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# Presence of the vomeronasal system in aquatic salamanders

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Previous reports have indicated that members of the proteid family of salamanders lack a vomeronasal system, and this absence has been interpreted as representing the ancestral condition for aquatic amphibians. I examined the anatomy of the nasal cavities, nasal epithelia, and forebrains of members of the proteid family, mudpuppies (*Necturus maculosus*), as well as members of the amphiumid and sirenid families (*Amphiuma tridactylum* and *Siren intermedia*). Using a combination of light and transmission electron microscopy, I found no evidence that mudpuppies possess a vomeronasal system, but found that amphiuma and sirens possess both vomeronasal and olfactory systems. Amphiumids and sirenids are considered to be outgroups relative to proteids; therefore, these data indicate that the vomeronasal system is generally present in salamanders and has been lost in mudpuppies. Given that the vomeronasal system is generally present in aquatic amphibians, and that the last common ancestor of amphibians and amniotes is believed to have been fully aquatic, I conclude that the vomeronasal system arose in aquatic tetrapods and did not originate as an adaptation to terrestrial life. This conclusion has important implications for the hypothesis that the vomeronasal organ is specialized for detection of non-volatile compounds.

**Keywords:** amphiuma; evolution; mudpuppy; olfaction; siren; tetrapod

## 1. INTRODUCTION

The vomeronasal system, or accessory olfactory system, is present only in tetrapods (amphibians, reptiles and mammals) and is lacking in fishes. When and how did this sensory system originate? Broman (1920) suggested that the vomeronasal system is homologous with the nasal chemosensory system of fishes, and that the olfactory system arose later as an adaptation to terrestrial life. This hypothesis was based largely on mistaken interpretations of the innervation of nasal sensory epithelia and must be incorrect, for the projections of the olfactory bulb are highly conserved across vertebrates (Eisthen 1997). Bertmar (1981) later inverted Broman's hypothesis, suggesting that the vomeronasal system arose in tetrapods as an adaptation to terrestrial life. This hypothesis fits well with data suggesting that the vomeronasal organ of snakes and rodents is specialized for the detection of high molecular weight, relatively non-volatile compounds (Halpern & Kubie 1980; Wysocki *et al*. 1980).

The proteid family of salamanders, all of which are permanently aquatic and do not metamorphose, has been reported to lack a vomeronasal organ (Seydel 1895; Anton 1911; Farbman & Gesteland 1974). This observation appears to support Bertmar's hypothesis, and suggests that the vomeronasal system may develop at metamorphosis in amphibians. Axolotls (Ambystomatidae: *Ambystoma mexicanum*) are also aquatic, non-metamorphosing salamanders, yet throughout their life cycle, axolotls possess a well-developed vomeronasal organ with microvillar receptor cells that project to an accessory olfactory bulb, as in other tetrapods (Eisthen *et al*. 1994). Which of these families represents the ancestral condition for salamanders?

According to the most recent well-corroborated phylogeny of salamander families, the proteid family is basal to the ambystomid family (Larson & Dimmick 1993). It is possible that axolotls represent the ancestral condition and that the vomeronasal system has been lost in proteids. In contrast, it is also possible that the development of the vomeronasal system in aquatic axolotls is an aberration, or that axolotls lack a true vomeronasal system but possess structures with convergent features. To determine which of these alternatives is correct, members of families that are outgroups relative to both proteids and ambystomids must be examined for the presence of both olfactory and vomeronasal systems. I therefore investigated the anatomy of the nasal cavities and forebrains in members of two families, Amphiumidae and Sirenidae, that are outgroups relative to both proteids and ambystomids. Like proteids, both families consist entirely of non-metamorphosing, aquatic salamanders. I also examined mudpuppies, which are members of the proteid family, to verify that peripheral elements of the vomeronasal system are not present in these animals. In each species, I sought evidence for the presence of anatomical features characteristic of the vomeronasal system: a separate, accessory chamber or diverticulum of the nasal cavity containing a sensory epithelium that is histologically distinguishable from the olfactory epithelium present in the main chamber of the nasal cavity; and an accessory olfactory bulb in the anterior telencephalon that is distinct from the main olfactory bulb.

 

## 2. MATERIAL AND METHODS

### (a) *Subjects*

Four mudpuppies (*Necturus maculosus*), snout–vent length (SVL) *ca.* 20 cm, were used to examine the anatomy of the nasal cavity; another three were prepared for electron microscopic examination of the nasal epithelia. One three-toed amphiuma (*Amphiuma tridactylum*), SVL = 51–64 cm, was used for examination of the nasal cavity and three for electron microscopy. Two lesser sirens (*Siren intermedia*), SVL = 14–19 cm, were used in examinations of the nasal cavity and five were prepared for electron microscopy. Wild-caught animals were purchased from licensed suppliers, and all experiments were conducted according to published animal care and use guidelines established by the US Public Health Service.

### (b) *Anatomy of the nasal cavity*

Subjects were anaesthetized by immersion in 0.1% tricaine methanesulphonate (pH 7.4) and transcardially perfused with 0.8% NaCl or amphibian Ringer's solution followed by a fixative of either 10% formalin or 4% paraformaldehyde–2.5% glutaraldehyde in phosphate or cacodylate buffer (pH 7.4). After perfusion, the snout was removed, post-fixed for four to ten days, then decalcified in a solution of 5% EDTA in 10% buffered formalin. The duration of the decalcification treatment ranged from six weeks for mudpuppies to five months for sirens. Snouts were embedded in paraffin and cut in transverse section at 10 µm. Alternate sections were stained with cresylecht violet for neuronal cell bodies or with thionin and picric acid for bone and cartilage.

### (c) *Ultrastructure of nasal epithelia*

Subjects were anaesthetized as described in § 2(b) and transcardially perfused with 0.8% NaCl followed by 2% paraformaldehyde–2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). The animals' entire nasal capsules were post-fixed for four days in paraformaldehyde–glutaraldehyde fixative at 4 °C, after which small pieces of tissue were removed. These pieces were rinsed in 0.1 M phosphate buffer, post-fixed in 2% osmium tetroxide, dehydrated in alcohols, stained with 2% uranyl acetate in 95% EtOH, dehydrated with propylene oxide, and embedded in Medcast™ resin (Ted Pella, Inc., Redding, CA, USA). Sections were cut perpendicular to the epithelial surface: thick 2 µm sections were used for light microscopic orientation, and silver sections (70–90 nm) were mounted on uncoated copper grids, stained with uranyl acetate and lead citrate, and examined with a Hitachi H-300 (Tokyo, Japan) transmission electron microscope at an accelerating voltage of 75 kV.

### (d) *Presence of main and accessory olfactory bulbs*

Light microscopic examination of the telencephalon was used to determine whether both main and accessory olfactory bulbs were present in each species. Brains were removed from each animal used in studies of the nasal cavity, post-fixed in 10% formalin, embedded in paraffin, cut in horizontal section at 10 µm, and stained with cresylecht violet.

## 3. RESULTS

### (a) *Anatomy of the nasal cavity*

In all three species, the main chamber of the nasal cavity resembled a tube extending from the external naris on the dorsal snout to the choana, or internal naris, in the roof of the mouth. Nasolacrimal ducts were not present.

The main chamber was lined with deep longitudinal grooves that contained patches of olfactory epithelium. The ridges between these grooves were covered with a non-sensory epithelium that was three to seven cells thick. The pseudostratified olfactory epithelium contained sustentacular cells, receptor cells, and basal cells. In amphiuma, Bowman's glands were present in the subepithelial mucosa; these glands were not present in mudpuppies or sirens. In all three species, nasal glands and intermaxillary glands were present in the connective tissue surrounding the nasal cavity.

In sirens, the vomeronasal epithelium was located in a narrow, dorsoventrally flattened organ that extended mediolaterally. The organ lay ventromedial to the main chamber of the nasal cavity, and was connected to the main chamber by a duct that extended between the medial wall of the nasal cavity, immediately anterior to the choana, and the midpoint of the vomeronasal organ. Vomeronasal epithelium was confined to the ends of the organ, and non-sensory epithelium lined the intervening portion and duct. Figure 1*a* illustrates the vomeronasal organ, duct, and main chamber of the nasal cavity. In amphiuma, vomeronasal epithelium was present in a large lateral evagination of the nasal cavity, approximately equidistant from the external and internal nares. Although Bowman's glands were present in the olfactory epithelium, none were observed in the vomeronasal epithelium. Regions of non-sensory epithelium lined the lateral portion of the nasal cavity both anterior and posterior to the vomeronasal region, and separated the medially located olfactory epithelium from the vomeronasal epithelium, as shown in figure 1*b*. In mudpuppies, the nasal cavity consisted of a simple, tube-like main chamber, without diverticula. Towards the anterior end of the nasal cavity, a slight lateral evagination contained only non-sensory epithelium like that in other regions of the nasal cavity; no vomeronasal organ was present. The main chamber and lateral evagination of the nasal cavity are shown in figure 1*c*.

### (b) *Ultrastructure of nasal epithelial surfaces*

In examining the nasal epithelia, the major goal was to determine whether both olfactory and vomeronasal receptor epithelia were present. Across tetrapods, the only consistently reported difference between olfactory and vomeronasal receptor cells is that vomeronasal receptor cells terminate in microvilli. In some amphibians, the vomeronasal organ contains a cell type not present in the olfactory epithelium; these cells have a large apical surface covered with motile cilia (Eisthen *et al.* 1994). Both features can be distinguished by electron microscopic examination of the superficial portion of the sensory epithelia.

In the olfactory epithelium of all three species examined, similar numbers of ciliated and microvillar receptor cells were present and intermixed. The receptor cell cilia contained pairs of microtubules in a 9 + 2 configuration; the presence of dynein arms indicated that these cilia were motile. The dendrites of ciliated receptor cells terminated in a central pair of cilia surrounded by a ring of outer cilia. The microvillar receptor cells contained

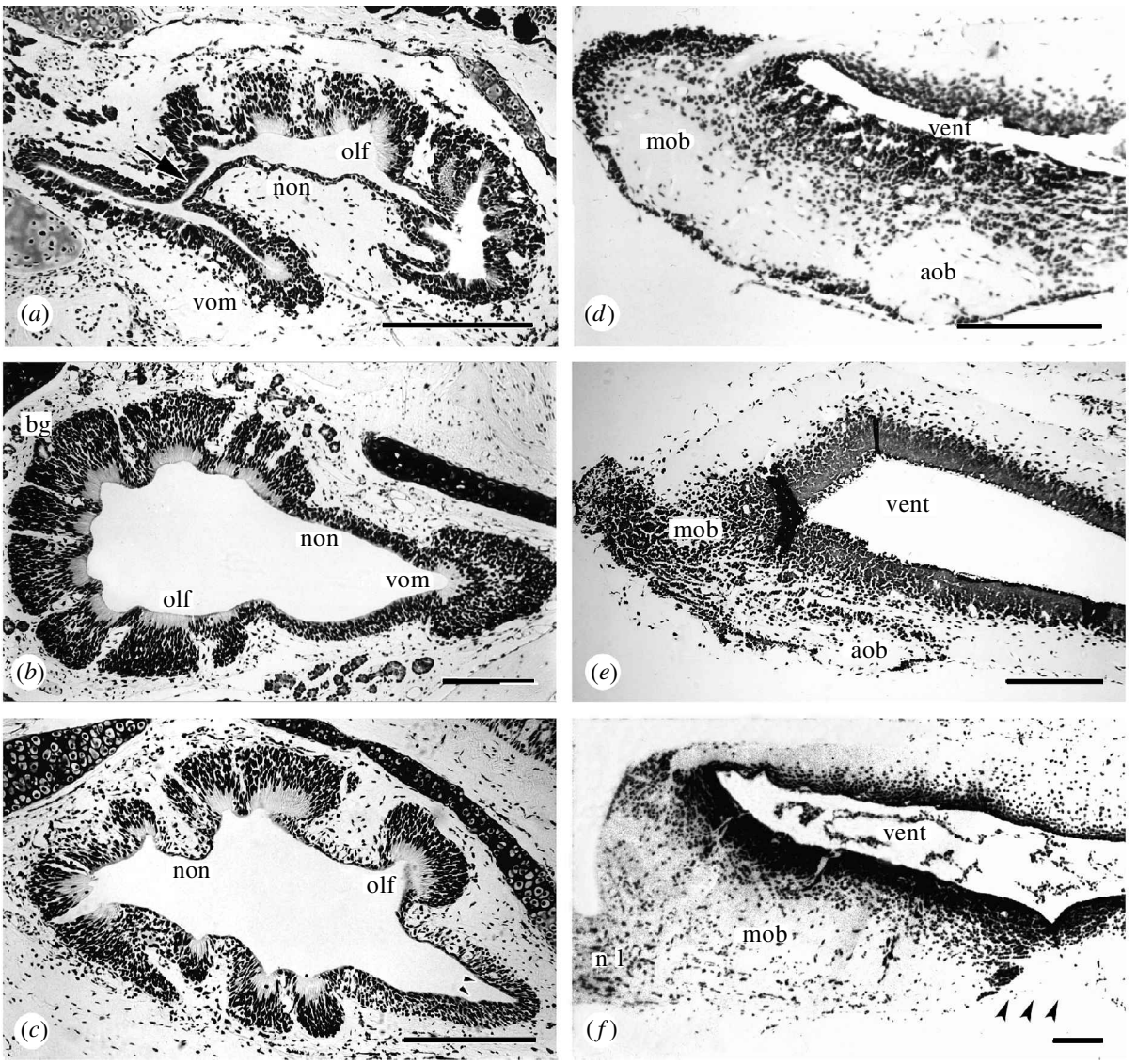


Figure 1. Photomicrographs of the nasal cavities and olfactory bulbs of adult sirens (*a*, *d*), amphiuma (*b*, *e*), and mudpuppies (*c*, *f*), stained with cresylecht violet. (*a–c*) Photographs of 10 µm transverse sections through the nasal cavity; dorsal is towards the top and medial is to the left. In all three species, patches of olfactory epithelium line longitudinal grooves in the main chamber of the nasal cavity. In sirens (*a*), the vomeronasal organ lies medial to the nasal cavity and is connected with the main chamber of the nasal cavity by a narrow duct (arrow). In amphiuma (*b*), the vomeronasal epithelium lines a lateral evagination of the nasal cavity. The nasal cavities of mudpuppies (*c*) contain a small lateral diverticulum (small arrowheads) that is lined with non-sensory epithelium and does not resemble the vomeronasal organs of other salamanders. (*d–f*) Photographs of 10-µm horizontal sections through telencephalon; anterior is to the left and medial is towards the top. Sirens (*d*) and amphiuma (*e*) possess both main and accessory olfactory bulbs, whereas mudpuppies (*f*) lack an accessory olfactory bulb (arrowheads). aob, accessory olfactory bulb; bg, Bowman's gland; mob, main olfactory bulb; n 1, olfactory nerve; non, non-sensory epithelium; olf, olfactory epithelium; vent, lateral ventricle; vom, vomeronasal epithelium. Scale bars, 500 µm.

multiple centrioles, but I did not observe any cells that terminated in both cilia and microvilli. The sustentacular cells of the olfactory epithelium lack cilia or microvilli and terminate in finger-like projections in all three species. The secretory granules of the sustentacular cells are larger and more numerous than in the cells of the non-sensory epithelium. The olfactory epithelia of amphiuma and mudpuppies are illustrated in figure 2*a*,*b*.

In both sirens and amphiuma, the vomeronasal receptor cells terminated in microvilli and contained multiple centrioles. The dendrites of vomeronasal receptors generally resembled those of microvillar olfactory receptor cells, containing many mitochondria and microtubules that extended the length of the dendrite. As in the olfactory epithelium, the sustentacular cells terminated in short (3–5 µm) finger-like projections. In addition to the sustentacular cells, the vomeronasal epithelium of amphiuma contained cells with wide surface processes that lacked secretory granules and terminated in dense clusters of cilia. These ciliated cells appeared to be present in much greater numbers than were sustentacular or receptor cells. The vomeronasal epithelium from an amphiuma is shown in figure 2*c*. The vomeronasal epithelium of sirens was similar, but lacked ciliated supporting cells.

The non-sensory epithelium in the lateral evagination of the mudpuppy nasal cavity, illustrated in figure 2*d*,

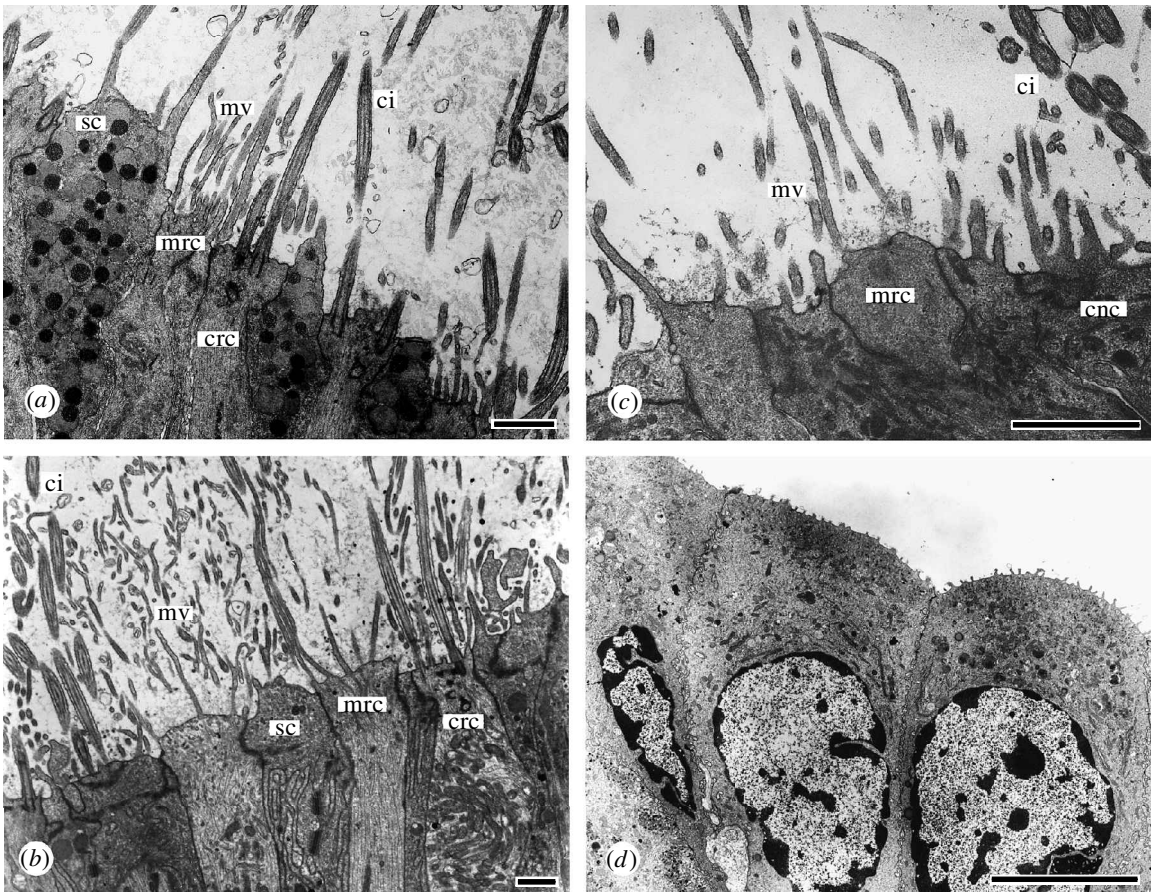


Figure 2. Transmission electron micrographs of the nasal epithelial surface in amphiuma (*a*, *c*) and mudpuppies (*b*, *d*). (*a*,*b*) The supranuclear region of olfactory epithelium in an amphiuma (*a*) and a mudpuppy (*b*). In both species, ciliated and microvillar receptor cells are present and are intermingled throughout the epithelium. The processes of the sustentacular cells terminate in short, finger-like projections. (*c*) Vomeronasal epithelium from the lateral evagination of the nasal cavity in an adult amphiuma, illustrating the surface processes of three cell types. The receptor dendrite terminates in microvilli and the sustentacular cells terminate in short, finger-like projections and contain small secretory granules. The ciliated processes of a second type of supporting cell are also present. (*d*) Non-sensory epithelium from the lateral diverticulum of the nasal cavity in a mudpuppy. These cells lack cilia and microvilli and contain small lipid droplets. ci, cilia; cnc, ciliated non-sensory cell; crc, ciliated receptor cell; mrc, microvillar receptor cell; mv, microvilli; sc, sustentacular cell. Scale bars, (*a–c*) 1 μm; (*d*) 10 μm.

contained cells resembling those in regions of non-sensory epithelium throughout the nasal cavity of all three species. These cells contained small, sparse secretory granules and lipid droplets, and terminated in short (< 1 μm) finger-like projections that may be artefacts of the fixation and embedding processes.

### (c) *Presence of main and accessory olfactory bulbs*

The main olfactory bulb was morphologically similar in all three species. The olfactory nerve entered the forebrain slightly laterally, at the rostral end of the telencephalon. The diverging fibres formed a plexus along the anterolateral edge of the olfactory bulb. Posteromedial to this plexus were the layers typically present in vertebrate olfactory bulbs.

In sirens and amphiuma, an accessory olfactory bulb was located dorsal and caudal to the main olfactory bulb, and protruded from the lateral margin of the telencephalon, as illustrated in figure 1*d*,*e*. In histological sections, the neuropil of the accessory olfactory bulb was clearly delineated by a surrounding layer of cells. Unlike sirens and amphiuma, mudpuppies appeared to lack an accessory olfactory bulb (figure 1*f*).

## 4. DISCUSSION

The results of the present study confirm previous reports that members of the proteid family of salamanders lack a distinguishable vomeronasal organ, vomeronasal epithelium, and accessory olfactory bulb (Seydel 1895; Anton 1911; Farbman & Gesteland 1974). Farbman & Gesteland suggested that the microvillar receptor cells in the olfactory epithelium of mudpuppies and teleosts are vomeronasal receptor cells integrated into the olfactory epithelium. This hypothesis, while interesting, is contradicted by the observation that both ciliated and microvillar receptor cells are present in the olfactory epithelia of amphiuma and sirens (present study), as well as axolotls (Eisthen *et al.* 1994), which possess both olfactory and vomeronasal systems.

Although proteids appear to lack a vomeronasal system, the system is present in members of the

amphiumid and sirenid families, which are outgroups relative to proteids (Larson & Dimmick 1993). The most parsimonious explanation of the data is that the vomeronasal system is generally present in aquatic salamanders, and has been lost in the proteid family. Given that all members of the Amphiumidae and Sirenidae are fully aquatic and non-metamorphosing, this finding refutes the hypothesis that the vomeronasal system develops at metamorphosis as an adaptation to terrestrial life and is lost in proteids either because they do not metamorphose or because they are aquatic.

An additional line of reasoning supports my contention that the vomeronasal system did not originate as an adaptation to terrestrial life. The vomeronasal system is present in modern amphibians as well as modern amniotes (reptiles and mammals), indicating that the vomeronasal system was present in the last common ancestor of these animals. This ancestor appears to have been fully aquatic (Panchen 1991; Lebedev & Coates 1995), indicating that the vomeronasal system could not have arisen as an adaptation to terrestrial life.

The results of the present study raise several questions concerning the function of the vomeronasal system, both within salamanders and among tetrapods in general. First, it is clear that the morphology and position of the vomeronasal organ vary considerably among salamander families. In axolotls and tiger salamanders, the vomeronasal organ is a blind-end sac protruding anteriorly from the lateral wall of the main chamber of the nasal cavity, and is completely lined with vomeronasal sensory epithelium (Eisthen *et al*. 1994; H. L. Eisthen, unpublished observations). In sirens, the vomeronasal organ lies ventral to the main chamber and is connected by a long, narrow duct, with sensory epithelium occurring only at the medial and lateral margins of the organ. In amphiuma, the vomeronasal organ is simply a lateral diverticulum of the main chamber of the nasal cavity. The sensory epithelium lining this diverticulum resembles the vomeronasal epithelium of other tetrapods, for it contains only microvillar receptor cells and lacks Bowman's glands. In mudpuppies, and probably all members of the proteid family, the vomeronasal organ is completely lacking. The functional implications of this diversity among salamander families remain to be explained.

Finally, based on studies of mammals and reptiles (Halpern & Kubie 1980; Wysocki *et al*. 1980), the vomeronasal system has been suggested to be specialized for detection and transduction of large, non-volatile odorants. Given that the system appears to have arisen in early aquatic tetrapods, and that volatility is irrelevant in an aqueous medium, the original function of the vomeronasal system was probably somewhat different. Nevertheless, the vomeronasal system could have been co-opted for this specialized function in modern, terrestrial mammals and reptiles.

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