# OLFACTORY ORGAN MORPHOLOGY AND ULTRASTRUCTURE OF THE LARVAL KOH TAO ISLAND CAECILIAN (*ICHTHYOPHIS KOHTAOENSIS*)

By

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# ABSTRACT

## OLFACTORY ORGAN MORPHOLOGY AND ULTRASTRUCTURE OF THE LARVAL KOH TAO ISLAND CAECILIAN (*ICHTHYOPHIS KOHTAOENSIS*)

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The olfactory system of living amphibians (Lissamphibia) undergoes major changes as it transitions from an aquatic to a terrestrial system during metamorphosis. Patterns of change in the cellular morphology of the nose have been examined for frogs (Anura) and salamanders (Caudata). However, it remains unknown if caecilians (Gymnophiona) have similar patterns of change in their nasal ultrastructure. In particular, no data on larval caecilian olfactory cell types are available. Here, using light microscopy and transmission electron microscopy, I examined the olfactory organ of larvae of the caecilian *Ichthyophis kohtaoensis*, to establish the ultrastructure of the epithelium and compare it to that of other amphibians. I found that there are microvillar receptor cells, ciliated receptor cells, and secretory supporting cells in the main olfactory epithelium (MOE) of *I. kohtaoensis*. However, in the posterior portion of the main olfactory cavity (MOC), the cells appear disorderly and "loose" with a haphazard orientation, in comparison to the anterior portion of the cavity where cells are neatly arranged and closely packed. There are only ciliated receptor cells and secretory supporting cells in the posterior MOC. The vomeronasal organ (VNO) of *I. kohtaoensis* has microvillar receptor cells, secretory supporting cells, ciliated supporting cells, and supporting cells with both

cilia and microvilli. Interestingly, similar cell types and a disorderly appearance of the posterior main olfactory epithelium have also been described in adult *Typhlonectes compressicauda,* the only other caecilian for which ultrastructural data exist. Apart from *I. kohtaoensis* not having any ciliated supporting cells in the main olfactory cavity, the epithelium of both the MOC and the VNO resembles that of other amphibian larvae.

# ACKNOWLEDGEMENTS

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# INTRODUCTION

The tetrapod group Lissamphibia (or Amphibia) consists of three orders: Anura (frogs and toads), Caudata (salamanders), and Gymnophiona (caecilians) (Frost et al., 2006). As the name suggests, most amphibians have aquatic (larval) and terrestrial (juvenile and adult) life stages. When amphibians metamorphose, many morphological changes occur in association with the change in environment, including the transition of the olfactory systems from an aquatic, water-smelling larval system to a terrestrial, air-smelling adult system (Duellman & Trueb, 1986). This anatomical remodeling of the olfactory system takes place on two major levels. First, the general morphology of the olfactory organ itself changes, usually from a smaller, simpler olfactory organ to a larger organ with more sophisticated features, including an expansion of the main cavities and addition of some features like ridges (Reiss & Eisthen, 2008). Second, the cellular morphology (ultrastructure) of the olfactory organ also changes, to accommodate the switch from smelling in water to smelling in air. The modality of olfaction in the two media are very different. Water is dense and viscous and the odorants it transports are hydrophilic and can be quite large. In comparison, odorant molecules in air are generally volatile and much smaller (Eisthen & Schwenk, 2008; Hemilä & Reuter, 2008).

At the gross morphological level, the main olfactory system of amphibians consists of a pair of nasal sacs. Each nasal sac begins with an external naris opening to a short vestibule, which leads into a larger main olfactory cavity (MOC). The MOC terminates at the internal naris, which is open ventrally to the buccal cavity (Reiss &

Eisthen, 2008). The accessory olfactory system consists of the vomeronasal organ (VNO) which is open to the MOC and generally is positioned along the ventrolateral side of it. The MOC and the VNO each contain sensory epithelium. They send axons to the olfactory bulb and the accessory olfactory bulb of the brain, respectively (Allison, 1953; Reiss & Eisthen, 2008; Schmidt & Wake, 1990).

At the ultrastructural level, amphibians—and tetrapods in general—have four possible main cell types in the main olfactory epithelium (MOE) lining the MOC: ciliated receptor cells, microvillar receptor cells, ciliated supporting cells, and secretory supporting cells (Allison, 1953; Bloom, 1954; Reiss & Eisthen, 2008). Various combinations of these cell types are found in the sensory epithelia of the MOC and the VNO during the different life stages, and across different species.

In larval anurans, all four main cell types are typically present in the MOE, while only microvillar receptor cells and ciliated supporting cells are present in the VNO (Benzekri & Reiss, 2011). There are few data on larval salamanders, but larval and neotenic *Dicamptodon* as well as a multitude of other species with neotenic adults have three general cell types in the MOE: ciliated receptor cells, microvillar receptor cells, and secretory supporting cells. Additionally, larval and neotenic *Dicamptodon* have ciliated supporting cells in their MOE. In the salamander VNO, when present, there are variable combinations of microvillar receptor cells, secretory supporting cells and ciliated supporting cells (Benzekri & Reiss, 2011; Stuelpnagel & Reiss, 2005). There are no larval ultrastructural data available for caecilians. In fact, the only caecilian ultrastructural data available are from an adult specimen of the secondarily aquatic

*Typhlonectes compressicauda*, from the family Typhlonectidae. In *T. compressicauda* there are ciliated receptor cells, microvillar receptor cells and secretory supporting cells in the anterior MOC, and only ciliated receptor cells and secretory supporting cells in the posterior MOC (Saint Girons & Zylberberg, 1992). The *T. compressicauda* VNO contains only microvillar receptor cells and secretory supporting cells (Saint Girons & Zylberberg, 1992).

The arrangement, pattern and presence of various cell types in the olfactory organ of amphibians can generally be correlated with lifestyle, though with a number of exceptions (reviewed by Benzekri & Reiss 2011, see their Supporting Information Table 1). Most commonly, the MOE of aquatic individuals contains two types of receptor cells, microvillar and ciliated; the supporting cells also are of two types, ciliated and secretory, and there are no (or poorly-developed) associated Bowman's glands (which function to secrete mucus). In contrast, the air-smelling MOE of terrestrial individuals typically has only ciliated receptor cells and secretory supporting cells, and has well-developed Bowman's glands. Species combining terrestrial and aquatic lifestyles as adults can have both types of epithelia present in the MOE. Unlike the MOE, the organization of the VNO epithelium shows no obvious pattern of correlation with lifestyle (Benzekri & Reiss, 2011).

Caecilians are much harder to find, collect, and study than other groups of amphibians, and little is known about their nasal ultrastructure. Moreover, the family Typhlonectidae, the only family for which ultrastructural data on the caecilian olfactory system are available, are a highly derived, secondarily aquatic, viviparous family. By

contrast, the family Ichthyophiidae is an early-diverging group that retains oviparity with an aquatic larval stage (Duellman & Trueb, 1986). This makes species in the family Ichthyophiidae important candidates for study of the caecilian olfactory organ, because their morphology can help us understand the ancestral condition within caecilians and amphibians in general (Carroll, 2009; Kamei et al., 2012; Nussbaum & Treisman, 1981). Here, I describe the general morphology and ultrastructure of the larval olfactory organ in the Koh Tao Island caecilian (*Ichthyophis kohtaoensis)*.

# MATERIALS AND METHODS

## Study Specimens

All *Ichthyophis kohtaoensis* specimens used in this study were a gift from Prof. Dr. Werner Himstedt (Department of Zoology, Technical University of Darmstadt, Germany). Care and maintenance of the embryos and larvae, before fixation, were carried out in the animal rooms of Humboldt State University in Fall 2001, and all specimens were fixed prior to the start of the study (see IACUC Protocol # 2020B90-A). A total of 14 specimens were used in this study (Appendix A); 12 larvae and two embryos in stages 31–32 (Dünker, Wake, & Olson, 2000).

## Tissue Preparation for Light Microscopy

Whole heads of *I. kohtaoensis* specimens, previously preserved in either 10% neutral-buffered formalin or aqueous Bouin's solution (Humason, 1979), were decalcified using RDO rapid decalcifier (Apex Engineering Products Corporation, Aurora, IL), dehydrated through an alcohol series, cleared with toluene and embedded in Paraplast® (Sigma Aldrich). Ten µm sections were cut with a rotary microtome and fixed on slides coated with Haupt Gelatin Fixative (Humason, 1979) and a 3% formalin solution. Slides were dewaxed, stained with hematoxylin and eosin, and cover slipped (Humason, 1979).

## Image Viewing and Capture for Light Microscopy

Slides were viewed on a Nikon Eclipse E400, and images were captured using a Nikon Coolpix 4500 digital camera. Adobe Photoshop 24.0.1 was used to adjust the brightness and contrast of the digital images as well as to crop, label, and remove artifact from the negative spaces of some images.

## Tissue Preparation for Electron Microscopy

Whole heads of *I. kohtaoensis* specimens (embryonic and larval) were fixed using 3% glutaraldehyde in 50 mM cacodylate buffer and stored until further processing in 0.035 M cacodylate buffer at 4$^{o}$C. Decalcification was done over a period of three days. Each day the specimen was submerged in a fresh 1:1 solution of 0.1 M ethylenediaminetetraacetic acid disodium salt (EDTA) and 0.035 M cacodylate buffer. After decalcification, specimens were returned to a 0.035 M cacodylate buffer solution and stored at 4$^{o}$C until further processing. Specimens were secondarily fixed using 1.5% osmium tetroxide, dehydrated through an ethanol series, and embedded in Spurr's epoxy resin (Spurr, 1969). Thin sections (75-95 nm) were cut using a diamond knife mounted in a Leica Ultracut R ultramicrotome and post-stained with 1% uranyl acetate and 0.4% lead citrate.

## Image Viewing and Capture for Electron Microscopy

A Phillips EM 208S transmission electron microscope (TEM) was used to examine *I. kohtaoensis* sections, and micrographs were taken at an accelerating voltage of 60kV. After development, the micrograph negatives produced in the TEM were directly scanned to produce digital images using an Epson V700 scanner. Adobe Photoshop 24.0.1 was used to adjust the brightness and contrast of the images as well as to crop, label, and to remove artifact from the negative spaces of some images.

# RESULTS

## General Morphology of the Larval Olfactory Organ

The larval olfactory organ (Figure 1A) extends from the external naris at its rostral end to the internal naris (choana) at its caudal end, where it meets the buccal cavity. At the rostral end of the snout a groove begins to form in the integument. The groove leads into the larger, circular opening of the external naris. Directly posterior to the external opening of the naris is the vestibule (Figure 1B), or entrance canal, of the olfactory organ. The short vestibule soon transitions into the main olfactory cavity (MOC) and the sensory epithelium becomes more prominent (Figure 1C). The MOC continues to widen until about midway through the organ. From here back, the cavity maintains its width until just before it terminates in the choana, at which point it quickly narrows to the size of the choanal opening. The height of the MOC is similar throughout the cavity.

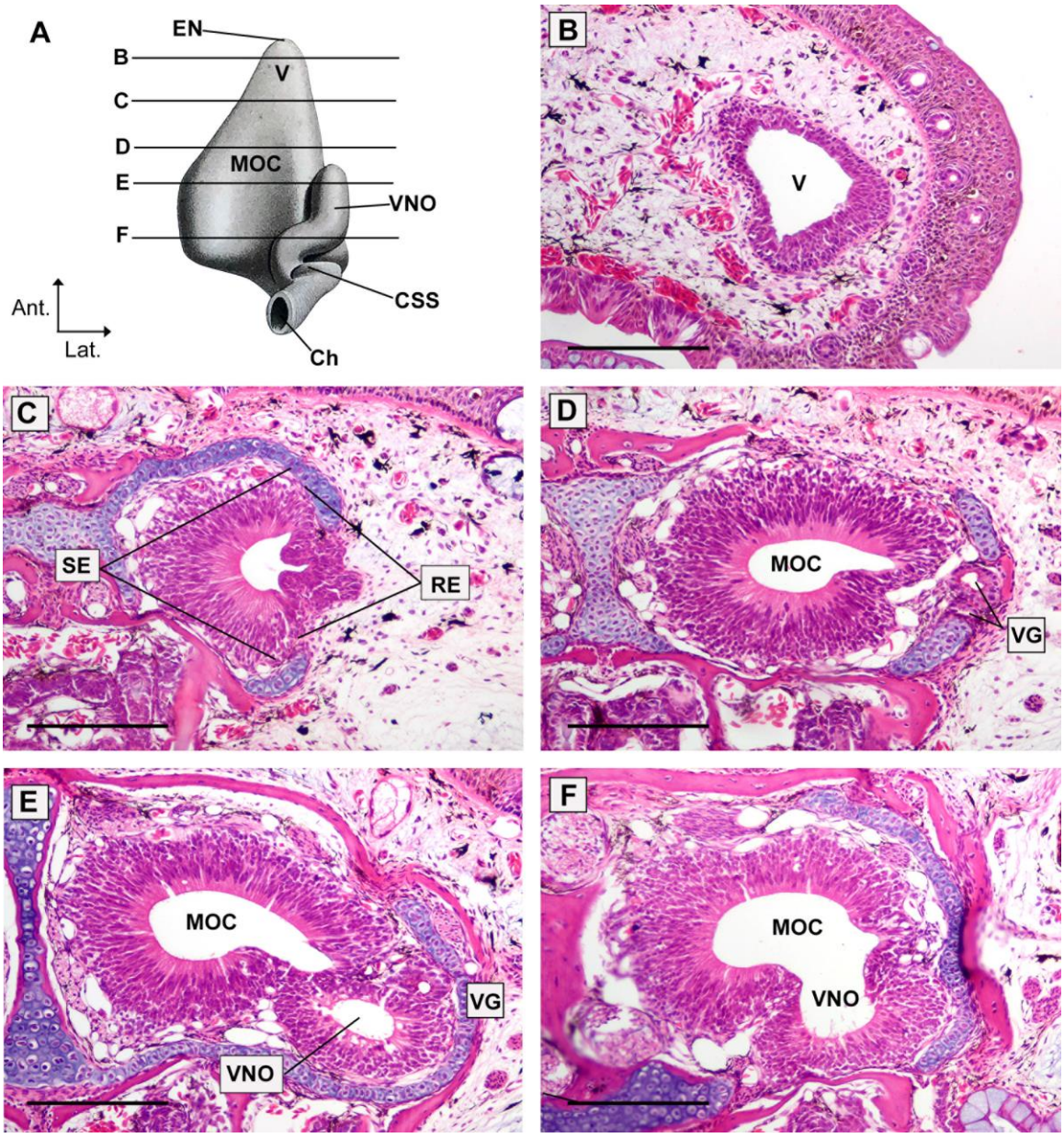


**Figure 1.** Light micrographs of transverse sections through the anterior portion of a larval olfactory organ of *Ichthyophis kohtaoensis*. (Specimen ID ICKO1) **A:** Illustrated ventral view of the left olfactory organ of *I. glutinosus*, adapted from Sarasin and Sarasin (1889). The labeled lines correspond with the approximate regions of the cross sections shown in panels B - F. **B:** The vestibule (V) posterior to the external nares (EN). **C:** The transitional area from vestibule to main olfactory cavity (MOC). Sensory epithelium (SE) begins medially and transitions laterally as the vestibule gives way to the MOC. The non-sensory respiratory epithelium (RE) recedes laterally as the sensory epithelium takes over. **D:** The MOC, posterior to the vestibule, fully transitioned into sensory epithelium. **E:** MOC and the anterior end of the vomeronasal organ (VNO). **F:** The VNO is open to the MOC and starting to transition medially. Additional abbreviations: CSS – choanal slime sac; Ch – choana, VG – vomeronasal gland. Scale bars = 0.5mm.

Approximately midway back through the organ, where the MOC has reached its full width, the sigmoid-shaped vomeronasal organ (VNO) begins. The anterior VNO is a blind pouch that lies ventrolaterally along the MOC (Figure 1E). As it continues posteriorly it soon opens to the MOC (Figure 1F) and stays open to the MOC as it shifts medially and eventually ends, still open to the MOC (Figure 2B). Tucked in posterior to the VNO but anterior to the choana is another nasal structure, the choanal slime sac (Figure 2B & C). The MOC and the choanal slime sac end posteriorly open to the choana, which opens into the buccal cavity (Figure 2D). Backflow from the buccal cavity into the choanae is prevented by the presence of a choanal valve (Figure 2D).

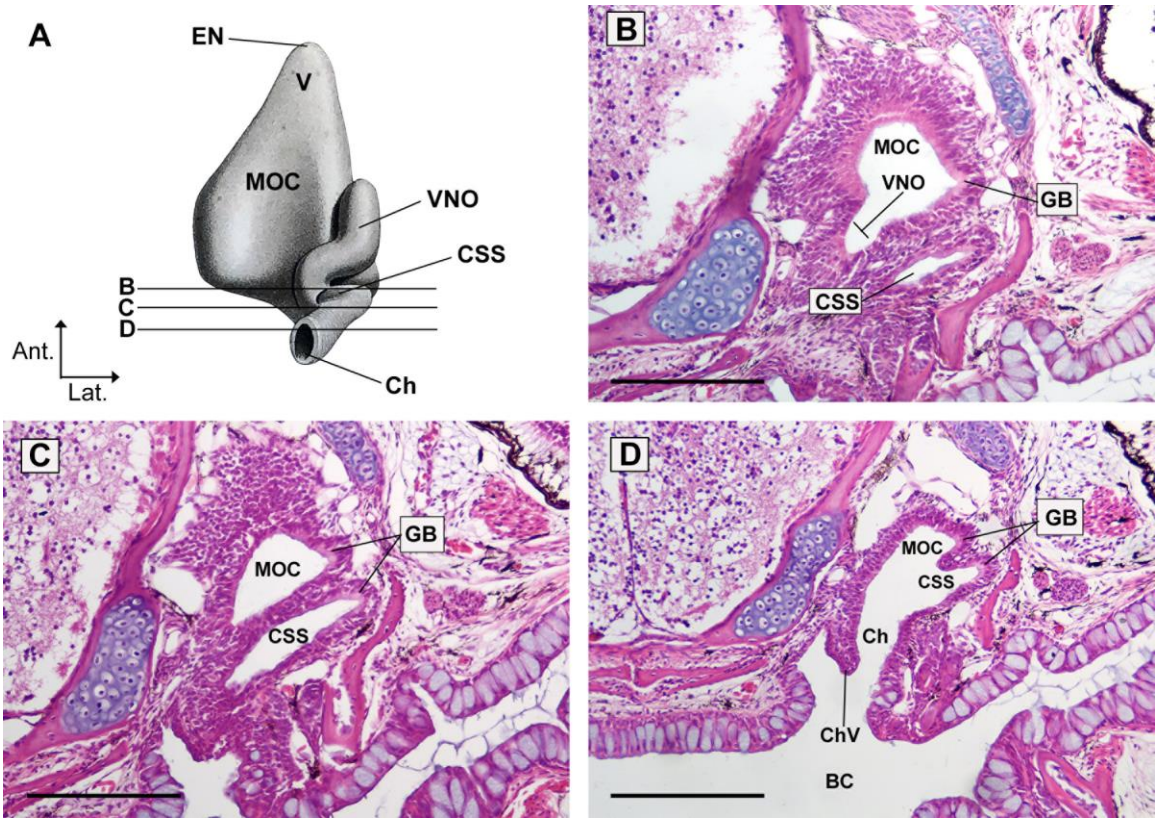


**Figure 2.** Light micrographs of transverse sections through the posterior portion of the larval olfactory organ of *Ichthyophis kohtaoensis*. Same individual as in Figure 1. **A:** Illustrated ventral view of the left olfactory organ of a larval *I. glutinosus*, adapted from Sarasin and Sarasin (1889). The labeled lines correspond with the approximate areas of the organ where cross sections shown in panels B, C, and D originated. **B:** The posterior end of the vomeronasal organ (VNO). The main olfactory cavity (MOC) is narrowing, and a few goblet cells (GB) are appearing in the ventrolateral "corner" of the MOC. **C:** The choanal slime sac can now be seen separate from and ventral to the MOC. There are a few goblet cells (GB) in the lateral portion of the CSS. The VNO can no longer be seen. **D:** The MOC and the CSS have opened to the choana (Ch) which is open to the buccal cavity (BC). The choanal valve (ChV) is present at this level, preventing back flow from the BC. Additional abbreviations: EN – external nares; V – vestibule. Scale bars = 0.5mm.

## Epithelium of the Larval Olfactory Organ

The lining of the olfactory organ includes sensory (olfactory) epithelium and non-sensory (respiratory) epithelium. Both are a pseudostratified columnar epithelium, but in the non-sensory areas the columnar cells are much shorter in comparison to the elongate, "skyscraper" columnar cells of the olfactory epithelium, whose nuclei are far removed from the apical end of the cells. The vestibule epithelium is non-sensory (Figure 1B). As the vestibule transitions into the MOC the epithelium also transitions and becomes primarily sensory (Figure 1C). The sensory tissue begins medially and extends dorsally and ventrally as one moves more posteriorly, restricting the non-sensory epithelium, which folds in on itself laterally and eventually gives way to the sensory epithelium almost entirely. At this level the MOC is teardrop shaped, with the point of the teardrop located laterally (Figure 1D). Only on the lateral part of the floor of the MOC, which eventually migrates ventrally to open into the VNO, does a patch of non-sensory epithelium remain. The epithelium of the VNO is sensory throughout, except on the dorsal portion of each side where it transitions into the MOC (Figure 1F). Both the choanal slime sac and the choanae contain only non-sensory epithelium (Figure 2C & D).

## Ultrastructure

### Respiratory Epithelium

At the ultrastructural level, the non-sensory respiratory cells present with a more electron-dense cytoplasm than the cells in the sensory tissue. Their nuclei are seen nearer

to the apical surface than the nuclei of the sensory epithelium and the cisternae of the Golgi apparatus can often be seen near the nucleus (Figure 3A–D). The apical surfaces of the respiratory cells can vary throughout the organ. In the vestibule, the cells are covered in loosely spaced short microvilli and contain small inconsistently shaped and spaced electron-lucent secretory granules near the apical end (Figure 3A). On the ventrolateral portion of the MOC, in addition to the typical respiratory cells, there are non-sensory cells that have secretory granules and few to no microvilli. The granules in these cells vary greatly in size, shape, and electron density (Figure 3B). In the non-sensory epithelium at the boundary of the VNO, found dorsally on both sides in the transitional areas from VNO to MOC, there are large ciliated respiratory cells (Figure 3C & D). Some of these respiratory cells (*REm* of Figure 3C) have electron lucent secretory granules that resemble those seen in the vestibule (Figure 3A).

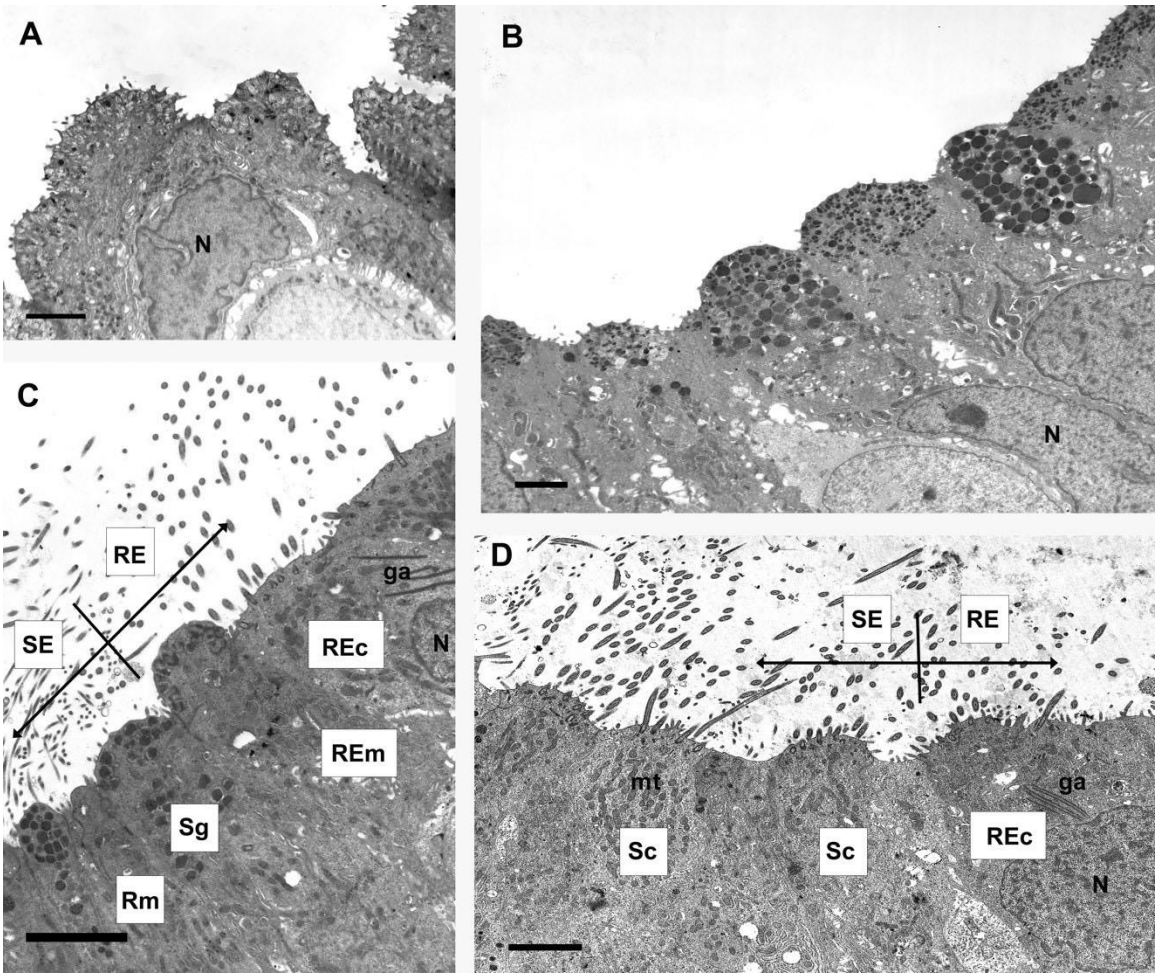


**Figure 3.** Transmission electron micrograph of respiratory epithelium in the vestibule, main olfactory cavity and VNO of larval *Ichthyophis kohtaoensis***. A**: Respiratory epithelium of the vestibule. **B**: Secretory cells of the non-sensory tissue in the main olfactory cavity (MOC). Note the different sizes and electron densities of the many secretory granules. **C**: Respiratory epithelium (RE) of the vomeronasal organ (VNO) towards the right and sensory epithelium (SE) towards the left. In the RE some respiratory cells have microvilli (REm) and others have cilia (Rec). In the SE there are microvillar receptor cells (Rm) and secretory supporting cells (Sg) with secretory granules and microvilli. **D**: Large ciliated supporting cells (Sc) with many mitochondria (mt) are found in the sensory epithelium of the VNO and ciliated (REc) cells in the respiratory epithelium (RE). Additionally, the cisternae of the Golgi apparatus (ga) can be seen near the nuclei of the ciliated respiratory cells in C and D. Scale bars = 3µm

Main Olfactory Cavity – Sensory Epithelium

The overall cellular composition of the sensory tissue in the anterior MOC is generally uniform. There are no large patches or highly concentrated areas of one specific cell type. There are, however, small clusters where one cell type is more numerous than another. The overall cellular composition of the sensory tissue in the posterior MOC appears disorderly and presents with a distinctive cellular composition.

Receptor Cells of the Anterior Main Olfactory Cavity. Two types of receptor cells are present here. Microvillar receptor cells are usually seen between two towering, secretory supporting cells (Figure 4A–C). Ciliated receptor cells are also seen between two supporting cells and often appear packed with mitochondria (Figure 4D). The ciliated receptor cells have numerous basal bodies anchoring the cilia into the cells (Figure 4D). A terminal web, made from sub-membranous filaments in the zonula adherens, is seen forming a band across the supporting cells (Figure 4D). A bulbous apical end of the cell, or olfactory vesicle, is present in both ciliated and microvillar receptor cells (Figures 4 and 5). The ciliated receptor cells are approximately 1.5–2 µm in width at their apical end, wider than the microvillar receptor cells, which measure about 1 µm, but are narrower than the supporting cells, which measure 2–3 µm (Figure 4).

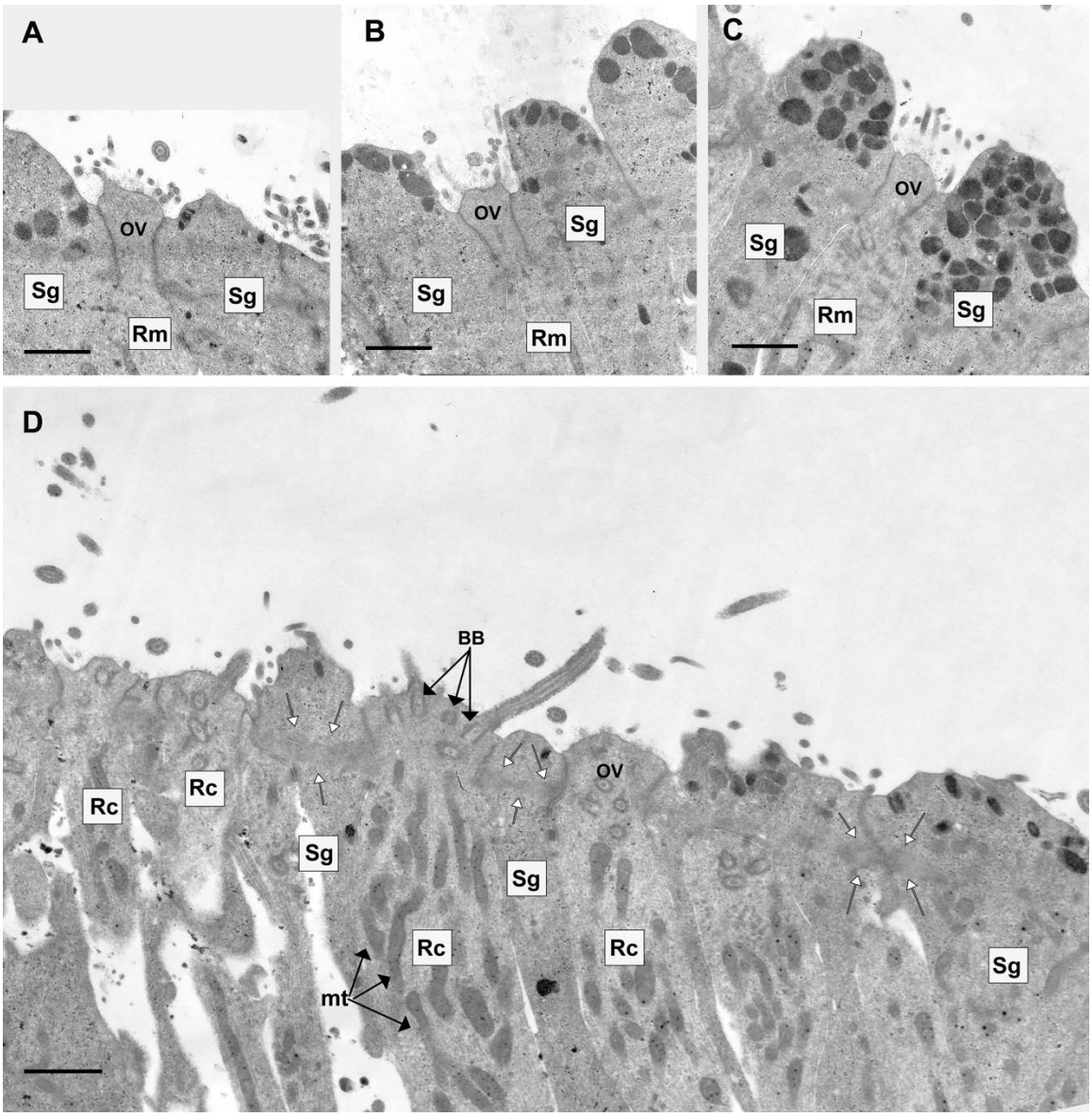


**Figure 4.** Transmission electron micrograph of receptor cells in the main olfactory cavity (MOC). **A, B, C:** All have a microvillar receptor cell (Rm) in between two large, secretory supporting cells with secretory granules (Sg). This is a prevalent situation in the anterior portion of the MOC. **D:** A multitude of ciliated receptor cells (Rc), each between secretory supporting cells (Sg). Note the presence of a terminal web in the supporting cells (white tipped arrows). Additional abbreviations: OV – olfactory vesicle, BB – basal body, mt – mitochondria. Scale bar = 1µm.

Supporting Cells of the Anterior Main Olfactory Cavity. The majority of the supporting cells in the anterior MOC are secretory, contain large secretory granules, and tower around the receptor cells (Figure 4A-C and Figure 5). However, in some areas, the supporting cells do not tower around the receptor cell, nor are there many granules present (Figure 4D). Some supporting cells of the MOC have short, infrequent microvilli and some do not.

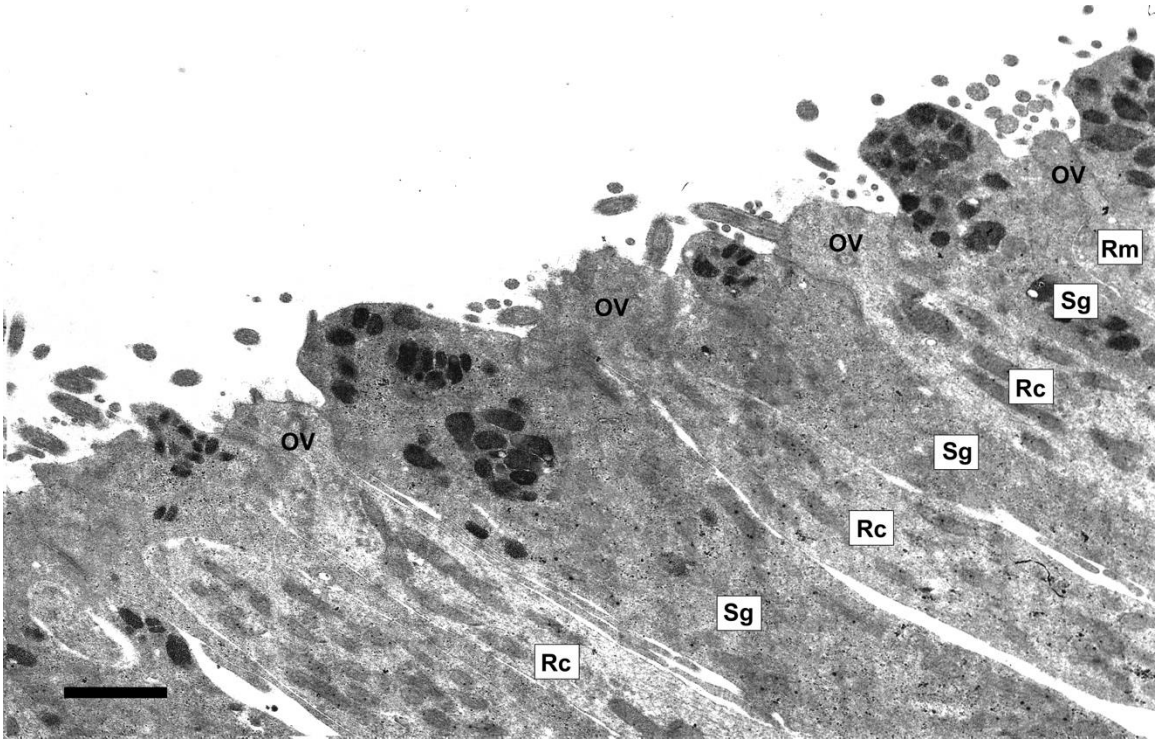


**Figure 5**. Transmission electron micrograph of ciliated (Rc) and microvillar (Rm) receptor cells in the main olfactory cavity (MOC). Receptor cells lie between secretory supporting cells (Sg). Additional abbreviation: OV – olfactory vesicle. Scale bar = 1µm.

Receptor Cells of the Posterior Main Olfactory Cavity. The only receptor cells found in the posterior MOC were ciliated receptor cells. In an angled section through the epithelium, they are seen extending up between supporting cells before they appear on

the apical surface of the tissue (Figure 6). In some areas of the posterior MOC there do not appear to be many, if any, receptor cells, but in other areas of the posterior MOC there appear to be receptor cells in equal abundance to the receptor cells of the anterior MOC.

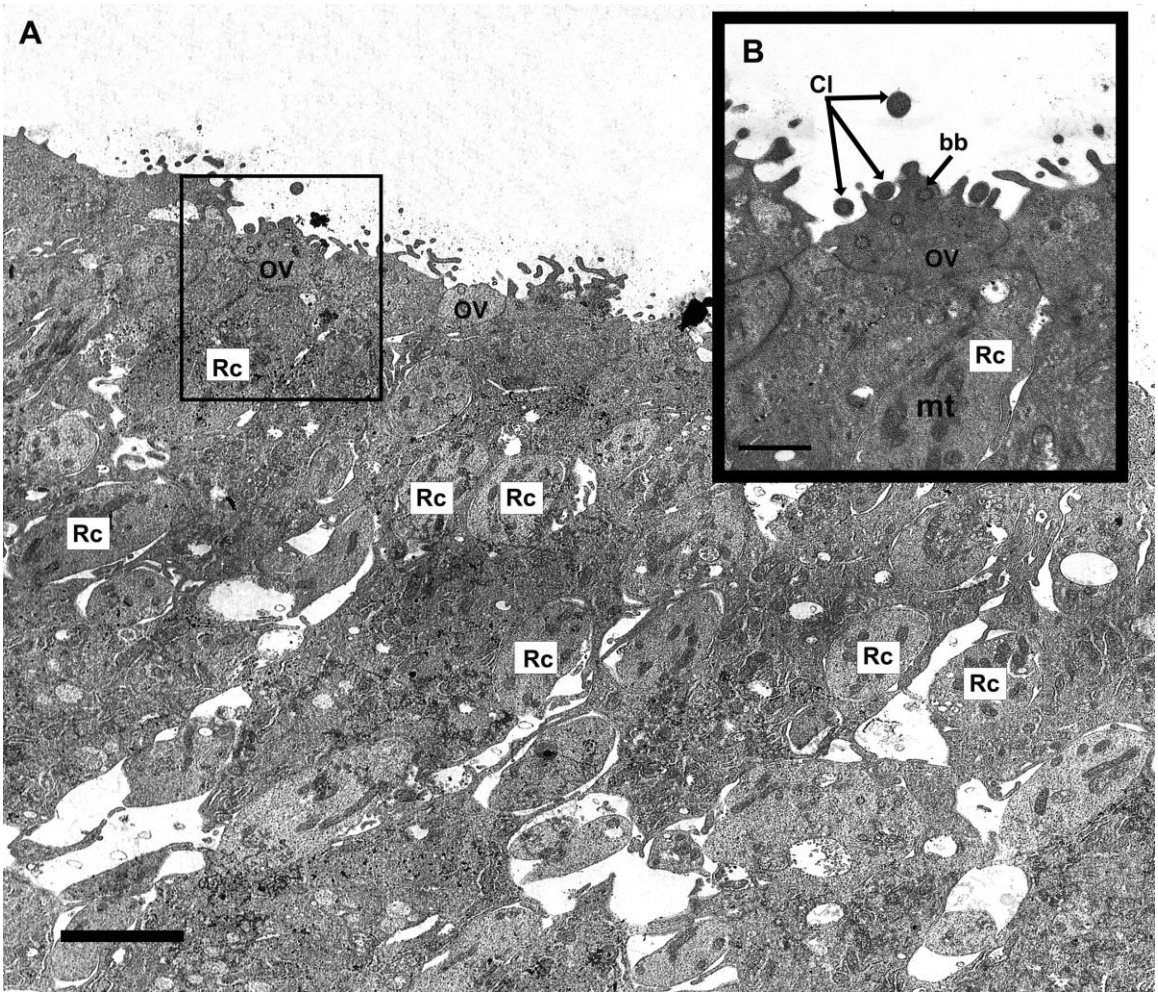


**Figure 6.** Transmission electron micrograph of the posterior portion of the MOC, passing at an angle through the epithelium. **A:** The ciliated receptor cells (Rc) appear disorderly. There are very few, if any, granules present in the secretory supporting cells (Sg). The rectangle corresponds with the inset panel B. Scale bar = 3µm. **B:** Present on the olfactory vesicle (OV) of the ciliated receptor cell (Rc) are basal bodies that correspond with cilia (Cl) and some cilia in cross section in the lumen. Mitochondria (mt) are also seen in abundance in the ciliated receptor (Rc) cells. Scale bar = 1µm.

Supporting Cells of the Posterior Main Olfactory Cavity. The only supporting cells in the posterior MOC are secretory supporting cells that feature short microvilli (Figure 6). Their overall appearance is disorderly, with a more haphazard orientation of the cells, in comparison to the tightly-packed uniform orientation seen the epithelium of the anterior MOC.

Vomeronasal Organ

The cellular layout of the sensory tissue in the VNO also appears uniform with no obvious large patches or concentrated areas of one specific cell type.

Receptor Cells. The only type of receptor cells present in the VNO are microvillar receptor cells (Figures 7 and 8A). They appear with regularity interspersed throughout the VNO epithelium and often show electron lucent vesicles near the apical end. The microvillar receptor cells in the VNO, like those of the MOC, are usually seen between two larger secretory supporting cells (Figures 7 and 8A).

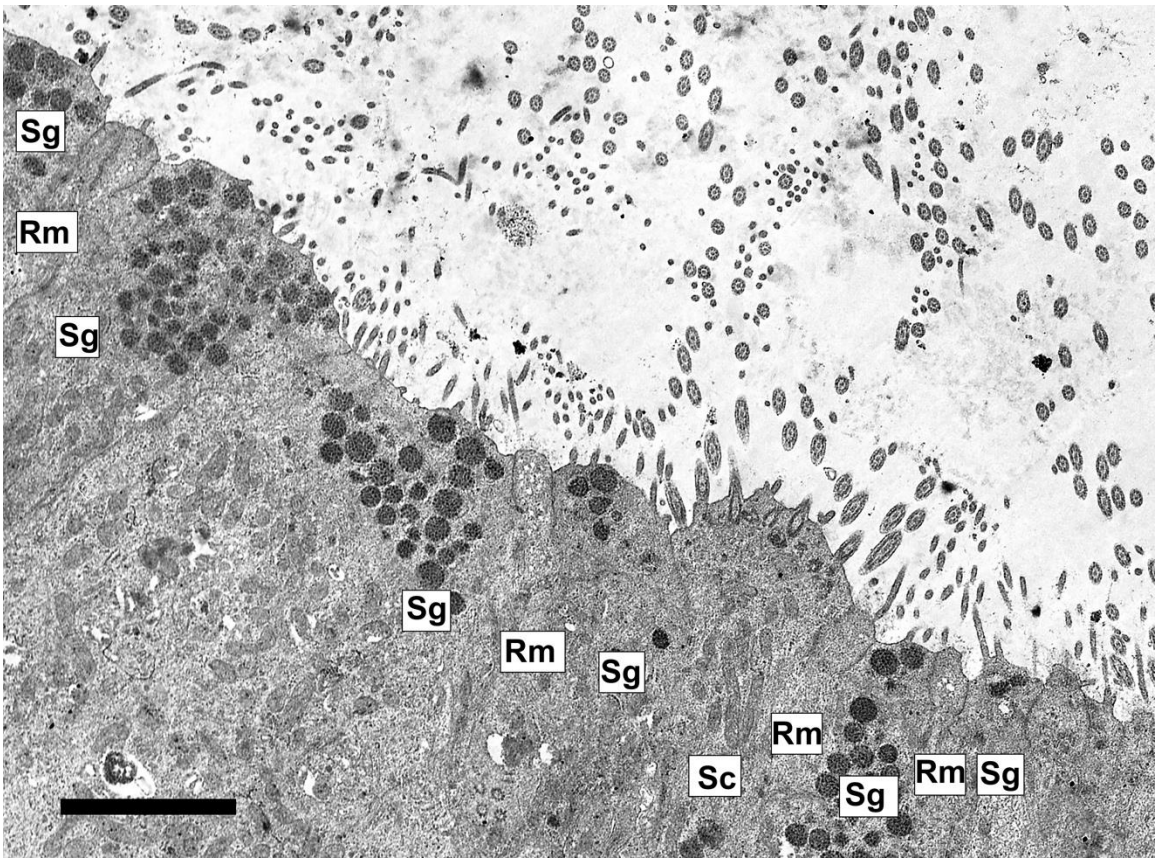


**Figure 7.** Transmission electron micrograph of receptor cells with microvilli in the vomeronasal organ (VNO). Each microvillar receptor cell (Rm) has a secretory supporting cell (Sg) on either side. The secretory supporting cells (Sg) here have microvilli as well. There is also one ciliated supporting cell (Sc) featured here and the Rm each have electron lucent granules. Scale bar = 3 µm.

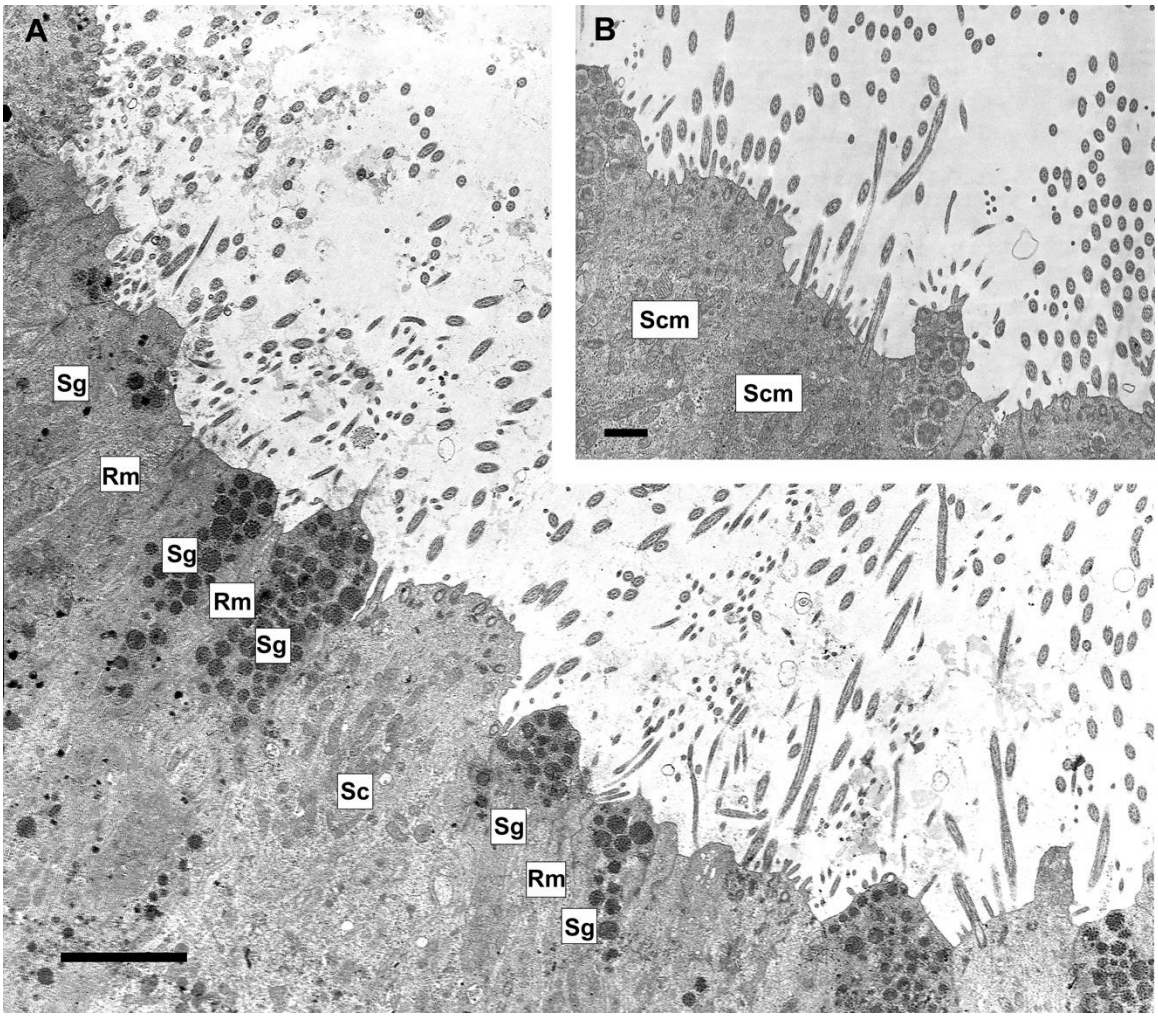


**Figure 8.** Transmission electron micrograph of cells in the VNO. **A:** Microvillar receptor cells (Rm) between secretory supporting cells (Sg). Note the many mitochondria in the ciliated supporting cell (Sc). Scale bar = 3 µm. **B:** Supporting cells with both cilia and short microvilli (Scm). Scale bar = 1 µm.

Supporting Cells. In the VNO there are ciliated supporting cells, secretory supporting cells, and a third supporting cell type: supporting cells with both cilia and microvilli. The ciliated supporting cells have broadly rounded apical ends and are packed with mitochondria (Figures 7 and 8A). Some but not all the ciliated supporting cells also bear microvilli between the cilia (Figure 8B). The microvilli on these cells do not project

more than 0.5 micrometers (µm) into the lumen of the VNO, while the microvilli on some of the secretory supporting cells project much farther into the lumen (Figure 9).

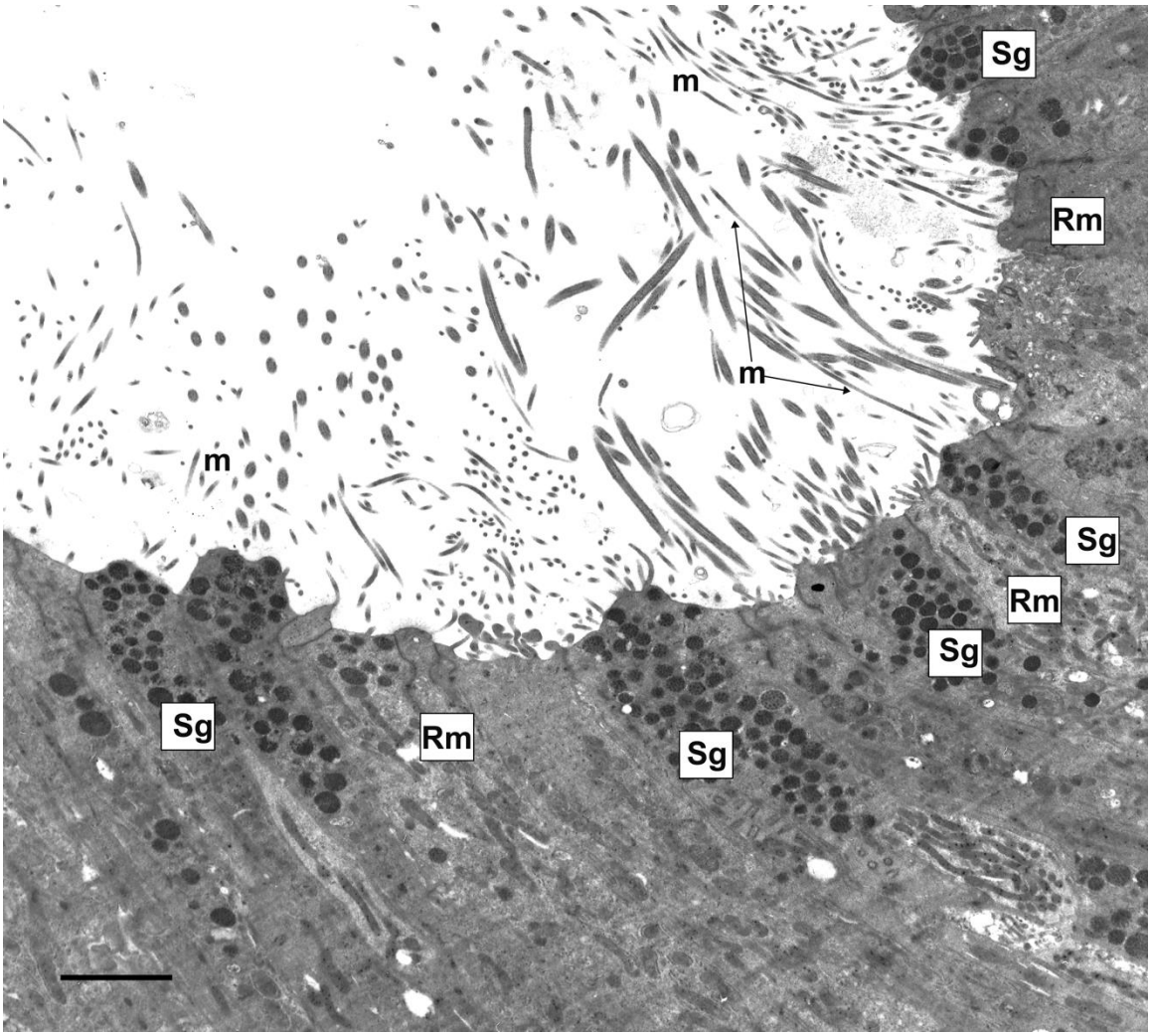


**Figure 9.** Transmission electron micrograph of the VNO epithelium. Many of the secretory supporting cells (Sg), surrounding the receptor cells with microvilli (Rm), have long microvilli (m) that project quite far into the lumen of the VNO in comparison to the microvilli on the ciliated supporting cells (Scm) seen in Figure 8B. Scale bar = 3 µm.

# DISCUSSION

## Comparative Morphology of the Larval Caecilian Nose

The structure of the larval nose of *Ichthyophis kohtaoensis* is generally similar in morphology to that of other known larval caecilians (Badenhorst, 1978; Bruner, 1914; Reiss & Eisthen, 2008; Sarasin & Sarasin, 1890; Wilkinson, 1992). The larval and adult nose of *I. glutinosus*, a member of the same genus, was described in detail in the late 19$^{th}$ century (Sarasin & Sarasin, 1890; Wiedersheim, 1879). The general size, shape, and features of the olfactory organ are almost indistinguishable between the two species. The only obvious distinction is that the choanal valve, found here in larval *I. kohtaoensis* (Figure 2D) and in many other larval amphibians (Bruner, 1914; Reiss & Eisthen, 2008; Stuelpnagel & Reiss, 2005; Wilkinson, 1992) was not described in *I. glutinosus* (Sarasin & Sarasin, 1890). Not surprisingly, there are no Bowman's glands, tentacular glands or organs, or lateral nasal glands present in the larval nose of *I. kohtaoensis*. These structures are known to develop during metamorphosis and are found only in the nose of adult caecilians (Badenhorst, 1978; Reiss & Eisthen, 2008; Sarasin & Sarasin, 1890; Schmidt & Wake, 1990; Wiedersheim, 1879). Likewise, there are only a few mucus secreting goblet cells in the larval nose, in the posterior lateral portions of the MOC and choanal slime sac. There are, however, abundant goblet cells lining the larval buccal cavity (Figure 2D) as well as in the adult respiratory epithelium (unpublished data) and buccal cavity.

The larval nose of *I. kohtaoensis* shares many features with those of salamander and frog larvae, including a defined vestibule, or entrance canal, an enlarged MOC (often referred to as a principal cavity (PC) in anurans), and a laterally situated VNO, all of which culminate in the choana that empties into the buccal cavity (Reiss & Eisthen, 2008). However, one unique feature of the caecilian olfactory organ, not found in other amphibians, is the choanal slime sac (Reiss & Eisthen, 2008; Sarasin & Sarasin, 1890; Wiedersheim, 1879). The choanal slime sac in the larval caecilian, seen here in *I. kohtaoensis* and first described for *I. glutinosus*, is small and tucked into the olfactory organ posterior to the VNO, as the VNO veers medially, and is open to the choana (Figure 2B & C) (Sarasin & Sarasin, 1890). The epithelium of the slime sac does not contain any sensory tissue. It does contain a few goblet cells in the lateral superior portion, at the same level as the goblet cells in the back of the MOC (Figure 2C & D). The choanal slime sac is significantly enlarged in adults (Sarasin & Sarasin, 1890), and while nothing is known regarding its adult function, based on its underdeveloped condition in the larva, it seems likely that the larval slime sac is merely a precursor to the adult structure and that it has no specific function at this stage.

## Ultrastructure of the Larval Caecilian Nose

The present study has shown that the MOE of *I. kohtaoensis* larvae is composed of microvillar receptor cells, ciliated receptor cells, and secretory supporting cells. The VNO contains microvillar receptor cells, ciliated supporting cells, secretory supporting cells, and supporting cells with both cilia and microvilli (Table 1). The only

ultrastructural data available for comparison from another caecilian are from an adult specimen of the secondarily aquatic *Typhlonectes compressicauda*, from the family Typhlonectidae (Saint Girons & Zylberberg, 1992). Two distinct types of sensory epithelium are found in the principal cavity of *T. compressicauda*. The first is found in the posterodorsal part of the principal cavity and is classic "adult" type olfactory epithelium with ciliated receptor cells and secretory supporting cells and Bowman's glands. The second is the anteroventral sensory epithelium, referred to as "vomeronasal-like" by Saint Girons and Zylberberg (1992), which is devoid of Bowman's glands, and contains both ciliated and microvillar receptor cells and secretory supporting cells. This "vomeronasal-like" epithelium described in *T. compressicauda* closely resembles the "larval type" MOC epithelium described here in larval *I. kohtaoensis*.

**Table 1.** Summary of olfactory organ ultrastructure in larval *Ichthyophis kohtaoensis*

| | Rm | Rc | Sg | Sc | Smc |
|---|---|---|---|---|---|
| MOC | + | + | + | - | - |
| VNO | + | - | + | + | + |

+ indicates present; - indicates not present; MOC – main olfactory cavity; VNO – vomeronasal organ; Rm – microvillar receptor cells; Rc – ciliated receptor cells; Sg – secretory supporting cells (with granules); Sc – ciliated supporting cells; Smc – supporting cells with both cilia and microvilli

The VNO of *T. compressicauda* contains only microvillar receptor cells and secretory supporting cells (Saint Girons & Zylberberg, 1992) whereas that of the larval *I.*

*kohtaoensis* has, in addition, ciliated supporting cells and supporting cells with both cilia and microvilli.

The epithelium of other larval amphibians varies from that of caecilians. Typically, in anurans all four main cell types are present in the MOE of the nose of aquatic larvae, while in the VNO there are microvillar receptor cells and ciliated supporting cells (Benzekri & Reiss, 2011; Hansen, Reiss, Gentry, & Burd, 1998; Taniguchi, Toshima, Saito, & Taniguchi, 1996). However, a few exceptions do exist. For example, in larval tailed frogs (*Ascaphus truei)* there are secretory supporting cells in the VNO in addition to the microvillar receptor cells and ciliated supporting cells (Benzekri & Reiss, 2011). Another exception is found in larvae of the toad *Rhinella arenarum*, Instead of all four cell types being present in the MOE, *R. arenarum* has only two types present; ciliated receptor cells and secretory supporting cells (Jungblut, Paz, López-Costa, & Pozzi, 2009).

In contrast to anurans, salamander larvae and neotenic adults typically have just three of the four main cell types present in the MOE: ciliated receptor cells, microvillar receptor cells, and secretory supporting cells (Benzekri & Reiss, 2011; Stuelpnagel & Reiss, 2005), the same types I observed in *I. kohtaoensis*. One exception to this general composition can be found in *Dicamptodon tenebrosus* larvae and neotenic adults, which display ciliated supporting cells in the MOE as well (Stuelpnagel & Reiss, 2005), like the majority of anurans.

Apart from *I. kohtaoensis* not having any ciliated supporting cells in the MOE, the epithelium of both the MOC and the VNO matches what is seen in *A. truei* and *D.*

*tenebrosus* (Table 2) (Benzekri & Reiss, 2011; Stuelpnagel & Reiss, 2005). While this correlation is suggestive there is not enough evidence to support inferences about early nasal ultrastructure. *Dicamptodon tenebrosus*, while a morphologically generalized salamander, is not an early-diverging representative of salamanders and there is no ultrastructural data available yet for earlier diverging families, such as the hynobiids.

**Table 2.** Cell types found in the larval olfactory organ of a representative from each Lissamphibian group. Shading, lighter for the MOE and darker for the VNO, indicates sameness for each nasal region. Absence of shading indicates the only deviation from the pattern. Boxes around the + indicate a deviation in character from the remainder of the group.

| Species Name | Region | Rc | Rm | Sc | Sm |
|---|---|---|---|---|---|
| *Ascaphus truei* (Benzekri & Reiss, 2012) | MOE | + | + | + | + |
| | VNO | - | + | + | [+] |
| *Dicamptodon tenebrosus* (Steulpnagel & Reiss, 2005) | MOE | + | + | [+] | + |
| | VNO | - | + | + | + |
| *Ichthyophis kohtaoensis* (This study) | MOE | + | + | - | + |
| | VNO | - | + | + | + |

+ indicates present; - indicates not present; MOE – main olfactory epithelium; VNO – vomeronasal organ; Rm – microvillar receptor cells; Rc – ciliated receptor cells; Sm – secretory supporting cells; Sc – ciliated supporting cells

## Posterior MOC Epithelium of the Larval *Ichthyophis* Olfactory Organ

One feature seen in the larval olfactory organ was rather surprising. As described above, the overall cellular composition of the sensory tissue in the posterior MOC appears disorderly, with the cells oriented haphazardly. It also only contains ciliated

receptor cells and secretory supporting cells (Figure 6). At this level, the cells are not compact and neatly arranged as is typical of sensory epithelium, but instead show large intercellular spaces. The area does not resemble the sensory tissue found in the anterior portion of the cavity. Interestingly, the posterior part of the MOC in adult *T. compressicauda* likewise appears "not well-organized" and disorderly despite its being a sensory area (Saint Girons & Zylberberg, 1992). The posterior portion of the *T. compressicauda* MOC only contains ciliated receptor cells and microvillar (secretory) supporting cells (Saint Girons & Zylberberg, 1992) which matches what I found in *I. kohtaoensis*. This anomaly may be a shared characteristic of the caecilian nose, but clearly further investigation is needed because my data are limited to only two distantly related species, and two distinct life stages.

# CONCLUSIONS

There are notable similarities between the ultrastructure of the larval nose of *I. kohtaoensis*, described here, and that of the nose of adult *T. compressicauda*, the only caecilian that has been previously characterized (Saint-Girons & Zylberberg, 1992). Both species have microvillar and ciliated receptor cells in the anterior portion of the MOC and only ciliated receptor cells in the posterior portion. The next question is whether these similarities can be further correlated with smelling in water, since both animals are entirely aquatic. A comprehensive look at the nose of adult *I. kohtaoensis*, which are terrestrial (fossorial), would be a valuable complement to this study. Comparing the adults to the larvae studied here will reveal the epithelial changes to the olfactory organ when transitioning from an aquatic smelling individual to an air smelling individual in this species. In terrestrial amphibians, the MOE typically contains ciliated receptor cells and secretory supporting cells with microvilli (Benzekri & Reiss, 2011; Reiss & Eisthen, 2008), similar to the posterior MOE of *Typhlonectes* and *Ichthyophis* larvae. Will this be the case for the entire MOE of adult terrestrial caecilians? Or will there be some surprises?

Traditionally Ichthyophiidae (at that time including rhinatrematids) was considered the earliest-diverging group of caecilians (Taylor, 1968), but further morphological investigation (Nussbaum, 1977) and more recent molecular evidence (San Mauro et al., 2014; Wilkinson, Mauro, Sherratt, & Gower, 2011), puts Rhinatrematidae in the earliest-diverging position. A comparison between rhinatrematids and

ichthyophiids could provide additional morphological characters for phylogenetic reconstructions.

Finally, the similarities in receptor and supporting cell types shared between larval *I. kohtaoensis*, *A. truei*, and *D. tenebrosus* are suggestive of a common ancestral larval lissamphibian pattern. Additional data, especially from early-diverging salamanders, would help to test this hypothesis.

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# APPENDIX

**Table of Specimens Examined**

| Specimen ID | Stage | Total Length | Date of Death | Usage: LM/EM |
|---|---|---|---|---|
| ICKO1 | Larva | 68 mm | 01/13/02 | LM |
| ICKO2 | Larva | 65 mm | 10/31/01 | LM |
| ICKO3 | Larva | 81 mm | 02/27/02 | LM |
| ICKO4 | Larva | 75 mm | 10/10/01 | LM |
| ICKO5 | Larva | Unknown | Unknown | LM |
| ICKO6 | Larva | 84 mm | 03/07/02 | LM |
| ICKO7 | Larva | 77 mm | 03/18/02 | LM |
| ICKO8 | Larva | 62 mm | 04/13/02 | LM |
| ICKO9 | Larva | Unknown | Unknown | LM |
| IKE2 | Embryo | 55 mm | Fall 2001 | EM |
| IKE3 | Embryo | 54 mm | Fall 2001 | EM |
| IKL10 | Larva | Unknown | 03/18/02 | EM |
| IKL11 | Larva | Unknown | 03/18/02 | EM |
| IKL12 | Larva | Unknown | 03/18/02 | EM |

Abbreviations: ID – identification, LM – light microscopy, EM – electron microscopy