POST-DIVE GAS RECOVERY AND THE TRANSITION BETWEEN METABOLIC STATES AS PHYSIOLOGICAL LIMITS TO DIVING IN STELLER SEA LIONS (*EUMETOPIAS JUBATUS*)

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submitted by Aaron Scott Purdy in partial fulfillment of the requirements for

the degree of Master of Science

in Zoology

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Abstract

Marine mammal diving behaviour is influenced by multiple physiological processes, both at depth and at the surface. To date, the majority of research in diving physiology has focused solely on how quickly marine mammals utilize their O_2 during a dive, as seen in the numerous studies of the aerobic dive limit (ADL) and calculated aerobic dive limit (cADL). In this thesis I investigated other physiological limits, namely how long it takes for marine mammals to recover after a dive, and how these animals transition between aerobic and anaerobic metabolism at depth. Specifically, I 1) determined how post-dive rates of O_2 and CO_2 gas exchange are affected by dive behaviour, and 2) measured how lactate accumulates with increased dive time, and examined how this indicator of metabolic transition affected post-dive recovery times. To measure gas exchange, I used flow-through respirometry to determine the time required for Steller sea lions (*Eumetopias jubatus*) to reach within 5% of stable rates of O_2 uptake and CO_2 excretion following a dive. These times were interpreted as the O_2 and CO_2 recovery times, respectively. CO_2 recovery time was longer and became more extended with increasing dive time when compared to O_2, requiring an extra 44 sec per minute submerged for CO_2 as opposed to 33 sec per minute submerged for O_2. This indicates that recovery time was limited by CO_2 as opposed to O_2, and this difference became greater with increased dive time. Contrary to traditional models, plasma lactate concentration was present even after short dives, and increased linearly with dive duration. Neither O_2 nor CO_2 recovery rates were affected by levels of blood lactate. This indicates that anaerobic metabolism may be used long before the body's total O_2 stores have been consumed. These results support the idea that there is not a distinct threshold between aerobic and anaerobic pathways, but rather a progressive transition, which casts doubt on the usual interpretations of the ADL and cADL. My thesis challenges long-held assertions in diving physiology, and underlines the need to further examine how CO_2 and lactate accumulation may act as limits to diving behaviour.

Lay Summary

Marine mammals must balance two important behaviours, diving underwater to hunt for food, and breathing air at the surface. While diving mammals have many physiological adaptations to help them maximize their time at depth and then recover efficiently, these behaviours are still limited by their physiology. My thesis explores two key physiological limits that affect diving behaviour in Steller sea lions (*Eumetopias jubatus*). Specifically, I examined how long it took to recover from dives, and how these animals transition from aerobic to anaerobic metabolism at depth. My results provide a greater understanding of how diving behaviour is limited by physiology. This insight is valuable for conservation and population management and will help us to better predict how marine mammals might react to a changing environment.

Preface

I acted as the lead investigator for the experiments described in this thesis. All research questions were created in collaboration with Dr. David Rosen who contributed suggestions to research design, and manuscript edits in his supervisory position. I collected and analysed all data outlined below, and prepared all manuscripts. Blood chemistry and hematology analysis as described in Chapter 3 were completed by Idexx Laboratories (Delta, BC, CAN).

All research in this thesis was approved by the Vancouver Aquarium Animal Care Committee and was conducted under UBC Animal Care Permits A11-0397 and A16-0027. Prior to starting on this research, I completed the ethics training requirements of the Canadian Council on Animal Care (CCAC) / National Institutional Animal User Training (NIAUT) Program (Certificate #: 7774 – 16).

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List of Abbreviations

ADL	Aerobic dive limit
BV	Blood volume
cADL	Calculated aerobic dive limit
DMR_{cycle}	Diving metabolic rate, measured over the 'dive cycle' (dive & recovery period)
DMR_{dive}	Diving metabolic rate, measured as excess O_2 consumption contributed to solely dive duration
EPOC	Excess post-exercise oxygen consumption
Hct	Hematocrit
LDH	Lactate dehydrogenase
PCO_2	Partial pressure of carbon dioxide
PO_2	Partial pressure of oxygen
RER_I	Respiratory exchange ratio, as measured at a single point in recovery
RER_T	Respiratory exchange ratio, as calculated over the entire recovery period
TBO	Total body oxygen stores
ΣVO_2	Total O_2 consumed during the recovery period

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To Laura Purdy

For inspiring me to seek out wonder in this amazing world

Chapter 1: Introduction

The field of diving physiology, while diverse in its scope, primarily aims to answer a single question succinctly expressed by Gerald L. Kooyman, one of the "fathers" of marine mammal physiology: "how [do] marine tetrapods 'work' under natural conditions." The field of inquiry encompasses many topics, but the majority of research has explored how diving animals are capable of remaining submerged for such long periods. More recently, some studies have begun to appreciate the significance of the time that animals spend at the surface between diving episodes, recovering from the physiological exertion of submergence. Investigating the physiology of "recovery" not only sheds light on aspects of diving physiology, but also has important implications for understanding the foraging ecology of marine mammals.

To optimize foraging efficiency, marine mammals must find a balance between two competing behaviours—diving at depth to find food, and subsequently recovering from this activity at the surface. Inevitably, this balance will be determined by the interaction of environmental conditions such as prey characteristics, and the physiological limits that constrain diving and recovery behaviour. Understanding the physiology of marine mammals both during a dive, and the subsequent recovery is therefore the foundation for understanding both their "normal" foraging ecology, and their capacity for adapting to changing circumstances. In the modern environment, many marine mammals can face rapid changes to the distribution or abundance of their prey. These changes have the potential to drastically alter marine mammal behaviour when diving to obtain their food. By studying diving physiology, we can both gain an appreciation for what shapes marine mammal foraging behaviour, and be better able to predict how marine mammals may respond to future environmental changes.

Researchers have long been interested in exploring the diving capability of marine birds and mammals, with research extending as far back as the mid-1800s. However, much of our initial understanding of the field comes from the 1930s and 1940s from studies by Per Scholander, Laurence Irving and their colleagues in laboratory studies (Irving *et al*., 1941; Scholander, 1940, 1963; Scholander *et al*., 1942a). These studies laid the groundwork for the field that eventually became "diving physiology", illustrating key foundations which, due to their importance, are now nearly considered common knowledge. In their investigations they described a series of

physiological responses to breath-hold diving in marine vertebrates that consist of three main adaptations: apnea, bradycardia, and peripheral vasoconstriction. Apnea delays the urge to return to the surface, allowing diving mammals to maximize the use of stored O_2 in the body. Peripheral vasoconstriction leads to hypoperfusion of "non-essential" peripheral tissues, diverting blood to the heart, brain, and other organs that require a constant oxygen supply. To avoid hypertension from this vasoconstriction, diving mammals also display decreased cardiac output through a profound bradycardia. This maintains a reasonably stable central arterial blood pressure in spite of an appreciable increase in peripheral vascular resistance. Together, these adaptations have become known as the mammalian "dive response." As a whole, this response allows marine mammals to direct most of their stored O_2 to organs and tissues that are vitally important, reducing diving animals to, as stated by Scholander in 1963, "heart, lung, and brain machines."

While these ground-breaking studies provided invaluable insights into how marine mammals can withstand such long durations submerged, they have been criticized for not truly showcasing the response to a natural dive. In studying the dive response, many animals were submitted to a "forced dive" where only the researcher was in control of the dive duration. This raises the spectre that the observed physiological response was either an unnatural maximum, or even a fear response. Later studies with voluntary dives (where dive duration is controlled by the animal) explored the plasticity of this dive response (Fahlman *et al*. 2008; Gerlinsky *et al*. 2014; Sparling & Fedak, 2004). Instead of an automatic reflex, marine mammals appear to respond to dives with more graded adjustments to different factors, such as the extent of bradycardia, which may even be under voluntary control to some extent (Butler, 1988; Butler & Jones, 1997). Further research revealed other adaptations to prolonged diving. For example, marine mammals have high O_2 stores compared to their terrestrial counterparts, allowing them to extend their aerobic potential (Butler & Jones, 1997; Castellini *et al*., 1992; Lydersen *et al*., 1992; MacArthur *et al*., 2001; Richmond *et al*., 2006; Weise & Costa, 2007). Studies have also shown that the rate at which oxygen is consumed during a dive is lower compared to terrestrial mammals exercising at similar intensities. Studies have also quantified how metabolic rate changes during a single dive; for example, transiting between the surface and the foraging site is more energetically costly and consumes more O_2 than swimming at depth (Goundie *et al*., 2015; Hindle *et al*., 2010). Behavioural adaptations can therefore allow these animals to avoid excess O_2 consumption, increasing their diving potential (Williams, 2001; Williams *et al*., 2000). Finally, external factors such as season

or prey quality can have an effect on body condition, which in turn can physiologically affect the diving ability of marine mammals (Boyd, *et al*., 1993; Gerlinsky *et al*., 2014). Together this suite of physiological, anatomical, and behavioural adaptations help to optimize O_2 supply and demand, and provide marine mammals with the tools to greatly extend dive duration before they must return to the surface.

However, even with these many adaptations, dive behaviour is not infinitely plastic. While diving capacity may be enhanced by an animal's physiology, this physiology ultimately places constraints on behaviours as well. It is important to remember that diving behaviour is not only constrained by physiology at depth, but also at the surface. Any O_2 stores consumed during a dive must eventually be replenished during a post-dive recovery period. This time at the surface is key to determining an animal's foraging efficiency, as every minute spent at the surface delays when it can dive again in search of food. In general, longer dives will incur longer recovery periods, and this link between dive duration and recovery is an important consideration when modeling optimal foraging behaviour of marine mammals (Carbone & Houston, 1996; Doniol-Valcroze *et al*., 2011; Halsey & Butler, 2006; Kramer, 1988; Mori, 1998, 2002; Thompson & Fedak, 2001). However, the exact nature of the relationship between dive duration and recovery time has largely been unstudied. If O_2 consumption at depth is considered a prime determinant of diving capacity, then the rate at which O_2 can be subsequently replenished should limit future dives. Basic principles of gas exchange help to explain how marine mammals may recover upon surfacing. At the end of a dive, the large difference in the partial pressure of oxygen (PO_2) between a diving mammal's blood and the environment will initially allow for rapid replenishment of O_2 stores when the animal returns to the surface according to Fick's Equation of diffusion. However, this difference greatly decreases as surface time extends, decreasing the efficiency of recovery. While these general mechanics of O_2 gas exchange are well understood, how this may change with differing dive duration has yet to be explored.

Replenishing O_2 stores is not the only post-dive physiological challenge that must be overcome at the surface. Throughout a dive, marine mammals accumulate metabolites that also must be eliminated during the post-dive recovery period. One of these key metabolites is carbon dioxide (CO_2), which generally accumulates in direct proportion to O_2 consumption at depth (Kooyman *et al*., 1980; Qvist *et al*., 1986). In terrestrial mammals CO_2 serves as a primary stimulus

for ventilation and heart rate (Phillipson *et al*., 1981), so it is logical to assume a similar effect in marine mammals. If accumulated CO_2 stimulates respiratory drive, dive duration may ultimately be dependent on a diving mammal's ability to store this CO_2, and/or withstand high partial pressures of CO_2 in the blood (PCO_2), to delay the drive to breathe (Craig & Påsche, 1980; Gerlinsky *et al.*, 2014; Påsche, 1976a, 1976b). In addition, stored CO_2 must be cleared effectively upon surfacing prior to the next dive (Boutilier, Reed, & Fedak, 2001). Despite its potential importance, relatively few studies have examined how marine mammals recover from CO_2 accumulation after diving.

There are also reasons to believe that CO_2 may actually prove to be more limiting to surface recovery than O_2. While CO_2 exchange at the lungs follows the same basic principles of diffusion outlined in Fick's Equation, the physiology of CO_2 removal is somewhat more complicated (for review see Piiper, 1990). The vast majority of CO_2 in mammals is transported in the blood as bicarbonate. When circulating blood enters the lungs, this bicarbonate must first be converted back to CO_2 for exchange. It is first transported into the red blood cell in exchange for chloride ions via the chloride-bicarbonate exchanger. The bicarbonate can then be converted to CO_2 by carbonic anhydrase and returned to the serum before finally diffusing into the lung. As previously stated, the rate of O_2 exchange is limited largely by partial pressure differences between a marine mammal's blood and the environment. In comparison, CO_2 is limited more by the rate of chemical reactions and transport, particularly the chloride-bicarbonate exchange across the membrane of the red blood cell (Bidani & Crandall, 1988; Wieth *et al*., 1982). It seems likely that after extensive dives, marine mammal recovery will be limited not by O_2, but by CO_2 exchange. While these principals are relatively well understood, the exact rate at which diving mammals recover from dives remains to be seen.

Another metabolite that can accumulate during prolonged diving is lactate. A by-product of anaerobic metabolism, lactate is produced primarily in the muscles through glycogenolysis. Many diving physiologists cite lactate accumulation as a key factor that limits dive duration. High lactate levels are thought to impair muscle function, and clearing it from the muscles is assumed to result in higher post-exercise oxygen consumption. There is a known O_2 cost to removing lactate if it is cleared from the muscles and converted back to glucose through the Cori cycle and gluconeogenesis. This O_2 cost would then be added to the post-dive replenishment of O_2 stores.

Therefore, the requirement to clear lactate after a dive is thought to represent both a physiological and behaviour constraint on diving behaviour.

The potential impacts of lactate, and its relation to O_2 utilization, has resulted in the most well-known and well-studied proposed limit to dive duration, the aerobic dive limit (ADL). Defined as the dive duration where lactate begins to accumulate in the body (Kooyman *et al*., 1983), the ADL has been used to mark the transition from aerobic to anaerobic metabolism. Historically, dives shorter than the ADL were considered to be far more efficient than longer ones, the presumption being that lactate recovery is more costly than O_2 store replenishment. Anaerobic dives are associated with lactate accumulation and increasing surface recovery time, suggesting a decrease in overall foraging efficiency. Laboratory studies show that the time required for lactate to return to resting levels increases drastically for dives that surpass the ADL (Kooyman *et al*., 1980; Ponganis *et al*., 1997). In addition, field studies of several marine mammals show that surface recovery time drastically increases after probable anaerobic dives when compared to shorter aerobic dives (Acevedo-Gutiérrez *et al*., 2002; Horning, 2012; Kooyman *et al*., 1983; Ponganis, 1992). Given this increase in recovery times (and the resulting decrease in foraging efficiency), the ADL is often used as a measurement of diving ability; animals with longer ADLs are considered to be better divers.

However, very little is known about how marine mammals actually transition between aerobic and anaerobic pathways during a dive. This is likely due to the difficulty of measuring this process in wild, or even laboratory environments; to date only six species have defined measurements of their ADL via monitoring of circulating lactate levels (Kooyman *et al*., 1983; Ponganis *et al*., 1997; Ponganis *et al*., 1997; Ponganis *et al*., 1997; Shaeffer *et al*., 1997a; Williams *et al*., 1993). Instead, most researchers rely on the calculated aerobic dive limit (cADL), which is derived by dividing an animal's total body oxygen store by their diving metabolic rate. The cADL therefore makes an important assumption about the transition between metabolic pathways: namely that a diving animal uses all of its stored O_2 before switching to anaerobic metabolism. In the past, the cADL and ADL have been used interchangeably, or at least inconsistently (Costa *et al*., 2001; Davis, 2014; Davis & Kanatous, 1999; Lydersen *et al*., 1992). However, many details of how O_2 is used at depth are undiscovered. Further research is required to determine how marine mammals transition between metabolic pathways, and how this might affect diving behaviour.

Determining the physiological limits to diving provides important information on marine mammals' ability to respond to environmental changes, particularly for species of concern such as the Steller sea lion (*Eumetopias jubatus*). The largest member of the family Otariidae (sea lions and fur seals), these animals are top predators along the coastlines of the North Pacific, spanning from California all the way to Japan. The western distinct population segment of Steller sea lions (Gulf of Alaska to Russia) were listed as endangered under the US Endangered Species Act in 1990 after seeing declines of over 80% (Trites & Larkin, 1996). While many factors may have contributed to the decline of this population, the Nutritional Stress Hypothesis remains one of the most prominent (Rosen, 2009; Trites & Donnelly, 2003). This theory proposes that environmental factors such as shifts in prey abundance, quality, or type, may have affected Steller sea lion health, reducing population productivity. To test this hypothesis, it is important to study the physiological limits for Steller sea lions diving under experimental conditions so we can extrapolate to wild populations. If dive behaviour of wild animals is close to their measured physiological limits, then they may be at greater risk of nutritional stress. Environmental changes that result in shifts of pretty abundance or distribution could force sea lions to dive beyond their means, requiring them to spend more energy finding food, and possibly hindering population growth.

To date, the majority of research on the physiological limits to diving has investigated the storage and use of O_2 at depth. Comparatively little research has explored the physiology of recovery at the surface, or how diving mammals transition between metabolic pathways at depth. My thesis aims to explore these understudied physiological processes to determine how they might affect overall diving behaviour in Steller sea lions, a species of importance in terms of conservation.

My primary objectives were to determine a) how post-dive rates of O_2 and CO_2 gas exchange are affected by dive behaviour in Steller sea lions; b) how Steller sea lions transition between aerobic and anaerobic metabolism; and c) how this transition affects surface recovery time. The first data chapter of this thesis (Chapter 2) focuses on post-dive recovery rates of O_2 uptake and CO_2 release as measured through flow-through respirometry of freely diving animals. The second data chapter (Chapter 3) examines the same freely diving animals to explore how post-dive blood lactate accumulates with increasing dive duration and how this correlates with changes in post-dive recovery rates of O_2 uptake and CO_2 release.

Chapter 2: O_2 and CO_2 recovery rates as limits to diving behaviour in Steller sea lions (*Eumetopias jubatus*)

2.1 Summary

Marine mammals have evolved to maximize their amount of time submerged while foraging, and minimize their time recovering at the surface. While the physiological adaptations that permit the former are relatively well studied, relatively few studies have examined how marine mammals recover their O_2 stores after a dive. Even fewer have explored CO_2 recovery, despite the vital role CO_2 plays in the control of ventilation in terrestrial mammals. I examined how dive time affects physiological recovery in marine mammals by determining the times for O_2 consumption rate and CO_2 excretion rates to return to stable levels in four freely-diving, adult Steller sea lions. CO_2 recovery times proved to be more of a limiting factor in recovery than O_2, with every extra minute of dive time increasing the required recovery time by 44 s. In comparison, O_2 recovery time increased with dive time at a slower rate, such that for every extra minute of dive time, O_2 recovery time increased by 33 s. As a result, CO_2 recovery times were significantly greater than O_2 recovery times for longer dives. The proportionally longer recovery times for CO_2 are likely due to either rate-limiting steps in the elimination of CO_2 that aren't involved in O_2 replenishment, or due to an increase in CO_2 release driven by hyperventilation or a decrease in pH due to anaerobiosis. These recovery rates are important in the development of optimal foraging models, and can be used to determine when Steller sea lions are diving close to their physiological limits.

2.2 Introduction

Diving physiologists often envision a dive as a compromise between two competing demands: the necessity of foraging at depth, and the requirement to recover at the surface. Extending dive duration permits the efficient exploitation of more resources, and scientists have long studied the numerous physiological adaptations that allow these animals to maximize dive duration. Marine mammals are well known for their particularly large O_2 stores and the various physiological responses that allow them to extend its use during dives (Butler & Jones, 1997; Fahlman *et al*., 2008; Kooyman & Ponganis, 1998; Reed *et al*., 1994; Sparling & Fedak, 2004). However, the longer an animal dives, the more time it must subsequently remain at the surface to recover by replenishing O_2 stores and removing metabolic by-products such as CO_2. Marine

mammals must therefore find an optimal balance between time spent consuming O_2 while foraging at depth, and time devoted to gas exchange and recovery at the surface.

This compromise between two fundamental requirements is the basis for the development of optimal foraging models that are specific to air-breathing vertebrates. The quality and distribution of food resources, the rate at which O_2 is depleted throughout the dive, and how rapidly (and the degree to which) recovery occurs at the surface are all taken into account to determine how long an animal should dive, and how long they should then remain at the surface (Carbone & Houston, 1996; Doniol-Valcroze *et al*., 2011; Halsey & Butler, 2006; Kramer, 1988; Mori, 1998, 2002; Thompson & Fedak, 2001). Researchers presume that much of the variation observed in dive duration among wild sea lions is a reflection of this optimization in conjunction with other environmental factors. Our ability to properly model foraging behaviour, and our interpretation of observed behaviour in wild animals, is therefore dependent upon our understanding of the dynamics of respiratory gas management.

Previous research on how diving animals manage respiratory gases has largely focused on understanding how marine mammals consume their O_2 supply at depth. Extended periods of apnea during a dive inevitably result in a decrease in blood O_2 content and partial pressure (PO_2). The mammalian dive response, well characterized by localized vasoconstriction and ensuing decreased heart rate, has also been hypothesized to result in a decrease in metabolic rate (Fahlman *et al*., 2008; Gerlinsky *et al*., 2014; Irving *et al*., 1941; Scholander, 1940; Scholander *et al*., 1942a; Scholander *et al*., 1942b; Sparling & Fedak, 2004). This decrease would help marine mammals to stretch their O_2 stores for extended periods. However, the rate at which O_2 consumption occurs can change during a dive; for example, transiting between the surface and the foraging site is more energetically costly than swimming at depth, and therefore increases how much O_2 is consumed (Goundie *et al*., 2015; Hindle *et al*., 2010).

While a great deal of progress has been made in understanding how O_2 is managed over a dive, relatively few studies have examined the dynamics of gas exchange once the animal returns to the surface. Upon surfacing, marine mammals immediately begin to replenish their spent O_2 stores. This is facilitated by the large PO_2 difference between the atmosphere and the animal's blood according to Fick's law of diffusion. Initially, the PO_2 difference, and thus rate of gas diffusion is high; however, as recovery progresses, the PO_2 difference becomes smaller, reducing

the efficiency of gas exchange. The final stage of gas exchange is therefore much slower, increasing the time to fully renew O_2 stores. Kramer (1988) first presented optimal diving models using this concept of O_2 loading curves, and it has been further developed to represent the kinetics of O_2 exchange (Carbone & Houston, 1996; Doniol-Valcroze *et al*., 2011; Halsey & Butler, 2006; Kramer, 1988; Mori, 1998, 2002; Thompson & Fedak, 2001). However, how O_2 replenishment time changes under differing dive durations is poorly understood, despite its clear importance in determining how long an animal must remain at the surface.

In addition to the depletion of O_2, marine mammals must also deal with the accumulation of CO_2 during a dive. Small increases in the partial pressure of CO_2 (PCO_2) typically stimulate the respiratory drive in mammals, increasing ventilation and heart rate (Phillipson *et al*., 1981). This clearly has the potential to limit submergence time for diving mammals, as CO_2 accumulates over the course of a dive in tandem with depletion of O_2 stores (Kooyman *et al*., 1980; Qvist *et al*., 1986). Early experimental studies show dive duration may be, at least partially, determined by blood PCO_2 levels in marine mammals (Gallivan, 1980; Påsche, 1976a, 1976b). In addition, CO_2 may play an important role in determining surface recovery time as well, possibly even more so than O_2 (Boutilier *et al*., 2001; Stephenson, 2005).

While PCO_2 levels may ultimately influence dive duration, there are indications that marine mammals have adaptations to tolerate increased PCO_2 levels. Ventilation rates of marine mammals increase to the same degree as their terrestrial counterparts in response to CO_2 exposure, but only after exposures to higher PCO_2 levels (Bentley *et al*., 1967; Butler, 1982; Craig & Påsche, 1980; Gallivan, 1980; Irving, 1938; Kohin *et al*., 1999; Milsom *et al*., 1996; Påsche, 1976a, 1976b; Robin *et al*., 1963). Marine mammals also have higher blood CO_2 carrying capacities with higher haemoglobin concentrations, and increased buffering capacity to deal with acidosis induced by CO_2 build-up (Boutilfier *et al*., 1993; Castellini & Somero, 1981; Lenfant *et al*., 1970; Mirceta *et al*., 2013). These adaptations allow marine mammals to tolerate higher levels of CO_2 production than their terrestrial counterparts, thus extending their dive durations. However, there clearly is a limit to the degree of build-up, and marine mammals must eventually return to the surface to eliminate this accumulated CO_2.

Post-dive gas exchange of CO_2 is far less studied than that of O_2 exchange in marine mammals, a somewhat surprising point given the central role CO_2 plays in ventilatory control in

mammals. Elimination of CO_2 follows somewhat similar dynamics as O_2 in relation to partial pressure differences. At the onset of gas exchange CO_2 is rapidly removed, followed by a slower process as the partial pressure difference becomes smaller. However, the process of CO_2 elimination takes longer than O_2 replenishment as described in several studies of marine mammals (Boutilier *et al*., 2001; Fahlman *et al*., 2008; Gerlinsky, Rosen, *et al*., 2014; Stephenson, 2005), although the reason for this is not entirely understood. It may be due to longer transit times in venous as opposed to arterial blood, or perhaps due to the comparatively larger number of biochemical steps that are involved in CO_2 gas exchange (Piiper, 1990). While O_2 exchange is limited largely by partial pressure differences, CO_2 release is instead limited largely by chloride-bicarbonate exchange at the membrane of the red blood cell (Bidani & Crandall, 1988; Wieth *et al*., 1982). This step involves the transport of bicarbonate into the cell so it can be converted to CO_2. It only then can be returned to the serum, and finally being exchanged at the lung. The rate-limiting step of the chloride-bicarbonate exchange likely influences the rate diving mammals recover from the CO_2 accumulated during extended dives.

In essence, CO_2 recovery time is a function of how much CO_2 was accumulated during a dive, and the rate at which it can be removed from the body. Similarly, O_2 recovery time is determined by the degree of O_2 depletion and the rate at which it can be replenished. Currently, the rate at which these two gases are replenished and removed during recovery is poorly understood, as well as how the dynamics are affected by dive time. Yet proper knowledge of these processes is essential for understanding the underlying physiological controls of diving behaviour and the development of accurate optimal foraging models. This study explores how post-dive rates of O_2 replenishment and CO_2 elimination are impacted by dive duration in four adult Steller sea lions (*Eumetopias jubatus*) performing voluntary, realistic dives.

We know that O_2 and CO_2 recovery will inevitably increase with dive time, though at what exact rate is unknown. However, given the known decrease in DMR with increased dive time from previous studies (Alboni *et al*., 2011; Castellini *et al*., 1992; Costello & Whitlow, 1975; Craig & Påsche, 1980; Jones *et al*., 1973), I hypothesized that the ratio of recovery time to dive time would decrease with increasing dive duration. I also hypothesized that CO_2 would prove to be more limiting in recovery than O_2, and that this difference would increase with dive time. As a result, I also predicted that these rate-limiting steps would result in increasingly greater differences

between O_2 and CO_2 recovery times with longer dives. This information will provide insights into respiratory gas management strategies and, ultimately, on how physiology informs optimal foraging strategies.

2.3 Methods

2.3.1 Study Animals

To determine the relationship between recovery time of O_2 and CO_2 and dive time, I used four trained, adult female Steller sea lions that were collected as 2-week old pups from rookeries in the Triangle Islands, BC. These animals were raised at the Vancouver Aquarium (Vancouver, BC) and subsequently housed at the Open Water Research Laboratory (Port Moody, BC). All individuals were routinely fed a diet of herring, squid, capelin, and supplementary vitamins. The animals had been previously trained using positive reinforcement to use the experimental equipment for this study and to perform similar diving trials for a number of previously reported studies (see Rosen *et al*., 2017). This study was conducted under UBC Animal Care Permits A11-0397 and A16-0027.

2.3.2 Field sampling

The overall goal of the study was to determine rates of O_2 uptake and CO_2 release before, during, and after a series of voluntary dives of varying length. Trials were conducted from April 19th – September 6th, 2016 in Indian Arm, a deep water fjord, located north of the Open Water Research Laboratory where the animals were housed. Sea lions were transported to the dive site by boat. Prior to the trials, including during transport to the dive site, animals were fed less than 0.5 kg of fish to minimize any potential effect of the heat increment of feeding on metabolism (Rosen & Trites, 1997). The experimental set up consisted of a floating platform that was moored in an isolated portion of the fjord. The platform housed a floating respirometry dome constructed from transparent Plexiglas which rested over a submerged cage (1.52m x 1.52 m x 2.5 m). At the beginning of a trial, a sea lion entered the cage and surfaced within the respirometry dome. The cage acted as behavioural reinforcement for the sea lion to remain within the respirometry dome when required, but did not physically constrain the animal. Within the dome, O_2 uptake and CO_2 excretion rates were measured using flow through respirometry while the animal floated calmly at the surface. Air was drawn through the dome and over the animal at 350 L min^{-1} using a Sable System model 500H mass flow generator and controller (Sable Systems Inc., Las Vegas, NV,

USA). The excurrent airstream was subsampled and water vapour was removed by drawing it over desiccating crystals (Drierite). I then measured fractional concentrations of O_2 and CO_2 using Sable System FC-1B and CA-1B analyzers and a Sable Data Acquisition system. Gas concentrations were recorded every 0.5 s.

For each trial, an animal remained in the dome until they reached a constant rate of O_2 uptake and CO_2 release (5-10 min duration). The last two minutes of this period were used to calculate pre-dive metabolic rate while resting quietly at the surface. When signalled by the trainer, the animal performed a dive to the end of two 40 m deep tubes attached to the platform, a relevant depth for feeding behaviours seen in the wild (Loughlin *et al*., 1998; Merrick *et al*., 1994; Rehberg *et al*., 2009). Pieces of fish (~20 g, herring or capelin) were alternately delivered to the sea lions every 5 sec at the bottom of the two feeding tubes. To simulate a foraging event, the two tubes were positioned on either ends of the platform ~9 m apart, forcing the animal to swim between the tubes to receive fish.

The sea lions controlled the duration of the dive. Upon completion of the dive, animals surfaced directly into the respirometry dome where instantaneous rates of O_2 uptake and CO_2 release were measured. The animals remained in the dome until the rates of both of these processes were constant. The last two minutes of this period were used to calculate post-dive metabolic rate. Relative humidity and air temperature were also recorded. Animals completed between 1-3 dives per session, but did not begin a new dive until they had reached stable rates of O_2 uptake and CO_2 release.

For all trials, animals wore a specially built, tight-fitting harness with a VHF transmitter as per Animal Care requirements. Animals were also equipped with one of three joint accelerometer and time-depth recorders (TDR10-DD & TDR10-X, Wildlife Computers; OpenTag, Loggerhead Instruments). As part of the experimental protocol, we wanted to differentiate between the closely related effects of dive time and total O_2 uptake. For a subset of the dives, we affixed two layers of 43cm x 13cm x 4 cm Astroturf-like material to the harness to induce additional drag, as a means of increasing total O_2 consumption rate for a given dive duration. This allowed us to validate whether total O_2 consumption can be used as a measure of dive effort.

2.3.3 Data Analysis

Respirometry data were analyzed using the program Expedata. Electronic drift in the gas analyzers was corrected by calibrating gas concentrations to ambient air at the beginning and end of each trial. Several times throughout the study the gas analysis system was calibrated with known concentrations of gases. The system was also leak-checked using standard nitrogen dilution techniques. Rates of O_2 uptake and CO_2 release were calculated using equations 11.7 and 11.8 from Lighton (2008).

O_2 recovery times were calculated by fitting a least squares regression to the instantaneous rate of O_2 uptake rate data from the peak rate of consumption to the point the animal reached a constant post-dive metabolic rate (See Fig. A.1 for an example trace of O_2 recovery and least squares regression). Recovery time was defined as the time between when the sea lion surfaced, until the point where O_2 uptake was within 5% of either pre- or post-dive rate of O_2 uptake, whichever was lower. We chose this point to represent when the animal was 95% recovered in terms of O_2 balance. Similar calculations were done for CO_2 release rate, with CO_2 recovery time defined as the time it took to reach 95% of resting CO_2 excretion rate. I had greater confidence in defining the 95% recovery versus 100% given the inherent variation in post-dive respiration.

Calculating the rate of O_2 uptake in a breath-hold diver is complicated by the fact that internal O_2 uptake is temporally separated from subsequent external gas exchange. As per accepted practices, the volume of O_2 consumed during a dive (ΣVO_2) was defined as the total O_2 consumed in excess of the pre- or post-dive baseline during the recovery period, whichever was lower. Diving metabolic rate (DMR) was calculated by dividing the total O_2 consumed in excess of these baseline levels by the dive duration as per Hastie *et al.* (2007). This calculation assumes that any excess O_2 consumed is strictly attributable to the dive itself; while this may not be physiologically accurate, it does provide a standard, comparative measure.

The respiratory exchange ratio for the total recovery period (RER_T) was calculated by dividing the total volume of CO_2 produced throughout the recovery period by the total volume of O_2 produced over the recovery period, including baseline levels. In addition, instantaneous RER (RER_I) was calculated at 10 second intervals to examine changes in the ratio of CO_2 released to O_2 consumed throughout the recovery period. Total volume of CO_2 released in each interval was

divided by the total O_2 consumed over the same time and this calculation was repeated through the entire surface time.

All data were analyzed using R software (R Development Core Team, 2015). I performed a linear mixed effects analysis on the relationship between dive time and recovery time for both O_2 and CO_2 using the *lme4* package (Bates *et al*., 2015). Fixed factors included dive duration (between 2 and 7 min) and dive type (with or without added drag). While previous research shows that depth may have an effect on diving metabolic rate (Hastie *et al*., 2006; Rosen *et al*., 2017), all trials were conducted at the same depth of 40 m. This minimized variation in maximum depth and was therefore excluded as a fixed effect. The same fixed factors were used in mixed effects models to examine how DMR and RER_T changed with dive duration. In modeling how recovery time changes with total O_2 uptake, ΣVO_2 and dive type were used as fixed effects. The repeated measures nature of the data was accounted for by including animal ID as a random effect in all models. I ran models using the maximum likelihood method to determine which model best represented the data. In cases where multiple fixed factors were significant, I used log likelihood ratio tests (LRT) on the nested models to determine the best overall model to fit the data. P-values were obtained by running LRTs of the full models with and without the effects in question. I visually reviewed all residual plots for deviations from homoscedasticity or normality.

To determine whether CO_2 or O_2 recovery times were longer after extended dives, I conducted a paired t-test on the recovery times of the two gases for dives that exceeded 4 min. Values are reported as means ± standard deviation. Statistical significance was set at $\alpha = 0.05$.

2.4 Results

Dive durations across all animals ranged from 1.69 – 6.55 min (mean ± SD = 4.26 min ± 1.29) and maximum dive depths were between 40 – 52 m (44 m ± 2). Recovery times for O_2 ranged from 3.88 min – 7.98 min (4.95 min ± 0.97). The results from the linear mixed effects analysis determined that dive time had a significant effect on the O_2 recovery times, increasing for longer dives (LRT = 188.96, $p < 0.001$). The relationship between O_2 recovery time and dive duration had a slope of 0.52 (Fig. 2.1). This means that for every extra minute of dive time, recovery time increased by 31 s. As expected, the presence of drag increased recovery time for a given dive duration, though it did not affect the rate of O_2 recovery. This indicates that drag increased the energy output required for a given dive. Oxygen recovery time also increased linearly with

increases in ΣVO_2 (LRT = 111.93, $p < 0.001$; Fig. 2.2). Recovery times therefore changed only in relation to the level of O_2 depletion, and not dive duration *per se*.

DMR was not significantly correlated with increased dive time (LRT = 3.84, $p = 0.14$). However, visual examination of the data indicated that this may have been due to the high level of variability in DMR calculations during short dives. This is supported by the fact that a greater proportion of recoveries from short dives (< 2.25 min) could not have a recovery slope fit mathematically to the data. When only considering dives > 2.25 min, DMR significantly decreased with increasing dive time (LRT = 18.235, $p = 0.00011$) as would be expected. The presence of drag increased DMR without affecting the slope, providing further evidence that drag did indeed increase the energy output required for a given dive.

CO_2 recovery times ranged from 2.87 min – 12.62 min (6.84 min ±1.93 min). Similar to oxygen, CO_2 recovery time increased significantly with increased dive time (LRT = 70.76 $p = <0.001$). The relationship between CO_2 recovery time and dive duration had a slope of 0.74 which means that for every extra minute of dive time, recovery time increased by 44 s. As with O_2, drag was deemed to be a significant fixed factor for this model, though the best model included an interaction term between dive time and dive type. Adding drag increased the slope of recovery to 1.31, meaning every extra minute of dive time increased recovery time by 1 min 21 s.

CO_2 recovery times were generally longer than O_2 recovery times for a given dive duration, particularly for longer dive durations. The fitted regression lines seem to suggest that dives shorter than ~3.5 min had greater O_2 recovery times; however, this was likely an artefact of the long recovery times for CO_2 after long dives. These long recovery times likely drive the overall relationship, forcing a lower intercept for CO_2 than is likely truly the case. For dives that exceed 4 min, CO_2 recovery times were significantly greater than O_2 recovery times ($t = 7.63$, $p = <0.001$).

RER_T increased significantly with increased dive time (LRT = 30.4, $p = < 0.001$), and ranged from 0.417-0.921 (0.679 ± 0.106; Fig. 2.5). RER_I was calculated to examine how the ratio between CO_2 release and O_2 uptake changed over the recovery period. RER_I for all dives started relatively low at the onset of recovery only to increase as recovery time increased, peaking ~250 s into recovery (see Fig. A.2 for an example trace of RER_I over the recovery period). For all of the above models, visual inspection of residual plots revealed no obvious deviations from linearity, homoscedasticity, or normality.

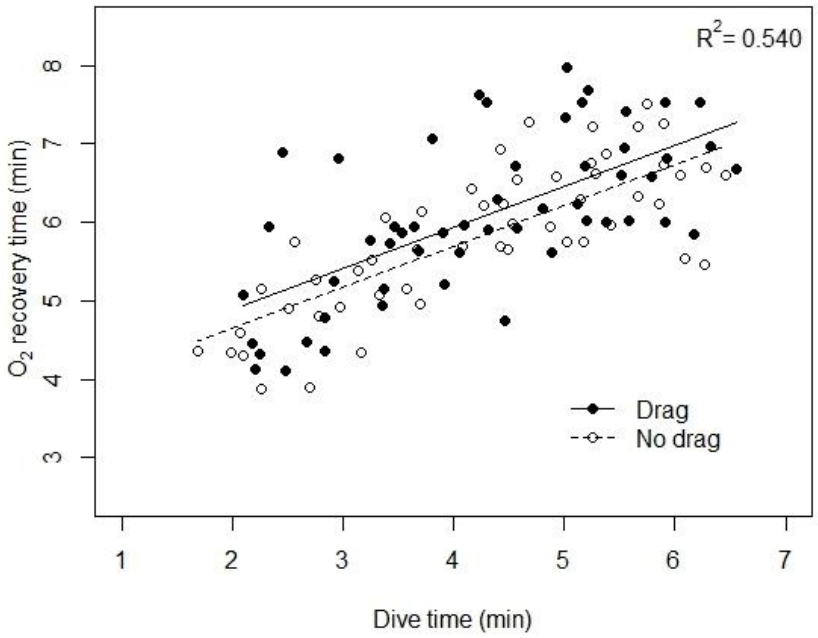

Figure 2.1 Time for the rate of O_2 uptake to return to within 5% of resting levels (O_2 recovery time) for varying dive durations in Steller sea lions both with and without added drag. O_2 recovery time was significantly affected by dive time ($p = < 0.001$), increasing linearly with increased time submerged. The conditional R^2 is associated with both fixed and random effects. Presence of drag was a significant fixed factor in the linear mixed effects model.

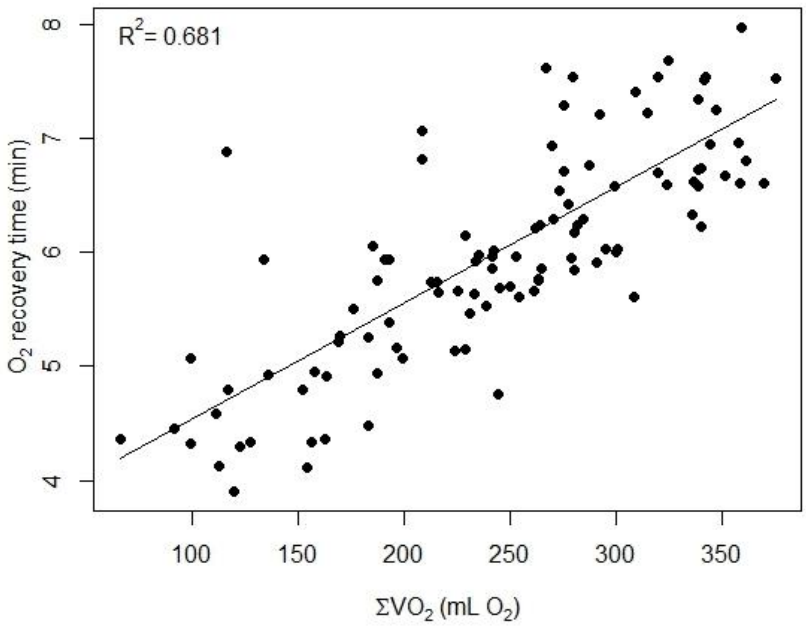

Figure 2.2 Time for the rate of O_2 uptake rate to return to within 5% of resting levels (O_2 recovery time) in relation to the total volume of O_2 consumed during the dive (ΣVO_2). O_2 recovery time increased linearly with greater ΣVO_2 ($p = < 0.001$). Dives with and without added drag are included together as drag was not a significant model factor. The conditional R^2 is associated with both fixed and random effects.

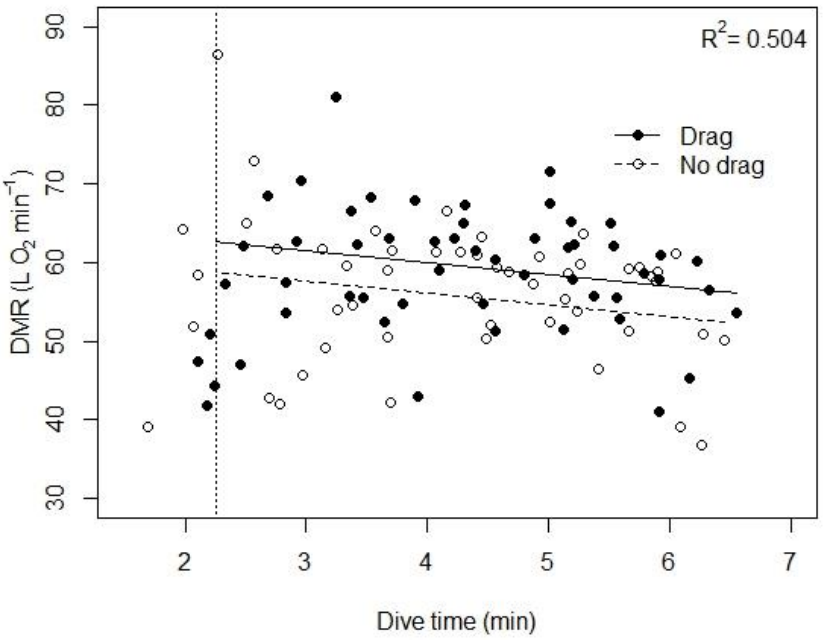

Figure 2.3 Diving metabolic rate (DMR) measured as O_2 consumption rate adjusted for body size as a function of dive duration of Steller sea lions. Dives less than 2.25 min (8 dives to left of the dotted line = 7.5% of dives) were excluded from the regression due to high degree of variability. For dives greater than 2.25 min, DMR decreased significantly with increased dive duration ($p = < 0.0001$). Added drag increased DMR for a given dive time, but did not affect the rate of decrease with increased dive time. The conditional R^2 is associated with both fixed and random effects.

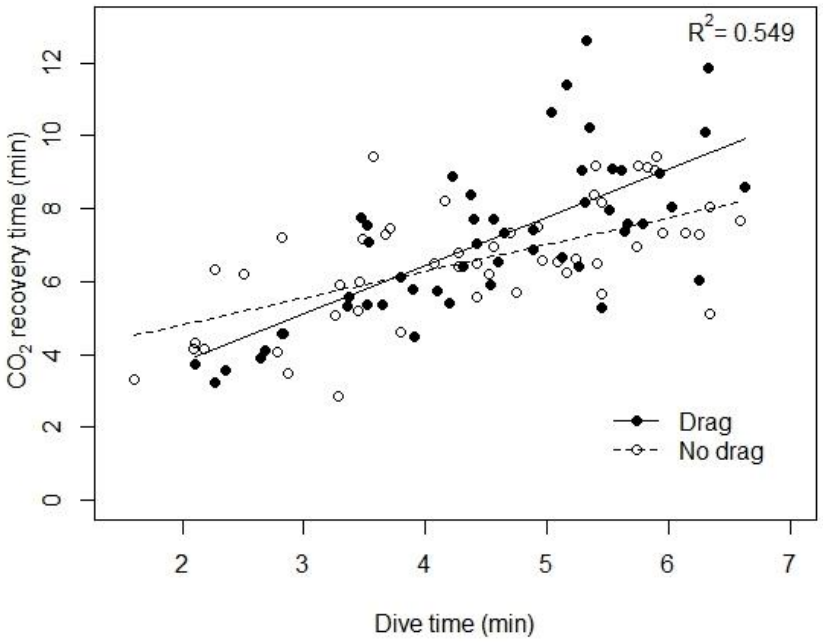

Figure 2.4 Time for CO_2 excretion rate to return to within 5% of resting levels (CO_2 recovery time) for varying dive durations in Steller sea lions both with and without added drag. CO_2 recovery time was significantly affected by dive time ($p = < 0.001$), increasing linearly with increased time submerged. Presence of drag was a significant fixed factor in the linear mixed effects model. The conditional R^2 is associated with both fixed and random effects.

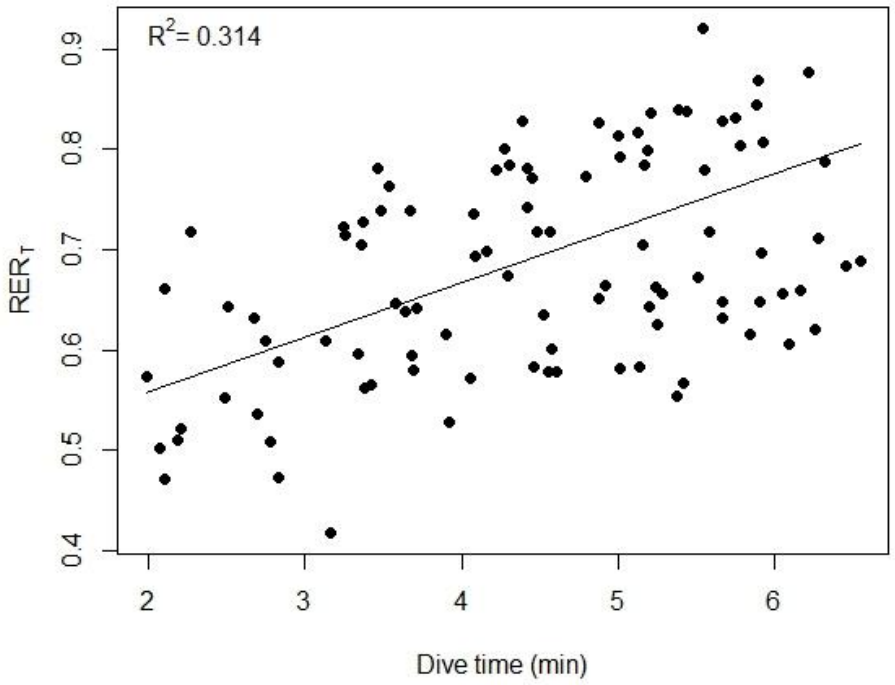

Figure 2.5 Respiratory exchange ratio calculated over the total recovery time (RER_T) after dives of varying durations in free diving Steller sea lions. RER_T increased significantly with increased dive time ($p = < 0.001$). The conditional R^2 is associated with both fixed and random effects.

2.5 Discussion

This study shows that Steller sea lions can replenish their O_2 stores relatively quickly after long dives. Recovery time (relative to dive duration) actually decreases with longer dives, partially due to a decrease in diving metabolic rate (DMR) during longer dives. However, post-dive CO_2 recovery time was generally much longer than O_2 recovery, which was most clearly seen in dives with longer durations. This was likely due to a rate limiting step in elimination of CO_2, or due to an increase in CO_2 release as a result of anaerobiosis induced acidosis. These results indicate that CO_2 recovery may actually play a more important role in determining dive behaviour than recovery of O_2, and therefore needs to be incorporated into optimal foraging models.

For this study I defined recovery time as the amount of time it took for post-dive rates of respired CO_2 or O_2 to be within 5% of stable rates. I found that CO_2 recovery times were consistently longer than O_2 recovery times for dives that exceeded 4 min. These dive durations more accurately reflect foraging dives seen in the wild. In addition, the difference between CO_2 and O_2 recovery times increased with dive time. This suggests that CO_2 recovery time may be a more important limiting factor for surface recovery time post-dive. The relationship between O_2 recovery time and dive time had a slope of 0.52, indicating that for every additional minute of dive time, recovery time increased by only 31 s. In comparison, for CO_2 recovery times the relationship had a slope of 0.74. This means for every extra minute of dive time, recovery time increased by 44 s. Thus, as dive time increased, the difference in recovery times for O_2 and CO_2 increased as well, with CO_2 taking disproportionately longer to recover. For example, for a 4-min dive, the difference in recovery times for O_2 and CO_2 was only 35 s (341 vs 376 s, respectively), but for an 8 min dive the difference was over 87 s. This difference in recovery rates was even more dramatic when the animals had to work harder while submerged. While wearing the drag harness, CO_2 recovery time increased drastically, further increasing the difference in recovery times from O_2.

The longer observed recovery times for CO_2 are consistent with my predictions, as well as with previous research on marine mammals. In a study using the same animals as this one, Gerlinsky *et al.* (2014) noted that CO_2 recovery took notably longer than O_2 after both single dives and dive bouts, however, this difference was not quantified. Similarly, Boutilier *et al*. (2001) observed that CO_2 recovery in the harbour porpoise (*Phocoena phocoena*) was relatively slow after experimental dives, continuing even after O_2 stores had stabilized. They hypothesized that the

animals remained at the surface after dives to eliminate CO_2 before the next dive rather than to replenish O_2.

The longer recovery times for CO_2 may be due to differences in how respiratory gases are stored in the body. Oxygen storage is relatively simple; the majority is stored in the lungs or bound to respiratory pigments in the blood and muscles (Lenfant *et al*., 1970; Mirceta *et al*., 2013). The rate of replenishment is therefore largely driven by the rate of diffusion across the respective membranes, and thereby the degree of O_2 depletion. CO_2 storage is more biochemically complex with the majority (~70%) existing as bicarbonate in the body's tissues (Piiper, 1990). The greater the proton buffering capacity, the greater the bicarbonate concentration for a given PCO_2. This allows for a greater total CO_2 content to be stored not only in the blood or muscles, but also in any tissue with proton accepting compounds (Piiper, 1990). The rate of CO_2 elimination is therefore not only determined by the rate of CO_2 diffusion, but also by the rate of transport and chemical reactions. With CO_2 largely being stored in the blood as bicarbonate, it must first be transported into the red blood cell, and then converted into CO_2 before exchange can occur in the lung. These rate limiting steps (particularly the chloride-bicarbonate exchange) result in longer times to remove CO_2 than to replenish O_2, particularly when CO_2 has become elevated during long dives (Bidani & Crandall, 1988; Piiper, 1990; Wieth *et al*., 1982). Falke *et al*. (2008) observed evidence of this by examining gas concentrations in the first few breaths following a dive in Weddell seals (*Leptonychotes weddellii*). They found that these first breaths had very low concentrations of CO_2, and that concentrations didn't peak until 2-3 min into the recovery period. Similar studies of harbour porpoises, Steller sea lions, and grey seals (*Halichoerus grypus*) found respiratory exchange ratio values (RER; rate of CO_2 release divided by rate of O_2 uptake) to be low at the onset of recovery only to increase as time progressed to values far higher than the animals' metabolic respiratory quotient (RQ > 1; Boutilier *et al*., 2001; Fahlman *et al*., 2008; Reed *et al*., 1994). Studies on diving humans found similar trends in RER during the recovery period with CO_2 excretion not reaching a maximum until ~5 min post-dive (Schaefer, 1965). While I was unable to measure breath-by-breath O_2 consumption or CO_2 production, I was still able to get a measurement of instantaneous RER (RER_I) through rates of uptake and release. In this study RER_I for all trials started low and increased over the first portion of recovery, consistent with the other studies. In a number of cases RER_I exceeded 1.0 during my trials. This increase in RER_I throughout recovery indicates a progressive increase in CO_2 excretion rate, a progressively slowing rate of O_2 uptake,

or a combination of the two. Should CO_2 excretion be driving this pattern in RER_I, it would further confirm its role in the prolongation of total recovery time.

The observation that it only takes O_2 on average 32 s extra to recover for every additional minute spent submerged simply indicates that the rate of replenishment at the surface is, on average, twice as fast as the rate of consumption at depth. However, this statement may be an oversimplification, as there are several reasons to expect that the relationship is not constant (i.e., linear). First, diving for longer periods should make replenishing O_2 stores more efficient on average. During a dive, sea lions deplete body O_2 stores which results in a PO_2 difference between their blood and the environment. This PO_2 difference drives gas exchange when the animal returns to the surface. As per Fick's Equation, a greater PO_2 difference will increase the rate of diffusion across the respiratory membranes in the lungs, as well as into the tissues. Thus, the greater O_2 depletion from longer dives will result in a faster initial rate of O_2 replenishment. In addition, haemoglobin has a greater affinity for O_2 at higher levels of depletion (e.g., after long dives) compared to when only partially depleted. Therefore, as sea lions deplete more of their O_2 stores during longer dives, initial replenishment at the onset of recovery would be more rapid. This would proportionally decrease the time it takes to recover from longer dives. If this were the case we would expect to see a curvilinear relationship between the total O_2 consumed in excess of their surface resting metabolic rate post dive (ΣVO_2) and recovery time. However, this likely was not the case— residual plots of this relationship did not indicate a deviance from linearity. We know that PO_2 differences are integral to gas exchange and inevitably play a role in the relationship between O_2 depletion and recovery time. However, the linear relationship between ΣVO_2 and recovery time in the data suggest that these processes did not result in longer dives having proportionally shorter recovery times.

A second factor that could affect the relationship between dive time and O_2 recovery time would be changes in DMR with dive duration. A decrease in average rates of O_2 consumption is a common characteristic of the dive response in marine mammals (Alboni *et al*., 2011; Castellini *et al*., 1992; Costello *et al*., 1975; Craig & Påsche, 1980; Jones *et al*., 1973), including Steller sea lions (Fahlman *et al*., 2008; Gerlinsky, Trites, *et al*., 2014; Hindle *et al*., 2010). This decrease in DMR during prolonged dives would cause sea lions to consume proportionally less O_2 while submerged—they would therefore need to replenish proportionally less O_2 after a longer dive. This

would result in a proportionally shorter recovery time for longer dives, without affecting the relationship between recovery time and total oxygen consumed. In this study, DMR does significantly decrease with increased dive time, suggesting that this physiological response is contributing to the proportionally lower recovery times for longer dives. Under this model, we would expect recovery time to increase curvilinearly with dive time, but residual plots did not indicate any deviance from the assumption of linearity. Further research may provide insight into the mechanisms that influence this pattern of O_2 and CO_2 recovery in marine mammals.

As previously discussed, the relatively slower recovery time of CO_2 is primarily due to limitations in their ability to void accumulated CO_2 as dive time increases. However, as with O_2, the relationship between dive time and CO_2 recovery time may not be linear. While CO_2 accumulation will naturally increase as dive time increases, the rate at which this occurs may vary depending on possible changes in the physiological processes occurring during a dive. Given a constant ratio of CO_2 production to O_2 consumption, decreases in DMR will affect CO_2 recovery rates by producing proportionally less CO_2 during longer dives, although they would not alter the fact that recovery times for CO_2 will still be longer than for O_2.

Steller sea lions may also be producing proportionally more CO_2 relative to their rates of O_2 consumption. This would clearly affect the difference in recovery times seen for O_2 and CO_2. While the previous examination of RER_I at the onset of recovery can inform us on the kinetics of gas exchange, RER_T (RER over the entire recovery period) can be used to determine how much CO_2 is released compared to the O_2 consumed over the entire dive. Since RER_T increased with dive time, we can assume more CO_2 was accumulated during longer dives – and therefore needed to be excreted during recovery – relative to the level of O_2 consumed. This indicates that rates of CO_2 release did not decrease in parallel with rates of O_2 consumption (i.e., DMR) with increased dive duration.

There are multiple factors that could result in an increase in RER_T for prolonged dives. Changes in fuel source (fats, proteins, or carbohydrates) can alter the proportion of CO_2 produced relative to O_2 consumed at the tissue level during a dive. During intense exercise, mammals typically shift from utilizing fat to relying more on carbohydrates, a shift that is also associated with an increase in the RQ (Brooks & Mercier, 1994; Costill, 1999). While RQ is a measure of cellular metabolism at the tissue level, this shift may still be measurable in the RER, the ratio of

CO_2 release to O_2 uptake of the whole animal. As marine mammals increase their reliance on anaerobic metabolism during long dives, their RER may increase as well, reflecting this shift towards carbohydrates as a preferential fuel source. However, changes in RER related to fuel sources can only be measured during steady state gas exchange, and are therefore undetermined in this study. Physiological changes related to acid-base regulation may also cause a change in RER_T. Upon surfacing, swimming muscles are quickly reperfused, releasing their accumulated CO_2 into the blood stream. This large release of CO_2 results in a decrease in blood pH promoting bicarbonate dehydration and elevating PCO_2, facilitating CO_2 excretion. While changes in bicarbonate have not been expressly measured in sea lions post dive, decreases in bicarbonate concentrations are well documented in humans post-exercise, particularly when subjects surpassed their aerobic threshold (Péronnet & Aguilaniu, 2006; Sutton & Jones, 1979; Wasserman *et al*., 1973). In addition, marine mammals have high bicarbonate stores in comparison to their terrestrial counterparts (Lenfant *et al*., 1970). This suggests that these animals rely heavily on these stores for acid-buffering after extended dives. This decrease in bicarbonate, paired with a subsequent rise in exhaled CO_2 through hyperventilation, likely explains the increase in RER_T during recovery seen in this study. Lactate induced acidosis has also been proposed as a cause for increased CO_2 release. By similarly decreasing the pH of tissues, lactate produced during intense exercise would cause a decrease in bicarbonate buffers and a stoichiometric increase in CO_2. Longer dives would be expected to have higher amounts of lactate accumulation, possibly leading to an increase in CO_2 and RER_T. However, the assumption of lactate's role in decreasing pH has recently come under question—the degree to which lactate acts in acidifying blood may have previously been overstated, and may in fact hinder acidosis (Hall, Rajasekaran, Thomsen, & Peterson, 2016; Robergs, Ghiasvand, & Parker, 2004). In addition, lactate is not necessarily buffered by bicarbonate, and would therefore not contribute to an increase in CO_2 exhalation (Péronnet & Aguilaniu, 2006). Further explorations on the accumulation of lactate are described in Chapter 3, and describe the relationship between increased lactate and CO_2 production in Steller sea lions. However, further research is required to fully understand how these two metabolites might interact to affect dive behaviour in marine mammals.

Marine mammals appear to be better able to cope with hypercapnia when compared to their terrestrial counterparts, suggesting they may be able to withstand the inevitable accumulation of CO_2 during long dives. When subjected to hypercapnia, marine mammals exhibit a blunted

ventilation response, increasing their breathing frequencies to the same degree as other mammals, but only under higher levels of PCO_2 (Bentley *et al*., 1967; Gallivan, 1980; Gerlinsky *et al*., 2014; Irving, 1938; Påsche, 1976a, 1976b; Robin *et al*., 1963). Steller sea lions are also able to maintain consistent surface intervals, even when exposed to hypercarbia before and after dives, compensating for the accumulated CO_2 with hyperventilation (Gerlinsky *et al.,* 2014). This reduced sensitivity suggests that marine mammals are better able to tolerate accumulated CO_2 than terrestrial mammals. Elevated haemoglobin levels give marine mammals a greater CO_2 carrying capacity, due to the formation of carboxyhaemoglobin, as well as the high buffering capacity of haemoglobin. Thus, for a given CO_2 accumulation, the increase in PCO_2 and reduction in pH will be reduced relative to non-diving mammals. Previous studies note that orcas (*Orcinus orca*) and grey seals (*Halichoerus grypus*) have non-bicarbonate blood buffering capacities that are two- to fourfold that of terrestrial mammals (Boutilier *et al*., 1993), and that marine mammal muscle has comparatively higher buffering capacity as well (Castellini & Somero, 1981; Mirceta *et al*., 2013). This further speaks to marine mammals' ability to respond to the potential acidosis caused by CO_2 accumulation.

Optimal foraging models assume that marine mammals maximize their time at depth (given that this is where foraging occurs), and minimize their time at the surface (Carbone & Houston, 1996; Doniol-Valcroze *et al*., 2011; Halsey & Butler, 2006; Kramer, 1988; Mori, 1998, 2002; Thompson & Fedak, 2001). They also predict that marine mammals should attempt to minimize the proportion of time spent in transit between the surface and depth as it is unproductive and energetically expensive (Carbone & Houston, 1996; Doniol-Valcroze *et al*., 2011; Halsey & Butler, 2006; Kramer, 1988; Mori, 1998, 2002; Thompson & Fedak, 2001). The results from this study provide insight into the most effective strategy Steller sea lions should use to maximize foraging effort. The proportionally short amount of time it takes to recover O_2 stores suggests that sea lions should rely on long dives, depleting their body oxygen stores as far as possible, and then rapidly replenish them at the surface between dives. However, the strength of this strategy is lessened when considering CO_2 recovery. Sea lions may need to forego maximizing O_2 management and instead focus on CO_2, shortening dive durations as to minimize surface recovery time for eliminating CO_2, or even choosing to not fully return to CO_2 homeostasis between dive bouts.

This brings up several important considerations for extrapolating the results of this study to wild Steller sea lions. First, wild sea lions are likely expending more energy that those tested in this study, and are therefore operating much closer to their physiological limits. This has the potential to change optimal foraging strategies, as demonstrated by dives in our study with induced drag. By increasing energy expenditure, the presence of drag also increased the CO_2 recovery times required for a given dive duration. This may alter the optimal foraging strategy, emphasizing short aerobic dives to proportionally decrease the amount of time spent at the surface. This is more similar to observations of marine mammal dives in the wild; Steller sea lions conduct very few dives that exceed 4 min (Merrick & Loughlin, 1997; Merrick *et al*., 1994; Pitcher *et al*., 2005). This behaviour is consistent with Steller sea lions that are constrained by CO_2 recovery rates (rather than O_2), forcing them to conduct short dives to reduce surface time and remain efficient in their foraging.

Second, in my investigation of O_2 and CO_2 recovery rates, I treated dives as individual events. Notably, I measured the time it took to recover from a single dive to stables rates of O_2 uptake and CO_2 release. This means that the sea lions would have theoretically fully replenished their O_2 stores and completely eliminated accumulated CO_2 from the preceding dive. However, this does not capture the complexity of "natural" dive behaviour. In the wild, sea lions regularly conduct bouts composed of multiple dives with short inter-dive intervals. In this situation, individual dives would likely impose a cumulative physiological cost on each subsequent dives (Fahlman *et al*., 2008; Kooyman *et al*., 1971; Ponganis *et al*., 1993). Previous studies suggest that it may in fact be beneficial for diving mammals to forgo full recovery during dive bouts due to the diminishing rate of O_2 replenishment as blood approaches full saturation (Fahlman *et al*., 2008; Goundie *et al*., 2015; Kooyman *et al*., 1971; Kramer, 1988). The animals would then pay off the excess "debt" at the end of the dive bout. However, with longer recovery times for CO_2, this strategy inevitably has the cost of progressively increasing CO_2 accumulation. Fahlman *et al*. (2008) suggested that the duration of a surface interval is a balance between replenishing O_2 and voiding CO_2, with CO_2 accumulation proving to be the ultimate factor in determining when to end a dive bout. The optimal diving strategy would therefore be one where marine mammals can quickly restore O_2 while eliminating enough CO_2 to allow tolerable levels to accumulate. Marine mammals' increased tolerance to high PCO_2 may allow them to use this strategy of partial recovery to increase foraging efficiency, deferring the costs of increased surface time.

Finally, it should be noted that, despite the animals of this study seemingly reaching stable rates of O_2 uptake and CO_2 release, true homeostasis may not occur until hours later. A thorough review from Wood (1991) describing acid-base, and metabolism recovery shows that fish may take hours to fully recover gas exchange homeostasis after exhaustive exercise. More specifically, when measuring O_2 uptake in Atlantic salmon (*Salmo salar*), Zhang *et al*. (2018) observed that recovery occurred in three phases (rapid, plateau, and slow). The initial rapid phase occurred quickly, restoring O_2 stores until the animal reached a somewhat stable rate of O_2 consumption. However, they determined that this rapid phase only contributed to 16% of the metabolic cost of recovery. The plateau and subsequent slow phases contributed far more (53 and 31% respectively), and was not complete until a full 8 h after the exercise event. For trained fish, the slow phase wasn't completed until nearly 16 h after exercise. If marine mammals follow a similar trend in recovery, then our study likely only measured the initial rapid phase of recovery. The "homeostasis" the sea lions displayed would only be the plateau phase with the majority of O_2 required for recovery going unmeasured. Zhang *et al*. (2018) determined that the slow phase in salmon was temporally associated with several biochemical processes important to recovery, namely lactate oxidation, pH regulation, and gluconeogenesis. It is possible the sea lions had not completed these processes of recovery and wouldn't do so until several hours later. Researchers have proposed "recovery" as an explanation for long periods of surface times often observed following extended series of dive bouts in some marine mammals. However, wild Steller sea lions very rarely have surface times longer than 8 min (Merrick *et al*., 1994), far shorter than the required time to fully complete recovery under this hypothetical pattern. Still, while our measurements of recovery cover this biologically relevant time, it is possible that we are not measuring complete metabolic recovery. These animals may forego complete recovery until long after foraging bouts, possibly not until the animal has hauled out. Further research into long term recovery of marine mammals is required to determine how much these animals truly recover during diving and foraging events.

Previous research on marine mammal diving has largely focused on how these animals most effectively manage O_2 stores throughout the dive cycle, and the role of CO_2 management has been largely ignored. By studying the patterns of gas exchange in adult Steller sea lions, I found that CO_2 recovery times were not only longer than O_2 recovery times (particularly for longer dive durations), but increased to an even greater degree with increased dive time. Whether CO_2

recovery takes longer due to increased production for long dives, or due to rate-dependent steps in CO_2 removal remains to be seen. Regardless, these longer recovery times suggest that increasing PCO_2 levels likely play an important role in the regulation of dive behaviour. The idea of CO_2 as a key regulator of dive duration becomes even more prominent when considering the behaviour of wild animals—partial recovery during dive bouts would likely lead to even higher levels of CO_2 accumulation. Further insights into the recovery time during dive bouts, as well as how dive time is affected by accumulated CO_2 after multiple dives may provide insights into what controls diving and foraging behaviour in sea lions and other marine mammals.

Chapter 3: The transition from aerobic to anaerobic metabolism and its effect on recovery time in Steller sea lions (*Eumetopias jubatus*)

3.1 Summary

The transition between aerobic and anaerobic metabolism is thought to act as an important threshold in diving behaviour. This transition, which is associated with many physiological changes, including the accumulation of lactate, is defined as the aerobic dive limit (ADL). To date, the majority of researchers ascribe to the traditional model of a rapid, distinct metabolic transition that occurs after an animal uses its entire aerobic capacity. This can be seen in numerous studies that utilize the calculated aerobic dive limit (cADL) as a physiological "break point". Under this model, lactate appearance would only occur when an animal had completely exhausted their O_2 stores. Alternately, the mixed metabolism model suggests that aerobic and anaerobic metabolism frequently occur in tandem during a dive, and that lactate accumulation occurs as the rate of its production exceeds its rate of removal. I measured post-dive lactate levels in freely diving Steller sea lions (*Eumetopias jubatus*) over varying dive durations as an indication of the transition from aerobic to anaerobic metabolism. In addition, I measured total body oxygen stores and diving metabolic rate to obtain cADL estimates for all study animals. Finally, I measured the time for O_2 uptake and CO_2 release to reach stable post-dive levels to determine how transitioning between metabolic pathways affects gas recovery time. Contrary to past studies, post-dive blood lactate was present even after relatively short dives, demonstrating a progressive increase with dive time. This supports the mixed metabolism model, indicating that, at least in Steller sea lions, anaerobic metabolism is likely occurring early in the course of a dive and in tandem with aerobic metabolism. Recovery times for O_2 and CO_2 increased at a steady rate in proportion to increased dive time, with no apparent inflection point. This suggests that anaerobic metabolism likely does not impose an additional metabolic burden that must be repaid during post-dive recovery at the surface. With these results it appears that Steller sea lions conform to a mixed metabolism model, utilizing both aerobic and anaerobic metabolism in tandem while diving.

3.2 Introduction

Marine mammals must maximize their time underwater for required behaviours such as foraging and predator avoidance. As such, these animals have adaptations to tolerate extended periods of apnea, many of which have long been studied (Butler & Jones, 1997; Kooyman & Ponganis, 1998; Reed *et al*., 1994; Sparling & Fedak, 2004). However, despite these physiological adaptations, marine mammals are required to return to the surface to replenish oxygen reserves and remove metabolic end products, ultimately limiting the time they spend conducting essential behaviours. This need to balance time spent between diving and recovery is the basis for optimal foraging models which aim to determine how marine mammals should best divide their time between the surface and depth (Carbone & Houston, 1996; Doniol-Valcroze *et al*., 2011; Halsey & Butler, 2006; Kramer, 1988; Mori, 1998, 2002; Thompson & Fedak, 2001). At the core of these models is an attempt to understand how an animals' physiology has the potential to influence their behaviour, both in terms of their ability to remain underwater and the subsequent time required to remain at the surface. Understanding these physiological constraints can provide valuable insights into how these animals utilize their environment.

The field of diving physiology has few thresholds more central than the aerobic dive limit (ADL). This cornerstone concept, first described by Kooyman *et al*. (1983), is defined as the dive duration at which there is a "point of inflection where lactate values rise above the resting levels". Physiologically, the ADL was conceived to serve as an indication of the switch between two different metabolic states— aerobic and anaerobic metabolism—each of which is limited by an animal's physiology. Aerobic metabolism is limited by the animal's ability to generate energy using stored O_2, while anaerobic metabolism is limited by their tissues' glycolytic capacity. The metabolic transition between these two mechanisms acts as an important physiological threshold to marine mammals, and has the potential to restrict certain diving behaviours. While maximizing time at depth allows marine mammals to obtain more resources while foraging during a single dive, this may not always be the best overall strategy. It is generally believed that short aerobic dives are preferable to longer anaerobic dives, as the latter are expected to require proportionally greater post-dive surface recovery times. Spending more time at the surface recovering will decrease overall time spent at depth during the course of several dives, thereby decreasing an animal's overall foraging efficiency. Despite the importance of this topic, it currently remains unclear how and when marine mammals transition between metabolic states. Defining this key

physiological point will shed light on how marine mammals manage dives within the realities of their physiological limits.

To date, the majority of researchers have utilized the traditional model of diving physiology where marine mammals are assumed to experience a distinct switch between metabolic pathways. In the initial part of their dive, an animal is presumed to use solely aerobic metabolism to supply energy. It's not until the animal has entirely consumed their total body oxygen stores (TBO) that they then switch to solely anaerobic metabolism to power the dive. The ADL under this physiological model is the point where all O_2 is consumed, and the animal must switch to anaerobic metabolism and rapidly accumulate lactate. This model leads to several predictions of both physiology and subsequent behaviour. First, if an animal dives without exceeding their ADL, we would expect no accumulation of lactate, and for recovery time to increase proportionally with increased dive time. This recovery time is needed to replenish O_2 stores depleted during the dive and to eliminate accumulated CO_2. However, after the animal surpasses its ADL, any additional recovery time will be due solely to changes brought on by anaerobic metabolism. Lactate would accumulate proportional to the dive duration in excess of the ADL which must be cleared at the surface, potentially adding to recovery time. Therefore, O_2 recovery time would continue to increase with dive duration past the ADL. This wouldn't be due to O_2 utilization while submerged (which theoretically ceases at the ADL) but due to the additional O_2 required upon surfacing to process accumulated lactate through the Cori cycle and its conversion back to glycogen. Anaerobic dives would ultimately require more O_2 than aerobic, resulting in an upwards inflection point at the ADL in the relationship between dive duration and O_2 recovery time.

The diving behaviour of marine mammals in the wild provides some evidence to support this model. Generally, marine mammals avoid surpassing their ADL, suggesting there is a negative effect of diving beyond this point. Less than 5% of dives completed by Weddell seals (*Leptonychotes weddellii*) exceed their ADL (Kooyman *et al*., 1983, 1980). Post-dive surface intervals for these animals also increase disproportionately to dive time for dives that exceed the ADL (Butler & Jones, 1997; Costa *et al*., 2001; Kooyman *et al*., 1980). Galapagos fur seals (*Arctocephalus galápagoensis*) have an abrupt change in the minimum duration of their post-dive surface durations as dive time increase, providing indirect evidence as well (Horning, 2012). The disproportional increase in recovery time for longer dives in comparison to shorter ones is

consistent with this traditional model of a distinct switch from aerobic to anaerobic metabolism at a given dive duration.

However, current knowledge of the physiological processes that occur during a dive suggest that this "complete switch" model is likely inaccurate. Like their terrestrial counterparts, marine mammals have key systems (e.g. central nervous system, heart) that are incapable of functioning using completely anaerobic metabolism. Logically, marine mammals must retain some amount of O_2 to be able to keep these systems functioning when surpassing their ADL. To facilitate this, marine mammals use localized peripheral vasoconstriction to prioritize O_2 use in these key systems during dives (Butler & Jones, 1997; Davis, 2014), transferring the burden of lactate accumulation to tissues that are better able to withstand it (e.g., the swimming muscles). The proposal that certain areas become anaerobic before others seems incompatible with the traditional model of a definitive switch from aerobic to anaerobic metabolism.

An alternate to the traditional model of diving physiology is a mixed metabolism model. This suggests that energy used throughout a dive is supplied from both aerobic and anaerobic stores in tandem. The ratio of energy produced between these two stores may change as dive time progresses—as O_2 stores become depleted with increased dive time, anaerobic metabolism would provide a greater degree of energy. In the early stages of diving under this model, the minimal lactate that is produced may be processed while submerged, either reduced through gluconeogenesis or utilized as a fuel source in the Krebs cycle. The ADL can therefore be redefined as the point where the rate of lactate production surpasses its rate of removal, resulting in net accumulation. Under this model, instead of a rapid accumulation of lactate at a given dive duration, we would instead expect to see a progressive increase with increased dive time. As the ratio of aerobic to anaerobic metabolism increases with dive time, lactate would build slowly at first, followed by a quicker accumulation over time. As accumulated lactate will also need to be metabolised at the surface, we would expect to see a similar increase for both O_2 and CO_2 post-dive recovery as well. The slope of this recovery curve (describing the relationship between recovery time and dive time) will depend on the proportion of aerobic to anaerobic metabolism at the beginning of the dive and how this ratio changes over time submerged.

While the mixed metabolism model has been suggested in the past (Butler, 2006; Carbone & Houston, 1996; Mori, 2002), the majority of studies tend to continue to use the paradigm of the

traditional model of a distinct switch between metabolic states. This is exemplified in the importance placed on the calculated aerobic dive limit (cADL), an estimate of the ADL that is obtained by dividing an animal's total body oxygen stores (TBO) by their diving metabolic rate (DMR). Due to the difficulty of obtaining post-dive blood lactate from marine mammals, researchers often rely instead on the cADL as an estimate of the transition between metabolic states. In these studies, the cADL is implied to measure the same thing as the ADL when, in fact, they may measure two different processes. The ADL is operationally defined as a measurement of when blood lactate rises above resting levels, which may signify different physiological processes under these two different models. The cADL, on the other hand is defined as the time it takes to consume all available O_2 stores during a dive, assuming only aerobic metabolism is used. Hence, ADL and cADL will only be equal if there is a distinct switch between metabolic states during a dive. A comparison between the timing of cADL and ADL should shed light on the underlying metabolic processes during a dive.

Few studies have tested whether aerobic and anaerobic metabolism are being used concurrently during dives. This study explores the timing of the transition between these two metabolic states in freely diving Steller sea lions (*Eumetopias jubatus*). I directly measured post-dive blood lactate concentrations and looked at the relationship to diving duration. I also compared the rate of lactate emergence to the sea lions' cADL, quantified from measures of total body oxygen (TBO) and diving metabolic rate (DMR). I also measured how O_2 and CO_2 recovery times were affected by the underlying shift from aerobic to anaerobic metabolism during dives. Measurements of lactate accumulation and gas recovery times allowed me to determine which of the two models of metabolic transition is used by Steller sea lions. Understanding how and when these animals transition between metabolic pathways can provide insights into the costs of diving behaviour. Ultimately, this can be used to better understand how marine mammals utilize their environment, and allow us to determine optimal foraging strategies.

3.3 Methods

3.3.1 Study Animals

I conducted trials from April 19th – September 6th, 2016 to measure post-dive blood lactate in reference to dive times and to O_2 and CO_2 recovery times (Chapter 2) from four adult female Steller sea lions. All animals were collected as pups from rookeries off the coast of the northern

tip of Vancouver Island and raised at the Vancouver Aquarium (Vancouver, BC). All research was conducted at the Open Water Research Laboratory (Port Moody, BC) where the animals were housed. For between 8 and 13 years, the sea lions have been actively involved in research by diving unrestrained in the open ocean (for review, see Rosen *et al*., 2017). All animals were trained through these past studies to use the experimental equipment and follow experimental procedures. During these studies, the animals were fed a diet of herring, squid, capelin, and supplementary vitamins. All measurements were conducted under UBC Animal Care Permits A11-0397 and A16-0027.

3.3.2 Lactate Measurements

To measure lactate emergence, I collected blood samples from the animals after they performed a series of voluntary dives of varying duration. Animals were transported by boat to the study site: a floating platform located in the Indian Arm fjord (Port Moody, BC). During transport they were fed less than 0.5 kg of fish in order to minimize any potential effect of the heat increment of feeding on metabolism (Rosen & Trites, 1997). This transport period also served to ensure pre-dive lactate levels reflected resting conditions. Once at the site, the trainers signaled for the animals to perform a dive to the end of two 40 m tubes attached to the platform ~9 m apart. Pieces of fish (~20 g, herring or capelin) were alternately delivered through the tubes, forcing the animals to swim between each tube. This simulated a foraging event at a depth relevant to these types of behaviours seen in the wild (Loughlin *et al*., 1998; Loughlin *et al*., 2003; Merrick *et al*., 1994; Rehberg *et al*., 2009). While the sea lions controlled the overall duration of the dive, the frequency of fish being pumped through the tubes allowed me to have a marginal degree of control over dive duration. Upon surfacing, the trainers signaled for the animals to climb onto the platform. The trainers then collected voluntary blood samples from the interdigital vessel of the hind flipper of the animal. Preliminary trials were completed in an effort to account for the delay in blood lactate emergence due to the reperfusion of muscles post dive. In practice, the ideal method was to collect blood samples as quickly as possible post-dive. The voluntary nature of the blood collection allowed us to forego anesthesia, such that blood collection occurred within 6 min of the animal surfacing (mean ± SD = 2.82 min ± 0.86). Blood samples were analyzed for lactate levels within 5 min of blood extraction (1.21 min ± 0.77) with the exception of one sample that was measured 24 min post-extraction. I analyzed lactate levels using a Lactate Plus meter (Nova Biomedical,

Waltham MA, USA). While developed for use in humans, the Lactate Plus meter has been validated for use in other mammals (Hauss *et al*., 2014; Nye & Mariani, 2018).

3.3.3 Metabolic Measurements

In a separate set of trials conducted over the same time period, I measured the post-dive rates of O_2 uptake and CO_2 release to determine recovery times from varying dive durations. These trials were conducted at the same location and research platform as the lactate emergence trials. The trials have been previously detailed (Chapter 2). Briefly, the work platform housed a floating Plexiglass respirometry dome. While an animal was in the dome I measured O_2 uptake and CO_2 release using flow-through respirometry, drawing air over the animal at 350 L min^{-1} using a model 500H mass flow generator and controller (Sable Systems Inc., Las Vegas, NV, USA). I subsampled the excurrent airstream, removing vapour in the process by drawing it over desiccating Drierite crystals. Using Sable System FC-1B and CA-1B analyzers (Sable Data Acquisition system, Sable Systems Inc.), I measured fractional concentrations of O_2 and CO_2 and recorded measurements every 0.5 s. The system was leak-checked using standard nitrogen dilution techniques. To account for electronic drift in the gas analyzers, I baselined gas concentrations to ambient air at the beginning and end of each trial. In addition to this, the gas analysis system was periodically calibrated with known concentrations of gases.

As with the lactate trials, the sea lions were transported to the site by boat. Upon arriving at the platform at the beginning of a trial, an individual animal surfaced in the respirometry dome. They remained in the dome between 5-10 min until they reached a constant rate of O_2 uptake and CO_2 release. Pre-dive metabolic rate was calculated using the last 2 min of this period while the animal was floating calmly at the surface. On a signal from the trainer the animal performed a dive to the end of the feeding tubes as described above. Similar to lactate dives, animals controlled the duration of the dive, but instead of surfacing onto the platform, the animals surfaced directly within the respirometry dome. Here I collected post-dive measurements of instantaneous O_2 uptake and CO_2 release. These rates were calculated using equations 11.7 and 11.8 in Lighton (2008) and analyzed with the program Expedata. Animals remained in the dome until the animals reached a constant rate of exchange for both O_2 and CO_2.

As per Animal Care requirements, all animals wore a specially built, tight-fitting harness equipped with a VHF transmitter. In addition, animals were equipped with one of three joint

accelerometer and time depth recorders (TDR10-DD & TDR10-X, Wildlife Computers; OpenTag, Loggerhead Instruments). For all metabolic trials animals completed between 1-3 dives per session while for the lactate emergence dives animals completed 1 dive per session.

3.3.5 Recovery times

To calculate O_2 recovery times, I fit a least squares regression to the instantaneous O_2 consumption data from the peak rate of consumption to the point the animal exited the dome. This allowed me to determine the when the animal had reached a near-constant post-dive metabolic rate. I then defined O_2 recovery time as the time post-surface to reach within 5% of this constant metabolic rate. This point represents when the animal had 95% recovered in terms of O_2 balance. Instantaneous CO_2 release rates were also fit with a least squares regression, and CO_2 recovery time was similarly defined as the time it took to reach within 5% of a constant rate of CO_2 release. A 95% recovery threshold was used as opposed to 100% as inherent variation in post-dive respiration may misrepresent the time it takes to reach constant rates of O_2 uptake and CO_2 production.

3.3.4 Total Body Oxygen and cADL

In order to calculate the cADL for different dives, a single estimate of total body oxygen stores (TBO) was determined for each sea lion at roughly the midpoint of the trial period through estimates of lung, muscle, and blood oxygen stores. To estimate lung O_2 stores, I assumed a diving lung volume of 55 ml kg^{-1} (Lenfant *et al*., 1970), that the air in the lungs had a 15% O_2 content (Kooyman *et al*., 1971), and that animals could use the entirety of this O_2 during dives. Thus, lung O_2 stores were estimated incorporating body mass measurements (M_b) into the following equation:

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

In calculating muscle O_2 stores, I assumed that muscle mass was 37% of total body mass based on measurements from juvenile California sea lions (*Zalophus californianus*; Ponganis *et al*., 1997; Richmond *et al*., 2006), and that 52% of this muscle was swimming and 48% was non-swimming muscle (as measured in one-month old pups; Richmond *et al*., 2006). I assumed myoglobin concentrations [Mb] were 28.7 mg g^{-1} and 20.0 mg g^{-1} of wet swimming and non-swimming muscle respectively (Kanatous *et al*., 1999). Myoglobin O_2 binding capacity was assumed to be 1.34 mL O_2 g^{-1} Mb (Kooyman & Sinnett, 1982). Muscle O_2 stores were therefore calculated as such:

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

I estimated blood O_2 stores through direct measurements of several blood parameters. Hematocrit (Hct) and haemoglobin concentrations [Hb] were determined by collecting blood samples extracted under veterinary supervised anaesthesia (maximum 5% isofluorane gas) from the caudal gluteal vein. These samples were analysed by a commercial laboratory (Idexx Laboratories, Delta, BC) to determine Hct and [Hb].

I determined plasma volume (PV) in each study animal using Evans blue dilution procedure as per Gibson & Evans (1937). Background blood samples were collected while the animal was anaesthetized prior to injecting a 0.5 mg kg^{-1} dose of Evan's blue dye (Alfa Aesar; EC 206-242-5) via intravenous catheter in the rear flipper. Serial blood samples were collected via this same catheter at around 7, 14, 22, 28, and 34 min post-injection (exact times were recorded) and centrifuged to separate plasma. I then used a simplified spectrometry procedure to measure the relationship between the optical densities of two different wavelengths (624 and 740 nm). This enabled me to correct absorbance measurements for the presence of plasma in dye samples. By plotting absorption values for the serial samples over time, I was able to use a linear regression to extrapolate the concentration of dye at the time of injection (concentration at the y-intercept). Using standard curves created from stock solutions of Evans blue, I calculated instantaneous dilution volume which was equivalent to PV. This, in turn, was used with Hct to calculate blood volume (BV) using the following equation:

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

I calculated final blood O_2 stores based on the sum of available O_2 in the arterial and venous blood compartments. I considered one-third of total BV to be arterial, and assumed 95% saturation at the start of a dive and 20% saturation by the end of the dive (Ponganis *et al*., 1993; Richmond *et al*., 2006). The remaining two-thirds of total BV was considered to be venous blood, and contained 5 vol% less O_2 than the initial arterial saturation (Ponganis *et al*., 1993; Richmond *et al*., 2006). Assuming an O_2 binding capacity of haemoglobin of 1.34 ml O_2 g^{-1} Hb (Kooyman & Sinnett, 1982), blood O_2 stores were calculated as follows:

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

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

These measurements of TBO could be used in conjunction with the measured rate of O_2 consumption (DMR) during a dive to calculate cADL. However, with diving mammals we face the problem that internal O_2 consumption during a dive is temporally separated from external gas exchange following this dive; i.e., we are only capable of measuring O_2 uptake rate during the post-dive recovery period. To address this issue I calculated DMR in two different ways as per Gerlinsky *et al*. (2014), which also allowed for easier comparison to previous papers. For the first method I determined the volume of O_2 consumed in excess of the post-dive baseline during the recovery phase (volume of O_2 attributed solely to the dive). This was divided by dive duration to obtain DMR_{dive} (as per Hastie *et al*., 2007; Hurley & Costa, 2001). For the second I calculated the average rate of O_2 uptake over the entire dive event (total volume of O_2 consumed divided by the total duration of the dive and subsequent recovery period) to obtain DMR_{cycle} (as per Fahlman *et al*., 2008; Kooyman *et al*., 1980). These two DMR measurements were then used to determine cADL for each animal by dividing TBO by DMR.

3.3.5 Data Analysis

I analyzed lactate emergence data using R software (R Development Core Team, 2015), performing a linear mixed effects analysis on the relationship between dive time and post-dive lactate levels using the lme4 package (Bates *et al*., 2015). This analysis allowed me to include post-dive collection time as a potential fixed factor and to account for the repeated measures nature of the data by including animal ID as random effect. I then performed a piecewise linear regression of lactate emergence and post-dive lactate levels, estimating the breakpoint using the package "segmented" with the R software. I then used a Davie's test to determine whether the slope change in the piecewise regression was statistically significant. Finally, to determine which model best represented the data, I used the maximum likelihood method, and performed a repeated measures ANOVA on the two models (linear vs piecewise) to determine if they were statistically different.

I performed a similar analysis to examine the relationship between dive time and recovery time for both O_2 and CO_2. A linear mixed effects model was fit to the relationship between O_2 recovery times and dive time with dive duration (between 2 and 7 min) as the fixed factor. Animal ID was once again included as a random effect. I performed a piecewise linear regression on the same data, estimating the breakpoint using R package "segmented", and performed a Davie's test to determine whether the slope change was statistically significant. These piecewise models were compared to linear models using the maximum

likelihood method to determine which best represented the data, and a repeated measures ANOVA was performed to determine if the models were statistically different. I visually inspected all residual plots to determine if there were any obvious deviations from homoscedasticity or normality. Values are reported as means ± standard deviation. Statistical significance was set at $\alpha = 0.05$.

3.4 Results

I determined the calculated aerobic dive limit (cADL) by dividing the total body O_2 stores (TBO) of each animal by their diving metabolic rate (DMR). TBO consists of three components: lung, muscle, and blood O_2 stores. As lung and muscle O_2 stores are assumed to be derived from allometric relationships with a slope of 1.0, they have the same mass-specific values for all sea lions: mass-specific lung and muscle O_2 for the four animals were 8.25 and 12.2 mL O_2 kg^{-1} respectively. Blood volume ranged between 63.54 – 93.22 mL kg^{-1} (80.68 mL kg^{-1} ± 12.67), resulting in blood O_2 stores averaging 13.3 ± 2.15 mL O_2 kg^{-1}. The mean TBO for all four sea lions was therefore 33.72 ± 2.15 mL O_2 kg^{-1} (Table 3.1).

I measured DMR from 105 dives that ranged in duration from 1.69 – 6.55 min (4.20 ± 1.31 min). Mean DMR_{dive} was 2.91 ± 0.71 L O_2 min^{-1} while mean DMR_{cycle} was 2.22 ± 0.36 L O_2 min^{-1}. Application of the higher DMR_{dive} value resulted in a lower cADL ($cADL_{dive}$), ranging from 1.95 – 2.64 min (2.24 ± 0.30 min; Table 3.2). In comparison, $cADL_{cycle}$ calculated from DMR_{cycle} for the four animals ranged from 2.62 – 3.11 min (2.89 min ± 0.23). Both of these estimates are lower than previous measurements of cADL in the same group of Steller sea lions (3.0 – 3.3 min for nutritionally stable and stressed animals respectively; Gerlinsky *et al*., 2014).

To examine the pattern of lactate emergence, I collected blood samples after 43 dives ranging in duration from 1.08 – 6.77 min (3.79 min ± 1.48) from three of the four sea lions. Generally, for all three animals, post-dive lactate levels increased with dive duration, ranging from 0.8 to 6.3 mmol/L (2.4 mmol L^{-1} ± 1.5; Fig. 3.1). For each animal, I estimated the breakpoint to determine if there was a change in the relationship between dive duration and rate of lactate emergence around the cADL using the package "segmented" in R (R Development Core Team, 2015), and fit a piecewise regression to the data. Using a Davies' test, I determined whether the change in slope from this piecewise regression was statistically significant (i.e., were slopes of the two fitted lines different), and then compared these models to linear regressions for each animal

to evaluate the significance of any change in slope using an ANOVA test (i.e., whether calculated breakpoint was statistically significant).

Breakpoints in lactate levels for the three animals ranged from 3.22 - 4.89 min, higher than cADL measurements from this or previous studies. There was no significant change in slopes after the breakpoint for any of the animals ($p > 0.05$; Table A.1). In two of the three animals, linear regression models received substantially more support than the piecewise regressions based on model averaging ($\Delta AIC > 2$; Table A.1). While the piecewise regression received more support in the final animal (F97BO), the low ΔAIC between the linear and piecewise model suggests that these two models are equally supported. In addition, ANOVA tests determined all linear regression models were not significantly different from piecewise regressions ($p > 0.05$). Thus, the data indicate that there was not a distinct breakpoint in lactate emergence for any of the Steller sea lions. Instead, lactate appeared to increase linearly with increased dive time (Fig. 3.1). Post-dive collection time was eliminated as a fixed factor in all linear models.

To analyse the change in O_2 recovery time with dive duration, I similarly fit a piecewise regression for each animal with breakpoints estimated in R. These models were once again compared to linear regressions for all four animals. A similar procedure was done with CO_2 recovery times. See Chapter 2 for further detail on recovery time results.

Breakpoints in O_2 recovery ranged from 3.77 - 5.78 min for the four animals. In three of the four animals, piecewise regressions received substantially more support than linear models ($\Delta AIC > 2$; Table A.1). However, O_2 recovery slopes before and after estimated breakpoints were not deemed to be significantly different according to Davie's tests in any of the animals ($p > 0.05$; Table A.1). In addition, ANOVA tests determined that piecewise regression models were not significantly different from linear models ($p > 0.05$).

Similar results were found with CO_2 recovery times. Breakpoints in CO_2 recovery for the four animals ranged from 2.29 - 6.22 min. In three of four animals a piecewise regression best suited the data in comparison to a linear regression (Table A.1), but the change in slopes were not deemed to be significant ($p > 0.05$). In addition, an ANOVA test determined that piecewise regression models were not significantly different from linear models ($p > 0.05$). This data suggests that there was no significant breakpoint in O_2 recovery or CO_2 recovery times. Instead, O_2 recovery

times and CO_2 recovery times increased at a constant linear rate with increased dive time (Fig. 3.2; Fig 3.3).

Table 3.1. Blood values for four captive adult female Steller sea lions, including hematocrit (Hct), plasma volume (PV), blood volume (BV), hemoglobin concentration [Hb], blood O_2 stores, and total body oxygen stores (TBO). Body mass on day of blood O_2 store measurement is also provided.

Animal	Mass (kg)	Hct	PV (mL kg^{-1})	BV (mL kg^{-1})	[Hb] (g L^{-1})	Blood O_2 (mL kg^{-1})	TBO (mL kg^{-1})
F97SI	225	0.49	47.5	93.2	0.168	15.4	35.8
F00YA	220	0.48	33.0	63.5	0.166	10.4	30.8
F00HA	161	0.46	43.1	79.8	0.169	13.3	33.7
F97BO	157	0.48	44.8	86.2	0.167	14.2	34.6
Mean ± SD	**191 ± 36.8**	**0.48 ± 0.012**	**42.1 ± 6.33**	**80.7 ± 12.7**	**0.168 ± 1.13E-3**	**13.3 ± 2.15**	**33.72 ± 2.15**

Table 3.2. Diving metabolic rates (DMR) and aerobic dive limits of four adult Steller sea lions. DMR was calculated both as a function of the time submerged (DMR_{dive}) and over the course of a complete dive cycle, including recovery time (DMR_{cycle}). Calculated aerobic dive limit (cADL) was calculated by dividing TBO by these two DMR measurements to obtain two estimates ($cADL_{dive}$ and $cADL_{cycle}$). Mass is averaged over the course of the study.

Animal	Mass (kg)	Blood O_2 (mL kg^{-1})	TBO (mL kg^{-1})	DMR_{dive} (L O_2 min^{-1})	DMR_{cycle} (L O_2 min^{-1})	$cADL_{dive}$ (min)	$cADL_{cycle}$ (min)
F97SI	226	15.43	35.84	3.52	2.59	2.32	3.14
F00YA	220	10.37	30.78	3.47	2.43	1.98	2.79
F00HA	164	13.30	33.71	2.59	2.07	2.16	2.69
F97BO	158	14.16	34.57	2.06	1.79	2.70	3.07
Mean ± SD	**193 ± 36.0**	**13.31 ± 2.15**	**33.72 ± 2.15**	**2.91 ± 0.710**	**2.22 ± 0.360**	**2.28 ± 0.307**	**2.92 ± 0.217**

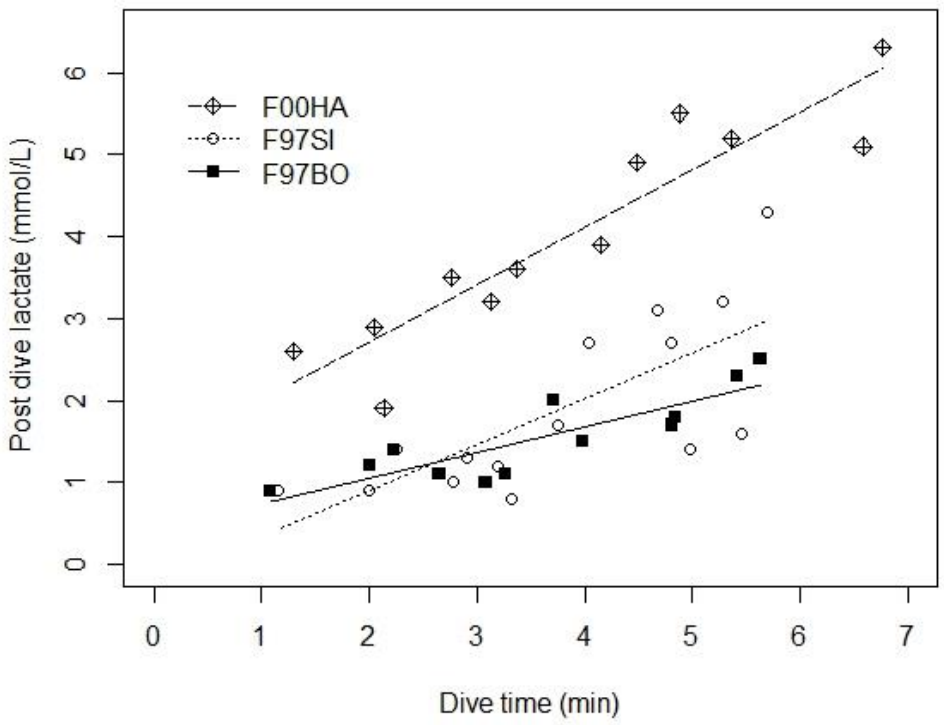

Figure 3.1. Post-dive blood lactate from three adult Steller sea lions after varying dive durations. For all three animals, blood lactate levels were measureable even after short dive durations. Linear regressions for animals F00HA and F97SI received greater support than piecewise regression indicating that, for these animals, blood lactate accumulated linearly with increased dive time. A piecewise regression received more support in the final animal (F97BO), however there existed a low ΔAIC, suggesting the two models were equally supported. In addition, ANOVA tests determined linear regressions were not significantly different from piecewise regressions for any of the animals ($p > 0.05$).

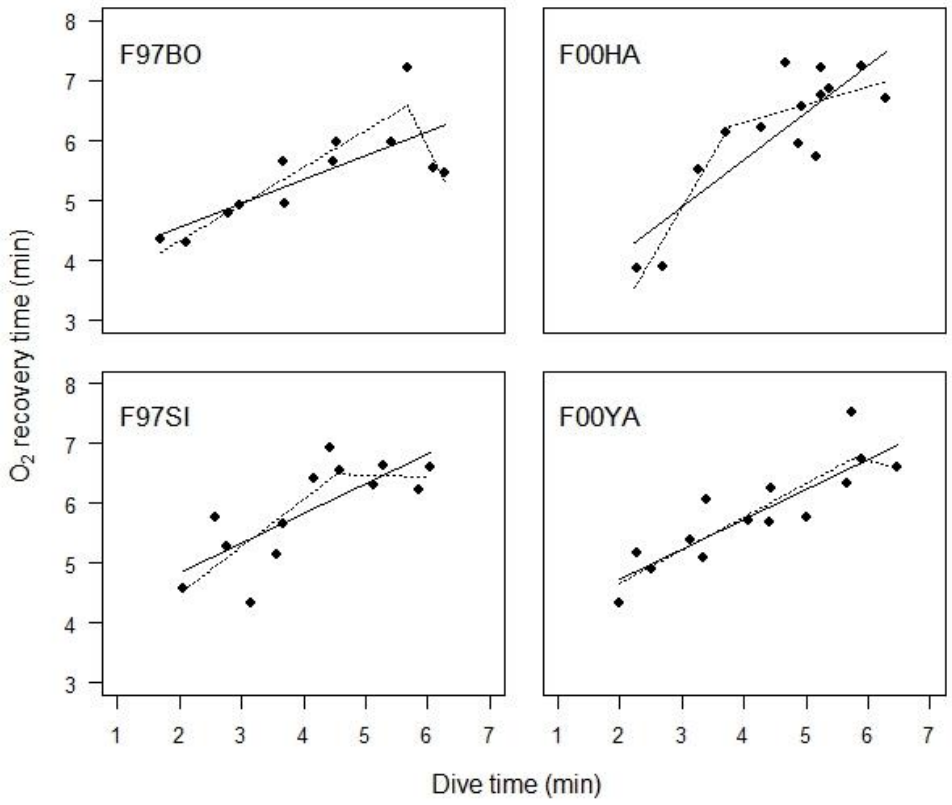

Figure 3.2. Time for the rate of oxygen uptake to return to within 5% of resting levels (O_2 recovery time) for varying dive durations in four adult female Steller sea lions. For three of four animals (F97BO, F00HA, F00YA), piecewise regressions receive substantially more support than linear models (ΔAIC > 2), however, according to Davie's test, slopes before and after the estimated breakpoints were not significantly different ($p > 0.05$). Piecewise regressions (broken lines) were not significantly different from linear models (solid lines) according to ANOVA test for any animal ($p > 0.05$).

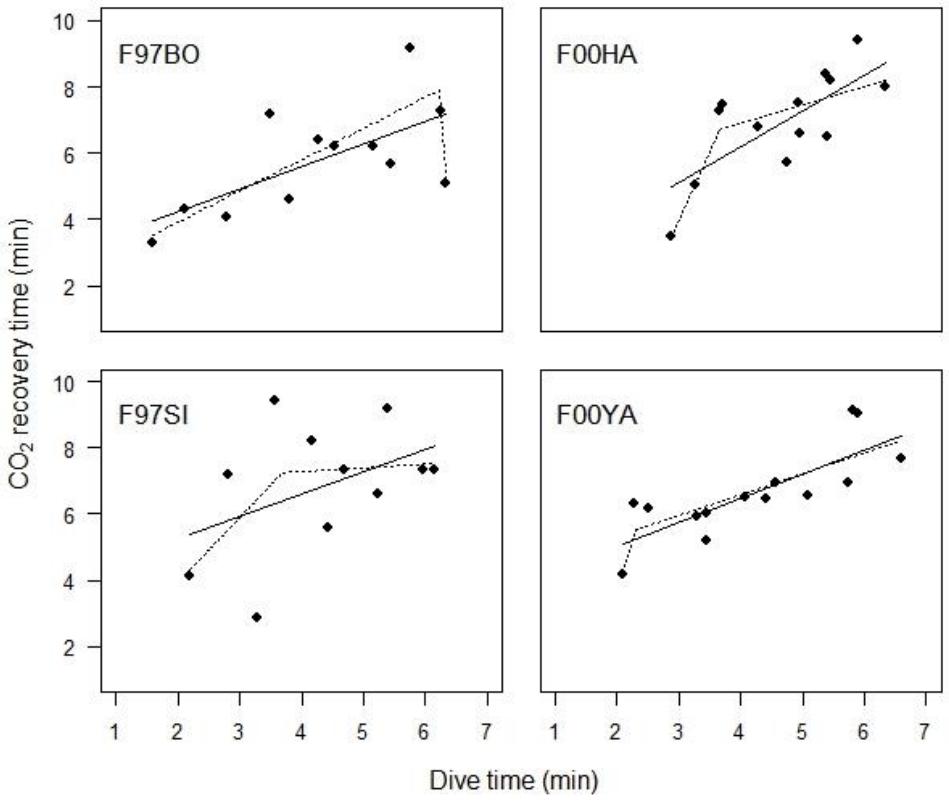

Figure 3.3. Time for the rate of CO_2 release to return within 5% of resting levels for varying dive durations in four adult female Steller sea lions. While for two animals (F97BO & F00HA), piecewise regressions received substantially more support than linear models (ΔAIC > 2), slopes before and after the estimated breakpoints were not significantly different (Davie's tests, $p > 0.05$). Piecewise regressions (dotted lines) for these two animals were not significantly different from linear models (solid lines; ANOVA, $p > 0.05$). For the remaining animals (F97SI & F00YA) CO_2 recovery time increased linearly with increased dive time ($p < 0.05$).

3.5 Discussion

The traditional model of the aerobic dive limit (ADL) is based on a key underlying assumption— that a diving animal uses all of its aerobic capacity at depth before making the distinct switch to a reliance on anaerobic metabolism. This paradigm is reinforced in the concept of the calculated aerobic dive limit (cADL), an estimate of how long an animal can remain submerged using only aerobic metabolism. The findings of this study do not conform to the predicted patterns of the generally accepted, traditional two-phase model of the ADL. Blood lactate appeared during dives that were much shorter than the cADL for these Steller sea lions. This lactate accumulated linearly with increasing dive, with no clear inflection point. Additionally, O_2 and CO_2 recovery times increased linearly, once again with no clear breakpoint or inflection. These results instead support a mixed metabolism model that assumes a slow transition between metabolic pathways as dive duration increases, with both aerobic and anaerobic metabolism occurring in tandem. This appears to indicate that the underlying mechanisms and potential constraints of increasing dive time are more complicated than originally treated in previous studies of diving physiology.

The pattern of accumulation seen in Steller sea lions raises important questions when considering other studies of diving mammals. In this study, lactate appeared even after relatively short dives, and increased linearly with dive time. In comparison, other marine mammals exhibit low levels of blood lactate after short dives followed by rapid accumulation at a given dive duration (Kooyman *et al*., 1980; Ponganis *et al*., 1997; Ponganis *et al*., 1997; Ponganis *et al*., 1993; Shaeffer *et al*., 1997b; Williams & Cruz, 1993). Why do animals in these previous studies exhibit such different patterns in lactate accumulation? First, it is important to realize that net lactate accumulation (i.e., circulating levels) is a function of both the rates of lactate production and an animal's ability to recycle this metabolite. Different species of marine mammals may differ in both the rate of production and removal, which will affect how lactate accumulates over the course of a dive. If aerobic and anaerobic metabolism are occurring in tandem during a dive, then an increase in post-dive lactate signifies not when lactate production begins, but when lactate production surpasses the rate of its removal.

As relatively poor divers with higher proportions of fast-twitch fibers (Kanatous *et al*., 1999) and lower aerobic capacity than other marine mammals (Reed *et al*., 1994), it seems

plausible that Otariid seals such as Steller sea lions may rely on anaerobic metabolism to a greater extent than some other diving mammals. If Steller sea lions rely more heavily on anaerobic metabolism to power short dives, they will have higher rates of lactate production than other marine mammals, and lactate will therefore appear in the blood stream more quickly. In my study, lactate levels appeared to be elevated after relatively short dives, suggesting they may indeed be utilizing anaerobic metabolism to a greater extent for these short dives.

However, if Steller sea lions are relying on anaerobic metabolism early on during a dive, this raises the question as to why we did not see incredibly high concentrations of lactate after longer dives. The highest lactate concentration in my study was 6.3 mmol L^{-1}. In comparison, Weddell seals have the greatest measured concentrations of post dive blood lactate amongst marine mammals, with measurements as high as 25 mmol L^{-1} post-dive, nearly four times higher than the highest measurement of lactate in this study (Kooyman *et al*., 1980). Emperor penguins have similarly high reported blood lactate levels, reaching as high as 13 mmol L^{-1}, twice the highest levels reported in this study (Ponganis *et al*., 1997). If Steller sea lions are relying so heavily on anaerobic metabolism, why would we not see similarly high lactate concentrations? It is possible that lactate levels are driven primarily by dive time. Peak lactate in emperor penguins emerges after dives of around 10 min, and Weddell seals experience their peak lactate after dives of nearly an hour. With Steller sea lions reaching peak lactate concentrations after only 6.77 min, it's possible that if these animals remain submerged for longer, they too would accumulate lactate to these high concentrations. However, it is important to compare the full patterns of lactate accumulation between species. Weddell seals take longer to reach similar lactate levels seen in Steller sea lion—while the sea lions took less than 7 min to reach 6.5 mmol L^{-1} of lactate, Weddell seals take over 30 min to reach similar concentrations. This implies that the physiology of Weddell seals is inherently different from Steller sea lions, and factors other than dive time are driving lactate accumulation. Instead, the comparatively low peak concentrations in Steller sea lions suggest that, although lactate is being produced early on, it is only doing so at a rate that is slightly higher than the animal's ability to remove it. Further study is required to assess whether these differences in lactate emergence patterns are due to differences in production or clearance rates, and what this means in regard to the pattern of simultaneous use of aerobic and anaerobic metabolism during dives.

Differences in lactate clearing capacity can also explain why Steller sea lions lack an apparent inflection point in their lactate accumulation curve. The inflection point seen in previous studies is assumed to reflect some sort of physiological tipping point during a dive. If during short dives Steller sea lions have reached their lactate recycling capacity, even a small increase in anaerobic metabolism will cause lactate to accumulate. Ecologically, a low clearing capacity seems reasonable; as previously mentioned, Steller sea lions rarely dive in excess of 8 min (Merrick *et al*., 1994), and therefore are not likely to experience high concentrations of lactate build up. They would therefore not have a large need for a high lactate clearing capacity. In comparison, an inflection in the lactate curves of more extreme divers may be the result of an increasing reliance on anaerobic metabolism, and the physiological point where recycling capacity reaches its maximum. It is telling that a species of marine mammal with moderate diving abilities like Steller sea lions would display an intermediate pattern; there is a slow increase in lactate accumulation with increasing dive time, and a less pronounced inflection point compared to species with longer average dive times.

More extreme divers such as Weddell seals and emperor penguins dive well in excess of their ADL, accumulating lactate to very high concentrations (Kooyman *et al*., 1980; Ponganis *et al*., 1997). Having a high lactate clearing capacity would help these extreme divers delay the onset of lactate accumulation during long dives. Minimal lactate would accumulate during short dives, only beginning to build up when production finally exceeds the high removal rate. While there has been limited research into the lactate clearing capacity of marine mammals, there is some evidence that differences exist between species. Lactate dehydrogenase (LDH), the enzyme which catalyzes part of both the production and removal of lactate, can be used as one measure of an animal's clearing capacity. Castellini *et al*. (1981) found that muscle LDH activity was highest in Weddell seals, the most extreme diver of animals tested, and LDH levels were also elevated in marine mammal livers (a major site of lactate gluconeogenesis) compared to terrestrial mammals. This suggests that marine mammals may have a greater ability to metabolize lactate, and that extreme divers are even better adapted to this strategy. In addition, a study of hooded seal (*Cystophora cristata*) brains shows higher concentrations of LDH isoenzymes which preferentially convert lactate to pyruvate (Leivas Müller Hoff *et al*., 2016). Further research into LDH isoenzymes within the muscle tissue of various marine mammal species may shed light on how well different species

can metabolize lactate and could provide insight into how differences in metabolite accumulation may ultimately affect behaviour.

O_2 and CO_2 recovery times, like lactate, increased linearly with dive time in our sea lions, with no apparent inflection point. This linear increase, while contrary to the traditional understanding of the ADL, is compatible with the mixed metabolism model. Under the traditional model, O_2 and CO_2 recovery times are expected to increase when an animal surpasses its ADL due to the increased metabolic burden of processing lactate at the surface. However, given the linear increase in accumulated lactate with dive time across almost all dives, it is not surprising that there was a parallel linear increase in O_2 and CO_2 recovery times. These results lend further support to the model of a gradual transition between metabolic states rather than a distinct switch at a given point.

The proportion of energy produced via aerobic to anaerobic metabolism and how this ratio changes during the dive determines how much lactate is produced during a dive. This, in turn, is assumed to affect post-dive O_2 recovery times. However, this relationship is also determined by how lactate is processed once the animal returns to the surface. Traditionally, lactate is assumed to be processed largely through gluconeogenesis. As the animal returns to the surface after a dive, vasodilation would allow lactate to enter the blood stream. It would then be transported to the liver and converted to glucose, requiring additional O_2 to supplement the ATP required for this process. Surface recovery times would therefore increase drastically when an animal surpasses its ADL—O_2 is required not only to replenish stores spent during a dive, but also to provide energy for processing lactate. The reason anaerobic dives have traditionally been considered to be less efficient than anaerobic ones is due to this increased surface time needed to metabolize lactate.

However, this traditional model fails to acknowledge the different ways lactate may be utilized during recovery. Gluconeogenesis is not the only process for eliminating this metabolite; if an animal is in the presence of O_2, lactate can also be oxidized as a fuel source in mitochondrial respiration. This process requires no extra O_2, and we would therefore not see an increase in recovery rates for O_2 or CO_2. It is not possible for marine mammals to metabolize lactate during a dive under the traditional model of diving physiology, as lactate would only accumulate once O_2 stores have been completely depleted. Under the mixed metabolism model, lactate may not only be utilized as a fuel source during recovery, but it may also be utilized at depth. A slower transition

between metabolic pathways allows for accumulating lactate to be cleared from the muscles through mitochondrial respiration as the dive progresses. If lactate can be oxidized both during a dive and while recovering, it stands to reason that anaerobic dives would not impose as great a metabolic cost on recovery as has been previously described by diving physiologists. The lack of breakpoint in O_2 and CO_2 recovery times in this study may not only reflect a slow transition between metabolic pathways but also that a significant amount of lactate is removed via oxidation (versus through gluconeogenesis). Unfortunately, only one study has examined how lactate is recycled in an exercising, but not diving, marine mammal (Davis *et al*., 1991). Further research is required to determine how marine mammals clear lactate, and whether this relates to their diving capability.

It is important to consider that, while we defined recovery under certain terms in this study, the animals may not have fully reached a steady state of O_2 uptake or CO_2 release. A recent study of post-exercise O_2 consumption in exercising Atlantic salmon (*Salmo salar*) by Zhang *et al*. (2018) found that O_2 consumption during recovery followed the typical steps of recovery seen in previous studies. This involves an initial rapid recovery where relatively little of the total excess O_2 was consumed followed by a slower plateau recovery where a greater portion of excess O_2 was consumed. However, they discovered that Atlantic salmon experienced a third slow recovery that took up to 15 h after the exercise event to complete. They noted that this phase corresponded temporally with a substantial decrease in blood and muscle lactate, as well as a recovery to normal pH. With direct measurements of blood PCO_2 and lactate, we know that rainbow trout (*Oncorhynchus mykiss*) can similarly take up to 10 and 12 h respectively to reach full recovery (Milligan & Wood, 1986; Wood, 1991). If Steller sea lions also required such long recovery times to clear metabolites, we would not have captured the animal's full recovery, as measurements of O_2 consumption and CO_2 production were conducted only for ~20 min after surfacing. While this may be the case, when comparing our study to the behaviour of wild animals, it is important to consider that Steller sea lions rarely have surface times greater than 8 min between dives (Merrick *et al*., 1994). While we may not have measured the animals' full metabolic recovery we still determined the patterns of O_2 and CO_2 recovery during a biologically relevant duration.

With early accumulation of lactate, and no breakpoints in lactate accumulation or recovery times for O_2 and CO_2, there is clear evidence that Steller sea lions are using both aerobic and

anaerobic metabolism in tandem for at least portions of their dives. While this evidence is helpful in supporting the mixed metabolism model, it is not a new hypothesis in the field of diving physiology. The capacity for both Weddell seals and emperor penguins to dive beyond their ADL is well documented. As pointed out by Butler (2006), they are therefore clearly capable of supplying O_2 to aerobic dependent organs during these times, even as they are accumulating lactate. In other words, both aerobic and anaerobic metabolisms must be working in tandem during the dive, and that a greater reliance on the latter likely occurs as the dive progresses. In addition, we now know that lactate production is not confined solely to a condition where O_2 stores are exhausted. Even while in the presence of O_2, vertebrate muscle receives some of its energy from anaerobic metabolism; indeed, it can even occur when the animal is not exercising at all (Kemper *et al*., 2001; Richardson *et al*., 1998; Stanley *et al*., 1986). While lactate has historically been treated solely as a waste product of glycolysis, we now know that it serves a much more nuanced role in the metabolic process. Lactate produced by anaerobic metabolism in one tissue can act as an important energy source in cellular respiration in another tissue (Wyss *et al*., 2011) and can even act as a signaling molecule (Philp *et al*., 2005; Sola-Penna, 2008). For example, the brain and heart prefer lactate to glucose as an aerobic fuel (Baltan, 2016; Riske *et al*., 2017; Smith *et al*., 2003). Likewise, some lactate produced glycolytically in white skeletal muscles is thought to diffuse into local red muscle fibres for oxidation in mixed muscle types (Brooks, 1985). The concept of a metabolic transition has been considered in the development of optimal foraging models as well. Carbone and Houston (1996), Mori (2002), and Boyd (1997) have all presented marine mammal foraging models that include a mixed metabolism hypothesis where both aerobic and anaerobic metabolism are contributing to energy production during a dive.

Yet, the traditional concept of the ADL as a definitive switch persists, partly due to confusion in what it is supposed to define. At its conception, the ADL was intended to refer solely to the dive duration at which post-dive blood lactate concentrations rise above resting levels. In this definition, the ADL concept is not specific to either the two-phase or mixed metabolism models. However, the ADL is often misconstrued to be equivalent to the dive duration at which an animal has used all of its oxygen stores, leading to the prevalent belief that the cADL was equivalent to the ADL (Costa *et al*., 2001; Davis, 2014; Davis & Kanatous, 1999; Lydersen *et al*., 1992). The continued use of the terms aerobic and anaerobic dives, or the construct that dive duration is determined solely by their O_2 stores and the rate at which they are depleted leads to the

idea that a dive is powered by only one form of metabolism at a time. Treating the ADL as a definitive switch between metabolic states fails to acknowledge that anaerobic metabolism can, and is likely occurring in the early stages of diving, and possibly even when the animal is at rest. The evidence provided in this study lends support to the idea that a mixed metabolism model should be more widely considered both in the field of diving physiology, and suggests that we reconsider the ways we refer to the ADL and cADL.

The patterns of lactate emergence and gas recovery in Steller sea lions documented in my study shed light on how marine mammals transition between metabolic pathways during a dive. Instead of a distinct and substantial increase in blood lactate at a given dive time, lactate in our study accumulated at a consistent linear rate with increased dive duration. Recovery times for O_2 and CO_2 followed similar trends, increasing linearly with no distinct breakpoint, even around the cADL as calculated from this or previous studies. This implies that the transition between metabolic states is not a distinct switch from aerobic to anaerobic metabolism as is expected under the traditional model of diving physiology. Instead, the data support a mixed metabolism model with a slower transition between metabolic pathways where anaerobic metabolism is occurring at least to some degree much earlier in the dive. Further research into the proportion of aerobic to anaerobic metabolism that is occurring during dives will further elucidate how these animals are utilizing energy at depth. In addition, studies exploring how lactate is eliminated both at depth and during recovery will help to determine how much of a metabolic burden anaerobic metabolism places on diving marine mammals. Using these results, researchers attempting to create optimal foraging models will be better able replicate how marine mammals utilize energy at depth during an important transitionary stage between metabolic pathways.

Chapter 4: Conclusions

This thesis explored the physiological constraints that shape diving behaviour in Steller sea lions, both those important at depth and those which come into play at the surface. More specifically, I quantified the post dive rates of O_2 and CO_2 recovery, and determined how these change with varying dive duration. In addition, I explored how marine mammals transition between metabolic pathways during a dive by measuring how lactate accumulates with increased dive time, and how this accumulation affected post-dive recovery times. I also estimated the calculated aerobic dive limit (cADL) by measuring oxygen stores and diving metabolic rate, to determine how this measure corresponded to measured lactate levels. With a greater understanding of the physiological limits of marine mammals, we can better ascertain how they might behave in the wild and, more importantly, be better able to predict how they might respond to environmental change.

4.1 CO_2 elimination as a limit to recovery rates

The vast majority of research on the physiological limits to diving has focused on the extent and use of O_2 stores at depth; comparatively little has examined the physiology of recovering at the surface. What little research there is almost exclusively focuses on O_2 exchange (i.e., post-dive replenishment at the surface), despite the well-known importance of CO_2 in respiratory control in terrestrial mammals. My goal was to determine the rates of O_2 and CO_2 recovery after varying dive durations to determine how the time to reach a stable rate of gas exchange is affected by dive time. I measured the rates of recovery for both O_2 and CO_2 in freely diving Steller sea lions using flow-through respirometry. As predicted, I demonstrated that post-dive rates of O_2 uptake generally returned to stable levels before similar stabilization in rates of CO_2 release. This indicates that O_2 recovers more quickly than CO_2 after diving. More importantly, I determined how the time for Steller sea lions to reach gas homeostasis changes with dive duration, an important finding that can be used in foraging models to define optimal strategies for obtaining food. These, in turn, can be used to assess the effect of changing dive durations on foraging efficiency of wild animals, an important consideration for understanding potential effects of changing environmental conditions on Steller sea lion populations. By observing dive times and surface intervals of wild populations, we can determine how close they are to their physiological limits, providing key insights into how they are utilizing their environment.

The rate of O_2 replenishment at the surface was, on average, twice as fast as the rate of consumption at depth; for every extra minute of dive time, O_2 recovery time increased by only 31 s. However, there are theoretical reasons to expect that recovery would be proportionally faster for longer dives due to either a decrease in rate of oxygen use and/or an increase in the rate of O_2 exchange. With increased oxygen consumption during a longer dive, a greater partial pressure difference would develop between the blood and atmosphere. This would theoretically increase the rate of diffusion early in the recovery (re-oxygenation) process, thereby decreasing the O_2 recovery times (relative to total dive duration) for long dives. However, in our study recovery time increased linearly with ΣVO_2, as opposed to curvilinearly, suggesting a negligible effect of initial partial pressure differences on time for final O_2 recovery. This lack of change in recovery rate with dive duration was also observed in spite of the observed decrease in rate of oxygen depletion (diving metabolic rate) with increasing dive duration, a hallmark of the mammalian dive response. It is possible that the observation that significant changes in DMR only occurred in the longer dives may have obscured the overall effect on the relationship between dive duration and recovery time.

The rate of CO_2 elimination was slower in comparison to O_2 replenishment—for every extra minute of dive time, CO_2 recovery time increased by 44 s. The difference between O_2 and CO_2 recovery for a given dive increased even more for dives where the sea lions had to work harder to overcome additional drag effects. This slower recovery is either caused by a slower rate of exchange, or due to an increase rate of CO_2 production as dive time increases, both of which are theoretically possible. Based on general mammalian physiology, it would be expected that the rate of exchange for this gas is slower than O_2 as much of the CO_2 produced during exercise is stored via the bicarbonate buffering system. While O_2 exchange is limited solely by the rate of diffusion, CO_2 exchange is additionally limited by the rate of transport into the RBC and conversion from bicarbonate. In this study, the instantaneous ratio of CO_2 release to O_2 uptake (RER_I), increased over the initial moments of recovery, indicating a slower release of CO_2 from the body in comparison to O_2 uptake. There is also evidence that longer dives have an increased rate of CO_2 production. Large amounts CO_2 accumulated during longer dives would decrease blood pH which in turn is buffered by bicarbonate. With this decrease in blood bicarbonate, PCO_2 would stoichiometrically increase, resulting in an even greater CO_2 blow off once the animal returns to the surface. This study showed that the ratio of all CO_2 produced to all O_2 consumed during

recovery (RER_T), increased with dive time, supporting the idea that longer dives would result in an increase in rate of CO_2 production with dive time. While both a slower rate of exchange and an increase in CO_2 production can explain the slower recovery rates for CO_2 vs O_2, this study is unable to determine which had a greater effect. Further research is required to determine how CO_2 recovery is affected by these physiological processes.

While this is not the first study to suggest that CO_2 is more important as a limitation to dive behaviour, very few researchers have directly examined the rate of recovery for this gas (Boutilier *et al*., 2001). Previous reports show that CO_2 takes comparatively longer to recover than O_2 in Steller sea lions, though they did not report the rate at which these occurred (Gerlinsky *et al*., 2014). CO_2 is well known to stimulate respiratory drive in terrestrial mammals (Phillipson *et al*., 1981), and hypercapnia is known to have a similar, though comparatively blunted, effect in marine mammals (Gallivan, 1980; Gerlinsky *et al*., 2014). This thesis, along with the above studies, clearly suggest that more focus should be placed on the study of CO_2 accumulation and removal as possible limitations to dive behaviour both at depth and at the surface.

The underlying assumption is that the time required to regain stable rates of O_2 consumption and CO_2 release limits when an animal is physiologically capable of commencing subsequent dives. However, this is not to say that sea lions in the wild must "fully recover" to undertake additional dives. It bears repeating that this study only consisted of individual dives, something that is rarely seen in wild marine mammals. Dive bouts where an individual completes several dives in succession, are far more common. In these instances, the animal is likely not reaching full recovery during these short surface intervals. In fact, by minimizing these surface intervals marine mammals would maximize partial pressure differences of subsequent recovery periods. This would allow for faster O_2 uptake during this truncated recovery period, thereby increasing the efficiency of a foraging event by spending proportionally more time at depth. However, it remains unclear how CO_2 recovery would be affected by this strategy. If CO_2 elimination is constrained by rate-limiting chemical processes, marine mammals may be unable to efficiently rid themselves of CO_2 if using a strategy of short surface periods. This would result in increasingly large amounts of CO_2 accumulation with repeated dives. Further research into CO_2 accumulation and recovery during dive bouts with varying surface intervals may provide key insights into how marine mammals should optimally forage in the wild.

Further, it's possible that the point at which the animals reached stable O_2 uptake and CO_2 release (point of recovery) may not actually be a true state of homeostasis. Excess post-exercise oxygen consumption (EPOC) can continue far past this point, with the animal not entering a true state of homeostasis until hours after exercise (Wood, 1991; Zhang *et al*., 2018). While I argued that the recovery times measured in this study still act as ecologically relevant measurements, the EPOC seen in other animals still raises interesting questions in the field of diving physiology. If present in marine mammals, this extended duration of increased O_2 consumption would clearly have an effect on a diving animal's ability to remain submerged. We know that DMR decreases with increased dive duration, which could possibly counteract or delay this increase in O_2 consumption, but this remains to be seen. Conversely, dive bouts with incomplete recovery have the potential to exacerbate this extended period of O_2 consumption. Long-term post-dive recovery of marine mammal physiology remains largely unstudied, and could perhaps provide valuable insights into how these animals balance the trade-offs of diving at depth and recovery at the surface.

4.2 Transition between aerobic and anaerobic metabolism

The aerobic dive limit (ADL) was conceived as an indication of the transition point from aerobic to anaerobic metabolism. Defined as the dive duration at which post-dive blood lactate rises above pre-dive levels, surpassing the ADL is considered to impose considerable physiological constraints to a marine mammal (Kooyman *et al*., 1980). Lactate, the end product of anaerobic metabolism, is traditionally thought to hinder foraging efficiency by inducing a metabolic burden on a mammal by increasing surface recovery times to process the metabolite, a process that requires excess O_2. This transition between metabolic pathways has therefore been of great interest to diving physiologists due to the potential restrictions it places on marine mammal behaviour. However, relatively few studies have explored how this metabolic transition occurs during a dive. The traditional model assumes that a diving animal makes a distinct switch from using solely aerobic metabolism to power a dive to using solely anaerobic metabolism. This paradigm is reflected in the use of the calculated aerobic dive limit (cADL), obtained by dividing an animal's total body O_2 stores by their diving metabolic rate, and routinely used as an estimate of the ADL. By definition, surpassing the cADL would mean an animal has completely exhausted their body oxygen stores and would be diving completely anaerobically. However, the mixed metabolism model proposes that a dive may be powered by both aerobic and anaerobic metabolism in tandem

during a dive, the proportions of which may change as dive time progresses. My goal was to determine how and when Steller sea lions transition between metabolic pathways during a dive by measuring post-dive blood lactate, and to examine how this transition affects surface recovery time as measured by O_2 and CO_2 gas exchange.

I determined that lactate is present even after short dives, and that it accumulates linearly with increased dive time. This suggests that anaerobic metabolism is occurring early in the dive in tandem with aerobic metabolism. This mixed metabolism model is more combatable with our current understanding of other aspects of diving physiology. We know in many terrestrial animals that anaerobic metabolism can occur in the presence of O_2, and can even occur at rest (Kemper *et al*., 2001; Richardson *et al*., 1998; Stanley *et al*., 1986). In addition, the marine mammal dive response, characterized by peripheral vasoconstriction would result in certain areas of a diving animal's body becoming hypoxic earlier than others. To continue powering a dive, a marine mammal would have to rely on anaerobic metabolism in these O_2 deficient areas as they maintain blood flow – and oxygen supplies - to vital areas.

The linear accumulation of lactate with increased dive time seen in this thesis is contrary to our expectations. All other marine mammals with measured lactate accumulation curves display a clear breakpoint—lactate levels remain low initially only to rapidly increase at a given dive duration (their ADL). Such data seemingly support the traditional metabolism model, with the first portion of the dive representing solely aerobic metabolism and the latter a reliance on anaerobic metabolism. Under the mixed metabolism model, the ADL simply represents the point where the rate of lactate production exceeds the rate of its removal. Within this model paradigm, the reason we do not see the same trend in lactate accumulation in Steller sea lions compared to other marine mammals may be due to a higher level of glycolytic activity early on during dives or due to differences in their lactate clearing capacity. As an otariid with a higher proportion of fast-twitch muscle fibers in comparison to phocid seals, Steller sea lions may be designed to utilize anaerobic metabolism during short dives (Kanatous *et al*., 1999). The lactate curves demonstrated in past studies tend to be from phocid species that are exemplary divers, and may therefore have unusually high lactate catabolism abilities that keep initial lactate levels low during dives. However, this is largely speculative; relatively little is known about the differences in anaerobic potential within different marine mammals. There is similarly little known about how lactate clearing may differ

between divers. A cross-species comparison of lactate mechanics is required to determine why Steller sea lions exhibit such a different trend in lactate accumulation from previously studied species.

Both O_2 and CO_2 recovery times increased linearly with no apparent breakpoint, contrary to the predictions under the traditional model of a rapid metabolic transition during dives. In the past, researchers often referred to the consequences of surpassing the ADL in terms of greater post-dive rates of oxygen consumption, and the associated increase in surface recovery times. The premise was that lactate accumulated during the anaerobic portion of a dive was cleared upon surfacing using gluconeogenesis, a process that uses O_2 and therefore places an additional metabolic burden on the animal. However, if marine mammals are capable of oxidizing lactate without using O_2, we would not expect to see this increase in recovery time associated with lactate accumulation. The lack of breakpoint in O_2 and CO_2 recovery times in this study may indicate that significant amounts of lactate are being removed via oxidation. This is supported by studies of lactate clearance in terrestrial mammals. Dogs remove half of the lactate produced at rest via oxidation, increasing to 75% during exercise (Depocas *et al*., 1969). In humans, only 25% of lactate is used in gluconeogenesis during exercise, with the remaining 75% being oxidized (Brooks, 1985). Unfortunately, only one study has examined how lactate is recycled in marine mammals. In this study it appears that harbor seals rely more heavily on gluconeogenesis than terrestrial mammals oxidizing 27% of lactate accumulated during rest, a proportion that increases only to 31% when the animal is exercising (Davis *et al*., 1991). However, it should be noted that the animals in this study were only swimming at the surface as opposed to diving; seals remained submerged for only an average of 50 sec, only to return to the surface for 20 sec over the course of a 30 min exercise trial. Lactate accumulation likely differs substantially when an animal is completing extended duration dives, and this may in turn affect how the metabolite is removed. Further studies on what pathways of lactate removal are primarily utilized by marine mammals may provide insights into how lactate accumulation will affect dive behaviour.

As previously noted, lactate accumulation has historically been touted as something marine mammals should avoid. Surpassing the ADL was associated with increased surface times due to the increased O_2 debt from metabolizing accumulated lactate (Horning, 2012; Iverson *et al*., 2010; Kooyman & Ponganis, 1998). In fact, the concept of an O_2 debt caused by lactate metabolism is a

somewhat antiquated view of O_2 recovery. While historically researchers have used post-exercise O_2 consumption during recovery as a means to measure the extent of anaerobic energy use, more recent experiments have disassociated these two phenomena. Both direct and indirect calorimeter experiments have definitively shown that when lactate is oxidized back to pyruvate for use in mitochondrial respiration, no heat consumption takes place, and therefore no O_2 is used (Scott & Kemp, 2005). In humans, any excess post-exercise O_2 consumption is now thought to be attributed to other factors such as ATP or creatine resynthesis, or temperature regulation in response to changes induced by exercise (Bahr & Sejersted, 1991; Gaesser & Brooks, 1984; Scott & Kemp, 2005). We would therefore not see any increase in O_2 recovery time related to how much anaerobic metabolism is occurring. In our study, there was no discernable increase in post-dive rates of oxygen consumption despite measurable lactate accumulation. A more thorough and accurate understanding of lactate metabolism and exercise physiology will help future studies more accurately predict how diving mammals will behave under the extreme conditions of long dives.

Another commonly reported consequence of surpassing the ADL is the assumed negative effect of accumulated lactate, which is associated with acidosis and fatigue in the swimming muscles (Blix, 2018; Muth *et al*., 2005). Yet marine mammals are known to dive without clearing accumulated lactate (Castellini *et al*., 1988; Le Boeuf *et al*., 1988). If lactate was hindering muscle action, would we not expect marine mammals to remain at the surface to fully clear this metabolite? Current studies show that, while lactate accumulation is correlated with muscle fatigue, there is little to no evidence of any causal effect. As reviewed by Robergs *et al*. (2004), there is more support for lactate slowing muscle acidosis than causing it, and while there is currently debate as to the cause of muscle fatigue, acidosis is considered an unlikely candidate. In fact, instead of contributing to muscle fatigue, there is some evidence that lactate may actually hinder its onset by acting as an alternative source of fuel (Davis *et al*., 1991; Gladden, 2011; Wyss *et al*., 2011). If lactate does not, in fact, contribute to acidosis or muscle fatigue in diving mammals, the emphasis on the importance of clearing lactate after anaerobic dives may be overstated. Further research should be done on the accumulation of other metabolites associated with muscle fatigue, and how their balance is restored during recovery.

4.3 Future role of the ADL and cADL

The results of this thesis support a reconsideration of several traditional pillars of diving physiology. First, it provides evidence to support a more gradual transition between aerobic and anaerobic metabolism with increased dive duration, rather than a rapid switch between the two physiological states. This was seen in both the gas recovery curves of the sea lions, as well as the rapid appearance and constant increase in blood lactate demonstrated in the sea lions lactate concentrations. Second, the concept that lactate concentrations may not directly hinder either muscular activity at depth or increase post-dive rates of oxygen consumption (as also demonstrated by the sea lions) suggests that accumulation of lactate may not serve as a physiological constraint. These findings then raise the larger question of what role the ADL and cADL serve in furthering our understanding of diving physiology. Historically, these two measurements have been used as an estimate of diving capability—animals with longer ADLs or cADLs were considered "better" divers. However, things become less clear when we consider a mixed metabolism model of diving physiology. The ADL – an actual measurement of circulating lactate levels - can still provide valuable insight into the relative contribution of the two metabolic pathways. Technically, the ADL measures when lactate begins to accumulate faster than it is recycled, and can therefore be affected by either of these rates. Yet defining the point where this occurs can still provide valuable information into underlying physiological processes.

In contrast, the cADL may not prove to be as useful. The cADL is intended to act as an estimate of how long an animal can remain submerged if it were to use the entirety of its O_2 stores. By definition, the cADL assumes that a diving animal uses solely aerobic metabolism before making a distinct switch to anaerobic metabolism. This thesis provides evidence that at least one marine mammal likely utilizes both aerobic and anaerobic metabolism concurrently to power their dives—all of the animals in this study dove well in excess of any cADL calculated for Steller sea lions in this or previous studies (Gerlinsky *et al*., 2014) with seemingly little metabolic consequence. If a diving animal is capable of using these two forms of metabolism in tandem, obviously the cADL will not be a reliable estimate of the ADL. The question remains whether the cADL can still serve as a useful comparative measurement of diving ability. While it might be argued that animals with greater oxygen stores (relative to rates of consumption) should still have a greater capacity for longer dives, true diving abilities must also take into consideration the anaerobic potential of a diving mammal.

4.4 Strengths and limitations

Our ability to explore the precise questions of diving physiology in this thesis is largely due to the unique experimental set up of the Open Water Research Laboratory. Using trained animals in an open water environment provides clear advantages when compared to both field and aquarium-based studies. In the field, researchers can be confident that their observations reflect the true behaviour of the study species. However, they are usually limited to observing animal behaviour and are generally incapable of manipulating factors that would allow one to test the limits of animal physiology. In aquarium-based studies, these factors are much easier to control; however, when limited by space, it is difficult to simulate dives like those an animal would experience in the wild. By working with trained animals in the open ocean, this study allowed us to explore diving behaviour in a much more natural environment than an aquarium, all while using more precise and in-depth techniques that field researchers are otherwise incapable of using.

While this experimental set up provides clear benefits, it does impose some limitations on our ability to study diving physiology. Like any study that utilizes animals under human care, it could be argued that the required training has the potential to affect natural behaviour and physiology. In the wild, Steller sea lions must dive to hunt and find food, and therefore are completing dives more frequently than the animals in this study. It could be argued that the trained animals would therefore not be as fit to diving as their wild counterparts. However, it should be noted that the animals used in this study dive regularly for research purposes, receiving a sizeable portion of their nourishment through these types of studies. While it is probable that there are differences between the animals used in this study and a wild population, they are still closer in activity to wild animals than typical individuals reared in an aquarium. We can assume that this study therefore does give us a reasonable estimate of the diving physiology of individuals from wild populations.

This experimental set up also provides challenges in regards to variation between dives. In an aquarium setting, researchers are able to constantly observe an individual during a trial. This would allow a researcher to not only observe, but potentially manipulate a greater number of factors, such as dive duration, distance or speed of travel underwater. By controlling these variations in dive behaviour, researchers can more soundly estimate how certain dive behaviours are affected by physiology. The open water trials used in this study did not allow us to control for

such variation between dives. This means that while two dives may have had similar dive durations, they are not guaranteed to have had similar energy outputs. In one dive the animal could have been working hard, and traveling longer distances, while in the other they put in relatively little effort. However, in the same vein, our lack of control over effort during individual dives facilitated separate analyses of the effects of dive duration and oxygen consumption.

Another limitation of this study is related to its small sample size, a common problem of many studies of marine mammalogy. The difficulty of rearing, training, and working with large animals under human care limits the number of animals we can test under these conditions. Only being able to work with four animals raises statistical concerns. However, by utilizing the repeated measures technique of the applied linear mixed effects model we were able to take inter-animal variation into account, minimizing this concern.

Due to animal training restraints, we were unable to consistently collect serial blood samples to ensure we were collecting blood at peak lactate levels. Lactate emergence is delayed at the onset of surfacing from a dive as lactate is constricted to peripheral tissues with minimal blood perfusion. Only being able to collect a single blood sample post-dive, it is possible that we missed peak post-dive lactate, potentially skewing the results. In an effort to determine when post-dive blood lactate reaches its peak, I conducted preliminary trials of sequential blood samples. This provided an estimated optimal window of between 3-6 min post-dive, to which we restricted our blood sampling, minimizing the risk of missing peak blood lactate.

Finally, it should be noted that, while we were able to get post-dive blood lactate measurements consistently after dives, we did not have pre-dive levels. Resting lactate measurements from marine mammals are sparse, and vary between species ranging from 0.5 – 2.9 mmol L^{-1} (Ponganis, *et al*., 1997; Shaeffer *et al*., 1997; Williams & Cruz, 1993). California sea lions (*Zalophus californianus*) are likely the closest marine mammal in behaviour and phylogeny to Steller sea lions with resting lactate measurements of >1 mmol L^{-1} (Ponganis *et al*., 2017). Even with this comparison, we cannot confidently say whether lactate levels rose above pre-dive levels as per the definition of the ADL. However, both the presence of lactate in early dives, and the linearly increasing trend lend credence to the hypothesis that anaerobic metabolism is occurring early during dives, and that there is a gradual transition to an increased reliance with dive time.

4.5 Applications, importance, and future research

Studying diving physiology not only gives us an understanding of how marine mammals are capable of remaining submerged for so long, but it helps us better understand how these animals use their environment in the wild. In determining how marine mammal diving is limited by their physiology, we can assess where, and for how long these animals can forage. This, in turn, can help us to predict how well individuals would be able to respond to environmental changes in their food distribution, something that is may be particularly important for population management and conservation.

My research quantified the recovery rates for both O_2 and CO_2 in Steller sea lions, with CO_2 reaching pre-dive levels at a proportionally slower rate than O_2 with increased dive time. These recovery rates can be used in the development of optimal foraging models. By comparing observations of wild Steller sea lions to these models, we are able evaluate how well these animals remain within their physiological limits while foraging. In addition, my research provides evidence that Steller sea lions utilize both aerobic and anaerobic metabolism in tandem during dives. This is an important finding that should be included in models of optimal foraging, but also opens up the door for a more realistic appreciation for how marine mammals utilize energy during dives. With declines of over 80% in the western population of Steller sea lions (Trites & Larkin, 1996), a nuanced understanding of their diving physiology is of particular importance. The Nutritional Stress Hypothesis predicts that environmental changes may have caused shift in prey abundance, quality, or type that has forced Steller sea lions to use suboptimal foraging strategies (Rosen, 2009; Trites & Donnelly, 2003). By comparing the behaviours of wild animals to optimal foraging models, we can determine whether these populations are diving beyond their means.

As CO_2 proves to be an important limit on surface recovery time, it is clear that further research should be done to understand the dynamics of CO_2 accumulation and removal during dives in marine mammals. Relatively little is known about CO_2 dynamics during dives in marine mammals. While this thesis provides an important step by determining CO_2 recovery rates from single dives, further research should investigate how this recovery rate changes with different factors. Dive bouts may not only increase recovery times but also change CO_2 exchange dynamics, thereby changing the rate of recovery. Further measures of CO_2 buffering and storage capacity

may be used to determine marine mammals' tolerance to CO_2 accumulation, and how this is related to diving ability.

With evidence that at least one marine mammal follows a mixed metabolism model during dives, more research should be undertaken to examine the anaerobic potential of diving mammals. It is likely that anaerobic potential may differ among marine mammal species, which may in turn be linked to different diving strategies. It is possible that otariids, with a greater proportion of fast-twitch muscle fibers, may be more suited to fast burst, anaerobic dives than other marine mammals (Kanatous *et al*., 1999). However, there are currently no studies that comprehensively compare anaerobic potential of different marine mammals. Cross-species comparisons of anaerobic capacity, both in lactate recycling and glycolytic potential through LDH activity may provide valuable insights into different diving ability and strategies.

Further research should also be conducted into the dynamics of lactate accumulation and circulation within marine mammals. Historically, this has proven to be difficult as the only way to get real time measurements of blood lactate is to surgically catheterize a marine mammal and collect blood during a dive. However, recent advancements in technology open the door to other possibilities of observation. Near-infrared spectroscopy (NIRS) has been well proven as a technology in the field of human physiology, being used to measure muscle oxygenation, haemodynamics, and even lactate accumulation (Chakravarti *et al*., 2009; Gladden, 2011; Mancini *et al*., 1994; Pollard *et al*., 1996; Williams *et al*., 2011) With similar studies in marine mammals we could determine not only how lactate accumulates in the muscles during a dive, but how this correlates to muscle oxygenation and blood flow. NIRS has the potential to shine light on many questions in the field of diving physiology, providing valuable insight into how marine mammals may utilize different forms of metabolism during a dive.

With our current understanding of exercise physiology, we know that while lactate is correlated with muscle fatigue, it is likely not the cause. This leads to questions of how muscle fatigue may actually be occurring in marine mammals, and whether they possess any physiological systems to limit its onset. It is now widely accepted that muscle fatigue has many causes such as changes in Ca^{2+} dynamics in muscle cells, increase in intracellular inorganic phosphate and ADP, or even increases in reactive oxygen species (for review, see Allen *et al*. 2008). Further study into

these processes and metabolite accumulations may be useful in determining how and when marine mammals experience fatigue at depth, and how they delay its onset.

All of these potential lines of inquiry will help us to better understand the nuances of diving physiology both for Steller sea lions, and marine mammals as a whole. Understanding how these animals "work" under natural conditions not only provides insight into what shapes diving behaviour, but also how these behaviours may be affected by the environment. In a changing world, a greater appreciation for the links between physiology and behaviour can be key to helping us develop effective strategies for conservation and population management.

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Appendix

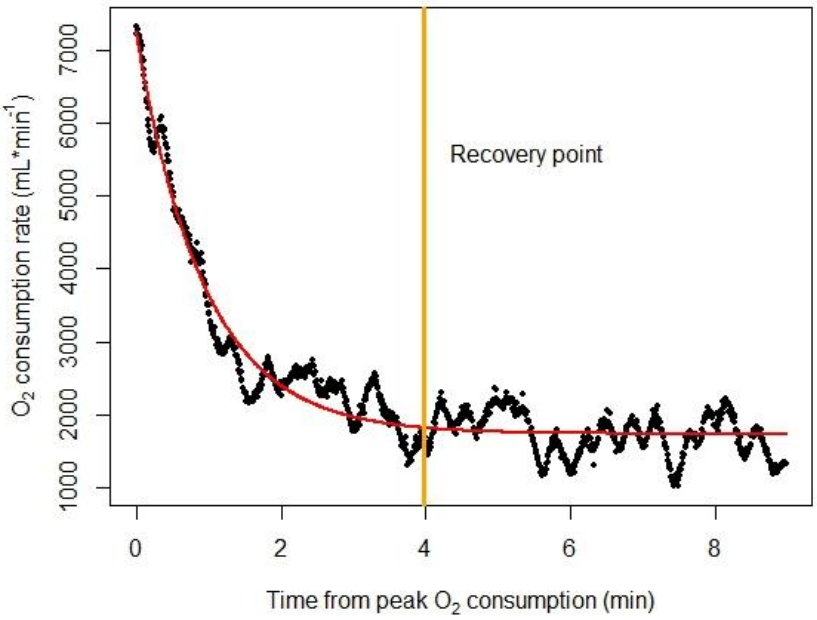

Figure A.1. An example trace of an individual Steller sea lion's (F97SI) rate of O_2 uptake during recovery from a single dive. A least-squares regression was used to calculate when the animal the animal had reached a constant rate of O_2 uptake as indicated by the red line. The animal was considered recovered when they had reached within 5% of this constant rate.

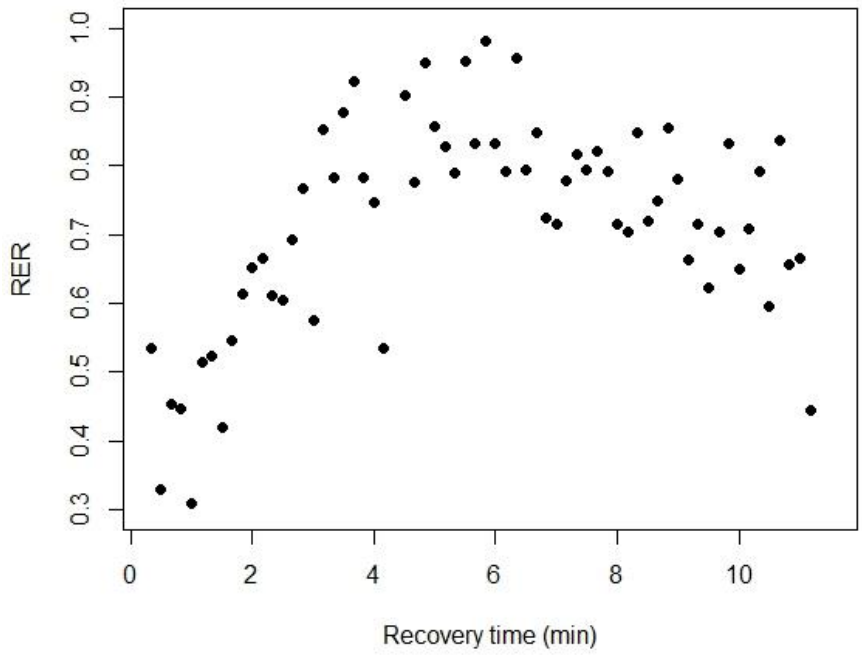

Figure A.2. An example trace of instantaneous RER_I calculated in 10 second intervals over the post-dive recovery period for an individual Steller sea lion (F97SI). RER_I generally increased during the initial portion of the recovery period, only to decrease slightly with increased time at the surface.

Table A.1. Model parameters for linear and piecewise regression models of lactate emergence, O_2 recovery times and CO_2 recovery times for four adult female Steller sea lions. Models with greater support are highlighted for each category. Degrees of freedom (df), Aikake Information Criterion (AIC), ΔAIC, Aikake weights (w_i), are included for each model with the calculated breakpoints ± SE and p-values from Davie's tests for piecewise regression models.

Animal		Model	Breakpoint ± SE	df	AIC	ΔAIC	w_i	p-value
F97SI	Lactate	Piecewise	3.222 ± 1.164	5	46.649	2.385	0.24	0.3367
		Linear	-	3	39.779	0	0.76	-
	O_2 recovery	Piecewise	4.532 ± 0.724	5	26.285	0.620	0.42	0.4775
		Linear	-	3	25.665	0	0.57	-
	CO_2 recovery	Piecewise	3.563 ± 1.471	5	51.835	2.904	0.11	0.6034
		Linear	-	3	48.930	0	0.48	-
F00HA	Lactate	Piecewise	4.886 ± 2.016	5	24.984	2.413	0.23	0.5537
		Linear	-	3	22.571	0	0.77	-
	O_2 recovery	Piecewise	3.769 ± 0.446	5	26.632	0	0.84	0.1769
		Linear	-	3	29.897	3.265	0.16	-
	CO_2 recovery	Piecewise	3.658 ± 0.643	5	41.398	0	0.29	0.3259
		Linear	-	3	43.164	1.766	0.70	-
F97BO	Lactate	Piecewise	4.815 ± 0.419	5	7.281	0	0.56	0.9221
		Linear	-	3	7.803	0.522	0.43	-
	O_2 recovery	Piecewise	5.672 ± 0.554	5	14.610	0	0.99	0.4429
		Linear	-	3	23.580	8.970	0.01	-
	CO_2 recovery	Piecewise	6.224 ± 0.068	5	42.671	0	0.64	0.9178
		Linear	-	3	43.985	1.315	0.33	-
F00YA	O_2 recovery	Piecewise	5.784 ± 0.730	5	14.610	0	0.99	0.7661
		Linear	-	3	23.580	8.970	0.01	-
	CO_2 recovery	Piecewise	2.288 ± 0.192	5	40.986	2.373	0.23	0.2696
		Linear	-	3	38.613	0	0.76	-