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Ultrastructural and Cytochemical Aspects of the Germarium and the Vitellarium in *Syndesmis patagonica* (Platyhelminthes, Rhabdocoela, Umagillidae)

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ABSTRACT The cytoarchitecture of the female gonad of the endosymbiont umagillid *Syndesmis patagonica* has been investigated using electron microscopy and cytochemical techniques. The female gonad consists of paired germaria and vitellaria located behind the pharynx in the mid-posterior region of the body. Both the germaria and the vitellaria are enveloped by an outer extracellular lamina and an inner sheath of accessory cells which contribute to the extracellular lamina. Oocyte maturation occurs completely during the prophase of the first meiotic division. Oocyte differentiation is characterized by the appearance of chromatoid bodies and the development of endoplasmic reticulum and Golgi complexes. These organelles appear to be involved in the production of round granules, about 2–2.5 μm in diameter, with a homogeneous electron-dense core surrounded by a granular component and a translucent halo delimited by a membrane. These egg granules migrate to the periphery of mature oocytes, are positive to the cytochemical test for polyphenol detection, are unaffected by protease and have been interpreted as eggshell granules. The mature oocytes also contain a small number of yolk granules, lipid droplets, and glycogen particles scattered throughout the ooplasm. The vitellaria are branched organs composed of vitelline follicles with vitellocytes at different stages of maturation. Developing vitellocytes contain well-developed rough endoplasmic reticulum and small Golgi complexes involved in the production of eggshell and yolk globules. Eggshell globules are round, measure 4–5 μm in diameter, and have a mosaic-like patterned content which contains polyphenols. The yolk globules, 2–3 μm in diameter, show a homogeneous protein content of medium electron density, devoid of polyphenols, and completely digested by protease. The mature vitellocytes also contain glycogen as further reserve material. The presence of polyphenolic eggshell granules in the oocytes and of polyphenolic eggshell globules with a mosaic-like pattern in the vitellocytes have been considered apomorphic features of the Rhabdocoela + Prolecithophora. J. Morphol. 275:703–719, 2014.

KEY WORDS: female gonad; oocyte; vitellocyte; accessory cell; Rhabdocoela

INTRODUCTION

Rhabdocoela is a large group of mainly free-living rhabdithophoran platyhelminths that have colonized marine, brackish water, limnic, and limnoterrestrial environments. These non-neodermatan flatworms have been subdivided in recent molecular studies into two large clades: Kalyptorhynchia and Dalytyphloplanida, the latter encompassing a group originating in marine environment and producing a major radiation into freshwater habitats (Willems et al., 2006; Van Steenkiste et al., 2013). Among these rhabdocoels are groups that have adopted a symbiotic life style, including the Umagillidae.

Because of the morphology of their female gonad, Rhabdocoela belong to the neoophoran level of organization (Karling, 1940; Ehlers, 1985), that is, they possess a heterocellular female gonad consisting of germaria (ovaries) with basically alecithal eggs, and vitellaria (vitelline glands) with specialized cells producing polyphenolic eggshell precursors and nutritive substances for the developing embryo. Vitellocytes are released from the vitelline follicles, pass through the vitelloduct, and are enclosed in the cocoon together with one or more fertilized eggs.

In the last three decades, ultrastructural investigations on the female gonad of Platyhelminthes have provided information on the reproductive biology and phylogeny of the group (Gremigni, 1988; Gremigni and Falleni, 1991; Sopott-Ehlers, 1997; Gremigni and Falleni, 1998; Falleni et al., 2012).

Although several studies have investigated the ultrastructure of the female gonad of free-living Rhabdocoela (Bunke, 1981; Falleni and Lucchesi, 1992; Lucchesi et al., 1995; Sopott-Ehlers, 1997;

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Falleni et al., 2005), little is known about the symbiotic and parasitic rhabdocoels. To our knowledge, the only extensive ultrastructural investigations available concern the female gonad of some temnocephalids belonging to Temnocephalidae (Falleni et al., 1998), and Scutariellidae (Falleni et al., 2002) and the eggshell formation of the umagillid *Syndisyrinx franciscanus* described by Shinn 1993.

We studied the ultrastructure and cytochemistry of the female gonad of the umagillid *Syndesmis patagonica*, an endosymbiont of the sea urchin *Arbacia dufresnii*. The aim was to obtain information on the genesis, structure and composition of oocyte and vitellocyte inclusions in order to compare them with those from other neoophoran platyhelminths, in particular with those of other members of the Rhabdocoela.

MATERIALS AND METHODS

Sampling

Sexually mature individuals of *Syndesmis patagonica* Brogger and Ivanov, 2010 ($n = 13$) were collected from the gut of seven specimens of the echinoid *Arbacia dufresnii*, out of a total number of 84 sea urchins examined, captured at a depth of 4–6 m in Puerto Madryn (42°46′S; 65°02′W, salinity 33.7–33.8‰), Argentina, in November 2010. After dissection of the echinoids, the coelomic fluid and the intestine were examined with a stereo microscope to find worms.

Transmission Electron Microscopy (TEM)

A total of eight worms were processed for TEM, and the remaining five worms were treated according to the cytochemical method to detect polyphenols. For TEM, specimens were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) containing 2% $CaCl_2$ and 8% sucrose at 4°C, postfixed in 1% osmium tetroxide in the same buffer for 2 h at room temperature, dehydrated in graduated series of ethanols, embedded in Epon-Araldite and polymerized at 60°C for 72 h.

For female gonad identification, 1–2 μm thick serial sections obtained with a Porter Blum MT-1 (USA) were stained with 1% toluidine blue and 1% methylene blue in 1% sodium tetraborate and observed with a Zeiss Axioplan light microscope (Germany). Ultrathin sections (50–80 nm thick), obtained with a Reichert-Jung Ultracut E (Austria) equipped with a diamond knife, were collected on 200-mesh formvar/carbon coated copper grids, double stained with aqueous uranyl acetate and lead citrate and examined with a Jeol 100 SX TEM operating at 80 kV. Composite micrograph plates were assembled using Adobe Photoshop CS6 (Adobe Systems, San Jose, CA).

Cytochemical Tests

Enzymatic protein extraction. Ultrathin sections obtained from blocks used for ultrastructural investigations were collected with mylar rings (3 mm in diameter) and treated with 2% H_2O_2 at 37°C for 15 min, rinsed in distilled water and then incubated for 6–12 h at 37°C in 0.5% protease (pronase E, Sigma) solution adjusted to pH 7.5. After rinsing in distilled water, the ultrathin sections were collected on 200-mesh formvar/carbon coated copper grids and counterstained with uranyl acetate and lead citrate.

Control sections were kept in distilled water without protease under the same conditions for the same time intervals.

Test for polysaccharides and glycoproteins. The Thiéry (1967) method was used on ultrathin sections obtained from the same blocks used for ultrastructural studies. They were treated with 1% periodic acid for 30 min, incubated with 0.2% thiocarbohydrazide (TCH) in 20% acetic acid for 8–72 h, and treated with 1% silver proteinate in the dark for 30 min at room temperature. They were observed without further staining.

Control sections were incubated in distilled water without periodic acid and processed as described above.

Test for polyphenolic substances. Whole specimens ($n = 5$) were fixed in 3% glutaraldehyde buffered with 0.1 M phosphate buffer and then treated according to the Locke and Krishnan 1971 methenamine silver method. In brief, the worms were treated with a 5% aqueous solution of sodium metabisulphite for 15 min, then incubated in freshly prepared methenamine silver reagent in the dark for 30 min at 60°C and finally treated with 5% sodium thiosulphate for 5 min at room temperature. The specimens were then dehydrated in ethanols and embedded in Epon-Araldite. Ultrathin sections cut from these blocks were observed without any other staining.

Control specimens, after treatment with sodium metabisulphite, were incubated at 60°C in distilled water without methenamine silver reagent and then processed as described above.

RESULTS

Gross Morphology

The female gonad of *Syndesmis patagonica* consists of paired germaria and vitellaria distributed posterior to the pharynx and testes in the mid-posterior region of the body. The germaria are lobed (from three to five lobes), about 100 × 250 μm in diameter, located posterior to the vitellaria. The vitelline glands, about 300 × 600 μm in diameter, are branched, with 3–6 primary branches, each divided once or twice, located posterior to the testis (see figure in Brogger and Ivanov, 2010, p. 63).

Germarium Ultrastructural Morphology

The germaria lie adjacent to the vitellaria but are well separated from them (Fig. 1A). They contain tightly packed oocytes at different stages of maturation (Fig. 1A,B). Each germarium is enveloped by an extracellular lamina, about 200–250 nm thick, composed of a finely granular material of medium electron density (Fig. 1B,C,D) and a monolayer of accessory cells (Fig. 1D) which remain confined to the periphery of the gonad (Fig. 2A,B). Accessory cells show a nucleus with mainly diffuse chromatin (Fig. 2B). The cytoplasm contains free ribosomes, mitochondria, cisternae of the endoplasmic reticulum, some Golgi complexes (Fig. 2C), and numerous elongated vesicles containing a material of the same medium electron density as the extracellular lamina (Fig. 2D,E). Some of these vesicles are visible in the process of fusing with the plasma membrane to discharge their content (Fig. 2D,E and inset). Occasionally large secondary lysosome-like bodies are visible in the accessory cell cytoplasm (Fig. 2A). Neither intercellular bridges nor specialized junctional complexes have been observed between oocytes and accessory cells or between adjacent accessory cells.

The early and young oocytes at the onset of differentiation are polygonal cells localized at the periphery of the gonadic lobe, just under the

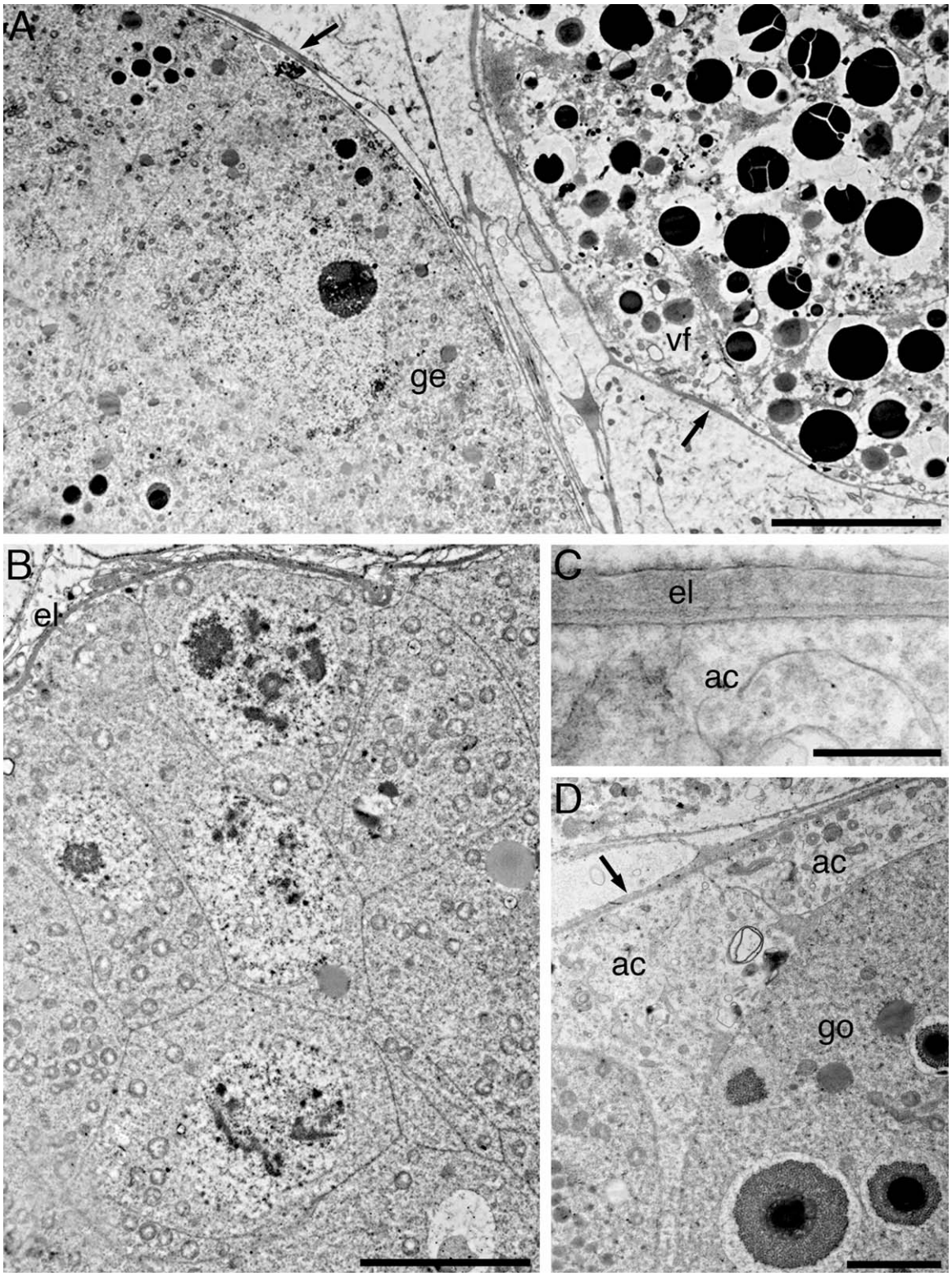

Fig. 1. *Syndesmis patagonica*. **A**: Portion of the germarium (ge) and an adjacent vitelline follicle (vf) both enveloped by an extracellular lamina (arrow). Scale bar, 10 μm. **B**: Germinative area of the germarium showing early and young oocytes. el, extracellular lamina. Scale bar, 5 μm. **C**: Higher magnification of the germarium extracellular lamina (el) showing a fibrillar content of medium electron density. ac, accessory cell cytoplasm. Scale bar, 0.5 μm. **D**: Outer portion of the germarium growth area showing the extracellular lamina (arrow) and two underlying accessory cells (ac) with scattered mitochondria. go, growing oocyte. Scale bar, 2 μm.

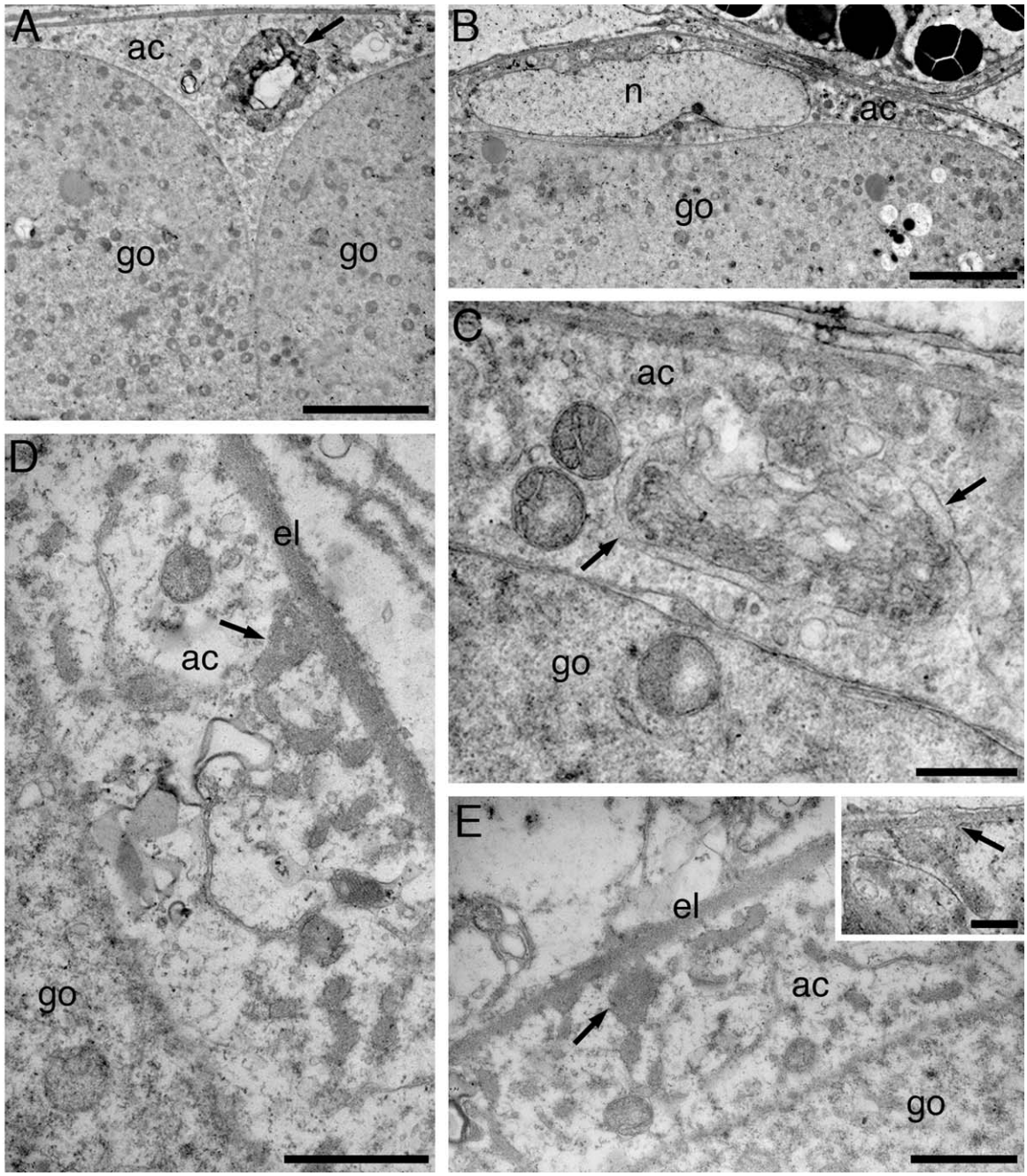

Fig. 2. *Syndesmis patagonica*. Details of the accessory cells of the germarium. **A**: Accessory cell cytoplasm (ac) surrounding two growing oocytes (go) and containing a large secondary lysosome-like body (arrow). Note that the cytoplasm of the accessory cell remains mainly confined to the periphery and penetrates only partially between oocytes. Scale bar, 5 μm. **B**: Accessory cell (ac) enveloping a growing oocyte (go) with a nucleus (n) showing mainly diffuse chromatin. Scale bar, 5 μm. **C**: Accessory cell cytoplasm (ac) displaying well-developed RER and Golgi complexes (arrow). go, growing oocyte. Scale bar, 0.5 μm. **D** and **E**: Elongated vesicles containing a material of the same electron density of the extracellular lamina (el) are visible close to the plasma membrane (arrow) and in the process of fusing with it (inset). ac, accessory cell; go, growing oocyte. Scale bars, 1 μm; inset, 0.25 μm.

extracellular lamina (Figs. 1B and 3A). They measure about 7 × 10 μm in diameter and have a large nucleus (about 5–7 μm in diameter) with small patches of condensed chromatin and a prominent, excentrally located nucleolus with intermingled or segregated fibrillar and granular components (Fig. 3A,C). Synaptonemal complexes, closely apposing homologous chromosomes and indicating the zygotene–pachytene stage of the first meiotic division, are visible in the nucleoplasm (Fig. 3B). The scarcely differentiated cytoplasm is packed with free and clustered ribosomes, contains numerous mitochondria, some ER profiles and lipids (Fig. 3C). Small, irregular, membrane-unbound aggregates of

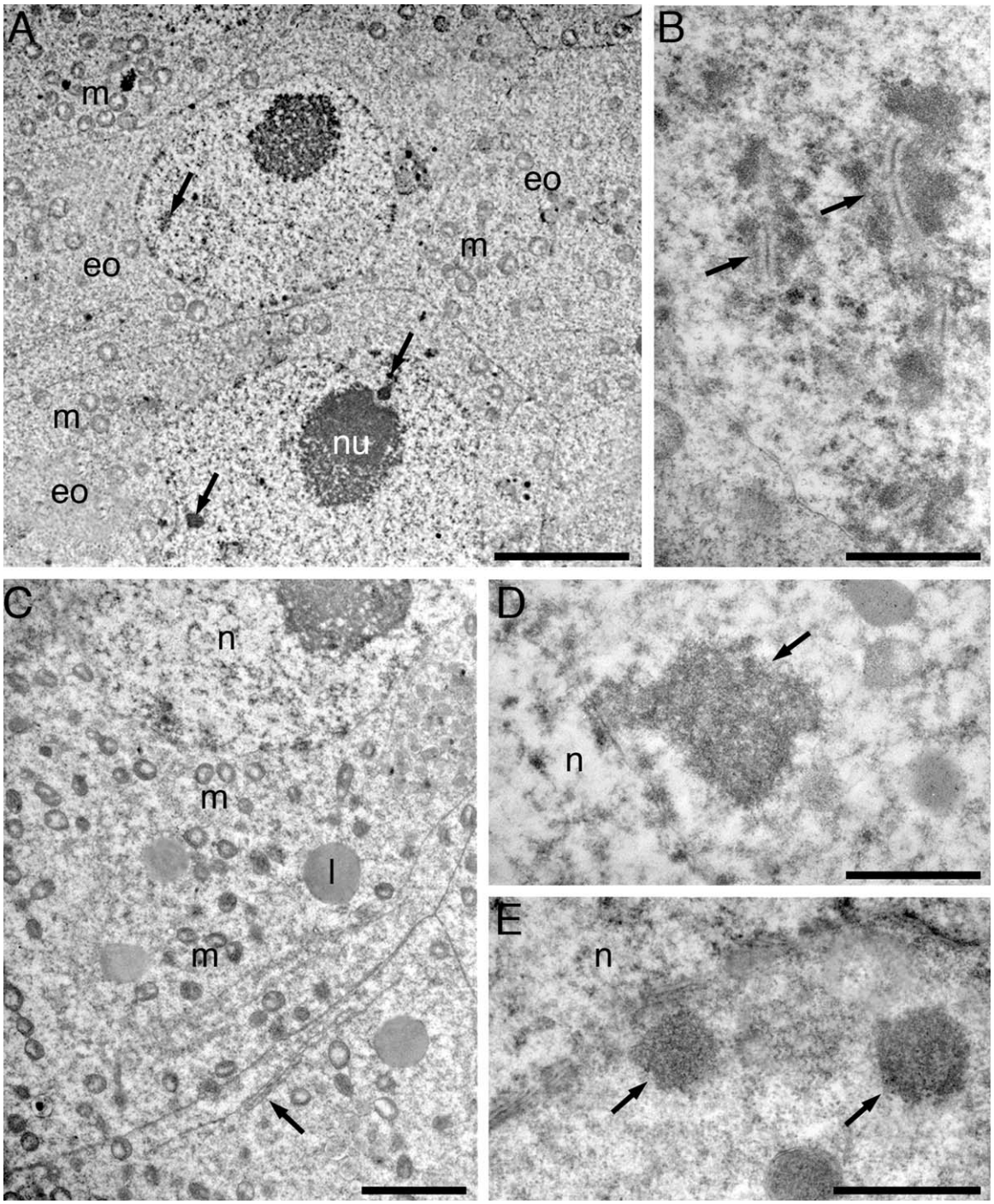

Fig. 3. *Syndesmis patagonica*. Ultrastructural features of early and young oocytes. **A**: Early oocytes (eo) showing a large nucleus with small patches of condensed chromatin (arrows) and a prominent nucleolus (nu). In the cytoplasm numerous free ribosomes and some mitochondria (m) are visible. Scale bar, 4 μm. **B**: Synaptonemal complexes with the typical tripartite structure (arrow) in the nucleus of a young oocyte. Scale bar, 1 μm. **C**: Young oocytes at the beginning of differentiation showing numerous mitochondria (m), some RER cisternae (arrow) and lipid droplets (l). n, nucleus. Scale bar, 2 μm. **D** and **E**: CBs (arrow) consisting of electron-dense granular/fibrillar material devoid of a limiting membrane in the perinuclear cytoplam. n, nucleus. Scale bars, 1 μm.

finely granular or fibrillar electron-dense material [chromatoid bodies (CB)], are observed in the perinuclear ooplasm of young germ cells (Fig. 3D,E) and in the deep cytoplasm of later oocytes (Fig. 4E).

The cytoplasmic differentiation of growing oocytes is characterized by an increase in the number of ER profiles, lipid droplets and the appearance of Golgi complexes and inclusions containing an electron-dense material (Fig. 4A). Golgi

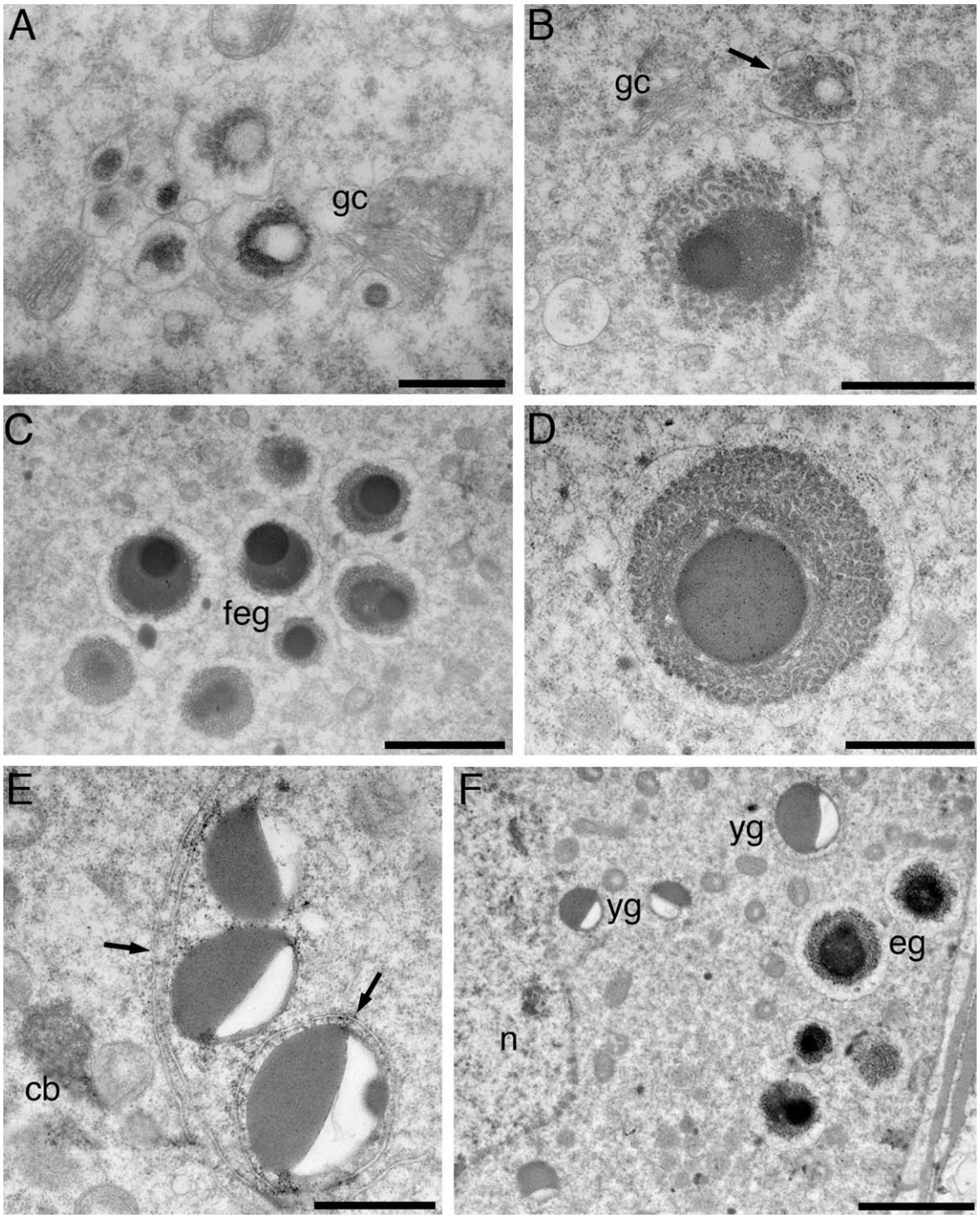

Fig. 4. *Syndesmis patagonica*. Ultrastructural features of growing and nearly mature oocytes. **A**: A Golgi complex (gc) and small vesicles containing an electron-dense granular material from a growing oocyte. Scale bar, 0.5 μm. **B**: A Golgi complex (gc) and a Golgi-derived vesicle (arrow) in the process of fusing with a nascent eggshell granule. Scale bar, 1 μm. **C**: Cluster of forming eggshell granules (feg) in a mid-stage oocyte. Scale bar, 2 μm. **D**: A mature eggshell granule with a complex substructure consiting of a homogeneous electron-dense core surrounded by a less dense granular/tubular component and a translucent halo delimited by a membrane. Scale bar, 1 μm. **E**: Forming yolk granules close to ER cisternae (arrow) in a growing oocyte. cb, chromatoid body. Scale bar, 1 μm. **F**: A nearly mature oocyte with eggshell granules (eg) in the cortical ooplasm and scattered yolk granules (yg). n, nucleus. Scale bar, 2 μm.

complexes appear to be involved in the production of vesicles containing a granular electron-dense material (Fig. 4A,B) which seems organized to form hollow convoluted tubules (Fig. 4B). The repeated coalescence of the content of these vesicles gives rise to larger inclusions, nascent

eggshell granules, showing a dense core, presumably deriving from a condensation of the hollow tubules. As a final result of this fusion and condensation process occurring in the Golgi areas large membrane-bound eggshell granules are formed (Fig. 4C). When completely mature they have a round shape, measure about 2–2.5 μm in diameter and show a complex substructure consisting of a central/subcentral electron-dense homogeneous core surrounded by a less dense granular/tubular component and a translucent halo delimited by a membrane (Fig. 4D). These eggshell granules are clustered and distributed throughout the ooplasm of growing oocytes (Fig. 4C) and migrate into the peripheral ooplasm of later oocytes (Fig. 4F). Long cisternae of the endoplasmic reticulum are often seen in close proximity with a second type of forming inclusions, the yolk granules (Fig. 4E). When mature, yolk granules are roundish in shape, measure about 1.4–1.6 μm in maximum diameter and show a content consisting of a homogeneous component of medium electron density and a translucent component. They are produced in a small quantity and remain scattered throughout the cytoplasm of late-stage and mature oocytes (Figs. 4F and 5B). Lipid droplets continue to be produced also in late stage oocytes. They show a content of low electron density, range in size (1–2 μm in diameter), and occur singly or in groups (Fig. 5A).

The mature oocytes have an oval shape and measure 45–50 μm in maximum diameter (Fig. 5B). The nucleus contains completely diffuse chromatin and the nucleolus is no longer visible. The electron-dense eggshell granules are located in the cortical ooplasm to form a discontinuous layer (Fig. 5B) under the plasma membrane while yolk granules remain scattered throughout the ooplasm.

Cytochemical Tests

The electron-dense eggshell granules, either scattered or peripherally located in the ooplasm react positively to the cytochemical test to detect polyphenols (Fig. 6A and inset). The fine silver precipitate is particularly condensed on the homogeneous core and loosely distributed on the surrounding granular component of the inclusions. No silver precipitate is observed on other oocyte inclusions or structures.

The cortex of the electron-dense eggshell granules is only partially extracted by the protease, whereas the homogeneous dense core appears inert to the enzymatic activity (Fig. 6B). The content of the yolk granules is negative to the cytochemical test to detect polyphenols, is not extracted by protease (Fig. 6C) and shows a fine silver precipitate, evidencing glycoproteins, which is better visible after 72 h incubation in TCH (Fig. 6D). A positive reaction to the Thiéry test, detecting the presence of widespread glycogen particles, is observed in the ooplasm and in the accessory cell cytoplasm (Fig. 6E) after 8 h incubation in TCH.

Vitellarium Ultrastructural Morphology

The vitellaria are branched organs showing a follicular organization (Fig. 7A). Each follicle is globose in shape, measures about 60 × 130 μm in diameter and contains vitellocytes at various stages of differentiation distributed along a maturation axis. Early

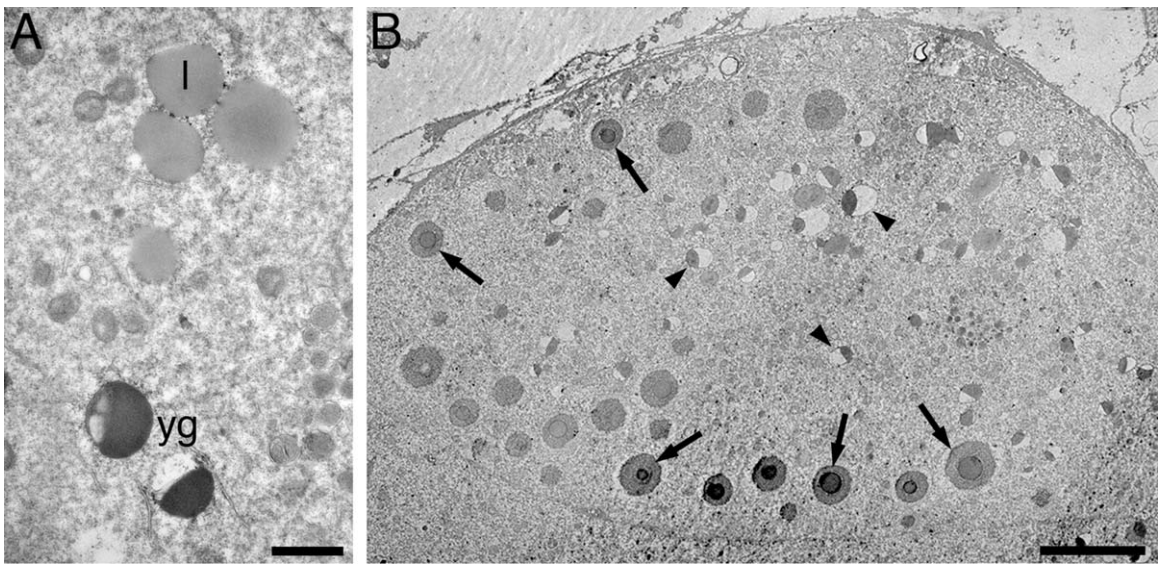

Fig. 5. *Syndesmis patagonica*. Ultrastructural features of nearly mature and mature oocytes. **A**: Nearly mature oocyte showing clustered lipids (l) and yolk granules (yg). Scale bar, 1 μm. **B**: A mature oocyte exhibiting eggshell granules located in the peripheral ooplasm (arrows) and scattered yolk granules (arrowheads). Scale bar, 5 μm.

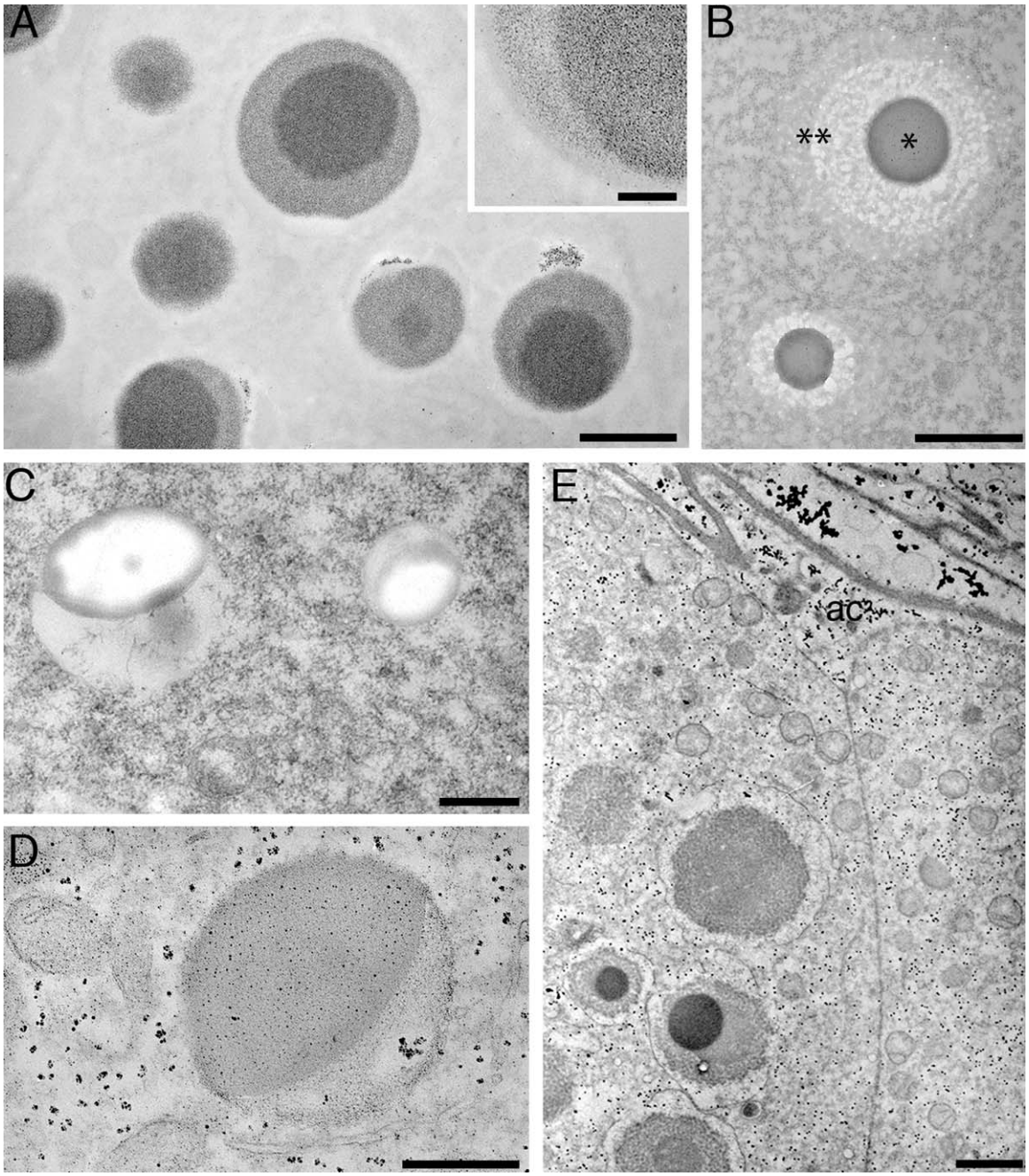

Fig. 6. *Syndesmis patagonica*. Oocyte aspects after cytochemical tests. **A**: Test for polyphenols. Unstained section. Peripheral eggshell granules showing the silver precipitate mainly concentrated on the central/subcentral homogeneous component. In the inset enlargement of a granule portion showing the high density of the silver precipitate. Scale bars, 1 μm; inset 0.25 μm. **B**: Protease extraction. The cortex (**) of the eggshell granules are only partially digested, while the homogeneous dense core (*) is completely unaffected. Scale bar, 1 μm. **C**: Protease extraction. The content of the yolk granule is not digested by pronase. Scale bar, 0.5μm. **D**: Thiéry test, 72 h incubation in TCH, unstained section. A fine silver precipitate detecting glycoproteins is present on the dense and less dense component of the yolk granule. Scale bar, 0.5μm. **E**: Thiéry test, 8 h incubation in TCH, unstained section. A silver precipitate is visible on the scattered glycogen particles in the oocytes and in the accessory cell (ac). Scale bar, 1 μm.

and young vitellocytes are located at the distal end of the follicle just under the extracellular lamina (Fig. 7B) whereas developing and mature vitellocytes occupy the central follicle area and its opposite end near the vitelloduct origin.

Each follicle is enveloped by a tunica consisting of an extracellular lamina, about 260–400 nm thick, which appears of medium electron density and finely granular in composition, and several accessory cells distributed peripherally under the

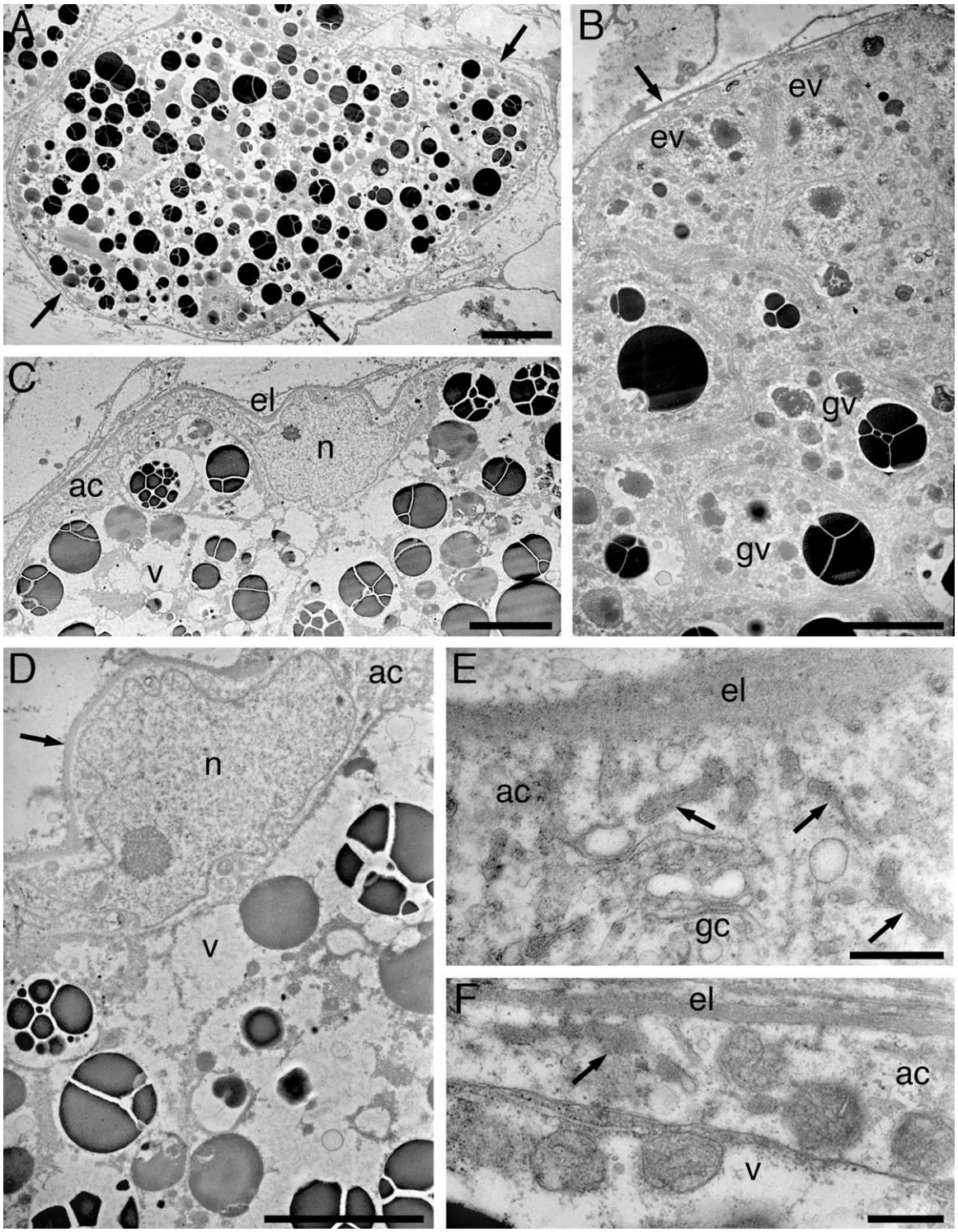

Fig. 7. *Syndesmis patagonica*. Ultrastructural features of vitelline follicles and accessory cells. **A**: Low magnification of a vitelline follicle with mature vitellocytes delimited by the extracellular lamina (arrow). Scale bar, 10 μm. **B**: Portion of a vitelline follicle showing early vitellocytes (ev) localized under the extracellular lamina (arrow) and growing vitellocyte (gv) with forming eggshell and yolk globules. Scale bar, 5 μm. **C**: Outer portion of a vitelline follicle surrounded by a peripheral oblong accessory cell (ac). el, extracellular lamina; n, accessory cell nucleus; v, mature vitellocyte. Scale bar, 5 μm. **D**: The irregularly shaped nucleus (n) of an accessory cell (ac) exhibiting diffuse chromatin and a small nucleolus is visible under the extracellular lamina (arrow). v, mature vitellocyte. Scale bar, 5 μm. **E** and **F**: Outer portion of the vitellarium showing an accessory cell cytoplasm (ac) with a Golgi complex (gc) and elongated vesicles (arrow), with the same electron density of the extracellar lamina (el), in the process of fusing with the plasma membrane. v, vitellocyte. Scale bars, 0.5 μm.

extracellular lamina (Fig. 7C,D). The irregularly shaped nucleus of the accessory cells shows mainly diffuse chromatin and a small nucleolus with intermingled fibrillar and granular component (Fig. 7D). The cytoplasm contains mitochondria with a compact matrix and a few cristae, some RER cisternae and Golgi complexes (Fig. 7E). Occasionally some elongated vesicles with a content of the same electron-density of the extracellular lamina are observed close to or fusing with the plasma membrane (Fig. 7E,F).

Early and young vitellocytes are tightly packed cells measuring about 5 × 9 μm in diameter located in the germinative area of the follicle. The nucleus, about 4 μm in diameter, displays large, irregular clumps of heterochromatin, and a prominent nucleolus (Fig. 8A). The cytoplasm contains a large number of free ribosomes, scattered mitochondria, and short cisternae of RER. The cytoplasmic differentiation is characterized by the appearance of Golgi complexes and an increase in the number and length of the RER profiles (Fig. 8B,C). The Golgi complexes consist of short cisternae with enlarged ends filled with an electron-dense material (Fig. 8C,D). The repeated fusion of Golgi-derived vesicles gives rise to large inclusions (eggshell forming globules) which progressively increase in size. At an intermediate stage of maturation, these membrane-bound inclusions are composed of 8–10 electron-dense polygonal granules of different size embedded in an electron-lucent material (Fig. 8E). When completely mature they are round in shape, measure about 4–5 μm in diameter, and show an electron-dense content consisting of 3–6 large granules forming the so-called mosaic-like pattern (Fig. 8F). RER cisternae and Golgi complexes are also observed in close proximity to nascent yolk globules having a medium electron-dense granular content delimited by a smooth membrane (Fig. 9A,B,C). The mature yolk globules have a roundish/oval shape, measure about 3 μm in maximum diameter and show a homogeneous content of medium electron density (Fig. 8F). Mature vitellocytes, located near the vitelloduct origin, show an increased volume due to the accumulation of different types of inclusions (Figs. 8F and 10D).

Cytochemical Tests

A positive reaction to the test to detect polyphenols is observed on the electron-dense eggshell globules with a mosaic content pattern (Fig. 10A). The reaction tests negative on every other inclusion or structure in the vitellocytes and in the accessory cells. The electron-dense polyphenolic component of the eggshell globules is unaffected by pronase while the yolk globule content is completely digested (Fig. 10B). A positive reaction to the Thiéry test is detected on the glycogen deposits in the vitellocyte cytoplasm after 8 h of incubation in TCH (Fig. 10C,D). Glycogen is absent or scarce in early vitellocytes (Fig. 10C) while it becomes abundant in mature vitellocytes (Fig. 10D). A small amount of glycogen is observed in the accessory cell cytoplasm (Fig. 10D).

DISCUSSION

The heterocellular female gonad of *Syndesmis patagonica* consists of well-separated germaria and vitellaria both enveloped by a tunica composed of an outer extracellular lamina/matrix and an inner sheath of accessory cells (saccular type gonad). This feature corresponds to that found in other Rhabdocoela (Lucchesi et al., 1995; Sopott-Ehlers, 1997; Falleni et al., 1998, 2002, 2005) and, more in general, in other rhabdithophorans such as Proseriata (Sopott-Ehlers, 1986, 1990, 1994, 1995), Tricladida (Falleni et al., 2006, 2009) and Neodermata (Xylander, 1987; Cifrian et al., 1993; Martinez-Alos et al., 1993; Bruňanská et al., 2005; Poddubnaya et al., 2006, 2012; Greani et al., 2012a). A different situation has been described in the Lecithoepitheliata where the germovitellarium is enveloped either solely by an extracellular lamina (Falleni et al., 1995) or by accessory cells (Falleni, 1997) and in Prolecithophora where only accessory cells surrounding the germarium have been reported (Nigro and Gremigni, 1987; Falleni et al., 2012).

In *S. patagonica* the accessory cells, which are localized under the extracellular lamina, remain confined to the periphery of the gonad or, occasionally, partially protrude their cytoplasmic processes between oocytes and vitellocytes as is typical of most rhabdocoels (Falleni and Lucchesi, 1992; Lucchesi et al., 1995; Falleni et al., 1998, 2005). Accessory cells completely enwrapping the growing oocytes and vitellocytes with their long cytoplasmic protrusions have been described in Proseriata (Sopott-Ehlers, 1994, 1995) and Tricladida (Gremigni and Nigro, 1983; Tekaya et al., 1999; Falleni et al., 2006, 2009) and in the rhabdocoel temnocephalid *Troglocaridicola* sp., belonging to Scutariellidae, which surprisingly shares other ultrastructural characteristics of female gonad with Proseriata and Tricladida (Falleni et al., 2002) as well as similarities to Proseriata in spermiogenesis (Iomini et al., 1994). As suggested in previous papers, accessory cells are thought to play a trophic role in transferring low- molecular-weight-precursors from the surrounding tissue to the developing germ cells besides having a supporting function (Falleni and Gremigni, 1992; Falleni et al., 2002, 2006, 2009). In addition, in *S. patagonica* the accessory cells contribute to the formation of the extracellular lamina of both the germarium and the vitellarium by releasing the content of elongated vesicles into the extracellular space through exocytosis, as has also been detected in some rhabdocoel Temnocephalida

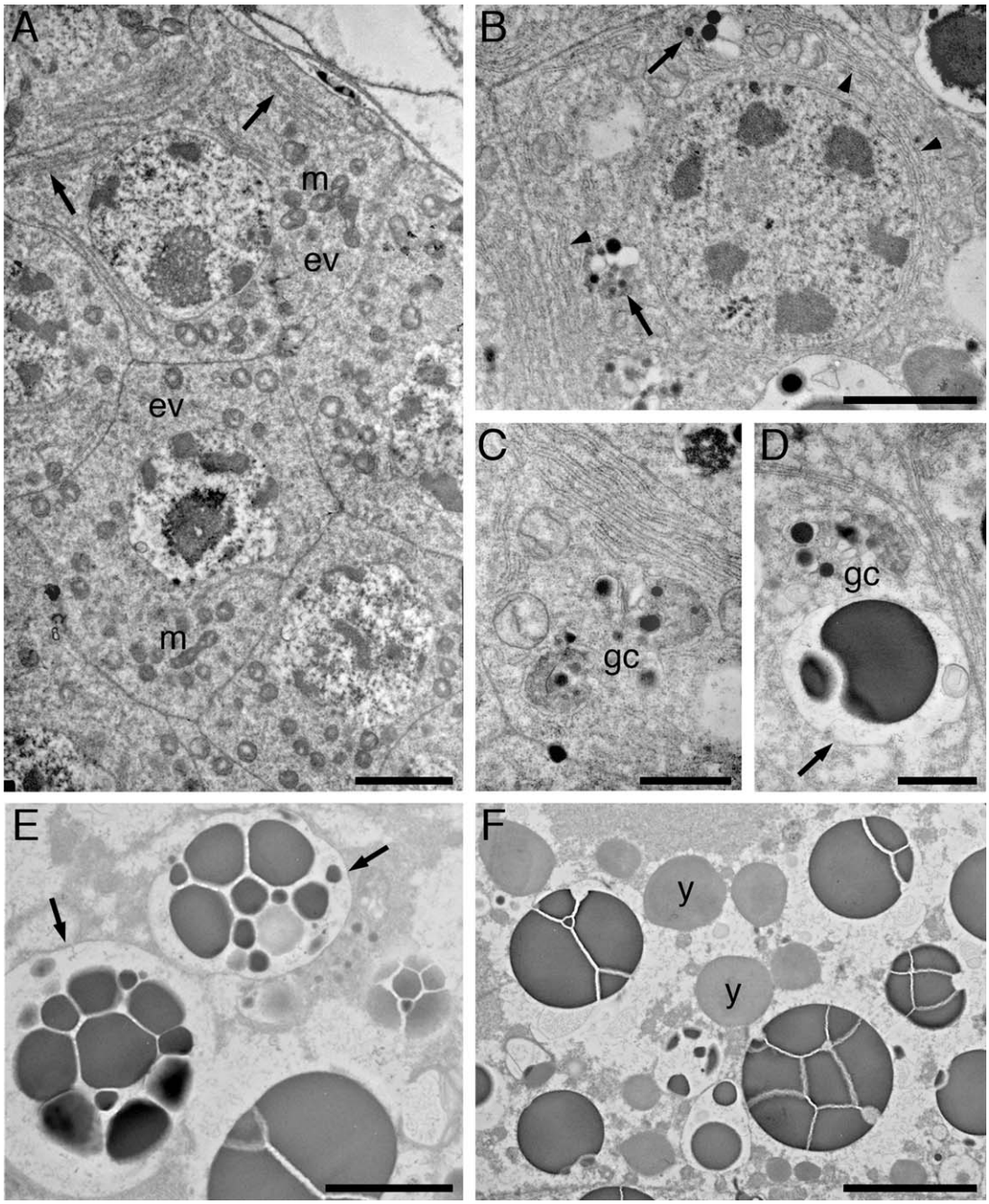

Fig. 8. *Syndesmis patagonica*. Ultrastructural features of vitellocyte differentiation. **A:** Early vitellocytes (ev) in the germinative area of the vitelline follicle showing a nucleus with large clumps of chromatin and a well-developed nucleolus. In the cytoplasm abundant ribosomes, scattered mitochondria (m) and RER profiles (arrows) are visible. Scale bar, 2 μm. **B**: A developing vitellocyte exhibiting some Golgi areas (arrows) and long RER cisternae (arrowheads). Scale bar, 2 μm. **C** and **D**: Small Golgi complexes (gc) with electron-dense vesicles and a nascent eggshell globule (arrow). Scale bars, 1 μm. **E**: Forming eggshell globules consisting of small polygonal electron-dense granules embedded in an electron transparent matrix (arrow). Scale bar, 2 μm. **F**: Eggshell globules showing an electron-dense mosaic-like content pattern and yolk globules (y) with a homogeneous content of medium electron density from a mature vitellocyte. Scale bar, 4 μm.

(Falleni et al., 1998, 2002) and in some terrestrial triclads (Falleni et al., 2006).

Oocyte maturation occurs during the prophase of the first meiotic division as shown by the presence of synaptonemal complexes even in large oocytes as is typical of other neoophoran platyhelminths (Falleni et al., 2005, 2006, 2009).

The presence of CBs, nonmembraneous aggregates of fibrous/granular material with high electron density, is a common feature of the germ line

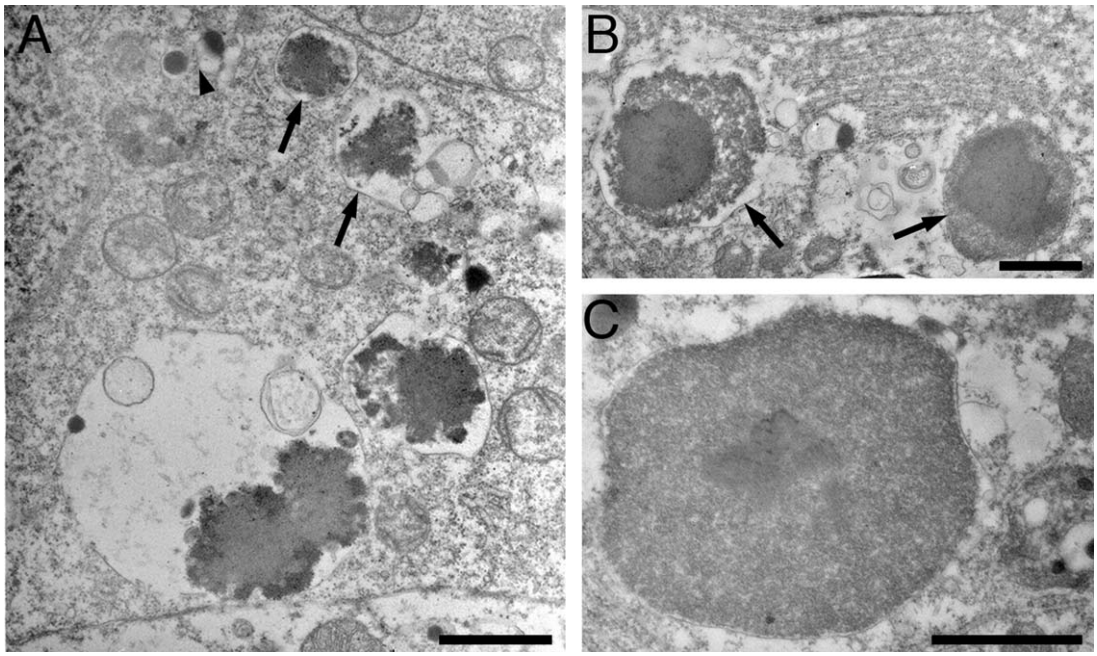

Fig. 9. *Syndesmis patagonica*. Ultrastructural features of vitellocyte differentiation. **A** and **B**: Nascent yolk globules (arrow) close to a small Golgi complex (arrowhead) and RER cisternae from developing vitellocytes. Scale bars, 1 μm. **C**: A nearly mature yolk globule with a finely granular content of medium electron density delimited by a smooth membrane. Scale bar, 1 μm.

cells of many invertebrates and vertebrates (Eddy, 1975; Saffman and Lasko, 1999). Although CBs were first described more than a century ago (Von Brunn, 1876), their components and functions have been remained obscure for decades and only recently are beginning to be revealed. It has been widely ascertained that RNAs, subunits of ribonucleoprotein and protein that acts as RNA chaperons are present in the CBs of male germ cells and these subcellular structures are now viewed as a storage/processing site of mRNAs (Parvinen, 2005; Kotaja and Sassone–Corsi, 2007), since they were found to contain the endonuclease Dicer, Argonaute proteins, and a number of microRNAs (Kotaja et al., 2006; Nagamori and Sassone-Corsi, 2008). Moreover, the role of degradation site, involving the ubiquitin–proteasome system and the lysosome system, has also been postulated for the CBs (Haraguchi et al, 2005; Yokota, 2008).

As far as the Platyhelminthes are concerned, CBs have been observed in the neoblasts or adult stem cells (Coward, 1974; Hori, 1982) and in male (Watson and Rohde, 1995; Rohde and Watson, 1995; Harrath et al. 2012) and female germ cells (Gremigni, 1976; Sopott-Ehlers, 1986; Falleni, 1997; Falleni et al., 1998, 2002, 2006, 2009; Charni et al., 2010) where they have been found both in the perinuclar cytoplasm and in the deeper cytoplasm sometimes associated with mitochondria. So far, however, investigations on the molecular composition and function of CBs in platyhelminths are mainly restricted to planarian neoblasts. These proliferating stem cells generate cells of all tissues during growth, differentiation, and tissue homeostasis and have, as their main feature, CBs that decrease in number and size during cytodifferentiation and disappear in completely differentiated cells except for germ-line cells. Sato et al. (2006) found transcripts of Djnos gene (nanos related gene from *Dugesia japonica*) in CBs of planarian germline stem cells. Nanos proteins are required for germ cell development in invertebrates and vertebrates (Tsuda et al., 2003). In addition, conserved posttranscriptional regulators required for regeneration in planarians, have also been discovered in neoblast CBs (Yoshida-Kashikawa et al., 2007; Solana et al., 2009; Fernandez-Taboada et al., 2010).

The development of RER and Golgi complexes in the oocytes of *S. patagonica* is mainly correlated to the production of electron-dense inclusions which become localized in the peripheral cytoplasm of mature oocytes. Our cytochemical tests show that these egg inclusions have a polyphenolic content as is the case in the rhabdocoels Dalyellioida, Typhloplanoida and Kalyptorhynchia (Falleni and Lucchesi 1992; Lucchesi et al., 1995; Falleni et al., 2005) and have been interpreted as eggshell granules. Peripheral polyphenolic eggshell granules with similar substructure have also been reported in the Prolecithophora belonging to Plagiostomidae (Nigro and Gremigni, 1987; Gremigni, 1988). The substructure, consisting of a dense homogeneous core surrounded by a less

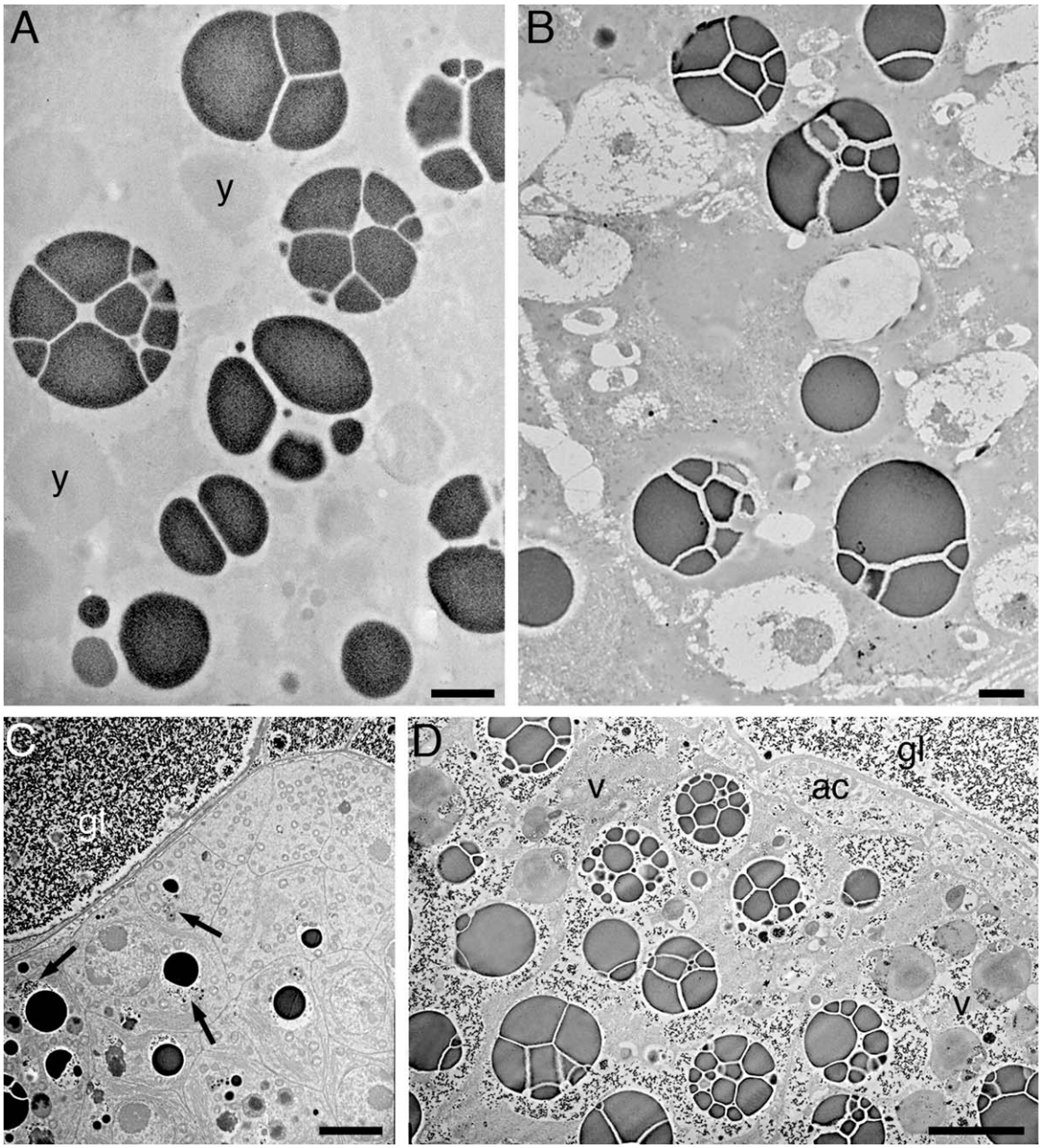

Fig. 10. *Syndesmis patagonica*. Vitellocyte aspects after cytochemical tests. **A**: Test for polyphenols. Unstained section. The silver precipitate is visible on the eggshell globules. y, yolk globules. Scale bar, 1 μm. **B**: Protein extraction. The content of yolk globules is completely digested while eggshell globules remain unaffected. Scale bar, 1 μm. **C** and **D**: Thiéry test, 8 h incubation in TCH, unstained section. Early and young vitellocytes contain little glycogen (arrow) while glycogen deposits markedly increase in mature vitellocytes (v). Note the large amount of glycogen (gl) in the parenchyma cells surrounding the vitelline follicle. ac, accessory cell cytoplasm. Scale bars, 4 μm.

dense granular/tubular component and a translucent halo, and the size (2–2.5 μm) of the eggshell granules of *S. Patagonica* are more similar to those described in the rhabdocoels Typhloplanoida and Kalyptorhynchia rather than in other dalyelliids belonging to Dalyelliidae, where polyphenolic shell granules measuring 0.5–1 μm in maximum diameter appear homogeneously granular (Lucchesi et al., 1995; Falleni et al., 2005, 2012). These eggshell granules can contribute, together with the eggshell globules produced by the vitellocytes and the material exocyted from the shell glands to the formation of the capsule-shell (cocoon-shell) (Nigro and Gremigni, 1987; Falleni and Lucchesi 1992; Lucchesi et al., 1995; Falleni et al., 2005). In the rhabdocoel, umagillid *Syndisyrinx franciscanus*, Shinn (1993) found that oocyte peripheral granules supply the ground material on which

polyphenolic substances from vitellocyte eggshell globules are deposited. In particular, they seem to be involved in the formation of the eggshell hatching sutures (Shinn and Cloney, 1986). A different situation has been observed in the oocytes of the rhabdocoel Temnocephalida belonging to Temocephalidae and Scutariellidae where peripheral egg granules, devoid of polyphenols and containing glycoproteins, have been interpreted as cortical granules (Falleni et al., 1998, 2002, 2005). Cortical granules have also been found in the oocytes of some Proseriata (Gremigni and Nigro, 1984; Gremigni et al., 1986; Sopott-Ehlers, 1995), Tricladida belonging to Planariidae and Dendrocoelidae (Gremigni, 1969, 1979; Gremigni and Domenici, 1975; Harrath et al., 2013) and in the parasitic Neodermata (Justine and Mattei, 1984, 1986; Cifrian et al., 1993; Justine et al., 1994; Yang et al., 2003; Poddubnaya et al., 2005, 2007, 2010; Greani et al., 2012a). These peripheral granules, which have a glycoprotein content and are devoid of polyphenols, are thought to be a tool to prevent polyspermy even though their role in the process of fertilization and disappearance in fertilized eggs has rarely been ascertained (Gremigni and Domenici, 1975; Sopott-Ehlers, 1994, 1995).

Ultrastructural aspects of fertilization in platyhelminths have been little studied so far. However, some valuable information derives from ultrastructural investigations performed in neodermatan worms where it has been demonstrated that, in most cases, it occurs in the oviduct lumen or in the fertilization canal proximal to the ootype by lateral fusion of oocyte and sperm plasma membrane (Świderski and Conn, 1999; Świderski et al., 2004).

The second type of inclusions, produced in small quantities in the oocyte of *S. patagonica*, has a glycoprotein content which is not extracted by protease, and remains scattered in the cytoplasm throughout oogenesis, as has been observed in other rhabdithophoran platyhelminths where such inclusions have been interpreted as yolk (Gremigni, 1969; Gremigni and Nigro, 1983, 1984; Falleni and Gremigni, 1992; Falleni et al., 2002, 2006, 2009). The appearance of vitelline glands in neoophoran Platyhelminthes has dispensed germaria from producing yolk and shell forming substances. However, the oocytes of some neoophorans continue to produce small amounts of yolk and shell granules (Falleni et al., 2012) as is the case of *S. patagonica*.

The maturation process of the vitellocytes in *S. patagonica* shows a pattern similar to that described in other rhabdocoels (Falleni et al., 2005) and more in general in other free-living and parasitic neoophoran Platyhelminthes (Rieger et al., 1991; Fried and Haseeb, 1991; Świderski and Xylander, 2000; Levron et al., 2010). Developing vitellocytes are biosynthetically active cells involved in the production of two types of membrane-bound inclusions as well as glycogen. The first type of membrane-bound inclusions to appear in the vitellocytes has a content consisting of round or polygonal electron-dense granules causing the so-called multigranular/mosaic like pattern. These electron-dense globules are positive to the polyphenol test and have been interpreted as eggshell globules. A similar substructure of the eggshell globules has been observed in the Prolecithophora (Gremigni, 1988; Gremigni and Falleni, 1991, 1992; Lanfranchi and Falleni, 1998) and in all the other rhabdocoels studied to date (Falleni et al., 2005, 2012) except for the temnocephalid *Troglocaridicola* sp. (Scutariellidae) which shows polyhenolic eggshell globules with the meandering/concentric content pattern typical of Proseriata and Tricladida (Falleni et al., 2002, 2005).

Cisternae of the rough endoplasmic reticulum and Golgi complexes are also involved in the formation of yolk, the second type of membrane bound inclusions in the vitellocytes. The yolk globules have a homogeneous protein content of medium electron density devoid of polyphenols and are similar in substructure and composition to those present in other rhabdocoels and in general in most neoophoran Platyhelminthes (Gremigni and Falleni, 1992; Falleni et al., 2005, 2006, 2012).

No lipid droplets have been detected in the developing and mature vitellocytes of *S. patagonica*. As reported by Greani et al. (2012b), the production of lipids varies among Platyhelminthes and this variability is particularly evident in the parasitic digeneans where several lipids per vitelline cell have been found in *Schistosoma mansoni*, only one lipid per cell in *Aphallus tubarium* and vitellocytes devoid of lipids have been described in *Fasciola hepatica*. The mature vitellocytes of *S. patagonica* also contain glycogen deposits whose synthesis increases during the last phase of the vitellocyte maturation process. This substance is considered as a source of energy for the developing embryo (Falleni et al., 2009; Greani et al., 2012b).

As discussed above, in the endosymbiotic *S. patagonica* vitellocyte differentiation show a similar pattern to that observed in other free-living neoophorans studied to date. The only significant difference, worthy of being highlighted, is the complete lack of lipid reserve in vitellocytes which, instead, show abundant glycogen deposits. This feature differs from that observed in the majority of free-living platyhelminths where large amounts of lipids are stored in the vitellocytes (Gremigni and Falleni, 1992; Falleni et al., 2002, 2006, 2009, 2012) and correlates with Jennings' observation (1981, 1997) that free-living platyhelminths generally have an energy source rich in lipids whereas endosymbionts generally are rich in glycogen. High glycogen levels reflect a stable, reliable, and adequate food supply conferred by the endosymbiotic habit, as is the case of *S. patagonica* which

lives in the gut of *A. dufresnii*, removing the need for long-term energy reserve (lipid storage). According to the Jennings (1981, 1997), there is a direct relationship between high fecundity, which is an automatic consequence of the nutrient-rich gut, and glycogen storage. Thus, the complete lack of lipids and the large amount of glycogen in the vitellocytes of *S. patagonica*, could be considered a result of the adaptation to symbiosis of this species. The presence of small peripheral polyphenolic granules in oocytes and polyphenolic eggshell globules with a mosaic-like content pattern in the vitellocytes of *S. patagonica* are considered apomorphic features as is the case in other dalyelliids, typhloplanids and kalyptorhynchids (Falleni et al., 2005, 2012). These ultrastructural characteristic of the female gonad are shared with members of the taxon Prolecithophora and can be considered synapomorphies of these two taxa.

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