Ultrastructure of vitellogenesis and vitellocytes in the trypanorhynch cestode *Aporhynchus menezesi*, a parasite of the velvet belly lanternshark *Etmopterus spinax*

Ultrastructure de la vitellogénèse et des vitellocytes chez le trypanorhynque Aporhynchus menezesi, *parasite du sagre commun* Etmopterus spinax

Zdzisław Świderski[a,b*], Jordi Miquel[c,d], Adji Mama Marigo[c,d], David I. Gibson[e]

[a]W. Stefański Institute of Parasitology, Polish Academy of Sciences, 51/55 Twarda Street, 00-818 Warsaw, Poland.

[b]Department of General Biology and Parasitology, Medical University of Warsaw, 5 Chałubińskiego Street, 02-004 Warsaw, Poland.

[c]Laboratori de Parasitologia, Departament de Microbiologia i Parasitologia Sanitàries, Facultat de Farmàcia, Universitat de Barcelona, Av. Joan XXIII, sn, E08028 Barcelona, Spain.

[d]Institut de Recerca de la Biodiversitat, Facultat de Biologia, Universitat de Barcelona, Av. Diagonal, 645, E08028 Barcelona, Spain.

[e]Department of Zoology, Natural History Museum, Cromwell Road, London, SW7 5BD, UK

[*]Corresponding author: Zdzisław Świderski, W. Stefański Institute of Parasitology, Polish Academy of Sciences, 51/55 Twarda Street, 00-818 Warsaw, Poland.

Email: z.swider@twarda.pan.pl

Phone: + 48 22 697 89 70

Fax: + 48 22 620 62 27

Abstract

This is the first TEM examination of vitellogenesis in the cestode *Aporhynchus menezesi*, a parasite of the velvet belly lanternshark *Etmopterus spinax* and a member of a little-studied trypanorhynch family, the Aporhynchidae. The synthetic activity of vitellocytes plays two important functions in the developmental biology of cestodes: (1) their shell globules serve in eggshell formation; and (2) their accumulated reserves of glycogen and lipids represent a food source for the developing embryo. In *A. menezesi*, vitelline follicles consist of cells at various stages of development, from peripheral, immature cells of the gonial type to mature cells towards the centre of the follicle. These stages are (I) immature, (II) early differentiation, (III) advanced maturation and (IV) mature. Gradual changes involved in this process occur within each stage. Vitellogenesis involves: (1) an increase in cell volume; (2) the development of a smooth endoplasmic reticulum and an accelerated formation and accumulation of both unsaturated and saturated lipid droplets, along with their continuous enlargement and fusion; (3) the formation of individual β-glycogen particles and their accumulation in the form of glycogen islands scattered among lipid droplets in the cytoplasm of maturing and mature vitellocytes; (4) the rapid accumulation of large, moderately saturated lipid droplets accompanied by dense accumulations of β-glycogen along with proteinaceous shell-globules or shell-globule clusters in the peripheral layer during the advanced stage of maturation; (5) the development of cisternae of granular endoplasmic reticulum that produce dense, proteinaceous shell-globules; (6) the development of Golgi complexes engaged in the packaging of this material; and (7) the progressive and continuous enlargement of shell-globules into very large clusters in the peripheral layer during the advanced stage of maturation. Vitellogenesis in *A. menezesi*, only to some extent, resembles that previously described for four other trypanorhynchs. It differs in: (i) the reversed order of secretory activities in the differentiating vitellocytes, namely the accumulation of large lipid droplets

accompanied by glycogenesis or β-glycogen formation during early differentiation (stage II), i.e. before the secretory activity, which is predominantly protein synthesis for shell-globule formation (stage III); (ii) the very heavy accumulation of large lipid droplets during the final stage of cytodifferentiation (stage IV); and (iii) the small number of β-glycogen particles present in mature vitellocytes. Ultracytochemical staining with PA-TCH-SP for glycogen proved positive for a small number of β-glycogen particles in differentiating and mature vitellocytes. Hypotheses, concerning the interrelationships of patterns of vitellogenesis, possible modes of egg formation, embryonic development and life-cycles, are commented upon.

Keywords: *Aporhynchus menezesi*, Trypanorhyncha, vitellogenesis, vitellocytes, ultrastructure

Resumé

Cette étude décrit pour la première fois, au microscope électronique à transmission, la vitellogénèse chez le cestode *Aporhynchus menezesi*, un trypanorhynque membre de la famille peu étudiée des Aporhynchidae, parasite du requin *Etmopterus spinax*, ou sagre commun. L'activité synthétique des vitellocytes joue deux rôles importants dans la biologie du développement des cestodes : (1) leurs globules coquilliers (appelés aussi parfois globules protéiques) servent à la formation de la coquille de l'œuf, et (2) leurs réserves accumulées de glycogène et de lipides constituent une source de nourriture pour l'embryon en développement. Chez *A. menezesi*, les follicules vitellins sont constitués de cellules à différents stades de développement, avec les cellules immatures de type gonial à la périphérie et les mûres au centre. Ces stades de développement sont : (I) immature, (II) début de différenciation, (III) maturation avancée et (IV) mûr. Des changements progressifs impliqués dans ce processus interviennent à l'intérieur de chaque stade. La vitellogénèse implique : (1)

une augmentation de volume cellulaire ; (2) le développement d'un réticulum endoplasmique lisse, une formation accélérée et accumulation des gouttelettes de lipides non-saturés et saturés, ainsi que leur croissance continue et leur fusion ; (3) la formation de particules individuelles de β-glycogène et leur accumulation sous forme d'ilots de glycogène dispersés parmi les gouttelettes lipidiques dans le cytoplasme du vitellocyte en maturation et du vitellocyte mûr ; (4) l'accumulation rapide de grandes gouttelettes de lipides modérément saturés, accompagnées d'accumulations opaques aux électrons de β-glycogène ainsi que de globules coquilliers protéiques ou de groupes de globules coquilliers dans la couche périphérique durant le stade avancé de la maturation ; (5) le développement de citernes de réticulum endoplasmique qui produit des globules coquilliers protéiques opaques aux électrons; (6) le développement de complexes de Golgi engagés dans l'emballage de ce matériel ; et (7) l'accroissement progressif et continu des globules coquilliers en de très grands amas au sein de la couche périphérique durant le stade avancé de la maturation. La vitellogénèse chez *A. menezesi*, d'une certaine façon, ressemble à celle décrite antérieurement chez quatre autres trypanorhynques. Elle en diffère par : (i) l'ordre inversé des activités sécrétrices lors de la différentiation des vitellocytes, à savoir l'accumulation de grandes gouttelettes lipidiques, accompagnées par la glycogenèse ou la formation du β-glycogène, durant le stade initial de la différentiation (stade II), c'est-à-dire avant l'activité sécrétrice, prédominée par la synthèse protéique pour la formation de globules coquilliers (stage III) ; (ii) la très forte accumulation de grandes gouttelettes lipidiques durant le stade final de la cytodifférentiation (stage IV) ; et (ii) le nombre réduit de particules de β-glycogène présentes dans les vitellocytes mûrs. Le test ultracytochimique pour le glycogène avec PA-TCH-SP est positif pour les particules de β-glycogène dans les vitellocytes en différentiation ou mûrs. Les hypothèses concernant les relations entre les modèles de vitellogénèse, le mode de formation des œufs, le développement embryonnaire et les cycles de vie sont commentées.

Mots clés : *Aporhynchus menezesi*, Trypanorhyncha, vitellogénèse, vitellocytes, ultrastructure

1. Introduction

The order Trypanorhyncha Diesing, 1863 is a large group of common marine polyzoic cestode parasites, the unique feature of which is the presence of a rhyncheal apparatus and whose adult stages are typically found in the stomach and spiral valve of elasmobranch fishes [sharks and rays]; their larval forms infect a wide variety of marine invertebrates and fishes [1]. In trypanorhynchs the attachment organ is a scolex which bears two or four bothria [2] and a rhyncheal apparatus consisting of four retractile tentacles armed with a complex array of hooks, which are linked via tentacle sheaths to four bulbs [3]. The rhyncheal apparatus forms a robust synapomorphy that supports the monophyly of this order. The Aporhynchidae Poche, 1926 is unique in the order in that species of its only genus, *Aporhynchus* Nybelin, 1918, have secondarily lost their rhyncheal apparatus [4,5].

The two important functions of cestode vitellocytes, egg-shell formation and nourishment of the embryo [6], can be intensified or reduced, depending on the characteristics of each species in terms of its degree of ovoviviparity and life-cycle. Several TEM studies are available on the structure and differentiation of vitellocytes in the Cestoda [for a review see 7]. Among the trypanorhynch cestodes, only four species have been examined in this respect: *Grillotia erinaceus* (Lacistorhynchidae), *Dollfusiella spinulifera*, *Parachristianella trygonis* (Eutetrarhynchidae) and *Progrillotia pastinacae* (Progrillotiidae) [8-11].

Using a member of a previously unstudied group, i.e. the aporhynchid *Aporhynchus menezesi* Noever, Caira, Kuchta & Desjardins, 2010, a parasite of the lanternshark *Etmopterus spinax*, the aims of the present study are: (1) to describe the ultrastructural and cytochemical aspects of vitellogenesis and the functional ultrastructure of mature vitellocytes;

(2) to compare the results with those of previous reports on the four trypanorhynch species examined previously; and (3) to discuss any possible functional, developmental, life-cycle or phylogenetic implications of the results obtained.

2. Materials and methods

Live adult specimens of *Aporhynchus menezesi* were collected by Prof. Janine Caira and her co-workers from the spiral valve of a naturally infected velvet belly lanternshark *Etmopterus spinax* (L.) (Elasmobranchii: Etmopteridae) captured off Faial Island (38°31'N, 28°37'W) (Azores Archipelago, Portugal).

Live cestodes were first placed in a 0.9% NaCl solution and then mature proglottids were fixed in cold (4°C) 2.5% glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2 for a minimum of 2 h, rinsed in a 0.1 M sodium cacodylate buffer at pH 7.2, 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 7.2, 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. Ultrathin sections were examined using a JEOL 1010 TEM operated at an accelerating voltage of 80 kV.

The Thiéry technique [12] was used for highlighting the presence of glycogen particles. Gold grids were treated in periodic acid, thiocarbohydrazide and silver proteinate (PA-TCH-SP) as follows: 30 min in 10% PA, rinsed in distilled water, 24 h in TCH, rinsed in acetic solutions and distilled water, 30 min in 1 % SP in the dark, and rinsed in distilled water.

3. Results

3.1. General topography of the vitellarium

In the trypanorhynch cestode *Aporhynchus menezesi*, the follicular vitellarium is composed of oval follicles which are circum-medullary and extend almost entire length of the proglottid but are less dense in the region of the uterus and ovary. Each vitelline follicle consist of cells in various stages of development, from immature cells of the gonial type close to the periphery to mature cells towards the centre of the follicle. Although vitellocyte cytodifferentiation represents a continuous process, in order to facilitate its description, it was subdivided into four discrete stages. These stages are: (I) immature, (II) early differentiation, (III) advanced maturation and (IV) mature. In fact, the gradual and continuous changes involved in this process occur clearly within each of the stages delineated herein [compare, for example, Fig. 1A and 1B, illustrating changes in the volume of the cytoplasmic layer and the number of its cell organelles which are already present at the gonial stem cell stage (I) and indicate the very beginning of the early maturation stage (II)]. The interstitial cells were not examined in detail, but their elongate cytoplasmic processes surround the periphery of the vitelline follicles and penetrate deeply between the differentiating vitellocytes (Figs 1B and 3B). The long, ramified cytoplasmic processes of interstitial cells of *A. menezesi* (Figs 1B and 3B) contain heavy accumulations of glycogen particles and several lipid droplets and mitochondria.

3.2. Stage I. Immature stem cells of the gonial type (Fig. 1A,B)

The undifferentiated cells of the gonial type (Fig. 1A), situated at the periphery of the vitelline follicles, represent the precursors of vitellocytes. They have a high nucleo-cytoplasmic ratio. Their large nuclei measure ~5 μm in diameter, whereas the diameter of the entire cell is ~6.5 μm. At the end of this stage, however, some increase in cell size takes place

(compare Fig.1A and 1B), reaching ~7-8 μm in diameter, especially in the volume of cytoplasm (Fig. 1B). The cell nucleus exhibits the presence of several electron dense islands of heterochromatin in addition to the nucleolus and numerous pores in its nuclear envelope (compare Fig.1A and 1B). The much thicker layer of granular cytoplasm contains numerous mitochondria and free ribosomes, a few Golgi complexes and a very few short profiles of granular endoplasmic reticulum (GER) (compare Fig.1A and 1B).

3.3. Stage II. Early differentiation: glycogenesis, lipid accumulation, shell globule formation (Fig. 2A-C)

Early cytodifferentiation of vitellocytes (Fig. 2A-C) is characterized by: (1) a distinct increase in cell size; (2) an increase in the number of mitochondria and both GER and SER (smooth endoplasmic reticulum) cisternae; and (3) the formation of the first β-glycogen particles, which are progressively grouped into small glycogen islands (Fig. 2B). Initiation of secretory activity by both the GER and SER results in (1) the appearance of lipid droplets associated with SER cisternae and in (2) the formation of shell globules within membrane-bound vesicles of Golgi origin within the numerous concentrically arranged Golgi complexes (Fig. 3A) which are in turn surrounded by concentric GER cisternae (Figs 2C and 3A). Fusion of small shell globules into much larger shell globules clusters starts very rapidly and has already begun by this stage (Fig. 2C). The glycogen islands are frequently situated around and between the lipid droplets (Figs 2B and 3A,B).

3.4. Stage III. Advanced maturation: rapid formation of shell globule clusters and accumulation of lipid droplets (Fig. 3A,B)

During the advanced phase of vitellocyte maturation (Fig. 3A,B), the cell doubles in size due to a great accumulation of large lipid droplets and numerous shell globule clusters,

accompanied by a progressive vacuolization of its cytoplasm. Adjacent shell globule clusters frequently become fused into much larger clusters (Figs 2C and Inset, and 3A,B). At the same time, the nucleus begins to change in form to the semilunar shape characteristic of the mature vitellocytes of *A. menezesi*. Vitellocyte maturation is characterized as a period of a very high secretory activity, resulting in shell globules and their rapid fusion into shell globule clusters (Fig. 3A,B).

3.5. Stage IV. Mature vitellocyte (Fig. 4A,B)

The mature vitelline cells (Fig. 4A) are ovoid or spherical in shape and measure ~12-14 μm in diameter. Their nuclei are semi-lunar or reniform and measure ~7 × 3.5 μm in diameter (Fig. 4A). The karyoplasm contains an elongate nucleolus and a few irregularly shaped heterochromatin islands of moderate electron density. The highly vacuolated cytoplasm contains numerous large lipid droplets of moderate electron density, being moderately osmiophilic, and great amounts of large, membrane-bound shell globule clusters composed of numerous shell-globules separated by a lucent phenolase component. Both of these cell inclusions (Fig. 4A,B) accumulate mainly in the peripheral layer of the cytoplasm of mature vitellocytes.

4. Discussion

Early TEM studies on cestode vitellogenesis, published during the 20th Century, were reviewed and analysed in relation to egg production, different types of embryonic development and a variety of parasite life-cycles [7]. TEM results in more recent studies on this subject have been dealt with in both the ‘Discussion’ and ‘Table I’ of a paper on vitellogenesis of the bothriocephalidean cestode *Clestobothrium crassiceps* [13].

Aporhynchus menezesi has been attributed to the trypanorhynch family Gilquiniidae [1,4,14] and, more recently, the Aporhynchidae [15-17). The general pattern of vitellogenesis in this species is essentially similar to that reported for other species of lower cestodes, i.e. gyrocotylideans [18], amphilinideans [19], spathebothriideans [20-22], caryophyllideans [23-27], diphyllideans [28], four other species of trypanorhynchs [8-11], rhinebothriideans [29] and bothriocephalideans [30-32].

During vitellogenesis in all of these cestode taxa, the synthetic activities of differentiating vitellocytes involve three processes: (1) protein synthesis, producing important material for eggshell formation, (2) glycogenesis, which assures glycogen storage and nutritive reserves for the developing embryos, and (3) lipid accumulation in the form of large lipid droplets of a more or less saturated chemical nature, which are considered to be another source of nutritive reserves for the embryo. There is, however, evident variation in the chronology of these three types of synthetic activity, as it is only very seldom that they take place simultaneously.

Protein synthesis is the predominant synthetic activity, which usually occurs in the very early stages of vitellogenesis in the great majority of tapeworms; however, in *A. menezesi* it is evidently delayed. Proteinaceous globules, which are progressively grouped together into larger subunits, such as shell-globules and then small globule clusters, are synthesised in the GER and packaged into small globules via the Golgi complex; this is a general feature of platyhelminth vitellocytes [7] and all also of other cell types involved in protein synthesis for external utilization [33].

However, variation in the formation and storage of embryonic nutritive reserves in the various cestode groups has been observed in relation to differences in the proportion and amounts of glycogen and lipids [see 7,13,28,34]. There are extreme cases, such as the caryophyllideans, where glycogen only is accumulated in both the vitellocyte cytoplasm and

its nucleus [23,24,26,27); another such extreme example occurs in rhinebothriidean vitellocytes [29], where generally lipids only are accumulated in both the cytoplasm and the nucleus. A recent comparison of both types of embryonic nutritive reserves in the vitellocytes of lower cestodes was presented [13].

In the present study, particular attention was paid to comparing the ultrastructural and cytochemical aspects of vitellogenesis and the functional ultrastructure of the mature vitellocytes of *A. menezesi* with those of the four other studied species of the Trypanorhyncha [8-11], which belong to three other families of this order. It appears that ultrastructural details of vitellogenesis, and in particular details of the mature vitellocytes, exhibit a great diversity in all five of these trypanorhynch species (Table I). Great variation was observed in the sequence of the two main synthetic activities, namely shell formation and the development and storage of nutritive reserves for the developing embryos; the latter occur in the form of glycogen and lipids, and are usually represented in different proportions in the five species. Both types of synthetic activity can take place at both the early and the advanced stages of vitellocyte differentiation, although they do not coincide in *Parachristianella trygonis* [11], slightly overlap in *Dollfusiella spinulifera* [9] and occur simultaneously in *Progrillotia pastinacae* [10]. Nevertheless, regardless of the group, proteinaceous globules (shell-globules) are evidently synthesized in the GER and packaged as small globules via the Golgi complex, as occurs in all studied platyhelminth species [7].

The most important differences observed in the mature vitellocyte of *D. spinulifera*, *Para. trygonis* and *Pro. pastinacae* [9-11] relate to glycogenesis (Table I). Of these three species, only *Para. trygonis* has large amounts of glycogen. In *D. spinulifera*, cytochemical overstaining with periodic acid-thiocarbazide-silver proteinate for polysaccharides indicated a strongly positive reaction for membrane-bound glycoproteins in all membranous structures, such as GER, mitochondria, Golgi complexes, nuclear and cell plasma membranes, where

there were only membrane-bound polysaccharides and very few granules of β-glycogen. Similar staining in *A. menezesi* revealed β-glycogen particles scattered in the cytoplasm of maturing vitellocytes. Typical cytoplasmic β-glycogen particles appeared only seldom during early vitellocyte maturation and were rarely visible in mature vitelline cells of *D. spinulifera* [9] and *Pro. pastinacae* [10]. Another important difference between the four species is the lipid content and the chemical nature of the lipid droplets (Table I). In *Para. trygonis*, there are massive concentrations of lipids which are, like those in *Grillotia erinaceus* and *Pro. pastinacae*, of the saturated type. In all five of the trypanorhynch species, lipid droplets were localised only in the vitellocyte cytoplasm, never inside the cell nucleus as reported for the tetraphyllidean *Echeneibothrium beauchampi* [29] or the spathebothriidean *Didymobothrium rudolphii* [22]. Studies by Smirnov and Bogdan [35] have shown that the differences in the level of lipid saturation between the former pseudophyllideans *Eubothrium crassum* (Bothriocephalidea) and *Diphyllobothrium dendriticum* (Diphyllobothriidea) are related to the body temperature of their definitive hosts. Although temperature difference among the hosts in the present study is not as great as between these two cestodes, it is of interest that highly unsaturated lipids were present only in *D. spinulifera* (Table I), which is from a warm, semi-tropical marine environment. The ultrastructural examination of the early cytodifferentiation of the vitellocytes (Fig. 2A) in *A. menezesi* exhibits numerous largely dilate cisternae of smooth endoplasmic reticulum (SER), which are usually associated with metabolism and the transport of lipids [33]. Lipids represent a highly diverse and heterogeneous group of chemical compounds, with a great variety of cellular functions. They are generally considered as important energy reserves, although this may not be always the case in cestodes [36]. In relation to the function of large lipid deposits, two theories prevail, i.e. they represent either an energy source or a waste product of metabolism. Studies on the ultrastructure of the free-swimming coracidial larve of *Bothriocephalus clavibothrium* [37] provide strong arguments

for their function as important energy reserves. The significance of such unusually high accumulations lipids and the wide variety of their chemical nature in cestode vitellocytes appears somewhat difficult to ascertain. In TEM studies, however, lipid droplets in cestode vitellocytes exhibit different degrees of saturation, which are manifest as differences in their affinity for osmium in ultrathin sections, e.g. unsaturated lipids are highly osmiophilic and appear black in the ultrathin sections, whereas saturated lipids are highly osmiophobic and appear white in these sections. The studies of Buteau et al. [38] and Beach et al. [39] on lipids of tetraphyllidean and trypanorhynch cestodes from sharks may offer some insights into the origin, metabolism and role of the lipids in these parasites. 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 (38,40). The fatty acid composition of cestodes is, therefore, similar to that of their immediate environment in the host [39] and the origin of these fatty acids in the host is the host's food chain. It can be concluded that the fatty acid patterns in all trypanorhynch lipids is host-related. According to these data, it is therefore not surprising that there is so much variation in the qualitative and quantitative aspects of lipids in the vitellocytes of trypanorhynchs (Table I), because those cestodes examined came from five different hosts and from four different marine environments, the Irish Sea, the Atlantic Ocean off the Azores, the Mediterranean Sea and the Coral Sea.

Lack of data on the life-cycles and developmental biology of the Trypanorhyncha [see 8,15,41] makes it difficult to speculate on the functional ultrastructure of their vitellogenesis and mature vitellocytes. No doubt, there is a close interrelationship between the pattern of vitellogenesis in parasitic platyhelminths and the type of embryonic development, the poly- or oligolecithality of their eggs and the different degrees of ovoviviparity [7,24,42-44]. Very little is known about trypanorhynch life-cycles due to difficulties in maintaining them alive and creating effective exposure conditions in the laboratory. In general, their life-cycles are

heteroxenous, involving several different invertebrate or vertebrate intermediate hosts and an elasmobranch definitive host [8,15,41]. The gravid segments may remain attached to the rest of strobila or may detach progressively from the mature proglottids. Two developmental pathways have been reported: (1) hexacanth larval development within the egg is arrested in the uterus after the first few cell divisions, i.e. at an early embryo stage, representing different degrees of ovoviviparity; development resumes almost immediately on contact with seawater and infective hexacanths, surrounded by a ciliated envelope, are produced within 5-8 days; and (2) intrauterine eggs contain fully-formed oncospheres [8,15,41,45]. Further studies may show a greater variety of trypanorhynch egg types than are known at present.

According to Noever et al. [46], the eggs of *A. menezesi* differ conspicuously from those of *Aporhynchus norvegicus*. In their opinion, the illustration of eggs published by Dollfus [3], which he attributed to *A. norvegicus*, closely resembles the eggs of *A. menezesi* which they examined. In their view, Dollfus' material consisted of a mixture of specimens of both *A. norvegicus* and *A. menezesi*, and that he in fact illustrated the eggs of *A. menezesi* rather than *A. norvegicus*. Our diagram of the egg of *A. menezesi* (Fig. 5) is a modification of Dollfus' illustration [3]. As described by Dollfus [3], the fusiform eggs of this species are very unusual. They are so delicate that they were only successfully examined and measured by him when observed directly in seawater. Any kind of fixation, dehydration or embedding resulted in their complete deformation and numerous artefacts. The fusiform eggs, measuring 155-165 μm in length by 45-55 μm in width, are surrounded by a very thin outer envelope, which is prolonged at one pole to form a short filament. Inside each egg (Fig. 5), there appear to be five or six compartments in line, and the embryo occupies only one, apparently any single one, of these compartments. Such an early embryo is composed of several blastomeres surrounded by a cluster of three or four entities, interpreted as being vitelline cells, arranged usually in tandem on either side of the embryo. The presence of such eggs with early embryos

may suggest that hexacanth development within the eggs is arrested in the uterus after several initial cleavage divisions, and that it resumes almost immediately upon contact with seawater, infective hexacanths typically being produced within a few days [46]. 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 transmission. However, until we have more information on the life-cycle of these cestodes, the variety of egg types and their adaptations to different hosts and environmental conditions, it will be difficult to judge to what extent the ultrastructure of the vitellocytes, and in particularly those with lipids, is a reflection of host influence, ecological adaptations or phylogenetic relationships.

Acknowledgements

Adult specimens of *Aporhynchus menezesi* used for present study were kindly collected, fixed and sent us by Prof. Janine Caira for the present TEM study. The collecting was partly supported by funds from NSF PBI award Nos DEB 0818696 and 0818823 to Janine N. Caira, Timothy J. Littlewood, Jean Mariaux and Kirsten Jensen. The authors also wish to thank Núria Cortadellas and Almudena García from the "Unitat de Microscòpia, Facultat de Medicina, Centres Científics i Tecnològics de la Universitat de Barcelona (CCiTUB)" for their support in the preparation of samples. Adji Mama Marigo benefits from MAEC-AECID doctoral grants (2009-10 0000448124 and 2010-11 0000538056.

References

[1] R.A. Campbell, I. Beveridge, Order Trypanorhycha Diesing 1863. In: L.F. Khalil, A. Jones, R.A. Bray (Eds.), Keys to cestode parasites of vertebrates. CAB International Publishing, Wallingford, 1994, pp. 51–148.

[2] A. Jones, R.A. Bray, L.F. Khalil, Key to the orders of the Cestoda. In: L.F. Khalil, A. Jones, R.A. Bray (Eds.), Keys to cestode parasites of vertebrates. CAB International Publishing, Wallingford, 1994, pp. 1–2.

[3] R.P. Dollfus, Études critiques sur les tétrarhynques du Muséum de Paris. Arch. Mus. natl. Hist. nat. (Paris) 6 ser. 19 (1942) 1–466.

[4] I. Beveridge, Revision of the family Gilquiniidae Dollfus (Cestoda: Trypanorhyncha) from elasmobranch fishes. Austr. J. Zool. 37 (1990) 481–520.

[5] P.D. Olson, J.N. Caira, K. Jensen, R.M. Overstreet, H.W. Palm, I. Beveridge, Evolution of the trypanorhynch tapeworms: parasite phylogeny supports independent lineages of sharks and rays. Int. J. Parasitol. 40 (2010) 223–242.

[6] Z. Świderski, H. Huggel, N. Schönnenberger, The role of the vitelline cell in the capsule formation during embryogenesis in *Hymenolepis diminuta* (Cestoda). Proc. 7th Int. Cong. Electron. Microsc, Grenoble (1970) 669–670.

[7] Z. Świderski, W.E.R. Xylander, Vitellocytes and vitellogenesis in cestodes in relation to embryonic development egg production and life cycles. Int. J. Parasitol. 30 (2000) 805–817.

[8] G. McKerr, The fine structure and physiology of a trypanorhynch tapeworm *Grillotia erinaceus*. PhD Thesis, The Queens University of Belfast, Northern Ireland, UK, 1985.

[9] Z. Świderski, J. Miquel, D. Młocicki, L. Neifar, B. Grytner-Zięcina, J.S. Mackiewicz, Ultrastructural and cytochemical studies on vitellogenesis in trypanorhynch cestode *Dollfusiella spinulifera* Beveridge, Neifar et Euzet, 2004 (Eutetrarhynchidae). Acta Parasitol. 51 (2006) 182–193.

[10] Z. Świderski, J. Miquel, D. Młocicki, L. Neifar, B. Grytner-Zięcina, J.S. Mackiewicz, Ultrastructure of the vitellocytes in the cestode *Progrillotia pastinacae* Dollfus, 1946 (Trypanorhyncha, Progrillotiidae). Acta Parasitol. 51 (2006) 194–199.

[11] Z. Świderski, J. Miquel, L. Neifar, J.S. Mackiewicz, Ultrastructural and cytochemical

studies on vitellogenesis in the trypanorhynch cestode *Parachristianella trygonis* Dollfus, 1946 (Eutetrarhynchidae). Acta Parasitol. 52 (2007) 114–126.

[12] J.P. Thiéry, Mise en evidence des polysaccharides sur coupes fines en microscopie electronique. J. Microsc. 6 (1967) 987–1018.

[13] Z. Świderski, D.I. Gibson, A.M. Marigo, E. Delgado, J. Torres, J. Miquel, 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). Acta Parasitol. 56 (2011) 392–405.

[14] I. Beveridge, J.-L. Justine, Gilquiniid cestodes (Trypanorhyncha) from elasmobranch fishes off New Caledonia with description of two new genera and a new species. Syst. Parasitol. 65 (2006) 235–249.

[15] H.W. Palm, The Trypanorhyncha Diesing, 1863. PKSPL IPB Press, Bogor, 2004.

[16] H.W. Palm, S. Klimpel, Evolution of parasitic life in the ocean. Trends Parasitol 23 (2007) 10–12.

[17] H.W. Palm, A. Waeschenbach, P.D. Olson, D.T.J. Littlewood, Molecular phylogeny and evolution of the Trypanorhyncha Diesing 1863 (Platyhelminthes: Cestoda). Mol. Phylogenet. Evol. 52 (2009) 351–367.

[18] W.E.R. Xylander, Ultrastructural studies on the reproductive system of Gyrocotylidea and Amphilinidea (Cestoda): II. Vitellarium, vitellocyte development and vitelloduct of *Gyrocotyle urna*. Zoomorphol. 107 (1987) 293–297.

[19] W.E.R. Xylander, Ultrastructural studies on the reproductive system Gyrocotylidea and Amphilinidea (Cestoda): I. Vitellarium, vitellocyte development and vitelloduct in *Amphilina foliacea*. Parasitol. Res. 74 (1988) 363–370.

[20] M. Bruňanská, L.G. Poddubnaya, B.S. Dezfuli, Vitellogenesis in two spathebothriidean cestodes. Parasitol Res 96 (2005) 390–397.

[21] L.G. Poddubnaya, J.S. Mackiewicz, Z. Świderski, M. Bruňanská, T. Scholz, Fine structure of egg-forming complex ducts, eggshell formation and supporting neuronal plexus in progenetic *Diplocotyle olrikii* (Cestoda: Spathebothriidea). Acta Parasitol. 50 (2005) 292–304.

[22] L.G. Poddubnaya, D.I. Gibson, Z. Świderski, P.D. Olson, Vitellocyte ultrastructure in the cestode *Didymobothrium rudolphii* (Monticelli, 1890): possible evidence for the recognition of divergent taxa within the Spathebothriidea. Acta Parasitol. 51(2006) 255–263.

[23] J.S. Mackiewicz, Vitellogenesis and egg-shell formation in *Caryophyllaeus laticeps* (Pallas) and *Caryophyllaeides fennica* (Schneider) (Cestoidea: Caryophyllidea). Z. Parasitenkd. 30 (1968) 18–32.

[24] Z. Świderski, J.S. Mackiewicz, Electron microscope study of vitellogenesis in *Glaridacris catostomi* (Cestoidea: Caryophyllidea). Int. J. Parasitol. 6 (1976) 61–73.

[25] L.G. Poddubnaya, J.S. Mackiewicz, B.I. Kuperman, Ultrastructure of *Archigetes sieboldi* (Cestoda: Caryophyllidea): relationship between progenesis, development and evolution. Folia Parasitol. 50 (2003) 275–292.

[26] Z. Świderski, M. Bruňanská, J.G. Poddubnaya, Ultrastructural and cytochemical studies on vitellogenesis in the caryophyllidean cestode *Caryophyllaeus laticeps*. Proc. 9th EMOP, Valencia, 2004, 602.

[27] Z. Świderski, M. Bruňanská, L.G. Poddubnaya, J.S. Mackiewicz, Cytochemical and ultrastructural study on vitellogenesis in caryophyllidean cestode *Khawia armeniaca* (Cholodkovski, 1915). Acta Parasitol. 49 (2004) 16–24.

[28] Z. Świderski, J.S. Mackiewicz, C. Eira, J. Miquel, Ultrastructural and cytochemical study on vitellogenesis in the diphyllidean cestode *Echinobothrium euterpes* (Echinobothriidae) and its phylogenetical implications. Acta Parasitol. 56 (2011) 180–190.

[29] F. Mokhtar-Maamouri, Z. Świderski, Vitellogénèse chez *Echeneibothrium beauchampi*

Euzet, 1959 (Cestoda: Tetraphyllidea: Phyllobothriidae). Z. Parasitenkd. 50 (1976) 293–302.

[30] Z. Świderski, F. Mokhtar, Étude de la vitellogénèse de *Bothriocephalus clavibothrium* Ariola, 1899 (Cestoda: Pseudophyllidea). Z. Parasitenkd. 43 (1974) 135–149.

[31] J.V. Korneva, Vitellogenesis and capsule formation during embryogenesis in *Triaenophorus nodulosus* (Cestoda, Pseudophyllidea, Triaenophoridea). Zool. Zh. 80 (2001) 1422–1428 (In Russian).

[32] C. Levron, L.G. Poddubnaya, R. Kuchta, M. Freeman, T. Scholz, Vitellogenesis and vitelline system in the pseudophyllidean tapeworm *Paraechinophallus japonicus*: ultrastructural and cytochemical studies. Folia Parasitol. 54 (2007) 43–50.

[33] D.W. Fawcett, An atlas of fine structure I. The cell: its organelles and inclusions. WB Saunders Company, Philadelphia, 1966.

[34] Z. Świderski, W.E.R. Xylander, Types of vitellocytes and vitellogenesis in the Cestoda in relation to different types of embryonic development, ovoviviparity and life cycles. Wiad. Parazytol. 44 (1998) 604.

[35] L.P. Smirnov, V.V. Bogdan, The phenomenon of the temperature preadaptation of the fatty acids spectra of lipids of some cestodes. Karelia and Norway, the main trends and prospects of scientific cooperation, Proc. Sci. Conf. Karelian Res. Cent. (1998) 51–54.

[36] J.D. Smyth, D.P. McManus, The physiology and biochemistry of cestodes. Cambridge University Press, Cambridge, 1989.

[37] Z. Świderski, J.S. Mackiewicz, Ultrastructural studies on the cellular organisation of the coracidium of the cestode *Bothriocephalus clavibothrium* Ariola, 1899 (Pseudophyllidea, Bothriocephalidae). Acta Parasitol. 49 (2004) 116–139.

[38] G.H. Jr. Buteau, J.E. Simmons, D.H. Beach, D.H. Jr. Holtz, I.W. Sherman, The lipids of cestodes from Pacific and Atlantic coast triakid sharks. J. Parasitol. 57 (1971) 1272–1278.

[39] D.H. Beach, I.W. Sherman, D.H.Jr. Holtz, Incorporation of docosahexaenoic fatty acid

into the lipids of a cestode of marine elasmobranchs. J. Parasitol. 59 (1975) 655–666.

[40] A. Nakagawa, T. Fukushima, S. Fukumoto, Fatty acid composition of diphyllobothriid cestodes with reference to their hosts. Yonago Acta Med. 30 (1987) 65–80.

[41] T.E. Mattis, Development of two tetrarhynchidean cestodes from the Northern Gulf of Mexico. PhD Thesis, University of Southern Mississippi, USA, 1986.

[42] Z. Świderski, Biodiversity of parasite eggs - their importance for disease dissemination and diagnostics: comparative TEM studies. Proc. Int. Symp. dedic. 130th Anniv. Acad. K.I. Skrjabin (2008) 453–459.

[43] Z. Świderski, J.S. Mackiewicz, Ultrastructure of polylecithal and oligolecithal eggs cestode parasites of fishes: comparative TEM study. Parassitologia 49 (2007) 394.

[44] Z. Świderski, J.S. Mackiewicz, Ovoviviparity in cestode parasites of fishes. Parassitologia 49 (2007) 394.

[45] L. Chervy, The terminology of larval cestodes or metacestodes. Syst. Parasitol. 52 (2002) 1–33.

[46] C. Noever, J.N. Caira, R. Kuchta, L. Desjardins, Two new species of *Aporhynchus* (Cestoda: Trypanorhyncha) from deep water lanternsharks (Squaliformes: Etmopteridae) in the Azores, Portugal. J. Parasitol. 96 (2010) 1176–1184.

Figure captions

Figure 1A-B. Undifferentiated stem cells of the gonial type or stage I. **(A)** Undifferentiated stem cells of the gonial type (stage I) situated at the periphery of the vitelline follicles. Note: (1) the high nucleo-cytoplasmic ratio of these precursor cells with a large nucleus containing a prominent, electron-dense nucleolus (n); and (2) a thin layer of granular cytoplasm surrounding the nucleus (N), which contains a very few small mitochondria (m). L: lipid droplets; sgc: shell globule clusters. (**B)** A distinct increase in cell size observed at the end of

stage 1 (compare with Fig. 1A). Note: (1) a much larger volume of cytoplasm containing numerous mitochondria (m), a few short profiles of GER and Golgi complexes (G); and (2) a large nucleus (N) with an electron dense nucleolus (n) and several islands of heterochromatin (Hch). gl: glycogen; IS: interstitial syncytium; L: lipid droplet; np: nuclear pore; sgc: shell globule cluster.

Figure 2A-C. An early differentiation stage: stage II and the beginning of stage III. **(A)** Details of two vitellocytes at an early stage of (stage II) situated in the left and right side corners, separated by a cell at more advanced stage of differentiation (stage III). Note: (1) numerous parallel cisternae of smooth endoplasmic reticulum (SER) in the perinuclear cytoplasm of the two cells at stage II, and (2) mitochondria (m), granular endoplasmic reticulum (GER), Golgi vesicles (G) and a large, irregularly shaped lipid droplet (L) apparently undergoing fusion in the middle cell at stage III. β-gl: beta-glycogen; N: nucleus. (**B)** Details of vitellocytes at the early differentiation stage (stage II) after Thiéry's cytochemical test for glycogen. Note: (1) a few heterochromatin islands (Hch) in the nucleoplasm; and (2) in the cytoplasm: (a) several accumulations of β-glycogen particles (β-gl); (b) numerous mitochondria (m); and (c) electron-dense lipid droplets (L). G: Golgi complexes; N: nucleus; v: vacuole. (**C)** Details of the vitellocyte cytoplasm (stage III). Note: (1) the development of parallel, concentrically arranged cisternae of the granular endoplasmic reticulum (GER) surrounded by a Golgi complex (G) and dilated Golgi vesicles that produce dense, proteinaceous shell-globule clusters (sgc) situated in the central part of the cell; (2) several lipid droplets (L), mitochondria (m) and two shell-globule clusters closely adjacent to the cell plasma membrane. Inset: Higher-power detail of the shell globule clusters and β-glycogen particles (β-gl) adjacent to cell plasma membrane.

Figure 3A-B. Advanced maturation of vitellocyte or stage III. **(A)** The peripheral region of the vitellocyte cytoplasm showing concentrically arranged Golgi complexes (G) composed of

numerous vesicles at various degrees of dilation; adjacent to them are shell globule clusters (sgc), large lipid droplets (L), one with a closely adjacent shell globule cluster, and several β-glycogen particles (β-gl) in the upper right corner of the micrograph. (**B)** Details of the vitellocyte cytoplasm (stage III). Note several shell globule clusters (sgc), lipid droplets (L) and β-glycogen particles (β-gl). The elongate cytoplasmic process of the interstitial cell (IS), with a few mitochondria (m), is shown in the lower right corner of the micrograph.

Figure 4A-B. Mature vitellocyte or stage IV. **(A)** A mature vitellocyte. Note: (1) the nucleus (N) with a semi-lunar shape and prominent nucleolus (n); (2) the highly vacuolated cytoplasm with a few parallel cisternae of granular endoplasmic reticulum (GER) in the perinuclear region, and numerous lipid droplets (L) and shell globule clusters (sgc) grouped mainly in the peripheral cytoplasm. v: vacuole. (**B)** High-power micrograph of the peripheral cytoplasm of two mature vitellocytes. Note: (1) numerous very large, moderately saturated lipid droplets (L); (2) a few large mitochondria (m); and (3) numerous membrane-bound shell globule clusters (sgc) grouped mainly in the peripheral region; each cluster is composed of numerous electron dense but rather small individual shell globules, and all are embedded in an electron lucent phenolase component.

Figure 5. Diagram of the egg of *Aporhynchus menezesi*. Modified after Dollfus [3]. Bl: blastomeres; C: capsule; EC: egg compartment.

Table / Tableau

Trypanorhynch families and species	Host species and families	Host locality	Glycogen: amount and type	Membrane bound glycoproteins	Lipid droplets: amount and chemical nature	Reference
Aporhynchidae						
Aporhynchus menezesi Noever et al., 2010	*Etmopterus spinax* (Etmopteridae)	Horta, Faial, Azores Islands (Atlantic Ocean)	moderate amount of β particles, mainly in immature vitellocytes	not studied	high amount; moderately saturated character	Present paper
Lacystorhynchidae						
Grillotia erinaceus (van Beneden, 1858)	*Raja clavata* (Rajidae)	Irish Sea	high amount; both α-rosettes and β particles	not studied	moderate amount; moderately saturated character	[8]
Eutetrarhynchidae						
Dollfusiella spinulifera (Beveridge and Jones, 2000)	*Rhinobatos typus* (Rhinobatidae)	Heron Island, Australia (Coral Sea)	only very few β particles, mainly in immature vitellocytes	attached to all membraneous structures such as GER, Golgi, mitochondria, nuclear and cell plasma membrane	very large amount and large size of unsaturated, highly osmiophilic droplets	[9]
Parachristianella trygonis Dollfus, 1946	*Dasyatis pastinaca* (Dasyatidae)	Sidi Mansour and Zarzis, Tunisia (Mediterranean Sea)	high accumulation of α-rosettes and β particles	not studied	high amount; moderately saturated character	[11]
Progrillotiidae						
Progrillotia pastinacae Dollfus, 1946	*Dasyatis pastinaca* (Dasyatidae)	Sidi Mansour, Tunisia (Mediterranean Sea)	only few traces of β particles adjacent to lipid droplets	not studied	very high acumulation; highly saturated, osmiophobic droplets	[10]

Table I: Comparison of the ultrastructural characters of vitelline material in five trypanorhynch species.

Figure 1

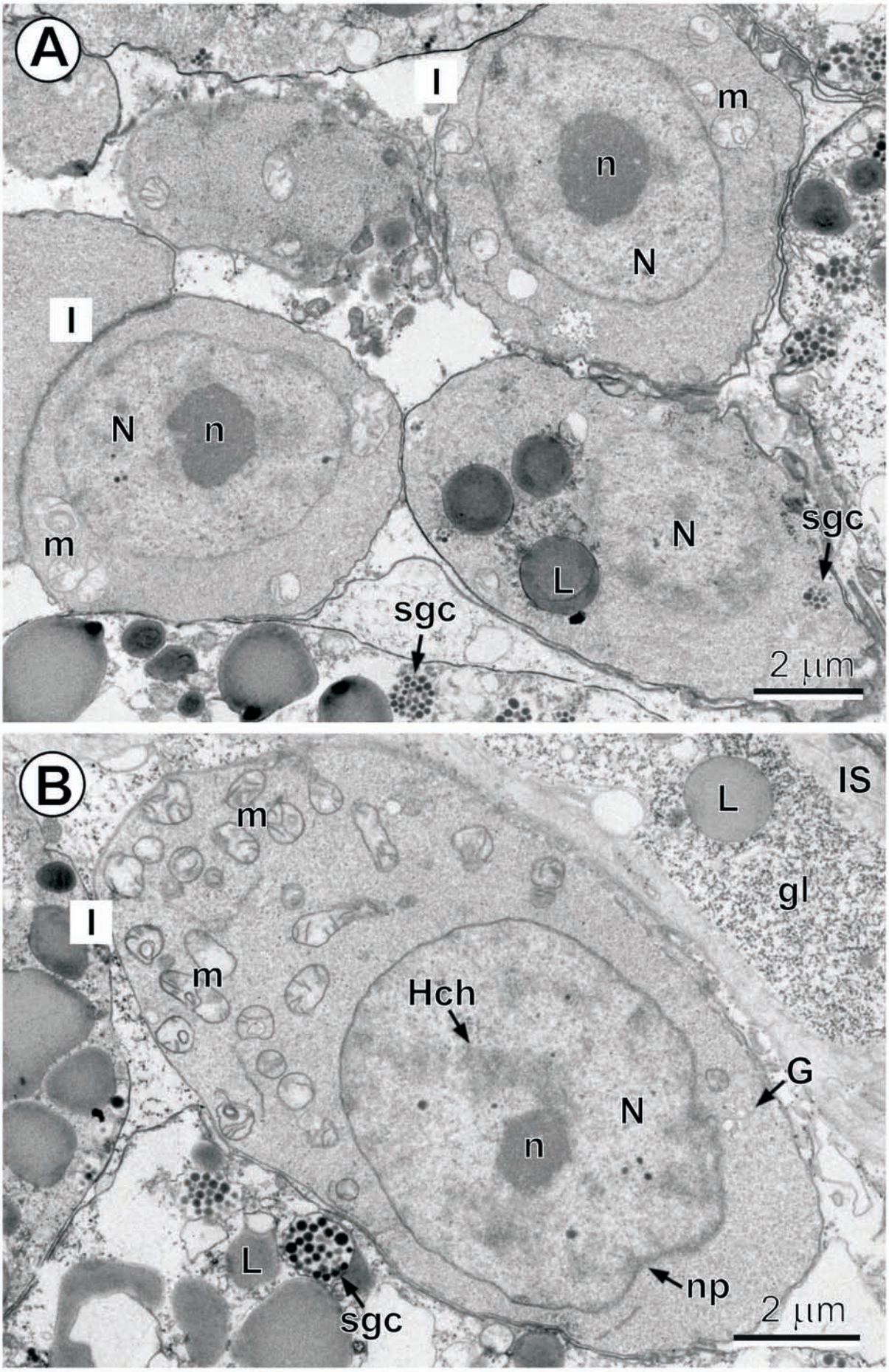

Figure 2

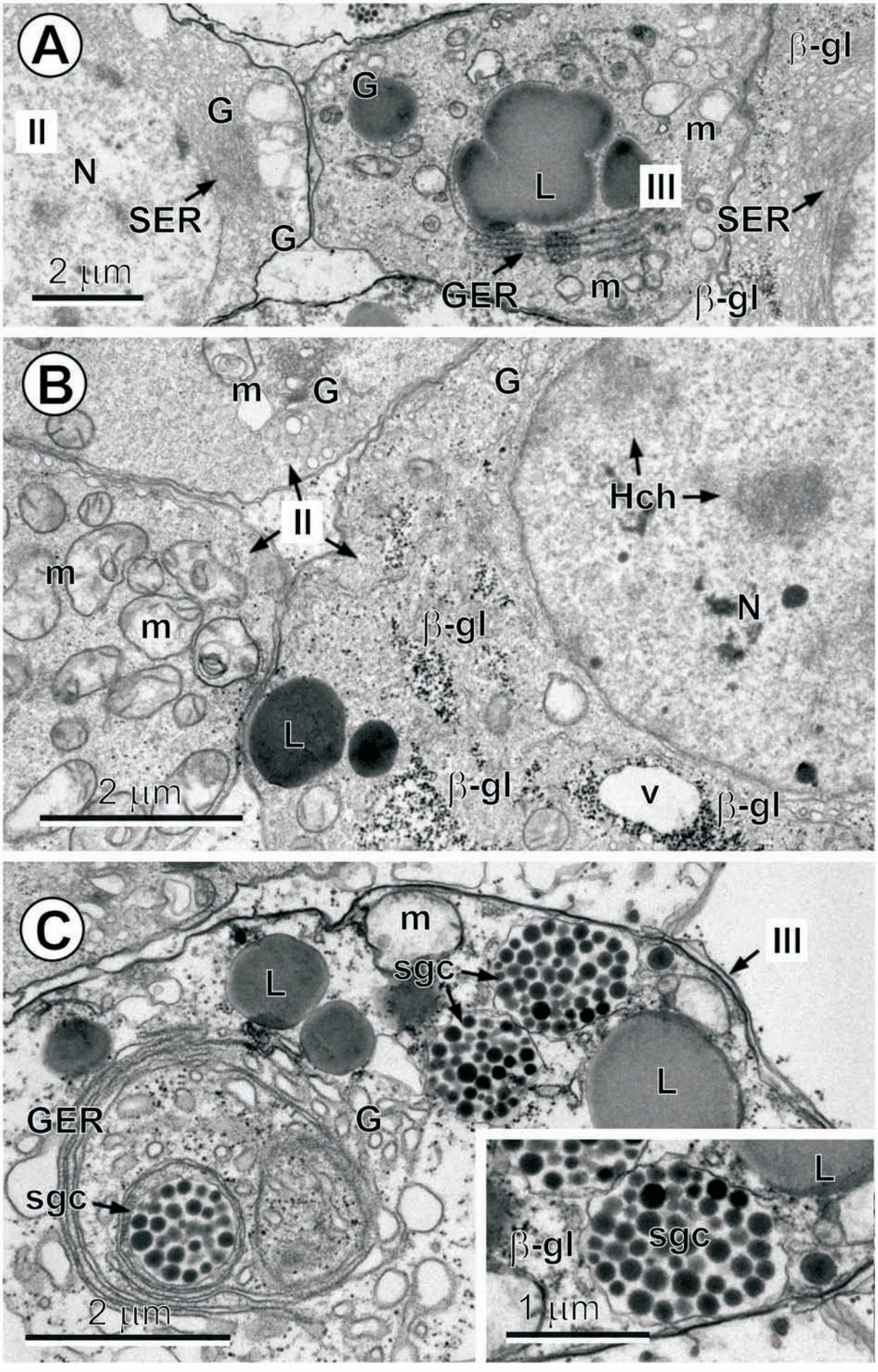
A
II
N
SER
G
G
G
L
III
m
β-gl
SER
GER
m
β-gl
2 μm
B
m
G
G
II
m
m
β-gl
Hch
N
L
β-gl
v
β-gl
2 μm
C
m
sgc
III
L
L
GER
G
sgc
2 μm
L
β-gl
sgc
1 μm

Figure 3

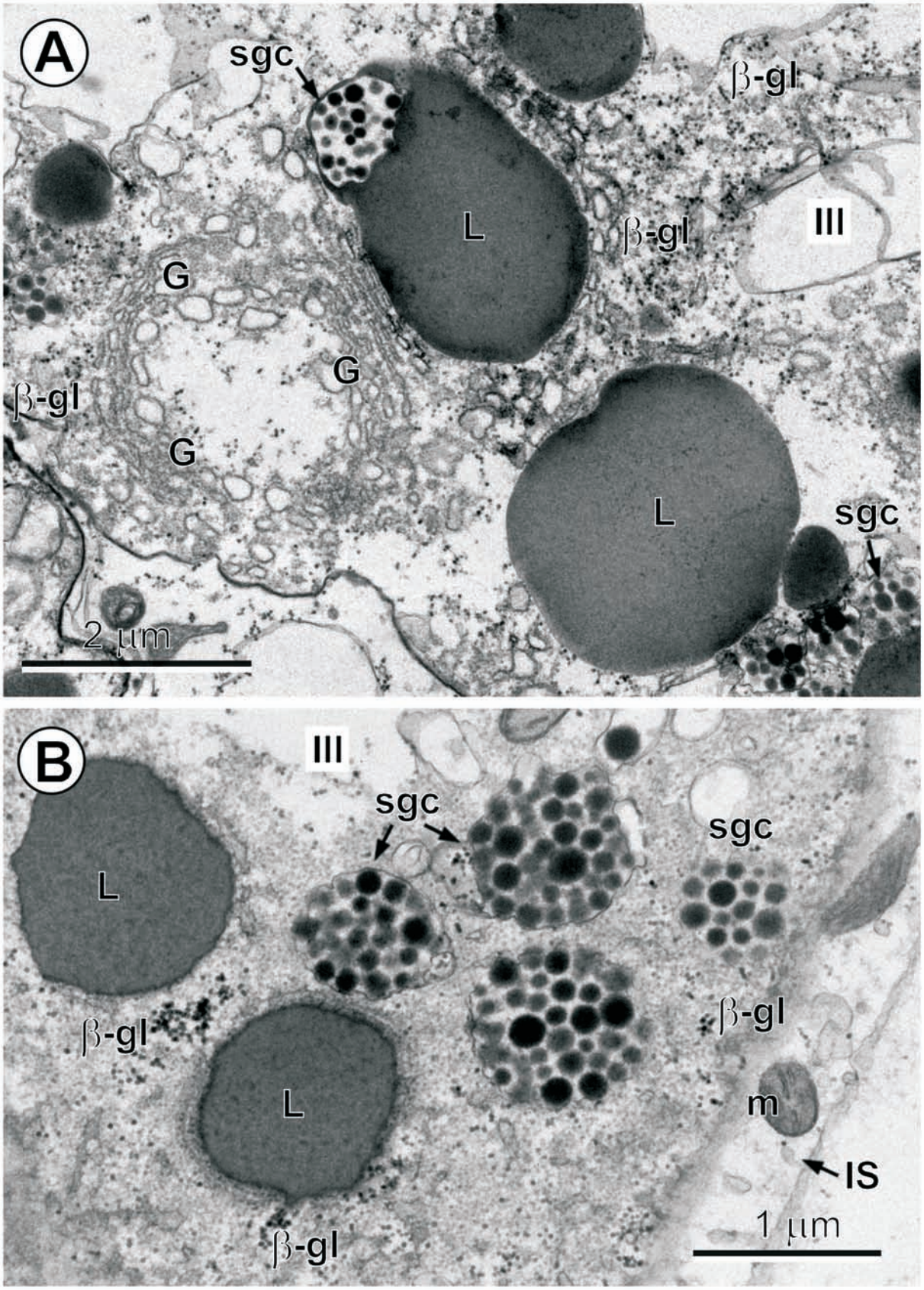
A
sgc
β-gl
L
β-gl
III
G
G
β-gl
G
L
sgc
2 μm
B
III
sgc
sgc
L
β-gl
β-gl
L
m
IS
β-gl
1 μm

Figure 4

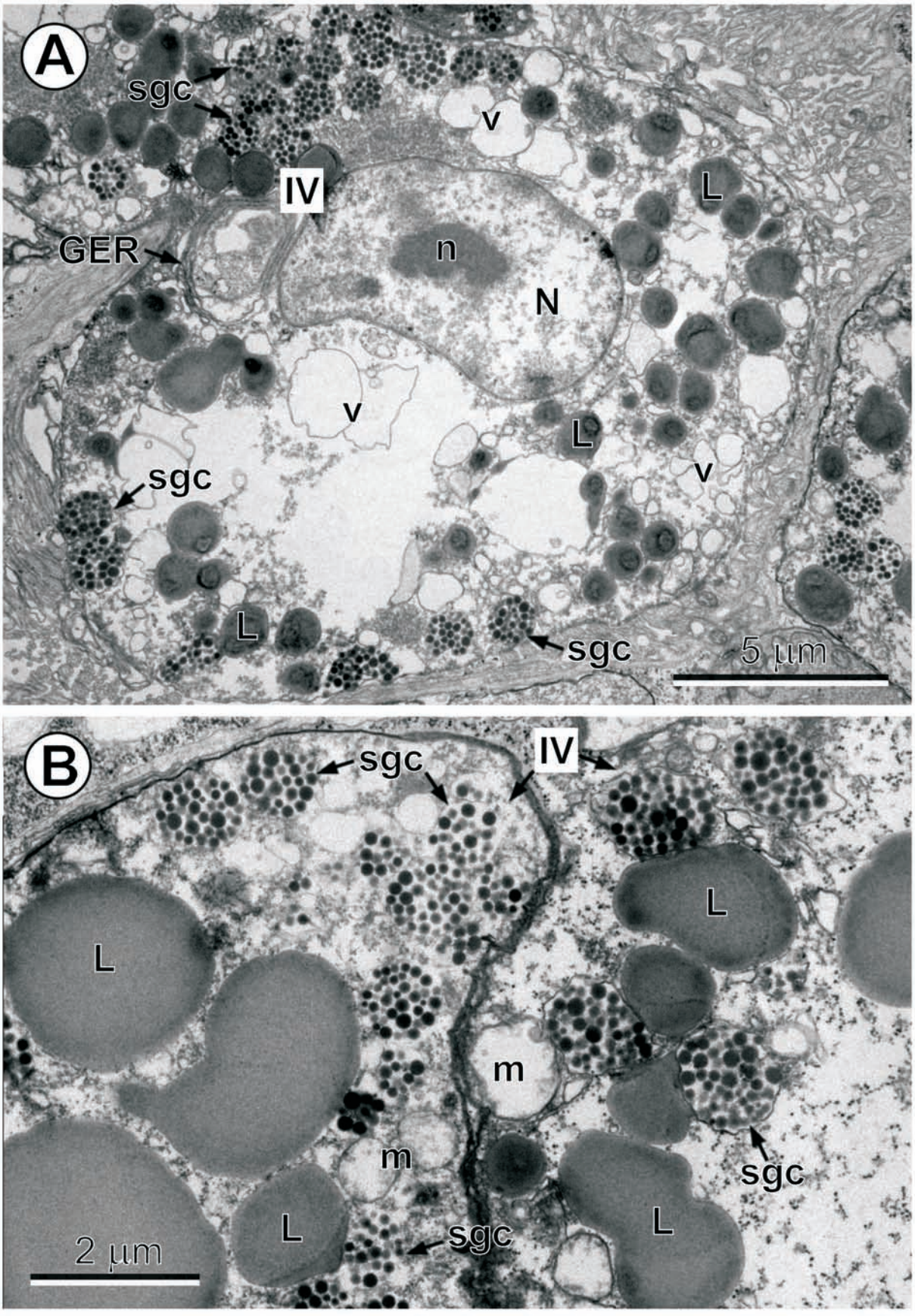

Figure 5