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Electron Microscopic Study on Adhesive Material of Pacific Herring (*Clupea pallasi*) Eggs

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Abstract A large number of electron dense granules less than 2 μm in diameter were found in granulosa cells in the pre-ovulatory follicles of the Pacific herring, *Clupea pallasi*, except in the region of the micropylar funnel where a micropylar cell and granulosa cells overlying the funnel were almost devoid of the granules. Granulosa cells in the post-ovulatory follicle had lost a large majority of cytoplasmic granules. On the chorion of ovulated eggs, a thin, electron dense layer newly occurred. This layer was sticky, and covered the whole surface of the chorion except for the micropylar region. These observations indicate that cytoplasmic granules existing in pre-ovulatory granulosa cells may be the origin of the adhesive material of the herring eggs. The micropylar region of ovulated eggs was, on the other hand, covered with two kinds of materials which seem to have been secreted from micropylar and granulosa cells covering the micropylar region. It is suggested that a micropylar cell and granulosa cells in the micropylar region are probably involved in the formation of material which may favor efficient fertilization of eggs in the herring.

It is well known that the chorion of ripe eggs of teleosts is often covered by adhesive material or adhesive fibrils, which have a role in attachment of the egg to various objects (see Laale, 1980). Eggs that are easily dislodged may sink into less desirable conditions, and a mechanism that produces an extremely adhesive membrane could enhance survival of the species (Shelton, 1978). In spite of the importance of adhesive devices of teleostean chorion, we have as yet little information as to what is involved in their formation.

Ripe eggs of the Pacific herring, *Clupea pallasi* Valenciennes, show high adhesiveness (Kanoh, 1949a, b, 1951). Kanoh (1949a, b) described the herring chorion as consisting of three layers including an outermost layer, which was sticky despite the fact that the layer did not show viscous properties in pre-ovulatory follicles. The present ultrastructural study was designed to ascertain the origin and distribution of the adhesive material on the chorion of the herring eggs.

Material and methods

The Pacific herrings, *Clupea pallasi*, used in the present study were caught in late April 1981 by a fixed net in Lake Notoro facing the Sea of Okhotsk, northern Hokkaido. They had migrated from the sea into the brackish lake for spawning. Five mature females with pre-ovulatory follicles in their ovaries, and three females containing ripe eggs in their ovarian cavities, were used in the study.

After the sacrifice by decapitation, pre-ovulatory follicles were carefully removed from the ovary and fixed in glutaraldehyde (5%)-paraformaldehyde (3.7%) mixture in a 0.2 M cacodylate buffer. Ripe eggs were stripped out directly into the fixative, and after squeezing them from ovaries, post-ovulatory follicles were fixed in the same fixative. After fixation for one or two days at room temperature, the specimens were post-fixed in 1% osmium tetroxide in the same buffer for 2 hours at 4°C, and embedded in Epon. Ultrathin sections stained double with uranyl acetate and lead citrate were observed with a Hitachi HU-12 electron microscope. Parallel sections of about 1 μm thick were stained with methylene blue for light microscopic observations.

In the present study, the term "chorion" is employed to indicate the envelopes surrounding ovulated eggs and the term "vitelline envelope" to indicate the envelope of oocyte before ovulation, in accordance with the proposal by Dumont and Brummett (1980).

Observations

Pre-ovulatory follicles. Female herring in the pre-ovulatory stage had ovaries containing two groups of oocytes; a group of oocytes of larger size was at the migratory nucleus stage, and the other groups of oocytes of smaller sizes were at the peri-nucleolus or less advanced stages.

The larger-sized oocytes, averaging 900 μm in diameter, were filled with a large quantity of minute yolk globules together with cortical alveoli arranged peripherally in the ooplasm (Fig. 4). The vitelline envelope of the oocytes was 45~50 μm in entire thickness and consisted of four distinct layers showing different stainability to methylene blue in 1 μm sections. The outermost layer, about 15 μm in thickness, which was described as the adhesive layer by Kanoh (1949a, b) and Yamamoto (1955), showed a palisade structure in cross sections because of prominent perforation of pore canals through the layer. This layer decreased in height gradually toward the outer orifice of the micropylar canal. Underlying this layer, there was a thin, darkly stained layer of about 1.2 μm thick. Inward from this layer, there were two layers measuring about 30 μm in total thickness which contained a series of alternating light and dark laminae (Figs. 1, 4).

The vitelline envelope was surrounded with a single layer of granulosa cells except for the micropyle area where the cells often formed two or more layers (Figs. 1, 4). The nuclei of the single-layered granulosa cells were ovoid in shape, often with irregular outlines (Fig. 2). Well-developed cisternae of the rough endoplasmic reticulum were usually lamellar and occasionally vesicular in form, and low electron dense material was contained in their cisternae. The Golgi apparatus consists of several long lamellae, a few vacuoles and many small vesicles. The most prominent feature of these cells was that they had a great number of granules measuring less than 2 μm in diameter and showing a strong affinity for methylene blue in their cytoplasm (Figs. 1, 2). The granules were round, oval or crescent in shape (Fig. 3). Most of the granules had a tight limiting membrane, and were made up of two or three concentric layers of different electron densities which tended to become progressively less electron dense toward the outer layer. The contents of the granules seemed to be highly condensed. Besides these, large granules with amorphous material of low electron density also appeared in the cytoplasm. They had generally homogeneous contents, but some of them had electron dense cores of irregular shapes and sizes (Fig. 2). The limiting membrane of such granules was often shown to be undulate (Fig. 3). In some instances Golgi vesicles containing opaque or highly electron dense material appeared to fuse with others to form larger vesicles or vacuoles (Fig. 3).

As reported previously (Ohta and Takano, 1982), the granulosa cells overlying the micropylar funnel in pre-ovulatory follicles were fairly large in size compared with those surrounding the other regions of the oocyte surface, and were mostly lacking in cytoplasmic granules in their cytoplasm (Fig. 4). The area occupied by these cells was confined within a radius of about 50 μm from the micropylar canal. In the granulosa cells outside the micropylar region, cytoplasmic granules were abundant, though they were rather small in number in the cells neighboring that region.

Post-ovulatory follicles. There existed a large number of post-ovulatory follicles and oocytes at the peri-nucleolus or less advanced stages in the ovaries examined after stripping them of ovulated eggs. Some of the post-ovulatory follicles possessed clear follicular lumina (Fig. 5), but in others follicular lumina were observed to be nearly absent due to complicated foldings of the granulosa cell layer.

Granulosa cells were columnar in shape, and had oval nuclei and large mitochondria with tubular cristae (Fig. 6). The cells also had dilated rough endoplasmic reticulum, which varied in shape and contained amorphous material of low electron density, and Golgi apparatus with many small vesicles and large vacuoles. Most of the granulosa cells lacked the electron dense granules that were prominent in the pre-ovulatory stage (Figs. 5, 6), though a few of the cells maintained a small number of highly electron dense granules.

Several follicular lumina contained a small quantity of dense material which seemed to be similar in appearance to the material covering the chorion (Fig. 6).

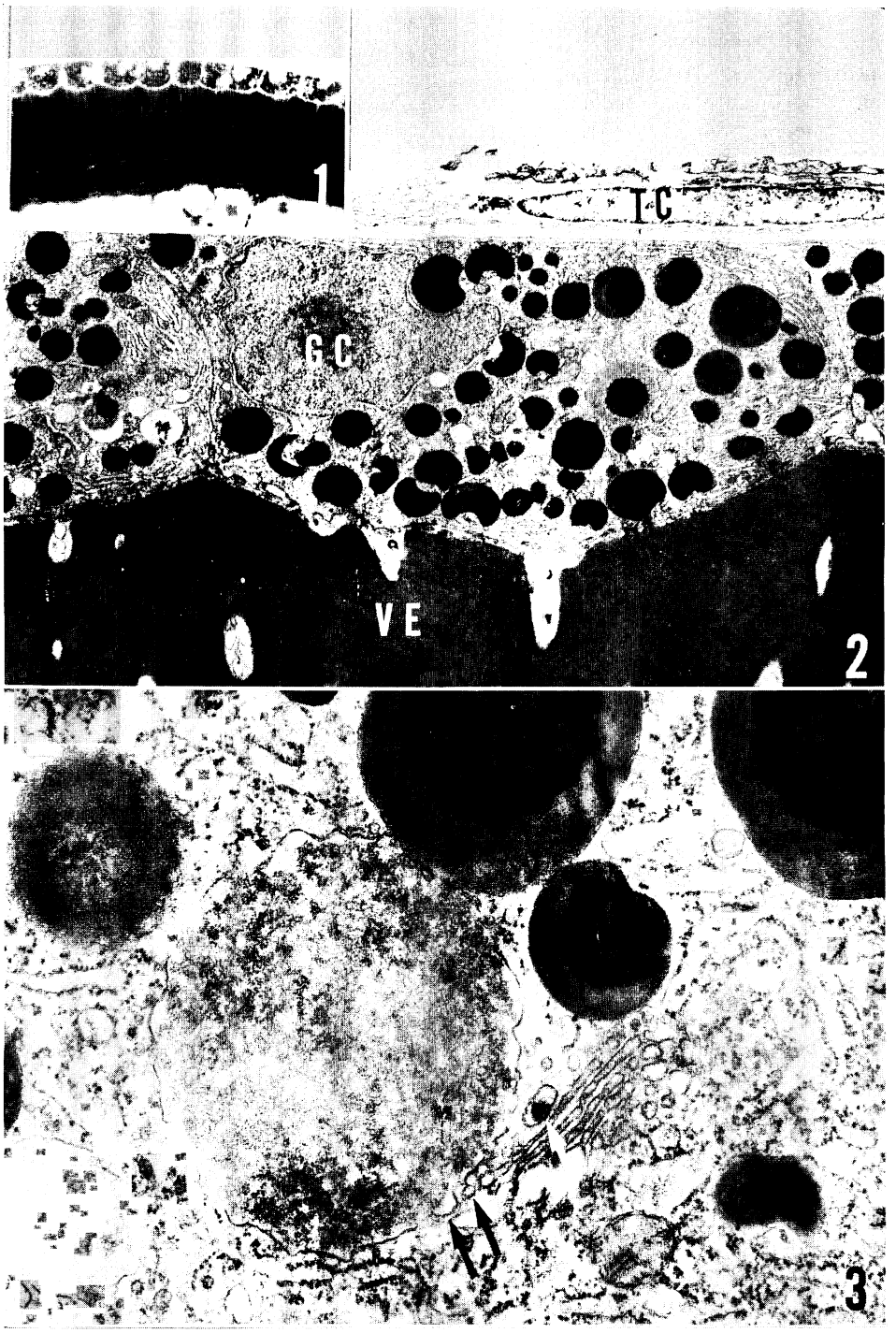

Fig. 1. Granulosa cells in the vegetal pole of a pre-ovulatory oocyte of the herring. Epon section (1 μm), methylene blue staining. ×320.
Fig. 2. Electron micrograph of granulosa cells (GC) in the vegetal pole area. TC, thecal cell; VE, vitelline envelope. ×5,600.
Fig. 3. Golgi apparatus and low electron dense granules of a portion of a granulosa cell in the vegetal pole area. Golgi vesicles often contain electron dense granules (white arrow). Black arrows indicate the fusion of Golgi vesicles to the low electron dense granule. ×29,700.

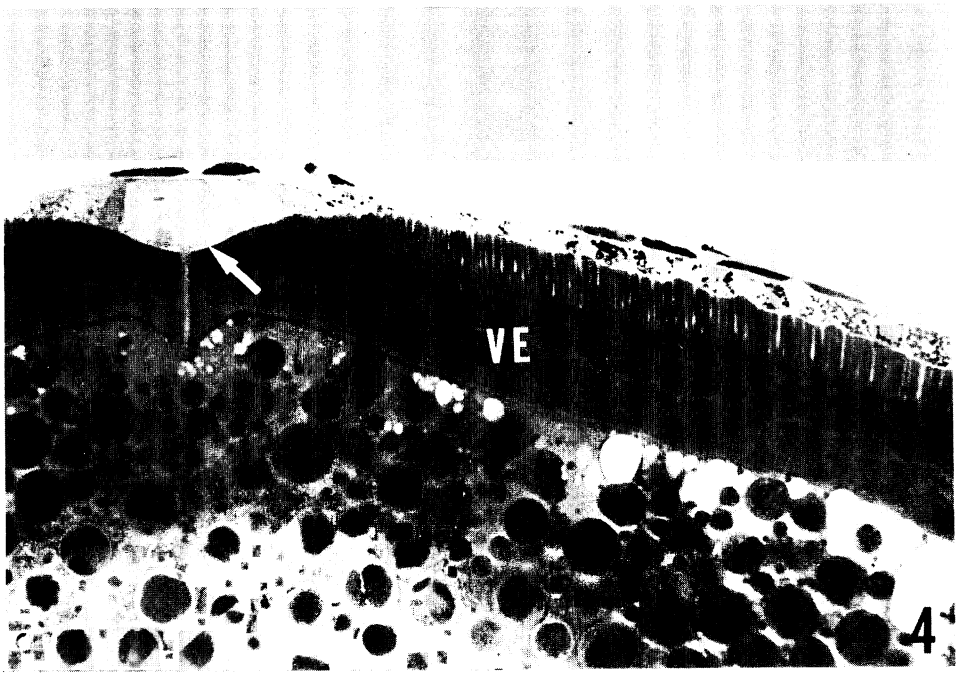

Fig. 4. Micropyle area of a pre-ovulatory oocyte of the herring. The vitelline envelope (VE) shows a shallow funnel-like depression, and granulosa cells in this area have few granules. Arrow indicates a micropylar cell extending a thick cytoplasmic process to the ooplasm. Epon section (1 μm), methylene blue staining. ×430.

Chorion of ovulated eggs. The outermost layer of the chorion of ovulated eggs was newly coated with electron dense material to a thickness of about 30~50 nm. The material was apparently sticky in nature, since the eggs came to adhere tightly to each other when immersed in seawater. This material was present also in depressions on the chorion surface, and reached a depth of 6~8 μm in the pore canal from its outer orifice (Fig. 7). The coating was absent in the animal pole region within a radius of 50 μm from the micropylar canal, and made its gradual appearance on the outside of this region. The location of the material on the chorion accorded well with the distribution of pre-ovulatory granulosa cells with highly electron dense granules in their cytoplasm.

On the other hand, the animal pole region of the chorion was newly covered with two kinds of materials (Fig. 8). One of them was relatively low in electron density and observed to cover the chorion rather homogeneously to a thickness of about 1 μm, while the other was a finely granular material of high electron density, which was scattered over the surface of the former (Fig. 8). The granular material was often aggregated to form a loose mass over the outer orifice of the micropylar canal (Fig. 9). Neither material was present within the micropylar canal or the pore canals (Fig. 9).

Discussion

In teleosts, although considerable attention has been paid to the formation of the vitelline envelope (Yamamoto, 1963; Hurley and Fisher, 1966; Anderson, 1967; Wourms, 1976; Tesoriere, 1977; Dumont and Brummett, 1980), little is known of the process of synthesis and secretion of adhesive material or adhesive chorionic fibrils of fish eggs (Tsukahara, 1971; Wourms and Sheldon, 1976; Busson-Mabillot, 1977; Shelton, 1978). There is general agreement among the authors that the chorion originates from ooplasm and the adhesive devices from granulosa cells. Therefore the vitelline envelope is thought to be a primary egg envelope and adhesive fibrils a secondary egg envelope (Anderson, 1967).

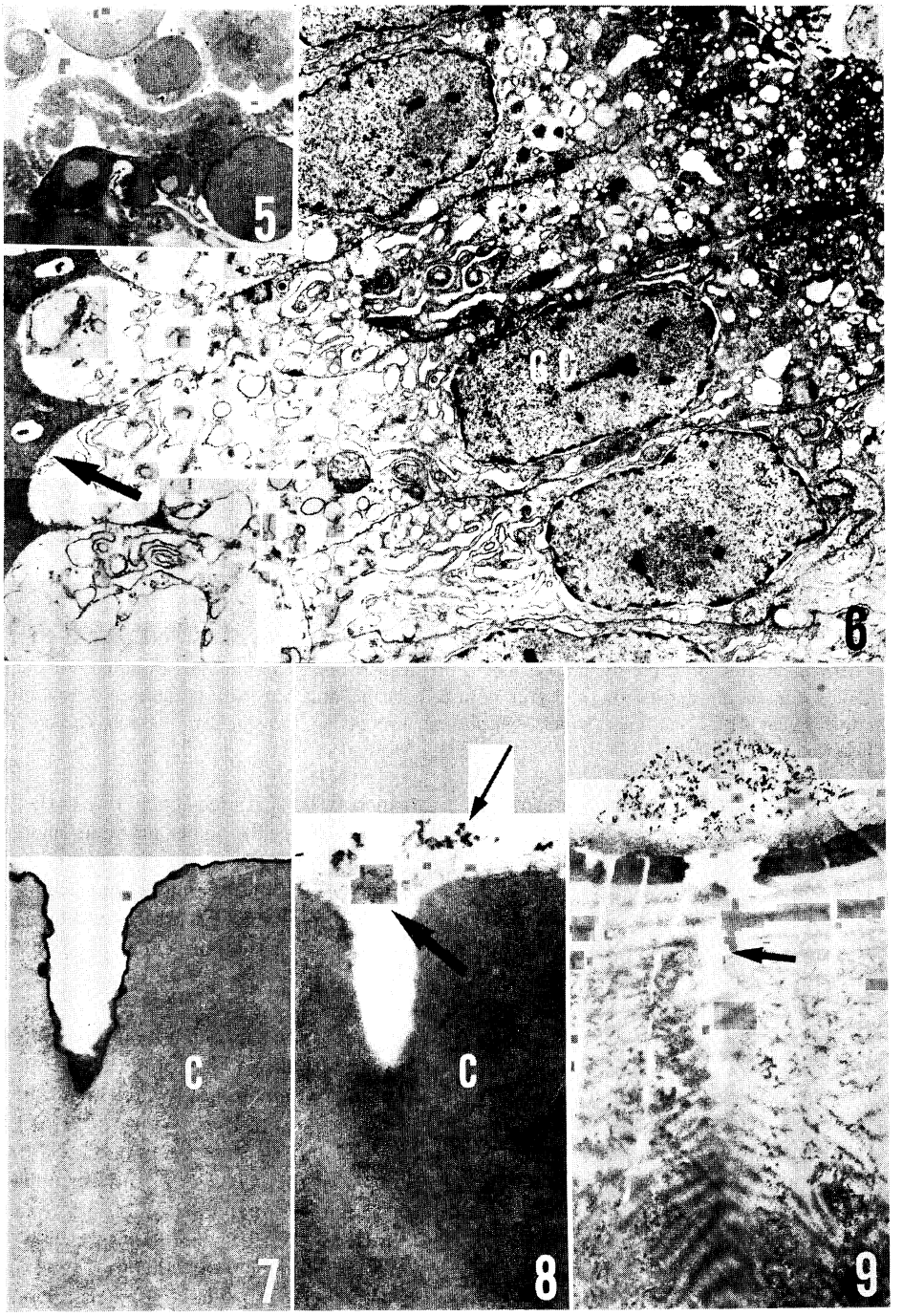

Fig. 5. Post-ovulatory follicle of the herring. Epon section (1 μm). ×120.
Fig. 6. Granulosa cells (GC) in a post-ovulatory follicle. Arrow indicates remnant of possible adhesive material in the follicular lumen. ×4,900.
Fig. 7. The chorion (C) surface of the vegetal area. Electron dense material is observed on the chorion as a thin layer. ×14,600.
Fig. 8. The chorion (C) surface of the micropylar funnel. An inner low electron dense material (large arrow) and an outer electron dense material (small arrow) cover the chorion surface. ×10,600.
Fig. 9. The outer opening of the micropylar canal. The electron dense material accumulates over the opening. Neither material is observed in the micropylar canal (arrow). ×4,500.

The present study indicates that post-ovulatory granulosa cells have lost most of the cytoplasmic granules that were abundant in the cells of pre-ovulatory follicles, and that material of an evidently sticky nature appears on the chorion surface of eggs immediately after ovulation. Further, the material does not exist on the micropylar region of the chorion where overlying granulosa cells generally lack the granules even in the pre-ovulatory follicles. Consequently it is apparent that the granules existing in large quantity in pre-ovulatory granulosa cells are the origin of the adhesive material, and that the contents of the granules are secreted and accumulate on the chorion surface just prior to ovulation. Polder (1961) observed oogenesis of the North Sea herring, *Clupea harengus,* light-microscopically, and found that granules stained dark-blue with Masson's stain were present in granulosa cells of the "Stage IV" oocytes during which the oocytes were actively accumulating yolk material. He also presumed that an adhesive layer was being formed by the secretion of the granulosa cells. The outermost layer of the chorion of the Pacific herring eggs was described as the adhesive layer by Kanoh (1949a, b) and Yamamoto (1955), though Kanoh (1949a) observed that the layer had no viscous property in pre-ovulatory follicles. From the results in the present study, it may be concluded that the "adhesive" layer of the herring eggs becomes functional only after the secretion of the granules from granulosa cells to the chorion surface.

Pre-ovulatory granulosa cells, which had a large number of granules in their cytoplasm, had well-developed rough endoplasmic reticulum and active Golgi apparatus. The Golgi vesicles and vacuoles, often containing electron dense material, were observed to be fused with low electron dense granules. This suggests that the precursor of the granules may be synthesized in the rough endoplasmic reticulum and then concentrated in the Golgi apparatus to form the granules. This interpretation corresponds to the typical scheme of protein synthesis in secretory cells (Caro and Palade, 1964; Jamieson and Palade, 1967). Busson-Mabillot (1977) observed pre- and post-ovulatory oocytes of *Cichlasoma nigrofasciata* ultrastructurally and histochemically, and found that a glycoprotein jelly-coat on the chorion surface was sticky and had an adhesive function in the attachment of eggs to the substrate. He showed that this glycoprotein material was synthesized and accumulated in the rough endoplasmic reticulum, and that an apocrine secretion of the material occurred during ovulation, bypassing the Golgi appparatus. Wourms and Sheldon (1976) also observed the synthesis and secretion of adhesive chorionic filaments in granulosa cells of oocyte follicles in *Cynolebias melanotaenia* and *C. ladigesi,* and similarly described these processes as being independent of the Golgi apparatus. In the present study, the dense granule contents was shown to be synthesized via the Golgi apparatus, though the present study could not determine the secretory process of the granules.

As described before, adhesive material of the herring eggs is absent only on the micropylar region of the chorion. This interesting phenomenon seems to be significant in relation to efficient fertilization since the adhesive material can trap spermatozoa as well. The fact that only micropylar vestibules are devoid of adhesive chorionic fibrils has been shown also in eggs of *Fundulus heteroclitus* (Kuchnow and Scott, 1977; Dumont and Brummett, 1980) and *Oryzias latiples* (Hosokawa, 1979).

Another point of interest is the occurrence of two kinds of materials on the micropylar region of the chorion. Because these materials could not be observed inside the micropylar canal and the pore canals, the materials did not seem to originate from ooplasm but from granulosa cells of the micropyle area containing a micropylar cell in the pre-ovulatory follicles. This view corresponds with the observation that the micropylar cell and the granulosa cells of the micropyle area in the pre-ovulatory follicles of the herring show an active feature of protein synthesis in their ultrastructure (Ohta and Takano, 1982). Although the physiological roles of the materials are as yet unknown, the possibility that they are a sperm activating substance can not be excluded, since a substance with such a function has been suggested to exist around the micropyle in the Pacific herring by Yanagimachi (1957a, b).

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Literature cited

Anderson, E. 1967. The formation of the primary envelope during oocyte differentiation in teleosts. J. Cell Biol., 35: 193～212.

Busson-Mabillot, S. 1977. Un type particulier de sécrétion exocrine: celui de l'appareil adhésif de l'œuf d'un poisson Téléostéen. Biol. Cell., 30: 233～244, pls. 1～5.

Caro, L. G. and G. E. Palade. 1964. Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study. J. Cell Biol., 20: 473～495.

Dumont, J. N. and A. R. Brummett. 1980. The vitelline envelope, chorion, and micropyle of *Fundulus heteroclitus* eggs. Gamete Res., 3: 25～44.

Hosokawa, K. 1979. Scanning electron microscopic observations of the micropyle in *Oryzias latipes*. Japan. J. Ichthyol., 26: 94～99. (In Japanese with English summary).

Hurley, D. A. and K. C. Fisher. 1966. The structure and development of the external membrane in young eggs of the brook trout, *Salvelinus fontinalis* (Mitchill). Can. J. Zool., 44: 173～190.

Jamieson, J. D. and G. E. Palade. 1967. Intracellular transport of secretory proteins in the pancreatic exocrine cell. I. Role of the peripheral elements of the Golgi complex. J. Cell. Biol., 34: 577～596.

Kanoh, Y. 1949a. On eggs of *Clupea harengus* L. Collecting and Breeding, 11: 162～164. (In Japanese).

Kanoh, Y. 1949b. Über den japanischen Hering (*Clupea pallasii* Cuvier et Valenc.). I. Morphologie des reifen Eies. Cytologia, 15: 138～144.

Kanoh, Y. 1951. Zweiartige Adhäsionstypen bei klebrigen Fisheiern. Zool. Mag. (Tokyo)., 60: 65～67. (In Japanese with German résumé).

Kuchnow, K. P. and J. R. Scott. 1977. Ultrastructure of the chorion and its micropyle apparatus in the mature *Fundulus heteroclitus* (Walbaum) ovum. J. Fish Biol., 10: 197～201.

Laale, H. W. 1980. The perivitelline space and egg envelopes of bony fishes: A review. Copeia, 1980: 210～226.

Ohta, H. and K. Takano. 1982. Ultrastructure of micropylar cells in the pre-ovulatory follicles of Pacific herring, *Clupea pallasi* Valenciennes. Bull. Fac. Fish., Hokkaido Univ., 33: 57～64.

Polder, J. J. W. 1961. Cyclical changes in testis and ovary related to maturity stages in the North Sea herring, *Clupea harengus* L. Netherlands J. Zool., 14: 45～60.

Shelton, W. L. 1978. Fate of the follicular epithelium in *Dorosoma petenense* (Pisces: Clupeidae). Copeia, 1978: 237～244.

Tesoriero, J. V. 1977. Formation of the chorion (zone pellucida) in the teleost, *Oryzias latipes*. I. Morphology of early oogenesis. J. Ultrastructure Res., 59: 282～291.

Tsukahara, J. 1971. Ultrastructural study on the attaching filaments and villi of the oocyte of *Oryzias latipes* during oogenesis. Develop. Growth and Differ., 13: 173～180.

Wourms, J. P. 1976. Annual fish oogenesis. I. Differentiation of the mature oocyte and formation of the primary envelope. Develop. Biol., 50: 338～354.

Wourms, J. P. and H. Sheldon. 1976. Annual fish oogenesis. II. Formation of the secondary egg envelope. Develop. Biol., 50: 355～366.

Yamamoto, M. 1963. Electron microscopy of fish development. II. Oocyte-follicle cell relationship and formation of chorion in *Oryzias latipes*. J. Fac. Sci. Univ., Tokyo. (Sect. 4), 10: 123～127, pls. 1～4.

Yamamoto, T. S. 1955. Ovulation in the salmon, herring and lamprey. Japan. J. Ichthyol., 4: 182～192. (In Japanese with English summary).

Yanagimachi, R. 1957a. Studies of fertilization in *Clupea pallasii*. III. Manner of sperm entrance into the egg. Zool. Mag. (Tokyo)., 66: 226～233. (In Japanese with English summary).

Yanagimachi, R. 1957b. Some properties of the sperm-activating factor in the micropyle area of the herring egg. Annot. Zool. Japon., 30: 114～119.

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ニシン卵粘着物質の電顕的観察

太田博巳

ニシン卵は強い粘着性を示す．この粘性物質の起源と排卵された卵の卵膜中での所在部位を明らかにする

目的で，排卵前後の卵濾胞および排卵された卵の卵膜を電顕的に観察した．

排卵前の濾胞の顆粒膜細胞中には，メチレンブルーに濃染され，種々の電子密度を示す直径 2 μm 以下の顆粒が多数存在した．また，この顆粒は卵門細胞とその周囲の顆粒膜細胞には存在しなかった．これに対して排卵後の濾胞の顆粒膜細胞では顆粒が消失しており，卵門部位を除く卵膜表面に電子密度の高い薄層が新たに認められ，この薄層は強い粘性を示した．また，卵門部位の卵膜表面には粘着物質を欠き，これとは異なる物質が観察された．

以上の観察結果より，ニシン卵の粘着物質は，排卵前濾胞の卵門域を除く顆粒膜細胞中に存在する顆粒に起源し，その顆粒は排卵直前に卵膜表面に分泌されることが明らかとなった．

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