

Ultrastructure of the vitellarium in the digeneans *Phyllodistomum angulatum* (Plagiorchiida, Gorgoderidae) and *Azygia lucii* (Strigeida, Azygiidae)

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Abstract

Fine structural features of the vitellarium of two digeneans, *Phyllodistomum angulatum* and *Azygia lucii*, are documented and compared with those of other digenean species. The cytodifferentiation of immature vitelline cells (vitellocytes) assumes the production and subsequent accumulation in their cytoplasm of several inclusions. Mature vitelline cells of *P. angulatum* are characterized by the presence of vitelline clusters (~2.7 µm in diameter, with ~100 vitelline globules of ~0.35 µm in diameter) and osmiophobic, saturated lipid droplets (~2–3 µm in diameter), and in *A. lucii* vitelline clusters of the same diameter include much fewer vitelline globules (~50 globules of ~0.5 µm in diameter), osmiophilic lipid droplets and α-glycogen. In both *P. angulatum* and *A. lucii*, interstitial cells are also present within the vitellarium. Two types of contact sites (septate and tight junctions) between adjoining interstitial cells also occur in both digenean species. Judging from the present and previous ultrastructural studies, it is suggested that there are three potential discriminatory characters of the digenean vitellarium (the number of different types of cell components within the vitellarium, the presence and type of junctional complexes between these cells, and the isolation of the vitellarium from the surrounding tissue) which may prove useful for a better understanding of the biology and evolutionary history of the different digenean groups.

Keywords

Digenea, ultrastructure, vitellarium, vitelline inclusions, interstitial cells, contact sites, discriminatory traits, phylogeny

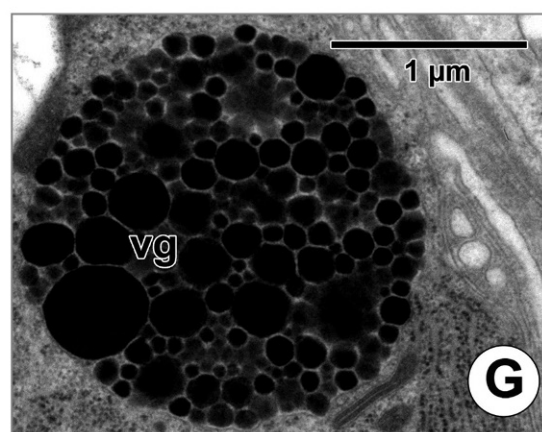
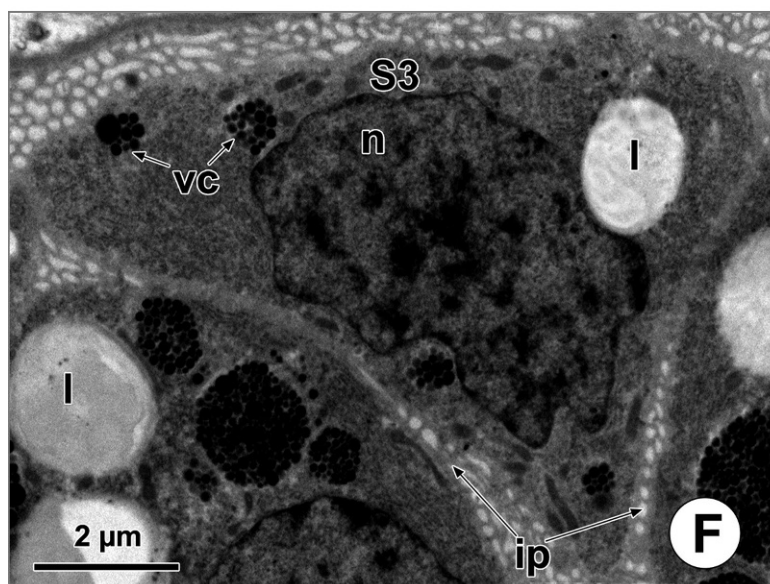
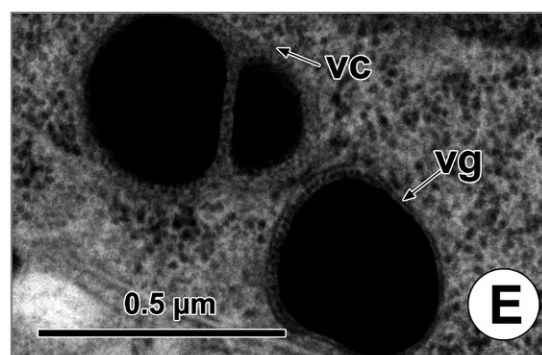
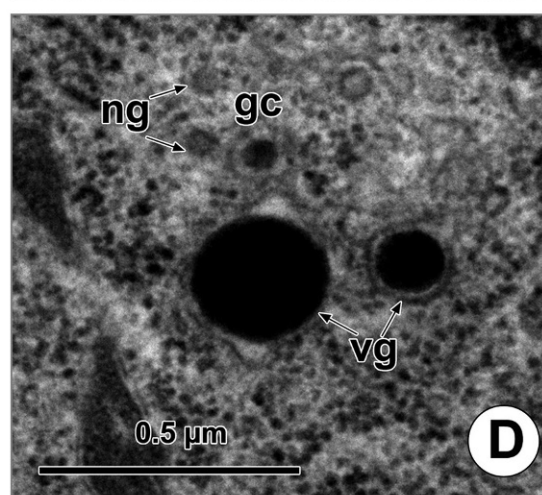
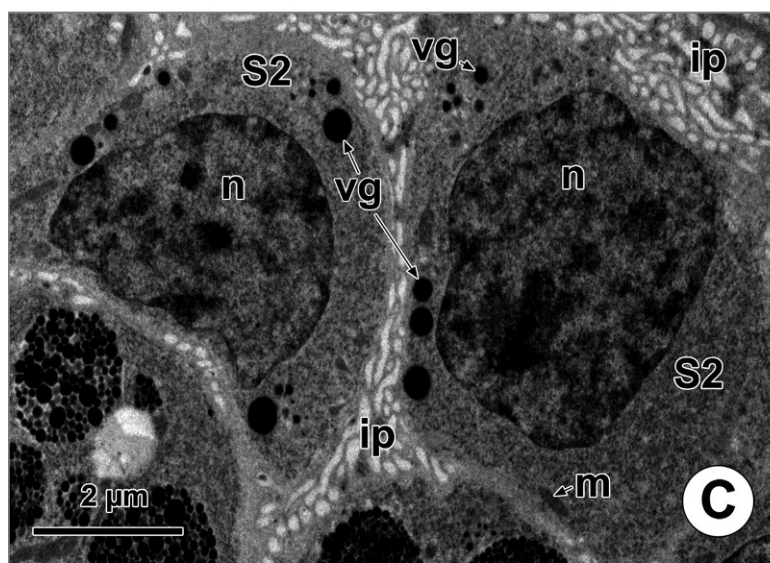
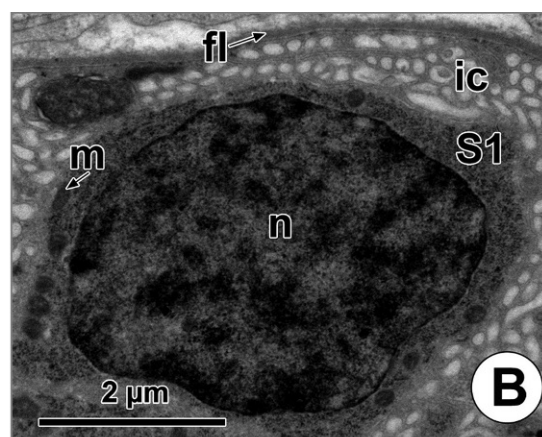
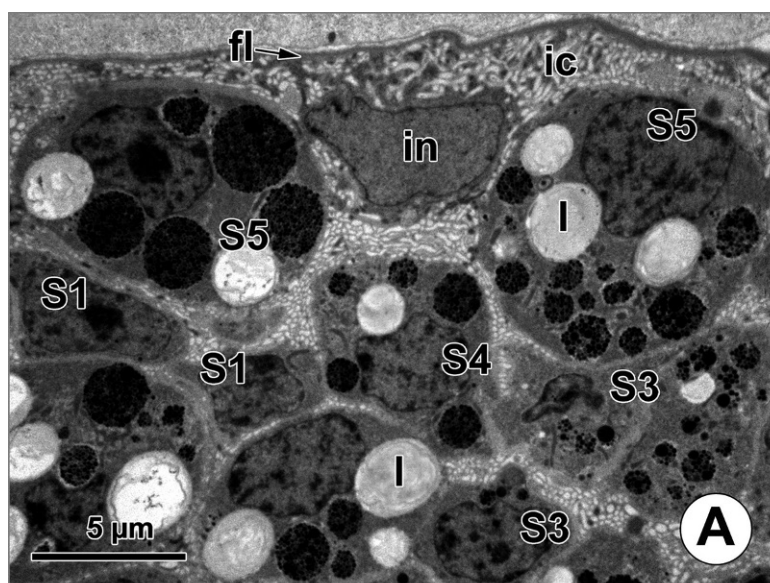
Introduction

The usefulness of ultrastructural investigations of the vitellarium of the Neodermata in terms of a better understanding of the relationships between the different taxa within this group has been commented upon by Świderski and Mackiewicz (1976), Świderski and Xylander (2000), Bruňanská *et al.* (2005), Poddubnaya *et al.* (2006) and Levron *et al.* (2007). Ultrastructural studies on the vitellarium of the trematode subclass Digenea have been presented by Tulloch and Shapiro (1957), Björkman and Thorsell (1963), Burton (1963), Koulis (1969), Irwin and Threadgold (1970), Erasmus (1973), Hanna (1976), Grant *et al.* (1977), Irwin and Maguire (1979), Erasmus *et al.* (1982), Fukuda *et al.* (1983), Holy and Witrock (1986), Hendow and James (1989), Podvyaznaya (1990, 2003), Sharma and Swarnakar (1992), Chaymardanov and

Tanyüksel (1995), Colhoun *et al.* (1998), Meepool *et al.* (2006), Sampour (2008) and Świderski *et al.* (2011).

In the Digenea, the vitellarium varies in form, occurring as a single mass, numerous masses, varying numbers of tubules, branched tubules and, most frequently, numerous follicles. This organ produces vitelline cells (vitellocytes), which are involved in egg formation, and most investigations have concentrated on the process of vitellogenesis. These have shown that this process tends to follow a similar pattern in all of the studied groups of digeneans, although the composition and amount of vitelline material (vitelline globules, lipid droplets and glycogen) vary (Świderski *et al.* 2011). However, there appears to be a distinct difference between the various digenean species investigated in the presence of different types of component cells within their vitellarium. In some digeneans a single type of cell (vitelline cells at different stages of de-

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velopment) is present (Grant *et al.* 1977, Holy and Wittrock 1986, Podvyaznaya 1990, Świdorski *et al.* 2011), whereas in others two types of cells (vitelline cells at different stages of development and what have been called 'interstitial', or 'nurse', cells) are present (Irwin and Threadgold 1970, Erasmus 1973, Hanna 1976, Irwin and Maguire 1979, Fairweather *et al.* 1988). Relatively little ultrastructural information is available on the nature of the vitellarium in digenean species of the family Gorgoderidae (Koulisch 1969, Irwin and Maguire 1979), and there has been no such ultrastructural study on species of the family Azygiidae.

The purpose of this study was to analyse the cytomorphology of the vitellarium of digenean species from these two distantly related families, *Phyllodistomum angulatum* (Plagiorchiida: Gorgoderidae) and *Azygia lucii* (Strigeida: Azygiidae), including: (1) cytodifferentiation of the vitelline cells; (2) the composition and amount of the vitelline material; (3) the structure of the interstitial cells; and (4) the occurrence of contact sites between different cells within the vitellarium. Our intentions were then to: (1) compare the findings with those known for other digeneans; and (2) highlight any discriminatory traits of the structure of the digenean vitellarium which may be useful for an understanding of the phylogeny of the different digenean groups.

Materials and methods

Adult *Phyllodistomum angulatum* were recovered from the urinary bladder of pike perch (*Lucioperca lucioperca*), and adult *Azygia lucii* from the stomach of northern pike (*Esox lucius*). These fishes were caught in the Rybinsk Reservoir, Russia. The live worms were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 for 20 days at 5°C. Small pieces of worm were rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4 and postfixed in cold (5°C) 1% osmium tetroxide in the same buffer for 1 h. The material was then dehydrated in a graded series of ethanol and acetone, and embedded in Araldite and Epon. Ultrathin sections (70–90 nm) were stained with uranyl acetate and lead citrate, and examined in a JEOL-1011 transmission electron microscope operating at 80 kV.

Results

Vitellarium of Phyllodistomum angulatum

The vitellarium of this species comprises two small, compact, symmetrical, oval masses situated just posterior to the ventral sucker. Each vitelline mass contains vitelline cells at various stages of development, which are surrounded by cytoplasmic projections of interstitial cells (Fig. 1A). The vitelline masses are delimited by a thin, fibrous sheath (Figs 1A, B; 2B, C).

Immature vitelline cells

This type of vitelline cell (S1) can be observed in the peripheral region of the vitelline mass (Fig. 1A, B). It is readily distinguished by a large, centrally situated nucleus, with evenly distributed chromatin within the nucleoplasm, and the presence of few mitochondria and many free ribosomes in the narrow region of its perinuclear cytoplasm (Fig. 1B).

Maturing vitelline cells

The second stage of vitelline cell development (S2) exhibits a relatively greater cytoplasmic volume around the nucleus (Fig. 1C). Zones with vesicles of Golgi complexes can be seen within this cytoplasm (Fig. 1D). Vitelline material is condensed in these vesicles in the form of vitelline globules of differing size (Fig. 1D). The first small, nascent globules are moderately electron-dense and have a diameter of ~0.05 µm. Later, these vitelline globules become more electron-dense and have a diameter of 0.1–0.2 µm. Larger, round globules (diameter ~0.4 µm) occur as single membrane-bound units throughout the cytoplasm (Fig. 1C, E).

The next step in vitelline cell development (S3) is characterized by the aggregation of single vitelline globules into clusters (Fig. 1F). Cluster formation can consist of different numbers of globules (2–20) embedded in a fine, fibrillar matrix (Fig. 1E, F). The diameter of clusters formed by the union of 10–20 globules is 0.4–0.7 µm. Single large (1.5 × 1.8 µm), oval, electron-lucid lipid droplets are present in the cytoplasm (Fig. 1E, F).

Fig. 1. Ultrastructural features of the vitellarium of *Phyllodistomum angulatum*. **A.** Section of a vitelline mass; note an interstitial cell and vitelline cells at different stages of maturation. **B.** Immature vitelline cell (S1) surrounded by vacuolated interstitial cytoplasm; note the fibrous layer delimiting the vitelline mass. **C.** Second stage (S2) of vitelline cell development, showing the appearance of individual vitelline globules within the cytoplasm. **D.** Vesicles of the Golgi complex; note nascent, moderately electron-dense globules within the Golgi vesicles. **E.** Single membrane-bound vitelline globule and forming cluster with two globules. **F.** Third stage of vitelline cell development (S3); note a few vitelline clusters composed of 10–20 globules and single electron-lucid lipid droplets present in the cytoplasm. **G.** Large vitelline cluster within the cytoplasm at the fourth developmental stage (S4) comprising about 50 globules. **Abbreviations to all figures:** cm – circular muscles; fl – fibrous layer; gc – Golgi complex; ger – granular endoplasmic reticulum; gl – glycogen; ic – interstitial cell cytoplasm; in – interstitial cell nucleus; ip – interstitial cell projections; l – lipid droplet; lm – limiting membrane of globule clusters; m – mitochondrion; md – moderately dense matrix within clusters; n – nucleus; ng – nascent globules; pc – perinuclear cytoplasm of interstitial cell; pm – plasma membrane of interstitial cell; sd – septate desmosome; ser – smooth endoplasmic reticulum; S1–S5 – developmental stages of vitelline cells; tj – tight junction; vg – vitelline globules; v – vacuoles; vs – vesicles

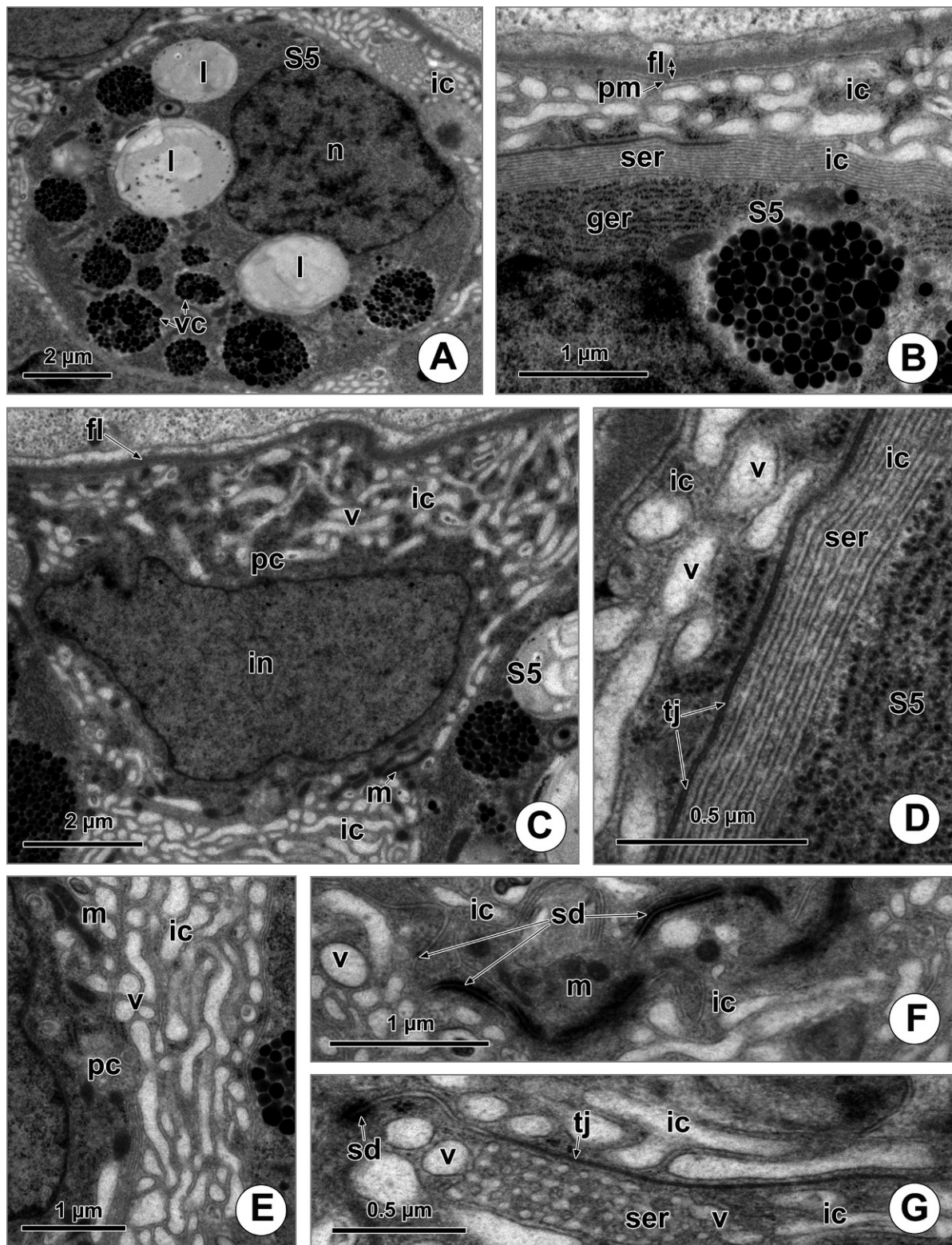


Fig. 2. Ultrastructure of mature vitelline cells and interstitial cells of *Phyllodistomum angulatum*. **A.** Mature vitelline cell showing vitelline clusters and large lipid droplets in the cytoplasm. **B.** Peripheral region of a vitelline mass, showing the plasma membrane of the interstitial projections surrounded externally by a fibrous layer; note the parallel concentration of smooth endoplasmic reticulum at the periphery of another interstitial projection. **C.** Nucleus of an interstitial cell; note the dense perinuclear and vacuolated areas of cytoplasm. **D.** Tight junction between two projections of interstitial cells. **E.** Dense perinuclear cytoplasm of an interstitial cell containing free ribosomes and mitochondria; note the vacuolated area of the cytoplasm. **F.** Septate desmosomes between projections of the interstitial cells. **G.** Two types of junction between adjacent membranes of two interstitial projections

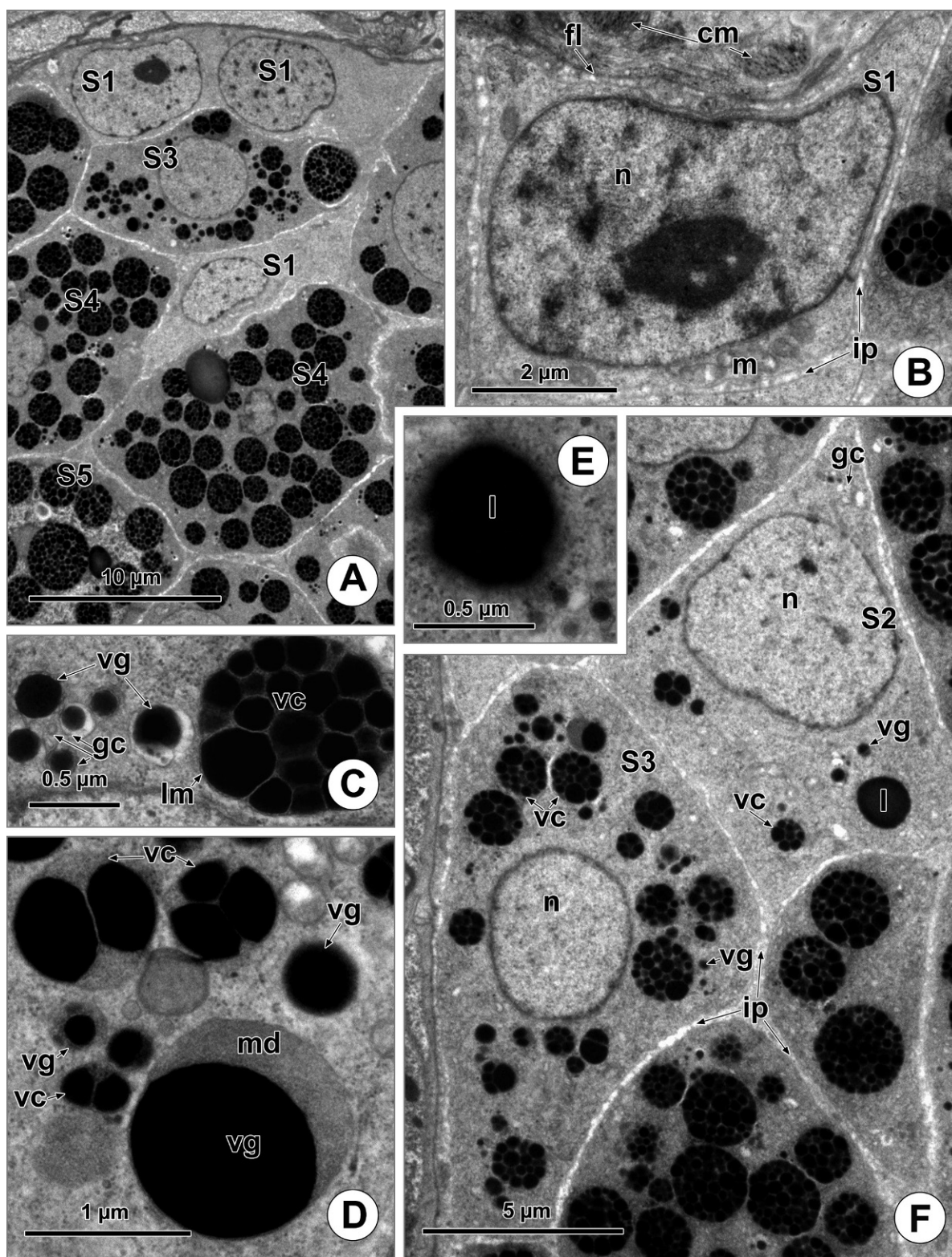


Fig. 3. Ultrastructural features of the vitelline follicles of *Azygia lucii*. **A.** Part of a vitelline follicle, showing closely packed vitelline cells at different stages of maturation. **B.** Immature vitelline cell at the periphery of the follicle; note the thin, fibrous layer surrounding the vitelline follicle and the neighbouring circular muscle fibres. **C.** Vesicles of the Golgi complex with individual vitelline globules; note the vitelline cluster limited by a membrane. **D.** Early stage of cluster formation with 2–3 globules embedded within a moderately electron-dense matrix; note a large individual globule that may have resulted from the fusion of smaller globules. **E.** Electron-dense lipid droplet within the cytoplasm at the second stage of vitelline cell development. **F.** Vitelline cells at the second and third stages of maturation

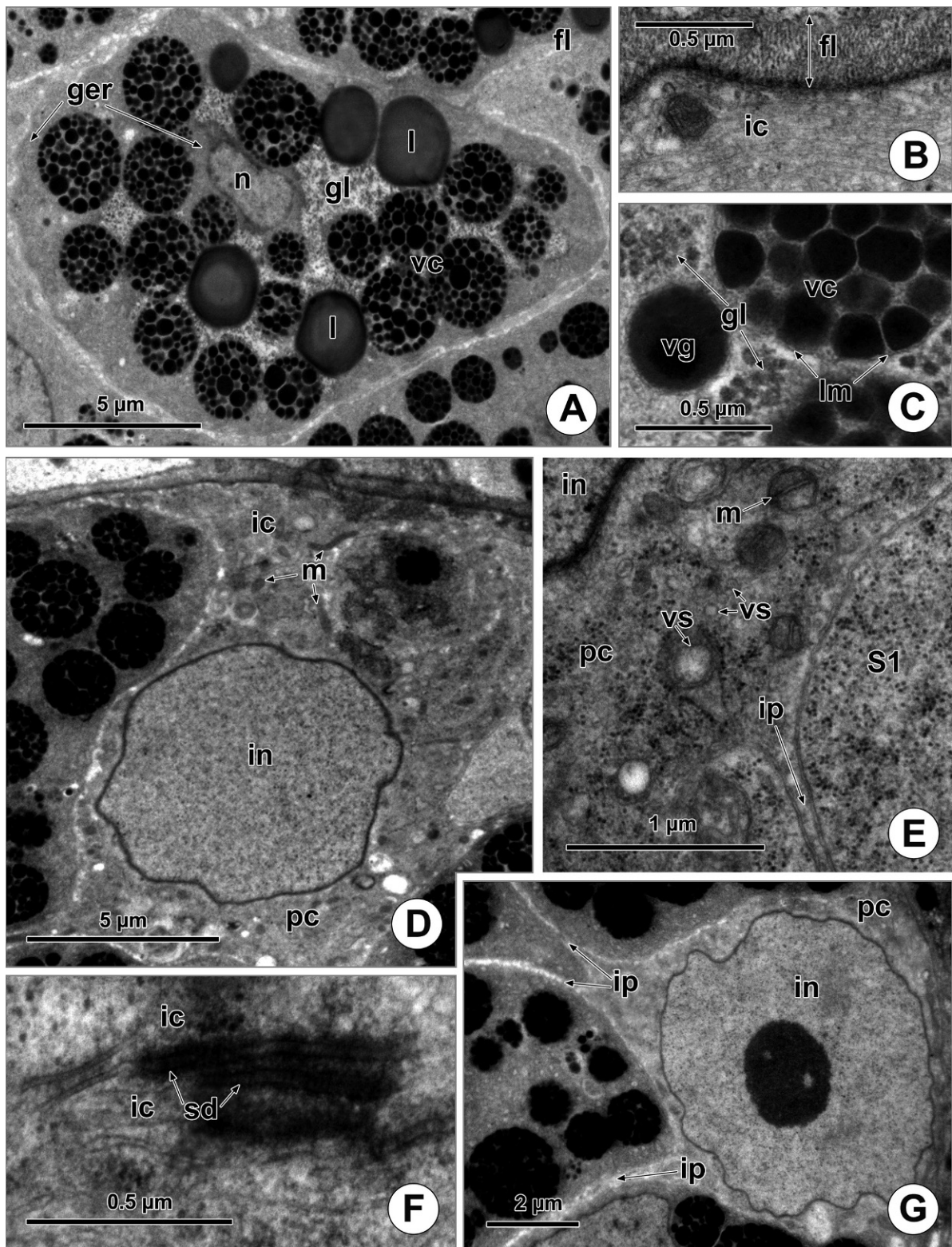


Fig. 4. Ultrastructure of mature vitelline cells and interstitial cells of *Azygia lucii*. **A.** Mature vitelline cell showing globule clusters, moderately electron-dense lipid droplets and glycogen; note the position of the granular endoplasmic reticulum at both the periphery of the cell and around the nucleus. **B.** Plasma membrane of an interstitial cell projection covered by a fibrous layer. **C.** Membrane-bound vitelline cluster and a large individual globule; note the accumulation of glycogen within the cytoplasm. **D** and **G.** Nucleus and perinuclear cytoplasm of interstitial cells at the periphery of the vitelline follicle. **E.** Organelles of the perinuclear cytoplasm of an interstitial cell. **F.** Septate desmosomes between the projections of interstitial cells

The advanced stage of maturation (S4) (Fig. 1A) can be recognized by the intensive formation of membrane-limited clusters of vitelline globules and the appearance of large clusters with a diameter of 1.5–2.7 μm (Fig. 1G). The number of globules within a single cluster varies widely between 50 and well over 100. The individual globules within the cluster measure 0.1–0.6 μm and are closely packed but do not coalesce. Each globule is embedded within an electron-lucent matrix. There are also some lipid droplets containing electron-lucent material within the developing vitelline cells (Fig. 1A).

Mature vitelline cells

Mature vitelline cells (S5) have a nucleus situated close to the cell plasma membrane (Fig. 2A). The cytoplasm is packed with large, membrane-bound clusters of vitelline globules and large, electron-lucid lipid droplets (Fig. 2A). The granular endoplasmic reticulum (GER) is confined to the perinuclear region and the periphery of the cytoplasm (Fig. 2B).

Interstitial cells

The interstitial tissue is composed of several cells within each vitelline mass. The long projections of these interstitial cells envelope each vitelline cell as well as the periphery of the vitelline mass (Figs 1A–B; 2B, C, E). Externally, the plasma membrane of the interstitial cells is surrounded by a thin, fibrous sheath (Figs 1B; 2B, C). The large nucleus of these cells is usually located at the periphery of the vitelline mass (Fig. 1A); it is irregular in shape and nucleoli are rare (Figs 1A, 2C). A thin layer of dense perinuclear cytoplasm, containing free ribosomes and mitochondria, surrounds the outer surface of the nucleus (Fig. 2C, E). Long, dendritic projections arise from the main body of these cells and extend throughout the vitelline mass (Fig. 1A). Their cytoplasm contains vacuoles of differing size and shape, free ribosomes, mitochondria and cisternae of smooth endoplasmic reticulum (Fig. 2B–F). The interstitial cytoplasm has a vacuolated appearance (Figs 1B, C; 2C, E). Projections of different interstitial cells are interwoven and, between their adjacent plasma membranes, two types of contact sites are present, septate desmosomes and tight junction-like structures (Fig. 2D, F, G).

Vitellarium of Azygia lucii

The vitellarium of this species is follicular, with the follicles occurring in two continuous lateral fields in the hindbody at the level of the uterus and gonads. Each vitelline follicle is composed of vitelline cells at different stages of maturation. Vitelline cells are closely packed within the follicle and have an irregular shape (Fig. 3A). The narrow spaces between these cells are occupied by thin projections of interstitial cells, the perikarya of which are localized at the periphery of the follicle (Figs 3A, F; 4D, G). The follicles are enveloped by a nar-

row fibrous outer layer (Figs 3B; 4B) and surrounded by isolated circular muscles (Fig. 3B).

Immature vitelline cells

Immature vitelline cells (S1) have a large nucleus with a distinct nucleolus (3B). Small amounts of perinuclear cytoplasm contain mitochondria and free ribosomes (Fig. 3B). As they begin to mature, the volume of the cytoplasm increases and the nucleoli disappear (Fig. 3A).

Maturing vitelline cells

The increased volume of cytoplasm in maturing vitelline cells (S2) includes round, electron-dense globules within electron-lucent vesicles in the Golgi complexes (Fig. 3C, F). These globules are $\sim 0.15 \mu\text{m}$ in diameter. In addition, round to oval electron-dense globules of variable diameter (0.15–1.0 μm) are embedded together within the moderately electron-dense matrix of the globule clusters (Fig. 3D). Individual globules within the clusters may be large, in comparison with solitary, isolated globules, and this may indicate a possible fusion of globules within the clusters. Early clusters comprise 2–5 electron-dense globules, which are often irregular in outline, enabling them to fit together closely within the clusters (Fig. 3D). The first electron-dense lipid droplets ($\sim 0.5 \mu\text{m}$ in diameter) occur in the cytoplasm of the early maturing vitelline cells (Fig. 3E, F).

In more advanced maturing vitelline cells (S3), the number of the clusters is increased, each comprising up to 25 globules of different sizes (Fig. 3C, F). Dense globules are irregular in outline and embedded within an amorphous matrix of medium density (Fig. 3C). These clusters, which measure $\sim 1.2 \mu\text{m}$ in diameter, are surrounded by an outer limiting membrane (Figs 3C; 4C).

The advanced stages of vitelline cell maturation (S4) are distinguished by an abundance of globule clusters in their cytoplasm (Fig. 3A, F). They can accumulate up to 50 globules in a single cluster, which may measure up to 2.5 μm in diameter. Large, moderately electron-dense lipid droplets (~ 2.0 –2.5 μm in diameter) are present in the cytoplasm.

Mature vitelline cells

In the cytoplasm of mature vitelline cells (S5) the GER is situated both at the periphery and in the perinuclear region (Fig. 4A). Large amounts of glycogen begin to accumulate within the cell, the appearance of which coincides with the disappearance of the GER (Fig. 4A, C). The cytoplasm is filled with globule clusters and lipid droplets (Fig. 4A).

Interstitial cells

The irregularly shaped nuclei and main cytoplasmic regions of the interstitial cells are localized in the peripheral areas of the

follicles (Fig. 4D, G). Prominent electron-dense nucleoli may or may not be present in the evenly distributed nucleoplasm of the nuclei (Fig. 4D, G). Cytoplasm of the interstitial cells surrounds each vitelline cell in the form of thin, dendritic projections visible in the narrow spaces between the vitelline cells (Fig. 3A, F). The majority of cytoplasmic organelles and inclusions (mitochondria, Golgi complexes, free ribosomes and small electron-lucent vesicles) are found in the perinuclear cytoplasmic regions of these cells (Fig. 4D, E). Junctional complexes (septate junctions) occur between the plasma membranes of adjacent interstitial cells (Fig. 4F).

Discussion

The vitellarium of the studied digenean species, *Phyllodistomum angulatum* (Gorgoderidae) and *Azygia lucii* (Azygiidae), contains vitelline cells (vitellocytes), at progressive stages of maturation, and interstitial cells. The sequential cytodifferentiation of immature vitelline cells assumes the production and subsequent accumulation in their cytoplasm of several inclusions, including vitelline globules, lipid droplets and glycogen. It is apparent from previous descriptions of vitelline cells that a number of differences exist between the composition and amount of these inclusions in various digenean species (Tulloch and Shapiro 1957; Koulis 1969; Irwin and Threadgold 1970; Hanna 1976; Grant *et al.* 1977; Irwin and Maguire 1979; Erasmus *et al.* 1982; Fukuda *et al.* 1983; Holy and Witrock 1986; Fairweather *et al.* 1988; Hendow and James 1989; Podvyaznaya 1990, 2003; Sharma and Swarnakar 1992; Chaymardanov and Tanyüksel 1995; Świdorski *et al.* 2011).

Vitelline globules

Vitelline globules appear during the first stages of secretory activity in the vitelline cells of both of the studied species. On the basis of an electron autoradiographic study of these globules, Hanna (1976) supported suggestions that globule protein is synthesized by the GER and transferred to the Golgi complexes for condensation, the formation of membrane-bound packages and the subsequent aggregation of individual globules into clusters. In the present study, large vitelline clusters (up to 2.7 µm in diameter) in *P. angulatum* contain up to 100 closely packed globules of ~0.35 µm in diameter, which are embedded within an electron-lucent matrix. In the case of *A. lucii*, the number of individual globules in clusters of about the same diameter and with a moderately-dense matrix is much less (up to 50 globules), and the diameter of individual globules is ~0.5 µm. It should be noted that in other studied species of the family Gorgoderidae, i.e. *Gorgoderina attenuata* and *G. vitelliloba*, the same morphology of the vitelline clusters as occurs in *P. angulatum* has been described by Koulis (1969) and Irwin and Maguire (1979). On the other hand, clusters with numerous heterogeneous vitelline globules have been indicated for species of the family Microphallidae,

i.e. *Maritrema linguilla* and *M. feliui*, by Hendow and James (1989) and Świdorski *et al.* (2011). Variation also occurs in the number and density of vitelline globules within clusters of different digenean species. For example, 20–25 tightly packed heterogeneous vitelline globules of irregular outline comprise every cluster in *Fasciola hepatica* (Fasciolidae) (Irwin and Threadgold 1970; Hanna 1976; Fairweather *et al.* 1988; Colhoun *et al.* 1998). Whereas, a much smaller number (7–12) of loosely packed globules are present in the electron-lucent matrix of the vitelline clusters of the diplostomid *Pharyngostomoides procyonis* and the schistosomatids *Schistosoma haematobium* and *S. japonicum* (see Grant *et al.* 1977; Erasmus *et al.* 1982).

It has been suggested that vitelline globules of digeneans represent building material for eggshell formation. Investigations of the mechanism of eggshell formation in the digeneans *S. mansoni* (see Wells and Cordingley 1991) and *F. hepatica* (see Threadgold 1982; Colhoun *et al.* 1998) have suggested that the precursor of the first thin eggshell is a complex of the enzyme phenol oxidase and eggshell precursor proteins. The egg capsule of digeneans consists of quinone-tanned protein (sclerotin), the synthetic pathway of which includes proteins and phenolic compounds (Burton 1963, Smyth and Halton 1983, Colhoun *et al.* 1998). Eggshell precursor enzymes, synthesized by the vitelline cells, are stored in vesicles and membrane-bound vitelline globule clusters (Wells and Cordingley 1991). Studies on the mechanism of eggshell formation in *F. hepatica* have shown that the membrane of the vitelline clusters disintegrates, releasing vitelline globules, which migrate to and are deposited on the developing eggshell (Irwin and Threadgold 1972, Colhoun *et al.* 1998).

In view of the fact that the eggshell formation involves vitelline globules, it is possible that the difference between the morphology of the vitelline clusters, their size and number, and the number of the vitelline globules within the clusters in different digenean species may be related to: (a) variations in the structure and size of the eggs of different species; and/or (b) the number of eggs produced per day.

An additional type of inclusion, granules with a concentric lamellar configuration, has been described in the vitelline cells of digenean species as: 'yolk globules' or 'labyrinthine shell globules' in *F. hepatica* (see Björkman and Thorsell 1963; Irwin and Threadgold 1970); 'ribosomal whorls' in *S. mansoni* (see Erasmus 1973); 'membranous whorls' in *Pharyngostomoides procyonis* (see Grant *et al.* 1977) and 'membrane bounded glycan vesicles' in *F. gigantica* (see Meepool *et al.* 2006; Schmidt 1998). The occurrence of these granules has been explained as an additional source of nutrition or as residual bodies by Irwin and Threadgold (1970) and Erasmus (1973). Schmidt (1998) suggested that such granules are involved in the process of egg hatching in *Echinostoma caproni* and *Fasciola gigantica*. In the present study, in both *Phyllodistomum angulatum* and *A. lucii*, such bodies are absent from the cytoplasm of the vitelline cells; this is also the situation in *Gorgoderina attenuata* (see Koulis 1969) and

G. vitelliloba (see Irwin and Maguire 1979). Hanna (1976) suggested that these 'yolk globules' may be autolysosomes, whereas Poddubnaya *et al.* (2006) referred to 'concentric bodies' in the cestode *Didymobothrium rudolphii* as representing a phase in vitelline globule transformation during the process of egg formation. According to Świdorski *et al.* (2009) and Młocicki *et al.* (2011), in the caryophyllidean cestode *Wenyonia virilis*, the most probable roles for such 'GER-bodies' are in the synthesis of glycoproteins and/or the formation of foci of cytoplasmic degradation. Differences in the appearance of these bodies in digenean vitelline cells may be related to the timing of the distribution of vitelline material during the process of egg formation.

Lipid droplets and glycogen

Lipid droplets and glycogen in cestode vitelline cells serve as nutritive reserves for the future embryo (Świdorski and Xylander 2000). The nutritive reserves of the vitelline cells of *P. angulatum* differ from those of *A. lucii* in relation to the different nature of the lipid droplets and glycogen present. Lipid droplets first appear at the second (in *A. lucii*) and the third (in *P. angulatum*) stages of vitelline cell development and, after coalescing, can reach dimensions of 2–3 µm. In *P. angulatum* they are osmiophobic and electron-lucid, a characteristic of saturated lipids (Świdorski and Xylander 2000), whereas in *A. lucii* small droplets appearing at the second stage of vitelline cell maturation are electron-dense, but, when they occur in the cytoplasm of subsequent stages of development, they are large, osmiophilic and moderately electron-dense, which are characteristics of unsaturated lipids (Świdorski and Xylander 2000). The presence of saturated lipid droplets has been also indicated for another gorgoderid species, *G. vitelliloba* (see Irwin and Maguire 1979), and for the microphallids *Maritrema linguilla* and *M. feliui* (see Hendow and James 1989, Świdorski *et al.* 2011). However, in a range of other digeneans unsaturated lipids have been found in their vitelline cells (Grant *et al.* 1977, Erasmus *et al.* 1982, Thulin 1982, Fairweather *et al.* 1988, Podvyaznaya 2003). Ginetsinskaya (1968) believed that the presence of a large amount of lipid droplets at the beginning of digenean larval development may be dependent on oxygen levels in the surrounding environment, such that many lipids occur when oxygen levels are low.

Glycogen in two forms is the last reserve substance to appear in vitelline cells of neodermatan worms (Świdorski and Xylander 2000, Świdorski *et al.* 2004). However, deposited glycogen present in the cells of *A. lucii* does not occur in *P. angulatum*. Glycogen of *A. lucii* is composed of large sacs of α -granules, large amounts of which begin to accumulate in the vitelline cell cytoplasm when active vitelline globules formation is completed. As in *F. hepatica* (Irwin and Threadgold 1970, Hanna 1976), glycogen synthesis in the vitelline cells of *A. lucii* is preceded by degeneration of the GER and is accompanied by growth. According to Hanna (1976), no evidence was found for the direct participation of any cell

organelle in this process, and it appears that glycogenesis is mediated by cytoplasmic enzymes within the vitelline cells. From the available literature, we can assume that, as in *P. angulatum*, mature vitelline cells of the other gorgoderids *Gorgoderina attenuata* (see Koulis 1969) and *G. vitelliloba* (see Irwin and Maguire 1979) do not contain glycogen, as is the case for the vitelline cells of *Paralecithodendrium* (as *Prosthodendrium*) *ascidia* (Lecithodendriidae) (see Podvyaznaya 1990) and the related *Gyrabascus* (as *Allassogonoporus*) *amphoraeformis* (Gyrabascidae) (see Podvyaznaya 2003). The presence of small numbers of β -glycogen particles has been noted in the peripheral cytoplasm of mature vitelline cells of *Maritrema feliui* (see Świdorski *et al.* 2011), which is a member of the same superfamily as the latter two species, as has α -glycogen in the vitelline cells of *Schistosoma mansoni* (see Erasmus 1973), *S. haematobium*, *S. japonicum*, *S. mattheei* (see Erasmus *et al.* 1982) and *Orthocoelium scoliocoelium* (Paramphistomidae) (see Sharma and Swarnakar 1992). Erasmus (1973) believed that such a difference in the amount of glycogen in various digenean species may be related to the retention of eggs within the host tissue for an extended period. If the nutrients necessary for the development of digenean larvae are derived from the host, it is possible that extensive glycogen reserves are not necessary for eggs which are fully developed within the uterus. For example, in *F. hepatica*, the vitelline cells of which contain a large amount of glycogen (Björkman and Thorsell 1963, Irwin and Threadgold 1970, Hanna 1976), the egg matures in the external aquatic environment. This is in contrast to vitelline cells of *S. mansoni* (where there is only a small amount of glycogen) and *Paralecithodendrium ascidia* (where there is no glycogen), where egg formation occurs within the uterine lumen (Erasmus 1973, Podvyaznaya 1990). In the cases of *A. lucii* and *Phyllodistomum angulatum*, in the present study, it seems that differences in the presence or absence of glycogen likely depend on variation in egg emission and the subsequent life-cycle. It is worth noting that in the developed egg of *P. angulatum* there is a ciliated miracidium which hatches as a free-swimming larva, whereas eggs of *A. lucii* contain an aciliate, non-swimming miracidium which is transmitted directly to the molluscan host without a free-living stage (Yamaguti 1975).

Interstitial cells

One of the most significant characters of the structure of the digenean vitellarium is the presence of either one or two different types of cell components and their interconnection with each other. The present results indicate that in both the gorgoderid *P. angulatum* and the azygiid *A. lucii* two types of cells, vitelline cells at different stages of development and interstitial cells, are present within the vitellarium. Interstitial, or 'nurse', cells in the vitellarium of digeneans have been shown to occur in another gorgoderid, *Gorgoderina vitelliloba*, by Irwin and Maguire (1979), and in the fasciolid *Fasciola hepatica* by Irwin and Threadgold (1970),

Hanna (1976) and Fairweather *et al.* (1988), in the schistosomatid *Schistosoma mansoni* by Erasmus (1973), in the gyrobascid *Gyrabascus* (as *Allassogonoporus*) *amphoraeformis* by Podvyaznaya (2003), in the paramphistomid *Orthocoelium scolicoelium* by Sharma and Swarnakar (1992) and in the haploporid *Haploporus lateralis* by Sampour (2008). Peripherally located cell bodies of interstitial cells give rise to a number of cytoplasmic projections that ramify around the vitelline cells and throughout the peripheral region of the vitellarium. The plasma membrane of these cells attaches basally to a thin, fibrous layer surrounding the vitellarium in *P. angulatum*, *A. lucii*, *Gorgoderina vitelliloba* and *Gyrabascus amphoraeformis* (present study, Irwin and Maguire 1979, Podvyaznaya 2003), but such a fibrous layer is absent around vitelline follicles of *F. hepatica* (see Irwin and Threadgold 1970). The cytoplasm of the interstitial cells exhibits different types of inclusions. In the case of *P. angulatum* and *A. lucii*, the most obvious difference between them is the presence in the former of a huge number of translucent vesicles, the fusion of which leads to an accumulation of irregularly-shaped cytoplasmic vacuoles. It is interesting to note that the same vacuolated cytoplasm of interstitial cells has been described in another gorgoderid, *Gorgoderina vitelliloba*, by Irwin and Maguire (1979). Hanna (1976), in his electron autoradiographic study of the vitellarium of *F. hepatica*, supported the view that interstitial cells select and transport precursors for protein and glycogen synthesis in the vitelline cells, and, if so, these pass through the interstitial cells prior to entering the vitelline cells in a soluble form.

An additional feature of those digeneans with a vitellarium consisting two different types of cells is the presence of intercellular junctional complexes. Our observations of both digenean species in the present study indicate the occurrence of two types of contact sites (septate junctions and tight junctions) between two adjoining interstitial cells. Previously, septate junctions between interstitial cells have also been found in another gorgoderid species, *G. vitelliloba*, by Irwin and Maguire (1979). Furthermore, heterologous tight junctions (junctions formed between cells of different types), between interstitial and parenchymal cells and between interstitial and developing vitelline cells, have been reported in *F. hepatica* by Irwin and Threadgold (1970) and Colhoun *et al.* (1998) and by Sharma and Swarnakar (1992) in the paramphistomid *Orthocoelium scolicoelium*.

Another group of digeneans is characterized by having a single type of cell within the vitellarium, which contains only vitelline cells at different stages of development. Such a vitelline structure has been found in the diplostomid *Pharyngostomoides procyonis* by Grant *et al.* (1977), the derogenid *Halipegus eccentricus* by Holy and Wittrock (1986), the microphallids *Maritrema linguilla* by Hendow and James (1989) and *M. feliui* by Świderski *et al.* (2011), the lecitodendriid *Paralecitodendrium ascidia* by Podvyaznaya (1990) and the dicrocoeliid *Dicrocoelium dendriticum* by

Chaymardanov and Tanyüksel (1995). According to Świderski *et al.* (2011), in *M. feliui*, nutritive processes, in the absence of interstitial tissue within the vitellarium of this species, are supplied by extensive, long, ramified processes of glycogen-rich parenchymal tissue which surrounds the entire vitellarium.

Discriminatory traits of the structure of the digenean vitellarium

Judging from the present and previous ultrastructural studies, the digenean vitellarium has three traits that can be considered as useful discriminatory characters of different digenean groups: (1) the number of different types of cell components within the vitellarium; (2) the presence and type of junctional complexes occurring between these cells; and (3) the isolation of the vitellarium from the surrounding tissue. For example, in two gorgoderid species, *Phyllodistomum angulatum* (present study) and *Gorgoderina vitelliloba* (see Irwin and Maguire 1979), the same basic structure of the vitellarium is found, including the presence of: (1) two types of cells (vitelline and interstitial cells, the latter with the same cytomorphology); (2) intercellular septate junctions between the interstitial cells; and (3) a fibrous layer surrounded the vitelline masses. Moreover, the vitellarium of two microphallids, *Maritrema linguilla* (see Hendow and James 1989) and *M. feliui* (see Świderski *et al.* 2011), is characterized by the presence of: (1) a single type of cell (vitellocytes); (2) the absence of junctional complexes; and (3) glycogen-rich parenchymal cells surrounding the vitellarium, the cytoplasmic extensions of which penetrate deeply into the vitelline follicles and surround each vitelline cell. Taking into account the small number of digenean species studied in relation to their vitelline structure (not just the process of vitellogenesis), it is not yet possible to comment on the above characters in terms of their usefulness as indicators of phylogeny, although there is evidence to suggest that at least the differences in the composition and amount of vitelline material (vitelline globules, lipid droplets and glycogen) are more closely related to the biology of the worms and especially the nature of their subsequent life-history. However, a broader view is required in terms of comparison with other members of the Neodermata, where, for example, interstitial cells are missing from the vitellarium of members of certain groups. A more detailed discussion on this topic is planned when we have completed similar studies on monogeneans.

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