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Ultrastructural and immunohistochemical characteristics of the olfactory organ Cardinal tetra, *Paracheirodon axelrodi* (Characiformes: Characidae).

Running title: Olfactory system in cardinal tetra

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ABSTRACT

The cardinal tetra *Paracheirodon axelrodi* (Schultz, 1956) belongs to the family Characidae, an economically important and morphologically diverse family of fishes. Information on the olfactory system of this species is scattered and scarce. Among teleost fishes, differences exist in the shape, number, and arrangement of the olfactory lamellae, in the distribution of the sensory and non-sensory epithelium, as well as in the abundance of various receptor cell types. Here, an anatomical and morphological description of the olfactory system was carried out using light microscopic histology, immunohistochemistry, scanning electron microscopy, and transmission electron microscopy. *Paracheirodon axelrodi* is a

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ditremous and isosmat species. It has an arrow-shaped olfactory rosette arrangement. The olfactory epithelium is covering the 12 to 14 lamellae of the olfactory rosette and, using SEM, we observed that the apical surface of the olfactory epithelium carries a dense layer of mucus. Based on the histological, immunohistochemical, and ultrastructural descriptions, all characteristic sensory and non-sensory cell types of the olfactory epithelium of teleost fish were identified. Three types of olfactory receptor neurons were identified: ciliated, microvilli, and crypt cells. The distribution of sensory and non-sensory cell types is like that described in *Aphyocharax anisitsi*, another species of the Characidae family. *Aphyocharax anisitsi* inhabits slow-flowing water bodies with high-density vegetation such as *P. axelrodi*.

Keywords:, SEM, TEM, olfactory epithelium,.

Research Highlights

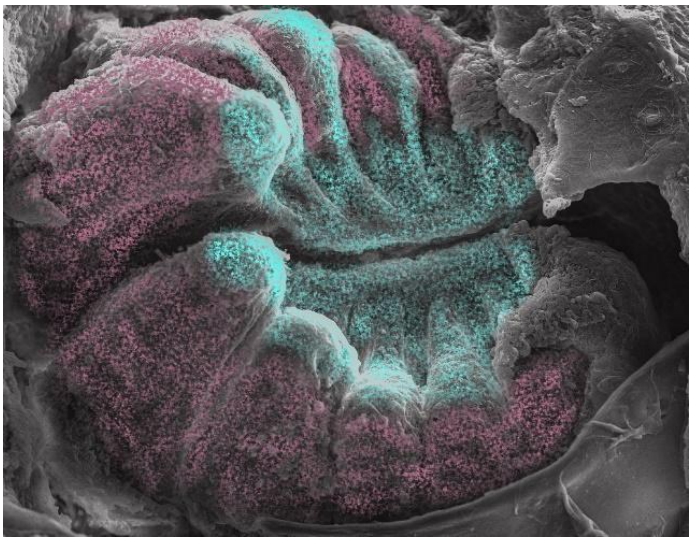
1. This work is the first characterization of the olfactory system in this species and the second description in Characiformes.

2. *P. axelrodi* is a ditremous species in which the olfactory chamber has a skin flap that separates the anterior and posterior nostrils. *P. axelrodi* is an isosmat species depending on the work of kinocilia for water flow into the olfactory chamber. The olfactory rosette (OR) has an arrow-shaped arrangement. The olfactory epithelium (OE) was found covering the 12 to 14 lamellae of the OR and was observed that the apical surface of the OE in *P. axelrodi* presents a dense layer of mucus.

3. The distribution of sensory and non-sensory cell types in *P. axelrodi* is like that described for *Aphyocharax anisitsi*, another Characiform that inhabits slow-flowing water bodies with high-density vegetation such as *P. axelrodi*.

Graphical Abstract

Topographic overview of the olfactory rosette of *P. axelrodi*, showing the areas where exclusively non-sensory cell types (blue) and intermixed sensory and non-sensory cell types (pink) were observed.



1. INTRODUCTION

Reproduction, feeding, the avoidance of predators, intra- and interspecific recognition, and migrations are part of several of the physiological processes on which the life and survival of a species depend. In fish, these functions can be mediated through the olfactory system, where different olfactory chemical stimuli participate, whether they are amino acids, bile salts, prostaglandins, or gonadal steroids (Hamdani & Døving, 2007; Hara 1994; Laberge & Hara, 2001).

The olfactory organ is the peripheral component of the olfactory system. Generally, it is a paired structure in the anterior region of the head and dorsal to the mouth that

consists of an olfactory chamber, which is connected to the outside through the nostrils, having direct contact with the flow of water (Hamdani & Døving, 2007). This general scheme of the olfactory organ has many different types and modifications in fishes (Zeiske, Theisen, & Breucker, 1992), such as the position of the inflow and outflow openings leading to the olfactory organ, their size, the presence of the nasal bridge dividing these openings, and the size of the ridge. For example, in carps (Cyprinidae), perches (Percidae), salmons (Salmonidae), and many other fish species, nostrils are conspicuous, located close to each other, and the nasal bridge bears a large ridge (or skin flap) forming a kind of funnel which directs water into the nasal cavity (Pashchenko & Kasumyan, 1983; Døving, 1986; Zeitske et al., 1992). Sturgeons (Acipenseridae) have a nasal bridge, but the skin flap is very small or absent. Sharksdo not form a nasal bridge, but the lateral and medial edges of theirolfactory openings have well-developed skin outgrowths (lamellae) directed toward each other (Shmalgauzen, 1962; Zeiske, Theisen, & Breucker, 1992).

Inside this olfactory chamber is the olfactory rosette (OR). There are several types of OR, differing by the position of lamellae: radial (Esocidae), arrow-like (Cyprinidae, Salmonidae), parallel (Pleuronectidae), and bilateral (Anguilliformes, Siluriformes), and numerous transitory forms exist between these basic types (Kasumyan, 2004). The central nervous part of the olfactory system comprises the olfactory nerve (ON) formed by the axons of olfactory receptor neurons (ORNs), the olfactory bulb (OB), and higher brain areas involved in the processing of olfactory information (Døving et al., 1977; Hansen & Reutter, 2004). The OBs, in most fish species, are located adjacent to the forebrain and the ONs are quite long. Such bulbs are called sessile. But in Cyprinids, Siluriformes and Gadiformes, for

example, OBs are remote from the forebrain and are adjacent to the olfactory organ (Døving, 1986). The olfactory nerves of these fish are short but the olfactory tracts, going from olfactory bulbs to olfactory centers in the forebrain, are of considerable length. Such bulbs are called stalked (Kasumyan, 2004).

The OR is composed of olfactory epithelium that covers each lamella, which has sensory and non-sensory cells that are interspersed throughout the entire lamella (Kasumyan, 2004; Hansen & Zielinski, 2005). The distribution of sensory cells at the lateral surface of lamellae in different fish species may differ. Continuous, large-zone, fine-zone, and irregular types of sensory epithelium distribution are usually distinguished (Yamamoto & Ueda, 1978; Yamamoto, 1982; Kasumyan, 2004). The non-sensory cell types include supporting cells (SC), goblet cells (GC), and basal cells (BC). The SCs and GCs provide protection and support of the olfactory epithelium, and the BCs guarantee the replacement of epithelial cells and ORNs (Chakrabarti & Ghosh, 2009; Ghosh & Chakrabarti, 2010).However, two different types of sensory cells are recognized in the olfactory epithelium, i.e., ciliated (cORN) and microvillar (mORN), through the identification of the cilia or microvilli extending from the apical olfactory knob (Evans et al., 1982; Hansen et al., 1999). A third type, i.e., the crypt cell (CC), has been observed in bony and cartilaginous fishes and in several teleost species (Hansen & Finger, 2000; Hansen, Anderson, & Finger, 2001; Ferrando et al., 2006).

Cardinal tetra *Paracheirodon axelrodi* is a characid species that lives in the Amazon, Orinoco, and Rio Negro basins (tropical habitat). It inhabits middle water layers with temperatures between 23°C and 27°C. Female spawning occurs in shaded territories (Anjos & Anjos, 2006; Brito & Bazzoli, 2009). The cardinal tetra has high market value and increasing global importance for ornamental

aquaculture. While most species of ornamental fish come from breeding facilities, some are still obtained from their natural habitat since it is difficult for them to grow in captivity (Evers et al., 2019).

The Characidae family, with more than 1352 described species, is the most diverse among Neotropical fish and the fourth most diverse in the world. In this family, a considerable morphological and behavioral diversity has been observed, which is related to the variety of environments that they inhabit (Mirande, 2010). Recently, the olfactory system of *Aphyocharax anisitsi*, another species of the Characidae family, was described (Pintos *et al.*, 2020). However, the information about the olfactory system of species of this taxon is scattered and scarce.

Considering the scarce or sometimes incomplete information on the structural organization of the olfactory system in species of the Characidae family, this work presents a complete description of *P. axelrodi* from an anatomical and morphological point of view, emphasizing the characteristics of the olfactory epithelium cell types by histology, immunohistochemistry, scanning and transmission electron microscopy.

2. MATERIAL AND METHODS

Animals. Adults of *Paracheirodon axelrodi* (Schultz, 1956) were obtained from local commercial aquaria. Animals were housed in community aquaria mimicking their natural conditions: 25°C, pH 5.0 to 6.0. Fish were fed twice a day with commercial fish pellets (Tetra ®) and acclimatized for at least one month. All procedures described in the following sections were conducted in accordance with international standards, the Guide for Care and Use of Laboratory Animals (NRC, 2011) on animal welfare as well as being compliant with local regulations

(Comisión Institucional para el Cuidado y Uso de Animales de Laboratorio, CICUAL, Protocol # 75b/2019).

Twenty adult specimens of *P. axelrodi*, of standard length (SL) of 2.86 ± 0.10 cm, total length (TL) of 3.36 ± 0.38 cm, and weight of 0.42 ± 0.17 g were used. Six individuals were used for light microscopis histology (hematoxylin-eosin and Masson trichrome), five for immunohistochemical assays, three for high-resolution optical microscopy and transmission electron microscopy, and three for scanning electron microscopy. Voucher specimens are deposited at the Ichthyological collection of the Museo Argentino de Ciencias Naturales "Bernardino Rivadavia" (MACN-Ict 12710), Buenos Aires, Argentina.

Histology. Animals (n=6) were anesthetized with 0.1 g/l of benzocaine and sacrificed by decapitation. Heads were fixed in Bouin solution for 24 hr at 4ºC in the dark. Afterward, they were dehydrated through an ascending ethanol series, clarified in xylene, and embedded in Paraplast® (Sigma). Embedded heads were sectioned at 7 µm thickness (sagittally and longitudinally orientated) and mounted on gelatin-coated glass slides. Then, sections were deparaffinized in xylene, rehydrated through a descending ethanol series, and stained with Masson trichrome (MT) or Hematoxylin-Eosin (H-E). Finally, sections were mounted in DPX (Sigma), examined using a Zeiss Primo Star microscope, and digitally photographed with a Canon PowerShot A640 camera.

High-Resolution Optical Microscopy (HROM). Animals (n=3) were anesthetized with 0.1 g/l of benzocaine and sacrificed by decapitation. Heads were fixed in 2.5% glutaraldehyde in phosphate buffer pH 7.2 for two days. Tissues were rinsed three times with phosphate buffer, followed by post-fixation with 2% osmium tetroxide in phosphate buffer for two hours. Tissue samples were rinsed again with distilled

water and dehydrated with ethanol 50%, 70%, 90%, 95%, 100%, a mixture of alcohol and acetone (1: 1), and acetone. After dehydration, tissues were embedded in Poly/Bed®812-acetone 1:2, 1:1, and 2:1, with a final 100% Poli/Bed®812. Samples were polymerized at 60°C. 1-µm-thick semithin sections were obtained with Microm Slee Cut 40650 rotatory microtome and stained with toluidine blue (Piloni et al., 2016).

Transmission Electron Microscopy. For ultrastructure analysis, 130 nm ultrathin sections were obtained using ultramicrotome Leica® EM UC6. The slides were contrasted with lead citrate/uranyl acetate. The samples were analyzed using a Jeol® JEM 1400 Plus 120 Kv transmission electron microscope. Photographs and morphometric measurements were taken with a GATAN® camera attached to this equipment and integrated with the GATAN® Digital Micrograph 1.80.70 program.

Scanning electron microscopy. Heads (n=3) were fixed by immersion in Bouin's solution for 24h at 4ºC in the dark and dehydrated through an ascending ethanol series. Then, the specimens were critical point dried in CO_2 using an EMS 850, Electron Microscopy Science. Most of the dried olfactory organs were dissected from the head to examine single lamellae and fractions. The specimens were coated with gold using a Quorum Technologies SC 7620 and examined with an Electron Microscopy Philips XL 30.

Immunohistochemistry. Animals (n=5) were processed as described for the histological study. After deparaffinization, sections were rinsed in phosphate buffer saline (PBS, pH=7.4) and treated with 3% H_2O_2 for five minutes to saturate endogenous peroxidase activity, followed by the blocking of nonspecific binding sites with 5% nonfat dry milk. Afterward, samples were incubated with primary antibodies (Table 1) overnight at room temperature in a moist chamber. Then,

sections were rinsed in PBS and incubated for 45 min with a biotinylated anti-rabbit IgG (1:600 Millipore-Chemicon). The reaction was developed with the 3,3-diaminobenzidine tetrahydrochloride (DAB) Staining Kit (Dako, Glostrup, Denmark). Sections were slightly counterstained with hematoxylin mounted in DPX and examined. Primary antibodies that recognize specific markers of olfactory neurons: OMP and GαO were previously tested in several fish species (Margolis, 1980; Chuah & Zheng, 1987; Hansen et al., 2004; Belanger et al., 2003; Pintos et al., 2020) and other non-mammalian and mammalian vertebrates (González et al., 2010; Jungblut et al., 2009). For the identification of all the ciliated structures in the lamellar surface, an acetylated tubulin antibody was used, which was also successfully tested in other fish species (Belanger et al., 2003, Dieris et al., 2017). Omission of the primary antibody (negative control) produced negligible background staining (data not shown).

3. RESULTS

3.1 Anatomical description of the olfactory organ

Like most teleost fish, the olfactory organ of *P. axelrodi* is paired and located in the nasal cavity, which connects to the exterior by anterior nostril (inflow) and posterior nostril (outflow) openings. Nostrils were found on the dorsal side of the head, anterior to the eyes, and both were separated by a skin flap (Figure 1A). Inside the olfactory chamber was a pair of symmetrical olfactory rosettes (ORs; Figure 1B, C), each of which leads to an olfactory nerve that connects with the olfactory bulb. This fish has sessile olfactory bulbs (OBs) attached to the telencephalon, so it does not show a long olfactory tract (Figure 1E).

The OR was formed by olfactory lamellae (OL) projecting outward from a midline raphe (Figure 2D). Several morphological types of olfactory rosettes exist in teleost fish, differing by the position of lamellae. Adults of *P. axelrodi* have an arrow-like rosette, which contained 12 to 14 lamellae in each rosette (Figure 1B, C). The anterior and posterior lamellae of *P. axelrodi* differ in lengths, the anterior lamellae being shorter than the posterior lamellae.

3.2 Morphological characteristics of the olfactory organ

The lamellae of the olfactory rosette consisted of two layers of pseudostratified epithelium (Figure 2A-C). These two layers are separated by a thin layer of connective tissue containing: fibroblasts, collagen fibers, nerve fibers, and blood vessels (Figure 2B, C, E). The olfactory epithelium in *P. axelrodi*, as in most teleost fish, contains sensory and non-sensory cells.

Cell types in the olfactory epithelium

Three different olfactory receptor neurons (ORNs) were identified, ciliated (cORNs), microvillous (mORNs), and crypt cells (CCs), which were distinguished by their general morphology by means of different techniques. The cORNs are bipolar, spindle-shaped neurons (Figure 3A, B; Figure 4A, B). Through observation in semithin sections and TEM, the cORNs are characterized by their apical dendritic ends that form a so-called olfactory knob, which extends into the lumen of the nasal cavity. From this olfactory knob, several cilia sprout radially (Figure 4A, B).

The mORNs were bipolar, spindle-shaped neurons too (Figure 3A, B, Figure 4C, D). Through observation in semithin sections and TEM, the mORNs are characterized by their apical dendritic ends that form an olfactory knob, which

extends into the lumen of the nasal cavity. The olfactory knobs of mORNs were often less pronounced than those of cORNs and two centrioles were observed at the olfactory knobs which is a common feature in mORNs (Figure 4C). The dendritic body of the mORNs was shorter and thicker than cORNs, and generally the neuronal soma of mORNs occupies the upper two-thirds of the epithelium. Crypt cells (CCs) are ovoid sensory cells located in the upper third of the olfactory epithelium (Figures 3A, B; 4D). In semithin sections and TEM, CCs had a large nucleus filling the lowest third of the cell. Above the nucleus, the cytoplasm is densely filled with organelles, especially the mitochondria. Crypt cells are difficult to find in TEM preparations since they are scarce (Figure 4D).

All three types of ORNs tend to be basophilic in histological preparations. In TEM preparations, these cells are much more electrondense than the surrounding supporting cells or non-sensory ciliated cells. In TEM preparation, long and longitudinally oriented mitochondria are abundant in the dendrite of all ORNs. The nuclei of all ORNs are often lobed and the chromatin is distributed in the typical "checker-board'' pattern (Figure 4A-D).

The immunoreactivity to OMP varies in the olfactory epithelium of *P. axelrodi* depending on the area of the lamellae. In some areas, most ir-OMP neurons were observed in the mid-basal zone, while in others, ir-OMP neurons were in the mid-apical zone, showing that there is no clear segregation of these neuronal types within the epithelium (Figure 3C-E). Similarly, ir-GαO was detected in ORNs in both neuronal somata located in the mid-basal and mid-apical parts of the olfactory epithelium. Again, this distribution does not show a clear segregation of the types of ORNs. (Figure 3F-G). In addition, some ovoid-shaped cells (coinciding with

CCs) located in the apical region of the olfactory epithelium were also positively stained for GαO (Figure 3H).

We find that in the central area of OR (i.e., the area closest to the raphe), ir-OMP and ir-GαO were observed in the medial zone of the lamella but were absent in the distal and interlamellar zone. In the peripheral area of the OR (i.e., the area furthest from the raphe), ir-OMP (Figure 3C) and ir-GαO (Figure 3F) were observed in the medial and interlamellar zone of the lamella but were absent in the distal zone, which was covered by non-sensory ciliated cells (cNSCs).

In *P. axelrodi*, supporting cells surrounding the receptor cells are represented in the olfactory epithelium by different types: (1) the supporting cells (SCs; Figure 4C), i.e., cells bearing shorts microvilli on the surface, and (2) non-sensory ciliated cells (cNSCs; Figure 4E), i.e., cells bearing numerous kinocilia on the surface facing the cavity of the nasal sac. Additionally, we found (3) goblet cells (GCs) and (4) basal cells (BCs). The SCs are cylindrical with a pronounced basal part resting on the basal lamina. Through observation in semithin sections and TEM, the cell body has a low affinity for staining, and abundant secretion granules are observed in the most apical area of the cytoplasm (Figures 4A-C, 5D).

The cNSCs are cylindrical and extend from the basal lamina to the epithelial surface and their nuclei are elongated (Figure 5D). Multiple kinocilia were identified in light microscopy (Figure 5A), SEM (Figure 5C), TEM (Figure 4E), and following acetylated tubulin immunodetection (Figure 5G). These kinocilia sprout from the broad, flat apex of the cell and are usually somewhat longer than the cilia of ORNs (Figures 4E, 5C). The cNSCs are characterized by a cytoplasm that has a lighter color in light microscopy and is more electron translucent in TEM than the ORN, (Figures 4E, 5D). As mentioned above, the cNSCs were observed

along the surface of the olfactory epithelium, and specifically, this was the only cell type observed in the olfactory epithelium of the distal zone of the lamella (Figure 4F, 5A).

Goblet cells were characterized by having a basal nucleus and having mucin granules in the apical region, which were weakly stained with H-E (Figure 5B, E). By observation in semithin sections and TEM, GCs were abundant on the olfactory epithelium surface, occupying its upper zone. They were mainly located at the interlamellar zone between lamellae, and secretions were exposed on the apical surface, covering the entire olfactory surface (Figure 5F). The mucin granules of GCs had a low affinity for staining too (Figure 5E). Finally, the BCs (Figures 4G, 5D, E) were found in the deepest part of the olfactory epithelium, just above the basal lamina. We observe that in the interlamellar or proximal zone of the lamellae these cells are arranged in several layers, and in the middle and distal part of the lamellae they are arranged in one or two layers only In semithin sections and TEM, BCs are small cells with different shapes. Their nuclei are large in relation to their cell size, some with abundant heterochromatin, others with less (Figure 4G).

4. DISCUSSION

the external morphology of the olfactory organ of teleost fish has either one opening, i.e., is monotremous, or has two openings, i.e., ditremous, which is the most typical among teleost fish (Døving, 1986). *Paracheirodon axelrodi* is a ditremous. Also, we observed a skin flap that separates the anterior and posterior nostrils, as in another species of the Characiformes family, e.g., the bloodfin tetra (*Aphiocharax anisitsi*; Pintos et al., 2020), and in other families such as Cyprinidae, Gadidae, Percidae, and Salmonidae (Døving, 1986; Hara, 1975; Pashchenko & Kasumyan, 1983; Zeiske, Theisen, & Breucker, 1992). This

arrangement seemed essential to achieve the unidirectional flow of water through the olfactory cavity (Kasumyan, 2004).

The olfactory chambers of fish may be ventilated by two different mechanisms for water flow (active ventilation); water flow is either caused by (synchronous) beating of the kinocilia of the ciliated non-sensory cells (cNSCs) of the olfactory epithelium (isosmats), or by active pumping of accessory sacs (cyclosmates) (Døving, 1986). *Paracheirodon axelrodi* is an isosmat species depending on the work of kinocilia for water flow into the olfactory organ and does not present an accessory olfactory sac. The function of these cells is very important because they are responsible for the transport of water which carries the signal to the receptor cells (Kasumyan, 2004). Further, *P. axelrodi* presented an arrow-shaped olfactory rosette arrangement according to the Teichmann classification (Teichmann, 1954), which coincides with the report for bloodfin tetra (*A. anisitsi*; Pintos et al., 2020). The anterior and posterior lamellae were of different lengths in *P. axelrodi*, like species of the families Salmonidae, Cyprinidae, and Clupeidae (Teichmann, 1954).

The morphology of the olfactory system of *P. axelrodi* is like the that of other teleost species (Kasumyan, 2004). *Paracheirodon* axelrodi has a sessile type of the olfactory system, i.e., the OBs are located adjacent to the forebrain (telencephalon) and the ON is quite long. This agrees with the previous partial description for this species (Obando-Bulla et al., 2013) and *A. anisitsi* (Rincón et al., 2016; Pintos et al., 2020), *Astyanax hubbsi* (Riedel, 1997), and *Astyanax altiparanae* (Gomes, Costa, & Borella, 2013). This anatomical arrangement was also observed in other species such as *Danio rerio* (Wullimann, Rupp, & Reichert, 1996), *Oryzias latipes* (Anken & Bourrat, 1998; Ishikawa, Yoshimoto, & Ito, 1999), and *Anguilla japonica* (Mukuda & Ando, 2003).

The olfactory epithelium (EO) covers the 12-14 lamellae of the OR, which seems to be a low number compared to other species such as *Wallago attu* and *Holopagus guentheri* of the Lutjanidae family, in which 164 to 168 and 230 lamellae have been described, respectively (Ghosh & Chakrabarti, 2009, Pfeiffer, 1964). These differences can be better understood when considering that W. *attu* is a demersal species that inhabits places with low luminosity, so the olfactory system may be designed for high sensitivity, resulting in a greater number of lamellae (Ghosh & Chakrabarti, 2009). There is substantial variability in the number of olfactory lamellae among the different species of teleost studied to date. Although there are some species with a similar number of lamellae to *P. axelrodi*, such as *Lampris guttatus*, with 12 to 14 lamellae (Mana & Kawamura, 2002), *Oncorhynchus keta*, showing 16 to 21 lamellae (Kudo et al., 2009), and *A. anisitsi* with 15 lamellae (Pintos et al., 2020). However, there are no further reports on the number of lamellae within other species in the Characidae family, besides those from *P. axelrodi* and *A. anisitsi*. Interestingly, there are some species of fish in which the olfactory lamellae are scarce or completely absent, such as the rainbow fish *Hemiramphus sajori,* in which one to three lamellae are found (Kasumyan, 2004).

As described for teleost, the OR is composed of a determined number of lamellae which is covered by the olfactory epithelium (Hansen & Zeiske, 1998; Kasumyan, 2004; Pintos et al., 2020). The distribution of sensory and non-sensory epithelium along the lamella varies according to species (Yamamoto, 1982) and according to the habitats in which they inhabit (Singh, 1994). For example, the OE of pelagic species can present two types of distribution: (1) a random one, where the sensory and non-sensory epithelium are intermixed along the lamellar surface, which is reported for the benthopelagic silurid *Channa punctatus* (Mandal, Roy, & Ghosh,

2005) and other pelagic species (Mana & Kawamura, 2002); and (2), another distribution in which the sensory epithelium is restricted to the distal zone of the lamella and non-sensory to the interlamellar zone (Singh, 1994, Zeiske, Theisen, & Breucker, 1992).

In *P. axelrodi*, the cNSCs are the only cell type in the distal zone of the lamella, while in the medial and interlamellar zone, the cNSCs and ORNs are intermingled in the epithelium, as was confirmed by immunodetection of OMP and GαO. This distribution in *P. axelrodi* is like that described for *A. anisitsi* (Pintos *et al.*, 2020), as well as for *C. punctatus*, *Carassius auratus*, and *D. rerio* (Hansen et al., 1999; Hansen & Zeiske, 1998; Mandal, Roy, & Ghosh, 2005). *Aphyocharax anisitsi* inhabits slow-flowing water bodies with high-density vegetation such as *P. axelrodi*. Taking this into account, the general organization of the olfactory system of *P. axelrodi* and what was reported in other Characids allow us to speculate that this family presents a structure of the olfactory system for species with similar habitats. However, more ecological, physiological, and evolutionary studies are needed in other species of Characiformes fish to confirm this.

Based on the histological, semithin sections, TEM, and immunohistochemical descriptions, the main cell types of the OE of teleost fish were identified in *P. axelrodi* and by SEM it was observed that the apical surface of the OE in *P. axelrodi* presents a dense layer of mucus. Regarding the ultrastructure of sensory cells, three types of ORNs were identified: ciliated (cORN) with their soma located in the mid-basal region of the epithelium, microvillus (mORN) in the middle region, and crypt cells (CC), in the apical region. The first two types of ORNs (ciliated and microvillous) have been reported in most teleosts (Laberge & Hara 2001). However, CCs have not been described for all the fish species studied

(Hansen et al., 1999, Hansen & Finger 2000; Belanger et al., 2003). In the OE of *P. axelrodi*, CCs were identified by their globose shape and their apical position in the epithelium. These cells may be related to the perception of sexual pheromones, therefore their presence in the epithelium is of great importance (Hamdani & Døving, 2007). In particular, the low number of CCs compared with the other neuronal types coincides with descriptions for other teleosts species (Hansen & Zeiske, 1998).

Regarding the distribution of sensory epithelium, in the central area of *P. axelrodi*'s OR, the ORNs were observed in the medial zone of the lamella but were absent in the distal and interlamellar zone. However, in the peripheral area of the OR, ORNs were observed in the medial and interlamellar zone of the lamella but were absent in the distal zone. In *D. rerio* (Hansen & Zeiske, 1998) and *A. anisitsi*, sensory cells cover the interlamellar valleys, as in *P. axelrodi*. In some species, a distinct pattern is detectable, for example in *Kuhlia sandvicensis* and *C. auratus*, where the sensory epithelium that borders the midline raphe is populated almost exclusively by mORNs (Hansen et al., 1999). A differential distribution of cORNs and mORNs has also been reported for the *Plotosus lineatus* (Theisen et al., 1991; Yamamoto & Ueda, 1978) and some salmonids (Thommesen, 1982, 1983).

In *P. axelrodi*, mORNs and CCs appear to be GαO immunoreactive; however, to confirm the identity and localization of ORN types within the EO, double immunohistochemistry or transmission electron microscopy immunohistochemistry assays are needed. Commercial GαO antibodies have been successfully employed for immunodetection of G proteins in a variety of vertebrates (Belanger et al., 2003; Hansen et al., 2004; Wakabayashi & Ichikawa, 2008). In this regard, some teleost species show differences in G protein expression. For example, in *C.*

auratus, *Ictalurus punctatus* and *Periophthalmus barbarous*, GαO allowed the identification of mORNs and CCs (Kuciel, et al., 2014; Hansen et al., 2003; Hansen et al., 2004), whereas in *Neogobius melanostomus*, GαO was only labeled in mORNs (Belanger et al., 2003).

Olfactory marker protein (OMP) is a protein originally described in mammals and expressed in mature olfactory and vomeronasal sensory neurons of vertebrates (Farbman & Margolis, 1980; Rössler, Mezler, & Breer, 1998). This protein has been suggested to be involved in neurogenesis, modulation of the olfactory signal, and olfactory epithelium renewal (Riddle & Oakley, 1992; Ferrando et al., 2007b). In *D. rerio* (Sato, Miyasaka, & Yoshihara, 2007) and *Oncorhynchus mykiss* (Riddle & Oakley, 1992), ir-OMP has been observed in cORNs, exhibiting the same morphology and location in both species. In *A. anisitsi*, OMP antisera labeled the CCs (Pintos et al., 2020) coinciding with observations in *Raja clavata* (Ferrando et al., 2007a). Likewise, in *P. axelrodi*, more than one neuronal type of ORNs was identified by the presence of OMP immunoreactivity, as described in *Scyliorhinus canicula* (Ferrando et al., 2007b). And, for example, in *Xenopus laevis*, it has been reported that two OMP subtypes are regionally expressed in the olfactory nasal epithelium. The lateral location of *XOMP1* and medial location of *XOMP2* correspond to the suggested locations of ORNs responsive to water-borne and air-borne odorants, respectively (Rössler, Mezler, & Breer, 1998). Although our immunoreactivity studies for GαO and OMP do not allow definitive conclusions as to the identity of ORNs within the EO of *P. axelrodi*, considering the morphological and immunohistochemical analysis of serial sections from the raphe and up to the most peripheral areas of the OR, we were able to clearly determine, which areas are covered by sensory and non-sensory epithelium in the OR.

The spatial organization of the sensory and non-sensory epithelium in *P. axelrodi* is similar to that described for *A. anisitsi* (Pintos *et al.*, 2020), another species of the family Characidae, as well as for *C. punctatus*, a pelagic species that develops in stagnant waters (Mandal, Roy, & Ghosh, 2005), and *C. auratus*, which also inhabits standing and slow-flowing bodies of water with high-density vegetation (Yamamoto & Ueda, 1978; Zippel & Hansen, 1997). Thus, that this spatial disposition might be associated with habitats that share the aforementioned characteristics but, more studies should be done on other species in similar environments to confirm these patterns.

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Table 1: Antibodies used for immunohistochemical detection.

	Primary AB	**Origin/ Dilution**	**Identified cellular structure**	**Secondary AB**	**Streptavidin**
OMP	Sigma-Aldrich (RRID: AB_796160)	Rabbit (1:800)	Olfactory mature neuron (Margolis, 1980; Chuah & Zheng, 1987)	Biotinylated anti-rabbit IgG, 1:600, Millipore-Chemicon (RRID: AB_916366)	HRP 1:600, Sigma
GαO	Santa Cruz Biotechnology Inc (RRID:	Rabbit (1:1000)	G protein associated to olfactory receptor	Biotinylated anti-rabbit IgG, 1:600, Millipore-	HRP 1:600, Sigma

	AB_2111638)		(Hansen, Anderson, & Finger, 2004)	Chemicon (RRID: AB_916366)	
Acetylated Tubulin	Santa Cruz biotechnology Inc (RRID: AB_628409)	Mouse (1:100)	Specific tubulin from cilia (Belanger et al., 2003)	Biotinylated anti-mouse 1:300, Dako (RRID: AB_2722538)	HRP 1:600, Sigma

FIGURE LEGENDS

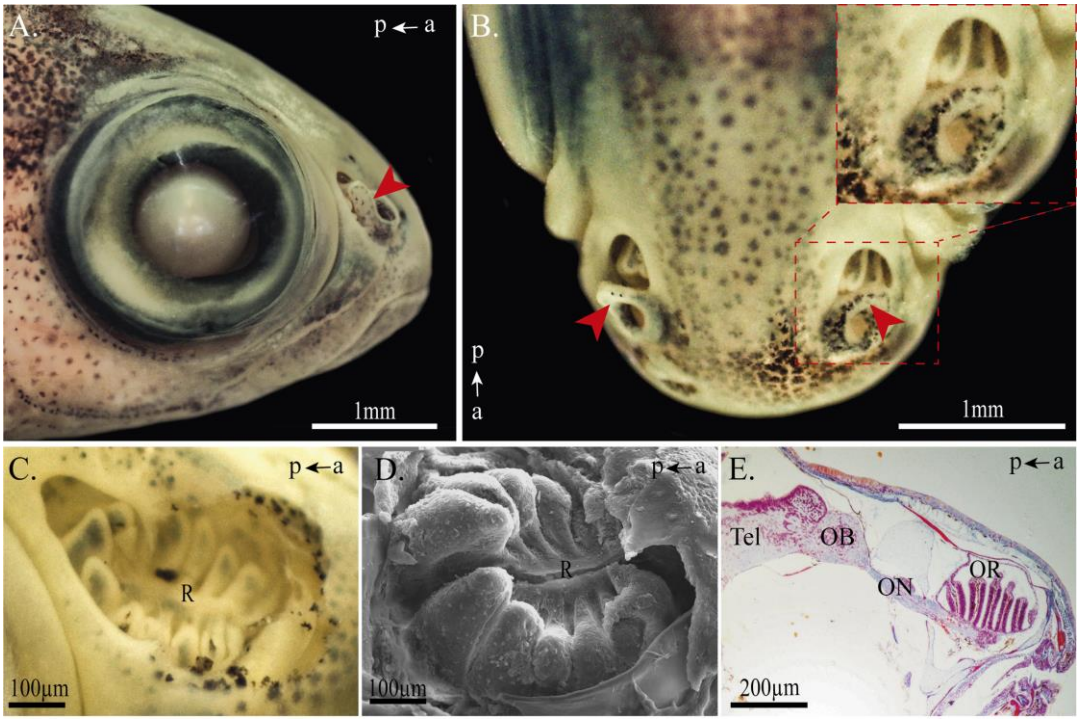

Figure 1. *Paracheirodon axelrodi*, anatomical arrangement of the olfactory chamber fixed in Bouin's solution (A-C). **(A)** Lateral view of the head under stereoscope showing the "skin flap" (red arrowhead) that separates anterior and posterior nostrils. **(B)** Dorsal view of the head under stereoscope showing the "skin flap" (red arrowheads) that separates anterior and posterior nostrils. Insert shows a detail of the "skin flap" of the left nostril. **(C)** Dissection of "skin flap" that shows the anatomy of the OR. **(D)** Scanning electron micrograph showing the OR inside the olfactory chamber and posterior lamellae radiating from the central raphe (R).

(E) The olfactory system in sagittal sections is colored with Masson Trichrome. The OB is adjacent to the telencephalon and the ON, projecting from the OR to the OB. a-p, antero-posterior axes; OB, olfactory bulb; ON, olfactory nerve; OR, olfactory rosette.

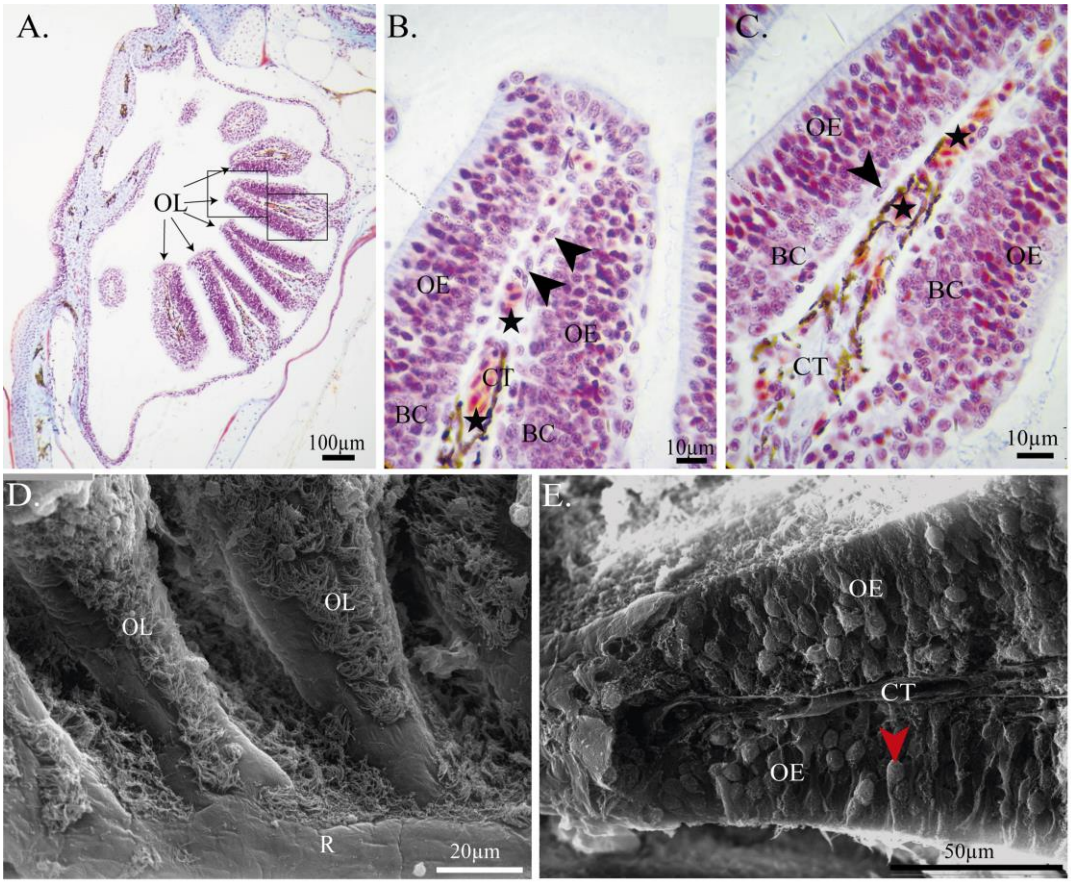

Figure 2. *Paracheirodon axelrodi*'s anatomy of the olfactory system. **(A-C)** Sagittal sections of OR stained with Masson Trichrome. **(A)** Panoramic view of the OR and OL in sagittal sections. **(B)** Detail of the medial and distal zone of OL indicated in A. Each lamella was composed by two layers of the OE and separated by a central core of CT. Fibroblast (black arrowhead) and blood vessels (black star) of the CT. **(C)** Detail of the medial and proximal zone of OL indicated in A. BCs in the deepest part of the OE. **(D)** Scanning electron micrograph showing kinocilia of

cNSCs in OL and areas of in the midline R characterized by epidermal cells with micro ridges. **(E)** Scanning electron micrograph showing a section of the OL. An ORN was observed (red arrowhead) with a dendrite reaching the upper part of the EO. BSc, basal cells; cNSCs, non-sensory ciliated cells; CT, connective tissue; OE, olfactory epithelium; OL, olfactory lamellae; OR, olfactory rosette; ORN, olfactory receptor neuron; R, raphe.

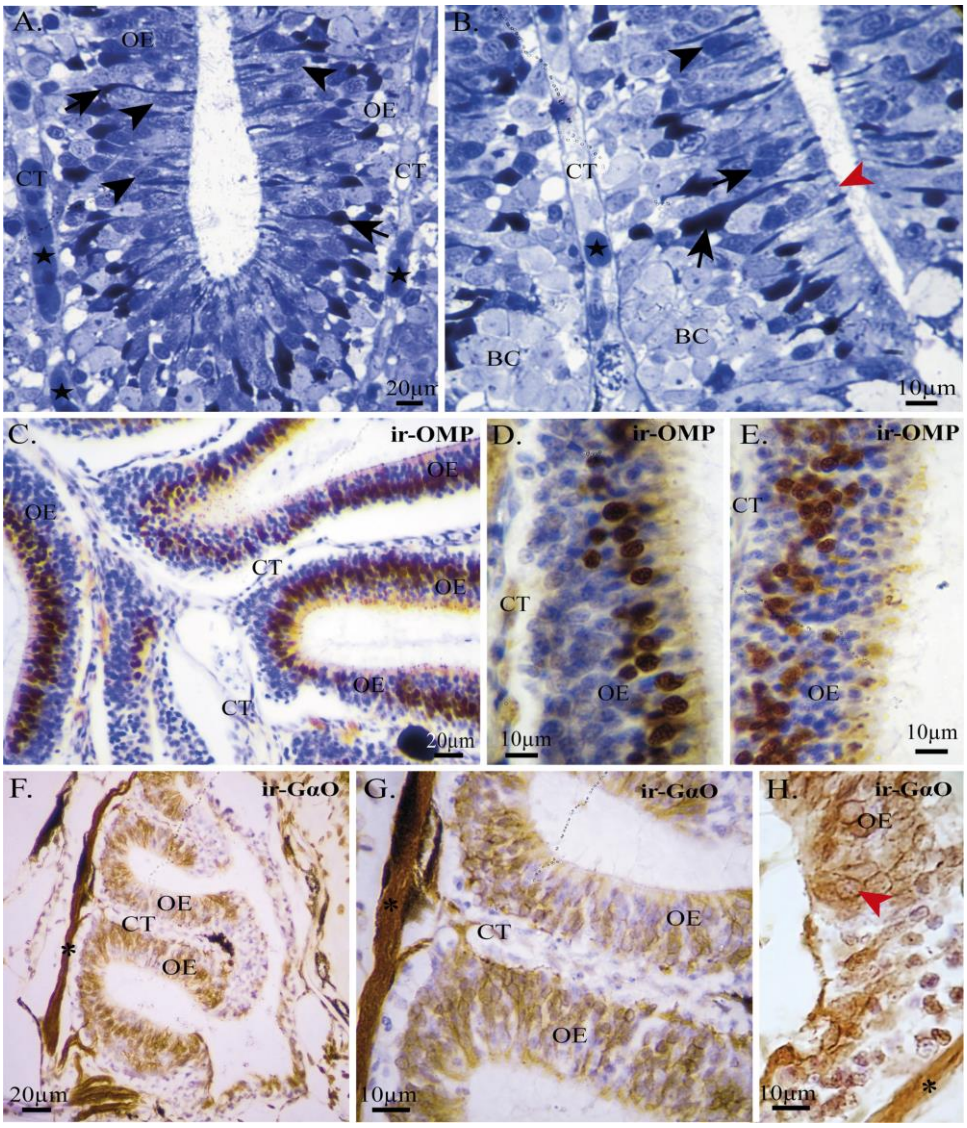

Figure 3. *Paracheirodon axelrodi*'s different types of ORNs in the olfactory epithelium. **(A, B)** Semithin sections stained with Toluidine Blue, showing

different types of ORNs in the OE. Ciliated olfactory receptor neurons (cORNs) (black arrows) showing long and darkly stained soma. Microvillous olfactory receptor neurons (mORNs) (black arrowheads) showing smaller nuclei than cORNs but equally darkly stained. Crypt cells (CCs) (red arrowheads) showing large nucleus filling the lowest third of the ovoid cell. **(C)** Sagittal section of the central area of OR (this is the area closest to the R), where ir-OMP ORNs show that they were located in the mid-basal region of the OE (lamellae on the right portion of the photograph, and detail in **D**), but also located in the mid-apical region of the EO (lamellae on the left portion of the photograph, and detail in **E**), showing that there is no clear segregation regarding the distribution of these cell types in the epithelium. **(F)** Horizontal section of peripheral area of the OR, where ir-GαO were observed in ORNs present in the medial and interlamellar zone of the lamella but were absent in the distal zone. **(G)** Detail of **F** where ir-GαO was observed in cells throughout the entire OE. **(H)** Horizontal section of the central area of OR, where an ir- GαO CCs (red arrowhead) was observed in the apical region of OE. Asterisk, ir- GαO in olfactory axon bundle; OE, olfactory epithelium; OR, olfactory rosette; ORNs, olfactory receptor neurons; R, raphe.

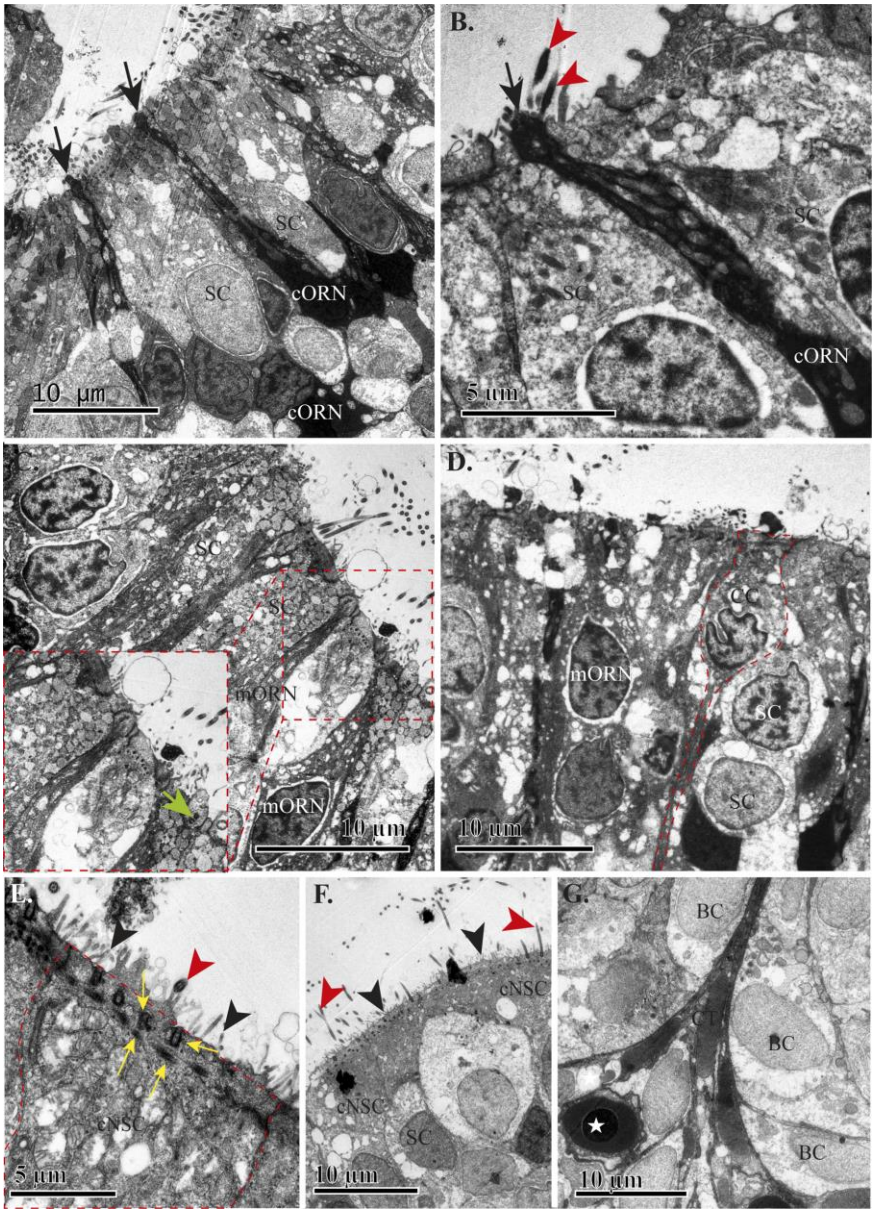

Figure 4. TEM micrographs of the olfactory epithelium of *P. axelrodi*. **(A, B)** Overview and detail of OE in the medial zone of the lamellae, where some cORNs were observed with their apical dendritic ends that form an olfactory knob (black arrows), which extends into the lumen of the nasal cavity. The olfactory knob had exposed apical cilia (red arrowheads). The cORNs are surrounded by some SCs. **(C)** Overview of OE in the medial zone of the lamellae, where some mORNs were observed with their apical dendritic ends and a dendritic body shorter. The insert shows a detail of two centrioles (green arrow) visible at the apical end (olfactory

knob) of a mORNs. The mORN was surrounded by some SCs with their characteristic granules in the cytoplasm. **(D)** CCs (segmented red line) in the upper third of the OE, that had a large nucleus filling the lowest third of the ovoid cell. The CC are surrounded by some mORNs and SCs. **(E)** Detail of the apical portion of a ciliated non-sensory cell (cNSCs) (segmented red line) in the OE in the medial zone of the lamellae. Red arrowheads indicate cilia and black arrowheads indicate short microvilli and yellow arrows indicate basal bodies. **(F)** Detail of the distal zone of the lamella where only SCs and cNSCs were observed. Red arrowheads indicate cilia and black arrowheads indicate short microvilli. **(G)** The basal portion of the OE with basal cells (BCs) and blood vessels (black star) of the adjacent CT. BCs, basal cells; CCs, crypt cells; cNSCs, non-sensory ciliated cells; cORNs, ciliated olfactory receptor neurons; CT, connective tissue; mORNs, microvillous olfactory receptor neurons (ORNs); OE, olfactory epithelium; R, raphe; SCs, supporting cells.

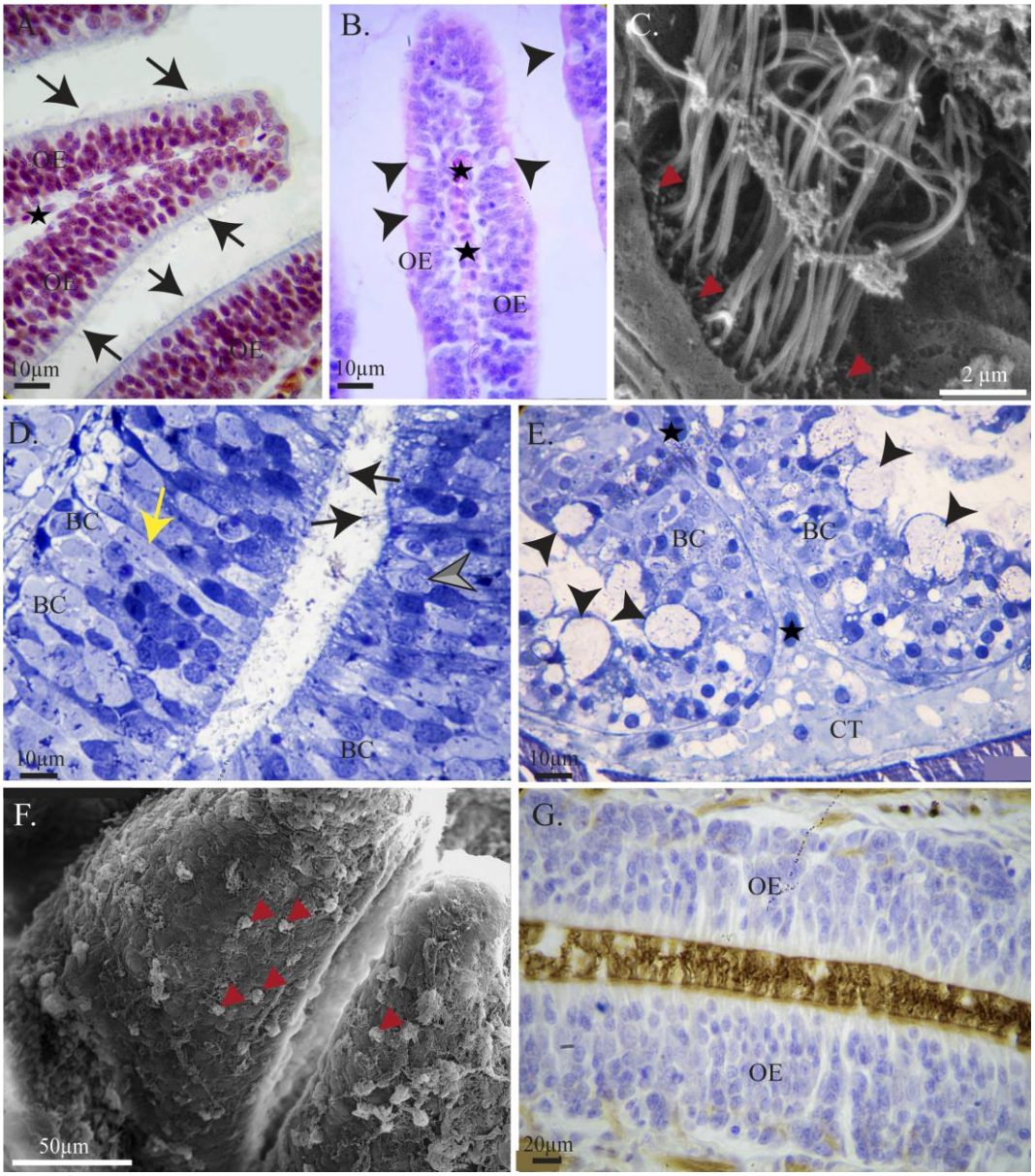

Figure 5. *Paracheirodon axelrodi*, non-sensory cells in the olfactory epithelium. **(A)** Sagittal section of OR, showing cNSCs with kinocilia (black arrow) of the OE stained with Masson Trichrome. **(B)** Sagittal sections of OR, showing GCs (black arrowhead) at the upper zone of the epithelium stained with H-E. **(C)** Scanning electron micrograph showing multiple kinocilia of cNSCs that sprout from the broad flat apex of the cell and that are surrounded by microvillous (red arrowhead). **(D)** Semi-thin sections stained with Toluidine Blue, showing cNSCs with kinocilia (black arrows), SCs (yellow arrow) which are polygonal epithelial cells with basal

nuclei, and CCs (gray arrowhead). **(E)** GCs (black arrowheads) in the upper zone of the epithelium with accumulated mucin granules within their cytoplasm. **(F)** Scanning electron micrograph showing GCs with abundant mucin granules on their apical surface (red arrowheads). **(G)** cORNs and cNSCs cilia were immunoreactive for acetylated tubulin all around the surface of the OE. BCs, basal cells; Black star, blood vessels; CCs, crypt cells; cNSCs, non-sensory ciliated cells; cORNs, ciliated olfactory receptor neurons; CT, connective tissue; GCs, goblet cells; OE, olfactory epithelium; OR, olfactory rosette; SCs, supporting cells.