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HETEROGENEITY OF MYOGLOBIN DISTRIBUTION IN THE LOCOMOTORY MUSCLES OF FIVE CETACEAN SPECIES

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Summary

Myoglobin is an important storage site for oxygen in the swimming muscles of diving marine mammals. However, little is known about its distribution within muscles since previous studies have relied on single samples. The goal of this study was to determine the distribution of myoglobin within the swimming muscles of five species of cetacean: dusky dolphin, false killer whale, striped dolphin, humpbacked dolphin and bottlenose dolphin. The entire dorsal (epaxial) and ventral (hypaxial) swimming muscles were removed from each animal and weighed. Transverse sections were taken from the cranial, middle and caudal regions of each muscle and sampled along a circular grid with a minimum of 30 sites per section. Spectrophotometric analysis was used to measure the myoglobin concentration of each sample. Contour maps of myoglobin concentration were made for each transverse section. Myoglobin concentration was found to be non-uniformly distributed within the muscle. The interior of the muscle lying closest to the vertebrae showed a significantly higher (11 %) mean myoglobin concentration than the exterior of the muscle for all five species. In the epaxial muscles, the mean myoglobin concentration was significantly higher in the caudal region closest to the flukes. The two deep-water species (false killer whale and striped dolphin) had significantly higher myoglobin concentrations than the three species (dusky, humpbacked and bottlenose dolphins) that occur in shallow, coastal waters. These results show that myoglobin is not homogeneously distributed in the locomotory muscle of cetaceans and that levels may be highest in those areas that produce greater force and consume more oxygen during aerobic swimming. Enhancing oxygen stores in those areas of the muscle that work the hardest would theoretically lengthen the aerobic dive limit of the animal during submerged swimming.

Key words: myoglobin, cetacean, muscle, dolphin, false killer whale, aerobic dive limit, heterogeneity, oxygen stores.

Introduction

Marine mammals and diving seabirds, especially those that make deep and long dives, have higher muscle myoglobin concentrations than their terrestrial counterparts (Kooyman and Ponganis, 1998). An elevated myoglobin concentration, which has been called the hallmark of air-breathing diving vertebrates, enhances the oxygen storage capacity of the muscle and increases the aerobic dive limit (ADL) of the animals (Kooyman and Ponganis, 1998). The ADL is the longest dive that an air-breathing animal can make while relying primarily on oxygen stored in the blood and muscle to sustain aerobic metabolism. Despite its importance as a source of oxygen for aerobic muscle metabolism, most estimates of muscle oxygen stores have been based on relatively few measurements of myoglobin concentration (Butler and Jones, 1997; Hochachka and Foreman, 1993; Dolar et al., 1999). Single measurements from a variety of skeletal muscles show that the myoglobin concentration in the muscles of diving mammals can be 3–10 times greater than in the muscles of terrestrial mammals (Kanatous et al., 1999; Kooyman and Ponganis, 1998; Robinson, 1939).

However, little is known about the distribution of myoglobin within the muscles of marine mammals, especially those muscles that generate thrust during locomotion. It has been assumed that the myoglobin concentration within muscles is relatively homogeneous, thus allowing the extrapolation of single biopsies to the entire muscle mass (Kanatous et al., 1999; Ponganis and Pierce, 1978; Reed et al., 1994). Up to 35 % of estimated body oxygen stores appear to occur as oxymyoglobin in seals and cetaceans (Kooyman, 1989). This oxygen is available to the muscle during dives when tissue perfusion is reduced and the muscle becomes hypoxic. The goal of the present study was to determine the distribution of myoglobin within the swimming muscles of five species of cetacean. Our null hypothesis was that myoglobin was homogeneously distributed. However, we observed a twofold variation in myoglobin concentration within the muscles, and this has important implications for the estimation of muscle oxygen stores and the ADL.

Table 1. *Species information*

Species	Mass (kg)	Length (cm)	Sex	Age class	Location
Humpbacked dolphin	79	183.5	Female	Subadult	Net caught in Natal
Dusky dolphin	56	162	Male	Adult	Net caught in Natal
Striped dolphin	134	225	Female	Adult	Stranded in Jefferys Bay
Bottlenose dolphin	61	178	Male	Subadult	Net caught in Natal
False killer whale	204	289	Male	Subadult	Stranded in Texas

Materials and methods

Animals

Skeletal muscle samples were obtained from a subadult Indo-Pacific humpbacked dolphin (*Sousa chinensis*), an adult dusky dolphin (*Lagenorhynchus obscurus*), an adult striped dolphin (*Stenella coeruleoalba*) and a subadult bottlenose dolphin (*Tursiops aduncus*), stranded or drowned after accidental entanglement in shark nets (Natal Shark Board, Durban, South Africa) (Leatherwood and Reeves, 1983; Table 1). The Natal Shark Board recovers animals entangled in the shark nets daily and immediately places them in a large freezer until necropsy. The animals collected for this study were sampled while still frozen and showed no signs of decay. Samples from a subadult false killer whale (*Pseudorca crassidens*) were collected from a stranded animal recovered by the Gulf of Mexico Marine Mammal Stranding Network located at Texas A&M University. This animal died after being brought to a rehabilitation facility and was sampled within 6 h of death. Age class determination was based on mass and standard length measurements (Leatherwood and Reeves, 1983).

Sampling protocol and analysis

The primary locomotory muscles of cetaceans lie along the vertebral column (Fig. 1). Several muscles work together to power the dorsal/ventral spinal flexion that creates thrust through the tail flukes during locomotion. For the purposes of this study, the m. semispinalis, m. multifindus, m. longissimus and m. intertransversarius caudae dorsalis will be collectively referred to as the epaxial muscles, and the m. intertransversarius caudae ventralis and m. hypaxialis lumborum will be referred to as the hypaxial muscles (Fig. 2) (Pabst, 1990). The epaxial and hypaxial muscles constitute the primary swimming muscles. All animals were sampled from both muscle groups with the exception of the striped dolphin, for which only the epaxial muscles were available. The epaxial and hypaxial muscles were removed in their entirety, weighed (Table 2), and three transverse sections taken along their length (Fig. 3). Each section of muscle was precisely labeled for its location and orientation within the animal. The sections were sampled at points on a circular grid using a stainless-steel borer, averaging 30 samples per muscle section.

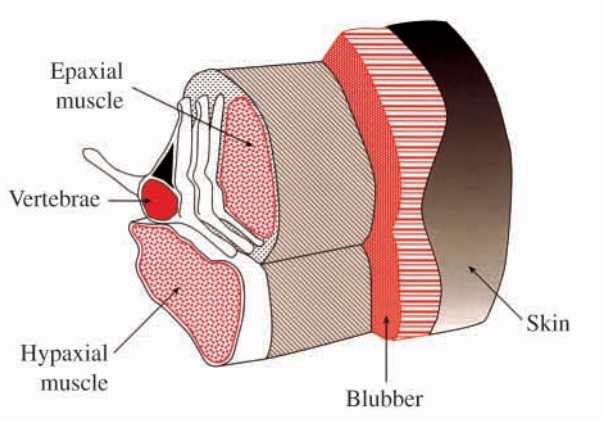

Fig. 1. Cross-sectional view of the locomotory muscles in a cetacean.

Myoglobin determination

The method of Reynafarje (1963) was used to determine myoglobin concentration. Muscle samples (200–300 mg) were cleaned of connective tissue and homogenized in a 15 ml of

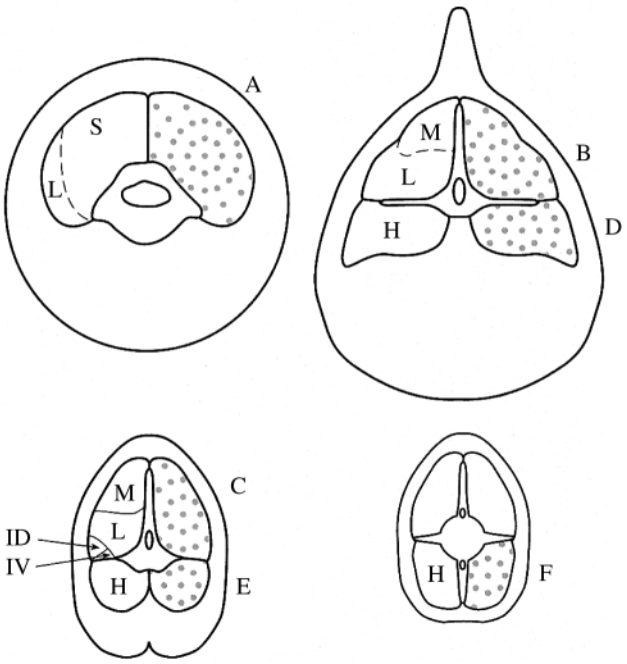

Fig. 2. Cross-sectional views of the muscle areas sampled. The right-hand half of each cross section contains lettering to denote the muscle groups sampled (A–F; see Fig. 3). The left-hand half of each cross section contains representative points from which cores were taken. Labels outside the cross sections correspond to sections labeled in Fig. 3. S, M. semispinalis; M, m. multifindus; L, m. longissimus; ID, m. intertransversarius caudae dorsalis; H, m. hypaxialis lumborum; IV, m. intertransversarius caudae ventralis.

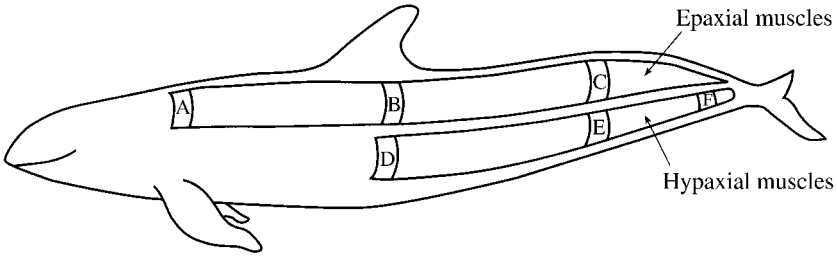

Fig. 3. Schematic representation of the three cross-sectional samples taken from both the epaxial and the hypaxial muscles.

0.04 mol l^{-1} phosphate buffer at pH 6.6 using a 15 ml Pyrex tissue grinder. The homogenate was centrifuged at 28 000 ***g*** for 50 min at 4 °C using a Sorvall RC5B refrigerated centrifuge. The supernatant was bubbled with 99.9 % carbon monoxide (CO) for 3 min to convert the myoglobin to carboxymyoglobin.

A series of tests was run to assess the minimum bubbling time necessary for complete conversion of myoglobin to carboxymyoglobin. Samples were bubbled for 1 min periods for 1–6 min, then spectrophotometric absorption readings were taken. The maximum reading for carboxymyoglobin occurred after 2.5 min of CO bubbling and remained constant for 3 min, then decreased slightly. On the basis of these results, samples were bubbled for a minimum of 3 min and a maximum of 5 min to ensure complete conversion to carboxymyoglobin.

After bubbling, the absorbance of the supernatant at 538 and 568 nm was measured using a Beckman DU-64 Spectrophotometer with a 1 cm lightpath. A myoglobin standard (horse myoglobin; Sigma-Aldrich, St Louis, MO, USA) was run with each set of samples. The myoglobin concentration was calculated as described by Reynafarje (1963) and expressed in milligrams per gram of fresh tissue. Lyophilization tests were conducted to ensure that the samples had not dehydrated during frozen storage. Mean water content was 74 %, which is similar to the value of 75 % for most mammalian muscle (Reynafarje, 1963).

Contours and statistical analyses

Contour maps of myoglobin concentration were made using Surfer (Golden Software, Inc., CO, USA). Kriging was used to contour the data because it generates the best interpretation of medium to small data sets (Keckler, 1997). Smoothing was not used because there was little or no difference in the kriging results when smoothed. GraphPad InStat (Version 3.00, GraphPad Software, CA, USA) was used for *t*-tests, and analyses of variance (ANOVAs) were used to test the significance of the myoglobin concentration within each section of muscle.

Three statistical comparisons were made. First, each transverse section was analyzed for homogeneity. This was done by dividing the data for each section into interior (I) and exterior (E) regions (Fig. 4). In section A, a diagonal line was drawn at 45 ° to divide the muscle in half, with the interior region being closest to the vertebrae. In section B, the division was made by drawing a line from the tip of the neural process to the tip of the transverse process. The angle of division used in section B was extended down from the neural process for section C. The same angle was used for sections D, E and F extending ventrally from the most exterior point of intersection with the epaxial muscle. Each transverse section was then divided into quarters by bisecting the interior and exterior regions. The mean myoglobin concentrations among the quarters were then compared using ANOVAs.

The results showed no consistent, significant pattern in myoglobin distribution except between the interior and exterior

Table 2. *Epaxial and hypaxial muscle mass with percentages of total body mass*

	Epaxial muscle mass		Hypaxial muscle mass	
Animal	(kg)	(%)	(kg)	(%)
Humpbacked dolphin	5.7	7.2	2.7	3.4
Dusky dolphin	5.4	9.6	2.7	4.8
Striped dolphin	18.2	13.6	–	–
Bottlenose dolphin	5.5	9.0	2.5	4.1
False killer whale	13	6.4	6.1	3.0

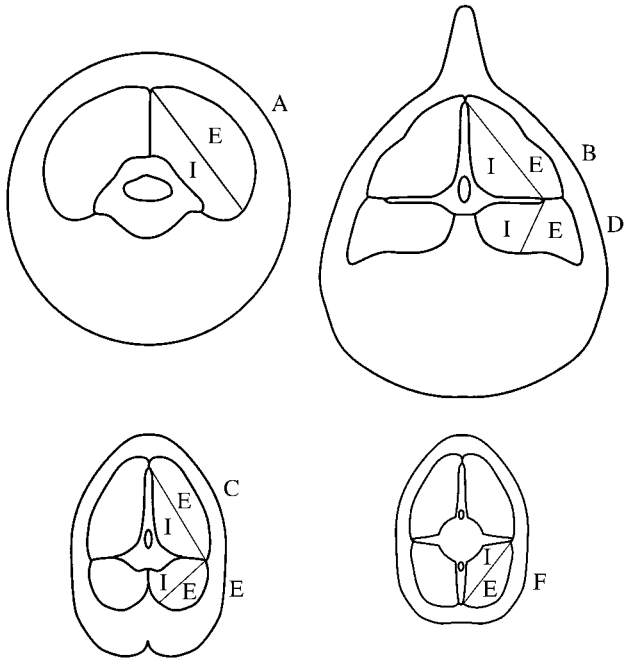

Fig. 4. Cross-sectional views of the muscles showing the separation of the exterior (E) portion of the muscle from the interior (I) used for statistical analysis. A–F, muscle groups sampled (see Fig. 3).

regions. A *t*-test was then used to test for differences in mean myoglobin concentration between the interior and exterior regions of the muscle. Physiologically, the deepest portion of the muscle (i.e. in the area of attachment to the vertebral column) should have higher concentrations of myoglobin because muscle contraction starts in the deep portion of the muscle and moves outwards as work load increases (Armstrong and Laughlin, 1985). Using ANOVAs to compare mean myoglobin concentrations among the sections for each animal, we determined whether significant differences were present along the length of the epaxial and hypaxial muscles. Finally, the mean myoglobin concentrations were compared between the epaxial and the hypaxial muscles. Because of some variation in numbers of cores taken per section, the mean myoglobin concentration was calculated for each section, and these values were used to calculate the means for the epaxial and hypaxial muscles used in the *t*-test. For all tests, a significance level of 0.05 was used, except where noted.

Results

All the muscle cross sections showed considerable heterogeneity in myoglobin distribution (Fig. 5; Table 3). However, the interior of the muscle closest to the vertebrae showed a significantly higher mean myoglobin concentration (11±6.9 %, mean ± S.D., P=0.05) than the exterior of the muscle for all five species (Table 3). For the epaxial muscles, there was a significant increase in myoglobin concentration along the cranial-to-caudal direction. However, for the hypaxial muscles, the cranial-to-caudal gradient was not significant (Table 4). There was no significant difference in the mean myoglobin concentration between the epaxial and the hypaxial muscles (P=0.2). On average, myoglobin concentrations were higher for the striped dolphin (69 mg g^{-1}) and false killer whale (62 mg g^{-1}) than for the bottlenose (24 mg g^{-1}), dusky (25 mg g^{-1}) and humpbacked (23 mg g^{-1}) dolphins. By comparison, the value for a dog was 7 mg g^{-1} (Reynafarje, 1963) and that for a rat was 2 mg g^{-1} (Reed et al., 1994).

Discussion

On the basis of the results from this study, we reject our null hypothesis that muscle myoglobin is homogeneously distributed within the locomotory muscles of dolphins. The interior of the epaxial and hypaxial muscles closest to the vertebrae showed significantly higher myoglobin concentrations than the exterior of the muscles for all five species. This heterogeneous distribution may reflect force generation and energy metabolism within the muscle during dorsal/ventral spinal flexion associated with thrust production during swimming. Armstrong and Laughlin (1985) found metabolic heterogeneity both within and among the skeletal muscles of terrestrial mammals. The deeper portions of muscle examined in that study were primarily composed of slow-twitch oxidative (SO) and fast-twitch oxidative-glycolytic (FOG) fibers, whereas fast-twitch glycolytic (FG) fibers predominate in the most superficial portions of the muscle. During locomotion, there is a progressive recruitment of SO and FOG fibers deep within the muscle and then of FG fibers in the more peripheral regions of the muscle as locomotory effort increases (Armstrong and Laughlin, 1985). In areas with increased numbers of SO fibers, there is an increased number of mitochondria and, therefore, an elevated demand for oxygen (Weibel, 1984; Sullivan and Pittman, 1987). Both SO and FOG

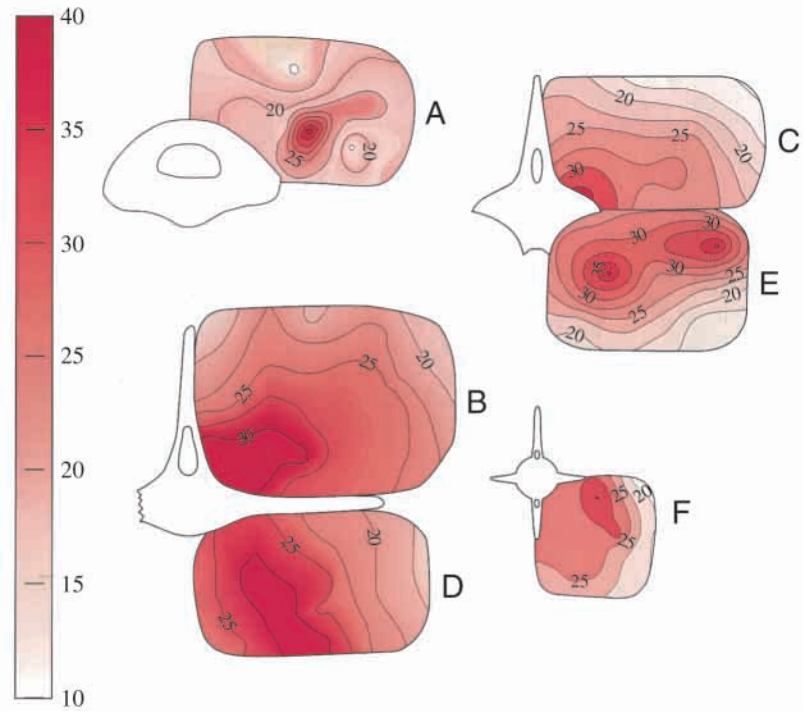

Fig. 5. Contours of myoglobin levels. The orientations of the contours are from the head looking towards the tail. The color scale is in units of milligrams of myoglobin per gram of muscle tissue. Labels outside the cross sections correspond to sections labeled in Fig. 3.

Table 3. *Mean myoglobin concentrations for each transverse muscle section*

Animal	Section	Mean myoglobin content (mg g^{-1} muscle)		
		Overall mean	Interior mean	Exterior mean
Humpbacked dolphin	A	20±3.6 (27–13)	21±3.7 (27–13)	19±3.0 (24–13)
	B	24±3.5 (33–18)	26±2.4 (33–23)	21±2.0 (28–18)
	C	20±3.0 (25–14)	21±2.9 (25–14)	19±2.8 (25–14)
	D	25±4.4 (32–18)	27±3.9 (32–19)	22±3.5 (30–18)
	E	24±4.1 (31–17)	26±3.6 (31–17)	21±3.0 (27–17)
	F	22±2.8 (27–17)	23±2.4* (27–19)	21±3.0* (27–17)
Dusky dolphin	A	24±2.0 (28–20)	24±1.9* (28–21)	24±2.0* (28–20)
	B	25±1.7 (29–22)	25±1.7 (29–22)	24±1.6 (28–21)
	C	27±1.5 (29–22)	28±0.7 (29–22)	26±1.5 (29–24)
	D	25±1.5 (28–22)	25±1.7 (28–23)	25±1.3 (28–22)
	E	25±1.6 (29–22)	25±1.8 (29–23)	25±1.4 (28–23)
	F	24±1.4 (27–21)	24±1.4 (27–21)	23±1.2 (27–21)
Striped dolphin	A	63±5.9 (75–51)	66±6.4 (75–51)	60±3.2 (67–53)
	B	76±4.5 (84–66)	78±3.7 (84–67)	74±4.1 (81–66)
	C	69±3.1 (81–63)	70±2.8 (82–66)	67±2.4 (72–63)
Bottlenose dolphin	A	21±2.8 (33–16)	21±2.9 (33–18)	20±2.7 (24–16)
	B	25±3.0 (33–18)	27±3.5 (33–19)	22±2.8 (27–18)
	C	23±4.9 (34–15)	27±3.2 (34–21)	20±3.3 (26–15)
	D	23±3.4 (29–17)	24±2.7 (29–18)	22±3.9 (29–17)
	E	25±5.7 (37–15)	28±3.9 (35–19)	21±5.2 (37–15)
	F	25±2.5 (29–19)	26±2.0 (29–20)	24±2.6 (28–19)
False killer whale	A	44±7.2 (64–33)	47±1.9 (64–37)	41±2.0 (53–33)
	B	61±8.7 (71–41)	66±1.7 (71–45)	57±1.6 (67–41)
	C	66±2.9 (76–60)	67±0.7 (73–60)	66±1.5 (76–60)
	D	68±5.9 (81–54)	72±1.3 (81–63)	64±1.7 (74–54)
	E	68±4.9 (83–53)	69±1.8 (74–59)	66±1.4 (83–53)
	F	62±4.3 (69–53)	64±1.4 (69–54)	61±1.2 (69–53)

The interior concentration was significantly higher than the exterior concentration (P<0.05) except where an asterisk denotes P<0.1.
Values are means ± S.D. (N=32–54); range is shown in parentheses.

fibers contain more myoglobin than FG fibers (Weibel, 1984). If deeper portions of the muscle are used during submaximal, aerobic exercise, then this may explain the increase in myoglobin concentration. The enhanced oxygen-storage capacity of skeletal muscles used for locomotion in marine mammals enables them to support aerobic metabolism during dives and to prolong their ADL.

There are conflicting results in the literature as to the relative thrust produced by the upstroke and downstroke in cetaceans (Videler and Kamermans, 1985; Fish and Hui, 1991; Fish and Rohr, 1999; Arkowitz and Rommel, 1985). We found a greater range in the myoglobin concentrations in the epaxial muscles than in the hypaxial muscles. There was a trend in the epaxial muscles to have the highest myoglobin levels in the most

Table 4. *Statistical results comparing the myoglobin concentrations in transverse muscle sections within each animal*

Animal	Section					
	A	B	C	D	E	F
Humpbacked dolphin	A<B	B<C	A<C*	D>E	E>F*	D>F
Dusky dolphin	A<B	B<C	A<C	D>E*	E>F	D>F
Striped dolphin	A<B	B<C	A<C			
Bottlenose dolphin	A<B	B>C*	A<C	D<E	E>F	D<F
False killer whale	A<B	B<C	A<C	D<E*	E>F	D<F

For all tests P<0.1; * indicates values that were not significantly different.
See Fig. 3 for the location of each transverse section.
A,B,C, epaxial muscle; D,E,F, hypaxial muscle.

Table 5. *Mean oxymyoglobin storage capacity of the epaxial and hypaxial muscles*

	Oxymyglobin storage capacity ($ml\,O_2\,kg^{-1}$ muscle)		
	Epaxial	Hypaxial	Epaxial and hypaxial
Bottlenose dolphin	31 (20–46)	32 (20–50)	32 (20–50)
Humpbacked dolphin	29 (17–44)	32 (23–43)	30 (17–44)
Striped dolphin	93 (68–113)	ND	ND
Dusky dolphin	33 (27–39)	33 (28–39)	33 (27–39)
False killer whale	76 (44–102)	88 (71–111)	82 (44–111)

The range of values is given in parentheses.
ND, not determined.

caudal regions closest to the flukes. This observation is in agreement with the results of Pabst (1993), who found that the anterior region of the dorsal muscles furthest from the tail contributes less to thrust generation during locomotion than the posterior region, which performs continuous work during swimming. In contrast, there was no significant cranial to caudal trend in the hypaxial muscle. The overall mean myoglobin concentrations in the epaxial and hypaxial muscles were not significantly different. This supports the hypothesis that the epaxial and hypaxial muscles produce similar propulsive forces during locomotion based on the similar arrangements of the fasciculi, tendons and muscle insertions (Arkowitz and Rommel, 1985). The fact that similar proportions of SO and FG fibers are found in the epaxial and hypaxial muscles of the bottlenose dolphin (*Tursiops truncatus*) and Pacific white-sided dolphin (*Lagenorhynchus obliquidens*) also indicates equivalent propulsive power (Bello et al., 1985; Ponganis and Pierce, 1978).

The swimming muscles examined in the present study showed a wide range of values for oxygen storage capacity. As myoglobin concentrations have been found to be heterogeneous throughout the muscle, a single spot sample from one area can result in a twofold difference in the calculated oxygen storage capabilities (Table 5). As a result, the sampling site and depth of the biopsy must be considered when using single biopsies to estimate muscle oxygen stores and the ADL.

Of the five animals tested, three occur in shallow, coastal waters and two occur in deeper, offshore habitats (Leatherwood and Reeves, 1983). The humpbacked, bottlenose and dusky dolphins are considered to be mainly shallow-water, near-shore species. The humpbacked dolphin feeds near shallow rocky reefs (Karczmarski et al., 2000). The major prey of bottlenose dolphins off Natal, South Africa, are common inshore species of fish (Cockcroft and Ross, 1990). Dusky dolphins prey both on many inshore species and on deep-water fish, although they feed primarily at night or in the early morning when deep-water fish migrate closer to the surface (Sekiyuchi, 1994). False killer whales and striped dolphin are more frequently found in offshore or oceanic environments. False killer whales feed primarily on deep-sea cephalopods and fishes (Madsen and Herman, 1980). Myctophids dominate in the stomach contents of striped dolphins from South Africa, and feeding may extend below 200 m because material in the stomach had organs of luminescence (Miyazaki et al., 1973; Ross and Bass, 1984). The offshore species in this study had, on average, significantly (P=0.0001) more (more than three times more) myoglobin than the coastal species, which may be an adaptation for deeper, longer dives. The overall size of the animal did not appear to be a factor. The striped dolphin is similar in size to the humpbacked dolphin, but they have very different habitats and myoglobin levels. Rather than body size, muscle myoglobin levels appear to be most strongly correlated with habitat (Blessing, 1972) and with prey preference, which probably reflects diving ability. The relationship between myoglobin concentration and habitat preference was also found by Dolar et al. (1999). Whether comparing individual muscle sections or entire muscle bundles, the two deep-water species had significantly higher muscle myoglobin concentrations than the three species that occur in shallow, coastal habitats.

In summary, myoglobin in the locomotory muscles of cetaceans was not homogeneously distributed. The highest myoglobin concentrations were located closest to the spine and, in the case of the epaxial muscles, closest to the flukes, which produce thrust during swimming. Myoglobin concentrations may be highest in those areas of the muscle that produce greater force and consume more oxygen during aerobic swimming. Enhancing oxygen stores in those areas of the muscle that work the hardest would theoretically prolong the ADL of the animal during submerged swimming.

This study would not have been possible without the assistance of Dr Victor Cockcroft and the Natal Shark Board in Durban for the cetacean samples they graciously made available to us.

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