DOI: 10.2478/s11686-012-0025-6

Acta Parasitologica, 2012, 57(2), 131–148; ISSN 1230-2821

VERSITA

Advanced stages of embryonic development and cotylocidial morphogenesis in the intrauterine eggs of *Aspidogaster limacoides* Diesing, 1835 (Aspidogastrea), with comments on their phylogenetic implications**

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Abstract

Ultrastructural aspects of the advanced embryonic development and cotylocidial morphogenesis of the aspidogastrean *Aspidogaster limacoides* are described. The posterior or distal regions of the uterus are filled with eggs containing larvae at advanced stages of morphogenesis and fully-formed cotylocidia. Various stages and organs of this larva are described in detail, including the aspects of the developing and fully-differentiated cotylocidium, the body wall (tegument and musculature), glandular regions and the protonephridial excretory system. Blastomere multiplication by means of mitotic divisions takes place simultaneously with the degeneration or apoptosis of some micromeres; this frequently observed characteristic is compared and discussed in relation to corresponding reports for other neodermatans. During the advanced stages of the embryonic development of *A. limacoides*, the vitelline syncytium disappears and the size of the embryo increases rapidly. Evident polarization of the differentiating larva was observed; towards one pole of the egg, cytodifferentiation of the mouth, surrounded by the oral sucker and cephalic glands, takes place, whereas, towards the opposite pole, differentiation of the posterior sucker (incipient ventral disc) occurs. The oral and posterior suckers are formed from numerous embryonic cells which have differentiated into myocytes. The central part of the oral sucker undergoes invagination and forms the future pharynx and intestine. Fully-developed cotylocidia of *A. limacoides* have a neodermatan type of tegument, flame cells and two types of glandular structures. These results suggest a sister relationship between the Aspidogastrea and the Digenea, although the systematic position of aspidogastreans in relation to other platyhelminth taxa remains somewhat equivocal.

Keywords

Aspidogastrea, Aspidogastridae, *Aspidogaster limacoides*, embryogenesis, cotylocidial morphogenesis, cotylocidium, intrauterine eggs, ultrastructure

Résumé

Les aspects ultrastructuraux du développement embryonnaire avancé et de la morphogenèse du cotylocidium de l'aspidogastre *Aspidogaster limacoides* sont décrits. Les régions postérieures et distales de l'utérus sont remplies d'oeufs qui contiennent des larves à des stades avancés de morphogenèse et des cotylocidia complètement formés. Des stades et organes variés de la larve sont décrits en détail, y compris les aspects du cotylocidium en développement et complètement développé, la paroi du corps (tégument et musculature), les régions glandulaires et le système excréteur protonéphridien. La multiplication des blastomères par des divisions mitotiques, prend place simultanément à la dégénération ou apoptose de certains micromères. Cette caractéristique fréquemment observée, est comparée et discutée par rapport à des études similaires correspondantes aux autres Neodermata. Pendant les stades avancés du développement embryonnaire de *A. limacoides*, le syncytium vitellin disparaît et la taille de l'embryon augmente rapidement. Nous avons observé une polarisation évidente de la larve en différentiation: vers un

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**Dedicated to the memory of Professor Robert Ph. Dollfus (1887–1976) on the occasion of the 125th Anniversary of his birthday

pôle de l'œuf prend place la cytodifférentiation de la bouche, entourée par la ventouse ventrale et les glandes céphaliques, alors que la différentiation de la ventouse postérieure (disque ventral en formation) a lieu au pôle opposé. Les ventouses orales et postérieures sont formées à partir de nombreuses cellules embryonnaires qui se sont différenciées en myocytes. La partie centrale de la ventouse orale s'invagine et forme le futur pharynx et le future intestin. Les cotylocidia complètement développés de *A. limacoides* ont un tégument de type Neodermata, des cellules-flammes et deux types de structures glandulaires. Ces résultats suggèrent que les Aspidogastrea et Digenea sont des groupes frères, bien que la position systématique des aspidogastres par rapport aux autres taxons de Plathelminthes reste quelque peu équivoque.

Introduction

The Aspidogastrea is a small subclass of exclusively parasitic flatworms which are generally considered to represent an ancient sister group of the subclass Digenea within the Trematoda. This group has retained some archaic characters of the hypothetical proto-trematodes (Rohde 1972, 1994, 2001, 2002; Gibson 1987) and has been considered to comprise two orders, four families, 12 genera and only about 80 species (Gibson and Chinabut 1984, Rohde 2001, 2002), although some doubt concerning the higher level classification has been expressed following a limited molecular study (Chen *et al*. 2007).

Aspidogastreans are relatively cosmopolitan in marine and freshwater environments. All of the species whose life-cycle is known use a mollusc as an obligate host and, in most cases, a lower vertebrate as a facultative or obligate final host (Rohde 1972, 1994, 2001, 2002; Gibson 1987). Parthenogenetic generations (asexual larval multiplication), such as occur in the Digenea, are absent in known life-cycles of aspidogastreans. Although some species can complete their life-cycle in one and the same mollusc, most exhibit a simple two-host life-cycle pattern. The vertebrate hosts are freshwater teleosts, and occasionally turtles, in the case of the Aspidogastrida and chondrichthyans in the case of the Stichocotylida (Aubert 1855; Voeltzkow 1888a, b; Monticelli 1892; Williams 1942; Brinkmann 1957; Dollfus 1958; Rohde 1972, 2001).

Rohde (1972, 2001), in his major reviews of the biology and relationships of the Aspidogastrea, has considered that a closer examination of this group may shed more light on its phylogenetic affiliations with other platyhelminth groups. According to this author (Rohde 2001, 2002), the more complex life-history patterns of the Digenea likely evolved from the simple life-cycle of the aspidogastreans.

Ultrastructural aspects of the embryonic development and the cytodifferentiation of the aspidogastrean larva (the cotylocidium) inside the intrauterine eggs have not previously been examined, with the exception of our previous report on the early embryonic development of *Aspidogaster limacoides* Diesing, 1835 (see Świderski *et al*. 2011). Our previous contribution, however, concentrated exclusively on the ultrastructural characteristics of the newly-formed eggshell, the origin of the operculum, the early embryo and the formation of the embryonic envelope. The cotylocidium differs from the digenean larva, the miracidium, which develops within the egg, in that it possesses the fundamental organisation of the adult form and thus more closely resembles the digenean metacercaria, i.e. the cotylocidium develops directly into an adult, either in the same host or in another individual of the same or a different species (Rohde 1972, 1994, 2002; Świderski *et al*. 2011).

The aims of the present study were: (1) to examine and describe the ultrastructure of the advanced stages of embryonic development and, in particular, cotylocidial morphogenesis inside the intrauterine eggs of the aspidogastrid aspidogastrean *Aspidogaster limacoides*; (2) to compare our results with previously reported data for other representatives of the Neodermata, and particularly for digeneans, cestodarians and lower eucestodes; and (3) to discuss any phylogenetic implication of the new results in relation to existing information for other parasitic flatworms.

Materials and methods

Adult specimens of *Aspidogaster limacoides* were obtained from the intestine of a cyprinid fish, the white bream *Blicca bjoerkna* (Linnaeus, 1758), in the Rybinsk Reservoir on the Volga River, Russia. Live aspidogastreans were rinsed in 0.9% NaCl solution, fixed in cold (4°C) 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 10 days, washed overnight in 0.1 M sodium cacodylate buffer at pH 7.4, postfixed in cold (4°C) 1% OsO_4 in the same buffer for 1 h, dehydrated in a graded series of ethanol and propylene oxide, and embedded in Epon epoxy resin. Ultrathin sections were cut on a Leica Ultracut UCT ultramicrotome, collected on copper grids and double stained with uranyl acetate and lead citrate. Sections were examined in JEOL 1010 and 1011 transmission electron microscopes operating at 80 kV.

Results

The developing cotylocidium (Figs 1–8)

During advanced stages of the embryonic development of *A. limacoides* (Figs 1 and 2), the material accumulated in the vitelline syncytium disappears progressively. Simultaneously,

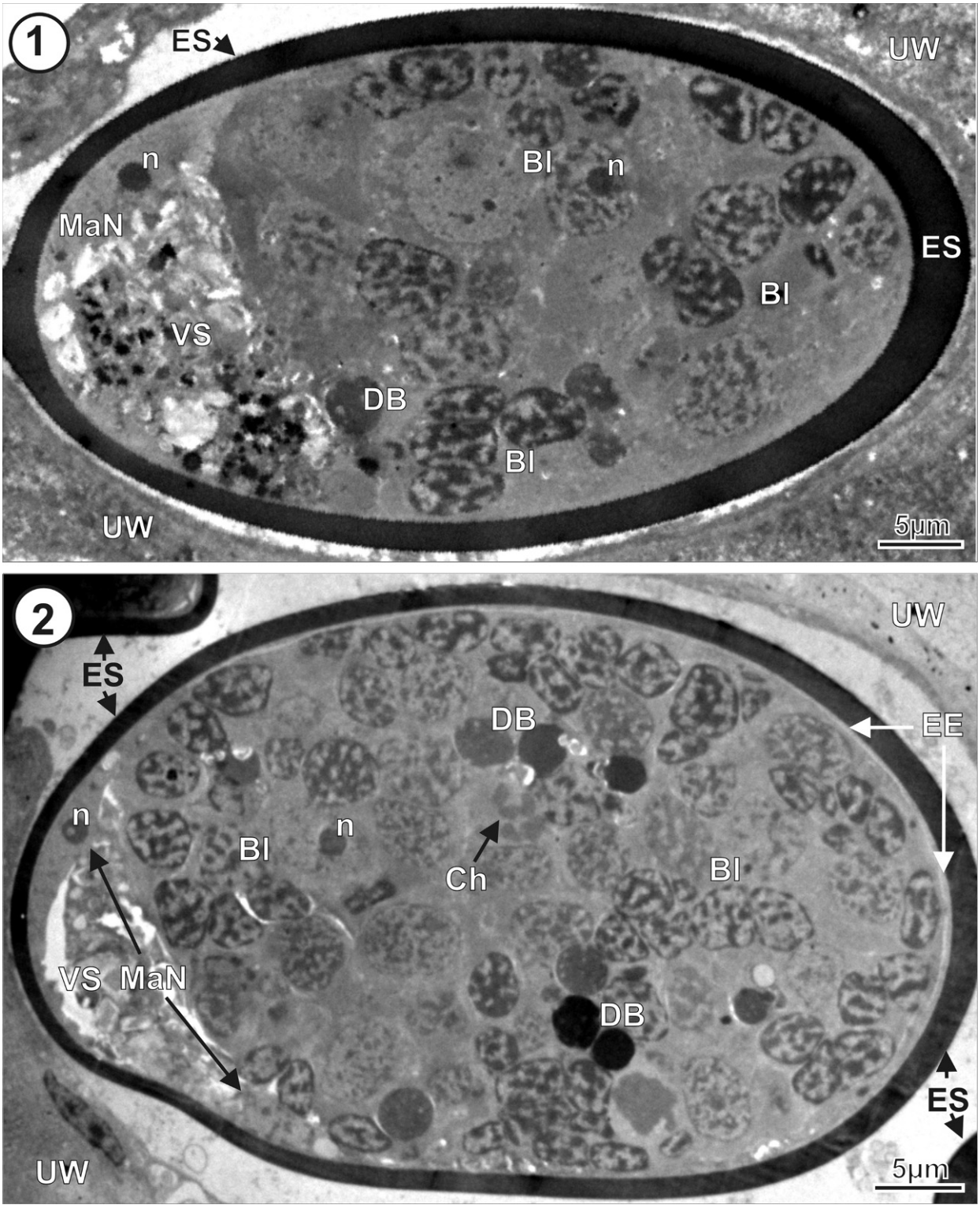


Fig. 1. Low magnification micrograph illustrating the general topography of advanced stage embryogenesis in the eggs of *Aspidogaster limacoides* situated in the distal region of the uterus. Note: (1) the reduced volume of the vitelline syncytium on the left side in comparison with the much greater volume of the numerous blastomeres of the embryo occupying the opposite pole of the egg; (2) the macromere nucleus with its prominent nucleolus, to the left, and adjacent to the vitelline syncytium which takes part in embryonic envelope formation; and (3) degenerating blastomeres in the form of dark, pycnotic nuclei of micromeres **Fig. 2.** A slightly more advanced stage of embryonic development than that illustrated in Fig. 1. Note: (1) the much more reduced volume of the vitelline syncytium and the more flattened nuclei of the two adjacent macromeres forming the future embryonic envelope; (2) the much greater number of blastomeres of different types; and (3) the presence of more numerous dark, pycnotic nuclei of degenerating micromeres undergoing apoptosis

the size of the embryo, which is composed of a growing number of blastomeres, increases rapidly (compare the size of the vitelline syncytium with that of the embryo in Figs 1 and 2). At this stage, the blastomeres undergoing early cytodifferentiation already exhibit obvious differences not only in their size but also in the ultrastructural characteristics of their nuclei and cytoplasm (Figs 1–4). Many of them, however, still remain actively engaged in two simultaneous morphogenetic processes, i.e. some of them are still undergoing mitotic division (Fig. 4, and its inset), which leads to an increasing embryonic cell number, whereas others, predominantly micromeres, undergo a progressive degeneration or apoptosis (Figs 1, 2 and 4) with the opposite result, a decrease in their number.

Apoptosis, or programmed cell death, starts in the early embryos of *A. limacoides*, as does the formation of a single embryonic envelope (Świderski *et al*. 2011). In the advanced stages of embryonic development, both of the macromere nuclei, which were involved in the formation of this envelope, become much flattened due to pressure caused by the increasing volume of other embryonic cells, but they always contain prominent, highly electron-dense nucleoli (Fig. 3).

During the final stages of blastomere cytodifferentiation (i.e., the early phase of cotylocidial morphogenesis) evident polarization of the differentiating larva was observed, which became folded back on itself within the egg (Figs 5–8). Towards one pole of the egg, cytodifferentiation of the mouth takes place (Figs 5–8), which is surrounded by the oral sucker and adjacent cephalic glands (Fig. 8) with their ducts, whereas, towards the opposite pole, differentiation of the posterior sucker (the incipient ventral disc) takes place (Figs 5–8). In the folded larva, primordia of the pharynx and intestine, which differentiate later (see below), are situated along the longitudinal axis of the larva between the oral and posterior suckers (Figs 5–8). Both of these large, spherical, apparently muscular suckers are formed from numerous embryonic cells which have differentiated into myocytes (Figs 5–8). In the eggshell-bound cotylocidium, it is sometimes difficult to differentiate myocytes of the oral sucker from those of the posterior sucker in ultrathin sections. Both are composed of irregularly-shaped, much infolded nuclei containing very numerous electron-dense heterochromatin islands and surrounded by moderately electron-dense layers of cytoplasm with muscle fibres and mitochondria (Figs 5–8).

During the advanced stages of cotylocidial morphogenesis, the two most substantial structures, the oral and posterior suckers, situated at opposite ends of the folded, eggshell-bound larva (Figs 5–8 and 17A), undergo rapid differentiation. They both originate from characteristic elongate blastomeres containing long, irregularly shaped nuclei (N1) with long, electron-dense chromatin islands. These blastomeres form myocytes and their muscle fibres (Figs 5–8). Nuclei (N2) of a second type are more spherical and contain less electron-dense heterochromatin; these are frequently situated under the tegumental cytoplasmic layer of the larva and may represent the nuclei of subtegumentary perikarya or those of developing parenchymal cells (Figs 5–8). A third type of nucleus (N3), usually situated adjacent to secretory regions and containing type sg1 secretory granules, occurs in the fully-differentated cotylocidium and may represent glandular perikaria of type 1 gland-cells (GC1) (Figs 9, 12, 14 and 17B). The central part of the oral sucker undergoes a progressive, very deep invagination and appears to differentiate into the future pharynx and intestine (compare Figs 5–8 and 17A, B).

The fully-differentiated cotylocidium (Figs 9–17A, B)

Egg-bound, folded, but fully-developed cotylocidia of *A. limacoides* present flame cells (Fig. 11), perforated nephropores (Figs 9 and 12) and two types of glandular structures which contain two evidently different types of secretory granules, sg1 and sg2 (Figs 10–15).

Larval body wall: *tegument and musculature*

The larval tegument of *A. limacoides* (Figs 15 and 16) is unciliated and of the neodermatan type, and composed of an outer syncytial layer of anucleate cytoplasm connected by cytoplasmic bridges to the more deeply located perikarya. Tegumental cytons are situated in the parenchyma beneath the longitudinal and circular muscle fibres of the body musculature, which are attached to myocytes (Figs 15 and 16). The tegumental surface of the cotylocidium bears short, blunt microvilli which arise from the more electron-dense outer part of the cytoplasmic syncytium (Figs 15 and 16). Several small mitochondria are situated below in less electron-dense layer of the tegumental syncytium (Fig. 16); their size is much smaller than that of myocyte mitochondria, seen localised in the left lower corner of Fig. 16.

Glandular regions

Two types of glandular regions with evidently different types of secretory granules were observed in the cotylocidia of *A. li-*

Abbreviations to all figures: Bl – blastomeres, **C** – flame cell cilia, **Ch** – chromosomes, **DB** – degenerating blastomeres, **EE** – embryonic envelope, **ES** – eggshell, **FCN** – flame cell nucleus, **GC1** – type 1 glandular region, **GC2** – type 2 glandular region, **GER** – granular endoplasmic reticulum, **gl** – glycogen rosettes, **Inv** – invagination, **Int** – intestine, **L** – lipid droplets, **M** – muscle fibres, **m** – mitochondrion, **MaN** – macromere nucleus, **Mo** – mouth, **Mv** – tegumental microvilli, **N1** – type 1 nucleus, **N2** – type 2 nucleus, **N3** – type 3 nucleus, **NP** – nephropore, **n** – nucleolus, **OS** – oral sucker, **Ph** – pharynx, **sg1** – type 1 secretory granules, **sg2** – type 2 secretory granules, **T** – tegument, **UW** – uterine wall, **VD** – ventral disc (posterior sucker), **VS** – vitelline syncytium

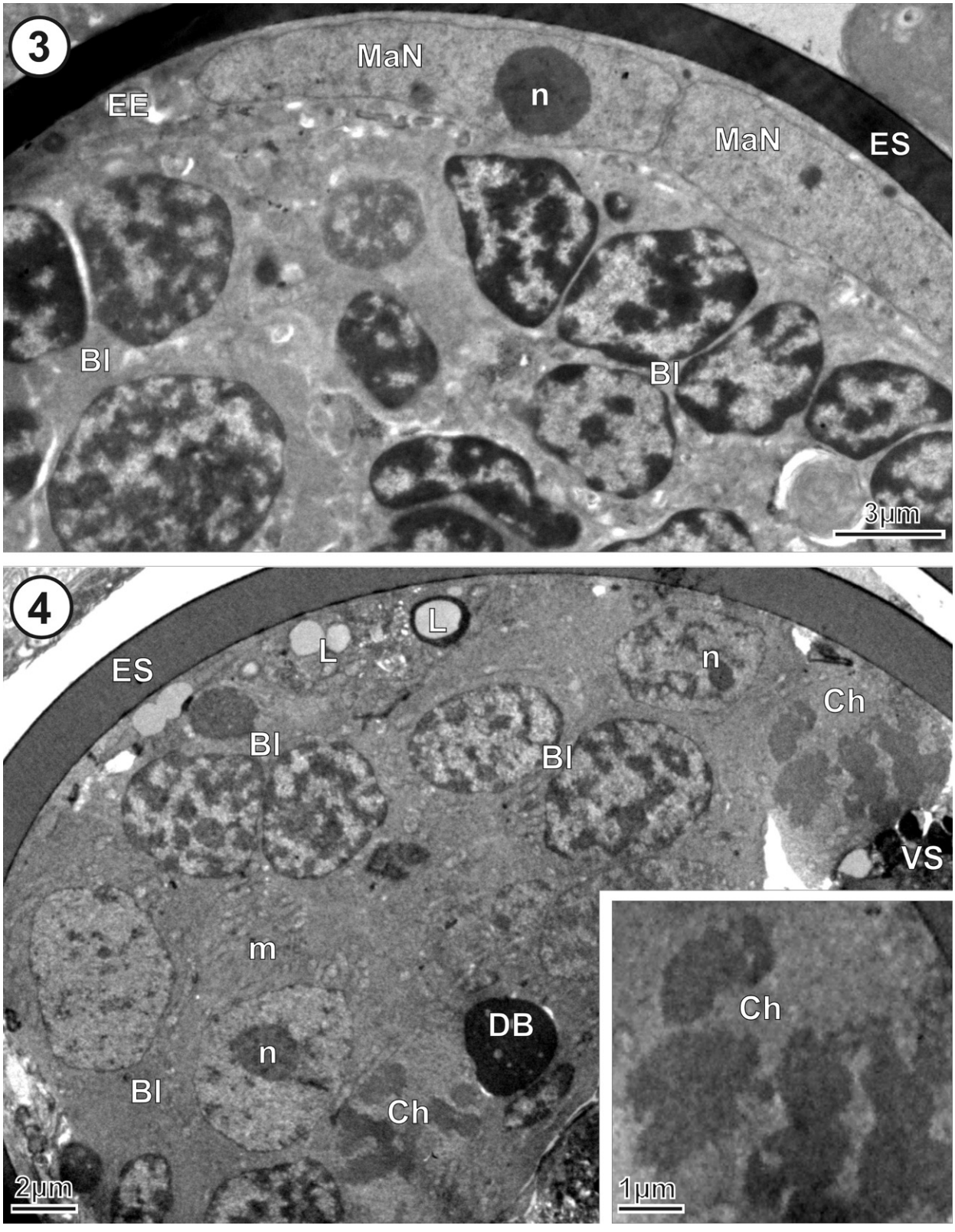


Fig. 3. High power micrograph showing a large, much flattened macromere nucleus, and its distinctive nucleolus, situated beneath the eggshell, which takes part in embryonic envelope formation. Note numerous blastomere nuclei of different ultrastructural types and sizes situated beneath the embryonic envelope **Fig. 4.** Part of the shell-bound embryo composed of numerous blastomeres of different types. Note that the mitotic division of some of these blastomeres occurs simultaneously with the degeneration of others. Inset: Enlarged detail of the mitotic division of the blastomere situated in the right upper corner, close to the small remnants of the vitelline syncytium

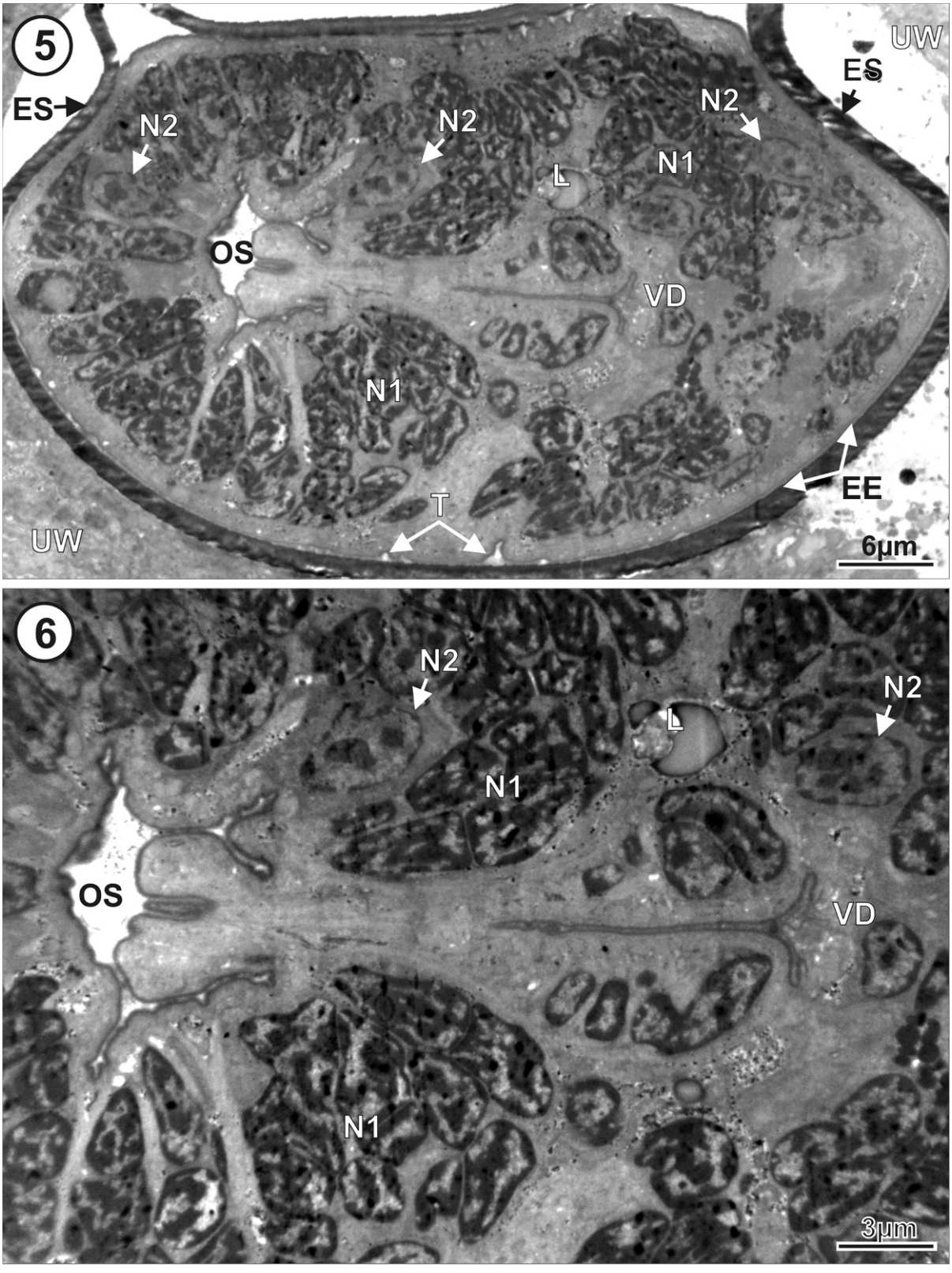
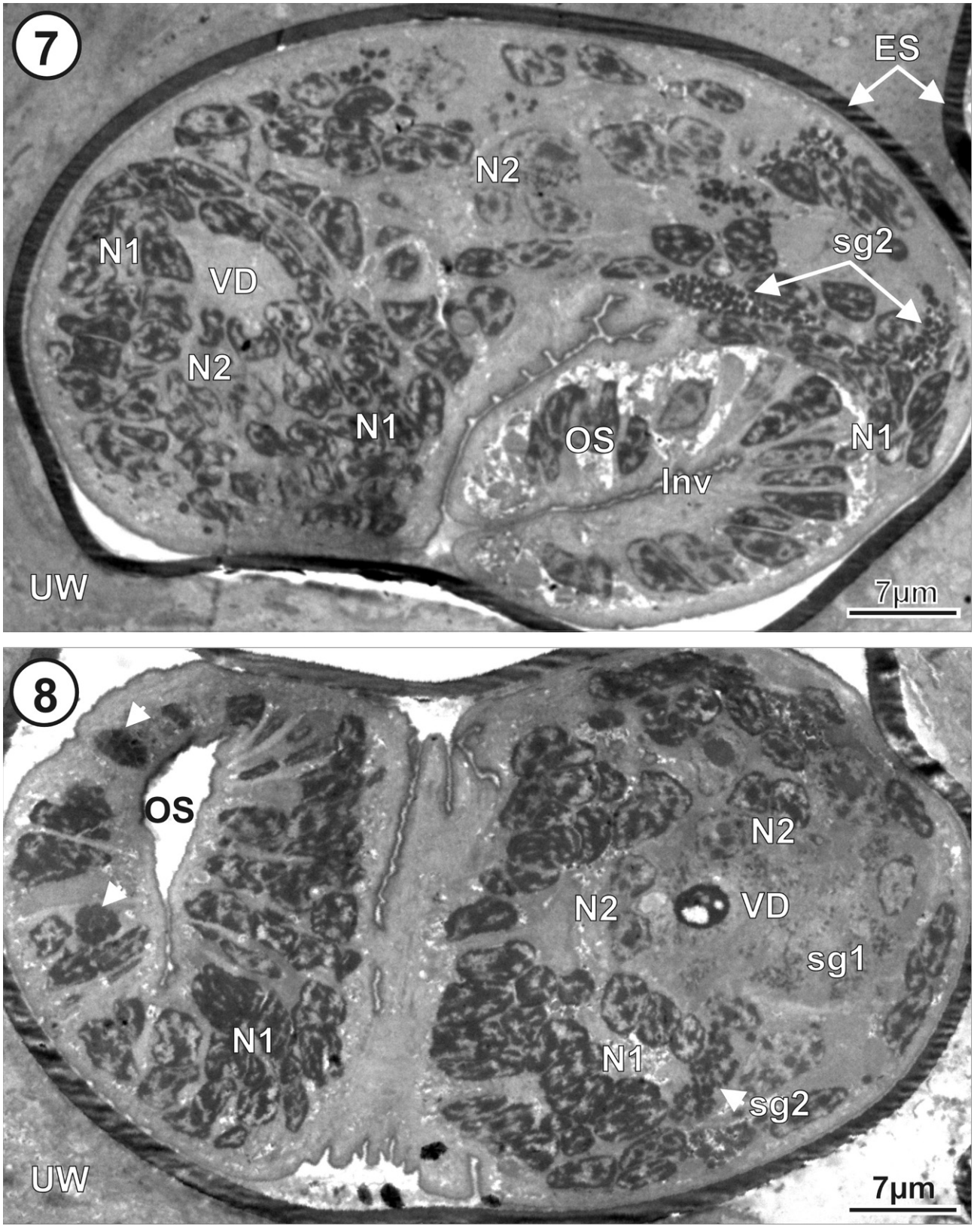


Fig. 5. Advanced stage of blastomere differentiation initiating cotylocidial morphogenesis. Note: (1) the presence of two ultrastructurally different types of nuclei, N1 and N2, of the former blastomeres; and (2) the beginning of larval tissue polarization, with the formation of the oral sucker towards one pole, on the left side, and the posterior sucker (incipient ventral disc) towards the other **Fig. 6.** Enlargement of the central region of Fig. 5, showing muscle bundles and myocyte perikarya forming the invaginated structure of the pharynx just to the right of the oral sucker, and the relatively thick layer of the musculature of the posterior sucker (ventral disc) further to the right



Figs 7 and **8.** Ultrathin sections through the cotylocidium at advanced stages of its morphogenesis. Note: (1) the oval shape of the oral sucker containing N1 cells that represent myocytes of its musculature; (2) a deep invagination in the region of the oral sucker which may represent the sucker lumen leading to the developing pharynx; (3) the accumulation of N1 cells in the region of the posterior sucker (ventral disc); (4) two types of secretory granules (sg1 and sg2); and (5) a cross-section through a gland-duct close to the oral sucker (Fig. 8, white arrowhead)

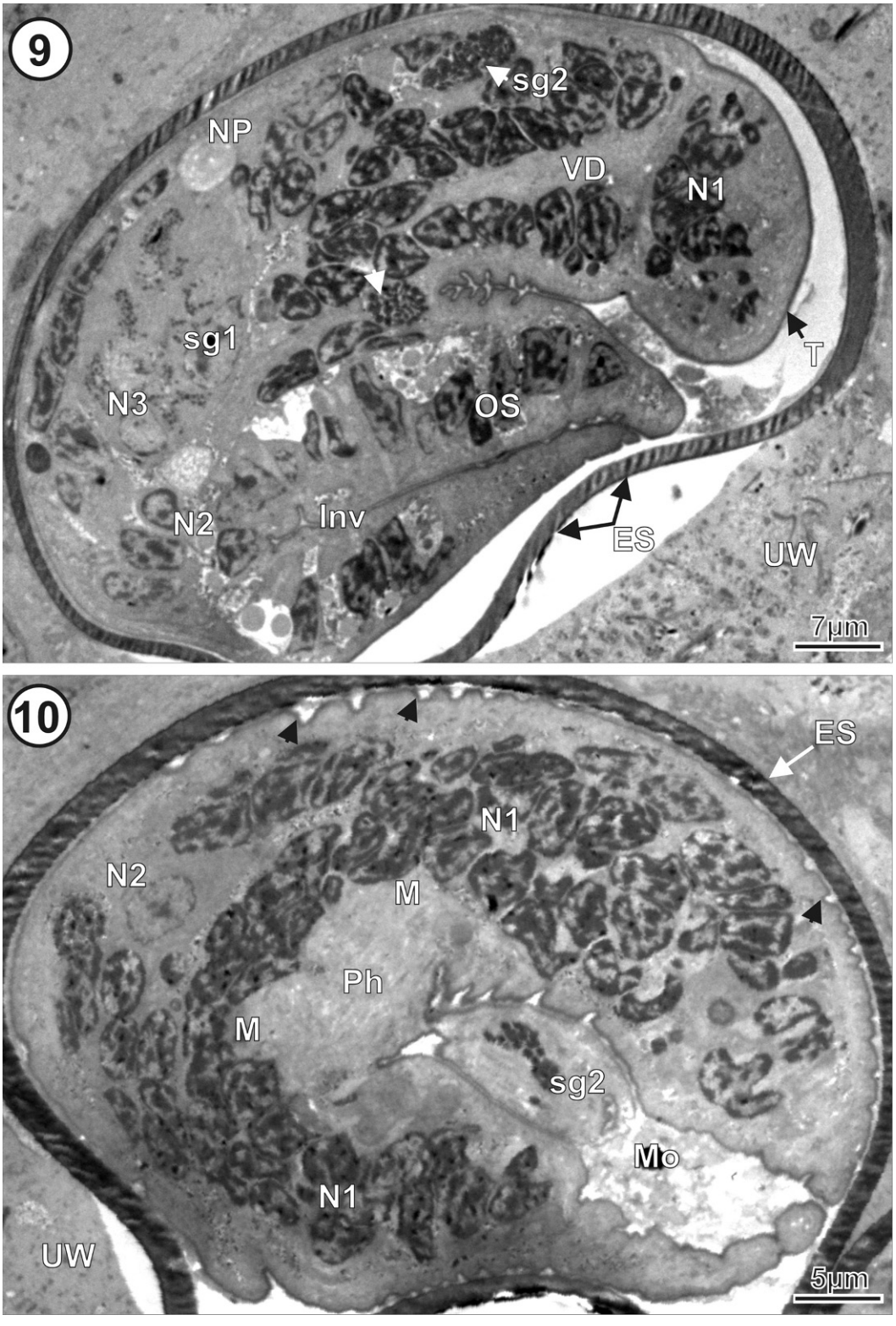
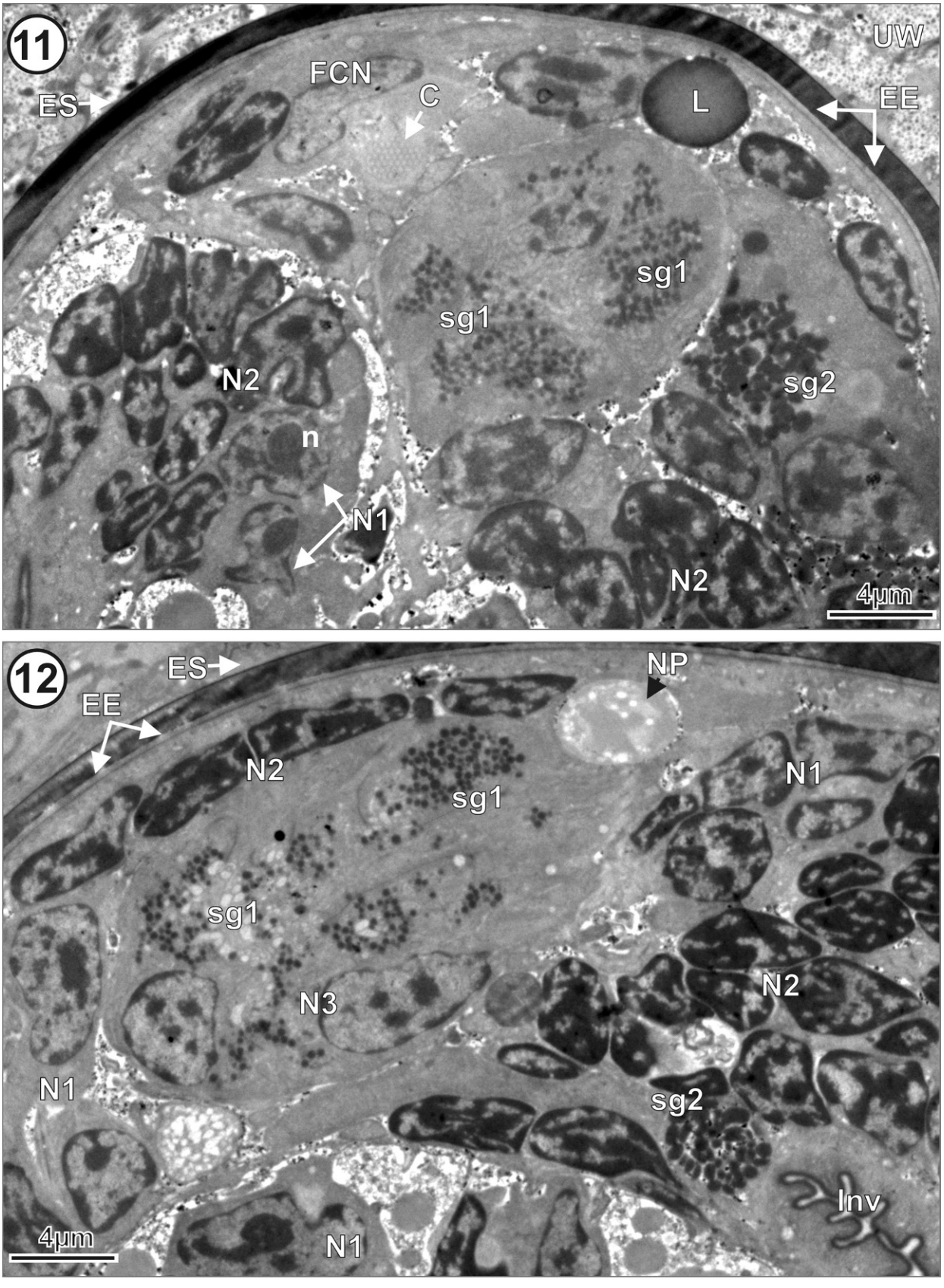
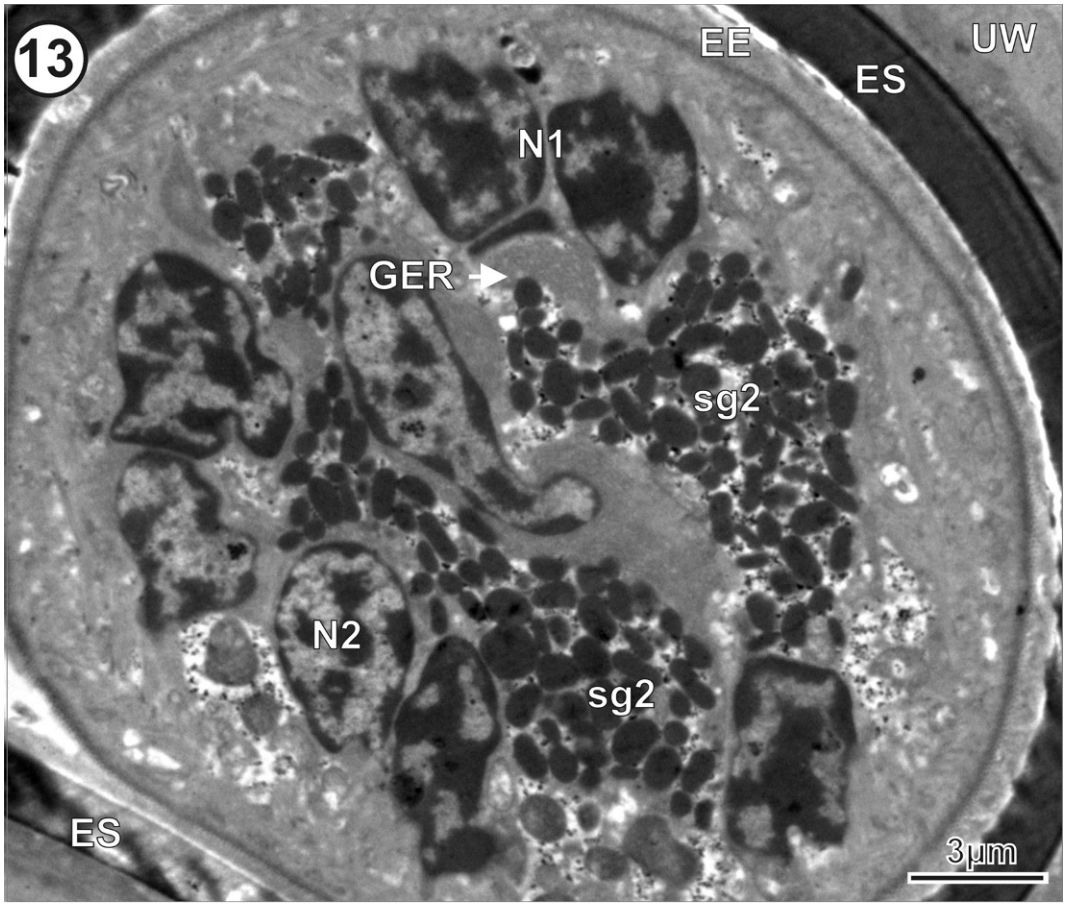
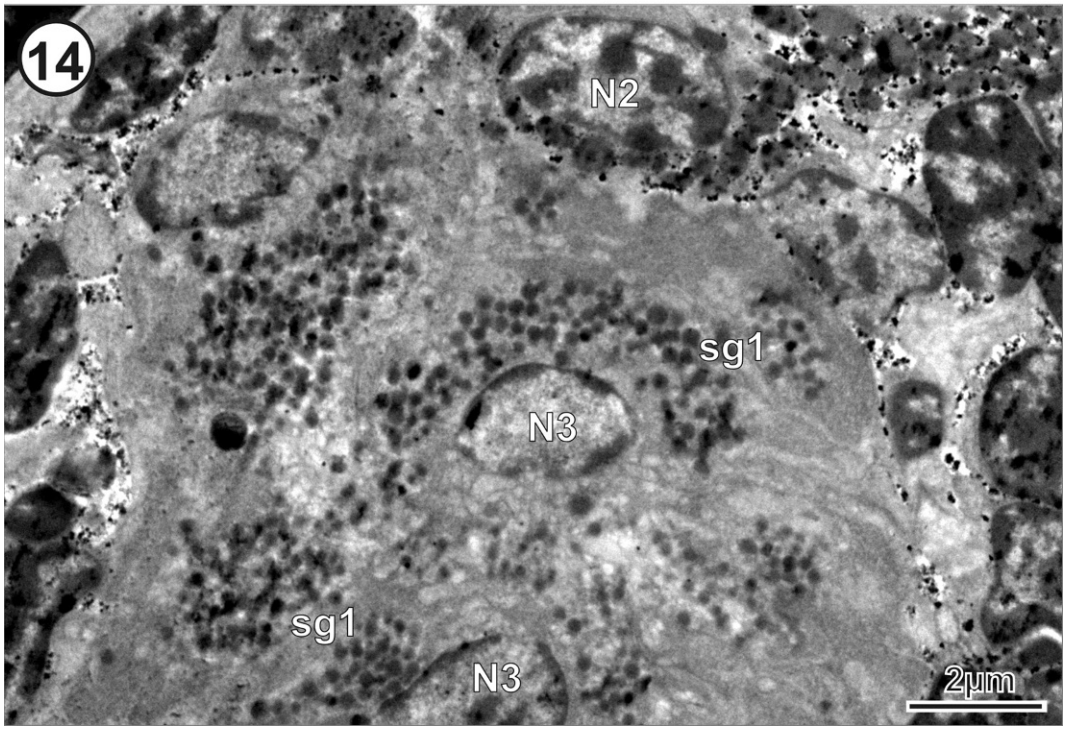


Fig. 9. Micrographs illustrating the structure of the fully-developed cotylocidium. Note: (1) a longitudinal section of the oral sucker, showing the long invagination which eventually results in the formation of the pharynx and intestine; (2) two types of secretory granules, the smaller sg1 type corresponding to neurosecretory granules localized within the cytoplasm of the cell type with an N2 nucleus in the central part of the cotylocidium, and the larger sg2 type to the secretory granules localized in the region of the posterior sucker (ventral disc) and within ducts frequently observed close to the oral sucker; (3) the thin tegument; and (4) a cross-section through a perforated nephropore forming part of the protonephridial excretory system **Fig. 10.** An oblique section through the oral sucker showing the thick bundles of pharyngeal musculature with adjacent mouth and sg2 type secretory granules. Note numerous invaginations of the tegument (black arrowheads)

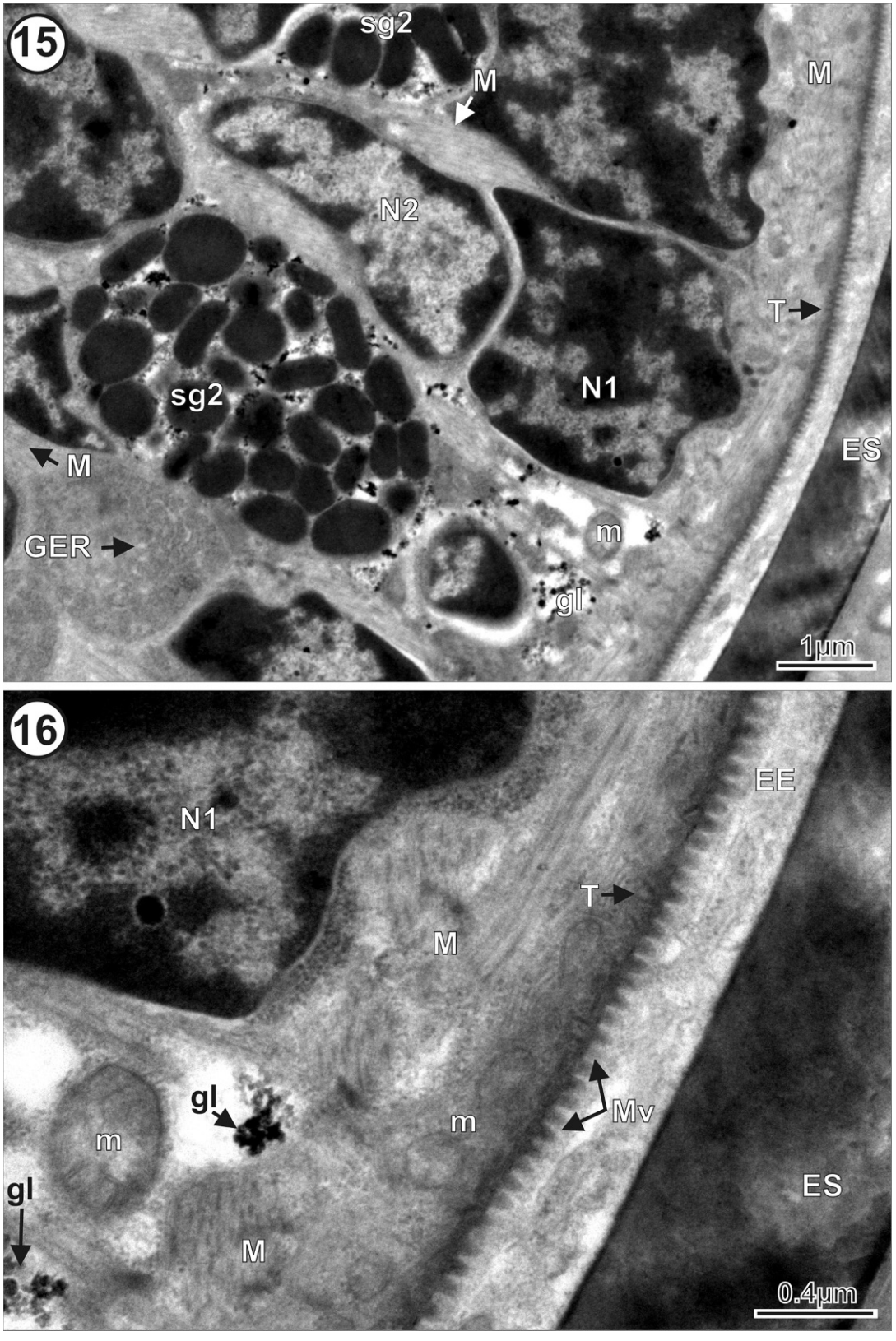


Figs 11 and **12.** Ultrastructural details of the mature cotylocidium showing: (1) a very thin, anucleate layer of the embryonic envelope; (2) a cross-section of the flame cell and nephropore, two subunits of the protonephridial excretory system; and (3) two types of secretory granules





Figs 13 and **14.** Micrographs of the glandular regions, indicating differences in the ultrastructure, electron-density and shape of the two types of the secretory granules. Note: the disc-like shape of the sg2 granules, which probably correspond to the secretion of the gland-cells, and the shape of the sg1 granules, which resemble neurosecretory granules



Figs 15 and **16.** High power details of the tegument and associated structures in the fully-developed cotylocidium. Note: (1) the thin, flocculent layer of remnants of the embryonic envelope; (2) the thin, electron-dense tegument with its microvilli; (3) myocytes, with attached muscle fibres of the longitudinal and circular body musculature and GER, mitochondria and glycogen particles embedded in their cytoplasm; and (4) highly electron-dense, disc-shaped sg2 granules

macoides (Figs 9–15). Differences in their ultrastructural details can be seen in Figs 13 and 14 (compare sg1 and sg2). The first type of glandular region, representing the primordium of a central ganglion and containing sg1 type of secretory granules, is situated in the central part of the larva (Fig. 17B). It is composed of a compact group of numerous small cells resembling neurosecretory cells. These cells have moderately electron-dense nuclei, with a few heterochromatin islands closely adjacent to their nuclear membrane, surrounded by a relatively thick cytoplasm filled with a great number of small, spherical, electron-dense granules characteristic of neurosecretory cells (Figs 11 and 12). Each granule

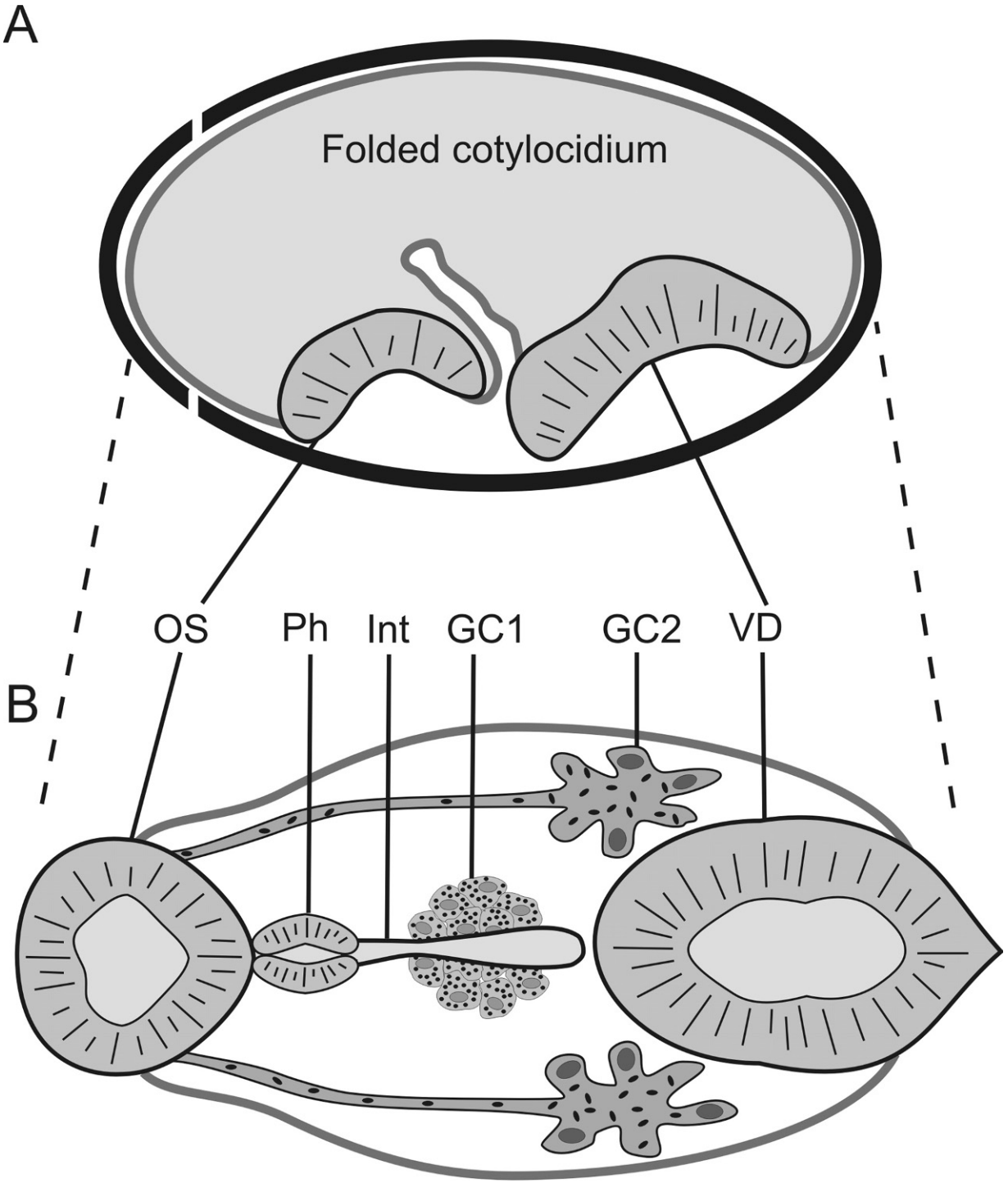


Fig. 17. Diagrams of *Apsidogaster limacoides*. **A.** An egg-bound, folded, but fully-developed cotylocidium. **B.** The general topography of the cotylocidium and the position of different larval structures within its body

of the sg1-type appears spherical, dense-cored and membrane-bound, and closely resembles a typical neurosecretory granule when examined under high magnification (Fig. 14). This classification is based exclusively on the above-mentioned ultrastructural features of these granules; a specific cytochemical test for acetylcholinesterase or analyses of serial sections impregnated with silver were not employed in this study.

Protonephridial excretory system

The evidence for two components of the fully-formed protonephridial system in differentiated cotylocidia, comprising flame-cells and nephropores, is presented in Figs 9, 11–12. In the *A*. *limacoides* cotylocidium, the twin excretory pores are situated on the surface of the larval tegument. As shown in Figs 9 and 12, they are covered with a diaphragm of cytoplasm punctured at intervals by small channels and holes. Debris larger than the diameter of the holes in the diaphragm was sometimes present in the atrial lumen, suggesting that the diaphragm may have a retaining function.

The exact numbers of flame cells and different types of excretory ducts were not established, as the results of this study were not based on the analysis and reconstruction of embryos from semithin and ultrathin serial sections.

Discussion

Comparison of the advanced stages of embryonic development in A. limacoides *with those of other neodermatans*

Ultrastructural aspects of the advanced stages of embryonic development in *A*. *limacoides* exhibit a number of similarities with those previously reported for other representatives of the Neodermata, and particularly for digeneans, cestodarians and lower eucestodes.

Such similarities include:

(1) The mode of formation and ultrastructure of the eggshell, as observed in digeneans such as schistosomes (Eklu-Natey *et al*. 1982a, b; Świderski 1984, 1985, 1994a) and some lower eucestodes (Świderski 1994b, Świderski and Mackiewicz 2004, Młocicki *et al*. 2010b).

(2) The early fusion of individual eggshell-bound vitellocytes to form a vitelline syncytium, followed by its progressive autolysis and reabsorption, as reported for schistosomes (Świderski 1984, 1985, 1994a) and lower eucestodes (Świderski 1981; Świderski 1994b, c; Świderski and Mackiewicz 2004; Młocicki *et al*. 2010b, c).

(3) The mitotic division of some blastomeres, which occurs simultaneously with the degeneration or apoptosis of other embryonic cells, such as micromeres (Rybicka 1961, 1966; Świderski 1968, 1972, 1994b; Bruňanská *et al*. 2012).

(4) The early fusion of the macromeres into the first syncytial layer of the embryonic envelope, as previously reported in schistosomes (Świderski 1984, 1985, 1994a) and eucestodes (Świderski 1968; Młocicki *et al*. 2005, 2010b, c).

(5) The presence of a protonephridial excretory system composed of flame cells, collecting ducts and nephrostomes, as observed in cotylocidia of *A*. *limacoides*, which have also been reported in the first larval stages of digeneans (Wilson 1969; Wilson and Webster 1974; Pan 1980; Eklu-Natey 1986), cestodarians (Xylander 1986, 1987, 1990, 2001) and most lower eucestodes so far studied (Świderski and Mackiewicz 2004).

(6) The presence of various types of glandular structures, which may have interesting homologies and analogies, associated with their different origins and/or different functions. Among them, in *A*. *limacoides* we distinguished two evidently different types of secretory regions: (1) neurosecretory-like cells connected with the larval nervous system and (2) gland cells, most probably of the merocrine type, with large electron-dense secretory granules which are comparable to the secretory granules described in the apical and/or lateral glands of digenean miracidia (Pan 1980; Eklu-Natey 1986) and to secretory granules reported in the penetration glands of cestodarian lycophores (Xylander 1986, 1990) and eucestode hexacanth larvae (Świderski 1972; Świderski and Tkach 2002; Świderski and Mackiewicz 2004; Młocicki *et al*. 2006, 2010a).

In the Neodermata, neurosecretory granules associated with larval nerve cells have been reported as dense-cored vesicles, resembling the so-called ‘elementary granules of neurosecretion (EG Ns)’ found in other organisms, in numerous taxa, including the oncospheres of *Hymenolepis nana* (see Fairweather and Threadgold 1981), *Inermicapsifer madagascariensis* (see Świderski and Tkach 2002), *Echinococcus granulosus* and *E*. *multilocularis* (see Świderski 1994d, 1997), *Mosgovoyia ctenoides* (see Młocicki *et al*. 2006) and *Eubothrium salvelini* (see Młocicki *et al*. 2010a), and also in the larval stages of digeneans, such as *Schistosoma mansoni* and *S*. *haematobium* (see Pan 1980; Eklu-Natey 1986; Cousin and Dorsey 1991; Halton and Gustaffson 1996). The most probable function of the larval neurosecretory cells of parasitic platyhelminths is stimulating and controlling coordinated body movements during hatching and host penetration (Świderski 1997; Młocicki *et al*. 2006).

The second, and evidently different, type of glandular cells in larval *A*. *limacoides* is similar, to some extent, to those of the merocrine type previously reported in: [a] the apical and lateral glands of digenean miracidia (Pan 1980; Świderski 1984, 1985) and also in digenean cercariae (Stirewalt 1973, 1974); [b] cestodarian lycophores, where about 12 gland cells of three or four different types have been described (Rohde 1986, 1987; Xylander 1986, 1990); and [c] the penetration glands of eucestode hexacanths which are directly involved in the mechanism of host invasion (Świderski and Tkach 2002; Świderski and Mackiewicz 2004; Młocicki *et al*. 2006, 2010a). Nevertheless, at present, there is not enough available information on these glandular structures

for any ultrastructural or functional comparison. Unfortunately, the chemical nature of this type of gland in cotylocidia of *A*. *limacoides* still remains unknown, as is the case for other aspidogastreans.

However, there are also differences between the embryonic development of *A*. *limacoides* and that of other neodermatans. These include the formation of only a single embryonic envelope, which disappears progressively in eggs containing a fully-developed cotylocidium, as opposed to the two embryonic envelopes reported for all digeneans and eucestodes so far examined (see Świderski 1972, 1994a, b, c; Młocicki *et al*. 2005, 2010c), where the outer envelope originates from the macromeres and the inner envelope results from the fusion of three mesomeres. Furthermore, the embryonic envelopes of the majority of digeneans and eucestodes undergo further differentiation into secondary envelopes (egg-envelopes), which surround the fully-developed larva of both groups, e.g. the digenean miracidium and the coracidium of some lower eucestodes (Świderski 1994a, b, c).

Comparison of the fully-formed cotylocidium of A. limacoides *with that of other aspidogastrean species*

As commented by Rohde (1972, 1994, 2001), a great deal of morphological and other variation exists among aspidogastrean cotylocidia. For example, some of these larvae are unciliated, as observed in the present study of *A*. *limacoides*, by both Voeltzkow (1888a) and Williams (1942) in *A*. *conchicola*, by Rai (1964) and Tang and Tang (1980) in *A*. *indica*, and by Brinkmann (1957) in *Multicalyx elegans*, although the latter represents a different family (see Fig. 18) and perhaps a different order to the others; whereas some, e.g. *Lophotaspis orientalis* (see Tang and Tang 1980), have a number of ciliary tufts situated on different parts of the larval body (see Rohde 1972, 1994, for details).

The tegument in *A*. *limacoides* is of the neodermatan type. Its surface is similar at both larval and adult stages, bearing the characteristic minute, rounded, microvillus-shaped tubercles of other aspidogastreans (Fried and Haseeb 1991, Littlewood 2006). The general topography of the aspidogastrean tegument, with its perikarya situated beneath the basement membrane and the body wall musculature present among the parenchyma, is therefore comparable with the neodermis of all of the major parasitic flatworm taxa.

In fact, the larva of *A*. *limacoides* is relatively simple in its morphology and resembles, to some extent, the larva of both *Lobatostoma manteri* (see Rohde 1973) and *Rugogaster hydrolagi* (see Schell 1973), even though the latter represents a different family, and perhaps order, to the former two species (see Fig. 18).

Comments on the phylogenetic implications of the morphogenetic results

In the light of the present TEM study and information on previous developmental and life-cycle studies of *A*. *limacoides* (see References above), the following conclusions can be drawn:

1. The life-cycle of *A*. *limacoides* is frequently direct, with this parasite occurring in a wide range of molluscs, but it may also be completed in a vertebrate host, as cyprinid fishes sometimes act as facultative definitive host when feeding on bivalves or snails.
2. The embryonated intrauterine eggs contain a fully-differentiated larva with incompletely developed characters of the adult.
3. Its development resembles the simple, direct life-cycle of the Monogenea.
4. There is a gradual transformation from the hatched larva to the adult.
5. There is an indication of the mollusc-vertebrate host association of the Digenea in this aspidogastrean (and, as indicated above, in others).
6. The metamorphosed larva, the cotylocidium, resembles in

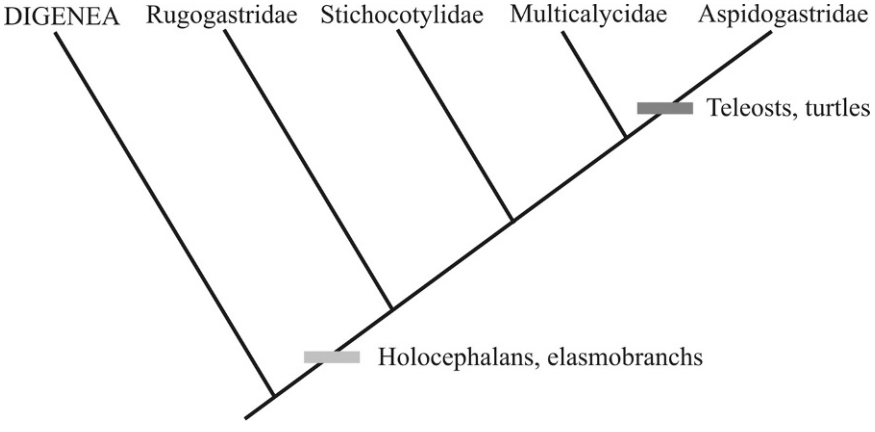


Fig. 18. The phylogeny of the Aspidogastrea at the family level based on a re-analysis of the characters used in Gibson (1987) by Brooks *et al*. (1989) and more recently by Zamparo and Brooks (2003). Modified after Littlewood (2006)

its general characters the digenean cercarial/metacercarial stage, especially in the nature of the oral sucker, gut, excretory system, primordia of the genital systems and posterior/ventral sucker.

7. Although the posterior sucker (incipient ventral disc) of the cotylocidium initially resembles the ventral/posterior sucker of digeneans, it subsequently undergoes subdivision into numerous alveoli.

Our TEM studies tend to support the early ideas of Burmeister (1856), Monticelli (1892), Furhmann (1928), Faust (1932), Faust and Tang (1936), Chauhan (1954) and Dollfus (1958) for treating the Aspidogastrea as a separate group occupying an intermediate position between the Monogenea and the Digenea (Timofeeva 1975, 2005; Poddubnaya *et al*. 2011, 2012). Rohde (1972) also indicated that the Aspidogastrea seem to combine features of both monogeneans and digeneans. Whereas their development is mainly direct, lacking an alternation of generations, as is the case for the Monogenea, they have features in common with the Digenea, such as the nature of their hosts (molluscs plus or minus vertebrates) and the sharing of several morphological characters by the cotylocidium and cercaria/metacercaria. On the other hand, aspidogastreans differ from a great majority of digeneans, apart from some rare exceptions, in that the ventral organ of attachment is normally subdivided into a number of alveoli. According to Stunkard (1946, 1970), digeneans acquired polyembryonic asexual reproduction in molluscs and their sexual maturation was secondarily progressively shifted to stages in vertebrates. The 'molluscan host first' hypothesis for digeneans has generally been accepted (e.g. Gibson 1987), but an alternative view has more recently been presented by Littlewood *et al*. (1999a, b) based on molecular data, according to which vertebrates and not molluscs were the original hosts of proto-trematodes. Furthermore, although in molecular studies, such as those of Littlewood *et al*. (1999a, b), Olson *et al*. (2003) and Littlewood (2006), the Aspidogastrea is treated the sister-group of the Digenea – this is a view not supported by Timofeeva (1975, 2005). It is clear that further studies are needed in the search for characters, which shed light on this and other, often contradictory, hypotheses on the status, origins and relationships of this group.

Data presented in Rohde's review (1972) of the group indicate that aspidogastreans are ill-adapted to parasitism, have many archaic features, form a group of their own and are closely related to the Digenea. Their direct life-cycle, without alteration of generations, and the use of a molluscan host appear not to be derived characters. Their poor adaptation to parasitism is indicated by the relatively long survival time outside a host in simple media (up to several week have been recorded for some species (Osborn 1903, Williams 1942) and by their apparently wide host and organ specificity (Rohde 1972, 2001, 2002; Gibson and Chinabut 1984). A long period of survival outside the host and a wide host-specificity tend to indicate that a parasite is only moderately dependent on its host(s) and for only general factors.

A phylogeny of the Aspidogastrea was first presented by Gibson (1987), who mapped key host associations and morphological changes. Later, these morphological characters were re-analysed at the family level by Brooks *et al*. (1989) and more recently by Zamparo and Brooks (2003), as summarised in Fig. 18. As reported by Littlewood (2006), a preliminary molecular analysis supports the basal position of the Rugogastridae, but the Aspidogastridae are paraphyletic with the Multicalycidae nested within (see also Olson *et al*. 2003). According to Littlewood (2006), gene sequencing of all of the aspidogastrean families is currently hampered by an inability to secure suitable tissue of *Stichocotyle nephropis*, the one and only member of the Stichocotylidae. Despite the fact that the Aspidogastrea, as a group, are of no known economic or medical importance, their status as the sister group of the Digenea is crucial to a better understating of digenean radiation, evolution and adaptation to the parasitic ways of life.

The results of the present investigation on the embryonic development and cotylocidial differentiation of *A. limacoides* confirm that members of the Aspidogastrea possess manyfeatures in common with the Digenea, which exhibit interesting analogies and homologies. This supports previous views of their close relationship and is in accordance with most molecular and other recent analyses which indicate a sister relationship between these two flatworm taxa (e.g. Gibson 1987, 2002; Littlewood *et al*. 1999a, b; Rohde 2001, 2002; Cribb *et al*. 2001; Zamparo and Brooks 2003; Lockyer *et al*. 2003).

Some unsolved problems for further investigation

The present study is the first, albeit modest, attempt, to elucidate a very complex morphogenetic process of larval differentiation. Detailed analysis and reconstruction of the differentiating cotylocidium based on serial semithin and ultrathin sections are needed for establishing the nature of the different cell types and details of other larval structures. For example, the group of cells situated in the central part of larval body, with characteristic sg1 granules of a neurosecretory type and considered to be a central neural ganglion, require their classification and functional ultrastructure be confirmed by using other more specific methods. Similarly, for ascertaining details of the differentiation of the nervous system and its ultrastructure in fully-developed cotylocidia, studies using histochemical and ultracytochemical methods are required.

Acknowledgements. We are grateful to B.B. Georgiev, J.-L. Justine, J.S. Mackiewicz and J. Miquel for kindly commenting on an earlier version of the manuscript. As this paper is dedicated to the memory of Prof. R. Ph. Dollfus on the occasion of the 125 Anniversary of his birthday, exceptionally a short Abstract in French kindly translated by J.-L. Justine (MNHN Paris, France) is included in this paper. The authors would like to thank the staff of the Centre of Electron Microscopy, Institute of Biology for Inland Waters, Borok, Russia, for technical assistance.

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(Accepted March 12, 2012)