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Effects of nasal parasite species in the small-spotted catshark *Scyliorhinus canicula* (Scyliorhinidae; Carcharhiniformes)

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Abstract. The presence of the parasitic copepod *Neoalbionella globosa* in the olfactory chamber of a specimen of the catshark *Scyliorhinus canicula* has been already reported in the literature, but this is the first record from the north-western Mediterranean Sea. Besides confirming this host-parasite association in the Ligurian Sea, the present study aims to describe some effects of the copepod's presence on the olfactory system of *S. canicula*, thus inferring potential effects of nasal parasites on olfaction. The copepod was accidentally found during a sampling campaign. The copepod, a mature female with well-developed egg sacs, parasitized the right olfactory rosette; the rosette presented visible swelling in some of the olfactory lamellae while, histologically, restricted edema was detectable close to the zone of attachment. The ipsilateral olfactory bulb, which receives the primary olfactory afferences, had a smaller number of cells and smaller neuron density compared to the contralateral bulb and to the average values for non-parasitized specimens of the same size. The results suggest that, although the olfactory rosette does not seem severely damaged, the presence of the parasite could deeply affect the highly efficient water flow within the nasal chamber, potentially causing partial olfactory impairment.

Key words: elasmobranch, *Neoalbionella globosa*, isotropic fractionator, olfactory rosette, olfactory bulb, north-western Mediterranean Sea

Introduction

As top predators in many marine food chains, sharks host a large range of parasites, with the most important taxa cestodes, nematodes and crustaceans. Cestodes and nematodes (e.g. *Anisakis* sp.) are endoparasites and the most common location to find these animals is the digestive system (Cislo & Caira 1993, Henderson et al. 2002). In contrast, parasitic crustaceans are typically ectoparasites and live on the host's body (Kabata 1979). Most parasitic crustaceans are hematophagous. However, in addition to obtaining nutrition from the host, some also take advantage of the host's movement in the environment (Hickling 1963, Costello 2006).

The Copepoda is an order of crustaceans that includes both free-living and parasitic species. The

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body shape and structure are substantially modified in parasitic species, with the three main adaptations to parasitism being: cephalization paired with a loss of segmentation; the development of the trunk, often paired with the loss of external appendages; the development of "prehensile appendages" for the attachment to the host (Boxshall 1974, Kabata 1979, 1982, Johnson et al. 2004). The Lernaepodidae is one of the most common families of parasitic copepods and their representatives parasitize both cartilaginous and teleost fishes globally (Youssef et al. 2016).

A key trait of the Lernaepodidae is the organ of attachment of the adult female to the host's tissues, the bulla, which develops on two enlarged disks on the top of the maxillae (Kabata 1979, 1986, Ruiz & Bullard 2019). This organ is made up of a manubrium with a duct that connects the maxillae to the anchor. The morphology of the bulla is variable depending on the host and tissue and is a feature of evolution in the Lernaepodidae (Kabata & Cousens 1972, Benkirane et al. 1999). Kabata & Cousens (1972) distinguish three types of bulla and the most common sites of attachment, which are the skin, gills, oral cavity and cloaca.

Among the possible responses of fish hosts to parasitism, the immune response has been studied most in farmed teleost fishes. In the sockeye salmon *Oncorhynchus nerka* Walbaum, 1792, the presence of *Salmincola californiensis* Dana, 1852 (Copepoda, Lernaeopodidae) induces hyperplasia, gill fusion and occlusion of branchial circulation, but also a low reaction against bulla fixation (Fast 2004). In the blue shark *Prionace glauca* Linnaeus, 1758, the response against infection by *Phyllothyreus cornutus* Milne Edwards, 1840 (Copepoda, Pandaridae) is tissue hyperplasia, with the presence of papillomatous structures and mucous-secreting cells. In the host's gills, at the site of attachment of the parasite, the presence of eosinophils associated with fibrous tissue and necrosis along with an inflammation response was observed (Borucinska & Benz 1999). Gradual loss of eye function was observed in the case of *Ommatokoita elongata* Grant, 1827 (Copepoda, Lernaeopodidae) when infecting the eyes of the Greenland sleeper shark, *Somniosus microcephalus* Bloch & Schneider, 1801 (Borucinska et al. 1998).

In this study we describe for the first time the association of *Neoalbionella globosa* and the small spotted catshark *Scyliorhinus canicula* Linnaeus, 1758 from the north-west Mediterranean Sea. Our aim was to investigate the morphology and histology of the parasite-host interface in the peripheral olfactory organ (i.e. olfactory rosette) and the possible effect of parasite presence. We additionally investigated the effect of the parasite on the ipsilateral olfactory bulb (OB); i.e. the zone of the telencephalon where the primary olfactory afferences arrive. We focused on the cell composition of the OB using the isotropic fractionator technique, which allows evaluation of cell and neuron number in nervous tissue (Herculano-Houzel & Lent 2005).

The small-spotted catshark is a small shark that belongs to the family of Scyliorhinidae and lives on the continental shelf at a depth of around 200 meters. Crustaceans, cephalopods and fishes compose the diet of this benthic shark (Compagno 1984). The parasitic copepods of the family Lernaepodidae that have been found on *S. canicula* are *Lernaepoda galei* Krøyer, 1837 and *N. globosa* Leigh-Sharpe, 1918 (Moore 2001). Bakopoulos et al. (2018) also found another unidentified species of *Neoalbionella* sp. on *S. canicula*. The copepod *L. galei* is more common than *N. globosa* and parasitizes several shark species (Leigh-Sharpe 1919, Moore 2001). On *S. canicula*, *L. galei* parasitizes the pelvic fins (Leigh-Sharpe 1916). Conversely, *N. globosa* has been found in the nasal fossae of the small-spotted catshark and this association was reported only in the seas around the British Isles (Leigh-Sharpe 1918, 1919, Kabata 1979).

Material and Methods

Sampling and examination of specimens

The sample was collected in the Ligurian Sea (north-west Mediterranean Sea) during a sampling campaign for cartilaginous fishes in February 2018, as bycatch of professional fishermen, following the Italian law "Decreto Legislativo 4 marzo 2014, n. 26" implemented under European Directive 2010/63/UE. The copepod infected a female specimen of *S. canicula* Linnaeus, 1758 (TL = 290 mm) and the identification of the catshark was performed according to Compagno (1984).

The head of the shark was dissected from the body behind the second gill opening and fixed in 4% paraformaldehyde (PAF) in 0.1 M phosphate buffer saline (PBS; 8‰ NaCl, pH 7.4) at 4 °C overnight. After three washes in PBS for 1 hour each, the head was placed in 70% ethanol. The copepod was found in the right olfactory rosette (Fig. 1a). After the dissection, the brain, the olfactory rosettes and the copepod were stored in 70% ethanol for further analyses. The copepod was dissected from the olfactory rosette using

entomological needles under a stereomicroscope (Stemi 2000 Stereo Microscopes, Zeiss, Germany, equipped with a Cellpad E camera, TiEsseLab s.r.l., Milan, Italy). Both olfactory rosettes and the OBs of the specimen were processed as indicated below. The data from the left OB, which receives the afference from the non-parasitized rosette, as well as some morphological data from the left rosette, were included in another study (Aicardi et al. 2020). Conversely, the histology of both the rosettes and the data from the right OB are the object of the present study. The crustacean (Fig. 1b) was dissected under a stereomicroscope. The identification of the species was based on the descriptions by Leigh-Sharpe (1918) and Kabata (1979) and a dichotomous key for the family Lernaepodidae (Kabata 1986).

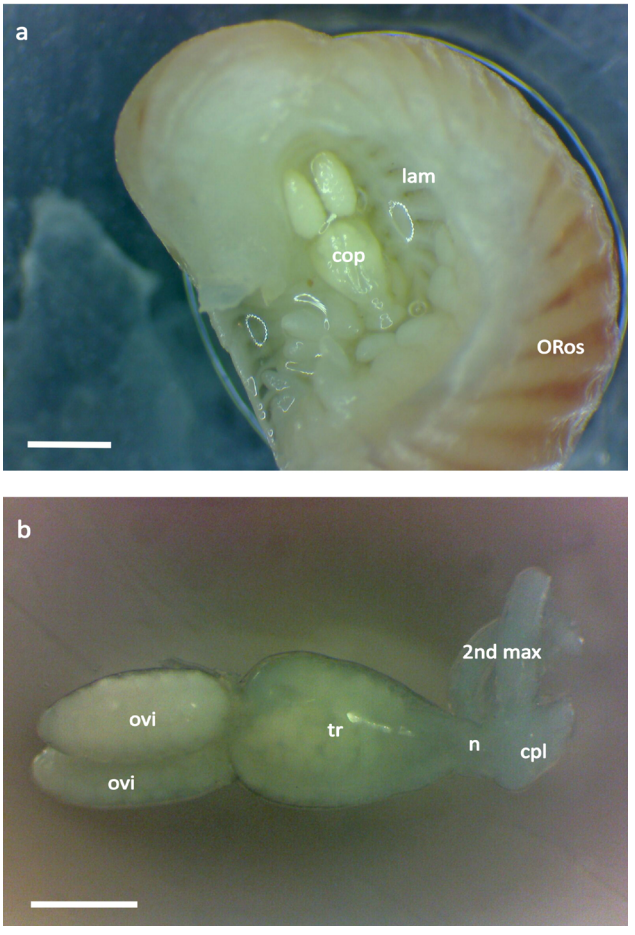

Fig. 1. The copepod specimen *Neoalbionella globosa*. a) location of the parasite copepod on *Scyliorhinus canicula* right olfactory rosette, scale bar 2 mm; b) isolated *N. globosa*, scale bar 1 mm. Legend: 2nd max – maxillae, cop – copepod specimen, cpl – cephalothorax, lam – olfactory lamellae, n – neck, ORos – olfactory rosette, ovi – egg sac, tr – trunk.

Histological observation

The olfactory rosettes were embedded in paraffin and 5 µm thick sections were used to evaluate if any histological difference was visible between the left (without parasite) and right (with parasite) rosette. Sections were stained with standard hematoxylin-eosin (H&E; Bio-Optica, Italy) and Masson's trichrome for connective tissue (MT; Bio-Optica, Italy).

Isotropic fractionator technique

The isotropic fractionator technique was used to evaluate the number of cells and the neuron percentage in the OBs of the shark specimen (Herculano-Houzel & Lent 2005). The two OBs were separately weighed and then fractioned in a detergent solution (40 mM trisodium citrate and 1% Triton-X) to dissolve the cell membrane. The solution was centrifuged and the nuclei pellet was resuspended in a known volume of PBS. The nuclei were stained with a fluorescent DNA marker, 4-6-diamidino-phenylindole-dihydrochloride (DAPI; Molecular Probes Europe BV) with a working dilution of 1:1,000 (1 mg/L). The quantification of the number of cells was performed using a hemocytometer. To discriminate the neuronal and non-neuronal nuclei, indirect immunofluorescence analysis was performed using a mouse anti-Neuronal Nuclei primary antibody (anti-NeuN; Millipore Mab377 – final working dilution 1:100) and a goat anti-mouse Dylight® 488 – conjugated secondary antibody (Immunoreagent, GtxMu-003-D488NHSX, lot 61-69-031618). The nuclei were centrifuged and rinsed with PBS after each step. The nuclei were resuspended in PBS and analyzed with an epifluorescence microscope to evaluate the percentage of neuronal nuclei. The total number of neurons was obtained as a proportion of the total number of cells.

Observation and photograph acquisition of histological sections, nuclei in the hemocytometer, and of nuclei after immunofluorescence were performed using a light and epifluorescence microscope (DMRB Leica, Germany) equipped with a Moticam 3+ camera (Motic, Europe). The images were managed using the ImageJ open source software (Schneider et al. 2012).

Results

The parasitic copepod was located in the right olfactory rosette of the shark and the entire body appeared whitish (Fig. 1a). We identified the subdivision between cephalothorax and trunk, separated by the neck and two developed egg sacs (Fig. 1b), and observed the maxillae with two terminal disks. Morphological measurements were taken and compared with those of Leigh-Sharpe (1919) and Kabata (1979) (Table 1). The copepod was

Table 1. Comparison of morphometric measurements of *Neoalbionella globosa* extracted from a small-spotted catshark *Scyliorhinus canicula* in the north-western Mediterranean Sea (present study) and those published in Leigh-Sharpe (1919) and Kabata (1979). All measurements are in mm. // – measurements not available.

		Leigh-Sharpe (1919)	Kabata (1979)	Present study
1 Cephalothorax		2	1	1.6
2 Trunk		> 2-3	2	3.1
	(1) to (2) ratio	2/3	1/2	1/2
3 Maxillae		4	3	2.5
4 Egg sacs		> 2	//	2.4
	(4) to (2) ratio	3/4	//	7/9
5 Uropods		0.5	0.5	0.9
	(5) to (2) ratio	1/4	1/4	2/7
Eggs	Rows	6-8	//	6
	# in a row	12	//	8

identified as *N. globosa* (Leigh-Sharpe 1919) based on the main body characters. The maxillae end with two enlarged disks with a vestigial bulla (Fig. 2a) and their length was comparable with body length (Table 1). The antennule supported six apical setae, one of which was digitiform (Fig. 2b). The mouth possessed mandibles with the primary teeth (Fig. 2c). The maxillipeds are like those of other Lernaepodidae and hold a spinulose pad (Fig. 2d). Egg sacs were located on the body with numerous egg rows and two sausage shaped posterior processes (modified uropods; Fig. 2e, f).

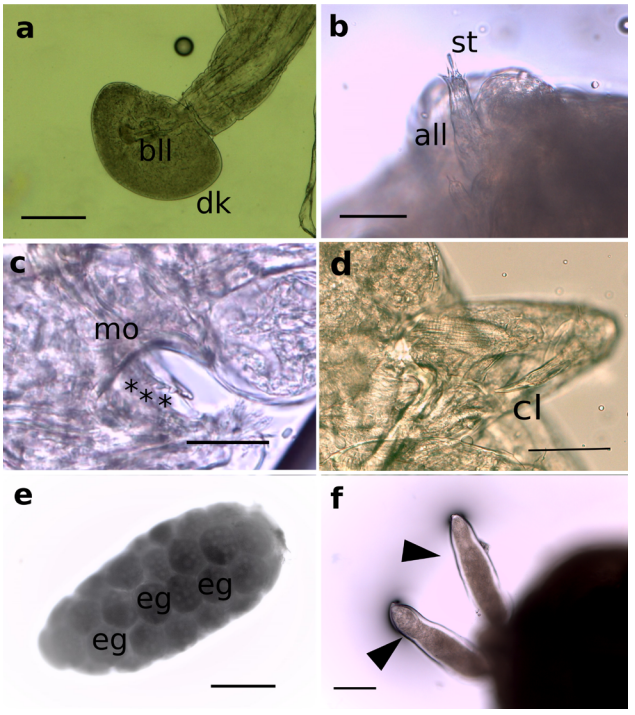

Fig. 2. Organs of *Neoalbionella globosa*. a) disk of the maxillae with bulla, scale bar 500 µm; b) antennule, scale bar 100 µm; c) mouthpart of *N. globosa*, with mandible showing primary teeth (black asterisks), scale bar 50 µm; d) maxilliped with rounded claw, scale bar 100 µm; e) egg sacs with numerous egg rows, scale bar 1 mm; f) uropods (black arrows), scale bar 500 µm. Legend: bll – bulla, dk – disk, all – antennule, st – apical setae, mo – mouth, cl – claw, eg – eggs.

The gross analysis of the right olfactory rosette showed the presence of swelling close to the site of attachment of the copepod (Fig. 3a). The histological sections show a large wound with accumulation of connective tissue (Fig. 3b, c). This wound was a similar shape to the copepod attachment organ. Cells of the immune system, monocytes and eosinophils, are also visible in the tissue around the wound (Fig. 3d). The comparison between the sensory epithelium of both the olfactory rosettes does not show any damage or hyperplasia. Indeed, the thickness of both epithelia was similar (Fig. 4).

The total number of neurons, measured by the isotropic fractionator technique, differed between the two OBs, the uninfected bulb had 1.5 million neurons and the parasitized OB 0.5 million (Table 2). Neuron density in the parasitized OB was also lower (14,000 N/mg) than the uninfected OB (40,000 N/mg) and in comparison with results from Aicardi et al. (2020).

Discussion

Raibaut et al. (1998) drafted a list of the parasitic copepods of fish in the Mediterranean Sea. In that list *S. canicula* is host to only two species, *L. galei* and *Albionella globosa,* in accordance with Leigh-Sharpe (1919) and Kabata (1979), the latter now assigned to genus *Neoalbionella* (Özdikmen 2008). Hitherto, a description of the relationship between *N. globosa* and *S. canicula* in the Mediterranean Sea, with a focus on

Table 2. Number of cells and percentage of neurons in the olfactory bulbs (OBs) of the small-spotted catshark infected with parasitic copepod *Neoalbionella globosa*. LOB/ROB – values from the left (uninfected) and right (parasitized) OB from the specimen in the present study; AOB – average values from OBs from Aicardi et al. (2020).

	LOB (Aicardi et al. 2020)	ROB (present study)	Percentage differences LOB-ROB	AOB (Aicardi et al. 2020)	Percentage difference AOB-LOB	Percentage difference AOB-ROB
OB mass (mg)	26	21	–19.2	31.5	–17.5	–33.3
Total cells (× 10^5)	15.3	4.5	–70.6	9.8	56.1	–54.1
Neuron percentage (%)	68.3	63.9	–6.4	72.7	–6.1	–12.1
Total neurons (× 10^5)	10.5	2.9	–72.4	7.1	47.9	–59.2
Cell density (× 10^4)	5.9	2.2	–62.7	3.1	90.3	–29.0
Neuron density (× 10^4)	4	1.4	–65.0	2.2	81.8	–36.4
Total non-neuron cells (× 10^5)	4.8	1.6	–66.7	2.7	77.8	–40.7

the effect of the parasite on the olfactory structures of the host has never been reported. In the twenty *S. canicula* specimens collected during the study, only one parasitic copepod was inadvertently observed; the detection of the presence of parasites was not a specific aim of the study. The potential loss of ectoparasites during fishing could explain the low frequency occurrence (Kvach et al. 2016).

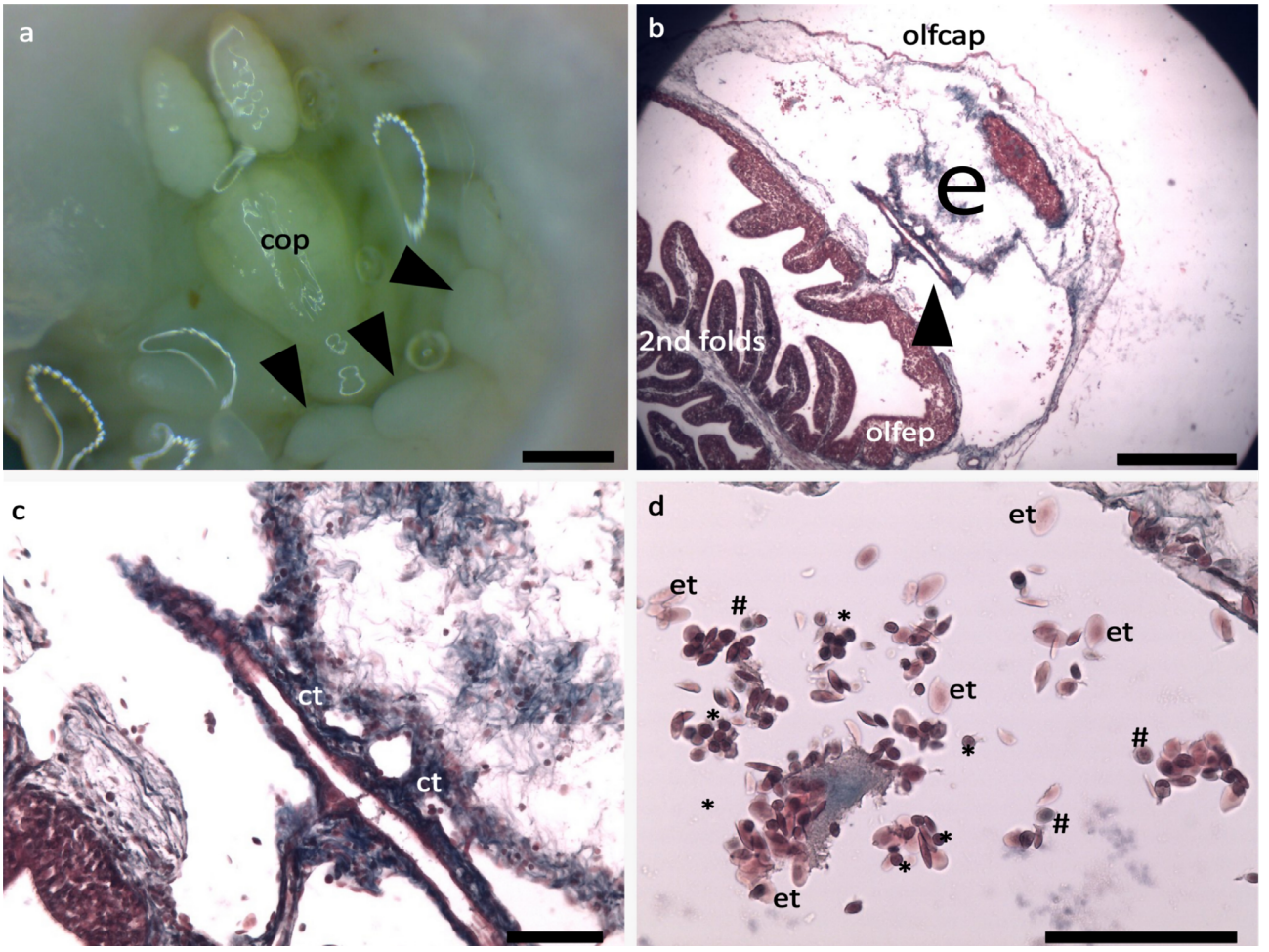

Fig. 3. a) black arrows show the edema on the olfactory lamellae of the olfactory rosette, scale bar 1 mm; b) and c) the wound (black arrow) on the periphery of the olfactory rosette, scale bar 500 µm (b) and 100 µm (c); d) groups of blood cells in the tissue and only in the sections with the wound. Erythrocytes are observed with eosinophils (black asterisk) and monocytes (black hashtag), scale bar 100 µm. Legend: cop – copepod specimen, 2nd folds – secondary folds on the primary olfactory lamellae, e – edema, olfep – olfactory epithelium, olfcap – olfactory capsule, ct – connective tissue, et – erythrocyte.

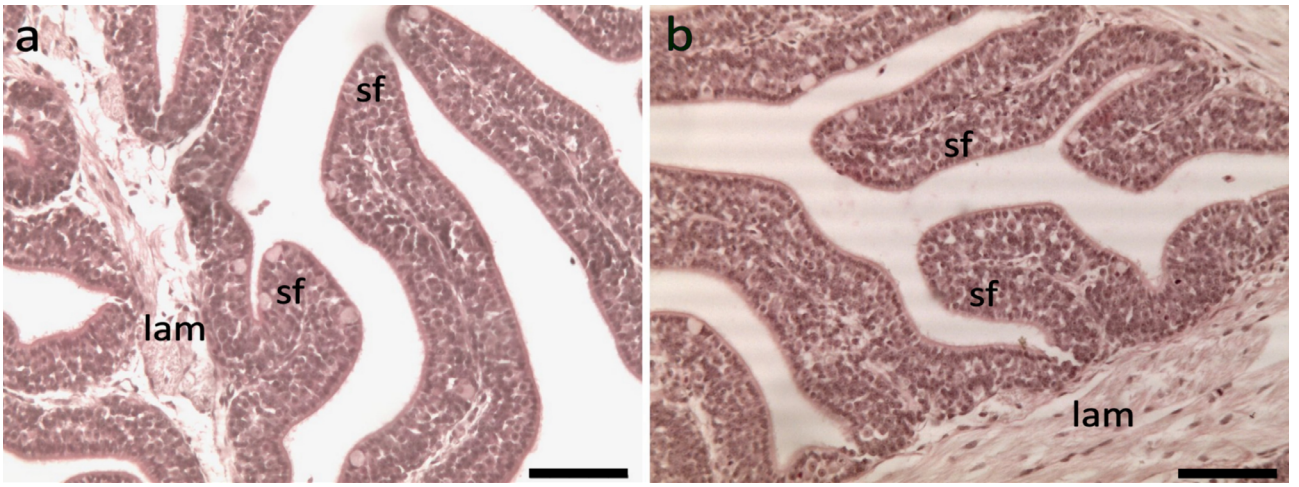

Fig. 4. Olfactory epithelium in both olfactory rosettes: a) uninfected; b) parasitized. The histological sections were taken on the same side of both rosettes. Legend: lam – primary olfactory lamella, sf – secondary lamellae, scale bar 100 μm.

In the Mediterranean Sea, small-spotted catsharks spend their early life on the continental slope at about 200 meters depth. When they reach a size of around 300 mm TL, they start to move to the continental shelf. The specimen in this study was 290 mm TL and was 2.3 years old (Ivory et al. 2005, Aicardi et al. 2020). Initial interactions between hosts and parasitic copepods, usually occurs in deep water (Boxshall 1998), with the adult parasitizing *S. canicula* between summer and autumn (Bakopoulos et al. 2018). The shark examined in this study was sampled in February and the copepod species was identified as *N. globosa* using the key structures, as reported in the results. The length of the cephalothorax, egg sacs and uropods are comparable with the measurement ranges reported by Leigh-Sharpe (1918) and Kabata (1979), except for the length of the maxillae. The difference could be due to the effects of PAF fixation (Põllupüü 2007). The site of parasite attachment confirmed previous observations in the literature, with *N. globosa* parasitizing the olfactory rosette of *S. canicula.*

The olfactory epithelium of the right olfactory rosette did not show damage, at least at a histological level (Fig. 4), and the presence of immune cells was localized close to the site of attachment of the parasite. Macroscopically the olfactory rosette showed swelling at the site of attachment. This swelling could suggest edema on the primary olfactory lamella (Fig. 1), indeed the tissue around the wound, probably due to the copepod's attachment, was fibrous and disorganized. The histological alteration was visible only in proximity to the site of parasite attachment, as suggested by Borucinska & Benz (1999). Alternatively, the disruption of the tissue could have been caused by the removal of the specimen from the olfactory chamber.

Water flow in the olfactory chamber is an important aspect of shark olfaction. Water flow is facilitated by the position of the nostril, the shape of the raphe, the olfactory lamellae and the presence of the cilia on the epithelial cells. The swimming rate and movements of the mouth also play an important role. The water flow ensures that odorants reach the olfactory receptors in the proper way (Cox 2008). In some species the swimming rate is enough to ensure water flow and the right pressure in the olfactory chamber (Johnsen & Teeter 1985, Zeiske et al. 1987). Timm & Fish (2012) observed that the habitat preferences shape nostril morphology. Benthic species, such as *S. canicula,* have well developed nasal flaps, with movements of the mouth ensuring water flow in the nasal chamber even when the animal is stationary. The presence of a foreign body on the rosette, in this case the ectoparasitic copepod, could severely affect the water flow and olfaction. For this reason, we propose that the presence of the parasite could be considered as having the potential for olfactory deprivation. In mammals, and particularly in mice, a significant loss of cells and neurons in the OB was observed due to olfactory deprivation (Meisami 1976, Meisami & Safari 1981), with the main loss in the granular layer; conversely, when one of the nares is occluded, the contralateral OB did not show any hypertrophy or compensatory effect (Corotto et al. 1994). In the teleost *Danio rerio* Hamilton, 1882, the disruption or the total removal of the olfactory organ induced a loss in volume of the OB and a reduction in neuron density and mitral cell arborization. Olfactory deprivation affected the juveniles more than the adults, due to the greater rate of neurogenesis in the early life stages of fishes. Moreover, in fishes with large olfactory brain areas, the effect of deprivation was more evident. However, after three weeks, in the

treated specimens, the olfactory capability recovered normally (Poling & Brunjes 2000, Paskin et al. 2011, Paskin & Byrd-Jacobs 2012).

The evaluation of the number of cells in the parasitized OB showed a lower number of neurons and non-neuron cells compared to the contralateral OB, and also to the average OB of unparasitized *S. canicula* of a similar size range (Table 2). The differences are noteworthy and unlikely could be due to error associated with the isotropic fractionator technique (Neves et al. 2019, Aicardi et al. 2020). If the copepod had hindered the shark's olfaction, the effect would be comparable to olfactory deprivation and it could be the cause of the neuron number decrease in the OB, like that seen in mammals and bony fishes.

The cell density in the uninfected OB was higher than the average of the OB in unparasitized specimens (Aicardi et al. 2020; Table 2), and three times higher than the contralateral, parasitized OB. This finding suggest that the contralateral OB may be able to compensate for the cell loss due to olfactory deprivation on the corresponding side thanks to the high neurogenesis rate in sharks (Docampo-Seara 2020). Nevertheless, this effect has not been observed in other vertebrates and warrants further investigation in sharks using an experimental approach (Corotto et al. 1994).

The mass of the parasitized OB was not reduced to the extent the low number of cells might suggest (Table 2). The volume of the OB depends to a large extent on the presence of glomeruli, i.e. the large bundles of fila olfactoria from the olfactory epithelium. As the parasitized rosette does not show any reduction in the olfactory epithelium, we could reasonably suppose that the glomeruli in both the OBs of specimens of *S. canicula* have a normal and comparable volume. A higher arborization of neurons to compensate for a low level of olfactory stimuli could increase cell size. Indeed, neurons in the OB, at least in mammals, also form a large arborization in the case of total anosmia prior to apoptosis (Petreanu & Alvarez-Buylla 2002). The presence of the parasite in the olfactory chamber was unlikely to cause total anosmia, and the response of the neurons in the OB could be attributed to an alternative reason and needs further clarification.

The number of non-neuronal cells in the parasitized OB was lower than normal (Aicardi et al. 2020; Table 2). In gerbils, after a damage to the OB, the number of glial cells has been shown to increase as a compensation for neuron loss (Hwang et al. 2004). As glial increase was not visible in the parasitized OB of *S. canicula*, the lower number of neurons could be interpreted as a developmental delay instead of neuronal loss. Indeed, the number of neurons in the OB of the one-year younger small-spotted catshark (Aicardi et al. 2020; Table 2) was similar to the number of neurons in the parasitized OB. This finding could be evidence that the host-parasite interaction could have occurred in the previous year, based on the lifespan information of Moore (2001).

In conclusion, the presence of the copepod parasite *N. globosa* infecting the olfactory rosette of the small-spotted catshark could influence the olfactory capability of the host, possibly affecting the development of the OB and the fitness of the parasitized specimen.

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