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Study of the Olfactory Epithelium in the Developing Sturgeon. Characterization of the Crypt Cells

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Abstract

In acipenserids, crypt cells (CCs) have only been observed in juvenile specimens, and it has not been clarified whether they differentiate along with olfactory receptor neurons (ORNs) during the lecithotrophic stage or during later development stages. Furthermore, no detailed optical microscopy (OM) or electron microscopy study on the development of CCs has been published to date. In the present study, we used OM and electron microscopy to follow the development of CCs in *Acipenser naccarii* from hatching to the establishment of exogenous feeding. Based on these observations, we can affirm that CCs are present from the first few posthatching (PH) days. CCs appear with their nucleus close to the basal lamina of the epithelium and enveloped by supporting cells. In addition, from the beginning of day 2 PH, we observed cells with highly similar characteristics to those of CCs (absence of knob, abundant mitochondria and filamentous material in apical cytoplasm, numerous microtubules, and envelopment by supporting cells) but with cilia still remaining on their noninvaginated apical surface. We conclude that these cells may correspond to immature CCs in which the crypt, the final feature of their morphological differentiation, has not yet formed.

Key words: *A. naccarii*, crypt cells, olfactory organ, olfactory receptor neurons, sturgeon

Introduction

Olfactory information is extremely important in fish participating in essential life processes such as migrations, alarm situations, sexual behavior, and feeding. As in other vertebrates, olfactory receptor neurons (ORNs) are the receptor element of the olfactory system. They are bipolar neurons whose dendrites end in an olfactory knob, which bears cilia in ciliated ORNs and only microvilli in microvillous ORNs. Crypt cells (CCs), a third type of ORN present only in fish, were described in teleosts in 1996 (Morita and Finger 1996). They are ovoid cells without dendrites and with a crypt-like invagination as their outstanding feature. The crypt contains various short cilia and an apical margin bounded by microvilli. The presence of CCs in the olfactory epithelium is considered a common characteristic of all Actinopterygii (Hansen and Finger 2000).

Olfaction is the first chemosensory organ to appear in the ontogenic development of fish, preceding the solitary chemosensory cell system (Kotrschal et al. 1997) and taste (Hansen, Reutter, and Zeiske 2002). The development of the olfactory organ in vertebrates (see Farbman 1992, 1994; Crews and Hunter 1994; Baker and Bronner-Fraser 2001) begins with the formation of olfactory placodes in the anterior region of the head. These ectodermal thickenings are formed by an apparently homogeneous population of cells that separate from the neural plate in its anterior region (Landacre 1910; Verwoerd and Van Oostrom 1979; Northcutt and Gans 1983; Farbman 1992; Whitlock 2004).

Before accessing the surface, placodal cells covered by epidermis start to differentiate into neurons and lengthen, with their axons crossing the basal lamina and growing toward brain structures that will give rise to the olfactory bulb (Zielinski and Hara 1988; Hansen and Zeiske 1993). In the differentiation of the apical pole, ciliated knobs appear first and then knobs bearing microvilli. CCs have not yet been reported in embryos of any Actinopterygii, although they have been presumed to be present and the last to differentiate (Zeiske et al. 2003; Hansen and Zielinski 2005). Given the absence of observations, the question arises as to whether

these CCs appear and develop along with the other ORNs during the first days posthatching (PH) or not until the juvenile stage when they have been documented.

The objective of our study of the morphology and development of olfactory organs in *Acipenser naccarii*, using transmission electron microscopy (TEM) and field emission scanning electron microscopy (FESEM), was to determine the ultrastructure of CCs at their first appearance and during subsequent development. It is of great interest to establish the degree of maturation and the composition of the olfactory organ at the transition to juvenile stage because this is the most critical period in the life of sturgeons (Buddington and Christofferson 1985).

Materials and methods

The fingerlings used in this study formed part of an F2 generation produced at Sierra Nevada Fish Farm Ltd from an F1 raised from wild parentals and reproduced at the same farm. After hatching, fingerlings were kept in water at 15 ± 1 °C in polyester pools with a photoperiod of 12:12 h light:dark and fed with live prey after consumption of the yolk sac. From 2 days before hatching (day 5 postfecundation), 11 specimens were taken for examination by optical microscopy (OM) (5 specimens), TEM (3 specimens), and FESEM (3 specimens) every 8 h until 15 days PH and subsequently on alternate days until day 30 PH, with a final sample on day 36 PH. Embryos were anesthetized in phosphate-buffered 0.02% 2-amino-benzoic acid ethyl ester (Sigma Chemical Co.).

Optical microscopy

For the OM study, whole *A. naccarii* specimens were fixed with Bouin's fluid, embedded in paraffin, and cut into serial sections of 5 μm thickness. Sections were stained with Harris hematoxylin and 1% aqueous eosin solution, Alcian Blue, pH 2.5 (AB) (Lev and Spicer 1964), and periodic acid-Schiff (Lillie 1954).

Confocal microscopy

1,1′-Dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) was used to identify the different types of ORNs in the olfactory epithelium of *A. naccarii* and to study their morphology and localization. Heads from specimens of different ages were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 12 h and then divided in half along the sagittal plane. Small crystals of DiI were inserted under visual control into the entire surface area of the bulbs using a sharpened insect needle. Bulbs were covered with liquid agar to prevent inadvertent spread of the dye. The tissue block was then placed in buffered 4% paraformaldehyde at room temperature for 14–30 days to permit diffusion of the dye. After the appropriate diffusion time, bulbs and olfactory organs were dissected from the tissue block and embedded in 15% gelatin (Sigma). The block was fixed in 4% paraformaldehyde overnight, and 50-μm sections were cut on a vibratome the following day. Sections were viewed under a confocal laser scanning microscope (Leica DMI6000).

Transmission electron microscopy

For the TEM ultrastructural study, fish were fixed in 4% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.4; Watson 1958). The head was then separated, postfixed in 1.5% osmium tetroxide in cacodylate buffer, dehydrated with acetone, and embedded in Epon 812 (Burke and Geiselman 1971). Ultrathin sections obtained with a Reichert-Jung ultramicrotome were contrasted with uranyl acetate and lead citrate (Reynolds 1963) for study under EM-902 transmission electron microscope at the Scientific Instrumentation Center of the University of Granada (SIC-UGR).

Scanning electron microscopy

For the FESEM study, fish were fixed with 4% glutaraldehyde, dehydrated with acetone, rinsed in amyl acetate, critical point dried with CO_2, coated with carbon, and visualized from different angles using a LEO field emission GEMINI-1530 microscope at the SIC-UGR. Some samples had to be lightly coated with gold to increase the conductivity.

Results

As illustrated in Figure 1, we distinguished 4 stages of development in the life cycle of sturgeons: embryo, free embryo, juvenile, and adult.

Localization and study of olfactory organs under OM

The olfactory system of these juvenile specimens of *A. naccarii* had an olfactory pit connected with the exterior through 2 openings, separated by a nasal bridge, which direct the water flow through the organ (Figure 2A). The olfactory rosette was formed by a central raphe and around 20 strongly pigmented radial lamellae (Figure 2B,C). The largest part of each lamella was coated by a pseudostratified sensory epithelium of around 60 μm thickness that contained ORNs, supporting cells, nonsensory ciliated cells, and basal cells (Figure 2E). At the apex of lamellae, the epithelium was nonsensory and monostratified, with a thickness of around 30 μm (Figure 2D).

In hematoxylin/eosin-stained sections, the apex of the sensory epithelium showed abundant apical membrane specializations (Figure 2E). With the AB technique, the cytoplasm

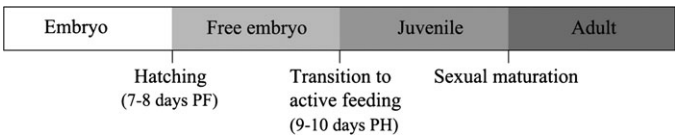

Figure 1 Timing of 4 stages of development in the life cycle of sturgeons. Stage definitions are from Balon (1986).

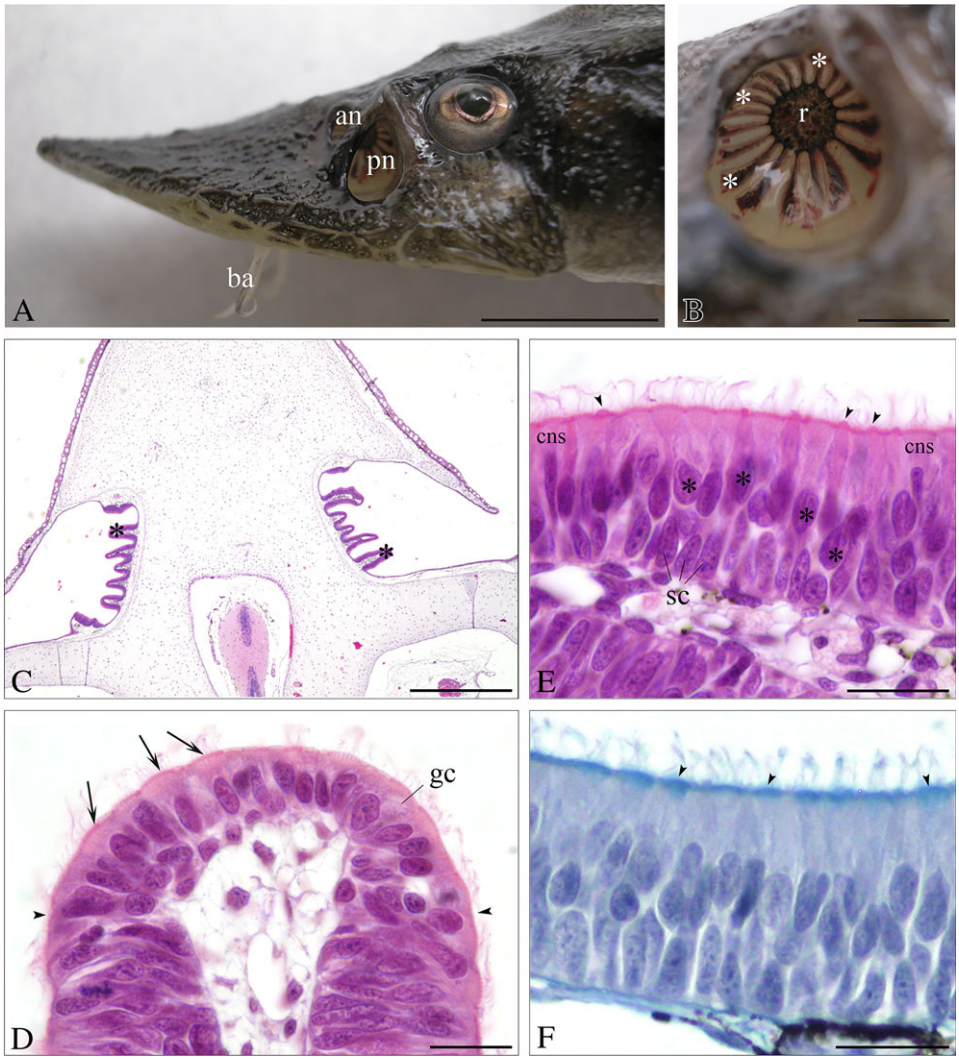

Figure 2 Different aspects of the olfactory organ of *Acipenser naccarii*. **(A)** Lateral view of the head of a juvenile specimen. Observe anterior (an) and posterior nostril (pn) of the olfactory pit separated by nasal bridge. Barbels, ba. **(B)** Olfactory pit devoid of nasal bridge. The olfactory rosette seen on the floor of the olfactory cavity comprises olfactory lamellae radially arranged (asterisks) around a raphe (r). **(C)** Histological section through the olfactory pits of *A. naccarii* at day 36 PH. Several lamellae (asterisks) are observed within olfactory pits. Hematoxylin and eosin stain (H&E). **(D)** Apical portion of a lamella under OM, showing monostratified nonsensory epithelium (between arrowheads) with a predominance of ciliated cells (arrows). Hematoxylin and eosin stain (H&E). Goblet cell, gc. **(E)** Sensory epithelium of a lamella under OM. Note the different height of ORN nuclei (asterisks) and the presence of some knobs (arrowheads). H & E. Ciliated nonsensory cells, cns; supporting cells, sc. **(F)** Sensory epithelium under OM stained with AB. The apical cytoplasm of nonsensory ciliated cells is clearly AB positive (arrowheads). Scale bars = 1 cm in (A), 2 mm in (B), 1 mm in (C and F), 20 μm in **(**D, E, **G**, and **H)**.

of the sensory epithelium was intensely stained, indicating a content rich in acid mucopolysaccharides (Figure 2F).

Application of the neuronal tracer DiI

Retrograde labeling with DiI injections into the olfactory bulb was used to examine ORNs in the olfactory sensory epithelium (Figure 3A). This study confirmed that *A. naccarii* have 3 types of ORNs, that is, ciliated, microvillous, and CCs, which are easily distinguished by the overall morphology and the position of the soma in the olfactory epithelium. Both ciliated and microvillous ORNs had a long dendritic process that projected above the epithelial surface as a knob with apical membrane specializations (cilia or microvilli) (Figure 3B). The dendrite of the ciliated ORN was longer and thinner than that of microvillous ORN, and the soma was consequently closer to the base of the epithelium. In contrast to these other ORNs, CCs showed a slightly sunken apex, and because they had no dendrite, the soma was closer to the luminal surface (Figure 3C).

Ultrastructural characteristics of the olfactory epithelium

Olfactory receptor neurons

TEM study of our juvenile sturgeons allowed ORNs to be distinguished from other cell types (basal cells, supporting cells, and nonsensory ciliated cells) by the characteristic

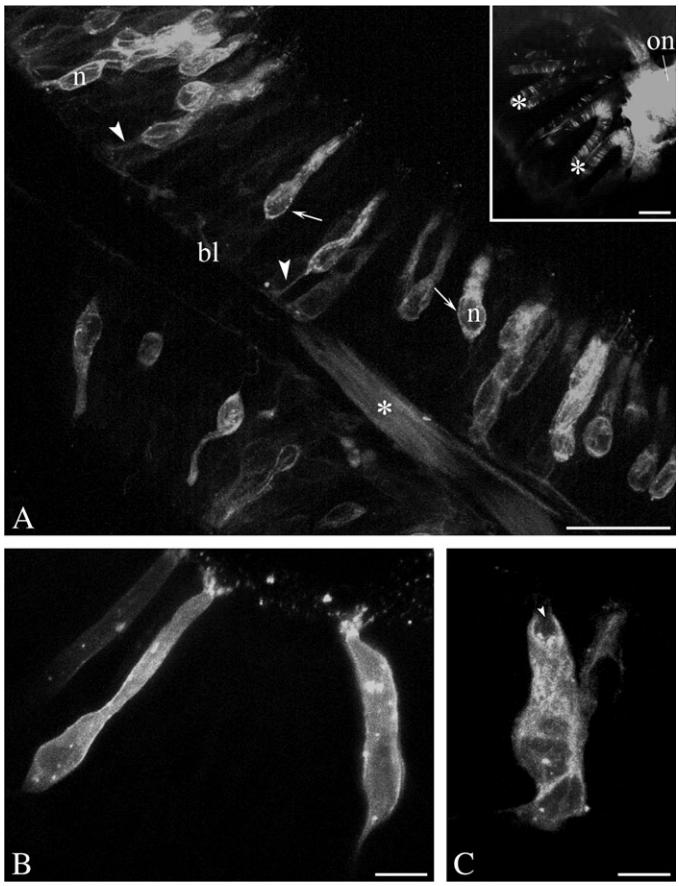

Figure 3 DiI-labeled ORNs in the olfactory sensory epithelium. **(A)** Micrograph of the sensory epithelium of *Acipenser naccarii* with different types of ORN labeled after placement of DiI crystals in the olfactory bulb. The cell somas are found at different depths and their axons (arrowheads) form bundles (asterisk) that flow within lamellae. Confocal microscopy. Basal lamina, bl; dendrite (arrow); nucleus, n. Inset: Panoramic image of a sagittal section of the olfactory rosette. Lamellae (asterisks); olfactory nerve, on. **(B)** On the left, a ciliated ORN that can be distinguished here only by the long, slender dendrite and cell soma in the basal portion of the olfactory epithelium; on the right, a microvillous ORN that is distinguishable only by its shorter, stout dendrite and the position of the soma nearer the epithelium surface. **(C)** CC with an apical invagination (arrowhead) and soma proximal to the epithelium surface. Scale bars = 30 μm in (A), 150 μm in inset, 10 μm in (B and C).

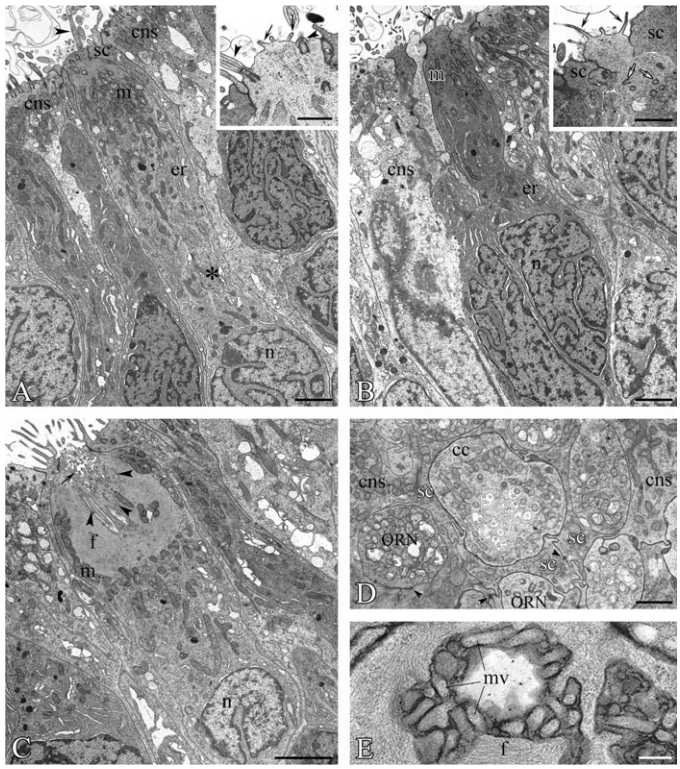

Figure 4 TEM images showing different types of ORNs of a juvenile sturgeon. **(A)** Ciliated ORN. It has a long dendrite, and the nucleus (n) is distant from the epithelium surface. Ciliated nonsensory cells, cns; rough endoplasmic reticulum, er; mitochondria, m. Inset: Detail of a knob bearing cilia (arrowheads) and short microvilli (arrows). **(B)** Microvillous ORN. It has a shorter dendrite than ciliated ORNs, and long and thin microvilli project from the knob (arrows). Inset: Detail of a knob. Groups of centrioles are observed in the dendrite localized below the knob (white arrows). Ciliated nonsensory cells, cns; rough endoplasmic reticulum, er; mitochondria, m; supporting cells, sc. **(C)** CC. The apex shows an invagination containing various cilia (arrowheads), and its margin is bounded by a large number of ramified microvillus-like protrusions (arrow). There is a thick net of filaments (f) around the crypt. As observed in (A and B), there are numerous filamentous mitochondria (m) in the most apical region of the cytoplasm and invaginations in the nucleus (n). **(D)** Cross-section of a crypt surrounded by cytoplasmic extensions of supporting cells (sc). Profiles of several ORNs, which are occasionally surrounded by ciliated nonsensory cells (cns). Desmosomes (arrowheads). **(E)** Detail of apical margin of a crypt bounded by microvilli (mv) and abundant filamentous material (f). Scale bars = 2 μm in (A and B), 1 μm in inset of (A), 1.5 μm in inset of (B) and in (D), 2.5 μm in (C), and 0.2 μm in (E).

appearance of their nuclei, with numerous narrow invaginations and small lumps of heterochromatin dispersed all around their interior (Figure 4A,B,C). Two knob types were distinguished at the apical margin of the epithelium: knobs with numerous cilia and short microvilli corresponding to ciliated ORNs (Figure 4A) and knobs with only long (6–7 μm) and thin (diameter, 75–100 nm) microvilli (Figure 4B) corresponding to microvillous ORNs. Although the microvillous ORNs lacked cilia, groups of centrioles were observed in the dendrite beneath the olfactory knob (inset Figure 4B). Apart from this difference, the 2 neuronal types had similar cytoplasmic characteristics: a large amount of filamentous mitochondria arranged parallel to the major axis of the cell, abundant endoplasmic reticulum, Golgi complexes, and microtubules (Figure 4A,B). CCs could be readily identified by their deeply invaginated apical region containing various cilia. The crypt margin was bounded by a large number of ramified microvillus-like protrusions (Figure 4C,D). The cytoplasm around the crypt contained a thick net of filaments surrounded by numerous filamentous mitochondria parallel to the long axis of the cell, as observed in ciliated and microvillous ORNs (Figure 4C,D). At large magnifications, we observed a certain similarity and continuity between actin filaments present in microvilli at the margin of the crypt and the filamentous material that covered it (Figure 4E).

Supporting cells

These were long cells that extended throughout the thickness of the epithelium. In longitudinal section, they had a narrow profile and were localized among ORNs (Figure 5, also see Figure 8A,C). In cross-section, they adapted to hollows among ORNs, evidencing their enveloping function (Figure 4D). Their free surface showed short microvillus-like protrusions that were occasionally ramified. (Figure 5)

Nonsensory ciliated cells

These cells contributed to the isolation of ORNs, with which they established junctional complexes (Figure 5). They had a broad apical surface (Figure 4D) from which short microvilli projected and intermingled with numerous cilia with long ciliary rootlets (Figure 5). The most apical cytoplasmic region showed numerous secretory granules and coincided with the AB-stained area (Figure 5). Just below this region, numerous mitochondria were distributed with no preferential orientation (Figure 5).

Basal cells

These were small cells with abundant free polysomes and numerous mitochondria in their cytoplasm. They were supported on the basal lamina and did not reach the free surface of the epithelium. They were difficult to identify due to their undifferentiated characteristics and because they are intermingled with the basal portion of other cell types in the epithelium and with groups of axons progressing toward the underlying conjunctive tissue.

Study of the development of olfactory epithelium from hatching

OM observations

On day 1 PH, the olfactory epithelium on the floor of the pit was pseudostratified, and ORNs could be identified by the presence of oval and euchromatic nuclei, knobs at the apical margin of the epithelium, and axons crossing the basal membrane and entering the olfactory bulb. Nonhatched specimens showed these same characteristics at 6/7 days PF (Figure 6A,B). Toward day 5/6 PH, the first lamella

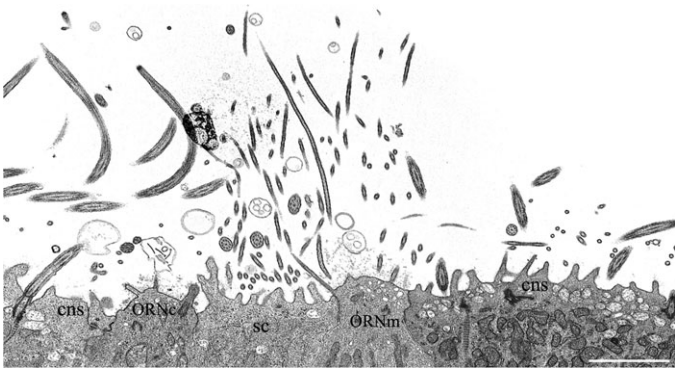

Figure 5 Olfactory sensory epithelium of a juvenile specimen under TEM. **(A)** Apical region of the epithelium. A supporting cell (sc) and 2 ciliated nonsensory cells (cns) can be observed with a ciliated ORN (ORNc) and a microvillous ORN (ORNm) interspersed among them. Note the length of microvilli of ORNm and the numerous secretory vesicles in ciliated nonsensory cells and, to a lesser extent, in the supporting cells. Mitochondria, m. Scale bar = 2 μm.

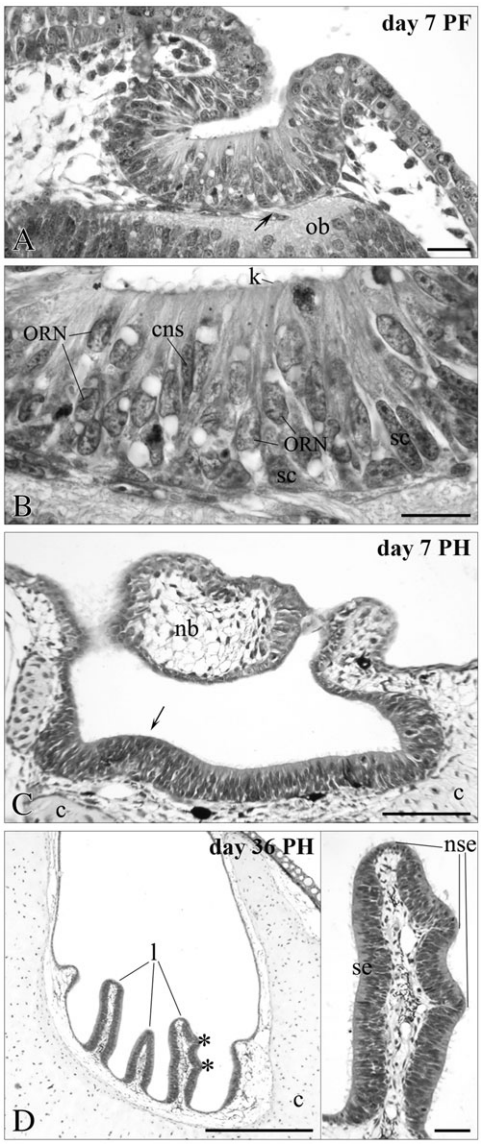

Figure 6 Micrographs under OM of an olfactory organ at different development stages. **(A)** Day 7 postfecundation (hours before hatching). At this stage, the olfactory organ is formed by a chamber covered with the olfactory epithelium, forming a flat surface. Note an axon bundle (arrow) crossing the basal membrane and ending in the prospective olfactory bulb (ob) area. **(B)** Detail of the olfactory epithelium. Different cell types can be distinguished according to the position and characteristics of the nucleus. Ciliated nonsensory cell, cns; knob, k; supporting cells, sc. **(C)** Day 7 PH, first lamella anlages are visible (arrow). Hyaline cartilage, c; nasal bridge, nb. **(D)** On the left, olfactory organ at day 36 PH. Several lamellae (l) can be observed, with several secondary folds in one of them (asterisks). Hyaline cartilage, c. On the right, detail of the lamella with secondary folds. Nonsensory epithelium, nse; sensory epithelium, se. Scale bars = 50 μm in (A) and inset of (D), 20 μm in (B), 100 μm in (C), and 500 μm in (D).

anlages were visible as faint undulations on the epithelium (Figure 6C), which accentuated and continued multiplying over subsequent days. At around 1 month after hatching, these lamellae began to develop folds perpendicular to their axis, designated secondary folding, until they formed the olfactory rosette found in adult specimens (Figure 6D).

Scanning electron microscopy observations

Examination by FESEM of unhatched specimens at 5/6 days PF showed a small (40–50 μm) oval orifice that lengthened rostrocaudally with development. At day 1 PH, the orifice had continued to lengthen in this direction, keeping a narrow rostral end but a wider caudal end (Figure 7A,C). At days 2 and 3 PH, it continued to grow longer, adopting an oblong morphology (Figure 7B,D). From day 3 PH, it acquired an hourglass shape through a narrowing of the central region. Toward day 6 PH, the orifice margins in this region fused, forming a partition between an anterior pore and a larger posterior pore (Figure 7E). Images showed a marked development of the epithelium surface at this time (see Figure 7F).

Observations by TEM

TEM images verified that neurons in the olfactory epithelium on day 1 PH were exclusively ciliated and microvillous. Both neuronal types still contained numerous yolk granules, but they also showed ultrastructural characteristics typical of ORNs. They were perfectly isolated from each other by supporting cells with which they maintained junctional complexes (Figure 8A). Their axons aggregated, forming bundles in the proximities of the basal lamina, and they were also surrounded by supporting cells. The first CCs identifiable by the presence of the crypt were observed toward days 2–3 PH. Except for this crypt, they shared the characteristics of the other ORNs being surrounded by supporting cells and having their nuclei at a similar height in the olfactory epithelium (Figure 8C). Until day 6 PH, the nuclei of the 3 types of ORNs remained at approximately the same height, that is, nearer the base than the luminal surface. They segregated from day 6 PH onward, with the nuclei of ciliated ORNs staying close to the basal epithelium and the nuclei of CCs and microvillous ORNs migrating toward the upper half of the epithelium (Figure 8B,D).

From day 2 PH on, some sensory cells were observed in the sensory epithelium that did not correspond to any of the reported cell types but showed some characteristics of ORNs. Thus, they were always surrounded by supporting cells and showed abundant microtubules and filiform mitochondria parallel to the long axis of the cell in apical cytoplasm. They were ciliated cells, with no knob, and their short cilia projected along a narrow apical margin at the same level as the rest of the epithelial surface. The most outstanding feature of many of these cells was a thick filamentous net occupying a large part of the apical cytoplasm (Figure 9B′). This

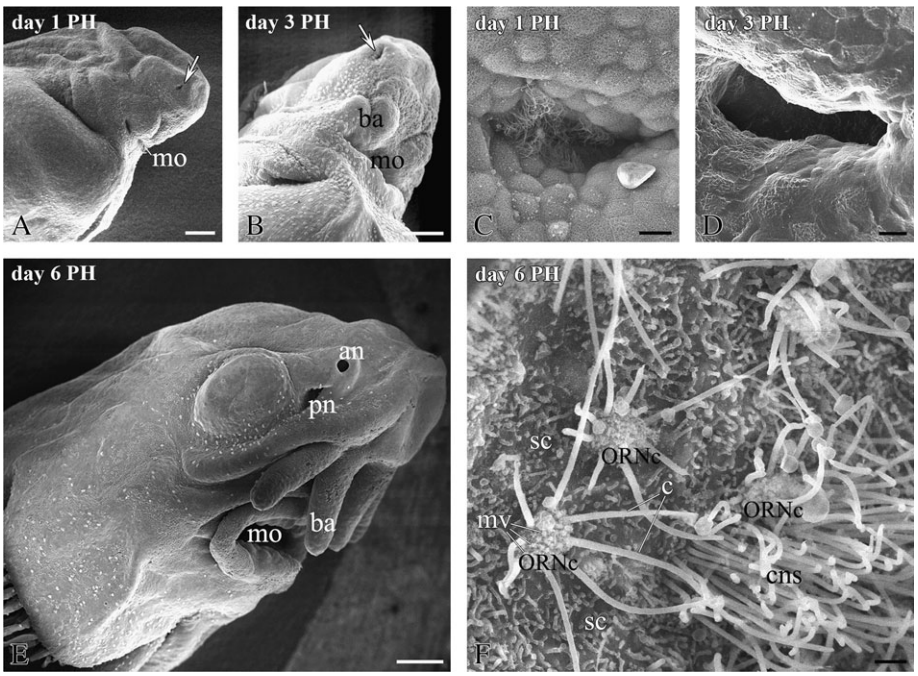

Figure 7 Formation of the nasal pits under scanning electron microscopy. **(A**, **B**, and **E)** Images of the surface of the head of *Acipenser naccarii* at days 1, 3, and 6 PH, respectively. Anterior nostril, an; barbels, ba; mouth, mo; olfactory pit (arrows); posterior nostril, pn. **(C)** Detail of pit organ at day 1 PH. **(D)** Pit organ at day 3 PH showing the approximation of its lateral borders **(F)** Detail of the olfactory epithelium at day 6 PH. Note several knobs of ciliated ORNs (ORNc) between the supporting cells (sc) and ciliated nonsensory cells (nsc). The knobs bear long cilia (c) and short microvilli (mv). Scale bars = 200 μm in (A, B, and E), 10 μm in (C and D), and 1 μm in (F).

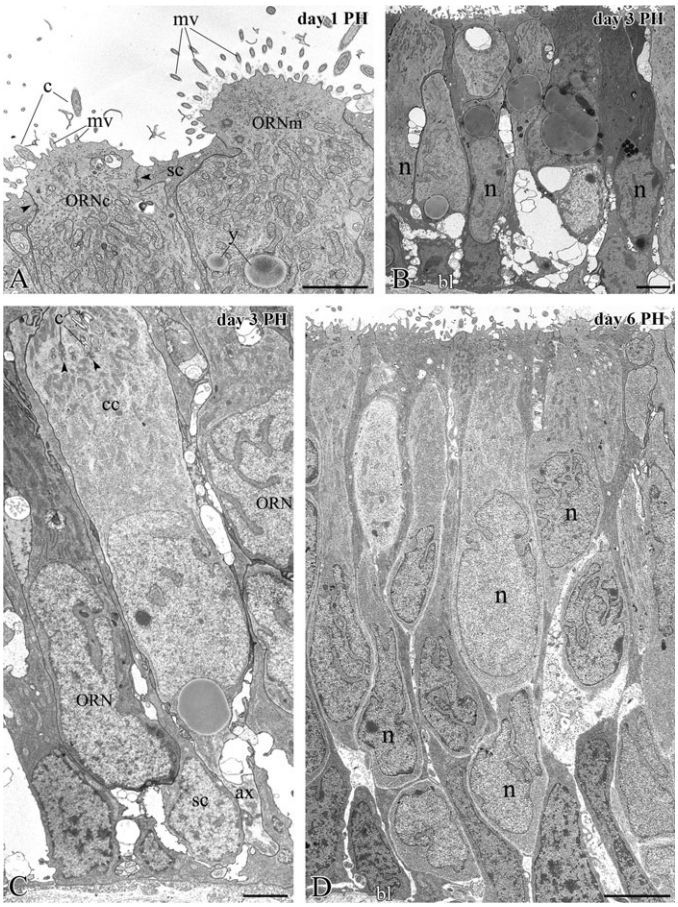

Figure 8 Transmission electron micrographs of the olfactory sensory epithelium at different development stages. **(A)** Day 1 PH. Knobs with numerous cilia (c) and short microvilli (mv), corresponding to ciliated ORNs (ORNc), and knobs bearing only long microvilli, corresponding to microvillous ORNs (ORNm), can be observed on the free epithelial surface. Supporting cells, sc; yolk granules, y. **(B)** Day 3 PH. bl, basal lamina. **(C)** Image of a CC of a specimen at day 3 PH showing a slightly oblique section of a CC with cilia (c) and basal bodies (arrowheads) sunken within the crypt. Short ciliary rootlets are observed in some of the basal bodies. The site from which the axon (ax) starts its projection toward the basal lamina can also be seen. The CC is surrounded by a narrow band of cytoplasm belonging to the supporting cell (sc). Compare the density of CC and supporting cell (sc) electrons. **(D)** Day 6 PH. Compare the segregation of ORNs in the olfactory epithelium with that in a specimen at day 3 PH. Basal lamina, bl. Scale bars = 2 μm in (A), 7 μm in (B and D), and 2.5 μm in (C).

filamentous material was scarce or absent in the others, and their cytoplasm contained abundant polysomes in its place. The apical margin of these cells always bores cilia, which were occasionally bordered by ramified microvilli (Figure 9A′).

Discussion

The main finding of this study was that CCs are present in the olfactory epithelium of fingerlings of *A. naccarii* from day 2 to 3 PH. We also report, for the first time, TEM and FESEM findings on the ultrastructure and development of CCs and other ORNs.

Our TEM observations also produced a novel finding with respect to the nonsensory ciliated cells, not the main object of this study. Their apical cytoplasm showed abundant secretory vesicles stained with AB, indicating that they were rich in acid mucopolysaccharides. To date, the function ascribed to these cells has been the transport of water and mucus secreted by goblet cells in the nonsensory epithelium (Zeiske et al. 1992). According to our observations of their ultrastructural characteristics, these cells also appear to have a secretory function.

Furthermore, whereas the knob of ciliated ORNs bears only cilia in the majority of fish species, we found that they also bore short microvilli in *A. naccarii*, as reported in *Polypterus* (Hansen and Zielinski 2005) and prolarval stage of the sea lamprey (Zielinski et al. 2005). We observed only microvilli on the knob of microvillous ORNs, which were long and thin in *A. naccarii*, as in *Pimephales promelas* (Hansen and Zielinski 2005).

Because CCs were first reported and verified to be ORNs in 1996 (Morita and Finger 1996), they have been subjected to numerous in-depth studies (Hansen and Zeiske 1998; Hansen and Finger 2000; Hansen, Nikonov, et al. 2002). The CCs in our juvenile specimens of *A. naccarii* share all the distinctive characteristics of CCs reported in other fish species: they showed a crypt containing numerous cilia, their nucleus was in the apical third of the epithelium, and they were surrounded by several supporting cells. In the majority of Actinopterygii fish species studied (Hansen and Finger 2000; Hansen and Zielinski 2005; Schmachtenberg 2006), CCs are accompanied by 1 or 2 specialized supporting cells with a low electron density. However, in *A. naccarii*, the 2 or 3 supporting cells surrounding each CC from the time of their emergence have a higher electron density and are structurally similar to the other supporting cells of the epithelium (see Figure 4).

Our study demonstrated the presence of CCs in the olfactory epithelium of fingerlings from day 2 to 3 PH. To date, no CCs have been observed in embryos of Actinopterygii. In 2003, Zeiske et al. (2003) studied the embryonic development of the olfactory organ in 2 species of the genus *Acipenser*, *Acipenser ruthenus and Acipenser baerii*. They concluded that most of its development took place between hatching and the start of active feeding while embryos were still supplied from yolk sac reserves. Although they could not identify CCs in the embryonic olfactory epithelium, they were convinced of their presence. CCs were only previously reported at this site in a recent OM study of embryos of the *Raja clavata* ray species (Ferrando et al. 2006), and no data were given on their ultrastructural characteristics during development or on the formation of their characteristic crypt. CCs have been readily differentiated from the other ORNs in most fish species studied due to the position of their nucleus in the upper third of the olfactory epithelium (Hansen et al. 1999, 2003, 2004; Hansen and Finger 2000; Catania et al. 2003; Zeiske et al. 2003; Germana et al. 2004; Ferrando et al. 2006;

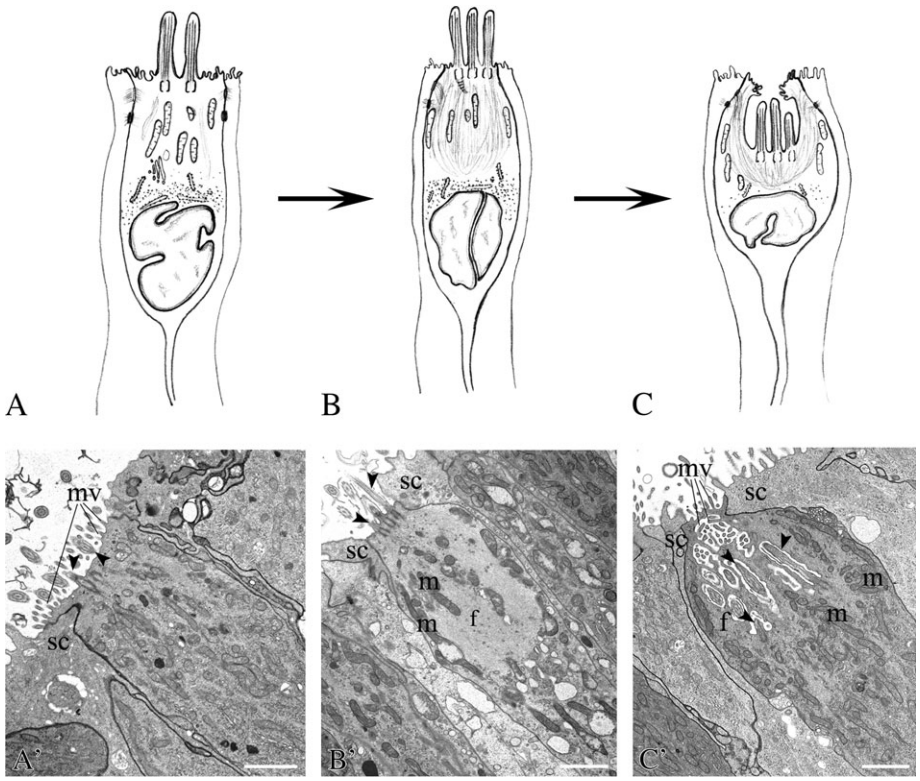

Figure 9 **(A–C)** Diagram illustrating the sequence of basic modifications proposed to take place in the structure of CCs in the final stage of their differentiation (see text for detailed explanation). **(A′** and **B′)** Atypical cells in the olfactory epithelium of specimens at 2 and 21 days PH. (A′) Note the apical portion of the cell whose characteristics do not exactly correspond to any of the established types in the olfactory epithelium. It is localized among supporting cells (sc). It lacks a knob, and its free surface bears both cilia (arrowheads) and microvilli (mv). The apical cytoplasm contains numerous filamentous mitochondria (m). (B′) It can be seen that, apart from the crypt, the cell in the image shares numerous characteristics with CCs. See CC in **(C′)**. Cilia (arrowheads), filamentous material, f; mitochondria, m; supporting cells, sc. Scale bars = 1.5 μm in (A′–C′).

Lazzari et al. 2007). However, our study of *A. naccarii* embryos has demonstrated that this feature is only found in juvenile and adult specimens. The segregation of nuclei is a slow process, which begins on day 6 PH in *A. naccarii* and is not evident until the second part of the juvenile stage. In the above-mentioned OM study of the olfactory epithelium of embryos of *R. clavata* (Ferrando et al. 2007), CCs' nuclei appear at the same height as the nuclei of the other ORNs.

CCs may emerge later than the other ORNs for the simple reason that they are absent in the epithelium until day 2/3 PH. It is also possible that they are very scarce or we are unable to detect them because their earlier structure is very different. Thus, the absence of a crypt would greatly hinder their identification.

From day 2 PH, we observed cells that do not correspond to any of the cell types reported to be present in the olfactory epithelium of *A. naccarii*. They possess some features that are characteristic of ORNs and others that are not. Among the former, we highlight numerous filamentous mitochondria parallel to the long cell axis in the apical cytoplasm, abundant microtubules, and complete isolation from neighboring ORNs due to surrounding supporting cells. Although they might be regarded as ciliated ORNs, because there are various cilia on the free cell surface, this is ruled out by the absence of a knob and the numerous ramified microvilli. Alternatively, they might be considered nonsensory ciliated cells because our atypical cells share some of their characteristics, such as the presence of cilia and a few short microvilli on the apical surface and the presence of abundant mitochondria in the supranuclear cytoplasm. However, nonsensory ciliated cells are not surrounded by supporting cells and do not have the other characteristics of ORNs listed above. Finally, they could be atypical cells belonging to some type that is characteristic of the olfactory epithelium but difficult to classify because their differentiation is not completed. This last possibility is supported by our finding of a thick filamentous net in the apical cytoplasm of some of these typical cells, similar to that observed in the CCs.

To summarize, we found some atypical cell forms in the olfactory epithelium of *A. naccarii* from day 2 PH immediately before the observation of CCs between days 2 and 3 PH. These atypical cells share some of the characteristics of ORNs; therefore, we consider these atypical cells to be intermediate stages of CCs' differentiation that undergo a drastic transformation before acquiring their definitive form.

Figure 9 depicts the main events in this transformation. Cilia genesis takes place before crypt formation, the final stage of this proposed differentiation process. Between these events, abundant filamentous material, known to contain actin, accumulates in the apical cytoplasm. As in other morphogenic processes, this filamentous net could participate in crypt formation by interacting with the plasmatic membrane of the apical portion of the cell and inducing its invagination. The apical surface that sinks into the cytoplasm bears cilia, which would therefore appear within the crypt that is then formed. Likewise, the ramified microvilli of the apical surface, localized at the periphery before formation of the crypt, would end up bounding its margin or opening. We detected cells representing immature stages of CCs throughout the juvenile period, indicating that the cell regeneration process from basal cells operates from the outset for CCs as well as for the other ORNs being replaced after 2–3 weeks with new neurons (Zeiske et al. 1992).

As reported above, all cell types are present in the olfactory epithelium at around days 2–3 PH, including CCs, and all show their distinctive morphological characteristics. However, the different cell types do not take up their definitive position in the epithelium until later, as also observed in *A. ruthenus and A. baerii* (Zeiske et al. 2003), and the olfactory rosette is far from its definitive organization. In *A. naccarii*, the septum forms at around day 6 PH, a short time before the transition to exogenous feeding, as also found in *Acipenser gueldenstaedtii*, *Acipenser stellatus*, *Acipenser ruthenus*, *Pseudoscaphirhynchus kaufmanni*, *Huso huso* (Dettlaff et al. 1993), and *A. baerii* (Zeiske et al. 2003). Dettlaff et al. (1993) reported that lamellae were largely covered by sensory epithelium from the beginning of their formation except at their apex, where the epithelium was nonsensory. In *A. naccarii*, the first lamella anlages are observable 1 day earlier, on day 6 PH, when the different cell types are already segregated within the thickness of the epithelium. It can be concluded from the above data that the development pattern of the olfactory organ is virtually identical among the different sturgeon species. However, teleosts differ in the time sequence of olfactory organ development. In the zebra fish (*Brachydanio rerio*), for instance, the olfactory epithelium differentiates very early; the different cell types are present in a very short time period and completely differentiated before hatching, by the beginning of day 4 postfecundation, when large axon bundles move from the epithelium toward the olfactory bulb. Nevertheless, the olfactory rosette develops very slowly, and the first fold is observed at around day 29 postfecundation (day 25 PH), whereas the septum is complete between days 40 and 42 postfecundation (around day 37 PH) (Hansen and Zeiske 1993).

Finally, our follow-up of juvenile specimens of *A. naccarii* revealed the appearance of soft secondary folds in olfactory lamellae at around day 36 PH. This method of augmenting the olfactory surface has been reported in only a few teleosts, such as salmonids and *Prionurus microlepidotus* (Yamamoto and Ueda 1977), although it has also been observed in the burbot (*Lota lota*) (Devitsyna 1972), garfish (*Lepisosteus*) (Bashor et al. 1974), and shark (*Iago omanensis*) (Fishelson and Baranes 1997).

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References

Baker CVH, Bronner-Fraser M. 2001. Vertebrate cranial placodes. I. Embryonic induction. Dev Biol. 232:1–61.

Balon EK. 1986. Types of feeding in the ontogeny of fishes and the life-history model. Environ Biol Fishes. 16:11–24.

Bashor DP, Beuerman RW, Easton DM. 1974. Ciliary action and normal movement of odorant wavefronts in garfish nasal capsule of *Lepisosteus osseus*. Experientia. 30:777–779.

Buddington RK, Christofferson JP. 1985. Digestive and feeding characteristics of the chondrosteans. Environ Biol Fishes. 14:31–41.

Burke CN, Geiselman CW. 1971. Exact anhydride epoxi percentages for electron microscopy embedding (Rpon). J Ultrastruct Res. 36:119–126.

Catania S, Germanà A, Laura R, González-Martínez T, Ciriaco E, Vega JA. 2003. The crypt neurons in the olfactory epithelium of the adult zebrafish express TrkA-like immunoreactivity. Neurosci Lett. 350:5–8.

Crews L, Hunter D. 1994. Neurogenesis in the olfactory epithelium. Perspect Dev Neurobiol. 2:151–161.

Dettlaff TA, Ginsburg AS, Schmalhausen OI. 1993. Sturgeon fishes developmental biology and aquaculture. Berlin (Germany): Springer-Verlag.

Devitsyna GV. 1972. Morphology of the organs of olfaction in the Gadidae. J Ichthyol. 12:994–1002.

Farbman AI. 1992. Cell biology of olfaction. Cambridge (UK): Cambridge University Press.

Farbman AI. 1994. Developmental biology of olfactory sensory neurons. Semin Cell Biol. 5:3–10.

Ferrando S, Bottaro M, Gallus L, Girosi L, Vacchi M, Tagliafierro G. 2006. Observation of crypt neuron-like cells in the olfactory epithelium of a cartilaginous fish. Neurosci Lett. 403:280–282.

Ferrando S, Bottaro M, Gallus L, Girosi L, Vacchi M, Tagliafierro G. 2007. First detection of olfactory marker protein (OMP) immunoreactivity in the olfactory epithelium of a cartilaginous fish. Neurosci Lett. 413:173–176.

Fishelson L, Baranes A. 1997. Ontogenesis and cytomorphology of the nasal olfactory organs in the oman shark, *Iago omanensis* (Triakidae), in the Gulf of Aqaba, Red Sea. Anat Rec. 249:409–421.

Germana A, Montalbano G, Laura R, Ciriaco E, del Valle ME, Vega JA. 2004. S100 protein-like immunoreactivity in the crypt olfactory neurons of the adult zebrafish. Neurosci Lett. 371:196–198.

Hansen A, Anderson KT, Finger TE. 2004. Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. J Comp Neurol. 477:347–359.

Hansen A, Finger TE. 2000. Phyletic distribution of crypt-type olfactory receptor neurons in fishes. Brain Behav Evol. 55:100–110.

Hansen A, Nikonov A, Anderson KT, Morita Y, Caprio J, Sorensen PW, Finger TE. 2002. Olfactory receptor neurons: functional, structural, and molecular correlates. Soc Neurosci Abstr. 847:12.

Hansen A, Reutter K, Zeiske E. 2002. Taste bud development in the zebrafish, *Danio rerio*. Dev Dyn. 223:483–496.

Hansen A, Rolen SH, Anderson KT, Morita Y, Caprio J, Finger TE. 2003. Correlation between olfactory receptor cell type and function in the channel catfish. J Neurosci. 23:9328–9339.

Hansen A, Zeiske E. 1993. Development of the olfactory organ in the zebrafish, *Brachydanio rerio*. J Comp Neurol. 333:289–300.

Hansen A, Zeiske E. 1998. The peripheral olfactory organ of the zebrafish, *Danio rerio*: an ultrastructural study. Chem Senses. 23:39–48.

Hansen A, Zielinski BS. 2005. Diversity in the olfactory epithelium of bony fishes: development, lamellar arrangement, sensory neuron cell types and transduction components. J Neurocytol. 34:183–208.

Hansen A, Zippel HP, Sorensen PW, Caprio J. 1999. Ultrastructure of the olfactory epithelium in intact, axotomized, and bulbectomized goldfish, *Carassius auratus*. Microsc Res Tech. 45:325–338.

Kotrschal K, Krautgartner WD, Hansen A. 1997. Ontogeny of the solitary chemosensory cells in the zebrafish, *Danio rerio*. Chem Senses. 22: 111–118.

Landacre FL. 1910. The origin of the cranial ganglia in *Ameiurus*. J Comp Neurol. 20:309–411.

Lazzari M, Bettini S, Ciani F, Franceschini V. 2007. Light and transmission electron microscopy study of the peripheral olfactory organ of the guppy, *Poecilia reticulata* (Teleostei, Poecilidae). Microsc Res Tech. 70:782–789.

Lev R, Spicer SS. 1964. Specific staining of sulphate groups with alcian blue at low pH. J Histochem Cytochem. 12:309.

Lillie RD. 1954. Histopathological technique and practical histochemistry. New York: The Blakiston Company.

Morita Y, Finger TE. 1996. Olfactory receptor cell morphology correlates with site of axon termination in the olfactory bulb. Soc Neurosci Abstr. 22:1072.

Northcutt RG, Gans C. 1983. The genesis of neural crest and epidermal placodes: a reinterpretation of vertebrate origins. Q Rev Biol. 58:1–28.

Reynolds ES. 1963. The use of lead citrate at high pH as an electron opaque stain in electron microscopy. J Cell Biol. 17:208–212.

Schmachtenberg O. 2006. Histological and electrophysiological properties of crypt cells from the olfactory epithelium of the marine teleost *Trachurus symmetricus*. J Comp Neurol. 495:113–121.

Verwoerd CDA, Van Oostrom CG. 1979. Cephalic neural crest and placodes. In: Brodal A, editor. Advances in anatomy, embryology and cell biology. Berlin (Germany): Springer-Verlag. p. 1–75.

Watson ML. 1958. Staining of tissue sections for electron microscopy with heavy metals. J Biophys Biochem Cytol. 4:475–478.

Whitlock KE. 2004. A new model for olfactory placode development. Brain Behav Evol. 64:126–140.

Yamamoto M, Ueda K. 1977. Contemplative morphology of fish olfactory epithelium-I Salmoniforms. Bull Jpn Soc Sci Fish. 43:1163–1174.

Zeiske E, Kasumyan AO, Bartsch P, Hansen A. 2003. Early development of the olfactory organ in sturgeons of the genus *Acipenser*: a comparative and electron microscopic study. Anat Embryol. 206:357–372.

Zeiske E, Theisen B, Breucker H. 1992. Structure, development, and evolutionary aspects of the peripheral olfactory system. In: Hara TJ, editor. Fish chemoreception. London: Chapman and Hall. p. 13–39.

Zielinski B, Fredricks K, Mcdonald R, Zaidi A. 2005. Morphological and electrophysiological examination of olfactory sensory neurons during the early developmental prolarval stage of the sea lamprey *Petromyzon marinus* L. J Neurocytol. 34:209–216.

Zielinski B, Hara TJ. 1988. Morphological and physiological development of olfactory receptor cells in rainbow trout (*Salmo gairdneri*) embryos. J Comp Neurol. 271:300–311.