

ULTRASTRUCTURAL CHANGES IN OOCYTES AND FOLLICLE CELLS DURING TADPOLE DEVELOPMENT

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ABSTRACT

J. J. Wang and C. Y. Hsü (1974). *Ultrastructural Changes in oocytes and follicle cells during tadpole development*. Bull. Inst. Zool., Academia Sinica 13(2): 75-86. Changes of some important organelles in oocytes and follicle cells in *Rana catesbeiana* at various stages between young tadpoles and newly metamorphosed froglets have been studied by electron microscopy. Mitochondrial masses, intramitochondrial yolk-crystals, nucleolus-like bodies-mitochondria complexes, intercellular bridges and annulate lamellae are traced during development and their significances discussed. An intranuclear mitochondrion in an oogonium and smooth ER, round mitochondria with tubular cristae and cilia in follicle cells are reported for the first time in amphibians.

Development of the amphibian ovary involves mainly the differentiation, growth and maturation of oocytes and follicle cells. Organelles within these cells, consequently, show a series of changes. Investigation dealing with fine structures in amphibian oocytes have been numerous^(3, 8-10, 17-19, 22-30, 35, 41, 43, 45, 47-49). However, whereas most of them are related to oocytes of adult animals, those concerning tadpoles are few. When literatures relating to ultrastructures in oocytes of *Rana catesbeiana* are reviewed, only two organelles were studied, cytoplasmic cylinders in tadpoles⁽²⁷⁾ and intramitochondrial yolk-crystals in adults^(28, 29). In our laboratory, tadpoles of *R. catesbeiana* have been used exclusively as the experimental animals for the study of sex transformation. Therefore, the present investigation pertains to structural changes of some important organelles in germ cells and follicle cells during normal development of *R.*

catesbeiana tadpoles. The result will serve not only as a basic understanding of the ultrastructural development but also as criteria for comparison with that in abnormal sex differentiation.

MATERIALS AND METHODS

Tadpoles of *R. catesbeiana* were reared in dechlorinated tap water at 20°C. Ovaries were excised from pithed tadpoles at the ages of 3, 6, 10 and 12 months and of 15 month-old froglets. The relevant data of the animals used are summarized in the table I.

Small pieces of ovaries were fixed with ice-cold 1.2% glutaraldehyde in 0.05 M phosphate buffer at pH 7.4. The fixed materials were washed with several changes of 0.1 M phosphate buffer and post fixed in buffered 1% osmium tetroxide. After washing, dehydration with graded ethanols and passage through propylene oxide, the specimens were embedded in epon 812. Thick sections were stained with toluidine

TABLE I
Relevant data of the tadpoles

Mating no. of parent frogs	Date of fertilization	Age in month	Average body weight in gm	Metamorphic stage*
FR 61-6	Jun. 10, '72	3	0.50	III
FR 61-6	Jun. 10, '72	6	0.86	V
FR 61-6	Jun. 10, '72	10	9.26	X
FR 61-1	Apr. 20, '72	12	8.82	XVIII
FR 60-9	Jun. 15, '71	15	7.98	XXV (Froglet)

* Stage of metamorphosis by Taylor and Kollros (44).

blue and examined under a light microscope for orientation and normality of the ovary. Thin sections were cut with Sorvall MT-2 ultramicrotome and doubly stained with uranyl acetate and lead citrate. They were observed under a JEM-100 B electron microscope.

OBSERVATION AND DISCUSSION

The ovary of a 3-month-old tadpole reared at 20°C is beginning to differentiate. It contains a prominent cortex and a reduced medulla. Three types of germ cells are identifiable in the cortex: primary oogonia, with diameter about 25 μ , have large, pale and irregularly shaped nuclei surrounded with a narrow rim of lightly stained cytoplasm; secondary oogonia are smaller cells, 12 μ in diameter with large but round or oval nuclei; and lastly, synaptic cells, with diameter of 15 μ , are young primary oocytes just entering into the prophase of the first meiosis with chromosomes in synapsis.

In the 6-month-old ovary, medulla cells have disappeared, but primary and secondary oogonia are still numerous. The latter and synaptic cells cluster respectively into nests. The primary oocytes appear much larger now with diameter about 80 μ ; they are encapsulated by a thin layer of small spindle-shaped follicle cells.

The oocytes are increasing in number and become the dominating cell type in the 10-month-old tadpole ovary. Their diameter increases to 100 μ . Cell nests are not found now,

yet few oogonia are still situating in the periphery of the ovary. The 12-month-old ovary is larger than before due to growth of oocytes which have a diameter of 150 μ now. The ovary of a 15-month-old froglet presents a similar condition as that in the previous stage.

The changes of some important ultrastructures in oogonia, oocytes and follicle cells during tadpole development are presented and their significances discussed as follows:

I. Mitochondrial Differentiation

Oocytes in amphibians usually harbor two types of mitochondria, the typical ones and those containing yolk-crystals. The former type may disperse in the ooplasm or aggregate in a mass form, while the latter are usually located at the periphery of the ooplasm. Sometimes, mitochondria have connections with the characteristic nucleolus-like bodies, probably originating from the nucleus. Therefore, mitochondria in oocytes appear to possess unique features when compared with those in somatic cells. The following paragraphs trace the changes of mitochondrial heterogeneity.

1. Mitochondrial Mass

Mitochondria in oogonia, synaptic cells and oocytes of all stages of *R. catesbeiana* tadpoles are usually found as cigar-shaped bodies either straight or slightly curved, possessing crosswise laminar cristae. The fine structure of this organelle conforms well to that described by Massover^(28,29) in adult oocytes of the same species. Mitochondria are fairly scarce and dis-

persed in the cytoplasm of oogonia and synaptic cells in 3-month-old tadpoles. This pattern remains unchanged in young oocytes of 6-month-old tadpoles. In oocytes of 10-month-old animals, mitochondria begin to increase in number rapidly and aggregate to form the first appearance of mitochondrial mass near the nucleus (Fig. 1). This association may be in the form of a single mass or several clumps. The mitochondrial mass has also been observed in oocytes of 12-month-old tadpoles. At this stage, a few mitochondria are found to migrate out of the mass and move toward the periphery of the oocyte where they may contain yolk inclusions. In froglet oocytes, the mitochondrial mass is still present. It is not certain when the mitochondrial mass will break up and become dispersed again.

As mitochondria are carriers of oxidative enzymes, the increase of mitochondrial number in oocytes is necessary for the demand of the increase of oxygen consumption during the growth of germ cells. However, the reason of their association into masses is unknown.

Mitochondrial mass was reported by Ward⁽⁴⁶⁾ in oocytes of *R. pipiens*, Wartenberg⁽⁴⁹⁾ in *R. esculenta* and Balinsky and Devis⁽³⁾ in *Xenopus laevis*.

In this study, mitochondria are sometimes observed in a branched form (Fig. 1) which could be in the process of binary fission. Massover^(28,29), Balinsky and Devis⁽³⁾ and Kessel⁽²⁵⁾ described this condition in oocytes of *X. laevis* and *R. pipiens* respectively and considered that binary fission might be one of the means to increase mitochondrial number, resulting in the formation of mitochondrial mass.

2. Intramitochondrial yolk-crystals

Intramitochondrial yolk-crystals appear first in oocytes of 12-month-old tadpoles, which coincides with Kessel's⁽²⁵⁾ observation that yolk inclusions within mitochondria began to appear in oocytes of *R. pipiens* tadpoles at Taylor and Kollros' stage XVII⁽⁴⁴⁾. Sometimes more than one crystals could reside in one mitochondrion. The crystals are in the form of hexagonal plate,

situating intracrystally. They are rather small but increase in size in 15-month-old froglets (Fig. 1). The yolk-containing mitochondria generally appear at the peripheral site of the ooplasm.

According to Wallace⁽⁴⁶⁾, protein kinase is found in the ovary of *R. pipiens*; this enzyme is capable of promoting the incorporation of phosphate into partially phosphorylated phosphatidylcholine, one of the two components of the yolk-crystal. The result of phosphorylation renders phosphatidylcholine insoluble, enabling the formation of yolk-crystals. Since protein kinase is a mitochondrial enzyme, it is natural that yolk-crystal formation could take place inside a mitochondrion.

Lanzavecchia⁽²⁶⁾ first reported intramitochondrial yolk-crystals in oocytes of *R. esculenta* tadpoles in 1958. Ward⁽⁴⁷⁾ confirmed this finding in oocytes of adult *R. pipiens*. Since then, similar reports were made in adults of other ranids^(3,25,28-30,48,49). The present finding of intramitochondrial yolk-crystals is in conformation morphologically with those described in the literatures.

Massover⁽²⁸⁾ observed in oocytes of *R. catesbeiana* several forms of modified mitochondria which could be arranged in a series of structurally intermediate forms between typical oocyte mitochondria and those with yolk-crystal inclusions. This finding led him to propose that yolk-crystals originated from some of the oocyte mitochondria by a complicate process of mitochondrial differentiation. This suggestion challenged the old concept that mitochondria containing yolk-crystals were an intermediate stage in the transformation of yolk platelets into mitochondria. The present observation of intramitochondrial yolk-crystals traces the history of yolk inclusion in mitochondria back to the tadpole stage of the same animal, and also gives an additional evidence to support Massover's view on genesis of the intramitochondrial yolk-crystals.

3. Association between mitochondria and nucleolus-like bodies

Nucleolus-like bodies are darkly stained

cytoplasmic materials of irregular contours near the nucleus (Fig. 1). They tend to lie on the flat or shallow surface of the nucleus and their density is similar to that of the nucleolus. These bodies first appear in primary oogonia of 3-month-old tadpoles and are also found in secondary oogonia, synaptic cells and oocytes of all age groups of tadpoles so far studied. They may increase in size and adhere to each other to form large aggregates. Sometimes they are found to associate with mitochondria to form nucleolus-like bodies-mitochondria complexes which first exist in secondary oogonia of 3-month-old tadpoles. The complexes develop most prominently in oocytes (Fig. 2).

Nucleolus-like bodies are also named dense bodies or "nuage" materials. They were described by Pollister *et al.*⁽¹⁵⁾ in frog oocytes in 1954. Kemp⁽¹⁶⁾ observed these bodies as nucleolar fragments released from the nuclear envelope into the ooplasm of oocytes of *R. pipiens*. Takamoto⁽¹⁸⁾ also found them as a transfer through nucleopores of nuclear RNA into the ooplasm during development of oocytes in *Triturus pyrrhogaster* and *R. nigromaculata*. Franke and Scheer⁽¹⁰⁾ claimed a similar finding in Alpine newts. Electron-dense, granular materials were described by Kawakib *et al.*⁽¹⁷⁾, probably passing between the nucleus and cytoplasm via nuclear pores in all stages of germ cell differentiation in *Xenopus*. Eddy and Ito⁽⁹⁾, working on tadpole oocytes of various frogs, held that the origin of nucleolus-like bodies was attributed to 350 Å nuclear granules forming cytoplasmic streamers of fibrous materials through nuclear pores into the cytoplasm. Therefore, these bodies are generally interpreted as materials transferring RNP from the nucleus to the ooplasm.

On account of the facts that mitochondria and nucleolus-like bodies are sometimes closely associated; that mitochondria are so unique as to be capable of making yolk inclusions intracristally; and that mitochondria can rapidly propagate their population, it seems that nucleolus-like bodies-mitochondria complex may deserve some degree of significance. Eddy and Ito⁽⁹⁾ demonstrated a negative result of a significant amount of RNA in nucleolus-like bodies with cytochemical reactions and autoradiography. However, due to technical limitation, they could not rule out the possibility of the existence of a minute quantity of RNA in these bodies. Therefore, nucleolus-like bodies mitochondria complex may indicate a functional relationship that nucleolus-like bodies could take some message from the nucleus to certain mitochondria for their specific activities in developing oocytes.

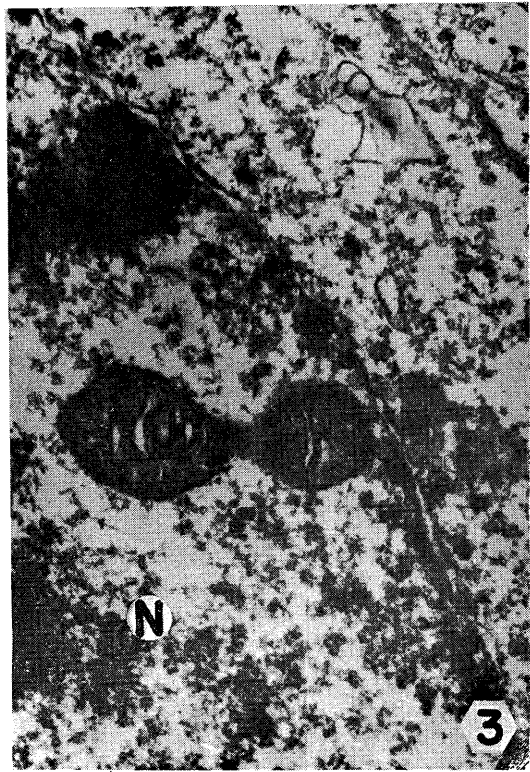
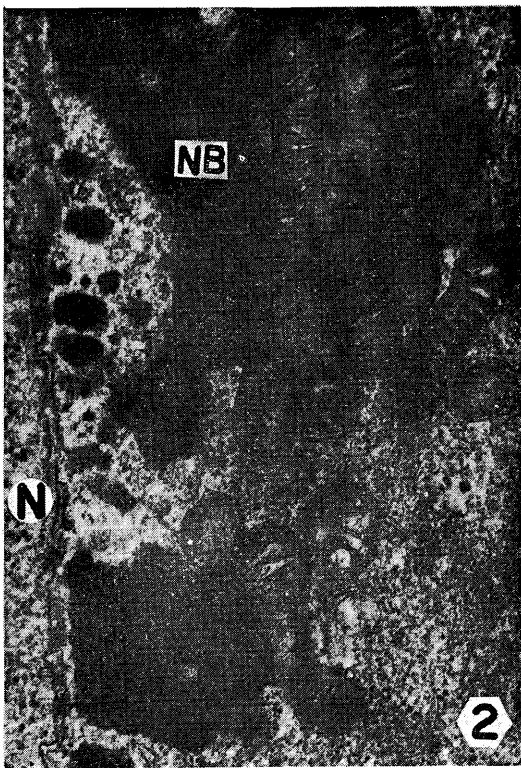
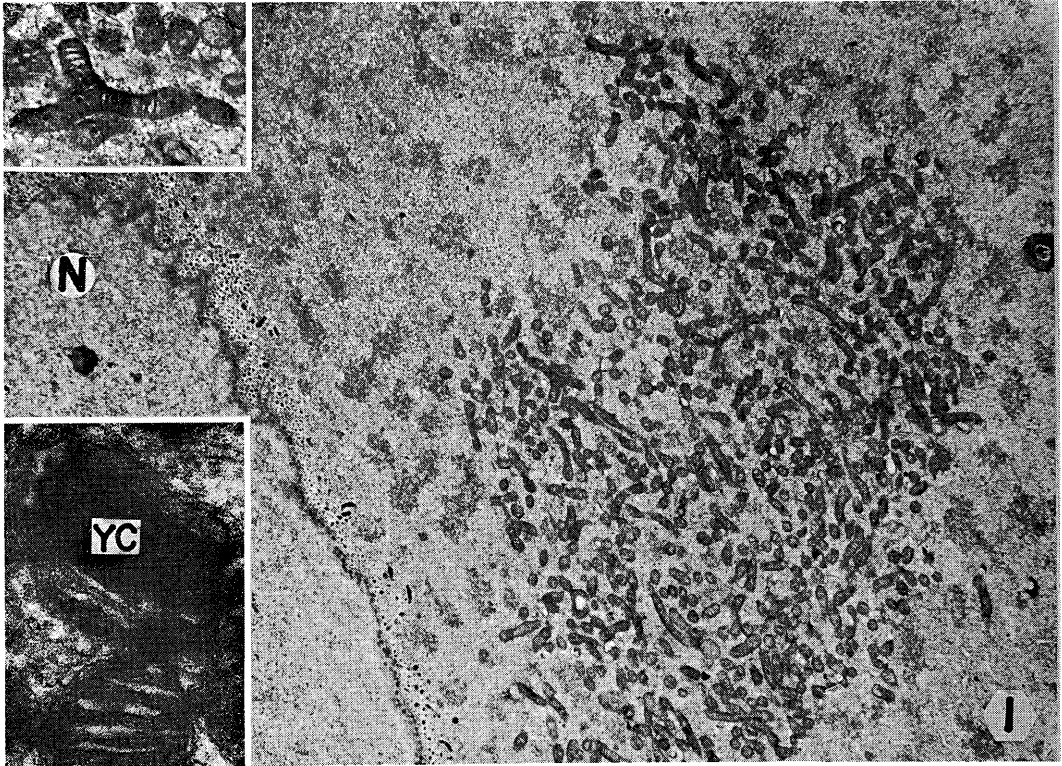
4. Intranuclear mitochondrion

It is of extreme interest to find an intact mitochondrion within the nucleus, close to the nuclear envelope of a secondary oogonium in a 6-month-old tadpole in this study (Fig. 3). The ultrastructure of this intranuclear mitochondrion was typical like any other oocyte mitochondria in developing germ cells of *R. catesbeiana* tadpoles. Thus it is clear that this unique mitochondrion could not be an artifact. However, it poses a problem as to the possible origin of this extraordinarily situated organelle.

Karasaki⁽¹⁹⁾ reported the presence in preneoplastic rat liver cells of intranuclear lipid droplets which were interpreted as being evaginated from the cytoplasm into the karyoplasm and thus being enclosed with a layer of the unit membrane. In the present case, the mitochondrion was naked besides its own outer membrane.

Explanation of Figures

1. Mitochondrial mass in oocyte of 10-month-old tadpole, numerous nucleolus-like bodies seen near nuclear envelope; N, oocyte nucleus, $\times 3,000$; upper inset showing branched mitochondrion, $\times 7,500$; lower inset indicating intracristal yolk-crystal in a mitochondrion of froglet oocyte, $\times 40,000$.
2. Nucleolus-like bodies (NB), small and large, near nuclear envelope, the large one associating with mitochondria to form complex; N, oocyte nucleus of froglet, $\times 20,000$.
3. Intranuclear mitochondrion of 6-month-old tadpole $\times 25,000$.



Therefore, the origin of cytoplasmic evagination was not likely.

It might not be impossible that during the mitosis of this particular primary oogonium, one of the mitochondrion could have gone astray and trapped in the daughter nucleus. In this foreign environment, the fate of this mitochondrion would probably be doomed. Therefore the occurrence of the intranuclear mitochondrion could have been temporary.

On the other hand, one would tend to think of theories relating to the membranous origin of mitochondria. The outer layer of the nuclear envelope might play a role in generating mitochondria as observed by Bell and Mühlethaler⁽⁴⁾ in egg cells of a plant. While challenging the symbiotic theory for the origin of mitochondria, Raff and Mahler⁽³⁷⁾ proposed that mitochondria could have arisen from the pinched off vesicles of invaginated cell membrane possessing respiratory assemblies. Could it be possible that inner layer of the nuclear envelope would also perform such a duty?

II. Significance of Intercellular Bridges

Intercellular bridges are often observed between primary oogonia, between secondary oogonia (Fig. 4) and between synaptic cells in tadpoles at all age groups and in froglets. However, connections between oocytes have not yet been found. The bordering membrane of the cylinder-shaped bridge is densely stained and possesses an underneath sleeve-like subsurface cistern. Mitochondria, ribosomes, vesicles and tubular-like bodies are found within the bridge (Fig. 5).

It is of interest to report a cilium (Fig. 5), protruding toward the intercellular space between

two oogonia near the intercellular bridge in a 3-month-old tadpole. Its function is unknown.

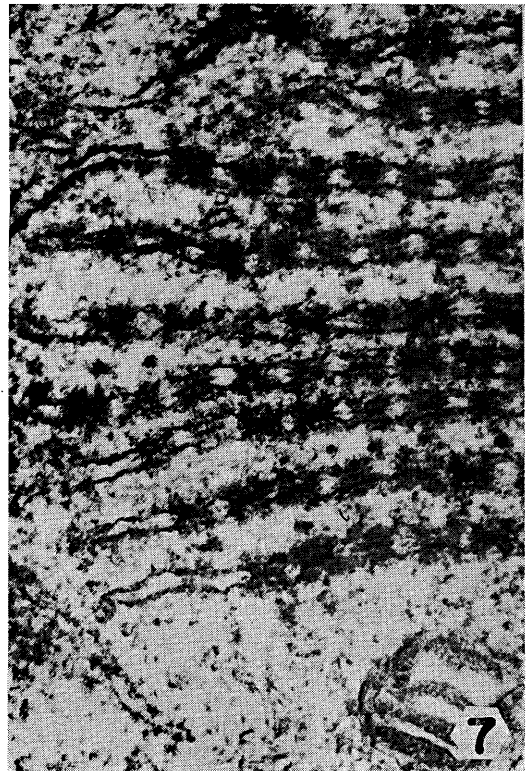
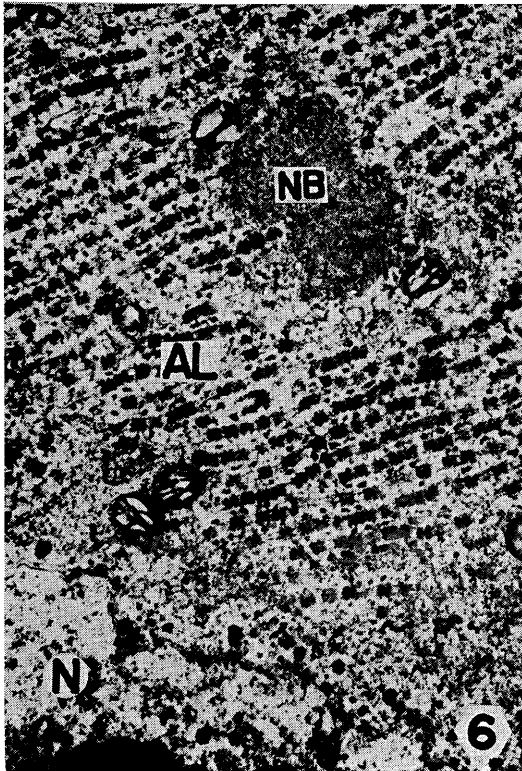
According to Ruby *et al.*⁽⁴¹⁾, intercellular bridges between germ cells in developing ovaries of various mammalian and invertebrate species were described by previous investigators. Ruby *et al.*⁽⁴¹⁾ also reported similar connections between the developing germ cells in ovaries of *R. pipiens* tadpoles. In all cases, intercellular bridges are true connections with similar ultrastructures regardless of species difference. The present observation in *R. catesbeiana* tadpoles also indicates a basic resemblance to the previous description. However, in ovaries of tick nymphs and adults, intercellular bridges show, besides the usual fine structures, a unique feature of narrow, elongated invaginations of the plasma membrane around both bases of the bridge⁽⁷⁾. This characteristic was interpreted by the author to provide an anchoring support for the bases of the bridge to the cells.

Functionally, these bridges are suggested by various authors for synchronous differentiation of germ cells by communication of various organelles through the connection. Ruby *et al.*⁽⁴¹⁾ speculated that since little or no synchronization of germ cells in human and tadpole ovaries was evident, it was difficult to ascribe such a function to these cellular connections. They further held the possibility that the intercellular bridges might facilitate to supply nutrients so that one of the connecting cells could be a nurse cell.

According to Swingle⁽⁴²⁾, in his description of ovarian development of *R. catesbeiana* tadpoles, egg nests (cell nests), peculiar to anurans, are formed by dividing oogonia at the stage when they are entering a period of rapid proliferation; the cells in each nest are sister cells and

Explanation of Figures

4. Intercellular bridge (IB) between two secondary oogonia of 10-month-old tadpole, $\times 18,000$.
5. Intercellular bridge (IB) between two secondary oogonia of 3-month-old tadpole with a mitochondrion near its lower border; a part of cilium (c) lying between two cells above the upper border of the bridge; Golgi complex (G) and nucleolus-like body (NB) discernible in each oogonium, $\times 24,000$.
6. Annulate lamellae (AL) in oocyte of 6-month-old tadpole with mitochondria and nucleolus-like body (NB) among lamellae; N, oocyte nucleus, $\times 12,000$.
7. Enlarged annulate lamellae with ends connecting smooth ER, $\times 50,000$.



generally pass through their cyclic changes simultaneously, so they are usually in the same stage of maturation.

In our hands, germ cells in tadpole ovaries conform to the same organization described by Swingle⁽⁴²⁾ that secondary oogonia and synaptic cells often gather respectively into cell nests. It is during this stage that intercellular bridges are found. When cell nests begin to break up and cells grow rapidly into oocytes, intercellular bridges are no longer observed. In this respect, we do not agree with Ruby *et al.*⁽⁴¹⁾ but consider that intercellular bridges are most likely related to synchronized cell development. Furthermore, oocytes in a tadpole ovary generally keep on growing and seldom show atresia as mammalian oocytes do; the possibility of intercellular bridges acting as connection between a nurse cell and a destined oocyte may not be valid in case of anurans.

III. Annulate Lamellae and Oocyte Growth

Annulate lamellae make up an unique system of cytoplasmic membranes arranged closely in stacks; each lamella consists essentially of two parallel membranes, interrupted by dense annular structures. In profile view, they look like rows of membranes cut sectionally; in surface view, they appear similar to the nuclear envelope cut tangentially with pore-like structures.

In this study, annulate lamellae first appear as a single row of fused vesicles near the nucleus in synaptic cells of 6-month-old tadpoles. They develop conspicuously in oocytes of the same age group, exhibiting typical multilayered membranes with mitochondria and nucleolus-like bodies intermingling between the lamellae (Fig. 6). The ends of the lamellar membrane may

fuse or appear as connected with the smooth endoplasmic reticulum (Fig. 7). Annulate lamellae are also abundant in oocytes of 10-month-old and 12-month-old tadpoles but they disappear in 15-month-old froglets.

Kessel⁽¹⁹⁾ reported annulate lamellae in oocytes of *Necturus* and also in oocytes of *R. pipiens*^(23,24). The fine structures are comparable to those of ours in tadpoles of *R. catesbeiana*.

The short life span of annulate lamellae of this study coincides well with the primary growth period of oocytes. The finding is compatible with the generally accepted view that annulate lamellae occur in young differentiating cells of both germinal and somatic lines and of cultured cells^(2,12,19-21,31,32). The function of annulate lamellae is still in speculation; suggestion has been made that annulate lamellae could contribute to the formation of membranous system^(2,12,19,21). Assumptions for the origin and morphogenesis of the organelle remain equally confusing; some emphasize nuclear origin⁽¹⁹⁻²¹⁾ while others hold that annulate lamellae might arise from dictyosomes of the Golgi complex^(12,32).

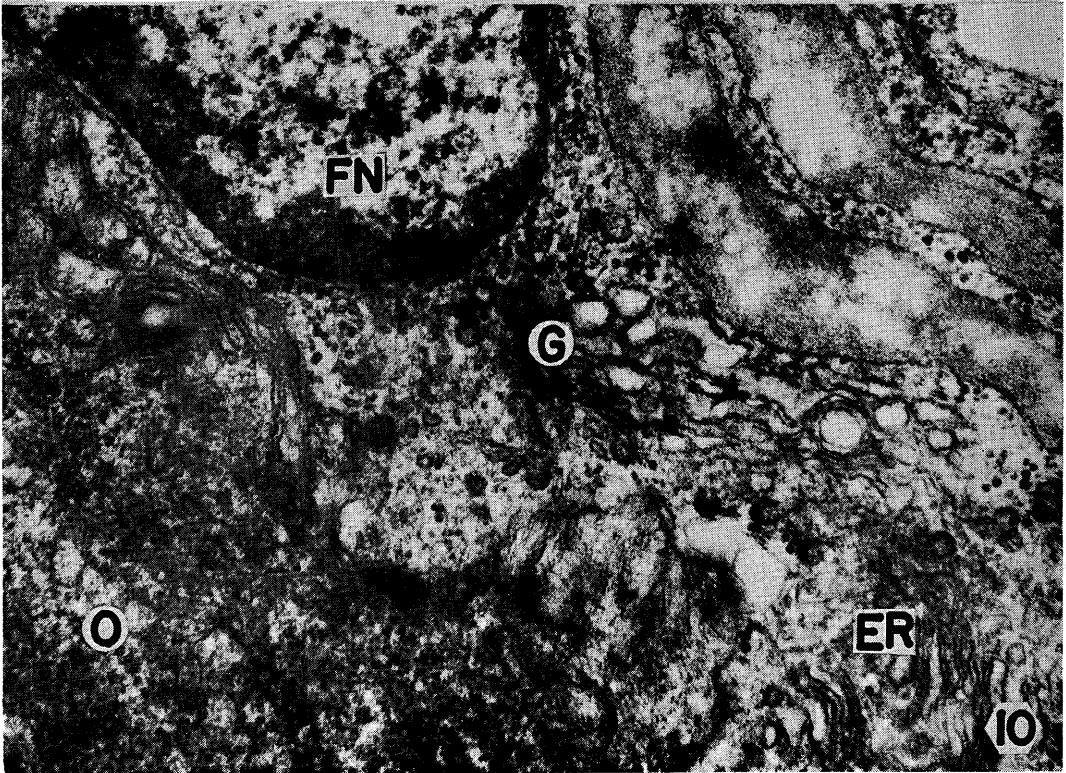
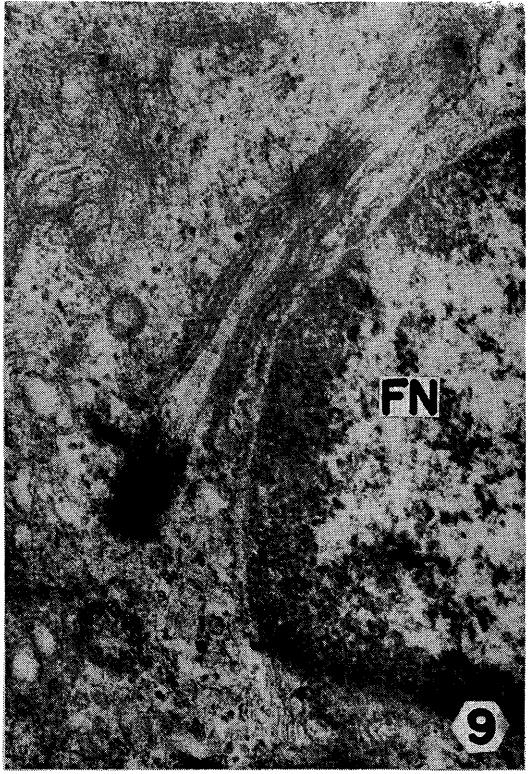
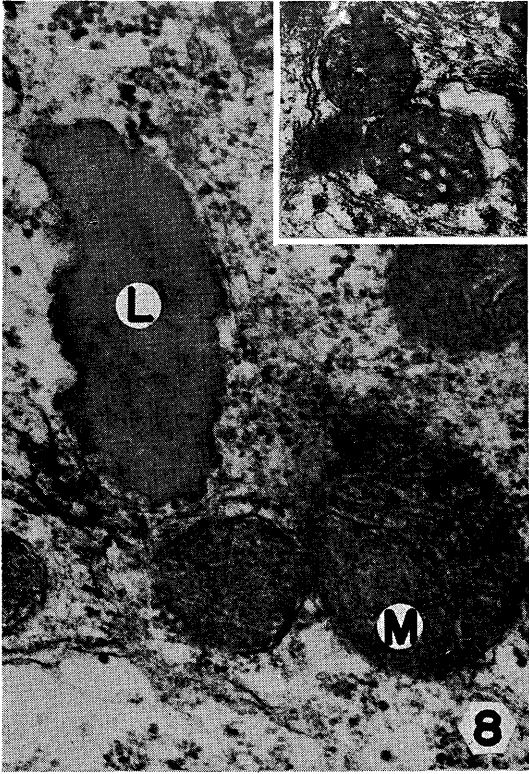
IV. Development of Some Fine Structures in Follicle Cells.

Follicle cells in 3-month-old tadpoles are rarely seen. When observed, the cytoplasm is scarce, mostly distributed at both ends of the spindle-like cell. Lipid droplets, centrioles, solitary cilia and Golgi complex are found occasionally while other organelles are also few.

The organelles develop gradually and become prominent in follicle cells of 12-month-old tadpoles. Mitochondria are numerous now. Golgi complex are widely distributed and some-

Explanation of Figures

8. Follicle cell of froglet, showing lipid droplet (L) and round mitochondria (M), $\times 34,000$; inset exhibiting cross section of mitochondria tubular cristae, $\times 15,000$.
9. Follicle cell of 12-month-old tadpole, showing cilium with its basal body and centriole with "9-0" structure; FN, nucleus of follicle cell, $\times 40,000$.
10. Golgi complex (G) and smooth ER in follicle cell of 12-month-old tadpole; FN, nucleus of follicle cell; O, ooplasm, $\times 40,000$.



times closely associated with the smooth endoplasmic reticulum (Fig. 10). Lipid droplets (Fig. 8) are present and so do centrioles and cilia. The cilium is seen as a solitary one lying lengthwise between the cytoplasmic folds of the cell while a cross section of the centriole shows 9-0 feature (Fig. 9).

The organelles keep on growing in froglets. Mitochondria appear as slightly ovoid or round bodies with tubular cristae (Fig. 8), similar to those in follicle cells of 3-month-old tadpoles but different from those in oocyte mitochondria.

Among ultrastructures found in follicle cells, abundant smooth endoplasmic reticulum, conspicuous Golgi complexes, lipid droplets, round mitochondria with tubular cristae, centrioles and cilia are often thought as characteristics in steroid-producing cells in mammals^(1,5,11,33,36,50). In amphibians, fine structures in follicle cells have been rarely reported. However, synthesis of estrogens was indicated in follicle cells of *X. laevis* with the methods of in vitro incubation and histochemistry⁽³⁹⁾, and release of progesterone from follicle cells of the toad with electron microscopy was claimed by Thornton and Evennett⁽⁴⁵⁾. Redshaw⁽⁴⁰⁾ made a review on the hormonal control of the amphibian ovary in which synthesis and secretion of estrogens by ovarian follicle cells was mentioned. It appears, thus, that steroidogenesis occurs in follicle cells of amphibians.

The possibility of estrogen synthesis in tadpoles has not been clarified with certainty. Indirect evidence from biochemical studies^(6,34,38) collaborated with histochemical findings⁽¹⁵⁾ showed that developing ovaries in tadpoles acquired the capability of synthesizing estrogens. The present result that the characteristic fine structures relevant to steroid production first appear but scarcely in follicle cells of differentiating ovaries and then develop in growing ovaries substantiates the possibility that frog tadpoles may be able to synthesize sex steroids.

Based on the present observations of organelles in oogonia, oocytes and follicle cells, it

is suggested that development of cytoplasmic fine structures in these cells correlates functionally to differentiation and growth of the tadpole ovary.

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牛蛙蝌蚪發育過程中卵巢細胞微細構造的變遷

王長君 許織雲

取正常發育過程中各期牛蛙蝌蚪之卵巢，作電子顯微鏡處理，觀察卵母細胞及卵泡細胞微細構造的變遷。獲知線粒體羣、線粒體內卵黃結晶、類核仁體-線粒體複合物、胞間橋及環孔頁膜等隨卵巢發育而變化，其意義曾予討論。

此外在一卵原細胞之核內發現一完整的線粒體；於卵原細胞胞間橋附近見有纖毛；又於卵泡細胞中亦察見平滑內質網、纖毛、圓形線粒體及其管狀嵴。此等結構在兩棲類中尚屬首次報告。