THE LOCOMOTOR MUSCLE OF CETACEANS:
A COMPARISON OF DEEP & SHALLOW DIVERS,
KOGIA BREVICEPS & TURSIOPS TRUNCATUS

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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.
Thus, the morphology and physiology of a marine mammal’s skeletal muscle provides
insight into its diving capabilities. This study investigated the locomotor muscle of a long
duration, deep-diving cetacean, the pygmy sperm whale (*Kogia breviceps*), and compared
it to that of the short duration, shallow-diving coastal form of the Atlantic bottlenose
dolphin (*Tursiops truncatus*). The of *m. longissimus dorsi*, an epaxial swimming muscle,
was collected from five adult stranded, or incidentally caught, individuals of each
species. Spectrophotometry was used to assess myoglobin content, and histochemical
assays (myosin ATPase and succinic dehydrogenase) coupled with stereological analyses
were used to determine muscle fiber type and fiber diameter. A photometric analysis of
sections stained for succinic dehydrogenase was used to measure an index of
mitochondrial density. The *m. longissimus dorsi* of *K. breviceps* displayed a significantly
higher myoglobin content (*Kb*=5.92±0.408, *Tt*=3.21±0.118 g Mb/100 g wet muscle;
p=0.0009), a significantly larger proportion of Type I (slow oxidative) fibers by area
(*Kb*=56.0±1.4%, *Tt*=48.2%±3.0%; acidic myosin ATPase, pH=4.1-4.15; p=0.0300),
significantly larger mean fiber diameters (Type I, *Kb*=86.2±2.2, *Tt*=49.2±1.6µm,
p<0.0001; Type II, *Kb*=81.7±2.1, *Tt*=59.7µm; p=0.0001), and significantly lower
index of mitochondrial density (Type I, *Kb*=151±1, *Tt*=208±2 mean fiber staining intensity,
p<0.0001; Type II, *Kb*=92±2, *Tt*=133±2; p<0.0001) than that of *T. truncatus*. These
results suggest that the locomotor muscle of *K. breviceps* (1) exhibits greater oxygen
storage capacity, (2) has reduced ATP demand, and (3) likely has a reduced rate of
oxygen consumption relative to that of *T. truncatus*. Thus, *K. breviceps* locomotor muscle appears able to ration its high onboard oxygen stores, a feature that may allow this species to conduct relatively long duration, deep dives aerobically.
ACKNOWLEDGEMENTS

Numerous individuals contributed to the development of this project and to my personal growth as a scientist. I wish to thank my advisor, Dr. D. Ann Pabst, for her enthusiasm, expertise and tireless hours editing this thesis. I would also like to thank my committee members, Dr. Richard Dillaman and Dr. Stephen Kinsey, for their insights, encouragement and open doors. I also acknowledge Bill McLellan, whose field skills and camaraderie have been invaluable. I owe a special thanks to Michelle Schisa for running myoglobin assays and exploring marine mammal physiology with infectious eagerness. I would also like to thank both the Virginia Aquarium and North Carolina Stranding Programs for providing the samples that made this project possible. I would like to also acknowledge Dr. Jenn Dearolf for supplying antibodies, her histochemistry savvy, and interest in my work. Thanks to Laura Bagge, Erin Cummings, Brandy Velten, Ryan McAlarney, Butch Rommel and the rest of the VAB lab for their constant support. Thank you also to Carolina Priester, Mark Gay and Ana Jimenez for their tutelage and patience.

I must also share my complete gratitude for my parents and closest friends. Mom, thank you for being my greatest advocate; my persistence is rooted in your faith in my potential. Dad, thank you for planting the seeds of scientific inquiry in my mind (here is my harvest, pun intended). I would also like to thank my boyfriend, Yossi Shirazi, for his absolutely endless support; you are my rock when I have am stuck in a hard place, and the first person I want to share my successes with. I would also like to thank my dearest friend, Sara O’Donnell, who has been there since (nearly) the beginning, defining friendship in its finest form.

Thank you all for helping me along the journey; onto the next destination!
DEDICATION

I would like to dedicate this work to two tremendously influential people in my life. For my late grandfather, Stuart J. F. Landa, who embodied excellence and left his mark indelibly on my heart and spirit. And for my undergraduate advisor, Dr. John Hudson, who saw my potential and held up the mirror until I saw it too; tenacity.
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INTRODUCTION

When a marine mammal dives, breathing and locomotion are mechanically uncoupled, and its locomotor muscles must power swimming when oxygen is limited (Butler and Jones, 1997; Davis and Kanatous, 1999; Cotten et al., 2008). Bradycardia and peripheral vasoconstriction, which occur as part of a marine mammal’s dive response, reduce convective oxygen delivery to the locomotor muscles during a dive (Scholander, 1940; Kooyman, 1973; Castellini and Kooyman, 1989; reviewed in Butler and Jones, 1997). These muscles must depend primarily upon endogenous stores of oxygen to fuel locomotion underwater (Kooyman, 1973; Kooyman, 1983; Polasek and Davis, 2001; reviewed in Kanatous et al., 1999).

Most marine mammals are thought to remain within their aerobic dive limit (ADL) for the majority of their dives (Kooyman, 1989; Williams et al., 2011). ADL is the maximum time an animal can dive while maintaining aerobic metabolism and is a function of both oxygen storage capacity and the rate of oxygen consumption (Kooyman, 1980). Because skeletal muscle mitochondria consume more than 90% of an animal’s total body oxygen during maximal oxygen consumption (VO$_{2\text{max}}$) (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; Kanatous et al., 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. Dolar et al., 1999; Noren and Williams, 2000; Hochachka and Somero, 2002). A muscle’s fiber type profile provides information regarding the predominant metabolic pathway (aerobic vs. anaerobic) utilized
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; reviewed in Burpee et al., 2010). Because the maximal rate of oxygen consumption within mitochondria is highly conserved across mammalian species, skeletal muscle mitochondrial volume density ($V_{mt}$) serves as a proxy for aerobic capacity (Hoppeler and Lindstedt, 1985; Hoppeler et al., 1987; Taylor, 1987; Leary et al., 2003; Burpee et al., 2009).

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). 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 because anaerobic metabolism powers contraction (Bello et al., 1985; Dearolf et al., 2000; Jimenez et al., 2008). Large muscle fibers also have a lower surface area to volume ratio (SA:V) than do smaller fibers. The lower the SA:V, the lower relative energy invested in maintaining a cell’s membrane potential (Johnston et al., 2006; Jimenez et al., in review). Thus, fibers with larger diameters should have relatively reduced cell maintenance costs.

The relationships between dive behavior, ADL, and locomotor muscle physiology have been well characterized in pinnipeds (e.g., Kanatous et al., 1999; Kanatous et al., 2001; Kanatous et al., 2002; Watson et al., 2003; Polasek et al., 2006; Watson et al., 2007; Kanatous et al., 2008). 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 (10.2 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 (Kooymans et al., 1966; Kooymans et al., 1980; Kooymans, 1981; Castellini et al., 1992; reviewed in Schreer and Kovacs, 1997; Williams et al., 2000).

The locomotor muscles of these two seals reflect their different dive behaviors. 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* (Kanalous et al., 1999; Kanalous et al., 2002). The locomotor muscles of both species are composed of exclusively Type I and Type IIa fibers; the absence of Type IIb fibers suggests that both species rely heavily on aerobic, rather than anaerobic, metabolism during a dive (Kanalous et al., 2002 Watson et al., 2003). However, the locomotor muscles *P. vitulina* display predominantly Type IIa fibers, while those of *L. weddellii* display predominantly Type I fibers (Kanalous et al., 2002; Watson et al., 2003). The locomotor muscles of *P. vitulina* have relatively higher aerobic capacity, as measured by high mitochondrial volume densities, similar to those of terrestrial endurance athletes (e.g., dog and pony) (Kanalous et al., 1999). The locomotor muscles of *L. weddellii*, by comparison, have lower aerobic capacity (Kanalous et al., 1999; Kanalous et al., 2002; Watson et al., 2003; Watson et al., 2007; Kanalous et al., 2008). The fiber diameters of *P. vitulina* locomotor muscle also appear to be smaller than those of *L. weddellii* (Kanalous et al., 2001, Kanalous et al., 2002).
Enhanced oxygen storage capacity, coupled with reduced oxygen consumption rate, extends the ADL of *L. weddellii*, as compared to *P. vitulina*. Thus, comparing differences in skeletal muscle design provides insight into the dive behavior of different marine mammal species. While there are considerable comparative data on locomotor muscle morphology and physiology for pinnipeds, there are fewer such data available for cetaceans, in large part because access to high-quality specimens is limited.

Myoglobin content within locomotor muscle has been assessed for a wide variety of cetaceans (Schaffer *et al*., 1997; Dolar *et al*., 1999; Noren and Williams, 2000; Polasek and Davis, 2001; reviewed in Hochachka and Somero, 2002). Although considerable interspecies variation exists, deep-diving species tend to possess higher myoglobin contents than do shallow-diving species (Noren and Williams, 2000).

Most literature regarding cetacean muscle physiology focuses on bottlenose dolphins (*Tursiops truncatus*) (Bello *et al*., 1985; Dearolf *et al*., 2000; Noren *et al*., 2001; Noren *et al*., 2002; Etnier *et al*., 2004; Cotten *et al*., 2008). The well-studied coastal ecotype of *T. truncatus* dives for an average of 20-40 seconds to depths between 2-10 m (Irvine *et al*., 1981; Connor *et al*., 2000; reviewed in Piscitelli *et al*., 2010); it is a relatively short duration, shallow diver. Recently features of the locomotor muscle of the long duration, deep-diving narwhal, *Monodon monoceros*, have been described (362 m mean dive depth, 12-13 min mean dive duration, wintering grounds) (Heide-Jorgensen 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 contents, 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 is 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 to 1000 m deep (reviewed in Scott et al., 2001; Clarke et
al., 2003; NOAA Stock Report, 2005). Although the dive behavior of kogiids is not well-
studied, the gut contents of stranded individuals reveal that their diet consists of
predominantly mid- to deep-water species of cephalopods (McAlpine, 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 minutes and
maximum dive duration of 52 minutes (Barlow et al., 1997). Additionally, a tagging
study by Scott et al. (2001) of a single, rehabilitated *K. breviceps* sub-adult reports a
maximum dive duration of 18 minutes. While data are limited, these studies strongly
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 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 epaxial locomotor muscle of the relatively long
duration, deep-diving *K. breviceps* will display higher oxygen storage capacity, a more
aerobic fiber type profile, lower aerobic capacity, and larger diameter muscle fibers than
those of *T. truncatus*. To date, the only published information on the skeletal muscle of *K. breviceps* is the myoglobin content of the locomotor muscle (*m. longissimus dorsi*) of a single individual, which was approximately twice that of the *T. truncatus* (Noren and Williams, 2000). This study reexamines myoglobin concentration in both *K. breviceps* and *T. truncatus* and provides a larger sample size to supplement published studies. This study also expands upon the literature regarding cetacean muscle fiber profile and fiber size by describing the skeletal muscle of a poorly-documented species, *K. breviceps*. Literature regarding muscle aerobic capacity is lacking for any cetacean.

This study relied upon an archive of high-quality frozen skeletal muscle samples from stranded or incidentally caught individuals. Spectrophotometry was used to assess myoglobin content of a primary epaxial locomotor muscle, the *m. longissimus dorsi*. Histochemical techniques (myosin ATPase, succinic dehydrogenase, and immunohistochemical stains) were used to determine muscle fiber type and mean muscle fiber diameter. While the frozen tissue quality was appropriate for light microscopy studies, the quality of these samples did not permit direct evaluation of mitochondrial volume density at the ultrastructural level. Thus, a photometric analysis of sections stained for succinic dehydrogenase, a mitochondrial-bound enzyme (Nachlas, 1957; Peter *et al.*, 1972), was used to develop a relative index of mitochondrial density. This study was the first to assess such a broad suite of morphological characters in cetacean locomotor muscle.
METHODS

Specimens

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). These specimens represented high-quality carcasses (Smithsonian Institute Code 1 or 2) that were in good body condition at the time of stranding. All *K. breviceps* specimens utilized were sexually mature. All *T. truncatus* specimens were >225 cm total length; Dearolf et al. (2000) demonstrated that individuals >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 ventrad to the superficial tendon (Pabst, 1990), were used (Figure 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), in a ratio of 38.5 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.
**TABLE 1. Specimens utilized in this study***

<table>
<thead>
<tr>
<th>Identification Number</th>
<th>Total Length (cm)</th>
<th>Sex</th>
<th>SI Code</th>
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<tr>
<td><em>Tursiops truncatus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CJH 003</td>
<td>229.5</td>
<td>F</td>
<td>2</td>
</tr>
<tr>
<td>WAM 633</td>
<td>244.0</td>
<td>F</td>
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<td>2</td>
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<tr>
<td>BRF 061</td>
<td>275.0</td>
<td>M</td>
<td>2</td>
</tr>
<tr>
<td><em>Kogia breviceps</em></td>
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<td></td>
<td></td>
</tr>
<tr>
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</tr>
<tr>
<td>KMS 429</td>
<td>283.0</td>
<td>M</td>
<td>2</td>
</tr>
</tbody>
</table>

*Specimens identified by their collector’s field number. SI code = Smithsonian Institution Code, 1=live stranding, 2=fresh carcass*
Figure 1. Schematic diagram of muscle sampling site. A cross-section of the epaxial muscle mass was taken at the level of the dorsal fin. For all analyses, samples *m. longissimus dorsi* were taken just ventrad to the superficial tendon. **A**=bottlenose dolphin (*Tursiops truncatus*), **B**=pygmy sperm whale (*Kogia breviceps*) (Cross-section for **B** is *K. sima*).
Approximately 5 mL of clear supernatant was 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 replicate and the mean value was reported for each specimen. Values are reported as means ± standard errors and data were statistically analyzed using a one-tailed t-test to compare myoglobin content across species.

*Muscle fiber type and diameter*

A roughly 1 cm³ 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. Non-sequential 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 Type II fibers, following the methods of Hermanson and Hurley (1990), as adapted by Dearolf et al. (2000). One series of sections was pre-incubated in an alkaline solution (pH 10.3; 18 mM CaCl₂, 100 mM 2-amino-2-methyl-1-propanol) for 10 min. Another series of sections was pre-incubated in an acidic solution (pH 4.1-4.15; 50 mM KAc, 18 mM CaCl₂) for 5 minutes. All sections were then incubated for 30 min in a freshly prepared ATP solution (18mM calcium chloride, 100 mM 2-amino-2-methyl-1-propanol, 50 mM KCl, 3 mM ATP, pH 9.4) 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 serial 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 (NBT) incubating media (NBT in 0.2M phosphate buffer/sodium succinate solution, 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.

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, 1989; J. Dearolf, pers. comm.). Frozen, prepared serial sections were brought to room temperature. A mini PAP pen (Invitrogen) was used to outline mounted sections. Slides were rinsed in a phosphate-buffered solution (PBS, 1X) for 15 min and then sequentially incubated in horse serum (Vector) (20 min) and primary antibody (SC-71) (1 hr) in a hydrated box. Slides were rinsed three times in 1X PBS for 5 min intervals. Following PBS rinses, slides were incubated in secondary antibody (1:1 IgG/1X PBS) for 30 min in a hydrated box. Slides were rinsed three times in 1X PBS for 5 min intervals. Diaminobenzadine (DAB) (Vector) was applied to slides until optimal staining occurred (approximately 1 min). Stained samples were rinsed three times in 1X PBS for 5 min intervals, and coverslips were mounted with Kaiser’s glycerine jelly.

Muscle sections were viewed using a light microscope (Olympus BX60) operated in brightfield mode at x20 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 gathered from 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 on 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 Type 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 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), Etnier et al. (2004), and Cotten et al. (2008). 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 for mean size by fiber area using the mean diameter tool in Media Cybernetics ImagePro Plus® 6.0 software. Values are reported as means ± standard errors and data were statistically analyzed using 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 microscopy (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. An approximately 0.5 g sample of m. longissimus dorsi was excised from the frozen cross-section at a position just ventrad to the superficial
tendon and thawed slightly. Each sample was cut into smaller subsamples to allow for rapid penetration of fixatives. Subsamples were immersed in a glutaraldehyde fixative (1.5 % paraformaldehyde in PBS, 2.5 % glutaraldehyde solution, pH of 7.4) at room temperature, then stored in a refrigerator at 4°C. Once fixed, the subsamples were rinsed in 0.1 M cacodylate buffer (two 15-min rinses), and the were fascicles teased apart and post-fixed for 2 h in a 1% osmium tetroxide solution (pH 7.4). Fascicles were then rinsed in cacodylate buffer and deionized water, dehydrated in an ethanol series, and embedded in Spurrs epoxy.

Embedded fascicles were trimmed from the resin blocks, sectioned at 90 µm using an ultramicrotome (Reichert Ultracut E) fitted with a diamond knife, and mounted on Formvar-coated grids. Mounted sections were post-stained for 15 min with a 2 % uranyl acetate in 50 % ethanol solution, rinsed with deionized water, post-stained with Reynolds’ lead citrate in a chamber with sodium hydroxide pellets (Reynolds, 1963) for 15 min, and allowed to dry prior to viewing.

Fibers were examined with a Philips CM-12 transmission electron microscope (TEM) operated at 80kV. Regions of interest were identified and 10 micrographs per section were taken using Kodak 4489 EM 3¼” X 4” plate film and developed. A Microtek Scanmaker 1900 was used to digitize negatives and all digital micrographs were processed using Adobe Photoshop 7.0. 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 (volume fraction of mitochondria per unit volume of muscle fiber, \(V_{mt}\)).
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 for SDH staining as described above. SDH is a mitochondrial-bound enzyme, and its staining intensity provides a metric of mitochondrial density within a cell (Nachlas et al., 1957; Peter et al., 1972). Thin 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 oversaturating the sections from this species at the 1hr incubation time that was required for optimal staining of *K. breviceps* tissue. Following incubation, slides were processed as described above. Stained muscle sections were viewed using a light microscope (Olympus BX60) at x20 magnification. Digital micrographs were captured (Diagnostic Instruments SPOT RT camera) for all specimens in a single sitting, to ensure identical imaging conditions. Camera settings were optimized for the darkest *T. truncatus* slide; a gain of 1, adjustment factor of 1 and a gamma of 2.5 were applied in the SPOT 3.5.6 software (Diagnostic Instruments, Inc.) of the SPOT RT camera. The first field of view image was exposed using the auto exposure camera setting. Each subsequent field of view was imaged using the previous exposure camera setting. All captured images were stored as uncompressed files (TIFFs).

Digital micrographs were converted to grayscale 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 were 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 one-tailed t-tests to compare fiber staining intensity within and across species.

RESULTS

*Myoglobin*

Myoglobin concentration is reported as g Mb/ 100 g wet muscle. Mean myoglobin concentration for *T. truncatus* (3.21 ± 0.118) was significantly lower than that of *K. breviceps* (5.92 ± 0.408) (p=0.0009) (Figure 2).

*Muscle fiber type*

Myosin ATPase differentiated two fiber populations in both *T. truncatus* and *K. breviceps* (Figure 3, Table 2). For both the alkaline and acidic pre-incubation treatments, *K. breviceps* displayed a higher percentage of Type I fibers, and a lower percentage of Type II fibers, by area, than did *T. truncatus*. These species specific differences were not significant for the alkaline pre-incubation treatment (p=0.1240, one-tailed t-test), but were for the acidic pre-incubation treatment (p=0.0300, one-tailed t-test).
**Figure 2.** Myoglobin concentration of the *m. longissimus dorsi* of *T. truncatus* and *K. breviceps*. Myoglobin content expressed as mean value ± S.E. Asterisk denotes significant difference (p=0.0009, one-tailed t-test).
Figure 3. Representative cross-sections of *m. longissimus dorsi* after myosin ATPase staining. A and C are bottlenose dolphin (*Tursiops breviceps*), 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 pre-incubation (pH 4.15) (C and D). Type I fibers appear light in A and B, dark in C and D.
TABLE 2. Mean (± S.E.) fiber-type composition by area of the m. longissimus in adult bottlenose dolphins (Tursiops truncatus, n=5) and adult pygmy sperm whales (Kogia breviceps, n=5).

<table>
<thead>
<tr>
<th>Stain</th>
<th>Fiber Type</th>
<th>% Fiber by Area</th>
<th>T. truncatus</th>
<th>K. breviceps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Myosin ATPase</td>
<td>Type I</td>
<td>47.0 ± 4.2</td>
<td>53.1 ± 2.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>53.0 ± 4.2</td>
<td>46.9 ± 2.0</td>
<td></td>
</tr>
<tr>
<td>Acidic Myosin ATPase&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Type I</td>
<td>48.2 ± 3.0</td>
<td>56.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>51.8 ± 3.0</td>
<td>44.0 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Succinic Dehydrogenase&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Type I</td>
<td>44.8 ± 2.2</td>
<td>55.7 ± 1.8</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>55.2 ± 2.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>44.3 ± 1.8</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Significant species difference for both Type I & II fibers (p=0.0300, one-tailed t-test)

<sup>b</sup> Significant species difference for both Type I & II fibers (p=0.0027, one-tailed t-test)

<sup>*</sup> For *T. truncatus* Type IIa (6.4% ± 1.8) and Type IIb (48.8% ± 2.0) fibers combined
For *T. truncatus*, the SDH assay differentiated three fiber populations (Figure 4A, Table 2). The fiber profile by area was $44.8 \pm 2.2\%$ Type I, $6.4 \pm 1.8\%$ Type IIa, and $48.8 \pm 2.0\%$ Type IIb. SDH differentiated only two fiber populations for *K. breviceps* (Figure 4B, Table 2). Attempts to identify the light-staining fibers of *K. breviceps* as Type IIa or IIb using immunohistochemistry were unsuccessful. SC-71 is an antibody purported to be specific to Type IIa myosins in other mammalian species (Schiaffino, 1989). The SC-71 antibody, though, broadly stained fibers in both *T. truncatus* and *K. breviceps* sections, suggesting that specificity for Type IIa fibers was not achieved in *T. truncatus*, which served as a positive control. Therefore, neither histochemical nor immunohistochemical assays could definitively identify the specific Type II myosin of *K. breviceps*. To permit statistical comparisons of fiber type profiles across species Type IIa and Type 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* ($p=0.0027$, one-tailed t-test).

The second method for quantifying fiber type profiles, that of direct fiber count, yielded no significant differences across species for any of the histochemical treatments ($p>0.05$, one-tailed t-tests) (Table 3). The different results across these two quantification methods are likely due to the species-specific differences in fiber diameters, and, thus, fiber area (see below).
Figure 4. Representative cross-sections of *m. longissimus dorsi* after succinic 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, while 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**.
TABLE 3. Mean (± S.E.) fiber-type composition by count of the m. longissimus dorsi in adult bottlenose dolphins (Tursiops truncatus, n=5) and adult pygmy sperm whales (Kogia breviceps, n=5).

<table>
<thead>
<tr>
<th>Stain</th>
<th>Fiber Type</th>
<th>% Fiber by Count</th>
<th>T. truncatus</th>
<th>K. breviceps</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaline Myosin ATPase</td>
<td>Type I</td>
<td>51.6 ± 1.8</td>
<td>52.7 ± 2.5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>48.4 ± 1.8</td>
<td>47.3 ± 2.5</td>
<td></td>
</tr>
<tr>
<td>Acidic Myosin ATPase</td>
<td>Type I</td>
<td>54.6 ± 1.4</td>
<td>55.5 ± 2.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>45.4 ± 1.4</td>
<td>44.5 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>Succinic Dehydrogenase</td>
<td>Type I</td>
<td>51.7 ± 1.7</td>
<td>54.5 ± 1.7</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Type II</td>
<td>48.3 ± 1.7*</td>
<td>45.5 ± 1.7</td>
<td></td>
</tr>
</tbody>
</table>

*For T. truncatus Type IIa (6.3% ± 2.2) and Type IIb (42.0% ± 1.5) fibers combined
Fiber diameter

For *T. truncatus*, the mean fiber diameter of Type I fibers (49.2 µm ± 1.6) was significantly smaller than that of Type II fibers (59.7 µm ± 1.9) (p<0.0001, one-tailed t-test) (Figures 3A, 3B and 5). For *K. breviceps*, the mean diameter of Type I fibers (86.2 µm ± 2.2) was similar to that of Type II fibers (81.7 µm ± 2.1) (p=0.9286, one-tailed t-test) (Figures 3A, 3B and 5). 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, one-tailed t-tests).

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* (Figure 6). Fiber distributions for individuals demonstrate intraspecific variation (Figures 7 and 8).

*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, ANOVA) (Figure 9A). *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, one-tailed t-test) (Figure 9B).
Figure 5. Mean fiber diameter (µm) of Type I and Type II fibers in the bottlenose dolphin (*Tursiops truncatus*) and the pygmy sperm whale (*Kogia breviceps*). Values with different letters are significantly different from each other.
Figure 6. Overall distribution of fiber stain 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.
Figure 7. Distributions of fiber stain intensity values for individual bottlenose dolphins (*Tursiops truncatus*) as indicators of mitochondrial density. Sections from all individuals were incubated simultaneously for 30 min. A=CJH003, B=BRF061, C=WAM628, D=WAM633, and E=WAM642.
Figure 8. Distributions of fiber stain intensity values for individual pygmy sperm whales (*Kogia breviceps*), as indicators of mitochondrial density. Sections from all individuals were incubated simultaneously for 30 min. \(A=\text{KMS429}, \ B=\text{BRF092}, \ C=\text{KMS427}, \ D=\text{KLC051}, \) and \(E=\text{MDB056}.\)
Figure 9. Mean (± S.E.) stain intensity values of Type I and Type II fibers for the (A) bottlenose dolphin (*T. truncatus*, n=5) and the (B) pygmy sperm whale (*K. breviceps*, n=5). Values with different letters are significantly different from each other.
A

Fiber Type

Type I

Type Ila

Type IIb

Stain Intensity

100

120

140

160

180

200

A

B

C

208

182

133

B

Fiber Type

Type I

Type II

Stain Intensity

100

120

140

160

180

200

A

B

A

B
To examine interspecific variation, a random subset of Type I and Type II (*T. truncatus*=Type IIb) fibers (n=275, n=250, respectively) from each species was compared (Figure 10). Type I fibers of *T. truncatus* stained significantly more intensely (208±2) than those of *K. breviceps* (151±1) (p<0.0001, one-tailed t-test). Type II fibers of *T. truncatus* also stained significantly more intensely (133 ± 2) than those of *K. breviceps* (92±2) (p<0.0001, one-tailed t-test).

**DISCUSSION**

The goal of this study was to compare the locomotor muscle morphology of two cetacean species with different dive behaviors: the bottlenose dolphin, *Tursiops truncatus*, and the pygmy sperm whale, *Kogia breviceps*. Biochemical and histochemical techniques were used to characterize the epaxial muscle, *m. longissimus dorsi*, of five individuals from each species.

The locomotor muscle of *K. breviceps* had significantly higher mean myoglobin concentration, generally more Type I fibers, significantly larger mean fiber diameters, and significantly lower mean SDH staining intensity than that of *T. truncatus*. This discussion will compare the muscle morphology of *T. truncatus* and *K. breviceps* 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 in diving mammals.
Figure 10. Mean (± S.E.) stain intensity values of Type I and Type II fibers across species. Asterisk and cross denote significant difference across species for each fiber type (p<0.001, one-tailed t-tests).
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; Kooymen et al., 1989; Lydersen et al., 1992; Ponganis et al., 1993; Schaffer et al., 1997; Dolar et al., 1999; Kanatous et al., 1999; Noren and Williams, 2000; Polasek and Davis, 2001; Kanatous et al., 2001; reviewed in Hochachka and Somero, 2002; Williams et al., 2011). While all diving marine 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; Kooymen, 1973; Castellini and Kooymen, 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; Kanatous et al., 2002; Williams et al., 2011) (Figure 11a). How these enhanced oxygen stores are utilized during a dive is dependent, in part, upon locomotor muscle fiber type 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 Type II fibers. Type I fibers contract slowly and are more resistant to fatigue than Type II fibers because they have a lower ATP demand during contraction (Pette et al., 1999). Dive duration is limited, in part, by the ability of skeletal muscle to resist fatigue.
Figure 11. 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 Type 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.
A

Myoglobin (g/100 g wet muscle)

3.2 4.6 5.9 7.8

B

% Type I fibers

47 53 67* 88

C

Fiber diameter (µm)

55 62 84 94
However, because Type I fibers are aerobic, they typically consume more oxygen than anaerobic Type II fibers. Type IIb fibers contract and fatigue quickly, and the intermediate fiber, Type IIa, contracts quickly like Type IIb fibers, but resists fatigue, similar to Type I fibers (Peter et al., 1972; Gauthier, 1986).

The fiber type profile of marine mammal skeletal muscle is often quantified as a percentage of total fibers counted (e.g. Bello et al., 1985; Kanatous et al., 2002; Watson et al., 2003). Stereological methods permit a measure of the cross-sectional area occupied by each fiber type, which takes into account differences in fiber size. Because a muscle fiber’s contractile force is proportional to its cross-section (Close 1972), this metric likely also provides a more functionally relevant description of a fiber type profile. Fiber proportion by count and area will yield similar results if the different fiber types are of similar size. For example, the Type I and Type II fibers of M. monoceros are of similar mean diameter (62.6 µm and 60.3 µm, respectively) and the percentage of Type I fibers by count and area for this species are also similar (86.8% and 87.8%, respectively) (Williams et al., 2011). This same pattern is observed in K. breviceps (see Tables 2 and 3), which possesses similarly sized Type I and II fibers. In contrast, in T. truncatus, the Type I fibers are ~18% smaller than Type II fibers. Thus, the percentage of Type I fibers by count (~53%) was higher than the percentage of the muscle fiber area composed of Type I fibers (~47%) for this species. Reporting fiber type by area, in addition to standard point counting, allows for a more comprehensive comparison of skeletal muscle profile across species.

The relative proportions of specific fiber types within the skeletal muscle of marine mammals provide insight into their diving and/or swimming capabilities (dive
duration and activity level). 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 myoglobin contents within their locomotor muscles (Kanatous *et al.*, 2002; Williams *et al.*, 2011) (Figure 11b). *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 (Tables 2 and 3). Thus, this species, found in temperate to tropical waters, does not display as extreme a reliance on slow fibers as these two other deep divers. While direct measurements of dive behavior are not available in the literature for *K. breviceps*, the predominance of Type I fibers may reflect relatively low levels of activity (Williams *et al.*, 2000). Further comparative study of endurance divers across both temperate and polar environments is merited.

In the *m. longissimus dorsi* of *T. truncatus*, the cross-sectional area occupied by Type II fibers was significantly larger than that of Type I fibers, although they were found in similar proportions by count (Tables 2 and 3). Higher proportions of Type II fibers (by count) have been reported for two other short-duration shallow divers, *P. vitulina* and *Eumetopias jubatus* (each approximately 53%) (Kanatous *et al.*, 1999; Watson *et al.*, 2003). However, the majority of Type II fibers in *T. truncatus* and *E. jubatus* were Type IIb, while those of *P. vitulina* were exclusively Type IIa (Kanatous *et al.*, 1999; Watson *et al.*, 2003). Interestingly, like *P. vitulina*, the Type II fibers of *L. weddellii* are only Type IIa. While active-swimming, short duration, shallow divers have high proportions of Type II fibers, differences in proportions of Type IIa and Type IIb fibers may indicate differences in diving and/or swimming behavior.
SDH staining distinguished three fiber types in *T. truncatus*, but only two fiber types were observed in the *m. longissimus dorsi* of *K. breviceps*. Neither histochemical nor immunohistochemical assays could definitively identify the specific Type II myosin in this species. However, the Type I and Type II fibers of *K. breviceps* had similar mean fiber diameters, suggesting that the fast fibers are Type IIa fibers (Lundgren and Kiessling, 1988; Cobb *et al*., 1994; Stegall, 2001). Williams *et al.* (2011) described the presence of both Type I and Type II fibers in the *m. longissimus dorsi* of *M. monoceros*, but did not identify the specific Type II fiber form(s) present. The mean diameter of Type II fibers reported for this species (60.3 µm) is similar to that of its Type I fibers (62.6 µm) (Williams *et al*., 2011), suggesting that they, too, may be Type IIa fibers. The identity of Type II fibers across these (and other) divers warrants further study. The use of alternative molecular techniques, such as gel electrophoresis, may help identify the fast fiber type(s) present in the locomotor muscle of diving mammals.

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, 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 (reviewed in Jimenez *et al*., 2008). 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). Similarly, in mammals,
aerobic Type I fibers tend to have smaller diameters than Type IIb anaerobic fibers (Lundgren and Kiessling, 1988; Cobb et al., 1994). Recently, Jimenez et al. (in review) 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, 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 can reduce overall metabolic rate (reviewed in Boutilier, 2001; reviewed in Johnston et al., 2006; reviewed in Jimenez et al., in review).

In the muscles of diving mammals, oxygen is bound to endogenous myoglobin; thus, the limits imposed on cell size by oxygen diffusion distance may be diminished in these species. Under these conditions, large fiber diameter, and the concomitant lower metabolic costs associated with maintaining ionic homeostasis, would appear to be beneficial to reducing the overall rate of oxygen consumption.

Muscle collection methods vary across marine mammal studies and tissues may be obtained in different contractile states. To estimate the effect different states of contraction may have on fiber diameter, I applied a simple geometric model. Modelling a muscle fiber as a right, circular cylinder of constant volume (Kier and Smith, 1985), a 10% contractile change in length (shortening) (a maximal contractile strain reported in the literature, 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.

*T. truncatus* and *P. vitulina* possess mean skeletal muscle fiber diameters that are within the range reported for terrestrial mammals (34-60 µm) (Figure 11c) (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). The high myoglobin content of the locomotor muscles of *K. breviceps* and *L. weddellii* likely reduces the size constraints associated with oxygen diffusion distances, allowing the fibers to be “optimally” large.

*L. weddellii* are known to utilize energy saving locomotor strategies during diving, such as gliding, and stroke-and-gliding (Williams *et al.*, 2000; Williams *et al.*, 2004). During gliding, which can occur for up to 78% of the total descent duration (Williams *et al.*, 2000), the metabolic costs of their locomotor muscles are reduced to primarily those of cell maintenance. Thus, large skeletal muscle fiber size, which contributes to reduced muscle metabolic maintenance costs, may contribute to lower overall rates of oxygen consumption during a dive. The similarities in muscle fiber diameter between *L. weddellii* and *K. breviceps* may suggest similarities in diving behavior. However, *M. monoceros*, which has been characterized as a very slow-swimming, deep diving species, and which possesses one of the highest myoglobin concentrations ever recorded, does not possess extremely large fibers (60-63 µm) (Williams *et al.*, 2011). Thus, large fiber size is not a universal trait of deep diving
marine mammals. The relationships between myoglobin content, fiber type profile and size, and diving behavior warrants further investigation.

A comprehensive comparison of skeletal muscle mitochondrial volume density across cetaceans is lacking, in part because adequate samples of fixed, fresh tissue of high ultrastructural quality are difficult to obtain from these protected species. This study developed a photometric assay for comparing mitochondrial density based on differential staining intensity of succinic dehydrogenase, a mitochondrial-bound enzyme. While this method did not permit direct quantification of mitochondrial volume density, it did provide an index to assess the relative mitochondrial abundance across fiber types and species.

The locomotor muscle of *K. breviceps* had a significantly lower index of mitochondrial density than that of *T. truncatus*, across both fiber types. These findings indicate that the *m. longissimus dorsi* of *K. breviceps* has a lower aerobic capacity compared to that of *T. truncatus*. This finding is similar to that observed in pinnipeds; the locomotor muscle of *L. weddellii* displayed lower mitochondrial volume density compared to *P. vitulina* (Kanatous *et al.*, 1999; Kanatous *et al.*, 2001). To better compare the skeletal muscle oxygen consumption rates of diving marine mammals, a concerted effort to obtain high-quality fixed, fresh tissue from stranded and by-caught cetaceans, to directly assess mitochondrial volume density, is needed. Alternatively, utilization of the photometric assay described herein, across a broad range of species, could provide a comparative index of mitochondrial density.

Although most marine mammals are believed to dive aerobically for the majority of their dives (Kooymann, 1989; Williams *et al.*, 2011), this information is not definitively
known for *K. breviceps*. There are few diving 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 ventilates rapidly (Scholander, 1940; Ridgeway and Johnston, 1966; Kooyman, 1973; Cotten *et al*., 2008; reviewed in Piscitelli *et al*., 2010).

The extended post-dive surface times of *K. breviceps* could be interpreted as a period required to compensate for the metabolic acidosis that occurs with the clearance of anaerobic metabolites, following an anaerobic dive. I hypothesize instead, 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 post-dive surface intervals, is believed to dive aerobically for the majority of its dives. It is plausible 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 aerobic 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 post-dive surface interval to adequately reperfuse its tissues and reoxygenate its high skeletal muscle myoglobin stores.

Across marine mammals for which skeletal muscle morphological characters and dive behavior have been reported, the results of this study suggest that short duration, shallow divers, like *T. truncatus*, possess muscle morphologies (relatively lower myoglobin content, lower proportions of Type I fibers, smaller muscle fiber diameters and higher 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. The collective results of this study suggest that the locomotor muscle of *K. breviceps* is able to ration high stores of onboard oxygen, which may permit this species to conduct prolonged, deep dives aerobically. These results illustrate how morphological characters of skeletal muscle may be utilized 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.
LITERATURE CITED


