

The Structure and the Formation of Egg Shells in the Parthenogenetic Species *Dactylobiotus dispar* Murray, 1907 (Tardigrada: Eutardigrada)

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The eggs of *Dactylobiotus dispar*, similar to other Tardigrada eggs, are covered with two shells: the vitelline envelope and the chorion. Ultrastructural studies have shown that the oocyte actively participates in the formation of both shells. The process of egg capsule formation begins at the midpoint of vitellogenesis. The chorion at first appears as isolated cones resulting from the exocytotic activity of the oocyte and the ovarian epithelium. Subsequently, connections between the cones are formed. Three layers can be distinguished in the completely developed chorion: (1) the inner layer of medium electron density; (2) the middle, labyrinthine layer; (3) the outer layer of medium electron density with cones (future conical processes). After chorion formation, a vitelline envelope is secreted by the oocyte. The *Dactylobiotus dispar* egg is covered with small, conical processes with hooked tips. The surface of the chorion is covered with a mesh-like network consisting of elongated interstices. The egg capsule has no micropylar opening.

Key words: Eutardigrada, egg capsule, chorion, vitelline envelope.

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The eggs of most animals are covered with egg shells (egg capsules) which protect them against the adverse influences of the environment (loss of water, radiation, etc.). The egg shells or some of their layers can be formed by the oocyte, by the follicular cells or by the cells of various parts of the reproductive system (ROŚCISZEWSKA 1994). Rarely, the precursors of egg shells are synthesized outside the reproductive system, for example in the fish liver.

Today, the most often applied egg shell classification is that of LUDWIG (1874), which distinguishes three types of egg shells: primary, secondary and tertiary.

The egg capsules, especially the chorion, harbour important traits for Tardigrada taxonomy. It is often impossible to identify certain species without collecting and examining their eggs (WĘGLARSKA 1982).

Egg capsule structure was the subject of numerous compilations both detailed and reviewed (DASTYCH 1973, 1975, 1980, 1988; GRIGARICK *et al.* 1973; TOFFNER *et al.* 1975; WĘGLARSKA 1975; BERTOLANI & REBECCHI 1993, 1996; NELSON *et al.* 1999), however, the process of the

formation of the egg shell is poorly known. Data concerning egg shell formation are known only from *Macrobotus richtersi* (WĘGLARSKA 1982).

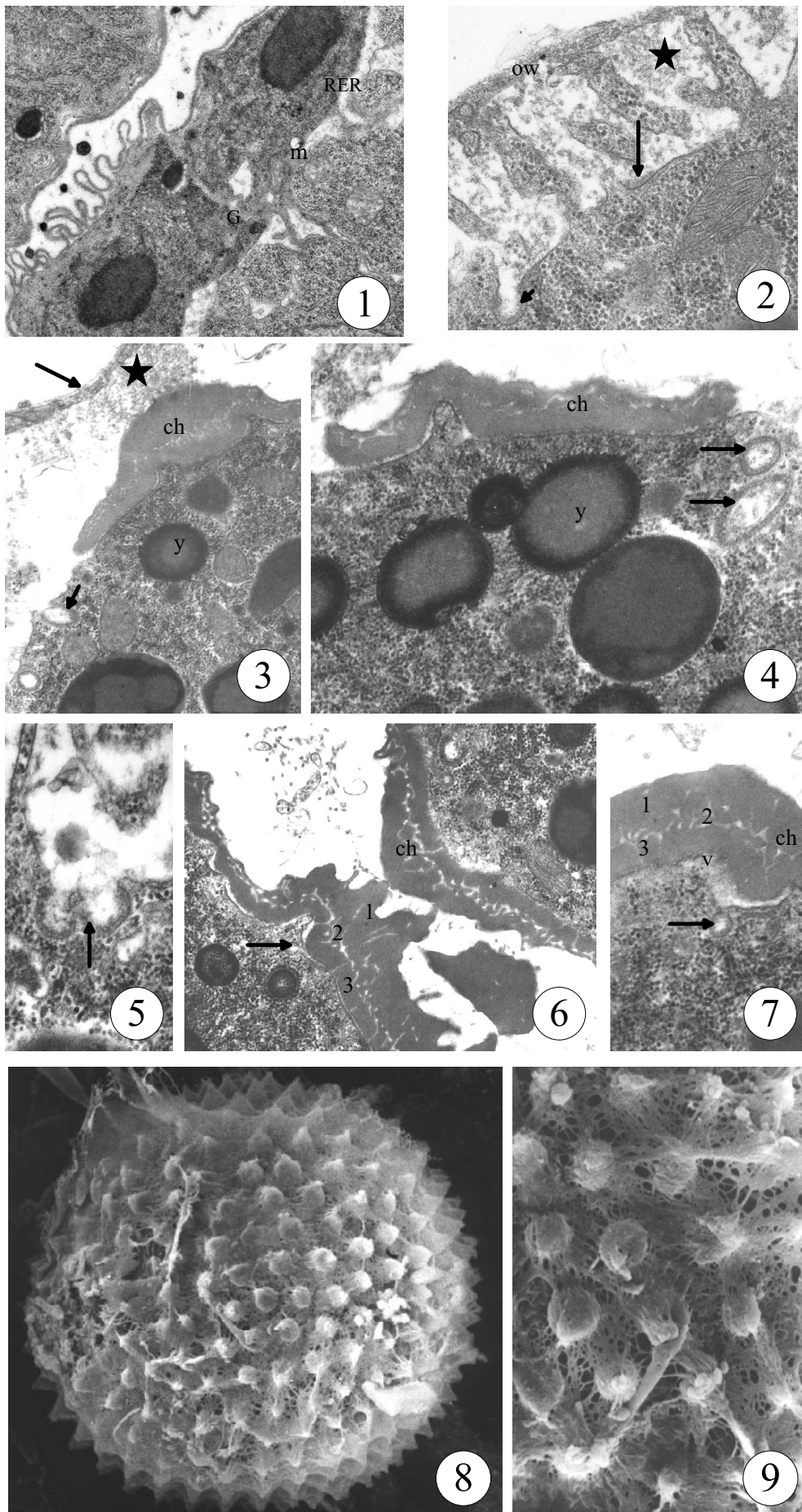
The aim of this study was to describe the choriogenesis and the egg capsule structure of the parthenogenetic species *Dactylobiotus dispar* Murray, 1907 (Eutardigrada).

Material and Methods

The material for this study originated from a laboratory culture of *Dactylobiotus dispar*. The culture was kept at room temperature (20°C) and pH = 7.0 (SZYMAŃSKA 1995).

Light and Transmission Electron Microscopy

Adult specimens of *Dactylobiotus dispar* were fixed with 2.5% glutaraldehyde (2h) and postfixed with 2% OsO₄ (2h). After dehydration in a graded ethanol series (50, 70, 90, 95 and 100%, each for 15 min), followed by acetone treatment (15 min), the material was embedded in Epon 812. Semi- and ultrathin sections were cut on a Leica Ultracut



UCT25 ultramicrotome. Semithin sections were stained with 1% methylene blue in 0.5% borax (DYKSTRA 1992) and observed using an Olympus BX60 light microscope.

Ultrathin sections were stained with uranyl acetate and lead citrate (REYNOLDS 1963) and examined with a Hitachi H500 transmission electron microscope at 75 kV.

Adult specimens of *Dactylobiotus dispar* were fixed with 4% paraformaldehyde (40 min). After dehydration in a graded ethanol series (50, 70, 90, 95 and 100%, each for 15 min), the material was embedded in histocryl. Semithin sections were cut on a Leica Ultracut UCT25 ultramicrotome and stained with bromophenol blue (BPB) to localise proteins, with Sudan black B to localise lipids or with Schiff (PAS method) to localise polysaccharides (LITWIN 1985). Semithin sections were observed using an Olympus BX60 light microscope.

Scanning Electron Microscopy

Some eggs, after fixing with 2.5% glutaraldehyde (2h), postfixing with 2% OsO₄ (2h), and dehydration in a graded alcohol series (30, 50, 70, 80, 90, 95, and 100% each for 10 min) and acetone (10 min), were dried at critical point Pelco CPD2 and coated with gold in a Pelco SC-6 duster. These preparations were examined with a Tesla BS340 scanning electron microscope.

Results

The process of egg capsule formation (choriogenesis) in *Dactylobiotus dispar* begins during the middle of vitellogenesis. The oocyte and the ovary wall cells participate actively in this process. The ovary wall cells possess organelles testifying to their intensive secretion activity such as cisterns of rough endoplasmic reticulum (RER), Golgi complexes, mitochondria and rybosomes (Fig. 1). These cells synthesize and secrete flocculent middle electron dense material inside the ovary (Figs 2, 3). This material is deposited on the oocyte surface forming successive layers of chorion (Figs 2, 3). Initially, the precursors of chorion are depos-

ited on the surface of the oocyte as solid processes forming characteristic cones (Figs 3, 4). After cone formation the basic chorion layer connecting the cones is formed. At the same time, vesicles containing fibrous, middle electron dense material can be observed (Fig. 4). The membranes of these vesicles fuse with the oolemma, and the fibrous material is secreted inside the ovary through exocytosis (Fig. 5). This material is built into the forming chorion. After chorion formation the oocyte secretes flocculent middle electron dense material into the space between the oolemma and the chorion (Figs 6, 7). This material is composed of precursors of the vitelline envelope.

The completely formed egg capsule of *Dactylobiotus dispar* is composed of two envelopes: a very thin vitelline envelope and a thick chorion (Fig. 7). The chorion has a multilayered structure (Figs 6, 7). It consists of a homogenous, middle electron dense internal layer, a middle, labyrinthine layer and an external layer with a structure similar to the structure of the internal layer.

Regularly arranged conical processes can be observed on the surface of the chorion (Fig. 8). Their height is from 4 to 5 μm and width of the base is from 3 to 4 μm . The distance between the bases of the processes varies from 2 to 3 μm . The tips of the conical processes are hook-like (Fig. 9). The conical processes are connected with thin and thick slats forming a network structure on the surface of the chorion consisting of elongated interstices (Fig. 9). There is no micropylar opening on the surface of the egg capsule. The operculum and attachment structures were not observed.

The histochemical treatments showed that the material building the chorion is PAS-positive, BPB-positive and Sudan Black B-negative (not shown). The vitelline envelope is PAS-, BPB- and Sudan Black B- positive (not shown).

Discussion

The process of choriogenesis and the morphology and the ultrastructure of the egg capsule in Tardigarda are relatively poorly known as compared with other groups of invertebrates. Up till

Figs. 1-9. *Dactylobiotus dispar* choriogenesis. Fig. 1. A fragment of the longitudinal section through the ovary wall: Golgi complex (G), mitochondria (m). TEM, $\times 16500$. Fig. 2. A fragment of the longitudinal section through the ovary: ovary wall (ow), exocitotic vesicles (arrowhead), precursors of chorion (asterisc), the forming layer of chorion (arrow). TEM, $\times 16600$. Fig. 3. A fragment of the longitudinal section through the ovary: chorion (ch), yolk (y), ovary wall (arrow), exocitotic vesicles (arrowhead), precursors of chorion (asterisc). TEM, $\times 16000$. Fig. 4. A fragment of the longitudinal section through the oocyte: chorion (ch), yolk (y), vesicles containing flocculent middle electron dense material (arrows). TEM, $\times 30000$. Fig. 5. The vesicles opening on the oocyte surface (arrow). TEM, $\times 34500$. Fig. 6. A fragment of the longitudinal section of the ovary: chorion (ch) consisting of three layers: homogenous, middle electron dense external layer (1), labyrinthine layer (2), homogenous, middle electron dense internal layer (3), exocitotic vesicle (arrow). TEM, $\times 17300$. Fig. 7. A fragment of the longitudinal section of the oocyte: chorion (ch) consisting of three layers: homogenous, middle electron dense external layer (1), labyrinthine layer (2), homogenous, middle electron dense internal layer (3), vitelline envelope (v), exocitotic vesicle (arrow). TEM, $\times 32800$. Fig. 8. The egg. SEM, $\times 1230$. Fig. 9. A fragment of the chorion surface with conical prosesses. SEM, $\times 2350$.

now, the process of egg shell formation was studied only in *Macrobiotus richtersi* (WEGLARSKA 1982). The egg capsule of this species is composed of two shells: a vitelline envelope adhering to an oolemma and a three-layered chorion. The internal layer of the chorion has a fibrous structure, the middle layer is labyrinthine, the external layer is of high electron density. The *Dactylobiotus dispar* egg capsule is composed of a vitelline envelope and a chorion as well. The vitelline envelope is very thin. The chorion, similar to the chorion of *Macrobiotus richtersi*, is three-layered, but the labyrinthine layer is more pronounced and the internal and external layers have the same electron density. In both species, the oocyte participates actively in the formation of the shells. The cells of the ovary wall also take part in chorion formation. Thus, according to the widely accepted classification of LUDWIG (1874), the vitelline envelope in both *Macrobiotus richtersi* and *Dactylobiotus dispar* is of the primary type, but the chorion should be regarded as a mixed type: primary – secreted by the oocyte, and secondary – produced by the cells of the ovary wall. The participation of the oocyte in egg shell formation occurs commonly in Annelida (OLIVE 1983; SPAŁEK-WOŁCZYŃSKA 2002), Crustacea (KUBRAKIEWICZ & KLIMOWICZ 1994; ANDO & MAKIOKA 1999), Arachnida (WITALIŃSKI & ŻUWAŁA 1981; WITALIŃSKI 1993) and in some insects (BILIŃSKI 1993; KISIEL *et al.* 1994; POPRAWA *et al.* 2002; ROŚCISZEWSKA 2003). Oocyte activity during choriogenesis is a primitive feature and suggests that Tardigrada are primitive animals.

The manner of chorion formation in Tardigrada is controversial. Material synthesized by the cells of the ovary wall is secreted inside the ovary, then it is deposited on the surface of the appropriate cells (oocytes). This material is not deposited on the surface of the trophocytes. This suggests that receptors of chorion precursors are on the surface of oocytes, and are absent from the surface of trophocytes. This is in need of additional research.

The sequence of events during the formation of egg shells is interesting. Generally, during choriogenesis the vitelline envelope is produced first, followed by the appearance of the chorion. In Tardigrada the chorion is formed first, after which the oocyte secretes material which forms the vitelline envelope (WEGLARSKA 1982; own observations). A similar sequence of egg shell formation has been described in *Tetradontophora bielensis* (Collembola) (KRZYSZTOFOWICZ & KISIEL 1989). In this case, the vitelline envelope is formed after oviposition. The formation of the vitelline envelope after oviposition in *Tetradontophora bielensis* is an adaptive? feature because in this species fertilization takes place inside the ovary. Probably

this is an adaptative feature in Tardigrada as well. This is suggested by the absence of a micropylar opening in the egg capsule and by the manner of chorion formation (at first isolated cones and then connections between cones are formed). In *Dactylobiotus dispar*, which reproduces parthenogenetically in laboratory cultures, this sequence of egg shell formation may be the remnant (lub legacy) of bisexual reproduction. On the other hand, it suggests that males and females may occur in natural populations and that fertilization takes place inside the ovary.

The egg capsule surface of Tardigrada has an abundant sculpture that is important in the taxonomy of Eutardigrada. Species and subspecies can be discerned on the basis of egg capsule morphology (DASTYCH 1980, 1988; BERTOLANI & REBECCHI 1996; GRIGARICK *et al.* 1973; TOFFNER *et al.* 1975). The eggs of *Macrobiotus richtersi* are spherical and belong to the “areolatus” type. Their chorion is covered with conical processes usually having flattened tips. There are areolations on the surface of the chorion between the processes. The processes are from 12 to 14 μm high, and width of the base is from 11 to 13 μm . Their surface is covered by a thin network (WEGLARSKA 1975; DASTYCH 1980, 1988). The eggs of *Dactylobiotus dispar* are also spherical. Their chorion is covered with small conical processes (height 4–5 μm , base width 3–4 μm), on the surface of which a network structure consisting of small elongated interstices is formed. The tips of conical processes are hook-like. A similar network, covering the processes, occurs on the surface of the basic chorion connecting the processes. The egg capsule of *Dactylobiotus dispar* does not reveal any regional differentiation such as an operculum and hatching line, micropyle, hydropyle or attachment structures. The whole surface of the chorion is covered with regularly arranged conical processes. Similar structures are observed in insects which lay their eggs in packets (Neuroptera, Hymenoptera). In these cases their role is to protect air-spaces around each egg in order to facilitate adequate gas exchange inside the packet (HINTON 1981). In Tardigrada the conical processes may have a similar function if eggs are laid among decomposing vegetation.

Histochemical investigation revealed that the vitelline envelope of *Dactylobiotus dispar* consists of polysaccharides, peptides and lipids. The presence of the lipids in the vitelline envelope is closely connected with the function of this layer. This species lays eggs in a freshwater habitat where embryos develop. The vitelline envelope, including the lipid component, is hydrophobic and protects the developing embryo against an excess of water. The chorion is composed of polysaccha-

rides and peptides. It does not consist of a lipid component. Up till now, no literature data concerning the histochemical structure of Tardigrada egg capsules existed.

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