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Oogenesis in the Medaka *Oryzias latipes* —Stages of Oocyte Development

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ABSTRACT—An ovary of *Oryzias latipes* contains developing oocytes of various sizes and morphology during the breeding season. Formation of the egg membrane (chorion), changes in cell organelles and the volume of the developing oocyte as well as changes in follicle cells were investigated by ordinary light and electron microscopy. Basic morphological distinctions were primarily used to prepare a table of the developmental stages of *Oryzias latipes* oocytes. In addition to these distinctions, differences in proteins analyzed by SDS–polyacrylamide gel electrophoresis and the capacity for steroid production assayed by radioimmunoassay were examined in follicles at different stages. On the basis of these results, oogenesis is grossly classified into five phases (early and late previtellogenic phases, early and late vitellogenic phases and postvitellogenic phase) which are divided in detail into ten stages (Stage I–X) of oocyte development. The present classification of developing oocytes was compared with early ones by other investigators.

INTRODUCTION

In teleost fishes, many investigations on fine structures of the oocyte during oogenesis have been reviewed [1–3]. In the medaka, *Oryzias latipes*, developing oocytes have already been investigated [4–17]. Since these investigators fragmentally described developing oocytes with different viewpoints toward oogenesis, they have not always provided sufficient details on the defined developmental stages of oocytes that are required for various physiological and biochemical studies [18, 19] of medaka oocytes.

In order to establish a standard table of the developmental stages of medaka oocytes that can be used for cytological, physiological and biochemical studies, we investigated the characteristics of live and fixed follicles by light and electron microscopy and by a few biochemical methods, we also reviewed briefly the cytological and histological observations by early investigators.

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MATERIALS AND METHODS

Adult medakas, *Oryzias latipes* (orange-red type), used in the present investigation were obtained from a fish farm in Yatomi (Aichi Pref. Japan). They were kept in glass aquaria under artificial reproductive conditions (light 14 hr, 26–28°C) and fed mixed powders of shrimps and roasted wheat-grains at least three times a day.

To test the ability of large oocytes to mature in response to hormone stimulation, intrafollicular oocytes isolated as described elsewhere [20] were incubated for 10 hr (26°C) in culture medium (90% Earle's medium 199, Dainippon-seiyaku, Osaka, Japan) containing 17α, 20β-dihydroxy-4-pregnen-3-one (17α, 20β-diOHprog, 100 ng/ml). Ten to fifteen oocytes were incubated in 5 ml of culture medium in a sterilized glass Petri-dish. At the end of incubation, maturation of oocytes was determined by observing the breakdown of the germinal vesicle (GVBD) and the responsiveness to insemi-

nation. In the present study, the diluted Earle's medium 199 was supplemented with 30 mg/l penicillin G potassium (Meiji-seika, Tokyo, Japan) and 60 mg/ml streptomycin sulfate (Meiji-seika) and adjusted to pH 7.4 with 1 M $NaHCO_3$.

For light microscopic observations, some ovaries were fixed for 3–12 hr with Bouin's fixative or glutaraldehyde dissolved in regular saline buffered to pH 7.3 (4°C) with 0.1 M phosphate buffer. Fixed samples were dehydrated in a graded ethanol series and embedded in paraffin. Paraffin sections 7 μm in thickness were stained with Delafield's haematoxylin. For transmission electron microscopy, oocytes were fixed with modified Karnovsky's fixative (pH 7.3) for 12 hr and post-fixed in 1% solution of buffered (0.1 M phosphate buffer, pH 7.3) osmium tetroxide for 2 hr (4°C). These samples were rinsed in 0.1 M phosphate buffer and then embedded in epoxy resin. Examination of ultrathin sections stained with uranyl acetate and lead citrate was conducted with a JEM–100 B and a JEM–100 CX electron microscopes. Fixed and dehydrated samples shrank as in Table 1.

Follicles (80–90 per sample) of various sizes were homogenized in 10 mM Tris-HCl buffer (pH 6.8) at a ratio of 35 volumes of buffer to one volume of follicle or egg with 10 strokes of a teflon microhomogenizer. The homogenate was incubated for 3 min at 60°C, and was centrifuged at 1,000 *g* for 10 min (room temp.).

The supernatant was mixed with an equal volume of the sample buffer containing 2% SDS, β-mercaptoethanol, 20 mM Tris-HCl (pH 6.8) and 40% glycerin and incubated for 10 min at 60°C. It was then analyzed by SDS-polyacrylamide gel electrophoresis (SDS–PAGE). The protein samples (about 25 μg) prepared above were loaded on polyacrylamide gels (12%) containing 0.1% SDS, 0.0025% fresh ammonium persulfate, 0.00025% riboflavin, and 0.05% Temed. Gels were run at a constant 10 A until the tracking dye (bromophenol blue) had reached the bottom of the gel, which took approximately 6.5 hr (20°C). A common electrophoresis buffer (0.1% SDS–0.05 M Tris–0.38 M glycine) was used. The gels were fixed for 10 hr in 45% methanol–10% acetic acid, stained for 6 hr with 0.05% Coomassie brilliant blue R–250 dissolved in the above solution, and destained by diffusion in 5% methanol–10% acetic acid for 6–12 hr. Molecular weights were determined after SDS–PAGE comparing log-relative electrophoretic mobilities (Log-Rm) of standard proteins as references: carbonic anhydrase, ovalbumin, albumin, phosphorylase, β-galactosidase and myosin (a MW-SDS-200 Kit from Sigma Chemicals, St. Louis, MO., USA).

17α, 20β-DiOHprog and estradiol-17β (E_2) were measured in conditioned culture medium (15 follicles/ml) with or without PMS (pregnant mare serum gonadotropin: Serotropin, Teikoku-zoki, Tokyo) by radioimmunoassay (RIA), as described in detail by Kagawa *et al.* [21] and Young *et al.* [22]. The samples of culture medium were assayed after extraction with five volumes of diethyl ether for 30 sec. The anti-E_2 and anti-17α, 20β-diOHprog sera were provided by Dr. Y. Nagahama. The level of cross reactivity of anti-E_2 serum, with E_2, estradiol-17α, estrone, estriol and testosterone was 100.0, 0.80, 3.20, 1.77 and 0.29%, respectively. The anti-17α, 20β-diOHprog serum was highly specific, cross-reacting less than 0.01% with most of a wide range of ovarian steroids such as progesterone (Δ^4-pregnen-3, 20-dione), 17α-hydroxy-4-pregnen-3, 20-dione and 20β-hydroxy-4-pregnen-3-one. Only 17α, 20β-dihydroxy-5β-pregnan-3-one (2.4%) showed a cross reactivity above 1%. (2, 4, 6, 7-^{3}H)E_2 and (1, 2, 6, 7-^{3}H)17α, 20β-diOHprog were used as antigens. In the present radioimmuno-assay system, both E_2 and 17α, 20β-diOHprog standards of 20 pg/ml could be believably distinguished from the buffer blank with

TABLE 1. Change in size of follicles due to dehydration after fixation

Diameter (μm) of live follicles	<100	200	300	400	500–700	800–900	1100–1200
Bouin's fixative	< 66	152	267	356	450–630	720–810	880–960
Modified Karnovsky's fixative	< 75	164	264	364	460–644	728–818	979–1068

Numbers in the table indicate diameter (μm) of dehydrated follicles after fixation.

a 98% confidence limit, but for practical purposes, portions of media reading less than 30 pg/ml were considered to have nondetectable levels.

RESULTS

In a medaka during the reproductive season, an ovary contains oocytes in all phases of oogenesis (Fig. 1). Oocytes can be grossly divided into five phases and further into ten stages, based on major morphological characteristics of developing oocytes and follicles (Fig. 2). Figure 3 diagramatically illustrates these stages, based on the present observations obtained using light microscope. Extracts of various sized follicles and eggs form numerous protein bands with SDS–PAGE (Fig. 4), which reveals some characteristic features. The capacity of follicles to produce E_2 and 17α, 20β-diOHprog in respone to gonadotropin stimulation is also different in follicles of different sizes (Fig. 5). The developmental pattern of E_2 secretion by follicles is different from that of 17α, 20β-diOHprog.

Early previtellogenic phase

Oocytes (Stages I and II of Fig. 3) in this phase (follicles approx. 20–90 μm) are the smallest growing oocytes in the ovary of spawning females. These oocytes usually form clusters in the peripheral region of the ovary.

Stage I (chromatin-nucleolar stage): This group of very small and transparent oocytes (follicles approx. 20–60 μm, Figs. 1A, 2 and 3), possesses poorly developed tubular endoplasmic reticulum (ER) and mitochondria. The roughly spherical nucleus with chromatin-threads with a network appearance has many spherical nucleoli, unlike that of the very small (about 10 μm) oogonia [6, 23] which have a large nucleolus in the nucleoplasm and aggregations of germinal dense bodies

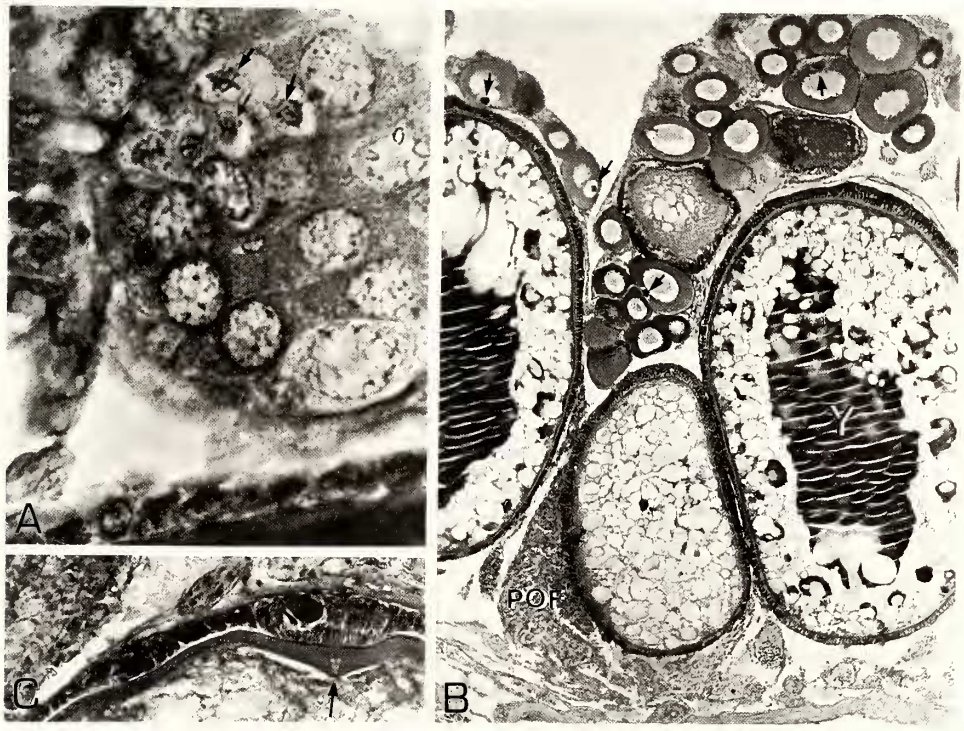


FIG. 1. Oocytes at the various stages of oogenesis in the *Oryzias* ovary.
A: Smallest oocytes at Stage I. ×600.
B: A section of ovary during the spawning season. Note large yolky oocytes, postovulatory follicles (POF), and previtellogenic oocytes with a yolk nucleus (arrows). Y, yolk mass. ×150.
C: Follicle layers and chorion at the animal pole side of an oocyte (micropyle, arrow). ×60.

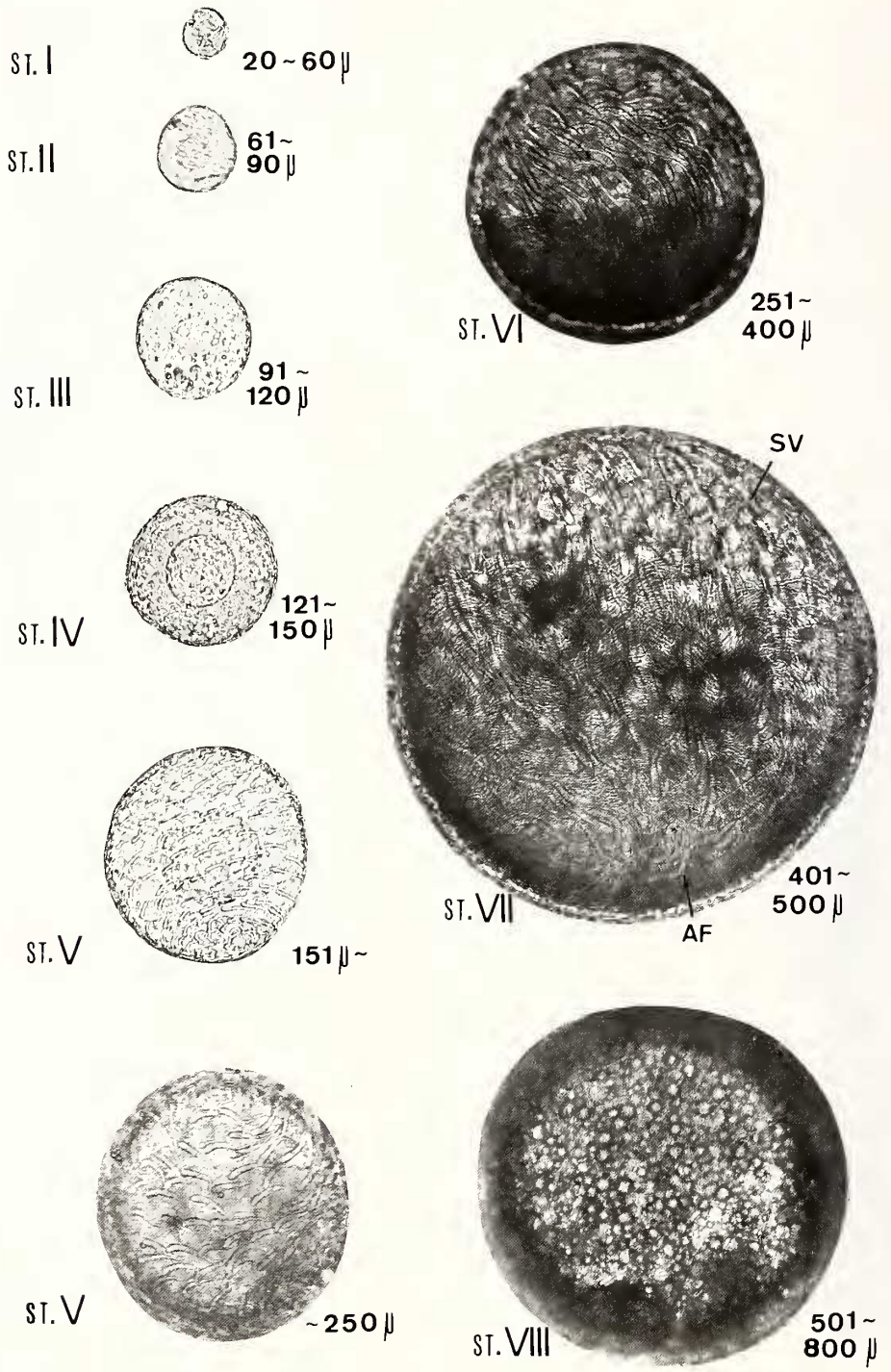


FIG. 2. Living follicles at various stages of oocyte development. Small- and medium-sized follicles (Stage I–VII). ×150. Large-sized follicles (Stage VIII). ×80. SV, short villi on the chorion; AF, attaching filaments on the chorion.

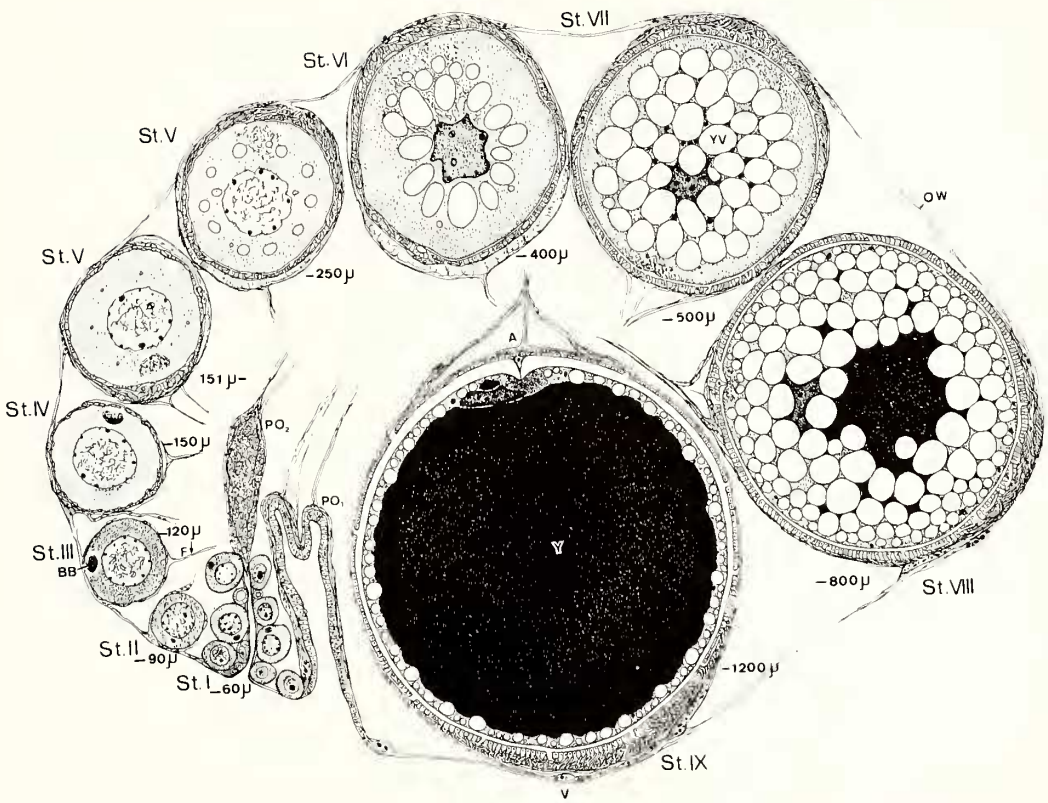
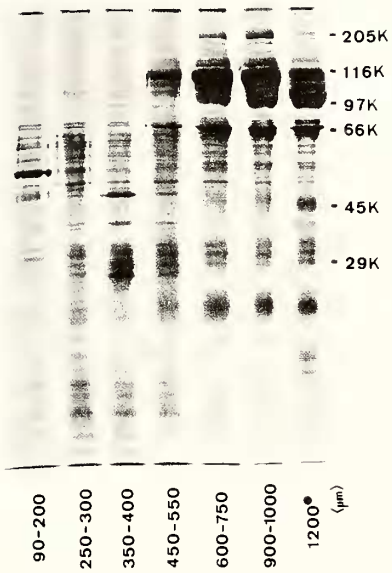


FIG. 3. Diagramatic illustration of growing oocytes at various stages of oogenesis. OW, ovarian wall; PO_1, postovulatory follicle just after ovulation; PO_2, postovulatory follicle 24 hr after ovulation; A, animal pole; V, vegetal pole; Y, yolk mass; YV, yolk vesicle.



and mitochondria in the cytoplasm. Oocytes are covered with an extremely thin single layer of flattened follicle cells.

Stage II (perinucleolar stage): The small and transparent oocytes in the follicles vary from 61 μm to 90 μm in diameter (Fig. 2) and have a relately large nucleus with many spherical nucleoli arranged in the peripheral region (Fig. 3) and haematoxylin-stainable cytoplasm containing a yolk nucleus (Balbiani's body, Fig. 1B). An electron-dense matrix free from cytoplasmic organelles is situated in the cortical region of the ooplasm (Fig. 6A). Mitochondria are arranged in the marginal region of the electron-opaque area of the cytoplasm that contains poorly developed

FIG. 4. SDS–polyacrylamide gel electrophoresis of extracts of various sized follicles and eggs. The size of the follicles and eggs (dot) is indicated at the bottom of each lane. Molecular weight values on the right indicate standard protein bands.

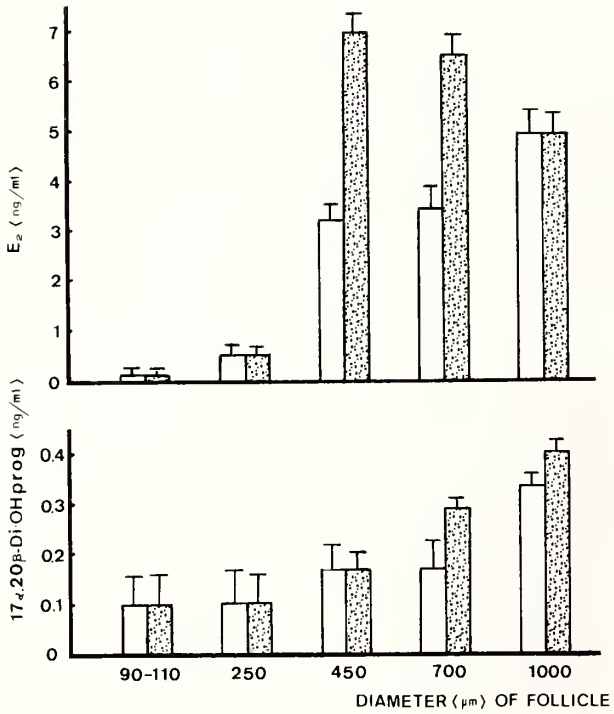


FIG. 5. Production of E_2 and 17α,20β-diOHprog by follicles of various sizes. Follicles were incubated with (shaded columns) or without PMS. Twenty follicles were incubated in 1 ml of culture medium. The medium was collected for determination of E_2 and 17α,20β-diOHprog by radioimmunoassay. Bars represent means$\pm$SE of four incubations.

tubular and smooth ER. The oocytes are covered with extremely flattened granulosa and thecal cells, which are separated by a basement membrane. Clustered microvillus-like projections increase on the restricted area of the oocyte surface facing the junctions of granulosa cells, which usually attach to each other by a number of desmosomes. Granulosa cells possess few microprojections, a small number of mitochondria, very poorly developed ER and flattened nuclei with a nucleolus.

Late previtellogenic phase (chorion-formation)

In this phase, follicles containing transparent oocytes (Fig. 2) vary in diameter from 91 μm to 150 μm (Stages III and IV of Fig. 3). The protein band pattern formed by SDS-PAGE is characterized by 53 k M_r as a major band and 42 k, 63 k and 67 k M_r as minor bands (Fig. 4).

Stage III (chorion-rudiment stage): The oocytes in these follicles (91–120 μm, Figs. 2 and 3) exhibit rudiments of short villi prior to chorion formation. In the cortical cytoplasm, the electron-dense matrix (Fig. 6B) has already disappeared from the cortical region, in which mitochondria and tubular ER are located. The monolayer of granulosa cells is interconnected tightly and circumferentially with many well developed desmosomes (Fig. 6B). Thecal cells are separated from the granulosa cells 1–1.5 μm in width by a basement membrane. The cytoplasm of the oocytes has a large yolk nucleus (Fig. 1B) and is stained less intensely with haematoxylin.

Stage IV (attaching filament and oil-droplet

FIG. 6. Sections of ovarian follicles containing Stage II and Stage III oocytes. A: Oocyte (in a follicle about 65 μm in diameter) surrounded by a very thin follicular epithelium consisting of flattened granulosa (G) and thecal (T) cell layers which are separated by a basement membrane (B). Note that there are only a few microprojections from the surface of the oocyte (O). The cortical cytoplasm of the oocyte is electron dense (DL). $\times$6,600. B: Oocyte (in a follicle about 100 μm in diameter) is surrounded by thick granulosa (G) and thecal (T) cell layers. Clusters of microprojections from the oocyte are found where adjacent granulosa cells joined by desmosomes (arrows) meet. Masses of long mitochondria are frequently observed. $\times$10,000.

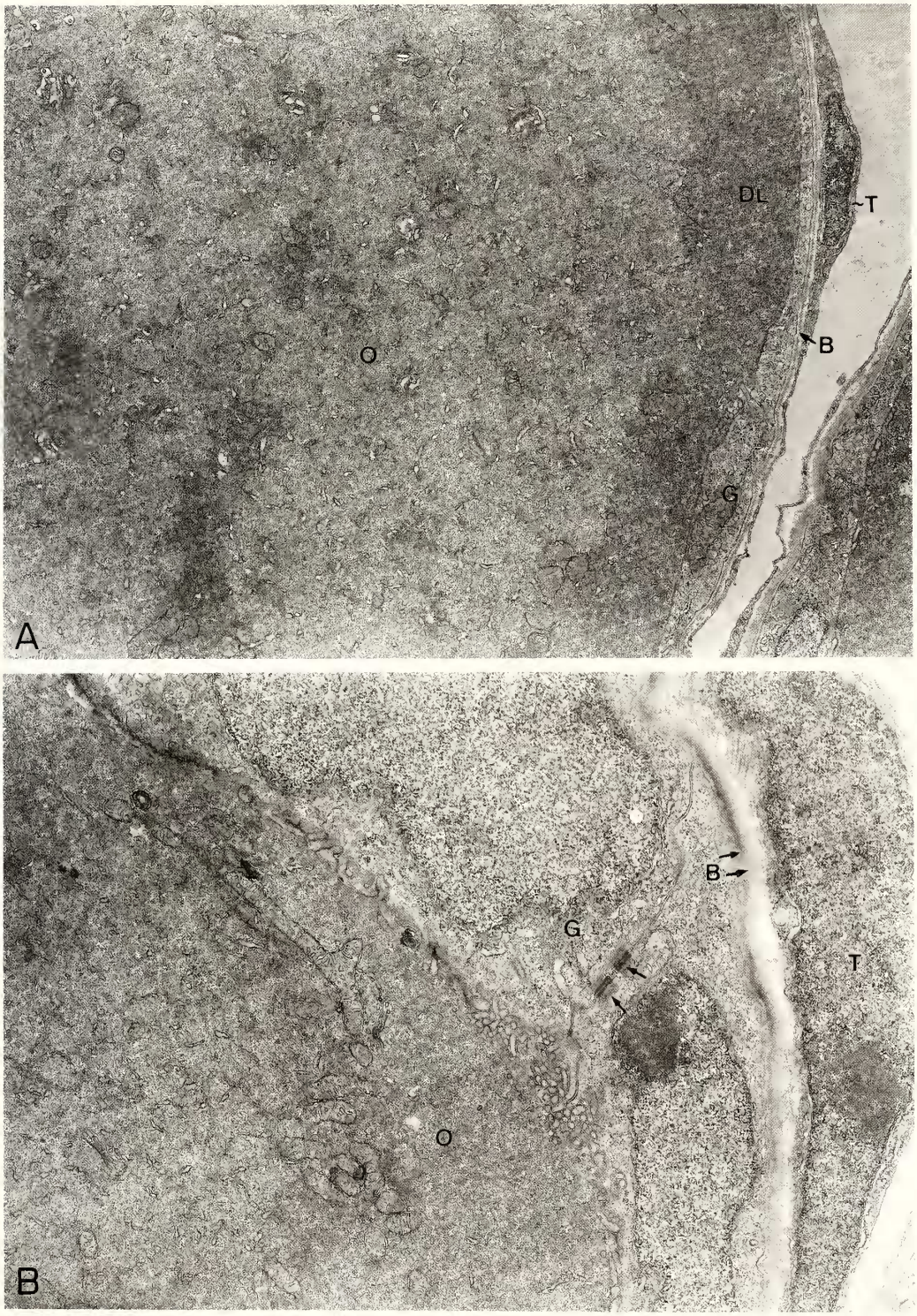


FIG. 6.

formation stage): The oocytes in these follicles (121–150 μm, Figs. 2 and 3) possess minute bumps as rudiments of short villi (Fig. 7A) on thin rudiments of chorion (about 0.1 μm in thickness) among ooplasmic projections (Fig. 7B). The oocyte surface possesses long microvillus-like projections among which very thin and electron-dense rudiments of the chorion have begun to appear. In the cortical region of the oocyte, scattered tubular ER, mitochondria (approx. 0.3–0.5 μm in diameter) and Golgi complexes have increased markedly in number (Fig. 7B). The oocytes belonging to this stage exhibit the differentiation of long chorion-villi (attaching filaments) at the vegetal pole region of the chorion (0.2–0.3 μm in thickness) and still possess the yolk nucleus (Fig. 1) in the cytoplasm. The most developed yolk nucleus consists of thread-like bodies [24], mitochondria, ER, Golgi lamellae, vesicles and multivesicular bodies (internal small vesicles, about 50 nm) (Fig. 8). Granulosa cells with desmosomes at intercellular junctions have become thicker than in the previous stage, and their cytoplasmic organelles such as mitochondria, ER and Golgi complexes have increased.

Early vitellogenic phase (pre-yolk formation stage)

The size of the oocytes markedly increases in this phase. The oocytes in those follicles (151–400 μm, Stages V and VI of Figs. 2 and 3) possess yolk vesicles forming from one to several layers and oocupying the greater part of the cytoplasm. Minute fatty or oil droplets appear mainly in the deeper region of the cytoplasm at the end of this phase.

Stage V (early yolk vesicle stage): The oocytes in these follicles (151–250 μm) are characterized by the appearance of 1–2 layers of small vesicles which contain granular materials with a lower electron density than the surrounding cytoplasm. The yolk nucleus is obscure or not observed in the cytoplasm. Oil droplets appear adjacent to the indented nuclear envelope. The nucleoplasm containing shortened lamp-brush chromosomes and ring-shaped nucleoli is deeply stained with haematoxylin. A new innermost layer has been found on the inside of the outermost layer of the chorion by deposition of electron-dense and amorphous material (0.2–0.6 μm in thickness) that is detectable as small vesicles in the vicinity of the oocyte surface (Fig. 9A). Numerous vesicular ER, lamella bodies [25] and mitochondria with minute electron-dense granules are distributed throughout the cortical cytoplasm of the oocytes (Fig. 9A). A special cell, presumably the micropylar cell, is observed among the granulosa cells with their desmosome junctions.

Stage VI (late yolk vesicle stage): The oocytes in these follicles (251–400 μm) before yolk formation have 2–6 layers of large yolk vesicles which occupy the greater part of the cytoplasm. Lipid granules are found in the perinuclear region and small yolk granules appear in the cytoplasm at the end of this stage. The protein band pattern is characterized by several bands (less than 20 k, 29 k, 37 k, 42 k, 53–80 k M_r; Fig. 4). The nucleoplasm has a high affinity for haematoxylin and contains shortened chromosomes and ring-shaped nucleoli. As the oocyte enlarges, the innermost layer of the chorion thickens from 1.5 μm to 3 μm (Fig. 9B). Electron-lucent vesicles are seen in the cortical cytoplasm (Fig. 9B), probably blebbing inward as pinocytotic vesicles (see [10, 26]). Granulosa cells have developed rough ER, dilated smooth lamellae and lysosome-like bodies, and are in contact with ooplasmic projections via gap junctions. The ability to produce E_2 is first recognized in follicles of this stage, but these follicles do not increase their secretion of E_2 and 17α, 20β-diOHprog in response to gonadotropin.

Late vitellogenic phase

In the oocytes within follicles (401–800 μm, Stages VII and VIII of Fig. 3) of this phase, yolk

FIG. 7. Follicles containing Stage IV and V oocytes. A: Follicle (about 125 μm in diameter, Stage IV) containing an oocyte that possesses bumps of short villi on the developing rudiment of the chorion. Note numerous microprojections derived from the oocyte at the oocyte-follicle cell interface. B, basement membrane; D, desmosome; G, granulosa cell; T, thecal cell. ×16,600. B: Follicle (about 155 μm in diameter containing Stage V oocyte) showing formation of a very thin outermost chorion layer (COL) as an electron-dense layer. Mitochondria and Golgi complex (GO) are located at the peripheral region of oocyte (O). ×26,000.

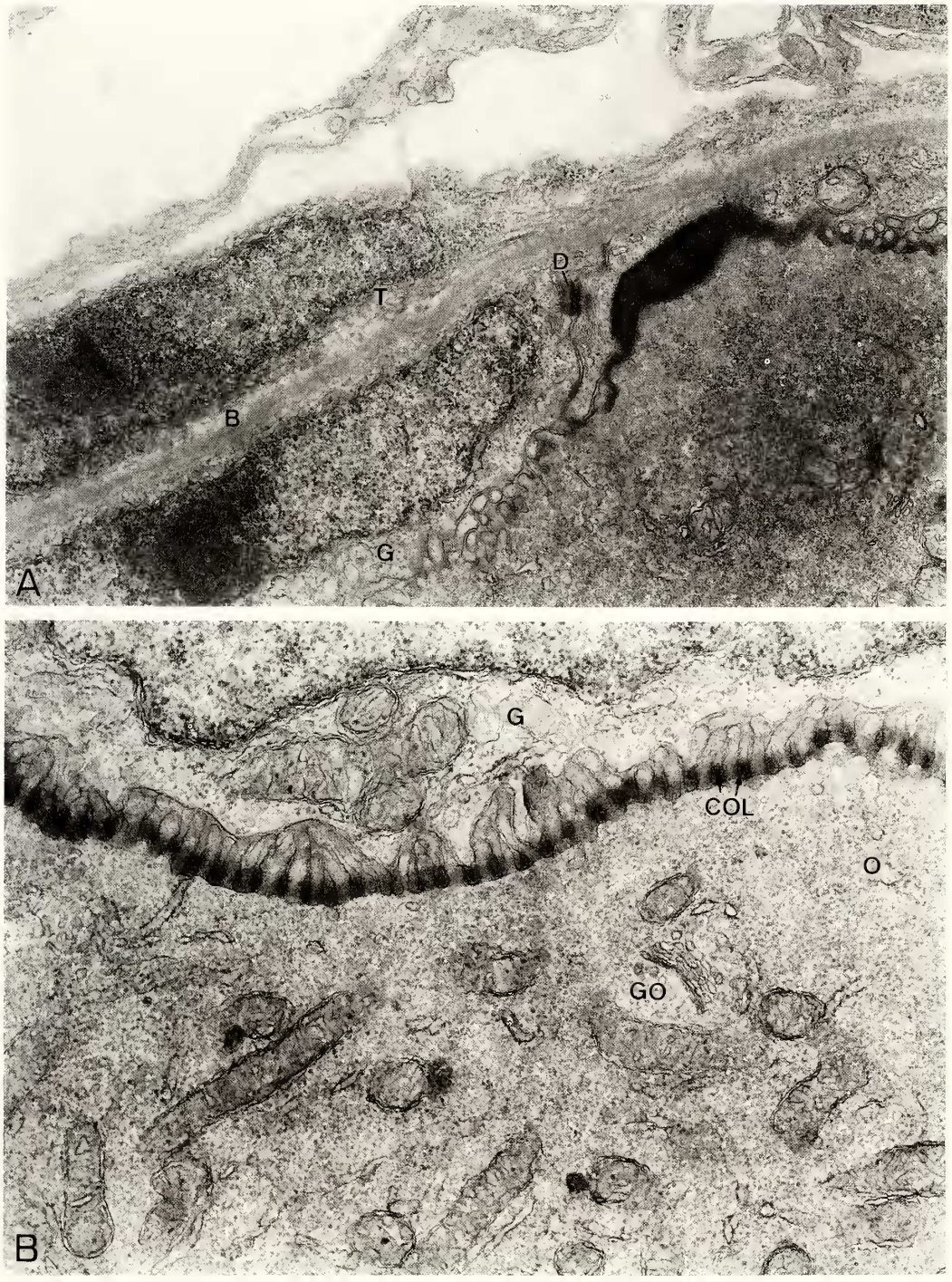


FIG. 7.

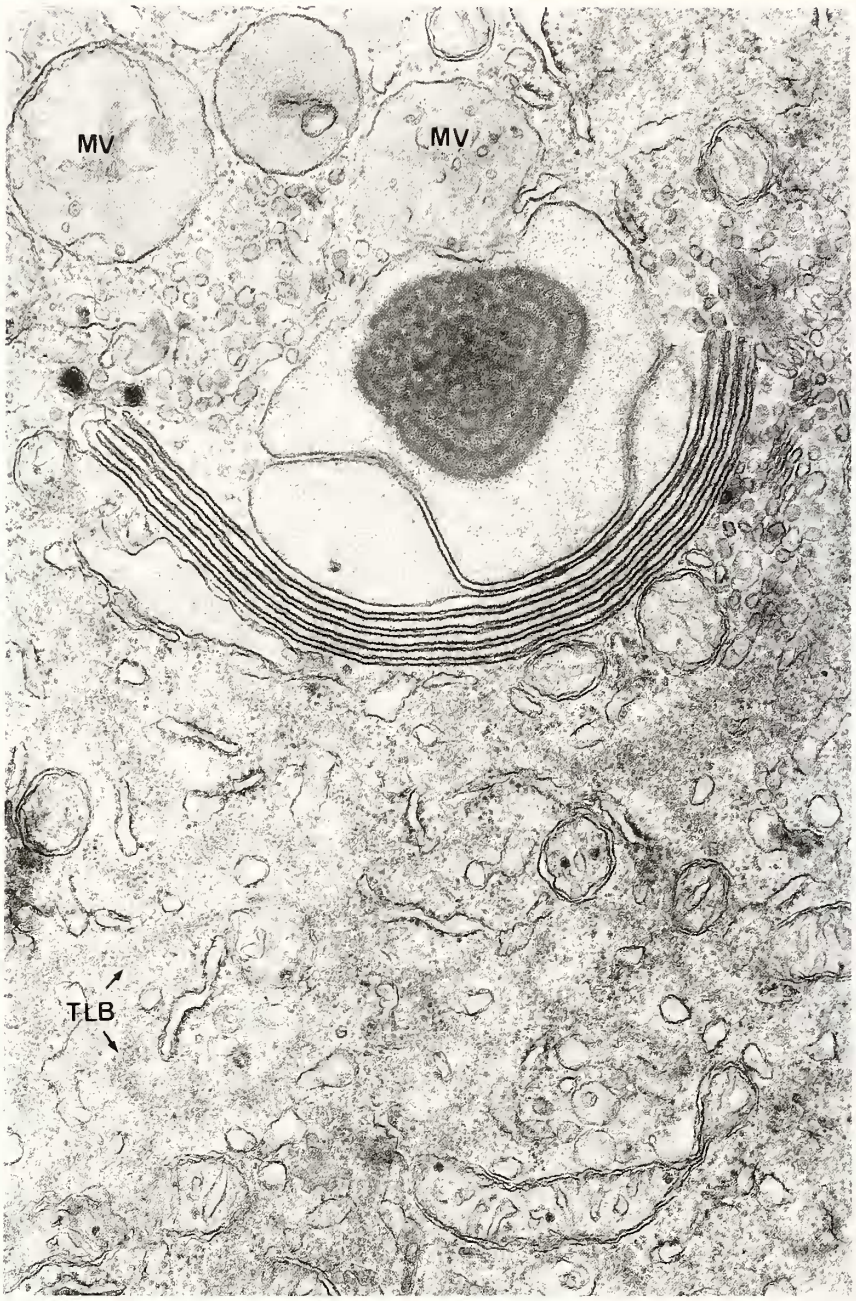


FIG. 8. Part of the yolk nucleus of an oocyte (in a follicle about 100 μm in diameter) showing numerous thread-like structures (TLB), tubular endoplasmic reticulum, Golgi lamellae, small Golgi vesicles and primitive multivesicular bodies (MV). ×31,000.

platelets first appear and fuse with each other to form yolk masses in the cytoplasm among yolk vesicles. Cytoplasmic inclusions are pushed toward the peripheral region of the oocyte as the mass of yolk enlarges in the central part of the oocyte. The protein band pattern is characterized by the new appearance of 26 k, 27 k, 30 k, 32 k, 53 k, 78 k, 98–116 k (yolk proteins), 175 k and 205

k M_r bands and the disappearance of bands less than 20 k M_r.

Stage VII (early yolk formation stage): The oocytes in these follicles (401–500 μm, Fig. 2) enlarge rapidly as fusion of small yolk platelets to form yolk masses among yolk vesicles advances. Protein bands of 98 k–116 k and 175 k M_r first appear in this stage. The cortical cytoplasm contains a number of mitochondria, ER, yolk granules (YG), cortical alveoli (CA) and oil droplets (L) (Fig. 10A). A nearly formed micropyle (Fig. 1C) is easily recognized at the animal pole of the chorion. Cytoplasmic projections of oocytes are 0.2–0.3 μm in width, and their cytoplasmic matrix is more electron-dense than that of the follicle cell projections. At this stage, the egg nucleus (germinal vesicle) begins to migrate toward the animal pole where the chorion (about 5 μm in thickness) is thicker than at the vegetal pole region.

Gap junctions between granulosa cells (Fig. 10B) are observed. Follicles of this stage are most active in secreting E_2 and secrete slightly detectable ($P<0.05$) 17α, 20β-diOHprog. Gonadotropin stimulates an apparent increase in E_2 secretion by follicles 450 μm in diameter, while it does not increase their 17α, 20β-diOHprog secretion (Fig. 5).

Stage VIII (late yolk formation stage): The oocytes which have formed a large yolk sphere in these follicles (501–800 μm, Figs. 1B and 2) show morphological differences between the animal and the vegetal poles. The chorion is thicker in the animal hemisphere than in the vegetal hemisphere, the micropyle is located at the animal pole, attaching filaments exist at vegetal pole (Fig. 1B), and the nucleus is located at the animal pole of the oocyte. The cortical ooplasm with the most elongated microprojections is filled with Golgi complexes, aligned ER, CA, yolk granules (YG) and ribosomes (Fig. 10D). Isolated these oocytes still fail to resume meiosis in response to maturation-inducing steroids (MIS; progesterone, or 17α, 20β-diOHprog) during incubation. Granulosa cells, which display pronounced mitochondria with well-developed tubular cristae and a dense matrix, actively produce E_2 and 17α, 20β-diOHprog. Production of these steroids is stimulated by gonadotropin ($P<0.05$) (Fig. 5).

Postvitellogenic phase

The fully grown oocytes within preovulatory follicles (801–1200 μm) have the potential to initiate their maturation events in response to MIS. Follicles are capable of producing E_2 and 17α, 20β-diOHprog (Fig. 5).

Stage IX (maturation stage): The oocytes within the largest follicles (801–1200 μm) 0–24 hr before ovulation are capable of undergoing maturation when incubated with MIS. The oocytes approaching the resumption of meiosis clearly exhibit a large germinal vesicle in the vicinity of the micropyle. Before initiation of maturation, the cortical cytoplasm exhibits many mitochondria, annulate lamellae, rough ER and Golgi complexes (Fig. 11). In maturing oocytes the nucleus changes with GVBD leading to formation of the metaphase II spindle of meiosis. The first polar body is extruded at the animal pole. In the granulosa cells of this stage, the nucleus is located in the vicinity to the basement membrane (Fig. 12A). Marked features of the granulosa cells before initiation of oocyte maturation are mitochondria with highly developed tubular cristae and dense-matrix, many vacuoles among the Golgi complexes, transport vesicles and dilated ER (Fig. 12B). All cytoplasmic projections of both the oocyte and granulosa cells have withdrawn completely from the chorion by the time of ovulation. At the end of this stage, ovulation takes place: oocytes are squeezed from the vegetal pole side out of the follicular layers.

Stage X (postovulatory stage): Ovulated oocytes (eggs, approx. 1200 μm) exposing short villi and long attaching filaments on the chorion are in the ovarian lumen. Under the light microscope, the whole egg is semitransparent, and numerous cortical alveoli (CA, 0.4–40 μm) and oil droplets (1–50 μm) are visible throughout the entire egg surface, except for a restricted area at the animal pole. A few Golgi complexes and no annulate lamellae are observed in the cortical cytoplasm, as reported in previous notes [9, 27]. The protein band pattern is characterized by faint protein bands of 175 k and 205 k M_r, and reappearance of 20 k, 21 k and 45 k bands (Fig. 4).

DISCUSSION

The present study has summarized cytological and histological characteristics of developing oocytes of *Oryzias latipes* which were assigned to five phases and ten stages (Table 2). Phases were assigned from the viewpoint of vitellogenesis. The stages of oocyte development, which were classified on the basis of observations of changes in the nucleus and the cytoplasm, and of the formation of the egg membrane (chorion), were compared with those reported in early investigations (Table 3).

Oocytes less than 20 μm in diameter, which were designated as the chromatin-nucleus stage by Yamamoto and Yoshioka [17], seem to correspond to oogonia [23] with a large nucleolus and chromatin-threads in the nucleus. The perinucleolar stage (Pn) oocytes in the classification of Yamamoto and Yoshioka [17] are divided into Stage I (small oocytes less than about 20 μm with the nucleus displaying several nucleoli and chromatin-threads) and Stage II (slightly larger oocytes, which have a large nucleus with many perinucleoli, as in *Fundulus heteroclitus* [28]). Yamamoto [15] and the present study, which employed electron microscopy, also divided the stage A oocytes of an early classification [16] into two stages based on fine structural differences in the ooplasm. Our classification of the oocytes, based on changes in oocyte volume as well as cytoplasmic and nuclear morphology of oocytes surrounded by extremely flattened granulosa cells with well developed desmosomes, is as a whole consistent with the observations of Yamamoto [16]. Thus, the early previtellogenic phase before formation of the chorion is conveniently divided into Stage I (chromatin-thread stage) and Stage II (perinucleolus stage).

Concomitant with development of follicles, the growing oocytes in which the cytoplasm faintly stains with haematoxylin can be distinguished from those of the previous stage. The late previtellogenic phase was established for two developmental stages of oocytes (Stages III and IV) with both the developing chorion and the most developed yolk nucleus present before formation of yolk vesicles. The present criterion for classification of these stages was the bipolar differentiation of the follicle, which was not studied in detail by any previous investigators. With advancing differentiation of the chorion, the egg polarity is recognized by appearance of attaching filaments on the chorion at the vegetal pole side in the vicinity of the yolk nucleus. Fine structural studies have revealed the yolk nucleus as an extensive aggregate of cell organelles such as thread-like bodies, mitochondria, Golgi complexes, smooth ER, vesicular bodies and plate structures. The thread-like bodies especially characterize the yolk nucleus [14]. Quite similar bodies in *Xenopus* oocytes (100 μm) have been described by Takamoto [24]. During the late phase of previtellogenesis, another typical feature is the most highly-developed lamp-brush chromosomes that possibly correlate with the most highly-developed yolk nucleus. In addition at this stage the maximal development of pinocytosis is seen at the oocyte surface, and the yolk precursors are actively incorporated. Yamamoto [16] and Yamakawa [13] assigned the yolk formation stage to oocytes that enlarged as yolk vesicles and mass increased.

The central zone of the cytoplasm has small yolk vesicles in the oocytes designated as being at the stage characterized by the dispersing yolk nucleus. The oocytes of Stage V are characterized by disappearance of the yolk nucleus and appearance of both a layer of yolk vesicles in the central part of the cytoplasm and two layers of the chorion. In addition to these features, other characteristics are appearance of oil droplets adjacent to the nucleus

FIG. 9. Follicles containing Stage V and VI oocytes. A: Follicle (about 200 μm in diameter) containing oocyte (Stage V) with thick outermost layer of the chorion. Microprojections from granulosa cells are observed at a low frequency. Arrows indicate electron-dense material. ×16,600. *Inset*: Lamellar body in the ooplasm. ×32,000. B: A part of the chorion of an oocyte at Stage VI. It consists of outermost (COL) and innermost (CIL) layers, into which microprojections (GP, from the granulosa cell; OP, from the oocyte) of both the oocyte (O) and the granulosa cell (G) insert. The granulosa cell surface is in contact with an ooplasmic projection (arrow heads). Arrows indicate small electron-lucent vesicles intact with the oocyte surface. ×20,000.

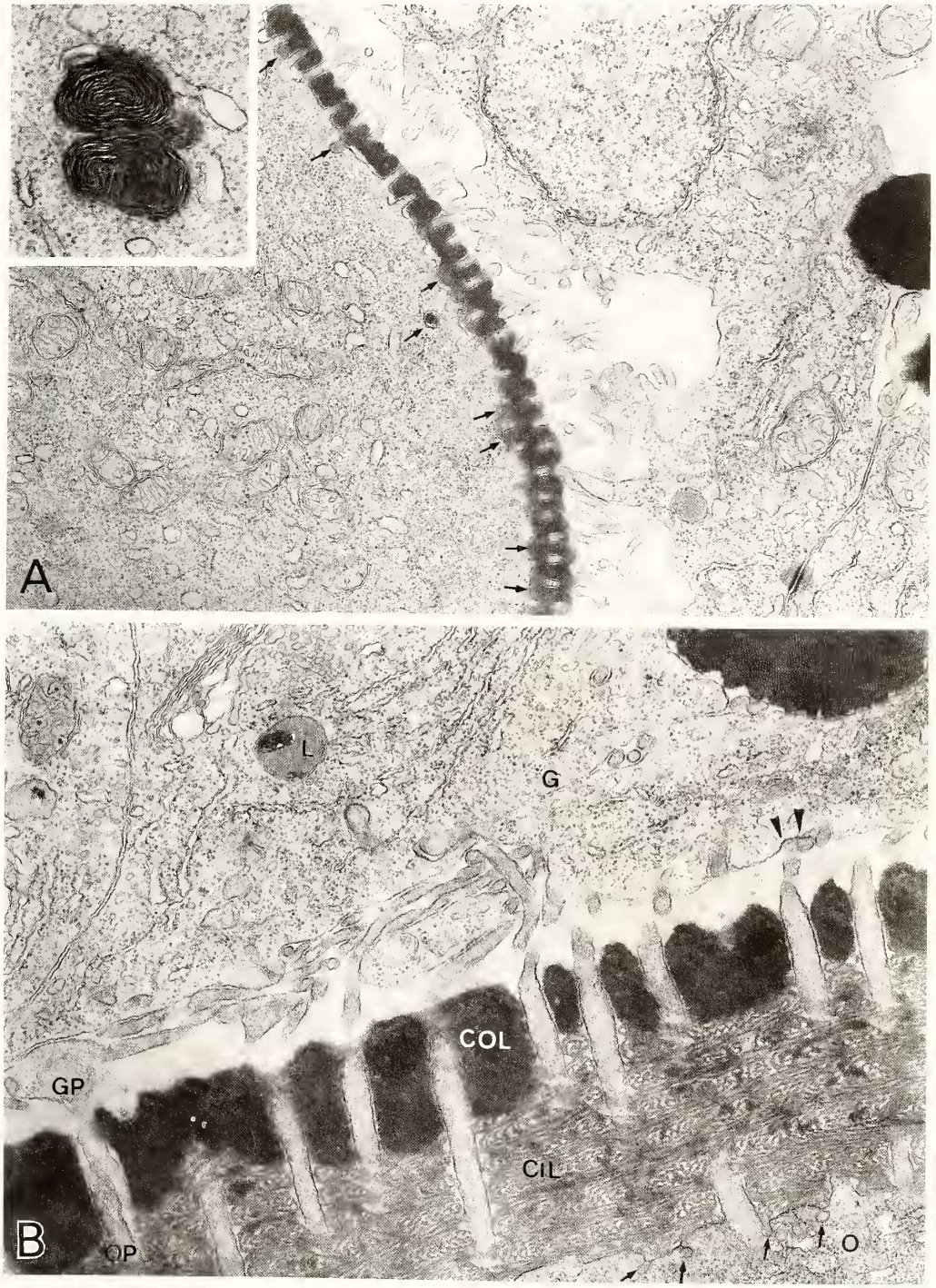


FIG. 9.

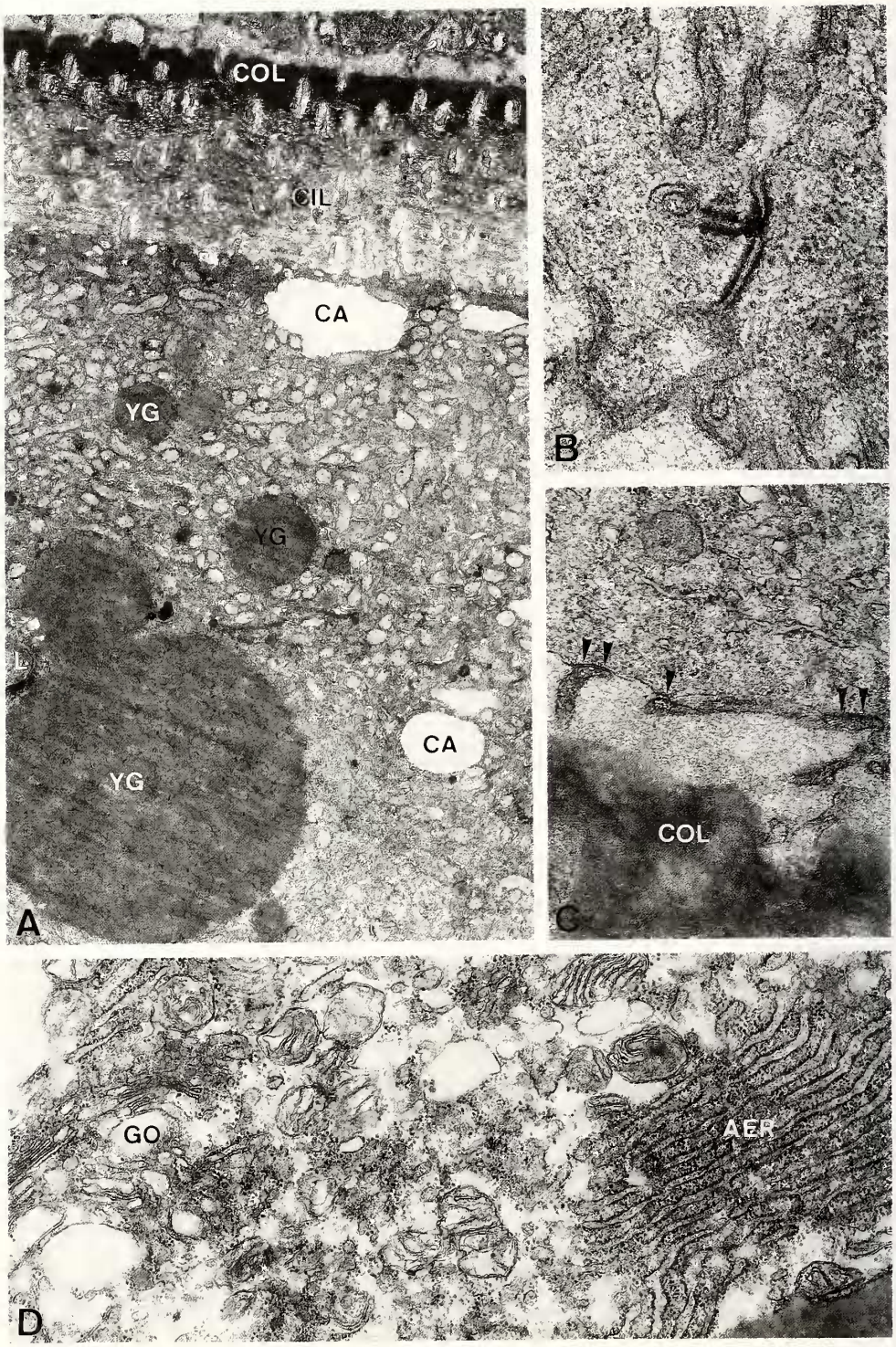


FIG. 10.

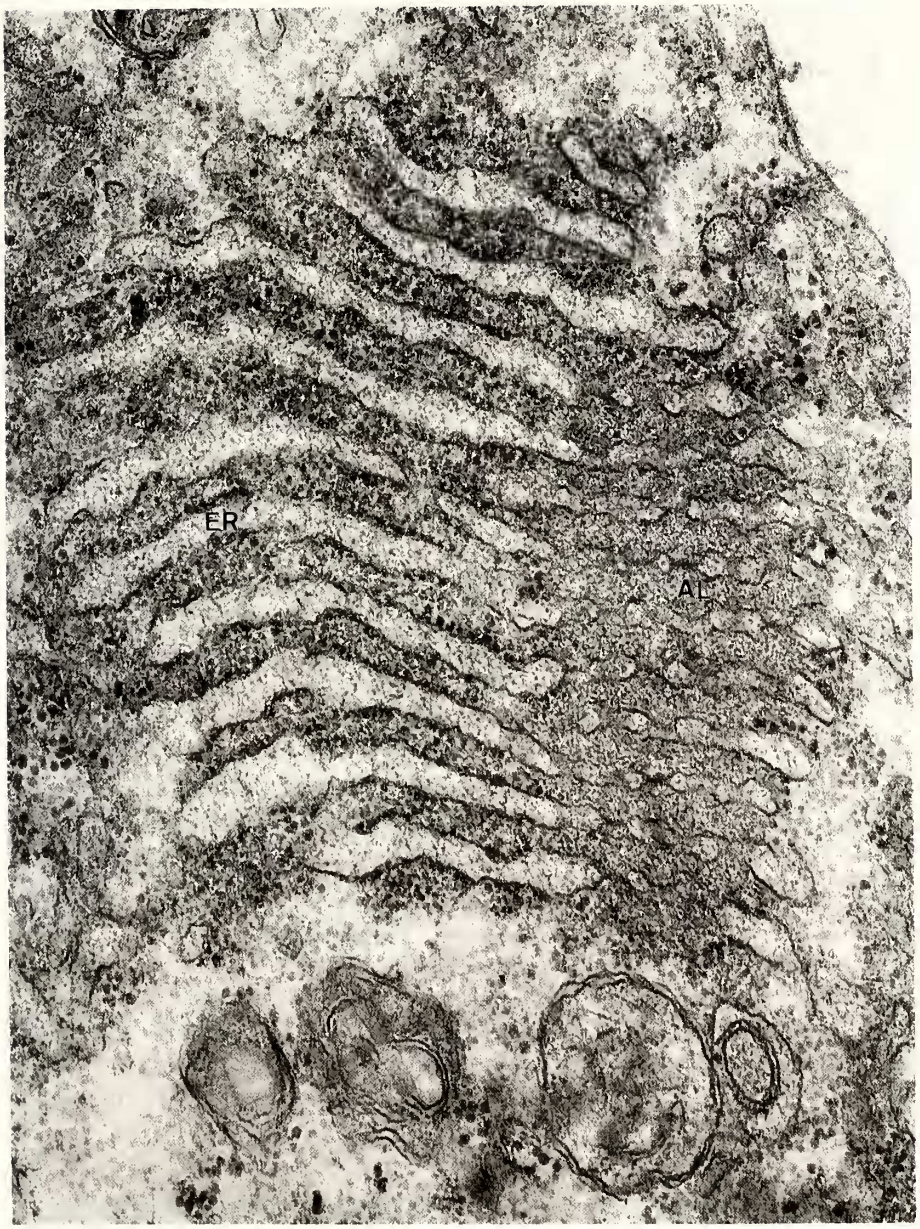


FIG. 11. Annulate lamellae in an oocyte at the maturation stage (Stage IX). Note the lamellae (AL) that are continuous at the end with lamellae of rough endoplasmic reticulum (ER). ×55,200.

FIG. 10. Portions of follicles containing Stage VII and VIII oocytes. A: Cortical cytoplasm of Stage VII oocyte. Note ooplasmic inclusions such as cortical alveoli (CA), numerous mitochondria, yolk granules (YG) and oil droplet (L). ×7,000. B: Gap junctions among granulosa cells surrounding Stage VII oocyte. ×44,800. C: Gap junctions (arrow heads) between Stage VII oocyte microprojections (electron dense) and granulosa cell. ×25,700. D: Cortical cytoplasm of Stage VIII oocyte. Note piled endoplasmic reticulum (AER) and many Golgi complexes (GO). ×23,100.

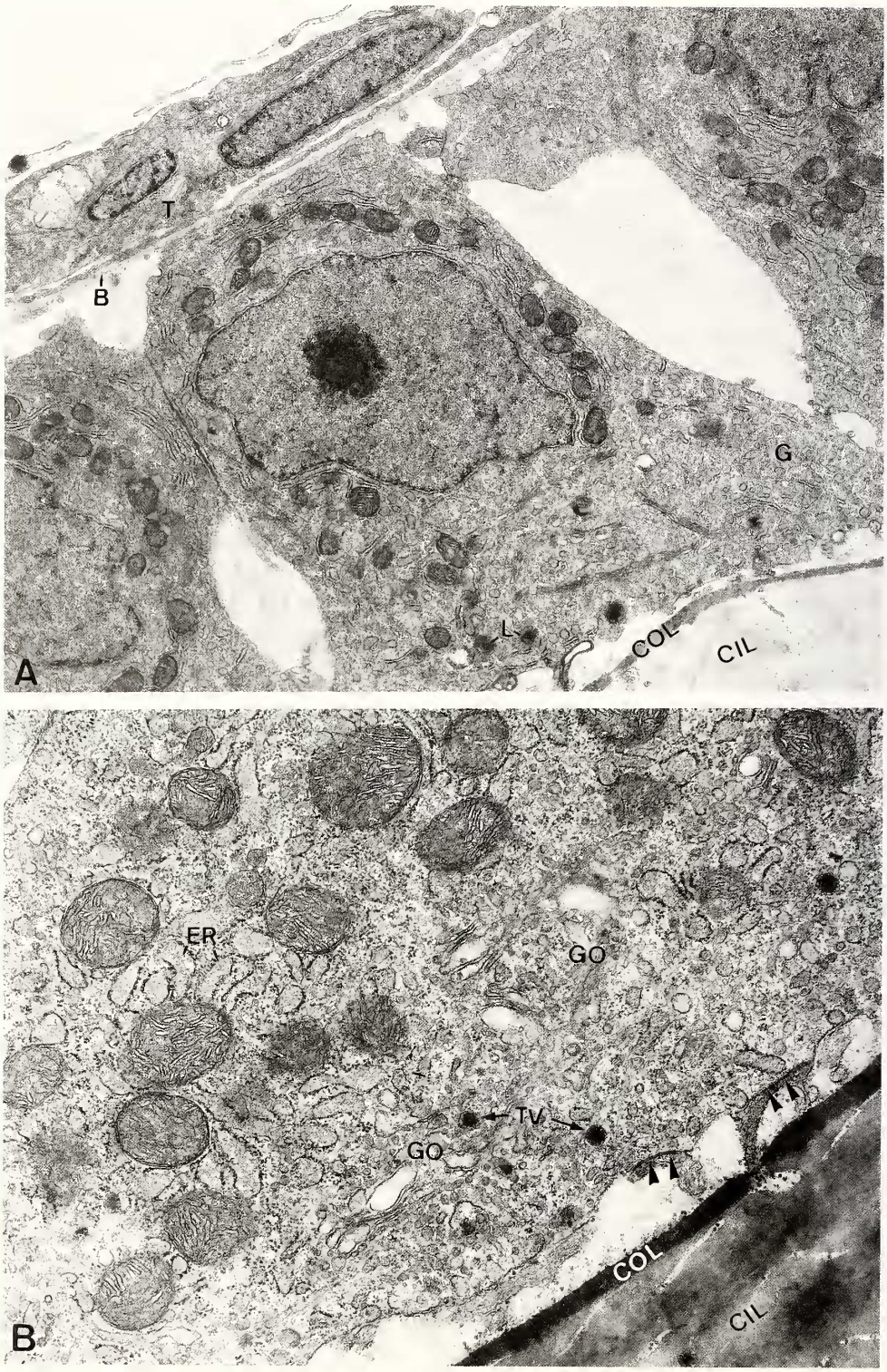


FIG. 12.

and ring-shaped nucleoli in the nucleoplasm. Our observations of Stage V oocytes are almost consistent with those reported by Yamamoto [14] in which irregularly shaped nucleoli become voluminous. At Stage V the two or more innermost layers are piled inside the outermost layer of the chorion. This mode of chorion formation has been proposed by Tesoriero [29]. The innermost layers are produced by the oocyte. Another characteristic of this stage is that yolk vesicles (or goutte claires, [30]), which contain PAS positive material [4, 13, 16, 31], are observed in the ooplasm. These vesicles correspond to the cortical alveoli (vesicles) in the mature egg. A change in the ER from a tubular to a vesicular form with ribosomes seems to reveal the beginning of active protein synthesis.

In hypophysectomized females [32], which are responsive to E_2, the ovary contains only young oocytes earlier than those of this stage. Oocyte development beyond Stage V depends on stimulation by gonadotropin.

At the yolk vesicle stage of Yamamoto and Yoshioka [17], the oocytes that possess several layers of yolk vesicles in the cytoplasm are designated as Stage VI. In this stage, oocyte volume rapidly increases, but yolk globules (small yolk masses) are hardly observed. The oocytes in which small yolk globules appear between large yolk vesicles are designated as Stage VII, belonging to the late vitellogenic phase. The appearance of yolk globules is quite consistent with that of yolk proteins of 98–116 k M_r, as shown in *Fundulus* oocytes [33]. In this stage, small yolk masses are found in the peripheral region of the cytoplasm among yolk vesicles and numerous oil droplets.

Beyond this stage, oocytes in which the yolk mass has rapidly enlarged in the central region are designed as Stage VIII, as described by Yamamoto [16], Yamakawa [13] and Yamamoto and Yoshioka [17]. In these growing oocytes, there are markedly increased microprojections of the oocyte surface toward the granulosa cells through the pore canals of the chorion, as well as projections of granulosa cells toward the oocyte surface. Gonadotropin stimulates an apparent increase in E_2 secretion by follicles of both Stages VII and VIII, but follicles of Stage VII do not secrete increased 17α, 20β-diOHprog in response to gonadotropic stimulation while those of Stage VIII do. Thus, follicles containing oocytes of Stage VII are different from those of Stage VIII in their steroid synthesis capacities.

As the yolk mass enlarges, the yolk vesicles and the nucleus (germinal vesicle) shift toward the peripheral region of the oocyte and the nucleus are finally located at the cortical cytoplasm (the animal pole) near the micropyle. Most investigators have reported the late stage of yolk formation as the maturation stage. However, oocytes within follicles about 800 μm in diameter are the first that are able to respond to the MIS reinitiating meiosis [20]. Therefore, we assigned Stage VIII to large oocytes that have not yet obtained the capacity for resuming meiosis. During the maturation stage, the nuclei of the granulosa cells move from the side facing the chorion to the opposite side of the cell. Additionally, cytoplasmic protrusions of both the follicle cells and the oocyte into the chorion have shrunk and withdrawn. The oocytes rapidly enlarge up to about 1200 μm in diameter, primarily due to hydration [5, 34], and ovulate from the vegetal pole region [8].

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FIG. 12. Preovulatory follicles (about 900 μm in diameter). A: Special granulosa cells as progesterone-producing cells contain characteristic mitochondria with tubular cristae and dense matrix. L, lysosome-like body. ×6,600. B: A portion of typical granulosa cell exhibits dilated Golgi lamellae (GO), endoplasmic reticulum and round mitochondria with many tubular cristae. Note gap junctions (arrow heads) between oocyte projections and granulosa cell. TV, transport vesicle. ×21,000.

TABLE 2. Characteristics of the

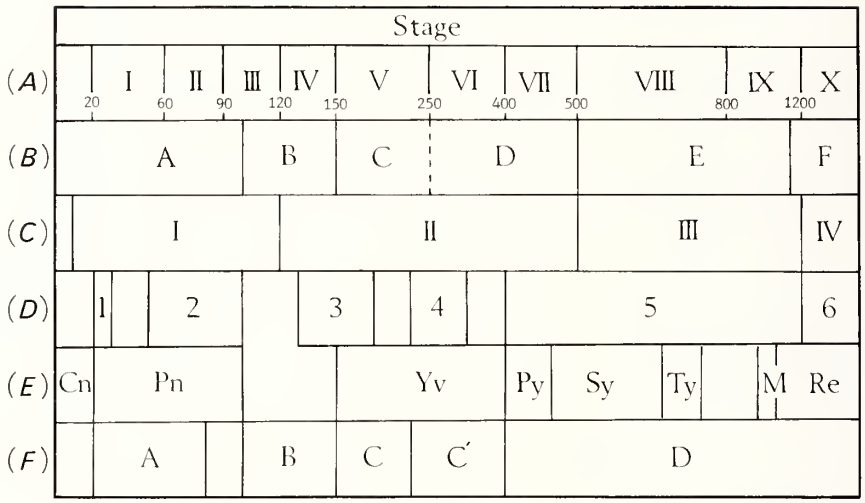
Phase		Stage	Follicle size (μm)	General appearance	Nucleus
Previtellogenic	Early	I	20 to 60		Centrally located and roughly spherical. Nucleoli and chromatin-threads with network appearance
		II	61 to 90	Transparent cytoplasm and nucleus with round nucleoli clearly visible	Centrally located with peripheral nucleoli and thin lamp-brush chromosomes
	Late	III	91 to 120		Centrally located. Folded envelope. Voluminous nucleoli irregular in shape. Developed lamp-brush chromosomes
		IV	121 to 150	Transparent cytoplasm and nucleus with irregular shaped nucleoli clearly visible. Rudiments (bright spots) of short villi on chorion	Centrally located. Folded envelope. Voluminous nucleoli ring-shaped. Developed lamp-brush chromosomes
Vitellogenic	Early	V	151 to 250	Transparent cytoplasm and nucleus with ring-shaped nucleoli. Elongated short villi and attaching filaments on chorion	Folded envelope. Nucleoplasm strongly stainable with haematoxylin. Lamp-brush chromosomes thicker and shorter. Ring-shaped nucleoli
		VI	251 to 400	Transparent cytoplasm. Attaching filaments opaque in vegetal hemisphere	
	Late	VII	401 to 500	Attaching filaments opaque in vegetal hemisphere. Dark lipid granules in perinuclear region of cytoplasm filled with vacuoles (yolk vesicles) and oil droplets	Very irregular in shape. Displaced toward animal pole. Shortened chromosomes. Reduced in volume. Nucleoplasm strongly stainable with haematoxylin.
		VIII	501 to 800	Cortical alveoli (yolk vesicles) and oil droplets dimly visible in translucent cytoplasm surrounding yolk sphere	Located at animal pole. Condensed chromosomes massed in center of nucleoplasm
Post-vitellogenic	Maturation	IX	801 to 1200	Cortical alveoli and oil droplets clearly seen in light cortical cytoplasm. A large germinal vesicle visible near micropyle until resumption of meiosis	Apparent large vacuole within outer nuclear envelope at ovarian surface side. Nucleoli and nuclear envelope disappear with resumption of meiosis
	Ovulated	X	1200*	Projecting short villi and attaching filaments on chorion. Transparent and light cytoplasm clearly showing cortical alveoli and oil droplets	Second metaphase figure at animal pole in cortical cytoplasm

*Egg.

various stages of oocyte development

Cytoplasm	Chorion	Follicle cells
A few microvilli and small number of poorly developed ER and mitochondria	Absent	Extremely flat
Clusters of long microvilli (cytoplasmic projections). Yolk nucleus, a small number of mitochondria and ER. Electron-dense cortex	Absent	A single layer each of thecal and granulosa cells. Desmosomes developed between adjacent granulosa cells
Cytoplasmic projections evenly distributed on whole surface. Yolk nucleus, increased mitochondria and ER. Faintly stainable with haematoxylin	Outermost layer rudiments among cytoplasmic projections of oocyte. Short villi bumps appear	
Most developed yolk nucleus. Increased mitochondria in cortex. Faintly stainable with haematoxylin	Outermost layer (0.2–0.3 μm) rudiments among long cytoplasmic projections. Elongated short villi. Differentiation of attaching filaments	Granulosa cells thick at attaching filament side
Elongated cytoplasmic projections. Broken yolk nucleus and a single layer of small yolk vesicles	Outermost layer 0.2–0.6 μm in thickness. Two layers at end of this stage	Micropylar cell detectable
Very elongated cytoplasmic projections. Two to several layers of large yolk vesicles occupy most of cytoplasm. Lipid granules at perinulear region and dense yolk granules	Outermost layer 0.5–0.6 μm and innermost layer 1.5–3 μm. Micropyle incomplete	Thick granulosa cells in vegetal hemisphere. E_2 production
Cytoplasmic projections irregularly contact with granulosa cells by gap junctions. Yolk vesicles fully occupy cytoplasm. Small yolk masses among yolk vesicles. Increased oil droplets	Thick in animal hemisphere. Outermost layer 0.6 μm and innermost layer about 4 μm	E_2 production responsive to gonadotropic stimulation
Most elongated cytoplasmic projections. Yolk vesicles and oil droplets aligned in a single layer in cortex. Central yolk mass occupies most of cytoplasm.	Very long attaching filaments at vegetal pole region, coiled around vegetal hemisphere. Ten μm in total thickness	17α, 20β-DiOHprog and E_2 production responsive to gonadotropic stimulation
Cortical alveoli (yolk vesicles), oil droplets, mitochondria, vesicular ER, Golgi complexes, annulate lamellae and multivesicular bodies in thin cortex	Cytoplasmic projections withdrawn at end of this stage	Nucleus located at side of basement membrane. Sifted off at end of this stage (micropyle open). E_2 and 17α, 20β-diOHprog production
Transparent and light cytoplasm showing clearly cortical alveoli and oil droplets	Projecting short villi and attaching filaments on chorion. Innermost layers showing a stratiform state (12–14 layers)	Absent

TABLE 3. Comparison of the present classification of the stages of oocyte development with those of early investigations of *Oryzias latipes* oogenesis



(A) The present classification
(B) Yamamoto, T. S. (1955)
(C) Yamakawa, Y. (1959)
(D) Yamamoto, M. (1964)
(E) Yamamoto, K. and Yoshioka, H. (1964)
(F) Iwamatsu, T. (1973)

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