OLFACTORY ULTRASTRUCTURE COMPARISON OF HERBIVOROUS AND CARNIVOROUS TERRESTRIAL GASTROPODS

By

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ABSTRACT

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The study of gastropod neuroanatomy and behavior has contributed greatly to the understanding of various subjects across a wide range of phyla and disciplines. Unfortunately, a vast number of these studies focus on only a few model organisms, which may not be representative of gastropods as a whole. One deficiency of the current research is the focus on herbivorous species while all but ignoring what data carnivorous species could yield. This study aimed to examine the ultrastructure of the three main chemosensory areas - the posterior and anterior tentacle tips along with the labial palps - in two herbivorous and two carnivorous species to see if the differences in structures could account for the differences in foraging behavior. Though several previously undescribed structures were noted and described - including a matrix layer, circular membrane structures and ciliated tufts - these structures did not appear to correlate with foraging behavior. Additional study is needed to discern the nature of these structures and to further describe the possible differences between herbivorous and carnivorous land snails.

Dedication

I would like to dedicate this publication to my wonderful husband and my loving parents who have supported me throughout this whole experience.

Acknowledgements

I would like to thank all of the staff at the Center for Advanced Microscopy, including Dr. Stanley Flegler, Carol Flegler, Alicia Pastor, Melinda Frame and Ewa Danielewicz, for all of their help and guidance throughout my research. Without your assistance I would not have made it half so far in this journey for my degree. I would also like to thank my committee members, Heather Eisthen, Weiming Li, and Alicia Pastor for advice and support. Lastly, I would like to thank Dr. James Atkinson for allowing me this chance to pursue this degree and to gain so many useful skills. Also, I would like to thank Dr. Ron Caldwell for collecting and sending me the *Haplotrema concavum* specimens and for Dr. Eugene Keferl for collecting and sending me the *Euglandina rosea* specimens.

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Chapter 1: Introduction

Escherichia coli, Chlamydomonas reinhardtii, Caenorhabditis elegans, Drosophila melanogaster and Mus musculus is a short list of organisms familiar to most scientifically minded persons. These few species belong to a small group of organisms that are instrumental to numerous fields of research and are close to many researchers' hearts. They are model organisms; they are easily cultured in labs, typically of small size, and have a short life cycle. These traits lend themselves to the laboratory and experimental design; therefore these key species are used by scientists worldwide in every imaginable aspect of research including genetic analysis, behavioral studies and medical research. Large amounts of data have been compiled on each of these species and great advances in knowledge can be attributed to the use of model organisms. Sometimes the use of the organism itself has spurred the growth of new fields. However, there is a danger of having so much understanding of only a few, or in some cases a single species. The danger of dependence on a few model organisms rests on over generalization and overlooking biological phenomena that could provide significant insights. What holds true for one species, may not hold true in even closely related species, let alone apply to distantly related taxa. Without proper investigation, false assumptions can stifle future scientific study. A common example is a positive clinical test of a drug in mice may not yield the same results with other rodents, primates or human subjects. The dangers of biased models was recently discussed in *Science* (Wald and Wu, 2010). Primarily male rodents were used for a wide range of studies because of their

ease of use and the increase in cost and time associated with including females in the study design. They note one high profile problem that was created by the bias: several drugs tested only on male rodents have been pulled from the market by the FDA because of their adverse reaction in women.

Though less alarming to the general public, the field of gastropod research is no exception when it comes to similar biases. Species like *Aplysia californica, Helix pomatia, Limax maximus, Cornu aspersus* and *Achatina fulica* have been used for decades for behavioral and neural research providing important information. For example, the study of *Aplysia'*s giant neurons has led to a better understanding of basic neuronal function in both invertebrates and vertebrates (Chase, 2002). Another major component of gastropod research has been in the field of learning and feeding behavior (Croll and Chase, 1977). The focus on *A. fulica* in such research is driven by the fact that *A. fulica* is an agricultural pest and invasive species (Civeyrel and Simberloff, 1996).

Gastropods are particularly well-suited for the study of chemoreception as their feeding behavior relies heavily upon it. Gastropods sense their world primarily through gustatory and olfactory means. Their eyes, if they have them at all, range from patches of light sensitive simple optic cups to eyes containing lenses. In most cases, light sensing areas seem to be mostly related to circadian rhythms (Chase, 2002) and light/dark recognition (Andrew and Savage, 2000). The field of gastropod vision has some of the same model organism issues as gastropod chemoreception in that research underrepresents pulmonate species, and those terrestrial gastropods that have been researched are all from a single

genus, *Cornu* (formerly in the genus *Helix*) (Zieger and Meyer-Rochow, 2008). While chemosensory studies have been concerned with more than a single genus, the number of species investigated has remained too low (*Achatina*, *Limax*, *Cornu*). The chemosensory capabilities of snails has been studied through various means, including lesions studies, studies that isolate and directly stimulate the sensory areas, electrophysiological and behavioral studies (Chase 1986; Chase and Croll,1981; Ermentrout *et al*., 2004; Friedrich and Teyke, 1998). These conclusions about behavior and sensory capabilities that are based on a few herbivorous species have been generally assumed to fit other species. However, those species where diet requires other methods for gathering food may experience a different sensory world.

A hint that there may be some behavioral and physiological differences between animals with carnivorous and herbivorous strategies can be found in the work of Shearer and Atkinson (2001). They found that the herbivore *Anguispira alternata* (Say) could navigate around scent-permeable barriers to find a food source, while the carnivore *Haplotrema concavum* (Say) could not. Additionally, the carnivore had a tendency to follow prey's slime trails to a food source, while the herbivore rarely followed conspecifics' slime trails to a food source. Even a marine carnivorous species, *Navanax inermis*, was found to track prey via slime trails and not distance chemoreception (Murray, 1971). This suggests that there may be some structural differences in sensory structures located in the three main chemosensory organs in herbivorous and carnivorous land snails. In these organisms the principal chemosensory structures are the tips of the large optic or

posterior tentacles, the tips of the short anterior tentacles and thickened areas associated with the mouth, the labial palps (Chase, 2002). Therefore these are logical places to perform a comparative study seeking structural components of the differences in behavior between herbivorous and carnivorous gastropods (Figure 1).

The aim of the research reported here was to examine the surface epithelium of three key areas involved in snails' chemoreception: the surface of the posterior tentacle tips, anterior tentacle tips and labial palps of four terrestrial snail species. Two herbivorous species, *Anguispira alternata* (Say) and *Arion subfuscus* (Draparnaud), and two carnivorous species, *Haplotrema concavum* (Say) and *Euglandina rosea* (Ferussac), were examined by means of transmission electron microscopy (TEM), scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM) to better understand if snails with different foraging behaviors present morphological differences in their chemosensory structures.

TEM and SEM were used to study the structure of the endothelial surface (Kunz and Haszprunar, 2001) of the cephalic tentacles in other gastropod species.

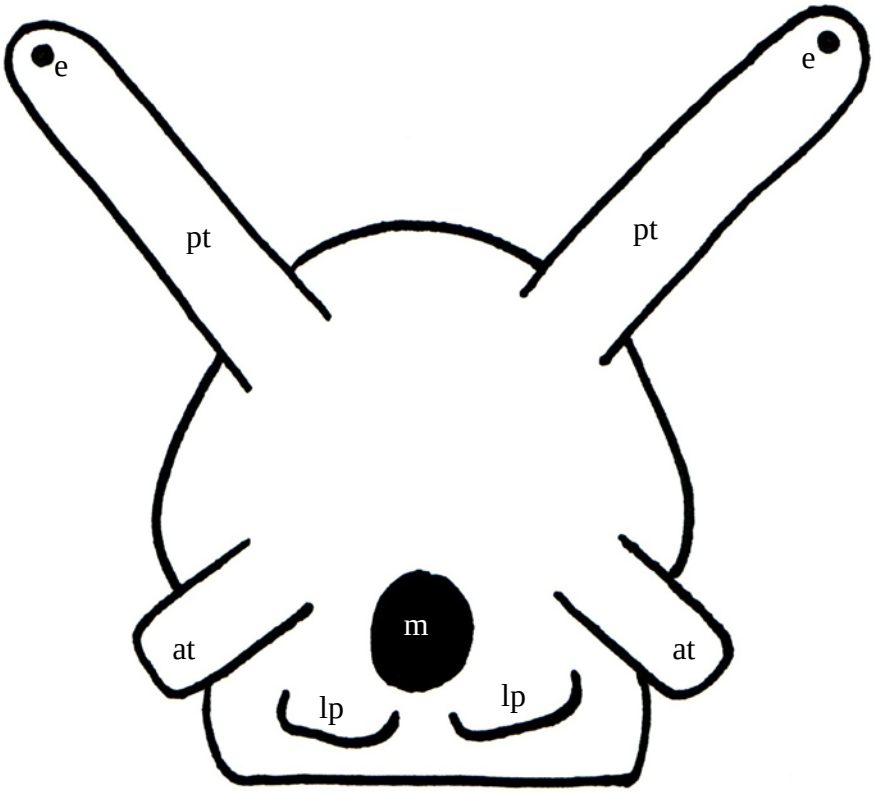

Figure 1 General Anatomy of a Snail Head: This diagrammatical representation of a snail's head as seen from the front shows the posterior tentacles (pt), anterior tentacles (at), labial palps (lp), mouth (m) and eyes (e).

Chapter 2: Materials and Methods

Specimen Collection

The herbivorous species, *A. subfuscus* and *A. alternata*, were collected in local wood lots in Ingham County, Michigan, and used to create a lab colony. The carnivorous species, *H. concavum* and *E. rosea*, were generously collected by Dr. Ron Caldwell in Arkansas and Dr. Eugene Keferl in Georgia.

Cultures

Both herbivorous species were cultured in either clear plastic containers (9.4cm x 15.0cm x 30.0 cm) or small glass aquaria (7.5cm x 16.0cm x 24.5cm). Approximately ½ inch of potting soil was used as a substrate, while a piece of broken flower pot was placed in the enclosure for cover. A small piece of wood or dry leaf litter was also included to help maintain proper moisture levels and to create a more natural environment. The carnivorous species were kept in similar cultures, but with one individual per culture to eliminate the chance of cannibalism.

Herbivorous species were fed sliced carrot and the soil moistened once a week. Any uneaten carrot was removed at the end of the week before new food was added. *A. alternata* was separated into three cultures: adult, juvenile and young. When a juvenile grew large enough, it was moved into the adult culture. Juveniles were also used to feed the carnivores (see below). The *A. alternata* adult culture also produced fertile eggs several times during this period. Any live young snails were removed from the adult culture and placed in a separate

young culture. When they grew to juvenile size, they were placed in the appropriate culture. About a dozen specimens were used for this study. *A. subfuscus* did not reproduce in the standard culture and was, therefore, used for analysis within a month or two of collection. *A. subfuscus* was very abundant. These were used to develop and standardize techniques for processing all specimens.

The carnivores were fed either a single *A. subfuscus* or *A. alternata* every 2-3 weeks but still watered weekly. Occasionally other locally available gastropods such as the garden slug *Deroceras reticulatum* were used to supplement their diet. Due to limited numbers, two specimens of *H. concavum* and three specimens of *E. rosea* were examined.

Anesthesia

Proper extension of the areas of interest (posterior and anterior tentacles and labials palps) is of particular importance in the scanning electron microscope (SEM) investigation of the morphology of the olfactory epithelium, and to a lesser extent transmission electron microscopy (TEM) and confocal laser scanning microscopy (CLSM). Several techniques were attempted before satisfactory procedures were reached (several of these are described in the appendix).

A combination of the submersion in chilled water and 5% succinylcholine chloride was devised for proper extension and anesthesia. Approximately 5 drops of the 0.5% succinylcholine chloride in a small finger bowl filled with chilled water had a fairly good result, with some extension of the tentacles; the animal's

reaction time was usually decreased sufficiently enough for careful dissection. Interestingly, neither cold water nor 5% succinylcholine chloride seemed to have any effect on *E. rosea*, which contracted in their shells and would not emerge while immersed. Once removed, however, the *E. rosea* actively extended their tentacles and started to explore the surrounding area. This allowed rapid removal of the head with a sharp razor blade. Once the head was removed, if the tentacles were partially retracted, it was possible to force extension with careful use of a pair of fine forceps by slowly and gently moving from the base toward the tip of the tentacle so as to not damage the tissue. It was essential not to use the tips of the forceps, as they tended to crush or tear the tissue. The fixative acrolein (Jongeblored *et al.*, 1999) facilitated tentacle extension when used after the removal of the head and before removal of mucus.

Scanning Electron Microscopy (SEM)

One challenge to producing clear imaging of gastropods is the mucus coat that can block the electron beam from the surface of the organism. This was particularly a problem with *Arion subfuscus*, which secretes copious amounts of thick mucus when agitated. A method using 16% glycerol (Zaitseva and Bocharova, 1981) to remove the mucus coat produced satisfactory results. Removal of the mucus was improved by sonicating the samples for 5 minutes.

Because of the large size of the samples studied, three different fixation techniques were used. Acrolein (Jongeblored *et al.*, 1999) was used to initially fix the cells and facilitate tentacle extension followed by 2.5% paraformaldehyde

and 2.5% glutaraldehyde in 0.1M sodium cocadylate buffer (pH = 7.4) to fix proteins. After fixation, the samples were washed in 0.1M sodium cacodylate buffer (pH = 7.4) and postfixed with 1% osmium tetroxide in 0.1M sodium cacodylate buffer (pH = 7.4) to fix lipids. Once all fixation was completed, the samples were dehydrated in an ethanol series and critical point dried. The dehydration steps had to be increased because of the large size of the samples to at least 6 hours. To increase resolution and minimize charging, the samples were osmium coated using a NEOC-AT Osmium Coater (Meiwafosis Co., Ltd., Osaka, Japan). Samples were observed using JEOL 6300f and JEOL 6400V Scanning Electron microscopes (JEOL, Japan).

Transmission Electron Microscopy (TEM)

Although not necessary for TEM, full extension of the tentacles made interpretation easier. The main areas of interest (posterior tentacles, anterior tentacles, and labial palps) were dissected and fixed with 2.5% paraformaldehyde and 2.5% glutaraldehyde in 0.1M sodium cocadylate buffer (pH = 7.4) and postfixed with 1% osmium tetroxide in 0.1M sodium cocadylate buffer (pH = 7.4). After postfixation the samples were washed with sodium cocadylate buffer to remove any excess osmium tetroxide. The samples were then dehydrated using an acetone series and infiltrated and embedded in Poly/Bed 812 epoxy resin (Polysciences, Warrington, PA). Standard resin infiltration times were increased to 5-6 hours to insure complete infiltration. The blocks containing the samples were polymerized at 60º C for 24 hours. Thick

and thin sections were obtained with a MTX Ultramicrotome (RMC, Boeckeler Instruments, Tuscon, AZ) using a diamond knife.

The thick sections (1000 nm) were placed on a glass slide and stained with Ready-to-Use Epoxy Tissue Stain® (Electron Microscopy Sciences, Hatfield, PA) and were observed with a compound light microscope to select areas of interest that would be observed with the TEM. Thin sections (~90 nm) were collected on 200 mesh copper grids. These grids were then stained using 2% uranyl acetate in 50% ethanol and lead citrate (Reynold's formulation). Stained grids were viewed on a JEOL100 CXII (JEOL, Japan) Transmission Electron microscope at an accelerating voltage of 80 kV.

Chapter 3: Results

General Epithelial Cell Morphology – All Species

SEM imaging did not reveal any general cell morphology, even with satisfactory mucus removal. Areas covered with microvilli appeared as a solid mass, except where damaged areas allowed for a deeper view. In general, the tips of the tentacles at low power appeared smoother than the surrounding tissue (See Figures 4B, 8A, 10 and 12). There were only a few cases where general morphological differences could be seen. Those cases include lone tufts of microvilli or specialized ciliary structures that extended past the microvillar mass (see Figures 5, 12C and 13).

TEM revealed more detailed characteristics. In general, at low magnification, all of the tissue examined seemed to share the same basic structures. The epithelial layer is a single layer of simple columnar cells (Figure 2). The distal edge of the cells contained a continuous brush border of microvilli. Large nuclei, taking up roughly 1/2 to 1/3 of the cell, are most often located near the basal edge of the cells. This single layer rested upon a basement membrane. Neighboring epithelial cell membranes were tightly interdigitated. The thickness of the epithelial layer varied and appeared to be the thickest at the distal tip of the tentacle, while becoming thinner proximally (Figure 17). Epithelium containing a microvillar brush border was found only at the distal end of the tentacles and labial palps, and was not found on adjacent areas of the snail body. At the tip, the surface bearing microvilli appeared to be smooth, but proximally

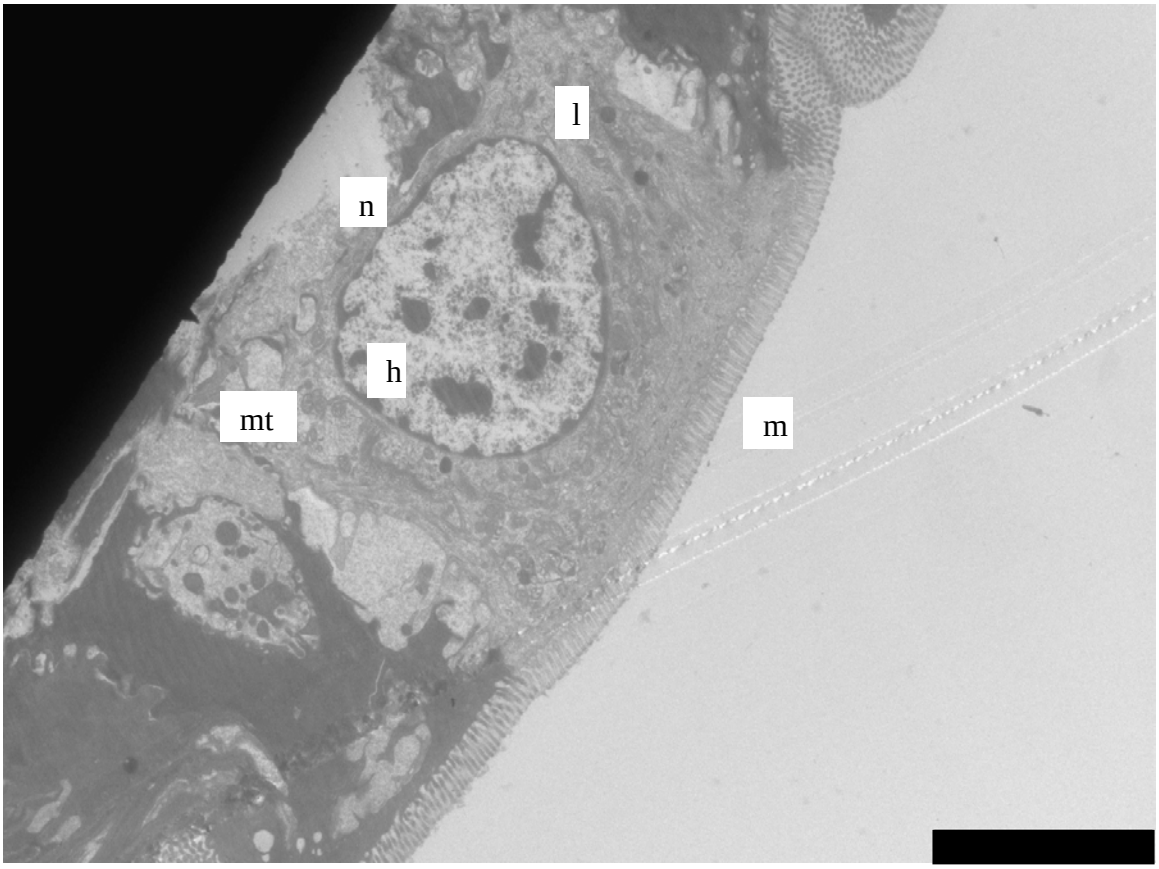

Figure 2 General Epithelial Cell Morphology: TEM micrograph of the epithelial cells showed cellular structures including the nucleus (n), heterochromatin (h), microvilli (m), lysosomes (l), mitochondria (mt) from an *E. rosea*. The center cell is uncharacteristically lightly stained but is shown because the lighter stain makes the cellular structures easier to see. More typical staining can be seen on its neighbors. Scale bar = 5µm.

invaginations were found, which coincide with the appearance of these structures seen with the SEM.

The epithelial cells containing microvilli stained darkly in the TEM preparation (uranyl acetate/lead citrate stain). Neighboring cells and tissues did not appear to stain overly dark, so it is unlikely that the sections were stained improperly. Although dark appearance occasionally made it difficult to make out distinct cellular structures, heterochromatin was seen within the nucleus, and cytoplasmic structures including smooth and rough endoplasmic reticulum, lysosomes, Golgi apparatus and mitochondria were seen (Figure 2).

Anguispira alternata

SEM analysis of the surface of the tentacles showed that the tips appear smoother than the surrounding tissue on both anterior and posterior pairs (Figure 4). Areas where microvilli were separated showed tightly packed microvilli, but they do not appear to be very long. Distinct structures were visible on either side of the mouth on the labial palps. These structures were first clearly visible at 300x magnification as lighter specks. At higher magnification, the structures appeared to be cilia emerging from the microvilli border and were grouped together in small clumps in a concentric circular pattern (Figure 5). These groups of cilia did not have any overall pattern of appearance but were rather irregularly scattered across the area.

The TEM revealed that the tip of the posterior tentacle contained microvilli that extended from the cell surface at the epithelial cell edge through a

convoluted and tightly packed matrix-like layer. The matrix layer had a well defined sharp edge, easily distinguished between the edge of the epithelial cells and the brush border. The matrix material stained lighter than the microvilli and internally had a grainy texture when compared with the dark, evenly stained microvilli. A membrane can be seen around the individual strands of matrix material and each microvillus (see Figure 2, Note: *E. rosea* is the example of matrix material because of the clarity of the images). Where the microvilli extended out of the matrix layer, they were discretely organized parallel to one another at the outer surface of the tentacle tip. This matrix layer contained circular structures consisting of concentric layers of cell membrane. The circular structures were completely covered by the matrix layer and do not appear in any other layer. Proximally along the tentacle, the matrix disappeared and regular microvillar bush border was found to dominate the outer edge of the epithelial cells. No circular structures were found along the tentacle shaft. Moving more proximally, the brush border disappeared completely, leaving a smooth membrane surface.

The above described matrix material was not found on the anterior tentacle tips. Rather the microvillar brush border of the tip was somewhat longer than that found on the epithelium of the tentacle shaft. Otherwise the anterior tentacles were similar to the posterior tentacles.

The labial palps also contained a microvillar brush border. TEM images revealed that the ciliary projections seen in the SEM micrographs contained the typical 9+2 microtubular doublet pattern in cross section (Figure 5). The number

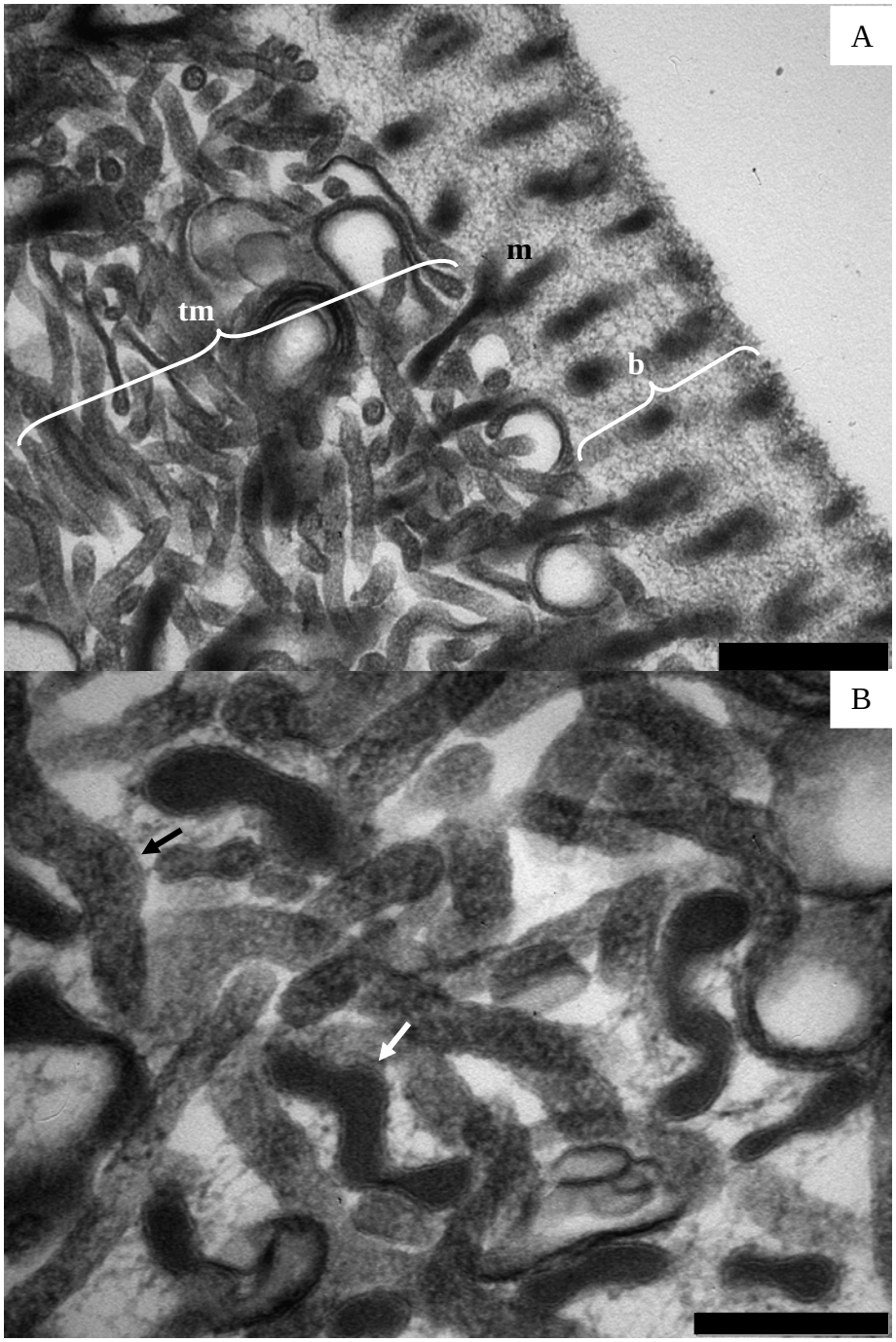

Figure 3 Detail of Matrix of *Euglandina rosea*: TEM of the tight matrix. Image A shows the concise edge of the tight matrix (tm) and the brush border (b). A single microvillus can be seen emerging from the matrix and branching into the border (m). In B the matrix material stains lighter and has a grainy texture (black arrow) when compared with the microvilli (white arrow). The arrows also show the membrane that binds the structures. A scale bar =500nm, B scale bar = 200nm.

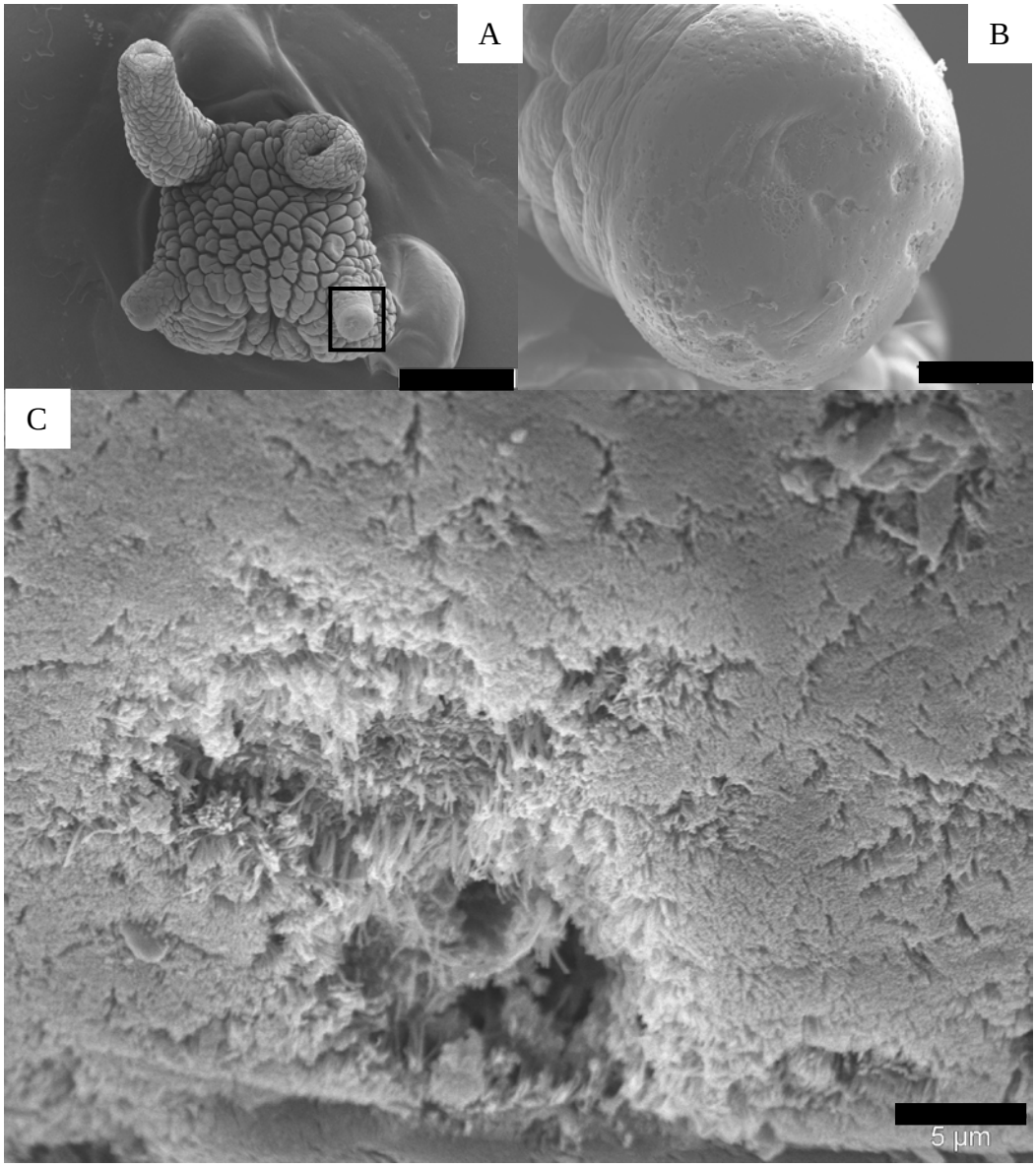

Figure 4 *Anguispira alternata* SEM Images: Image A shows a whole mounted *A. alternata* head. Note the smooth appearance of the partial right posterior (left posterior tentacle is contracted) and both anterior tentacles. B is a left anterior tentacle at higher magnification, while C shows the high magnification of another *A. alternata* specimen of the smooth surface that has been fractured during processing, revealing the underlying microvilli. A scale bar = 1mm; B scale bar = 100µm; C scale bar = 5 µm.

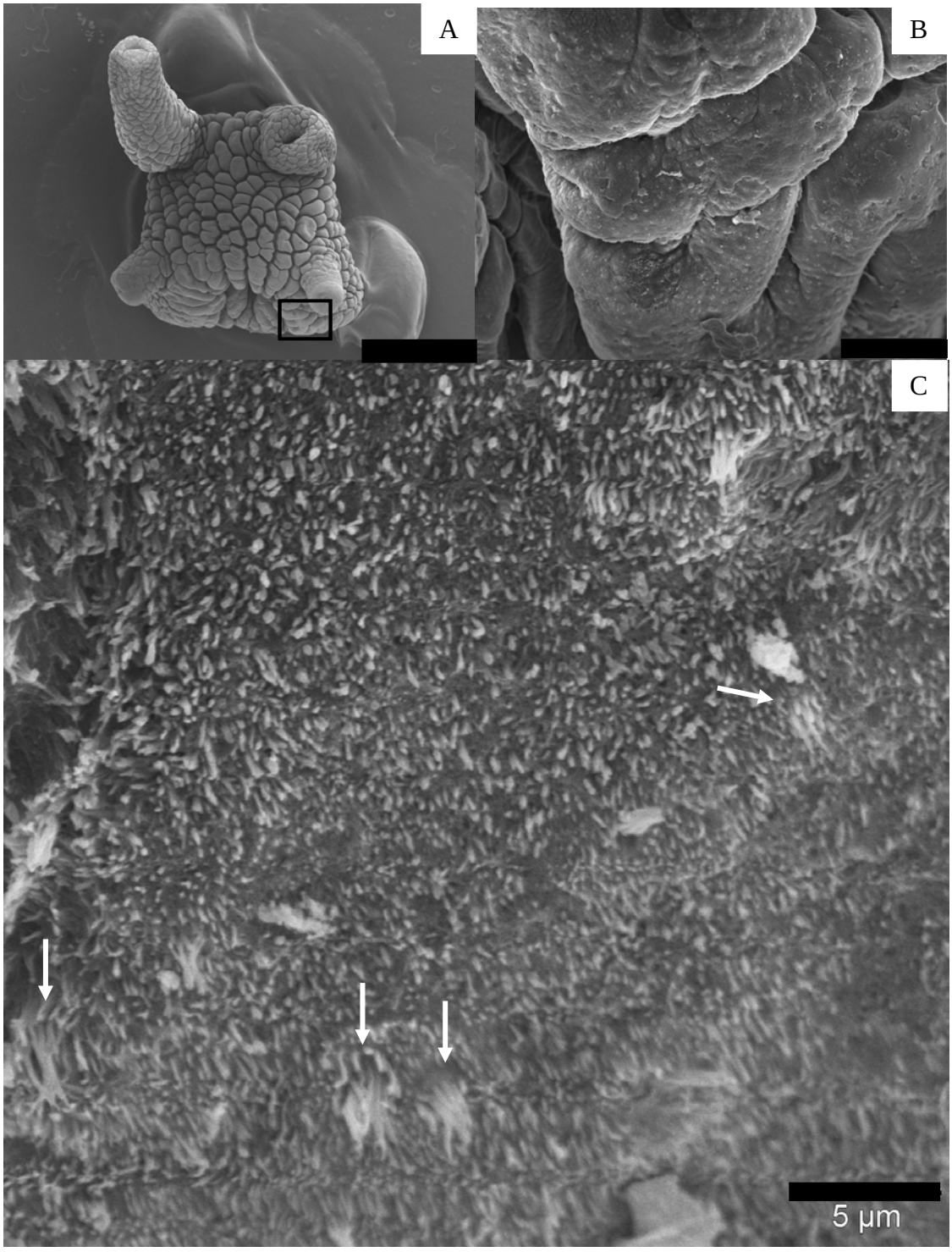

Figure 5 *Anguispira alternata* SEM Images of Ciliated Structures: A shows the labial palp area at low magnification (box). B shows box A at higher magnification, the cilia tufts look like light specks. C shows individual tufts (arrows). A scale bar = 1mm; B scale bar = 100µm; C scale bar = 5 µm.

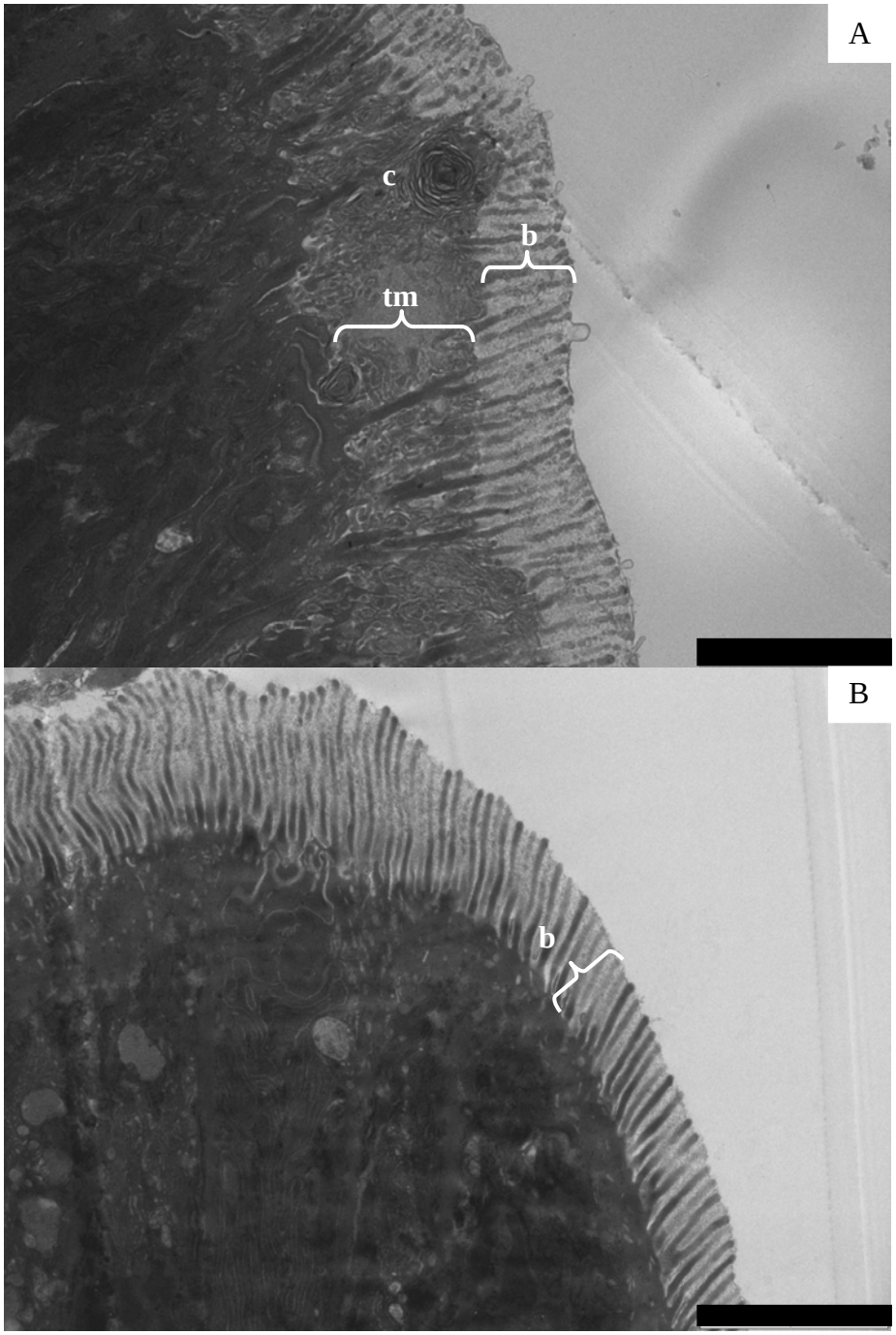

Figure 6 *Anguispira alternata* TEM Images: Image A shows the tight matrix (tm) of the posterior tentacles tip, complete with circular structure (c) just beneath the brush border (b). Image B shows the general microvilli of brush border (b) which is located proximal to the distal tip. A and B scale bar = 2µm.

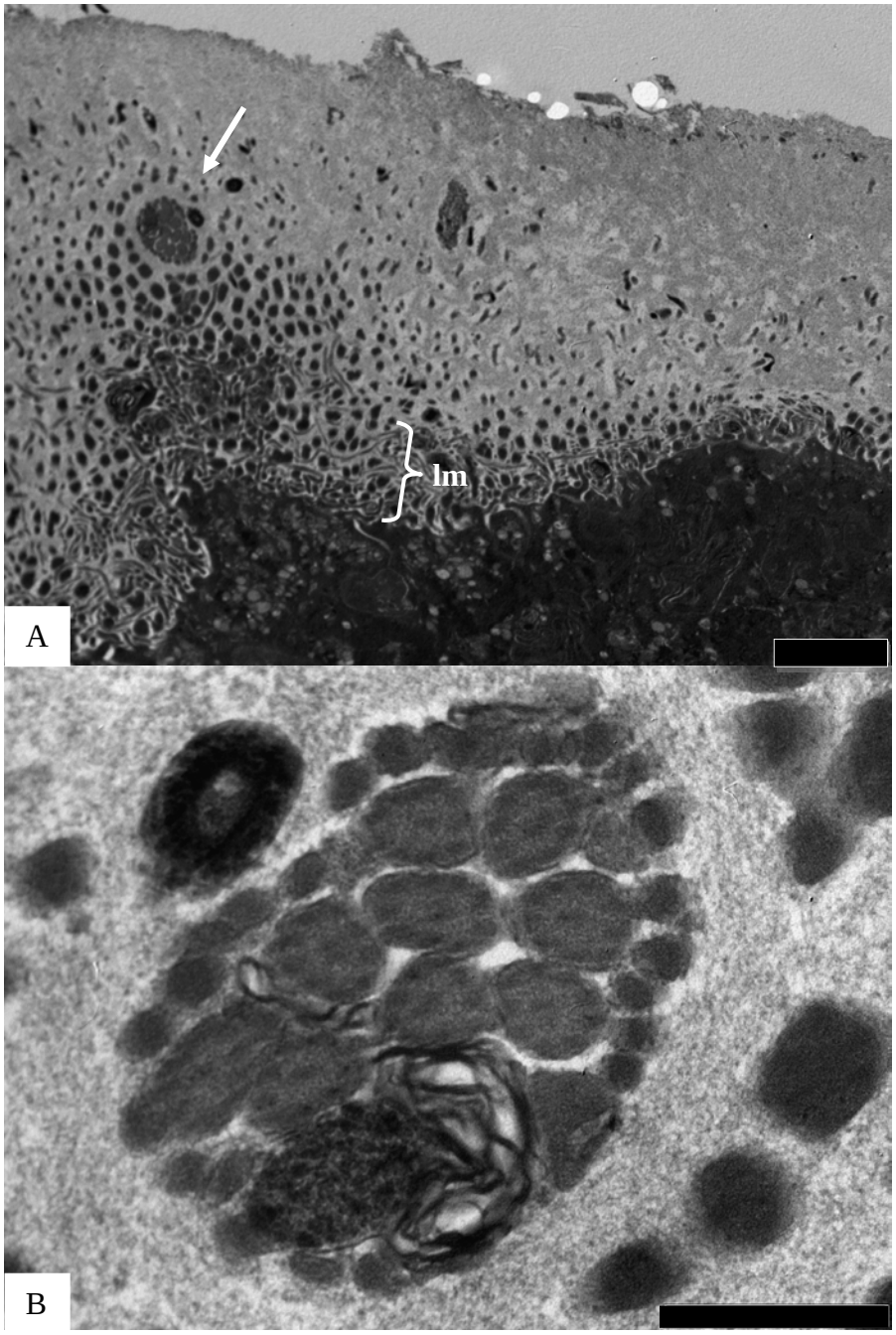

Figure 7 *Anguispira alternata* TEM Images of Ciliated Structures of the Labial Palps: Image A shows the loose matrix (lm) with embedded ciliated tufts (arrow). Notice the ring of microvilli around the tuft in the upper left corner. Image B is the cross-section of a tuft, showing the internal structure of the 9+2 microtubule doublet. A scale bar = 2µm; B scale bar = 500nm.

of cilia contained in each tuft and in each ring was not constant and no numerical pattern could be easily deciphered. The total number of cilia in each tuft varied from 5 to 15. The tufts also appeared to have a ring of microvilli surrounding them. These microvilli were of slightly smaller diameter than those in the nearby brush border but not associated with ciliary tufts. These ciliated tufts and the microvilli extended to the surface through a matrix layer, similar to that described above for the posterior tentacle. This matrix layer is much less organized and not as densely packed as that described in the posterior tentacle. The edge of this loose but uniform matrix layer was less sharply defined than that of the posterior tentacle, unlike the closely packed nature of the tight matrix which had a discrete outer layer of tightly packed parallel microvilli. The loose layer also contained a few small circular structures consisting of concentric layers of membrane; however, these were less numerous and smaller than those found in the posterior tentacles. As described in the posterior tentacles, this loose matrix layer disappeared proximally from the area with the ciliary tufts, leaving only a microvillar brush border, which also shortened and disappeared proximally.

Arion subfuscus

SEM analysis of *A. subfuscus* revealed what appeared to be a covering of small microvilli on the surfaces of interest, similar to that seen in *A. alternata* (Figure 6). No ciliary structures were seen in the areas examined, including the labial palps. TEM revealed a substructure similar to *A. alternata*, with the

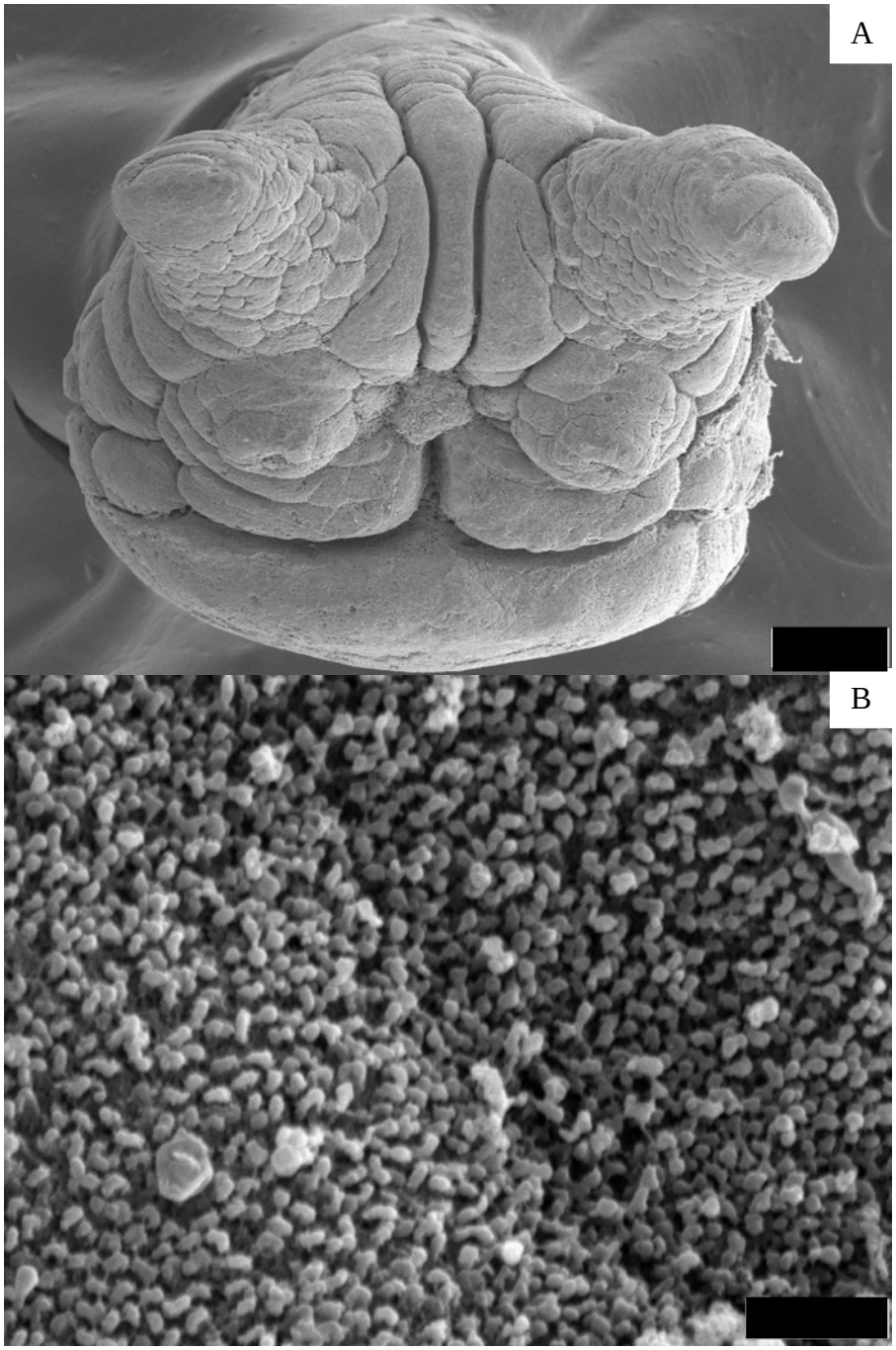

Figure 8 *Arion subfuscus* SEM Images: Image A shows a whole *A. subfuscus* head with the tentacles in various states of extension. Image B is the typical sensory surface, showing the tips of the microvillar brush border found at the tips of all four tentacles and labial palps. A scale bar = 500 µm; B scale bar = 1 µm.

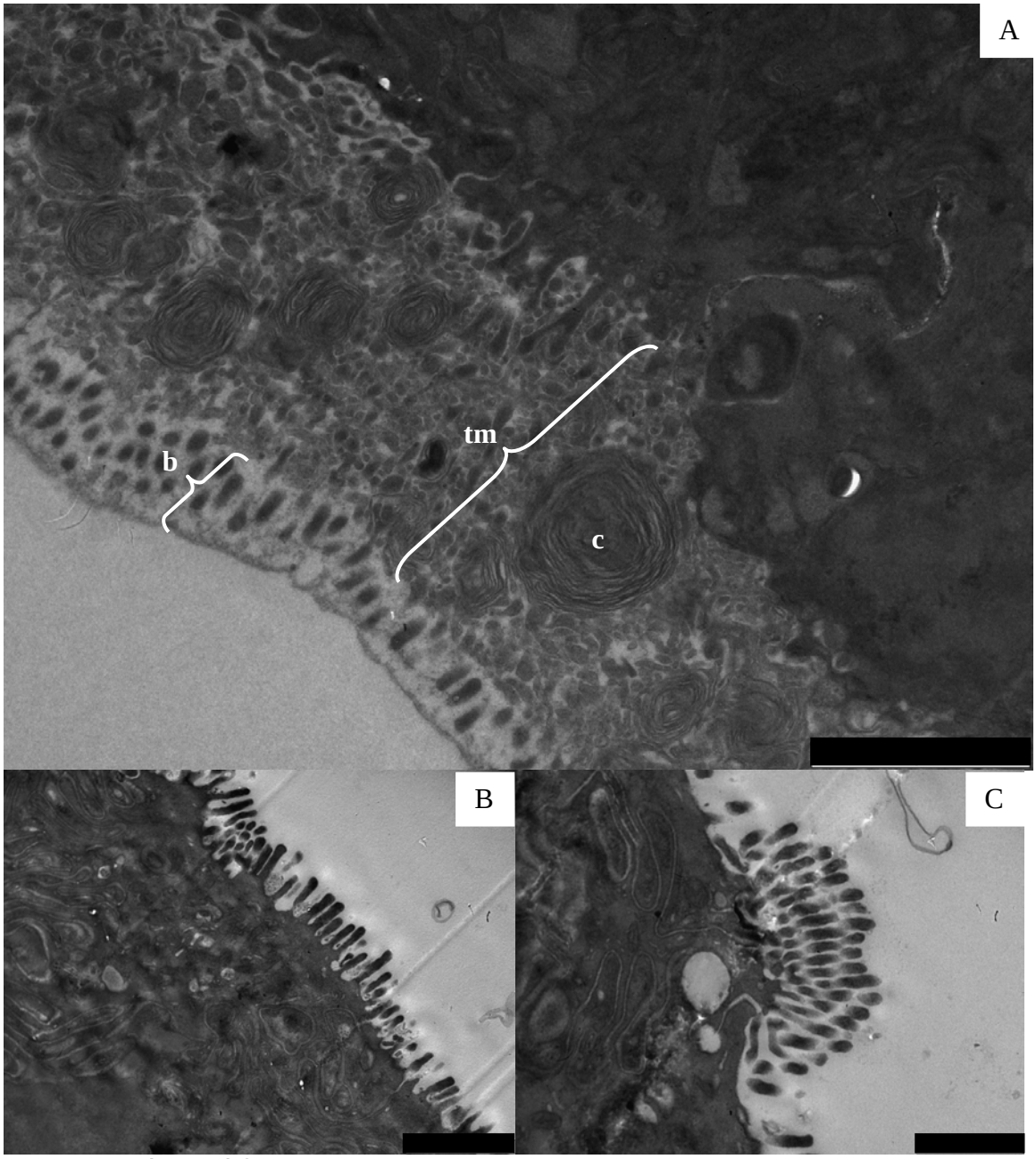

Figure 9 *Arion subfuscus* TEM Images of the Posterior Tentacles: Image A shows the tight matrix (tm), brush border (b) and circular structures (c) of the posterior tentacle tip of *A. subfuscus*. Image B shows the typical non-matrix containing microvilli proximal to A. C shows a cluster of microvilli. A and B scale bar = 2 µm; C scale bar = 1 µm.

posterior tentacle containing a tight matrix layer with embedded large circular membrane structures (Figure 9). Microvilli extended through the matrix at the tip, emerging in parallel rows. Characteristically, the matrix thinned and disappeared proximally; the microvilli reduced in size, and eventually they too disappeared.

The anterior tentacle contained no matrix or circular membrane structures, only a microvillar border. Unlike in other species, microvilli occasionally seemed to be grouped together, but this difference in orientation was not seen in SEM and the clustered microvilli did not appear to be different from other microvilli in the same area.

Unlike in *A. alternata*, the labial palps in *A. subfuscus* did not have any matrix or ciliary structures. The labial palps appeared remarkably like the anterior tentacles. The only structures seen extending from the cell surface were microvilli.

Haplotrema concavum

Unfortunately, the only *H. concavum* specimen prepared for the SEM was destroyed through a laboratory accident before much of any analysis could be made. The only image collected using the SEM was that of the whole head. It can be seen in Figure 10. The tips of the tentacles (but not the labial palps) appeared smoother than the surface of the surrounding tissue. No other differentiation in surface morphology could be seen at low magnification; therefore, all additional analysis for *H. concavum* was done using only the TEM data.

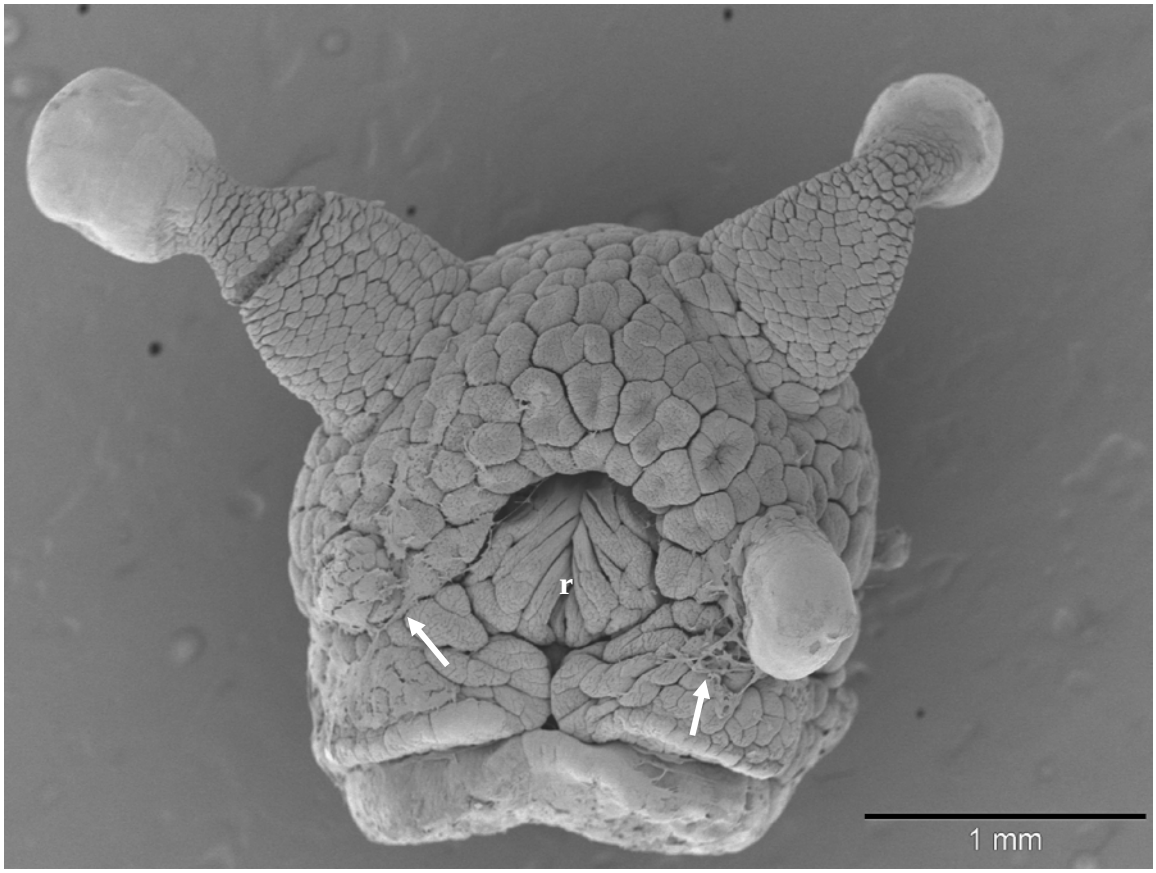

Figure 10 *Haplotrema concavum* SEM Image: SEM image of *H. concavum* showing the smooth appearance of the tentacle tips. Mucous strands (arrows) can easily be seen on some of the surface areas along with the open mouth containing the radula (r) with long slicing teeth characteristic of carnivorous species. Scale bar = 1mm.

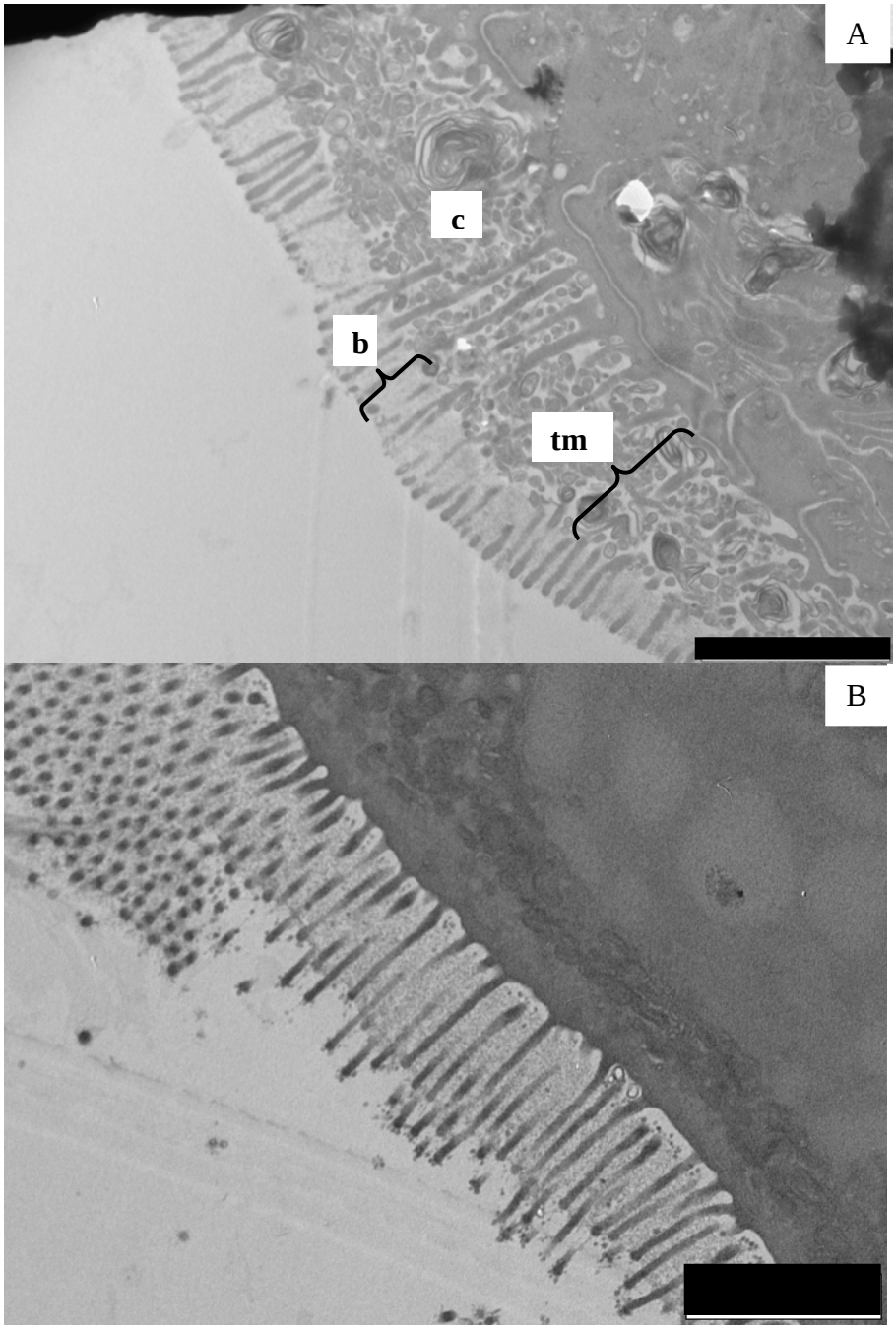

Figure 11 *Haplotrema concavum* TEM Images: Image A shows the matrix layer found on the posterior tentacle tip. Note the highly organized top brush border (b) over the convoluted tight matrix layer (tm) with circular structures (c). Image B shows the normal brush border of the labial palp, anterior tentacle and posterior tentacle proximal to the tip. A and B scale bar = 2 µm.

Cross sectional views of the tip of the posterior tentacle in *H. concavum* revealed microvilli that extended from the surface of the epithelial cell and extended through a dense convoluted middle tight matrix layer to emerge at a highly organized top layer. In this tight matrix layer, circular structures with what appeared to be many layers of concentric circular cell membranes are seen (Figure 11), similar to those seen in the other species. Some microvilli are branched near the middle-top layer border. As one examined medially towards the body, the matrix layer diminished and normal non-matrix containing microvilli appeared. Proximally, the microvilli shortened and eventually disappeared.

The anterior tentacle contained no matrix layer, but was covered in normal microvilli border. No other structures are seen on the cell surface.

The labial palps of *H. concavum* also appeared to be without a matrix layer. The microvilli are very similar in appearance to the anterior tentacle surface. No additional cell surface structures were found.

Euglandina rosea

Overall, the SEM analysis revealed that the tips of the posterior and anterior tentacle of *E. rosea* had a smooth texture similar to the other species examined. At higher magnifications, similar microvillar brush surfaces were seen (Figure 12). Tufts were occasionally seen on both the posterior and anterior tentacles (Figure 12C). Unfortunately these areas were not seen in TEM samples and therefore the internal structure could not be examined; thus it is unknown if

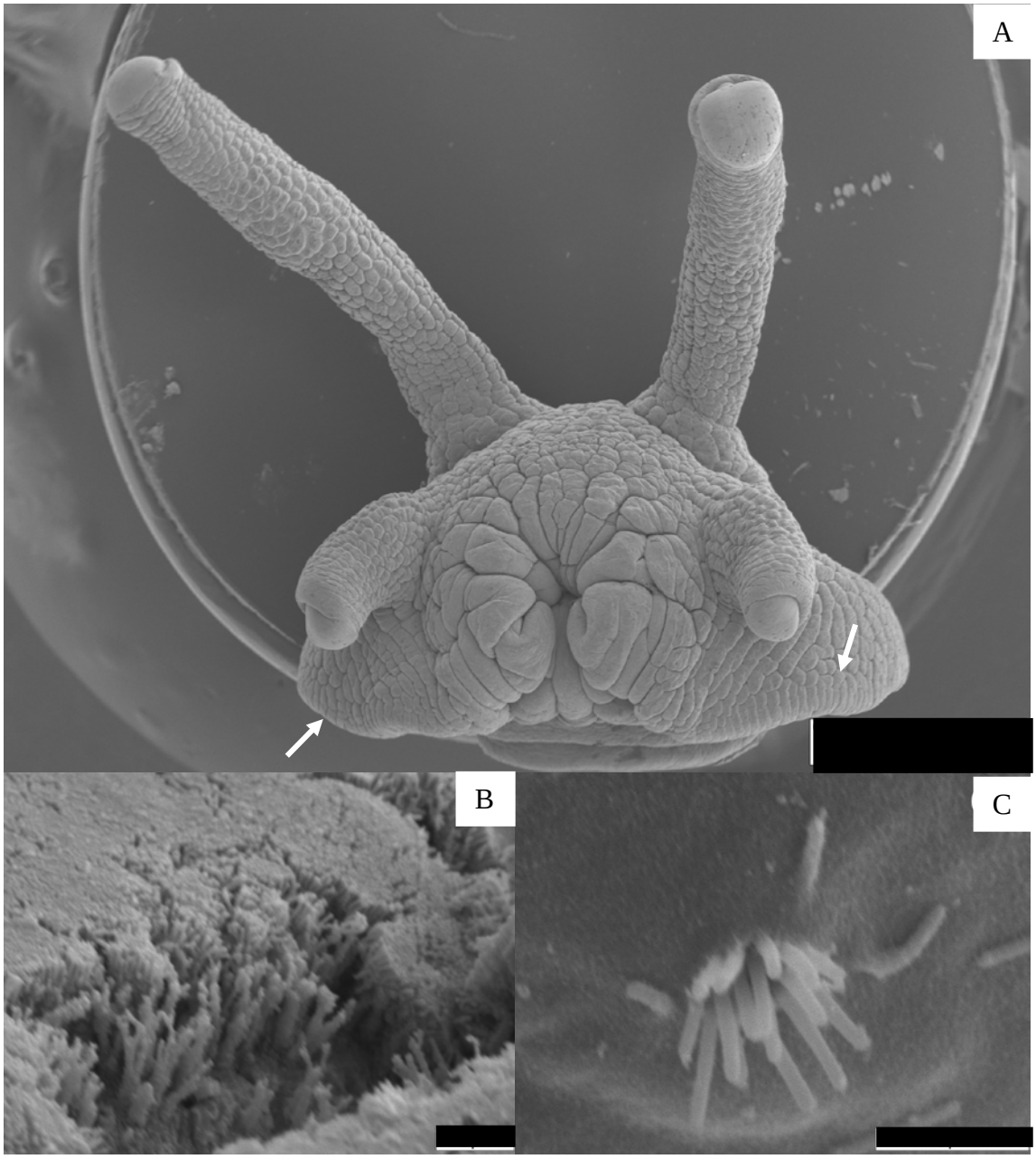

Figure 12 *Euglandina rosea* SEM Images: Image A shows a whole *E. rosea* head with its tentacles at near full extension, the labial palps lay curled along the side of the face with the pleats along the ventral surface (arrow). Image B shows the microvilli at a disruption of the brush border. Image C shows a tuft from the tentacle shaft, which was only seen in SEM. A scale bar = 2mm; B and C scale bar =2 µm.

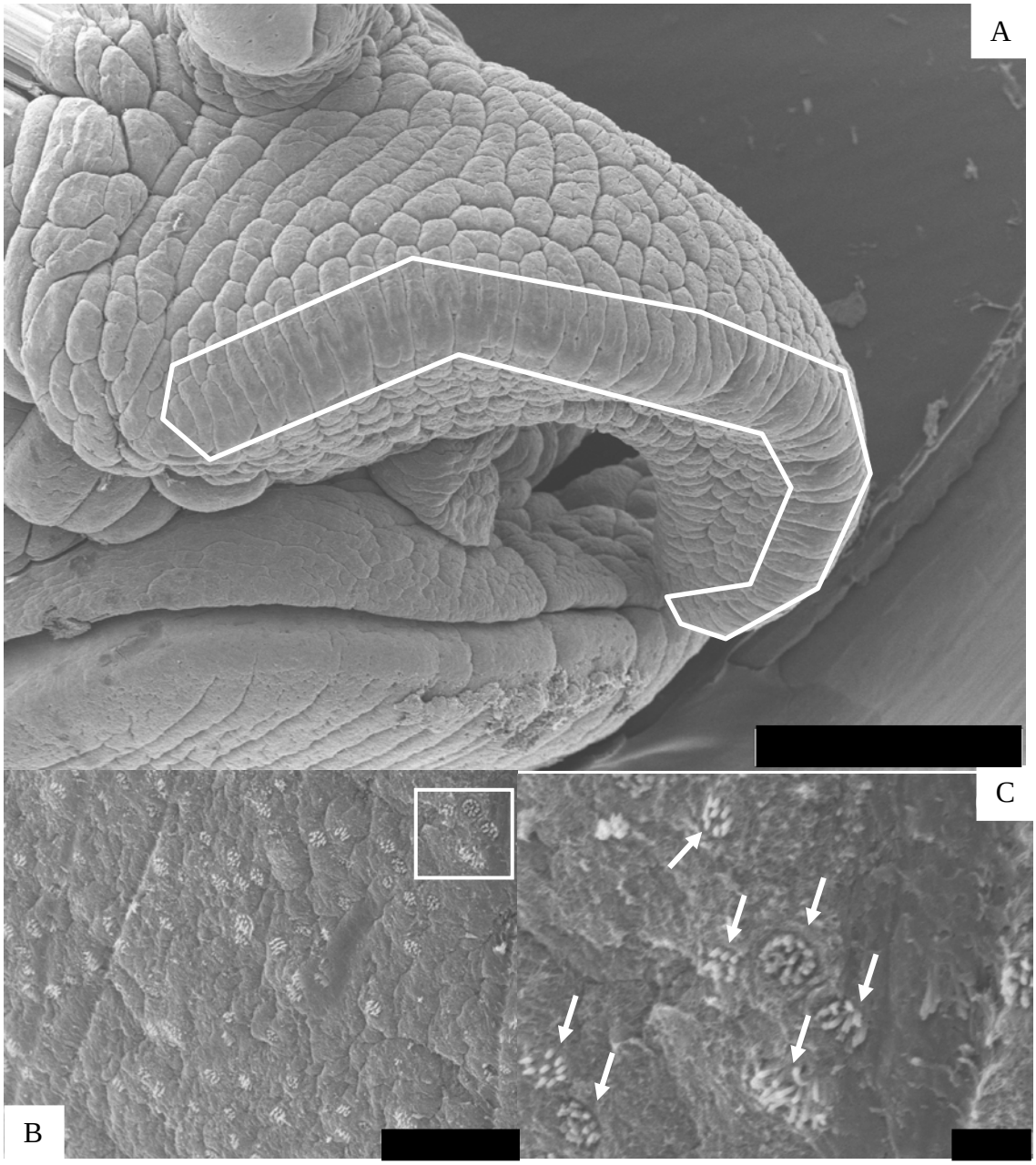

Figure 13 *Euglandina rosea* SEM Images of Ciliated Structures: Image A shows a single labial palp. The highlighted area is where the ciliated tufts were located. Note the difference in surface texture between the highlighted area (described as pleated) and surrounding pebble-like surface. Image B shows the density of the ciliated tufts. C shows the roughly circular grouping pattern of the cilia (arrows) found in box B. A scale bar = 1000 µm; B scale bar = 10 µm; C scale bar = 2 µm.

they are longer microvilli or cilia. SEM images of the elongated labial palps showed tufts of cilia arranged in concentric circular patterns like that of *A. alternata*. The tufts appear in a concentrated strip along the entire leading anterior-ventral edge of the labial palps (Figure 13). This edge, in the relaxed state viewed during SEM analysis, exhibited a distinct texture when compared with the surrounding skin. This area appears to have a vertical rectangular pleat pattern, while the surrounding areas were irregular and pebble-like (Figure 13A). Whether or not this particular epithelial folding pattern is maintained when the labial palps are extended is unknown.

Using the TEM, the tip of the posterior tentacle is shown to have a thick, tight and dense matrix at the tip through which long microvilli extend. The matrix did not appear to contain any circular membrane structures as described in the other species. Areas containing dense concentrations of short microvilli completely covered by matrix were seen in the distal tip (Figure 14). Matrix material also was seen within intercellular pockets contained in the cytoplasm of the epithelial cells, some of which opened to the extracellular space (Figure 16). These areas were only found on the posterior tentacles of *E. rosea*.

The anterior tentacle, unlike in the other species, was found to have a tight matrix layer similar in density and structure to the posterior tentacle. More proximal to the body, the matrix eventually disappeared and the microvilli appeared shorter until completely absent.

The TEM revealed images of the labial palp structures that resembled those found in *A. alternata*. The loose matrix of the labial palps appeared less

tightly organized and packed than those found in the tentacles. There appeared to be no circular membrane structures in either the matrix or non-matrix containing areas. Cross sections of the tufts show characteristic cilia morphology. The ciliary roots extend into the cell, but the depth to which they penetrate could not be determined (Figure 15). These tufts also appear to be surrounded by a ring of thinner microvilli, similar to those seen in *A. alternata*. The ciliated tufts on *E. rosea* do not appear to have a specific number of cilia, as those seen ranged from 6 to 17. Distal to the surface of the matrix the microvilli are highly organized and parallel to each other forming a brush border. The matrix is thinner close to the base of the labial palp where disappears, leaving the brush border which also becomes thinner and disappears proximally.

A comparison of the structures and areas found across the species examined is summarized in Table 1.

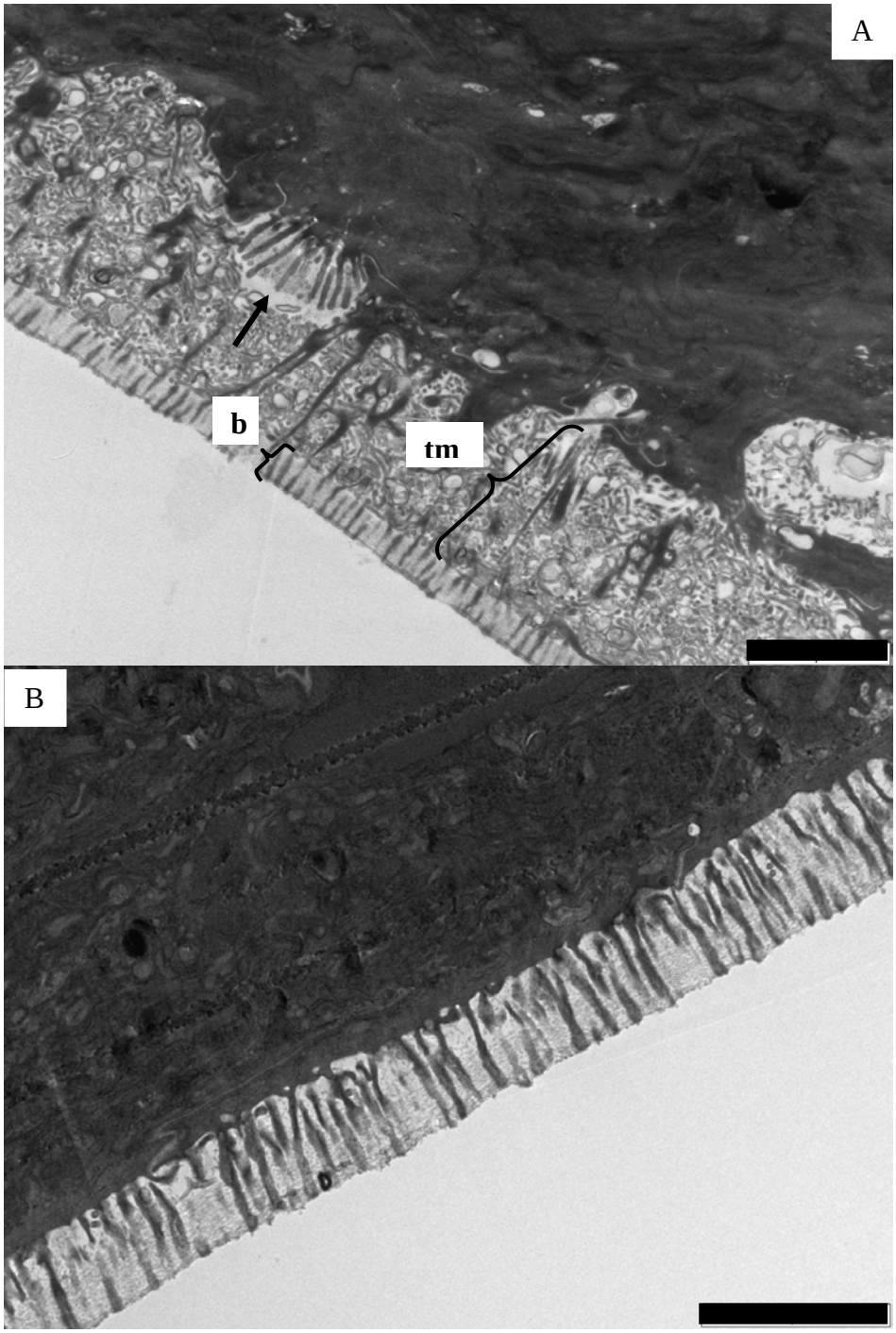

Figure 14 *Euglandina rosea* TEM Images of the Posterior Tentacles: Image A shows the tight brush border (b) and matrix layer (tm) of the posterior tentacle tip, but without circular structures. Note the short microvilli covered by matrix (arrow). B shows the brush border without any matrix proximal to A. A and B scale bar = 2 µm.

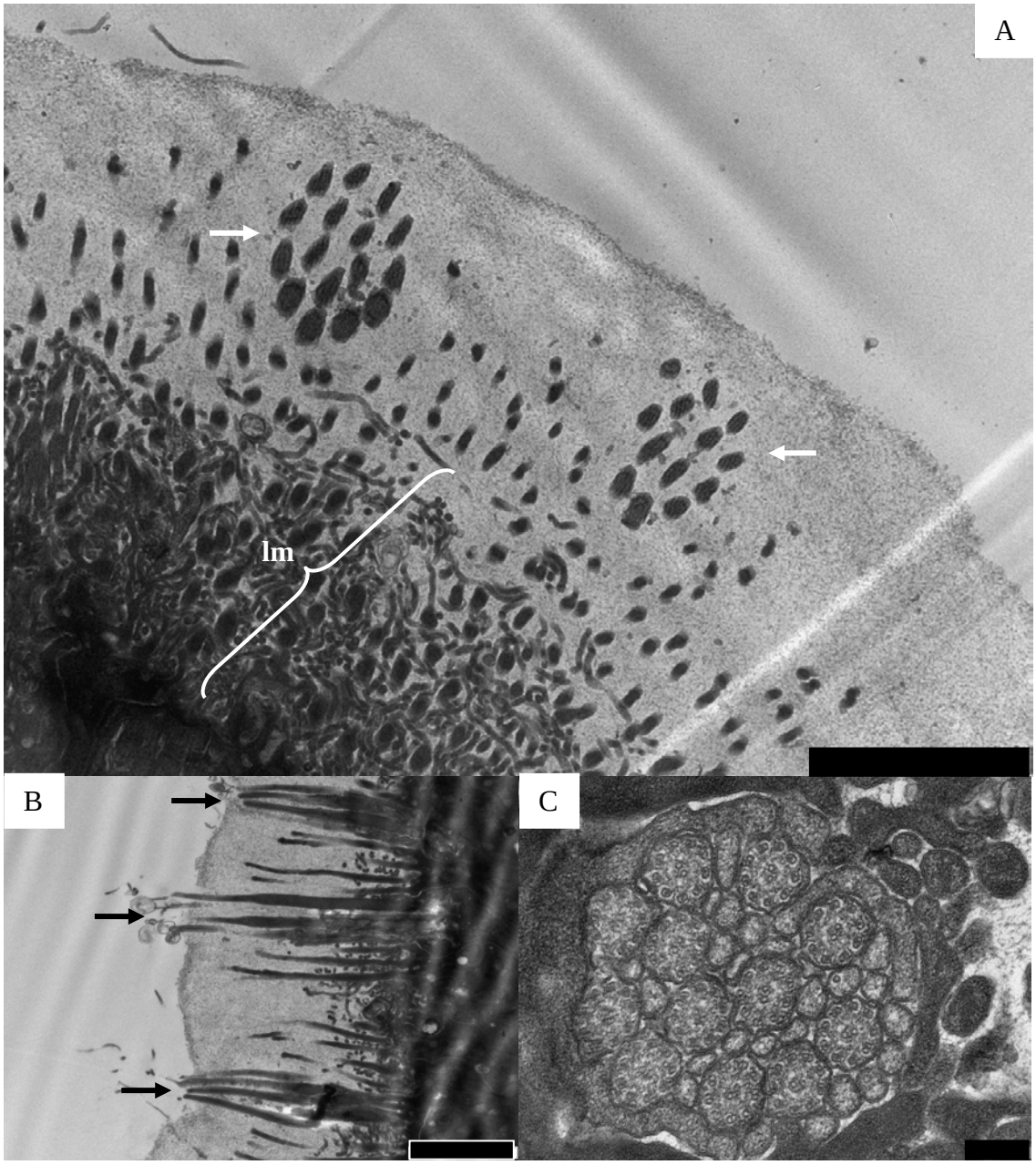

Figure 15 *Euglandina rosea* TEM images of Ciliated Structures of the Labial Palps: A shows a cross sectional view of the ciliated tufts (arrows) above the loose matrix (lm) of the labial palp. B shows the ciliated tufts cut longitudinally (arrows). C shows a high magnification image of the cross section of the ciliated tuft with stereotypical 9+2 doublet internal structures. A and B scale bar = 2 µm; C scale bar = 200 nm.

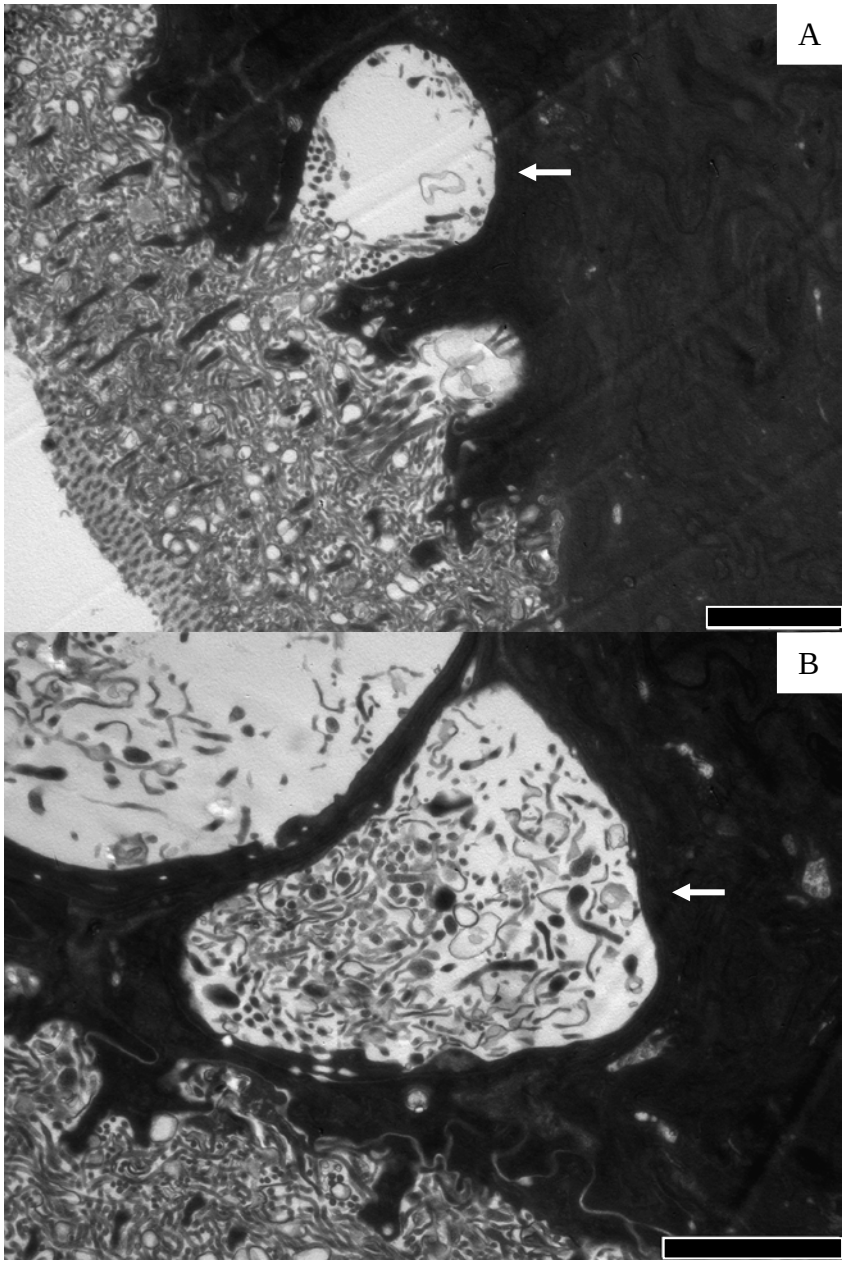

Figure 16 *Euglandina rosea* TEM images of Cellular Spaces Containing Matrix Material in the Posterior Tentacles: A and B: TEM images of *E. rosea*, showing the possible cellular origin of the extracellular matrix found on the posterior tentacles. These kinds of structures (arrows) with matrix looking material were not found in any of the other species examined. A and B scale bar = 2µm.

	Posterior Tentacle			Anterior Tentacle		Labial Palp		
	Matrix	Circular Structures	Microvillar Border	Matrix	Microvillar Border	Matrix	Ciliated Tufts	Microvillar Border
Aa	Yes tip	Yes	Yes	No	Yes	Loose tip	Yes tip	Yes
As	Yes tip	Yes	Yes	No	Yes	No	No	Yes
Hc	Yes tip	Yes	Yes	No	Yes	No	No	Yes
Er	Yes tip	No	Yes tip	Yes	Yes	Loose tip	Yes tip	Yes

Table 1 Table of Ultrastructural Elements by Species: This table shows which ultrastructural elements were found by species and area of interest. Aa = *Anguispira alternata*, As = *Arion subfuscus*, Hc = *Haplotrema concavum*, Er = *Euglandina rosea*

Figure 17: Diagram of Ultrastructural Elements Examined Across Species

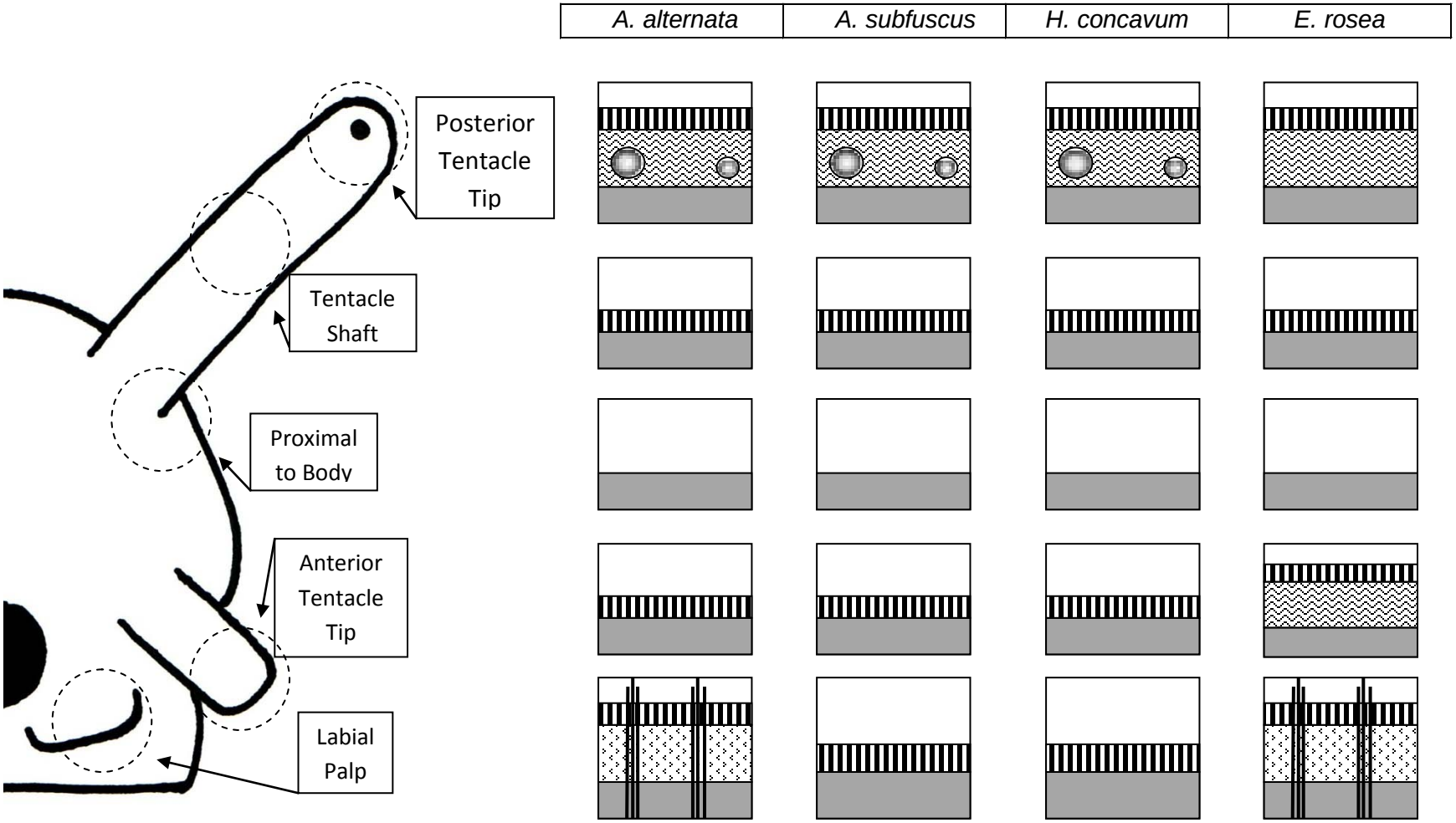

Figure 17: Key : Epithelial Cells Microvilli Tight Matrix Loose Matrix Circular Structures Ciliated Tuft Exterior

Chapter 4: Discussion

The hypothesis that snails with similar feeding strategies would have similar epithelial morphology was not supported in the species examined in this study. Every sensory area examined had similar microvilli. Other structures not previously described in land snails were found including a matrix layer with two different densities, the circular membranous structures and tufts of cilia. These three structures were found in different areas in different species (Table 1), but they did not correlate with feeding behavior. Even though the original hypothesis was not supported by the evidence found, many new and interesting findings suggest further study.

Microvilli of the Tentacles and Labial Palps

In all of the species used, a general pattern was seen in the tentacles and labial palps. The distal tip of the tentacles' epithelial cells stain darkly and the tip surface is smooth and includes a matrix layer in all of the posterior tentacles examined. *E. rosea* also exhibits a matrix layer in the anterior tentacle. The labial palps showed a similar staining pattern at the leading ventral edge. The labial palps of *A. alternata* and *E. rosea* contain a loose matrix. The microvilli and cilia (when present) seem to pass through the matrix layer. Once free of the matrix, the microvilli align and form a tight brush border. The microvilli could occasionally be traced from the edge of the cell through the matrix, sometimes branching where they extend through the matrix (Figures 6A, 11A and 14A). Proximal to the tip of the tentacle tip (and proximal to the leading edge of the

labial palps in species that have matrix) the matrix is reduced and replaced by a brush border of microvilli. The microvilli also reduce in length proximally until only a smooth microvilli-free epithelium remains.

Several functions for microvilli of sensory structures have been suggested. They may serve as chemo- or mechanoreceptors, protection for receptors deeper in the tissue or they could merely increase cell surface area (Chase, 2002). This range of the possible functions can suggest different interpretations of the results. Carnivores appear to follow slime trails to find a food source and do not follow scent cues carried through the air when hunting prey (Shearer and Atkinson, 2001). If it is assumed microvilli are involved with chemoreception, then it would be expected to find microvilli prominently on the labial palps and, to a lesser extent on the anterior tentacle tips as these structures brush along the ground during trail following. Conversely, the microvilli on the posterior tentacle tips would be reduced or nonexistent as they almost never come in contact with the ground when trail following. The presence and morphology of microvilli did not correlate with the preconceived assumptions about the chemosensory areas. This suggests the function of the microvilli may not be chemosensory but may be mechanosensory or serve as a protective covering for underlying receptors. Until more research is done and the exact function microvilli perform on the tips of gastropod sensory structures is determined, these questions cannot be satisfactorily answered nor can interpreting their presence or absence in a behavioral context be assessed. This could be accomplished by tracing the sensory neurons from patches of microvilli to see if they are processed in known

regions of the brain that pertain to olfaction like the procerebrum, though exactly what olfactory information the procerebrum processes is debated (Chase, 2002). Emin Oztas' (2003) paper *Neuronal Tracing* compiles several tracing techniques that could accomplish this task. A more plausible method to find if chemoreception is occurring in the microvilli is to search for some of the proteins needed for chemical signal transduction (Hofer *et al*, 1999). These proteins could be tagged using immunogold labeling techniques and then viewed using TEM. Physiological data then could be correlated with behavioral studies in more herbivorous and carnivorous species to see if these possible chemoreceptors are used for just locating food, locating potential mates/chemical signals or a combination of both. Determining what kind of chemicals these receptors specifically detect, while outside the scope of the above described techniques, could also be another avenue of research to better understand the sensory world of these organisms.

Matrix

A matrix layer at the distal tip of the posterior tentacles is present in all species. *E. rosea* also had such a matrix on the tip of the anterior tentacles. *A. alternata* and *E. rosea* were the only two to have matrix on their labial palp regions, though this matrix appeared to be a less dense than that found on the posterior tentacle (Table 1). When present, the matrix was only found on the distal tip of the tentacles or leading edge of the labial palps, not along the shaft of the tentacles or in the surrounding tissue of the labial palps. The matrix forms a

layer between the edge of the epithelial cells and the brush border of microvilli. The matrix can be very tightly and densely packed material with discrete edges or loosely organized in a semi-indistinct layer. The matrix layer may originate from within the epithelium (Figure 16). However, evidence for this was found only in *E. rosea*. Thus further investigation is needed to see if the origin is intracellular and if this holds true for other species and other matrix containing areas.

Within the matrix, membranous circular or ciliary structures can be found. In all but two instances (*E. rosea* posterior and anterior tentacle tips), the presence of the matrix coincided with the presence of either circular or ciliary structure. The matrix may serve as a cushion or support system for long microvilli, perhaps also protecting the circular and ciliary structures from damage. If the matrix adds a cushion between these structures and the environment, a likely area to find the matrix would be the posterior tentacles' tips, as they are more likely to brush against objects first in the environment, given their length and location. During hunting/trail following, the handlebar-shaped labial palps of *E. rosea* are actively being touched to the ground and other species trail follow to find mates etc; but the matrix was only found on the labial palps in half the species examined. The anterior tentacles sometimes come in contact with the ground during foraging by all of the species examined, but only *E. rosea* had matrix in this area. An extracellular matrix containing tethers increased sensitivity to sheering forces for mechanoreception in invertebrates such as nematodes (Fritzsch *et al*, 2007). This may explain the presence of the matrix in areas important to mechanoreception, but none have yet been described in this

capacity for gastropods. The matrix may also play a role in scent capture similar to that of a coating of mucus. If the presence of a matrix layer could be correlated with confirmed olfactory structures, this would support such a hypothesis.

Ciliated Tufts and Circular Structures

In addition to the previously undocumented matrix, the ciliated tuft structures of the labial palps of *A. alternata* and *E. rosea* had not previously been reported in either species. Both species had groups of cilia arranged roughly in two rings (the unit here being called a 'tuft'). The tuft was surrounded by another ring of microvilli specifically associated with the tuft. The cilia extend through the matrix layer and past the brush border. How deep the cilia and ciliary root extend into the tissue is not clear. If subsequent research finds that the root extends beyond the epithelial layer into neural tissue, this may strengthen the case for a sensory role for these cells. These structures are found only on the labial palps of *A. alternata* and *E. rosea*, and not in the tissue surrounding the oral cavity itself. In *E. rosea* they are specifically located on the area of the labial palps that comes in contact with the ground most often when the animal is actively trail following. The function has yet to be determined, but examination of behavior between species with these tufts and those without may help clarify their role. Perhaps they are specifically used for circulating mucus for tasting as the animal is trail following in general, either for hunting, following others to stationary food sources or for finding receptive mates. It should be noted that only in *E. rosea* the areas on the labial palps that contained tufts took on a pleated appearance, but

this was not the case for *A. alternata*. Whether these specialized cilia function in a chemo- or mechanoreceptor role is unknown. Ciliary tufts were described by Kunz and Haszprunar (2001) in the gastropod *Patella caerulea*; however, their role was not fully investigated but was postulated to be used to circulate water. Tracing these ciliary structures to neurons excited by odor cues would strengthen the hypothesis that they are used in olfaction and not for generating water currents. To generate currents, the cilia would have to be motile. Motile cilia contain dynein arms which link the microtubule doublets and allow for movement. (Cross and Mercer, 1993). Dynein arms can be imaged with TEM, but the clarity of the images captured in this study were not sufficient to determine their presence in these cilia.

Circular structures were also found in the matrix layer. They appear to be layers of concentric membrane superficially similar to the layers of an onion. These structures were found in the posterior tentacles of three out of the four species examined (*A. alternata*, *A. subfuscus* and *H. concavum*). These structures do not appear to be associated with cilia or the surrounding microvilli. Superficially they resemble mechanoreceptors found in mammals or insects, specifically Meissner's or Pacinian corpuscles (Freeman and Bracegirdle, 1976), although these structures lie deeper in the tissue and have a connection to the nervous system. It is not believed that they are cellular debris or dead cells, given their very specific location (only tentacle tips and outside of the cells) and their highly structured internal layering. Behaviorally, the tentacles can be retracted extremely quickly in response to even very light touch stimuli. Thus, it is

reasonable that these structures serve some kind of mechanoreceptor, but further study is needed to fully characterize their function. Why these structures are not seen in *E. rosea* remains a puzzle. Given the density and ease of identification of these structures in the other species examined, it is unlikely that they were missed via human error, though the low number of *E. rosea* samples examined may have led to their being missed through random chance. Barring that, *E. rosea* could have lost its circular structures through the course of its evolution, thus specializing its behavior towards hunting. The eyes of *E. rosea* are also located at the tentacle tip. The eyes of gastropods are not thought to be image forming and are not associated with foraging or hunting behavior but with phototaxis, circadian locomotion and detection of movement (Chase, 2002). Protection of the eyes via mechanoreceptors may not have been as evolutionarily important as developing specialized olfactory receptors and/or structures that greatly improve food gathering abilities for some species. This needs to be researched further by examining other known carnivorous species such as those in the families Rhytidae (*syn.,* Paraphantidae) and Streptaxidae.

Conclusion

The focus of research on model organisms has left gaps in the understanding of gastropod chemosensory capabilities. It is no longer enough to use a handful of species to generalize across a multitude of clades. This tactic overlooks specialized structures, leaving the larger picture incomplete without their possible behavioral and neuroanatomical significance. In depth study of the

ultrastructure of epithelial tissue seems to be overlooked in favor of gross neuroanatomical data like that of *Achantina fulica* (Chase, 1986). Research correlating ultrastructure or neuroanatomical findings to directly observable behavior is rare. There are a few instances where specialized sensory structures were directly linked to behavior. Murray (1971) found that direct chemical stimulation of the *Navanax*'s anterior lateral folds evoked turning behaviors. Upon further investigation, specialized cilia-containing structures called the phalliform organ were discovered only in this area. Matera and Davis (1982) described dense fields of cilia in behaviorally significant areas ending with a paddle-shaped bulb on the rhinophores and tentacles of the marine gastropod *Pleurobranchaea californica.* These were theorized to be chemoreceptors. With more unique structures being discovered, particularly those containing cilia like the ciliated tufts of *E. rosea* and *A. alternata*, the possible link to behavior needs to be more concretely established. Conversely, these structures may relate more to mate finding and recognition than to foraging for some species; thus a reexamination of a wider range of behaviors and the presence of specialized structures is warranted.

This connection between behavior and structure can be difficult to confirm because of the nature of the delicate and time-consuming research techniques needed to examine sensory areas. A multifaceted approach involving descriptive morphology, behavior, neuroanatomical mapping and various molecular biological surveys, including immuno-gold labeling for proteins needed for chemical signal transduction, should be employed to create a truly

comprehensive understanding of the relationship among microvilli, specialized ciliated structures and matrix. Also, discovering the origin of the matrix, either extra- or intra-cellular, and its makeup may help explain its function and relationship to sensory structures.

One explanation for the pattern of specialized structures seen in the species examined may be the result of the close phylogenetic relationship between *A. alternata* (Enodontidae) and *E. rosea* (Oleacinidae), while *A. subfuscus* (Ariondae) and *H. concavum* (Haplotrematidae) are further apart (Barker, 2001). The presence of loose matrix and ciliated tufts may have arisen from sharing a common ancestor and not because of any behavioral differences. The classification scheme used by Barker (2001), while including a wide range of 57 genetic and morphological characteristics including alimentary, renal, neurological, muscular, genital, developmental, reproductive and cytological characteristics, the scheme does not include any consideration of microvilli or sensory areas other than eye position. This is probably because there are not enough data available in enough different species to include it in the evaluation. A second phylogenetic tree appears in Barker (2001), which uses the same characteristics but also constrains the data to conform to the 28S rDNA sequence phylogeny. In this version, the two closest related groups are those that include *E. rosea* and *A. subfuscus,* not *A. alternata*. To clarify, an investigation of other species, both closely and distantly related to *E. rosea, A. subfuscus and A. alternata* is needed to see if they share similar structures because of a shared common ancestor. Kunz and Haszprunar (2001) previously

suggested that a more thorough examination of ciliary tuft and sensory structure morphology of the epidermis may help with phylogenetic reconstructions. Expanding the focus from a few key species to a broader scope encompassing many clades will create a clearer picture of the relationships and traits shared among groups of organisms. Although the original hypothesis that differences in sensory structures are correlated with differences in foraging behavior was not borne out, analysis of a variety of different gastropod species from a wide range of different clades is necessary to determine if such differences correlate with phylogenetic position.

APPENDIX

Appendix

Unsatisfactory Methods

Anesthesia

The use of diluted chloretone in water (Lee, 1950) appeared to result in satisfactory extension of the head and foot. However, when touched, the snail immediately retracted into the shell. Chilling the solution to 10° Celsius helped to slow the animal's reaction times. Nevertheless, tentacle extension was only moderately successful. Dissolving a few chloral hydrate crystals into water containing the specimen (Lee, 1950) met with little success. A 5% solution of ethanol in water (Flores, Salas and Saavedra, 1983) was used. Although there was some relaxation with some tentacle extension while in the solution, removal of the animal from the water to begin dissection resulted in contraction. Although on some occasions, waiting for the animal to re-extend its tentacles was possible, this usually required several hours, making it impractical. Methanol was tried (Lee, 1950) with unsatisfactory extension. Mechanical restraint was also considered, but abandoned. A commercially available topical anesthesia (benzocaine) was tried, but was not successful. Use of liquid nitrogen did not successfully preserve extension and caused major tissue damage visible with the naked eye. Injection with Ringer's solution plus 0.5% succinylcholine chloride was used on several animals (Chase and Croll, 1981). This method worked well for animals without shells, as the combination of anesthesia and increased internal pressure from added fluids expanded the tentacles nicely. Animals with shells were more difficult; if they retracted into the shell, any further benefit from

anesthesia was rendered useless. This method also seemed to have a high mortality rate for those that did not extend well.

Mucus Removal

To remove the mucus coating, several methods were considered including using glycosidase (Monteiro-Riviere and Jiang, 1986), glucosidase (Nilsson, Hellstrom and Hedlund, 1992), and pronase (Saito *et al*., 2002). These methods proved to be too cost prohibitive to pursue and may have the unwanted side effect of damaging the microstructure of the underlying cells.

Confocal Laser Scanning Microscopy (CLSM)

1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI) manufactured by Invitrogen (Carlsbad, California) was determined to be the best neural tracing dye to study the path the olfactory signal may take into the brain. Using an investigation of DiI penetration in cortical slices of rats (Kim *et al.,* 2007) as a basis, a study of what temperature and amount of fixation was best for crystal dye penetration in the gastropod brain was performed.

An entire brain was dissected from an *A. alternata*. The cerebrum and visceral loop were removed and bisected to test the following parameters on identical tissue. The cerebral ganglia were placed in buffered Ringer's solution, with one half kept at room temperature (21°C) and the other cooled to 10° Celsius. The visceral loop was used to test 1.5% paraformaldehyde solution in 0.1 M phosphate buffer with one half kept at room temperature and the other at

10º Celsius. Each quarter of the brain was placed in its fixative solution for one hour. After this incubation, each section was quickly dried by carefully whisking away the liquid with an absorbent Kimwipe, but not touching the tissue. The tissue was then placed in a depression slide and a crystal of DiI was positioned next to the cut end of the connective of interest with a small amount of petroleum jelly: the optic connective for the cerebrum halves and the parietal nerves on the visceral loop (depending on which one was longer and easier to manipulate). The depression was then filled with treatment solution. A ring of petroleum jelly secured the cover slip. This step proved to be very important not only for the use with the inverted confocal microscope but also to prevent evaporation of the liquid. The samples were examined after 12 hours and again after nine days. Although the Ringer solution worked better than the paraformaldehyde, the dyeing was inconsistent. Olympus FluoView FV1000 Laser Scanning Confocal Microscope (Center Valley, PA) and Zeiss LSM 5 Pascal Laser Scanning Confocal Microscope (Thornwood, NY) were used to image the results.

To increase consistency, a gel version of DiI was tested along with a one hour fixation in 1.5% paraformaldehyde solution followed by mounting in buffered Ringer's on another cerebral ganglia preparation. This method yielded exciting results, with dye seeming to be drawn into the brain rather consistently along the connective, with definition of structures increasing up to two weeks post incubation, with no bacterial growth. There did appear to be some lysis of cells at this point, but it did not appear to affect the dye's progress. Unfortunately, due to the thickness of the brain, the fluorescence could not to be resolved once the

connective entered the cerebrum. A solution for this issue would be to fix, dye and then embed the dyed brain; then, three-dimensionally scan several thickly sliced brain sections and reconstruct the path of the dyed nerves through stacking and reconstructing of the full brain. Such an analysis was not undertaken in this study.

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