The contribution of TEM to solving issues in the oogenesis of lower metazoans: a comparison between Acoela and rhabditophoran Platyhelminthes

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The chapter reports data on the oogenesis and eggshell formation in some Acoela obtained by our group over the years by means of transmission electron microscopy (TEM) and cytochemical techniques. In this review we show how TEM and appropriate cytochemical methods have been helpful in clarifying issues related to female germ cell differentiation and more in general to the cellular aspects of the reproductive biology in these lower metazoans. The ultrastructural findings have been compared with those from other Acoelomorpha (Acoela + Nemertodermatida) and rhabditophoran Platyhelminthes

Keywords: Acoela; oogenesis; accessory cells; egg envelope; ultrastructure; cytochemistry

1. Introduction

Acoela are bilaterally symmetric, acoelomate, hermaphroditic worms of microscopic size that are predominantly found in benthic marine habitats. They are characterized by the presence of a statocyst at the anterior end, a net-like nervous system, a blind digestive gut and by the absence of protonephridia and oviducts. They were first included in the taxon Acoelomorpha (Acoela + Nemertodermatida) within the phylum Platyhelminthes which, at the same time, were divided into three major lineages (Acoelomorpha, Catenulida, and Rhabdithophora). Then, with the advent of molecular phylogenetic investigations the Acoelomorpha were removed from Platyhelminthes and considered as a separate phylum, presumably, as the majority of molecular studies seem to suggest, at the base of bilaterians, as sister group to the rest of Bilateria [1-3]. More recently another genus of small marine worms, *Xenoturbella*, was found to be closely related to Acoelomorpha [4] and the new phylum Xenacoelomorpha was created [5]. Some molecular phylogenetic investigations seem now to suggest that Xenacoelomorpha is the sister group of Nephrozoa (i.e. all the remaining Bilateria, namely protostomes and deuterostomes) [6, 7]. Some years ago our research group investigated the female gonad and oogenesis of some Acoela by means of transmission electron microscopy and cytochemical tests. In this review we report the main data we have obtained over the years on the oogenesis of three species belonging to different families: *Synsagittifera psammophila* (Sagittiferidae), *Actinoposthia beklemischevi* (Childiidae) and *Paratomella rubra* (Paratomellidae) with the aim of comparing the mechanisms involved in the production of eggshell precursors and yolk with those from other animals with entolecithal eggs, in particular the rhabditophoran Platyhelminthes with the archoophoran (homocellular) condition of the female gonad, like Macrostomida and Polycladida.

2. Female gonad morphology

Acoela have paired or unpaired homocellular female gonads (ovaries), known as diffuse or asaccular gonads, i.e. oocytes do not form a compact gonad but lie free in the parenchyma and are not lined and separated from the surrounding tissue by a tunica [8]. The ovaries consist of entolecithal eggs (Figs 1, 2) closely associated with accessory cells, and generally developing on an anterior-posterior axis with mature oocytes located posteriorly (Fig. 1). The female reproductive system of acoels may consist only of ovaries but in many cases also comprises accessory organs such as seminal bursae for storing sperm from a mating patner and bursal nozzles, tubiform outlets of the seminal bursae, to transfer sperm to the oocytes (Fig. 1).

3. Oocytes differentiation

Oogenesis in acoels can be divided into two periods, the previtellogenic and the vitellogenic phase [9-13] as is typical in the platyhelminths with archoophoran (homocellular) organization of the female gonad [14-16, 18, 19] and in general in animals with entolecithal eggs [20, 21]

3.1 Previtellogenic oocytes

Early previtellogenic oocytes are small roundish or elliptical cells measuring about 5-10 μm in diameter with a high nucleus/cytoplasmic ratio. At the beginning of differentiation, which occurs during the prophase of the first meiotic

division, as indicated by the presence of synaptonemal complexes, oocytes gradually increase in volume reaching up 20-30 µm (Figs 3, 4).

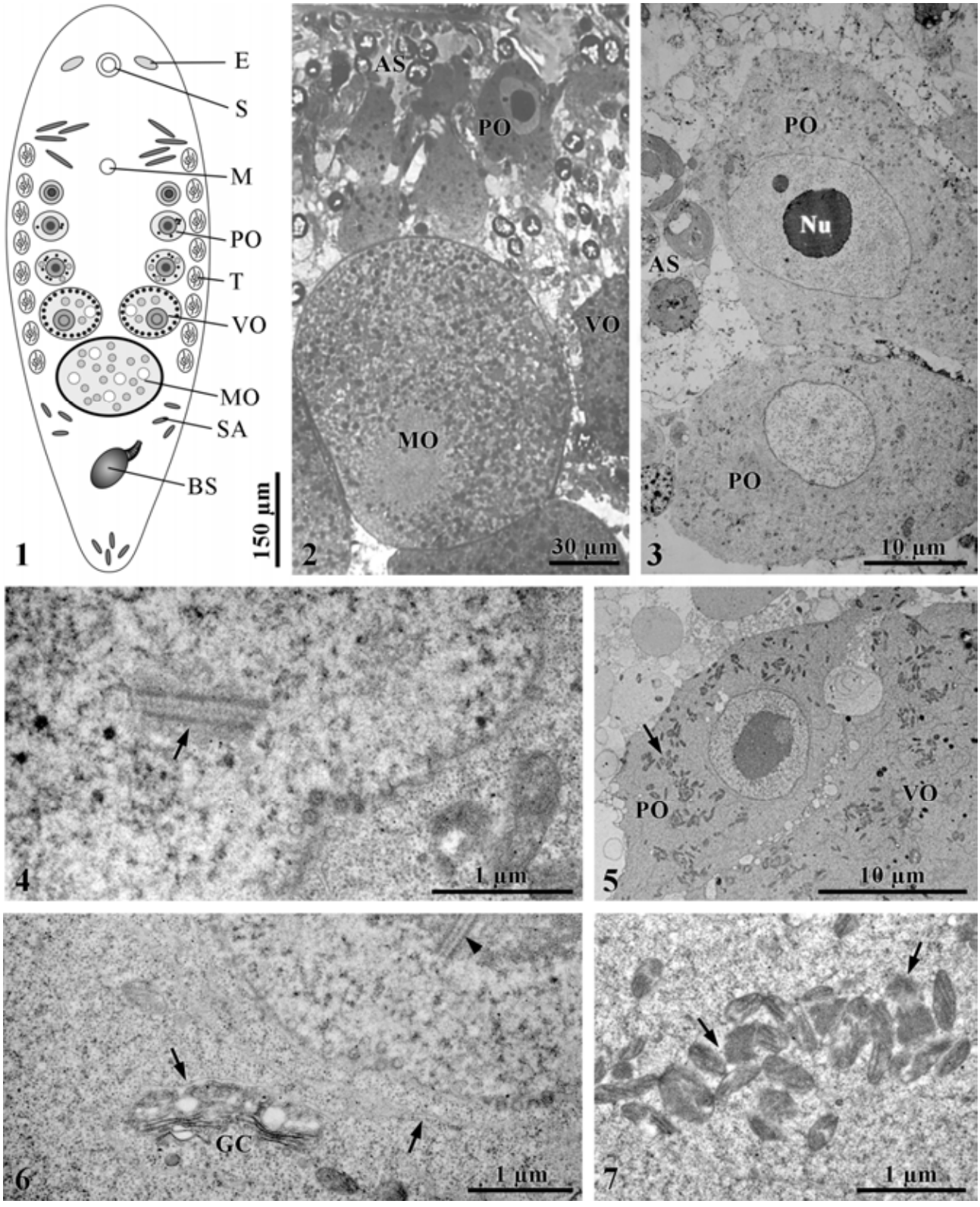


Fig. 1. Schematic drawing of *Synsagittifera psammophila*. BS, bursa seminalis; E, eye; M, mouth; MO, mature oocyte; PO, previtellogenic oocyte; S, statocyst; SA, sagittocyst; T, testis; VO, vitellogenic oocyte. **Fig. 2**. *S. psammophila*. Light microscopy. Asaccular gonad. AS, algal symbiont; PO, previtellogenic oocyte; VO, vitellogenic oocyte; MO, mature oocyte. **Fig. 3.** *S. psammophila*. Two previtellogenic oocytes (PO). AS, algal symbiont; Nu, nucleolus. **Fig. 4.** *P. rubra*. A synaptonemal complex (arrow) with the typical tripartite structure in the nucleus of a previtellogenic oocyte. **Fig. 5.** *P. rubra*. A previtellogenic oocyte (PO)

at the onset of differentiation with the nucleus showing a prominent nucleolus and the cytoplasm with clustered mitochondria (arrow). VO, vitellogenic oocyte. **Fig. 6.** *P. rubra*. Differentiating previtellogenic oocyte showing numerous free ribosomes, some RER profiles (arrows) and Golgi complexes (GC). Arrowhead points to a synaptonemal complex. **Fig. 7.** *P. rubra*. Chromatoid bodies (arrows) surrounded by mitochondria in the cytoplasm of a previtellogenic oocyte.

The nucleus, about 7-15 μm in diameter, shows mainly diffuse chromatin, and a prominent compact nucleolus with intermingled fibrillar and granular components (Figs 3, 5). The cytoplasm contains a large number of free ribosomes, some mitochondria, some cisternae of rough endoplasmic reticulum, Golgi complexes and chromatoid bodies, often surrounded by mitochondria (Figs 6, 7). At this time RER and Golgi complexes are involved in the production of small vesicles of moderate electron density. Repeated fusions of these vesicles give rise to membrane-bound granules, called type I inclusions (Figs 8-10). In all the species investigated, these granules are round or slightly elliptical in shape, measure about 0.4-0.6 μm in diameter, show a homogeneous content of medium/high electron density and remain distributed throughout the ooplasm of previtellogenic oocytes. Some lipid droplets are also visible at this stage of oocyte differentiation (Figs 8, 9).

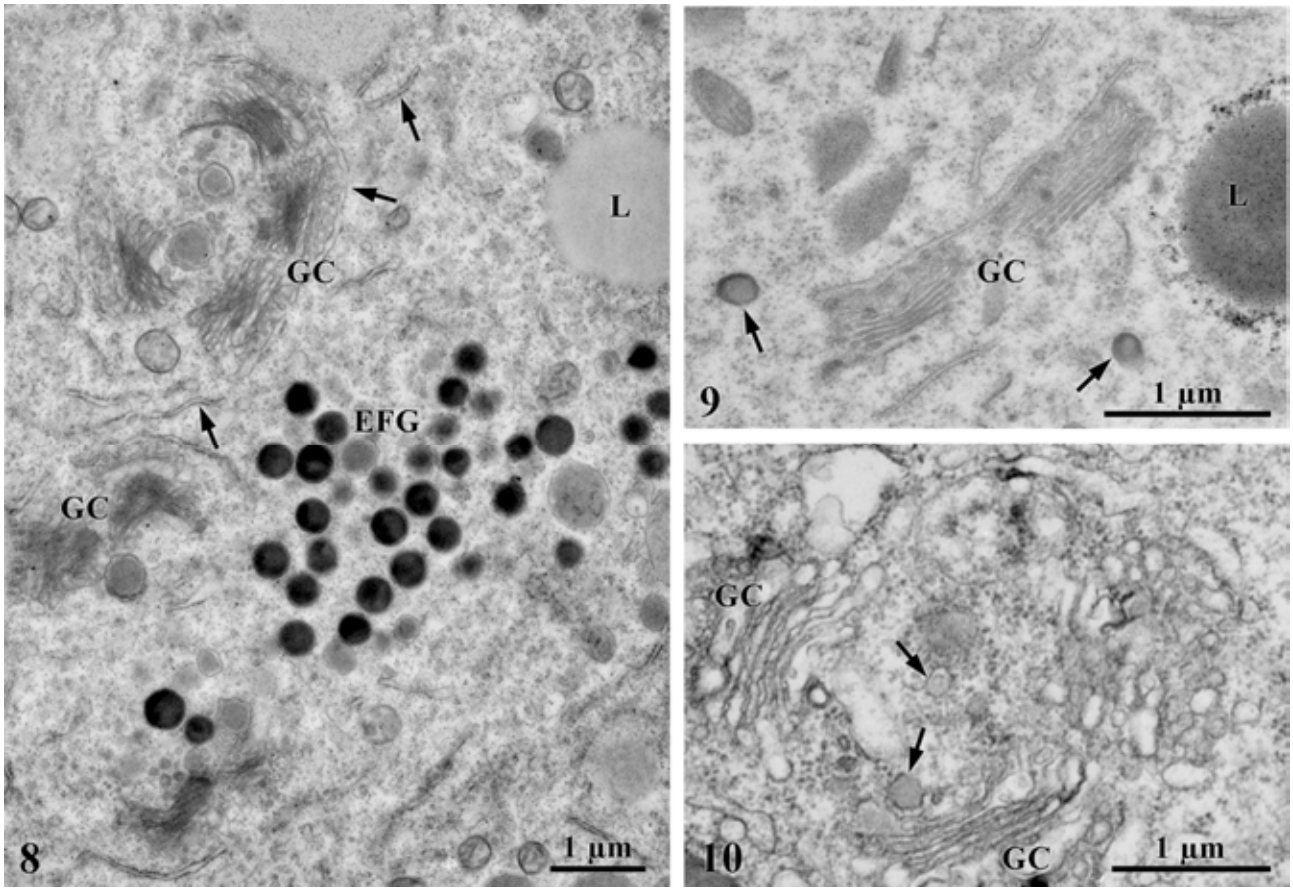


Fig. 8. *S. psammophila*. RER profiles (arrows), Golgi complexes (GC) and clustered eggshell-forming granules (EFG, type I inclusions) in the cytoplasm of a previtellogenic oocyte. L, lipid. **Fig. 9.** *P. rubra*. RER, Golgi complexes (GC) and two eggshell-forming granules (arrows). L, lipid. **Fig. 10.** *A. beklemischevi*. Golgi complexes (GC) and nascent eggshell-forming granules (arrows) in a previtellogenic oocyte.

3.2 Vitellogenic oocytes

Vitellogenesis begins in the middle of the meiotic prophase I when synaptonemal complexes are no longer visible and is characterized by the rapid increase in volume of the oocyte due to the accumulation of different types of inclusions with the consequent decrease of the nucleo/cytoplasmic ratio (Fig. 11). The oocyte nucleus contains mainly diffuse chromatin and the nucleolus loses its compact structure, at first showing areas of low electron density, then assuming a ring shape (Fig. 12) and finally disappearing in the late stages of vitellogenesis (Fig. 15). RER and Golgi complexes are always well-developed and continue to produce type I inclusions which, at the onset of vitellogenesis, are still scattered throughout the ooplasm. During the vitellogenic phase RER and Golgi complexes also appear to be involved in the production of a second type of membrane-bound inclusion, known as type II inclusions (Figs 11, 12, 14). These are larger than type I inclusions and measure about 1-2 μm. Type II inclusions remain scattered in the ooplasm of late vitellogenic and mature oocytes and have been interpreted as yolk [9-13]. The shape, substructure and diameter of yolk

globules vary according to the species [13, 22]. A peculiar substructure has been observed in *Paratomella rubra* where yolk globules are elliptical in shape (about 1.8 µm in maximum diameter) and show a narrow electron-dense lamellar cortex and a less dense paracrystalline core [12] (Fig.13). In late vitellogenic oocytes, which can reach up to 100-130 µm in diameter, type I inclusions move to the cortical ooplasm and become localized under the oolemma where they participate in the formation of the egg envelope (Figs 24-26). On the basis of their localization and function in late vitellogenic oocytes type I inclusions have been interpreted as eggshell-forming granules (EFGs) [9-13].

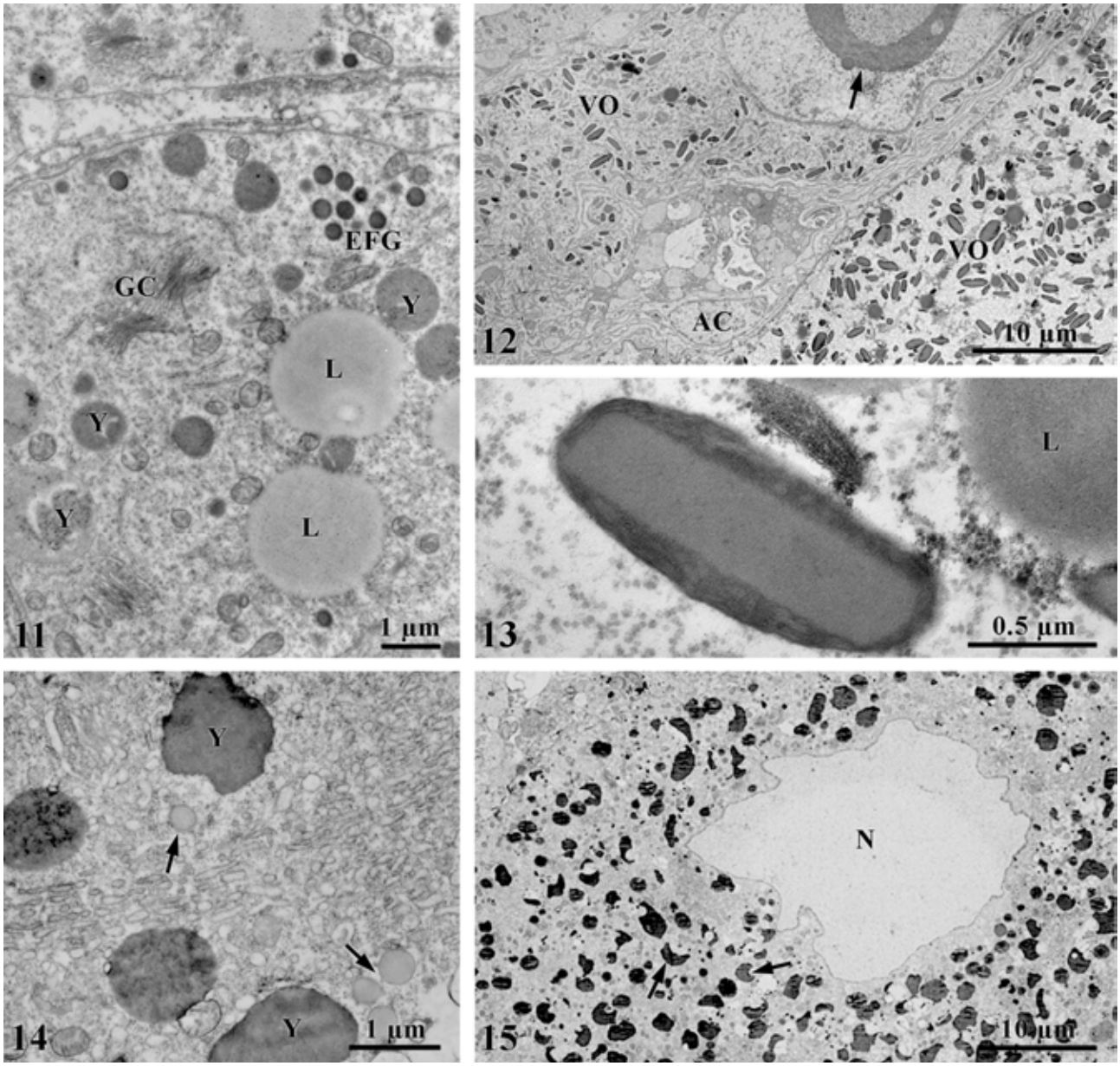


Fig. 11. *S. psammophila*. A vitellogenic oocyte showing three types of inclusion: yolk globules (Y, type II inclusions), eggshell-forming granules (EGF) and lipids (L). **Fig. 12.** *P. rubra.* Two vitellogenic oocytes (VO) at different stages of maturation surrounded by accessory cells (AC). Note the ring-shaped nucleolus (arrow) and the cytoplasm containing numerous elliptical yolk globules. **Fig. 13.** *P. rubra.* A mature yolk globule showing a narrow electron-dense lamellar cortex and a less dense paracrystalline core. L, lipid. **Fig. 14.** *A. beklemischevi*. A large area of smooth and rough ER with close yolk globules (Y) and eggshell-forming granules (arrows) in the cytoplasm of a vitellogenic oocyte. **Fig. 15.** *A. beklemischevi.* A late vitellogenic oocyte with a large amount of yolk globules some of which have a crescent shape (arrows). N, nucleus.

4. Egg envelope formation

4.1 Accessory cells, primary egg envelope (outer layer of the egg envelope)

In all the acoel species investigated to date differentiating oocytes are associated with accessory cells [9-13, 22]. Accessory cells completely envelope female germ cells with their long cytoplasmic processes (Figs 12, 16-18, 20, 23) from the beginning of oocyte differentiation in some species [9] or in later stages of development in others [11].

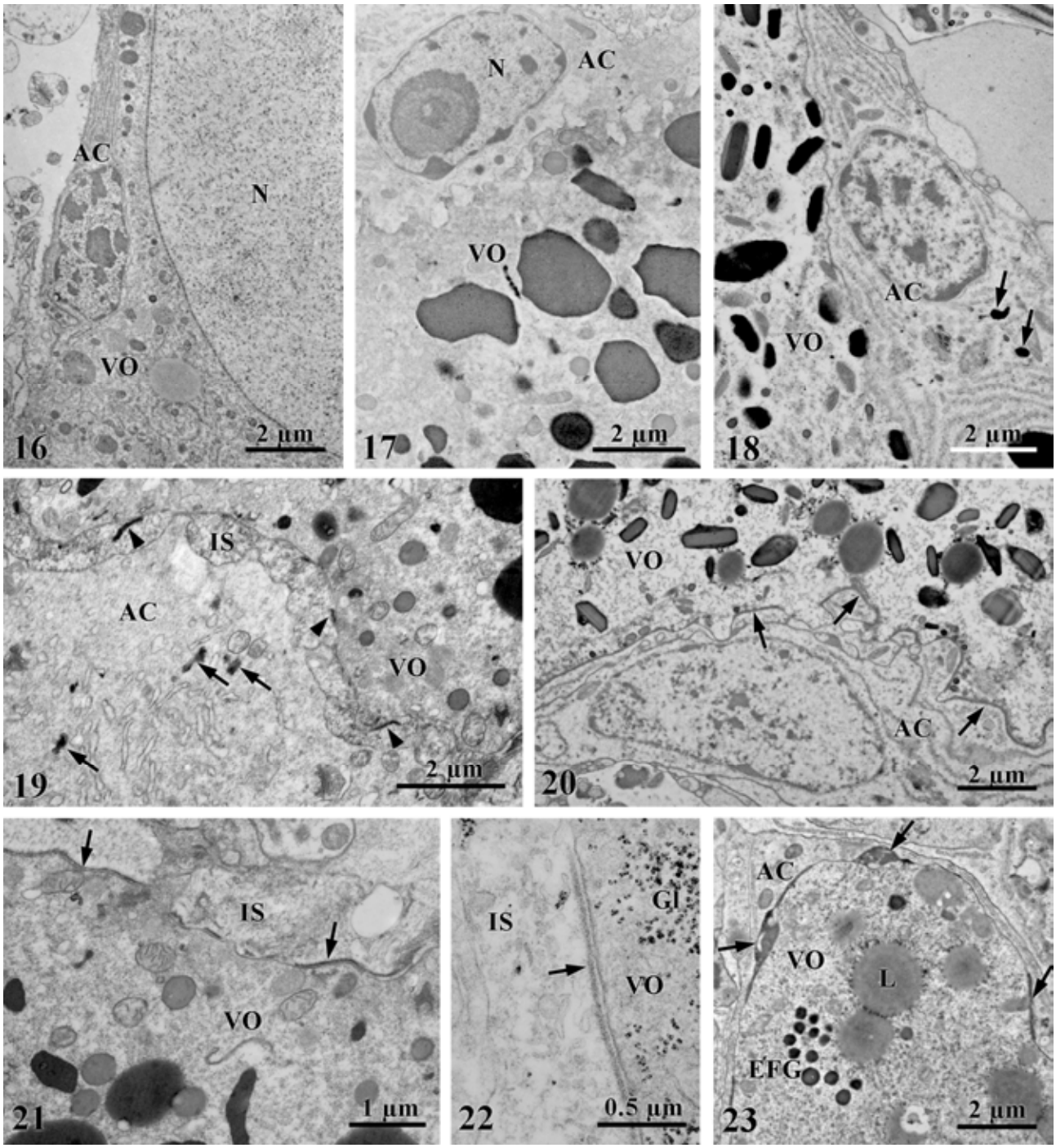


Fig. 16. *S. psammophila.* Accessory cell (AC) enveloping a vitellogenic oocyte (VO). The AC nucleus shows patches of condensed chromatin and a prominent nucleolus, the cytoplasm contains elongated RER cisternae. N, oocyte nucleus. **Fig. 17**. *A. beklemischevi*. Accessory cell (AC) associated with a vitellogenic oocyte (VO). The nucleus (N) shows a well-developed nucleolus. **Fig. 18**. *P. rubra*. Accessory cell (AC) surrounding a vitellogenic oocyte (VO). Long cisternae of RER and electron-dense inclusions (arrows) are visible in the AC cytoplasm. **Fig. 19**. *A. beklemischevi*. Portion of an accessory cell cytoplasm (AC) with RER cisternae and dense inclusions (arrows). Arrowheads point to short stripes of the primary egg envelope with the same electron density of the small inclusions of the accessory cell. IS, intercellular space. **Fig. 20**. *P. rubra*. Accessory cells (AC) surrounding a late vitellogenic oocyte (VO). Arrows point to portions of the primary egg envelope. **Figs 21, 22**. *A. beklemischevi*. Primary egg envelope formation. Note long stripes of the primary egg envelope adjacent to the oolemma (arrows). IS, intercellular space; VO, vitellogenic oocyte; Gl, glycogen. **Fig. 23**. *S. psammophila*. Accessory cell (AC) encompassing a vitellogenic oocyte (VO). Note the dense material (arrows), presumably exocyted by the accessory cell, placed against the oolemma. EFG, cluster of eggshell forming granules; L, lipid.

Intercellular junctions between accessory cells and oocytes have only been detected in *Paratomella rubra* [12]. The ovoid/elongated nucleus (about 5-8 μm in diameter) exhibits scattered clumps of heterochromatin mainly adjacent to the inner nuclear membrane and a prominent nucleolus (Figs 16-18). The cytoplasm contains numerous RER cisternae, some Golgi complexes, and electron-dense granules probably deriving from fusions of Golgi derived vesicles (Figs 16, 18, 19). These granules are occasionally visible close to or fusing with the plasma membrane of the accessory cells. Their content is discharged into the space between the accessory cells and the oocytes giving rise to the primary egg envelope (Figs 19-23). This process has been well documented in *Convoluta pulchra* now *Isodiametra pulchra* [10] and *Actinoposthia beklemiscevi* [13] where the granule content, released from accessory cells, forms small electron-dense clumps adjacent to the oolemma that spread along the membrane and give rise, at first, to a discontinuous layer, the primary egg envelope (Figs 21, 22).

4.2 Late vitellogenic oocytes, secondary egg envelope (inner layer of the egg envelope)

In late vitellogenic oocytes, the EFGs are located under the oolemma (Figs 24-26) where they can either fuse to form larger shell granules, as is the case in *S. psammophila* [9] or not undergo fusion, as in *I. pulchra* and other species [10]. The content of EFGs is released into the intercellular space between the oocytes and the primary egg envelope, where it coalesces giving rise to the secondary egg envelope (Figs 26-28). Mature oocytes in the process of being laid show a homogeneous egg envelope measuring about 250-300 nm in thickness where the outer and inner layers of the egg envelope are no longer visible, as in *S. psammophila* (Figs 2, 29, 30). Laid eggs are encapsulated in a transparent, flexible, non-sclerotized shell [10].

5. Cytochemical tests

Among all the acoel species investigated to date, cytochemical tests have been performed only in a small number of species and prevalently by our research group [10, 13, 23, 24]. We believe that, in order to have a complete picture of the ultrastructural features of oogenesis, it is also important to carry out cytochemical tests. In particular, if we want to make comparisons between homologous oocyte inclusions in different organisms, the chemical composition of such inclusions must be ascertained through careful cytochemical tests. As an example, we report the data obtained in *S. psammophila*, *A. beklemishevi* and *P. rubra* by means of cytochemistry.

In *S. psammophila* the EFGs and the egg envelope surrounding mature oocytes tested negative to the Locke and Krishnan test [25] to detect polyphenols as well as yolk globules (Figs 31, 33). The content of the EFGs and the egg envelope was completely digested by the action of pronase as was that of yolk globules (Figs 32, 34, 35). In *A. beklemishevi* the content of EFGs as well as the egg envelope was negative to the polyphenols test while a positive reaction was observed on the yolk globules (Figs 36, 37), where, the silver precipitate was more concentrated on mature than young yolk globules (Figs 36, 37). After incubation of ultrathin sections either in pronase or pepsin a partial extraction of the peripheral content of the EFGs was observed while the yolk globules and the egg envelope were unaffected (Fig. 38). In *P. rubra* the content of the EFGs and the egg envelope was negative for polyphenols while a light positive reaction was visible on the lamellar cortex of yolk globules (Fig. 39). The paracrystalline core of yolk globules was completely digested by pronase and pepsin while the content of EFGs and the egg envelope was completely unaffected (Fig. 40).

In all the three species examined a positive reaction to the Thiéry method [26] revealing the presence of glycogen was observed in the ooplasm after 8 h of treatment in thiocarbohydrazide. The amount of glycogen was low in previtellogenic and early vitellogenic oocytes while it was higher in late vitellogenic oocytes (Figs 41, 42).

6. Conclusions

Transmission electron microscopy has shown that the pattern of oogenesis is similar in all the species of acoels studied to date. The main feature of oocyte differentiation is the appearance of two distinct types of membrane-bound inclusion: type I and type II. This feature is similar to that observed in the acoelomorph Nemertodermatida [23] and in the rhabditophoran Platyhelminthes Macrostomida and Polycladida, which possess an archoophoran organization of the female gonad and entolecithal eggs [27, 28]. In acoels type I inclusions are the first to appear during the previtellogenic phase, are roundish and smaller than type II inclusions, contain glycoproteins and lack polyphenols [12, 13]. During the vitellogenic phase they move to the cortical ooplasm, become localized under the oolemma and their content is discharged through exocytosis into the space between the oocyte and the accessory cell where the coalescence of their contents, gives rises to the secondary egg envelope (inner layer of the eggshell). On the basis of their localization and function, and due to the fact that they are no longer visible in mature eggs, all the authors have agreed to consider type I inclusions as eggshell-forming granules (EFGs).

The feature of EFGs without polyphenols in acoels is similar to that found in Nemertodermatida [23] and differs from that observed in the archoophoran platyheminths Macrostomida and Polycladida, where EFGs containing

polyphenols have been found in the oocytes [15, 16] (Figs 43, 44) and in most neoophoran Platyhelminthes, where eggshell globules rich in polyphenols have been observed in the vitelline cells [28-30].

The mechanism of eggshell formation in acoels seems to be similar in all the species studied to date and involves the contribution of the accessory cells. The primary egg envelope (outer layer of the eggshell) is formed by the accessory cells and the secondary egg envelope (inner layer of the eggshell) is formed by fusion of material deriving from EFGs that is exocyted by the vitellogenic oocytes.

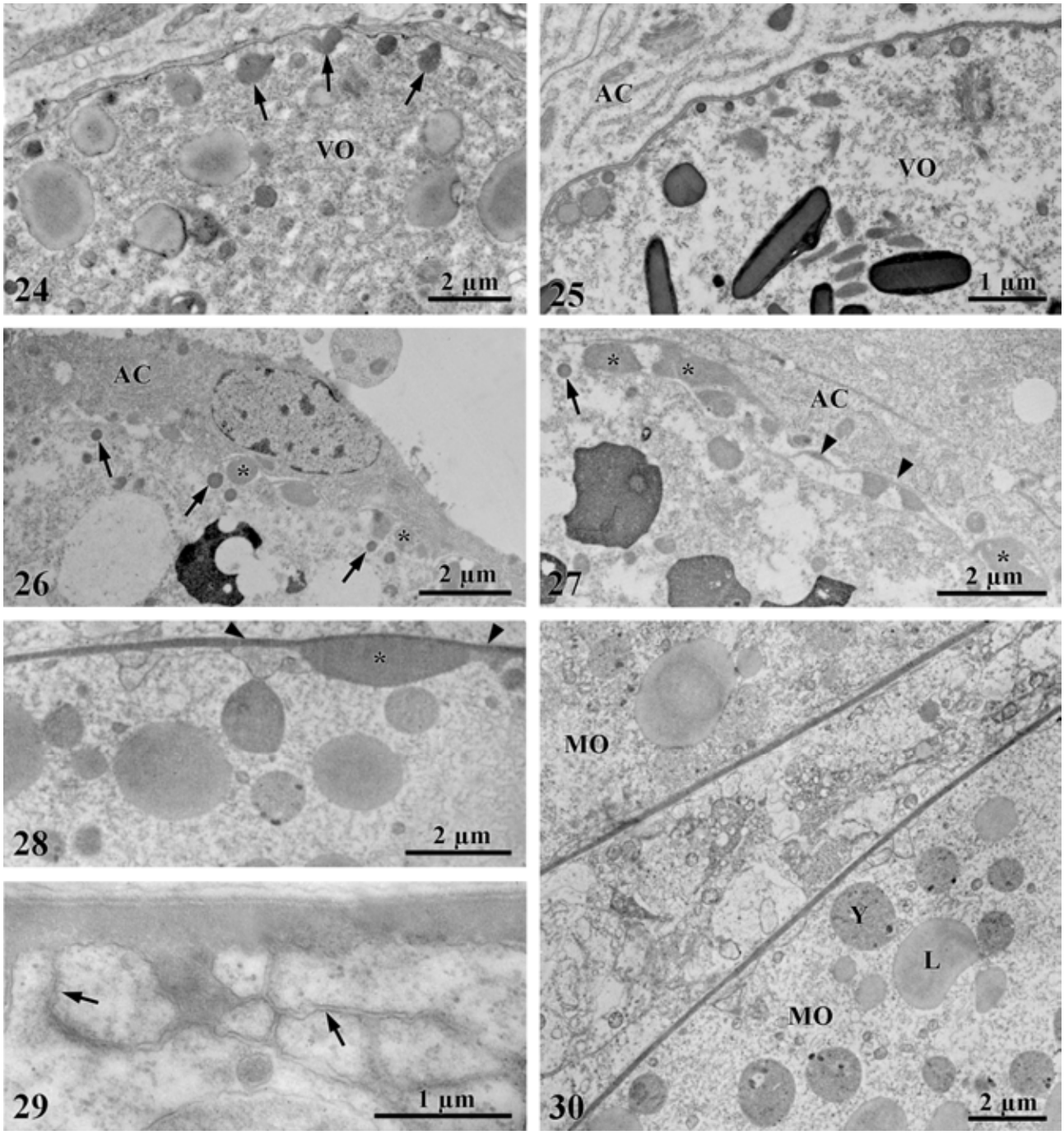
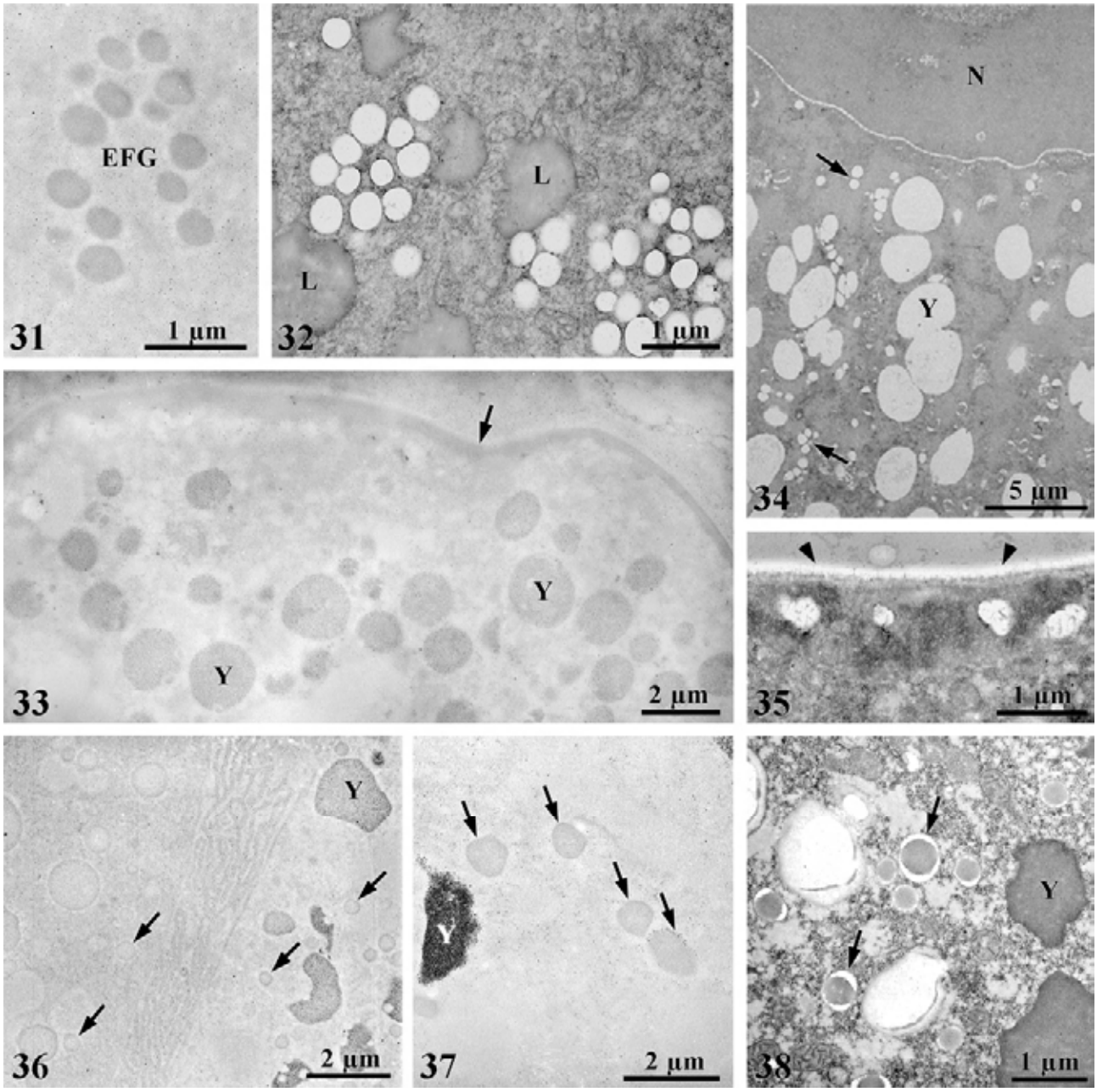


Fig. 24. *S. psammophila*. Eggshell-forming granules under the oolemma (arrows) in the process of releasing their contents to form the secondary egg envelope. VO, late vitellogenic oocyte. **Fig. 25**. *P. rubra*. The same as in Fig. 24. AC, accessory cell. **Figs 26, 27**. *A. beklemischevi*. Eggshell-forming granules (arrows) in the process of releasing their contents in the intercellular space between the primary egg envelope (arrowheads) and the oolemma where the contents coalesce (*) giving rise to the secondary egg envelope. **Figs 28-30**. *S. psammophila.* Secondary egg envelope formation and fully formed eggshell. Fig. 28 shows that the material released from the EFGs (*) is placed against the primary egg envelope which is still visible (arrowheads). Fig. 29 shows a magnification of the egg envelope where arrows indicate vestiges of EFGs membrane and Fig. 30 exhibits portions of two mature eggs (MO) in the process of being laid with a fully formed egg envelope. Note that EFGs are no longer visible inside the eggs. Remnants of accessory cells are visible between the eggs. L, lipid; Y, yolk.

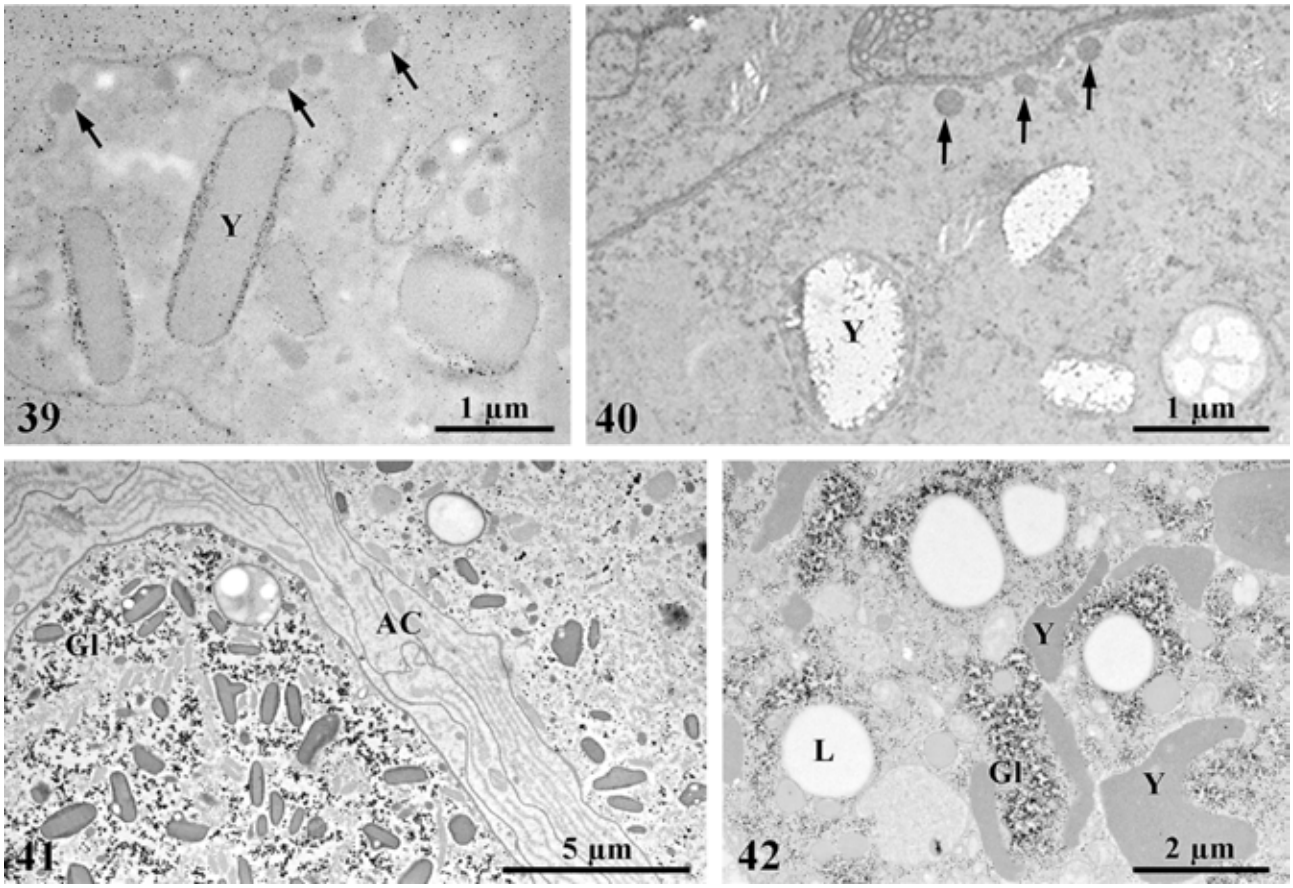
The presence of flattened accessory cells surrounding the oocytes seems to be a common feature in acoels and, apart from their role in the formation of the primary egg envelope which has been clearly demonstrated, these cells could have also a supporting function in providing the oocytes with a cellular envelope and a defensive function in preventing oocytes from being digested by the enzymatic activity of the neighbouring digestive cells [12]. Accessory cells have also been observed to enwrap differentiating oocytes of archoophoran and neoophoran Platyhelminthes [8, 31-35].



Cytochemical tests. Test of Locke and Krishnan to detect polyphenols (Figs 31, 33, 36, 37). Enzymatic protein extraction (Figs 32, 34, 35, 38). **Fig. 31**. *S. psammophila.* No silver precipitate indicating the presence of polyphenols is present on the eggshell-forming granules (EFG). **Fig. 32**. *S. psammophila.* (6 h incubation in pronase). The content of the EFGs is completely digested by the action of protease. L, lipid. **Fig. 33**. *S. psammophila.* No silver precipitate detecting polyphenols is present either on the egg envelope (arrow) or on the yolk globules (Y). **Figs 34, 35**. *S. psammophila* (6 h incubation in pronase). The content of the yolk globules (Y) as well as that of the EFGs (arrows) and the egg envelope (arrowheads) is completely digested. N, nucleus. **Figs 36, 37**. *A. beklemischevi*. No silver precipitate detecting polyphenols is present on the EFGs (arrows) while a positive reaction is visible on the yolk globules (Y). **Fig. 38**. *A. beklemischevi*. After pronase incubation (12 h), the peripheral content of the EFGs (arrows) is digested while the yolk globules (Y) are unaffected.

However, in these flatworms, accessory cells show a low biosynthetic activity not having a well-developed rough endoplasmic reticulum, as is the case instead in acoels, and no contribution of these cells to the formation of the

eggshell has been described to date. Ishida and Teshirogi [36] found a dual origin for the eggshell of the archoophoran polyclads in which part of the egg envelope is synthesized by the shell glands of the female reproductive system and the remaining part of the eggshell is formed by the release of the eggshell-forming granules from the oocyte. According to Ishida [37] the eggshell substance released by the shell glands covers the egg during its passage into the vagina and the eggshell formation is completed only after ovoposition when the content of eggshell-forming granules is discharged between the oolemma and the newly formed shell gland envelope.

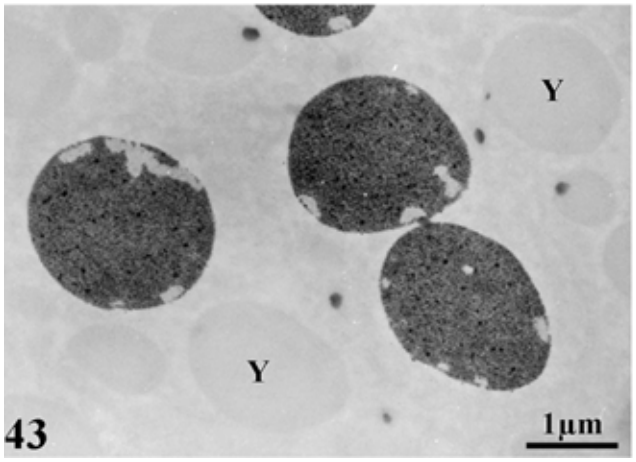


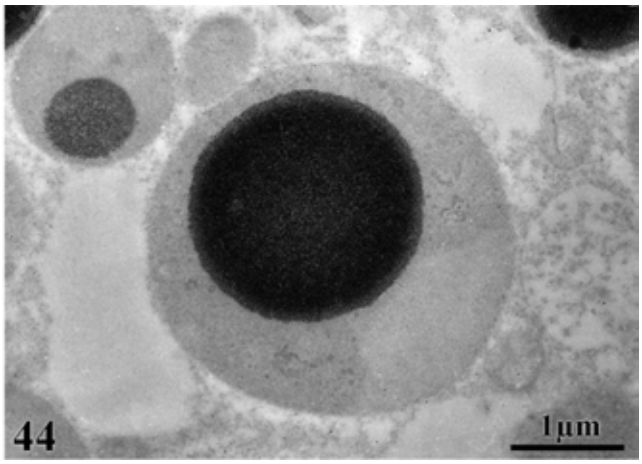
Cytochemical tests. **Fig. 39**. *P. rubra*. Test of Locke and Krishnan to detect polyphenols. No positive reaction is visible on the EFGs (arrows) while a fine silver precipitate is visible on the lamellar cortex of the yolk globules (Y). **Fig. 40**. *P. rubra*. Enzymatic protein extraction (pronase incubation, 6 h). The paracrystalline core of yolk globules (Y) is completely digested while the lamellar cortex is unaffected as well as the EFGs (arrows). **Fig. 41.** *P. rubra*. Thiéry test to detect polysaccharides and glycoproteins. Early vitellogenic oocyte with a few glycogen particles and a late vitellogenic oocyte with a large amount of glycogen (Gl). Note that accessory cells (AC) are devoid of glycogen. **Fig. 42**. *A. beklemischevi*. Thiéry test to detect polysaccharides and glycoproteins. Large aggregates of glycogen (Gl) are visible in the cytoplasm of a late vitellogenic oocyte. L, lipid; Y, yolk.

In acoels type II inclusions are the second to appear in the ooplasm where they rapidly accumulate thus characterizing the vitellogenic phase. Type II inclusions are produced by the cooperation of RER and Golgi complex or mainly by the RER as it is in some acoels [13]. Type II inclusions have a proteinaceous content, remain scattered in the ooplasm even in late vitellogenic oocytes, do not take part in the egg envelope formation, and have been considered yolk globules, i.e reserve material for the embryo. The light positive reaction of the yolk globules to the Locke and Krishnan test for polyphenols in *P. rubra* and *A. becklemiscevi* is due to the fact that the yolk of these two species is composed of glycoproteins presumably rich in tyrosine and basic aminoacids [12, 13]. Lipids and glycogen represent further important sources of nutrients for the developing embryo.

To date the majority of ultrastructural studies show that an autosynthetic mechanism involving the RER and Golgi complex is responsible for yolk production in acoels [10, 12, 13, 22, 27]. This feature is similar to that observed in some Nemertodermatida [23] and archoophoran platyhelminths Macrostomida [15] and Polycladida [14, 35]. However, in the acoel *Childia groenlandica*, belonging to the Childiidae an autoheterosynthetic mechanism of yolk synthesis has been described by Stricker et al. [11]. According to the authors the uptake of exogenous yolk precursors presumably deriving from somatic cells (accessory cells) is demonstrated by the presence of endocytotic activity and endocytotic vacuoles in the cortical ooplasm of vitellogenic oocytes. By contrast, no evidence of internalization of high molecular weight precursors by endocytosis has been observed in the oocytes of *A. becklemiscevi*, which belong to the same family Childiidae as *C. groenlandica* and where the autosynthetic mechanism is the only mode of yolk production observed in

this species during the vitellogenetic phase [13]. Thus, further comparative studies are needed in acoels to ascertain whether different modes of yolk synthesis exist in different species belonging to the same family or in members of different families. Indeed, as is the case in some anellids [38], a correlation between the mode of yolk production and the animal life history pattern (i.e., speed of egg formation, frequency of spawning) cannot be excluded.





Cytochemical test to detect polyphenols. **Fig. 43**. *Macrostomum* sp. Eggshell-forming granules positive to the test for polyphenols as shown by the dense silver precipitate on their content. Y, yolk. **Fig. 44**. *Notoplana alcinoi*. A dense silver precipitate indicating polyphenols is present on the core of the eggshell-forming granules from a mature oocyte.

The use of transmission electron microscopy has allowed us to highlight some differences between Acoela and archoophoran Platyhemlminthes. In particular in acoels: 1) the lack of a tunica surrounding the female gonad, 2) the presence of eggshell precursors not containing polyphenols and 3) a non-sclerotized egg envelope. These features are similar to those found in the Nemertodermatida [8] and differ from those observed in the archoophoran platyhelminths Macrostomida and Polycladida whose ovary is delimited by a tunica [10, 11] and contains entolecithal eggs producing EFGs with polyphenols and whose egg envelope is sclerotized [12]. More generally the absence of polyphenols in the EFGs of the Acoelomorpha is in contrast with the presence of polyphenols in the EFGs of rhabdithophoran Platyhelminthes and lends further strength to the molecular data demonstrating phylogenetic distance between Acoelomorpha and Rhabditophora. In conclusion, transmission electron microscopy continues to be a reliable technique for discovering ultrastructural characteristics of potential value for phylogenetic considerations which, when assembled with molecular data, may help in understanding the evolutionary and phylogenetic relationships of bilaterian animals.

References

[1] Haszprunar, G. Plathelminthes and Plathelminthomorpha-paraphyletic taxa. Journal of Zoological Systematics and Evolutionary Research. 1996; 34:4-48.
[2] Achatz, JG, Chiodin M, Salvenmoser W, Tyler S, Martinez P. The Acoela: on their kind and kinships, especially with nemertodermatids and xenoturbellids (Bilateria incertae sedis). Organisms Diversity & Evolution. 2012; 13:267-286.
[3] Ruiz-Trillo I, Paps J. Acoelomorpha: earliest branching bilaterians or deuterostomes? Organisms Diversity & Evolution. 2016; 16:391–399.
[4] Nielsen C. After all *Xenoturbella* is an acoelomorph! Evolution & Development. 2010; 12:241-243.
[5] Philippe H, Copley RR, Moroz, LL, Nakano H, Poustka A J, Wallberg A, Peterson KJ, Telford MJ. Acoelomorph flatworms are deuterostomes related to *Xenoturbella*. Nature. 2011; 470:255-258.
[6] Haszprunar G. Review of data for a morphological look on Xenacoelomorpha (Bilateria incertae sedis). Organisms Diversity & Evolution. 2015; 16:363-389.
[7] Cannon JT, Vellutini BC, Smith J, Ronquist F, Jondelius U, Hejnol A. Xenacoelomorpha is the sister group to Nephrozoa. Nature. 2016; 530:89-93.
[8] Rieger RM, Tyler S, Smith JPS, Rieger GE. Platyhelminthes: Turbellaria. In: Harrison FW, Bogitsh BJ, editors. Microscopic Anatomy of Invertebrates, vol. 3: Platyhelminthes and Nemertinea. New York: Wiley-Liss; 1991. p. 1-140.
[9] Falleni A, Gremigni V. Ultrastructural study of oogenesis in the acoel turbellarian *Convoluta.* Tissue and Cell. 1990; 22:301-310.
[10] Chandler RM, Thomas MB, Smith JPS III. The role of shell granules and accessory cells in eggshell formation in *Convoluta pulchra* (Turbellaria, Acoela). The Biological Bulletin. 1992; 182:54-65.
[11] Stricker SA, Welford AM, Morris CA. Somatic cell-oocyte interactions during oogenesis in the acoel flatworm *Childia groenlandica*. Invertebrate Reproduction and Development. 1992; 21:57-77.
[12] Falleni A, Raikova O, Gremigni V. Ultrastructural and cytochemical features of the ovary in *Paratomella rubra* (Platyhelminthes, Acoela). Journal of Submicroscopic Cytology and Pathology. 1995; 27:511-523.

[13] Raikova O, Falleni A, Gremigni V. Oogenesis in *Actinoposthia beklemishevi* (Platyhelminthes, Acoela): an ultrastructural and cytochemical study. Tissue and Cell. 1995; 27:621-663.
[14] Boyer BC. Ultrastructural studies of differentiation in the oocyte of the polyclad turbellarian *Prostheceraeus floridanus*. Journal of Morphology. 1972; 136:273-295.
[15] Gremigni V, Falleni A, Lucchesi P. An ultrastructural study of oogenesis in the turbellarian *Macrostomum*. Acta Embryologiae et Morphologiae Experimentalis. 1987; 8: 257-262.
[16] Domenici L, Galleni L, Gremigni V. Electron microscopical and cytochemical study of egg-shell globules in *Notoplana alcinoi* (Turbellaria Polycladida). Journal of Submicroscopic Cytology and Pathology. 1975; 7:239-247.
[17] Liana MK, Litvaitis MK. Anatomy and ultrastructure of the female reproductive system of *Pleioplana atomata* (Platyhelminthes: Polycladida). Journal of Morphology. 2009; 270:337-343.
[18] Gammoudi M, Ahmed RB, Ahmed M, Sayed SR, Alwasel SH, Tekaya S, Harrath AH. Ultrastructural study of oogenesis in the acotylean *Echinoplana celerrima*, (Platyhelminthes Polycladida). Zoologicher Anzeiger. 2016; 260:72-77.
[19] Gammoudi M, Salvenmoser W, Tekaya S, Egger B. Ultrastructure of the ovary and oogenesis in the flatworm *Prosthiostomum siphunculus* (Polycladida, Cotylea). Cell Biology International 2016; 9999:1-13.
[20] Adiyodi KG, Adiyodi RG, editors. Reproductive Biology of Invertebrates. Vol. 1. Oogenesis, Oviposition, and Oosorption. John Wiley & Sons, 1983.
[21] Elbarhoumi M, Scaps P, Djediat C, Zghal F. Ultrastructural study of oogenesis in *Marphysa sanguinea* (Annelida: Polychaeta: Eunicida) from the Lagoon of Tunis. Scientia Marina. 2014; 78:99-113.
[22] Zabotin YaI, Golubev AI. Ultrastructure of oocytes and female copulatory organs of Acoela. Biology Bulletin. 2014; 41:722-735.
[23] Smith JPS III, Thomas MB, Chandler R, Zane SF. Granular inclusions in the oocytes of *Convoluta* sp., *Nemertoderma* sp. and *Nemertinoides elongatus* (Turbellaria, Acoelomorpha). Fortschritte der Zoologie. 1988; 36:263-269.
[24] Falleni A, Gremigni V. Egg covering formation in the acoel *Convoluta psammophila* (Platyhelminthes, Turbellaria): an ultrastructural and cytochemical investigation. Acta Embryologiae et Morphologiae Experimentalis n.s. 1989; 10:105-112.
[25] Locke M, Krishnan N. The distribution of polyphenoloxidases and polyphenols during cuticle formation. Tissue and Cell. 1971; 3:103-126.
[26] Thiéry JP. Mise en évidence des polysaccharides sur coupes fines en microscopie électronique. Journal of Microscopy. 1967; 6:987-1018.
[27] Gremigni V, Falleni A. Mechanisms of shell-granule and yolk production in oocytes and vitellocytes of Platyhelminthes Turbellaria. Animal Biology. 1992; 1:29-37.
[28] Gremigni V, Falleni A. Ultrastructural features of cocoon-shell globules in the vitelline cells of neoophoran platyhelmenths. Hydrobiologia. 1991; 227:105-111.
[29] Gremigni V, Falleni A. Characters of the female gonad and the phylogeny of Platyhelminthes. Hydrobiologia. 1998; 383: 235-242.
[30] Falleni A, Lucchesi P, Ghezzani C. Comparative analysis of female gonad characters in neoophoran Platyhelminthes: an ultrastructural and cytochemical overview. In: Méndez-Vilas A, editor. Current Microscopy Contributions to Advances in Science and Technology, vol 1. 2012; p. 711-722.
[31] Falleni A, Lucchesi P. Ultrastructural and cytochemical aspects of oogenesis in *Castrada viridis* (Platyhelminthes, Rhadbocoela). Journal of Morphology. 1992; 213:241-250.
[32] Falleni A, Lucchesi P, Ghezzani C, McDonalds JC, Jones HD. The female gonad in two species of *Microplana* (Platyhelminthes, Tricladida, Rhynchodemidae): ultrastructural and cytochemical investigations. Journal of Morphology. 2009; 270:1042-1054.
[33] Falleni A, Lucchesi P, Ghezzani C, Brogger M I. Ultrastructural and cytochemical aspects of the germanium and the vitellarium in *Syndesmis patagonica* (Platyhelminthes, Rhabdocoela, Umagillidae). Journal of Morphology. 2014; 275:703-719.
[34] Charni M, Ammar AB, Jaafoura MH, Zghal F, Tekaya S. Ultrastructure of germaria and vitellaria in *Dugesia sicula* Lepori, 1948 (Platyhelminthes, Tricladida, Paludicola). Belgian Journal of Zoology. 2010; 140:111-118.
[35] Liana MK, Litvaitis MK. Anatomy and ultrastructure of the female reproductive system of *Pleioplana atomata* (Platyhelminthes: Polycladida). Journal of Morphology. 2009; 270: 337-343.
[36] Ishida S, Teshirogi W. Eggshell formation in polyclads (Turbellaria). Hydrobiologia. 1986; 132:127-135.
[37] Ishida, ST. Further studies on the shell-forming granules and eggshell formation in polyclads (Turbellaria, Platyhelminthes). Scientific Reports Hirosaki University. 1989; 36: 55-72.
[38] Eckelbarger KJ. Oogenesis and oocytes. Hydrobiologia. 2005; 535/536:179-198.