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Oogenesis in the viviparous phoronid, *Phoronis embryolabi*

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Abstract

The study of gametogenesis is useful for phylogenetic analysis and can also provide insight into the physiology and biology of species. This report describes oogenesis in the *Phoronis embryolabi*, a newly described species, which has an unusual type of development, that is, a viviparity of larvae. Phoronid oogonia are described here for the first time. Yolk formation is autoheterosynthetic. Heterosynthesis occurs in the peripheral cytoplasm via fusion of endocytosic vesicles. Simultaneously, the yolk is formed autosynthetically by rough endoplasmic reticulum in the central cytoplasm. Each developing oocyte is surrounded by the follicle of vasoperitoneal cells, whose cytoplasm is filled with glycogen particles and various inclusions. Cytoplasmic bridges connect developing oocytes and vasoperitoneal cells. These bridges and the presence of the numerous glycogen particles in the vasoperitoneal cells suggest that nutrients are transported from the follicle to oocytes. *Phoronis embryolabi* is just the second phoronid species in which the ultrastructure of oogenesis has been studied, and I discuss the data obtained comparing them with those in *Phoronopsis harmeri*. Finally, I discuss the distribution of reproductive patterns across both, molecular and morphological phylogenetic trees in Phoronida proving that parental care has evolved independently several times in this phylum.

KEYWORDS

autoheterosynthesis, germ plasm, oogenesis, Phoronida

1 | INTRODUCTION

The study of gametogenesis and gonadal structure can provide insight into the physiology and reproductive biology of a species and can also generate data that are useful for phylogenetic analysis (Drozdov, Vinnikova, Zezina, & Tyurin, 1992). Studies of gametogenesis in invertebrates generally consider three main characteristics. The first is the location and mechanism of origin of the primordial germ cells (Extavour & Akam, 2003). In many invertebrates, including phoronids, the location and development of primordial germ cells have been traditionally described using light microscopy rather than transmission electron microscopy (TEM) or immunocytochemistry and histocytochemistry. The second characteristic concerns the mode of oogenesis: solitary, nutrimentary, or follicular (Aisenstadt, 1984; Anderson, 1974; Raven, 1961; Wourms, 1987). The third characteristic describes whether yolk is formed via autosynthesis, heterosynthesis, or both mechanisms (Aisenstadt, 1984; Eckelbarger, 2005). The mode of oogenesis governs the developmental and larval types, which can differ among clades within a phylum (James, 1997; Ostrovsky, 2013a, 2013b).

The Phoronida is a small phylum of filter-feeding aquatic invertebrates with biphasic life cycles (Emig, 1982; Temereva & Malakhov, 2015). Currently it includes 15 species of adult representatives (Emig, 1979; Temereva & Chichvarkhin, 2017; Temereva & Neklyudov, 2017). All phoronids have benthic adults and planktotrophic larva, except *Phoronis ovalis* which has creeping lecithotrophic larvae. In six species the entire development occurs in the water column whereas most other phoronids brood their embryos between the tentacles of the adults. Two species (including *P. ovalis*) incubate embryos inside mother's tubes (reviewed in Emig, 1977; Silén, 1954; Temereva & Neklyudov, 2017; Zimmer, 1991). Finally, viviparity was recently reported in the newly described phoronid species *P. embryolabi* which incubates embryos in the trunk coelom to produce feeding actinotroch larvae (Temereva & Chichvarkhin, 2017; Temereva & Malakhov, 2016). These reproductive patterns are connected with the modes of oogenesis. Small (less 100 μm in diameter) and numerous eggs are produced by broadcasting phoronids, whereas eggs are larger (about 100 μm) and less numerous in brooders. *Phoronis ovalis* produces relatively few, large (125 μm) eggs (summarized in Emig, 1977; Zimmer, 1991). Eggs in

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P. embryolabi are small (60 μm) and numerous like in broadcasters, but embryos are incubated in the parental coelom.

Oogenesis has been understudied in Phoronida, and few existing descriptions deal with fragmentary data on various stages of oocytic development. All of them were made using histological method (summarized in Temereva, Malakhov, & Yushin, 2011). The first and only detailed study of oogenesis that includes ultrastructural data is that of *Phoronopsis harmeri* (Temereva et al., 2011). This article further describes oogenesis in the viviparous phoronid *P. embryolabi* aiming its comparative analysis in respect to what is known in other phoronids.

2 | MATERIAL AND METHODS

2.1 | Habitat and sampling

Adult *Phoronis embryolabi*, Temereva and Chichvarkhin (2017) were collected in July 2015 in the inlet Tihaya Zavod, Vostok Bay, Sea of Japan. The phoronid habitat is characterized by fluctuation of the salinity from 11 to 32.5‰ depending on the flows of two rivers and the tide level. Adult phoronids live as commensals in the burrows of the shrimp *Nihonotrypaea japonica*. Adult phoronids with sediment were removed from the burrows of the shrimp *Nihonotrypaea japonica* with a vacuum pump, which was driven into the burrow holes. Tubes with animals were separated from sediment by washing through a sieve with 2-mm openings.

2.2 | Transmission electron microscopy

For TEM, tubes with adult phoronids inside were fixed without dissection, at 4°C in 2.5% glutaraldehyde in 0.05 mol L^{-1} cacodylate buffer. Then the animals were peeled from the tubes and dissected. Parts of the bodies were then washed several times in 0.05 mol L^{-1} cacodylate buffer and postfixed in 1% osmium tetroxide in the same buffer. The specimens were dehydrated in ethanol followed by an acetone series and were then embedded in EMBed-812. Thick (1,000 nm) and thin (60 nm) sections were cut with a Leica UC6 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Thick sections were stained with methylene blue, observed with a Zeiss Axioplan 2 microscope, and photographed with an AxioCam HRm camera. Thin sections were stained with uranyl acetate and lead citrate and observed with the JEOL JEM 100B electron microscope (JEOL Ltd., Tokyo, Japan).

3 | RESULTS

3.1 | Vasoperitoneal cells

As in all other phoronids, oocytes in *Phoronis embryolabi* develop within the vasoperitoneal tissue (VPT) together occupying the entire lumen of the posterior portion of the body (Figure 1a–c). The oocytes and vasoperitoneal cells are associated with blood vessels (Figures 1d, 2a,b, and 3a). The vasoperitoneal cells are generally considered as originating from peritoneal cells, which form the wall of blood capillaries (Figure 2a,b). Initially, vasoperitoneal cells retain the typical ultrastructure of

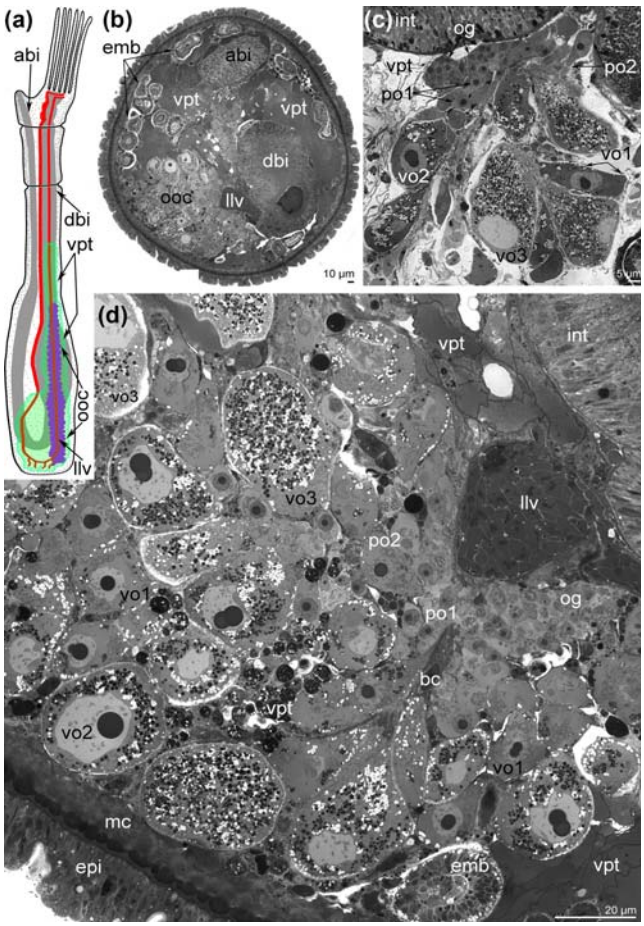

FIGURE 1 *Phoronis embryolabi*, oogenesis. (a) Scheme of general view of the adult animal. The vasoperitonael tissue (green) with developing oocytes (violet) is mostly located in the posterior part of the body. Blood system is simplified and shown in red. (b) Cross semithin section of the posterior part of the body. (c) Cluster of oogonia and oocytes at various developmental stages; semithin section. (d) Accumulation of oogonia and developing oocytes around the left lateral blood vessel; semithin section. Abbreviations: abi, ascending branch of intestine; bc, blood capillary; dbi, descending branch of intestine; emb, embryo; epi, epithelium; int, intestine; mc, muscle cells; llv, left lateral blood vessel; og, oogonia; ooc, oocytes; pbp, posterior body part; po1, early previtellogenic oocyte; po2, late previtellogenic oocyte; vo1, early vitellogenic oocyte; vo2, mid-staged vitellogenic oocyte; vo3, late vitellogenic oocyte; vpt, vasoperitoneal cells

peritoneal cells: having a rudimentary cilium with a basal apparatus (Figure 2c), and myofilaments in the basal part. The basal side shows several elongated finger-like protrusions toward the basal-lamina lining the blood vessel (Figures 3c and 4a). During reproduction, the vasoperitoneal cells and their processes are convoluted around blood capillaries (Figure 2a,b). The size of vasoperitoneal cells increases and their shape changes: they form thin, long processes, which envelop oogonia and oocytes and contact the neighbouring blood vessels (Figures 2a and 5a). The processes of adjacent vasoperitoneal cells are connected by belt-like desmosomes that are also seen between processes and developing oocytes (Figures 4 and 5c,d). The cytoplasm of the vasoperitoneal cells was electron-translucent in one studied specimen (Figure

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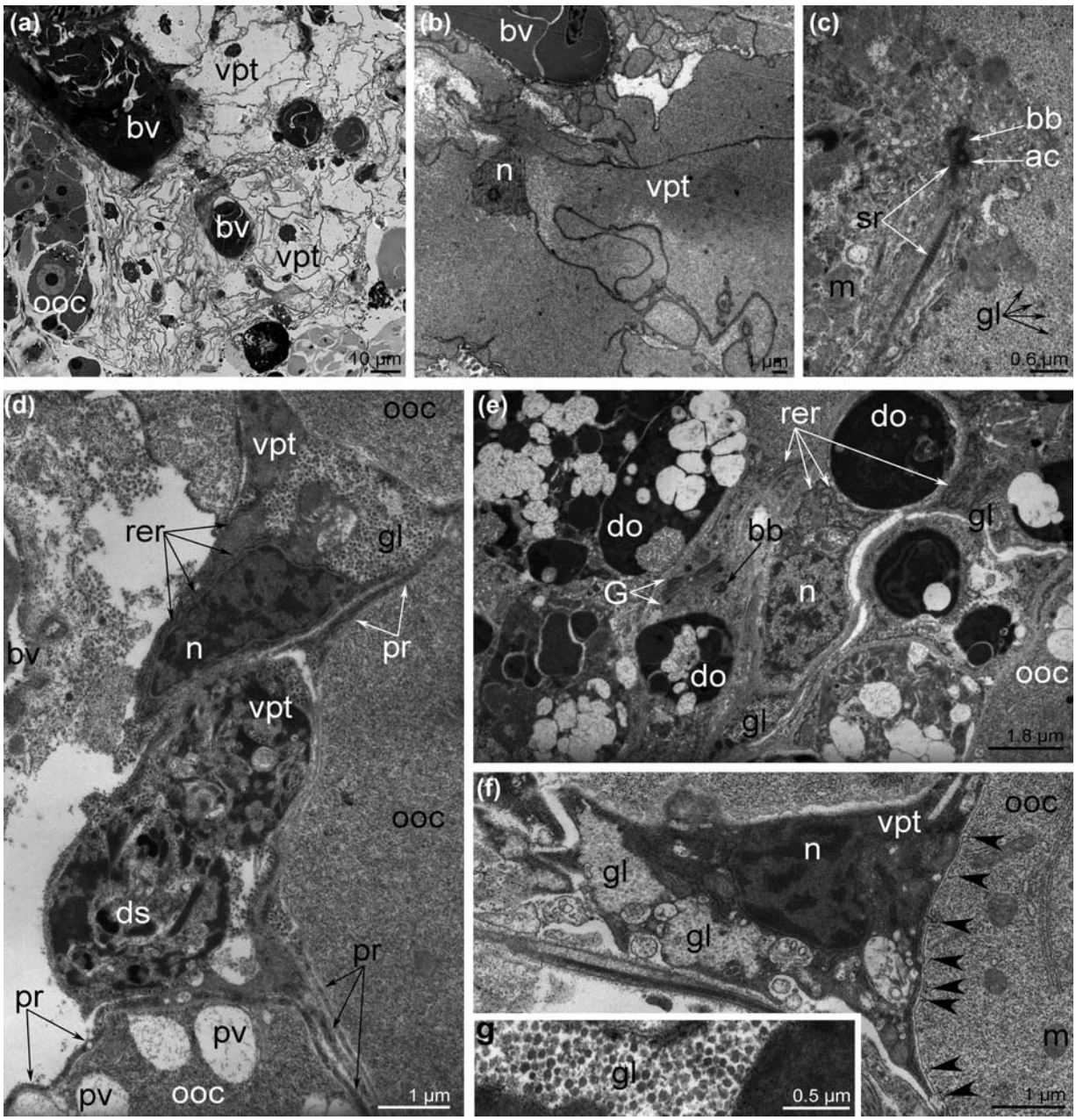

FIGURE 2 *Phoronis embryolabi*, vasoperitoneal tissue; semithin (a) and ultrathin (b-g) sections. (a) Vasoperitoneal cells in semithin section: the rosettes of cells around blood vessels are visible. (b) Electron dense cytoplasm of vasoperitoneal cells in thin section of low magnification. (c) A portion of cytoplasm with basal apparatus and striated rootlets. (d) Cell bodies and thin projections of vasoperitoneal cells. Phagosomes containing the sperm debris are visible. (e) Aggregation of vasoperitoneal cells: inclusions with debris of oocytes, rough endoplasmic reticulum, and Golgi apparatus are evident. (f) Body of vasoperitoneal cells in contact with the oocyte: invaginations of oolemma are indicated by arrowheads. (g) Glicogen is represented by monoparticulate beta-glycogen particles. Abbreviations: ac, accessory centriole; bb, basal body; bv, blood vessel; do, debris of oocytes inside phagosome; ds, debris of spermatozoa inside phagosome; G, Golgi apparatus; gl, glycogen; n, nucleus; m, mitochondria; ooc, oocyte; pr, projections of vasoperitoneal cells; pv, pale vesicles; rer, rough endoplasmic reticulum; sr, striated rootlet; vpt, vasoperitoneal cells

2a) but electron-dense in others (Figure 2b). In both cases, the cytoplasm of vasoperitoneal cells was filled with numerous monoparticulate beta-glycogen particles 20–30 nm in diameter (Figure 2d–g). The nucleus of the vasoperitoneal cell is irregular in shape, electron-dense, and contains a nucleolus (Figures 2e,f and 5a). The vasoperitoneal cells also contain a well-developed synthetic apparatus, which is mostly represented by rough endoplasmic reticulum (RER; Figure 2d–f). In some cells, the activity of the Golgi apparatus can be detected: the terminal portions of dictyosomes are swollen and filled with dense material (Figure 2e). During the reproductive period, numerous inclusions appear in the cytoplasm of vasoperitoneal cells. Most of these inclusions appear to be phagosomes that contain the desintegrated sperm (Figure 2d) and oocytes (Figure 2e). Multilamellar bodies are present in the cytoplasm (Figure 4d).

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3.2 | Oogenesis

3.2.1 | Oogonia

Oogonia and developing oocytes at different stages are associated with the left lateral blood vessel, from which blind ending capillaries extend. Oogonia form a layer directly adjacent to the wall of the blood vessel (Figure 3a,b). Oocytes and developing vasoperitoneal cells are seen between oogonia too (Figure 3c). Oogonia are roundish cells 7 ± 1 μm in diameter, each containing a large, round nucleus (3.7 ± 0.1 μm). The nucleus lacks a nucleolus, and the chromatin is arranged into several stripes, such that the nucleus appears to be striped (Figure 3a, b). Primary oogonia divide forming doublets temporarily connected via a wide cytoplasmic bridge (Figures 4a and 5b). The inner side of the plasmalemma of this bridge contains electron-dense amorphous material (Figure 5b). The electron-dense cytoplasm of oogonia contains a few cisternae of the RER (Figure 3c), a centriole (Figure 5e), and germ plasm. The germ plasm is represented by a cluster of several electron-dense granules (Figures 3c and 5d) or by roundish aggregations of electron-dense material that are scattered in the cytoplasm and that are not accompanied by mitochondria (Figure 5b). The plasmalemma of some oogonia is dense and thick, suggesting the presence of cell junctions between them and adjacent vasoperitoneal cells.

3.2.2 | Oocytes

Previtellogenic oocyte I

The earliest previtellogenic oocytes are elongated cells (10 ± 0.5 μm in length); each has a large roundish nucleus (5 ± 0.3 μm in diameter) with a large nucleolus (2 ± 0.1 μm; Figure 6a,b). The nucleolus is flocculent on its periphery and dense in the centre (Figure 4b). The cytoplasm contains rare cisternae of the RER (which abuts the nucleus), mitochondria, and germ plasm, which is represented by aggregations of electron-dense granules of various sizes. In some previtellogenic oocytes, the Golgi apparatus is evident (Figure 6b). The oocyte is attached to the basal lamine of the blood vessel.

Previtellogenic oocyte II

The late previtellogenic oocyte (about 15 ± 2 μm in length) has a nucleus with predominantly central position (7 ± 0.5 μm in diameter). The nucleolus (4 ± 1 μm in diameter) has two visible parts: one is dense, and the other is flocculent (Figures 4c, 7a, and 8a). In some oocytes, a strongly developed synthetic apparatus is readily observed by light microscopy (Figure 7a) and are represented by numerous cisternae of RER (Figure 7b). The mitochondria are numerous and are usually arranged as a circle (Figures 4c and 8a). The germ plasm is represented by numerous electron-dense granules (Figure 7c). In some oocytes, the centriole is visible in the ooplasm (Figures 7d and 8a). Vesicles with pale, flocculent content usually appear in the cortical ooplasm and in the basal portion of the oocyte, which abut against the blood vessel (Figures 4c and 8a). These vesicles are 0.8–1 μm in diameter, and could be a result of the merging of small vacuoles in the cytoplasm (Figure 8c). The basal part of the oocyte forms folds, which are underlain by the extracellular matrix (basal lamina) of the blood vessel. In the cytoplasm of the basal part, a few vesicles, which are probably endocytotic, are found (Figure 8a,b).

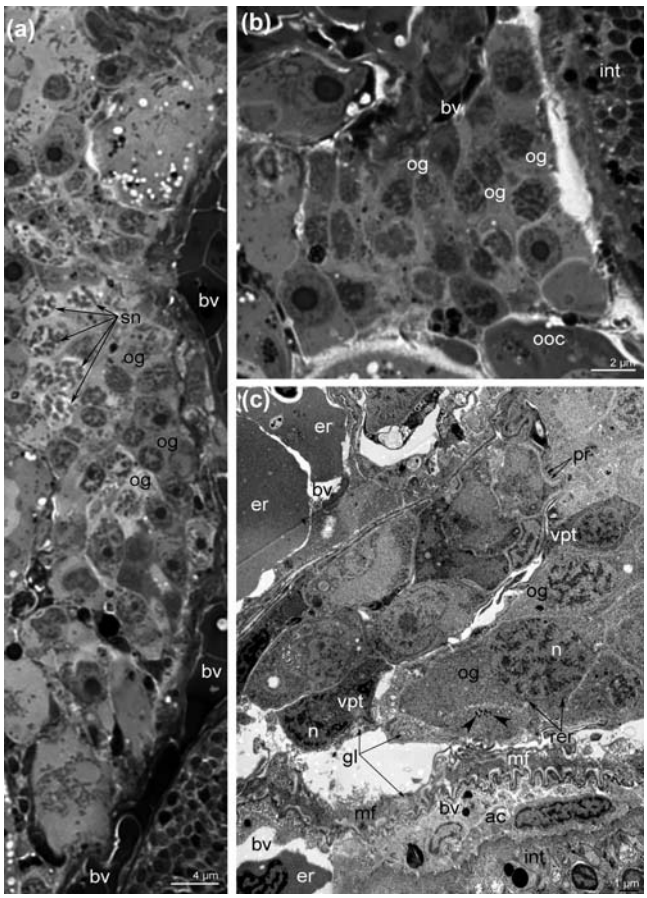

FIGURE 3 *Phoronis embryolabi*, oogonia. (a) Accumulation of oogonia (og) abuts the blood vessel; semithin section. (b) A cluster of oogonia; semithin section. In oogonia, the nucleus appears to be striped. (c) Fine section of oogonia and vasoperitoneal cells (vpt). Germ plasm is represented by a cluster of several electron-dense granules and is indicated by arrowheads. Abbreviations: ac, amoebocyte; bv, blood vessel; er, erythrocyte; gl, glycogen; int, intestine; mf, myofilaments; n, nucleus; og, oogonia; ooc, oocyte; pr, projections of vasoperitoneal cells; sn, "striped" nucleus of oogonia; vpt, vasoperitoneal cells

Vitellogenic oocyte I (= early vitellogenic oocyte)

Early vitellogenic oocytes have a maximal length of 22 ± 2 μm and have a nucleus and nucleolus with diameters of 12 ± 1 and about 5 ± 0.5 μm, respectively. Oocytes have a prominent apical-basal polarity; the nucleus is shifted from the central to the apical part of the cell (Figure 4d). In some oocytes, the nucleolus is bilobular (Figure 9a). At this stage, vitellogenesis begins (Figures 4d and 10). In early vitellogenic oocytes, the first aggregations of yolk granules are located in the peripheral ooplasm and are readily observed in semithin sections (Figures 4d, 9a, and 11a). Two types of yolk are detected in the mid-stage oocytes. Type I yolk is formed heterosynthetically, and Type II yolk is formed autosynthetically.

Endocytosis is involved in the Type I yolk formation. In some instances, the pits pinch off forming coated vesicles ranging from 130 to 160 nm in diameter (Figure 10a). In these vesicles, the content undergoes condensation, and the central dense core appears (Figure 10b). The vesicles move into the cytoplasm and appear to fuse with each other or

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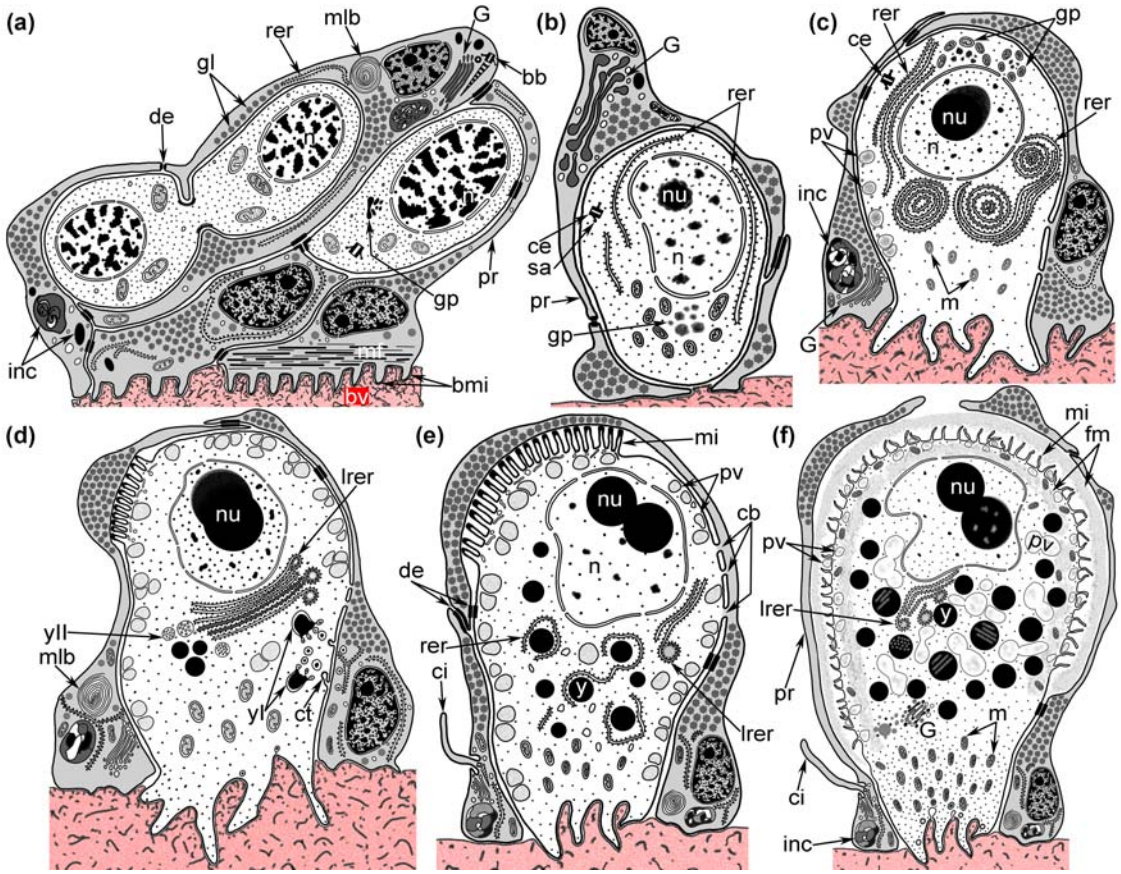

FIGURE 4 *Phoronis embryolabi*, schematic drawings of consecutive stages of oogenesis. Vasoperitoneal cells are shown in grey; blood vessel is shown in pink. (a) Oogonia. (b) Early previtellogenic oocyte. (c) Late previtellogenic oocyte. (d) Early vitellogenic oocyte. (e) Mid-staged vitellogenic oocyte. (f) Late vitellogenic oocyte. Abbreviations: bb, basal body; bmi, basal finger-like protrusions; bv, blood vessel; cb, cytoplasmic bridge; ce, centriole, ci, cilium; ct, coated tip; de, desmosome; fm, flocculent material; G, Golgi apparatus; gp, germ plasm; inc, inclusion; lrer, loop of rough endoplasmic reticulum; m, mitochondrion; mf, myofilaments; mi, microvilli; mlb, multilamellar body; n, nucleus; nu, nucleolus; pr, process of vasoperitoneal cell; pv, vesicles with pale, flocculent material (=pale vesicles); rer, rough endoplasmic reticulum; sa, satellite; y, yolk granule; yI, type I of yolk formation (heterosynthesis); yII, type II of yolk formation (autosynthesis)

with previously formed Type I yolk droplets (Figure 10a,b). The sites of endocytosis are along the adjacent projections of the vasoperitoneal cells, whose dense cytoplasm contains spherical inclusions (Figure 10b).

The Type II yolk forms simultaneously or begins its formation slightly later than the Type I yolk. Two morphologically different mechanisms were detected in formation of the Type II yolk. In the first, multivesicular-like bodies are formed (about 0.7 ± 0.1 μm in diameter) containing many vesicles (about 70 ± 5 nm), which appear disk-shaped in certain sections (Figure 10c). Some multivesicular-like bodies have an incomplete membrane. In these bodies, condensation of their content occurs (Figure 10a), and their coalescence results in a formation of the yolk granule. The aggregation of such vesicular bodies and terminal yolk granules are usually located near the RER (Figure 10a).

The second mechanism of the Type II yolk formation is connected with the activity of the RER. Some cisternae of the RER form terminal loops (Figure 9c), which further on detach and lie in the ooplasm as double-membraned vesicles. Initially, they are 350 ± 50 nm in diameter and have electron-translucent content. The peripheral content becomes denser and then occupies the entire volume of the vesicle. The transformation of the dense content into yolk granule was not detected in the current study, but the yolk granules, which are encircled by RER, are abundant in middle oocytes (Figures 4e and 9e) and rare in late oocytes (Figure 11c). The diameter of these yolk granules ranges from 0.5 to 1 μm (Figure 9e).

In some oocytes, short (0.2–0.3 μm long) branchless microvilli with electron-dense tips were recorded being in contact with the vasoperitoneal cells (Figures 4d and 9d). The canals and vesicles including pale vesicles with flocculent content are evident under the oolemma (Figure 9d).

At this stage of oogenesis, the connection between oocytes and vasoperitoneal cells becomes evident (Figure 9f–h). The desmosomes between vasoperitoneal cells and oocytes as well as between the processes of adjacent vasoperitoneal cells are recorded (Figure 9f,g). In addition to desmosomes, short and narrow cytoplasmic bridges connect oocytes and vasoperitoneal cells (Figure 9g,h). The diameter of these connections ranges from 150 to 300 nm (Figure 9g). In many cases, these cytoplasmic bridges occur in places where glycogen deposits are located in the vasoperitoneal cell (Figure 9h). The presence of electron-dense material in the cytoplasmic bridges suggests that the bridges enable the exchange of nutritive material between cells (Figure 9h).

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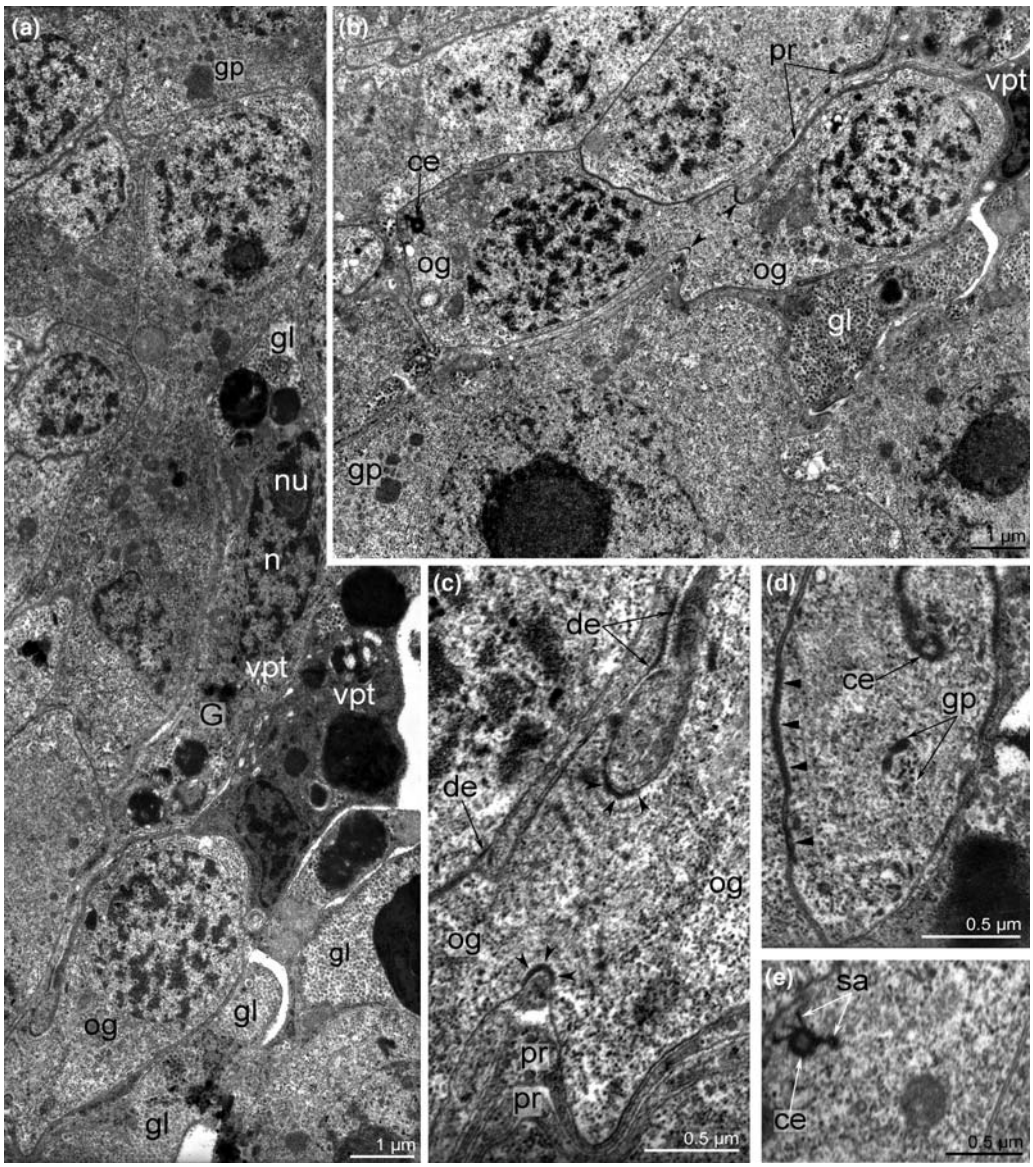

FIGURE 5 *Phoronis embryolabi*, ultrastructure of oogonia. (a) Group of oogonia associated with vasoperitoneal cells. (b) Two oogonia with cytoplasmic bridge in between (the electron dense material along edges of this bridge is shown by arrowheads). (c) Details of cytoplasmic bridge between two oogonia: electron dense material is shown by arrowheads; thin projections of vasoperitoneal cells bear desmosomes. (d) Some details of oogonia organization: electron dense oolemma abuts on vesaperitoneal cell (shown by arrowheads); germ plasm (gp) is represented by group of electron dense granules. (e) Centriole in cytoplasm of oogonia. Abbreviations: ce, centriole; de, desmosome; G, Golgi apparatus; gp, germ plasm; gl, glycogen; n, nucleus; nu, nucleolus; og, oogonia; pr, projection of vasoperitoneal cell; sa, satellite; vpt, vasoperitoneal cells

Vitellogenic oocyte II (= mid-staged vitellogenic oocyte)

Mid-staged vitellogenic oocytes are 30 ± 5 μm in length having a nucleus and a nucleolus with diameter of 15 ± 1 and about 6 ± 0.5 μm, respectively (Figure 9a). The cytoplasm contains many large pale vesicles with flocculent content, yolk granules, including developing ones surrounded by RER, multivesicular bodies, mitochondria; and numerous canals of RER (Figures 4e and 9e). Annulate lamellae and typical Golgi complexes consisting of dictyosomes were not detected at this stage.

Vitellogenic oocyte III (= late vitellogenic oocyte)

Late vitellogenic oocytes are macrolecithal. They are 60 ± 5 μm in length and having a nucleus and nucleolus with diameter of 25 ± 2 μm and about 8 ± 1 μm, respectively (Figure 11a). The nucleoli lose their bipartite character and and become vesiculated (Figure 11a). Some oocytes contain two nucleoli of the similar size. The fine structure of the nucleoli is heterogeneous (Figures 11a and 12a). The nucleus abuts the oolemma and acquires an irregular shape with numerous lobes (Figures 4f and 11b). F12

The cytoplasm of the late oocytes contains many large vesicles with pale, flocculent content. These vesicles fuse with each other, forming spacious lucent areas in the cytoplasm of the oocyte (Figure 12c). Some of them border the oolemma (Figure 11c) and obviously ejected from the cytoplasm, being visible in the perioocytal space (Figure 11f). Moreover, the flocculent material fills the perioocytal space

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and peripheral ooplasm (Figure 11f). The oolemma forms slightly branched microvilli that are ≤ 0.5 μm long (Figure 11c). Numerous yolk granules at different stages of formation are scattered in the cytoplasm (Figure 11d). Cisternae of the RER are abundant; some of them form circles around the proliferated yolk granules. Mitochondria are concentrated along the periphery under the oolemma and in the basal part of the oocyte. The Golgi complex, which is represented by several short dictyosomes with dense content, is evident in the cytoplasm of the late oocyte (Figure 12b). Some yolk granules show paracristalline structure (Figures 4f and 12c).

The late vitellogenic oocyte is still attached to the basal lamina of the blood vessel. Its basal part forms a "pedicle": almost without yolk granules, vesicles, or other inclusions. Instead the dense cytoplasm of the "pedicle" contains many mitochondria (Figure 11b) and a few small vesicles (Figure 11g). Vesicles of 0.2 μm are detected being filled with dense material, whose structure seems to be similar to that of the extracellular matrix of the blood vessel (Figure 11g). Vesicles of 0.1 μm are detected here too seemingly being coated endocytotic vesicles. The basal surface of the oocyte forms a few processes and lobes, which are exposed to the blood vessel and being covered by a dense extracellular matrix (Figure 11e,g).

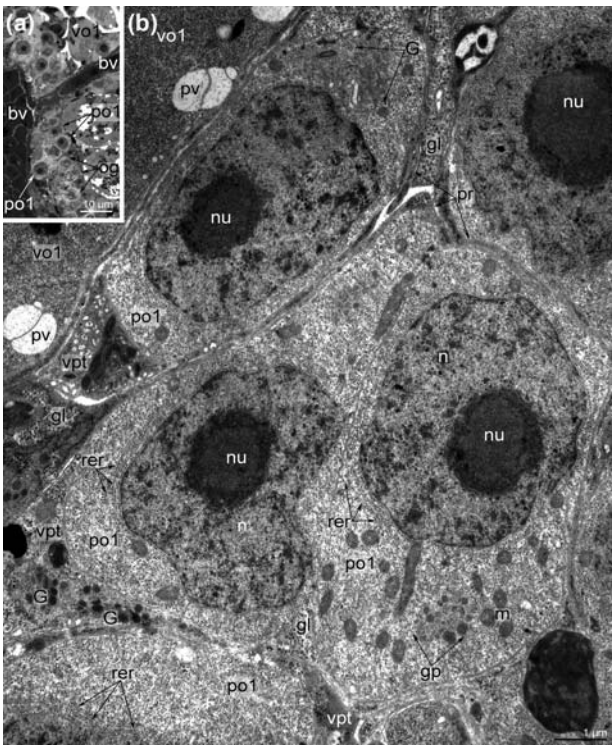

FIGURE 6 *Phoronis embryolabi*, early previtellogenic oocytes. (a) Semithin section of oogonia and previtellogenic oocytes. (b) Group of three previtellogenic oocytes. Abbreviations: bv, blood vessel; G, Golgi apparatus; gl, glycogen; gp, germ plasm; m, mitochondria; po2, late previtellogenic oocyte; pr, process of vasoperitoneal cell; pv, vesicles with pale, flocculent material (=pale vesicles); rer, rough endoplasmic reticulum; vo3, late vitellogenic oocyte; vpt, vasoperitoneal cells

4 | DISCUSSION

4.1 | Vasoperitoneal tissue

In all phoronids, VPT occupies the trunk coelom around the blood vessels, and includes the developing sexual cells. It is assumed that VPT originates from cells of the coelomic lining of blood vessels (Benham, 1889; Cori, 1939; Emig, 1982, 1983; Forneris, 1959; Ikeda, 1903; Marcus, 1949; Rattenbury, 1953; Selys-Longchamps, 1907; Silén, 1952). The degree of VPT development varies annually and depends on the reproductive period (Rattenbury, 1953; Silén, 1952). Early light microscopy studies revealed the presence of various inclusions with different structure in vasoperitoneal cells (Pixell, 1912; Selys-Longchamps, 1907). Since then, some additional data were obtained (e.g., Rattenbury, 1953; Silén, 1952), but the ultrastructure of vasoperitoneal cells has been described in only one phoronid species, *Phoronopsis harmeri* (Temereva et al., 2011). Its vasoperitoneal cells remain ciliated as typical cells of the coelomic lining, and contain various large inclusions, mostly phagosomes. Cilia and large phagosomes have also been found in the vasoperitoneal cells of *P. embryolabi* that are also characterized by abundant glycogen granules and strongly developed synthetic apparatus. Both these characters suggest suggests that the cells may have a nutritive function.

The size and number of the inclusions in the vasoperitoneal cells increase at the time of spawning. In *P. embryolabi*, phagosomes contain parts of degrading oocytes and spermatozoids, that suggest phagocytosis utilizing degenerating and/or excessive sexual cells. Because portions of the VPT are removed from the body of phoronids during spawning (Temereva & Malakhov, 2012), the organism eventually ejects excretes that are kept in the vasoperitoneal cells. It follows then that vasoperitoneal cells in phoronids function in a manner similar to that of the chloragogenous tissue in some annelids or the fat body of insects.

4.2 | Oogenesis

The earliest stages of oogenesis, that is, primordial germ cells and oogonia, have not been described in phoronids. Based on light microscopy, researchers have previously inferred that primordial germ cells develop in the peritoneal epithelium during late embryogenesis and due to epigenesis (Extavour & Akam, 2003). According to data presented herein, the wall of the left lateral vessel during the reproductive period consists of the sexual cells (oogonia and oocytes), specialized vasoperitoneal cells, and peritoneal cells of usual structure. I suggest that precursors of oogonia, that is, primordial female germ cells, occur among them too, but they are not distinguishable from the latter using light microscopy or TEM. A similar problem has been mentioned in respect to Bryozoa (Ostrovsky, 2013a, 2013b). Such a relationship between somatic cells and germ cells raises questions regarding the mechanisms of epigenesis, which is suggested to be the basal mode of germ cell specification for the Metazoa (Extavour & Akam, 2003), including phoronids and many other bilaterians. The origin of germ cells

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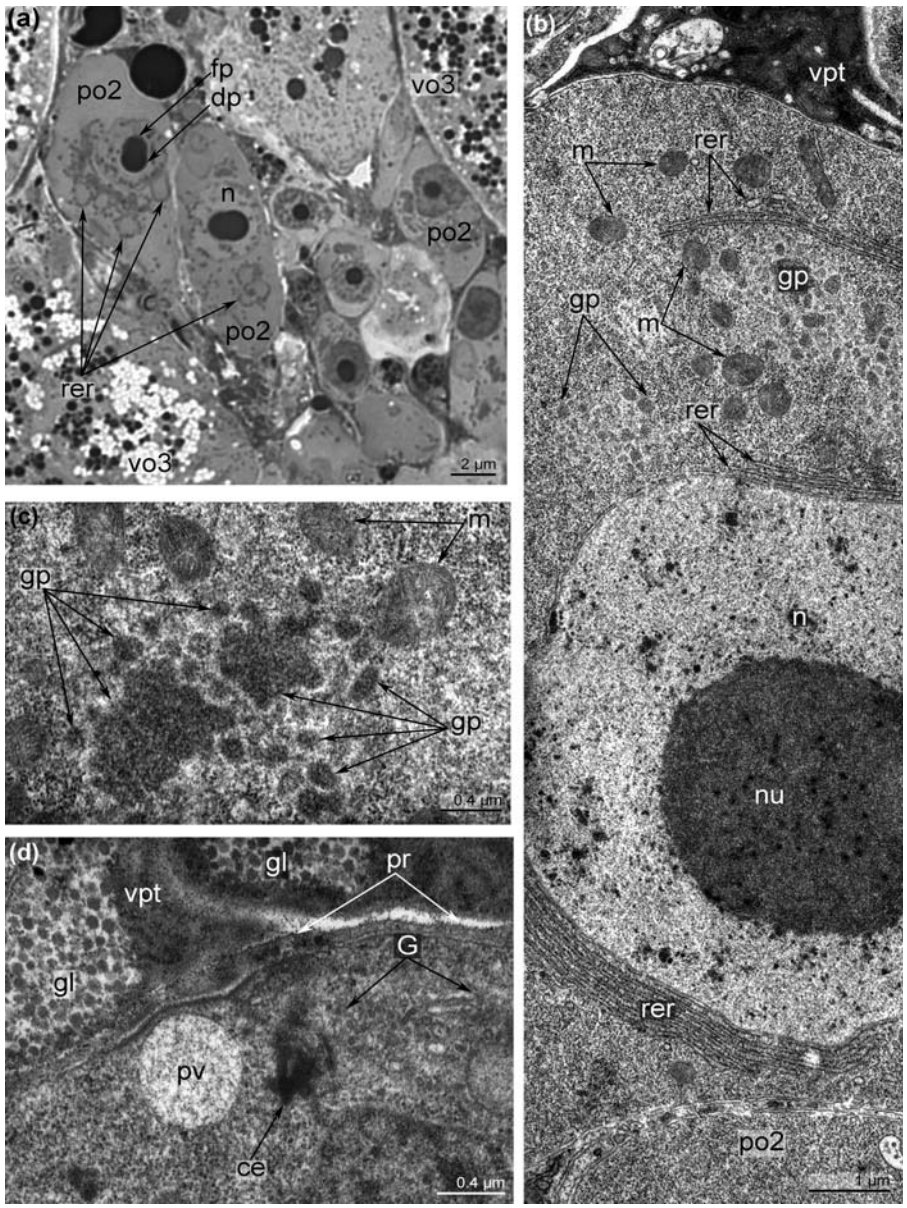

FIGURE 7 *Phoronis embryolabi*, later previtellogenic oocytes. (a) Semithin section of late previtellogenic oocytes: strongly developed synthetic apparatus are readily observed. Dense and flocculent portions of nucleolus are visible. (b) A central portion of later previtellogenic oocyte: nucleus with nucleolus, mitochondria, germ plasm, and rough endoplasmic reticulum are evident. (c) Germ plasm is represented by large aggregation of electron dense material. (d) Centriole and vesicles of Golgi apparatus in cytoplasm of late previtellogenic oocyte. Abbreviations: ce, centriole; dp, dense portion of nucleolus; fp, flocculent portion of nucleolus; G, Golgi apparatus; gl, glycogen; gp, germ plasm; m, mitochondria; n, nucleus; nu, nucleolus; po1, early previtellogenic oocyte; pr, projection of vasoperitoneal cell; pv, vesicles with pale, flocculent material (=pale vesicles); rer, rough endoplasmic reticulum; vo1, early vitellogenic oocyte; vpt, vasoperitoneal cells

from epithelial cells of the coelomic lining is indirectly supported by the presence of a centriole in oogonia and oocytes at different stages. This centriole may be regarded as rudimentary basal body of epithelial cells of blood capillaries, which are monociliar in phoronids (Temereva & Malakhov, 2004a).

The ultrastructure of phoronid oogonia is described here for the first time. In particular, the oogonia of *P. embryolabi* contain germ plasm or "nuage," which is represented by electron-dense granules and which is not associated with mitochondria till the late stages of oogenesis. The clusters of germ plasm and mitochondria are well known in many other bilaterians (Eckelbarger, 2005; Eddy, 1975; Extavour & Akam, 2003; Wourms, 1987). Interestingly, the ultrastructure of germ plasm varies among families and even among species within genera. Thus, it is represented by large "clouds" of electron-dense material in the previtellogenic oocytes of *P. embryolabi* (our data) but by small electron-dense granules in such oocytes of *P. harmeri* (Temereva, Malakhov & Yushin, 2011).

Three types of oogenesis can be recognized based on morphology: solitary (oocytes develop without accessory cells), nutrimentary (each oocyte develops with support of special nurse cells), and follicular (each

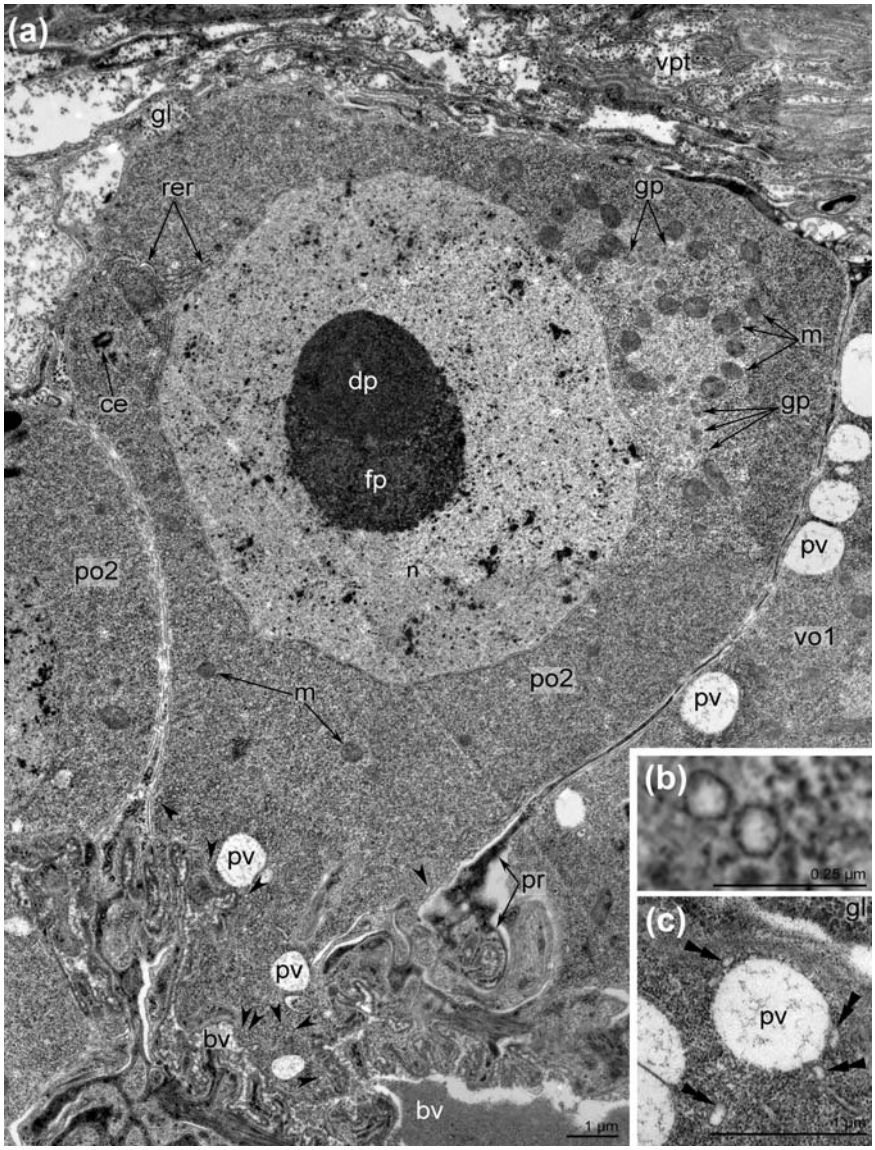

FIGURE 8 *Phoronis embryolabi*, ultrastructure of late previtellogenic oocyte. (a) Late previtellogenic oocyte attached to the blood vessel and surrounded by thin processes of vasoperitoneal cells. Vesicles (shown by arrowheads), which are probably endocytotic, are observed in basal cytoplasm. (b) Vesiclec, which are probably endocytotic, from the basal cytoplasm of oocyte. (c) Vesicle with pale, flocculent material is probably result of fusion of small vesicles (shown by double arrowheads). Abbreviations: bv, blood vessel; ce, centriole; gl, glycogen; gp, germ plasm; m, mitochondria; po2, late previtellogenic oocyte; pr, process of vasoperitoneal cell; pv, vesicles with pale, flocculent material (=pale vesicles); rer, rough endoplasmic reticulum; vo1, early vitellogenic oocyte; vpt, vasoperitoneal cells

oocyte develops in a follicle, formed by somatic cells) (Aisenstadt, 1984; Anderson, 1974; Raven, 1961). According to this terminology, phoronid oogenesis is follicular. Follicles of vasoperitoneal cells adjacent to developing oocytes have been described using both, light and electron microscopy (Emig, 1982; Herrmann, 1997; Pixell, 1912; Selys-Longchamps, 1907; Temereva et al., 2011). Follicular oogenesis is known from many groups of invertebrates and can occur in two modes depending on the involvement of the follicular cells in the vitellogenesis. In the first mode, follicular cells form an envelope around the developing oocyte; the envelope serves a mechanical and supporting function rather than a nutritive one. In this case, the yolk is formed by autosynthesis in the oocyte. This form of follicular oogenesis is well known in a variety of invertebrates including other lophophorates, for example, Brachiopoda (James, 1997; James, Ansell & Curry, 1991; Williams, James, Emig, Mackay, & Rhodes, 1997). Follicular autosynthetical oogenesis has been suggested to occur in the phoronid *P. harmeri* for the following reasons: (1) presence of highly developed RER, annulate lamellae, and Golgi apparatus in oocytes; (2) absence of the traces of endocytosis on oolemma; (3) absence of developed synthetic apparatus and glycogenic granules in vasoperitoneal cells; and (4) absence of special contacts between the follicle cells and oocytes (Temereva et al., 2011).

In the second mode of follicular oogenesis, the follicle cells provide nutrition to the developing oocytes. Among lophophorates this mode is known, for example, from gymnolaemate Bryozoa being also added by the activity of nurce-cell (Moosburgger, Schwaha, Walzl, Obst, & Ostrovsky, 2012; Ostrovsky, 2013a; Reed, 1988). Low molecular

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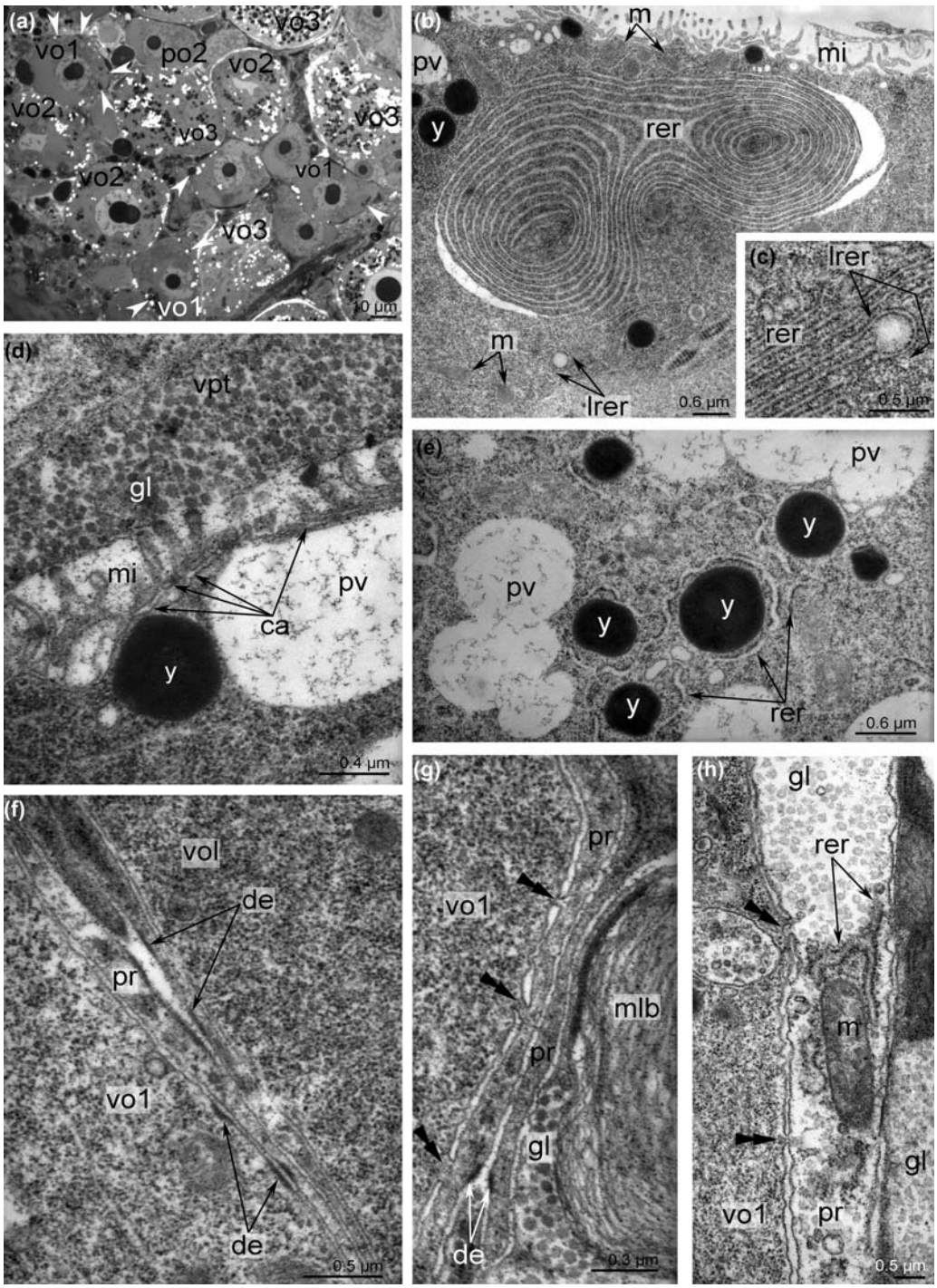

FIGURE 9 *Phoronis embryolabi*, vitellogenic oocytes. (a) Semithin section of oocytes at different developmental stages. Peripheral yolk granules in early vitellogenic oocytes (vo1) are shown by arrowheads. (b–d, f–h) Early vitellogenic oocyte; (e) mid-staged vitellogenic oocyte. (b) Large aggregation of cisternae of rough endoplasmic reticulum. (c) Terminal loop of rough endoplasmic reticulum. (d) Microvilli and cortical cytoplasm of oocyte: vesicles and canals are visible under the oolemma. (e) Cytoplasm of mid-staged vitellogenic oocyte: yolk granules are surrounded by cisternae of rough endoplasmic reticulum. (f) Desmosomes between oocytes and processes of vasoperitoneal cells. (g) Cytoplasmic bridges (double arrowheads) between oocyte and processes of vasoperitoneal cells. Desmosome between two processes of vasoperitoneal cells. (h) Cytoplasmic bridge (double arrowheads) between oocyte and processes of vasoperitoneal cells contains electron dense material. Abbreviations: ca, canals under oolemma; de, desmosome; gl, glycogen; lrer, loop of rough endoplasmic reticulum; m, mitochondria; mi, microvilli; mlb, multilamellar body; po2, late previtellogenic oocyte; pr, projection of vasoperitoneal cell; pv, vesicles with pale, flocculent material (=pale vesicles); rer, rough endoplasmic reticulum; vo1, early vitellogenic oocyte; vo2, mid-staged vitellogenic oocyte; vo3, late vitellogenic oocyte; vpt, vasoperitoneal cells; y, yolk granule

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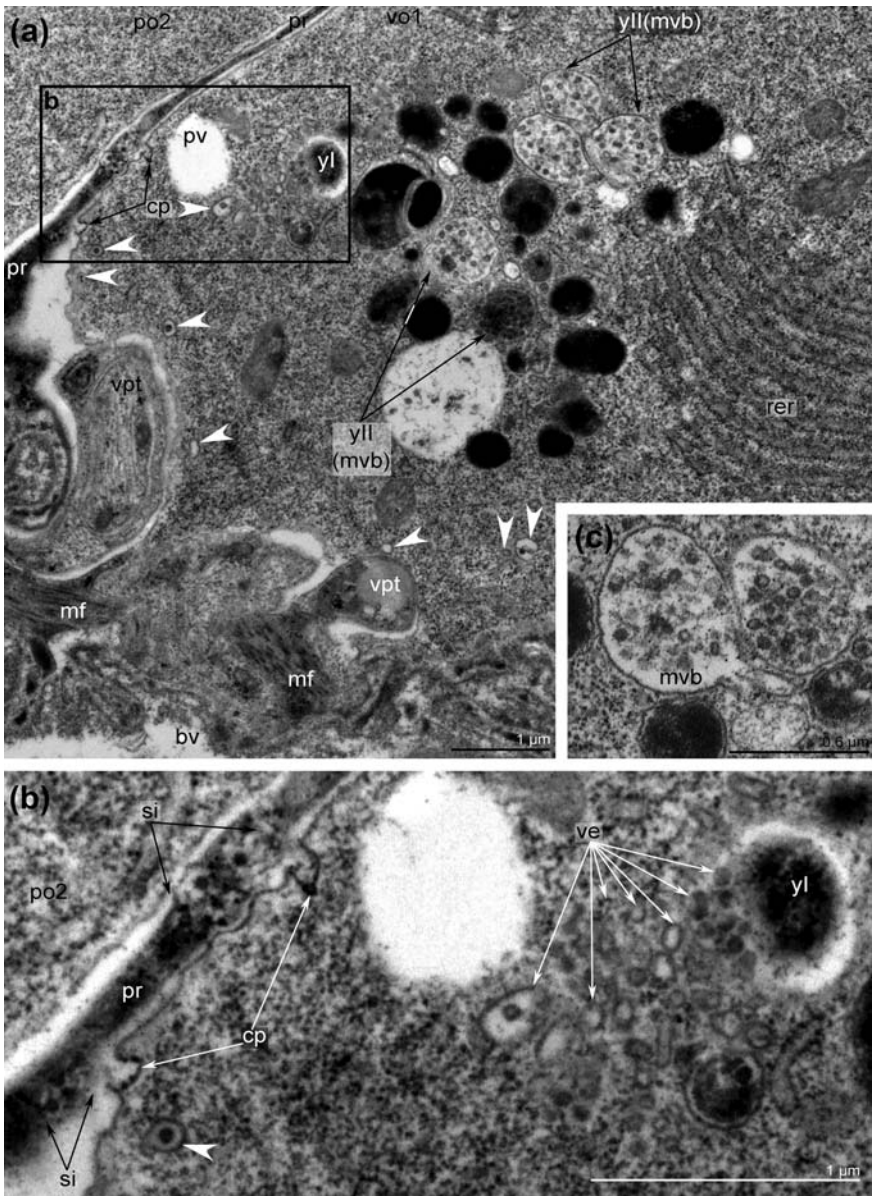

FIGURE 10 *Phoronis embryolabi*, early vitellogenic oocyte: yolk formation. (a) Peripheral cytoplasm with two types of yolk. Type I is formed due to appearance of coated pits and endocytotic vesicles (arrowheads), which later fuse to form a yolk granule. Type II yolk forms due to multivesicular-like bodies. (b) Detail of the area indicated via rectangle in (a). Coated pits of the oolemma and the spherical inclusions in cytoplasm of projection of vasoperitoneal cell are evident. (c) Multivesicular-like bodies contain many disk-shaped vesicles. Abbreviations: bv, blood vessel; cp, coated pit; mf, myofilaments; mvb, multivesicular-like body; po2, late previtellogenic oocyte; pr, process of vasoperitoneal cell; pv, vesicles with pale, flocculent material (=pale vesicles); si, spherical inclusions; ve, vesicles; vo1, early vitellogenic oocyte; vpt, vasoperitoneal cells; yI, type I of yolk formation (heterosynthesis); yII (mvb), type II of yolk formation (autosynthesis)

weight nutrients may be absorbed through the oolemma by diffusion or active transport, or both. The presence of coated pits in the oolemma and of coated vesicles in the cytoplasm of an oocyte is typically regarded as an evidence of nutritional transport. The presence of extensive glycogen deposits and various inclusions in follicle cells also indicates their nutritional function of follicle cells (Eckelbarger, 1975, 1979).

In *P. embryolabi*, several ultrastructural features in both, the developing oocytes and follicle cells indicate a nutritional function of the latter. First, the vasoperitoneal cells have a well-developed synthetic apparatus, and their cytoplasm is filled with many glycogen granules and various inclusions. Second, the presence of coated vesicles is evidence of endocytosis. And third, there are cytoplasmic bridges between developing oocytes and vasoperitoneal cells.

The presence of cytoplasmic bridges in *P. embryolabi* is an unusual feature among phoronids. Such cytoplasmic bridges between oocytes and accessory cells are the main feature of nutrimentary oogenesis that connect developing oocytes and one or more sibling cells or nurse cells, which are termed trophocytes. The nurse cells provide an oocyte with RNA and ribosomes during vitellogenesis (Wourms, 1987).

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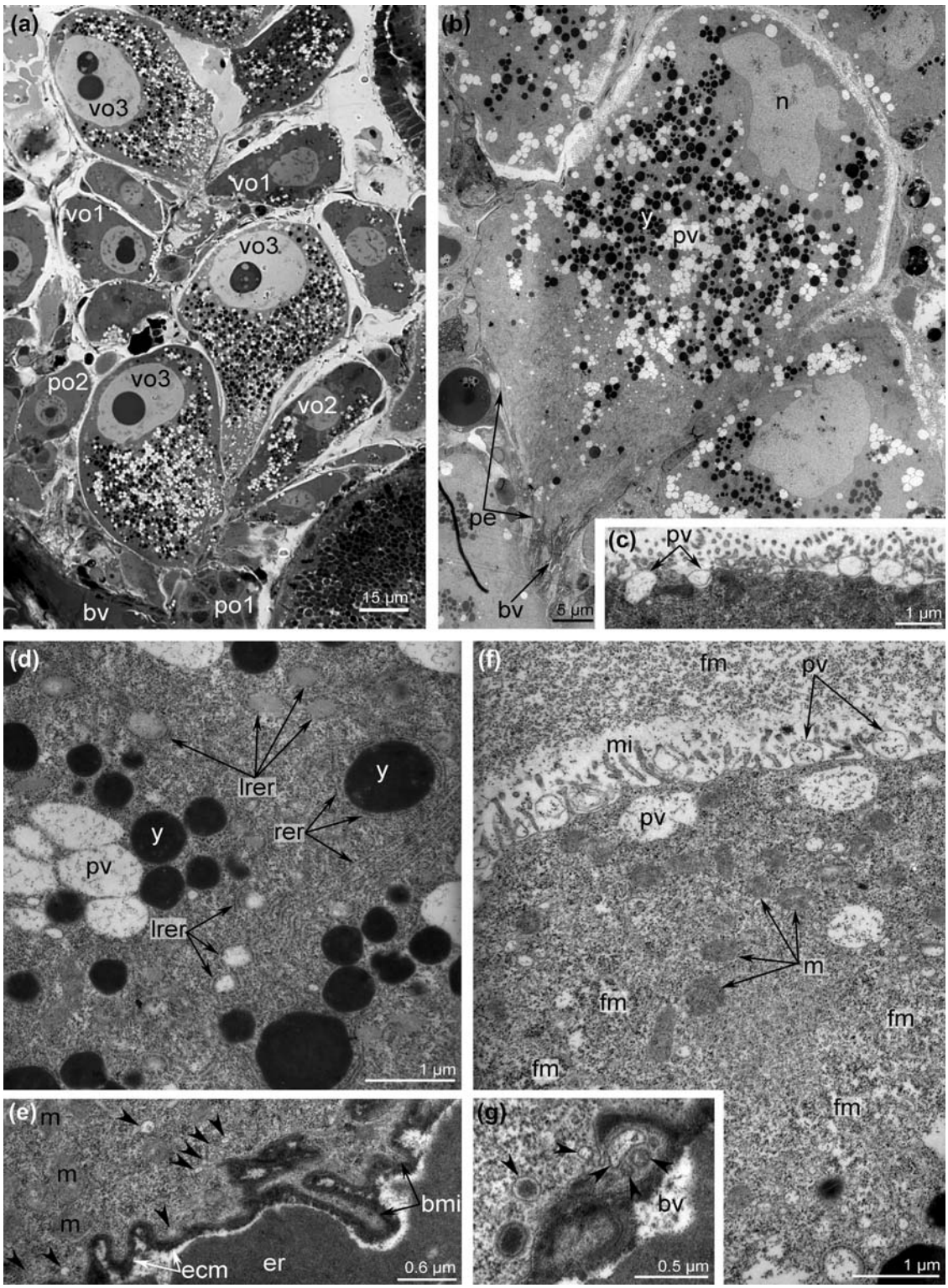

FIGURE 11 *Phoronis embryolabi*, late vitellogenic oocytes. (a) Semithin section of oocytes at different developmental stages. (b) Late vitellogenic oocyte: apical portion of the cell is occupied by nucleus, ooplasm contains pale vesicles and yolk granules, the basal part of cell forms a "pedicle" containing numerous mitochondria. (c) Oolemma: pale vesicles contact the oolemma aslo being visible in ooplasm and in perioocytal space. (d) Ooplasm containing yolk granules, loops of rough endoplasmic reticulum with material of different electron density, and pale vesicles. (e) Basal surface of oocyte forms a few basal microvilli covered by extracellular matrix. Presumed endocytotic vesicles, are shown by arrowheads. (f) A portion of cortical cytoplasm: the flocculent material is evident in perioocytal space, in pale vesicles, and in ooplasm. (g) Basal ooplasm with vesicles of different size and different content (arrowheads). Abbreviations: bv, blood vessel; ecm, extracellular matrix; er, erythrocyte; fm, flocculent material; lrer, loop of rough endoplasmic reticulum; m, mitochondria; mi, microvilli; n, nucleus; pe, basal "pedicle" of oocyte; po1, early previtellogenic oocyte; po2, late previtellogenic oocyte; pv, vesicles with pale, flocculent material (=pale vesicles); rer, rough endoplasmic reticulum; vo1, early vitellogenic oocyte; vo2, mid-staged vitellogenic oocyte; vo3, late vitellogenic oocyte; y, yolk granule

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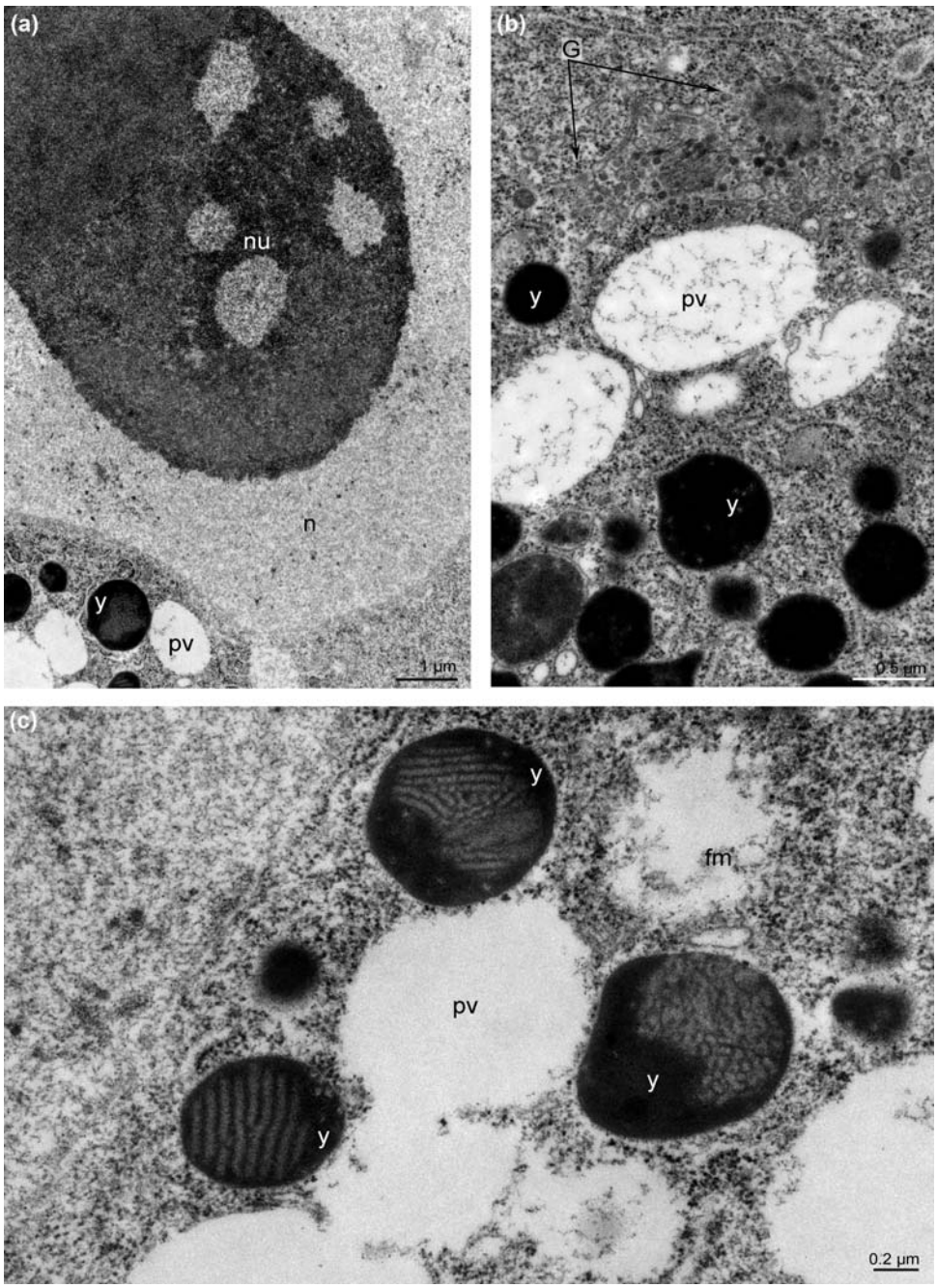

FIGURE 12 *Phoronis embryolabi*, some ultrastructural details of late vitellogenic oocytes. (a) Nucleolus with heterogeneous structure. (b) A portion of ooplasm with Golgi apparatus, which is mostly represented by vesicles and short cisternae, but not by typical dictyosomes. (c) Yolk granules with paracrystalline structure. Abbreviations: fm, flocculent material; G, Golgi apparatus; n, nucleus; nu, nucleolus; pv, vesicles with pale, flocculent material (=pale vesicles); y, yolk granule

Our study showed that vitellogenesis in *P. embryolabi* involves both autosynthesis and heterosynthesis. During autosynthesis, the yolk is produced by the oocytes involving different organelles. The first granules of the autosynthetic yolk typically appear in the central cytoplasm of the oocyte. Usually, they are surrounded by RER cisterna. During heterosynthesis, macromolecules (vitellogenin) are produced by other cells being absorbed by oocytes from the perioocytal space or from the blood via formation of coated vesicles. The granules of the heterosynthetic yolk first appear in the peripheral cytoplasm of the oocyte, and this was determined to be the case for *P. embryolabi* in the current study.

The presence of features of both autosynthesis and heterosynthesis indicates that vitellogenesis of *P. embryolabi* is best described as autoheterosynthetic. This type of yolk formation is known in many groups of Bilateria (for review see Aisenstadt, 1984; Anderson, 1974; Eckelbarger, 1994). Autosynthesis was suggested for *P. harmeri* (see above), which is the only other phoronid for which vitellogenesis has been studied. This difference in vitellogenesis between the two species

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TABLE 1 Organization of late vitellogenic oocyte and vasoperitoneal cells in the phoronids *Phoronopsis harmeri* and *Phoronis embryolabi*

Species Character	*Phoronopsis harmeri* (Temereva et al., 2011)	*Phoronis embryolabi* (this study)
1. Vitellogenic oocyte		
1a. Length of cell (μm)	90	60
1b. Size of nucleus (μm)	40	25
1c. Synthetic apparatus	RER, annulate lamellae, Golgi apparatus	RER, few Golgi apparatus
1d. Storage inclusions	lipid droplets, yolk granules	yolk granules
1e. Basal cytoplasm	foam-like because of numerous vesicles	contains mitochondria and few coated vesicles
1f. Presence of large, pale vesicles with flocculent content	+	+
1g. Endocytosis	-	+
1h. Microvilli	2 μm, branched, covered by glycocalix	0.5 μm, slightly branched, without glycocalix
1i. Cortical granules	-	-
2. Organization of VPT cells		
2a. Synthetic apparatus	–	+ RER, Golgi apparatus, multilamellar bodies
2b. Glycogen	–	+
2c. Inclusion bodies	+	+

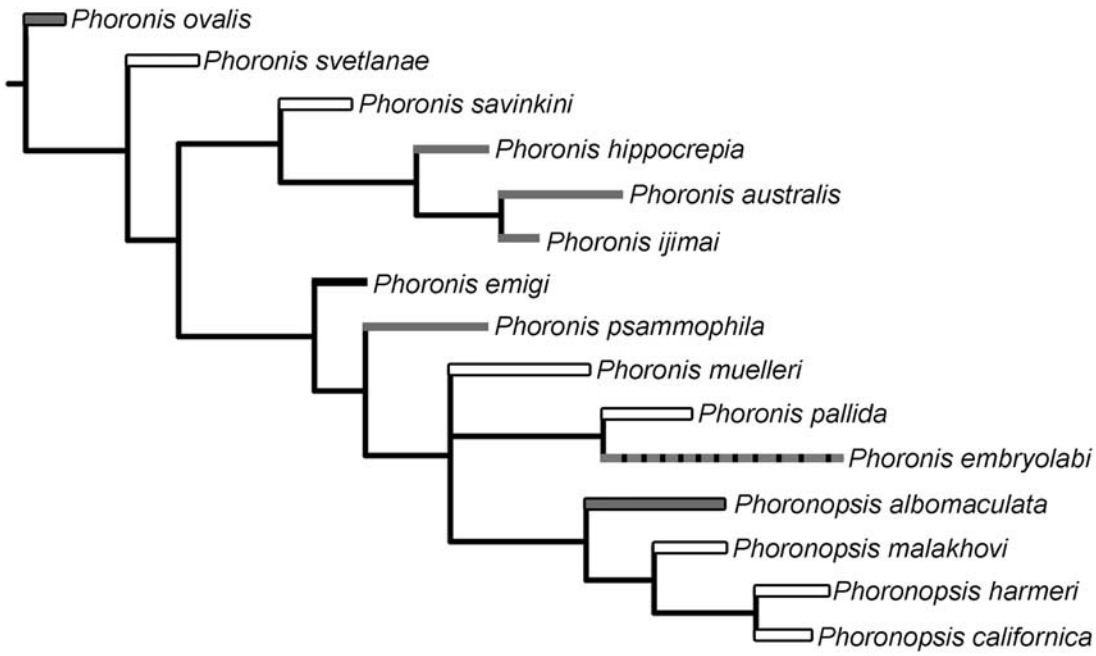

FIGURE 13 Distribution of type of development within phoronids. The phylogenetic tree is based on results of cladistic analysis (Temereva & Neklyudov, 2017). Species with parental care are shown in grey, including: tube brooders (in black boxes); species brooding embryos in lophophoral concavity (grey), and vivipaous species (black stripers). Broadcasters are shown in white. For *Phoronis svetlanae*, *Phoronis savinkini*, *Phoronopsis malakhovi* and *Phoronopsis californica* the broadcasting is supposed due to absence of nidamental glands in lophophoral concavity. Species, for which the information is unknown, are shown by black boxes

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is associated with differences in the organization of the late oocyte in *P. embryolabi* versus *P. harmeri*.

Although *P. harmeri* and *P. embryolabi* show some differences in both, oocyte structure and vitellogenesis, they share some unusual features (Table 1). Oogenesis mode is the same, that is, small macrolecithal oocytes are produced. The oocytes of both species, for example, have large, vesicles that contain pale flocculent material. The function of these vesicles is unknown, but their location in the cortical cytoplasm and their interaction with the oolemma as well as the presence of flocculent material around the late oocyte suggest that these vesicles could be involved in the formation of the fertilization membrane and egg envelope, which prevent polyspermy during fertilization. This function is typically supplied by cortical granules, which differ in size and structure and which are usually observed as dense filamentous or heterogeneous bodies bordered by the oolemma (Schuel, 1985). Because the large, pale vesicles with flocculent material have little in common with typical cortical granules, their function should be investigated in detail. Processes of fertilization and formation of fertilization membrane, which are still not described in phoronids, should also be studied in the future.

4.3 | Phylogenies and evolution of reproductive patterns

Four known patterns of phoronid reproduction could be considered as a morphoseries showing a transition from a typical broadcasting pattern with larval planktotrophy to brooding (either within a tentacle crown or in a tube) followed by feeding or nonfeeding larval period. Broadcasters possess holoplanktonic "Actinotrocha 1" larva, living up to three months (Temereva, 2009; Temereva & Malakhov, 2007; Temereva & Neretina, 2013) (some, possibly less, e.g., four weeks, according to Zimmer, 1964; Santagata & Zimmer, 2002). The only possible exception is *P. albomaculata* that is probably a tube brooder (Gilchrist, 1907, 1919). All other brooders produce feeding "Actinotrocha 2" that lives in plankton from three weeks to 1.5 months (Temereva, 2009; Temereva & Malakhov, 2004a, 2004b). Similarly, the feeding larva ("Actinotrocha 3") of the only known viviparous *P. embryolabi* lives one month (larvae of this species were long known before the actual adult; see Temereva & Neretina, 2013). Finally, duration of the larval life of creeping nonfeeding larva of the tube brooder *P. ovalis* is nine days (Silen, 1954; Emig, 1977, 1982; Temereva, 2009; Temereva & Chichvarkhin, 2017; Temereva & Malakhov, 2004b,2012,2016; Temereva & Neretina, 2013; Zimmer, 1991).

Oogenesis generally corresponds to this morphoseries. Broadcasters possess small (50–90 μm) and numerous (500–1,000 per individual in given time) eggs whereas brooders have 100–400 eggs with a diameter about 80–130 μm. Tube brooder *P. ovalis* produce 40 large (125 μm) eggs (Emig, 1982). Intriguingly, viviparous *P. embryolabi* forms hundreds of small (60 μm) small eggs (Temereva & Chichvarkhin, 2017).

While an evolutionary vector is obvious (see Emig, 1974), both morphological and molecular phylogenetic trees suggest that parental care originated independently several times in Phoronida (Temereva & Neklyudov, 2017). Moreover, because the trees contradict each other, distribution of reproductive patterns on them could give additional arguments in our understanding how reproduction could evolve in this phylum.

Having a basalmost position in all recently published trees (Hirose et al., 2014; Temereva & Chichvarkhin, 2017; Temereva & Neklyudov, 2017) *P. ovalis* shows the first example of independently evolving tube brooding and a shift to nonfeeding larva (Figure 13). In the generally similar molecular trees by Hirose et al. (2014) and Temereva and Chichvarkhin (2017), species of the genus *Phoronopsis* that are mostly broadcasters, have the basal position pointing to the ancestral reproductive pattern. Noteworthy, in the morphological tree by Temereva and Neklyudov (2017) the basal position is occupied by presumed broadcaster too (*Phoronis svetlanae*; Figure 13).

Further branching in both trees results in two large clades of the differing composition. The most important outcome is that in both trees, molecular and morphological, these clades include brooders and broadcasters, pointing to at least two more independent origins of the parental care. In fact, morphological tree supports four additional origins, and the molecular one six. Importantly, the origin of vivipaity in *P. embryolabi* was connected with broadcasting ancestor in both trees that is also supported by the fact that this species produces numerous small eggs (Figure 13).

Unfortunately, there are almost no data on the oogenesis mode (oligolecithal vs. macrolecithal) in Phoronida. They could greatly add to our understanding of the evolution of their reproductive patterns. For instance, despite eggs are small in the broadcaster *P. harmeri* and viviparous *P. embryolabi*, they are macrolecithal in both species. In contrast, eggs are always oligolecithal in broadcasting bryozoans while they could be either macrolecithal or oligolecithal in the matrotrophic species illustrating various evolutionary trajectories accompanied by the shifts in oogenesis (Ostrovsky, 2013a, 2013b). We need more data on phoronid oogenesis to develop such scenarios.

Finally, the differences in the maternal provisioning of the offspring may reflect differences in phoronid biology. Thus, the viviparous *P. embryolabi* is a commensal living in the burrows of the axiid shrimp *Nihonotrypaea japonica*. The extraembryonic nutrition combining growth and embryonic development provides the faster larval production and, thus, could favour the faster recruitment. This could be important in the nonstable seasonal environment, for example, during occupation of the vacant niches after overwintering (see Ostrovsky, 2013a, 2013b; Ostrovsky, Gordon, & Lidgard, 2009). Whether the larval production of *P. embryolabi* is somehow connected with the life cycle of the shrimp is unknown, and requires future investigation.

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CONFLICT OF INTEREST

The author declares she does not have any conflict of interest.

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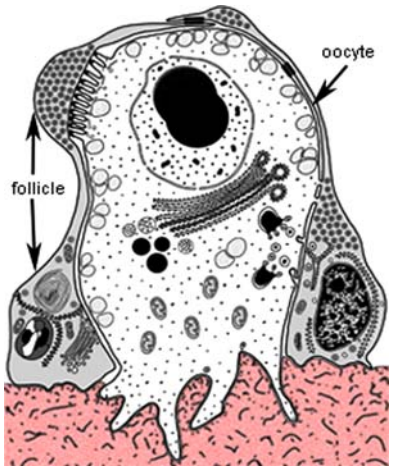

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Oogenesis of viviparous phoronid *Phoronis embryolabi* is autoheterosynthetic and involves follicles that appear to provide nutrients to the developing oocytes. Parental care has evolved independently several times in Phoronida.