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Immunohistochemical fiber typing of harbor seal skeletal muscle

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Summary

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 re-examine 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% 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 us to conclude that harbor seal swimming muscle is entirely composed of oxidative fibers. Our 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.

Key words: harbor seal, *Phoca vitulina*, diving, skeletal muscle, muscle fiber, pinniped, fiber type, immunohistochemistry.

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 (Family Carnivora, Subfamily 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, we hypothesized that there would be a higher proportion of type I and type IIa fibers than type IIb fibers. We also hypothesized that the fiber type distribution within the muscles would be heterogeneous (i.e. we expected to see fast twitch fibers located superficially and slow twitch fibers located deeper in the muscle). Our 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, 1928). 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.

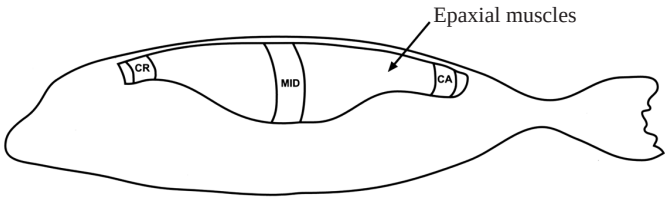

Fig. 1. Schematic diagram illustrating the location of the three transverse sections taken from the epaxial muscles. CR, cranial; MID, middle; CA, caudal.

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

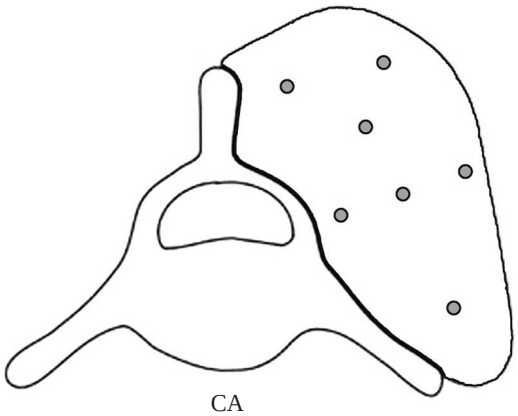

Fig. 2. Representative transverse section showing the coring pattern of samples collected from the epaxial muscles. CA, caudal.

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., 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 (e.g. Fig. 5B,C) 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

We used a combination of mouse anti-rat primary antibody and goat anti-mouse secondary antibody 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. We have also verified antibody reactivity 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

We analyzed fiber type distribution 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, we divided the seven samples of the CR, MID and CA sections into one of two categories, 'proximal' or 'distal'. We then applied a 3-factor analysis of variance (ANOVA) using Minitab statistical software. By using a 3-factor ANOVA, we were able to analyze simultaneously the distribution of fibers in both the lateral and longitudinal planes of the epaxial muscles. 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', we grouped the samples of each section 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 'proximal' or 'distal' to the vertebral column (Fig. 4). 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 muscle 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 1, Fig. 5). 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 48.3%, respectively. The mean numerical populations of type IIa fibers as a percentage of total fibers for the three transverse sections were 54.5%, 52.3% and 52.2%, respectively.

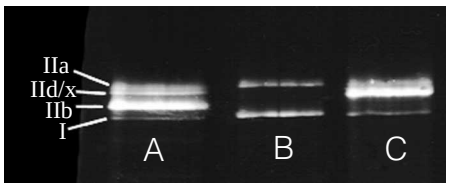

Fig. 3. Silver stain (reverse western blot) of (A) rat vastus medialis muscle, (B) seal epaxial muscles, and (C) seal pectoralis muscle, showing differentiation of myosin heavy chain isoforms based on molecular mass. The type IIb band is absent in both seal muscles.

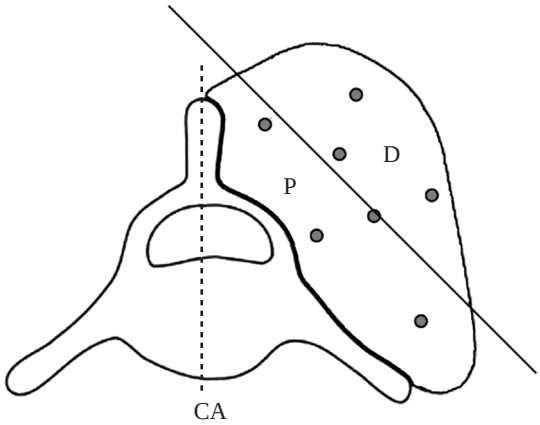

Fig. 4. Representative transverse section showing the statistical division used for testing homogeneity within transverse sections of the epaxial muscles. The broken line represents the natural vertical axis of the animal and the solid line the line drawn at a 45° angle to the vertical axis to divide the epaxial muscles into proximal (P) and distal (D) sections. CA, caudal.

Table 1. *Mean population percentages of fiber types from three cross sections of the harbor seal epaxial muscle and pectoralis*

	Fiber type (%)		
	Type I	Type IIa	Type IIb
Epaxial muscles			
Cranial (10)	45.3±5.1	54.5±4.7	0
Middle (9)	47.7±3.3	52.3±3.6	0
Caudal (9)	48.3±5.8	52.2±5.7	0
Mean of all sections (9)	47.4±4.7	52.8±4.6	0
Pectoralis (6)	16.2±5.6*	84.3±5.4*	0

Values are means ± 1 S.D. (*N*), where *N* is number of animals averaged for each cross section.

*Significant difference from the epaxial muscles at $P<0.01$.

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 long axis of the epaxial muscle fibers (Table 1) or proximity to the vertebrae. The distribution of type I fibers was not significantly different with respect to the long axis of the epaxial muscle fibers (Table 1). 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 muscle (Table 1) ($P<0.0001$).

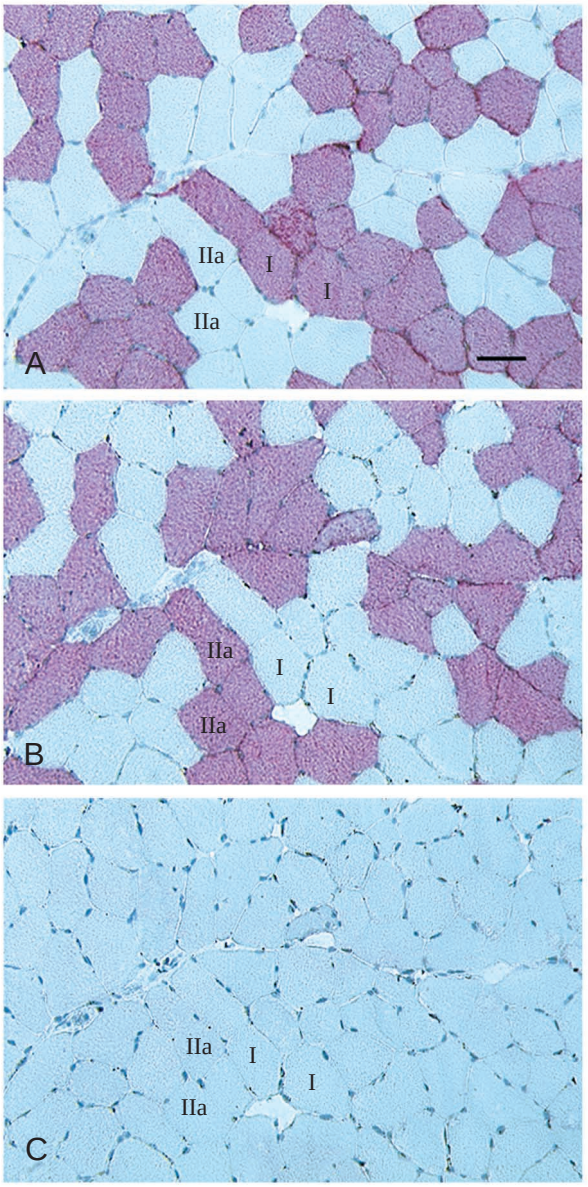

Fig. 5. Representative serial cross sections from the epaxial muscles of a harbor seal *Phoca vitulina* stained for myosin heavy chain isoforms using a series of monoclonal antibodies. (A) Type I (slow-twitch, oxidative) fibers, (B) type IIa (fast-twitch, oxidative) fibers and (C) type IIb (fast-twitch, glycolytic) fibers. Lack of stain in C indicates an absence of type IIb fibers in this cross section of the muscle. Bar, 50 μm.

Discussion

Although published data based on traditional histochemical techniques agree with our results for type I fiber populations, there is a substantial difference in the results for the type IIa and type IIb fibers. Our results 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 fiber type distribution of 20% slow, oxidative fibers, 27% fast oxidative-glycolytic fibers, and 53% fast glycolytic fibers in an unspecified muscle of one Stellar sea lion. Our results 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 we have found in the harbor seals. However, based on the variable staining intensity of the histochemically stained cells shown in the figures, we believe that 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, we 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. We are confident that the IHC fiber typing technique demonstrates a lack of classical type IIb fibers in the locomotory muscles of harbor seals. However, we cannot rule out the presence of other type II MyHC isoforms, in particular type IId/x and type IIc. SDS-PAGE 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, we did not have an antibody capable of specifically differentiating the type IId/x isoform and therefore we analyzed the type IId/x fiber population 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, we found that the fiber type population 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 (L. K. 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 1). 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. (Castellini et al., 1992; Davis et al., 1985; 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).

Our fiber typing results show that harbor seal skeletal muscle is made exclusively of slow-twitch and fast-twitch oxidative 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.

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