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Diaphragm Muscle Development in Bottlenose Dolphins (*Tursiops truncatus*)

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ABSTRACT Being born directly into the aquatic environment creates unique challenges for the breathing muscles of neonatal cetaceans. Not only must these muscles be active at the instant of birth to ventilate the lungs, but their activities must also be coordinated with those of the locomotor muscles such that breathing takes place only at the water's surface. At least one major locomotory muscle of bottlenose dolphins (*Tursiops truncatus*) has been demonstrated to be well developed and, therefore, able to power the neonatal dolphin's early movements (Dearolf et al. [2000] J Morphol 244:203–215). Thus, because of the demands for coordinated behavior with the locomotor muscles, it is hypothesized that the breathing muscles of bottlenose dolphins, represented in this study by the diaphragm, will also demonstrate adult morphology at birth. However, histochemical and biochemical analyses demonstrate that neonatal dolphins have immature diaphragms, with only 52% of the adult slow fiber-type profile (neonates: 34% slow-twitch fibers; adults: 66% slow-twitch fibers). The developmental state of the dolphin diaphragm is compared to those of other neonatal mammals, using a muscle development index (% slow-twitch fibers in neonatal muscle / % slow-twitch fibers in adult muscle). Fiber-type profiles reported in the literature are used to calculate index values for the diaphragms of altricial rats, rabbits, and cats, intermediate baboons and humans, and precocial sheep and horses. The dolphin is not unique in having an immature diaphragm at birth; however, there is a positive relationship between the developmental state of the diaphragm and the overall developmental state of the neonate. The presence of type IIc ("undifferentiated") fibers in the diaphragms of altricial developers (e.g., rats, rabbits, and cats) is correlated with the slow contraction speeds recorded from their diaphragms. The diaphragms of neonatal horses and dolphins express little to no type IIc fibers and, thus, may have the ability to contract at the speeds required for their increased ventilation rates. These results lead to the modification of the criterion for evaluating the developmental state of a muscle at birth. Thus, the developmental state of a neonatal muscle should be based on both its value of Dearolf et al.'s (2000) developmental index, as well as the percentage of type IIc fibers found in that muscle. J. Morphol. 256:79–88, 2003.

KEY WORDS: dolphin; diaphragm; development; histochemistry; biochemistry

Most mammals, whether they are born in a relatively helpless (altricial) or well-developed (precocial) state (Ewer, 1973; Eisenberg, 1981), have the ability to ventilate their lungs from the moment of birth (Mortola, 1987; but see Frappell and Mortola, 2000). The early activity of neonatal ventilatory muscles suggests that they must be well developed; however, in the species that have been studied (rats, rabbits, cats, humans, baboons, sheep, and horses), the fiber-type profiles of neonatal diaphragms, the main muscle of inspiration, differ from those of their adult counterparts (Keens et al., 1978; Maxwell et al., 1983; Le Souef et al., 1988; Sieck et al., 1991; Finkelstein et al., 1992; Watchko and Sieck, 1993; Cobb et al., 1994a,b; Fratacci et al., 1996). These studies reveal that the ventilatory muscles of neonatal mammals are not adult-like in morphology and, therefore, not well developed.

All of the neonatal mammals studied to date are terrestrial, and because of their terrestrial habitat, these young animals are able to breathe at any time their bodies demand the exchange of respiratory gases. In comparison, neonatal cetaceans (whales, dolphins, and porpoises) are born directly into the marine environment, and therefore, they must coordinate the activities of their ventilatory and locomotor muscles such that breathing occurs only at the water's surface. The extensor caudae lateralis of neonatal bottlenose dolphins (*Tursiops truncatus*), an important epaxial locomotor muscle, has been demonstrated to be well developed at birth (Dearolf et al., 2000). Therefore, it is hypothesized that the breathing muscles of cetaceans will also be well developed at birth because of the demands for coordinated activity with the locomotor muscles. To date, no ventilatory muscle in any cetacean has been investigated to determine its developmental state at birth.

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Many muscles are considered to function in the breathing mechanics of mammals (Lieberman et al., 1992). However, because of its central role in inspiration, most of the investigative work on breathing muscles has focused on the diaphragm. The large comparative database available for the diaphragm (Gordon et al., 1989; Bramble and Jenkins, 1993; Cobb et al., 1994a), as well as information about its development (Keens et al., 1978; Maxwell et al., 1983; Le Souef et al., 1988; Sieck et al., 1991; Finkelstein et al., 1992; Watchko and Sieck, 1993; Cobb et al., 1994b; Fratacci et al., 1996), and the longstanding hypothesis that the diaphragm plays a substantial role in the breathing mechanics of dolphins (Slijper, 1962; Ridgway, 1972) lead to its choice as the focus of this study.

To test the hypothesis that the bottlenose dolphin's diaphragm is well developed at birth, histochemical (Hermanson and Hurley, 1990) and biochemical (Reiser et al., 1985) techniques are used to determine the fiber-type profiles of the diaphragms of adult and neonatal dolphins. The muscle development index proposed by Dearolf et al. (2000) is then utilized to calculate the developmental state of the dolphin diaphragm, as well as the developmental states of the diaphragms of other mammals, using fiber-type profile values reported in the literature. In a review of phylogenetically diverse mammalian muscle development, it was found that altricial developers maximally exhibit 70% of the adult slow-twitch fiber-type profile (Dearolf et al., 1998, 2000). Thus, diaphragms are considered well developed if they exhibit a developmental index of 75% or more. Finally, the values of the muscle development index are compared to determine if the dolphin has a well-developed diaphragm in comparison to other mammals.

MATERIALS AND METHODS

Specimens

Fresh carcasses of adult and neonatal bottlenose dolphins (*Tursiops truncatus* Montagu) were collected by the Northeast and Southeast regional marine mammal stranding networks and the National Marine Fisheries Service (NMFS) and were made available through the University of North Carolina at Wilmington's Marine Mammal Stranding Program (Table 1). Five animals were frozen within hours of their stranding and remained frozen for up to several months before thawing and dissection. The remaining animals were either dissected within 3–5 h of their stranding or placed in ice baths and dissected within 24 h.

Neonatal dolphins, all less than 132 cm in length (Mead and Potter, 1990; Read et al., 1993), were aged using a suite of neonatal characteristics derived from the literature (Dearolf et al., 2000). All neonates utilized in this study had floppy dorsal fins and tailflukes, suggesting that they were less than 2 weeks of age (Mcbride and Kritzler, 1951). Dolphins greater than 200 cm were considered to exhibit mature muscle characteristics; however, these specimens represented both sexually mature and immature animals (Mead and Potter, 1990; Read et al., 1993).

TABLE 1. Bottlenose dolphin (Tursiops truncatus) specimens utilized in this study

Identification number[a]	Total length (cm)	Sex	Muscle age category[b]
KMT 062	101.5	F	Neonate
VMSM 20001020[c]	106.5	M	Neonate
NC98-079	108	F	Neonate
CALO 99-13[c]	109.5	F	Neonate
VMSM 991086[c]	115	F	Neonate
VMSM 20001031[c]	127	M	Neonate
VMSM 20001049	207	F	Adult
JLH 001	212	F	Adult
PTM 047[c]	212	M	Adult
NEFSC 5451[c]	218	M	Adult
WAM 559[c]	239	F	Adult
WAM 545[c]	246	F	Adult

[a]Specimens are identified by their collector's field number.
[b]Neonate category (<132 cm and floppy dorsal fins and tailflukes); adult category (>200 cm).
[c]Diaphragm collected whole.

Muscle Histochemistry

Muscle samples were collected from 12 bottlenose dolphins: four adult females, two adult males, four neonatal females, and two neonatal males (Table 1). Diaphragms were collected whole from four adults and four neonates (Table 1), wrapped in Saran wrap, placed into plastic bags, and frozen flat at −20°C. Samples were taken from the center of the costal region (Fig. 1) of the diaphragms of the remaining animals (Table 1), wrapped in Saran wrap, placed into plastic bags, and frozen flat at −20°C. The central region of the costal diaphragm was chosen as the sampling site for this study because it was identifiable in both neonatal and adult diaphragms. In addition, most of the comparative literature includes values only for this site (Keens et al., 1978; Maxwell et al., 1983; Le Souef et al., 1988; Sieck et al., 1991; Finkelstein et al., 1992; Fratacci et al., 1996).

Small (1 cm^2) blocks were then removed from the center of the costal region and were allowed to thaw at room temperature. Once the muscle blocks completely thawed, they were mounted on cork blocks with 5% gum tragacanth and frozen in isopentane cooled to −160°C with liquid nitrogen. Tissue blocks from each diaphragm were then serially sectioned (10 μm thickness) in a cryostat (Minotome) at −19°C and mounted on glass slides.

Muscle sections were then stained for myosin ATPase (Hermanson and Hurley, 1990). One series of serial sections from each diaphragm block was preincubated in an alkaline medium (52 mM NaCl, 52 mM glycine, 24 mM $CaCl_2$, 46 mM NaOH, pH 10.3) for 10 min at 37°C. Alternate serial sections were preincubated in an acidic medium (44 mM barbitol acetate, 44 mM HCl, pH 4.3, 4.4, and 4.5) for 5 min at 37°C. All samples were then incubated for 30 min in a freshly prepared ATP solution (0.02 mM sodium barbitol, 13.5 mM $CaCl_2$, 2.7 mM ATP, pH 9.4) at 37°C. Fibers were classified as type I (slow-twitch), type II (fast-twitch: included both IIa, fast-twitch oxidative glycolytic, and IIb, fast twitch glycolytic), or IIc (fast-twitch "undifferentiated") on the basis of acidic and basic preincubation protocols for myosin ATPase following the classification scheme of Brooke and Kaiser (1970).

Immunocytochemistry for Myosin Heavy Chain Isoforms

To verify the histochemical results, additional serial sections from each diaphragm block were stained with antibodies to fast and slow myosin (Novocastra, Newcastle upon Tyne, UK). These sections were mounted on glass slides and stored at −20°C. To begin staining, the sections were allowed to warm to room tem-

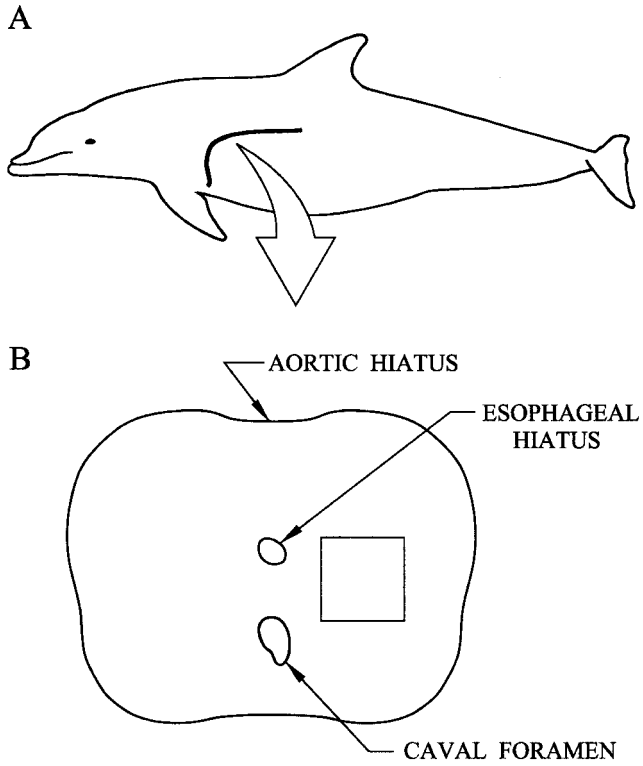

Fig. 1. Schematic diagram of the diaphragm muscle sampling location in a bottlenose dolphin (*Tursiops truncatus*). **A:** Position of the diaphragm muscle within the thoracic cavity of the dolphin. **B:** Thoracic surface of the diaphragm showing the sampling location (box). Samples were taken from the midcostal region of the diaphragm in both adult and neonatal dolphins. Original drawing produced using FastCAD (Evolution Computing, Tempe AZ) by S.A. Rommel and modified in AutoCAD LT 97 (Autodesk Press, Albany, NY) by D. Gallo.

perature for 5 min and then preincubated in blocking solution (Histostain Plus Kit, Zymed, South San Francisco, CA) for 10 min at room temperature. Following preincubation, the blocking solution was removed and a mouse anti-fast or anti-slow myosin primary antibody (Novocastra) was applied and allowed to incubate overnight in a humid chamber at 4°C.

The following day, the primary antibodies were removed, and the sections were washed for 10 min in 0.1 M phosphate buffer. Samples were then reacted with a biotinylated rabbit antimouse secondary antibody for 1 h in a humid chamber at room temperature, horseradish peroxidase enzyme conjugate for 10 min, and a DAB chromagen solution for 3 min in the dark. Between each of these steps, the samples were washed for 10 min with 0.1 M phosphate buffer. After staining, the slides were washed for 10–20 min in distilled water and coverslipped.

Myosin Heavy Chain Isoform Electrophoresis

Myosin heavy chain electrophoresis was performed following the protocol of Blough et al. (1996). Myosin was extracted from minced muscle samples (10–30 mg) at 4°C for 60 min in 40 volumes of a urea/thiourea gel sample buffer (8.0 M urea, 2.0 M thiourea, 0.08 M dithiothreitol, 0.04 M Trizma base, 0.12 M SDS, 0.004% bromophenol blue, pH 6.8) (Blough et al., 1996). Extracts were then centrifuged at 13,000 rpm for 30 min at room temperature in an IEC/Micromax microfuge. Supernatants were boiled for 2 min and then diluted 1:100 with the urea/thiourea gel sample buffer (Blough et al., 1996).

Electrophoresis was performed in an 8% acrylamide separating gel with 30% (v/v) glycerol and a 4% stacking gel with 5% (v/v) glycerol. Thirty to 35 μl aliquots of diluted myosin were electrophoresed for 24 h at 275V and 8°C. Separating gels were silver-stained according to the protocol of Blough et al. (1996) with modifications by P.J. Reiser (pers. commun.). Separating gels were fixed in a solution of 50% ethanol / 10% acetic acid (v/v) for 1 h, followed by a 2 h incubation in a 10% solution of glutaraldehyde (v/v). Fixation was followed by four 1 h rinses in Milli-Q water (Millipore, Bedford, MA).

The silver staining solution was made using the following protocol: silver nitrate was added to Milli-Q water to a concentration of 0.045 M and allowed to dissolve. Ammonium hydroxide was then quickly added to the silver solution to a concentration of 1.4% (v/v). Finally, a 180-mM NaOH solution was added to a concentration of 0.017 M (Blough et al., 1996, with modifications by P.J. Reiser, pers. commun.). Gels were incubated in the staining solution for 10 min at room temperature. They were then rinsed three times in Milli-Q water (two 5-min rinses, one 2-min rinse). Finally, gels were developed using a 0.2 mM citric acid, 1.9% formaldehyde (v/v) solution until the myosin bands were dark (approximately 30 sec). Developing was stopped with a 5% acetic acid (v/v) solution, and the gels were rinsed with Milli-Q water. The stained gels were then photographed on a light table.

Data Collection and Statistical Methods

Digital images of the histochemically stained sections were captured using an Olympus BH-2 light microscope, a Dage MTI CCD72S video camera system (Diagnostic Instruments, Sterling Heights, MI), and NIH Image (v. 1.62; National Institutes of Health, Bethesda, MD) on an Apple Macintosh Quadra 840AV. For each sample (adult and neonate costal diaphragm), images were taken from identical regions of each of the treatments (myosin ATPase acid and alkaline preincubations, and anti-fast and anti-slow myosins) described earlier for fiber-type determination. These images were then printed for analysis.

To determine the percentage of area occupied by type I and II fibers in both adult and neonatal diaphragms, a Mertz-curvilinear test system was placed onto the images (myosin ATPase acid preincubation), and the points residing in each fiber-type (P_I and P_{II}) and in the white space (P_S) were counted (Russ, 1986). This process was repeated on multiple images (usually n = 4) from one cross-section per sample until the least-represented fiber-type was counted 100 times. To calculate the percentage area occupied by each fiber-type, P_S was subtracted from the total number of points counted, P_T. The resulting value was equal to the number of points residing in muscle tissue, P_M. The points residing in each fiber-type were then divided by P_M and multiplied by 100%, resulting in the percent area for each fiber-type, where $P_P = A_A$ (P_P – points per unit point [point fraction]; A_A – area per unit area [area fraction]; and $A_A \times 100\%$ = area percentage [Russ, 1986]).

The images from the acid preincubation protocol of the myosin ATPase assay were also analyzed to obtain fiber-type percentages by number. The number of each type of fiber on each image was counted, and this process was repeated for each sample until a minimum of 1,000 fibers was counted (usually images = 4). The numbers of fibers of each type were then summed, divided by the total number of fibers counted, and multiplied by 100% to express fiber number as a percentage of the total number of fibers. In addition, 50 fibers of each type were also measured for least diameter and area using NIH Image.

Histochemical datasets were statistically analyzed using Minitab release 11 (Minitab, State College, PA). Adult and neonatal area and fiber number percentages were compared using ANOVAs. These datasets were also compared using paired Students *t*-tests to determine if differences existed between the two fiber-type profile assessments for adults and neonates. The fiber diameters of the adult and neonatal diaphragms were compared using an ANOVA. Significance was correlated with $P \leq 0.05$.

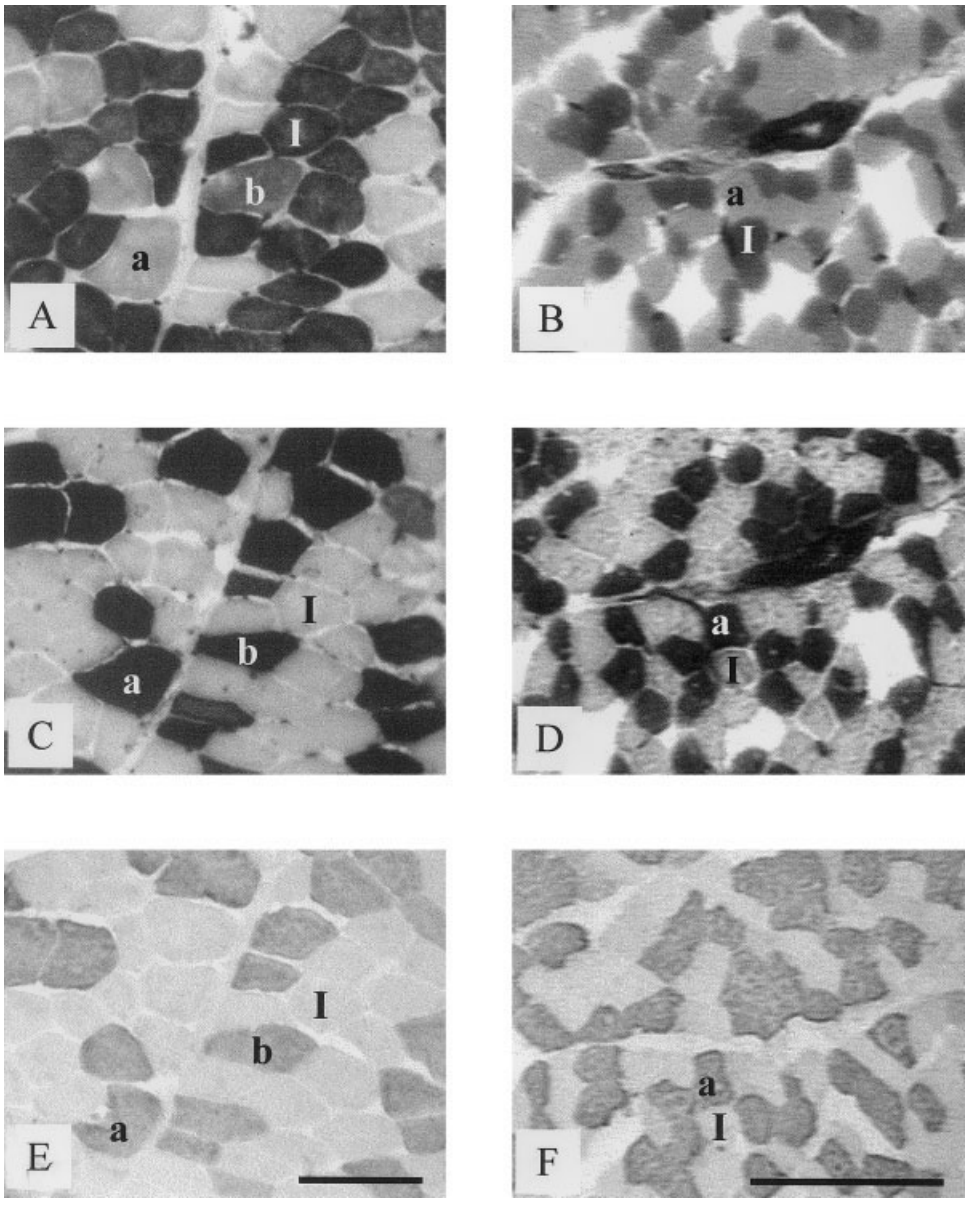

Fig. 2. Representative cross-sections of the diaphragm muscle from bottlenose dolphins (*Tursiops truncatus*) after histochemical and immunocytochemical staining. The diaphragm was stained for myosin ATPase activity after acidic (**A,B**) and alkaline (**C,D**) preincubation, and for its reaction to anti-fast myosin antibody (**E,F**). **A,C,E:** Adult muscle (VMSM 20001049). **B,D,F:** Neonatal muscle (WAM 550). Scale bar = 100 μm. The labels I (type I, slow-twitch), a (IIa, fast-twitch oxidative glycolytic), and b (IIb, fast-twitch glycolytic), indicate the same fibers on each of the three images for the adult and neonatal muscle.

Developmental Index

To compare the developmental state of the dolphin diaphragm to those of other mammals, values of the muscle development index of Dearolf et al. (2000) were calculated. For the dolphin diaphragm, the mean fiber number percentages (adult and neonate) reported in this study were used, and for the diaphragms of other mammals, values reported in the literature (Keens et al., 1978; Maxwell et al., 1983; Sieck et al., 1991; Finkelstein et al., 1992; Watchko and Sieck, 1993; Cobb et al., 1994a,b; Fratacci et al., 1996) were utilized. Neonatal fiber-type profiles taken from the literature were averages calculated from specimens less than 2 weeks of age, and adult profiles were calculated from specimens either identified as adults by the study they were taken from (Maxwell et al., 1983; Sieck et al., 1991; Finkelstein et al., 1992; Watchko and Sieck, 1993; Cobb et al., 1994a; Fratacci et al., 1996), or from sexually mature specimens (Keens et al., 1978). If more than one set of adult and neonatal fiber-type profile data were available for the same species, index values were calculated for each dataset and then an average of the index values was used (rats: Watchko and Sieck, 1993; Fratacci et al., 1996). The index was calculated by dividing the percent slow-twitch fibers in the neonatal muscle by the percent slow-twitch fibers in the adult muscle. The muscle development indices were then plotted with descriptors of the overall developmental states of the neonatal mammals, based on Eisenberg's (1981) values of degree of precociality. In a review of muscle development from a broad range of taxa, it was discovered that no altricial developer had muscles with developmental indices greater than 70% (Dearolf et al., 1998, 2000). Thus, a value of 75% was chosen to define a well-developed muscle.

RESULTS

Muscle Histochemistry and Immunocytochemistry

The adult diaphragms contained predominantly type I fibers (Fig. 2), and the mean fiber-type profile

TABLE 2. Fiber-type percentages (± one SD) and ratios of type I (slow-twitch) to type II (fast-twitch) diameters in adult and neonatal bottlenose dolphin (Tursiops truncatus) diaphragms

Specimen	% Type I fibers by area	% Type I fibers by count	Dia I/II
Neonate category[a]			
KMT 062	26	38	0.86
VMSM 20001020	25	36	0.62
NC98-079	26	35	0.79
CALO 99-13	25	26	0.93
VMSM 991086	26	42	0.72
VMSM 20001031	22	27	0.79
Mean	25 (1.5)	34 (6.3)	0.78 (0.1)
Adult category[a]			
VMSM 20001049	52	64	0.81
JLH 001	52	59	0.76
PTM 047	52	66	0.73
NEFSC 5451	56	64	0.94
WAM 559	72	76	0.82
WAM 545	62	68	1.00
Mean	58 (8.0)	66 (5.7)	0.84 (0.1)

[a]Neonate category (<132 cm and floppy dorsal fins and tail-flukes); adult category (>200 cm).

for all five adults was 58% (±8.0 [SD]) slow-twitch by area and 66% (±5.7) slow-twitch by number (Table 2). In comparison, the diaphragms of neonatal dolphins were composed mainly of type II fibers (Fig. 2). The mean fiber-type profile for all five neonates was 25% (±1.5) slow-twitch fibers by area and 34% (±6.3) slow-twitch fibers by number (Table 2). Thus, neonatal dolphins had significantly fewer slow-twitch fibers, both by area ($P \ll 0.05$) and by number ($P \ll 0.05$) in comparison to adults. In addition, the two assessments of fiber-type profile, the area and number fiber-type profiles, were found to be significantly different in both neonatal ($P = 0.009$) and adult ($P = 0.003$) diaphragms.

To compare the relative diameters of the two fiber-types, a ratio of the diameter of type I to type II fibers was calculated (Table 2). In adults, the average ratio was 0.84 (±0.1), which was not significantly different ($P = 0.37$) from the ratio in neonatal diaphragms (0.79 ± 0.1) (Table 2). In all animals, except for the largest adult (WAM 545), in which the fiber-types were approximately equal in size, type II fibers were larger than type I (Table 2). The larger size of the type II fibers in both adult and neonatal diaphragms led to a greater area of these diaphragms being invested in type II fibers (area percentage fiber-type profiles), although in adults, there were less of these fibers making up the diaphragms (number percentage fiber-type profiles).

Myosin Heavy Chain Isoform Electrophoresis

Both adult and neonatal dolphin diaphragms demonstrated three isoforms subsequent to heavy chain electrophoresis (Fig. 3). One of the bands migrated at the same rate as the type I isoform in rat costal diaphragm described by LaFramboise et al. (1991), and adult and neonatal dolphins differed in their level of expression of this isoform. Adult diaphragms expressed more (darker band) of the type I isoform than the neonatal diaphragms, a result that confirms the histochemical results (Table 2).

The other two dolphin myosin bands migrated differently than the myosin isoforms expressed in rat costal diaphragm. One of the other bands migrated more slowly than the rat type IIa and IIx (fast-twitch intermediate oxidative glycolytic) (Schiaffino et al., 1990) isoforms, while the other migrated more quickly than the rat type I isoform (Fig. 3). Again, adults and neonatal dolphins differed in their expression of these two isoforms. Neonatal dolphins expressed more of the slowest running band (Fig. 3: *), and this adult expressed more of the fastest running band (Fig. 3: +). However, expression of the + band was variable across all specimens, and there was no correlation between the level of expression of this isoform and age.

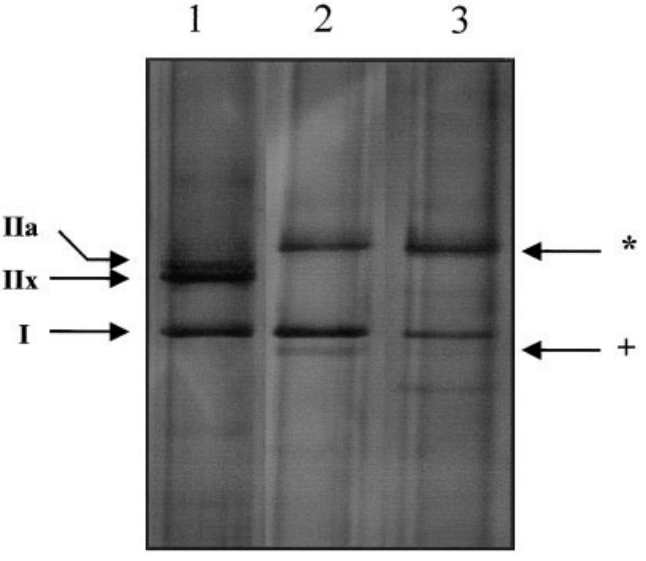

Fig. 3. Six percent SDS-PAGE of myosin heavy chains from the costal diaphragm of a rat (lane 1), an adult (lane 2), and a neonatal (lane 3) dolphin. The three myosin heavy chains labeled in rat diaphragm are shown for comparison with the dolphin samples (see LaFramboise et al., 1991). Adult and neonatal dolphin diaphragms have only one myosin heavy chain isoform that comigrates with an isoform of the rat diaphragm (type I), and they differ in the levels of expression of this isoform. Adult dolphin diaphragm contains more (darker band) type I isoform than the diaphragm of neonates. Dolphins also express two isoforms that do not comigrate with the isoforms found in rat diaphragm (labeled * and +). Neonates express more of the * isoform in their diaphragms, and this adult expresses more of the + isoform. However, expression of the + isoform was variable and showed no correlation with age. Bands below the dolphin + isoform are not considered to represent myosin heavy chain isoforms.

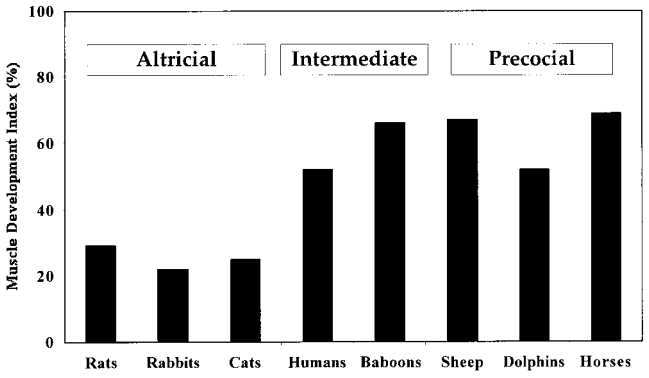

Fig. 4. Developmental states of the diaphragm muscle in selected neonatal mammals as determined by the muscle developmental index of Dearolf et al. (2000). Index values were calculated using fiber-type percentage count data from this study (bottlenose dolphin) and from values reported in the literature (Keens et al., 1978; Maxwell et al., 1983; Le Souef et al., 1988; Sieck et al., 1991; Finkelstein et al., 1992; Watchko and Sieck, 1993; Cobb et al., 1994a,b; Fratacci et al., 1996). If more than one set of adult and neonatal fiber-type profile data were available for the same species, index values were calculated for each dataset, and then an average of the index values was used (rats: Watchko and Sieck, 1993; Fratacci et al., 1996). The index was calculated by dividing the percent slow-twitch fibers by count found in the neonatal diaphragm by the percent slow-twitch fibers in the adult muscle, and the resulting value was reported as a percentage. Note that none of the neonatal mammals investigated has a well-developed (developmental index $\geq$ 75%) diaphragm and that there is a general increase in the developmental state of the diaphragm with the overall developmental state of the neonate (Eisenberg, 1981).

Developmental Index

The neonatal dolphin possessed 52% of the adult profile in its diaphragm and, thus, was similar in developmental state to the diaphragms of neonatal sheep (67%) (Finkelstein et al., 1992) and horses (69%) (Cobb et al., 1994a,b), both precocial terrestrial mammals (Fig. 4). It was also similar to the developmental states of diaphragms of intermediate developers, baboons (66%) (Maxwell et al., 1983) and humans (52%) (Keens et al., 1978). However, the diaphragms of altricial rats (29%) (Watchko and Sieck, 1993; Fratacci et al., 1996), rabbits (22%) (Le Souef et al., 1988) and cats (25%) (Sieck et al., 1991) had lower values of the developmental index in comparison to dolphins (Fig. 4). The plot also demonstrated an increase in developmental state of the diaphragm with an increase in the neonate's overall developmental state (Fig. 4).

DISCUSSION

Bottlenose dolphin neonates have a mean fiber-type area profile of 25% slow-twitch fibers in comparison to 58% in adults (Table 2). In addition, their mean profile by number is also significantly different ($P \ll 0.05$) from that of adults (34% and 66% respectively) (Table 2), and these differences in fiber-type profiles between neonatal and adult diaphragms are confirmed by the results of the myosin heavy chain electrophoretic analysis (Fig. 3). Thus, the diaphragm of neonatal bottlenose dolphins does not express 75% of the adult slow-twitch fiber-type profile and, therefore, is not well developed ("adult-like") at birth. This result leads to the rejection of the hypothesis that the diaphragm of neonatal bottlenose dolphins is well developed.

Developmental State of Neonatal Mammalian Diaphragms

Although the neonatal dolphin diaphragm is not found to be well developed, according to the fiber-type criterion established by Dearolf et al. (2000), it is similar in developmental state to the diaphragms of intermediate and precocial terrestrial neonates (Fig. 4). Interestingly, none of the mammals investigated in this developmental comparison meet the criteria for having a well-developed ("adult-like") diaphragm at birth. This result is surprising and leads to a consideration of the breathing mechanics of young animals. If there are differences in the ventilation behavior of young animals in comparison to their adult counterparts, one or more of these differences might explain the disparity in fiber-type profiles seen between these different-aged mammals.

Breathing Mechanics of Neonatal Mammals

The act of ventilation can be described using a large number of variables, including breathing frequency (breaths/min), inspiratory time (sec), expiratory time (sec), tidal volume (ml), etc. (Mortola, 1987). However, neonatal mammals appear to modify only one of these variables, breathing frequency, in order to achieve the greater minute ventilations (ml of O_2/min) that they require to fuel their higher mass specific metabolic rates in comparison to adults (Mortola, 1984). Thus, the breathing mechanics of the neonatal mammals in this study are compared using this variable.

All of the neonatal mammals in this study, except for the rabbit, breathe faster than their adult counterparts (Table 3). For example, neonatal horses breathe 30–40 times per minute, while adults only

TABLE 3. Breathing frequencies of selected adult and neonatal mammals

Species	Neonatal breathing frequency	Adult breathing frequency	Reference
Rats	109	91	Mortola and Noworaj, 1985
Rabbits	76	79	Mortola and Noworaj, 1985
Cats	80	26	Mortola and Noworaj, 1985
Humans	43	13	Mortola and Noworaj, 1985
Sheep	50	42	Terra, 1990
Dolphins	3.8	2.6	Mann and Smuts, 1999
Horses	35	16	Smith, 1990

breathe 12–20 times per minute (Smith, 1990). Thus, on average, neonatal horses are breathing at 1.9 times the rate of adults. In comparison, neonatal dolphins are breathing at 1.5 times the rate of their adult counterparts (Mann and Smuts, 1999). To relate the relatively high breathing frequencies exhibited by neonates to the developmental state of their diaphragms, the fiber-type profile data must be analyzed in a different way.

The muscle developmental index of Dearolf et al. (2000) relies on differences in the percentages of slow-twitch fibers between the fiber-type profiles of neonatal and adult mammals to evaluate the developmental state of a muscle at birth. This method is based on a developmental pathway that is shared by most muscles, including ventilatory muscles (Dearolf et al., 2000). In brief, most muscles progress through three developmental states as they mature to their adult fiber-type profiles, "undifferentiated," fast, and mixed, and muscles differ in where they are along this pathway when the neonate is born. During this maturation process, muscles gain histochemically identifiable slow-twitch fibers. Thus, Dearolf et al. (2000) base their developmental index on the percentage of the adult slow-twitch fiber-type profile that a neonatal muscle expresses.

However, this method may not be the most appropriate way to examine the developmental state of breathing muscles. These muscles are not functioning in an adult-like manner at birth, and may, in fact, be contracting faster than the muscles in adults in order to power the high breathing frequencies seen in neonatal mammals. Thus, it is more appropriate to investigate the percentage of fast-twitch fibers in neonatal ventilatory muscles in comparison to adults, as well as the types of fast-twitch fibers found in neonatal and adult muscles.

Fast-Twitch Fiber-Type Profile Comparison

All of the neonatal diaphragms in this study exhibit more fast-twitch fibers than their adult counterparts (Fig. 5). Thus, there appears to be a relationship between the primarily fast fiber-type profile of neonatal diaphragms and their relatively high breathing rates. However, the relationship between these two variables is not strong. For example, dolphins and humans have approximately the same difference in the proportion of fast-twitch fibers between adult and neonatal diaphragms (Fig. 5). However, neonatal humans are breathing at 3.3× the rates of adults (Mortola and Noworaj, 1985), while neonatal dolphins are breathing at 1.5× the rate of their adult counterparts (Mann and Smuts, 1999).

The lack of a strong relationship between the proportion of fast-twitch fibers in neonatal diaphragms and their breathing frequencies may be related to the small scope of this comparative study. The fiber-type profiles of only one ventilatory muscle, the diaphragm, are being compared. In addition, although

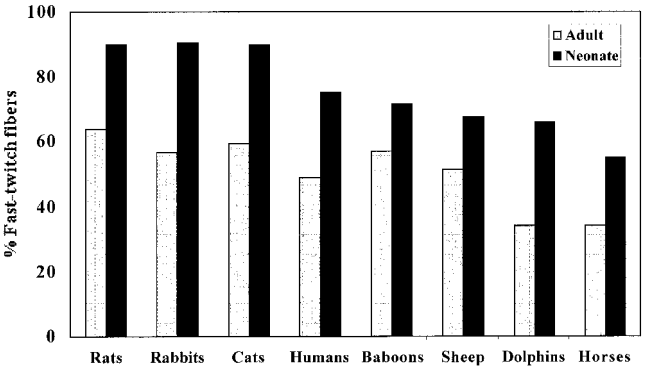

Fig. 5. Percent fast-twitch fibers in the diaphragms of selected neonatal (black bars) and adult (gray bars) mammals. Values of percent fast-twitch fibers by count were taken from this study (bottlenose dolphin) and from the literature (Keens et al., 1978; Maxwell et al., 1983; Le Souef et al., 1988; Sieck et al., 1991; Finkelstein et al., 1992; Watchko and Sieck, 1993; Cobb et al., 1994a,b; Fratacci et al., 1996). When two values were available for the same species, means of these values were used (rats: Watchko and Sieck, 1993; Fratacci et al., 1996). Note the larger percentage of fast-twitch fibers in the diaphragms of all the neonatal mammals in comparison to their adult counterparts.

mean breathing frequency varies between neonates and adults, the breathing behaviors of neonatal mammals are highly variable (Mortola, 1984). Thus, by focusing on the mean differences between the resting breathing frequencies of neonatal and adult mammals, a whole range of behaviors that might be better correlated to the differences in fiber-type profiles seen between adult and neonatal diaphragms could be missed.

Muscle Contraction Speeds and Fast Fiber-Types

The majority of the available data on muscle contraction speeds do not support the relationship between the fast-twitch histochemical profile of the neonatal diaphragm and the faster breathing frequencies of neonatal mammals (Table 3). In most of the neonatal mammals that have been investigated (rats: Martin-Caraballo et al., 1988; Johnson et al., 1994; rabbits: Moore et al., 1993; cats: Sieck et al., 1991), the contraction speeds of neonatal diaphragm fibers are slower than those of adults. Thus, although the histochemical profile of most of the fibers in the diaphragms of these neonates is fast, the fibers are not functioning like adult fast-twitch fibers.

The discrepancy between the histochemical reaction of neonatal muscle fibers and their functional capabilities is attributed to the presence of type IIc or "undifferentiated" fibers in neonatal muscle (Maxwell et al., 1983). Type IIc fibers demonstrate the histochemical reaction of adult fast-twitch fibers (acid preincubation) but express neonatal and sometimes fetal forms of myosin (Whalen et al., 1981). The presence of neonatal isoforms has been shown to

correlate with slower force–velocity relationships compared with adult myosin heavy chain isoforms (Reiser et al., 1985; Johnson et al., 1994). Therefore, neonatal muscles with large percentages of type IIc fibers would be expected to have slower contraction velocities than adult muscles and, conversely, neonatal muscles with slow contraction speeds would be expected to have large percentages of type IIc fibers. As predicted, the diaphragms of the neonatal mammals found to have slow contraction speeds contain a large percentage of type IIc fibers (rats: 88% [Watchko and Sieck, 1993; but see Fratacci et al., 1996]; cats: 90% [Sieck et al., 1991]; and rabbits: 40% [Le Souef et al., 1988]).

The diaphragms of neonatal dolphins have no type IIc fibers (this study) and those of horses have only 3% type IIc fibers at birth (Cobb et al., 1994b). Thus, little to no neonatal myosin isoform is being expressed in the diaphragms of these neonates, a conclusion that is supported by the electrophoretic analyses of myosin heavy chains in both species (Fig. 3; Cobb et al., 1994b). Thus, it is hypothesized that the diaphragms of neonatal dolphins and horses contain fibers with adult contraction capabilities and, therefore, that they could contract more quickly because of their greater percentage of fast-twitch fibers relative to adults (Fig. 5).

The results of a study on the contraction capabilities of the diaphragm in neonatal baboons (McCarter et al., 1987) support this hypothesis. In baboons, the fibers of neonatal diaphragms have similar contraction capabilities as those of adults (McCarter et al., 1987). Neonatal baboons have 66% of the adult slow-twitch fiber-type profile (Fig. 4) and only 26% type IIc fibers at birth (Maxwell et al., 1983; McCarter et al., 1987). Thus, the diaphragms of precocial mammals like horses and dolphins, with even smaller proportions of type IIc fibers than in baboon diaphragms, could have contraction capabilities similar to those of adults.

Altricial developers also breathe faster than their adult counterparts (Mortola and Noworaj, 1985), despite the fact that their diaphragms are the least developed, both in terms of the muscle development index (Fig. 4) and the proportion of type IIc fibers that make up their diaphragms (Le Souef et al., 1988; Sieck et al., 1991; Watchko and Sieck, 1993). Although determining the mechanism of ventilation in altricial developers is beyond the scope of this study, it is interesting to note that the diaphragms of these neonates appear unable to power their increased breathing frequencies (Martin-Caraballo et al., 1988; Sieck et al., 1991; Moore et al., 1993; Johnson et al., 1994). No study has addressed the mismatch between the contraction speeds of the diaphragm and the breathing frequencies of these young mammals. Thus, to date, it is unknown how altricial developers power their high breathing frequencies.

All of these findings serve to support the correlation between the overall developmental state of the neonate as determined by Eisenberg (1981) and the developmental state of its diaphragm (Fig. 4). Altricial developers have only 22–29% of the adult fiber-type profile in their diaphragms and a large percentage of type IIc fibers (Le Souef et al., 1988; Sieck et al., 1991; Watchko and Sieck, 1993). Intermediate developers have a greater percentage of the adult fiber-type profile than altricial neonates (mean, 59%) (Fig. 4), but also have a greater percentage of type IIc fibers than precocial mammals (Maxwell et al., 1983). Finally, precocial neonates have a similar percentage of the adult fiber-type profile (mean, 63%) as intermediate developers, but also possess the smallest percentage of type IIc fibers (0–3%) (Cobb et al., 1994b).

CONCLUSION

In conclusion, the neonatal dolphin diaphragm is found to be immature, according to the criterion of having an "adult-like" fiber-type profile, as determined by the developmental index of Dearolf et al. (2000). It is demonstrated that the neonatal dolphin diaphragm is not unique in having a fiber-type profile that differs from that of the adult (Fig. 4). However, there is a generally positive relationship between the developmental state of the diaphragm in the neonatal mammals investigated and their overall developmental state (Eisenberg, 1981).

The results of the comparative study led to an investigation of the breathing mechanics, specifically, the breathing frequency of neonatal mammals. The relatively high breathing frequencies of young mammals are found to be related to the greater proportion of fast-twitch fibers in their diaphragms (Fig. 5). However, the relationship between these two variables is not strong, and it is also not supported by the majority of the available data on muscle contraction speeds (Martin-Caraballo et al., 1988; Sieck et al., 1991; Moore et al., 1993; Johnson et al., 1994).

The fibers of the diaphragms of altricial developers cannot contract as quickly as those of adults, and their slower contraction speeds are correlated with the presence of type IIc fibers. Because the diaphragms of precocial developers (horses and dolphins) contain few to no type IIc fibers, it is concluded that the fibers of their diaphragms will have adult contraction capabilities, and this hypothesis is supported by contraction speed data from neonatal baboon diaphragms (McCarter et al., 1987). Thus, the diaphragms of precocial neonates, like the horse and dolphin, should have the ability to power the increased breathing frequencies seen in these young animals.

The diaphragms of neonatal horses and dolphins were not found to be well developed, according to the

adult-like criterion. However, these neonatal muscles must have the contraction abilities of adult muscle, because they have to contract quickly to power the elevated breathing frequencies of horse and dolphin neonates. These results lead to the modification of the criterion for evaluating the developmental state of a muscle at birth. The developmental state of a neonatal muscle should be based on both its value of Dearolf et al.'s (2000) developmental index, as well as the percentage of type IIc fibers found in that muscle. Therefore, precocial mammals do have "well-developed" diaphragms in comparison to altricial developers, because they have a greater proportion of the adult slow-twitch profile than altricial neonates and a very small percentage (0–3%) of type IIc fibers.

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