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Organization of phoronid larval tentacles, their metamorphic remodeling, and a scenario of the lophophore origin

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ABSTRACT: Phoronida is the phylum within stem Spiralia comprising marine worm-like animals with biphasic life cycle: adult phoronids live as benthic organisms in soft or hard substrata, whereas larvae (actinotrochs) are planktonic. Both adults and larvae have tentacle apparatus, which supplies the capture of food particles. Organization of phoronid tentacles may shed light on the problem of appearance and evolution of appendages in Bilateria in general. Different methods were used in order to study the morphology, ultrastructure, proliferative activity, and metamorphic remodeling of larval tentacles in different phoronid species. In phoronid larvae, tentacle apparatus differs in tentacle number, shape, diameter, and length. The postoral ciliated band extends along the lateral sides of the tentacles. Its cilia beat from the top to the down, i.e. band works as it does in tornaria larva. Interesting, the preoral ciliated band works as in trochophore; thereby, in actinotrochs, the mechanism of the ciliated bands processing combines features of protostomes and deuterostomes. Larval tentacles exhibit prominent zonality: there are six differently ciliated zones, which are accompanied with the nerve tracts and muscle bundles. Such type of tentacles can be regarded as "highly specialized". During metamorphosis, larval tentacles undergo great transformation, which occurs in different ways: partial or complete reduction. In newly formed juvenile, tentacles mostly lack prominent zonality and look like "less specialized type". This type of organization may recapitulate an evolutionary step when the last common bilaterian ancestor had been benthic suspense feeder and had non-specialized or less specialized tentacles. Because all recent lophophorates have highly specialized lophophore, which might be evolved in only water column, I have suggested the origin of the lophophore at pelagic stage of the life cycle of the last common lophophorate ancestor. This scenario should suggest that all lophophorates are descendants of the pelagic juvenile of the last common bilaterian ancestor.
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KEY WORDS: lophophore, metamorphosis, larvae, proliferation, coelomic lining, EdU, filter feeding, upstream-collecting mechanism.

Devoted to memory of Claus Nielsen.

Организация личиночных щупалец у форонид, их трансформация в метаморфозе и эволюционный сценарий происхождения лофофора

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РЕЗЮМЕ: Phoronida — это отдельный тип животных в составе ствола Spiralia, включающий морских червеобразных животных с бифазным жизненным циклом: взрослые форониды живут как бентосные организмы в мягких или твердых субстратах, в то время как личинки (актинотрохи) плавают в планктоне. И взрослые особи, и личинки имеют щупальцевый аппарат, который обеспечивает захват частиц пищи из толщи воды. Организация и развитие щупальцевого аппарата форонид может пролить свет на проблему возникновения и эволюции щупалец и других придатков у Bilateria в целом. Различные методы были использованы для изучения морфологии, ультраструктуры, пролиферативной активности и метаморфоза личиночных щупалец у разных видов форонид. У личинок форонид щупальцевый аппарат различается количеством щупалец, формой, диаметром и длиной. Вдоль латеральных сторон щупалец проходит посторальный ресничный шнур, реснички которого бьют сверху вниз и, таким образом, работают как у торнарии. Интересно, что предротовой ресничный шнур актинотрох тоже бьет сверху вниз, как прототрох у трохофоры. Таким образом, работа ресничных шнуров актинотрох сочетает признаки как первичноротых, так и вторичноротых. Личиночные щупальца демонстрируют выраженную зональность: имеется шесть ресничных зон, которые сопровождаются нервными трактами и мышечными пучками. Щупальца такого типа можно рассматривать как «высоко специализированные». В ходе метаморфоза личиночные щупальца подвергаются существенной трансформации, которая происходит разными путями и ведет к частичной или полной утрате щупалец. У молодой ювенильной особи щупальца в основном лишены выраженной зональности и их можно рассматривать как «слабо специализированный тип». Наличие такого неспециализированного типа строения щупалец, вероятно, рекапитулирует эволюционные события, когда последний общий предок всех Bilateria был бентосным организмом, который собирал пищу с поверхности грунта при помощи слабо специализированных или неспециализированных щупалец. Поскольку все современные лофофораты имеют высоко специализированный лофофор, который мог сформироваться только в толще воды и нигде больше, я предполагаю происхождение лофофора на пелагической стадии жизненного цикла последнего общего предка лофофорат. Из этого предположения вытекает, что все лофофораты — группа, произошедшая в результате педоморфоза от пелагических ювенильных особей общего предка Bilateria.
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КЛЮЧЕВЫЕ СЛОВА: лофофор, щупальца, метаморфоз, личинки, пролиферация, EdU, фильтраторы, апстрим.

Introduction

Tentacles are present in cnidarians as well as in representatives from two major lineages of Bilateria: protostomes and deuterostomes. This distribution raises important questions regarding the origin, evolution, and diversification of tentacles in Metazoa. Did the last common bilaterian ancestor possess a tentacle apparatus inherited from a coelenterate-like diploblastic ancestor? This inquiry aligns with the fundamental issue of the origin and morphological characteristics of the last common bilaterian ancestor (Malakhov, Gantsevich, 2022).

Phoronida is a small phylum within the protostome lineage. Thogether with two other phyla of invertebrate s — Brachiopoda and Bryozoa — phoronids form a unified clade known as Lophophorata. Although the monophyly of lophophorates has been supported by various morphological (Temereva, Tsitrin, 2015; Temereva, Kosevich, 2016, 2018; Temereva, 2017; Temereva, Kuzmina, 2022; Temereva *et al*., 2023) and molecular data (Jang, Hwang, 2009; Nesnidal *et al*., 2013; Zverkov *et al*., 2019; Laumer *et al*., 2019), it remains a topic of ongoing debate (Khalturin *et al*., 2022). Most phoronids exhibit a biphasic life cycle, which includes a pelagic larval stage and a sessile benthic adult stage. The tentacle apparatus appears during the larval stage, serving not only to capture food particles but also to facilitate movement and swimming in the water.

In different phoronid species, the larval tentacle apparatus varies in number, length, and thickness of the tentacles, as well as the presence or absence of primordial definitive tentacles (Emig, 1982; Temereva, 2009). The number of tentacles in phoronid larvae is used as a criterion for defining specific developmental stages (Silen, 1954). Furthermore, the maximum number of tentacles in a competent larva is a distinguishing feature for identifying species. However, the data on fine structure of phoronids larval tentacles are limited by organization of their coelomic lining (Temereva, Malakhov, 2006), musculature (Temereva, Tsitrin, 2013), and nervous system (Hay-Schmidt, 1989; Temereva, Tsitrin, 2014a; Temereva, 2017). During metamorphosis, larval tentacles undergo significant transformation to form the adult tentacle apparatus (Temereva, Tsitrin, 2014b; Temereva, Malakhov, 2015). The definitive tentacle apparatus, known as the lophophore, exhibits considerable variation in both morphology and structure across various phoronid species (Temereva, Malakhov, 2009; Temereva, 2019a, b, 2020). While the morphology of phoronid lophophores has been documented in several studies, the specific organization of the tentacles has not been described in detail, unlike the investigations conducted on other lophophorates (Tamberg, Shunatova, 2017; Tamberg *et al*., 2021; Kuzmina, Temereva, 2022, 2024).

This study presents original information regarding the organization of larval tentacle apparatus and its transformation during metamorphosis within the phoronid life cycle, across different phoronid species.

Material and methods

Larvae, metamorphic animals, and juveniles of seven phoronid species were used for this study. Animal were collected in different areas in different times (Table 1). Young larvae of *Phoronopsis viridis* were growth in laboratory (for details see Temereva, Wanninger, 2012). Larvae of other species were gathered from the plankton samples, which were collected with a plankton net (mesh size is 0.4 mm) and a deep net (mesh size is 0.2 mm). All live or fixed larvae were examined with an Olympus CX22 (or Olympus XZ7) light microscope and were photographed with a Panasonic DMCTZ10 (or Toupcam) digital camera. Animals were fixed in different ways accordingly to future studies (Table 1).

Scanning electron microscopy (SEM)

To study the fine morphology, phoronid larvae, which were fixed in 2.5% glutaraldehyde and ten postfixed in 1% OsO4, were dehydrated in increasing concentrations of ethanol and acetone and underwent critical point drying in CO2. Then material was mounted on stubs and sputter coated with platinum-palladium. Specimens were examined with a JEOL JSM-6380LA scanning electron microscope (JEOL Ltd., Tokyo, Japan).

Histology and transmission electron microscopy (TEM)

Larvae and metamorphic animals, which were fixed in 2.5% glutaraldehyde and ten postfixed in 1% OsO4, were dehydrated in increasing concentrations of ethanol and isopropanol and then were embedded in Embed-812 resin (Electron Microscopy Science, USA). Semi-thin (500 nm) and ultra thin (40 nm) sections were prepared with a Leica UC7 ultramicrotome

Table 1. Studied phoronid species, localities, dates of collections, and used methods.

Species, locality, ontogenetic stage, fixative solution, and date of collection / Methods	Light microscopy	SEM	TEM and histology	(Immuno) cytochemistry and CLSM	EdU
Phoronopsis viridis, Coos Bay, Oregon, USA: young larvae (05.2010; 4% paraformaldehyde)	+	–	–	+	–
Phoronopsis harmeri, Vostok Bay, Sea of Japan: larvae, metamorphic animals, juveniles (07–08.2015; 11.2015; 11.2022; 4% paraformaldehyde, 2.5% glutaraldehyde)	+	+	+	+	+
Unknown species, Vostok Bay, Sea of Japan: larvae (11.2022; 96% ethanol)	+	–	–	–	–
Unknown species, Sakhalin Island near Nevelsk: larvae (09.1987; 4% paraformaldehyde)	+	+	–	–	–
Phoronis embryolabi, Vostok Bay, Sea of Japan: larvae (07–08.2015; 4% paraformaldehyde)	+	–	–	–	–
Phoronis ijimai, Vostok Bay, Sea of Japan: larvae (07.2015; 4% paraformaldehyde)	+	+	–	–	–
Unknown species, Nha Trang Bay, South China Sea: competent larva, metamorphic animals, and juvenile (05.2013; 96% ethanol)	+	–	–	–	–

"+" is addressed if the method is used
"–" is addressed if the method is not used

(Leica Microsystems GmbH, Wetzlar, Germany). Semi-thin sections were stained with methylene blue, viewed in an Olympus BX51 microscope (Olympus), and photographed with a Toupcam camera (ToupTek Photonics Co LTD). Ultrathin sections were stained with uranyl acetate (0.5%) and lead citrate (0.4%) and then examined with JEOL JEM-1400 120 kV electron microscope ((JEOL Ltd., Tokyo, Japan), equipped with tungsten filament electron source. Images were taken with JEOL Flash CMOS 2k××2k camera and SerialEM software.

(Immuno)cytochemistry and confocal laser scanning microscopy (CLSM)

Larvae were fixed with 4% paraformaldehyde in 0.2 M phosphate-buffered saline (PBS) (pH 7.4) for 8 h at 4 °C. After fixation, specimens were washed in PBS with Triton X-100 (10%) (ThermoFischer) (PBT): 8 times for 20 min each. Nonspecific binding sites were blocked with 12% normal goat serum (Jackson ImmunoResearch, Newmarket, Suffolk, UK) in PBT overnight at 4 °C. The specimens were incubated overnight in primary antibodies mouse against acetylated-alfa-tubulin (1:700) in PBT at 4 °C overnight. Material then was washed in PBT three times for 5 h each and exposed to the secondary antibody donkey anti-mouse 647 (TermoFisher Scientific, A-31571, 1:1000) in PBT for 24 h at 4 °C. The specimens were then incubated in a 1:40 dilution of AlexaFluor 488 phalloidin (Molecular Probes, Eugene, OR) in PBT for 4 h at room temperature. Following incubation, the specimens were washed in PBS (four times for 60 min each), and embedded in glycerin. Specimens were examined with a Nikon Eclipse Ti confocal microscope (Nikon, Thermo Fisher Scientific, Waltham, MA, USA).

Cell proliferation investigations

The 5-ethynyl-2′-deoxyuridine (EdU; Thermo Fisher Scientific), which incorporates in nucleolar DNA during its synthesis in S-phase, was used as a label for proliferating cells. The EdU stock solution was prepared in filtered sea water (FSW). The optimal EdU concentration of 50 μM and incubation time of 0.5 hr were applied for larvae and metamorphic animals of *Phoronopsis harmeri*. After the incubation period,

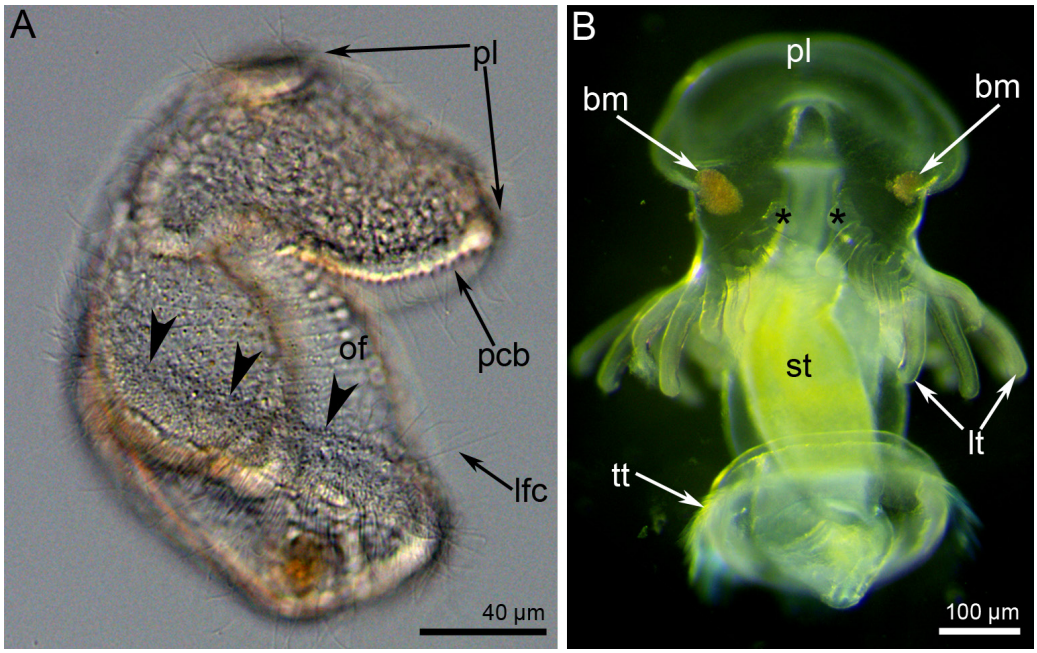

Fig. 1. Different stages of larval development in phoronids; photographs of live animals. A — young larva of *Phoronopsis viridis* viewed from the right. The upper border of ciliated ridge (postoral band) is pointed by arrowheads; B — advanced larva of *Phoronopsis harmeri* viewed from the dorsal side. The youngest tentacles are marked by asterisks.
Abbreviations: bm — blood masses; lfc — cilium of laterofrontal cell; lt — larval tentacles; pcb — preoral ciliated band; pl — preoral lobe; st — stomach; tt — telotroch.

all individuals were rinsed twice with FSW and fixed with 4% PFA (Sigma-Aldrich) in phosphate-buffered saline (PBS; Amresco, Inc., Cleveland) for 8 hr at 4°C. Fixed specimens were rinsed for 1 hr in with PBT and treated with the Click-iT EdU Alexa Fluor 555 Imaging Kit (Thermo Fisher Scientific, Walthan) for the visualization of EdU labeling according to the manufacturer's instructions. Finally, the specimens were rinsed several times with PBS, stained with 4′,6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich), and embedded in glycerin. Specimens were examined with a Nikon Eclipse Ti confocal microscope (Nikon, Thermo Fisher Scientific, Waltham, MA, USA).

Image processing

Z-projections were generated using Image J version 1.43 software. Volume renderings were prepared using Amira version 5.2.2 software (ThermoFischer, Waltham MA, USA). Schemes, photographs, and Z-projections were processed in Adobe Photoshop CS3 (Adobe World Headquarters, San Jose, CA, USA).

Measurements

Measurements of larval tentacles are done for four specimens of each species. The standard error is not calculated.

Results

Development and organization of tentacle apparatus in phoronid larvae

The appearance of tentacle apparatus starts from formation of tentacle ridge at stage early actinotroch (Figs 1A; 2A). The tentacle ridge looks like a swelling of the epithelium of posterior portion of the oral field that extends along the ventral and lateral parts of the body (Fig. 2A), but is disrupted on the dorsal side (Fig. 3A). Thus, the tentacle ridge is horseshoe-liked. Exactly on the dorsal side of a larva, the zone of appearance of novel tentacles is located (Figs 1B; 3A). Here, tentacles are the shortest. The epithelium of tentacle ridge contains specialized cells, each of which bears long immobile cilium that is surrounded by eight thick microvilli (detailed description is below). These cells are well visible in staining with phalloidin due to presence of fibrillar actin in the microvilli (Fig. 2A–C). In advanced actinotroch, small protrusions appear along the tentacle ridge (Fig. 2B). These protrusions become longer and turn into

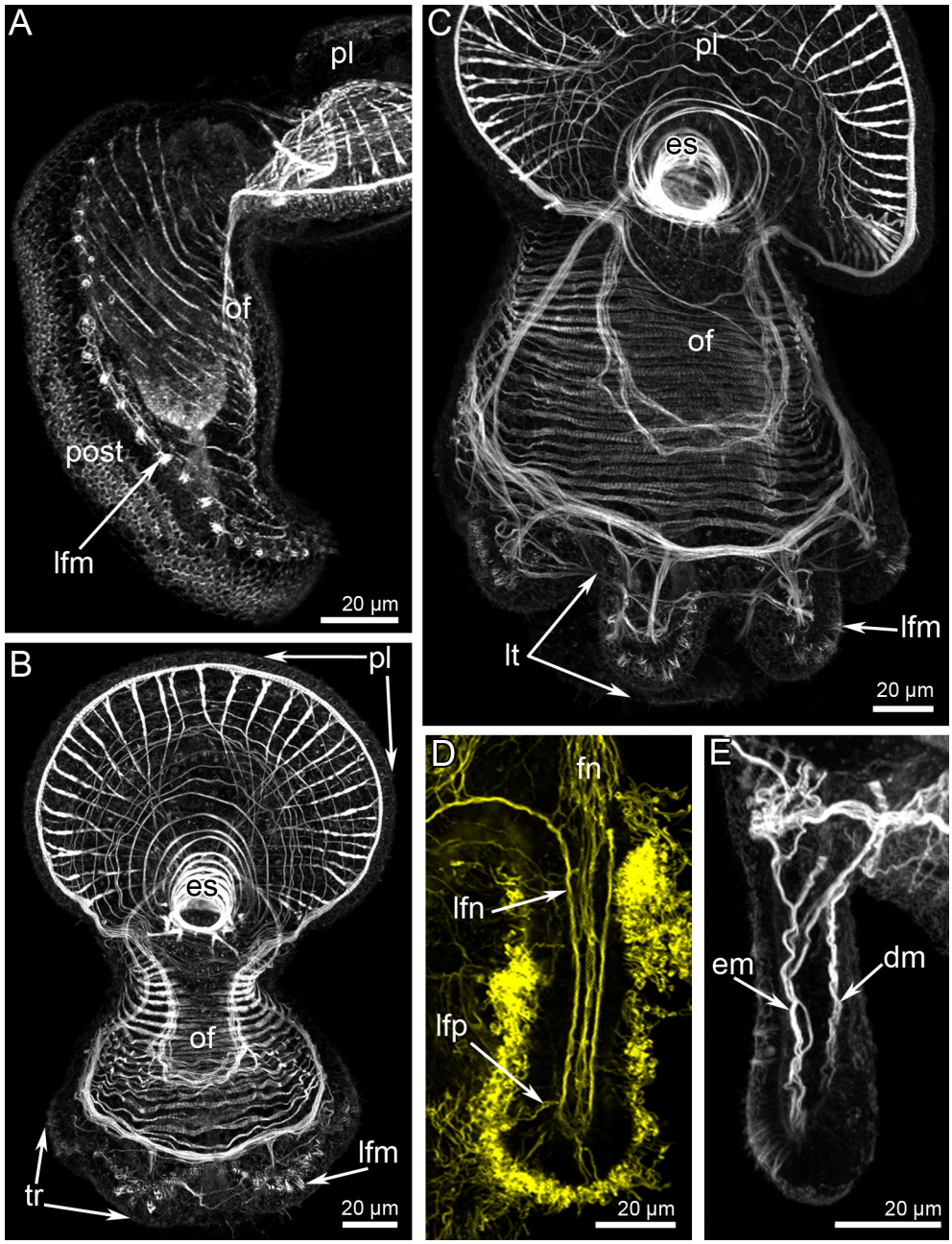

Fig. 2. Details of larval development in *Phoronopsis viridis*: Z-projections; cytochemistry and immunocytochemistry after staining with phalloidin (gray: A–C, E) and acytilated-alpha tubulin (yellow: D). A — young larva, viewed from the right: the laterofrontal cells are well visible due to the presence of long microvilli, in which the thick bundles of fibrillar actin extend; B — larva at first steps of the tentacles formation; C — larva with primordial tentacles; D — a single tentacle viewed from the frontal side; E — a single tentacle viewed from the lateral side.

Abbreviations: dm — depressor muscle; em — elevator muscle; es — esophagus; lfm — microvilli of laterofrontal cell; lfn — laterofrontal neurite bundle; lfp — laterofrontal cell basal projection; lt — larval tentacle; of — oral field; pl — preoral lobe; post — postoral ciliated band; tr — tentacle ridge.

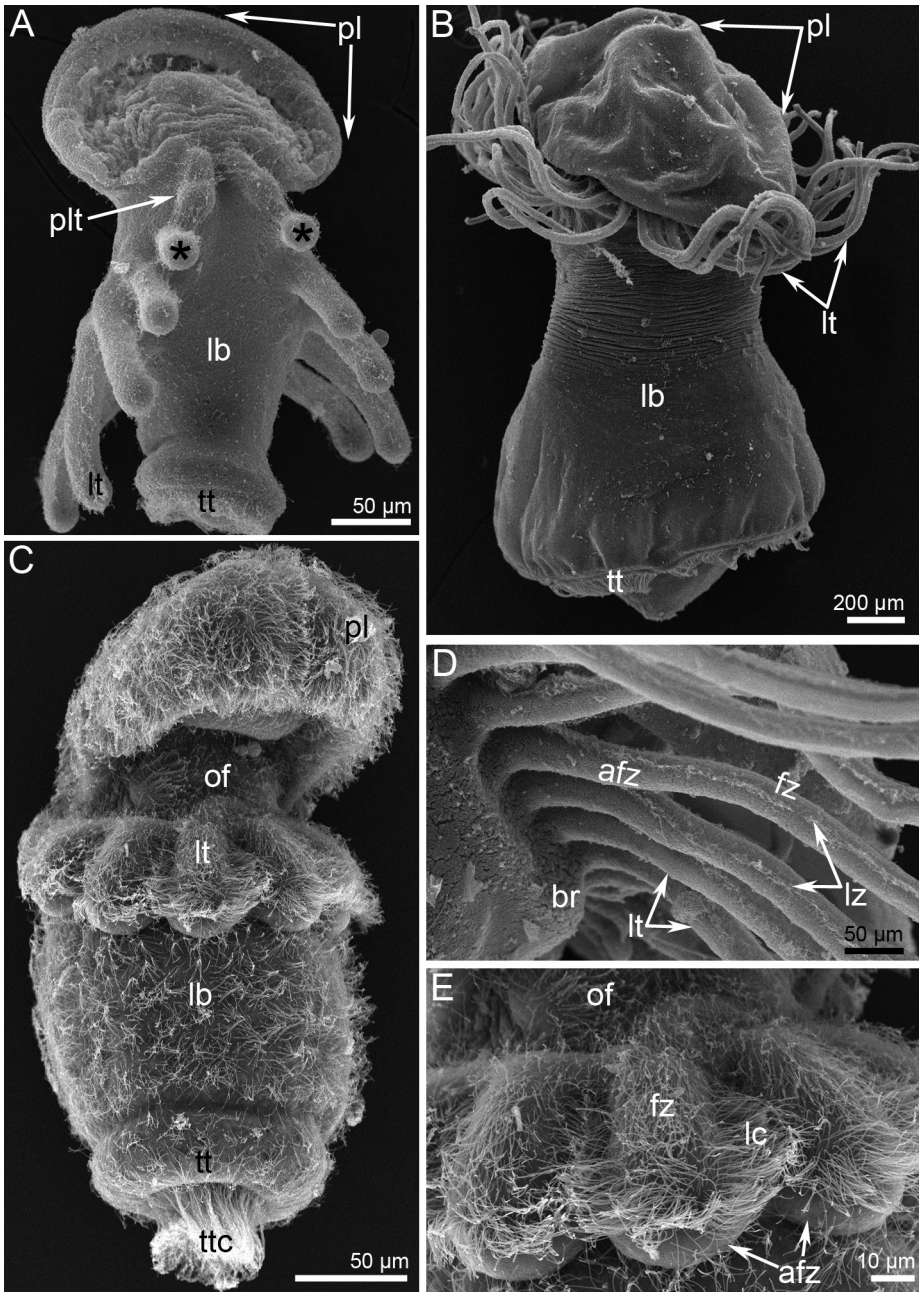

Fig. 3. Organization of the tentacle apparatus in different phoronid larvae (SEM). A — young larva of *Phoronopsis harmeri* viewed from the dorsal side. The youngest larval tentacles are marked by the asterisks; B — competent larva collected near Nevelsk (Sakhalin) viewed from the right; C — competent larva of *Phoronis ijimai* vied from the laterofrontal; D —united basal ridge under the larval tentacles (primordia of definitive tentacles); E — several larval tentacles with prominent zonality of *Phoronis ijimai* vied from the frontal.
Abbreviations: afz — abfrontal zone; br — united basal ridge under the larval tentacles (primordia of definitive tentacles); fz — frontal zone; lb — larval body; lc — lateral cilia; lt — larval tentacle; lz — lateral zone; of — oral field; pl — preoral lobe; plt — primordium of larval tentacle; tt — telotroch; ttc — telotroch cilia.

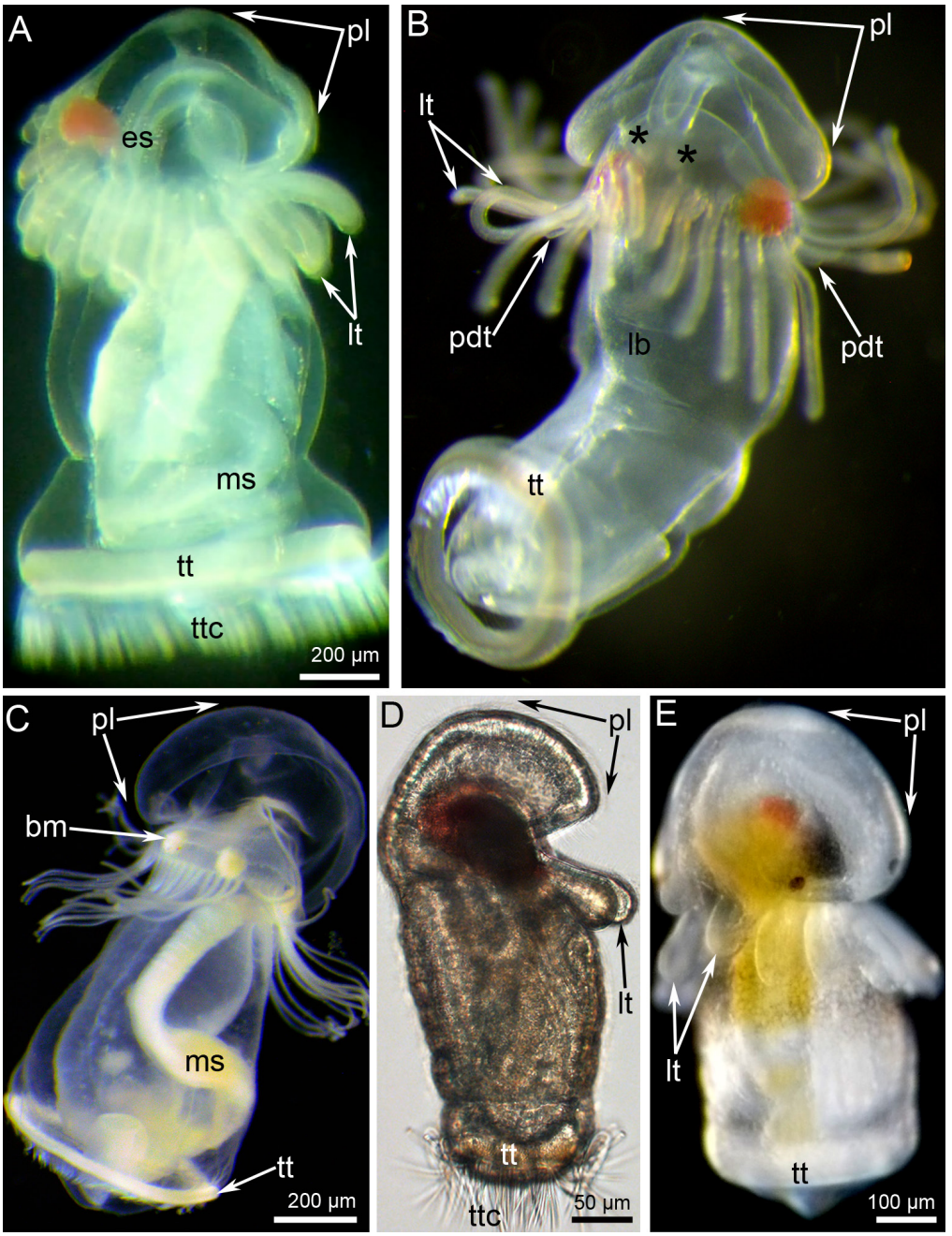

Fig. 4. Organization of the tentacle apparatus in different phoronid larvae: photographs of live (A–B, D–E) and fixed (C) animals. A — competent larva of *Phoronopsis harmeri* viewed from the right; B — competent larva of unknown species collected in Vostok Bay, Sea of Japan; dorsal view. The length of larva is about 1.7 µm; C — competent larva collected near Nevelsk (Sakhalin) viewed from the ventral side; D — competent larva of *Phoronis embryolabi* vied from the right; E — competent larva of *Phoronis ijimai* vied from the right. Abbreviations: bm — blood mass; es — esophagus; lb — larval body; lt — larval tentacle; ms — metasomal sac; pl — preoral lobe; pdt — primordium of definitive tentacle; tt — telotroch; ttc — telotroch cilia.

tentacles (Fig. 2C). The number of tentacles increases gradually with time (Fig. 1B).

In competent phoronid larvae, which belong to different species, the tentacle apparatus has different organization (Figs 3, 4). The difference concerns the number of tentacles, their length and diameter, and the presence (or absence) of definitive tentacle's rimordial. Thus, competent larva of *Phoronopsis harmeri* has 24 tentacles, which are relatively short and thick (Fig. 4A). Larvae of unknown phoronid species from the Sea of Japan have about 30 long tentacles (Fig. 4B). Undefined phoronid larvae from the vicinity of Nevelsk (Sakhalin) have about 40 thin long tentacles (Figs 3B; 4C). Competent larvae of *Phoronis embryolabi* have 8 thick short tentacles, which are swollen on their terminal end (Fig. 4D). Similarly, larvae of *Phoronis ijimai* have 14 thick short tentacles (Figs 3C; 4E). In larvae of some species, the protrusions originate under the base of each larval tentacle. At first steps of formation, these protrusions form a basal ridge (Fig. 3D). In future, these protrusions give rise to the definitive tentacles.

Fine morphology of larval tentacles looks similar in different phoronid species. Thus, SEM revealed the presence of several zones, which extend along each tentacle. There are two lateral zones, which bear the longest cilia, heavy ciliated frontal zone, a few ciliated abfrontal zone, and two laterofrontal zones, which are composed from specialized cells with motionless cilia (Figs 3E; 5). All these zones are well visible in transverse section of a tentacle due to the difference in organization of the epithelium. Thus, the epithelium of lateral zones is highly pseudostratified and composed of spindle like cells bearing large nucleus with a lot of dense heterochromatine (Fig. 6A). Apical cytoplasm of cells of lateral zones is filled with numerous mitochondria of large diameter. Lateral zones of all tentacles altogether form the postoral ciliated band.

In all other zones, organization of epithelium differs from that of lateral zones in density and location of nuclei and abundance of mitochondria. Moreover, abfrontal epithelium includes glandular cells, which extend as two latero-abfrontal longitudinal rows along a tentacle (Fig. 6A, B). In competent larvae, which are ready for metamorphosis, epithelium of abfrontal zone undergoes remodelling due to the loss of some epithelial cells (Fig. 6B). Laterofrontal zones consist of specialized sensory cells (Fig. 7). These cells have a single motionless cilium, which is surrounded by eight thick microvilli (Fig. 7A, B). Thin microvilli are located along outer perimeter of the apical surface of the cell (Fig. 7C). Three longitudinal striated rootlets extend from the basal body of cilium (Fig. 7D). The basal part of cell is transformed to the long projection, which is filled with synaptic vesicles (Fig. 7A). This projection is connected with the laterofrontal neurite bundles, which extend along the laterofrontal sides of each tentacle and form a continuous nerve (Fig. 2D).

Larval tentacles contain the cavity (blastocoel = haemocoel), in which coelomic canal and muscle bundles extend (Figs 5; 8A, B). In transverse section, the coelomic canal is horseshoe-shaped and its edges are close on the frontal side (Fig. 5). Here, the coelomic lining consists of myoepithelial cells, which bear longitudinal myofibrils (Fig. 6D). Myoepithelial cells can be observe in coelomic lining of the abfrontal side (Fig. 6E), whereas on the lateral sides coelomic lining consists of epithelial cells only (Fig. 6C). Myoepithelial cells of abfrontal side have fanciful shape and form basal projections, which are interdigitated in adjacent cells (Fig. 6E). The coelomic canal does not reach the end of tentacle (Fig. 8B). The inner walls of horseshoe-shaped coelomic canal surround the haemocoel, which thereby forms here the blood vessel (Figs 5; 8A). Coelothelial cells, which form the wall of blood capillary, contain transverse myofibrils. In each tentacle, there are two main muscle bundles: frontal (elevator muscle) and abfrontal (depressor muscle) (Fig. 2E). Both these muscles include myoepithelial cells of coelomic lining as well as thick bundles, which extend in the haemocoel (Fig. 8B).

Experiments with EdU revealed that larval tentacles growth in young and competent larvae (Fig. 9A, B). There is a single zone of proliferative activity in tentacles. This zone is associated with the postoral ciliated band and is located at the base of tentacles. There are proliferated cells, which are located between tentacles, as well as in each tentacle, there are two lateral zones of proliferation (Fig. 9A, B). Cells, which are located between tentacles, are also visible in histological sections: they are submerged under the epithelium and form the groups with 5–8 cells (Fig. 8A).

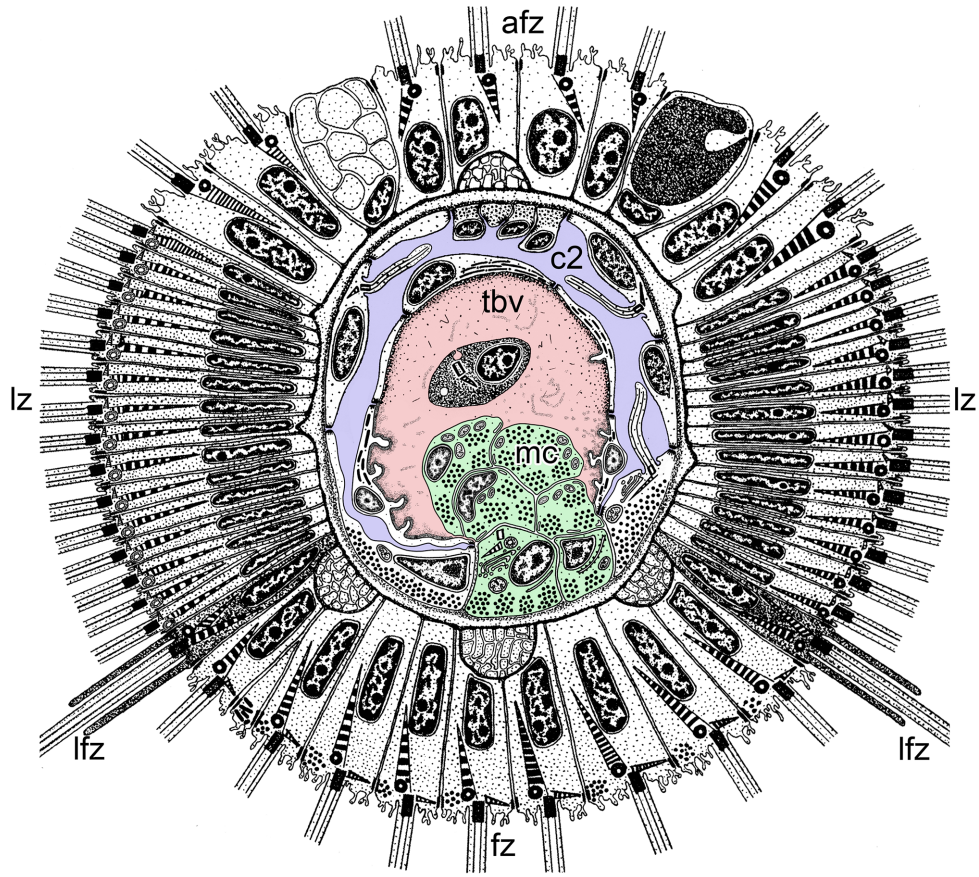

Fig. 5. The scheme of *Phoronopsis harmeri* larval tentacle organization in the transverse section.
Abbreviations: afz — abfrontal zone; c2 — mesocoel (tentacle coelom); fz — frontal zone; lfz — laterofrontal zone; lz — lateral zone; mc — muscle cell; tbv — tentacle blood vessel.

Metamorphic remodelling of larval tentacle apparatus

In this paper, two different ways of tentacles remodelling are observed: partial transformation (in *P. harmeri*) and complete transformation (in unknown phoronid species from Nha Trang Bay). In *P. harmeri*, transformation of tentacles occurs at early stages of metamorphosis and starts from expulsion of epithelium of lateral and laterofrontal zones, which form a continuous rope along larval tentacles (Fig. 10A). In early metamorphic animals, this rope can be observed in transverse sections of tentacles as two lateral bundles (Figs 10B; 11A). The rope consists of apoptotic and degenerated cells, which lose the contact with basal lamina (Fig. 11B). Among these cells specialized laterofrontal cells are still recognizable. Frontal and abfrontal musculature undergo great remodeling (Fig. 11C–E). In both bundles, parts located in the haemocoel are completely destroyed (Fig. 11C, D). Thereby the large rapture is formed on the frontal side (Fig. 11C). On the abfrontal side, degenerated muscle cells are visible within cells of coelomic lining (Fig. 11E). At first steps of metamorphosis proliferative activity is registered at the base of tentacles, but lacks along the tentacles (Fig. 9C).

In *P. harmeri*, 3–4 days-old juvenile has tentacle apparatus, which consists of 28–30 tentacles (Fig. 10C). Although tentacles look like completely developed, their changes continue (Figs 10D; 12A). First of all, juvenile tentacles do not exhibit prominent zonality, which is characteristic of larval tentacles (Fig. 12A). The most obvious transformation concerns the lateral zones: here, epithelial cells undergo cell

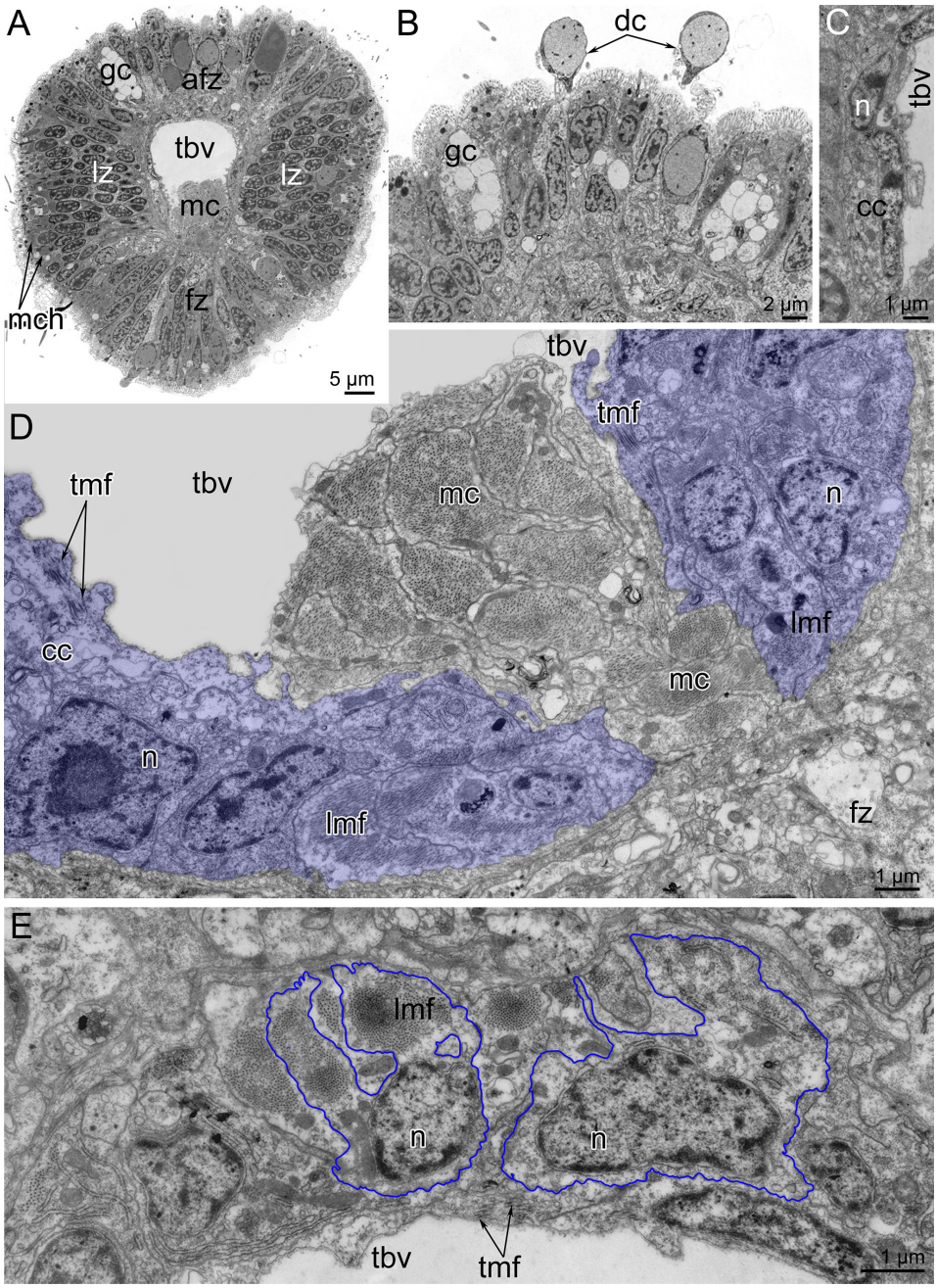

Fig. 6. Details of ultrastructure of larval tentacles in *Phoronopsis harmeri*; TEM. A — general view of the tentacle transverse section; B — specificity of the abfrontal zone, in which the extrusion of cells occurs; C — coelomic lining of the lateral side of the tentacle coelom: myoepithelial cells are absent; D — coelomic lining (blue) and muscle bundle on the frontal side of tentacle; E — coelomic lining of the abfrontal side of tentacle: the border of two myoepithelial cells are shown by blue line.

Abbreviations: afz — abfrontal zone; c2 — mesocoel (tentacle coelom); cc — coelomic lining; dc — degenerated cells; gc — gland cell; fz — frontal zone; lfz — laterofrontal zone; lmf — longitudinal myofilaments in cells of coelomic lining; lz — lateral zone; mc — muscle cell; mch — mitochondria; n — nucleus; tbv — tentacle blood vessel; tmf — transverse myofilaments in cells of the blood vessel wall.

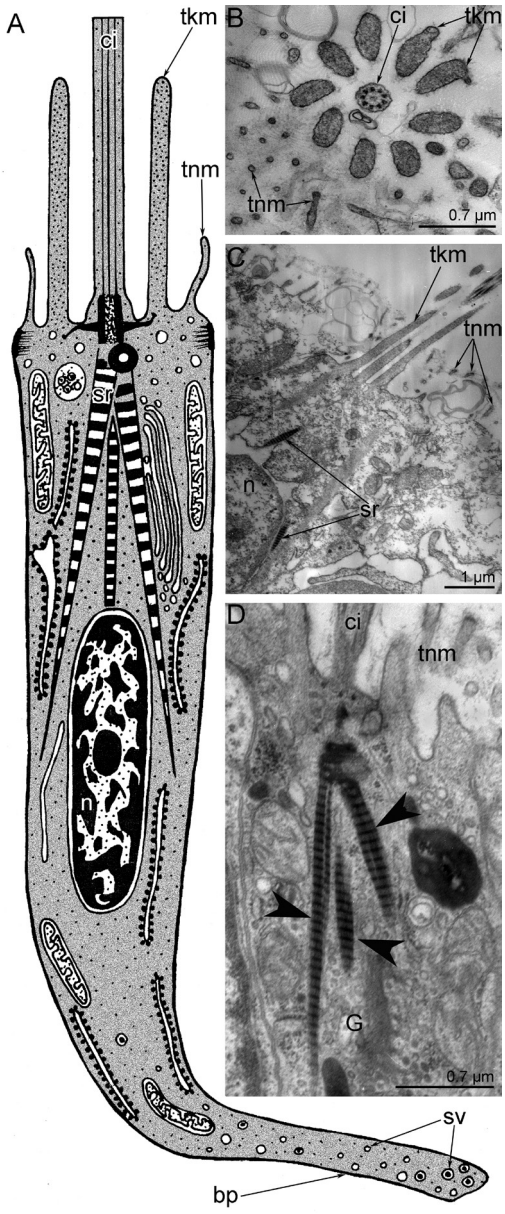

Fig. 7. Organization of laterofrontal cells of larval tentacle in *Phoronopsis harmeri*; scheme (A) and TEM (B–D). A — the scheme of a cell; B — transverse section of the cilium, which is surrounded by eight thick microvilli; C — longitudinal section of apical part of cell: thick microvilli with bundles of fibrillar actin are visible; D — the rootlet apparatus; rootlets are pointed by arrowheads.

Abbreviations: bp — basal projection; ci — cilium; G — Golgy apparatus; n — nucleus; sr — striated rootlet; sv — synaptic vesicle; tkm — thick microvilli; tnm — thin microvilli.

division (Fig. 12A). This data are confirmed by experiments with EdU: great proliferative zone extends along the lateral sides of tentacles (Fig. 9D). Newly formed lateral epithelium differs from that of larval tentacles: cells lack large mitochondria in their apical cytoplasm and nuclei form a single row (Fig. 12B). Musculature of frontal side is formed by myoepithelial cells of coelomic lining (Fig. 12C), whereas abfrontal musculature is not already developed (Fig. 12D).

In unknown phoronid species from Nha Trang Bay, larva has 20 larval tentacles and short definitive tentacles under them (Fig. 13A). When the metamorphosis begins, larval tentacles seem to be broken: they are curved and clasped to the body (Fig. 13B). Then, larval tentacles start to submerge into the mouth and their number decreases (Fig. 13C). Short definitive tentacles are visible as knobs. The number of larval tentacles constantly decreases (Fig. 13D, E) and then all larval tentacles are eaten by newly formed juvenile (Fig. 13F).

Discussion

Development and organization of tentacle apparatus in phoronid larvae

Development of tentacles in phoronid larvae is known for many species, but not in detail: only appearance of novel tentacles and the maximal number of tentacles are known for larvae of some phoronid species due to importance of these features for identification of larvae (Silen, 1954; Emig, 1982; Temereva, Malakhov, 2004, 2007; Temereva, Kulikova, 2007; Temereva, 2009; Temereva, Neretina, 2013). The number of larval tentacles is also important for ascertainment of the stages of larval development (Silen, 1954; Emig, 1982). The origin of larval tentacles from ciliated ridge of early larva has been shown for some phoronid species: *Phoronopsis harmeri* (Zimmer, 1964; Temereva, Malakhov, 2007), *Phoronis ijimai* (Zimmer, 1964; Temereva, Malakhov, 2000), *Phoronis muelleri* (Silen, 1954), *Phoronis embryolabi* (Temereva, 2017). Interesting, this ciliated ridge contains specialized cells with movementless cilium and thick long microvilli at early stage of development that means the transformation of true larva (early gastrula) to the advanced organism, which should be regarded not as true larva, but as young juvenile (Malakhov *et al*., 2019; Temereva, 2022).

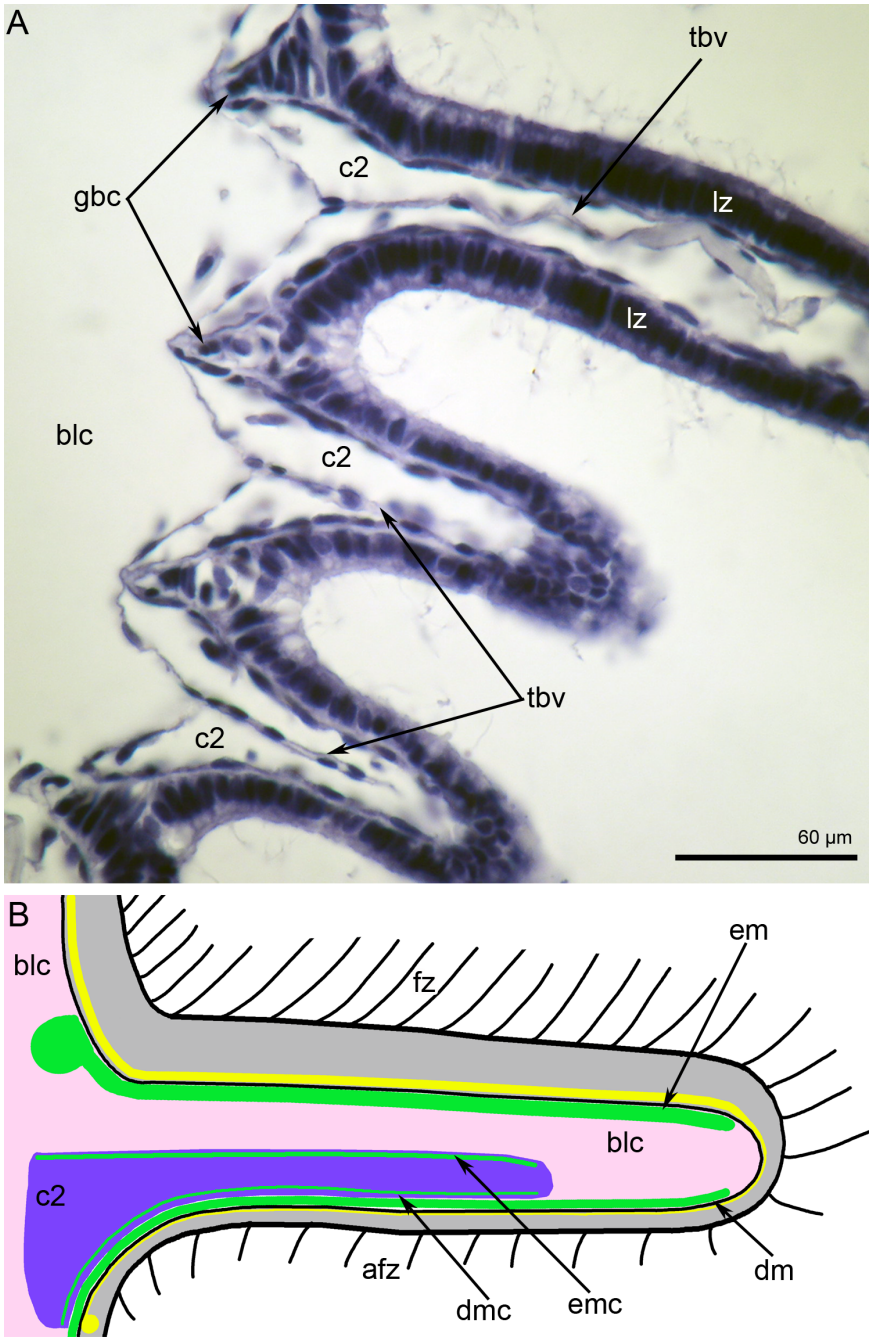

Fig. 8. Organization of larval tentacles of *Phoronopsis harmeri* in longitudinal section. A — histological section; B — the scheme. Color code: blue — mesocoel; gray — epidermis; green — muscles; pink — blastocoel; yellow — nerve elements.

Abbreviations: afz — abfrontal zone; blc — blastocoel = heamocoel = blood vessels; c2 — mesocoel (tentacle coelom); dm — depressor muscle; dmc — depressor muscle formed by cells of coelomic lining; em — elevator muscle; emc — elevator muscle formed by cells of coelomic lining; fz — frontal zone; gbc — groups of basal cells; lz — lateral zone; tbv — tentacle blood vessel.

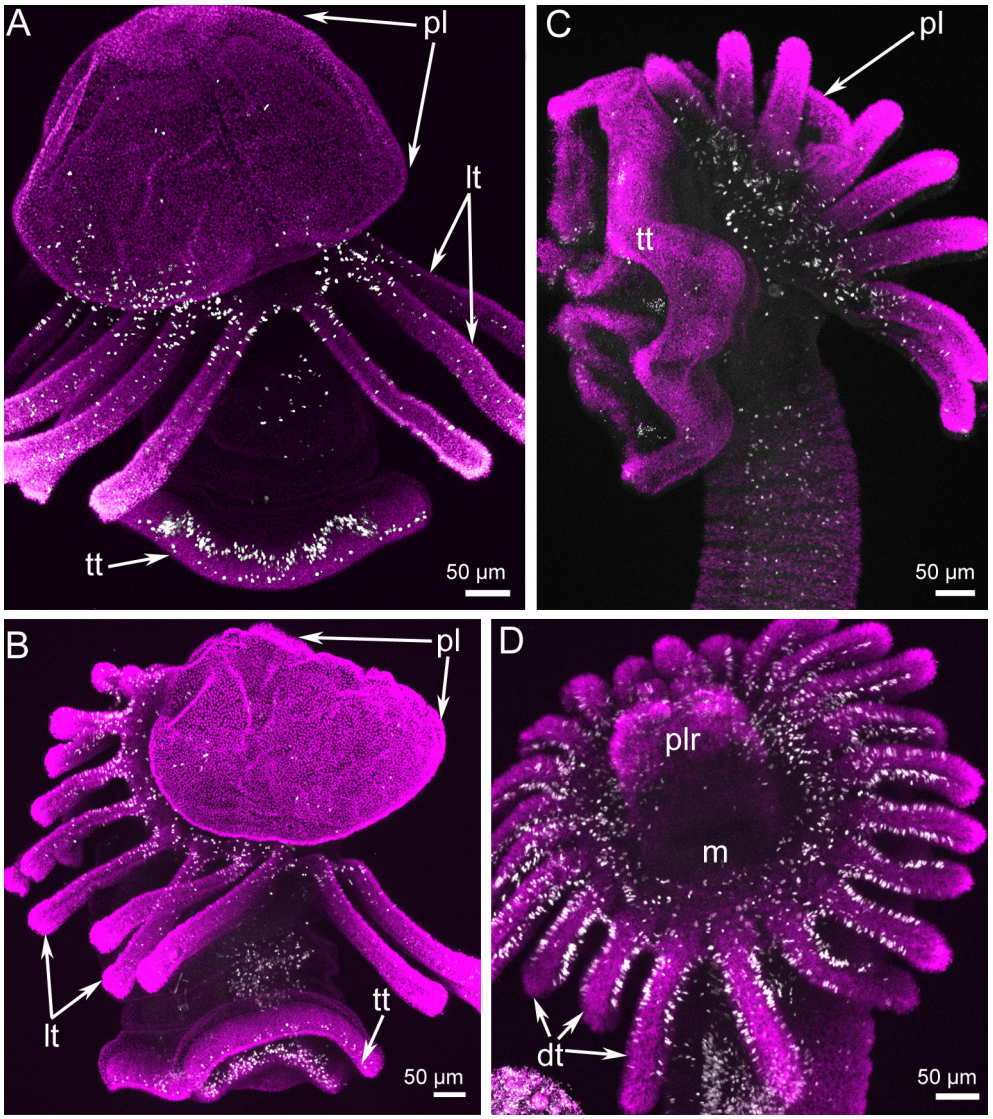

Fig. 9. Different stage of *Phoronopsis harmeri* life cycle; Z-projections after staining with DAPI (nucleus are shown in magenta) and Click-iT EdU Alexa Fluor 555 (proliferating cells are shown in gray); CLSM. A — young larva; B — competent larva; C — metamorphic animal (5 min after metamorphosis starts); D — newly formed juvenile (30 min after metamorphosis starts).
Abbreviations: dt — definitive tentacles; lt — larval tentacles; m — mouth; pl — preoral lobe; plr — preoral lobe remnant; tt — telotroch.

The organization of larval tentacles in phoronids is described in the light of two fundamental problems: the mechanism of filter feeding and the origin of tentacles in Bilateria. Accordingly to some researchers, including Claus Nielsen, there are two main mechanisms of water direction in bilaterians with tentacles: upstream and downstream, thereby there are "upstream" and "downstream" filter feeders (Jørgensen *et al.*, 1984; Nielsen, 1987, 2002; Nielsen, Riisgård, 1998; Larsen, Riisgård, 2002; Riisgård, Larsen, 2010; Riisgård *et al.*, 2015). In upstream filter feeders, the water current directs from the top and escape the tentacle crown between tentacles. All lophophorates and deuterostomians are upstream filter feeders (Nielsen, 1987, 2002;

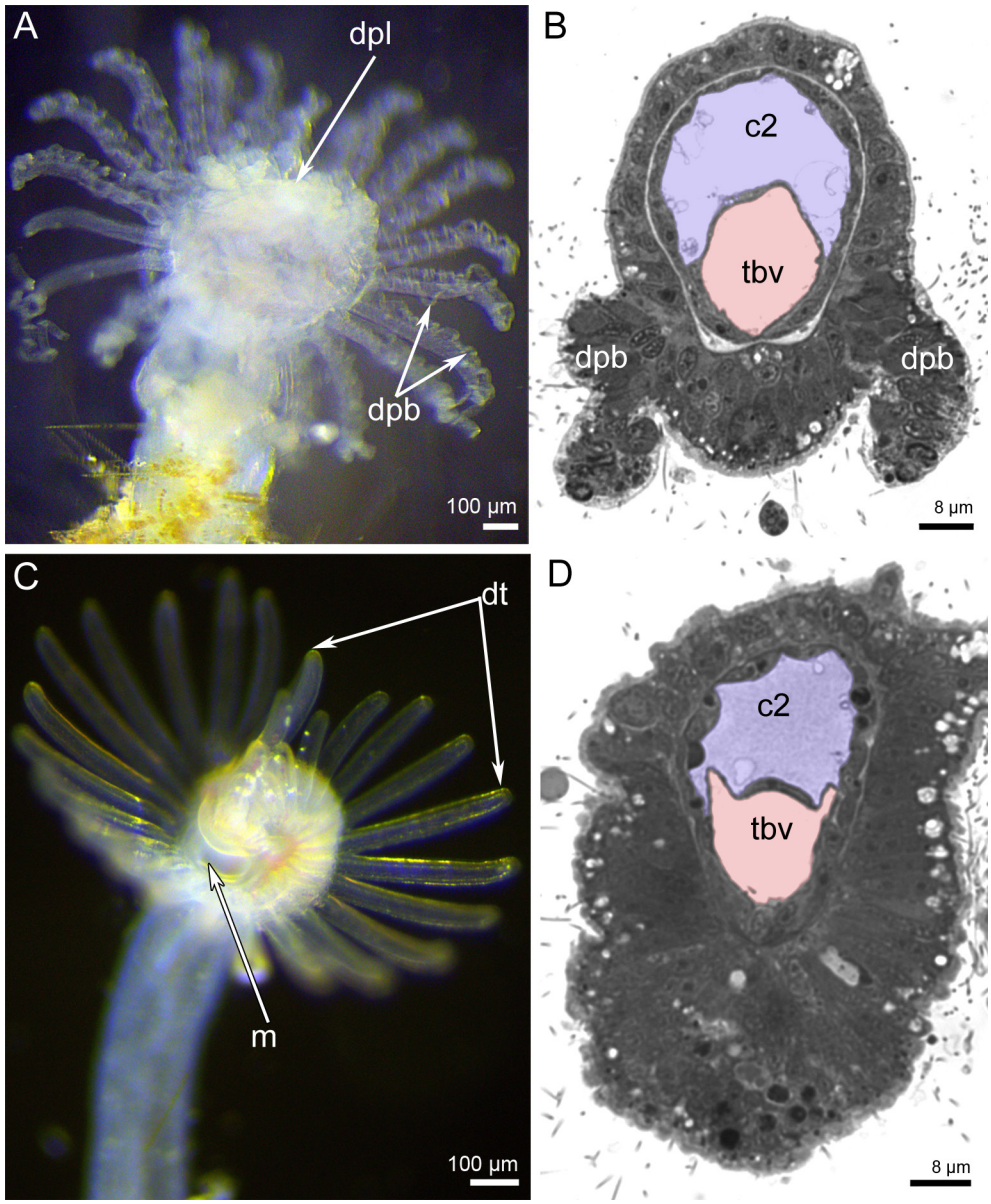

Fig. 10. Tentacles apparatus in juveniles of *Phoronopsis harmeri*. A — metamorphic animal with extruded postoral ciliated band; live animal; B — transverse semithin section of a tentacle in metamorphic animal; C — juvenile with completely renovated tentacle apparatus; live animal; D — transverse semithin section of a tentacle in juvenile.

Abbreviations: c2 — mesocoel (tentacle coelom); blc — blastocoel = heamocoel = blood vessels; dpb — degenerated postoral ciliated band; dpl — degenerated preoral lobe; dt — definitive tentacles; m — mouth; tbv — tentacle blood vessel.

Nielsen, Riisgård, 1998; Ruppert *et al*., 2004). In downstream filter feeders, i.e. spiralians: annelids, mollusks, some rotifers, and entoprocts, the water current passes in the tentacle crow from the down between tentacles and escapes at the top (Riisgård, Ivarsson, 1990; Mayer, 1994, 2000; Riisgård *et al.*, 2000, 2002; Riisgård, Nielsen, 2006; Riisgård, Larsen, 2010). Such patterns of mechanism of filtration are presence not only in adults, but in larvae as well. In deuterostomian

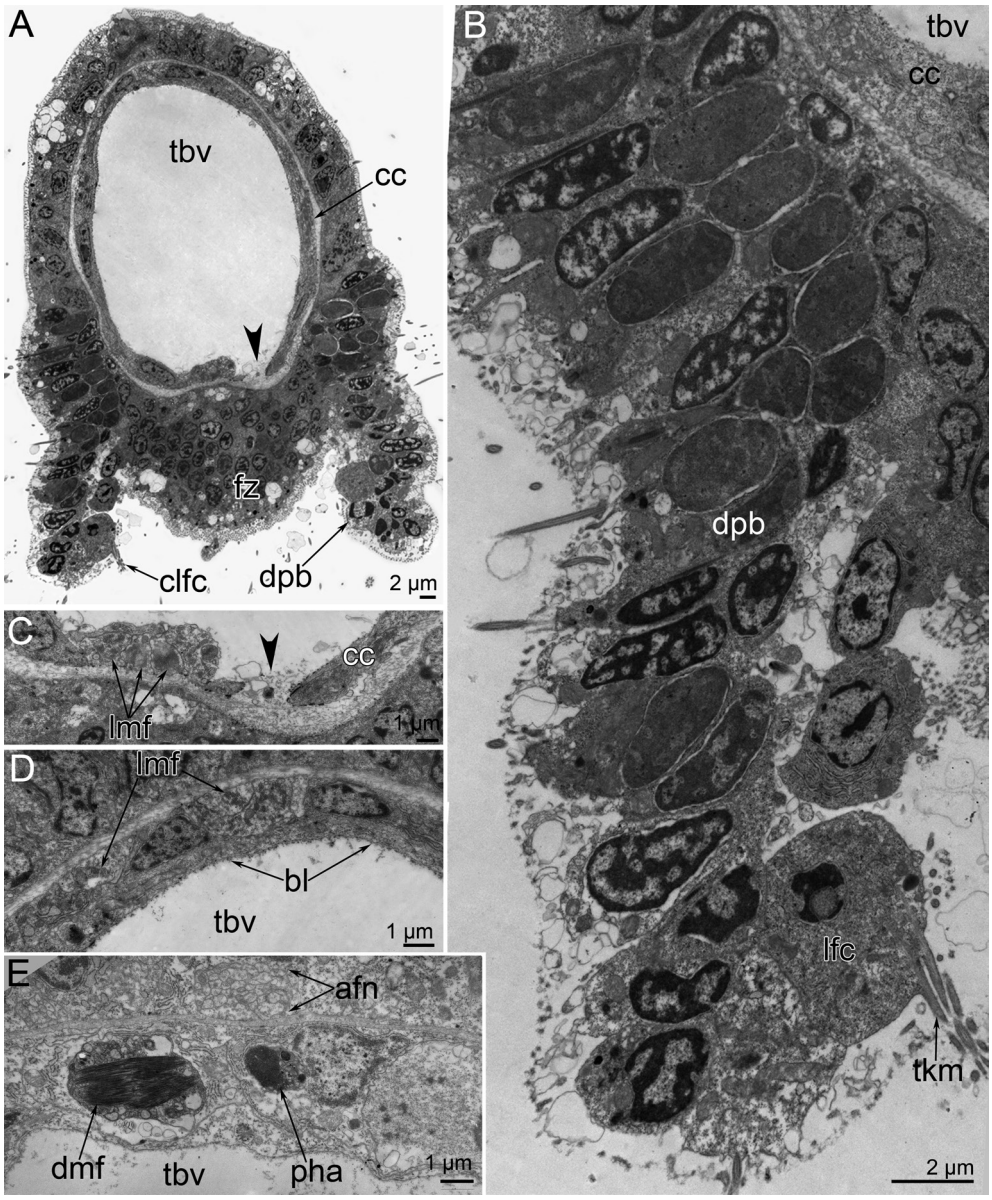

Fig. 11. Details of a tentacle ultrastructure in metamorphic *Phoronopsis harmer*; TEM. A — general view of transverse section of tentacle; B — a portion of degenerated postoral ciliated band; C — coelomic lining of the frontal zone; the place of location of degenerated muscle elevator is pointed by arrowhead; D — coelomic lining of abfrontal zone; D — coelomic lining of abfrontal zone with prominent evidence of phagocytosis. Abbreviations: afn — abfrontal neurite bundle; bl — basal lamina; cc — coelomic lining; clfc — cilium of laterofrontal cell; dpb — degenerated postoral ciliated band; dmf — degenerated myofilaments; fz — frontal zone; lmf — longitudinal myofilaments in cells of coelomic lining; pha — phagosome; tbv — tentacle blood vessel; tkm — thick microvilli.

larvae, cilia of both preoral and postoral ciliated band beat in opposite directions: to the top (preoral band) and to the down (postoral band) (Strathmann, 1975). In contrast, in trochophore larvae, cilia of both prototroch and metatroch beat towards each other (Pernet, Strathman, 2011). When the larvae growth in time, the ciliated zone around the mouth growths as well, and the both ciliated bands form numerous projections – future tentacles (Nielsen, 2012). These tentacles then

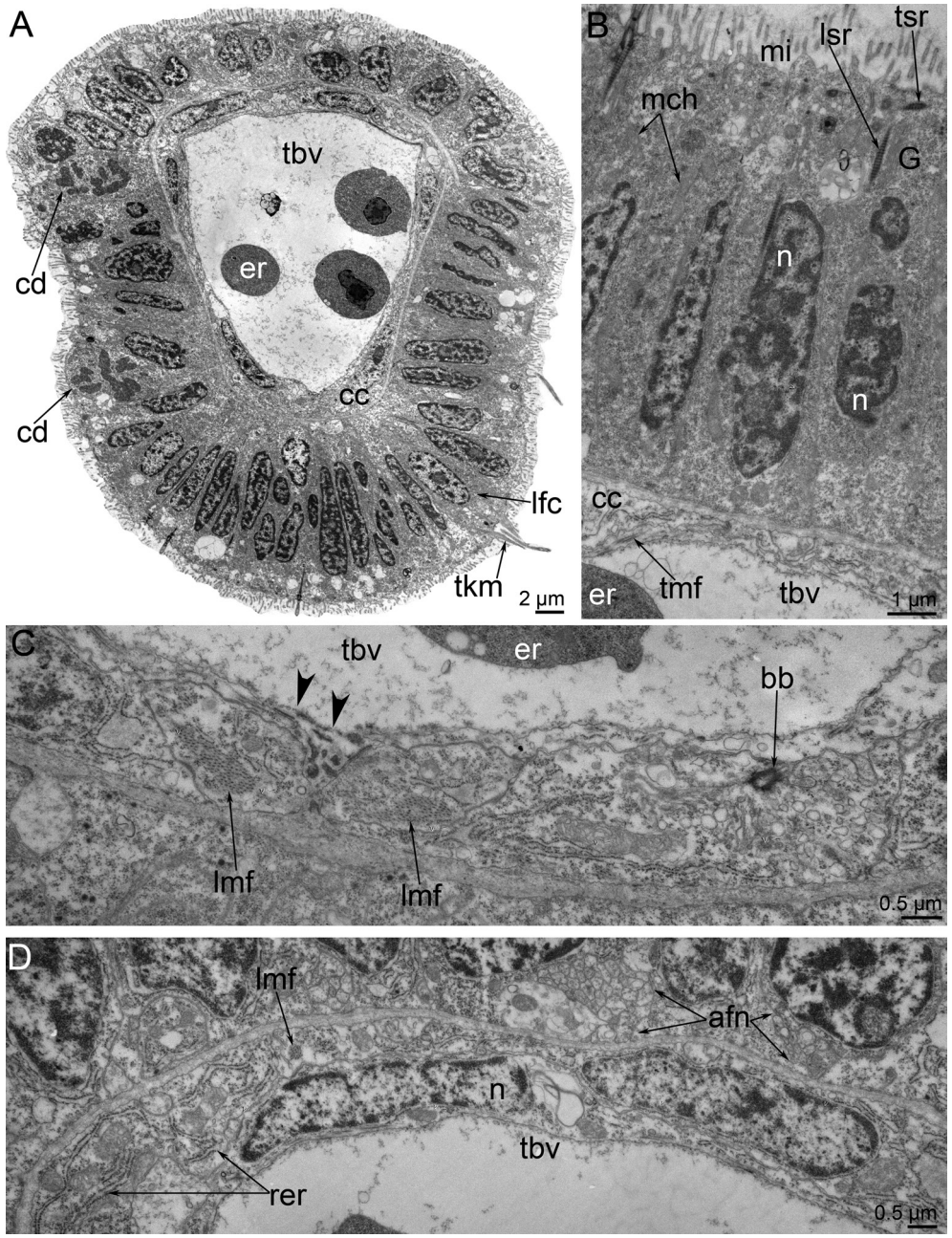

Fig. 12. Details of a tentacle ultrastructure in juvenile of *Phoronopsis harmer*; TEM. A — general view of transverse section of tentacle; B — cells of lateral zone; C — coelomic lining of the frontal zone; the place of location of degenerated muscle elevator is pointed by arrowheads; D — coelomic lining of abfrontal zone. Abbreviations: afn — abfrontal neurite bundle; bb — basal body; cc — coelomic lining; cd — cell division; er — erythrocyte; G — Golgi apparatus; lfc — laterofrontal cell; lmf — longitudinal myofilaments in cells of coelomic lining; lsr — longitudinal striated rootlet; mch — mitochondria; mi — microvilli; n — nucleus; rer — rough endoplasmic reticulum; tbv — tentacle blood vessel; tkm — thick microvilli; tmf — transverse myofilaments in cells of the blood vessel wall; tsr — transverse striated rootlet.

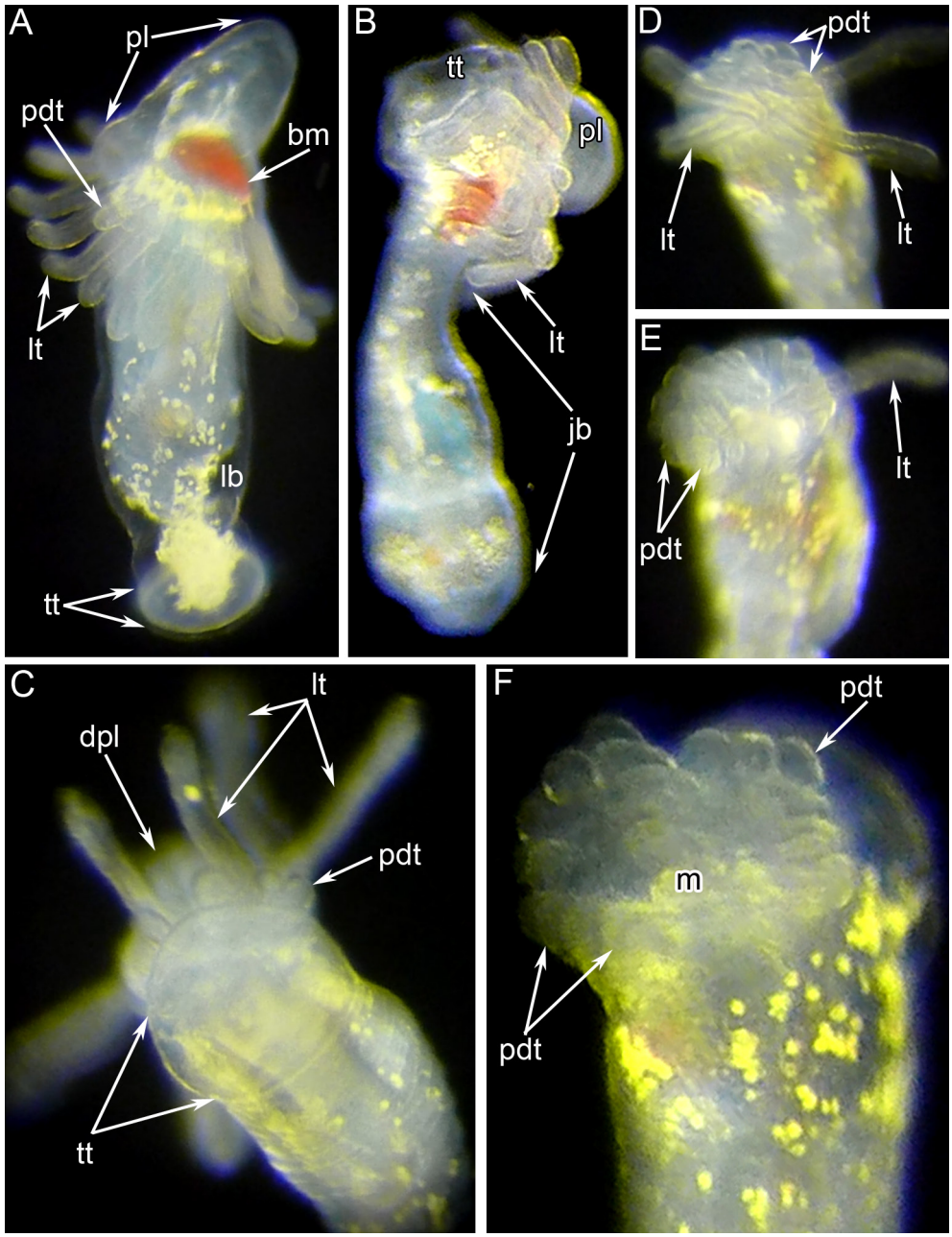

Fig. 13. Consecutive stage of metamorphosis of unknown phoronid species from the Nha Trang Bay (South-China Sea); photographs of live animals. A — competent larva viewed from the right; B — animal with everted metasomal sac viewed from the right; C — animal in active metamorphic process viewed from the posterior side (the telotroch side); D — animal in active metamorphic process viewed from the oral side: there are four long larval tentacles; E — animal in active metamorphic process viewed from the oral side: there is one long larval tentacles; E — juvenile after 7 min after metamorphosis starts.

Abbreviations: bm — blood mass; dpl — degenerated preoral lobe; jb — juvenile body; lb — larval body; lt — larval tentacle; m — mouth; pdt — primordia of definitive tentacles; pl — preoral lobe; tt — telotroch.

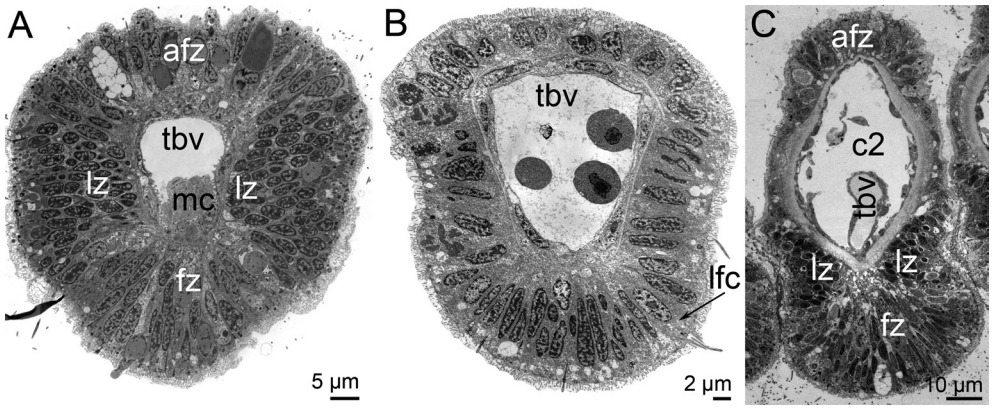

Fig. 14. Ultrathin transverse sections of tentacles of *Phoronopsis harmer* at different stage of life cycle; TEM. A — competent larva; B — juvenile; C — adult animal.
Abbreviations: afz — abfrontal zone; c2 — mesocoel (tentacle coelom); fz — frontal zone; lfc — laterofrontal cell; lz — lateral zone; mc — muscle cell; tbv — tentacle blood vessel.

give rise to the tentacles of adult animals and do not undergo great transformation, thereby the main principle of the ciliated bands operation remains and is inherited by the juvenile.

Phoronid larvae demonstrate the unique type of filter feeding mechanism, which does not occur in any other planktotrophic larvae with ciliated bands (Temereva, Malakhov, 2012). Phoronid larvae combine deuterostome-like and spiralian-like features in processing of the filter feeding apparatus: both preoral and postoral ciliated bands beat from the top to the down, thereby preoral ciliated band works as in trochophore, whereas the postoral ciliated band works as in dipleurula. Another one specificity of phoronid larval tentacle apparatus is the absence of the preoral tentacles, which exist in late metatrochophore and dipleurula. In phoronid larvae, preoral ciliated band corresponds to the preoral tentacles of the last common bilaterian ancestor.

Claus Nielsen and some other researchers have regarded the specificity of mechanism of filtration as phylogenetically important feature (Nielsen, 1987, 2012; Rouse, 1999, 2000). However, in the light of evolutionary perspectives, philogenetic significance of the direction of water current in tentacle apparatus of the last common bilaterian ancestor is highly unlikely (Malakhov *et al*., 2019). The mechanism of filtration might occur in different large stems independently; phoronids larvae, for example, exhibit unique mechanism of filtration. The most important thing is the presence (or absence?) of the ciliated tentacles in the last common bilaterian ancestor. This question is directly correlates with the fundamental problem of the origin of Bilateria and morphological appearance of the last common bilaterian ancestor (Malakhov *et al*., 2019; Malakhov, Gantsevich, 2022).

The mechanisms of development and renovation of the tentacle apparatus in lophophorates and in phoronids, in particular, is poorly studied. We only know about great possibility of some phoronid species to regenerate the lophophore very quickly: in 72 or even in 48 hours (Emig, 1972; Gomzhin, Temereva, 2022). Phoronids do not have specialized stem cells; during the regeneration, cell of both ectoderm and mesoderm exhibit proliferative activity and are involved in the lophophore renovation (Gomzhin, Temereva, 2022). In adult bryozoans, the maintenance of the lophophore tentacles is supplied by groups of cell, which are located at the base of the lophophore between tentacles (Shunatova, Borisenko, 2020). Accordingly to this study, in phoronid larvae and juveniles, the zone of tentacle growth and renovation is located at the base of tentacles as well. The similarities in lophophore reconstruction in both phoronids and bryozoans may evidence the monophyly of the lophophorates and the homology of the lophophore (Temereva, Kosevich, 2018; Temereva *et al*., 20023).

Metamorphic remodelling of larva tentacle apparatus

The fate of larval tentacles in phoronid metamorphosis has never been described in detail neither in first studies (Schneider, 1862;

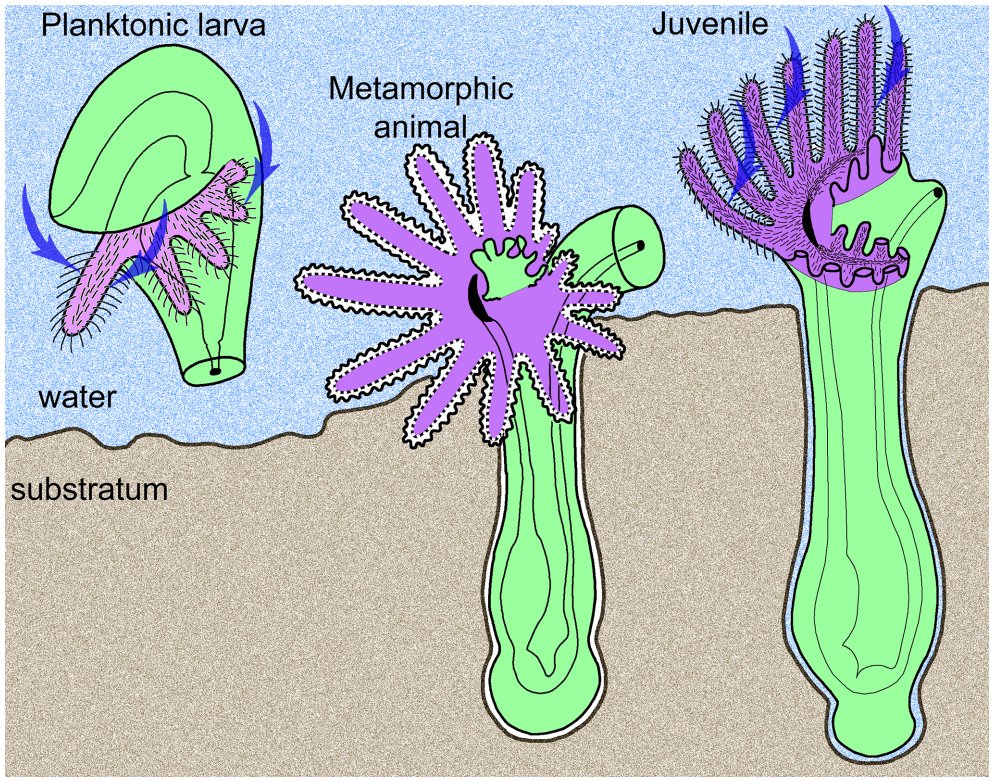

Fig. 15. Ontogenetic transformation of the tentacle apparatus in phoronids. Blue arrows show the currents of water in larva and juvenile. In metamorphic animal, tentacles do not work; thereby arrows are absent.

Kovalewsky, 1867; Metschnikoff, 1871; Wilson, 1881; Roule, 1896, 1900; Ikeda, 1901; Selys-Longchamps, 1907) nor in recent papers (Siewing, 1974; Herrmann, 1976, 1980, 1986; Zimmer, 1978, 1980; Bartolomaeus, 1989, 2001; Temereva, Tsitrin, 2013, 2014; Temereva, Malakhov, 2015; Temereva *et al*., 2016). In most studied phoronids, larvae have primordia of definitive tentacles, thus, the larval tentacles are consumed completely and the definitive tentacles develop *de novo*. The same scenario has been observed in this study in the larva of unknown phoronid species from Nha Trang Bay.

At the same time, in phoronids there is another way of larval tentacles transformation, in which larval tentacles undergo great changes, but are not completely consumed (Fig. 14). This pattern has been discovered and described in only *P. harmeri* (Temereva, Malakhov, 2015). Despite general maintenance of larval tentacles, their organization changes significantly: tentacle muscles, which are located in the haemocoel, reduce (Temereva, Tsitrin, 2013); tentacle epithelium and coelomic lining undergo remodelling (Temereva, Malakhov, 2015). Such great transformation leads to appearance of "less specialized tentacles" (Temereva *et al*., 2021) in juvenile: newly formed juvenile tentacles (Fig. 14B) mostly lack prominent zonality in comparison with larval (Fig. 14A) and definitive tentacles (Fig. 14C).

As the mechanism of operation of the tentacles in both larval and adult phoronids is fundamentally similar, there seem to be no grounds for replacement of the tentacles during metamorphosis, so this may be evidence that the larval tentacles are no more than a provisional organ and the adult tentacles appear *de novo* (Fig. 15) This interpretation does not allow a continuous sequence to be made from larval tentacles to the tentacles of the adult animal and casts doubt on the presence of tentacles in the last common bilaterian ancestor. It is, however, possible that transformation of larval tentacles is evidence

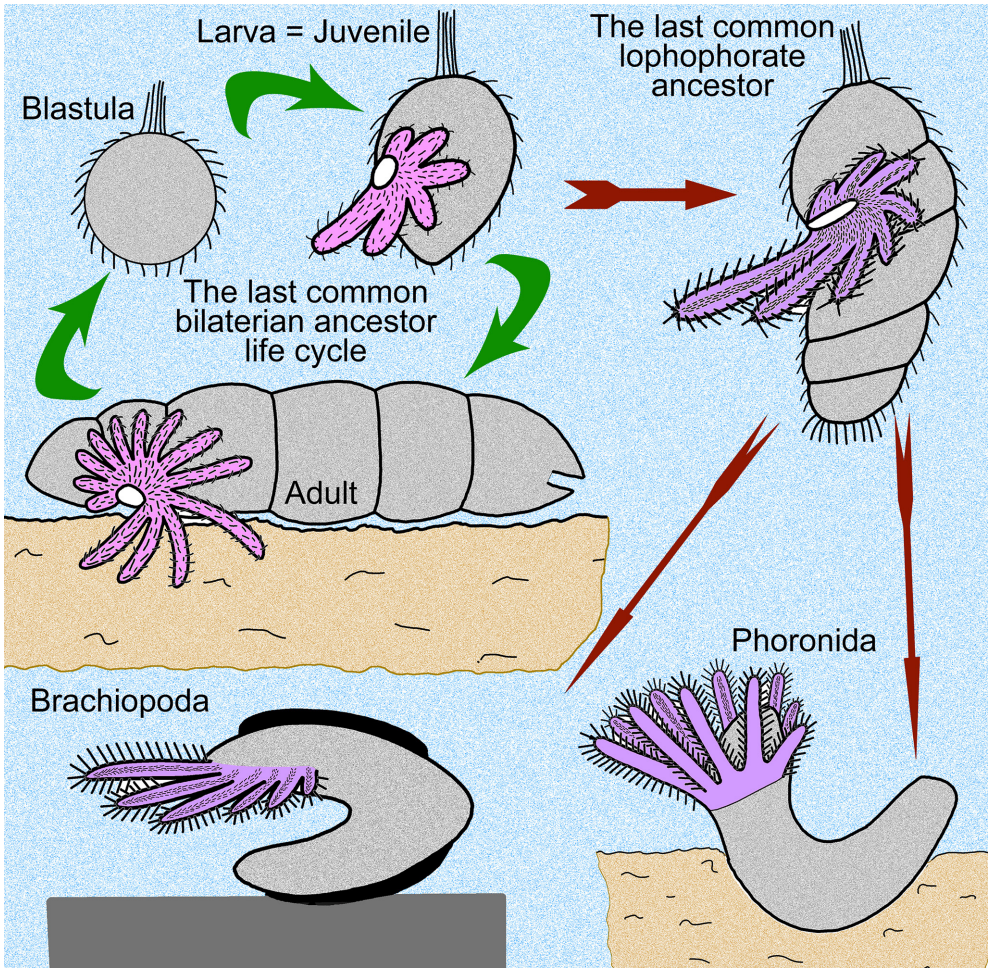

Fig. 16. Evolutionary transformation of the tentacle apparatus in phoronids and the origin of the lophophore in the pelagic last common lophophorate ancestor.

for specialization of the phoronid lophophore in comparison with non-specialised tentacle apparatus of the last common bilaterian ancestor, which had served to collect food particles from the surface of soft substratum (Fig. 16).

The origin of the lophophore

Phoronids as other lophophorates have the lophophore, which is extremely specialized tentacle apparatus. The specialization is reflected in presence of prominent zonality of tentacles: ciliated and non-ciliated zones are accompanied with nerve tracts and muscle bundles. Such high specialization is characteristic of only filter feeders, which catch food particles from the water column (Temereva *et al*., 2021). Thus, the lophophore, which is the homologous structure in all lophophorates (Temereva, Kuzmina, 2022), might be evolved in the water column only. It means that the lophophorates ancestor was either a sessile organism with tentacles exposing into the water or planktonic organism. This last common lophophorates ancestor firstly acquired the lophophore and then changed in the body plan: phoronids had "folded" on the dorsal side (Temereva, Malakhov, 2011, 2015) and brachiopods had folded on the ventral side (Nielsen, 1991; Cohen *et al*., 2003; Kuzmina *et al*., 2019; Plandin, Temereva, 2023). Such great transformation of the body is highly unliked in organism, which is already sessile, because its body plan is already formed and changed in

comparison with creeping last common bilaterian ancestor (Temereva, Malakhov, 2011; Malakhov *et al*., 2019; Malakhov, Gantsevich, 2022). In this consideration, it is more reasonably to suggest the appearance of the lophophore in planktonic juvenile, which had settled on the substrate and then had transformed in different ways (Fig. 16). This suggestion leads us to conclusion about appearance of all lophophorates due to the paedomorphosis, which is the magistral way of metazoan evolution (Iordansky, 2005; Isaeva, Rozhnov, 2021).

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