Classification of Developing Oocytes, Ovarian Development and Seasonal Variation in *Rana tigerina*

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ABSTRACT Developing oocytes in adult *Rana tigerina* can be divided into six stages based on size, color and histology. The stage I oocyte (50-350 µm) is characterized by translucent cytoplasm and a smooth nuclear membrane. The major portion of its cytoplasm contains a large quantity of free ribosomes. Cytoplasmic organelles confined to the peripheral part of oocyte include a few mitochondria, Golgi apparatus, primary and secondary lysosomes, and a few lipid droplets. The stage II oocyte (360-550 µm) contains alcian-blue positive cortical alveoli at the periphery and large central nucleoli in the nucleus. The stage III oocyte (560-900 µm) is characterized by the deposition of yolk platelets and formation of pigmented granules. The cortical alveoli are greatly increased in number as well as in size. Endocytotic activity on the surface of oocyte can be observed. The stage IV oocyte (910-1300 µm) is characterized by a large number of main yolk bodies, cortical alveoli and melanin granules. High endocytotic activities are observed. In the stage V oocyte (1310-1500 µm), most of the melanin granules migrate to the animal pole, while the cortical granules are concentrated underneath the oolemma. The stage VI or fully grown oocyte (1510-1700 µm) is characterized by a complete absence of melanin granules in the vegetal pole and by the cessation of endocytotic activity. Studying the development of ovaries in frogs at various ages reveals that definitive ovaries are formed in the one-month-old frog. Ovaries of two- to four-month-old frogs contain only stage I oocytes, while the ovaries of twelve-month-old frogs contain oocytes of all stages, which indicate the maturity of female frogs. Studying the seasonal variation of ovaries throughout the year reveals that there are no stage VI oocytes in ovaries collected from November to February, while these oocytes are present during the period from March to October.

KEYWORDS: *Rana tigerina*, developing oocytes, ultrastructure, ovarian development, seasonal variation.

INTRODUCTION

The classification of developing oocytes of anurans has been carried out by many researchers.[1-5] The most studied is *Xenopus laevis* whose oocytes have been classified into six stages based on their external appearance, color and size.[5] Similarly, in *Rana pipiens*, the same criteria have been used to classify developing oocytes.[2] In addition, oocytes can be classified by the uptake of vitellogenin or yolk protein into three stages: previtellogenesis, vitellogenesis and postvitellogenesis or mature oocytes.[1] There is currently little morphological data of the ovary and the classification of oocytes in *Rana tigerina.* Thus, one of the aims of the present study was to classify the developing oocytes in *R. tigerina* based on their morphological and histological features.

Detailed studies on the morphological changes in the oocyte cytoplasm, the oocyte-follicle cell relationships and the process of vitellogenesis have been carried out in *R. esculenta*,[6] *R. pipiens*,[7] *Triturus viridescens*[8] and *X. laevis*.[9,10] By comparison in *R. tigerina* there is still a lack of information on the ultrastructure of developing oocytes. Hence, another aim of the present study was to investigate the ultrastructure of developing oocytes of this species.

Reproductive cyclicity seems to be correlated with the climatic condition prevailing in the habitats. Slight annual variations in environmental conditions could disrupt breeding cycles. Annual variation in the precipitation seems to be the main factor in inducing periodicity in breeding activity. In habitats within equatorial regions with a constant warm and humid climate, amphibians may reproduce throughout the year, such as the frog *Rana erythraea* in Borneo,[11] and the toad *Bufo melanostictus* in Singapore and Jarkata.[12,13] In those regions with pronounced wet and dry seasons, the main breeding season

coincides with monsoon rains.[14] Even in the same species, a high variation in breeding period occurs, such as in the African toad *Bufo regularis*, whose breeding period coincides with the beginning of the rainy season from November to April in Tanzania[15] and in March in Kenya.[16] In Thailand where the climate is sharply split into wet and dry seasons, native frog species including *R. tigerina* are expected to be seasonal breeders. Therefore, another aim of this study was to study the histological changes of the ovarian cycle of *R. tigerina* during different months of the year. In addition, the development of ovaries in various ages of the female frogs and the reproductive maturity of this species were also investigated.

MATERIALS AND METHODS

Experimental Animals

R. tigerina, the rural rice field frogs of Thailand, were cultured in the concrete tanks at the Faculty of Science, Mahidol University, Bangkok, Thailand. They were maintained in a natural environment with an approximate 12 hours light/dark cycle. The ambient temperature was 25-35°C, and the relative humidity ranged from 80 to 100%. Pelleted frog feeds were given daily in the afternoon. The water in the culture tanks was changed on alternate days.

Classification of ovarian follicles/ oocytes

Frogs were anesthetized by hypothermia and the ovaries were removed and transferred to 50% frog's Ringer solution.[17] Follicles with various sizes were randomly isolated from fragments of ovaries, and the diameters of follicles were measured with an eyepiece micrometer fitted in an Olympus stereomicroscope. The follicles/oocytes were classified into various stages based on their size and color.[5]

Histological study

Both ovarian fragments and isolated follicles were fixed either in Bouin's solution, or 10% buffered formalin for 3 hours. Then they were dehydrated through a graded series of ethanol, cleared in dioxane, and embedded in paraffin. Large-yolky oocytes were fixed for 6-8 hours in Bouin's solution or overnight in 10% buffered formalin. Then they were dehydrated in increasing concentrations of ethanol for 30 minutes each, and immersed in methyl benzoate for about 3 days before clearing for 1 hour with two changes of benzene. The tissues were infiltrated in a mixture of benzene and paraffin. Six-micron-thick sections were deparaffinized and stained with Harris's hematoxylin and eosin. Finally, they were examined under an Olympus MT-2 light microscope.

In addition, several staining techniques were employed for the detection of lipid, acid mucopolysaccharide, protein and glycoprotein in the oocytes. For the detection of lipid, small pieces of ovary were fixed in Ciaccio's solution and stained either with saturated oil red-O or Sudan III.[18] For the detection of acid mucopolysaccharide, small pieces of ovary were fixed in 2.5% glutaraldehyde and stained with 0.1% alcian blue, concomitantly with 0.1% Kernechtrot nuclear fast red.[18] For the detection of protein and glycoprotein, the ovarian tissue was fixed either in Bouin's solution or in buffered formalin. Sections were stained with Lee-Brown's modified Mallory trichrome dye.[18]

Ultrastructural study

For transmission electron microscopy (TEM), all stages of oocytes were prefixed for 18-24 hours in 2.5% glutaraldehyde in 0.05M cacodylate buffer pH 7.4 at 4°C and washed in the same buffer. Thereafter, they were postfixed in 1% osmium tetroxide in the same buffer at 4°C for 1 hour and stained *en bloc* with 0.5% aqueous uranyl acetate. Then they were dehydrated through increasing concentrations of ethanol and embedded in Araldite resin. In case of large-yolky oocytes, a low viscosity Spurr's resin[19] was used instead of Araldite. Ultrathin sections were cut and stained with uranyl acetate, followed by Reynold's lead citrate.[20] Finally they were examined under a Hitachi H-300 transmission electron microscope at 75 kV.

Development and seasonal variation of ovarian follicles

Four to six developing frogs from one month to 14 months old were collected at each month for this study. Ovarian follicles of various sizes were isolated, classified, and counted under a stereomicroscope as previously described. For each frog at least 300-500 follicles from right and left ovaries were counted to establish the percentage of various stages of oocytes at each month. In addition, pieces of ovaries from frogs collected at each month were also prepared and examined by conventional light microscopy as previously described.

For the study on the seasonal variation of the ovary, adult frogs aged more than 12 months were used. The ovaries from 4-6 frogs were obtained every month during the year. Various stages of oocytes were classified and counted under a stereomicroscope as previously described.

RESULTS

Stages of oocytes/follicles

The multilobed ovary of an adult frog contains developing oocytes which can be classified into six stages based on size, color and histology.

Stage I oocyte: previtellogenic stage

Under the stereomicroscope, the stage I oocyte exhibits a translucent cytoplasm with a diameter ranging from 50-350 µm (Fig 1A). The nucleus is clearly visible through the cytoplasm and occupies a large portion of the oocyte. At light microscopic level, the cytoplasm of the previtellogenic stage I oocyte appears heavily basophilic. In addition, it also acquires a smooth nuclear membrane and nucleoli of various sizes (Fig 1B). In the late stage I oocyte, the cytoplasm stains paler when compared to the early stage.

Stage II oocyte: previtellogenic stage

This stage develops an opaque ring around its concentric nucleus. Its size ranges from 351-550 µm (Fig 1A). Towards the end of this stage, the cytoplasm is almost completely opaque so that the nucleus becomes inconspicuous under a stereo-microscope. Hence, the translucent stage I oocyte can be easily separated from the dark opaque stage II oocyte. Histologically, the presence of a few rows of peripheral vacuoles (cortical alveoli) seems to be the most predominant characteristics of stage II oocyte (Fig 1B). In addition, numerous nucleoli which vary in size can be observed in each cell.

Stage III oocyte: vitellogenic stage

The opacity is complete in the stage III oocyte as it appears intensely white. The diameter of stage III oocyte is 560-900 µm (Fig 1A). Histologically, the number of vacuoles gradually increases, and they become dispersed towards the central area (Fig 1C). Yolk platelets are formed and rapidly replace the central vacuoles. The vitelline envelope also becomes conspicuous under the follicle cells. Pigmented granules first appear in this stage and are located at the periphery of the oocyte. The nucleus of the stage III oocyte possesses a convoluted nuclear membrane and numerous nucleoli.

Stage IV oocyte: vitellogenic stage

The distinct morphological feature of the stage IV oocyte is the pigmentation of the surface as light-brown to brown. The oocyte is 910-1300 µm in diameter (Fig 1A). Yolk platelets completely replace the central vacuoles, while the remaining vacuoles are located around the periphery of oocyte (Fig 1C). The nucleus is surrounded by a highly convoluted nuclear membrane and contains a large number of nucleoli.
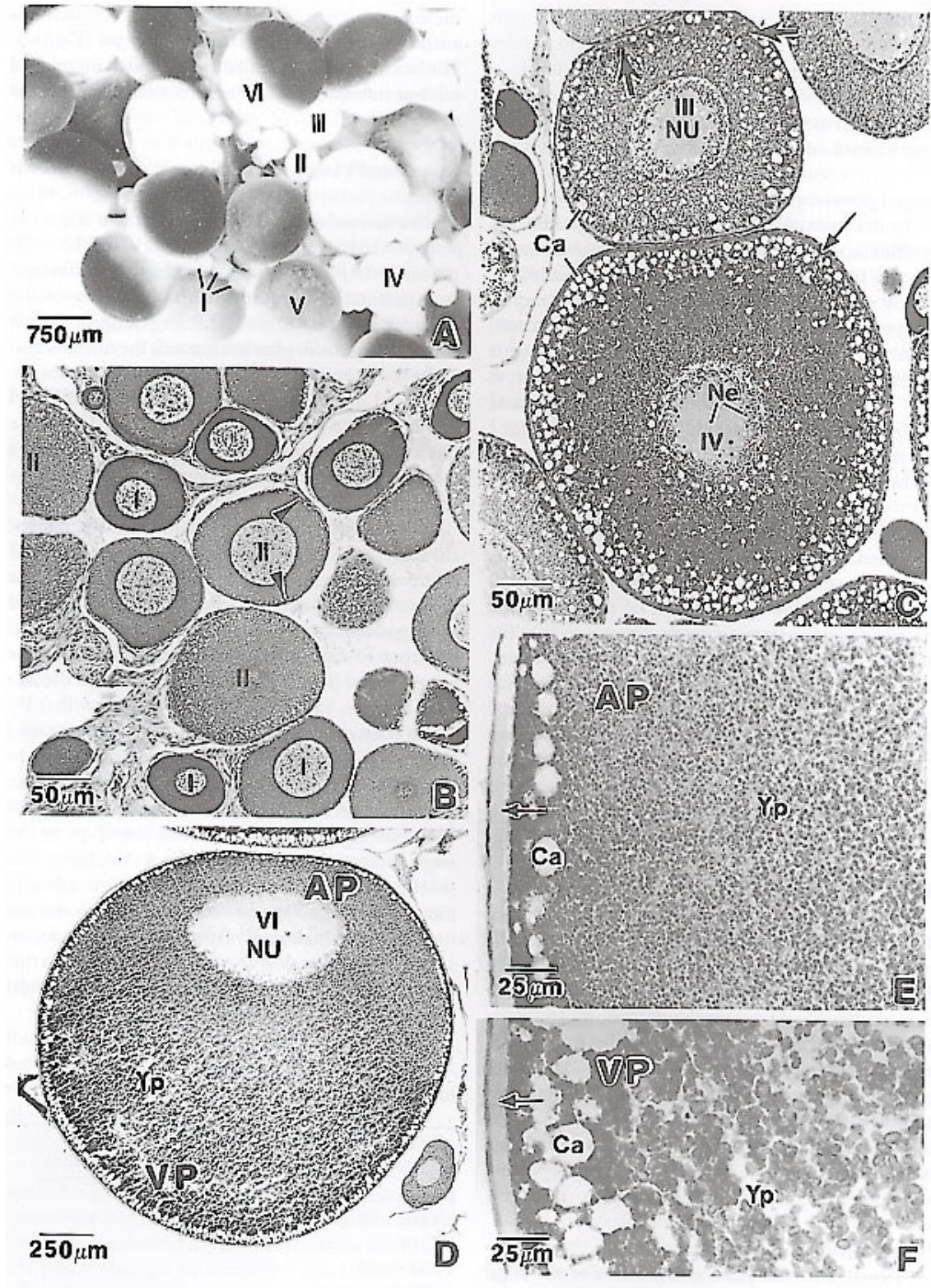
Stage V and VI oocytes: vitellogenic and fully grown stages

Distinct polarity occurs in the last two stages, *ie,* stage V (1310-1500 µm) and stage VI (1510-1700 µm) (Fig 1A). This is manifested by the difference in pigmentation underneath the oolemma of the animal pole in contrast to the vegetal pole which contains large-yolk platelets instead; the nucleus also shifts to the animal pole (Fig 1D). The vacuoles are decreased in number while the yolk accumulation increases. The animal pole in the stage VI oocyte has only one row of vacuoles on the periphery, whereas two or three rows of vacuoles are present in the vegetal pole (Fig 1E, F).

Cytochemical studies of the oocytes are shown in Table 1. These revealed that there is no lipid component present in the vacuoles. Most of the bright red and brown lipid droplets are intermingled among yolk platelets and vacuoles. The distribution of lipid droplets at the periphery is more concentrated than that in the inner region of the late stage oocytes. Vacuoles, positively stained with 0.1% alcian blue, are developed in the stage II oocyte, and they become scattered throughout the cytoplasm in the stage III oocyte, and then accumulate at the periphery of stage IV to VI oocytes. Mallory trichrome dye stained the vitelline envelope in the stage II oocyte and revealed it as a thin layer. The thickness of the vitelline envelope gradually increases in stage III and IV oocytes and then reaches its maximal thickness in the stage V oocyte. In addition, yolk platelets also stained positive with this dye and appeared first in the stage III oocyte as bright red clusters on the cell periphery.

Table 1. Histochemistry of staging oocytes .

Structure	Stain		
	Oil Red-O/ Sudan III	Alcian blue	Mallory trichrome
Lipid droplet	+	-	-
Vacuole	-	+	-
Yolk platelet	-	-	+
Vitelline envelope	-	-	+

Fig 1. Stereomicrographs of ovarian fragments displaying randomly arranged follicles at stages I-VI (A). B-F Light micrographs of ovarian fragments showing histology of stage I,II, III,IV and VI oocytes. Arrowhead, cortical alveolus (Ca); thick arrows, pigmented granules; thin arrows, vitelline coat; Nu, nucleus; Ne, nucleolus; AP, animal pole; VP, vegetal pole; Yp , yolk platelets.

Ultrastructure of developing oocytes

All stages of oocytes are covered with three cellular layers (Fig 2A). The outermost layer is a simple squamous surface epithelium. Beneath this is a layer of fibroblasts which secrete ground substance and make collagen fibers. The innermost layer is made up of follicle cells which project their cytoplasmic processes into the perivitelline space. These cells are joined together with desmosomes. In addition, the oocyte coat consists of a non-cellular layer, the vitelline envelope, whose fibers are arranged in three directions interposed between microvilli extending from the oocyte surface (Fig 2B).

The stage I oocyte contains a large quantity of free ribosomes and clusters of lysosomes. Mitochondria are loosely distributed throughout the cytoplasm (Fig 2C, C1). Within the perivitelline space, there are short cytoplasmic projections both from follicular cells called follicular processes, and from the oocyte surface called microvilli. The nucleus is surrounded by a wavy nuclear membrane and contains euchromatin and various sized electron-dense nucleoli (Fig 2D).

The stage II oocyte is characterized by the presence of vacuoles or cortical alveoli on the periphery (Fig 2E). Some cortical alveoli are coalesced and some are closely associated with lysosomes. The Golgi complex is well developed. In this stage, the vitelline envelope begins to form as isolated bundles of fine filaments within the perivitelline space. Both the microvilli of the oocyte and the cytoplasmic processes of the follicle cell become longer. The number of elongated to spherical shaped mitochondria is increased, and these are distributed around the nucleus (Fig 2F).

As previously mentioned, two major distinct features of the stage III oocyte are the deposition of yolk platelets and the formation of pigmented granules. Cortical alveoli become larger in size (Fig 3A). Groups of mitochondria are observed among the yolk platelets of vitellogenic oocytes, and endocytotic activity on the surface of the oocyte is initiated (Fig 3A1). Newly synthesized yolk platelets exhibit bipartite characteristics: the central paler compact body which is arranged into a crystalline lattice, and the peripheral electron-dense portion (Fig 3A2). Although pigmentation begins in this stage, most oocytes still contain only a few membrane-bound melanin granules called premelanosomes. The stage IV oocyte is characterized by coloration of the surface which is due to the presence of membrane-bound melanin granules (Fig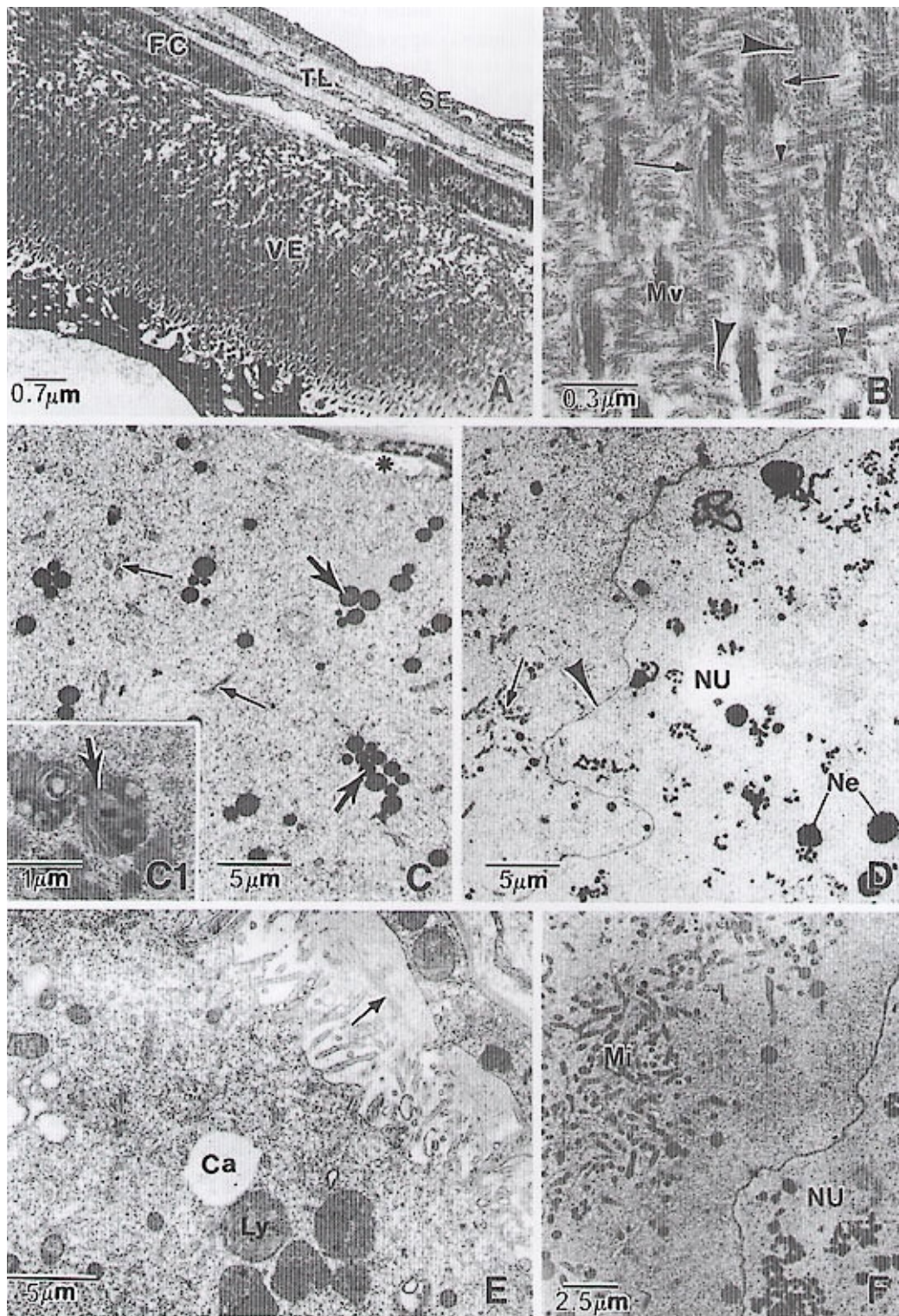 3B). Abundant endocytotic vesicles could be observed under the oolemma. A large number of endosomes appear close to lysosomes and some are fused with these lysosomes which contain electron-dense material (Fig 3C). More electron-dense main yolk bodies were observed in addition to the newly formed yolk platelets. Groups of mitochondria and lipid droplets are intermingled among yolk platelets (Fig 3D). Later, there is an increase in the number of yolk platelets in the perinuclear cytoplasm, while microvilli are increased in length and number. A highly folded nuclear membrane results in a sacculated nuclear outline (Fig3E).

In the stage V oocyte, most pigmented granules migrate toward the animal pole (Fig 4A), leaving only a few of them at the vegetal pole and perinuclear zone (Fig 4B). During this stage, the cortical alveoli with their translucent content are conspicuous underneath the oolemma. Some endocytotic activity could still be observed (Fig 4C). Although the general ultrastructures of the stage VI oocyte are quite similar to the stage V oocyte, the pigmented granules are completely absent from the vegetal pole, but endocytotic activity could be rarely observed (Fig 4D).
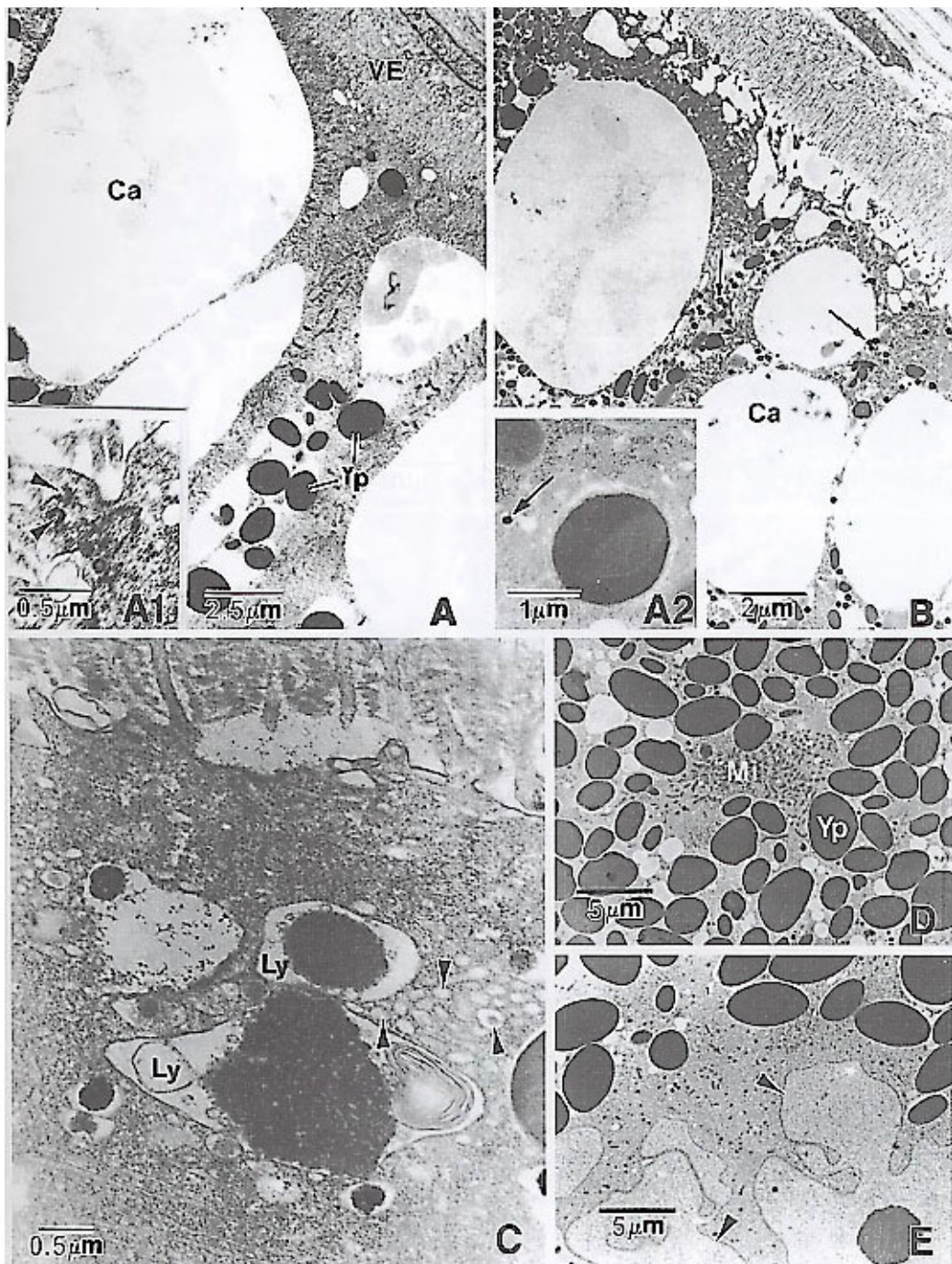
Development of ovarian follicle/ovary

A definitive ovary can also be observed in the one-month-old frog. It appears as a small oval organ and consists of a large number of primodial germ cells which undergo intense mitotic division. Most of the central region is occupied by mesenchymal cells referred to as the primary germinal cavity. This later develops into the secondary germinal cavity (Fig 5A, B). The presence of some stage I oocytes among the oogonia, primodial germ cells and stromal cells was observed. Lobulation of the ovary appears in the second month of development which results in multilobed ovaries. The number of stage I oocytes increases in the ovary of two-month-old frogs (Fig 5D). No oogonia were observed in the ovary of the four-month-old frog (Fig 5E). Stage II, III and IV oocytes become evident in the fifth, seventh and eleventh month of development, respectively (Fig 5F, G, H) while the last two stages (V and VI) appear when the frogs reach the age of 12 months.
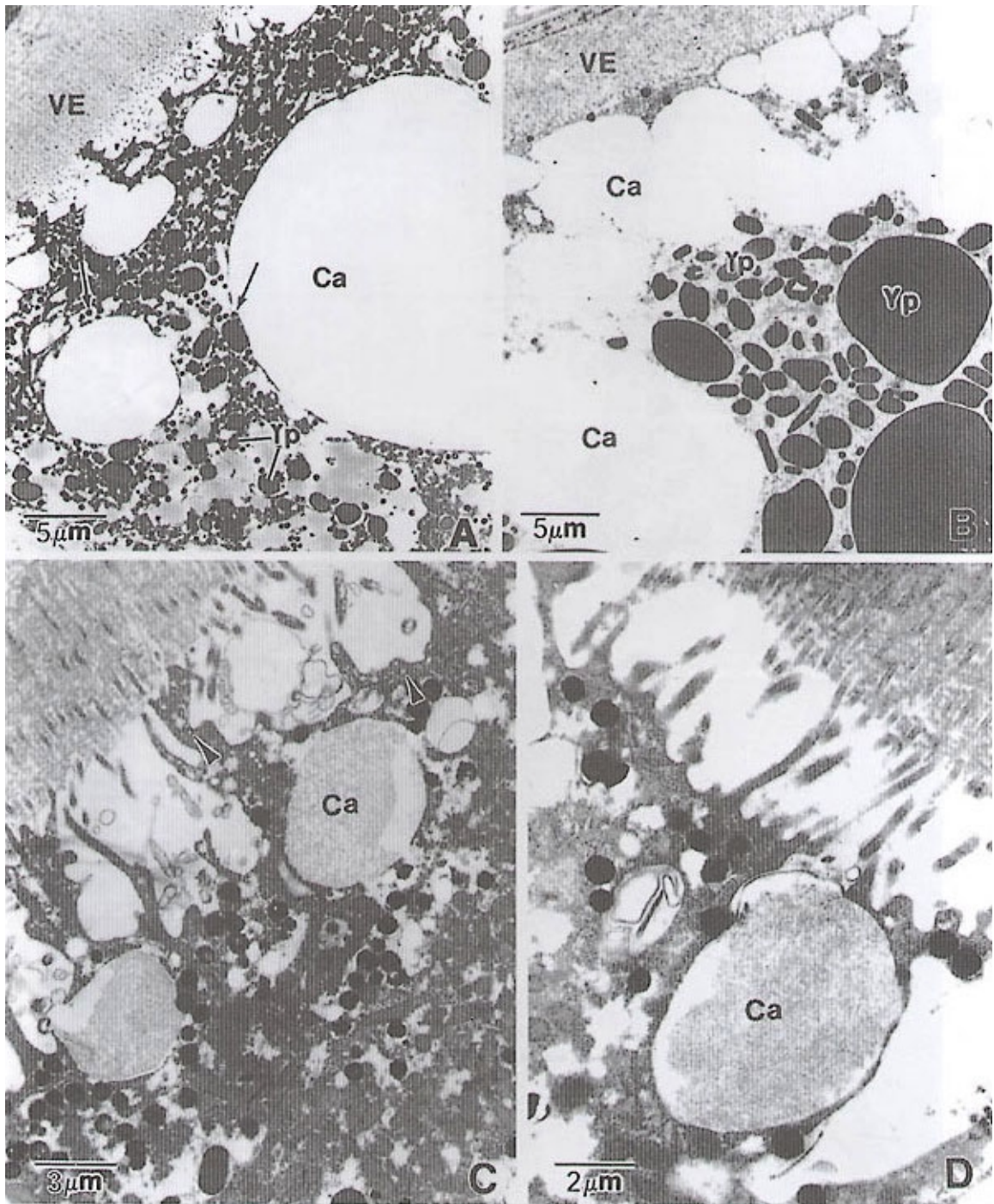
The histograms in Figure 6 illustrate the percentages of various stages of oocytes during development, corresponding to the histological appearances as previously described. There are only stage I oocytes in the ovaries of frogs between one and four months of age. In the fifth, seventh and eleventh month, the proportions of stage II, III and IV oocytes are 1.5, 0.7, and 10.1%, respectively. The
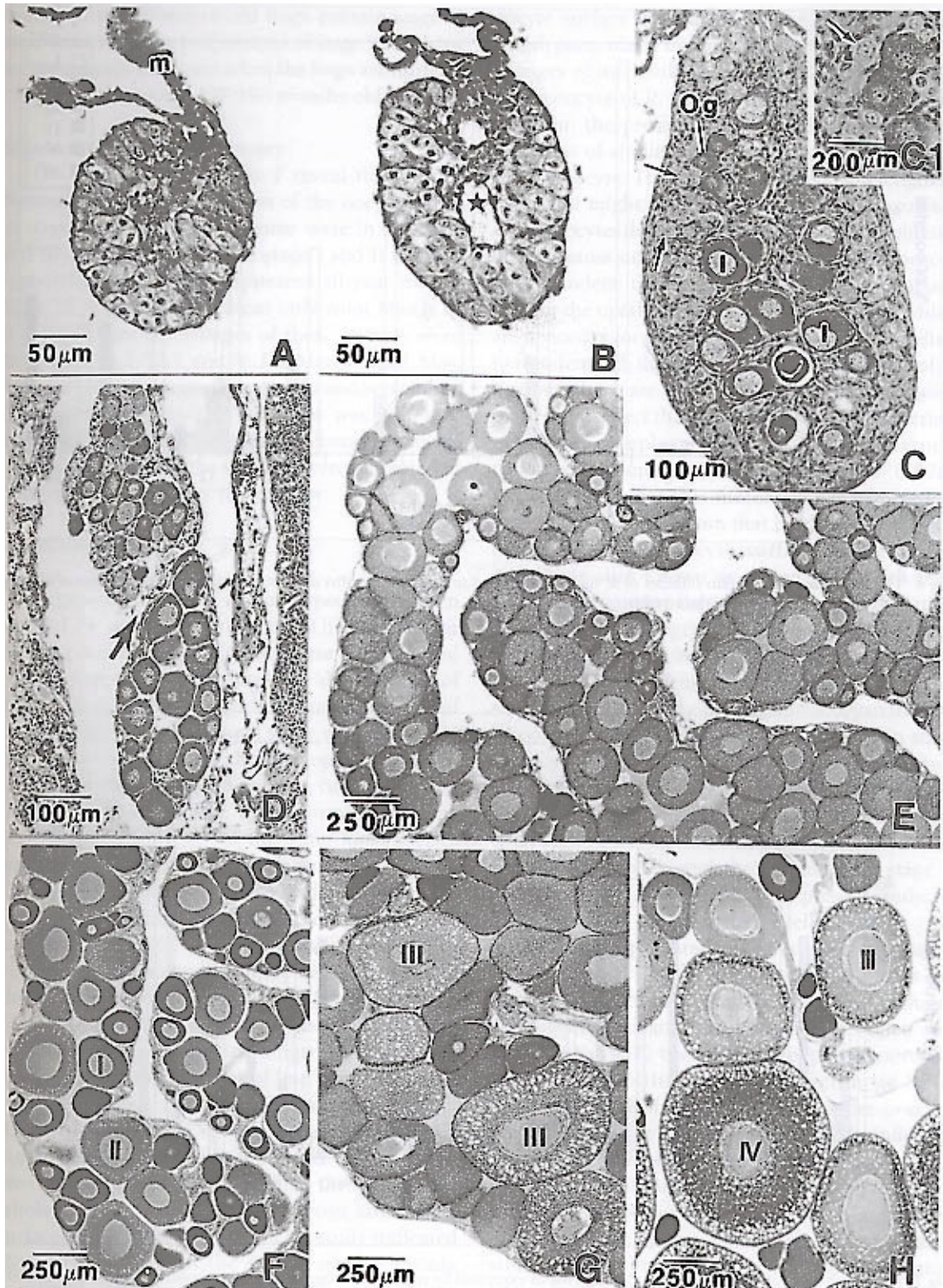
Fig 2. A. Electron micrographs illustrating the organization of the oocyte coat. SE,surface epithelium; TL, theca layer; FC, follicle cells; VE, vitelline envelope. B. High magnification of vitelline envelope displaying the intermingling of fibers arranged parallel (arrows), across (arrowheads) and perpendicular (smaller arrows) to the microvilli (Mv). C,C1,D. Stage I oocyte showing the distribution of mitochondria (arrows) and aggregation of lysosomes (large arrows). NU, nucleus; arrowhead, nuclear membrane; asterisk, perivitelline space. E,F. Stage II oocytes demonstrating the presence of cortical alveoli (Ca) associated with lysosomes (Ly), a bundle of fine filaments (arrow) and the group of mitochondria (Mi). NU, nucleus.
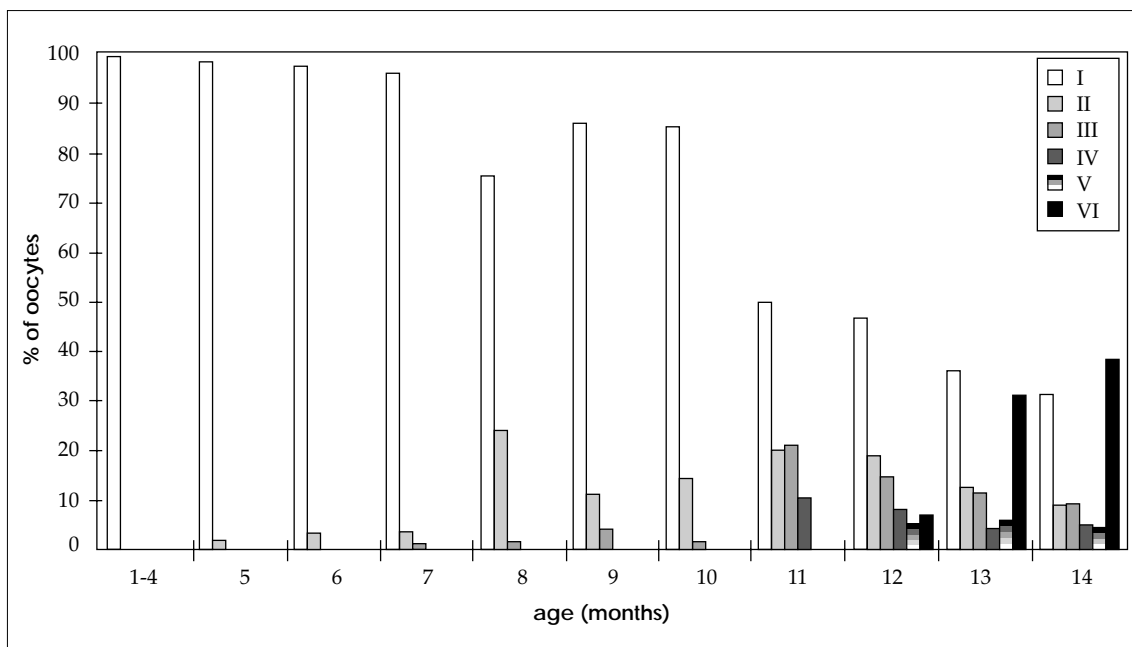
Fig 3. A. Electron micrographs of stage III oocytes showing the presence of yolk platelets (Yp), coated pits and vesicles (arrowheads) (A1) and pigmented granules (arrow) (A2). A2. Yolk platelet shows a bipartite structure. VE, vitelline envelope. B-D. Stage IV oocytes displaying a large number of yolk platelets, pigmented granules (arrows), endosomes (arrowheads) close to the lysosomes (Ly) containing electron-dense material, and the mitochondrial cluster. E. Nuclear membrane of stage IV oocyte showing numerous folds (arrow heads).
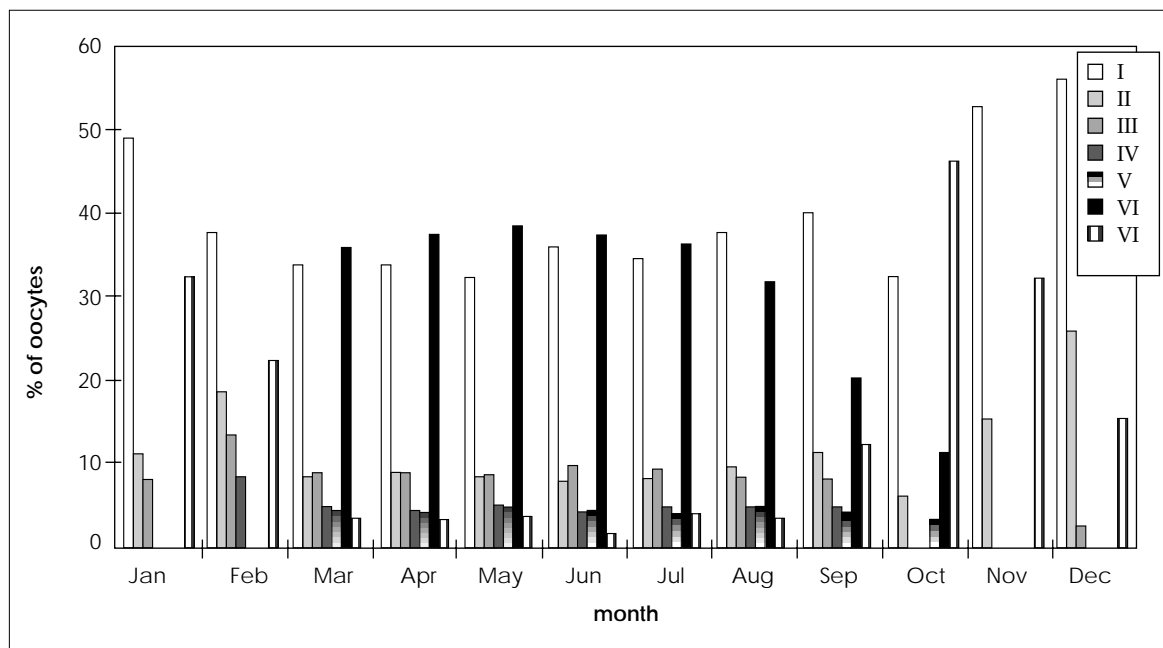
Fig 4. Electron micrographs of stage V oocytes displaying the presence of pigmented granules (arrows) and cortical alveoli (Ca) at the animal pole (A) whereas a few pigmented granules and 2-3 layers of cortical alveoli appear at the vegetal pole (B). Yp, yolk platelets; VE, vitelline envelope. Some endocytotic vesicles (arrowheads) are present in the stage V oocytes (C) while they are rare or absent in the stage VI oocytes (D).

Fig 5. A-C. Sections of one-month-old ovaries showing the primary (empty star) and secondary germinal cavities (dark star) and the mitotic division of primordial germ cells (PGC)(A,B). Oogonia (Og), PGC (arrows) and diplotene stage I oocytes (I) are present (C) Higher magnification of oogonia and PGC are illustrated in C1. D. Two-month-old ovaries displaying the stage I oocytes intermingled with PGC and oogonia. E. Four-month-old ovary consisting of only stage I oocytes. F,G,H. Ovaries aged five, seven and eleven months displaying stage II, III and IV oocytes, respectively.

Fig 6. The appearance of ovarian follicles of *R. tigerina* aged one to fourteen months as expressed by mean percentage of all stage oocytes (observed during 1993-1995).

Fig 7. Annual changes of ovarian follicles of *R. tigerina* as expressed by mean percentage of all stage oocytes (observed during 1993-1995).

ovaries of twelve-month-old frogs contain stage V and VI oocytes. The proportions of stage VI oocytes are remarkably increased when the frogs are thirteen (30.4%) and fourteen (37.5%) months old.

Seasonal variation of the ovary

The histograms in Figure 7 reveal that during November and February most of the oocytes were in stage I (>40%) whereas some were in stages II and III. It was observed that stage I and II oocytes (previtellogenic oocytes) appeared all year round. Stage VI oocytes were present only from March to October, where percentages of these oocytes were 35.5, 37.2, 38.1, 37.1, and 36.1 in March, April, May, June, and July, respectively. In August and September, the percentage of stage VI oocytes was 20% and decreased to 11.2% in October. Degenerated oocytes appeared throughout the year and were increased in number during October to February.

DISSCUSSION

Oogenesis in ovaries of *Rana* species has been studied by many investigators. The criteria for dividing oocytes into many stages are mainly based on the size, the amount and the distribution of yolk and pigment,[2,3,6] and the morphology of chromosomes.[21] In the present study, we used several criteria for characterizing the developing oocytes of *R. tigerina.* These included the size, color, as well as the internal morphology, in conformity with the classification of oocytes in the other *Rana* species and *X. laevis* as previously described.[1-5]

The outer coat of oocytes of *R. tigerina* is composed of surface epithelium, a connective tissue layer and a follicular cell layer as well as a layer of vitelline fibers arranged in three directions (called the vitelline envelope or coat). This feature is common in most species of anurans. The formation of the vitelline envelope in *R. tigerina* can be first detected as isolated bundles of fine filaments in the stage II oocyte similar to those found in oocytes of *X. laevis*[5]. However, TEM studies employing IgG-conjugated colloidal gold revealed that vitelline envelope antigens were distributed throughout the whole cytoplasm and began to deposit around the surface of the stage I oocyte.[22] This study indicated that the cytoplasm of the stage I oocyte already contained components reactive with anti-vitelline coat antibodies[22], thus suggesting that the oocyte may play a major role in synthesizing its own vitelline envelope.

In *X. laevis*,[10] the microvilli extending from the oocyte surface gradually increase in number and length particularly in stage III and IV oocytes. These changes of microvilli are similar to those observed in the oocyte of *R. tigerina* in the present study. In addition, the present study revealed that the full thickness of vitelline envelope was observed in the stage V oocyte. The increased number and length of microvilli might be needed to increase the surface area of oocytes during development. Since amphibian oocytes must store nutrient materials in the form of yolk platelets, they need a relatively large surface area for the uptake of the nutritive substances which are necessary for the yolk formation. Corresponding to this demand, during stage III and IV oocytes of *R. tigerina*, there are extensive pinocytotic activities, which may reflect the mechanism whereby materials enter the cytoplasm through the endocytotic pathway as reported in *X. laevis*.[10] Dumont[23] (1978) has suggested that this mechanism is involved in the uptake of vitellogenin that binds to the specific receptors on the oocyte surface at preformed membrane sites known as coated pits which then give rise to coated vesicles. The evidence supporting the uptake of vitellogenin *in vitro* was demonstrated by the injection of 1% vital dye trypan blue into the dorsal lymph sac of female anurans.[24-27] These studies showed that the uptake of trypan blue began in stage III oocytes and reached the maximal level in stage IV oocytes, then the activity declined in the last two stages. These results are similar to those reported by Wallace[28] (1970) using an *in vitro* study, and are also compatible with the increased pinocytotic vesicles in state III oocytes, and their decrease in stage V and VI oocytes as observed in the present study.

It has been reported that vitellogenin or yolk protein is the precursor of yolk platelets during vitellogenesis.[29] The term vitellogenesis means the process of synthesizing yolk platelets and includes vitellogenin formation from the liver.[30] Follett and Redshaw[30] (1967) used serum lipophosphoprotein (SLPP) to substitute for vitellogenin. SLPP is transported *via* blood circulation to the ovaries, where it is taken up by the oocytes under the influence of gonadotrophic hormones.[31] After transport into the oocytes, SLPP is dissociated into two components, phosvitin and lipovitellin, and these are finally reconstituted to form yolk platelets. The present study has demonstrated that the yolk formation begins in stage III, as in *X. laevis*.[5] Perhaps this occurs through the increased receptor-mediated endocytosis as discussed earlier, since a large number of endosomes and electron-dense lysosomes could be observed in stage IV oocytes.

Mitochondrial clusters observed in vitellogenic oocytes in this study are commonly observed in many anurans.[32] This might be due to the need for a large energy supply for the synthesis of new macro-molecules and for the assembly of various structures in the oocytes. The increase of mitochondria numbers during vitellogenesis may be due to their rapid proliferation, as it has been suggested that out of the total of 16-17 rounds of mitochondrial DNA replication during oogenesis, 12 rounds take place before the onset of vitellogenesis.[33]

The present study also revealed the abundance of free ribosomes in the cytoplasm of all stages of oocytes especially around the nucleus, while only a little rough endoplasmic reticulum was observed. This is thought to be a basic characteristic of anuran oocytes,[33] since free ribosomes are the site where various proteins are synthesized for usage within the cell. During vitellogenesis, mRNA molecules are actively synthesized but they are mainly stored in an inactive form. When the synthesis of the large mRNA population stops, the production of rRNA begins and continues during the whole oogenesis. Whether these changes in RNA synthetic patterns are directly due to the uptake of vitellogenin or to the appearance of yolk platelets is still unknown.[33] During development of oocytes of *R. tigerina*, the nuclear envelope changes from having a smooth contour in the earlier stages to being more highly folded in later stages. This change may be in response to the need for a large surface area for the transport of the various classes of RNA to the cytoplasm, and the reverse transport of proteins into the nucleus across the nuclear membrane via nuclear pores.[33]

Our cytochemical study using alcian-blue staining agrees with TEM results that the vacuoles first appear in the stage II oocyte, and their number is increased in stage III and IV oocytes. However, they are reduced to one or two layers at the periphery of the fully grown oocyte. These vacuoles are similar to the cortical alveoli of teleost oocytes as reported by Selman *et al*.[34,35] They appear empty in routine histological sections due to the extraction of their content during the tissue preparation. Alcian-blue staining of these vacuoles indicates the presence of mucopolysaccharide content. In *X. laevis*, it was found that N-acetyl-β-D-glucosaminidase activity was associated with similar cortical granules.[36] An autoradiographic study also demonstrated the incorporation of [³H] glucose into the content of cortical alveoli of teleost oocytes.[34] Moreover, we found that they disappear in the fertilized egg (unpublished data).

Histological observations of developing ovaries in this study revealed that the structures of the one-month-old ovary are quite variable because each frog with the same age has a different growth rate, and this affects the ovarian development. All oogonia had differentiated into stage I oocytes by the time the frogs reached four months. These stage I oocytes cease development at the diplotene stage of first meiosis.[33] Stage VI oocytes which occur in 12-month-old frogs may represent the age which female frogs reach maturity. This is in contrast to *Bufo bufo* in Europe, which reach maturity after the age of 3-4 years.[37,38] This finding implies that the growth rate of anurans living in the tropics is much faster than those living in a cold climate.

One of the most remarkable characteristics of amphibians is the change of ovarian cyclicity in correlation with the variation in environmental or seasonal conditions,[39,40] especially the seasonal climatic cycle of temperature and precipitation. In tropical countries, where there is pronounced wet and dry seasons, the breeding and non-breeding periods are clearly separated. In India (in Dharwad), the main breeding season of frogs coincides with the peak of monsoon rain during June to August.[41] The climate in Thailand is similar, and the breeding season of *R. tigerina* coincides with the period of monsoon rain that generally extends from May to September. The appearance of stage I and II or previtellogenic oocytes throughout the year as in other anurans,[42] suggests that there is always a reserved pool of oocytes. The decline in their number is associated with the recruitment of oocytes from this pool to final vitellogenic oocytes as occurs in *R. tigrina* between April-June.[43] This study revealed that the degenerated oocytes also appear all year round and increase during October to February, which correlates with the decline of stage VI oocytes. This suggests that atresia during that period is due to the degeneration of the stage VI oocytes, which could be due to the lower level of gonadotropins.[44,45]

In the present study, it was found that the mature-stage VI oocytes are observed only during March to October. The presence of stage VI oocytes slightly precedes the onset of rainy season, but their termination is close to the end of the season. Thus, this period is referred to as the breeding season while the period during November to February is referred to as the non-breeding season. Moreover, the presence of stage VI oocytes during March to October is correlated with the large amount of spermatozoa in the testis in male frogs[46] and abundance of basophils which produce gonadotropins in the pars distalis of *R. tigerina*.[47]

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