

Microscopic Characteristics of the Olfactory Organ in the Gluttonous Goby *Chaenogobius gulosus* (Pisces, Gobiidae), Compared to Sympatric Intertidal Gobies

By Hyun Tae Kim and Jong Young Park*

Department of Biological Science and Institute for Biodiversity Research, College of Natural Sciences, Jeonbuk National University, Jeonju 54896, Republic of Korea

ABSTRACT Using stereo, light, and scanning electron microscopes, we researched the anatomical and histological structure of *Chaenogobius gulosus*'s olfactory organ and compared it to those of sympatric gobies *Luciogobius guttatus* and *Favonigobius gymnauchen*. Results revealed the following common characteristics: i) tubular anterior nostril (AN) and flat posterior nostril (PN), ii) a single longitudinal lamella, iii) two accessory nasal sacs (ANS, ethmoidal and lacrimal), iv) abundant sensory epithelium lymphatic cells (LC), v) an eosinophil cell, and vi) a ciliary length a quarter of the knob diameter in the olfactory receptor neuron (ORN). Some characteristics are specific to *C. gulosus* and different from the other two gobies: i) 0.5~1.0 mm AN and 0.2~0.5 mm PN (vs. 0.2~0.3 mm and 0.2~0.3 mm in *L. guttatus*; 0.2~0.4 mm and 0.1~0.3 mm in *F. gymnauchen*), ii) two ANS (vs. absence in *L. guttatus*; two in *F. gymnauchen*), iii) abundant LC (vs. low in *L. guttatus* and *F. gymnauchen*), iv) low density non-sensory cilia on the lamellar surface (vs. high in *L. guttatus*; low in *F. gymnauchen*), and v) a quarter ciliary length to knob diameter ratio in the ORN (vs. mixture of a quarter to equal ratio in *L. guttatus*; two or three times in *F. gymnauchen*). From these results, we confirmed the *C. gulosus* olfactory organ has adapted anatomically and histologically to the sand-rock tidal zone.

Key words: Olfactory organ, nostril diameter, lymphatic cell, non-sensory cilia, a quarter ciliary length

INTRODUCTION

The olfactory organ is a sensory system used for smell. It enables fish to perform ecologically important behaviors such as prey searching, predator avoidance, reproduction, homing migration, habitat cognition, and fright reaction (Hara, 1986). Its morphology and histology varies in teleost fishes allowing them to adapt to diverse aquatic environments. The olfactory organ displays diverse lamellar structures from a lack of folded lamellae (e.g., whitespotted pygmy filefish *Rudarius arcodes*, yellow boxfish *Ostracion tuberculatus*, Japanese medaka *Oryzias latipes*, and Asian swamp eel *Monopterus albus*) (Kim, 2018) to many folded lamellae (120 in the whitespotted conger *Conger myriaster*

and 112 in *Microdonophis erado*) (Kasumyan, 2004). Structural differences in the olfactory organ, such as the nostril's position and shape, the number of nasal sacs, olfactory epithelium cell contents, and the olfactory receptor neuron, (ORN)'s dendrite shape, length and number are also found in taxonomically related and sympatric species (Kim, 2018).

Korean intertidal gobies show habitat preferences for bottom structure, water depth and volume, salinity, and spatial distribution (Kim *et al.*, 2005). Their body specialization and physiological habitat adaptation may also apply to olfactory organ morphological differences (Kim, 2018; Kim *et al.*, 2019). Three mudskippers, the shuffles mudskipper *Periophthalmus modestus* (Kim *et al.*, 2019), the blue spotted mudskipper *Boleophthalmus pectinirostris* (Kim, 2018), and the gigas goby *Scartelaos gigas* (Kim *et al.*, 2014), all intertidal mudflat sympatric fishes, show olfac-

저자 직위: 김현태 (박사 후 연구원), 박종영 (교수)

*Corresponding author: Jong Young Park Tel: 82-63-270-3344,

Fax: 82-63-270-3362, E-mail: park7877@jbnu.ac.kr

tory organ morphological variation among these species. In this study, we focused the olfactory organ of the gluttonous goby *Chaenogobius gulosus* and compared it to the flat headed goby *Luciogobius guttatus* (Kim and Park, 2016) and the sharp-nosed sand goby *Favonigobius gymnauchen* (Kim *et al.*, 2016). *C. gulosus* is an omnivorous species distributed along the western and southern coast of the Korean peninsula and northeastern Japan. It inhabits intertidal zones and estuaries with sandy, rocky, and gravel bottoms (Kim *et al.*, 2005). Other than some ecological studies (Kim *et al.*, 2004; Baeck *et al.*, 2010) and molecular biological analyses (Hirase and Ikeda, 2014; Oh *et al.*, 2016), there has not been a lot of morphological or histological *C. gulosus* research. This study investigates *C. gulosus*'s olfactory organ and analyzes structural differences between sympatric gobies.

MATERIALS AND METHODS

1. Specimen preparation

We collected 20 adult gobies, *C. gulosus* (Fig. 1A, 63 to 94 mm standard length) using a scoop net (4 × 4 mm mesh) from an intertidal pool at Gyeokpo-ri, Byeonsan-myeon, Buan-gun, Chollabuk-do, South Korea in April 2020 (Fig. 1B). We took the fishes to the laboratory and anesthetized

them with MS-222 (Sigma, St Louis, MO, USA). We fixed six in 10% buffered formalin solution (pH 7.4) and the rest were fixed in 2.5% buffered glutaraldehyde (GA) solution (pH 7.4). All procedures followed methods outlined by the Jeonbuk National University Institutional Animal Care and Use Committee.

2. Microscopic investigation

We anatomized and photographed the olfactory organs using a surgical blade, a stereo microscope (SM; Stemi DV4, Carl Zeiss, Germany), and a digital camera (TG-3, Olympus, Japan).

For light microscopy, we washed the buffered formalin organs with tap water for 24 hours. Next, we passed the organs through an ascending alcohol series (70%, 80%, 90%, 95%, 100%), cleaned with xylene, and embedded them in paraffin wax. We cut the block into 5 μm thick slices. We stained the tissue with hematoxylin and eosin (H&E) and Masson's trichrome, and observed the samples using a light microscope (LM, Carl Zeiss, Germany). For the scanning electron microscopy, we washed the buffered GA organs with tap water for 20 minutes three separate times. Next, we post-fixed the organs with 2% OsO₄ in 0.1 M potassium phosphate buffer, dehydrated them in an ascending alcohol series (50%, 60%, 70%, 80%, 90%, 95%, 100%), and immersed them in tert-butyl alcohol. We dried

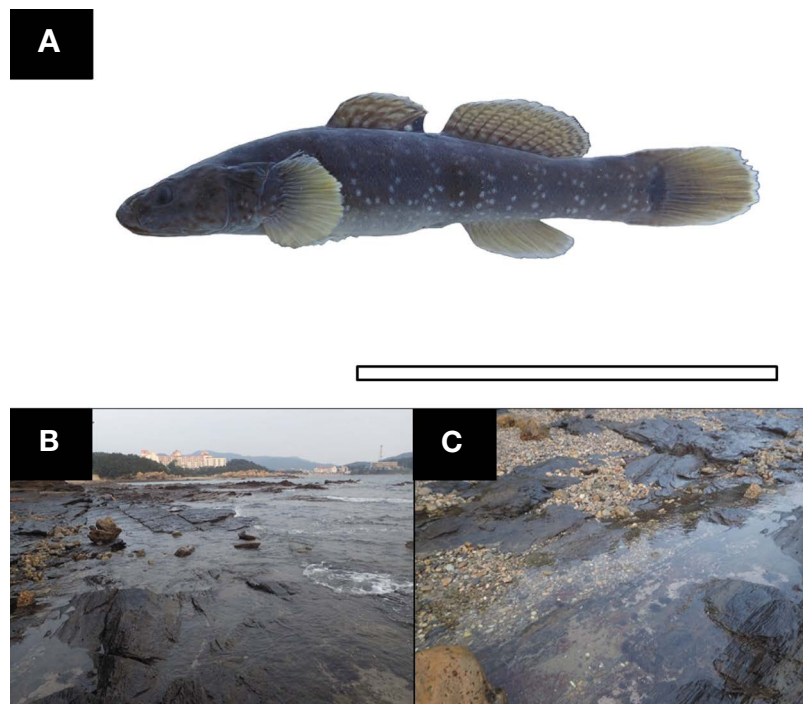


Fig. 1. The photograph (A) of *Chaenogobius gulosus* and its habitat (B) and bottom view (C), 35°38'05"N, 126°27'59"E. The bar indicates 5 cm.

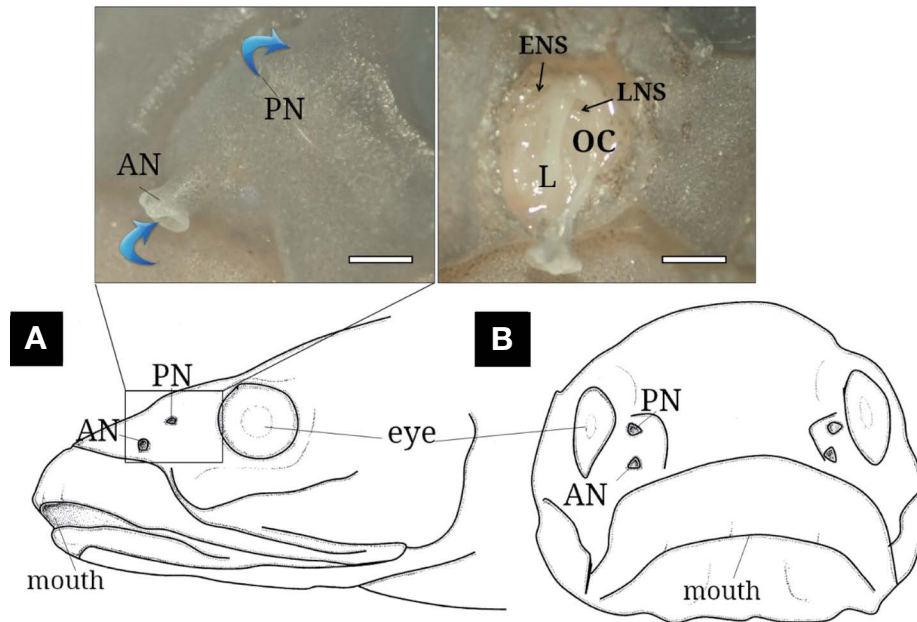


Fig. 2. The schematic diagram of lateral (A) and front (B) views of *Chaenogobius gulosus* and its photographs (above) of the external (left) and inner (right) structures. The blue arrows indicate water flow. AN, anterior nostril; ENS, ethmoidal nasal sac; L, lamellae; LNS, lacrimal nasal sac; OC, olfactory chamber; PN, posterior nostril. The bars indicate 1 mm.

the organs using a freeze-dryer (VFD-21S, Vacuum Device Co., Ltd., Ibaragi, Japan), coated them with OsO₄ powder, and observed them under a scanning electron microscope (SEM; SUPRA40VP, Carl Zeiss, Germany).

RESULTS

1. Anatomy

Externally, the *C. gulosus* olfactory organ is paired on both snout sides and composed of anterior (AN) and posterior (PN) nostrils (Fig. 2A and B). The AN (0.5 to 1.0 mm diameter) is a tubular opening protruding outward. The PN (0.2 to 0.5 mm diameter) lies flat against the skin surface. Internally, the olfactory chamber (OC) is oval with only one longitudinal lamella and leads to the posterior ethmoidal and lacrimal accessory nasal sacs (ACS).

2. Histology

In a cross-sectional view, the OC shows one longitudinal lamella covered by sensory epithelium (SE) on both sides (Fig. 3A and B). The ORN's SE consists of basal cells (BC), supporting cells (SC), lymphatic cells (LC), and eosinophil cells (ELC) (Fig. 3C and D). The ORN is an elongated bipolar neuron with a flattened nucleus located at the cell's middle and colored dark red from the H&E stain and dark

violet from the Masson trichrome stain. Its cytoplasm extends from the bottom to the superficial surface. The SC is an extending cell with cytoplasm that H&E stained weakly. Its nucleus is larger than ORN. The round BC is cell situated at the epithelia's bottom. The LCs are small, dense, and abundant in the basal area. They are dark violet from the H&E stain and dark blue from Masson's trichrome stain. The ELCs are observable white blood cells in the SE's middle layer and have two divided nuclei. They appear as a deep scarlet color in the cytoplasm.

Under the SEM, the longitudinal lamella is exposed inside the OC (Fig. 4A). Its surface shows the ORN's dendrites, which consist of 3~5 short cilia on the knob (Fig. 4B).

DISCUSSION

The intertidal zone is a turbulent environment where the periodic tidal cycle causes constant physical and chemical changes in salinity, water volume, and degree of air exposure (Horn *et al.*, 1999). Based on bottom sediment, these extreme areas can be subdivided into three types, rock, sand, and mud (Masselink and Gehrels, 2014). Our collection site was a sand-rock tidal zone inhabited by three goby species: *C. gulosus*, *L. guttatus*, *F. gymnauchen* (Kim *et al.*, 2005). To survive such extreme surroundings, gobies

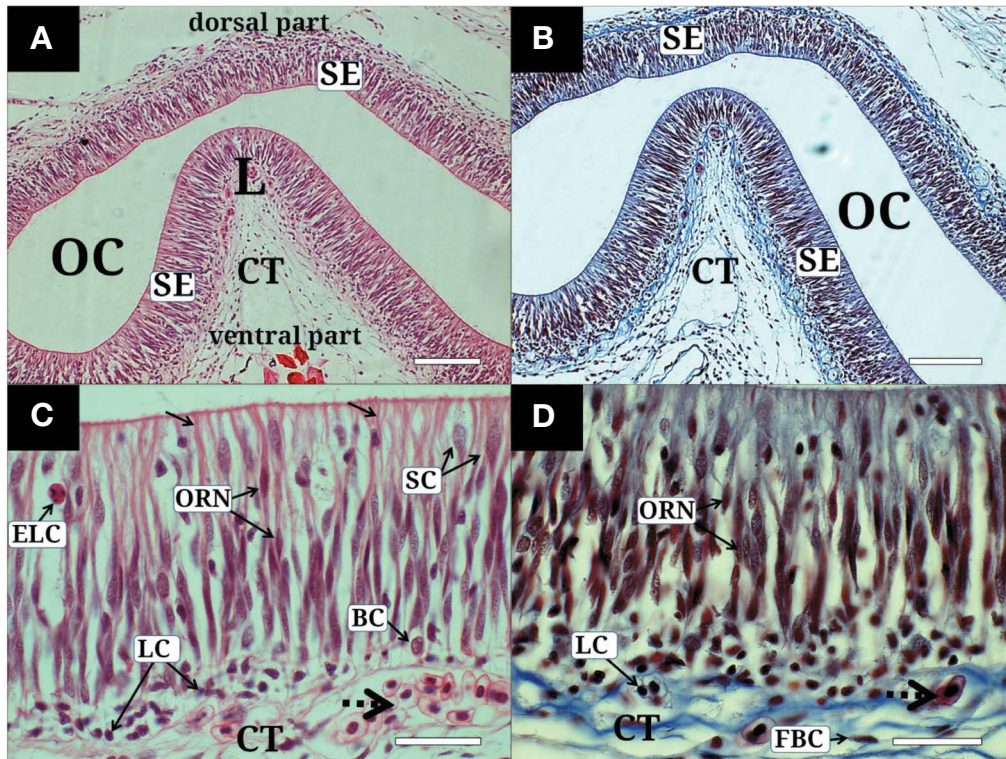


Fig. 3. Histological characteristics of the olfactory epithelium of *Chaenogobius gulosus*, stained with hematoxylin and eosin (A, C) and Masson's trichrome (B, D). A and B, the single lamella covering the sensory epithelium; C and D, the sensory epithelium consisting of olfactory receptor neurons, basal cells, supporting cells, lymphatic cells and eosinophil and the connective tissue with blood capillaries and fibroblast cells. Broken arrow, red blood cell; BC, basal cell; CT, connective tissue; ELC, eosinophil; FBC, fibroblast cell; L, lamella; LC, lymphatic cell; OC, olfactory chamber; ORN, olfactory receptor neuron; SE, sensory epithelium; SC, supporting cells. The bars indicate 200 μm in A and B, 50 μm in C and D, respectively.

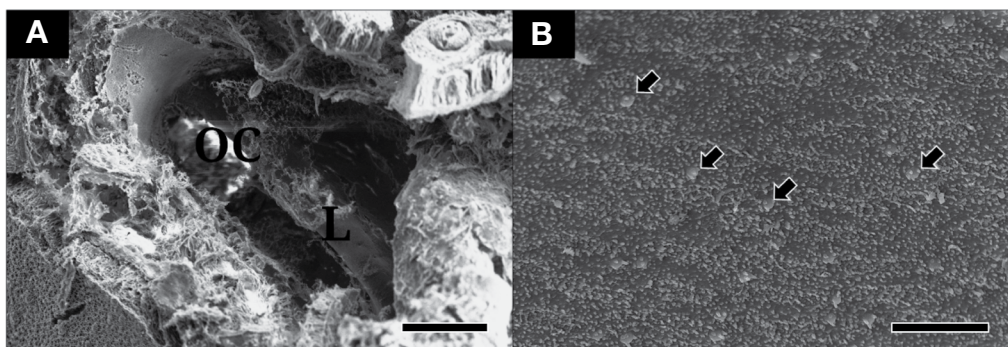


Fig. 4. The olfactory lamella and lamellar surface of *Chaenogobius gulosus*. A, the lamella inside the olfactory chamber; B, the olfactory receptor neuron with 3~5 short cilia. L, lamella; OC, olfactory chamber. The bars 200 μm in A and 5 μm in B.

possess the ability to tolerate rapid salinity changes and occasional body desiccation from air exposure. They can withstand ocean waves and often hide under substances (Horn *et al.*, 1999). To better understand the relationship between *C. gulosus* and its environment, we studied how its olfactory organ adapted to the intertidal zone (viewed in Fig. 1B and C) and compared it with other gobiid fish

(Table 1).

The *C. gulosus* olfactory organ has the following characteristics: i) tubular AN and flat PN lying on the surface, ii) single longitudinal lamella, iii) two ANS (ethmoidal and lacrimal) cells, iv) SE with abundant LCs, v) ELC presence, and vi) ciliary length that is a quarter of the knob diameter. Although all three gobies have tube-forming AN and flat

Table 1. Anatomical and histological differences of the olfactory organ between three sympatric gobies living in the intertidal zone

	<i>Chaenogobius gulosus</i> (this study)	<i>Luciogobius guttatus</i> (Kim and Park, 2016)	<i>Favonigobius gymnauchen</i> (Kim <i>et al.</i> , 2016)
Anatomy			
Diameter of AN (mm)	0.5~1.0 (n=20)	0.2~0.3 (n=13)	0.2~0.4 (n=9)
Diameter of PN (mm)	0.2~0.5 (n=20)	0.2~0.3 (n=13)	0.1~0.3 (n=9)
The number of ANS	2 (ethmoidal and lacrimal)	0	2 (ethmoidal and lacrimal)
Histology			
The density of LC	***	*	*
Ultrastructure of the OE surface			
The density of NSC	*	***	*
The ciliated ORN's ciliary length of KD	A quarter	Mixture of a quarter to equal ratio	Two or three times

min-max (n = number); AN, anterior nostril; ANS, accessory nasal sac; KD, knob diameter; LC, lymphatic cell; NSC, non-sensory cilia; ORN, olfactory receptor neuron; PN, posterior nostril; *, low or lacking; ***, high

PN on their snouts, the AN and PN diameter sizes are different. *C. gulosus* and *F. gymnauchen*'s AN are larger than their PN whereas *L. guttatus*'s AN and PN are similar. Chernova (2008) divided the AN and PN diameter difference into two categories: i) AN larger than PN and ii) AN similar to PN. These morphological variations occurred in fishes with similar ecological habits and can therefore be applied to systematic characterizations. Cox (2008) hypothesized that the sterlet's wider PN compared to the pike is because the fish is more active and benefits from viscous environments. Therefore, *C. gulosus* and *F. gymnauchen*'s larger AN diameter may reflect more active swimming and smoother olfactory cavity ventilation than *L. guttatus*.

C. gulosus's two ANS are the same as *F. gymnauchen* but higher than *L. guttatus*. Generally, ANS of teleost allows an entire olfactory organ volume change through its relaxation and contraction by skeletal movements (Kasumyan, 2004). Kim (2019) states that *P. modestus*'s single ANS is olfactory evidence of terrestrial adaptation compared to the more aquatic mudskipper *B. pectinirostris*'s two. The two ANS of *C. gulosus* may be structural indicators of more active olfactory ventilation than *L. guttatus*. This is supported by non-ANS *L. guttatus*'s abundant non-sensory cilia, which help water flow over the lamella (Table 1; Doving *et al.*, 1977).

C. gulosus contains abundant LCs at the OC's basal area whereas *L. guttatus* and *F. gymnauchen* are lacking (Kim *et al.*, 2016; Kim and Park, 2016). Most teleost fishes produce lymphocytes or LCs to fight infective pathogens as part of a more advanced and acquired immune system (Press and Evensen, 1999). They have also been discovered in other immune cells, plasma cells and macro-

phages, inner organs such as thymus, spleen, kidney, heart and skin (Press and Evensen, 1999). Kim (2018) stated that SE and NSE LCs in five estuary or coastal species are associated with brackish and seawater species survival. In our study, *C. gulosus* have many LCs. *L. guttatus* and *F. gymnauchen*, however, did not. This may mean *C. gulosus* is more sensitive to the intertidal environment.

The ORN's ciliary length varies by fish species: over 0.3~5 µm in the goldfish *Carassius carassius* (Barbar and Boyde, 1968), 2~3 µm in the Zebrafish *Danio rerio* (Hansen and Eckart, 1998), 10 µm in *Acipenser baeri* (Hansen and Zielinski, 2005), 5~6 µm in the European river lamprey *L. fluviatilis* (Thornhill, 1967), and 0.2~0.5 µm in *L. reissneri* (Kim and Park, 2020). Menco (1992) suggested the intertwined, longer cilia increase the interaction area and probability of external odor contact. Measuring exact ciliary length is difficult due to their curved and intertwined structure and position under the SEM. Instead, we measured the ORN's ciliary length by comparing its ratio to the knob. *C. gulosus*'s quarter ciliary length reflects a lower olfactory dependence than the other two species (Table 1).

With some counter staining techniques, a study on anatomy and histology of cell, epithelium, and components of fish olfactory organ well provide how fish has been adapted to fully-aquatic habitat as well as semi-aquatic life in aspect of its morphology and ecology (Kim *et al.*, 2014, 2019; Kim and Park, 2020).

In conclusion, we confirm *C. gulosus*'s olfactory organ, compared to olfactory organs from two sympatric gobies, shows a morphological structure demonstrating 1) its sand and rock bottom, non-turbid water habitat 2) its active ventilation, and 3) its aggressive immunity.

ACKNOWLEDGEMENTS

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (2020R1A6A3A0109608211).

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별망둑 the gluttonous goby *Chaenogobius gulosus* 후각기관의 해부, 조직학적 특성 및 동소 망둑어과 출현종들과의 비교연구

김현태 · 박종영

전북대학교 자연과학대학 생명과학과, 전북대학교 부설 생물다양성연구소

요 약 : 본 연구는 우리나라 서해의 모래와 암반 조간대에 서식하는 별망둑 *Chaenogobius gulosus* 후각기관의 해부 및 조직학적 구조를 확인하고 그 특징을 동소종(미끈망둑 *Luciogobius guttatus*, 날개망둑 *Favonigobius gymnauchen*)들과 비교하였다. 별망둑은 튜브형 전비공, 표면과 평행하는 후비공, 세로배열의 한 개 후판, 두 개 비낭, 후상피 하부에 풍부한 림프구, 호산구, 후감각뉴런에서 후돌기 직경의 1/4의 섬모 길이의 일반적 결과를 보여 주었다. 이러한 특징들 중 1) 0.5~1.0 mm의 전비공 직경, 0.2~0.5 mm의 후비공 직경 (vs. 미끈망둑의 0.2~0.3 mm, 0.2~0.3 mm; 날개망둑의 0.2~0.4 mm, 0.1~0.3 mm의 전비공과 후비공 직경), 2) 감각상피에서의 상대적으로 풍부한 림프구, 3) 비감각섬모의 부재 (vs. 미끈망둑에서 높은 분포; 날개망둑에서 낮은 분포), 4) 후감각뉴런의 후돌기 대비 1/4의 섬모 길이 (vs. 미끈망둑의 1/4에서 1:1 비율 혼재; 날개망둑의 2~3배)는 두 동소종들과 비교되는 특이적인 결과였다. 결론적으로, 별망둑 후각기관의 해부 및 조직학적 특징들은 모래와 암반 조간대에서 다른 동소종들보다 더 적극적인 면역반응과 활동적인 움직임에 적응된 결과로 간주된다.

찾아보기 낱말 : 후각기관, 비공 직경, 림프구, 비감각 섬모, 후감각뉴런 섬모 길이