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Eggs and Ovaries of the Stickleback, *Pungitius tymensis*, with a Note on the Formation of a Jelly-like Substance Surrounding the Egg[1,2)]

By

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(*With 4 Plates*)

In studying the development of the stickleback egg, Ichikawa, Yamamoto and Yoshida ('56) found that the newly spawned eggs of this fish are surrounded by an amorphous, jelly-like substance which makes separation of the eggs difficult. A similar substance was observed by Ransom (1867) in the *Gastrosteus* egg but has not been described in the eggs of other fish, as far as the author knows. In echinoderms and amphibians, the jelly layer or jelly envelopes of the egg are reported to be important in the fertilization reaction (Sugiyama '58, Katagiri '62, '63) and the jelly-like substance of the stickleback egg may also play a role in the fertilization of this fish.

The present study is part of a series of studies of the activation or fertilization of fish eggs. The structure of the eggs and ovaries was investigated morphologically in order to find the source of the jelly-like substance surrounding the newly spawned eggs of the stickleback.

Material and method

The materials used in this study were taken from the stickleback, *Pungitius tymensis*, which was captured in the stream running through the campus of the University. Mature fishes and spawned egg masses were found in this stream from March to May of every year, thus the spawning period of this species seems to be during these months. A long cut was made along the belly and the ovary was removed and placed in a petri-dish. For observation in the fresh state, the ovary was then immersed in Ringer's solution of following constitution; M/7 NaCl 100 cc+ M/7 KCl 2.0 cc+M/10 $CaCl_2$ 2.1 cc (pH 7.3). For detailed study, the materials were fixed in Bouin's or Helly's fluid and embedded in paraffin in the ordinary manner. The specimen thus prepared was sectioned (5–10μ) and stained

1) This paper is dedicated to Professor Atsuhiko Ichikawa, Zoological Institute, Hokkaido University, Sapporo, in honor of his sixtieth birthday, May 20, 1964.

2) Contribution No. 620 from the Zoological Institute, Faculty of Science, Hokkaido University, Sapporo, Japan.

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with Delafield's hematoxylin-eosin or Heidenhain's azan stain. Hotchkiss' PAS test for polysaccharides and a devised method of ferric-ferricyanide test for SH- or SS-groups (cf. T.S. Yamamoto '57a) were adopted for the cytochemical purpose of the study.

Results

I. *Structure of ovary and oviduct*: The ovary is unpaired but is bilobed in its anterior part and has a mid-ventral groove that separates it superficially into right and left portions (Fig. 1). It is covered with a thin peritoneum beneath which there is a thin tunica albuginea of connective tissue, in which may be found smooth muscle fibers. Within the tunica albuginea are many ovigerous lamellae which project into the ventral lumen of the ovary (Figs. 2 and 3). These lamellae and the inner surface of the tunica albuginea are covered with an epithelial lining continuous with that of the oviduct which is a single, short structure leading from the lumen of the ovary to the genital opening posterior to the anus but anterior to the urinary opening. There is a urogenital papilla between the genital opening and the anus.

Sections of the ovary show that the ovigerous lamellae contain a large number of oocytes in various stages of growth, each of which is surrounded by a follicular cell layer. In fish which had spawned, these oocytes are small in size and have no yolk granules, and there are a number of empty follicular structures in the ovary from which the eggs probably had been spawned (Fig. 2).

The lumen occupies the ventral part of the ovary, and is narrow in immature fish and expanded in mature one. In the ovulating ovary, the lumen containing mature eggs and a jelly-like substance is easily recognized through the ovarian wall as shown in Fig. 4. When observed in fresh materials, the ventral wall of the lumen, which has no ovigerous tissue, shows peristaltic movement toward the oviduct, which movement may bring the eggs contained in the lumen into the oviduct.

The ventral wall of the ovarian lumen is primarily connective tissue, tunica albuginea, which is rich in blood vessels and muscle fibers. The inner surface is covered by a single layer of cubic cells which are homogeneously stained with hematoxylin (Fig. 5). Careful observation reveals that a drastic morphological change occurs in this cubic cell layer with the advancement of ovarian maturation. The cubic cells covering the inner surface of the tunica albuginea are compactly arranged in the immature ovary and deeply stained with hematoxylin (Fig. 5), but they become disarranged as ovulation approaches. In ovaries whose lumen contain mature eggs, the cubic cells completely lose their affinity for hematoxylin and it appears as if the cytoplasm of these cells had flowed into the ovarian lumen (Fig. 6). It is worthy of note that in such ovaries, the jelly-like substance is found in the ovarian lumen (Fig. 7). The cubic cells seem to regain their compact arrangement and basophilic property following the spawning period.

The oviduct is a thick structure of connective tissue and is rich in muscle

fibers. The inner surface is folded and covered with columnar cells which are easily distinguished from the cubic cells covering the tunica albuginea in the ovarian lumen (Fig. 8). These columnar cells do not show any morphological change with advancement of ovarian maturation. In immature fish, the terminal part of the oviduct is closed with a plug of loose connective tissue, which may separate the ovarian lumen externally (Fig. 9).

II. *Immature oocytes*: Ovaries taken from fish captured in the early spawning period contain a number of oocytes in various stages of growth. The smallest of these oocytes measure less than 8μ in diameter and have spherical nucleus, in which there are chromatin threads deeply stained with hematoxylin and one or two indistinct nucleoli stained with eosin. The cytoplasm is scarcely stained with any of the dyes used.

In oocytes measuring about 40μ in diameter, which are surrounded by discontinuous follicular cells, the staining affinity of chromatin threads to hematoxylin decreases. There is a single, large hematoxylin stained nucleolus in the nucleus but no eosinophilic nucleoli. The cytoplasm shows a weak basophila, suggesting a beginning of accumulation of RNA (Fig. 10).

In the next stage of growth the oocytes are surrounded by a continuous layer of follicular cells. Just beneath the inner surface of this follicular layer is a thin, indistinctly structured membrane stained with aniline blue in the azan stain. This membrane may grow into the chorion in the next stage of oocyte growth. The nuclei of the oocytes are irregular in form, and show so-called "sacculation". The chromatin threads in the sacculated nuclei are only faintly stained with hematoxylin and take an appearance of "lamp-brush chromosomes" (Fig. 11). In azan-stained preparations, the chromatin threads are indistinctly observed in the nuclear sap which is faintly stained with azocarmine. The nucleoli, deeply stained with hematoxylin and markedly increased in number, are peripherally arranged in the nucleus. The cytoplasm is also deeply stained with hematoxylin, suggesting an intense accumulation of RNA.

The oocytes, which are about 250μ in diameter, are characterized by the appearance of small vesicles in their peripheral cytoplasm (Fig. 12). These vesicles are positive to the PAS test as the vesicles of the oocytes of many other teleost fishes (K. Yamamoto '55, '56, T.S. Yamamoto '55a, etc.) and contain polysaccharides other than glycogen. Shortly after the first appearance of the vesicles, a similar polysaccharide reaction appears in the cytoplasm. The membrane, which in the preceding stage of oocyte growth had been stained with aniline blue, is now distinct, thick chorion, clearly stained with azocarmine of the azan stain. It is radial in structure and shows the so-called "radial striae" (Fig. 13). The PAS and the ferric-ferricyanide tests are positive in this chorion. In the nucleus, the staining property of the nuclear sap is clearly changed from pink of the preceding stage to red in the azan stain. The lamp-brush chromosomes retain their indistinct appearance in the sap. There are many basophilic

nucleoli in contact with the nuclear membrane in the nucleus which are now positive to the ferric-ferricyanide test.

In the succeeding stage of growth, the PAS positive vesicles increase in number in the peripheral cytoplasm of the oocyte and finally the cytoplasm of the oocyte is almost filled with these vesicles except in the narrow, outermost region which is about 10μ thick. In these oocytes therefore the cytoplasm is only found in that narrow outermost region, the intervesicular space and the perinuclear region (Fig. 14). The striated chorion is clearly visible between the follicular cell layer and the surface of the oocyte. In this stage the chorion has some small protrusions on the outer surface of its restricted area (Fig. 15). These small protrusions may correspond to Ransom's "buttons", the accessary of the chorion found on the animal hemisphere of the mature egg (Ransom 1867). The follicular cells found near these protrusions do not differ from other follicular cells in their morphological and staining properties.

The oocytes measuring about 450μ in diameter are characterized by the presence of yolk granules (Fig. 16). The cytoplasm of the oocyte is filled with the PAS-positive vesicles and, near the periphery, with fine yolk granules. The most peripheral region of the oocyte is free from vesicles and yolk granules and is about 3μ thick. The yolk granules increase in number in succeeding stages and also appear in the intervesicular cytoplasm of the oocyte. With further increase of the yolk granules gradual changes occur in the distribution of the cytoplasm and in the property of the nucleus. The intervesicular cytoplasm gradually disappears, so that in the later stages the cytoplasm may only be seen in the peripheral and perinuclear regions of an oocyte. In the nucleus, the nuclear sap becomes deeply stained with azocarmine of the azan stain, and nuclear membrane becomes very indistinct. And two or more highly refractive particles appear in each peripheral nucleolus. When the cytoplasm of an oocyte is completely filled with PAS-positive vesicles and yolk granules except in the peripheral and perinuclear regions, the yolk granules begin to fuse and form spherules. These yolk spherules appear first in the vegetal hemisphere of the oocyte and later in the animal hemisphere.

In oocytes measuring about 550μ in diameter, the nucleus begins to move toward the animal pole with the perinuclear cytoplasm (Fig. 17). The staining property of the nucleus is the same as that of the preceding stages and indistinct lamp-brush chromosomes are still observed in it. The peripheral nucleoli move toward the center of the nucleus from the nuclear membrane. With the translocation of the nucleus to the animal pole, the yolk spherules begin to fuse and form a large mass of yolk. The fusion of the yolk spherules first takes place in the central region of the oocyte in the former nuclear site, which is now the vegetal hemisphere of the translocated nucleus, and proceeds gradually outward but never occurs along route which the nucleus will travel toward the animal pole (Fig. 17).

With oocyte growth, morphological and cytochemical changes occur in the

chorion. The radial structure of the chorion fades and the radial striae are only faintly observable in those oocytes with translocated nuclei. The PAS-positive property of the chorion decreases with this morphological change until it becomes negative.

As in other teleostean eggs, the animal pole of the chorion of the stickleback egg is also perforated by a pore. This pore is called a micropyle and may be found in ovarian oocytes which are in the stage of the formation of the yolk granules. The chorion of these oocytes is perforated by a special cell called a micropylar cell which inserts its relatively long process into the micropylar canal (Figs. 18 and 19). The micropylar cell is easily distinguished from the other follicular cells surrounding the oocyte by its low affinity to dyes. Before the nuclear translocation begins, the animal pole of the oocytes may easily be recognized by the presence of this micropylar cell and the button-like protrusions of the chorion.

III. *Mature egg*: The mature egg is an elastic, semitransparent spherical body about 1.2 mm in diameter (Fig. 20). Newly spawned eggs are surrounded by an amorphous jelly-like substance which makes separation of the eggs difficult. After a time in water, the jelly-like substance coagulates regardless of the activation or fertilization of the egg. In this way, the eggs seem to be fixed on supporting materials in water. Before it coagulates the fresh jelly-like substance is deeply stained with methylene blue but not with neutral red (Fig. 21).

In fresh eggs, the animal pole is easily recognized by the presence of the distinct micropyle (Fig. 22). Many oil drops may be seen through the transparent chorion enclosing the egg. They remain regardless of the polarity of the egg and move easily under the influence of gravity. The chorion consists of two layers and has a reticular pattern on its outer surface. The radial structure of the chorion indicated by the radial striae in the immature oocyte is not found in the mature egg. In the animal hemisphere, the chorion has protrusions on its outer surface (Fig. 23). These are the button-like protrusions previously described in the ovarian oocytes and are not found in the vegetal hemisphere. Cytochemically, the chorion is positive to the ferric-ferricyanide test and negative to the PAS test except in a special case which will be mentioned later. Beneath the chorion there is a cortical cytoplasmic layer with cortical alveoli. As previously reported by Kusa ('53), the alveoli are positive to the PAS test and negative to the ferric-ferricyanide test (Fig. 24). From these cytochemical properties it is supposed that the alveoli are derived from the PAS-positive vesicles in the ovarian oocytes. The yolk mass is surrounded by the cortical cytoplasmic layer and does not show any granular structure when observed in sections. The oil drops are found in the boundary region between the cortical cytoplasmic layer and the yolk mass.

The cortical cytoplasmic layer thickens somewhat at the animal pole of the egg, forming a rudiment of a blastodisc (Fig. 25). A polar view of the egg shows that this thickened part of the cytoplasm appears as a round disc on the yolk mass,

the center of which is thicker than the peripheral area. The micropyle is found in the chorion covering this thickened layer of the cytoplasm and is a funnel-shaped indentation of the chorion with a small inner opening. The thickened chorion forming the margin of the inner end of the micropylar canal protrudes inward. The canal does not open on the center of the thickened cytoplasmic disc mentioned above but opens eccentrically. This may also be observed in the sections and is shown in Fig. 25. In this figure, the cytoplasmic layer seems to be thickest along a line A to A', but the micropyle diverges from this line.

In most cases, the staining property of the chorion in the region of the micropyle is the same as that of the rest of the chorion but in some cases it is different. For example, Fig. 26 is a section through the micropyle from an egg fixed in Bouin's fluid and stained with azan stain. In this case, the chorion is stained with azocarmine except in the inner margin of the micropylar canal, where the chorion protrudes into the cytoplasm and is stained with aniline blue. Peculiar properties of the chorion in the inner margin of the micropylar canal are also found in the sections tested with ferric-ferricyanide or PAS. In the former test, the reaction is weak in the inner margin of the canal, though it is distinct in the rest of the chorion (Fig. 27). In PAS-treated preparations, the reaction is positive in the inner margin of the canal and negative in the rest of the chorion (Fig. 28). The most striking feature of the micropylar region is shown in Fig. 29. In this figure, the protruded part of the chorion around the inner opening of the canal has disappeared and there is a large empty space in the cortical cytoplasm just beneath the inner opening of the micropylar canal. When these figures are compared with the micropylar structure revealed in fresh material (Fig. 22), it is clear that these are the micropyles deformed by fixation and dehydration of the egg.

Discussion

The newly spawned eggs of the stickleback are surrounded by a jelly-like substance which had been secreted into the ovarian lumen of nearly matured fish but which was not found in the immature ovary. The substance does not originate from the follicular cells surrounding the oocyte in the ovary or from the oocytes themselves since it, or its precursor, was never found in any part of the ovigerous lamellae. It may be secreted from the epithelial cells lining the inner surface of the ovarian lumen since the cubic cells covering the inner surface of the tunica albuginea showed a drastic morphological change during ovulation. They lost their basophilic property and it appeared as if their cytoplasm had flowed into the ovarian lumen. Although the jelly-like substance was not compared cytochemically with the cytoplasm of the cubic cells, it is most likely that the substance is a secretion of the cubic cells covering the tunica albuginea. A similar observation has been made in *Gastrosteus* ovary by Ransom (1867) who considered that the jelly-like substance is a secretion from the inner surface of the ovarian lumen and the oviduct. Since in the present study the inner surface of the oviduct did not show

any morphological change during the maturation of the ovary, the oviduct may not participate in the secretion of the substance. K. Yamamoto ('63) recently studied the ovary of the medaka and noticed that with the advancement of ovarian maturation there was a marked change in the inner epithelial lining of the ovarian wall (which may correspond to the cubic cells of the present study) and the tunica albuginea. He observed that the cells of the epithelial lining elongate with the advancement of ovarian maturation and actively secrete a liquid substance which facilitates extrusion of the eggs in spawning. There have been no studies which explain the significance of the jelly-like substance in the development of stickleback egg, but the substance may also be useful in facilitating extrusion of the eggs in spawning as K. Yamamoto ('63) has pointed out in the medaka. It may also be useful in fixing the eggs on supporting materials in water and may possibly aid in maintaining the fertilizability of the egg in water since unpublished experiments show that the unfertilized eggs of the stickleback retain their fertilizability in water for more than 30 minutes. This long period of time is surprising since in most freshwater fishes the unfertilized eggs are known to lose the capacity of fertilization within a few minutes (T. Yamamoto '58).

In the animal hemisphere the chorion of the stickleback eggs has small button-like protrusions on its outer surface which have the same staining and cytochemical properties as the chorion. In the ovaries, the protrusions appeared following the distinct formation of the chorion and the follicular cells near the protrusions had the same staining property as those further from the protrusions. These observations may possibly suggest that the button-like protrusions are products of cells which participate in the formation of the chorion. The significance of these protrusions in the development of the stickleback egg is still unknown. They are morphologically similar to the villi of the medaka egg which serve to fix the egg on supporting materials in water. However the villi of the medaka egg have been reported to be formed on the surface of the ovarian oocyte prior to the distinct appearance of the chorion (T.S. Yamamoto '55a). The difference in the manner of their formation may suggest that the button-like protrusions of the stickleback egg do not correspond functionally to the villi of the medaka egg. The function of the villi in the development of the medaka egg may be performed by the jelly-like substance in the stickleback egg, though Ransom (1867) had considered that the protrusions of the *Gastrosteus* egg are organs of adhesion and serve to fix the egg.

It has been reported that the chorion of many teleostean eggs is positive to the PAS test (T.S. Yamamoto '55a, etc.). In the stickleback egg the test was positive in the artificially deformed region of the micropyle but it was usually negative in the chorion of the mature egg. This and the positive reaction of the chorion of the ovarian oocyte suggest that polysaccharides are one of the components of the chorion which have been cytochemically masked in the test used in the mature egg.

As is observed in the ovarian oocytes of many teleosts, the micropylar cell was found in the micropylar region of the stickleback oocytes. It was distinguished from other follicular cells by its achromatic property and its relatively long cytoplasmic process inserted into the micropylar canal (Eigenmann 1890, T.S. Yamamoto '55a, b, '57b, Tchou-Su & Wang '62). Probably this process of the micropylar cell is in close contact with the ooplasm in the canal. Electron microscopic studies of vertebrate ovarian oocytes have revealed that the follicular cells insert their cytoplasmic branches into the canal of zona radiata, or canal of the chorion, closely contacting the microvilli extended from the surface of ooplasm (Kemp '56, '57, '58, Kemp & Allen '56a, b, Kemp & Hibbard '57, Wartenberg '62). In this manner the nutritives seem to infiltrate into the growing oocyte through the chorion. These canals, revealed in the above mentioned electron microscopic studies, may be compared with the radial striae of the chorion observed in the present study. Microvilli extended from the surface of the oocyte have not been actually observed in the stickleback but their presence may be inferred from the electron microscopic observations of *Fundulus* egg (Kemp and Hibbard '57, etc). The micropylar cell may contact its cytoplasmic process with the cortical cytoplasm in the micropylar canal, although the significance of this is unclear at present.

The micropylar region of the chorion was artificially deformed by fixation and dehydration of the eggs. It is important to note here that the deformation always occurs in that part of the chorion which forms the margin of the inner end of the micropylar canal because, in the course of fertilization, supernumerary spermatozoa are prevented from penetrating into the egg at the inner end of the canal. Kanoh has obtained a differential staining of the chorion of the micropylar region of the herring egg and has discussed it in relation to the monospermic fertilization of the egg (Kanoh '49, '57). In experiments with the removal of the chorion of the herring egg, the author reported a differential susceptibility of the chorion of the micropylar region to hypotonic acidulated Ringer's solution (T.S. Yamamoto '58). The results obtained in the present study show that the susceptibility of the chorion of the micropylar region to fixative or alcoholic dehydration differs from that of the rest of the chorion. Considering these facts, it may be safe to say that the chorion is highly susceptible to physico-chemical agents in the margin of the inner end of the micropylar canal. Probably this property of the chorion of the micropylar region is concerned with the mechanism of monospermic fertilization of fish egg, as has been previously noted by Kanoh ('57).

Summary

1. The morphological structure of the eggs and ovaries of the stickleback, *Pungitius tymensis*, is reported.

2. The ovary is unpaired but bilobed in its anterior part.

3. The inner epithelial lining of the ventral wall of the ovary shows

morphological changes with advancement of ovarian maturation.

4. In immature fishes the oviduct is closed with a plug of loose connective tissue.

5. The staining property of the nucleus of the oocyte is clearly altered with growth.

6. PAS-positive vesicles appearing in ovarian oocytes are precursor of the cortical alveoli of the mature eggs.

7. The yolk first appears as fine granules in the peripheral cytoplasm of the oocyte. As the oocyte grows, the granules fuse forming spherules which finally grow into the large yolk mass. This fusion first occurs in the central region of the oocyte and proceeds outward.

8. In the animal hemisphere the chorion has button-like protrusions on its outer surface.

9. The mature eggs are surrounded by an amorphous, jelly-like substance, stained with methylene blue, which is secreted by the inner epithelial lining of the ventral wall of the ovary.

10. The micropylar cell inserts its relatively long process into the micropylar canal where it appears to contact the cortical cytoplasm of oocyte.

11. In the mature egg the chorion forming the margin of the inner end of the micropylar canal is highly susceptible to physico-chemical agents.

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Explanation of Plates VI-IX

Plate VI

Fig. 1. Ovary from fish captured April 13, 1961. Ventral view. ca. × 4.

Fig. 2. Transverse section of the ovary from fish captured July 18, 1958. Note the empty follicular structures (*f*) and the narrow ovarian lumen (*l*). Hematoxylin-preparation. ca. × 15.

Fig. 3. Sagittal section of the ovary and the oviduct (*v*) from fish captured February 24, 1958. *i*, intestine; *l*, ovarian lumen. Azan-preparation. ca. × 8.

Fig. 4. Mature eggs in the ovarian lumen. Fresh material. *ov*, ovigerous tissue; *w*, ventral wall of the lumen. ca. × 25.

Fig. 5. Section of the ventral wall of the ovarian lumen (*l*) showing thin peritoneum (*p*), tunica albuginea (*t*) and epithelial lining of cubic cells (*c*). Hematoxylin-preparation. ca. × 560.

Fig. 6. Section of the ventral wall of the ovarian lumen showing the change in the epithelial lining of its inner surface. Compare with figure 5. *c*, cubic cells. Hematoxylin-preparation. ca. × 560.

Fig. 7. Section of the ovary of nearly matured fish showing the jelly-like substance (*j*) in the ovarian lumen. The substance has been precipitated by fixative and appears as a fibrous structure. Hematoxylin-preparation. ca. × 115.

Plate VII

Fig. 8. Section of the wall of the oviduct showing epithelial lining of columnar cells. Hematoxylin-preparation. ca. × 590.

Fig. 9. Section through the cloaca of an immature fish. Note pulg (*pl*) of loose connective tissue in the oviduct. *a*, anus; *u* urinary opening. Azan-preparation. ca. × 10.

Fig. 10. Section of young oocyte with a basophilic nucleolus in the nucleus. Hematoxylin-preparation. ca. × 770.

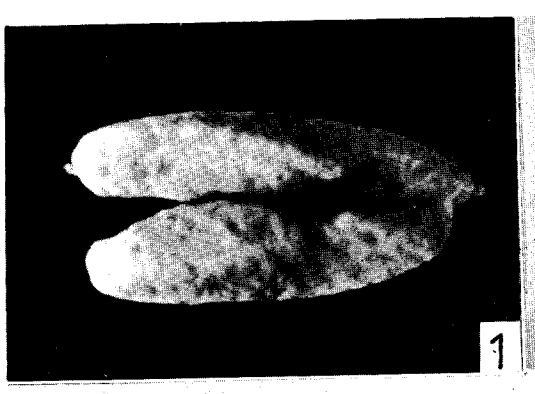
Fig. 11. Section of sacculated nucleus showing lamp-bursh chromosomes. The nucleus has been artificially shrunken by fixation and dehydration. Hematoxylin-preparation. ca × 560.

Fig. 12. Section of oocytes with small vesicles in the peripheral cytoplasm. Hematoxylin-preparation. ca. × 130.

Fig. 13. Section of oocytes showing positive ferric-ferricyanide reaction in the chorion. ca. × 130.

Fig. 14. Section of oocytes filled with vesicles. Hematoxylin-preparation. ca. × 90.

Fig. 15. Section of oocyte showing the button-like protrusions of the chorion. *b*, button-like protrusion; *m*, micropyle. Ferric-ferricyanide-preparation ca. × 670.



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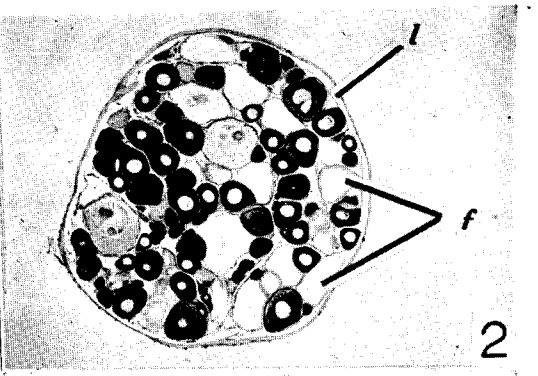

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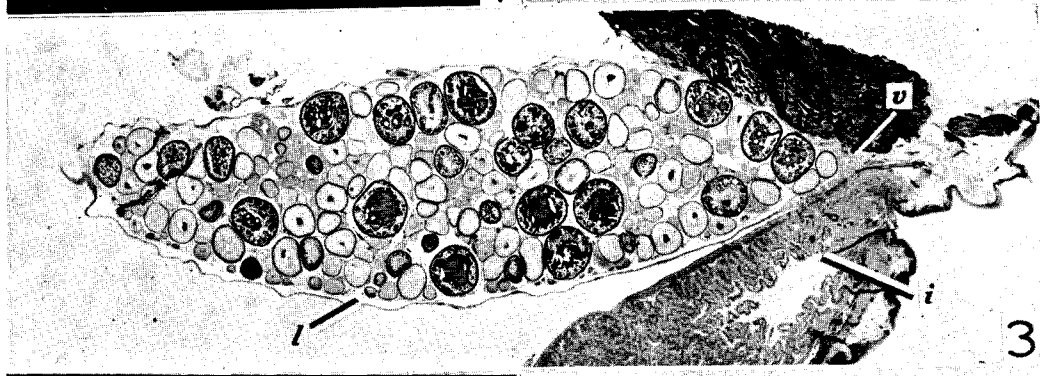

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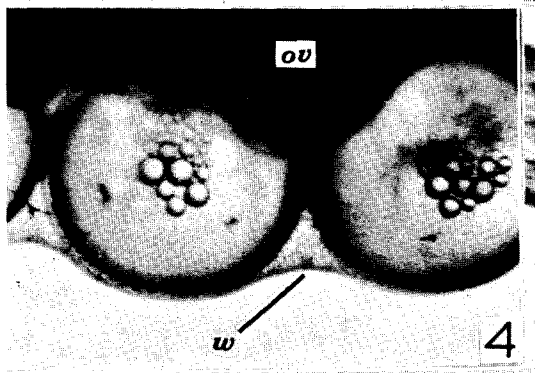

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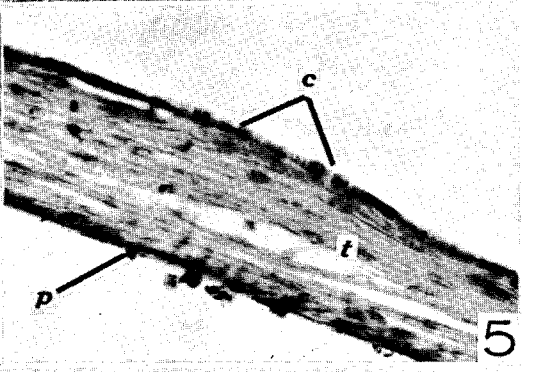

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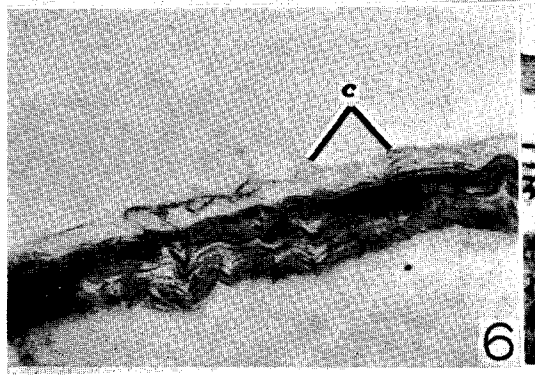

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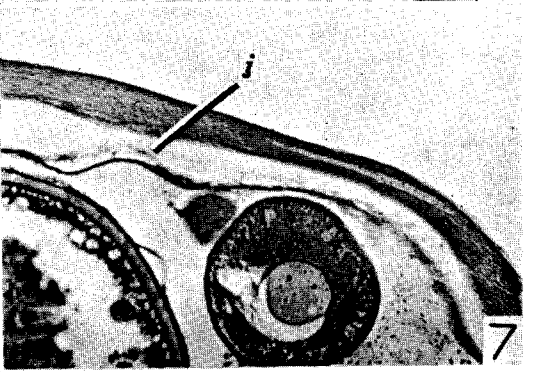

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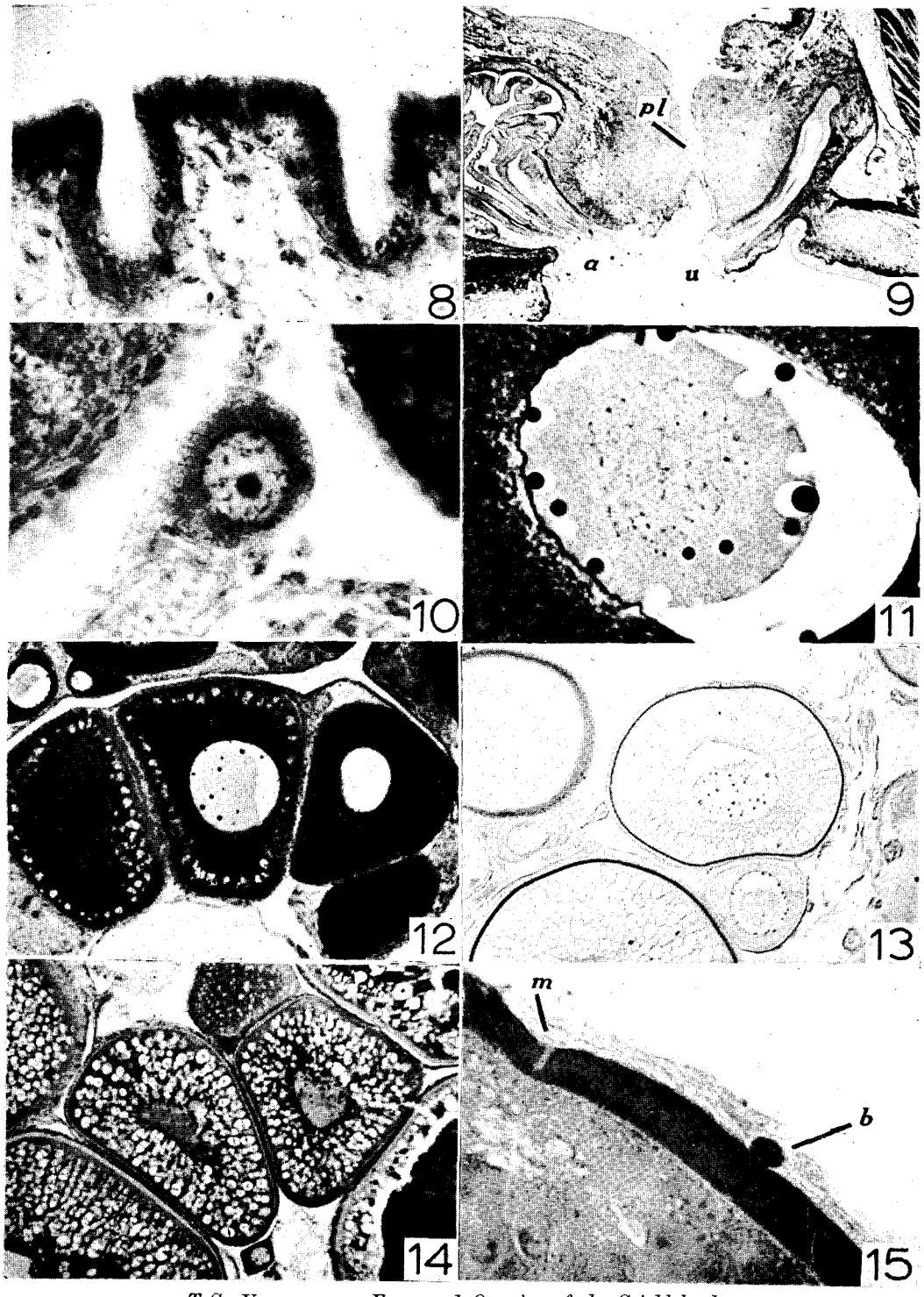
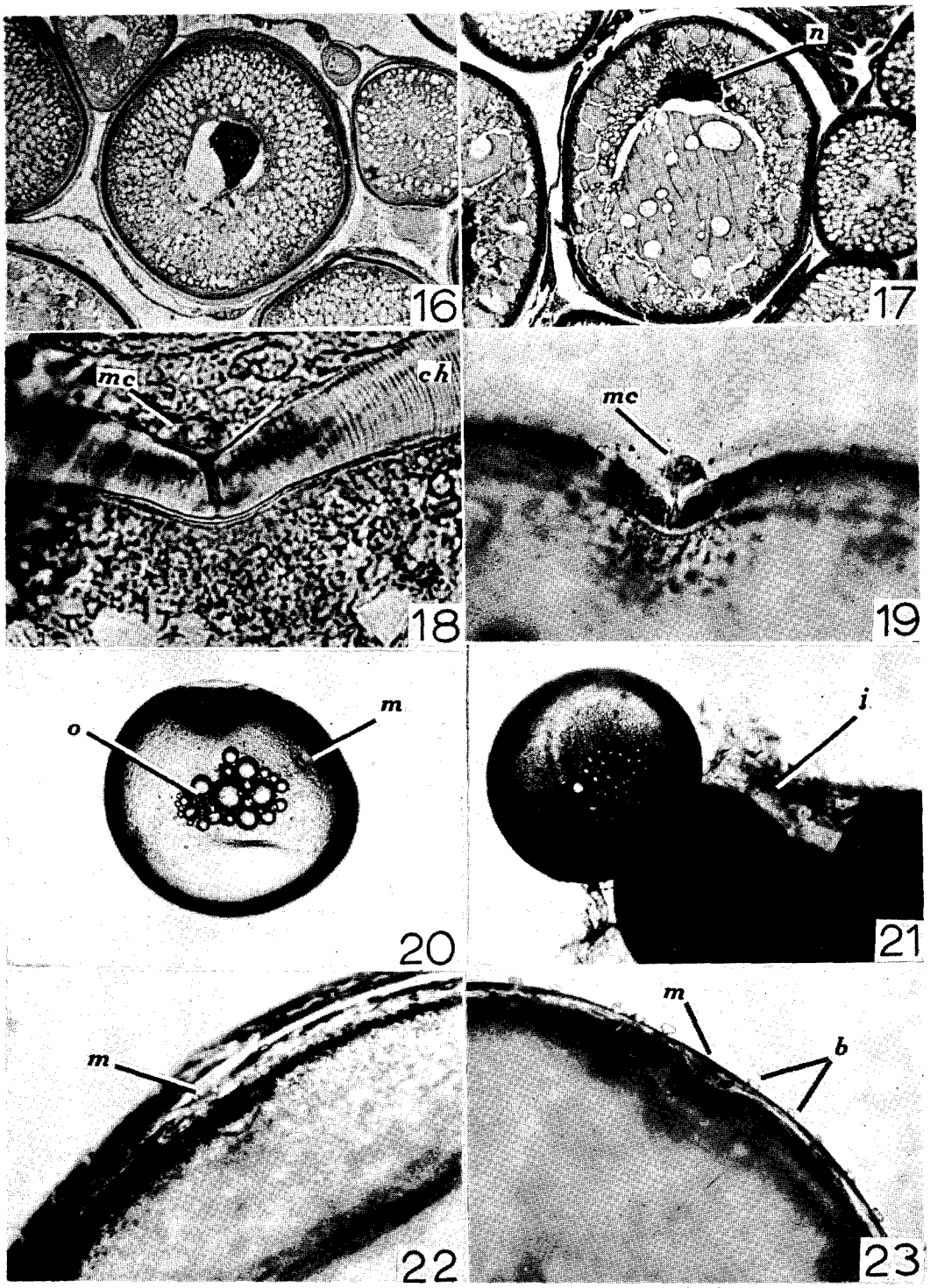
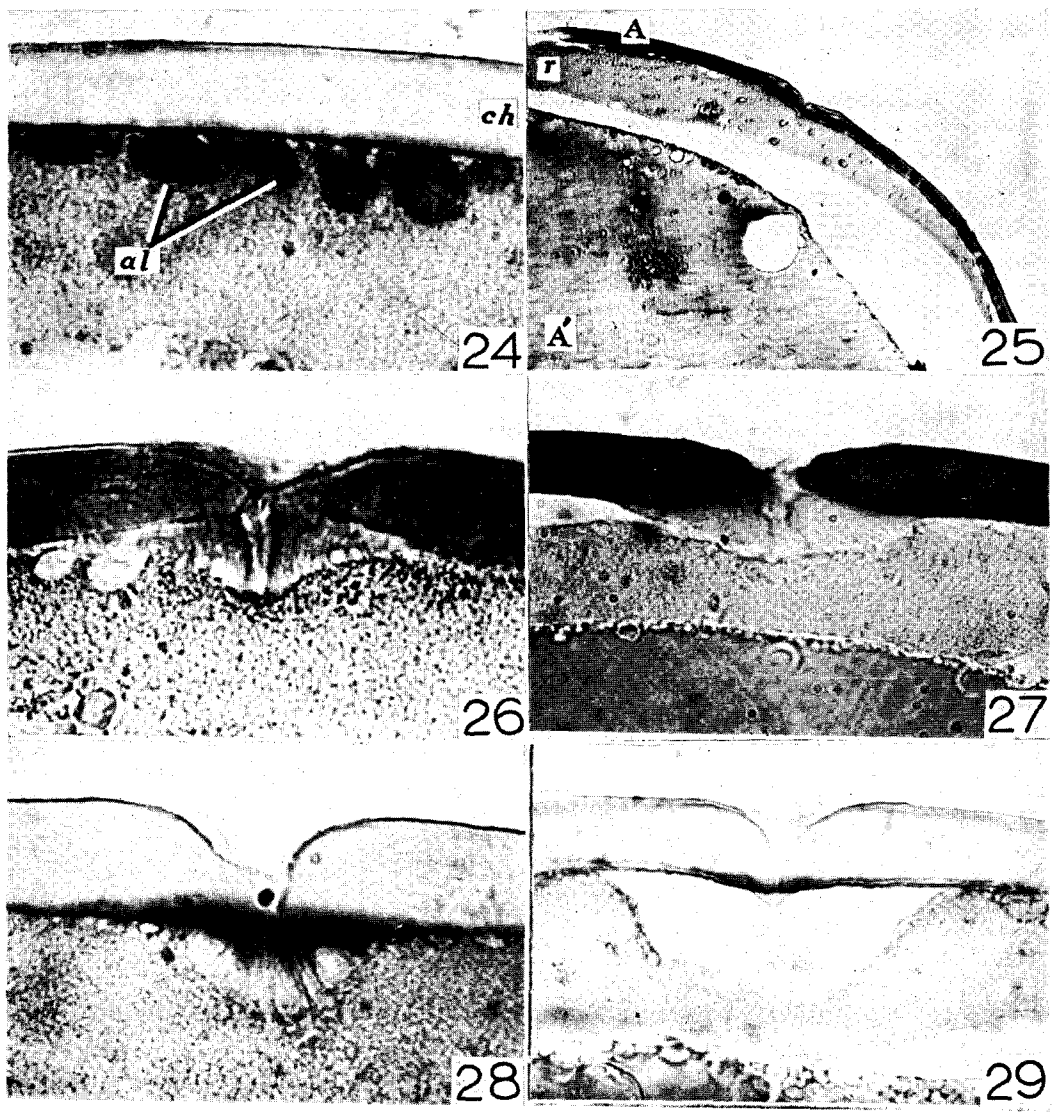
T.S. Yamamoto: Eggs and Ovaries of the Stickleback



T.S. Yamamoto: Eggs and Ovaries of the Stickleback



T.S. Yamamoto: Eggs and Ovaries of the Stickleback



T.S. Yamamoto: Eggs and Ovaries of the Stickleback

Plate VIII

Fig. 16. Section of oocytes showing the formation of yolk granules in the peripheral cytoplasm. Hematoxylin-preparation. ca. × 80.

Fig. 17. Section of oocytes with translocated nucleus. Note the yolk mass situated in the center and the yolk spherules distributed in the peripheral region of the oocyte. *n*, nucleus. Hematoxylin-preparation. ca. × 60.

Fig. 18. Section through the micropyle of an immature oocyte. Note the micropylar cell (*mc*) and the radial striae in the chorion (*ch*). Azan-preparation. ca. × 930.

Fig. 19. Section through the micropyle of an immature oocyte. The follicular cells were artificially removed from the surface of the oocyte. Note the cytoplasmic process of the micropylar cell inserted into the micropylar canal. *mc*, micropylar cell. Hematoxylin-preparation. ca. × 770.

Fig. 20. Mature egg. Fresh material. *m*, micropyle; *o*, oil drop. ca. × 28.

Fig. 21. Photomicrograph showing the jelly-like substance (*j*) stained with methylene blue. Fresh material. ca. × 24.

Fig. 22. Micropyle (m) of mature egg. Fresh material. ca. × 125.

Fig. 23. Button-like protrusions of the chorion distributed in the animal hemisphere. Fresh material. *b*, button-like protrusion; *m*, micropyle (out of focus). ca. × 100.

Plate IX

Fig. 24. Section of mature egg showing cortical structure. PAS-preparation. *al*, cortical alveoli; *ch*, chorion. ca. × 1100.

Fig. 25. Section of mature unfertilized egg showing rudiment of the blastodisc. The cortical cytoplasmic layer is thickest along the line A to A'. Hematoxylin-preparation. ca. × 115.

Figs. 26–29. Sections through the micropyle of mature egg showing deformation in the micropylar region. Figure 26, azan-preparation, ca. × 730; figure 27, ferric-ferricyanide-preparation, ca. × 730; figures 28 and 29, PAS-preparation, ca. × 600.