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Neuronal Fiber Composition of the Corpus Callosum Within Some Odontocetes

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ABSTRACT

Odontocetes (toothed whales) evolved from terrestrial mammals approximately 55 million years ago and have since remained on a unique evolutionary trajectory. This study used formalin-fixed tissue and light microscopy to quantify the size and number of fibers along the corpus callosum of the bottlenose dolphin (n = 8). Two other species, the Amazon River dolphin (n = 1) and the killer whale (n = 1), were included for comparison. A large amount of variation in the shape and area of the corpus callosum was observed. The odontocete corpus callosum is a heterogeneous structure with variation in fiber size and density along the length of the corpus callosum in all specimens examined. Using the species with the largest sample size, the bottlenose dolphin, comparisons by sex and age (sexually mature verses immature) were made for the area of the corpus callosum, five subregions, and fiber densities. Although no sex differences were detected, age appeared to affect the size, shape, and fiber composition of the bottlenose dolphin corpus callosum. Anat Rec, 291:781–789, 2008. © 2008 Wiley-Liss, Inc.†

Key words: dolphin; *Tursiops*; brain; corpus callosum

Modern cetaceans evolved from terrestrial mammals that re-entered the seas approximately 55 million years ago, followed by an expansive adaptive radiation beginning 30–35 million years ago (Fordyce, 2002; Arnason et al., 2004). Since diverging from hippopotamid artiodactyls, cetaceans have been on a distinct evolutionary trajectory. The pressures of the marine environment have led to numerous adaptations, including loss or reduction of limbs, telescoping of the skull, and development of echolocation. Additionally, odontocete brains have several remarkable features compared with other mammals of similar size, including significantly larger brain size relative to body size (Marino et al., 2004); highly convoluted cerebral and cerebellar cortices (Striedter, 2003); reduced pyramidal tract of the medulla (Glezer, 2002); and a smaller corpus callosum area relative to brain mass (Tarpley and Ridgway, 1994). Of the cetaceans, the largest relative brain sizes are found within the Delphinidae, resulting in high encephalization indices in extant species (Ridgway and Brownson, 1984; Worthy and Hickie, 1986; Marino, 1998). Ridgway (2002) suggests that the reduction of the corpus callosum in cetaceans is associated with unihemispheric slow wave sleep (one hemisphere of the brain remains awake while the other is asleep; Serafetinides et al., 1970; Mukhametov and Supin, 1975; Mukhametov et al., 1976, 1977; Mukhametov, 1984, 1987; Oleksenko et al., 1992).

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TABLE 1. Specimen life history information, corpus callosum area (CCA), and corpus callosum area to brain mass ratio (CCA:BM)

Specimen	Sex	Mature	Body length (cm)	Brain mass (g)	CCA (mm^2)	CCA:BM
Orcinus orca						
OoA	M	M	–	6052.0	534.98	0.088
Inia geoffrensis						
IgB	M	M	–	630.0	139.27	0.221
Tursiops truncatus						
TtC	F	M	256	1606.2	233.67	0.145
TtD	M	M	277	1397.5	190.87	0.136
TtE	F	M	252	1609.0	320.17	0.199
TtF	M	M	260	1509.0	287.70	0.191
TtG	F	M	-	1609.3	305.64	0.190
TtH	M	M	260	1320.8	236.45	0.179
TtI	F	I	153	993.0	131.03	0.132
TtJ	M	I	234	1519.3	160.13	0.105

Large diameter fiber sizes have been reported in the auditory (Jacobs and Jensen, 1964), visual (Dawson et al., 1982), and terminal nerve (Demski, et al., 1990) systems of odontocetes. Probably because it is difficult to acquire and fix dolphin brains, ours is the first study to investigate the fiber composition of the corpus callosum of odontocetes. The current study adds to the limited knowledge on the cellular structure of the odontocete brain and provides some basis for functional studies of hemispheric connectivity in dolphins.

Even in terrestrial mammals, few studies have investigated fiber size or number within the corpus callosum (Tomasch, 1954; Berbel and Innocenti, 1988; Nakamura and Kanaseki, 1989; Lamantia and Rakic, 1990; Aboitiz et al., 1992a,b; Kim et al., 1996) and even fewer studies have compared callosal fibers across species (Olivares et al., 2001). Given the small size of the odontocete corpus callosum relative to the large brain size and unique evolutionary history of these odontocetes, we hope the current study will add insight on the evolution of the mammalian brain.

MATERIALS AND METHODS

This study used a total of 10 specimens from two families of odontocetes (Table 1): Iniidae (1) and Delphinidae (9). The iniid representative was *Inia geoffrensis* (Amazon River dolphin). Within Delphinidae, two genera were represented: *Tursiops* and *Orcinus*. The specimens were eight *Tursiops truncatus* (bottlenose dolphin), including six adults (three males, three females) and two juveniles (one male, one female) and one adult of the largest member of the Delphinidae, *Orcinus orca* (killer whale; male).

Whole brains were opportunistically collected during necropsies from odontocetes that died of nonneurological diseases. All brains were removed within 6 hours of death, immediately weighed and immersion-fixed whole in 10% buffered formalin (Fig. 1A). After 24–72 hr of fixation, all brains except the *Inia geoffrensis* were sectioned along the midsagittal plane, exposing the corpus callosum to the fixative (Fig. 1B). The *Inia geoffrensis* brain was fixed whole in 10% buffered formalin for 4 years before it was midsagittally sectioned. Shrinking and changes in brain mass have been reported with increasing fixation time (Frontera, 1959). Within odontocetes, Tarpley and Ridgway (1994) determined brain mass was reduced with increasing fixation time ranging from 1.6–7.8%. To assess the impact of the increased fixation time, the *Inia* brain was re-weighed before midsagittal sectioning for this study and compared with the brain weight taken before fixation. The extended fixation time resulted in a change in brain mass of 1.62%. Given the small change in brain mass and the high quality of tissue preservation as demonstrated by an magnetic resonance imaging (MRI) scan (data not shown), the *Inia* brain was included in this study.

Because of the length of time preserved and diffusion of the fixative, thin-diameter fibers would have been lost during passive fixation resulting in an underestimation of the total number of fibers and the number of the smaller fiber categories (Olivares et al., 2001). Our observations suggest that fiber sizes down to the limit of practical resolution with light microscopy were preserved in all specimens. To reduce the loss of fibers, every attempt was made to use brains of high quality and to reduce the time between death and fixation of the corpus callosum. This study used similar fixation and light microscopy methods that have been successful in determining fiber size and number within the corpus callosum of other large brained mammals including humans (Aboitiz et al., 1992a,b; Aboitz, 1992) and cows and horses (Olivares et al., 2001).

After fixation and before the removal of the corpus callosum, a digital photograph was taken of the brain sectioned along the midsagittal plane (Fig. 1B). Each brain was also photographed with a centimeter scale positioned in the same midsagittal plane allowing the area of the corpus callosum to be calculated. The photographs were analyzed with ImageJ freeware from NIH (Rasband, 1997–2007) and all measurements of area were performed by the same rater in triplicate and averaged. Midsagittal area of the corpus callosum was determined according to the methods of Tarpley and Ridgway (1994). These photographs were used to partition the corpus callosum into subregions using methods described by Clarke and Zaidel (1994) and Olivares et al. (2000). Briefly, the corpus callosum was subdivided into halves, thirds, and the posterior fifth based on fractions of the maximum anterior–posterior length for a total of five subregions (Fig. 2A).

A 2- to 3-mm-thick section of the entire length of the corpus callosum was removed, paraffin embedded, and

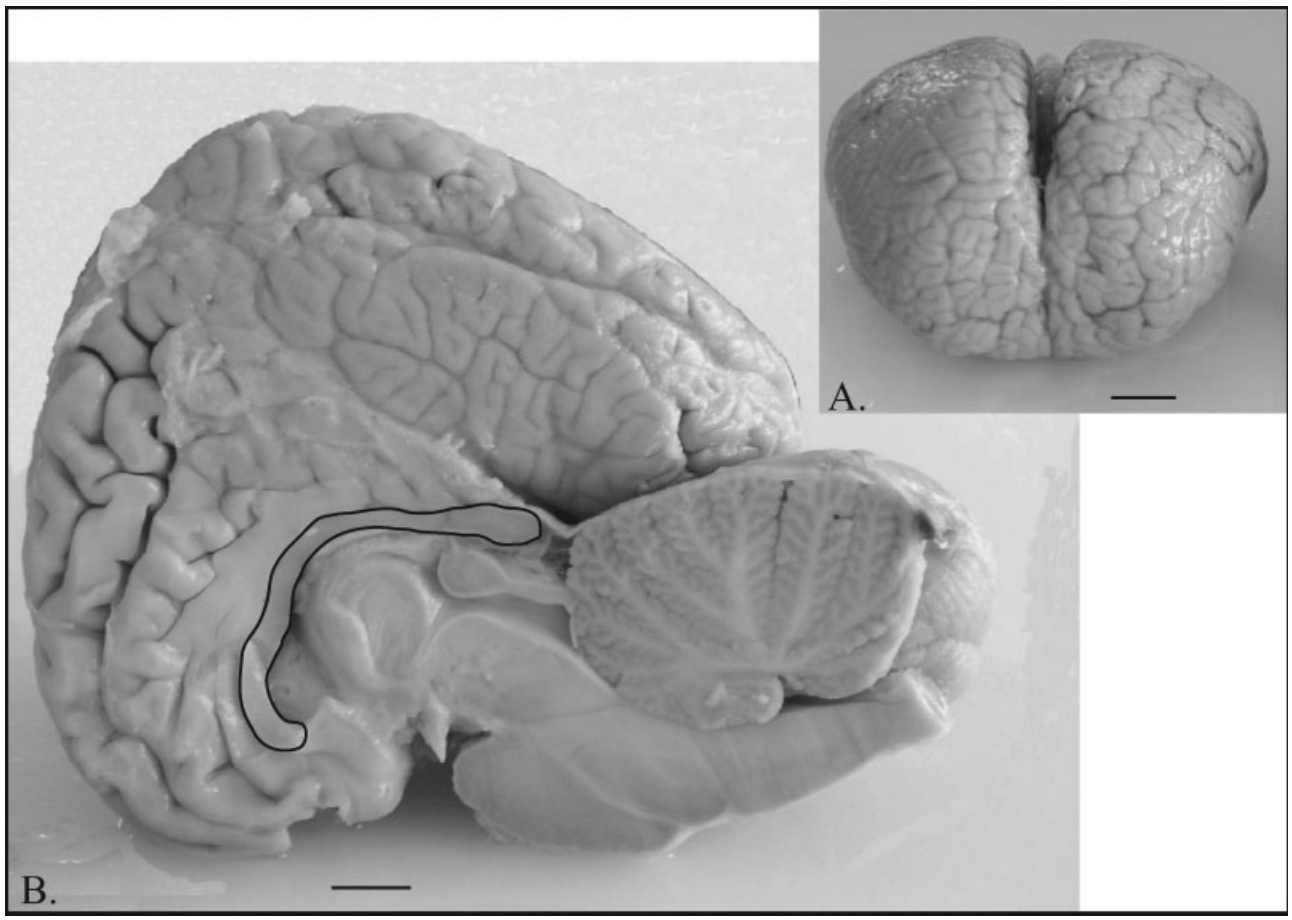

Fig. 1. **A:** Anterior view of a whole brain from a *Tursiops truncatus* (bottlenose dolphin). **B:** Brain from a *Tursiops truncatus* (TtE) midsagittally sectioned exposing the corpus callosum outlined in black. Scale bar = 1 cm.

sectioned at 4–6 μm along the sagittal plane. Slides were deparafinized and stained with Luxol Fast Blue stain following standard histological techniques (Prophet et al., 1994). The slides were examined using an Olympus BX41 microscope with a ×60 high dry objective. Color images were captured with an Optronics Macrofire digital camera from 10 loci equally spaced along the midsagittal plane of the corpus callosum based on the maximum anterior–posterior length (Fig. 2B). A 10 × 10 grid was superimposed over the image and every distinguishable fiber was measured. The area of each counting field was 304 μm × 304 μm.

The criterion used to identify fibers was the presence of a pink fiber within a complete or nearly complete blue circle (myelin sheath). Figure 3 is a section stained with Luxol Fast Blue showing the myelin sheath (blue) and the nerve fibers and support cells (pink). Three example fibers are outlined in black boxes. Fibers were divided into four categories (0.4–0.99 μm; 1.0–2.99 μm; 3.0–4.99 μm; >5.0 μm) based upon maximum diameter. There is a limit to light-microscopic resolution at approximately 0.4 μm. Therefore, as with previous studies on mammals with relatively large brains (Aboitiz et al., 1992a,b; Olivares et al., 2001), the population of fibers less than 0.4 μm could not be taken into account. Fiber measure-

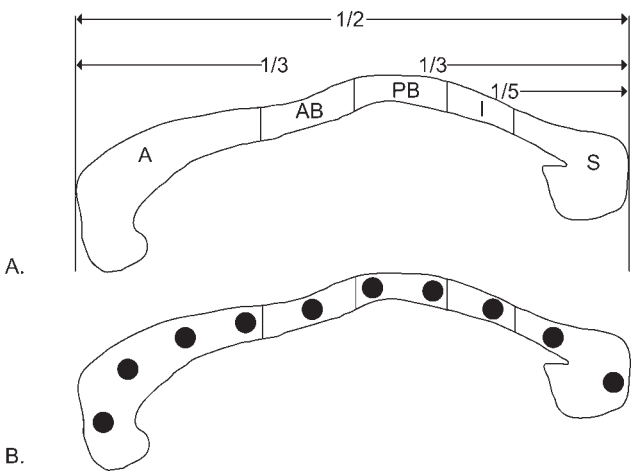

Fig. 2. **A:** Schematic showing the corpus callosum divided into the following subregions: (A) anterior one-third, (AB) anterior midbody, (PB) posterior midbody, (I) isthmus, and (S) splenium. The anterior midbody and posterior midbody make up the middle one-third and the isthmus and splenium make up the posterior one-third. **B:** Schematic of the 10 loci equally spaced along the midsagittal plane of the corpus callosum based on the maximum anterior–posterior length.

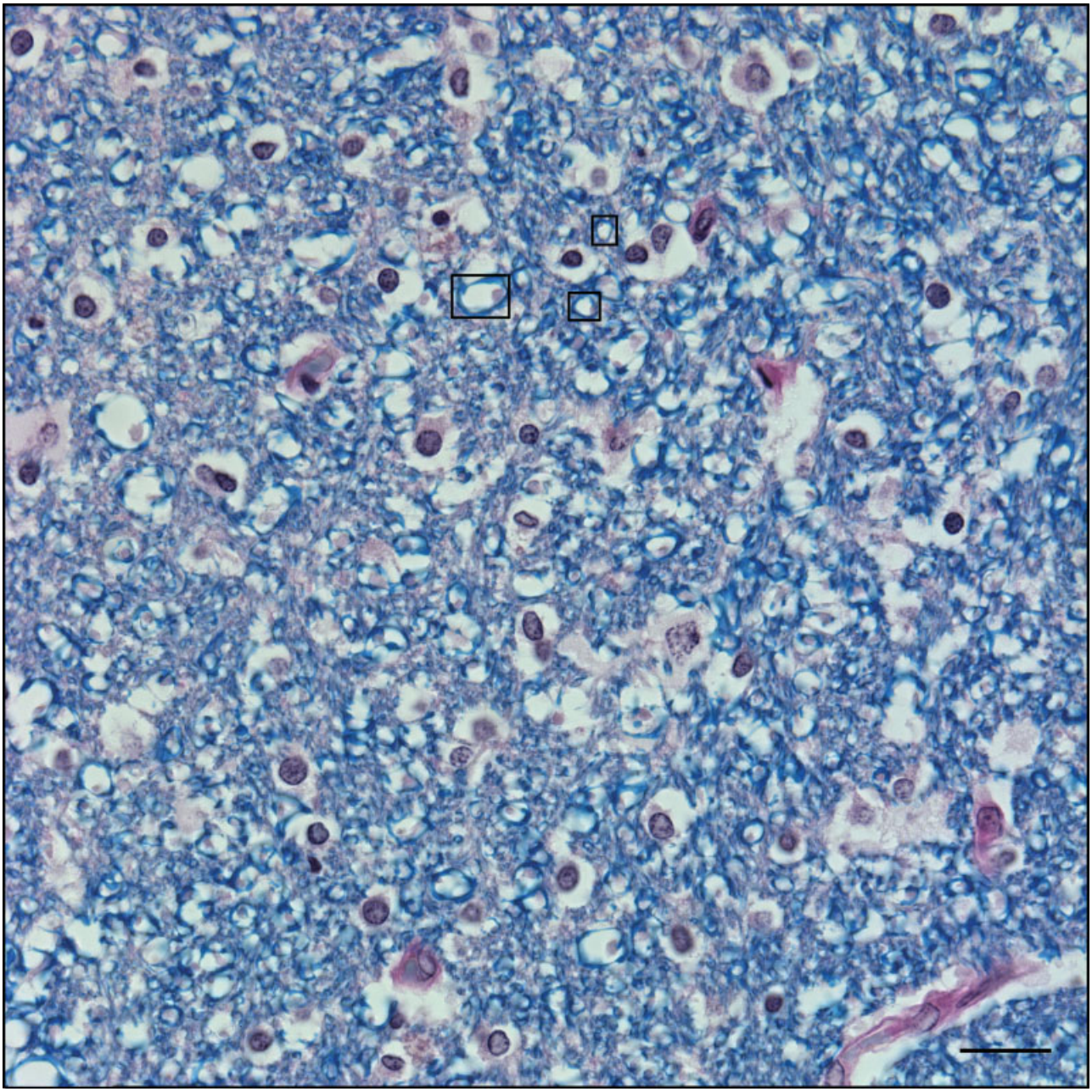

Fig. 3. A section stained with Luxol Fast Blue showing the myelin sheath (blue) and the nerve fibers and support cells (pink) from TtG. Boxes outline three fiber examples. Scale bar = 25 μm.

ments were performed twice and were highly reproducible. The first count was accepted when there was less than 10% difference between the two counts.

Statistical Analysis

Statistical analyses were performed using SYSTAT 10 (Systat Software, Inc, Point Richmond, CA). For the adult *Tursiops truncatus*, *t*-tests were used to make comparisons between males and females for brain mass, total callosal area, and total callosal fiber density. To determine the fiber density per callosal region, the calculated area of each region was multiplied by the average fiber density of the region. Fiber density of the adult *Tursiops truncatus* was compared by two-way analysis of variance with sex (male, female) and callosal region (anterior third, anterior midbody, posterior midbody, isthmus, and splenium) as independent variables. Probabilities less than 0.05 were considered significant.

RESULTS

Table 1 shows the brain mass (BM; g), corpus callosum area (CCA; mm^2), and the CCA: BM ratio for all

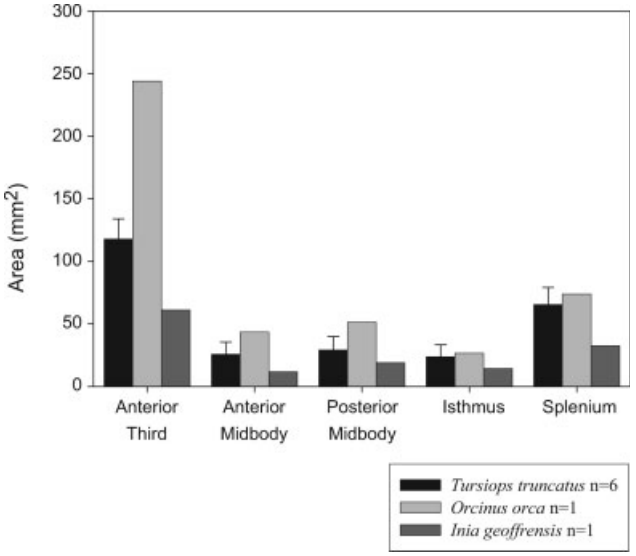

Fig. 4. Area of the callosal subregions (anterior third, anterior midbody, posterior midbody, isthmus, and splenium) of all adult specimens from the three species.

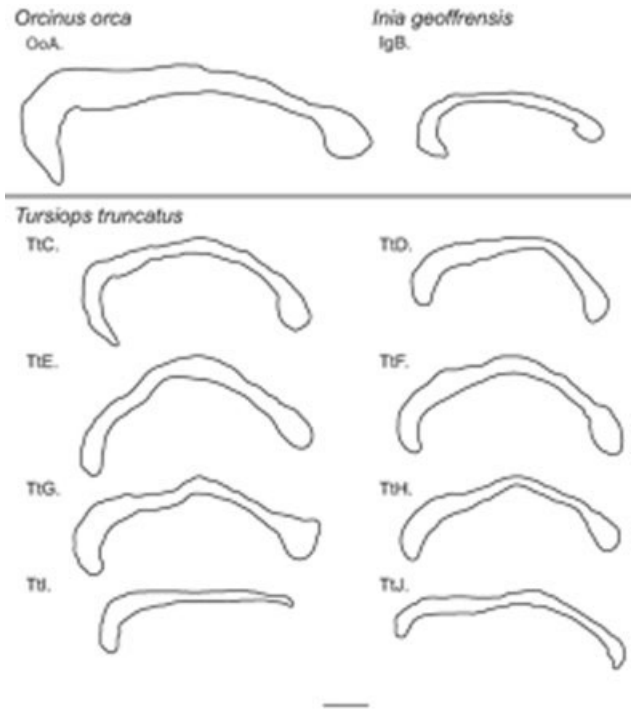

Fig. 5. Outlines of the corpus callosum along the midsagittal plane for all specimens used in this study. *Orcinus orca* (OoA); *Inia geoffrensis* (IgB); Adult *Tursiops truncatus* (TtC, TtD, TtE, TtF, TtG, and TtH); Immature *Tursiops truncatus* (TtI and TtJ). Scale bar = 1 cm.

specimens. Brain mass ranged from 630 g (*Inia geoffrensis*) to 6,052.0 g (*Orcinus orca*) and the CCA:BM ratio ranged from 0.088 (*Orcinus orca*) to 0.221 (*Inia geoffrensis*). Both were within the ranges previously reported for odontocetes by Tarpley and Ridgway (1994).

Figure 4 shows the area of the five subregions of the corpus callosum from adult specimens averaged by species. A large amount of variation exists in the shape and area of the corpus callosum both between and within species (Fig. 5). For each species, the average area of the anterior third accounted for the largest region compared with the middle third (anterior and posterior midbody) and the posterior third (isthmus and splenium). The splenium showed a dramatic increase in area in all adult specimens and often appeared bulbous. This finding is especially striking in the *Orcinus orca* brain (Fig. 5 OaA). It is interesting to note the small area and the apparent lack of bulbous shape of both immature *Tursiops truncatus* specimens (Fig. 5 TtI and TtJ) compared with the adults (Fig. 5 TtC, TtD, TtE, TtF, TtG, and TtH).

Variation in fiber size and density was observed along the length of the corpus callosum in odontocetes. Figure 6 is the Luxol fast blue staining for a portion of each of the 10 loci from one *Tursiops truncatus* (TtH). The distribution of fibers in the adult *Tursiops truncatus*, *Inia geoffrensis*, and *Orcinus orca* specimens showed some similar patterns in all specimens examined. Larger sample sizes, particularly for the *Inia geoffrensis* and *Orcinus orca* are needed to make definitive conclusions about differences across species. The 1.0–2.99 μm fibers were generally the most prevalent fibers and were fairly uniformly distributed along the corpus callosum, while 3.0–4.99 μm fibers tended to increase from the anterior-most region of the corpus callosum until the anterior midbody (Fig. 7). The *Inia geoffrensis* had very few large diameter fibers (>5.0 μm) throughout the entire corpus callosum and had no fibers >5.0 μm in the isthmus or the splenium regions. Fibers of 0.4–0.99 μm and 1.0–2.99 μm represented 38.95 ± 3.2% and 59.00 ± 2.10%, respectively, of the total fibers and remained consistent across all specimens. The greatest amount of variation was found in the large diameter fibers (3.0–4.99 μm and >5.0 μm), which comprised 3.50 ± 3.12% and 2.04 ± 3.05%, respectively.

Brains from the adult *Tursiops truncatus* specimens (n = 6) ranged in mass from 1,320.8 to 1,609.3 g, and the CCA:BM ratio ranged from 0.136 to 0.199. There was not a significant difference in brain mass (df = 4; t = 3.63; $P = 0.068$) between the adult females (1608.17 ± 1.71 g) and males (1409.10 ± 94.63 g), nor was there a difference in the total callosal area (df = 4; t = 1.24; $P = 0.281$) between the adult females (286.49 ± 46.3 mm^2) and males (238.3 ± 48.4 mm^2). There was a significant difference in the area of the subregions by sex (F1, 4 = 4.650; $P = 0.043$). However, there was no difference in fiber density of any fiber categories in the subregions of the corpus callosum (F1, 4 = 0.164; $P = 0.690$).

This study included two sexually immature *Tursiops truncatus*, a 4-month-old female (TtI) and a 4-year-old male (TtJ). While TtJ was considered an immature animal it is important to note that its brain mass (1,519.3 g) was within the range of the adult *Tursiops truncatus* (1,320.8–1,609.3 g). Additionally, TtJ had a similar fiber pattern to those of the adult *Tursiops truncatus* (Fig. 8). The overall patterns in fibers for TtI did not follow the patterns seen in the adults. The fiber densities for small diameter fibers (0.4–0.99 μm and 1.0–2.99 μm) were higher than the densities found in the adults and the 4-year-old male (TtJ). Unlike the adults, TtI showed a continual increase in the smallest fibers (0.4–0.99 μm) along

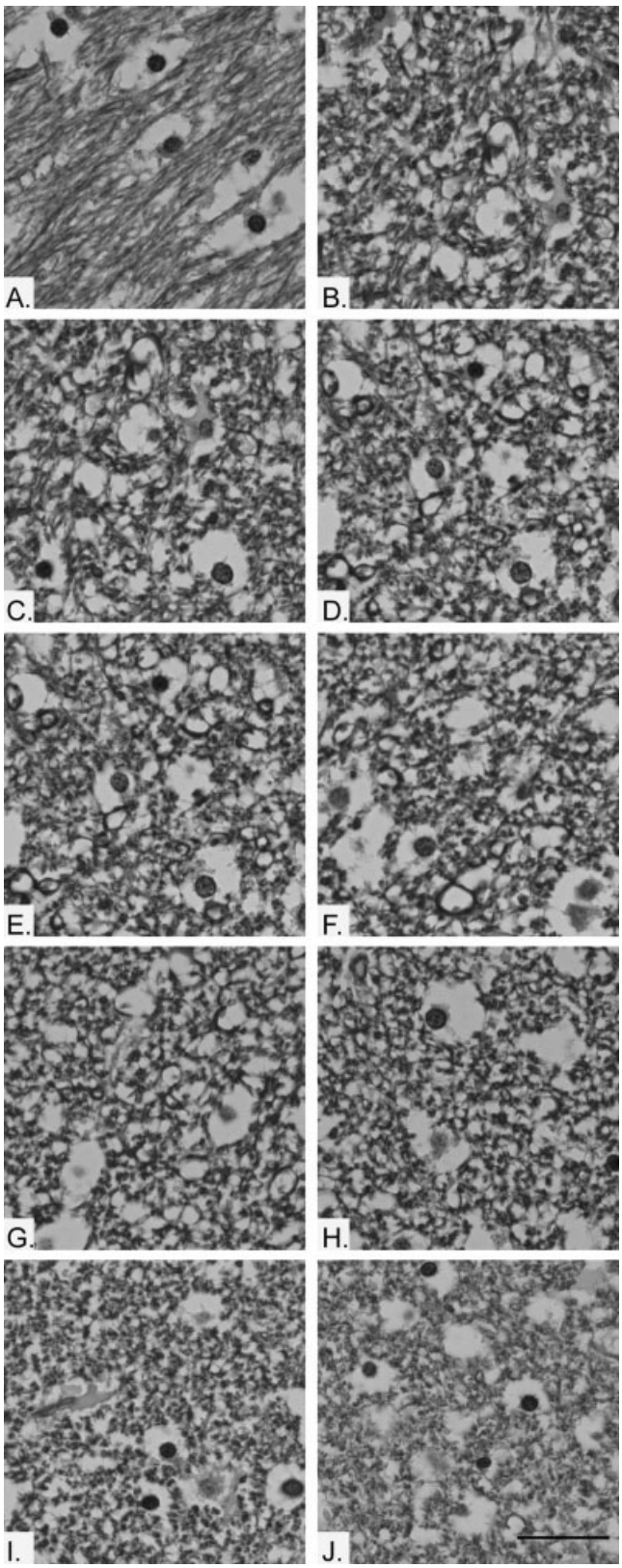

Fig. 6. Luxol fast blue stain for a portion of the 10 loci along the midsagittal plane of the corpus callosum from the anterior (A) to posterior (J) from a *Tursiops truncatus* (TtH). Scale bar = 25 μm.

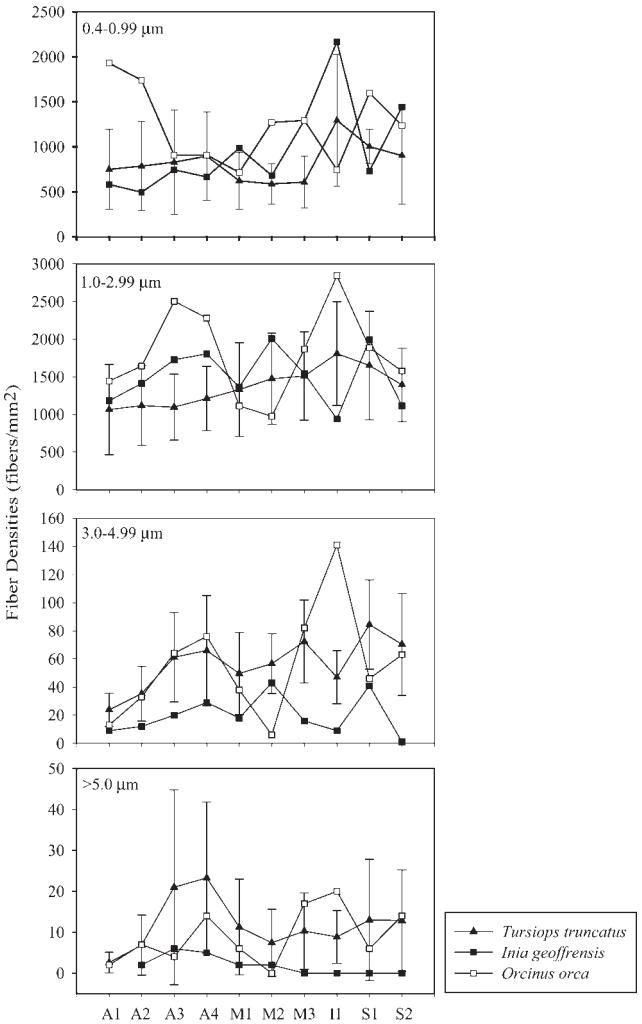

Fig. 7. Average fiber densities per loci (fibers/mm^2) from adult *Tursiops truncatus* (n=6), *Inia geoffrensis* (n=1), and *Orcinus orca* (n=1) for the 10 loci along the midsagittal plane of the corpus callosum for each class of fibers. Subregions are as follows: anterior third (A1, A2, A3, A4); anterior midbody (M1); posterior midbody (M2, M3); isthmus (I), and splenium (S1, S2).

the length of the corpus callosum. Furthermore, TtI had very few fibers >5.0 μm throughout the corpus callosum and showed a fairly continual decrease in the density of fibers between 3.0 and 4.99 μm from anterior to posterior of the corpus callosum.

DISCUSSION

The results of this study show that the corpus callosum of these odontocetes is not a homogeneous structure. There is variation in fiber size and density along the length of the corpus callosum. Studies on terrestrial mammals have determined, to varying degrees, a rough topographic representation of the different cortical areas along the corpus callosum in humans (Abiotz and Montiel, 2003), cats (Imig and Bruge, 1978; Lomber et al., 1994), Mongolian gerbils (Scheich et al., 1993; Pallas, 2001), and rhesus monkeys (Rockland and Pandya,

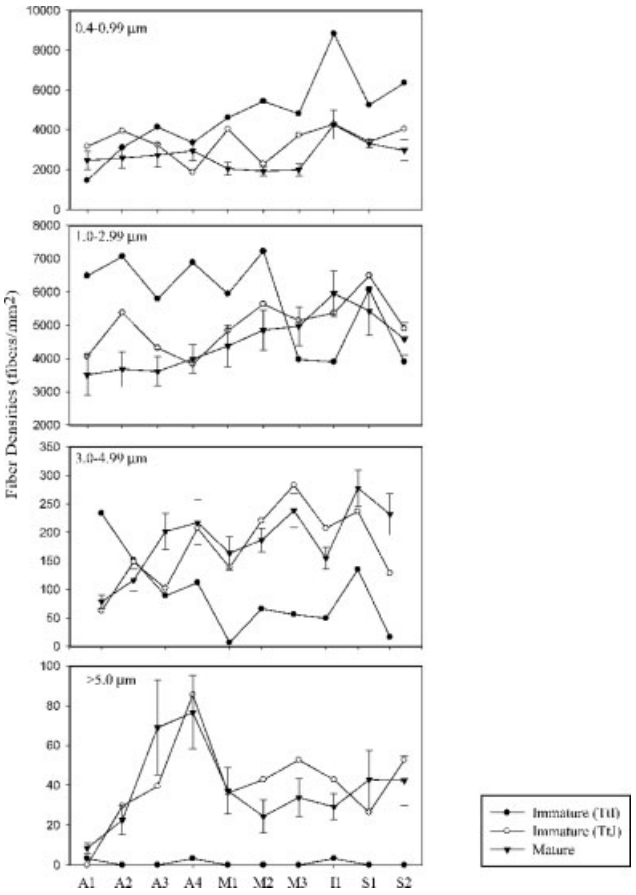

Fig. 8. Fiber densities (fibers/mm^2) for mature (three male, three female) and two immature *Tursiops truncatus* TtI (female, 4 months) and TtJ (male, 4 years) for the 10 loci along the midsagittal plane of the corpus callosum for each class of fibers. Subregions are as follows: anterior third (A1, A2, A3, A4); anterior midbody (M1); posterior midbody (M2, M3); isthmus (I), and splenium (S1, S2).

1986). However, functional maps of the odontocete brain are incomplete (Kesarev and Malofeeva, 1969; Lende and Welker, 1972; Ladygina et al., 1978; Supin et al., 1978; Morgane et al., 1988, 1990; Fung et al., 2005), and to date, there has been no mapping of function across the corpus callosum. Additionally, the primary areas of the cortex that have been identified were mapped to different cerebral lobes in dolphins than terrestrial mammals (Lende and Welker, 1972; Morgane and Jacobs, 1972; Glezer et al., 1992). Therefore, caution should be taken when comparing known functional area of terrestrial mammals and their corresponding location across the corpus callosum and extrapolating it to odontocetes.

The largest population of small diameter fibers (0.4–0.99 μm and 1.0–2.99 μm) was found in the anterior third of the corpus callosum. In other mammalian studies, the anterior corpus callosum connects the frontal lobes and likely plays a role in frontal lobe function (Eacott and Gaffan, 1990). The frontal lobes of the *Tursiops truncatus* have been identified as the primary somatosensory and primary motor functional areas (Kesarev and Malofeeva, 1969; Lende and Welker, 1972; Glezer, 2002).

Previous studies on humans found the largest fiber diameters within the corpus callosum were located in the posterior midbody and splenium which connect the primary and secondary auditory, visual, and motor–spatial systems across the hemispheres (Lamantia and Rakic, 1990; Abiotz et al., 1992b). The concentration of fast-conducting, largediameter callosal fibers in the auditory, visual, and motor systems indicates the importance of merging these systems across the hemispheres. This study found that the largest population of fibers >5.0 μm in the splenium (except *Inia geoffrensis*) and near the interface between the anterior third and anterior midbody, a region of the corpus callosum which is anatomically close to the parietal lobes of the brain. The parietal lobes of *Tursiops truncatus* have been identified as the primary vision and auditory functional areas (Morgane et al., 1988).

Given that odontocetes use vocalizations and echolocation to forage and communicate and that the primary auditory field encompasses a substantial region of the neocortical surface (Ladygina and Supin, 1978; Oelschlager and Oelschlager, 2001; Poth et al., 2005), it seems reasonable that the presence of large-diameter fibers near auditory and visual regions indicates a relatively high degree of connection between the parietal lobes, highlighting the importance of these sensory systems in the odontocete brain. Furthermore, it may be significant that, while the regions of the corpus callosum with the largest diameter fibers are slightly different in humans and dolphins, the functional areas those fibers connect are likely the same, specifically the primary visual and auditory functional areas.

Inia geoffrensis had very few large diameter fibers (3.0–4.99 μm and >5.0 μm) throughout the entire corpus callosum. While the low occurrence of large fibers may be related to the low brain mass (630.0 g), it is interesting to note the complete lack of large diameter fibers (>5.0 μm) in the splenium. Previous studies on terrestrial mammals have shown that fibers crossing through this area connect the primary and secondary visual cortex of the two hemispheres (Houzel and Milleret, 1999; Aboitiz and Montiel, 2003) and have found an increase in large diameter fibers in this region (Kim et al., 1996; Olivares et al., 2001). The lack of these fibers may have functional significance, because *Inia geoffrensis* normally lives in turbid river waters of South America, have small eyes, and are thought to have relatively poor vision.

Age appears to influence the odontocete corpus callosum, resulting in a reduced area, low occurrence of fibers >5.0 μm, and a lack of bulbous shape in the splenium of immature animals. The differences in shape and fiber composition found in the corpus callosum of the immature dolphin brain would result in functional differences between the immature and mature dolphins because there is an established relationship between morphological and functional development of the corpus callosum (Galin et al., 1977; O'Leary, 1980; Quinn and Geffen, 1986). The current findings, while based on only two specimens (TtI and TtJ) suggest that increases in fiber sizes in the corpus callosum are occurring in the brain of *Tursiops truncatus* until the brain reaches full adult size. Although there is considerable individual variation, the average dolphin of this species attains a full brain size around the age of 9 years (Ridgway, 1986). The 4-year-old (TtJ) in this study had a brain that was

apparently near full size with a fiber distribution similar to an adult. However, this specimen's relative callosal area was still small compared with the adults in this study suggesting the possibility of further growth of the corpus callosum.

This relatively long period of development is emphasized by the observation that the overall pattern of fibers in the 4-month-old dolphin (TtI) did not follow the pattern of the adults. As previously described in terrestrial mammals (Innocenti, 1986, 1991), the fiber composition of the juvenile is more uniformly distributed and has larger numbers of small diameter fibers (0.4–0.99 μm and 1.0–2.99 μm). While this pattern is prominent in the 4-month-old female (TtI), the 4-year-old male (TtJ) had a similar fiber composition to the adults. In terrestrial mammals, the proportions of large-diameter fibers in the corpus callosum tend to increase with age (Godlewski, 1991; Aboitiz et al., 1996). Myelination of the human brain continues through childhood and into early adulthood (Brizzolara et al., 1994; Fields, 2005). As the corpus callosum develops, the number of total fibers present is reduced and becomes more heterogenic (Innocenti, 1994). While this sample size is small, it indicates that the corpus callosum of odontocetes may follow a similar developmental pattern found in other mammals. Specifically, that the corpus callosum of odontocetes changes in shape and fiber composition with age, likely the result of the myelination of fibers after birth.

The findings of this study add to the limited knowledge of the cellular components of the odontocete brain and highlight the need for further research in functional maps of the odontocete cortex and across the corpus callosum. Noninvasive investigation of dolphin brain function, previously not feasible, is now possible with modern imaging methods (positron emission tomography and MRI scans) as demonstrated by Ridgway et al. (2006). Studies aimed at applying similar techniques are needed to complete the functional map of the odontocete cortex enabling this and previous studies to be put into context to reveal the extent of functional connectivity between specific areas of the two brain hemispheres of odontocetes.

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