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Early ontogeny of the olfactory organ in a basal actinopterygian fish: *Polypterus*

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Abstract

The present study employed light and electron microscopic methods to investigate the ontogenetic origin of the olfactory organ in bichirs (Cladistia: Polypteridae) and explore its evolution among osteichthyans. In former studies we demonstrated that in teleosts a subepidermal layer gives rise to the olfactory placode which in turn builds all types of olfactory cells (basal, receptor, supporting, ciliated non-sensory cells). In contrast, the olfactory placodes in sturgeons (Chondrostei: Acipenseridae) as well as in the clawed frog *Xenopus laevis* (Anura: Pipidae) originate from two different layers. Receptor neurons derive from cells of the subepidermal (sensory) layer and supporting cells from epidermal cells. As sturgeons and amphibians in some characters show a more primitive condition than teleosts, we extended our study to *Polypterus* to allow for an approach at the basic osteichthyan pattern. In *Polypterus*, an internal lumen occurs in early ontogenetic stages surrounded by the epithelium of the olfactory placode. Two different populations of supporting cells follow one another: a primary population derives from the subepidermal layer. Later supporting cells develop from epidermal cells by transdifferentiation. The primary opening of the internal lumen to the exterior develops by invagination from the epidermal surface and simultaneously by a counter-directed process of cell dissociation and fragmentation inside the olfactory placode. Our results indicate the following features to be plesiomorphic actinopterygian character states: The primary olfactory pit (prospective olfactory cavity) is formed by invagination of the epidermal and the subepidermal layer (as in *Acipenser* and *Xenopus*). The incurrent and excurrent nostrils derive from a single primary opening which elongates and is then separated by an epidermal bridge into the two external openings (as in *Acipenser* and many teleosts). The olfactory epithelium derives from an epidermal and a subepidermal layer (as in *Acipenser* and *Xenopus*). Apomorphic (derived actinopterygian) features are: (1) an internal lumen as primordium of the future olfactory chamber; (2) a subepidermal layer gives rise to the olfactory epithelium and its constituents (*Polypterus* and teleosts). As to the origin of the olfactory supporting cells in *Polypterus* we assume a combination of plesiomorphic and apomorphic characters. We conclude that *Acipenser* and *Xenopus* exhibit the most widely distributed features among basal osteognathostomes and thus ancestral character states in the development of the olfactory organs.

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Keywords

Actinopterygii; Cladistia; Olfactory Placode; Ontogeny; Phylogeny; Ultrastructure

Introduction

Ray-finned fish (Actinopterygii) show considerable morphological variability in the olfactory organ. Location and shape of the olfactory openings vary greatly among taxa. Arrangement, structure, and development of the olfactory epithelium also differ to a surprising extent [Yamamoto, 1982; Zeiske et al. 1992]. Variation in the morphology of the olfactory organ correlates with the enormous diversity of life-styles among fish species, the long, divergent evolutionary history of primary aquatic vertebrates, and their inferred actual ecological adaptations.

There is still considerable lack of information concerning the basic structure, composition, and development of the olfactory organ in many basal taxa, as we have emphasized and attempted to remedy in a previous study [Zeiske et al., 2003]. This information is crucial for reconstruction of the evolution of this sensory system and the phylogeny of osteognathostomes (the monophyletic group composed of osteichthyan fishes and Tetrapoda). Accordingly, here we focus on the study of structural and developmental characteristics of the olfactory organ in those “primitive” extant osteognathostome groups that are rather close to the dichotomy of Actinopterygii (ray-finned fishes) and Sarcopterygii (lobe-finned fishes and tetrapods). These groups are expected to be highly informative of ancestral character states and character combinations that are indispensable to establish a phylogenetic framework of plesiomorphic and apomorphic features. Sturgeons (Acipenseridae, Chondrostei: a rather basal clade of Actinopterygii) show close resemblance to anuran tetrapods in several characters of early olfactory development, rather than similarities to some known teleostean conditions [Zeiske et al., 2003]. Except for Acipenseridae, however, knowledge of the development of the early olfactory organ is still limited to a few highly derived teleostean taxa of Actinopterygii [Hansen and Zeiske, 1993; Whitlock and Westerfield, 2000]. As to basal Sarcopterygii, the African clawed frog, *Xenopus laevis*, is at present the sole example of an adequate study on the structure and development of the olfactory epithelium [Klein and Graziadei, 1983; Costanzo and Graziadei, 1987; Reiss and Burd, 1997a/b].

Description of the formation and ultrastructure of the olfactory organ during early ontogeny is particularly insufficient in the Cladistia (Polypteriformes), another basal clade of Actinopterygii and usually considered to represent the sister-group of all other Recent Actinopterygii. A few studies are available concerning the gross morphology of the adult condition [Pfeiffer, 1968, 1969; Theisen, 1970], the detailed anatomy of a larval nasal sac [Bjerring, 1988] in *Polypterus*, and the ultrastructure of the adult olfactory epithelium in *Erpetoichthys calabaricus* [Schulte and Holl, 1971]. Some remarks on the early development of the olfactory placode are mentioned in the ontogenetic and microscopical study of [Kerr, 1907] based on Budgett's work [Budgett, 1902] and material collected at the Gambia River.

Ontogenetically, the complex structures of the olfactory epithelium arise from paired olfactory placodes [von Kupffer, 1894], ectodermal thickenings that originate during early embryogenesis. As to the formation of the olfactory epithelium we found a considerable difference between sturgeons and teleosts, the latter at present still exemplified in ultrastructural studies on zebrafish, *Danio rerio* [Hansen and Zeiske, 1993, 1995; Whitlock and Westerfield, 2000], and 6 species of atherinomorph fishes [Zeiske and Hansen, 2005]. Whereas in the teleosts studied a subepidermal layer of cells forms all cell types of the olfactory epithelium, i.e. basal cells, receptor neurons, supporting as well as ciliated non-sensory cells,

in sturgeons only olfactory receptor neurons develop from cells of the subepidermal layer. Supporting and ciliated non-sensory cells derive from epithelial surface cells [Zeiske et al., 2003]. Exactly this type of formation has been described for the African clawed frog, *Xenopus laevis* [Klein and Graziadei, 1983; Costanzo and Graziadei, 1987; Reiss and Burd, 1997a/b]. Consequently, as a working hypothesis we assumed that the type found in sturgeons and *Xenopus* represents the plesiomorphic condition among osteognathostomes. In order to elucidate which mode of formation shows plesiomorphic or apomorphic characteristics or perhaps merely convergently evolved, we here extend our ontogenetic studies to *Polypterus* (bichirs). The description of the major characteristics is based on a combined technique of scanning electron microscopy, semithin-serial sectioning, and transmission electron microscopy of selected sites and stages through a closely staged ontogenetic series. This enables comparison of the structure and derivation of different cell types as well as formation of the olfactory cavity, openings and olfactory nerve. It is this approach we have taken for the present study that led to new insights into the ontogeny of the peripheral organ of *Polypterus*.

Material and Methods

Acquisition of the proper study material alone was quite a task in polypterid fishes and requires time and a good knowledge of general biology of the group. Briefly, *Polypterus senegalus* Cuvier, 1829, and *Polypterus ornatipinnis* Boulenger, 1902, were bred in aquarium tanks according to techniques reported in [Bartsch et al., 1997]. Stage determination follows [Diedhiou and Bartsch, 2009].

A certain problem arises from the fact that spawning occurs in portions over a longer time interval ranging from few hours to one day and the adult fishes are rather sensible to disturbance during this time. Accordingly, several spawns were needed for a fairly complete ontogenetic series. For our study we concentrated on *P. senegalus* because handling was generally easier and overall productivity higher. *P. ornatipinnis* was mainly used for comparison of general external characters in SEM pictures. Principles of laboratory animal care (NIH publication No. 86-23, revised 1985) and current German animal care regulations were followed.

Light (LM) and Transmission Electron Microscopy (TEM)

After removal of the egg-envelope (zona radiata), specimens of early ontogenetic stages were fixed by immersion in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for several hours for electron microscopic analysis. After rinsing in phosphate buffer, the animals were postfixed with 1% osmium tetroxide for 2 h. The fixed specimens were dehydrated in a graded series of ethanol and embedded in Glycid ether 100 (Serva). Semithin sections of 1 µm were stained with toluidine blue and examined under the light microscope. Ultrathin sections (silver to gold) were stained with uranyl acetate and lead citrate and examined with a Philips 420 (Eindhoven, Netherlands) transmission electron microscope.

Scanning Electron Microscopy (SEM)

Specimens were fixed by immersion in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2). After rinsing in phosphate buffer, the specimens were dehydrated in a graded series of acetone and isoamyl acetate, critical-point-dried in CO₂, coated with gold, and examined with a CamScan DV4 and a LEO 1525 scanning electron microscope.

All images were prepared in Adobe Photoshop CS 2 (Adobe Systems, Inc.). In some figures dirt spots that did not affect the image accuracy were removed with the eraser tool.

Results

The primordium of the olfactory placode in *Polypterus* is found in embryos of stages 23 to 24 ['tail bud stage', 34 h after fertilization (AF)], long before hatching. The anlage consists of paired thickenings of a cell layer which extends as a continuous sheet between the epidermis and the forebrain in the front of the head (fig. 1A, B). This subepidermal layer is closely attached to the epidermis but separated from the brain by primordial cells of prospective loose connective tissue. In the beginning, the subepidermal layer as well as the covering epidermis consists of only one layer of cells. There is no basal lamina between these two monolayers. The placodes further thicken by expansion and addition of new cells in the triangular region between forebrain, eyes, and attachment glands (or 'cement glands'). These latter prominent and paired structures develop early in ontogeny and provide excellent markers for orientation and analysis of the histological sections (see fig. 1C,D). The subepidermal cell layer in these early embryonic stages separates the tissue of the attachment glands from the epidermis covering them.

Embryos of stage 26 (40 – 52 h AF (depending on temperature of incubation) have cone-shaped olfactory placodes (fig. 1C). These protrude inwards from the subepidermal layer and form a solid accumulation of cells positioned above the stalk of the attachment glands and a little in front and below the eye vesicles. The epidermal cells above the subepidermal layer contain masses of light vesicles in their apical region. Single cells bear bundles of long cilia. Such ciliated epidermal cells originate already during neurulation and are distributed over the entire body surface of early embryos (fig. 3A) [Bartsch et al., 1997]. In embryos 48 AF, a basal lamina is formed (not shown). It separates the cells of the subepidermal layer from cells of the underlying prospective loose connective tissue which later becomes the *lamina propria*. The basal lamina adheres to collagen fibrils. The early placode grows towards the forebrain, but, except for axon connections of the olfactory nerve, remains separated from it by cells of the prospective loose connective tissue (fig. 1D). The placodal cones may also approach the subepidermal layer on the opposite side of the stalk of the attachment gland, but later they are invariably separated by fibroblasts of the growing loose connective tissue. The superficial epidermis is continuous and shows signs neither of invagination nor of differentiation above the olfactory placode.

In embryos of stage 27 (62 h AF, close to hatching time), the placode has become roundish in shape. Its cells are radially arranged, with their nuclei positioned close to the periphery where the cells rest on the basal lamina. The placode exhibits prominent cellular interdigitations or, in the more central region, cellular connections by a net of junctional complexes. The junctional complex consists of tight junctions, intermediate junctions, and desmosomes. Occasionally, intermediate filaments are present at the level of the junctional complex. Some cells exhibit centrioles containing the triplet pattern of microtubules as well as sections of cilia showing the doublet pattern of microtubules.

In hatchlings of stage 28 (68-72 h AF), a lumen appears in the centre of the olfactory placode (fig. 1D). The lumen represents the primordium of the developing olfactory cavity. One day later at stage 30 (4 days AF) the lumen has increased in size. It contains abundant cell debris. The surrounding olfactory epithelium shows the typical pseudostratified epithelial structure (fig. 2A). It already consists of cells that show characteristics of supporting cells (e.g. terminal web, tonofilaments, and vesicles) or olfactory receptor neurons (e.g. neurotubules). Both cell types possess numerous centrioles, procentrioles or their precursor bodies, especially in the apical region (fig. 2A). In yolk-sac larvae 4 d 2 h AF, the lumen has further widened but cilia are not present at the epithelial surface that faces the lumen (fig. 2B). At this stage, merely irregularly shaped protrusions project into the future olfactory cavity. Cilia are only found inside the cells as already seen in stage 30 (fig. 2A). These so-called primary cilia are present

not only in the distal, but also in the proximal part of the cells. The basal bodies of these cilia are associated with rootlets which are deeply anchored in the cytoplasm. Dynamics of growth of the olfactory epithelium is demonstrated by abundant mitoses.

Significant progress in development is evident in early yolk-sac larvae of stage 31, 5 days (120 h) AF. Short cilia of olfactory receptor neurons (ORN, orn) project into the lumen which is still closed up from the exterior (fig. 2C). However, an extension of the internal lumen becomes visible in the direction of the surface of the snout. This extension is apparent even at the light microscopic level. TEM micrographs show that this extension is achieved by cell lysis. The expanding cavity is filled with cell debris. As deduced from serial ultrathin sections, the tissue disintegrates by loosing the cell contacts at the junctional complexes (fig. 2D). Thereby the cellular interstitium increases in size followed by cell dissociation and fragmentation. In yolk-sac larvae of this stage (120 h AF) the extension of the internal lumen has not reached the epidermis and not even penetrated the whole olfactory placode. At the surface of the head, however, exactly above the placode, a counter-directed process of invagination occurs, which initially starts with a tiny shallow depression visible in SEM micrographs and in microscopic sections (fig. 3A). The presence of small undifferentiated cells at this groove indicates activity of cell division. The epidermal surface cells possess the fingerprint-like pattern of microridges typical of fish epidermis. In addition, superficial cells are present which bear a tuft of kinocilia. These cells are the ciliated epidermal (non-sensory) cells already described for early embryos (see above).

In late yolk-sac larvae following stage 32, (7 days, 168 h) AF, the internal lumen of the placode opens to the exterior. This opening of the olfactory organ results from two developmental processes: the epidermal invagination above the olfactory placode and the extension of the internal lumen of the placode which grows towards the invagination (fig. 4A). The connection opens when both extensions have fused. Epidermal surface cells line the entrance (primary olfactory opening) and also its open connection to the lumen of the placode (developing olfactory cavity). Single surface cells around and inside the connecting duct possess basal bodies in the apical region indicating that the cells may become ciliated non-sensory cells. SEM studies imply that ciliated non-sensory cells from the epidermal surface found in more advanced stages of the developing olfactory epithelium are shifted into the growing olfactory organ through the process of invagination (see fig. 3B,C,D). Progressively, the primary opening continues to elongate and later becomes constricted by an epidermal bridge that separates anterior (incurrent) and posterior (excurrent) nostrils (fig. 3D).

The epithelium which lines the invaginated olfactory pit and the duct to the olfactory cavity also contains growing ORNs whose dendrites reach the free surface. The dendritic endings are surrounded by large cells which show features typical for the epidermal surface cells, e.g. large numbers of vesicles which are densely packed in the apical region. The vesicles are filled with either finely dispersed or densely packed material. Some of them contain an electron-dense core.

Cells which are in cell contact with ORNs stretch along the proximal-distal axis of the ORNs. In the course of this process they acquire a horizontally oriented terminal web and longitudinally arranged tonofilaments. Sections of larvae 190 h (~ 8 days, stage 33) and 226 h (~ 9.5 days, stage 35) AF show that the epidermal surface cells which have cell contacts with ORNs become supporting cells of the olfactory epithelium. Their origin from epidermal surface cells can be recognized by the filled vesicles still present in the upper region of the cell (fig. 4B).

At 190 h AF, a second type of neuron appears in the region of the placodal lumen in addition to ciliated ORNs, the microvillous ORN (fig. 5C). The dendritic ending of this receptor cell

type bears microvilli instead of cilia. Numerous basal bodies associated with a basal foot and rootlets are located deep inside their dendrites as also seen in ciliated ORNs (fig. 4B). Supporting cells in the placode display a high number of vesicles distributed throughout the apical region. Quite a few of these vesicles are located close to the membrane of the free cell surface. The vesicles of these placodal supporting cells are morphologically different from the vesicles found in the supporting cells which derived from epidermal cells (see above). Most of them are smaller and of higher electron density. Each supporting cell usually contacts between four and six olfactory receptor neurons (fig. 4C,D). When the lumen of the placode opens, both types of supporting cells can be found in the olfactory epithelium which lines the lumen. The placodal supporting cells are predominantly found in the region at the closed end of the sac; the epidermal supporting cells occur more frequently at the end that opens to the exterior. Later in ontogeny a third type of non-sensory cells becomes abundant in the olfactory epithelium, ciliated non-sensory cells (not shown) that remain similar to the ciliated cells of the epidermal surface which in polypterids serve a ventilatory function inside the olfactory organ.

As the olfactory epithelium further matures, the ciliated olfactory neurons become so-called “mixed” receptor cells [Graziadei and Bannister 1967], i.e. they bear both cilia and microvilli (fig. 5A,B). A short rootlet inserts at the base of the basal body of the cilia (fig. 5A). Olfactory knobs with only microvilli are still present (fig. 5C). Also crypt ORNs appear. These olfactory neurons do not have an olfactory knob but bear microvilli at the outer rim of an invagination. Several cilia grow in this invagination (“crypt”) but do not reach into the lumen of the olfactory organ (fig. 5D).

Discussion

The present study investigated the ontogenetic origin of the olfactory organ in bichirs in comparison to ontogenetic features described for other groups of fish and amphibians (table 1).

The adult olfactory organs of Cladistia (*Polypterus* spp. and *Erpetoichthys* (syn *Calamoichthys calabaricus*) are large and have a complicated structure, unique among fish [Pfeiffer, 1968; Theisen, 1970; Schulte and Holl, 1971; Bjerring, 1988; Zeiske et al., 1992]. The olfactory epithelium consists of basal cells, supporting cells, olfactory receptor neurons (ORNs) and ciliated non-sensory cells [Schulte and Holl, 1971]. In addition, we here demonstrate the presence of three types of ORNs, ciliated, microvillous and crypt cells. Longitudinal and horizontal sections of the olfactory epithelium show that ciliated ORNs are far more frequent than the microvillous type. Microvillous ORNs appear later than ciliated ones during ontogeny. Crypt-cell ORNs are quite rare in the olfactory epithelium of *Polypterus* and previously escaped detection [Hansen and Finger, 2000]. Despite the unique and complicated arrangement of the olfactory organ, the olfactory epithelium of *Polypterus* contains 3 types of olfactory neurons and thus resembles the olfactory epithelium of other fishes [Hansen and Finger, 2000].

Our study on the development of the olfactory organ of *Polypterus* shows that the placode originates from a subepidermal layer (fig. 6). This layer gives rise to supporting cells as well as to ORNs. An internal lumen formed by cell lysis appears inside the placode and is the primordium of the future olfactory cavity. The primary olfactory opening develops by invagination from the epidermal surface and simultaneously by a counter-directed process of cell dissociation and fragmentation inside the olfactory placode. Ciliated non-sensory cells derive from the epidermal layer and are shifted into the olfactory epithelium through the primary opening of the olfactory organ.

The development of the olfactory epithelium from a subepidermal layer is also found in teleosts as has been reported for the zebrafish [Hansen and Zeiske, 1993] as well as for 6 different species of atherinomorph fishes (rainbowfish, sailfin silversides, rivulines, live-bearers, and garfish/needlefish) [Zeiske and Hansen, 2005]. Earlier observations of the development of the olfactory organ in the salmon [Gawrilenko, 1910; Reinke, 1937] seem to correspond to the formation described for zebrafish and atherinomorph fishes. If so, salmon, zebrafish, and atherinomorph fishes are likely to represent the model type of formation of the olfactory epithelium in ‘modern teleosts’ from an initially monolayered placode [Hansen and Zeiske, 1993; Hansen and Zielinski, 2005; Zeiske and Hansen, 2005]. The epidermal epithelium which transiently covers the olfactory placode disappears when the first dendritic endings of the ORNs and other apical cell endings appear on the surface of the olfactory epithelium. An internal lumen or at least a small gap between epidermis and subepidermal layer occurs before the epidermis disappears and forms by cell lysis or retraction of cells [Zeiske and Hansen, 2005]. Also the opening of the internal lumen to the exterior results from cell death or cell retraction within the epidermis [Zeiske and Hansen, 2005]. A similar developmental morphology of the olfactory organ is described for the African lungfish *Protopterus*. Here an internal lumen is formed in the subepidermal layer by cytolysis, and the superficial ectoderm opens above the cavity [Fullarton, 1933]. However, Fullarton’s studies as well as essential parts of a study by Fishelson and Baranes [1997] on the viviparous shark *Iago omanensis* are based on light microscopy. Thus the exact derivation of supporting cells in the olfactory epithelium of lungfishes and sharks is still unknown.

Whereas in teleosts the olfactory placode is situated underneath the epidermal layer as a solid cell mass, it surrounds an internal lumen in *Polypterus*. Consequently, the internal lumen has to stretch through the placodal tissue during the opening process before connecting to the counter-directed epidermal invagination. Formation of the primary olfactory pit by invagination of only the epidermal layer as seen in *Polypterus* has not been described for teleostean fishes. We interpret the invagination as a different mode of opening of the primary nasal pit to the exterior compared to teleosts. This mode might result from the different timing of the ontogenetic processes involved: in *Polypterus*, opening of the pit occurs at an advanced stage when the olfactory placode is already large and rounded into the sac-like structure.

Moreover, in teleosts all supporting cells arise from the subepidermal olfactory placode. In *Polypterus*, the placode gives rise to an initial generation of supporting cells which we call ‘placodal supporting cells’. Morphologically, they can clearly be distinguished from ‘epidermal supporting cells’ which transdifferentiate from epithelial cells of the epidermal layer. The epidermal supporting cells form when epithelial cells come into contact with dendritic profiles of differentiating ORNs. They populate the olfactory epithelium secondarily when the internal lumen opens. Thus, *Polypterus* combines the potency of the epidermal as well as subepidermal layer to proliferate supporting cells.

Supporting cells originating from the epidermal layer above the placode are the only type of supporting cells present in the African clawed frog *Xenopus*. The underlying subepidermal layer gives rise only to the ORNs [Klein and Graziadei, 1983; Costanzo and Graziadei, 1987; Reiss and Burd, 1997a/b; Hansen et al., 1998]. According to Klein and Graziadei [1983], the ‘olfactory placode proper’ derives from the so-called ‘nervous layer’ also termed ‘subepidermal layer’ [Hansen and Zeiske, 1993]. We recently found this same mode of development in sturgeons (*Acipenser* species). Ciliated non-sensory cells in the latter also secondarily populate the olfactory epithelium [Zeiske et al., 2003]. In *Xenopus* embryos, incorporation of ciliated epidermal cells into the olfactory epithelium is also likely [Hansen et al., 1998].

Generally, similarities may be based on symplesiomorphies (shared primitive structures), synapomorphies (shared derived structures), or convergencies (independently evolved structures). Formation of the olfactory epithelium with an exclusively epidermal contribution of supporting cells as seen in *Acipenser* and *Xenopus* is phylogenetically considered a plesiomorphic (primitive) osteichthyan or at least a basal actinopterygian character, as opposed to the apomorphic (derived) mode found in teleosts [Zeiske et al., 2003]. Likewise, the formation of olfactory pit and cavity through invagination of both epidermal and subepidermal components (as opposed to the situation in *Polypterus* where only the epidermal layer invaginates) we described for *Acipenser* [Zeiske et al., 2003] is obviously primitive for osteichthyans. It is similar in selachians [Berliner, 1902], *Xenopus* [Costanzo and Graziadei, 1987] and some urodeles [Schuch, 1934; Bertmar, 1966 (with further references to the development of the olfactory organ in amphibians)]. The same holds true for the contribution of ciliated non-sensory cells to the olfactory epithelium from the invaginating epidermal layer. It may be speculated that this plesiomorphic developmental type has been conserved in the branch that leads from the fish-like to the tetrapodean grade. The process of invagination, however, which is restricted to the epidermis above the placode in *Polypterus*, only bears some resemblance in comparison to this plesiomorphic condition.

The formation of the incurrent and excurrent olfactory opening by division of a single primordial opening as described for *Polypterus* [Bartsch et al., 1997] is termed the ‘salmonid type’ [Reinke, 1937]. It is widespread among actinopterygians, e.g. paddlefish, sturgeons, and teleosts [Bemis and Grande, 1992; Zeiske et al., 1992; Hansen and Zeiske, 1993; Olsen, 1993; Zeiske et al., 2003]. It is considered a primitive or plesiomorphic character of actinopterygians [Kleerekoper, 1969; Zeiske et al., 1992].

Acipenserids are considered a basic group of actinopterygian fish and thus rather close to the common origin of actinopterygians and sarcopterygians, including tetrapods [Zeiske et al., 2003]. This is also true for polypterids. In the development of the olfactory organ, *Polypterus* shows a mixture of primitive and derived similarities to teleosts, but also to *Acipenser* and *Xenopus*. It is evident that the incorporation of ciliated non-sensory and supporting cells from the epidermal layer as well as the division of the primary olfactory opening into the incurrent and excurrent nostrils are plesiomorphic characters of actinopterygians. It is unclear, whether the primary generation of supporting cells of subepidermal placodal origin in *Polypterus* is homologous to the teleostean condition or a convergency. The corresponding character state in other relevant actinopterygian groups, e.g. *Lepisosteus*, *Amia* and basal groups of teleosts, is unknown. It would apparently contradict actual phylogenetic hypotheses of gnathostome evolution [cf. Bartsch and Britz, 1997; Northcutt, 1997; Venkatesch et al., 2001] to assume that the formation of an internal lumen as the primordium of the olfactory cavity is a synapomorphic character of lungfish, polypterids and teleosts. Significant structural differences as discussed above for *Polypterus* and teleosts point rather to a convergent evolution. Further justification for this interpretation lies in the unique, complicated and highly specialized structure of the olfactory organ in the adult polypterids.

Conclusions

Following the principles of the Phylogenetic Systematics we consider similarities either as symplesiomorphies (shared primitive structures), synapomorphies (shared derived structures), or convergent features (independently evolved structures). This method makes characters of the developing olfactory organ available for phylogenetic analysis and also allows a scenario demonstrating the crucial changes in the evolution of the olfactory organs in basal vertebrates. Based on our present and previous studies we sum up and postulate the following.

Plesiomorphic or basal osteognathostome character states: The primary olfactory pit (prospective olfactory chamber) originates through invagination of the epidermis and the subepidermal layer (in *Acipenser* and *Xenopus*). - The two olfactory openings (anterior incurrent and posterior excurrent nostrils) derive from a single primary opening which elongates and is then constricted by a bridge that separates the two external openings (in *Acipenser*, *Polypterus*, and many teleosts). — The olfactory epithelium derives from the two-layered placode consisting of an epidermal and subepidermal layer (in *Acipenser* and *Xenopus*).

Apomorphic or derived osteognathostome character states: An internal lumen is the primordium of the prospective olfactory chamber. It results from local cell death in the placode or by refraction of cells that lose their contacts (in *Polypterus* and teleosts, possibly also in the lepidosirenid lungfish *Protopterus*). A subepidermal layer gives rise to the olfactory epithelium and all its cellular constituents (teleosts).

As to the two different origins of the olfactory supporting cells in *Polypterus* we assume either a combination of plesiomorphic and apomorphic character states or an independently evolved third type of development. The most frequent features and thus ancestral osteognathostome character states in the development of the olfactory organs are found in *Acipenser* and *Xenopus*. *Polypterus* and teleosts show a mixture of plesiomorphic, apomorphic, and convergent features. The development of the olfactory organ in teleosts, however, is more derived from the ancestral type than the development of the olfactory organ seen in *Polypterus*. Major gaps in the phylogenetic analysis still concern the lack of adequate ultrastructural studies and knowledge of the early development of the olfactory organ in *Lepisosteus* and *Amia* among Actinopterygii, Dipnoi, and Urodela among Sarcopterygii with respect to these character states.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

agl, attachment gland
 an, anterior nostril
 cc, cilia
 corn, ciliated olfactory receptor neuron
 de, dendrite
 ep, epidermal layer
 esc, epidermal supporting cell
 fb, forebrain
 fbl, fibroblasts
 ipc, intracellular primary cilia
 morn, microvillous olfactory receptor neuron
 nce, non-sensory ciliated cell
 no, neuromast organs
 ok, olfactory knob
 opl, olfactory placode
 orn, olfactory receptor neuron

pn, posterior nostril
 poc, primary olfactory cavity = primary lumen of the nasal sac
 pop, primary olfactory pit
 psc, placodal supporting cell
 sc, supporting cell
 se, subepidermal layer

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Fig. 1.

Early development of the olfactory placode in *P. senegalus*. Light Microscopy, semi-thin sections. **A,B** Early embryo 34 h AF, parasagittal section showing early placode formation. **C** Cone-shaped ingrowth of the thickened placode in 52 h AF (transversal section). **D** Formation of the primary nasal sac and internal lumen 72 h AF (transversal section). agl—attachment gland, fb — forebrain, opl — olfactory placode, poc — primary olfactory cavity or lumen of the nasal sac.

Fig. 2. Developing olfactory epithelium after hatching in *P. senegalus* (A — 96 h AF, B — 98 h AF, C,D — 120 h AF), TEM. **A** Olfactory epithelium with primary (placodal) supporting cells (psc) and developing olfactory receptor neurons (orn) with intracellular primary cilia (ipc). **B** Extension of the primary olfactory cavity of the nasal sac (poc). **C** Dendritic ending of ciliated olfactory receptor neuron (corn) with projecting cilium. **D** Disintegrating junctional complex of the olfactory epithelium close to the primary lumen.

Fig. 3.

Development of the olfactory organ in *P. ornatipinnis* and *P. senegalus*, SEM. **A** *P. ornatipinnis*, stage 28, embryo just before hatching. Formation of the primary olfactory pit by epidermal invagination. **B,C** *P. senegalus*, stage 34 (8d 23h AF, yolk-sac larva). Process of division of the primary olfactory opening. **D** *P. senegalus*, stage 35 (9d 22h AF, latest yolk-sac larval stage before onset of extrinsic feeding). Anterior (incurrent) and larger, posterior (excurrent) olfactory opening formed by complete epidermal bridge. agl — attachment glands, an — anterior nostril, nce — non-sensory ciliated cell, no — neuromasts organs of cephalic lateral lines, pn — posterior nostril, arrows — primary olfactory pit.

Fig. 4.

Advanced development of the olfactory epithelium in *P. senegalus*, TEM. **A** Stage 32 (168 h AF). Formation of the primary olfactory cavity (poc) and its open connection with the primary olfactory pit. **B** Ciliated olfactory receptor neurons (corn) accompanied by secondary (epidermal) supporting cells (esc) in the yolk-sac larval olfactory epithelium (Stage 33, 190 h AF) in cross-section. **C, D** Apical region of the olfactory epithelium close to the placodal lumen and its duct to the exterior (Stage 33, 190 h AF). Horizontal sections through two different levels. Olfactory knobs (ok) (in C) and dendrites (de) (in D) of olfactory neurons surrounded by secondary (epidermal) supporting cells (esc).

Fig. 5.

Receptor types of the mature olfactory epithelium in *P. senegalus* (larvae and juveniles older than 30 days). **A** Ciliated (or “mixed”) receptor neurons (corn) accompanied by supporting cells (sc). Arrow indicates basal body and rootlet of a cilium. TEM. **B** Juvenile olfactory epithelium showing the olfactory knobs of ciliated olfactory neurons (corn). SEM. **C** Microvillous receptor neuron (morn) among supporting cells (sc) and ciliated receptor neurons (corn). TEM. **D** Rare crypt olfactory receptor neuron in a cross section through the juvenile olfactory epithelium. Cilia (cc) shown included in “crypt” spaces of the cell body.

Fig. 6. Schematic diagram of major steps in early development of the olfactory organ in *Polypterus*. **A** Early embryonic stage with epidermis (ep) and sole subepidermal (se) component of the olfactory placode. **B** Formation of the primary olfactory pit (pop) and cavity (poc), fibroblasts of the loose connective tissue (fbl) and first forebrain (fb) connections. **C** Opening of the internal lumen to the exterior and shaping of the olfactory cavity. Ciliated olfactory receptor neurons (corn) and primary (placodal) supporting cells (psc) of the subepidermal placode shaded. Epidermal supporting cells (esc).

Taxon	Genus/species	Formation of primary olfactory cavity and opening mechanism	Origin of ORNs	Origin of supporting cells	References
Chondrichthyes					
Elasmobranchii	<i>Iago omanensis</i>	epidermal and subepidermal invagination	subepidermal	subepidermal?	Fishelson & Baranes, 1997
Sarcopterygii					
Dipnoi	<i>Protopterus</i>	"epidermis disappears"	subepidermal	subepidermal?	Fullerton, 1933
Amphibia					
Anura	<i>Xenopus laevis</i>	epidermal and subepidermal invagination	subepidermal	epidermal	Klein & Graziadei, 1983; Hansen et al. 1998
Actinopterygii					
Cladistia	<i>Polypterus</i>	epidermis separates and retracts	subepidermal	epidermal and subepidermal	this study
Chondrostei	<i>Acipenser baeri</i> , <i>Acipenser ruthenus</i>	epidermal and subepidermal invagination	subepidermal	epidermal	Zeiske <i>et al.</i> , 2003
Teleostei	<i>Danio</i> (<i>Brachydanio</i>) <i>rerio</i>	epidermis separates and retracts	subepidermal	subepidermal	Hansen & Zeiske, 1993