

Ultrastructure of the Olfactory Organ in the Clawed Frog, *Xenopus laevis*, During Larval Development and Metamorphosis

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ABSTRACT

Development of the olfactory epithelia of the African clawed frog, *Xenopus laevis*, was studied by scanning and transmission electron microscopy. Stages examined ranged from hatching through the end of metamorphosis. The larval olfactory organ consists of two chambers, the principal cavity and the vomeronasal organ (VNO). A third sensory chamber, the middle cavity, arises during metamorphosis. In larvae, the principal cavity is exposed to water-borne odorants, but after metamorphosis it is exposed to airborne odorants. The middle cavity and the VNO are always exposed to waterborne odorants.

Electron microscopy reveals that in larvae, principal cavity receptor cells are of two types, ciliated and microvillar. Principal cavity supporting cells are also of two types, ciliated and secretory (with small, electron-lucent granules). After metamorphosis, the principal cavity contains only ciliated receptor cells and secretory supporting cells, and the cilia on the receptor cells are longer than in larvae. Supporting cell secretory granules are now large and electron-dense. In contrast, the middle cavity epithelium contains the same cell types seen in the larval principal cavity. The VNO has microvillar receptor cells and ciliated supporting cells throughout life.

The cellular process by which the principal cavity epithelium changes during metamorphosis is not entirely clear. Morphological evidence from this study suggests that both microvillar and ciliated receptor cells die, to be replaced by newly generated cells. In addition, ciliated supporting cells also appear to die, whereas there is evidence that secretory supporting cells transdifferentiate into the adult type. In summary, significant developmental additions and neural plasticity are involved in remodeling the olfactory epithelium in *Xenopus* at metamorphosis. *J. Comp. Neurol.* 398:273–288, 1998. © 1998 Wiley-Liss, Inc.

Indexing terms: amphibia; chemosensory; ontogeny; scanning electron microscopy; transmission electron microscopy

As their name implies, amphibians typically lead a dual life, with an aquatic larva and a terrestrial adult. In this way, they are intermediate between fishes and amniotes. The transition from water to land clearly places many demands on sensory systems, including the olfactory system. Classical studies at the light microscopic level have shown that the olfactory organ remodels at metamorphosis in all living amphibian groups: salamanders (Seydel, 1895; Schuch, 1934), caecilians (Sarasin and Sarasin, 1890; Badenhorst, 1978), and frogs (Born, 1876; Hinsberg, 1901; Föske, 1934; Rowedder, 1937; Khalil, 1978). This remodeling is far more extensive in frogs than in the other groups. However, little is known about the ultrastructural changes that accompany the changes in gross morphology.

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Xenopus laevis, the African clawed frog, is a favored animal for studies of development and morphology, and a great deal of information exists on various aspects of the *Xenopus* olfactory system (reviewed by Reiss and Burd, 1997b). The olfactory organ of adult *Xenopus* comprises a complicated system of chambers: the principal cavity, the middle cavity, and the vomeronasal organ (VNO; Föske, 1934; Paterson, 1939a,b, 1951; Altner, 1962). Terminology for these chambers has varied among authors; however, we prefer to use the same terminology as for other frogs (Scalia, 1976). The two divisions of the olfactory organ proper (principal and middle cavities) appear to be used alternately by the frog, depending on the position of flap-like specialized skin folds within the nasal cavities. One of the divisions, the middle cavity, is always filled with water and serves the animal when it is under water (Altner, 1962); the other division, the principal cavity, is filled with air and appears to function when the animal is in terrestrial surroundings (Weiss, 1986). Like other members of the family Pipidae, *Xenopus* is unusual among frogs in being secondarily aquatic as an adult. The presence of sensory epithelium in the middle cavity appears to be unique to *Xenopus* and other pipids (Paterson, 1951) and is clearly functionally correlated with the aquatic lifestyle of adults. Most frogs have only a main olfactory epithelium (located in the principal cavity) and a vomeronasal epithelium (Scalia, 1976), like the majority of other tetrapods (Eisthen, 1992).

Interestingly, a recent study has shown that *Xenopus* has two distinct classes of odorant receptor genes: one class of receptors is closely related to those of teleost fishes, the other class clusters with those of mammals (Freitag et al., 1995). The fish-like odorant receptors are expressed in the middle cavity, which detects water-soluble odorants; the mammalian-like odorant receptors are expressed in the principal cavity, which is used to smell airborne odorants.

Previous work on the development of the olfactory organ in *Xenopus* includes Föske (1934), who examined morphogenesis from the early embryo through the adult, by using wax models based on paraffin sections (see also Paterson, 1939a,b, 1951), and Klein and Graziadei (1983), who examined the early development (stages 23–37/38) of the olfactory placode with both light and electron microscopy. The development and structure of the olfactory nerve and the olfactory bulb have also been studied thoroughly at the level of both light and electron microscopy (Burd, 1991; Byrd and Burd, 1991, 1993a,b). The ultrastructure of the adult olfactory organ was briefly examined by Saint Girons and Zylberberg (1992) and Reiss and Burd (1997b), and

both adults and larvae were examined in two unpublished dissertations (Weiss, 1986; Key, 1986).

The olfactory organs of *Xenopus*, like those of other vertebrates, derive from paired olfactory placodes that form as thickened regions of ectoderm at the anterior end of the embryo (Föske, 1934; Klein and Graziadei, 1983). The placode is first distinguishable from the surrounding ectoderm at about stage 23 (Nieuwkoop and Faber, 1994). In subsequent development, it becomes better defined and begins to invaginate to form an olfactory pit. At about stage 40, the olfactory pit begins to segregate into the anteromedial VNO and posterolateral principal cavity (Föske, 1934; Nieuwkoop and Faber, 1994). The VNO and principal cavity become progressively more distinct. At the same time, the lumen of the principal cavity extends down toward the roof of the mouth, breaking through to form the choana at about stage 50 (Nieuwkoop and Faber, 1994). In slightly later larval stages (about stage 51–52), the middle cavity begins to form at the anterior edge of the olfactory organ, dorsal to the VNO. The middle cavity expands markedly during metamorphosis. At the same time, the larval principal cavity is remodeled into the principal cavity of the juvenile. Thus, the principal cavity, which is used to smell waterborne odorants in the larva, is used to sense airborne odorants in the adult, while at the same time the middle cavity develops and assumes the role of detecting water-soluble odorants. This seemingly nonsensical developmental pattern is understandable in terms of the secondarily aquatic nature of the adults.

The differentiated olfactory epithelium of *Xenopus* has the same basic structure as in other vertebrates: bipolar neurons and supporting (sustentacular) cells lie in a pseudostratified epithelium, with the nuclei of the supporting cells located above several rows of receptor cell nuclei (Föske, 1934; Saint Girons and Zylberberg, 1992). Thin axons or groups of axons travel through the epithelium towards the basal lamina. In the lamina propria, they gather to form fila olfactoria, which in turn gather and form the olfactory nerve. In addition to receptor and supporting cells, basal cells lie along the basement membrane.

The work of Klein and Graziadei (1983) showed that the early olfactory placode is made up of a superficial layer and a deep (nervous or sensory) layer, like the rest of the embryonic ectoderm. Olfactory receptor cells derive from the nervous (sensory) layer, whereas the supporting cells derive from the superficial layer. At stage 37/38 (just after hatching), the last stage examined by Klein and Graziadei (1983), the developing receptor cells are all ciliated, and the supporting cells are all microvillar.

In the present study, we describe the morphology and development of the peripheral olfactory organ of *Xenopus laevis* from stage 39, immediately following the last stage studied by Klein and Graziadei (1983), through the juvenile, with both light and electron microscopy. We focus in particular on the stages before and during metamorphosis, to examine the changes that convert the larval to the juvenile organ. We show that during metamorphosis, a marked change in the ultrastructure of the olfactory epithelium occurs in the principal cavity, whereas the middle cavity only differentiates more fully, and the VNO undergoes no appreciable changes.

Abbreviations

k	kinocilia
MC	middle cavity
p	pigment granule
PC	principal cavity
Rc	ciliated receptor cell
Rm	microvillar receptor cell
Sc	ciliated supporting cell
SEM	scanning electron microscopy
St	stage
Sv	secretory supporting cell
Sw	Schwann cell
TEM	transmission electron microscopy
VNO	vomeronasal organ

MATERIALS AND METHODS

Embryos were obtained by mating adult *Xenopus laevis* of our own breeding colonies after chorionic gonadotropin stimulation (New, 1966). Embryos and larvae were reared in tap water or rearing solution (Burd, 1991), and the larvae were fed with boiled nettle leaves with some also receiving yeast. Embryos and larvae were staged according to the table of Nieuwkoop and Faber (1994). Postmetamorphic frogs were fed liver. Before fixation, embryos, larvae, and juvenile animals were anesthetized with 0.02 % 3-aminobenzoic acid ethyl ester (MS 222, Sigma, St. Louis, MO). Prior to our study, all protocols for animal care and treatment were approved by the University Animal Care and Use Committee at the University of Arizona. Ten animals per stage were processed for both forms of electron microscopy (see below) and at least five animals per stage were examined. Semiquantitative analyses, for example, the length of cilia, were determined from at least five animals.

Scanning electron microscopy

Embryos and larvae from different breedings and young adults 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 electron microscope.

Transmission electron microscopy

Embryos, larvae, and young adults from different breedings and populations were fixed by several different procedures. Some were fixed by immersion in 5% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for several hours or overnight. Others were fixed by immersion or (for juveniles) by perfusion followed by immersion with 0.6% paraformaldehyde, 2% glutaraldehyde, 0.02% CaCl₂ in 0.035 M cacodylate buffer (pH 7.3) overnight. After rinsing in buffer, specimens were postfixed in 1% or 2% osmium tetroxide for 1–2 hours. Finally, some specimens were fixed in the 0.6% paraformaldehyde, 2% glutaraldehyde fix in a Model 3450 microwave oven (Ted Pella, Inc., Redding, CA) according to previously published procedures (Kok and Boon, 1992; Giberson, et al., 1997). The procedure produced the least shrinkage of tadpole olfactory tissue. The tissue was dissected out into 600 µl cold (5°C) fixative solution in a microfuge tube, then processed through three cycles of microwaving for 30 seconds followed by cooling 5 minutes in an ice water bath. After rinsing in cold buffer, these specimens were postfixed for three cycles of 25 seconds microwaving followed by 10 minutes cooling.

The fixed specimens were dehydrated in a graded series of ethanol and propylene oxide and embedded in glycid ether 100 (SERVA Feinbiochemica/Boehringer Ingelheim Bioproducts, Heidelberg, Germany) or Eponate 12 (Ted Pella, Redding, CA). Semithin sections of 1 µm were stained with toluidine blue and examined with light microscopy. Ultrathin sections (silver to gold) were stained and contrasted with uranyl acetate and lead citrate and examined with a Philips 300 or 420 or a JEOL 1200 electron microscope.

RESULTS

Early larval development (stages 39–45)

At stage 39, just after hatching, the olfactory pits are shallow depressions dorsolateral to the developing mouth (Fig. 1A,B). Only some cells at the edge of the olfactory pit still show the domed apices of epithelial cells in the process of transdifferentiation into supporting cells that are abundant at earlier stages (Klein and Graziadei, 1983). The microvilli-like protrusions of the supporting cells, seen in earlier stages, are now considerably smaller. Between these newly formed supporting cells, a few olfactory knobs bearing receptor cilia as well as tiny protrusions are visible (Fig. 1C). Between 18 and 23 olfactory knobs of ciliated receptor cells are present per olfactory organ.

At stage 40, the interface between the olfactory pit and the surrounding epidermis has become sharper, and the olfactory pit has begun to invaginate (Fig. 2A). About 250 olfactory knobs with receptor cilia are present per olfactory organ. In addition, the first few microvillar receptor cells can now be seen at the surface of the epithelium (Fig. 1D). Their olfactory knobs are less pronounced than those of the ciliated receptor cells. In TEM micrographs of stage 40, the ciliated receptor cells and the few microvillar receptor cells already have the typical features of olfactory receptor cells (Fig. 3). The microvillar supporting cells are now joined for the first time by supporting cells bearing kinocilia (Fig. 1D). These appear between the olfactory knobs, especially at the dorsomedial end of the pit, where a region entirely covered with this type of cilia has developed. The supporting cells still contain pigment granules and yolk droplets, showing their origin from the nonsensory layer of the ectoderm (Figs. 2A, 3). Receptor cilia, receptor microvilli and kinocilia are still short (1–1.5 µm, 0.5–0.8 µm, and 3 µm, respectively).

At stage 41, the dorsomedial area covered exclusively by kinocilia begins to form a small tube (Fig. 1E); this is the beginning of the VNO. The remaining surface of the olfactory pit is now densely covered by receptor cilia and microvilli and by kinocilia, which have all increased in length (3 µm, 2 µm, and 6 µm, respectively). The olfactory knobs of the ciliated receptor cells that are added at this stage and later in development (Fig. 1F) bear 6–10 cilia, fewer than the pioneering olfactory knobs of the early stages, which often showed up to 14 cilia.

During the next few stages, the olfactory pit widens and the tube-like form of the VNO becomes more distinct. Differentiated receptor cells are present in the VNO by stage 42. Larval feeding begins at stage 45 (Nieuwkoop and Faber, 1994).

Later larval development (stages 45–55)

Later larval development involves mainly growth and further differentiation of the olfactory organ. In a stage 50 animal, corresponding approximately to stage 7 of Föske (1934), the principal cavity is a deep cup, broadly open above (Fig. 2B). The borders of the external naris overhang this cup laterally and caudally. A passageway extends down from the anterolateral corner of this cup to the choana (internal nostril), which is guarded by a valve-like flap. The VNO is a flattened sac that extends medially from the anteromedial wall of this passageway. It lies just deep to the skin at the anterior border of the external naris. Jacobson's gland has begun to branch off of the caudal end of the VNO (Nieuwkoop and Faber, 1994).

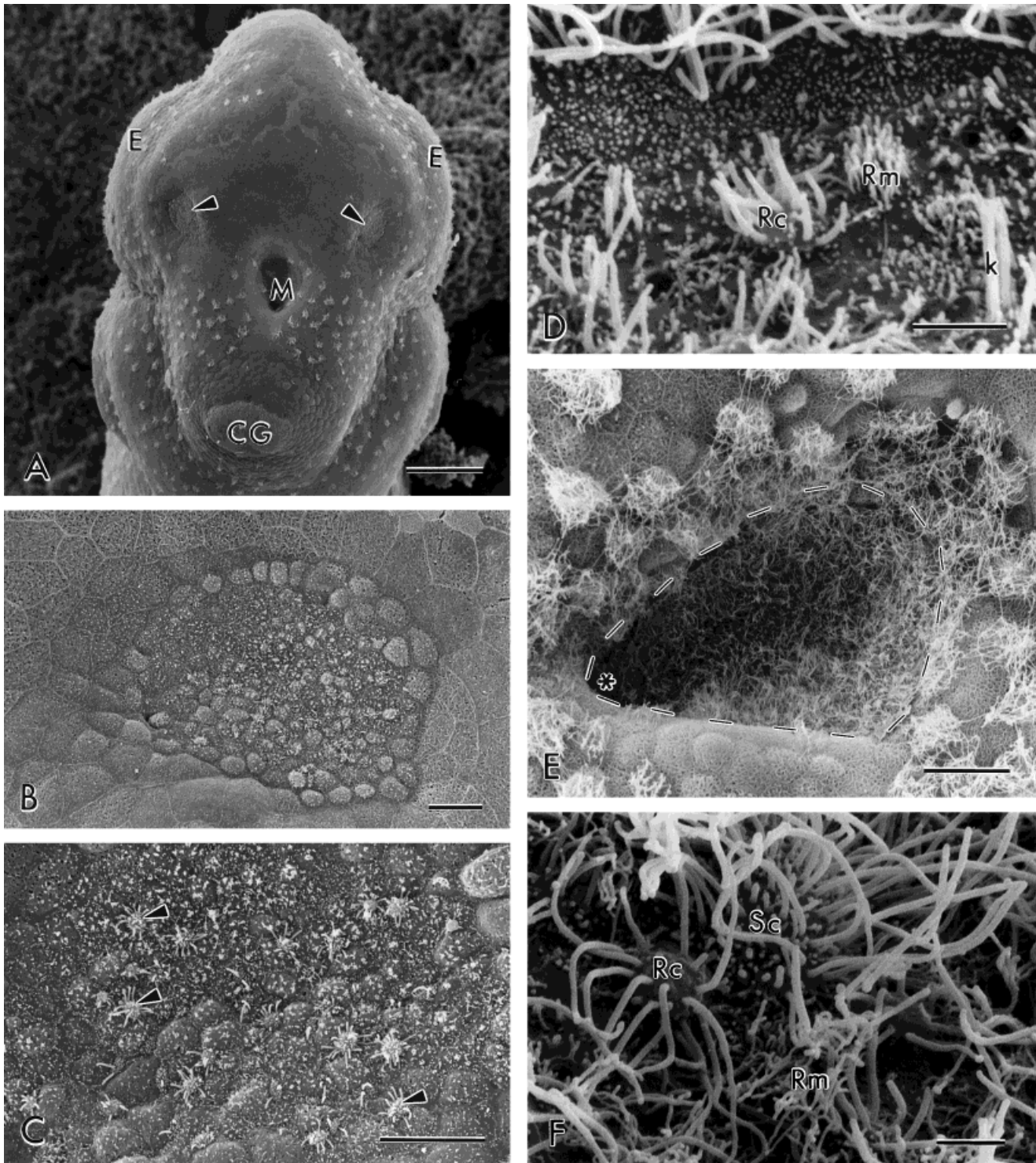


Fig. 1. Scanning electron microscopy (SEM) of the developing olfactory organ. **A:** Stage (St.) 39. Head in anterior view illustrating the eyes (E), developing mouth (M), and cement gland (CG). Arrowheads indicate olfactory placodes. **B:** St. 39. Olfactory placode and surrounding epidermis. **C:** St. 39. Higher power of olfactory placode showing examples of olfactory knobs of receptor cells with cilia (arrowheads). **D:** St. 40. Microvillar receptor cells (Rm) are now

present in addition to ciliated receptor cells (Rc). Kinocilia (k) of ciliated supporting cells also present. **E:** St. 41. The olfactory pit (dashed line) has deepened, particularly in the region of the future vomeronasal organ (asterisk). **F:** St. 46. Cilia of receptor cells and supporting cells (Sc) and microvilli of receptor cells become much longer by this stage. Scale bars = 100 μ m in A, 30 μ m in B, 10 μ m in C, E, 2 μ m in D, F.

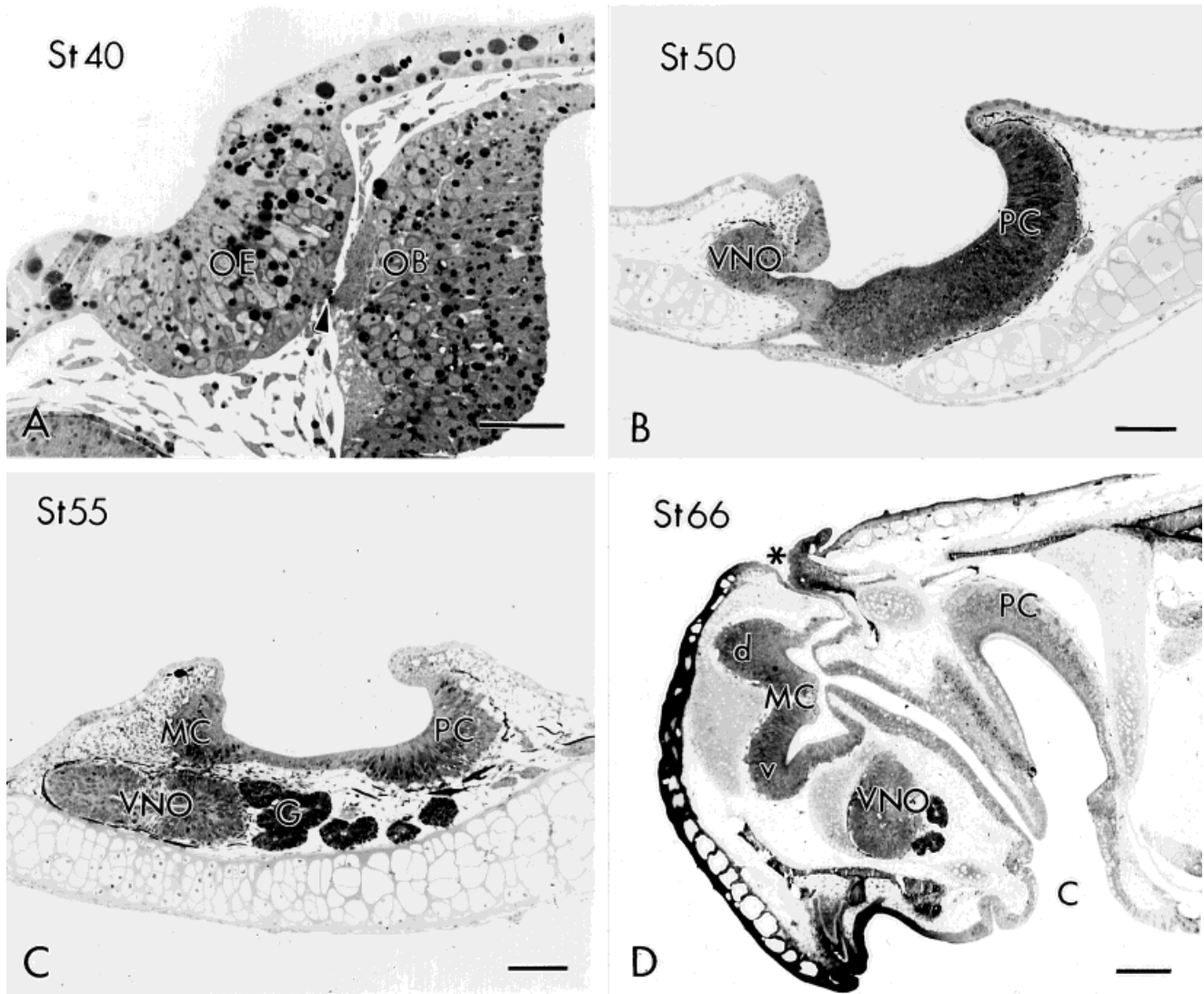


Fig. 2. One- μ m-thick sections of the olfactory organ stained with toluidine blue. **A:** Stage (St.) 40. Horizontal section, anterior at top. OE, developing olfactory epithelium; OB, developing olfactory bulb. Arrowhead points to olfactory axons. **B–D:** Sagittal sections, anterior to left. **B:** St. 50. VNO, vomeronasal organ; PC, principal cavity.

C: St. 55. Middle cavity (MC) is now evident. G, Jacobson's glands. **D:** St. 66 (young frog). Middle cavity is divided into dorsal (d) and ventral (v) sections. Note external naris (asterisk) and choana (C). Scale bars = 50 μ m in A, 100 μ m in B, C, 200 μ m in D.

At stage 50, the olfactory epithelium in the principal cavity (Fig. 4) contains two types of receptor cells, ciliated and microvillar, both of which were already forming in the early larval stages described above. The ciliated receptor cells bear 6–8 cilia on their olfactory knobs. The cilia show the typical 9+2 doublet system of microtubules, have a basal body with a basal foot, no basal plate and no rootlets, and are 8- to 10- μ m-long. Between the cilia, tiny membrane protrusions may occur. The less pronounced olfactory knobs of microvillar receptor cells bear 15–25 microvilli, which are almost as long as the cilia of ciliated receptor cells. The microvilli do not branch. Beneath the olfactory knob of the microvillar receptor cells, sometimes deep down in the dendrite, up to 6 centrioles can be found; these were never noted in ciliated receptor cells. Both receptor cell types have many long mitochondria and microtubules arranged parallel to the axis of the cell.

Microvillar and ciliated receptor cells appear to be distributed randomly throughout the epithelium.

At this stage, the principal cavity also has two different types of supporting cells, one type containing small apical secretory vesicles and short microvilli, and one type with kinocilia and no vesicles (Fig. 4). Both secretory and ciliated supporting cells are cylindrical and have oval, electron-lucent nuclei. Lateral folds reach between the receptor cells, separating them. Ridge-like protrusions surround the collars of the olfactory knobs. The apical end of the ciliated supporting cell bears numerous kinocilia. The kinocilia are as long as the receptor cilia (approximately 10 μ m) and also show the 9+2 system of microtubule doublets. They have a basal body with a basal foot and one or two striated rootlets. The second type of supporting cell has no kinocilia, but has tiny microvilli-like protrusions on its domed apical surface, which occasionally

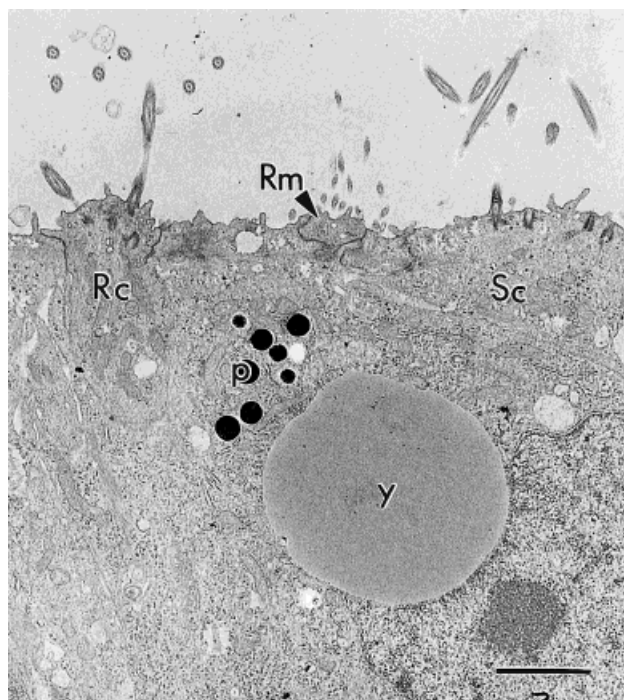


Fig. 3. Transmission electron microscopy (TEM) of the larval principal cavity at stage (St). 40. Ciliated and microvillar receptor cells and a supporting cell with cilia are illustrated. Note pigment granules (p) and yolk droplets (y). Rc, ciliated receptor cell; Rm, microvillar receptor cell; Sc, ciliated supporting cell. Scale bar = 2 μ m.

towers above the olfactory knobs. The domes are filled with secretory vesicles. Both receptor and supporting cells have junctional complexes close to their surfaces. Filaments attaching to the zonula adherens often span the supporting cells, thereby forming a "terminal web." Desmosomes are also found deeper in the epithelium between neighboring supporting cells, and less frequently, between supporting cells and receptor cells.

Two types of basal cells are present in the larval principal cavity: oblong or triangular cells close to the basal lamina and round cells (cf. Fig. 6B). Bowman's glands are not yet developed. The larval VNO has one type of receptor and one type of supporting cell (Fig. 5A,B). VNO receptor cells have olfactory knobs bearing 20–30 microvilli of 5–8 μ m in length. Their dendrites may contain up to 6 centrioles. VNO supporting cells bear typical kinocilia (approximately 10- μ m-long) with broad striated rootlets. The basal cells show the same characteristics as those of the olfactory epithelia proper.

Towards the end of premetamorphic development, the future middle cavity begins to differentiate at the rostral end of the principal cavity, dorsal to the VNO. DiI back-labeling from the olfactory bulb first begins to label receptor cells in this region at stage 52 (data not shown). Over the next few stages, the middle cavity epithelium continues to enlarge and differentiate.

Prometamorphosis and metamorphosis (stages 55–65)

By stage 55, the middle cavity epithelium is well developed (Fig. 2C). It consists of the same cell types found in

the larval principal cavity. These include microvillar and ciliated receptor cells, secretory and ciliated supporting cells, and basal cells (Fig. 6B–D). The middle cavity continues to enlarge in the following stages, developing into a complicated system of chambers (Föske, 1934), but its epithelium shows no important changes in structure.

While the middle cavity is elaborating, signs of degeneration develop in the principal cavity. At stages 55–56 (Fig. 6A), receptor cells may have the same appearance as at earlier stages, but some receptor cells develop large vacuoles. By stage 57, receptor cells are found that show surface blebbing and degenerating organelles (Fig. 7A). Meanwhile, small vesicles can be observed just below the basal bodies of some ciliated supporting cells (Fig. 7A). Secretory supporting cells, in contrast, begin to show a mixed population of vesicles (Fig. 7A), with some vesicles larger and more electron-dense than the typically lucent vesicles in larvae. By stage 59, some of the secretory supporting cells contain vesicles that are extremely electron-dense (Fig. 7B). At the same time, ciliated receptor cells begin to appear that have much longer cilia than those found in larvae, though not yet as long as those in adults. These changes continue, and by stages 63–65, the epithelium of the principal cavity resembles, in all respects, that of a postmetamorphic animal.

Postmetamorphic structure

In postmetamorphic animals, the complicated olfactory organ fills most of the upper jaw apparatus. Compared with the larval stages, the most notable change is the marked separation between principal and middle cavities (Fig. 2D). The principal cavity is now a large, elongate sac located close to the nasal septum. The rostral end of the principal cavity is a pointed cupula anterior to the external naris, and the caudal end is a flat cupula extending posteriorly slightly beyond the choana. The middle cavity lies anterolateral to the principal cavity, and has become much more complicated. It is now divided into dorsal and ventral sections, which each have a number of subdivisions and diverticulae, many of which are lined by respiratory epithelium. The principal and middle cavities are connected anteriorly only by a narrow slit, the infundibulum, formed between two flaps (the ventral and dorsal folds). The external naris communicates with both the principal and middle cavities, whereas the choana communicates only with the principal cavity. The VNO is a narrow sac extending transversely ventral to the principal cavity. It communicates with the principal cavity by a slit known as the isthmus, which is the caudal continuation of the infundibulum.

Three sets of glands are now present. The large compound tubular Jacobson's gland empties into the VNO (Föske, 1934). The compound tubular glandula oralis interna empties into the principal cavity. Bowman's glands are now found throughout the olfactory epithelium of the principal cavity. Unlike those of mammals, they are located primarily within the sensory epithelium, and only some reach down into the lamina propria. These latter two glands, together with the supporting cells, are presumably responsible for a thick layer of mucus that now coats the sensory epithelium of the principal cavity (Fig. 8A). As in the larval principal cavity, no glands are associated with the middle cavity.

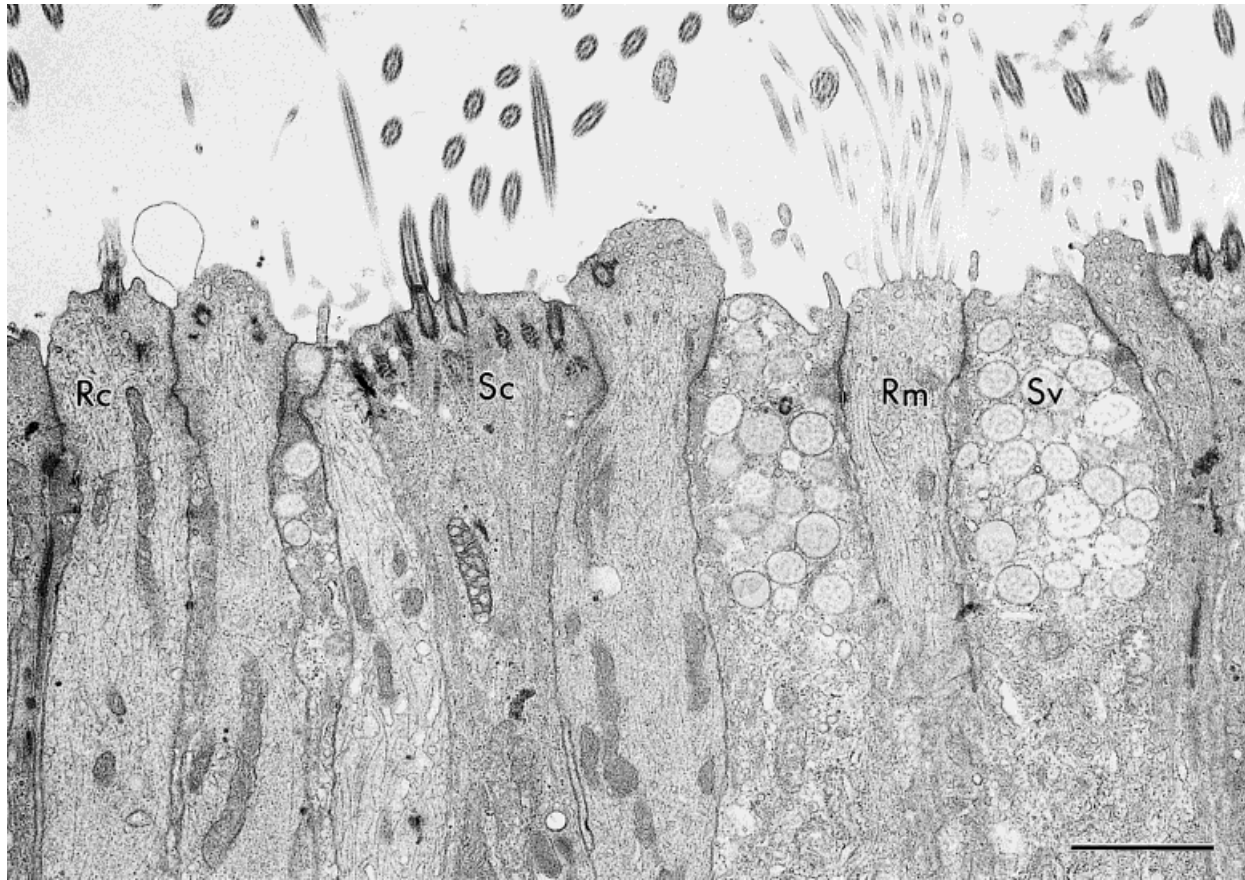


Fig. 4. Transmission electron microscopy (TEM) of the larval principal cavity at Stage 50. All four cell types are well differentiated. Rc, ciliated receptor cell; Rm, microvillar receptor cell; Sc, ciliated supporting cell; Sv, secretory supporting cell. Scale bar = 2 μ m.

The ultrastructure of the principal cavity epithelium has changed completely from the larval condition (Figs. 8A, 9A,B). Only ciliated receptor cells are now present. These cells resemble those of the larval organ in most respects, but their cilia are much longer. The cilia at first extend directly toward the lumen, but after about 12–20 μ m they bend and run parallel to the epithelial surface within the mucus layer. Because the cilia are embedded in mucus, it is difficult to measure their length, but we estimate that they are 80 to 100 μ m. Spindle-shaped swellings (Reese, 1965) have been found only infrequently. Proximally, the ciliary microtubules are arranged in 9+2 doublets, but distally, the cilia taper and cross sections show a single pair of microtubules.

Only secretory supporting cells are now present in the principal cavity. These supporting cells bear microvilli on their apical surfaces (Fig. 9). The apical ends of these cells are sometimes dome-shaped and protrude into the lumen so that they may envelop the olfactory knobs. The secretory granules that fill the supranuclear region (Figs. 8A, 9A,B) are now large and electron-dense, unlike those in the larval principal cavity or postmetamorphic middle cavity. The shape of the supporting cell is more or less cylindrical in its apical portion. The basal portion tapers between the layer of receptor cell nuclei and basal cell nuclei towards the basal lamina.

As in larval stages, junctional complexes with zonula occludens, zonula adherens, and macula adherens connect the cells close to the lumen. More basally, desmosomes connect supporting cells to each other, but only infrequently connect supporting cells and receptor cells. Two types of basal cells are still present: a dark cell type that lies adjacent to the basal lamina and a light type located between the dark basal cells and the layer of receptor cell nuclei.

The sensory epithelium of the middle cavity (Figs. 8B,C, 10A) is quite different from that of the postmetamorphic principal cavity, but identical in most respects to that of the larval principal cavity. Both ciliated and microvillar receptor cells are present (Figs. 8C, 10A) as are both secretory and ciliated supporting cells (Fig. 10A). The ultrastructure of the postmetamorphic VNO resembles that of advanced larvae in all respects (Fig. 10B), though the region of sensory epithelium is now much enlarged.

The areas between the sensory epithelia of the various chambers are lined with nonsensory epithelia of different types. Some areas are completely covered by nonsensory cells with numerous cilia that are 3- to 5- μ m-long. Other areas show ciliated nonsensory cells together with plain-surfaced cells that are filled with tiny electron-dense granules. Near the borders of the middle cavities and the VNO, typical goblet cells are interspersed in the nonsensory

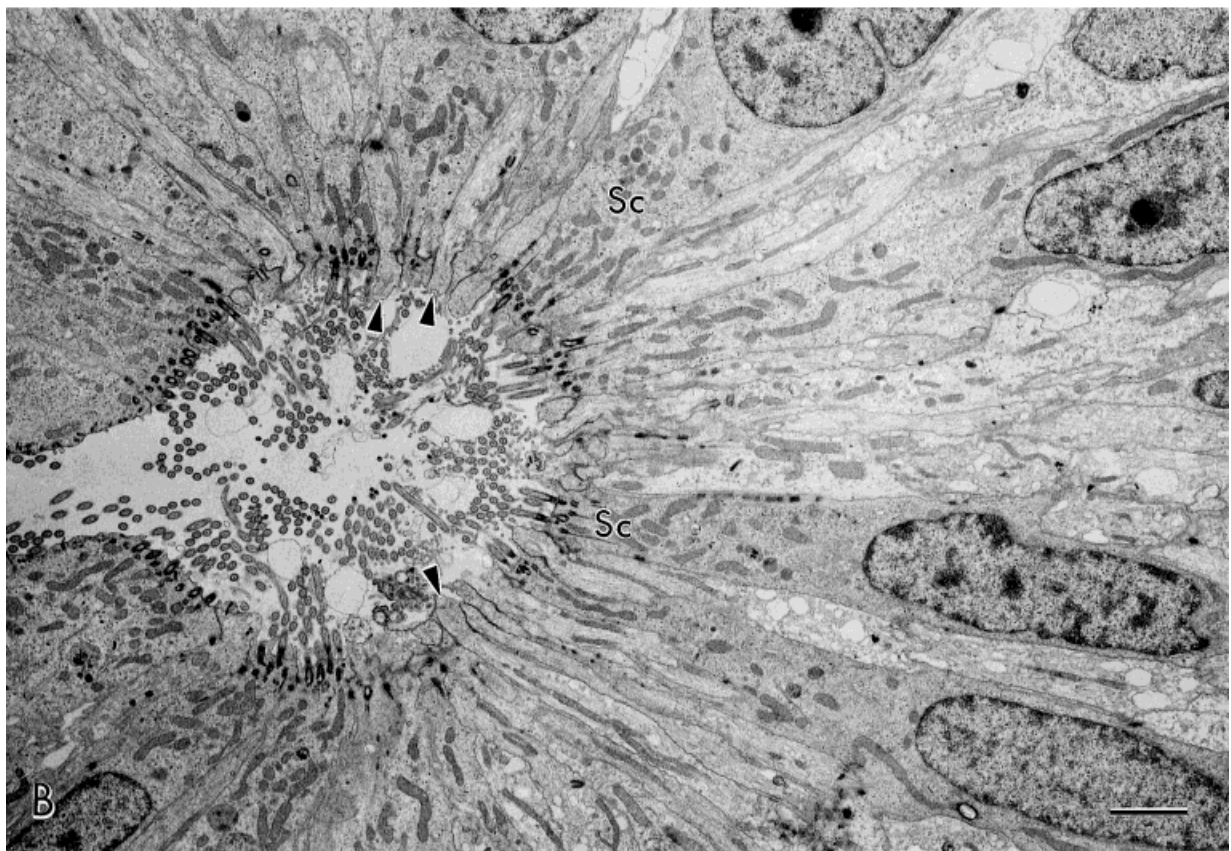
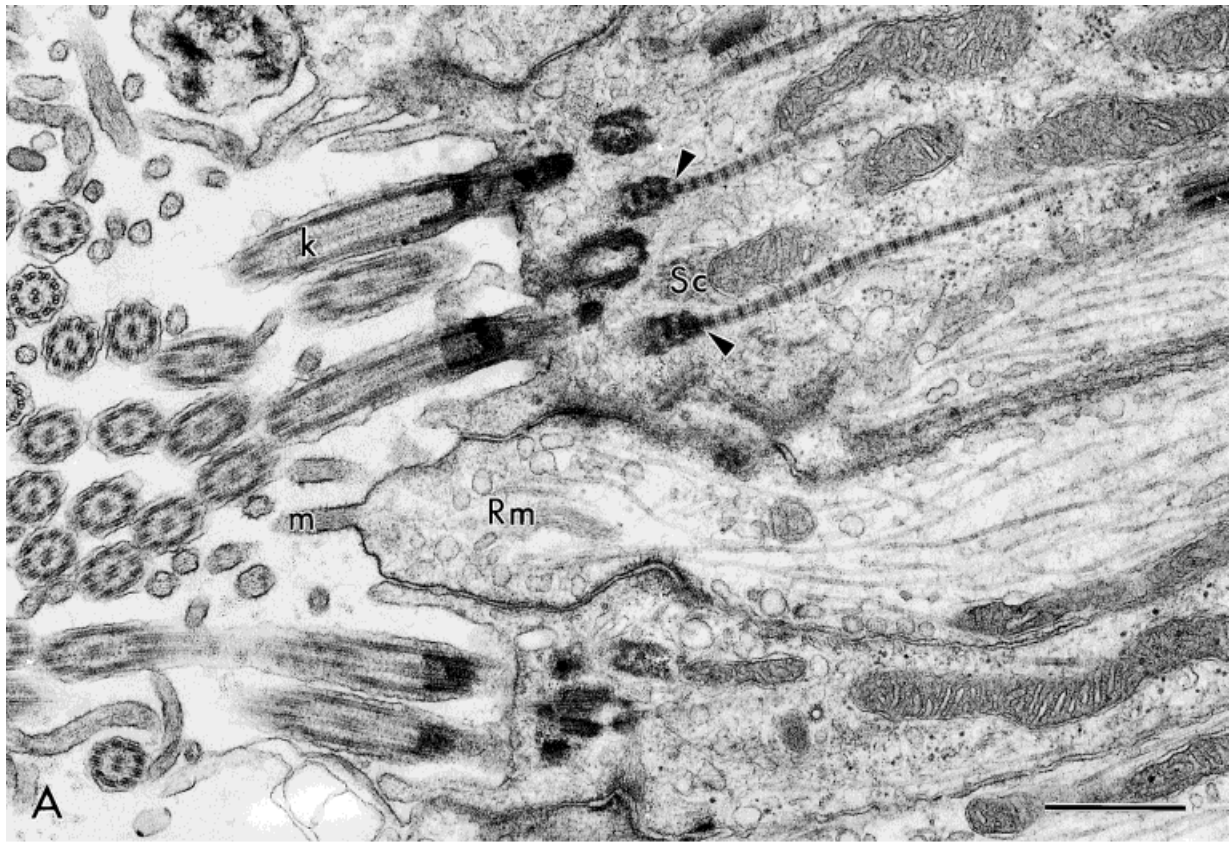


Fig. 5. Transmission electron microscopy (TEM) of the vomeronasal organ at stage (St.) 55. **A:** Microvillus (m) on a microvillar receptor cell and kinocilia (k) on a supporting cell. Note ciliary rootlets (arrowheads). **B:** Lower magnification to show overall form of cavity, as

well as microvillar receptor cells (arrowheads) and ciliated supporting cells. Rm, microvillar receptor cell; Sc, ciliated supporting cell. Scale bars = 0.5 μ m in A, 2 μ m in B.

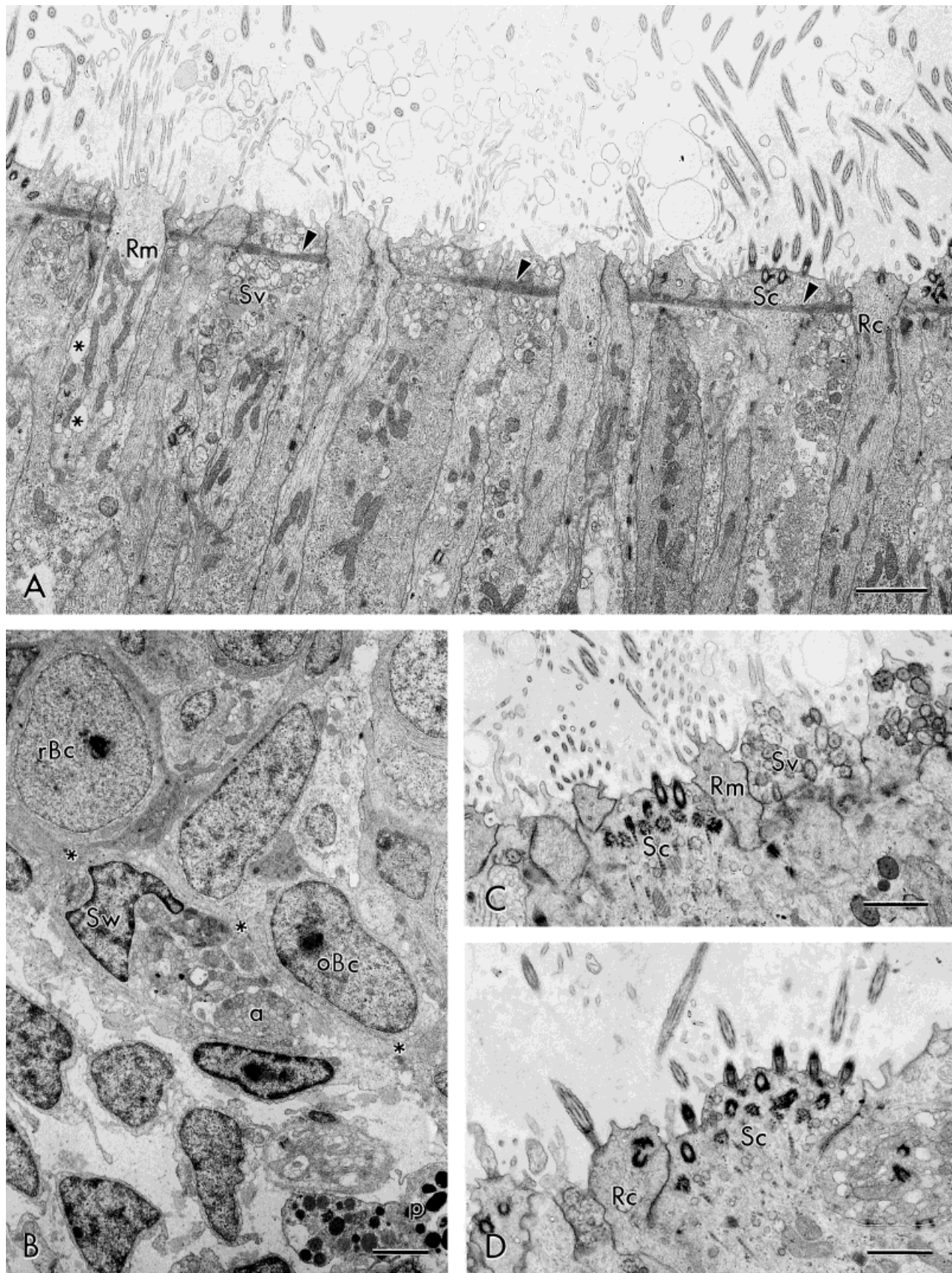


Fig. 6. Transmission electron microscopy (TEM) of principal and middle cavities at prometamorphosis. **A:** Stage (St.) 56 principal cavity. Microvillar and ciliated receptor cells are present, as are ciliated and secretory supporting cells. Note prominent terminal web (arrowheads) spanning supporting cells, also vacuoles in microvillar receptor cell (asterisks). **B:** St. 55 middle cavity, basal region of epithelium. Note a round cell presumed to be a basal cell (rBc), and an oblong basal cell (oBc), a Schwann

cell (Sw), and bundle of olfactory axons (a). Asterisks mark the location of the basal lamina; above this is olfactory epithelium, below is the lamina propria. Note also pigment granules (p) in lamina propria. **C,D:** St. 55 middle cavity, apical surface. Note presence of all cell types seen above in the larval principal cavity. Rc, ciliated receptor cell; Rm, microvillar receptor cell; Sc, ciliated supporting cell; Sv, secretory supporting cell. Scale bars = 2 μ m in A,B, 1 μ m in C,D.

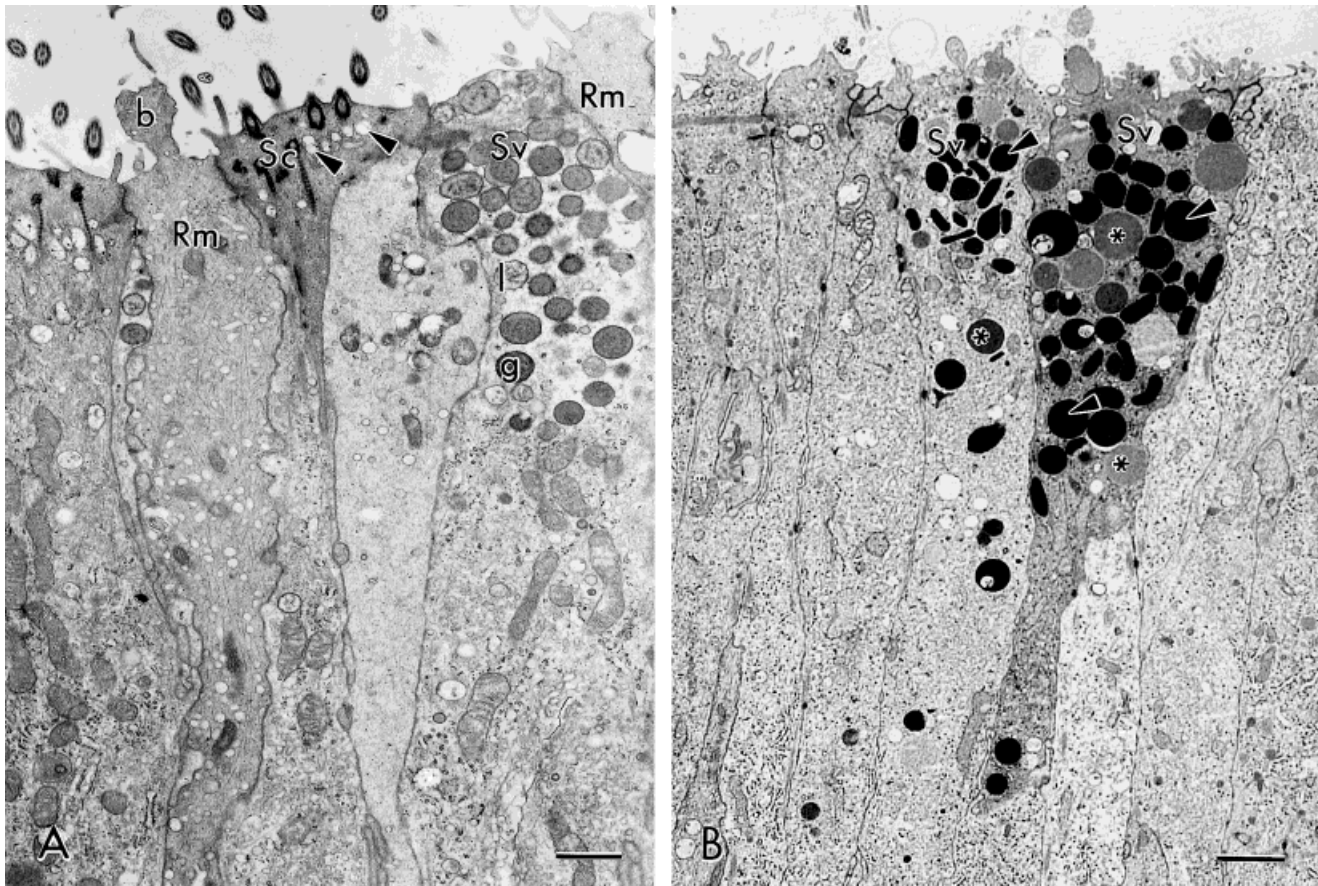


Fig. 7. Transmission electron microscopy (TEM) of the principal cavity during metamorphosis. **A:** Stage (St.) 57. Note blebbing (b) of the surface of a microvillar receptor cell, vacuoles (arrowheads) in a ciliated supporting cell, and variation in vesicle density (light [l] and dark gray [g]) in secretory supporting cells. **B:** St. 59. Note less dense

vesicles (asterisks) scattered among typically dense, adult type vesicles (arrowheads) in secretory supporting cells. Rm, microvillar receptor cell; Sc, ciliated supporting cell; Sv, secretory supporting cell. Scale bars = 1 μ m in A, 2 μ m in B.

epithelium (data not shown). They are especially abundant close to the infundibulum.

DISCUSSION

Early differentiation of the olfactory placode

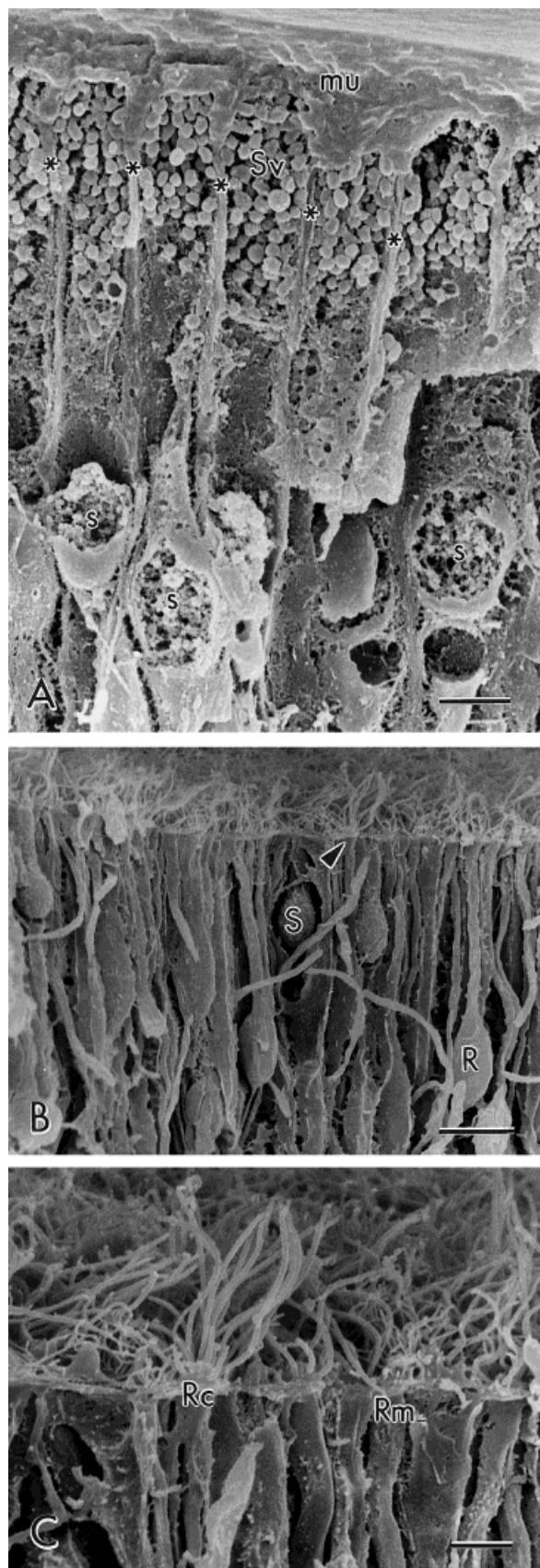
The present study has shown that all cell types do not appear simultaneously as the olfactory placode differentiates. At stage 37/38, the last stage examined by Klein and Graziadei (1983), only ciliated receptor cells and microvillar (secretory) supporting cells are present. Microvillar receptor cells and ciliated supporting cells of the principal cavity arise for the first time at stage 40. Interestingly, the microvillar receptor and ciliated supporting cells of the vomeronasal epithelium also appear around this time. This pattern shows both similarities and differences from that found in the only other frog in which olfactory development has been studied, *Rana japonica* (Taniguchi et al., 1996). In the principal cavity of *R. japonica*, the microvillar supporting cells also develop before the ciliated supporting cells, but vomeronasal receptor cells apparently develop only in later larval stages.

When the olfactory organ of *Xenopus* becomes functional is not clear. At least some receptor neurons of the principal

cavity appear morphologically differentiated by stage 40, although their cilia are still short. Other important features of the olfactory system also appear to be present by this time. In particular, synapses of receptor cell axons in the olfactory bulb first appear at stage 37/38, and are well developed by stage 40 (Byrd and Burd, 1991). However, the typically layered structure of the main olfactory bulb is not recognizable until stage 44 (Byrd and Burd, 1991). The vital dye DASPEI, which is thought to stain selectively mature olfactory receptor cells (Collins and Ross, 1992), first labels receptor cells only at stage 45 (data not shown). In addition, odorant receptor mRNA is detected with in situ hybridization by stage 45 (Hansen, personal observation), the stage at which feeding begins. It is possible that the first olfactory neurons become functional only at this time.

Metamorphic changes in the olfactory organ

Metamorphic changes in the *Xenopus* olfactory organ are extensive. On a gross level, the two-part larval nose, consisting of principal cavity and VNO, is converted to the three-part juvenile nose, consisting of principal cavity, middle cavity, and VNO (Föske, 1934; Reiss and Burd, 1997b). At the ultrastructural level, our observations show



that extensive metamorphic changes are largely restricted to the principal cavity. The larval principal cavity epithelium contains both ciliated and microvillar receptor cells and both ciliated and secretory supporting cells. In post-metamorphic animals, only ciliated receptor cells and secretory supporting cells are present in the principal cavity, and these differ in a number of features from the corresponding larval cell types. Neither the middle cavity nor the VNO epithelium undergo any major change during metamorphosis. When it first appears, and throughout all later stages and in adulthood, the middle cavity epithelium contains the same cell types as the larval principal cavity. The VNO epithelium always contains microvillar receptor cells and ciliated supporting cells. These observations confirm the unpublished findings of Weiss (1986) and Key (1986).

During metamorphosis, the epithelium of the principal cavity undergoes a dramatic restructuring. How is this transformation accomplished? At least three different scenarios are possible: (1) all receptor and supporting cells of the larval epithelium die, to be replaced by newly differentiating adult-type receptor and supporting cells; (2) some receptor or supporting cells survive and transdifferentiate into the corresponding adult cell type; (3) all receptor and supporting cells survive and transdifferentiate into the adult cell types. Although our observations cannot definitively resolve this question, the observation of dying receptor cells (both ciliated and microvillar) and supporting cells (only ciliated) during metamorphosis suggests that death of these cell types plays an important role in the transformation of the epithelium. In contrast, the observation of secretory supporting cells containing both electron-lucent and electron-dense vesicles at early metamorphic stages argues that at least some of these supporting cells transdifferentiate into the adult type.

If transdifferentiation of secretory supporting cells is occurring, it is not clear whether cell division is a prerequisite. In general, it is thought that all supporting cells derive from other supporting cells (Farbman, 1985). Receptor cells, on the other hand, are thought to derive from a stem-cell-like subpopulation of basal cells, so that it is possible that all larval receptor cells die. Additional evidence for the role of cell death in the replacement of receptor cells comes from the observations of the olfactory nerve during metamorphosis (Burd, 1991). Nevertheless, labeling olfactory receptor cells prior to metamorphosis and following the labeled cohort through metamorphosis may be necessary to fully resolve this question.

Comparative ultrastructure of the amphibian nose

The living amphibians consist of three distinct groups: frogs (Anura), salamanders (Caudata), and caecilians

Fig. 8. Scanning electron microscopy (SEM) fractures through the olfactory epithelia of juvenile frogs. **A:** Principal cavity. Note thick layer of mucus (mu) at surface of epithelium. Secretory supporting cells are densely packed with secretory vesicles. Receptor cell dendrites (asterisks) are separated by supporting cells. s, soma. **B:** Middle cavity. Arrowhead indicates olfactory knob of ciliated receptor cell. S, supporting cell soma; R, receptor cell soma. **C:** Middle cavity. Higher magnification to show olfactory knobs of ciliated and microvillar receptor cells. Rm, microvillar receptor cell; Rc, ciliated receptor cell; Sv, secretory supporting cell. Scale bars = 3 μ m in A, 20 μ m in B, 6 μ m in C.

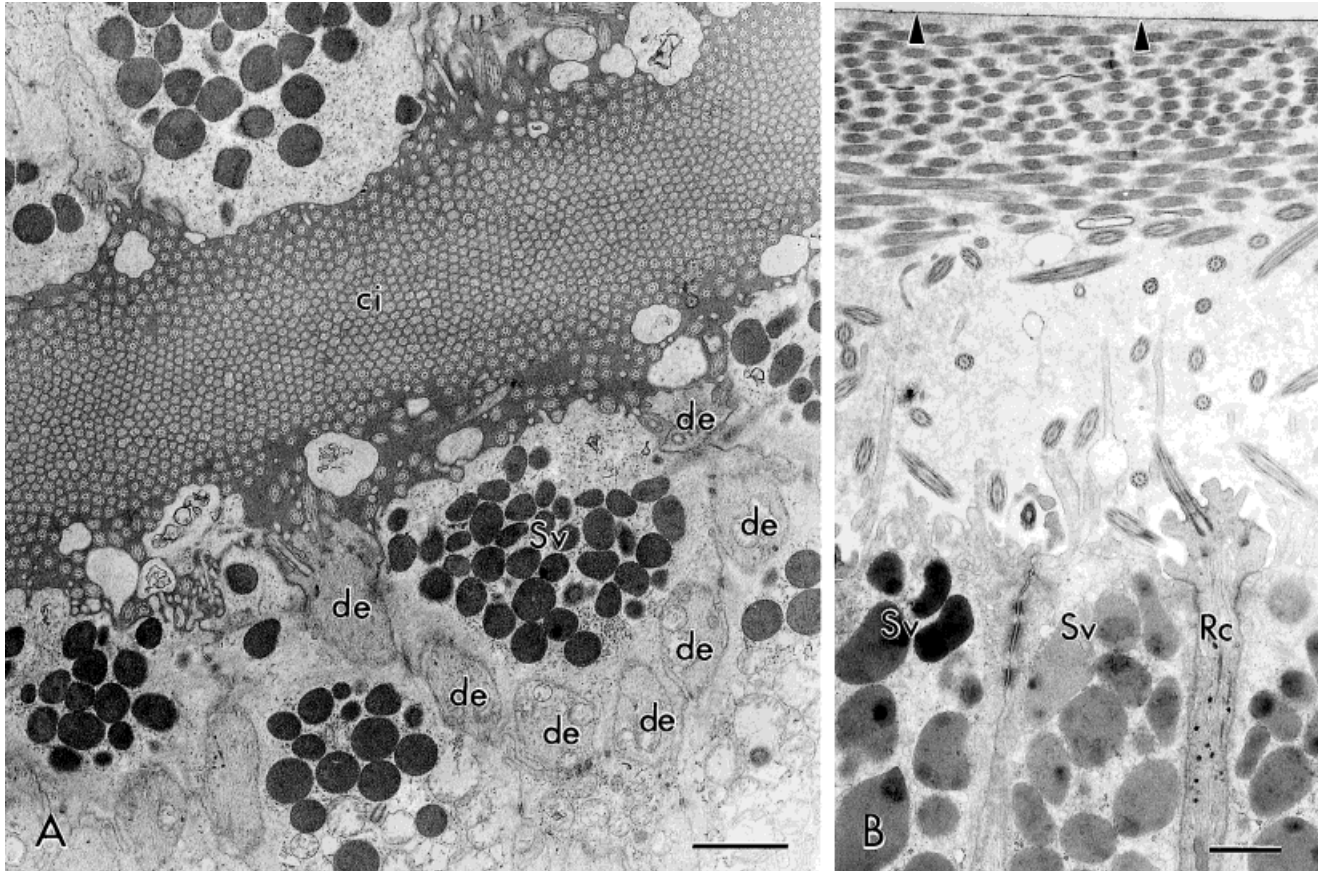


Fig. 9. Transmission electron microscopy (TEM) of the principal cavity epithelium in a juvenile frog. **A:** Tangential section through a fold at surface of epithelium. Note abundant olfactory cilia (ci) in lumen, and ringlike arrangement of receptor cell dendrites (de)

separated by processes of the supporting cell. **B:** Section through apical region of epithelium. Arrowheads indicate top of thick mucus layer within which the cilia run. Rc, ciliated receptor cell; Sv, secretory supporting cell. Scale bars = 2 μ m in A, 1 μ m in B.

(Gymnophiona; Duellman and Trueb, 1986). Of these, frogs have the most complicated olfactory organ. In typical frogs of the genus *Rana*, the olfactory organ consists of three chambers: (1) a principal (or superior) cavity, containing the main olfactory epithelium, (2) a middle cavity, which is nonsensory and receives the nasolacrimal duct, and (3) an inferior cavity, which contains the vomeronasal epithelium (Scalia, 1976). In spite of much variation in detail, the same organization exists in almost all anurans (reviewed by Helling, 1938; Jurgens, 1970). The presence of olfactory epithelium in the middle cavity appears to be unique to *Xenopus* (Föske, 1934; Paterson, 1939a,b) and other pipid frogs (Bancroft, 1895; Trahms, 1936; Paterson, 1951). In *Xenopus*, there is thus a tripartite olfactory system, in which the main olfactory bulb is divided into a part that receives principal cavity afferents and a part that receives middle cavity afferents, and the vomeronasal epithelium connects to the accessory olfactory bulb (Weiss, 1986; Key, 1986; Reiss and Burd, 1997a).

Ultrastructural observations on the principal cavity epithelium of other adult frogs show a remarkable uniformity of structure among species (Bloom, 1954; Reese, 1965; Graziadei, 1973; Burton, 1985; Saint Girons and Zylberberg, 1992; Taniguchi et al., 1996). A single type of ciliated receptor cell and a granule-filled supporting cell with microvillar protrusions have been described. These

cell types closely resemble the cells of the principal cavity of *Xenopus*, although receptor cilia can be significantly longer than those of *Xenopus* (Reese, 1965). The vomeronasal epithelium of other frogs also closely resembles that of *Xenopus* (Kolnberger, 1971; Franceschini et al., 1991; Saint Girons and Zylberberg, 1992; Taniguchi et al., 1996). In contrast, sensory epithelia containing both ciliated and microvillar receptor cells and both secretory and ciliated supporting cells, as in the middle cavity of *Xenopus*, have not been described for other adult anurans (Eisthen, 1992; Saint-Girons and Zylberberg, 1992).

Like *Xenopus*, all anurans show extensive metamorphic changes in olfactory organ morphology (Born, 1876; Hinsberg, 1901; Föske, 1934; Rowedder, 1937; Khalil, 1978). These changes consist largely in remodeling of the principal cavity and VNO, the loss of larval diverticuli of the principal cavity, and the development of the middle cavity. Ultrastructure of the larval nose has recently been examined in *Rana japonica* (Taniguchi et al., 1996). Interestingly, as in *Xenopus*, the principal cavity of larval *R. japonica* contains both ciliated and secretory supporting cells (Taniguchi et al., 1996). Moreover, both ciliated and microvillar receptor cells appear to be present (e.g., Taniguchi et al., 1996, Fig. 11), although only ciliated receptor cells are mentioned in the text. The same four cell types are also present in the principal cavity of larvae of the

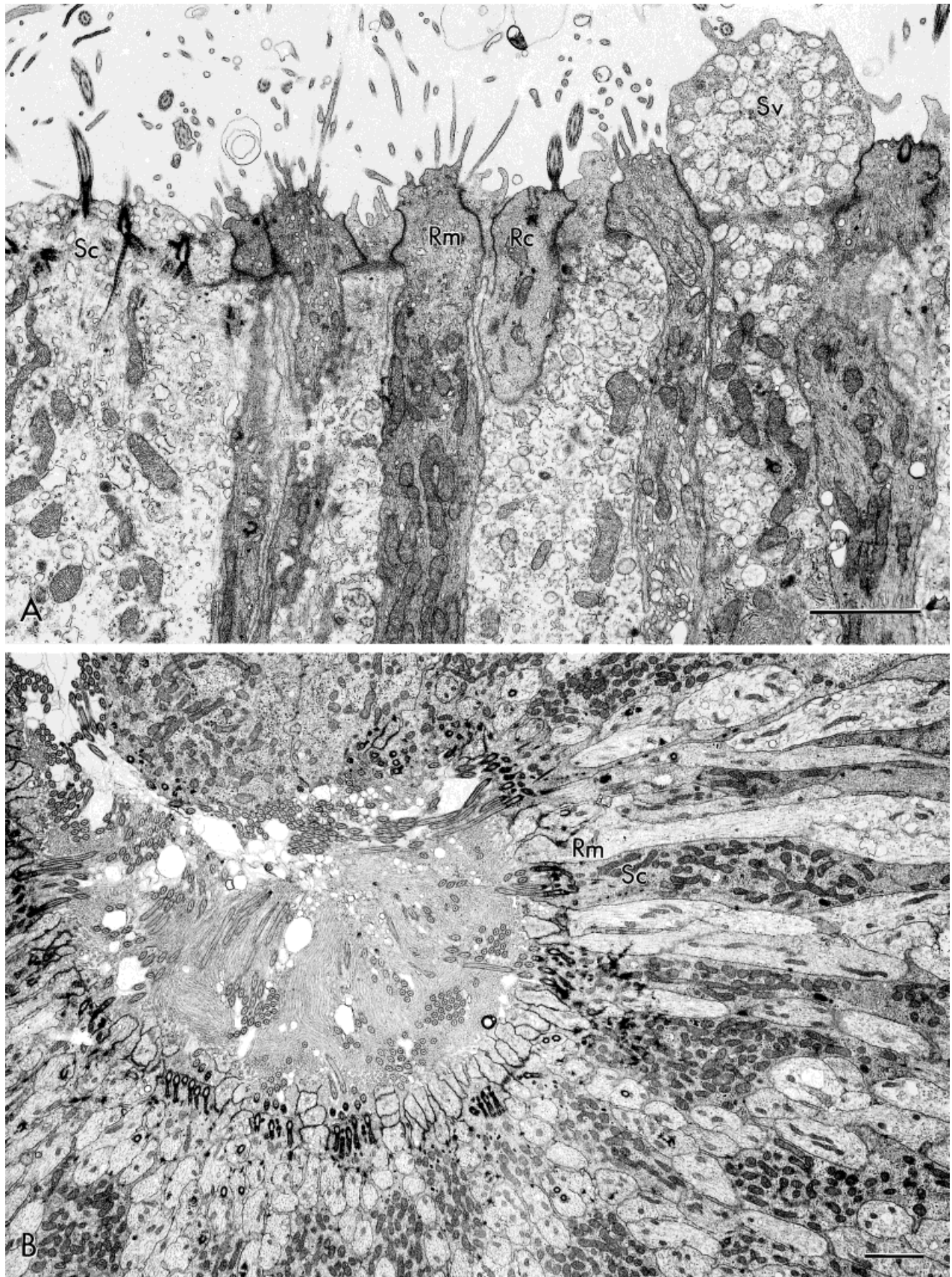


Fig. 10. **A:** Transmission electron microscopy (TEM) of the middle cavity epithelium in a juvenile frog. Note similarity to larval principal cavity. **B:** TEM of the vomeronasal organ epithelium in a juvenile frog.

Note lack of change from larval condition. Rc, ciliated receptor cell; Rm, microvillar receptor cell; Sc, ciliated supporting cell; Sv, secretory supporting cell. Scale bars = 2 μ m.

Oriental fire-bellied toad, *Bombina orientalis* (J. Reiss, unpublished observations). It is likely, then, that the presence of these cell types is typical of larval anuran olfactory organs, and that *Xenopus* is unusual only in retaining this type of sensory epithelium after metamorphosis. As in *Xenopus*, in both *R. japonica* (Taniguchi et al., 1996) and *B. orientalis* (J. Reiss, unpublished observations), the VNO epithelium of larvae resembles that of adults.

The olfactory organ of salamanders generally has a much simpler gross morphology than that of frogs, consisting only of a large principal cavity and a lateral outpocketing containing vomeronasal epithelium (reviewed by Jurgens, 1970; Saint Girons and Zylberberg, 1992). However, at the ultrastructural level, the salamander olfactory epithelium seems to show a much greater variety than that of anurans (Farbman and Gesteland, 1974; Graziadei and Monti Graziadei, 1976; Eisthen et al., 1991, 1994; Eisthen, 1992; Eisthen and Schroeder, 1992; Saint Girons and Zylberberg, 1992; Jones et al., 1994). Conflicting reports regarding the same species (e.g., Farbman and Gesteland, 1974; Graziadei and Monti-Graziadei, 1976) lead one to doubt that all of this variation is real, but some may be, and may relate to differences in life history among salamanders. Unlike anurans, many salamanders, such as the mudpuppy (*Necturus*) and axolotl (*Ambystoma mexicanum*), are paedomorphic (neotenic), becoming sexually mature with an otherwise larval morphology. Others, like the newts (Salamandridae), are secondarily aquatic as adults, returning to water for an extended period of time after metamorphosis.

In adults of terrestrial salamanders, such as the tiger salamander (*Ambystoma tigrinum*), olfactory epithelium of the principal cavity typically occurs in narrow grooves separated by ridges of nonsensory epithelium (Breipohl et al., 1982). The olfactory epithelium of these grooves has three types of cells: ciliated receptor cells, microvillar receptor cells, and secretory supporting cells (Breipohl et al., 1982; Eisthen, 1992; Eisthen and Schroeder, 1992). Ciliated supporting cells do not occur in the olfactory epithelium, although ciliated cells are present in the nonsensory epithelium of the ridges. The vomeronasal epithelium of terrestrial tiger salamanders, by contrast, has only microvillar receptor cells, but both ciliated and secretory supporting cells (Eisthen, 1992; Eisthen and Schroeder, 1992). Saint Girons and Zylberberg (1992) report the presence of ciliated as well as microvillar receptor cells in vomeronasal epithelium of the salamanders *Pleurodeles* and *Triturus*.

The olfactory epithelium of neotenic adult salamanders, such as the axolotl (Eisthen et al., 1994), and of secondarily aquatic adult salamanders, such as the newt, *Cynops pyrrhogaster* (Jones et al., 1994), appears to have the same receptor and supporting cell types described above for metamorphosed salamanders. Bowman's glands are present. In contrast, neotenic salamanders of the family Proteidae (*Necturus* and *Proteus*) lack Bowman's glands in the olfactory epithelium (Farbman and Gesteland, 1974; Eisthen et al., 1991; Saint Girons and Zylberberg, 1992). This is reminiscent of the middle cavity epithelium of *Xenopus*. The vomeronasal epithelium of the axolotl resembles that of metamorphosed salamanders (Eisthen, 1992), but the vomeronasal epithelium described by Jones et al. (1994) in *Cynops* is unusual in having alternating stripes of typical olfactory epithelium with ciliated and microvillar recep-

TABLE 1. Summary of Receptor and Supporting Cell Types in Amphibian Nasal Cavities

	Frogs		Salamanders Adults	Caecilians Adults
	Larvae	Adults		
Principal cavity	Rm Rc Sv Sc ²	Rc Sv	Rm Rc Sv	Rm Rc Sv
Middle cavity (<i>Xenopus</i> only)	Rm Rc Sv Sc	Rm Rc Sv Sc	0 ¹	0
VNO	Rm Sc	Rm Sc	Rm (Rc?) Sv Sc	Rm Sv

¹0, this cavity is not formed in these animals.

²For references and abbreviations, see the text.

tors (though lacking Bowman's glands) and stripes of an exclusively microvillar epithelium. Finally, proteids completely lack a vomeronasal system (Farbman and Gesteland, 1974; Eisthen et al., 1991; Saint Girons and Zylberberg, 1992).

No information is available on the ultrastructure of the olfactory epithelium in larval salamanders. If the epithelium of neotenic adults represents a larval morphology, then, unlike frogs, no important changes occur in the salamander nose during metamorphosis. However, light microscopic studies of *Triturus alpestris* (Schuch, 1934) and *Ambystoma tigrinum* (Getchell et al., 1984) make it clear that the presence of Bowman's glands, at least, is not typical of larvae; it may be that ultrastructural features also differ between larvae and neotenic adults. Schuch (1934) observed the cilia of receptor cells to be shorter in larvae than metamorphosed juveniles, and Getchell et al. (1984) found supporting cell granules to be more basophilic in larvae.

Little is known about the third group of living amphibians, the caecilians. The olfactory organ consists of a large principal cavity and a sac-like vomeronasal organ (Schmidt and Wake, 1990; Saint Girons and Zylberberg, 1992). The only species that has been examined at the ultrastructural level is *Typhlonectes compressicaudum*, a derived, secondarily aquatic form. In *Typhlonectes*, the principal cavity epithelium contains both microvillar and ciliated receptor cells and secretory supporting cells (Saint Girons and Zylberberg, 1992). Bowman's glands are absent. The vomeronasal organ contains only microvillar receptor cells and secretory supporting cells. Thus, available information shows surprising differences in olfactory ultrastructure among the three living amphibian groups (Table 1).

Metamorphic changes in olfactory function

Little is known about olfactory abilities of *Xenopus* or other amphibians. A recent review by Elepfandt (1996) notes that "the crucial problem seems to be to find not only substances that affect the olfactory system, but odorous stimuli that have some biological meaning in the animals' natural environment" (p. 112). Olfaction is clearly important in the feeding response of adult *Xenopus*. Altner (1962) found that he could elicit a feeding response of blinded adults to *Tubifex* worms or worm extract. This response occurred under water, but not in air. Because the middle cavity is exposed to waterborne odorants and the principal cavity to airborne odorants, he concluded that the middle cavity is responsible for all olfaction, with the principal cavity only serving as a conduit for air to the lungs. However, the presence of olfactory epithelium in the principal cavity makes it clear that this view is incorrect, and the significance of airborne odors remains unclear. Du Plessis (1966) suggested that airborne odors may be used

by *Xenopus* to locate new ponds during overland migrations.

The biological role of olfaction in larvae is even less clear. Kiseleva (1989) reported an increase in activity of tadpoles after addition of nettle tea (a typical laboratory food) to the rearing solution. In other tadpoles, olfactory cues have been shown to be important in mediating kin recognition based schooling (reviewed by Waldman, 1986).

In general, amino acids are thought to be important olfactory stimuli in water, whereas volatile organic compounds are important stimuli in air. Arzt et al. (1986) compared electro-olfactograms (EOGs) generated by volatiles (in air and water) and amino acids (in water) between larval and adult tiger salamanders. They found a greater response to amino acids in larvae than in adults, and a greater response to volatiles (either in water or air) in adults than in larvae. Dorries et al. (1997) have recently shown that adult tiger salamanders can be conditioned to some of the same volatiles. Unfortunately, as discussed above, it is not clear whether there are any differences in receptor cell type between larvae and adults that correlate with these physiological changes. A recent study by Kruzhalov (1995) compared the EOGs elicited by various amino acids between the middle and principal cavities of *Xenopus*. He found differences in the response pattern of the two cavities, but responses were generally of the same order of magnitude. He did not test responses to volatile compounds.

The relation between morphological characteristics of receptor cells and odorant response is not clear. In zebrafish development, the morphological appearance of ciliated receptor cells (Hansen and Zeiske, 1993) coincides with the first expression of putative odorant receptors (Barth et al., 1996; Byrd et al., 1996) and staining with DASPEI (Collins and Ross, 1992), but the onset of physiological response to odorants is unknown. Zippel et al. (1993, 1997) have shown that in adult goldfish, ciliated receptor cells mediate behavioral responses to amino acids, whereas microvillar receptor cells mediate responses to pheromones. The present observations support a direct connection between the ultrastructural characteristics of the olfactory epithelia and the olfactory medium in *Xenopus*. The larval principal cavity epithelium and adult middle cavity epithelium are used to smell waterborne odorants (Altner, 1962) and have two receptor and two supporting cell types, whereas the adult principal cavity is used to smell airborne odorants and has only one receptor and one supporting cell type. Freitag et al. (1995) found fish-like odorant receptor genes expressed in the middle cavity and mammalian-like odorant receptor genes in the principal cavity of *Xenopus* adults. However, contrary to what one would expect, the principal cavity of larvae expresses both fish-like and mammal-like odorant receptor types (Hansen, unpublished observations). The relation between the morphological changes of metamorphosis and changes in the physiological characteristics of the epithelium clearly needs further study.

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