JOURNAL OF MORPHOLOGY 274:663–675 (2013)

Locomotor Muscle Profile of a Deep (*Kogia breviceps*) Versus Shallow (*Tursiops truncatus*) Diving Cetacean

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ABSTRACT When a marine mammal dives, breathing and locomotion are mechanically uncoupled, and its locomotor muscle must power swimming when oxygen is limited. The morphology of that muscle provides insight into both its oxygen storage capacity and its rate of oxygen consumption. This study investigated the *m. longissimus dorsi*, an epaxial swimming muscle, in the long duration, deep-diving pygmy sperm whale (*Kogia breviceps*) and the short duration, shallow-diving Atlantic bottlenose dolphin (*Tursiops truncatus*). Muscle myoglobin content, fiber type profile (based upon myosin ATPase and succinate dehydrogenase assays), and fiber size were measured for five adult specimens of each species. In addition, a photometric analysis of sections stained for succinate dehydrogenase was used to create an index of mitochondrial density. The *m. longissimus dorsi* of *K. breviceps* displayed significantly a) higher myoglobin content, b) larger proportion of Type I (slow oxidative) fibers by area, c) larger mean fiber diameters, and d) lower indices of mitochondrial density than that of *T. truncatus*. Thus, this primary swimming muscle of *K. breviceps* has greater oxygen storage capacity, reduced ATP demand, and likely a reduced rate of oxygen consumption relative to that of *T. truncatus*. The locomotor muscle of *K. breviceps* appears able to ration its high onboard oxygen stores, a feature that may allow this species to conduct relatively long duration, deep dives aerobically. J. Morphol. 274:663–675, 2013.

KEY WORDS: skeletal muscle; morphology; histochemistry; cetaceans

INTRODUCTION

When a marine mammal dives, breathing and locomotion are mechanically uncoupled, and its locomotor muscles must power swimming when oxygen is limited (e.g., Butler and Jones, 1997; Davis and Kanatous, 1999; Cotten et al., 2008). Bradycardia and peripheral vasoconstriction reduce convective oxygen delivery to swimming muscles, which must, therefore, depend primarily upon endogenous stores of oxygen bound to myoglobin, to fuel locomotion underwater (Scholander, 1940; Kooyman, 1973; Kooyman et al., 1983; Castellini and Kooyman, 1989; Butler and Jones, 1997; Kanatous et al., 1999; Polasek and Davis, 2001). The aerobic dive limit (ADL) is the maximum time an animal can dive while maintaining aerobic metabolism and is a function of both whole body oxygen storage capacity and the rate of oxygen consumption (Kooyman et al., 1980). Because skeletal muscle mitochondria consume more than 90% of an animal's total body oxygen during maximal oxygen consumption (Mitchell and Blomqvist, 1971; Hoppeler et al., 1987; Taylor, 1987; Weibel, 2002), the morphology and physiology of a marine mammal's skeletal muscle provides insight into its ADL (Kanatous et al., 1999, 2002; Watson et al., 2003; Williams et al., 2011).

The concentration of myoglobin ([Mb]), the oxygen-binding protein within muscle, provides a measure of oxygen storage capacity (e.g., Scholander, 1940; Kooyman, 1989; Dolar et al., 1999; Noren and Williams, 2000; Hochachka and Somero, 2002). A muscle's fiber type profile provides information regarding the contractile function (slow vs. fast) and the predominant metabolic pathways (aerobic vs. anaerobic) used by active swimming muscle (Peter et al., 1972). Mitochondrial volume density within the skeletal muscle provides information on an animal's aerobic capacity, defined as the maximal rate of oxygen consumption (Schwerzmann et al., 1989; Hoppeler and Weibel, 2000; Burpee et al., 2010).

Muscle fiber size may also offer insight into the metabolic costs of cell maintenance. The smaller the muscle fiber diameter, the shorter the distance oxygen must diffuse into the cell to reach a mitochondrion, the site of cellular metabolism (reviewed in Kinsey et al., 2007, 2011). Aerobic, slow oxidative (Type I) fibers are generally smaller in diameter than anaerobic, fast glycolytic (Type IIb) fibers, which are not constrained by oxygen diffusion rates during active contraction (Bello

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Received 3 July 2012; Revised 31 October 2012; Accepted 16 December 2012

Published online 28 February 2013 in Wiley Online Library (wileyonlinelibrary.com)
DOI: 10.1002/jmor.20124

et al., 1985; Dearolf et al., 2000; Jimenez et al., 2008). Because a large muscle fiber also possesses a relatively low surface area to volume ratio (SA:V), it has been proposed that it experiences lower metabolic costs associated with maintaining the muscle fiber membrane potential than does a smaller fiber (Johnston et al., 2004, 2006). Jimenez et al. (2011) have recently provided experimental data on crustacean muscle that supports this hypothesis. If this phenomenon is broadly applicable, then large muscle fiber size may reduce cell maintenance costs in diving mammals.

The relationships between dive behavior, ADL, and locomotor muscle morphology have been well characterized in pinnipeds (e.g., Kanatous et al., 1999, 2001, 2002, 2008; Watson et al., 2003, 2007; Polasek et al., 2006). For example, the harbor seal, *Phoca vitulina*, is a relatively short duration, shallow-diving species (5–19 m mean dive depth, 1–3 min mean dive duration) (Gentry and Kooyman, 1986; reviewed in Schreer and Kovacs, 1997), with a relatively short calculated ADL ($\sim$10 min) (Burns et al., 2005). Conversely, the Weddell seal, *Leptonychotes weddellii*, is a long duration, deep-diving species (100–350 m mean dive depth, 10–12 min mean dive duration) with an extended ADL of 20–24 min (Kooyman, 1966; Kooyman et al., 1980, 1981; Castellini et al., 1992; reviewed in Schreer and Kovacs, 1997; Williams et al., 2000).

Although all marine mammals have elevated levels of myoglobin compared to terrestrial mammals, the myoglobin concentration of the locomotor muscle of *L. weddellii* is 1.5 times higher than that of *P. vitulina* (Kanatous et al., 1999, 2002). *L. weddellii* possess skeletal muscle composed predominantly of slow, oxidative (Type I) fibers, whereas those of *P. vitulina* are composed predominantly of fast, oxidative glycolytic (Type IIa) fibers (Kanatous et al., 2002; Watson et al., 2003). The locomotor muscles of *L. weddellii* have low aerobic capacities (Kanatous et al., 1999, 2002, 2008; Watson et al., 2003, 2007). Those of *P. vitulina*, by comparison, have relatively high aerobic capacities, similar to those of terrestrial endurance athletes (e.g., dog and pony; Kanatous et al., 1999). The muscle fiber diameters of *L. weddellii* are reported to be larger than those of *P. vitulina* (Kanatous et al., 2001, 2002).

The data summarized above suggest that skeletal muscle's enhanced oxygen storage capacity, coupled with a slower twitch muscle profile and reduced oxygen consumption rate, extends the ADL of *L. weddellii*, as compared to *P. vitulina.* While there are considerable comparative data on locomotor muscle morphology for pinnipeds, fewer such data are available for cetaceans, in large part because access to high quality specimens has been limited. Myoglobin content, the most commonly reported feature of cetacean locomotor muscle, has been assessed for a variety of species (Shaffer et al., 1997; Dolar et al., 1999; Noren and Williams, 2000; Polasek and Davis, 2001; reviewed in Hochachka and Somero, 2002). Although considerable interspecific variation exists, deep-diving species tend to have higher skeletal muscle myoglobin contents than do shallow-diving species (e.g., Noren and Williams, 2000), a pattern similar to that observed in pinnipeds.

Most literature regarding cetacean muscle morphology focuses on bottlenose dolphins (*Tursiops truncatus*; Bello et al., 1985; Dearolf et al., 2000; Noren et al., 2001, 2002; Etnier et al., 2004; Cotten et al., 2008). The well-studied coastal ecotype of *T. truncatus* dives for an average of 20–40 s to depths between 2–10 m (Irvine et al., 1981; Connor et al., 2000; reviewed in Piscitelli et al., 2010), making it a relatively short duration, shallow diver. Recently, features of the locomotor muscle of the long duration, deep-diving (362 m mean dive depth, 12–13 min mean dive duration, wintering grounds) narwhal, *Monodon monoceros*, have been described (Heide-Jørgensen and Dietz, 1995; Williams et al., 2011). Similar to the pattern described for pinnipeds, the locomotor muscles of the short duration shallow-diving *T. truncatus* have lower myoglobin content, and proportionately fewer aerobic (Type I) fibers than do those of the long duration, deep-diving *M. monoceros* (Bello et al., 1985; Dearolf et al., 2000; Noren and Williams, 2000; Noren et al., 2001; Williams et al., 2011). To date, the Arctic narwhal is the only deep-diving cetacean for which such muscle data exist.

The goal of this study was to investigate the muscle morphology of another deep-diving cetacean, the pygmy sperm whale (*Kogia breviceps*), and compare it to the well-studied *T. truncatus*. Both species have a wide geographic range, inhabiting temperate to tropical waters (Mead and Potter, 1995; reviewed in Hoelzel et al., 1998; NOAA Stock Report, 2005; reviewed in Beatson, 2007). In the North Atlantic, *K. breviceps* are most commonly sighted in waters 400–1000 m deep (reviewed in Scott et al., 2001; Clarke, 2003; NOAA Stock Report, 2005). Although the dive behavior of kogiids is not well studied, investigations of stranded individuals reveal that their diet consists of predominantly mid- to deep-water species of cephalopods (McAlpine et al., 1997; Santos et al., 2006; Beatson, 2007; reviewed in Piscitelli et al., 2010). Dive times obtained from a study of visually tracked animals report an average dive duration of 13.1 min and maximum dive duration of 52 min (Barlow et al., 1997). Additionally, a tagging study by Scott et al. (2001) of a single, rehabilitated and released *K. breviceps* subadult reports a maximum dive duration of 18 min. While data are limited, these studies suggest that *K. breviceps* is a relatively long duration, deep-diving species as compared to *T. truncatus*. ADL has not been directly measured in either species but the

TABLE 1. Specimens utilized in this study

Identification number	Total length (cm)	Mass (kg)	Sex	SI code*
Tursiops truncatus				
CJH 003	229.5	138.0	F	2
WAM 633	244.0	180.0	F	1
WAM 628	246.0	213.0	F	2
WAM 642	250.0	226.0	M	2
BRF 061	275.0	257.0	M	2
Kogia breviceps				
MDB 056	263.5	–	M	2
BRF 092	267.0	316.6	F	1
KMS 427	267.0	363.6	F	1
KLC 051	271.0	–	M	1
KMS 429	283.0	371.8	M	2

*Specimens identified by their collector's field number. SI code = Smithsonian Institution Code, 1 = live stranding, 2 = fresh carcass.

calculated ADL for *T. truncatus* is 4.8–5.4 min (Noren et al., 2002).

Using the comparative observations for diving pinnipeds as a predictive model, this study tested the hypotheses that the locomotor muscle of the relatively long duration, deep-diving *K. breviceps* will display higher oxygen storage capacity, a slower fiber type profile, lower aerobic capacity, and larger diameter muscle fibers than those of *T. truncatus*. To date, the only published information on skeletal muscle composition of *K. breviceps* is the myoglobin content of the locomotor muscle (*m. longissimus dorsi*) of a single individual, which was approximately twice that of *T. truncatus* (Noren and Williams, 2000). This study reexamines myoglobin concentration in both *K. breviceps* and *T. truncatus* and increases sample size to supplement published studies. Published values regarding muscle aerobic capacity are lacking for any cetacean.

METHODS

Specimens

This study relied upon an archive of frozen samples obtained between November 2004 and December 2009, collected along the North Carolina coastline. All assays were conducted between June 2010 and May 2011. All research was carried out under UNCW IACUC Protocols #2003-013, #2006-015, #A0809-019, and under a NOAA Stranding Agreement to UNCW.

Muscle samples were collected from adult *Kogia breviceps* ($n = 5$) and *Tursiops truncatus* ($n = 5$) that had either stranded or been taken incidental to fishing operations (Table 1). Specimens were high-quality carcasses (Smithsonian Institute Code 1 or 2) in good body condition at the time of stranding. All *K. breviceps* specimens used were sexually mature. All *T. truncatus* specimens were greater than 225 cm total length; Dearolf et al. (2000) demonstrated that individuals greater than 200 cm total length have mature skeletal muscle. An entire cross-section of epaxial locomotor muscle from each specimen was taken at the position of the dorsal fin, wrapped in Saran™ wrap, double-wrapped in Ziploc® freezer bags, and stored at −20°C until analyzed. For all analyses described below, samples of the *m. longissimus dorsi*, at the position just ventral to the superficial tendon (Pabst, 1990), were used (Fig. 1).

Myoglobin Content

The myoglobin content ([Mb], g Mb/100 g wet muscle mass) of each muscle sample was obtained using methods adapted from Reynafarje (1963) by Noren and Williams (2000) and Etnier et al. (2004). Briefly, frozen tissue samples of approximately 0.5 g were thawed, minced, and fat and connective tissue removed. Three 0.5 g replicates were subsampled for each specimen. Minced samples were added to a 0.04 M phosphate buffer (4°C, pH 6.6), to a final dilution of 39.25 ml buffer per gram of tissue. Muscle was homogenized (Kinematica® Polytron PT 2100) completely and centrifuged at 28,000*g* for 50 min, at a temperature of 4°C. Approximately 5 ml of clear supernatant were drawn from each centrifuge tube and bubbled at room temperature with pure CO for 8 min. Following bubbling, approximately 0.02 g of sodium dithionite was added, the solution was vortexed for 10 s to ensure complete reduction of chromoproteins, and bubbled with CO for an additional 2 min. Approximately 2 ml of solution was then transferred to a cuvette and the absorbance of each sample was read using a spectrophotometer (Ultraspec 3000, Ultraspec 4000, Pharmacia Biotech) at room temperature (∼ 25°C). The difference in absorbance at 538 and 568 nm was multiplied by a constant (117.3) (Reynafarje, 1963) to determine mean myoglobin concentration. Three sequential readings were obtained for each of the three replicates and the mean value was reported for each specimen. Values are reported as means ± standard errors and data were statistically analyzed using an unpaired, one-tailed *t*-test to compare myoglobin content across species.

Muscle Fiber Type and Diameter

A 1 cm^3 block of the *m. longissimus dorsi* was cut from the center of each frozen epaxial muscle cross-section and partially thawed. Each sample block was mounted on a microtome chuck with Optimum Cutting Temperature (OCT) compound (Sakura Finetek), coated with additional OCT compound, and flash-frozen in liquid nitrogen to −160°C. Flash-frozen tissue blocks were then stored in a Leica Cryocut 1800 freezing microtome at −19°C for at least 2 h prior to cutting, to allow the tissues to warm to an appropriate temperature for sectioning. Nonsequential sections (10 μm) were mounted on "Plus" glass slides (Fisher Scientific Superfrost®/Plus).

Muscle sections were stained for myosin ATPase, under both alkaline and acidic conditions to differentiate Type I and II fibers, following the methods of Hermanson and Hurley (1990), as adapted by Dearolf (2003). One series of sections was preincubated in an alkaline solution (pH 10.3; 32 mmol^{-1} $CaCl_2$, 53 mmol^{-1} NaCl, 53 mmol^{-1} glycine, 45 mmol^{-1} NaOH) for 10 min. Another series of sections was preincubated in an acidic solution (pH 4.1–4.15; 43.5 mmol^{-1} barbital acetate, 43.5 mmol^{-1} HCl) for 5 min. All sections were then incubated for 30 min in a freshly prepared ATP solution (pH 9.4; 0.02 mmol^{-1} sodium barbital, 18 mmol^{-1} $CaCl_2$, 2.7 mmol^{-1} ATP) at 37°C. Sections were subsequently run through a series of 3 min rinses with deionized water (pH 8.5–9.0), 2% calcium chloride, and 1% cobalt chloride; stained for 3 min (1% ammonium sulfide); and rinsed in cold deionized water for 5 min. Sections were dehydrated and coverslips were mounted onto the slides with Permount mounting media.

Additional sections were stained for succinate dehydrogenase (SDH) to differentiate between Type I, IIa, and IIb fibers following the methods of Peter et al. (1972), as adapted by Dearolf et al. (2000). Sections were incubated at 37°C in nitro blue tetrazolium in 0.2 mol l^{-1} phosphate buffer containing 0.32 mol l^{-1} sodium succinate (pH 7.6). Optimal staining was achieved with incubation times of 30 min for *T. truncatus* and 1 h for *K. breviceps*. Following incubation, slides were rinsed in saline for 2 min and fixed in a 10% formalin–saline solution for 10 min. Once fixed, slides were rinsed in 15% ethanol for 5 min and coverslips were mounted with Kaiser's glycerine jelly (Presnell and Schriebman, 1997).

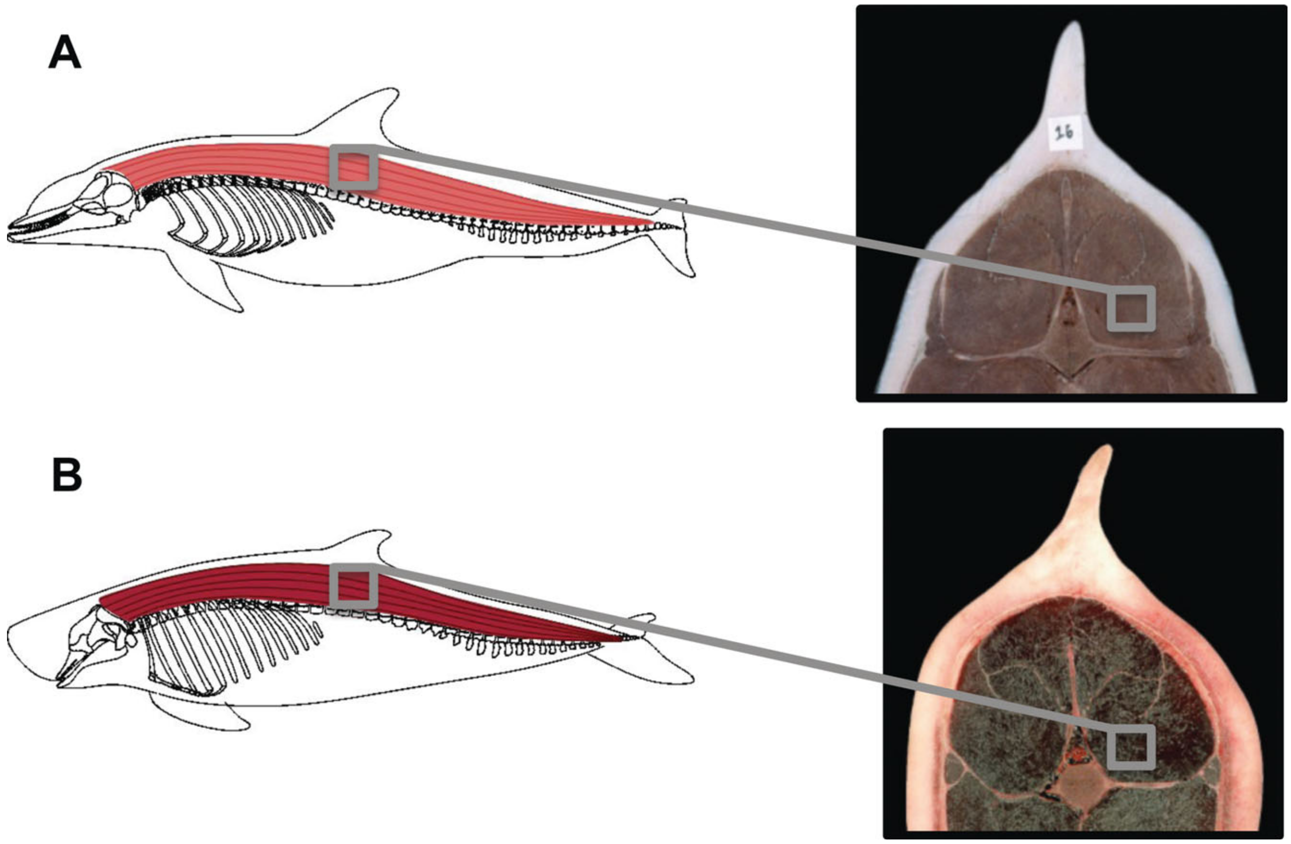

Fig. 1. Schematics and cross-sections of muscle sampling sites. The cross-section of the epaxial muscle mass was taken at the level of the dorsal fin. For all analyses, samples of *m. longissimus dorsi* were taken just ventral to the superficial tendon. **A** = bottlenose dolphin (*Tursiops truncatus*), **B** = pygmy sperm whale (*Kogia breviceps*) (Cross-section for B is *K. sima*).

Additional sections underwent immunohistochemical staining, using SC-71, a Type IIa-specific antibody, in an effort to further differentiate between Type IIa and IIb fibers (Schiaffino et al., 1989). Serial frozen sections were brought to room temperature and a mini PAP pen (Invitrogen) was used to outline mounted sections. Slides were rinsed with phosphate-buffered solution (PBS, 1 ×) for 15 min and then sequentially incubated at room temperature in horse serum (2.5%) (Vector MP-7402 ImmPRESS Anti-Mouse Ig peroxidase detection kit) (20 min) and primary antibody (SC-71) (1 h). Slides were rinsed three times in 1 × PBS for 5 min intervals. Following PBS rinses, slides were incubated in secondary antibody (1:1 antimouse IgG/1 × PBS) (30 min) in a hydrated box. Slides were rinsed three times in 1 × PBS for 5 min intervals. Diaminobenzadine (Vector MP-7402 ImmPRESS Anti-Mouse Ig peroxidase detection kit) was applied to slides until optimal staining occurred (~1 min). Stained samples were rinsed three times in 1 × PBS for 5 min intervals, and cover slips were mounted with Kaiser's glycerine jelly.

Muscle sections were viewed using a light microscope (Olympus BX60) operated in brightfield mode at ×20 magnification and digital micrographs were captured (Diagnostic Instruments SPOT RT camera) and stored as uncompressed files (TIFFs). Quantitative measures of fiber type (area and count) and size were determined using these images. A Mertz curvilinear grid was used to determine the percent area occupied by each muscle fiber type, which accounted for size differences between fiber types (Bozzola and Russell, 1999; Russ and Dehoff, 2000). A digital micrograph was projected onto a computer screen and overlaid with a Mertz curvilinear grid. Points residing within each specific fiber type and those that were within white space were counted, and this process was repeated for subsequent fields of view until a minimum of 500 total fibers were counted for each specimen. Points that resided in space were deducted from the total point count. To determine the percentage of each specific fiber type by area, the number of points counted for each fiber type was divided by total muscle points counted.

A standard point count method was used to quantify the relative number of Type I and IIa/IIb fibers by projecting a digital micrograph on a computer screen, overlaying a 15 cm^2 grid and counting each fiber type until a minimum of 150 total fibers were counted for each specimen (e.g., Dearolf et al., 2000). The percentage of each specific fiber type was calculated as the specific fiber count divided by total fiber count. Area and point count values are reported as means ± standard errors and data were statistically analyzed using unpaired, one-tailed *t*-tests to compare fiber profiles across species.

Alkaline ATPase-stained fibers were used to measure cross-sectional area and diameter of individual fibers following Dearolf et al. (2000). Ten fibers of each type with uniform, circular cross-sections were arbitrarily selected and measured. Each fiber from this subset was outlined individually in Adobe Photoshop® 7.0, saved as a TIFF and analyzed in Media Cybernetics ImagePro Plus® 6.0 software. The "mean diameter tool," which reported the mean of diameters measured at 2° intervals around its circumference, was used. Values are reported as means ± standard errors and data were statistically analyzed using unpaired, one-tailed *t*-tests to compare fiber size within and across species.

Index of Mitochondrial Density

This study relied upon an archive of fresh-frozen muscle samples. Matched samples, fixed for transmission electron micros-

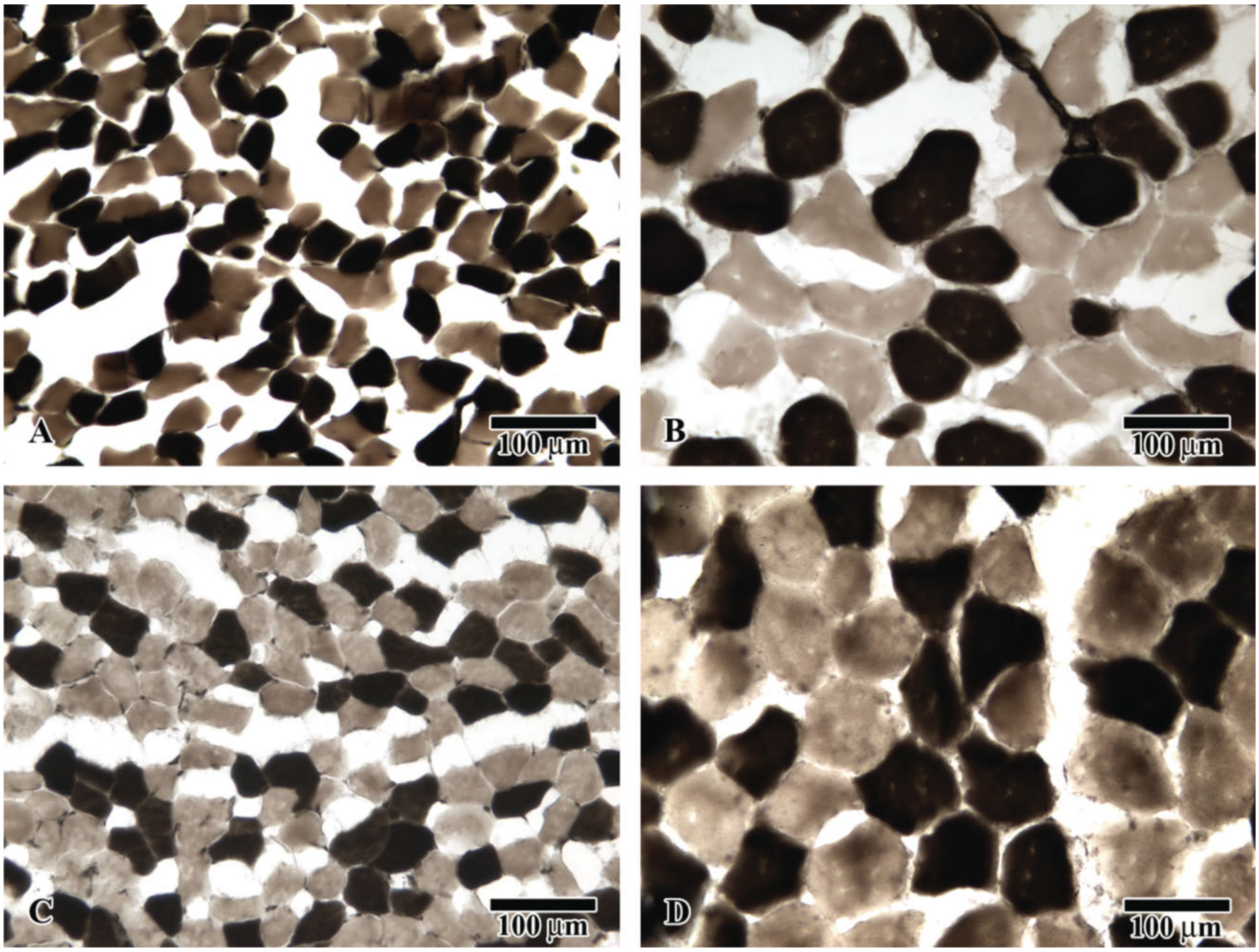

Fig. 2. Nonsequential cross-sections of *m. longissimus dorsi* after myosin ATPase staining. **A** and **C** are bottlenose dolphin (*Tursiops truncatus*), **B** and **D** are pygmy sperm whale (*Kogia breviceps*) muscle. The *m. longissimus dorsi* was stained for myosin ATPase activity after alkaline (pH 10.3) (A and B) and acidic preincubation (pH 4.15) (C and D). Type I fibers appear light in A and B, dark in C and D.

copy (TEM) studies, were not available. Frozen muscle tissues from both species, though, were prepared for TEM, using standard methods to explore whether they were of sufficient quality to be used to calculate mitochondrial volume density. Although mitochondria, myofibrils, and other cell features were visible in samples from both species, ultrastructural quality was deemed insufficient to support quantifying mitochondrial volume density.

In lieu of directly measuring mitochondrial volume density using TEM, an index of mitochondrial density was developed using photometric analyses of histochemically treated thin sections. Samples were prepared and stained for SDH as described above. SDH is a mitochondrial-bound enzyme, and its staining intensity provides a measure of mitochondrial density within a cell (Nachlas et al., 1957; Peter et al., 1972). Sections (10 μm) of *T. truncatus* and *K. breviceps* muscle were incubated simultaneously for 30 min. This incubation time was optimal for *T. truncatus*, and was chosen to avoid saturating the sections from this species, which stained more intensely than *K. breviceps* tissue. Following incubation, slides were processed as described above. Stained muscle sections were viewed using a light microscope (Olympus BX60) at ×20 magnification. Digital micrographs were captured with a SPOT RT camera (Diagnostic Instruments) under identical conditions, so that comparisons could be made among images using Media Cybernetics ImagePro Plus® 6.0 software.

Adapting the methods of Hardy et al. (2010), cells were outlined using the "area of interest" software tool, taking care to trace within the cell boundary to avoid edge effects. A mean pixel density value (range 0–255 gray values; 0 = black; and 255 = white) was determined for each fiber using the measurement menu. For each individual, a minimum of 50 fibers of each type was measured; because the Type IIa fibers, which were only observed in *T. truncatus*, represented a small percentage of the total (see below), fewer of these fibers (7–36 per individual) were measured. Raw data were exported to Microsoft Excel and each density value was converted to a staining intensity value (calculated as 255 minus mean pixel density value, so darker staining fibers would have higher intensity values). Values are reported as means ± standard errors and data were statistically analyzed using unpaired, one-tailed *t*-tests to compare fiber staining intensity for *K. breviceps* and across species. An ANOVA was used to compare the staining intensities of the three fiber types identified in *T. truncatus*.

RESULTS

Myoglobin

Myoglobin concentration is reported as g Mb/100 g wet weight muscle. Mean myoglobin concentration for *T. truncatus* (3.21 ± 0.118) was

TABLE 2. Mean (±S.E.) fiber-type composition by area and by count of the m. longissimus dorsi in adult bottlenose dolphins (Tursiops truncatus, n = 5) and adult pygmy sperm whales (Kogia breviceps, n = 5)

		% Fiber by total fiber area		% Fiber by total number	
Stain	Fiber type	*T. truncatus*	*K. breviceps*	*T. truncatus*	*K. breviceps*
Alkaline myosin ATPase	Type I	47.0 ± 4.2	53.1 ± 2.0	51.6 ± 1.8	52.7 ± 2.5
	Type II	53.0 ± 4.2	46.9 ± 2.0	48.4 ± 1.8	47.3 ± 2.5
Acidic myosin ATPase	Type I	48.2 ± 3.0[a]	56.0 ± 1.4	54.6 ± 1.4	55.5 ± 2.1
	Type II	51.8 ± 3.0[a]	44.0 ± 1.4	45.4 ± 1.4	44.5 ± 2.1
Succinate dehydrogenase	Type I	44.8 ± 2.2[b]	55.7 ± 1.8	51.7 ± 1.7	54.5 ± 1.7
	Type II	55.2 ± 2.2[b,c]	44.3 ± 1.8	48.3 ± 1.7[d]	45.5 ± 1.7

[a]Significant species difference for both Type I and II fibers ($P = 0.0300$, one-tailed *t*-test).
[b]Significant species difference for both Type I and II fibers ($P = 0.0027$, one-tailed *t*-test).
[c]For *T. truncatus* Type IIa (6.4% ± 1.8) and Type IIb (48.8% ± 2.0) fibers combined.
[d]For *T. truncatus* Type IIa (6.3% ± 2.2) and Type IIb (42.0% ± 1.5) fibers combined.

significantly lower than that of *K. breviceps* (5.92 ± 0.411) ($P = 0.0009$).

Muscle Fiber Type

Myosin ATPase differentiated two fiber populations in both *T. truncatus* and *K. breviceps* (Fig. 2, Table 2). For both the alkaline and acidic preincubation treatments, *K. breviceps* displayed a higher mean percentage of Type I fibers, and a lower mean percentage of Type II fibers, by area, than did *T. truncatus*. These species-specific differences were not significant for the alkaline preincubation treatment ($P = 0.1240$), but were for the acidic preincubation treatment ($P = 0.0300$).

For *T. truncatus*, the SDH assay differentiated three fiber populations, which permitted Type II fibers to be identified as either IIa or IIb based upon staining intensity (Fig. 3A, Table 2). The fiber profile by area was 44.8 ± 2.2% Type I, 6.4 ± 1.8% Type IIa, and 48.8 ± 2.0% Type IIb. In contrast, SDH differentiated only two fiber populations for *K. breviceps* (Fig. 3B, Table 2). Immunohistochemistry did not differentiate the light-staining, fast fibers of *K. breviceps* as Type IIa or IIb. SC-71, an antibody purported to be specific to Type IIa myosins in other mammalian species (Schiaffino et al., 1989), did not specifically stain Type IIa fibers in *T. truncatus*, but rather broadly stained fibers in both species. Because neither histochemical nor immunohistochemical assays could definitively identify the specific fast myosin type of *K. breviceps*, these fibers are referred to only as Type II. To permit statistical comparisons of fiber type profiles across species, Type IIa and IIb fibers of *T. truncatus* were combined as Type II fibers. *K. breviceps* displayed a higher percentage of Type I fibers and a lower percentage of Type II fibers, by area, than did *T. truncatus* ($P = 0.0027$).

The second method for quantifying fiber type profiles, that of fiber count, yielded no significant differences across species for any of the histochemical treatments ($P > 0.05$; Table 2). The different results across these two quantification methods

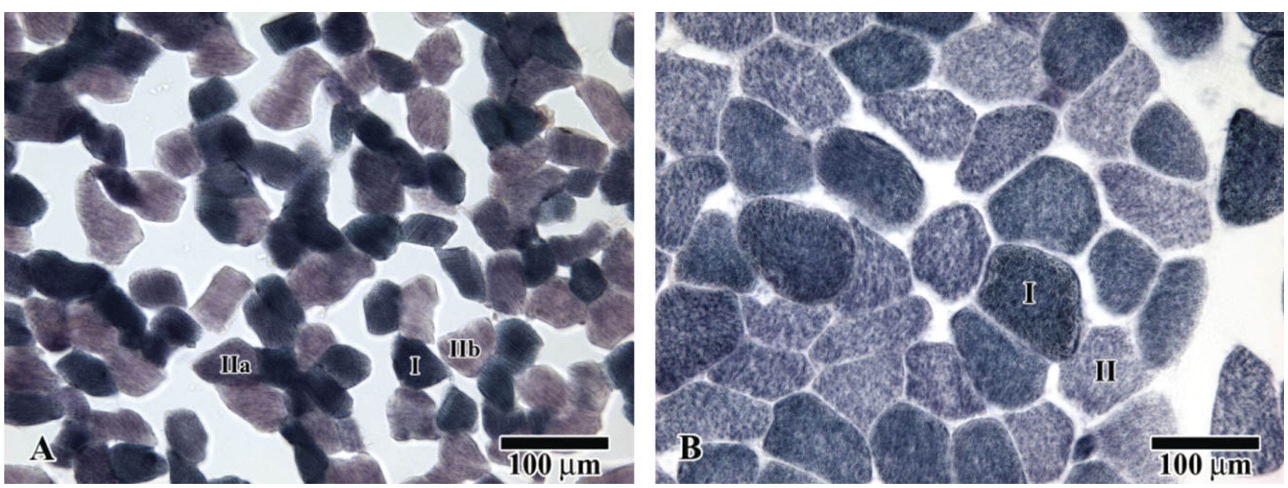

Fig. 3. Cross-sections of *m. longissimus dorsi* after succinate dehydrogenase staining. **A** is bottlenose dolphin (*Tursiops truncatus*), **B** is pygmy sperm whale (*Kogia breviceps*) muscle. Sections of *T. truncatus* muscle were stained for 30 min, whereas sections of *K. breviceps* muscle were stained for 1 h. Type I fibers are darkly-stained in A and B. Type IIa fibers are intermediately-stained and Type IIb fibers are lightly-stained in A. Type II fibers are lightly-stained in B.

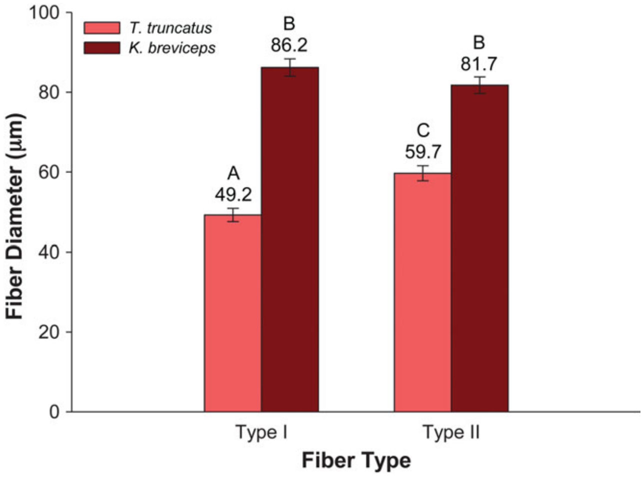

Fig. 4. Mean fiber diameters (µm) (±S.E.) of Type I and II fibers in the bottlenose dolphin (*Tursiops truncatus*) and the pygmy sperm whale (*Kogia breviceps*). Values with different letters are statistically different from each other.

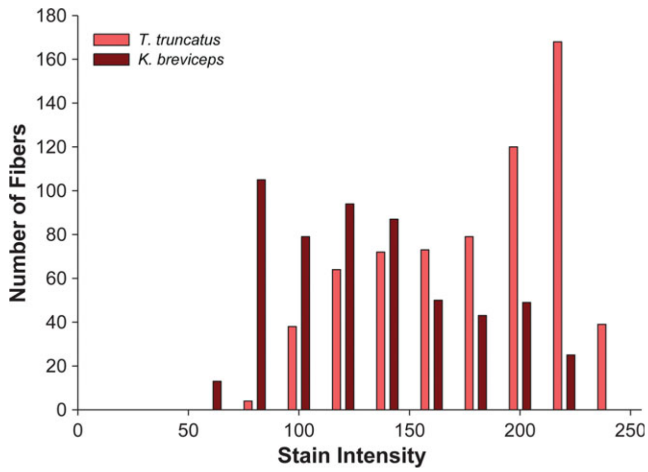

Fig. 5. Distribution of fiber stain (succinate dehydrogenase) intensity values for the bottlenose dolphin (*Tursiops truncatus*, $n = 5$) and the pygmy sperm whale (*Kogia breviceps*, $n = 5$), as an indicator of mitochondrial density. Sections from both species were incubated simultaneously for 30 min.

are likely due to the species-specific differences in fiber diameters, and thus, fiber area (see below).

Fiber Diameter

For *T. truncatus*, the mean fiber diameter of Type I fibers across specimens (49.2 µm ± 1.6) was significantly smaller than that of Type II fibers (59.7 µm ± 1.9; $P < 0.0001$; Figs. 2A,B, and 4). For *K. breviceps*, the mean diameter of Type I fibers across specimens (86.2 µm ± 2.2) was similar to that of Type II fibers (81.7 µm ± 2.1; $P = 0.9286$; Figs. 2A,B, and 4). Across both fiber types, the mean diameters of *T. truncatus* muscle were significantly smaller than those of *K. breviceps* (Type I fibers, $P < 0.0001$; Type II fibers, $P = 0.0001$).

Mitochondrial Density

An index of mitochondrial density was developed by comparing the SDH staining intensity of fibers from *T. truncatus* and *K. breviceps*. Although absolute values of staining intensities for all fibers overlapped across species, fibers from *T. truncatus* tended to stain more intensely than those of *K. breviceps* (Fig. 5).

T. truncatus muscle displayed three significantly distinct fiber populations: the mean staining intensity values were 208 ± 1 for Type I fibers, 182 ± 2 for Type IIa fibers, and 133 ± 2 for Type IIb fibers ($P < 0.0001$). *K. breviceps* muscle displayed two significantly distinct fiber populations: the mean staining intensity values were 151 ± 2 for Type I fibers and 92 ± 2 for Type II fibers ($P < 0.0001$).

To examine interspecific variation, a random subset of Type I and II (*T. truncates* = Type IIb) fibers ($n = 275$, $n = 250$, respectively) from each species was compared (Fig. 6). Type I fibers of *T. truncatus* stained significantly more intensely (208 ± 1) than those of *K. breviceps* (151 ± 2) ($P < 0.0001$). Type II fibers of *T. truncatus* also stained significantly more intensely (133 ± 2) than those of *K. breviceps* (92 ± 2) ($P < 0.0001$).

DISCUSSION

This study compared the locomotor muscle morphology of two cetacean species with different dive behaviors. As predicted, the *m. longissimus dorsi* of the relatively long duration, deep-diving pygmy sperm whale, *Kogia breviceps*, had a significantly

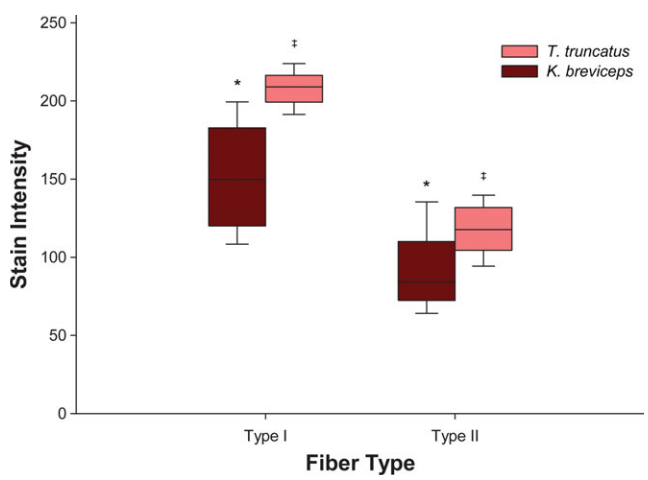

Fig. 6. Box-and-whisker plot of succinate dehydrogenase stain intensity values of Type I and II fibers across species. Asterisks and cross hatches denote significant differences across species for each fiber type ($P < 0.001$, one-tailed t-test).

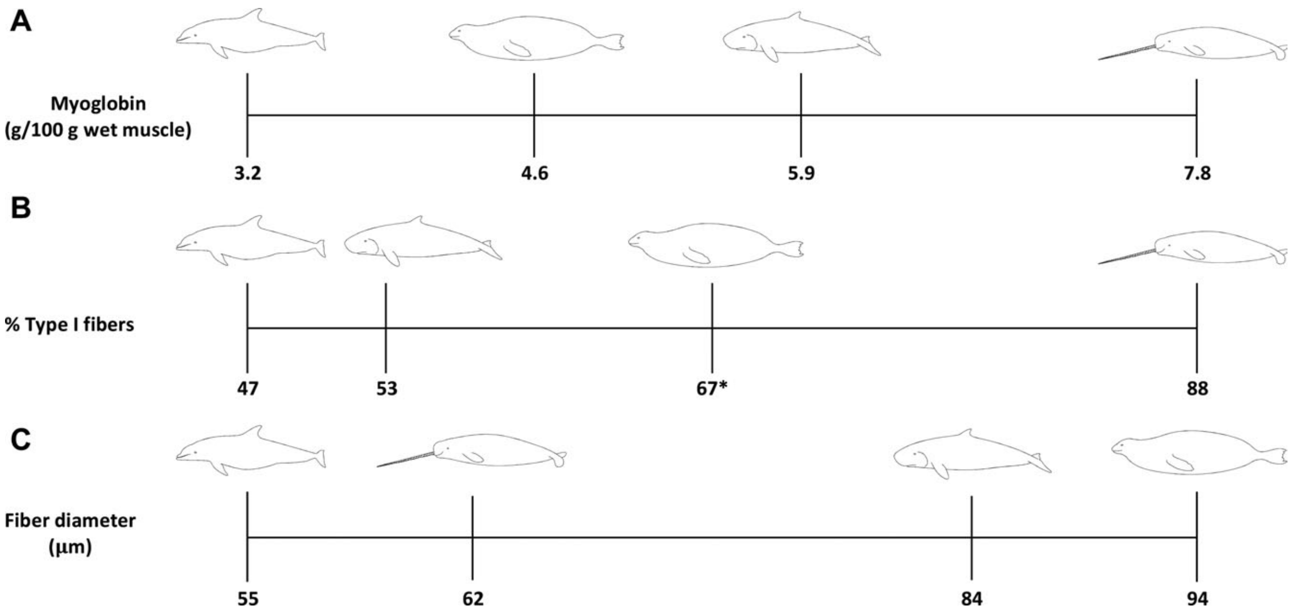

Fig. 7. Scales of skeletal muscle morphological features across several diving marine mammals (in order along scale **A**): *T. truncatus*, *L. weddellii*, *K. breviceps,* and *M. monoceros*. Scale A depicts myoglobin content. Scale **B** shows the proportion of Type I fibers, by area. Asterisk denotes that the proportion of Type I fibers for *L. weddellii* was determined by count because proportion data by area were unavailable. Scale **C** depicts mean fiber diameter. For *T. truncatus*, *K. breviceps,* and *M. monoceros*, mean fiber diameters were determined as a weighted average of Type I and II fiber diameters, accounting for the relative proportion of each fiber type, by area. For *L. weddellii*, only one mean fiber size measurement was reported, which did not specify fiber type. Data for *L. weddellii* from Kanatous et al. (2002); data from *M. monoceros* from Williams et al. (2011).

higher myoglobin concentration, greater area of Type I fibers, larger mean fiber diameters, and lower indices of mitochondrial density than did that of the short duration, shallow-diving bottlenose dolphin, *Tursiops truncatus*. This discussion will compare the muscle morphology of these two species with those of other divers, with a special focus on the short duration, shallow-diving harbor seal (*Phoca vitulina*) and two long duration, deep divers, the Weddell seal (*Leptonychotes weddellii*) and the narwhal (*Monodon monoceros*). The results of these comparisons suggest that there are multiple morphological approaches to balancing skeletal muscle oxygen storage capacity with oxygen consumption demands in diving mammals.

Myoglobin content has been measured in a wide variety of marine mammals (e.g., Scholander, 1940; Lenfant et al., 1970; George et al., 1971; Castellini and Somero, 1981; Kooyman, 1989; Lydersen et al., 1992; Ponganis et al., 1993; Shaffer et al., 1997; Dolar et al., 1999; Kanatous et al., 1999, 2001; Noren and Williams, 2000; Polasek and Davis, 2001; reviewed in Hochachka and Somero, 2002; Williams et al., 2011). While all diving mammals experience bradycardia and peripheral vasoconstriction as part of the dive response, the associated reduction of convective oxygen delivery to skeletal muscle is hypothesized to be especially profound in species that perform long duration, deep dives (Scholander, 1940; Kooyman, 1973; Castellini and Kooyman, 1989; reviewed in Butler and Jones, 1997). Thus, the muscles of deep, endurance divers are particularly reliant upon high concentrations of endogenous oxygen bound to myoglobin (e.g., Noren and Williams, 2000). The mean myoglobin concentration (g Mb/100 g wet muscle) of the locomotor muscle of *K. breviceps* (~5.9) is nearly twice that of *T. truncatus* (~3.2), nearly 1.5 times that of *P. vitulina* (~3.7), and falls within the range of the two other deep, long duration divers, *L. weddellii* (~4.6) and *M. monoceros* (~7.8) (Kanatous et al., 1999, 2002; Williams et al., 2011) (Fig. 7A). How these enhanced oxygen stores are used during a dive is dependent, in part, upon locomotor muscle fiber type profile and mitochondrial densities.

The locomotor muscles of all diving mammals studied to date possess a mixed fiber profile, but differ in their proportions of Type I and II fibers. Type I fibers predominate in *L. weddellii* (by count) and *M. monoceros* (by both count and area), polar species that are slow, endurance divers with high muscle myoglobin contents (Kanatous et al., 2002; Williams et al., 2011) (Fig. 7B). *K. breviceps* also possesses a higher proportion of Type I than Type II fibers by area, although they were found in similar proportions by count (Table 2). These results suggest that a predominantly slow muscle fiber profile is a feature common to long duration, deep-diving marine mammals. *K. breviceps*, found in temperate to tropical waters, does not display as extreme a reliance on slow fibers as do the two polar species. Direct measurements of the dive behavior are not yet available for *K. breviceps* and, to date, the functional consequences of these differences in fiber composition are not known. Further comparative study of endurance divers across both temperate and polar environments is warranted.

Of these three deep-diving species, the identity of the fast myosin type is only known with certainty for *L. weddellii*; the skeletal muscles of this species contain only Type IIa fibers (Kanatous et al., 2002). In both *K. breviceps* and *M. monoceros*, the Type I and II fibers are of similar diameter, suggesting that the myosin type of their fast fibers is also IIa (Lundgren and Kiessling, 1988; Cobb et al., 1994; Stegall, 2001; see also Fig. 3, Kanatous et al., 2002). Thus, possessing oxidative fast fibers may be a feature shared by deep-diving mammals. Utilization of alternative antibodies, or other molecular techniques such as gel electrophoresis, may help identify the fast fiber type(s) present in the locomotor muscle of these and other diving mammals.

In contrast to the deep divers, *T. truncatus* possesses a higher proportion of Type II than Type I fibers by area, although they were found in similar proportions by count (Table 2). Higher proportions of Type II fibers (by count) have been reported for two other short-duration shallow divers, *P. vitulina* (approximately 53% for epaxial muscle; Watson et al., 2003) and the Stellar sea lion, *Eumetopias jubatus* (approximately 80%, muscle not identified; Kanatous et al., 1999). Interestingly, the majority of Type II fibers in *T. truncatus* and *E. jubatus* were Type IIb, whereas those of *P. vitulina* were exclusively Type IIa (Kanatous et al., 1999; Watson et al., 2003).

In this study, the locomotor muscle of both *T. truncatus* and *K. breviceps* displayed small percentage differences in the counts (% fiber by total number) and cross-sectional areas (% fiber by total fiber area) occupied by a specific fiber type across staining methods (alkaline vs. acidic myosin ATPase); approximately 1–3% of muscle fibers displayed characteristics of both Type I and II fibers (see Table 2). Utilization of alternative antibodies, or other molecular techniques such as gel electrophoresis, may help identify the fast fiber type(s) present in the locomotor muscle of these and other diving mammals, and aid in identifying fibers that display mixed staining patterns.

Across both Type I and II fiber types, *K. breviceps* displayed a significantly lower index of mitochondrial density than did *T. truncatus*, indicating a reduced aerobic capacity of its locomotor muscle. This result is similar to that observed in pinnipeds; the locomotor muscle of *L. weddellii* displayed lower mitochondrial volume density compared to *P. vitulina* despite having enhanced myoglobin content (Kanatous et al., 1999, 2002). Kanatous et al. (2002) hypothesized that the reduced aerobic capacity of *L. weddellii* swimming muscle, coupled with its enhanced oxygen storage capacity reflected this species' reliance upon energy-saving swimming strategies during a deep dive. Gliding, and alternating periods of stroking with gliding, reduce the metabolic costs of swimming, and are utilized more extensively by deep than by shallow divers (Williams et al., 2000, 2004). While the diving behavior of *K. breviceps* is not known, the lower mitochondrial density indices of their swimming muscle, coupled with enhanced myoglobin content, relative to that of *T. truncatus*, suggest a profile similar to that of this well-studied, deep-diving seal. Enhanced myoglobin content likely serves multiple purposes in the muscle of diving mammals, from enhancing oxygen storage capacity, to ensuring adequate oxygen transport into active muscle cells throughout the course of each dive via facilitated diffusion (Kinsey et al., 2011).

The last morphological feature of muscle investigated here was that of fiber size. While the metabolic consequences of skeletal muscle fiber size have not previously been explored in marine mammals, they have been extensively studied in fishes and crustaceans (e.g., Johnston et al., 2004, Kinsey et al., 2007, 2011; Nyack et al., 2007; Hardy et al., 2009). Johnston et al.'s (2004) "optimal maximum fiber diameter hypothesis" states that a muscle should achieve a size that reduces the metabolic costs of cellular ionic homeostasis, while balancing the limits of oxygen diffusion. In fishes and invertebrates, aerobic (Type I) fibers, which are dependent upon oxygen diffusion rates into and across the cell, tend to have smaller diameters than anaerobic (Type II) fibers, which are not reliant on the diffusive flux of oxygen to power contraction (reviewed in Kinsey et al., 2007, 2011). Similarly, in mammals, aerobic Type I fibers tend to have smaller diameters than anaerobic Type IIb fibers (Lundgren and Kiessling, 1988; Cobb et al., 1994). In a recent study on crustacean muscle, Jimenez et al. (2011) provided the first empirical evidence that the energetic costs of the sarcolemmal membrane-bound Na+-K+ ATPase pump scale with fiber size as predicted by the surface area to volume ratio. Thus, in invertebrates, large fibers have relatively reduced costs of cell membrane maintenance. Because the cellular costs of maintaining the cytoplasmic gradient can represent 40–50% of the resting skeletal muscle metabolic rate, large fiber size may reduce overall metabolic rate (reviewed in Boutilier, 2001; reviewed in Johnston et al., 2006; reviewed in Jimenez et al., 2011).

The shallow-diving *T. truncatus* and *P. vitulina* possess mean skeletal muscle fiber diameters that are within the range reported for terrestrial mammals (34–60 μm; Fig. 7C) (Kanatous et al., 1999; reviewed in Ross and Pawlina, 2006; reviewed in Kinsey et al., 2007). In contrast, *K. breviceps* and

L. weddellii both possess extremely large mean fiber diameters (82 μm, 94 μm, respectively; Kanatous et al., 2002)[1] In the muscles of diving mammals, oxygen is bound to myoglobin and convective delivery of oxygen to working muscles during a dive is reduced; both of these features are especially pronounced in deep divers. Thus, the limits imposed on cell size by oxygen diffusion distance in working muscle may be diminished in these species. Under these conditions, large fiber diameter, and the potential concomitant lower metabolic costs associated with maintaining ionic homeostasis, would appear to be beneficial to reducing the overall rate of oxygen consumption. These features may be especially important to deep divers that utilize the energy-saving locomotor (stroke and glide) strategies described above. For example, during gliding, which can occur for up to 78% of the total descent duration for *L. weddellii* (Williams et al., 2000), the metabolic costs of their locomotor muscles are reduced to primarily those of cell maintenance. Large skeletal muscle fiber size, which may contribute to reduced muscle metabolic maintenance costs, could contribute to lower overall rates of oxygen consumption during a dive.

A recent study by Noren et al. (2012) reports that in *T. truncatus*, heart rate is modulated by activity level throughout the course of a dive. Cardiac output increases during more active portions of a dive cycle, but bradycardia resumes during more sedentary intervals, a finding that is consistent with rationing limited oxygen stores (Noren et al., 2012). The results of this study, in concert with these recent insights into cardiac function, suggest there may be multiple mechanisms to use limited oxygen stores more effectively and reduce the metabolic costs of diving, resulting in a more efficient aerobic dive.

Although most marine mammals are believed to dive aerobically for the majority of their dives (Kooyman, 1989; Williams et al., 2011), this information is not definitively known for *K. breviceps*. There are few dive data for *K. breviceps* but extended dives of up to 18 (Scott et al., 2001) and 52 min (Barlow et al., 1997) have been reported. *K. breviceps* has been characterized to be a "slow" breather (Scholander, 1940; Kooyman, 1973) and has been reported to log at the surface for up to 11 min following long dive intervals (Scott et al., 2001). This surface behavior contrasts starkly with that of *T. truncatus*, which is highly active, spends little time at the surface and respires rapidly (Scholander, 1940; Ridgway and Johnston, 1966; Kooyman, 1973; Cotten et al., 2008; reviewed in Piscitelli et al., 2010).

The extended postdive surface times of *K. breviceps* could be interpreted as a period required to recover from the metabolic acidosis that occurs following an anaerobic dive. An alternative hypothesis is that *K. breviceps* may require this surface time to reperfuse oxygen to its tissues. Scaled to body size, *K. breviceps* has small lungs, similar to that observed in the long duration, deep-diving sperm whale (*Physeter macrocephalus*; Omura, 1950; Piscitelli et al., 2010). Like *K. breviceps*, *P. macrocephalus* has been reported to surface for long intervals (9 min) following extended dives (Watwood et al., 2006). Studies of *P. macrocephalus* tracked with time-depth recorders report a mean dive duration (45 min) that falls within its calculated ADL (43–54 min; Watwood et al., 2006). Thus, *P. macrocephalus*, despite long postdive surface intervals, is believed to dive aerobically for the majority of its dives. It is expected that *K. breviceps* may also conduct the majority of its dives aerobically. The morphological features of its locomotor muscle characterized in this study, high oxygen storage capacity, a large proportion of slow fibers, large fiber diameters, and likely reduced muscle oxygen consumption rate, suggest that this species may be adapted to dive aerobically for extended intervals. Because *K. breviceps* has small lungs and is a slow breather (Scholander, 1940; Kooyman, 1973; reviewed in Piscitelli et al., 2010), it may require an extended postdive surface interval to adequately reperfuse its tissues and reoxygenate its high skeletal muscle myoglobin stores.

Across marine mammals for which skeletal muscle morphological characters have been reported, the results of this study suggest that short duration, shallow divers, like *T. truncatus*, possess muscle morphologies (relatively low myoglobin content, more Type II fibers, small muscle fiber diameters, and high mitochondrial densities) distinctly different than those of long duration, deep divers, including *K. breviceps, L. weddellii*, and *M. monoceros*. Within the deep divers, though, there appear to be multiple skeletal muscle designs (*sensu* Lauder, 1982) to support prolonged, endurance dives.

These results illustrate how morphological characters of skeletal muscle may be used to gain insight into the diving behavior of cryptic species, like *Kogia breviceps*, for which there exist few behavioral data in the wild, as well as to gain a broader understanding of muscle morphology across species with distinctly different dive regimes.

[1]Because muscle collection techniques varied across the studies reported here, and muscles may have been collected in different contractile states, a muscle fiber was modeled as a right cylinder of constant volume to estimate the effect of contractile state on fiber diameter (Kier and Smith, 1985). A 10% contractile change in length (shortening) (e.g. Gordon et al., 1966; Close, 1972; Muhl, 1982; Dimery, 1985; reviewed in Pabst, 1993) would result in an approximately 5% increase in muscle diameter. Thus, reported values for fiber diameter may vary by up to ±5% depending upon contractile state. The wide range of fiber diameters reported across marine mammal species suggests that contractile state alone cannot account for these differences.

ACKNOWLEDGMENTS

Specimens were collected by the Marine Mammal Stranding Program at the University of North Carolina Wilmington (IACUC permits A0809-019, 2006-015, 2003-013; NOAA Southeast Stranding Agreement); the Cetacean and Sea Turtle Team at the National Marine Fisheries Service Laboratory in Beaufort, North Carolina; the National Park Service; and the North Carolina Wildlife Resources Commission. The SC-71 monoclonal antibody developed by Dr. S. Schiaffino was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by The University of Iowa, Department of Biology, Iowa City, IA 52242.The authors wish to thank Michelle Schisa, Erin Cummings, Ryan McAlarney, Laura Bagge, Brandy Velten, Cally Harper, Carolina Priester, Ana Jimenez, VABLAB members past, and UNCW Marine Mammal Stranding Program volunteers for their assistance on this project. For access to specimens, the authors thank Bruce Ferrier and Gretchen Lovewell from NMFS NOAA Beaufort Laboratory, Michelle Bogardius with National Park Service, and Karen Clark with NC Wildlife Resource Commission.

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