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## Ultrastructure of Micropylar Cells in the Ovarian Follicles of the Pond Smelt, *Hypomesus transpacificus nipponensis*

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### Abstract

Morphological changes of micropylar cells during the course of vitellogenesis and maturation of oocytes of the pond smelt, *Hypomesus transpacificus nipponensis*, were examined by electron microscopy. In micropylar cells, a bundle of tonofilaments was well developed throughout the stages of oocyte development examined. An established micropylar cell extended a cytoplasmic process through a micropylar canal, and the apical part of the process extended into the surface of ooplasm. A characteristic of the cell was the bundle of microtubules extending linearly through the cytoplasm of the process. This structure was considered to be concerned with the formation and support of the process. In addition to numerous mitochondria, moderately developed Golgi bodies and dilated cisternae of rough endoplasmic reticulum were observed in the cytoplasm of the micropylar cells in the earlier stages of development of ovarian oocytes. In the later stages, numerous smooth-surfaced vesicles were noticed. Possible functions of the micropylar cells are discussed in connection with the development of these cytoplasmic organelles.

### Introduction

It is generally accepted that in teleost eggs, spermatozoa can reach the surface of ooplasm through a micropylar opening located at the animal pole of the egg membrane. The micropyle is occupied by a micropylar cell until matured oocytes are discharged from their follicular envelopes at the time of ovulation. Many studies focused on the light microscopic morphology of micropyles and micropylar cells, which show different morphological aspects in different species of fishes (cf. Riehl and Götting<sup>1</sup>), Laale<sup>2</sup>).

In recent years, many observations on the ultrastructure of micropyles have been made in teleost fishes such as the rainbow trout<sup>3</sup>), *Fundulus*<sup>4),5)</sup>, the starry flounder and the pink salmon<sup>6</sup>), *Oryzias*<sup>7</sup>), some salmonids<sup>8</sup>), the common carp<sup>9</sup>), and the porgy<sup>10</sup>). There is, however, little ultrastructural information on the origin and morphogenesis of micropylar cells except for Riehl's study on *Noemacheilus* and *Gobio*<sup>11</sup>). This lack may be attributed to the difficulty of detecting a single, small micropylar cell in each ovarian follicle, especially in its early stages of development. In the pond smelt, *Hypomesus transpacificus nipponensis*, it has been known that an outer adherent membrane overlies the egg membrane along its animal hemisphere<sup>12</sup>). As the micropyle occupies the central portion of the adherent membrane, it is rather easy to search for micropylar cells in this fish

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in order to make ultrastructural studies on the cells.

The present study deals with observations on ultrastructural changes of micropylar cells during the course of vitellogenesis and maturation in the pond smelt.

### Material and Methods

The pond smelt, *Hypomesus transpacificus nipponensis* McAllister, used in this study were captured from Lake Onuma in the vicinity of Hakodate, southern Hokkaido. In this lake, the fish begin to accumulate yolk globules in their oocytes in October, and spawning takes place in early April. In addition, some samples were obtained from stocks of fish which had been maintained in outdoor ponds during the winter. Ovaries were taken from freshly captured females monthly from October 1978 to April 1979. Small pieces of the ovary were prefixed with Karnovsky's glutaraldehyde-paraformaldehyde mixture in 0.2 M cacodylate buffer (pH 7.4) for about 3 hours at room temperature, and postfixed in 1% osmium tetroxide in the same buffer for about 2 hours at 4°C. After dehydration through a graded ethanol series, the specimens were embedded in Epon. Ultrathin sections were stained with uranyl acetate and lead citrate, and examined with a Hitachi HU-12 electron microscope. Parallel sections of the Epon-embedded ovaries of about 1  $\mu$ m thick were stained with methylene blue for light microscopy.

### Results

Females captured in early October had ovaries containing many oocytes, averaging 280  $\mu$ m in diameter, in the primary yolk stage. In the peripheral ooplasm, a few minute yolk globules with high electron density were detectable among the yolk vesicles which were spherical and filled with electron-lucent material. On the outer surface of the oocyte, a dense, homogeneous material was deposited to form the future outer layer of the vitelline envelope.\* Short microvilli of the oocyte perforated this thin layer (Fig. 1b).

The micropylar cell was first observed ultrastructurally among granulosa cells which were arrayed as a single layer of densely packed cuboidal cells. In semithin sections, the micropylar cell was distinguished from neighbouring granulosa cells by its large size and its limited stainability with methylene blue (Fig. 1a). The large nucleus which occupied the center of the cell showed a somewhat irregular shape with depressions of the nuclear envelope. Ultrastructurally, the most prominent feature was a large cluster of tonofilamentous bundles in contact with the inner surface of the nuclear depressions. Although similar tonofilamentous bundles were also observed in the granulosa cells, they were poorly developed. Mitochondria were round to oval, sometimes elongated in shape, and scattered throughout the cytoplasm. Their cristae were irregularly elongated and often tubular in form. The cisternae of the rough endoplasmic reticulum were slightly developed in

\* In this study, the term 'vitelline envelope' was employed, in accordance with the proposal by Dumont and Brummet<sup>5)</sup>, to indicate the structure of the developing envelope of oocytes.

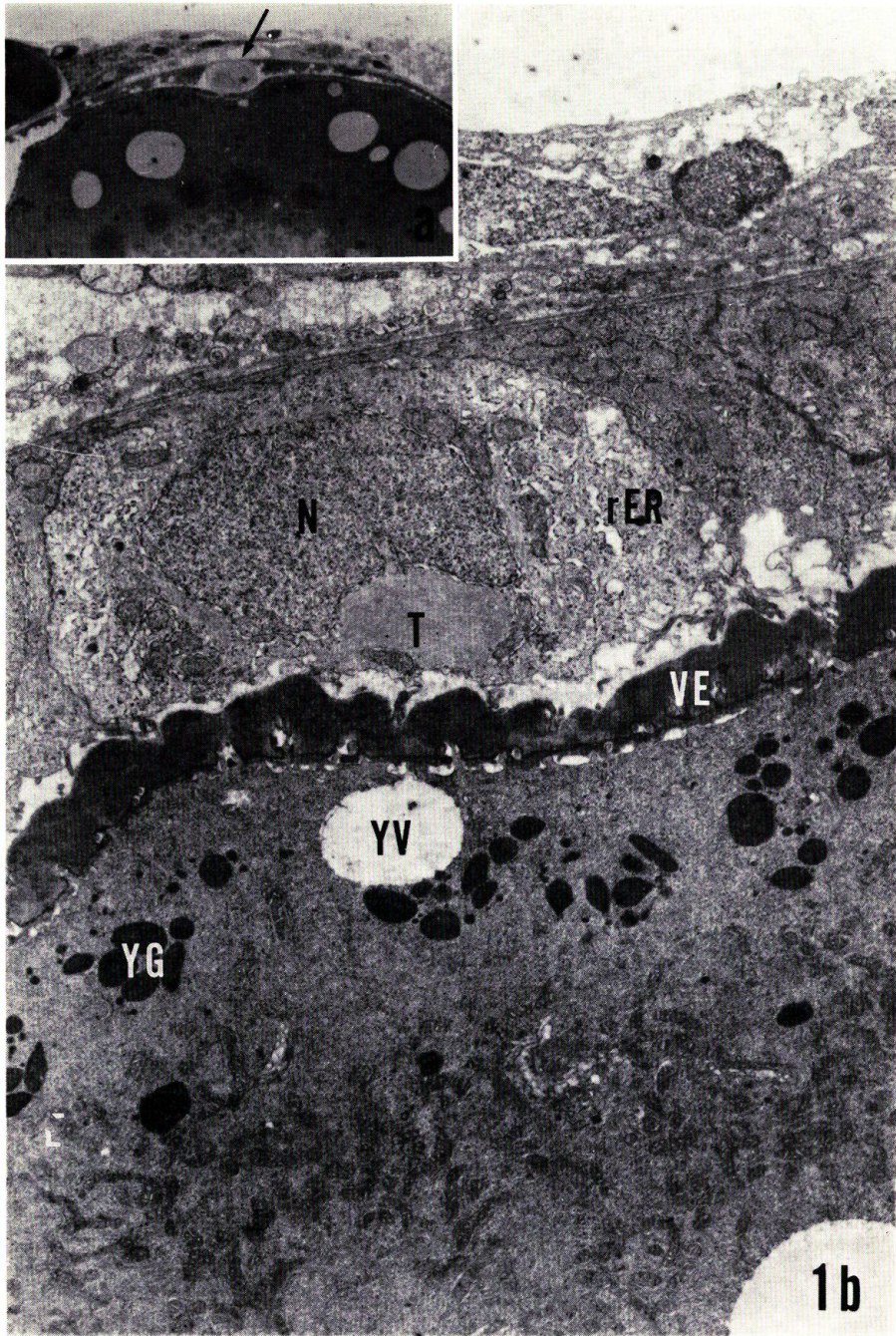
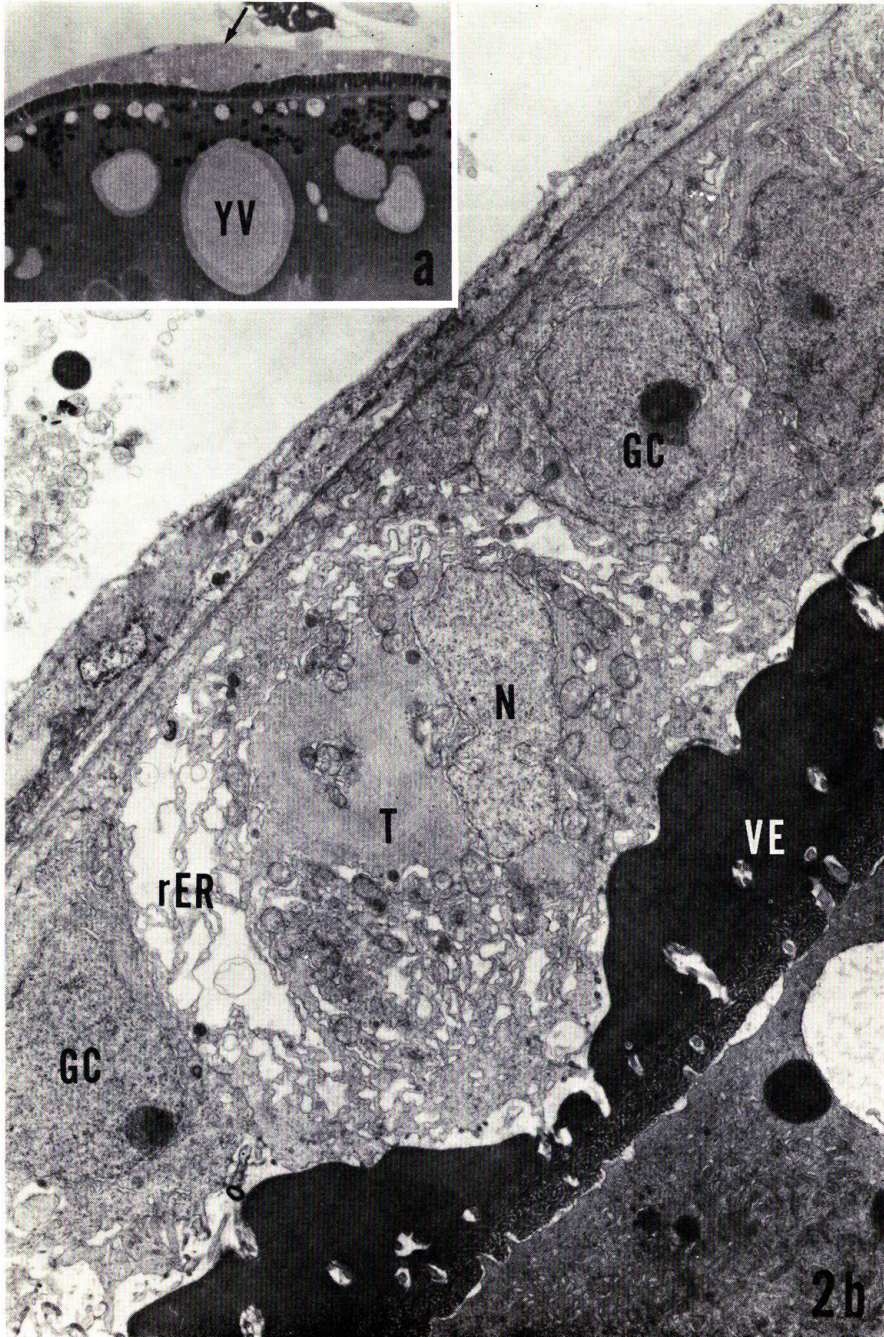


Fig. 1. Animal pole of an oocyte of a smelt collected in October. A micropylar cell (arrow) is found among granulosa cells (Fig. a). A large nucleus (N), a cluster of tonofilamentous bundles (T), and slightly developed rough endoplasmic reticulum (rER) are found in the micropylar cell (Fig. b). VE, vitelline envelope; YG, yolk globule; YV, yolk vesicle. a,  $\times 80$ ; b,  $\times 7,400$ .



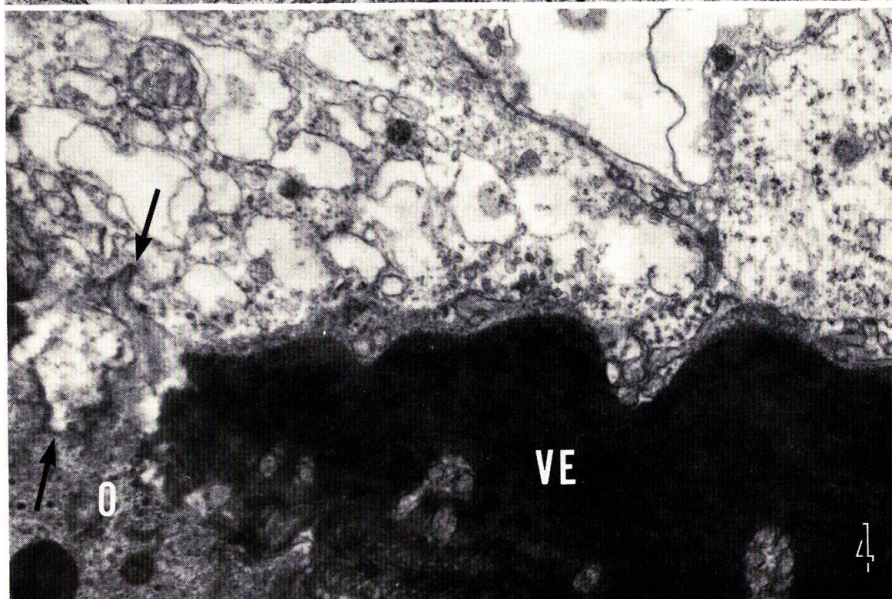
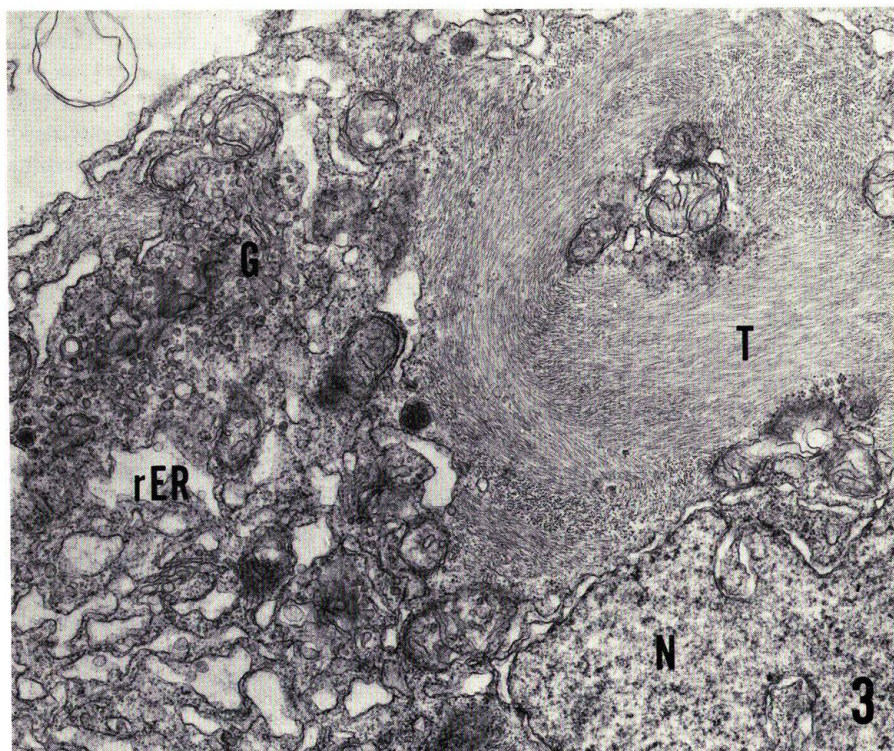
contrast to those of the granulosa cells. Moderately developed Golgi bodies and a large number of free ribosomes were also observed in the cytoplasm. The inner surface of the micropylar cells was indented and provided with many microvilli projecting into a narrow space between the granulosa cell layer and the vitelline envelope (Fig. 1b). Although a cytoplasmic process was shown by light microscopy to extend through a pore of the thin vitelline envelope at this stage of development (Fig. 1a), this profile could not be observed by electron microscopy.

Oocytes obtained in early November were at almost the same developmental stage as in October, but were larger in size, averaging  $300\ \mu\text{m}$  in diameter. Yolk globules and yolk vesicles increased in size and number in the peripheral ooplasm, and oil droplets occupied the peri-nuclear ooplasm. The vitelline envelope surrounding the oocyte not only increased in thickness, but showed a two-layered organization in this stage (Figs. 2a, b); its inner layer was of uniform thickness and was composed of loosely packed bundles of filaments covering the whole surface of the ooplasm, while the outer layer was homogeneous with high electron density and decreased in thickness gradually from the animal pole to the opposite pole. The border between the outer and the inner layer was obscure in this stage.

During this period, micropylar cells were detected among granulosa cells at the animal pole where the vitelline envelope formed a shallow cup-like depression (Figs. 2a, b). A large array of tonofilamentous bundles occupied the central portion of the micropylar cell (Figs. 2b, 3). The cisternae of the rough endoplasmic reticulum were widely dilated and contained electron-lucent material. The Golgi bodies consisted of several long lamellae and many small vesicles with moderately electron-dense contents (Fig. 3). Small fractions of microtubules were detected in the distal area of cytoplasmic expansion which ended on the surface of the cup-like depression of the envelope. In another ultrathin section, however, the apex of the cytoplasmic expansion was observed to attach directly and to interdigitate with the surface of the ooplasm through an orifice of the vitelline envelope (Fig. 4), but microtubules were not detected in that region. The inner surface of micropylar cells showed marked indentations with several microvilli projecting into pore canals of the vitelline envelope. The outer surface of the cells, on the other hand, was smooth and made direct contact with the thecal layer without the intervention of the overlying granulosa cells (Fig. 2b).

In December, many oocytes of the secondary yolk stage appeared in the ovary. The diameter of oocytes increased to about  $360\ \mu\text{m}$ , and the accumulation of yolk globules proceeded centripetally, spreading to the peri-nuclear zone. The outer layer of the vitelline envelope was absent in the area near the vegetal pole, while the inner layer covered the whole surface of the oocyte. The micropylar cell occupied a typical funnel-shaped cavity and was covered with a layer of granulosa cells. A micropylar canal terminated internally on the surface of the ooplasm at the bottom of the cavity (Fig. 5).

Fig. 2. Animal pole of an oocyte of a smelt collected in November, showing an enlarged micropylar cell (arrow) (Fig. a). The vitelline envelope (VE) shows a two-layered organization and a shallow cup-like depression. The micropylar cell is characterized by an eccentric nucleus (N), widely dilated rough endoplasmic reticulum (rER), and tonofilamentous bundles (T) (Fig. b). GC, granulosa cell; YV, yolk vesicle; VE, vitelline envelope. a,  $\times 80$ ; b,  $\times 5,500$ .



In the cytoplasm of the micropylar cells, thick bundles of tonofilaments developed consecutively around the nucleus. Mitochondria and moderately expanded cisternae of rough endoplasmic reticulum were found in the supra- and para-nuclear region of the cells. In addition to these organelles, numerous smooth-surfaced vesicles of irregular shape occupied the cytoplasm immediately beneath the nucleus adjacent to the ooplasm. Through the micropylar canal, an enlarged cytoplasmic process extended to the surface of the ooplasm (Fig. 5). The most conspicuous feature was a bundle of microtubules, about 25 nm in diameter, in the cytoplasmic process. The microtubules were arrayed parallel to the long axis of the process, and spread into the cytoplasm of the basal region of the process (Figs. 5, 6a). A centriole-like structure was detected in the apical portion of the process. Around the bundle of microtubules, numerous smooth-surfaced vesicles of various sizes were arranged in the cytoplasm, which was without other organelles. Often some of them were closely attached to the cytoplasmic membrane which showed flask-like invaginations. The apical part of the process was wedged into the ooplasm. No special junctional apparatus could be found between the apical surface of the process and the ooplasmic membrane (Fig. 6b).

During the long freezing season from middle December to early April, oocytes grew slowly with an accumulation of yolk material. In March, oocytes enlarged, measuring about 500  $\mu\text{m}$  in diameter, and were filled with a large bulk of yolk globules. The two layers of the vitelline envelope were now distinctly separated by a high electron-dense material deposited between them (Figs. 7a, b). The outer layer, corresponding to the adherent membrane, covered about two-thirds of the whole surface of oocyte. This layer, about 12  $\mu\text{m}$  in thickness at the animal pole, was homogeneous and moderately dense. The inner layer, also about 12  $\mu\text{m}$  in thickness, which invested the whole surface of the oocyte, was rather coarse and moderate in density.

The micropylar cell was covered by two or more layers of granulosa cells which were characterized by a rather distended, rough endoplasmic reticulum. The enlarged process of the micropylar cell extended through the micropylar canal, and a bundle of microtubules extended smoothly through the cytoplasm of the process. The cytoplasmic organelles of the micropylar cells showed no conspicuous changes compared with the previous stage, except for the appearance of an abundance of smooth-surfaced vesicles (Fig. 7b).

Finally, micropylar cells were observed in oocytes which had been incubated for 6 hours in Ringer's solution containing progesterone (0.1  $\mu\text{g}/\text{ml}$ ) for the induction of *in vitro* maturation of oocytes (Yamauchi et al., unpublished). In the micropylar cells of the mature oocytes just before ovulation, it was noted that a bundle of microtubules became markedly loose and meandered through the cytoplasm of the enlarged cytoplasmic process (Fig. 8). Meandering bundles of

Fig. 3. A part of a micropylar cell, from the same section as showed in Fig. 2. Well-developed tonofilamentous bundles (T), dilated cisternae of rough endoplasmic reticulum (rER), and Golgi bodies (G) are found in cytoplasm. N, nucleus.  $\times 14,800$ .

Fig. 4. A part of a micropylar cell found in ovarian follicle in November. An apex of cytoplasmic expansion of the cell interdigitates with the surface of ooplasm (O) (arrows) through an orifice of vitelline envelope (VE).  $\times 11,800$ .

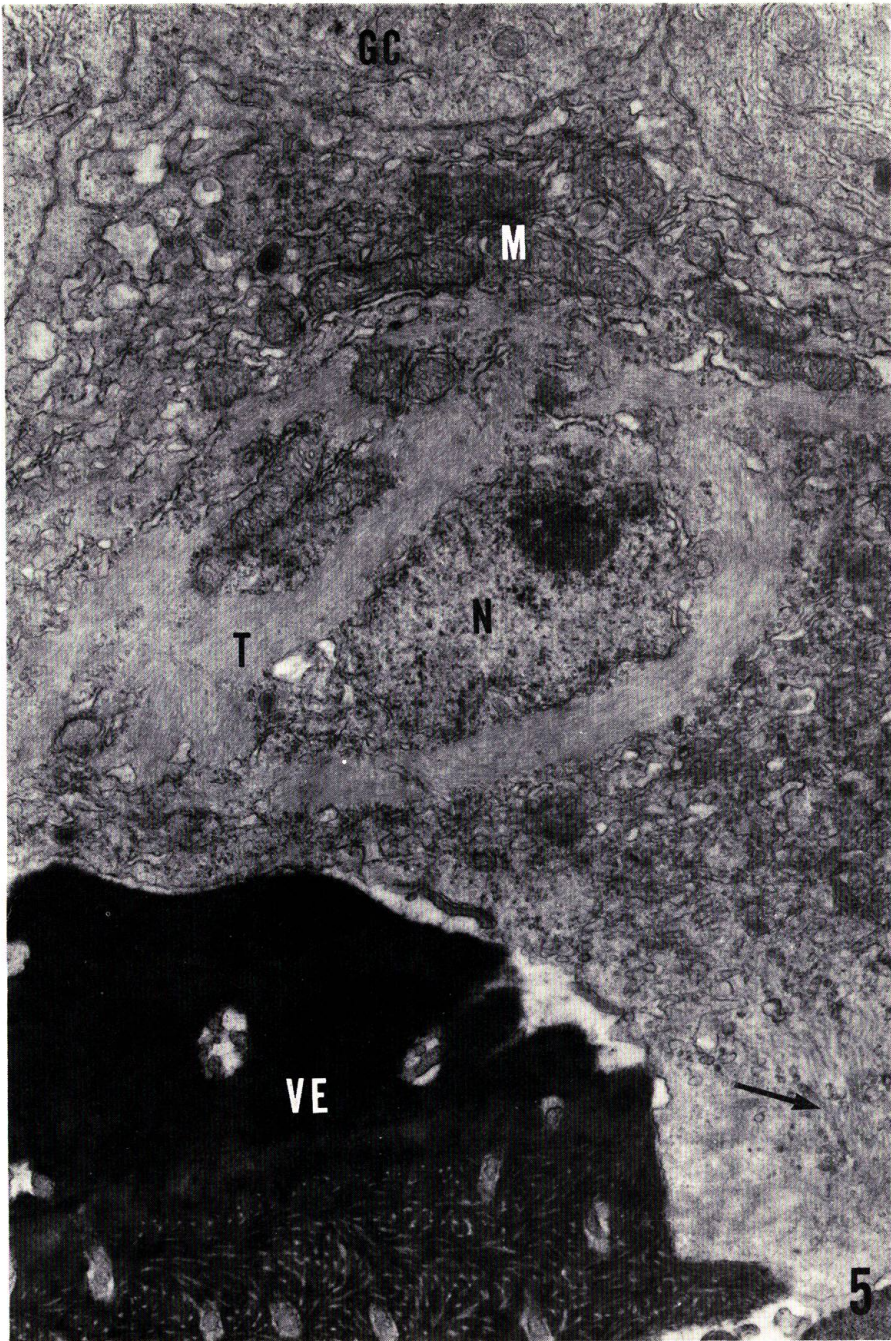


Fig. 5. A part of a micropylar cell found in ovarian follicle in December. Thick bundles of tonofilaments (T) have developed around the nucleus (N), and numerous smooth-surfaced vesicles are abundant in cytoplasm beneath the nucleus. Note a bundle of microtubules (arrow) in an enlarged process extending through the micropylar canal. GC, granulosa cell; M, mitochondria; VE, vitelline envelope.  $\times 12,000$ .

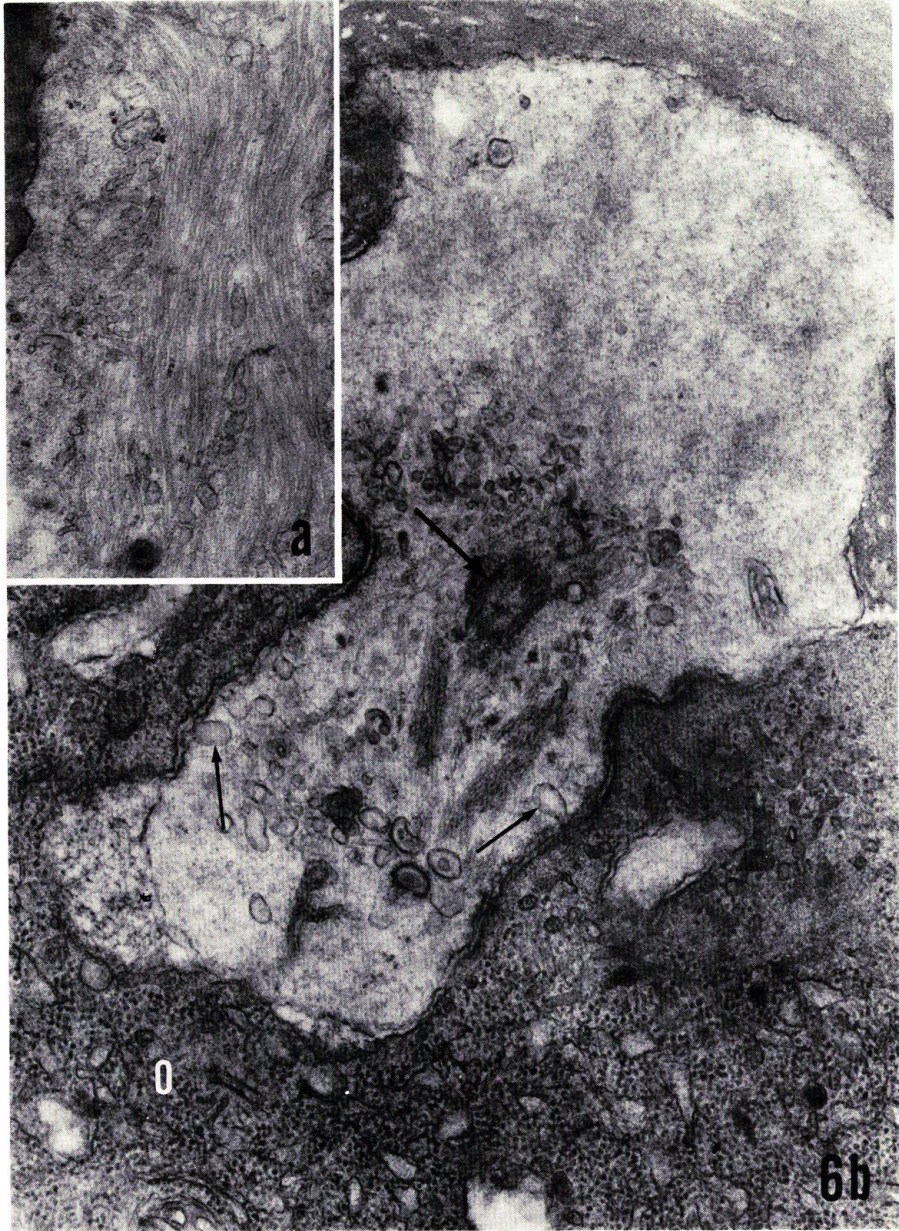
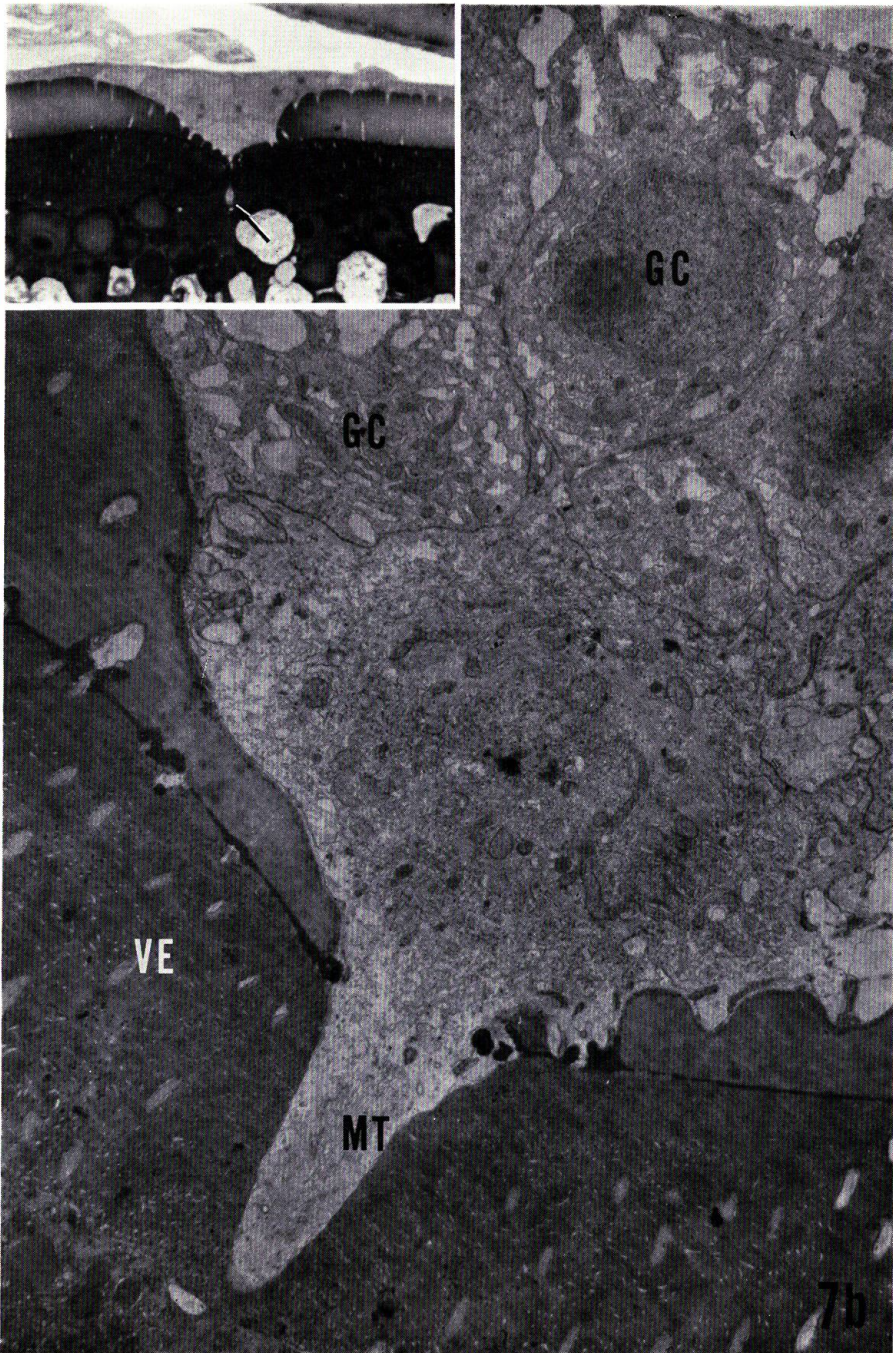


Fig. 6. Microtubules arranged parallel to the long axis of the process (Fig. a). A centriole-like structure (large arrow) and smooth-surfaced vesicles showing flash-like invaginations (small arrows) are found in the apical part of the process extending into the ooplasm (O) (Fig. b). a,  $\times 30,000$ ; b,  $\times 33,300$ .



microtubules with similar features were also observed in the oocytes obtained in March in vivo, though they were not so loose as those of oocytes in vitro. Just before ovulation, the internal opening of the micropylar canal was about  $2\ \mu\text{m}$  in diameter.

### Discussion

In the first demonstration of micropyles in the ovary of teleost fishes by transmission electron microscopy, Riehl<sup>11)</sup> found the micropyle established in the late stage of yolkless oocytes of *Noemacheilus barbatulus*. He described that the micropyle was formed by a modified follicle cell ("Zapfenzelle") as indicated in many earlier light microscopic studies (cf. Laale<sup>2)</sup>). In the pond smelt, micropylar cells were first observed ultrastructurally in oocytes at the early stage of vitellogenesis. The cells were distinguished from granulosa cells on the basis of their larger cellular and nuclear sizes. No essential differences in cytoplasmic organelles, however, were noted between the micropylar cells and the granulosa cells at this stage of oocyte development, except for the greater abundance of tonofilaments in the former. Although this feature suggested that the micropylar cell might be derived from a modified granulosa cell, we could not obtain clear evidence for the origin of the micropylar cell in this study.

The large mass of tonofilaments detected in the micropylar cells at the earliest stage examined continued to increase until the final stage of oocyte maturation. Tonofilaments are generally accepted to act as a kind of cytoskeleton, observed universally in various epithelial cells. The unusual development of the tonofilamentous bundles in the micropylar cells of the pond smelt might, therefore, play an important role in supporting the great bulk of the cells.

A cytoplasmic expansion which was regarded as a cytoplasmic process was observed in semithin sections to project from micropylar cells of oocytes at the primary yolk stage. In *Noemacheilus barbatulus*, Riehl<sup>11)</sup> found that a plug-like process projecting toward the ooplasm pushed in the cortex radiatus of the oocytes at stage II, the yolk vesicle stage of development. In the pond smelt, a clear, enlarged cytoplasmic process was found ultrastructurally to pass through the micropylar canal in oocytes at the secondary yolk stage. Its most characteristic feature was a well-developed bundle of microtubules extending linearly along the long axis of the process. This structure was maintained until just before ovulation. It is generally agreed that microtubules may function as the principal cytoskeleton in the formation and support of the asymmetrical contours of cells<sup>13)</sup>. The microtubules of the micropylar cells observed in this study may, therefore, act as a cytoskeleton which supports the cytoplasmic process extending into the ooplasm from the cells. Although detailed information about the ultrastructural characteristics of micropylar cells at earlier stages could not be obtained in this study, it is presumed that microtubules may develop in close parallel with the extension of the

Fig. 7. Animal pole of an oocyte of a smelt collected in March. Arrow indicates an apex of the process of the micropylar cell (Fig. a). Inner and outer layers of vitelline envelope (VE) are separated by a thin, electron-dense layer. The micropylar cell is covered by two or more layers of the granulosa cells (GC) (Fig. b). MT, a bundle of microtubules. a,  $\times 80$ ; b,  $\times 4,500$ .



Fig. 8. A part of a micropylar cell observed in an ovarian follicle just before ovulation. Note the bundle of microtubules which is loose and meanders in the cytoplasm of the process (arrow). GC, granulosa cell; O, ooplasm; VE, vitelline envelope.  $\times 7,400$ .

cytoplasmic process during the course of the gradual increase in thickness of the vitelline envelope. In any case, the process extended into the ooplasm is important in anchoring the micropylar cell to the bottom of the micropylar cavity.

It is also an interesting fact that the bundle of microtubules in the process became markedly loose and meandering at the stage just before ovulation under *in vitro* conditions. This may be a characteristic of the process of the micropylar cell as it emerges from the micropylar canal of the vitelline envelope at the time of ovulation. It is well known, however, that microtubules are unstable with regard to fixation procedures. Further observations on the ultrastructural changes of the micropylar cells during the course of spontaneous ovulation are necessary before drawing any final conclusion.

In addition to the filamentous structures mentioned above, various kinds of organelles such as mitochondria, rough endoplasmic reticulum, smooth-surfaced vesicles, and Golgi bodies were commonly observed in the cytoplasm of micropylar cells. Although Riehl<sup>12)</sup> found many mitochondria and endoplasmic reticula with vesicles in micropylar cells in *Noemacheilus barbatulus* and *Gobio gobio*, he could not detect the Golgi bodies in the cells of either species of teleost. In the pond smelt, on the contrary, moderately developed Golgi bodies were clearly observed in micropylar cells of oocytes at early developmental stages. A marked expansion of rough endoplasmic reticulum was also seen in earlier stages. As oogenesis advanced further, smooth-surfaced cytoplasmic vesicles of various shapes gradually increased in number in the micropylar cells. Often, some of them were closely attached to the area of cytoplasmic membrane showing flask-like invaginations. These features suggest that synthesis and secretion of proteinaceous material are carried out in these cells. Yanagimachi and Kanoh<sup>14)</sup> and Yanagimachi<sup>15)</sup> observed that sperm were activated around the micropyle in *Clupea pallasii*, and suggested that a sperm-activating substance was present in that area. Suzuki<sup>16)-19)</sup> also observed aggregation and activation of sperm around the micropyle area in *Acheilognathus lanceolata*, *Acheilognathus tabira*, *Rhodeus ocellatus* and *Sarcocheilichthys variegatus*. He suggested that the phenomena were ascribed to a certain factor which originated in the micropylar cell<sup>20)</sup>. Thus, the characteristics of some of the cytoplasmic organelles observed in micropylar cells of the pond smelt may be involved in the production of such a factor. It must also be considered that the micropylar cells may be involved in the production of unknown materials which block the formation of the vitelline envelope around the processes of the cells.

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