IMMUNOHISTOCHEMICAL FIBER TYPING, ULTRASTRUCTURE, AND MORPHOMETRY OF HARBOR SEAL SKELETAL MUSCLES

A Dissertation

by

REBECCA REIKO WATSON

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

May 2004

Major Subject: Wildlife and Fisheries Sciences

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ABSTRACT

Immunohistochemical Fiber Typing, Ultrastructure, and Morphometry of Harbor Seal Skeletal Muscles. (May 2004)

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There is strong evidence that the skeletal muscles of pinnipeds are adapted for an aerobic, lipid-based metabolism under the hypoxic conditions associated with breath-hold diving. However, regional variations in mitochondrial density are unknown, and the few fiber typing studies performed on pinniped skeletal muscles are not consistent with an aerobic physiological profile. Thus, the objectives of this study were to (1) reexamine the fiber type distribution throughout the primary locomotory muscles of the harbor seal, and (2) to better understand the density and distribution of mitochondria in the locomotory muscles. Multiple samples from transverse sections of the epaxial muscles and a single sample of the pectoralis muscle of wild harbor seals were analyzed using immunohistochemical fiber typing and electron microscopy. Fiber typing results indicated that harbor seal epaxial muscles are composed of 47.4% type I (slow twitch, oxidative) fibers and 52.8%, IIa (fast twitch, oxidative) fibers. No fast twitch, glycolytic (type IIb) fibers were detected in the epaxial muscles or the pectoralis muscle. Mean volume density of mitochondria [V_v(mt,f)] was 5.6%, which is elevated over what would be predicted for a terrestrial mammal of similar mass. The elevated V_v(mt,f) had a high proportion of intermyofibrillar

mitochondria, a trait not normally found in the muscles of terrestrial mammals with elevated V_v(mt,f). These results provide further evidence that the elevated mitochondrial volume density in pinniped muscle decreases the oxygen diffusion distance between myoglobin and mitochondria to facilitate aerobic respiration in working muscles. In addition, analyses of heterogeneity revealed that the regions of the epaxial muscles that were located deep within the muscle showed a significantly higher V_v(mt,f) relative to those regions that were superficially-located. In contrast, there was no significant heterogeneity of fiber type detected in either plane of the epaxial muscles. Thus, there was a fine-scale pattern of spatial heterogeneity of V_v(mt,f) within the epaxial muscles that does not manifest in fiber type distribution, indicating that the fibers have similar oxidative capacities.

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CHAPTER I

INTRODUCTION

The aquatic environment imposes a host of physiological constraints on air-breathing mammals that dive and swim at depth. Yet there are a myriad of behavioral, morphological, and physiological adaptations exhibited by marine mammals that enable them to efficiently exploit the underwater habitat. Current knowledge of marine mammal diving physiology is largely based on data collected from studies involving pinnipeds (Otariidae, Phocidae, and Odobenidae). Of the pinnipeds, the phocids (the earless or "true" seals) are the most elite divers in both dive depth and duration. In phocids, the components of the classical dive response triad of apnea, bradycardia, and peripheral vasoconstriction (Scholander, 1940) are now known to be graded events that are correlated with dive duration and and appear to be under limited voluntary control. During a dive, seals reduce cardiac output by effecting a decrease in heart rate and, in some species, stroke volume (Ponganis et al., 1990). Diving heart rate can slow to 5-10 beats·min^{-1} or less in some of the deeper- and longer duration-diving species, but typical diving heart rates in phocids are normally 2-5 times lower than normal eupneic heart rate, regardless of swim velocity during the dive (Jones et al., 1973; Thompson and Fedak, 1993; Butler and Jones, 1997). Associated with bradycardia is the readjustment of circulation by peripheral vasoconstriction to maintain central arterial blood pressure. Thus,

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during the dive response, most organs and tissues undergo ischemic hypoxia due to oligaemia. The exceptions are the heart and central nervous system.

Vasoconstriction complicates the partitioning of oxygen and fuel substrates among organs and tissues during the inevitable progressive hypoxic hypoxia that occurs throughout a dive's duration. Thus, early experimentation with forcibly submerged pinnipeds ('simulated' dives) invoked an extreme dive response that resulted in elevated post-dive concentrations of circulating lactic acid (Butler and Jones, 1997). To explain these observations, it was hypothesized that pinnipeds were able to dive for extended periods of time because they possessed an enhanced anaerobic metabolic capacity compared to that of terrestrial mammals (Butler and Jones, 1997). However, Castellini et al. (1981) cast initial doubt on this theory when key glycolytic enzyme activities measured in the organs and muscles of a variety of marine mammals were similar to the activities measured in tissues collected from a variety of terrestrial mammals. Evidence of enhanced anaerobic metabolism, if present, was not manifest at the tissue level.

The introduction of new technology provided the opportunity to study diving physiology in seals under semi-natural conditions. Research on Weddell seals (*Leptonycotes weddellii*) diving freely from isolated ice-holes in Antarctica enabled Kooeyman et al. (1980, 1983) to establish a maximum dive duration that could be accomplished without incurring a net post-dive elevation in plasma lactic acid concentration. This concept became known as the aerobic dive limit (ADL) and varies according to species. Subsequent research on a variety of

pinnipeds showed that routine dives were largely aerobic in nature, with few exceptions (e.g., elephant seals (Boyd and Croxall, 1996)).

The observation that seals maintain aerobic metabolism during diving places emphasis on the ability of pinnipeds to efficiently manage their body oxygen stores. Aside from coping with the large hydrostatic pressures associated with depth, the ability to partition blood and muscle oxygen stores among tissues to balance oxygen supply with metabolic demand is paramount to successful diving and survival. Investigations of the structural and biochemical adaptations in locomotory muscles of pinnipeds that enable the efficient storage, transport, and utilization of oxygen provide empirical evidence that furthers the understanding of diving physiology.

Phocid swimming and the swimming muscle architecture

Kinematics

Aquatic locomotion in phocids is dissimilar to that of other mammals, with the exception of the odobenids (walrus). Thus knowledge of their swimming mechanics and the gross anatomy of their locomotory musculature aids in the establishment of structure-function relationships related to oxygen management. Unlike the cetaceans, which generate thrust *via* oscillating dorsiventral sweeps of the tail flukes, the phocids and the odobenids swim primarily by lateral undulations of the hind end. Phocids were previously thought to fall into the carangiform mode of fish swimming classifications, but kinematic analyses of seals swimming in flumes revealed propulsive characteristics that were more similar to thunniform swimming motions (Fish et al., 1988). During steady

swimming in seals, the lateral undulations of the posterior half of the body is accompanied by alternating dorsiflexion of the hind flippers, whose associated tibia-fibula complex resembles the caudal peduncle or tailstock of thunniform swimmers such as tuna (Fish et al., 1988). The foreflippers are normally adducted against the body during forward propulsion, however, they may be abducted to achieve directional changes and thus appear to serve as rudders. Howell (1929) also notes that captive seals that are loafing in the water use their pectoral flippers in a manner that bears resemblance to the water treading motions of humans. However, forward locomotion in phocids is primarily achieved by the well-developed epaxial musculature.

Locomotory muscle gross anatomy

A detailed dissection of the ringed seal (*Phoca hispida*) musculoskeletal system, accompanied by a comparison with otariid anatomy, was described in the early 20th century by Howell (1929). The dominant swimming muscles of the seal are primarily the erector spinae muscles (or long system of the back), which appear to be well-adapted for flexion in the lateral plane due to the prominent iliocostalis and longissimus muscles (Howell, 1929). Moreover, the vertebral spinous processes are low and the surface area of the vertebral articulations is high; both of these features restrict dorsiventral movement of the vertebral column to some degree (Howell, 1929). During swimming, the neck and anterior thorax of phocids act as a fulcrum for the active epaxial muscles of the posterior thorax and lumbar region (Howell, 1929). The posterior third of the epaxial musculature is particularly massive and originates at the ilium. Craniad, they

become less discrete and separate into slips that insert into various places along the ribs and vertebral processes (Howell, 1929).

Historically, the relative length and angle of pinnation of the epaxial muscles in pinnipeds have not been studied beyond qualitative observations during dissections. In bottlenose dolphins (*Tursiops truncatus*), the epaxial musculature appeared to be optimized for tendon displacement and velocity (angle of pinnation with respect to tendon= 15º), while the hypaxial musculature appeared organized to maximize force generation (Goforth, 1983, Roy et al., 1983). These distinct differences between the dorsal and ventral muscle systems lead the authors to hypothesize that the power stroke in dolphins is in the downward motion. Kinematic analyses and hydrodynamic modeling of dolphin locomotion later provided supporting evidence for this theory, at least during slow swimming speeds (<3.2 $m \cdot s^{-1}$) (Videler and Kamermans, 1985). In comparison, Howell's (1929) description and illustration of the phocid epaxial musculature indicate elongate fibers that are oriented approximately parallel to the vertebral column in a manner that is roughly analogous to the arrangement of the dolphin epaxial muscles. This anecdotal observation implies little evidence of muscle pinnation in the seal. Thus gross morphology of the epaxial muscles appears to be organized for high muscular excursion and contraction velocity rather than enhancement of force production.

Fiber type composition and distribution

In general, there is a dearth of information regarding fiber type composition and distribution in the skeletal muscles of pinnipeds. Two separate studies

found that harbor seal epaxial muscles have approximately 45% type I or slow oxidative fibers (SO), 45% type IIb or fast-glycolytic fibers (FG), and 10% type IIa or fast oxidative-glycolytic fibers (FOG) (Table 1) (Hochachka and Foreman, 1993; Reed et al., 1994). Deep divers such as the grey seal and Weddell seal had a higher population of type I or SO fibers compared to the harbor seal, whereas the swimming muscles of the more active otariids possessed a higher population of FG fibers (Table 1) (Reed et al., 1994; Kanatous et al., 2002).

All of these studies applied a variant of the original Padykula and Herman (1955) histochemical staining technique, which differentiates muscle fibers into type I (slow) and type II (fast) fibers by the activity of myofibrillar ATPase. Further division of the type II classification (into type IIa and IIb or FOG and FG) was achieved by staining for enzymatic indicators of aerobic capacity, such as NADH tetrazolium reductase and succinate dehydrogenase (Ponganis and Pierce, 1978; Hochachka and Foreman, 1993; Reed et al., 1994). Kanatous et al. (2002) applied a novel procedure by using an ATPase-based metachromatic stain in conjunction with immunohistochemistry to classify fiber types in the muscles of Weddell seals. Immunohistochemical (IHC) fiber typing exploits the antigenic properties of the myosin protein to differentiate between myosin heavy chain isoforms, which vary according to rate of contraction (Pette and Staron, 1990). The results of IHC fiber typing of the longissimus dorsi of the Weddell seal showed 67% type I fibers, 33% type IIa fibers, and no type IIb fibers (Table 1). The pectoralis muscle likewise did not have type IIb fibers. These results contradict all previous pinniped fiber typing data and the resolution of this discrepancy forms the basis of the first manuscript of this dissertation.

Table 1. Fiber types in pinniped locomotory skeletal muscles. Compilation of results from the literature. Fiber type populations are expressed as a percent of total count. Values in parentheses are the number of individuals sampled. An asterisk (*) denotes a secondary swimming muscle.

Species (n)	Common name	muscle	fiber type			Reference
			Slow twitch	Fast twitch		
Phoca vitulina (4)	Harbor seal	longissimus dorsi, deep	48% type I	9% type IIA	43% type IIB	Hochachka and Foreman, 1993
		longissimus dorsi, superficial	43% type I	9% type IIA	49% type IIB	Hochachka and Foreman, 1993
		iliocostalis, deep	48% type I	3% type IIA	49% type IIB	Hochachka and Foreman, 1993
		iliocostalis, superficial	40% type I	12% type IIA	49% type IIB	Hochachka and Foreman, 1993
		pectoralis*, deep	13% type I	35% type IIA	52% type IIB	Hochachka and Foreman, 1993
		pectoralis*, superficial	27% type I	20% type IIA	53% type IIB	Hochachka and Foreman, 1993
Halichoerus grypus (7)	Grey seal	longissimus dorsi	55% SO	6% FOG	36% FG	Reed et al., 1994
Phoca vitulina (6)	Harbor seal	longissimus dorsi	47% SO	6% FOG	47% FG	Reed et al., 1994
Arctocephalus gazella (1)	Antarctic fur seal	pectoralis	10% SO	28% FOG	61% FG	Reed et al., 1994
		latissimus dorsi	32% SO	11% FOG	58% FG	Reed et al., 1994
Arctocephalus gazella (1)	Antarctic fur seal	pectoralis	14% SO	30% FOG	57% FG	Reed et al., 1994
		latissimus dorsi	29% SO	19% FOG	52% FG	Reed et al., 1994
Eumetopias jubatus (1)	Stellar sea lion	unspecified	20% SO	27% FOG	53% FG	Kanatous et al., 1999
Leptonychotes weddelli (13)	Weddell seal	longissimus dorsi, deep	67% type I	33% type IIA	0% type IIB	Kanatous et al., 2002
		pectoralis*, deep	41% type I	59% type IIA	0% type IIB	Kanatous et al., 2002

Hochachka and Foreman (1993) found that fiber type composition in pinniped skeletal muscles varied with sampling depth, indicating intramuscular heterogeneity of fiber types. Such regional heterogeneity has been described in the skeletal muscles of a variety of terrestrial mammals and may function to partition force generation during the activation of motor units (Hoppeler et al., 1981; Armstrong et al., 1982; Armstrong and Phelps, 1984; Suzuki and Tamate, 1988; Rivero et al., 1993; Grotmol et al., 2002).

Metabolic characteristics of pinniped skeletal muscle metabolism

One of the physiological hallmarks of diving endotherms (birds and mammals) is the presence of elevated myoglobin (Mb) concentrations within their muscles (Cherepanova et al., 1993; Hochachka and Foreman, 1993; Reed et al., 1994; Ponganis et al., 1997; Kanatous et al., 1999, 2002; Polasek and Davis, 2001). In seals, there is a strong correlation between body mass, Mb content, and diving ability; the higher the body mass and Mb concentrations the, longer the maximum dive duration (Kooyman and Ponganis, 1998). Myoglobin is an intramuscular, monomeric oxygen-binding pigment related to hemoglobin and also binds oxygen reversibly. However, Mb has a higher affinity for oxygen compared to Hb, so when partial pressures of oxygen (P_{O2}) are high, Mb loads oxygen at a higher rate than Hb. Conversely, Mb dissociates from oxygen at a lower P_{O2} compared to Hb. This characteristic prevents the transfer of oxygen from muscle Mb to blood Hb during systemic circulation (Wittenberg and Wittenberg, 1989).

The high intramuscular Mb concentrations found in pinnipeds allows the animal's own muscles to serve as oxygen depots during diving. Thus, despite the hypoxia and ischemia associated with diving, evidence indicates that locomotory muscles function using primarily ATP that has been derived from aerobic metabolism. Analysis of tissue homogenates shows that the biochemical pathways of pinniped skeletal muscles are poised for oxidative metabolism derived from fatty acids. Elevated Mb concentrations and elevated enzymatic activity of citrate synthase (a key enzyme in the Kreb's cycle) in pinniped skeletal muscles are consistent and indicate a high capacity for oxidative metabolism (Ponganis and Pierce, 1978; Hochachka and Foreman, 1993; Reed et al., 1994; Kanatous et al., 1999, 2002; Polasek and Davis, 2001; Polasek et al., in prep). Likewise, elevated activities of enzymes involving fatty acid transport into the mitochondria (carnitine palmitoyltransferase and carnitine acetyltransferase) and β-oxidation (β-hydroxyacyl CoA dehydrogenase) in the skeletal muscles of phocids and otariids indicate that fatty acids are the preferred substrate for oxidation (Ponganis and Pierce, 1978; Hochachka and Foreman, 1993; Reed et al., 1994; Kanatous et al., 1999, 2002; Polasek and Davis, 2001; Polasek et al., in prep;).

Patterns in the maximal activities of enzymes involved with the glycolytic pathway are less consistent. As previously mentioned, some studies showed that there was no evidence of elevated lactate dehydrogenase (LDH) capacity in the muscles of marine mammals compared with terrestrial mammals (Castellini et al., 1981; Reed et al., 1994). Conversely, evidence of elevated LDH activity in the skeletal muscles of pinnipeds has also been demonstrated (Hochachka and Foreman, 1993; Polasek et al., in prep). Hochachka and Foreman (1993)

assayed LDH in both directions (towards pyruvate production and lactate production) and concluded that the LDH enzyme kinetics favored flux towards pyruvate oxidation. However, Davis (1983) showed that in harbor seals, lactate oxidation contributed very little (<6%) to resting energy production and concluded that lactate was preferentially recycled rather than oxidized. Although there remains no general consensus about the subject, it is possible that elevated anaerobic capability in the skeletal muscles of pinnipeds may function to provide energy when aerobic metabolic sources are insufficient during 'emergency' situations, such as an unexpected delay in emergence or escape from predators.

Concurrent with elevated mitochondrial enzyme activities is the elevation of the volume density of mitochondria in the skeletal muscles of pinnipeds (Kanatous et al., 1999, 2001, 2002). Although measured volume densities are not elevated to the degree of some highly aerobic terrestrial athletes such as foxes and pronghorn antelope (Lindstedt et al., 1991; Bicudo et al., 1996;), in seals, the epaxial musculature has a mitochondrial volume that is elevated over that found in the locomotory muscles of less active mammals of similar mass (Hoppeler et al., 1981; Mathieu et al., 1981b). The apparent paradox is that there is a mismatch between pinniped locomotory muscle mitochondrial volume density and maximum oxygen consumption, which is suspected to be low in seals (Davis et al., 1991, Kanatous et. al., 1999). In addition, behavioral evidence shows that seals use cost-efficient modes of locomotion (such as burst-and-glide swimming) to maintain a low level of exertion while diving and have a low cost of transport that is comparable to that of terrestrial runners (Williams et al., 1999, 2000). Thus it has been suggested that seals have an

elevated mitochondrial volume density to reduce the intramuscular diffusion distance of oxygen from oxymyoglobin stores to the mitochondria, thereby enhancing convective oxygen transport within the muscle (Kanatous et al., 1999). Implicit in this supposition is that mitochondria are dispersed throughout the muscle without displaying any regional clustering. In the latter case, measured mitochondria volume density may be high, but oxygen diffusion is not enhanced because the distribution of mitochondria does not facilitate diffusion by reduction of diffusion distance. Quantitative mitochondrial distribution has not been studied in any marine mammal, and the second manuscript of this dissertation seeks to describe the relationship between elevated mitochondrial volume density and mitochondrial distribution within the locomotory muscles of a phocid.

CHAPTER II
IMMUNOHISTOCHEMICAL FIBER TYPING OF HARBOR SEAL SKELETAL MUSCLE

Introduction

The skeletal muscles of marine mammals are well adapted for maintaining aerobic metabolism under the hypoxic conditions that occur during diving. In previous studies, investigators reported high mitochondrial volume densities and elevated enzyme activities that support an aerobic, lipid-based metabolism in the skeletal muscles of some pinnipeds (Order Carnivora, Family Pinnipedia: seals, sea lions and walrus) (Hochachka and Foreman, 1993; Reed et al., 1994; Kanatous et al., 1999, 2002). Myoglobin concentrations in the skeletal muscles of both cetaceans (Family Cetacea: whales and dolphins) and pinnipeds are elevated 10–20× compared to terrestrial mammals and provide an endogenous source of oxygen during dives (Cherepanova et al., 1993; Kanatous et al., 1999, 2002; Polasek and Davis, 2001). The high mitochondrial volume densities and myoglobin concentrations enhance the intracellular diffusion of oxygen into mitochondria under low oxygen partial pressure (Kanatous et al., 1999). In contrast, there appears to be little enhancement of glycolytic enzyme activities in pinniped skeletal muscle and other organs compared to terrestrial mammals (Castellini et al., 1981). These observations are in agreement with the well-accepted theory that marine mammals maintain aerobic metabolism during most voluntary dives (Kooyman et al., 1981, 1983; Davis, 1983; Thompson and Fedak, 1993; Butler and Jones, 1997).

There have been a few attempts to quantify fiber type composition in marine mammal skeletal muscles. Histochemical ATPase staining of the swimming muscles of seals has shown an average numerical composition of approximately 46% type I (slow twitch, oxidative fibers), 46% type IIb (fast twitch, glycolytic fibers), and the balance type IIa (fast twitch, oxidative fibers) (Hochachka and Foreman, 1993; Reed et al., 1994). These results appear to conflict with the suggestion that skeletal muscles of seals are adapted for aerobic metabolism, since type IIb fibers characteristically do not possess high concentrations of mitochondria or myoglobin. Although the two studies measured the oxidative capacity of the fibers by staining for NADH diaphorase and succinate dehydrogenase (SDH) activity, neither reported the results. Fiber typing of biopsies taken from the locomotory (epaxial) muscles of one Pacific white-sided dolphin *Lagenorhynchus obliquidens* and the hypaxial and epaxial muscles of one live and one dead bottlenose dolphin *Tursiops truncatus* showed approximately 50% fast twitch, glycolytic fibers and 50% slow twitch, oxidative fibers (Ponganis and Pierce, 1978; Bello et al., 1983; Goforth, 1983). Of these, only Goforth (1983) performed SDH staining and verified that fast twitch, oxidative-glycolytic fibers were rare or absent due to the lack of staining overlap between SDH activity and fast twitch fibers. Recently, Kanatous et al. (2002) performed metachromatic histochemical staining of Weddell seal skeletal muscles and verified the results of the stain with immunohistochemical (IHC) fiber typing. They found that the epaxial muscles were composed of approximately 67% type I fibers, 33% type IIa fibers and no type IIb fibers (Kanatous et al., 2002). However, only 1–2 muscle samples from five animals were analyzed using immunohistochemistry, and it is possible that the biopsy

samples (ca. 0.5 g each) were not representative of the entire musculature. Kanatous et al. (2002) provides the only evidence to date to suggest a lack of type IIb fibers in a pinniped.

The purpose of this study was to collect multiple samples from the primary (epaxial muscles) and secondary (M. pectoralis) swimming musculature of the harbor seal *Phoca vitulina* and apply IHC techniques to determine the fiber types present, quantify fiber type populations, and determine the distribution of fiber types within the muscle. Based on previous studies of enzyme activities, fiber typing and mitochondrial volume density, it was hypothesized that there would be a higher proportion of type I and type IIa fibers than type IIb fibers. The second hypothesis was that the fiber type distribution within the muscles would be heterogeneous (i.e. there would be fast twitch fibers located superficially and slow twitch fibers located deeper in the muscle). The results showed that all fibers in both of the muscles sampled were either type I or type IIa, which supports the fiber typing results of Kanatous et al. (2002). The pectoralis muscle possessed significantly more type IIa fibers than the epaxial muscles. In addition, fiber type distribution within the locomotory muscle did not show pronounced spatial heterogeneity.

Materials and Methods

Animals and sample collection

Samples from locomotory and non-locomotory muscles were collected within 6 h of death from adult and subadult harbor seals *Phoca vitulina* L. (two males and eight females; mean mass=46.1±13.5 kg) during a native subsistence hunt

in eastern Prince Williams Sound, Alaska. The epaxial muscles lie along the vertebral column (Fig. 1) and alternately contract and stretch to produce the lateral spinal flexions that generate thrust by the hind flippers during swimming (Fish et al., 1988). Muscle fibers of the epaxial musculature originate at the ilium, run approximately parallel to the spine, and terminate at various attachments to the ribs and vertebrae (Howell, 1929). The degree of muscle pinnation was not measured. The entire epaxial musculature along one side of the spine was removed (mean length=52.5±1.9 cm), weighed (mean mass=2.88±0.4 kg), and three transverse sections were taken in the cranial (CR), middle (MID) and caudal (CA) regions (Fig. 1). The CR transverse section was taken at the seventh cervical vertebra, MID was taken at the fourteenth thoracic vertebra, and CA was from the lower lumbar region. Seven samples (ca. 0.5 g) per transverse section were taken at points on a circular grid using a 6 mm stainless steel biopsy punch (Fig. 2). Muscle samples were placed in a phosphate-buffered saline (PBS) solution containing 7% glycerol and 4% sucrose for 30 min prior to freezing in 3-methylbutane cooled with liquid nitrogen. After sample processing, a rectilinear grid was overlaid on the cross section, and the location of each of sample was determined relative to a true dorso–ventral and medio–lateral orientation in the animal. Samples were transported back to Texas A&M University in liquid nitrogen and kept frozen at –70°C until analysis. For comparison with a secondary swimming muscle, a single sample was taken from the center of the intact m. pectoralis.

Immunohistochemical analysis of muscle fiber types

Serial cross sections (7 μm thick) of frozen epaxial and pectoralis muscle samples were cut on a cryostat microtome and mounted on glass slides. Sections of muscle were fixed with cold AFA (50 ml of 37% zinc formalin + 370 ml 95% ethanol + 25 ml glacial acetic acid) for 5 min and then hydrated for 10 min in PBS prior to blocking. PowerBlock (InnoGenex, San Ramon, CA, USA) was added to the sections and incubated for 5 min at room temperature. Following removal of excess blocker, primary antibodies to the myosin heavy chains, type I (BA-D5), type IIa (SC-71) and type IIb (BF-F3) were added to the appropriate sections, and the slides were incubated at 4° C overnight in a humid chamber. Following incubation, slides underwent two 10 min washes in PBS with gentle rotation. After washing, a biotinylated goat anti-mouse Ig secondary antibody was added to the sections for 20 min at room temperature. After washing the slides as described above, streptavidin–alkaline phosphatase conjugate was added, and the sections were incubated for 20 min at room temperature. The conjugate was removed by washing (as in prior steps), and a solution of naphthol phosphate buffer and Fast Red dye was added. The sections were then incubated until adequate color development was observed, counterstained with Mayer's Hematoxylin and mounted with Glycergel (DakoCytomation California Inc., Carpinteria, CA, USA). Fibers containing the myosin heavy chains expressed a red color following exposure to the immunohistochemical staining procedure. Samples were analyzed using a BIOQUANT image analysis system (R&M Biometrics, Inc., Nashville, TN, USA). This system consists of an Olympus BX-60 microscope (Olympus America Inc.,

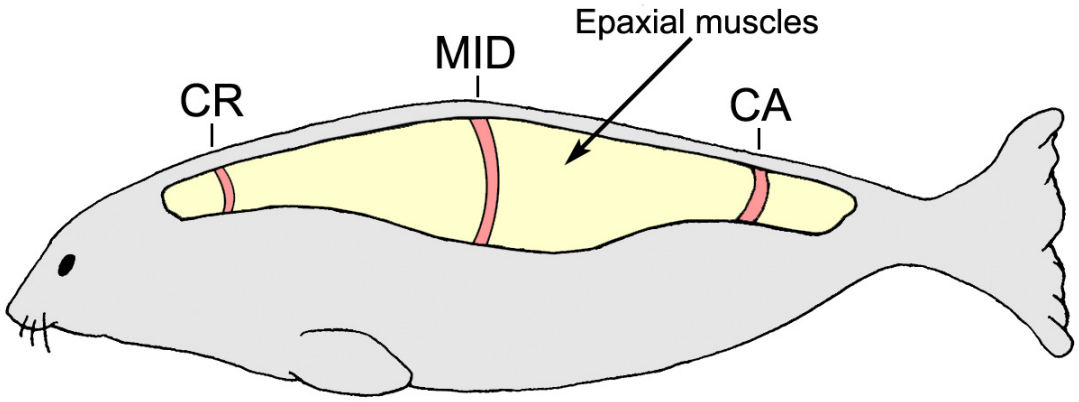

Fig. 1. Schematic diagram illustrating the approximate locations of the three transverse sections taken from the epaxial muscles. CR, cranial transverse section; MID, middle transverse section; and CA, caudal transverse section. (Image not drawn to scale).

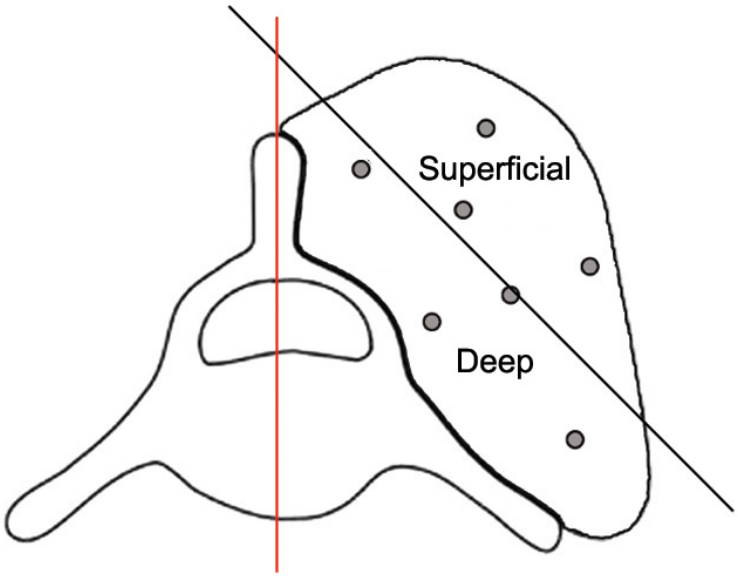

Fig. 2. Representative caudal transverse section showing the coring pattern of samples (grey circles) collected from the epaxial muscles and the statistical division used for testing homogeneity within the transverse section. The red line represents the natural vertical axis of the animal and the black line represents the line drawn at a 45 degree angle to the vertical axis to divide the epaxial muscles into deep and superficial regions.

Melville, NY, USA) with an attached Optronics (Goleta, CA, USA) DEI 470 camera interfaced with a personal computer. All artifact-free fibers were counted at a total magnification of 100× for each serial section (between 300–1500 fibers per section) and characterized as type I, type IIa, type IIb or 'unstained', as described by Schiaffino et al. (1989). Cells that showed inconsistent, light staining due to non-specific binding of the antibody were considered 'unstained'. The relative abundance of fiber types for each section was determined and is presented as a percentage of the total number of fibers counted. Serial muscle sections were also examined for IIx fibers (i.e. fibers that expressed no staining following exposure to any of the heavy chain antibodies).

Verification of antibody reactivity

A combination of mouse anti-rat primary antibody and goat anti-mouse secondary antibody was used to differentiate between three myosin heavy chain isoforms. Western blot analysis (data not shown) and SDS-PAGE (Fig. 3) verified that the fiber types of seals matched the electrophoretic properties of rat fiber types. Antibody reactivity verification was performed in serial sections of Weddell seal skeletal muscle subjected to both IHC fiber typing and a traditional histochemical staining procedure. Fiber type populations were similar for both methodologies (Kanatous et al., 2002).

Data analysis

Fiber type distribution was analyzed along the length of the epaxial muscles by comparing average fiber type percentages among the CR, MID and CA sections. To analyze fiber type distribution with respect to proximity to the vertebrae, the

seven samples of the CR, MID and CA sections were divided into one of two categories, ‘deep’ or ‘superficial’. A 3-factor analysis of variance (ANOVA) was applied using Minitab statistical software. By using a 3-factor ANOVA, the distribution of fibers in both the lateral and longitudinal planes of the epaxial muscles could be simultaneously analyzed. The fixed factors were ‘section’ (either CR, MID or CA) and ‘proximity’, and the random effects factor was the individual animal. For fixed factor ‘proximity’, the samples of each section were grouped into two categories in the following manner. The grid used to identify the seven sample locations was divided into two sections delineated by the equation y=–1x+0. Samples that fell on either side of the line were pooled into two categories depending on their location within the muscle *in vivo*: either ‘deep’ or ‘superficial’ to the vertebral column (Fig. 2). Samples that fell on the line were discarded. Type I and type IIa percentages were analyzed separately. Comparisons of mean percentages of fiber types in the epaxial muscles and the pectoralis were analyzed using a Student’s paired t-test. Fiber type populations were also analyzed with respect to seal sex and mass. All results are expressed as means ± 1 standard deviation (S.D.) and tested at a level of significance of $P<0.05$.

Results

The epaxial and pectoralis muscles were composed of type I and type IIa fibers, with type IIb fibers completely absent (Table 2, Fig. 4). The mean numerical populations of type I fibers as a percentage of total fibers for the CR, MID and CA transverse sections of the epaxial muscles were 45.3%, 47.7% and

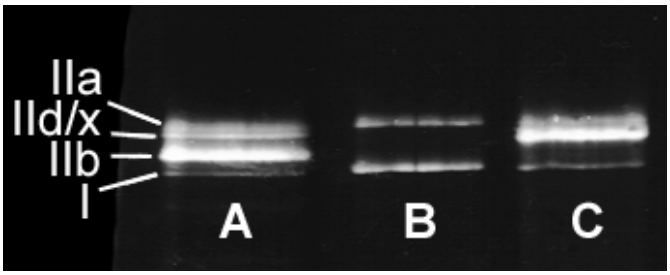

Fig. 3. Silver stain (reverse western blot) of (A) rat *vastus medialis* muscle, (B) seal epaxial muscles, and (C) seal pectoralis muscle showing differentiation of myosin heavy chain isoforms based on molecular weight. Type IIb band is absent in both seal muscles.

and 52.2%, respectively. The pectoralis muscle was composed of 16.2% type I fibers and 84.3% type IIa fibers. The distribution of type IIa fibers was not significantly different with respect to the dorsiventral axis of the epaxial muscles (Table 2) or proximity to the vertebrae. The distribution of type I fibers was not significantly different with respect to the dorsiventral axis of the epaxial muscles (Table 2). However, statistics describing the distribution of type I fibers with respect to the vertebrae were inconclusive (3-factor ANOVA, $P=0.05$; paired t-test, $P=0.06$). Fiber type composition was not significantly different between females (N=8) and males (N=2), nor was there a mass-specific relationship. There was a significant difference between fiber type percentages of the epaxial muscles and the pectoralis. The pectoralis was composed of significantly less type I fibers and significantly more type IIa fibers when compared to the epaxial muscles (Table 2) ($P<0.0001$).

Discussion

Although published data based on traditional histochemical techniques agree with these results for type I fiber populations, there is a substantial difference in the results for the type IIa and type IIb fibers. The results in this research show no type IIb fibers and a high percentage of type IIa fibers (approximately 53%) in the epaxial muscles of the harbor seal. Previous histochemical fiber typing of the harbor seal epaxial muscles showed a high percentage of type IIb fibers (approximately 45–47%) and few (<10%) type IIa fibers (Hochachka and Foreman, 1993; Reed et al., 1994). Additionally, Kanatous et al. (1999) found a

Table 2. Mean population percentages of fiber types ± one standard deviation from three cross sections of the harbor seal epaxial muscles and pectoralis. The value in brackets represents the number of animals averaged for each cross section. An asterisk (*) indicates a significant difference from the epaxial muscles at $p < 0.01$.

	Type I %	**Type IIa %**	**Type IIb %**
Epaxial muscles			
Cranial [10]	45.3 ± 5.1	54.5 ± 4.7	0.0 ± 0.0
Middle [9]	47.7 ± 3.3	52.3 ± 3.6	0.0 ± 0.0
Caudal [9]	48.3 ± 5.8	52.2 ± 5.7	0.0 ± 0.0
Mean of all sections [9]	47.4 ± 4.7	52.8 ± 4.6	0.0 ± 0.0
Pectoralis [6]	16.2 ± 5.6*	84.3 ± 5.4*	0.0 ± 0.0

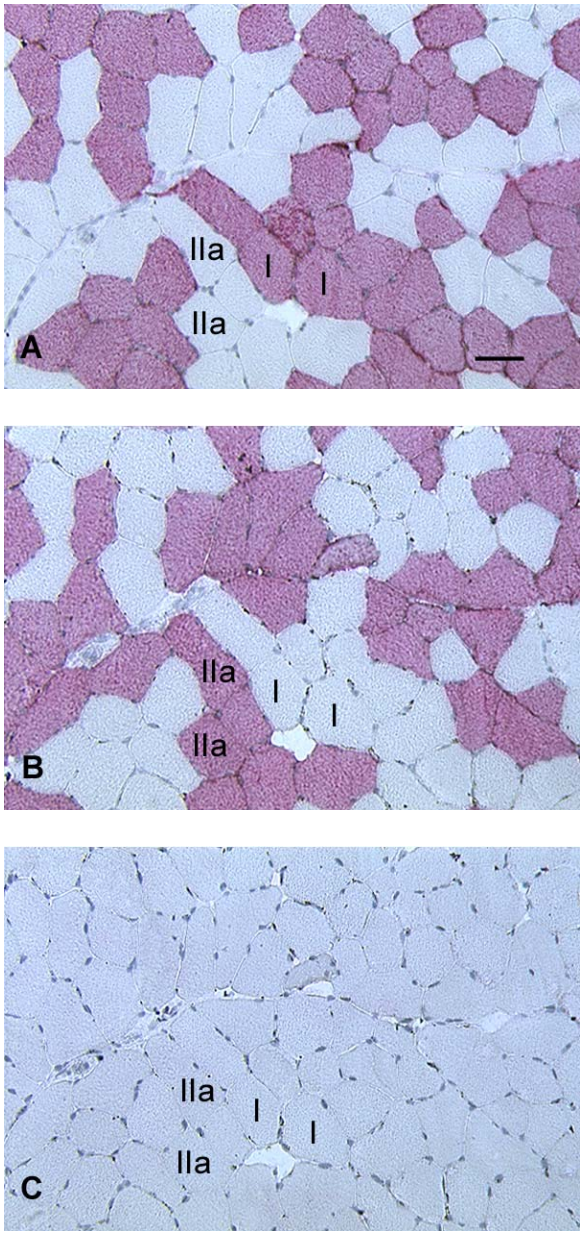

Fig. 4. Representative serial cross sections from the epaxial muscles of a harbor seal (*Phoca vitulina*) stained for myosin heavy chain isoforms using a series of monoclonal antibodies. (A) Type I (slow-twitch, oxidative) fibers, (B) type IIa (fast-twitch, oxidative) fibers, and (C) type IIb (fast-twitch, glycolytic) fibers. Lack of stain in C indicates an absence of type IIb fibers in this cross section of the muscle.

fiber type distribution of 20% slow, oxidative fibers, 27% fast oxidative-glycolytic fibers, and 53% fast oxidative-glycolytic fibers in one unspecified muscle of a Stellar sea lion. The results of this research were also dissimilar to the fiber type composition of the epaxial and hypaxial muscles of dolphins, which were composed of approximately 50% type I and 50% type IIb fibers (Ponganis and Pierce, 1978; Bello et al., 1983; Goforth, 1983).

Traditional histochemical staining procedures use acidic and alkaline preincubations to selectively inhibit the ATPase of the different fiber types, allowing for differentiation (Brooke and Kaiser, 1970). This procedure is based on the correlation between the velocity of muscle contraction and the concentration of actomyosin ATPase within each fiber type and has been used reliably and extensively in both research and clinical settings. However, under some applications, ATPase staining may have limitations. Of primary concern is the inability of the ATPase technique to reliably differentiate between the types of fast twitch fibers (types IIa, IIb, IIc and IId/x) in some species and the variability of optimal ATPase staining conditions from species to species (Green et al., 1982; Gorza, 1990; Amann et al., 1993; Rivero et al., 1996). ATPase staining also misrepresents cases of fibers coexpressing two different fiber types (hybrid fibers) because the most dominant isoform is histochemically stained (Gorza, 1990). Finally, the technique itself is sensitive. Inaccuracies may result from small changes in preincubation and incubation time, temperature, pH, preincubation buffer type and the ionic composition of the preincubation medium (Matoba and Gollnick, 1984). Taken together, these considerations potentially make the results of actomyosin ATPase fiber typing difficult to qualify, variable and irreproducible when making interspecies comparisons or fiber typing a

species for the first time (Green et al., 1982; Amann et al., 1993), and raise questions about the interpretation of the results obtained from previous histochemical fiber typing of seal muscles (Ponganis and Pierce, 1978; Bello et al., 1983; Goforth, 1983; Hochachka and Foreman, 1993; Reed et al., 1994). The exception is the aforementioned study by Kanatous et al. (2002), which used an ATPase-based metachromatic stain in combination with IHC fiber typing on Weddell seal skeletal muscles. Their results showed a fiber type profile similar to what was found in the harbor seals. However, based on the variable staining intensity of the histochemically stained cells shown in the figures, without the accompanying IHC fiber typing, the differentiation of type II fibers may have been difficult (Kanatous et al., 2002). Thus, to maximize the accuracy of histochemical staining techniques in novel or controversial fiber typing applications, multiple staining protocols are recommended (Braund et al., 1978; Snow et al., 1982; Amann et al., 1993; LaTorr et al., 1993; Kanatous et al., 2002).

There is strong evidence that myosin heavy chain (MyHC) composition directly corresponds to the shortening velocity of muscle fibers, subsequent ATPase activity, and thus, ATPase staining intensity (Reiser et al., 1985; Betto et al., 1986; Staron and Pette, 1986; Termin et al., 1989; Gorza, 1990). To circumvent potential ATPase staining difficulties, this study used IHC fiber typing to characterize the fiber composition of seal muscle. Since IHC fiber typing utilizes the specific antigenicity of MyHC isoforms to differentiate between the fiber types, antibody binding capacity is binary in pure fibers. Thus, IHC staining eliminates the subjective determination of ‘stain intensity’ to separate fiber type, making quantification more accurate. IHC fiber typing may be used on a wide

range of mammalian species because MyHC genes in striated muscle are highly conserved in a variety of animals spanning several phyla, from nematodes to man (Nguyen et al., 1982). Furthermore, the MyHC genes themselves have a highly conserved organization and primary structure (Mahdavi et al., 1986). Although IHC fiber typing is not a new technique (Arndt and Pepe, 1975), it has not been widely used other than on laboratory animal and human tissues. The IHC fiber typing technique definitely demonstrates a lack of classical type IIb fibers in the locomotory muscles of harbor seals. However, the presence of other type II MyHC isoforms, in particular type IId/x and type IIc cannot be ruled out. SDSPAGE of harbor seal and rat skeletal muscle yielded corresponding bands for type I and type IIa fibers in the epaxial muscles, but in the pectoralis muscle the correspondence for the type IIa band is not clear and appears to be associated with the type IId/x MyHC isoform found in the rat (Fig. 3). For this study, an antibody capable of specifically differentiating the type IId/x isoform was not available and therefore the type IId/x fiber population was analyzed by process of elimination. Based on the results of the electrophoresis, further investigation on the presence of type IId/x fibers in the harbor seal pectoralis is warranted. In addition, the presence of type IIc fibers, which are considered 'undifferentiated' or 'transitional' fibers (Betto et al., 1986), were not found nor analyzed electrophoretically. Type IIc fiber population as a percentage of total fibers counted is usually small (<3%) and probably does not contribute significantly to the total muscle fiber population (Betto et al., 1986; Amann et al., 1993).

In general, the fiber type composition within the epaxial muscles matched the myoglobin and enzyme activity data of tissue samples collected from the same

seals and from the same locations in the transverse muscle sections. Harbor seal myoglobin (Mb) concentrations, citrate synthase activities (CS; an indicator of aerobic metabolism) and β -hydroxyacyl CoA dehydrogenase activities (HOAD; an indicator of fatty acid metabolism) were either the same or elevated compared to rat and dog (Polasek et al., manuscript in preparation). Moreover, in a separate study, mitochondria volume density in harbor seal swimming muscle was elevated compared to the density in locomotory muscles of sedentary terrestrial mammals of comparable size (Kanatous et al., 1999). These results are consistent with the characteristics of type I and type IIa fibers, which are both oxidative. In addition, Polasek et al. (manuscript in preparation) likewise did not find pronounced spatial heterogeneity of enzyme activities or Mb concentrations within the CR, MID or CA transverse sections. Since the cross sections of the harbor seal epaxial muscles were observed to be a uniform deep red color during dissection, these results are not surprising. However, Polasek et al. (manuscript in preparation) found significantly higher CS and LDH activities in the CA and the MID transverse sections compared to the CR section. These results indicate that a longitudinal gradient for the physiological indices of aerobic capacity exists in the harbor seal epaxial muscles, but it may not be manifest in the fiber type distribution.

Fiber type distribution in the primary locomotory muscles (epaxial muscles) of the seal was significantly different from the secondary locomotory muscle (pectoralis). Whereas the epaxial muscles were composed of approximately 50% type I fibers and 50% type IIa fibers, the pectoralis possessed approximately 15% type I fibers and 85% type IIa fibers (Table 2). Seals swim using lateral undulations of their hind flippers to propel themselves through the

water, and are characterized as thunniform swimmers (Fish et al., 1988). The foreflippers, which act as rudders and are used during burst swimming, do not significantly contribute to forward propulsion. Thus, to generate force during swimming, the epaxial muscles are alternately contracted and stretched. Stretch–shortening cycles in the locomotory muscles of some terrestrial mammals and fish maximize muscle force and power output during each stroke by absorbing and storing potential energy during the lengthening phase of the cycle for utilization during the shortening phase (Altringham and Johnson, 1990; Curtin and Woledge, 1993; Lou et al., 1999; Lindstedt et al., 2002). In mammals, muscles that undergo active stretch (eccentric) contractions and isometric contractions generally have more type I fibers than muscles that perform concentric contractions (Armstrong and Phelps, 1984; Delp and Duan, 1996). Seal pectoralis may perform mostly concentric contractions during foreflipper movement and therefore contain fewer type I fibers than the epaxial muscles, which perform eccentric contractions during the stretch–shortening cycle of thunniform locomotion.

The physiological profile of the harbor seal skeletal muscle appears to be similar to that of terrestrial mammals adapted for sustained, aerobic exercise (e.g. horses and dogs). This physiological profile includes an elevated mitochondrial volume density, increased enzymatic capacity to oxidize fatty acids, elevated tricarboxylic acid cycle enzyme capacity, and a fiber type distribution of primarily type I and type IIa fibers in locomotory muscles. However, in seals, routine metabolic rate during diving is generally less than twice the resting, predive levels. (Davis et al., 1985; Castellini et al., 1992;

T. M. Williams et al., manuscript in preparation). Additionally, behavioral studies indicate that seals are not active swimmers and may not maximize their aerobic capability *in vivo*. Rather, seals use energy-saving locomotory strategies. Recent evidence shows that when seals dive, they often alternate between an active stroke phase and a passive glide phase to conserve energy and oxygen stores, a pattern that is demonstrated in a variety of diving mammals (Williams et al., 2000; Davis et al., 2001). Consequently, this behavioral information coupled with physiological data suggests that the elevated mitochondrial volume density found in seal skeletal muscle may have a primary function of decreasing the diffusion distance of oxygen stores in myoglobin to the site of oxidation at the mitochondria (Kanatous et al., 1999).

The fiber typing results show that harbor seal swimming muscles are made exclusively of type I and type IIa fibers. These results are consistent with seal behavioral data and the theory that diving in marine mammals is an aerobic activity. Fiber type distribution did not show pronounced spatial heterogeneity along the dorso–ventral and medio–lateral axes of the epaxial muscles. Finally, differences in fiber type distribution in the epaxial muscles vs. the pectoralis muscle may be related to contraction velocity and ability to store elastic energy.

Overview

There is strong evidence that pinnipeds maintain a lipid-based, aerobic metabolism during diving. However, the few fiber-typing studies performed on pinniped skeletal muscles are not consistent with an aerobic physiological profile. The objective of this study was to reexamine the fiber type distribution

throughout the primary locomotory muscles of the harbor seal *Phoca vitulina*. Results from immunohistochemical (IHC) fiber typing indicated that harbor seal swimming muscles (the epaxial muscles) are composed of 47.4% type I (slow twitch, oxidative) fibers and 52.8% type IIa (fast twitch, oxidative) fibers, which are homogeneously distributed throughout the muscle. Harbor seal pectoralis, a secondary swimming muscle, was composed of 16.2% type I and 84.3% type IIa fibers. No fast twitch, glycolytic (type IIb) fibers were detected in either muscle, in contrast to published data on fiber typing of harbor seal epaxial muscles using traditional histochemical techniques. The extreme specificity inherent in the IHC fiber typing procedure leads to the conclusion that harbor seal swimming muscle is entirely composed of oxidative fibers. These results are consistent with the enzymatic analyses of pinniped skeletal muscle that support the use of lipid-derived aerobic catabolism to fuel working muscle during diving in these marine mammals.

CHAPTER III

ULTRASTRUCTURE AND MORPHOMETRY OF HARBOR SEAL SKELETAL MUSCLE

Introduction

Despite experiencing hypoxia during subsurface swimming, available evidence indicates that pinnipeds (Order Carnivora, Suborder Pinnipedia: seals, sea lions, and walrus) maintain aerobic respiration during the majority of dives (Butler and Jones, 1997). Likewise, their tissues appear to be adapted for aerobic metabolism. Single biopsies taken from the locomotory muscles of a variety of pinnipeds showed elevated mitochondrial volume densities compared to terrestrial mammals of similar mass (Kanatous et al., 1999, 2001). Activities of citrate synthase (CS, a mitochondrial matrix enzyme of the tricarboxylic acid cycle) and β-hydroxyacyl CoA dehydrogenase (HOAD, a β-oxidation enzyme) were also elevated, indicating that the oxidation of fatty acid-derived acetyl CoA was an important source of energy in these tissues (Hochachka and Foreman, 1993; Reed et al., 1994; Kanatous et al., 1999; Polasek et al., in preparation). Similarly, Fuson et al. (2003) showed that in harbor seal (*Phoca vitulina*) heart, kidney, and splanchnic organs, mitochondrial volume density and HOAD enzyme activity were elevated, although the authors did not find a concomitant increase in CS activity. Finally, myoglobin concentrations in the skeletal muscles of pinnipeds are elevated 10-20 times compared to that of terrestrial mammals and provide an endogenous source of oxygen in the muscles during dives (Cherepanova et al., 1993; Hochachka and Foreman, 1993; Kanatous et al., 1999; Polasek et al., in preparation). The elevated mitochondrial volume

densities and myoglobin concentrations enhance the intracellular diffusion of oxygen into mitochondria under low oxygen partial pressures that occur towards the end of a dive (Kanatous et al., 1999).

Immunohistochemical fiber type analysis of locomotory muscles is consistent with the aforementioned morphological and metabolic characteristics. Harbor seal epaxial muscles were composed of fibers with myosin heavy chain isoforms analogous to type I (slow twitch, oxidative) and type IIa (fast twitch, oxidative) fibers, but no type IIb (fast twitch, glycolytic) fibers are present (Watson et al., 2003). Although fiber types cannot be reliably distinguished based on metabolic properties, type I and type IIa fibers typically have a higher oxidative capacity and mitochondrial volume density than type IIb fibers (Pette and Staron, 1990; Mattson et al., 2002). Biochemical assays performed on samples taken from the same harbor seals and at the same locations as those for fiber type analysis showed elevated myoglobin concentrations and an enzyme profile that supports lipid-derived fatty acid oxidation (Polasek et al., in preparation). These results indicate that the immunohistochemically-defined type I and IIa fibers in the epaxial muscles are highly oxidative in nature.

The spatial relationships between specific fiber types, enzyme activities and myoglobin concentration in harbor seals are less apparent. Fiber type composition, Mb concentration, and CS, LDH, and HOAD enzyme activities do not show significant variability within transverse sections of the epaxial muscles (Watson et al., 2003; Polasek et al., in preparation). This muscular characteristic is unlike the locomotory muscles of many terrestrial mammals, in which “deep” portions of the muscle located closer to the bone possess a higher percentage of type I fibers and a higher enzymatic capacity for oxidative

metabolism compared to “superficial” portions of the muscle located near the limb periphery (Armstrong et al., 1982; Kline and Bechtel, 1988; Rivero et al., 1993).

The purpose of this study was to examine mitochondrial volume density and distribution throughout transverse sections of the epaxial muscles of harbor seals using electron microscopy. This approach allows the examination of these two morphological variables on a larger and more detailed scale than past studies that were performed on single biopsy samples. As a result, this research was able to address questions about spatial heterogeneity within the muscle. Based on previous fiber type analysis and enzymology performed on harbor seal swimming muscles, it was hypothesized a lack of significant spatial heterogeneity within transverse sections of the epaxial musculature. It was also hypothesized that mitochondrial volume density would be higher in the harbor seal’s locomotory muscles compared to that of the terrestrial controls.

Materials and Methods

Animals and sample collection

Primary and secondary locomotory muscles were sampled within six hours of death from five adult and subadult harbor seals (one male and four females; average weight = 51.6 ± 3.4 kg) collected during a native subsistence hunt in eastern Prince Williams Sound, Alaska. The epaxial muscles lie along the vertebral column (Fig. 5) and alternately contract and stretch to produce the lateral spinal flexions that generate thrust by the hind flippers during swimming (Fish et al., 1988). Muscle fibers of the epaxial muscles originate at the ilium,

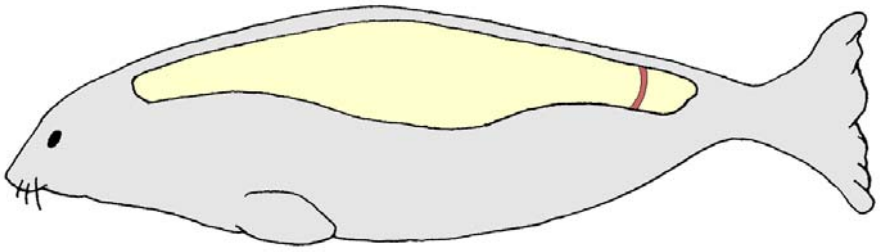

Fig. 5. Schematic diagram illustrating the approximate location of the caudal transverse section sampled from the epaxial muscles. The epaxial muscles are yellow and the approximate location of the caudal transverse section is shown as a red band. (Image not drawn to scale.)

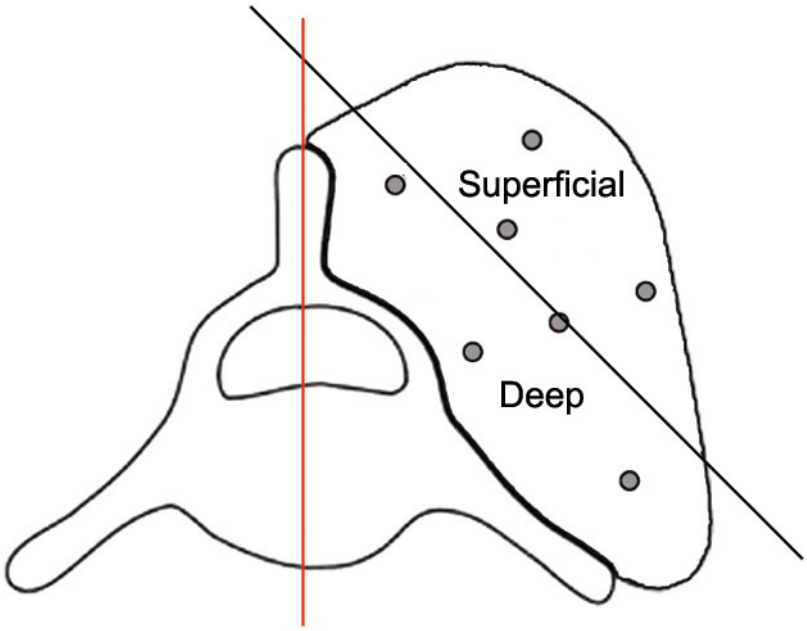

Fig. 6. Representative caudal transverse section showing the coring pattern of samples (grey circles) collected from the epaxial muscles and the statistical division used for testing homogeneity within the transverse section. The red line represents the natural vertical axis of the animal and the black line represents the line drawn at a 45 degree angle to the vertical axis to divide the epaxial muscles into deep and superficial regions

run approximately parallel to the spine, and terminate at various attachments to the ribs and vertebrae (Howell, 1929). The degree of muscle pinnation was not measured. The entire epaxial musculature along one side of the spine was removed (average length = 55.0 ± 2.0 cm), weighed (average weight = 1.72 ± 0.2 kg), and a caudal transverse section was taken from the lower lumbar region. Seven samples (ca. 0.5 g) from the transverse section were excised at points on a circular grid (Fig. 6) using a 6-mm stainless steel biopsy punch. Muscle samples were immersed in a 6.25% gluteraldehyde solution in cacodylate buffer (ph 7.4) and placed on ice. A rectilinear grid was overlaid on the transverse section, and the location of each of sample was determined relative to a true dorso-ventral and medio-lateral orientation in the animal. For comparison with a secondary swimming muscle, a single sample was taken from the center of the intact m. pectoralis. Samples were transported to Texas A&M University on ice and refrigerated at 4°C until analysis.

Sample preparation for electron microscopy

Within 90 days of collection, tissue samples were cut into small blocks using a dissecting microscope and stored in 0.1 M cacodylate buffer (ph 7.4) at 4°C for six months at Texas A&M University. All further sample processing was performed in the Electron Microscopy Laboratory of the Department of Pathology at the University of Texas Medical Branch in Galveston. Samples were rinsed in fresh cacodylate buffer, post-fixed for 1 hour in 1% osmium tetroxide, and block-stained with 2% uranyl acetate for 30 minutes at 60°C. The samples were then passed through stepwise dehydration in increasing

concentrations of ethanol (50-100%) and rinsed twice with 100% propylene oxide. Samples were subject to increasing concentrations of Poly/Bed 812 (Polysciences, Inc., Warrington, PA) before being embedded in flat molds with fresh Poly/Bed 812 and allowed to polymerize for 24 hours at 60°C. At least 10 blocks were prepared for each tissue sample.

Morphometry

Four blocks were randomly chosen from each sample for stereology. The blocks were thick sectioned (1 μm), stained with toluidine blue, and viewed under a light microscope to verify that the orientation of the sections was transverse or slightly oblique to the muscle fiber axis. Ultrathin sections were cut from each block with a Reichert/Leica Ultracut S ultramicrotome (Reichert Division of Leica Co., Vienna, Austria), placed on 150-mesh copper grids, and stained with Reynold's lead citrate (0.4%) to improve contrast. Thin sections were photographed at a magnification of 6,600x with a Phillips CM-200 transmission electron microscope (TEM) (FEI Company, Eindhoven, Netherlands) at 60 Kv. Carbon grating replica calibration was performed on the TEM to confirm that the measured magnification was within 5% of nominal magnification. Ten images were taken per block on 70 mm film (Weibel, 1979). This yielded 40 images per sample for stereological analysis. Negatives were converted to high resolution (1,200 dpi) digital images with a Microtek Scanmaker 8700 flatbed scanner (Microtek USA, Carson, CA) for subsequent quantification. Standard point counting procedures (Weibel, 1979; Mathieu et al., 1981a) were performed in Adobe Photoshop 7.0 with the "grid" feature enabled to replicate a B-36 square grid (144 test points) superimposed on the

center of the digital image at a final magnification of approximately 28,935x. Care was taken to maintain the aspect ratio of the image during all digital manipulations to eliminate distortion. Points that intersected subsarcolemmal mitochondria, interfibrillar mitochondria, lipid droplets, and myofibrils were counted separately. The relative standard error (RSE) of the volume density counts was calculated by pooling the data from all four blocks of a sample (i.e. 40 micrographs) and applying the RSE equation for binomial counts (Mathieu et al., 1981a). RSE values calculated for volume densities of total mitochondria, interfibrillar mitochondria, and myofibrils were <15%. The RSE values for the volume densities of subsarcolemmal mitochondria and lipid droplets in both seal muscles were considerably higher. No attempts were made to further decrease the RSE for these variables.

Statistics

Differences among muscles were analyzed using a paired Student's t-test. To test for heterogeneity within the epaxial muscles, each transverse section was divided into two sections delineated by the equation $y = -x$. Samples that fell on either side of the line were grouped into either a "deep" category or a "superficial" category with respect to the vertebral column. Samples lying on the origin or on the line were discarded (Fig. 6). Total mitochondrial volume densities for deep and superficial categories were combined for all seals, and the two groups were compared using a binomial generalized linear model. All results are expressed as means ± standard error and tested at a level of significance of $p<0.05$.

Methodological control

Control samples were collected from the soleus muscle of 3 male Sprague-Dawley rats sacrificed for research in the Department of Health and Kinesiology at Texas A&M University, College Station. Eight-month-old rats were anesthetized with pentobarbital sodium (60 mg/kg IP), decapitated, and the entire soleus muscle was excised, trimmed, and processed using the identical protocol as seals. Electron microscopy and point-counting procedures were also the same. Morphometric analysis of the rat soleus yielded results similar to that in the literature (mean V_v (mt, f) = 4.7±0.3%).

Results

At 6,600 times normal magnification, morphological characteristics of the seal epaxial muscles and pectoralis appeared similar to that of other mammals. Individual muscle fibers were separated by connective tissue and possessed peripherally-located nuclei located within the plasma membrane, although satellite cells were not differentiated from true muscle fiber nuclei. Fibers were primarily composed of myofibrils which were roughly polyhedral or elliptical in cross section and surrounded by the sarcoplasm, which contains sarcoplasmic reticulum, organelles, myoglobin, and endogenous energy depots (Figs. 7, 8). On average, myofibrils occupied approximately 85.6% of harbor seal epaxial and pectoralis muscle fiber volume (Table 3). Myofibrils sectioned transversely across the Z disc were separated by thicker bands of sarcoplasmic reticulum compared to myofibrils sectioned across the thick and thin filaments (Figs. 7, 8). Glycogen granules were not observed in any of the micrographs. However,

glycogen is more apparent in longitudinally-sectioned muscle. Lipid droplets were uncommon (mean volume density = 0.2 ± 0.04%) (Table 3), but normally closely associated with mitochondria when present.

The electron-dense mitochondria appeared as discrete, ellipsoid structures located either between myofibrils (interfibrillar mitochondria) or adjacent to the sarcolemma (subsarcolemmal mitochondria). Mitochondria that were compressed into irregular shapes due to close proximity of myofibrils, lipid droplets, or other mitochondria were common (Figs. 7, 8). Evidence of a mitochondrial reticulum, as has been described in other mammalian skeletal muscles, was not consistently observed in the seal muscle ultrastructure, unlike the rat soleus ("control") micrographs (Kirkwood et al., 1986). Internal mitochondrial structures were not visible in all micrographs, but mitochondria typically displayed cristae that extended perpendicularly from the outer mitochondrial wall to the organelle's center and lay parallel to adjacent cristae (Figs. 7, 8).

Total mitochondrial volume density [V_v (mt, f)] of the epaxial muscles and pectoralis muscle was 5.6±0.3% and 5.2±0.3%, respectively (Table 3). Interfibrillar mitochondria of the epaxial muscles and pectoralis (mean volume density = 4.6 ± 0.2% for both muscles) were approximately 5-7 times more abundant than subsarcolemmal mitochondria (Table 3). Subsarcolemmal mitochondria (mean volume density = 1.0 ± 0.1% in the epaxial muscles and 0.6 ± 0.1% in the pectoralis) were usually distributed in clumps that were sometimes associated with capillaries. Interfibrillar mitochondria accounted for 82.1% and 88.5% of the total mitochondrial volume density in the epaxial muscles and pectoralis muscle, respectively, with the balance classified as subsarcolemmal

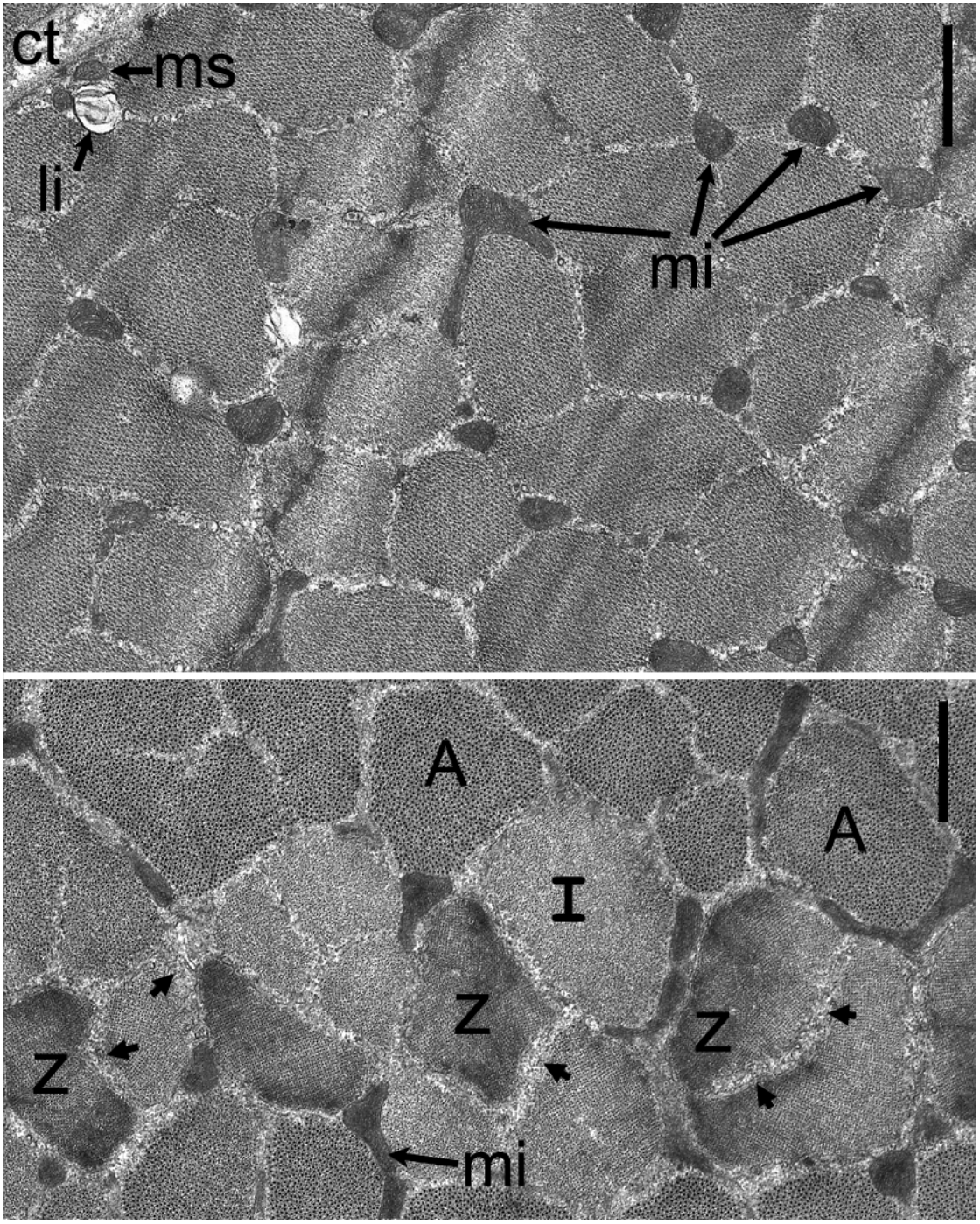

Fig. 7. Representative micrographs of harbor seal epaxial muscles. Li, lipid droplet; ct, connective tissue; mi, interfibrillar mitochondria; ms, subsarcolemmal mitochondria; A, I, Z, myofibrils sectioned through the A band, I band, and Z-disc, respectively; arrowheads, sarcoplasmic reticulum. Bar, 1 micron.

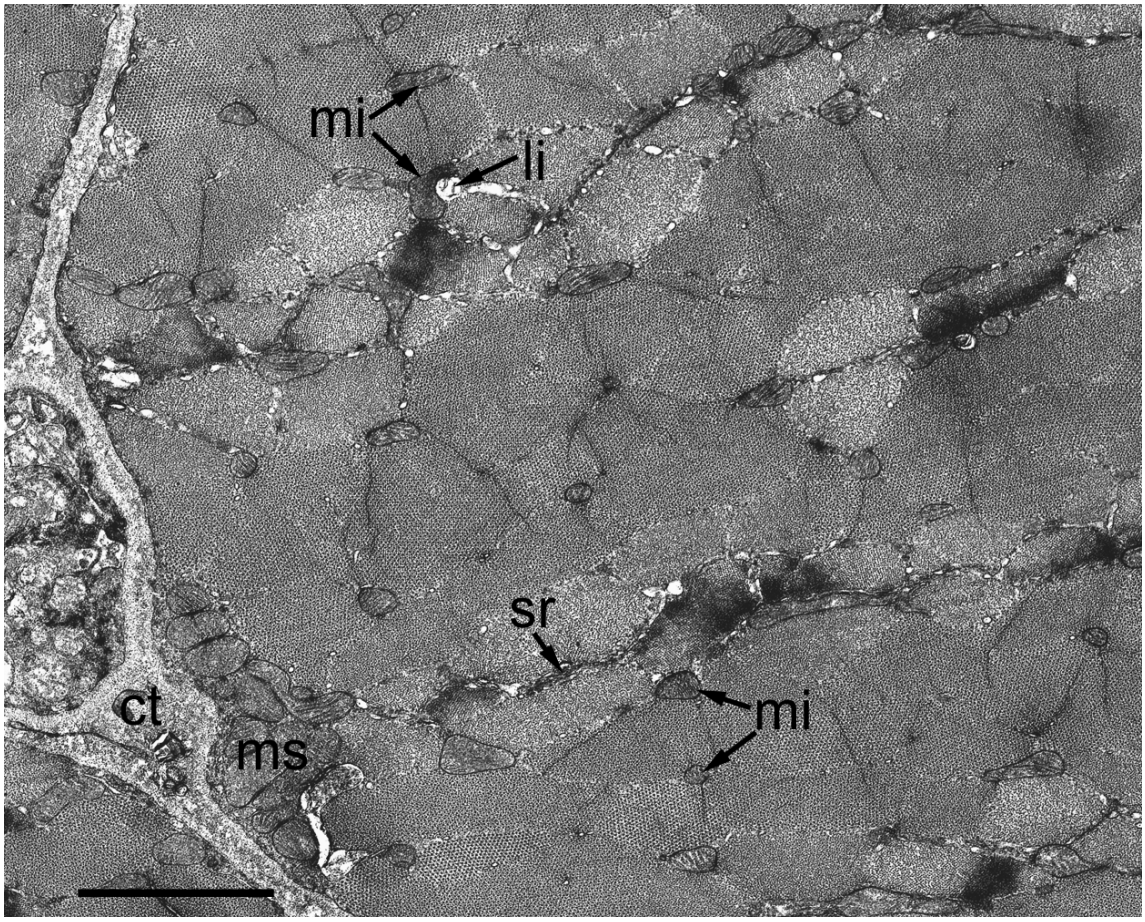

Fig. 8. Representative micrograph of harbor seal pectoralis muscle. Li, lipid droplet; ct, connective tissue; mi, interfibrillar mitochondria; ms, subsarcolemmal mitochondria; and sr, sarcoplasmic reticulum. Bar, 2 microns.

Table 3. Summary data for muscle morphometry of the harbor seals. Values are means ± SE. M_b, body mass; N, number of samples analyzed; V_v(mt, f), volume density of total mitochondria; V_v(ms, f), volume density of subsarcolemmal mitochondria; V_v(mi, f), volume density of interfibrillar mitochondria: V_v(li, f), volume density of lipid droplets; and V_v(fi, f), volume density of myofibrils.

	M_b (kg)	muscle	N	V_v (mt, f) %	V_v (ms, f) %	V_v (mi, f) %	V_v (li, f) %	V_v (fi, f) %
Seal 4	53.1	epaxial	7	6.19	1.06	5.13	0.18	85.1
Seal 5	43.6	epaxial	7	5.86	1.14	4.70	0.19	87.4
Seal 6	48.7	epaxial	7	5.33	0.92	4.40	0.11	85.2
Seal 7	63.9	epaxial	7	4.58	0.68	3.91	0.32	86.6
Seal 9	48.6	epaxial	7	5.89	1.14	4.75	0.15	83.8
mean (n=5)	51.6±3.4	epaxial	35	5.6±0.3	1.0±0.1	4.6±0.2	0.2±0.04	85.6±0.6
		pectoralis	5	5.2±0.3	0.6±0.1	4.6±0.2	0.2±0.1	85.6±1.2

Table 4. Mean percentages ± standard error of the volume density of total mitochondria expressed per fiber volume in deep and superficial regions of harbor seal epaxial muscles. See text for region explanation. *Significantly different from superficial.

Seal	Region	Mean V_v(mt, f) %
4	Deep	6.51±0.30
	Superficial	5.94±0.34
5	Deep	6.36±0.46*
	Superficial	5.18±0.40
6	Deep	5.56±0.43*
	Superficial	4.31±0.27
7	Deep	5.17±0.32*
	Superficial	3.95±0.08
9	Deep	7.29±0.52*
	Superficial	4.80±0.16
mean	Deep	6.03±0.26
	Superficial	4.95±0.23

mitochondria.

There were no significant differences between the epaxial and pectoralis muscles of the seals for any of the measured volume density variables (Table 3). Individual variation was high in all of the measured parameters.

There was evidence of heterogeneity within transverse sections. In four seals, the samples collected from the deep region of the muscle showed a significantly higher V_v (mt, f) than those samples collected more superficially (deep mean, 6.03%; superficial mean, 4.95%) ($p < 0.005$) (Table 4). In one seal (Seal 7), there was no significant difference between V_v (mt, f) measured in the deep region (5.17%) and superficial region (3.95%)of the epaxial musculature, and this seal also had a significantly lower (4.6%) mean V_v (mt, f) compared to the other seals (Table 4).

Discussion

Allometry

Muscle mitochondrial volume density scales inversely to body mass, although it is dependent on the muscle type (Mathieu et al., 1981b). The mean body mass (M_b) of the harbor seals was applied to the allometric equations for two muscles of the hindlimb (the semitendinosus and vastus medialis) that were generated from the V_v (mt, f) measurements of a variety of wild and domestic African mammals spanning several orders of magnitude in M_b (Mathieu et al., 1981b). Although these two muscles are functionally dissimilar to the epaxial muscles, the combined data represent the most comprehensive mitochondrial volume density analysis of

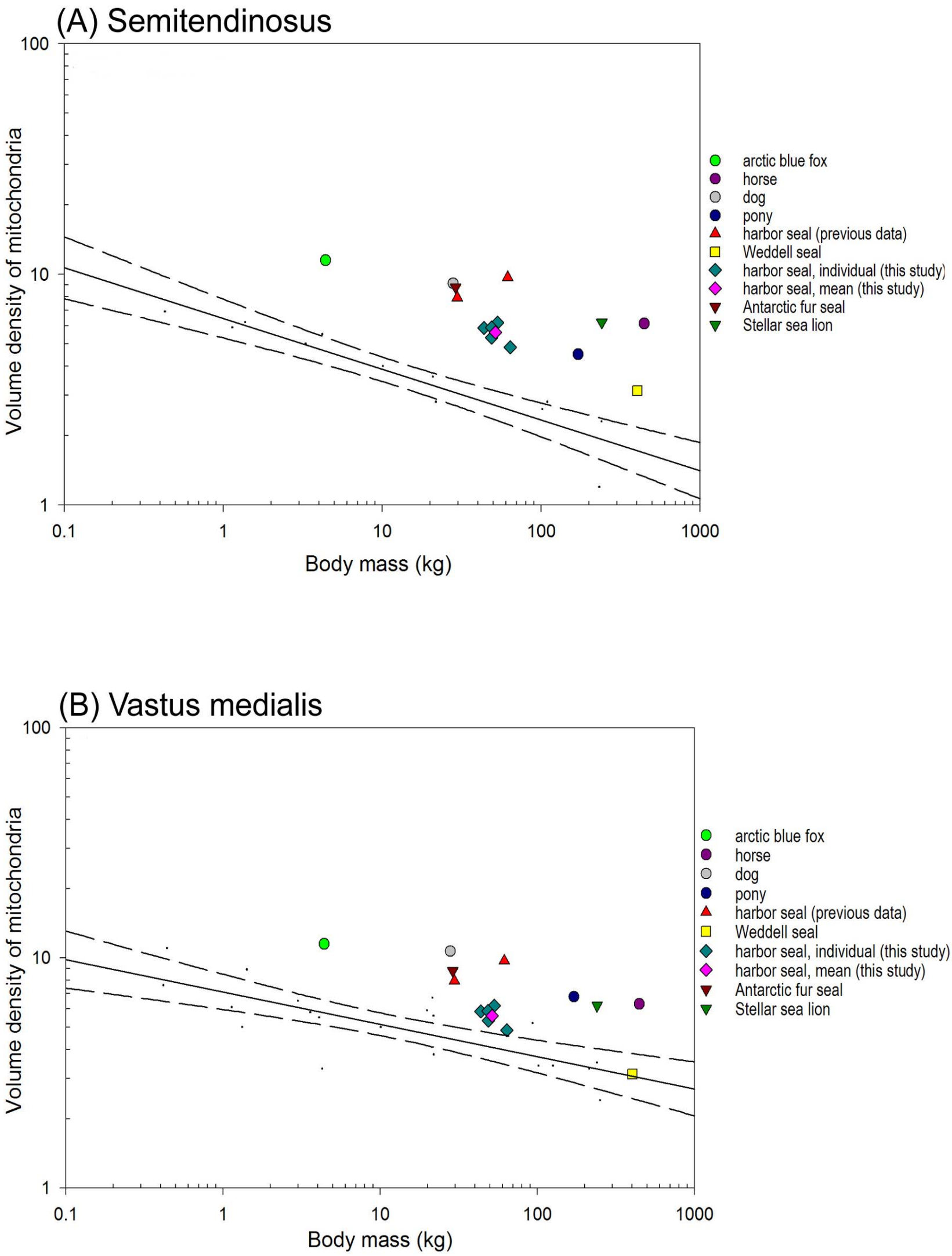

Fig. 9. Allometric plots of total mitochondrial volume density [V_v (mt, f)] (unitless) vs. body mass in kilograms for locomotory muscles of different function in terrestrial mammals and pinnipeds. V_v (mt, f) was analyzed in muscles from a variety of wild and domestic African mammals and plotted against body mass (black dots) to generate a regression line (—) with 95% confidence intervals (---) (Mathieu et al., 1981b). The V_v (mt, f) for the same muscle used to generate the regression line is also shown for some 'athletic' species (colored circles) to compare with the V_v (mt, f) of pinniped muscles, and is not included in the regression equation (Hoppeler et al., 1987; Kayar et al., 1989, 1992; Bicudo et al., 1995; Kanatous et al., 1999, 2001, 2002;). (A) Allometric plot for the semitendinosus (locomotor flexor) with the equation V_v (mt, f) = 0.065 x $M_b^{-0.231}$, (B) Allometric plot for the vastus medialis (locomotor extensor) with the equation V_v (mt, f) = 0.071 x $M_b^{-0.139}$.

locomotory muscles in wild mammals and the only muscle-specific allometric regressions of mitochondrial volume in the literature. In quadrupeds, the vastus medialis is a muscle of the quadriceps extensor group that undergoes active stretch prior to contraction during locomotion (Goslow et al., 1981), while the semitendinosus is a member of the hamstring group that flexes the knee in addition to extending the thigh. Neither muscle has a fiber type composition similar to that of the epaxial muscles of the seal. In a survey of the literature for the basic fiber types of the vastus medialis and the semitendinosus of different mammals with a variety of locomotory habits, the range of fiber type compositions for type I, type IIa, and type IIb fibers of the semitendinosus was 7-50%, 6-55%, and 30-58%, respectively, and the fiber type ranges of the vastus medialis was 0-62%, 5-53%, and 4-69%, respectively (Ariano et al., 1973; Hoppeler et al., 1981; Delp and Duan, 1996; Grotmol et al., 2002; Mattson et al., 2002). These fiber type compositions are distinctly different from that of the seal epaxial muscles, which are composed of approximately 50% type I fibers, 50% type IIa fibers and 0% type IIb fibers (Watson et al., 2003). Clearly, a degree of caution is necessary when interpreting the following comparative data.

The mean V_v (mt, f) of all five harbor seal epaxial muscles exceeded the upper 95% confidence interval (CI) for the predicted V_v (mt, f) of the vastus medialis and semitendinosus muscles of the terrestrial African mammals (Fig. 9) (Mathieu et al., 1981b). The mean V_v (mt, f) of each harbor seal also exceeded the upper 95% CI of both muscles. Thus, all five seals showed mean values of V_v (mt, f) that were moderately elevated over what would be predicted for an animal of equivalent mass. In contrast, the V_v (mt, f) measured in the muscles of untrained horses, ponies, foxes, and dogs and in the primary swimming muscles

of wild otariids (Stellar sea lion and Northern fur seal) were substantially elevated over the 95% CI (Fig.9). Earlier harbor seal muscle research measured V_v (mt, f) of spot biopsies taken from the midbelly portion of the longissimus dorsi of seals captured in the same region of Alaska as this study (Kanatous et al., 1999). Their results were likewise substantially higher than the 95% CI prediction for the semitendinosus and vastus medialis using the same allometric equation (Fig. 9). Subsequent mitochondrial volume density data collected from different harbor seals by the same author was slightly lower (V_v (mt, f) = 7.9%) (Kanatous et al., 2001). In this study, the V_v (mt, f) value plotted for each seal represents the mean V_v (mt, f) of all seven samples (both the deep and superficial fractions) taken from the transverse section of the seal (Fig. 9). A mean V_v (mt, f) of 6.4% calculated for the samples collected exclusively in the deep portion of the muscles is higher than the values presented for the mean of the transverse sections in Fig. 9, and provides a more accurate comparison to the published harbor seal morphometric data.

Therefore, from these data and published data, harbor seals possess varying degrees of elevated mitochondrial volume density in their muscles, although the elevation is not as consistently high with what has been measured in the locomotory muscles of animals bred for aerobic endurance. In the context of marine mammals, it has been proposed that seals have an elevated mitochondrial volume density in their skeletal muscles to reduce the diffusion distance between mitochondria and intracellular oxygen stores (oxymyoglobin) during subsurface swimming (Kanatous et al., 1999). At low partial pressures of oxygen that are incurred towards the end of a dive, the elevated mitochondrial volume density enhances intracellular convective oxygen transport and ensures

an adequate supply of oxygen to maintain aerobic metabolism in working muscles. This data supports this argument by providing evidence that harbor seals have an elevated V_v (mt, f) in their primary locomotory muscles compared to sedentary terrestrial mammals of equivalent mass. However, the elevation does not appear to be as extreme as what is found in terrestrial mammalian athletes.

Mitochondrial distribution

In this study, mean V_v (mi, f) of the epaxial muscles of the harbor seals was 82.1% of V_v (mt, f) (Table 3). In two previous studies, mean V_v (mi, f) in harbor seal epaxial muscles was approximately 85% and 94% of V_v (mt, f), respectively (Kanatous et al., 1999, 2001). These results indicate that a substantial proportion of the mitochondrial volume is found distributed among the myofibrils. Data from the literature was compiled to compare the relative percentages of interfibrillar and subsarcolemmal mitochondria in two different types of locomotory muscles among different animals (Fig. 10). The terrestrial non-athletic mammals had similar percentages of interfibrillar and subsarcolemmal mitochondria compared to the marine mammals. However, the terrestrial non-athletic mammals do not possess comparable mass-specific V_v (mt, f) (Fig. 9). Marine mammals have a mass-specific V_v (mt, f) that is similar to that of terrestrial athletes (Fig. 9), yet there appears to be a higher proportion of interfibrillar mitochondria in the marine mammals compared to that of the terrestrial athletic mammals (Fig. 10). It has been shown that 'athletic' terrestrial mammals typically have an elevated V_v (mt, f) in their skeletal muscles compared to the muscles of 'sedentary' mammals due to an elevation of both V_v

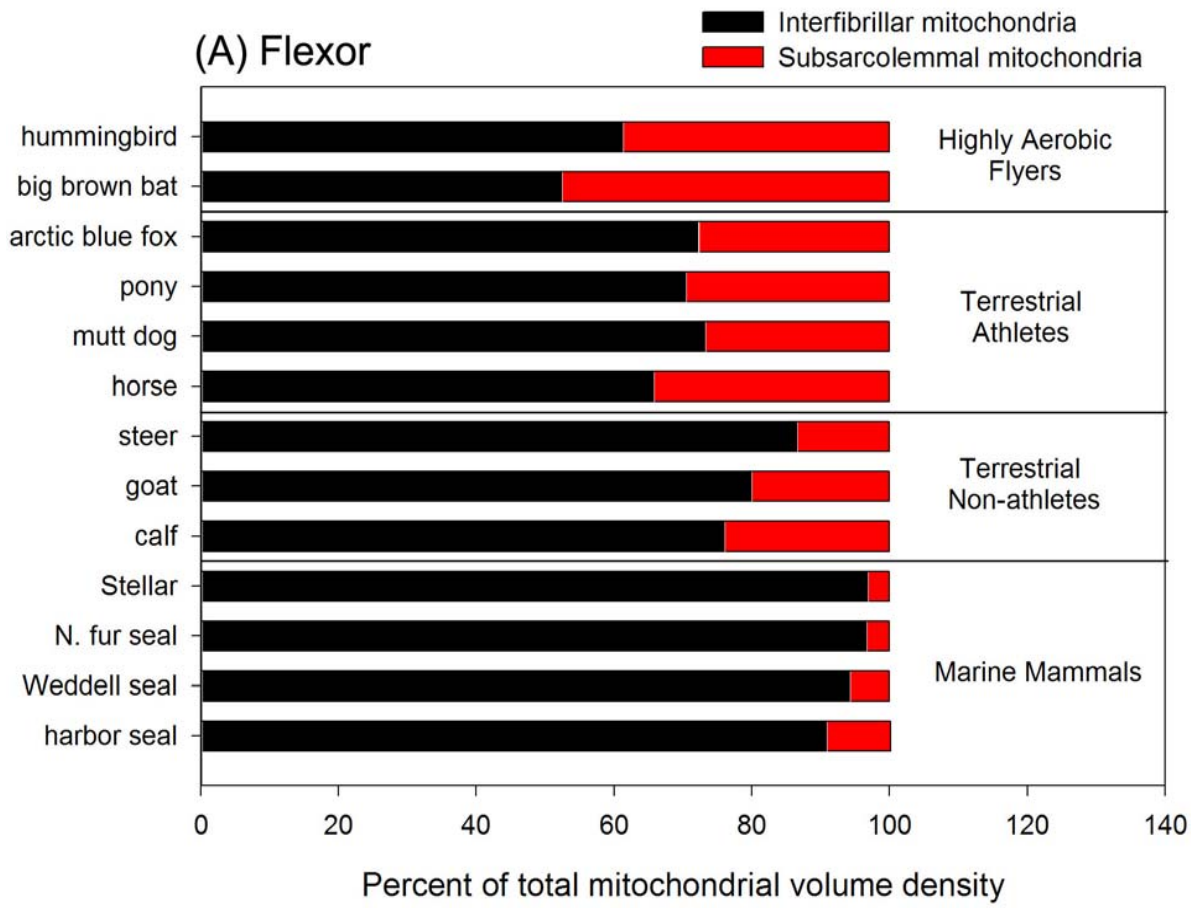

Fig. 10. Stacked bar charts showing the percent interfibrillar and percent subsarcolemmal mitochondria composition of total mitochondrial volume density in two locomotory muscle types of various animals. (A) The flexor muscles were the pectoralis and the semitendinosus for the marine mammals and the terrestrial mammals, respectively; (B) the extensor muscles were the epaxial muscles and the vastus medialis for the marine mammals and the terrestrial mammals, respectively; and in both charts the pectoralis muscle is shown for the highly aerobic flyers. In both muscle types, the marine mammals appear to have a high percentage of interfibrillar mitochondria compared to the other groups. Data for harbor seals compiled from this study, Kanatous et al. (1999, 2001), and Davis et al. (unpublished data); Stellar sea lion and northern fur seal, Kanatous et al. (1999); Weddell seal, Kanatous et al. (2002); goat, dog, calf, and pony, Hoppeler et al. (1987); steer, Kayar et al. (1989); fox, Bicudo et al. (1996); bat, Mathieu-Costello et al. (1992); hummingbird, Suarez et al. (1991).

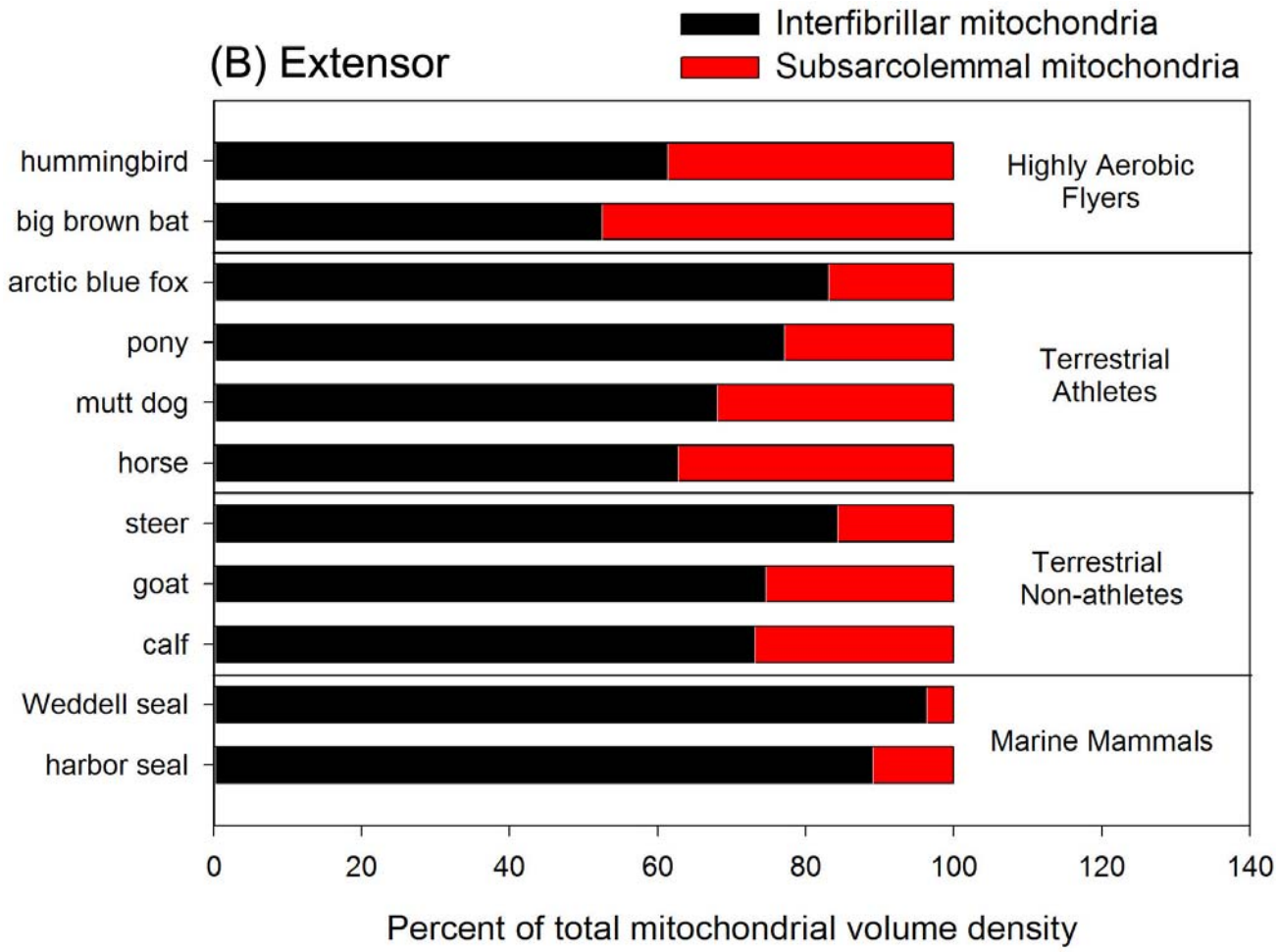

Fig. 10. Continued.

(ms, f) and V_v (mi, f) (Hoppeler et al., 1987; Kayar et al., 1989). Moreover, with the addition of the data for the bat and the hummingbird, which have among the highest measured V_v (mt, f) in locomotory muscles, it appears that locomotory muscles which rely on convective oxygen transport to support aerobic metabolism have a higher proportion of subsarcolemmal mitochondria, possibly to reduce the diffusion distance from the capillary to the mitochondria, thereby increasing oxygen flux (Mathieu-Costello et al., 1992). In contrast, pinniped skeletal muscle has an elevated proportion of interfibrillar mitochondria compared to athletic terrestrial mammals with similar V_v (mt, f). The elevated V_v (mt, f) and high proportion of interfibrillar mitochondria reduce the diffusion distance between mitochondria and the large stores of endogenous oxygen bound to myoglobin, especially under conditions of reduced convective oxygen transport (ischemic hypoxia) and reduced partial pressure of blood oxygen (hypoxic hypoxia) that occur during diving (Davis and Kanatous, 1999). Since a large proportion (ca. 33%) of the total oxygen store and even larger proportion (50%) of the oxygen used by the muscle comes from oxymyoglobin, an elevated mitochondrial volume density coupled with a homogenous distribution of mitochondria in the muscle fibers can function to maximize the diffusive flux of oxygen into muscle mitochondria under the hypoxic conditions associated with diving. Evidence in support of this theory is also available in experimental research of mammals acclimatized to hypoxia. Terrestrial mammals subject to chronic hypoxia may show morphological changes in their skeletal muscles that are comparable to the adaptations observed in the seal muscles. For example, in rats exercising in chronic hypoxia, the oxidative soleus muscle showed an increase in total mitochondrial volume density due to an increase in interfibrillar

mitochondria, while subsarcolemmal mitochondria decreased (van Ekeren et al., 1992). In the soleus muscle of guinea pigs subject to 14 weeks of chronic hypoxia, myoglobin concentrations were significantly higher (Sillau et al., 1980).

Thus, in pinnipeds, the diffusive flux of O_2 into the skeletal muscle mitochondria during breath-hold diving may be enhanced by two functional adaptations: (1) an elevated mitochondrial volume density (Kanatous et al., 1999), and (2) a relatively high proportion of interfibrillar mitochondria, which is manifest as a V_v (mt, f) that is distributed throughout the muscle fibers (Table 3, Fig. 10).

Heterogeneity of mitochondrial volume density

In the caudal transverse section of the epaxial muscles of the harbor seals, it was found that the V_v (mt, f) was significantly greater in samples collected in the deep region of the epaxial muscles compared with the superficial region (Table 4). This indicates heterogeneity of mitochondrial volume density in the lateral plane of the lumbar region of harbor seal swimming muscle. In quadrupedal mammals and man, metabolic heterogeneity is evident both within and among locomotory skeletal muscles. “Deep” muscles (closer to the bone) and postural (e.g., soleus) muscles tend to have higher populations of oxidative muscle fibers (type I and type IIa) than superficial muscles or muscles that are activated primarily during very energetic activities (e.g., white gastrocnemieus) (Armstrong et al., 1982; Armstrong and Phelps, 1984). Likewise, within each muscle, the compartmentalization pattern is similar: histochemical fiber typing and enzyme analyses have demonstrated that muscle fibers located close to the bone are characteristically more oxidative than those located on the muscle periphery

(Armstrong and Laughlin, 1985; Kline and Betchel, 1988; Rivero et al., 1993). Thus, in cat and cow locomotory muscles biopsied both superficially and from a deep portion of the muscle, the deep samples contained a higher proportion of oxidative fibers and had a significantly higher V_v (mt, f) compared to the superficial samples (Hoppeler et al., 1981). The regional heterogeneity of oxidative capacity and fiber twitch velocity relates to functional differences between the various sections of the muscle. Deeper areas of the muscle may be recruited for sustained, low intensity movements or postural maintenance, while the superficial regions are activated only during short duration, high velocity or high power activities (Armstrong, 1981; Armstrong and Laughlin, 1985).

In the harbor seals, although evidence of heterogeneity exists in the epaxial muscles, the aerobic capacity *in toto* is high. Immunohistochemical (IHC) fiber typing of samples collected from the identical locations in the epaxial muscles of the same harbor seals showed the presence of oxidative fibers only (type I and type IIa) and an absence of glycolytic fibers (IIb) (Watson et al., 2003). These results agree with the mitochondrial volume density data. However, there was no significant heterogeneity of muscle fiber type distribution found in the harbor seal epaxial muscles (Watson et al, 2003). The method of IHC fiber typing categorizes fiber types according to specific myosin isoform without consideration of the metabolic properties of the fiber (Pette and Staron, 1990). Because V_v (mt, f) was significantly higher in deeper regions compared to the muscle periphery, it may be concluded that the oxidative capacity of the epaxial muscles may be distributed within the muscle in a heterogeneous fashion,

whereas the distribution of the physical components of force generation do not follow the same trend.

There was also evidence that type I and type IIa fibers in the harbor seal may have similar oxidative capabilities. Like the epaxial muscles, seal pectoralis muscle consisted of only type I and type IIa fibers, but differed in that the pectoralis had a significantly higher numerical percent of type IIa fibers compared to the epaxial muscles (Watson et al., 2003). However, V_v (mt, f) was not significantly different in the two muscles (Table 3). These results indicate that both fiber types share a high oxidative capacity, although the relative oxidative capacity of the individual fibers was not measured in this study.

In a separate study, mitochondrial matrix enzymes and myoglobin content of the epaxial muscles and pectoralis muscle of the same harbor seals used in this study were analyzed. In general, the mitochondria volume density data within the epaxial muscles matched the enzyme activity data of tissue samples collected from the same locations in the transverse muscle sections. Citrate synthase activity (CS; an indicator of aerobic metabolism), β-hydroxyacyl CoA dehydrogenase activity (HOAD; an indicator of fatty acid metabolism), and lactate dehydrogenase activity (LDH; an indicator of anaerobic metabolism) were either comparable to or elevated in harbor seal muscle scaled to rat and dog enzyme activity (Polasek et al., in preparation). These data indicate a reliance on lipid-derived aerobic metabolism in seal swimming muscle. Elevated LDH activity in seals shows an increased anaerobic metabolic capacity and does not necessarily preclude the maintenance of a high aerobic capability. Polasek et al. (in preparation) also showed that myoglobin concentrations in both the epaxial muscles and the pectoralis muscle were elevated, which supports the

hypothesis that an elevation of the interfibrillar mitochondrial population in harbor seal skeletal muscle decreases the diffusion distance between mitochondria and endogenous oxygen stores.

Endogenous lipid depots

Mean percentages of V_v (li, f) in harbor seal epaxial muscles were similar (<0.3%) in all three studies that analyzed volume density of lipid droplets (Table 3, Kanatous et al., 1999, 2001). Endogenous triacylglycerol droplets were therefore not a large component in the muscles by volume, in contrast to the dorsal muscles of bottlenose dolphins (*Tursiops truncatus*), which were observed to have numerous, large lipid droplets (Tulsi, 1975). However, it is important to note that the point counting technique (with the B-36 test lattice) employed by all of the studies is subject to a potentially large RSE (Weibel, 1979; Mathieu et al., 1981b). Furthermore, lipid concentrations can be significantly affected by the individual's nutritional state and exercise history prior to muscle sample collection (Hoppeler, 1986). Despite the gross estimate of volume density of lipid droplets, these structures were clearly present in the muscle tissues, and three different studies independently measured similar volume density values. Thus, the potential energy available to the epaxial muscles from endogenous lipids was estimated. The calculations showed that the mean volume density of lipid (0.18%) in the seals' epaxial muscles provides enough energy to fuel aerobic metabolism in the muscle mass for 5-6 hours when a seal is exercising at 4-5 times resting muscle metabolic rate. These calculations assume that the density of muscle and lipid is 1.06 $g \cdot ml^{-1}$ and 0.94 $g \cdot ml^{-1}$, respectively (Mendez and Keys, 1960; Schmidt-Nielson, 1997), and that

the specific resting metabolic rate (RMR) of muscle = $125 \cdot Mb^{-0.17}$ (Wang et al., 2001). Thus, small amounts of endogenous lipid by volume may nevertheless provide substantial amounts of energy for muscle metabolism. Similarly, Vock et al. (1996) showed that the mean volume density of lipid (0.46%) measured in the muscles of dogs provides enough energy to fuel 3 hours of exercise at 60% maximum molar oxygen consumption rate. These authors suggested that endurance-adapted terrestrial mammals have higher intracellular energy depots (in the form of glycogen and lipid droplets) in their muscles compared to non-athletes to ensure adequate substrate supply all exercise intensities (Vock et al., 1996). However, in the athletic arctic blue fox, no endogenous lipid depots were detected in any of the tissues, indicating that their mitochondria receive substrate from an exogenous pathway (Hoppeler et al., 1987; Bicudo et al., 1995).

These data show that the caudal region of the harbor seal epaxial muscles possesses a moderately elevated V_v (mt, f) relative to terrestrial mammals of similar size. This appears to result from an elevation of V_v (mi, f) and not V_v (ms, f). These results provide further evidence that the elevated mitochondrial volume in seals functions to reduce intracellular diffusion of oxygen during diving. V_v (mt, f) of the seal epaxial muscles was not significantly different from that of the pectoralis muscle, indicating that the type I and type IIa fibers found in both muscles do not differ substantially in oxidative capacity. The V_v (mt, f) in the lateral plane of the muscle was greater deep to the vertebral column compared to samples collected more superficially, indicating some heterogeneity that may reflect the muscle's function or pattern of force generation. The results from this study are consistent with enzyme activities and myoglobin

concentrations performed on the same samples collected from the same animals (Polasek et al., in preparation). Taken together, the data show that the harbor seal's swimming muscles are morphologically adapted to maintain an aerobic, lipid-based metabolism under hypoxic conditions associated with subsurface swimming.

Overview

To better understand the density and distribution of mitochondria in seal muscles, samples distributed throughout a transverse section of the lumbar region epaxial musculature from of five wild harbor seals were analyzed using electron microscopy. Mean volume density of mitochondria [V_v(mt, f)] was 5.6%, which is slightly elevated over what would be predicted for a mammal of similar mass, but not as high as previously reported values. Mean V_v (mt, f) of single samples collected from the pectoralis muscle of the same seals was not significantly different from the epaxial muscles. The elevated V_v (mt, f) of the locomotory muscles of seals appeared to have a higher proportion of interfibrillar mitochondria compared to the muscles of terrestrial mammals with elevated V_v (mt, f). In addition, deeper regions of the epaxial muscles had a significantly higher V_v (mt, f) than more peripheral regions. These results are consistent with those for aerobic enzyme activities and fiber typing performed on the same muscle samples, which, taken together, provide further evidence that the elevated mitochondrial volume density in pinnipeds serves to decrease the oxygen diffusion distance between myoglobin and mitochondria to facilitate aerobic respiration in working muscles.

CHAPTER IV

SUMMARY

There is strong evidence that pinnipeds rely on aerobic metabolism during the majority of dives and are therefore well-adapted for aerobic respiration. Single biopsies taken from harbor seal epaxial (swimming) muscle have shown elevated mitochondrial volume densities, elevated aerobic enzyme activities, and an enhanced capacity for lipid catabolism. These results indicate adaptations for maintenance an aerobic, lipid-based metabolism under the hypoxic conditions associated with breath-hold diving. However, regional variations in mitochondrial density are unknown, and the few fiber typing studies performed on pinniped skeletal muscles are not consistent with an aerobic physiological profile. The objectives of this study were: (1) To reexamine the fiber type distribution throughout the primary locomotory muscles of the harbor seal (*Phoca vitulina*), and (2) To better understand the density and distribution of mitochondria in the locomotory muscles. To accomplish this, multiple samples from transverse sections of the primary swimming muscles (the epaxial muscles) of wild harbor seals were analyzed using immunohistochemical (IHC) fiber typing and electron microscopy. Fiber typing results indicated that harbor seal epaxial muscles are composed of 47.4% type I (slow twitch, oxidative) fibers and 52.8%, type IIa (fast twitch, oxidative) fibers, which are homogeneously distributed throughout the muscle. No fast twitch, glycolytic (type IIb) fibers were detected in the epaxial muscles or the pectoralis muscle, in contrast to the published data on fiber typing of harbor seal muscles using traditional histochemical techniques. Mean volume density of mitochondria [V_v(mt, f)] was

5.6%, which is slightly elevated over what would be predicted for a mammal of similar mass. The elevated V_v(mt,f) had a high proportion of intermyofibrillar mitochondria, a trait not normally found in the muscles of terrestrial mammals with elevated V_v(mt,f). These results are consistent with enzymatic data performed on the same muscle samples, which, taken together, provide further evidence that the elevated mitochondrial volume density in pinnipeds serves to decrease the oxygen diffusion distance between myoglobin and mitochondria to facilitate aerobic respiration in working muscles. In addition, V_v (mt, f) was analyzed for evidence of heterogeneity along the transverse plane of the epaxial musculature, and fiber type was analyzed for evidence of heterogeneity along both the longitudinal and transverse planes of the musculature. The results showed that the deeper regions of the epaxial muscles (located close to the vertebral column) showed a significantly higher V_v (mt, f) relative to those regions that were superficially located. In contrast, there was no significant heterogeneity of fiber type detected in either plane of the epaxial muscles. Thus, a fine-scale pattern of spatial heterogeneity of V_v (mt, f) was found within the epaxial muscles that does not manifest in fiber type distribution, indicating that the fibers have a similar oxidative capacity.

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