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VERSITA

Vitellocyte ultrastructure in the cestode *Didymobothrium rudolphii* (Monticelli, 1890): possible evidence for the recognition of divergent taxa within the Spathebothriidea

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Abstract

In the spathebothriidean tapeworm *Didymobothrium rudolphii* (Monticelli, 1890) the fine structure of the vitellocytes at different stages of their development within the vitelline follicles, vitelline ducts and uterus was studied for the first time using transmission electron microscopy. The vitellocyte inclusions of *D. rudolphii* are: shell globule clusters containing tightly packed shell globules associated with a matrix of moderate electron density, glycogen granules, large electron-lucent lipid droplets (up to 3 µm in diameter), and, occasionally, a lipid droplet may occur in the nucleus of the vitellocytes. The diameter of the clusters ranges from 0.4 to 2.5 µm, the number of shell globules in the clusters varies from 8 to 45, and the size of the globules ranges from 0.12 to 0.25 µm and they are of approximately homogeneous sizes within a cluster. Most vitellocyte lipid droplets have a heterogeneous configuration with a 'cavity' inside them when they are within vitelline ducts and intrauterine eggs. Vitellocytes of the eggs contain dark concentric bodies and lipid droplets. The interstitial tissue has a syncytial structure. The morphological parameters of the diameter and shape of shell globule clusters, arrangement of shell globules in clusters, number and diameter of globules within clusters, types of lipid droplets and presence of dark concentric bodies are compared with those of two other spathebothriidean genera, *Cyathocephalus* and *Diplocotyle*. The comparative data demonstrate that vitelline material morphology has unique features in three spathenothriidean genera and may be used as evidence for the recognition of separate taxa.

Key words

Didymobothrium rudolphii, Spathebothriidea, Cestoda, vitellocytes, ultrastructure

Introduction

Spathebothriidean cestodes are enteric parasites of disparately related groups of freshwater and marine teleost (Salmoniformes, Pleuronectiformes and Scorpaeniformes) and chondrostean (Acipenseriformes) fishes. They lack external segmentation, but have multiple sets of reproductive organs (proglottids) along the body. Molecular phylogenetic analyses (for a review see Olson and Tkach 2005) indicate a basal position of the group (potentially the most basal lineage in the Eucestoda), and thus they are a pivotal group for understanding the origin of segmentation in tapeworms. The group comprises five or six monotypic taxa, the taxonomic rank of which has been controversial (e.g. Burt and Sandeman 1969, Gibson 1994, Protasova and Roytman 1995), as they exhibit few gross morphological differences.

Didymobothrium rudolphii (Monticelli, 1890) is a parasite of soleid teleosts from the western Mediterranean and the Atlantic coast of Spain and Portugal. *Didymobothrium* Nybelin, 1922 was erected on the basis of genital apertures alternating between the dorsal and ventral surfaces. Burt and Sandeman (1969) considered both *Didymobothrium* and *Diplocotyle* Krabbe, 1874 as synonyms of *Bothrimonus* Duvernoy, 1842, and Protasova and Roytman (1995) considered *Didymobothrium* a *nomen dubium* and *D. rudolphii* a potential synonym of *Diplocotyle olrikii* Krabbe, 1874. On the other hand, Gibson (1994) recognised three independent genera: *Didymobothrium* from soleids, *Diplocotyle* from salmonids and pleu-

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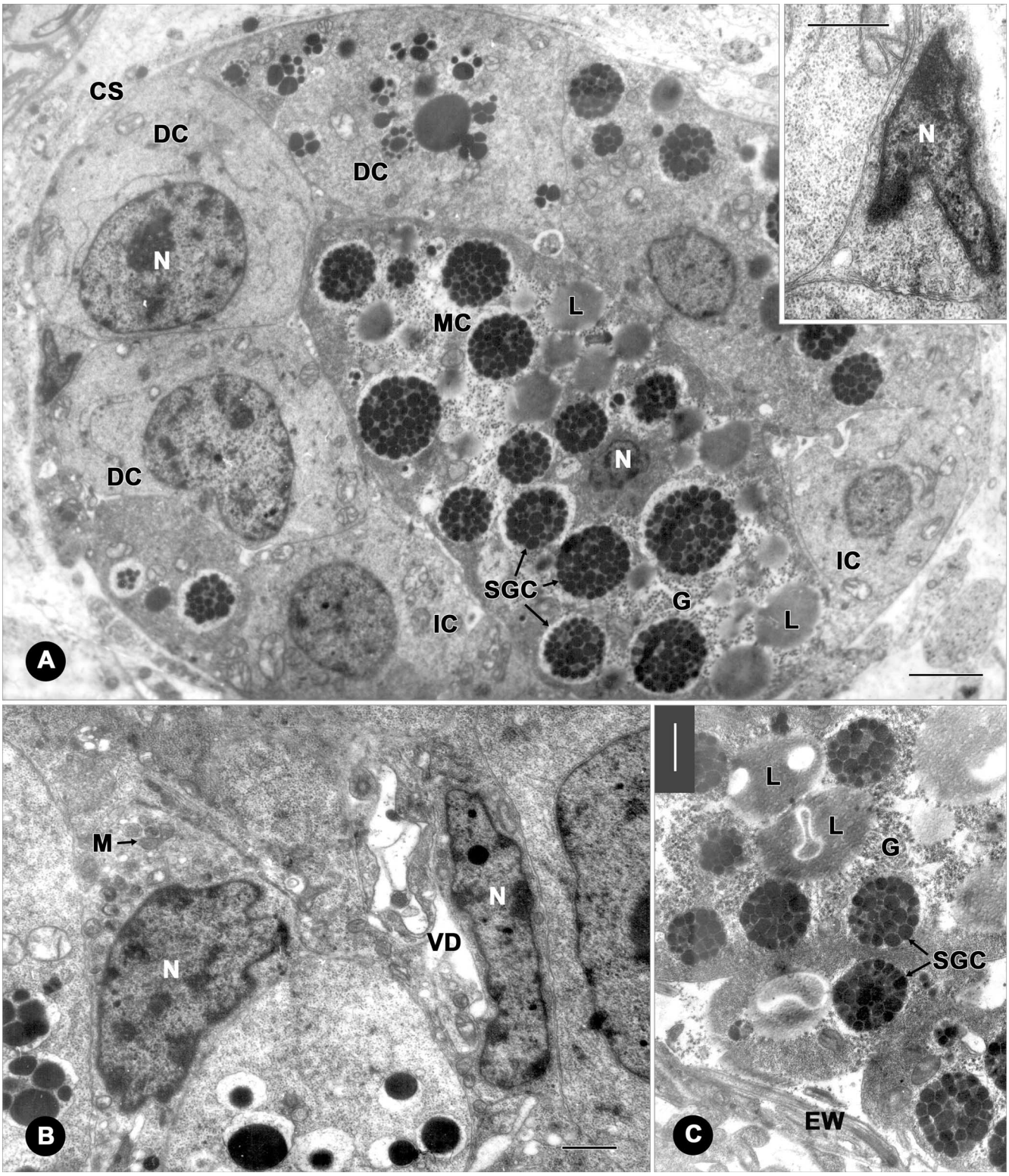

Fig. 1. General topography of a vitelline follicle and vitelline ducts of *Didymobothrium rudolphii*. **A.** Different stages of vitellocyte development (inset: higher magnification showing peripheral nucleus of interstitial tissue). **B.** Vitelline duct and central nucleus of the interstitial tissue. **C.** Vitelline material within a vitelline duct. Scale bars = 3 μm (A), 0.5 μm (inset and B), 1 μm (C). **Abbreviations to all figures:** CB – concentric body, CS – cytoplasmic sheath, DC – developing cell, E – egg, ES – eggshell, EW – epithelial wall, FVC – fragments of the vitelline cytoplasm, G – glycogen, GC – Golgi complex, GER – granular endoplasmic reticulum, IC – immature cell, L – lipid droplet, M – mitochondrion, MC – mature cell;, N – nucleus, OV – ovum, SG – shell globule, SGC – shell globule cluster, VD – vitelline duct, UW – uterine wall

ronectids and *Bothrimonus* from acipenserids based on the form of the scolex and the dorsoventral arrangement of the genital pores. Adding to the disagreement over the validity and taxonomic rank of spathebothriidean taxa, it is likely that each may comprise a complex of cryptic species, as was shown by the allozyme studies by Renaud and Gabrion (1988) in which *D. rudolphii* (referred to as *Bothrimonus nylandicus* Schneider, 1902) from soleids in the western Mediterranean and eastern Atlantic was found to comprise two sympatric, sibling species. More recently, Marques *et al*. (in review) demonstrated the presence of two distinct genotypes of *D. rudolphii* along the Portuguese coast using two molecular markers (large-subunit and ITS rDNA) that correlated with statistically significant differences in the overall length and width of the otherwise cryptic forms. As distinguishing such forms in the field is virtually impossible, it is almost certainly the case that previous reports were based on confounded identifications.

In the present work, the ultrastructure of the vitellocytes of *D. rudolphii* is studied at different stages of their development both within the vitelline follicles and in the different female ducts. Although vitellogenesis tends to follow a similar pattern throughout the Eucestoda, the composition and amount of vitelline material, differences in the morphology of shell globule clusters, the types of lipid droplets and the localisation of these inclusions in the cytoplasm of the vitellocytes vary among genera and species (Świderski and Mackiewicz 1976; Bruňanská 1997; Świderski and Xylander 2000; Świderski *et al*. 2004, 2005, 2006a, b). Such differences were also shown recently in the spathebotriidean genera *Cyathocephalus* and *Diplocotyle* (see Bruňanská *et al*. 2005).

The aim of this study is: (a) to characterize the ultrastructure of the vitellocytes in *D. rudolphii*, with particular attention to the vitelline material morphology within mature vitellocytes of the vitelline follicles, vitelline ducts and intrauterine eggs; (b) to compare the morphology of the vitelline material among spathebothriideans and other parasitic flatworms; and (c) to establish whether or not unique features of the vitelline material morphology may be used as further evidence for the recognition of divergent taxa.

Materials and methods

Adult *Didymobothrium rudolphii* were collected from the intestine of *Solea lascaris* from the Atlantic coast of northern Portugal off Aveiro during September, 2005. All specimens examined correspond to the 'common' form of *D. rudolphii* as defined by Marques *et al*. (in review). The worms were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 20 days at 5°C. Postfixation followed in 1% osmium tetroxide in 0.1 M phosphate buffer for 1 h at 5°C. The material was then dehydrated in a graded series of ethanol and acetone and embedded in Araldite and Epon. Ultrathin sections were stained with uranyl acetate and lead citrate. They were examined in a JEM-100 C transmission electron microscope operating at 80 kV.

Results

Vitelline follicles

The numerous vitelline follicles of *Didymobothrium rudolphii* are arranged as a continuous series in the cortical parenchyma with some solitary follicles in the medullary parenchyma. Within a follicle, the immature cells tend to be located towards the periphery (Fig. 1A). As well as vitelline cells, each follicle contains interstitial tissue, and each vitellocyte is completely surrounded by the interstitial cytoplasm (Fig. 1A, B). In the centre of each follicle is a small vitelline duct (Fig. 1B). The vitelline follicles are surrounded by a thin cytoplasmic sheath delimited by small amounts of fibrous material (Fig. 2B).

Interstitial tissue

In the centre of each follicle is one irregular nucleus with a dense area of heterochromatin, and along the follicle sheath there are several (2–3) additional nuclei (Fig. 1A, B). The cytoplasm of this syncytium surrounds each vitelline cell (Fig. 1A, B). The syncytial cytoplasm includes free ribosomes, mitochondria and small vesicles.

Vitellocyte development

Immature cells possess a small amount of cytoplasm filled with free ribosomes and mitochondria around the large nucleus (Figs 1A and 2A).

Maturing vitelline cells are highlighted by the appearance of GER and Golgi complexes in the cytoplasm. The first membrane-bound individual globules associated with the vesicles of the Golgi complexes begin to appear. Single rounded, electron-dense globules (ca. 0.3 μm in diameter) within the vesicles occur close to the cell plasmalemma (Fig. 2B). The single globules increase in quantity and occur throughout the cytoplasm (Fig. 2C). The aggregation of globules in the form of membrane-limited clusters also occurs. At this stage of vitellocyte development, the clusters are made up 2–3 shell globules of a smaller size than isolated individual globules (Fig. 2C, D). A few lipid droplets of ca. 0.75 μm in diameter are found in the cell cytoplasm; on rare occasions, single lipid droplets may be observed inside concentric rows of GER (Fig. 2E).

Mature cells consist of little else than a central nucleus surrounded by parallel arrays of GER (Figs 1A and 3A). Mature vitelline cells display mixed deposits of shell globule clusters, large lipid droplets and glycogen that are distributed evenly throughout most of the cell cytoplasm. Within the clusters shell globules are embedded in a matrix filled with material of moderate electron density (Fig. 3C-E). The number of shell globules contained within a single cluster varies widely, from 8 to 45 (Fig. 3A, C), but most clusters have more then 35 individual globules inside them. The diameter of the rounded clusters ranges from 0.4 to 2.5 μm, and the individual globules in clusters may measure from 0.12 to 0.25 μm. Individual shell globules within a moderately electron-dense matrix of the clusters are of approximately homogeneous size in a single

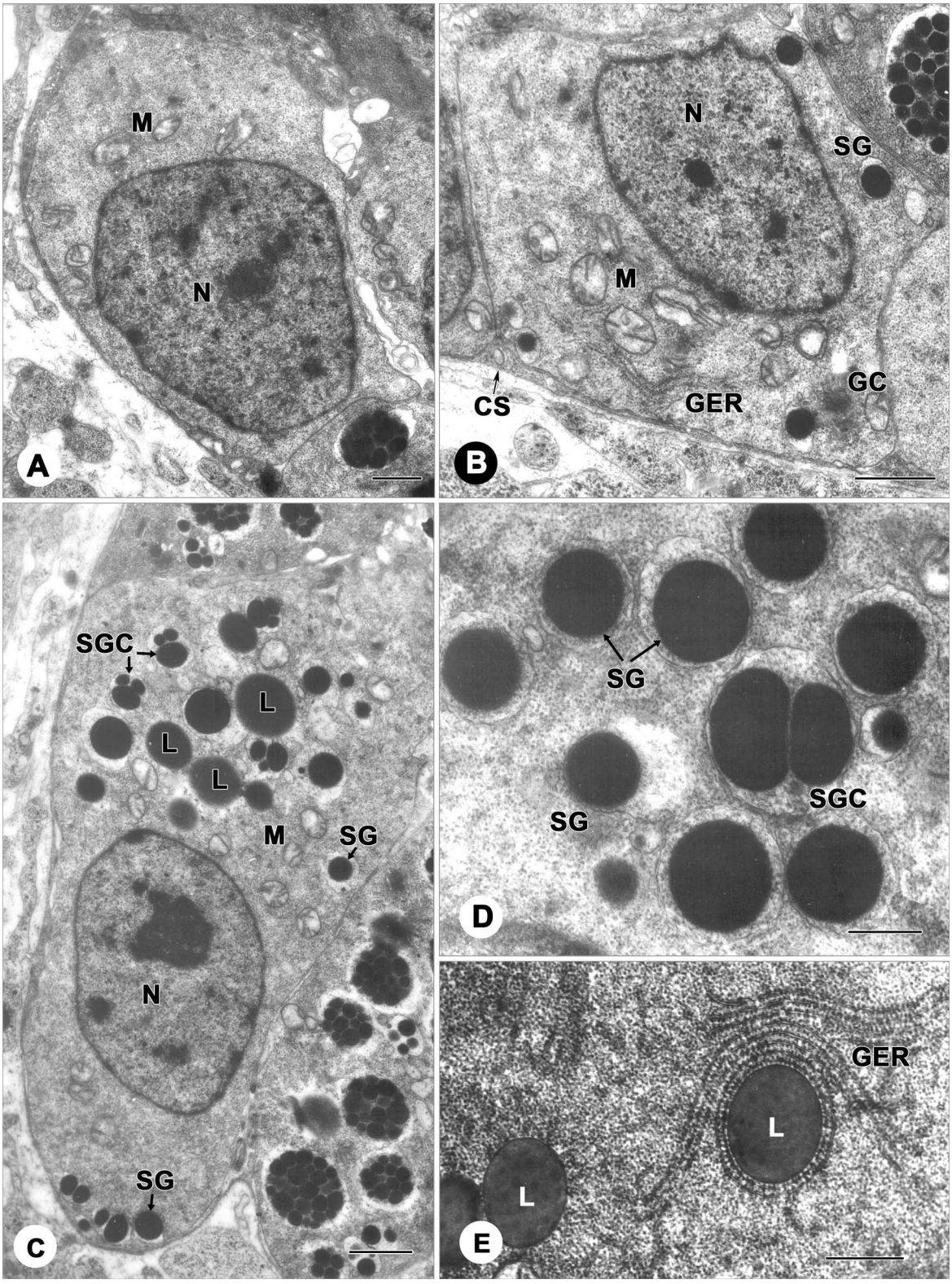

Fig. 2. Developing vitelline cells of *Didymobothrium rudolphii*. **A.** Immature cell. **B.** Early stage of vitelline cell maturation and appearance of the first individual shell globules. **C.** Advanced stage of vitelline cell maturation and appearance of the first shell globule clusters. **D.** Individual shell globules and beginning of their aggregation in clusters. **E.** Formation of lipid droplet inside concentric rows of GER. Scale bars = 1 μm (A, B), 2 μm (C), 0.5 μm (D, E)

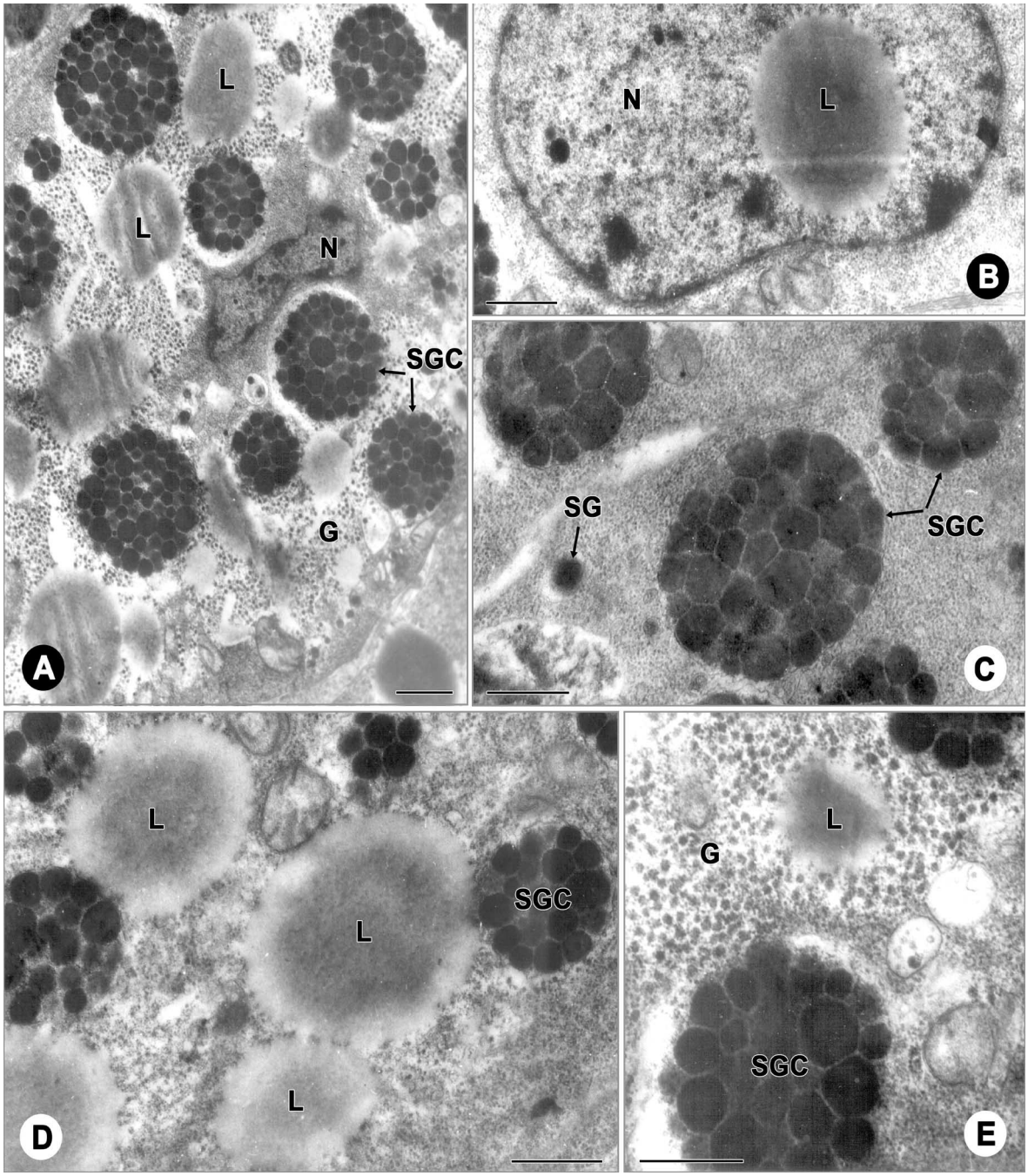

Fig. 3. Mature vitellocytes of *Didymobothrium rudolphii*. **A.** Three types of inclusions. **B.** Lipid droplet within the nucleus. **C.** Tightly packed shell globules within the moderately electron-dense matrix of the clusters. **D.** Pale lipid droplets. **E.** Glycogen granules in the vitelline cytoplasm. Scale bars = 1 μm (A), 0.5 μm (B-E)

cluster. There are large, electron-lucent lipid droplets, the majority of which are up to 3 μm in diameter and similar but smaller droplets (0.8 μm in diameter) (Fig. 3D). Occasionally, a single electron-lucent lipid droplet (1.5 μm in diameter) may be present in the nucleus (Fig. 3B). Large amounts of glycogen granules are distributed throughout much of the cytoplasm (Fig. 3A, E).

Vitellocytes within the female ducts

Within the vitelloduct lumen the vitelline cytoplasm contains clusters similar in morphology to those in the cytoplasm of the mature vitellocytes, including large electron-lucent lipid droplets, most of which have a heterogeneous configuration with a 'cavity' inside them, and glycogen granules that surround these inclusions (Fig. 1C).

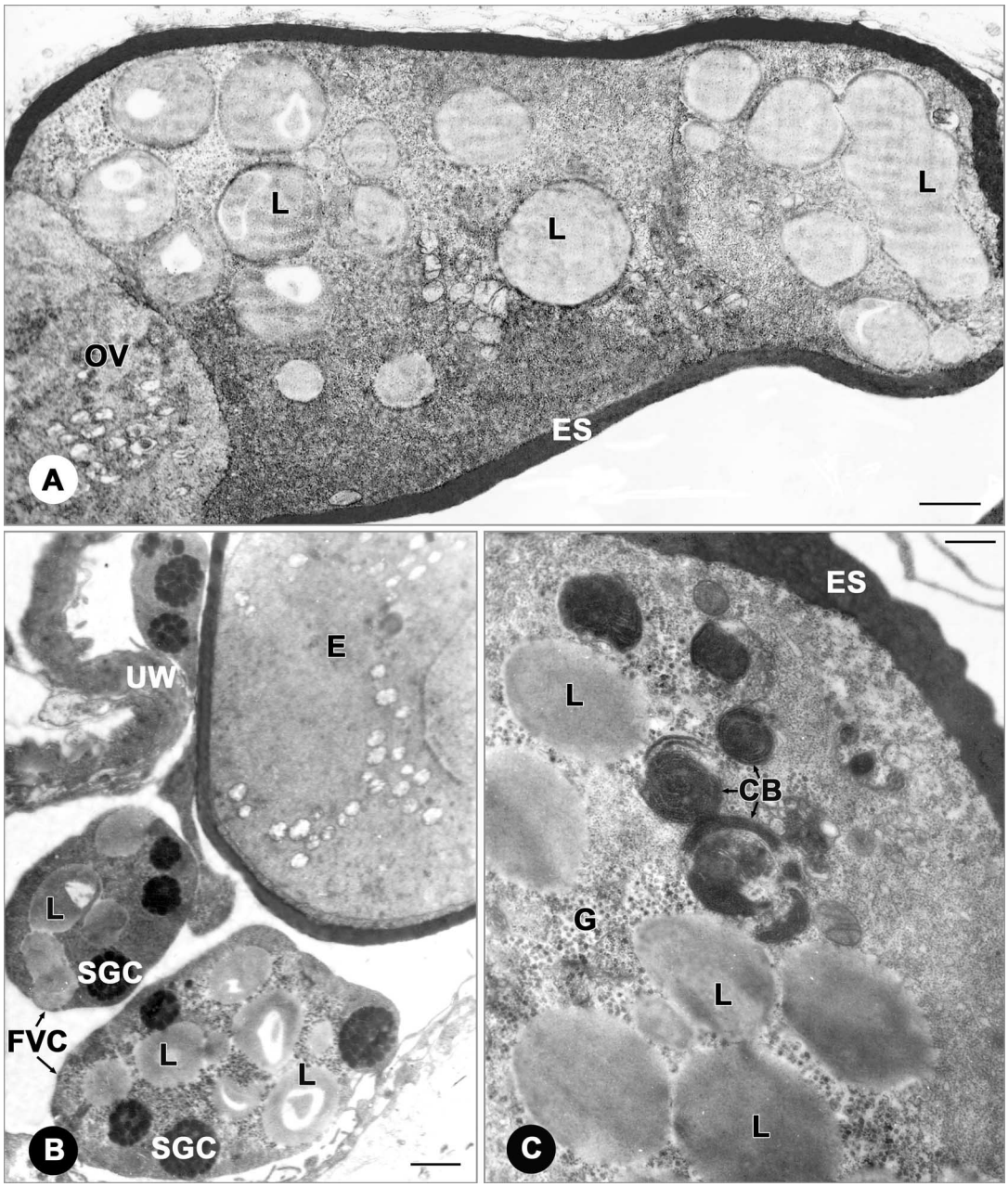

Fig. 4. Vitelline inclusions in the eggs of *Didymobothrium rudolphii*. **A.** Part of the egg showing pale lipid droplets in the vitelline cytoplasm. **B.** Uterine lumen with the egg and unutilised vitelline material within which are shell globules clusters, lipid droplets and glycogen. **C.** Part of vitelline cytoplasm with pale lipid droplets, glycogen and concentric bodies inside the egg. Scale bars = 2 µm (A), 1 µm (B), 0.5 µm (C)

The uterine lumen is filled with polylecithal eggs in a formed eggshell and within which are numerous vitellocytes and an ovum. The egg vitelline cytoplasm contains large electron-lucent lipid droplets, some of which also exhibit a 'cavity', glycogen granules and dark concentric bodies with concentric configurations (Fig. 4C). The uterine lumen also has numerous fragments of vitelline cytoplasm including vitelline material of similar morphology to that found in the vitelline ducts (Fig. 4B).

Discussion

Vitelline material morphology in three spathebothriidean genera

Based on the present study of the vitellocyte structure of *Didymobothrium rudolphii*, investigations of vitellogenesis in *Cyathocephalus truncatus* and *Diplocotyle olrikii* by Bruňanská *et al*. (2005), and unpublished data (LGP) on *C. trunca-*

tus and *D. olrikii*, ultrastructural differences exist in all of the characterized features of the vitelline inclusions among the three spathebothriidean taxa (Table I). Firstly, shell globule clusters may contain (1) loosely packed shell globules situated in an electron-lucent matrix (*Diplocotyle*), (2) tightly packed shell globules associated with a matrix of moderate electron density (*Didymobothrium*), or (3) closely packed globules which do not coalesce (*Cyathocephalus*) (including differences in the quantity and morphology of the globules inside the vitelline clusters (see Table I). Secondly, the mature vitellocytes may contain electron-lucent lipid droplets (*Didymobothrium)*, electron-dense droplets (*Diplocotyle*), or both types of lipid droplets (*Cyathocephalus*). Thirdly, the dark concentric bodies occur in the vitelline cytoplasm of the vitelline follicles, vitelline ducts and within the eggs in *C. truncatus*, in the vitelline duct lumen and within the eggs in *D. olrikii* and within the eggs in *D. rudolphii*.

Dark concentric bodies

For the first time in tapeworms, dark concentric bodies ('lamellar bodies') were described in the mature vitellocytes of the vitelline follicles of *C. truncatus* (Bruňanská *et al*. 2005). Similar bodies were described in the vitellocytes of two trematode species as 'membranous whorls' (see Grant *et al*. 1977) and as 'labyrinthine shell globules' or 'yolk globules' in *Fasciola hepatica* (L.) (see Björkman and Thorsell 1963, Irwin and Threadgold 1970), and as 'endoplasmic reticulum whorls' in *Schistosoma mansoni* Sambon, 1907 (see Erasmus 1973). Granules with a concentric lamellar configuration were also mentioned as occurring in the vitellocytes of the monocotylid monogenean *Calicotyle kroyeri* Diesing, 1850 (see Halton *et al*. 1974). The occurrence of this type of granule in the vitelline cells of different platyhelminth groups has been explained either as an additional source of nutrition or as residual bodies (Irwin and Threadgold 1970, Erasmus 1973). According to Halton *et al*. (1974), the appearance of such a vitelline body type coincides with the breakdown of GER and the start of glycogenesis. In *D. rudolphii* the dark concentric bodies are observed as principal bodies in vitellocytes of intrauterine eggs, in *Cyathocephalus truncatus* they occur as sparse clusters or sparse bodies in the vitellocytes from the vitelline follicles, the vitelline ducts and as principal bodies in intrauterine eggs, and in *D. olrikii* they are found in the vitelline duct lumen as sparse bodies and within the eggs as principal bodies. We suggest that in the Spathebothriidea dark concentric bodies represent a phase of shell globule transformation during the process of egg formation. Unutilised vitelline material for egg formation in the uterine lumen of spathebothriidean genera is similar to that from their vitelline follicles. The differences in the appearance of the dark concentric bodies in the vitellocytes of the three spathebotriideans may be related to differences in the timing of the disruption of vitelline material during the process of egg formation.

Comparisons with other cestode groups

In comparison with other parasitic flatworms, the developmental stages of vitellocytes in the Spathebothriidea are similar in general to those observed throughout the Eucestoda and in some trematode and monogenean species (see Björkman and Thorsell 1963, Irwin and Threadgold 1970, Erasmus 1973, Halton *et al*. 1974, Grant *et al*. 1977, Erasmus *et al*. 1982, Fairweather *et al*. 1988, Świderski and Xylander 2000).

In the Spathebothriidea, the interstitial tissue of the vitelline follicles has a syncytial structure with one nucleus in the centre of each follicle and several nuclei (two or three) at the periphery. The syncytial structure of the interstitial tissue has also been noted in the Caryophyllidea (Świderski and Mackiewicz 1976) and Pseudophyllidea (Levron *et al*. 2006, in press).

Shell globule clusters, lipid droplets and glycogen granules are present in the mature vitellocytes of spathebothrideans. Such vitelline inclusions are also characteristic of the Amphilinidea, Gyrocotylidea, Pseudophyllidea and Trypanorhyncha, as well as of some Monogenea (Halton *et al*. 1974)

Table I. Comparison of the ultrastructural characters of vitelline material in three spathebothriidean species

Characteristic	*Didymobothrium rudolphii*	*Diplocotyle olrikii*	*Cyathocephalus truncatus*
Diameter of clusters	0.4–2.5 µm	0.7–1.95 µm	0.6–2.0 µm
Shape of egg shell globule clusters	rounded	rounded	oval
Arrangement of shell globules in clusters	tightly packed within moderately electron-dense matrix	loosely packed within electron-lucent matrix	closely packed which do not coalesce
Number of shell globules within a single cluster	8–45	3–30	4–20
Diameter of individual globules within clusters	0.12–0.25 µm, of approximately homogeneous size in one cluster	0.25–0.75 µm, of heterogeneous size in one cluster	0.2–0.9 µm, of heterogeneous size in one cluster
Location of dark concentric bodies	intra-uterine eggs	vitelline ducts, intra-uterine eggs	vitelline cells, vitelline ducts, intra-uterine eggs
Types of lipid droplets	electron-lucent with 'cavity' in female ducts	electron-dense	electron-lucent and electron-dense

and some Trematoda (Björkman and Thorsell 1963, Irwin and Threadgold 1970, Erasmus 1973, Grant *et al*. 1977). However, quantitative and qualitative differences in the composition of these inclusions have been indicated in the vitellocytes of all eucestode groups studied (Świderski and Xylander 2000; Świderski *et al*. 2006a, b).

Rare characteristics of vitellocyte structure: intranuclear lipid droplets

The unusual appearance of a single lipid droplet in the nucleus of vitellocytes has only been reported in the tetraphyllidean species *Echeneibothrium beauchampi* Euzet, 1959 (see Mokhtar-Maamouri and Świderski 1976). We suggest two ways in which lipid droplets get into the nucleus: the first may be a result of the fact that they are synthesized in the endoplasmic reticulum. This organelle possesses the ability to actively transport different compounds and, in particular, lipid droplets in the cells of the intestinal epithelium of animals, and it has a structural and functional relationship with the nuclear membrane (Swanson and Webster 1980). The second may be the result of the penetrability of the nuclear membrane. It is known that different compounds enter the nucleus from the cell cytoplasm through pores in the nuclear membrane and there are cytological observations *in vitro* demonstrating the transport of large globules across nuclear pores from the cell cytoplasm into the nucleus by extension of the globules (Chentsov 1984). Lipid droplets also have the ability to form elongate filaments, as has been observed in *C. truncatus*, where, in prominent lipid droplets, long thin lamellar processes are formed during the association of the vitellocytes with fertilized oocytes in the ovovitelline duct (Poddubnaya *et al*. 2005).

The presence of intranuclear (in the form of glycogen granules) and cytoplasmic inclusions (shell globule clusters and glycogen) has been shown for caryophyllidean vitellocytes (Mackiewicz 1968, Świderski and Mackiewicz 1976, Świderski and Xylander 2000, Świderski *et al*. 2004). According to the above authors, progenesis and the presence of bottom-dwelling annelids as intermediate host might be determinant factors for the appearance of such types of nutritive reserves in the Caryophyllidea. However, in view of observations on parallelism and analogies between both the adaptations to the parasitic way of life in different groups of cestodes and the nature of the nutritive reserves of their vitellocytes (Świderski and Xylander 2000), it is worth noting that the life cycle of spathebothriidean tapeworms also includes bottom-dwelling invertebrates (amphipods) as their intermediate host. Furthermore, progenesis may be common in the group, as both *D. olrikii* and *C. truncatus* can produce gravid worms in amphipods (Gibson and Valtonen 1983, Leontovich and Valovaya 1989, Protasova and Roytman 1995, Okaka 2000). As in the caryophyllideans, spathebothriidean eggs are gradually released into the lumen of the intestine and develop after deposition in water, embedded within the host's faecal material (Protasova and Roytman 1995). Once in the water, such eggs remain there for a long time, until embryonation of the oncosphere is complete and they become infective to the amphipod host. Although caryophyllideans and spathebothrideans have the same pattern of egg production and development and the capacity to accumulate nuclear inclusions, these two orders have different types of vitelline material in their vitellocytes.

Thus, the evolutionary significance of the presence of intranuclear vitelline inclusions remains obscure for both cestode groups. We only suggest that in the Spathebothriidea and other groups the presence of nuclear inclusions in the vitellocytes is a plesiomorphic trait.

Evolutionary and taxonomic comments

The different pattern of the vitelline cytoplasmic inclusions in the Caryophyllidea and the Spathebothriidea may support the idea that these tapeworms form independent lineages within the Eucestoda, as was recognized in the most recent systematic treatment of the group (Khalil *et al*. 1994) and subsequently by molecular phylogenetic analyses (Olson and Caira 1999, Kodedová *et al*. 2000, Olson *et al*. 2001, see also Olson and Tkach 2005). On the other hand, comparing the results of vitellocyte structure of the Spathebothriidea with previous such studies of different members of the Cestoda (see Świderski and Xylander 2000) suggests a close similarity between the vitellocyte morphology of the Spathebothriidea, the Pseudophyllidea and the Gyrocotylidea.

The ultrastructural observations of the nature of the vitelline material of three spathebothriidean genera (see Table I) show clear differences in the morphology of the vitelline cytoplasmic inclusions distinctive enough to represent criteria that can be used for the recognition of distinct taxa and, potentially, when more species are studied, as indicators of generic boundaries.

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