GREAT LAKES FISHERY COMMISSION
Research Completion Report *

INTERACTION OF THE OLFACTORY AND ENDOCRINE SYSTEMS IN SEA LAMPREY DEVELOPMENT: TOWARD AN INTEGRATED BIO-CHEMICAL CONTROL: MORPHOLOGICAL DEVELOPMENT

by

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SUMMARY The objectives of this study are to provide a systematic and detailed study of the development olfactory organ in the sea lamprey, *Petromyzon marinus*. The focus has been on olfactory receptor cell (ORC) localization and density (the number of ORC per 100 μm length of olfactory epithelium. The report is divided into seven parts that report on specific aspects of the developmental stages. There will be five publications in peer-reviewed journals from this work. One paper is in press, one is in revision, and three are in the final stages of preparation.

1. The report of our discovery that sea lamprey ammocoetes have ORC (accepted for publication in *Brain, Behavior and Evolution*).
2. We have examined embryos and prolarvae, to determine at what stage olfactory receptor cells (ORC) differentiate, and if there are migrating nerve fibers from the olfactory placode to the brain, that may regulate sexual development. This manuscript is in revision for publication.
3. The olfactory mucosa in ammocoetes was examined to locate regions of the larval olfactory organ with high surface densities of ORC for electrophysiological recordings. We have included preliminary results from electrophysiological recordings, and enzyme histochemistry for nitric oxide synthase, which suggest that nitric oxide may be associated with chemosensory transduction of L-arginine. These results have a potential application in directing ammocoete chemosensory behaviour. A publication is in preparation.
4. Sea lampreys that were undergoing metamorphosis were analyzed to serve as a basis for future correlations of changes in the transformation of the olfactory organ with endocrine and physiological factors. The major re-organization of the olfactory organ occurs between metamorphic stages 3

and 5. This work was part of an M.Sc. thesis (Mr. Jamie Vandenbossche), and is the basis of a manuscript in the final stages of preparation.

5. The relative distribution of ORC of sea lamprey in the juvenile (parasitic) stage was analyzed. There are variations in ORC distribution and ORC density that may be associated with pathological conditions, population differences or other factors.
6. In the summer of 1993, we monitored the structure of the olfactory organ and ORC density in upstream migrants from Hammond Bay and the St. Mary's River. The ORC density was slightly lower in lampreys that were trapped at the St. Mary's River site. Moreover, the ORC density dropped in specimens trapped at the St. Mary's River site, at the end of upstream migration, and there were frequent morphological characteristics of ORC apoptosis (programmed cell death). This study will be published.
7. Sea lampreys that were trapped at sites of upstream migration and had survived in captivity from 3 to 11 months, were called post-upstream migrants. The olfactory organ and olfactory nerve was present, but did not contain ORC dendrites, cells bodies or axons. These results suggest that following the period of upstream migration, ORC degenerate.

The morphology of the olfactory epithelium in the larval, juvenile and upstream migrant stages of the sea lamprey, *Petromyzon marinus*

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ABSTRACT

The structure of the olfactory epithelium in the larval, juvenile and upstream migrant stages of the sea lamprey, *Petromyzon marinus*, was investigated by light microscopy and by scanning and transmission electron microscopy. Ciliated olfactory receptor cells (ORC) were present in all stages. In larval specimens, the number of ORC was 20±8 ORC per 100 μm length of olfactory epithelial surface. In juveniles and in upstream migrants the ORC density dropped to 9±2 and 6±2 ORC/100 μm, respectively. Sustentacular cells were microvillar in the smallest larval stage (with a body length of 15 mm) and ciliated in larger larvae and later life stages. The morphological characteristics of the olfactory mucosa suggest that the sea lamprey's capacity for use of the olfactory system extends into the larval stage, and that there are specific changes associated with metamorphosis.

INTRODUCTION

The importance of the olfactory system to the lampreys' survival has been suggested by several investigators (e.g. Kleerekoper, 1969; Bertmar, 1985). The benthic filter feeding ammocoete larvae retain filter feeding, which is interpreted as a conservative protochordate character (Gans and Northcutt, 1983). The ammocoetes are able to select specific diatoms as food (Chappius, 1939), and may communicate by releasing a growth inhibiting substance (Mallatt, 1983). It certainly would not be surprising if larvae use chemical signals for a variety of behavioral mechanisms, yet there have been very few studies of the larval olfactory system (e.g., Leach, 1951; Kleerekoper, 1969; Gorbman and Tamarin, 1985). The olfactory organ is highly developed in the parasitic juvenile stage (Kleerekoper and van Erkel, 1960), and appears to be associated with location of prey (Chappius, 1939, Kleerekoper and Mogensen, 1959, 1963). Upon reaching a specific stage of sexual maturation, sea lampreys use pheromone communication (Teeter, 1980).

Chemostimulatory compounds flow into the lamprey's single medial nostril on the dorsal surface of the head by pumping of the nasal chamber (Kleerekoper and Van Erkel, 1960), and become mixed with mucous secretions. Odor molecules reach the olfactory epithelium, a specialized region of the nasal tube, and stimulate the olfactory receptor cells (ORC), which transmit their signals to higher brain centers. The sensory portions of vertebrate ORC are located on bulbous dendritic endings, (the olfactory knob) that extend into the nasal cavity. Thornhill (1967) showed that the olfactory knobs of adult freshwater lampreys *Lampetra fluviatilis* are ciliated. A recent review by Eisthen, (1992) points out that lampreys are unique among fish groups in having only ciliated ORC. The second extant agnathan group, the hagfishes have both ciliated and microvillar ORC, as do the teleosts; Chondrichthyes have only microvillar ORC.

Previous researchers have described the shape and structure of the larval olfactory organ with low power light microscopical (Leach, 1951; Kleerekoper, 1969) and scanning electron microscopical (Gorbman and Tamarin, 1985) techniques. We have extended these studies to investigate the localization, ultrastructure and relative ORC density in the olfactory epithelium in three stages of the sea lamprey life cycle: the larval, juvenile and upstream migrant stages (Youson, 1980).

MATERIALS AND METHODS

Specimens of landlocked sea lampreys were obtained from the Hammond Bay Research Station and the St. Mary's River System (Sea Lamprey Control Center, Department of Fisheries and Oceans, Sault Ste Marie, Canada). These included ten larvae (with body length from 15 mm to 80 mm), three juveniles, and three upstream migrants. The original research reported herein was performed under guidelines established by the Canadian Council of Animal Care.

For scanning electron microscopy (SEM), larval specimens were fixed by immersion into modified Karnovsky's fixative (Zielinski et al., 1988), split along the sagittal plane with a razor blade, immersed into buffered osmium tetroxide, and dehydrated in ethanol. Specimens were mounted on metal stubs, sputter coated with gold, and viewed with a Hitachi scanning electron microscope at the Harrow Research Station, Agriculture Canada.

Light microscopy (LM) was used to identify the olfactory epithelium, and cellular ultrastructure was determined by transmission electron microscopy (TEM). For LM and TEM analyses, the dissected pieces of modified Karnovsky-fixed olfactory mucosa were osmicated and dehydrated, then immersed into propylene oxide and embedded into epoxy resin. Sections with a thickness of 1 μm were made with a RMC ultramicrotome, stained with a modified Richardson's stain, and viewed on a Nikon Optiphot light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate prior to examination on a Philips 201 transmission electron microscope.

The relative number of ORC per unit length was estimated in larvae that were longer than 18 mm, in juveniles and in upstream migrants. The number of ORC olfactory knobs per 100 μm length of olfactory mucosa was counted at the LM level (X1300). The following criteria were used to identify ORC at the LM level: ciliated olfactory knob, basal bodies at the outer margins of the olfactory knob, and a palely stained distal dendritic cytoplasm.

RESULTS

To explore the shape of the developing nasal tube in the sea lamprey, tissue was viewed by SEM and LM (figs. 1A, B). In the larvae, the nasal tube is partially paired by an incomplete septum, as previously described by Leach (1951) in the larvae of *Ichthyomyzon fossor*. The posterior and lateral surfaces of the nasal tube are lined by pseudostratified epithelium, characteristic of olfactory epithelium (fig. 1B). Definitive identification of ORC was made at high power LM and by TEM. The ORC were recognizable at high power LM by their ciliated olfactory knobs with basal bodies at the outer margins (figs 1C, D). The ORC in 15 mm larvae are closely spaced (fig. 1C), compared to the more widely spaced ORC in larger larvae (fig. 1D). The ORC cytoplasm is generally lighter than that of the surrounding sustentacular cells (SC). The ciliated olfactory knobs are prominent, and the proximal portions of the ORC cilia contain specialized ladder-like structures (fig. 1E). These are not unique to ORC, as they are present in some SC cilia (data not shown) and in renal tubule cilia (Kluge and Fischer, 1991). Larval olfactory knobs contain ciliary basal bodies and microtubules that extend into the distal dendritic cytoplasm (figs. 1E, F). The SC in 17 mm ammocoetes are microvillar, and the apical cytoplasm contains secretory vesicles with flocculent material (fig. 1F). The SC in larger larvae, in comparison, are ciliated (fig. 1D).

The surface of the olfactory organ in juvenile sea lampreys is covered by lamellar folds that are lined with olfactory epithelium (fig. 2A). Previously identified gland-like structures (Kleerekoper, 1969) are present in the lamina propria at the base of the lamellae (fig. 2A). The ORC mitochondria stain darkly and appear proximal to the level of the junctional complex. In comparison, the SC mitochondria reach within 0.2 μm of the apical membrane. The SC apical cytoplasm also contains striated rootlets and small secretory vesicles. When comparing the mucociliary complex in the juvenile and upstream migrant stages, two features stood out: the olfactory knobs in the migrants appeared more prominent, and the mucociliary complex was more heavily ciliated (figs. 2C, D).

The number of ORC per length of olfactory epithelium appeared to be higher in larval specimens than in juveniles and migrants. To substantiate our finding, we estimated the ORC density by counting the number of olfactory knobs per epithelial length of 100 μm. In larvae, the ORC density was 30±9 ORC per 100 μm length (130 lengths counted, N=3). The density was lower in juveniles (8.1±2.3, 260 lengths counted, N=3,) and in upstream migrants (5.8±1.8 ORC per 100 μm length, 36 lengths counted, N=2). These results show that ORC density is greater in larval stages, than the larger juvenile and upstream migrant stages.

DISCUSSION

Our investigation has shown that the olfactory mucosa of the sea lamprey contains ciliated ORC in the larval, juvenile and upstream migrant stages and that the values for ORC density are greater in larval ammocoetes than in juveniles and upstream migrants.

The abundance of ORC that are morphologically mature suggests that the filter feeding larvae, which represent protochordate simplicity (Gans and Northcutt, 1983), are capable of olfaction. Since the development of embryonic ciliated olfactory knobs goes hand-in-hand with the ORC's physiological function in rainbow trout (Zielinski and Hara, 1988) and in rats (Gesteland et al., 1982), the ORC in larval ammocoetes may be physiologically active. In contrast, visual receptor cells are not morphologically mature in larval lampreys (Rubinson, 1990).

Thornhill (1967) reported that the olfactory epithelium in adult *Lampretra fluviatilis* has one morphological form of ORC, the ciliated form, and our study extends this observation to the larval ammocoete and adult stages. In her recent review, Eisthen (1992) concluded that of all fishes, only lampreys have exclusively ciliated ORC. All other fishes have microvillar ORC. For example, the Atlantic hagfish, another agnathan has both microvillar and ciliated ORC (Thiesen, 1976), as do teleosts (e.g. Yamamoto, 1982, Zielinski and Hara, 1988). Chondrichthyes have only the microvillar ORC (e.g. Holl, 1973, reviewed by Eisthen, 1992).

The ciliated SC appear to be phylogenetically primitive compared to the microvillar supporting cells in other species. Ciliated SC are present in three developental stages in sea lampreys, and were previously observed by Thornhill (1967) in adult freshwater lampreys *Lampretra fluviatilis*. Elasmobranchs have ciliated SC (Holl, 1973), whereas teleosts have microvillar SC as well as ciliated nonsensory cells that are similar to the SC in sea lampreys, but are not adjacent to ORC (e.g. Yamomoto, 1982). In the adult stages of sea lampreys, the SC are wider and more heavily ciliated than in the larvae. Narrow SC appear to account for the ORC density values in larvae, that were more than double the ORC density values in adults. The maturation of SC lags behind differentiation of ORC by only a few days in embryonic

rainbow trout (Zielinski and Hara, 1988), in mice (Cuschieri and Bannister, 1975), and in regenerating catfish olfactory mucosa (Cancalon, 1982). The sea lamprey is unique with a prolonged period of high ORC density. The closely spaced ORC in larvae may compensate for the confined surface area of the olfactory epithelium compared to the large surface area of the olfactory epithelium on lamellar folds in juveniles and upstream migrants. In rats the number of ORC per 100 μm length of epithelial surface quickly rises from approximately 5 at birth to a mean of 30 ORC after 20 days, and 35 at 60 days after birth (Walker et al., 1990). These values appear to be the reverse of developmental changes in the sea lamprey.

The secretory vesicles in larval SC appear to be smaller and less abundant than in juvenile and upstream migrant SC. Evolutionary comparison of the SC secretory vesicles is complex. SC with small endosome-like vesicles are present in olfactory mucosae that lack glands (e.g. teleosts, Yamamoto, 1982; Zielinski and Hara, 1988) and in some animals with glandular olfactory mucosae (e.g., humans, (Moran et al., 1982) and rats, (Mendoza and Kuhnel, 1991)). SC with prominent large secretory vesicles are present in aquatic larval phase salamanders which lack glands, and in the glandular salamander olfactory mucosa of adults (Getchell et al., 1988). The secretory products of gland-like structures below the lamellae in juveniles and upstream migrants (Jacobson's organ reviewed by Kleerekoper, 1969) may also be added to the nasal cavity. The presence of complex glandular structures in the olfactory mucosa of adult sea lampreys does not support the common belief that a glandular olfactory mucosa evolved with terrestrial habitat. Clearly, sea lamprey olfactory mucosal glands should be further investigated.

This report has shown that the sea lamprey olfactory mucosa is intriguing from an evolutionary aspect and for future behavioral experiments of ammocoete olfaction.

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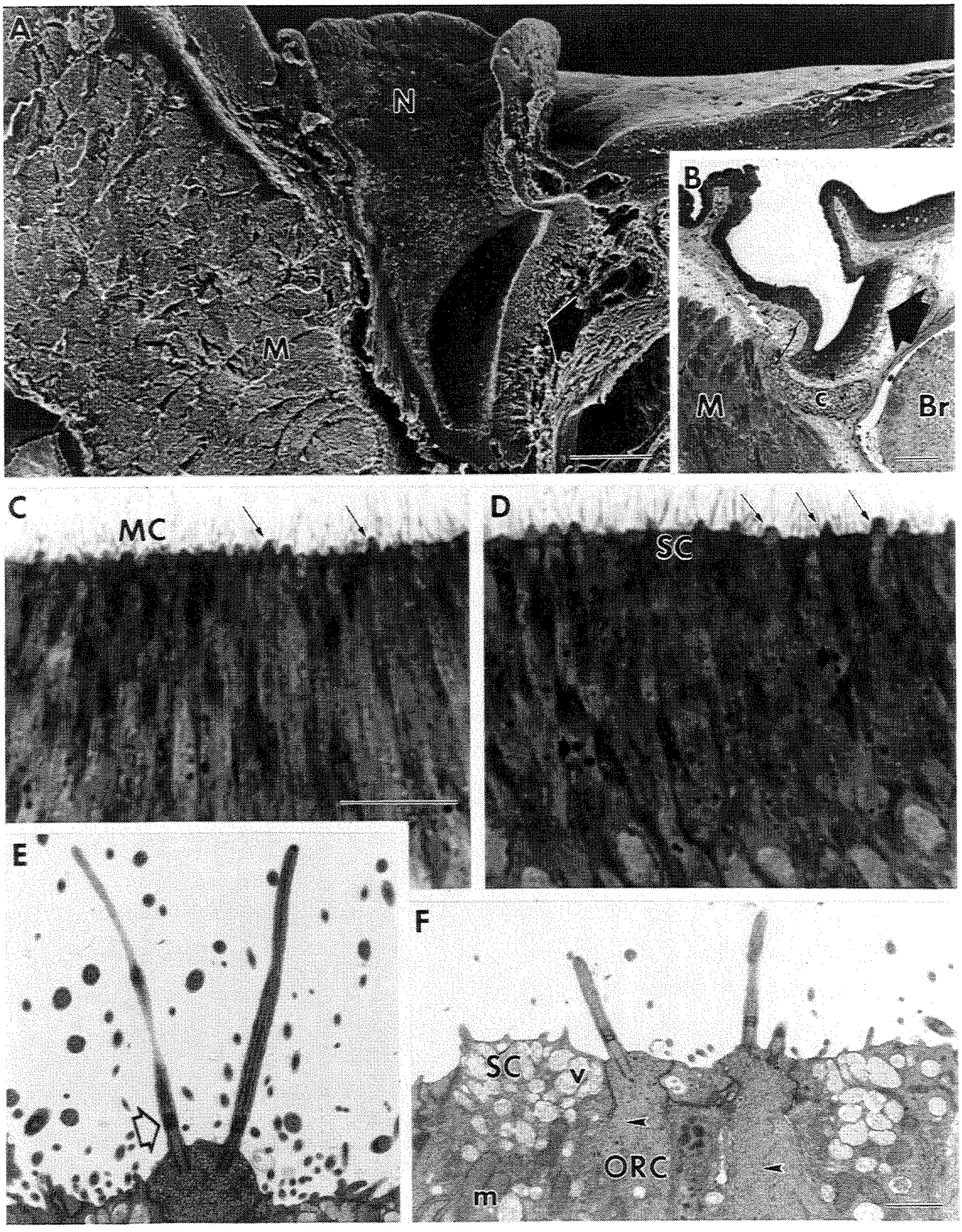

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Figure 1 The olfactory epithelium in larval sea lampreys.

A) The scanning electron micrograph shows the nasal tube and olfactory epithelium (arrow) from a larva (length 75 mm). The external nostril (N) is on the larva's dorsal surface and the olfactory epithelium is located on the ventro/caudal bend of the nasal tube. Large fascicles of muscular tissue (M), viewed as cross-sections, extend rostrad to the nasal tube. Bar=100 μm.

B) In this light micrograph from a larva (length 80 mm) the olfactory epithelium (arrow) is recognized by its pseudostratified arrangement. Muscular tissue is prominent anterior to the nasal tube (M), a small piece of cartilage (C) is located dorsal to the nasal tube and the brain (Br) fills the bottom right region of the micrograph. Bar=100μm.

C) The high power light micrograph from olfactory epithelium of a 15 mm larva, shows closely spaced ORC olfactory knobs (arrows). Cilia extend from the olfactory knobs into the mucociliary matrix (MC). The bar in figures C and D is 10 μm.

D) In the olfactory epithelium of a 55 mm ammocoete, ciliated sustentacular cells (SC) with flat apical surfaces are located between the ORC olfactory knobs (arrows). Darkly stained granules are located in the supranuclear cytoplasm of the olfactory epithelial cells (small, arrowheads).

E) The transmission electron micrograph shows an olfactory receptor cell from a 15 mm larva. There are two prominent cilia extending from the olfactory knob. Note the densely stained structures in the proximal portion of each cilium (arrow). Bar=0.5 μm

F) ORC dendritic cytoplasm contains microtubules (arrowheads), and the apical region of the sustentacular cells (SC) contains secretory vesicles (v) and mitochondria (m). Bar=0.5μm.

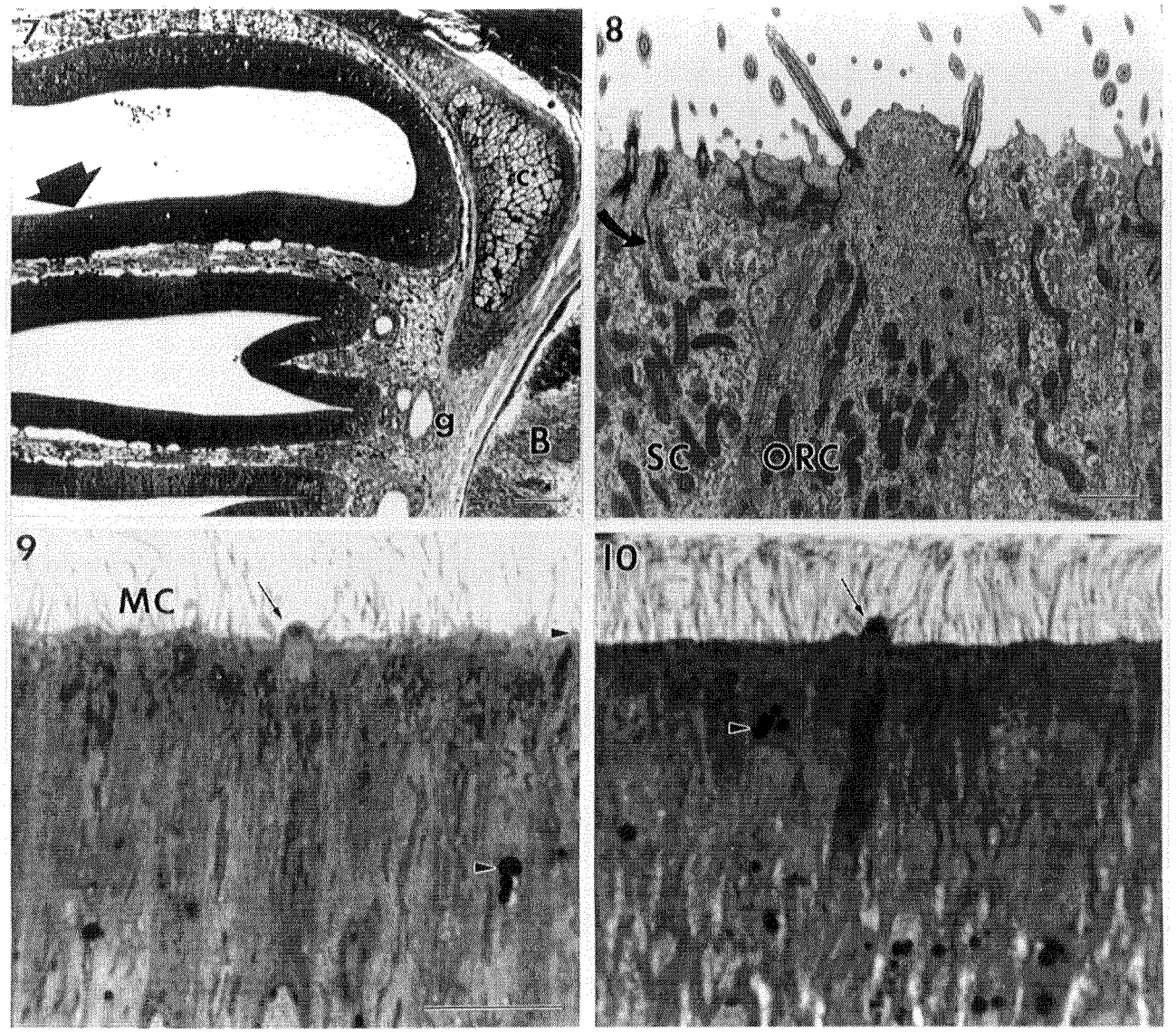

photo B

Figure 2 The olfactory epithelium in parasitic and spawning sea lampreys.

A) The low power light micrograph shows the lamellar folds of the olfactory organ from a juvenile sea lamprey. Olfactory epithelium is located on opposite facing lamellar surfaces (arrow). Gland-like structures (g) are located on the basal surface of the lamellae. Cartilage (c) encloses the peripheral portion of the olfactory organ. The olfactory bulb (Br) is located adjacent to the olfactory organ.

B) At the TEM level, the ORC have a well-formed olfactory knob with cilia extending from the basal surface of the olfactory knob. The olfactory knob contains very small endosome-like vesicles (surrounded by a circle, O) and microtubules (arrowheads). Mitochondria (m) are abundant in the dendrite, but are absent from the area of the tight junction (•) with neighboring sustentacular cells (SC). The mitochondria of the SC, on the other hand are abundant throughout the apical cytoplasm. The SC apical cytoplasm also contains striated rootlets (curved arrow) and small vesicles (v). The bar equals 0.5 μm.

C) The light micrograph shows the supranuclear portion of the olfactory epithelium from a juvenile sea lamprey. The mucociliary matrix (MC) contains cilia from sustentacular cells and from ORC (arrow). The short black arrow points to darkly stained granules located within the olfactory epithelium. Bar = 10 μm.

D) The mucocilary matrix in an upstream migrant sea lamprey is densely ciliated. The ORC olfactory knob (arrow) is prominent. There are many darkly stained granules within the olfactory epithelium (short black arrows). The scale is the same as in figure 2C.

EMBRYOS AND PROLARVAE Sea lamprey eggs were collected from the St. Mary's River following the spawning run of August '92, with the assistance of Rod McDonald at the Sea Lamprey Control Center in Sault Ste Marie. They were prepared for SEM, light microscopy and TEM and analyzed by Ella Wong and B. Zielinski at U. of Windsor, during the autumn '92 and winter '93. The results were been submitted for publication to the Journal of Comparative Neurology in Aug. '93, and are now undergoing revision for resubmission. Our overall conclusions are

1. The olfactory placode is present in Piavis stage 14 embryos.
2. There are ORC after hatching in stage 15 prolarvae.
3. In addion to ORC axons, there are fibers with peptidergic-type vesicles that may be nerve fibers that are migrating into the brain.

The olfactory organ is located in a shallow nasal cavity on the dorsal surface of the head during early development, making it accessible for future electrophysiological recordings. Recording electrodes could be placed directly onto areas with ORC. Water flow could be maintained during experiments without surgery since the olfactory organ is very close to the surface of the head.

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DEVELOPMENT OF THE OLFACTORY ORGAN IN EMBRYONIC SEA LAMPREY, *PETROMYZON MARINUS*.

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ABSTRACT

To determine if olfaction may be used by sea lamprey embryos and prolarvae prior to abandoning their nests and moving downstream to feeding areas, these early life stages were collected from nests and analyzed by light microscopy and by scanning and transmission electron microscopy. The olfactory placode was present from stage 14 (Piavis, 1976), morphologically mature olfactory receptor cells were observed at hatching (stage 15), and ORC were abundant in the oldest by the time of downstream migration (stages 17 and 18). Asymmetric synapses with agranular vesicles, characteristic of olfactory nerve synaptic contacts were present in the telencephalon adjacent to the olfactory nerve. Agranular vesicles in fibers in the basal portion of the olfactory epithelium may be *"en passage"* to terminals in the telencephalon. Fibers with agranular vesicles and dense cored vesicles within the olfactory placode, in the olfactory nerve and in the telencephalon may represent peptidergic fibers migrating into the central nervous system. These results show that the olfactory organ of early stages of the sea lamprey, that preclude feeding, processes morphologically mature ORC and has characteristics of growing and migrating fibers.

INTRODUCTION

The olfactory system is a significant medium that animals use to communicate amongst each other and with the environment. Early life stages of various vertebrates appear to use the olfactory system (e.g. Pedersen et al., '86). Evolutionary patterns of the development of the olfactory mucosa is therefore of substantial interest to comparative neurobiologists. The present investigation is intended to explore the development of the olfactory organ in Agnathan jawless fish, which have originated from the most primitive sources in the vertebrate evolutionary line. The Agnathan fish *Petromyzon marinus*, the sea lamprey, has a complex life cycle. In the Great Lakes, the land-locked sea lamprey hatch in nests in stream gravel beds, abandon these approximately two weeks after hatching (Piavis, 1971) and move downstream to silty eddies where they remain in the filter feeding larval (ammocoete) stage for two to seven years. The larvae undergo metamorphosis to the juvenile stage and move to open water and feed upon fish such as salmonids and whitefish. Upon reaching sexual maturity, sea lamprey move inshore and migrate upstream to gravel spawning areas.

The adult lamprey has a single dorsal midline nostril, a nasal cavity with two olfactory nerves and two olfactory bulbs (Kleerekoper and van Erkel, '60; reviewed by Nieuwnhuys, '67). We have previously observed olfactory receptor cells (ORC) in the ammocoete, juvenile and spawning stages of the sea lamprey and have found that the olfactory receptor cells (ORC) are ciliated (Vandenbossche et al., '93). The absence of microvillar ORC in lamprey, previously observed by Bronshtein and Ivanov (1965) and by Thornhill (1967), and reveiwed by Eisthen ('92) emphasizes their evolutionary isolation. The olfactory epithelium of all other fish, including the hagfish which belong to a sister Agnathan group, and all fish of the Chondrichthyes (Yamamoto, 1984) contain microvillar ORC (Theisen, 1973)

Both the lamprey and the hagfish have a single dorsally located nostril, and a single nasal sac. The isolated phylogenetic location of the lamprey is further illustrated by the ontogenetic origin of the adenohypophysis. It is derived in the lamprey embryo as a posterior projection from a single median epithelial pit (olfactory pit) on the front of the embryonic head (e.g. Scott, 1883, reviewed by Gorbman and Tamarin, '85).

The primary aim of our study is correlate the differentiation of the olfactory mucosa to usage of the system by lamprey. Additionally, we present ultrastructural characteristics of the developing placode of presumed growth cones and synaptic junctions between the developing axons and sheath cells. Embryonic and prolarval stages were collected from nests and were analyzed by light microscopy, and by scanning and transmission electron microscopy.

MATERIALS AND METHODS

For this study, eggs and prolarvae were collected from nests following the spawning run of August, 1992 and immersed immediately into modified Karnovky's fixative (Zielinski et al., 1988). Unhatched embryos were removed from their egg capsules before further tissue processing. Sea lamprey embryos in the St. Mary's River system hatch approximately two weeks after the adults have spawned. The embryos and prolarvae were staged according to Piavis's review of embryonic development ('71). The eggs hatch during stage 14, and remain in their nests until stage 17 or stage 18. SEM specimens were then post-fixed in 1% osmium tetroxide, dehydrated in ethanol, critical point dried and viewed on a Hitachi scanning electron microscope. For TEM, specimen were passed through propylene oxide following ethanol, then embedded into epoxy resin, One micrometer sections or 70 nm sections were prepared on a RMC ultramicrotome, and viewed on a Nikon Optiphot or Philips 201 transmission electron microscope, respectively.

RESULTS

Our results show that the olfactory placode started differentiation prior to hatching (stage 14; fig. 1 and 2), and became recognizable as the olfactory epithelium after hatching, and by stage 16, the ORC were prominent.

The stage 14 olfactory placode was located on the ventral surface of the head, adjacent to the presumptive telencephalon (fig 1). The olfactory placode cytoplasm contained yolk granules, and the nuclei were pale, and microvilli extended from the surface of the placode (fig. 2). In hatched embryos, the olfactory organ was situated within the nasal cavity and located on the anterior surface of the head (stages 15 and 16, figs. 3, 4 and 5) . Eyes were not visible on the surface of the head of the embryos or the prolarvae, and do not differentiate until the ammocoete stage (e.g. De Miguel et al., '90). The olfactory epithelium, recognizable by its pseudostratified cellular arrangement, was located on the caudal surface of the nasal cavity. Yolk granules were still present in the cytoplasm of the olfactory epithelium in stage 15 (fig. 3). The olfactory epithelium maintained its location adjacent to the telencephalon throughout these early stages. By stage 17, the nasal pore was located on the dorsal surface of the head (fig. 6). It had a triangular shape; the anterior edge was narrower than the posterior portion, and the floor was covered by cilia (fig. 7). Two germinal areas of olfactory epithelium were located on lateral surfaces on the anterior portion of the nasal cavity (figs. 8 and 9), and a single sheet of olfactory epithelium remained on the posterior surface, as observed in earlier stages (figs. 2 and 5) .

The ORC in embryonic and prolarval sea lamprey were ciliated; the olfactory knob contained microtubules, endosome-like particles and ribosomes (figs. 10 to 15). The apical region of the dendrite below the level of the tight junction contained a variety of vesicles and electron dense mitochondria. The cytoplasm of cells which were presumably were young ORC which were immature, because of their low olfactory knobs contained numerous vesicles. (fig. 12). Microvilli extended from the olfactory knob of these ORC (figs 10 to 12). The cilia contained a ladder-like structure, seen also in the cilia of the nonsenseory in the olfactory epithelium and other body regions in the lamprey, such as the pronephros (figs. 14 and 15; e.g. Kluge and Fisher '91). Tight junctions and asymmetric desmosomes joined the ORC to adjacent sustentacular cells. The cytoplasm of sustentacular cells surrounding immature ORC

with low olfactory knobs, contained ribosomes, mitochondria and intermediate filaments. The SC contained secretory vesicles that emptied their secretion into the nasal cavity by endocytosis (figs. 11 and 12).

Axons formed aggregates at the base of the olfactory epithelium (fig. 16). Some of these contained large groups of agranular vesicles, and mitochondria; other profiles have ribosomes (fig. 17). There was a parallel membrane apposition adjacent to the clusters of agranular vesicles, suggesting immature synapses. The post-synaptic surface of these contained ribosomes and filaments, suggesting that they were glial-like cells (fig. 17). Also present on the surface of the fascicle was a fiber that extended from the olfactory epithelium along the olfactory nerve to the olfactory bulb (figs. 18 and 20). The fiber contained many mitochondria, synaptic vesicles at all locations (figs 19 and 21). The ORC made synaptic contact of in the telencephalon, immediately beside the olfactory nerve (fig. 22). These were asymmetric synapses, with agranular vesicles, and mitochondria in the presynaptic cytoplasm. Young synapses *en passant* were also present.

DISCUSSION

Our results demonstrate that sea lamprey embryos had morphologically mature ORC before abandoning their nests for feeding sites downstream. We observed the olfactory placode in early stage 14 embryos; and by stage 17, there were abundant ciliated ORC with axons extending to the telencephalon where synaptic contacts were made. The rapid development of the peripheral olfactory organ suggests that the sense of smell is used by these early life stages, possibly to acquire an odor memory and to recognize conspecifics in downstream feeding areas. When compared to the visual system, with the photoreceptor and bipolar cells differentiating during metamorphosis (Rubinson and Cain, '89) and larval phototactic responses in the tail (e.g. Young, '36), the embryonic olfactory receptors seem precocious. A possible explanation is that the benthic ammocoetes do not require visual input for survival, and olfactory cues suffice for habitat and food selection, and communication. ORC in hatching rainbow trout respond to chemical stimulation (Zielinski and Hara, 1988), and juvenile salmonids are able to recognize siblings and conspecifics (Quinn and Hara, '86; Olsen '89). In comparison, very little is known about the developing sea lamprey nervous system.

The early differentiation of the ORC is not surprising. In *Xenopus laevis*, axons begin to cross from the olfactory placode during the tail bud stage of development (Klein and Grazadei, '83), and ORC axons begin to grow out at E14 in rats (Farbman and Squinto, '85) and E12 in mice (Marin-Padilla and Amiera, '89). The lamprey is particularly interesting since it hatches shortly before ORC appear, and has distinct behavioral activity that may be guided by the newly formed olfactory system.

From stages 14 to 16, the olfactory placode/epithelium moved from the ventral surface of the head to its final position on the dorsal surface. The ventral location of a single placode was described by previous studies (Kupffer, '06; de Beer, '23; Gorbman and Tamarin, '85) in specimens that, just as ours, were fixed shortly before hatching, and were 3 mm long. At stage 15, when the tissue was located on the anterior surface of the head, it became recognizable as olfactory epithelium. By stage 16, when the olfactory epithelium reached its final position on the dorsal surface of the head. there were abundant ORC and ciliated sustentacular cells.

The olfactory placode/epithelium was immediately adjacent to the brain from stage 14 to 17. This close association could be significant for the migration of neurons such as the GnRH neurons, (e.g. Schwanzel-Fakuda, '89) from the placode to the brain. Migrating neurons in lamprey are of additional significance since the adenohypophysis originates from the anterior surface of the head and close to the rudiment of the olfactory organ (Scott, 1883).

The single stretch of olfactory epithelium persisted in later stages, although there were two germinal areas in the anterior region of the nasal cavity. In later stages, the single sheet of olfactory epithelium is interrupted by an incomplete septum of nonsensory tissue (shown in Leach, '51, personal observations). The monorhinous condition appears to apply to the olfactory epithelium's origin from a single placode, and to the lamprey's single nasal cavity. The olfactory epithelium enlarges at two separate germinal regions, has two olfactory nerves and two olfactory bulbs.

Our observations of the timing of the differentiation of the embryonic olfactory system appears to coincide with liver bile synthesis (Piavis, '71). The first sign of the liver, and its yellow-colored bile is in late stage 14; the gall bladder is bile-green color at stage 17 when the olfactory epithelium has differentiated. Larvae produce and release a unique bile alcohol, petromyzonol sulfate (Hasselwood,), which is an odorant for migratory adults (Li et al., 1993). Prolarvae may be able to identify conspecifics from the bile acids of siblings within a nest. Prolarvae that have left their nests and are moving downstream to feeding sites may recognize these locations by the scent of the conspecifics. Previous studies with salmonids have shown that they are able to discriminate conspecifics and siblings (Quinn and Hara, '86; Olsen, '89)

The sea lamprey embryonic and prolarval olfactory placode/epithelium is unusual in several ways. Firstly, the cells of the olfactory placode contained yolk granules, which were previously observed by embryonic pronephros (Ellis and Youson, '90). Secondly, the embryonic and prolarval ORC olfactory knobs were microvillar as well as ciliated. In comparison, only cilia extend from the olfactory knobs of larval and adult ORC (Vandenbossche et al., 1993). These may represent a morphological subtype, since both ORC with microvilli and ORC without microvilli were present in the olfactory epithelium. The ciliated ORC of teleosts do not have microvilli on the surface of the olfactory knob (Yamamoto, 1982;

Zielinski and Hara, 1988). Other animals with both microvillar-like structures as well as cilia on olfactory knobs include the dipnoi fish *Protopterus annectens*, which has small microvilli and cilia (Derivot et al., 1979), frogs (Burton, 1985) and birds (e.g. Graziadei and Bannister, 1967). In addition to the regular complement of organelles that include microtubules, basal bodies, mitochondria; the ORC in sea lamprey embryos have numerous vesicles. These may be fragments of plasma membrane that are moving to sites to be incorporated into the membrane. Axons within the olfactory epithelium have groups of both agranular and granular synaptic vesicles. Since synaptic contacts: (i.e. pre and post synaptic densities) were not observed in the olfactory epithelium, the vesicles may be *en passage* to the synaptic sites in the olfactory bulb. Alternatively, the vesicular contents may be emptied by endocytosis within the olfactory epithelium, and their contents may diffuse to receptors on nearby targets. In this way the growing axons may modulate the activity of the differentiating olfactory epithelium.

There were mitochondria-rich fibers located within axons in the olfactory epithelium, on the surface of the olfactory nerve, and within the telencephalon that resembled the terminalis in the shark (Demski, Fields, '88). This may represent the sea lamprey terminal nerve, which has been identified in adult silver lamprey (Northcutt and Puzdrowski, '88). Some fibers within the olfactory epithelium and olfactory nerve may contain growth cones, which were characterized by smooth endoplasm-like sacs (Cheng and Reese, '85) and by vesicles with various sizes and electron densities (e.g. Lance-Jones and Landmeeser '81; Tennyson, '70). The cells surrounding these structures included basal cells, narrow granular profiles, and other axons. The filipodia, and enlarged, varicose structure that are usually associated with growth cones(e.g. Skoff and Hamburger, '74; Linke and Frotscher, '93) were not observed in our studies.

The early development of the olfactory organ that is directly exposed to the ambient environment when barely out the placode stage, and long period of larval development that follows, offers many advantages for the future study of the development of the vertebrate olfactory system.

A survey of the sea lamprey embryonic and prolarval olfactory organ.

Figures 1, 3 and 5 are at the same magnification, the bar is 50 μm.

Figure 1. Low power light micrograph of an early stage 14 embryo. The olfactory placode is located on the ventral surface of the head, directly adjacent to the neural tissue. .

Figure 2. High power light micrograph of an early stage 14 embryo. The arrows point to intracellular yolk particles. The nuclei are pale. The bar is 10 μm.

Figures 3 . A frontal section of a stage 15 embryo shows that the olfactory epithelium lines a depression on the front surface of the head, directly adjacent to the brain. Small yolk granules are present in the cytoplasm of cells of the olfactory epithelium, as well as of other cells.

Figures 4 and 5 show the location of the olfactory epithelium in the stage 16 prolarva. The scanning electronmicrograph (fig. 4) shows that the nasal cavity is a single triangular shaped pit on the anterior surface of the head. Figures 4 and 6 are at the same magnification, the bar, shown in figure 4 is 100 μm. The light micrograph of a 1μm section shows that the olfactory epithelium is located in the base of the nasal cavity and directly adjacent to the presumptive telencephalon.

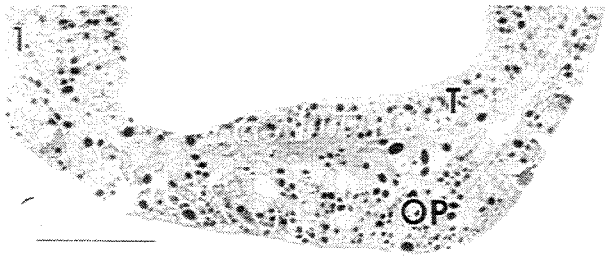
Figures 6 and 7 show the location of the nasal cavity in the stage 17 embryo. The low power scanning electron micrograph (fig. 6) shows that the nasal cavity is located on the dorsal surface of the head. The area enclosed by a square is shown at higher magnification in figure 7.

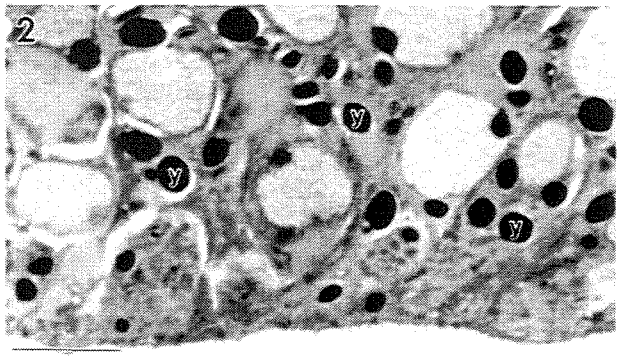
Figure 7. The triangular shape of the nasal cavity is clearly shown. The anterior edge is narrower than the rostral portion. Cilia are visible on the floor on the nasal cavity on the posterior, wider surface. The bar is 10 μm.

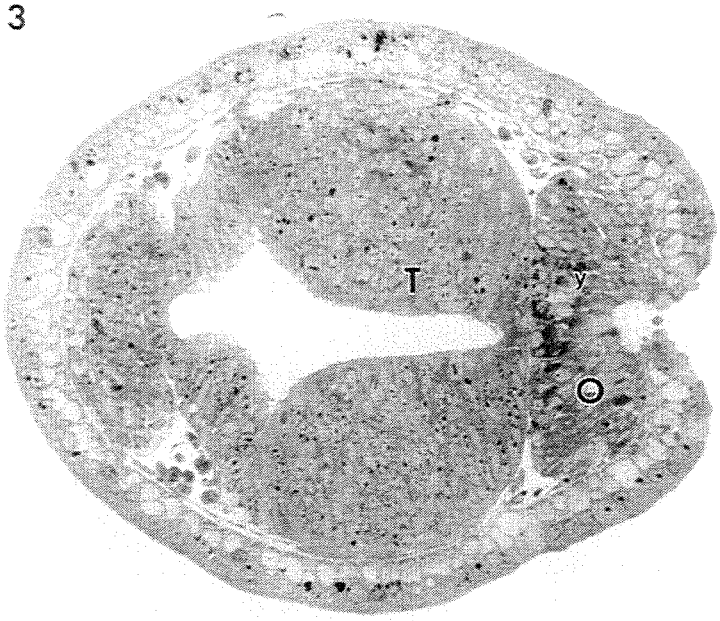
Figures 8 and 9 are cross-sectional views from the anterior narrow portion of the nasal cavity in a stage 17 prolarva, the bar length is 50 μm..

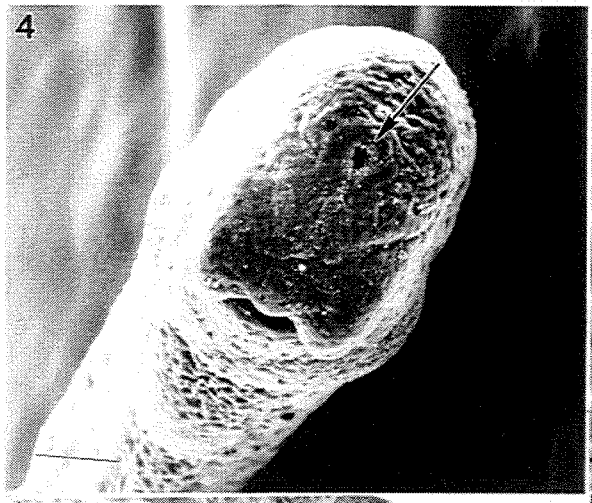
Figure 8. The lateral surfaces on the anterior region of the nasal cavity has two germinal regions, one on each surface of the nasal cavity. (thick, black arrows). Muscle fascicles are located at the base of the nasal cavity, and surround the mouth.

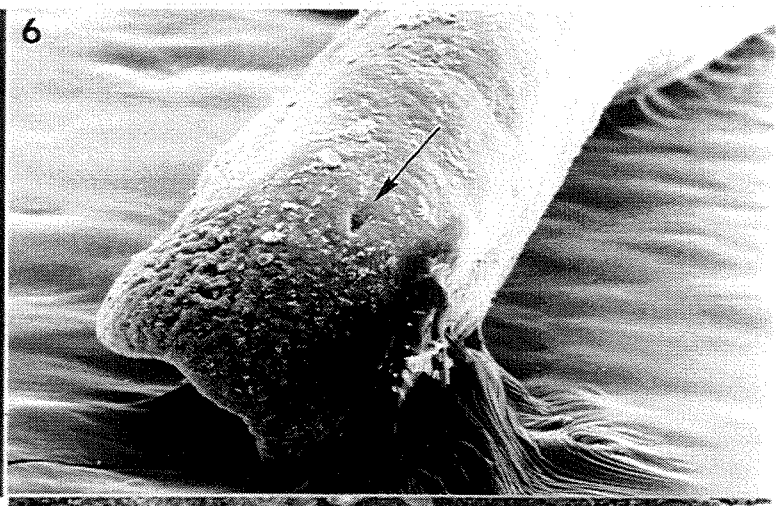
Figure 9. In a more posterior section, olfactory epithelium lines the lateral surfaces of the nasal cavity.

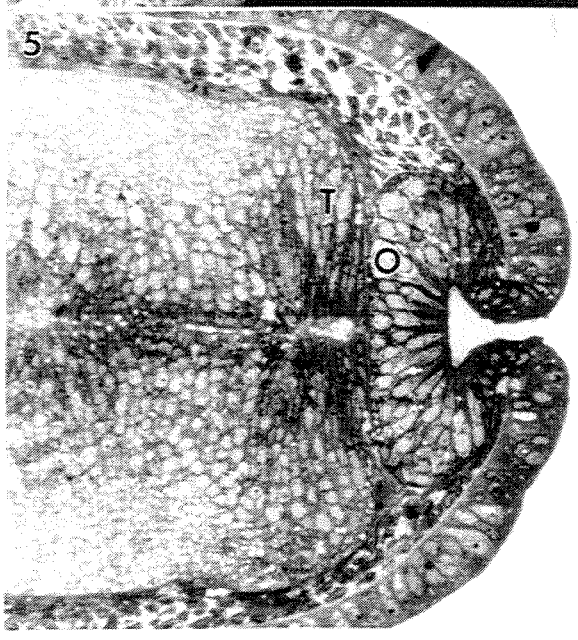












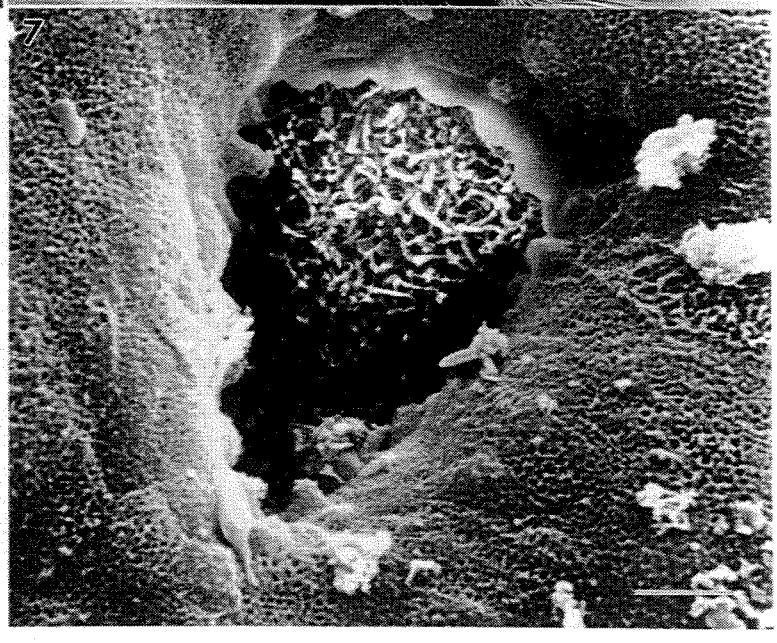


Photo C

Bars are 0.5 μm.

Figures 10 to 15 show olfactory receptor cell and sustentacular cell ultrastructure.

Figure 10. The olfactory receptor cell (ORC) has microtubules (arrowheads), a cilium with a basal body. the olfactory knob has microvillar-like protrusions (slightly bent arrow) and ribosomes. A tight junction is located between the ORC and the adjacent sustentacular cell. The sustentacular cell (SC) has intermediate-sized filaments (V-shaped arrows), ribosomes and mitochondria.

Figure 11. The thin section of the ORC has cilia, and microvillar-like protrusions (slightly bent arrows) extending from the surface of the olfactory knob. The olfactory knob is densely packed with microtubules (arrowheads) and has sparse ribosomes. A centriole is visible subjacent to the tight junctions (small, thin arrow). Sustentacular cells are joined by prominent desmosomes. The SC cytoplasm has prominent vesicles filled with flocculent material (sv). A sustentacular cell secretory vesicle is directly apposed to the luminal surface of the membrane (asterisk).

Figure 12. The ORC has microvillar projections extending fro the olfactory knob (curved arrow). A centriole is seen in the dendritic region (small arrow). The sustentacular cell contains secretory vesicles (sv). The asterisk shows a bleb-like profile between two sustentacular cell microvilli.

Figure 13. The apical cytoplasm of the immature olfactory receptor cell is packed with microtubules and centrioles are in the dendritic region.

In figures 14 and 15, an electron-dense structure is located at the base of an ORC cilium (short, thick arrow).

Figure 14. A single cilium is seen extending from the apex of the olfactory knob. Three centrioles are visible in the dendritic cytoplasm.

Figure 15. The filaments associated with SC desmosomes extend across the width of the SC (V-shaped arrow), with the distal region containing a clump of secretory vesicles. The mitochondria do not extend beyond the level of the tight junctions in both cell types.

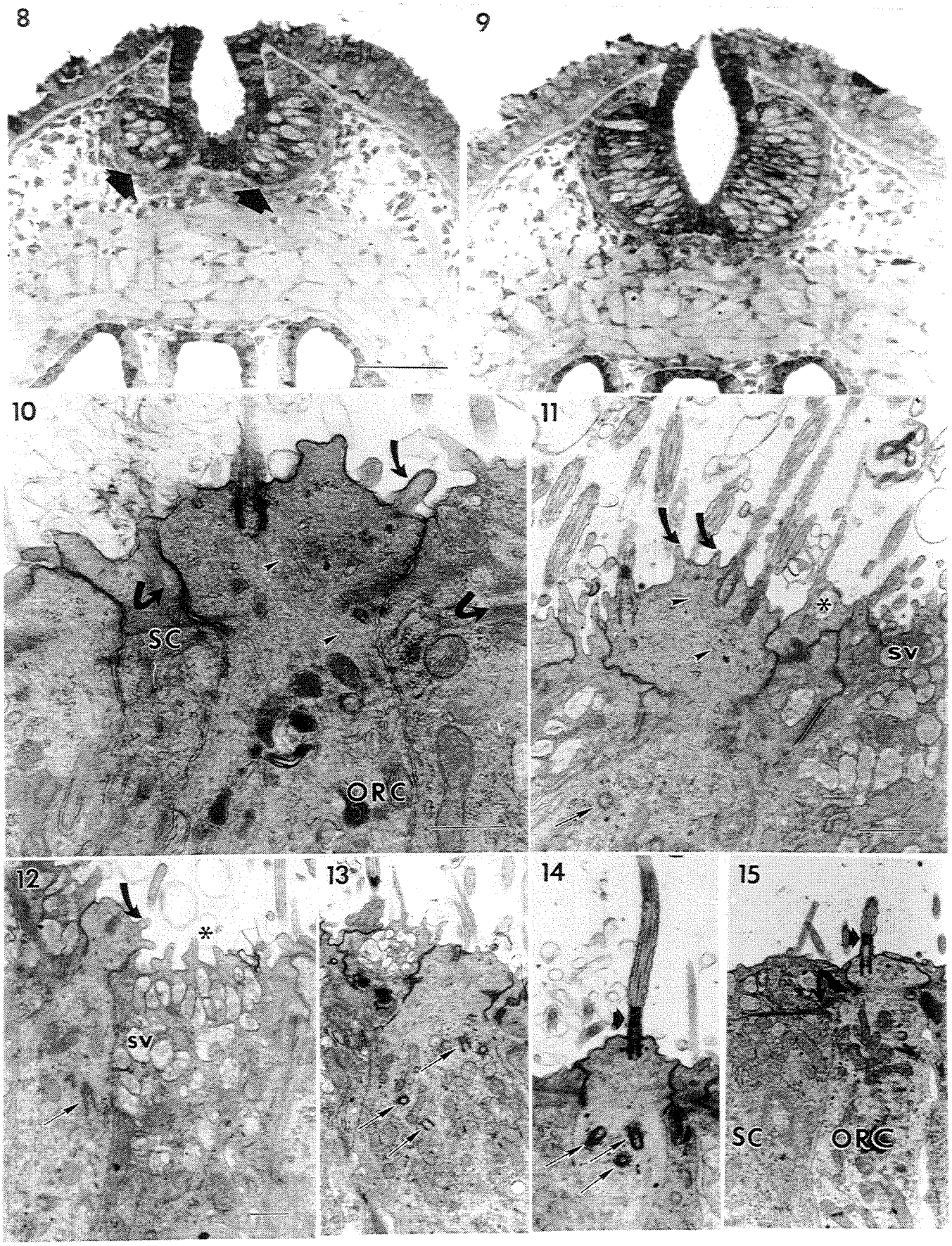


Photo D

Fibers with synaptic vesicles in the olfactory mucosa.

Figure 16. A group of axons within the olfactory epithelium. The lamina propria is indicated by a row of arrowheads. The upper portion of the axonal grouping contains profiles with mitochondria and agranular synaptic vesicles. The area enclosed by brackets is shown at higher magnification in figure 17.

Figure. 17. A fiber with a group of axons within the olfactory epithelium has an cluster of agranular vesicles (agv) of uniform size. The vesicles face parallel membrane apposition with a profile that contains a mitochondrion, ribosomes and vesicular structures.

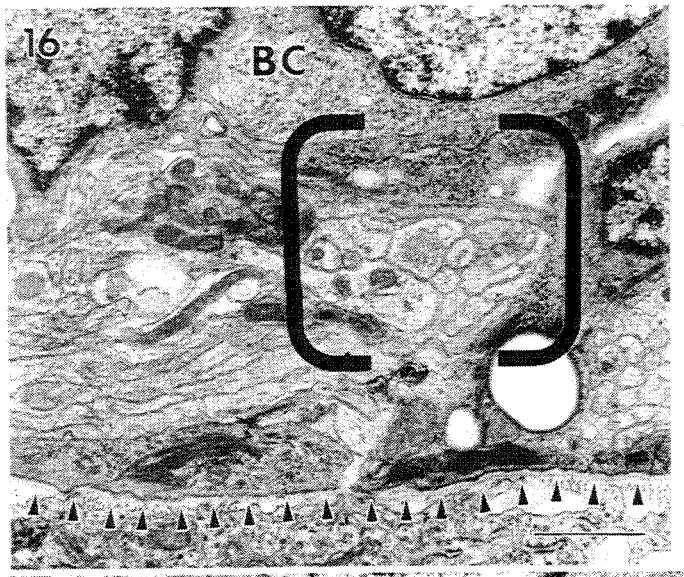
Figure 18. A nerve bundle extends from the olfactory epithelium to the lamina propria. The basement membrane is outlined by arrowheads. Profiles within upper portion of the nerve bundle are packed with mitochondria. The area enclosed by a square is shown at higher magnification in figure 19.

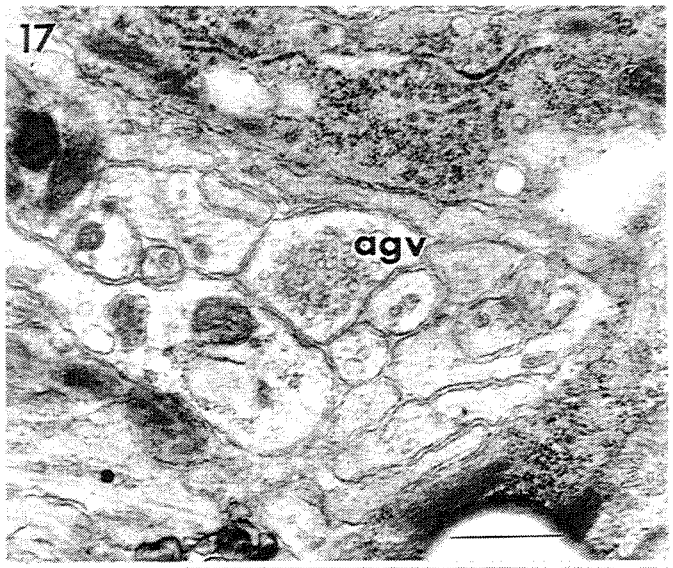
Figure 19. A profile within the olfactory epithelium has a cluster of agranular vesicles of uniform size (agv) that are adjacent to the plasma membrane, and a cluster of small mitochondria. The surrounding profiles have a similar composition.

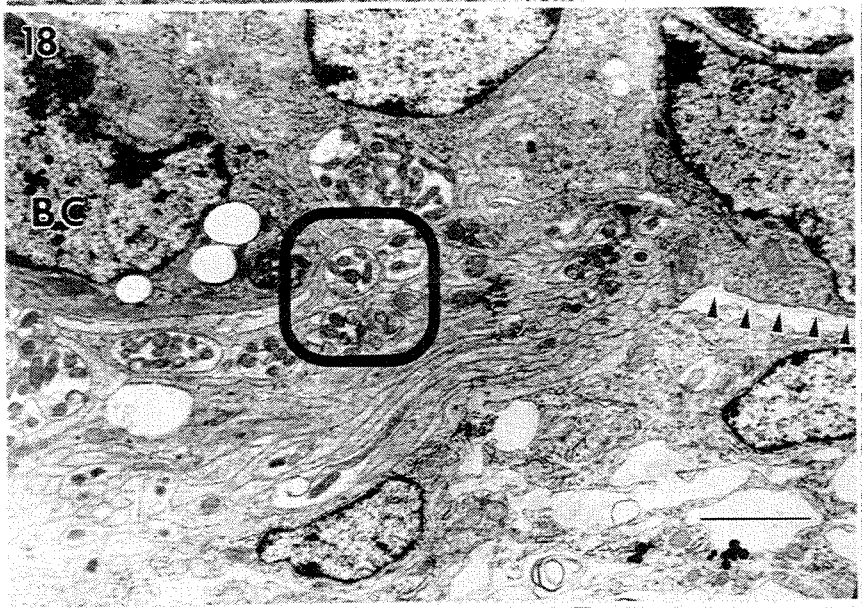
Figure 20. The region of the olfactory nerve between the olfactory epithelium and the telencephalon contains numerous nonmyelinated axons (lower left), which appear as typical olfactory nerve axons. An electron lucent varicosity is located directly below the olfactory epithelium is surrounded by brackets and enlarged in figure 21.

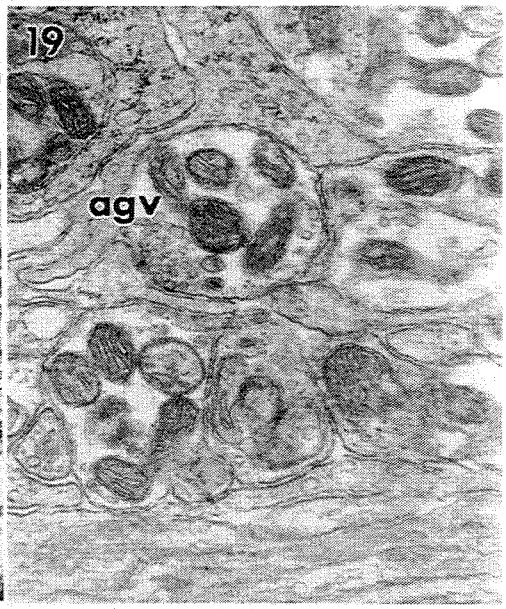
Figure 21. The varicosity contains vesicles of various sizes, mitochondria, and membranous material. It is adjacent to the basement membrane (arrowheads). A portion of the basement membrane (small thin arrow) appears to incorporate the region with the varicosity. .

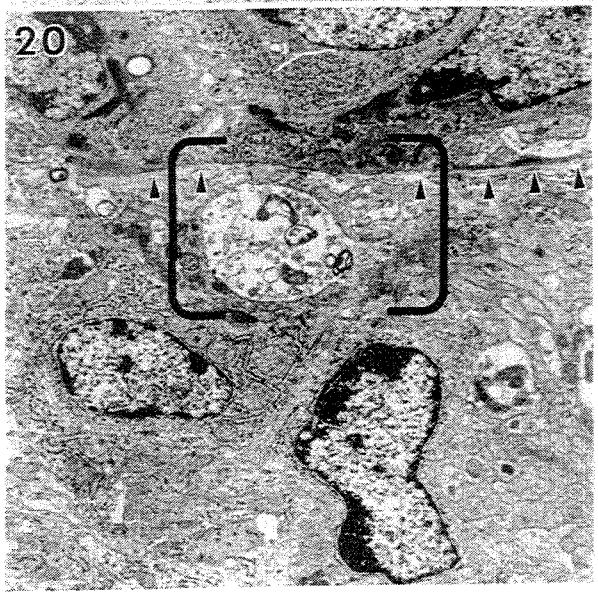
Figure 22. The stage 16 prolarval telencephalon contains synaptic contacts. The contacts are characterized by clusters of agranular synaptic vesicles (agv), parallel membrane apposition, postsynaptic density (large arrow), The post synaptic contact is probably axodendritic since the postsynaptic profile contains ribosomes. A less mature contact in the upper portion of the micrograph has a less pronounced postsynaptic density (small arrow). Bar is 0.5 μm.

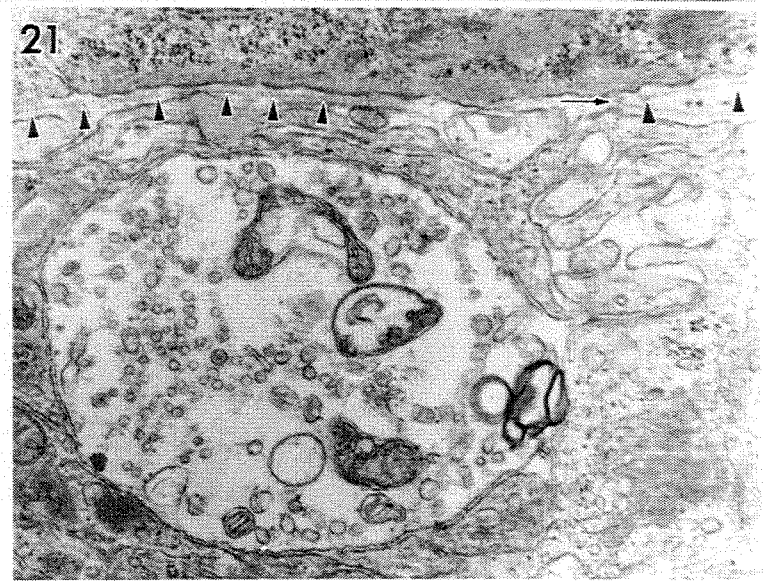












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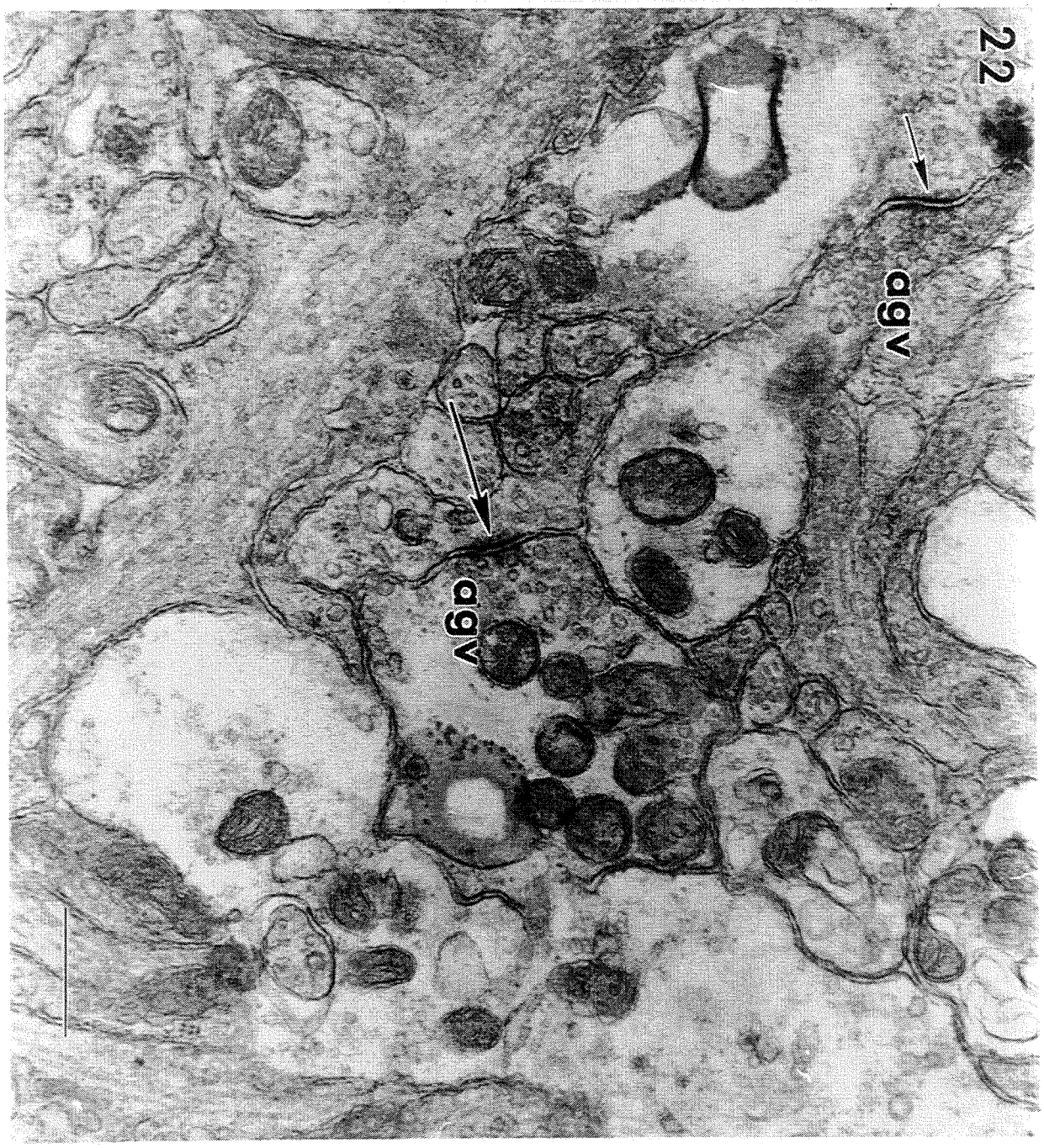

photo F

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3. **Ammocoetes**

3.1 Morphometric analyses of the olfactory organ. The results of our morphometric analysis of the olfactory organ in ammocoetes indicate the extent of olfactory organ development, and serve as a basis for comparison for later stages, for experimental treatments, and for electrode placement for future electrophysiological experiments. We chose one year old larvae (5 to 6 cm) from Hammond Bay (courtesy of Mr. Bill Swink). The nasal cavity appeared shallow enough for the passage of stimulatory compounds during electrophysiological experiments, after minimal surgery to remove a skin flap covering the olfactory organ. Morphometric analyses were determined with an image analysis system purchased with funds from the GLFC (Nikon Labophot microscope and a computer assisted with *JAVA Video Analysis Software (Jandel Scientific)* and *TARGA Frame Grabber (Truevision)*). A video camera and monitor were borrowed from the University of Windsor. The following values were calculated by image analysis:

(A) ***ORC density***, the number of ORC per 100 μm of olfactory epithelium. The number of ORC olfactory knobs were counted at the LM level (X1000). The following criteria were used to identify ORC at the LM level: ciliated olfactory knob, basal bodies at the outer margins of the olfactory knob, and a palely stained distal dendritic cytoplasm.

(B) SA_T, the total surface area of olfactory epithelium within an ammocoete that contains ORC.

(C) Total number of ORC.

1. Serial sections were taken of the nasal cavity. The ultramicrotome (*RMC 6000XL* is able to prepare sections of uniform and precise thickness, and we assumed each section was 1 μm wide.

2. Identified regions with olfactory epithelium (followed the criteria for olfactory epithelium).
3. Counted total number of sections with olfactory epithelium.
4. Using *Java Video Analysis*, we measured the length of olfactory epithelium from every tenth section.
5. Taking into account the above, the following formula was used to calculate the total number of ORC.

Calculation of total number of ORC in a lamprey:

$$\text{Total \#ORC} = \text{SA}_\text{T} \times \frac{\#ORC}{mm^2}$$

Surface Area = (LENGTH OF OLFACTORY EPITHELIUM) X (# OF SECTIONS WITH THAT LENGTH)

SA = L X W

L = randomly measured length of olfactory epithelium in one set. (μm)
W = width of section (always = 1μm)

A set of 8 to 10 sections contained the same length of olfactory epithelium.

Surface area total (SA_T) is the total surface area of the nasal cavity that is covered by olfactory epithelium.

$$\text{SA}_\text{T} = \sum_{i=I}^{M} \text{SA}_i$$

where *M* = total is # of sets of surface area that were summed to calculate the total surface area
i = individual number of sets of SA that were used to calculate the total surface area.

Calculation of ORC density (no. of ORC per mm^2)

Table 3.1 ORC density values were calculated from three ammocoetes that were 5.5 cm long. ORC density was calculated from twenty five regions in the olfactory epithelium of each ammocoete.

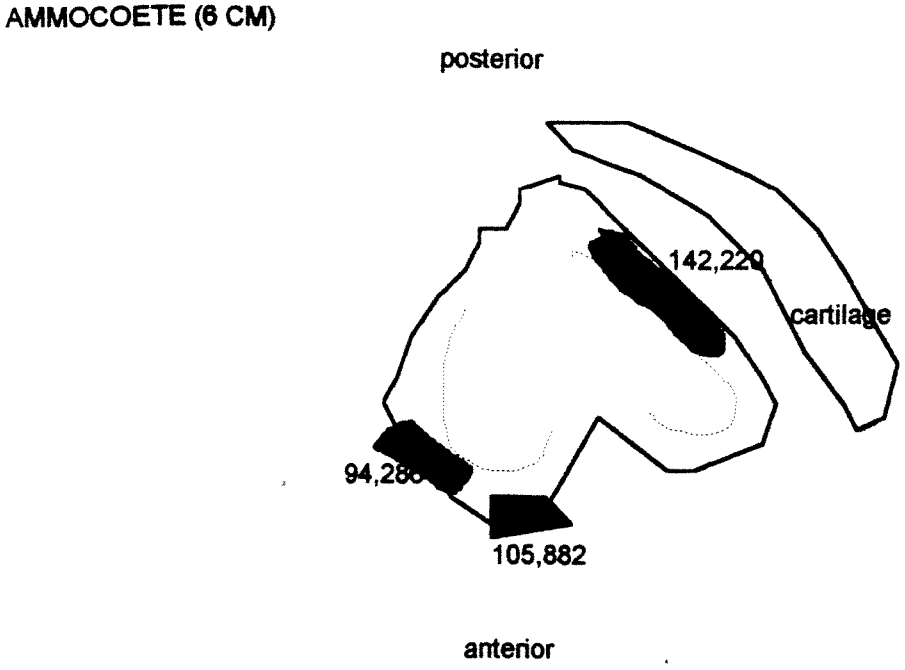
Specimen number	Range of ORC density per 100 μm	Average ORC density per 100 μm	ORC density, per mm^2 of surface area
HB55A	10 to 21	16.3	162,644
HB55C	7 to 20	12.5	102,778
HB55B	7 to 15	10.3	125,000

In the 5.5 cm ammocoetes, the average ORC density was 13 ORC/100 μm length of olfactory epithelium, and 130,141 ORC per mm^2 of olfactory epithelial surface area.

In an ammocoete that was 5 cm long, the surface area with olfactory epithelium was 0.07 mm^2, the ORC density per mm^2 was 102,778, and the total number of ORC was 7,194.

The following diagrams illustrate regions were ORC density was determined. The areas were chosen because the ORC were clearly discernible in a serial sequence of 30 sections for quantification by image analysis. We could resolve smaller regions with ORC, These also had the same ORC density.

Fig. 3.1 ORC density per mm^2 surface area in three different regions of the olfactory mucosa of a 6 cm ammocoete.



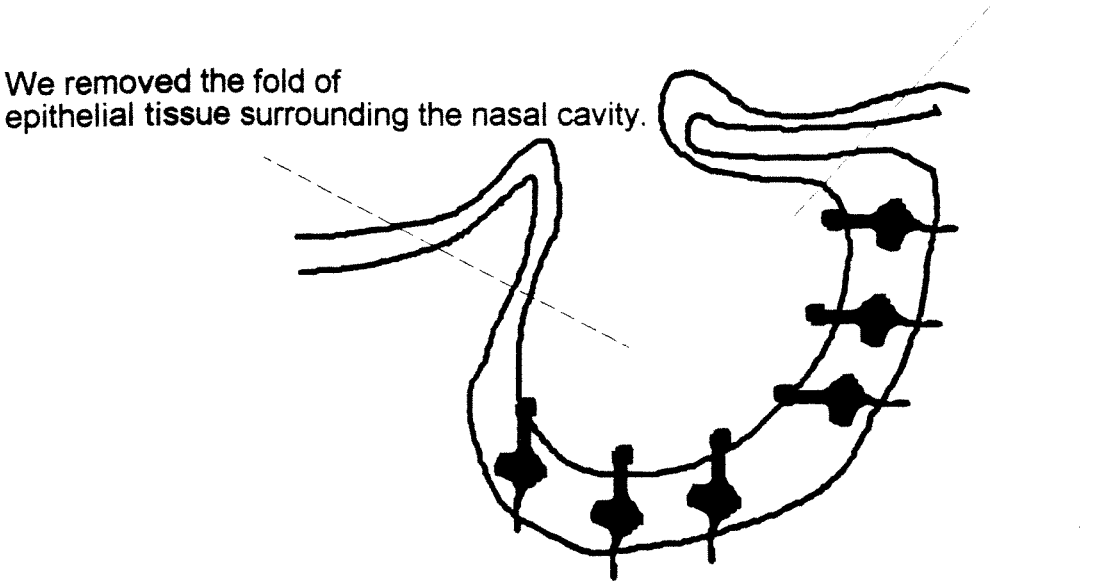
In the 6cm ammocoete, the ORC density was slightly higher deep within the olfactory organ, which is surrounded by cartilage; than the density values that are located more toward the periphery, which is not associated with cartilage.

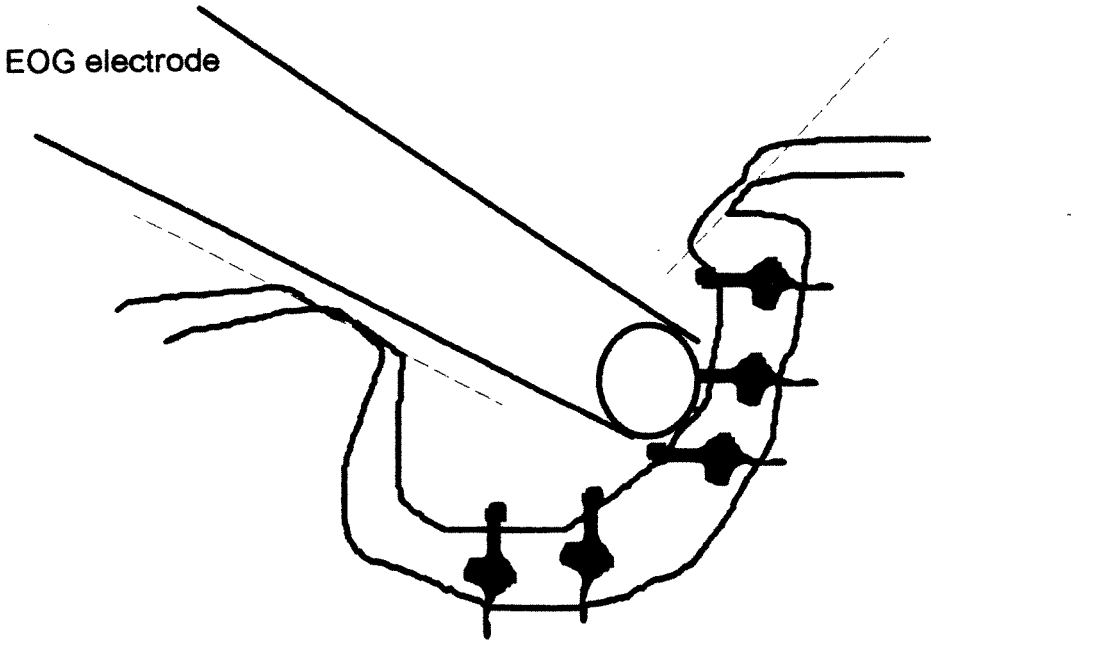
Table 3.2 A comparison of the total number of ORC in various species.

Sea lamprey		
Ammocoete (5 cm)	7,194	This study
Adult animals		
bull frog	3,900,000	Matsuzaki et al., 1980
Reeve's turtle	1,300,000	"
dog	220,000,000	Takahashi, 1979
Humans	10,000,000	"

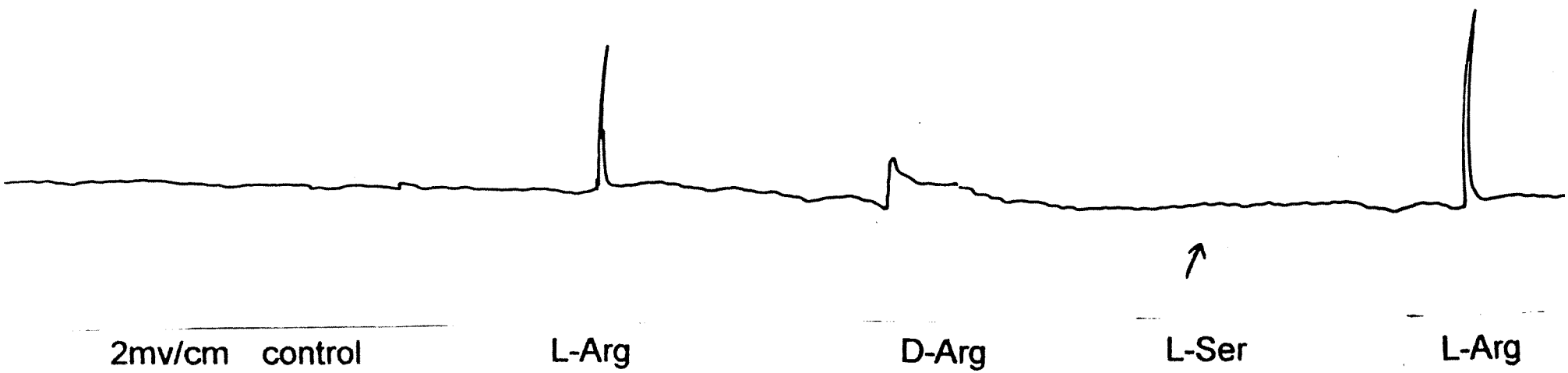
3.2 **EOG recordings** were initiated in the autumn of 1993, on the population of sea lamprey ammocoetes that were analyzed morphometrically. The recordings were made from the posterior, ventral surface of the olfactory organ, where the ORC density was highest (Fig. 3.2). The exact concentrations of stimulatory solutions was equal (stock solution of 10^{-5} M for each) is not known because of dilution effects from the experimental apparatus that was available to us. Since the apparatus was the same for all experiments, we assume that the dilution was equal for all the physiological recordings (Fig. 3.3).

The results show that L-arginine is a stimulatory amino acid. D-arginine is much less chemostimulatory, and L-serine is not chemostimulatory.





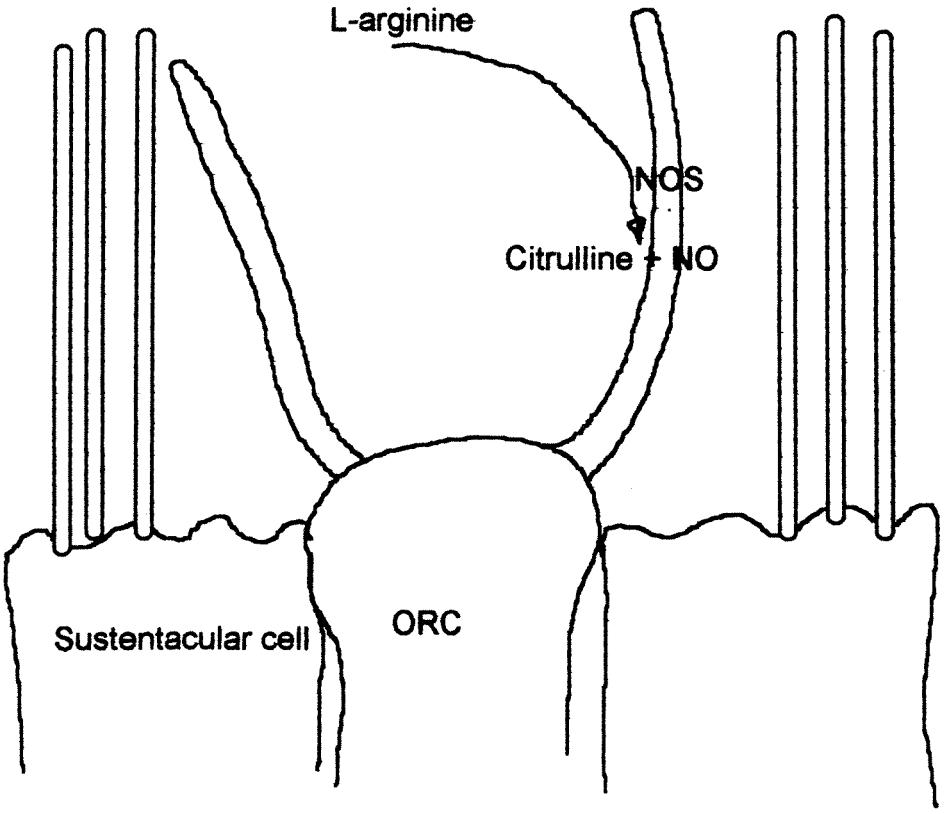
olfactory epithelium of 8 cm ammocoete


2mv/cm control
L-Arg
D-Arg
L-Ser
L-Arg

3.3 Staining for nitric oxide synthase in ammocoete olfactory mucosa

In larval sea lampreys, L-arginine was a potent stimulant when EOG responses were recorded from the posterior surface of the olfactory organ where ciliated olfactory receptor cells are located. We have investigated the possibility that the L-arginine response is mediated by nitric oxide (NO), a signaling molecule produced from L-arginine by the enzyme, NO synthase. N-monomethyl-L-arginine, a blocker of NO synthase competitively inhibited the EOG response. We localized NO synthase in the dendrites and olfactory knobs of olfactory receptor cells by light microscopic examination of tissue reacted by the NADPH diaphorase technique. Histochemical specificity for NO synthase was demonstrated by enhancing the labeling with L-arginine, calcium and calmodulin, and by diminishing the staining intensity following inhibition of the reaction with L-arginine and N^G-nitro-L-arginine. Our results suggest that in sea lamprey larvae, NO mediates olfactory receptor cell responses to L-arginine. We are currently using behavioural preference/avoidance studies with L-arginine and NO inhibitors. Further studies are to test arginine as an attractant to areas that are feeding areas, and potentially to block NO synthase in prolarvae that are moving from nests to feeding sites.

Fig. 3. 4 A model for the mechanism of transduction of L-arginine by ammocoete olfactory receptor cells. The stimulatory amino acid L-arginine binds onto a membranous receptor protein that has the enzymatic activity of nitric oxide synthase (NOS). The membrane permeable second messenger nitric oxide (NO) diffuses across the cell membrane where it activates intracellular mechanisms that open ion channels.



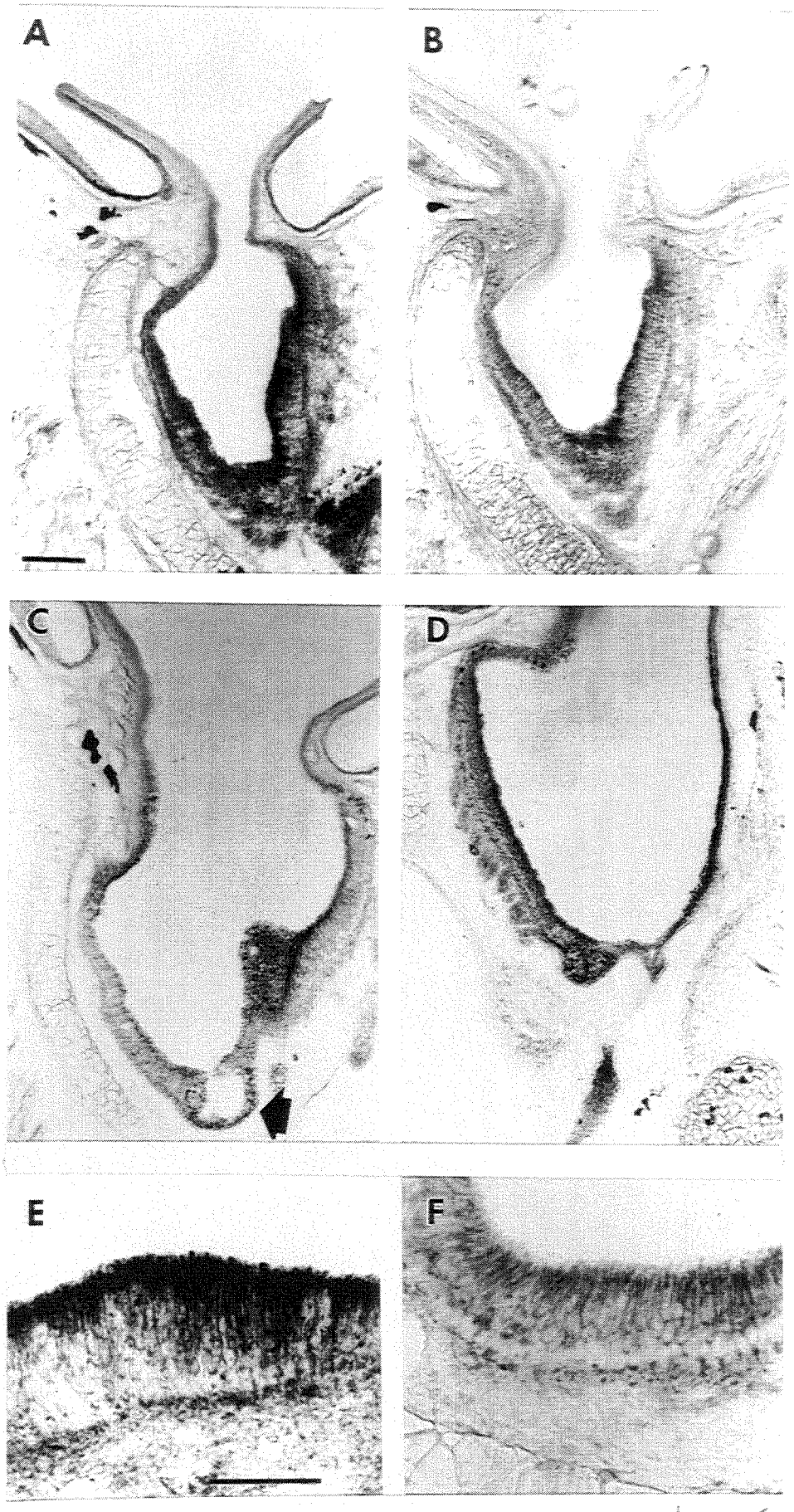


Photo 6

Fig. 3.5 NADPH diaphorase staining of the olfactory mucosa from ammocoete sea lamprey.

A. Control, a cross-sectional view The bar is 100 μm. The same magnification is in B,C and D

B. With Nω-nitro-L-arginine (10^{-6}M), a cross-sectional view

C. With Nω-nitro-L-arginine (10^{-5}M), a sagittal view, shows the accessory olfactory organ.

D. Nω-nitro-L-arginine (10^{-6}M), a parasagittal view

E. control The bar is 50 μm. The same magnification is in F.

F. L-arginine, 10^{-3}M

The olfactory organ of the sea lamprey, *Petromyzon marinus* L. during metamorphosis: Morphological changes and morphometric analysis.

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ABSTRACT:

During the metamorphic process of the agnathan vertebrate, the sea lamprey (*Petromyzon marinus*), the olfactory organ changes from being a relatively inconspicuous sac with small densely packed cells to a prominent lamellar structure with large distinct olfactory epithelial cells. The timing of the transformation is important in that it may reveal information about factors that regulate the development of the olfactory organ. Leach (1951) suggested that in the lamprey *Ichthyomyzon fossor*, the transfomation of the olfactory organ leads that of other structures. We have used scanning electron microscopy and light microscopy to examine the timing of the transformation of the olfactory organ and process by which it aquires specific adult characteristics. The scale of expansion of the olfactory organ during the seven stages of metamorphosis, is evident from doubling of the relative weight of the nasal sac, and of the surface area covered by olfactory epithelium (0.5 mm^2 to 1.1 mm^2). The total number of olfactory receptor cells almost doubled (43,248 to 76,084) and nonmyelinated nerve bundles became increasingly prominent in the lamina propria. The olfactory organ expanded during early metamorphosis (stages 1 and 2), remodeled during midmetamorphosis (stages 3 to 5), and the transformed olfactory organ expanded during late metamorphosis (stages 6 and 7). During the remodelling period specific structures formed: a nasal valve separating the nasal tube from the olfactory organ, lamellar folds lined by olfactory epithelium, and diverticuli of the accessory olfactory organ. The trough-like depressions between adjacent lamellae contained narrow cells and mitotic figures, suggesting that these were germinal areas. These results show that during metamorphosis, the larval olfactory organ precedes the expansion of the brain and expresssion of the peptide hormone GnRH which occurs during late

metamorphosis. This timing suggests that there may be a trophic relationship between the expanding olfactory nerve and subsequent transformation in the CNS.

INTRODUCTION:

During the early development of vertebrates, the olfactory epithelium grows from an embryonic placode, to an olfactory epithelium in a sac-like structure. In fish, such as salmonids the olfactory epithelium folds to a lamellar like structure forms shortly after hatching. In the sea lamprey *Petromyzon marinus* L., **a** representative of the most ancient class of vertebrates, the Agnatha, or jawless fish, **the formation of the lamellar structure occurs during metamorphosis from the larval to the juvenile stage. This sudden expansion and change in form is undoubtably influenced by specific regulatory mechanisms. The extent of the differences between the larvae and adults: lamellar folds, increased size of olfactory epithelial size, nasal valve, offers a model for investigating factors that regulate the development of the olfactory organ. The purpose of the present investigation is to** systematically describe changes that occur during metamorphosis of the olfactory organ. The timing of the transformation of the olfactory organ and process by which it aquires specific adult characteristics, serves as a preliminary study for future examination of the effects of regulators of metamorphosis on the expression of specific olfactory epithelial phenotypes. Leach ('51) observed that in *Icthyomyzon fossor*, the olfactory organ transforms during early metamorphosis.

Lampreys have a single dorsal nostril, which in adults, houses a large multilamellar olfactory organ (e.g. Kleerekoper, '71). Physiological (Li and Sorensen, '93) and behavioral (Kleerekoper and Mogensen '63; Teeter, '80) investigations have shown that the olfactory system in adult sea lampreys responds to chemostimulation by odorants. Our preliminary studies show that larval sea lampreys respond to chemostimulation (Zielinski et al., '94).

As in other vertebrates, the lampreys' olfactory receptor cells (ORC)

extend from a specialized region of the nasal sac, the olfactory mucosa (Thornhill. '67) onto the olfactory bulb (Schober, 1964). In the small nasal cavity of larvae, the olfactory mucosa is located on the caudo-lateral surface (Vandenbossche et al., 1993). A small indentation, the accessory olfactory organ extends from the ventral surface of the olfactory organ. In adults, a valve separates the nasal tube from the olfactory organ which is a relatively large capsular structure, with many epithelial folds (Kleerekoper and van Erkel '60). The ORC are bipolar neurons, with dendritic tips (olfactory knobs) that bathe in the mucus of the nasal cavity. The nuclei are located in the olfactory epithelium and the axons form synapses in the olfactory bulb. In lampreys, the ORC are adjacent to ciliated sustentacular cells (SC), elongate cells that span the width of the epithelium with nuclei near the luminal surface. The ORC density (number of ORC per 100 mm length of olfactory epithelium) varies considerably during the life cycle of sea lampreys. In the larval period the density is high (20 ORC/100 mm) but drops in juveniles to 10 ORC/100 μm (VanDenbossche et al., '94). In adults, diverticuli of the accessory olfactory organ fill the caudal space of the olfactory mucosa behind the folds of the olfactory epithelium and the duct-like opening of the accessory olfactory organ is posterior to the olfactory organ.(Leach, '51; Kleerekoper, '69). The function of this accessory organ is unknown, it may be sensory or glandular (reviewed by Hagelin and Johnels, '55).

Larvae pass into the adult form during a highly synchronized metamorphic process with seven distinct stages that are associated with dramatic changes in both external and internal features (Youson and Potter, '79; Youson, '80; 88). Modifications of the nostril during metamorphosis involve a reduction of the flaring and production of an elevated, tube-like channel with a serrated edge (Youson, 1980; Manion and Stauffer 1970). Early descriptions by Lubosch ('05)

and Immamura ('28) and later by Leach ('51) have provided some insights into the details of transformation of the larval lamprey olfactory organ during metamorphosis. Most recently , Youson (';80) described DNA synthesis in the connective tissue and epithelium of the early stages of metamorphosis of the anadromous sea lampreys. However there has never been a detailed quantitative analysis or electron microscopic description of the olfactory organ during metamorphosis. In this study we used scanning electron microscopy and light microscopy of semi-thin epoxy sections to follow the dramatic development of olfactory organ through metamorphosis of the sea lamprey *P. marinus*.

MATERIALS AND METHODS:

Sea lamprey ammocoetes were captured from the Great Chazy River in the Lake Champlain region, New York, U.S.A. between May 18-24, '92. All animals were transported to holding tanks at the Scarborough Campus, University of Toronto, Scarborough, Canada. and were kept at 15-21 °C in a dechlorinated, flow-through water facility. The animals were fed baker's yeast once per week. The animals that were used for this study began metamorphosis in July '92, and from 3 to 10 lampreys of each of the metamorphic stages, (1 - 7), and post metamorphic sea lampreys (PM) were obtained between July '92 (M1) and February '93, (PM). The original research reported herein was performed under guidelines established by the Canadian Council of Animal Care. Lampreys were anaesthetized with tricaine methone sulphonate (MS222), and staged by the method of Youson and Potter, ('79). The lampreys were weighed and measured, then decapitated around the third branchiopore region and immediately fixed by immersion into a modified Karnovky's fixative (Zielinski et al., 1988).
The nasal sac was carefully dissected and weighed. The olfactory organ including the nares and nasal tube was dissected as one piece for larval, M1 and M2 and PM specimens. The larger, M3 to M7 samples of were cut sagittally down through the middle of the nares.

For scanning electron microscopy (SEM) the tissue was immersed into 1% osmium tetroxide buffered with cacodylate buffer (pH 7.4) and dehydrated in ethanol. Specimens were mounted on metal stubs, sputter coated with gold and viewed with a Hitachi S-510 scanning electron microscope at the Harrow Research Station, Agriculture Canada.

Light microscopy (LM) was used to identify the olfactory epithelium throughout the different stages of metamorphosis. For LM analyses, tissue that had been immersed into modified Karnovky's fixative, was osmicated and dehydrated, then immersed in propylene oxide and embedded into epoxy resin. Sections with a thickness of 1 μm were made with a RMC ultramicrotome and stained with Toluidine blue stain.

All morphometric analysis was carried out using a Nikon Labophot microscope with a Hitachi CCTV camera and a computer assisted with JAVA Video Analysis Software (Jandel Scientific) and TARGA Frame Grabber (Truevision). To determine the relative change in ORC density for each stage, the number of ORC per 100 μm length of olfactory epithelium was determined from three specimens at each developmental stage (except M2, 2 specimens were available). The number of ORC olfactory knobs were counted at the LM level (X1000). The following criteria were used to identify ORC: a ciliated olfactory knob, basal bodies at the outer margins of the olfactory knob, and a pale-stained distal dendritic cytoplasm.

The total number of ORC was obtained from one M2 and one M7 specimen to estimate overall increase in the number of ORC during metamorphosis. The analysis was made from olfactory organs that were serially sectioned at a thickness of 1 μm. The surface area included only regions that

contained olfactory epithelium (with ORC). Java Video Analysis was used to measure the length of the olfactory epithelium, and to record ORC tallies. The following formula was used to calculate the total number of ORC in a lamprey:

$$\text{Total \#ORC} = SA_T \times \frac{\#ORC}{mm^2}$$

Surface area total (SA_T) is the total surface area of the nasal cavity that is covered by olfactory epithelium.

$$SA_T = \sum_{i=I}^{M} SA_i$$

SA_i = a set of 8 to 10 sections that contain the same length of olfactory epithelium.

M = total # of sets of surface area that were summed to calculate the total surface area.

i = individual number of sections that were used to calculate SA_i (usually 8 to 10 sections)

$SA_i = L \times W$

L = randomly measured length of olfactory epithelium in one set. (μm)

W = width of set (i.e., the number of sections in the set, since one section is always 1μm thick)

The number of ORC per mm^2 was determined from serial sections by the disector method (Gundersen et al., '88). Thirty, 1 μm serial sections were cut and stained. The ORC were tallied from the same region on each serial section. The value was then extrapolated to a surface area of 1 mm^2.

RESULTS:

During metamorphosis the olfactory organ grew dramatically and transformed, as some structures were reorganized and others were added. The growth of the nasal sac was a continual process, between M1 and PM, the percent relative weight doubled (Fig. 1). Changes in the overall structure of the nasal cavity and olfactory organ were evident by scanning electron microscopy (Fig. 2). In larval ammocoetes, the nasal tube was J-shaped, with the olfactory epithelium on the caudolateral surface (Fig. 2A). A midline partial septum separated the nasal cavity into two symmetrical regions ventral/caudad of the nasal tube (Figs. 2A) previously observed in *I. fosor* (Leach, '51) and *P. marinus* (Vandenbossche et al. '94). A flared ridge of tissue that surrounded the naris was prominent in larvae (Fig. 2A), shortened during early metamorphosis (Manion and Stauffer, 1970; M1 and M2; Fig. 2B) as the olfactory organ deepened and expanded. At M4, a small infolding at the junction of the nasal tube and the olfactory organ was evident and the surface of the olfactory organ had short lamellar folds (Fig. 2C). By M5, there was a prominent nasal valve is at the junction of the nasal tube and the olfactory organ and the lamellar folds were prominent (Fig. 2D).

Changes associated with the fomation of lamellae were evident by light microscopy. Duirng M1 and M2, as the olfactory organ expanded, the cellular organinzation of the olfactory mucosa remained as that observed in larvae. The thickness of the olfactory epithelium was uniform to the thickness of the larval olfactory eptihtelium, the nerve bundles were uniformly stained small circular bundles and blood vessels were scattered throughout the lamina propria (Figs 3A-D). The cartilage that surrounded the olfactory mucosa contained chondrocytes, with small nuclei (Fig.3C). Several changes were evident by M3, as the lamellae started to form. The olfactory epithelium thickened. with the

addition of nuclear layers, the olfactory nerve fascicles widened and were crowded with vacuolated fibers, blood vessels were aligned along the basement membrane (Fig. 3E). The chondrocyte nuclei were large and round and extracellular matrix had accumulated between the chondrocytes (Fig. 3E). From M5 to PM, the olfactory epithelium narrowed, nonmyelinated nerve fascicles were even more prominent (Fig. 4). Diverticuli of the accessory olfactory organ became prominent in the lamina propria of M4 lamprey and in later stages (Fig. 5). In *I. fossor*, which transforms directly to sexually mature stage rather than trophic as in *P. marinus*, the accessory olfactory organ in the late ammocoete stage was prominent where it attached to the nasal sac, during early transformation branches are present (leach, '50), late metamorphosis, essentially adult, secretions of the accessory organ are evident. tilney (19?) the accessory olfactory organ in P. marinusis not as well developed as in I. fossor.which is phylogenetically more primitive. It is less well developed in the predatory forms of the lampreys than in the predatory forms. It may be glandular and clear away muddy water that has been taken up into the nasal cavity. It may be endocrine, Woerdeman suggested that it represent a a division of the anterior hypophysis and represent early gonadotrophic activity these are probably those that we observed from M4 onward. These gland-like structures were located in the caudal space at base of the lamellar folds as described by Hagelin and Johnels ('55) in adult specimens of *Lampetra planeri* and *L. fluviatilis*. At M7 and PM, the diverticula were abundant, some were immediately adjacent to the base of the olfactory epithelium (Fig. 5). The diverticuli, were acinar-like arrangements of simple low columnar epithelium, and were surrounded by blood cells and some appeared immediately adjacent to nonmyelinated nerve bundles (Fig. 5A). Some diverticuli, which may be developing regions, lacked a lumen, and had cells with little cytoplasm (Fig. 5C).

A closer view of the diverticuli revealed two types of epithelial cells previously identified by Hagelin and Johnels ('55). The first type was large with a pronounced nucleus and patches of palely stained areas within the cytoplasm (Fig. 5B, black asterisk), and cilia that extended into the lumen. The second type existed on the outer edge of the diverticulum, It was smaller and more darkly stained relative to the first (Fig. 5B, white asterisk).

DISTRIBUTION OF ORC. The ORC were not localized in the anterior region of the nasal cavity, and on lateral lamellae there are very few ORC. The distribution that was shown in figure ?? are from the caudo-ventral lamellae. The crests of the olfactory lamellae did not have ORC.

MORPHOLOGY OF OLFACTORY RECEPTOR CELLS AND SUSTENTACULAR CELLS. There are several morphometric changes in the olfactory epithelium during metamorphosis. In larvae, the ORC olfactory knobs and SC, were narrow (approximately 2 μm wide), and the apical region of the olfactory epithelium was tightly packed with cells (Fig. 6A). By M2, the SC appeared to be broader and the ORC were more widely spaced and the mitotic figures of unidentified cells appear in the apical region of the olfactory epithelium (Fig. 6B), reflecting the rapid growth at this stage that precedes the formation of olfactory lamellae. As metamorphosis progressed, the cells of the olfactory epithelium along the slopes of the olfactory lamellae became increasingly wider and more widely spaced (Fig. 6C-E). In M7 and PM lampreys, the supranuclear regions of the ORC and SC lengthen and the nuclear layer is relatively narrow (Fig.6C-E). Before the formation of olfactory lamellae (larvae to M2), ORC density was homogenous along the entire olfactory epithelium. During early metamorphosis, as the olfactory organ enlarged, the ORC density decreases (Fig. 7). With the formation of lamellae at M3, the ORC density becomes greater in the troughs between adjacent lamellae, compared ORC density to lamellar slopes (Fig. 10).

The troughs, which contain densely packed, small cells may be areas of cell proliferation during this period. By M6, both areas had relatively low ORC densities, and the average ORC density remaind more or less stable after M5 (Fig. 10,11). The values for ORC density per unit surface area decreased from 84,800 ORC per mm^2 at M2 to 69,167 ORC/mm^2 at M7. From M2 to M7, the surface area of the olfactory organ that is covered by olfactory epitheium doubled from 0.51 mm^2 at to 1.1 mm^2. The total number of ORC increased from 43,248 ORC at M2 to 76,084 ORC at M7. Therefore, during metamorphosis, when both the surface area of the olfactory epithelium, and percent weight of the nasal cavity to the total body weight double, and ORC density drops, the total number of ORC almost doubles. The proliferation is reflected by lamellar expansion, germinal regions in the troughs between the lamellae and the expasion of the olfactory nerve. The metamorphic process appears to have three major periods (Table 3). From M1 to M2, the larval-like olfactory organ expands, remodeling occurs during M3 to M5, and the transformed structure enlarges during M6 and M7.

Figure 1. Relative weight of nasal sac

Percent wet weight of nasal sac (which includes naris and nasal tube) per total body weight o

the lamprey during metamorphosis

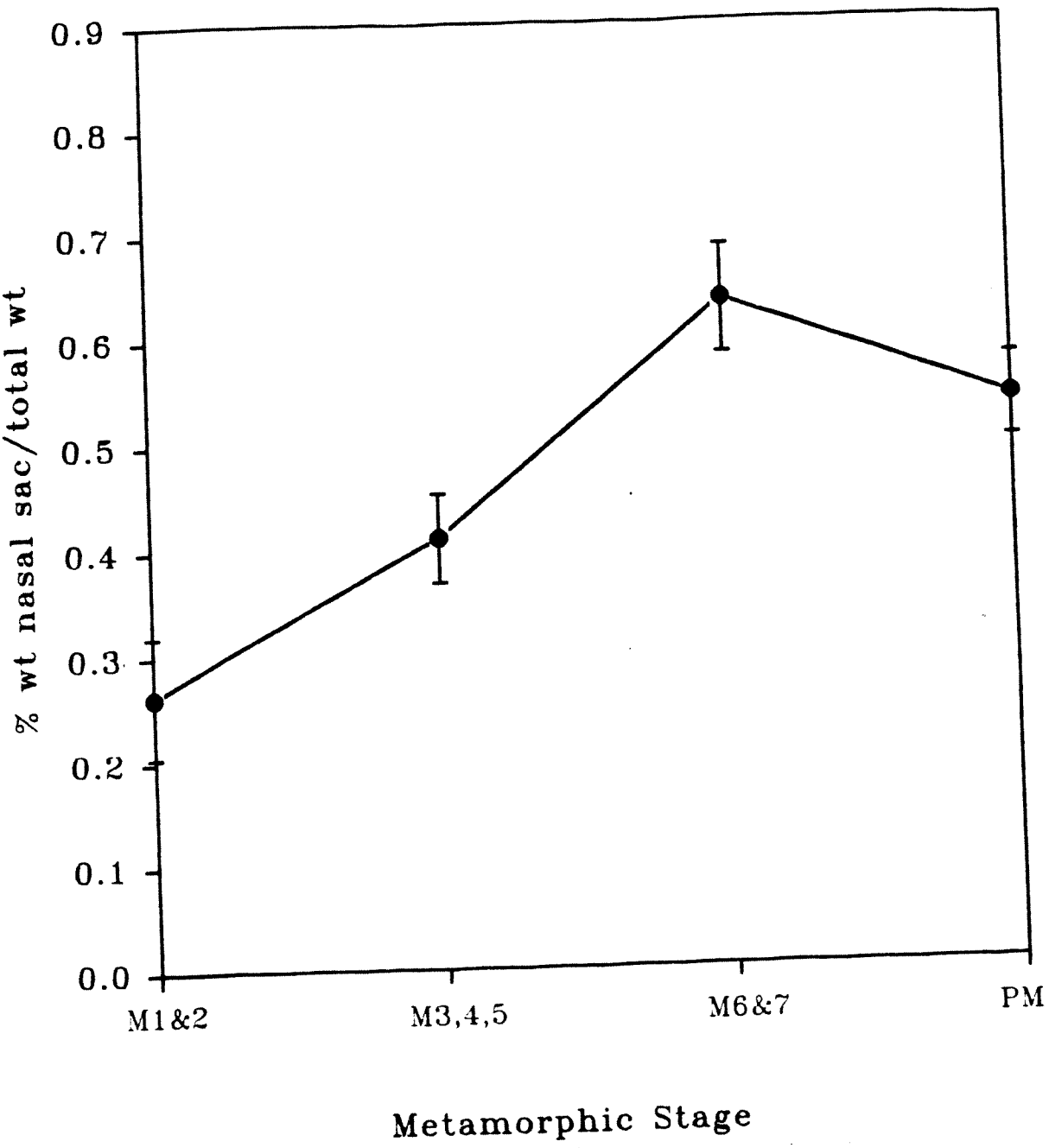
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
% wt nasal sac/total wt
M1&2
M3,4,5
M6&7
PM
Metamorphic Stage

Figure 2. Scanning electron micrographs of the nasal sac from larvae and from metamorphic stages 3, 4, and 5 (M3, M4 and M5).

A. The nasal tube in a 10 cm ammocoete is J-shaped, with the external nostril (naris) located on the dorsal surface of the larvae. A tall ridge of tissue surrounds the naris (white arrow). Lining the ventro/caudal bend of the nasal tube (nt) is the olfactory epithelium (indicated by stippling). Adjacent to the olfactory epithelium is the partial septum (arrowhead). Large fascicles of muscular tissue (M), viewed as cross-sections, extend rostral to the nasal tube. Small pieces of cartilage (c) are located behind and below the nasal sac. The brain (br) is located in the lower left portion of the micrograph .

B. In M3, the nasal tube (nt) appears perpendicular to the small olfactory lamella (L) and is visible on the dorsal surface of the olfactory organ. The ridge of tissue that surrounds the naris is short (white arrow).

C. This view from M4 shows the lamellae folds (L) on the lateral walls of the developing olfactory organ. A small ridge of tissue (white arrow) representing the early developing nasal valve in M4.

D. The olfactory organ of M5 possess 6 clearly visible lamellar folds (L). A nasal valve (nv) is located between the nasal tube (nt) and the entrance of the olfactory organ.

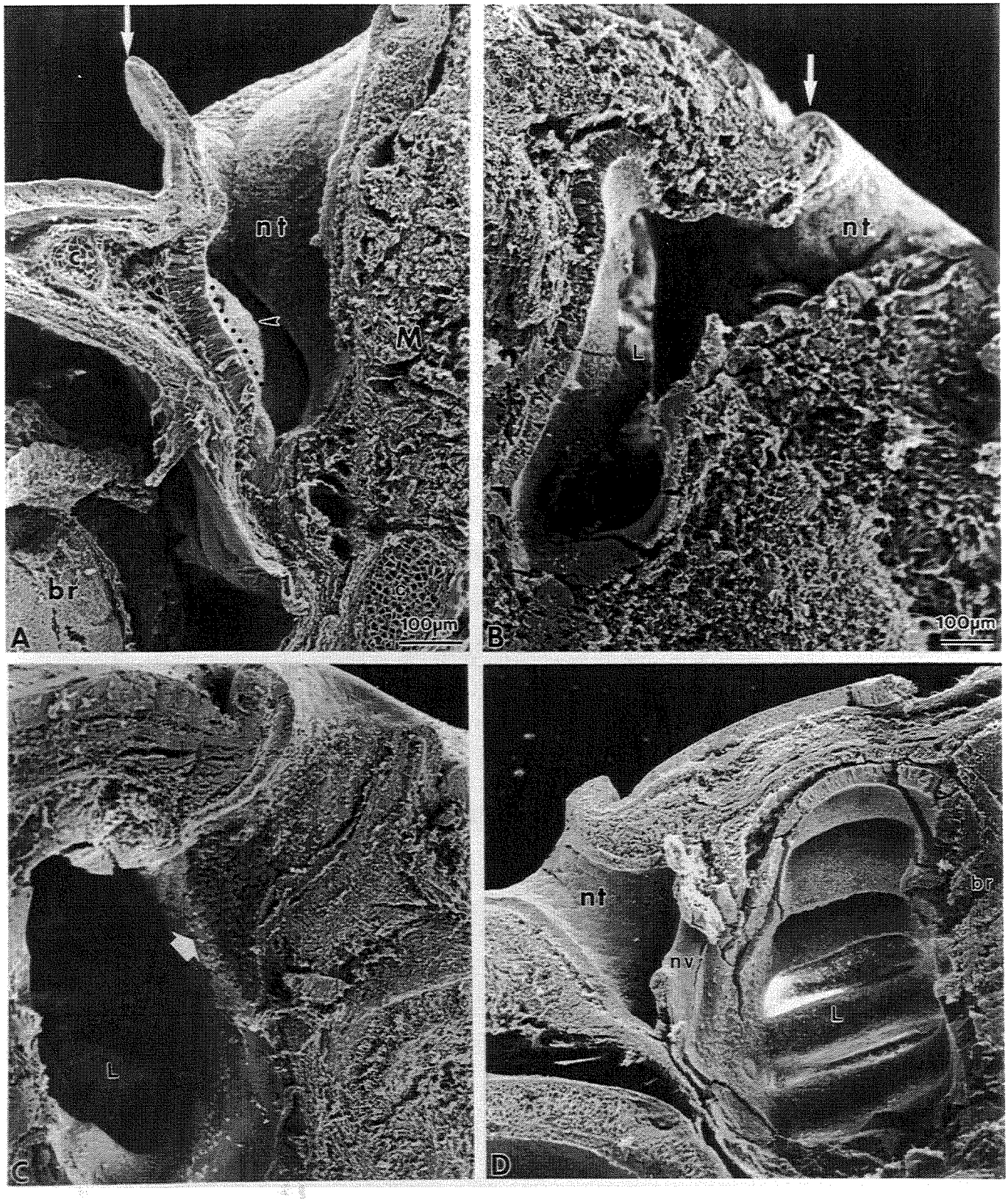

photo H

Figure 3. Low power scanning electron micrographs of the olfactory organ from metamorphic stages 5 and 7 (M5 and M7), and high power views of the olfactory mucosal surface of metamorphic stages 3 and 6 (M3 and M6).

A. There are 8 lamellae (L) visible in the lateral portion of the olfactory organ at M7. The nasal valve (nv) separates the olfactory organ from the nasal tube (nt).

B. The surface of the olfactory lamellae (L) in a M5 animal is densely covered by cilia. Membranous material lies on portions of the lamellar surface.

C. The high power scanning electron micrograph taken from the transitional area between olfactory and nasal tube epithelium in a M3 animal shows olfactory knobs of olfactory receptor cells (ORC). Short cilia (arrowheads) protrude from the lateral edges of the olfactory knobs. Short microvilli (mv) are visible in the lower right portion of the micrograph. Mucus and microvilli cover portions of the surface.

D. In the center of a lamella, long cilia protrude from ORC of a M6 sample. Some extend from the basal surface of the olfactory knob (outlined by arrowheads). The cluster of upright cilia in the upper left area of the micrograph are from sustentacular cells. Spread out intermittently over the cilia are mysterious stellate structures (*).

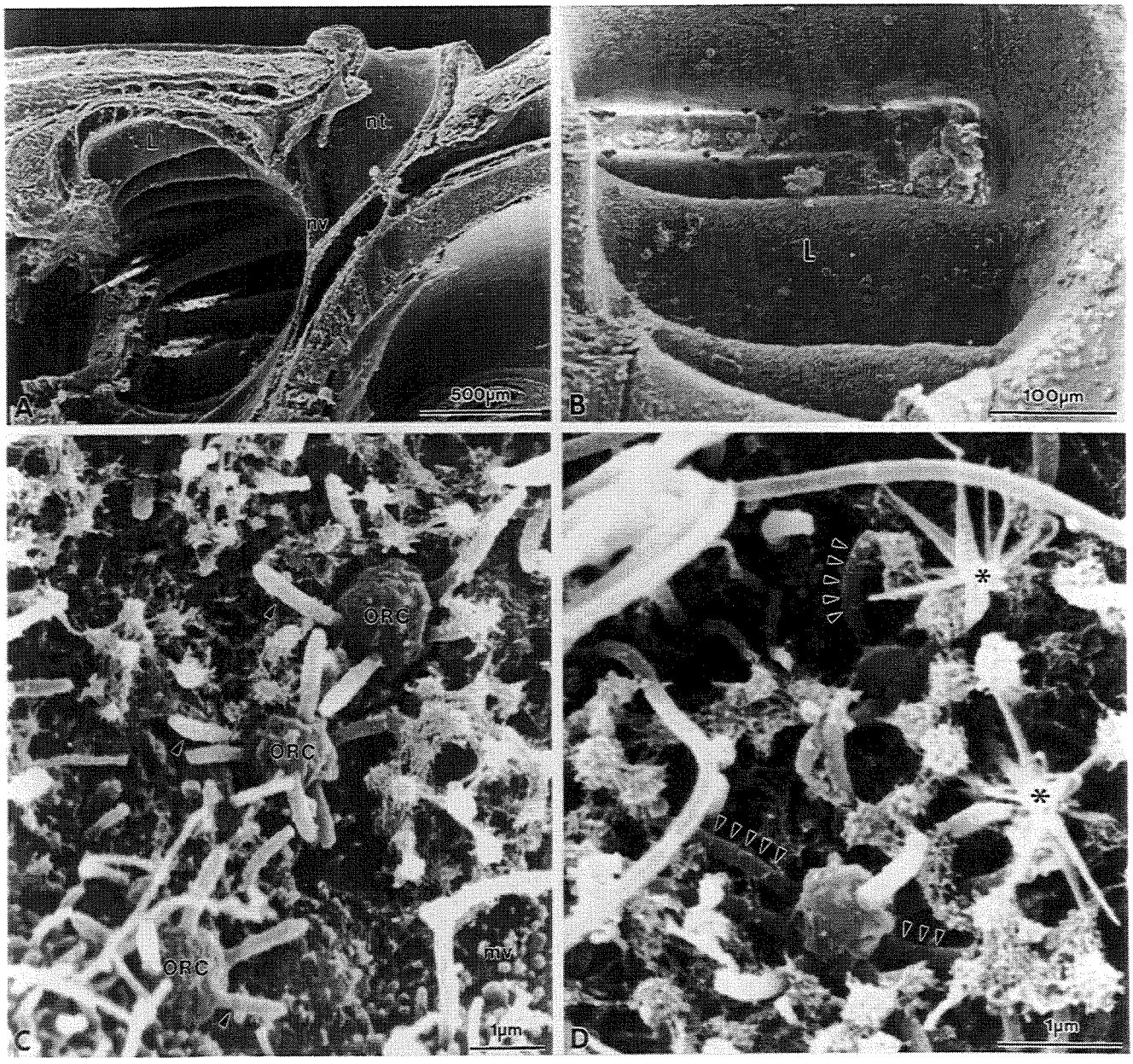

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Figure 4. A light microscopic survey of the olfactory organ from ammocoete through to stage 3 (M3) of metamorphosis.

The length of micrometer bars are in equal in figures 4 A, B, D and E.

A. A low power light micrograph of a cross section of an entire nasal sac from an ammocoete. A partial septum (arrowhead) divides the nasal sac into two distinct areas. The posterior regions of the nasal sac (indicated by stippling) is olfactory epithelium, recognizable by the pseudostratified columnar arrangement.

B. A cross-sectional view of the nasal sac from M1 shows bilateral symmetry with a very low ridge (arrowhead) at the saggital midline. The nasal sac has a slightly asymmetrical shape. The olfactory epithelium (indicated by stippling) is located on the posterior surface. Two semi-circular strips of cartilage are located posterior to the olfactory epithelium. Small circular nerve bundles are located between the olfactory epithelium and the cartilage. This area is enclosed by brackets and shown at higher magnification in figure 3C.

The length of micrometer bars are equal in figures 4C and F.

C. This micrograph shows the composition of the olfactory mucosa during M1. The olfactory epithelium (OE) clearly shows the pseudostratified epithelial arrangement. Blood vessels (bv) of various sizes are scattered throughout the lamina propria and small circular nerve bundles (small curved arrows) are arranged in a row. The largest nerve fascicles are located between the two pieces of cartilage (c). The chondrocytes have small fusiform nuclei (small straight arrows).

D. During M2, the partial septum (arrowhead) is a low ridge, giving the nasal sac a scalloped appearance. The cartilage is a semi-circular ring encasing the posterior portion of the nasal sac. The length covered by olfactory epithelium is outlined with stippling.

E. This micrograph shows a cross section of half of the nasal cavity in M3. The area closest to the region of the partial septum at the midline of the nasal sac is shown with an arrowhead. The length of the posterior surface of the nasal sac that is covered by olfactory epithelium is

Figure 4 continued

indicated by stippling. The olfactory epithelium has a lamellar fold (L) on the posterior surface. An adjacent section of the area enclosed by brackets is shown at high power in figure 4F.

F. The olfactory epithelium (OE) of M3 has many layers of nuclei, and vacuolated spaces between the nuclei. The nerve fascicles (curved arrows) appear large and vacuolated. Blood vessels (bv) are lined up against the base of the olfactory epithelium and dispersed throughout the lamina propria. Chondrocytes within the cartilage (c) have large, round nuclei (small straight arrows).

G. The high power micrograph shows the homogenous stain of a nonmyelinated nerve fascicle in lamina propria during M2.

H. At M3, there are many vacuoles (arrows) in the fibers of the nonmyelinated nerve bundle within the lamina propria.

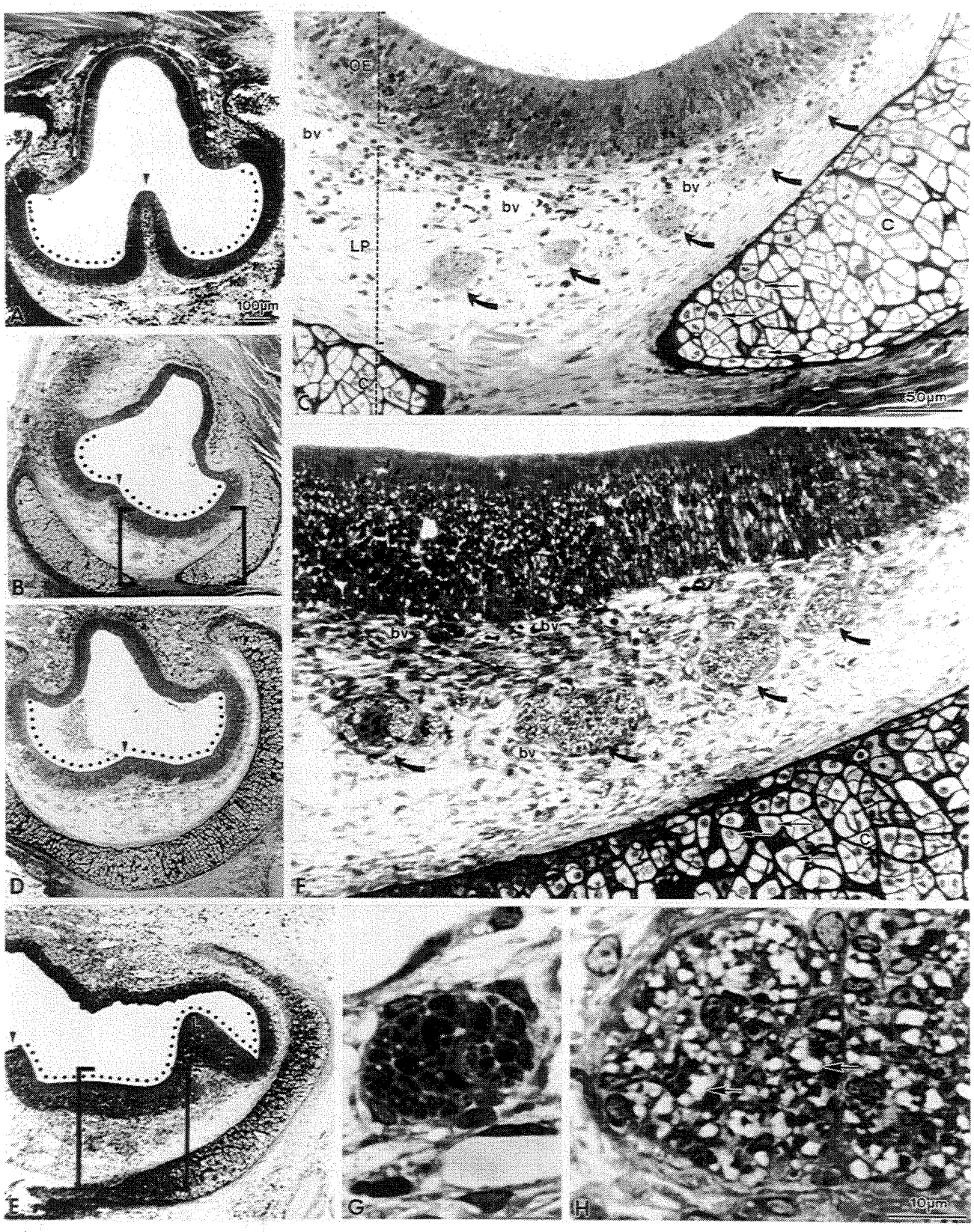


Photo J

Figure 5. Light micrographs of the olfactory mucosa in stage 5 (M5) and post metamorphic (PM) sea lampreys.

The length of micrometer bars are equal in figures 5A and C.

A. The lamellar development is seen in this cross-sectional view of half of the nasal sac in M5. The partial septum is indicated by the arrowhead. The brain (br) is located posterior to the olfactory epithelium. Two lamellae (L) are clearly visible; the lamellar structure on the extreme left was sectioned obliquely and appears as an enclosed cavity. The nerve bundles in the median part of the animal appear to be large. The cartilaginous ring (c) appears thick at the posterior end and narrows outwards to the anterior side as it encapsulates the nasal organ. A small diverticulum (a) of the accessory olfactory organ is visible in the lamina propria.

The length of micrometer bars are equal in figures 5B and D.

B. The high power micrograph shows the composition of the olfactory mucosa. Blood vessels (bv) are lined up against the base of the olfactory epithelium. The fascicles of the nerve bundles appear large and have a few vacuolated areas.

C. A section from the basal portion of the olfactory organ of a PM lamprey. The septum (arrowhead) completely divides the olfactory organ. Outer lamellae appear to join in this basal region and form a septum-like structure (arrowheads). The anterior and posterior portions of lamellar-like structures on the periphery (*) are in close apposition. Diverticula (a) of accessory olfactory glands are located at the caudal borders of the folds of the olfactory epithelium. This area is enclosed by brackets and shown at higher magnification in figure 5D.

D. The higher power micrograph shows the large size of the nonmyelinated nerve fascicles. The diverticula (a) of the accessory olfactory organ are in close proximity to nerve fascicles. The diverticula always appear in the lamina propria between the olfactory epithelium and the brain (br).

Photo K

Figure 6. Light micrographs of the accessory olfactory organ in metamorphic stages 4 and 7 (M4 and M7), and in post metamorphic (PM) sea lampreys.

The length of micrometer bars are equal in figures 6A and C.

A. A low power cross-sectional view of the lateral portion of the olfactory organ during M4. The arrowhead points to the partial septum. Three diverticular structures of the accessory olfactory organ (a) are located adjacent to the basal surface of the olfactory epithelium.

The length of micrometer bars are equal in figures 6B, D, and E.

B. The high power micrograph shows individual diverticula. The diverticula have an acinar structure and are surrounded by darkly stained cells within the lamina propria. The olfactory mucosa surrounding the diverticula is inundated with blood cells and vessels (bv).

C. The low power micrograph of the olfactory organ from the ventral portion of a PM sea lamprey shows the location of clusters of diverticula (a) of the accessory olfactory organ posterior to the olfactory epithelium.

D. A small diverticulus of the accessory olfactory organ (a) is seen immediately adjacent to the trough-like depression between adjacent lamellae in a M7 animal.

E. A PM sea lamprey shows a cluster of diverticula of the accessory organ reaching from the base of the olfactory epithelium to the dense connective tissue that surrounds the cartilage. Axon fascicle is adjacent to the cluster (curved arrow). Blood vessels (bv) are located around the periphery of the diverticula.

F. The high power micrograph shows the two cell types of the accessory olfactory organ. On the outer edge of the accessory organ are small cells with patches of dark staining particles (white asterisks). The large cells lining the lumen of the organ are ciliated with nuclei located basally and their cytoplasm are filled with vesicular material (black asterisks). Cilia protrude from basal rootlets into the lumen of the organ.

Figure 6 continued

G. The diverticulum of the accessory olfactory organ (a) lacks a lumen, and is directly adjacent to the olfactory epithelium (OE) in a PM sea lamprey. An ORC (arrow) is visible in the olfactory epithelium. The broken line shows the basement membrane that separates the olfactory epithelium from the diverticulum of the accessory organ.

Figure 7. High power light micrographs showing the height of olfactory epithelium and mucociliary complex and the width, density and arrangement of ORC and SC in larval, metamorphic stages 2, 7 (M2 and M7), and post metamorphic (PM) sea lampreys.

The length of micrometer bars are equal in figures 7A - E.

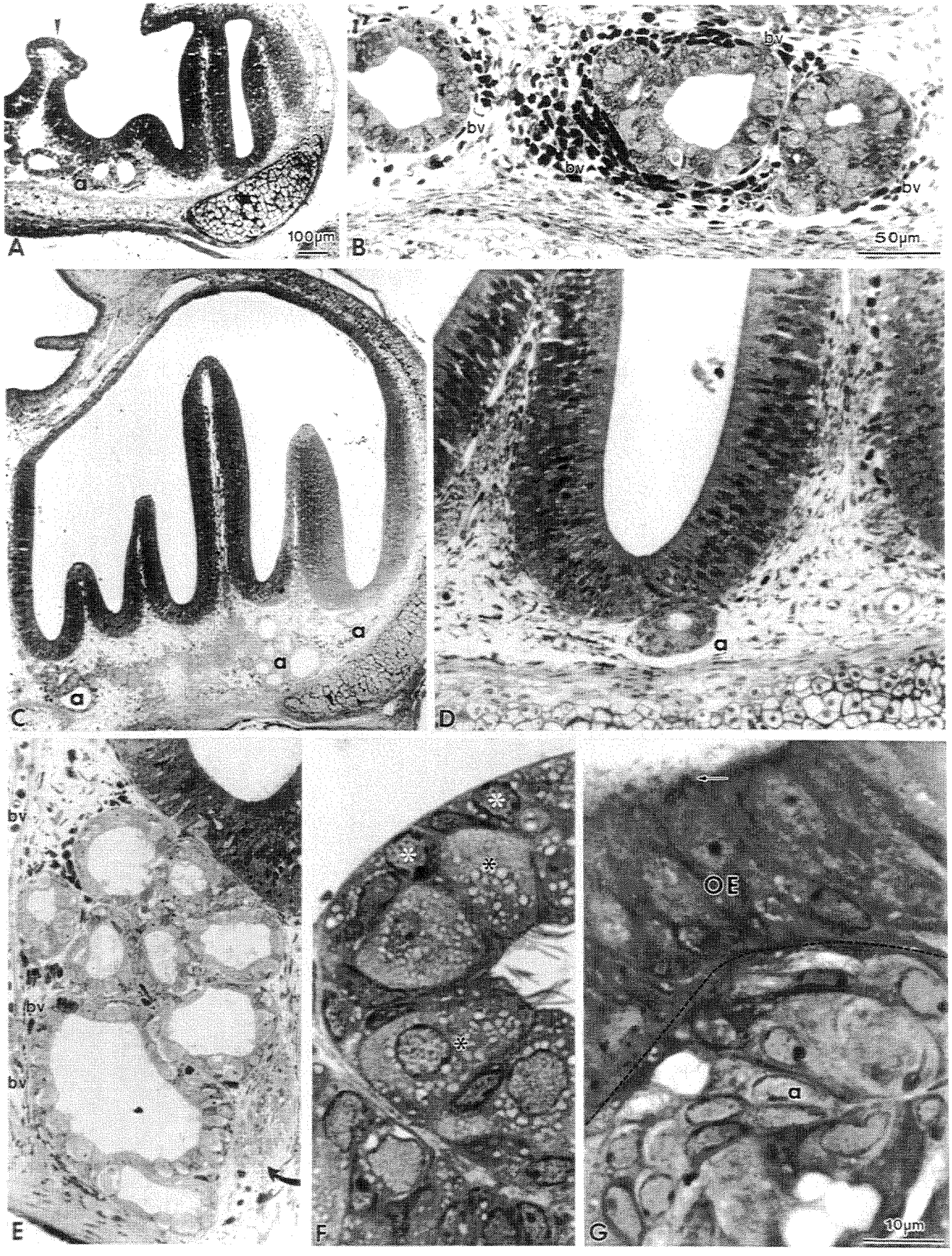
A. The larval olfactory knobs of the ORC (black arrows), and closely spaced SC (white arrows) are located between the ORC.

B. In a M2 sea lamprey, relatively wide SC (white arrows) separate the ORC (black arrows). Nuclei are visible in the lower portion of the micrograph. Two mitotic figures (*) are located in the distal portion of the olfactory epithelium.

C. In the M2 sea lamprey, the cells of the olfactory epithelium (OE) appear very long in length and have a wavy disposition to the medial region of their axons. The mucociliary complex (MC) consists of short sparsely arranged cilia covering both ORC and SC.

D. In the M7, cells of the olfactory epithelium, have a straight upright appearance. Numerous white granules appear in the cytoplasm of the epithelial cells.

E. In the PM animal, the medial region of the ORC and sustentacular cells appear very straight. The mucociliary complex appears very densely covered with long thick cilia. The ORC and SC appear thick in along the width of their processes.



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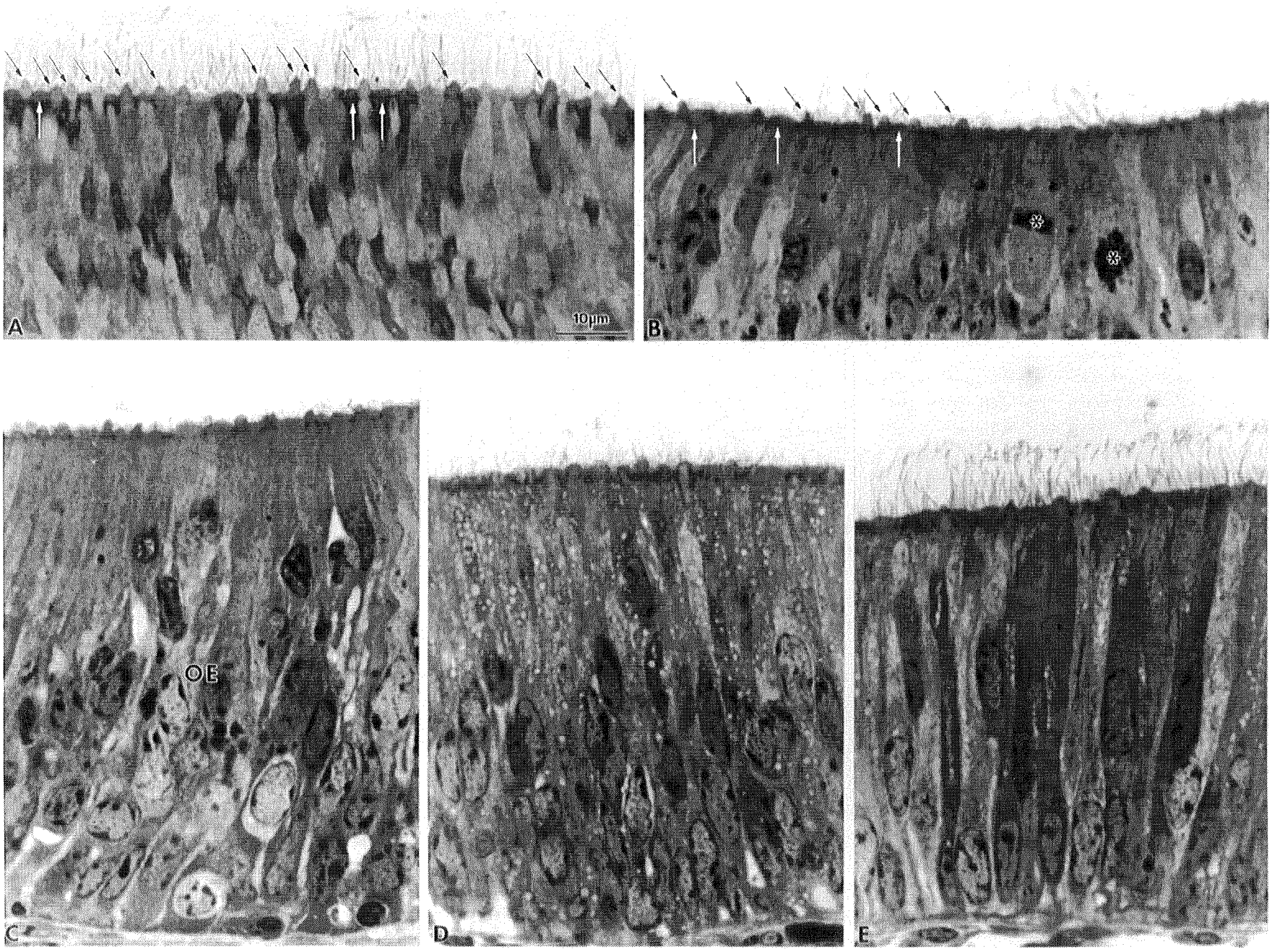


Photo M

Figure 8. The appearance of the olfactory epithelium at different locations in the olfactory organ.

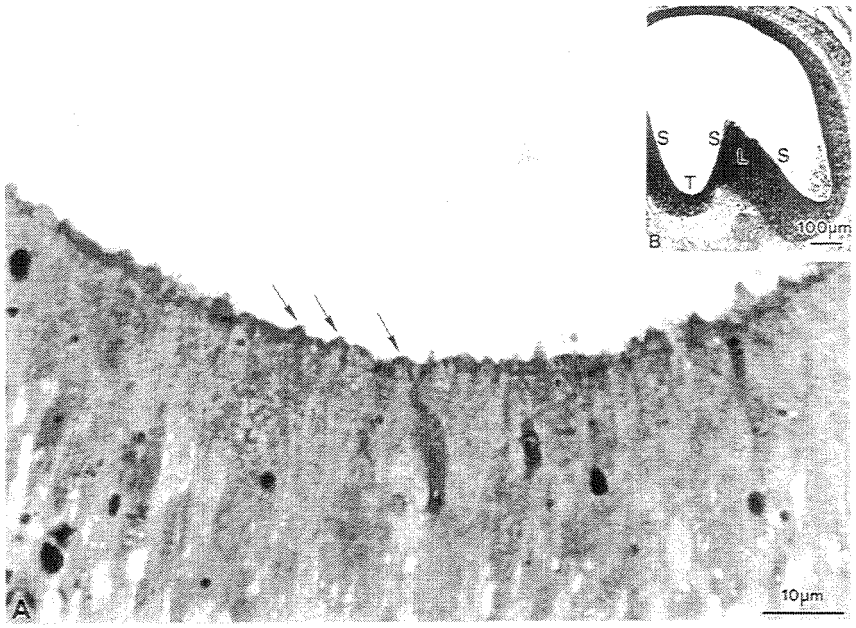
The length of micrometer bars are equal in figures 8A, C and D.

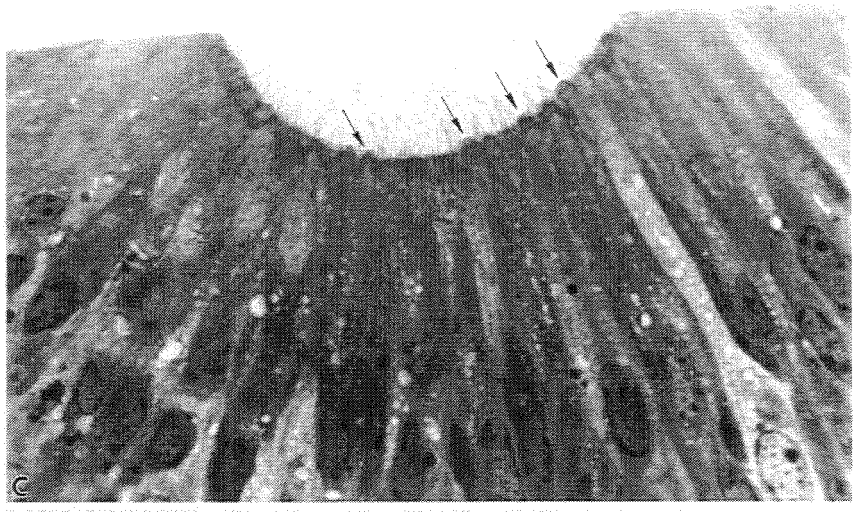
A. The olfactory epithelium in the trough-like depression between lamellae in a M3 animal possessing ORC with dendritic endings appear with wide or narrow (arrows) apical swellings.

B. The low power micrograph shows the shape of the olfactory organ in a M3 sea lamprey. There is a trough-like (T) depression between adjacent lamellae (L). The adjacent lamellar surfaces have been called slopes (S).

C. The olfactory knobs (arrows) are clearly visible and are thick in appearance in the trough region of a PM sea lamprey.

D. Olfactory knobs (arrows) in the slope region of a lamella from a PM animal are found in a loose arrangement. The mucociliary complex (MC) in the PM has a dense arrangement of cilia. SC (white arrows) separating the ORC are thick and appear white in color.





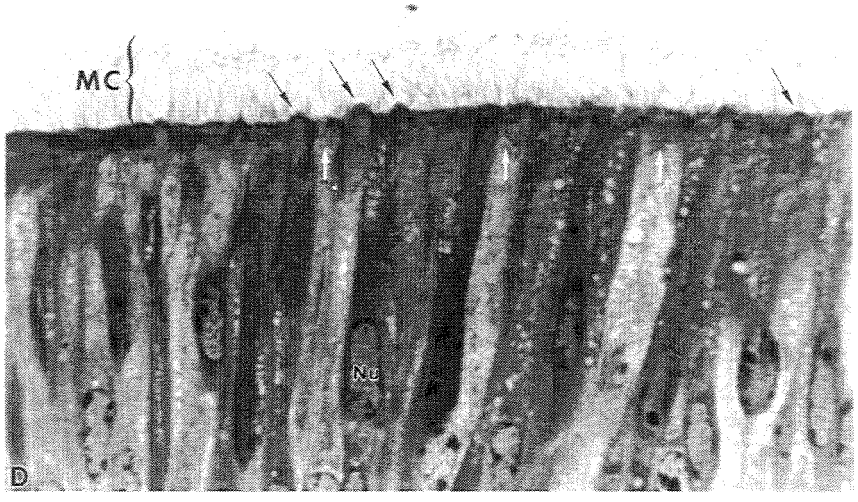


Photo N

Figure 9. Total ORC density.

Total ORC density (ORC/100 μm) of all areas of olfactory epithelium containing ORC (troughs and slopes combined) during various stages of life; starting with the larval stage (L), and proceeding through the 7 stages of metamorphosis (M1-M7), and ending with the post metamorphic (PM) and the juvenile (J). n = 3; Bars = error bars.

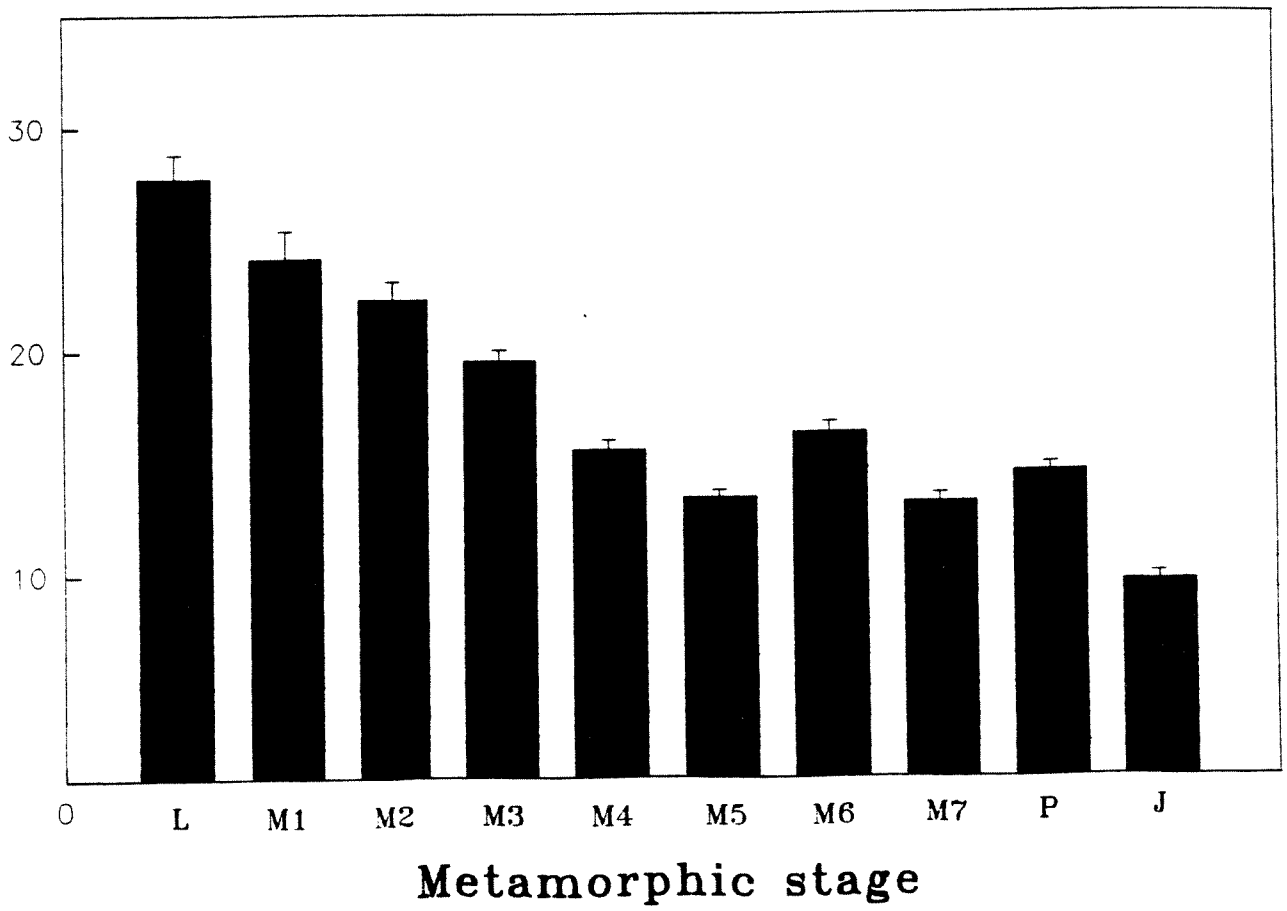
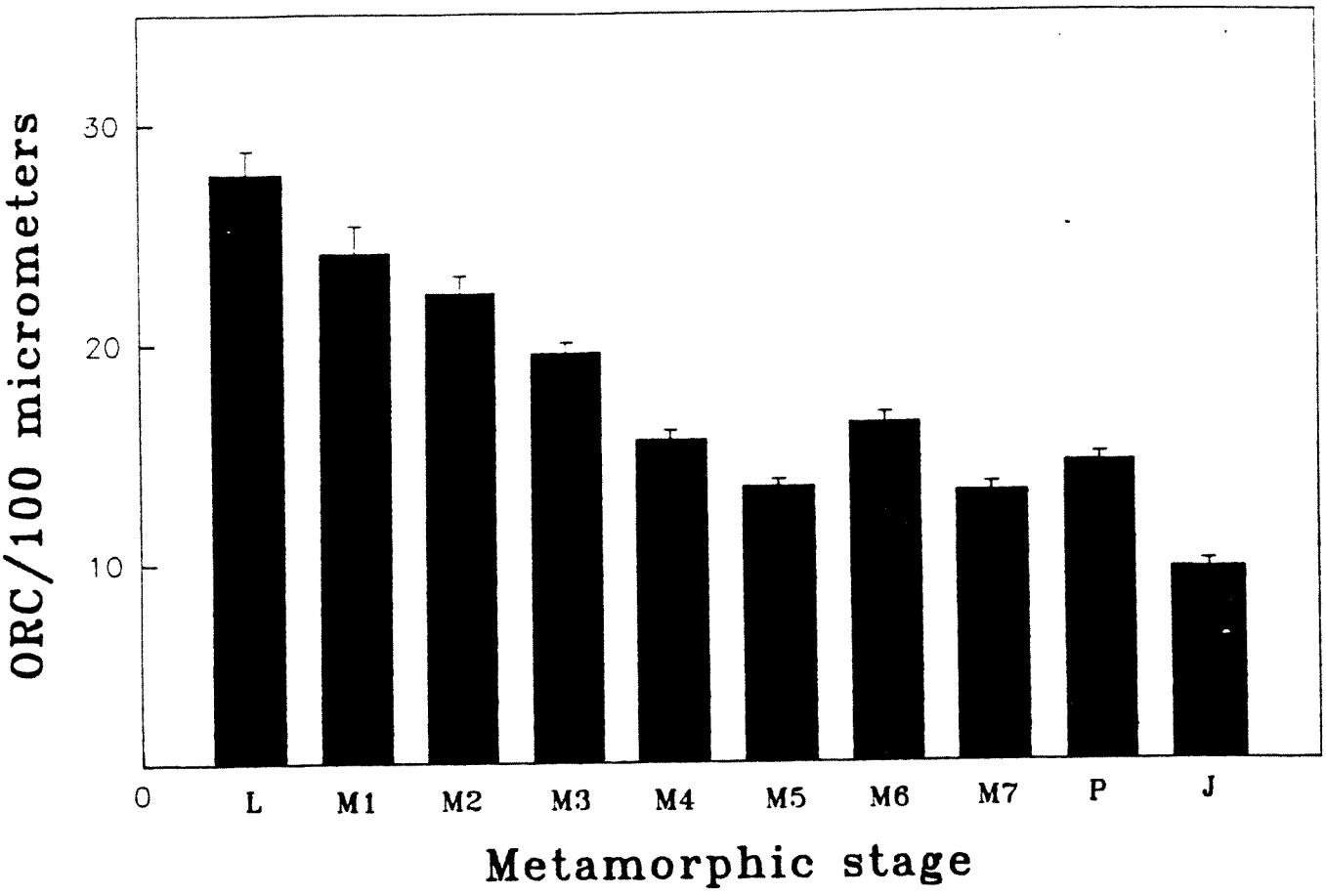

ORC/100 micrometers
30
20
10
0
L
M1
M2
M3
M4
M5
M6
M7
P
J
Metamorphic stage

Figure 10. ORC density in slope and trough regions.

ORC density (ORC/100 μm) in the slope (dark bars) and trough (white bars) regions of the olfactory organ from larval (L) to juvenile (J) stages. Beginning at M3, the trough regions have the same density as the slope regions of the olfactory epithelium. n = 3; Bars = error bars.

Fig 9.


ORC/100 micrometers
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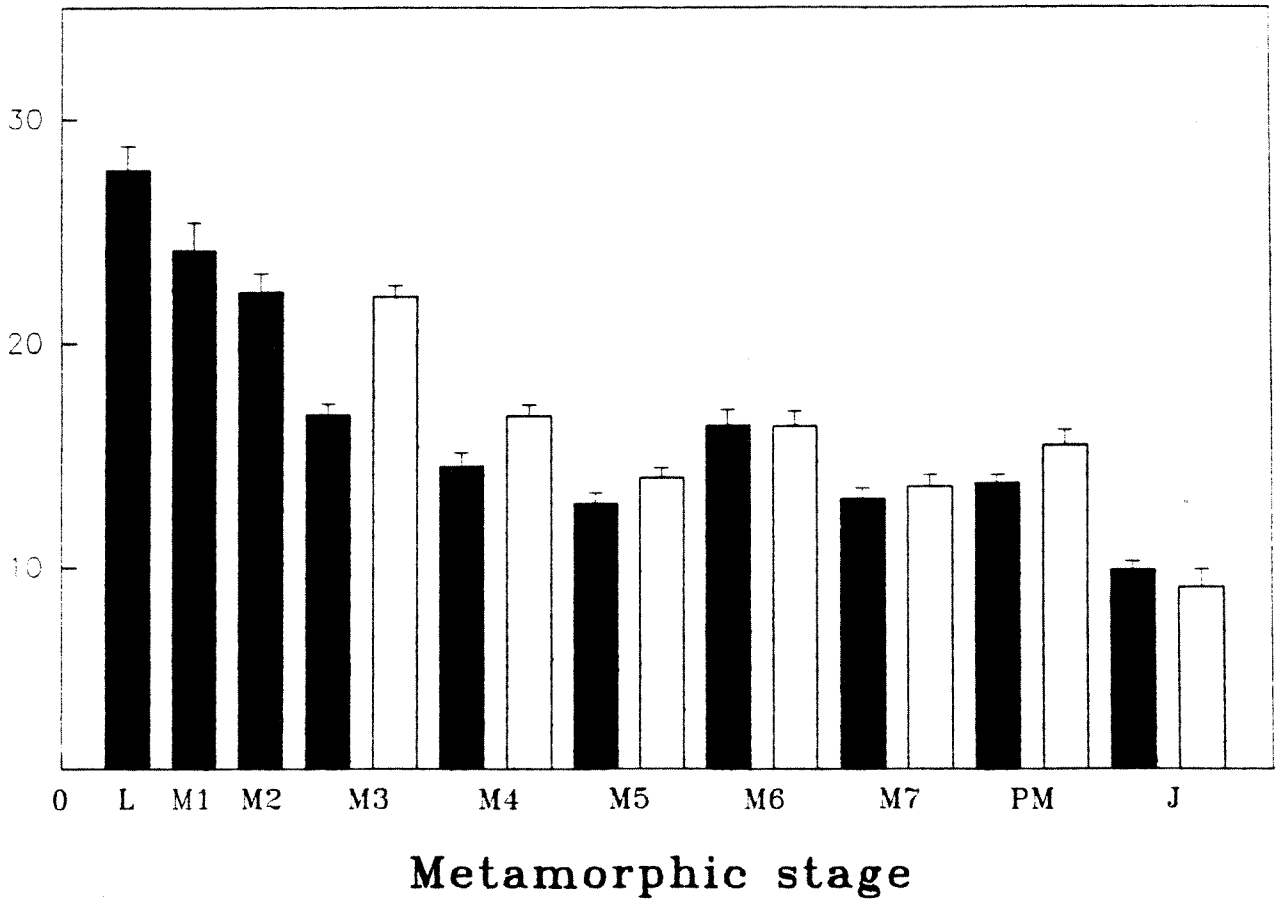
Metamorphic stage


ORC/100 micrometers
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PM
J
Metamorphic stage

Table 3. Summary of the changes in the olfactory organ during metamorphosis.

Olfactory organ	Ammocoete	M1 and M2	M3 to M5	M6 and M7	PM and Juvenile
Percent weight of nasal sac	NA	0.26	0.38	0.63	0.51 (PM)
Nasal valve	-	-	present in M5	present	present
Olfactory lamellae	-	-	+	++	++
Thickness of olfactory epithelium (μm)	73	80	93	85	80 (PM)
Average ORC density (number of ORC/100μm)	27.7±1.1	23.2±1.0	16.2±0.4	14.8±0.4	12.1±0.4
Surface Area (mm^2)		0.51 (M2)		1.1 (M7)	
Total number of ORC		43,248		76,084	
ORC density in troughs between lamellae (number of ORC/100μm)	NA	NA	17.6±0.5	14.9±0.6	12.3±0.8
Relative diameter of nonmyelinated nerve fascicles	small	small	medium	large	very large
Accessory organ diverticuli in the lamina propria	-	-	+	+	+

This is a partially finished discussion.

Discussion

During metamorphosis of the sea lamprey, the the larval form of the olfactory organ enlarged initially (during M1 and M2), changed abruptly during mid-metamorphosis (M3 to M5), and the transformed olfactory organ expanded during late metamorphosis (M6 and M7). This pattern varies from Leach's ('51) observation of an *Icthyomzon fossor* ammocoete with a transformed olfactory organ and hypophysis during the early stages of transformation. Unfortunately, he did not specify the criteria that were used to identify exactly how far it had been metamorphosized. Since *I.fossor* transforms directly to the sexually mature stage, it may have to use the adult ORC immediately, whereas sea lampey wait several months before feeding (Potter...), and even longer to spawn.

The change in the structure of the olfactory organ is probably associated with divergence in feeding and habitat between the burrowing larval stage and the post-metamorphic sea lamprey that assumes a predatory habit and leaves the streams for open water. Juveniles use olfaction to locate prey (Kllerekoper,); and during the upstream spawning migration, adults respond to the bile alcohol petromyzonol sulfate that origningates from larval bile. The larval form of the olfactory organ is essentially a transitional developmental structure that benefits the larva. Since the larva is relatively sedentary, the small volume of water sampled in the nasal sac suffices in comparison to the larger volume sampled by the actively predating post metamorphic lamprey. In ammocoetes the dorsal epidermis folds around the naris, creating a ciliated funnel-like structure that may direct water flow into the nasal sac. It is probably effective in removing the silty substrate to prevent passage of material into the oflactory organ. This became ineffective for the wider nasal tube, following metamorphosis which requires rapid exchange of relativiely large volumes of water. After the ridge flattens at M3, the nasal valve grows. The flexible nasal valve may regulate water flow efficiently from the nasal tube into the olfactory lamellae and prevents the outflow of nasal secretory products through the nasal tube. the enlarged postmetamorphic olfactory organ probably enables the adult to sample a large quantinty of water, the olfactory organ becomes more efficient at chemoreception: more water sampled, more odors can be detected. Juveniles pursue mobile prey and probably rely on the accuracy of olfactory sense to attach onto their prey successfully (Kleerekoper, '72). The precision and timing of "hunting" needs a system that effectively controls the inflow and outflow of water between the nasal tube and the lamellae of the olfactory organ.

The changes that occurred during the transformation of the olfactory organ included both a series of progressive phenomena and discrete events. Progressive events during metamorphosis included increase in the size and weight of the olfactory organ and enlargement of the olfactory nerve. Discrete events commences with the cancellation of specific larval characteristic the funnel-shaped nostril. The initial specific event was the lowering and widening of the nostril. At M3, other metamophic events that included the formation of the

nasal valve, eruption of lamellar folds and invasion of the lamina propria by diverticuli of the accessory olfactory organ were visible. Three processes that occur during sea lamprey metamorphosis that were outlined by Youson, '?? were present in the sea lamprey olfactory organ during metamorphosis. These included:

1. transformation of larval tissues and organs into those of the adult. This occurs in the olfactory epithelium. Tissue regression and poliferation in functioning larval structure. The folding of the olfactory epithelium, the ....accessory olfactory
2. regression and loss of larval structures. As the olfactory organ gradually enlarged, the flared funnel-like nostril became lower and wider, representing the degeneration of tissue, and was replaced by a lower structure. The ammocoete cells of the nostriil may have degenerated and become replaced by adult cells as in the secretory cells of the intestinal lumen (Youson, 1980).The lip height deminishes.
3. formation of new adult structures from anlagen which may have persisited since embryonic life, such as the kidney and intestine; the valve between the nasal passage and the olfactory organ forms.

The processes involed in some of the mophognentic events are also represented in various phases of ontogeny of other vertbrates?
In salmonids, the formation of the olfactory lamellae after few weeks after hatching (see Evans and Hara, '82). co-incides with the onset of feeding Olsen, ref?). In mammals, the olfactory mucosa folds into nasal turbinates and ORC increase in diameter during early embryonic development (ref). The sea lamprey has delayed the folding considerably, to accomodate for the larval stage. The very large lamprey ORC are unique product of lamprey transformation (Suzuki, ...), and are paralled in the brain by the postmetamorphic enlargement of Mauthner cells (Raivenen, '67). The opposite change occurs during Amphibian metamorphosis, larvae have a folded olfactory mucosa, appears scalloped. During metamorphosis, the salamander olfactory epithelium flattens to sheet-lke arrangement, and that the opposite occurs in the lamprey. Interestingly, the thyroxine levels in amphibioan are reversed.
The first signs of metamorphosis is the enlargment of the nasal cavity and lowering of lip.

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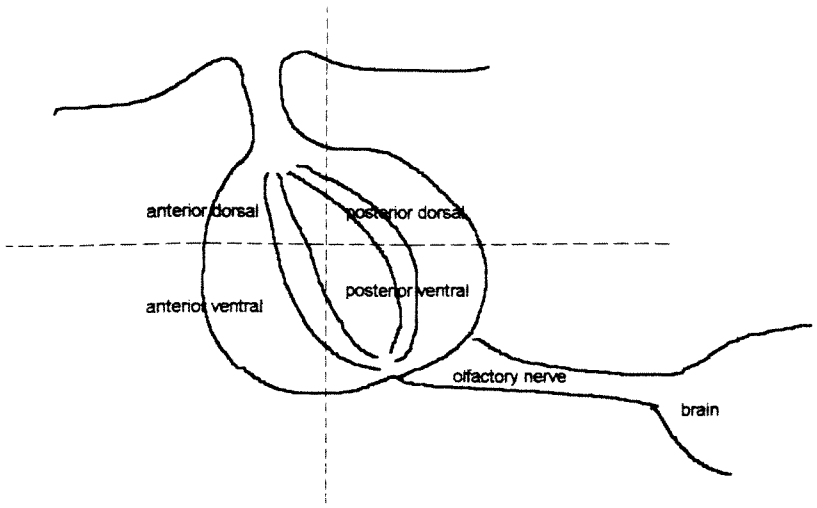
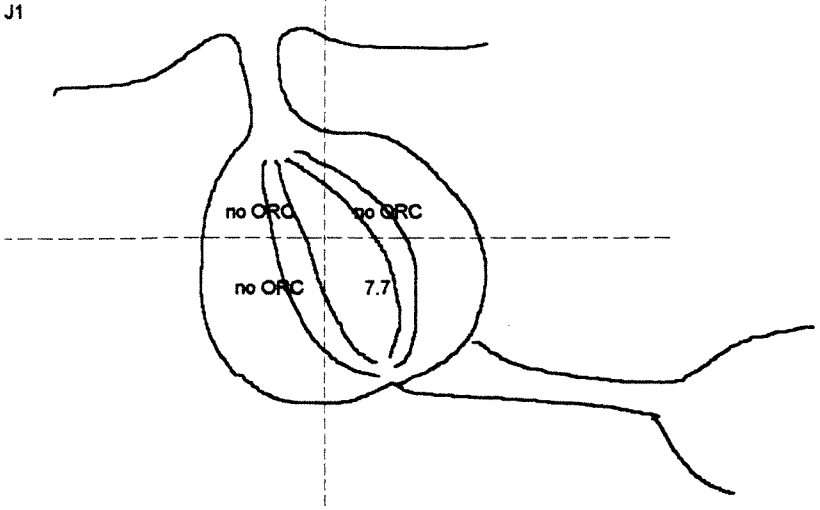
5. Juveniles (the parasitic phase) The ORC density was examined in the entire olfactory organ of 5 specimens. In the two specimens examined in October, 1993, no ORC were located in the dorsal region of the olfactory organ. One specimen had substantial ORC density only in the posterior ventral quadrant of the olfactory organ (7.7 ORC/100 μm). The other specimen had high ORC densities in the ventral anterior (7.2 ORC/100 μm). The posterior ventral quadrants could not be counted because of poor section angles. Of the three lampreys examined in January, 1994, one had low ORC densities in the anterior dorsal and ventral quadrants (1.7 and 2.1 ORC/100 μm) and high ORC densities in the posterior dorsal and ventral quadrants (7.1 and 7.9 ORC/100 μm). In 2 lampreys, there was high ORC density throughout the organ. In all three lampreys, ORC density was high in the ventral area and low in the dorsal olfactory organ.

Table 5.1 A summary of ORC density (number of ORC/100 μm length of olfactory epithelium in four quadrants of the olfactory organ in parasitic phase lampreys from the Hammond Bay Biological Station.

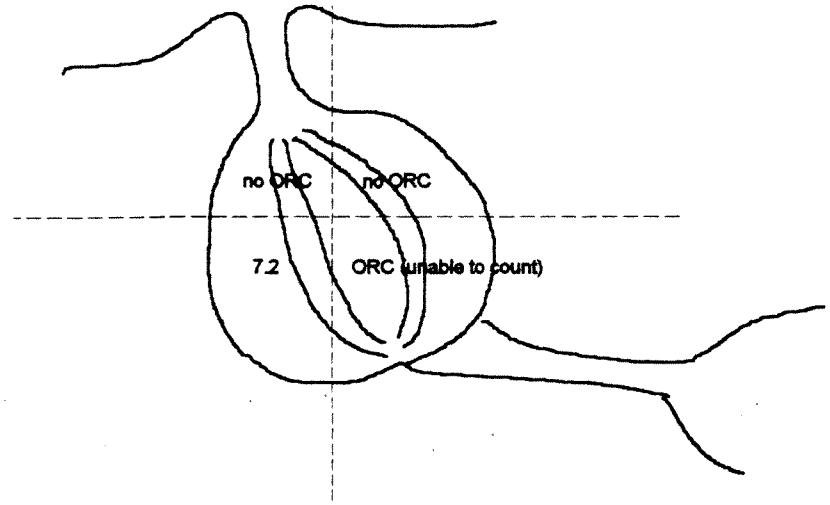
date	anterior dorsal	anterior ventral	posterior dorsal	posterior] ventral
Sept. 1993	too low to count	too low to count	too low to count	7.7
Sept. 1993	too low to count	7.2	too low to count	7.5
Jan. 1994	5.6	5.6	5.3	5.0
Jan. 1994	1.7	2.1	7.1	7.9
Jan. 1994				

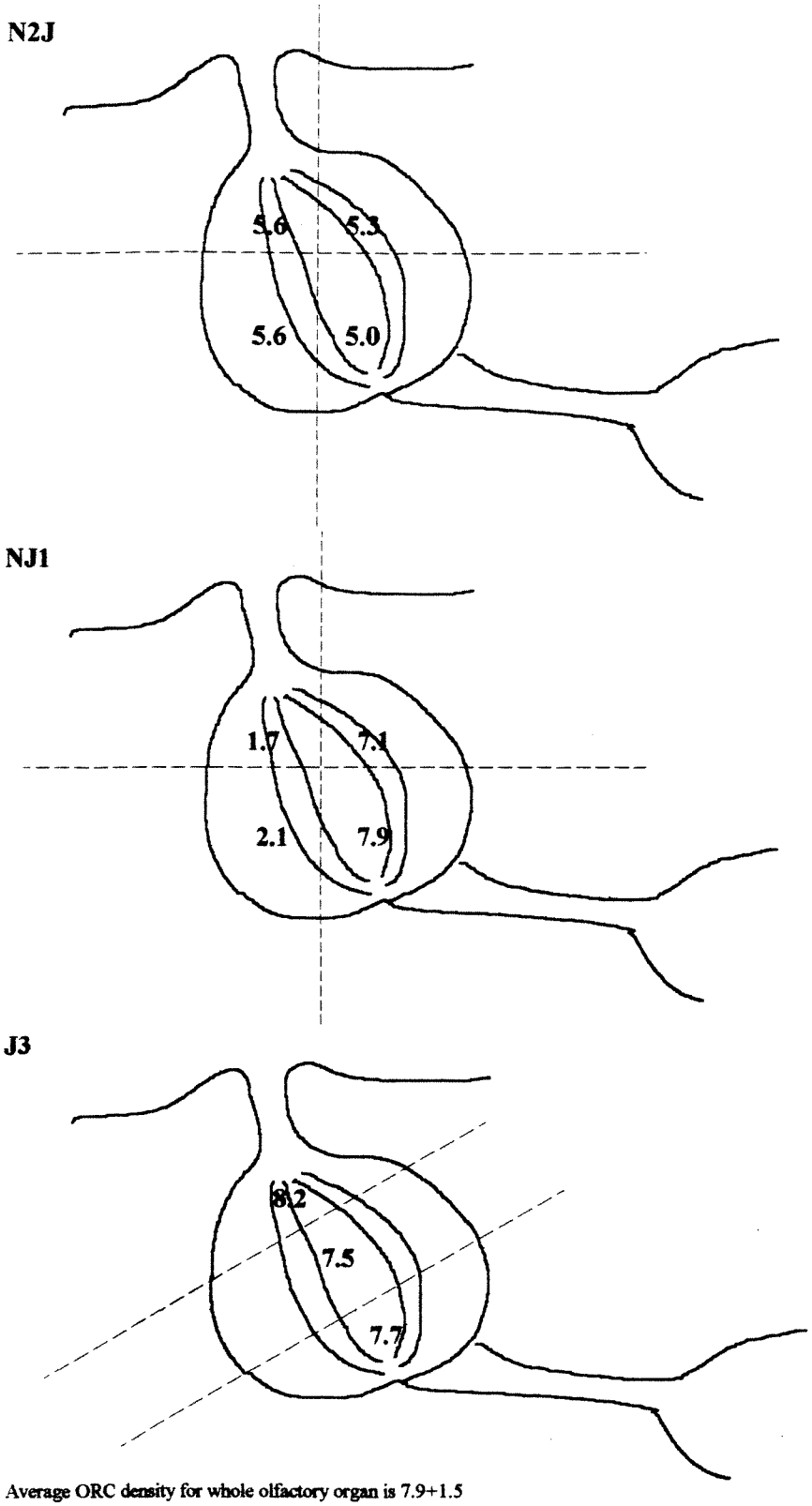

anterior dorsal
posterior dorsal
anterior ventral
posterior ventral
olfactory nerve
brain

J1


no ORC
no ORC
no ORC
7.7

J2


no ORC
no ORC
7.2
ORC (unable to count)



Average ORC density for whole olfactory organ is 7.9+1.5

Fig. 5.1 **Composition of the olfactory organ in juvenile (parasitic) sea lamprey.**

A. The lamellar folds in the anterior portion of the olfactory organ is surrounded by a cartilaginous capsule (arrowheads). . Bar is 100 μm.

B. The lamellar fold from is lined by stratified epithelium, the lamina propria between the lamellae contains sinuses. Bar is 100 μm.

C. The posterior region of the olfactory organ has nonmyelinated nerve bundles (N), diverticuli of the accessory organ (d). The brain (Br) is posterior to the olfactory organ. Same magnification as B.

Photo O

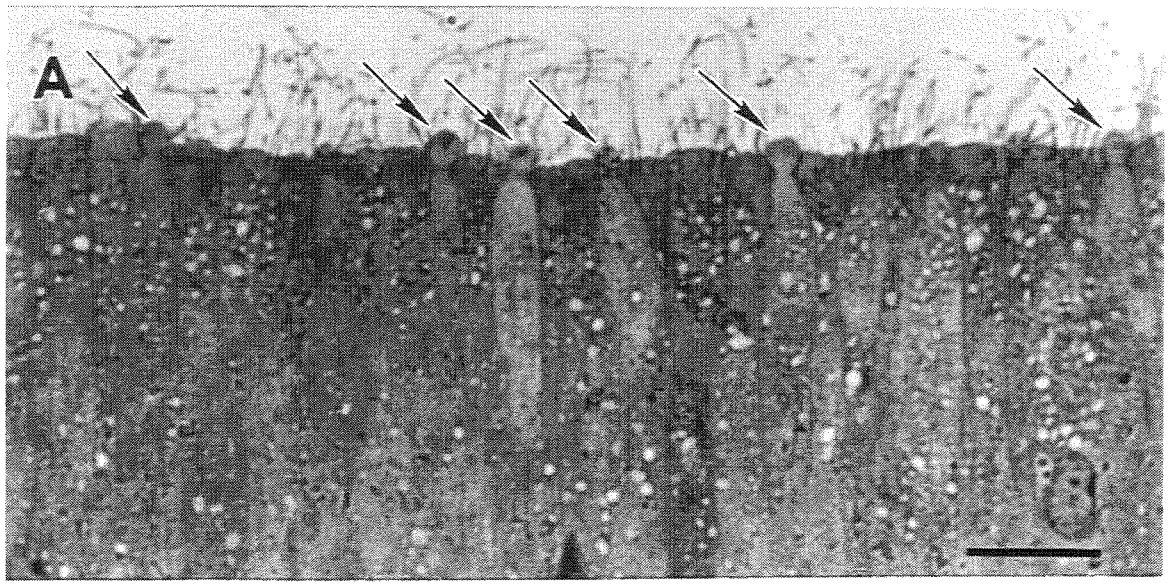
Fig. 5.2 **High power micrographs of the olfactory organ in juvenile (parasitic) sea lamprey.**

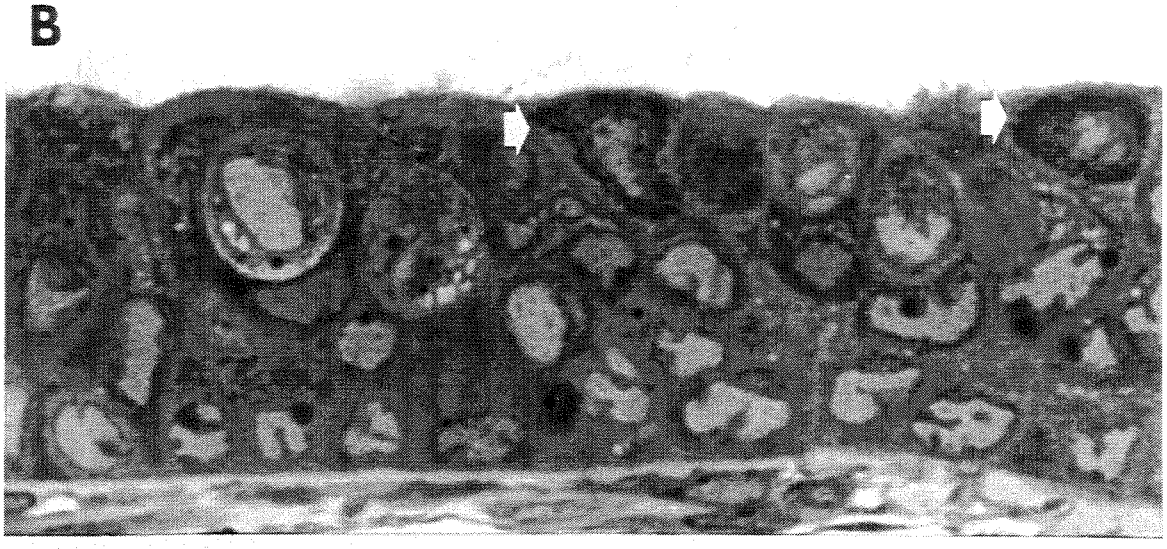
A. In the olfactory epithelium, ORC are recognizable by round olfactory knobs (arrows) and slight indentation below the free surface. ORC are separated by ciliated sustentacular cells. The cytoplasm of all the cells appear vacuolated. Cilia (Bar is 10 μm. All micrographs are at the same magnification)

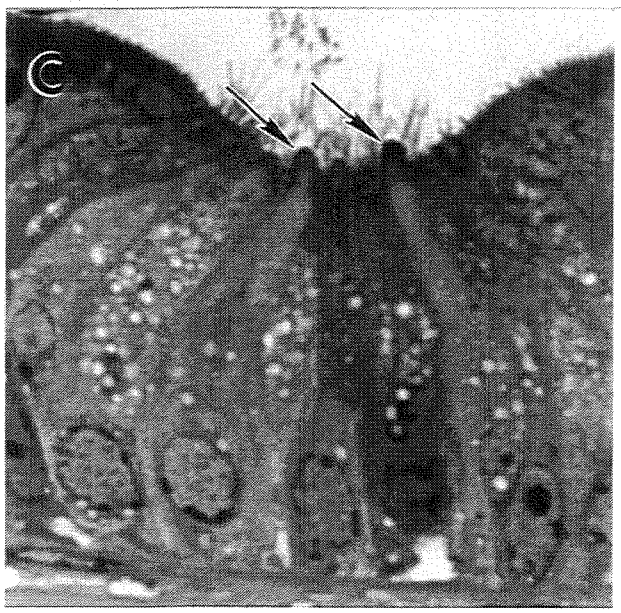
B. The epithelium that lines some lamellae is stratified cuboidal. Some cells have electron dense granules (white arrow).

C. Along some lamellae, there are small grooves with narrow ORC.

D. There are mitotic figures (curved arrows) in the apical region of the stratified cuboidal epithelium that lines the anterior lamellae.







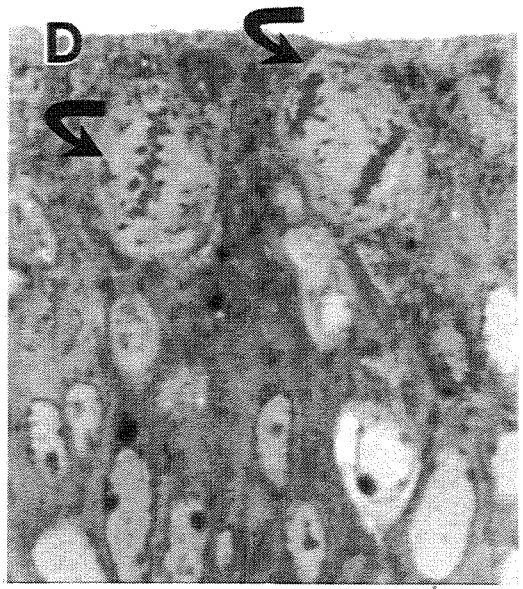


Photo P

6. Upstream migrants

The objective was to determine if there is any change in the morphological composition of the olfactory organ and ORC density in sea lampreys captured at two sites during the upstream migration.

Materials and Methods Sea lamprey specimens were obtained at the following dates from the weir on Ochqueoc Lake Rd., Hammond Bay, Michigan in 1994 on May 11, 14, 17, 19, 21, 25, 28, June 3, 8, 16, 18, 22, 25. From the St. Mary's River in 1994, on July 2, 5, 8, 12, 15, 19, 22, 26, Aug. 3, Aug. 6.

Analysis of covariance was used to determine if there is any variation in number of ORC among times at each site and furthermore to test if the ORC density vs. time relationships are the same at both sites.

Results

- The ORC density at the Hammond Bay site was higher than at the St. Mary's River site.
- There were no ORC on lamellae with a narrow, irregular shape.
- There were regions with no ORC in some specimens during the upstream migration.
- There were regions with ORC undergoing cellular degeneration by apoptosis. These lamellae contained nerve bundles, but did not contain diverticuli of the accessory olfactory organ.
- In some late migrants, there were no ORC located in the dorsal olfactory organ. (e.g. July 19, from St. Mary's River). In upstream migrants that were

- At the St. Mary's River site, males and females were sampled. There did not appear to be any difference in the ORC density between males and females.
- The ORC density at the Hammond Bay site was higher than at the St. Mary's River site.

ANOVA analysis. Is there any difference among groups from Hammond Bay?

The T value is 1.39

Therefore

1. There is no significant difference in ORC values at the different times when group to group is compared.
2. There is no difference in ORC density with time. Therefore the rise in Hammond Bay are not significant.
3. For the St. Mary's River, the ORC density does not significantly decrease as a function of time. Significance is at 0.5 level.
4. The two slopes, Hammond Bay and St. Mary's River are different.
5. The ORC density at one site is significantly higher at one site than at the other. The Hammond Bay population has a significantly higher ORC density than the St. Mary's population.

Are the populations distinct? There may be population differences.

Table 6.1 The range of ORC density in the olfactory epithelium of upstream migrant specimens trapped from two sites in 1993. From ten to fifteen regions with olfactory epithelium were analyzed in each specimen. Females are indicated by (F). All others are males.

Hammond Bay (Weir on Ochqueoc Lake Rd.)		St. Mary's River	
Date	Range of ORC density (#ORC/100 μm)	Date	Range of ORC density (#ORC/100 μm)
May 11	6-12 6-14 6-12	July 2	2-8 6-10 (F) 2-6 (F)
May 14	4-10 2-12	July 5	4-10 4-10 (F) 4-10 (F)
May 17	4-8 4-12 4-10	July 8	6-10 6-10 4-10 (F)
May 19	4-8 6-12	July 12	4-12 (F) 4-8 (F)
May 21	6-10 4-6 4-8	July 15	2-10
May 25	4-8 6-12 6-10	July 19	4-10 (F) 4-8 (F)
May 28	10-14 6-10 8-12	July 22	2-6 4-10 (F) 4-8 (F)
June 3	4-12 (F) 6-18 (F) 4-8 (F)	July 26	2-6 4-10 (F) 4-10 (F)
June 8	6-12 6-10 4-14	Aug. 3	4-6 (F) 4-8(F) 4-12 (F)
June 16	6-12 4-14 8-12	Aug. 6	4-10 2-6 (F) 4-8 (F)
June 18	4-6 10-16 6-10		
June 22	2-12 8-12 8-12		
June 25	6-14 4-10		

Fig. 6.1 **Upstream migrants, low power micrographs.**

A. Most cells have the same intensity of stain. Lamina propria has some very dark cells and some nerve bundles. (Bar for A,B and C is 100 μm)

B. There are some dark cells in the epithelium. There are some secondary folds on the lamella.

C. At the posterior ventral portion of the olfactory organ, the lamina propria has large nerve bundles and diverticuli of the accessory olfactory organ.

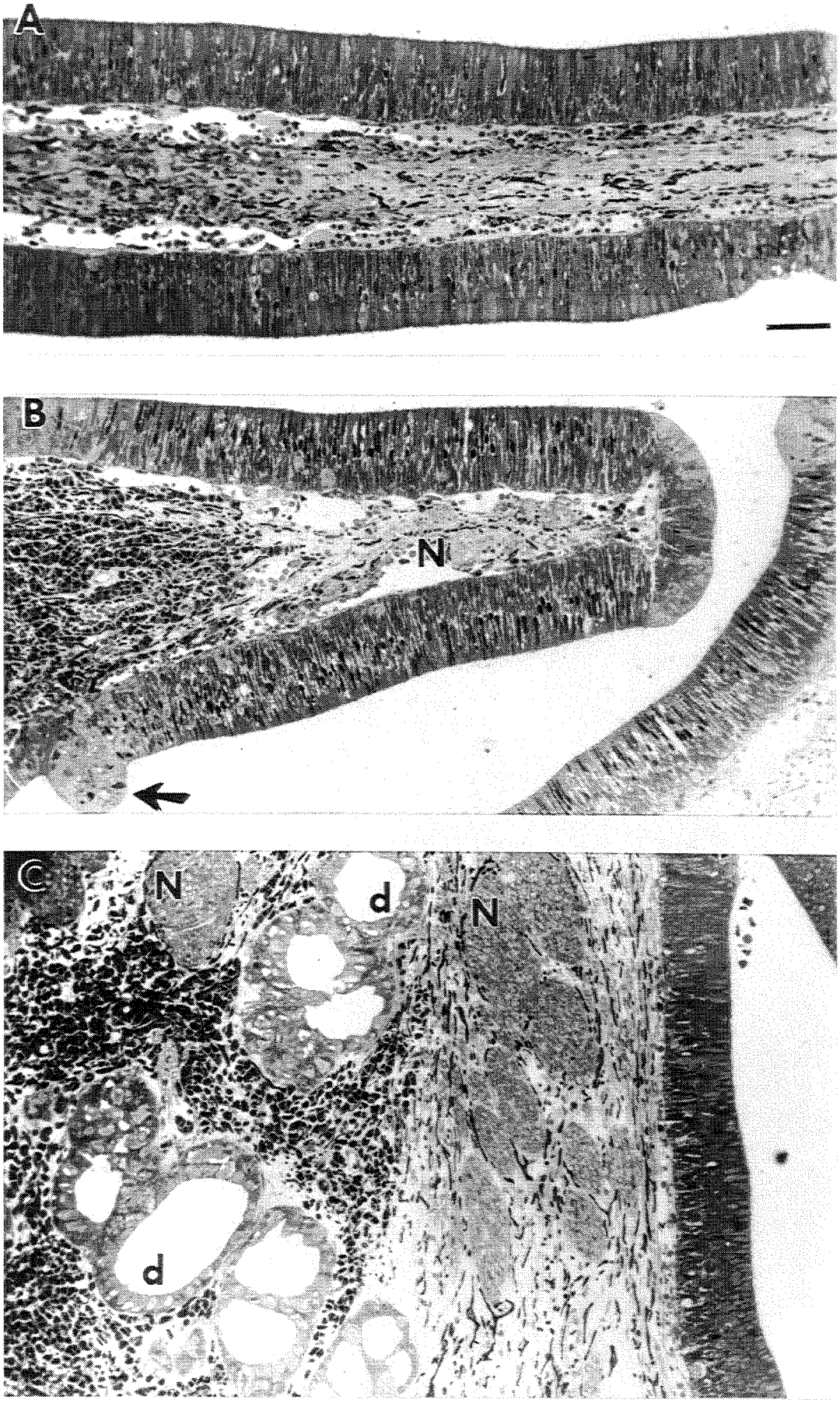


Photo 9

Fig. 6.2 Upstream migrants, high power micrographs.

The magnification is the same in all micrographs.

A. In a specimen that was taken in July has mitotic figures in the olfactory epithelium (short arrows). The ORC stain slightly paler than the sustentacular cells. The bar is 10 μm.

B. ORC in upstream migrants, some ORC are darkly stained.

C. Some areas have more darkly stained cells.

D. Regionswith very darkly stained cells and vacuolated cells.

E. Area with pyknotic very dark cells.

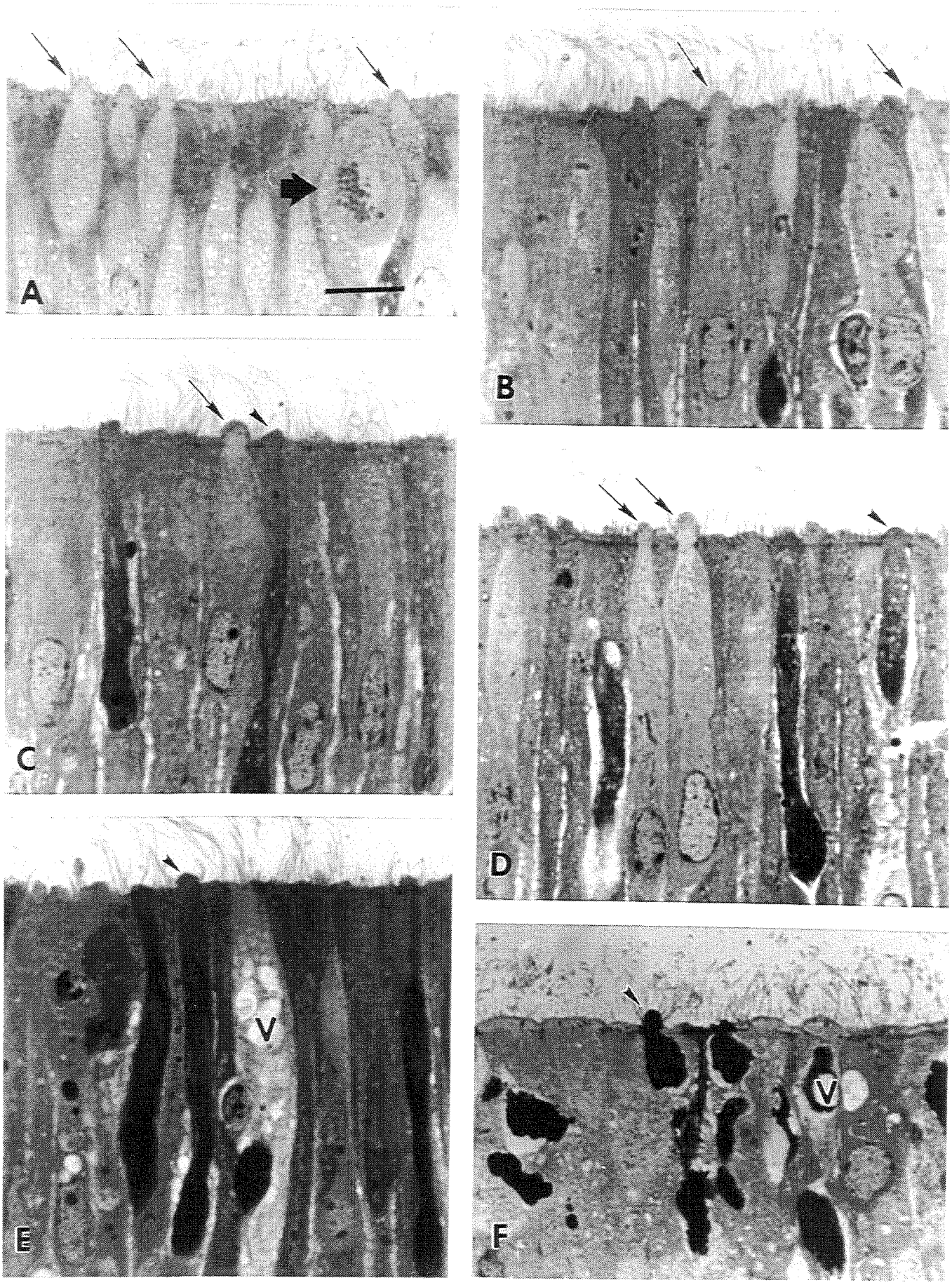


Photo R

Fig. 6.3 **Upstream migrant stage.**

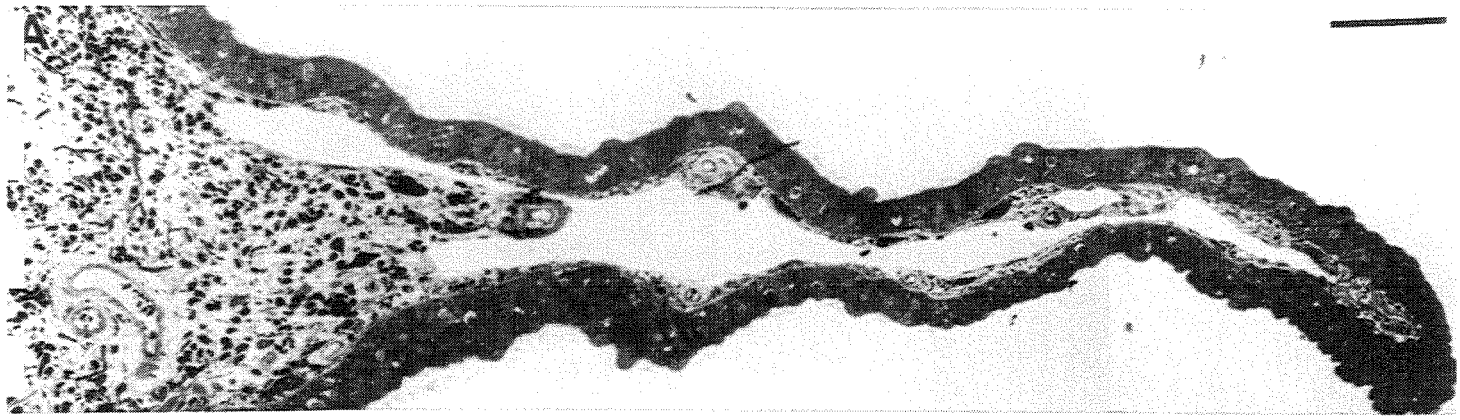
A. Lamella from the anterior region of the olfactory organ from August 1993 from the St. Mary's River. The lamellae without olfactory epithelium are usually thin and have an irregular shape. There are empty spaces (sinuses) in the lamina propria. Bar is 100 μm.

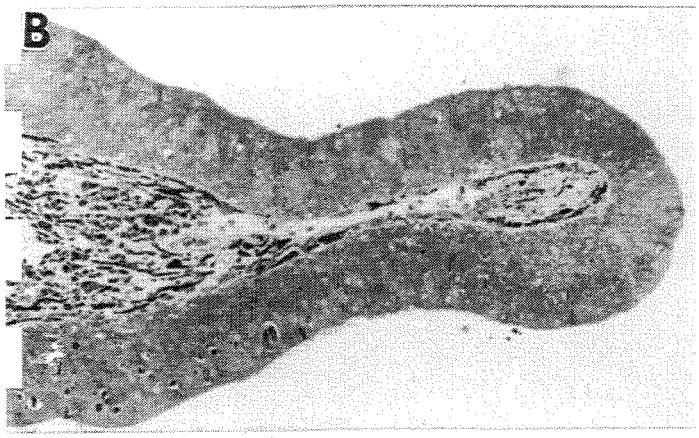
B. Region with no ORC. epithelium is thick and pseudostratified. Magnification is the same as in A.

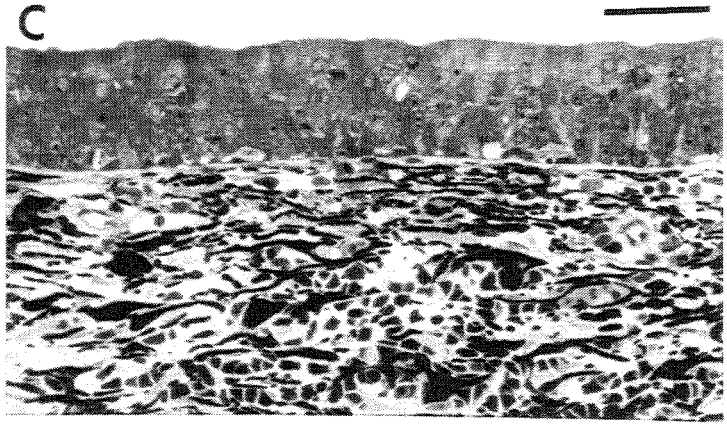
C. Pseudostratified epithelium. Lamina propria has very dark cells (presumably mast cells). Bar is 100 μm.

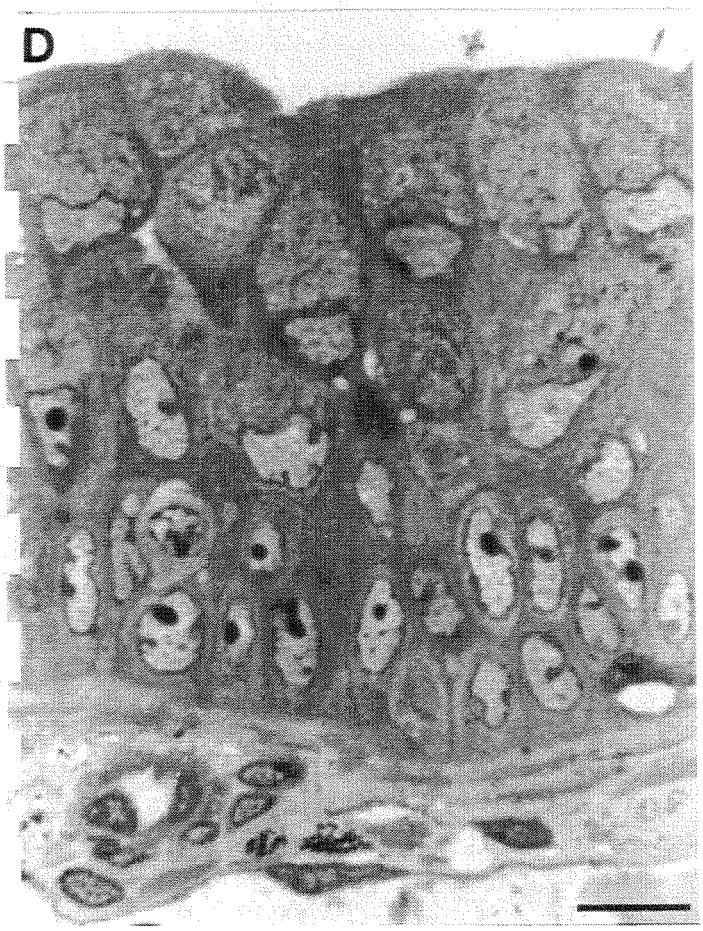
D. The top layer of the stratified cells that line an olfactory lamella have mucoid secretion and microvillar apical surface. Bar is 10 μm.

E. Some mucoid cells have a dome-shaped apex.









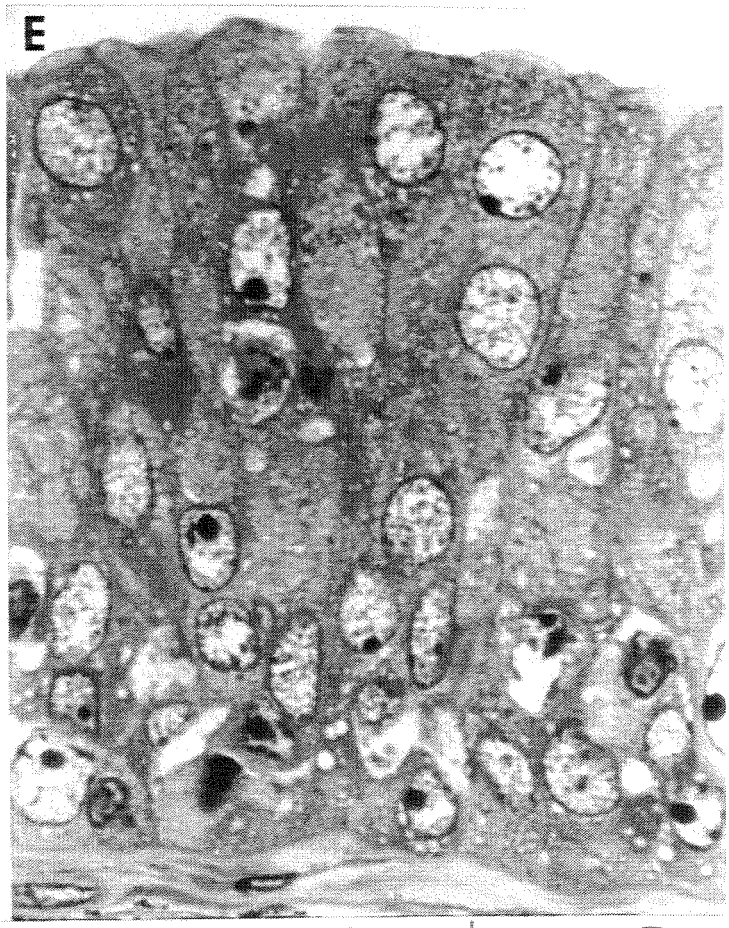
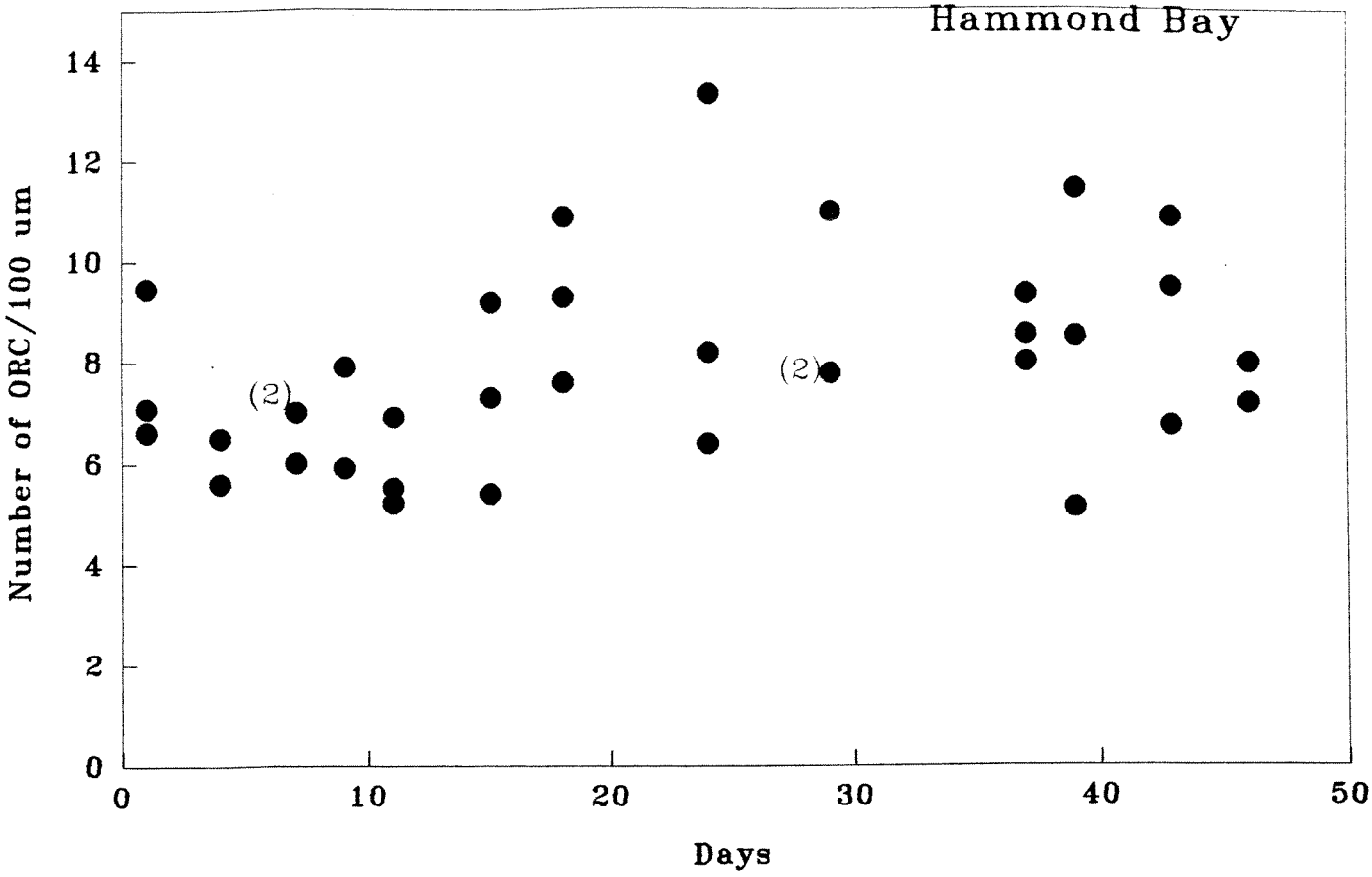
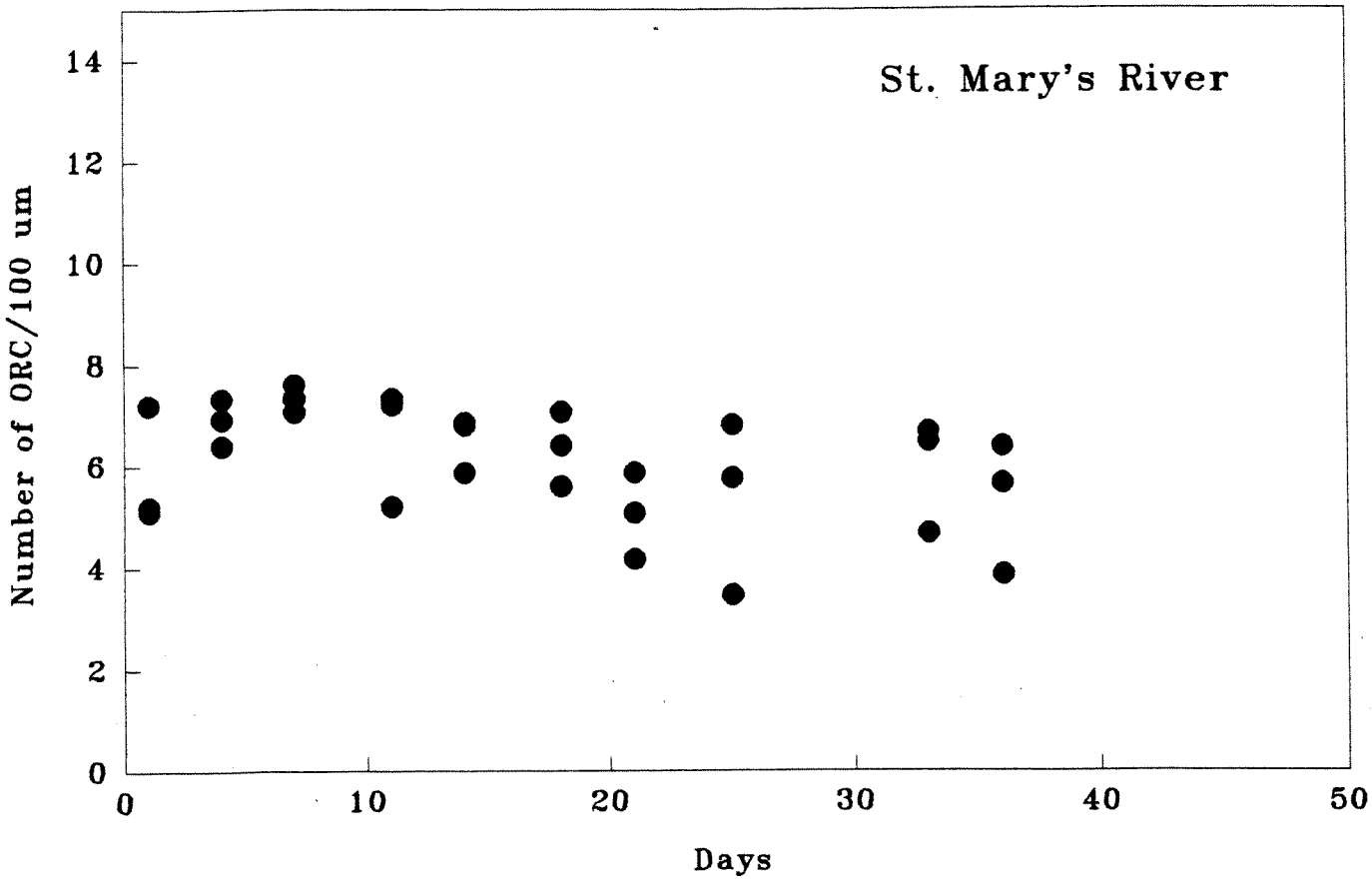


Photo 5

Fig. 6.4 Two graphs show ORC density (number of ORC per 100 μm length of olfactory epithelium). Each point represent the ORC density from a single lamprey olfactory organ. At least 10 different regions were counted to calculate ORC density for each lamprey.

(2) Two lampreys had the same ORC density value.


Hammond Bay
Number of ORC/100 um
Days
(2)
(2)
0
2
4
6
8
10
12
14
0
10
20
30
40
50


St. Mary's River
Number of ORC/100 um
Days
0
2
4
6
8
10
12
14
0
10
20
30
40
50

7. Post-upstream migrant lampreys

The olfactory organs from two sets of post-upstream migrants were examined.

1. May 1993 Three lampreys that were kept at the Hammond Bay Biological Station since the summer of 1992.
2. Oct. 1993 Two lampreys that were kept at the Hammond Bay Biological Station since the summer of 1993.

In both sets of sea lampreys, there were no ORC, the olfactory nerve fascicles did not contain axons, and the cells of the olfactory bulb appeared to be vacuolated. The olfactory lamellae were thin and irregularly shaped and were lined by cuboidal stratified epithelium, similar to the samples shown in Fig. 6.3

These results suggest that the ORC degenerate following the time of the upstream migration. Alternatively, the laboratory holding conditions or experimental manipulations may have caused ORC degeneration.