The follicle-sinus complex of the bottlenose dolphin (Tursiops truncatus). Functional anatomy and possible evolutional significance of its somato-sensory innervation

THE FOLLICLE-SINUS COMPLEX OF THE BOTTLENOSE DOLPHIN (*Tursiops truncatus*). FUNCTIONAL ANATOMY AND POSSIBLE EVOLUTIONAL SIGNIFICANCE OF ITS SOMATO-SENSORY INNERVATION.

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Abstract

Vibrissae are tactile hairs found mainly on the rostrum of most mammals. The follicle which is surrounded by a large venous sinus is called "follicle sinus complex" (FSC). This complex is highly innervated by somatosensitive fibers and reached by visceromotor fibers that innervate the surrounding vessels. The surrounding striate muscles receive somatomotor fibers from the facial nerve. The bottlenose dolphin (*Tursiops truncatus*), a frequently described member of the delphinid family, possesses this organ only in the postnatal period. However, information on the function of the vibrissal complex in this latter species is scarce. Recently, psychophysical experiments on the river-living Guiana dolphin (*Sotalia guianensis*) revealed that the FSC could work as an electroreceptor in murky waters. In the present study we analyzed the morphology and innervation of the FSC of newborn (n. = 8) and adult (n. = 3) bottlenose dolphins. We used Masson's trichrome stain and antibodies against neurofilament 200 kDa (NF 200), protein gene product (PGP 9.5), substance P (SP), calcitonin gene related peptide (CGRP) and tyrosine hydroxylase (TH) to characterize the FSC of the two age classes. Masson's trichrome staining revealed a structure almost identical to that of terrestrial mammals except for the fact that the FSC was occupied only by a venous sinus and that the vibrissal shaft lied within the follicle. Immunostaining for PGP 9.5 and NF 200 showed somatosensory fibers finishing high along the follicle with Merkel nerve endings and free nerve endings. We also found SP-positive fibers mostly in the surrounding blood vessels and TH both in the vessels and in the mesenchymal sheath. The FSC of the bottlenose dolphin, therefore, possesses a rich somatomotor innervation and a set of peptidergic visceromotor fibers. This anatomical disposition suggests a mechanoreceptor function in the newborns, possibly finalized to search for the opening of the mother's nipples. In the adult, however, this structure could change into a proprioceptive function in which the vibrissal shaft could provide information on the degree of rotation of the head. In the absence of psychophysical experiments in this species, the hypothesis of electroreception cannot be rejected.

(344 words)

Keywords:

Vibrissae; Whiskers; Follicle-Sinus Complex; Innervation; *Tursiops truncatus;* bottlenose dolphin

Introduction

Vibrissae, also called whiskers, are modified tactile hairs that occur in most mammals except monotremes, anteaters, rhinoceroses, and humans (Cave, 1969; Van Horn, 1970; Chernova, 2006; Muchlinski, 2010). They are mainly located around the muzzle but can be also present in other parts of the head and under the carpus, depending on the species (Fundin *et al.*, 1995; Sarko *et al.*, 2011). Their main function is to convey mechanical (tactile) stimuli to the somatosensory cortex (Woolsey and Van der Loos, 1970). The hair follicle of each vibrissa is surrounded by a large venous sinus, together forming the "Follicle-Sinus Complex" (FSC) (Rice *et al.*, 1986). The presence of vibrissae in Pinnipeds, Odobenids, Sirenids and otters, suggests that their somatosensory function is functional also in the water. However, vibrissae are present only in newborn cetaceans and generally disappear in adults. Therefore the question arises if the vibrissae of very young cetaceans perform a temporary function that is lost within a few weeks after birth, or are just a remnant of a structure that evolution discarded in these mammals.

The morphology and innervation of the vibrissae have been studied extensively in rodents and cats (Rice *et al.*, 1986, 1993; Ebara *et al.*, 2002; Park *et al.*, 2003), and thus our present knowledge of the structure and function of the FSC mostly derives from these species, although efforts have been developed in marsupials (Lyne, 1958; Hollis and Lyne; 1974; Marotte *et al.*, 1992). In general, the FSC of terrestrial mammals consists of epidermal and dermal components. The epidermal parts include the hair bulb, the vibrissal shaft (VS), the inner and outer root sheaths, surrounded by a glassy membrane. The latter separates these components from the dermal parts, that is the mesenchymal sheath (MS) and the venous sinus. The sinus is horizontally divided into a proximal ring sinus (containing the ringwulst and the inner conical body), and a distal cavernous sinus (that contains a large number of trabeculae, filled with venous blood). The last dermal part is the connective tissue capsule that limits the follicle and caps it above the inner conical body with the outer conical body. Finally, the rete ridge collar is a thickening of the epidermis where the VS protrudes (Rice *et al.*, 1986, Ebara *et al.*, 2002).

As mentioned above, marine mammals also develop vibrissae, and a description of their morphology and dimensions in seals and otter has been recently reported in comparison with several terrestrial species (Dougill et al., 2020). Walruses have the highest number of vibrissae (up to 350 on each side), while pinnipeds possess large and richly innervated FSCs, divided in three parts, with up to 1600 axons reaching it (Hyvärinen, 1989, 1995; Hyvärinen *et al.*, 2010; Ling, 1966, 1977; Marshall *et al.*, 2006). In manatees, extensive studies have described the vibrissae, which are spread out on the

muzzle and the body (Reep *et al.*, 1998, 2001; Sarko et al., 2007). Mysticetes have vibrissae in large quantity caudally to the blowhole and on the rostro-lateral sides of the upper and lower jaws with numbers up to 250 in the bowhead whale (*Balaena mysticetus*) (Slijper, 1962; Yablokov and Klevezal, 1964). On the contrary, most adult toothed whales have no facial hair and show 2-10 bilateral rows of vibrissae only during fetal life and the early postnatal period (Yablokov *et al.*, 1972; Ling, 1977; Reidenberg and Laitman, 2009). Toothed whales show fully developed vibrissae only in the early phases of their post-natal life (Czech-Damal *et al.* 2013; Cozzi *et al.*, 2017; Dehnhardt and Hanke, 2017). From morphological comparisons among odontocetes, a classification divided them into four groups based on the development of the FSC (Yablokov *et al.*, 1972). Following this classification, the bottlenose dolphin falls into a group comprising species in which the VS is still present in the early postnatal period but disappears in the majority of adult individuals. This is not the case in river dolphins such as the Guiana dolphin (*Sotalia guianensis*) of which a recent study described the FSC (Czech-Damal *et al.*, 2012). The FSC of this species was renamed *vibrissal crypt* because of its different anatomical structure, characterized by the absence of the VS, hair papilla, clear root sheaths, blood sinus and capsule (Czech-Damal *et al.*, 2012). The FSC lumen is filled with desquamated corneocytes and keratinous fibers, that together may be considered a highly electrically conductive biogel (Czech-Damal *et al.*, 2012), part of an electrosensory system that facilitates the hunt of small bottom-living prey in turbid water, where echolocation is not possible or potentially not efficient enough, by detection of their electric field (Czech-Damal *et al.*, 2012).

The somatosensory innervation of mystacial vibrissae is provided by three subdivisions of the maxillary branch of the trigeminal nerve. The deep vibrissal nerve, originates directly from the infraorbital nerve, supplies a single FSC, penetrates the capsule and arborizes dorsally at various levels. The superficial vibrissal nerves (SVNs) come from superficial cutaneous nerves and supply several FSCs. Small- to fine-caliber nerve fiber branches reach the FSC from the base and supply the hair papilla and hair bulb (Rice *et al.*, 1986; Ebara *et al.*, 2002). The deep vibrissal nerve ends in mechanoreceptors such as Merkel nerve endings (MNEs), lanceolate endings and free nerve endings (FNEs) along the follicle. The SVNs, instead, provide innervation to lanceolate endings at the level of inner conical body and MNEs at the level of the rete ridge collar (Fundin *et al.*, 1997a; Ebara *et al.*, 2002). The somatomotor innervation is provided by motoneurons placed in the lateral part of the facial nucleus and innervates the extrinsic (mimic) and intrinsic musculature of the mystacial pad (Haidarliu *et al.*, 2010; Herfst and Brecht, 2008). The visceral innervation (sympathetic and parasympathetic) regulates blood flow in the FSC, supplied by the deep vibrissal artery, and consequently regulates blood pressure, which is essential for the activation of receptors that respond to specific stimulation thresholds. (Fundin *et al.*, 1997b, Maklad *et al.*, 2004).

Here we describe the FSC in a series of postnatal and adult bottlenose dolphins, aiming at characterizing the changes in the anatomy and morphology of this structure at different life stages by histochemical and immunohistochemical techniques. Special attention was dedicated to the innervation of the FSC, the nature of the nerve fibers, and its functional potential.

Material and methods

a) Animals

The samples of vibrissae from 11 bottlenose dolphins (*Tursiops truncatus*, Montagu 1821) were obtained from the Mediterranean Marine Mammal Tissue Bank (MMMTB, http://www.marinemammals.eu), housed in the Department of Comparative Biomedicine and Food Science (BCA) of the University of Padova. The MMMTB is a CITES recognized institution (IT 020) that collaborates with the Italian Ministry of the Environment. The MMMTB collects, processes, and stores samples of tissues of various cetacean species that stranded along the Italian coastline since 2000. Additional samples derived from marine mammals that died at marine theme parks and aquaria, and whose bodies were delivered to BCA for diagnostic post-mortem. More details of the specimens used in this study can be seen in Table 1.

b) Sample processing

Each sample was obtained by carving out around the VS on both sides of the rostrum in the newborn and around the dimple containing the orifice in the adult (Figure 1a, b).

The samples were fixed by immersion in 4% neutral buffered paraformaldehyde and stored at 4 °C. Tissues for Masson's trichrome were then included in paraffin and cut in 5 µm- and 10 µm-thick sections either longitudinal or transversal to the main axis of the FSC by use of a rotatory microtome (Leica, Germany). Sections were mounted on gelatinized slides and air dried. Samples bound for immunocytochemistry were washed in standard phosphate buffer solution (PBS) overnight at 4 °C, stored in PBS containing 0.1% Na-azide and sucrose at 30%, immersed in OCT Compound (Tissue Tek, Sakura Finetek Europe, NL) and frozen at -80 °C in isopentane cooled with liquid nitrogen. 25 µm-thick sections of the longitudinal and transversal planes were subsequently taken with a cryostat (Leica, Germany).

Table 1: Origin of specimens

ID	Species	Sex	Age class	Origin
# 83	*T. truncatus*	M	Newborn	Died in a marine theme park
# 114	*T. truncatus*	M	Newborn	Died in a marine theme park
# 123	*T. truncatus*	F	Newborn	Died in a marine theme park
# 124	*T. truncatus*	M	Newborn	Died in a marine theme park
# 144	*T. truncatus*	M	Newborn	Died in a marine theme park
# 145	*T. truncatus*	M	Newborn	Died in a marine theme park
# 162	*T. truncatus*	M	Newborn	Wild

# 229	*T. truncatus*	M	Newborn	Died in a marine theme park
# 146	*T. truncatus*	M	Adult	Died in a marine theme park
# 159	*T. truncatus*	M	Adult	Died in a marine theme park
# 444	*T. truncatus*	M	Adult	Wild

c) Histological techniques:

The morphology of the FSC was stained using a Masson's trichrome staining protocol. Briefly, the sections were immersed in 3 baths of xylene for 5 minutes each and subsequently hydrated with a descending series of graded alcohol solutions (100 %, 95 %, 90 %, 80 %, 70 %, 50 %). Then, they were stained with Mayer's Emallume for 5-10 minutes and rinsed with tap water. Later, the sections were colored for 5 minutes in a solution of distilled water (300 ml) containing Ponceau 2R (0,2 g), acid fuchsin (0,1 g) and acetic acid (0,6 ml). After rinsing with a 1% acetic acid solution, the sections were put in a solution of distilled water (100 ml), phosphomolybdic acid (3-5 g) and orange G (2 g), for 5 minutes, and rinsed again in an acetic acid solution. The sections were then colored for 5 minutes in light green (0,1-0,2 g in 100 ml distilled water) and acetic acid (0,2 ml). After the last rinsing in a 1% acetic acid solution, the slides were dehydrated directly with absolute alcohol, and passed in xylene (3 x 3 minutes) and coverslipped with Entellan (Merck, Damstraldt, Germany).

The innervation of the FSC was characterized with immunocytochemistry, either *via* immunoperoxidase (IP) or immunofluorescence (IF), using the neuronal markers shown in Table 2.

For IP staining, contiguous sections were initially immersed in a 0,4% solution of Triton X-100 (Merck, Darmstadt, Germany) in PBS at 4 °C for 24 hours. They were then rinsed in PBS baths for 3 x 10 minutes Next, sections were treated with 1% H_2O_2 in PBS for 30 minutes. After three 10-minute washes in PBS, a 3% solution of normal goat serum (NGS, Sigma G-9023, Saint Louis, Missouri, USA) was applied for 2 hours, at room temperature. Thus, sections of each sample were incubated in a wet chamber for 48 hours, at 4 °C with the primary antibodies (Table 2a) in antibody diluent (1,8% NaCl in a 0.01 M sodium phosphate solution containing 0,1% Na-azide). After primary incubation, the sections were washed with PBS and incubated with the specific secondary antibodies (Table 2b) diluted in PBS in a wet chamber for 2 hours at room temperature. After further three washes in PBS, they were transferred for 30 minutes in an avidin-biotin complex solution (ABC Standard, ABC kit Vectastain, Vector Laboratories, Burlingame, CA, USA, PK 6100) and washed again in PBS. Finally, immunoperoxidase was developed using 3.3'-diaminobenzidine (DAB kit Vector Laboratories, Burlingame, CA, USA, BA-9200). The sections were dehydrated in ethanol, passed in xylene and covered with a coverslip using Entellan.

The slides of both Masson's trichrome and IP were observed with an optic microscope (Zeiss Axioplan, Carl Zeiss, Oberkochen, Germany), captured with the microscope Nikon Coolscope (Nikon, Japan) and subsequently elaborated with the programs Elipsenet 1.20.0 (Nikon, Japan) and GIMP 2 (GNU Image Manipulation Program 2.10).

For the IF procedure, slides were placed in a wet chamber. A first PBS wash was performed to rehydrate the sections. A Blocking Serum solution (0,5% Triton X-100, 10% Normal Goat serum, NGS, Vector, Burlingame, CA, USA, in PBS) or 10% Normal Donkey Serum (NDS, Jackson, Bar Harbor, Maine, USA) was used at room temperature for 2 hours. Then, the sections of each sample were incubated in a wet chamber for 48 hours, at 4 °C, with the primary antibodies (Table 2a) in antibody diluent. After 48 hours, the sections were washed with PBS and either pure NGS or 10% NDS, (5 x 10 minutes on a stirrer). Next, the sections were incubated for 3 hours, at room temperature, with specific secondary antibodies (Table 2b), diluted in PBS. After further five 10-minute washes in PBS, the slides were air dried and prepared with glycerol buffered with 0,5 M sodium carbonate (pH 8,6) to be finally sealed with nail polish.

The slides obtained were observed under an epifluorescence optical microscope (Axioplan, Carl Zeiss, Oberkochen, Germany), equipped with a system of filters that allowed the distinction of the fluorescence FITC (given by fluorescein) from Alexa 594 fluorescence. The images were acquired using a digital camera and DMC 2 software (Polaroid Corporation, Cambridge, MA, USA). The images were processed using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Table 2a: List of the primary antibodies used for immunoperoxidase (IP) or immunofluorescence (IF).

Primary antibody	Used for	Immunogen /host	Supplier	Dilution	Antibody RRID	Validation
Protein Gene Product 9.5 (PGP 9.5)	IP	Polyclonal rabbit	Millipore, Temecula, CA, USA	1:500	AB_91019	PMID:19296476
	IF			1:1000		
Substance P (SP)	IP	Polyclonal rabbit	Immunostar, Hudson, WI, USA	1:1000	AB_572266	PMID:10087030 PMID:10196365
	IF	Monoclonal rat	Fitzgerald Industries International, North Acton, MA, USA	1:200	AB_2313816	PMID:22740069 PMID:26713509
Calcitonin Gene Related Peptide (CGRP)	IP/IF	Monoclonal mouse	Santa Cruz Biotechnology Inc., CA, USA	1:200	AB_2259462	PMID:30971286 PMID:29943954
	IF	Polyclonal rabbit	Peninsula Laboratories Inc., San Carlos, CA, USA	1:1000	AB_2313775	PMID:18186028 PMID:28680400
Human Tyrosine	IP/IF	Monoclonal mouse	Monosan, Uden, Netherlands	1:50	ID: MONX10786*	PMID:29615733

Hydroxylase (TH)						
Neurofilament 200kDa (NF 200kDa)	IP/IF	Monoclonal rabbit	Sigma, Saint Louis, Missouri, USA	1:1000	AB_477272	PMID:18022951 PMID:19937712

Table 2b: List of the secondary antibodies used for immunoperoxidase (IP) or immunofluorescence (IF).

Secondary antibody	Used for	Immunogen /host	Supplier	Dilution	Antibody RRID	Validation
Biotinylated Anti-Rabbit	IP	Goat	Vector Laboratories, Burlingame, CA, USA	10 µg/ml	AB_2313606	PMID:19127523 PMID:23766132
Anti-Mouse	IP	Goat	Vector Laboratories, Burlingame, CA, USA	10 µg/ml	AB_2336171	PMID:23766132 PMID:25057794
Anti-Mouse Alexa 594	IF	Goat	Thermo Fisher Scientific, Waltham, MA, USA	1:200	AB_141372	PMID:23913443 PMID:25057190
Anti-Rat Alexa 594	IF	Donkey	Thermo Fisher Scientific, Waltham, MA, USA	1:200	AB_2535795	PMID:25933105 PMID:28089909
Anti-Rabbit-FITC	IF	Goat	Calbiochem, Darmstadt, Germany	1:100	ID: 401314*	PMID:29615733

*Antibody RRID are universally identified codes and were taken from the website the antibody registry (https://antibodyregistry.org/) which integrated the antibody database of the *Journal of Comparatve Neurology*. For each antibody, there is at least one publication correlated to a unique PMID (PubMed Identifier). For the antibodies whose lot number are MONX10786 and 401314, there are still no current RRID available, but the validation appears in one publication (Bombardi et al., 2010).

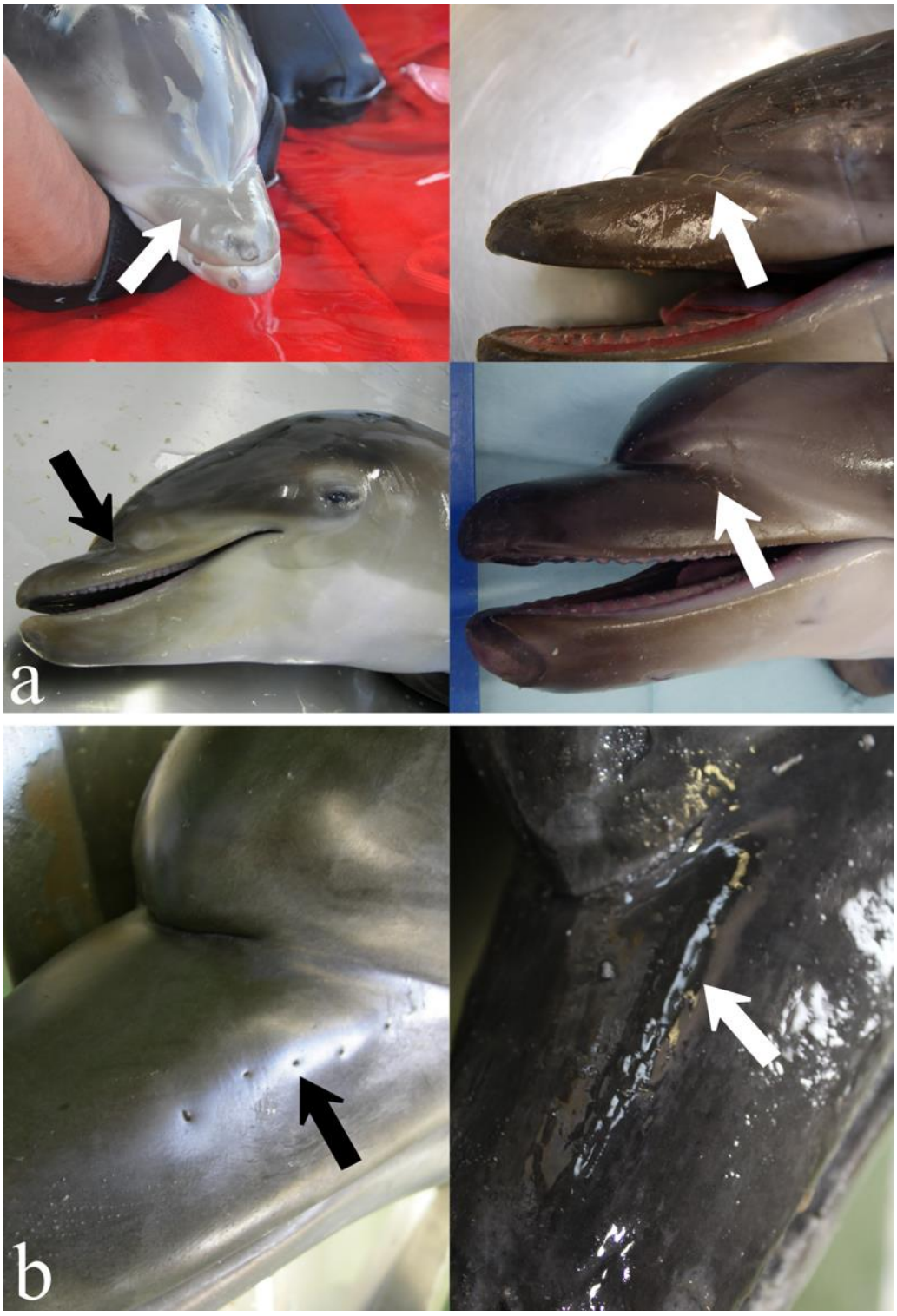


Figure 1: Macroscopic images of the rostrum of some specimens of (a, top four) newborn and (b, bottom two) adult bottlenose dolphin. The arrows indicate where the vibrissae emerge from the skin as can be seen in the newborns (a) or the concavity found in the adults (b).

Results

1. Morphology

Newborn dolphins

In the newborns, the external part of the VS was approx. 10 mm long. The FSC of all specimens consisted of an epidermal and dermal part. The epidermal part comprised the hair with its sheaths, overlying the dermal venous sinus. The VS originated from the bulb and consisted of three concentric layers which, from inside to outside, were identified as the medulla, the cortex, both made of keratinized cells, and the cuticle, which consisted of a simple squamous keratinized epithelium. The MS and the capsule were fused near the follicle outlet. At the base of the FSC, the bulb resembled a highly innervated and vascularized dermal papilla (Figure 2a). The VS was wrapped by the inner root sheath, attached to the cuticle, and the outer root sheath, surrounded by the glassy membrane (Figure 2b). The hair shaft was surrounded by a venous sinus and delimited by a connective tissue capsule. The sinus comprised internally the MS, in contact with the glassy membrane and externally by a capsule (Figure 2c).

In the slides analyzed, it was never possible to observe either a ringwulst or a ring sinus. Furthermore, no muscle fiber or gland was present around the follicle.

Adult dolphins

In the adults, the VS was present but did not reach the skin surface. Apart from this feature, the FSC of the adult dolphins showed the same structure of those of the newborns.

2. Innervation

Anti-PGP 9.5 immunoreactive (-ir), anti-NF 200-ir, anti-TH-ir and anti-SP-ir nerve fibers were evident in all the samples. No anti-CGRP-ir fibers were observed.

Newborn dolphins

PGP 9.5-ir fibers penetrated the FSC at the level of the hair bulb, and yielded an intricate arborized network of ramifications (Figure 3a, b). The nerve fibers protruded at various levels in the mesenchymal sheath, giving rise to button-like terminations characterizing MNEs (Figure 3c). They derived from large and medium-sized fibers that ran to form clusters of button-like endings with a smooth and regular surface, between which fine spiral-like fibers were present (Figure 3d).

NF 200-ir fibers were also detected penetrating the bulb (Figure 3e), first running parallel to the VS and then entering at different levels along the follicle (Figure 3f). Nerve fibers of different calibers

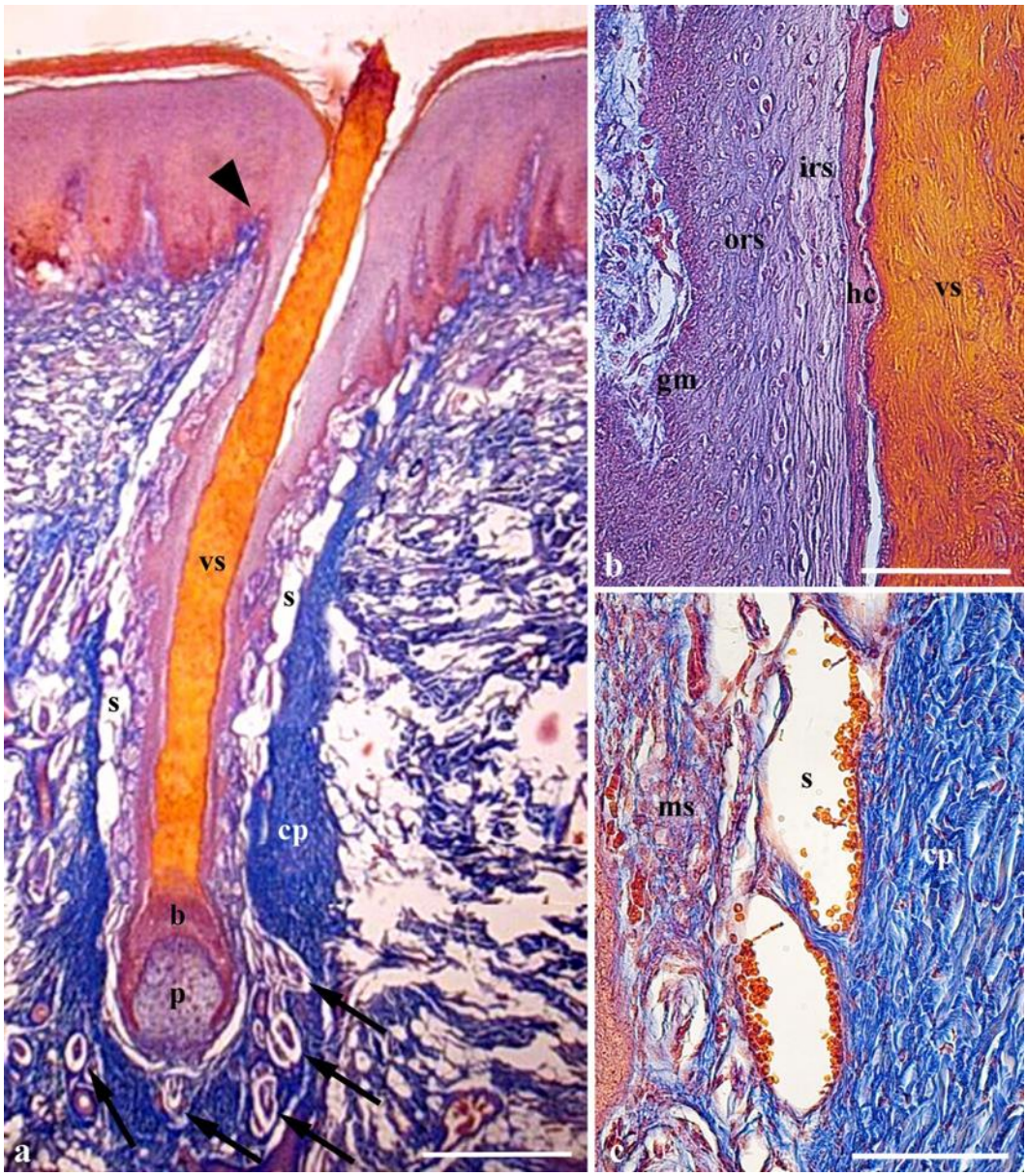
were distributed along the VS, progressing either in a straight line or along a winding path until they reached the top of the FSC (Figure 3f).

Numerous nerve fibers were observed in transversal sections of the FSC, from the hair bulb to the apex (Figure 4). These fibers innervated the hair bulb (Figure 4b, c) and sent small groups of axons to surround the follicle (Figure 4d, e), ending into MNEs (Figure 4f). Some of these axons penetrated the venous sinus, ran along the trabeculae (Figure 4g) and ended at the MS that wrapped the VS with MNEs (Figure 4h). This rich innervation was evident in all transverse sections up to the outlet of the vibrissa (Figure 4i). We did not identify other receptors with certainty and, as mentioned above, most fibers seemed to end as FNEs.

SP-ir fibers ran either grouped in bundles or alone close to blood vessels (Figure 5a, b). Double immunofluorescence for PGP 9.5 and TH showed that PGP 9.5-ir fibers were qualitatively four-fold the TH-ir fibers (Figure 5c, d). TH-ir fibers were mainly located around the blood vessels, and sometimes presented a tortuous pattern. They contained thin-caliber axons that ran first on the surface of the adventitia and then penetrated the wall (Figure 5e). Few TH-ir fibers were found at the base of the bulb and in the MS (Figure 5f).

Adult dolphins

Immunohistochemical results in adult dolphins showed the same general pattern of that of newborns, with some notable exceptions. PGP 9.5-ir and NF 200-ir nerve fibers were clear (Figure 6a-c) and the MNEs bound to the mesenchymal sheath were smaller in adults (Figure 6d). SP reactivity was found in large caliber fibers near the dermo-epidermal junction (Figure 7a), where they ran parallel to the skin before bending towards the FSC and ending as FNEs (Figure 7b). TH-ir fibers were rarer. Very thin TH-ir fibers were present in the trabeculae of the venous sinus and the mesenchymal sheath ending with isolated oval corpuscles (Figure 7c-e).



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254 **Figure 2** – Longitudinal section of a typical FSC in newborn bottlenose dolphin. a. The vibrissa is
255 surrounded by a venous sinus (s). A capsule (cp) envelops the complex. Several nerves (arrows) reach
256 the root of the vibrissa. The arrowhead indicates the fusion between the capsule and mesenchymal
257 sheat. b. Detail at higher magnification of the epidermal components. c. Detail at higher magnification
258 of the dermal components. b, bulb; cp, capsule; gm, glassy membrane; irs, inner root sheat; ms,
259 mesenchymal sheath; ors, outern root sheat; p, papilla; s, venous sinus; vs, vibrissal shaft. Masson's
260 Trichrome stain. Scale bars: a = 1 mm; b, c = 100 μm.
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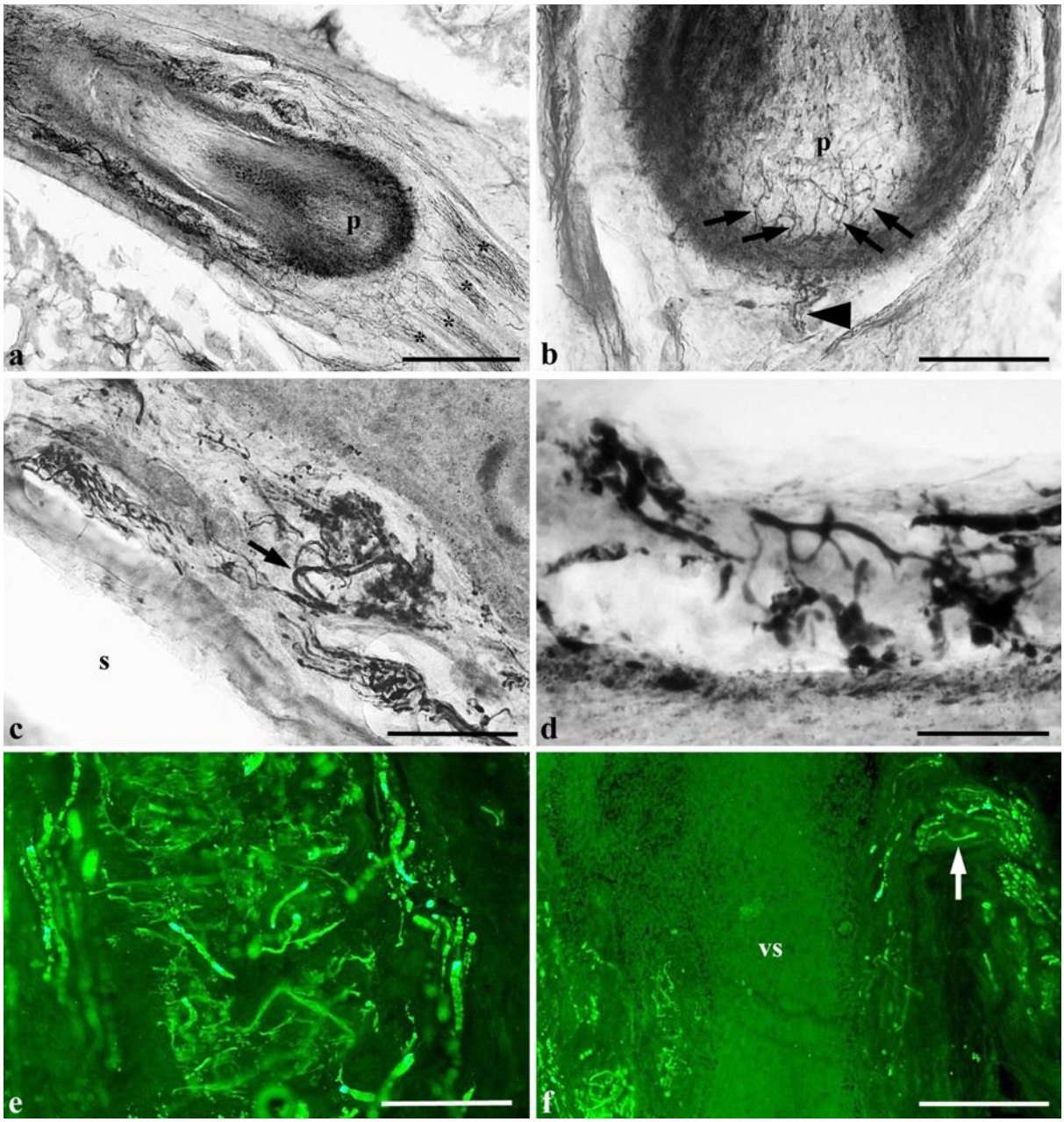

Figure 3 – Innervation of the FSC in a newborn bottlenose dolphin. The nerve fibers were immunolabelled for PGP 9.5 (a-d) and NF200kD (e, f). a. Several PGP 9.5-ir nerve bundles (asterisks) reach the root of the vibrissa. p, papilla. b. Few thin-caliber fibers (arrowhead) enter the papilla (p) and terminate as free nerve endings (arrows). c. In the mesenchymal sheath, some nerve fibers (arrow) give rise to MNEs (asterisks). d. High magnification showing MNEs. Note the characteristic button-like endings. e. The dense network of nerve fibers around the bulb. f. A nerve bundle penetrate the FSC laterally. vs, vibrissal shaft. Scale bars: a, e =100 μm; b, c, f = 200 μm; d = 50 μm.

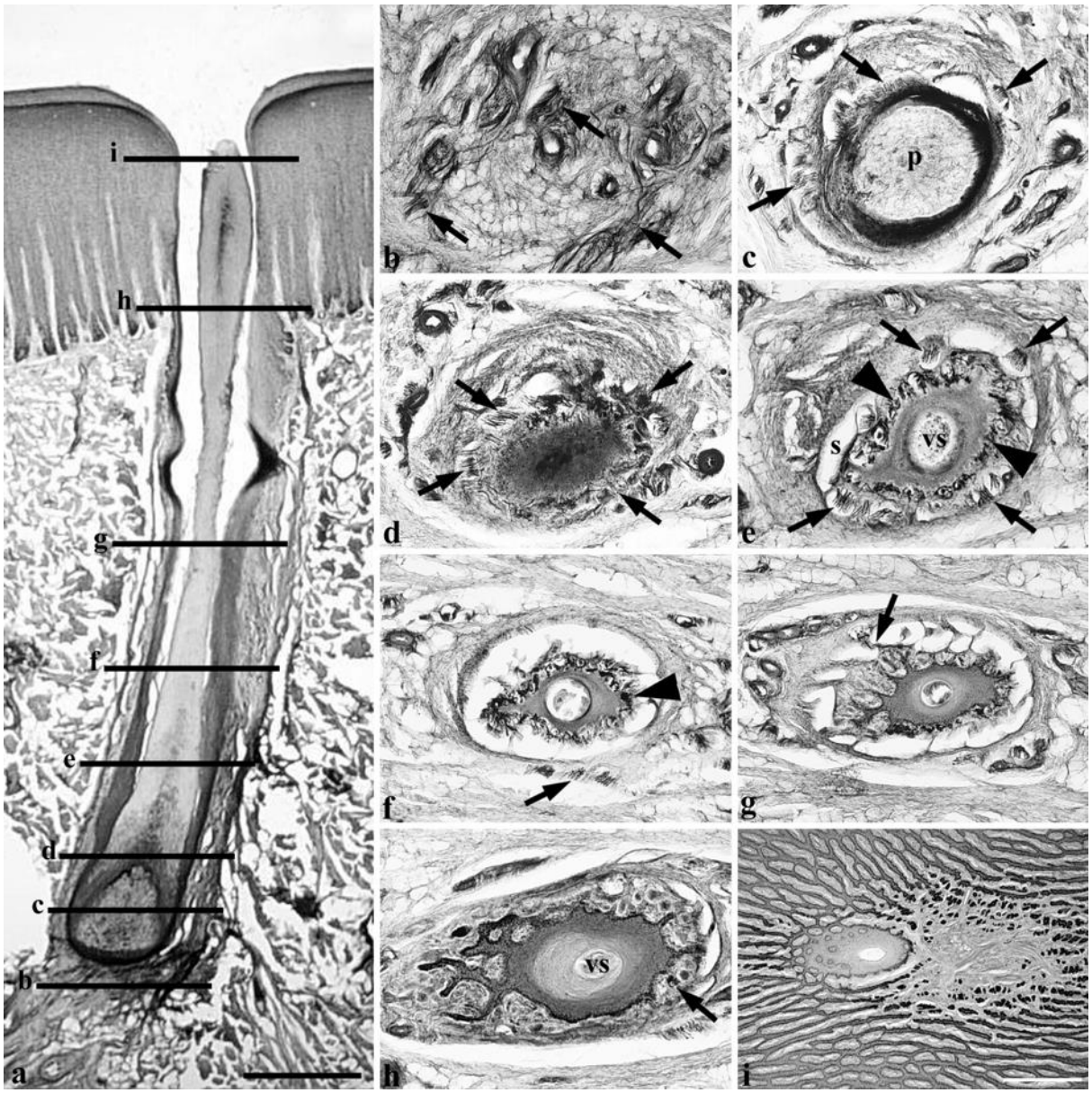

Figure 4 – Longitudinal (a) and transverse (b-i) sections (b) of FSC in newborn bottlenose dolphin showing the innervation at different levels from the basal (b) to the apical (i). The nerve fibers were immunolabelled with antibodies to PGP 9.5. b. Some nerves (arrows) reach the root of the vibrissa. c. The nerves break into several fascicles (arrows) that ascend close to the papilla (p). d. The nerve fibers (arrows) surround the follicle. e. Some nerve fibers (arrows) penetrate the venous sinus (s) and branch in the mesenchymal sheath (arrowhead). vs, vibrissal shaft. f. Some fibers terminate on MNEs (arrowhead), while others continue along the FSC (arrow). g. A nerve fiber (arrow) passes through one of numerous trabeculae of the venous sinus. h. At the level of dermo-epidermal border, the nerve fibers disappear but the MNEs are still present (arrow). i. Section through the skin and the dermal papilla. Scale bars: a = 1 mm; b-h = same magnification of i; i = 350 μm.

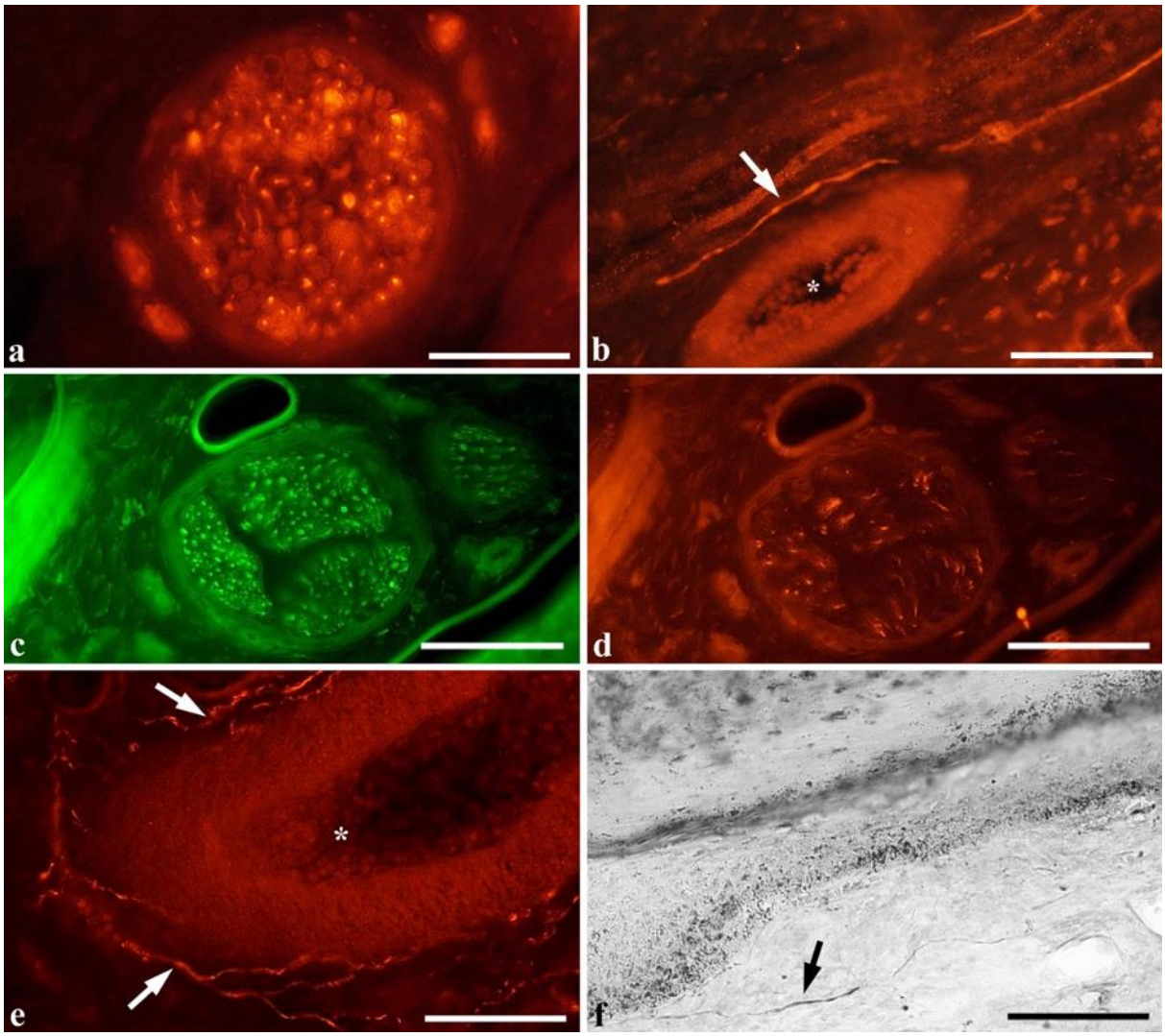

Figure 5 - SP- (a, b) and TH-(c-f) immunoreactive fibers in newborn bottlenose dolphin. a. Transverse section of a nerve bundle showing many immunoreactive fibers. b. A nerve fiber (arrow) runs parallel to a blood vessel (asterisk). c, d. Double immunofluorescence PGP 9.5-FITC (c) / TH-Alexa 594 (d) of a nerve bundle in transverse section. Note the TH immunoreactivity of some fibers. e. Several nerve fibers (arrows) reach the tunica adventitia of a vessel (asterisk). f. A thin fiber run within the ms. Scale bars = 100 μm.

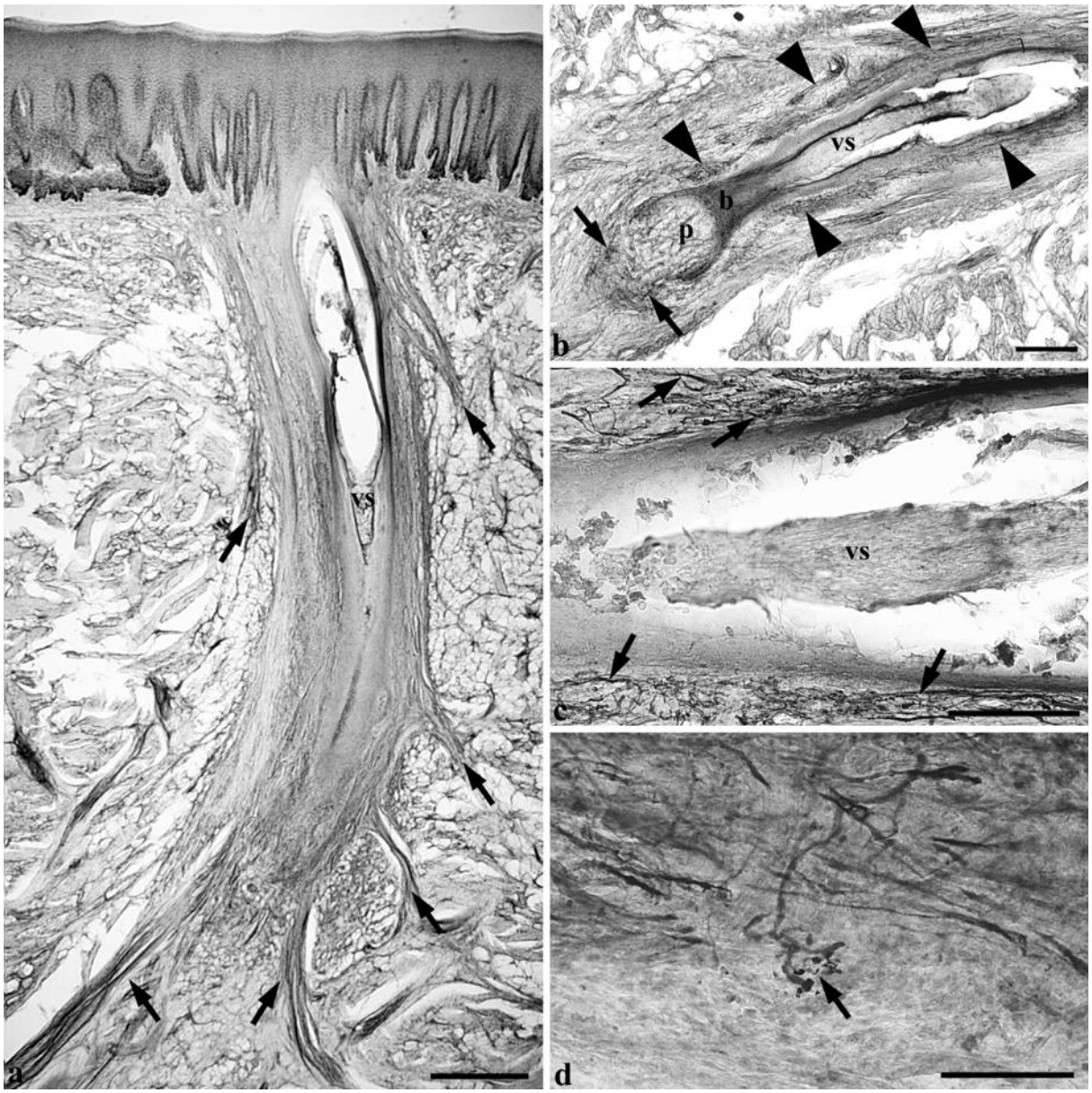

Figure 6 – Longitudinal sections of FSC in adult bottlenose dolphin. The nerve fibers were immunolabelled for PGP 9.5 (a, b) and NF200kD (c, d). a. Note the six nerve bundles (arrows) reaching the FSC. b. A vibrissa is clearly visible inside the follicle. Some nerve fibers (arrows) reach the bulb, others (arrowheads) run within the ms. b, bulb; p, papilla; vs, vibrissal shaft. c. High magnification showing the rich innervation (arrows) of the ms. d. Detail of a MNE (arrow). Scale bars: a, b = 1 mm; c = 200 μm; d =100 μm.

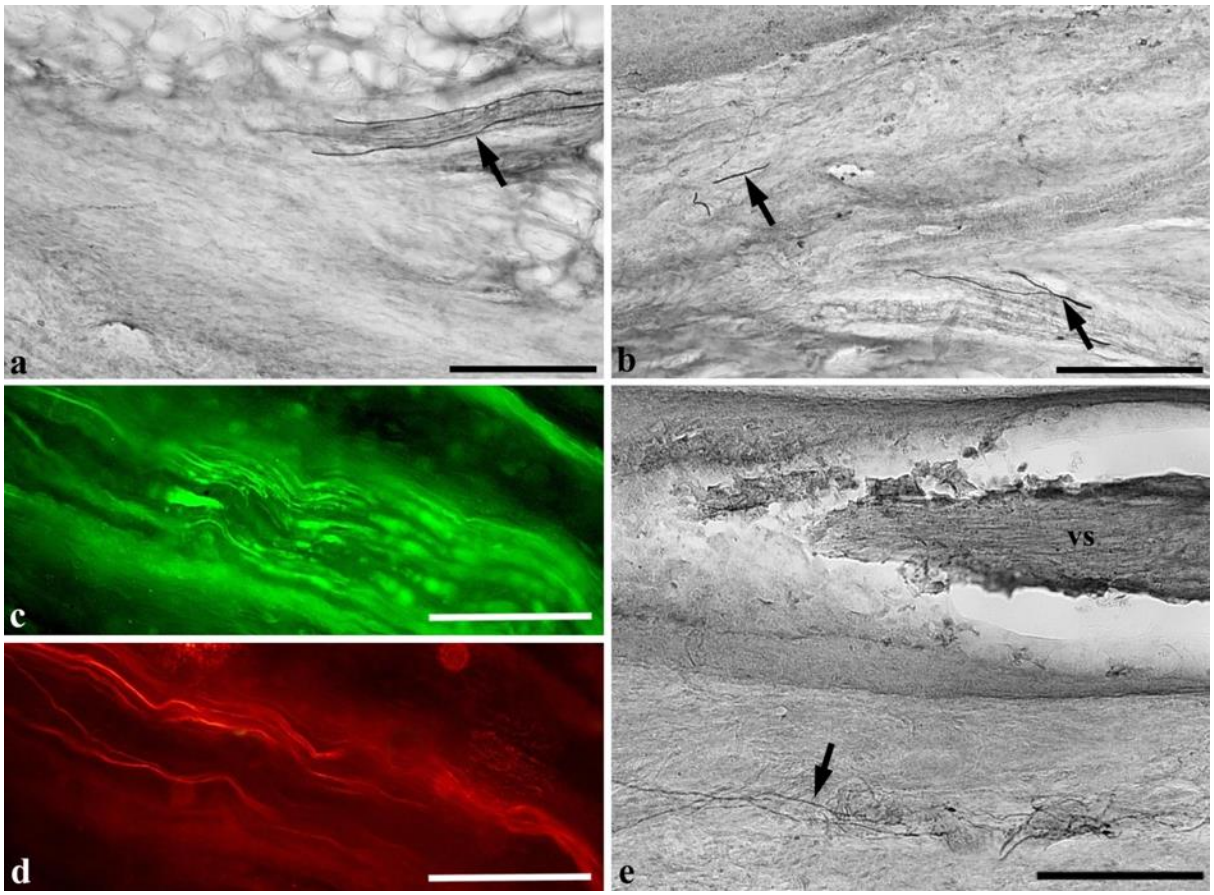

Figure 7 – SP- (a, b) and TH- (c-e) immunoreactive fibers in an adult bottlenose dolphin. a. Two positive fibers reach the FSC laterally. b. Few nerve fibers in the ms. c, d. Double immunofluorescence PGP 9.5-FITC (c) / TH-Alexa 594 (d) of a large nerve bundle in longitudinal section. Note the few TH-ir fibers. e. Few fibers (arrow) in the ms. Scale bars: a, b, e = 200 μm; c, d = 100 μm.

Discussion

In the present work, we describe the morphology and the innervation of FSC of newborn and adult bottlenose dolphins. Our findings reveal that newborn specimens possess a complete structure divided in an epidermal and a dermal part, hence the term "follicle-sinus complex" whereas, in adults, the VS lies within the follicle (Figs. 2, 6). The absence of a ring sinus (ringwulst) of the *erector pili* muscle and of any kind of associated gland constitutes differences with the vibrissae in terrestrial mammals. The blood sinus of the bottlenose dolphin consists of just one cavity forming a trabecular net just like the tammar wallaby (*Macropus eugenii*) (Marotte *et al.*, 1992) and is not divided into two parts as in terrestrial mammals (Rice *et al.*, 1986, Ebara *et al.*, 2002) or three parts as in pinnipeds (Marshall *et al.*, 2006) (Figure 8).

Anti-neuronal antibodies (anti-PGP 9.5, -NF 200, -TH, and -SP) helped to characterize the innervation of the FSC. Antibodies directed against proteins of the neurofilaments (anti-PGP 9.5, -NF 200, Figs. 3, 4, 6) identified several fiber bundles which bifurcated from the deep vibrissal nerve at the bottom of the FSC to reach various heights of the follicle, similarly to what was previously described in manatees (Sarko *et al.*, 2007). Few fibers were also found at the dermo-epidermal junction (Figure 4i). Merkel receptors were evenly distributed along the MS of the FSC, although, in the newborn they were also found at the dermo-epidermal junction (Figs. 3, 4). Since no striated muscle fibers and no glands were present in the FSC, the nature of the present innervation is likely somatosensory and derived from the maxillary nerve (V2) of the trigeminal nerve. The V2 runs – from its exit of the skull base – rostrally to enter the dolphin equivalent of the maxillary foramen and subsequent canal. The rostralmost fibers of the V2 run as the infraorbital nerve in maxillary canals. Some fibers of the latter penetrate the maxilla in dorsal direction to provide sensorial innervation to the skin of the rostrum (Rauschmann 1992). Dolphins possess no movable lips and have virtually no snout fascia or mimic muscles beyond those, more caudal, that act on the melon, thus implying a virtual absence of a somato-motor component in the facial nerve this far forward on the face. Comparisons with other mammals are difficult. Whisking rodents possess the mystacial pad, a thickening of the snout fascia where mimic muscles, sensory receptors and collagen structures form a highly developed motor-sensory organ (Haidarliu *et al.*, 2020). In the rat and mouse, a column of neocortical neurons in the whisker somatosensory cortex (wS1, or barrel cortex) corresponds to each FSC, with a highly developed layer IV receiving the thalamic afferent (Woolsey and Van der Loos, 1970; Van der Loos and Woolsey, 1973; Van der Loos, 1976; Rice and Van Der Loos, 1977; Jeanmonod *et al.*, 1981; Pearson *et al.*, 2006; Bosman *et al.*, 2011; Schröder *et al.*, 2020). However, the barrel cortex is typical of rodents, and is not present in any other species (i.e. mammals of the genus *Felis* or *Panthera*), even ones which present whisking behavior such as the short-tailed

opossum (Waite *et al.*, 1991; Ramamurthy and Krubitzer, 2016). Furthermore, the neocortex of dolphins and whales lacks a layer IV and it is currently hypothesized that thalamic projections reach layer II instead of IV (for general description see Cozzi et al., 2017), thus making any comparison with the highly specialized barrel cortex of rodents difficult. In particular the study by van Kann and colleagues (2017) pointed out the main differences in the primary neocortical areas layering between the common dolphin, the wild boar and humans.

The use of antibodies against peptides (SP, CGRP) and against a key enzyme in catecholamine synthesis (TH), allowed further characterization of the innervation. SP is involved in nociception, and a subpopulation of sensory neurons in the mammalian trigeminal ganglion contains SP, colocalized with CGRP (Alvarez *et al.*, 1988, Fundin *et al.*, 1997b; Waite and and Ashwell, 2012). SP-ir neurons have also been described in the dorsal root ganglia of bottlenose dolphins (Bombardi *et al.*, 2010).

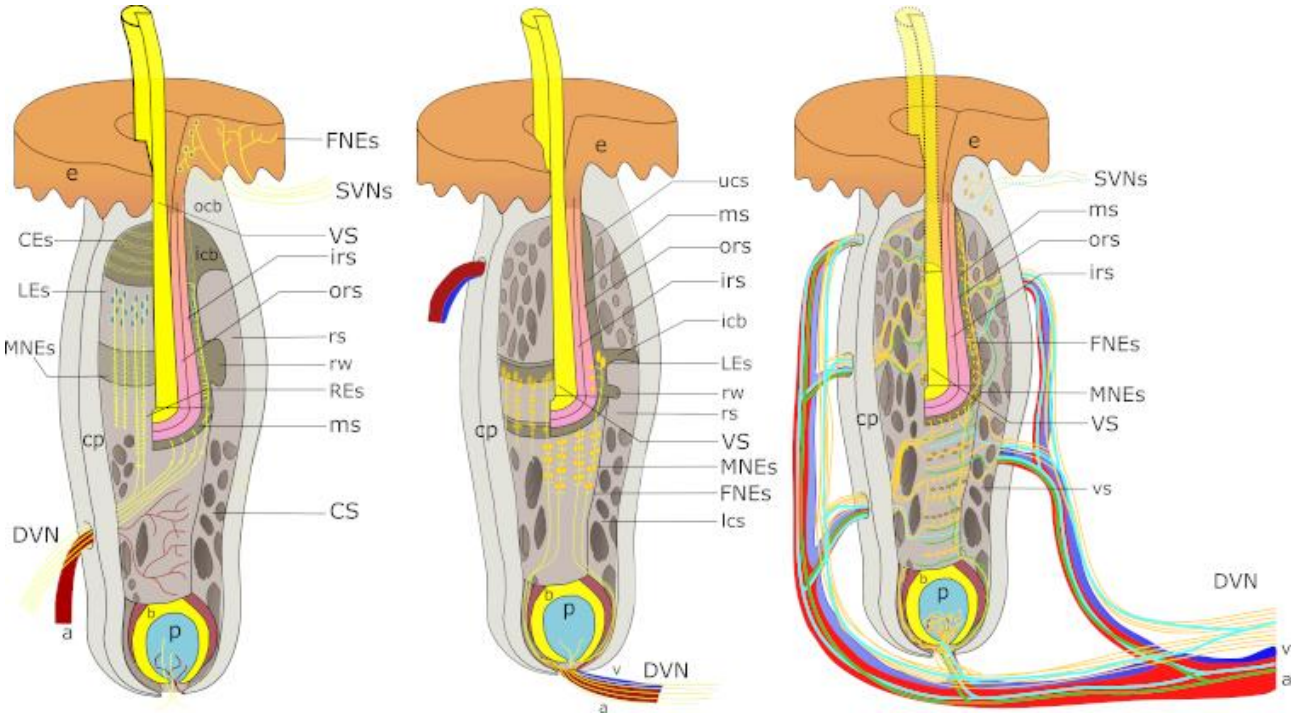

Figure 8 – Schematic drawn representing the main differences between the FSCs of a terrestrial mammal (left), pinniped (center) and dolphin (right). On the right, the follicle represented is that of the adult as the dotted lines and transparent areas (SVNs and MNEs in the dermo-epidermal junction) are of the newborn. In terrestrial mammals the FSC is divided into two halves, the inferior cavernous sinus, and the superior ring sinus. Receptors, coming mainly from the deep vibrissal nerve are positioned at different heights depending on their sensory nature. In pinnipeds it is instead divided into three portions, including an upper cavernous sinus. The fibers, which derive only from the deep vibrissal nerve, innervate up to the inner conical body, without reaching the epidermis. Finally, in the dolphin, a trabecular component forms a single venous sinus in which the receptors, also deriving mainly from the deep vibrissal nerve, are distributed along the follicle until they reach the epidermis in the newborn. Also note the TH-ir (green) and SP-ir (light blue) fibers which accompany the blood vessels. Abbreviations: VS, vibrissal shaft; e, epidermis; irs, inner root sheat; ors, outern root sheat; ms, mesenchymal sheat; ocb, outern conical body; icb, inner conical body; rw, ringwulst; rs, ring sinus; cs, cavernous sinus; lcs, lower cavernous sinus; ucs, upper cavernous sinus; c, capsule; p,

papilla; b, bulb; DVN, deep vibrissal nerve; SVNs, superficial vibrissal nerves; MNEs, merkell nerve endings; FNEs, free nerve endings; CEs, circular endings; LEs, lanceolate endings; REs, reticular endings; a, artery; v, vein; vs, venous sinus.

Several SP-ir were located in the dermo-epidermal junction and, deeper lateral to the follicle. Other SP-ir nerves were also found around the sinus' blood vessels. Both SP and CGRP are vasodilators. However, the presence of SP-ir fibers within big nerve bundles suggests a nociceptive function while the presence of SP in thin fibers around blood vessels might indicate a parasympathetic activity on the vessels of the FSC (Fundin *et al.,* 1997b). No CGRP-ir fiber was detected in the vibrissae of the dolphins in this study. This absence is difficult to explain from a functional point of view. Indeed, CGRP has been found in the gastrointestinal tract of the striped dolphin (*Stenella coeruleoalba*) (Domenighini *et al.,* 1997), and has been proposed to be present in CNS of the bottlenose dolphin (Rambaldi *et al,* 2016). Additionally, the presence of this molecule was demonstrated in the FSC of manatees (Sarko *et al.,* 2007). Therefore, the apparent absence of CGRP in the FSC of the bottlenose dolphin could be due to a loss of nociceptive function or different trigeminal organization.

TH-ir fibers consisted of thin branches located around the surface of the blood vessels connecting to the sinus, even though some elements were found also in the MS and trabeculae. Considering their peripheral location, TH-ir fibers are indicative of a noradrenergic sympathetic innervation derived from the cranial [superior] cervical ganglion. In mammals, sympathetic fibers follow both the external and internal carotid arteries. From the former, fibers then run along the infraorbital artery. However, the internal carotid artery obliterates early in postnatal life in dolphins (Boenninghaus, 1903; Cozzi *et al.,* 2017), as in many Cetartiodactyls. Therefore, the precise route of the TH-ir that was observed in the FCS of dolphins remains to be ascertained. It may be possible that the internal carotid artery develops in the odontocete embryo to guide the growing sympathetic fibers from the cervical ganglion since it is known that the axonal outgrowth of mammalian sympathetic precursors proceeds in dependence and thus in parallel to the development of the internal carotid artery (reviewed in Kameda 2020). Thus, possibly only after the sympathetic fibers have found their route, the internal carotid artery obliterates in odontocetes. Alternatively, these fibers in cetaceans may rely exclusively on the external carotid path.

Contrarily to what was reported by Yablokov *et al.* (1972), we were able to confirm the presence of the VS in adult specimens, as was previously described by Palmer and Weddell (1964). In all the adult animals analyzed in the present study, the FSC was complete, i.e. the epidermal and dermal components were discernable, and the VS was still present, albeit only in the follicle. In fact, the hair papillae maintained the same morphology found in the newborns. Moreover, the VS did not have the aspect of a formless cluster due to the epithelial regeneration. Since the papilla is responsible for the

production of the VS's components, a lack of this structure can justify the absence of the VS in the adult Guiana dolphin, in which it had transformed into an agglomeration of fat cells (Czech-Damal *et al.*, 2012). Interestingly, we found MNEs and FNEs but no other receptor, which is coherent with what was described in the Guiana dolphin (Czech-Damal *et al.*, 2012). In the absence of other markers (e.g. anti-S100 protein), it however remains impossible to rule out the existence of other nerve endings such as Pacini corpuscles.

The Guiana dolphin is in fact very similar to the ubiquitous bottlenose dolphin in general body morphology and proportions, although somewhat smaller. Yet, the preferred habitat of the Guiana dolphin is estuarine and coastal, while bottlenose dolphins are distributed worldwide and live both along coastlines and in the high seas. The FSC complex in Guiana dolphins has been linked to the sensitivity to electric fields generated by prey burrowed in sand or hidden by murky waters (Czech-Damal *et al.*, 2012, 2013). A progressive morphological adaptation to different specific environments over time may be the explanation to the differences in the structure of the FSC in those two similarly sized species. Whether the evolution was towards a loss or a gain of function remains unclear.

Based on the absence in dolphins of morphological features typical of terrestrial mammals with vibrissae, i.e. the absence of a wide range of receptors, the mystacial pad or the barrel cortex, we can infer that the FSC of dolphins are relatively rigid structures, with a sensitivity potentially reduced which does not allow perception of dynamic changes the way fully formed whiskers do. Yet, since the vibrissae persist in newborn dolphins, contrarily to other structures lost during fetal growth (pelvic limbs, to name the most striking difference with land mammals) it is possible that the vibrissae play a role in the early postnatal life, in the days immediately following parturition.

Pinniped mothers recognize their newborn mostly through olfaction while the newborn, from the first minutes of life, vocalizes for most of the first day, a behaviour that diminishes gradually afterwards (Trillmich, 1981). Underwater, this kind of recognition is almost impossible to dolphins since they cannot smell (Cozzi *et al.*, 2017). As proposed by Cozzi *et al.* (2012, 2015), an evolutionary adaptation in cetaceans to an immediate recognition is the early ossification of the tympanic bulla which would allow the newborn to locate the mother's vocalizations and help it in postpartum period. However, full echolocation capacities are likely not to fully develop until month 1-3 after birth (Harder *et al.*, 2016); and while it is difficult to think that vibrissae may act for this recognition, it is more plausible that it could allow the newborn to find the opening of the nipples immediately after birth, thanks to the rich SP innervation and vasomodulation regulated by the TH-ir plexus (Fundin et al., 1997b; Maklad, 2004).

A VS is still present within the FSC in the adult dolphin and the innervation remains relatively developed. The original tactile function of the FSC may lose value in the growing dolphins that, later

in life, may rely on different sensory modalities to interact with their mother and the rest of the pod. This organ could act as a proprioceptor as suggested by Yablokov *et al.* (1972). The VS could be sensitive to low frequency oscillations and water movements caused by head rotation and would consequently activate the receptors that provide this information to the central nervous system. This would allow the dolphin to always have a perception of the angular position of the head. Nervous fibers could modify the pressure inside the venous system and help maintain thermoregulation and modify the threshold of the receptors (Fundin *et al.*, 1997b). This idea does not preclude that the structure found by Czech-Damal and colleagues (2012) in the Guiana dolphin could function as an electroreceptor. However, to confirm or reject this hypothesis, further psychophysical experiments should be conducted in the bottlenose dolphin.

Authors contributions :

TG, AG, JMG and BC designed the study, TG, AG, CT and MDS acquired and analyzed the data. TG, AG, JMG and BC wrote the draft. AG, SdV and SH critically revised the manuscript. All authors approved the article.

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Tables:

Table 1: Origin of specimens

ID	Species	Sex	Age class	Origin
# 83	*T. truncatus*	M	Newborn	Died in a marine theme park
# 114	*T. truncatus*	M	Newborn	Died in a marine theme park
# 123	*T. truncatus*	F	Newborn	Died in a marine theme park
# 124	*T. truncatus*	M	Newborn	Died in a marine theme park
# 144	*T. truncatus*	M	Newborn	Died in a marine theme park
# 145	*T. truncatus*	M	Newborn	Died in a marine theme park
# 162	*T. truncatus*	M	Newborn	Wild
# 229	*T. truncatus*	M	Newborn	Died in a marine theme park
# 146	*T. truncatus*	M	Adult	Died in a marine theme park
# 159	*T. truncatus*	M	Adult	Died in a marine theme park
# 444	*T. truncatus*	M	Adult	Wild

Table 2a: List of the primary antibodies used for immunoperoxidase (IP) or immunofluorescence (IF).

Primary antibody	Used for	Immunogen /host	Supplier	Dilution	Antibody RRID	Validation
Protein Gene Product 9.5 (PGP 9.5)	IP	Polyclonal rabbit	Millipore, Temecula, CA, USA	1:500	AB_91019	PMID:19296476
	IF			1:1000		
Substance P (SP)	IP	Polyclonal rabbit	Immunostar, Hudson, WI, USA	1:1000	AB_572266	PMID:10087030 PMID:10196365
	IF	Monoclonal rat	Fitzgerald Industries International, North Acton, MA, USA	1:200	AB_2313816	PMID:22740069 PMID:26713509
Calcitonin Gene Related Peptide (CGRP)	IP/IF	Monoclonal mouse	Santa Cruz Biotechnology Inc., CA, USA	1:200	AB_2259462	PMID:30971286 PMID:29943954
	IF	Polyclonal rabbit	Peninsula Laboratories Inc., San Carlos, CA, USA	1:1000	AB_2313775	PMID:18186028 PMID:28680400
Human Tyrosine Hydroxylase (TH)	IP/IF	Monoclonal mouse	Monosan, Uden, Netherlands	1:50	ID: MONX10786*	PMID:29615733
Neurofilament 200kDa (NF 200kDa)	IP/IF	Monoclonal rabbit	Sigma, Saint Louis, Missouri, USA	1:1000	AB_477272	PMID:18022951 PMID:19937712

Table 2b: List of the secondary antibodies used for immunoperoxidase (IP) or immunofluorescence (IF).

Secondary antibody	Used for	Immunogen /host	Supplier	Dilution	Antibody RRID	Validation
Biotinylated Anti-Rabbit	IP	Goat	Vector Laboratories, Burlingame, CA, USA	10 µg/ml	AB_2313606	PMID:19127523 PMID:23766132
Anti-Mouse	IP	Goat	Vector Laboratories, Burlingame, CA, USA	10 µg/ml	AB_2336171	PMID:23766132 PMID:25057794
Anti-Mouse Alexa 594	IF	Goat	Thermo Fisher Scientific, Waltham, MA, USA	1:200	AB_141372	PMID:23913443 PMID:25057190
Anti-Rat Alexa 594	IF	Donkey	Thermo Fisher Scientific, Waltham, MA, USA	1:200	AB_2535795	PMID:25933105 PMID:28089909
Anti-Rabbit-FITC	IF	Goat	Calbiochem, Darmstadt, Germany	1:100	ID: 401314*	PMID:29615733

*Antibody RRID are universally identified codes and were taken from the website the antibody registry (https://antibodyregistry.org/) which integrated the antibody database of the *Journal of Comparatve Neurology*. For each antibody, there is at least one publication correlated to a unique PMID (PubMed Identifier). For the antibodies whose lot number are MONX10786 and 401314, there are still no current RRID available but the validation appears in one publication (Bombardi et al., 2010).

Figure legends

Figure 1 – Rostrum of some specimens of (a) newborn and an (b) adult bottlenose dolphin. The arrows indicate where the VS emerge from the skin (a) or the concavity (b).

Figure 2 – Longitudinal section of a typical FSC in newborn bottlenose dolphin. a. The vibrissa is surrounded by a venous sinus (s). A capsule (cp) envelops the complex. Several nerves (arrows) reach the root of the vibrissa. The arrowhead indicates the fusion between the capsule and mesenchymal sheat. b. Detail at higher magnification of the epidermal components. c. Detail at higher magnification of the dermal components. b, bulb; cp, capsule; gm, glassy membrane; irs, inner root sheat; ms, mesenchymal sheath; ors, outern root sheat; p, papilla; s, venous sinus; vs, vibrissal shaft. Masson's Trichrome stain. Scale bars: a = 1 mm; b, c = 100 μm.

Figure 3 – Innervation of the FSC in a newborn bottlenose dolphin. The nerve fibers were immunolabelled for PGP 9.5 (a-d) and NF200kD (e, f). a. Several PGP 9.5-ir nerve bundles (asterisks) reach the root of the vibrissa. p, papilla. b. Few thin-calibre fibers (arrowhead) enter the papilla (p) and terminate as free nerve endings (arrows). c. In the mesenchymal sheath, some nerve fibers (arrow) give rise to MNEs (asterisks). d. High magnification showing MNEs. Note the characteristic button-like endings. e. The dense network of nerve fibers around the bulb. f. A nerve bundle penetrate the FSC laterally. vs, vibrissal shaft. Scale bars: a, e =100 μm; b, c, f = 200 μm; d = 50 μm.

Figure 4 – Longitudinal (a) and transverse (b-i) sections (b) of FSC in newborn bottlenose dolphin showing the innervation at different levels from the basal (b) to the apical (i). The nerve fibers were immunolabelled with antibodies to PGP 9.5. b. Some nerves (arrows) reach the root of the vibrissa. c. The nerves break into several fascicles (arrows) that ascend close to the papilla (p). d. The nerve fibers (arrows) surround the follicle. e. Some nerve fibers (arrows) penetrate the venous sinus (s) and branch in the mesenchymal sheath (arrowhead). vs, vibrissal shaft. f. Some fibers terminate on MNEs (arrowhead), while others continue along the FSC (arrow). g. A nerve fiber (arrow) passes through one of numerous trabeculae of the venous sinus. h. At the level of dermo-epidermal border, the nerve fibers disappear but the MNEs are still present (arrow). i. Section through the skin and the dermal papilla. Scale bars: a = 1 mm; b-h = same magnification of i; i = 350 μm.

Figure 5 – SP- (a, b) and TH-(c-f) immunoreactive fibers in newborn bottlenose dolphin. a. Transverse section of a nerve bundle showing many immunoreactive fibers. b. A nerve fiber (arrow)

runs parallel to a blood vessel (asterisk). c, d. Double immunofluorescence PGP 9.5-FITC (c) / TH-Alexa 594 (d) of a nerve bundle in transverse section. Note the TH immunoreactivity of some fibres. e. Several nerve fibers (arrows) reach the tunica adventitia of a vessel (asterisk). f. A thin fiber run within the MS. Scale bars = 100 μm.

Figure 6 – Longitudinal sections of FSC in adult bottlenose dophin. The nerve fibers were immunolabelled for PGP 9.5 (a, b) and NF200kD (c, d). a. Note the six nerve bundles (arrows) reaching the FSC. b. A vibrissa is clearly visible inside the follicle. Some nerve fibers (arrows) reach the bulb, others (arrowheads) run within the MS. b, bulb; p, papilla; vs, vibrissal shaft. c. High magnification showing the rich innervation (arrows) of the MS. d. Detail of a MNE (arrow). Scale bars: a, b = 1 mm; c = 200 μm; d =100 μm.

Figure 7 – SP- (a, b) and TH- (c-e) immunoreactive fibers in an adult bottlenose dolphin. a. Two positive fibers reach the FSC laterally. b. Few nerve fibers in the MS. c, d. Double immunofluorescence PGP 9.5-FITC (c) / TH-Alexa 594 (d) of a large nerve bundle in longitudinal section. Note the few TH-ir fibers. e. Few fibers (arrow) in the MS. Scale bars: a, b, e = 200 μm; c, d = 100 μm.

Figure 8 – Schematic drawn representing the main differences between the FSCs of a terrestrial mammal (left), pinniped (center) and dolphin (right). On the right, the follicle represented is that of the adult as the dotted lines and transparent areas (SVNs and MNEs in the dermo-epidermal junction) are of the newborn. In terrestrial mammals the FSC is divided into two halves, the inferior cavernous sinus, and the superior ring sinus. The receptors are of various nature, are positioned at various heights depending on their receptor (sensory) nature and come mainly from the deep vibrissal nerve. In the pinniped it is instead divided into three portions, including an upper cavernous sinus. In this case, however, the fibers, which derive only from the deep vibrissal nerve, innervate up to the inner conical body, without reaching the epidermis. Finally, in the dolphin there is a trabecular component that forms a single venous sinus in which the receptors, deriving mainly from the deep vibrissal nerve, are distributed along the follicle until they reach the epidermis in the newborn. Also note the TH-ir (green) and SP-ir (light blue) fibers which accompany the blood vessels. Abbreviations: VS, vibrissal shaft; e, epidermis; irs, inner root sheat; ors, outern root sheat; ms, mesenchymal sheat; ocb, outern conical body; icb, inner conical body; rw, ringwulst; rs, ring sinus; cs, cavernous sinus; lcs, lower cavernous sinus; ucs, upper cavernous sinus; c, capsule; p, papilla; b, bulb; DVN, deep vibrissal nerve; SVNs, superficial vibrissal nerves; MNEs, merkell nerve endings; FNEs, free nerve endings;

CEs, circular endings; LEs, lanceolate endings; REs, reticular endings; a, artery; v, vein; vs, venous sinus.

Figure 9 – Schematic representation of the course of the maxillary nerve (V2) in adult (left) and newborn (right) bottlenose dolphin. The dotted ellipse approximates the area of the FSCs location.