ABSTRACT

Diving Physiology in Marine Mammals: Significant Findings
in Pinniped Muscle Physiology and Trachea Morphology

Colby D. Moore, Ph.D.

Mentor: Stephen J. Trumble, Ph.D.

The muscular biochemistry and respiratory morphology of diving mammals are closely intertwined through the utilization and allocation of inspired oxygen for metabolism. Marine mammal physiological mechanisms and adaptations are of great intrigue due to the heightened environmental pressures that these animals are routinely subjected. These species also experience varying degrees of ischemia, hypoxemia and gas tissue saturation, which are pathological in terrestrial mammals. Data included in this dissertation suggest a unique skeletal muscle fiber type profile for the deep-diving Northern elephant seal; a profile predominately comprised of enlarged aerobic type I myofibers. In addition, enzymatic data suggest that diving mammals maintain higher levels of aerobic enzymes in primary locomotory muscle and that muscle-based enzymes degrade rapidly and variably with temperature and time. Histological analysis of harbor seal tracheal rings microscopically describes a unique continuity of cartilage that correlates with lung compression, depth at which lungs collapse, as well as maximum dive depth. Cumulatively, biochemical and structural adaptations allow diving mammals to reach extensive depth, while maintaining homeostatic levels of on-board gasses and avoiding dive-related injury. Ultimately,

this research highlights the relationship between morphology, physiology and life history of these animals.

Diving Physiology in Marine Mammals: Significant Findings in Pinniped Muscle Physiology and Trachea Morphology

by

Colby Moore, B.S., M.Phil.

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Robert D. Doyle, Ph.D., Chairperson

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Approved by the Dissertation Committee

_____

Stephen J. Trumble, Ph.D., Chairperson

_____

Robert D. Doyle, Ph.D.

_____

Kenneth T. Wilkins, Ph.D.

_____

Bessie W. Kebaara, Ph.D.

_____

Darryn S. Willoughby, Ph.D.

Accepted by the Graduate School

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J. Larry Lyon, Ph.D., Dean

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To the strong men in my life:

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My late grandfather, Francis D. Moore, your discoveries in science continue to hold me to a higher standard every day

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My brothers, I am so proud of you and what you stand for

To the strong women in my life:

My mothers and sisters.
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CHAPTER ONE

Introduction

The diving physiology of marine mammals was introduced by Scholander (1940) and remains fundamental to what is known, and how research is conducted, to this day. Scholander was primarily interested in two problems: how diving mammals withstand apnea, and how they avoid the detrimental accumulation of gas bubbles in the bloodstream, also known as decompression sickness (DCS). The solutions to these dilemmas are still elusive, sparking research into the complex interactions of anatomy and physiology that allow dives to great depths. This thesis continues Scholander's inquiry with detailed research describing mechanisms of mammalian diving physiology. Thus, the two themes of withstanding apnea and avoiding DCS will be further explored using current biochemical and histological methods.

Maintenance of Prolonged Apnea

Research conducted by Irving and Bert (1870) advanced Scholander's (1940) studies on prolonged breath-hold diving. The cumulative works of the three men can be further delineated into the following subjects: metabolic rate, utilization of oxygen stores, and sensitivity to carbon dioxide.

Regulation of Metabolic Rate

In 1940, Scholander recorded the heart rate of a seal during forced diving using electrocardiograms. Data showed that during descent there was an abrupt decrease in heart rate from 150 to 10 beats per minute, which is termed bradycardia. As a vertebrate

response to breath-holding, bradycardia is a conserved mechanism among many species. This "profound" response of phocid seals is of particular interest to dive physiologists (Kooyman et al., 1981). Scholander (1940) also found a reduction in circulation and oxygen consumption during the dive, specifically to the flippers and musculature. The overall reduction in oxygen consumption, coupled with bradycardia, suggested a decreased metabolic rate during diving. However, a baseline diving metabolic rate proved difficult to measure in a restrained seal. Subsequent researchers have also had difficulty measuring diving metabolic rate, as results are impacted by foraging (Ponganis et al., 2011), prey introduction (Rosen, 2009; Trumble and Kanatous 2012) and hormonal variation (Weingartner et al., 2012). Ultimately, it is difficult to model the metabolic profile of a diving marine mammal.

Oxygen Storage

Aerobic Dive Limit

In Weddell seals (*Leptonychotes weddellii*), measurement of the aerobic dive limit (ADL), or maximum breath-hold dive maintained without an increase in blood lactate levels, clarified what had been previously obscure to researchers since Scholander's time: pinnipeds rely primarily on the aerobic pathway during a dive, exceeding their aerobic capacity on a mere 4% of dives (Kooyman et al., 1980). Therefore, researchers hypothesized that pinnipeds had no significant adaptation for anaerobic metabolism, but relied fully on flexible vascular blood redistribution, reduced function in some organs, and an overall reduction of energetic need to maintain an almost exclusively aerobic-based metabolism during breath-hold dives (Kooyman et al., 1981). Pinnipeds present a

good model for the study of low-level aerobic locomotion (Scholander, 1940; Kooyman et al., 1981; Davis and Kanatous, 1999). Specific ADL calculations have now been completed in many marine mammal species (Kooyman et al., 1983; DeLong and Stewart, 1991; Costa et al., 2001; Watwood et al. 2006). Species-specific data have been correlated with the Weddell seal model with scaling of oxygen stores and metabolic rate adjusted by mass (Kooyman 1989; Kleiber 1975; Watwood 2006) and the mass specific metabolic rate of the diving animal (Watwood et al., 2006). For example, a sperm whale ADL was calculated using the model formula, and determined to be between 43 and 54 min for this 8 to 20 ton animal, whereas the ADL was 21 min for a 450 kg Weddell seal (Kooyman 1989; Watwood 2006). Application of the ADL formula shows the theoretical trend of larger animals exhibiting a longer aerobic dive time due to increased oxygen stores (Castellini et al., 1992). The model allows ADL predictions of other diving mammals that are difficult to capture or hold in captivity. Comparison of calculated ADL with observed dive regiments aids in identification of species-specific adaptations for long-duration apneic diving.

Behavioral adaptations can contribute to a maximal ADL, specifically, gliding locomotion (Williams et al., 2000). Gliding is an energy-efficient movement that minimizes energy expenditure during apnea (Williams et al., 2000). Although, much of the diving mammal research has been focused on physiological mechanisms, such as metabolic suppression (Hochachka, 1992) and vasoconstriction (Elsner et al., 1966; Elsner et al., 1978; Kooyman et al., 1981, Zapol et al., 1979), activity-level alterations also have a significant effect on energy conservation and oxygen stores. The ability to

dive for prolonged durations is a result of behavioral, anatomical and biochemical adaptations.

Oxygen Storage in Blood

Oxygen stores, or "depots" as Scholander referred to them, were of particular interest, specifically the anatomical storage location, or sequestration of oxygen (Scholander, 1940). Greater oxygen storage capability was found in diving marine mammals compared to terrestrial counterparts, which was presumed to maximize dive duration (Scholander, 1940; Lenfant et al., 1970; Kooyman et al., 1980; Castellini and Castellini, 1993; Davis and Kanatous, 1999; Meir et al., 2009). Three general locations of oxygen storage were determined to be blood, muscle and lungs (Scholander, 1940). Irving (1939) determined the blood oxygen capacity of diving and terrestrial animals, including the duck, harbor seal and bottlenose dolphin (*Tursiops truncatus*). Results suggested that seals had an elevated capacity for oxygen storage in the blood. Elevated levels of hemoglobin and hematocrit have since been quantified in many pinniped species (Kooyman et al., 1980; Lenfant et al., 1970; Kanatous et al., 2002). For example, hematocrit levels were found to be elevated throughout the entire dive of Weddell seals (Castellini et al., 1988). A conceptual "scuba tank" or oxygen reservoir was also hypothesized, whereby seals could store large amounts of red blood cells in the spleen, and release oxygenated blood during hypoxia and apnea (Castellini et al., 1986; Hurford et al., 1996).

Oxygen Storage in Muscle

Oxygen storage in muscle tissue is primarily maintained through the oxygen binding protein, myoglobin. Most marine species, mammals and birds, have elevated levels of myoglobin in their primary swimming muscles (Scholander, 1940; Castellini and Somero, 1981; Kooyman, 1989; Noren and Williams, 2000; Kanatous et al., 2002, Moore et al., 2014). Studies show diving mammals and birds have 10 to 30 times greater myoglobin concentrations than their terrestrial or aerial-sized counterparts (Kooyman, 1989). Cetaceans have been shown to demonstrate an even greater reliance on oxygen-bound myoglobin than pinnipeds. For example, the bottlenose dolphin had 38% of its total body oxygen stored in myoglobin within skeletal muscle (Williams et al., 1993), whereas pinnipeds stored 33% of total body oxygen in skeletal muscle (Kooyman, 1989) and humans only 15% (Noren and Williams, 2000).

Although myoglobin plays a key role in the aerobic state of diving mammals, muscle fiber type and size is another significant factor in diving metabolism (Dearolf et al., 2000; Kanatous et al., 2002; Watson et al., 2003; Kielhorn et al., 2013; Velten et al., 2013; Williams and Noren, 2011; Trumble and Kanatous, 2012, Moore et al., 2014). An inverse relationship exists between the oxidative potential of a muscle fiber and its diameter, suggesting that rapid oxygen diffusion is associated with smaller fibers (Van Der Laarse et al., 1998; Van Wessel et al., 2010). However, contrary to this "rule", enlarged muscle fiber diameters have been found in beaked whales (*Mesoplodon* sp.), the short finned pilot whale (*Globicephala macrorhynchus*) (Velten et al., 2013), the pygmy sperm whale (*Kogia breviceps*) (Kielhorn et al., 2013), the Weddell seal (Kanatous et al. 2002) and the Northern elephant seal (*Mirounga angustirostris*; NES) (Moore et al.,

2014). These findings suggest that enlarged fiber size may be a common adaptation for diving mammals. Although larger fibers increase the diffusion distance for oxygen (Van Wessel et al., 2010; Kinsey et al., 2011), recent research (Kielhorn et al., 2013; Velten et al., 2013) suggested that the low surface area to volume ratio of enlarged fibers reduces the overall metabolic rate of the animal. Thus, enlarged fiber size serves as a beneficial adaptation for a deep and long-duration diver.

A metabolically homogeneous fiber profile is also an adaptation for dive maintenance (Kanatous et al., 2002; Trumble and Kanatous 2010; Moore et al., 2014). Typically, mammalian skeletal muscle profiles are heterogeneous, containing a diverse population of fiber sizes and metabolic capacities throughout the muscle bundles (Scott et al., 2001). However, the Weddell seal and NES show the predominance of type I oxidative muscle fibers throughout the longissimus dorsi (l. dorsi) muscle and with no heterogeneity (Kanatous et al., 2002; Trumble and Kanatous 2010; Moore et al., 2014). Oxidative fibers provide fatigue resistance (Van Wessel et al., 2010) and produce antioxidants (nitric oxide), which has been shown to protect against hypoxia (Yu et al., 2008). Overall, a predominately type I muscle fiber profile would be beneficial for a long-duration diver that is dependent on stored oxygen.

Although a higher volume density of mitochondria, and increased activity levels of citrate synthase and ß-hydroxyacyl CoA dehydrogenase, have been shown to play a role in the efficiency of oxygen utilization, these adaptations exist exclusively in muscles of short-duration divers, such as the harbor seal, Northern fur seal (*Callorhinus ursinus*) and Stellar sea lion (*Eumetopias jubatus*) (Davis and Kanatous, 1999; Kanatous et al., 1999). In contrast, the Weddell seal and NES maintain low-level lipid-based metabolism

with low mitochondrial volume density (Kanatous et al., 2002; Moore et al., 2014). This is comparable to a sedentary animal of similar size and indicative of low-level metabolism (Kanatous et al., 2002). Furthermore, in Weddell seals, the quantity of mitochondria in myofibers did not differ from a sedentary animal, although distribution was distinctly different. Mitochondria in Weddell seals were in greatest density in the interfibrillar areas, rather than in the subsarcolemmal placement seen in sedentary animals, indicating that Weddell seals have a more efficient system of oxygen uptake (Kanatous et al., 2002). This was an important finding to explain species-specific biochemical adaptations of a long-duration diver (Weddell seal, NES) compared with a short-duration diver (harbor seal, dolphins). Thus, more than one muscular model for diving marine mammals exists (Moore et al. 2014).

Oxygen Storage in Lungs

Pinnipeds store relatively smaller amounts of oxygen in lungs (Lenfant et al., 1970) indicating a heavier reliance on blood and muscle-bound oxygen. Although this may suggest a diminished respiratory role in diving, pinnipeds have a slightly larger lung volume than humans (Kooyman, 1973). A large lung volume may contribute to buoyancy (Kooyman, 1973). However, buoyancy does not support deep diving, and marine mammals cannot rely on oxygen acquisition from the lungs during a dive (Kooyman, 1973). With common dive depths of 300 to 400 m for a Weddell seal, the greatest force acting on the lungs is pressure, which can be immense at these depths (Kooyman, 1973). These dive depths also increase the solubility of nitrogen (Kooyman, 1973), a major concern in decompression sickness. A few physiological mechanisms for avoiding nitrogen absorption have been proposed, such as lung collapse and vasoconstriction,

facilitating consolidation of nitrogen away from peripheral tissue (Scholander, 1940; Kooyman, 1973). Blubber has been hypothesized to be a potential area of nitrogen consolidation, as fat is 5 times more soluble for nitrogen than other tissues (Kooyman, 1973).

Lung collapse causes the cessation of gas exchange, and increases dead space in the lungs, relative to total lung capacity (Kooyman, 1973). During the progression of a dive, elevated pressures force respiratory air from the alveoli up into the bronchial tubes and trachea, which are non-gas absorbing tissues (Kooyman, 1973). A number of theoretical models have been developed to estimate the depth at which lungs collapse (Bostrom et al., 2008, Fahlman et al., 2009), and the potential role of the trachea in respiratory gas management (Moore et al., 2014). Contrary to early research (Scholander, 1940), recent data indicate species-specificity in lung collapse depth (Fahlman et al. 2009).

The trachea tissue properties, and its internal air volume, present important parameters for lung collapse and gas management during a dive (Moore et al., 2014). Recent theoretical models suggest that the compliance, or compressibility, of the trachea is imperative when calculating both lung collapse depth (Bostrom et al., 2008) and gas exchange under pressure (Fahlman et al., 2009). Although quantitative data exist for lower portions of the respiratory system, considerably less is known about the upper airway, or trachea, of marine mammals (Sokolov et al., 1968; Cozzi et al., 2005; Bagnoli et al., 2011, Moore et al., 2014). Modeling airway dynamics has revealed confounding results. Results indicated that deeper lung collapse depth may allow lower end nitrogen levels, as continued gas exchange is able to occur (Fahlman et al., 2009; Hooker et al.,

2012). Compliance measurements indicate that shallow diving pinnipeds (harbor seals) have more rigid, less compliant tracheas, perhaps limiting the depth of collapse and ultimately dive depth (Moore et al., 2014). Alternatively, the more compliant (less rigid) trachea found in deeper diving pinnipeds (NES) may be an anatomical mechanism for deeper dives (Moore et al., 2014).

Dive Duration

Limitations of an aerobic dive are the size of the oxygen stores and the rate at which that oxygen is sequestered (Noren and Williams, 2000). For cetaceans, dive limitations can be predicted by muscle myoglobin content and body mass (Noren and Williams, 2000). For example, four long-duration (25-73 min) diving odontocete species all had high myoglobin concentrations compared to short-duration (5 to 18 min) divers (Noren and Williams, 2000). Thus, there is a direct correlation between myoglobin concentration and dive duration in odontocetes (Noren and Williams, 2000).

Body mass also influences the diving ability of marine mammals (Noren and Williams, 2000). A larger body mass correlates with increased total muscle mass, decreased mass specific metabolic rate, and increased oxygen storage capacity (Noren and Williams, 2000). Similar findings have also been recorded in the Weddell seal and other pinniped species (Kooyman et al., 1983; Costa, 1991). This evidence shows that a larger body size is better suited to prolonged hypoxia.

Sensitivity to Carbon Dioxide

For an exercising terrestrial animal, the normal response to increased levels of circulating carbon dioxide, or hypercapnia, is ventilation (Kohin et al., 1999).

Some evidence suggests that diving animals are sensitive to circulating levels of carbon dioxide (CO_2), and that may define the end point of a dive (Irving 1939(b)). Given the duration of the breath-hold dive of marine mammals, research into the threshold and tolerance to circulating levels of carbon dioxide was prompted early on. Scholander (1940) and Irving (1939(b)) found that seals were either non-sensitive to circulating CO_2 or demonstrated a blunted response. However, recent research indicates that marine mammals are in fact sensitive to CO_2, but the level that would stimulate respiratory drive to breathe is at a higher threshold compared to terrestrial animals (Butler and Jones, 1982; Milsom et al., 1996).

Lactic acid accumulation, which is closely correlated with circulating levels of carbon dioxide, was found to be elevated during post dive recovery, and at higher concentrations following longer dives (Scholander, 1940). The appearance of lactic acid in the recovery phase, as opposed to during the dive, prompted research focused on vasoconstriction. This process allowed seals to nearly cease blood flow to peripheral musculature, thereby "controlling" distribution of oxygen during a dive (Scholander, 1940). Subsequent research utilizing labeled microspheres, demonstrated the almost complete cessation of blood flow to specific tissues, such as the kidney and peripheral skeletal muscle (Elsner et al., 1966; Elsner et al., 1978; Kooyman et al., 1981, Zapol et al., 1979). Therefore, vasoconstriction is a key adaptation in the ability to extend breath-holding (Kooyman et al., 1981)

Given the prevalence of lactic acid in post dive blood samples, researchers asked if pinnipeds possess a heightened capacity for anaerobic metabolism (Andersen, 1966; Kooyman et al., 1981; Castellini and Castellini, 2004). Three muscle-based adaptations

have been hypothesized to explain hypoxic tolerance in diving mammals: enhanced activity of glycolytic enzymes, enzymatic ability to quickly switch from high to low activity, and tolerance for high levels of lactate (Hochachka and Somero, 1973). However, the levels of lactate dehydrogenase (LDH) and pyruvate kinase (PK) were determined to not significantly differ when compared to terrestrial counterparts, and eventually the scientific record concurred that diving mammals do not possess an elevated capacity for anaerobic metabolism (Castellini et al., 1981) and the direction of research trended away from this line of inquiry (Castellini and Castellini, 2004).

Avoidance of Decompression Sickness

To understand the adaptations of marine mammals to extreme depth and pressure, an outline of the underwater environment is necessary. The most important component is pressure. Both atmospheric and hydrostatic pressure act on a body underwater (NOAA, 1991). Atmospheric pressure (atm) increases with oceanic depth, such that 1 atm is equal to 14.7 psi (100 atm equal 1470 psi). Similarly, hydrostatic pressure also increases with depth, but at a rate of .445 psi per foot of seawater (NOAA, 1991).

In 2001 the record breath-hold dive for humans was 150 m (Ferretti, 2001). Marine mammals considered to be shallow divers, such as the harbor seal (*Phoca vitulina)*, have a mean dive depth of approximately 12-40 m (Boness et al., 1994), whereas an extreme deep diving species (NES) can reach a mean depth of approximately 466 m (Kuhn et al., 2009). According to Boyle's law, a mere 10 atmospheres, or approximately 90 m, would compress an air filled space into $1/10^{th}$ its original size, indicating even a "shallow" dive invites large amounts of pressure and is a taxing physiological event (NOAA, 1991).

The physiological consequences of dive pressure to breath-holding animals pertain to the compression of air spaces in the body, such as sinuses and the respiratory tract. Boyle's gas law describes changes in gas volume as a result of pressure at a constant temperature, such as the inspired gases that become compressed at depth, and alternatively expand with ascent. The nitrogenous "bubble" that forms as a byproduct of the gas volume expansion during rapid ascent (and reduction in pressure) was originally proposed by the French physiologist, Paul Bert, in *Barometric Pressure* (1878), and later emphasized as a detrimental disease called decompression sickness (DCS) (Bove, 2004). Scholander (1940) drew the connection between Dalton's gas law (nitrogen saturation with pressure) and Boyle's law, establishing early on that many marine mammal species reach depths of susceptibility to DCS. The plaguing research question was thus fueled, "How do diving mammals avoid DCS?"

One suggestion for the apparent avoidance of DCS was lung collapse (Scholander, 1940; Kooyman, 1973). The compression of the respiratory system forces air from the alveoli into the rigid, cartilaginous bronchi and trachea terminating gas exchange (Scholander, 1940; Kooyman, 1973). Later research confirmed that this process reduces the alveolar surface area and increases the thickness of the alveolar membrane, thus reducing overall gas diffusion (Bostrom et al., 2008), decreasing accumulation of nitrogen and susceptibility to saturation and bubble formation (Scholander, 1940). Marine mammals have therefore become a model organism for the study of decompression sickness and lung mechanics.

Marine mammals have been found with DCS-like symptoms (gas emboli) as a result of sonar induced stranding (Jepson et al., 2003; Fernandez et al., 2005). The

composition of gas found in stranded marine mammals was similar to DCS-associated emboli in terrestrial species, and not bacterial decomposition (De Quirós et al., 2012). Although a confounding result for a routine diver, this suggests that marine mammals can suffer from DCS, and are not protected from the detrimental effects of the disease. Furthermore, research is now focusing on how many marine mammals chronically live with elevated levels of inert gas (nitrogen) in their tissues (Dennison et al., 2012).

Specific Aims and Hypotheses

Specific Aim One

To test the hypothesis that NES have elevated levels of muscular myoglobin, and a muscle fiber profile of predominately large type I muscle fibers.

H_{01}: Myoglobin values for NES will not differ from terrestrial counterparts.

H_{02}: The fiber type of the NES l. dorsi muscle will not differ from the homologous muscle in terrestrial and marine mammals.

H_{03}: The fiber diameter of the NES l. dorsi muscle will not differ from terrestrial counterparts or other marine mammals, when scaled to body size.

Rationale for Specific Aim One

Myoglobin (Mb) and muscle fiber type play a key role in the metabolic state of diving marine mammals (Dearolf et al., 2000; Kanatous et al., 2002; Watson et al., 2003; Kielhorn et al., 2013; Velten et al., 2013; Williams and Noren, 2011; Trumble and Kanatous, 2012). This author, in collaboration with other researchers (Moore et al., 2014), hypothesize that the deep-diving NES (Le Boeuf et al., 2000; Kuhn et al., 2009; Robinson et al., 2012), would demonstrate elevated levels of Mb in the l. dorsi muscle. Since NES are long-duration exercisers, we also hypothesized that their skeletal muscle

components would be homologous to the Weddell seal, another long-duration diver. Skeletal muscle of the Weddell seal is known to have elevated levels of Mb, large type I fibers, as well as previously undescribed mitochondrial placement and lipid-based fuel utilization (Kanatous et al., 2002). Similarly, we hypothesized that NES also maintain aerobic metabolism on long-duration dives via three predominate mechanisms: 1. A predominately type I muscle fiber profile; 2. Enlarged muscle fibers; 3. Elevated Mb concentration. Cumulatively, these factors would act to increase muscle oxygen stores, decrease muscular and whole body metabolic rate, and increase resistance to fatigue.

A potential medical application of this research asks if the predominance of type I muscle fibers and complete absence of type IIb fibers in adults, protects these animals from a deleterious human disease, termed ischemia-reperfusion (IR) injury. Onset of IR is triggered by the perfusion of blood to previously occluded tissue and the subsequent immune system response involving reactive oxygen species (ROS). Mammalian dives lasting brief (15-20 min) or long (30-50 min) periods prompt a 90% decrease in blood flow to skeletal muscle (Zapol et al., 1979), denoting the extent of vasoconstriction and resultant ischemia to tissues. In mice, ischemic events lasting 20 min cause edema in tissue (Carattino et al., 2000). It is of particular interest why marine mammals do not suffer from IR injury. One hypothesis involves ROS maintenance mechanisms in skeletal muscle, whereby the release of ROS as a result of the reintroduction of oxygenated blood causes cellular damage (McCord, 1985; Saugstad, 1996) and substantial injury (Li and Jackson, 2002). The frequent exposure to ischemia from repetitive dives may also have an aspect of "preconditioning" pinniped tissue, thereby providing some protection against tissue damage (Elsner et al., 1998). Seals also have elevated activity of glutathione

peroxidase (GPx) activity in muscle (Vázquez-Medina et al., 2006) and superoxide dismutase (SOD) in myocardium (Elsner et al., 1998), acting as a "scavenging" mechanism for removal of oxygen radicals. Although these data lend evidence for a protective biochemical mechanism, recent research suggests that an adaptation for elevated levels of antioxidant scavenging is not sufficient for the ultimate avoidance of IR injury. While IR-related tissue necrosis is not the result of ROS (Chan et al., 2003), it is specifically linked to the inflammatory response (Austen et al., 2004; Chan et al., 2004; Suber et al., 2007) and the antibody-antigen binding interactions of the muscle tissue itself (Chan et al., 2004). Free radicals may remain a key player in the IR-injury pathway, but their role is secondary to the amplification of the initial immune protein mediated pathway (Chan et al., 2004). If muscular injury from ischemia has little to do with ROS damage, diving mammals must avoid injury through an additional adaption. Therefore, we hypothesized that the absence of type IIb fibers in adult locomotory muscle may provide an answer to this question.

IR-injury is non-uniform in muscle tissue (Chan et al., 2004). The deposition of the IgM and complement proteins, such as those involved in the inflammatory response, are localized to type IIb muscle fibers, which are lacking in NES and Weddell seals (Kanatous et al., 2002, Trumble and Kanatous, 2012; Moore et al., 2014). Without type IIb fibers, pinnipeds also lack the unique expression of epitopes that bind immune proteins during IR injury. Thus, the absence of type IIb muscle fibers acts as an adaption to avoid IR-injury in vasoconstricted peripheral skeletal muscle.

Specific Aim Two

To test the hypothesis that enzymatic assays and protein quantification are of value when evaluating post mortem tissue, including the rate and acceleration of decomposition achieved under various temperature conditions. We aim to produce a graph of expected degradation for stranding (post mortem) sample collection. We will also test the hypothesis that marine mammals have elevated activity levels of aerobic enzymes, such as citrate synthase (CS), in primary locomotory muscle, which result in an increased capacity for aerobic metabolism.

H_{01}: Citrate synthase enzyme activity levels will not differ in individual locomotory muscle samples stored at varying temperatures and time periods.

H_{02}: Myoglobin concentration will not differ in individual locomotory muscle samples stored at varying temperatures and time periods.

H_{03}: Lactate dehydrogenase activity level will not differ in individual locomotory muscle samples stored at varying temperatures and time periods.

H_{04}: Freeze-thaw events will not have a significant impact on the degradation of muscle tissue.

H_{05}: Citrate synthase enzyme concentration will not vary among locomotory muscles collected at different anatomical regions.

Rationale for Specific Aim Two

Both enzyme assays and Mb protein quantification are common methodologies for marine mammal physiological studies demonstrating the aerobic and anaerobic capabilities of marine mammals (Lenfant et al., 1970, Castellini et al., 1981, Kooyman et al., 1981, Reed et al., 1994, Kanatous et al., 1999, Polasek et al., 2006, Kanatous et al., 2008). Over the last fifteen years, samples collected from live biopsies have been double the number of samples acquired from post mortem stranded or bycaught animals

(Kanatous et al., 2002; Burns et al., 2005; Noren et al., 2005; Richmond et al., 2006; Clark et al., 2007; Spence-Bailey et al., 2007; Kanatous et al., 2008; Ponganis et al., 2002; Hindle et al., 2009; Prewitt et al., 2010; Shero et al., 2012). However, obtaining biopsy samples for research are time consuming and expensive to collect, starting with the acquisition of federal permits for research or government approved subsistence hunts (Reed et al., 1994, Kanatous et al., 1999, Polasek et al., 2006, Kanatous et al., 2008). Stranded and bycaught animals, however, are relatively accessible. So, we ask if biochemical data derived from intramuscular post mortem tissue is viable for physiological research, specifically enzyme assays and protein quantifications. The few post mortem samples recently collected were in a 6 hour post mortem window and are complementary to prior physiological research (Watson et al., 2003; Polasek et al., 2006; Watson et al., 2007; Lestyk et al., 2009). Should physiologists be suspect of biochemical assays performed on tissues collected 6 hours post mortem?

Temperature and other environmental factors affect the rate of muscle proteolysis, (Morita et al. 1996), disease (Costelli et al., 2005), pH (Eijsink et al., 2005) and enzymatic activity (Geesink et al. 2006). Proteolysis should be considered when assessing muscle for protein and enzyme quantification. For example, assays performed on biopsy samples have shown the muscle proteins myosin and actin to degrade in as little as 15 minutes in biopsy samples (Lecker et al., 1999).

We have mapped a 48-hour timeline of enzymatic activity and proteolysis for l. dorsi muscle at varying temperatures (room, refrigerator and body temperature). We then hypothesized that tissue integrity is optimized when stored at 4°C. However, we also noted that enzymatic activity can vary greatly among muscles of the same individual, and

also among conspecifics, therefore caution should be exercised when interpreting results. To better understand how thawing episodes affect muscle tissue degradation, we also mapped activity and degradation of an enzyme throughout the course of four freeze-thaw cycles. Repeated thaws did not affect enzyme activity or condition, provided that post mortem tissue is maintained at ≤4°C. However, stable proteins, such as Mb, can quantitatively interpret aerobic proficiency from post mortem tissue acquired from less optimal conditions.

Specific Aim Three

To test the hypothesis that when viewed under routine histology, harbor seals have tracheal cartilage rings that exhibits an anatomical transition as the trachea progresses towards the lungs. Alternatively, NES tracheal rings do not change anatomically as they progress towards the lungs. We also test the hypothesis that elastic fiber integration affects the overall compliance of the trachea, and that the internal volume of the trachea can change when factoring in the inconsistencies, or gaps, in tracheal cartilage rings.

H_{01}: The tracheal rings of the harbor seal will not differ as the trachea approaches the lungs.

H_{02}: NES tracheal rings will not change anatomy as the trachea approaches the lungs.

H_{03}: Harbor seals and NES do not have significantly greater elastic fiber integration in tracheal rings than a terrestrial animal.

H_{04}: The internal tracheal volume for NES and harbor seals does not vary.

Rationale for Specific Aim Three

The microscopic anatomy of the upper respiratory tract of marine mammals has not been well described, however, evidence suggests that these animals have a unique network of hyaline cartilage-reinforced airways that can compress and expand during a dive (Denison and Kooyman, 1973; Boyd, 1975; Stewardson et al., 1999; Gray et al., 2006; Smodlaka et al., 2006). Various respiratory diving adaptations, such as elastic fiber integration, specialized cells and airway reinforcements are visible at the microscopic scale. Species-specific tissue variations also convey functional adaptations that are important to dive regimes and life history. These anatomical descriptions also assist in guiding research questions regarding management of inhaled gases, prevention of DCS, and lung collapse depth.

The trachea was once believed to be made of incompressible tissue, however, radiography has revealed that under pressure, the tracheal diameter (air volume) compresses up to 20% (Kooyman et al., 1970). Furthermore, tracheal compression can dictate lung collapse depth, such that more compliant (pliable) tracheal tissues permit deeper depths of lung collapse (Bostrom et al., 2008; Fahlman et al., 2009). These results were supported in a subsequent study of gross tracheal anatomy and compliance measurements (Moore et al., 2014). Therefore, tracheal anatomy and the microscopic aspects of cartilage rings have a functional role in the compliance of the trachea, depth of lung collapse, management of onboard gases and mitigation of DCS symptoms (Fahlman et al., 2009).

We aim to utilize histology to create a model of tracheal compression in two age classes of the harbor seal. The hypothesis driving this aim is that the microscopic tracheal

morphology changes as the trachea progresses towards the lungs. Further, we also hypothesized that the microscopic tracheal anatomy of the deeper diving NES would differ from the shallow diving harbor seal (Le Boeuf et al., 2000; Gjertz et al., 2001; Kuhn et al., 2009; Robinson et al., 2012). We expected that histological findings would support earlier studies suggesting that the compliance of the harbor seal trachea is heterogeneous and that the tracheal compliance values of the NES are greater than shallow diving and terrestrial species (Moore et al., 2014). We also hypothesized that elastic fiber integration plays a role in overall tracheal compliance, both cross-sectionally and longitudinally. Finally, volume measurements using basic circumference and diameter were hypothesized to affect compliance values, particularly where discontinuities in the tracheal ring could impact compression thus changing internal air volume.

Summary and Theme

The greatest physiological challenge to a diving mammal is the efficient utilization of inspired gas for maintenance of metabolic function, and avoiding the detrimental consequences of gas supersaturation. Thus the theme of oxygen (specifically management, utilization and storage of oxygen) will permeate throughout this thesis. A diving mammal must strike a unique balance between adequate foraging time at depth and the biological limits of gas management. This dissertation aims to elucidate the story of gas management in a diving pinniped from oxygen inspiration and respiratory management to the pathways of oxygen utilization for aerobic metabolism and myoglobin binding for intramuscular oxygen storage. More specifically, results indicate that adult Northern elephant seals have enlarged type I muscle fibers in locomotory

musculature with elevated levels of myoglobin. In addition, muscle-specific enzymes were found to be an unreliable method of testing aerobic proficiency in post mortem specimens, as enzymatic activity can vary greatly within and between individuals. Finally, microscopic analysis of tracheas yielded evidence that even small anatomical differences, such as continuity of cartilage or elastic fiber integration, can have a large effect on respiratory gas management. Diving mammals must ultimately maintain a balance between gas inspiration, utilization and saturation. Therefore, marine mammals provide a unique opportunity to model aerobic efficiency and avoidance of deleterious human diseases. This thesis elucidates comparative functional physiological adaptations to depth and pressure, and provides relevant foundational data that can be applied to future research in ischemia reperfusion injury and decompression sickness.

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CHAPTER TWO

Ontogenetic Changes in Skeletal Muscle Fiber Type, Fiber Diameter and Myoglobin Concentration in the Northern Elephant Seal (*Mirounga angustirostris*)

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Abstract

Northern elephant seals (*Mirounga angustirostris*) (NES) are known to be deep, long-duration divers and to sustain long-repeated patterns of breath-hold, or apnea. Some phocid dives remain within the bounds of aerobic metabolism, accompanied by physiological responses inducing lung compression, bradycardia and peripheral vasoconstriction. Current data suggest an absence of type IIb fibers in pinniped locomotory musculature. To date, no fiber type data exist for NES, a consummate deep diver. In this study, NES were biopsied in the wild. Ontogenetic changes in skeletal muscle were revealed through succinate dehydrogenase (SDH) based fiber typing. Results indicated a predominance of uniformly shaped, large type I fibers and elevated myoglobin (Mb) concentrations in the longissimus dorsi (LD) muscle of adults. No type II muscle fibers were detected in any adult sampled. This was in contrast to the juvenile animals that demonstrated type II myosin in Western Blot analysis, indicative of an ontogenetic change in skeletal muscle with maturation. These data support previous hypotheses that the absence of type II fibers indicates reliance on aerobic metabolism during dives, as well as a depressed metabolic rate and low energy locomotion. We also

suggest that the lack of type IIb fibers (adults) may provide a protection against ischemia reperfusion (IR) injury in vasoconstricted peripheral skeletal muscle.

Introduction

Kooyman et al. (1980) defined the aerobic dive limit (ADL) as the maximum dive duration maintained with aerobic metabolism, while studying Weddell seals (*Leptonychotes weddellii*). These authors discovered that this pinniped species maintains oxygen stores in relation to metabolic rate (oxygen consumption) during diving. This maintenance of aerobic metabolism during breath-hold diving is accompanied by a reduction in cardiac output, vasoconstriction and lung collapse (Scholander, 1940; Kooyman, 1973; Kooyman et al., 1981). Deep-diving pinniped species also display physiological adaptations of their locomotor muscle that lengthen ADL, including elevated concentrations of Mb and aerobic enzymes, reliance on lipids for fuel and a prevalence of type I, slow twitch, oxidative muscle fibers (Lenfant et al., 1970; Hochachka and Foreman, 1993; Kanatous, 1999; Kanatous et al., 2002; Trumble and Kanatous, 2012). These adaptations, as well as energy-saving swimming behavior (Kooyman et al., 1980; Williams et al., 2000) contribute to extending ADL (Kooyman et al., 1981; Davis and Kanatous, 1999).

The focus of this study was to assess Mb concentrations, fiber type composition, and muscle fiber diameter and cross-sectional area of a primary locomotor muscle, the longissimus dorsi, in three age classes (pup, juvenile and adult) of NES. Terrestrial species, such as mice have comparatively smaller diameter oxidative (type I) fibers, which are interspersed between larger, anaerobic fibers (type II) in a heterogeneous fiber population, such that type I (slow twitch oxidative), type IIa (fast twitch oxidative-

glycolytic) and IIb (fast-twitch glycolytic) are present in fiber bundles, and were considered during this study (Scott et al., 2001). Comparatively, a deep-diving pinniped such as the Weddell seal, appear to have a skeletal muscle composition containing only large type I fibers (Kanatous et al., 2002; Trumble and Kanatous, 2010). Alternatively, California sea lions (CSL; *Zalophus californianus*) are relatively shallow divers (Feldkamp et al., 1989; Weise et al., 2006) and have a more heterogeneous fiber type distribution in the muscle given different dive durations and rates of muscular activity, all of which could determine recruitment of glycolytic fibers (Ponganis and Pierce, 1978).

Myoglobin concentrations also play a key role in the metabolic profile of marine mammals (Dearolf et al., 2000; Kanatous et al., 2002; Watson et al., 2003; Kielhorn et al., 2013; Velten et al., 2013; Williams and Noren, 2011; Trumble and Kanatous, 2012). As the oxygen binding protein in skeletal muscle, Mb controls the release and utilization of oxygen during hypoxia (Salathe and Chen, 1993), and elevated concentrations are correlated with increased breath-holding times (Kooyman, 1989; Noren and Williams, 2000). Both diving mammals and diving birds demonstrate elevated levels of Mb (Scholander, 1940; Castellini and Somero, 1981; Kooyman, 1989). For example, the NES, which is capable of repeated dives to mean depths of 516 ± 53.2 m for 23.1 ± 2.6 min (Le Boeuf et al., 2000; Robinson et al., 2012), have skeletal muscle Mb concentrations (7.5 ± 0.7 g/100g) among the highest reported in mammals (Hassrick et al., 2010). Previous research on Mb and associated muscle enzyme concentrations of the NES (Thorson and LeBoeuf, 1994), Weddell seal (Kanatous et al., 2008; Trumble et al., 2010), harp seal (*Pagophilus groenlandicus*), hooded seal (*Cystophora cristata*) (Lestyk et al., 2009), harbor seal (*Phoca vitulina*) (Jørgensen et al., 2001) and gray seal

(*Halichoerus grypus*) (Noren et al., 2005) has revealed that muscle development proceeds gradually during ontogeny (Noren et al., 2005; Burns et al., 2007; Kanatous et al., 1999; Picard et al., 2002). Specifically, blood-based oxygen storage calculations and measurement of muscle-based Mb in the NES during the10-week period post weaning, demonstrate an increase in Mb concentrations from 2.1 g/100g tissue in weaners to 5.7 g/100g tissue in juveniles (Thorson and LeBoeuf, 1994). This correlates with the deep dive depths (206 m) reached by juvenile seals during their first few weeks at sea and indicates ontogeny related changes in Mb expression (Thorson and LeBoeuf, 1994). More recent work has revealed that apnea stimulates the production of Mb protein expression by 60% in elephant seals (Vázquez-Medina et al., 2011) and Mb development may be increased in response to post natal signals such as exercise (Lestyk et al., 2009). Furthermore, it has been suggested that diving Weddell seals have a unique adaptation in the regulation of Mb expression, where hypoxia and lipids prime myocytes for optimal Mb expression (De Miranda et al., 2012). Coupled with muscular activity, elevated Mb concentrations are developed and maintained (De Miranda et al., 2012). The role of lipids as a driving force behind the regulation of Mb expression lends evidence to a correlation between reliance on aerobic lipid-based metabolism and the utilization of oxygen during a dive (De Miranda et al., 2012).

Fiber type distribution has been described in several pinniped species including the grey seal (Reed et al., 1994), harbor seal (Reed et al., 1994; Watson et al., 2003), Antarctic fur seal (*Arctocephalus gazella*) (Reed et al., 1994), and Weddell seal (Kanatous et al., 2002; Trumble et al., 2010). However, knowledge of the ontogeny of diving, as it relates to NES skeletal myofibrillar profile data are limited (Lestyk et al.,

2009). To date, skeletal muscle fiber type data are lacking for the NES. It was the aim of this study to determine the differences in the skeletal muscle composition from pup to adult in the NES. We compared fiber type profiles and cross-sectional diameter and area for three age groups (pups, juveniles and adults) and assessed the changes in Mb concentrations. We hypothesize that NES are adapted for aerobic long-duration exercise by having a predominately type I muscle fiber profile and elevated Mb levels

Material and Methods

Specimen Collection

We obtained muscle biopsies from 9 adults (1 female and 8 males), 4 juveniles (all males) and 8 recently weaned pups (3 females and 4 males). In addition, a CSL juvenile (n=1) and adult mouse (n=1) was sampled as a mixed fiber comparison and control. Average approximate mass (kg) ± SD for each NES age class and one CSL are reported in Table 2.1.

Table 2.1: Elephant seal average approximate weight (kg) ± sd for three age classes and one CSL average weight (kg) ± sd. N=22.

Species	Age class	Average weight (kg) ± sd
NES	Weaned pup	132 ± 11
NES	Juvenile	167 ± 28
NES	Adult	1385 ± 511
CSL	Juvenile	120

NES were sampled on Año Nuevo State Reserve, California during the breeding and molt haulouts for adults and fall haulout for juveniles in 2012 and 2013. Seals were

anesthetized using an intramuscular injection of Telazol (teletamine/zolazepam HCl) at a dose of 1.0 mg/kg and intravenously administered doses of ketamine and diazepam (as needed) to maintain immobilization (Fort Dodge Laboratories, Ft. Dodge, IA) (Crocker et al., 2012). To access the LD, a $2cm^2$ area was cleaned with betadine before each incision (2 cm). For standardization purposes, samples were taken from the mid-belly of the muscle and at the same location in all age classes (one-third of the body length from the tip of the tail). Muscle biopsies (connective tissue and blood dissected away) were collected (30–50mg) and taken under local anesthetic (1ml; Lidocaine®, Whitehouse Station, NJ, USA) using a 6mm biopsy cannula (Depuy, Warsaw, IN, USA). Muscle samples were stored in a liquid nitrogen dry vapor shipper (Thermo Scientific) until long-term storage in a -80°C freezer. Specimens were collected under NMFS marine mammal permit #14636 and all procedures were approved by the Sonoma State University IACUC. LD muscle samples were specifically chosen, as LD is the major locomotory muscle in NES. However, biopsies may represent a small subset of muscle from a large animal and can be considered a limitation to this study. Two other muscles were collected to act as control for the methods described. The locomotory muscle (pectoralis major) of the CSL (CSL10281), housed at The Marine Mammal Center in Sausalito, California was sampled immediately following death. For a terrestrial mammal comparison, the hindlimb locomotor muscle (biceps femoris) from a mouse was harvested (Jackson Laboratory in Bar Harbor, ME). NES samples were sent frozen in cryovials and kept in a -80°C freezer approximately one month prior to analysis.

Muscle Fiber Typing, Diameter and Cross-sectional Area

Muscle bundles were oriented to ensure that the long axis of the isolated myofibers were perpendicular to the cryostat blade. Cross-sections were sliced frozen (-20°C) at 10 µm using a cryostat (Bright Instrument Co., OTF). Serial sections were placed on glass slides and stained for SDH according to the methods of Dearolf et al. (2000). These methods have been successfully used in previous marine mammal fiber typing analyses (Watston et al., 2003; Cotten et al., 2008; Kielhorn et al., 2013; Velten et al., 2013).

Slides were incubated in a 0.2 M sodium phosphate buffer solution, sodium succinate (13.025 g/250 ml) and nitro blue tetrazolium chloride (NBT; 0.015g/30ml). NBT, a purple-colored salt, binds to the electron acceptor following the oxidation of succinate, resulting in a purple staining pattern within the mitochondria of each muscle cell, and thus offers a good marker for mitochondrial abundance in muscle fibers (DiMauro, 2012). Incubation time was approximately 60 min at 37°C, followed by a 2 min rinse in saline solution (1.96g/200ml), 10 min fixation step in formalin-saline solution (10ml/90ml) and a 5 min rinse in 15% ethanol. All slides were not incubated together which may reflect some coloration differences between slides. Stained cross-sections were dried and mounted with cover slips. Samples were analyzed in triplicate using a high-resolution camera-mounted microscope (Nikon Eclipse Ci; Nikon, Brighton MI, USA).

Ten fibers from each fiber bundle were counted and measured based on consistent orientation, where dissected fiber bundles were tightly arranged, round and whole (adapted from Velten et al., 2013). Only fiber types commonly found in mammalian

skeletal muscle can be determined by SDH staining methods, thus type I, type IIa and type IIb were considered during this study. SDH stain designates the relative oxidative potential of each fiber type via a colorimetric method. There is a positive linear correlation between color and oxidative potential. Type I fibers stained darkest, followed by a decreasing color spectrum of type IIa and type IIb. Given the similar staining intensity for all fibers within NES cross-sections, the SDH stained fibers were qualified as one fiber type, where all whole, round fibers were counted. This was in contrast to the terrestrial mouse where fibers were individually qualified based on their fiber type.

Average fiber diameter and area were measured to scale using a high-resolution microscope with accompanying camera (Nikon Eclipse Ci) and software (NIS element D) calibrated at 200X magnification. Data are reported here as mean μm ± standard deviation (SD) for each fiber sampled (Table 2.2). Freeze fracture was visible in some muscle cross-sections and deemed unavoidable due to field sampling protocol. These fibers were not utilized when assessing fiber size.

Western Blotting

Muscle tissue was homogenized using a Bullet Blender (Next Advance, NY USA) in CellLytic MT buffer (Sigma Aldrich) using 0.5 mm Zirconium oxide beads. The supernatant was aliquoted and utilized for Bradford assay (Beckman Coulter DU 730 spectrophotometer at 595 nm) to determine total protein content. Standard Western Blot protocol (Abcam) was performed using 8 ul of sample and ladder (Bio-Rad) loaded into SDS gel wells (ClearPAGE; 4-20%), run in a Tris-Tricine SDS running buffer (ClearPage) and transferred using Tris/Glycine buffer (Bio-Rad). Three primary antibodies specific to the myosin heavy chains I, IIa and IIb (Developmental Studies

Hybridoma Bank, University of Iowa, BA-D5 (1:750), SC-71 (1:500) and BF-F3 (1:1) respectively) and two secondary antibodies (KPL peroxidase labeled goat anti-mouse IgG (H+L) at 1:10,000, Invitrogen HRP goat anti-mouse IgG+A+M at 1:30,000) were used. Primary antibodies have been previously confirmed for fiber typing in pinnipeds (Watson et al., 2003; Kanatous et al., 2008). Protein bands on a nitrocellulous membrane were visualized using a chemiluminescent substrate kit (KPL International) sensitive to peroxidase-labeled antibodies and developed using a luminescent image analyzer (GE LAS 4000) and accompanying software (GE Healthcare Life Sciences). Western Blot analysis was completed in duplicate.

Myoglobin Quantification

Mb assays were completed using methods modified by Kanatous et al. (1999) from Reynafarje (1963). Homogenates (prepared as described above) were diluted in phosphate buffer (0.4 M KPO4 at pH 6.6) and centrifuged at 28,000 g for 50 min. Supernatant was bubbled with carbon monoxide for 3 min and spectrophotometric absorbance was determined. Absorbance was measured in triplicate at two wavelengths and extinction coefficients (14.7×10^3 $cm^{-1}M^{-1}$at 538 nm and 11.8×10^3 $cm^{-1}M^{-1}$at 568 nm). Mb concentration was calculated and reported as mg/g of muscle mass in Table 2.2.

Statistical Analysis

Data were analyzed and log transformed for normalization (homogeneity of variances was determined using the Brown-Forsythe test) and statistical significance was maintained at or below the 0.05 alpha level for analysis of variance (ANOVA) and

Tukey-Kramer HSD testing. Results are presented here as means ± standard deviation (SD).

Results

Muscle Fiber Type Distribution and Diameter

Visual examination of SDH stained cross-sections of LD myofibers for all age classes of NES revealed only one type of fiber (Fig. 2.1). Based on Western Blot analysis, the predominate fiber type was type I, where pups (n=3) had slight antibody specific binding to both type IIa and IIb myosin (Fig. 2.2) and adults (n=6) did not demonstrate any binding to type IIa and IIb antibodies, just type I (Fig. 2.3). Therefore, our analysis indicates that myosin fiber type changes over the maturation of the NES. Unlike the LD of the NES, pectoralis muscle of the CSL possessed three different muscle fiber types as did the mouse biceps femoris (Appendix A,B).

Table 2.2: Elephant seal fiber diameters (μm), average cross-sectional area (μm^2) per age class (mean ± SD) and average myoglobin (mg/g) concentrations (mean ± SD). Letters denote statistical significance, N=22.

Age Class	Average Fiber Diameter	Average Cross-Sectional Area	Average Mb concentration (mg/g)
Pup	41.9 ± 5.9	1406.8 ± 397.4	21.0 ± 7.2
Juvenile	64.4 ± 12.6	3373.8 ± 1327.3	51.8 ± 13.7
Adult	118.7 ± 21.1	11410.1 ± 3941.8	59.1 ± 4.1

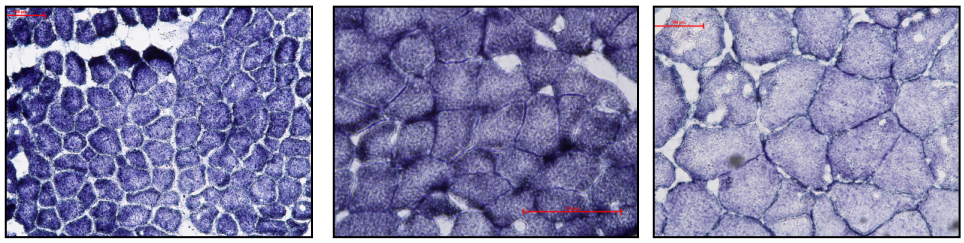

Figure 2.1 (**A**-**C**): Photomicrographs (100µm scale) of cross-sections of Northern elephant seal longissimus dorsi muscle stained with succinate dehydrogenase. The succinate dehydrogenase stain is known to correlate with muscle fiber type (Dearolf et al. 2000). Samples from pup (**A**)(6606), juvenile (**B**)(Ele#4) and adult (**C**)(3TC) are shown. Fibers uniformly demonstrate the same staining intensity and cross-sectional diameter within individuals indicative of the presence of a single fiber type. N=3.

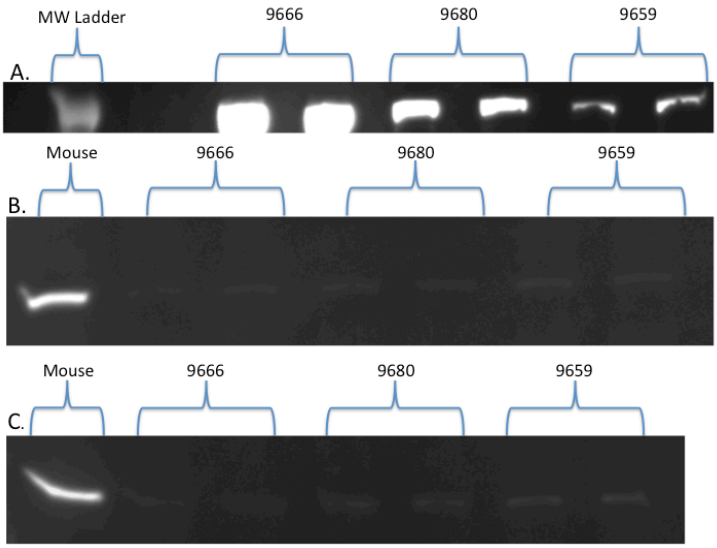

Figure 2.2 (**A**-**C**): Western Blot results for Northern elephant seal pups (N=3: 9666, 9680, 9659). Type I (**A**), type IIa (**B**) and type IIb (**C**) skeletal muscle myosin heavy chain antibodies are present in duplicate wells, with either a molecular weight (MW) ladder (**A**) or mouse control (**B** and **C**) shown for comparison. Qualitative comparisons can be seen between the bright type I (**A**) bands versus the light type IIa (**B**) and IIb (**C**) bands, indicative of the presence of all myosin types in pup longissimus dorsi muscle, where type I strongly binds and type IIa and IIb slightly bind.

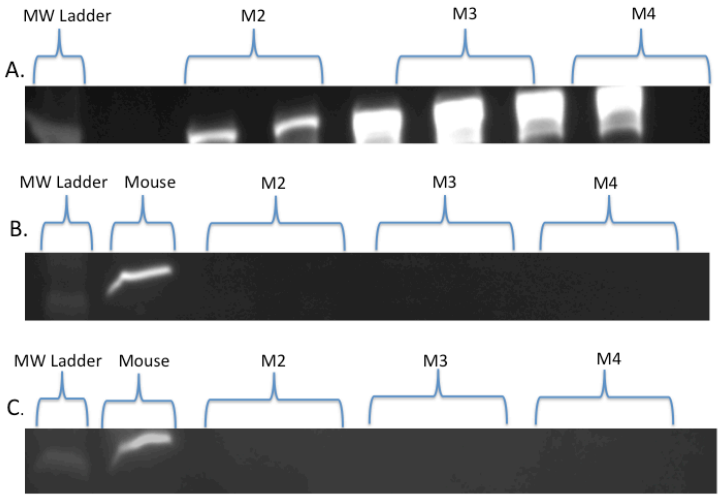

Figure 2.3 (**A-C**): Western Blot results for Northern elephant seal adults (N=3: M2, M3, M4). Type I (**A**), type IIa (**B**) and type IIb (**C**) skeletal muscle myosin heavy chain antibodies are present in duplicate wells, with either a molecular weight (MW) ladder (**A**) or mouse control (**B** and **C**) shown for comparison. Qualitative comparison, specifically absence versus presence, can be seen between the present bright bands of the type I (**A**) versus the absent bands of the type IIa (**B**) and IIb (**C**) myosin of the adults, indicating the presence of one fiber type (Type I) in adult longissimus dorsi muscle.

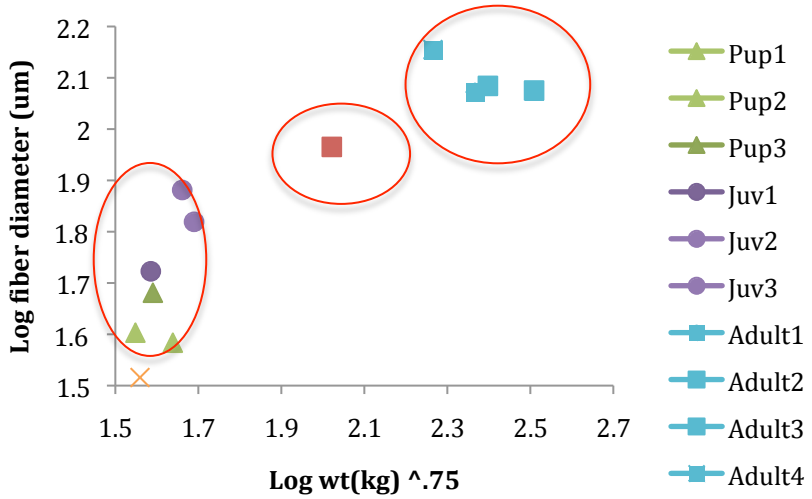

Figure 2.4: Fiber diameter (um) in correlation with increasing scaled animal mass ($kg^{.75}$). Pups (7515, 7606, 6606)=*M. angustirostris*, juv (ele4, FJ11, FJ13) =*M. angustirostris*, adult 1-4 all male (Male2, 6th, 3TC, 7728M08) = *M. angustirostris*, adult 5 only female (FemaleTrip2), CSL= *Z. californianus*. Red circles indicate large groups of similar animals, from top right: adult male, adult female, pup and juveniles. The California sea lion scales smaller than all Northern elephant seals and is not encircled. N=10.

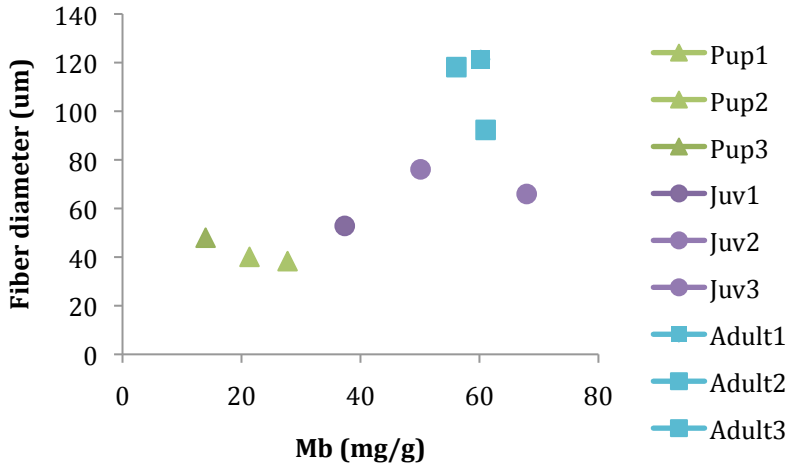

Figure 2.5: Myoglobin concentration (mg/g) in correlation with fiber diameter (um) in three age classes of the Northern elephant seal: Pups (7515, 7606, 6606), Juv (ele#4, FJ13, FJ11), and Adults (6th, Male2, FemaleTrip2). N=9.

There was a statistical difference in fiber diameters for NES pups (n=4), juveniles (n=4) and adults (n=5) among age classes (pups, juveniles < adults; ANOVA; $p<0.05$, Table 2.2) but not within individuals of each age class (ANOVA; $p>0.05$, Table 2.2). Fiber diameters were used to calculate average fiber cross-sectional area for each age class of NES. Pups had an average of 1406.8 ± 397.4 μm^2. Juvenile animals had an average of 3373.8 ± 1327.3 μm^2 with adults averaging 11410.1 ± 3941.8 μm^2. The fiber cross-sectional area was also representative of the increase in fiber size with age (Fig. 2.4, Table 2.2).

Fiber size also varied across species and fiber types (Table 2.3). Adult NES had relatively large LD fibers throughout cross-sections (Table 2.2). For both the control mouse and CSL, type I fibers had the smallest mean diameter and type IIb the largest mean diameter (Table 2.3). Fiber diameters for type I, IIa and IIb (within each animal) were significantly different (ANOVA; $p<0.05$, Table 2.3). The NES had a uniformly sized fiber type population in the LD muscle among each age class (Fig. 2.1; Table 2.2).

Table 2.3: Fiber diameters (μm) for mouse and California sea lion (mean ± SD). N=2.

Animal	Type I	Type IIa	Type IIb
Mouse	35.1 ± 4.8	48.4 ± 7.5	59.9 ± 7.3
California sea lion	32.8 ± 5.3	42.5 ± 6.2	54.6 ± 6.7

Myoglobin Concentrations

NES pups (n=3) had the lowest average concentration of Mb in LD musculature, averaging 21.0 ± 7.2 mg/g of protein while juveniles (n=3) had a concentration of 51.8 ± 13.7 mg/g (Fig. 2.5, Table 2.2; ($p<0.05$)). Adult (n=3) elephant seals had the highest average Mb concentration of 59.1 ± 4.1 mg/g of protein (Fig. 2.5, Table 2.2; pup < juvenile, adult, Tukey-Kramer HSD, $p<0.05$). Mb concentrations were calculated for NES age classes only; CSL and mouse Mb concentration was not determination due to sample size constraints. Mb concentration was compared to the fiber diameter, and indicated the positive correlation of Mb concentration with increasing fiber diameter (Fig. 2.5).

Discussion

This is the first study to describe muscle fiber profile changes across ontogeny in NES skeletal muscle. Here, we determined that the adult NES, a deep-diving phocid (Le Boeuf et al., 2000; Kuhn et al., 2009; Robinson et al., 2012), has uniformly large and metabolically uniform (SDH) type I fibers in the fiber bundles of the LD muscle investigated in this study. Western Blot analysis revealed pup muscle has binding for type IIa and IIb myosin heavy chain, demonstrating ontogenetic changes in fiber type. In conjunction, we show age-related increases in Mb concentration, where Mb is positively correlated to fiber diameter.

Ontogenetic Change in Fiber Type and Large Muscle Fibers

Western Blot analysis demonstrated a distinct fiber population in immature seals as compared to adult seals. Pups expressed myosin I antigens, and to a smaller extent IIa and IIb antigens, whereas adult muscle expressed solely type I antigens (Figs. 2.2, 2.3). The absence of type II fibers in adults in this study was similar to previous studies on harbor seal and Weddell seal locomotory musculature, where one or both anaerobic fibers are absent (Watson et al., 2003; Kanatous et al., 2002; Trumble et al., 2010). In contrast, the CSL (Appendix A) as well as some cetaceans have type IIa and type IIb fibers in locomotory musculature (Keilhorn et al., 2013; Velten et al., 2013), indicating different models for exercise. Low oxidative capacity (SDH color intensity) was observed in association with the type I fibers in adults. This result has also been seen in Weddell seals (Kanatous et al., 2008), and could be indicative of low mitochondrial densities within myofibers and a low rate of oxygen consumption (Kanatous et al. 2002).

The presence of type II myosin in pups, but absence in adults, indicates that muscle plasticity, or shifts in muscle myosin type, occur with age in NES. Previous research on Weddell seals confirms the existence of a juvenile fiber profile (Trumble et al., 2010), suggesting an element of dive training and shift in muscle myosin with age. Muscle fiber plasticity has been documented in mammals, and the change in contraction speed and metabolic basis (fiber type) is thought to occur in response to various stimuli (Pette and Staron, 1997; Grossman et al., 1998; Ricoy et al., 1998; Scott et al., 2001). Neonatal dolphins were found to have different mitochondria and lipid content than adults, indicative of a lower aerobic capacity (Dearolf et al., 2000) and demonstrating marine mammal ontogenetic changes in musculature. Fiber conversion, specifically, has

been seen between type IIa and IIb with type I to II also possible (Pette and Staron, 1997; Scott et al., 2001). Less common is the shift from type II to type I (Scott et al., 2001), although recent data show the activation of certain muscle-specific proteins can generate an "endurance athlete" mouse model with increased levels of aerobic enzymes, mitochondria and type I fibers (Wang et al., 2004). This would indicate that muscle plasticity, specifically transformation to type I could be promoted with endurance training. Muscle fiber type conversion in development for NES indicates that muscle cells in young animals are also plastic and muscle type may be dependent on nerve activity (Eken and Gundersen, 1988). In a more general sense, exercise stimuli based on extensive deep-diving as a juvenile after/during the first trip to sea might stimulate muscle fiber type plasticity. Similarly, Weddell seals also demonstrate muscle plasticity when exposed to muscular activity and hypoxia (De Miranda et al., 2012). Perhaps the expression of anaerobic antigens can be considered an intermediary developmental link between age groups, where anaerobic antigens are not present in the adult group when an optimal concentration of Mb is achieved. Thus, smaller diameter fibers of mixed composition are indicative of a more terrestrial dwelling pup and larger homogeneous fibers are indicative of a swimming adult. The adult elephant seal has high concentrations of Mb and relatively large oxidative fibers, with little necessity for anaerobic metabolism, a metabolic state attained through endurance training. Future work could be aimed at quantifying the changes in neural innervations stimulating fiber conversions and how this activity correlates with age and exercise training. Regardless, more than one qualifying/quantifying protocol should be used for fiber determination in diving mammals (Watson et al., 2003).

As a general rule, there is a positive correlation between muscle fiber diameter and oxygen diffusion distance in mammals (Van Der Laarse et al., 1998; Van Wessel et al., 2010). The limitation of having larger fiber diameters is a function of the diffusion distance across the cellular membrane and cell, with smaller fibers allowing for more rapid oxygen diffusion (Van Wessel et al., 2010; Kinsey et al., 2011; Keilhorn et al., 2013). The typically larger anaerobic type II fibers are not constrained by oxygen diffusion distance (Van Wessel et al., 2010; Keilhorn et al., 2013). This was evident in our control mouse (Appendix B) as well as the CSL pectoral muscle (Appendix A), where type I oxidative fibers were significantly smaller than both type IIa and IIb fibers (Table 2.3). NES had greater mean muscle fiber diameter regardless of age class than the CSL and the mouse control ($p<0.05$, Table 2.2, 2.3) as well as previously sampled cetaceans and pinnipeds (86.2 μm *K. breviceps*; 66.8 μm *G. macrorhynchus*; 90.5 μm M. europaeus; 94 μm *L. weddellii*) (Kanatous et al., 2002; Kielhorn et al., 2013; Velten et al., 2103). Previous myofiber studies in the beaked whale (*Mesoplodon* sp.), short finned pilot whale (*Globicephala macrorhynchus*)(Velten et al., 2013), pygmy sperm whale (*Kogia breviceps*) (Kielhorn et al., 2013) and the Weddell seal (Kanatous et al., 2002), report larger relative fiber diameters, suggesting an oxygen conserving adaptation for diving. According to the "optimal fiber size hypothesis" (Johnston et al., 2004; Jimenez et al., 2011), Velten et al. (2013) and Kielhorn et al. (2013) hypothesized that due to low surface area to volume ratios of enlarged muscle fibers and associated dampened metabolic cost for membrane potential, enlarged fibers may result in lower muscular energetics and thus lower metabolic rates (Kielhorn et al., 2013).

Elevated Myoglobin Concentrations

In humans, exercise prompts an increased consumption of oxygen that is compensated by increased blood flow (Blomstrand et al., 1997). This is counter to breath-hold diving pinnipeds, known to vasoconstrict and decrease blood flow to peripheral skeletal muscle (Zapol et al., 1979). Therefore, diving pinnipeds must store greater amounts of Mb in musculature as on-board oxygen storage during a dive (Kanatous et al., 1999**)** indicated by reports of increases in Mb expression after a juvenile's first trip to sea and achievement of deep dive depths (Thorson and LeBoeuf, 1994). In this study, we found increasing Mb levels with age and body mass (Fig. 2.5). Juveniles and adults had significantly higher levels of Mb than pups (Fig. 2.5, Table 2.2, $p<0.05$). Mean Mb values reported during this study for the adult NES (59.1 ± 4.1mg/g) are similar to other pinniped species such as the adult Weddell seal (45.9 ± 3.3 mg/g: Kanatous et al., 2002), harbor seal (38 ± 1 mg/g: Polasek et al., 2006; 37.4 ± 1.7: Kanatous et al., 1999; 41 ± 4: Reed et al., 1994) and Stellar sea lion (28.7 ± 1.5 mg/g: Kanatous et al., 1999). Compared to terrestrial species (domestic dog: 3.5 mg/g, Kanatous et al., 2002), NES and other species noted above have much greater Mb concentrations in their locomotory musculature. Thus, large intraspecific variation exists for Mb concentration as Mb values have been determined to vary with activity level (De Miranda et al., 2012). Noren and Williams (2000) found that diving proficiency is correlated with elevated Mb concentration and increased body mass in some odontocete species. This correlation appears to apply to pinnipeds as well (Fig. 2.5). Similarly, in this study, the correlation of Mb (mg/g tissue) and body mass suggests that a shift occurs during ontogeny, where juvenile animals demonstrate increased levels of Mb (Fig. 2.5). These data are concurrent

with other species of diving mammals where Mb expression in skeletal muscle is triggered through exposure to hypoxia (De Miranda et al., 2012).

Possible Connection to Ischemia Reperfusion Injury

Ischemia and subsequent restoration of blood flow to mammalian body tissue can cause harmful effects, cumulatively often termed ischemia reperfusion (IR) injury. A similar process involving vasoconstriction followed by restoration of blood flow progresses during a pinniped dive (Scholander, 1940), yet no injury has been described. In mice, ischemic events lasting as little as 20 min can cause edema in tissue (Carattino et al., 2000). The release of oxygen-free radicals, or reactive oxygen species (ROS), as a result of the reintroduction of oxygenated blood, causes cellular damage (McCord, 1985; Saugstad, 1996), and thus substantial injury (Li and Jackson, 2002). Diving mammals might avoid harmful effects of IR injury through frequent exposure to ischemia (repetitive dives), which may "precondition" muscle and provide protection (Elsner et al., 1998). Seals may also have enhanced scavenging capacity via elevated activity of superoxide dismutase (SOD) in myocardium (Elsner et al., 1998) and glutathione peroxidase (GPx) in muscle (Vázquez-Medina et al., 2006). Although free radicals are present and play a role in IR injury, their role is secondary to the amplification of the initial injury (Chan et al., 2004), contradicting early research suggesting that IR-related tissue necrosis was the result of ROS (Chan et al., 2003). IR injury has now been linked specifically and primarily to the inflammatory response (Austen et al., 2004; Chan et al., 2004; Suber et al., 2007) responsible for destruction of muscle tissue in mice (Chan et al., 2004). The site of IgM and complement protein deposition (inflammatory proteins primarily responsible for mediation of the injury) is on fast-twitch type IIb muscle fibers

(Chan et al., 2004) - absent in adult NES skeletal muscle. Due to the lack of type IIb fibers, deep-diving pinnipeds must also lack the unique expression of epitopes on type IIb fibers, singularly found to bind to immune proteins during the process of injury. We believe that this apparent lack of type IIb fibers in adult locomotory musculature may preclude IR injury in diving pinnipeds. This would suggest that diving cetaceans and even other pinniped species (CSL) represent a different muscular model for deep-diving, as they possess type IIb fibers in skeletal muscle (Keilhorn et al., 2013; Velten et al., 2013). A deletion or alteration to the activating immune pathway of the disease is possible, making the presence of type IIb fibers inconsequential. In addition, extent of ischemia in musculature (Meir et al., 2009; McDonald and Ponganis, 2013) may vary and should not be assumed for all marine mammals, as early experiments did not encompass all species (Scholander, 1940), which may cover a wide range of exercise preferences. Future research could also be aimed towards the culture of cells to identify the expression of epitopes or inhibitory proteins expressed in muscle tissue after an ischemic event, especially for animals with type IIb fibers (CSL) which also undergo ischemic insult to muscle tissue during a dive (McDonald and Ponganis, 2013).

In summary, the cross-sectional fiber type profile of the NES LD muscle shown in this study was complementary to what was previously known about mammalian divers. Previous research on the Weddell seal (Kanatous et al., 2002; Kooyman, 1981; Castellini et al., 1992; Davis et al., 1999; Kanatous et al., 2002) revealed a large predominance of type I fibers in locomotory musculature in contrast to short-duration divers with mixed fiber profiles that are able to rely on anaerobic metabolism (Kanatous et al., 1999). One benefit to having a type I fiber distribution is fatigue resistance (Van Wessel et al., 2010).

Additionally, type I oxidative muscle fibers produce increased levels of antioxidants (nitric oxide), which act as protection against hypoxic insult (Yu et al., 2008). Cumulatively, scaled larger type I muscle fibers would be beneficial for a long-duration diver wholly dependent on efficient utilization of stored oxygen and suppression of harmful effects of hypoxia. We suggest that the NES maintains a similar diving strategy to the Weddell seal, as low-level aerobic metabolism may be the best model for long, deep diving. The scaled enlarged fiber size, predominately type I fiber profile, and elevated Mb content of skeletal muscle in adult NES allows for an excellent model for adaptation to heightened capability of oxygen storage and utilization. Furthermore, these adaptations coupled with the lack of type IIb fibers may allow seals to avoid a deleterious mammalian disease (IR injury) and withstand repeated ischemia to peripheral tissue.

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Author Contributions

C.M. was primary author and completed data analysis. A.F, M.M., D.W. and S.J.T. contributed to concept, and method development; D.C., K.R., S.B.K and S.J.T. collected and handled biopsy and whole muscle samples.

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CHAPTER THREE

The Degradation of Proteins in Pinniped Skeletal Muscle: Viability of Post-mortem Tissue in Physiology Research

Abstract

As marine divers, pinnipeds have a high capacity for exercise at depth and pressure. A large component of that is being more fit for extended aerobic exercise and conservation of energy. Pinnipeds must deal with common physiological hurdles such as hypoxia, exhaustion and acidosis- common to all exercising mammals. These hurdles have sparked much research in the marine mammal field as to how these diving animals "deal" with onboard carbon dioxide and utilize oxygen stores. In some cases, stranded animals are used in marine mammal research, but can biochemical data derived from post mortem tissues be reliable? In this study, we mapped an enzymatic degradation time series from four biopsied Northern elephant seals (*Mirounga angustirostris*). We also compared the enzymatic activity of different muscle groups in relation to locomotion as well as measured the effects of four freeze-thaw cycles on a muscle tissue enzyme. Results indicated that enzymatic activity vary depending on the storage temperature, storage time, species and muscle group being assayed. In contrast, proteins, such as myoglobin (Mb), can remain stable at refrigerator temperature. Stranded animals can be a valuable source of biochemical data, but enzyme assays may not be a reliable assay in post mortem tissue. Here, we aim to provide a map of muscle degradation for stranding units collecting post mortem muscle samples and highlight recommendations for storage protocols for the best preservation of tissue. Ultimately, we aim to determine the

reliability of biochemical assays derived from post mortem tissue, and further promote the immediate sampling of stranded animals for the purpose of physiological research.

Introduction

Muscle tissue samples collected *in vivo* have provided a vast amount of knowledge on the physiology, exercise performance and basic muscle structure of marine mammals (Kanatous et al., 1999; Dearolf et al. 2000; Watson et al., 2003; Kanatous et al., 2008; Trumble et al., 2010; Kielhorn et al., 2013; Velten et al., 2013). Obtaining marine mammal specimens for research purposes is difficult and commonly small tissue biopsies from opportunistic captures during permitted research as well as subsistence hunts are utilized in physiology research (Reed et al., 1994, Kanatous et al., 1999, Polasek et al., 2006, Kanatous et al., 2008). For example, there are currently twice as many published papers, in the last fifteen years, utilizing biopsy sampling in marine mammal research as compared to post mortem specimens (Kanatous et al., 2002; Burns et al., 2005; Noren et al., 2005; Richmond et al., 2006; Clark et al., 2007; Spence-Bailey et al., 2007; Kanatous et al., 2008; Ponganis et al., 2002; Hindle et al., 2009; Prewitt et al., 2010; Shero et al., 2012**)**. However, the relative accessibility of stranded animals, whether from beach cast or incidental netting, promotes questioning of whether intramuscular biochemical data collected from post mortem species is feasible for tissue-based related physiological research in marine mammals. Previous studies using marine mammal tissues collected post mortem from strandings or beachcasts up to 30 hours post necropsy have been used and yielded publishable results (Moore et al., 2009; Hoffman et al., 2013). Most post mortem samples are collected within 6 h of death and samples from stranded animals have been shown to compliment data pertaining to physiological adaptations to depth and

pressure (Watson et al., 2003; Polasek et al., 2006; Watson et al., 2007; Lestyk et al., 2009). However, depending on the decomposition state of the tissue after even 6 h, data may be suspect due to integrity of the sample from potential decomposition.

Research utilizing marine mammal muscle tissue often focuses on aerobic and anaerobic capabilities commonly assessed by pathway enzymes and Mb in discrete skeletal muscle biopsies (Lenfant et al., 1970, Castellini et al., 1981, Kooyman et al., 1981, Reed et al., 1994, Kanatous et al., 1999, Kanatous et al., 2002; Ponganis et al., 2002; Burns et al., 2005; Noren et al., 2005; Richmond et al., 2006; Clark et al., 2007; Spence-Bailey et al., 2007; Kanatous et al., 2008; Hindle et al., 2009; Prewitt et al., 2010; Shero et al., 2012; Moore et al., 2014). Post mortem muscle proteolysis, however, may be a problem when assessing tissue for protein and enzyme quantification. There are a number of contributing factors to proteolysis, such as enzymatic activity (Geesink et al., 2006), temperature (Morita et al., 1996), disease state (Costelli et al., 2005), pH (Eijsink et al., 2005) and level of muscle atrophy (Kachaeva and Shenkman, 2012). It has been shown in skeletal muscle biopsies that actin and myosin proteins can degrade in as little as 15 minutes (Lecker et al., 1999). To determine the integrity of decomposed tissue and determine a time line of enzymatic activity and proteolysis at varying temperature regimes, skeletal muscle (l. dorsi: LD) was degraded in a controlled laboratory setting: standard storage temperature (4°C), room temperature (21°C) and mammalian body temperature (37°C) for up to 48 hours. Citrate synthase (CS) and lactate dehydrogenase (LDH) enzymes as well as the Mb protein were measured. Enzymes were chosen due to their common use in marine mammal literature as proxies for metabolic profiles (Castellini et al., 1981; Reed et al., 1994; Kanatous et al., 1999; Polasek et al., 2006;

Kanatous et al., 2008). Results indicated the stability of enzymes and proteins was greater in standard storage (4°C) when compared to higher temperatures (21°C, 37°C). Enzymatic activity varied between individuals of the same species, sex and age class, as well as between different muscle groups of individuals. Therefore, caution should be taken when using stranded skeletal muscle tissue for enzymatic assays. Proteins, such as Mb, appeared more stable when exposed to time and temperature. In addition, freeze-thaw cycles did not affect enzyme activity, indicating that acute thawing episodes less than 24 hours apart did not adversely impact protein quantification as well as indicating the necessity of immediate storage at freezing temperatures. In other words, data suggest that in order to maintain muscle integrity, immediate cold storage is necessary, and even risk of repeated freeze-thaw is less detrimental than exposure to temperatures above 4°C for even short periods of time. To our knowledge, this is the first study determining the degree and rate that marine mammal tissue becomes unusable for skeletal muscle physiological investigations.

Material and Methods

Animals

Skeletal muscle biopsies were obtained from 5 live adult male Northern elephant seals (NES, n=5). In addition, California sea lions (CSL, *Zalophus californianus*) (n=2), one NES (n=1) and one harbor seal (*Phoca vitulina*) (n=1) were sampled immediately post mortem. For a terrestrial mammal comparison, Biceps femoris skeletal muscle was extracted from a *Rattus rattus* (Petco, Waco, TX, USA). All samples were stored at -80°C for long-term storage. NES muscle samples were collected during muscle

physiology research on Año Nuevo State Reserve (California) during beach haulouts in 2013. Seals were anesthetized with an intramuscular injection of Telazol, a teletamine/zolazepam HCl, at 1.0mg/kg dosage (Crocker et al., 2012). Doses of ketamine and diazepam were also intravenously administered as needed to maintain immobilization (Fort Dodge Laboratories, Ft. Dodge, IA, Crocker et al., 2012). LD muscle was accessed via incision post sterilization of the outer skin area (2cm^2 area). Biopsies were administered (30-50mg) in the mid-belly of the muscle at identical locations in all NES, using local Lidocaine® (1ml; Whitehouse Station, NJ, USA) using a 6mm cannula (Depuy, Warsaw, IN, USA, Crocker et al., 2012). All muscle samples were immediately sealed in a cryovial and stored in a dry vapor shipper (Thermo Scientific). Samples were shipped overnight to Baylor University held in a -80°C ultrafreezer for long-term storage. Samples were collected under NMFS permit #14636 issued to D. Crocker. All procedures were approved by Sonoma State University IACUC.

Assay Protocols

NES skeletal muscle was subjected to two temperatures (4ºC, 21ºC) and four time intervals (hours 3, 12, 24, 48) during decomposition studies. Skeletal muscle was homogenized using a Bullet Blender (0.5mm Zirconium oxide beads, Next Advance, NY USA) in Sigma CellLytic MT buffer (Sigma Aldrich). Total protein content was determined using a Bradford assay (Beckman Coulter DU 730) or NanoDrop (NanoDrop Technologies, Inc. ND-1000 Spectrophotometer) procedure (mg/ml).

Citrate synthase assays were performed on a Beckman Coulter DU 730 spectrophotometer according to the Sigma Aldrich protocol (CS0720). Activity level (umole/min/g) was determined at a 412nm wavelength by combining the protein sample,

assay buffer, Acetyl CoA solution, dithiobis-nitrobenzoic acid (DTNB) solution and oxaloacetic acid (OAA) solution. The reaction of Acetyl CoA and OAA to citrate followed the colorimetric reaction of DTNB to TNB, forming a yellow color. The reaction was followed for 1.5min to measure the baseline. OAA was added, and after another 1.5min the total activity was measured. Results were based on the change in absorbance at 412nm over one minute and the extinction coefficient of TNB. Effects of four freeze-thaw cycles on skeletal muscle enzyme concentration were performed on the rat muscle only; where enzyme assays were compared and muscle was maintained at -80ºC during the freeze event and thawed for less than 10 minutes, just until muscle could be homogenized. Thaw cycle measurements were made 24 hours apart, thus muscle was fully refrozen before the subsequent measurement was taken.

Lactate dehydrogenase assays were performed according to Sigma Aldrich protocol (MAK066) on a spectrophotometric multiwell plate reader (Beckman Coulter, DTX880). The quantification of LDH was based on the catalyzation of the interconversion of pyruvate and lactate, which reduced NAD to NADH and was detected at 450nm. Protein samples were mixed with LDH assay buffer and a master reaction mix containing buffer and LDH substrate. Samples were then rotated between incubation at 37°C and measurements every 5min until activity surpassed the highest standard.

Myoglobin assays were completed using methodology modified by Kanatous et al., (1999) from Reynafarje (1963). Homogenates were diluted in phosphate buffer (.4 M KPhos at pH 6.6) and centrifuged at 28,000g for 50min. Supernatant was bubbled with carbon monoxide for 3min before being measured for spectrophotometric absorbance.

Absorbance was measured at two wavelengths (538 and 568nm) and Mb concentration was calculated in mg/g wet muscle mass.

Statistical Analysis

Results were analyzed with statistical significance at $p \leq 0.05$ alpha level for Tukey- Kramer HSD and Student's t testing. Results are presented as means ± standard deviation (SD).

Results

Citrate synthase activity level (umole/min/g) was measured for four adult male NES (Fig. 3.1, Table 3.1; n=4). Measurements were made over 48h at 5 different time points (0, 3, 12, 24, 48h) at 4ºC and 21ºC (Fig. 3.1). There was a significant difference for time 0 for the 21ºC group, where other time points were significantly lower in enzymatic activity (Fig. 3.1; Tukey-Kramer HSD, $p<0.05$). For biopsies maintained at 4ºC, CS enzymatic activity increased over time, where the12h sampling time was significantly higher than other sampling times (26.1 ± 5.0, Fig. 31; Tukey-Kramer HSD, $p<0.05$). Percent change in CS activity (4ºC) level over 4 sampling times (0-3, 3-12, 12-24 and 24-48h; Table 3.2) fluctuated from a 42.3% increase to a 46% decrease, demonstrating the enzyme's instability over time. Overall, there was a statistical difference between animals maintained at 4ºC versus 21ºC, from 3h to 48h (Fig. 3.1; ANOVA, $p<0.05$). Percent change in CS activity level at 21ºC decreased with the largest negative percent change from 0 to 3h (-78.1%) as compared to the 21ºC from 3 to 12h (82.4%). CS activity level was also measured in a rat locomotory muscle (Biceps femoris; Fig. 3.2). CS activity in the rat muscle was elevated at 4ºC when compared to 37

ºC (Fig. 3.2; Student's t test, $p<0.05$). Therefore, the CS activity level was greater in both NES and rat tissues maintained at 4ºC. For the 37ºC rat group, CS activity was significantly decreased after time 0 (Fig. 3.2; Tukey-Kramer HSD, $p<.05$).

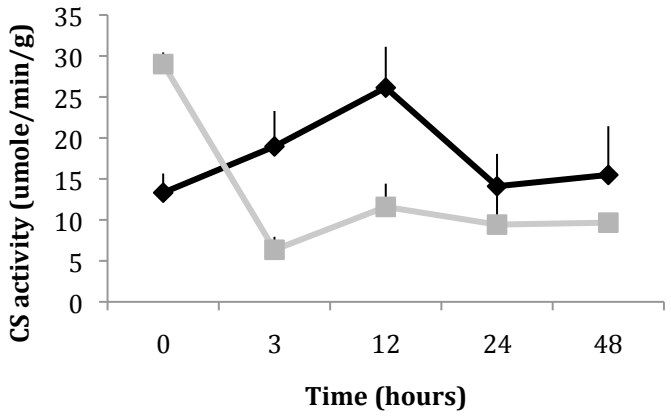

Figure 3.1: Citrate synthase activity level (umole/min/g) ± SD in the longissimus dorsi muscle of 4 Northern elephant seal adult males. Measurements were made over 48 hours at two different temperatures (black 4°C (M3, AM12) and gray 21°C (AM13, M4)) indicating the greater stability of the enzyme at 4°C versus 21°C in biopsied muscle tissue. N=4.

Lactate dehydrogenase activity level (miliunits/ml) was measured for four adult male NES (Fig. 3.3, Table 3.1; n=4), where sampling times (0, 3, 12, 24h) indicated a relative greater enzyme concentration at 4ºC when compared to 21ºC (Fig. 3.3). LDH activity levels were significantly greater at time 3h when compared among other times (Fig. 3.3; Tukey-Kramer HSD, $p<0.05$) indicative of a spike in the enzymatic activity at hour 3 for both temperatures. Overall, there was a statistical difference between animals maintained at 4ºC versus 21ºC, from time frame 0 to 24h (Fig. 3.3, Student's t test, $p<0.05$). Therefore, the LDH activity level was higher in tissues maintained at 4ºC over a 24-hour period.

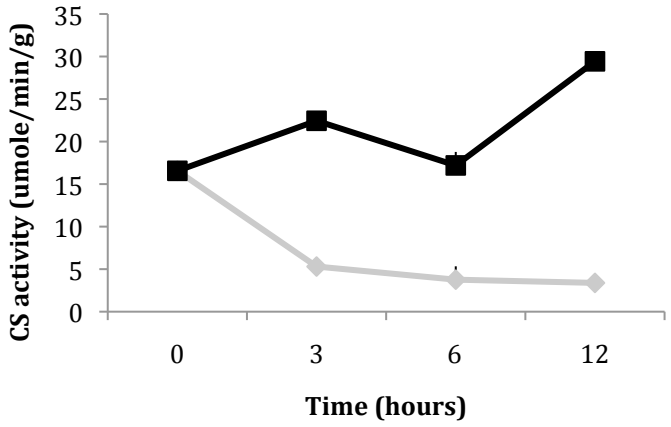

Figure 3.2: Citrate synthase activity (umole/min/g) ± SD level in a rat locomotory muscle (n=1). Measurements were made over 12 hours at two temperatures (black 4° C, gray 37° C), indicating the greater stability of the enzyme at 4° C as opposed to 37° C. N=1.

Table 3.1: Average citrate synthase (umole/min/g) ± SD and lactate dehydrogenase (milliunits/ml) ± SD for 4 adult Northern elephant seals over 24 and 48 hours at two different temperatures: 4°C (M3(CS), M2(LDH), AM12) and 21°C (M4, AM13). N=5.

Time	CS at 4° C	CS at 21°C	LDH at 4° C	LDH at 21°C
0	13.3 ± 2.3	29.0 ± 1.4	48.9 ± 11.0	39.6 ± 6.3
3	18.9 ± 4.3	6.3 ± 1.6	127.6 ± 63.2	54.1 ± 5.6
12	26.1 ± 5.0	11.6± 2.8	89.4 ± 13.4	50.6 ± 50.2
24	14.1 ± 3.9	9.4 ± 4.8	28.7 ± 17.9	30.4 ± 30.7
48	15.5 ± 5.9	9.7 ± 0.9	---	---

The effects of four freeze-thaw cycles on the degradation of rat skeletal muscle showed no statistical difference in CS activity when thawed 24 hours apart (Table 3.3;Tukey-Kramer HSD, $p>0.05$). Therefore, the CS activity level was relatively stable when the muscle is maintained at -80ºC, even after four consecutive freeze-thaw cycles. However, variability increased with consecutive freeze-thaw although there was no statistical difference among the mean values.

Table 3.2: Citrate synthase and lactate dehydrogenase activity percent change over four time frames at two temperatures (4°C, 21°C). N=5.

Time frame (hours)	CS 4°C %	CS 21°C %	LDH 4°C %	LDH 21°C %
0 to 3	42.3	-78.1	160.9	36.7
3 to 12	37.8	82.4	-29.9	-6.5
12 to 24	-46.0	-18.6	-67.9	-39.8
24 to 48	9.8	2.5	---	---

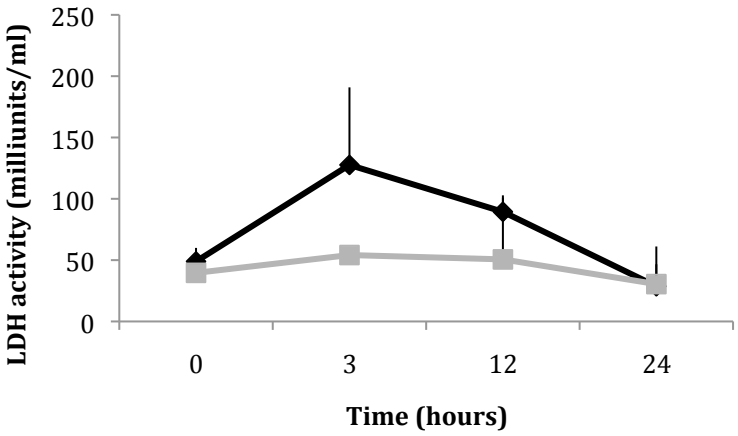

Figure 3.3: Lactate dehydrogenase activity level (miliunits/ml) ± SD in the longissimus dorsi muscle of 4 Northern elephant seal adult males. Measurements were made over 24 hours at two different temperatures (black lines at 4°C and gray lines at 21°C) indicating the greater stability of the enzyme at 4°C versus 21°C in biopsied muscle tissue. N=4.

The change in Mb concentration during decomposition studies was measured in one elephant seal (n=1, ES3289) over 48h (Fig. 3.4, Table 3.4). Samples were maintained at 4ºC for 48h. Mb values increased from time 0 to 48h (Fig. 3.4, Table 3.4; Tukey-Kramer HSD, $p<0.05$). The percent change in average Mb over 48h was 27.3% indicating the relative slow increase in the Mb protein concentration when muscle is maintained at 4ºC over 48h (Fig. 3.4, Table 3.4).

Table 3.3: Average citrate synthase (CS) activity level (umole/min/g) ± SD for a rat after three freeze-thaw cycles. N=1.

Freeze Times	CS activity (umole/min/g)
Baseline	14.3 ± .2
Freeze Cycle 1	13.9 ± .5
Freeze Cycle 2	13.9 ± .6
Freeze Cycle 3	14.3 ± 1.5

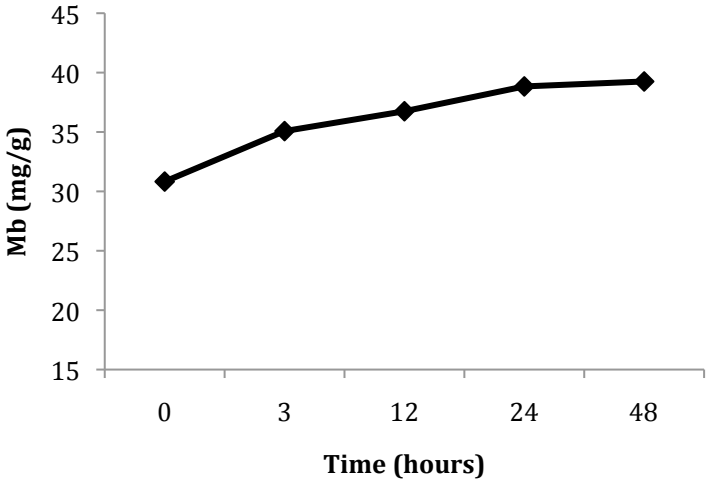

Figure 3.4: Northern elephant seal (ES3289) measured over 48 hours at 4° C for degradation of myoglobin (mg/g) ± SD. The figure indicates the relative stability of myoglobin when maintained at 4° C. N=1.

Table 3.4: Northern elephant seal (ES3289) average myoglobin (Mb) concentration (mg/g) ± SD over 48 hours, maintained at 4° C. N=1.

Time (hours)	Average Mb ± SD
0	30.8 ± 0
3	35.1 ± 1.2
12	36.7 ± .7
24	38.8 ± 0
48	39.2 ± .7

Citrate synthase activity (umole/min/g) was measured in both pectoralis and LD muscle in 4 marine mammals (n=8) to compare the relative activity level of the two muscles (Fig. 3.5). Both CSLs (CSL10281, CSL10305) demonstrated statistically increased levels of CS activity in pectoral muscle as compared to LD muscle (t test, $p<0.05$). The elephant seal (ES3289) showed elevated levels of CS in the LD muscle (t test, $p<0.05$). CS enzymatic activity in the harbor seal (HS2192) showed no significant difference in pectoral versus LD (t test, $p>0.05$). Therefore, for 3 of the 4 marine mammals the primary locomotory muscle utilized (pectoralis in CSL and LD in NES) had elevated CS activity (Fig. 3.5).

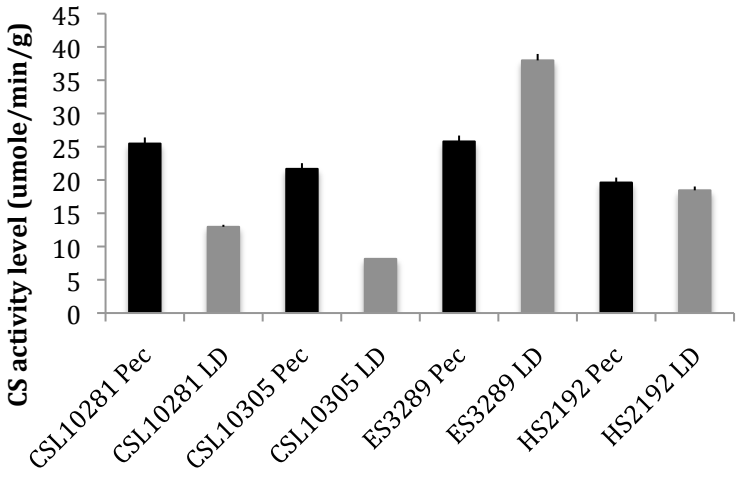

Figure 3.5: Citrate synthase activity level (umole/min/g) ± SD in the pectoral and longissimus dorsi muscle of 4 different marine mammals, indicating the general trend of higher values in pectoral muscle of those animals that predominately utilize pectoral for locomotion (California sea lions). N=4.

Discussion

Our results indicate that skeletal muscle tissue from post mortem specimens could be utilized for biochemical research, however enzymatic assays, such as for LDH, may not provide reliable data, as the enzyme tends to be relatively unstable over 24h at both room and refrigerator temperature. CS data were less variable than LDH when degraded

over time in post mortem tissue, although changes in the activity level may occur even when maintained at 4ºC. Storage at lower temperature (4ºC) provides more stability in enzymatic data overall. Mb showed less variability than enzymes and appeared to be relatively stable in comparison. In addition and unexpectedly, up to four freeze-thaw cycles did not negatively affect rat skeletal muscle tissue enzyme concentration. These data would indicate both the necessity for immediate (on-location) below 0ºC temperature storage of skeletal muscle tissue from live and recently deceased animals, as well as show that data derived from stranded animals or recently deceased marine mammals can be important for physiological research. During this study, every precaution was taken to ensure all biopsy samples were sterile and maintained in enclosed sterile cryovials.

The specific role of CS in the mammalian cell is to catalyze the condensation of acetyl coenzyme A and oxaloacetate, yielding citrate (Zhi et al., 1991). Enzyme stability is related to a number of factors, including pH, temperature and oxidative stress (Eijsink et al., 2005). Over 24h animals at 4°C (M3, AM12) demonstrated only a 6% change in enzymatic activity, whereas the animals maintained at 21°C (AM13, M4) showed a degradation of 68% over 24h. This would indicate the relative stability of CS at 4°C as compared to higher temperatures. When exposing rat tissue to an even higher temperature (37°C), CS activity level decreased significantly when compared to 4°C (Fig. 3.2), further indicating that increased temperature causes large percent degradation of the CS enzyme. These data complemented previous findings that temperature increases have been shown to influence the relative instability of the CS enzyme in swine (Zhi et al., 1991). CS activity varied among the NES regardless of time and temperature (Fig. 3.1).

This correlates well with previous research indicating that muscle tissue post mortem can be variable even within the same muscle (Bendall, 1973; Eriksson et al., 1980). Previous research suggests that CS activity decreases at temperatures above 40°C (Zhi et al., 1991) and inactivation of the enzyme is reached at 43°C (Jakob et al., 1995). Thus, CS is very sensitive to thermal stress (Jakob et al., 1995). Furthermore, the addition of substrates, or formation of complexes (CS-OAA) can affect the thermostability of the enzyme and even reactivate the enzyme (Zhi et al., 1991; Jakob et al., 1995), indicating the enzymes reactive nature to a number of influences. These data suggest that CS may not be a reliable enzyme as a proxy for aerobic metabolism in even fresh biopsy samples given the large amount of variability among individuals of the same species, age class and sex (Fig. 3.1). Although freezing temperatures can affect muscle in various ways, including formation of ice crystals, dehydration and denaturation of proteins (Jeong et al., 2011), in the current study it was shown that freezing is the best method of preservation. Evidence for frozen storage was demonstrated by rat skeletal muscle repeatedly frozen four times, resulting in no difference detected in mean CS activity among freeze-thaw cycles (Table 3.3). However, some caution should be taken with successive freezes as variability (SD) increases with increasing freeze event.

The LDH enzyme has been found to be sensitive to thermal conditions (Adler and Lee, 1999). LDH plays an integral role in anaerobic metabolism by catalyzing the conversion of pyruvate to lactate and oxidizing NADH (Coquelle et al., 2007). It was found that LDH in serum stored at 4°C for 4 days degraded 15% from its initial activity (Jacobs et al., 1986). These findings are similar to another study which found degradation of LDH to be 5% at 4°C after 24h (Wagner et al., 1992). In this study, for NES subjected

to both 4°C and 21°C, we found a much different result, where LDH demonstrated instability at 4°C (Fig. 3.3). Over 24h, NES maintained at 4°C had an enzymatic activity decrease of 41.3% (Table 3.1). For NES muscle maintained at 21°C, percent degradation was 23.2% over 24h (Fig. 3.3, Table 3.1). Any relative increases in activity level during the 24h period may indicate increased enzymatic activity level due to exposure to elevated temperatures. Although LDH was less variable at 4°C when compared to 21°C (Fig. 3.3) the high variability and high percent change of LDH at both temperatures should negate the use of this physiological measurement from post mortem tissue. In addition, and much like CS, the variability among animals of the same species, age range and sex may indicate that LDH as a proxy for metabolism in even fresh tissue may not be valuable.

Muscle biopsy sampling is currently the protocol most widely used to obtain fresh samples from live marine mammals (Reed et al., 1994, Kanatous et al., 1999, Polasek et al., 2006, Kanatous et al., 2008). This method is also widely used in the veterinary clinical setting, as samples maintained at low temperatures retain muscle architecture necessary for histochemistry (Stanley et al., 2009). In veterinary clinical cases, as with stranding response, tissue biopsy samples are often collected and stored at different temperatures for shipping and/or long-term storage (Stanley et al., 2009). Therefore, unfortunately, due to circumstances beyond the control of the researcher, frozen samples are often thawed during shipping and refrozen before analysis. It has been reported from studies in the food industry that the freeze-thaw process can be detrimental to the overall quality of the tissue sample (Jeong et al., 2011). These results focused primarily on lipid oxidation and maintenance of meat color in regards to temperature alteration (Tang et al.,

2006; Jeong et al., 2011) and did not quantify enzymes or protein. Previous data indicate that immediate storage of skeletal muscle (canine) at 0°C up to 30h showed little changes in histochemical properties (Braund and Amling, 1988), indicating that immediate storage at freezing temperatures is preferred over storing at room temperature or chilled temperatures over 24 h (Stanley et al., 2009). These data suggested that samples should be removed from the animal immediately and put into long-term storage at -80°C, as enzymatic activity can change at 4°C in as little as 3 hours, but tends to be relatively stable at -80°C, even when repeatedly thawed for biochemical analysis. However, some caution should be taken with increased variability in data (SD) with successive freezes.

In this study, we observed that Mb at 4°C for up to 48h remained relatively stable in comparison to the enzymes measured. The Mb concentration changed maximally by 8.4 mg/g as compared to LDH which fluctuated by 98.9 miliunits/ml at 4°C (Table 3.1, 3.4). In addition, the standard deviation of the Mb values were lower (1.2 mg/g maximum) than SD values for either CS or LDH, indicating less deviation and more consistency (Table 3.1, 3.4) The stability of Mb has been shown to be species-specific due to structural differences and more specifically to the differing amino acid sequences (Thiansilakul et al., 2011). Mammalian Mb has been shown to be thermally stable over a 24-hour period (Chen-Levy et al., 2005). During this study, we found a similar trend in Mb stability (Fig. 3.4); no statistical difference in Mb content for up to 48 h ($p>.05$). Relatively small increases in Mb concentration may be indicative of Mb oxidation (to metmyoglobin) which can give muscle a brown discoloration and has been shown to increase in freeze-thawed beef in the food industry (Jeong et al., 2011). We suggest that Mb is a relatively stable and reliable protein for post mortem biochemical analyses even

48h post sampling, when maintain at 4°C or below. Although, some small fluctuations in concentration should be expected as Mb is oxidized.

In this study, enzymatic activity varied between species as well as in different muscle groups of the same animal (Fig. 3.5). CS activity was measured in pectoral and LD muscle in 3 individual pinnipeds from different species, to determine if there was species-specificity and determine differences in CS activity levels between muscle groups. The greatest level of CS activity was found in the NES LD, a primary locomotory muscle of this deep diving phocid seal (Le Boeuf et al., 2000; Kuhn et al., 2009; Robinson et al., 2012; Fig. 3.5). For the CSL, a relatively shallow diving otariid species (Feldkamp et al., 1989; Weise et al., 2006), greater levels of CS were found in pectoral muscle when compared to the CSL LD (Fig. 3.5). This provides evidence of the primary locomotory muscle in this species (Feldkamp, 1987). No difference was found between CS activity in pectoral and LD muscles for the shallow diving phocid, the harbor seal (Fig. 3.5). This may be indicative of the relative equal reliance on fore and hind flippers of the harbor seal versus the elephant seal, or the relative lack of utilization of pectoral muscle as compared to the CSL. We suggest that enzymatic activity levels collected within the same animal can yield valuable data on the limb preference for locomotion, and although enzymatic data may not be reliable in a species wide comparison, on an individual level it is. These data also correlate well to what is know about the approximate total body oxygen stores of these animals: NES greatest at 97ml/kg (Kooyman, 2009), harbor seals at 64 ml/kg (Clark et al., 2007) and CSL least at 40ml/kg (Kooyman, 2009). In general, there was a difference in CS activity between species as

well as between muscle groups, indicative of the aerobic metabolism of the muscle group.

The aim of this study was to determine a map of protein degradation in marine mammal tissues collected post mortem to be used in subsequent physiological studies. We suggest that our findings substantiate the expedited use of post mortem tissue and provide concrete data that tissue is of greater value when refrigerated or frozen immediately following removal from an animal. Skepticism should be maintained for biochemical data when tissues are degraded at high temperatures, such as room temperature (21°C) and body temperature (37°C). Studies utilizing enzyme assays to determine aerobic capacity may find that values have a large range, even among biopsies of the same species and age class, potentially indicating the instability and fluctuating nature of these enzymes. We suggest that data collected from stranded (post-mortem) marine mammals can be valuable, and promote the quantification of stable proteins, such as Mb, if tissues have been maintained at 4°C for under 48 hours. In addition, for the most reliable data, we suggest the immediate storage of tissues at long-term temperatures (-80°C) onsite, immediately following removal from the animal. Even the imminent freeze-thaw cycle will maintain more integrity of tissues as compared to leaving tissues at refrigerator or room temperature for extended periods of time. In the case of an absence of a freezing method (liquid nitrogen or -80°C freezer) the next best method of transport for samples is chilled (ice packs) wrapped in dry gauze and enclosed in a plastic container (Staley et al., 2009). It is important to note the difficulty in obtaining marine mammal samples and the scientific value that stranded tissue can provide. Thus, it is imperative that we maintain the integrity of valuable samples for physiological research.

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CHAPTER FOUR

Macroscopic and Microscopic (Histological) Descriptions of the Tracheal Rings of Harbor Seals and Northern Elephant Seals, with Special Attention to Elastic Fiber Quantification and Volume Changes

Abstract

Excised tracheas from two species of diving pinnipeds, harbor seals (*Phoca vitulina*) and Northern elephant seals (*Mirounga angustirostris*; NES), and one dog were evaluated for width, continuity of cartilage, volume and elastic fiber concentrations (N=7). Data indicated the importance of elastic fibers to the respiratory function of diving mammals. Pinnipeds had greater percentage of elastic as compared to the dog. The continuity of cartilage also differed between pinniped species. The large gap in the tracheal rings of the NES, compounded with the concentration of elastic, may aid in the large compliance values previously measured. In general, the macroscopic and microscopic data presented here provide further evidence of compliant tracheal systems in diving pinnipeds and evidence that tracheal anatomy varies among even phocid seals and should be considered when making estimates for lung collapse measurements and alveolar gas exchange models.

Introduction

Microscopic analysis of the respiratory anatomy of pinnipeds is not widely represented in the literature, but evidence supports a network of cartilage-reinforced small airways for compression and expansion during dives and rapid ventilation at the surface (Denison and Kooyman, 1973; Boyd, 1975; Stewardson et al., 1999; Gray et al.,

2006; Smodlaka et al. 2006). The microscopic anatomy of diving mammals is important from a histopathological and comparative perspective by elucidating respiratory diving adaptations such as reinforced airways or elastic fiber integration among different species (Gray et al., 2006).

The suborder Pinnipedia (Carnivora) encompasses the Family Phocidae (earless seals), Otariidae (seal lions and fur seals) and Odobenidae, with one distinct difference being the presence of the ear pinnae (Berta, 2008). Morphological and molecular data suggest that the pinnipeds are monophyletic, of single origin, where the closest relatives are the ursids (Perrin et al., 2009). However, there is some evidence for an ursid-mustelid ancestry as well as a sister relationship between odobenids and otariids (Perrin et al., 2009). The evolutionary split between ursids and pinnipeds was approximately 35 million years ago (mya), although the region is debatable between the North Pacific and southern North America (Arnason et al., 2006; Higdon et al., 2008; Perrin et al., 2009). The split between Phocidae (earless) and Otariidea (eared) did not occur until approximately 12 million years later (23 mya) (Higdon et al., 2008). Important to this study is the evolutionary relatedness of the Phocid family, as both harbor seals and NES belong to this family of pinniped. The phocid subfamilies are monophyletic and divergence (at approximately 14.6-16 mya) occurred in a short time frame (Arnason et al., 2006; Higdon et al., 2008) separating the harbor seal into one subfamily, which includes Atlantic species like the hooded seal (*Cristophora cristata*) and the gray seal (*Halichoerus grypus*) and the NES into another subfamily encompassing Pacific species like the Weddell seal (*Leptonychotes weddellii)* and the Hawaiian monk seal (*Monachus schauinslandi*) (Higdon et al., 2008).

Despite similar monophyletic lineage (Higdon et al., 2008), there is a wide variety of tracheal morphology in the pinniped suborder (Moore et al., 2014). Trachea anatomical differences even exist among the phocid subfamilies, from the horseshoe-shaped gross tracheal ring anatomy of the leopard seal (Gray et al., 2006) and Northern elephant seal (present study; Moore et al. 2014), to the overlapping cartilage ring of the harp seal (Moore et al. 2014) and the whole tracheal rings of the harbor seal and gray seal (Moore et al. 2014).

The compliance, or compressibility, of the trachea may have implications for the amount of air volume that can be contained in the tracheal dead space during a deep dive (Bostrom et al., 2008). During deep diving, the chest of the seal compresses under pressure, forcing air from the alveoli up into the reinforced upper airways (Scholander, 1940; Leith, 1979). It was previously thought that the pinniped trachea was incompressible, however, radiography has shown a different result, where the trachea can compress in diameter and air volume up to 20% (Kooyman et al., 1970). This tracheal compression may dictate the depth of lung collapse, where a more compressible or compliant trachea allows for deeper lung collapse depth (Bostrom et al., 2008; Fahlman et al., 2009). Lung compression and subsequent cessation of gas exchange is of great importance for an animal diving with onboard gas, otherwise subject to dangerous levels of nitrogen, oxygen and carbon dioxide (Fahlman et al., 2009).

Morphological differences present among the pinniped tracheas may not be a result of evolutionary pressure for dive depth, but due to differences in the ability to forcibly respire post dive. Deeper diving species such as Weddell seals and NES are known to exhale prior to diving (Kooyman et al., 1970) whereas shallower diving

species, sea lions and fur seals, have been reported not to exhale prior to a dive (Kooyman, 1973). In addition, some pinniped species have been observed exhaling during underwater descent (Hooker et al., 2005), which may prevent arterial oxygen levels from becoming lower than venous levels and depleting tissues of oxygen, ultimately resulting in unconsciousness (Fahlman et al., 2009). Therefore, different species may progress through a dive with very different alveolar gas volumes (Bostrom et al., 2008) which may greatly affect gas exchange and lung collapse. While recent data are emerging describing the gross morphological differences among pinniped tracheas (Moore et al. 2014), the basic histological details of the trachea for many species is still unknown. Gaining insight pertaining to the histology or morphology of pinniped tracheas in correlation to dive behavior may lend insight into dive depth and lung collapse mechanisms.

The aim of this study was to describe the macroscopic and microscopic (histological) tracheal anatomy of both the harbor seal and NES. A specific focus was on the integration of elastic fibers and the quantification of elastic in different locations along the trachea, cross-sectionally and longitudinally. Here, we aimed to utilize tracheal histology to support previous compliance data suggesting pinniped tracheas are compressible. We also described the volume change from the relaxed state to the compressed state of the trachea. Results indicated a greater displacement of volume for NES tracheas, which complemented previous research using pressure-volume measurements. Overall, we determined that microscopic anatomy could be valuable in support of pressure-volume based compliance data, as well as lend evidence to models for respiratory gas exchange and lung collapse depth.

Material and Methods

Animals

Three age classes of harbor seals, (pup, juvenile, adult) (N=3), were collected by the International Fund for Animal Welfare (IFAW) in Yarmouth Port, MA, and the NOAA Observer program. Three pup/weaner NES (N=3) were collected by the Marine Mammal Center in Sausalito, California. All animals were necropsied according to each stranding unit's procedure and tracheas were extracted and frozen at -20°C prior to shipping on ice packs. One canid trachea was used for a terrestrial mammal control (female chow mix, N=1).

Histology

Excess tissue was dissected and removed from the trachea and not considered in analysis. Tracheas were weighed (g) and measured for length (mm) just distal to the cricoid cartilage and proximal to the first bifurcation of the trachea into primary bronchial airways (Fig. 4.1,4.2). The total number of cartilage rings were counted and the trachea was sectioned into equal portions: proximal, mid and distal for each cross-section (Fig. 4.1, 4.2) and longitudinal trachea analysis (Fig. 4.3 A,B,C). Tracheal rings from each section were dissected from the whole trachea, oriented in cassettes, and fixed in a solution of 10% formalin for approximately 24h followed by submergence in ethanol (90%) for shipping and long-term storage. Tracheal rings were paraffin-embedded and sectioned into 5um-thick sections according to methods from Massachusetts Histology Services. Sections were mounted to glass slides and stained for hematoxylin and eosin (H&E), Trichrome and elastic stain (Massachusetts Histology Services). H&E was used

for orientation and sample adequacy, Trichrome for continuity and quantity of cartilage and the elastic stain for the evaluation and quantification of elastic fibers.

Histomorphometry

From each stained cross section, the circumference was divided into four equal quadrants. Among each quadrant, 12 areas from the lumen to the exterior cartilage were evaluated. Each image was digitized using a Nikon camera mounted to a microscope (Ci90). NIS elements software (calibrated for magnification) was used for morphometric analysis, yielding basic quantitative histomorphometrics. The arithmetic mean, minimum and maximum were used to obtain accurate values for width in the four quadrants of the trachea analyzed. Tracheal width was measured in um at a magnification of 20X.

Elastic Fiber Quantification

The surface area (um) of elastic fibers in each of the four tracheal quadrants (40x) was further evaluated using a superimposed grid of squares (each box with an area of .27mm^2). Five rectangular areas overlay the lumen and were analyzed for pixilation density using image analysis software (Photoshop, Fig. 4.4). The five areas made up approximately 20% of the total ring area. Images were scored and accuracy was determined by counting pixel percentages in each test area three times. The coefficient of variation was calculated to be less than 1%. Elastic fiber values are reported in % to allow comparison of surface area between tracheal locations (proximal, mid, distal), age class (pup, juvenile, adult) and species (harbor seal, elephant seal, dog) scaled for mass.

Statistical Analysis

To ensure normality, elastic fiber percentages were arcsign transformed whereas tracheal widths were log transformed. The statistical significance was maintained at or below the .05 alpha level for analysis of variance (ANOVA) and Tukey-Kramer HSD testing. Results are presented here as means ± standard deviation (SD) or standard error (SE).

Results

The tracheal length and weight of three age classes of harbor seals are as follows: 180mm (20.88g) in the pup, 190mm (24.41g) in a juvenile and 300mm (58.8g) in an adult. Each portion of the trachea was approximately equal in length (60mm in pup and juvenile and 100mm in adult) with each (proximal, mid and distal) section consisting of 20-22 rings, making the tracheas 60 to 64 rings in total (Fig 4.1, 4.2). The trachea of the harbor seal has discontinuous and heterogeneous cartilage rings as the trachea progresses towards the lungs (Fig 4.1; Moore et al., 2014). Proximal rings can be continuous or whole and subsequent rings tend to have one or two gaps in the cartilage, where gaps consist of connective tissue and often elastic fibers. Mid and distal rings also tend to have "slips" (Moore et al. 2014) where one side of the cartilage overlays another to form a compliant anatomy (Fig. 4.1; Moore et al. 2014). The length of the gap or "slip" can vary considerably from location in the tracheal length and also the animal's age, where the minimum gap found was 214um and the largest was a "slip" approximately 6 mm in length. Thus, there was large variability in the extent of the discontinuity of cartilage for harbor seal tracheas. Whole tracheal rings were found in proximal sections of the tracheas but were not consistently found in association with one specific cartilage ring.

Histologically, the submucosa has mixed glands and merges with the perichondrium just adjacent to the hyaline cartilage (Fig 4.1; Young et al., 2006). The cells of the trachea of the harbor seal also have elastic fibers that can be found in the lamina propria, similar to a human trachea (Fig 4.1; Young et al., 2006). However, elastic fibers seem to be thicker and more diffuse in the harbor seal as compared to the human (Young et al., 2006). As in humans, the area between the cartilage and the submucosa can also contain a significant amount of elastic fibers (Fig 4.1; Young et al., 2006). On the external portion of the tracheal ring there is also a large band of elastic fibers that can be seen on the lengthwise micrographs of the harbor seal (Fig. 4.3). The lengthwise description relates to the tracheal length and is opposite to the cross-sectional aspect of tracheal ring histology. Lengthwise, tracheal rings were variably spaced within each location on the trachea (proximal, mid, distal) and among age classes, with maximum values reaching 977um and minimum values of 139 um (Fig. 4.3). The adult harbor seal (IFAW13-177) had significantly greater distance between tracheal rings (ANOVA, Tukey-HSD, $p>0.05$) as compared to juvenile animals, which is consistent with scaling, but no statistical difference existed between spacing of rings in the proximal, mid and distal regions. Although ring spacing was not statistically significant for tracheal location, there were morphological differences, such as in distal locations where the cartilage overlapped in an overlay fashion (Fig. 4.3) indicative of a more compressible region.

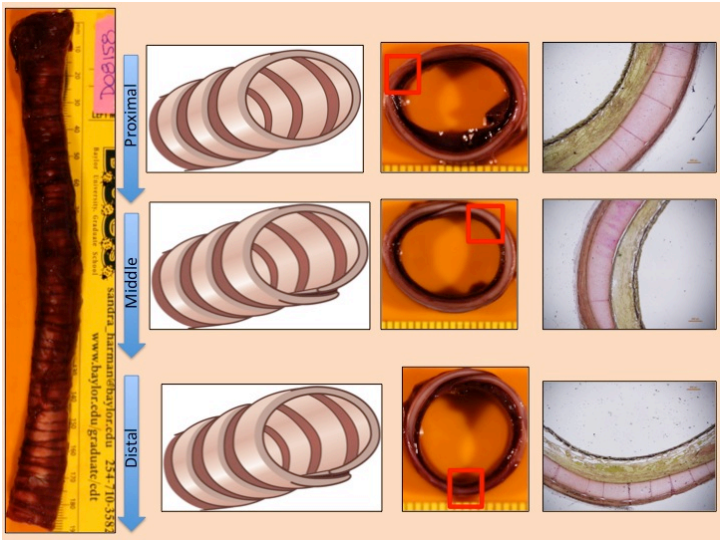

Fig. 4.1: Full trachea (left), graphical representations (Moore et al., 2014) and associated gross and corresponding microscopic (20X) images stained for elastic (black coloration) of tracheal rings in the proximal, mid and distal regions. Pictures demonstrate the inconsistency of cartilage and change in continuity as the harbor seal (IFAW13-141) trachea progresses towards the lungs. In addition, large quantities of elastic fibers can be seen most frequently in longitudinal bundles in the lamina propria. N=1.

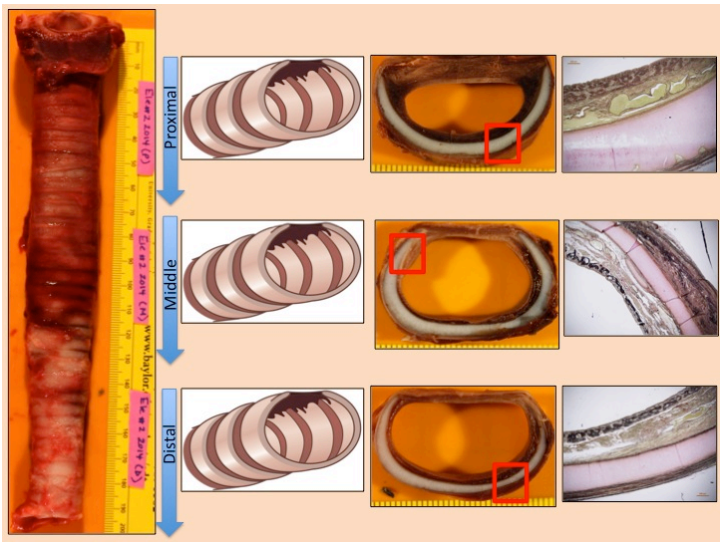

Fig. 4.2: Full trachea (left), graphical representations (Moore et al., 2014) and associated gross and corresponding microscopic (20X) images stained for elastic (black coloration) of tracheal rings in the proximal, mid and distal regions in the juvenile Northern elephant seal (Ele2). Images demonstrate the morphological consistency as the trachea progresses towards the lungs as well as the large quantity of elastic bundles longitudinally throughout the lamina propria and external to the hyaline cartilage. N=1.

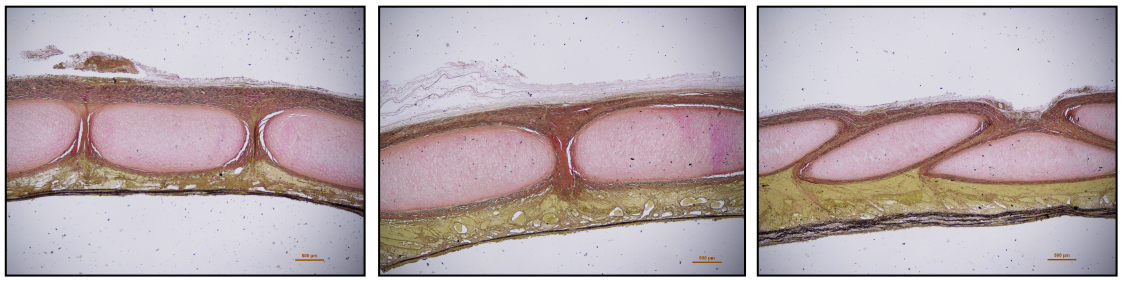

Fig. 4.3 A,B,C: Lengthwise cartilage sections of the trachea in three general locations (proximal (A), mid (B), distal (C)) in a juvenile harbor seal (DO8158). Microscopic images (20X) demonstrate a change in compliance as the trachea progresses towards the lungs, as proximal and mid rings are spaced with a gap as compared to distal rings, which overlap. The presence of dark elastic fibers throughout the length of the trachea can also be seen (elastic stain). N=1.

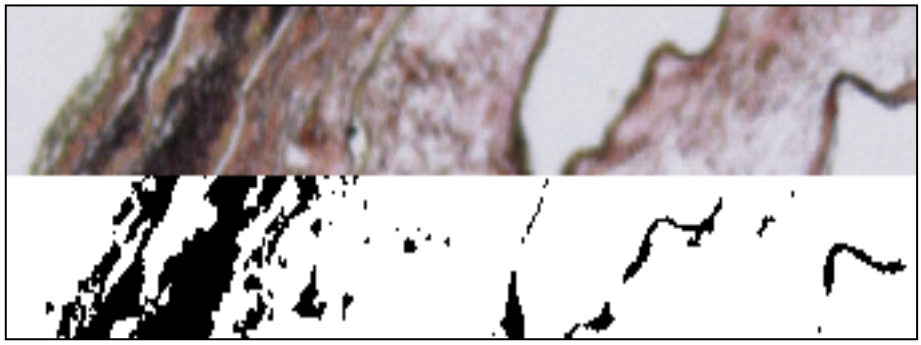

Fig. 4.4: Light micrograph (top; 40X) and complementary pixel representation (bottom; Photoshop) of elastic fibers, demonstrating the accuracy of counting the elastic fibers in a trachea cross section. N=1.

The Northern elephant seal tracheas had weight and length in the same range as the harbor seal, weighing 45.3 g and measuring 185 mm, but with fewer total rings (36) (N=1). The trachea of the NES forms a C-shape or "horse-shoe shape" (Moore et al., 2014) and does not change in morphology as the trachea progresses towards the lungs (Fig. 4.2). All portions (proximal, mid and distal) consisted of similar morphology, where rings formed an incomplete circle separated by a thick muscular paries membranaceus (Fig 4.2). Elephant seals also share the typical mammalian tracheal cell structures of a pseudostratified columnar cell inner lining, a lamina propria thick with elastic fibers, a submucosa with apparent glands, and a second layer of elastic forming just above or with

the perichondrium (Fig. 4.2; Young et al., 2006). Elephant seals also have a dense layer of elastic fibers externally on the trachea (Fig. 4.2).

Tracheal cartilage width (Table 4.1, 4.2) was analyzed in four distinct quadrants among the circumference of each ring. Widths varied greatly for both the harbor seals (minimum mean value of 581 ± 224 um (pup), maximum mean value of 963 ± 87 um (pup), Table 4.1) and elephant seals (minimum mean value of1168 ± 339, maximum mean value of 2172 ± 176 um, Table 4.2) as tracheal rings taper to gaps and "slips", forming an overall inconsistently wide structure. For harbor seals, there was no statistical difference between the tracheal widths of all three animals (ANOVA, Tukey-Kramer HSD, $p>.05$) but there was a significantly greater width for proximal and mid rings as compared with distal rings (proximal=mid>distal, ANOVA, Tukey-HSD, $p<0.05$). In addition, the canid had significantly larger tracheal ring width than all harbor seals (ANOVA, Tukey-HSD, $p<0.05$) when scaled for mass (Fig. 4.5). For the NES, proximal rings were bigger than both the mid and distal rings and NES tracheal widths were all larger than the dog (p>m=d, ANOVA, Tukey-HSD, $p<0.05$) when scaled for mass (Fig 4.5).

Table 4.1: Tracheal average, SD, minimum and maximum widths (um) for three morphological locations (proximal, mid and distal) and three age classes (pup: IFAW13-141, juvenile: DO8158, adult: IFAW13-177) of harbor seals. N=3

	Proximal			Mid			Distal		
	Pup	Juv	Adult	Pup	Juv	Adult	Pup	Juv	Adult
Mean	869	914	728	963	823	806	581	594	686
SD	232	310	186	87	226	137	224	96	212
Minimum	412	343	306	874	479	593	347	342	426
Maximum	1099	1188	1068	1136	1099	1016	994	702	974

Table 4.2: Tracheal average, SD, minimum and maximum widths (um) for three morphological locations (proximal, mid and distal) of three juvenile Northern elephant seals. N=3

	Proximal			Mid			Distal		
	Ele2	ES3247	ES3208	Ele2	ES3247	ES3208	Ele2	ES3247	ES3208
Mean	2172	1347	1601	1667	1224	1309	1475	1168	1208
SD	176	344	217	689	258	339	391	339	215
Minimum	1823	614	1300	636	897	680	933	585	826
Maximum	2388	1687	1892	2386	1568	1686	2068	1544	1568

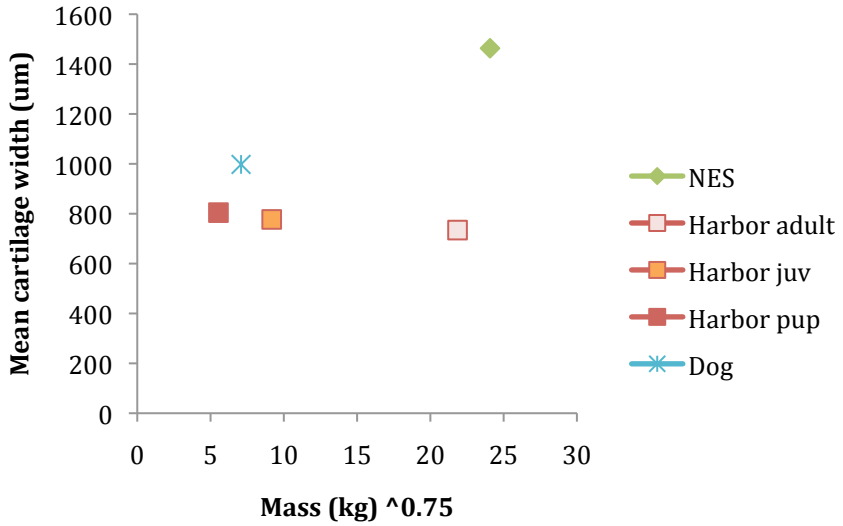

Fig. 4.5: Mean cartilage width in two species of pinniped and one dog. Elephant seals have a larger mean cartilage width than both the dog and harbor seal when scaled for mass. N=7.

Percent elastic fibers were quantified in all species (n=7) using pixel counts from Photoshop, where black pixels were used to represent elastic fibers (Fig 4.4, Table 4.3, 4.4). Both harbor seals and elephant seals had elastic fibers present in tracheal rings, although the pattern of fibers throughout the circumference of the tracheal rings was patchy in distribution, lending to high variability in data. Both the elephant seals and harbor seals showed significantly more elastic fibers in the trachea than the dog (ANOVA, Tukey-HSD, $p<0.05$) when scaled for mass (Fig. 4.6). Although elephant seals

showed no statistical difference ($p>.05$) in elastic per location on the trachea (proximal, mid, distal), harbor seals showed significantly more elastic in proximal rings in all age classes (Fig. 4.7; ANOVA, Tukey-HSD, $p<0.05$). Harbor seals also showed a scaling relationship between elastic fibers, where adults had significantly larger amounts of elastic fibers than pups and juveniles (Fig 4.8, ANOVA, Tukey-HSD, $p<0.05$). Because elastic fibers were variable between animal and location, a coefficient of variation was calculated (35%) and indicated a large variation in data. However, the large test area (approximately 20% of the tracheal space) indicates that variability is consistent with elastic distribution and not methodology. The quadrant system used in this study was also considered a variable, and thus was statistically evaluated, but no difference was detected among quadrants (ANOVA, Tukey-HSD, $p>0.05$) for any species.

Table 4.3: Percent elastic fibers (%) in the trachea of three age classes (pup: IFAW13-141, juvenile: DO8158, adult: IFAW13-177) of harbor seals. N=3

	Proximal			Mid			Distal		
	Pup	Juv	Adult	Pup	Juv	Adult	Pup	Juv	Adult
Mean	11.1	11.9	45.7	11.3	12.4	14.5	15.6	13.9	12.5
SD	4	7	16	3.6	2	3	4	13	5

Table 4.4: Percent elastic fibers (%) in the trachea of three juvenile Northern elephant seals. N=3

	Proximal			Mid			Distal		
	Ele2	ES3208	ES3247	Ele2	ES3208	ES3247	Ele2	ES3208	ES3247
Mean	19.0	9.2	12.7	8.0	13.6	15.1	11.5	14.8	15.6
SD	9	6	6	4	8	3	2	6	6

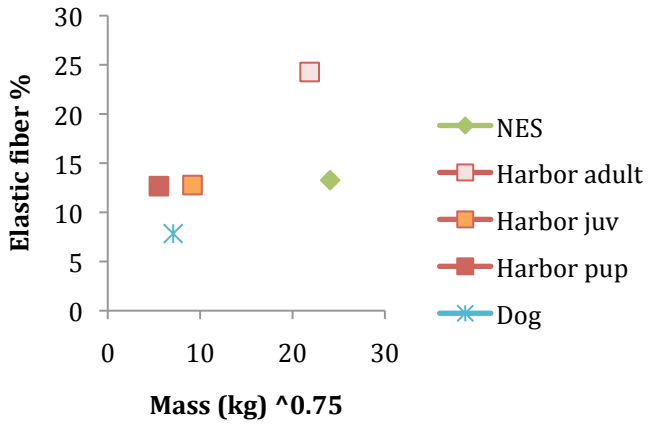

Fig. 4.6: Percent elastic fibers in two species of pinniped (harbor seal and Northern elephant seal) and one dog. The harbor seal has a larger mean average elastic fiber concentration (for all age classes) than both the Northern elephant seal and dog when scaled for mass. N=7.

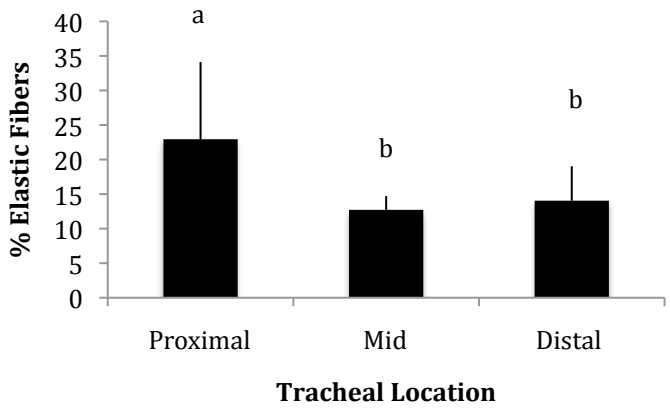

Figure 4.7: Percent elastic fibers ± SE in three locations on three harbor seal tracheas (proximal, mid and distal). Letters denote the statistical difference between locations, demonstrating the significantly large amount of elastic fibers in the proximal location of the tracheas as compared to the mid and distal locations. N=3.

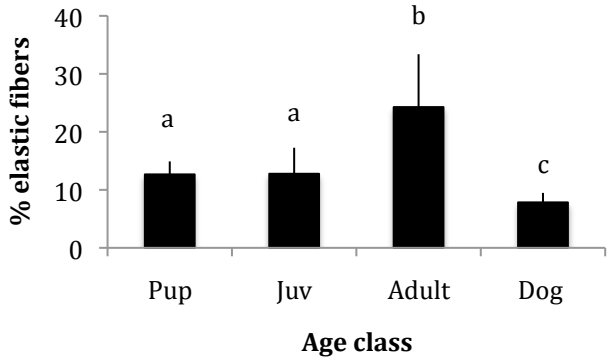

Figure 4.8: Percent elastic fibers ± SE in three age classes of harbor seal as well as one dog. Letters denote statistical differences, where pups and juveniles are similarly lower in percent elastic than adults and all seals are larger than the adult dog. N=4.

Both the radius of each ring and the length of the tracheal section (proximal, mid, distal) were measured and used to determine the estimated tracheal section volume (ml; Table 4.5, 4.6). Given section volume, a whole tracheal volume was also calculated (ml; Table 4.5,4.6). Comparisons in volume were made between the "normal" state at the time of histology versus a hypothetical "deflated", or compressed, state under pressure (Table 4.5, 4.6). Deflated volumes were determined by subtracting the distance of the gap in cartilage from the total circumference. A smaller volume was then calculated and representative of a "deflated" trachea (i.e. represents a trachea with no gap in cartilage) (Table 4.3, 4.4). The opposite value, expansion or inflation, was not considered, as a numeric value designated to the amount of expansion the gap can allow cannot be construed from the histology. Thus, only the normal state and the deflated state were evaluated and compared in this study.

Basic respiratory variables were calculated for all animals (Table 4.7) based on the allometric equations known for respiration in mammals (Stahl, 1967; Schmidt-Nielsen, 1984). From these lung calculations, and given that tracheal dead space is thought to be a constant fraction of lung volume in mammals (Schmidt-Nielsen, 1984),

data was extrapolated for tracheal elastic work per minute (g cm min^{-1}) and total compliance (ml(cm H_2O^{-1})) for all animals (Table 4.7, Fig. 4.9, 4.10).

Table 4.5: Estimated tracheal volume (ml) for three age classes of harbor seals (pup: IFAW13-141, juvenile: DO8158, adult: IFAW13-177). N=3

Estimated Volume (ml)	IFAW13-141		DO8158		IFAW13-177	
Tracheal fill	Normal	Deflated	Normal	Deflated	Normal	Deflated
Proximal	7.96	6.92	6.56	6.56	43.00	42.51
Mid	8.59	7.50	9.90	9.15	37.39	36.22
Distal	6.23	6.07	6.56	4.42	49.76	47.65
Total tracheal volume	22.78	20.50	23.01	20.12	130.13	126.37

Table 4.6: Estimated tracheal volume (ml) for one Northern elephant seal (Ele#2, juvenile). N=1.

Estimated Volume (ml)		
Tracheal fill	Normal	Deflated
Proximal	22.81	10.6
Mid	26.17	17.01
Distal	25.33	14.17
Total tracheal volume	74.32	41.8

Table 4.7: Calculations for each animal given the known respiratory variables for the lungs (Stahl ,1967; Schmidt-Nielsen, 1984) and extrapolated data for the trachea of the harbor seal (three ages) and Northern elephant seal given that the trachea is thought to be a constant fraction of the lung volume in mammals. N=4.

Animal	Lung volume (ml)	Lung Elastic work (g cm min^{-1})	Lung Compliance ml(cm H_2O^{-1})	Trachea volume (ml)	Trachea Elastic work (g cm min^{-1})	Trachea Compliance ml(cm H_2O^{-1})
NES	4795.8	26298.0	128.5	74.3	407.4	2.0
Harbor adult	4183.7	23784.1	112.4	130.1	739.6	3.5
Harbor juv	1226.5	9641.6	33.7	23.01	369.0	1.3
Harbor pup	601.2	5706.0	16.7	22.78	216.2	0.6

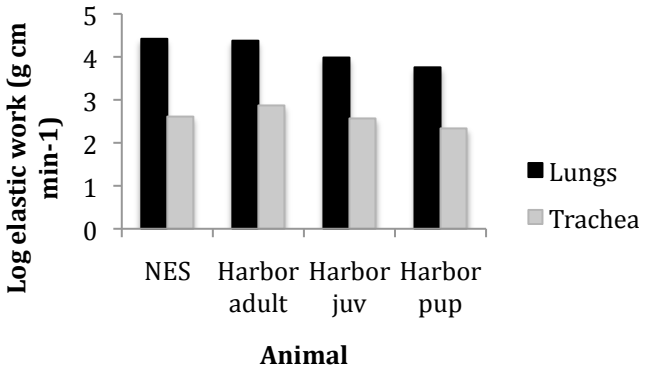

Figure 4.9: Elastic work (g cm min^{-1}) for the lungs (black columns) and trachea (grey columns) of three harbor seals and one Northern elephant seal. Tracheal elastic work (g cm min^{-1}) data are extrapolated from known respiratory variables (Stahl, 1967; Schmidt-Nielsen, 1984) based on mass and the concept that the trachea is a constant fraction of lung volume in mammals (Stahl ,1967; Schmidt-Nielsen, 1984). N=4.

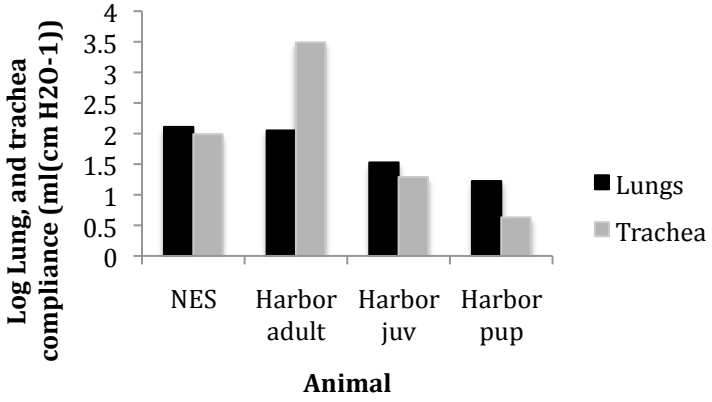

Figure 4.10: Tracheal compliance (ml(cm H_2O^{-1})) for the lungs (black columns) and trachea (grey columns) of three harbor seals and one Northern elephant seal. Tracheal compliance (ml(cm H_2O^{-1})) data are extrapolated from known respiratory variables (Stahl ,1967; Schmidt-Nielsen, 1984) based on mass and the concept that the trachea is a constant fraction of lung volume in mammals (Stahl ,1967; Schmidt-Nielsen, 1984). N=4.

Discussion

The gross morphology of the harbor seal trachea as it progresses towards the lungs is variable in the continuity of cartilage (Fig 4.1; Moore et al., 2014). Although this has been previously described, histological evidence of the cartilage and associated elastic fibers is lacking. In addition, the lengthwise histomorphology of the harbor seal

trachea (Fig. 4.3) and its potential affect on tracheal compliance has not been described. Discontinuous gaps in the cartilage rings and "slips" (Moore et al. 2014) were found throughout the length of the harbor seal trachea, however, proximal rings tended to be more whole than distal regions. Differences in individual ring morphology may be consistent with different rings, where alternate sampling locations could yield variable results, or ring morphology may vary with age as has been described in the Cape fur seal (Stewardson et al., 1999). Previous findings have described distal portions of the trachea as more compliant than proximal (Moore et al., 2014). Here, in the lengthwise anatomy (Fig 4.3) we see evidence for this finding, as distal rings are more overlapping (Fig 4.3C) than proximal and medial rings (Fig. 4.3A,B), likely indicative of a different and more complaint anatomy distally, as opposed to the more evenly spaced rings proximally (Fig 4.3 A,B).

The NES trachea has also been described in gross terms (Moore et al., 2014) but has not been investigated histologically in reference to quantification of elastic fibers or volume measurements. Cartilage rings tended to be more discrete lengthwise than the harbor seal and homogeneous in morphology (Fig. 4.2). C-shaped or "horseshoe" shaped rings comprised the entire length of the NES trachea (Moore et al., 2014). Histologically, the trachea was very similar to a human trachea with the presence of a large trachealis muscle (Fig 4.2), which joined the free ends of the rings (Young et al., 2006) and appeared to attached to cartilage rings on the external region of each end (Fig 4.11 B,C). The attachment location of the muscular membrane is important as under compression and expansion it can pull ends of the C-shape together allowing for a smaller or larger internal diameter (Fig. 4.11 B,C).

Figure 4.11 A,B,C: Discontinuities in cartilage in a harbor seal (A; small gap stained with elastic) and Northern elephant seal (stained for trichrome (B) and elastic (C)) at 20X (B,C)and 100x (A) showing the large trachealis muscle (red in the trichrome stain; B) in the paries membranaceus of the elephant seal and the presence of elastic fibers in the small gap of the harbor seal trachea (A) as well as in the elephant seal trachea (C). N=2.

The width of the tracheal rings varied considerably within the circumference of an individual and among individuals of the same species (Fig. 4.4, Table 4.1). For example, there was no difference in the width of harbor seal tracheal rings regardless of age. However, there was a significantly larger width for proximal and mid rings as opposed to distal rings, in the harbor seal trachea (Fig. 4.7). This finding lends evidence to the hypothesis that distal portions of the harbor seal trachea are different morphologically and are more complaint than proximal portions (Moore et al., 2014).

Elastic fibers in the harbor seal trachea consistently ran throughout the circumference of the ring in the lamina propria as longitudinal bundles, as well as at the boundary of the submucosa and perichondrium and throughout the external circumference (Fig. 4.1, 4.3, 4.5, 4.11 A). Therefore, the tracheal rings were heavily invested with elastic fibers cross-sectionally as well as longitudinally (Fig. 4.5). Elastic fibers were patchy in distribution, and the percentage in each ring quadrant varied. This statistical variability may be consistent with an irregular pattern to elastic integration, where fibers run longitudinally as well as horizontally and diagonally. More elastic was found in proximal sections of the harbor seal tracheas, which was counter to the concept

that distal regions are more compliant and would therefore need more "stretch" (Fig. 4.7). The greater distribution of elastic in proximal areas was also seen in the NES, however, and thus may be a pinniped-wide adaptation for high ventilation rates.

Some discontinuities in cartilage in the harbor seal trachea were consistent with elastic fibers, where fibers stretch across the distance of the gap (Fig. 4.11 A). This finding suggests that elastic fibers may aid in the compliant nature of the tissue, as elastic may allow a degree of stretching and compression at the gap. Lengthwise portions of the harbor seal trachea (in proximal, mid and distal regions) demonstrated a prominent boundary of elastic fibers on the external portion of the trachea (Fig. 4.3). This suggests a compliant nature to the trachea lengthwise (as opposed to cross-sectional expansion and compression). These are valuable data in the determination and discussion of tracheal compliance, as it adds another dimension to the overall change in air volume. In other words, the trachea is capable of both longitudinal and cross-sectional expansion and compression.

The percentage of elastic fibers was larger in the harbor seals as compared to the NES (Fig. 4.6, Table 4.3, 4.4). This finding may seem counter to the compliance data previously described (Moore et al., 2014), which found NES tracheas to be more compliant. If elastic fibers were correlated to compliance, the NES would have more elastic. However, the NES has a large muscular paries membranaceus, with elastic fibers present at the end of the ring and throughout the membrane, in addition to a much larger gap between cartilage rings in general (Fig 4.11 C). We suggest that although the harbor seal had more elastic fibers, its mechanism of compression is different. The harbor seal relies more on elastic compression between gaps in cartilage and longitudinal

compression, whereas the NES trachea compresses primarily through the paries membranaceus muscle.

Elastic fibers also tended to be greater in adult animals (harbor seals) as opposed to younger animals (Fig. 4.8). This finding was consistent with other mammals, where elastin increases with age regardless of overall age-related degradation of elastic properties, not only in the trachea but also in arteries and the larynx (Kahane et al., 1987; Palecek and Jezová, 1988; Hammond et al., 1998; De Andrade et al., 2012). Another reason for the disparity in elastic could be artifact of histology and is a limitation to this study. Some lamina propria with quantifiable elastic may be missing due to histology processing, which would affect the total elastic percentage calculations. However, authors counted whole tissue sections to prevent this error. In addition, both pinniped species had greater elastic fiber percentages than the dog in this study (Fig. 4.6), which correlates well to known behavioral differences. Although the trachea is utilized for the same physiological purpose in all mammals, seals have high ventilatory rates (Denison and Kooyman, 1973) and deep dive depths that would require more tracheal recoil and compression (Kooyman et al. 1970), both physiological events which demand elastic fibers in the respiratory tract.

Given the compliant nature of the pinniped trachea, the change in volume from the normal state (at histology) versus the pressurized "deflated" state was measured to determine the percent change in tracheal air volume during a dive (Table 4.5, 4.6). Calculated volumes were consistent with floodable volumes previously measured with water displacement (Moore et al., 2014). Differences in the volume for normal versus deflated state for harbor seals ranged from 10% in the pup, 12% in the juvenile and 3% in

the adult. This may indicate that juvenile animals have a more compliant trachea than adults based on the length of gaps in cartilage. Due to sampling constraints, only one NES was measured for volume and demonstrated a decrease of 44% from the normal state to the pressurized/deflated state. The larger percent change of the NES is consistent with its larger gap in cartilage (Fig 4.3, 4.11), its large quantity of elastic fibers (Fig 4.6), and its compliant trachea as compared to a harbor seal (Moore et al., 2014). The large percent volume change of the NES is also consistent with previously reported radiographs showing compression of the tracheal diameter of NES to be 54% (Kooyman et al., 1970). This represents a very large percent change in volume from the normal state to the deflated state, indicating that the trachea is highly compressible in NES. This compressibility would have great implications for lung collapse depth, where a more compliant trachea allows for greater depth of lung collapse (Bostrom et al., 2008, Fahlman et al., 2009). Therefore the harbor seal has a relatively shallow depth of collapse as compared to the NES. A deeper depth of lung collapse for the NES (Bostrom et al., 2008; Fahlman et al., 2009) may be indicative of the deep dive depths these animals reach (Le Boeuf et al., 2000; Kuhn et al., 2009; Robinson et al., 2012) and the need to manage onboard gases and avoid symptoms of decompression sickness. The counter intuitive concept of collapsing lungs later in a dive may actually allow the NES to avoid decompression sickness (Fahlman et al., 2009). In contrast, the harbor seal would be constrained by its physiology and collapse its lungs at shallower depths, which correlates well with their routinely shallow dive regime (Gjertz et al., 2001).

Basic respiratory variables were also calculated for three harbor seals and one NES (N=4). Variables considered were lung volume, lung elastic work and lung

compliance, all values that have a determined allometric relationship to mass (Stahl,1967; Schmidt-Nielsen, 1984). Given the measured mass of the animals, lung parameters could be determined (Table 4.7) and yielded consistent results where the largest animal, the NES, also had the largest lung volume, elastic work and compliance. Utilizing these calculations, along with the concept that the tracheal dead space is a constant fraction of the lung volume (Schmidt-Nielsen, 1984), we also determined the elastic work (Fig. 4.9) and compliance for the trachea (Fig. 4.10). Although Schmidt-Nielsen (1984) and others have determined the tracheal volume (dead space) to have the same affect on ventilation in all mammals, we disagree. Here, we find inconsistency in what should be a scaling relationship of body mass to tracheal elastic work and compliance. In other words, the larger NES, with higher lung values for elastic work and compliance, should therefore also have higher values for tracheal work and compliance. This theoretical result is not reflected in our data. Tracheal values for the adult harbor seal are larger than the NES in both tracheal work and compliance (Fig. 4.9, 4.10). However, among just harbor seals the tracheal values scale consistently with mass indicating a species-specific correlation (Fig. 4.9, 4.10). The conflicting results between theoretical data (here) and previous measurements using pressure-volume transducers (Moore et al., 2014), indicates that the microscopic anatomy of the trachea might play a large role in the determination of tracheal respiratory variables, as well as have an affect on lung ventilation. In other words, the concept that the trachea is a constant fraction of lung volume also assumes that all tracheas compress and expand similarly. However, given differing microscopic anatomy, we see that calculating tracheal elastic work and compliance using a given equation does not correlate to real life results (Moore et al., 2014). Allometric respiratory

calculations may, however, be valuable among individuals of the same species. If small anatomical differences such as elastic fiber integration and continuity of cartilage can disrupt the theoretical tracheal volume to lung volume constant, than the vast anatomical differences seen in the pinniped family (Moore et al., 2014) may demand a recalculation of this fractional relationship for pinnipeds. Therefore, species-specific microscopic differences in pinniped tracheas can affect volume and compliance measurements and should be taken into account for allometry and modeling.

The data gathered in this study through histological investigation further supports the concept that the compliance of the harbor seal trachea changes as it approaches the lungs (Moore et al., 2014). Through distal lengthwise morphological differences (Fig 4.3C) and unequal distributions of elastic fibers through the tracheal length (Fig. 4.7), we determine that the pressure-volume measurements previously made for the harbor seal (Moore et al., 2014) are further supported by the histology here. In addition, the NES trachea, demonstrated greater volume changes from the normal state to the deflated state, indicative of greater compliance and in support of previous modeling data (Moore et al., 2014). The tracheal morphology of pinnipeds is important to understanding the overall respiratory functions of pinnipeds during a dive and further histomorphological investigations encompassing more species could yield more information about how these animals manage gas at depth and avoid decompression sickness. Additional histomorphological data could also be in support of lung volume modeling and aid in elucidating small species-specific differences in diving respiratory adaptations.

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CHAPTER FIVE

Conclusions

Limitations and Contributions to the Field of Marine Mammalogy

Data collection in the field of marine mammalogy has improved vastly in the last 50 years due to advances in data logging instrumentation, which allows for more advanced modeling capabilities for aerobic dive limit (ADL), lung collapse estimates and behavioral analysis (Goldbogen and Meir, 2014). However, a constant limitation continues to arise in every research project: sample size. This is also a limitation to this thesis. Quality of tissue samples is also of concern in marine mammalogy, as discussed in Chapter Three, and although many studies utilize fresh samples, any time post mortem has the potential to alter the physiological status of the muscle. This dissertation makes a major contribution to the field of marine mammalogy in that the time frame for muscle tissue degradation is now mapped for future comparisons. These data could be utilized as a baseline for stranded or bycaught samples. In addition, the recommendations for sample storage based on degradation results will provide sample-handling instructions to stranding units. Even given these research limitations, marine mammal samples are unique and invaluable to the study of hypoxia and repeat exposure to environmental stressors.

The second contribution to the field of marine mammalogy was made through the novel description of the fiber type population of the deep-diving Northern elephant seal (NES) (Kuhn et al., 2009). The diving patterns of this species has been well described (LeBoeuf et al., 2000; Robinson et al., 2012), but the intramuscular biochemical

adaptation to deep long-duration diving, specifically the fiber type profile, had until now not been described. We additionally linked the absence of ischemia reperfusion (IR) injury post dive to a specific muscular adaption. This is a novel comparison in the field, as the previous belief was that the absence of IR injury was solely due to enhanced reactive oxygen species scavenging (Elsner et al., 1998; Vázquez-Medina et al., 2006). Although a bridge to human clinical research does not ultimately dictate value for data, the introduction of a novel mode for avoidance of IR in the field of marine mammalogy is a major contribution, and connecting human and animal research can improve funding avenues and introduce complementary work for the future.

Finally, contributions were made to the smaller, yet growing, field of marine mammal respiratory physiology. The depth at which marine mammals collapse their lungs is highly debatable, as species-specific differences are common and therefore absolute depth determination cannot be definitive. In addition, tracheal anatomy can be highly variable in different species, which ultimately dictates different depths of lung collapse. This makes modeling projects difficult and results variable. Even though "marine mammals" are often grouped colloquially together, they have different lineages, which may indicate that these animals have different ways of dealing with onboard gases. Any insight into the process of lung collapse/reinflation, via tracheal volume and anatomy, in these animals is important to understanding overall body gas management, dive routine and avoidance of pathology.

Future Aims

The work completed in Chapter Two indicates that pinnipeds and cetaceans have different muscle fiber profiles in primary locomotory musculature. This is intriguing

because both groups face similar exposure to repeat ischemia and hypoxia. These data would indicate that pinnipeds and cetaceans have unique and different ways of dealing with these physiological stressors.

Some pinnipeds, like the NES, are deep long-duration divers with low-level muscular metabolic rates and predominately type I muscle fiber profiles. On the other hand, some cetaceans, like dolphins, and other pinnipeds (California sea lions) are sprinters with many type II fibers integrated into the fiber profile. This indicates that two muscular models for diving exist for mammals. If deep-diving seals like the NES (and Weddell seal) avoid IR injury via absence of type IIb muscle fibers, how are the sprinters (dolphins, California sea lions) avoiding injury? This dilemma is a future aim for research. A number of scenarios may play a role in answering this question; such as, perhaps cetaceans are not exposed to the same discrete level of ischemia during a dive. We, as researchers, sometimes assume that diving to depth translates into the same physiological adaptations, but with different lineages cetaceans and pinnipeds may have adapted different mechanisms. This dissertation only hints at the vast world of mammalian adaptations and only discovers the partial story of the incredible evolutionary tale that the anatomy and physiology of marine mammals can reveal.

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APPENDICES

APPENDIX A

California Sea Lion Fiber Typing Data

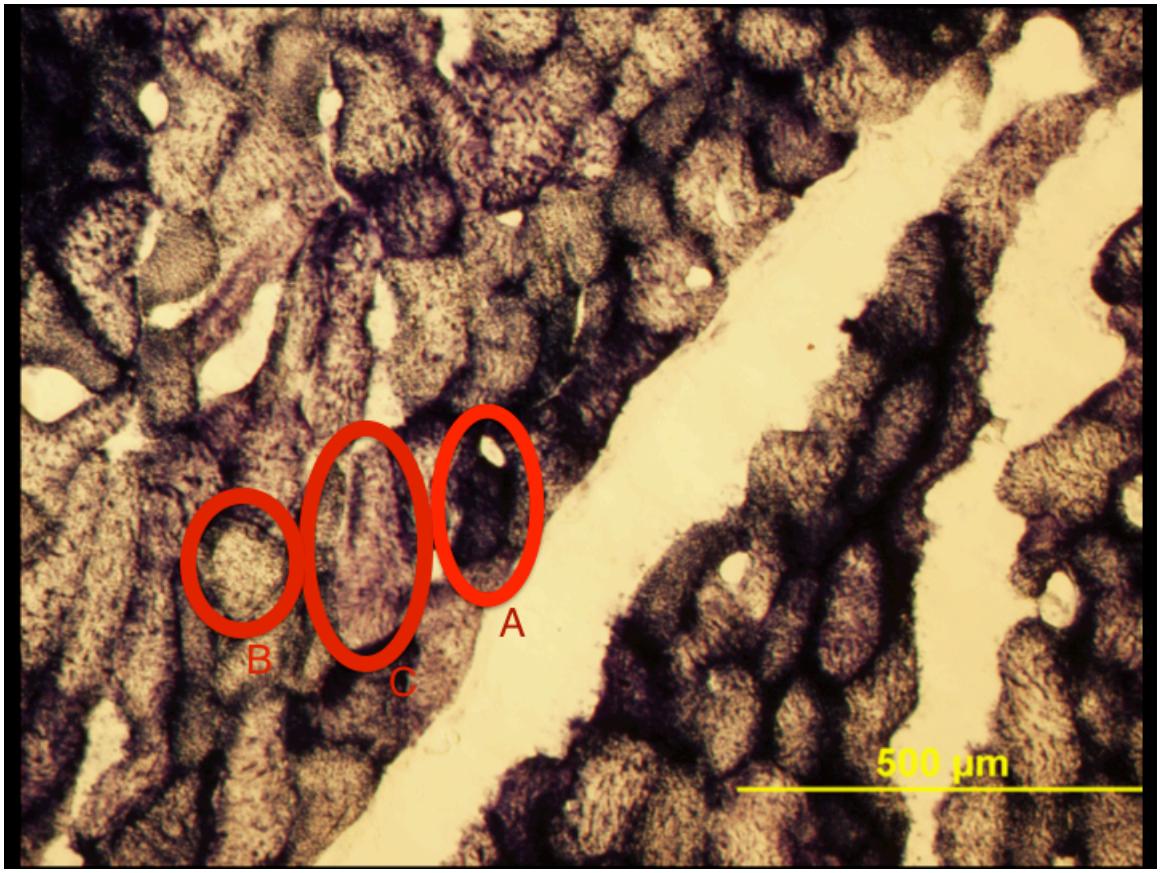

Figure 1 (**A**-**C**): Succinate dehydrogenase fiber typing profile from a California sea lion pectoral muscle revealing three fiber types: type I (**A**), IIa (**B**) and IIb (**C**), each designated with circles. The succinate dehydrogenase stain demarks each fiber type based on color intensity correlated with oxidative capacity, where type I oxidative fibers stain darkest and type IIb anaerobic fibers are lightest.

APPENDIX B

Mouse Fiber Typing Data

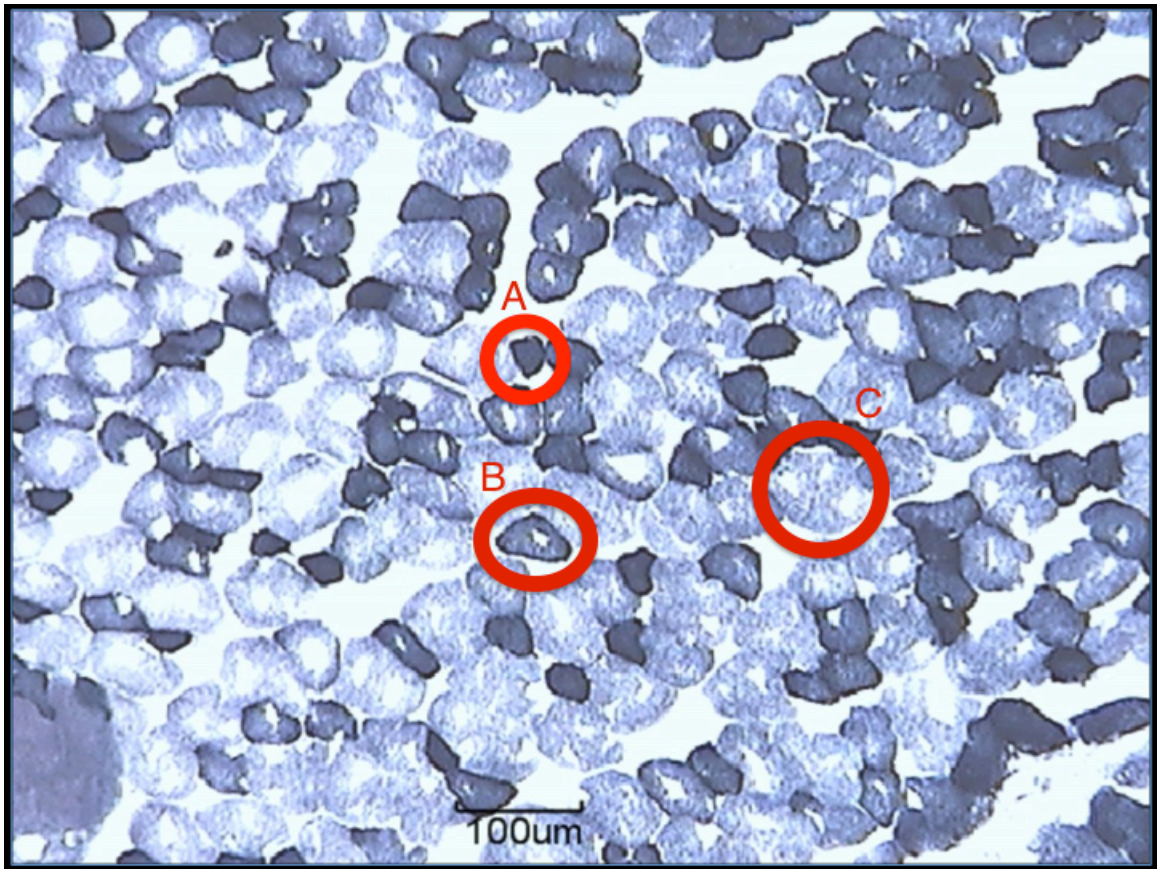

Figure 2 (**A-C**): Succinate dehydrogenase fiber typing profile from a mouse revealing three fiber types: type I (**A**), IIa (**B**) and IIb (**C**), each designated with circles. The succinate dehydrogenase stain demarks each fiber type based on color intensity correlated with oxidative capacity, where type I oxidative fibers stain darkest and type IIb anaerobic fibers are lightest.

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