ABSTRACT

Beaked whales (*Mesoplodon* spp.) are some of the deepest-diving cetaceans (including whales, dolphins, and porpoises), but little is known about their surfacing behaviors and ventilation. In addition, to date, no ventilatory muscle of a beaked whale has been studied. Knowing the morphology of their breathing muscles may lead to insights about the ventilation of beaked whales and help explain their extreme breath-hold diving abilities. Thus, the purpose of this study is to characterize the beaked whale diaphragm in order to better understand its role in their breathing and breath-hold diving behaviors. To accomplish this goal, diaphragm muscles were collected from four beaked whales [*M. densirostris* (n=1), *M. europaeus* (n=2), and *M. mirus* (n=1)], which varied in body length (range: 227 to 465 cm). Samples from the costal regions of these diaphragms were serially sectioned and stained for their myosin ATPase and NADH (oxidative enzyme) activities. The stained sections were imaged, and the images were used to determine the percentages of slow-twitch fibers in the diaphragms. We also measured the NADH staining densities and diameters of the slow- and fast-twitch fibers in the muscles using ImageJ. These techniques allowed us to compare the proportions, sizes, and NADH staining densities of the fast- and slow-twitch muscle fibers in the diaphragms. The characteristics of the beaked whale diaphragm will also be compared to those of other cetacean diaphragms in order to highlight which features may be responsible for the ventilation and breath-hold abilities of beaked whales.

INTRODUCTION

Due to their so-called “skittish” behavior around boats, interspecific resemblance, and thus challenge to study in the wild, the behaviors of the deep-diving beaked whales (*Mesoplodon* spp.) are barely understood (NOAA Fisheries, 2012). Prior gut analyses of stranded beaked whales have identified their major prey items as small fish and squid belonging to a deep-water environment. Limited tagging data show these cetaceans use their deep-diving abilities to forage for their prey using echolocation at mesopelagic and bathypelagic depths (Tyack et al., 2006). Recently, a species of beaked whale (*Ziphius cavirostris*) was shown to dive for 137.5 min and down to 2,992 meters—deeper than any marine mammal known to date (Figure 1) (Schorr et al., 2014).

The aerobic dive limit (ADL) is the maximum time an organism can dive before significant lactic acid buildup occurs (Kooyman et al., 1980). It has been shown that several beaked whale species dive for periods of time up to twice as long as their estimated ADL, and it is inferred that the oxygen deficit resulting from such long, deep dives requires an extended span of shallow, short dives as a recovery period (Tyack et al., 2006).

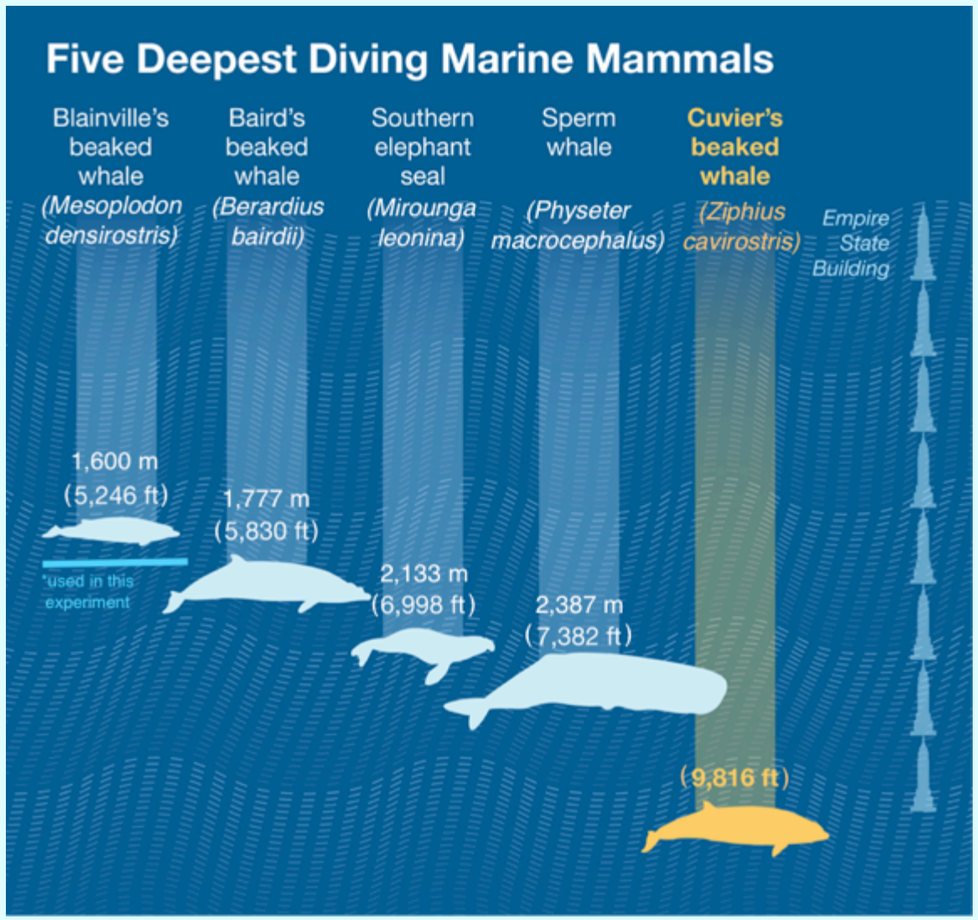


Figure 1. Deepest diving marine mammals. Adapted from National Geographic Staff (2014).

INTRODUCTION

While recent tagging data has provided novel understanding of the beaked whale (Figure 2) dive profile (Schorr et al., 2014), it does not reveal any ecological or morphological drivers of their breath-holding behavior patterns. Analyzing the fibers in a breathing muscle could offer insight into these behaviors. Slow-twitch (Type I) fibers are rich in myoglobin, aerobic, and oxidative—features that aid in increasing the ADL, and thus deep-diving when they dominate the fiber-type profile of locomotor muscles (Kielhorn et al., 2013; Velten et al., 2013). Fast-twitch fibers are primarily used in short burst activity, such as rapid inhalation, a behavior seen in most cetaceans (Cotten et al., 2008). However, the bottlenose dolphin (*Tursiops truncatus*) diaphragm has been shown to be composed of primarily slow-twitch fibers (66%) (Dearolf, 2003).

Figure 2. A breaching *Mesoplodon densirostis*. Photo by John Durban.

Here, we seek to characterize the morphology of the beaked whale diaphragm using histological staining techniques (myosin ATPase and NADH). Overall, our purpose is to attempt to elucidate the diaphragm's role as a physiological driver of beaked whale breathing behavior. Because they spend minimal time at the surface (Tyack et al., 2006) and inhale rapidly, we expect the beaked whale diaphragm to be composed of primarily fast-twitch fibers.

METHODS

Table 1. Beaked whale (*Mesoplodon* spp.) specimens utilized in this study*.

ID	Species	Total Length (cm)	Mass (kg)	Sex	Reproductive Status
MDB 064	*M. europaeus*	465	NE	Female	Mature
KLC 112	*M. mirus*	455	951	Male	Mature
WAM 593	*M. densirostris*	423	940	Male	Mature
KLC 110	*M. europaeus*	227	84.8	Female	Immature

* Collected by the Marine Mammal Stranding Program at UNC Wilmington, the Cetacean and Sea Turtle Team at the NMFS laboratory in Beaufort, NC, and the Virginia Aquarium Stranding Response Program in Virginia Beach, VA.

- Samples cut from the costal region of the diaphragm (DIA) of four beaked whales (Table 1) were covered in Tissue Freezing Medium, flash frozen (20 sec) in isopentane cooled by liquid nitrogen at -150° C, and stored at -80° in Nalgene vials until cutting.
- Eighteen 0.7-0.8 µm serial sections from each sample were cut using a Microm cryostat, which were placed onto six slides for staining.
- To stain for myosin ATPase (Hermanson and Hurley, 1990), five slides were incubated for 10 min at 37° C in a medium with a pH ranging from 10.1-10.5 at 0.1 increments and then in a freshly made ATP solution for 30 min at 37° C.
- The slides underwent a 3 min rinse/stain cycle—DI H_2O (pH=8.5-9.0), 2% $CaCl_2$, DI H_2O, 1% $CoCl_2$, DI H_2O, 1% $S(NH_4)_2$, running H_2O (5 min), 70%, 80%, 90%, and 100% EtOH, and xylene—before being mounted in xylene-based media. Type I muscle fibers appeared light and Type II appeared dark (Brooke and Kaiser, 1970) (Figure 3).

METHODS

- To stain for oxidative enzyme properties, one slide was incubated in an NADH solution for 35 min at 37° C, rinsed for 2 min in DI H_2O (Novikoff et al., 1961), dipped in eosin for 30s, rinsed in in DI H_2O again for 3 min and mounted in glycerin jelly.
- Four to five images were taken of each NADH-stained section, and then the same areas were imaged from the complementary ATPase slide.
- To determine percentages of type I and II muscle fibers, the images were printed, and the fibers were manually counted.
- The diameters and NADH-staining densities of types I and II muscle fibers were determined using Image J (Figure 4A).

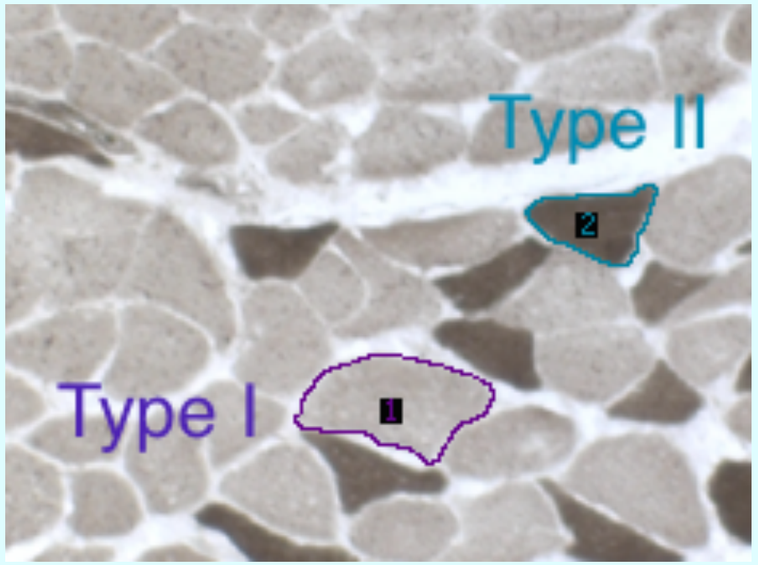
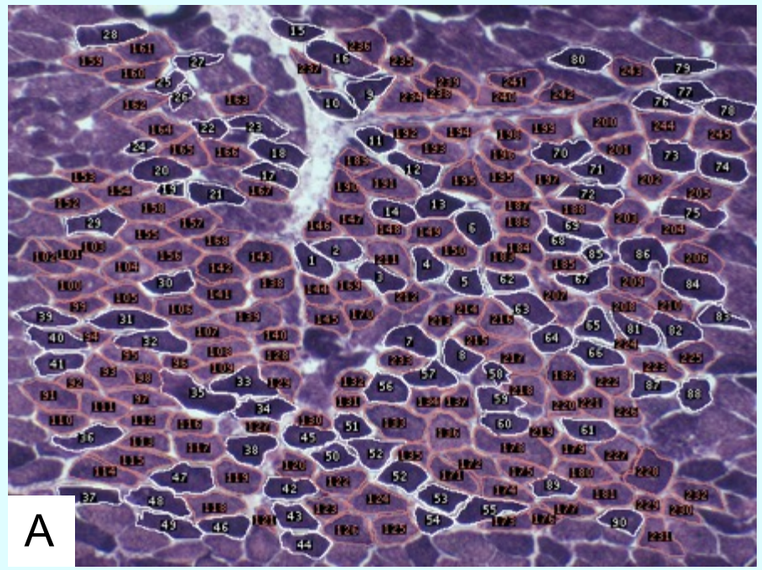


Figure 3. Alkaline myosin ATPase stain showing light Type I and dark Type II muscle fibers.



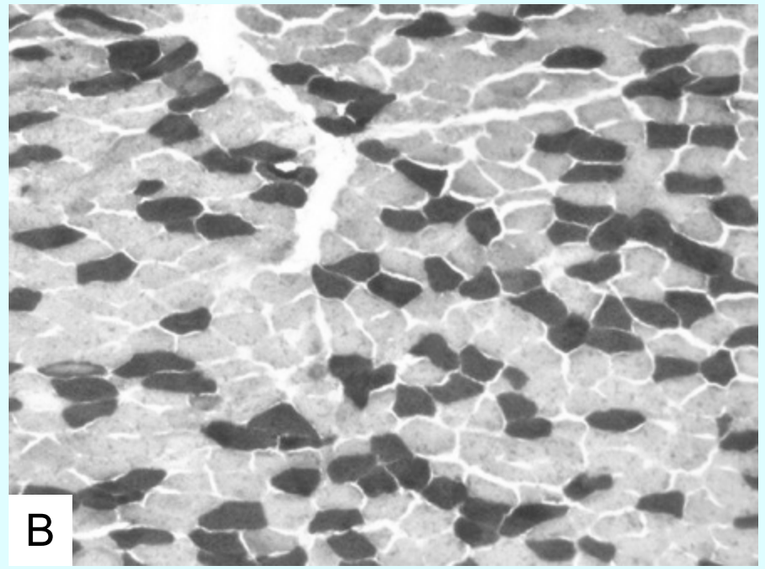


Figure 4. A) Beaked whale costal DIA section stained for NADH with Type I fibers circled in pink and Type II in white and B) corresponding ATPase stain of the same serial section.

RESULTS

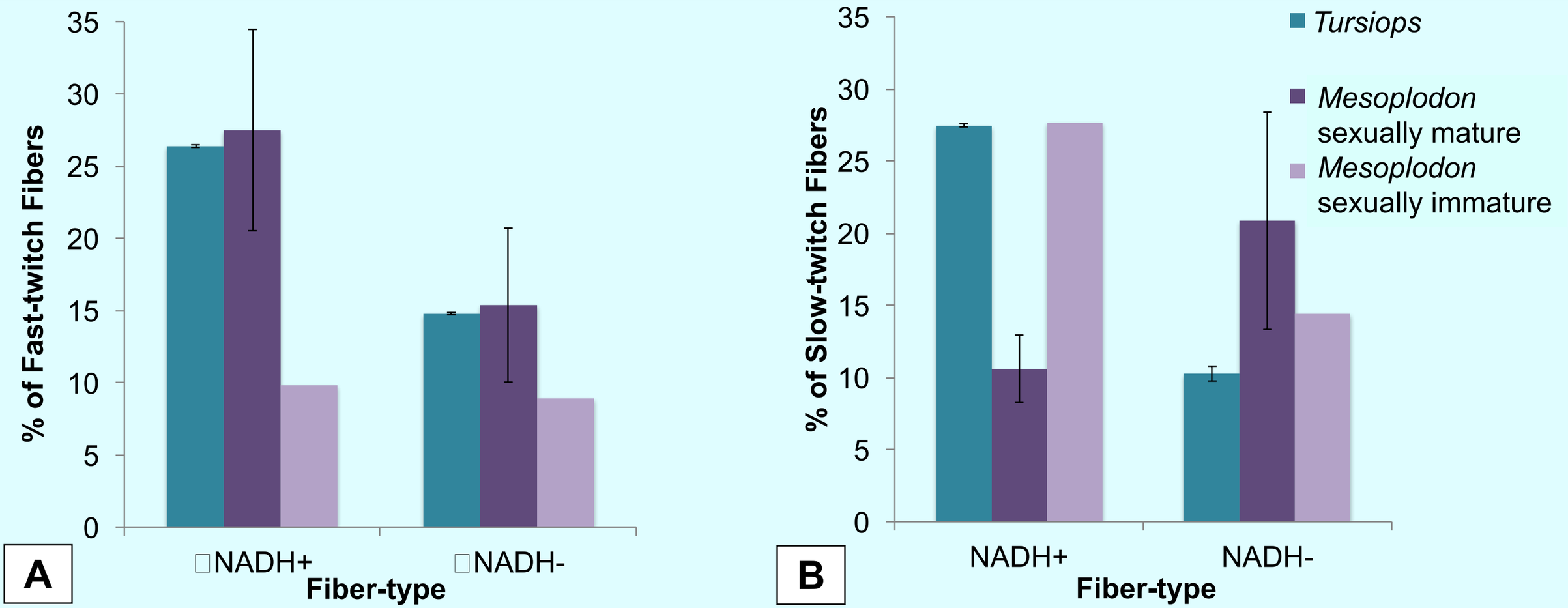


Figure 5. Percentages of NADH+ and NADH- (**A**) fast-twitch fibers and (**B**) slow-twitch fibers in the diaphragms of adult bottlenose dolphins (*Tursiops truncatus*) and sexually mature and immature beaked whales (*Mesoplodon* spp.). NADH+ indicates high oxidative/low glycolytic activity; NADH- indicates low oxidative/high glycolytic activity. Error bars are ±1 SE. (**A**) Diaphragms of adult bottlenose dolphins and mature beaked whales had the same percentages of NADH+ (26.4 ± 0.09; 27.4 ± 6.96) and NADH- (14.8 ± 0.08; 15.4 ± 5.32) **fast-twitch fibers**, all of which were greater than the percentages present in the immature beaked whale muscle. (**B**) Diaphragms of adult bottlenose dolphins and the immature beaked whale had similar percentages of NADH+ (27.5 ± 0.11; 27.6) **slow-twitch fibers**, which were greater than the percentage found in the muscle of the mature beaked whale and NADH- (10.3 ± 0.51; 14.4) **slow-twitch fibers**, which were less than the percentage in the mature beaked whale muscle.

RESULTS

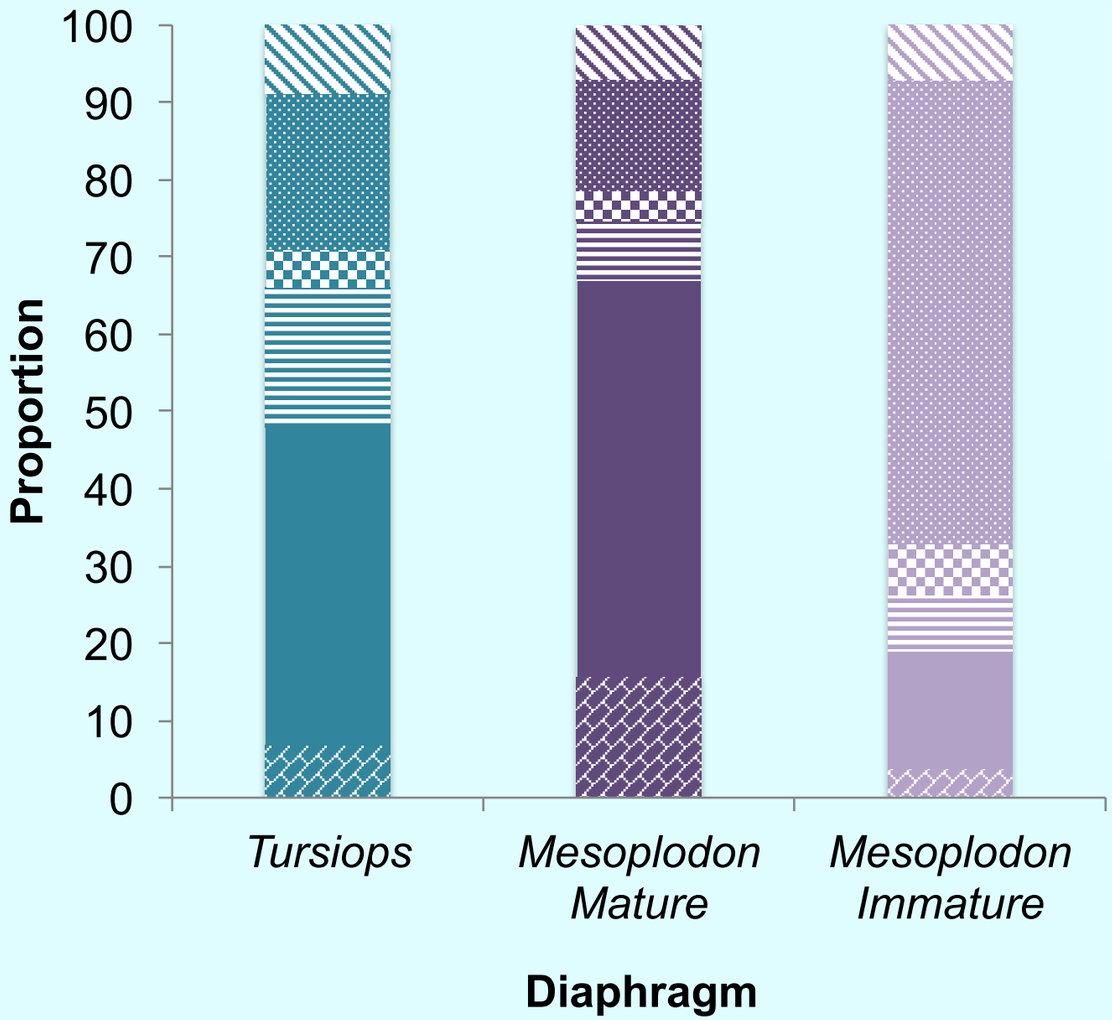


Figure 6. Proportions of each of the six types of muscle fibers (fast-twitch NADH+, fast-twitch NADH±, fast-twitch NADH-, slow-twitch NADH+, slow-twitch NADH±, and slow-twitch NADH-) for the diaphragms of each group of animals: adult bottlenose dolphins (*Tursiops truncatus*) and sexually mature and sexually immature *Mesoplodon* spp. Proportions of fast NADH+ and slow NADH+ were similar for all three groups, except slow NADH+ for *Tursiops* [18.2% vs. 7.6% (*Mesoplodon* average)]. The diaphragms of adult bottlenose dolphins and mature beaked whales expressed largely similar proportions of all fiber types, except for the proportions of slow NADH- fibers (6.8% vs. 15.6%). However, both adult muscles were mostly composed of slow-twitch fibers [66% (Dearolf, 2003) and 74.8% (this study), respectively]. The immature beaked whale diaphragm, in contrast, was composed mainly of fast-twitch fibers (73.8%), most of which were NADH±.

DISCUSSION

The percentage of slow-twitch muscle fibers in neonatal bottlenose dolphin (*Tursiops truncatus)* diaphragms (34 +/- 2.6%) (Dearolf, 2003) is similar to the percentage of slow-twitch muscle fibers in the diaphragms (26.2%) of sexually immature beaked whales (*Mesoplodon* spp.). Moreover, these percentages are significantly less than the percentages of slow-twitch muscle fibers in the diaphragms of adult/sexually mature bottlenose dolphins and beaked whales, which are statistically similar [66 +/- 2.3% (Dearolf, 2003) and 74.8 +/- 12.6% (this study), respectively]. Thus, this is the first study to demonstrate that a breathing muscle of a long-duration, deep-diving adult marine mammal is predominantly Type I (slow-twitch), and these results refute our hypothesis.

This fiber-type profile leads us to hypothesize that the adult bottlenose dolphin and mature beaked whale diaphragms are both used during breath-hold. However, we know that the diving habits of these two organisms are drastically different. The bottlenose dolphin is a shallow, short-duration diver (Schreer and Kovacs, 1997), while the beaked whale is a deep, long-duration diver (Tyack et al. 2006; Schorr et al. 2014).

Thus, we look to the differences in the NADH properties of their slow-twitch fibers to explain these results. The mature beaked whale diaphragm had a significantly greater percentage of NADH-, low oxidative activity slow-twitch fibers than the muscle of the bottlenose dolphin (Figure 5B and Figure 6). We hypothesize that the low oxidative activity corresponds to a high glycolytic activity, and that this enhanced glycolytic activity of their **slow-twitch fibers** is one feature that allows the diaphragms of beaked whales to continue to contract even if their oxygen stores are depleted during extended dives to depth.

DISCUSSION

A sexually immature beaked whale shares its maximum dive depth and breath-hold abilities with its adult counterparts (Tyack et al., 2006), despite its diaphragm being so morphologically different from the adult muscle (Figures 5A, 5B, and 6). In contrast, neonatal bottlenose dolphins do not possess the same diving abilities as adults, and the morphology of their diaphragms reflects these differences (Dearolf, 2003). However, immature beaked whales may breathe more frequently than adults, since most neonatal mammals have a greater ventilation frequency than older animals, including bottlenose dolphins (Dearolf, 2003). This breathing behavior would explain the primarily fast-twitch profile (73.8%) of the immature beaked whale diaphragm.

In the future, we plan to create an oxidative scaling factor to be able to better compare the oxidative capacity of fibers across animals. As of now, the staining densities are calibrated to each individual. Instead, we would stain sections of a dolphin, immature, and mature beaked whale diaphragm for their NADH activity at the same time and measure the staining density of a small number of fibers of each type using the same settings on the microscope and in the imaging software. This process would allow us to calculate a scaling factor and thereby compare the oxidative properties of fibers across species.

ACKNOWLEDGEMENTS

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