The Histology of *Nanomia bijuga* (Hydrozoa: Siphonophora)

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Abbreviated Title: Histology of *Nanomia bijuga*

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Abstract

The siphonophore *Nanomia bijuga* is a pelagic hydrozoan (Cnidaria) with complex morphological organization. Each siphonophore is made up of many asexually produced, genetically identical zooids that are functionally specialized and morphologically distinct. These zooids predominantly arise by budding in two growth zones, and are arranged in precise patterns. This study describes the cellular anatomy of several zooid types as well as of the stem and gas-filled float, called the pneumatophore. The distribution of cellular morphologies across zooid types enhances our understanding of zooid function. The unique absorptive cells in the palpon, for example, indicate specialized intracellular digestive processing in this zooid type. Furthermore, there are multiple areas of both endodermal and ectodermal epithelial complexity. Though cnidarians are usually thought of as mono-epithelial, we characterize at least two cellular populations in this species which are not connected to a basement membrane. This work provides a greater understanding of epithelial diversity within the cnidarians, and will be a foundation for future studies on *Nanomia bijuga*, including functional assays and gene expression analyses.

Introduction

Siphonophores are pelagic hydrozoans (Cnidaria) with a highly complex development and morphological organization. Many pressing questions regarding the biology of siphonophores remain unresolved (Pugh, 1999). In particular, there has not yet been a systematic examination of the histology of a mature siphonophore colony, so it remains unclear what cell types are present, how they are distributed across zooids, and how the tissues are organized. A better understanding of *Nanomia bijuga* histology is fundamental to questions of zooid function, colony organization, and differential gene expression.

The siphonophore *Nanomia bijuga* begins as a single sexually produced polyp, the protozooid. The pneumatophore, a gas-filled float, forms as an invagination at the aboral or anterior end (Carré, 1969). We follow the conventions of Haddock et al. (2005) for siphonophore axes and orientation. Two distinct growth zones arise along the body column of the protozooid. At these growth zones, the protozooid body elongates and develops into the stem. New zooids are added to form a colony (Carré and Carré, 1995), with repeated zooid morphologies that are functionally and structurally specialized for particular tasks such as feeding, reproduction, and defense. Each zooid is a modified polyp or medusa (Totton, 1965).

The two regions that arise from these growth zones are referred to as the nectosome, which carries the pneumatophore and nectophores and which is responsible for locomotion, and the siphosome, which carries all other zooids (Fig. 1). The siphosome contains cormidia, which are reiterated sequences of feeding zooids (gastrozooids), digestive zooids (palpons), male and female reproductive zooids (gonozooids), and protective bracts. The nectosomal growth zone is located posterior to the pneumatophore, and the siphosomal growth zone is located at the anterior end of the siphosome, adjacent to the posterior of the nectosome.

Cnidarians are generally described to have diploblastic tissue layer composition; they have an outer ectoderm (also known as epidermis) and an inner endoderm (also known as a gastrodermis), separated by extracellular matrix (Thomas and Edwards, 1991). The extracellular structure on which these cells rest is known as the mesoglea and is composed of fibers in an amorphous matrix. Each of these cnidarian tissue layers is generally thought to be only one cell thick (mono-epithelial). Stem cells, known as i-cells, were originally described in hydrozoans (Weismann, 1892). These stem cells have been shown to give rise to nematocytes, neural cells, gametes, secretory cells and, in some species, epithelial cells (Bosch and David, 1987; Plickert et al., 2012).

Several studies have touched on different aspects of the biology of *N. bijuga*, though many basic details remain unknown (Metschnikoff, 1870; Claus, 1878; Chun, 1891; Totton, 1965). Carré (1969) provided a detailed description of gametogenesis and the embryological origin of the pneumatophore, as well as descriptions of the gastrozooid. Dunn and Wagner (2006) described the organization of zooids within the colony and the budding sequence that gives rise to them. Features of the nervous system were described by Mackie (1973; 1978) and Grimmelikhuijzen et al. (1986). Siebert et al. (2011) quantified differential gene expression between zooids using RNA-seq, and demonstrated the first *in situ* mRNA hybridizations in the species. Siebert et al. (2014) described the distribution of stem cells throughout *N. bijuga*, finding that they are restricted to the growth zones, young zooids, and particular regions of some mature zooids.

This study provides a systematic investigation of the the microscopic anatomy of the mature *Nanomia bijuga* colony through the use of staining thick sections and transmission electron microscopy, as well as a study of neural cell distribution through *in situ* hybridization. Emphasis is placed on the pneumatophore, gastrozooid, and palpon, with additional insight into the stem and gonodendra. Bracts and mature nectophores are not examined, as their enlarged gelatinous mesoglea makes them difficult to investigate with the tools used here.

Methods

Thick Sections and Electron Microscopy

Specimens were collected in Monterey Bay on 29 Sep 2012 via blue-water diving from a depth of 10–20 m. The specimens examined here were also used for the histological examinations of stem cells by Siebert et al. (2014).

Specimens were relaxed in MgCl and fixed in 0.5 % glutaraldehyde and 4% paraformaldehyde in filtered seawater (FSW) for two minutes, transferred to 4% paraformaldehyde in FSW, and stored overnight at 4ºC. After five washes with phosphate buffered saline (PBS), tissue was stored at 4ºC in the presence of sodium azide [1ng/ml]. Specimens were postfixed in 2% glutaraldehyde, 4% paraformaldehyde, 100mM sucrose, and 100mM sodium cacodylate buffer. Specimens were treated with a postfix of 100mM sucrose, 100mM sodium cacodylate, and 1% osmium tetroxide overnight, after which they were washed with water and ethanol.

Tissue was processed for resin embedding according to the manufacturer’s instructions (Spurr’s Low Viscosity Resin, no. 14300). All washes and incubations were conducted at slow agitation on a rocker table.

Thick sections (0.5-0.75 μm) were prepared with glass knives, dried and counterstained using toluidine blue dye in a sodium borate buffer, which stains nucleic acids blue and polysaccharides purple. Ultra thin sections (90nm) were prepared using a Diatome size 6 diamond knife (no. HI 546g). TEM images were acquired on a Philips 410 electron microscope at 80 kV. For larger structures, multiple images of a single section were aligned and blended automatically.

In Situ Hybridization

We used tblastx (Johnson et al., 2008) against a *Nanomia bijuga* transcriptome assembly (Dunn et al., 2013) in order to identify a homolog of RFamide, a known neuropeptide in cnidarians. The RFamide sequence of Polyorchis penicillatus was used as a query (Accession number L14777). Primers were designed using Primer3 (Rozen and Skaletsky, 1999). The primers used for successful amplification were as follows: P1 - CAC ACG AAA CAG ACA TGA CAC; P2 - GTC CTT GGC TGA TTT CTC TTC. The Nb-RFamide sequence has been submitted to Genbank (Accesion number XXXXX).

Nanomia bijuga specimens for *in situ* hybridization were collected in Monterey Bay on July 11 and July 15, 2013 via blue-water diving from a depth of 10–20 m. After collection, specimens were kept in filtered seawater (FSW) overnight at 8ºC in the dark. Specimens were transferred into a Petri dish coated with Sylgard 184 (Dow Corning Corporation) and relaxed by adding isotonic 7.5 % $MgCl_2 \cdot 6H_2O$ in Milli-Q water at a ratio of approximately ⅓ $MgCl_2$ solution and ⅔ FSW. They were fixed in 0.5% glutaraldehyde/4% paraformaldehyde (PFA) in FSW for two minutes and incubated in 4% PFA in FSW overnight at 4ºC. Specimens were then washed for three times in phosphate buffer saline and 0.1% Tween (PTw). Dehydration was performed using ethanol (EtOH) with 15 min washes in 25% EtOH/PTw, 50% EtOH/PTw, 75% EtOH/Milli-Q water, 2x 100% EtOH and then transferred to MeOH and stored at -20ºC.

Dig-labeled probes were generated using Megascript T7/SP6 kits (Life Technologies). The length of the RFamide probe was 734. Working concentration of mRNA probes were 1ng/ml. In situ hybridizations were performed according to the protocol described by Genikhovich and Technau with a few deviations. Starting at step #27, the specimens were incubated in maleic acid buffer (MAB) instead of PTw. The blocking buffer composition was MAB with 1% BSA and 25% sheep serum. Anti-Digoxigenin-AP, Fab fragments (Cat.No.11093274910, Roche Diagnostics) were used in 1:2000 dilution in blocking buffer. After antibody binding the specimens were washed in MAB instead of PBT. Once the NBT/BCIP development was stopped with water, the samples were stored overnight in 100% ethanol followed by storage in PBS. Samples were stable in PBS for many weeks provided that the medium was exchanged regularly to prevent bacterial growth. After all photo documentation was completed, specimens were stored in 4% PFA/PBS. *In situ* hybridization with multiple specimens yielded consistent results.

Results and Discussion

Pneumatophore

Background:

The pneumatophore (Fig. 1A, B) is a gas filled float located at the anterior end of *Nanomia bijuga*. The development of this structure was described by Carré (1969) and provides a context for understanding the tissue composition. Carré shows that structure arises as an invagination at the aboral end of the planula stage (Carré, 1969). The bilayered epithelium pushes into the gastric cavity, forming an internal mass. The ectodermal cells divide and expand, forming two populations: a layer surrounding an internal cavity, which will become the gas chamber of the float, and a population of cells internal to this cavity which are separated from the basement membrane. The mature pneumatophore therefore has five distinct tissues. From outward in, these are the external ectoderm followed by its associated endoderm, followed by the invaginated endoderm, the invaginated ectoderm, and the population of ectodermal cells within the gas chamber that are not in contact with the basement membrane.

The gas chamber is surrounded by chitin and at the site of invagination there is a pore from which gas can be released (Carré, 1969). A mature pneumatophore also includes longitudinal septa that divide the gastric cavity which are composed of endodermal cells resting on mesoglea and connect the two gastrodermal layers. Projecting from the base of the gas chamber are blind tubes which are insinuated within the two gastrodermal layers of the septa.

The development and tissue composition of the mature pneumatophore has been cited as evidence in the discussion of whether the structure is medusoid or not. Most recently, Garstang (1946), Leloup (1935) and Carré (1969; 1971) established that the tissues of the pneumatophore are not homologous to those of a swimming bell, specifically because the structure is the product of invagination and not asexual budding. Additionally, the structure of the most internal population of ectodermal cells lack characteristics which would indicate homology with the entocodon of medusae (Garstang, 1946).

Results:

We confirm the described tissue layer arrangement of the pneumatophore and present additional details on the cellular features of each layer (Fig. 2). The outermost layer contains ciliated cells with small vesicles at their surfaces (ec1 and ci, Fig. 2F). The mesoglea between the outer ectoderm and first layer of endoderm is thick relative to the mesoglea in other parts of the colony (m, Fig. 2B-F).

The morphologies of the two endodermal cell layers are distinct (en1 and en2, Figs. 2B-D). The outermost endodermal cells (en1, Fig. 2B, D-E) are compact and densely arranged on the mesoglea. They are tall in shape - longer from basement membrane to the gastric surface than they are wide - and have round nuclei located at the gastric surface of the cell. The second endodermal cell layer, derived from the invagated tissue during pneumatophore formation, has large vacuoles and rests on the mesoglea between the gastric cavity and gas chamber (en2, Fig. 2B-D, G). The nuclei of this endodermal cell layer are flattened and located on the opposite side of the vacuole from the basement membrane. Both endodermal cell layers contain beta granules (Fawcett, 1967) (gr, Fig. 2G) and are ciliated. The morphology of the endodermal cells on the mesoglea of the septa is identical to that of the cells on the outermost endodermal layer, in that the cells are packed tightly next to one another, with round nuclei and no large vacuoles (en 1, Fig. 2D-E). The mesoglea of the septa connects directly to the mesoglea of the inner endodermal cell layer but branches to form multiple connections to the outer mesoglea (m, Fig. 2E).

A layer of chitin is located immediately interior to the layer of ectoderm derived from invagination (c, Figs. 2C, G). The inner chamber is surrounded entirely by chitin except at the base, where the chitin layer is not continuous (Fig. 2A). The invaginated ectoderm (ec2, Figs. 2B-C) is the only cell layer which is in contact with the chitin towards the apex of the pneumatophore, where the internal population does not extend, which supports the hypothesis that this ectoderm is responsible for the chitin secretion (Carré, 1969). The ectodermal cells are wider near the base of the gas chamber and thinner toward the middle of the gas chamber (ec2, Fig. 2B). In between the individual cells of this monostratified layer are extracellular projections which span the distance between the chitin and the mesoglea; these are especially visible near the base of the gas chamber (Fig. 2C, G). These connective tissues appear to attach to, but are not continuous with, the mesoglea of the invaginated ectoderm and endoderm (Fig. 2G). The function of this layer of tissue as well as the secreted chitin shell surrounding the gas chamber is likely structural support as well as to prevent diffusion of gas out of the pneumatophore.

The cells internal to the gas chamber have been referred to as aeriform (gas-producing) (Carré, 1969) or as giant cells (Garstang, 1946). The suggestion that these cells are responsible for gas production is supported by the observation that they are the only cells in direct contact with gas in the chamber, as all other cells are separated from the gas by a thick layer of chitin (ae, Fig. 2B). These cells are roughly spherical and contain many 1-2 μm granules (ae and gr, Fig 2B, H-I). The population is amassed near the base of the gas chamber with no clear organization in layers (Fig. 2B). The anterior-most cells on the surface of the population have a thin layer of extracellular matrix on the exposed surface which may act as a protective envelope for the population (Fig. 2I). The surface cells are separated from the mesoglea by both the chitin and multiple aeriform cells beneath them. Direct contact with mesoglea is made only by the aeriform cells at the base of the gas cavity, where there is no chitin layer, and by the cells continuous with the aeriform tissue present in the blind tubes connected to the chamber base (Fig. 2B).

Gastrozooid

Background:

The gastrozooid is a polyp that is specialized for feeding. It has two distinct regions: the oral hypostome and the aboral basigaster (Carré, 1969). The hypostome is further divided into the buccal region and the mid region, and has a thickened endoderm and thin ectoderm. The mouth can open to many times its resting size. Carré described the endodermal cells in the buccal region of the hypostome as epithelio-muscular-glandular in type (Carré and Carré, 1995). In contrast to the hypostome, the basigaster has a relatively thick ectoderm that is a site of nematocyst production. This region has been referred to as the cnidogenic swelling, and has been studied for insight into the development of the stinging capsule cells (Carré and Carré, 1995). The gastrozooid tentacle is attached to the base of the basigaster on the anterior side.

Results:

The endodermal cells of the hypostome are glandular and club shaped, with the head of the club adjacent to the gastric cavity where secretory vesicles are released (gc1, gc2, and gc3, Fig. 3E-G). In this study we find that the hypostome endoderm contains three types of glandular cells, similar to those found in other hydrozoans (Thomas and Edwards, 1991).

In the buccal region, there are two types of gland cells: those with smaller granules which here stain darkly (gc1, Fig. 3 E-H), and those with larger, more lightly stained granules (gc2, Fig. 3 E-H). The cells with smaller, tightly packed granules are similar to the granular mucous cells described in other hydrozoans (Siebert et al., 2008) and likely correspond to the spherical hypostomal gland cells described by Carré (1969). The buccal cells with larger, more lightly stained granules are the spumous cells. In the mid-region of the hypostome, a third gland cell type is present (gc3, Fig. 3 C-D, F) which contains larger granules of variable stain affinity. These appear to correspond with the zymogen gland cells described in other hydrozoans

(Siebert et al., 2008) as well as the gastric spherical cells described by Carré (1969). The buccal region of the hypostome contains a number of folds that presumably afford the zooid the ability to stretch around larger prey (f, Fig. 3B). These folds become more defined toward the mid region (f, Fig. 3C), and more numerous and less regularly arranged further down the zooid (f, Fig. 3D). Externally, these folds appear as longitudinal stripes. The folds extend almost to the center of the gastric cavity. (Fig. 3C, G). The gastrodermis is densely ciliated (ci, Fig. 3K).

The ectoderm of the hypostome contains simple epithelial cells as well as glandular cells (ec and eg, Fig. 3E). The glandular cells are only present in the buccal region and also contain small, darkly stained granules (eg, Fig. 3 B,E). Ciliated cells are present more densely toward the mouth (ci, Fig. 3, E-F). Nematocysts were not observed in the hypostome of the gastrozooid.

In the ectoderm of the basigaster there is a maturation sequence of nematocysts, with undifferentiated cells located near the mesoglea and mature nematocysts present distally (ca, Fig. 3I). Towards the surface of this basigaster region, fully formed nematocysts measure 10-25μm (n, Fig. 3, I). The cells of the nematocyte population reside between the mesoglea and a very thin monolayer of ectodermal cells (ec, Fig. 3I). Large capsules are present near the base of the gastrozooid, away from likely points of contact with prey, suggesting migration to other regions of deployment within the colony. The endoderm of the basigaster consists of absorptive cells with large vacuoles and small granules (ab, Fig. 3I).

Palpon

Background:

The palpon is a polyp and is thought to be homologous to the gastrozooid but having lost the ability to feed (Totton, 1965). Recent findings using molecular markers and histology are in support of this hypothesis (Siebert et al., 2014). The palpon attaches to the stem via a peduncle and has a single palpacle homologous to the tentacle of the gastrozooid. The palpon has been described as an accessory of the digestive system and can be seen inflating and deflating with gastric fluid (Mackie et al., 1988).

Results:

The gastrodermal surface of the palpon is populated by a unique cell type, previously described in *Apolemia* as ciliated funnel cells (Willem, 1894). These cells are ovoid and have at their apex a tuft of cellular projection (fc, Fig. 4B-C). Electron micrographs reveal that in *Nanomia bijuga* these projections are microvilli and not cilia (mi, Fig. 4G-H) (Fawcett, 1967), therefore we refer to these cells simply as funnel cells. The microvilli are arranged in rows at the apex of the cells (mi, Fig. 4G). Inside these cells are large vesicles containing visible particulate matter (v, Fig. 4H). Large absorptive cells, densely packed with vacuoles, are also present in the endoderm (ab, Fig. 4, B-D). The presence of these two cell types suggests that a key function of the palpon gastrodermis is particle capture and intracellular digestion. Many cells of the endoderm have two distinct nuclei which do not appear to be in the process of nuclear division (nu, Fig. 4C). Additionally, well developed endoplasmic reticula are visible in endodermal cells (er, Fig. 4G).

The ectoderm of the palpon contains small cells which are occasionally ciliated (ci, Fig. 4, C, F). These cells are thin and contain multiple beta granules (gr, Fig. 4F) (Fawcett, 1967). Developing nematocysts are only found in a region near the base of the palpon (ca, Fig. 4, B, E). This region contains capsules at multiple stages of development similar to what could be observed in the gastrozooid basigaster.

Some palpons contain a large droplet near the base of the palpon, which creates a pronounced protrusion of the body. This droplet resides within the endodermal tissue, adjacent and apically to the site of nematocysts formation (li, Fig. 4E). The droplet is highly affinate to the stain used here, suggesting that it is composed of lipids. Within the endodermal cells, small, homogeneous droplets can be observed which are also likely lipids (li, Fig 4G). The presence of these droplets in *Nanomia bijuga* suggests that they cannot be used as a distinguishing feature between *N. bijuga* and *N. cara*. The lipid droplet could have multiple functions, including nutrient storage, buoyancy, or accumulation of compounds that deter predation.

Male Gonodendra

Background:

The compound sexual reproductive structures found in siphonophores are called gonodendra. Nanomia bijuga has male and female gonodendra, each with multiple gonophores. Each gonodendron, like the gonophores they bear, can therefore be referred to as either male or female. The gonodendra are inserted close to the base of the palpon, with male and female gonodendra alternating positions left and right from palpon to palpon (Dunn and Wagner, 2006). On the male gonodendron, individual gonophores are borne on a stalk connected to the base of the palpon. While the gonophores are hypothesized to be greatly reduced medusae, it is not known whether the supporting stalk is a zooid or an evagination of the stem. The male gonophores of Nanomia bijuga do not maintain an umbrella in contrast to other closely related species of siphonophore (Carré, 1969). The male gonophore contains a blind internal cavity known as the spadix, and a saccular ectoderm (Daniel, 1985).

Results:

The male gonophores examined here contained a large population of ectodermal sperm progenitor cells (sp, Figs. 5B-D). Sperm progenitor cells are located beneath a thin monolayer of ciliated ectodermal cells, forming an envelope around the saccular structure (ec and ci, Figs. 5B-D). While it is unclear whether sperm maintain a connection to the underlying mesoglea, the ectodermal cells appear to be connected only to one another and not to the basement membrane (ec, Figs. 5B-C). The nuclei of sperm progenitor cells in small gonophores were round and filled the majority of the cytoplasm, while those in larger gonophores were elongated slightly (sp, Figs. 5B-D). Sperm progenitor cells were found at uniform stages of development in the gonophores (sp, Figs. 5B-D). No feeding structures were visible within the spadix of the gonophores, which approached but did not reach the surface of the gonophore in any specimen observed (Fig. 5D). The gastric cavity of the spadix is lined with compact, endodermal cells containing small granules (en, Fig. 5C-D).

Female Gonodendra

Background:

The female gonodendron is a compound structure composed of a stalk and gonophores. The stalk of the gonodendron contains an internal cavity that connects to the gastric cavity near the palpon base. Individual gonophores at various stages of development are connected to the gonodendron stalk by a pedicel (Carré, 1969). Each gonophore is a reduced medusa and contains a single oocyte of ectodermal origin (Carré, 1969). The cytoplasm of mature oocytes is spotted with large vacuoles filled with vitellus (Carré, 1969). Two large looped gastrodermal canals connect to the peduncle and wrap around the oocyte (Carré, 1969).

Results:

The stalk of a young female gonodendron, similar to that of the male gonophore, contains compact endodermal cells surrounding a tubular gastric cavity (en and g, Fig. 5E). Developing oocytes reside in between the mesoglea and ectodermal cells (ec, Fig. 5E-F). The mesoglea of the gonodendron is thin near the site of oocyte connection (m, Fig. 5E). In young oocytes the nucleus occupies nearly half of the cytoplasm (nu, Fig. 5F) and endodermal canals can be observed (cn, Fig. 5F).

Siphosomal Stem

Background:

All zooids of a colony are attached to the stem of the colony. The gastric cavity of the stem and the gastric cavities of each of the zooids are continuous. The stem of *N. bijuga* is highly contractile (Mackie, 1973). Mackie (1978) described the epidermis of the stem, focusing on the presence of myofibrils lining the mesoglea as well as the axons cells present at the dorsal midline.

Results:

The myofibrils along the stem are arranged in tightly packed columns along radial extensions of the mesoglea (my, Fig. 5G). These myofibrils are contained within ectodermal cells. The gastrodermis of the stem is made up of small epithelial cells which are thickened on the dorsal and ventral sides of the gastric cavity (en, Fig 5G). At the dorsal ridge of the stem are the cells of the giant axon, which are enclosed by a monolayer of ectodermal cells. (ax, Fig. 5G).

Neural Cells

Background:

Cnidarians show significant density variation in neuronal aggregation along the body axis, contrary to the initial expectation of a diffuse nerve net (Pantin, 1952). Many species show aggregations in the form of nerve rings (Spencer and Satterlie, 1980) as observed in the buccal region of hydrozoans (Passano, 1963; Kinnamon and Westfall, 1981; Koizumi et al., 1992). Immunohistochemistry studies in siphonophores indicate a complex nervous system with nerve rings and transverse bands in addition to a diffuse nerve net (Grimmelikhuijzen et al., 1986).

We characterized the expression of RFamide by *in situ* hybridization. RFamide is a neuropeptide (Plickert et al., 2003) and is used here as an indicator of a sub-population of mature nerve cells. It is a well-studied gene family in all major groups of cnidarians (Grimmelikhuijzen, 1985; Grimmelikhuijzen et al., 1988; Plickert, 1989; Grimmelikhuijzen et al., 1991; Koizumi et al., 1992; Moosler et al., 1996; Darmer et al., 1998; Mitgutsch et al., 1999; Anderson et al., 2004; Watanabe et al., 2009). We compared the *Nanomia bijuga* RFamide sequence to neuropeptides of this family described in other hydrozoans (Fig. 6H), as summarized by Grimmelikhuijzen (2004). The RFamide shown is most similar to Pol-RFamide-II which is the RFamide of *Polyorchis penicillatus* used as a query. We found evidence of an additional putative RFamide gene in *N. bijuga* which was not characterized in this study.

Immunohistochemistry using an RFamide antibody showed that *Nanomia bijuga* possesses ectodermal nerve nets in gastrozooids, tentacles, tentilla and the pneumatophore (Grimmelikhuijzen et al., 1986). Nerve rings are present at the base of the pneumatophore, gastrozooid, palpons, and gonozooids. Along the stem transverse bands of RFamide positive cells can be found, one per cormidium. Additionally, cells of the giant axon were identified as RFamide positive. No previous *in situ* hybridization studies have been conducted on neuronal genes in siphonophores.

Results:

The expression patterns obtained here correspond very well with Grimmelkhuijzen's (1986) immunohistochemistry studies. The pneumatophore shows scattered RFamide positive neurons, which are clustered near the apex and less dense near the base (ap, Fig. 6A). These cells are most dense in a ring around the apical pore.

The siphosomal stem shows scattered RFamide positive neurons, possibly corresponding to bracteal scars, as well as transverse bands at regular intervals indicating sphincter regions (st, Fig. 6B). Sphincters are regions of circular musculature where the stem can be constricted, and are located posterior to the gastrozooids. Under high magnification the transverse bands can be seen as two rings around the sphincters on the stem located posterior to each gastrozooid (Fig. 6F). The giant axon is visible along the ventral side of the nectosome and the dorsal side of the siphosome. The placement of the nectosomal zooids relative to the siphosomal has been identified as a shared feature of representatives from the family Agalmatidae, to which *Nanomia bijuga* belongs (Dunn et al., 2005). The shift in orientation suggests a 180° torsion between these two regions early during development.

Gastrozooids have large numbers of scattered RFamide positive cells in the hypostome (ga, Fig. 6B, D-E), with the highest density in the area surrounding the mouth. These cells were only observed in the ectoderm (ga, Fig. 6F) and the cell morphology suggests a sensory function (arrow, Fig. 6F). Rings of RFamide positive cells are present at the base of each gastrozooid (Fig. 6B).

The palpons also showed a dense collar of RFamide positive neurons at their base, near the junction with the stem (pa, Fig. 6C). No patterning of scattered cells was visible as was observed in the palpon, indicating the palpon does not function primarily as a sensory structure. The palpon lacked any RFamide signal near the tip of the palpon in the region homologous to the mouth of the gastrozooid.

Conclusion

The distribution of cellular morphologies furthers understanding about zooid structure and function. For example, multiple hypotheses have been provided for the function of palpons, including that they act as tactile or excretory structures (Leuckart, 1853; Mackie et al., 1988). The results here support instead the hypothesis that the palpon of *Nanomia bijuga* is primarily involved in digestion. The endoderm of the palpon is packed with specialized cells possessing structures for particle capture, such as the funnel cells and absorptive cells. The presence of lipid droplets also suggests a possible role in long-term energy storage. The lack of RFamide positive neurons near the tip suggests that the palpon of *Nanomia bijuga* is not primarily a sensory zooid. A deeper understanding of the microanatomy also reveals new questions for further investigation. For example, the function of the di-nucleate cells as well as relatively well developed endodermal endoplasmic reticula in the palpon remains unknown.

The microanatomy of the pneumatophore also provides a context for a discussion function. Pneumatophores are often cited as providing buoyancy for the colony (Pickwell et al., 1964), but they are in many species of siphonophore small relative to the total size of the organism, which makes this function unlikely (Jacobs, 1937). It may be that pneumatophores are sensory structures that use the buoyancy of the gas to detect orientation or the relative pressure of the gas to detect depth. In the case of *Nanomia bijuga*, the development of the tissue layers in this structure results in the gas chamber being surrounded by three ectodermal and two endodermal tissue layers as well as mesoglea, gastrovascular space, and chitin. These multiple layers may serve to prevent gas diffusion. The presence of an apical pore indicates that the overall pressure and buoyancy of the structure can be regulated, The morphology of the specialized endodermal layers of the the float, as well as the large population of gas producing cells within the gas chamber is evidence that the float is a center of dynamic cellular processing in a mature colony. The pneumatophore and the production of gas in the siphonophore colony merits further functional investigation.

Throughout the *Nanomia bijuga* colony there are regions of high cellular complexity present in both endoderm and ectoderm. As mentioned above, the cells in the pneumatophore derived from invaginated ectoderm are highly specialized. Part of these ectodermally derived cells remain in a tight mono-epithelial layer and, unlike any other cells in the siphonophore colony, are involved in chitin production. The additional ectodermally derived cells, the aeriform population, are not arranged in an epithelial layer and produce gas. Many of the cells of this population are clearly separated from a basement membrane. These two cell populations, derived from the same ectodermal region, represent complex and unique cellular processes

There are additional regions of epithelial complexity in other zooids. In the hypostome, the region of the gastrozooid adjacent to its mouth, the endoderm is many times thicker than the ectoderm. Multiple endodermal cell types are found in this region of highly expandable tissue, including three gland cell types. Adjacent to the hypostome, in the basigaster, is an area of ectodermal complexity where nematocysts are forming *en masse*. Many of these forming nematocysts, as well as the ectodermal cells which envelop them, also retain no connection with the mesoglea. Both these regions, the hypostome and basigaster, are located within a single zooid type, indicating that function is not only achieved through zooid specialization, but also through regional tissue specialization of both gastrodermis and epidermis.

It is commonly asserted that cnidarians are simple animals composed entirely of an extracellular matrix sandwiched between two single-layered epithelia. The results presented here show that this is clearly not the case, and that many siphonophore tissues are quite complex. The aeriform tissue, gastrozooid basigaster, and possible the male gonophore ectoderm all include cells that are not in direct contact with the mesoglea, and therefore are not mono-layered epithelia. It is likely that tissues with multiple cell layers are found in all siphonophores, and may also be present in other hydrozoans and cnidarian groups as well.

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Figure Legends

Figure 1. Schematic of *Nanomia bijuga* colony.

Adapted from http://commons.wikimedia.org/wiki/File:Nanomia_bijuga_whole_animal_and_growth_zones.svg, which was drawn by Freya Goetz. A-C are oriented with anterior to the top and ventral to the left. D-F are oriented with anterior to the left and ventral up. (A) Overview of the mature colony. All zooids are produced from two growth zones, one at the anterior end of the siphosome, immediately posterior to the pneumatophore, and one at the anterior end of the siphosome, immediately posterior to the nectosome. Zooids are organized on the siphonosome into reiterated units known as cormidia. (B) The pneumatophore and nectosomal growth zone at the anterior of the colony, showing forming nectophores. (C) The siphosomal growth zone and budding cormidia, with gastrozooids labelled showing cormidial boundaries. (D) Gastrozooid, connected at the base to the siphosomal stem, showing distinct regions of the mouth (buccal hypostome), the mid-region of the hypostome, and the basigaster. (E) Palpon, connected to the stem by its peduncle. (F) Female and male gonodendra. Multiple individual gonophores are borne on stalks which connect to the siphosomal stem.

Figure 2. Pneumatophore,

(A) Schematic of pneumatophore showing orientation of sections in other panels. (B) Longitudinal section of the pneumatophore (composite image). Thick section stained with toluidine blue. From outward in, there are five tissues and several acellular layers: ectoderm (ec1) resting on the mesoglea (m), endodermal cells (en1) packed tightly surrounding the gastric cavity (g), invaginated endodermal cells (en2) with vacuoles (v), mesoglea (m), invaginated ectodermal cells (ec2) which produce chitin (c), and ectodermally derived aeriform cell population (ae). At the base of the gas chamber blind tubes (t) connect and insinuate between the endodermal septa (s). (C) Enlarged image of region shown in B, showing internal endoderm, invaginate ectoderm with extracellular projection (pr) from mesoglea to chitin layer, and aeriform ectoderm. (D) Transverse section of pneumatophore, showing septa composed of endodermal cells, and blind tubes. (E) Enlarged image of endoderm showing tightly packed endodermal cells, as well as the branching connection of the mesoglea on the septa to the outermost mesoglea. (F) Transverse transmission electron micrograph of the transverse section of the ectoderm (ec1), showing cilia (ci) and granules (gr). (G) Transverse electron micrograph of chitin producing cells (ec2) and chitin layer, showing nuclei (nu) of individual cells. (H) Transverse electron micrograph of the aeriform ectoderm cell with electron dense granules. (I) Electron micrograph of the envelope separating aeriform ectoderm cells from gas chamber. Panels B-E are thick sections (0.5-0.75 μm) stained with toluidine blue, and panels F-I are electron micrographs (90nm).

Figure 3. Gastrozooid.

(A) Schematic of gastrozooid showing orientation of sections in other panels. (B) Transverse section of mouth region of hypostome showing ciliated ectoderm cells (ec) and club shaped gland cells of the endoderm (en), which are connected to the mesoglea (m) and line the gastric cavity (g) in folds (f) represented with the dashed line. (C) Transverse section of lower buccal region of hypostome, showing compact folds (f) of endodermal tissue. (D) Transverse section of mid-region of hypostome showing disorganized folds (f) of endodermal tissue in this region. (E) Enlarged image of mouth region from B. Two types of endodermal gland cells, one with more darkly stained granule (gc1) and one with larger granules (gc2), are present, as well as ectodermal cilia (ci). (F) Enlarged image of mid-region of hypostome from D, showing third type of gland cell (gc3) which contains granules which stain lightly and are much larger than those of gc2. The cilia on the ectoderm is less dense in the mid region of the hypostome. (G) Longitudinal section of the hypostome showing extended gland cells of all three types. (H) Transverse electron micrograph of the mid region of the hypostome, showing gland cells types gc1 and gc3. (I) Transverse section of the basigaster region. A maturation sequence of nematocysts (n) is visible, with undifferentiated cells near the mesoglea and capsules (ca) visible towards the cell surface. Only the undifferentiated cells nearest the gastric cavity are in contact with mesoglea; the ectodermal cell layer and all other capsules do not appear to have a connection to the basement membrane. Absorptive cells (ab) are present endodermally. (J) Transverse electron micrograph of the mid-region of the hypostome showing an additional gland cell type (gc2). (K) Transverse electron micrograph of the mid-region of the hypostome, showing the ciliated surface of the gland cells, with cilia oriented perpendicular to gastric surface. Panels B-G and I are thick sections (0.5-0.75 μm) stained with toluidine blue, and panels H, J, and K are electron micrographs (90nm).

Figure 4. Palpon

(A) Schematic of the palpon showing the orientation of sections in other panels. (B) Longitudinal section of the base of the palpon, showing both funnel cells (fc) and absorptive cells (ab) in the endoderm (en) lining the gastric cavity (g), the ectoderm (ec) including the reduced basigaster region in the ectoderm (ec) with forming capsules (ca), and the peduncle (pe) that connects to the stem. (C) Enlarged image of region boxed in C showing the absorptive and funnel cells with microvilli (mi), as well as the ectodermal cilia (ci). Pairs of nuclei (nu) within a single cell are visible. (D) Transverse section of the mid-region of the palpon. (E) Longitudinal section of the base of the palpon, showing the mesoglea (m) partially surrounding a lipid droplet. A reduced basigaster can be observed basal to the droplet. (F) Longitudinal electron micrograph of the ectoderm showing cilia and beta granules (gr). (G) Longitudinal electron micrograph of the endoderm showing rows of microvilli, a well developed endoplasmic reticulum (er), beta granules, and lipid droplets. (H) Longitudinal electron micrograph of the endoderm, showing funnel cells with microvilli (mi) arranged in clusters at the apex, with a large vacuole (v) internal to the cell. Panels B-E are thick sections (0.5-0.75 μm) stained with toluidine blue, and panels F-

H are electron micrographs (90nm).

Figure 5. Gonodendra and Stem.

(A) Schematic of the palpon showing the orientation of sections in other panels. B) Longitudinal section of the ectoderm of the male gonophore, showing cilia (ci) at regular intervals on thin ectodermal cells (ec). Beneath the ectodermal cells is a population of sperm progenitor cells (sp), which have nuclei (nu) that fill most of the cellular space. (C) Longitudinal section of the male gonophore showing the large volume of sperm progenitor cells located beneath the thin layer of ectodermal cells, as well as the mesoglea (m) of the spadix. Compact endodermal cells (en) line the gastric cavity (g) of the spadix. (D) Longitudinal section showing the tip of a large male gonophore. The spadix reaches nearly the surface of the ectoderm but no opening is visible. The nuclei of the sperm progenitor cells of the large gonophore are elongated toward the ectodermal surface. (E) Longitudinal section of the female gonodendron stalk showing the tissue organization of the oocytes (o) which form ectodermally. (F) Transverse section of young oocytes showing endodermal canal cells (cn) and large nuclei (nu). (G) Transverse section of the siphosomal stem, showing radial extentions of mesoglea lined with ectodermal cells containing myofibrils (my). The large axon (ax) is dorsal. Endodermal cells (en) are compact and thickened at the apical and dorsal sides of the gastric cavity (g). All panels are thick sections (0.5-0.75 μm) stained with toluidine blue.

Figure 6. Nerve Cells.

In situ RFamide hybridization. (A) The pneumatophore with the anterior facing upward, showing apical pore (ap). RFamide positive cells are scattered most densely in a ring posterior to the apical pore, and less densely toward the mid region of the pneumatophore. (B) The cormidia of the siphosome, anterior to the left, with gastrozooids (ga) and palpons (pa) ventrally attached and the giant axon (ax) on the dorsal side. RFamide expression can be observed in rings (r) around palpon and gastrozooid peduncles shortly above their point of attachment on the siphosomal stems and along bract scars (bsc). (C) Palpon showing rings (r) of RFamide positive cells at the base of the peduncle (pe) and at the base of the palpacle (pl). No RFamide positive cells are observed in the palpon body. (D) Gastrozooid hypostome showing ectodermal RFamide positive cells with higher densities near the mouth (mo) and lower density in the mid-region (mr) of the hypostome. (E) Enlarged image of ectodermal neuronal cells in the hypostome of the gastrozooid. (F) Whole mount of the siphosomal stem showing transverse bands of RFamide positive cells surrounding a sphincter region. (G) RFamide positive cells in the hypostome of the gastrozooid and absence of signal in the endoderm (en). (H) Table showing amino acid structures of various hydrozoan neuropeptides, modified from Grimmelikhuijzen (2004). Amino acids shared with Nb-RFamide are shaded.

Figures

Figure 1

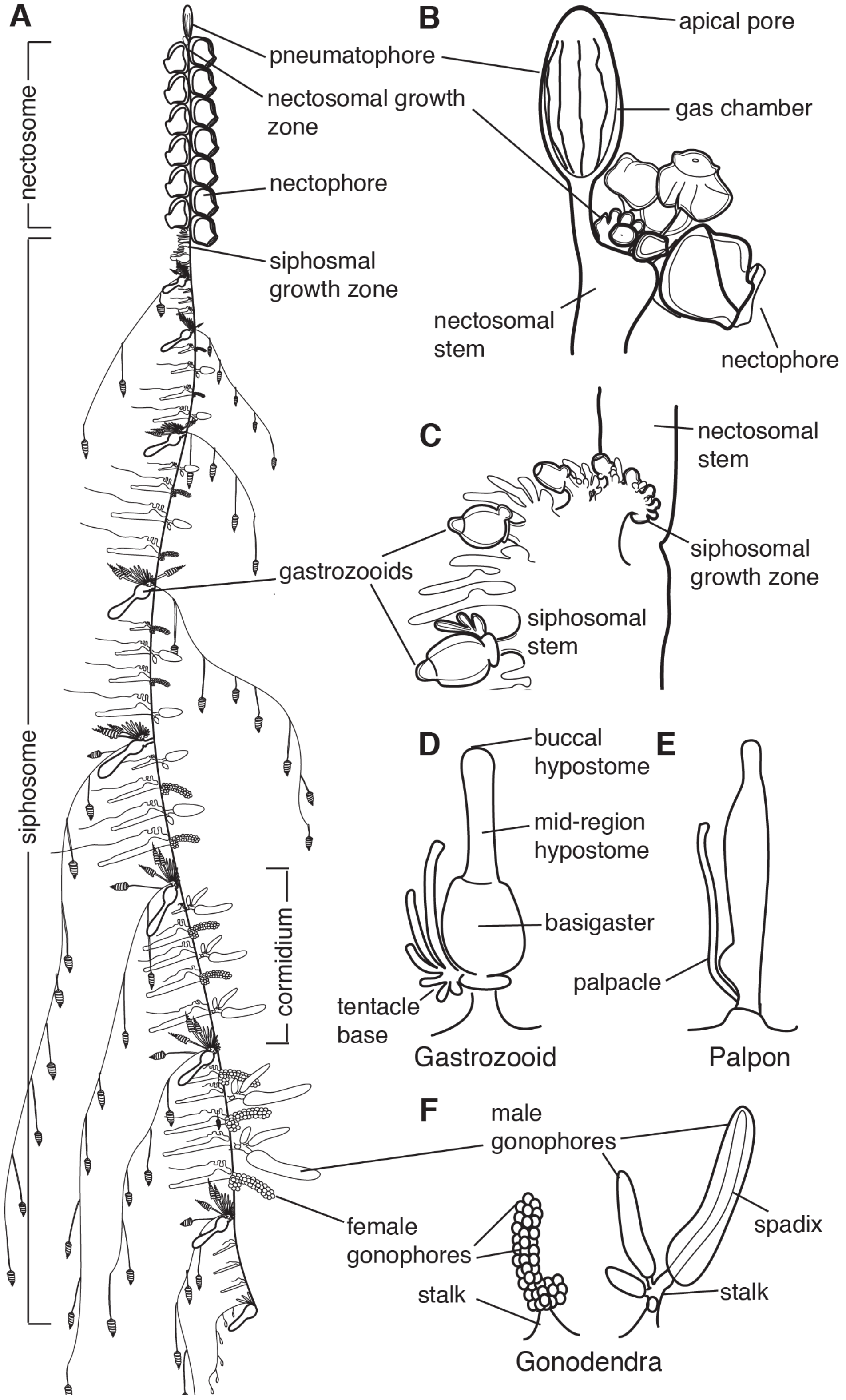

Figure 2

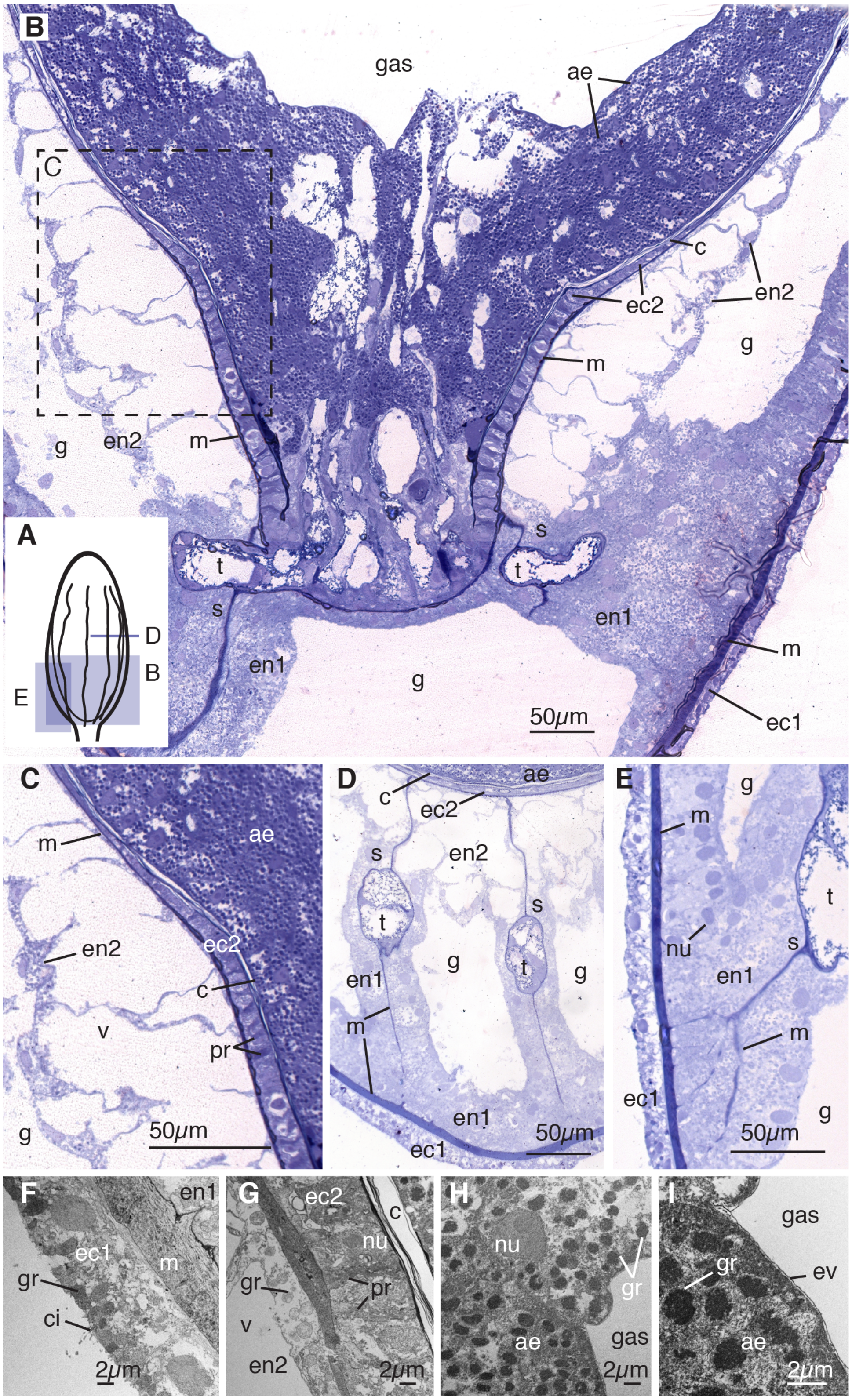
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Figure 3

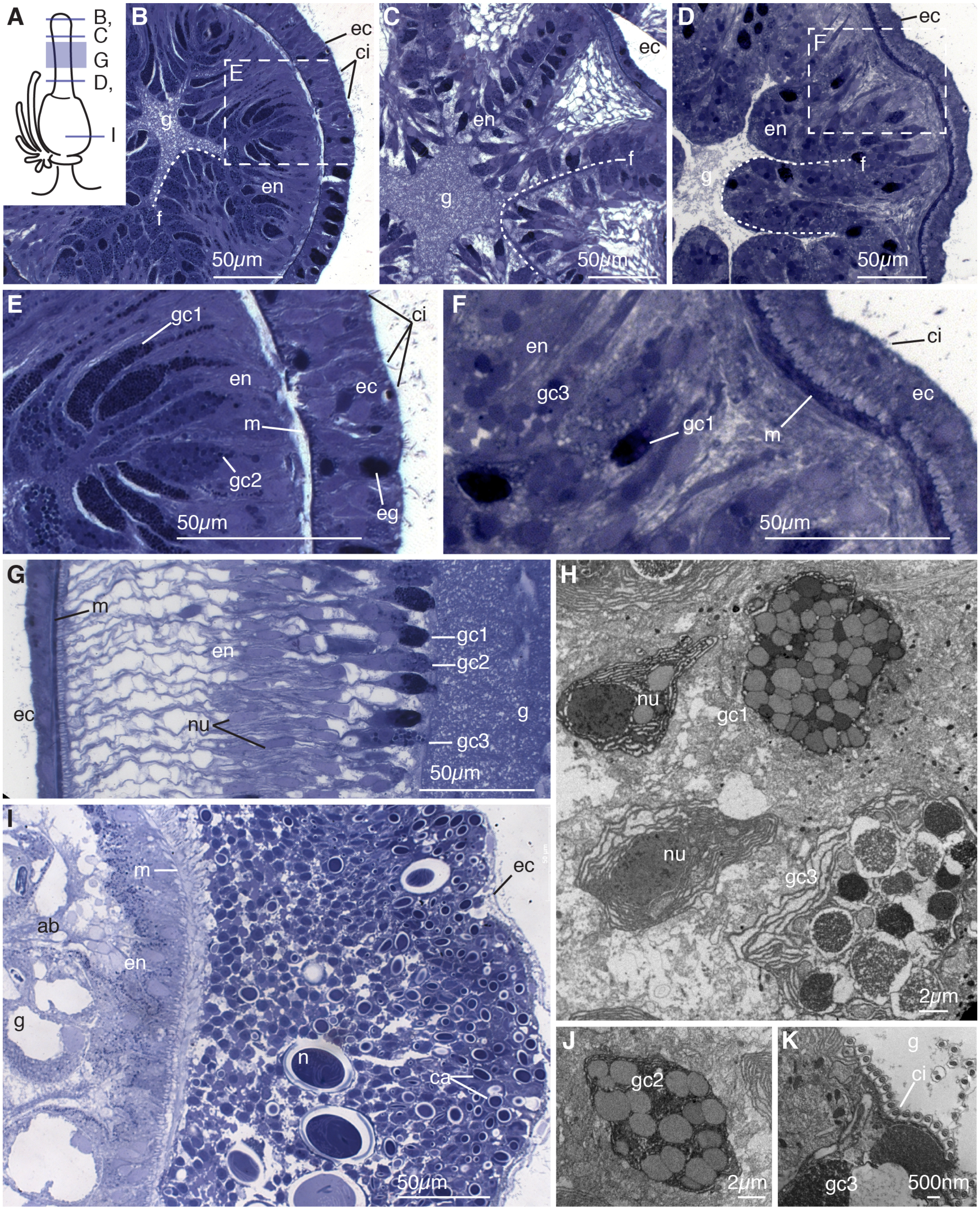
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Figure 4

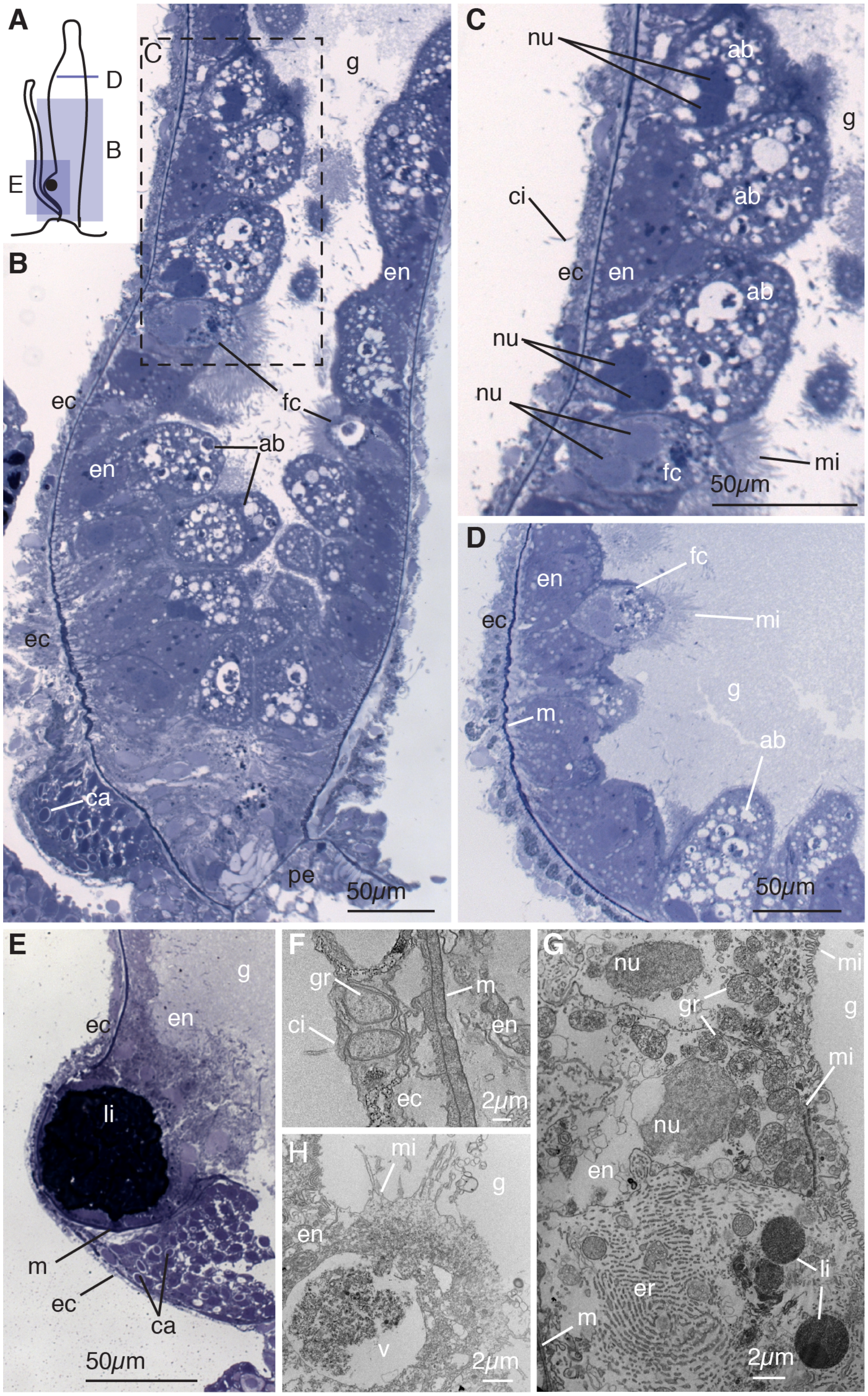
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Figure 5

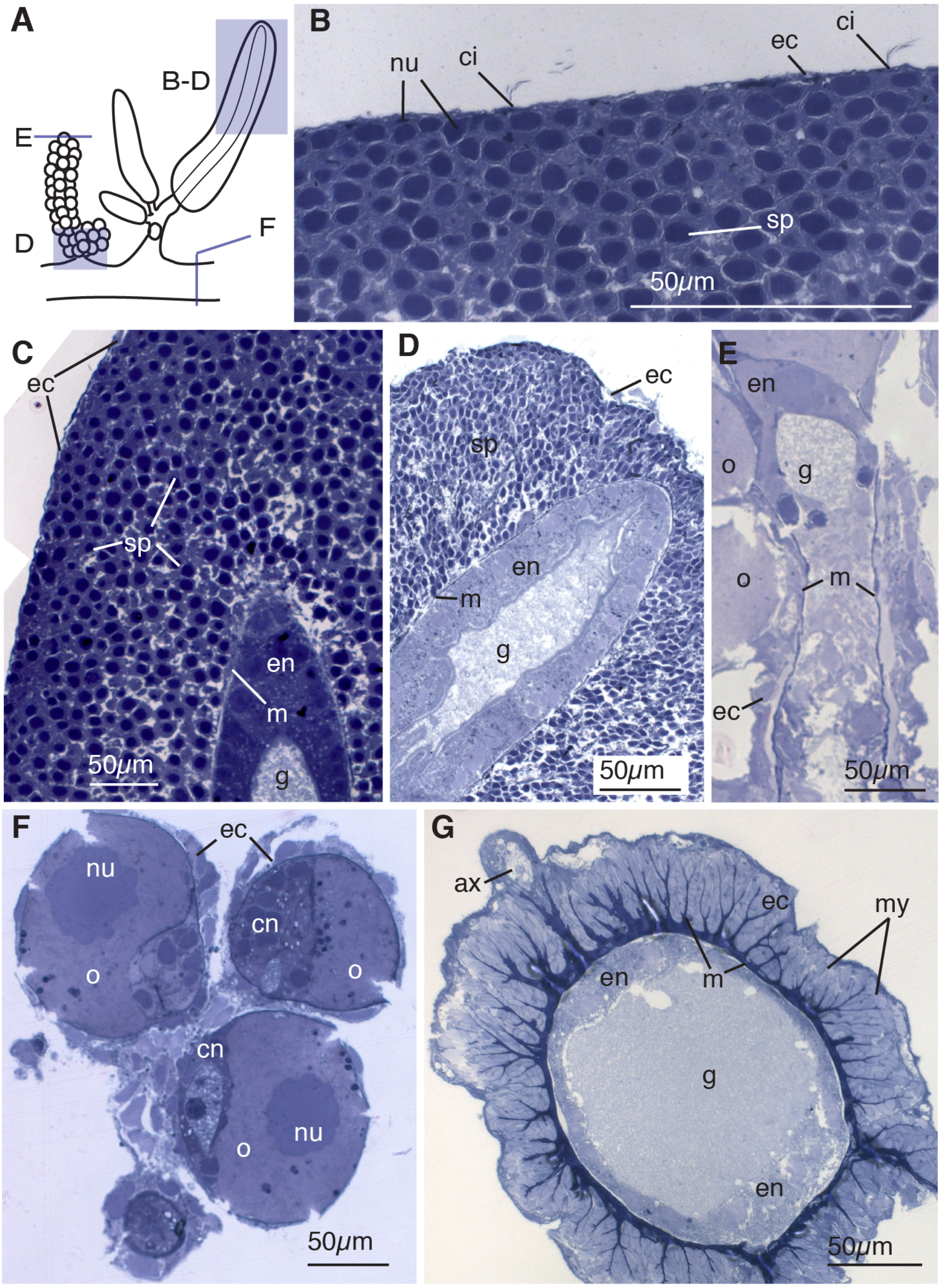
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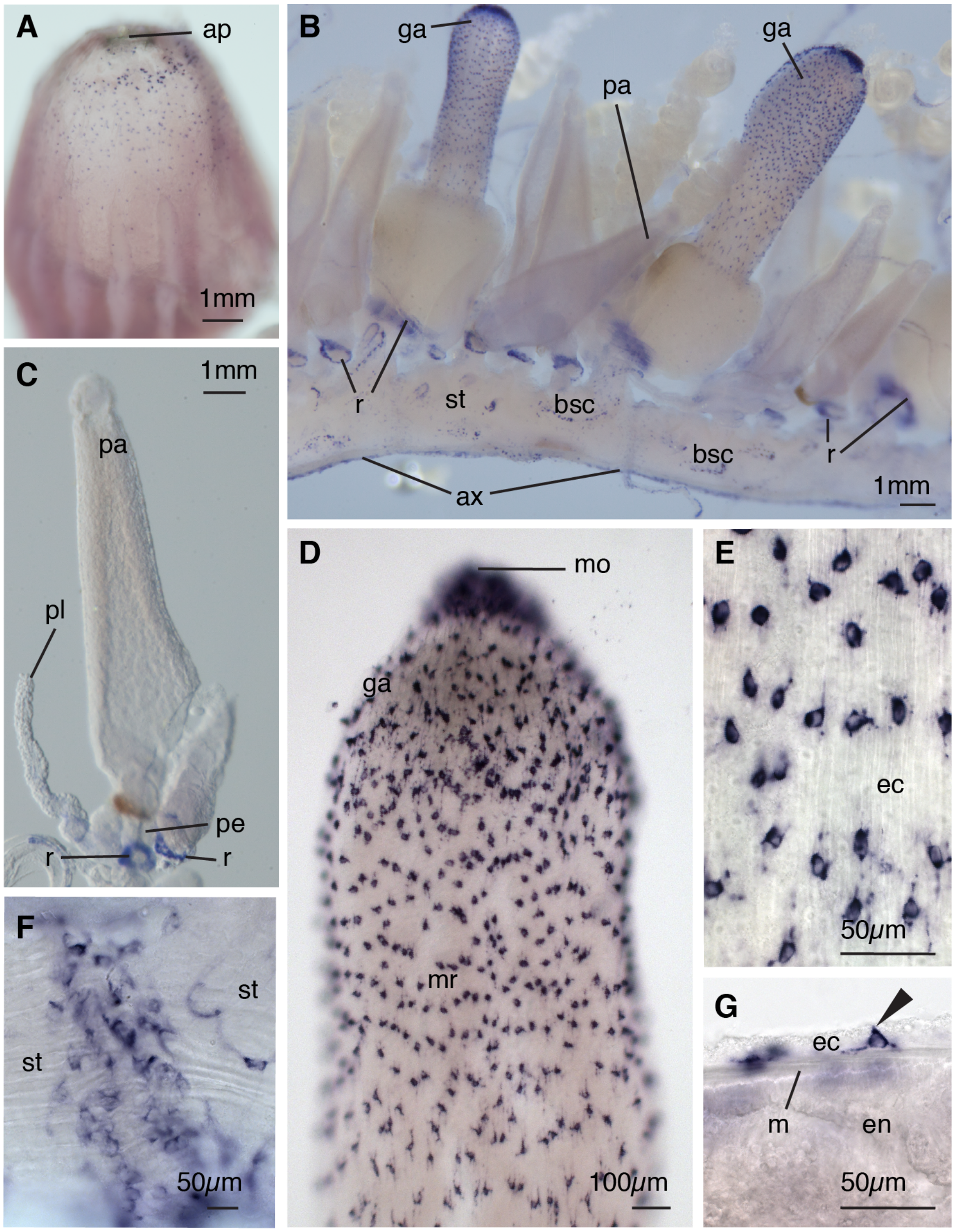
Figure 6



H

Species	**Structure**	**Name**
Cyanea lamarckii	Gly-Arg-Phe-NH2	Cyanea-RFamide III
Cyanea lamarckii	Glu-Pro-Leu-Trp-Ser-Gly-Arg-Phe-NH2	Cyanea-RFamide I
Polyorchis penicillatus	Glu-Leu-Leu-Gly-Gly-Arg-Phe-NH2	Pol-RFamide I
Polyorchis penicillatus	Glu-Trp-Leu-Lys-Gly-Arg-Phe-NH2	Pol-RFamide II
Nanomia bijuga	Gln-Trp-Leu-Lys-Gly-Arg-Phe-NH2	Nb-RFamide
Hydra magnipapillata	Lys-Pro-His-Leu-Arg-Gly-Arg-Phe-NH2	Hydra-RFamide III
Hydra magnipapillata	His-Leu-Arg-Gly-Arg-Phe-NH2	Hydra-RFamide IV
Hydra magnipapillata	Asn-Pro-Tyr-Pro-Gly-Leu-Trp-NH2	Hydra-RFamide IV
Hydra magnipapillata	Gly-Pro-Met-Thr-Gly-Leu-Trp-NH2	Hydra-RFamide V