Ultrastructure of germaria and vitellaria in *Dugesia sicula* Lepori, 1948 (Platyhelminthes, Tricladida, Paludicola)

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ABSTRACT. The female gonad of the diploid and sexual planarian *Dugesia sicula* Lepori, 1948 has been studied by means of transmission electron microscopy (TEM). The germaria consist of two ovaries occurring in the anterior body part behind the eyes. The vitellaria are composed of two lateral rows of vitelline follicles ranged dorsally and ventrally from the ovaries to the copulatory apparatus. During their growth, oocytes are provided with scattered residual yolk globules. The cytoplasm of maturing oocytes is filled with mitochondria, chromatoid bodies, Golgi complexes, RER, annulate lamellae and small yolk globules (2-3 µm in diameter) surrounded by a simple membrane, and lacks cortical granules. During the early stage of differentiation, vitellocytes enclose well-developed RER and Golgi complexes. Mature vitellocytes show yolk and eggshell globules, lipid droplets and glycogen particles. Golgi complexes and RER are involved in the production of eggshell and yolk globules respectively. Eggshell globules exhibit a concentric pattern typical for triclads. Results are discussed and compared with bibliographic data for other triclads.

KEY WORDS: Platyhelminthes, Paludicola, *Dugesia sicula*, oogenesis, vitellogenesis, ultrastructure.

INTRODUCTION

Previous ultrastructural studies of ovogenesis and vitellogenesis in Platyhelminthes have offered good insights into triclad phylogeny and classification. Among many works, the study of Gremigni (1979) strongly supported the classification proposed by Ball (1974) and confirmed that the primary taxonomic division in the Paludicola is between two groups; the first group comprises the Dugesiidae whose oocytes produce yolk globules of similar structure ranging from 2 to 7 µm in size with, for some species, a paracrystalline organization of the granular component (Gremigni, 1969a, 1974). The second group comprises the Planariidae and the Dendrocoelidae, whose oocytes produce a monolayer of cortical granules of almost 1µm diameter while yolk globules are absent (Gremigni & Domenici, 1975; Gremigni, 1974, 1979).

Dugesia sicula Lepori, 1948 is an important paludicolan species because it is represented by many asexual triploid populations distributed in the Mediterranean region that arose from diploid strains. These diploid strains are in danger of extinction especially in European countries and until now have not been the subject of fine ultrastructural studies. Gremigni (1979) presented only the structure of yolk globules occurring inside oocytes of an Italian strain of this species. So the aim of this study is to analyse, using ultrastructural techniques, the cytodifferentiation of oocytes and vitelline cells in a Tunisian diploid strain.

MATERIALS AND METHODS

Specimens of *Dugesia sicula* were collected from Mountain streams in North West Tunisia. It is a diploid sexual strain showing a high percentage of sexually-mature specimens and many fertile cocoons during the period of late spring to autumn. Worms were fixed in 3% glutaraldehyde in 0.2 M sodium-cacodylate buffer (pH 7.4) for about 4h at 4 ℃, washed in buffer for 30 min at 4 ℃, post fixed in 4% OsO_4 in cacodylate buffer for 1h at room temperature. They were dehydrated in an ethanol series, embedded in EPOX resin and polymerized at 60 ℃ for 12h. Ultrathin sections of the ovaries and vitellaria were stained with uranyl acetate and lead citrate and examined under a JEOL GEM-1010 at 80 kV.

RESULTS

Germarium morphology

Paired ovaries of *Dugesia sicula* are oval and located anteriorly to the pharynx in a ventral position behind the eyes. A distinct extracellular lamina is not observed, although a multilayered gonadal wall made by the extension of peripheral accessory cells separates each ovary from the surrounding parenchyma (Fig. 1). Accessory cells, usually associated with oocytes, are located at the periphery of the gonad and between germ cells. Peripheral accessory cells are elongated and their cytoplasm contains many mitochondria, a

flattened nucleus and dense nucleoplasm with small patches of heterochromatin mainly adjacent to the inner nuclear membrane (Fig. 1). Internal accessory cells located between germ cells present the same structure as the peripheral ones but they are larger and their nuclear membrane is irregular (Fig. 1).

Oocyte differentiation

Oogonia and young oocytes are elliptical cells with a diameter of 15 to 20 µm. The large nucleus is surrounded by a thin dense ooplasm. It contains a prominent nucleolus, the chromatin is diffuse with few clumps scattered in the nucleoplasm (Fig. 2). The nucleolus is about 2 to 2.5 µm; it contains granules and intermingled fibrils (Fig. 2). In the perinuclear ooplasm, dense and granular material (0.2 to 0.6µm) usually called 'chromatoid bodies' is located facing the nuclear envelope pores, which are increased in number (Fig. 3). Few elongated mitochondria, free ribosomes and glycogen particles are present in the ooplasm. Golgi complex and endoplasmic reticulum (RER) are rare in these maturing oocytes.

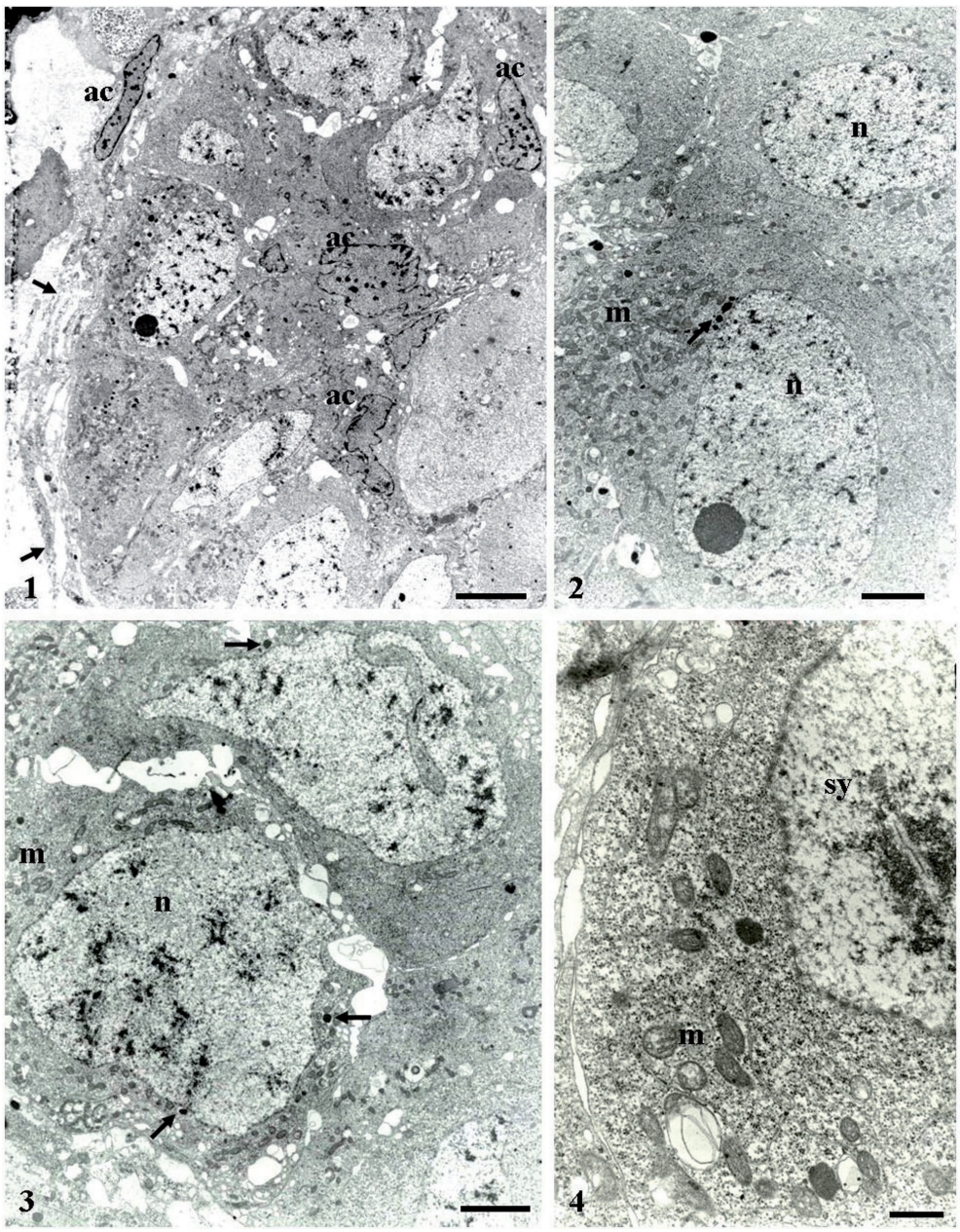

Fig. 1-4. – Ovogenesis of *Dugesia sicula*. Electron micrographs of sections through the ovary. **1. -** Thin tunic separates each ovary from the surrounding parenchyma (arrows). Elongated accessory cells (ac) associated with oocytes at the periphery of the gonad and between germ cells. Scale bar: 5µm. **2. -** Oogonia and young oocytes. The large nucleus (n) is surrounded by a thin basophilic cytoplasm rich in elongated mitochondria (m), free ribosomes, glycogen particles and chromatoid bodies (arrow). The nucleolus is prominent, the chromatin is diffuse. Scale bar: 2µm. **3. -** Maturing oocytes showing a small enlargement of cells, mitochondria (m) increase in number, chromatoid bodies are located facing the increasing nuclear envelope pores (arrow). Scale bar: 2µm. **4. -** Nucleoplasm of growing oocytes displaying synaptonemal complexes (sy) with the tripartite structure. Scale bar: 500nm.

In growing oocytes, nucleoplasm displays synaptonemal complexes with the tripartite structure typical of pachytene and diplotene stages (Fig. 4). As with Dugesiidae, endogenous vitellogenesis characterizes the oocyte differentiation. In fact this differentiation is marked by a slight enlargement of cells, a limited development of the RER and an increase of the Golgi complexes. The latter apparatus produces small vesicles, which undergo repeated and progressive coalescence giving rise to small round or oval yolk globules 2-3 µm in diameter surrounded by a simple membrane (Fig. 5). Mature yolk globules have a roundish, granular, electron-dense core surrounded by a thin layer of amorphous, translucent material (Fig. 6). Two points are worthy of note; firstly, the granular component occupies practically all the globule whereas the amorphous material is confined to the peripheral area. Secondly, no paracrystalline organization was observed in *D. sicula* oocyte yolk globules.

Yolk globules are scattered throughout mature oocyte cytoplasm, and they are surrounded by a great number of mitochondria clusters close to chromatoid bodies. This mitochondria proliferation constitutes a large 'Balbiani body' or 'yolk nucleus' (Fig. 8) which may include lipid droplets of 3.5 µm diameter and few bundles of annulate lamellae (Fig. 7).

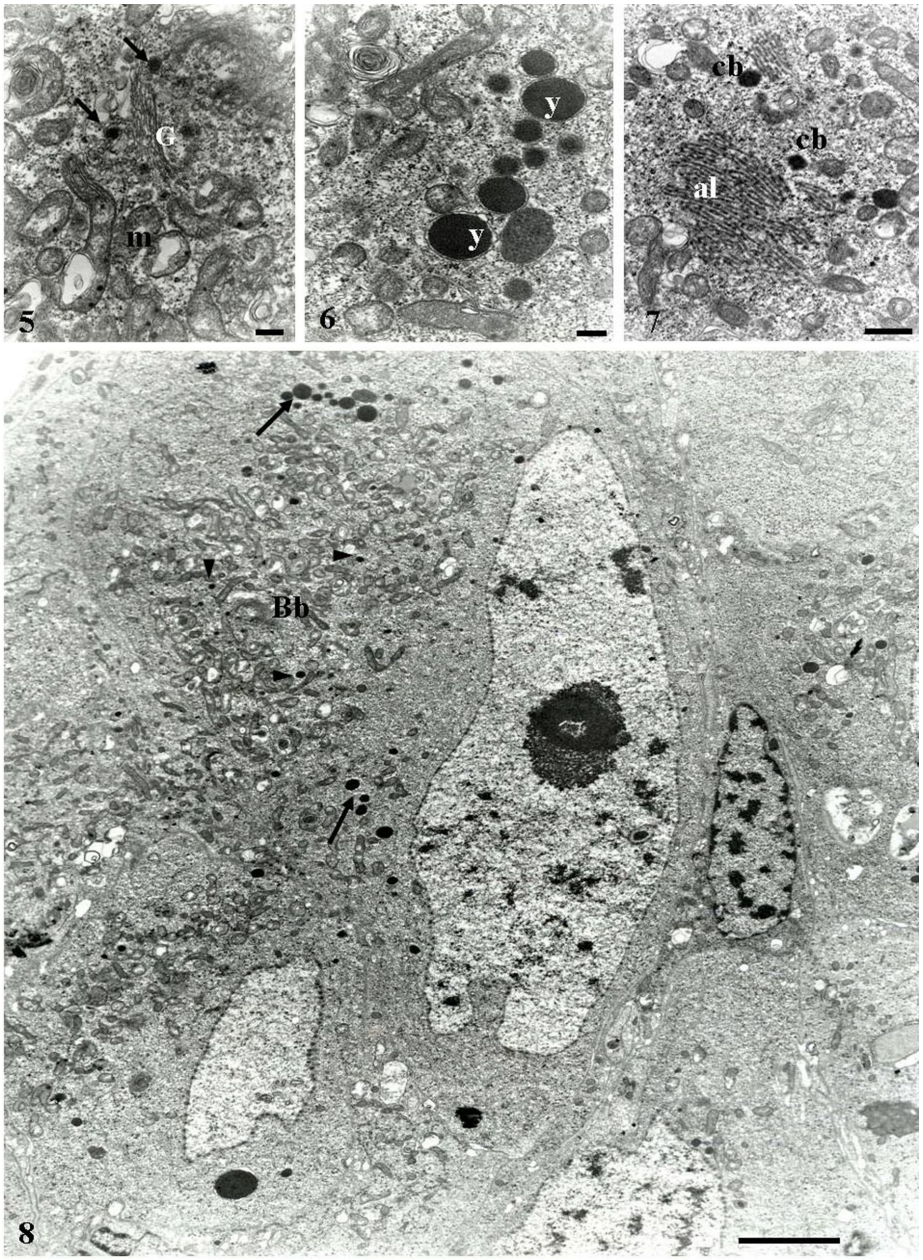

Fig. 5-8. – Ovogenesis of *Dugesia sicula*. Electron micrographs of sections through maturing oocytes. **5.** - Golgi complex (G) produces small vesicles that fuse to give rise to small yolk globules (arrows). Mitochondrion (m). Scale bar: 200nm. **6. -** Small round or oval yolk globules (y) 2-3µm diameter surrounded by a simple membrane accumulate in the ooplasm. Scale bar: 200nm. **7.** - High magnification within Balbiani body showing few bundles of annulate lamellae (al), mitochondria and chromatoid bodies (cb). Scale bar: 500nm. **8.** - Balbiani body (Bb) in maturing oocytes. Yolk globules (arrows) are scattered throughout ooplasm and surrounded by a great number of mitochondrial clusters close to chromatoid bodies (arrow heads). Scale bar: 2µm.

Vitellocyte structure

In vitelline follicles, young vitelline cells are peripheral and display the same shape as undifferentiated cells (neoblasts) scattered everywhere in the surrounding parenchyma. They are oval shaped, 10-12 µm long and have a large rounded nucleus (5-6 µm diameter) surrounded by a thin basophilic cytoplasm. In the nucleoplasm, the chromatin is diffused and the nucleolus displays fibrillar and granular components. The cytoplasm contains many free ribosomes, some mitochondria and few RER.

During their differentiation, vitelline cells are characterized by a very low nucleoplasmic ratio and have the typical structure of secreting cells: They show a notable increase in RER, Golgi complex and lipid droplets (Figs 9, 10). Differentiated vitelline cells are spherical or cylindrical and can reach 30-40 µm in diameter. Their cytoplasm contains low numbers of free ribosomes with mitochondria and very developed cisterns of RER (Figs 9, 11). The nucleus reduces

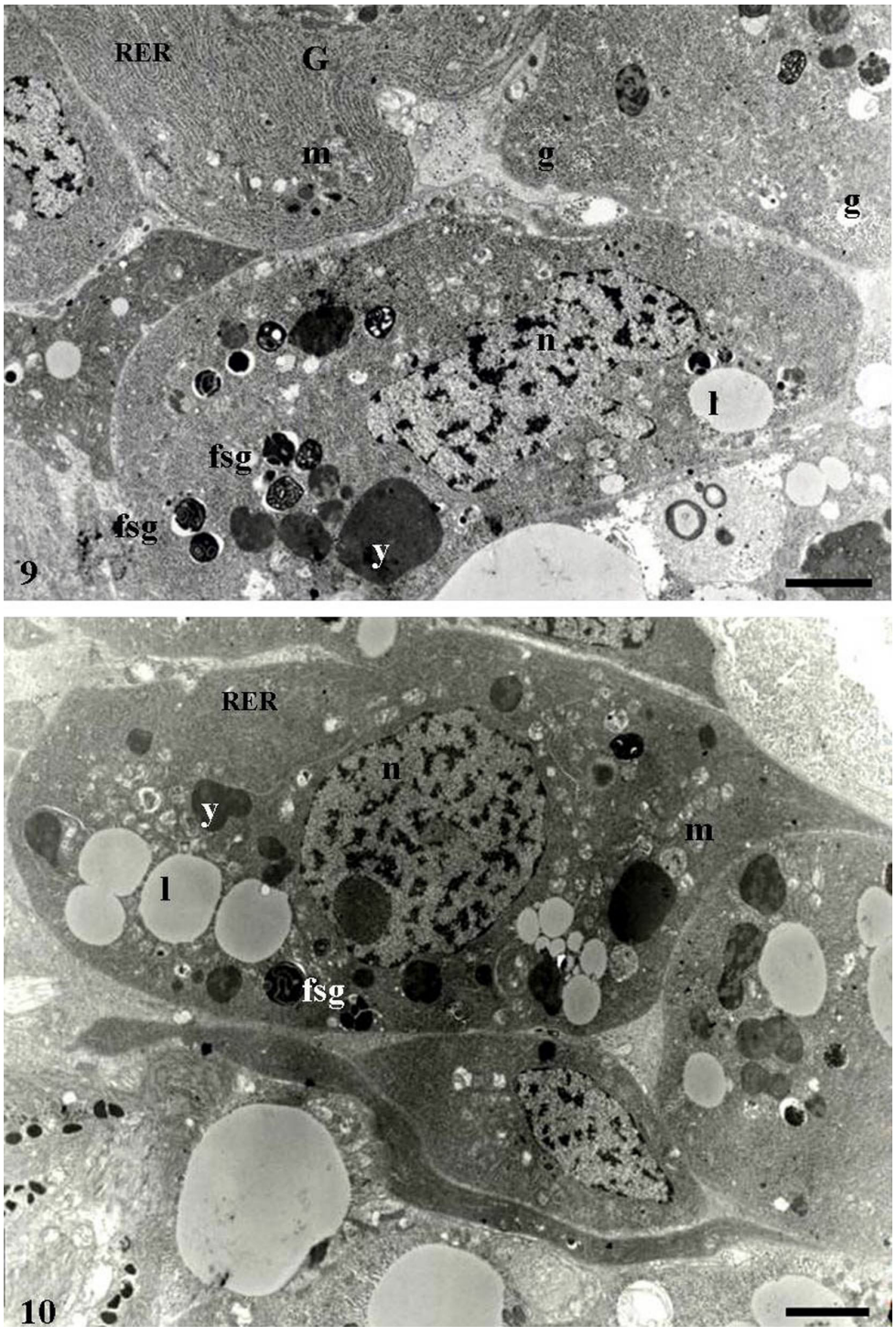

Fig. 9-10. – Vitellogenesis of *Dugesia sicula*. Electron micrographs of sections through maturing vitellocytes. The cytoplasm contains low numbers of free ribosomes, mitochondria (m), very developed cisterns of the RER, glycogen particles (g), lipid droplets (l), yolk (y) and forming eggshell globules (fsg). Nucleus (n). Scale bars: 5µm and 2µm.

in size, the chromatin is diffused and a small nucleolus displaying a granular component is present (Fig. 10). The cytoplasm of mature vitelline cells is almost entirely occupied by yolk and shell globules, lipid droplets and glycogen granules. Lipid droplets of 1-1.5 µm are spherical, larger and less numerous than yolk and shell globules (Figs 12, 13).

Yolk globules result from the coalescence and repeated fusion of vesicles of 0.2 µm diameter produced by the RER and containing only one type of homogeneous component of medium density. Their final diameter varies roughly between 1.5 and 2.5 µm (Fig. 12). Eggshell globules are less numerous than yolk globules and derive from fusion of Golgi-derived vesicles of medium electron density (Fig. 11). At the beginning of eggshell globule formation, these two types of vesicles agglomerate to give globules containing small dense bodies and broad masses of fairly dense homogeneous material (Figs 12, 13). Immature eggshell globules, limited by a simple membrane, show a multigranular pattern (Figs 12, 13). In mature eggshell globules, of 0.8-2 µm diameter, two components of different density are present giving it a typical organization of alternating dense and clear concentric rings (Fig. 14).

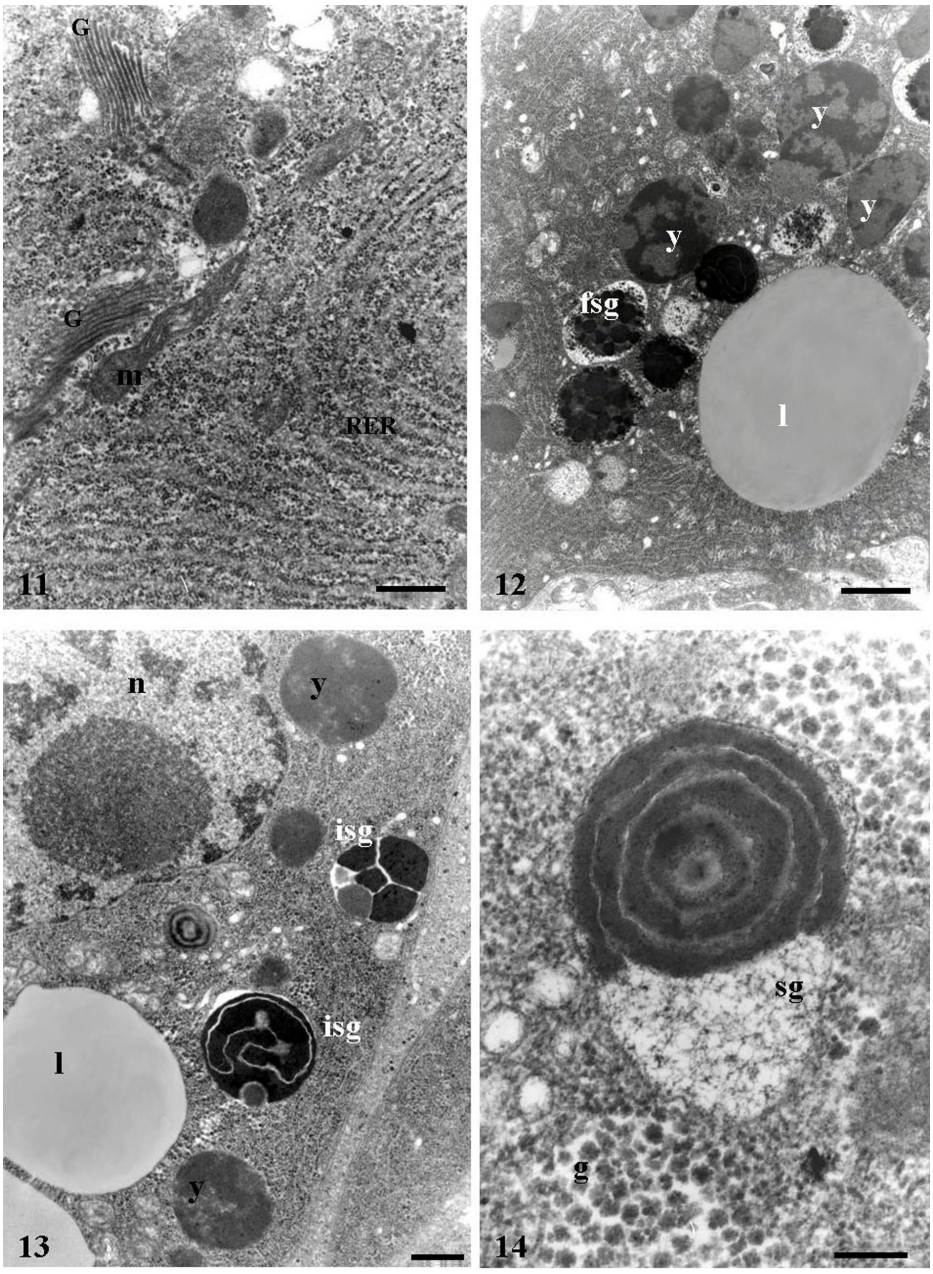

Fig. 11-14 – High magnification of cytoplasm of mature vitelline cells. **11.** - Note the development of Golgi complexes (G) producing small granules of medium electron density. Long cisterns of endoplasmic reticulum (RER) occupy a great part of the cytoplasm. Mitochondrion (m). Scale bar: 500nm. **12.** - Cytoplasm of mature vitelline cells showing yolk globules (y), lipid droplets (l) and forming shell globules (fsg). Scale bar: 1µm. **13.** - Cytoplasm of mature vitelline cells showing yolk globules (y) and immature eggshell globules (isg) limited by a simple membrane and displaying a multigranular pattern. n, nucleus. Scale bar: 500nm. **14.** - Maturing eggshell globule (sg) showing the typical organization of alternating dense and clear concentric rings. g, glycogen. Scale bar: 200nm.

DISCUSSION

The female gonad of *D. sicula* consists of well-separated germarian and vitellarian areas both enveloped only by extension of accessory cells; an extracellular lamina is lacking. The heterocellular female gonad of turbellarians can reportedly be enveloped by a tunica composed of an outer extracellular lamina and an inner sheath of accessory cells. This tunica has been found in some Proseriata (Sopott-Ehlers, 1986, 1994, 1995), Rhabdocoela (Lucchesi et al., 1995; Sopott-Ehlers, 1997; Falleni et al. 1998, 2002, 2005) and recently in three terricola; *Geoplana burmeisteri* Schultze & Müller, 1857 *Microplana scharffi* Graff, 1896 and *Microplana terrestris* Müller, 1774 (Falleni et al., 2006, 2009). This extracellular lamina is absent in the marine triclad *Sabussovia dioica* Claparède, 1863 (Tekaya et al., 1999) and in Lecithoepitheliata (Falleni et al., 1995; Falleni, 1997).

Accessory cells have been observed to surround oocytes and vitellocytes during their differentiation and they are thought to have a protective function and to play a trophic role in transferring precursors from the surrounding tissue to the maturing oocytes (Nigro & Gremigni, 1987; Falleni & Gremigni, 1992; Falleni et al., 2002, 2006, 2009). They have been reported throughout oogenesis in some Proseriata (Sopott-Ehelers, 1994, 1995), Tricladida (Gremigni & Nigro, 1983; Tekaya et al., 1999; Falleni et al., 2006, 2009) and Rhabdocoela (Falleni et al., 2002).

The synaptonemal complexes present in the nuclei of large oocytes indicate that their maturation is completed during the prophase of the first meiotic division. This phenomenon is marked also by the development of RER and Golgi complexes, which is correlated with the production of granules with a finely granular content of medium electron density. Such granules having a glycoprotein content remain scattered in the ooplasm throughout oogenesis and are interpreted as yolk; they have been observed in some proseriates (Falleni & Gremigni, 1992), freshwater triclads (Gremigni, 1969a, 1988) and terrestrial triclads (Falleni et al., 2006, 2009). This yolk production occurs only by an autosynthetic mechanism, as previously described in platyhelminths belonging either to the archoophoran or to the neoophoran level of organization of the female gonad (Gremigni & Falleni, 1992).

With respect to the shape of yolk globules produced within oocytes, Gremigni (1979) has noted that they are large in *D. sicula* and can reach a final diameter of 7-8 µm and more. However, in this work we have never observed such size. In fact, yolk globules produced in Tunisian *D. sicula* oocytes did not exceed 3µm as it was shown in *Dugesia dorotocephala* Woodworth, 1897 *Dugesia anceps* Kenk, 1930 and *Dugesia tigrina* Girard, 1850 studied by Gremigni (1979). As in *Dugesia benazzii* Lepori, 1951, *Dugesia biblica* Benazzi & Banchetti, 1973 and *Dugesia gonocephala* Dugès, 1830 (Gremigni, 1979), these yolk globules produced by *D. sicula* (this work) are scant and do not display the paracrystalline organization in the completely mature globules. The amorphous component appears to be rarefied, in the same way in both growing and mature globules, which thus allows the central granular component to appear surrounded by a thin translucent zone.

It can be noted that the structure of yolk globules produced in oocytes is quite different from those produced by vitelline cells. The oocyte yolk autosynthesis is considered as a character more primitive than the vitellaria yolk production and it is interpreted as a residue of an ancestral feature (Boyer, 1972).

Our ultrastructural study shows that a Balbiani body is present in growing oocytes of *D. sicula*. It consists of an accumulation in the ooplasm of many mitochondria, small electron-dense bodies and few annulate lamellae. This structure attracted the attention of many scientists who discussed its role and its composition in each zoological group. Previously it was believed that this cellular structure, which generally disappears after fecundation, intervenes in the yolk synthesis, but electron microscopy did not confirm this interpretation. Gremigni (1976) has indicated the presence of the Balbiani body in vitellogenic oocytes of *D. dorotocephala*. He noticed that this structure, with its typical organization, remains present in the prophasic oocytes ready to enter the oviducts. However, according to the same author, the Balbiani body cannot be regarded either as a constant structure with a well-defined function in the oocytes (since it is absent in *Dugesia lugubris* Schmidt, 1861, *D. gonocephala* and *D. benazzii* which produce yolk globules) or as possessing a direct role in yolk synthesis (since it is absent in vitelline cells of the neoophorans).

The presence of chromatoid bodies is a common aspect in differentiating cells, especially in the germline cells of many animals, as is the case in the oocytes of many Platyhelminthes (Gremigni, 1976; Justine & Mattei, 1986; Falleni & Gremigni, 1992; Falleni & Lucchesi, 1992; Tekaya, 1999; Falleni et al., 2002, 2006, 2009). Chromatoid bodies are maintained in germ cells during their differentiation from neoblasts and they are suggested to be concerned with the totipotency of theses cells (Shibata et al., 1999). It has been demonstrated that they contain many components as the transcript of genes implicated in germ cell development (Sato et al. 2006).

No types of cortical or peripheral granules have been found in the mature oocytes of *D. sicula* as in other Dugesiidae studied (Gremigni, 1969a, 1976, 1979, 1988). Such granules have been detected in the cortical ooplasm of marine (Gremigni & Nigro, 1983; Tekaya et al., 1999) and freshwater planarians belonging to the Planariidae and Dendrocoelidae (Gremigni, 1969b, 1979; Gremigni & Domenici, 1975) and in some proseriates (Gremigni & Nigro, 1984; Gremigni et al., 1986; Sopott-Ehlers, 1995).

Generally, the pattern of vitellocyte maturation in *D. sicula* is similar to that described in other neoophoran Platyhelminthes (Rieger et al., 1991) and especially in some marine, terrestrial and freshwater triclads (Domenici & Gremigni, 1974; Tekaya et al., 1998; Falleni et al., 2006, 2009). In fact the ultrastructural study of the vitellaria indicates that vitellocytes have the general structure of neoophoran vitelline cells. Young cells have a large nucleus with a promi-

nent nucleolus and little-differentiated cytoplasm. During maturation, the nucleoplasmic ratio decreases gradually while the Golgi complex and the RER develop. The mature vitellocytes have a differentiated cytoplasm due to various inclusions: lipid droplets, yolk and eggshell globules. The mature eggshell globules display a substructrure similar to that observed in some proseriates, freshwater, terrestrial and marine triclads (Gremigni, 1988; Tekaya et al., 1998; Falleni et al., 2006, 2009) where two components display concentric rings of respectively dense and clear materials. According to previous studies on freshwater planarians of the Dugesiidae family, the clear component of these eggshell globules corresponds to non-phenolic proteins while the dense component contains polyphenols (Gremigni & Domenici, 1974).

Three types of eggshell globules have been described in neoophoran Platyhelminthes: the homogeneous pattern typical of Lecithoepitheliata and some Proseriata (Gremigni, 1988; Falleni et al., 1995), the convoluted or concentric pattern typical of some Proseriata and Tricladida (Gremigni & Domenici, 1974; Gremigni & Falleni, 1991; Sopott-Ehlers, 1991, 1995; Tekaya et al., 1998; Falleni et al., 2006, 2009) and the multigranular pattern typical of the Prolecithophora and Rhabdocoela (Gremigni, 1988; Gremigni & Falleni, 1991, 1998; Sopott-Ehlers, 1997; Falleni et al., 2005).

It is known that vitelline cells produce proteins and polyphenols needed for the formation of the sclerotin eggshell through a tanning process (Marinelli, 1972; Gremigni & Domenici, 1974; Yanagita & Yamamoto, 1981). Mature vitellocytes contain other types of reserve material such as lipids, glycogen particles and yolk globules. These latter constitute the second type of membrane-bound inclusion and are produced by the RER and Golgi complex. They have a homogeneous content of medium electron density and they are similar to those described in other triclads (Gremigni & Falleni, 1992; Falleni et al., 2005, 2006, 2009).

In conclusion, the present ultrastructural investigation has provided evidence that the female gonad of *D. sicula* displays ultrastructural features typical of the basic pattern of Tricladida and especially of the freshwater planarians from the Dugesiidae family. Since many triploid asexual populations of this species are present in the Mediterranean region and can develop ex-fissiparous and sterile specimens, we envisage a similar study of their female gonad to compare it with that of the sexual strain.

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