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Diphyllobothrium latum: Scanning Electron Microscopic Study on the Eggshell Formation

YOSUKE YAMANE*, AKIO NAKAGAWA*, YUMIKO MAKINO* AND KAZUMITSU HIRAI†

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Introduction

The formation of cestode eggs has been extensively investigated (Lethbridge, 1971, 1976; Löser, 1965a, 1965b, 1965c; Pence, 1974; Rybicka, 1966, 1972, 1973a, 1973b; Smyth, 1951, 1954, 1969; Smyth and Clegg, 1959). The nature and mode of formation of eggs reflected the structure of the female genitalia, the life history and nutrition of the parasite, and the physico-chemical characteristics of the environment.

The studies of the egg formation in pseudophyllidea cestodes were concentrated on the formation of eggshell and the biochemistry of the processes involved (Bogomopova and Chavpova, 1963; Löser, 1965a; Smyth, 1956).

The scanning electron microscope (SEM) has recently come to be used in observing the topographical morphology, and this procedure was thought to be important in elucidating the surface structure of eggshell stereoscopically. The possible phylogenetic and taxonomic significance of the structure of eggshell in diphyllobothriid cestodes was discussed (Andersen and Halvorsen, 1978; Coil, 1977; Hilliard, 1972; Lethbridge, 1976; Yamane *et al.*, 1976).

The present study was designed to observe the mode of eggshell formation by means of SEM and to analyze the chemical composition of the eggshell of *Diphyllobothrium latum* by X-ray microanalysis.

* *Department of Environmental Medicine, Shimane Medical University, Izumo 693, Japan.*
† *Department of Parasitology, School of Medicine, Ehime University, Ehime 791-02, Japan*

Materials and Methods

An adult worm of *Diphyllobothrium latum* was expelled spontaneously from a man. Some mature proglottids were cut off from the strobila and were removed immediately to be processed for scanning electron microscopy. Specimens were washed in physiological saline, fixed for 6 hr at 4 C in 5% glutaraldehyde in phosphate buffer (pH 7.4) and post-fixed in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) for 2 hr at 4 C. They were dehydrated in an ascending series of ethanol and embedded in styren for 48 hr at 60 C (Tanaka *et al.*, 1874). After polymerization the specimens were cracked under a stereoscope to observe the process of eggshell formation in cross fractures. After the polymerized styren was dissolved with propylene oxide, the specimens were soaked in amylacetate for 30 minutes, dried by the critical point dry method, coated with Pt-Pd alloy, and observed by Hitachi HFS-2ST scanning electron microscope.

The cracked specimens were mounted on

stubs for analytical electron microscopy of eggshells, and subsequently coated with carbon for X-ray microanalysis. Other specimens of eggs were collected by pushing gently around the uterine pores with filter papers. The mature eggs which were released through uterine pores of gravid proglottids were washed ten times in distilled water, dehydrated in an ascending series of ethanol, soaked in amylacetate for 30 minutes, dried by the critical point dry method, and subsequently coated with carbon for microanalysis. Five eggs from each of the proximal, middle, terminal parts of uterus and five mature eggs collected through uterine pores were analysed using Hitachi S-450 with a dispersive X-ray microanalysis system (Horiba, EMAX-2200) at an accerelating voltage 20 kV.

Results

The formation of eggshells took place in the initial part of the uterus. The yolk glands beneath the tegumental layer exhibited an aciniform structure which consisted of many round vitelline cells. Numerous globules, 0.5–2.0 μm in diameter, and polygonal bodies were packed around a large nucleus in the cytoplasm of vitelline cells. The surface of polygonal bodies was relatively smooth (Fig. 1).

Mature vitelline cells were released from the yolk glands to the vitelline reservoir, where they were temporarily stored. The wall of the vitelline reservoir was surrounded with the honeycomb-like, interstitial tissue. Many round vitelline cells were packed densely in a vitelline reservoir which runs to the ootype.

Round vitelline cells had various sizes, approximately 3–10 μm in diameter, and consisted of a conglomeration of numerous globules. As the vitelline cells were wrapped with a thin capsule which had granular, uneven surface, some of them were broken and military globules burst into the vitelline reservoir (Fig. 2).

The fertilisation and the formation of eggshells took place in the ootype. Vitelline cells were conglomerated each other in the ootype and some of them began to form primitive eggshells (Fig. 3). The thin, porous texture of the primitive eggshell wrapped up the ovum and attached globules of various size (Fig. 4).

The first recognizable eggs, appearing in the proximal part of uterus near the ootype, showed coarse, fibrous textures, which were presumed to be the precursor of eggshells. Numerous round globules surrounded densely the eggshell. Some globules were observed burying themselves in the pores of eggshell surface (Fig. 5).

The porous, coarse texture of the eggshell was ultimately filled up with globules in the vitelline cells and showed an uneven, granular surface pattern. The eggshell in this stage did not exhibit surface specialization such as operculum, opercular suture or surface pits (Fig. 6). As the egg moved to the terminal part in the uterus, the porous texture was filled up with globules to complete a thicker and smooth eggshell. The surface of the eggshell showed a geometrical pattern as if pentagonal plates were arranged (Fig. 7).

In the cross section of the middle part of uterus, eggs with a characteristic operculum were observed in the numerous vitelline cells (Fig. 8). The surface of the eggshell exhibited fine furrows and shallow, small pits which were sporadically distributed on the whole surface. Some globules in the vitelline cells entered into the opercular suture to seal the operculum (Fig. 9). When the eggshell has been fully formed, the mature eggs showed operculum, opercular suture and surface pits. Pits were minute, widely separated and shallow (Fig. 10). Some globules attached to the surface of eggshell, and fused to form the homogeneous constituent of eggshell (Fig. 11).

The pits in fully developed eggs were

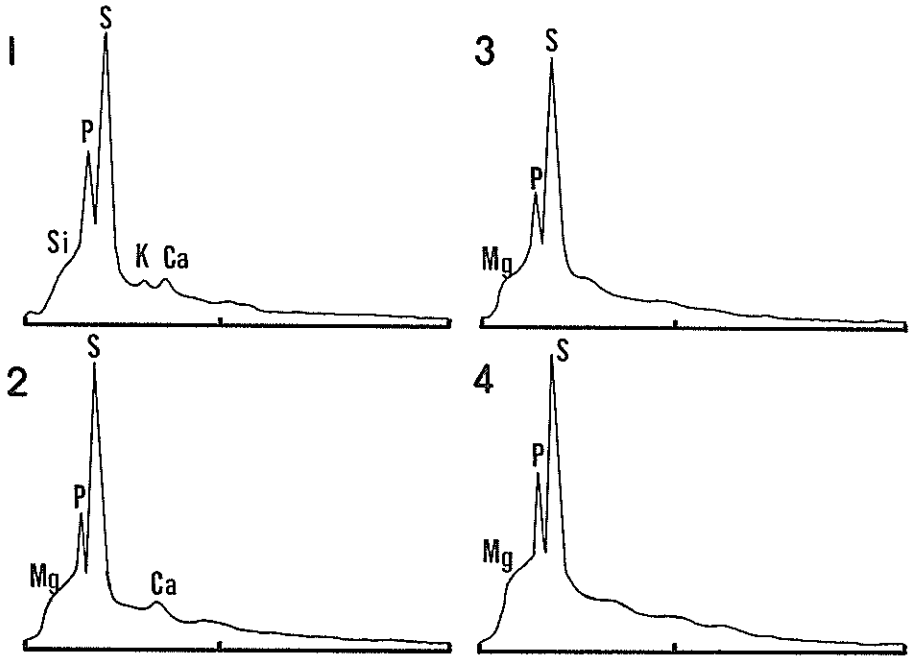


Fig. 14 Energy dispersive X-ray scan of eggshell. 1) eggs in the proximal part of the uterus, 2) eggs in the middle part of the uterus, 3) eggs in the terminal part of the uterus, and 4) eggs released from the uterine pore. Accelerating voltage 20 kV and counting time 200 seconds.

showed as shallow excavations, approximately 0.1 μm in diameter, and did not penetrate the wall of eggshells. Higher magnification revealed that small patches were attached to the entire surface of eggs (Figs. 12 and 13).

The composition of the eggshell was determined by the X-ray analysis, comparing the eggs at an proximal, middle and terminal parts of the uterus with the completely developed eggs which were released by pushing around the uterine pores of gravid proglottids. Phosphorus and sulphur were commonly described as prominent peaks in all the eggshells. Besides, magnesium, calcium and potassium peaks were slightly detectable (Fig. 14).

Discussion

Smyth (1956, 1969) described that ova are released periodically from the ovary and pass down the oviduct to the ootype where fertilization takes place; mature vitelline cells, which contain both shell and yolk precursor, pass from the vitelline glands to the vitelline reservoir, and temporarily stored there. Vitelline cells were then passed from the reservoir to the ootype where globules of shell precursor were released and coalesced to form an eggshell.

The fractographs of vitelline cells revealed a large nucleus and numerous globules in the cytoplasm, showing a mulberry-like inner structure. The atypical ellipsoidal and polygonal bodies were often mixed among globules. Swiderski and Mokhtar-Maamouri (1974), and Mokhtar-Maamouri and Swiderski (1976) described that mature vitelline cells were filled with shell globules, glycogen and lipid droplets in their cytoplasm. The

shell globules which contained protein substance were considered as a shell precursor and glycogen was considered as a food store for the developing embryo.

In an early stage of the eggshell formation, the globules coalesced in a coarse, fibrous texture to form primitive eggshell. These globules attached to the surface of the primitive eggshell had the same morphological feature as the globules which were found in the vitelline cells. It was supposed that the globules in the vitelline cells played an active role in forming the coarse texture of primitive eggshells.

The fibrous network which often wrapped an ovum in ootype may be protocollagenous. Bogomopova and Chavpova (1963) described that globules in the vitelline cells of *Diphyllobothrium latum* exhibited a positive reaction to α-aminoacids, proteins of acid nature and phenol. They suggested that the globules contained protein of the protocollagenous type. After the protocollagenous protein of vitelline cells wrapped ova in network, some globules in the vitelline cells sealed the meshes of the network to form coarse texture of the primitive eggshell. The globules sealed subsequently crevices on the primitive eggshell and ultimately formed the mature eggshell. Some of the remaining globules were enclosed into the eggs and served as true "yolk" for the nourishment of eggs during the development of coracidia. The rest of the globules gathered around the eggs, filling up the uterine loops.

In cyclophyllid cestodes, Coil (1977) observed with SEM that the early outer envelope surface of *Shipleya inermis* was covered with secretory granules, 0.1–0.6 μm in diameter. Lethbridge (1976) also described that the surface of the eggshell of *Hymenolepis diminuta* was covered with spherical masses, 0.14–0.98 μm in diameter, which often attached to the underlying shell material. These 'secretory bodies' and 'spherical masses' are probably identical with the globules in the vitelline cells.

The developed mature eggs showed numerous pits on the eggshell, and many patches which were found in the bottom of the pits and on the eggshell surface at the higher magnification of SEM. The function of the pits has not been elucidated, but Hilliard (1972) suggested that the embryos in the eggs may have metabolic activity or ion exchange through these numerous pits. The pattern and arrangement of the pits on the eggs had some taxonomic value. Eggs in marine species had much thicker shells with deeper, more numerous pits. The superficial pits on thin eggshells in such freshwater species as *D. latum* may be vestiges of structures that have undergone evolutionary alterations or adaptaiton to development in the habitat of freshwater. The differentiation between cestodes whose intermediate hosts are of freshwater origin or of sea origin is certainly possible by observing the surface pattern and the pits on their eggshells through SEM (Hilliard, 1960; Ishii, 1972; Yamane *et al.*, 1976).

Jones (1979) revealed a high sulphur content in the infective eggs of *Hydatigera taeniaformis* by means of X-ray microanalysis. The present study showed a high phosphorus, high sulphur and low magnesium content in the eggshell of premature eggs at the proximal part of the uterus. In the eggshell of mature eggs at the middle and the terminal part of the uterus a high phosphorus and sulphur, and a low calcium and magnesium contents were confirmed. The high content of phosphorus and sulphur in the eggshell seems to support that the eggshells consisted of precursor protein of eggshell in the globules of the vitelline cells.

Summary

The microtopographical details of eggshell formation of *Diphyllobothrium latum* as seen with the scanning electron microscope were described. The observations revealed the process of eggshell formation, the relationship

between eggshells and globules in the vitelline cells, the inner structure of the vitelline cells, and pits on the eggshell surface.

At an early stage of the eggshell formation, the surface structure of the eggshell showed fibrous, porous texture. The globules in the vitelline cells buried themselves into crevices on the fibrous texture of primitive eggshells. The X-ray microanalysis revealed a high phosphorus, a high sulphur peak and low peaks of magnesium, calcium and potassium in the eggshell.

References

1) Andersen, K. and Halvorsen, O. (1978): Egg size and form as taxonomic criteria in *Diphyllobothrium* (Cestoda, Pseudophyllidea). Parasitol., 76, 229–240.
2) Bogomopova, N. A. and Chavpova, P. E. (1963): Vitelline cells of *Fasciola hepatica* and *Diphyllobothrium latum* and their role in the formation of the eggshell and the nutrition of the embryo. Helminthologia 3, 47–59.
3) Coil, E. H. (1977): Studies on the embryogenesis of the tapeworm *Shipleya inermis* Fuhrmann, 1908 using transmission and scanning electron microscopy. Z. Parasitenkd., 52, 311–318.
4) Hilliard, D. K. (1960): Studies on the helminth fauna of Alaska. XXXVIII. The taxonomic significance of eggs and coracidia of some diphyllobothriid cestodes. J. Parasitol., 46, 703–717.
5) Hilliard, D. K. (1972): Studies on the helminth fauna of Alaska. LI. Observations on eggshell formation in some diphyllobothriid cestodes. Can. J. Zool., 50, 585–592.
6) Ishii, Y. (1972): Morphology of helminth ova through the scanning electron microscope. Fukuoka Acta Med., 63, 419–431.
7) Jones, B. R. (1979): Application of scanning electron microscopy and X-ray microanalysis to studies on the infective eggs of *Hydatigera taeniaformis*. IRCS Medical Science 7, 391–392.
8) Lethbridge, R. C. (1971): The chemical composition and some properties of the egg layers in *Hymenolepis diminuta* eggs. Parasitol., 63, 275–288.
9) Lethbridge, R. C. (1976): The architecture of the eggshell of *Hymenolepis diminuta*. Internat. J. Parasitol., 6, 87–90.
10) Löser, E. (1965a): Der Feinbau des Oogenotop bei Cestoden. Z. Parasitenkd., 25, 413–458.
11) Löser, E. (1965b): Die Eibildung bei Cestoden. Z. Parasitenkd., 25, 556–580.
12) Löser, E. (1965c): Die Postembryonale Entwicklung des Oogenotop bei Cestoden. Z. Parasitenkd., 25, 581–596.
13) Mokhtar-Maamouri, F. and Swiderski, Z. (1976): Vitellogénèse chez *Echeneibothrium beauchampi* Euzet, 1959 (Cestoda: Tetraphyllidea, Phyllobothriidae). Z. Parasitenkd., 50, 293–302.
14) Pence, D. B. (1974): Electron microscope and histochemical studies on the eggs *Hymenolepis diminuta*. J. Parasitol., 56, 84–97.
15) Rybicka, K. (1966): Embryogenesis in cestodes. In Advances in Parasitology, Vol. 4, ed. by Ben Daves, Academic Press, London and New York, 107–186.
16) Rybicka, K. (1972): Ultrastructure of embryonic envelopes and their differentiation in *Hymenolepis diminuta* (Cestoda). J. Parasitol., 58, 849–863.
17) Rybicka, K. (1973a): Ultrastructure of macromeres in the cleavage of *Hymenolepis diminuta* (Cestoda). Trans. Amer. Micros. Soc., 92, 241–255.
18) Rybicka, K. (1973b): Ultrastructure of the embryonic syncytial epithelium in a cestode *Hymenolepis diminuta*. Parasitol., 66, 9–18.
19) Smyth, J. D. (1951): Egg-shell formation in trematodes and cestodes as demonstrated by the methyl or malachite green techniques. Nature, 168, 322–323.
20) Smyth, J. D. (1954): A technique for the histochemical demonstration of polyphenol oxidase and its application to egg-shell formation in helminths and byssus formaiton in *Mytilus*. Quart. J. Micros. Sci., 95, 139–152.
21) Smyth, J. D. (1956): Studies on tapeworm physiology. IX. A histochemical study of eggshell formation in *Schistocephalus solidus* (Pseudophyllidea). Exp. Parasit., 5, 519–540.
22) Smyth, J. D. and Clegg, J. A. (1959): Eggshell formation in trematodes and cestodes. Exp. Parasit., 8, 286–323.
23) Smyth, J. D. (1969): The physiology of cestodes, Wolf Freeman and Company, San Francisco, 105–126.
24) Swiderski, A. and Mokhtar-Maamouri, F.

(1974): Étude de la vitellogénèse de *Bothriocephalus clavibothrium* Ariola, 1899 (Cestoda: Pseudophyllidea). Z. Parasitenkd., 43, 135–149.
25) Tanaka, K., Iino, A. and Naguro, T. (1974): Styren resin cracking method for observing biological materials by scanning electron microscopy. J. Electron Microsc., 23, 313–315.
26) Yamane, Y., Seki, R. and Okada, N. (1976): Comparative observation on surface topography of teguments and eggshells of diphyllobothriid cestodes by scanning electron microscopy. Yonago Acta Med., 20, 55–65.

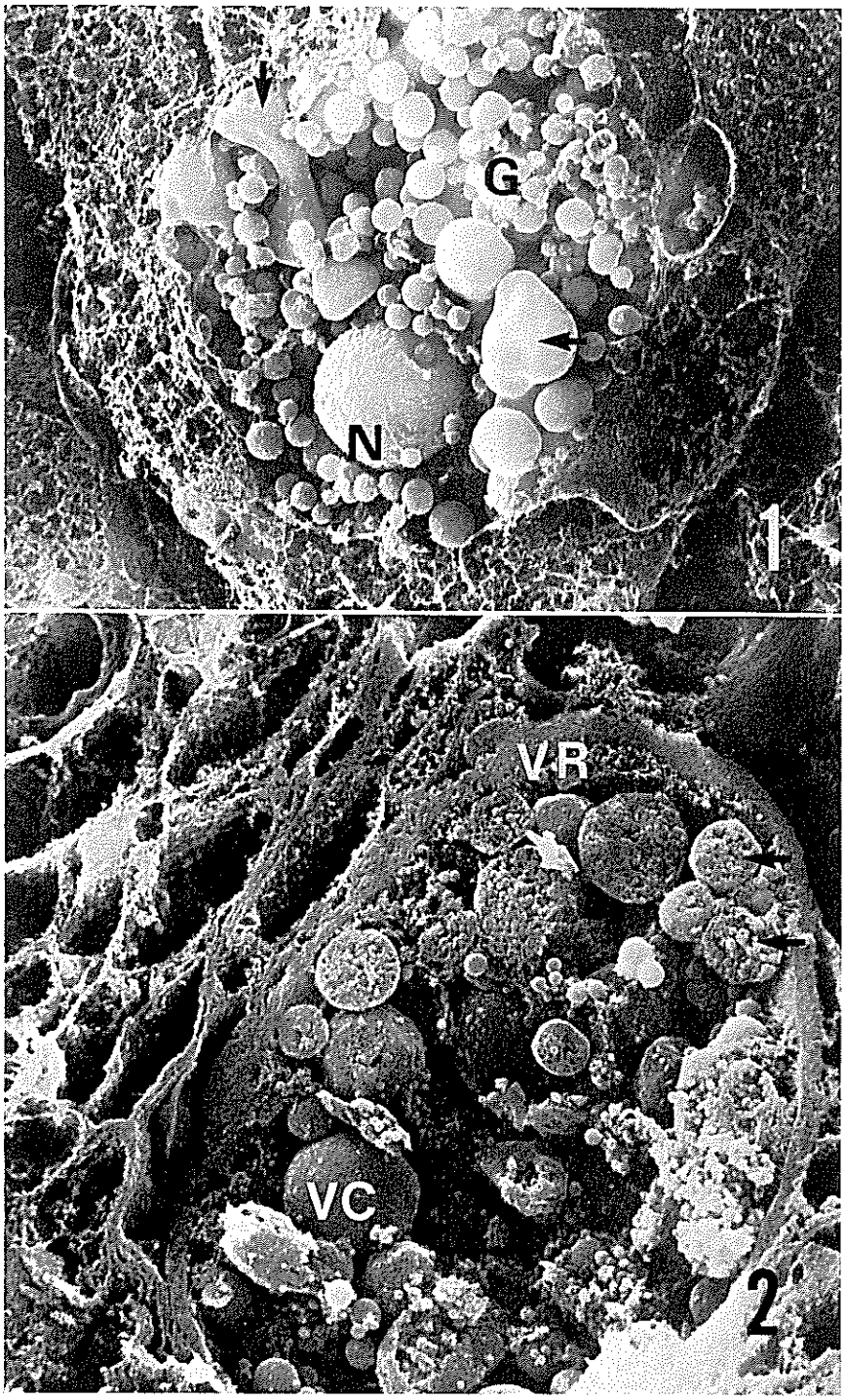
広節裂頭条虫（*Diphyllobothrium latum*）における卵殻形成に関する走査電子顕微鏡的研究

山根洋右　中川昭生　牧野由美子
（島根医科大学環境保健医学教室）
平井和光
（愛媛大学医学部寄生虫学教室）

広節裂頭条虫の卵殻形成過程を中心に，卵黄腺細胞の微細内部構造，卵黄腺細胞の細胞質内小顆粒の卵殻形成への関与，卵殻表面構造と点刻形成などを走査電子顕微鏡を用いて立体的に観察した．

卵殻形成初期には，卵殻は膠質性物質による線維性多孔性網状構造を形成し，その網目状間隙に卵黄細胞由来の顆粒が充満し，間隙をふさぐ．卵殻表面は凹凸不整の粗面構造から多角形の平滑板状構造を示し，やがて微小な点刻が分布する緻密な卵殻が完成する．

またエネルギー分散形X線分析により，多くの燐及び硫黄を含有し，微量のマグネシウム，カルシウムなどを含むことを明らかにした．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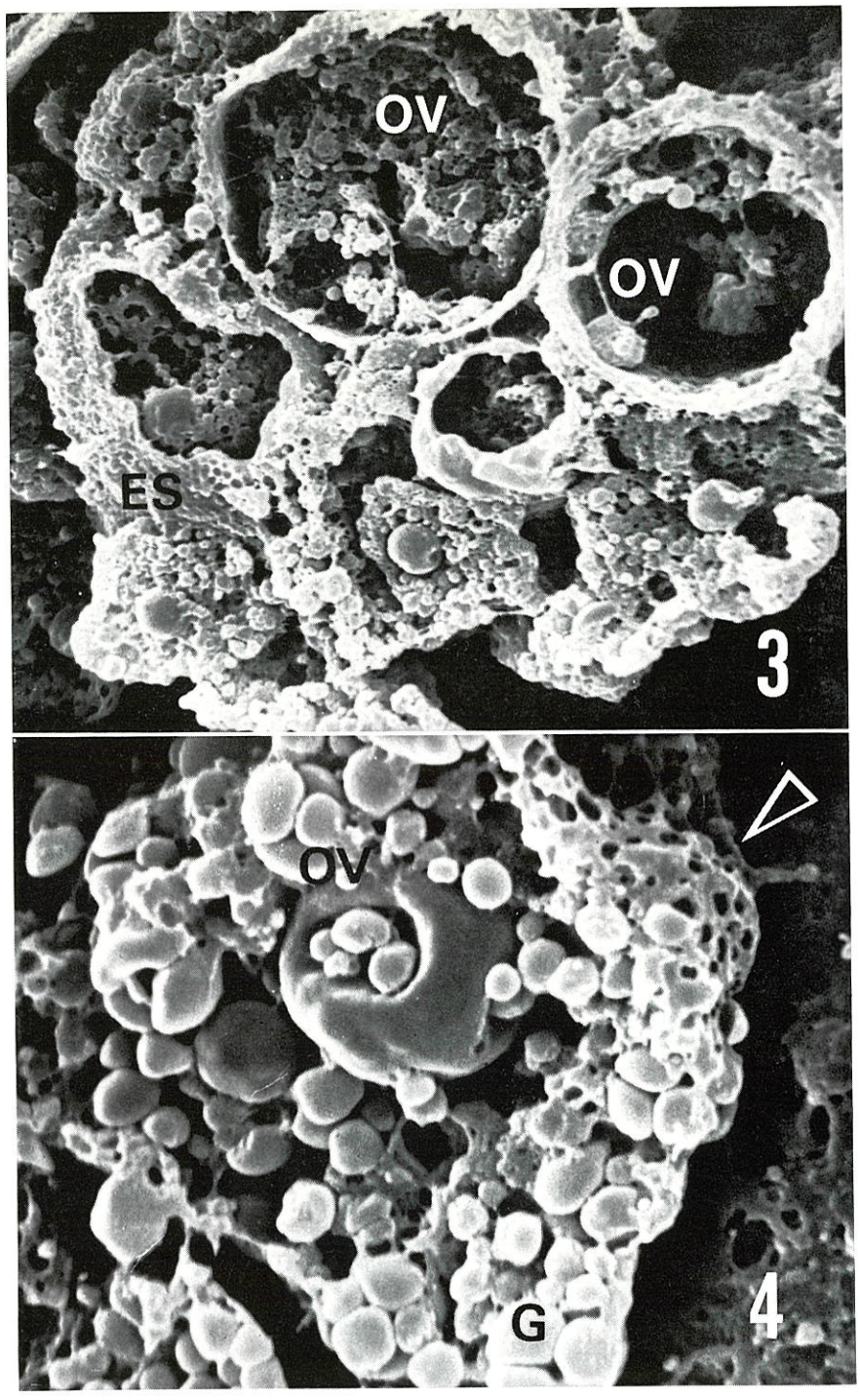
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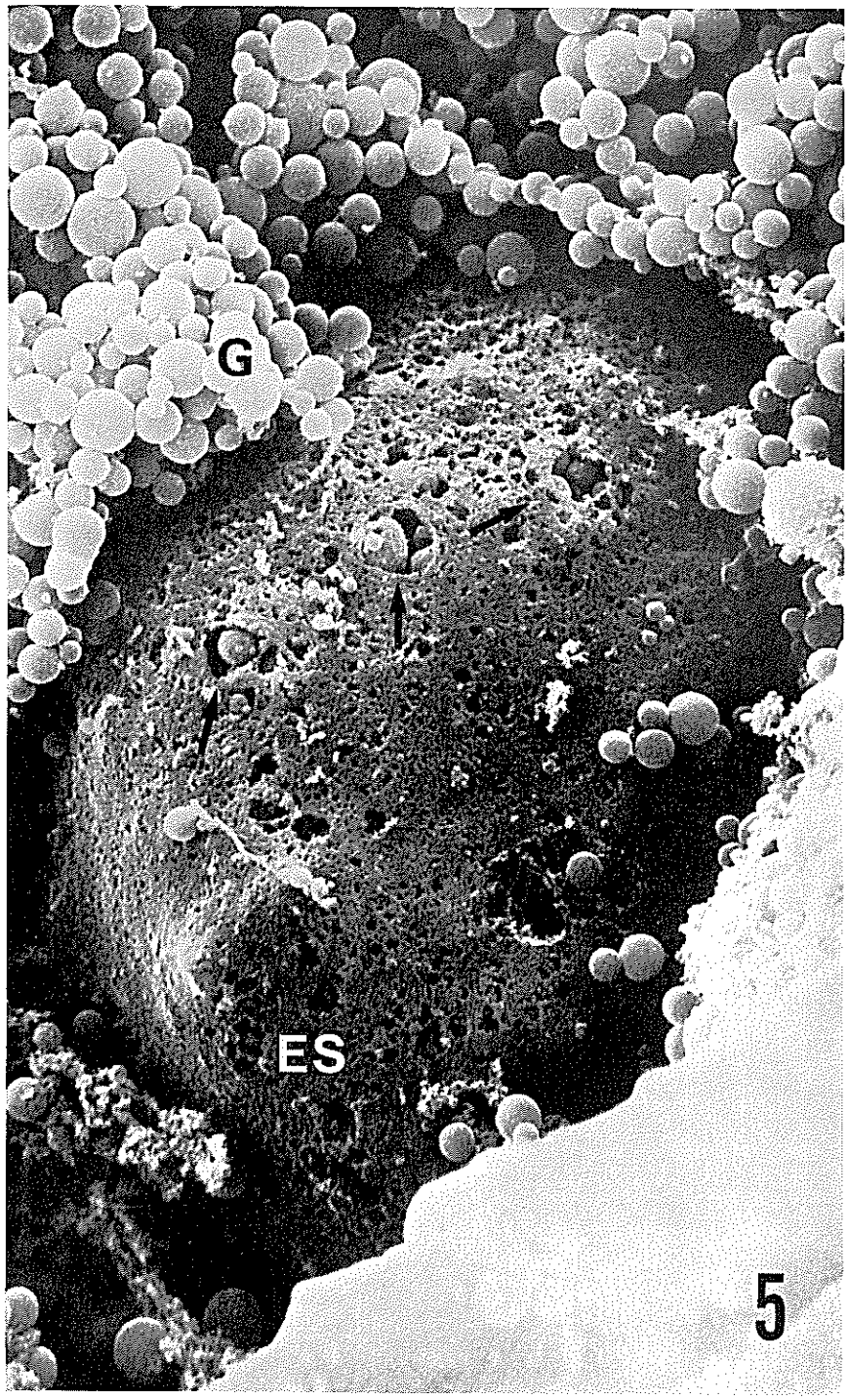
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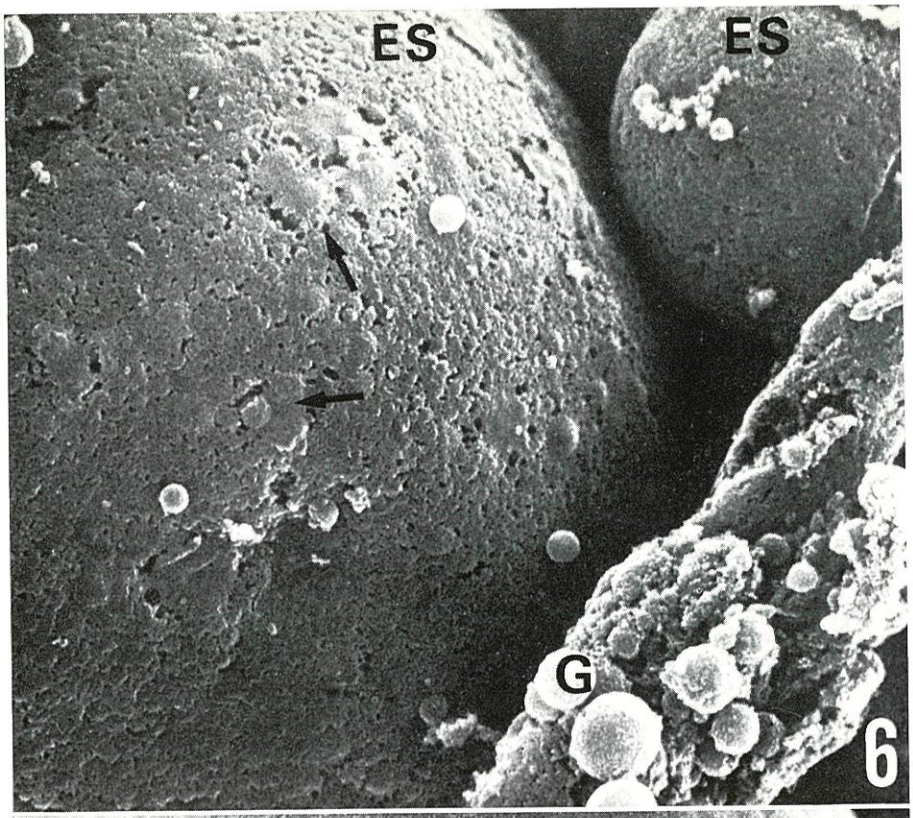
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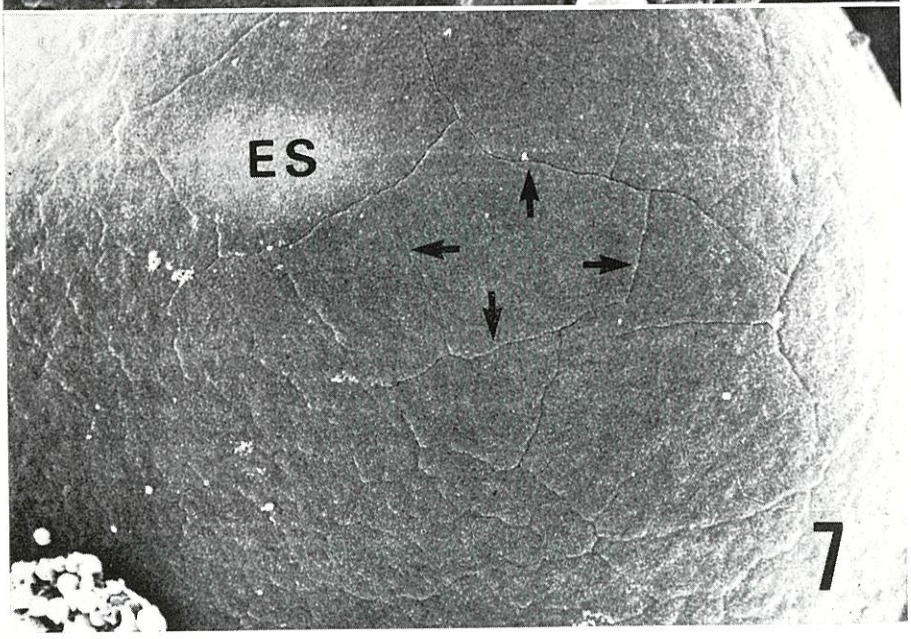
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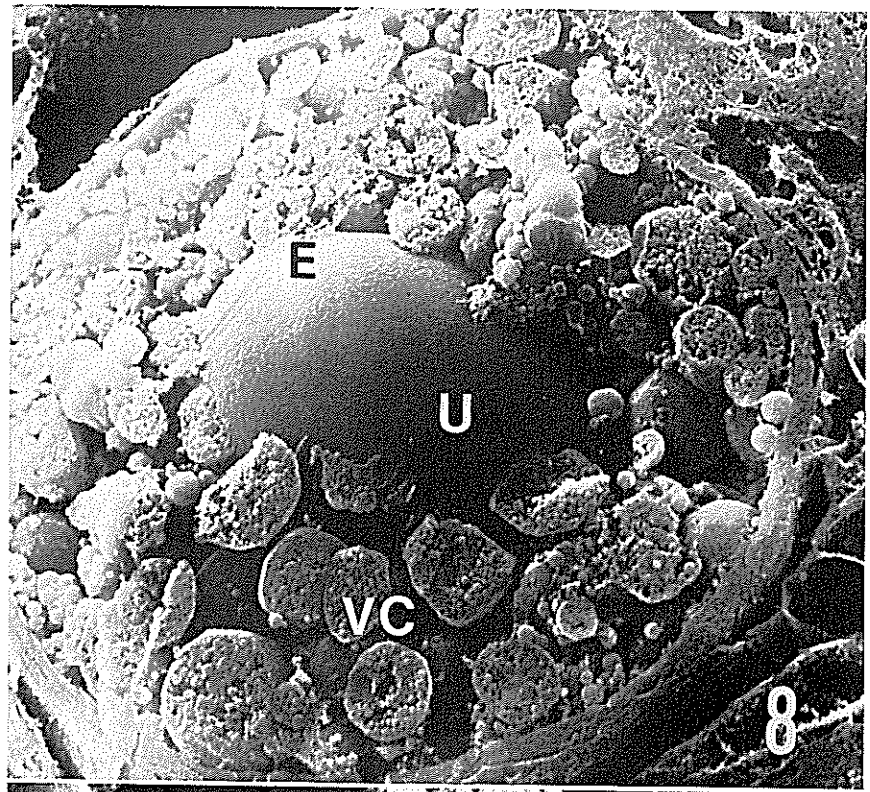
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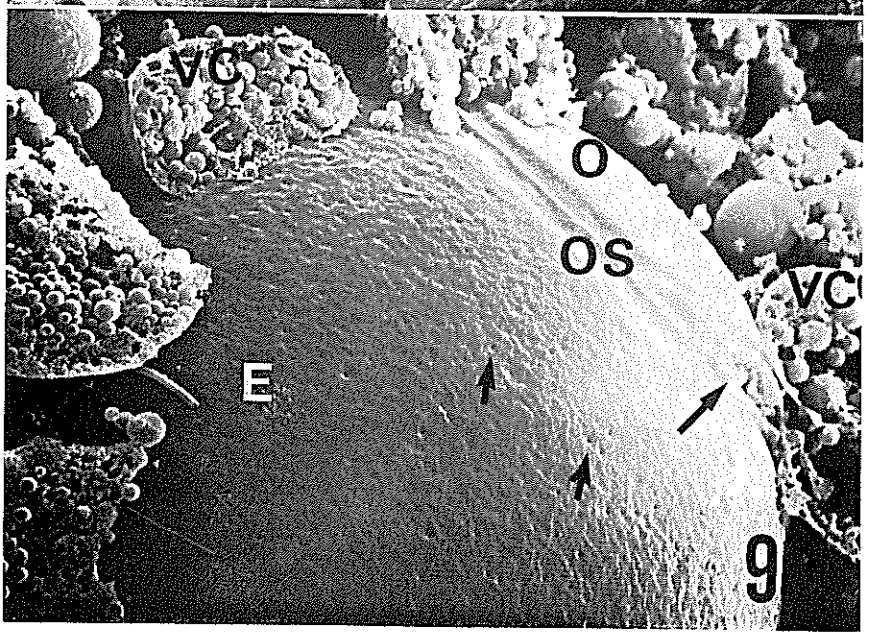
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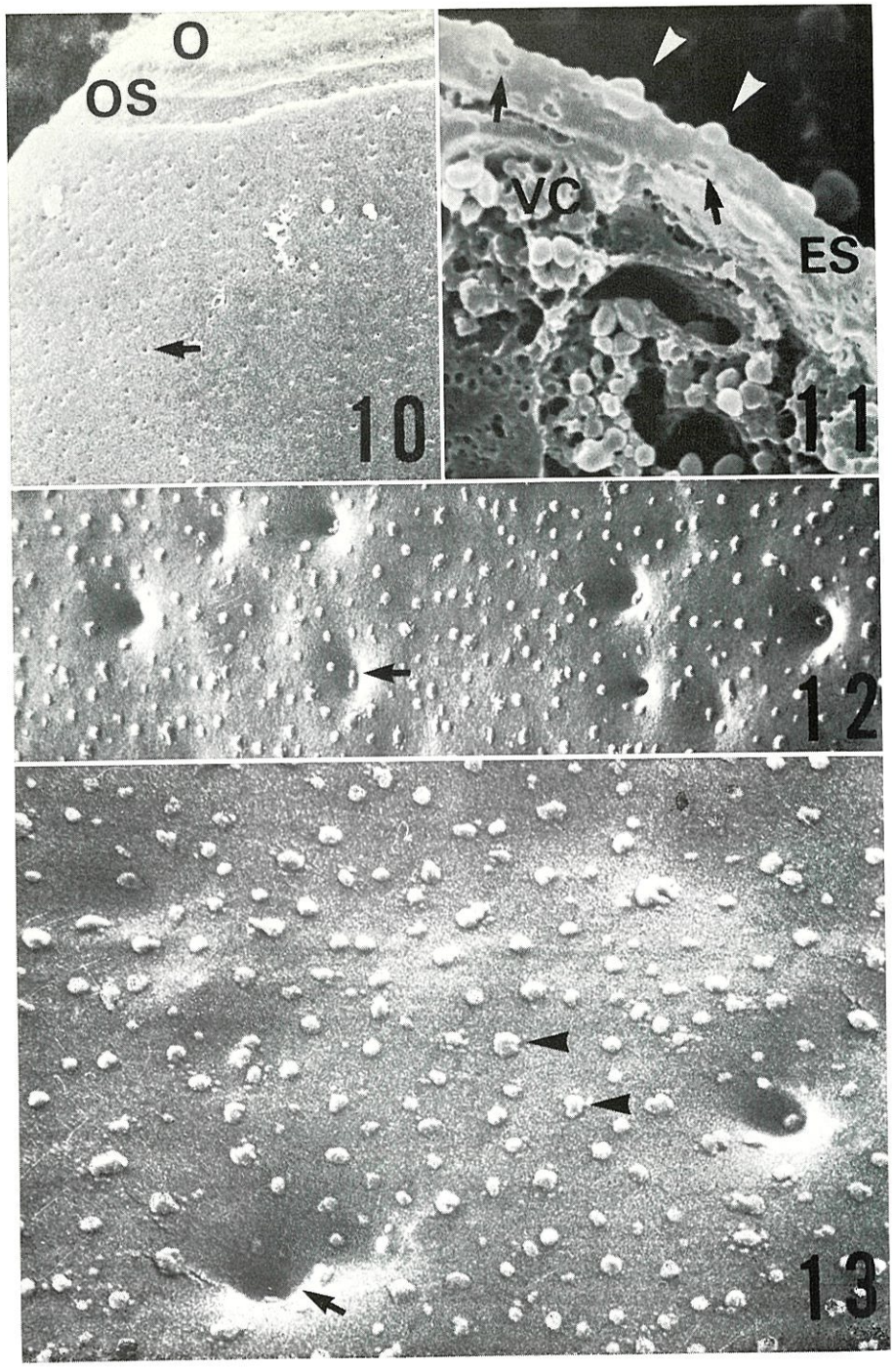
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Explanation of Figures

Fig. 1 The round globules (G) and polygonal globules (arrows) were densely packed around the nucleus (N) in the cytoplasm of the vitelline cells. (×16,000)

Fig. 2 The cross fracture of the vitelline reservoir (VR) in which many vitelline cells (arrows) were stored. Note the numerous military globules were densely packed and a part of them burst into the vitelline reservoir. (×3,000)

Fig. 3 The primitive eggshells (ES) were formed around the ova (OV) with the eggshell precursors of globules at an ootype. (×1,000)

Fig. 4 The fine porous network (arrowhead) wrapped an ovum (OV) and many globules (G), showing the beginning of eggshell formation. (×8,800)

Fig. 5 The primitive eggshell revealed the coarse, fibrous texture (ES), showing the globules (G) which burried themselves into the crevices on the eggshell (arrows). (×24,000)

Fig. 6 Th pores on the eggshell (ES) surface were almost filled up with globules (G) of the vitelline cells, showing uneven, granular surface pattern (arrows). (×15,600)

Fig. 7 The eggshell (ES) which completed a thicker shell, showing polygonal geometrical surface pattern (arrowheads). (×15,000)

Fig. 8 An egg (E) in the uterus (U) which were burried in the numerous vitelline cells (VC). (×3,000)

Fig. 9 The surface pattern of an egg (E), showing shallow, small pits (arrowheads) and furrows. Some granules (arrow) in the vitelline cells (VC) sealed the operculum (O) and the opercular suture (OS). (×9,000)

Fig. 10 The surface pattern of a developed egg, showing the operculum (O), the opercular suture (OS) and the surface pits (arrows). (×6,000)

Fig. 11 The fracture of an eggshell (ES), showing pores (arrows) in the eggshell wall, patched globules on the surface (arrowheads) and globules of vitelline cells (VC) inside of the eggshell. (×6,600)

Figs. 12, 13 Higher magnification of the pits (arrow) and eggshell surface of the developed eggs, showing numerous patches (arrowheads) on the eggshell surface. (×42,000; ×80,000)