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DISSERTATION

Titel der Dissertation

„Asexual organogenesis and neuro-muscular system of basal bryozoans“

Verfasser

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1. General Introduction

The phylum Bryozoa consists of benthic, colonial filter feeders that live on various substrates. It currently contains over 5.000 extant and approximately 20.000 described extinct species. They are currently divided into three taxa with the Phylactolaemata representing a small group of solely freshwater-inhabiting species while the Stenolaemata (with the only remaining extant taxon Cyclostomata) and the Gymnolaemata mainly constitute marine animals. Within the Gymnolaemata two distinct groups, the Ctenostomata and Cheilostomata, are recognized (Cheetham & Cook 1983). The phylogenetic position of bryozoans has been disputed for decades. So far molecular phylogenetic analyses have only been able to settle them within the Lophotrochozoa, but their position within the latter is still under debate (e.g. Halanych 2004). Moreover, the relationships between the higher bryozoan clades are currently also not well understood. In principle, two clades show a high potential for phylogenetic considerations: The Phylactolaemata are interesting since they are often regarded as the most basal bryozoans and show several morphological characters such as an epistome and body wall musculature that distinguish them from all remaining bryozoans (Wood 1983, Mukai et al. 1997). In particular their sexual development, however, is heavily altered, probably as an adaptation to freshwater habitats, and therefore impedes comparisons to other phyla and bryozoans (Reed 1991). Within the Gymnolaemata, the Ctenostomata are a group of uncalcified, comparatively simple species that are currently regarded as paraphyletic being ancestral to the species-richest Cheilostomata and perhaps even the Stenolaemata (Larwood & Taylor 1979; Taylor 1990, Todd 2000, Ernst & Schäfer 2006). Consequently, they represent the second important clade for addressing phylogenetic questions of bryozoans.

Most recent morphological studies focus on characters of the cystid, i.e. the protective part or housing of each individual zooid. In particular the calcified skeletons of the Stenolaemata and Cheilostomata are well studied in a phylogenetic context (e.g. Gordon 2000). Details on the

anatomy of the soft body (polypide) parts remain less investigated and are thus widely neglected in phylogenetic analyses. Still, there are a couple of more recent publications dealing with specific adult and developmental characters (Gruhl 2008, 2009, 2010a, b; Gruhl & Bartolomaeus 2008; Gruhl et al. 2009; Santagata 2008a, b; Ostrovsky & Schäfer 2003; Ostrovsky et al. 2003, 2006, 2007, 2009; Nielsen & Worsaae 2010).

In the present thesis I analyse two morphological aspects and their value for drawing phylogenetic conclusions in the Phylactolaemata and the Ctenostomata that were previously not analysed or are in need for revision: 1. Myoanatomical features and neurotransmitter distribution, for example serotonin or FMRF-amide, have recently been used for phylogenetic inferences among lophotrochozoans (e.g. Wanninger 2004, 2009, Wanninger et al. 2007). Previous studies on bryozoans using state-of-the-art techniques are currently restricted to larvae or developmental stages, whereas adult zooids were not analysed yet. Consequently, gaining insight into the myoanatomy and serotonergic nervous system of adult zooids for phylogenetic inferences was the first aspect of the present thesis. 2. As colonial organisms asexual reproduction by budding represents an essential part in bryozoan life cycles. The organogenesis within the polypide bud is identical in asexual and sexual development, but has only been subject of few studies and is in need for revision (Nielsen 1971). Accordingly, the second aspect analyses trends during this organogenesis and whether these are comparable to other phyla.

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2. Organogenesis in the budding process of the freshwater bryozoan *Cristatella mucedo* Cuvier, 1798 (Bryozoa, Phylactolaemata)

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Organogenesis in the Budding Process of the Freshwater Bryozoan *Cristatella mucedo* Cuvier, 1798 (Bryozoa, Phylactolaemata)

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ABSTRACT The phylogenetic position of bryozoans has been disputed for decades, and molecular phylogenetic analyzes have not unequivocally clarified their position within the Bilateria. As probably the most basal bryozoans, Phylactolaemata is the most promising taxon for large-scale phylogenetic comparisons. These comparisons require extending the morphological and developmental data by investigating different phylactolaemate species to identify basal characters and resolve in-group phylogeny. Accordingly, we analyzed the bud development and the organogenesis of the freshwater bryozoan *Cristatella mucedo*, with special focus on the formation of the digestive tract and differentiation of the coelomic compartments. Most parts of the digestive tract are formed as an outpocketing at the future anal side growing towards the mouth area. The ganglion is formed by an invagination between the anlagen of the mouth and anus. The lophophoral arms develop as paired lateral protrusions into the lumen of the bud and are temporarily connected by a median, thin bridge. All coelomic compartments are confluent during their development and also in the adult. The epistome coelom develops by fusion of two peritoneal infolds between the gut loop and overgrows the ganglion medially. The coelomic ring canal on the oral side develops by two lateral ingrowths and supplies the oral tentacles. On the forked canal, supplying the innermost row of tentacles above the epistome, a bladder-shaped swelling, probably with excretory function, is present in some adults. It remains difficult to draw comparisons to other phyla because only few studies have dealt with budding of potentially related taxa in more detail. Nonetheless, our results show that comparative organogenesis can contribute to phylactolaemate systematics and, when more data are available, possibly to that of other bryozoan classes and bilaterian phyla. J. Morphol. 272:320–341, 2011.

KEY WORDS: budding; organogenesis; Bryozoa; Phylactolaemata; *Cristatella*

INTRODUCTION

The phylogenetic position of bryozoans has been disputed for decades. So far, molecular phylogenetic analyzes have positioned them within the Lophotrochozoa, but their relationship within the Lophotrochozoa is still under debate. Traditionally, they have been united with phoronids and brachiopods into the Tentaculata (Hatschek, 1888) or Lophophorata (Hyman, 1959), but no support for this concept has been provided by molecular analyses (e.g., Hausdorf et al., 2007; Helmkampf et al., 2008). Within the Bryozoa, three classes are currently recognized: the exclusively marine Stenolaemata and chiefly marine Gymnolaemata, and the freshwater-inhabiting Phylactolaemata. Morphological studies usually place phylactolaemate bryozoans as the most basal group (Wood, 1983). In contrast, molecular studies are not so clear, but show some support for their basal placement (Fuchs et al., 2009). Only few studies on bryozoan morphology within a phylogenetic and evolutionary context have been conducted during the last decade (Wanninger et al., 2005; Gruhl, 2008, 2009, 2010; Gruhl and Bartolomaeus, 2008; Santagata, 2008a, 2008b; Gruhl et al., 2009). In Phylactolaemata, sexual development and larval morphology are modified, possibly due to the adaptation to freshwater habitats; this poses problems in comparisons with other bryozoan and lophotrochozoan larvae (Reed, 1991). As colonial organisms, their main reproduction occurs by two modes of asexual reproduction: germination of so-called statoblasts and budding of the colony. Statoblasts, sometimes referred to as internal buds, are phylactolaemate-specific dormant stages used for dispersal, overwintering and to outlive other adverse conditions. Colonies are formed by settling larvae or more frequently by germinating statoblasts followed by colonial budding. Some species are also capable of reproduction by fragmentation or fission (Wöss, 2005).

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In her extensive study on the budding of the cheilostome *Membranipora membranacea*, Lutaud (1961) classified previous works on budding as follows: 1) Experimental studies on ecological and biological factors influencing colony development; 2) Studies focusing on laws in the generation and succession of buds in the colony; 3) Anatomical investigations dealing with bud and cystid formation as well as organogenesis. Several anatomical studies on the budding process in phylactolaemate bryozoans have been conducted (Nitsche, 1875; Kafka, 1887; Davenport, 1890; Braem, 1890, 1913; Oka, 1891; Herwig, 1913; Aen den Boom, 1933; Brien, 1936, 1953, 1960a), but most of them dealt only with early bud formation. Merely the studies of Braem (1890, 1913), Davenport (1890), and Oka (1891) provide a detailed description of the development of the buds, including the formation and differentiation of the organs. Nielsen (1971) stressed that, for comparative phylogenetic purposes, bryozoan budding processes are in need of revision. This is because the formation of the digestive tract in early buds has been controversially described in almost all major bryozoan clades (Nielsen, 1971). Furthermore, the organization of coelomic cavities in phylactolaemates has been subject of debate, and several past authors who favored a deuterostome affinity of bryozoans suggested a trimeric organization (e.g., Hyman, 1959; Siewing, 1980). However, the coelomic cavities of three adult phylactolaemates (*Fredericella sultana*, *Plumatella emarginata*, and *Lophopus crystallinus*) were recently investigated in detail and were found to be confluent. In contrast to previous investigations, no epistome was reported in a species forming large gelatinous colonies (*Lophopus crystallinus*; Gruhl et al., 2009). This calls for reinvestigating the condition of the different parts of the coelomic system (i.e., lophophoral and epistome coelom) in other gelatinous species, both in the adult stage and during formation. This study focuses on bud development and the organogenesis of the freshwater bryozoan *Cristatella mucedo*, a species forming gelatinous colonies of approximately 3–4 mm width and one to several centimetres length.

MATERIALS AND METHODS

Cristatella mucedo colonies were collected from the Laxenburg pond, Lower Austria, in September 2007. Colonies were transferred to the laboratory, anesthetized with chloral hydrate and then fixed either in Bouin's solution (Böck, 1989) or following Gruhl and Bartolomaeus (2008) in a 2.5% glutaraldehyde solution in 0.01 mol l^{-1} PBS (pH 7.4) followed by postfixation with 1% osmium tetroxide.

For sectioning and 3D-reconstruction, specimens were dehydrated with acidified dimethoxypropane and embedded into Agar low-viscosity resin using absolute acetone as intermedium. Ribbons of serial semithin sections with a thickness of 1 μm were produced with a Histo Jumbo diamond knife on a Reichert Ultracut S microtome (Ruthensteiner, 2008). Sections were stained with toluidine blue and digitally photographed with a Nikon DS-5MU1 camera on a Nikon Eclipse E800 microscope. Microphotographs were enhanced in contrast and converted into gray-scales using Adobe Photoshop CS2 (Adobe, San Jose, CA) before being imported into the 3D-reconstruction software Amira 4.1 (Mercury Computer Systems, Chelmsford, MA). Image stacks of different budding stages were aligned with the Amira AlignSlices tool. Depending on the degree of differentiation, organ systems or their anlagen including lophophore, digestive tract, nervous system, funiculus, and coelomic epithelium were manually segmented with the brush tool. After segmentation, a surface was generated, which was subsequently reduced and smoothed (Ruthensteiner, 2008). Snapshots of the 3D models were taken with the Amira software.

For scanning electron microscopy, specimens were dehydrated with acidified dimethoxypropane, transferred into absolute acetone and chemically dried using hexamethyldisalazane (Walzl and Wöss, 2005). Dry specimens were mounted on stubs with Tempfix Leit C adhesive glue. Mounted pieces of *C. mucedo* colonies were manually broken with sharpened tungsten needles to display inner structures of the colony. Specimens were sputtered with gold in an Agar Sputtercoater 108 and afterward analyzed with a Philips XL 20 scanning electron microscope.

RESULTS

Cristatella mucedo forms elongated colonies that can creep with a gliding or creeping sole situated at the base of the colony (Fig. 1a). At the colony margin, the creeping sole forms a bulge (Fig. 1b and c). In cross-section, the colony is semicircular, with the creeping sole directed towards the substrate and the colony crest facing the opposite direction (Fig. 2). Fully grown zooids are positioned at the colony crest close to the longitudinal median plane of the colony (Figs. 1a and 2). All buds in *C. mucedo*, which are produced in large amounts and typically within a short time, arise in a budding zone located between the grown zooids and the colony margin (Fig. 1a–c). In the following, selected developmental stages are treated according to the degree of differentiation. For orientation purposes, in single zooids the terms left and right are applied with respect to a viewing direction from the anus to the mouth opening (Fig. 2).

Stage 1

Budding starts with a proliferation of cells in the outer epidermal layer of the colonial wall; this bulges the inner peritoneal layer towards the coelomic cavity (Fig. 3a,b). Subsequently, the proliferating cells form the inner budding layer, whereas the outer budding layer is formed by the peritoneum (Figs. 3c, 4a and b). In general, new buds always develop close to older buds. This is most obvious very early in development, when each bud already produces another bud, resulting in a so-called duplicate bud (Figs. 3a–c and 4b–d), which is connected to the colony wall by a single neck (Figs. 3b and 4b). Early duplicate buds show a common lumen, which is surrounded by the inner budding layer and later separated in the neck region. This gives early buds the shape of two-layered sacs (Fig. 4b). With the enlargement of the bud,

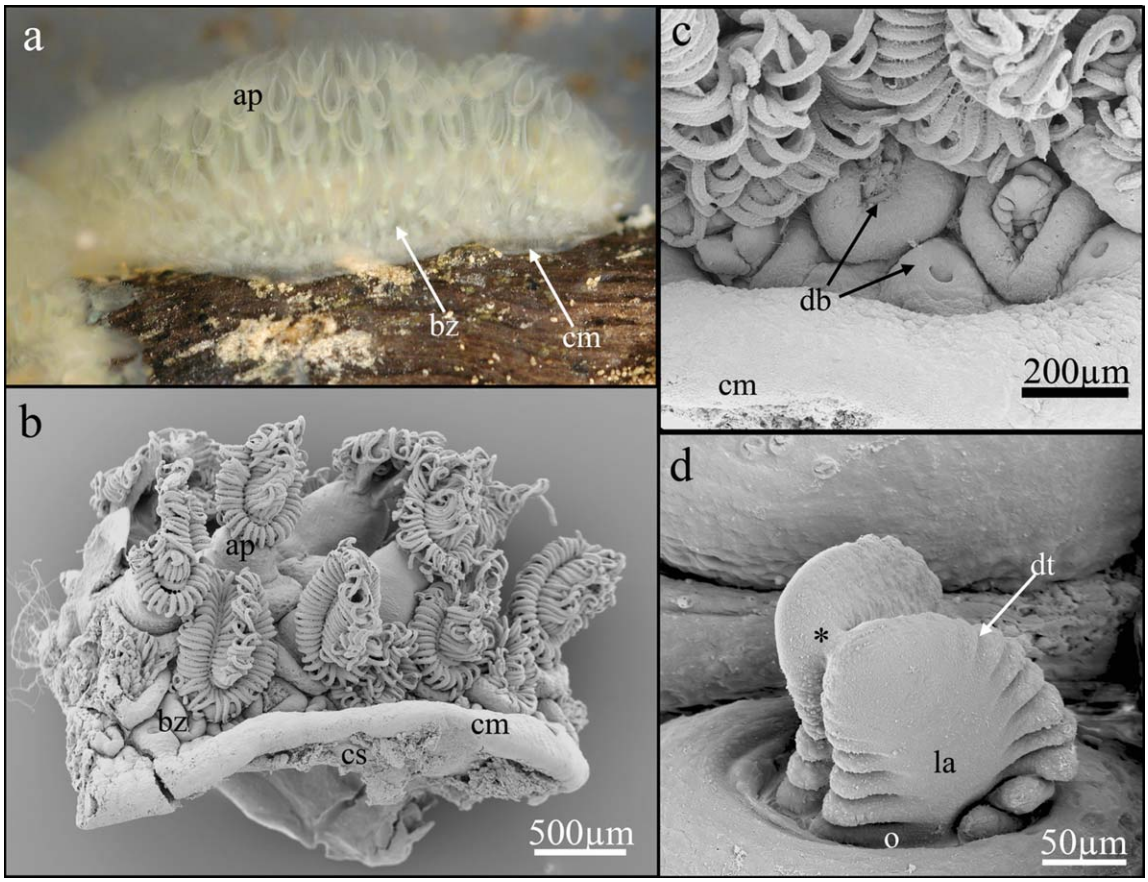

Fig. 1. Overview of *Cristatella mucedo* colonies and their budding zone. (**a**) Living colonies on a piece of wood. The colony is about 1.5 cm in length. (**b–d**) Scanning electron microscopic images. (b) Piece of a colony showing the bulge-shaped colony margin and the budding zone above it. (c) Detail of the budding zone with several developing buds. (d) Detail of a bud protruding from the colony. The asterisk marks the median connection of both lophophoral arms. ap, adult polypides; bz, budding zone; cm, colony margin; cs, creeping sole; db, developing buds; dt, developing tentacles; la, lophophoral arm; o, orifice.

the lumen expands to form a flat disc (Fig. 4d,e). The inner budding layer forms two indentations directed towards the colony center. The indentation closer to the colony margin is shallower and represents the prospective mouth area, while the second indentation, which is deeper and slightly bent towards the prospective mouth area, represents the anlage of the midgut and hindgut.

Stage 2

In the following, the younger bud has been omitted for clarity. The further developed bud and its lumen are more elongated (Fig. 5a,b), and the midgut and hindgut anlage has protruded further towards the mouth area (Fig. 5a). Between the future mouth and anus, the inner budding layer bulges slightly towards the gut anlage, indicating first signs of the invagination of the central nervous system, the ganglion (Fig. 5a). The outer budding layer shows no differentiation except at its proximal side, close to the prospective mouth area, where it forms a knot-like protuberance, the funiculus anlage (Fig. 5a,b).

Stage 3

The bud and its lumen have widened laterally, more distinctly distal to the prospective mouth–anus axis (Fig. 6a,b). The bud differs in its degree of differentiation on the right and left side. Both budding layers bulge towards the bud's lumen, more distinctly on the left lateral widening than on the right (Fig. 6b). These bulges represent the first sign of the lophophore anlage. The developing gut has almost reached the prospective mouth area (Fig. 6b), and the ganglion invagination is much deeper and more prominent than in the previous stage (Figs. 5a and 6b). At the oral side of the bud, the developing funiculus, which is partially attached to the outer budding layer, already extends over the whole length of the bud up to its neck (Fig. 6a).

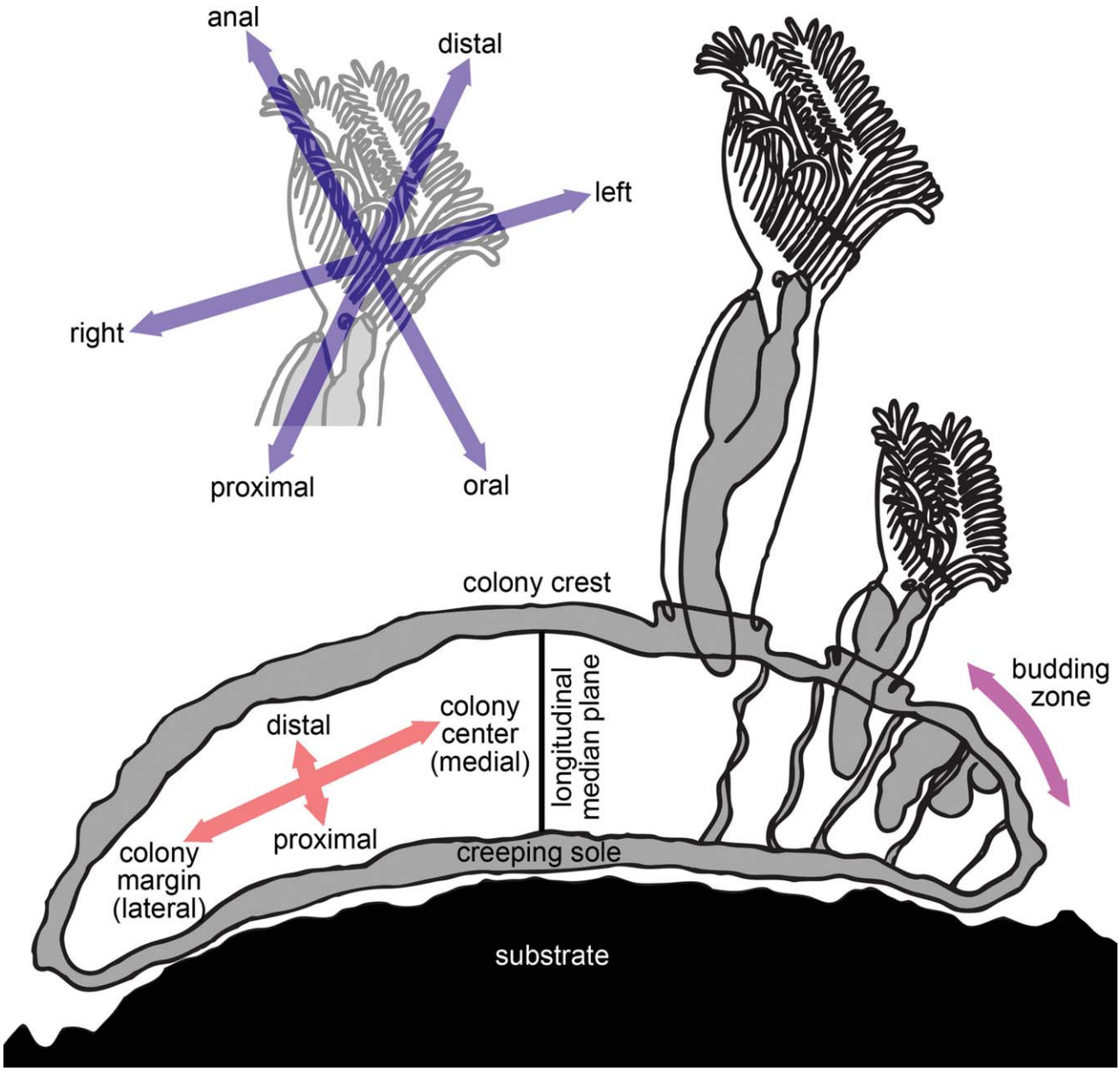

Fig. 2. Schematic cross-section of a *Cristatella mucedo* colony explaining the axis terminology used in this article, modified and redrawn from Brien (1960a).

Stage 4

The gut anlage has fused with the oral indentation, making the gut continuous (Fig. 7c). The two lophophore bulges are much more pronounced and have advanced in their growth further towards the median plane of the bud above the ganglion anlage (Fig. 7a,c,d). The latter has distinctly increased in size and is proportionally large compared with other developing organ systems; it almost entirely fills the space in the loop of the U-shaped digestive tract. It is widely open to the lumen of the bud, the invagination area facing towards the prospective foregut (Fig. 7c). Below the ganglion anlage, the outer budding layer indents laterally on both sides and protrudes between adjacent parts of the inner budding layer (Fig. 7b). The two budding layers extending from the lophophore base to the neck of the bud represent the developing tentacle sheath (Fig. 7d). The funiculus has separated from the outer budding layer except at the proximal oral side (Figs. 3c and 7b). It is a compact cord, which attaches to the colony wall slightly below the neck of the bud. In this stage, distinct developing retractor muscle fibres are visible for the first time. They attach to the outer budding layer on the oral side at the height of the lophophoral bulges, run towards the colony margin, and either attach close to

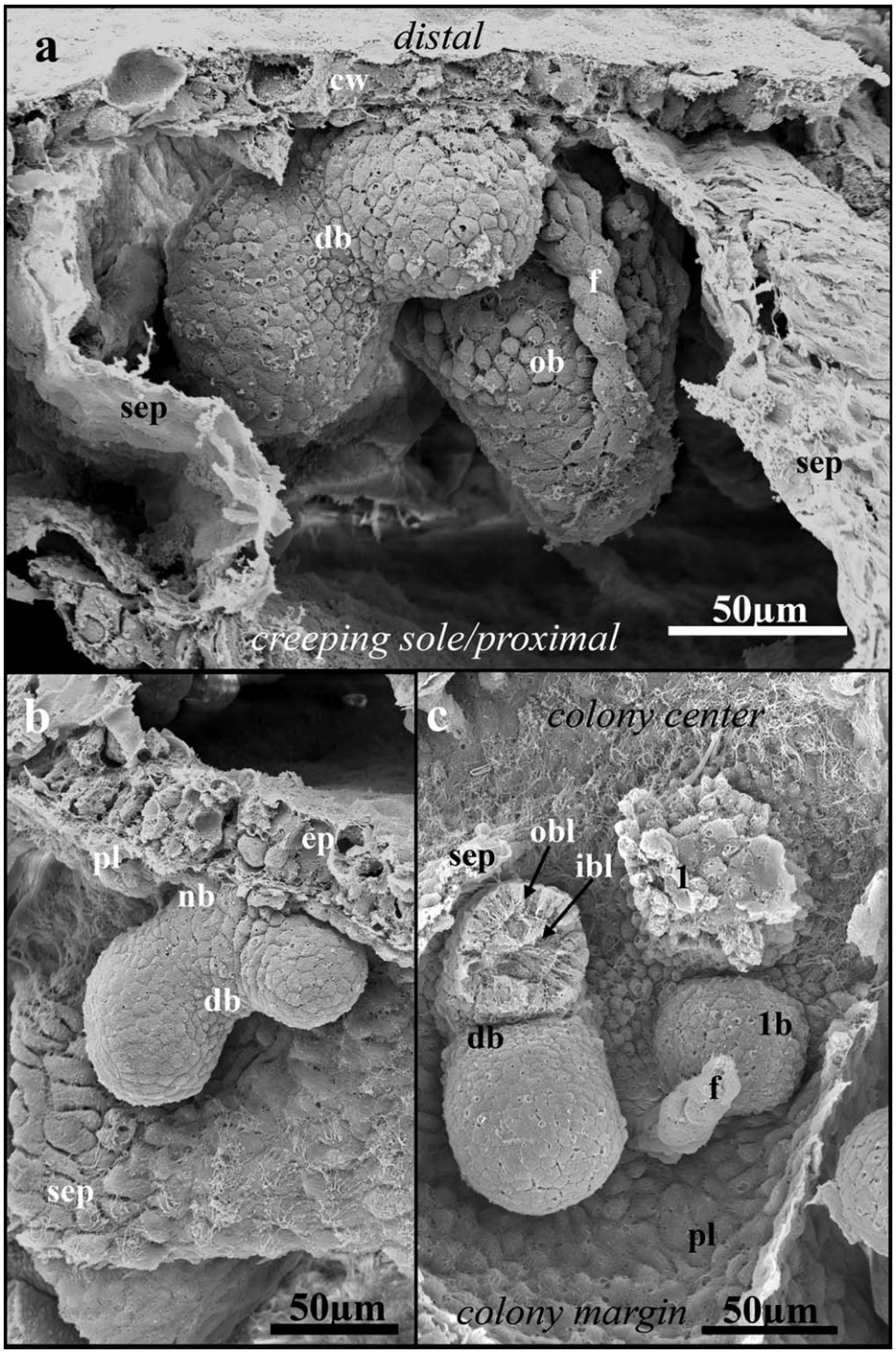

Fig. 3. Scanning electron microscopic images of early buds of *Cristatella mucedo*. (**a**) Lateral view (from the colony margin) showing a duplicate bud and an older bud with a funiculus approximately at budding stage 4. (**b**) Lateral view of a duplicate bud showing the more-progressed left bud next to the less-developed one on the right. (**c**) Proximal view (from the side of the creeping sole) of two duplicate buds with the upper buds broken off. The bud on the upper right (1) is more developed, with bud (1b) derived from it. db, duplicate bud; cw, colony wall; ep, epidermis; f, funiculus; ibl, inner budding layer; nb, neck of the bud attaching the bud to the colony wall; ob, old bud; obl, outer budding layer; pl, peritoneal layer; sep, septum.

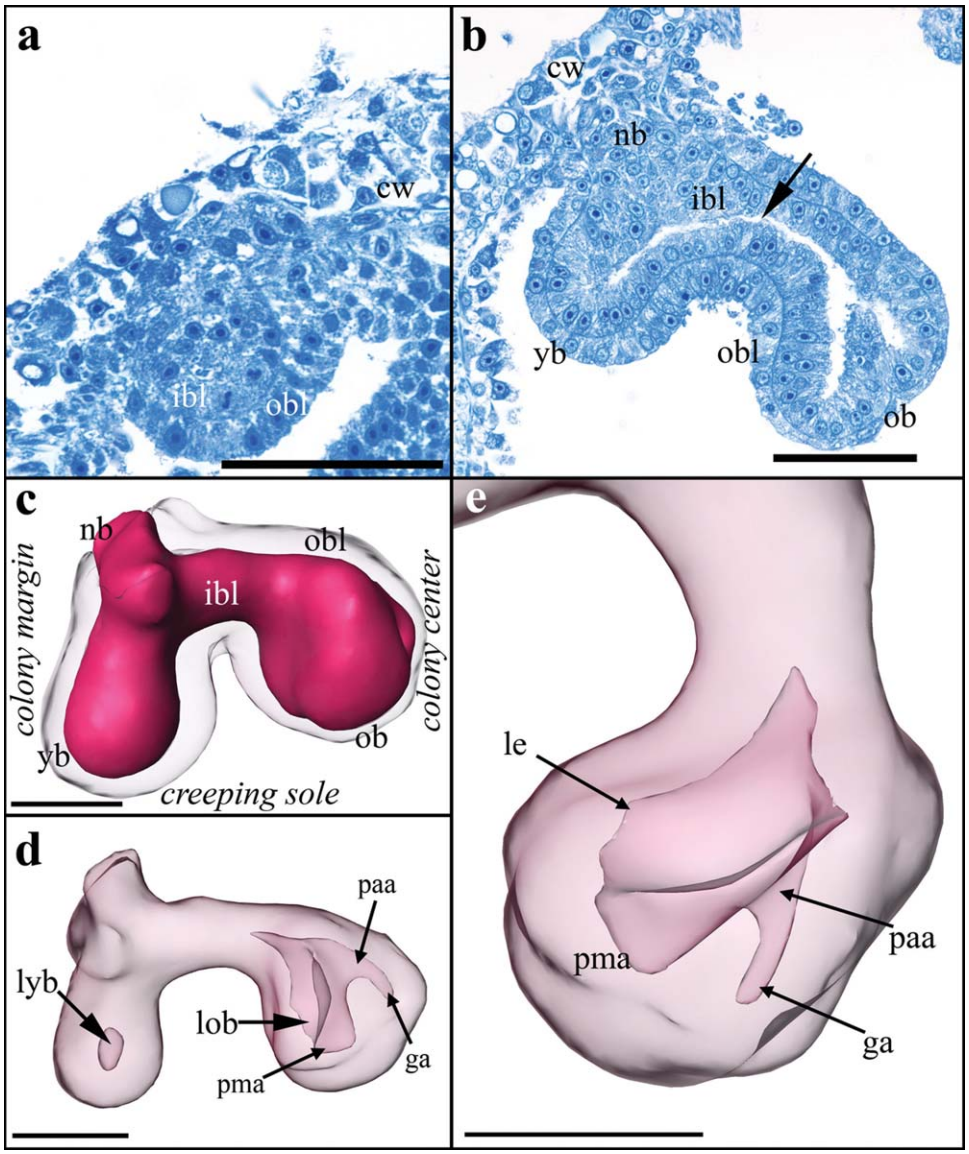

Fig. 4. Semithin section micrographs and 3D-reconstructions based on serial semithin sections of early budding stages of *Cristatella mucedo*. (**a**) Section micrograph of an early bud showing cell proliferation in the colony wall. (**b**) Section micrograph of an early duplicate bud showing a common lumen (arrow). (**c**) Lateral view of a 3D-reconstructed duplicate bud showing (transparently) the outer budding layer surrounding the shaded inner budding layer. (**d**) Same view as in (c), but solely showing (transparently) the inner budding layer. (**e**) Oblique view of the right, more progressed budding stage of the duplicate bud in (c) and (d). Outer budding layer omitted, inner budding layer displayed transparently. cw, colony wall; ga, gut anlage; ibl, inner budding layer; le, lateral extensions of the bud lumen; lob, lumen of the older bud; lyb, lumen of the young bud; nb, neck of the bud attaching the bud to the colony wall; ob, older bud; obl, outer budding layer; paa, prospective anal area; pma, prospective mouth area; yb, younger bud. Scale bar = 50 μm

the neck of the bud to the colony wall or to the so-called incomplete septa that develop from the peritoneum during the budding process (Fig. 7b).

Stage 5

The bud has further grown in length, approximately measuring 200 μm, and has come close to the proximal colony wall, the creeping sole (Fig. 8a). The tentacle sheath, where both budding layers have become very thin, has distinctly elongated. Regional differentiations of the developing digestive tract start: in the middle of the U-shaped anlage, a pouch-like protrusion indicates the developing stomach/caecum region, separating the oral cardiac portion from the intestinum (Fig. 8d). The lophophoral bulges have extended towards the neck of the bud; the outer budding layer now forming the lining of the coelomic cavity within the lophophoral arms. They occupy almost the whole lumen enclosed by the

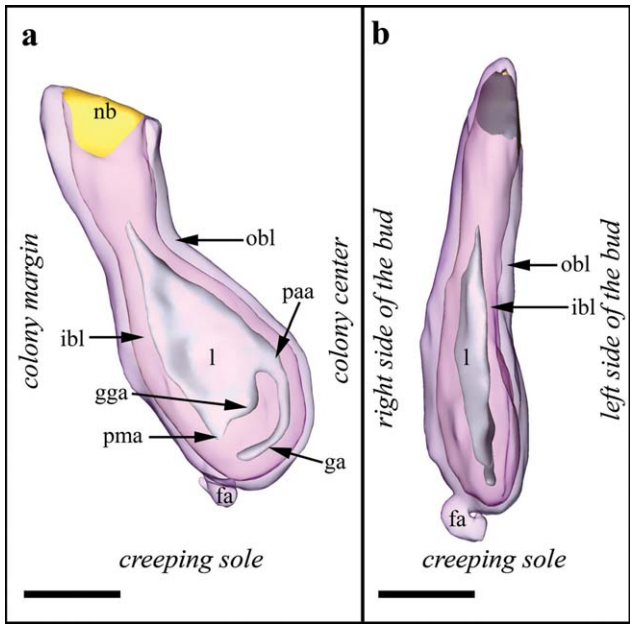

Fig. 5. Three-dimensional reconstructions based on serial semithin sections of budding stage 2 of *Cristatella mucedo*. (**a**) Lateral view of the bud with both budding layers displayed transparently. (**b**) View of the bud from the prospective oral side. Both budding layers displayed transparently. fa, funiculus anlage; ga, gut anlage; gga, ganglion anlage; ibl, inner budding layer; l, lumen of the bud; nb, neck of the bud attaching the bud to the colony wall; obl, outer budding layer; paa, prospective anal area; pma, prospective mouth area. Scale bar = 50 μm

tentacle sheath, the atrium. Along their whole length the two lophophoral arms are connected by a two-layered epithelial bridge (Fig. 8f). Furthermore, the lophophoral base connecting the lophophoral arms starts to differentiate as a circumoral ridge (Fig. 8c,f). On the oral side of the lophophore base, the outer budding layer invaginates from both lateral sides and starts to form the circumoral ring canal, a part of the coelomic cavity of the adult zooid (Fig. 8a). First signs of tentacle differentiation at the lophophore base and in the growing lophophoral bulges are discernable as small hillocks of the peritoneal layer (Fig. 8e). At the base of the lophophoral arms, a minute pore shows that the ganglion anlage is still in open connection to the prospective foregut (Fig. 8e,f). Two lateral outgrowths of the ganglion anlage, the ganglionic horns, protrude in between the epidermal and peritoneal layer of the lophophoral arms (Fig. 8d,e). Medially of the ganglionic horns, the peritoneal layer of each developing lophophoral arm protrudes over the ganglion towards the mouth area (Fig. 8e). Compared with the previous budding stage, the ganglion does not fill the entire space between the gut loop (Fig. 8c,d). Instead, the two lateral indentations of the outer budding layer observed in the previous stage (Fig. 7b) have fused medially and now form the lining of the part of the coelomic cavity that is situated between the gut loop and the ganglion of the developing zooid. From this part of the coelomic cavity, a small flap-shaped out-

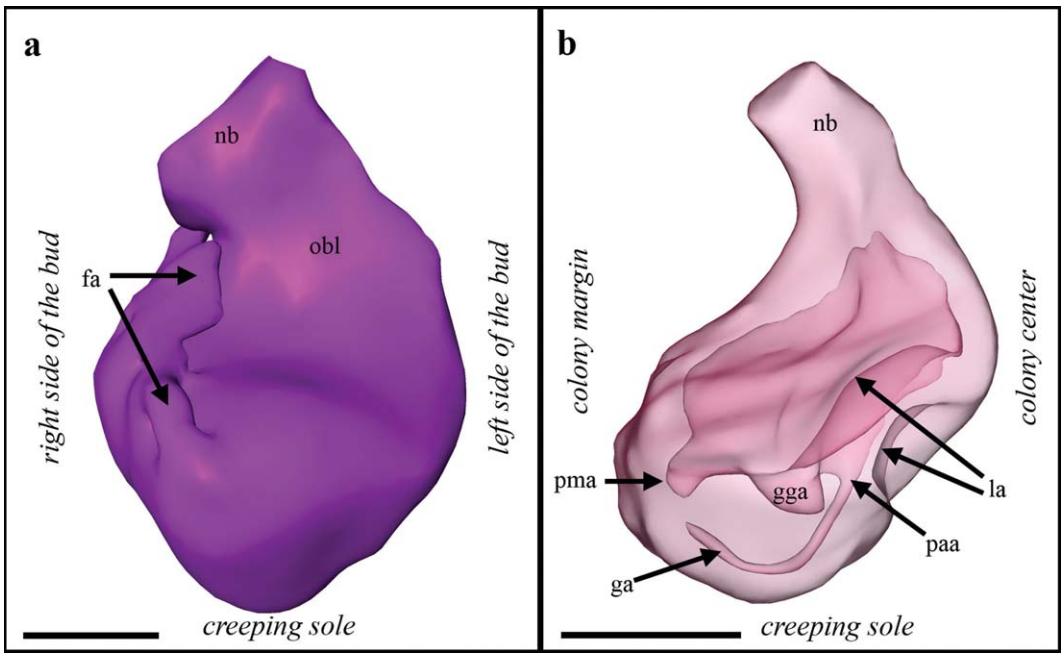

Fig. 6. Three-dimensional reconstructions based on serial semithin sections of budding stage 3 of *Cristatella mucedo*. (**a**) View of the bud from the prospective oral side. The outer budding layer is displayed shaded. (**b**) Lateral view of the transparently displayed inner budding layer. fa, funiculus anlage; ga, gut anlage; gga, ganglion anlage; la, anlage of the lophophoral arm; nb, neck of the bud attaching the bud to the colony wall; obl, outer budding layer; paa, prospective anal area; pma, prospective mouth area. Scale bar = 50 μm

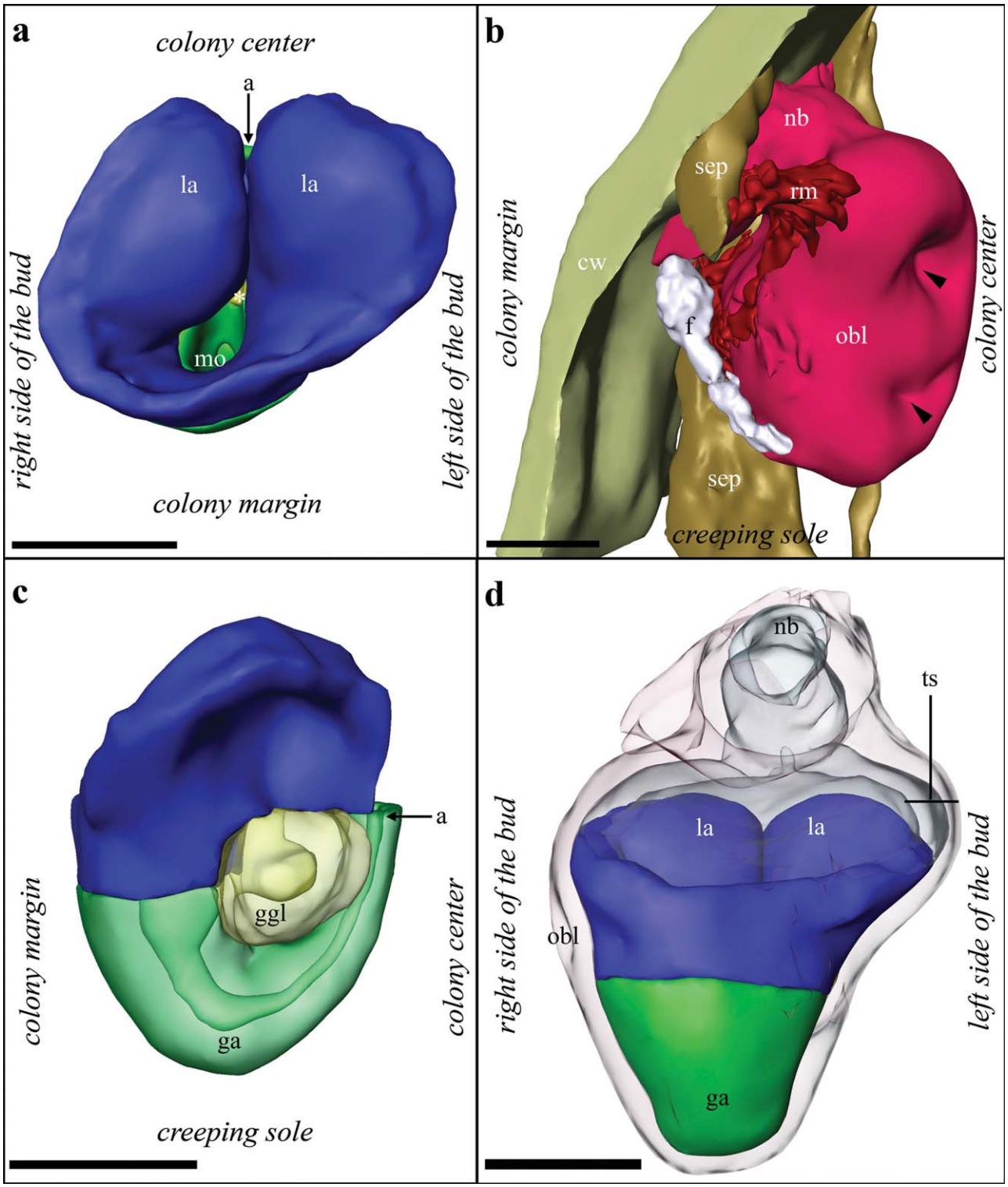

Fig. 7. Three-dimensional reconstructions based on serial semithin sections of budding stage 4 of *Cristatella mucedo*. (**a**) Distal view of the bud showing the developing lophophoral arms, parts of the digestive tract and the ganglion (asterisk). (**b**) Lateral view of the bud showing the outer budding layer attaching to the colony wall by the neck of the bud as well as the funiculus and retractor muscles. Arrowheads indicate indentations of the peritoneal outer budding layer to form linings of the digestive tract and the epistome coelom (lower arrowhead) as well as the lining of the lophophoral arms (upper arrowhead). (**c**) Lateral view of derivates of the inner budding layer, i.e., lophophore, ganglion, and digestive tract. Digestive tract displayed transparently. (**d**) View from the oral side of the bud. Outer budding layer and part of the inner budding layer contributing to the tentacle sheath displayed transparently. a, anus; cw, colony wall; f, funiculus; ga, gut anlage; ggl, ganglion; la, anlage of the lophophoral arm; mo, mouth opening; nb, neck of the bud; obl, outer budding layer; rm, retractor muscles; sep, septum; ts, tentacle sheath. Blue, lophophore; crimson, outer budding layer, i.e., peritoneum; dark brown, septa; dark red, muscles; green, digestive tract; light brown, colony wall; light grey, the part of the inner budding layer contributing to the tentacle sheath; white, funiculus; yellow, ganglion. Scale bar = 50 μm

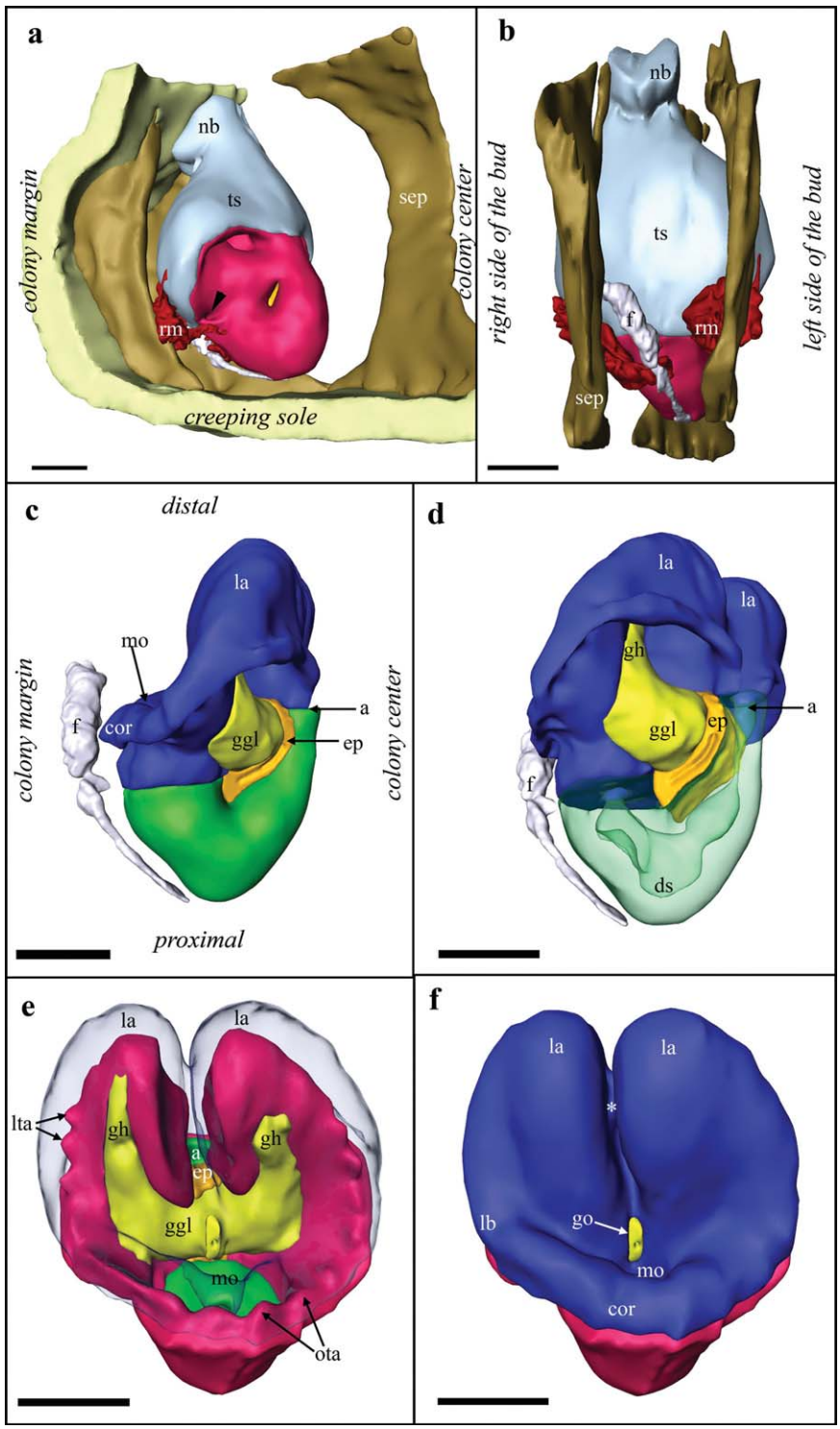

Fig. 8. Three-dimensional reconstructions based on serial semithin sections of budding stage 5 of *Cristatella mucedo*. (**a**) Lateral view of the bud showing the position of the bud within the colony. The arrowhead indicates the lateral infolding of the peritoneal layer showing the developing circumoral ring canal. (**b**) Oral view on the bud showing attachment of the funiculus and the developing retractor muscles on the septa. (**c**) Lateral view of internal structures and the funiculus of the developing bud. (**d**) Oblique view of internal structures and the funiculus. Digestive tract is displayed transparently. (**e**) Distal view of the bud showing the open ganglion with its ganglionic horns between the peritoneal layer and the lophophore surface. Lophophore displayed transparently. (**f**) Similar view as in (e) with the lophophore displayed shaded. The asterisk marks the median connection of both lophophoral arms. a –anus; cor, circumoral ridge; ds, developing stomach; ep, epistome coelom; f, funiculus; ggl, ganglion; gh, ganglionic horns; go, ganglion opening; la, lophophoral arm; lb, lophophoral base; lta, tentacle anlagen on the lophophoral arms; mo, mouth opening; nb, neck of the bud; ota, tentacle anlagen on the oral side; rm, retractor muscles; sep, septum; ts, tentacle sheath. Blue, lophophore; crimson, peritoneal layer; dark brown, septa; dark red, muscles; green, digestive tract; light blue, tentacle sheath; light brown, colony wall; orange, epistome coelom; white, funiculus; yellow, ganglion. Scale bar = 50 μm

growth, the anlage of the epistome coelom, encompasses the ganglion in the median plane (Fig. 8c–e). Close to the developing circumoral ring canal, the retractor muscle fibres attach to both sides of the tentacle sheath and to the peritoneal layer covering the digestive tract (Fig. 8a,b). The retractor muscle bundles attach to the transverse septa that border the developing bud laterally and, in contrast to the previous stage (Fig. 7b), show no contact to the lateral colony wall (Fig. 8a,b). The funiculus has elongated in its proximal part and attaches to a septum (Fig. 8b–d).

Stage 6

The developing zooid has grown larger, approximately measuring 300 μm, and its proximodistal axis lies almost parallel to the colony's creeping sole (Figs. 9a and 10a). At the distal end of the tentacle sheath, several short bands, the duplicature bands, have developed from the peritoneal layer (Figs. 9a–c and 10b). A small protrusion on the right side of the tentacle sheath attaches to a septum (Fig. 9b). The lophophoral arms fill the atrium almost completely, the left arm being slightly larger than the right. The two arms still possess the cellular connection in the median plane over almost the entire length (Fig. 9d). Developing tentacles are present as small hillocks of the epidermal layer of the lophophore, most prominent at the lophophoral base (Fig. 9d–f). Associated with the developing circumoral ring canal, six oral tentacles grow simultaneously. A coelomic cavity can be distinguished at the base of the first two outermost tentacles of each side (Fig. 9f), whereas the median tentacles harbor a compact peritoneal mass. On the lophophoral arms, tentacles develop in a proximodistal succession (Fig. 9d,e). The thick ganglionic horns have almost extended to the distalmost tip of the arms (Fig. 9e–g). With the closure of the pore of the ganglion anlage, the invagination process is completed, resulting in a ganglion that retains a central cavity. Two short protuberances have appeared on each lateroproximal side of the ganglion (Fig. 9g). The prospective epistome coelom has further progressed over the median plane of the ganglion towards the mouth (Fig. 9e–g). Laterally, it is adjacent to the peritoneal lining of the most proximal tentacle anlagen, which are situated medially of the ganglionic horns. Major changes in the digestive tract involve the growth and further differentiation of the future stomach. Instead of inserting at transverse septa as in the previous stage, the retractor muscles attach to the basal colony wall (Fig. 9a). The left portion of the retractor muscles is larger, and some of its fibres attach at the lower end of the tentacle sheath (Fig. 9a,c). The funiculus has become a thin, long strand extending from the oral side of the prospective stomach to the lateral wall of the colony (Figs. 9a,c, and 10a).

Stage 7

Major changes during further development mainly affect the differentiation of the lophophore, the nervous system, and the coelomic cavities. The atrium is still closed towards the vesitbulum anlage, which is indicated by a shallow indentation of the colony wall (Fig. 11a,b). The duplicature bands have elongated and attach to the colony wall around the prospective orifice (Figs. 10c,d, and 11d). Between the duplicature bands and the orifice, short vestibular dilatators have developed (Figs. 10d and 11a). The lophophoral arms have grown extensively and are bent towards the right side of the developing polypide. Their medial connection via a thin cellular bridge is still present (Fig. 11c). The tentacle anlagen at the lophophoral base and in its proximity have grown into stub-like protrusions, while more tentacles continue to develop in a proximodistal succession on the lophophoral arms (Fig. 11c,e). This stage has approximately 65 tentacles. The peritoneal layer of the tentacle anlagen is mostly compact, and coelomic lumina are detectable only at the base of each tentacle (Fig. 11f). In the lophophoral arms, the ganglionic horns extend to the growth zone of further tentacles at the distalmost tip of each arm (Fig. 11e). Nerve strands emanating from the ganglionic horns have grown in between neighboring tentacle anlagen (Figs. 11e and 12a,d). The small proximolateral protuberances of the ganglion seen in the previous stage have grown around the pharynx to form a circumoral nerve ring. As in the lophophoral arms, nerves extend from this nerve ring into the space between neighboring oral tentacles (the prospective intertentacular membrane) (Fig. 12a). Below the circumoral nerve ring, a dense nervous layer surrounds the pharynx (Fig. 12a). The anlage of the epistome appears as a small bulge slightly protruding towards the mouth opening (Fig. 12c). The epistome coelom has widened laterally and medially points finger-like into the epistomal bulge (Fig. 12a,b,d). On each side of the developing polypide, three distinct retractor muscle bundles insert at the proximal end of the tentacle sheath, while the remaining muscles insert at the lophophore base and various parts of the digestive tract (Figs. 10c,d and 11c,d).

Stage 8

The cellular bridge between the lophophoral arms has disappeared. The proximal parts of the coelomic cavity within both lophophoral arms fuse in the median plane above the epistome coelom to form the so-called forked canal. From the latter, coelomic processes extend into those tentacles located between the mouth and the anus (Fig. 13a,b). The two connections of the forked canal to the remaining coelomic cavity are densely ciliated (Fig. 13f). Nerves emerging from the laterodistal part of the ganglion

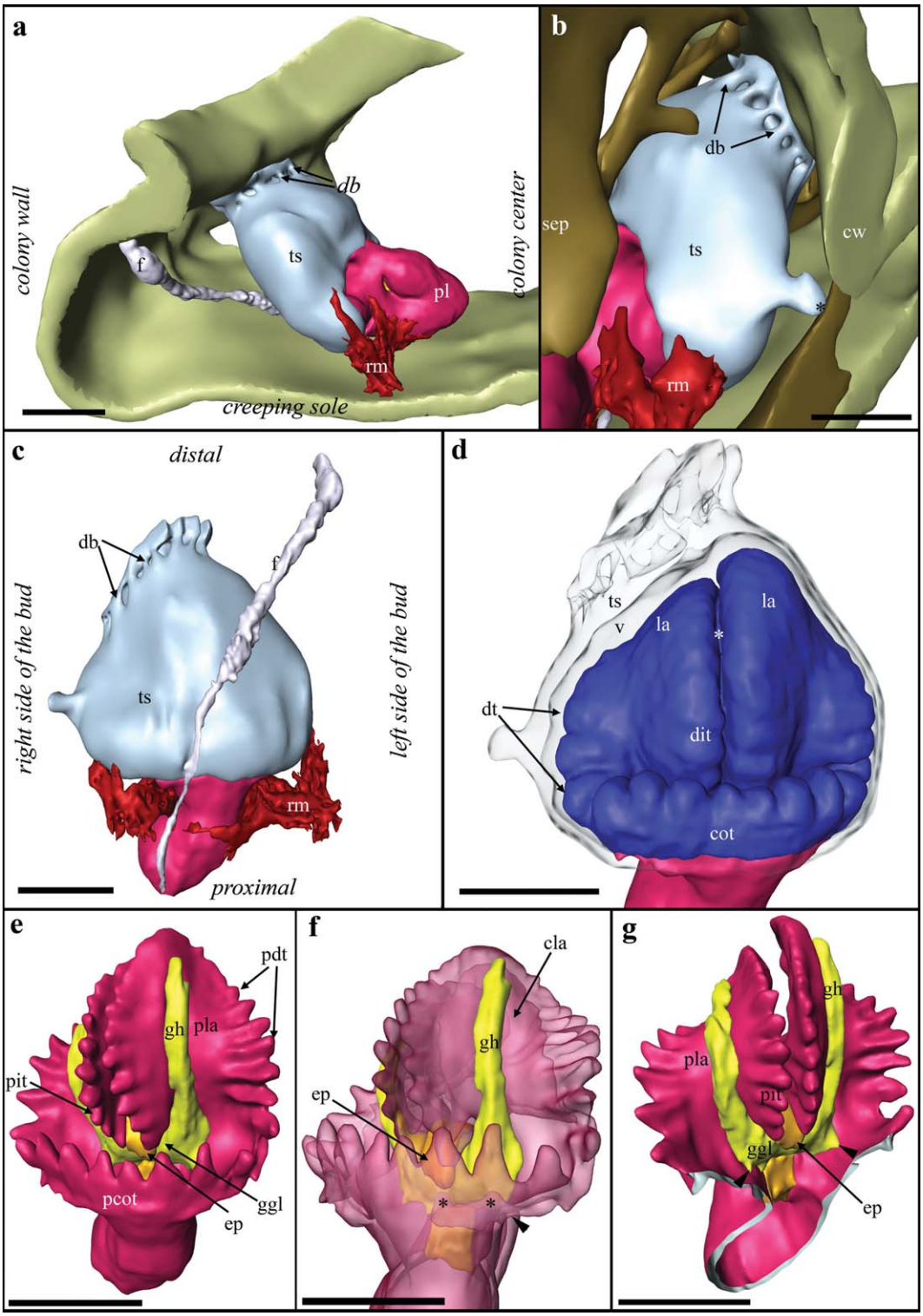

Fig. 9. Three-dimensional reconstructions based on serial semithin sections of budding stage 6 of *Cristatella mucedo*. (**a**) Lateral view of the bud within the colony. (**b**) Oblique view towards the colony margin showing the tentacle sheath's attachment to a septum (asterisk). (**c**) View of the bud from the oral side. (**d**) View of the bud from the oral side showing the developing tentacle crown. The tentacle sheath is displayed transparently. The asterisk marks the persisting median connection of the lophophoral arms. (**e**) Oblique view of the peritoneal layer with the nervous system and the epistome coelom. (**f**) Similar view as in (e) with the peritoneal layer displayed transparently. Asterisks mark the visible lumen at the base of two lateral tentacles on the ring canal. (**g**) Surface cut through the peritoneal layer showing the ganglion encompassed medially by the epistome coelom. Arrowheads point to the proximo-lateral outgrowths of the ganglion. cla, coelomic cavity within the lophophoral arms; cot, circumoral tentacles; cw, colony wall; db, duplicature bands; dit, developing inner row of tentacles; dt, developing tentacles; ep, epistome coelom; f, funiculus; ggl, ganglion; gh, ganglionic horns; la, lophophoral arms; pcot, peritoneal layer of the circumoral tentacles; pdt, peritoneal layer of developing tentacles; pit, peritoneal layer of the inner row of tentacles; pl, peritoneal layer; pla, peritoneal layer of the lophophoral arms; rm, retractor muscles; sep, septum; ts, tentacle sheath; v, vestibulum. Blue, lophophore; crimson, peritoneal layer; dark brown, septa; dark red, muscles; light blue, tentacle sheath; light brown, colony wall; orange, epistome coelom; white, funiculus; yellow, ganglion. Scale bar in a, c–g = 100 μm, in b = 75 μm.

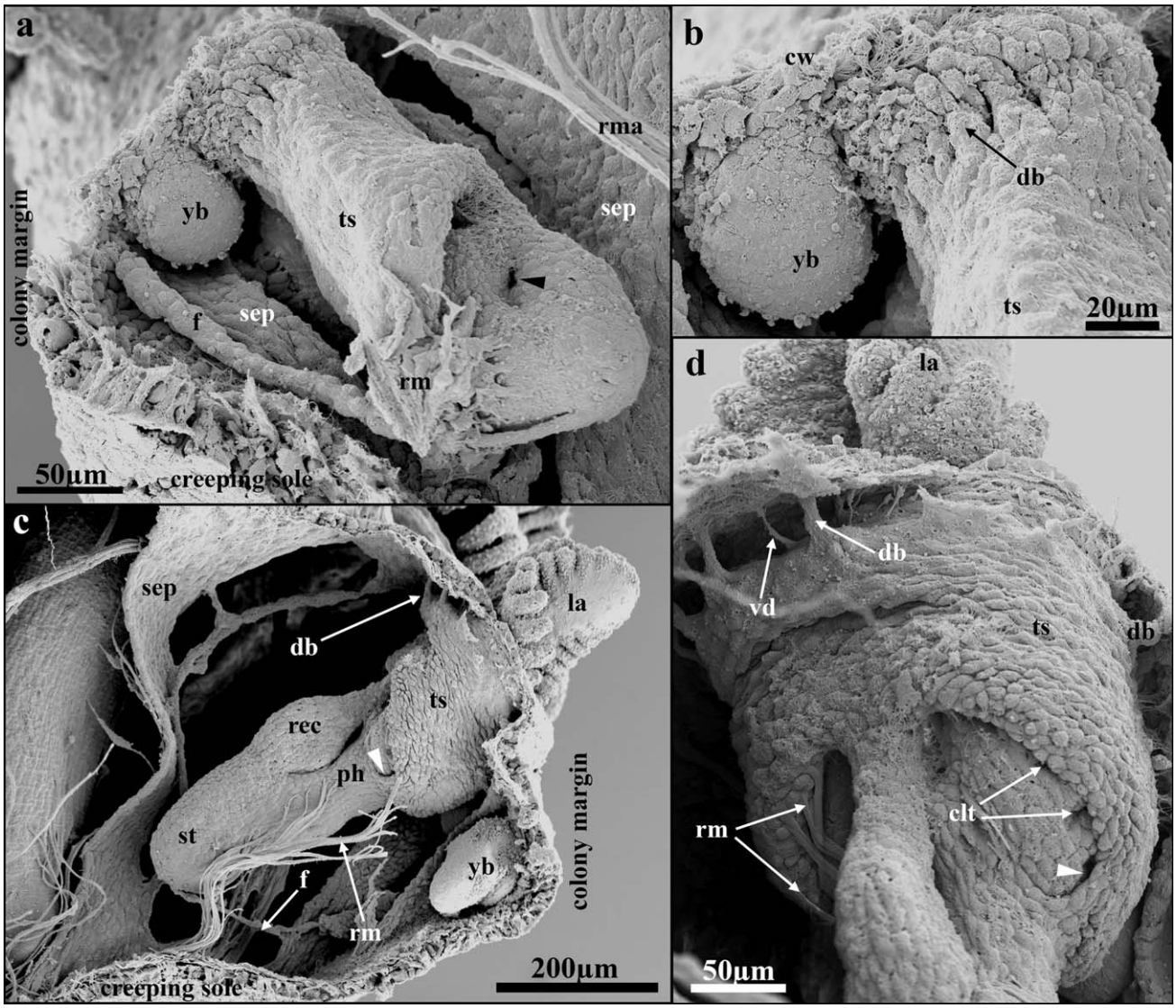

Fig. 10. Scanning electron microscopic images of progressed buds of *Cristatella mucedo*. (**a**) Lateral view of a developing bud approximately at budding stage 6. The arrowhead indicates the opening towards the epistome coelom. (**b**) Detail of (a) showing developing duplicature bands. (**c**) Lateral view of a developing bud approximately at budding stage 7. Note that in this bud the zooid is already evaginating. The arrowhead indicates the opening of the circumoral ring canal. (**d**) Close-up view from the anal side of the bud shown in (c). The arrowhead indicates the opening of the circumoral ring canal. clt, coelomic cavity leading into lateral tentacles; cw, colony wall; db, duplicature bands; f, funiculus; la, lophophoral arm; ph, area of the pharynx; rec, area of the rectum; rm, retractor muscles of the bud; rma, retractor muscles of an adult zooid; sep, septum; st, area of the stomach; ts, tentacle sheath; vd, vestibular dilatators; yb, young bud.

innervate the tentacles associated with the forked canal in the same manner as previously described for the other tentacles (Fig. 13b,c). The epistome forms a large, laterally broad flap above the mouth opening (Fig. 13a,b). Its coelomic cavity remains confluent with the remaining coelom. Within the epistome it is tongue-shaped, whereas it is widened laterally in the part adjacent to the ganglion (Fig. 13c,e).

The ganglion has differentiated: the anal situated part forms a dense nervous mass, whereas the part adjoining the pharyngeal epithelium has become very thin (Fig. 13e). This results in a displacement towards the oral side of the previously centrally located ganglionic cavity, which in this stage has acquired the shape of a flattened dumbbell with lateral portions bending, and a median portion protruding anally (Fig. 13d).

Adult Coelomic Organization

In adult specimens of *C. mucedo*, all coelomic compartments are confluent (Fig. 14b–d). Six to eight tentacles are associated with the ring canal on the oral side (Fig. 14b), while nine tentacles are linked to the forked canal (Fig. 15a,b). The epi-

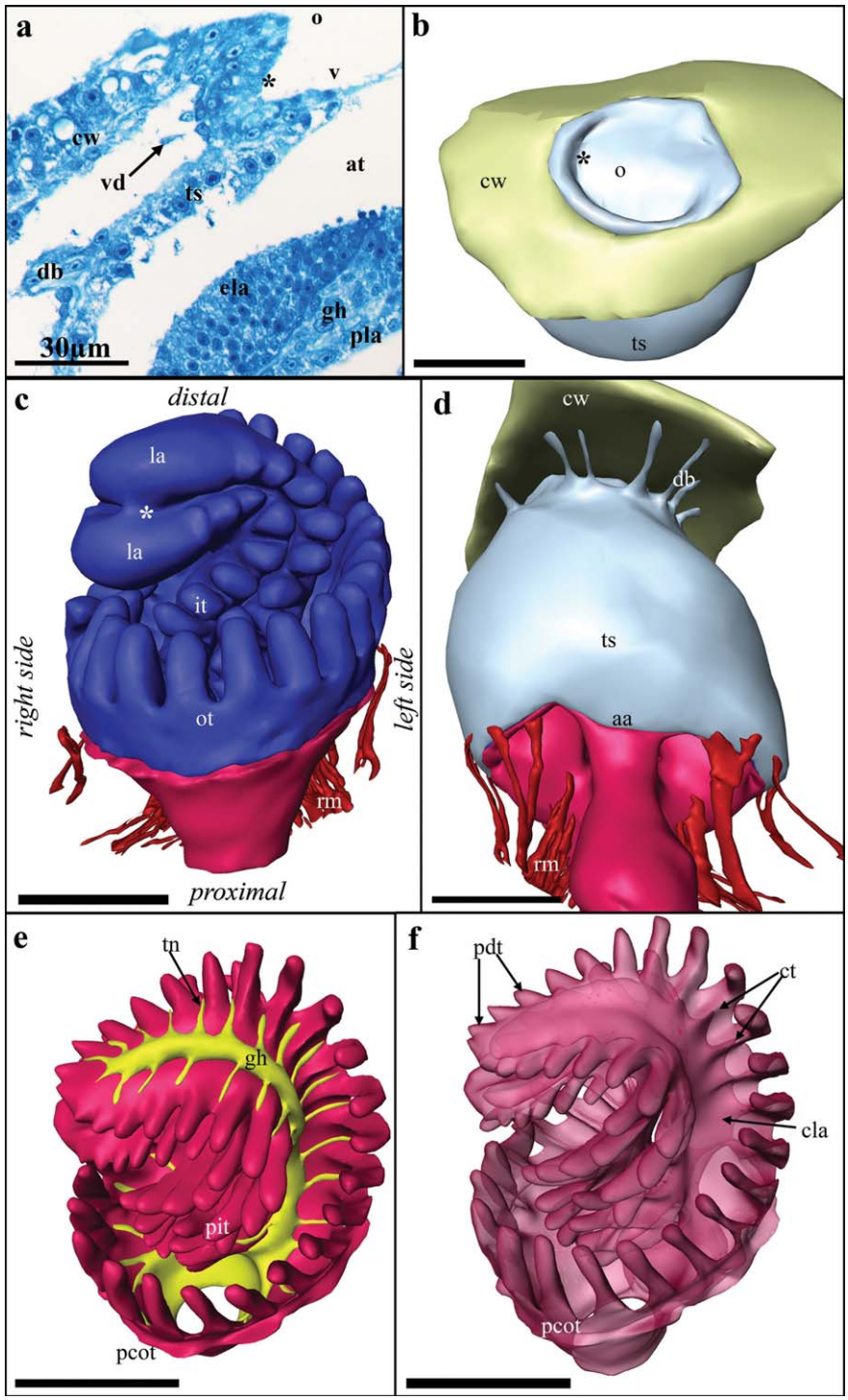

Fig. 11. Budding stage 7 of *Cristatella mucedo*. (**a**) Semithin section. The asterisk marks the indentation of the colony wall where the polypide will evaginate. (**b–f**) Three-dimensional reconstructions based on serial semithin sections. (**b**) View of the colony wall showing the indentation (asterisk) of (a). (**c**) View from the oral side on the developing tentacle crown. The asterisk marks the median connection of the lophophoral arms. (**d**) View from the anal side showing duplicature bands. (**e**) Oblique view of the peritoneal layer of the tentacle crown and the nervous system. (**f**) Similar view as in (e), but with the nervous system omitted and the peritoneal layer displayed transparently. aa, area of the anus; at, atrium; cla, coelomic cavity within the lophophoral arms; ct, coelomic cavity extending towards the tentacles; cw, colony wall; db, duplicature band; ela, outer epithelium of the developing lophophoral arm; gh, ganglionic horn; it, inner row of tentacles; la, lophophoral arm; o, orifice; ot, oral tentacles; pcot, peritoneal layer of the circumoral tentacles; pdt, peritoneal layer of developing tentacles; pit, peritoneal layer of the inner row of tentacles; pla, peritoneal layer of the lophophoral arms; rm, retractor muscles; tn, tentacle nerves; ts, tentacle sheath; v, vestibulum; vd, vestibular dilatators. Blue, lophophore; crimson, peritoneal layer; dark red, muscles; light blue, tentacle sheath; light brown, colony wall; yellow, ganglion. Scale bar in b–f = 100 µm.

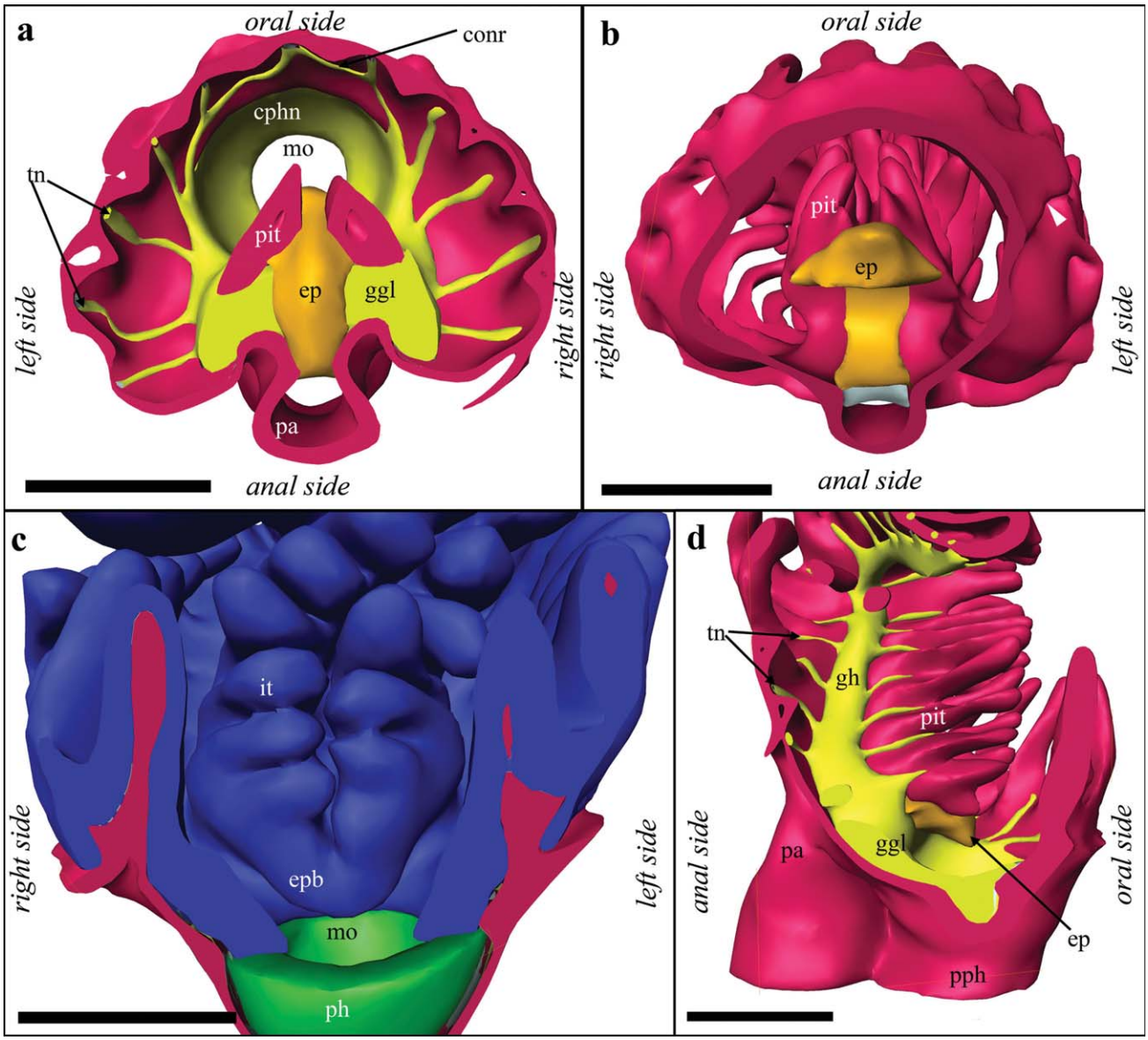

Fig. 12. Three-dimensional reconstructions of the developing epistome based on serial semithin sections of budding stage 7 of *Cristatella mucedo*. (**a**) Distal view of a cut through the oral–anal axis showing the peritoneal layer at the tentacle base, parts of the nervous system, and the epistome coelom. (**b**) View from the side of the digestive tract showing peritoneal layer and the epistome coelom. (**c**) Oral view on a cut through the circumoral tentacle region showing the epistome bulge. (**d**) Lateral cut through the peritoneal layer and the nervous system. conr, circumoral nerve ring; cphn, circumpharyngeal nerves; ep, epistome coelom; epb, epistome bulge; ggl, ganglion; gh, ganglionic horn; it, inner row of tentacles; mo, mouth opening; pa, peritoneal layer covering the rectum and anus; ph, pharynx; pit, peritoneal layer of the inner row of tentacles; pph, peritoneal layer covering the pharynx; tn, tentacle nerves. Blue, lophophore; crimson, peritoneal layer; green, digestive tract; orange, epistome coelom; yellow, ganglion. Scale bar in a = 50 μm, b–d = 75 μm.

stome coelom has formed a flat disc and remains in open connection to the trunk coelom, similar to the condition in stage 8 (Figs. 14b–d and 15a,b). In some adults, the forked canal possesses a distinct bladder below the tentacle that is situated in the median plane (Figs. 14a, 15a,b, and 16). The bladder protrudes anally and is externally visible as an epidermal bulge. It is filled with cells, some of them containing several vacuoles or dense inclusions and contorted, prominently staining thin threads (Fig. 16). On the anal side of the bladder, the peritoneum and the epidermis are very thin. On the oral side, the peritoneal epithelium adjacent to the epistome coelom resembles the epithelium of the ciliated ducts of the forked canal, except that the cells possess vacuoles (Fig. 16).

Emergence of the polypides. The lophophore of stage 8, shortly before polypide eversion, shows well-developed lophophoral arms that have lost the median cellular bridge. However, we also observed

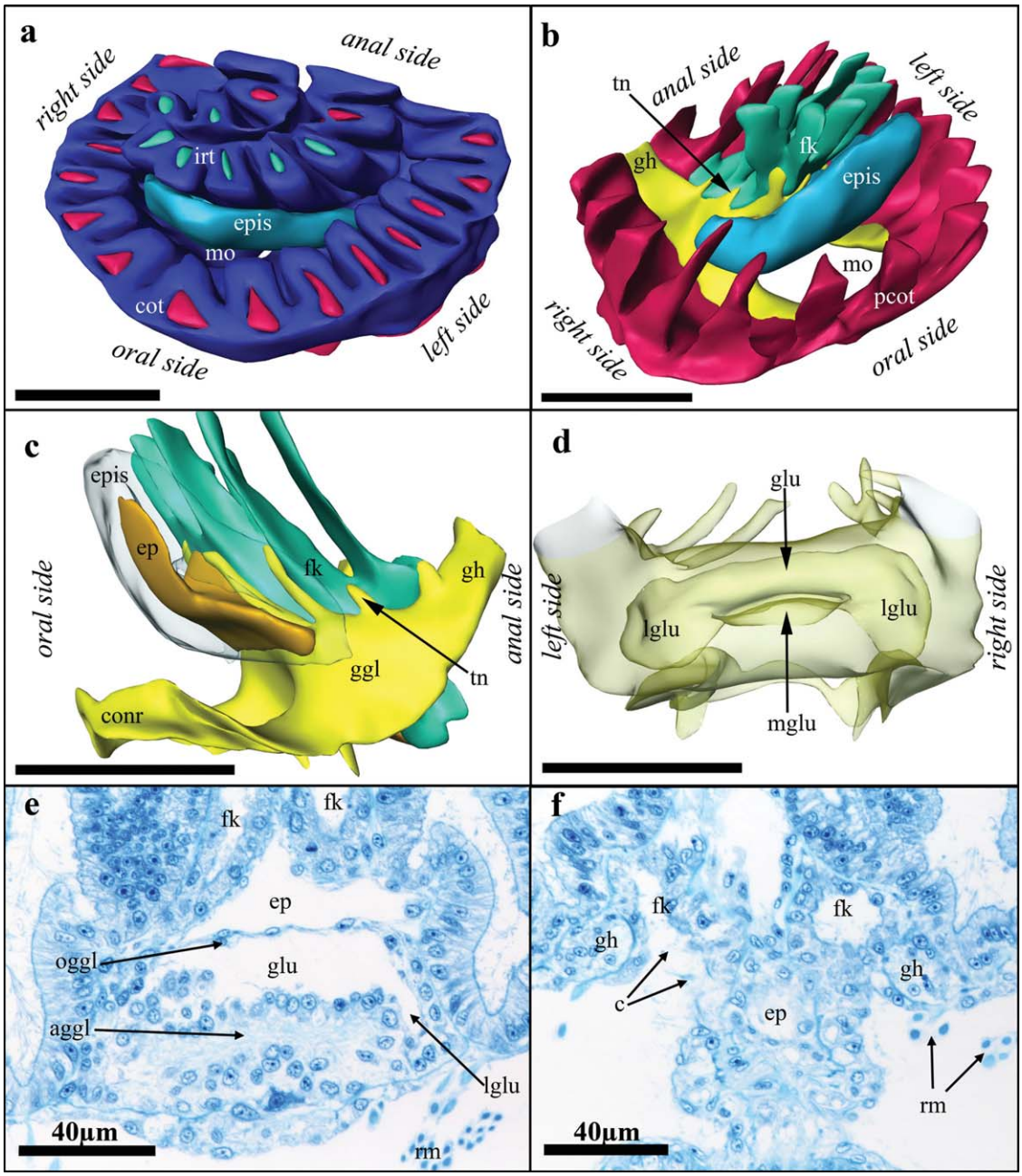

Fig. 13. Budding stage 8 of *Cristatella mucedo*. (**a–d**) Three-dimensional reconstructions based on serial semithin sections. (**a**) Oblique view of the lophophore base. (**b**) Oblique view showing the peritoneal layer of the lophophore base as well as the epistome and nervous system. (**c**) Lateral view on the nervous system with the peritoneal layer of the inner row of tentacles (forked canal) and the epistome coelom arching over the ganglion. (**d**) Transparent view of the ganglion showing its lumen. (**e** and **f**) Semithin sections. (**e**) Oblique section through the ganglion and epistome coelom. (**f**) Oblique section at the entrances of the forked canal showing its ciliation. aggl, anal side of the ganglion; c, cilia; conr, circumoral nerve ring; cot, circumoral tentacles; ep, epistome coelom; epis, epistome; fk, forked canal; ggl, ganglion; gh, ganglionic horns; glu, ganglion lumen; irt, inner row of tentacles; lglu, lateral parts of the ganglion lumen; mglu, median part of the ganglion lumen; mo, mouth opening; oggl, oral side of the ganglion; pcot, peritoneal layer of the circumoral tentacles; rm, retractor muscles; tn, tentacle nerv. Blue, lophophore; crimson, peritoneal layer; light green, peritoneal layer of the inner row of tentacles (forked canal); orange, epistome coelom; turquoise, epistome; yellow, ganglion. Scale bar in a–c = 100 µm, d = 75 µm.

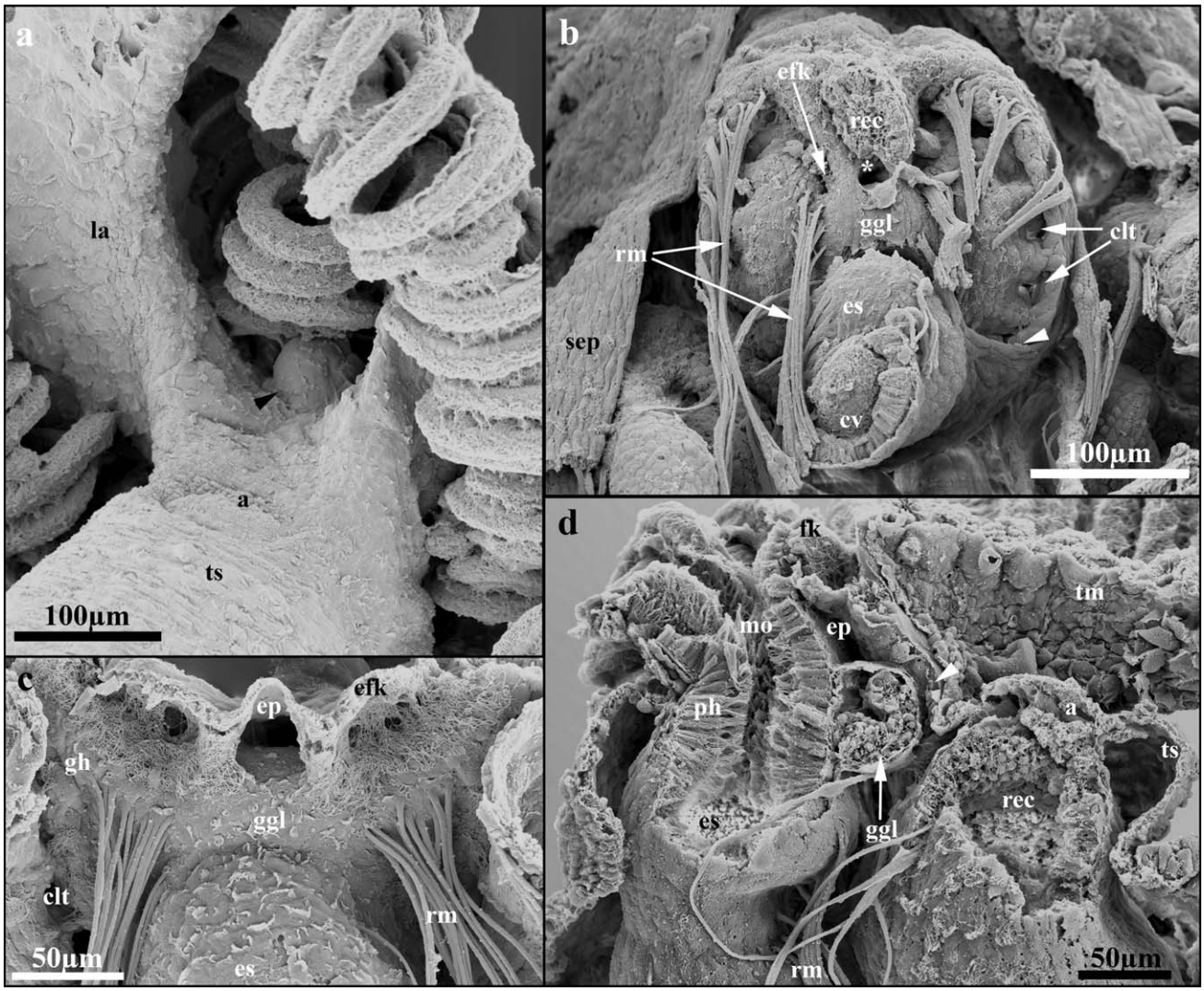

Fig. 14. Scanning electron microscopic images of adult zooids of *Cristatella mucedo*. (**a**) Extended zooid showing the large tentacle-bearing lophophoral arms and the bladder-shaped bulge (arrowhead) of the forked canal above the anus. (**b**) Retracted zooids with most parts of the digestive tract broken away showing the confluent coelomic cavity. The arrowhead marks the opening of the circumoral ring canal into the trunk coelom. The asterisk marks the opening of the epistome coelom into the trunk coelom. (**c**) Detail of another zooid viewed from the anal side showing the entrances of the densely ciliated forked canal and the epistome coelom above the ganglion. (**d**) Lateral view into a broken, extended zooid showing the connection of the epistome coelom with the remaining coelom (arrowhead). a, anus; clt, coelom of lateral tentacles; cv, cardiac valve; efk, entrance to the forked canal; ep, epistome coelom; es, esophagus (covered by the peritoneal layer) ; fk, ciliated forked canal; ggl, ganglion (covered by the peritoneal layer) ; gh, ganglionic horns (covered by the peritoneal layer) ; la, lophophoral arm; mo, mouth opening; ph, pharynx, rec; rectum, rm; retractor muscles, sep; septa, tm; tentacle membrane, ts; tentacle sheath.

polypides emerging with sparsely developed lophophoral arms still exhibiting a median cellular connection (Fig. 1d).

DISCUSSION

Comparison with Previous Descriptions and other Phylactolaemate Species

Phylactolaemates exhibit three different modes of reproduction, one sexual and two asexual modes. The latter are represented by budding of the colony and germination of statoblasts. Previous investigators concluded that polypide formation is identical in all three developmental pathways (see Nielsen, 1971 for a summary). Our results on the development of the bud in *C. mucedo* substantiate the previous works of Davenport (1890) and Braem (1890, 1913).

Differences among phylactolaemate species occur in the relative timing of the differentiation of organ systems. In a rather advanced stage of germinating statoblasts of *P. fungosa*, comparable with *Cristatella* budding stage 6–7, the ganglion is still in open connection with the lumen of the digestive tract (Handschuh et al., 2008), whereas the ganglion

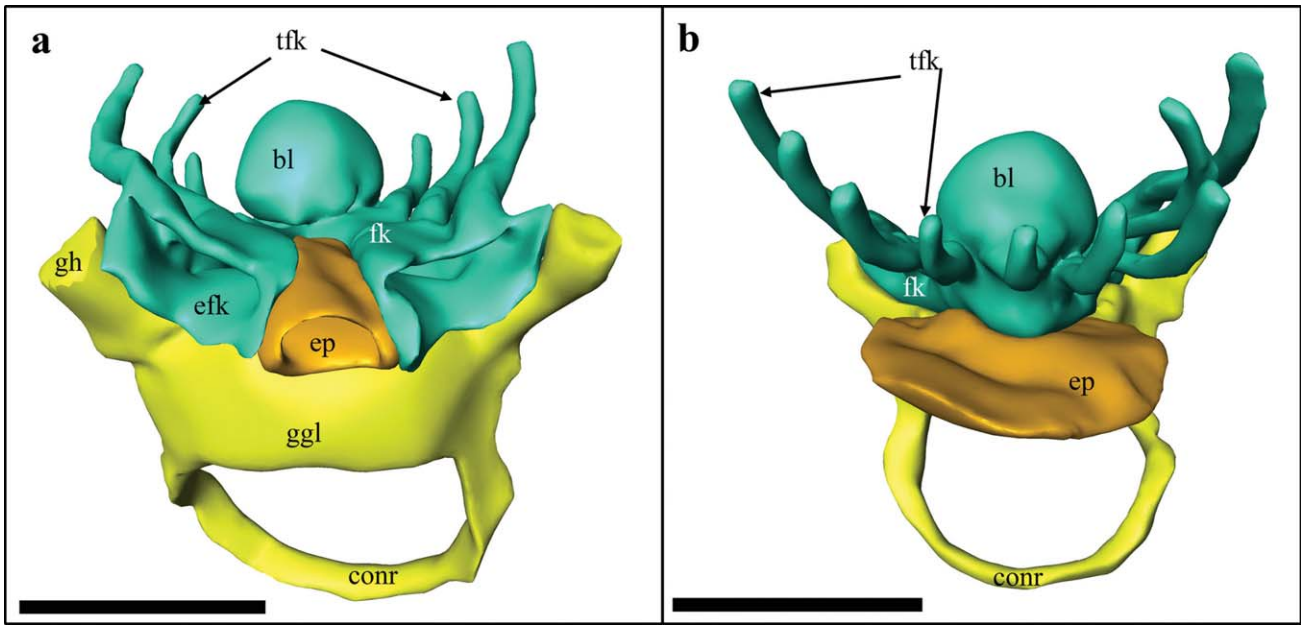

Fig. 15. Three-dimensional reconstructions based on serial semithin sections of the forked canal, the epistome coelom and the nervous system in an adult zooid of *Cristatella mucedo*. (**a**) View from the anal side on the openings of the forked canal and the epistome coelom above the ganglion. (**b**) View from the oral side on the forked canal with its distal bladder and the flat epistome coelom. bl, bladder; conr, circumoral nerve ring; efk, entrance to the forked canal; ep, epistome coelom; fk, forked canal; ggl, ganglion; gh, ganglionic horns; tfk, tentacles on the forked canal. Light green, peritoneal layer of the inner row of tentacles (forked canal); orange, epistome coelom; yellow, ganglion. Scale bar = 100 μm.

closes earlier in *C. mucedo*. A developmental stage in the germinating statoblast of *Hyalinella punctata*, similar to that of *P. fungosa* mentioned above, shows a gut still closed at the esophagus-cardia border (Schwaha, unpublished data), which in both *P. fungosa* and *C. mucedo* breaks through very early.

In *C. mucedo*, lophophoral arms medially connected during their development were previously recognized by Davenport (1890) and Braem (1913, in germinating statoblasts). Such "bridged" lophophoral arms are only known in one other species, *Pectinatella magnifica* (Davenport, 1890). This "bridge" connecting both arms was described to dissolve before orifice formation in *C. mucedo* (Davenport, 1890). We also found fully developed tentacle crowns at the time of polypide eversion. Nonetheless, we also observed several cases in which both arms were still connected at the time of emergence. We conclude that there is a high variability in the differentiation of the tentacle crown at the time of emergence.

In young buds of *Lophopodella carteri*, the tentacle crown is sparsely differentiated at the time of emergence. Moreover, each pair of developing tentacles on the median side of the lophophoral arms is connected in the middle (Rogick, 1937). This mode of tentacle development in *L. carteri* therefore differs considerably from the "bridge" joining both developing lophophoral arms over the whole length in *C. mucedo* and *P. magnifica*. The function of these different connections of lophophoral structures, which seems to be restricted to large, gelatinous phylactolaemates, remains unknown.

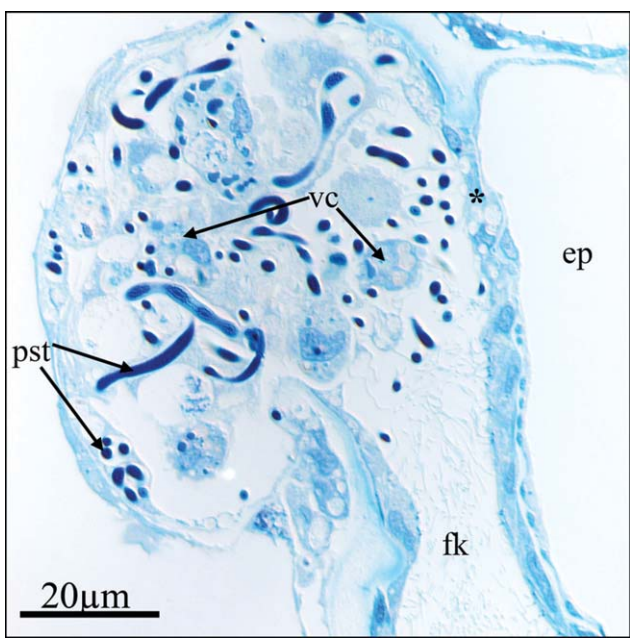

Fig. 16. Oblique semithin section trough the bladder-shaped bulge at the distal end of the forked canal. The asterisk marks vacuolated cells in the epithelium of the forked canal facing the epistome coelom. ep, epistome coelom; fk, ciliated forked canal; pst, prominently staining threads; vc, vacuolated cells.

Formation of the Funiculus

To describe the organogenesis during the budding process, we analyzed a series of 10 developmental stages that show the major morphological changes in the differentiation of the lophophore, digestive tract, and central nervous system. Accordingly, the selection of the treated developmental stages was focusing on the structures originating from the epithelial inner budding layer and associated coelomic cavities. While these developments take place gradually throughout the budding process, other organ systems such as the funiculus or retractor muscles develop more rapidly and during a comparatively short time frame. Consequently, their development was treated in less detail in this study, and the descriptions are thus only fragmentary.

The formation of the funicular strand begins in our budding stage 2, whereas in stage 4 it already resembles the adult condition in that it connects the digestive tract to the colony wall. In budding stage 2, the funiculus anlage is a mere protuberance at the proximal side of the outer budding layer, as similarly described by Davenport (1890), Kraepelin (1892), Rabito (1897), and von Buddenbrock (1910). The latter authors all described the funiculus at one point of its development to be a short compact cord hanging freely into the coelomic cavity. Contrarily, Braem (1890) described the funiculus to originate from a longitudinal ridge that arises from the median part on the oral side of the outer budding layer, thus bearing similarity to our budding stage 3. Furthermore, he never found the funicular strand to be hanging freely into the coelomic cavity of the colony. Our current results on funiculus formation do not favor one of the two diverging descriptions. However, we did find indications that funiculus and retractor muscle formation are highly variable processes that are apparently often associated with amoeboid coelomocytes within the colonial coelom. This variability appears to be correlated to the spatial and temporal succession of the buds and consequently to the position of the bud within the colony in regard to the colony margin and zooidal septa. This variability is also reflected in the diverging attachment of the funiculus to either a septum or the colony wall (compare stages 5 and 6).

Formation of the Digestive Tract

As described in previous studies on *C. mucedo* (Davenport, 1890; Braem, 1890, 1913), most parts of the digestive tract are formed by an outpocketing of the prospective anal side of the early bud; this outpocketing grows in a U-turn towards the oral side of the polypide anlage, while a shallow indentation forms the future esophagus and pharynx. In *P. magnifica* (Braem, 1913), *Plumatella fungosa* (Kraepelin, 1892) and *H. punctata* (Schwaha, unpublished data), gut formation is similar. Only in *Asajirella gelatinosa* gut formation is described as involving a protrusion of the oral side of the bud (Oka, 1891). In its first description, *A. gelatinosa* was placed in the genus *Pectinatella* (Oka, 1891), but based on morphological investigations, the new genus *Asajirella* was created (Oda and Mukai, 1989). This monotypic genus is placed within the Lophopodidae, which is also corroborated by recent molecular phylogenies (Okuyama et al., 2006; Hirose et al., 2008; Fuchs et al., 2009). That family is remotely related to Pectinatellidae or Cristatellidae. Accordingly, there is no reason to doubt the correctness of either Braem's (1913) or Oka's (1891) results on digestive tract formation in *P. magnifica* and *A. gelatinosa*, respectively. Nonetheless, Oka (1891) probably missed the first stages of germination (see Mukai, 1982), calling for a reinvestigation of the gut formation in *A. gelatinosa*.

Similar discrepancies of the gut formation are described within cyclostome and ctenostome bryozoans. In cyclostome budding, it was described as being similar to most phylactolaemate species as an outpocketing of the anal region which later fuses with a small indentation at the oral side (Borg, 1926). In contrast, the gut was found to be formed as an outpocketing of the oral side in a more recent study on metamorphosis and ancestrula formation in cyclostomes (Nielsen, 1970). Although polypide formation is essentially the same in colonial budding and at metamorphosis in phylactolaemates and gymnolaemates, differences could occur within cyclostomes. This would explain the controversial descriptions of gut formation. In ctenostomes, descriptions diverge as well. In several species, the gut is formed as an anal outpocketing (*Flustrellidra hispida*: Prouho, 1890; *Paludicella articulata*: Davenport, 1891; *Pottsiella erecta*: Braem, 1940; *Hislopia malayensis*: Schwaha, unpublished data), whereas formation by the prospective mouth area was described for *Zoobotryon verticillatum* (Zirpolo, 1933), *Pherusella*, and *Bowerbankia* (Soule, 1954). Note that an intermediate type of two more or less simultaneous invaginations at the oral and the anal side, which later fuse in the midline, has been described for the ctenostomes *Alcyonidium mytili* (Barrois, 1877) and *Hypophorella expansa* (Ehlers, 1876). The same mode has also been reported for the cheilostomes *Bugula avicularia* (Seeliger, 1890) and *M. membranacea* (Nitsche, 1871), contrary to the observations of Calvet (1900) and Lutaud (1961), respectively, who described the process as an anal outpocketing. The gut in *Schizoporella unicornis* also forms from the anal side (Barrois, 1886).

Kamptozoans (entoprocts) in particular have been considered as sister group to bryozoans in past (Nitsche, 1869; Marcus, 1958; Nielsen, 1971; Cuffey, 1973) and recent (molecular) phylogenies (Hausdorf et al., 2007; Helmkampf et al., 2008; Hejnol et al., 2009). On the other hand, similarities in the organogenesis during budding of bryozoans and kamptozo-

ans were considered to be analogies (Brien, 1960b). Early in development, kamptozoan buds become two-layered sacs that give rise to new individuals. However, in kamptozoan budding, the whole gut is formed by an invagination of the prospective mouth area (Brien, 1957), thereby differing from the process described for several bryozoans including *C. mucedo*.

Because of similarities in their adult bodyplan, phoronids are another group that has been closely associated to phylactolaemate bryozoans (e.g., Mundy et al., 1981). Asexual reproduction in phoronids occurs in several species by transverse fission, but budding is restricted to the shell-inhabiting *Phoronis ovalis* (Zimmer, 1991). Buds arise as protrusions of the body wall on the oral side of the animal. The gut is formed from a tubular outgrowth of the intestinal tract of the mother individual, and the mouth is formed before the anus (Du Bois-Reymond Marcus, 1949). Thus, the mode of gut formation fundamentally differs from both bryozoans or kamptozoans. Note, however, that the budding process in *Ph. ovalis* is only poorly investigated and comparisons are difficult to draw with this scant information. Details on the formation of other organ systems such as of the lophophore could prove to be valuable for comparative investigations.

Formation of the Central Nervous System

The central nervous system or ganglion in *Cristatella mucedo* forms by an invagination of the future pharyngeal area, as already reported in previous studies (Davenport, 1890; Braem, 1890, 1913; Mukai, 1982). The same process has been described for *Lophopus crystallinus* (Graupner, 1930) and *P. fungosa* (Handschuh et al., 2008). In cyclostomes and gymnolaemates, the ganglion forms more or less identically (Davenport, 1891; Nielsen, 1970, 1971). However, Phylactolaemates are the only bryozoan group that as adults still exhibit a central cavity inside the ganglion. The outgrowth of the ganglionic horns from the ganglion into the lophophoral arms has been previously described by Braem (1890) and Davenport (1890). These and other authors (Oka, 1891 for *A. gelatinosa*, Gewerzhagen, 1913a for *C. mucedo*, Marcus, 1934 for *L. crystallinus*) reported a continuation of the ganglionic cavity into the horns, which our study did not find. A recent study on the ultrastructure of the ganglion shows that the cavity is definitely present, but that its extent and size are probably much smaller than previously assumed. Descriptions of the large size of the ganglionic cavity probably reflect fixation artifacts (Gruhl and Bartolomaeus, 2008). Accordingly, the described lumen within the ganglionic horns perhaps represents a similar artefact, although future transmission electron microscopic studies are required to determine this.

Comments on the Coelomic Organization in Phylactolaemate Bryozoans

We show that the coelomic cavities in *Cristatella mucedo* are all in open connection at the anal side of the lophophore base. In accordance with Gruhl et al. (2009), we reject a trimeric organization of the phylactolaemate body plan demonstrated by the coelomic cavities. At no point during budding in *C. mucedo* does the formation of the different coelomic compartments indicate any sign of trimeric organization. While the coelomic organization in adult *C. mucedo* is identical to other recently investigated chitinous species such as *Fredericella sultana* and *Plumatella repens*, the gelatinous form *L. crystallinus* lacks an epistome (Gruhl et al., 2009). Where other species possess an epistome, however, the forked canal in *L. crystallinus* medially forms a bulge, reminiscent of the bladder on the anal side of the forked canal in *C. mucedo*. As in *C. mucedo*, the forked canal of *L. crystallinus* is confluent with the remaining trunk coelom and is densely ciliated. Based on their position and dense ciliation, the funnels of the forked canal have been previously considered as nephridia, similar to those of phoronids (Verworn, 1887; Cori, 1890, 1893, 1941). This interpretation has been rejected by subsequent authors (Braem, 1890; Oka, 1895; Schulz, 1901; Willem, 1910a, 1910b; Gewerzhagen, 1913b; Marcus, 1934; Mano, 1964). Still, the dense ciliation was interpreted to transport variably termed coelomocytes (leucocytes: Delage and Herouard, 1897; phagocytes: Marcus, 1934; amoebocytes: Cori, 1941); these coelomocytes were thought to carry excretory substances from the body cavity into the unpaired part of the forked canal, below the bases of the innermost tentacles. Only *C. mucedo* possesses the thickened bladder at the unpaired part of the forked canal, where cells and substances accumulate. The bladder is present only in some adult specimens and its occurrence is probably related to the age of individual zooids, as previously described (Willem, 1910b). We found no sign of any pore or opening that could remove excretory coelomocytes from the polypide, an observation in accordance with Braem (1890), Gewerzhagen (1913b), and Schulz (1901). In contrast, several authors described a permanent pore in the epithelial lining of the bladder or close-by at the innermost tentacles (Verworn, 1887; Delage and Herouard, 1897; Cori, 1893; Willem, 1910b). Marcus (1934) reported a gap (rather than a permanent pore) in the basement membrane at the anal side of the bladder in *C. mucedo*, but we conclude that the linings of the bladder are too thin and delicate to determine whether a gap is present using light microscopy alone. A similar gap located at the median, distal-most part of the forked canal was reported for *L. crystallinus* (Marcus, 1934), but seems to be absent in *P. magnifica* (Marcus, 1934) and *L. carteri* (Rogick, 1937). In *A. gelatinosa*, no definite pore could be demonstrated, although it was assumed to be present (Oka, 1891, 1895).

The bladder in *C. mucedo* probably has an excretory function. We did not observe the discharge of its contents, but a rupture of its thin epithelial linings, as reported by Gewerzhagen (1913b), seems plausible. A sphincter muscle on the anal side of the bladder as described by Delage and Herouard (1897) is absent.

The current state of knowledge permits no conclusion about whether the ciliated canals are rudimentary nephridial tubules. Particularly the absence of a well-founded phylogeny of phylactolaemates makes the interpretation of characters difficult. However, coelomocytes are also discharged by the nephridia in brachiopods (James, 1997), and sperm cells have been observed in the bladder of *C. mucedo* (Braem, 1890). Consequently, the ciliated funnels in *C. mucedo* act as gonoducts, similar to the nephridial ducts in phoronids and brachiopods (Zimmer, 1991; Long and Stricker, 1991).

CONCLUSION AND OUTLOOK

We confirm the results of previous studies on the organogenesis during budding in *Cristatella mucedo* (Davenport, 1890; Braem, 1890, 1913) using modern visualization techniques. These yield a clearer picture of the whole process and will simplify future comparisons with other species. It remains difficult to draw comparisons to other phyla because only few studies have dealt with budding of potentially related taxa in more detail. Nonetheless, the "bridged" lophophoral arms during budding unite two related families, showing that comparative organogenesis can contribute to phylactolaemate systematics. When more data become available, this approach may also contribute to the systematics of other bryozoan classes and bilaterian phyla.

The considerable discrepancies in gut formation reported by previous studies in all bryozoan classes calls for reinvestigations and for including more species to draw a clearer picture. One possibility is that both modes (i.e., gut mainly from mouth or anal area) coexist within a single species and are controlled by unknown mechanisms. If both modes of gut formation do exist, then their distribution pattern could perhaps be allocated to certain systematic taxa. Thus, it might confirm or refine current views.

Of particular interest for phylactolaemate and bryozoan evolution is the organogenesis in *L. crystallinus*, which lacks an epistome (Gruhl et al., 2009). Recent phylogenetic studies indicate a rather basal position of lophopodids within phylactolaemate bryozoans (Wood and Lore, 2005; Okuyama et al., 2006; Hirose et al., 2008). Organogenesis during budding can show whether an anlage for an epistome is present or not. If the latter is the case, the epistome may represent an apomorphy for more advanced phylactolaemate families.

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3. Myoanatomy and serotonerg nervous system of plumatellid and fredericellid Phylactolaemata (Lophotrochozoa, Ectoprocta)

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Myoanatomy and serotonerg nervous system of plumatellid and fredericellid Phylactolaemata (Lophotrochozoa, Ectoprocta)

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Abstract:

The phylogenetic position of the Ectoprocta within the Lophohotrochozoa remains controversially discussed. According to the Lophophorata concept they are related to the Brachiopoda and Phoronida. From a phylogenetic standpoint the Phylactolaemata among the Ectoprocta represent one of the most interesting clades being commonly considered as the sister-group to the remaining ectoproct clades. Only few morphological recently had dealt with phylactolaemate soft-body morphology for gaining more insight into the ectoproct relationships and comparing it with other possibly related phyla. In the present study, we analysed the myoanatomy and serotonergic nervous system of adult representatives of fredericellid and plumatellid Phylactolaemata. The body wall contains a regular mesh of outer circular and inner longitudinal muscles. On its distal end the orifice possesses a prominent sphincter and continues into the vestibular wall showing longitudinal and circular musculature. The tentacle sheath carries mostly longitudinal muscle fibres in *Plumatella* sp., whereas *Fredericella sultana* also possesses regularly distributed circular muscle fibres. Three groups of muscles associated with the lophophore can be distinguished: 1. lophophoral arms muscles (missing in *Fredericella*), 2. epistome musculature and 3. tentacle musculature. The epistome-flap is encompassed by smooth muscle fibres. A few fibres extend medially over the ganglion to the proximal floor of the epistome. Abfrontal tentacle muscles are of similar arrangement over the whole lophophore with proximal diagonally arranged muscle fibres followed more distally by a stack of muscles arranged as an inverted ‘V’. In *F. sultana* a gap is present between the proximal and more distally located abfrontal tentacle muscles. The medially facing frontal tentacle muscles are arranged similarly over the whole length of the lophophore in *F. sultana* with two proximal rootlets at the lophophoral base that fuse medio-distally into a single longitudinal bundle extending into each tentacle. The oral row of the frontal tentacle muscles in *Plumatella* is similar to *Fredericella*, but with the roots being adjacent to the pharyngeal ring musculature. The lateral frontal tentacle muscles at the

lophophoral base and lophophoral arms bend orally and terminate before reaching the fibres of neighbouring tentacles. The frontal tentacle muscles facing the inner lophophoral concavity possess two more prominent rootlets in *Plumatella*. The digestive tract possesses circular musculature which is striated except at the intestine where it is composed of smooth muscle fibres. The serotonergic nervous system is concentrated in the central nervous system. From the latter a serotonergic nerve extends to each tentacle base. In *Plumatella* the inner row of tentacles at the lophophoral concavity show no serotonergic nerves. Body-wall musculature is a common feature in many other lophotrochozoan phyla, but among other typical filter-feeders like the Ectoprocta is only present in the ‘lophophorate’ Phoronida. The longitudinal tentacle musculature is reminiscent of the condition found in the Phoronida and the Brachiopoda, but differs to the condition of entoproct tentacles. Although the current study shows some support for the ‘Lophophorata’, more comparative analyses using state-of the-art techniques on adult specimens of the possibly related phyla are required.

Introduction:

Because of similarities in their adult morphology - particularly the filter-feeding structure, the lophophore - the phylum Ectoprocta was traditionally united with the Phoronida and the Brachiopoda into the clade Lophophorata (Hyman 1959, Mukai et al. 1997). Recent molecular analyses showed support for a close relationship of the Phoronida and Brachiopoda, but failed to reconstruct the 'Lophophorata' as a whole and variously placed the Ectoprocta into the Bilateria (Helmkampf et al. 2008, Hausdorf et al. 2010, Nesnidal et al. 2010). Some of these studies argue for a sister-group relationship to the Entoprocta, even though both groups only show superficial resemblance (Jebram 1986). On a morphological basis, in particular the Phylactolaemata among the Ectoprocta share several similarities with phoronids and consequently were considered to have a common ancestor with the latter (Mundy et al. 1981, Jebram 1986, Backus & Banta 2002).

Among all clades of the Ectoprocta, the Phylactolaemata are easily distinguishable from the remaining clades showing several morphological characters as body-wall musculature, an epistome, usually a horse-shoe shaped lophophore and specific dormant stages, statoblasts, used for dispersal and enduring rough environmental conditions (Mukai et al. 1997). They are a small group of approximately 70 species and solely inhabit freshwater habitats (Massard & Geimer 2008). The Phylactolaemata are considered monophyletic (e.g. Mukai 1999), but their relation to the remaining ectoproct clades is ambiguously discussed. Morphologists considered them as the earliest branch being sister-group to all the remaining clades, the Cyclostomata and Gymnolaemata (Hyman 1959; Jebram 1973, 1986; Wood 1983). Recent molecular studies are not so clear, but show some support for the basal position of the Phylactolaemata (Fuchs et al. 2009). Consequently, the Phylactolaemata represent an important taxon for gaining more insight into the ground pattern of the ectoproct bauplan and its evolution. Recent studies using modern immunocytochemical techniques on complex three-dimensional structures such as muscular or nervous systems have proven to be

particularly valuable for this matter (e.g. Wanninger 2009, Gruhl 2008, 2009, 2010). Since these features have not been investigated in adult Phylactolaemata, we focused on the myoanatomy and serotonergic nervous system of several representatives from two phylactolaemate families for comparison with other, possibly related phyla.

Material and Methods:

Material

In total four species, one fredericellid (*Fredericella sultana*) and three plumatellids (*Plumatella emarginata, P. fungosa and P. vaihiriae*) were collected and used for the current study. *F. sultana*, *P. emarginata* and *P. fungosa* were collected at the Laxenburg pond in Lower Austria in June and July 2009. The plumatellid *P. vaihiriae* was collected from the pond of the Faculty of Fisheries of the Kasetsart University in Bangkok, Thailand.
Some samples were relaxed in 2% MgCl or chloralhydrate prior to fixation. All specimens were fixed in 4% paraformaldehyde in 0.01M PBS (pH 7.4) for 1-2 hours followed by several rinses with the buffer for an hour. Until further preparation, samples were stored in 0.01M PBS containing 0.01% NaN^3.

Immunocytochemistry and confocal microscopy

Prior to staining, single zooids of a colony were dissected from the colonies. Because of the large size of each zooid (approximately 300-400µm), several zooids were dissected to increase depth of scanning in the z-axis. Dissection often required the removal of the opaque ectocyst. With the exception of *P. fungosa,* the ectocyst forms a pergament-like sheath closely attaching to the remaining endocyst or body-wall. Consequently, dissection frequently resulted in the distortion or destruction of the body wall or other tissues.
For F-actin staining, specimens were permeabilized in PBS containing 4% Triton-X (PBT) for 1 hour, followed by overnight incubation in a 1:40 dilution of AlexaFluor 488 phalloidin

(Molecular Probes, Eugene, OR, USA) in PBT at 4°C. Then, the specimens were rinsed three times in PBS. For staining of the serotonergic nervous system, single zooids were transferred to 6% normal goat serum (NGS; Sigma-Aldrich, St. Louis, MO, USA) in PBT (block-PBT) overnight at 4°C. Subsequently, a polyclonal rabbit anti-serotonin antibody (Zymed, San Francisco, CA, USA) was applied at a concentration of 1:400 in block-PBT for 24 hours at 4°C. Then, the specimens were rinsed several times in block-PBT for 6 hours at 4°C prior to application of a secondary fluorochrome-conjugated antibody (goat anti-rabbit AlexaFluor 594, Molecular Probes) in block-PBT at a concentration of 1:200 for 24 hours at 4°C. Specimens were then washed three to four times in PBS for about 6 hours. Nuclei were stained by adding a few drops of DAPI (Invotrogen, 3µg/ml) for 15-20 minutes, followed by three short washes in PBS. Specimens were mounted in Fluoromount G (Southern Biotech, Birmingham, AL, USA) on standard microscope slides.

Analysis and image acquisition was performed on a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit (Leica Microsystems, Wetzlar, Germany). Confocal image stacks were recorded with 0.5-1µm step size along the Z-axis. Images stacks were captured as maximum intensity projections or further processed as volume renderings with Amira 4.1 software (Mercury Computer Systems, Chelmsford, MA, USA). For isolating specific muscle systems they were first roughly labelled with the Amira segmentation editor. Afterwards, grey-scale information within the labels were separated from the original grey-scale image stack with the Amira Arithmetic tool (expression: (a==1)*b, where ‘a’ represents the Amira label file and ‘1’ is the Id of the labelled material; b is the original image stack file).

Results:

The following description will primarily focus on the *Plumatella* species which did not show any significant differences among each other. Differences in *Fredericella sultana* will be mentioned when present.

Each zooid consists of a protective cystid and a retractable polypide. The cystid is mainly represented by the body-wall consisting of the cellular endocyst and the acellular protective ectocyst. The polypide itself contains all major organ systems of the zooid and consists of the tentacle crown and essentially the digestive tract (Figs. 1, 2a). The body-wall possesses a regular grid of outer circular and inner longitudinal musculature (Fig. 3a). At the distal end of the zooid the orifice is situated and shows a denser aggregation of circular muscle fibres, the orifice sphincter (Fig 3a, c). From the latter, the vestibular wall extends proximally and continues into the tentacle sheath (Figs. 1; 3c). The vestibular wall contains a set of thick circular muscle bands and thinner longitudinal ones. At its proximal side it ends at the diaphragm with a diaphragmatic sphincter (Fig. 3c). Several delicate muscle fibres, the vestibular dilatators, traverse the body cavity in between the body wall and the vestibular wall (Figs. 1; 3c). Proximally of the vestibular dilatators several, radially arranged peritoneal bands supplied with longitudinal muscles, the duplicature bands, traverse in a similar manner as the dilatators (Figs. 1; 3b, c). In protruded zooids the tentacle sheath extends distally to the vestibular wall, while in retracted zooids it extends proximally of the latter into the body cavity and envelops the retracted lophophore. In plumatellids, the tentacle sheath almost exclusively shows longitudinal muscle fibres over its length. Only at the site where it attaches to the lophophoral base few circular muscle fibres are present (Fig. 3b). On the contrary, *Fredericella sultana* shows prominent circular muscle bands over the entire length of the tentacle sheath (Fig. 4d) (in retracted condition the circular muscles are facing the atrium; in protruded state, they resemble the body-wall musculature).

At the lophophoral base three types of muscles are present: tentacle muscles, muscles associated with the epistome and muscles associated with the lophophoral arms. Tentacles are

supplied with two longitudinal muscle bands, one on the frontal or inner side and a second on the abfrontal or outer side. The roots of the abfrontal tentacle muscles at the lophophoral base are identical over the whole length of the lophophore. They are located within the intertentacular membrane and proximally show a series of diagonally arranged smooth muscle fibres (Fig. 4a, e). A single muscle straight bundle extends medially into the longitudinal plane of each tentacle (Fig. 4e). More distally, but still within the intertentacular membrane a stack of tightly adjoining smooth muscles fibres in the shape of an inverted 'V' extend into the tip of each tentacle (Fig. 4e). The roots of the frontal tentacle musculature on the inner side of the lophophore show three different forms. On the oral side, these arise from a ring of smooth muscle fibres adjacent to the pharynx epithelium. From the former, two rootlets branch off and fuse medially in the longitudinal plane of each tentacle (Fig. 4b). On the lateral sides next to oral row of tentacles until the distal-most tip of each lophophoral arm, the frontal tentacle muscles traverse the length of each tentacle in a similar way as seen on the oral tentacles. Proximally at the lophophoral base and lophophoral arms, the muscle fibres bend orally and terminate before reaching the fibres of neighbouring tentacles (Figs. 4c; 5). On the median tentacles bordering the lophophoral concavity, the frontal tentacle muscles originate from two rootlets situated in the lophophoral arms and over the epistome (Figs. 4a, c, d; 5). The latter is flap-like protruding towards the mouth opening below the most medial tentacles in the lophophoral concavity. Several smooth muscle bands encompass the epistome-flap forming a muscular basket (Fig. 4f). A second set of muscles originates from the lophophoral concavity on the anal side of the lophophore, passes over the ganglion and inserts on the proximal lower side of the epistome (Fig. 4a, b). On the proximal end of the lophophore, lophophoral arms musculature in form of four to five smooth muscle fibres extend on the anal side of the lophophoral arms (Fig. 4a).

In *F sultana* the lophophore is circular and lacks distinct lophophoral arms. The muscular system of the lophophore slightly differs in this species: The lophophoral arms muscles

observed in *Plumatella* are not present in *F. sultana*. The frontal tentacle muscles are similar to those of *Plumatella*, but each fibre bifurcates into the intertentacular membrane on the proximal side. Additionally, they are not adjacent to the musculature of the pharynx (Fig. 4d). The abfrontal tentacle musculature in *F. sultana* is less prominent when compared to *Plumatella*: On the proximal side only few smooth muscle fibres with diagonal orientation are present. Distally of these muscles there is a short gap without any musculature before smooth, longitudinal muscle fibres extend into each tentacle (Fig. 4d).

The mouth opening is located at the lophophoral base surrounded by the tentacle crown. The whole digestive tract solely shows circular musculature. In the pharynx and the esophagus it forms a dense layer of cross to obliquely striated fibres (Figs. 2b-d). The cardia below the cardiac valve and most parts of the stomach contain striated, but more loosely arranged circular musculature as well (Fig. 2c, d). Only at the proximal end of the caecum the musculature forms a dense layer. The funiculus, which is attached to the proximal tip of the caecum, contains several smooth and longitudinal muscle fibres (Fig. 2e, f). The intestine at the anal side of the zooid is ovoid to bulb-shaped and possesses smooth ring-musculature (Fig. 2d). At its distal end it terminates via an anal sphincter into the tentacle sheath.

The prominent retractor muscles originate from the colony wall and inserts proximally at the lophophoral base and the orally situated parts of the digestive tract, i.e. pharynx, esophagus and cardia (Fig. 1). They consist of smooth muscle fibres in *Plumatella*. In *Fredericella*, some of the more distally located parts of the fibres appear regularly striated (Fig. 4d).

Serotonergic nervous system

The serotonergic nervous system is concentrated in the neuropil of the central nervous system or ganglion on the anal side of the lophophoral base (Fig. 6 a-c). From the central nervous system, serotonergic nerves extend to the oral tentacles and the outer, lateral tentacles of the lophophoral arms (Fig. 6 a, b). Within the traverse of these nerves, one or two serotonergic

perikarya are intercalated with one usually lying proximally, still at the intertentacular membrane (Fig. 6 a-c). The inner row of tentacles facing the lophophoral concavity in *Plumatella* sp. shows no serotonergic innervation at all.

Discussion:

Body wall musculature and tentacle sheath

A body wall musculature consisting of a regular grid of an outer layer of circular and an inner layer of longitudinal musculature is found in several vermiform lophotrochozoan phyla such as molluscs (Haszprunar & Wanninger 2000), platyhelminthes (Hooge 2001), annelids (Purschke & Müller 2006) and sipunculans (Wanninger et al. 2005). Tentacle-bearing filter feeders like entoprocts and brachiopods lack such a distinct regular body wall musculature (Wanninger 2004, Fuchs et al. 2006, Altenburger & Wanninger 2009) and only the 'lophophorate' phylum Phoronida possesses a set of body wall musculature of outer circular and inner longitudinal musculature (Herrmann 1997). The longitudinal musculature of phoronids is prominent and extends as several vanes into the body cavity. Among the Ectoprocta, the Phylactolaemata are the only clade possessing distinct body wall musculature (Mukai et al. 1997, Gruhl et al. 2009). It is similar to those of phoronids, but with the longitudinal muscle layer less prominent. In the Phoronida this prominent layer is used for retracting the animal into their tube upon external disturbances. Individual zooids in all Ectoprocta are composed of an outer protective cystid and a retractable polypide that in principle consists of the lophophore and the digestive tract. Retraction in all ectoproct clades is accomplished by the prominent retractor muscles inserting on the polypide. According to Jebram's evolutionary scenario (1986), these muscles derived from the longitudinal body wall musculature of phoronids resulting in a more economized and effective defensive mechanism in the Ectoprocta. The question remains whether these two phyla are actually related or this similarity in this body wall musculature evolved convergently. In both, the Phylactolaemata

and Phoronida, the circular musculature acts in increasing pressure within the coelom. In the Phylactolaemata, this results in the polypide eversion which is always effectuated by an increase of hydrostatic pressure in the coelom, but the mechanism for the latter differs among the Ectoprocta (Taylor 1981, Mukai et al. 1997). The Phylactolaemata use the aforementioned body-wall musculature for this purpose. The Cyclostomata use a series of annular muscles in the membranous sac (Nielsen & Pedersen 1979), whereas the Gymnolaemata use so-called parietal muscles which traverse the coelom laterally of the polypide (Mukai et al. 1997). These muscles in the latter two clades are regarded as a modification of circular muscles of the body-wall increasing the efficiency of the protrusion and giving way for reinforcing the cystid wall in form of a thicker cuticle or calcification (Jebram 1986). Considering this body-wall musculature in the Phylactolaemata as a plesiomorphic condition, it seems tempting to assume a 'pro-ectoproct' state as assumed by Jebram (1986) with only parts of the fore-body retractable, similar to the condition found in phoronids. Especially *Phoronis ovalis* possesses a highly retractable fore-body and also resembles phylactolaemates in being the sole phoronid showing colonial budding (Harmer 1917). Consequently, this species represents the most interesting candidate for gaining more insight into the probable 'pro-ectoproct' state and the 'lophophorate' problem.

The tentacle sheath in the Phylactolaemata was reported to contain only longitudinal muscle fibres (Marcus 1934; Rogick 1937, Mukai et al. 1997). On the contrary, in particular in *F. sultana* and in less extent in *Plumatella* (only at the lophophoral base) circular muscles were observed in the current study. Traditional and modern phylogenies always regard the Fredericellidae more basal than the Plumatellidae (Mukai 1999, Hirose et al. 2008). Considering the similar arrangement of the body-wall musculature to the musculature of the tentacle sheath with outer layer of ring musculature and inner layer of longitudinal musculature, it appears likely that the tentacle sheath musculature in *F. sultana* represents the plesiomorphic state and reflects an ancestral body-wall musculature. In particular, the

condition in the Lophopodidae, which are often regarded as a basal family (Wood & Lore 2005; Okuyama et al. 2006; Hirose et al. 2008), should be investigated with more precise techniques for asserting a probably basal condition of the tentacle sheath musculature in the Phylactolaemata.

Tentacle musculature

The tentacle musculature consisting of frontal and abfrontal longitudinal musculature within the tentacles is in accordance to previous descriptions on the Phylactolaemata (Mukai et al. 1997, Gruhl et al. 2009). In *Fredericella sultana* only the frontal tentacle muscle was recently described by sectioning methods (Gruhl et al. 2009). However, in the present study we were able to confirm an abfrontal tentacle muscles for *F. sultana*. Since the abfrontal tentacle muscles are not continuous on the proximal lophophoral base, it is likely that sections were only conducted where the gap is present and thus overlooked.

Whereas detailed information on the muscular system of the lophophoral base of the remaining lohophorate clades is missing, several studies dealt with the muscle system of their tentacles. In both, phoronids (Pardos et al. 1993) and brachiopods (Reed & Cloney 1977), the musculature within the tentacles is similar to the condition found in the Phylactolaemata and other ectoprocts and consists of longitudinal musculature concentrated on the frontal and abfrontral side. These muscles are always part of myoepithelial peritoneal cells lining the inner lumen of the tentacles (cf. references above). The muscle fibres of the abfrontal tentacle musculature of the brachiopod *Terebratalia transversa* are oriented at an angle of about 12.5° from the longitudinal axis of the tentacle (Reed & Cloney 1977). This resembles our observations on the phylactolaemate abfrontal tentacle musculature which appeared like a pile of inverted Vs.

Entoproct tentacle musculature differs from all the ‘lophophorate’ phyla: On each lateral side of the tentacle is a single, more prominent outer longitudinal muscle and more medially

paired, inner tentacle muscles (Wanninger 2004, Fuchs et al. 2006, Schwaha et al. 2010). Whereas the inner tentacle muscles are myoepithelial (but epidermal), the outer ones are separate fibres traversing the tentacles (Nielsen & Jespersen 1997). Consequently, tentacle musculature seems to be similar among the 'lophophorate' phyla, but not at all show any resemblance to those of the filter-feeding entoprocts.

Epistome musculature

The presence of an epistome and an enclosed separate coelomic compartment in the Phylactolaemata previously was considered to support the 'Lopohophorata' concept in sharing this feature with the Brachiopoda and Phoronida (e.g. Hyman 1959). However, more recent analyses have shown that phoronids (Bartolomaeus 2001, Gruhl et al. 2005) and brachiopods (Lüter 1996, 2000) do not possess any coelomic cavity within the epistome. The Phylactolaemata, however, possess a coelomic cavity within the epistome that is in open connection to the remaining coelom (Gruhl et al. 2009, Schwaha et al. 2011). Despite this difference in being underlain peritoneally or not, the epistome always contains musculature. In the brachiopod *Lingula anatina* the epistome is present as a small median tentacle which is filled with isolated smooth muscle cells and functions as a sensory organ (Lüter 1996). In the phoronid *Phoronis ijimai* a series of crossing muscle bundles through the entire epistome was described (Pross 1974, 1978), whereas the epistome of *Ph. ovalis* is filled with myoepithelial cells that are continues with the lining of the lophophoral coelom. In the latter, the myofibrils extend laterally into the extracellular matrix beneath the epidermal layer of the epistome. In the Phylactolaemata, Gruhl et al. (2009) described thick muscle bundles on the lateral walls of the epistome in *F. sultana* and *Pl. emarginata*. This mostly coincides with our findings on the epistome musculature, except that we did not detect any irregular striation in these muscles as mentioned by Gruhl et al. (2009). The proximal epistome musculature has to our knowledge not been described so far. In *Lophopodella carteri*, several isolated muscle fibres were

mentioned to traverse the coelomic cavity of the epistome in oral-anal direction (Rogick 1937), thus differing from aforementioned condition found in other phylactolaemates. However, the closely related genus *Lophopus* – which is essentially only distinguishable from *Lophopodella* from statoblast features – lacks an epistome (Gruhl et al. 2009). Consequently, it should be considered that *Lophopodella* lacks an epistome as well and the reported muscles are not comparable to those phylactolaemate species possessing an epistome.

Functionally the epistome is interpreted to be involved in feeding especially in sorting particles and directing water currents (Gilmour 1978). In *Ph. ijimai*, the epistome is reported to shut the mouth opening when the animal retracts thus preventing loss of food items that otherwise could be expelled (Pross 1978). The epistome of the Phylactolaemata never closes the mouth opening (Wood 1983). Possible food reflux in this clade is hindered by the cardiac valve at the esophagus-cardia border. A similar valve (infundibuliform valve) is described in *Phoronis ovalis* (Du Bois-Reymond Marcus 1949). The latter is regarded as sister-taxon to all remaining phoronid species (Santagata & Cohen 2009) and solely shows asexual reproduction by budding, a feature present in all ectoprocts (Du Bois-Reymond Marcus 1949). The epistome of *Ph. ovalis* is only small bulb that with its few lateral muscle bundles seems unlikely to close the mouth opening. Accordingly, the situation resembles to those of the Phylactolaemata, but analyzing the three-dimensional arrangement of its musculature for better comparison with the phylactolaemate condition calls for immunocytochemical techniques.

Serotonergic nervous system

The nervous system of ectoprocts has been subject of several studies (cf. Lutaud 1977, Mukai et al. 1997), but the serotonergic nervous system was hardly analysed. To date, two reports on more or less adult zooids are present: one on the juvenile condition of the serotonergic nervous system of the lepraliomorph cheilostome *Triphyllozoon mucronatum* (Wanninger et

al. 2005) and the second on the larva of the phylactolaemate *Fredericella sultana*, which already contains an adult zooid (Gruhl 2010). In the latter, the larva possesses a transitory serotonergic nervous hull without contact to the nervous system of the enclosed polypide. When comparing the serotonergic nervous system of the polypide of the *F. sultana* larva to our results in adult *F. sultana* and *Plumatella*, it is clear that this current study effectually corroborates the results of Gruhl (2010), showing a similar arrangement of this part of the nervous system in the Phylactolaemata. The situation of the serotonergic nervous system of the young polypide of *Tr. mucronatum* is similar to those of the analysed Phylactolaemata. It consists of a concentration in the central nervous system from where several nerves emanate to each tentacle base terminating with a serotonergic perikaryon. However, these perikarya and nerves run in between the tentacles, whereas in the analysed Phylactolaemata these appear directly on the axis of the tentacles. Nonetheless, the basic construction of the serotonergic nervous system in these clades is similar, but comparison with the other lophophorate phyla currently is not possible, because of lack of adult data in the Phoronida and Brachiopoda.

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Figure Legends:

Figure 1: Schematic overview of *Plumatella* sp. showing the general structure of a single zooid. The polypide consists of the lophophore carrying the tentacles. The tentacle crown encloses the mouth opening situated before the pharynx which leads into a short esophagus. A cardiac valve separates the esophagus from the following cardia which leads to the sac-shaped caecum. At the proximal bottom of the caecum, a peritoneal strand, the funiculus connects the caecum with the body wall. The remaining digestive tract consists of the intestine which terminates via an anus situated at the tentacle sheath. Upon retraction through the prominent retractor muscles, the latter encloses the tentacle crown. Further protection from is given by

the closure of the orifice bordered distally by the vestibular wall, which itself is part of the cystid. The remaining cystid consists of the body wall covering the polypide.

Abbreviations: a – anus, ca – cardia, cae – caecum, cw – cystid wall, db – duplicature band, es – esophagus, f – funiculus, int – intestine, lb – lophophore base, p – pharynx, rm – retractor muscles, tc – tentacle crown, ts – tentacle sheath, vd – vestibular dilatators, vw – vestibular wall.

Figure 2: Muscular system of the digestive tract in the Phylactolaemata visualized by F-actin staining.

(**a**) Overview of two protruded zooids of *Plumatella fungosa* showing from the lateral side on the left and the anal side on the right. (**b**) Magnification of the left zooid in (a) showing distal parts of the gut in vicinity of the lophophore. The gut on the oral side, i.e. the pharynx and esophagus and cardia show striated ring musculature, whereas the intestine shows smooth ones. Note that longitudinally oriented fibres visible on the intestine belong to the tentacle sheath (**c**) Lateral view of the digestive tract musculature of a dissected zooid of *Plumatella vaihiriae* showing the foregut (pharynx, esophagus) entering the cardia on the oral side of the zooid. The cardia continues into the sac-like caecum. The hindgut consists of the intestine on the anal side. (**d**) Higher magnification of details of the fore- and hindgut of (c). (**e**) Proximal part of the caecum of *Plumatella fungosa* showing the dense circular musculature and the funiculus with longitudinal muscle fibres. (**f**) Proximal part of the caecum of *Fredericella sultana* showing the striated musculature of the caecum and parts of the funiculus.

Abbreviations: a – anus, ca – cardia, cae – caecum, dg – digestive tract, dst – developing statoblast, es – esophagus, f – funiculus, int – intestine, l – lophophore, o – orifice, ph – pharynx, rm – retractor muscles

Figure 3: Muscular system of the body wall, tentacle sheath and vestibular area in the Phylactolaemata visualized by F-actin staining.

(a) Lateral view of a retracted zooid of *Plumatella emarginata* showing the regular net of longitudinal and circular musculature as well as the orifice on the distal side. (b) Retracted zooid of *Plumatella fungosa* with the body wall turned over to show musculature of the tentacle sheath. (c) View from the lateral side on the vestibular wall muscular as well as the vestibular dilatators and duplicature bands of *Plumatella vaihiriae*.

Abbreviations: bw – body wall, cts – circular muscles of the tentacle sheath, db – duplicature band, ds – diaphragmatic sphincter, lt – longitudinal muscles of the tentacle sheath, o – orifice, tm – tentacle muscles, ts – tentacle sheath, vd – vestibular dilatators, vm – vestibular wall musculature. Scale bar in (a) + (b) = 150µm, in (c) = 110µm.

Figure 4: Muscular system of the lophophore in the Phylactolaemata visualized by F-actin staining.

(**a**) View of the lophophoral base of *Plumatella fungosa* from the anal side showing the lophophoral arms muscles and proximal epistome muscles. (**b**) View of the lophophore of *Pl. fungosa* from the oral side showing the frontal tentacle muscles on the oral side with two proximal rootless that conjoin at the pharynx musculature (asterisk). (**c**) View of the frontal tentacle musculature on the median and lateral sides of the lophophore in *Pl. fungosa*. (**d**) Lateral view of the lophophoral base of *Fredericella sultana* showing the slightly different condition when compared to *Plumatella*. All frontal tentacle muscles show proximally two rootlets which proximally meet. The abfrontal tentacle shows a less pronounced proximal musculature which is separated from the distal longitudinal muscle bands. Also note the prominent circular muscular of the tentacle sheath as well as the ‘striation’ in several of the retractor muscle bundles (arrowheads). (**e**) Detail of the proximal abfrontal tentacle

musculature of *Pl. emarginata*. (**f**) Volume rendering of the epistome musculature of *Pl. fungosa* viewed from the proximal side.

Abbreviations: aft – abfrontal tentacle musculature, ct – circular muscles of the tentacle sheath, daft – distal abfrontal tentacle musculature, ep – epistome musculature, ftm – frontal tentacle musculature, int – intestine, itm – intertentacular membrane, la – lophophoral arm, lam – lophophoral arms muscles, lft – frontal tentacle musculature on the lateral side of the lophophore, lt – longitudinal muscles of the tentacle sheath, mft – frontal tentacle musculature on the median side of the lophophore, mm – median muscle fibres on the proximal abfrontal tentacle muscles, oft – oral row of frontal tentacle muscles, paft – proximal abfrontal tentacle muscles, pep – proximal epistome muscles, ph – pharynx, rm – retractor muscles. Scale bar in (a) and (b) = 75µm, in (c) = 60µm, in (d) and (e) = 50µm, in (f) = 25µm.

Figure 5: Schematic drawing of the arrangement of the frontal tentacle musculature in plumatellid Phylactolaemates viewed from the distal side. Grey indicates the area in between the tentacles, the intertentacular membrane. The tentacles on the oral side possess two rootlets directly in connection to a circular muscle adjacent to the pharynx below the mouth opening. The frontal musculature of the lateral tentacles shows a similar arrangement, but with only one rootlet that is not in contact with any of its neighbouring tentacles. The inner tentacles facing the lophophoral concavity at the anal side of the lophophore possess two short rootlets anchored on the lophophoral arms or above the epistome.

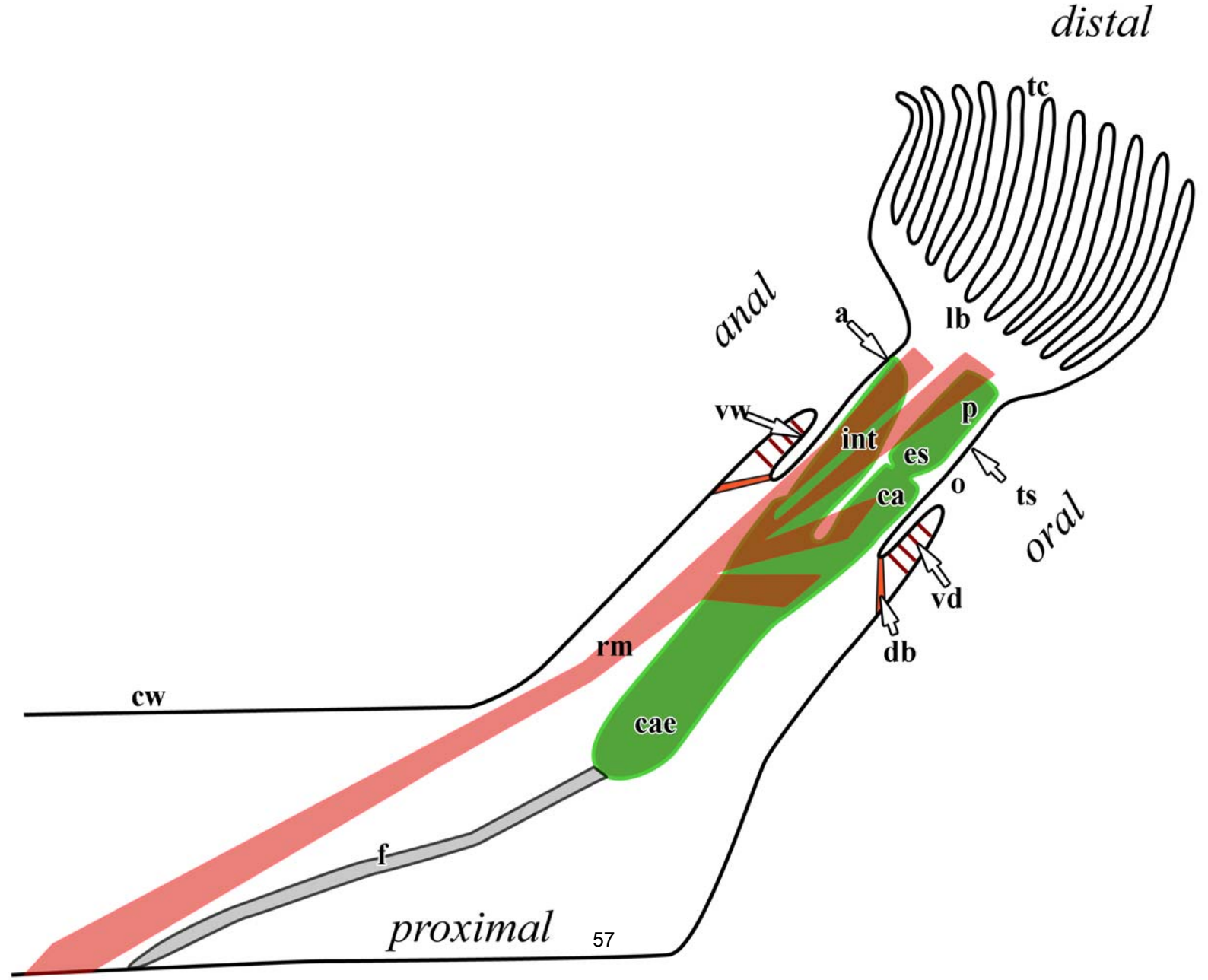
Abbreviations: ep – epistome, la – lophophore arm, loc – lophophoral concavity, mo – mouth opening.

Figure 6: Serotonergic nervous system in the Phylactolaemata.

(**a**) View of the oral side of the lophophore of *Plumatella fungosa* showing the serotonergic nervous system (yellow) and cell nuclei (blue). The serotonergic nervous system has its

greatest concentration in the central nervous system from where nerves emanate to each tentacle and terminate in serotonergic perikarya at the base of each tentacle except at the inner row of tentacles. (**b**) Lateral view of the lophophore of *P. fungosa* solely showing the serotonergic nervous system. In the top right corner the serotonergic nervous system of a young bud is displayed. On the left side the nerves extend distally onto the lophophoral arms. (**c**) Lateral view of the lophophore of *Fredericella sultana* indicating the much smaller size of the species, fewer tentacles and absence of lophophoral arms.

Abbreviations: cns – central nervous system, la – lophophore arm, ph – pharynx, sp – serotonergic perikarya at the tentacle base, t – tentacle, Scale bar in (a) and (b) = 100µm, in (c) = 75µm.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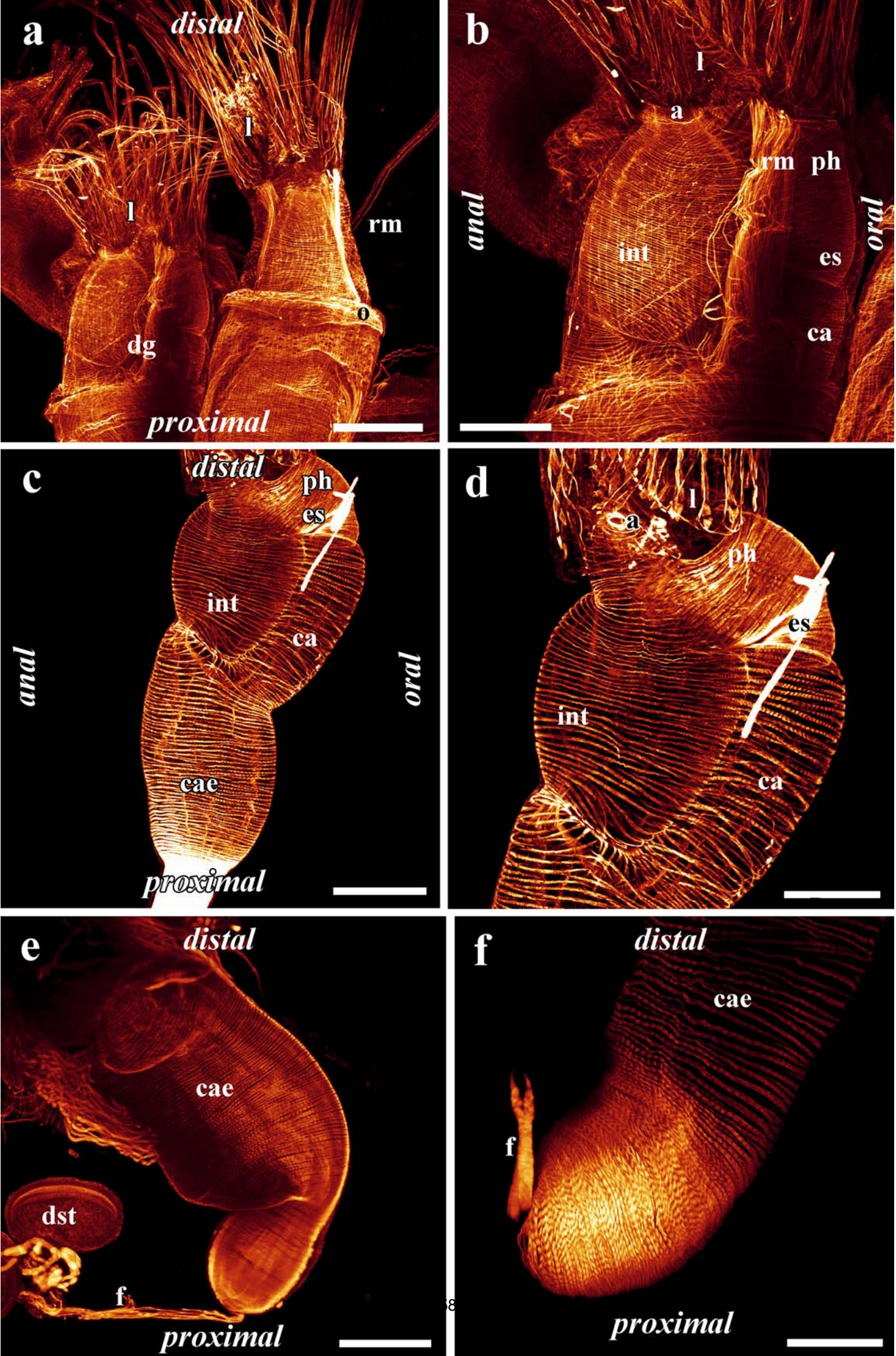
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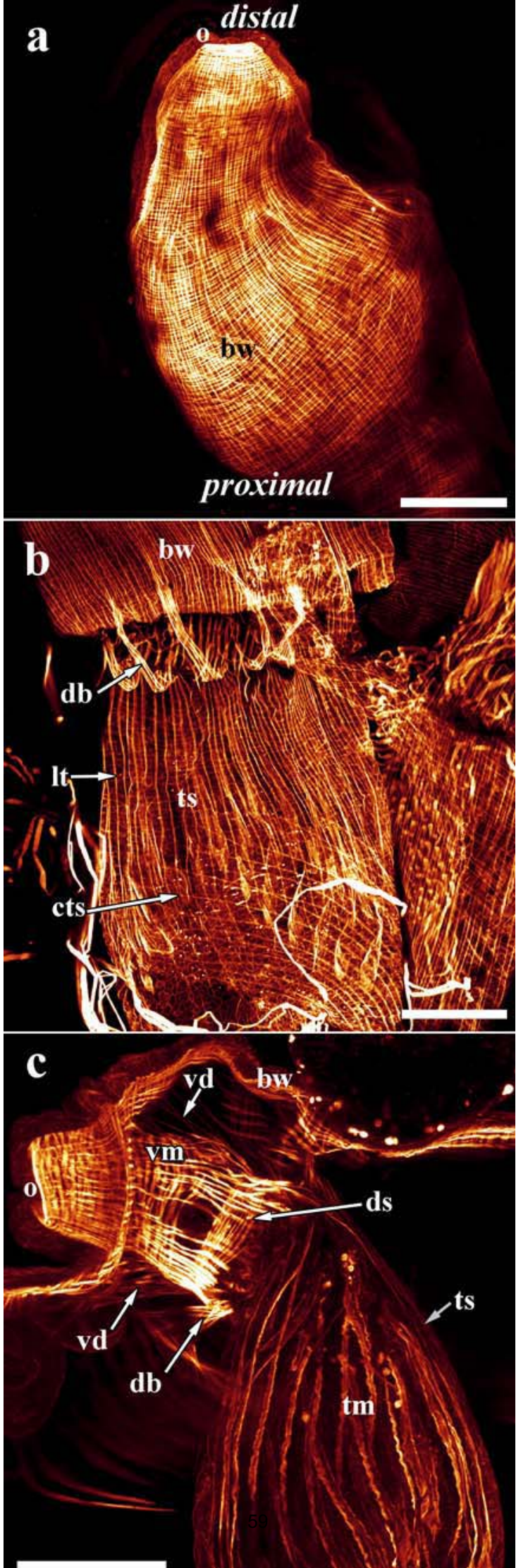
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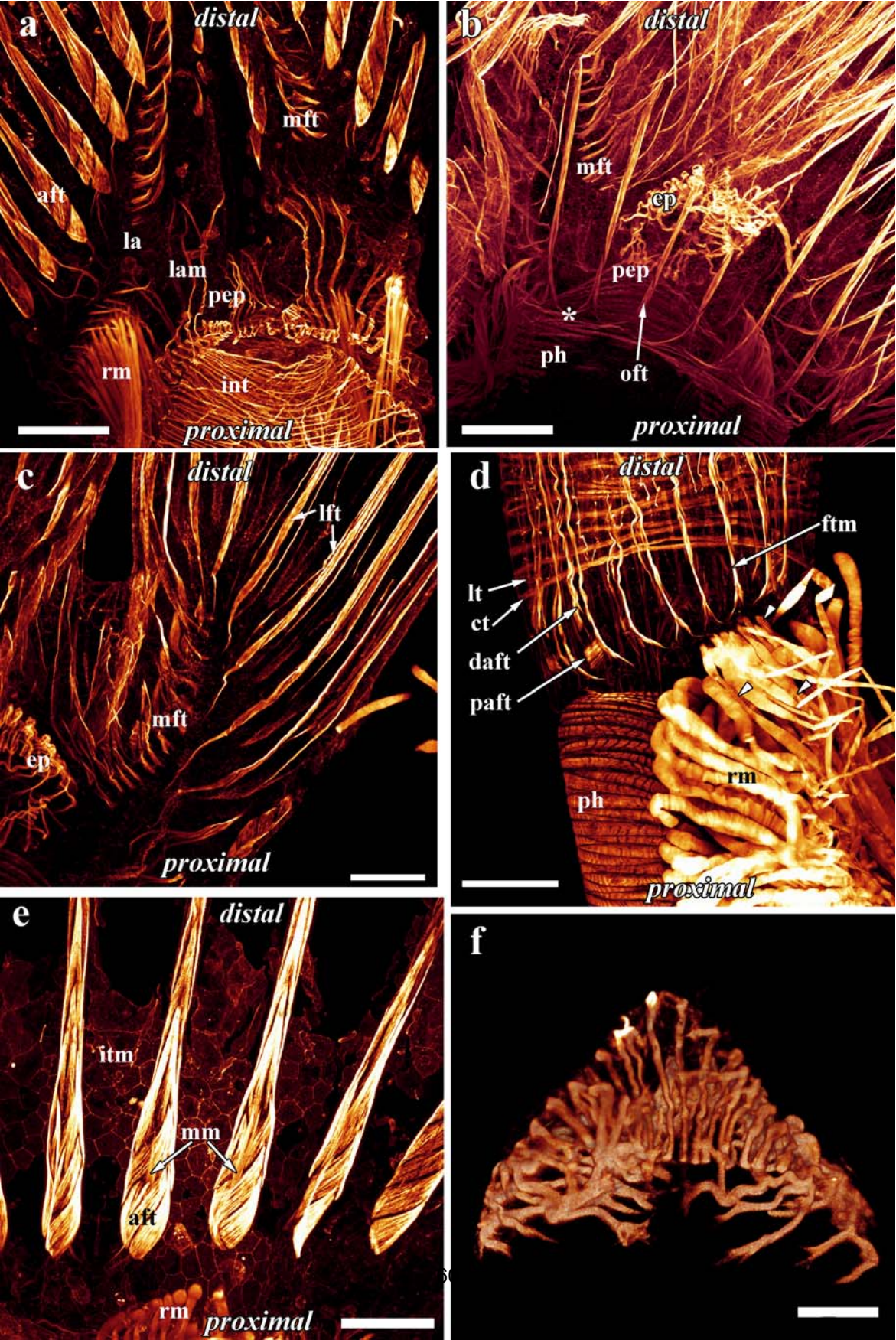
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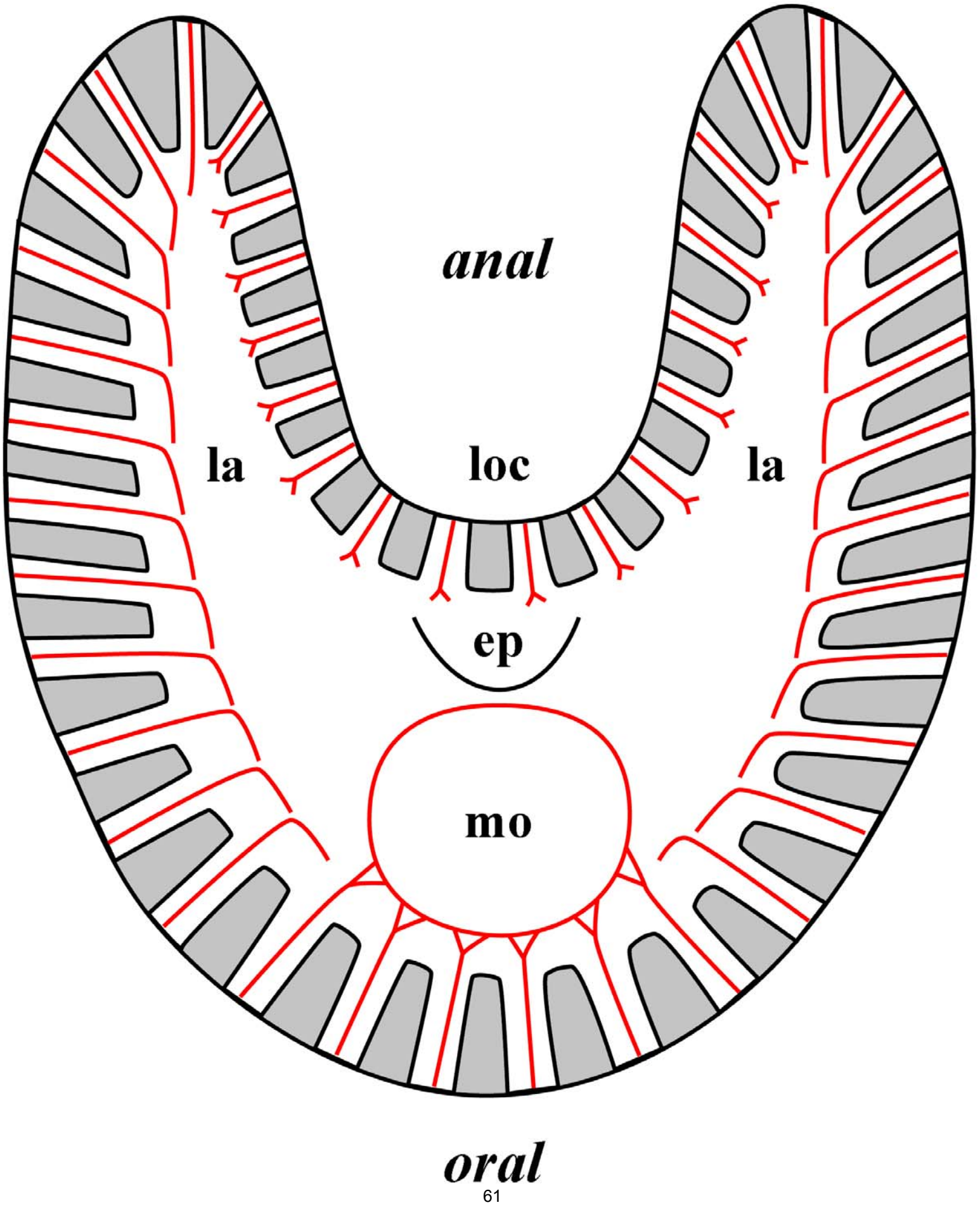
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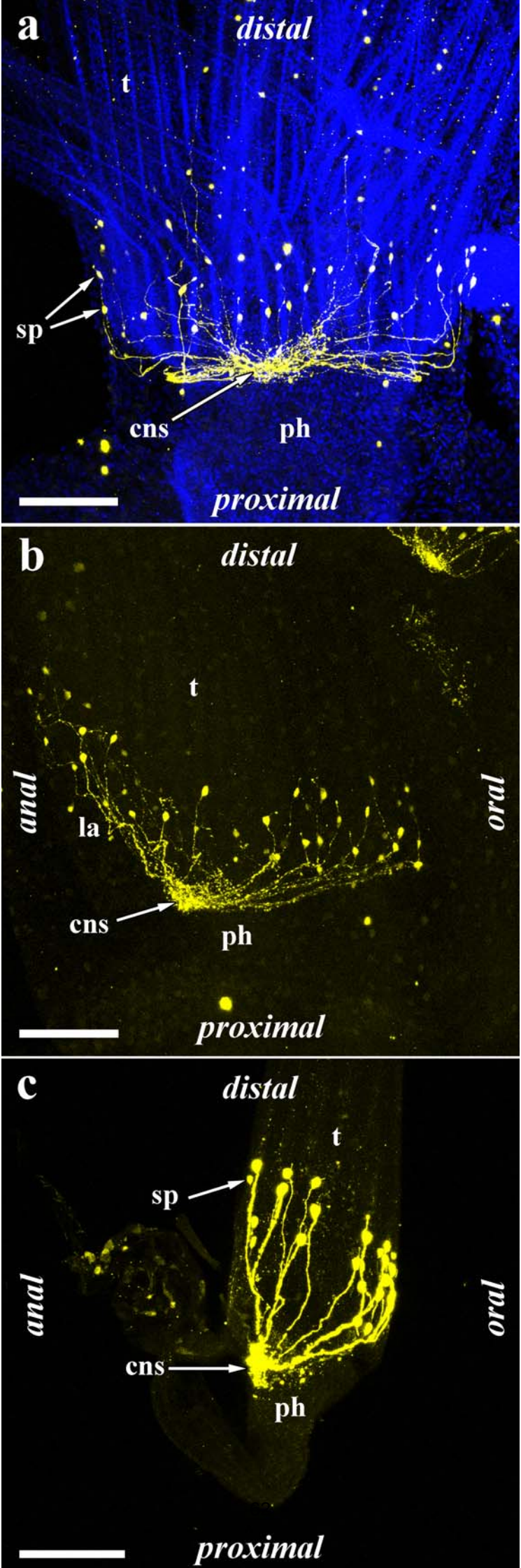
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4. Organogenesis in the budding process of *Hislopia malayensis* Annandale, 1916 (Bryozoa, Ctenostomata)

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Organogenesis in the budding process of *Hislopia malayensis* Annandale, 1916 (Bryozoa, Ctenostomata)

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Abstract

Background

Bryozoans represent a large lophotrochozoan phylum with controversially discussed large-scale and in group relationships. Developmental processes during the budding of bryozoans are in need for revision. Just recently a study on a phylactolaemate bryozoan analysed with modern techniques gave a comprehensive basis for further comparisons among bryozoans. The aim of this study is to gain more insight into developmental patterns during polypide formation in the budding process of bryozoans. For this purpose we studied organogenesis in the budding process of the ctenstome bryozoan *Hislopia malayensis*.

Results

Polypide buds develop on the frontal side of the developing cystid as proliferation of the epidermal and peritoneal layer. Early buds develop a lumen bordered by the inner budding layer resulting in the shape of a two-layered sac or vesicle. The hind- and midgut anlagen are first to develop as outpocketing of the prospective anal area. These grow towards the prospective mouth area where a comparatively small invagination marks the formation of the foregut. In between the prospective mouth and anus the ganglion develops as an invagination protruding in between the developing gut loop. Lophophore development starts with two lateral ridges which form tentacles very early. At the lophophoral base, intertentacular pits, previously unknown for ctenostomes, develop. The ganglion develops a circum-oral nerve ring from which the tentacle nerves branch off in adult zooids. Tentacles are innervated by medio-frontal nerves arising directly from the nerve ring, and medio-frontal and abfrontal nerves which originate both from an intertacular fork.

Conclusions

This is the second recent study on polypide development of a bryozoan and thus extends the available information for comparative analyses on organogenesis within the phylum. We are able to show distinct similarities in the formation of the different organ systems: a two-

layered vesicle-like early bud, the ganglion forming as an invagination of the epidermal layer in between the prospective mouth and anal area, the digestive tract mainly forming as an outpocketing of the prospective anal area, and the lophophore forming from two lateral anlagen that first fuse on the oral and afterwards on the anal side. Future studies will concentrate on cyclostome budding to complement our knowledge on developmental patterns of bryozoans.

Background

The Bryozoa represent a large lophotrochozoan phylum consisting of sessile filter-feeders comprising over 6000 extant species. The phylum consists of three large clades: The Phylactolaemata, the Stenolaemata and the Gymnolaemata (Ctenostomata and Cheilostomata) [1]. The relationship in between the different clades and also to other phyla remains controversially discussed [2]. The Phylactolaemata represents a small group of freshwater inhabiting species. From a phylogenetic perspective they are interesting, since they are often regarded as the most basal bryozoans and show several morphological characters that distinguish them from all remaining bryozoans, such as an epistome and body wall musculature [1, 3]. In particular their sexual development, however, is heavily altered, probably as an adaptation to freshwater habitats, and therefore impedes comparisons to other phyla and bryozoans. Within the Gymnolaemata, the Ctenostomata is a group of uncalcified, comparatively simple species that are currently regarded as paraphyletic being ancestral to the species-richest Cheilostomata and perhaps even the Stenolaemata [4-7]. Consequently, they represent an important clade for addressing phylogenetic questions of bryozoans.

As previously mentioned by Nielsen [8], budding in bryozoans, in particular organogenesis, is only poorly known. Schwaha et al. [9] recently studied the organogenesis in the budding process of the phylactolaemate *Cristatella mucedo* and established a first comprehensive study for further comparative purposes. Detailed investigations on the polypide development during the budding process of ctenostome bryozoans were only carried out by Davenport [10] for *Paludicella articulata*. Soule [11] studied several species, but only gave generalized and short descriptions with poor documentation. Accordingly, ctenostome budding requires new data to gain more insight into general trends and patterns in bryozoan budding. This study focuses on the organogenesis in the budding process of the ctenostome *Hislopia malayensis*, a species occurring in freshwater habitats of South East Asia [12]. Since it shows many primitive traits among ctenostomes [13], it represents a suitable species for the current study.

Material and Methods

Specimens of *Hislopia malayensis* Annandale, 1916 were collected from the pond of the Faculty of Fisheries of the Kasetsart University in Bangkok (see [13]). Colony pieces were fixed in 1.5 % glutaraldehyde in 0.01 M sodium cacodylate buffer (pH 7.4) for about 1 hour. Specimens were afterwards rinsed three times for 20 minutes in the buffer. Postfixation was conducted with 1% Osmium tetroxide solution in distilled water for 1-2 hours, followed by rinsing in distilled water for about 1 hour. Specimens were afterwards dehydrated with a graded alcohol series prior to embedding the samples into Agar Low-viscosity resin using acetone as intermedium. Ribbons of serial semi-sections (1µm thickness) were conducted as described by Ruthensteiner [14]. Sections were stained with toluidine blue and afterwards analysed and photographed with a Nikon DS5M-U1 digital camera mounted on a Nikon Eclipse E800 light microscope. Image stacks from the serial section micrographs were enhanced in contrast and converted to greyscales using Adobe Photoshop CS3 (Adobe, San Jose, CA, USA) before being imported into the 3D reconstruction software Amira 4.1 (Mercury Computer Systems, Chelmsford, MA, USA). Alignment of the image stacks was conducted with the AlignSlice tool of Amira. Segmentation of different structures was conducted manually with a brush. A surface for each structure was generated followed by iterated steps of triangle reduction and smoothing (see [14]). Snapshots were taken with the Amira software.

Results

Hislopia malayensis forms flat, encrusting colonies on various substrates (Fig 1.). Each individual zooid is oval-shaped and consists of an outer cystid that protects the polypide which consists of the lophophore carrying the tentacles and the digestive tract (Fig. 1b, d). Buds of *H. malayensis* arise on the distal or lateral sides of each zooid (Fig. 1a, c). In the following selected developmental stages of the budding process will be treated according to their degree of differentiation.

Stage 1

Early buds arise from the frontal side of the developing cystid. The cystid wall consists of an epidermal and a peritoneal layer (Fig. 2a). The epidermal layer of the cystid is easily recognized, whereas the peritoneum is very thin and only sporadically visible on light microscopical level (Fig. 2a, b). The early bud probably originates from a proliferation of these layers. However, both of the two involved budding layers, the inner budding layer deriving from the epidermal layer and the outer budding layer from the peritoneum, are prominent and consist of a much thicker epithelium when compared to the remaining cystid wall (Fig. 2a). In many instances, peritoneal cells seem to dislocate from the peritoneal epithelium of the cystid and appear as round, amoeboid coleomocytes within the body cavity (Fig. 2a). The earliest analysed stage, stage 1, has the shape of a two-layered sac or vesicle that is connected to the cystid frontally via the neck of the bud (Figs. 2a; 3a, b). The bud contains a central lumen, bordered by the inner budding layer (Figs 2a; 3a, b). In our stage 1, the lumen is almost club-shaped in its proximo-distal direction, but flat in its lateral dimensions (Fig. 3a, b). It extends basally and terminates in a short u-turn (Fig. 3a). This extension of the lumen represents the developing gut anlage, which has developed from an invagination from the distally-oriented prospective anal area.

Stage 2

The following budding stage has only slightly altered in its lateral size, but compared to the first budding stage is twice as large in the proximo-distal axis. The lumen of the bud has expanded in this axis (Fig. 4a). The gut anlage has grown into the proximal direction towards the prospective mouth area on the proximal side of the bud (Fig. 4a, b). From the latter a slight indentation indicates the anlage of the prospective mouth area and foregut (Fig. 4a). In between the prospective anal and mouth area, the inner budding layer invaginates and forms the anlage of the ganglion, the central nervous system (Fig. 4a). The outer budding layer remains comparatively thin, except at both lateral sides of the bud where it pushes in form of two indentations medially into the inner budding layer representing the developing inner peritoneal lining of the gut and the ganglion (Fig. 4b).

Stage 3

The following budding stage does not show any distinct increase in size, but is characterized by differentiation of the developing organs. Most prominent is the lophophore anlage with the developing tentacles. Two lateral ridges bulge into the lumen of the bud (Fig. 5a, b). On each ridge, five tentacle anlagen protrude medially (Fig. 5c). Each of them consists of both the inner budding layer (the future tentacle epidermis) and the outer budding layer (the future peritoneal lining). The peritoneal cells are present as a dense mass without any lumen. At the prospective lophophoral base 2-3 cells layers of peritoneal cells representing the future circum-pharyngeal ring coelom are wedged in between the prospective foregut and outer peritoneal lining (Fig. 2b). On the distal side of the bud, the anal area continues into the anlage of mid- and hindgut (Fig. 5a). Compared to budding stage 2, the former now forms a voluminous sac (Fig. 5b). On the proximal side of the bud, the anlage of the foregut has advanced from the prospective mouth area towards the anlage of the mid- and hindgut (Fig. 5b). In between the prospective mouth and anal area, the ganglion has further invaginated, but

it still widely open and in contact with the remaining lumen surrounded by the lophophore anlage (Fig. 5b, c). The two lateral indentations of the outer budding layer seen in the previous budding stage have fused medially. As a consequence, the epithelium of the ganglion and the digestive tract are not adjacent anymore (Fig. 5b, c). At the neck of the bud on the frontal side, both budding layers have formed the vestibular wall enclosing a small globular cavity, the vestibulum, which is not in communication with the remaining lumen of the bud, i.e. prospective atrium of the zooid (Fig. 5a).

Stage 4

The developing polypide has bent almost 90° and its longitudinal axis lies in the same plane as the proximo-distal axis of the zooid. The two lateral ridges of the developing lophohore anlage in budding stage 3 have fused on the oral and anal side of the bud and thus form an oval tentacle crown (Fig. 6b). The tentacle anlagen, 15 in the analysed stage, are present as small stubs of equal-size projecting distally into the atrium, the space enclosed by the two-layered tentacle sheath (Fig. 6a-c). The latter has approximately doubled in size and both layers have become very thin. Distally the tentacle sheath passes into the vestibular wall, which on the frontal side attaches the bud to the cystid. The vestibulum is x-shaped and is closed towards the exterior and the atrium (Fig. 6a). Proximally, the tentacle sheath terminates at the tentacle bases. In between each pair of tentacles, the tentacle epidermis extends proximally of the tentacle sheath in form of intertentacular pits (Fig. 6a). Each of these pits contains a hollow canal (Fig. 6c). Medially of the intertentacular pits, the outer budding layer has formed a circum-pharyngeal coelomic compartment, the lophophoral ring coelom (Fig. 6a, c). The latter forms an almost complete ring around the pharynx except at the ganglion at the anal side of the lophophoral base where it is confluent with the remaining body cavity. In between the intertentacular pits the coelom extends from the lophophoral ring into each tentacle. Externally, the lophophoral ring coelom and also the intertentacular pits are covered

by a thin peritoneal layer (because of its thinness not shown in 3D-reconstructions). The ganglion at the lophophoral base has formed two lateral outgrowths that start to form a circum-oral nerve ring (Fig. 6d). The ganglion is still in open connection with the mouth/pharyngeal area, but the opening is comparatively smaller than in the previous budding stage (Fig. 6b, d). The digestive tract has for the most part differentiated into the regions found in adult zooids. At the lophophoral base the mouth opening continues into a broad pharynx (Fig. 6a, c). From the latter, the digestive tract continues into a much smaller and still short esophagus (Fig. 6a). The esophagus ends blindly and its epithelial wall is in intimate contact with those parts of the digestive tract which derived as an outgrowth from the prospective anal area (Fig. 6c). These parts begin with a short tube-like region which quickly broadens into the bulb-shaped proventriculus (Fig. 6a, c). The proventriculus or cardiac portion of the stomach is thick walled and supplied with a prominent muscular layer in between the peritoneal covering and the epithelium of the digestive tract. The remaining stomach consists of the caecum. In this budding stage, it has formed a large sac that has bent on the basal side slightly towards the right side of the developing bud (Fig. 6a-d). Distally, the digestive tract continues into a short ovoid intenstine which terminates via the anus into the tentacle sheath (Fig. 6a-d).

Stage 5

The following budding stage is mainly characterised by further differentiation of the different organ systems. The vestibulum has slightly expanded and exhibits no open connection towards the atrium or the exterior (Fig. 7a). A collar has started to form within the vestibulum and on sections is present as deeply staining material that is continuous with the ectocyst (Fig. 2c). Along with the elongation the tentacle sheath, the tentacles have approximately doubled in their length. At the lophophoral base, the intertentacular pits have grown to measure about 35µm in length and retain their central canal (Fig. 7a, b). The lophophoral ring coelom has not

distinctly changed when compared to budding stage 4 and remains confluent with the remaining coelom at the anal side of the ganglion. The latter has closed towards the mouth/pharyngeal area and forms a rather flattened disc distally of the lophophoral ring coelom (Fig. 7c). With the exception of the foregut, the digestive tract is characterized by growth and widening of the different regions of the digestive tract, i.e intestine, caecum, proventriculus (Fig. 7a, b, d). The pharynx is cone-shaped opening distally with the mouth opening (Fig. 7b, d). Proximally, the esophagus adjoins the pharynx. It has more become elongated and like in the previous budding stage ends blindly towards the cardiac portion of the stomach. At the terminal end it is slightly expanded to the shape of a bulb. From the cardiac portion of the stomach or proventriculus, the thin tube extending to the esophagus has grown longer and is more delimited towards the proventriculus than in the previous budding stage (Fig. 7b, d).

Condition of the lophophoral base in adult zooids

The adult lophophoral base clearly represents the most complex part of the polypide. The lophophoral ring coelom is similar as in budding stage 5. The intertentacular pits range from 50-60µm in their length and the epithelium bordering the pits is covered by a weakly staining layer (Figs 8a, b; Fig. 9a). In between the intertentacular pits, the tentacle coelom extends from the lophophoral ring coelom into each tentacle (Fig. 9a, c). A considerable extracellular matrix (ECM) lies between the epidermal layer and the peritoneum of the tentacles. On light-microscopical cross-sections the medial side of this ECM stains more prominently: More proximally on the lophophoral base it appears either like a zigzag at the tentacle coelom or three-lobed with a pointed median lobe and two large lateral ones which extend towards the median side of the intertentacular pits (Fig. 9b). Distally on the lophophoral base only the two lateral lobes are visible as flap-like latero-medial border of the tentacle coelom (Fig. 9a).

The nervous system at the lophophoral base forms a circum-oral/pharyngeal nerve ring (Fig. 8c, d). The compact central nervous mass, the ganglion, situated at the anal side of the lophophoral base (Fig. 8c) contains numerous perikarya and nerve fibres. A single conspicuous perikaryon that is distinctly large than all remaining nerve cells is situated centrally within the ganglion (Fig. 9b). Opposite to the ganglion the circum-oral nerve trunks are connected by a thin bridge (Fig. 8d). From the circum-oral nerve ring two principal types of nerves emanate that ultimately innervate the tentacles. The medio-frontal nerve of each tentacle directly emanates in the median plane of each tentacle from the circum-oral nerve ring (Figs. 8d, 9a, b). The roots of the latero-frontal and abfrontal tentacle nerves originate from an intertentacular junction which is connected to the circum-oral nerve ring (Fig. 8b, d). On the frontal side of the tentacles the medio-frontal tentacle nerves bifurcate from the intertentacular junction and innervate two neighbouring tentacles (Figs. 8d, 9a). Similarly, the abfrontal tentacle nerve roots bifurcate on the abfrontal side of the tentacles (Figs. 8b, 9a). Proximally of the bifurcation of the abfrontal tentacle nerve roots are conspicuous perikarya that are connected with the intertentacular nervous junction and lie within the wall of each intertentacular pit (Figs. 8b, 9a). The abfrontal tentacle nerve roots expand in their diameter along their traverse towards the tentacles. Proximally of the tentacle sheath they fuse into a single abfrontal nerve body (Fig. 8a, b). From the latter a single abfrontal tentacle nerve extends into each tentacle (Fig. 8b, d). Besides the regular innervations of the tentacles, additional nerve fibres come from the circum-oral nerve ring and innervate cells (probably sensory cells) in the area of the mouth opening. On light microscopical sections, these cells are readily distinguishable by their bright and more translucent cell plasma (Fig. 9a).

Discussion

Origin of the budding layers

Like in all other bryozoans, the polypide in *H. malayensis* develops from two budding layers; the inner budding layer from the epidermis and the outer budding layer from the peritoneum [15]. Some previous investigations described the outer budding layer to form from proliferating epidermal cells [11, 16]. More recent observations [17-18] found both layers of the body wall directly involved in the formation of buds. Although this study did not focus on the early bud formation, we never found any peritoneal cells derive from the epidermal layer. In addition, it should be mentioned that the peritoneal layer of the body wall in *H. malayensis* is always inconspicuously thin, even in adult zooids. As a consequence, it is more reasonable to assume a separate thin peritoneal layer to form the outer budding layer. Whether coelomocytes liberated from the peritoneal layer, as observed in the current study in early buds, participate in the formation of the outer budding layer remains unanswered. Different kinds of coelomocytes within the body cavity have been reported in representative of all bryozoan clades. In adult zooids, they possibly act in phagocytosis of excretory substances [1]. Their role during budding could be similar in accumulation of metabolic waste created during budding. On the other hand, coelomocytes are perhaps involved in the formation of peritoneally derived tissues, such as muscles. A similar function has been indicated for phylactolaemate coelomocytes [9].

Formation of the lophophore

The initial lophophore anlage develops as two lateral ridges in *H. malayensis*. A similar formation has been described for all other bryozoan clades (Cyclostomata: [19], Cheilostomata: e.g. [18, 20], Ctenostomata: [10-11], Phylactolaemata: [9]). In *Paludicella articulata* the lateral ridges first unite at the oral side, while the tentacles on the anal side are the last to form [10]. In the current study on *H. malayensis*, a stage showing a U-shaped

arrangement of the developing tentacles was not encountered. However, in our budding stage 3 of *H. malayensis*, the lophophoral ridges bulge slightly inward on the oral side, whereas they abruptly end on the anal side as described in *P. articulata*. A similar formation of the lophophore is described for the cheilostome *Membranipora membranacea* [20] and the phylactolaemate *Cristatella mucedo* [9]. The Phylactolaemata, however, show some differences regarding the formation of the lophophore which are also reflected in their adult condition. The lateral lophophoral ridges or more precisely bulges form the large lophophoral arms giving this clade the typical horse-shoe shaped lophophore. At first these do not carry tentacles in phylactolaemates [9] as seen in members of the Ctenostomata [10] and the Cheilostomata [18, 20]. In contrast to the remaining, pre-dominantly marine clades, the oral tentacles are the first to be formed in the Phylactolaemata [9, 21-22]. However, these differences are again reflected in the condition of the coelomic compartments of the adults. In the Phylactolaemata the ring-canal on the oral side of the lophophore base is comparatively short supplying only few tentacles [9], whereas the ring canal in the Ctenostomata (*H. malayensis*, this study) and Cheilostomata (*Cryptosula pallasiana*, [23]) encompasses almost the entire lophophoral base. Accordingly, two major patterns in the development of the lophophore can be recognized from the currently available data: 1. it starts with paired lateral anlagen that first close on the oral side and later also on the anal side and 2. the first tentacles arise on the area of the prospective ring canal with the most medial ones on the oral side to appear last.

Intertentacular pits of the lophophoral base

As stated by Gordon [23], the lophophoral base represents the most complex structure of the polypide. Surprisingly, his detailed study of the situation in *Cryptosula pallasiana* currently remains the only study. In *H. malayensis* we describe intertentacular pits at the lophophoral base for the first time in a ctenostome. A similar, most probably homologous structure is

present in *C. pallasiana*. In the latter they are called ciliated pits and measure 25 – 30 µm in length [1]. In adult specimens of *H. malayensis* they are approximately two times longer than in *C. pallasiana* and consequently much more noticeable. In *C. pallasiana* the pits are covered by a cuticle and the lining cells possess cilia projecting into the lumen of the pit. In *H. malayensis* a thin acelluar layer, most likely cuticle, lines the intertentacular pits as well, but the presence of cilia requires additional electron microscopic observations. Conspicuous intertentacular pits also occur in several other ctenostome bryozoans (Schwaha, unpublished data). Consequently, it seems likely to expect them in more cheilostomes as well and thus might be a synapomorphy for these two clades. As in *C. pallasiana* [23], we currently can give no indication about the function of the intertentacular pits.

Formation of the central nervous system and adult condition

In *H. malayensis* the central nervous system forms by an invagination of the inner budding layer (epidermal layer) in between the prospective mouth and anus, thus being identical to all other bryozoan classes [8-9]. However, in contrast to the Phylactolaemata, the ganglion in *H. malayensis* never contains an enclosed lumen and is compact in late budding stages (stage 5) and in adults.

The nervous system, in particular at the lophophoral base and the tentacle innervation, has been subject of several studies [1, 24]. Detailed studies on the central nervous system of the Phylactolaemata were conducted by Gewerzhagen [25], Marcus [26] and more recently by Gruhl & Bartolomaeus [27]. Ctenostomes were mainly studied by Graupner [28] and Bronstein [29] and cheilostomes by a series of papers by Lutaud [30-31] and a study by Gordon [23]. So far, the central nervous system in cyclostome bryozoans remains unstudied. Tentacle innervation, however, is briefly mentioned by Nielsen & Riisgard [32]. All studied bryozoans possess a circum-oral/pharyngeal nerve ring. Tentacles are innervated by 4-6 nerves. Four of these tentacle nerves (the abfrontal, frontal and the paired latero-frontal

nerves) are located subepidermally, while the remaining two are located subperitoneally. Only the phylactolaemate *Asajirella gelatinosa* shows a slightly different configuration of the subepidermal nerves [1]. In the current study on *H. malayensis*, we were only able to locate the four subepidermal nerves and not the paired subperitoneal ones. Only in the cheilostome *Cryptosula pallasiana*, the full set of six tentacle nerves was detected [23]. In the cyclostome *Crisia eburnea* [32] and the cheilostome *Electra pilosa* [33] the four subepidermal nerves were confirmed, whereas only the two subperitoneal and the latero-frontal tentacle nerves were found in *Flustrellidra hispida*, *Membranipora membranacea* [28], *Farrella repens* and *Alcyonidium* sp. [29]. In phylactolaemate bryozoans radial nerves extend from the nerve ring in between the tentacles, in the intertentacular membrane. Towards the tentacles, the radial nerves bifurcate within the intertentacular membrane and branch off the tentacles nerves [1, 27]. This intertentacular origin of the tentacles nerves resembles the abfrontal and latero-frontal tentacle nerves of *H. malayensis*. However, the medio-frontal tentacle nerves branch off directly from the circum-oral nerve ring in *H. malayensis*. In the ctenostomes *Flustrellidra hispida* [28], *Alcyonidium* sp., *Farrella repens* [29] and the cheilostomes *Membranipora membranacea* [28] and *Electra pilosa* [33] only one pair of tentacle nerves were found to originate from an intertentacular origin, i.e. the latero-frontal nerves. In *E. pilosa* the abrontal nerve extends directly from the circum-oral nerve ring, whereas it was not detected at all in the other aforementioned species – most likely a result of methodological problems. Nonetheless, summing up the little information available the following trend seems to be present in bryozoans: In the Phylactolaemata all tentacle nerves are of intertentacular origin, while gymnolaemates subsequently branch off tentacle nerves directly from the nerve ring, first the medio-frontal nerve (Ctenostomata: *H. malayensis*, this study) and then the abfrontal nerve as well (Cheilostomata: *E. pilosa* [33]). This trend coincides with current opinions of bryozoan phylogeny, with the Phylactolaemata as most basal branch and the Ctenostomata being paraphyletic as ancestors of the Cheilostomata. However, a broader range of taxa

(including the neglected Cyclostomata) need to be studied to confirm this trend. Additionally, the basic set of tentacle nerves needs to be ascertained. In several Phylactolaemata [1, 34] and the cheilostome *E. pilosa* [33], 'subperitoneal' or 'enclosed' peritoneal cells that are topologically identical to the position of the subperitoneal nerves described for *C. pallasiana* [23] and several ctenostomes [28-29] were described. Consequently, it seems probable to expect them in most if not all bryozoans, also in *H. malayensis*, but their detection requires detailed electron microscopic or state-of-the-art immunocytochemical studies.

Formation of the gut and the esophagus-cardia length

The mid- and hindgut in *H. malayensis* form as anal outpocketing as described for the ctenostomes *Flustrellidra hispida* [35], *Paludicella articulata* [10] and *Pottsiella erecta* [36]. As recently summarized by Schwaha et al. [9], diverging descriptions of gut formation have been described. Some authors claim an oral outpocketing to give rise to these parts of the digestive tract [11, 37]. Considering the similarities in the formation of all other organ systems during budding of bryozoans, it appears more probable that the mid- and hindgut of bryzoans always develop from an anal outpocketing. Ultimately, reinvestigating and increasing the number of species in all clades needs to be conducted to confirm this suggestion.

As mentioned by Rogick [38], the gut terminology of bryozoans is in a 'nice state of confusion'. Only Silen [39] attempted to give a general terminology to the various parts of the digestive tract by considering all bryozoan classes. Two valve-like constrictions, one at the end of the foregut and a second before the intestine (or rectum), are important criteria for assigning terms for specific gut regions [39]. The valve at the end of the foregut is commonly termed cardiac valve or esophageal valve and represents the border between the esophagus and the cardia. As seen in the current study on *H. malayensis*, previous studies on the Phylactolaemata [9] as well as the Cyclostomata [19] this valve develops at the border of the

two anlagen assembling the gut during budding. In the Phylactolaemata and the Stenolaemata the digestive tract from the caecum to the mouth opening is short, while it is usually elongated in gymnolaemates. Based on the distal position of the cardiac valve in the latter, this elongated tube was considered to be a result of the elongation of the cardia. Consequently, an esophagus was stated to absent in gymnolaemates, because no proper differentiation towards the pharynx is given [39]. While this might be true for several cheilostome species (e.g. *Membranipora*: [39]; *Bugula:* [16]; *Cryptosula*: [16, 40]; *Electra*: [16]; *Hippothoa*: [41]; *Lageneschara*: [42], also see [1, 43], ctenostome bryozoans show a larger variation. Our results on *H. malayensis* show that the cardiac valve is situated far proximally and most of the tube-like elongation develops and consists of the foregut, the esophagus. Only a comparatively small part of the tube is composed of the cardia distally of the muscular proventriculus. An identical arrangement is present in the hislopiids *H. corderoi* [44] and *Echinella placoides* [45]. On the contrary, in the only ctenostome superfamily showing similar flattened box-shaped zooids, the Alcyonidioidea, the esophagus is negligibly small and the cardiac tube elongated [46-47]. An elongated esophagus is generally considered to be present in 'stoloniferan' ctenostomes [48] in which the polypide bud becomes dislocated into an elongated peristome that later is separated from the remaining 'stolon' [49-50]. However, in other ctenostomes with elongated peristomes but lacking true stolons, like the Victorellidae, the esophagus and cardia are both present as almost equally long tubes. In addition, the relative size, particularly of the cardiac tube, is affected by the state of its contraction [51]. It seems worthwhile to investigate whether the differences in the morphology of the gut prove to be valuable for drawing phylogenetic inferences. Comparative data is currently sparse, because the location of the cardiac valve is only given for very few species. Since the cardiac valve hinders reflux of food particles from the cardiac stomach, it seems more reasonable that the anatomy of the gut is influenced by the diet and the mode of digestion.

Conclusions and Outlook

This is the second recent study on polypide development of a bryozoan and thus extends the available information for comparative analyses on organogenesis among the phylum. Compared with the recent study of the phylactolaemate *Cristatella mucedo* and older studies, we are able to show that the development of the polypide shows distinct similarities in the formation of the different organ systems. These include the early polypide bud formation as a proliferation of epidermal cells bulging towards the peritoneal layer of the bud, a two-layered vesicle-like early bud, the central nervous system or ganglion forming as an invagination of the epidermal layer in between the prospective mouth and anal area, the digestive tract mainly forming from an outpocketing of the prospective anal area that grows towards a comparatively small anlage of the foregut (pharynx and esophagus), and the lophophore forming from two lateral anlagen that first fuse on the oral and afterwards on the anal side. The point where the anlage of the mid/hind-gut and the foregut meet is represented in adult zooids by the cardiac valve. A comparison of different bryozoan species and superfamilies shows that its location is not identical in gymnolaemates which always possess an elongated tube-shaped gut connecting the pharynx with the caecum. With the current paucity of comparative data, it is more appropriate to consider the diet and the mode of digestion to be decisive on the variable location of the cardiac valve.

At the complex lophophoral base of adult zooids intertentacular pits of unknown function are described for the first time in a ctenostome. Similar structures were only reported in the cheilostome *C. pallasiana* [23]. It is likely that they are present in more if not all gymnolaemate species, but have escaped the attention of previous investigators. Along with structures of the nervous system at the lophophoral base and the tentacle innervation, these characters appear promising for further analysis for comparative phylogenetic purposes on bryozoans.

With the polypide development of the Phylactolaemata [9] and Ctenostomata (this study) studied in more detail with modern visualisation techniques, the Cyclostomata remain an essential taxon for further study. Organogenesis in the budding process of the later was only studied by Borg [19] and Nielsen [37], but is only poorly documented. In cyclostome bryozoans the polypide is formed first and the cystid later. This formation of buds is also found in the basal Phylactolaemata, in contrast to budding of the Cteno- and Cheilostomata where the cystid is formed first and the polypide later. Accordingly, future studies will concentrate on cyclostome budding to complement our knowledge on developmental patterns of bryozoans.

Competing interests

The authors declare that they have no competing interesting.

Authors' contributions

TS conducted all practical work and wrote the manuscript. TW coordinated research in Thailand, collected and identified the animals and contributed significantly to the manuscript. All authors read and approved the final version of the manuscript.

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Figures

Figure 1 - Overview of *Hislopia malayensis*.

(a) View from the basal side of colony detached from the substrate showing the arrangement of the flat encrusting zooids and the communication sites to neighbouring zooids (asterisks). (b) Fragment of a colony viewed from the frontal side to show the arrangement of the zooids and the polypides within each zooid. (c) Detail of an older bud already showing the typical oval shape of the adult zooids with a new bud arising as a slender process on its distal side. (d) Detail of a single zooid showing most of the polypides morphological features.

Abbreviations: a – atrium enclosed by the tentacle sheath, cae – caecum, cw – cystid wall, es – esophagus, int – intestine, lb – lophophoral base, nz – neighbouring zooid, p – polypide, ph – pharynx, pv – proventriculus, o – orifice, z – zooid

Figure 2 - Micrographs of semithin sections of *Hislopia malayensis* buds.

(a) Cross-section through an early bud showing the prominent inner and outer budding layer. Asterisk marks a coelomocyte within the body cavity. (b) Slight oblique section through budding stage 3 showing dense peritoneal cells accumulating at the developing lophophoral base. (c) Cross-section through the vestibular wall of budding stage 5 showing the first signs of collar formation in the vestibulum.

Abbreviations: am – apertural muscles, c – developing collar, cw – cystid wall, epl – epidermal layer of the cystid, ga – gut anlage, ibl – inner budding layer, la – lophophore anlage, lca – lophophoral coelom anlage, lb – lumen of the bud, nb – neck of the bud, obl – outer budding layer, pl – peritoneal layer of the cystid, pma – prospective mouth area, ts – tentacle sheath, vw – vestibular wall. Scale bar in (a) and (c) = 30µm, in (b) 50µm.

Figure 3 - 3D-reconstruction based on serial semithin sections of budding stage 1 of *Hislopia malayensis*. Inner and outer budding layer displayed transparently.

(a) Lateral view of the bud. (b) View on the proximal side of the bud.

Abbreviations: ga – gut anlage, ibl – inner budding layer, lb – lumen of the bud, nb – neck of the bud, obl – outer budding layer, paa – prospective anal area. Scale bar = 50µm.

Figure 4 - 3D-reconstruction based on serial semithin sections of budding stage 2 of Hislopia malayensis. Inner and outer budding layer displayed transparently.

(a) Lateral view of the bud. (b) View on the proximal side of the bud. Asterisks mark the lateral indentations of the budding layers towards the median plane of the bud.

Abbreviations: ga – gut anlage, gla – ganglion anlage, ibl – inner budding layer, lb – lumen of the bud, nb – neck of the bud, obl – outer budding layer, paa – prospective anal area, pma – prospective mouth area. Scale bar = 50µm.

Figure 5 - 3D-reconstruction based on serial semithin sections of budding stage 3 of *Hislopia malayensis*.

(a) Lateral view of the bud with the outer budding layer displayed transparently. (b) Similar view as in (a) from the lateral side of the bud with the outer budding layer omitted and the developing gut-, lophophore and ganglion displayed transparently. (c) View from the frontal side of the bud showing the developing tentacles on the lateral lophophoral ridges.

Abbreviations: aa – anal area, ga – gut anlage, ggl – ganglion, la – lophophore anlage, nb – neck of the bud, pma – prospective mouth area, ta – tentacle anlagen, ts – tentacle sheath, v – vestibulum.

Colors: blue – lophophore anlage, green – gut anlage, purple – outer budding layer, yellow – ganglion anlage. Scale bar in in (a) and (b) = 100µm, and in (c) = 50µm.

Figure 6 - 3D-reconstruction based on serial semithin sections of budding stage 4 of *Hislopia malayensis*.

Because of their thinness, the peritoneal lining of the digestive tract and the lophophoral base were not reconstructed. (a) View from the basal side of the bud. The tentacle sheath and vestibular wall are displayed transparently. (b) View from the distal side of the bud showing the digestive tract as well as the developing lophophore and ganglion. The asterisk marks the opening of the ganglion towards the mouth opening. (c) View from the basal side showing the digestive tract and epidermal layer of the lophophore anlage transparently. (d) View from the distal side showing the epidermal layer of the lophophore anlage transparently.

Abbreviations: a – anus, at – atrium, cae – caecum, con – circum-oral nerve trunks, dt – developing tentacles, es – esophagus, int – intestine, itp – intertentacular pits, lc – lophophoral ring coelom, mo – mouth opening, ph – pharynx, pv – proventiculus, ts – tentacle sheath, v – vestibulum, vw – vestibular wall

Colors: blue – lophophore anlage, crimson – lophophoral ring coelom, green – gut anlage, turquoise – vestibular wall, yellow – nervous system. Scale bar = 100µm.

Figure 7 - 3D-reconstructions based on serial semithin sections of budding stage 5 of *Hislopia malayensis*.

Because of their thinness, the peritoneal lining of the digestive tract and the lophophoral base were not reconstructed. (a) View from the frontal side of the bud. The tentacle sheath and vestibular wall are displayed transparently. (b) Similar view as in (a) with the tentacle sheath and vestibular wall omitted and the digestive tract and the epidermal layer of the lophophore displayed transparently. Asterisk marks the border of the still separated fore- and mid-gut. (c) Distal view on the lophophoral base showing the lophophoral coelom with the ganglion situated above it and the mouth opening. The surface of the tentacles has been cut. (d) View

of the digestive tract displayed transparently. The shape of the pharynx is better visible than in (b). Asterisk marks the border of the still separated fore- and mid-gut.

Abbreviations: a – anus, at – atrium, cae – caecum, es – esophagus, ggl – ganglion, int – intestine, itp – intertentacular pits, lc – lophophoral ring coelom, mo – mouth opening, ph – pharynx, pv – proventiculus, t –tentacles, ts – tentacle sheath, v – vestibulum, vw – vestibular wall

Colors: blue – epidermal layer of the lophophore, crimson – lophophoral ring coelom, green – gut anlage, turquoise – vestibular wall, yellow – nervous system. Scale bar in (a), (b) and (d) = 100µm, in (c) = 50µm.

Figure 8 - 3D-reconstructions based on serial semithin sections of the adult lophophoral base of *Hislopia malayensis*.

(a) Lateral view on the lophophoral base showing its epidermal layer, the lophophoral ring coelom and parts of the nervous system. (b) Similar view as in (a) but with the epidermal layer of the lophophoral base displayed transparently. (c) Slight oblique view from the distal side of the lophophoral base showing the lophophoral coelom and the anally situated central nervous system. (d) Detail of the nervous system showing the junction of the circum-oral nerve ring (asterisk) and the tentacle nerves.

Abbreviations: afn – abfrontal tentacle nerve, afnb – abfrontal nerve body, afnr – abfrontal tentacle nerve root, cgl – cerebral ganglion, con – circum-oral nerve trunk, if – intertentacular nerve fork, itnc – intertentacular nerve cell, itp – intertentacular pit, lfn – latero-frontal tentacle nerve, mfn – medio-frontal tentacle nerve, t – tentacle, tc – tentacle coelom

Colors: blue – epidermal layer of the lophophoral base, crimson – lophophoral coelom, yellow – nervous system. Scale in (a-c) = 50µm, in (d) = 20µm

Figure 9 - Semithin sections of the adult lophophoral base of *Hislopia malayensis*.

(a) Near-cross-section through the mouth opening distally of the lophophoral base. (b) Near-cross-section through the pharyngeal area more proximally of the lophophoral base than in (a).

Abbreviations: afnr – abfrontal nerve root, cc – conspicuous central nerve cell of the cerebral ganglion, cgl – cerebral ganglion, cont – circumoral nerve trunk, cw – cystid wall, ecm – prominently staining extracelluar matrix, es – esophagus, itnc – intertentacular nerve cell, itp – intertentacular pits, lc – lophoral ring coelom, litp – lumen enclosed by the intertentacular pits, lfn – latero-frontal tentacle nerves, mfn – medio-frontal tentacle nerve, mo – mouth opening, n – nerve fibres, phl – pharynx lumen, pt – thin peritoneal layer surrounding the lophophoral base, sc – presumed sensory cells, st – stomach. Scale bar = 30µm.

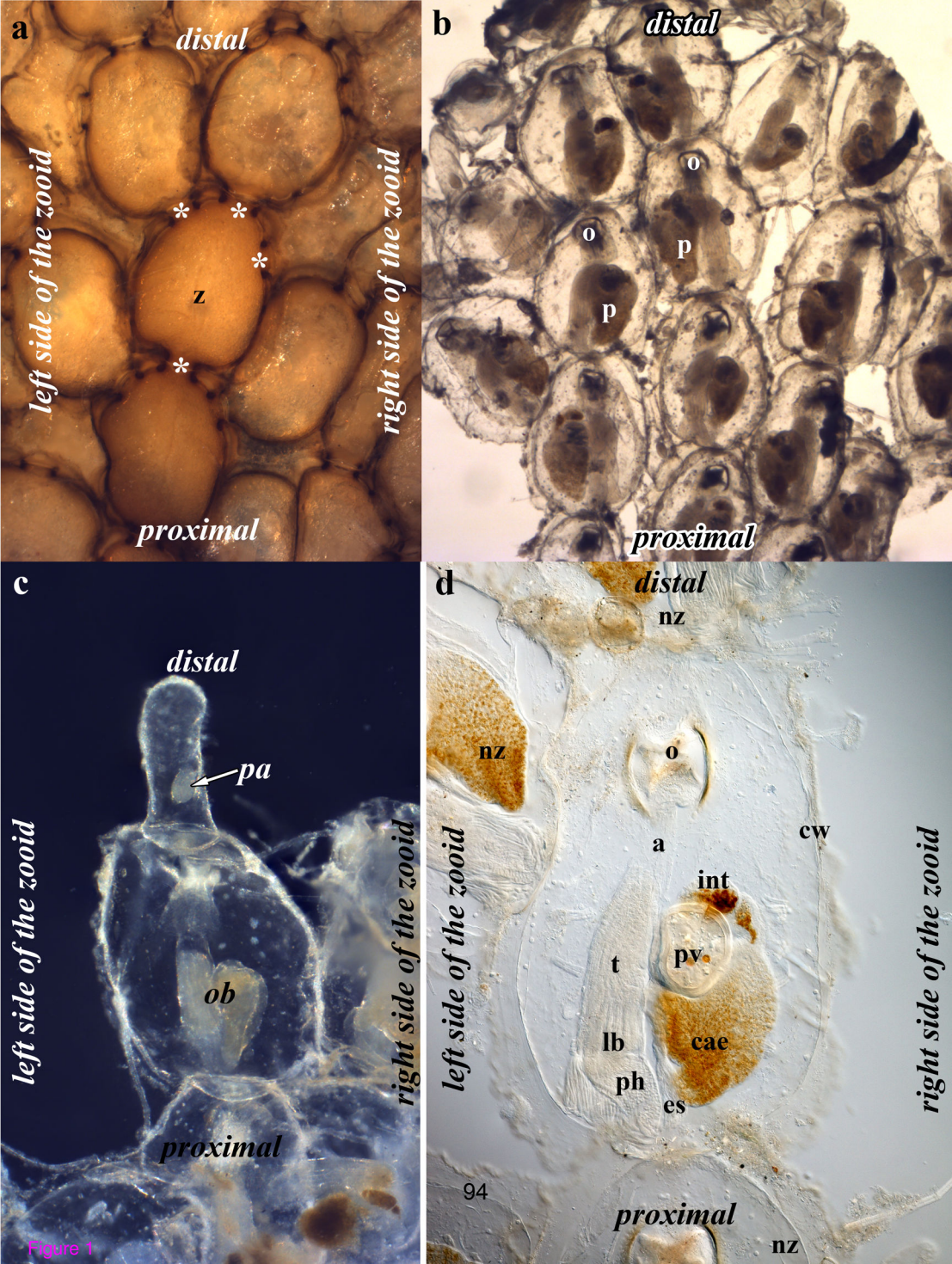
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Figure 1

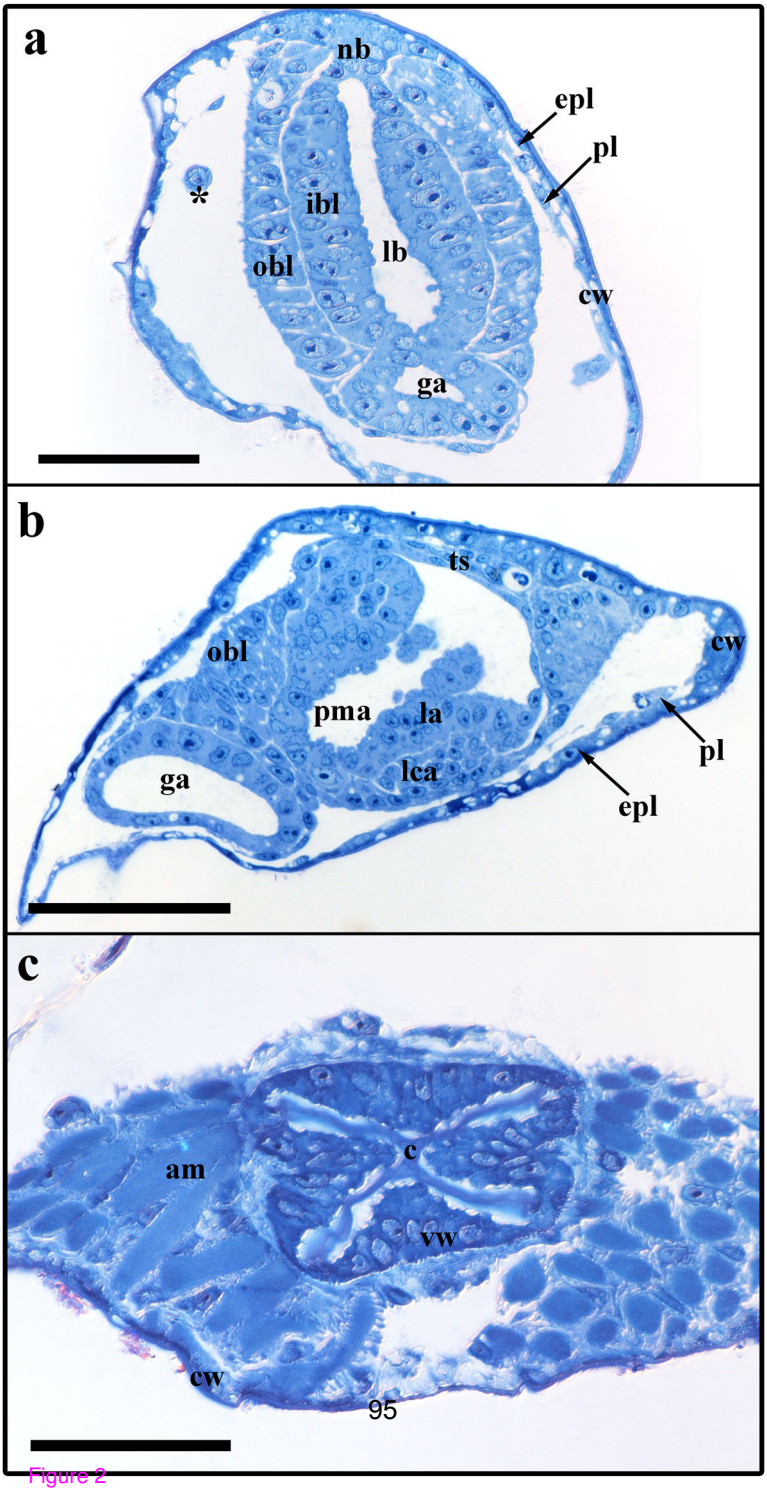
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Figure 2

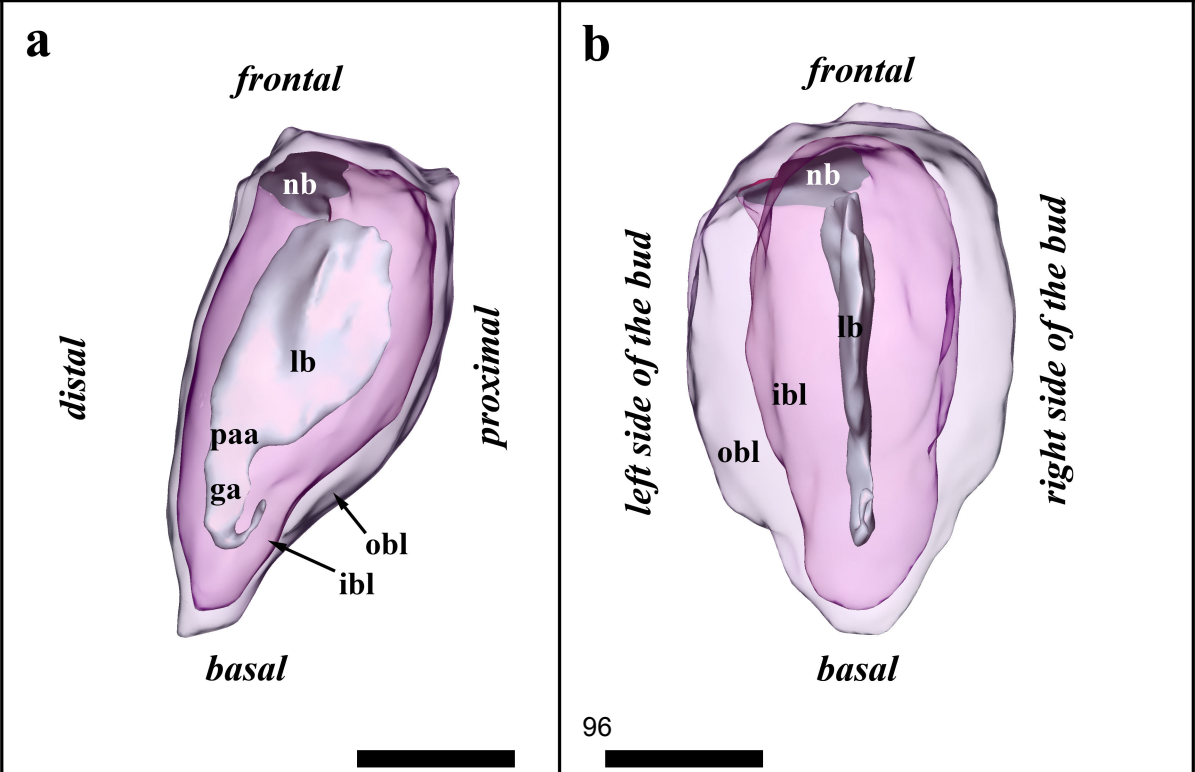
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Figure 3

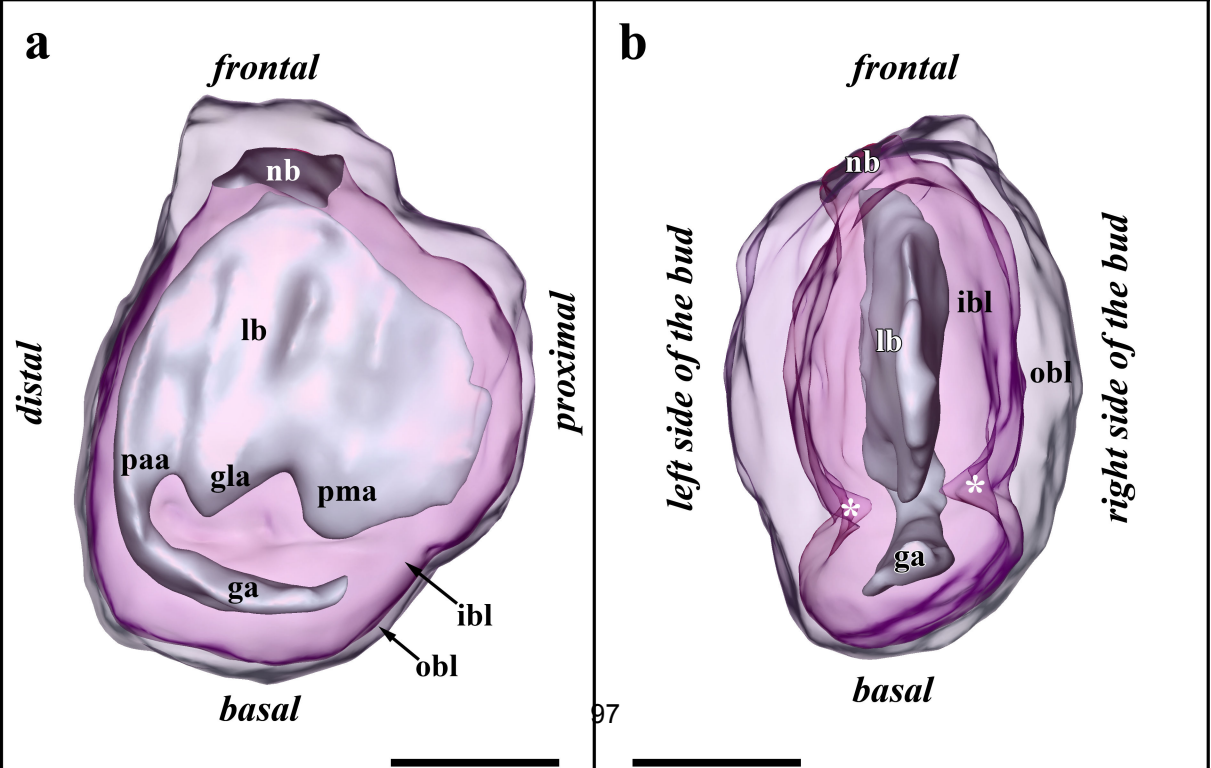
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Figure 4

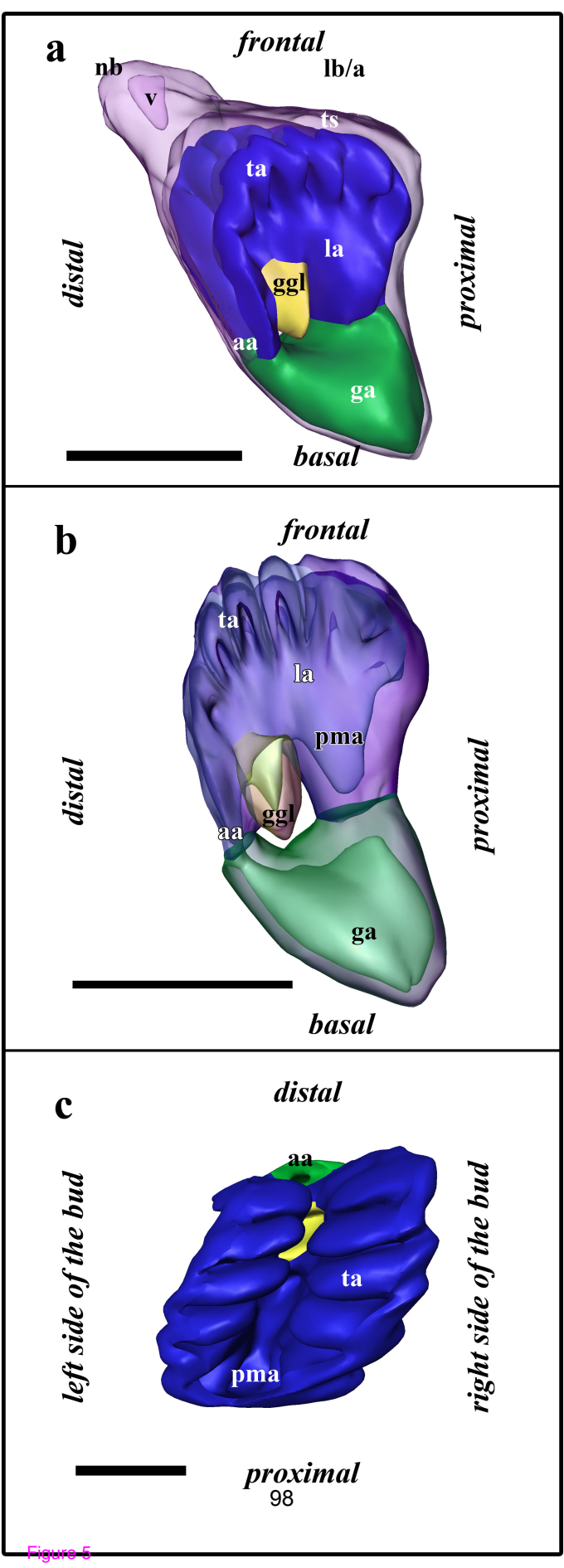
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Figure 5

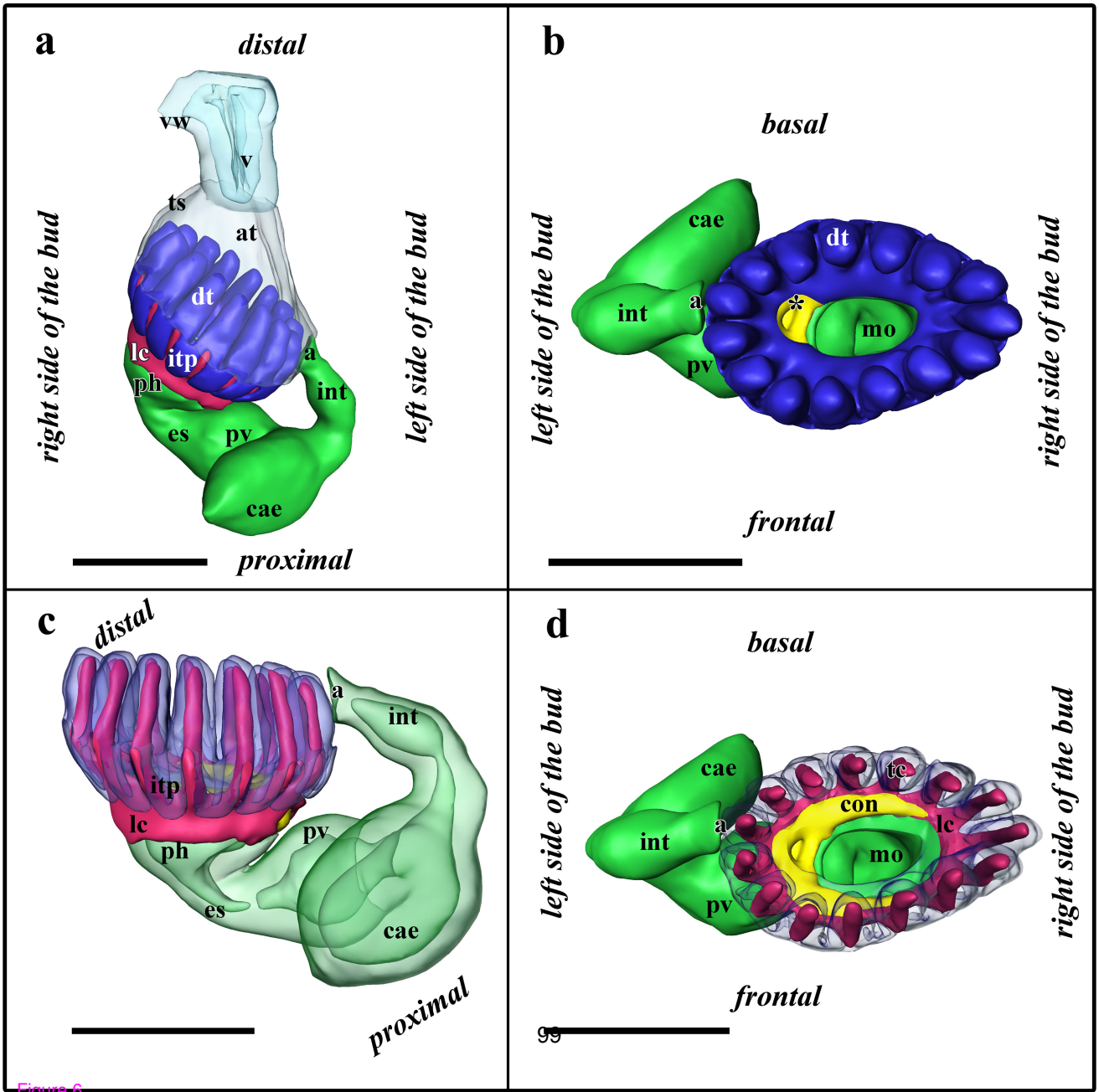
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Figure 6

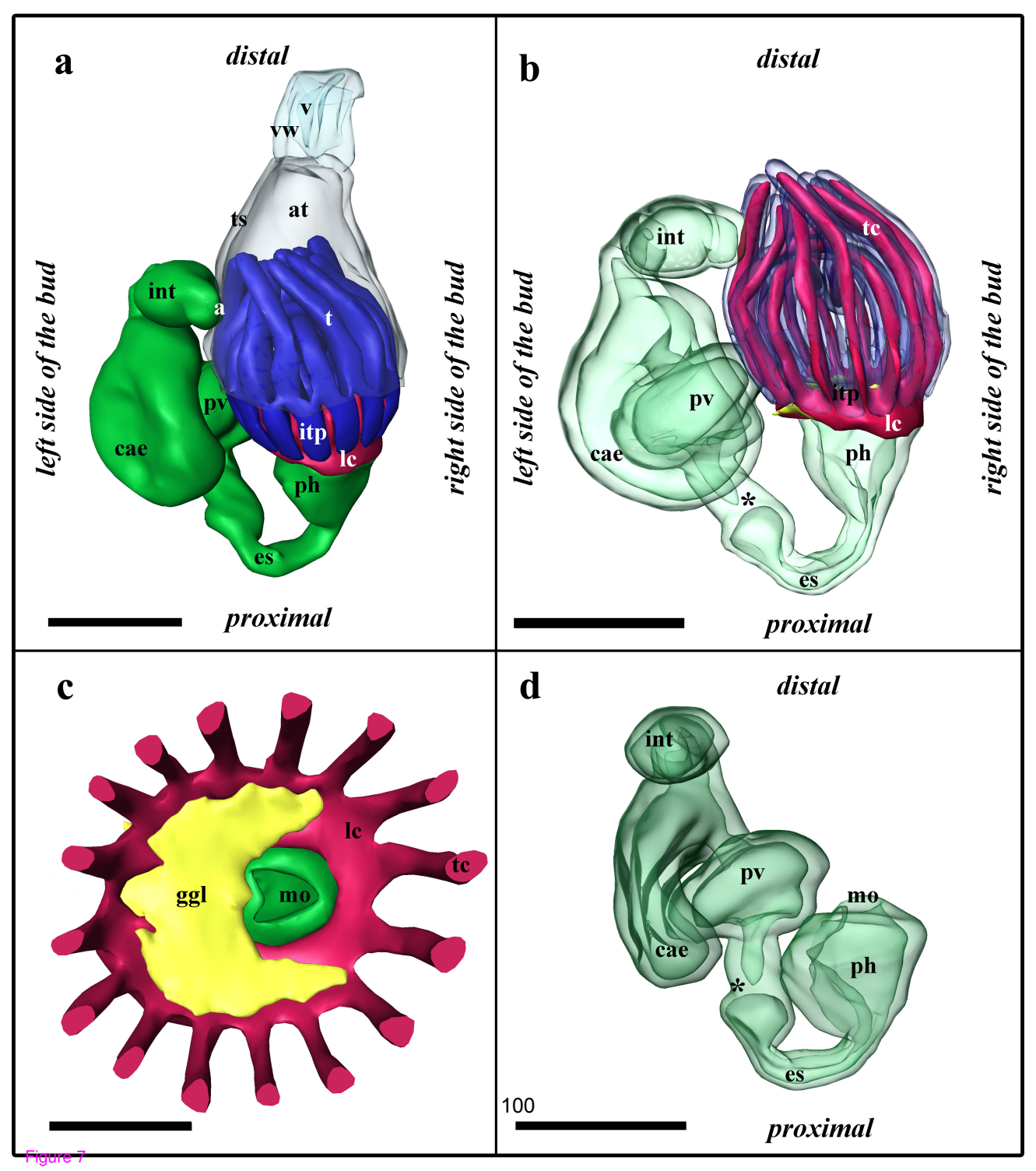
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Figure 7

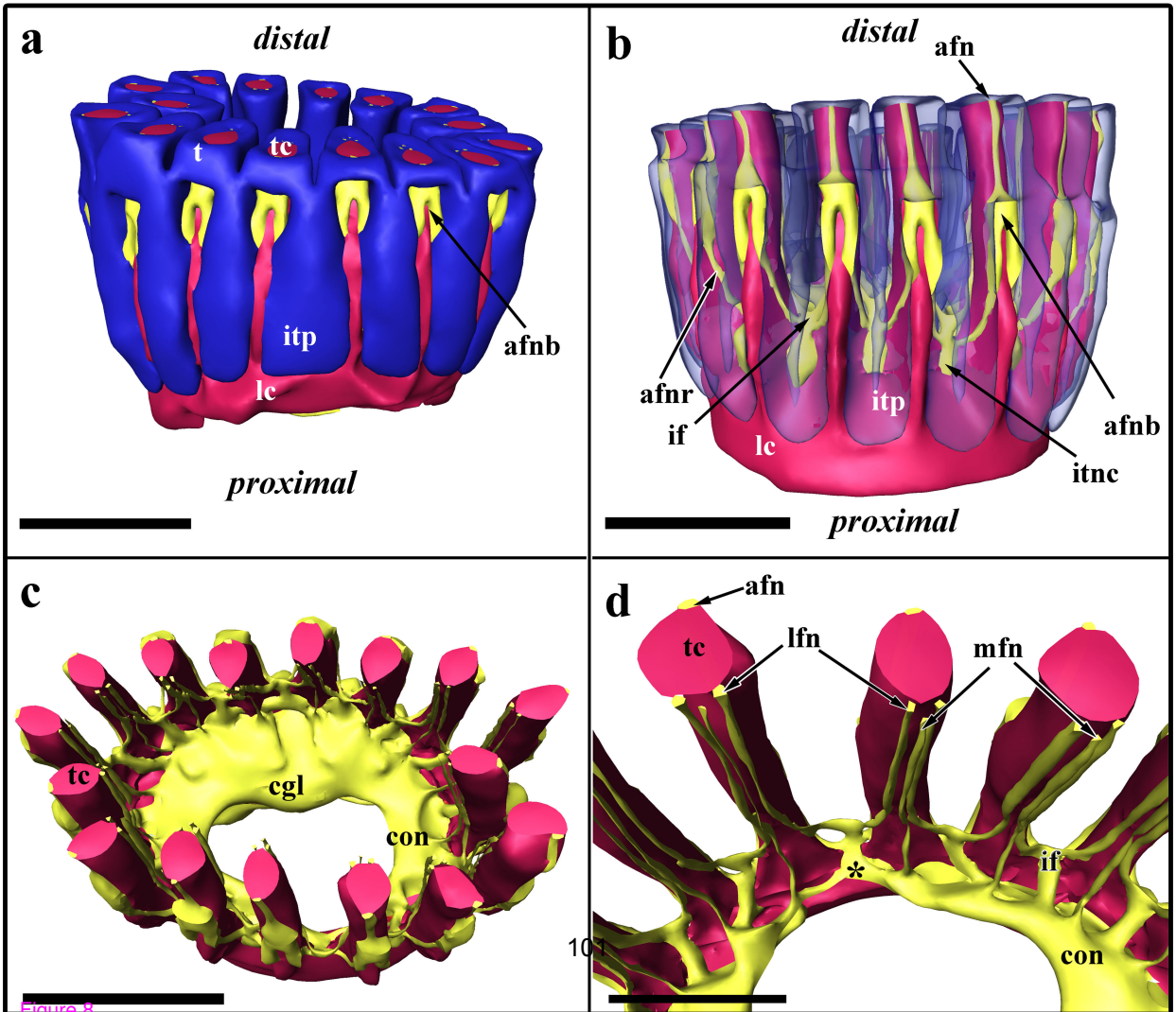
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Figure 8

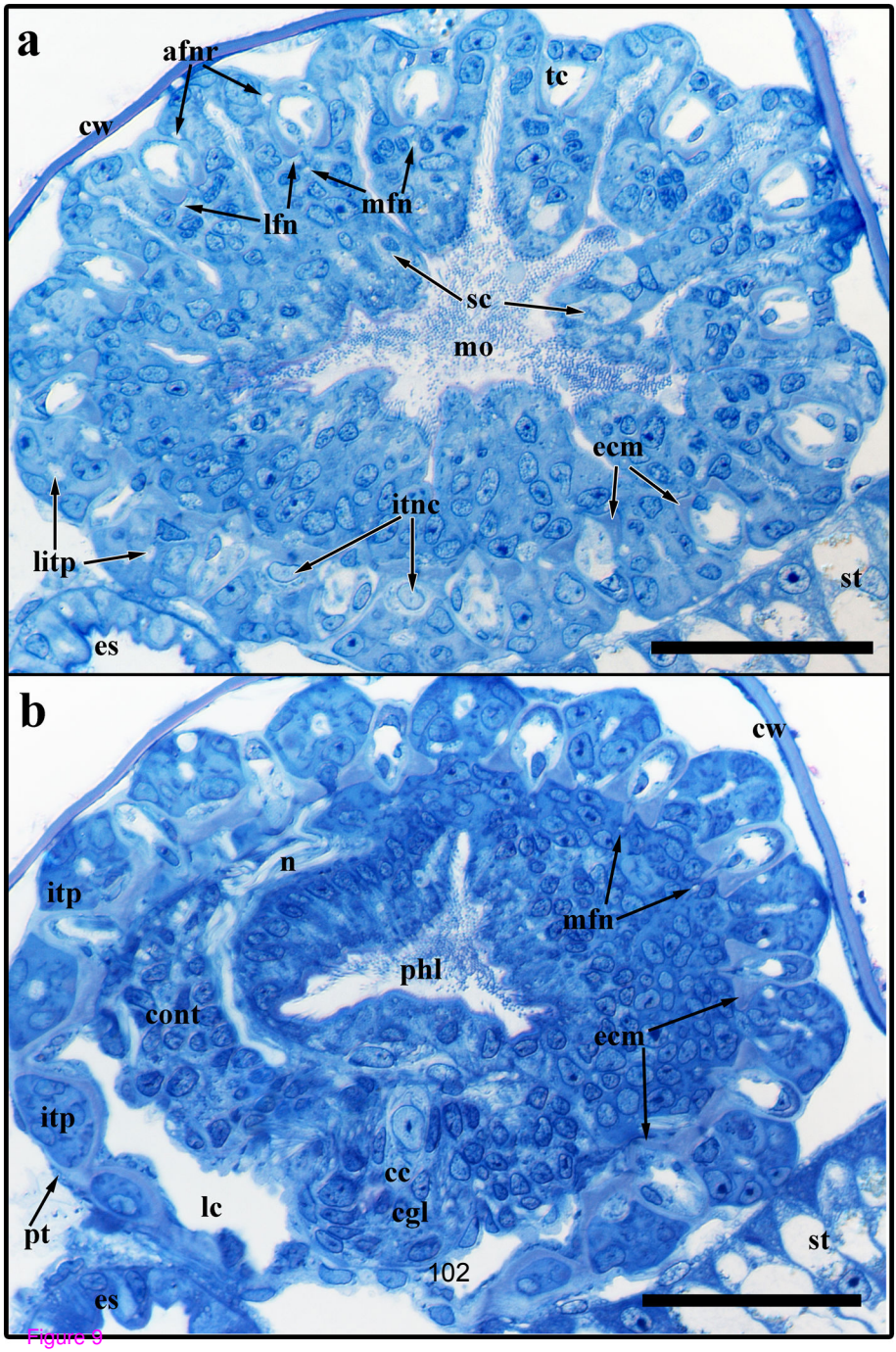
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Figure 3

5. Myoanatomy and serotonergic nervous system of the ctenostome *Hislopia malayensis*: Evolutionary trends in bodyplan patterning of Ectoprocta

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Myoanatomy and serotonergic nervous system of the ctenostome *Hislopia malayensis*: Evolutionary trends in bodyplan patterning of Ectoprocta

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Abstract

Background:

Ectoprocta is a large lophotrochozoan clade of colonial suspension feeders comprising over 5.000 extant species. Their phylogenetic position within the Lophotrochzoa remains controversially discussed, but also the internal relationships of the major ectoproct subclades –Phylactolaemata, Stenolaemata, and Gymnolaemata - remains elusive. To gain more insight into the basic configuration of ectoproct muscle systems for phylogenetic considerations, we analysed the adult myoanatomy and the serotonergic nervous system as well myogenesis in budding stages of the ctenostome *Hislopia malayensis*.

Results:

In adults, the serotonergic nervous system is restricted to the lophophoral base with a high concentration in the cerebral ganglion and serotonergic perikarya between each pair of tentacles. Prominent smooth apertural muscles extend from the basal cystid wall to each lateral side of the vestibular wall. The musculature of the tentacle sheath consists of regular strands of smooth longitudinal muscles. Each tentacle is supplied with two bands of longitudinal muscles that show irregular striation. At the lophophoral base several muscles are present: (i) Short muscle fibres that proximally diverge from a single point from where they split distally into two separate strands. (ii) Proximally of the first group are smooth, longitudinal fibres that extend to the proximal-most side of the lophophoral base. (iii) Smooth muscle fibres, the buccal dilatators, traverse obliquely towards the pharynx, and (iv) a circular ring of smooth muscle fibres situated distally of the buccal dilatators. Retractor muscles are mainly smooth with short distal striated parts. The foregut consists mainly of striated ring musculature with only few longitudinal muscle fibres in the esophagus, while the remaining parts of the digestive tract solely exhibits smooth musculature. During budding, apertural and retractor muscles are first to appear, while the parietal muscles appear at a later stage.

Conclusions:

The apertural muscles show high similarity within Ectoprocta and always consist of two sets of muscles. Gymnolaemates and Phylactolaemates show clear differences within their digestive tract musculature, the former showing smooth and longitudinal muscles to a much greater extent than the latter. The complex musculature at the lophophoral base appears promising for inferring phylogenetic relationships, but sufficient comparative data are currently lacking.

Introduction

The lophotrochozoan phylum Ectoprocta consists of benthic, colonial filter feeders that live on various substrates. It currently contains over 5.000 extant and approximately 20.000 described extinct species. They are currently assigned to three taxa, whereby the Phylactolaemata represent a small group of solely freshwater-inhabiting ectoprocts, while the Stenolaemata (with the only remaining extant taxon Cyclostomata) and the Gymnolaemata mainly constitute marine animals. Within the Gymnolaemata two distinct groups, the Ctenostomata and Cheilostomata, are recognized [1]. The relationships between these higher taxa are currently not well understood: The Phylactolaemata are considered monophyletic, while the Cheilostomata and Stenolaemata have been considered both monophyletic [2] or polyphyletic [3], [4]. Due to their calcified protective skeletons, the latter two taxa show a long fossil record yielding more insight into their evolution. In contrast, the fossil record of the non-mineralized Ctenostomata is poor [5] and only represented by casts of borings (e.g. [6]) and bioimmurations (i.e., overgrowth by encrusting, mineralized organisms, cf. [7]). There is consensus that “Ctenostomata” is paraphyletic and that ctenostome-like ancestors have lead independently to the origin of the calcified exoskeletons of Cheilostomata and Stenolaemata [4-5, 8-9]. Accordingly, they are of particular interest for ectoproct evolution. Due to the scarcity of their fossil record, the study of extant ctenostome ectoprocts appears particularly promising for further insights into ectoproct relationships and evolution. To date, ctenostome phylogenies are mostly based on features of the cystid and colony morphology [5, 10], whereas details on the anatomy of the soft body (polypide) remain less investigated and are thus widely neglected in phylogenetic analyses.

Myoanatomical features and neurotransmitter distribution, for example serotonin or FMRF-amide, have recently been used for phylogenetic inferences among lophotrochozoans [11-13]. Thereby, several immunocytochemical investigations have dealt with the neuromuscular system of different ectoproct larval types [14-20], whereas no such study is as of yet available

for adult ectoprocts. Data on the development of the zooids and the ontogenetic appearance of certain muscle systems have been used to elucidate internal ectoproct relationships [3, 21-24]. Myoanatomical details of the digestive tract have also been proposed to be useful for discriminating certain subtaxa [25-26]. By contrast, characters of the nervous system have never been considered on a broad, comparative scale for systematic or phylogenetic deductions.

Hislopiid ctenostomes comprise only seven freshwater species and are the sole family within the superfamily Hislopioidea. The latter belongs to the paraphyletic 'carnosans', which are regarded as primitive within the Euctenostomata (sensu [24]), and which have retained a simple colonial morphology similar to the proposed cheilostome-like ancestor (see e.g. [5, 9, 21, 24, 27]). Their supposedly relatively basal position within Euctenostomata (Fig. 1; [5, 10, 21, 24]) renders the Hislopiidae an important model taxon for inferring ectoproct phylogeny and evolution. To gain more insight into the basic configuration of ectoproct muscle systems, we studied the adult myoanatomy and serotonergic nervous system as well as myogenesis during budding in *Hislopia malayensis* Annandale, 1916.

Material and Methods

Animals

Colonies of *Hislopia malayensis* Annandale, 1916 were collected from the pond of the Faculty of Fisheries of the Kasetsart University in Bangkok (see [28]). Specimens were fixed in 4% paraformaldehyde in 0.01M phosphate buffer (PBS) containing 0.01% NaN_3 for 1 hour at room temperature. Subsequently, they were rinsed three times for 20 min and stored in the same solution.

Immunocytochemistry and confocal microscopy

Some colonies were dissected prior to staining to increase permeability. For F-actin staining, specimens were permeabilized in PBS containing 4% Triton-X (PBT) for 1 hour, followed by overnight incubation in a 1:40 dilution of AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR, USA) in PBT at 4°C. Then, the specimens were rinsed three times in PBS. For staining of the serotonergic nervous system, pieces of *H. malayensis* colonies were transferred to 6% normal goat serum (NGS; Sigma-Aldrich, St. Louis, MO, USA) in PBT (block-PBT) overnight at 4°C. Subsequently, a polyclonal rabbit anti-serotonin antibody (Zymed, San Francisco, CA, USA) was applied at a concentration of 1:400 in block-PBT for 24 hours at 4°C. Then, the specimens were rinsed several times in block-PBT for 6 hours at 4°C prior to application of a secondary fluorochrome-conjugated antibody (goat anti-rabbit AlexaFluor 594, Molecular Probes) in block-PBT at a concentration of 1:200 for 24 hours at 4°C. Specimens were then washed three to four times in PBS for about 6 hours. Nuclei were stained by adding a few drops of DAPI (Invotrogen, 3µg/ml) for 15-20 minutes, followed by three short washes in PBS. Specimens were mounted in Fluoromount G (Southern Biotech, Birmingham, AL, USA) on standard microscope slides.

Analysis and image acquisition was performed on a Leica DM IRBE microscope equipped with a Leica TCS SP2 confocal unit (Leica Microsystems, Wetzlar, Germany). Confocal image stacks were recorded with 0.5-1µm step size along the Z-axis. Images stacks were captured as maximum intensity projections or further processed as volume renderings with Amira 4.1 software (Mercury Computer Systems, Chelmsford, MA, USA).

Results

Myoanatomy of adult *Hislopia malayensis*

Colonies of *Hislopia malayensis* form simple encrusting sheets on a variety of artificial or natural substrates [29]. Its individual zooids are flat and oval-shaped and are interconnected by rosette-shaped communication pores. Each zooid consists of a more or less rigid, chitinous

to hyaline protective cystid and a flexible polypide that contains all major organs of the zooid (Fig. 2). On each side of the zooid a series of four to five parietal muscle bundles are associated with the cystid wall. They originate from the basal attachment site and traverse the coelomic cavity to the frontal cystid wall, where the orifice is situated (Fig. 3a, b). The inner plug of each communication pore shows a high number of actin-filaments, which is highest in its periphery and decreases towards the center (Fig. 4h).

In retracted zooids the polypide is attached to the cystid by an almost rectangularly invaginated vestibulum that extends from the orifice on the frontal cystid wall to the diaphragm at the distalmost end of the invaginated tentacle sheath (Fig. 2). Prominent apertural muscles extend from the basal cystid wall to each lateral side of the vestibular wall. They consist of smooth muscle fibres and extend along the entire length of the vestibular wall (Fig. 3a, b; 4b). On the frontal and lateral side, the vestibular wall shows a regular net of smooth ring and longitudinal musculature, whereas diagonal and longitudinal muscles are present on the basal side (Fig. 4b). A sphincter at the diaphragm (diaphragmatic or atrial sphincter) separates the vestibulum from the space enclosed by the tentacle sheath, the atrium (Fig. 2; 3a; 4f). The tentacle sheath ranges from the diaphragm to the lophophoral base. Its musculature consists of regular strands of smooth longitudinal muscles that run from its distalmost end approximately until the region of the anal area (Fig. 4f). At its proximal end, the tentacle sheath continues into the lophophore. Each tentacle is supplied with two bands of longitudinal muscles that show irregular striation (Fig. 4b, c, e). A distinct muscular knot is present at the distal tip of each tentacle (Fig. 4c). Proximally, the longitudinal tentacle musculature extends to the lophophoral base. At the lophophoral base four groups of muscles are present: Below the musculature of each tentacle, short muscle fibres are present that proximally diverge from a single point from where they split distally into two separate strands. These muscle elements are approximately V-shaped with some fibres traversing medially (Fig. 4a, e). A second set of muscles is situated proximally to the first group and

consists of smooth, longitudinal fibres that extend to the proximal-most side of the lophophoral base (Fig. 4a, e). From that point smooth muscle fibres, the buccal dilatators, traverse obliquely towards the pharynx (Fig. 4a, e). At the site of the cerebral ganglion, two pairs of buccal dilatators are present. The first pair inserts more proximally on the pharynx whereas the second pair traverses inserts more distally at the pharynx (Fig. 4e). A circular ring of smooth muscle fibres is situated in the region of the mouth, medially to the proximal-most part of the lophophoral base, and slightly above the pharynx (Fig. 4e). The retractor muscles of the polypide originate from the proximal cystid wall and insert at the entire lophophoral base except for the ganglion area (Fig. 3a; 4a, e). The fibres of the retractor muscles appear smooth for most of their length. In some cases, however, the distal-most part of the fibres appears cross-striated (Fig. 4g). The pharynx below the mouth opening is provided with several narrow bands of circular musculature which shows distinct cross-striation (Fig. 3a; 4e). The pharynx continues into the esophagus which is an elongated tube. Similar to the pharynx, its musculature is mainly composed of obliquely striated ring musculature (Fig. 2; 3a; 4a, e), but few delicate longitudinal muscle fibres could be observed as well (Fig. 4d). The following cardia or proventriculus is bulbous and possesses densely packed smooth ring musculature (Fig. 3a; 4a, i). The digestive tract continues into the voluminous caecum which for the most part carries bands of smooth ring musculature (Fig. 3a, b; 4a, e, i). From the proximal end of the caecum two prominent longitudinal muscles extend distally. Halfway of the stomach they split into two bundles (Fig. 3a, b; 4e). A few sparse longitudinal muscle fibres are also present between the circular muscle bands (Fig. 4i). The intestine, which adjoins the stomach, carries smooth longitudinal muscles over its entire length and terminates in the tentacle sheath (Fig. 3a; 4a, f, i).

Myogenesis in budding zooids

Buds start as distal or lateral outgrowths of the cystid of a zooid that soon become constricted off from the mother zooid (Fig. 3b). Further development leads to a tube-like elongation of the cystid slightly broadened at its distal tip. In the middle of the bud the polypide anlage develops. The retractor and apertural muscles are first to appear in the bud. The anlagen of the retractor muscles is V-shaped: Proximally, they originate from the cystid wall from a single site, whereas distally the bundles split and insert at the developing polypide. The apertural muscles form as two small bands on each side of the prospective aperture (Fig. 5a).
In a later budding stage with distinct tentacle anlagen, the cystid has broadened distally. The retractor and apertural muscles have only slightly changed, but possess more muscle fibres (Fig. 5b). Additional muscles have not been formed at this stage of development. Further in development, the cystid widens even more on its lateral sides. Compared to the previous stage, the widening has also progressed towards the proximal pole of the zooid. Three to four distinct parietal muscle bands appear laterally of the developing polypide. Besides the much enlarged retractor and apertural muscles, musculature of the digestive tract has started to form (Fig. 5c). In the final analysed stage the cystid exhibits the oval shape, characteristic of adults. The developing polypide already exhibits most of the adult musculature. In addition, differentiation and regionalization of the digestive tract has started and its corresponding musculature has started to form. The lophophoral base and the tentacles already show all muscular elements that occur in the adults. The apertural muscles are prominent and ring musculature of the duplicature as well as the diaphragmatic sphincter is present. Only the tentacle sheath is still devoid of muscles (Fig. 5d).

Serotonergic nervous system

The serotonergic nervous system in adult zooids is restricted to the lophophoral base of each polypide (Fig. 6a, b). The highest concentration is found in the cerebral ganglion, from where nerves extend circumpharyngeally. On the oral side there are three serotonergic perikarya at

the base of each pair of tentacles, which are connected to the circumpharyngeal nerves to form a nerve ring. At the remaining tentacles, neuronal perikarya are present as well. These are, however, more apart than the three oral ones and are directly connected to the ganglion via delicate neurites. From each perikaryon short neurites extend into each of the two tentacles.

Discussion

Homology of apertural muscles and their phylogenetic significance

Apertural muscles are present in all three ectoproct subtaxa, the Phylactolaemata, the Stenolaemata (with the sole extant taxon Cyclostomata) and the Gymnolaemata, which include the Cteno- and Cheilostomata. A review of the existing literature shows that their terminology is utterly confusing and inconsistent. Common terms found for apertural muscles are for example “parieto-vaginal muscles” [25], “parieto-diaphragmatic muscles” [30], “parieto-atrial muscles” [31], “parieto-vestibular muscles” [32], “pyramidal muscles” [33] or “longitudinal parietal muscles” [34]. The latter term has been established according to the notion that apertural muscles are derived parietal muscles [27, 32] and is also frequently used in more recent compendia on ectoprocts [34-36].

A comparison of these muscles is most easily performed among retracted zooids (Fig. 7). In all ectoproct subtaxa, retracted zooids show a distalmost invaginated portion of the cystid wall, termed the vestibular wall, which is separated from the proximally adjoining tentacle sheath by a diaphragm. At the diaphragm a strong sphincter is present in all three subtaxa (Fig. 7).

The Phylactolaemata, the suggested sistergroup of the remaining ectoprocts [37-38], have two different apertural muscle systems: the duplicature bands and the vestibular dilatators (Fig. 7a, b). The duplicature bands are peritoneal bands containing longitudinal muscles fibres that emerge from the lateral body wall and insert either directly at the diaphragm (as in the

lophopodids: Fig. 7b; *Lophopus*: [33, 39]; *Lophopodella*: [40]) or at the tentacle sheath below the diaphragm in the remaining taxa (Fig. 7a; *Cristatella*: pers. obs., *Fredericella*: [33], *Pectinatella*: [32-33, 41]; *Plumatella*: [33, 42]; *Stolella*: [43]). Vestibular dilatators consist of separate muscle fibres that traverse the coelom distally of the duplicature bands. In all phylactolaemates they are loosely arranged and run from the lateral body wall towards the vestibular wall. The latter attach to the entire area of the vestibular wall, starting at the area of the the diaphragm and projecting up to the distal parts of the vestibular wall.

Cyclostome ectoprocts show a peculiar coelomic condition. In contrast to all other ectoprocts, the peritoneal layer of the endocyst is detached from the epidermis to form a coelomic sac, the membranous sac, around the polypide (Fig. 7c; [32, 44]). However, topographically similar to the duplicature bands of phylactolaemates, cyclostomes possess an attachment organ. It consists of ligaments that attach the polypide and membranous sac to the skeletal walls of the zooid (Fig. 7c; [44-45]). Supposedly homologous structures of the phylactolaemate vestibular dilatators are present as few muscle fibres ("musculi extensores vestibuli" *sensu* [46], "longitudinal ectodermal muscle" *sensu* [44]), that run from the distalmost bodywall (i.e., terminal membrane in cyclostomes) to the diaphgram (Fig. 7c).

In principle, the Gymnolaemata (Cteno- and Cheilostomata) also possess two muscular systems. First, homologs of the duplicature bands, which are most commonly termed "parieto-vaginal bands", and second, vestibular muscles that are always prominent in cteno- and cheilostomes.

Protoctenostome, i.e. benedeniporoidean [24], polypide morphology is only insufficiently known. 'Parieto-vaginal muscles' were mentioned for *Benedenipora catenata* [47], but their description and illustration are too incomplete for drawing any comparisons. The vestibular muscles in ctenostomes usually form two portions, a small proximal portion of muscular bundles that insert at the diaphragm, the parieto-diaphragmatic muscles, and a large distal portion that attaches at the vestibular wall. The latter muscles are often referred to as parieto-

vaginal muscles. To avoid confusion with the parieto-vaginal muscle bands, we refer to them as “distal vestibular muscles”. An erect and simple, uniserial colony morphology is regarded as ancestral among ctenostomes [5, 21], a condition exhibited among “carnosan” ctenostomes only by the paludicelloideans. Accordingly, we consider the arrangement of their apertural muscle systems as plesiomorphic state for ctenostomes. They possess parieto-vaginal bands and their vestibular muscles are present as parieto-diaphragmatic muscles that consist of few fibres closely adjoining the distal vestibular muscles (Fig. 7d; *Paludicella*: [48-51]).

The ‘carnosan’ superfamilies Alcyonidioidea and Hislopioidea are both considered as early offshoots within ctenostomes (Fig. 1). Alcyonidioideans possess parieto-vaginal bands (*Alcyonidium*: [52-54]; *Elzerina*: depicted, but not labelled by [1]) and their vestibular muscles show a distinct separation into parieto-diaphragmatic and distal vestibular muscles (Fig. 7g; e.g. Alcyonidium: [52]). Within the Hislopioidea the current study on *Hislopia malayensis* shows that parieto-vaginal bands are reduced and only vestibular muscles are present, as reported by Annandale [51]. A similar arrangement of muscles is present in *H. corderoi* [55-56]. Only for *H. lacustris* a set of muscles resembling parieto-vaginal bands has been illustrated [57]. However, Carter’s specimens were not well preserved and differences in the muscular system were stated to be absent among different species of *Hislopia* [51]. Accordingly, it is appropriate to assume the absence of parieto-vaginal bands in hislopiid ectoprocts. In contrast to the Alcyonidioidea, vestibular muscles of *Hislopia* show no separation into parieto-diaphragmatic and distal vestibular muscles, but instead extend over the entire length of the vestibular wall (Fig. 7h).

The second trend is evident in the remaining clades. Arachnidioidean ctenostomes display a wide range regarding the length of the peristome and are mainly characterized by cystid appendages which often anastomise among individual zooids of a colony. Details on their polypide morphology are mostly restricted to the genus *Nolella* [58-59], which always has an

elongated peristome. So far, no parieto-vaginal bands have been recorded for this genus (Fig. 7e).

The Victorelloidea, Walkerioidea, and Vesicularioidea are characterized by peristome-elongation/trophon or stolon formation [21, 24] and often have reduced the parieto-vaginal bands. In the latter two clades, the distal parieto-vaginal muscles are spatially more displaced from the parieto-diaphragmatic muscles (Fig. 7f; e.g. *Bowerbankia*: [25]) than in the victorelloideans (*Victorella*: [25]) or the arachnidioideans (e.g. *Nolella*: [58]). Within the Walkerioidea only *Hypophorella expansa*, a species considered to be basal within this clade [24], possesses distinct parieto-vaginal bands [52, 60]. Indications for the latter are also present in *Farrella repens* [61], while all other walkerioideans have reduced them (*Aeverillia*: [62]; *Harmeriella*: [63]; *Walkeria*: [64-65]). Similarly, a single species among victorelloideans, *Sundanella sibogae*, possesses parieto-vaginal bands [66], while other genera have reduced them (*Pottsiella*: [67-68]; *Victorella*: [25, 69]). From our current knowledge, all Vesicularioidea lack parieto-vaginal bands (*Bowerbankia*: [25, 51, 58, 70-71]; *Buskia*: [72]; *Spathipora*: [73]; *Terebripora*: [74]; *Vesicularia*: [75]).

Parieto-vaginal bands are present in malacostegan ectoprocts (Table 1), which are commonly regarded as a basal, paraphyletic group within the cheilostomes [76]. Accordingly, parieto-vaginal bands are probably part of the ancestral cheilostome bauplan and may have been present in the last common cheilostome ancestor (Fig. 7i). Since they have also been recorded in species from almost all "higher" groupings of cheilostomes (Table 1 and references therein [30, 58, 77-91]), it seems reasonable assume that they are present in most cheilostomes.

Cheilostomes have retained the parieto-diaphragmatic muscles. Distally to these muscles, opercular occlusors are situated (Fig. 7i). We consider the occlusors as a modification of the distal vestibular muscles. As a consequence, we reject the notion that apertural muscles, including the opercular occlusors, are phylogenetically derived from parietal muscles [27, 32].

As shown above, apertural muscles are present in all three ectoproct subclades including the Phylactolaemata and Stenolaemata, which both lack parietal muscles.

Several authors have regarded the cheilostome ancestors to be *Arachnidium*-like [1, 27], but as previously mentioned, soft-body morphology is almost unknown for the genus. The most precise data are available for the genus *Nolella*, which forms elongated peristomes. Similar to *Nolella*, species with long peristomial tubes that belong to other superfamilies commonly lack the parieto-vaginal bands. Since parieto-vaginal bands were probably present in the ctenostome-like ancestor of cheilostomes, it would be of particular interest to study arachnidioidean species with short peristomes, such as the genera *Arachnidium* or *Arachnoidea*.

Lophophoral and digestive tract musculature

The tentacle musculature consists of two longitudinal muscle bands in most ectoprocts investigated to date [32]. Within Phylactolaemata, *Fredericella* is an exception in having only one longitudinal muscle band [92]. In general, the tentacle musculature is smooth in phylactolaemates and cheilostomates, whereas striated myofibrils have been reported for ctenostomes and cyclostomes. However, only for phylactolaemates several species where analysed in detail by electron microscopy [32, 92]. Accounts on cyclostome tentacle muscles rely on the classical study of Borg [46], whereas only a single species of both cteno- [93] and cheilostomes [94] were analysed on the ultrastructural level. We observed striation of the longitudinal tentacle musculature of *Hislopia malayensis* which supports the previous notion that ctenostomes possess striated tentacle musculature.

As the center of the nervous system and source of feeding and sensory structures, the lophophoral base represents the most complex part of the polypide [32]. However, most of our current knowledge resides in its description of the cheilostome *Cryptosula pallasiana* [94]. Buccal dilatators are present in this species, but were also described for the cheilostome

Bugula simplex [58], the ctenostome *Bowerbankia pustulosa* [95], in *Crisia eburnea*, and other cyclostomes [46, 96]. They are smooth in *H. malayensis*, *C. pallasiana* and *B. simplex*, while they were reported striated for *B. pustulosa*. A second pair of dilatators inserting above the ganglion as in *H. malayensis* has not been reported for any other species. In *C. pallasiana*, so-called "basal transverse tentacle muscles" are present at the base of each pair of tentacles that probably act as antagonist to the tentacle musculature [94]. These muscles are absent in *H. malayensis*, but possibly the circular ring muscle at the lophophoral base of this species may be a homologous structure. The two muscle groups at the lophophoral base below each longitudinal tentacle muscle have to our knowledge not been reported for any other ectoproct so far. Functionally, they remain difficult to interpret, but they could act in lophophore movement and rotation. To clarify their function, more detailed observations on living specimens, e.g., by video microscopy, are required.

In several ctenostomes the vacuolated cells of the pharyngeal epithelium possess distinct striated muscle fibres on their lateral walls. For *Zoobotryon verticillatum* [97] and *Alcyonidium polyoum* [98] ultrastructural analysis revealed that the pharyngeal cells along with these fibres represent a prominent myoepithelium. Light microscopical observations on such fibres were also conducted for the ctenostomes *Alcyonidium hirsutum* [99], *Victorella pavida* [100], and *Bowerbankia pustulosa* [95]. This specialized pharynx acts as a strong sucking pump for particle capture and has also been observed in the cheilostomes *Bugula flabellata* [99], *B. neritina* [101], and *Cryptosula pallasiana* [102]. In the current study we found no muscular elements in the pharyngeal cells of *Hislopia malayensis*. The only musculature associated with the pharynx consists of striated ring muscles surrounding the pharyngeal epithelium. Pharyngeal ring musculature has been described by the above mentioned authors for the respective species and is, when mentioned, striated. This musculature is also present in *Flustrellidra hispida* [103] and *Zoobotryon verticillatum* [104]. Longitudinal muscles in the pharynx were only found in *B. pustulosa* [95]. Most

phylactolaemate bryozoans possess striated ring musculature at the pharynx as well [39, 105]; only in *Asajirella gelatinosa* longitudinal muscles have been observed [32]. Cyclostome pharyngeal musculature consists of striated ring musculature and few longitudinal muscle fibres [46]. In conclusion, striated ring musclulature appears to be a common trait of the ectoproct pharynx, whereas longitudinal fibres only occur in a few of the species studied so far. However, drawing the border between the pharynx and adjoining esophagus is often difficult in many ectoprocts and mostly only discernable by the ciliation pattern [106]. Accordingly, some of the observed longitudinal pharyngeal musculature could actually belong to the esophagus, which possesses few longitudinal fibres in *H. malayensis* (this study), *Fl. hispida* [103], and *B. pustulosa* [95].

The differentiation of the cardiac portion of the stomach or proventriculus into a gizzard occurs in some cheilostomes and cyclostomes, but is more frequently found in ctenostomes [32, 107]. In the latter, the gizzard was previously considered to have evolved only once [24], while other authors argue for its multiple independent origin [107]. Internally, it is lined by cuticular plates or teeth used for crushing ingested food particles. In hislopiids the proventriculus carries a smooth cuticular lining in all species except for *Echinella placoides*, where it contains several spirally arranged cuticular ridges [108]. Functionally, it was either interpreted to act as a mere storage organ or as crushing organ of ingested food particles [109]. In *H. malayensis* the inner walls of the proventriculus never actually come together and food particles are very small that do not require grinding. In the proventriculus, there is a very active exchange of undigested whole particles: the distal sphincter closes; then a slow wave of tight constriction starts from the proximal end and moves distally. It squeezes the contents and forces them to spurt back through the narrow constriction, between cuticular ridges, towards the proximal end. In addition, particles are pushed back and forth between the lower proventriculus and the upper caecum. This is achieved mostly by cilia in these two regions but also by spasmodic contractions of the caecum (Wood, personal observation). The purpose of

all this movement is unclear. The effect may be to break apart any particles that may be clinging together and not to grind any particles.

In *H. malayensis* the remaining stomach or caecum possesses mainly smooth circular muscle bands with two prominent longitudinal muscles at the proximal side and few longitudinal fibres between the circular muscles. A similar muscular system of the caecum has been described for *Fl. hispida* [103], *Z. verticillatum* [104], *B. pustulosa* [95], and the cheilostome *C. pallasiana* [102]. The circular muscle bands in these species are not adjoining, but keep a distance to each other. On the contrary, phylactolaemates solely possess a dense layer of circular, striated muscle fibres in the caecum [32]. The remaining digestive tract, i.e the intestine and rectum, possess only smooth longitudinal musculature in *H. malayensis* (this study), *Z. verticillatum* [104], and *B. pustulosa* [95], while additional ring musculature was described for *Fl. hispida* [103]. In contrast, most phylactolaemates possess mainly densely packed smooth ring musculature in the intestine [39]. Among phylactolaemates, only *A. gelatinosa* shows few additional longitudinal muscle fibres in the intestine [32].

Retractor muscles and the striation problem

The partitioning of the zooid into the cystid and a retractable polypide is a characteristic feature of all ectoprocts. Accordingly, polypide retractors are present in all clades, and usually constitute the most prominent somatic muscles. Their traverse within the coelom is usually simple and unidirectional from the proximal or lateral cystid wall to the lophophore base [110]. This condition is also present in *H. malayensis* and other species of *Hislopia* [51, 55-56].

Retractor muscle fibres have been controversially described as either striated (e.g. [53, 111]) or smooth (see [112]). Thereby, all ultrastructural studies found the fibres to be smooth [32]. In the current study on *Hislopia malayensis* we found the retractor muscle fibres to be mainly smooth, while the distal-most parts appeared ‘striated’. A similar appearance of the retractor

muscle fibres was recently observed in *Pottsiella erecta* [68]. Such striations were attributed as 'pseudostriations', resulting from contraction folds or helically coiled fibrils [72, 95, 112]. However, other ctenostomes also show striations in expanded zooids with relaxed retractors (Schwaha: unpublished observations). Consequently, it remains difficult to fully interpret these striation patterns, which inevitably requires ultrastructural analyses.

Myogenesis during the budding process

The temporal appearance of the retractor, apertural and parietal muscles, as well as the polypide anlage during budding has previously been analysed in several ctenostomes and has been used for phylogenetic inferences. The earliest analyses tried to reconstruct the suborders 'Carnosa' and 'Stolonifera' as monophyletic taxa [22-23], which was later rejected [21, 24]. The latter author, Jebram [21], analysed myogenesis during the budding process of a hislopioid, *Hislopia corderoi*. Accordingly, parietal muscles are the first to appear during budding in *H. corderoi*, whereas the polypide anlage is second, followed by the retractor muscles and the apertural muscles, respectively - a succession also found in specimens of the closely related superfamilies Alyconidiodea and Arachnidioidea [21]. The results of this study on *H. malayensis* show that the parietal muscles are last to appear during budding, which is not in accordance with the observations on *H. corderoi*. However, it has to be considered that the observations by Jebram [21] were conducted on old preserved material and that the specimens were mainly analysed as whole mounts. With these methods, the delicate first anlagen of the retractor and apertural muscles are rather difficult to distinguish. Considerable variation in the asexual muscle succession occurs among numerous other ctenostome species [21], and might also be present among species of *Hislopia*. As promising these muscle successions might be for ctenostome phylogeny, their value should perhaps not be overestimated until analysed with modern methods.

The serotonergic nervous system

The serotonergic nervous system in Ectoprocta was previously analysed in different kinds of planktotrophic and lecitotrophic larvae [14-16, 113]. During ectoproct metamorphosis, larval organ systems undergo histolysis and adult organs, including the nervous system, are formed anew ("catastrophic metamorphosis") [16, 114]. Accordingly, larval nervous systems are ontogenetically not homologous to those of the adults. So far, the adult serotonergic nervous system has only been investigated in the lepraliomorph cheilostome *Triphyllozoon mucronatum* [16]. The latter shows a similar condition to *Hislopia malayensis*. The highest concentration of serotonin in both species is located in the circumpharyngeal nerve ring or "cerebral ganglion". Additional serotonergic perikarya are present at the lophophoral base and are connected to the ganglion by fine neurites. *Hislopia malayensis* possesses additional serotonergic nerves, which run from these perikarya into the tentacles. Such serotonergic neurites have not been found in *T. mucronatum*. These results currently support a common serotonergic nervous system in gymnolaemates, i.e cteno- and cheilostomes, but for estimating its value for phylogenetic considerations, more species need to be analysed.

Conclusions and Outlook

This study presents the first data on the myoanatomy and serotonergic nervous of an adult ctenostome ectoproct, *Hislopia malayensis*. Comparative analysis revealed that the Phylactolaemata show several morphological differences to the remaining ectoprocts by, e.g., being the only ectoproct taxon with a distinct bodywall musculature. Only few synapomorphies for all ectoproct taxa are identifiable (cf. [92]). As an example, our study demonstrates that the apertural muscles are highly similar among the major ectoproct subclades and consist of two principal sets of muscles, the parieto-vaginal bands and the vestibular muscles. These have been modified among the different ectoproct taxa according to the different morphology of the aperture. Their phylogenetic derivation from parietal muscles

seems unlikely. Concerning the apertural muscles in ctenostomes, two main evolutionary trends are apparent: 1. Formation of dense encrusting colonies with almost box-shaped zooids and the aperture and its associated musculature shifted towards the frontal side (Fig. 7g, h). 2. Peristome elongation or even stolon formation usually accompanied by loss of parieto-vaginal bands and with distal parieto-vaginal musculature being more distantly situated from the parieto-diaphragmatic musculature (Fig. 7e, f). Parieto-vaginal bands most likely were present in the cheilostome ancestor and appear to be present in most extant Cheilostomata.

Concerning the musculature of the digestive tract, there are clear differences between the Phylactolaemata and the Gymnolaemata. Most species of the former possess tightly packed, mostly cross-striated ring musculature, whereas the digestive tract musculature of the Gymnolaemata contains longitudinal muscles that are more loosely arranged. Among the phylactolaemates, only *Asajirella gelatinosa* possesses few longitudinal muscles in the wall of the digestive tract. This species belongs to the family Lophopodidae, which is currently regarded as the most basal family within Phylactolaemata [115-116]. Accordingly, we assume longitudinal and ring musculature in the wall of the digestive tract a basal ectoproct feature. Similarly, two longitudinal muscle bands in the tentacles of the lophophore seem to a basal ectoproct feature. The lophophoral base connecting the tentacles shows a complex set of several muscle groups, but due to the lack of comparative data, conclusions on the plesiomorphic state for Ectoprocta are currently not possible.

Cyclostome ectoprocts possess annular ring muscles in their membranous sac that might have evolved from the ring musculature of the phylactolaemate bodywall [44]. However, additional investigations using state-of-the-art technology are needed to further address this issue, since most available data rely on the classical work by Borg [46].

Competing interests

The authors declare that they have no competing interesting.

Authors' contributions

TS conducted all practical work and drafted the manuscript. TW coordinated research in Thailand, collected and identified the animals and contributed significantly to the manuscript. AW provided necessary facilities for providing research and contributed significantly to the writing of the manuscript. All authors read and approved the final version of the manuscript.

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Figures

Figure 1 - Phylogenetic system of the Ctenostomata, modified after (a) Jebram (1986) and (b) Todd (2000).

The phylogenetic reconstruction of Jebram (a) is mainly based on cystid and muscle differentiation, whereas the work of Todd (b) has in particular included characters of fossil ctenostomes.

Figure 2 - Schematic overview of a retracted *Hislopia malayensis* zooid showing the main components of the polypide.

The orifice at the distal end of the zooid heads in to the vesitbulum, which is separated from the proximally situated tentacle sheath by the diaphragm. Within the tentacle sheath the tentacle crown, the lophophore is situated. The mouth opening is situated at the lophoral base and leads into the broad pharynx followed by an elongated, tube-like esophagus. The latter continues into the prominent proventriculus from where the digestive tract leads into the voluminous caecum. From the caecum the intestine leads into the anus, which terminates in the tentacle sheath.

Abbreviations: a – anus, at – atrium, cae – caecum, cw – cystid wall, d – diaphragm, es – esophagus, int – intestine, lb – lophophore base, o – orifice, pv – proventriculus, t – tentacle, ts – tentacle sheath, v – vestibulum, vw – vestibular wall.

Figure 3 - Maximum-intensity projections of confocal laserscanning image stacks providing an overview of the muscle system of a single zooid of *Hislopia malayensis*.

(a) F-actin staining. Associated with the cystid are solely the parietal muscles. Most muscles of the zooid are present at the distally situated aperture which continues proximally into the polypide. Prominent retractor muscles run from the distal end of the zooid to the lophophoral base. From the lophophoral base the digestive tract starts with the pharynx, followed by the

esophagus, which both possess mostly striated ring musculature. The adjoining proventriculus is the most prominent region of the digestive tract and possesses only smooth ring musculature. The caecum carries several distinct ring muscles and two longitudinal muscles at its proximal tip. The intestine possesses only smooth, longitudinal musculature. (b) Different zooid of H. malayensis similar as in (a) but also with cell nuclei stained with DAPI to provide a clearer picture on the cystid wall and its outlines. Also note the early bud on the right side. Abbreviations: am – apertural muscles, cae – caecum, clm – caecal longitudinal muscle, cp – communication pore, cw – cystid wall, ds – diaphragmatic sphincter, es – esophagus, (cardia), int – intestine, lb – lophophore base, o – orifice, pm – parietal muscles, pv – proventriculus, rm – retractor muscles, ts – tentacle sheath, v – vestibulum, vwm – vestibular wall musculature, yb – young bud. Scale bar in (a) 50µm, (b) 150µm

Figure 4 - Myoanatomical details of the polypide of *Hislopia malayensis*.

All but (a) are maximum-intensity projections of confocal laserscanning image stacks. (a) Volume rendering of the confocal stack of the myoanatomy of a dissected polypide showing most of the musculature of the digestive tract. The intestine is separated from the remaining gut by preparation. (b) Oblique view on the vestibular wall showing apertural muscles and vestibular wall muscles. Diagonal vestibular wall muscles can be dinstinguished on the basal side. (c) Detail of the tentacle musculature and muscular knot at the distal tips of the tentacles (open arrowheads). (d) Detail of the musculature of the esophagus showing thin longitudinal muscle fibres. (e) Musculature of the lophophoral basis and parts of the digestive tract. Asterisk marks a second pair of buccal dilatators. (f) Detail of the tentacle sheath musculature and apertural muscles. (g) Magnified view of the distal portion of the retractor muscles displayed in (e), showing their striated/banded appearance. (h) Detail of the muscular elements of a rosette plate between individual zooids. (i) Detail of the proventriculus and adjacent parts of the digestive tract musculature. Double-arrowheads marks fine longitudinal

muscle fibres in the caecum.

Abbreviations: a – anal area, am – apertural muscles, bd – buccal dilatators, brm . ring muscle at the lophophore base, cae – caecum, clm – caecal longitudinal muscle, ds – diaphragmatic sphincter, dvm – diagonal vestibular wall musculature, es – esophagus, int – intestine, lb – lophophore base, les – longitudinal musculature of the esophagus, llb – longitdunal muscles at the lophophore base, pm – parietal muscles, pv – proventriculus, rm – retractor muscles, tm – longitudinal tentacle muscles, ts – tentacle sheath, vlb – v-shaped muscles at the lophophore base, vwm – vestibular wall musculature, yb – young bud. Scale bar in (a) 100µm; (b, d, f) 75µm; (c) 25µm; (e, i) 50µm (h) 15µm.

Figure 5 - Myogenesis during the budding process of *Hislopia malayensis*.

Maximum-intensity projections of confocal laserscanning image stacks. (a) Early budding stage showing the polypide anlage and the first anlagen of the developing apertural and retractor muscles at its distal and proximal side, respectively. (b) More advanced budding stage with distinct tentacle anlagen (asterisk) where the cystid of the bud has widened distally and the apertural and retractor muscle anlagen have grown and are more pronounced. (c) Advanced budding stage where first anlagen of the digestive tract musculature have formed and also the parietal muscles are present laterally of the polypide anlage. Both, the apertural and the retractor muscle anlagen are most prominent. The former consists of loosely smooth muscle fibres, whereas the prospective retractor muscles have differentiated into two elongated muscle fibre bundles. (d) Almost completely developed zooid where most parts of the digestive tract, especially the most prominent proventriculus, are formed similar to the adult. Comparate to Fig. 3 and 4 for details on the musculature of adult specimens.

Abbreviations: am – apertural muscles, ama – aperutral muscle anlage, cae – caecum, ds – diaphragmatic sphincter, dt – distal tip of the cystid of the bud, dtm – developing digestive tract musculature, ep – epizooic organism, int – intestine, lb – lophophore base, pa – polypide

anlage, pma – parietal muscle anlage, pm – parietal muscles, pv – proventriculus, rm – retractor muscles, rma – retractor muscle anlage, vwm – vestibular wall musculature. Scale bar: 150µm.

Figure 6 - The serotonergic nervous system of adult specimens of *Hislopia malayensis*.

Maximum-intensity projections of confocal laserscanning image stacks (a) Lateral view of the lophophoral base showing the cerebral ganglion at the proximal side and parts of the circumpharyngeal nerves emanating to serotonergic perikarya at the lophophoral base. From the latter two nerves are distinguishable at the base of each adjacent tentacle (b) Top view of the serotonergic nervous system at the lophophoral base. The highest concentration of serotonin is found in the cerebral ganglion at the left side. At the oral side, three serotonergic perikarya are present on the circumpharyngeal nerve. Further nerves extend to serotonergic perikarya from the ganglion or the circumpharyngeal ring.

Abbreviations: cg – cerebral ganglion, cpn – circum-pharyngeal nerves, bp – perikarya at the lophophore base, obp – perikarya on the oral side of the lophophoral base, nt – nerves extending into the tentacles. Scale bar in (a) 50µm; (b) 30µm.

Figure 7 - Schematic representation of apertural areas of retracted ectoproct zooids with their associated muscles.

The distal zooidal part always shows upwards. Epidermal layers are drawn in solid lines, whereas coelomic epithelia are dashed. Tentacles are displayed in dark grey and the diaphragm is displayed in orange. In all retracted ectoprocts the cystid at the distal end invaginates into the vestibulum, bordered by the vestibular wall. The vesitbulum terminates proximally into the diaphragm, which in retracted zooids is constricted by a circular sphincter. It continues into the atrium which is bordered by the tentacle sheath enclosing the retracted

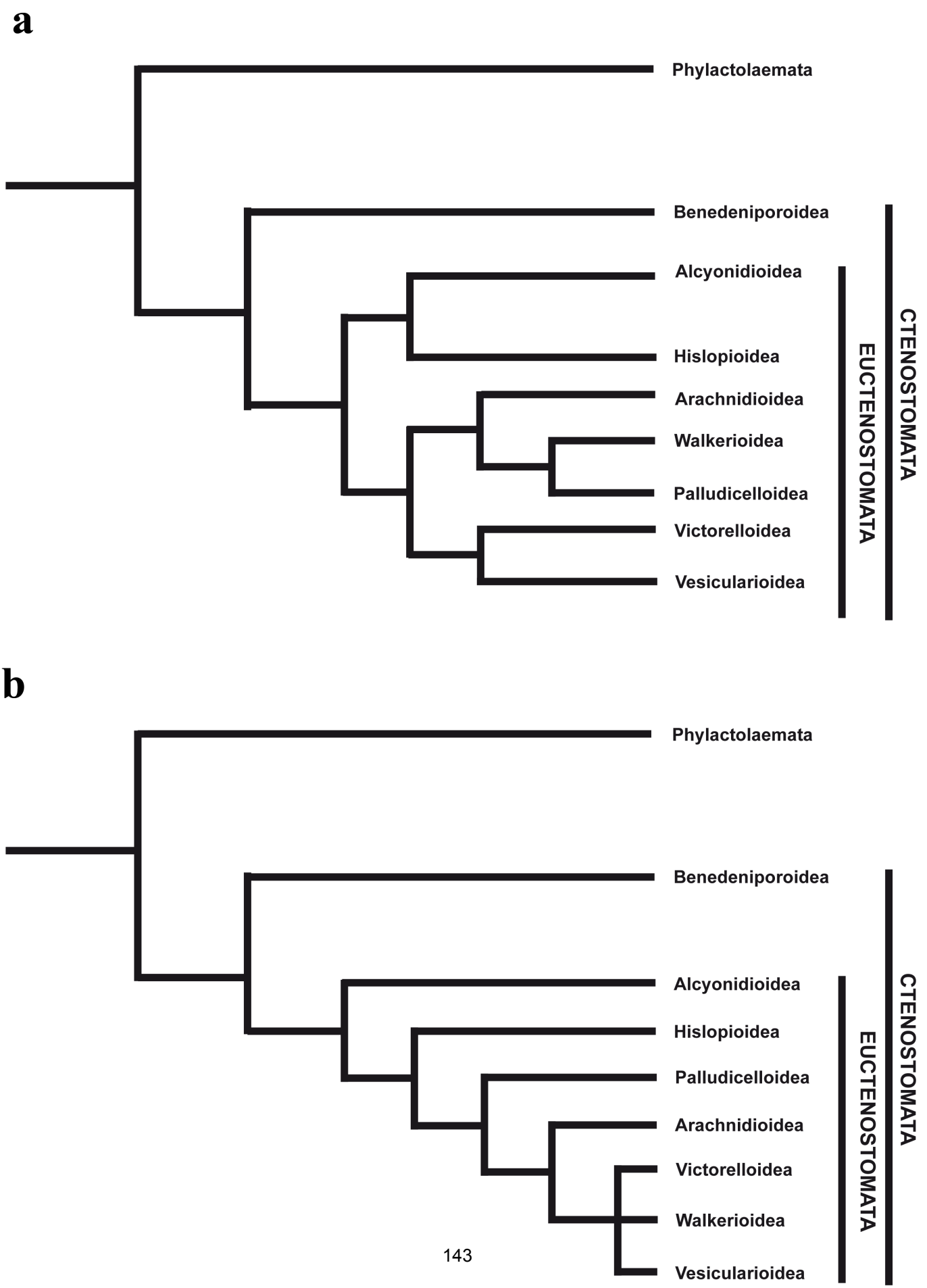
tentacles. (a + b) Apertural muscles in phylactolaemates consist of sparse vestibular dilatators that extend over the whole length of the vestibular wall. The duplicature bands are muscular peritoneal bands that either insert at the diaphragm in lophophodids or at the tentacle sheath in the remaining families. (c) The aperture in cyclstomes possesses main bundles of vestibular dilatators that extend from the distal body wall to the diaphragm. Topographically comparable to the duplicature bands of phylactolaemates (a + b) cyclostomes possess a ligamentous attachment organ built by peritoneal strands. (d – h) Ctenostomes (d) Paludicelloidean ctenostomes such as *Paludicella* show a simple condition that has retained both apertural muscle systems: homologues of phylactolaemate duplicature bands, commonly termed as parieto-vaginal bands and the vestibular dilatators which are prominently developed. The latter can be distinguished into a proximal, smaller portion, the parieto-diaphragmatic muscles and the distal parieto-vestibular muscles. (e + f) Ctenostomes forming elongated peristomes or stolons usually lack parieto-vaginal bands and show a strong separation of the parieto-diaphragmatic and distal parieto-vestibular musculature. (g) Alcyonidioidean and (h) hislopioidean ctenostomes commonly form dense encrusting colonies with the aperture shifted to the frontal side. Alcyonidioideans possess parieto-vaginal bands whereas hislopioideans lack them. The vestibular dilatators consists of a single portion along the vestibular wall in hislopioideans. In Alcyonidioideans they are separated into the parieto-diaphragmatic and distal vestibular musculature. (i) Cheilostomes possess parieto-vaginal bands and the parieto-diaphragmatic musculature as found in ctenostomes. The aperture in cheilostomes is closed by an a thickened lid, the operculum. Closure of the operculum is accomplished by the opercular occlusors which are most likely modified distal vestibular muscles .

Abbreviations: a – atrium, ao – attachment organ of cyclostomes, db – duplicature bands, dvm – distal vestibular muscles, ms – membranous sac of cyclostomes, oocl – operculum

occlusors, op – operculum, pdm – parieto-diaphragmatic muscles, pvb – parieto-vaginal bands, v – vestibulum, vd – vestibular dilatators,

Tables

Table 1 - List of cheilostome genera where parieto-vaginal bands are present.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Phylactolaemata
Benedeniporoidea
Alcyonidioidea
Hislopioidea
Arachnidioidea
Walkerioidea
Palludicelloidea
Victorelloidea
Vesicularioidea
EUCTENOSTOMATA
CTENOSTOMATA
b
Phylactolaemata
Benedeniporoidea
Alcyonidioidea
Hislopioidea
Palludicelloidea
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Victorelloidea
Walkerioidea
Vesicularioidea
EUCTENOSTOMATA
CTENOSTOMATA

Figure 1

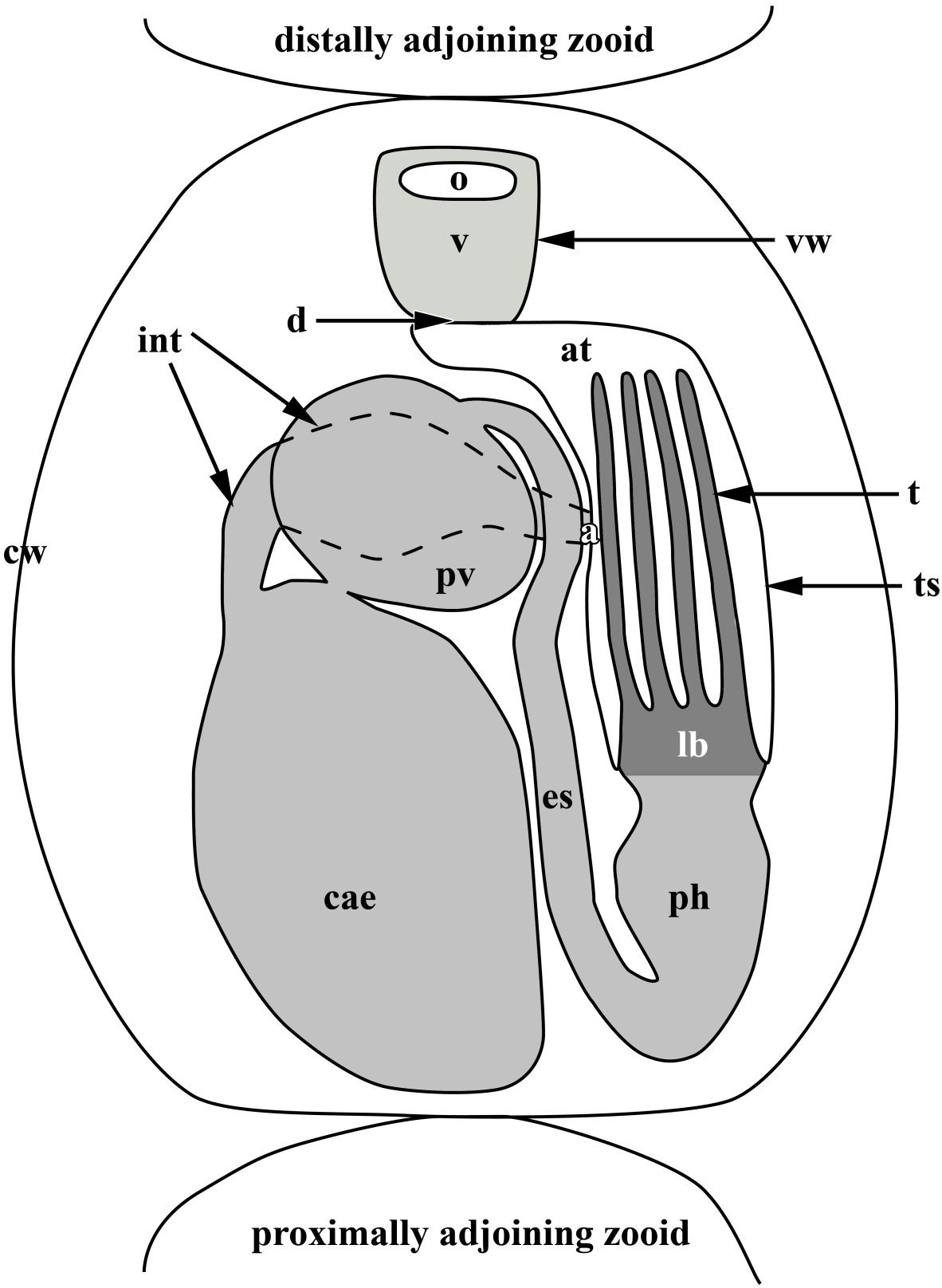
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Figure 2

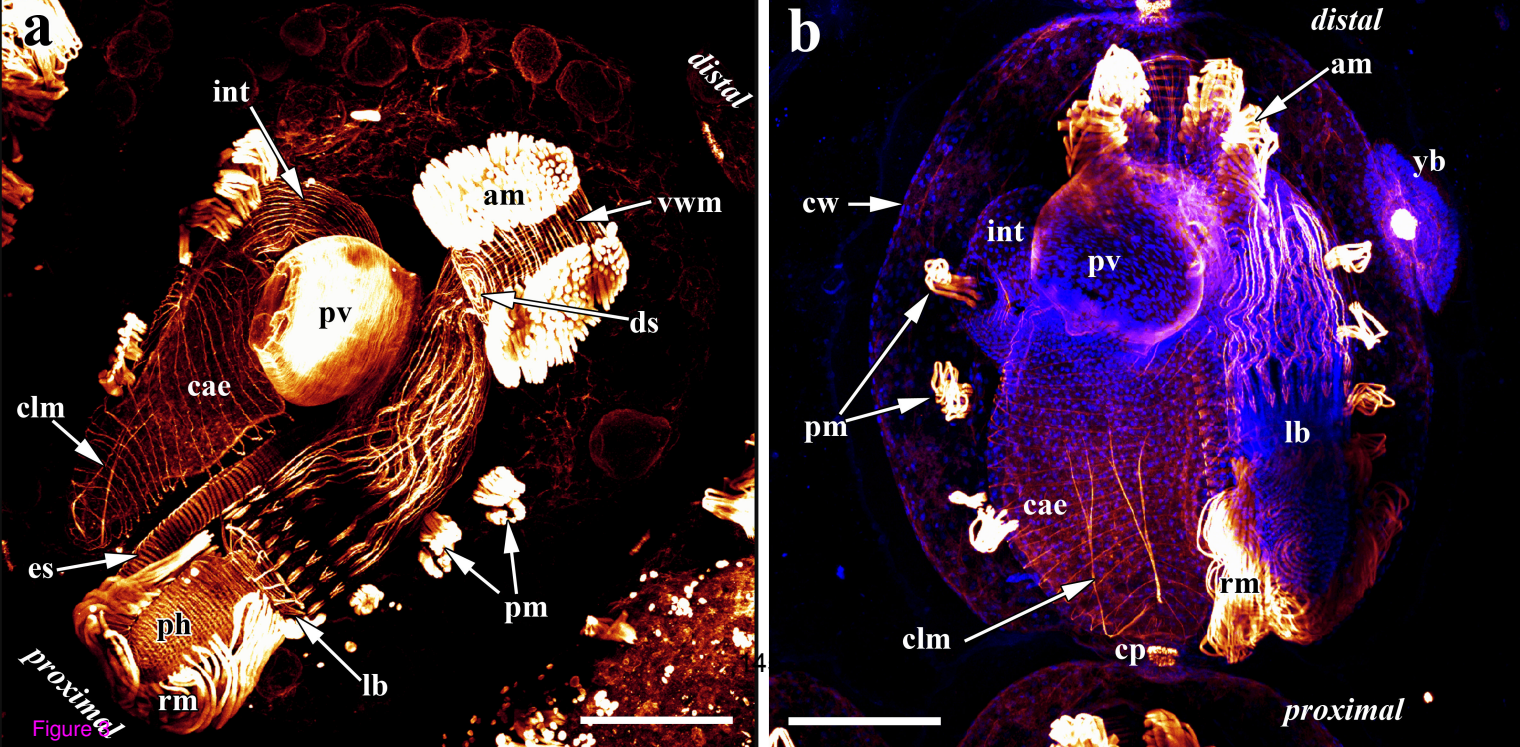
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Figure 4

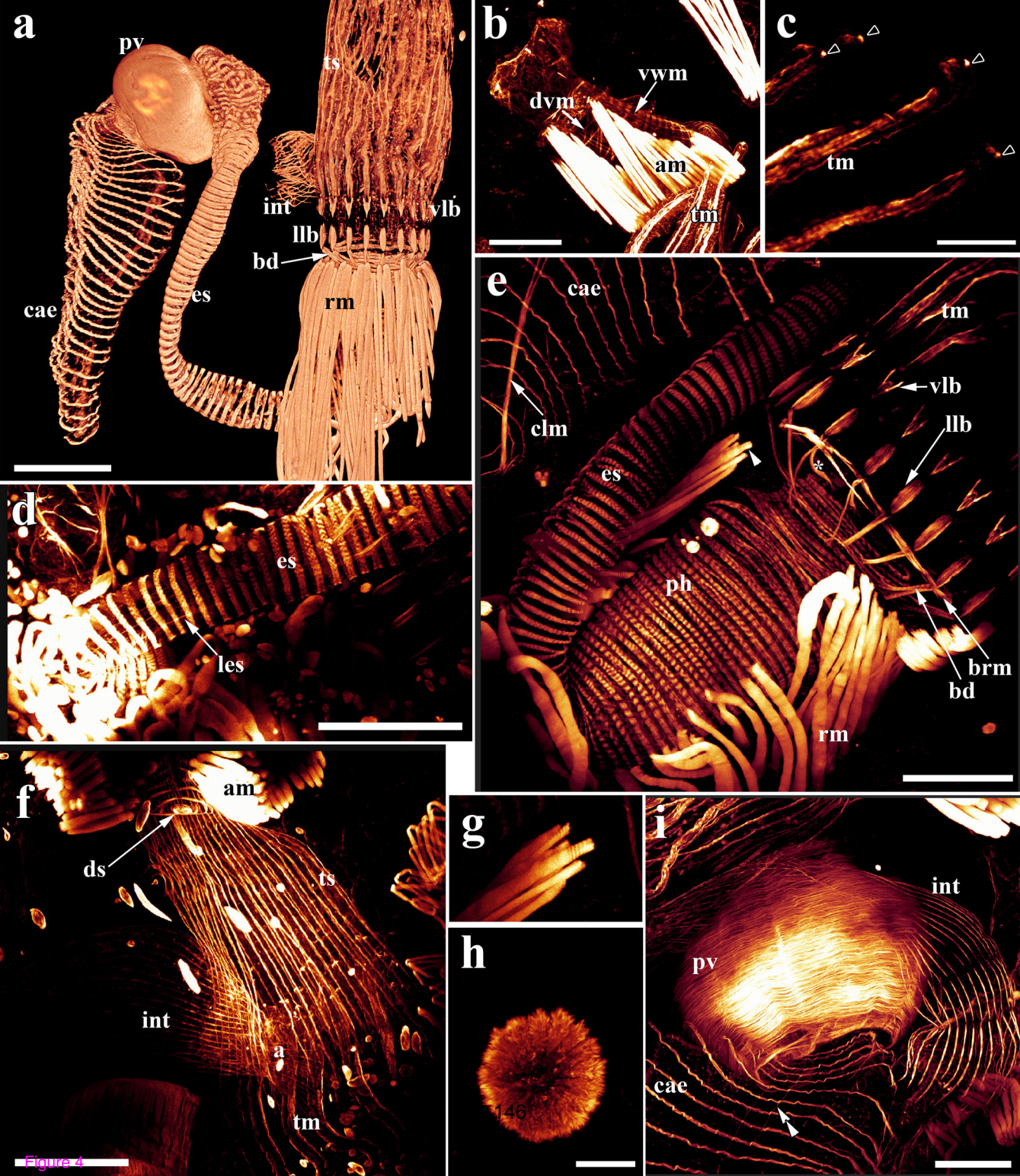
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Figure 4

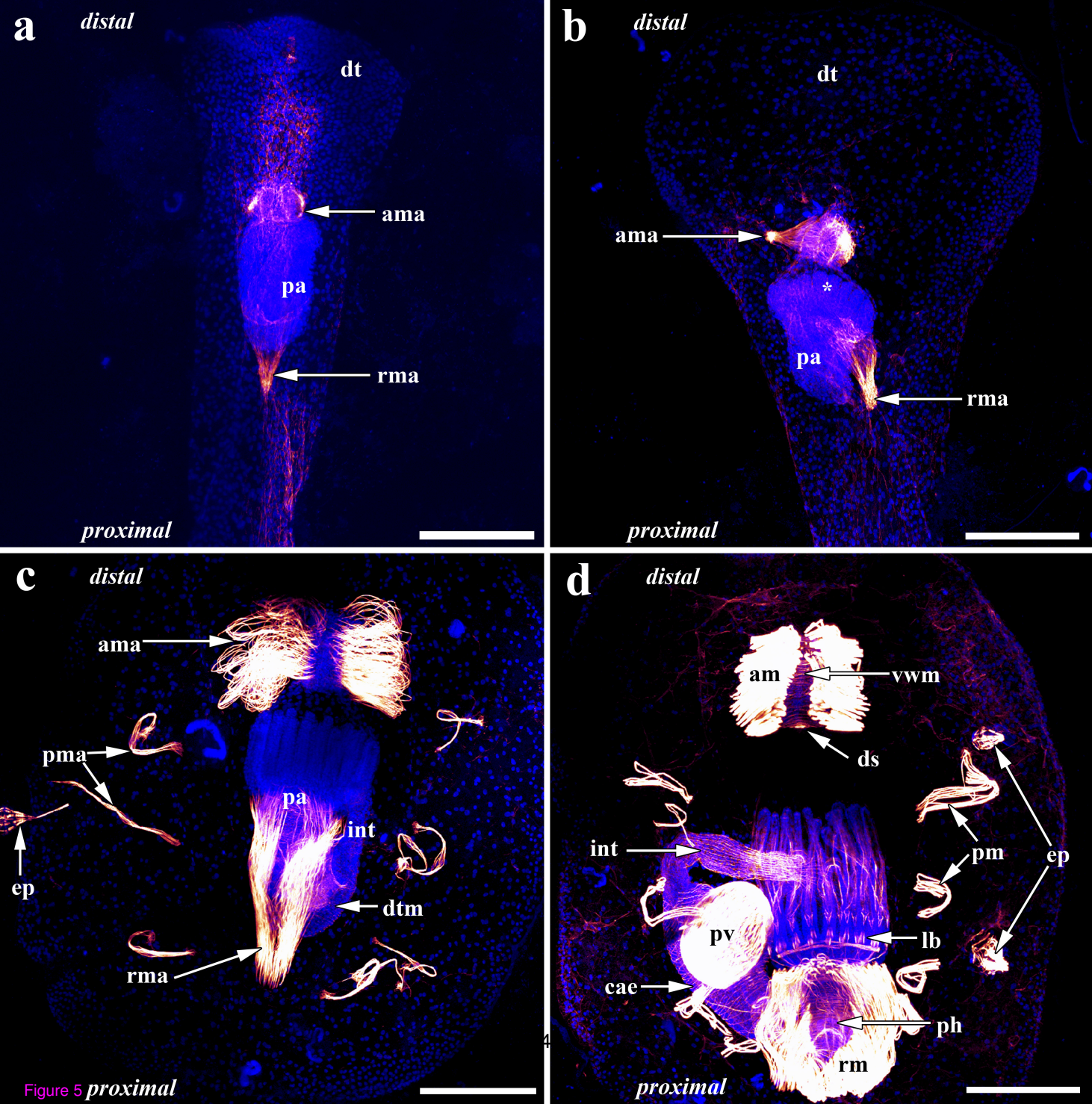
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Figure 5

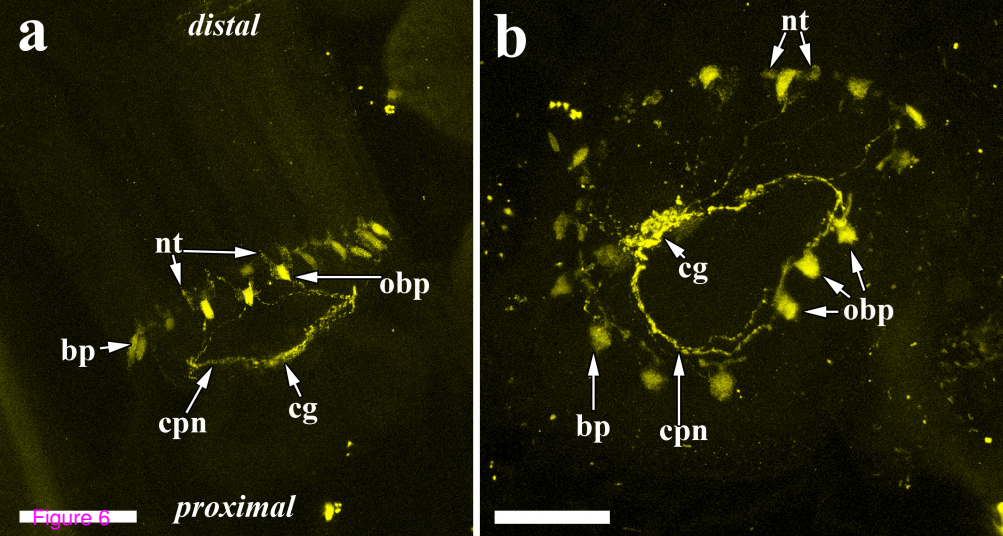
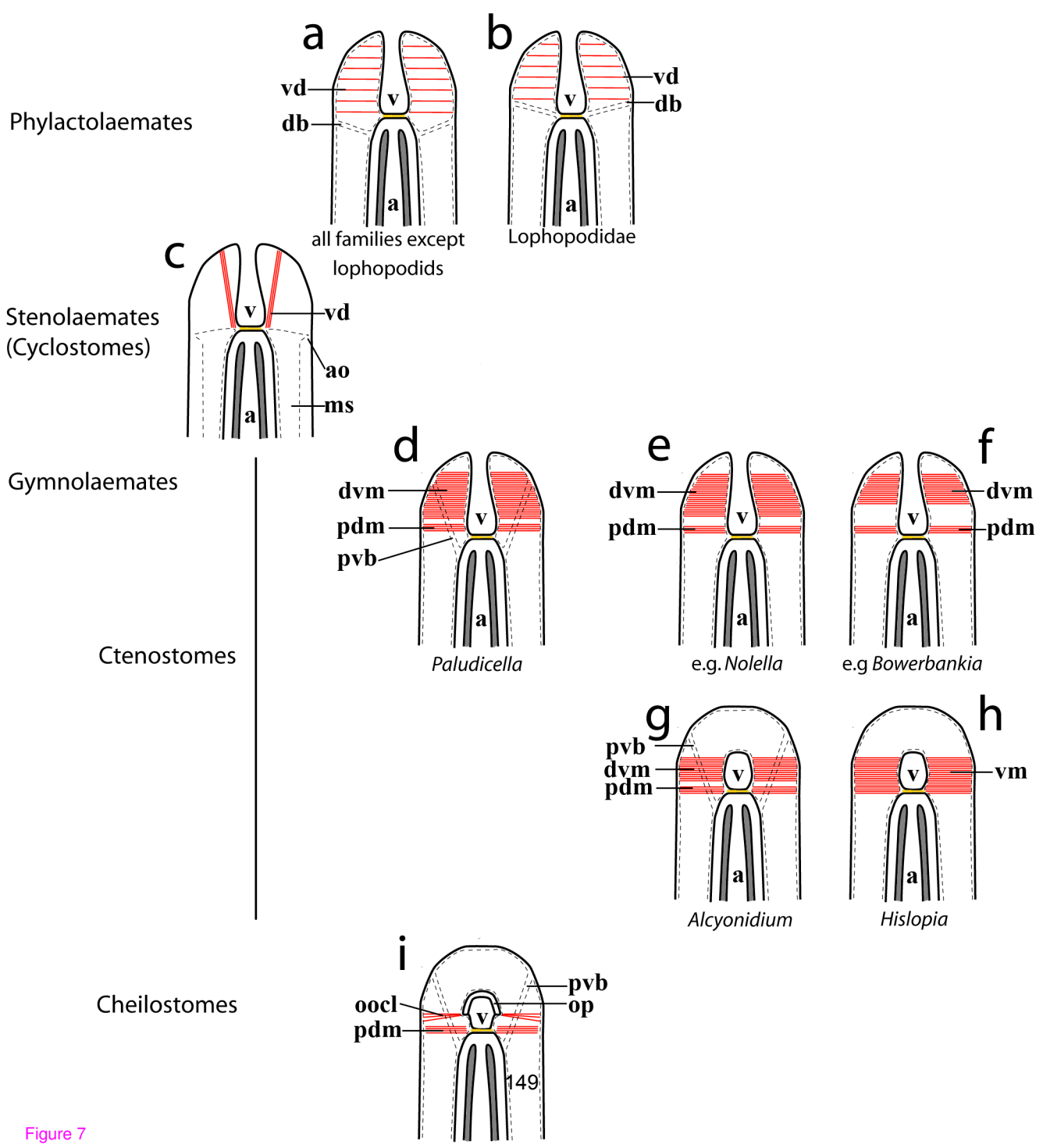


Figure 6


Phylactolaemates
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db
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all families except lophopodids
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Stenolaemates (Cyclostomes)
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vd
ao
ms
a
Gymnolaemates
Ctenostomes
d
dvm
pdm
pvb
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Paludicella
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dvm
pdm
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e.g. Nolella
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pdm
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Alcyonidium
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Hislopia
Cheilostomes
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Figure 7

Additional files provided with this submission:

Additional file 1: Table1.xls, 16K
http://www.frontiersinzoology.com/imedia/1291906450475865/supp1.xls

6. Summarizing discussion

In this work, two previously neglected morphological aspects in bryozoan research, the organogenesis during the budding process as well as the neuromuscular system of adult zooids, are analysed in a phylogenetic context for the first time. Some of the recent molecular analyses support bryozoan monophyly (e.g. Hausdorf et al. 2010, Nesnidal et al. 2010), whereas other don't (e.g. Helmkampf et al. 2008, Passamaneck & Halanych 2006). With few exceptions (e.g. Mundy et al. 1981), morphological analyses generally regard the Bryozoa as a monophyletic phylum. On a morphological basis, the largest discrepancies are between the solely freshwater-inhabiting Phylactolaemata and the remaining, chiefly marine taxa, Stenolaemata and Gymnolaemata (Mukai et al. 1997). The Phylactolaemata along with the few gymnolaemate representatives living in freshwater are considered as a relic groups that preserved basal features, because of the lower rate of competition of other filter-feeders (Jebram 1973). From a phylogenetic standpoint only few synapomorphies, such as brown bodies (remnants of decayed polypides) and the feeding mechanism, are present for the whole phylum (cf. Gruhl et al. 2009). Characters such as 'retractable lophophore' are questionable since at least the phoronid *Phoronis ovalis* is reported to be able to retract the forebody or lophophore quite well into the remaining proximal body (Harmer 1917). Consequently the latter character should be complemented to read 'lophophore retractable by prominent reatractor muscles traversing the coelomic cavity'. Other characters such as 'absence of nephridia' are in a strict phylogenetic sense not proper, since the absence of a character can never be regarded as an apomorphic feature of a clade, however, can aid in defining the latter. In the current thesis two characters – the serotonergic nervous system and the similarities in the organogenesis during budding – can be added to support bryozoan monophyly.

The serotonergic nervous system in the previously (Wanninger et al. 2005, Gruhl 2010) and currently, in this thesis, studied bryozoan representatives shows a principally similar condition. In all representatives it merely shows a strong concentration within the central

nervous system at the lophophoral base with few serotonergic nerves extending to the tentacle bases. Few variations exist among the studied representatives, but the general architecture is identical.

Concerning organogenesis during the budding process, the present thesis shows that the development of the polypide shows distinct similarities in the formation of the different organ systems. These include the following: 1. early polypide bud formation as a proliferation of epidermal cells bulging towards the peritoneal layer of the bud; 2. a two-layered vesicle-like early bud; 3. the central nervous system or ganglion forming as an invagination of the epidermal layer in between the prospective mouth and anal area; 4. the digestive tract mainly forming from an outpocketing of the prospective anal area that grows towards a comparatively small anlage of the foregut (pharynx and esophagus); and 5. the lophophore forming from two lateral anlagen that first fuse on the oral and afterwards on the anal side. In addition, the point where the anlage of the mid/hind-gut and the foregut meet is represented in all bryozoans by the cardiac valve. A comparison of different bryozoan species and superfamilies shows that its location is not identical in gymnolaemates which always possess an elongated tube-shaped gut connecting the pharynx with the caecum. With the current paucity of comparative data, it is more appropriate to consider the diet and the mode of digestion to be decisive on the variable location of the cardiac valve.

Deducing a basal set of bryozoan muscle systems remains difficult with the currently analysed phylactolaemate and ctenostome bryozoans. However, as chapter 5 shows particularly apertural muscles of bryozoans are very similar among the different clades and consist of two basic muscle sets: more distally located separate muscle fibres and proximally peritoneal bands supplied with longitudinal muscle fibres. Concerning the musculature of the digestive tract, there are clear differences, but also similarities between the Phylactolaemata and the Gymnolaemata. Most species of the former possess tightly packed, mostly cross-striated ring musculature, whereas the digestive tract musculature of the Gymnolaemata contains

longitudinal muscles that are more loosely arranged. Among the phylactolaemates, only *Asajirella gelatinosa*, a proposed basal representative of the Phylactolaemata, possesses few longitudinal muscles in the wall of the digestive tract. Accordingly, a set of longitudinal and ring musculature in the wall of the digestive tract currently appears as a basal bryozoan feature. Similarly, two longitudinal muscle bands in the tentacles of the lophophore seem to a basal bryozoan character. In contrast, the muscular system of the lophophoral base significantly differs when comparing the Phylactolaemata and Ctenostomata. However, this is not too surprising considering the general difference in the structure of the lophophore in these two clades.

As an additional aspect for future bryozoan research, structures of the nervous system at the lophophoral base including tentacle innervation as well as intertentacular pits appear promising for further analysis for comparative phylogenetic purposes on bryozoans. In chapter 4 some of these features were three-dimensionally reconstructed for the first time in a bryozoan. The same applies for the intertentacular pits at the complex lophophoral base which are described for the first time in a ctenostome. Similar structures were only reported in the cheilostome *Cryptosula pallasiana* (Gordon 1974) and their function remains unknown. It is likely that they are present in more if not all gymnolaemate species, but have escaped the attention of previous investigators.

In particular morphological studies on the soft-body parts of the Cyclostomata remain an essential clade for future studies. The latter are commonly regarded in close association with the Gymnolaemata, sometimes as sister group (e.g. Brien 1960) or even regarded to have originated from them (Todd 2000). Some of their morphological characters are intermediate between the Phylactolaemata and the Gymnolaemata, while few are similar to the Phylactolaemata: Organogenesis in the budding process of cyclostomes was only studied by Borg (1926) and Nielsen (1970), but is only poorly documented. In cyclostome bryozoans the polypide is formed first and the cystid later. This formation of buds is also found in the

Phylactolaemata, in contrast to budding of the Cteno- and Cheilostomata where the cystid is formed first and the polypide later (Reed 1991). Regarding the insertion of the retractor muscles on the polypide cyclostome bryozoans show an intermediate between the Phylactolaemata and the Gymnolaemata. In addition to their insertion at the lophophoral base, the retractor muscle fibres in the Phylactolaemata insert almost over the whole length of the orally situated digestive tract (i.e. pharynx, esophagus, cardia and parts of the stomach) (e.g. Hyatt 1865-1866; pers. observ.). Cyclostomes possess two larger bundles, one essentially inserting at the lophophoral base and a second which inserts at the cardia (Nielsen & Pedersen 1979). In the Gymnolaemata the retractors solely insert at the lophophoral base (e.g. Brien 1960, pers. observ.). Thus, a gradual decrease in the number of retractor muscle bundles is present in these clades. This is also reflected in the mode of protrusion of each clade. The Phylactolaemata possess body-wall musculature for protruding retracted polypides (Mukai et al. 1997), whereas the Cyclostomata possess annular ring muscles in their membranous sac that might have evolved from the ring musculature of the phylactolaemate bodywall (Nielsen & Pedersen 1979). The Gymnolaemata possess a series of so-called parietal muscles which traverse the coelomic cavity from the basal to the frontal side of the zooid (Taylor 1981). According to Jebram's evolutionary scenario (1986), these modes of protrusion reflect a subsequent or gradual economisation of the protrusion process. Along with the formation of calcareous protective cystid walls, this economisation of muscle groups used for protrusion and retraction also seems to be evidenced in the large diversity of the species-richest Cheilostomata.

So far the present thesis contributes to resolve internal relationships of bryozoans and to identify basal features. Comparisons with other potentially related lophotrochozoan phyla remains difficult because comparative data from these phyla remains very limited or is not existent or sometimes inaccurate and old. Most data for an often suggested sister-group of the Bryozoa is available from the Kamptozoa where several studies were conducted on budding

(cf. Nielsen 1971) and the neuro-muscular system (Wanninger 2004, Fuchs et al. 2006, Schwaha et al. 2010). Their budding process, however, only shows superficial resemblance to those of bryozoans, the myo-anatomy of the adults shows no resemblance at all to those of bryozoans and the serotonergic nervous system shows only slight similarities. However, based on similarities in the calyx morphology and trochophore larvae, adult Kamptozoa have been considered as neotenous trochophore that have lost their original metamorphosis (Emschermann 1995). Consequently, a direct comparison of the adult organisation of kamptozoans and bryozoans seems to be inappropriate and only larval characters should be considered for comparisons. The plesiomorphic creeping-type larva of the Kamptozoa, however, shows several synapomorphies with basal molluscs suggesting a close relationship between these two groups (Wanninger et al. 2007, Haszprunar & Wanninger 2008, Wanninger 2009). In principle, marine bryozoans exhibit two larval types, the planktotrophic cyphonautes and lecitotrophic coronate type of larva (Zimmer and Woollacott 1977, Taylor 1988). Both types are considered to be highly specialized forms, with the coronate type to have evolved multiple times (Taylor 1988, Temkin & Zimmer 2002). The Phylactolaeamta possess larvae – often referred to as ‘swimming colonies’ – that are considered highly derived and difficult to compare with the remaining larval types (Reed 1991, Gruhl 2010). Consequently, identifying basal characters in bryozoan larvae remains difficult. Some morphology traits of bryozoan larvae resemble more those of other spiralian taxa than to other ‘lophophorates’(Gruhl 2009). Some authors argued for a close resemblance to kamptozoan larvae (Nielsen 1971). Still, the large discrepancies such as for example different cleavage modes, particle-collection mechanisms or coelomic vs. acoelomate condition between the Kamptozoa and the Bryozoa represents a rather unparsimonious solution for a sister-group relationship.

As an outlook of this thesis, it can be said that the aforementioned Cyclostomata seem most important for clarifying internal relationships of the Bryozoa. Concerning the placement of

the phylum within the Bilateria, I regard the analysis of other adult Brachiopoda and Phoronida with modern immunocytochemical methods to be crucial for gaining more insight into their possible relationship to the Bryozoa. In particular *Phoronis ovalis* requires more attention since it shows several morphological characters such as budding, a retractable fore-body/lophophore and the infundibuliform valve reminiscent of bryozoans.

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7. Summary

The phylogenetic position of bryozoans has been in dispute for decades. Traditionally they were considered related to the Brachiopoda and Phoronida as ‘Tentaculata’ or Lophophorata. More recent analyses found no evidence for this relationship and a sister-group of bryozoans is currently wanting. Molecular phylogenetic studies have so far not been able to aid very much for this cause and recent morphological studies with phylogenetic background are few. In addition to the questionable position of the whole phylum within the Bilateria, internal relationships of the three different clades of bryozoans are not clear as well. In this thesis I analyse two morphological traits of bryozoans previously not studied or considered in need for revision: The organogenesis during the budding process and the myoanatomy and serotonergic nervous system of adult specimens. Both of these characters were analysed in representatives of the Phylactolaemata and the Ctenostomata. From a phylogenetic point of view the latter two clades are the most promising ones for gaining more insight into their basal evolution. The current results add new characters such as trends in the formation of organ systems during the asexual budding process and the distribution of the serotonergic nervous system that support the monophyly of the Bryozoa. Concerning muscular systems of bryozoans, the apertural muscles show high similarity within Bryozoa and always consist of two sets of muscles. Gymnolaemates and Phylactolaemates show clear differences within their digestive tract musculature, the former showing smooth and longitudinal muscles to a much greater extent than the latter. The complex musculature at the lophophoral base appears promising for inferring phylogenetic relationships, but sufficient comparative data are currently lacking. Comparing the analysed traits with potentially related phyla remains difficult because of a lack of data. From the commonly suggested candidate phyla, the Kamptozoa are the best studied phylum. However, their asexual development only superficially resembles those of bryozoans and their neuro-muscular system is significantly different.

8. Zusammenfassung

Seit Jahrzehnten steht die phylogenetische Stellung der Bryozoen unter Debatte. Traditionell wurden sie gemeinsam mit den Brachiopoden und Phoroniden als ‚Tentakulata' oder ‚Lophophorata' zusammengefasst. Neuere Analysen konnten diese Beziehung nicht bestätigen und eine Schwestergruppe fehlt derzeit. Bisher konnten molekulare phylogenetische Analysen zu dieser Thematik kaum etwas beisteuern und nur wenige neuere morphologische Arbeiten mit phylogenetischer Fragestellung sind vorhanden. Zusätzlich zu der fragwürdigen Stellung des Stammes innerhalb der Bilateria, sind auch die internen Beziehungen der drei Bryozoenklassen zueinander nicht geklärt. In dieser Arbeit wurden zwei morphologische Merkmale von Bryozoen analysiert, die bisher nicht untersucht wurden oder revidiert werden müssen: Die Organogenese während des Knospungsvorgangs und sowohl die Myoanatomie als auch das serotonerge Nervensystem adulter Vertreter. Diese beiden Charaktere wurden bei Vertretern der Phylactolaemata und Ctenostomata untersucht. Von einem phylogenetischen Blickwinkel stellen diese beiden Gruppen die vielversprechendtsen dar. Die gegenwärtigen Ergebnisse liefern neue Merkmale, wie zum Beispiel Ähnlichkeiten während der Bildung der Organe im asexuellen Knospungsvorgang und die Verteilung des serotonergen Nervensystems, welche die Monophylie der Bryozoen untermauern. Bezüglich der Muskelsysteme der Bryozoen zeigen vor allem die Apertur-Muskeln eine große Ähnlichkeit innerhalb der Bryozoen und bestehen immer aus zwei Gruppen von Muskeln. Die Gymnolaemen und Phylactolaemen besitzen deutliche Unterschiede in ihrer Darmtraktmuskulatur, wobei Erstere wesentlich mehr glatte Muskulatur aufweisen als Letztere. Die komplizierte Muskulatur im Bereich der Lophophorbasis scheint vielversprechend für phylogenetische Rückschlüsse zu sein, jedoch liegt ein Mangel an vergleichbaren Daten vor. Ein Vergleich der in dieser Arbeit untersuchten Merkmale mit jenen anderer, möglicherweise verwandten Stämmen erweist sich als schwierig, da ebenfalls vergleichbare Daten fehlen. Von all den Stämmen, die öfters in nähere Verwandschaft mit

Bryozoen gezogen werden, sind die Kamptozoen die am besten untersuchten. Deren asexuelle Entwicklung zeigt jedoch nur oberflächliche Ähnlichkeiten und deren Neuro-muskuläres System weist deutliche Unterschiede auf.

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10. Curriculum vitae

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Date of birth: 30.7.1983
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Education

Summersemester 2008	Beginning of the PhD thesis „ Asexual organogenesis and neuro-muscular system in basal Bryozoans.“ at the University of Vienna
10/2001 – 05/09/2007	Study of Biology with the focus on Zoology at the University of Vienna, Diploma thesis titled „Zur Embryonalentwicklung der Milbe *Sancassania berlesei*“ (On the embryonic development of the mite Sancassania berlesei) under supervision of Prof. Dr. Manfred Walzl, graduated with distinction
09/1993 – 10/2001	public school Wasagasse 10, 1090 Wien Graduation with Matura on 9.10.2001
09/1989 – 06/1993	grammar school St.Elisabeth Obere Augartenstraße 34, 1020 Wien

Working experience

Since July 2010	research assistant within the FWF project ‘*Evolution of matrotrophy and placental analogues in Bryozoa*’ granted to A. Ostrovsky
since 2008	Lecturer at the University of Vienna, Department of theoretical biology, Morphology Section: Summer 2008: - Practical courses in zoology, part 2 - Advanced practical course on comparative embryology Winter 2008 - Practical Courses in Zoology, part 1

- Imaging and visualization in developmental biology - Principles and applications, including 3D Methods
- Microscopic anatomy of vertebrates

Summer 2009:
- Practical courses in zoology, part 2

Winter 2009
- Practical courses in zoology, part 1
- Microscopic anatomy of vertebrates
- Advanced practical course on comparative anatomy of vertebrates

Summer 2010:
- Practical courses in zoology, part 2
- Advanced practical course on comparative embryology

Winter 2010:
- Practical courses in zoology, part 1
- Microscopic anatomy of vertebrates
- Projectcourse for functional anatomy of vertebrates

01/2008 – 12/2008 Member of the laboratory staff of the histological research at the „Bernhard Gottlieb“ Dental clinic, Medical university-Vienna

06/2006 – 01/2008 Machine service at the harness racing track in Krieau and Baden

2005 - 2007 teaching assistant at the University of Vienna, Department of theoretical biology:

Summer 2005:
Practical courses in Zoology B

Winter 2005/2006:
Morphological practical course on vertebrates

Summer 2006:
Practical courses in Zoology B
Practical course on comparative embryology

Winter 2006/2007:
Comparative anatomy for oecologists

Summer 2007:
Practical courses in Zoology B

Winter 2007:
Morphological practical course on vertebrates
Comparative anatomy for oecologists

July 1998, August 1999 cable laying for the “Leopold Jordan Systemtechnik” company

Personal Abilities:

Languages

English
French, basic knowledge

Qualification

Lightmicroscopy: Brightfield, Darkfiled, Differential-Interference contrast microscopy (Nomarski), Fluorescence microscopy (epifluorescence and confocal laser scanning microscopy including immunolabeling).
Embedding techniques: paraffin, Technovit, resin (Araldit, Epon, Spurr, Ultra-low viscosity resin, AGAR – Low viscosity resin),
Sectioning skills: paraffin, resin (semithin, ultrathin), cryostat
Digital Photography: Macro- and Microphotography, digital image editing
Electronmicroscopy: Scanning electron microscopy (including BSE–detection), Transmissionselectronmicroscopy, experiences with ESEM, coating of slot-grids, qualitative and quantitative EDX-measurement, diverse contrasting techniques (Thiery test, Short-Thiery, etc.)
Miscellaneous: Micropreparation, histological staining techniques, Microwave-praeparations, clearing and staining of bone and cartilage, basic knowledge of molecular techniques (DNA-extraction, PCR, cloning)

Others skills

Computer applications: MS-Office (Word, Excel, Powerpoint), Adobe Photoshop, Adobe Premiere, Adobe Illustrator, Amira (3D-reconstructionen), Adobe Acrobat 3D, basic knowledge of Blender, EndNote, basic knowledge of hardware-specifics

Drivers license
Rearing and keeping animals
Education and teaching of students

Professional memberships

Gesellschaft für biologische Systematik (Society for biological Systematics)
Deutsche Zoologische Gesellschaft (DZG)
International Bryozoology Association (IBA)
International Society for Invertebrate Morphology (ISIM)

Grants and Prizes

- travel grant from the ÖFG (Austrian Research Community) (300€)
- Short Research Stays Abroad (KWA) of the University of Vienna (650€)
- Larwood Prize for Best Student Poster at the 15th International Bryozoology Association Conference at Kiel, Germany, August 2-6, 2010

Research visits/field trips

- February 2009: Sampling freshwater bryozoans in Thailand in cooperation with Timothy Wood, Department of Environmental Science, Kasetsart University, Bangkok

- April 2009: Immunohistochemical fluorescence staining of freshwater bryozoans and kamptozoans in cooperation with Andreas Wanninger, Research Group for Comparative Zoology at the University Copenhagen,

- June 2009: Participation in the Bryozoan workshop in Kristineberg, Sven Lovén Centre, Sweden, hosted by Matthias Obst

- September 2009: Immunohistochemical fluorescence staining of bryozoans and kamptozoans in cooperation with Andreas Wanninger, Research Group for Comparative Zoology at the University Copenhagen

- May 2010: Immunohistochemical fluorescence staining of bryozoans and kamptozoans in cooperation with Andreas Wanninger, Research Group for Comparative Zoology at the University Copenhagen

- June 2010: Sampling bryozoans at Orkney Islands, Scotland (UK)

- July 2010: Sampling bryozoans and kamptozoans at Rovinj marine biological station, Croatia

Organisation of scientific meetings

Co-organiser (together with Emanuel Redl) of the 3. Graduiertenforum der Fachgruppe Morphologie der DZG, 21-24 October 2010 Vienna, Austria

Co-supervision of diploma thesis

Szalai Noemi Katalin (2008)
Beschreibung der Knochenqualität und histomorphometrische Charakterisierung der Trabekelarchitektur im durch Micromovements behandelten Frakturkallus

International and National Cooperations

Dr. Emmy Wöss
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Dr. Andrey Ostrovsky
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Mag. Emanuel Redl
Department of Evolutionary Biology, Emerging Focus Molecular Phylogenetics, University of Vienna, Austria

Mag. Stephan Handschuh
Department of theoretical biology, University of Vienna, Austria

Assoc. Prof. Andreas Wanninger
Research Group for Comparative Zoology Institute of Biology, University of Copenhagen, Denmark

Prof. Timothy Wood
Department of Biological Sciences, Wright State University, USA

Publications

Peer reviewed

11. Schwaha T., Wanninger A. (to be submitted): Myoanatomy and serotonerg nervous system of plumatellid and fredericellid Phylactolaemata (Lophotrochozoa, Ectoprocta).

10. Schwaha T., Wood T.S. (submitted): Organogenesis in the budding process of Hislopia malayensis Annandale, 1916 (Bryozoa, Ctenostomata). *BMC Developmental Biology*

9. LECHNER W., HEISS E., **SCHWAHA T.**, GLÖSMANN M., LADICH F. (submitted) Ontogenetic development of Weberian Ossicles and hearing abilities in the African Bullhead Catfish. *PlosONE*

8. HEISS E., NATCHEV N., **SCHWAHA T.**, SALABERGER D., LEMELL P., BEISSER C., WEISGRAM J. (submitted): The tortoise is different: On the oropharyngeal morphology in the basal testudinid *Manouria emys emys*.

7. SCHWAHA T., WOOD T.S., WANNINGER A. (in revision): Myoanatomy and serotonergic nervous system of the ctenostome *Hislopia malayensis*: Evolutionary trends in bodyplan patterning of Ectoprocta. *Frontiers in Zoology*

6. SCHWAHA T., HANDSCHUH S., REDL E., WALZL M.G (2011): Organogenesis in the budding process of the freshwater bryozoan *Cristatella mucedo* Cuvier, 1798 (Bryozoa, Phylactolaemata). *Journal of Morphology*, 272:320-341.

5. CYRAN N., KLINGER L., SCOTT R., GRIFFITHS C., **SCHWAHA T.**, ZHEDEN V., PLOSCZANSKI L., VON BYERN J. (2010): Characterization of the adhesive systems in cephalopods. In: Von Byern J. & Grunwald I. (Eds): Biological Adhesive Systems: From Nature to Technical and Medical Applications. Springer Wien, New York. Pp. 54-82.

4. HANDSCHUH S., **SCHWAHA T.**, METSCHER B. (2010): Showing Their True Colors: A Novel Approach to Volume Rendering from Serial Sections. *BMC Developmental Biology*, 10:41.

3. SCHWAHA T., WOOD T.S., WANNINGER A. (2010): Trapper in freshwater: The internal anatomy of the entoproct *Loxosomatoides sirindhornae*. *Frontiers in Zoology*, 7:7

2. SCHWAHA T., GITH R., WALZL M.G. (2008): The nutritive chamber in the ovaries of astigmatic mites. *Soil Organisms*, 80 (2): 249-259

1. HANDSCHUH S., **SCHWAHA T.**, NESZI N. Z., WALZL M. G., WOESS E. R. (2008): Advantages of 3D-Reconstruction in bryozoan development research: Tissue formation in germinating statoblasts of *Plumatella fungosa* (Pallas, 1768) (Plumatellidae, Phylactolaemata). In: *Bryozoan Studies 2007*. Proceedings of the 14th International Bryozoology Association Conference, Boone, North Carolina. Hageman S.J., Key M.M. & Winston J.E. (eds.) *Virginia Museum of Natural History Special Publication* 15: 49-55**.**

Other publications

4. NATCHEV N., HEISS E., LEMELL P., KUMMER S., **SCHWAHA T.**, WEISGRAM J. (2009): Kinematical analysis of animal performance – the challenge to frame rate in high-speed cinematography. Biotechnology & Biotechnological Equipment, 23: 117-120.

3. HEISS E., WOLFRAM M.T., REDL E., **SCHWAHA T.**, WEISGRAM J. (2008): Defense mechanisms of *Pleurodeles waltl* (Amphibia, Urodela) against predators. *Annual of the Konstantin Preslavsky Univeristy, Shumen*, 18, B6: 99-108

2. REDL E., **SCHWAHA T.**, HANDSCHUH S., HEISS E., WOLFRAM M.T. (2008): Reconstruction techniques in morphological and developmental research. Part I: Historical overview, traditional methods and general principles. *Annual of the Konstantin Preslavsky Univeristy, Shumen*, 18, B6: 201-203

1. SCHWAHA T., REDL E., HANDSCHUH S., WOLFRAM M.T., HEISS E. (2008): Reconstruction techniques in morphological and developmental research. Part II: computergenerated 3D – reconstruction. *Annual of the Konstantin Preslavsky Univeristy, Shumen*, 18, B6: 214-221

Published Abstracts

4. REDL E., **SCHWAHA T.**, HANDSCHUH S., SALVINI-PLAWEN L. (2008): The ctenidial retractors of Caudofoveata (Mollusca): a comparative study. *Journal of Morphology*, 269, (12): 1474

3. SIDERITS D., HANDSCHUH S., REDL E., **SCHWAHA T.**, METSCHER B. (2008): X-ray microtomography and histological based 3-D reconstruction in invertebrate morphology: comparing different techniques of visualisation. *Journal of Morphology*, 269, (12): 1474

2. SCHWAHA T., HANDSCHUH S., REDL E., WALZL M.G. (2008): Asexual development in phylactolaemate bryozoans. *Journal of Morphology*, 269, (12): 1475

1. HANDSCHUH S., **SCHWAHA T.**, REDL E., WALZL M.G. (2008): The larva of *Plumatella fungosa* (Pallas, 1768) (Bryozoa, Phylactolaemata): a 3D perspective. *Journal of Morphology*, 269, (12): 1490

Congress contributions

25. LECHNER W., HEISS E., **SCHWAHA T.**, GLÖSMANN M., LADICH F. (**2010**): Ontogeny of the Weberian apparatus and hearing abilities in the African claroteid catfish *Lophiobagrus cyclurus*. *9th International Congress of Vertebrate Morphology, Punta del Este (Uruguay).*

24. SCHWAHA T., WOOD T.S., WANNINGER A. (**2010**): Larval morphology of the freshwater entoproct *Loxosomatoides sirindhornae*, Wood 2005. (poster) *15th Meeting of the International Bryozoology Association, Kiel (Germany)*

23. SCHWAHA T., WANNINGER A. (**2010**): Ectoproct muscle systems and their significance for phylogenetic considerations. (talk) *15th Meeting of the International Bryozoology Association, Kiel (Germany)*

22. REDL E., **SCHWAHA T.,** TODT C., SCHANDER C., SALVINI-PLAWEN L. (**2010**): The nervous system of *Scutopus ventrolineatus* (Caudofoveata): A study using histology and 3D-reconstruction. *17th World congress of Malacology, Phuket (Thailand)*

21. HANDSCHUH S., SCHWAHA T., METSCHER B. (**2010**): Volume-Visualisierungen basierend auf histologischen Schnitten: Ein Zugang zu einer drei-dimensionalen Histologie. 3. Graduiertenforum Morphologie (DZG), Wien (Austria)

20. LECHNER W., HEISS E., **SCHWAHA T.**, GLÖSMANN M., LADICH F. (**2010**): Ontogenetische Entwicklung der Weberschen Knöchelchen und des Hörvermögens beim Tanganjika-Stachelwels *Lophiobagrus cyclurus.* 3. Graduiertenforum Morphologie (DZG), Wien (Austria)

19. HEISS E., NATCHEV N., **SCHWAHA T.**, WEISGRAM J. (**2010**): Lieber morgen leben als heute sterben: Über die ungewöhnliche Verteidigungsstrategie des Spanischen Rippenmolches, *Pleurodeles waltl* (Amphibia, Salamandridae). 3. Graduiertenforum Morphologie (DZG), Wien (Austria)

18. NATCHEV N., HEISS E., HANDSCHUH S., WEISGRAM J., **SCHWAHA T.** (**2010**): Defensive response in the ctenostome bryozoan *Paludicella articulata*: First kinematics of polypide retraction and associated muscles. 3. Graduiertenforum Morphologie (DZG), Wien (Austria)

17. SCHWAHA T., HANDSCHUH S., REDL E., WALZL M.G. (**2009**): Development of the bud in *Cristatella mucedo*. (talk) *Larwood Meeting 2009, Oslo (Norway)*

16. SCHWAHA T. (**2009**): Morphologische Untersuchungen an Bryozoen (talk). *2. Graduiertenforum Morphologie (DZG), Greifswald (Germany)*

15. REDL E., **SCHWAHA T.**, HANDSCHUH S., METSCHER B., TODT C., SCHANDER C., SALVINI-PLAWEN L. (**2009**): Vergleichend-anatomische und organogenetische Studien an aplacophoren Mollusken. *2. Graduiertenforum Morphologie (DZG), Greifswald (Germany)*

14. REDL E., **SCHWAHA T.**, HANDSCHUH S., SALVINI-PLAWEN L. (**2009**): In search of phylogenetic signals in the soft body of Caudofoveata (Mollusca). *Third annual meeting of NOBIS Austria, Vienna (Austria)*

13. SCHWAHA T., HANDSCHUH S., REDL E., METSCHER B., WALZL M.G. (**2008**): The affinities of bryozoans – searching for phylogenetic characters with new techniques. (talk) *Larwood Meeting 2008, Vienna (Austria)*

12. REDL E., **SCHWAHA T.**, HANDSCHUH S., SALVINI-PLAWEN L. (**2008**): The ctenidial retractors of Caudofoveata (Mollusca): a comparative study. *First international congress on invertebrate morphology. Copenhagen 2008*

11. SIDERITS D., HANDSCHUH S., REDL E., **SCHWAHA T.**, METSCHER B. (**2008**): X-ray microtomography and histological based 3-D reconstruction in invertebrate morphology: comparing different techniques of visualisation. *First international congress on invertebrate morphology. Copenhagen 2008*

10. SCHWAHA T., HANDSCHUH S., REDL E., WALZL M.G. (**2008**): Asexual development in phylactolaemate bryozoans. (talk) *First international congress on invertebrate morphology. Copenhagen 2008*

9. HANDSCHUH S., **SCHWAHA T.**, REDL E., WALZL M.G. (2008): The larva of *Plumatella fungosa* (Pallas, 1768) (Bryozoa, Phylactolaemata): a 3D perspective. (poster) *First international congress on invertebrate morphology, Copenhagen 2008*

8. SCHWAHA T., REDL E. (2008): The position of the bryozoans in the animal kingdom – past and present. (talk) *Natural Sciences 2008, Scientific Conference, Varna (Bulgaria)*

7. REDL E., **SCHWAHA T.** (**2008**): The position of the aplacophorans in the animal kingdom – past and present. *Natural Sciences 2008, Scientific Conference, Varna (Bulgaria)*

6. HANDSCHUH S., **SCHWAHA T.**, NESZI N. Z., WALZL M.G., WOESS E.R. (**2007**): Advantages of 3D-Reconstruction in bryozoan development research: Tissue formation in germinating statoblasts of *Plumatella fungosa* (Pallas, 1768) (Plumatellidae, Phylactolaemata). *14th Meeting of the International Bryozoology Association, Boone, North Carolina (USA).*

5. SCHWAHA T., WALZL M.G., GITH R. (**2007**): Die zentrale Nährkammer im Ovar der astigmaten Milben. (The central nutritive chamber in the ovaries of astigmatic mites) (talk). *6. Milbenkundliches Kolloquium, Kiel (Germany).*

4. HEISS E., WOLFRAM M.T., REDL E., **SCHWAHA T.**, WEISGRAM J. (**2007**): Defense mechanisms of *Pleurodeles waltl* (Amphibia, Urodela) against predators. *Natural Sciences 2007, Scientific Conference, Varna (Bulgaria)*

3. REDL E., **SCHWAHA T.**, HANDSCHUH S., HEISS E., WOLFRAM M.T. (**2007**): Reconstruction techniques in morphological and developmental research. Part I: Historical overview, traditional methods and general principles. *Natural Sciences 2007, Scientific Conference, Varna (Bulgaria)*

2. SCHWAHA T., REDL E., HANDSCHUH S., WOLFRAM M.T., HEISS E. (**2007**): Reconstruction techniques in morphological and developmental research. Part II: computergenerated 3D – reconstruction. (talk) *Natural Sciences 2007, Scientific Conference, Varna (Bulgaria)*

1. SCHWAHA T., WALZL M. G. (**2006**): The early development of *Sancassania berlesei* (Michael, 1903) (Acaridida, Acaridae) (poster). *12th International Congress of Acarology; Amsterdam, (Netherlands)*