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VERSITA

Ultrastructure and cytochemistry of vitellogenesis and the vitellocytes of the bothriocephalidean cestode *Clestobothrium crassiceps* (Rudolphi, 1819), a parasite of the teleost fish *Merluccius merluccius* (L., 1758) (Gadiformes, Merlucciidae)

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Abstract

Vitellogenesis and vitellocytes of the bothriocephalidean cestode *Clestobothrium crassiceps* (Rudolphi, 1819), a parasite of the teleost fish *Merluccius merluccius* (L., 1758), were studied by means of transmission electron microscopy (TEM) and cytochemistry. During vitellogenesis, four developmental stages were distinguished at the TEM level: (I) a stem cell stage of the gonial type; (II) an early differentiation stage, predominantly exhibiting lipid and protein synthetic activity; (III) an advanced differentiation or vitellocyte maturation stage, primarily exhibiting active glycogenesis still accompanied by an increase in lipid accumulation; and (IV) a mature vitellocyte stage. Vitellogenesis involves: (1) an increase in cell volume; (2) an extensive development of parallel, frequently concentrically arranged, cisternae of granular endoplasmic reticulum (GER) that produce dense, proteinaceous shell-gobules; (3) the development of Golgi complexes engaged in the packaging of this material; (4) an accelerated accumulation of unsaturated and saturated lipid droplets, along with their continuous enlargement and fusion; (5) the formation of individual β-glycogen particles and α-glycogen rosettes and their accumulation in the form of glycogen islands scattered among lipid droplets in the cytoplasm of maturing and mature vitellocytes; and (6) the rapid accumulation of large, saturated lipid droplets accompanied by dense accumulations of α- and β-glycogen along with proteinaceous shell-globules or shell-globule clusters in the peripheral layer during the advanced stage of vitellocyte maturation. Vitellogenesis in *C. crassiceps* generally resembles that previously described for three other bothriocephalideans, but differs from that of other cestode orders. Cytochemical staining with periodic acid-thiocarbazide-silver proteinate for glycogen indicates a strongly positive reaction for β-glycogen particles and α-glycogen rosettes, which form several large glycogen accumulations around the large, saturated lipid droplets of maturing and mature vitellocytes. Some hypotheses concerning the interrelationships between patterns of vitellogenesis, the possible modes of egg formation, embryonic development and life cycles in cestodes, and their phylogenetic implications are commented upon.

Keywords

Cestoda, Bothriocephalidea, *Clestobothrium crassiceps*, vitellogenesis, vitellocytes, ultrastructure, cytochemistry

Introduction

The cestode genus *Clestobothrium* comprises five species, *C. crassiceps*, the types-species, *C. gibsoni*, *C. neglectum*, *C. spendidum* and *C. cristinae*. *Clestobothrium* has recently been transferred from the suppressed order "Pseudophyllidea" to the newly erected order Bothriocephalidea by Kuchta *et al*. (2008a, b). In their studies, involving morphological, molecular and eco-

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logical data, Kuchta *et al*. (2008a, b) showed that the order "Pseudophyllidea" in fact consists of two unrelated clades. Members of the order Bothriocephalidea are intestinal parasites of teleost fishes and comprise 46 genera distributed in four families (Bothriocephalidae, Echinophallidae, Philobythiidae and Triaenophoridae) (Kuchta *et al.* 2008b). *Clestobothrium* belongs to the Bothriocephalidae, which includes 13 other genera.

Several TEM studies have been published on the ultrastructure and differentiation of vitellocytes in cestodes (for a review: see Świderski and Xylander 2000). Vitellocytes in cestodes have two important functions, i.e. eggshell formation and the nourishment of the early embryo (Świderski *et al*. 1970a, b; Świderski and Xylander 1998, 2000). During cestode evolution, both of these two functions have been intensified or much reduced in different taxa, depending on the type of their embryonic development, degree of ovoviviparity and life cycles (Świderski and Mokhtar 1974; Świderski and Mackiewicz 1976; Świderski and Xylander 2000; Świderski *et al.* 2000, 2004a, b, 2005, 2006a, b, 2007). Among the bothriocephalidean cestodes, as far as we are aware, only three species, all from different families, have been studied in this way: *Bothriocephalus clavibothrium* by Świderski and Mokhtar (1974), *Triaenophorus nodulosus* by Korneva (2001) and *Paraechinophallus japonicus* by Levron *et al*. (2007). In addition to providing data on the reproductive biology, the usefulness of ultrastructural data on vitellogenesis and vitellocytes for elucidating phylogenetic relationships within the platyhelminth groups has been commented upon by several authors (Świderski and Mokhtar 1974; Świderski and Mackiewicz 1976; Świderski and Xylander 1998, 2000; Bruňanská *et al*. 2005).

The aim of the present study is to describe ultrastructural and cytochemical aspects of the vitellogenesis and functional ultrastructure of the mature vitellocytes of *Clestobothrium crassiceps* (Rudolphi, 1819), a parasite of gadoid fishes, to compare it with the results of previous reports of vitellogenesis and vitellocyte structure in other cestode taxa and to discuss any possible phylogenetic implications.

Materials and methods

Live adult specimens of *Clestobothrium crassiceps* (Rudolphi, 1819) were collected from the intestine of the teleost fish *Merluccius merluccius* (L., 1758) (Gadiformes, Merlucciidae) caught off Roses, Girona, Spain. The live worms were first placed in a 0.9% NaCl solution, and mature proglottids were fixed in cold (4°C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.4 for a minimum of 2 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4, postfixed in cold (4°C) 1% osmium tetroxide with K_4FeCn_6 in the same buffer for 1 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.4, dehydrated in an ethanol series and propylene oxide, and finally embedded in Spurr's resin. Ultrathin sections were obtained using a Reichert-Jung Ultracut E ultramicrotome, placed on copper grids and double-stained with uranyl acetate and lead citrate. They were examined using a JEOL 1010 TEM operated at an accelerating voltage of 80 kV.

The presence of glycogen was detected at the ultrastructural level by means of the specific cytochemical method of Thiéry (1967). Ultrathin sections collected on gold grids were treated with the periodic acid-thiocarbohydrazide-silver proteinate (PA-TCH-SP) as follows: 30 min in 10% periodic acid (PA), rinsed in distilled water, 24 h in thiocarbohydrazide (TCH), rinsed in acetic solutions and distilled water, 30 min in 1% silver proteinate (SP) in the dark, and rinsed in distilled water.

Results

General topography of the vitelline follicles

Vitelline follicles in *Clestobothrium crassiceps* are situated in the cortical parenchyma and are continuous around the lateral margins of the proglottids. Each mature follicle contains vitellocytes at different stages of vitellogenesis and the so-called 'interstitial syncytium' (Figs 1, 3, 4 and 6). During vitellogenesis, four developmental stages can be distinguished at the TEM level: (I) a stem cell stage of the gonial type; (II) an early differentiation stage, predominantly exhibiting lipid and protein synthetic activity; (III) an advanced differentiation or vitellocyte maturation stage, primarily exhibiting active glycogenesis, which is still accompanied by an increase in lipid accumulation; and (IV) the mature vitellocyte stage. This subdivision into four stages, although arbitrary, is used here only to facilitate the description of the developmental process. It is clear that the cytodifferentiation of vitellocytes is a continuous process and that gradual changes occur throughout these four stages. Generally, the stem or gonial cells are situated at the periphery of the vitelline follicles, whereas the differentiating and mature vitellocytes occur in their central regions (Figs 1 and 2).

Interstitial syncytium

The interstitial syncytium of the vitelline follicles (Figs 1, 3, 4 and 6) is characterised by elongate, flattened or spindle-shaped nuclei of a vesicular type, with small nucleoli (Fig. 6) and a few small heterochromatin islands adjacent to their nuclear membranes (Fig. 8). Several small mitochondria are present within the thin layer of perinuclear cytoplasm (Figs 4 and 9). Elongate cytoplasmic processes of the interstitial cells surround the periphery of the vitelline follicles and penetrate deeply between the differentiating vitellocytes. These processes contain elongate, densely packed accumulations of glycogen and a few small lipid droplets (Figs 1, 4 and 8).

Stage I: *Gonial stem cell stage*

Stem cells of the gonial type, measuring *c*. 7–9.5 μm in diameter, are generally situated at the periphery of the follicles.

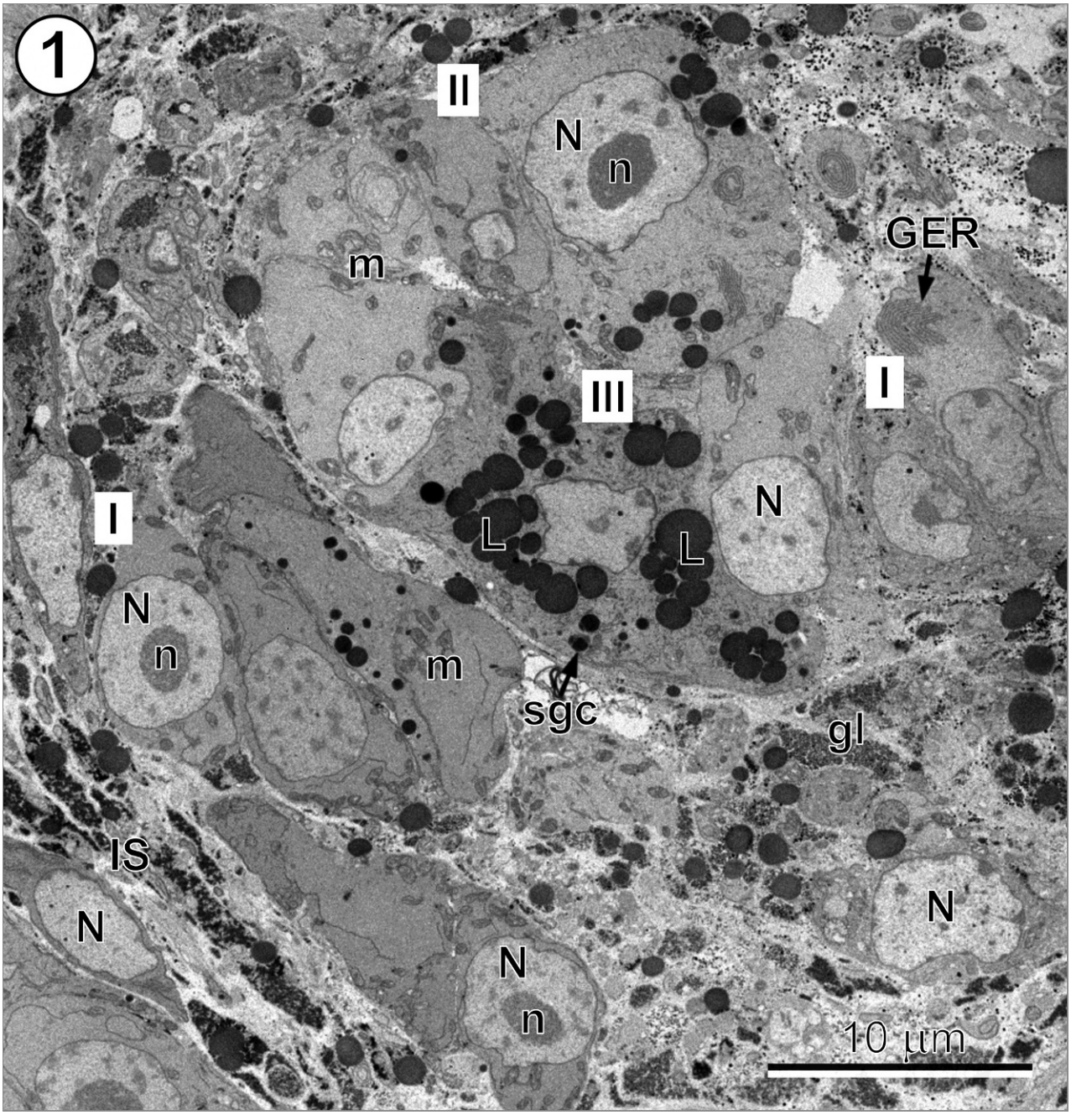


Fig. 1. General topography of the peripheral part of the vitelline follicle showing the localisation of vitellogenesis developmental stages I–III within the follicle: I – stem cell of gonial type; II – early stage of vitellocyte differentiation; III – advanced stage of vitellocyte differentiation. **Abbreviations to all figures:** GC – Golgi complex, GER – granular endoplasmic reticulum, gl – glycogen, Hch – heterochromatin islands, IS – interstitial syncytium, L – lipid droplet, m – mitochondria, N – nucleus, n – nucleolus, sgc – shell-globule clusters, I–IV – consecutive stages of vitellogenesis: I stem cell of gonial type; II early and III advanced stages of vitellocyte differentiation; IV mature vitellocyte

They exhibit a high nucleo-cytoplasmic ratio and a large concentration of free ribosomes in a relatively thin layer of granular cytoplasm containing a few mitochondria and a very few short cisternae of GER (Figs 1, 3 and 4). The large nuclei contain prominent spherical nucleoli and numerous large islands of heterochromatin adjacent to the nuclear envelope (Figs 1, 3 and 4).

Stage II: *Early differentiation stage – predominantly protein synthesis for shell-globule formation and the beginning of a rapid accumulation of lipid droplets*

At this stage, cells are distinguished by their increased size, *c*. 10–12 µm in diameter, and by an increased number of cytoplasmic organelles, particularly long, parallel cisternae of GER,

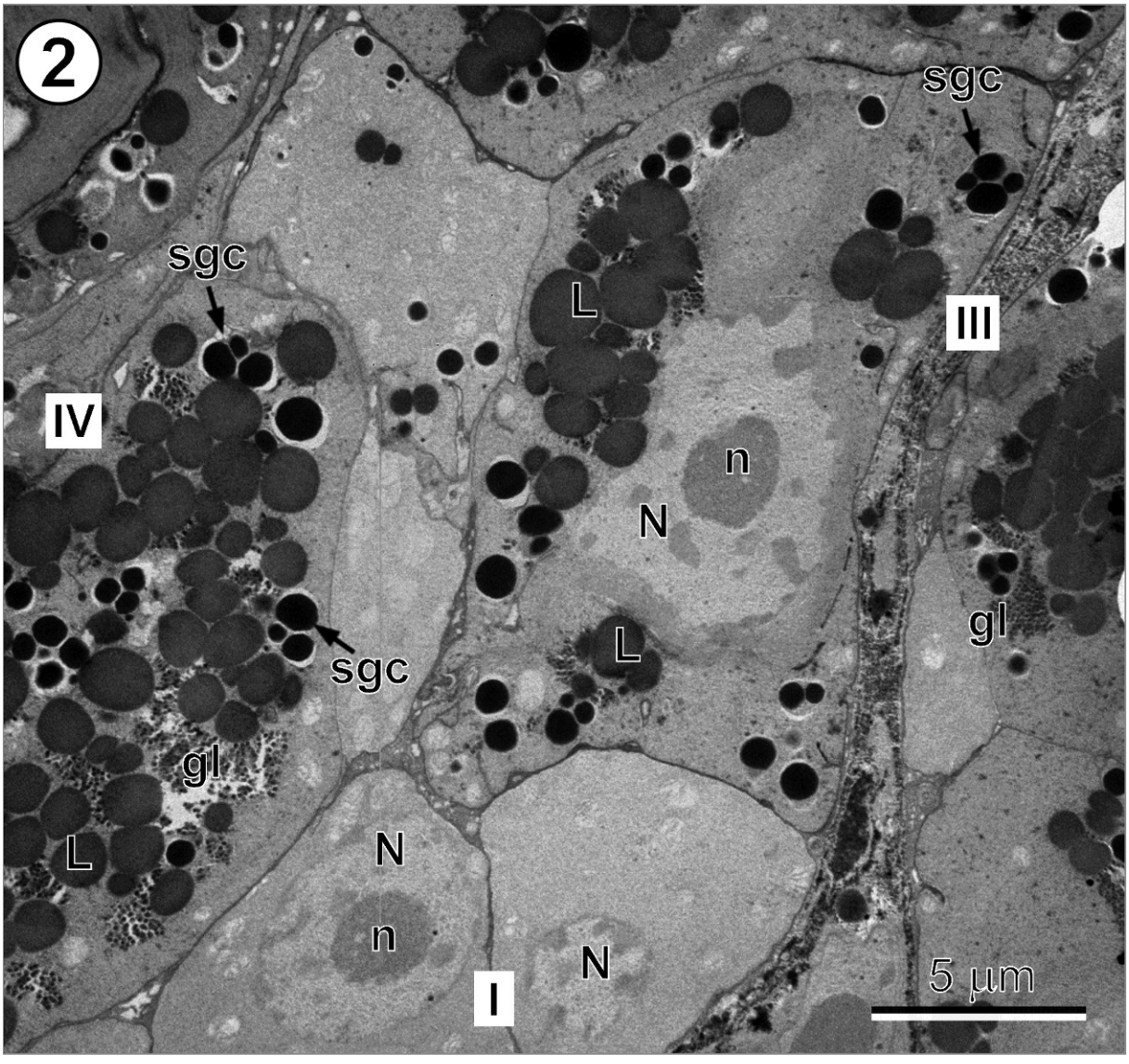
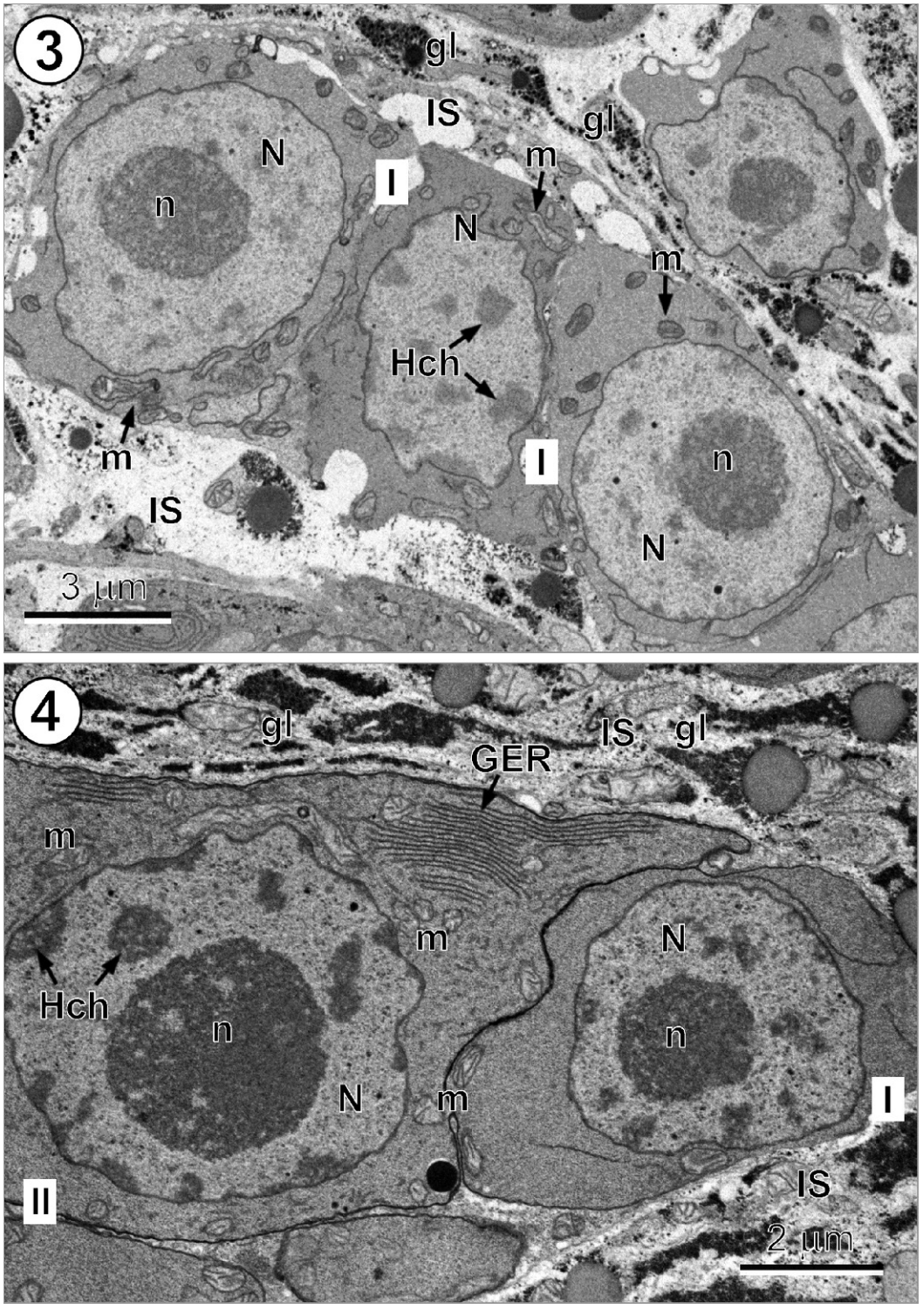
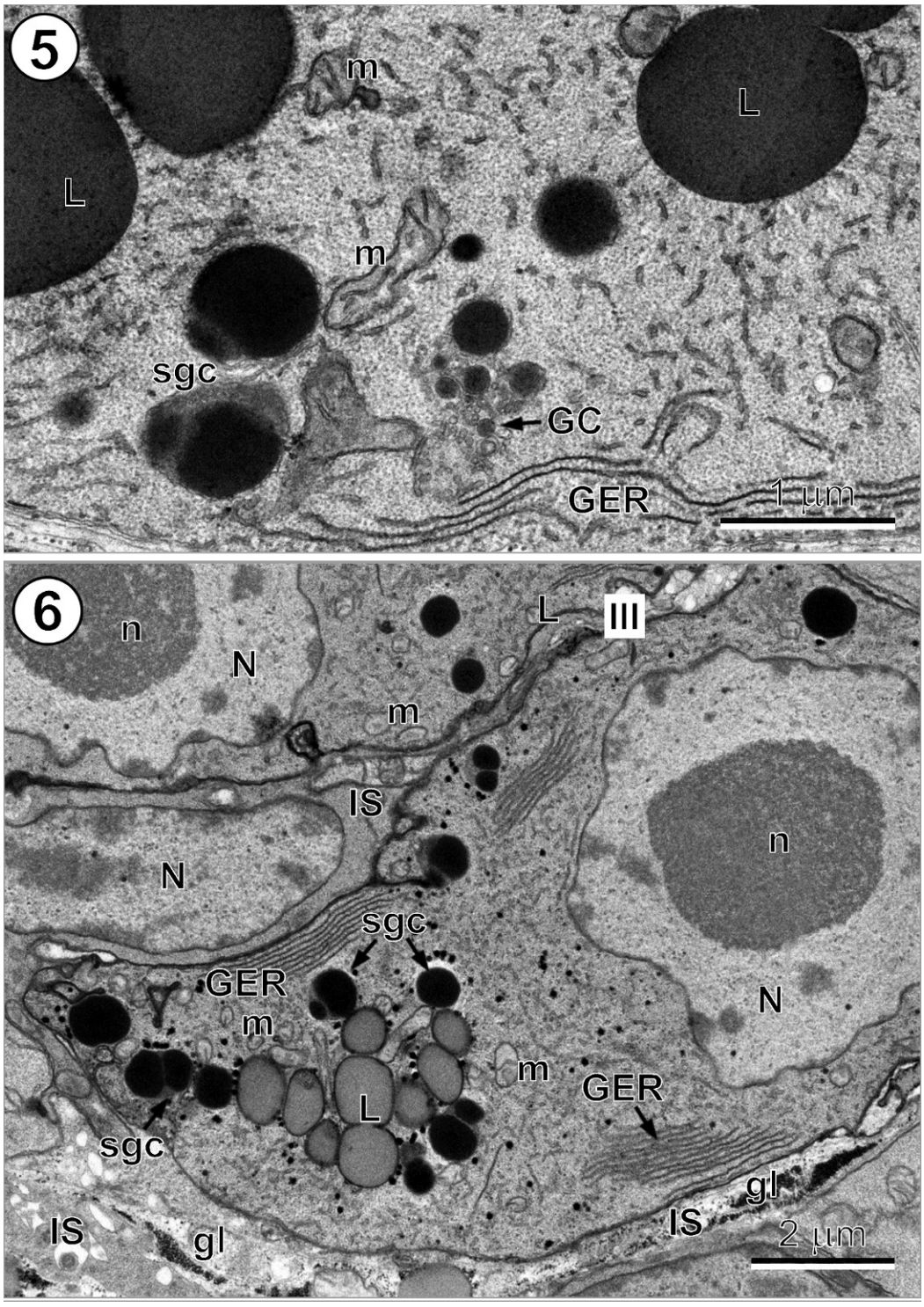


Fig. 2. Central part of the vitelline follicle showing three different stages (I, III and IV) of vitellogenesis: I – stem cell of gonial type; III – advanced stage of vitellocyte differentiation; and IV – mature vitellocyte which also show two stem cells of the gonial type in the peripheral region (see lower part of the micrograph)

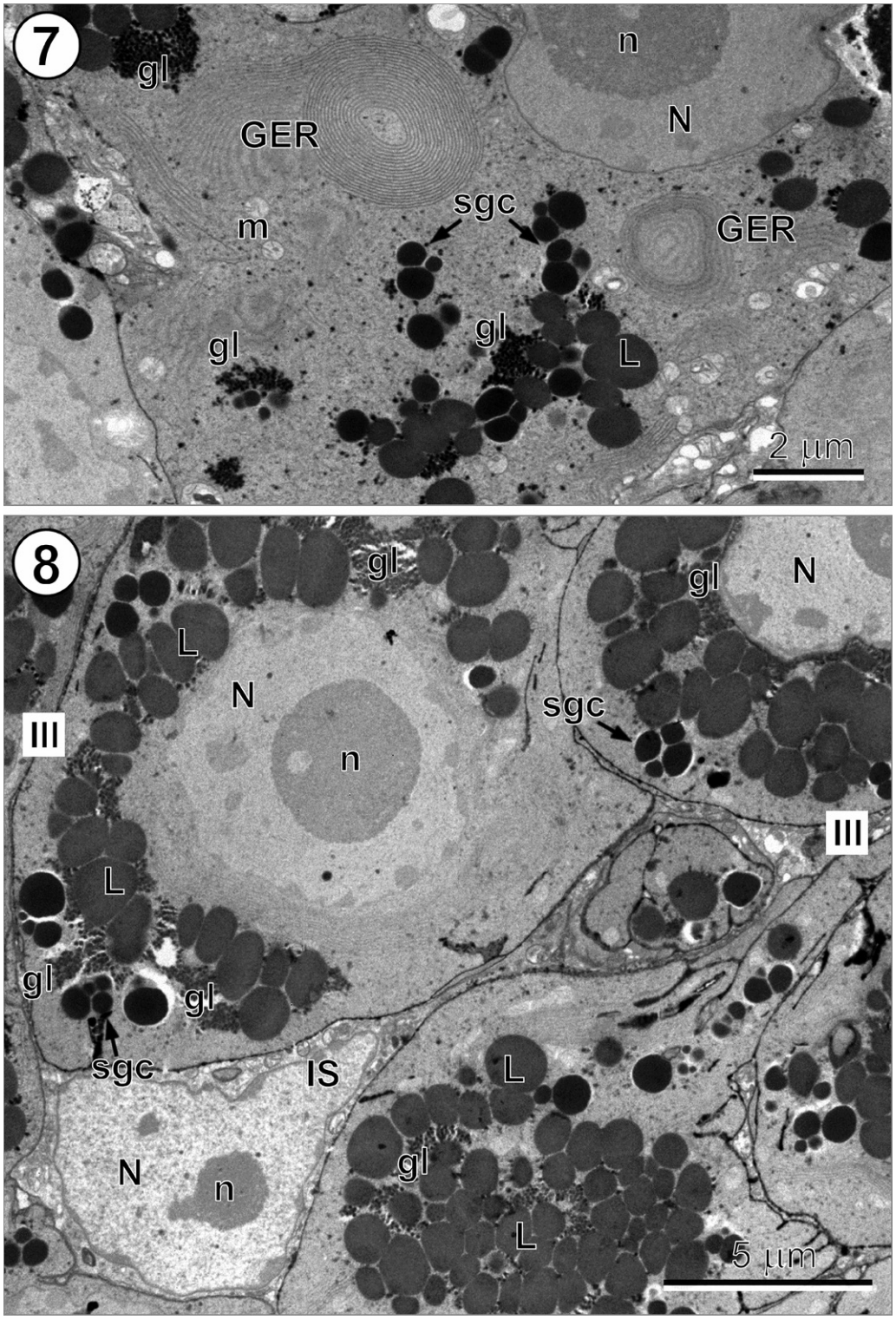
which are frequently arranged in concentric rings, mitochondria and Golgi complexes (Figs 5–7). Simultaneously with the increase in cell size and the development of cell organelles, there is a very rapid increase in the number of large, moderately saturated lipid droplets (Figs 5–7). The smallest individual proteinaceous shell-globules are synthesised in the GER and packaged as small globules with membrane-bounded vesicles via the Golgi complexes (Figs 5 and 7). Initially, as shown in Figure 5, very small spherical granules of Golgi origin and filled with an electron-lucent material appear inside the vesicles. Further growth and differentiation results in the formation of a moderately dense matrix and several small islands of a more electron-dense material (Figs 5 and 7). The consecutive stages in the development of proteinaceous granules and their transformation into shell-globule clusters are illustrated in Figures 5–7. A cluster is composed of several tightly packed electron-dense globules of various sizes embedded in a much less electron-dense matrix (Figs 1 and 4). This early stage of vitellocyte maturation is apparently completed when a large number of the shell-globule clusters, surrounded by significantly more numerous lipid droplets, are formed within the vitellocyte cytoplasm (Figs 1, 4 and 5). However, in this species, very active protein synthesis and shell-globule formation continue at the advanced stage of vitellocyte differentiation (Figs 6 and 7), when the cytoplasm is already filled with a high accumulation of α-glycogen rosettes and β-glycogen particles. These observations, therefore, indicate that both synthetic activities of differentiating vitellocytes, i.e. protein synthesis, leading to shell-globule formation, and carbohydrate synthesis and storage, overlap each other.



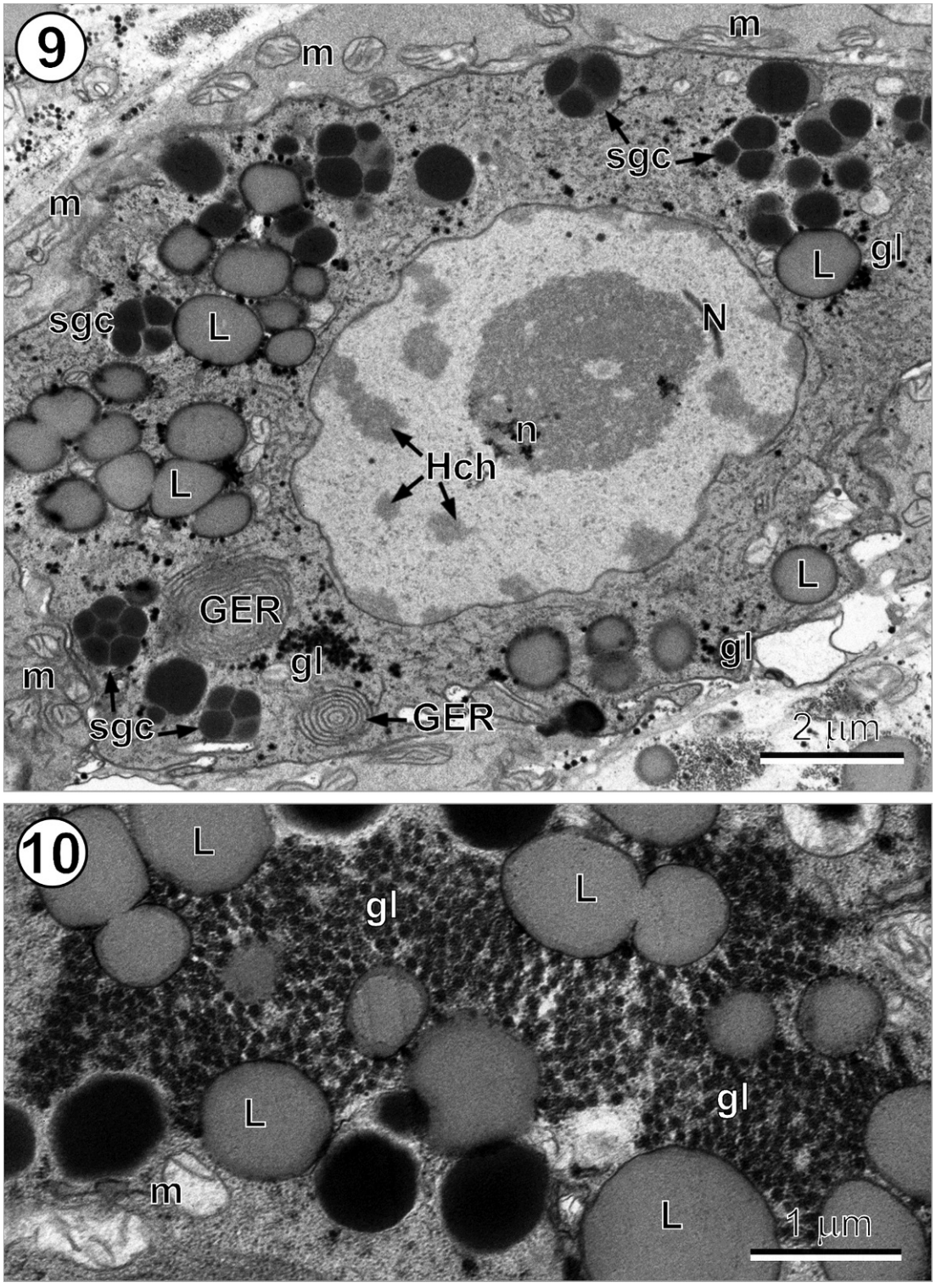
Figs 3 and **4.** Ultrastructural details of a stem cell of the gonial type (stage I) and an early stage (II) (Fig. 4 only) of vitellocyte cytodifferentiation, both showing very long cytoplasmic processes of the interstitial syncytium rich in glycogen in the peripheral parts of both micrographs. In Figure 3 note: (1) that the large nuclei contain prominent spherical nucleoli and numerous large islands of heterochromatin adjacent to the nuclear envelope; and (2) there is a very high nucleo-cytoplasmic ratio and a large concentration of free ribosomes in a relatively thin layer of granular cytoplasm containing a few mitochondria and a very few short cisternae of GER. In Figure 4 note: (1) a large nucleus (N) with a prominent electron-dense nucleolus and several islands of heterochromatin randomly dispersed in the nucleoplasm; (2) differences in the size and ultrastructural details of both nuclear components at stages I (on the right side) compared with those at stage II (on the left); and (3) variations in the granular cytoplasm, with only one or two elongated profiles of GER at stage I compared with that of the extended numerous parallel cisternae of GER and several mitochondria at stage II



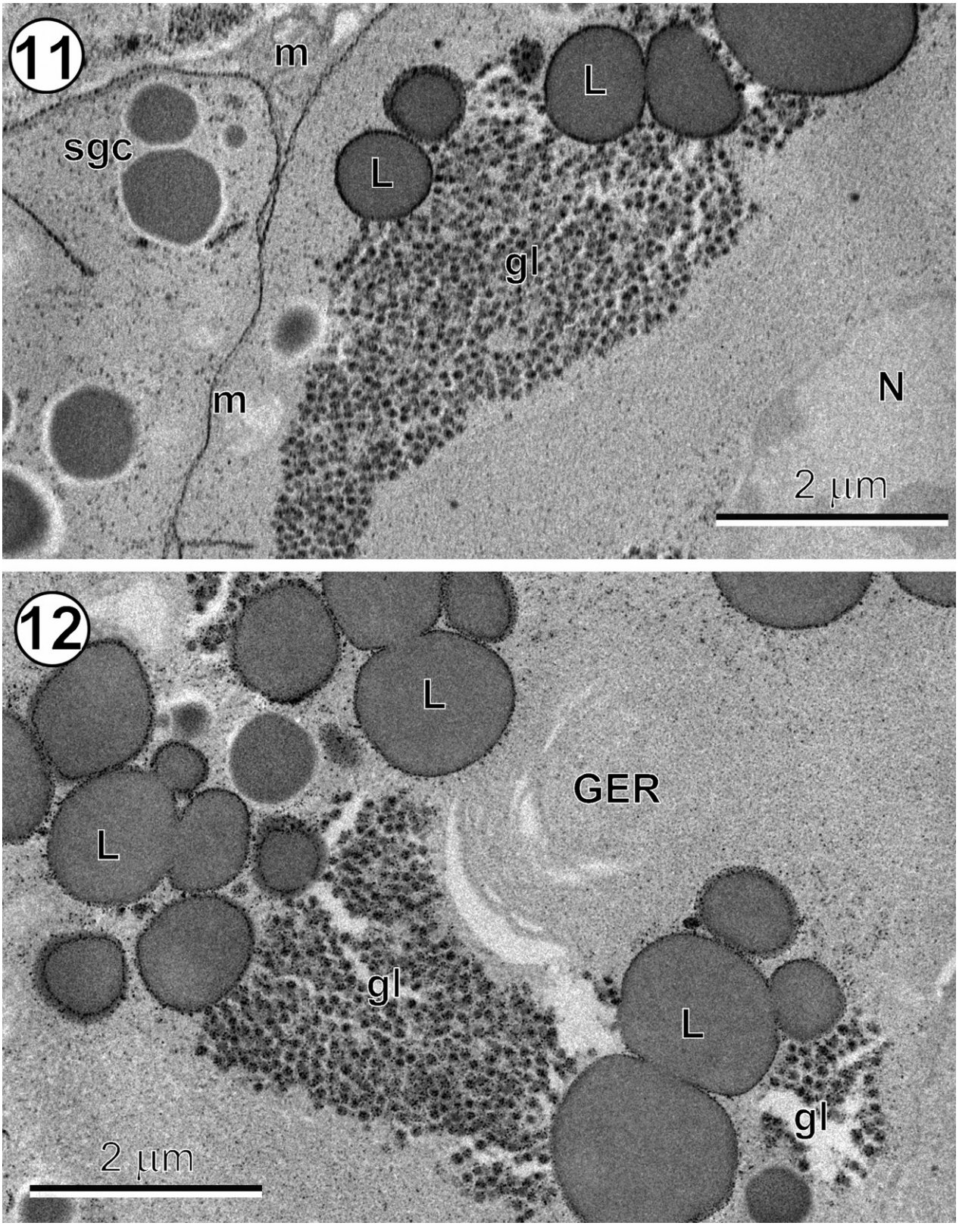
Figs 5 and **6.** TEM micrographs illustrating the beginning of the early stage II of vitellocyte differentiation at both high (Fig. 5) and low magnifications (Fig. 6**)**. In Figure 5 note: (1) the ultrastructural details of the GER adjacent to the Golgi complex composed of a few membrane-bounded Golgi vesicles containing dense material of shell-globules primordia of different sizes and electron density; (2) several large, unsaturated lipid droplets with a similar electron-density to that of the largest adjacent shell-globules; and (3) several mitochondria of different shapes and sizes. In Figure 6 note: (1) the large, slightly lobate nucleus with a predominant spherical nucleolus of the homogeneous type and a few heterochromatin islands; (2) a granular cytoplasm containing several long, parallel profiles of GER, numerous osmiophilic, unsaturated and osmiophobic, saturated lipid droplets, and a few shell-globules of similar electron-density to the osmiophilic, unsaturated lipids; and (3) a flattened nucleus and long cytoplasmic process of the interstitial syncytium situated between two vitellocytes on the left side of the micrograph



Figs 7 and **8.** Figure 7 shows the beginning of the advanced stage of vitellocyte maturation (early stage III), with clear signs of glycogenesis and numerous glycogen islands situated among the lipid droplets and shell-globules. Note: the characteristic arrangement of GER cisternae forming several concentric rings. Figure 8 shows the advanced stage of vitellocyte maturation (advanced stage III). Note: (1) a large nucleus with a predominant spherical nucleolus and several large islands of heterochromatin adjacent to the nuclear envelope; (2) a heavy accumulation of moderately saturated lipid droplets and several shell-globule clusters in the peripheral layer of the cytoplasm; (3) several sites of glycogenesis, with numerous glycogen islands situated among the lipid droplets and shell-globule clusters; and (4) the perikaryon of an interstitial syncytium, with a long cytoplasmic process, situated between two vitellocytes in the lower left corner of the micrograph



Figs 9 and **10.** Figure 9 shows ultrastructural details of a mature vitellocyte (stage IV). Note: (1) a large, slightly lobate nucleus with a predominant spherical nucleolus and several large islands of heterochromatin adjacent to the nuclear envelope; (2) two large rings of spherical, concentrically arranged parallel cisternae of GER and agglomerations of glycogen particles dispersed between them; (3) numerous electron dense shell-globule clusters in the peripheral layer of the cytoplasm; and (4) numerous large, less electron dense, saturated lipid droplets, a concentration of glycogen particles, composed mainly of α-glycogen rosettes, dispersed between the shell-globules, and heavy accumulations of large lipid droplets. Figure 10 is a high power magnification showing details of the peripheral cytoplasm of a mature vitellocyte. Note: a heavy accumulation of large, saturated lipid droplets surrounded by densely packed α-glycogen rosettes and β-glycogen particles, several shell-globule clusters and mitochondria



Figs 11 and **12.** The results of a cytochemical test (Thiery 1967) for glycogen using periodic acid-thiocarbazide-silver proteinate (PA-TSC-SP). This indicates a strongly positive reaction for β-glycogen particles and α-glycogen rosettes forming large accumulations around large unsaturated lipid droplets. Note: a thin electron dense layer at the surface of all lipid droplets and a white layer at the surface of the shell-globules; these are very helpful for distinguishing these two types of cell inclusion

Stage III: *Advanced differentiation stage – predominantly active glycogenesis accompanied by increasing accumulation of lipids and shell-globule clusters*

At this stage of vitellocyte maturation, there is intense glycogen synthesis (= glycogenesis), i.e. the formation and storage of α-glycogen rosettes and β-glycogen particles grouped together in tightly packed islands within the cytoplasm (Figs 6 and 7). In fact, this had commenced at the end of stage II, when a small number of predominantly β-glycogen particles appeared dispersed within the cytoplasmic matrix. At stage III (Figs 6 and 7), and in particular at stage IV (Figs 9–12) individual β-glycogen particles accumulate in several small densely-packed β-glycogen islands (Figs 10–12) and become surrounded by high concentrations of α-glycogen rosettes. Despite this intense glycogenesis, protein synthetic activities and the differentiation of proteinaceous granules into shell-globule clusters still take place (Figs 7–12). At the advanced stages of glycogenesis (Figs 7–12), enlarged glycogen islands become progressively fused, forming heavy glycogen accumulations surrounding large lipid droplets. These two types of cell inclusion occupy almost the entire central region of the mature vitellocyte cytoplasm (Figs 10–12). Consequently, the remaining cytoplasm, containing long, parallel cisternae of GER and several mitochondria, becomes displaced to the cell periphery by the massive accumulation of large lipid droplets and glycogen (Figs 10–12).

Stage IV: *Mature vitellocyte stage*

This cell stage (Figs 9 and 10–12), measuring *c*. 25 μm in diameter, is characterised by a great accumulation of three cell inclusions: (1) heterogeneous shell-globule clusters; (2) numerous large lipid droplets; and, adjacent to them, (3) compact aggregates of glycogen, predominantly in the form of α-glycogen rosettes. The elongate, slightly irregular-shaped nuclei of the mature vitellocytes contain prominent nucleoli and several dense heterochromatin islands in their karyoplasm. All cell organelles embedded in the remaining cytoplasm, namely the long, parallel cisternae of the GER and several mitochondria, become displaced towards the periphery of the cell (Figs 9 and 11).

Discussion

The general pattern of vitellogenesis in *Clestobothrium crassiceps* is essentially similar to that reported for other species of lower cestodes, i.e. gyrocotylideans (Xylander 1987), amphilinideans (Xylander 1988), spathebothriideans (Bruňanská *et al*. 2005; Poddubnaya *et al*. 2005, 2006), tetraphyllideans (Mokhtar-Maamouri and Świderski 1976), caryophyllideans (Mackiewicz 1968, Świderski and Mackiewicz 1976, Poddubnaya *et al*. 2003, Świderski *et al*. 2004a, b, 2009), trypanorhynchs (Świderski *et al*. 2006a, b, 2007) and diphyllideans (Świderski *et al*. 2011), in particular, the three other bothriocephalidean species listed below (Świderski and Mokhtar 1974, Korneva 2001, Levron *et al*. 2007). In all of these cestode taxa, the proteinaceous globules (i.e. shell-globules) are synthesised in the GER and packaged into small globules via the Golgi complex, as generally occurs in cells, such as platyhelminth vitellocytes (Świderski and Xylander 2000), which are involved in protein synthesis for external utilisation (Fawcett 1966). There is, however, evident variation in the chronology and predominant synthetic activity that occurs during both the early and advanced stages of vitellocyte differentiation in the various cestode groups. A comparison of the available TEM data on cestode vitellocytes is presented in Table I.

Ultrastructural data on vitellogenesis and vitellocytes of bothriocephalidean cestodes are presently available for the three following species: *Bothriocephalus clavibothrium* (Bothriocephalidae) by Świderski and Mokhtar (1974), *Triaenophorus nodulosus* (Triaenophoridae) by Korneva (2001) and *Paraechinophallus japonicus* (Echinophallidae) by Levron *et al.* (2007). Despite their general similarity, the ultrastructural details of the vitellocytes of each of these three species exhibit considerable variation and are thus characteristic for each species. Such details include: (a) the type of shell-globule clusters, which may exhibit a loose or tight packaging of their shell-globules; (b) the chemical nature of the lipid droplets, which exhibit considerable variation between two extremes, i.e. unsaturated (highly osmiophilic) and saturated (highly osmiophobic); and (c) the different amounts and proportions of the two types of glycogen reserves, namely single β-glycogen particles (for rapid utilisation) and α-glycogen rosettes (for long-term storage). In *C. crassiceps*, in comparison with the three above-mentioned species of the Bothriocephalidea, large lipid droplets already appear in the cytoplasm of differentiating vitellocytes by stage II, i.e. much earlier than in other bothriocephalideans, and their number in maturing and mature vitellocytes is much greater and clearly predominant in relation to the amount of glycogen. These differences may be associated to some extent with subsequent variations in their embryonic development, degree of ovoviviparity and life cycle details which still remain unknown.

In parasitic flatworms, lipids represent a heterogeneous and highly diverse group of chemical compounds, with a great variety of cellular functions. They are generally considered as important energy reserves, although this may not be the case in cestodes (Smyth and McManus 1989). With regard to the function of large lipid deposits, two hypotheses prevail, i.e. they represent (1) an energy source or (2) the waste products of metabolism. Our studies on the ultrastructure of the coracidial larva of *Bothriocephalus clavibothrium* (see Świderski and Mackiewicz 2004) provide strong arguments for their functioning as important energy reserves. Furthermore, the histochemical study of Moczoń (2006), on the accumulation and utilisation of lipids during the development of the cysticercoid metacestode of the cyclophyllidean *Hy-*

Table I. Characteristic features of mature vitellocytes in cestodes

Systematic position and species examined	General characteristic of mature vitellocytes	References
Amphilinidea		
Amphilina foliacea	Nuclei absent. Glycogen absent. Great number of heterogeneous shell-globules. Numerous large lipid droplets	Xylander (1988)
Gyrocotylidea		
Gyrocotyle urna	Nuclei present. Cytoplasmic glycogen present in α and β forms. Heterogeneous shell-globules. Numerous lipid droplets	Xylander (1987)
Spathebothriidea		
Cyathocephalus truncatus	Nuclei present. Cytoplasmic glycogen present. Heterogeneous shell-globules and "lamellar granules". Numerous lipid droplets	Bruňanská *et al.* (2005)
Didymobothrium rudolphii	Nuclei absent. Cytoplasmic glycogen present. Heterogeneous shell-globules. Numerous lipid droplets	Poddubnaya *et al.* (2006)
Diplocotyle olrikii	Nuclei present. Cytoplasmic glycogen present. Heterogeneous shell-globules and "lamellar granules". Numerous lipid droplets	Bruňanská *et al.* (2005)
Caryophyllidea		
Atractolytocestus huronensis	Nuclei present. Great amount of both cytoplasmic and nuclear glycogen (= "nuclear vacuole"). Presence of heterogeneous shell-globules and "lamellar granules". Lipids absent	Bruňanská (2009)
Caryophyllaeus laticeps	Nuclei present. Great amount of both cytoplasmic and nuclear glycogen (= "nuclear vacuole"). Presence of heterogeneous shell-globules and "lamellar granules". Lipids absent	Świderski *et al*. (2004a)
Glaridacris catostomi	Nuclei present. Great amount of both cytoplasmic and nuclear glycogen (= "nuclear vacuole"). Presence of heterogeneous shell-globules and "lamellar granules". Lipids absent	Świderski and Mackiewicz (1976)
Khawia armeniaca	Nuclei present. Great amount of both cytoplasmic and nuclear glycogen (= "nuclear vacuole"). Presence of heterogeneous shell-globules and "lamellar granules". Lipids absent	Świderski *et al*. (2004b)
Wenyonia virilis	Nuclei present. Small amount of nuclear glycogen and moderate accumulation of cytoplasmic glycogen present in both in α and β forms; but "nuclear vacuole" absent. Lipid absent	Świderski *et al*. (2009)
Diphyllidea		
Echinobothrium euterpes	Nuclei present. Small accumulations of glycogen particles scattered between numerous, large lipid droplets in the cytoplasm of maturing vitellocytes. Great vacuolization of vitellocyte cytoplasm	Świderski *et al*. (2011)
Trypanorhyncha		
Dollfusiella spinulifera	Nuclei present. Moderate amount of shell-globule clusters. Presence of membrane bounded glyco-proteins and/or very few β-glycogen particles, but absence of α-glycogen rosettes. Great number of large lipid droplets	Świderski *et al*. (2006a)
Grillotia erinaceus	Nuclei present. Moderate amount of shell-globule clusters. Presence of membrane bounded glyco-proteins and/or very few β-glycogen particles, but absence of α-glycogen rosettes. Great number of large lipid droplets	McKerr (1985)
Parachristianella trygonis	Nuclei present. High accumulation of cytoplasmic α- and β-glycogen and large lipid droplets	Świderski *et al*. (2007)
Progrillotia pastinacae	Nuclei present. Moderate amount of shell-globule clusters. Presence of membrane bounded glyco-proteins and/or very few β-glycogen particles, but absence of α-glycogen rosettes. Great number of large lipid droplets	Świderski *et al*. (2006b)
Rhinebothriidea		
Echeneibothrium beauchampi	Nuclei present. Cytoplasmic glycogen in β form. Great number of heterogeneous shell-globules. Numerous large lipid droplets of different sizes in the cytoplasm, but sometimes also in the nuclei	Mokhtar-Maamouri and Świderski (1976)
Bothriocephalidea		
Bothriocephalus clavibothrium	Nuclei present. Numerous shell-globules and heterogeneous shell-globule clusters. Great amount of cytoplasmic α- and β-glycogen and large lipid droplets	Świderski and Mokhtar (1974)
Clestobothrium crassiceps	Nuclei present. Glycogen particles (α and β forms) scattered between and among lipid droplets in the cytoplasm of maturing and mature vitellocytes. Great amount of large saturated (=osmiophobic) lipid droplets	Present study

Systematic position and species examined	General characteristic of mature vitellocytes	References
Paraechinophallus japonicus	Nuclei present. Perinuclear cytoplasm with lipid droplets and glycogen. Shell-globule clusters are present in cytoplasm of maturing and mature vitellocytes	Levron *et al.* (2007)
Triaenophorus nodulosus	Nuclei present. In the cytoplasm shell-globules and shell-globule clusters, lipids and glycogen	Korneva (2001)
Proteocephalidea		
Proteocephalus exiguus	Nuclei present. Numerous heterogeneous shell-globules, glycogen particles and few lipid droplets.	Bruňanská (1997)
Proteocephalus longicollis	Nuclei present. Cytoplasm with numerous heterogeneous shell-globules, glycogen in both α and β forms and a few lipid droplets	Świderski and Subilia (1978)
Cyclophyllidea		
Catenotaenia pusilla	Nuclei present. Three large vitelline vesicles present	Świderski *et al*. (2000)
Inermicapsifer madagascariensis	Nuclei present. One large vitelline vesicle present	Świderski (1973)
Moniezia expansa	Nuclei present. One large vitelline vesicle present	Li *et al.* (2003)
Mosgovoyia ctenoides	Nuclei present. One large vitelline vesicle present	Świderski *et al*. (2005)

menolepis diminuta, confirmed their important role in cestode morphogenesis. In addition, according to his data (Moczoń 2006, p. 152), "the utilization of neutral lipids proves both the presence of a lipase-type enzyme(s) and an operative β-oxidation pathway in the cells of the cysticercoids, the latter feature being highly indicative of oxidative metabolism of these larvae".

The significance of such an unusually wide variation in the products of mature vitellocytes is difficult to ascertain. Since cestodes are incapable of *de novo* synthesis of non-volatile saturated and unsaturated fatty acids, the biosynthesis of lipids depends on the host for the fatty acids (Buteau *et al*. 1971, Nakagawa *et al*. 1987). Because of this relationship, the fatty acid composition of cestodes is quite similar to that in their immediate environment within the host (Beach *et al*. 1975), and the host's fatty acids are derived from its food chain. Consequently, it can be concluded that the quantity of lipids in a parasite's tissues is generally host-related. It is, therefore, not surprising that there is so much variation in the qualitative and quantitative aspects of the lipids observed in the vitellocytes of the four studied bothriocephalidean species (Table I), because these cestodes come from three totally unrelated freshwater, euryhaline and marine host groups. A similar relationship was previously found in trypanorhynch cestodes by Świderski *et al*. (2007). In *C. crassiceps,* lipids represent the most abundant cell inclusion and appear at a very early stage of vitellogenesis, in fact much earlier than reported for the three other bothriocephalidean species (Świderski and Mokhtar 1974, Korneva 2001). As in the other three species, the chemical nature of the lipid droplets changes progressively from unsaturated to saturated and their constant fusion results in the formation of very large droplets. The very high accumulation of lipids observed in the vitellocytes of all four bothriocephalidean species raises important questions regarding the factors determining lipid type, their functional significance and what role they might have in assessing evolutionary relationships at any level.

The suggested functions of vitellocytes are egg-shell formation and nourishment of the embryo. Azzouz Draoui and Maamouri (1997) reported, in their research note on egg development and hatching of *Clestobothrium crassiceps*, that freshly laid eggs are initially anoperculate and unembryonated when released into the external aquatic environment. The operculum appears only after 11–13 days of development in water, and only about three days before the hatching of the coracidium larva. These observations clearly indicate that in *C. crassiceps* the numerous vitellocytes within these polylecithal egg must provide abundant nutritive reserves which are important not only for the nourishment of the developing embryo but also serve as an energy source for the free-swimming coracidium until it is ingested by a suitable intermediate host. Undoubtedly, the high accumulation of α-glycogen in the form of conspicuous rosettes of varying size represents a long-term energy reserve, whereas single β-glycogen particles represent a short-term source of carbohydrate for a more rapid enzymatic degradation into glucose.

Two types of egg have so far been described for bothriocephalidean cestodes: (a) those which are operculate and unembryonated *in utero*, as occur in *Bothriocephalus clavibothrium* (see Świderski 1994), and (b) those which are anoperculate and embryonated *in utero*, as occur in *Eubothrium salvelini* (see Świderski *et al.* 2005, Młocicki *et al.* 2010). It is likely, however, that future studies will show that there is a greater variety of egg types in this order. As suggested by Jarecka (1961) and Świderski (1981, 2008), cestode eggs may exhibit various morphological adaptations that aid the transmission to various intermediate hosts in different aquatic environments. There are many factors that influence and promote successful cestode life cycles (Mackiewicz 1988). Differing quantitative and qualitative concentrations of glycogen and lipids, as described in the present study, may be associated with physical and physiological adaptations that enhance the parasite's transmission to the next host. Until we have more information on cestode life cycles and the variety

of egg types and their adaptations to different hosts and environments, it will be difficult to judge to what extent the ultrastructure of the vitellocytes, and particularly those with a large accumulations of lipids, is a reflection of host influence, ecological adaptation or phylogenetic relationships. Variations in vitellogenesis and egg development in the various cestode groups were discussed in a review by Świderski and Xylander (2000). However, even now, studies on most groups are still too few to make any definite judgements in terms of their phylogenetic implications, especially in the cases of apparently distinctive features, such as the absence a nucleus in mature vitellocytes of the only amphilinidean species studied (Xylander 1988) and of one of the three spathebothriideans (Poddubnaya *et al.* 2006), and the absence of lipids in mature vitellocytes of the caryophyllideans (e.g. Świderski *et al.* 2004a, b). It seems more likely that vitellocyte development and composition reflects the subsequent nature of the life history of the worm and the length of time that the egg/larva must remain viable.

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