by increasing ventilation rate and heart rate. However, this response would be disadvantageous for aquatic mammals, as it would severely limit submergence times. Marine mammals must therefore have adaptations to compensate for the buildup of CO_2 they produce while diving.

Early studies found marine mammals were less responsive to hypercapnic conditions (high inspired CO_2) than terrestrial mammals, leading to suggestions that their relative insensitivity to CO_2 allowed them to dive longer than their terrestrial counterparts (Irving et al., 1935). Ventilation rates of restrained seals were observed to increase only slightly when the inspired CO_2 concentration was 5%, and breathing frequency doubled when CO_2 was 10% (Irving, 1938). Subsequent studies revealed that unrestrained marine mammals were nearly as sensitive to CO_2 as terrestrial mammals (i.e. increases in CO_2 resulted in the same relative increase in ventilation), but that instigation of the ventilatory response may be blunted; i.e. occur at a higher P_{CO_2} levels than for terrestrial mammals (Robin et al., 1963; Bentley et al., 1967; Påsche, 1976a; Craig and Pasche, 1980; Gallivan, 1980; Butler, 1982; Milsom et al., 1996; Kohin et al., 1999). Stimulation of the dive response (via trigeminal nerve input from facial receptors during submergence) may inhibit the response of the carotid bodies to rising P_{CO_2} levels while diving, contributing to the apparent insensitivity of marine mammals to CO_2 (de Burgh Daly et al., 1977; Elsner et al., 1977).

In addition to tolerating higher levels of CO_2, marine mammals also possess adaptations that increase the CO_2 carrying capacity of their blood. Elevated hemoglobin levels, which increase O_2 stores, can also carry more CO_2. Marine mammal blood may also have a higher buffering capacity to protect against acidosis resulting from CO_2 build-up

(Lenfant et al., 1969; Lenfant et al., 1970; Castellini and Somero, 1981; Boutilier et al., 1993), which may also contribute to the apparent blunted response of marine mammals to CO_2 compared to terrestrial mammals.

A number of studies conducted in aquarium tanks suggest that breath hold duration of marine mammals is affected by hypercapnia—indicating a potential effect of CO_2 on dive behaviour (Påsche, 1976b, a; Craig and Pasche, 1980; Gallivan, 1980). CO_2 may also affect and determine the duration of inter-dive surface intervals when diving mammals are thought to be primarily replenishing their O_2 stores (Boutilier et al., 2001). O_2 stores are generally restored faster than CO_2 is eliminated because of the longer time it takes to mobilize and remove CO_2 stores that have dissolved in tissues or have been buffered out of the blood (Boutilier et al., 2001). Hence, removal of CO_2 might regulate when the surface interval can end and the next dive can begin, thereby affecting overall foraging efficiency (percent of total time available for foraging).

Most studies on the effect of CO_2 have been undertaken with restrained animals or in restricted environments (i.e. on land or in small tanks). No studies have examined the effect of hypercapnia on voluntarily diving animals—or on ventilation in an otariid. I therefore used a unique experimental set-up to examine the effect of hypercapnia in Steller sea lions (*Eumetopias jubatus,* Schreber, 1776) that were actively foraging at depths typical of wild counterparts. I sought to examine the effects of hypercapnia and hypoxia on ventilation (through changes in breathing frequency) in non-diving animals, and determine whether hypercapnia directly affected dive behaviour (dive duration, inter-dive surface interval) of animals diving naturally in an open ocean environment. I hypothesized that hypercapnia would significantly increase breathing rates in Steller sea lions and this would result in either a decrease in dive durations or an increase in surface durations. Finally, I considered the role that CO_2 plays in controlling the dive behaviour of Steller sea lions by examining the effects of hypercapnia or hypoxia on the temporal relationship between CO_2 elimination and O_2 uptake following a dive (recovery).

Methods

Data collection

I used eight adult, female Steller sea lions that were raised at the Vancouver Aquarium (British Columbia, Canada). All animals were previously trained to use experimental equipment and performed all trials voluntarily under trainer control. Four of the sea lions (between 12 – 15 years old) were housed at the Open Water Research Station (Port Moody, BC), and have been actively diving in the open ocean for research purposes since 2003 (F00SI, F00BO), 2005 (F00HA) or 2008 (F00YA). Animals were fed a diet of herring (*Clupea pallassi*) and market squid (*Doryteuthis opalescens*) supplemented with vitamins. All experiments were conducted under UBC Animal Care Permits #A07-0413 and #A11-0397.

Ventilation in resting animals

The effect of hypoxia and hypercapnia on ventilation rate (measured as breaths per minute) was initially examined on non-diving animals (n = 4) at the Vancouver Aquarium. This was done to determine levels of hypoxia and hypercapnia to be tested on diving animals, and to understand baseline changes in a resting, non-diving animal. Animals were fasted overnight prior to any trials and the water temperature was within their assumed thermoneutral zone. Trials were carried out in a small covered pool using flow through respirometry, where breathing was restricted to a Plexiglas dome with air drawn through at a known rate of 200 - 350 l/min (depending on inspired gas) using a Sable Systems 500H mass flow generator and controller (Sable Systems Inc., Las Vegas, NV, USA). Animals were breathing either ambient air (control) or an altered inspired gas mixture. I created hypoxic (17%, 18%, 19% or 20% O_2) or hypercapnic (1%, 2%, 3%, 4% or 5% CO_2) conditions by adding either nitrogen or carbon dioxide gas, respectively, at known rates (monitored with a mass flowmeter; Omega, FMA-2322) to the incurrent air being drawn through the metabolic dome. Required flow rates for each level of inspired gas were experimentally determined prior to trials. Excurrent air was sub-sampled and scrubbed of water vapor, then fractional concentrations of oxygen and carbon dioxide were measured using Sable system FC-1B and CA-1B analyzers and recorded every 0.5 seconds (Sable Data Acquisition system, Sable Systems Inc.). Barometric pressure, relative humidity, and air temperature were also recorded.

Metabolic data was analyzed using Lab Analyst X (Warthog systems, Mark A. Chappell, University of California). Data were corrected for electronic drift by baselining gas concentrations to ambient air, or to the added gas for hypercapnic and hypoxic conditions, at the beginning and end of the trial. Rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were calculated using equations 11.7 and 11.8 in Lighton (2008).

During the first set of trials (short duration increase in F_iCO_2), animals breathed ambient air (20.94% O_2 and 0.04% CO_2) for the first ten minutes followed by a stepwise increase in F_iCO_2 or decrease in F_iO_2. Breathing frequency (in breaths per minute; bpm) was calculated using the last ~6 minutes (when bpm was constant) of a 10 minute period for each concentration exposure following a two-minute equilibration period for the inspired concentration in the metabolic dome to stabilize. In a subsequent set of trials (long duration increase in F_iCO_2) I repeated the hypercapnic trials (2% and 3% CO_2) where the animals breathed a single concentration for 40 minutes (which was a comparable time frame for the dive trials – see following). Each animal completed one trial per day, with treatments (ambient, 2% or 3% CO_2) in a random order. For these trials (and dive trials), gas was added prior to the animal entering the chamber; hence, metabolic rate could be more accurately calculated from these added gas baselines (versus; Kohin et al., 1999). Estimates of metabolic and ventilation rates were calculated as the lowest 20 minute average of a 40 - 45 minute trial period (when $\dot{V}_{O_2}$ and bpm had reached a steady state).

Diving trials

Dive trials were conducted over a 3 month period (May – July 2011) with each animal typically doing 2 trials per week (never more than one per day). Using the same protocol as above, the sea lions breathed either ambient air or an altered inspired gas mixture of 2% CO_2, 3% CO_2, 19% O_2, or 20% O_2. Animals would not reliably enter the metabolic dome when CO_2 concentrations were >3%; and only 3 of the 4 animals completed the 40 m bout dive at 3% CO_2. Dive trials were also not undertaken under conditions of <19% O_2 due to the logistics of the respirometry set up (high flow rates required for dive trials precluded lower O_2 concentration beyond 19%) as well as animal safety, given the open ocean conditions of the

trials. Two sets of trials were conducted under each set of inspired gas conditions with animals diving either to 10 or 40 m depth (see below).

The experimental set-up consisted of a floating platform with a square opening in the middle containing a floating transparent Plexiglas respirometry dome (100 L). Metabolic rate was measured in the dome using flow through respirometry as described above.

Animals were fasted overnight prior to trials and weighed each morning. They were fed <0.5 kg during transport to the dive site to minimize any effect of heat increment of feeding on metabolic rate. During trials, the animals wore a harness with a VHF transmitter and time depth recorder. Pre-dive metabolism was measured at the start of each trial while animals rested inside the metabolic dome for 5-10 minutes until $\dot{V}_{O_2}$ is constant (~3 minute period). Animals then dove voluntarily to a pre-determined depth (10 or 40 m), to the bottom of feeding tubes that delivered ~0.2 gram pieces of herring every 5 seconds for the duration of the dive. Fish were delivered alternately between two tubes ~6 m apart to create a "prey patch" at depth that encouraged the animals to remain active during the dive.

For each trial, the animals were directed to complete a single long dive followed by a 4-dive bout cycle, in which the animals chose both their dive and inter-dive surface interval duration. Following both the single dive and the dive bout, the sea lions were kept at the surface in the respirometry dome for a post-dive 'recovery' measurement (defined as the time it took for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ to return to within 5% of baseline values). Diving metabolic rate (DMR) was calculated as the average $\dot{V}_{O_2}$ over the dive and following recovery period. For bout dives, DMR was the average $\dot{V}_{O_2}$ for the entire cycle of dives (including inter-dive surface intervals) and recovery period.

Statistical analysis

All data were analyzed using R (R Development Core Team, 2011). Data from each animal were treated as repeated measures by including animal ID as a random effect, using linear mixed-effects models (lme) from the nlme package (Pinheiro et al., 2011). Models were run using the maximum likelihood method. A repeated measures ANOVA was used to determine whether inspired gas affected breathing rate in resting and diving animals. For post-dive breathing rate, O_2 recovery time, CO_2 recovery time and the difference in recovery

time ($CO_2 - O_2$), an ANCOVA was performed to determine whether inspired gas (as a categorical variable) co-varied with dive duration (as a continuous variable). Type of dive (single or bout) and depth (10 or 40 m) were also tested as fixed factors. Finding significant multiple fixed factors resulted in comparing the nested models (with or without a fixed effect) using a log likelihood ratio test to determine the best overall model to fit the data (Pinheiro and Bates, 2000). For significant categorical factors, post-hoc tests (using the Bonferroni method) were performed to compare the means between multiple groups. Values are reported as means (± st. dev.) and significance was set at $\alpha = 0.05$.

Results

Hypercapnia and hypoxia on ventilation rate

Hypercapnia significantly affected breathing frequencies (Fig. 4.1). For the non-diving animals, short step-wise increases in F_iCO_2 (every 10 minutes) resulted in a significant increase in breathing frequency when concentrations reached 4% CO_2 (9.5 ± 1.6 breaths per minute; bpm) and 5% CO_2 (11.8 ± 1.4 bpm) compared to ambient air (0.04% CO_2; 6.8 ± 1.5 bpm). I did not observe any effect of lower F_iCO_2 on breathing frequency during these trials, presumably because the baselines were artificially elevated due to the general decrease in breathing frequency that occurred over the duration of each trial. Alternatively, the short (~10 min) exposure to hypercapnia in these trials may not be long enough for blood P_{CO_2} levels to be affected in a resting animal. However, there was a significant increase in breathing frequency during the longer 40-minute exposure trials at lower concentrations of 2% (6.4 ± 1.4 bpm; $p = 0.008$) and 3% CO_2 (9.0 ± 0.8 bpm; $p < 0.001$) compared to when the sea lions were breathing ambient air (4.9 ± 1.2 bpm).

Similar levels of hypercapnia affected the breathing frequency of the diving animals (Fig. 4.2), where pre-dive breathing frequency at 2% and 3% CO_2 was significantly higher than when the animals were breathing ambient air (5.41 ± 1.5 bpm, 7.28 ± 1.7 bpm and 8.03 ± 1.7 bpm for ambient air, 2% and 3% CO_2, respectively, $p < 0.001$) as was post-dive breathing frequency (9.00 ± 1.8 bpm, 12.1 ± 2.6 bpm and 13.6 ± 2.1 bpm for ambient air, 2% and 3% CO_2, respectively, $p < 0.001$). Post dive breathing frequency (following a single dive) also significantly depended on dive duration ($p < 0.001$). There was also a significant increase in

breathing frequency during the inter-dive surface intervals of a bout dive, ranging from 22.3 (± 3.9) in ambient air to 28.9 (± 4.1) bpm in 3% CO_2 (Fig. 4.2; $p = 0.007$).

Hypoxia (to 17% O_2) had no effect on the breathing frequency of either diving or non-diving sea lions (Fig. 4.1). Unfortunately, I was unable to measure tidal volume, which may have been adjusted to facilitate changes in the overall ventilation rate.

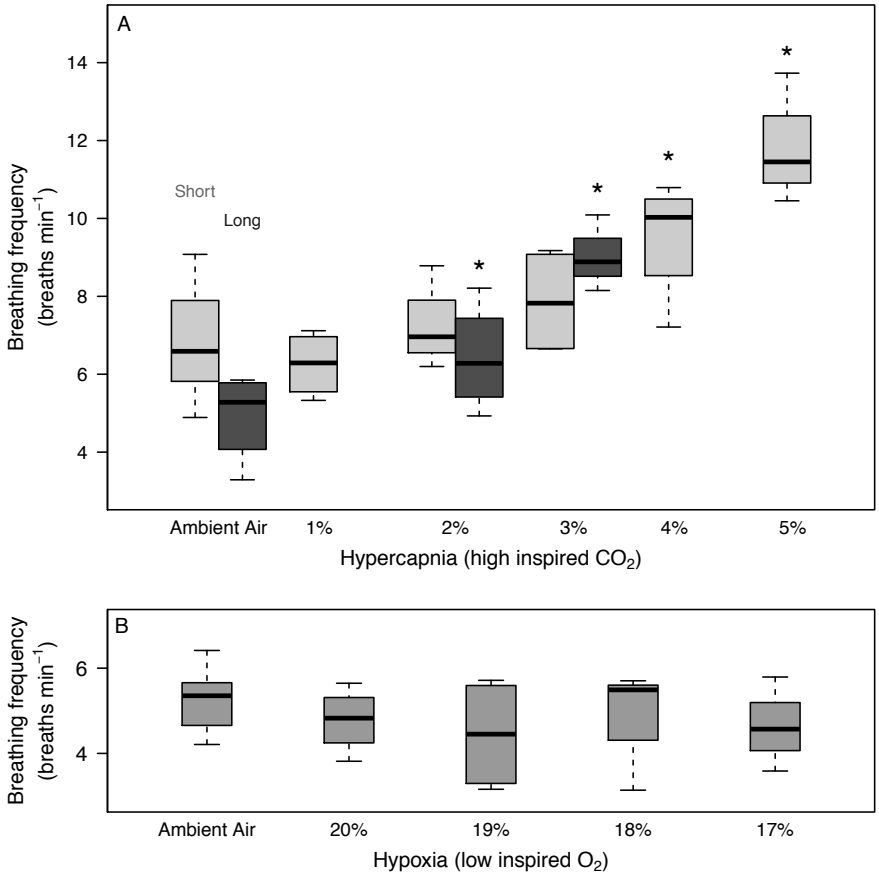

Figure 4.1 Breathing frequency in non-diving, resting Steller sea lions (n = 4) as a function of inspired gas composition, including ambient air (20.9% O_2 and 0.04% CO_2). (A) Hypercapnic conditions significantly increased breathing frequency when sea lions were subjected to either short duration step-wise increases in F_iCO_2 (lt. grey, $p < 0.001$) or long duration (40 minute; dk. grey). Asterix indicates a significant difference from ambient conditions based on a linear mixed effects model accounting for repeated measures between animals. (B) Hypoxia (short step-wise decrease in F_iO_2) had no effect on breathing frequency within the range used in this study.

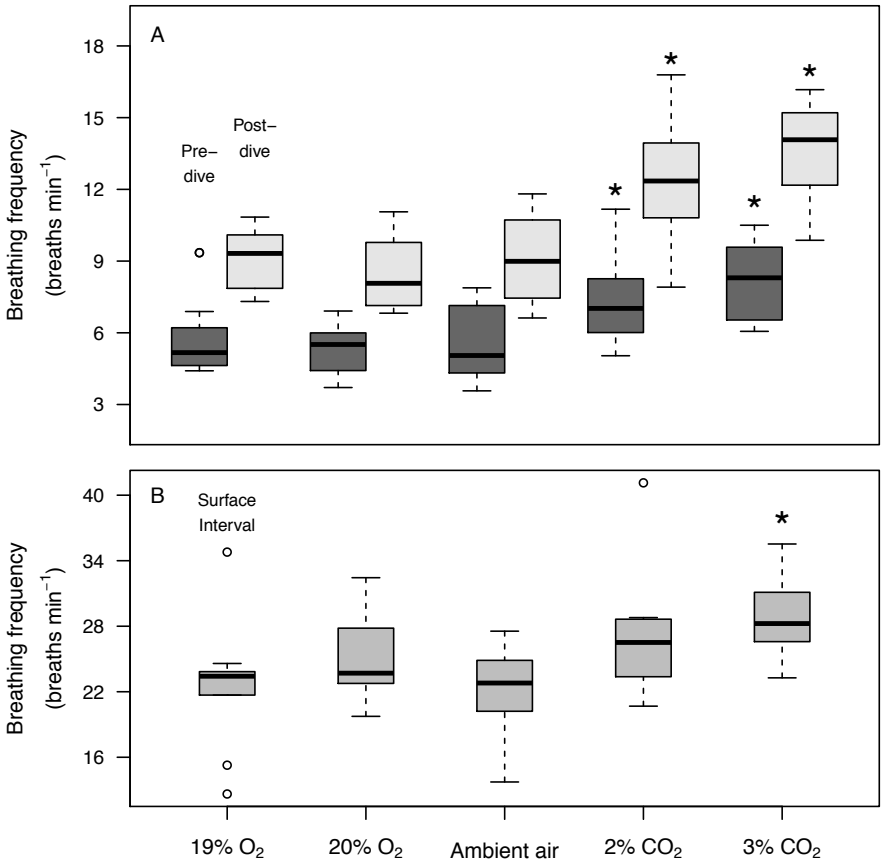

Figure 4.2 Breathing frequency in diving Steller sea lions (n = 4) as a function of inspired gas composition. (A) Comparing pre-dive (3 minute average, $p < 0.001$) and post-dive (over duration of recovery period, $p < 0.001$). (B) Breathing frequency during surface intervals between bout dives ($p = 0.007$). Asterix indicate a significant difference from ambient conditions based on a linear mixed effects model that accounted for repeated measures between animals and the significant effect of dive duration ($p < 0.001$) on post-dive values.

Hypercapnia and hypoxia on dive behaviour and metabolism

There was no effect of hypercapnia or hypoxia on diving or resting metabolic rate. There was also no measurable effect of hypoxia or hypercapnia on dive behaviour (dive or surface interval duration) at levels up to 3% CO_2 or down to 19% O_2—despite there being a significant effect of hypercapnia on breathing rate and an observed initial behavioural response (adverse to staying in the metabolic dome) to increases in F_iCO_2 even as low as 1%.

Hypercapnia and hypoxia on post-dive recovery of CO_2 production and O_2 consumption

Following all bout dives, and all but two (96%) of the single dives, it took longer for the $\dot{V}_{CO_2}$ of the sea lions to return to baseline levels (recover) than $\dot{V}_{O_2}$ (Fig. 4.3). $\dot{V}_{O_2}$ recovery time was dependent on dive duration for single dives (longer dives resulted in longer recovery times; $p = 0.002$). However, $\dot{V}_{O_2}$ recovery time was not related to the duration of dive bouts and was not affected by F_iO_2. In contrast, $\dot{V}_{CO_2}$ recovery time was dependent on depth, dive duration and inspired gas concentrations when both single and bout dives were combined (recovery did not depend on dive type). Specifically, $\dot{V}_{CO_2}$ recovery times were higher at both 2% ($p = 0.018$) and 3% ($p = 0.002$) CO_2 than in ambient air. $\dot{V}_{CO_2}$ recovery times also increased with increasing depth and dive duration (which were inter-related) for both single and bout dives.

The differences between O_2 and CO_2 recovery time increased with dive duration ($p < 0.001$), depth ($p = 0.0319$) and F_iCO_2. Hypercapnia (2% and 3% CO_2, $p < 0.001$) resulted in a greater difference between recovery times (generally due to an increase in CO_2 recovery time but no change in O_2 recovery); hypoxia (to 19% O_2) had no effect on $\dot{V}_{O_2}$ recovery, $\dot{V}_{CO_2}$ recovery, or the difference between the two (Fig. 4.4). It is important to note that I was unable to determine recovery time in 7 out of 55 trials (5 of which were hypercapnic), as $\dot{V}_{CO_2}$ did not reach pre-dive levels or a low enough slope to be considered stable and recovered. Hence, $\dot{V}_{CO_2}$ recovery of Steller sea lions is likely to be longer than shown by my study.

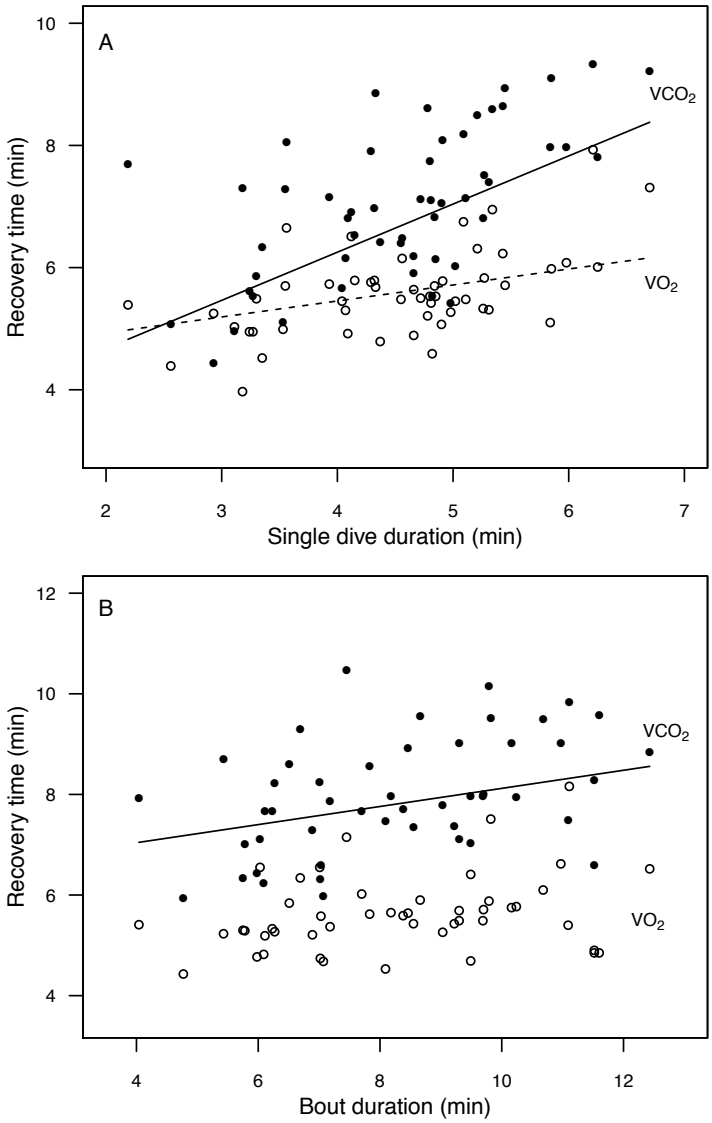

Figure 4.3 Time for $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ to return to resting levels (recovery time) in Steller sea lions as a function of: (A) single dive duration and (B) duration of a bout of four consecutive dives. CO_2 recovery was longer than O_2 recovery in all but two dives (96%). Both O_2 ($p = 0.002$) and CO_2 ($p < 0.001$) recovery times increased with the duration of single dives. Following multi-dive bouts, only CO_2 recovery increased with duration ($p = 0.004$) and O_2 recovery was independent of duration ($p = 0.125$).

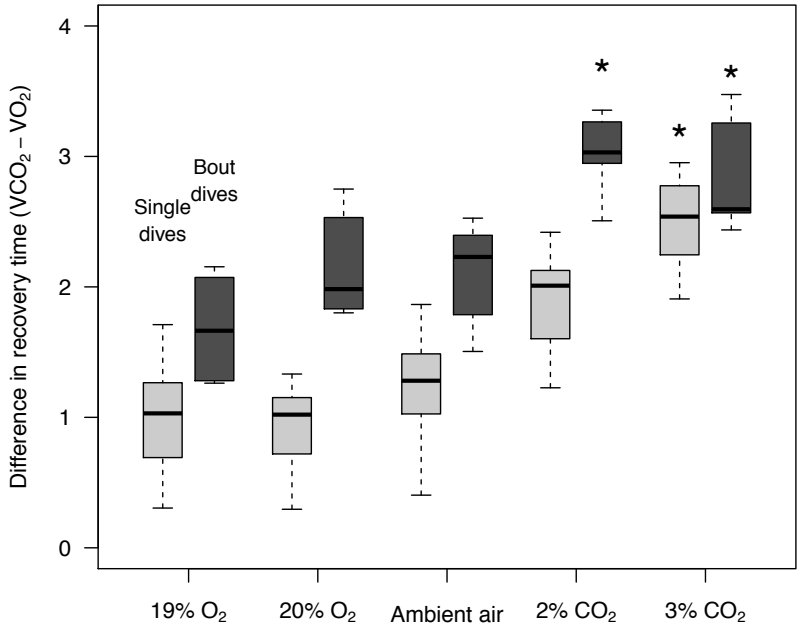

Figure 4.4 Difference in recovery (time required for CO_2 and O_2 levels to return to pre-dive levels) after completing a single dive (lt. grey) or a bout of 4 consecutive dives (dk. grey). All variables show differences ($\dot{V}_{CO_2} - \dot{V}_{O_2}$) greater than zero, indicating that it took the sea lions longer to remove CO_2 than it did to replenish O_2 stores. Asterix indicate a significant difference from ambient air conditions. Each box plot contains 6-8 data points that were derived from a linear mixed effects model that accounted for the effect of dive duration and repeated measures between animals.

Discussion

Ventilation and dive behaviour

Past studies of how gas exchange affects dive behaviour have usually focused on the role that oxygen storage and utilization rates play in limiting dive duration as well as how the inter-dive surface durations are defined by the time required to the refill those oxygen stores. However, control of dive behaviour must also depend to some extent on respiratory drive, and therefore on P_{CO_2} levels (Butler and Jones, 1997; Stephenson, 2005). My expectation was that hypercapnia or hypoxia would affect blood P_{CO_2} or P_{O_2} levels by altering the rate of diffusion of these gases between the blood and lungs in accordance with Fick's Law. If disfacilitation of respiratory drive is needed to initiate diving (i.e. P_{CO_2} levels must be reduced below some

"threshold" value; Stephenson, 2005), then hypercapnia should have increased the required surface intervals relative to the duration of dives. This additional surface time would have enabled the sea lions to eliminate sufficient CO_2 to allow P_{CO_2} levels in their blood to return to pre-dive levels or the "threshold" value needed before being able to continue diving. Failing to change the surface interval duration during hypercapnia suggests the animals were diving before P_{CO_2} had returned to resting levels that should, in turn, have limited subsequent dive durations. Surprisingly, I saw neither of these two results—both dive and surface interval duration chosen by the study animals were unaffected by hypercapnia.

I did observe significant changes in breathing frequency during hypercapnia before and after diving, indicating a potential mechanism to compensate for the hypercapnic conditions to maintain dive behaviour. While the diffusion gradient for offloading of CO_2 was decreased by hypercapnia, animals may have used increased breathing rates to sufficiently lower P_{CO_2} levels such that respiratory drive while diving (and diving behaviour) was unaffected. Given that I also observed a change in ventilation in resting animals, hyperventilation must be in part simply a response to higher P_{CO_2} levels in the blood caused by breathing hypercapnic gas (and not solely a mechanism to maintain dive behaviour). Hence, the hyperventilation I saw in the diving animals was likely due both to higher P_{CO_2} levels caused by hypercapnia, and reflects their normal hyperventilatation prior to diving to further lower P_{CO_2} before they can initiate a dive (Stephenson, 2005). In addition to hyperventilation, a greater amount of CO_2 than normal was likely stored in body tissues (due to the increased concentration gradient of CO_2 between the lungs, blood and body) and blood. The added CO_2 storage (along with hyperventilation) likely contributed to maintaining blood P_{CO_2} below a level at which it would affect dive behaviour despite the hypercapnic conditions.

I saw no effect of hypoxia on breathing frequency with inspired O_2 levels down to 17%. This level of hypoxia may not have been low enough to elicit a ventilatory response—and likely would have been manifested as changes in tidal volume had there been an effect. Previous studies have rarely found any effect of hypoxia above ~13% O_2 (100 mmHg), below which there is, in phocid seals, generally a decrease in time spent in apnea until apneic periods are eliminated altogether (Milsom et al., 1996; Kohin et al., 1999). Ventilation increased

when alveolar P_{O_2} fell below ~30 mmHg in manatees (*Trichechus inunguis*; Gallivan, 1980) and Weddell seals (*Leptonychotes weddellii*; Parkos and Wahrenbrock, 1987) and diving and ventilation were affected below ~80 mmHg in harbour seals (*Phoca vitulina*; Påsche, 1976b).

In general, I saw an effect of hypercapnia on breathing rates at lower F_iCO_2 than seen in previous studies. This is expected given previous studies were on non-diving animals and the stress of hypercapnia in an actively diving animal is likely much greater than in a resting animal, due to increasing CO_2 in the body during apnea and the added exercise of diving to depth. This is also reflected in the greater effect of hypercapnia I observed on pre-dive breathing rates as opposed to in animals at rest (Fig. 4.1). All previous studies were also on phocid seals, which are generally considered better divers with more developed adaptations to diving than otariids (Kooyman, 1989). Unfortunately, most studies only report one level of hypercapnia or report changes versus alveolar P_{CO_2} ($P_{A_{CO_2}}$) instead of F_iCO_2, making direct comparisons difficult. Kohin et al. (1999) found that an inspired CO_2 of 7% in northern elephant seals doubled breathing frequency (and completely eliminated periods of apnea on land in 10 of 28 animals studied). Påsche (1976a) also found an increase in ventilation occurred somewhere between 3% and 6% inspired CO_2 in harp (*Phoca groenlandica*) and hooded seals (*Cystophora crystata*), whereas in my study breathing frequency increased at an inspired CO_2 of 2%. In general, for studies on phocid seals when alveolar $P_{A_{CO_2}}$ was determined, there was a linear increase in breathing frequency or ventilation with increasing $P_{A_{CO_2}}$ above resting levels (Robin et al., 1963; Bainton et al., 1973; Påsche, 1976a; Craig and Pasche, 1980; Gallivan, 1980; Parkos and Wahrenbrock, 1987). The slope of this response ($P_{A_{CO_2}}$ vs. ventilation rate) indicates phocids have a level of sensitivity to CO_2 that is similar to most terrestrial mammals (except possibly humans)—although the response of phocids may occur at a higher threshold $P_{A_{CO_2}}$ level (Bentley et al., 1967; Parkos and Wahrenbrock, 1987; Butler and Jones, 1997). I did not have $P_{A_{CO_2}}$ values in my study, but given the observed increases in breathing frequency at 2% CO_2 the response of Steller sea lions to hypercapnia is probably more similar to terrestrial mammals than to phocid seals.

It is unclear exactly how F_iCO_2 levels translate to $P_{A_{CO_2}}$ values and it is possible that I did not see changes in dive behaviour of the sea lions because I did not actually alter arterial

P_{CO_2}. Data from Påsche (1976a) indicate that an inspired CO_2 of 3% resulted in a $P_{A_{CO_2}}$ of around 55-70 mmHg in harp and hooded seals. Hence my inspired CO_2 levels likely altered $P_{A_{CO_2}}$. However, alveolar ventilation may have increased enough to mediate increases in arterial P_{CO_2} and minimize any increases in the amount of CO_2 in the body as compared to what would normally be produced during a dive.

A further complication to making simple comparisons between studies is that overall changes in ventilation can be independently achieved through changes in breathing frequency, tidal volume or decreases in time spent apneic when at rest. Gallivan (1980) showed that most of the variation in ventilation rate in manatees was due to changes in breathing frequency (corresponding to a decrease in breath-hold time) as opposed to tidal volume. In contrast, Påsche (1976b, a) reported that decreases in tidal volume during hypercapnia resulting in a change in ventilation that was over-estimated by breathing frequency alone. However, tidal volume in their study was highly variable and also increased with similar magnitude in one animal due to swimming activity alone (Påsche, 1976a). Hence, in my study there may also have been a change in tidal volume that affected the overall ventilatory response to hypercapnia in Steller sea lions.

I saw significant ventilatory responses among the sea lions to hypercapnia at rest, but no response when actively diving (dive and inter-dive duration), suggesting there were other mechanisms involved in control of ventilation (respiratory drive) during diving. This is consistent with earlier studies employing experimental forced "dives" in harbour seals that showed the dive response may inhibit the response of carotid bodies to elevated P_{CO_2} (de Burgh Daly et al., 1977; Elsner et al., 1977). Carotid bodies normally respond to increased blood P_{CO_2} with an increase in respiratory drive (stimulation to breathe), but trigeminal nerve input while diving (stimulated by immersion of the facial receptors in water) has an inhibitory influence on chemoreceptor activity (de Burgh Daly et al., 1977; Elsner et al., 1977). In tufted ducks, denervation of carotid bodies resulted in significantly longer dive durations (Butler and Woakes, 1982). Hence, an inspired CO_2 of 2% and 3% in my study could have resulted in an increase in arterial P_{CO_2}, but not great enough to "override" the inhibitory influence of the dive response on carotid body chemoreceptors. A similar inhibition of carotid bodies to

hypercapnia (up to a critical point) was demonstrated during REM sleep in elephant seals (Milsom et al., 1996).

Although hypercapnia did not affect dive behaviour in my study, other studies of marine mammals have shown that breath-hold duration decreases at CO_2 levels as low as 3% (Påsche, 1976a; Gallivan, 1980). The differences in results may have been due to the artifical nature of the "dives" in these other studies that affected both motivation to extend dive durations and the extent of the dive response. Only one other study examined hypercapnia on actively diving animals, and it found that "increasing alveolar CO_2 always completely inhibited diving behaviour" (Parkos and Wahrenbrock, 1987) but was not specific as to what $P_{A_{CO_2}}$ or F_iCO_2 this occurred, suggesting there would likely be an effect in a freely diving sea lion at high enough levels of hypercapnia.

It might be suggested that the sea lions were not diving close enough to their physiological limits for hypercapnia to limit dive behaviour. However, their average dive duration for single dives was 4.4 minutes, which is significantly longer than their calculated aerobic dive limit of 3 minutes (Chapter 2) and much longer than the typical dive durations (2.0 – 2.4 minutes) of Steller sea lions in the wild (Merrick et al., 1994; Merrick and Loughlin, 1997; Loughlin et al., 1998). Based on previous studies (Påsche, 1976b, a; Craig and Pasche, 1980; Gallivan, 1980; Parkos and Wahrenbrock, 1987), I would likely have seen an effect on dive duration at higher CO_2 levels. As animals voluntarily participated in trials, it is interesting to note that the threshold F_iCO_2 that might affect diving appeared to be the same concentration at which the animals would no longer voluntarily enter the breathing dome with hypercapnic gas (~3%); i.e. instead of increasing surface intervals or decreasing dive durations, animals chose not to breathe hypercapnic gas—despite the fact that this resulted in the end of their feeding opportunities. In fact, sea lions breathing ambient air were able to increase dive duration when motivated to dive more than normal (when nutritionally stressed). However, they were unable to increase their dive durations when inspired CO_2 was 2% suggesting that CO_2 was limiting dive duration under these more strenuous conditions.

Recovery from diving

Although there was no direct effect of hypercapnia on dive behaviour, I saw evidence that CO_2 could affect diving indirectly by altering the surface intervals and effective recovery

time following a dive. In nearly all dives, I observed that the $\dot{V}_{CO_2}$ of the sea lions took longer to return to baseline levels than their $\dot{V}_{O_2}$ regardless of whether it followed a single long dive or a bout of 4 consecutive dives with short surface intervals. This increased CO_2 recovery time as compared to O_2 suggests it could limit dive behaviour by imposing minimum surface interval durations. Boutilier et al. (2001) also showed the return of CO_2 production to baseline levels took much longer than replenishment of O_2 stores in harbour porpoises (*Phocoena phocoena*). Their animals continued to breathe at the surface after O_2 stores were fully refilled, presumably to eliminate built up CO_2 before their next dives, and they suggested that CO_2 may be limiting their dive behaviour. Additional data from grey seals further shows the respiratory quotient (RQ = $\dot{V}_{CO_2} / \dot{V}_{O_2}$) before the next dive in a surface interval is typically very high (>1; Reed et al., 1994), suggesting that the extent of last portion of the surface interval is determined by CO_2 removal. This suggests that P_{CO_2} is a potential signal to end the surface interval rather than P_{O_2} (Boutilier et al., 2001).

It takes longer for accumulated CO_2 stores to be removed from the body as CO_2 is sequestered in the blood and tissues as bicarbonate and needs to be converted back to CO_2 gas and bound to hemoglobin to be removed from the body (Boutilier et al., 2001). This is reflected in a study by Falke et al., (2008) that found very low levels of exhaled CO_2 in the first few breaths following diving in Weddell seals, with CO_2 concentration peaking 2 – 3 minutes into the recovery period. $\dot{V}_{CO_2}$ took ~9 – 15 minutes to reach resting values (following 29 – 43 min dives) in Weddell seals. In my study, $\dot{V}_{CO_2}$ took ~5 – 10 minutes to return to resting values following much shorter dives that were ~2 – 6 minutes long.

Although changes in dive behaviour were not observed under my experimental conditions, I predict that an increase in the number of consecutive dives in a bout would have resulted in a decrease in dive durations as CO_2 further accumulated in the body. Indeed, there was some indication that the last dives in the bout were limited (i.e., surface intervals became slightly longer relative to dive durations), but this was not statistically significant and was probably confounded by variation in individual dive behaviour. Another potential reason I did not see longer surface intervals (relative to dive duration) as expected may be that this physiological response was counter to the animals' adverse behavioural response to the

hypercapnic conditions (to decrease breathing duration in the metabolic dome, which would result in decreased surface time relative to dive durations).

Dive duration significantly affected $\dot{V}_{O_2}$ recovery following single dives only, unlike $\dot{V}_{CO_2}$ recovery, which was affected by the duration both single dives and dive bouts (of 4 consecutive dives with short surface intervals). This suggests that O_2 stores during bouts were restored to a similar level after each surface interval, such that the animals always ended the bout at a similar level of O_2 depletion independent of duration. However, CO_2 probably continued to build up in their bodies over the course of the dive series as it could not be cleared at the same rate of O_2 store replenishment, resulting in a longer recovery at the end. This is consistent with the higher buffering capacity of the muscle and blood for CO_2 (Lenfant et al., 1969; Castellini and Somero, 1981) and suggests the animals were accumulating more of a "CO_2 load" over several repetitive dives. Under normal diving, CO_2 likely does not determine dive duration per se, but has more of an effect on the overall foraging efficiency by limiting the time an animal can spend in breath hold over the course of a foraging bout. While it is likely that O_2 stores limited individual dives, "CO_2 load" may eventually limit overall foraging for an animal performing several repetitive dives, such as in Steller sea lions. The difference between recovery time for $\dot{V}_{CO_2}$ and $\dot{V}_{O_2}$ was amplified with increasing F_iCO_2 concentrations and decreased slightly with decreasing F_iO_2. The increase in $\dot{V}_{CO_2}$ recovery time further supports that off-loading of CO_2 was compromised during the recovery period by hypercapnia. The difference in recovery time was also significantly greater following the longer dives undertaken by the sea lions when they were nutritionally stressed (and had greater metabolic rates; Chapter 3) with hypoxia resulting in a much smaller difference and hypercapnia in a much greater difference (Fig. A.4).

The limit to which I could increase the inspired CO_2 level and still have the Steller sea lions voluntarily participate in the experimental trials is probably similar to the physiological conditions under which CO_2 would affect diving. This may mean that, in the wild (unless there is unusually high motivation to extend dive durations), animals are unlikely to stress themselves beyond a "CO_2 dive limit". Steller sea lions (and probably most marine mammals) appear to be just as sensitive to CO_2 as terrestrial mammals, but their increased capacity to

store CO_2 and inhibition of carotid bodies during diving, contribute to the apparent "blunted" ventilatory response to CO_2 during forced dives and to some extent uncouple the control of ventilation by CO_2 while diving.

What terminates a dive is undoubtedly a combination of factors that includes behavioural and physiological limitations such as low O_2, high CO_2, and even rising pH levels from anaerobic metabolism (Noren et al., 2012). My data support Stephenson's (2005) model that it is disfacilitation of respiratory drive that initiates diving. When exposed to hypercapnia, the sea lions were able to compensate with hyperventilation instead of needing to increase surface intervals or decrease dive durations. Additionally, my data suggest that, while the storage of O_2 is probably the main factor limiting single dive durations, the accumulation of CO_2 over several dives in a foraging bout could ultimately limit the time the animal spends in apnea and therefore overall foraging duration.

Chapter 5: Conclusion

I investigated the physiological control of dive behaviour in Steller sea lions and determined what factors limit their diving ability in order to infer limits to their foraging behaviour. Specifically, I sought to define the aerobic dive limit (ADL) of Steller sea lions by quantifying body O_2 stores and their rate of depletion, determine how episodes of nutritional stress affected these limits, and investigated the role of carbon dioxide on controlling dive behaviour. To do this I directly measured blood oxygen stores and body composition (to estimate total body O_2 stores), metabolic rate and dive behaviour in four voluntarily diving animals before and during a period of nutritional stress. I also tested whether CO_2 levels limit dive behaviour by determining the sensitivity of Steller sea lions to CO_2 and examined how accumulation and elimination of CO_2 affected dive behaviour and recovery. To examine sensitivity to CO_2, I exposed both resting and diving animals to altered inspired gas compositions of either hypercapnic or hypoxic air, and measured changes in breathing rate, dive behaviour and recovery from diving (CO_2 elimination and O_2 uptake rates).

Body O_2 stores and the effect of nutritional stress

Direct measurements of blood oxygen stores and body composition allowed me to estimate total body oxygen stores. Combining these with measured rates of diving metabolism allowed me to calculate an ADL of 3 minutes for adult, female Steller sea lions. This cADL was lower than previously reported, resulting from both slightly lower TBO and higher DMRs than previously reported for both Steller sea lions and other otariids. These previous calculations of ADL were likely overestimated as they used values of TBO for younger animals. They were also based on lower DMRs that were variously estimated from studies of other otariids, derived from allometric relationships, or measured on animals that were less active at depth.

Although lower than previously reported, the cADL of Steller sea lions in my study corresponds with the dive behaviour of wild animals that typically undertake short, shallow dives (<4 min at depths ~20 m). Additionally, my cADL of 3 minutes is about 25-30% of their maximum dive durations, which also aligns with the pattern from the few direct measures of ADL as compared to dive behaviour. The only directly measured ADL in an otariid is 2.3 minutes in a juvenile California sea lion (Ponganis et al., 1997). Steller sea lions may be

capable of lowering their DMR in some circumstances, resulting in a longer ADL. However, it is unlikely they dive regularly with a lower metabolism, particularly in the wild where they are actively foraging. More direct measurements of DMR and post-dive lactate concentrations in Steller sea lions are needed to be confident of their true ADL.

As nutritional stress has been hypothesized to contribute to the decline of wild Steller sea lions, I wanted to determine the impact it has on their diving ability and subsequent foraging behaviour. I predicted that nutritional stress would lower the ADL of Steller sea lions, mainly because of a reduction in lean body mass and hence muscle O_2 stores. I measured significant losses of LBM and concluded that nutritionally stressed Steller sea lions likely lost muscle mass and therefore muscle oxygen stores through decreased myoglobin. However, significant increases in blood volume with parallel increases in haemoglobin resulted in an overall increase in TBO, both on an absolute and mass-specific basis. While absolute DMR did not change during periods of nutritional stress, it did increase on a mass-specific basis, but to a lesser degree than the increases in body oxygen stores. The combined changes in TBO and DMR resulted in a longer cADL (increased from 3 to 3.3 minutes) when nutritionally stressed, inferring that diving ability (in terms of ADL) was not limited and may have actually been enhanced. The dive behaviour of nutritionally stressed animals paralleled these changes in aerobic capacity, with all individuals increasing the duration of their single dives.

The higher DMR during the period of nutritional stress was likely a result of the 'hunger' response previously demonstrated in Steller sea lions on a restricted diet of high fat fish, purported to be representative of increased foraging drive (Rosen and Trites 2002). Loss of insulative lipid stores may also have resulted in greater thermoregulatory costs (Rosen, 2009) and the higher proportion of LBM also contributed to higher mass-specific metabolic rates. Although aerobic diving ability was not limited when nutritionally stressed, animals experienced a higher cost of diving due to these higher DMRs. This resulted in animals spending a greater proportion of a diving bout at the surface recovering from their dives, in order to refill O_2 stores (and offload CO_2) that would have been depleted at a higher rate while diving. As a result, the Steller sea lions had reduced foraging efficiency and net energy intake for a given cycle of diving when nutritionally stressed.

Significant increases in total body O_2 stores were due to higher blood volume that more than compensated for potential losses of muscle O_2 stores. The overall increase in TBO could actually be much higher than measured in this study given potential increases in myoglobin concentration, and (contrary to my conservative calculations) that skeletal muscle may not be the sole contributor to losses in LBM. It is unclear why blood volume was so much higher in nutritionally stressed animals, but it was also paralleled by increases in total body water. One possibility is that the high blood volume was a result of conserving water in response to limited food intake and catabolism of protein and lipid stores, rather than an adaptation to diving per se—but it is important to note that haemoglobin concentration in the blood did not change.

In summary, although diving and foraging capacity was not limited during episodes of nutritional stress, foraging efficiency (measured as net energy intake for a given duration of foraging) could be significantly lower depending on the abundance and distribution of prey available to wild animals.

Effects of CO_2 on breathing rates, dive behaviour and recovery

The role of O_2 stores in determining diving ability is fairly well understood, but the effect of accumulating CO_2 on respiratory drive and dive behaviour has never been studied in an otariid, and is unclear in diving vertebrates in general. My goals were to determine the sensitivity (i.e., the ventilatory response) of resting and diving Steller sea lions to CO_2 to investigate the role of CO_2 and control of ventilation in shaping dive behaviour. Specifically, I wanted to determine if CO_2 limited diving either directly—through the effect of hypercapnia on dive behaviour, or indirectly—if CO_2 elimination following a dive limited surface durations.

I found a significant effect of hypercapnia on breathing frequency in both diving and resting animals at inspired CO_2 levels as low as 2%, suggesting blood P_{CO_2} levels had been altered. However, there was little evidence that CO_2 limited dive behaviour at concentrations up to 3% CO_2. There was, nevertheless, a greater effect on breathing frequency in diving animals (pre- and post-dive) than in resting animals—suggesting that the increase in ventilation in diving animals was a means to compensate for the hypercapnic conditions as well as reflected normal hyperventilation before diving to lower P_{CO_2} levels below resting

levels. This would be consistent with the hypothesis by Stephenson (2005) that it is disfacilitation of respiratory drive (rather than inhibition) that ends the surface interval and initiates diving.

It is also possible that P_{CO_2} was elevated in animals breathing hypercapnic gases but was not high enough to override the apnea induced by the dive response. This is consistent with studies showing the dive response inhibits respiratory drive (at least up to a certain threshold level of pCO_2) (de Burgh Daly et al., 1977; Elsner et al., 1977). The combination of hyperventilation and inhibition of respiratory drive by the dive response likely contributed to the animals being able to maintain normal dive behaviour in the face of hypercapnic conditions.

The ventilatory response in Steller sea lions was seen at F_iCO_2 levels much lower than previously found to affect ventilation in marine mammals. Previous studies have only been undertaken with phocid seals that generally have different diving patterns. These studies were also conducted with animals that were restricted to breathe hypercapnic gases only in aquarium tanks or under "forced" dive experimental protocols. Hence, the response to CO_2 found in these studies is likely confounded by a survival response and could be very different in an unrestrained animal. The dive response is usually much stronger in forced dive studies (Butler and Jones, 1997) and these studies may not be representative of a normal response to CO_2 when animals are diving naturally.

My inability to detect quantitative changes in dive behaviour with differences in inspired gas concentrations can be partially attributed to the voluntary nature of the trials. Observationally, an initial adverse response to entering the metabolic dome made it clear that the animals were able to detect the hypercapnic conditions. My study tested the effect of hypercapnia in animals that were not only voluntarily diving, but also voluntarily breathing hypercapnic gases. Animals had to enter the metabolic dome with hypercapnic air both pre- and post-dive. Hence, the point at which the animals refused to participate in trials represents the level of hypercapnia that they would no longer endure to continue diving—which, given the same level of motivation to dive, may represent the point at which hypercapnia would affect their dive behaviour. They had both behavioural and physiological responses to hypercapnia, suggesting they may also have both behavioural and physiological adaptations to dealing with high pCO_2 levels while diving.

Diving behaviour, including surface recovery time, is often thought of in terms of oxygen store management. However, CO_2 elimination is also an important process. I found that elimination of accumulated CO_2 took significantly longer than refilling of O_2 stores under all inspired gas conditions (including ambient air). This occurred following all but 2 dives (which were quite short, <3 minutes) and shows CO_2 elimination could be the limiting process regulating surface intervals and therefore minimum surface duration. However, I did not find that CO_2 elimination regulated surface duration under the conditions tested. At an inspired CO_2 of 3% there was a trend for increasing time spent at the surface relative to dive durations in a bout, but this was not significant. The difference between CO_2 and O_2 recovery times was also greater with increasing dive durations. Hence, there is evidence I was approaching a limit at which CO_2 elimination would have affected surface duration but, unfortunately, this likely corresponds to the point at which animals would no longer voluntarily participate in trials.

From my study, I found that although hypercapnia affected respiratory drive at rest, it did not alter dive behaviour. I therefore concluded that the dive response may alter the control of respiratory drive by CO_2 while diving. This has been demonstrated in laboratory experiments, but never in an actively diving animal. I also found it takes longer for sea lions to remove stored CO_2 than to refill O_2 stores—and can speculate that CO_2 may become limiting over several dives. CO_2 is probably more important in repetitive, short duration divers (with short surface intervals) that do not have long ADLs or in dives that extend beyond the ADL and rely on anaerobic metabolism. Under natural diving conditions it is plausible that rising pCO_2 is indeed responsible for signaling the end of a dive (Stephenson, 2005). However if prevented from ending their dive (or motivated to extend their dive duration due to environmental circumstances such as prey availability) animals can use anaerobic metabolism or adjust management of O_2 stores—at which point low pO_2, high pCO_2 or increasing acidosis from anaerobic metabolism may all contribute to what ultimately terminates a dive. It is clear that diving animals can extend their diving well beyond their aerobic limits, but there may be a CO_2 limit beyond which animals cannot further adapt to increasing CO_2 stress.

Strengths, weaknesses & study limitations

A significant advantage of my study over others on diving physiology was the opportunity to work with trained animals diving under natural settings. While the training

itself affected the natural behaviour of the animals, it also allowed specific aspects of that behaviour to be controlled and examined. Many variables (especially physiological) that could not be measured or observed in wild individuals could be tested. Hence, although some consider captive animals to not accurately represent their wild counterparts, I believe it is clear from my study that captive animal programs are an invaluable tool in the field of diving physiology, as significant amounts of data could not be collected otherwise. Although raised in captivity, the animals in my study were diving regularly for research purposes and obtained a significant proportion of their food from such diving trials. Hence, they were closer in activity level to wild animals than they were to animals held in an aquarium or laboratory. Also, although training and behavioural responses can be significant in some respects, the physiological responses I directly measured (such as oxygen consumption rates) could not be adjusted by behavioural changes.

Working in the open ocean as opposed to an aquarium permits more natural dive behaviour and conversely, results in less control over animal behaviour. My thesis aimed to push the limits of voluntarily diving animals in which the training to use experimental equipment (and influence on natural behaviour) was kept as minimal as possible. In my study, the animals were allowed to determine their dive behaviour (duration of dives and surface intervals). Hence it was difficult to tease apart whether behavioural effects were indeed attributable to changes in physiology. Also, variable behavioural responses by the sea lions could have masked physiological effects. Despite these challenges, the ability to obtain this type of data that cannot be examined in the wild highlights the importance of my research.

A significant strength of my study was the ability to directly measure O_2 consumption rates alongside dive behaviour. Direct measures of $\dot{V}_{O_2}$ are considered one of the best measures of energy expenditure, allowing me to obtain a very precise measure of diving metabolic rates as compared to those obtained during field studies. When combined with measures of body O_2 stores, these resulted in the most accurate estimate of the cADL. In most other studies, field metabolic rate — measured using the doubly labeled water technique — is commonly used to estimate DMR in free ranging animals. However, this technique only provides a single average value of energy expenditure that cannot distinguish between different activity states and periods when the animal is on land (Costa and Gales, 2000; Costa and Gales, 2003; Fowler et al., 2007), and therefore cannot take into account potential

reductions in DMR while diving as compared to other activity states. Other studies estimate DMR through measures of activity using proxies such as accelerometry or heart rate, or as a multiple of resting or basal metabolic rate (BMR) based upon Kleiber's (1975) allometric equation. Hence, when comparing diving metabolic rate between species or individuals or when using DMR estimates to calculate dive limits, it is essential to consider how DMR is calculated and what assumptions most accurately reflect the true metabolic rate while diving in order to get the most accurate results.

A weakness of my study was the small sample size, which is fairly typical of studies on marine mammals (even those conducted in the wild) that commonly have data from only a few individuals. This reflects the difficulty of working with large, animals (that are voluntarily participating in dive trials) that spend significant portions of their lives underwater and for studies on detailed aspects of physiology in which limited biological samples can be obtained. However, my statistical analysis using linear mixed effects models added quantitative strength by enabling me to account for inter-animal variation through repeated measures techniques.

A significant limitation in my study was the extent to which inspired gas composition could be manipulated. It was logistically difficult to get steady initial concentrations using the experimental equipment (flow through respirometry). Hence, I was unable to alter gas concentrations within a trial, such as changing inspired gas concentrations for only certain portions of a dive cycle. The high flow-through rates required by the respirometry system (partly to keep inspired gas concentrations constant) also reduced the degree to which I could alter F_iCO_2 and F_iO_2, which was particularly limiting for my studies on hypoxia. I was unable to test hypoxic conditions at O_2 levels below 19% for diving trials, and therefore I could not examine hypoxic conditions to the same extent as I did for hypercapnia. Furthermore, the voluntary nature of the diving trials, combined with the sea lion's unwillingness to enter the metabolic dome at concentrations >3% CO_2 further limited the scope of my experimental conditions.

Applications & importance of study

Although there have been recent advancements in bio-logging studies that allow remote collection of biological data from wild animals (Ponganis, 2007), studies on

physiological control of diving are still essentially limited to laboratory settings due to the precision and nature of the measurements and manipulations required. In my thesis I had the opportunity to combine aspects of both lab and field studies in order to get precise physiological measurements under circumstances that would be experienced by wild animals. This ability to combine components of both lab and field studies is invaluable in understanding diving physiology in an unrestrained animal. I used this paradigm in my study to understand the physiological control of dive behaviour and the plasticity in these controls, to determine dive limits and infer how these may be shaped by environmental demands.

Knowledge of the diving limits of marine mammals is vital to understanding their foraging abilities. Dive limits will constrain where (depth) and how long animals can forage in the wild, and therefore the amount and type of prey accessible to them, allowing better models to be developed to assess the effects of changing prey abundance and distribution on wild populations. This also allows modeling the impact of resultant physiological changes on subsequent foraging success.

Results from my research suggest that animals need to expend more energy to dive when nutritionally stressed (due to higher metabolic rates) and that the proportion of a dive bout spent foraging would be lower. This would result in an overall lower foraging efficiency, and has implications for Steller sea lions in Alaska if nutritional stress is indeed affecting the declining populations of sea lions. The resulting decrease in net energy gain could further affect the nutritional status of wild animals and exaggerate the potential consequences of changing prey abundance and distribution and type of prey accessible to the animal.

Future research

Surprisingly, I found that nutritional stress did not limit the dive durations of Steller sea lions (although resulted in greater surface durations), and actually resulted in greater body O_2 stores due to significant increases in blood volume. The mechanism responsible for this increase is unclear and warrants further study. Observed changes could be triggered by several factors including the sudden reduction in food intake, the resulting change in body composition or simply the mass loss itself, and may be further affected by other circumstances such as the type (quality) of prey or season. Extended periods of nutritional stress as opposed to acute periods of limited food intake or fasting could have different effects on body

composition, oxygen stores and metabolic rates. These, in turn, could have very different implications for management decisions regarding the conservation of wild sea lions. Determining the direct causes of changes in diving capacity — and whether these are long or short term — are of immediate value to conservation efforts.

The physiological factors that ultimately limit an animals' diving ability are still unclear. Many animals end their dives well in advance of running out of O_2. Observing animal behaviour, such as the decisions that animals make in relation to prey availability would help to understand foraging behaviour and to better inform management decisions. Additionally, while many animals dive within aerobic limits, they are all capable of diving well beyond these limits using anaerobic metabolism, eventually resulting in metabolic acidosis. There is surprisingly little research investigating the anaerobic diving capacity of marine mammals and the environmental circumstances under which animals choose to extend dive durations and use anaerobic metabolism. While it is fairly clear what factors determine total body O_2 stores, the parameters determining total body CO_2 stores or the capacity to store metabolic byproducts from anaerobic metabolism are much more difficult to measure. It would be interesting to determine an "anaerobic dive limit" or total amount of CO_2 storage a diving mammal is capable of.

The ADL is generally calculated using a DMR averaged across the dive (and generally surface interval) and can only represent the total cost of a dive. The methods in my study did not allow me to look at the costs of diving vs. recovering at the surface. Nor was I able to distinguish between the cost of exercise vs. simply being at depth. Clearly, some aspects of a dive may be more costly than others, and changes in dive behaviour could affect DMR and the amount of time animals can remain in apnea. More accurately separating the costs of diving into its inherent components such as travelling, remaining at depth and breathing in between dives, may help to explain why animals can dive so much longer than their ADL. Management of oxygen stores goes beyond the degree to which animals choose to dive aerobically or anaerobically; animals must also be capable of altering O_2 use in different parts of the body depending on the type and duration of their dive. Future studies should attempt to determine the pattern and rate of O_2 use while diving, incorporate activity levels and examine differences in metabolic rate at the surface vs. being at depth. The measured ADL or "diving

lactate threshold" also has yet to be measured in Steller sea lions. Comparing this limit to the calculated ADL could be useful in determining what O_2 management strategies sea lions use.

It is interesting to note that $\dot{V}_{CO_2}$ did not rise following some short dives; hence DMR (in terms of CO_2 production) was essentially zero and could not be measured. As I was interested in determining the physiological limits of the diving ability of Steller sea lions there were very few short dives in my study, and I could not quantify the dive duration after which a measurable "CO_2 debt" (rise in $\dot{V}_{CO_2}$) occurred following diving. This rise in $\dot{V}_{CO_2}$ seemed to occur around 2 – 3 minute dive durations, curiously close to the ADL. Future studies could examine the point at which $\dot{V}_{CO_2}$ following a dive rises above baseline levels, in comparison to metabolic rate. This could be combined with other measures of buffering (CO_2 capacity) of blood and tissues to determine how much CO_2 the body can actually hold, especially over multiple dives.

Lastly, it is clear that individual sea lions have a significant behavioural capacity to ignore or withstand certain stresses while diving (i.e., the animal must be "ignoring" the urge to breathe to some extent during very long dives). More quantitative studies of the behavioural responses (as opposed to the physiological responses) of the animals to hypercapnia at varying levels and under various conditions could be very informative of CO_2 sensitivity and would be interesting to study further. There are many aspects of physiological control of dive behaviour that are still largely unknown, particularly the role of CO_2 and the extent of respiratory drive in an actively diving mammal. There could also be significant variation in body O_2 stores that needs further exploration to best estimate changes in diving ability and foraging behaviour. Understanding the behavioral and physiological limits to diving has significant implications for the population management and conservation of Steller sea lions.

References

Arnould, J. P. Y., Boyd, I. L. and Speakman, J. R. (1996). Measuring the body composition of Antarctic fur seals (*Arctocephalus gazella*): validation of hydrogen isotope dilution. *Physiol Zool* **69**, 93-116.

Bainton, C. R., Elsner, R. and Matthews, R. C. (1973). Inhaled CO_2 and progressive hypoxia: Ventilatory response in a yearling and a newborn harbour seal. *Life Sci* **12**, 527 - 533.

Benson, A. J. and Trites, A. W. (2002). Ecological effects of regime shifts in the Bering Sea and eastern North Pacific Ocean. *Fish Fish* **3**, 95-113.

Bentley, P., Herreid, C. and Schmidt-Nielsen, K. (1967). Respiration of a monotreme, the echidna, *Tachyglossus aculeatus*. *Am J Physiol* **212**, 957-961.

Biuw, M., McConnell, B., Bradshaw, C. J. A., Burton, H. and Fedak, M. (2003). Blubber and buoyancy: monitoring the body condition of free-ranging seals using simple dive characteristics. *J Exp Biol* **206**, 3405-3423.

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

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

Boyd, I. L., Woakes, A. J., Butler, P. J., Davis, R. W. and Williams, T. M. (1995). Validation of heart rate and doubly labelled water as measures of metabolic rate during swimming in California sea lions. *Funct Ecol* **9**, 151-160.

Burns, J., Lestyk, K., Folkow, L., Hammill, M. and Blix, A. (2007). Size and distribution of oxygen stores in harp and hooded seals from birth to maturity. *J Comp Physiol B* **177**, 687-700.

Burns, J. M., Costa, D., Frost, K. and Harvey, J. T. (2005). Development of body oxygen stores in harbor seals: effects of age, mass, and body composition. *Physiol Biochem Zool* **78**, 1057-1068.

Butler, P. J. (1982). Respiratory and cardiovascular control during diving in birds and mammals. *J Exp Biol* **100**, 195-221.

Butler, P. J. (2006). Aerobic dive limit. What is it and is it used appropriately? *Comp Biochem Physiol A Mol Integr Physiol* **145**, 1-6.

Butler, P. J. and Woakes, A. J. (1982). Control of heart rate by carotid body chemoreceptors during diving in tufted ducks. *J Appl Physiol* **53**, 1405-1410.

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

Butler, P. J., Green, J. A., Boyd, I. L. and Speakman, J. R. (2004). Measuring metabolic rate in the field: the pros and cons of the doubly labelled water and heart rate methods. *Funct Ecol* **18**, 168-183.

Castellini, M. A. and Somero, G. N. (1981). Buffering capacity of vertebrate muscle: Correlations with potentials for anaerobic function. *J Comp Physiol B* **143**, 191-198.

Castellini, M. A., Kooyman, G. L. and Ponganis, P. J. (1992). Metabolic rates of freely diving Weddell seals: correlations with oxygen stores, swim velocity and diving duration. *J Exp Biol* **165**, 181-194.

Cherel, Y., Gilles, J., Handrich, Y. and Le Maho, Y. (1994). Nutrient reserve dynamics and energetics during long-term fasting in the king penguin (*Aptenodytes patagonicus*). *J Zool* **234**, 1-12.

Collier, G. (1969). Body weight loss as a measure of motivation in hunger and thirst. *Ann N Y Acad Sci* **157**, 594-609.

Cornish, E. R. and Mrosovsky, N. (1965). Activity during food deprivation and satiation of six species of rodent. *Anim Behav* **13**, 242-248.

Costa, D. P. and Gales, N. J. (2000). Foraging energetics and diving behavior of lactating New Zealand sea lions, *Phocarctos hookeri*. *J Exp Biol* **203**, 3655-3665.

Costa, D. P. and Gales, N. J. (2003). Energetics of a benthic diver: seasonal foraging ecology of the Australian sea lion, *Neophoca cinerea*. *Ecol Monogr* **73**, 27-43.

Costa, D. P., Gales, N. J. and Crocker, D. E. (1998). Blood volume and diving ability of the New Zealand sea lion, *Phocarctos hookeri*. *Physiol Zool* **71**, 208-213.

Costa, D. P., Gales, N. J. and Goebel, M. E. (2001). Aerobic dive limit: how often does it occur in nature? *Comp Biochem Physiol A Mol Integr Physiol* **129**, 771-783.

Costa, D. P., Kuhn, C. E., Weise, M. J., Shaffer, S. A. and Arnould, J. P. Y. (2004). When does physiology limit the foraging behaviour of freely diving mammals? *Int Congr Ser* **1275**, 359-366.

Craig, A. B. and Pasche, A. (1980). Respiratory physiology of freely diving harbor seals (*Phoca vitulina*). *Physiol Zool* **53**, 419-432.

Crocker, D. E., LeBoeuf, B. J. and Costa, D. P. (1997). Drift diving in female northern elephant seals: Implications for food processing. *Can J Zool* **75**, 27-39.

Davis, R. W., Polasek, L., Watson, R., Fuson, A., Williams, T. M. and Kanatous, S. B. (2004). The diving paradox: new insights into the role of the dive response in air-breathing vertebrates. *Comp Biochem Physiol A Mol Integr Physiol* **138**, 263-268.

de Burgh Daly, M., Elsner, R. and Angell-James, J. E. (1977). Cardiorespiratory control by carotid chemoreceptors during experimental dives in the seal. *Am J Physiol Heart Circ Physiol* **232**, H508-H516.

Dierauf, L. A. and Gulland, F. M. D. (2001). CRC Handbook of Marine Mammal Medicine. Boca Raton, Florida: CRC Press Inc.

El-Sayed, H., Goodall, S. R. and Hainsworth, F. R. (1995). Re-evaluation of Evans blue dye dilution method of plasma volume measurement. *Clin Lab Haematol* **17**, 189-194.

Elsner, R., Angell-James, J. E. and Daly, M. B. (1977). Carotid body chemoreceptor reflexes and their interactions in the seal. *Am J Physiol* **232**, H517-H525.

Fahlman, A. L., Svard, C., Rosen, D. A. S., Jones, D. R. and Trites, A. W. (2008). Metabolic costs of foraging and the management of O_2 and CO_2 stores in Steller sea lions. *J Exp Biol* **211**, 3573-3580.

Falke, K. J., Busch, T., Hoffmann, O., Liggins, G. C., Liggins, J., Mohnhaupt, R., Roberts Jr, J. D., Stanek, K. and Zapol, W. M. (2008). Breathing pattern, CO_2 elimination and the absence of exhaled NO in freely diving Weddell seals. *Respir Physiol Neurobiol* **162**, 85-92.

Feldkamp, S. D., DeLong, R. L. and Antonelis, G. A. (1989). Diving patterns of California sea lions, *Zalophus californianus*. *Can J Zool* **67**, 872-883.

Foldager, N. and Blomqvist, C. G. (1991). Repeated plasma volume determination with the Evans blue dye dilution technique: The method and a computer program. *Comput Biol Med* **21**, 35-41.

Fowler, S. L., Costa, D. P., Arnould, J. P. Y., Gales, N. J. and Burns, J. M. (2007). Ontogeny of oxygen stores and physiological diving capacity in Australian sea lions. *Funct Ecol* **21**, 922-935.

Gallivan, G. J. (1980). Hypoxia and hypercapnia in the respiratory control of the Amazonian manatee (*Trichechus inunguis*). *Physiol Zool* **53**, 254-261.

Galster, W. and Morrison, P. (1975). Seasonal changes in body composition of the arctic ground squirrel, *Citellus undulatus*. *Can J Zool* **54**, 74-78.

Gerlinsky, C. D., Rosen, D. A. S. and Trites, A. W. (2013). High diving metabolism results in a short aerobic dive limit for Steller sea lions (*Eumetopias jubatus*). *J Comp Physiol B* **183,** 699-708.

Gibson, J. G. and Evans, W. A. (1937). Clinical studies of the blood volume. I. Clinical application of a method employing the azo dye "Evans Blue" and the spectrophotometer. *J Clin Invest* **16**, 301.

Green, J. A. (2011). The heart rate method for estimating metabolic rate: Review and recommendations. *Comp Biochem Physiol A Mol Integr Physiol* **158**, 287-304.

Guppy, M. and Withers, P. (1999). Metabolic depression in animals: physiological perspectives and biochemical generalizations. *Biol Rev* **74**, 1-40.

Halsey, L. G., Shepard, E. L. C. and Wilson, R. P. (2011). Assessing the development and application of the accelerometry technique for estimating energy expenditure. *Comp Biochem Physiol A Mol Integr Physiol* **158**, 305-314.

Hastie, G. D., Rosen, D. A. S. and Trites, A. W. (2007). Reductions in oxygen consumption during dives and estimated submergence limitations of Steller sea lions (*Eumetopias jubatus*). *Mar Mamm Sci* **23**, 272-286.

Horning, M. (2012). Constraint lines and performance envelopes in behavioural physiology: the case of the aerobic dive limit. *Front Physiol* **3**.

Horning, M. and Trillmich, F. (1997). Development of hemoglobin, hematocrit, and erythrocyte values in Galapagos fur seals. *Mar Mamm Sci* **13**, 100-113.

Houston, A. I. and Carbone, C. (1992). The optimal allocation of time during the diving cycle. *Behav Ecol* **3**, 255-265.

Hurley, J. A. and Costa, D. P. (2001). Standard metabolic rate at the surface and during trained submersions in adult California sea lions (*Zalophus californianus*). *J Exp Biol* **204**, 3273-3281.

Irving, L. (1938). The insensitivity of diving animals to CO_2. *Am J Physiol* **124**, 729-734.

Irving, L., Solandt, O. M. and Solandt, D. Y. (1935). The respiratory metabolism of the seal and its adjustment to diving. *J. Cell. Comp. Physiol.* **7**, 137-151.

Jeanniard du Dot, T., Rosen, D. A. S. and Trites, A. W. (2008). Steller sea lions show diet-dependent changes in body composition during nutritional stress and recover more easily from mass loss in winter than in summer. *J Exp Mar Biol Ecol* **367**, 1-10.

Kanatous, S. B., DiMichele, L. V., Cowan, D. F. and Davis, R. W. (1999). High aerobic capacities in the skeletal muscles of pinnipeds: adaptations to diving hypoxia. *J Appl Physiol* **86**, 1247.

Kleiber, M. (1975). The Fire of Life: An Introduction to Animal Energetics. New York: Robert E. Krieger Publ. Co.

Kodama, A. M., Elsner, R. and Pace, N. (1977). Effects of growth, diving history, and high altitude on blood oxygen capacity in harbor seals. *J Appl Physiol* **42**, 852-858.

Kohin, S., Williams, T. M. and Ortiz, C. L. (1999). Effects of hypoxia and hypercapnia on aerobic metabolic processes in northern elephant seals. *Respir Physiol* **117**, 59-72.

Kooyman, G. L. (1985). Physiology without restraint in diving mammals. *Mar. Mamm. Sci.* **1**, 166-178.

Kooyman, G. L. (1989). Diverse divers: Physiology and behaviour. Berlin: Springer.

Kooyman, G. L. (2002). Diving physiology. In *Encyclopedia of Marine Mammals*, eds. W. F. Perrin B. Wursig and J. G. M. Thewissen), pp. 339-344. San Diego, CA: Academic Press.

Kooyman, G. L. and Sinnett, E. E. (1982). Pulmonary Shunts in Harbor Seals and Sea Lions during Simulated Dives to Depth. *Physiol Zool* **55**, 105-111.

Kooyman, G. L. and Ponganis, P. J. (1998). The physiological basis of diving to depth: birds and mammals. *Annu Rev Physiol* **60**, 19-32.

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

Kooyman, G. L., Kerem, D. H., Campbell, W. B. and Wright, J. J. (1971). Pulmonary function in freely diving Weddell seals, *Leptonychotes weddelli*. *Respir Physiol* **12**, 271-282.

Kooyman, G. L., Kerem, D. H., Campbell, W. B. and Wright, J. J. (1973). Pulmonary gas exchange in freely diving weddell seals Leptonychotes weddelli. *Respir Physiol* **17**, 283-290.

Kooyman, G. L., Castellini, M. A., Davis, R. W. and Maue, R. A. (1983). Aerobic diving limits of immature Weddell seals. *J Comp Physiol A* **151**, 171-174.

Kooyman, G. L., Wahrenbrock, E. A., Castellini, M. A., Davis, R. W. and Sinnett, E. E. (1980). Aerobic and anaerobic metabolism during voluntary diving in Weddell seals: evidence of preferred pathways from blood chemistry and behavior. *J Comp Physiol A* **138**, 335-346.

Lenfant, C., Johansen, K. and Torrance, J. D. (1970). Gas transport and oxygen storage capacity in some pinnipeds and the sea otter. *Respir Physiol* **9**, 277 - 286.

Lenfant, C., Elsner, R., Kooyman, G. L. and Drabek, C. M. (1969). Respiratory function of the blood of the adult and fetus Weddell seal *Leptonychotes weddelli*. *Am J Physiol* **216**, 1595-1597.

Lighton, J. R. B. (2008). Measuring metabolic rates: A manual for scientists. New York: Oxford University Press.

Loughlin, T. R., Perlov, A. S., Baker, J. D., Blokhin, S. A. and Makhnyr, A. G. (1998). Diving behavior of adult female Steller sea lions in the Kuril Islands, Russia. *Biosph Conserv* **1**, 21-31.

MacArthur, R. (1990). Seasonal changes in the oxygen storage capacity and aerobic dive limits of the muskrat (*Ondatra zibethicus*). *J Comp Physiol B* **160**, 593-599.

Maj, A. (2011). lmmfit: Goodness-of-fit measures for linear mixed models with one-level grouping. R package version 1.0.

Markussen, N. H. (1995). Changes in metabolic rate and body composition during starvation and semistarvation in harbour seals. In *Developments in Marine Biology 4: Whales, Seals, Fish and Man*, eds. A. S. Blix L. Walløe and Ø. Ulltang), pp. 383-391. Amsterdam: Elsevier.

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

Merrick, R. L. and Loughlin, T. R. (1997). Foraging behavior of adult female and young-of-year Steller sea lions in Alaskan waters. *Can J Zool* **75**, 776-786.

Merrick, R. L., Loughlin, T. R., Antonelis, G. A. and Hill, R. (1994). Use of satellite-linked telemetry to study Steller sea lion and northern fur seal foraging. *Polar Res* **13**, 105 - 114.

Milsom, W. K., Castellini, M. A., Harris, M. P., Castellini, J. M., Jones, D. R., Berger, R., Bahrma, S., Rea, L. and Costa, D. (1996). Effects of hypoxia and hypercapnia on patterns of sleep-associated apnea in elephant seal pups. *Am J Physiol* **271**, R1017 - R1024.

Mitani, Y., Andrews, R. D., Sato, K., Kato, A., Naito, Y. and Costa, D. P. (2010). Three-dimensional resting behaviour of northern elephant seals: drifting like a falling leaf. *Biol Lett* **6**, 163-166.

Nielsen, M. H. and Nielsen, N. C. (1962). Spectrophotometric determination of Evans blue dye in plasma with individual correction for blank density by a modified Gablers method. *Scand J Clin Lab Investig* **14**, 605-617.

Nordoy, E. S. and Blix, A. S. (1985). Energy sources in fasting grey seal pups evaluated with computed tomography. *Am J Physiol Regul Integr Comp Physiol* **249**, R471-R476.

Noren, S., Williams, T., Ramirez, K., Boehm, J., Glenn, M. and Cornell, L. (2012). Changes in partial pressures of respiratory gases during submerged voluntary breath hold across odontocetes: is body mass important? *J Comp Physiol B* **182**, 299-309.

Noren, S. R. and Williams, T. M. (2000). Body size and skeletal muscle myoglobin of cetaceans: adaptations for maximizing dive duration. *Comp Biochem Physiol A Mol Integr Physiol* **126**, 181-191.

Noren, S. R., Iverson, S. J. and Boness, D. J. (2005). Development of the blood and muscle oxygen stores in Gray seals (*Halichoerus grypus*): Implications for juvenile diving capacity and the necessity of a terrestrial postweaning fast. *Physiol Biochem Zool* **78**, 482-490.

Noren, S. R., Williams, T. M., Pabst, D. A., McLellan, W. A. and Dearolf, J. L. (2001). The development of diving in marine endotherms: preparing the skeletal muscles of dolphins, penguins and seals for activity during submergence. *J Comp Physiol B* **171**, 127-134.

Oritsland, N. A. (1990). Starvation survival and body composition in mammals with particular reference to Homo sapiens. *Bull Math Biol* **52**, 643-655.

Parkos, C. A. and Wahrenbrock, E. A. (1987). Acute effects of hypercapnia and hypoxia on minute ventilation in unrestrained Weddell seals. *Respir Physiol* **67**, 197-207.

Påsche, A. (1976a). The effect of hypercapnia on respiratory characteristics and diving behaviour of freely diving seals. *Respir Physiol* **26**, 183-193.

Påsche, A. (1976b). Hypoxia in freely diving hooded seal, *Cystophora cristata*. *Comp Biochem Physiol A Mol Integr Physiol* **55**, 319-322.

Phillipson, E., Duffin, J. and Cooper, J. (1981). Critical dependence of respiratory rhythmicity on metabolic CO_2 load. *J Appl Physiol* **50**, 45 - 55.

Pinheiro, J., Bates, D., DebRoy, S., Sarkar, D. and Team", R. D. C. (2011). nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-102.

Pinheiro, J. C. and Bates, D. M. (2000). Mixed-effects models in S and S-PLUS. New York: Springer-Verlag.

Pitcher, K. W., Rehberg, M. J., Pendleton, G. W., Raum-Suryan, K. L., Gelatt, T. S., Swain, U. G. and Sigler, M. F. (2005). Ontogeny of dive performance in pup and juvenile Steller sea lions in Alaska. *Can J Zool* **83**, 1214-1231.

Ponganis, P. J. (2007). Bo-logging of physiological parameters in higher marine vertebrates. *Deep Sea Research Part II* **54**, 183-192.

Ponganis, P. J., Kooyman, G. L. and Castellini, M. A. (1993). Determinants of the aerobic dive limit of Weddell seals: Analysis of diving metabolic rates, postdive end tidal PO_2's, and blood and muscle oxygen stores. *Physiol Zool* **66**, 732-749.

Ponganis, P. J., Meir, J. U. and Williams, C. L. (2011). In pursuit of Irving and Scholander: a review of oxygen store management in seals and penguins. *J Exp Biol* **214**, 3325-3339.

Ponganis, P. J., Kooyman, G. L., Winter, l. M. and Starke, L. N. (1997a). Heart rate and plasma lactate responses during submerged swimming and trained diving in California sea lions, *Zalophus californianus*. *J Comp Physiol B* **167**, 9-16.

Ponganis, P. J., Kooyman, G. L., Baranov, E. A., Thorson, P. H. and Stewart, B. S. (1997b). The aerobic submersion limit of Baikal seals, *Phoca sibirica*. *Can J Zool* **75**, 1323-1327.

Qvist, J., Hill, R. D., Schneider, R. C., Falke, K. J., Guppy, M., Elliot, R. L., Hochachka, P. W. and Zapol, W. M. (1986). Hemoglobin concentrations and blood gas tensions of free-diving Weddell seals. *J Appl Physiol* **61**, 1560-1569.

R Development Core Team. (2011). R: A language and Environment for Statistical Computing. Vienna, Austria: R Foundation for Statistical Computing.

Reed, J. Z., Chambers, C., Fedak, M. A. and Butler, P. J. (1994). Gas exchange of captive freely diving grey seals (*Halichoerus grypus*). *J Exp Biol* **191**, 1-18.

Reilly, J. J. and Fedak, M. A. (1990). Measurement of the body composition of living gray seals by hydrogen isotope dilution. *J Appl Physiol* **69**, 885-891.

Richmond, J. P., Burns, J. M. and Rea, L. (2006). Ontogeny of total body oxygen stores and aerobic dive potential in Steller sea lions (*Eumetopias jubatus*). *J Comp Physiol B* **176**, 535-545.

Robin, E. D., Murdaugh, H. V., Pyron, W., Weiss, E. and Soteres, P. (1963). Adaptations to diving in the harbor seal— gas exchange and ventilatory response to CO_2. *Am J Physiol* **205**, 1175-1177.

Rosen, D. A. S. (2009). Steller sea lions *Eumetopias jubatus* and nutritional stress: evidence from captive studies. *Mammal Rev* **39**, 284-306.

Rosen, D. A. S. and Trites, A. W. (1997). Heat increment of feeding in Steller sea lions, *Eumetopias jubatus*. *Comp Biochem Physiol A Mol Integr Physiol* **118**, 877-881.

Rosen, D. A. S. and Trites, A. W. (1999). Metabolic effects of low-energy diet on Steller sea lions, *Eumetopias jubatus*. *Physiol Biochem Zool* **72**, 723-731.

Rosen, D. A. S. and Trites, A. W. (2001). Effect of diet composition and feeding regime on body mass and composition in captive Steller sea lions. In *14th Biennial Conference on the Biology of Marine Mammals*, pp. 183. Vancouver, BC.

Rosen, D. A. S. and Trites, A. W. (2002). Changes in metabolism in response to fasting and food restriction in the Steller sea lion. *Comp Biochem Physiol B Biochem Mol Biol* **132**, 389-399.

Rosen, D. A. S. and Trites, A. W. (2010). Split personalities: Seasonal energetic priorities in young northern fur seals. In *Proceedings of the Canadian Zoological Society, May 17-21, 2010*. Vancouver, BC, Canada.

Rosen, D. A. S., Hastie, G. D. and Trites, A. W. (2004). Searching for stress: hematological indicators of nutritional inadequacies in Steller sea lions. In *Symposia of the Comparative Nutrition Society*, pp. 145-149. Hickory Corners, Michigan.

Rosen, D. A. S., Winship, A. J. and Hoopes, L. A. (2007). Thermal and digestive constraints to foraging behaviour in marine mammals. *Philos Trans, R Soc Lond B Biol Sci* **362**, 2151-2168.

Saunders, D. K. and Fedde, M. R. (1991). Physical conditioning: Effect on the myoglobin concentration in skeletal and cardiac muscle of bar-headed geese. *Comp Biochem Physiol A Mol Integr Physiol* **100**, 349-352.

Scholander, P. F. (1940). Experimental investigations on the respiratory function in diving mammals and birds. Oslo: Det Norske Videnskaps-Akademi i Oslo.

Schreer, J. and Kovacs, K. (1997). Allometry of diving capacity in air-breathing vertebrates. *Can J Zool* **75**, 339-358.

Sease, J. L., Taylor, W. P., Loughlin, T. R. and Pitcher, K. W. (2001). Aerial and Land Based Surveys of Steller Sea Lions (*Eumetopias jubatus*) in Alaska. June and July 1999 and 2000. In *NOAA Technical Memorandum NMFS-AFSC-122*, (ed. U. S. D. O. Commerce). Seattle, WA.

Shaffer, S. A., Costa, D. P., Williams, T. M. and Ridgway, S. H. (1997). Diving and swimming performance of white whales, *Delphinapterus leucas*: an assessment of plasma lactate and blood gas levels and respiratory rates. *J Exp Biol* **200**, 3091-3099.

Sparling, C. E., Fedak, M. A. and Thompson, D. (2007). Eat now pay later? Evidence of deferred food-processing costs in diving seals. *Biol Lett* **3**, 94-98.

Stephenson, R. (2005). Physiological control of diving behaviour in the Weddell seal *Leptonychotes weddelli*: a model based on cardiorespiratory control theory. *J Exp Biol* **208**, 1971-1991.

Stephenson, R., Turner, D. L. and Butler, P. J. (1989). The relationship between diving activity and oxygen storage capacity in the tufted duck (*Aythya fuligula*). *J Exp Biol* **141**, 265-275.

Svärd, C., Fahlman, A. L., Rosen, D. A. S., Joy, R. and Trites, A. W. (2009). Fasting affects the surface and diving metabolic rates of Steller sea lions *Eumetopias jubatus*. *Aquat Biol* **8**, 71-82.

Trites, A. W. and Larkin, P. A. (1996). Changes in the Abundance of Steller sea lions (*Eumetopias jubatus*) in Alaska from 1956 to 1992: how many were there? *Aquat Mamm* **22**, 153-166.

Trites, A. W. and Donnelly, C. P. (2003). The decline of Steller sea lions, *Eumetopias jubatus,* in Alaska: a review of the nutritional stress hypothesis. *Mamm Rev* **33**, 3-28.

Trites, A. W., Miller, A. J., Maschner, H. D. G., Alexander, M. A., Bograd, S. J., Calder, J. A., Capotondi, A., Coyle, K. O., Lorenzo, E. D., Finney, B. P. et al. (2007). Bottom-up forcing and the decline of Steller sea lions (*Eumetopias jubatus*) in Alaska: assessing the ocean climate hypothesis. *Fish Oceanogr* **16**, 46-67.

Villegas-Amtmann, S. and Costa, D. P. (2010). Oxygen stores plasticity linked to foraging behaviour and pregnancy in a diving predator, the Galapagos sea lion. *Funct Ecol* **24**, 785-795.

Villegas-Amtmann, S., Atkinson, S., Paras-Garcia, A. and Costa, D. P. (2012). Seasonal variation in blood and muscle oxygen stores attributed to diving behavior, environmental temperature and pregnancy in a marine predator, the California sea lion. *Comp Biochem Physiol A Mol Integr Physiol* **162**, 413-420.

Weise, M. J. and Costa, D. P. (2007). Total body oxygen stores and physiological diving capacity of California sea lions as a function of sex and age. *J Exp Biol* **210**.

Williams, T. M., Friedl, W. A. and Haun, J. A. (1993). The physiology of bottlenose dolphins (*Tursiops truncatus*): heart rate, metabolic rate and plasma lactate concentration during exercise. *J Exp Biol* **179**, 31-46.

Williams, T. M., Haun, J. E. and Friedl, W. A. (1999). The diving physiology of bottlenose dolphins (*Tursiops truncatus*). I. Balancing the demands of exercise for energy conservation at depth. *J Exp Biol* **202**, 2739-2748.

Young, B. L., Rosen, D. A. S., Hindle, A. G., Haulena, M. and Trites, A. W. (2010). Dive behaviour impacts the ability of heart rate to predict oxygen consumption in Steller sea ions (*Eumetopias jubatus*) foraging at depth. In *Proceedings of the Canadian Zoological Society, May 17-21, 2010*.

Appendices

Appendix A Supplementary figures and data tables

Table A.1 Blood measurements and nutritional status for diving (summer) and non-diving (spring) animals before and after a period of restricted food intake

Animal	Season	Nutritional state	Diet	Mass	Hct	PV (L)	BV (L)	BV% Mass	[Hb] (g/l)	Blood O_2 (L)	Lean body mass (kg)	Lipid Mass (kg)
F00YA	Summer	Normal	Herring	218	0.43	12.1	21.1	9.70%	141	2.82	183	35.2
F00BO	Summer	Normal	Herring	157	0.42	8.74	15.1	9.60%	155	2.26	133	24.0
F97SI	Summer	Normal	Herring	225	0.41	12.8	21.7	9.63%	150	3.13	197	28.4
F97HA	Summer	Normal	Herring	175	0.44	9.85	17.6	10.1%	160	2.74	150	24.9
				194	**0.43**	**10.9**	**18.9**	**9.75%**	**152**	**2.74**	**166**	**28.1**
F00YA	Summer	Stressed	Herring	197	0.46	13.7	25.4	12.9%	162	4.03	174	23.5
F00BO	Summer	Stressed	Herring	140	0.43	10.3	18.1	12.9%	152	2.65	122	17.5
F97SI	Summer	Stressed	Herring	201	0.47	15.8	29.8	14.9%	166	4.87	186	14.3
F97HA	Summer	Stressed	Herring	159	0.47	10.3	19.4	12.2%	162	3.07	141	18.2
				174	**0.46**	**12.5**	**23.2**	**13.2%**	**161**	**3.65**	**156**	**18.4**
F03RO	Spring	Normal	Herring	171	0.49	8.77	17.2	10.1%	170	2.89	146	24.7
F03WI	Spring	Normal	Herring	178	0.50	9.45	18.9	10.6%	166	3.08	148	29.7
F03AS	Spring	Normal	Atka mackerel	171	0.40	9.64	16.1	9.40%	137	2.07	146	24.6
F03IZ	Spring	Normal	Atka mackerel	180	0.40	10.5	17.6	9.76%	137	2.26	153	27.1
				175	**0.45**	**9.6**	**17.4**	**9.96%**	**153**	**2.58**	**148**	**26.5**
F03RO	Spring	Stressed	Herring	157	0.50	9.21	18.4	11.7%	170	3.09	139	18.6
F03WI	Spring	Stressed	Herring	166	0.48	9.70	18.7	11.2%	166	3.04	149	17.3
F03AS	Spring	Stressed	Atka mackerel	152	0.51	9.41	19.2	12.7%	171	3.25	144	7.74
F03IZ	Spring	Stressed	Atka mackerel	161	0.40	8.11	13.5	8.41%	138	1.76	153	8.11
				159	**0.47**	**9.1**	**17.4**	**11.0%**	**161**	**2.79**	**146**	**12.9**

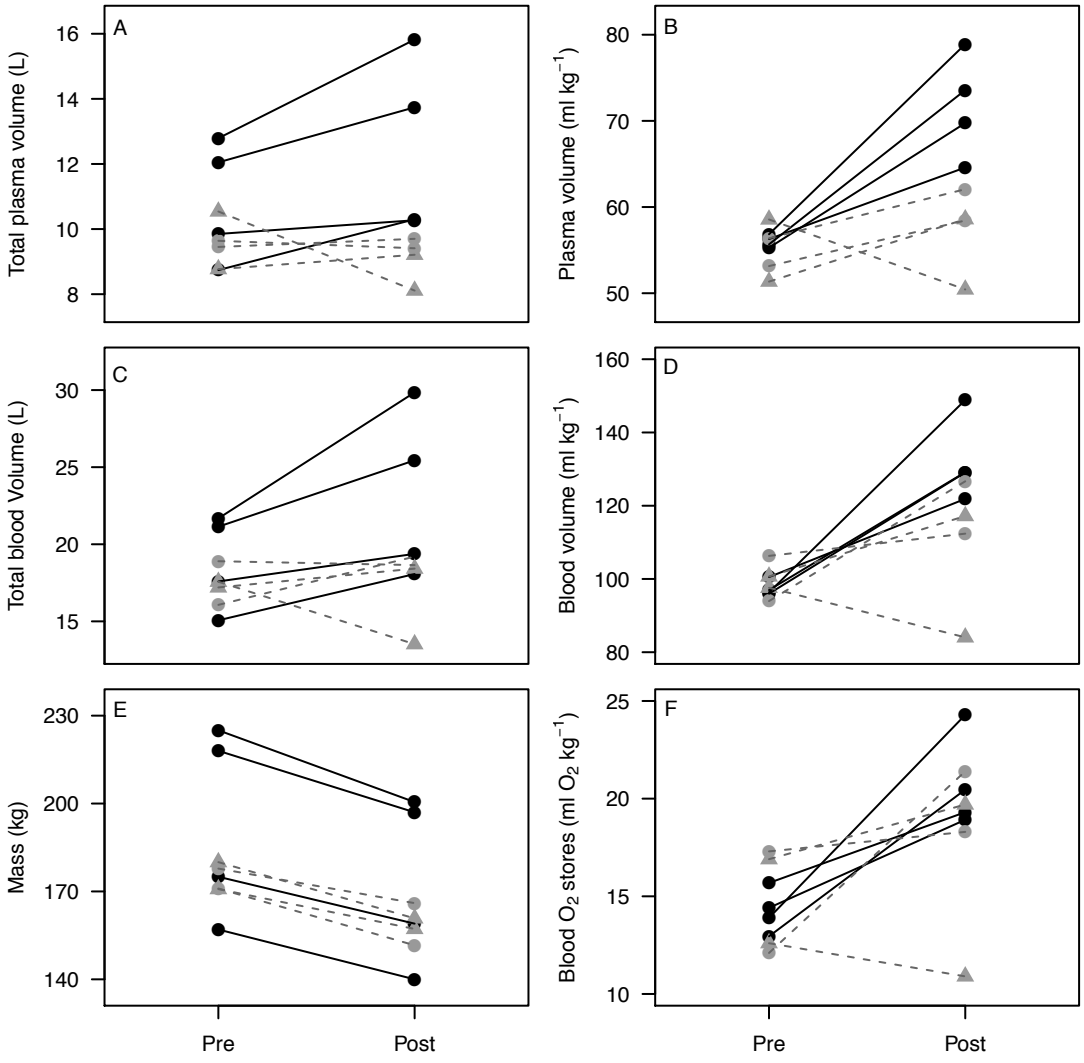

Figure A.1 Parameters related to blood volume measurements before and after nutritional stress. Each line represents an individual diving (black, summer) and non-diving (grey, spring) animal. Individuals were on a diet of either herring (circles) and or Atka mackerel (triangles).

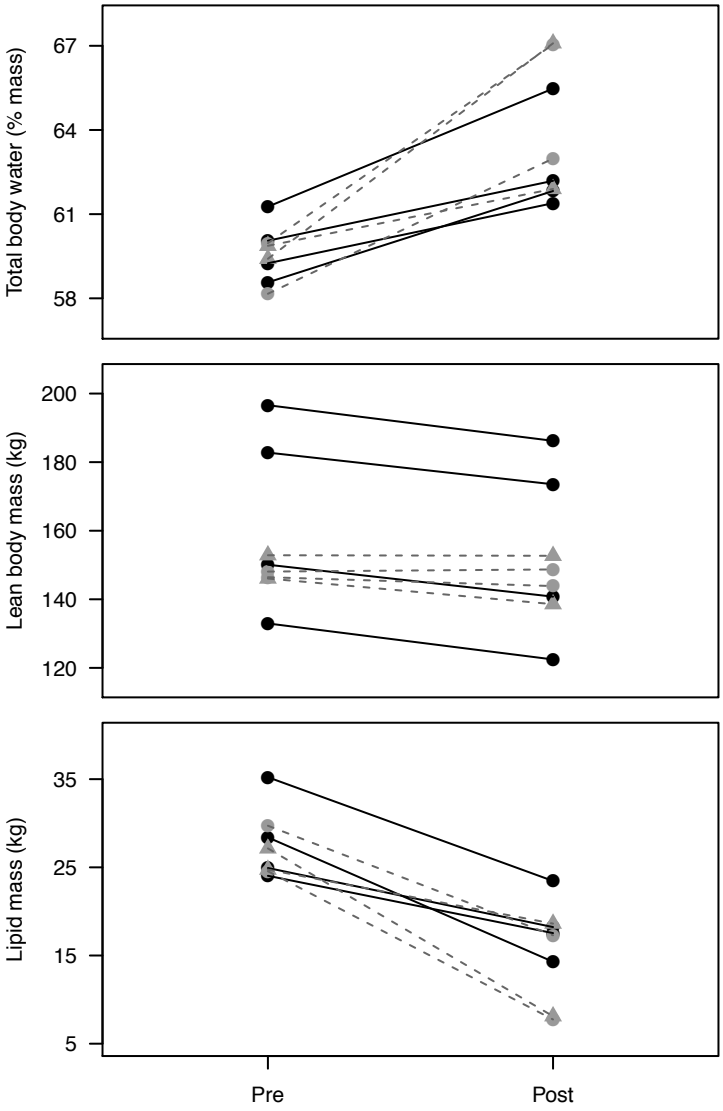

Figure A.2 Body composition of diving (black) and non-diving (grey) animals before and after nutritional stress including lean body mass (kg), total body lipid (kg) and total body water as a % of body mass. Animals were on a restricted diet of either Herring (circles) or Atka mackerel (triangles)

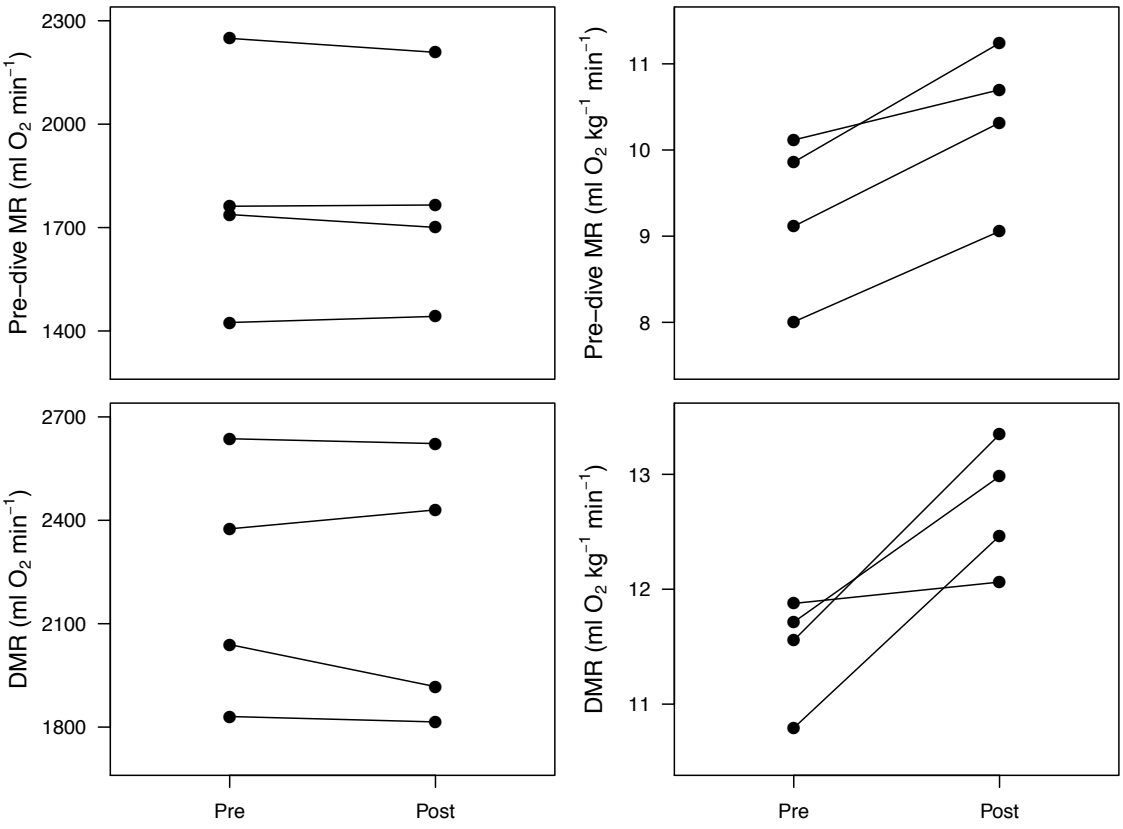

Figure A.3 Pre-dive (resting, top) and diving (bottom) metabolic rate before and during nutritional stress on an absolute (left) and mass-specific (right) basis.

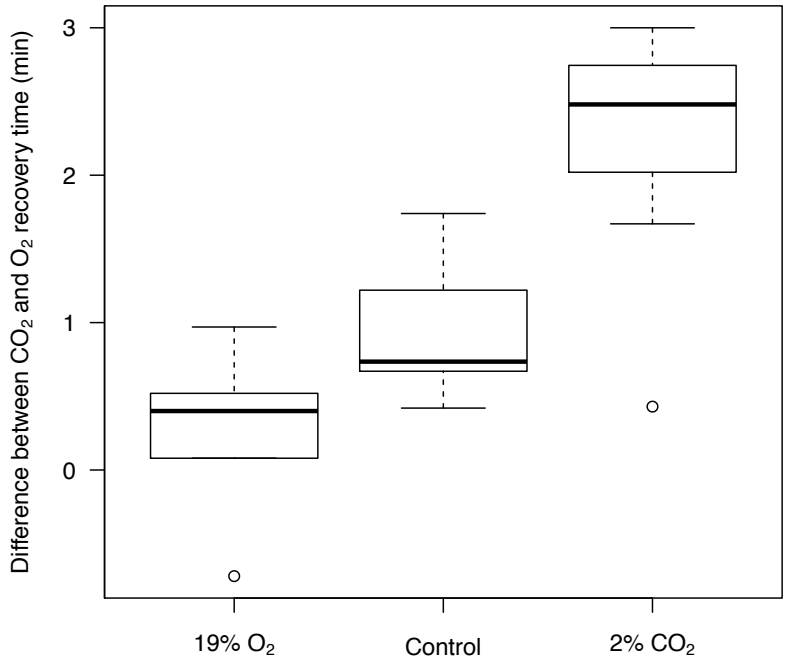

Figure A.4 Difference between CO_2 and O_2 recovery time (time to eliminate accumulated CO_2 vs. refill O_2 stores) following single long dives (average 5.2 minutes) in nutritionally stressed animals as a function of inspired gas.