

## THE OLFACTORY ORGAN OF *TORPEDO MARMORATA* RISSO, 1810: MORPHOLOGY, HISTOLOGY, AND NOS-LIKE IMMUNOREACTIVITY

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### ABSTRACT

The olfactory organ of Chondrichthyes is characterized by a central raphe and several lamellae covered by the sensory olfactory epithelium; usually the lamellae have secondary folds on their surface. It is an overall complex shape and very variable among the species of this vertebrate class. The sensory olfactory epithelium, in turns, has a general organization which is conserved across species. We here describe for the first time the morphology and histology of the olfactory organ of a juvenile male of marbled electric ray *Torpedo marmorata*. It is constituted by an elongated raphe and 37 primary lamellae. As the lamellar number does not change ontogenetically and as a little variability among individuals, the number 37 is likely to be indicative as the lamellar number of the species, and it is the smaller to date reported in the order of Torpediniformes. NOS1-like immunoreactivity allowed to observe a basal cell population possibly involved in immune defense or in the regulation of cell renewal.

**KEYWORDS:** Electric ray, nitric oxide, olfactory epithelium, olfactory rosette

### INTRODUCTION

Although the olfactory organ has a very different morphology in different vertebrate taxa, the olfactory epithelium that covers the organ, and that represents the actual sensory surface, is quite similar in all the species. It is a pseudostratified neuroepithelium, where the olfactory sensory neurons are intermingled with proper epithelial cells (namely, the supporting cells); the olfactory sensory neurons are in contact with the olfactory chamber lumen which is, in fact, the external environment (e.g. Firestein, 2001). The olfactory epithelium is continuously renewed mostly, but not only, by means of the proliferation of basal cells, which are a not-homogeneous population, and which have been gaining much interest, because of their neurogenic potential (e.g. Ferrando et al., 2010; Schwob et al., 2017). Although a large piece of literature attempted to correlate the anatomical features of the olfactory system to the olfactory capability of vertebrates, which parameters should be considered is still matter of debate (McGann, 2017). Among fish, where the shape of the olfactory organ varies from a smooth cavity to a complex multilamellar organ, such a correlation between form and function is uncertain (Zielinski and Hara, 2006). The class of Chondrichthyes, the cartilaginous fish (shark, rays and chimaeras), is of interest in the matter of vertebrate evolution, representing the more basal Gnatostomata class. All the species to date investigated in the class of Chondrichthyes possess a complex multilamellar organ, still very variable among the species in relative size, number of primary lamellae, size and shape of secondary folds. As for other vertebrates classes, these anatomical data have been matched to ecological aspects only partially (Schuessel et al., 2008; Meredith & Kajiura, 2010; Ferrando et al., 2017a; Ferrando et al., submitted). In this frame, morphological data on the olfactory organ

from the most phylogenetically and ecologically diverse species of Chondrichthyes will be valuable.

To the best of our knowledge, the only information available in the literature about the olfactory system of the genus *Torpedo* regards the presence of mucous cells in the olfactory epithelium of the marbled electric ray *Torpedo marmorata* (Ferrando et al., 2017b). In the present work, the gross morphology and histology of the olfactory organs of a juvenile of *T. marmorata* is described.

Besides olfactory receptor neurons, supporting cells, and basal cells, which are the three general cell population in the olfactory epithelium of all vertebrates, a third population of cells, characterized by the nitric oxide synthase type 1-like (NOS1-like) immunoreactivity was described in the small spotted catshark *Scyliorhinus canicula* (Ferrando et al., 2012). In order to individuate this further cell type also in *T. marmorata*, the NOS1-like immunoreactivity in the olfactory epithelium of this species was detected. The NOS enzyme is present in three isoforms in mammals, two are constitutively expressed (NOS1 and NOS3) and one is inducible (NOS2). Only two isoforms are present in fish, one constitutively expressed and one inducible (Andreakis et al., 2011). The presence of the constitutive NOS (which is considered NOS1-like) in the mucosal surface of fish was correlated to the defense from bacterial challenge (Dong et al., 2016). On the other hand, specific roles for nitric oxide in the olfactory epithelium of vertebrates, such as an involvement in olfactory transduction or in receptor neuron development, were suggested (Dellacorte et al., 1995; Sánchez-Islas & León-Olea, 2001).

## MATERIALS AND METHODS

### *Morphology*

One juvenile of *T. marmorata* (Fig. 1A, B) with total length =  $178 \pm 1$  mm and disk width =  $118 \pm 1$  mm, was accidentally caught by professional fishermen in the Ligurian Sea (North-West Mediterranean Sea) on May 2015. It came on board lifeless and it was cold preserved until sampling. The olfactory organs were dissected from the specimens, fixed in 4% paraformaldehyde in 0.1 M phosphate-buffered solution (PBS, pH 7.4), washed in PBS and stored in ethanol (70% in distilled water). The left olfactory organ was used to count the lamellar number according to Ferrando et al. (2017a) and to calculate the surface area of the lamellae.

### *Histology*

The right olfactory organ, after observation in order to highlight notable morphological differences from the left one, was paraffin embedded, and sectioned at 5  $\mu$ m thickness. Histological analyses were performed with hematoxylin-eosin, Alcian blue and periodic acid-Schiff (Alcian-PAS), and Masson's trichrome stain (Bio-Optica, Italy).

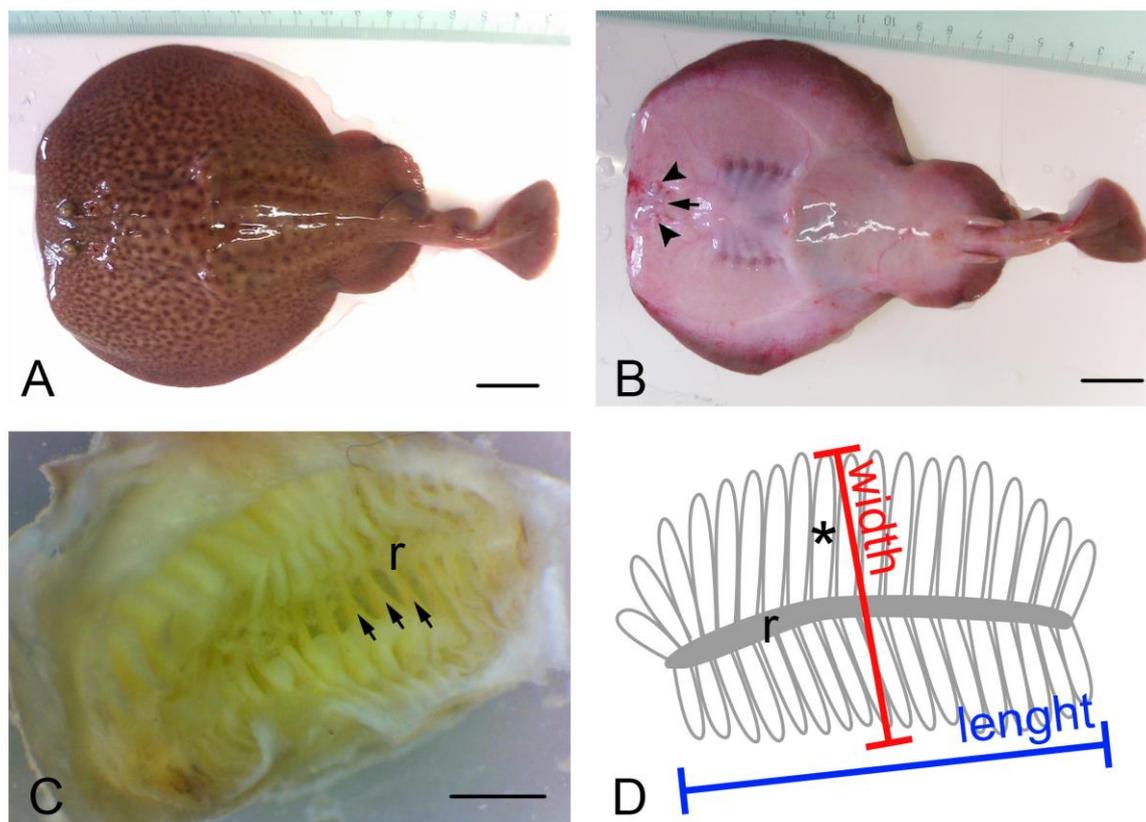


Figure 1. *Torpedo marmorata* A) Dorsal view of the specimen. Scale bar 2 cm. B) Ventral view of the specimen. The mouth (arrow) and the nostrils are visible (arrowheads). Scale bar 2 cm. C) Olfactory organ. The raphe (r) is elongated; lamellae (arrows) are arranged in two arrays at the sides of the raphe. Scale bar 1 mm. D) Scheme of the olfactory organ. The length and width of the organ reported in the Results were measured as indicated. The surface area of one lamella indicated in the Results was calculated in the central part of the raphe (r), in the zone indicated by the asterisk.

### Immunohistochemistry

Immunohistochemistry was performed using a rabbit anti-nNOS (H-299) polyclonal antiserum (1:100 in PBS, Santa Cruz Biotechnology, USA, Cat. sc-8309). The polyclonal antiserum was raised against an epitope corresponding to the aminoacids 2-300 at the N-terminus of human NOS1. The EnVision System anti-rabbit HRP-DAB (Dako Cytomation, Denmark) was used as secondary antiserum. In order to support the possibility that the antiserum could have recognized NOS1 in *T. marmorata*, being absent the genome and any detection of this protein in this species, we aligned the human epitope to NOS1 putative protein sequence from two chondrichthyan species. In particular, the sequence of predicted NOS1 is available for the holocephalan elephant shark *Callorhynchus milii* (NCBI Reference Sequence: XM\_007902342.1) and for the elasmobranch whale shark *Rhincodon typus* (NCBI Reference Sequence: XM\_020522241.1). The N-terminus of the chondrichthyan predicted proteins were aligned to the N-terminus of human NOS-1 (UniprotKB – P29475) using the online resource Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>). The obtained alignment is showed in Figure 2G.

### Microscopy

The whole olfactory organs were observed through a Zeiss Stemi 2000 stereomicroscope (Zeiss Microscopy, Jena, Germany) equipped with a digital camera (Cell- Pad E; Microptik BV, Schondijke, Netherlands). Sections were examined using Leica DMRB light microscope, and images were acquired with a Leica CCD camera DFC420C (Leica, Switzerland).

### Image analysis

Micrograph measurements were performed using ImageJ2 open source software (Rueden et al., 2017).

## RESULTS

### Morphology

The olfactory organs of the juvenile *T. marmorata* were characterized by an elongated raphe, with two arrays of primary lamellae at the sides. The left olfactory organ (Fig. 1C) was measured according to the Fig. 1D and it resulted 2.6 mm wide and 4.7 mm long. The lamellar number, according to Ferrando et al. (2017a), was 37. The surface area of a single lamella from the medial zone of the raphe (Fig. 1D) was 1.7 mm<sup>2</sup>.

### Histology

The histological investigation showed that the primary lamellae had only faint secondary folds on their faces (Fig. 2A). The sensory epithelium covered the lamellae and folds and had average thickness of  $111 \pm 17 \mu\text{m}$ . The shape and position of the nuclei allowed to recognize the main cell types in the epithelium: basal cells, olfactory receptor neurons, supporting cells and mucous cells (Fig. 2B). Alcian-PAS staining showed scattered, mainly-alcianophilic goblet cells (Fig. 2C). Masson Trichrome, highlighting the edge between connective tissue and epithelium, allowed to better appreciate the position of basal cells (Fig. 2D), and the appearance of the basal lamina, especially in correspondence of the secondary folds (Fig. 2E).

### Immunohistochemistry

The alignment of human NOS1 and two chondrichthyan predicted NOS1 showed that the aminoacids 2-125 at the N-terminus are very conserved (Fig. 3A); this part of the protein falls within the epitope of human NOS1 used to obtain the anti-NOS1 antiserum and suggest that the antiserum could recognize the NOS1 in Chondrichthyes.

In the olfactory organ of *T. marmorata*, NOS1-like immunoreactivity was observed only in the olfactory epithelium (Fig. 3C, D). In particular, the immunoreactivity was detected in scattered basal cells, with a quite large cytoplasm. About one or two immunoreactive cells were detectable each 200  $\mu\text{m}$  of linear epithelium in histological section. The immunoreactive cells seemed in contact or in the proximity to the basal lamina (Fig. 3C, D). The omission of the primary antisera gave negative results (Fig. 3B).

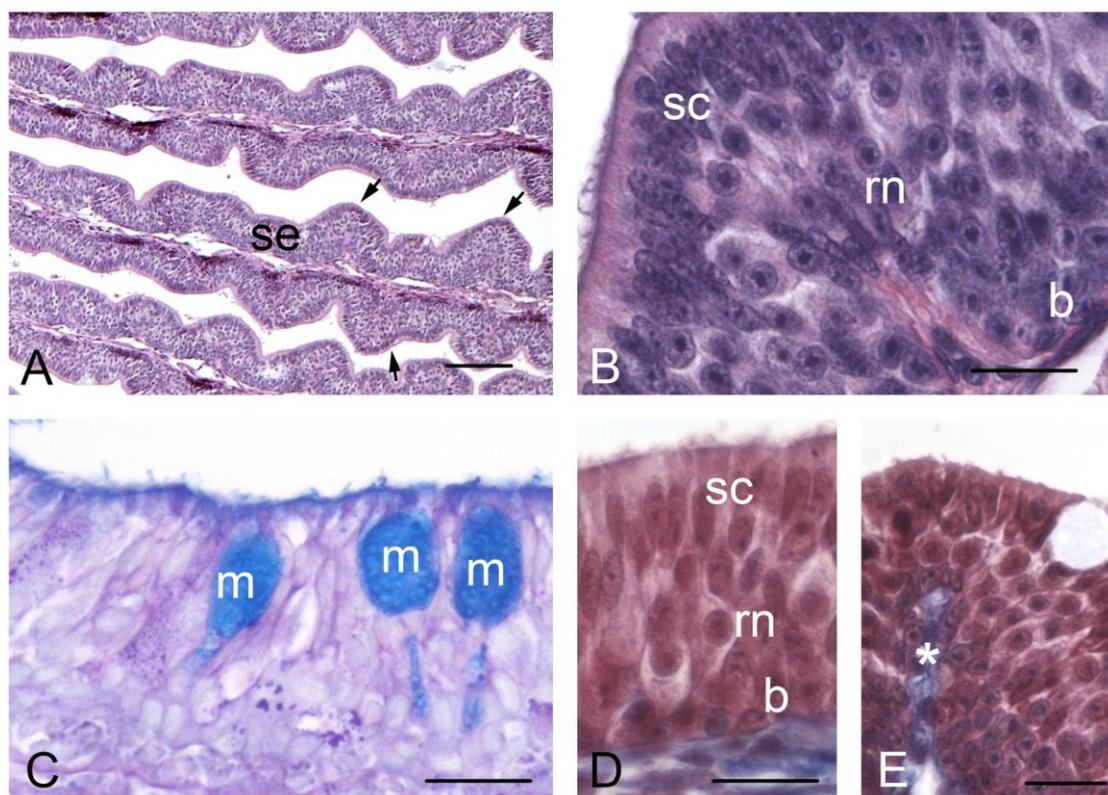


Figure 2. Histology of the olfactory organ of *Torpedo marmorata*. A) Hematoxylin-Eosin. The olfactory lamellae are covered by sensory epithelium (se). Small, faint secondary folds are visible on the lamellae (arrows). Scale bar 200  $\mu\text{m}$ . B) Hematoxylin-Eosin. In the epithelium, nuclei with different shapes are visible and indicate basal zone (b), middle zone with the nuclei of the olfactory receptor neurons (rn), and apical zone with the elongated nuclei of the supporting cells (sc). Scale bar 30  $\mu\text{m}$ . C) Alcian-PAS. Mucous cells (m) are scattered in the olfactory epithelium; they are mainly Alcian stained. Scale bar 30  $\mu\text{m}$ . D) Masson Trichrome. The blue staining of the connective tissue allows to observe the shape of the basal cells (b) lying on the basal lamina. The nuclei of the olfactory receptor neurons (rn) and of the supporting cells (sc) are visible as well. Scale bar 30  $\mu\text{m}$ . E) Masson Trichrome. In correspondence of the secondary folds, the connective tissue follows the trend of the epithelium, forming a fold (asterisk) perpendicular to the lamellar axis. Scale bar 30  $\mu\text{m}$ .

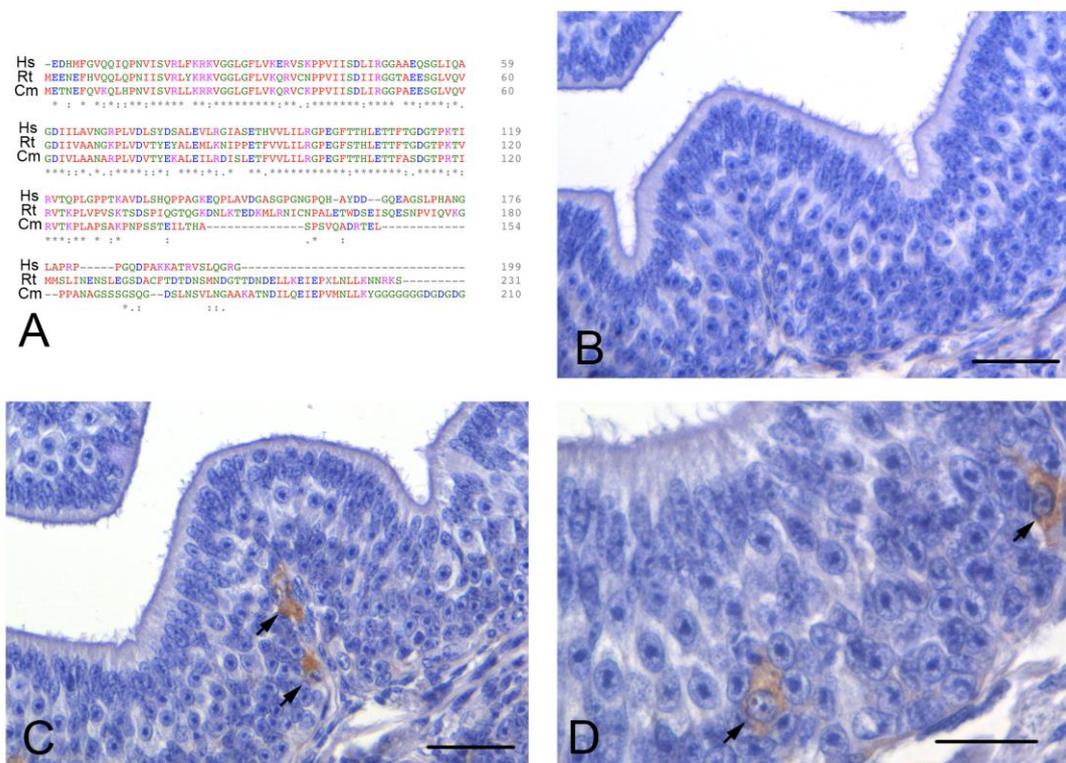


Figure 3. A) Alignment of the aminoacids 2-199 from human NOS1 and two chondrichthyan predicted NOS1. Hs = *Homo sapiens* UniprotKB P29475-1; Rt = *Rhincodon typus* NCBI Reference Sequence: XM\_020522241.1; Cm = *Callorhinchus milii* (NCBI Reference Sequence: XM\_007902342.1). B) Immunohistochemistry for NOS1-like, negative control. Nuclei are stained with hematoxylin. Omitting the primary antiserum no immunoreactivity is detectable. Scale bar 50 μm. C, D) Immunohistochemistry for NOS1-like. Nuclei are stained with hematoxylin. Immunoreactive cells (arrows) are present in the basal layer of the epithelium, possibly in contact with the basal membrane. C) Scale bar 50 μm. D) Scale bar 30 μm.

DISCUSSION

In chondrichthyan olfactory organ, an elongated raphe with two arrays of primary lamellae is the more common organization. In fact, among the species investigated to date, only the holocephalan *Chimaera monstrosa* and two species of Hexanchidae, *Notorhynchus cepedianus* and *Heptranchias perlo*, are characterized by primary lamellae arranged around a roundish raphe (Holl, 1973; Meng and Yin, 1981a; Howard et al., 2013; Ferrando et al., 2017b). Thus the shape of the olfactory organ of *T. marmorata* is consistent with most of elasmobranch species.

The number of lamellae in the olfactory organ is not affected by age or size in Chondrichthyes (Theiss et al., 2009). Although this lamellar number in the olfactory organ of Chondrichthyes is not so regular to be considered as a taxonomic character, average values differ sufficiently among species (Ferrando et al., 2017a). To date, the lamellar number in the order of Torpediniformes, has been investigated only in four species by Meng and Yin (1981b): the lamellar numbers of *Narke japonica*, *Crassinarke dormitor*, *Narcine ligula*, *Narcine maculata*, and *Narcine timlei* are respectively 52, 41, 51, 53, and 59. Thus the lamellar number of 37, here detected in *T. marmorata*, is the lowest among this order. It is noteworthy that the lamellar

number 37 is one of the lowest among the about 120 species of Chondrichthyes with a known lamellar number to date (Ferrando et al., 2017a). The number of olfactory lamellae is usually considered as a strategy to increase the sensory surface area. Although a large sensory surface area is intuitively related to a better olfactory performance, actually the relationship between sensory area and olfactory sensitivity has not been demonstrated in fish (Zielinski & Hara, 2006). Still the low lamellar number of *T. marmorata*, together with the relative small olfactory organ compared to other elasmobranch species (personal observation), could indicate that this species rely more on other senses, such as electroreception.

The histology of the olfactory organ of *T. marmorata* did not showed peculiarities that can distinguish this species from other elasmobranches to date investigates. The NOS-like immunoreactive cells strictly resembles those already described in the small spotted catshark *Scyliorhinus canicula* (Ferrando et al., 2012). The role of NO in the olfactory epithelium could be related to immune response (Dong et al., 2016), or to the regulation of other processes, such as cell proliferation (Gibbs, 2003).

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