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„Organogenesis in the budding process of the freshwater bryozoans *Fredericella sultana* and *Plumatella casmiana* (Bryozoa, Phylactolaemata)“

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Contents

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Abstract

Freshwater-inhabiting phylactolaemates of the phylum Bryozoa are characterised by some prominent features such as a flap-like extension above the mouth opening called epistome, a horseshoe-shaped lophophore and ganglionic horns, which are ganglionic extensions into the lophophoral arms. Species of the family Fredericellidae differ from other phylactolaemates with respect to their circular lophophore. Since Fredericellidae share this trait with marine bryozoans, this family was classified as the earliest branch within phylactolaemates. However, this hypothesis has been rejected using morphological and molecular data. In order to assess whether traces of a horseshoe-shaped lophophore occur during development, organogenesis during the budding process was analysed in *Fredericella sultana* (Fredericellidae) and the closely related *Plumatella casmiana* (Plumatellidae). Lophophore development starts with two lateral ridges, which connect on the oral side and later on the anal side. The lophophore of *F. sultana* is "heart-shaped" in early budding stages. Anlagen of ganglionic horns emanating from the cerebral ganglion are also discernible in early stages, which only remain as unilateral rudiments in adults. *F. sultana* has only four oral tentacles instead of six found in *P. casmiana*. These differences may be due to reduction of the lophophoral arms and the lophophore itself in *F. sultana*. This study shows that the early formation of the lophophore anlage is similar in all phylactolaemates and considerably different to gymnolaemate and stenolaemate bryozoans. Anlagen of the ganglionic horns also provide evidence that fredericellids once possessed a horseshoe shaped lophophore. Consequently, the superficial appearance of a circular lophophore in this clade has to be considered a secondary condition.

Keywords: Bryozoa, Phylactolaemata, Fredericellidae, Plumatellidae, Budding, Organogenesis, Lophophore

Introduction

The phylum Bryozoa (Ectoprocta) belongs to the monophyletic Lophotrochozoa (e.g. Kocot et al., 2017), which comprises 'Lophophorata' (brachiopods, phoronids and bryozoans) and the monophyletic 'Trochozoa' (e.g. annelids, molluscs) (e.g. Aguinaldo et al., 1997; Passamaneck and Halanych, 2006; Kocot, 2016). Putative monophyly of 'Lophophorata' is based on conspicuous morphological characters such as the food-gathering structure, the lophophore (Emig, 1984). However, most molecular studies reject the monophyly of this assemblage (e.g. Halanyck et al., 1995; Hausdorf et al., 2007; Helmkampf et al., 2008; Mallatt et al., 2012). Bryozoans are a group of colonial and sessile suspension-feeders, whereby a single individual is called zooid. Each zooid consists of a cystid and a polypide. The cystid consists of the body wall and cuticle and acts as protective layer encasing the retractable polypide. The zooid is composed of the gut and the ciliated tentacle crown, also referred to as lophophore. Suspension-feeding is mediated by the lophophore, which creates feeding currents via metachronal beat of the cilia located on the tentacles. The U-shaped gut is divided into pharynx, oesophagus, cardia, caecum and intestine. When retracted, the lophophore is surrounded by the tentacle sheath, which is connected with the body wall by the vestibular wall. The prominent retractor muscles, which are situated on both sides of the polypide and originate from the proximal body wall, are responsible for polypide retraction. The muscle bundles are connected with the polypide at several positions on the oral side of the polypide. (e.g. Wood, 1983; Mukai et al., 1997)

There are three class-level taxa within the phylum Bryozoa: the Phylactolaemata, whose members live exclusively in freshwater and generally possess a horseshoe-shaped lophophore, and the Gymnolaemata and Stenolaemata, which are both chiefly marine and characterized by a circular lophophore (Mukai et al., 1997). The colonies of the three classes differ in size and structure. Gymnolaemanta and Stenolaemata build erect or encrusting colonies. Gymnolaemate colonies may be mineralized or chitinized, whereas stenolaemate colonies are always mineralized (Bishop, 1989). The cystid and the colonies of phylactolaemates are always uncalcified (Mukai et al., 1997).

Phylactolaemata comprises six family-level taxa with about 80 species: Stephanellidae, Lophopodidae, Pectinatellidae, Cristatellidae, Fredericellidae and Plumatellidae (Massard & Geimer, 2008). Bryozoans of the family Pectinatellidae form large gelatinous compound colonies, whereas Cristatellidae constitute vermiform colonies with a creeping sole. Both Plumatellidae and Fredericellidae form branched colonies colonizing various substrates (Marcus, 1926; Wood, 1983). In contrast to the circular lophophore of Gymnolaemanta and Stenolaemata, the tentacle crown in phylactolaemates is usually horseshoe-shaped and carries two extensions into the anal direction of the zooid, the lophophoral arms. The only exception within phylactolaemates are the Fredericellidae, which have a circular lophophore (Wood, 1983; Mukai et al., 1997). The phylactolaemate lophophore has different coelomic canals: the oral tentacles are provided by the ring canal at the lophophoral base and the anal tentacles in the lophophoral concavity by the forked canal (Schwaha et al., 2011). The tentacles of the lophophoral arms and the lateral tentacles on the lophophoral base are widely confluent with the remaining coelomic cavity of the zooid. The lophophore of phylactolaemates possesses a flap-like epistome above the mouth opening, which is used for sorting food particles and presumably also for excretion (Schwaha and Wanninger, 2012). The ganglion is located proximally of the epistome and adjacent to the pharynx. In phylactolaemates, the ganglion has a typical extension into the lophophoral arms called ganglionic horns (Mukai et al., 1997; Gruhl et al., 2009).

On the proximal side of the caecum, the elongated funicular cord runs to the body wall. This is the site of male gametogenesis where testes are attached in clusters along the cord (Wood, 1983). Phylactolaemates also form characteristic statoblasts on the funiculus. These are dormant bodies and are produced for dispersal and to endure unfavourable environmental conditions. Their shape and structure are very diverse with two basic types, the floatable type called floatoblast and the sessile type, which is called sessoblast. Some statoblasts additionally have hooked appendages (Mukai, 1982; Wood, 2014). The main asexual reproduction used for colonial growth is the production of buds. Budding is the most common way of asexual reproduction in bryozoans (Wood, 1983). Non-phylactolaemates primarily form their buds on the anal side of the zooid (anal growth direction), whereas phylactolaemates almost exclusively bud on the oral side (oral growth direction) (Jebram, 1973).

Traditionally, Fredericellidae had been regarded as the earliest branch within Phylactolaemata because of the simple branching colony type, the simple statoblasts, the circular lophophore and the low number of tentacles (Mukai, 1999; Hyman, 1959; Lacourt, 1968; Toriumi, 1956; Brien, 1953). In contrast, molecular studies indicate that Fredericellidae is sister group to the Plumatellidae and both are late branches within the Phylactolaemata (Hirose et al., 2008), indicating that the mentioned characteristics may be derived. This implies that the circular lophophore of fredericellids is secondarily reduced and originally derived from a horseshoe-shaped tentacle crown, contrary to the traditional view that this trait is shared with non-phylactolaemates. Morphologically, there are few investigations and observations that indicate traces of a horseshoe-shaped lophophore in fredericellids (e.g. Marcus, 1926).

Most recent studies found evidence that the structure of the central nervous system in fredericellids is similar to other bryozoans with a horseshoe-shaped lophophore (Gruhl and Bartolomaeus, 2008; Shunkina et al. 2015), while an earlier study found a single budding stage to be heart-shaped and reminiscent of plumatellid stages (Marcus, 1926). Hence, there are indications that organogenesis during budding shows traces of a horseshoe-shaped lophophore, but studies on budding are restricted to the ontogenetic sequences of the formation of buds (Braem, 1890). Recent data on organogenesis during the budding process are only present for the phylactolaemates *Cristatella mucedo* CUVIER, 1798 (Schwaha et al, 2011) and *Lophopus crystallinus* (PALLAS, 1768) (Schwaha, 2018) as well as for the ctenostome *Hislopia malayensis* ANNANDALE, 1916 (Schwaha and Wood, 2011). Data on fredericellids, but also on the closely related plumatellids, the largest phylactolaemate family (Hirose et al. 2008), are entirely missing. This study documents the budding process of *Fredericella sultana* (BLUMENBACH, 1779) and *Plumatella casmiana* OKA, 1907 in order to assess whether budding stages of fredericellids show any sign of a horseshoe-shaped lophophore during development and to analyse how the lophophore develops in this clade. Since only gelatinous forms have been recently studied in more detail (Schwaha et al. 2011, Schwaha 2018), another aim of this study is to compare the budding-process of the gelatinous and branched-colony phylactolaemates. Consequently, this study will also provide data for reconstruct the ground pattern of budding in different forms of phylactolaemate families showing different colonial growth forms.

Material and methods

Specimens of *Fredericella sultana* were collected in Laxenburg (Lower Austria), whereas samples of *Plumatella casmiana* were collected in Bangkok or Kanchanaburi (Thailand). Fixation of the specimens was done with a 2% glutaraldehyde solution in 0.01 mol l^{-1} PBS. Postfixation followed with 1% osmium tetroxide. Fixed specimens were dehydrated with dimethoxypropane (DMP) and rinsed with 100% acetone, which also acted as intermediate to embed specimens in Agar Low Viscosity Resin (Agar Scientific, Stansted, Essex, Great Britain). Serial semithin sections were cut with a Leica UC6 ultramicrotome (Leica Microsystems, Wetzlar, Germany) at a thickness of 1 µm. The sections were stained with toluidine blue for about 15 seconds and sealed with Agar Low Viscosity Resin. After the curing process, the serial sections of the area of interest were photographed with a Nikon E800 light microscope equipped with a Nikon Ri1 microscope camera (Nikon, Tokyo, Japan). The image sequences were imported to Amira 6.3 (FEI Visualization Sciences Group, Mérignac Cédex, France). After image alignment, the developing organ systems were manually segmented. Snapshots of the reconstructions were taken with the Amira software and figures were prepared with Photoshop CS5 (Adobe Inc., San Jose, California, US).

Results

Both *Fredericella sultana* and *Plumatella casmiana* form branched and sand-encrusted colonies. In adult animals, the body wall consists of an outer epidermis and a peritoneum that is situated on the inner side. The budding process starts with an invagination of the epidermal and peritoneal layer of the body wall on the oral side of the zooids. After the invagination process of the body wall, the epidermal part of the bud constitutes the inner budding layer and is surrounded by the outer peritoneal budding layer. Phylactolaemates always form their buds on the oral side, which means that the oral side of the bud is directed towards the body wall and the anal side towards the mother animal. For reconstructing the complete organogenesis, six budding stages according to their degree of differentiation from the earliest stage to the seventh, adult stage were analysed. Schematic illustrations of the most important changes during organogenesis are given in figure 1 (third, fifth and adult stage).

Stage 1

The first and earliest stage is identical in both species. The buds are small sac-like structures, which are connected to the mother animal by a single neck. The bud consists only of the outer (peritoneal) and the inner (epidermal) layer (Fig. 2). In the first development stage the inner epidermal layer encloses a small lumen. The buds of both species have a lumen with different extensions. The extension of the lumen towards the oral side is the prospective mouth area and the one on the anal side is the prospective anal area. Both areas are easily visible in the bud of *Plumatella casmiana* because the bud is slightly more differentiated (Fig. 2 D) than in *Fredericella sultana,* where only the prospective anal area is apparent (Fig. 2 B).

Stage 2

The shape of both distinct budding layers, the outer peritoneal and inner epidermal part (Fig. 3 A-D), has changed compared to the first stage. The difference is mostly discernible in the epidermal layer, which starts to form anlagen of the organs. However, these are not yet distinct yet. The proximal area, which is the future digestive tract, is laterally compressed. The distal area between the neck of the bud and the compressed proximal part is laterally widened. This part constitutes the prospective lophophore. The lumen extends over the entire future lophophore and to the anal side into the prospective anal area of the gut. The lumen of the gut anlage on the oral side is longer in proximal direction, but too thin for an adequate reconstruction. The reconstructed specimen of *Plumatella casmiana* is folded in the neck area (Fig. 3 C, D).

Stage 3

From this stage onwards, the different anlagen of the developing organ systems in the bud can be differentiated more easily. In both species, anlagen of the lophophore, gut, ganglion, epistome and the ring canal have formed. The shape of the two buds differs because of individual variability and superficial folds depending on the location within the colony (Fig. 4 A and 5 A). The lophophore anlage already shows the typical lateral lobes. They are located on the distal oral-anal axis of the bud. The more prominent bulges are situated on the anal side where the future lophophoral arms will develop. There are no indications of individual tentacle anlagen in this stage. On the oral side, the ring canal anlage is present in both species and develops from an invagination of the peritoneal layer that protrudes medially from both lateral sides on the lophophoral base (Fig. 4 C and 5 B). The U-shaped gut is also evident, but a continuous lumen is not present. The ganglion that develops by an invagination of the prospective mouth/pharyngeal area is very prominent. It is situated between the two shanks of the gut and nearly fills the whole space between them (Fig. 4 C). The size and shape of the ganglion is similar in both species (Fig. 4 B and 5 C). In this budding stage, anlagen of ganglionic horns are present only in *Fredericella sultana* (Fig. 4 B). Both species possess the typical lumen inside the ganglion, but only in the bud of *F. sultana* the ganglion is still open and the lumen is discernible (Fig. 4 A, D). The anlage of the epistome coelom is situated between the anal gut shank and the ganglion. It develops from two lateral invaginations of the peritoneal budding layer similar to the ring canal, but between the gut shanks.

The retractor muscles have already formed in both species but are more prominent in *F. sultana* than in *Plumatella casmiana*. They are situated laterally on both sides of the bud, originate from the proximal body wall and are connected with the bud at several positions. The tentacle sheath is well recognisable and has the shape of a thin-walled structure. Except for growing longer and thinner, it does not change during further development. The funiculus anlage is present in the bud of *P. casmiana* (Fig. 5 A) but absent in *F. sultana* (Fig. 4 A). In *P. casmiana* it connects the proximal part of the U-shaped gut with the body wall and runs on the lateral side in oral direction.

Stage 4

In both species, stage 4 is characterized by a considerable size increase, especially concerning the lophophore. In addition to the two lobes, small bulges on the lophophore represent the anlagen of the future tentacles (Fig. 6 B, D and 7 A, B). The bud of *Fredericella sultana* shows a heart-shaped lophophore (Fig. 6 A, B, D). The heart-shaped lophophore is less conspicuous (Fig. 7 A, B), because *Plumatella casmiana's* bud is laterally compressed. The ring canal is more developed but still consists of two parts and there is no median connection between the two lateral parts (Fig. 6 B). The lumen of the gut is distinguishable on the anal and oral side of the gut. The lumen of the U-shaped gut is not continuous at this stage, lacking a connection in the area of the future cardia (Fig. 6 C). An additional cellular layer is present between the epidermal and the peritoneal layer in *P. casmiana* (Fig. 7 C).

The ganglion of both species has a similar morphology and is completely closed towards the mouth area. Anlagen of ganglionic horns are present in *F. sultana* and *P. casmiana* (Fig. 6 D and 7 B). The epistome anlage is present and arches over the distal area of the ganglion between the ganglionic horns into the direction of the mouth opening (Fig. 6 C).

The retractor muscles are very prominent in both species and stretch across the whole lateral length of the bud (Fig. 6 A and 7 A). The funiculus of *P. casmiana* is more elongate than in *F. sultana*. In both species it is situated medially on the oral side and extends to the oral body wall (Fig. 6 A and 7 A).

Stage 5

The bud of both species has a more elongate shape than in previous stages and the shape of the buds is similar. The lophophore of both species is well developed. In comparison to the previous stage, the lateral width of the lophophore is not increased, but the lophophore is twice as long in the proximo-distal axis. Individual tentacles are recognisable (Fig. 8 A and 9 A). Both species have 12 distinct tentacle anlagen, the future anal tentacles have not formed yet. In general, the budding stage of *P. casmiana* is slightly more developed. The ring canal in both species is continuous and supports four oral tentacles (Fig. 8 D and 9 B). On the anal side of the lophophore above the ganglion, the paired anlagen of the forked canal have developed. These anlagen originate from anal invaginations of the peritoneal budding layer and will provide the future anal tentacles with coelomic fluid. At this stage, the forked canal is not continuous, and the anal tentacles are not fully developed. The openings are situated on both lateral sides laterally of the epistome coelom anlage (Fig. 8 B, D and 9 C). The lumen of the U-shaped gut is continuous (Fig. 8 B).

The ganglion in both species is of similar size. Well-developed ganglionic horns are present in the bud of *P. casmiana* (Fig. 9 D). The ganglion of *F. sultana* shows a rudiment of ganglionic horns only on one side of the ganglion (Fig. 8 C). The anlage of the epistome is similar in both species and more developed than in earlier stages. In comparison to the early anlage of former stages, the characteristic flap-like extension of the epistomal coelom above the ganglion has formed (Fig. 8 B, C and 9 D).

Retractor muscles are well developed in both species. The funiculus of both buds is situated on the same lateral side (Fig. 8 A and 9 A). Overall, the tentacle sheath remains unchanged. However, the size increase of the lophophore results in an elongation of the tentacle sheath. Consequently, the width of epithelium decreases with increasing growth.

Stage 6

The size of the buds has increased in comparison to the previous stage. The shape of the bud of *Fredericella sultana* is elongated (Fig. 10 A), whereas the bud of *Plumatella casmiana* is more compact (Fig. 11 A). Especially the gut of the latter has multiple foldings. The lophophore in both species possesses more and longer tentacles. Altogether, *F. sultana* has 20 tentacles, four of which belong to the ring canal and six to the forked canal. The budding stage of *P. casmiana* has 21 tentacles, whereas the ring canal supplies also four tentacles and the forked canal eleven. Both ring canal and forked canal are continuous from this stage onwards. Consequently, the anal tentacles have started to form as well (Fig. 10 C and 11 C). In comparison to the fifth stage, the lophophore of the bud of *F. sultana* is ring-shaped.

In both species the circumoral nerve ring is visible and very prominent. It is situated around the mouth opening at the edge of the lophophoral base (Fig. 10 B, C and 11 B, C). The ganglion of both species is similar in size. Only *P. casmiana* possesses well-developed ganglionic horns (Fig. 11 B, C). The bud of *F. sultana* has only rudiments of ganglionic horns on one side (Fig. 10 B). The epistome with its flap-like extension above the ganglion is more prominent than in earlier stages and extends in the direction of the mouth opening (Fig. 10 B and 11 B).

The U-shaped gut and its lumen as well as the tentacle sheath are unchanged. The retractor muscles are prominent and grow with increasing size of the bud. Like the whole bud, the funiculus of *F. sultana* is elongate, whereas *P. casmiana* features a compact and short funiculus (Fig. 10 A and 11 A).

Stage 7 (adult)

Differences between the last budding stage and the adult state are only present in the lophophore and lophophoral base, because the gut morphology did not change from the fifth to the sixth stage. Therefore, only the lophophoral base was reconstructed in detail. *Fredericella sultana* possesses 22 tentacles altogether, four of them are provided by the ring canal and eight by the forked canal (Fig. 12 B). Since the original opening of the forked canal is situated more internally than in previous stages in

Plumatella casmiana, the differentiation between the forked canal and the tentacles of the lateral lophophoral arms was not possible. The ring canal supplies six oral tentacles. Overall, 31 tentacles were found in *P. casmiana* (Fig. 13 B). The ganglion of *P. casmiana* has large and conspicuous ganglionic horns, which extend into the lophophoral arms (Fig. 13 A). In general, the lophophore of *F. sultana* is smaller. As a consequence of the absence of lophophoral arms, the ganglionic horns are also reduced and only its rudiments are visible on one side (Fig. 12 A). In both species the circumoral nerve ring is prominent and well developed. In comparison to the last budding stage, the epistome extends farther towards the mouth opening (Fig. 12 A and 13 A).

Discussion

General aspects of organogenesis during bryozoan asexual reproduction

Organogenesis of bryozoans is as of yet poorly understood and there are only a few studies, which are concerned with the budding process of Phylactolaemata (e.g. Allmann, 1879; Braem, 1890; Davenport, 1890; Nitsche 1871; Oka, 1891). Most of these studies deal with early bud formation, their sequence and colonial growth pattern. Organogenesis during the budding process was only investigated and documented for the phylactolaemate *Cristatella mucedo* (Schwaha et al., 2011) and *Lophopus crystallinus* (Schwaha, 2018). Detailed analyses for gymnolaemates are available only for the ctenostome *Hislopia malayensis* (Schwaha and Wood, 2011) and the cheilostomes *Membranipora membranacea* (LINNAEUS, 1767) (Lutaud, 1961) and *Carbasea carbasea* (ELLIS & SOLANDER, 1786) (Haddon, 1883). Organogenesis during budding of different cyclostomes, which represent the only extant order of the Stenolaemata, was only investigated by Borg (1926). All in all, detailed studies using modern imaging techniques, such as 3D reconstructions based on histological sections, are lacking so far.

A new bud originates from a proliferation and invagination process of the body wall of a zooid, which consists of an outer epidermal and inner peritoneal part. As a result, the young bud consists of two different layers, with the outer part being derived from the peritoneum and the inner part from the epidermis. The position of a new bud differs in Phylactolaemata and Gymnolaemata. Phylactolaemates have an oral budding direction, i.e. buds are always formed on the oral side of zooids, which consequently also represents the colony growth margin. Steno- and gymnolaemates primarily form buds on their anal side and thus have anal growth direction (Jebram, 1973).

Development of the lophophore

Together with the digestive tract, the early anlage of the lophophore is the first recognizable structure during organogenesis. Although all bryozoans possess a lophophore, the development in Phylactolaemata differs from the development of the mainly marine groups.

The development of the lophophore in all bryozoans starts with two lateral ridges that form from the epidermal budding layer. First, these lateral ridges are connected with each other on the oral side and later on the anal side. Species with a circular lophophore, e.g. the ctenostomes *Paludicella articulata* (EHRENBERG, 1831) (Davenport, 1891) or *Hislopia malayensis* (Schwaha and Wood, 2011) and the cheilostome *Membranipora membranacea* (Lutaud, 1983), possess tentacle anlagen on the lateral lophophoral ridges in early budding stages. In the Phylactolaemata the lateral lophophoral ridges form the lophophoral arms of adult zooids, which cause the characteristic horseshoe-shaped lophophore. The first differentiable tentacle anlagen of phylactolaemate bryozoans are the oral ones (Schwaha et al., 2011).

The lophophore of *Fredericella sultana* appears circular in adult protruded specimens and there is no indication of a horseshoe-shaped structure (Pyttel, 1981). However, in the retracted state, a kind of horseshoe-shaped lophophore can be recognized (this study). Especially in young individuals of *F. sultana*, a “heart-shaped” lophophore is present (Marcus, 1926). The horseshoe-shaped lophophore anlage in the third and fourth budding stage, which was also found during this study, confirms this situation. Although the size of the buds of *Plumatella casmiana* and *F. sultana* differs, the lophophore shows a similar morphology in the early stages. Only in later stages, a difference in the shape and number of tentacles is evident. The typical lophophoral arms of phylactolaemates are reduced in *F. sultana* (Braem, 1890; Marcus 1926).

Traditionally, the absence of lophophoral arms and supposed circular lophophore was considered ancestral and shared with non-phylactolaemates. Accordingly, fredericellids were regarded the earliest branch within the Phylactolaemata (e.g. Brien, 1953). This interpretation has been rejected by several authors based on morphological (e.g. Braem, 1890; Marcus, 1926) and molecular data (Okuyama et al., 2006; Hirose et al., 2008). Because of the reduction of the lophophoral arms, fredericellids have the lowest number of tentacles within the Phylactolaemata. In addition, in comparison with other phylactolaemate species, *F. sultana* possesses the smallest zooids. These findings suggest a correlation between body size and the number of tentacles, which was also proven for many marine bryozoans (e.g. Winston, 1977).

Furthermore, the lophophoral development varies within Phylactolaemates. *Cristatella mucedo* and *Pectinatella magnifica* (LEIDY, 1851) possess a cellular median bridge between the two lophophoral arms (Schwaha et al., 2011), which is missing in *P. casmiana*, *F. sultana* and *Lophopus crystallinus* (Schwaha, 2018; this study). In general, both *C. mucedo* and *P. magnifica* have a higher number of tentacles (Lacourt, 1968). During development, the lophophore of these two species is folded. These foldings contribute to a compact appearance of the lophophore and the median bridge may serve to stabilise it (Schwaha et al. 2011). The lophophore of *P. casmiana*, *F. sultana* (this study) and *L. crystallinus* does not show any foldings (Schwaha, 2018). Although the lophophore of *P. casmiana* is horseshoe-shaped, the lophophoral arms are shorter than those of *P. magnifica* and *C. mucedo* (Wood, 1983). In *P. casmiana* and *F. sultana* the right lophophoral arm is slightly larger than the left. This difference is only visible in stage 3. In all later stages, the lophophoral arms have the same size. In contrast, *C. mucedo* possesses a larger left arm (Schwaha et al., 2011). The left-right asymmetry may be the result of individual variability. Size differences between the two sides of the arms result in folding of the two arms within the developing buds of *C. mucedo* and *P. magnifica*. In addition to the smaller lophophore, Plumatellidae and Fredericellidae have a similar branched colony type with zooids spatially more separated than gelatinous colony types such as Pectinatellidae or Cristatellidae. Zooids are also smaller in these branching forms including lophophore size and the number of tentacles. Fredericellids have a small zooidal size and reduced lophophoral arms. The circular lophophore is reminiscent of most marine bryozoans, which are also characterized by smaller zooids compared to phylactolaemates (Jebram, 1986).

Development of coelomic systems

The development of the ring canal supplying the oral tentacles is similar in all investigated phylactolaemates (Schwaha et al., 2011; Schwaha, 2018, this study). It develops in early stages (this study: stage 3) from lateral invaginations of the outer budding layer that medially fuse in later stages (this study: stage 5) to provide the oral tentacles with a coelomic inner lining. Adult specimens of *Plumatella casmiana* and *Cristatella mucedo* possess six oral tentacles, whereas *Fredericella sultana* has only four oral tentacles (Schwaha et al., 2011, this study). In the earlier stages the ring canal is associated with four tentacles in both species studied herein. The total number of tentacles is higher in *P. casmiana* than in *F. sultana*. The investigation of more individuals would be necessary to ascertain a possible correlation. The number of oral tentacles is not always an even number, since species like *Lophopus crystallinus* possess five oral tentacles (Schwaha, 2018).

The forked canal is a coelomic canal, which constitutes a unique feature of phylactolaemates. It is situated above the epistome and supplies the anal tentacles (Braem, 1890; Gruhl et al., 2009). Its opening towards the main body cavity is densely ciliated and its function is controversially debated. The forked canal has previously been considered as vestigial metanephridium because of the position, the ciliated structure of the forked canal and the assumed close relationship of bryozoans with phoronids (Verworn, 1887). This assumption has been rejected by several authors owing to the lack of a pore and was regarded merely as a necessity to supply tentacles above the epistome (e.g. Braem, 1890; Marcus, 1934; Gruhl et al., 2009).

The forked canal starts to develop later than the ring canal. In this study, the anlage of the forked canal is visible from stage 5 onwards. In *F. sultana*, the number of tentacles that are associated with the forked canal increases from the last budding stage to the adult specimen from six to eight tentacles. Since the opening of the forked canal in adult *P. casmiana* is situated more internally, it was not possible to determine how many tentacles belong to the forked canal. In the previous stage, *P. casmiana* possesses eleven tentacles which are associated with the forked canal, the same number as in *L. crystallinus* (Schwaha, 2018). *C. mucedo* has nine, also an uneven number, of forked canal associated tentacles (Schwaha et al., 2011). This indicates that phylactolaemates normally have an uneven number of tentacles associated with the forked canal. The general lower number of tentacles in *F. sultana* correlates with the low number of forked canal tentacles.

The epistome develops from two lateral invaginations from the outer budding layer that further proceed distally. In the early stages these invaginations, which are situated between the two shanks of the U-shaped gut, only represent the epistome coelom. From the fourth budding stage onwards, the epistome coelom protrudes above the ganglion and extends towards the mouth opening. The development of the epistome and its coelom is similar in all bryozoans (Braem, 1890; Mukai et al., 1997; Gruhl et al., 2009; Schwaha et al., 2011; Schwaha, 2018). The epistome differs only in size among the families. The epistome of *C. mucedo* is much larger and has a long, broadened shape as opposed to *P. casmiana* and *F. sultana* (Schwaha et al., 2011; this study). The epistome of *L. crystallinus* is much smaller compared to the one of *P. casmiana* and *F. sultana*. The function of the epistome is not completely resolved, but it is probably involved in feeding (e.g. Gruhl et al., 2009). The size differences of the epistome can be expected to have an impact on the feeding process. Detailed studies on the functional morphology of this structure are necessary to shed light on the role of the epistome during feeding.

All phylactolaemates possess a ring canal and a forked canal. During organogenesis of *C. mucedo, L. crystallinus, P. casmiana* and *F. sultana* the formation of the ring canal and forked canal takes place at the same time (Schwaha et al., 2011; Schwaha, 2018; this study). The ring canal always differentiates in early stages (this study: stage 3) and initially fuses on the oral side, followed by the later-forming forked canal (this study: stage 5), which completes the lophophore on the anal side. Consequently, the chronology of the development of the ring and forked canal follows a general pattern and is reminiscent of the formation of lophophores in other phylactolaemates (Schwaha et al., 2011; Schwaha, 2018; this study).

Development of the cerebral ganglion

The cerebral ganglion is formed by an invagination of the inner epidermal budding layer in the prospective mouth/pharyngeal area. It is formed very early (this study: stage 3) and belongs to the first developing organs during organogenesis. Especially in early stages, the ganglion occupies most space between the developing shanks of the U-shaped digestive tract. As a result of the invagination, the ganglion is still open at the beginning of its development. In addition to *Plumatella casmiana* and *Fredericella sultana*, the same process has been described for the phylactolaemates *Cristatella mucedo* (Schwaha et al., 2011) and *Lophopus crystallinus* (Schwaha, 2018) and for the ctenostome *Hislopia malayensis* (Schwaha and Wood, 2011). The cerebral ganglion possesses a central cavity, which is a result of the invagination process during early gangliogenesis of bryozoans in general. Probably, only phylactolaemates are characterized by the presence of this cavity in the adult stage. Detailed studies on the ontogeny of the central cavity are scarce so far, but Weber et al. (2014) and Temereva and Kosevich (2016) recently showed at least the existence of a central cavity of unknown origin in marine bryozoans.

Another unique feature of Phylactolaemata is the existence of ganglionic horns. These are extensions of the ganglion into the lophophoral arms (Braem, 1890; Davenport, 1890). The ganglionic horns of *P. casmiana* are shorter than in *C. mucedo* and *L. crystallinus* (Schwaha et al., 2011; Schwaha, 2018; this study). The length of the ganglionic horns corresponds to the size of the lophophoral arms. *F. sultana* possesses anlagen of ganglionic horns during the budding stages 3 to 5. Unilateral rudiments of ganglionic horns appear during budding stage 6 and the adult stage. The reduction of the ganglionic horns is a consequence of the reduction of the lophophoral arms. These results support the findings of earlier studies (Braem, 1890; Shunkina et al., 2015). The central cavity of the cerebral ganglion extends into the ganglionic horns (Braem, 1890; Davenport, 1890; Gewerzhagen, 1913; Marcus, 1934; Gruhl and Bartolomaeus, 2008). This extended cavity is absent in the ganglion of *F. sultana*, *P. casmiana* and *C.*

mucedo (Schwaha et al., 2011) and only the cerebral ganglion possesses a continuous cavity. Ultrastructural evidence has shown a central cavity in the ganglionic horns as extension of the lumen of the ganglion (e. g. Gruhl and Bartolomaeus, 2008). However, light microscopical investigation lack the resolution for resolving this cavity. Future ultrastructural studies should confirm whether or not phylactolaemates possess a cavity extending into the ganglionic horns.

Development of the digestive tract

The digestive tract of *Plumatella casmiana* and *Fredericella sultana* is mostly formed from an outpocketing of the prospective anal side. This outpocketing grows in direction of the prospective mouth area and forms the hindgut (intestine) and midgut (caecum and cardia). On the oral side, there is also a proximally directed extension which forms the pharynx and oesophagus. These two parts merges at the border of esophagus and cardia, which is marked by the cardiac valve. Most bryozoans form the digestive tract in a similar way (e.g. Braem, 1890; Davenport, 1890; Schwaha et al., 2011; Schwaha and Wood, 2011; Schwaha, 2018).

An additional cell layer is present between the gut epithelium and the outer peritoneal cover during the fourth budding stage of *P. casmiana*. This additional layer is currently unique for *P. casmiana*. In adult specimens, the epithelium of the digestive tract is surrounded by circular musculature that is located between the gut epithelium and the peritoneal layer that lines the body cavity (Schwaha and Wanninger, 2012; Gawin et al., 2017). Consequently, the observed cell layer between these epithelia in the bud consists of differentiating musculature.

Conclusion

The present work is the first complete ontogenetic study of representatives of the family-level taxa Fredericellidae and the closely related Plumatellidae. The analysis of *Fredericella sultana*´s organogenesis including lophophore development confirms the existence of a horseshoe-shaped lophophore during budding. Despite the adult stage reflecting a circular lophophore similar to non-phylactolaemates, this study supports previous molecular data that the Fredericellidae do not constitute the earliest branch within phylactolaemates. Instead, the results of this study support the notion that fredericellids have reduced several morphologies features secondarily, and that they are later-branching. In addition to the horseshoe-shaped lophophore and its development, fredericellids share more features with phylactolaemates than with gymnolaemates or stenolaemates, namely (1) the presence of an epistome, (2) a forked canal, (3) rudiments of ganglionic horns and (4) a central cavity in the cerebral ganglion. In general, the budding process of the phylactolaemate Fredericellidae, Plumatellidae, Cristatellidae and Lophopodidae is similar except for size and shape differences of the bud and some details of lophophore development. Fredericellidae and Plumatellidae share characters such as their similar lophophore development and especially by the branched colony type, which supports their closer phylogenetic relationship. The reduction of the horse-shoe shaped lophophore in Fredericellidae is probably a consequence of the small size of their zooids.

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Figures

Figure 1: Schematic view of the most important budding stages. **A** Lateral view of the bud representing the third budding stage. **B** Lateral view of the bud representing the fifth budding stage. **C** Lateral view of the bud representing the adult state. *ea* epistome anlage, *eca* epistome coelom anlage, *ep* epistome, *fca* forked canal anlage, *fc* forked canal, *ggl* ganglion, *ga* gut anlage, *gu* gut, *la* lophophore anlage, *lo* lophophore, *rca* ring canal anlage, *rc* ring canal. Blue, lophophore (anlage); green, gut (anlage); mauve, ring canal (anlage); purple, forked canal (anlage); red, epistome (coelom anlage); yellow, ganglion.

Figure 1

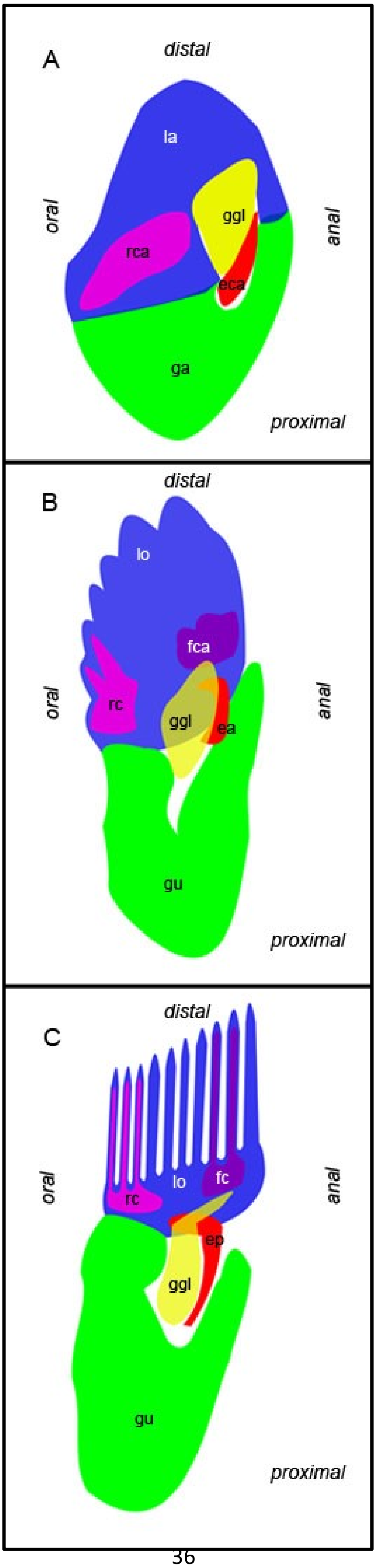
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Figure 2. Segmentation-based 3D-reconstruction of the first budding stage with two layers. **A** Lateral view of *Fredericella sultana*. Peritoneal layer (violet transparent) and epidermal layer (green). **B** Lateral view of *Fredericella sultana*. Peritoneal layer and epidermal layer with lumen. **C** Lateral view of *Plumatella casmiana*. Peritoneal layer and epidermal layer. **D** lateral view of *Plumatella casmiana*. Peritoneal layer and epidermal layer with lumen. *ne* neck of the bud, *paa* prospective anal area, *pma* prospective mouth area.

Figure 2

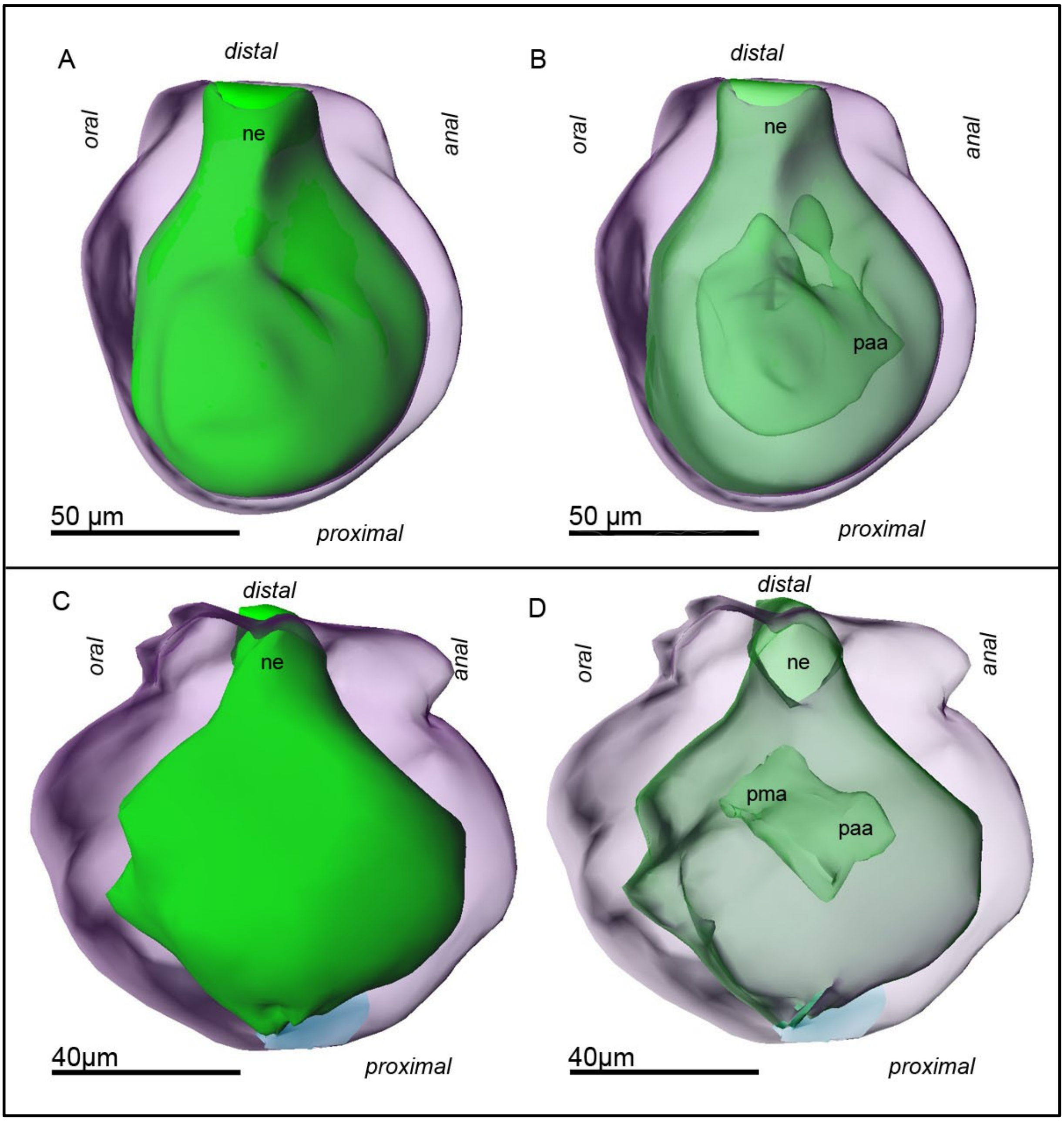
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Figure 3. Segmentation-based 3D-reconstruction of the second budding stage with two layers. **A** Oral view of *Fredericella sultana*. Peritoneal layer (violet transparent) and epidermal layer (green transparent). **B** Lateral view of *Fredericella sultana*. Epidermal layer with lumen. **C** Oral view of *Plumatella casmiana*. Peritoneal layer and epidermal layer. Superficial fold is marked with an arrow. **D** Lateral view of *Plumatella casmiana*. Epidermal layer with lumen. *ne* neck of the bud, *paa* prospective anal area, *pma* prospective mouth area.

Figure 3

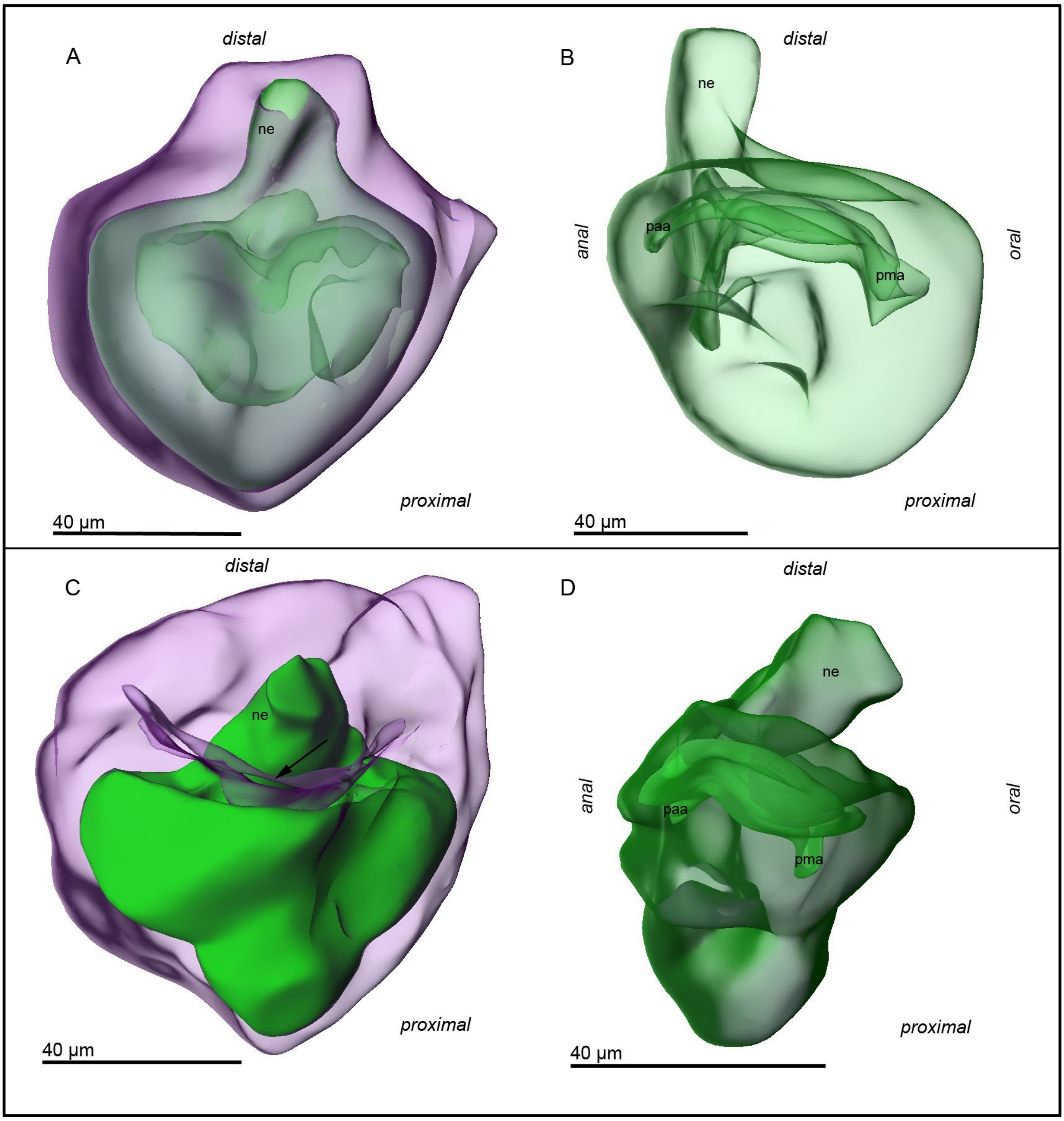
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Figure 4. Segmentation-based 3D-reconstruction of the third budding stage of *Fredericella sultana*. **A** Oral view of the bud showing the two lobes of the lophophore without tentacles. The open ganglion is marked with an arrow. Peritoneal budding layer (violet transparent). On both sides are the retractor muscles. The proximal gut anlage with mouth opening and the lateral ring canal anlage. **B** Oral view of the bud showing the lophophore transparent and the ganglion with rudiments of ganglionic horns. Gut anlage with mouth opening. **C** Lateral view of the bud without peritoneal layer showing lophophore with ring canal anlage and transparent gut anlage. Between the oral and anal part of the gut the ganglion and epistome coelom anlage are situated. **D** Lateral view of the bud with lophophore and transparent gut anlage. The ganglion is transparent and shows the lumen (arrow). Between the ganglion and the anal part of the gut the epistome coelom anlage is situated. *eca* epistome coelom anlage, *ga* gut anlage, *ggl* ganglion, *gh* ganglionic horns, *la* lophophoral arms, *mo* mouth opening, *rca* ring canal anlage, *rm* retractor muscle. Blue, lophophore; green, gut anlage; mauve, ring canal anlage; orange, retractor muscles; red, epistome coelom anlage; yellow, ganglion.

Figure 4

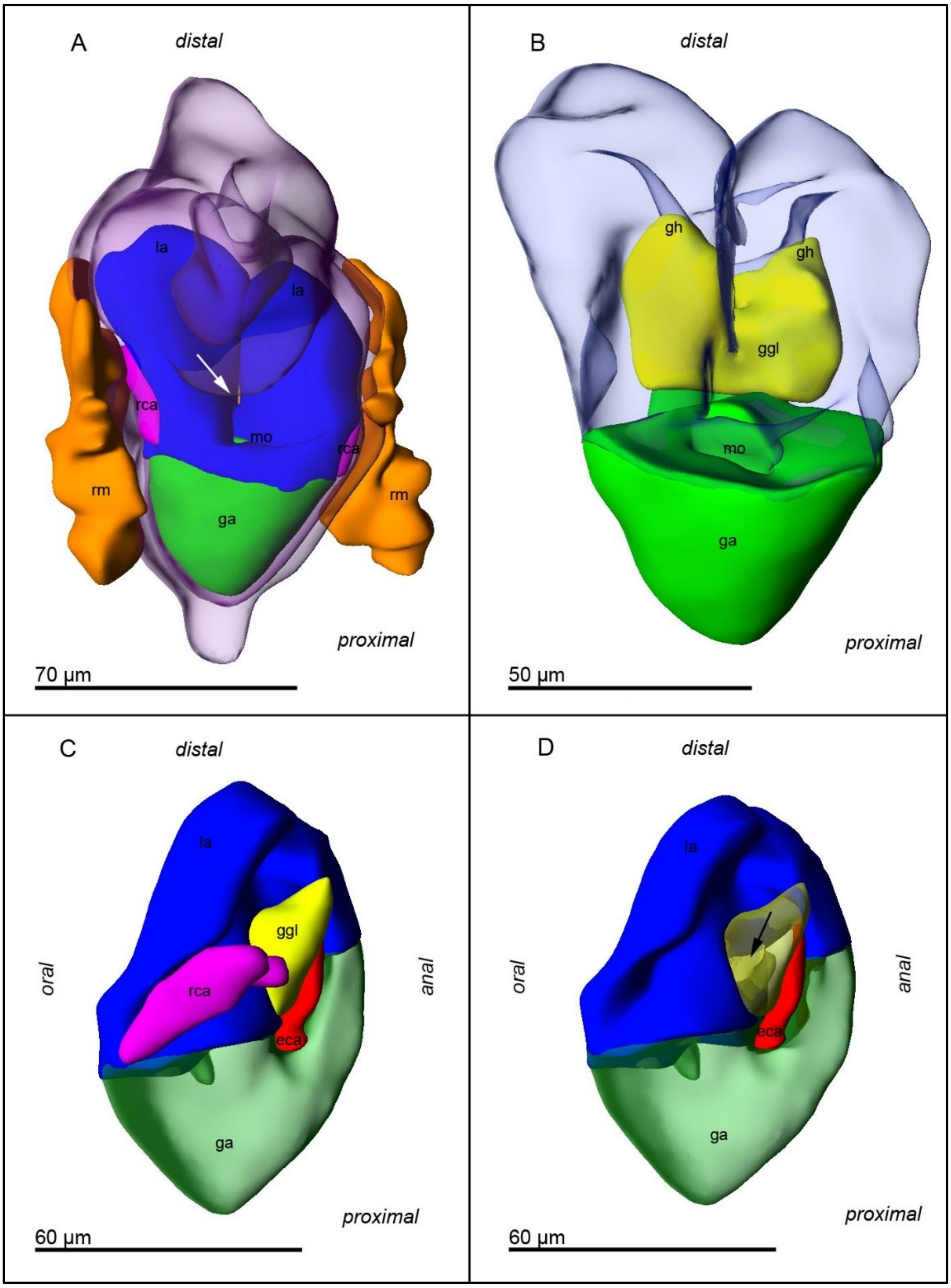
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Figure 5. Segmentation-based 3D-reconstruction of the third stage of *Plumatella casmiana*. **A** Oral view of the bud showing the two lobes without tentacles, the gut anlage with the mouth opening, the funiculus and the retractor muscles. Peritoneal budding layer with the neck of the bud (violet transparent). **B** Lateral view of the bud with transparent gut anlage and lumen which is not continuous. The lophophore anlage with the ring canal anlage is also visible. Between the oral and anal part of the gut anlage the ganglion and epistome coelom anlage are situated. **C** Anal view showing the anal part of the gut anlage, lophophore anlage and the ganglion. Note that there are no ganglionic horns. *eca* epistome coelom anlage, *ga* gut anlage, *ggl* ganglion, *la* lophophoral arms, *mo* mouth opening, *ne* neck of the bud, *rca* ring canal anlage, *rm* retractor muscle. Blue, lophophore; green, gut anlage; mauve, ring canal anlage; orange, retractor muscles; red, epistome coelom anlage; turquoise, funiculus; yellow, ganglion.

Figure 5

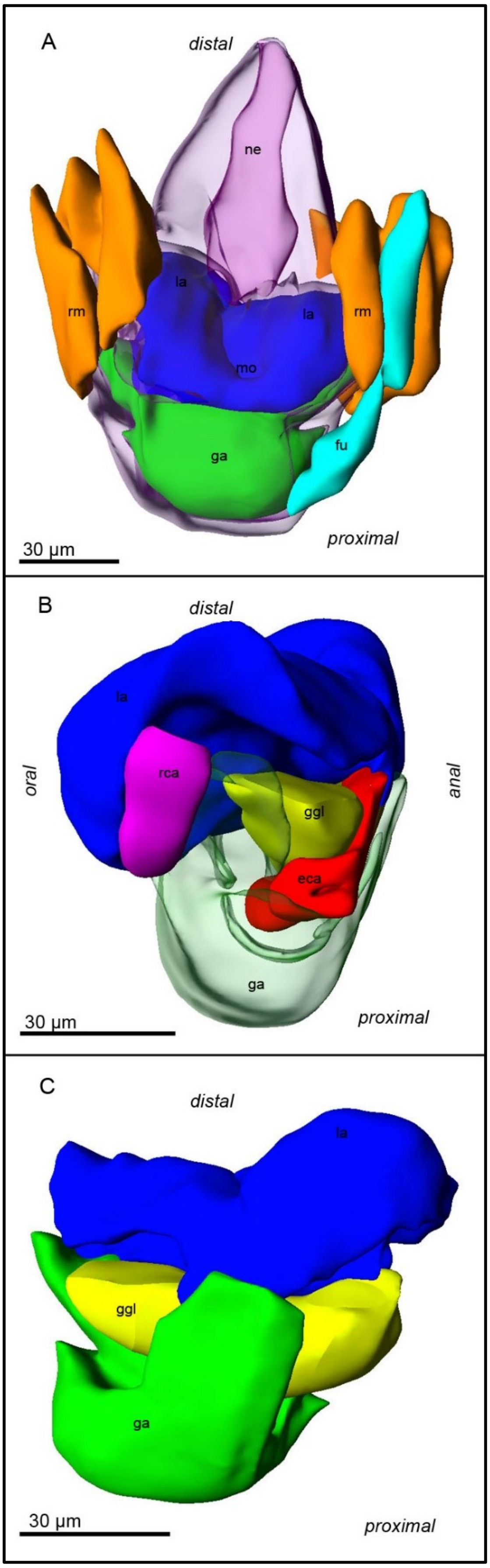
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Figure 6. Segmentation-based 3D-reconstruction of the fourth stage of *Fredericella sultana*. **A** Oral view of the bud with heart-shaped lophophore, gut anlage, retractor muscles and funiculus. Outer budding layer displayed transparent. **B** Oral view with ring canal anlage not medially fused yet. Bulges of the future tentacle anlagen marked with an arrow. **C** Lateral view with transparent gut anlage. Note the missing connection of the lumen at the oral side. Ganglion displayed transparent to have a look at the characteristic ganglion lumen. Epistome grew above the ganglion. **D** Anal view with transparent gut anlage and lophophore anlage for showing anlagen of the ganglionic horns. Marked with an arrow are the tentacle anlagen. *ea* epistome anlage, *ga* gut anlage, *ggl* ganglion, *gh* ganglionic horns, *la* lophophoral arms, *mo* mouth opening, *ne* neck of the bud, *rca* ring canal anlage, *rm* retractor muscle. Blue, lophophore; green, gut anlage; mauve, ring canal anlage; orange, retractor muscles; red, epistome anlage; turquoise, funiculus; yellow, ganglion.

Figure 6

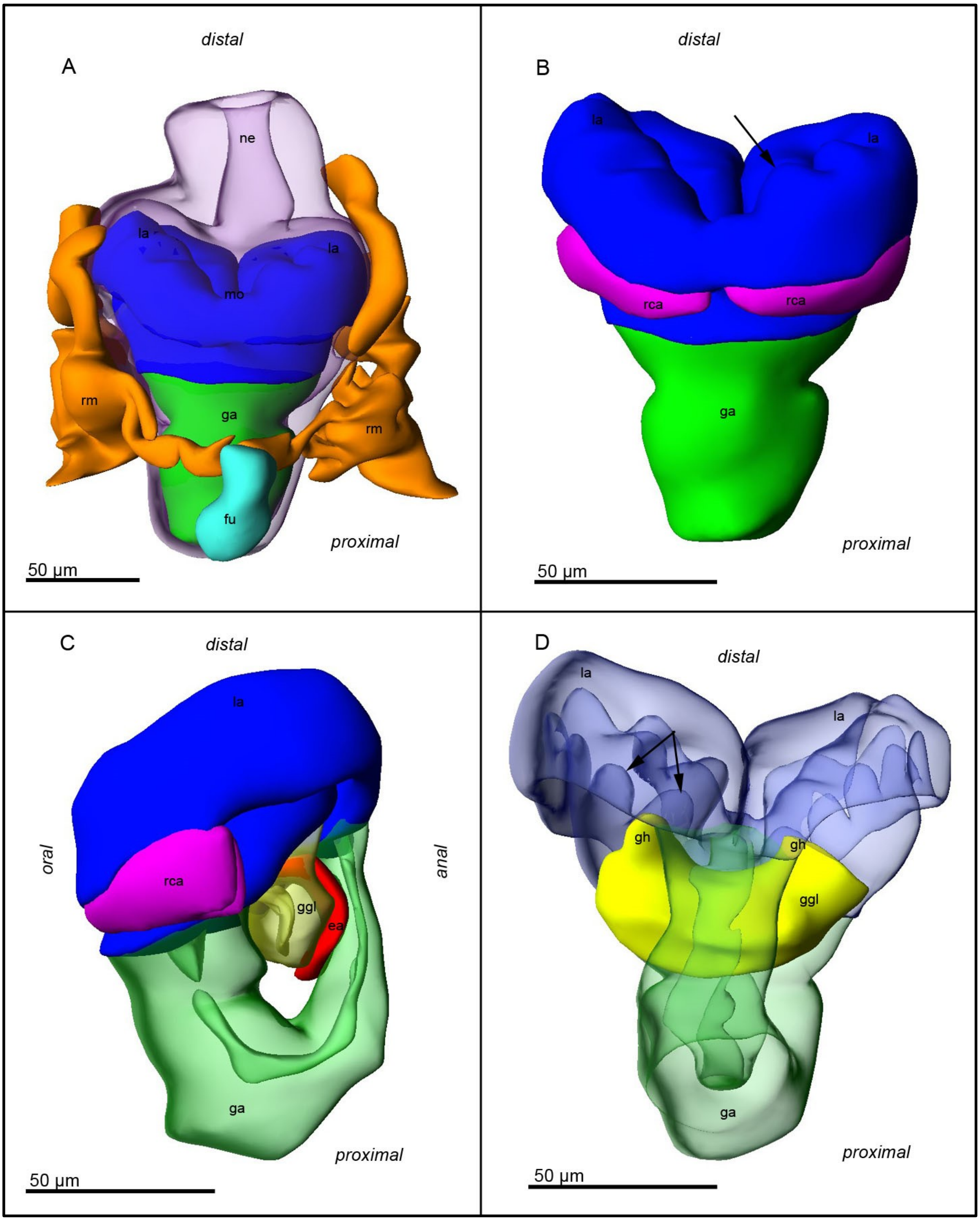

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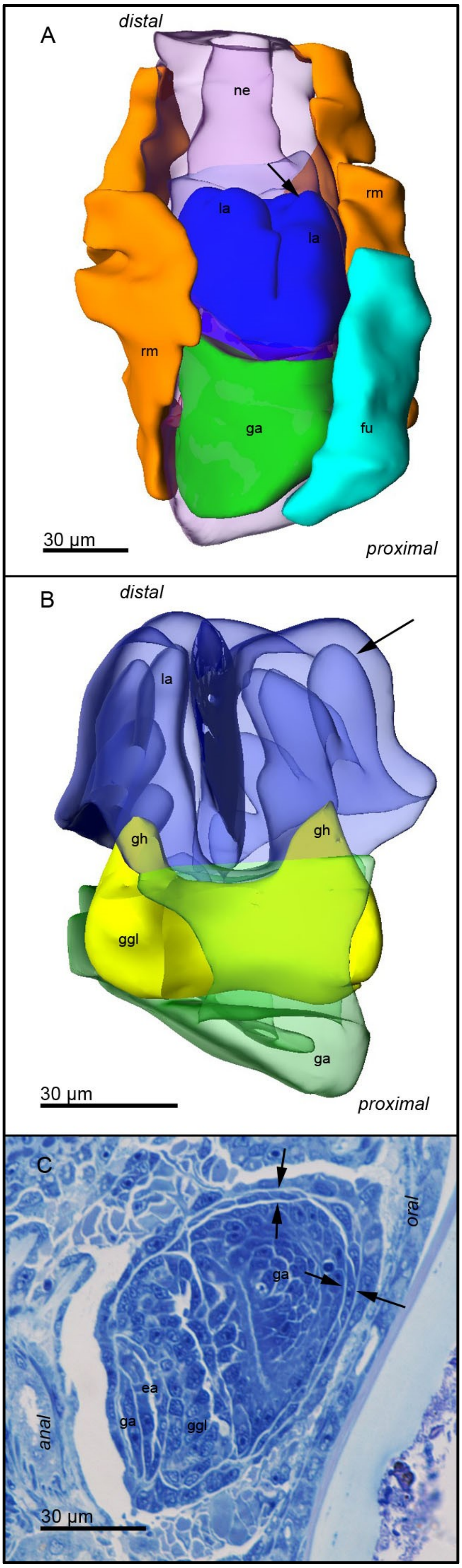
Figure 7. Segmentation-based 3D-reconstruction of the fourth stage of *Plumatella casmiana*. **A** Oral view with gut anlage and lophophore anlage with tentacle anlagen (arrow). On both sides the retractor muscles are situated. Note the very prominent funiculus. **B** Anal view with transparent gut and lophophore to show the ganglion with the anlagen of ganglionic horns. Tentacle anlagen are marked with an arrow. **C** Histological slide of the middle region through the gut, ganglion, epistome anlage. Undifferentiated cell layer around the gut anlage in the region of cardia is between the arrowheads. *ea* epistome anlage, *ga* gut anlage, *ggl* ganglion, *gh* ganglionic horns, *la* lophophoral arms, *mo* mouth opening, *ne* neck of the bud, *rca* ring canal anlage, *rm* retractor muscle. Blue, lophophore; green, gut anlage; orange, retractor muscles; turquoise, funiculus; yellow, ganglion.

Figure 7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ga
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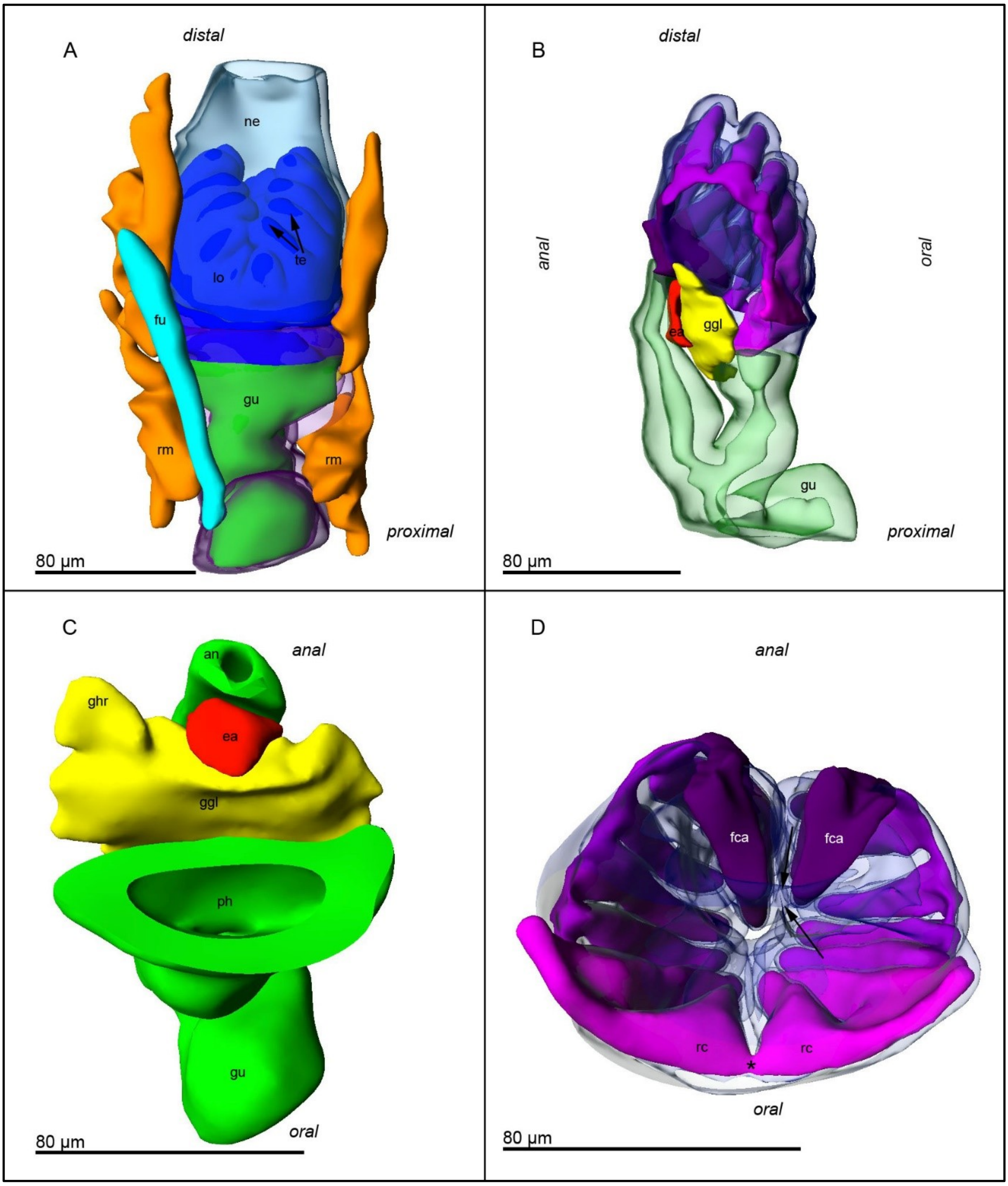
Figure 8. Segmentation-based 3D-reconstruction of the fifth stage of *Fredericella sultana*. **A** Oral view of the bud for orientation. Lophophore with developed tentacles (arrows), lateral retractor muscles and funiculus. **B** Lateral view with transparent lophophore. Gut is displayed transparent to have a look at the continuous lumen. Ganglion between the oral and anal part of the gut and epistome anlage, growing above the ganglion. **C** Distal view without lophophore to see the rudimental ganglionic horn and the small flap-like extension of the epistome anlage above the ganglion. **D** Proximal view of the lophophore without gut to see the continuous ring canal (median connection marked with an asterisk) and the missing connection between the two parts of the forked canal anlage (marked with arrows). *an* anus, *ea* epistome anlage, *fca* forked canal anlage, *gu* gut, *ggl* ganglion, *ghr* rudiments of ganglionic horns, *lo* lophophore, *ne* neck of the bud, *ph* pharynx, *rc* ring canal, *rm* retractor muscle, *te* tentacle. Blue, lophophore; green, gut; mauve, ring canal; orange, retractor muscles; purple, forked canal anlage; red, epistome anlage; turquoise, funiculus; violet, lateral tentacle coelom; yellow, ganglion.

Figure 8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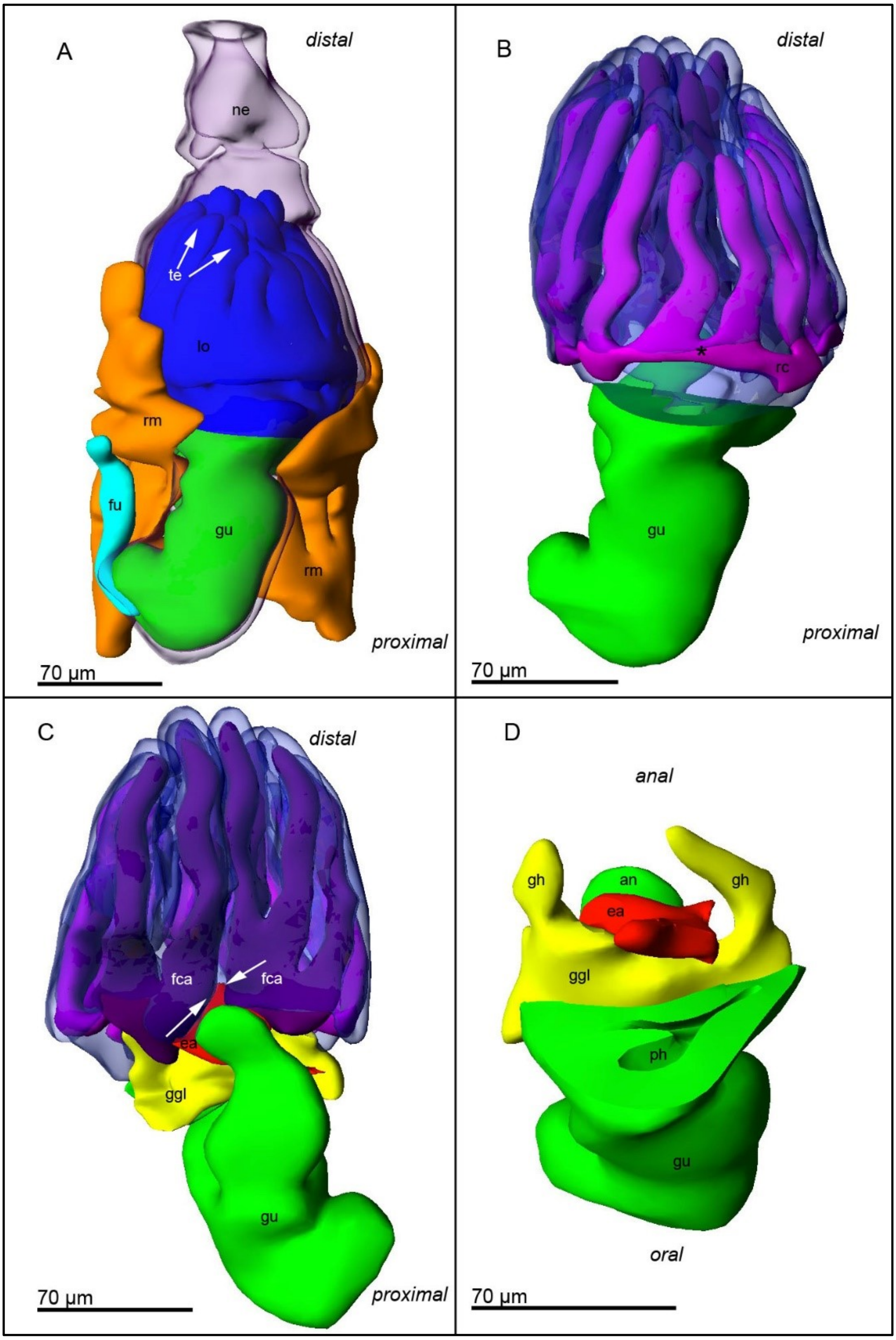
80 µm
D
anal
fca
fca
rc
*
rc
oral
80 µm

Figure 9. Segmentation-based 3D-reconstruction of the fifth stage of *Plumatella casmiana*. **A** Oral view of the bud with gut, lophophore with developed tentacles (arrows), retractor muscles and funiculus. **B** Oral view with gut and transparent lophophore to show the continuous ring canal. The median connection is marked with an asterisk. **C** Anal view with gut, ganglion and epistome anlage. Lophophore is displayed transparent to see the two parts of the unconnected forked canal anlage. Missing connection are marked between the arrowheads. **D** Distal view without lophophore to see the ganglion between the shanks of the gut. Between ganglion and anal part of the gut, the epistome anlage with the small flap-like extension is situated. Note the prominent ganglionic horns. *an* anus, *ea* epistome anlage, *fca* forked canal anlage, *gu* gut, *ggl* ganglion, *gh* ganglionic horns, *lo* lophophore, *ne* neck of the bud, *ph* pharynx, *rc* ring canal, *rm* retractor muscle, *te* tentacle. Blue, lophophore; green, gut; mauve, ring canal; orange, retractor muscles; purple, forked canal anlage; red, epistome anlage; turquoise, funiculus; yellow, ganglion.

Figure 9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gu
proximal
70 µm
D
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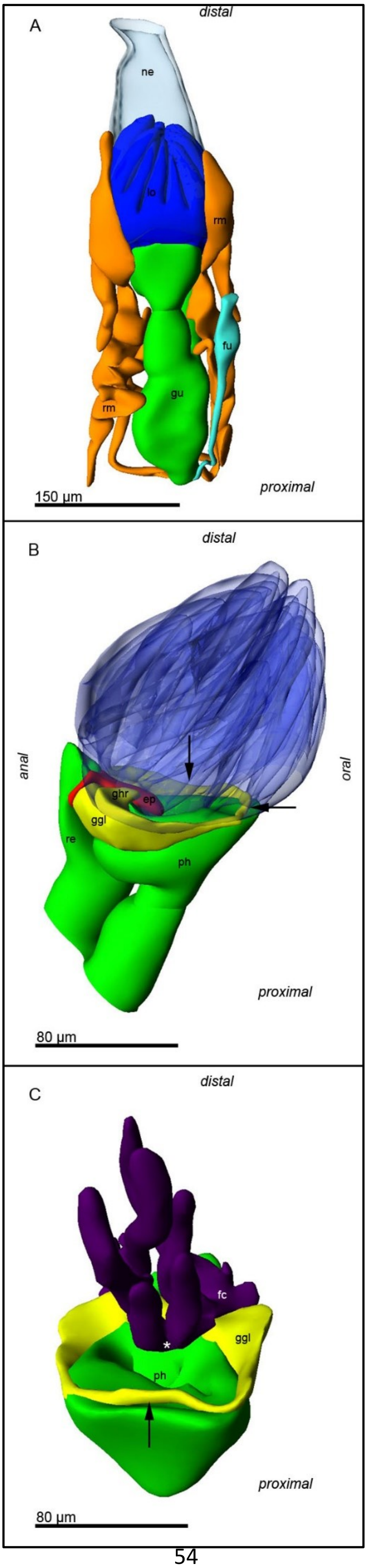
gh
an
gh
ea
ggl
ph
gu
oral
70 µm

Figure 10. Segmentation-based 3D-reconstruction of the sixth stage of *Fredericella sultana*. **A** Oral view of the bud for an overview. **B** Lateral view of the bud with partially removed gut. Additionally to the ganglion and rudiments of ganglionic horns, the circumoral nerve cord is completely developed (arrows). Note the flap-like extension of the epistome above the ganglion. **C** Oral view with partially removed gut. Circumoral nerve cord is marked with an arrow. Note the forked canal is continuous and the median connection is marked with an asterisk. *ep* epistome, *fc* forked canal, *gu* gut, *ggl* ganglion, *ghr* rudiments of ganglionic horns, *lo* lophophore, *ne* neck of the bud, *ph* pharynx, *re* rectum, *rm* retractor muscle. Blue, lophophore; green, gut; orange, retractor muscles; purple, forked canal; red, epistome; turquoise, funiculus; yellow, ganglion.

Figure 10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B
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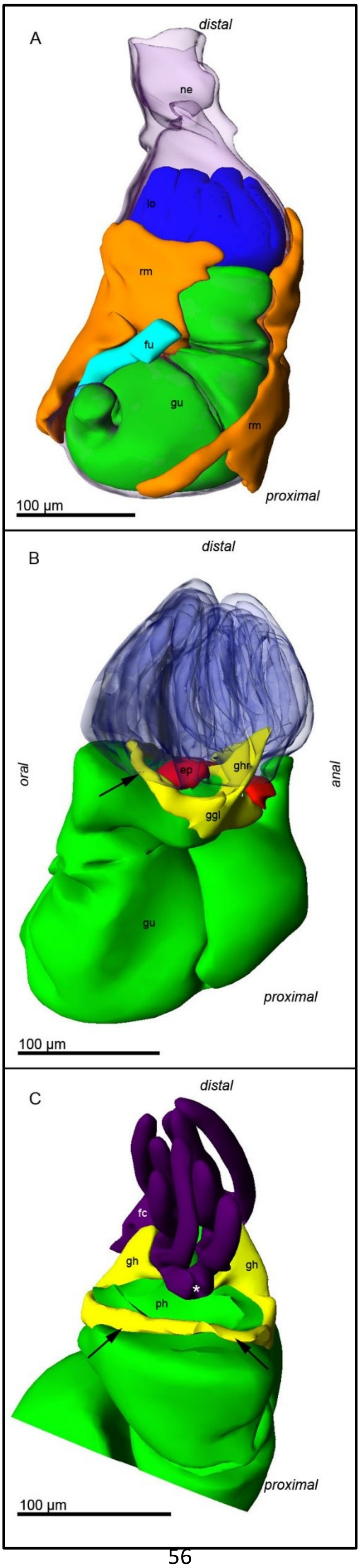
ph
proximal
80 µm
C
distal
fc
ggl
*
ph
proximal
80 µm

Figure 11. Segmentation-based 3D-reconstruction of the sixth stage of *Plumatella casmiana* **A** Oral view of the bud for an overview. Note that the reconstructed individuum has many foldings. **B** Lateral view with partially removed gut. The circumoral nerve cord is marked with an arrow. Note the well-developed ganglionic horns and the epistome with the typical flap-like extension. **C** Oral view with partially removed gut. Forked canal is continuous, and the median connection is marked with an asterisk. Both ganglionic horns are visible and are situated next to the forked canal. *ep* epistome, *fc* forked canal, *gu* gut, *ggl* ganglion, *gh* ganglionic horns, *lo* lophophore, *ne* neck of the bud, *ph* pharynx, *rm* retractor muscle. Blue, lophophore; green, gut; orange, retractor muscles; purple, forked canal; red, epistome; turquoise, funiculus; yellow, ganglion.

Figure 11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C
distal
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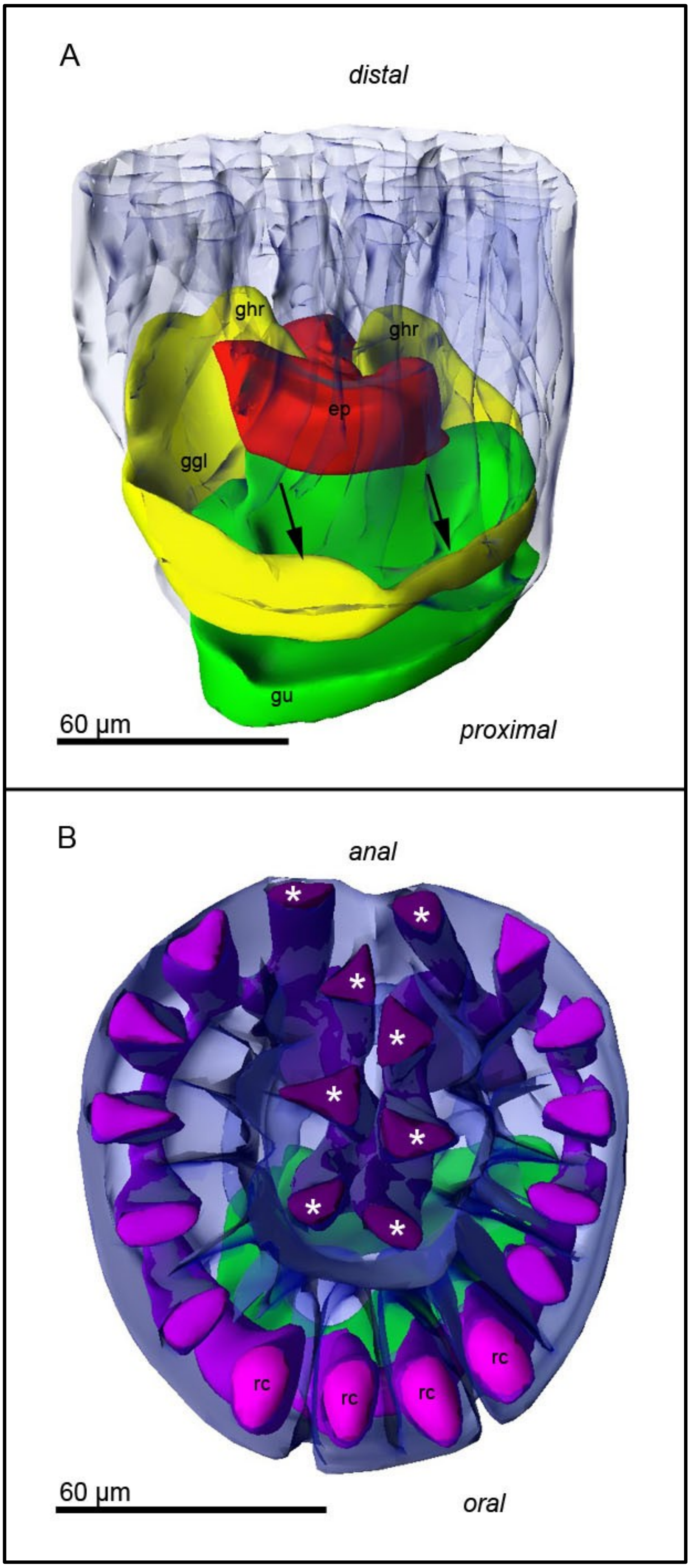
proximal
100 µm

Figure 12. Segmentation-based 3D-reconstruction of the adult lophophoral base of *Fredericella sultana*. **A** Oral view with lophophore displayed transparent. Circumoral nerve cord is very prominent and marked with arrows. The ganglionic horns are only as rudiment visible and the epistome is completely developed. **B** Distal view with lophophore displayed transparent shows the coelomic supply of the tentacles. Four oral tentacles are provided by the ring canal. The eight anal tentacles are provided by the forked canal (asterisks). The lateral unlabelled coelomic supply is not connected to a separated canal. *ep* epistome, *ggl* ganglion, *ghr* rudiments of ganglionic horns, *rc* ring canal. Green, gut; mauve, ring canal; purple, forked canal; red, epistome; violet, lateral tentacle coelom; yellow, ganglion.

Figure 12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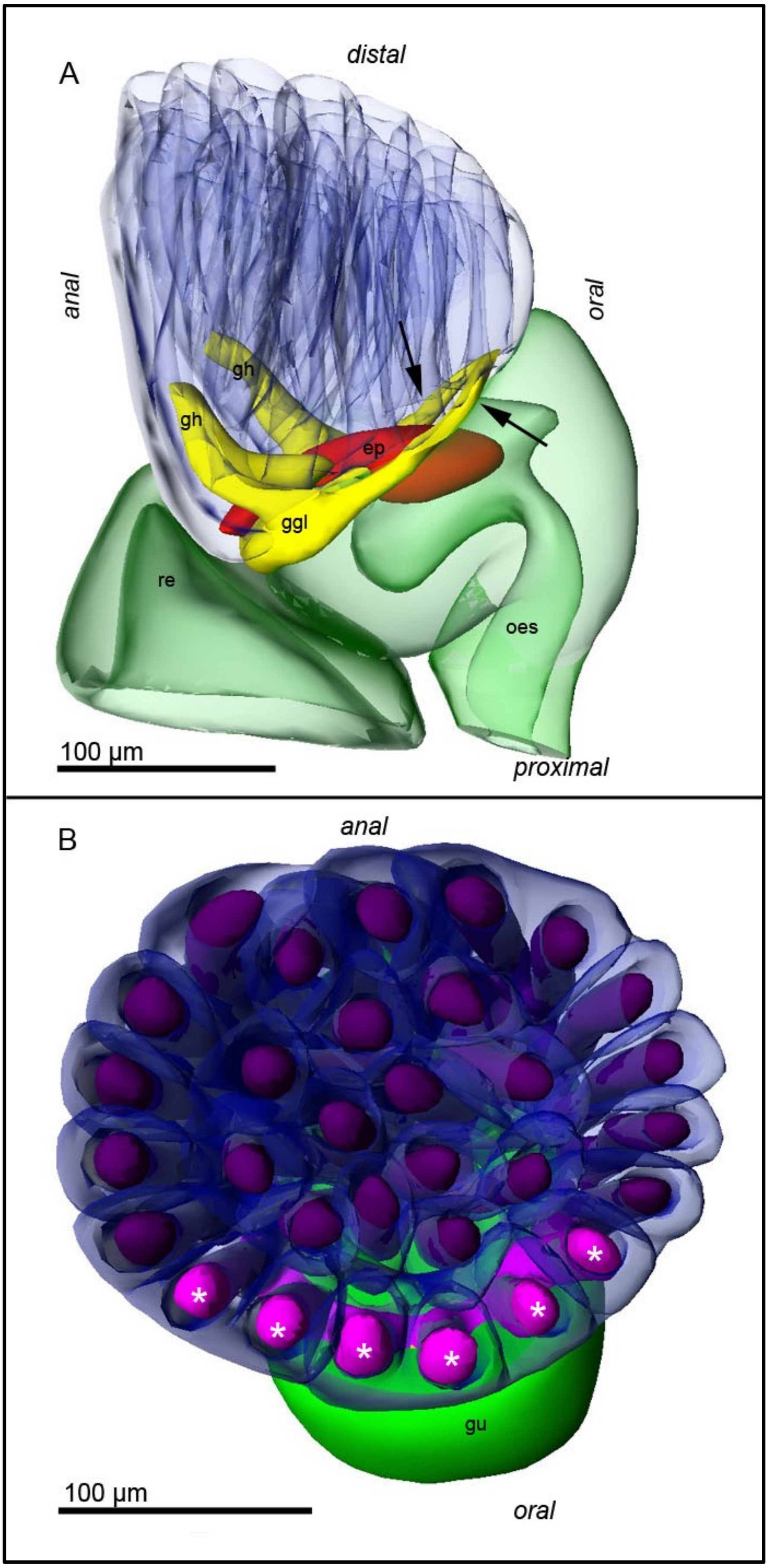
rc
rc
60 µm
oral

Figure 13. Segmentation-based 3D-reconstruction of the adult lophophoral base of *Plumatella casmiana.* **A** Lateral view with lophophore and gut displayed transparent. Ganglionic horns and circumoral nerve cord are well-developed. The epistome of this individuum is wide extended into the mouth opening. **B** Distal view with lophophore displayed transparent shows the coelomic supply of the tentacles. There are six oral tentacles which are provided by the ring canal (asterisks). The coelomic supply of the anal tentacles (forked canal) and the lateral tentacles are reconstructed together. *ep* epistome, *fc* forked canal, *gu* gut, *ggl* ganglion, *gh* ganglionic horns, *oes* oesophagus, *re* rectum. Green, gut; mauve, ring canal; purple, forked canal; red, epistome; yellow, ganglion.

Figure 13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Zusammenfassung

Die Klasse der Phylactolaemata lebt ausschließlich im Süßwasser und gehört zum Stamm der Bryozoa. Diese Süßwasser-Bryozoen besitzen einzigartige Merkmale: (1) ein Epistom, eine lappenartige Struktur oberhalb der Mundöffnung, (2) einen hufeisenförmigen Lophophor und (3) Ganglionhörner, Verlängerungen des Ganglions in die Lophophorarme. Die einzige Ausnahme innerhalb der Phylactolaemata stellt die Familie Fredericellidae dar. Diese verfügen über einen runden Lophophor, was eher auf eine Zugehörigkeit zu den größtenteils marin lebenden Bryozoen hindeutet. Diese Tatsache, gemeinsam mit der geringen Größe der einzelnen Zooide, stellte die Familie der Fredericellidae an die Basis der Phylactolaematen-Phylogenie. Aufgrund morphologischer und molekularer Untersuchungen wurde diese Annahme bereits widerlegt. Um festzustellen, ob während der Entwicklung eines Vertreters der Fredericellidae, *Fredericella sultana*, ein hufeisenförmiger Lophophor vorhanden ist, wurde die gesamte Organogenese während des Knospungsprozesses rekonstruiert. *Plumatella casmiana* wurde als Vertreter der nahe verwandten Familie Plumatellidae vergleichend untersucht, wobei ein besonderes Augenmerk auf die Lophophorentwicklung gelegt wurde. Der Lophophor entsteht aus zwei lateralen Wölbungen, welche zunächst auf der oralen Seite und später auch auf der analen Seite zusammenwachsen. Die Untersuchung zeigte, dass *F. sultana* in frühen Knospungsstadien einen herzförmigen Lophophor, sowie Ganglionhörner besitzt. Im adulten Zustand sind lediglich unilaterale Rudimente der Ganglionhörner vorhanden. Weiters wurde nachgewiesen, dass die Lophophorentwicklung von *F. sultana* der Phylactolaematen-Entwicklung folgt. Diese unterscheidet sich erheblich von der Entwicklung der Gymnolaematen und Stenolaematen. Zusätzlich beweist das Vorhandensein von Ganglionhörnern, wenn auch im adulten Zustand nur noch rudimentär vorhanden, dass Fredericellidae einst einen hufeisenförmigen Lophophor hatte. Es wird daher angenommen, dass der rund aussehende Lophophor durch eine sekundäre Reduktion der Lophophorarme entstanden ist.