An ultrastructural study of reproduction in the parthenogenetic metacercariae of *Cercaria margaritensis* Ching, 1982 (Digenea: Gymnophallidae)

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SUMMARY

The parthenogenetic metacercarial stages of the gymnophallid trematode *Cercaria margaritensis* are found in the extrapallial cavity of the subtidal prosobranch mollusc *Margarites helicinus*. The primary metacercariae (M1) produce second-generation metacercariae (M2) which become independent and give rise to M3 metacercariae which are infective to the definitive host, the common eider (*Somateria mollissima*). This study used transmission electron microscopy to follow the development of M2 inside M1 organisms and M3 inside M2 organisms. The process is similar in both cases with embryos developing from individual cells from the parent body walls. In each case the brood sac was divided into brood chambers by multilaminated cells and both M2 and M3 embryos developed inside embryonic membranes that originated from specialized blastomeres. The tegument of M2 and M3 embryos developed in a similar manner underneath the embryonic membrane. Both the multilaminated cells and the embryonic membranes possessed features that indicated that they are involved in transport of nutrients. It is suggested that the continuous nature of M2 and M3 embryo development may well be similar to that postulated for ancestral digeneans.

Key words: ultrastructure, parthogenetic metacercaria, Gymnophallidae.

INTRODUCTION

Gymnophallid digeneans are parasites of coastal birds and they utilize marine invertebrates as intermediate hosts. Most gymnophallids have 3 hosts in their life-cycles and, unlike the majority of digeneans, they use a bivalve mollusc as a first intermediate and a gastropod or bivalve as a second intermediate host. Some are also peculiar in that, inside the second intermediate host, they have primary metacercariae (M1 metacercariae) which parthenogenetically produce secondary metacercariae (M2 metacercariae) which in turn give rise to a further generation (M3 metacercariae) which are infectious to the final host. Gymnophallid parthenogenetic metacercariae have been described from the bivalve *Mytilus platensis* and the gastropods *Littorina* spp. and *Margarites* spp. (Szidat, 1962; James, 1964; Chubrik, 1966; Ching, 1982). James (1964) described the larval stages of *Parvatrema homoeotecnum* from the periwinkle *Littorina saxatilis* and he concluded that this mollusc was the only intermediate host for that parasite. This point of view has been called into question many times (Cable, 1965; Ching, 1982, 1995; Pearson, 1992) and it clearly illustrates that life-cycles of this complexity may well require experimental validation.

The life-cycle of gymnophallid *Cercaria margaritensis* Ching, 1982 has been studied and established by Galaktionov (1996). The first intermediate host of this parasite is the sublittoral bivalve *Turtonia minuta* which is common in many areas including the Barents Sea. The sporocysts develop in *T. minuta* and the daughter sporocysts produce furcocercariae that have a typical gymnophallid cercarial shape. They are shed from *T. minuta* and following a brief free-living period they penetrate the subtidal prosobranch *Margarites helicinus*, the second intermediate host. Each cercaria migrates to the extrapallial cavity of the *M. helicinus,* discards its tail and changes into a metacercaria. This primary metacercaria (M1) gives rise to the second generation of metacercariae (M2) inside a brood sac that is divided into brood chambers. The mechanism for production of the M2 metacercariae is open to debate but is probably by parthenogenesis as suggested for other digenean intramolluscan stages by James (1964), James & Bowers (1967), Ginetsinskaya (1968), Pearson (1972) and Gibson (1987). The M2 metacercariae leave the

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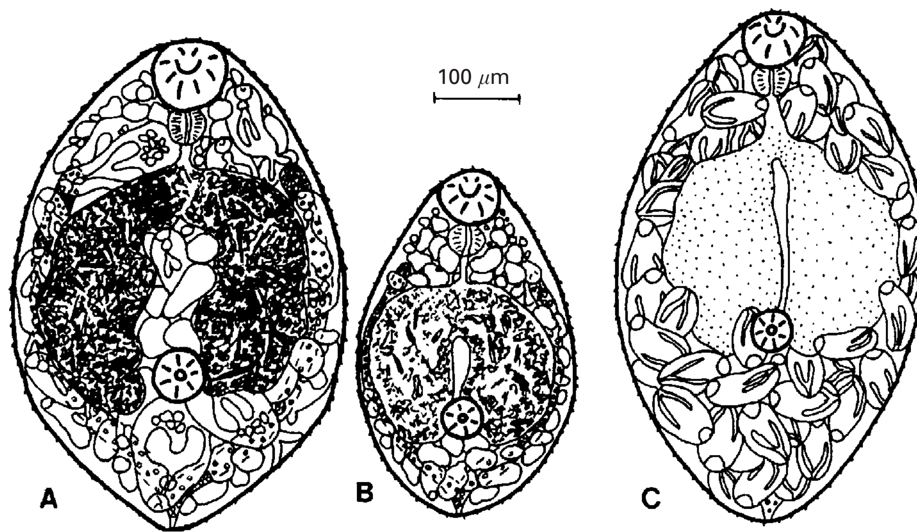

Fig. 1. Drawings of *Cercaria margaritensis* metacercariae. (A) An M1 metacercaria containing M2 metacercariae at various stages of development. (B) A young M2 from the extrapallial cavity of its host. It contains developing M3 metacercariae. (C) An M2 metacercaria containing fully formed M3 metacercariae.

M1 and independently parasitize the extrapallial cavity of the *M. helicinus*. The M2 stage is probably also parthenogenetic and it produces the M3 metacercariae which are infective to the definitive host, the common eider (*Somateria mollissima*). If a mollusc containing M3 metacercariae is eaten by an eider, the M3 metacercariae will develop into adult digeneans belonging to the genus *Parvatrema* (Galaktionov, 1996). Details of the life-cycle will be published in English as a separate paper.

The present article deals with the ultrastructure of M1 and M2 metacercariae and investigates the formation and development of the successive generations within them.

MATERIALS AND METHODS

Cercaria margaritensis Ching, 1982 first (M1) and second (M2) generation metacercariae were taken from the extrapallial cavity of *Margarites helicinus* collected on the upper sublittoral zone of the Barents Sea near the Dalnie Zelentsy Biological Station of the Murmansk Marine Biological Institute. The molluscs were dissected under a stereomicroscope, and metacercariae were accumulated and fixed for transmission electron microscopy following the methods described by Galaktionov *et al.* (1996). The morphology of the metacercariae was also studied *in vivo* using light microscopy.

RESULTS

No very immature M1 metacercariae were found in the *M. helicinus* collected. The brood sac of the least mature M1 metacercariae (Fig. 1A) was divided into brood chambers already containing developing embryos and young M2 metacercariae. Transmission electron microscopy revealed that numerous embryos, at various stages of development, occupied a very large proportion of each M1 body. The relationship between the embryos and the surrounding tissues of the M1 metacercariae is represented in the drawing, Fig. 2. Cells, sometimes referred to as blastomeres, forming the early embryos had fairly electron-lucid cytoplasm containing large numbers of small mitochondria, free ribosomes and some RER cisterna (Fig. 3A). Similar cells, which might have given rise to these embryos, were located in the metacercarial body wall (Fig. 3B). Like the early embryo cells these germinal cells had large nuclei, well dispersed chromatin and very distinct nucleoli. Again the cytoplasm was fairly transparent and contained mitochondria, unattached ribosomes and some RER. The relatively few germinal cells that were located were found in the posterior end of the M1 metacercariae. Searches for early M2 embryos (the stages referred to as 'naked cell aggregates' by Cheng (1961) and Cheng & Bier (1972)) revealed occasional groups of closely adhering cells which were very similar in composition (Fig. 3C). In slightly more developed embryos the outer cells were flattened and seemingly continuous, forming a surface syncytial layer or embryonic membrane around each individual (Fig. 3D, E). This membrane was referred to as the 'primitive epithelium', 'embryonic sheath', 'enveloping membrane' by different authors (see Dunn *et al.* (1992) for synonymy). Careful inspection showed that the nuclei and cytoplasm of the embryonic membrane were similar in composition to those of the other blastomeres and each M2 embryo that had developed past the 'naked cell aggregate' stage to those containing developing M3 embryos were ensheathed in this manner.

The brood sac was separated from the body wall and all the organs of the M1 metacercariae by tissue composed of very distinctive, relatively

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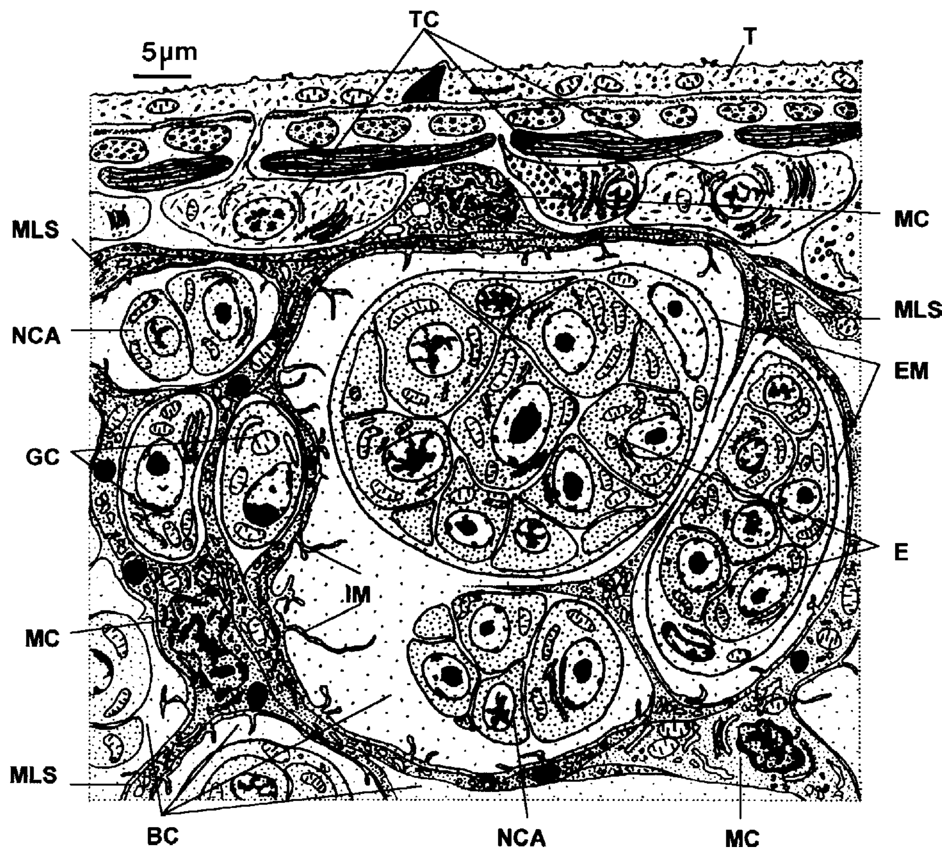

Fig. 2. Drawing of part of a young M1 to illustrate the relationship between its developing M2 embryos and the tissues of the M1. BC, brood chamber; E, M2 embryo covered by embryonic membrane; EM, embryonic membrane; GC, germinal cell; IM, interstitial material; MC, multilaminated cell; MLS, multilaminated structure composed of extensions of multilaminated cells; NCA, early embryo referred to as 'naked cell aggregate'; T, tegument; TC, tegument cell.

electron-dense cells. The surface area of these cells was greatly increased by the presence of flattened extensions that were often folded resulting in them having a multilaminate appearance. For this reason we refer to them as multilaminate cells (Fig. 3C–F). In their simplest form (Fig. 3C) it was apparent that they contained a system of branched narrow channels that contained electron lucid material. In some cases these channels could be traced to the periphery of the cells where it became apparent that they were continuous with a layer of surrounding fibrous interstitial material (see Smyth & Halton (1983) for a description of this material). The extensions of these cells spread around and between developing embryos and often interdigitated with those of other laminated cells to form multilaminated structures and septa dividing the brood sac into chambers. Although some seemingly adjacent nuclei in these cells appeared not to be separated by plasma membranes (Fig. 3C) this could have been due to their large and irregular shape and no other evidence that they formed a syncytial tissue was found. In the more complex of these cells (Fig. 3D–F) the surrounding interstitial material, and its delineating membranes, were still apparent and extensions could be seen not only penetrating into the cytoplasm of the multilaminate cells but also into the surrounding brood chambers. It was noticeable that some of the laminated cells maintained close contact with individual young embryos and/or small groups of young embryos (Fig. 3C–F). At this stage of their development these cells possessed surface cavities and vacuoles containing material with the same appearance as the contents of the brood chambers and they also contained lipid droplets, numerous mitochondria and occasional Golgi complexes.

Inside the very young M2 within the M1 (Fig. 1A) germinal cells, naked cell aggregates and early M3 embryos were recorded. Features of the M1 body wall and the relationship between the developing stages of the M3 and the young M2 metacercariae are illustrated in Fig. 4. The M2 still retained their enveloping embryonic membranes and close inspection revealed that in some cases their embryonic membranes were thicker and contained more mitochondria than those of immature embryos (Fig. 5A). In addition, a thin syncytial tegument containing spines could be observed under the embryonic membrane (Fig. 5A–C). Beneath the tegument, among the free germinal cells that would give rise to M3 embryos, the early stages of the more dense multilaminated cells that would eventually separate the brood chambers of the developing M2 larvae were apparent (Fig. 5A). In these larvae undifferentiated cells,

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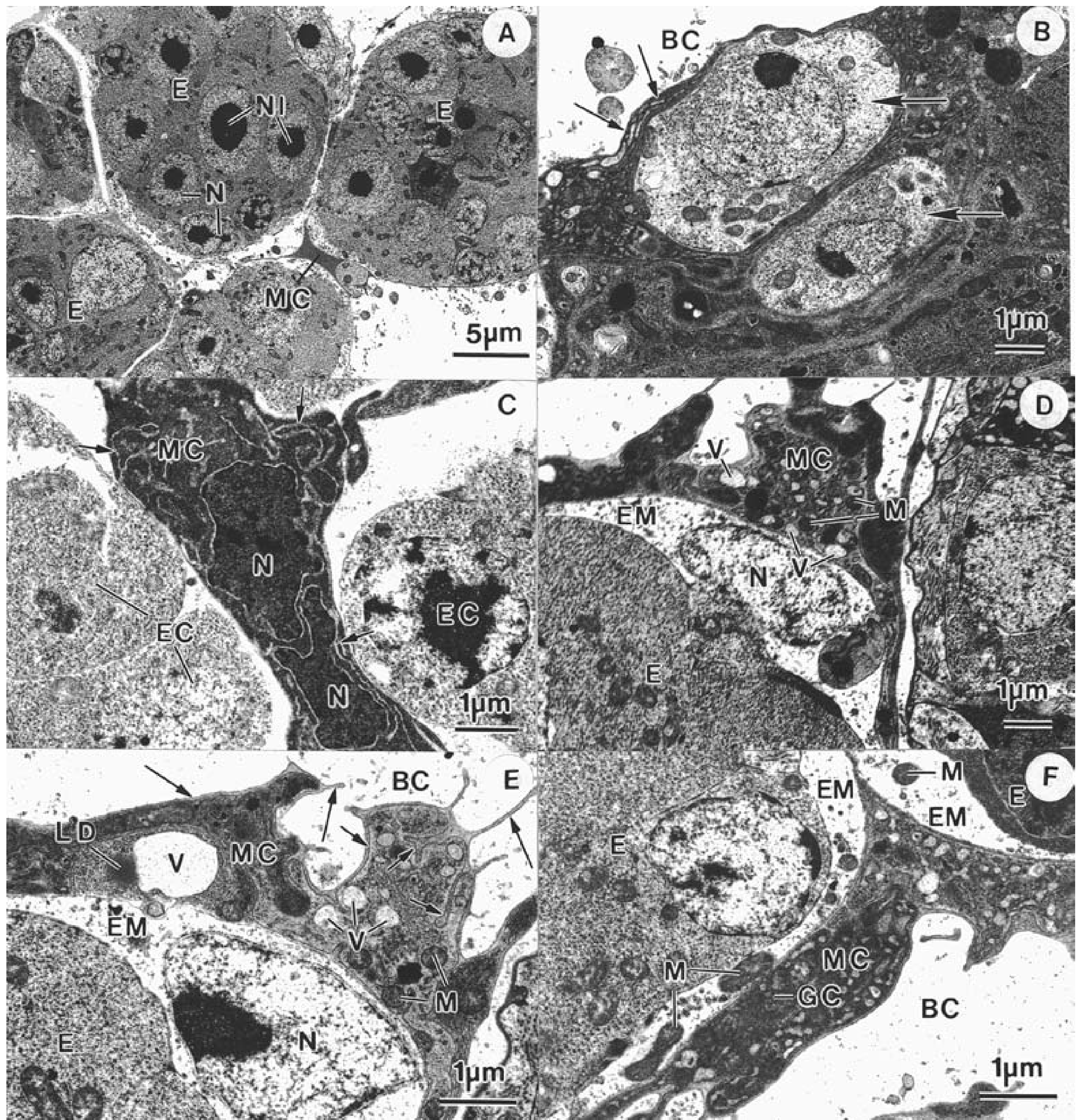

Fig. 3. Sections through M1 metacercariae showing early M2 embryo development. (A) A number of early embryos (E). They are composed of cells with large spherical nuclei (N) with very distinct nucleoli (NI). Early multilaminated cell (MC). (B) Two cells (large arrows) in the body wall with features similar to those of early embryo cells. These are assumed to be germinal cells. The cells lining the brood chamber (BC) are highly folded into lamellae (small arrows). (C) Embryo cells (EC) comprising 'naked cell aggregates' separated by a multilaminated cell (MC) that has 2 apparently adjacent nuclei not separated by plasma membrane. Note channels filled with interstitial material (arrows). (D) An embryo (E) surrounded by an embryonic membrane (EM) with a nucleus (N). This is in close contact with a developing multilaminated cell (MC). V = vacuoles. M = mitochondria. (E) Nucleus (N) and some of the cell body of an embryonic membrane (EM) surrounding an embryo (E). The embryonic membrane is in close contact with a developing multilaminated cell (MC) which is surrounded by its own plasma membrane (small arrows) and a membrane delaminating the brood chamber (large arrows). The membranes are separated by fine fibrous interstitial material. Folds of this material extend into the multilaminated cell and also into the surrounding brood chamber (BC). LD, lipid droplet; M, mitochondria; V, vacuoles. (F) Parts of two embryos (E), each surrounded by an embryonic membrane (EM) containing mitochondria (M). Developing multilaminated cell (MC) and brood chamber (BC) can be seen. GC, possible Golgi complex.

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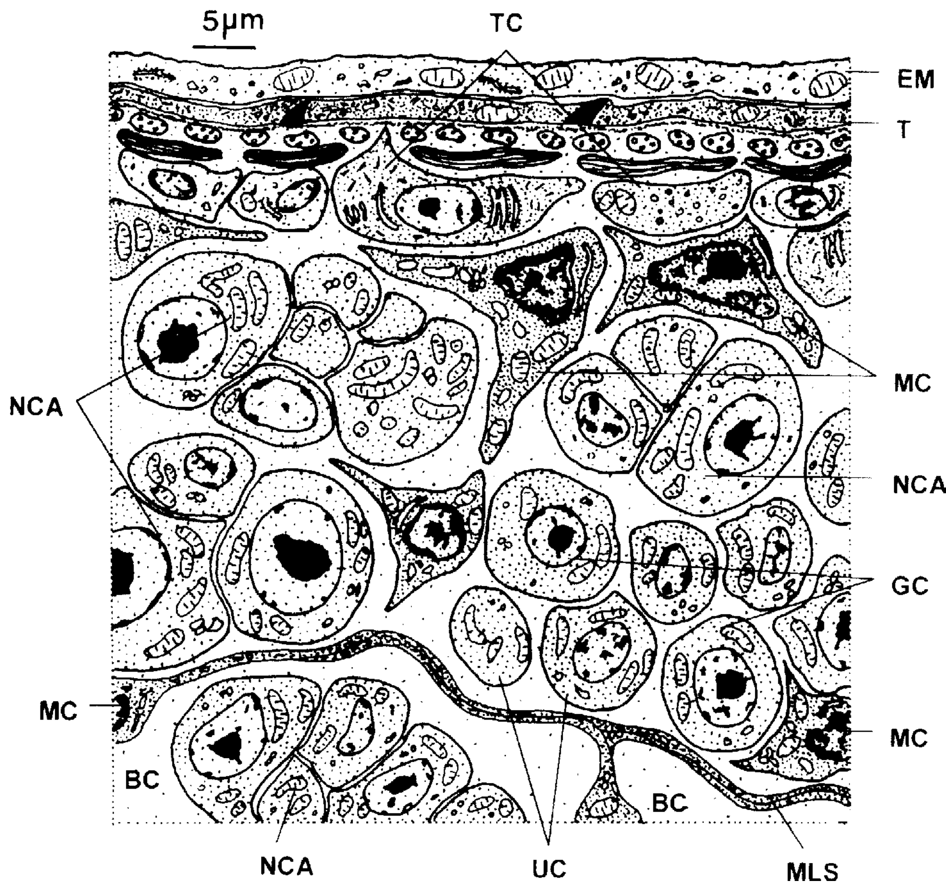

Fig. 4. Drawing of part of a young M2 inside an M1 to show early development of M3 embryos and their relationship with the tissues of the M2. UC, undifferentiated cells. All other abbreviations as in Fig. 2.

germinal cells and early embryos filled the entire brood sac, surrounding organ systems such as the excretory system of the M2 parents (Fig. 5B, C). The germinal cells were uniformly electron lucid, mostly round in outline, with large nuclei and dense spherical nucleoli. In some early M2 embryos the multilaminated cells were already partially or wholly flattened and formed partitions which extended from beneath the tegument to the organs such as the excretory ducts. In slightly more developed M2 individuals some of these cells were more flattened and their surface membranes were extended and folded to produce a multilaminated appearance and formed septa dividing the brood sac into brood chambers (Fig. 5D). They were found ramifying throughout the brood sac and they often enveloped either individual embryos (Fig. 5E) or groups of embryos (Fig. 5F). As was the case in the M1 metacercariae, in the more developed M2 metacercariae these multilaminated cells comprised a layer that lined the entire M2 body wall, covered the organ systems and delineated the brood chambers. Again, the possibility that they had fused to form syncytial tissue could not be ruled out. It should be noted, however, that development of the multilaminate cells did not appear to be synchronous. Different developmental stages of these cells could be observed within each developing M2 metacercaria.

M2 metacercariae at roughly the same stage of development were observed both inside M1 metacercariae and free in the extrapallial cavity of the host (Fig. 1B). In fact, a proportion of the free metacercariae appeared to be less well developed than some inside the M1 metacercariae. Features such as the oral sucker were very apparent (Fig. 6A) and the brood sacs of these M2 metacercariae were divided into brood chambers by the thin laminate cellular extensions seen in earlier stages (Fig. 6B). In all cases the brood chambers contained embryos at different stages of development, from 'naked cell aggregates' (Fig. 5D) up to those covered with embryonic membrane (Fig. 6B, C) and a thin syncytial tegument (Fig. 6D, E). The occasional single embryo cell could be found in the centrally located brood chambers but many more, along with 'naked cell aggregates', were present in the anterior region, near the oral sucker (Fig. 6A). Significantly, M3 embryos with fully formed embryonic membranes were found alongside very much less mature embryos in small, multilaminated cell-lined cavities underlying M2 body wall through to the progressively larger brood chambers that constituted the brood sac. The layer of multilaminated cells that divided the brood sac into chambers and lined the other organs (gut diverticula, excretory system etc) appeared to be contiguous with those that lined the inside of the body wall (Fig. 6B).

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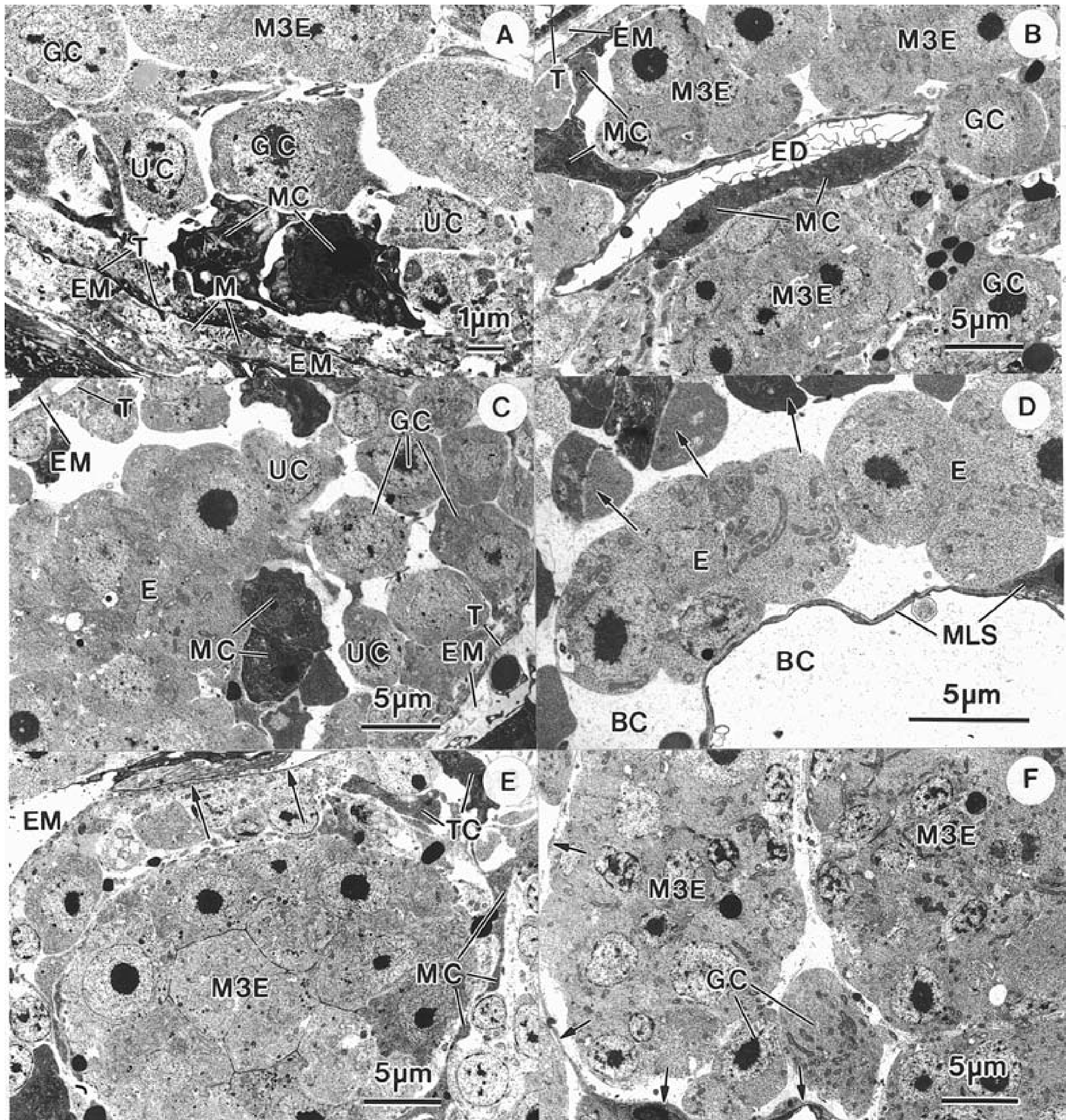

Fig. 5. Sections showing early M3 development inside M2 metacercarial embryos still within M1 metacercariae. (A) Young M2 embryo surrounded by embryonic membrane (EM) containing mitochondria (M). This embryo has a thin tegument (T). It contains undifferentiated cells (UC), germinal cells (GC) and a young M3 embryo (M3E) as well as multilaminated cells (MC) at an early stage of specialization. (B) Young M2 embryo showing tegument (T) and embryonic membrane (EM) as well as germinal cells (GC) and numerous developing M3 embryos (M3E). Developing multilaminated cells (MC) extend into and surround excretory duct (ED). (C) An M2 metacercarial embryo containing M3 germinal cells (GC), undifferentiated cells (UC) and a young M3 embryo (E) as well as early multilaminated cells (MC). The M2 is surrounded by a thin tegument (T) and an embryonic membrane (EM). (D) An M2 embryo in which some M3 embryos (E) are free between cells of the M2 body wall (arrows) and now well-developed multilaminated structures (MLS) dividing the brood sac into brood chambers (BC). (E) An M2 embryo containing an M3 embryo (M3E). The electron-lucid embryonic membrane (EM) of the M2 is apparent, as is the thin tegumental layer (arrows) and associated tegumental cells (TC). Multilaminated cells (MC) surround much of the M3 embryo. (F) Multilaminated structures (arrows) delineating an M2 brood chamber containing a number of M3 embryos (M3E) and germinal cells (GC).

As the purpose of the present study was to investigate and compare the formation and development of M2 and M3 metacercariae, the ultrastructure of fully developed M3 is not described in detail. However, it is relevant to point out that inside the most mature M2 (Fig. 1C) the vast majority of M3 metacercariae were fully formed and possessed a tegument that bore all the features of that of a mature digenean worm

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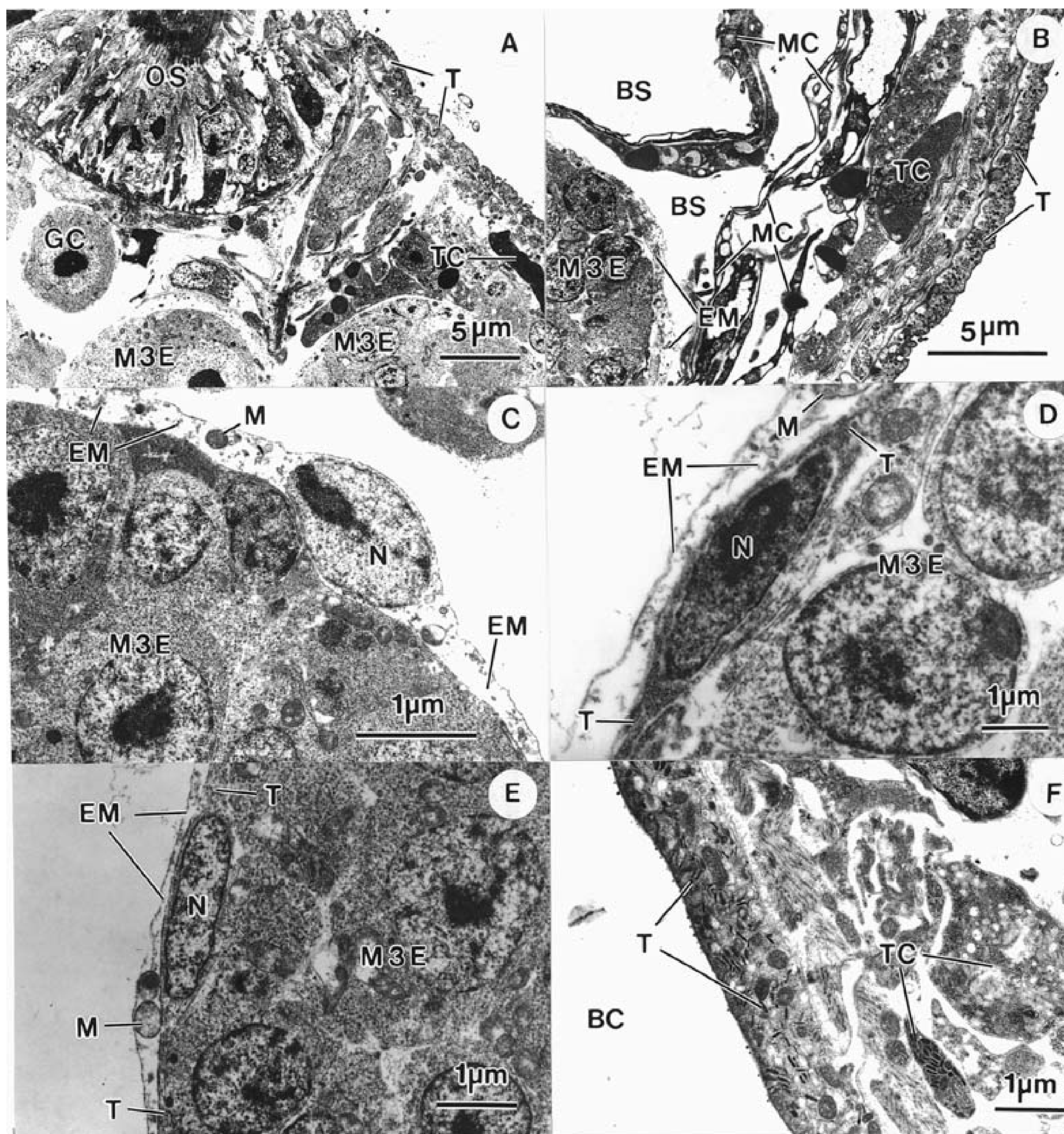

Fig. 6. Sections of M2 metacercariae from the host extrapallial cavity showing various stages of M3 metacercarial development. (A) A germinal cell (GC) and young M3 embryos (M3E) in the region of the oral sucker (OS) of an M2 metacercaria. Note tegument (T) and tegument cell (TC). (B) The body wall of an M2 metacercaria with well developed tegument (T) and a tegument cell (TC). The underlying multilaminated cells (MC) extend into the brood chamber dividing it into brood sacs (BS). An M3 embryo (M3E) with embryonic membrane (EM) is visible. (C) Part of an M3 embryo (M3E) surrounded by its embryonic membrane (EM) containing a nucleus (N) and a mitochondrion (M). (D and E) M3 embryos (M3E) surrounded by early tegument (T) containing a nucleus (N). Outside this the embryonic membrane (EM) containing mitochondria (M) is present. (F) Mature tegument (T) on the surface of a fully developed M3 metacercaria located inside the brood chamber (BC) of a fully developed M2 metacercaria. TC, tegument cells.

(Fig. 6F). No germinal cells or early embryos were located. Multilaminated cells still lined the inside of the body wall and formed partitions that divided the brood sac into brood chambers.

During this study the entire range of M3 embryo development was identified. As was the case for M2 embryos, only the earliest M3 embryos ('naked cell aggregates') lacked evidence that the outer cells had become flattened to give rise to the 'embryonic membrane' (Figs 3D, E, 5B, 6A, C). In slightly more mature individuals the early embryonic membranes no longer appeared to be composed of individual cells. They did, however, retain nuclei with nucleoli and evenly dispersed chromatin and the cytoplasm

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was relatively electron lucid and contained large mitochondria, unattached ribosomes and some RER cisternae (Figs 3D–F and 6C, D). In M3 development the embryonic membrane appeared to undergo less hypertrophy than was observed in M2 development. Tegument formation could be traced in cells underlying the embryonic membrane. First evidence of this process was the presence of some cells that were flattened towards the periphery and possibly joined together. These cells were characterized by having more condensed nuclear chromatin and denser cytoplasm than those constituting the embryonic membrane (Fig. 6D, E). At a later stage of development, the tegument had become a continuous layer over the entire surface of the M3.

DISCUSSION

The fact that *Cercaria margaritensis* produces its metacercariae in a similar manner to other digeneans has greatly influenced its morpho-functional organization so that it shares some features that are found in rediae and daughter sporocysts. Certainly, development of gonads and reproductive system ducts has been completely suppressed. Instead, germinal cells appear to arise from undifferentiated cells in the body walls of young M1 and M2. The fact that the majority of the germinal cells were observed in the posterior portions of M1 and M2 metacercariae might suggest that genital primordium development has been suppressed at a very early stage when only undifferentiated cells are present. The undifferentiated cells could give rise to the germinal cells that, in turn, cleave to give rise to embryo cells. The same can be seen in developing rediae and daughter sporocysts (up to the point of brood sac development), when some cells in the undifferentiated central cell mass become specialized as germinal cells (the so called primary germinal cells) and begin to cleave (Cort, Ameel & Van der Woude, 1954; Galaktionov & Dobrovolskij, 1998).

In many digeneans that have rediae in their life-cycles, and in the majority of those having daughter sporocysts, embryo development from primary germinal cells is accompanied by the formation of a specialized reproductive organ known as the germinal mass (Cort *et al*. 1954; Galaktionov & Dobrovolskij, 1998). Nothing structurally similar to the germinal masses of rediae and sporocysts was found in the M1 and M2 investigated in this study. They seem to have adopted the strategy seen in other gymnophallids and in the daughter sporocysts of Spirorchidae which, during the whole period of their functional activity, continuously produce new germinal cells in the body walls. These cells are thought to arise from undifferentiated cells that are available for that purpose (Cort *et al*. 1954; James & Bowers, 1967). This fact greatly increases their individual fecundity. Evidence from the present study suggests that a similar process takes place in the body walls of M1 and M2 throughout their existence. Again, it probably serves to increase the individual fecundity of the parthenogenetic metacercariae.

Another morphological feature that has a parallel in the parthenogenetic metacercariae of *C. margaritensis* and in rediae and daughter sporocysts, is the formation of a brood sac divided into individual brood chambers. In all cases the lining of the brood sac appears to arise in a similar fashion. That is, some cells become multilaminated and specialized, perhaps even forming a syncytium, for that function. The process by which the multilaminated cells extend and develop around individuals and groups of embryos has been interpreted in different ways. This has resulted in a diversity of opinion on the origin of envelopes that surround hermaphroditic generation embryos developing within daughter parthenites (Galaktionov & Dobrovolskij, 1998). The essence of the dispute is whether the embryonic membrane is produced by the embryo itself, or by the daughter parthenite. Significantly, the latter point of view is supported by those researchers who used only electron microscopy (Rifkin, 1970; Meuleman & Holzman, 1975; Gobel & Pan, 1985; Halton & McCrae, 1985; Dunn *et al*. 1992). Practically all researchers who have studied cercarial embryogenesis by light microscopy, however, have described the formation of the embryonic membrane from superficial blastomeres (macromeres) (Ishii, 1934; Cheng, 1961; James & Bowers, 1967; Cheng & Bier, 1972; Gerasev & Dobrovolskij, 1977). Their opinion has also been supported by electron microscopy studies including those of Matricon-Gondran (1971); Hockley (1972); Rees & Day (1976); Al-Salman & James (1988), Galaktionov & Dobrovolskij (1998). Assuming that in all cases parthenogenic development follows the pattern observed in the present study, it seems possible that supporters of the parthenite-origin theory may have confused the embryonic membrane with the laminated structures that result from the transformation of undifferentiated redial and sporocyst cells. The multilaminated structures sometimes form several layers over the true embryo-produced embryonic membrane making it difficult to distinguish. The embryonic membrane may also have been confused with the underlying developing tegument.

It is likely that the embryonic membrane protects early embryos from mechanical injury before their own tegument has formed. Moreover, it probably provides for transport of nutrients to the growing embryos. The presence of large mitochondria in its cells suggests that it is metabolically very active and the fact that it is still present around well-developed individuals suggests that it has an important role to play. It probably provides for the transport of nutrients necessary for the normal development of the M2 and M3s from the M1 and M2 brood chambers. (The fact that these nutrients have already had to be

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absorbed from the M1 brood chambers may be significant). The role of the M2 alimentary tract in this process is still uninvestigated. Possibly the gut system only begins to function after the M2 leave the M1 and begin to feed in the extrapallial cavity of the molluscan host.

The function of the multilaminated structures that line the brood sac of M1s and M2s, dividing it into individual chambers, is probably to spatially distribute the embryos and prevent mechanical damage from impact between freely suspended individuals in one common cavity. It is also probable that the multilaminated structures play a role (at least during the initial stages of embryo development) in the transport of nutrients absorbed by parent tegument to the developing embryos. This suggestion is supported by the abundance of large mitochondria observed in the multilaminated structures around young M2. It is also significant that the early multilaminated cells in the M1 metacercariae are bounded by two membranes separated by interstitial material. Because no very early M1 were investigated in this study, the origin of the outer of the two membranes was not established. Assuming that this membrane must have previously enclosed a cell, it would appear that the remainder of that cell has degenerated, perhaps to provide the area to become the brood sac. Meuleman, Holzmann & Peet (1980) described degenerating parenchymal cells surrounding early cercarial embryos inside *Schistosoma mansoni* mother sporocysts and suggested that these represented redundant cells from the previous larval stage, the miracidium. Perhaps in *C. margaritensis* similar cells from the preceding larval stage (the cercaria) may also degenerate. The fact that the surface area between the early multilaminated cells and the surrounding brood chamber is increased by folds and invaginations suggests that nutrient material is transferred between the two. Interestingly, no evidence of a similar arrangement of membranes and interstitial material was observed around early multilaminated cells in M2 metacercariae. This is consistent with the fact that, unlike miracidia and cercariae that are involved in locomotion and distribution, the M2 metacercariae only ever have one function, i.e. the multiple production of M3 metacercariae. M2 metacercariae therefore do not have redundant cells available for degeneration and resource reallocation.

Gymnophallid metacercariae do not encyst and, unlike most digenean metacercariae, are able to make use of their oral suckers and gut caeca to actively ingest and digest their molluscan host tissues. This contrasts with the majority of digenean metacercariae that depend on provision of their energy resources from their molluscan hosts through their teguments (Bibby & Rees, 1971; Stein & Lumsden, 1971 *a*, *b*; Strong & Cable, 1972; Popiel, 1976; Higgins, 1979; Halton & Johnson, 1982; Galaktionov *et al.* 1996). The period during which gymnophallid metacercariae may feed on second intermediate host tissue is not limited and that could be a reason why they have adopted parthenogenetic reproduction. Parthenogenetic metacercariae have been recorded in three gymnophallid species (*Gymnophallus australis*, *Parvatrema homoeotecnum*, *Cercaria margaritensis*) and are absent from other modern digeneans. In many ways the life-style of these gymnophallid metacercariae resembles that postulated for ancestral proto-digeneans, and hypothesized to underlie the origin of parthenogenetic generations in digenean trematode life-cycles (Cable, 1965, 1974; Pearson, 1972; Ginetsinskaya, 1968; Rohde, 1971, 1994; Gibson, 1987; Gibson & Bray, 1994; Galaktionov & Dobrovolskij, 1998). According to that hypothesis, proto-digeneans possessing a typical turbellaria-like structure adopted commensalism before becoming tissue parasites in gastropods. That provided them with access to practically unlimited energy resources that provoked earlier maturation and a gradual passage to parthenogenesis. These gymnophallid metacercariae feed in the same manner as the proposed proto-digeneans. Ching (1982) emphasized that *C. margaritensis* metacercariae ingested molluscan extrapallial fluid. They may therefore be considered borderline between commensals and true tissue parasites. Analysis and interpretation of our material would suggest that the parthenogenetic mode of reproduction may have originated in association with retardation and eventual cessation of reproductive organ formation. The same processes may also have taken place in the evolution of proto-digenean parthenogenetic generations.

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