universität wien

MASTERARBEIT / MASTER'S THESIS

Titel der Masterarbeit / Title of the Master's Thesis

„The embryonic development of the phylactolaemate bryozoan *Plumatella casmiana*“

verfasst von / submitted by

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angestrebter akademischer Grad / in partial fulfilment of the requirements for the degree of

Master of Science (MSc)

Wien, 2020 / Vienna 2020<

Studienkennzahl lt. Studienblatt / degree programme code as it appears on the student record sheet:	A 066 831
Studienrichtung lt. Studienblatt / degree programme as it appears on the student record sheet:	Masterstudium Zoologie / Master's degree programme Zoology
Betreut von / Supervisor:	Univ.-Prof. DDr. Andreas Wanninger

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Abstract

Bryozoans are a chiefly marine group of colonial sessile filter feeders within the Lophotrochozoa. All bryozoans are hermaphrodites, with an ovary developing from the body wall and testes often forming on peritoneal strands. In the exclusively freshwater inhabiting phylactolaemates, reproduction involves brooding in form of an invagination of the body wall, the embryo sac. Detailed studies on the sexual development of phylactolaemates date back to the late 19th and early 20th century and many aspects of development such as oocyte transfer and placentation need a reinvestigation owing to previous contradictive findings. This study focuses on the embryogenesis of the fresh-water species *Plumatella casmiana* by using serial-section 3D-reconstruction techniques and transmission electron microscopy. Early embryos develop into a single-layered blastula that forms a small central cavity. Subsequently, a mesodermal layer delaminates inside the blastula, following a gradient along the longitudinal axis from aboral to oral. In *Plumatella* two polypides form successively via ingression and proliferation of both tissues, resulting in a ciliated “mantle larva” that houses two zooids. The embryo temporarily forms two different contacts to the maternal tissues. Ultrastructural details show that ‘placental’ cells do not necessarily provide extra embryonic nutrition, and should thus instead be referred to as primary and secondary contact cells. Furthermore, cytological features like abundance of mitochondria, RER and increased surface area indicate that the embryo is nourished as the brood sac takes up nutrients from the maternal coelom and releases them into its internal lumen. Overall, this study confirms the general process of sexual development in plumatellids and shows small differences e.g. the timing of bud formation. Moreover, the modern methods and better preservation in the present study reveal the delaminating process of mesoderm formation and allow a sophisticated visualisation of developmental stages. Eventually, the present study shows, that ‘placental cells’ form differently from previous descriptions. Furthermore, a different way of matrotrophy (histotrophy) and hence revision of ‘placental cells’ is suggested. Consequently, independently formed ‘placental cells’ should rather be referred to as primary and secondary contact cells.

Introduction

Bryozoans are a clade of hermaphroditic, aquatic, colonial, sessile filter-feeding lophotrochozoans, which inhabit primarily marine but also freshwater habitats (Mukai et al, 1997). Although the phylogenetic position of bryozoans are still controversally discussed, recent studies position bryozoans within the lophophorates (Marlétaz et al., 2019; Laumer et al., 2019). Individual animals of a colony, called zooids, are subdivided into the polypide and cystid. The polypide is the soft-body part of the animal and includes, e.g., the tentacle-bearing lophophore with the mouth opening at its base and the u-shaped digestive tract, which terminates outside of the lophophore. The cystid constitutes the body wall and is composed of an outer, acellular ectocyst that is secreted by an inner endocyst. The endocyst is of cellular nature and consists of an ectodermal and mesodermal (peritoneal) layer. The polypide can retract into the cystid via prominent retractor muscles. Increase of the coelomic pressure leads to protrusion of the polypide through the cystid opening, named orifice (Mukai, 1982; Wood, 1983; Schwaha, 2019). In general, three taxa are distinguished: Phylactolaemata, a group restricted to freshwater; the marine Stenolaemata, with the Cyclostomata as the only extant clade, and the Gymnolaemata. The latter include the exclusively marine, most speciose group of Cheilostomata, which evolved from the paraphyletic “Ctenostomata” (Waeschenbach et al, 2012), of which some species occur in brackish/ freshwater (Reed, 1991; Mukai et al., 1997). In contrast to the dominant marine bryozoans (Cheilostomata, Cyclostomata), phylactolaemates (and ctenostomes) possess a non-calcified ectocyst of gelatinous, chitinous, or sand-encrusted nature. Phylactolaemates are larger in zooid size and are characterized by a horseshoe-shaped lophophore with a protruded lobe above the mouth opening (epistome) and a regular grid of body wall musculature between both layers of the endocyst (Wood, 1983; Gruhl et al, 2009).

Bryozoans reproduce mainly via asexual budding from the body wall. Most phylactolaemates form new buds exclusively on the oral side of the zooid with the first (oldest) bud developing furthermost (proximal) from the orifice (Braem, 1897). The first bud is followed by additional buds that form consecutively in direction of the orifice, which results in a budding direction from proximal to distal on the oral side of each zooid. Another apomorphic character of the phylactolaemates are statoblasts, i.e. internal buds which serve for overwintering and dispersal (Wood, 2014) and are adaptations to freshwater habitats (Mukai, 1982). Besides asexual budding, bryozoans also show sexual reproduction which results in distinct larval types in different groups of bryozoans. Many bryozoans brood their embryos (Ostrovsky, 2015). In phylactolaemates, embryos are brooded in internal brood sacs that originate from the endocyst on the oral body wall. With respect to asexual buds and the ovary its position is closest to the orifice. Consequently, a preserved arrangement of reproductive structures is present along the oral body wall in phylactolaemates: a series of buds, followed by the ovary and the brood sac (from proximal to distal on the oral side of the body wall). Most previous studies focused on the asexual reproduction whereas many questions on the sexual development, e.g., gametogenesis, fertilisation, oocyte transfer requires a reinvestigation, also on ultrastructural level (Mukai, 1982). In addition, an essential process in embryogenesis, the formation of the mesoderm, needs to be revised in order to clarify descriptions of early authors (Hyman, 1959).

In general, six families belong to the phylactolaemates (Schwaha, in press). The most speciose taxon are the Plumatellidae comprising approximately six genera with a branching colony morphology. Besides gelatinous ectocysts, (*Gelatinella*, *Hyalinella)*, also transparent, sand encrusted or chitinous ectocysts occur (many species of *Plumatella)*. Although most *species* of the family are creeping, erect branching colonies also occur (Braem, 1890; Toriumi,

1952). Plumatellids are regarded as sister-group to the Fredericellidae (Waeschenbach et al., 2012). In addition, they also are the best studied taxon and most morphological analyses focused on *Plumatella repens* or *fungosa*.

Sexual reproduction was studied mainly in the plumatellids, *Plumatella fungosa* (Braem, 1897; Brien, 1952), *Plumatella casmiana* (Mukai, 1982), and *Fredericella sultana* (Braem, 1908), as well as the lophopodid *Lophopus crystallinus* (Marcus, 1934) and the cristatellid *Cristatella mucedo* (Davenport 1981). The most profound study on sexual development in phylactolaemate bryozoans is on the globally distributed and common species *Plumatella fungosa* (Braem, 1897). Since bryozoans are hermaphrodites, gametes of both sexes form in each zooid. Spermatogenesis occurs on the funicular cord and mature sperm has been observed to leave the coelom via the vestibular pore (Wiebach, 1953; Oda, 1958). Although it is not clear how they enter zooids of different colonies, cross fertilisation has been shown (Joy, 1994). Sperm was observed to enter the ovary in *L. crystallinus*, which led to the conclusion that fertilisation takes place in the ovary (Marcus, 1934). In contrast, however, in *P. fungosa,* fertilisation occurs when an oocyte is directly transferred into the embryo sac (Braem, 1897). The fertilised oocyte is brooded within the brood sac. Within bryozoans, the cleavage of the phylactolaemates seems to be most derived of the spiralian cleavage, but only little information is available. In general, their cleavage is holoblastic and irregular and results in a hollow blastula (Braem, 1897; Santagata, 2015.). The developing embryo is temporarily accompanied by a supposed placenta-like structure that potentially serves for extra embryonic provisioning (Braem, 1897; Ostrovsky et al., 2009; Ostrovsky, 2013a, b). The sexual development of phylactolaemate bryozoans results in a short-lived “mantle larva” that contains a ciliated hull that encloses two functional polypides. (Schwaha et al.2015; Reed, 1991 and Mukai, 1982).

The development from the zygote to the larva has only been studied in the late 19th and early. 20th century and requires reinvestigation with more modern methods. Several questions especially concerning early development, remain open: location and moment of fertilisation have been contradictorily described for various families. Ultimately, there is no evidence for fertilisation in plumatellids (Kraepelin, 1892; Braem, 1897, 1908; Marcus, 1934). Direct transfer of oocytes into the embryo sac has not been described except for two species (Braem,1897; Braem, 1908). In addition, the formation of a placenta remains ambiguous. The origin of the cells referred to as 'placental cells', which form a ring around the embryo in *P. fungosa* (Braem, 1897) or an aboral disc in *Fredericella sultana* (Braem, 1908), remains unclear. Comparison of placentas in phylactolaemate bryozoans (Braem, 1897, 1908) requires reinvestigation. Also, the function of these 'placental cells' remains unclear. The term placenta assumes a nutritive function, however it could simply serve as an anchoring structure to keep the embryo in place (Braem, 1897; Hyman, 1959).

The present study aims to provide the first reconstruction of embryogenesis of a phylactolaemate bryozoan, exemplified by *Plumatella casmiana* using state-of-the-art techniques. Embryogenesis is described based on semi thin sections and 3D-reconstructions. Questions such as fertilisation and oocyte transfer are addressed. Furthermore, it aims to clarify how the placenta is formed and infer the function of the 'placental cells.'

Material methods

Specimen and sample preparation

Samples of colonies of *Plumatella casmiana* were collected from a pond of the Faculty of Fisheries at the Kasetsart University in Bangkok, Thailand. Colony pieces were fixed in 2% glutaraldehyde (GA) in 0.01 mol l^{-1} phosphate buffer (PB) for one hour and rinsed several times in PB. Samples were post fixed in 1% osmium tetroxide (OsO_4) in double distilled water for 1 hour and rinsed in double distilled H_2O. Then, samples were dehydrated with acidified 2-2-dimethoxypropane (DMP) before being embedded in Agar Low Viscosity resin (Agar Scientific Ltd, Essex, UK) using acetone as intermediate.

Sectioning and 3D-Reconstruction

Ribbons of serial semithin sections of 1.5 µm thickness were produced on a Leica UC6 Ultramicrotome (Leica Microsystems GmbH, Weitzlar, Germany) using a Histo-Jumbo diamond knife (Diatome AG, Biel, Switzerland), for details see Ruthensteiner (2008). Sections were stretched at ~70°C and attached to a glass slide. For resectioning of semithin sections for TEM, the slide was kept on the heat plate just shortly until the remaining water had evaporated in order to prevent too strong adhesion of sections to the glass slide. Sections were stained with toluidine blue for 10 to 15 seconds at 60°C. Before sealing slides with a cover slip, single sections were chosen for transmission electron microscope investigations.

Embryos of different developmental stages were analysed and photographed on a Nikon Ni-U compound microscope with a Nikon DS-Ri2 camera (Nikon, Tokyo, Japan). Image stacks were then converted to grey scales with Photoshop CS6 (Adobe, San Jose, CA) and imported into the reconstruction software Amira 6.3 (Mercury Computer Systems, Chelmsford, USA). Images were automatically aligned using the alignment tool of Amira with some manual

corrections. Segmentation of the structures of interest was done manually using the brush tool and interpolation between sections. Afterwards, a polygonal surface was created based on the segmentation with the SurfaceGen module of Amira. Optimisation of surfaces was conducted by repeated triangle reduction and smoothing steps. Finally, snapshots of reconstructed embryos were edited with Photoshop.

Electron microscopy

For ultrastructure investigations of 'placental cells' and other structures of interest ultra-thin sections of 60 nm were resectioned from semi-thin sections (see Handschuh et al, 2013). First, uncovered semi thin ribbons were checked for embryos. Chosen sections were separated from the ribbon by cutting the borders of the section with a razor blade. Afterwards a drop of MQ water (Merck KGaA, Darmstadt, Germany) or double distilled water was placed next to the section of interest. One edge of the section was lifted with the tip of a needle in order for water to flow underneath the section. From that point on the section was repeatedly lifted to drag the water under the section until the section is fully detached from the object slide. The detached section was then mounted on an empty resin block. Prior to that the latter had been trimmed to a surface area just larger than the section to be mounted. The empty block surface was polished by cutting with a Histo-Jumbo diamond knife or an ultra 45° diamond knife (Diatome AG, Biel, Switzerland) on a Leica UC6 Ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Before the detached section was transferred onto the resin block, a droplet of dd H_2O was placed on the polished surface. Then, the detached section was picked up with the needle tip and released on the water drop on the block surface. Excess water was removed with filter paper to position the section without wrinkles. Next a plastic cup was placed over the block holder to reduce the amount of dust that adheres to the surface while the block was in the oven for at least 30

minutes (up to several hours) at 60°C. The mounted semithin section was trimmed to a small area of interest with the entire section properly attached to block surface. Due to inevitable dust particles on the section, narrowing of the knife worked best under dry conditions. The ultrathin sections were placed on copper grids. Dry sections were contrasted with 1% Uranyl-III-Acetate (UA) for 8 minutes, followed by 8 minutes in 2.5% lead citrate (Laurylab, Brindas, France) with at least three rinses in dd H_2O after each medium. Transmission electron microscopy was performed on a Zeiss Libra 120 (Zeiss, Oberkochen, Germany). Images were taken with a post column ccd camera (Sharp:eye TRS (2 × 2 k); Tröndle, Moorenwies, Germany) and the image SP software (Sysprogs OÜ, Kaiserslautern, Germany).

Results

General overview of reproductive structures

Single zooids show a preserved arrangement of organs involved in sexual as well as asexual reproduction (Fig. 1A). Asexual buds, the ovary and the embryo sac are located on the oral cystid wall in a certain pattern. The first and largest bud develops most proximally of the orifice. Additional buds from the mother zooid form distally of the first bud distally towards the orifice. The sexual reproductive structures, the ovary and embryo sac, are always located distally to the buds and are closest to the orifice. The ovary develops from the mesodermal layer of the maternal endocyst, adjacent to the youngest bud of the mother zooid: Some peritoneal cells successively enlarge and differentiate into oocytes protruding into the coelomic cavity (Fig. 1B; 2A-E). Formation of oocytes occurs in the mesodermal part of the body wall (Fig. 1B; 2A), with the thin peritoneal layer surrounding the differently developed oocytes (Fig. 2C, D, E), which in summary constitute the ovary. It is grape-shaped and protrudes from the body wall into the coelomic cavity with the oldest (hence largest) oocytes situated at its suspended end (Fig. 2C; 3A).

The ovary with several oocytes is formed from the mesodermal layer of the body wall, slightly delayed an embryo sac including maternal ectoderm develops. The embryo sac develops closest to the lophophore (Fig. 1A) and in contrast to the ovary is formed by the ectoderm and the mesoderm of the maternal cystid (Fig. 2A-D). At the beginning of its formation, cells of the ectoderm become high prismatic causing the body wall to bulge into the coelomic cavity. On the inside the thickened ectoderm is limited by a little differentiated mesoderm (Fig. 2A). The cells of the ectoderm continuously proliferate with the regular body musculature leaving a gap in the zone of the embryo sac, similar to normal buds (Fig. 4A-C). In

this way a distinctive cell mass grows into the coelomic cavity of the mother zooid. This cell mass first fills the lumen of the embryo sac. It will be referred to as embryo sac cavity (esc) herein and stains less intense than the surrounding tissue (Fig. 3C; 4B). The embryo sac cavity is not in contact with the coelomic fluid but is surrounded by a flattened layer of the peritoneum (Fig. 2D) which is part of the embryo sac. The cells of the embryo sac increase in volume and change from a flat to a cubic epithelium, which grows with the brooded embryo (Fig.4B, C).

Gametes

Mature oocytes are found in the well-developed ovaries, with oocytes close to the body wall being smaller than mature ones at the end of the ovary (Fig. 3A). Each oocyte is surrounded by a thin peritoneal layer and often accompanied by follicle-like ovarian cells (Fig. 3A). Oocytes possess a large nucleus with two nucleoli when they are mature. They are comparatively large and include small dark granules, referred to as yolk droplets, in the periphery of the cytoplasm (Fig. 3A). Fully developed ovaries persist until late developmental stages and can reach sizes comparable to the embryo sac in advanced developmental stages. Sperm differentiates in grape-like arrangement along the funiculus (fig. 3B). Cross sections of sperm cells were sporadically found in the coelom. Occasionally, single oocytes were encountered in the coelomic cavity of the maternal zooid. There were, however, still located within the ovary. Although the ovary is situated in close association with the embryo sac (Fig. 3D) a direct contact between embryo sac and ovary was not found. No indications of fertilisation or oocyte transfer were observed. A sole finding of a zygote that had divided into several blastomeres within the embryo sac is the earliest evidence of embryonic development (Fig. 3C).

Embryology

Early stage

The youngest developmental stage found in the present study is an embryo consisting of approximately 20 cells (Fig. 4C, D) that are situated within a rather thick-walled embryo sac. In this stage the cells have formed a blastula with a small central cavity (Fig. 3D; 4C, D; 5B). The cells appear voluminous and show large nuclei that contain intensely stained nucleoli. The embryo is surrounded by a thin, unicellular layer of maternal ectoderm, (Fig. 3D; 4C, D). From this point on, the ectodermal lining degenerates, starting from the suspended end of the embryo sac (Fig. 4D). In general, the surrounding ectoderm represents remnants of the prominent embryo sac cavity, which once filled the entire lumen of the forming embryo sac and later is situated on the distal top of the embryo (Fig. 4B, C; 5B, C; 6B, C). Externally, the mesodermal layer of the embryo sac separates the embryo from the coelomic fluid of the maternal zooid. Occasionally, dividing nuclei of the mesoderm of the embryo sac are present. Also, different sizes of nuclei and number of nucleoli suggest high activity within mesodermal parts of the embryo sac. Already at this stage, cells of the embryo show extensions towards the mesoderm as well as the ectoderm of the embryo sac (Fig. 4D; 6B, D). Although a direct cell contact is not (yet) always verifiable, a possible contact area is present on the embryo sac side (Fig. 4C, D; 6B, D). Cells of the embryo are tightly opposed to the maternal, especially mesodermal tissue, with possible cytoplasmatic extensions sometimes more abundant at the suspended hemisphere. Only in some early embryos, densely stained cytoplasmatic inclusions were present within both layers of the embryo sac (Fig. 4C, D). They appear to be in close association with the embryo at first but then are located within the maternal ectoderm (Fig. 4D). Such dark inclusions are also found within and along the whole mesoderm of the embryo

sac (Fig.4C) and occasionally a coelomocyte is found on the outside of the brood pouch (Fig. 8F).

Mesoderm formation

Concerning the orientation of the embryo, the upper part close to the embryo sac cavity is referred to as aboral/ posterior side owing to the swimming direction of the fully developed larva, whereas the opposite side, on the bottom of the embryo sac, is referred to as oral/ anterior side. Once the embryo has formed a blastula, cells at the aboral pole divide and produce a second layer of embryonic tissue via delamination (Fig. 3D; 6D). Delamination starts from the aboral pole (Fig 5C) and continues proximally along the longitudinal axis. In total, the mesoderm of the embryo delaminates from the outer maternal ectoderm following a gradient from aboral (top) to oral, while the embryo simultaneously grows and elongates. As a result, the mesoderm is well developed on the aboral side whereas at the bottom the mesoderm forms a very flat epithelium (Fig. 7; 8B). At the end of mesoderm formation a bilayered embryo is present (Fig. 5D; 7; 8B).

The ectodermal lining of the embryo sac further decreases but still surrounds most of the embryo (Fig. 7B). There are also indications of contact between cells of the maternal ectoderm and the ectoderm of the embryo (Fig.6B, D): mainly in the aboral part of the embryo, weakly stained cells of the maternal ectoderm project towards the embryo and vice versa. The mesodermal layer of the embryo sac remains unaltered and still consists of voluminous cells which are sometimes in close association with the embryo where the ectodermal lining has vanished (Fig. 7B, D)

Bud formation

In the course of development several processes occur simultaneously. As the embryo continuously grows, the ectoderm forms the mesoderm that further divides on its own, after it initially derives from the ectoderm. When the mesoderm lines nearly the entire interior of the embryo except the still forming oral side, the cells on the aboral side are isoprismatic and together with the ectoderm form a bud via ingression (Fig. 8A, B; 9). Cells of the ectoderm divide (Fig. 9A, B; 10C) and thereby protrude into the lumen of the embryo (Fig. 9A-C). Since ingression takes place between both embryonic tissues, ingressed cells of the ectoderm are surrounded by proliferating mesoderm (Fig. 9D; 10C). In this way both layers of the embryo grow inwards and form a bud attached to the aboral pole. In total two buds, the anlagen of future polypides, develop. When the first anlage has formed a cup-like structure that protrudes into the embryonic cavity, a second bud develops in identical way next to it (Fig. 10, 11).

Late stages

Developing buds continuously grow and it is difficult to assess which of the buds has formed first. Both layers of the buds participate in the formation of the organs of the differentiating adult polypides (Fig. 12). Simultaneously, the embryo and the embryo sac enlarge. In earlier stages the cells of the embryo sac divide, whereas in later stages no mitotic activity was detected and they seem to stretch with the growing embryo. Thus, the embryo sac lining becomes very thin, composed of only a few cells (Fig. 12). However, several cells of the embryo sac (mostly on the oral side) remain thicker and more prominent (Fig.12, 13A, B).

The oral hemisphere of the bilayered embryo, which is not involved in budding, thickens (Fig 12, 13A , B) and becomes the mantle of the late embryo/early larva. The

previously mentioned embryonic cells with possible cytoplasmatic extensions have established contact to the mesoderm of the embryo sac and are now located halfway along the oral-aboral axis. The cells connecting embryonic ectoderm and maternal mesoderm are referred to as 'placental cells'. The mantle invaginates and forms a groove directly below the 'placental cells' (Fig. 11C, 12A, 13A, B). This groove, referred to as mantle fold, overgrows the embryo in direction of the aboral pole, ultimately leaving just a small orifice (Fig. 12B, 13C, D) through which the two zooids are connected to the environment when the larva hatches. During all these processes, the embryo/larva increases its size by a hundredfold and ultimately results in a ciliated ancestrula/mantle larva that contains two fully functional zooids.

Maternal contact

Contacts with embryonic and both maternal tissues were observed. As mentioned above, a close association of the maternal ectoderm of the embryo sac and the embryo was found during mesoderm formation (Fig. 6B, D) and is indicated by possible contact areas of maternal and embryonic tissue in earlier stages (Fig. 4C, D). Evidence of direct contact between the embryo sac cavity and the embryo is present from an early, gastrula-like stage onwards (Fig. 14A). These connections were mostly observed at the aboral/apical pole. They were not confined to the embryo sac cavity but were also observed connecting the embryo to the ectodermal lining of the the embryo sac (Fig. 14B, C). Since the ectoderm continuously vanishes from the oral side on, there are no connections to the ectoderm found in the oral half at later stages. However, there is contact of several of the most aboral embryonic cells and the degenerating embryo sac cavity when the second bud forms (Fig. 14D). Irrespective of the developmental stage of the embryo, cells that originate from the maternal ectoderm

and that are in contact with the embryo possess a smaller nucleus and nucleolus when compared to embryonic cells (Figure 14). A single cell is in contact with the embryo, which appears brighter than the remaining ones (Fig. 14A). Starting in early embryogenesis, the embryo is connected to the inner lining of the embryo sac and a clear contact to the embryo sac cavity persists until later stages.

Besides connections between the embryo and the maternal ectoderm, connections to the mesoderm of the embryo sac are also present. Similar connections were previously observed and were considered to nourish the embryo and therefore referred to as 'placental cells'. Consequently, the term 'placental cells' is used herein for connections of the embryo and the mesoderm of the embryo sac. These 'placental cells' were first observed at the stage when mesoderm formation is almost complete and the first bud is about to develop (Fig. 8D-F). Initially, emerging contacts are not restricted to certain regions of the embryo but are patchy distributed around the embryo (Fig. 14) until the inner lining vanishes. Depending on the section plane, single cells of most likely the embryo are shown to form a contact to the embryo sac (Fig. 8D-F; 13G, H; 15A, B). However, also several neighbouring embryonic cells can establish contact from the embryo to the embryo sac on a wider contact area (Fig. 13B, C; 15A). These interconnections seem to be formed by cytoplasmatic extensions of embryonic cells (Fig. 8E, 15A,). In addition, corresponding cells of the embryo sac (mesoderm) often seem to project towards the embryo which links these two tissues. Occasionally, the cytoplasm of cells of the embryo sac appears brighter than the embryonic ones, similarly to the linked ectoderm cells (Fig. 8D-F). In most investigated embryos, 'placental cells' also form at the area where buds are formed (Fig. 10A, D), approximately halfway along the longitudinal axis (equatorial region) of the embryo. As the embryo and the embryo sac grow all 'placental cells' come to lie in this region. At later developmental stages the thickened body wall of the embryo

constitutes the mantle, which forms a circular groove proximal of these ‘placental cells’ (Fig. 13A, B). Later this mantle fold overgrows the entire embryo in oral direction and breaks through these cells, resulting in loss of direct contact from the embryo to its mother zooid (Fig. 13 C, D). On first sight it seems that mainly embryonic cells are responsible for the established connections (Fig. 13E, F), but it is not always clear whether the ‘placental cells’ belong to the embryo or the embryo sac (Fig. 13G, H). In some embryos it appears as if they would originate solely from the embryo sac (Fig. 13I). In these rather late stages, the ‘placental cells’ are often bordered by several others on the side of the embryo sac (Fig. 13E, H). These cells of the embryo sac remain quite distinct when compared to the very thin remaining mesoderm of the embryo sac. Also, these cells stain less intense and sometimes include dark spots as reported for early ontogenetic stages (Fig. 13F). In conclusion, it can be stated that ‘placental cells’ establish all over the embryo as soon as the inner lining of the embryo sac decreases. In the course of development, the contact to the maternal zooid increases and is restricted to the equatorial region before it breaks down due to the overgrowing mantle.

In summary, two forms of contact between mother and offspring are established during embryonic development. At first the embryo is linked to the maternal ectoderm, which decreases with progressed development. A well-established contact to the also decreasing embryo sac cavity persists until late embryonic stages. While the ectoderm disappears, single cells of the embryo project to the remaining mesoderm of the embryo sac and vice versa. In this way ‘placental cells’ establish contact between mother and offspring. This contact is maintained until the mantle extends and covers the entire larva at latest stages.

Ultrastructural details of contact areas

As stated above, the term ‘placental cells’ is used on the basis of pioneering studies from the late 19th century, which concluded a possible nutritive function of these cells. The first analysed stage is of a bilayered embryo whose mesoderm has formed just recently. Details of both layers of the embryo as well as the embryo sac reveal ultrastructural differences of maternal and embryonic tissues (Fig. 16). The embryonic tissue shows that both layers consist of high prismatic cells with large nuclei at the basal side of the cells (Fig. 16A). On the inner side, two vacuoles with unknown content are present at the apical side of the embryonic mesoderm (Fig. 16D). Besides numerous large mitochondria, the same cell shows formation of vesicles on its basal side, although it cannot be distinguished whether it is exo-/or endocytosis. (Fig. 16A, D). In the cells of the embryonic ectoderm several mitochondria are present. Apart from this the ectoderm appears rather inconspicuous. It is full of ribosomes and some multivesicular bodies (Fig.16B). Moreover, it shows filiform extensions towards the embryo sac. When compared to the mesoderm of the embryo sac, the latter has a lower density of ribosomes, but highly abundant endoplasmatic reticulum (Fig. 16C). In addition, exocytosis occurs from the embryonic mesoderm to the ectoderm of the embryo (Fig. 16D, arrow).

Different sections of the bilayered, medium-sized embryo show again numerous filiform extensions from the embryonic ectoderm that extends towards the embryo sac. Cells of the latter form massive outgrowths, sometimes including vesicles (Fig. 17A). In addition, several secretions of the embryo sac mesoderm into the lumen of the embryo sac were observed (Fig. 17A, upper insert). The extensions of the embryo sac do not only project towards the embryo, but also seem to interdigitate (Fig. 17B). Although no direct cell contact

was found between mother and embryo there is a high probability that they are interconnected (Fig. 17B, lower insert).

As indicated on a light microscopic level for early stages, there is electron microscopic evidence for the close association of maternal and embryonic tissue at a stage when the embryo is about to develop its first bud (Fig. 18). Three cells of the embryonic ectoderm are firmly interconnected and collectively form a pit. (Fig. 18A, D). On the corresponding position at the embryo sac side, a multivesicular body secretes its content almost directly in the thin gap between embryo sac and embryo (Fig. 18D). In general, the embryo sac appears active with some autophagosomes and numerous vesicles, partial in open contact with the lumen of the embryo sac (Fig. 18A, C). However, not only the inner side of the embryo sac facing the embryo shows high activity but also the outer side that is in contact with the coelomic fluid of the maternal zooid (Fig. 18B, E). Plenty of small, pit-like invaginations are found on the outer side of the embryo sac, potentially indicating endocytosis (Fig. 18B). Moreover, large multi vesicular bodies are present on both sides of the embryo sac (Fig. 18C, E).

In total, the ultrastructural data show that the embryo forms filiform extensions towards the embryo sac. In addition, vesicles on the apical side of its ectoderm are in open contact with the lumen of the embryo sac. The latter shows numerous situations in which multi vesicular bodies release their content into the same lumen. Also, outgrows of the embryo sac towards the embryo appear more massive when compared to their embryonic counterparts. The mesoderm of the embryo sac appears more active than the embryonic ectoderm and shows numerous indications of endocytosis on its side facing the coelomic cavity (apical pole).

Discussion

The sexual reproduction and development of phylactolaemates is poorly investigated. The little information available comes from few studies that mainly deal with *Plumatella fungosa* and *Fredericella sultana* (e.g. Braem 1890, 1897, 1908), which is why present findings will be discussed on the basis of the pioneering studies and more recent reviews (e.g. Mukai, 1982; Mukai et al., 1997; Schwaha, 2019).

Overview of reproductive structures in phylactolaemates

All phylactolaemate bryozoans with the exception of *Stephanella* form the buds exclusively on the oral side of the cystid wall (Schwaha, in press), which also applies to the investigated species *Plumatella casmiana*. Further, the investigated species shows a preserved arrangement of reproductive organs along the oral body wall, namely several buds, followed by the ovary and the embryo sac located closest to the orifice. This situation is concordant with previous investigations (Braem, 1897). This preserved arrangement occurs in *Plumatella casmiana* and is described for *Plumatella fungosa* (Braem, 1897) but also in the close related *Fredericella sulatana* (Braem, 1908). With some exceptions concerning the sequence of bud formation, this arrangement represents the ground pattern in phylactolaemates (Braem, 1880, 1890).

Gametes

In *Plumatella fungosa* oocytes differentiate from the mesodermal/peritoneal tissue. At first, they are not distinguishable from other peritoneal cells until their nucleus enlarges and

nucleoli become visible (Braem, 1987). The oocytes of *P. casmiana* develop successively with the first and hence oldest oocyte closest to the embryo sac; consecutive oocytes differentiate towards the youngest bud (fig. 2A), so that in the initial phase the youngest oocyte neighbours the youngest bud. Since the oocytes differentiate from mesodermal cells, they also surround by mesoderm. As a result, the oocytes are in close contact to the latter, so that when the oocytes grow, they are accompanied by follicle-like ovarian cells (Fig. 3A; Mukai, 1982), although a complete follicular envelope is generally absent. As the oocytes mature, they protrude into the coelom of the reproductive zooid (Fig. 2 B-D), whereas supply of new oocytes comes solely from the distal side. At the end of oogenesis of *P. casmiana*, a thin epithelial sac protrudes from the cystid wall and includes several differently matured oocytes, giving the ovary a grape-like shape as in *P. fungosa* (Braem, 1897). The matured oocyte of *P. casmiana* is rather large, with a big nucleus including up to two nucleoli, although mostly just one evidentially present, which is consistent with descriptions of *P. fungosa* (Braem, 1897). In the initial phase of oogenesis, the nucleolus appears rather large in comparison the nucleus that, in turn, is rather small in relation to the cytoplasmatic part of the oocyte. In addition, the cytoplasm appears homogenous at the beginning (e.g. Fig. 7C, 8B), whereas yolk droplets occur essentially in the periphery when the oocyte matures. This is congruent with other studies (Braem, 1897; Mukai et al., 1997), whereby the initially homogenous cytoplasm stratifies in the course of ripening, resulting in a large oligolecithal oocyte with yolk granules in the periphery. This is essentially true for all investigated phylactolaemates (e.g. fredericellids (Braem, 1908), plumatellids (Braem, 1897; Mukai; 1982), *Lophopus* (Marcus, 1934)). Interestingly, changes of the nucleoli (in size, form, degree of vacuolisation) in *Asajirella gelatinosa* (Tazima et al, 1984) which are extrapolated for all phylactolaemates (Mukai et al., 1997), are characteristic for macrolecithal oocytes. The ultimate function of

these changes are not clear (Tazima, 1984). In total, numerous (more than ten) large, oligolecithal oocytes develop. This is slightly different from the fredericellid *Fredericella sulatana* which produces a maximum of five oocytes (Braem, 1908) that are half the size of *Plumatella* (although the relation of the diameters of cell and nucleus remains 2:1). Consequently, the ovary of the former does not protrude from the body wall in a grape-like shape, but rather forms a knot in the body wall on a broader area but still close to the embryo sac (Braem, 1908).

Fertilisation and oocyte transfer

As the spermatogenesis was excessively studied by other authors (e.g. Braem, 1897; Franzèn, 1977), and is of little relevance in this study, it is just mentioned that the formation of the testes is observed along the funiculus in *Plumatella casmiana*, which is true for most phylactolaemates (Mukai et al., 1997). Mature sperm leaves the coelom through pores at the tips of the tentacles (Wiebach, 1953; Okamura, 1995; Ostrovsky, 2013). Nevertheless, sperm occurs within the coelom implicating the possibility of self-fertilisation. However, genetic fingerprinting of *Cristatella mucedo* has shown that outcrossing occurs (Jones et al, 1994). The mechanisms how sperm can enter a zooid are so far unknown; also, the feasibility of self-fertilisation is not fully declined (Oka and Oda 1948, citied in Mukai 1982). The earliest result of sexual reproduction found in the present study is a very early embryo consisting of several blastomeres (Fig. 3C), located at the suspended end of the embryo sac. The question remains how a mature oocyte is transferred from the ovary into the embryo sac, including timing and location of syngamy. In fact, there is barely any evidence of fertilisation except for some data

on *Lophopus crystallinus* (Marcus, 1934). Ultimately, many questions on fertilisation and oocyte transfer remain unanswered.

Sexual development

The general process of sexual development in *Plumatella casmiana* is in accordance to previous descriptions on the same species and *P. fungosa* (e.g. Braem, 1897; Mukai et al., 1997). The smallest and youngest embryo found in this study is of approximately 20 cells and has formed a blastula with a small central cavity. Earlier stages have not been encountered in this study. Early cleavage-stages of *Lophopus crystallinus* increase rapidly in size and are encountered for just a short time (e.g. Marcus 1934). Mesoderm formation starts approximately at a 30-36 cell stage (Braem, 1987) in *Plumatella fungosa*. In *P. casmiana* mesoderm formation begins when the embryo is about the same size as in *P. fungosa*. Its differentiation from the ectoderm starts from the aboral pole of the embryo as previously described (Kraepelin, 1892; Braem, 1897; Mukai 1982). However, differentiating cells do not just protrude into the blastocoel and then move to the ectoderm as previously assumed (Kraepelin, 1892; Braem, 1897). Instead, they delaminate from the ectoderm (Fig. 6D) in a gradient from aboral to oral. The cells of the fully formed mesoderm are isoprismatic on the aboral pole but still rather flat on the oral side (Fig. 7, 8B). A second blastocoel, which gradually disappears in the course of development, is considered as the result of different growth rates of both embryonic tissues in *P. fungosa* (Braem, 1897). Equivalent cavities could not be confirmed in this study, although the embryonic mesoderm is only loosely associated to the embryonic ectoderm in the oral hemisphere of the embryo when compared to the aboral side. Possibly mechanical manipulation or shrinkage owing to fixation and dehydration of the specimen could have led to an artificially prominent cavity as described by Braem (1897). A similar formation has been described for *P. casmiana* (Mukai 1982), but probably is a

misinterpretation of a budding process in later embryonic stages. The two anlagen of future polypide buds form consecutively in *P. casmiana* as described for *P. fungosa* (Braem, 1897), whereas only a single zooid forms in *Fredericella sultana* (Braem, 1908). In concordance with previous investigations (Braem, 1897) bud formation starts when the mesoderm formation is almost completed. The current study shows that the embryonic ectoderm initially proliferates and ingresses towards the mesoderm in *P. casmiana* (Fig 9, 10C), which is then accompanied by an invagination (Fig. 9) and further proliferation (Fig. 10A) of the latter. This differs from previous descriptions according to which both embryonic tissues simply thicken and fold inwards as found in asexual buds (Braem, 1897). A distinct endoderm is absent. However, few cells of the aboral pole of the embryo ingress at early embryogenesis in *Plumatella* but degenerate shortly afterwards. These cells are considered as temporary endodermal cells (Braem, 1897), but were not encountered in the present nor previous studies (Mukai, 1982).

Maternal contact

Since embryos develop from oligolecithal oocytes additional nutrients are necessary in the course of further development. In *Plumatella fungosa* a placental ring connecting the embryo to the embryo sac at the equatorial region was described to provide extra embryonic nutrition (Kraeplin, 1886; Korotneff, 1887) or function as anchoring structure (Braem, 1897). The origin and development of this placental ring was investigated in detail in *P. fungosa*: As the embryo grows, it adjoins the ectodermal embryo sac cavity at the aboral side of the embryo sac. At the stage of a bilayered embryo, the embryo sac is in direct contact with the aboral pole of the former. This contact does not fully persist. Cells of the embryonic ectoderm enlarge at the margin of the contact area and remain in contact with the embryo sac during further

development. A placental ring forms in the equatorial region of the embryo as the embryo grows more in the aboral than in the oral direction (Braem, 1897). A placental ring is also present in the plumatellid genus *Hyalinella* (Mukai, 1982), whereas in *Fredericella* a placental disc is present at the aboral pole of the embryo (Braem, 1908). In *Lophopus* the same cells that form the initial contact in *Plumatella* and *Fredericella* do not form a ring but just loosely migrate in the gap between embryo and embryo sac (Marcus, 1934).

First indications of contact between embryo and maternal tissue in *P. casmiana* is at the blastula with cytoplasmic extensions towards the embryo sac cavity (Fig. 14A, arrow). When the embryo of *P. casmiana* is about to form the mesoderm, its aboral hemisphere is connected to the ectodermal lining that has partly disappeared from the oral hemisphere (Fig. 6B, D) as described for *P. fungosa* (Braem, 1897). In *P. casmiana* several, mostly aboral situated cells of the embryonic ectoderm show cytoplasmic extensions towards the remains of the embryo sac cavity when the embryo is already forming the second bud (Fig. 14D). The ectodermal lining of the embryo sac has disappeared and the embryo sac cavity has shrunk as described for *P. fungosa* (Braem, 1897). However, a prominent contact of maternal and embryonic ectoderm has not been previously described at such late developmental stages. Ultimately, contact of maternal and embryonic ectoderm establishes very early in embryogenesis and persists far longer than previously known. Contact is not established over the whole surface but rather by single cells that are patchily distributed predominantly in aboral hemisphere of the embryo.

As aforementioned, the initial broad contact zone partly disappears and only a ring of embryonic ectoderm cells persists as placental ring. The placental ring connects the embryonic ectoderm to the remaining mesoderm of the embryo sac. Hence, the placental ring develops from the initial aboral contact zone in *P. fungosa* (Braem, 1897). The present study shows that

cells which connect the embryonic ectoderm to the mesoderm of the embryo sac do not form from an aboral contact zone in *P. casmiana*.

Owing to better comparability to previous studies (Braem 1897, 1908), the term 'placental cells' is used herein for cells that connect the embryonic ectoderm to the maternal mesoderm. The term placenta was first introduced by Kraepelin (1886) assuming a nutritive function of these cells in phylactolaemates. When the mesoderm starts to form in *P. casmiana*, protrusions of the embryo towards the embryo sac indicate the formation of 'placental cells' (Fig. 6B, C). The 'placental cells' do not form a complete ring, but are patchily distributed cells extending all around the embryo, predominantly in the oral hemisphere where the ectodermal lining has already disappeared (Fig. 15). However, at later stages the 'placental cells' form consistently in regions of the oral hemisphere where buds develop (Fig. 10A, D). As the embryo grows, 'placental cells' are only present in the equatorial region of the embryo/ larva and often it is a broad area in which several neighbouring/stacked cells connect mother an offspring (Fig. 13B). As mentioned before, it is not always clear where cells in *P. casmiana* originate from, whereas in *P. fungosa* ectodermal cells of the embryo extend towards the inside of the embryo sac and appear granularly. Corresponding cells of the embryo sac change similarly and form a 'placental cell' with maternal and embryonic parts becoming indistinguishable, too (Korotneff, 1887).

In total, two sorts of contact to maternal tissues establish independently in *P. casmiana*. First, contact between embryonic and maternal ectoderm forms predominantly in the aboral hemisphere. Later, contact between the embryonic ectoderm and maternal mesoderm develops ('placental cells') predominantly in the oral hemisphere. Since both sorts of contact form independently, and 'placental cells' do not emerge from aboral contact zones, cells that connect the embryonic and maternal ectoderm are referable as primary 'placental

cells'. In contrast, cells that link the embryonic ectoderm to the maternal mesoderm are referred to as secondary 'placental cells'. Both terms would assume that the connecting cells exchange substances and provide extra embryonic nutrition.

Matrotrophy

The term matrotrophy defines parental, extra-vitelline nutrition during offspring gestation (e.g. brooding) (Ostrovsky, 2016). Different variants of matrotrophy evolved independently in several animal phyla including bryozoans (Ostrovsky, 2013). Several different modes of matrotrophy are described across all animal phyla: ingestion of sibling oocytes (oophagy) or of siblings (embryophagy), absorbtion of nutrients from the parental surroundings of the offspring (histotrophy) or ingestion of parental secretions, floating cells, organs, etc. (histophagy) (Ostrovsky, 2016). The most elaborated form of matrotrophy is placentotrophy with a placenta referred to as any intimate apposition or fusion of embryonic and maternal tissue for the purpose of substance exchange (Mossmann, 1937). During incubation, a prominent increase in embryonic size and the formation of temporary structures (e.g. placentas) are often indicative for matrotrophy (Ostrovsky, 2016). Oocytes of *P. casmiana* show only little yolk and the embryo increases in size significantly, indicating an external nutrient supply. In fact, all analysed phylactolaemates are placental brooders (Braem, 1890; Ostrovsky, 2016). In *P. casmiana*, ultrastructure of the embryo sac and a corresponding bilayered embryo reveals cytoplasmic bridges between the maternal mesoderm and embryonic ectoderm, which strongly indicates contact of mother and offspring (Fig. 17B). This is not true for all cytoplasmic extensions, as other protrusions of the embryo sac in *P. casmiana* appear thicker and include numerous vesicles (Fig. 17A), which empty their content into the

gap between embryo sac and embryo. An increased surface in the placental analogue and exocytosis towards the incubated embryo have been reported in a matrotrophic cheilostome (Moosbrugger et al., 2012). Besides an increased surface area, other ultrastructural indications of matrotrophy in bryozoans are an increased amount of organelles such as mitochondria and endoplasmatic reticulum (mostly in the nourishing structure) during development and the formation of multi vesicular bodies (Moosbrugger et al., 2012; Nekuliudova et al., 2019). In *P. casmiana* cytoplasmic protrusions on the embryonic side are mostly filiform and in general less prominent than those of the embryo sac (Fig. 17A). In addition, the protrusions do not contain any vesicles in contrast to the embryonic cells themselves that show many small vesicles in their periphery (Fig. 17A). It appears that the embryo sac secretes substances from rather large vesicles into its lumen and the embryo takes them up via endocytosis indicated by clathrin-coated pits (Fig. 18D). Although the latter could not be properly located, endocytosis is very likely when compared to *Bicellariella ciliata* (Moosbrugger et al., 2012). At the outer side of the embryo sac where it is in contact with the coelomic cavity of the mother zooid, vesicles form from numerous infoldings (Fig. 18B, E). In addition, several multi vesicular bodies occur on both sides of these cells (Fig. 18B), whereas the presence of multi vesicular bodies corresponds to the situation in *Bicellariella ciliata* and nearby exocytosis of the embryo sac mesoderm and a corresponding event of endocytosis of the embryo takes place (Fig. 18D). In total, the mesoderm of the embryo sac transfers coelomic fluid and its contents from the maternal coelom into the lumen of the embryo sac, where the embryonic ectoderm absorbs them. Autophagosomes mostly close to the embryo sac (Fig. 17A, 18A, C, E) are involved in recycling substances and digest excess cell membrane. These indicate a high cellular activity as observed in other bryozoans (Moosbrugger et al., 2012).

In addition, intensely stained cytoplasmic inclusions are present in both linings of the embryo sac (Fig. 4D) and also in the mesoderm of the early embryo (Fig. 4C). Coelomocytes are occasionally present on the outer side of the embryo sac. This indicates that possibly embryonic waste products might be disposed through the embryo sac via coelomocytes in early embryonic stages, which fits to the assumption of the excretory function of some coelomocytes (Mano, 1964). Waste products of the embryo are disposed by multi vesicular bodies that release their content into the brood chamber in the cheilostome *Bicellariella ciliata* (Moosbrugger et al., 2012). In total, several indications for matrotrophy are present in *P. casmiana*: mitochondria are the most abundant cell organelles in the embryo as well as in the embryo sac, high amounts of rough endoplasmatic reticulum in the embryo sac, several events of exo-/endocytosis between embryo sac and embryo, increased surface area of both named structures and potential transport of waste product. All these findings are in accordance with previous investigations of cheilostome placental brooders (Moosbrugger et al., 2012; Nekliudova et al., 2019). In *P. casmiana* placentotrophy could not be verified, however matrotrophy via histotrophy (absorbtion of nutrients directly from the surroundings) seems highly likely due to reported signs. Since it is possible that histotrophy gradually becomes placentotrophy (Ostrovsky, 2016), more TEM investigations of later stages are needed to assess whether placentotrophy develops. This would further allow to see how embryo sac and embryo change on ultrastructural level since matrotrophy is also accompanied by increase of certain organelles like RER (Moosbrugger et al., 2012)

Conclusion

This study provides the first detailed analysis of the development of a phylactolaemate bryozoan with modern methods including 3D-reconstructions of approximately seven different embryonic stages. The embryonic development of *P. casmiana* is concordant with other plumatellids but features some small differences: in the investigated species the mesoderm and bud formation occurs earlier than in *P. fungosa*. Concerning the 'placental cells', this study found two distinctive sorts of contact between mother and offspring: ectodermal contact, restricted to the aboral hemisphere of the embryo and mesodermal contact initially restricted to the oral hemisphere and referred to as 'placental cells' in other studies. Unlike in *P. fungosa*, this study shows that ectodermal and mesodermal contact cells do not form from the same contact zone but form independently. In addition, nutritive function of the 'placental cells' (placentotrophy) could not be verified. There are clear indications of matrotrophy (histotrophy) in *P. casmiana*, which leads to the conclusion that reported 'placental cells' serve as anchoring structure and the term 'placental cells' should be rejected. However, it is still possible that these cells have a nourishing function in later development. Eventually, very early stages should be investigated in order to clarify questions of fertilisation and oocyte transfer. However, those stages are hard to find, as they seem very inconspicuous and persist only for a rather short time, meaning investigations on the early development will be time consuming and depend on coincidence. Ultimately, it is to say, that the preservation of the material in the present study resembles a more natural state compared to the old material that underwent a rougher treatment. In order to clarify whether mentioned differences occur due to smoother treatment and better preservation or are in fact differences occurring in the embryogenesis, a reinvestigation of related species and families would be of interest.

Acknowledgments

I want to thank Andreas Wanninger (Head of Department Evolutionary Biology, University of Vienna) for providing all necessary laboratories, supervising this thesis and offering his scientific expertise on many occasions. Thanks to Andrey Ostrovsky (University of Vienna, University of St. Petersburg) for his comments and advices in order to improve this manuscript and special thanks to Thomas Schwaha (University of Vienna) for introducing me into the world of bryozoans and his extensive help in the lab and in every other part of this thesis. Lastly, I want to thank everybody who cheered me up during this intense period.

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Zusammenfassung

Bryozoen sind eine Gruppe kolonialer, sessiler Filtrierer innerhalb der Lophotrochozoa, die vorwiegend in marinen Lebensräumen vorkommen. Alle Bryozoen sind hermaphroditisch und besitzen demnach sowohl ein Ovar, welches sich an der Körperwand formt, also auch Hoden, die meist entlang eines peritonealen Gewebsstranges auftreten. Phylactolaemata stellen eine Gruppe reiner Süßwasser-Bryozoen dar, die aus rund 80 Arten besteht. Die sexuelle Reproduktion jener Gruppe beinhaltet Brutpflege in einem Brutsack, der aus einer Invagination der Körperwand hervorgeht. Detaillierte Studien über die sexuelle Entwicklung der Phylactolaematen gehen auf das späte 19. bzw. frühe 20. Jahrhundert zurück. Einige Aspekte, wie etwa der Transfer der Oozyte in den Brutsack oder auch die Plazentation wurden in der Vergangenheit widersprüchlich beschrieben und verlangen daher nach einer erneuten Untersuchung. Diese Studie untersucht die Embryogenese der Süßwasser-Art Plumatella casmiana anhand von 3D-Rekonstruktionen basierend auf Schnittserien und Elektronenmikroskopie. Frühe Embryonen entwickeln sich in eine einschichtige Blastula mit zentralem Hohlraum. Innerseitig setzt sich ein Mesoderm via Delamination ab und folgt dabei einem Gradienten entlang der Körperlängsachse vom aboralen zum oralen Ende. In der Gattung Plumatella entstehen nacheinander zwei voll funktionsfähige Polypide durch Ingression und Proliferation des Ektoderms und des Mesoderms. Das Resultat der sexuellen Entwicklung ist eine bewimperte „Mantellarve“, die zwei Zooide, also eine Gründerkolonie, beherbergt. Im Laufe der Entwicklung ist der Embryo durch unterschiedliche „Plazentazellen“ zeitweilig in Kontakt mit dem mütterlichen Zooid. Ultrastruktur-Daten zeigen, dass diese „Plazentazellen“ den Embryo nicht zwingend mit Nährstoffen versorgen, weshalb der Ausdruck primäre und sekundäre Kontaktzellen passender erscheint. Zytologische Befunde, wie etwa die Anzahl und Häufigkeit von Mitochondrien, dem endoplasmatischen Retikulum

also auch eine vergrößerte Zelloberfläche, deuten dennoch auf eine extra-embryonale Nährstoffversorgung hin. Dies geschieht vermutlich, indem der Brutsack Nährstoffe von dem Coelom des Mutterzooids in sein Lumen transferiert, wo sie vom Embryo absorbiert werden (Histotrophie). Insgesamt bestätigt diese Studie den allgemeinen Entwicklungsablauf von Plumatella, konnte jedoch auch kleine Unterschiede im zeitlichen Ablauf aufzeigen. Zudem konnte durch die bessere Konserverierung und modernen Methoden gezeigt werden, dass das Mesoderm sich durch Delamination vom Ektoderm abgrenzt, ebenso erlaubt die 3D-Rekonstruktion eine elaboriertere Darstellung der verschiedenen Entwicklungsstadien. Letztlich zeigt die vorliegende Studie, dass die Bildung der „Plazentazellen“ anders als bisher angenommen abläuft. Außerdem geht aus dieser Studie hervor, dass Matrotrophie durch Histotrophie und weniger wahrscheinlich durch Placentotrophie erfolgt. Folglich sollten die separat entstandenen „Plazentazellen“ viel mehr als primäre und sekundäre Kontaktzellen angesprochen werden.

Figures

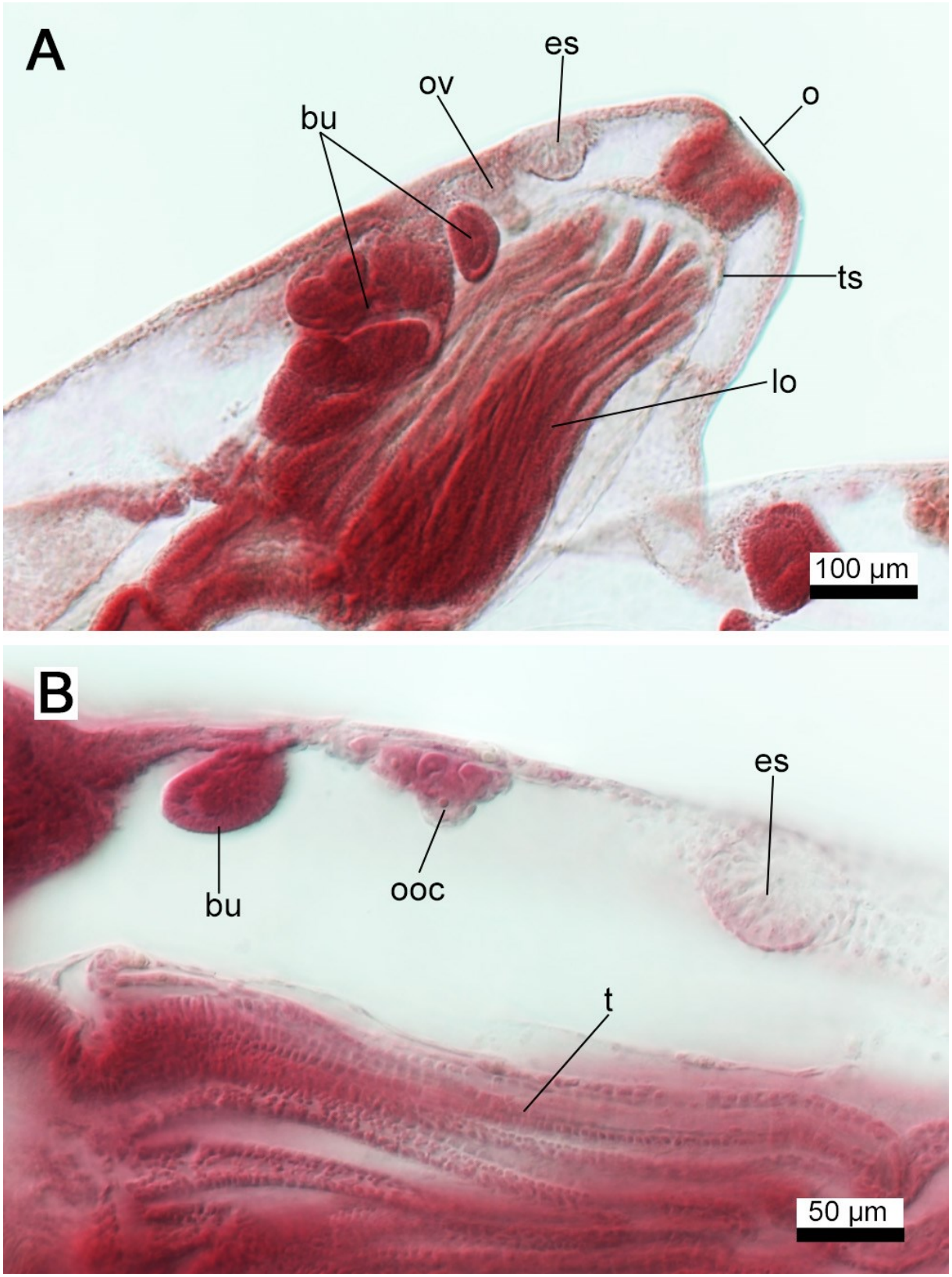

Figure 1: Whole mounts of colony pieces of *Plumatella* sp., stained with borax carmine. **A** Overview of single autozooid with retracted polypide. Most reproductive organs are located in the oral body wall. Most distant from the orifice two asexual produced buds are situated. Asexual buds are followed by a still small ovary including few oocytes. Closest to the orifice an

embryo sac forms from both layers of the cystid. **B** Close up of proximal cystid wall. Oocytes differentiate within and from the mesoderm. All oocytes are limited by thin layer of mesoderm. The embryo sac develops from the hypertrophied ectoderm of the endocyst that bulges into coelom of the reproductive zooid and is also lined by the flattened layer of mesoderm that belongs to the embryo sac, too. Abbreviations: **bu** bud; **es** embryo sac; **lo** lophophore; **o** orifice; **ooc** oocytes; **ov** ovary; **t** tentacles; **ts** tentacle sheath.

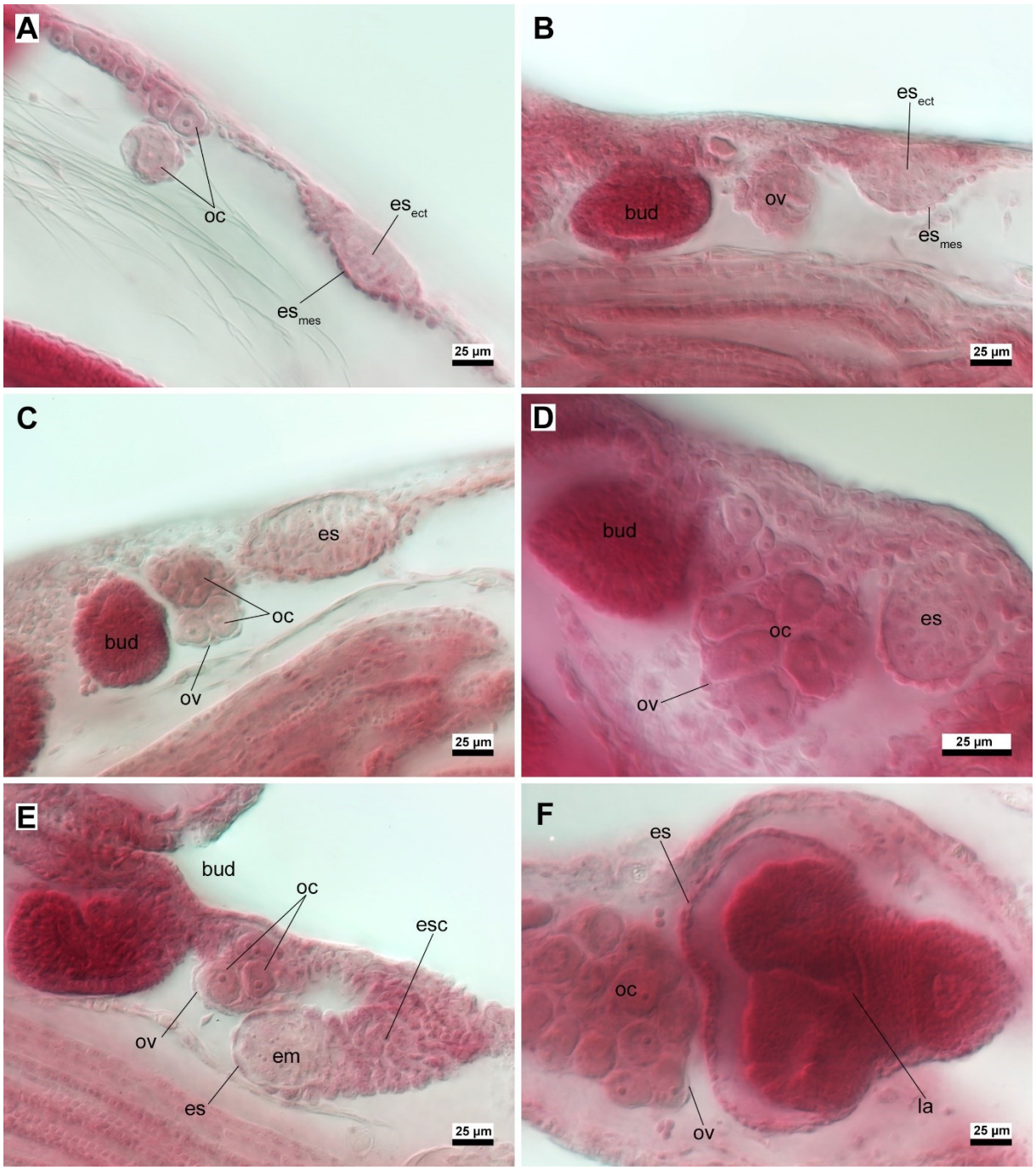

Figure 2: Whole mounts of borax carmine stained colony pieces of *Plumatella* sp. **A-D** Ovary and embryo sac formation within the oral body wall in detail. **A** Several cells of the mesoderm consecutively enlarge and differentiate into oocytes. The oocytes form within the mesoderm and are lined by a flat mesodermal epithelium. The embryo sac becomes apparent next to the ovary. **B** Arrangement of reproductive organs, slightly further developed than in A. Most proximal (left) an asexual produced (youngest) bud forms as invagination from both tissues of the maternal endocyst. **C** More advanced embryo sac formation. The largest and hence ripest oocytes are located at the free end of the ovary towards the coelom. **D** Late stage of embryo

sac formation with distinctive ectoderm lined by thin mesoderm. The ovary outreaches the adjoining bud in size and includes several ripe oocytes with a large nucleolus. **E** Beginning of embryogenesis. The embryo lies at the suspended end of the embryo sac. Towards the fixed end of the embryo sac, the embryo is bordered by the embryo sac cavity. In total one embryo is brooded, the adjoining ovary is still full of ripened oocytes. **F** Late embryonic/ larval stage. Embryogenesis results in ciliated larva. Also at this late developmental stage a prominent ovary including even more mature oocytes is present. Abbreviations: **bu** bud; **em** embryo; **es** embryo sac; $\mathbf{es}_{\mathbf{ect}}$ embryo sac ectoderm; $\mathbf{es}_{\mathbf{mes}}$ embryo sac mesoderm; **esc** embryo sac cavity; **la** larva; **oc** oocyte; **ov** ovary.

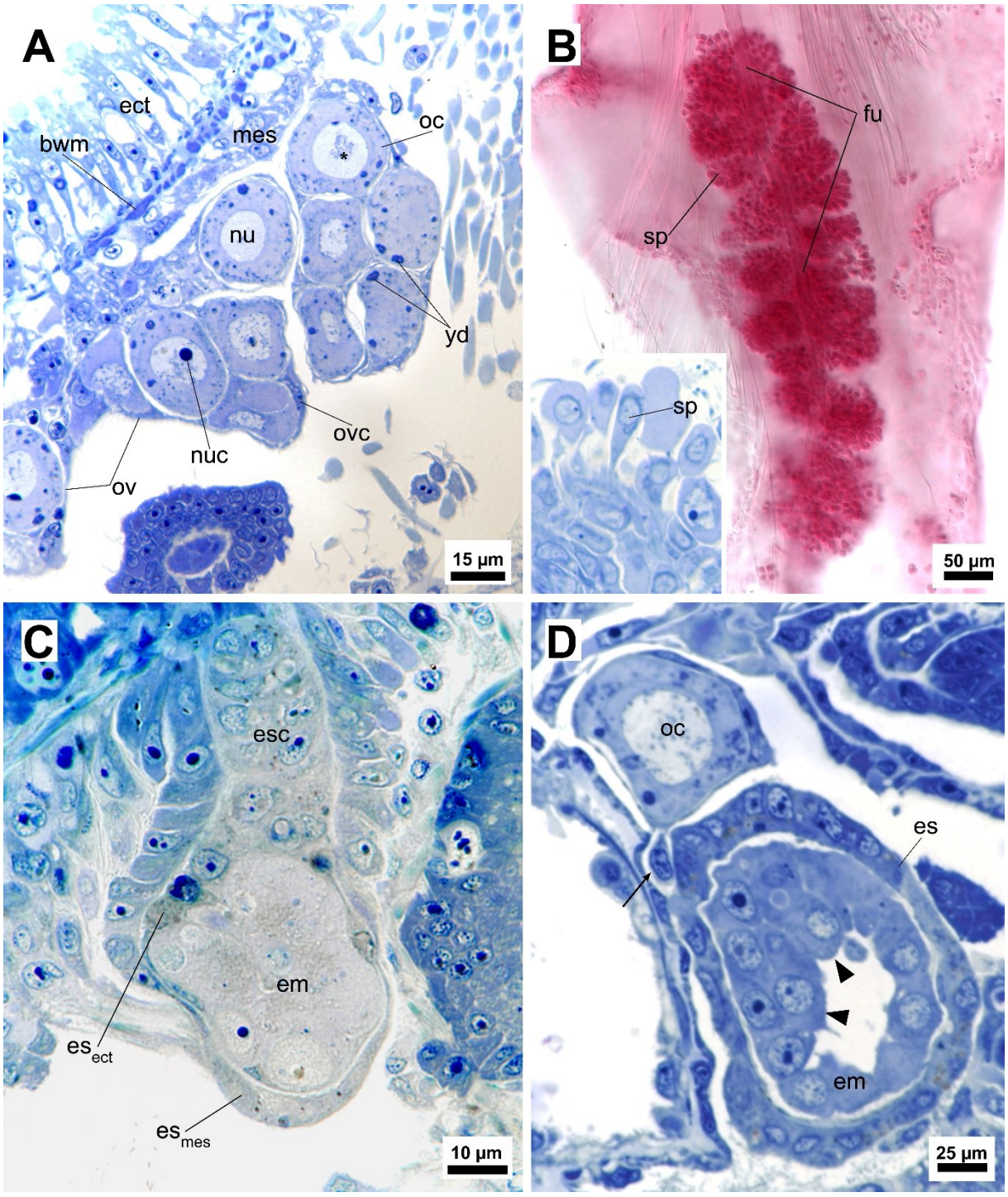

Figure 3: Semithin sections of *Plumatella casmiana*, stained with toluidine and whole-mount of *Plumatella* sp. stained with borax carmine (B). **A** An ovary dangling from the body wall. Several oligolecithal oocytes matured within the ovary. A very thin mesodermal lining of the ovary surrounds every single oocyte. **B** Overview of testes as a grape-like structure along the funiculus. Middle stage of spermatogenesis at which the nucleus fills most of the head of the spermatids (insert). **C** Early developmental stage. The embryo sac hangs from the body wall right next to the ovary. An embryo consisting of several blastomeres is located at the free end

of the embryo sac. **D** Oblique section of early staged embryo. The single layered embryo has formed a small central cavity and on one side cells undergo cell division and a second layer of embryonic tissue delaminates. Abbreviations: **bwm** body wall musculature; **ect** ectoderm of zooid; **em** embryo; **es** embryo sac; **es_{ect}** embryo sac ectoderm; **es_{mes}** embryo sac mesoderm; **esc** embryo sac cavity; **fu** funiculus; **mes** mesoderm of zooid; **nu** nucleus; **nuc** nucleolus; **oc** oocytes; **ov** ovary; **ovc** follicle-like ovarian cell; **sp** sperm; **yd** yolk droplets

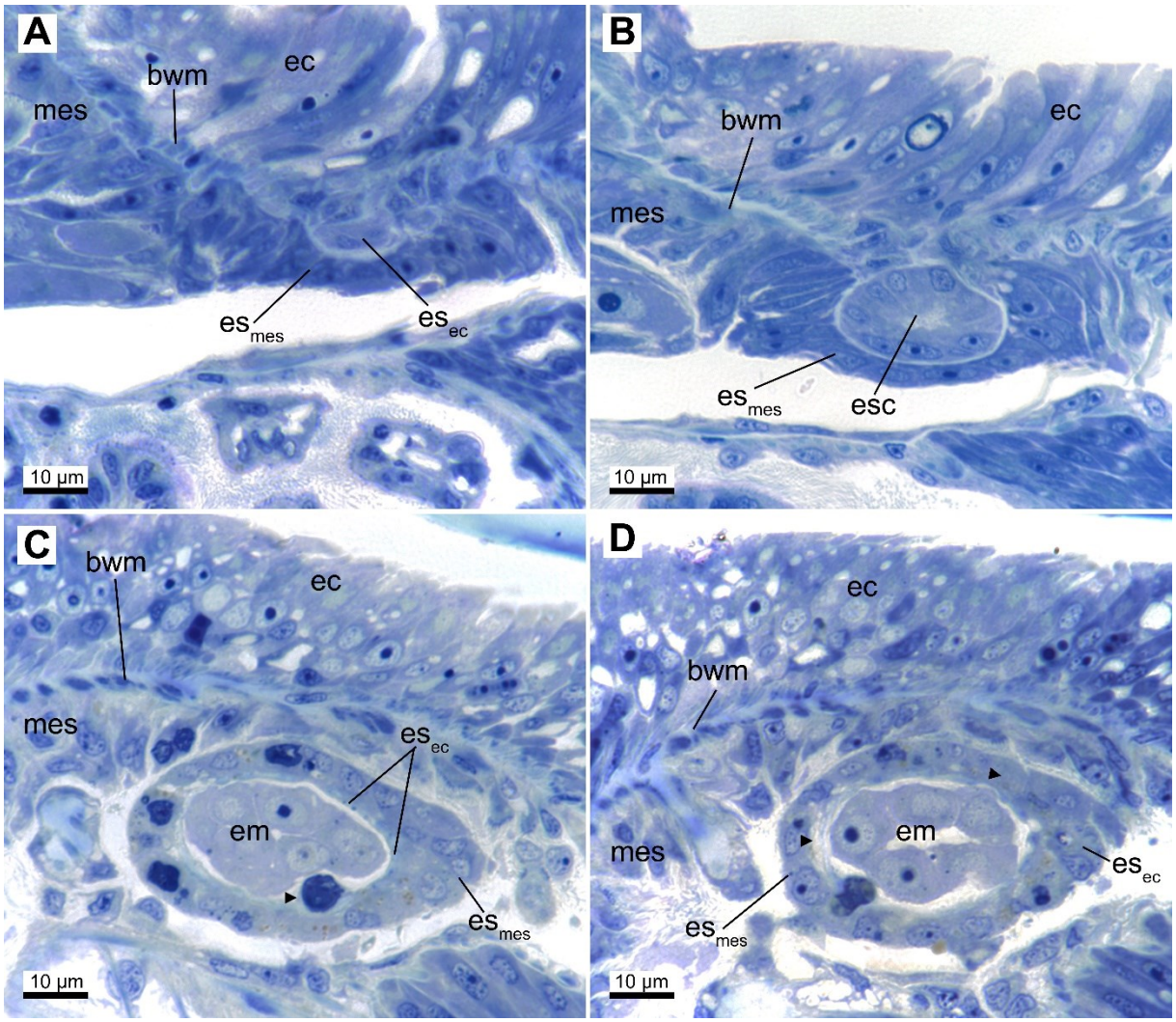

Figure 4: Semithin sections of embryo sacs of *Plumatella casmiana*. **A** Section through the fixed ("apical") end of the embryo sac, where it is attached to/ formed from the body wall of the maternal zooid. The endocyst is composed of the outer ectoderm and the inner mesoderm of zooid. **B** Section through fixed end of the embryo sac with distinctive embryo sac cavity. As the ectoderm protrudes towards the coelom it demarcates itself and forms a distinct cell mass that fills the apical part of the embryo sac and is referred to as embryo sac cavity. **C** Cross section of the embryo sac including a young, single layered embryo that just formed a small central cavity. At this stage, intensely stained cytoplasmic inclusions (arrow) occur in the embryo sac ectoderm and mesoderm. **D** Cross section closer to the suspended end of the embryo sac. The embryo buckles at some points (right arrowhead) towards the embryo sac. The ectodermal lining of the embryo sac begins to vanish (left arrowhead) from the suspended end of the embryo sac. In addition, a cytoplasmatic inclusion of unknown origin is present. Abbreviations: **bwm** body wall musculature; **ect** ectoderm of zooid; **em** embryo; **es** embryo

sac; **es$_{ect}$** embryo sac ectoderm; **es$_{mes}$** embryo sac mesoderm; **esc** embryo sac cavity; **fu** funiculus; **mes** mesoderm of zooid.

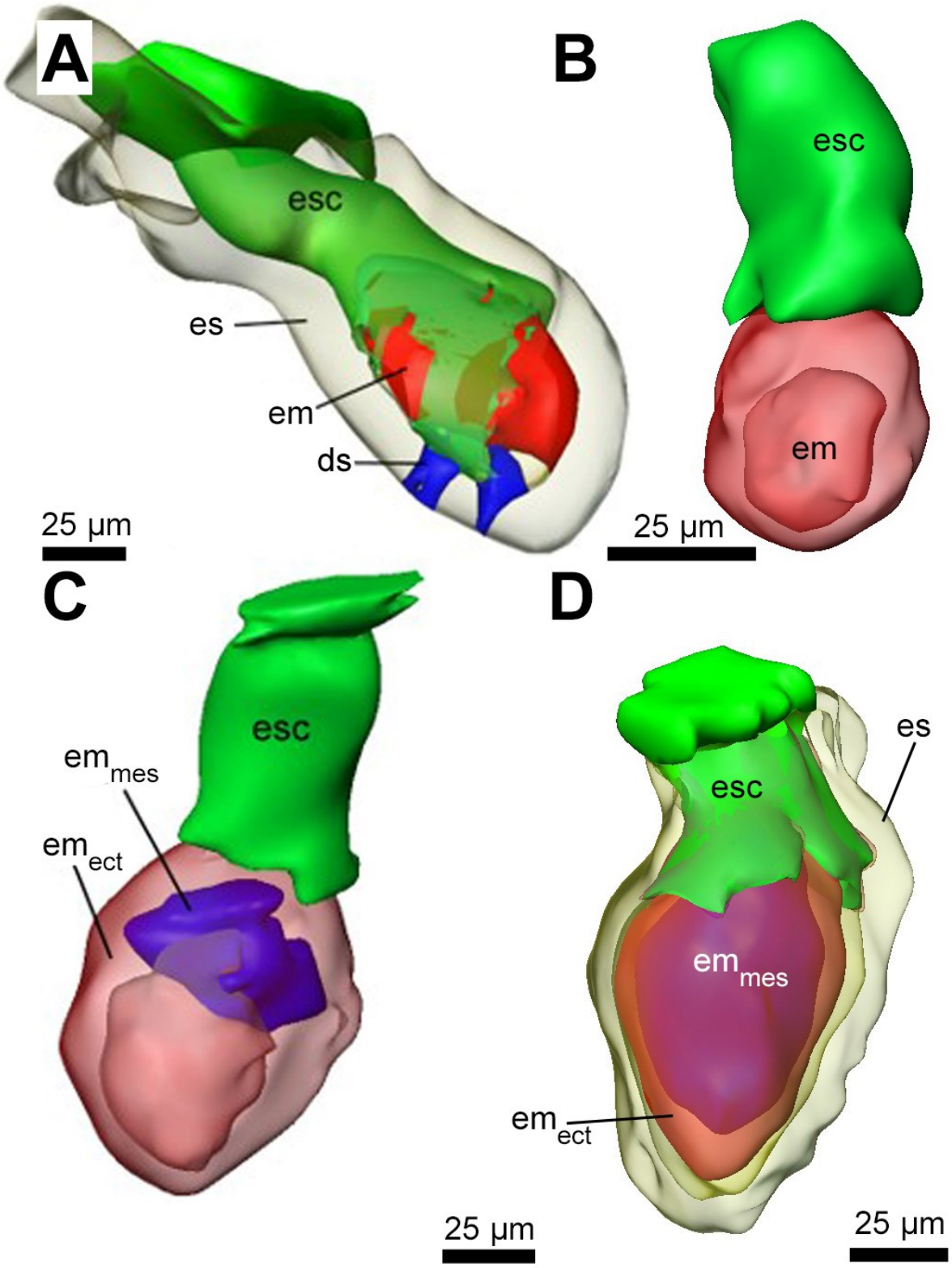

Figure 5: 3D-reconstruction of early staged embryos of *Plumatella casmiana* based on serial semi thin sections. **A** Embryo consisting of several blastomeres lies at the suspended end of the bilayered embryo sac. The inner, ectodermal lining of the embryo sac begins to vanish from the suspended end towards the body wall of the mother zooid. **B** The embryo has further

developed and formed a central cavity, on top of the embryo, towards the body wall, the embryo sac cavity is located. **C** The embryo has increased in size with the shrinking embryo sac cavity still on top. In addition, in the central cavity of the embryo a second tissue differentiates via delamination. The delamination of the embryonic mesoderm begins from the aboral side (close to the body wall of the mother zooid) and continues towards its oral side (at the suspended end of the embryo sac). **D** A bilayered embryo is located within the embryo sac. The decreasing embryo sac cavity (including cup like extensions that represent remnants of the inner ectoderm of the embryo sac) sits on top of the embryo. Formation of the embryonic mesoderm is completed and the ectoderm of the embryo sac has almost entirely vanished. Abbreviations: **em** embryo; **em_{ect}** embryonic ectoderm; **em_{mes}** embryonic mesoderm; **es** embryo sac; **esc** embryo sac cavity; **ds** dark spots.

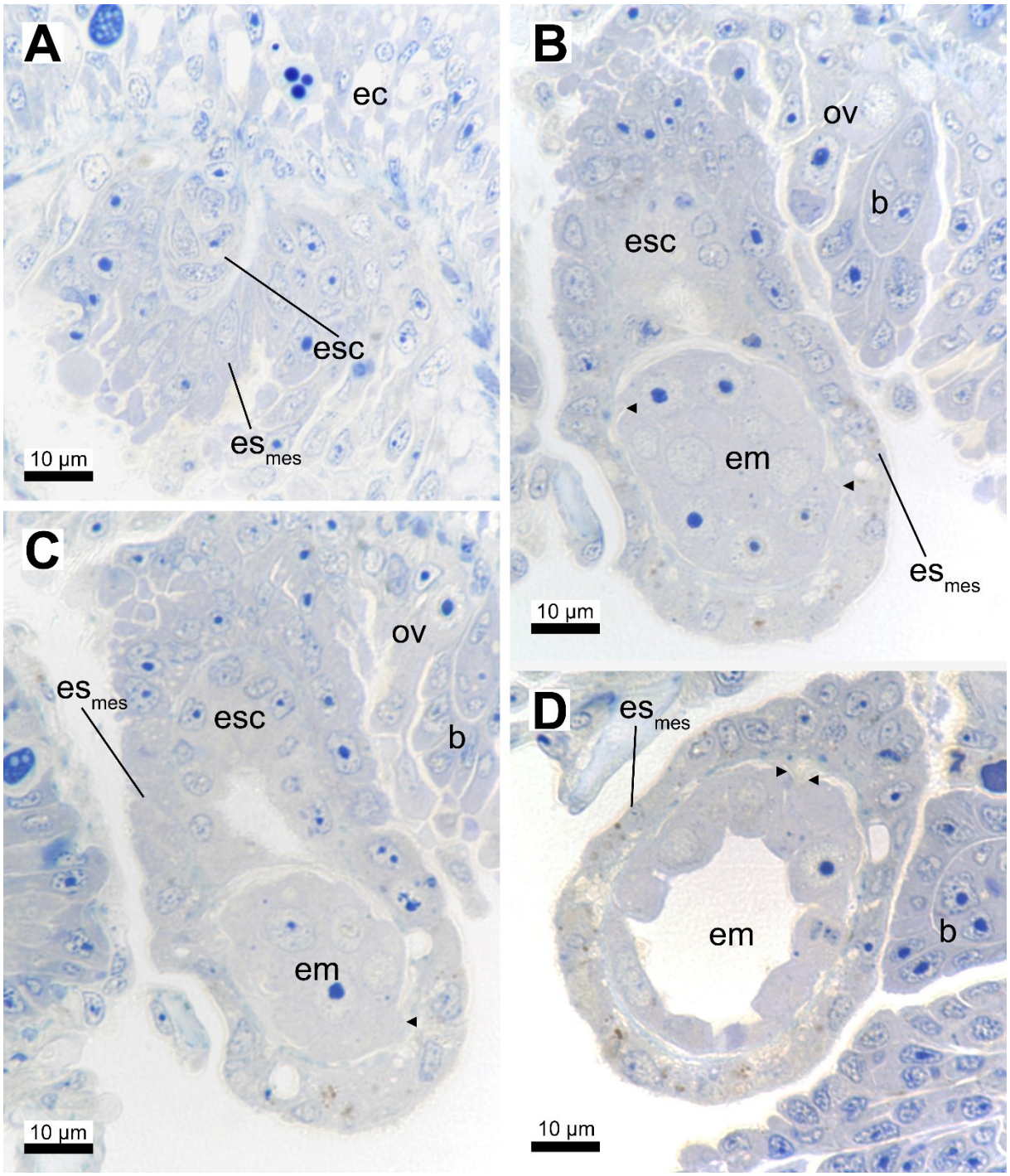

Figure 6: Semi thin sections of a blastula of *Plumatella casmiana,* stained with toluidine blue. **A** Section through fixed end of the embryo sac showing that the embryo sac cavity originated from the maternal ectoderm of the body wall. **B** Oblique longitudinal section of entire embryo sac. The double layered embryo sac has a rather thick outer mesoderm and on the inside a very thin layer of ectoderm almost all around the embryo with a prominent embryo sac cavity above the embryo. The embryo interconnects with the ectoderm of the embryo sac (left arrowhead) and also protrudes towards the mesoderm of the latter (right arrowhead). **C**

Longitudinal section of the middle of the embryo sac. The cells of the embryo sac cavity form a hollow space as it cavity seems to degenerate. Isoprismatic cells of the mesoderm of the embryo sac occasionally show two nucleoli within their nucleus and also large vacuoles are present or in open contact with the lumen of the embryo sac. **D** Oblique section through the embryo sac and including embryo, beneath the embryo sac cavity. On the aboral pole a connection of the embryo and the ectoderm of the embryo sac is present. The rest of the ectoderm is rather thin and has already vanished as the section plane is closer towards the suspended end of the embryo sac. The embryo itself has formed a rather large central cavity and its cells are flatter on the oral side and isoprismatic on the aboral side. Embryonic cells on the aboral side show delamination of the second embryonic tissue, the mesoderm of the embryo. Abbreviations: **bu** bud; **ec** ectoderm of mother zooid; **em** embryo; **es**$_{\mathbf{mes}}$ mesoderm of the embryo sac; **esc** embryo sac cavity.

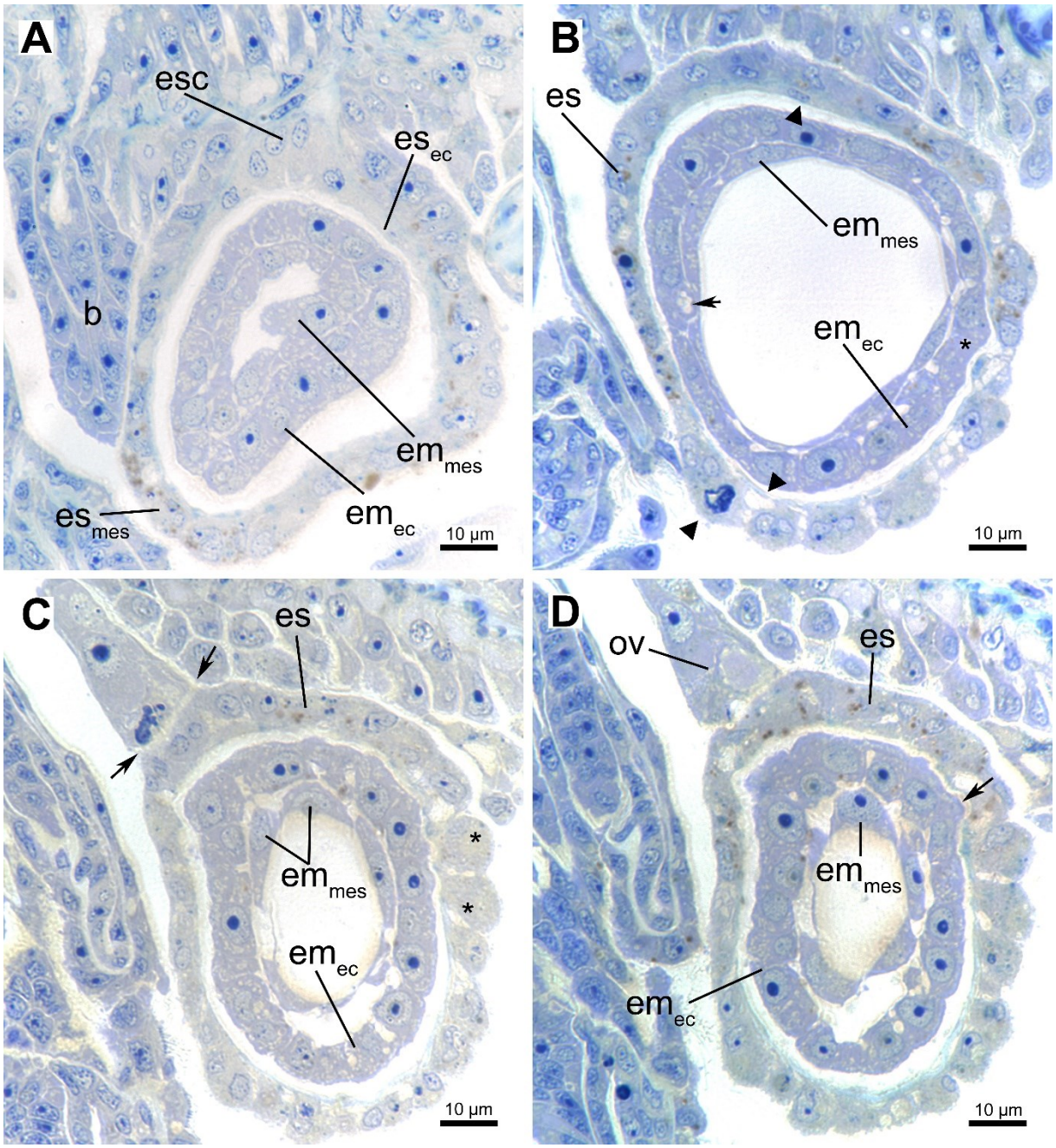

Figure 7: Semi thin sections of a bilayered embryo of *Plumatella casmiana.* **A** Oblique cross-section of fixed end of the embryo sac. Extensions of the embryo sac cavity, the ectodermal lining is thicker when closer to the embryo sac and is very thin on the opposite side. Both embryonic tissues consist of voluminous isoprismatic cells on the aboral side with single cells of the mesoderm preparing for further proliferation. **B** Section of the equatorial region of the embryo sac embryo. The mesoderm of the embryo sac is rather thick whereas the ectodermal lining is very thin but still cellular. The mesoderm of the embryo sac includes a cell containing a dark cytoplasmatic inclusion. While the embryonic mesoderm appears inconspicuous, the ectoderm occasionally shows vacuoles and is tightly opposed to the embryo sac ectoderm. **C**

Section through the oral hemisphere of the embryo. The mesoderm of the embryo sac appears similar to equatorial regions, additionally some very voluminous cells are present (asterisks). The ectoderm of the embryo sac is barely existent or visible distant from the body wall. Also, the ovary is in tight association to the embryo sac with likely, though not verifiable contact (arrows). **D** Section through oral side of the embryo. The embryo sac appears as described in (C). The mesoderm of the embryo protrudes towards the mesoderm of the embryo sac. In contrast to the flat mesoderm of the equatorial region, at the oral pole its cells are rather voluminous with a large nucleus and also it is not as tightly opposed to the embryonic ectoderm, as it is in the equatorial region. Abbreviations: **bu** bud; **em_{ect}** embryonic ectoderm; **em_{mes}** embryonic mesoderm; **esc** embryo sac cavity; **es_{ect}** ectoderm of the embryo sac; **es_{mes}** mesoderm of the embryo sac; **ov** ovary.

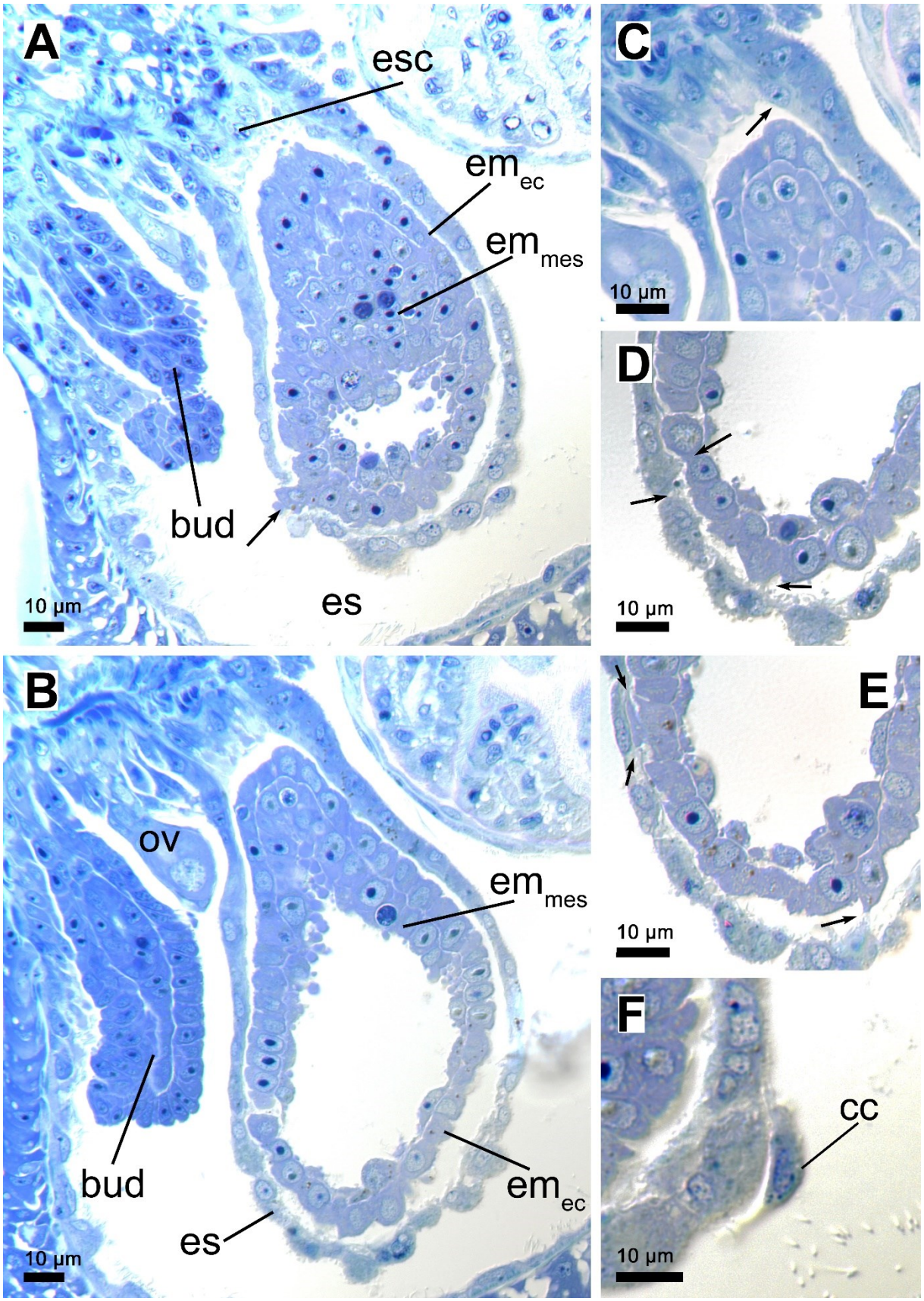

Figure 8: Semi thin sections of a bilayered embryo of *Plumatella casmiana* at the beginning of bud formation. **A-B** Oblique longitudinal section of embryo sac and neighbouring reproductive organs. The

ovary follows asexual bud. The embryo sac hangs from the oral body wall with its mesoderm becoming flatter except for the suspended end where it stays isoprismatic. The embryo sac cavity decreases but still shows ectodermal extensions that represent remnants of the ectodermal lining. At one point the embryo sac shows a hole through which the embryo appears growing out (A). At the suspended end a coelomocyte is in close association to the embryo sac (A). The embryo itself has increased in volume. Its ectodermal cells are isoprismatic all over the embryo and the mesoderm has fully formed. On one side in the aboral hemisphere, cells further proliferate and produce a bud (A). In addition, the mesoderm is thicker than on the oral hemisphere, where it is almost lacking. (B). Indications of connections from the embryonic ectoderm to the maternal one are restricted the aboral hemisphere (B), whereas in the oral hemisphere the embryonic ectoderm projects towards the mesoderm of the embryo sac. **C** Detail of the fixed end of the embryo sac. The embryo sac cavity and the rest of the ectodermal lining stain less intense than the embryo and the embryo sac mesoderm. In addition, the embryo sac ectoderm shows a cellular nature at the fixed end, displaying the reduction of the ectodermal lining. Reduction begins from the suspended end and continues towards the fixed end. **D, E** Details of the embryo in close association to the embryo sac on the oral hemisphere. Single cells of the embryonic ectoderm show cytoplasmatic extensions towards the embryo sac. The mesoderm of the embryo sac that also projects toward the embryonic ectoderm. In this way a contact is established between embryonic and maternal tissue. **F** Detail of the embryo sac. At the suspended end of the embryo sac a coelomocyte is associated to the embryo sac at its outer side, in the maternal coelom. Abbreviations: **bu** bud; **cc** coelomocyte; **em_{ect}** embryonic ectoderm; **em_{mes}** embryonic mesoderm; **es** embryo sac; **esc** embryo sac cavity.

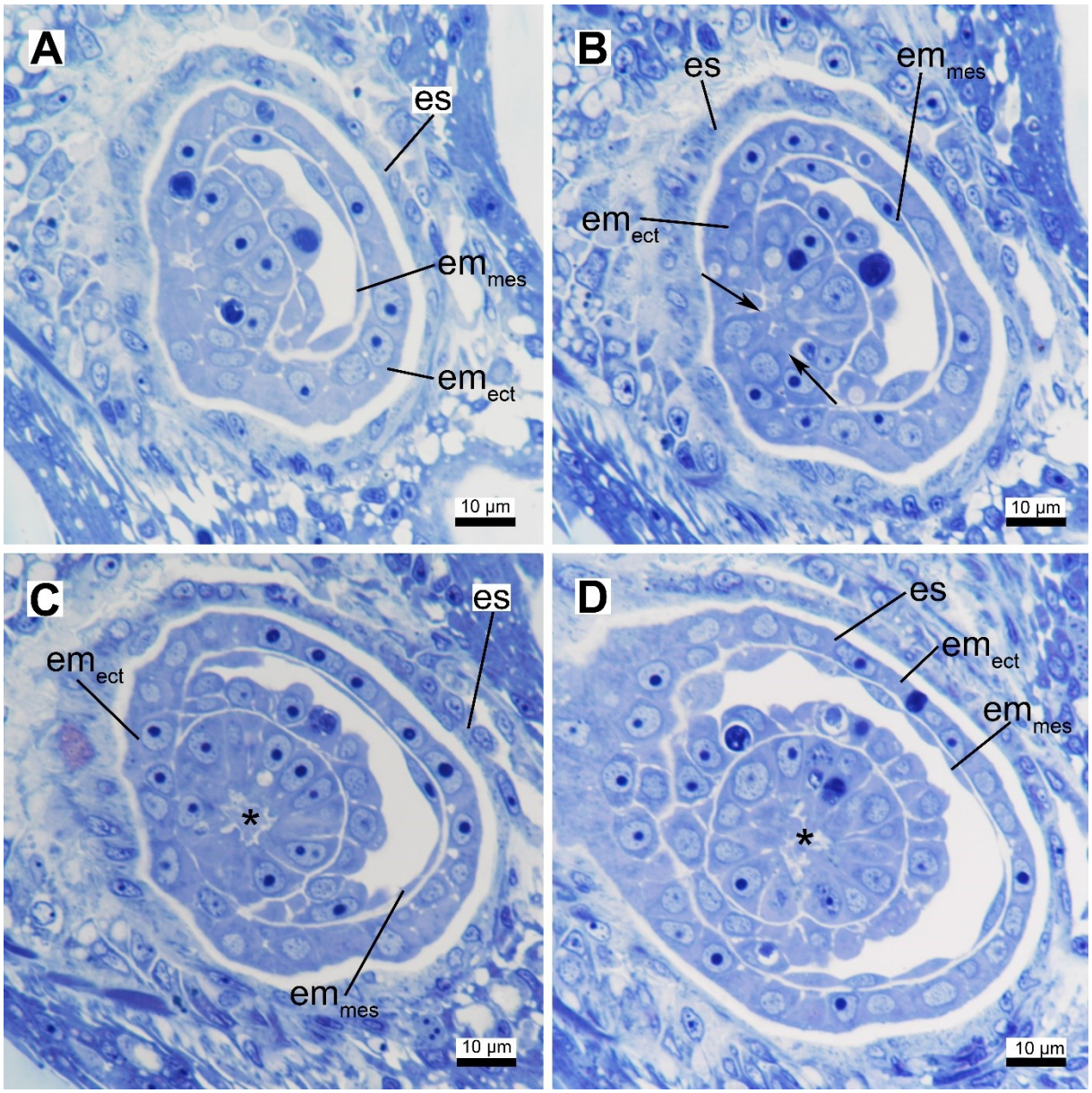

Figure 9: Semi thin cross sections of an embryo of *Plumatella casmiana* in the process of bud formation. The embryo consists of the outer ectoderm and also the inner mesoderm has fully formed when cells of the ectoderm proliferate and start to protrude into the lumen of the embryo while still limited by the embryonic mesoderm (**A**). The ingressing cells originate from dividing cells of the ectoderm of the aboral hemisphere (B, arrows). The mesodermal tissue that lines the embryo inside is rather thin except for the regions where it invaginates along with the ingressing cells of the ectoderm (**B, C**). The thickened mesoderm surrounding the latter is the part of the mesoderm involved in the formation of the future polypide. In further sections closer to the equatorial region of the embryo, the ingressed ectoderm constitutes a concrete cell mass separated from the rest if the embryonic mesoderm, limited by a thickened mesoderm (**D**). Out of both tissues the anlagen of a future polypide differentiated. In addition,

especially the mesoderm but also ectoderm involved in the bud formation show intensely stained cytoplasmatic inclusion (A-D). Abbreviations: **em_{ect}** embryonic ectoderm; **em_{mes}** embryonic mesoderm; **es** embryo sac; asterisk: polypide anlagen.

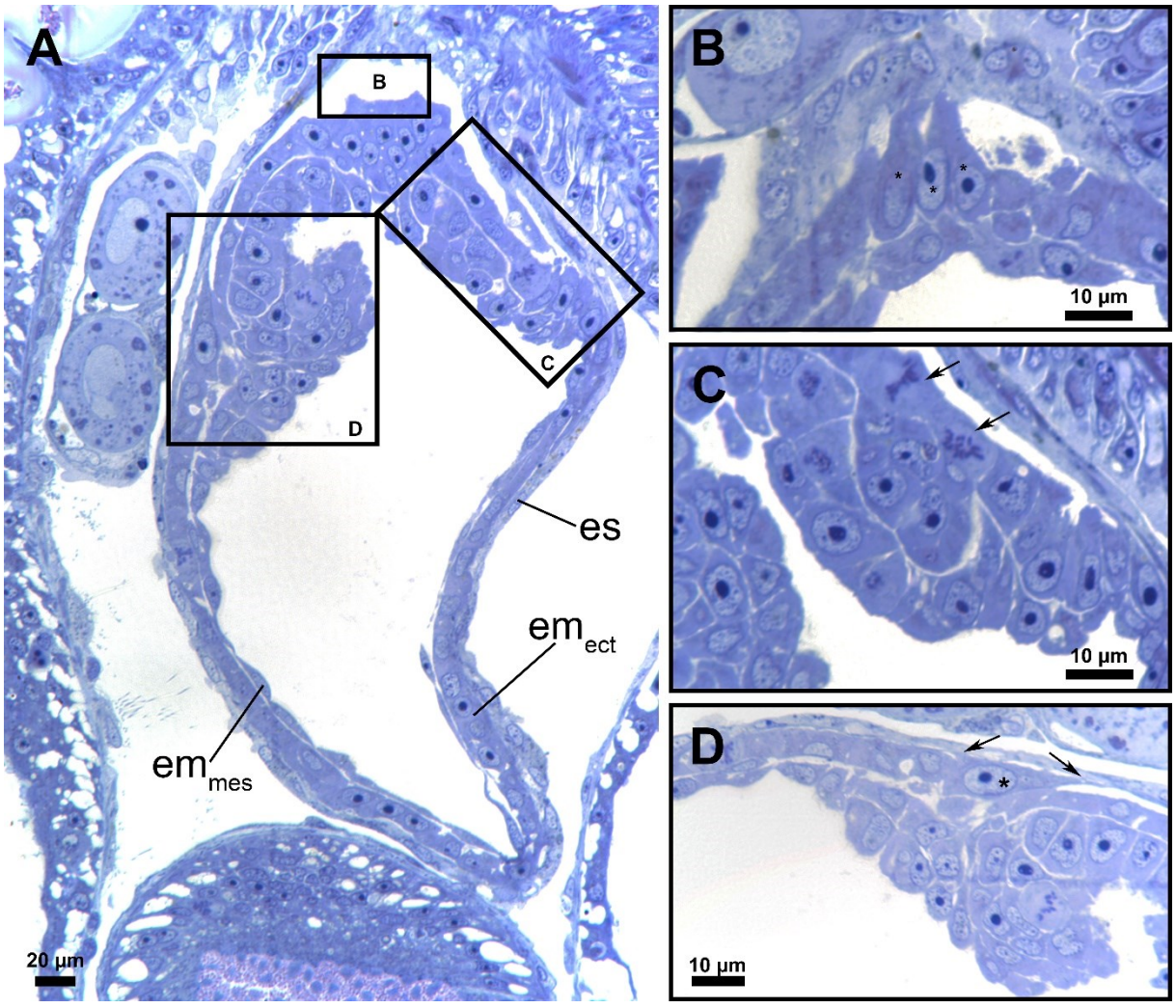

Figure 10 Longitudinal section of a late embryo of *Plumatella casmiana.* **A** Overview of the large, thin embryo sac dangling from the oral body wall next to the ovary that includes several ripe oocytes. The mesoderm of the embryo sac has become a rather thin epithelium that passively stretches along with the growing embryo. The ectodermal lining has completely vanished. The embryo increased in size and is about to form a second bud opposite to the first one. **B** On the fixed end of the embryo sac there are remnants of the once prominent embryo sac cavity. Aboral cells of the embryonic ectoderm are in direct contact to the remnants of the embryo sac cavity at this late stage. **C** Formation of the second bud within the aboral hemisphere of the embryo. Ectodermal cells proliferate (arrows) and ingress while the adjoining mesoderm thickens and forms a cup around the ectodermal ingression. **D** Enduring formation of the older bud. Especially cells of the mesoderm proliferate and borders between ectoderm and mesoderm blur. In addition, single cells of the embryonic ectoderm, referred to as 'placenta cells', establish contact to the mesoderm of the embryo sac. Abbreviations: **em$_{ect}$**

embryonic ectoderm; **em$_{mes}$** embryonic mesoderm; **es** embryo sac; **esc** embryo sac cavity; **pc** placenta cell.

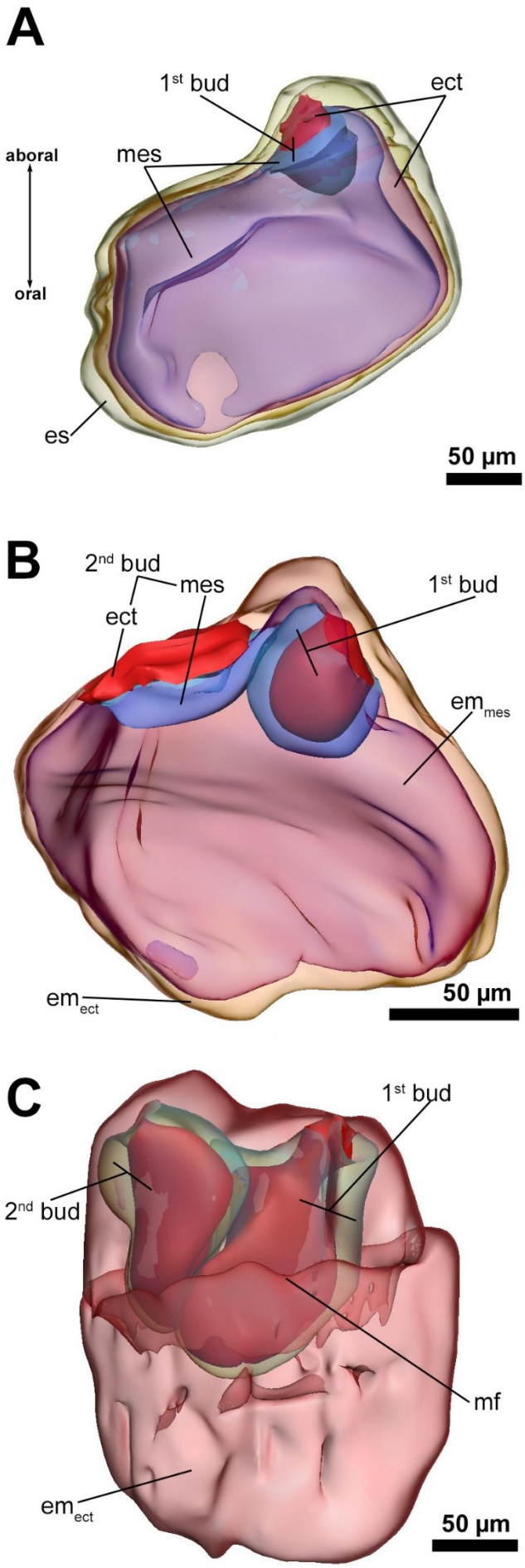

Figure 11: 3D-reconstruction of advanced staged embryos of *Plumatella casmiana* based on serial semi thin sections. **A** Bilayered embryo within thin embryo sac. The first bud forms out of both embryonic tissues in the aboral hemisphere. Mesoderm invagination and ectoderm ingression take place simultaneously. **B** Bilayered embryo with two buds. Two buds develop opposite to each other in the aboral hemisphere The first bud became a cup-like structure, while the mesoderm of the second bud (left) starts to invaginate and is accompanied by the proliferation of the ectoderm. **C** Embryo at late stage with both buds hanging from the aboral pole. The first bud is slightly larger than the second one, apart from that it is indistinguishable what bud formed first. In addition, mantle fold, forms halfway the longitudinal axis.

Abbreviations: **em_{ect}** embryonic ectoderm; **em_{mes}** embryonic mesoderm; **es** embryo sac; **mf** mantle fold.

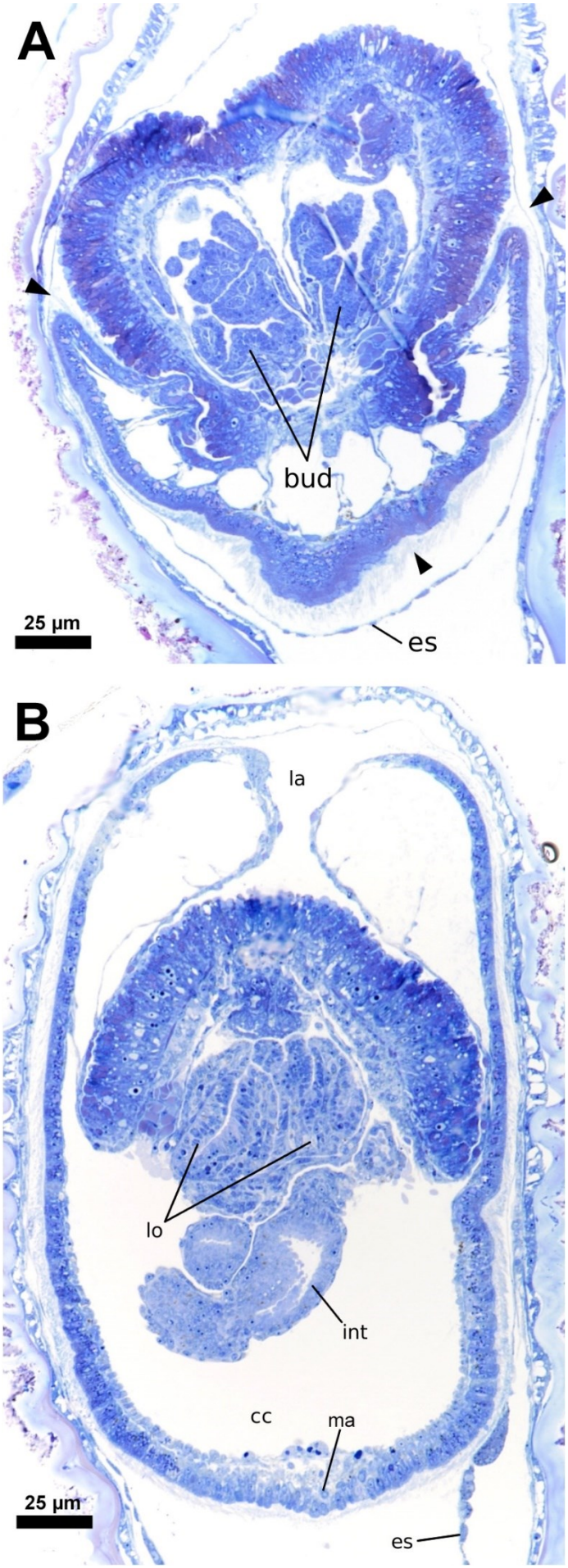

Figure 12: Longitudinal sections of larval stages of *Plumatella casmiana*. **A** The embryo sac has become an extremely thin line, occasionally with flattened cell. Embryonic tissues thickened enormously and now constitute the mantle. At the oral hemisphere, the mantle is thinner and ciliated on the outside (arrowheads). Halfway along the longitudinal axis the mantle forms a deep groove. Inside the larva the two buds increased in size started to differentiate into zooids. **B** Late staged larva. The embryo sac that is barely visible but includes single, voluminous cells. The mantle fold of the equatorial region of the larva overgrew the entire larva towards the aboral pole leaving just a small aperture. The ciliated mantle overgrew the entire larva and now encloses the coelomic cavity. The buds have differentiated the lophophores and digestive tracts, which are suspended in the coelomic cavity. Abbreviations: **b** bud; **cc** coelomic cavity; **es** embryo sac; **int** intestine; **la** larval aperture; **lo** lophophore; **ma** mantle; arrowheads: mantle fold; arrow: cilia.

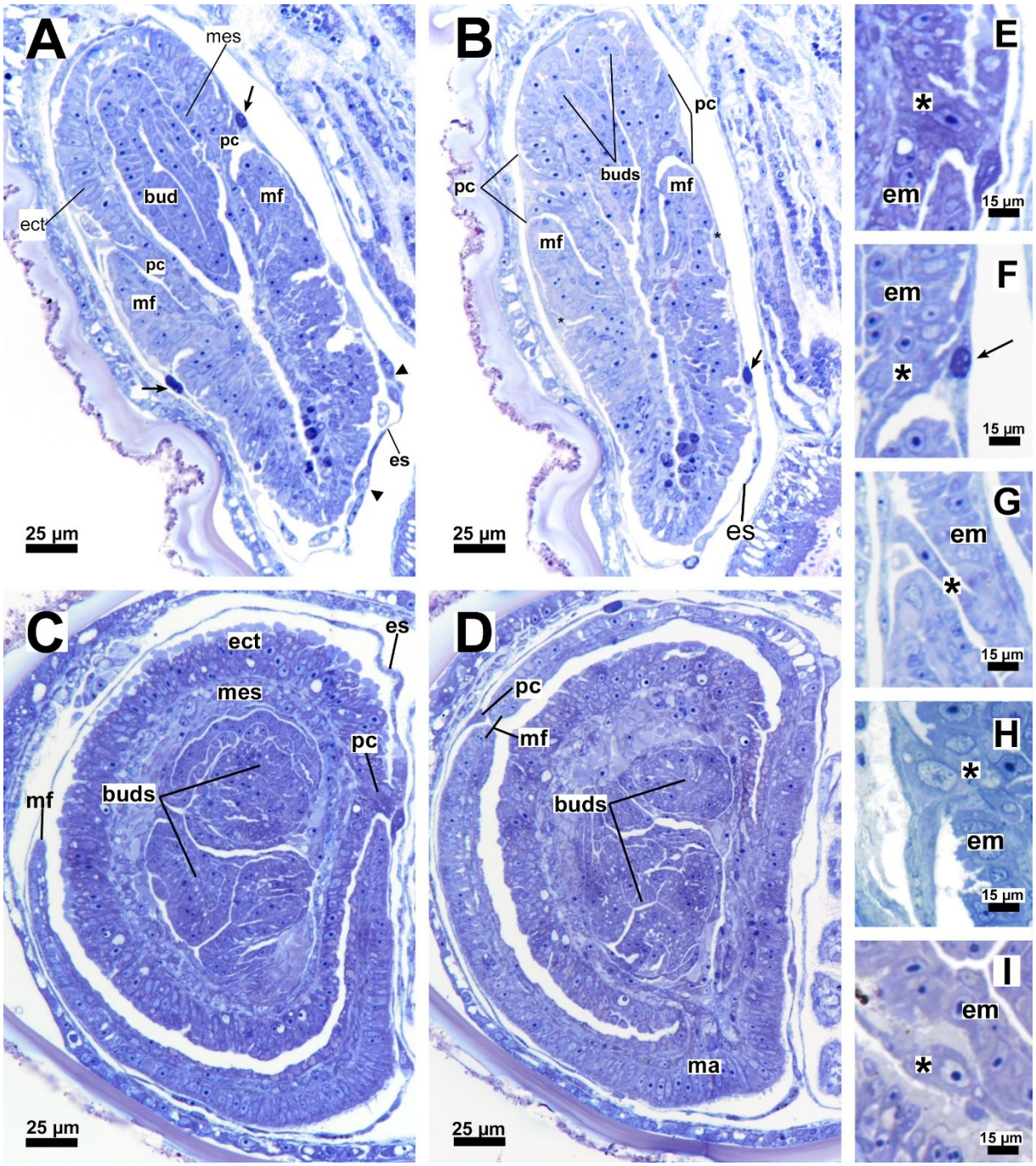

Figure 13: Semi thin sections of larval stages of *Plumatella casmiana.* **A-B** Longitudinal sections of early larva. The coelomic cavity is rudimental and the mantle is not ciliated in contrast to later stages. However, a mantle fold in the equatorial region already exists. Aboral of the mantle fold, single (A) or several adjoining (B) cells of the larval ectoderm establish contact to the mesoderm of the embryo sac and become 'placental cells'. The buds have a formed a lumen via invagination of both tissues that follows the initial phase of bud formation. The embryo sac is rather thin but clearly of cellular nature. Some cells of the embryo sac have intensely stained cytoplasmatic inclusions. Such an inclusion is also present in the 'placental

cell' of the larva (A, F). **C-D** Oblique cross section of the aboral half of an advanced larva showing contact areas of several adjoining 'placental cells' of the larva connected to remaining cells of the embryo sac (C). Such 'placental cells' get disrupted when the mantle fold overgrows the larva in aboral direction (D). Both buds inside the larva differentiate into functional zooids. Both tissues are involved in forming the zooid, however it is not possible which cells originate from what tissue. **E-I** 'Placental cells' of different sections of different larvae. Comparison of those 'placental cells' shows, that it is not always clear where the placenta cell originated. Mostly, 'placental cells' appear as if formed by cytoplasmatic extensions of embryonic/ larval cells (E, F), whereas in other situations it is impossible to tell what the origin of the cell is (G, H). Also, sometimes the cell looks like clearly belonging to the embryo sac (I). Abbreviations: **b** bud; **ect** ectoderm; **em** embryo/ larva; **es** embryo sac; **ma** mantle; **mes** mesoderm; **mf** mantle fold; **pc** placenta cells; arrows: cytoplasmatic inclusions; asterisks: 'placenta cells' in detail.

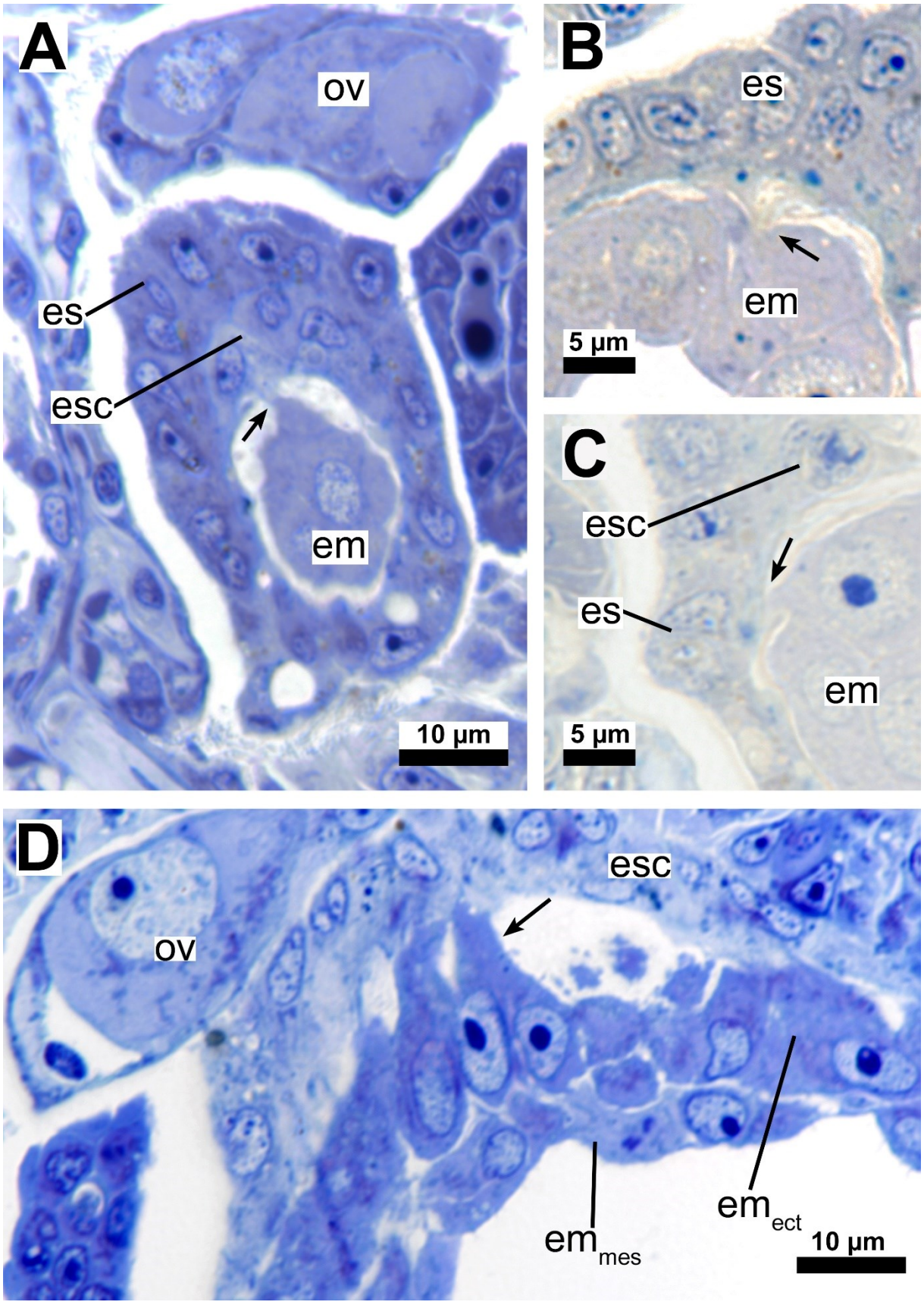

Figure 14: Semi thin sections of different embryonic stages of *Plumatella casmiana* showing contact to the maternal ectoderm. **A** Oblique section through blastula-stage within the

bilayered embryo sac. The latter consists of a thick (outer) mesoderm and a thin inner lining of maternal ectoderm. On the aboral end of the embryo sac the embryo sac cavity has differentiated. A single blastomere of the embryo, is connected to the embryo sac cavity. The cytoplasm of the connected embryo sac cavity cell stains less intense than adjoining cells. **B-C** Detail of single layered embryo in contact with the maternal ectoderm that lines the inside of the embryo sac. Cells of the maternal ectoderm are rather thin and are situated close to the embryo sac cavity. In addition, their cytoplasm stains less intense than the embryo and the mesoderm of the embryo sac. **D** Detail of aboral pole of a late embryo that is about to form the second bud. Most aboral cells of the embryonic ectoderm are clearly in contact with the remaining embryo sac cavity that is very reduced at this late stage. Abbreviations: **em** embryo; **em_{ect}** embryonic ectoderm; **em_{mes}** embryonic mesoderm; **es** embryo sac; **esc** embryo sac cavity; **oc** ovary.

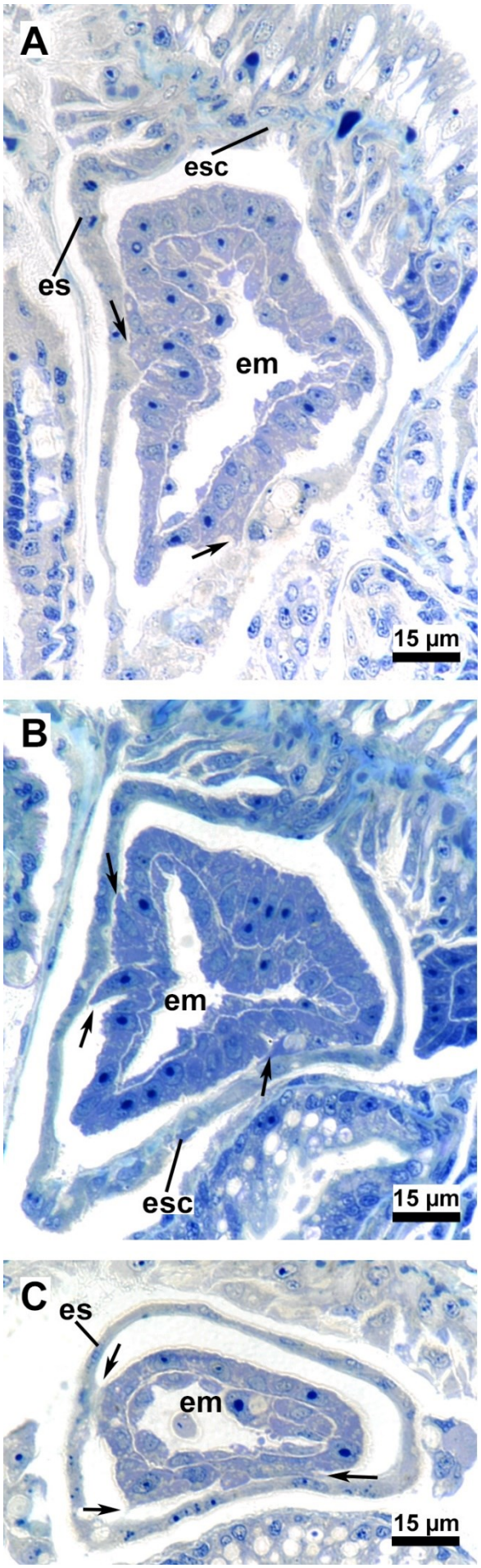

Figure 15: Semi thin oblique cross sections of different regions of an embryo of *Plumatella casmiana*. The embryo sac consists of a rather thick mesoderm and an inner lining of ectoderm. The embryo has just formed the entire mesoderm. On one side of the embryo, the mesoderm thickened (A) and invaginates accompanied by the ingression of the ectoderm (B). The bilayered embryo is about to form its first bud when cytoplasmatic extensions of the embryonic ectoderm establish contact to the mesoderm of the embryo sac. The contact between the mesoderm of the embryo sac and the embryonic ectoderm is referred to as 'placental cells' (arrows). Initially, the 'placental cells' form in the oral hemisphere (C) as well as in the equatorial region (B), where the ectodermal lining has vanished (A). Although concentrated in the equatorial region at later embryonic stages these 'placental cells' form patchy distributed contact areas all around the oral hemisphere of the embryo. **em** embryo; **es** embryo sac; **esc** embryo sac cavity; arrows: contact areas of embryo and embryo sac.

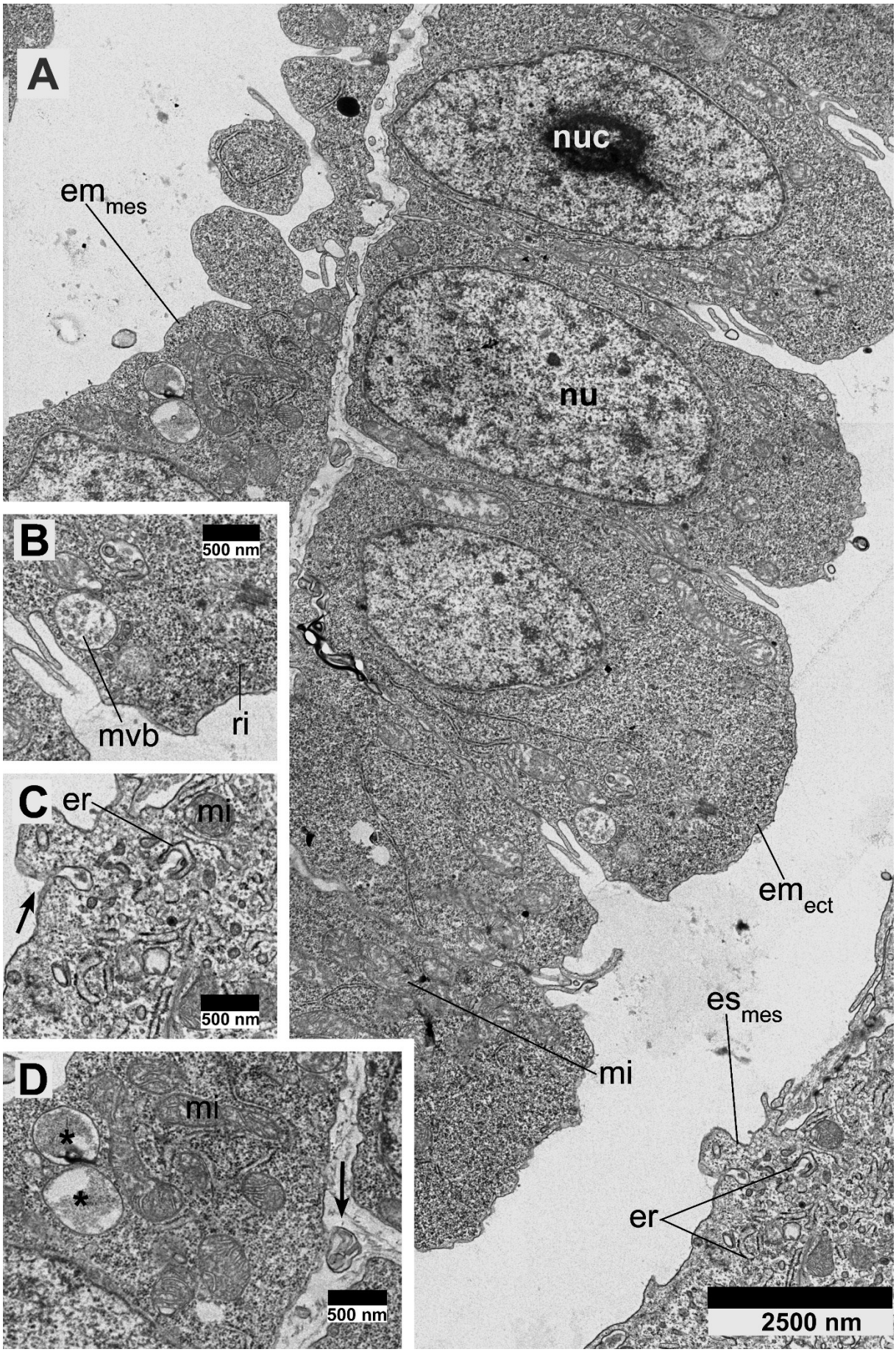
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Figure 16*:* Detail of the oral hemisphere of a bilayered embryo and embryo sac just before bud formation (TEM). **A** Overview of embryonic epithelia and embryo sac. Both embryonic tissues consist of high prismatic cells with rather large nuclei located at the basal pole of the cells. Large vacuoles with unknown content are present at the apical side of the cell. The thick embryonic ectoderm hast several filiform extensions towards the embryo sac. The mesoderm as well as the ectoderm of the embryo show a high density of ribosomes. **B** Detail of the embryonic ectoderm. The ectodermal cell includes several mitochondria as well as mulivesicular bodies especially on the apical side of the cell. **C** Detail of the embryo sac. In contrast to the embryonic tissues, the embryo sac shows a lower density of ribosomes, however much more vacuoles and endoplasmatic reticulum are present. Also, towards the lumen of the embryo sac, vacuoles are in open contact with the former, meaning exo- or endocytosis takes place (arrow). **D** Detail of the mesoderm of the embryo including large vacuoles with unknown content on their apical side (asterisks). In addition, exocytosis towards the ectoderm occurs (arrow). Abbreviations: **em$_{ect}$** embryonic ectoderm; **em$_{mes}$** embryonic mesoderm; **er** endoplasmatic reticulum; **es$_{mes}$** mesoderm of the embryo sac; **mi** mitochondria; **nu** nucleus; **nuc** nucleolus; **ri** ribosomes; asterisks: vacuoles.

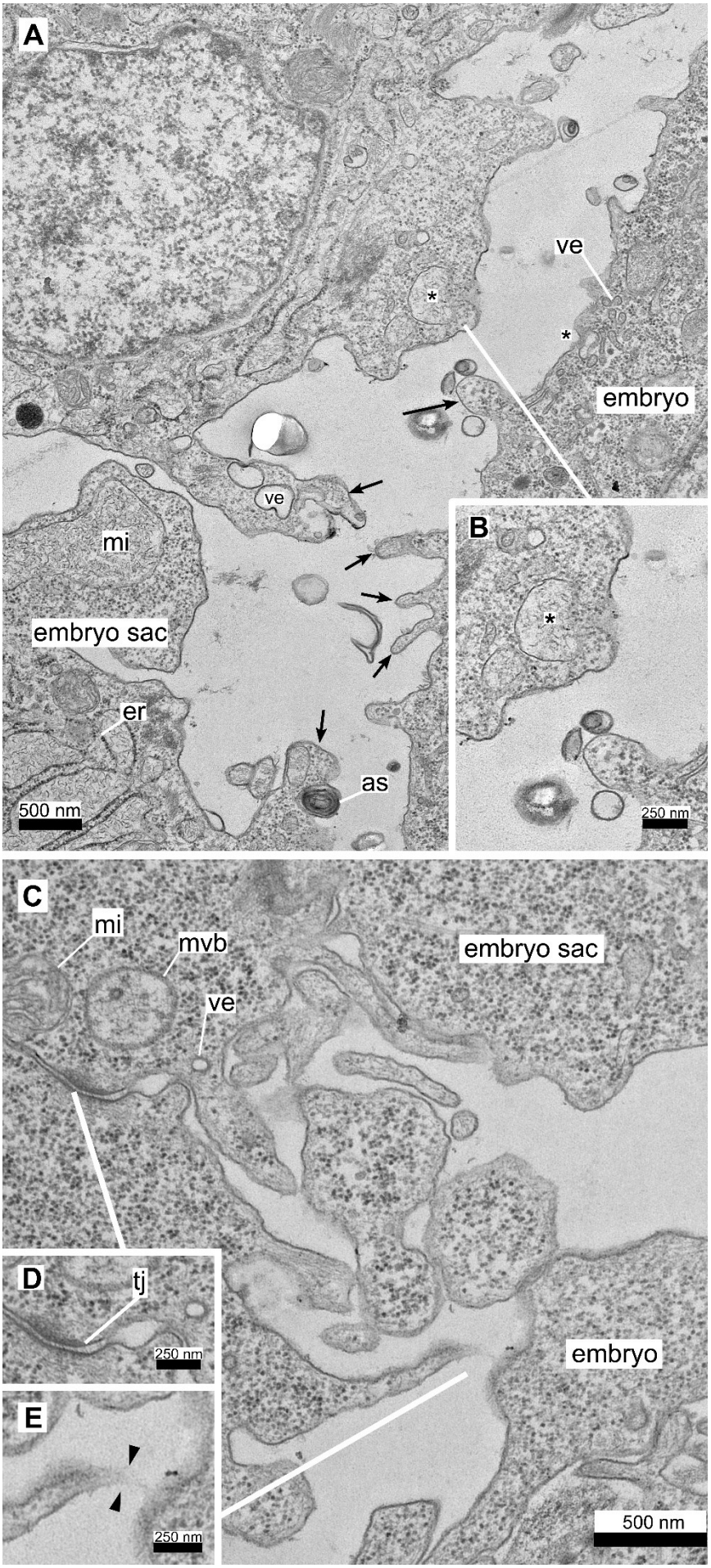
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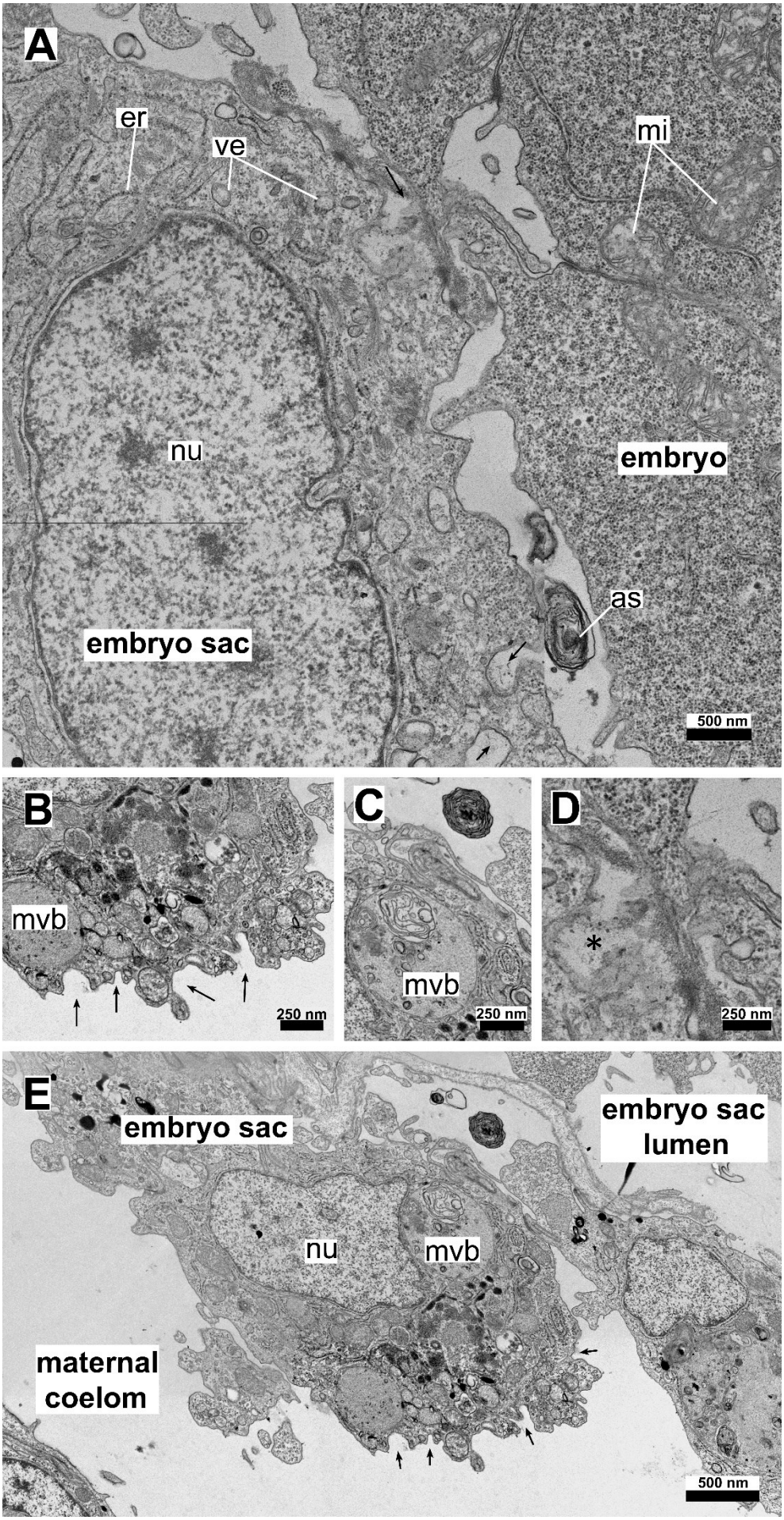
Figure 17: Ultrastructural detail of the gap between embryo and embryo sac (TEM). **A** The embryo shows several filiform extensions towards the embryo sac and vice versa (arrows). Extensions from the embryo sac are more massive and include large vesicles, partly in open contact to the lumen of the embryo sac (asterisk) (**B**). In general, vesicles are present in the embryonic ectoderm as well, however they are smaller and not seen within the extensions. Nevertheless, the ectoderm of the embryo occasionally forms pits indicating exo-/endocytosis. In addition, autophagosomes are present close to the embryo sac. **C** close up of the gap between embryo sac and embryo. Besides mitochondria and vesicles, the embryo sac shows large multi vesicular bodies and numerous, broad cytoplasmatic extensions that also interconnect cells of the embryo sac. Moreover, cells of the embryo sac are connected to each other via tight junctions (**D**). A direct contact between embryo sac and embryo is not verifiable in this section plane, the extensions from the embryo sac are in very close association to the embryo (arrowhead), indicating direct contact between maternal and embryonic tissues (**E**). Abbreviations: **as** autophagosomes; **er** endoplasmatic reticulum; **mi** mitochondria; **mvb** multi vesicular body; **tj** tight junctions; **ve** vesicles; arrows: cytoplasmatic extensions; asterisks: event of exo-/ endocytosis.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mvb
maternal coelom
500 nm

Figure 18: Ultrastructural detail of the embryo sac in close association with the embryo (A, C, D) and the embryo sac in contact with the coelomic cavity of the maternal zooid (B, E). **A** Combined image of the mesoderm of the embryo sac with a large nucleus and surrounding endoplasmatic reticulum. Also, numerous vesicles of different sizes are present around the nucleus. Several large vesicles are in open contact with the lumen of the embryo sac (arrows). Vacuoles are less present within the embryonic ectoderm. The three shown cells of the embryonic ectoderm are interconnected via tight junctions on their apical side. In addition, they possess cytoplasmic extensions on a broad area, so that they collectively form a large pit tightly opposed of a multi vesicular body of the embryo sac that empties its content into the lumen of the embryo sac (asterisk) **(D)**. In addition, a large autophagosome is present close to the embryo sac **(A, C)**. **E** Detail of the outer side of an embryo sac cell in contact with the coelom of the maternal zooid. The large nucleus of the cell appears less spherical than other nuclei of the embryo sac. On the coelomic side, several mitochondria are present whereas on the opposite side directed towards the lumen of the embryo sac, endoplasmatic reticulum and vesicles seem more abundant. One cell of the embryo sac shows several pits (arrows) that indicate endocytosis of coelomic fluid **(B).** Besides numerous intensely stained substance of unknown content, in the same area several vesicles and large multivesicular bodies are present, an especially large one is present on the side of the lumen of the embryo sac **(C, E)**. Abbreviations: **as** autophagosomes; **er** endoplasmatic reticulum; **mi** mitochondria; **mvb** multi vesicular body; **ve** vesicles; arrows: event of exo- /endocytosis.