

Ultrastructural Observations of Previtellogenic Ovarian Follicles of the Caecilians *Ichthyophis tricolor* and *Gegeneophis ramaswamii*

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ABSTRACT The ultrastructural organization of the previtellogenic follicles of the caecilians *Ichthyophis tricolor* and *Gegeneophis ramaswamii*, of the Western Ghats of India, were observed. Both species follow a similar seasonal reproductive pattern. The ovaries contain primordial follicles throughout the year with previtellogenic, vitellogenic, or postvitellogenic follicles, depending upon the reproductive status. The just-recruited primordial follicle includes an oocyte surrounded by a single layer of follicle and thecal cells. The differentiation of the theca into externa and interna layers, the follicle cells into dark and light variants, and the appearance of primordial yolk platelets and mitochondrial clouds in the ooplasm mark the transition of the primordial follicle into a previtellogenic follicle. During further development of the previtellogenic follicle the following changes occur: i) the theca loses distinction as externa and interna; ii) all the follicle cells become the dark variant and increase in the complexity of ultrastructural organization; iii) the nucleus of the oocyte transforms into the germinal vesicle and there is amplification of the nucleoli; iv) the primordial yolk platelets of the cortical cytoplasm of the oocyte increase in abundance; v) the mitochondrial clouds fragment and the mitochondria move away from the clouds, leaving behind the cementing matrix, which contains membrane-bound vesicles of various sizes, either empty or filled with a lipid material; vi) the perivitelline space appears first as troughs at the junctional points between the follicle cells and oocyte, which subsequently spread all around to become continuous; vii) macrovilli and microvilli develop from the follicle cells and oocyte, respectively; and viii) the precursor material of the vitelline envelop arrives at the perivitelline space. The sequential changes in the previtellogenic follicles of two species of caecilians are described. *J. Morphol.* 268:329–342, 2007. © 2007 Wiley-Liss, Inc.

KEY WORDS: caecilians; follicle cells; mitochondrial cloud; ovarian follicles; previtellogenic follicle

Ovarian follicular development in amphibians occurs in sequential stages of differentiation beginning with the initial establishment of follicles and ending with ovulation. A definite number of stages in this sequence has been identified and reviewed. Sanchez and Villecco (2003) broadly classified

these stages in the anurans as previtellogenesis, vitellogenesis, and postvitellogenesis and suggested that such a classification can serve as a framework for more studies in anuran oogenesis to compare this process in different species. Dumont (1972) divided oogenesis in *Xenopus laevis* into six stages, and each stage of oocyte development was correlated with histological, ultrastructural, physiological, and biochemical characteristics. According to Uribe (2003), follicular maturation in *Ambystoma mexicanum*, a urodele, can be divided into six stages—previtellogenic Stages 1 and 2, vitellogenic Stages 3–5, and preovulatory oocyte, Stage 6, which correlate with the morphological changes in the oocyte, zona pellucida, follicular cell layer, and theca. Exbrayat (2006) identified six stages in the follicular development of caecilians, the first (Stage A), in which oogonia are grouped into germinal nests, the second (Stage B), having an early stage primary oocyte, which may or may not be surrounded by follicle cells, the third (Stage C), consisting of previtellogenic follicles, the fourth (Stage D), consisting of vitellogenic follicles, the fifth (Stage E), having atretic follicles, and the sixth (Stage F), consisting of corpora lutea.

Among the different classifications above, that of Sanchez and Villecco (2003) into previtellogenesis, vitellogenesis, and postvitellogenesis is simple and can be applied to all amphibian groups. During previtellogenesis, the follicles undergo structural,

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biochemical, and molecular changes towards preparation for the accumulation of yolk material. The major precursor to this stage is vitellogenin, which is synthesized in the liver, transported in the blood and sequestered into the oocyte through/across the follicle/granulosa cells (Wallace and Dumont, 1968; Wallace and Bergink, 1974; Wallace and Jared, 1976; Brummet and Dumont, 1977; Dumont, 1978; Wallace and Selman, 1990; Polzonetti-Magni, 1998; Hamlett et al., 1999; Sretarugsa et al., 2001; Vилlecco et al., 2002; Sanchez and Vилlecco, 2003; Uribe, 2003; Exbrayat, 2006). The fully formed yolk platelet consists of glycogen and lipids, the acquisition of which may follow a different pattern (Vилlecco et al., 1999; Uribe, 2003). The ultrastructural changes in the follicles in relation to these stages have been described only for a few anuran species (Aybar et al., 1996; Vилlecco et al., 1996, 1999, 2000, 2002; Sanchez and Vилlecco, 2003). The ultrastructural description of previtellogenic follicles of urodeles and caecilians has not yet been attempted (Uribe, 2003; Exbrayat, 2006).

The caecilians, order Gymnophiona, are unique in several aspects of their anatomy and reproductive biology. All caecilians practice internal fertilization, making use of the eversible phallodeum as the phallus. There are species representing two different grades of female reproductive patterns, namely oviparity and viviparity. Several of the oviparous species lay eggs with embryos developed to different stages depending upon the species, thus shortening larval life. However, the sequential changes in the ovary towards the establishment of follicles in the caecilians have only been poorly studied (Wake, 1968, 1970a,b, 1972, 1977, 1980; Exbrayat and Sentis, 1982; Exbrayat and Colletot, 1983; Exbrayat, 1986, 2006; Masood-Parveez, 1987; Berois and De Sa, 1988; Masood-Parveez and Nadkarni, 1993a,b; Exbrayat and Anjubault, 2003). Thus, detailed ultrastructural descriptions of the follicular stages in the caecilians are highly pertinent. We describe the biology of female reproduction of these caecilians from anatomical, histological, ultrastructural, biochemical, and molecular biological perspectives. This article further describes the ultrastructural organization of previtellogenic follicles of *Ichthyophis tricolor* and *Gegeneophis ramaswamii*.

MATERIALS AND METHODS

Ichthyophis tricolor (Annandale, 1909) (Ichthyophiidae) and *Gegeneophis ramaswamii* (Taylor, 1964) (Caeciliidae) were collected from terraced plantations of mixed coconut and rubber from Thekkada (08° 37'N, 76° 57'E), in the Trivandrum district of Kerala, and Maramalai (08° 26'N, 77° 24'E), in the Kanyakumari district of Tamil Nadu, Southern India, from June 2004 to June 2005. Monthly samples (three animals from each species) were collected, euthanized with MS222 (tricaine methane sulfonate) and dissected to expose the female reproductive system. Tissues representing different phases of ovarian changes were

fixed in 2.5% glutaraldehyde prepared in cacodylate buffer, postfixed in 1% osmium tetroxide, and embedded in resin. Semi-thin sections (1 µm thick) were stained with toluidine blue O. In addition to making observations of the status of the ovaries, the diameters of oogonia, oocytes, pregranulosa cells, and granulosa cells, including the nuclei, were measured in five randomly selected cells of each type, using a research microscope supported with *Q-Win* software (Leica, Jena, Germany). Ultrathin sections, obtained by a Leica ultramicrotome (Jena, Germany), were stained with uranyl acetate and lead citrate and subjected to transmission electron microscopic analysis, using a Philips 201C transmission electron microscope (Amsterdam, Holland). The images were processed using Adobe Photoshop version 7.0.

RESULTS

The previtellogenic oocyte identifies the third stage (Stage C) of Exbrayat (2006) in caecilians (Stages A and B are described elsewhere) or Stages 1 and 2 (previtellogenesis) of Uribe (2003) in urodeles. At the previtellogenic stage the follicle is spherical or oval and has a distinct theca encompassing a single layer of follicle cells and the oocyte. The ooplasm has an outer granular cortical region and a clear medullary region (Fig. 1A,B). The latter surrounds the large nucleus, which is clear, and contains only one or two nucleoli (not shown). In a more developed previtellogenic oocyte the cytoplasm is highly granular and dense in the cortical region, whereas it is less granular and clear in the medullary region (Fig. 1C,D). The nucleus has transformed into a spherical germinal vesicle positioned at one pole of the cell, and possessing several nucleoli (Fig. 1C,E). A distinct nuclear envelope surrounds the germinal vesicle. The amplified nucleoli, randomly distributed within the nucleus (Fig. 1E) possess the classical fibrillo-granular constitution (Fig. 2A,B). The nuclear envelope is not folded and has a smooth profile (Figs. 1E,F and 2A,B). It is perforated by numerous pore complexes, which are the sites of local fusion of the inner and outer membranes of the nuclear envelope (Fig. 2B).

The theca is differentiated as the theca externa and theca interna (Fig. 3A). The theca externa consists of spindle-shaped fibroblast-like cells separated by large intercellular spaces that contain collagen fibrils. The theca interna is also formed of similar cells that are more prominent than in the theca externa. There are large collagen-filled intercellular spaces between the cells towards the outer side of the theca externa, but closer to the basement membrane of the follicular cells. Theca interna fibroblasts are closely packed (Fig. 3B).

In the early previtellogenic follicles the single layer of follicular epithelial cells is compactly arranged with no intercellular space between them (Fig. 4A). There are two types of follicular epithelial cells: dark and light. The dark cells possess highly elongated intense heterochromatin and an irregular nucleus (Fig. 4B) containing a distinct nucleolus (Fig. 4D). The light cells possess a finely

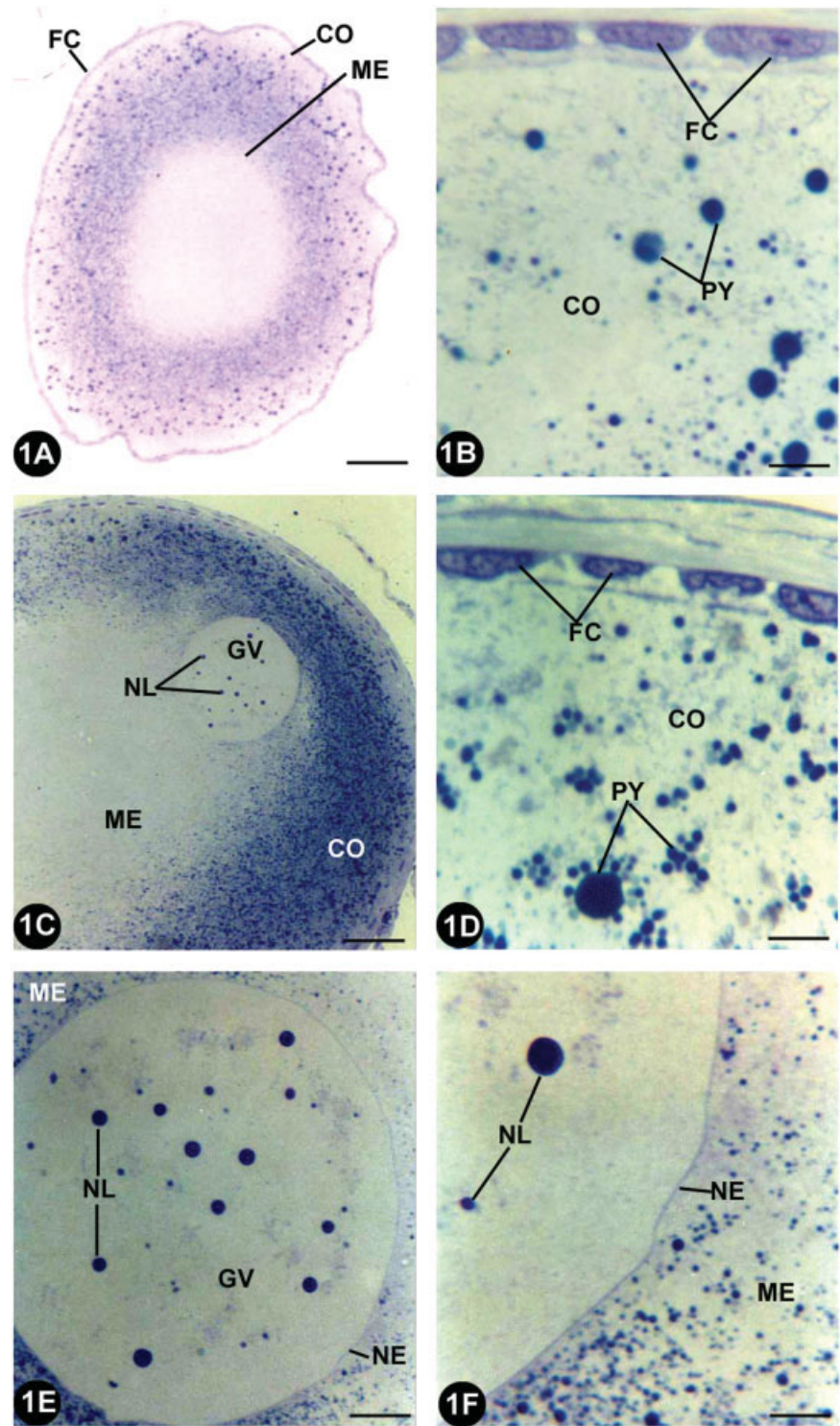


Fig. 1. A–F: Toluidine blue O-stained semi-thin section of previtellogenic follicles of caecilians. **A:** An early follicle of *Ichthyophis tricolor* showing the follicular epithelium and cortico-medullary differentiation of the ooplasm. **B:** A portion of (A) magnified to show a single continuous layer of follicular epithelium and oocyte cortical primordial yolk platelets. **C:** A follicle in a more advanced phase, in *Gegeneophis ramaswamii*, showing cortico-medullary differentiation of ooplasm and several nucleoli in the oocyte nucleus. **D:** A portion of (C) magnified showing follicular epithelial cells and cortical cytoplasm. **E:** Germinal vesicle in (C) magnified showing several randomly distributed nucleoli of different sizes. **F:** A portion of the germinal vesicle–medullary ooplasm interface of (E) magnified to show the nuclear envelope and a nucleolus. CO, cortex; FC, follicular epithelial cells; GV, germinal vesicle; ME, medulla; NE, nuclear envelope; NL, nucleoli; PY, primordial yolk platelets. Scale bar = 0.2 mm in (A,C); 15 μ m in (B,D,E); 2.5 μ m in F.

euchromatic irregular nucleus with a distinct nucleolus and having the classical fibrillo-granular architecture (Fig. 4C). In both the cell types there is only meager cytoplasm surrounding the nucleus. The most prominent cytoplasmic organelles are the mitochondria. In the light follicle the mitochondria are small and electron dense and several

have a prominent vacuolar space (Fig. 4C). The mitochondria of the dark follicle cells are large and less electron dense (Fig. 4B,D). There is little intercellular space between the follicle cells and there are tight and gap junctions between the membranes of adjacent cells (Fig. 4D). The follicle cells closely envelop the oocyte with some vacuolar

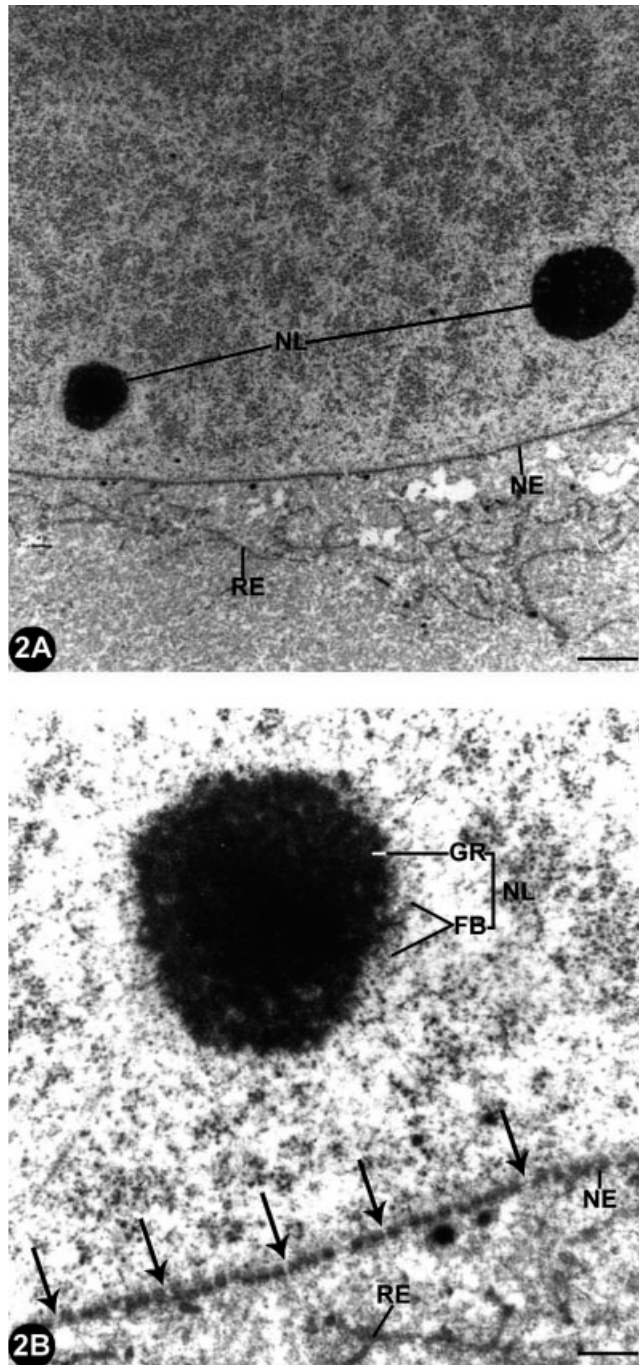


Fig. 2. A,B: The nucleus of the oocyte in Figure 1B (*Gegeneophis ramaswamii*). TEM. **A:** Two nucleoli, differing in size, are shown beneath the nuclear envelope; the medullary ooplasm is rich in rough endoplasmic reticulum. **B:** A portion of (A) magnified to show the fibrillo (FB)-granular (GR) nature of a nucleolus; there are numerous pores in the nuclear envelope (arrows). NE, nuclear envelope; NL, nucleolus; RE, rough endoplasmic reticulum. Scale bar = 0.5 μm in (A); 0.1 μm in (B).

spaces appearing between the two at the regions where the neighboring follicle cells associate through junctional complexes (Fig. 4A,D). The

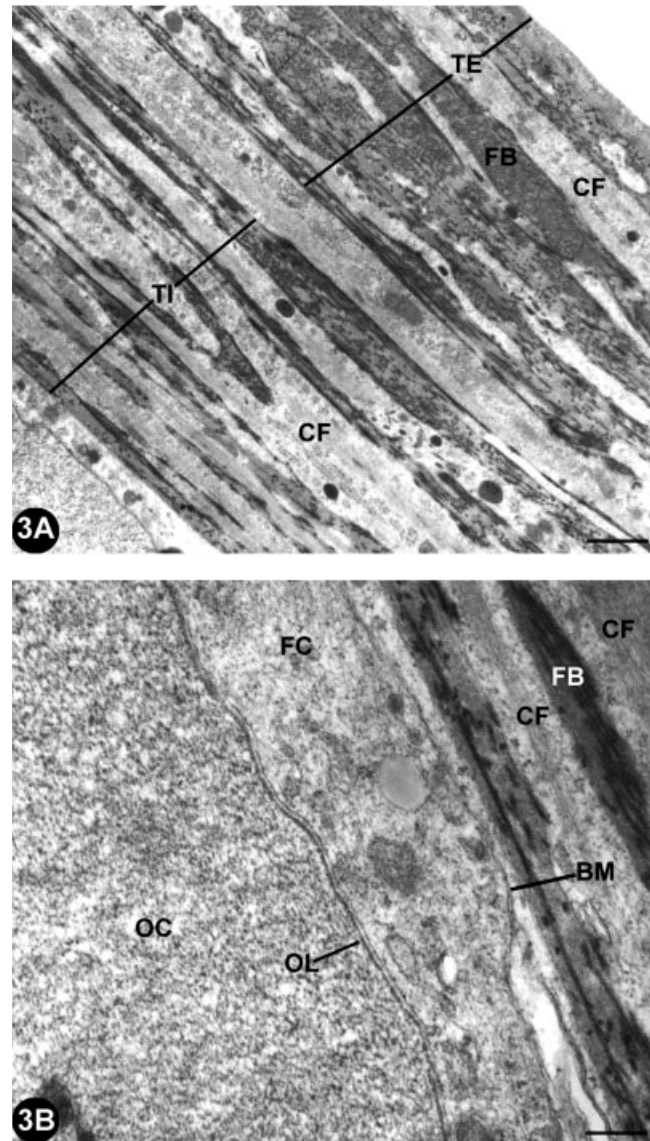


Fig. 3. A,B: A very early previtellogenic follicle of *Gegeneophis ramaswamii*. TEM. **A:** The theca is differentiated as theca externa (TE) and theca interna (TI). There are collagen fibrils (CF) between fibroblasts (FB) of both layers. **B:** The oocyte (OC) is bounded by the oolemma (OL), which is closely applied to a follicular epithelial cell (FC), which in turn rests on the basement membrane (BM). The theca interna consists of fibroblasts (FB) and collagen fibrils (CF). Scale bar = 6.5 μm .

oocyte has a distinct oolemma (Fig. 4A,D). The oocyte cytoplasm exhibits cortico-medullary differentiation and the cortex has scattered mitochondria, rough endoplasmic reticulum (RER), a few saccular elements and a finely granular material (Fig. 4B). The medullary cytoplasm contains abundant yolk precursors of various sizes (not shown).

With further development, the previtellogenic follicle, the theca externa and theca interna become less distinct and the collagen-filled intercellular space increases (Fig. 5A). The basement

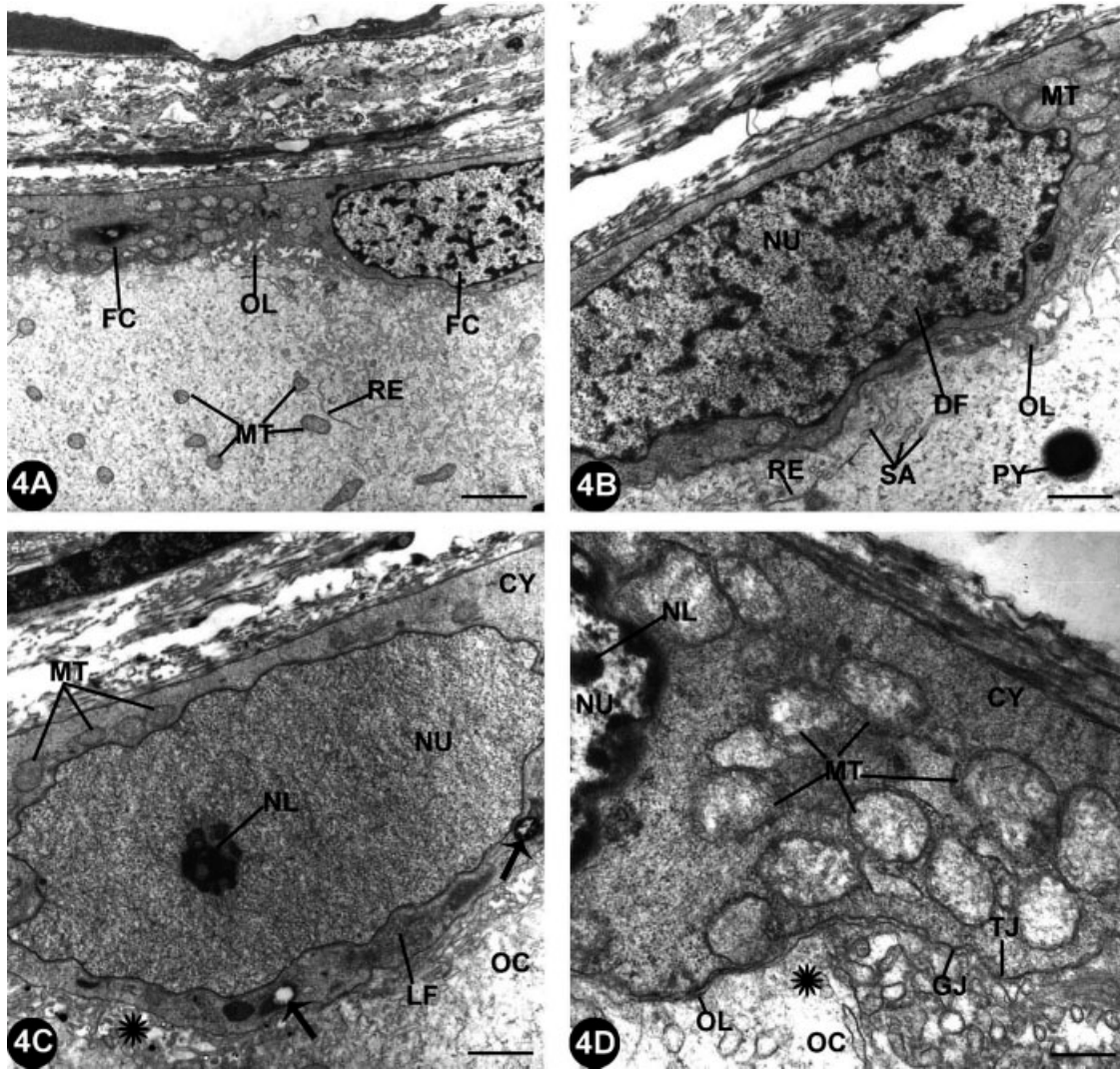


Fig. 4. A–D: The follicular epithelium–oocyte interface of a more advanced previtellogenic follicle (*Ichthyophis tricolor*). TEM. **A:** A low power image showing follicle cells overlying the oolemma. The cortical ooplasm includes scattered mitochondria and rough endoplasmic reticulum. **B:** A dark follicle cell with a dense heterochromatic nucleus. **C:** A light follicle cell with a euchromatic nucleus. Arrows point to vacuolated mitochondria. **D:** A portion of a dark follicle cell showing the mitochondria characteristic of this cell type. CY, cytoplasm of follicle cell; DF, dark follicle cell; FC, follicle cell; GJ, gap junction; LF, light follicle cell; MT, mitochondria; NL, nucleolus; NU, nucleus; OC, oocyte; OL, oolemma; PY, primordial yolk platelet; RE, rough endoplasmic reticulum; SA, saccular elements in cortical ooplasm; TJ, tight junction. Asterisks in (C,D) indicate the intercellular space appearing between the oocyte and follicle cells at specific regions. Scale bar = 4 μm in (A); 2 μm in (B,C); 0.8 μm in (D).

membrane of the follicle cells becomes prominent. The follicle cells become flat. There are both dark and light follicle cells with the characteristic ultrastructural organization as described (Fig. 5B). Trough-like gaps appear between the oocyte and the follicle cells, which are initially confined to the junctional points between the follicle cells (Fig. 5C,H) but later extend as a narrow strip elsewhere (Fig. 5B,D–F). The follicle cells produce finger-shaped macrovilli. Corresponding projections, the microvilli, less prominent than the macrovilli, develop from the oocyte. There is diffuse material in the troughs, surrounding the macro- and micro-villi (Fig. 5C–F). The prominence of the RER (Fig. 5D)

in the cytoplasm of the dark-follicle cells is increased. The highly tortuous intercellular junctions between the follicle cells develop wide gaps between the membranes, particularly at the regions of desmosomes and tight junctions (Fig. 5D,G). The mitochondria of the light follicle cells are compact, highly electron dense and possess saccular cristae (Fig. 5E) whereas those of the dark follicle cells are less electron dense and the cristae are less prominent (Fig. 5F,G). The cytoplasm of the oocyte still maintains the same kind of cortico-medullary differentiation, and the cortex contains highly elongated mitochondria with cristae in the ladder pattern (Fig. 5H) and yolk precursors (not shown).

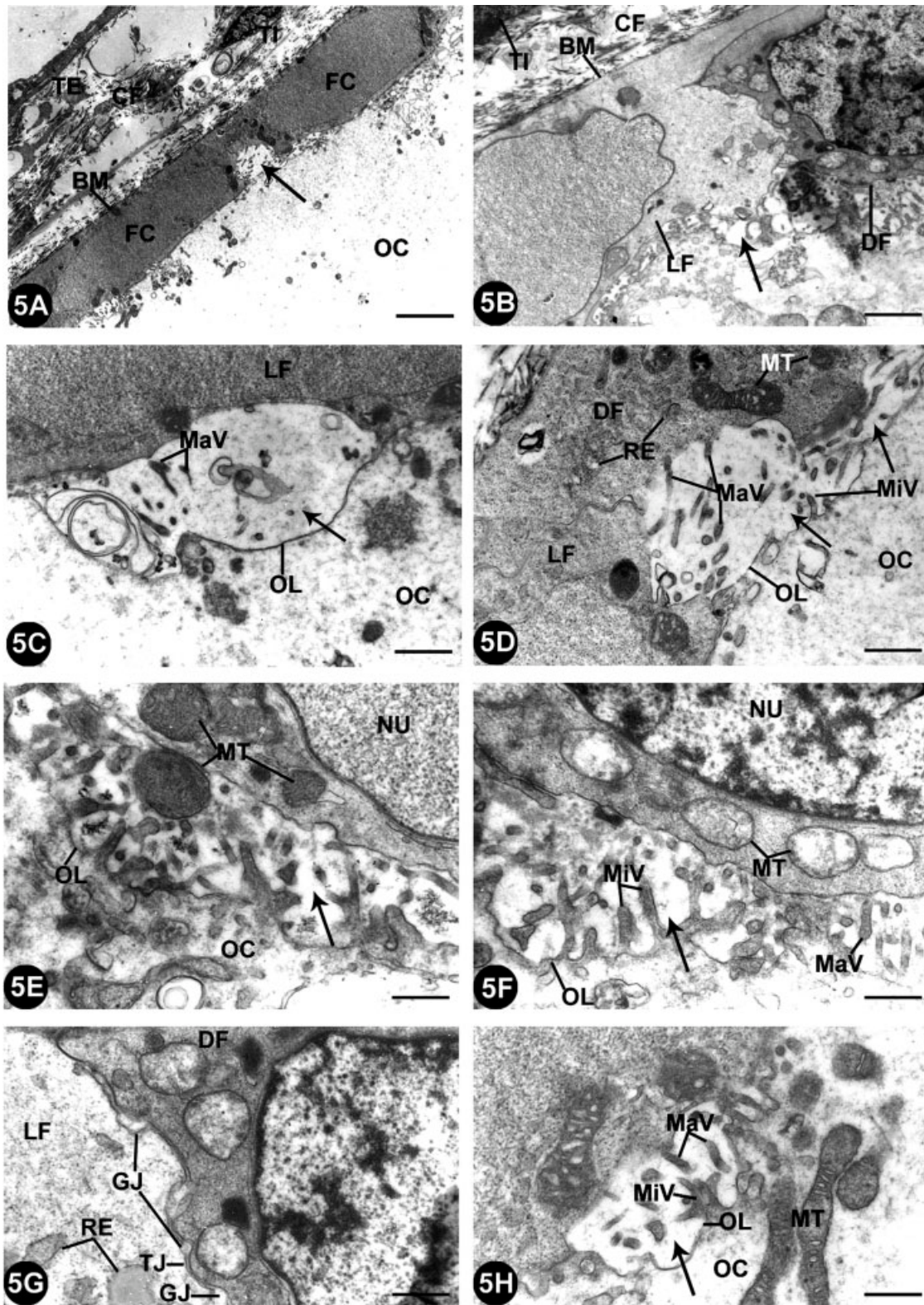


Fig. 5. A-H: The follicular epithelium-ooocyte interface of a more advanced previtellogenic follicle (*Gegeneophis ramaswamii*). TEM. **A:** Low power image showing follicle cells overlying the oocyte. The perivitelline space has started appearing (arrowhead). **B:** Light and dark follicle cell junction showing the forming perivitelline space (arrowhead). **C:** The trough-like nature of the forming perivitelline space (arrowhead). **D:** The trough-like nature of the perivitelline space appearing at the junction between dark follicle cell and light follicle cell and the oocyte (arrowhead). **E:** A portion of the forming perivitelline space (arrow) and a light follicle cell. **F:** A portion of forming perivitelline space (arrow) and a dark follicle cell. **G:** Light follicle cell-dark follicle cell interface showing tight and gap-junctions. **H:** Image shows the nature of macro- and micro-villi appearing in the developing perivitelline space (arrowhead). Mitochondria, with cristae in the ladder pattern, in the cortical ooplasm, are also present. BM, basement membrane of follicle cells; CF, collagen fibrils; DF, dark follicle cell; FC, follicle cells; GJ, gap junction; LF, light follicle cell; MaV, macrovilli; MiV, microvilli; MT, mitochondria; NU, nucleus; RE, rough endoplasmic reticulum; TE, theca externa; TI, theca interna; TJ, tight junction; OC, oocyte; OL, oolemma. Scale bar = 6 μm in (A); 2 μm in (B); 1.1 μm in (C,D); 0.8 μm in (E-H).

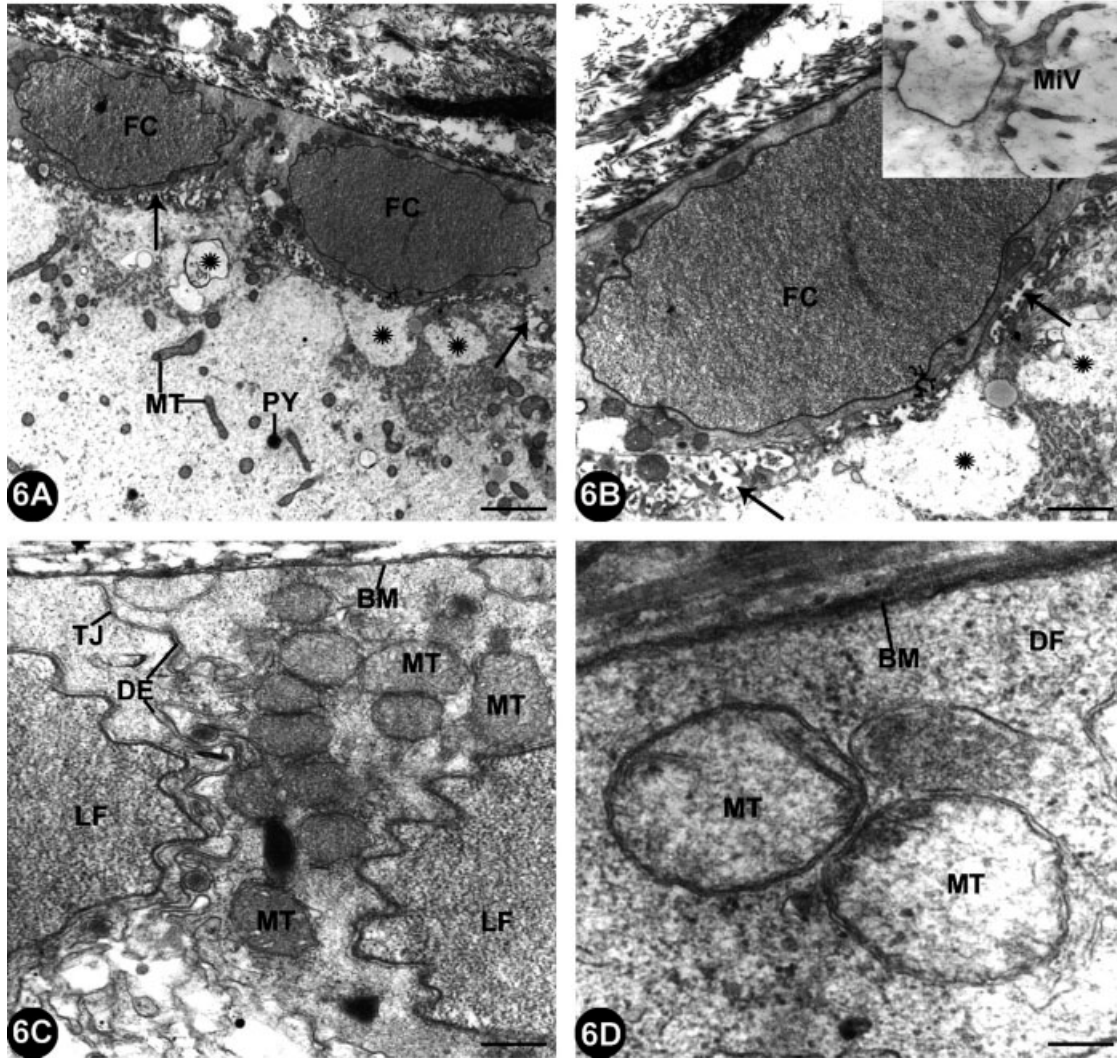


Fig. 6. A–D: The follicular epithelium–oocyte interface at a more advanced stage of previtellogenic follicle development (*Gege-neophis ramsawamii*). TEM. **A:** Appearance of fine granule-filled vesicles in the cortical ooplasm of a previtellogenic follicle (asterisks). The mitochondria of the oocyte have increased in abundance and are scattered. **B:** A portion of (A) magnified. The developing perivitelline space is spreading to neighboring areas (arrowheads). The insert is a magnified view of a branching microvillus (MiV). **C:** Tortuous intercellular junction between follicle cells. **D:** Mitochondria of a dark follicle cell. BM, basement membrane; DE, desmosomes; FC, follicle cells; LF, light follicle cell; MT, mitochondria; PY, primordial yolk platelet; TJ, tight junction. Scale bar = 5 μm in (A); 2.5 μm in (B); 0.3 μm in insert; 0.8 μm in (C); 0.3 μm in (D).

In subsequent development the theca contains fewer fibroblasts but more collagen fibrils. The most characteristic feature of this stage is the appearance of fine granule-filled vesicles in the cortical ooplasm closer to the oolemma (Fig. 6A,B). The oocyte mitochondria of the cortical cytoplasm increase in abundance. The intercellular space between the follicle cells and the oocyte increases in prominence but not yet continuous. The macrovilli of the follicle cells appear to be simple structures but the microvilli of the oocyte become prominent and branched (Fig. 6B insert). Due to flattening of the follicle cells, the junctions between the neighboring cells become more tortuous and the membranes are held together by desmosomes

and tight junctions (Fig. 6C). As in the earlier stage the mitochondria of light follicle cells are compact and electron dense and possess saccular cristae (Fig. 6C), whereas in the dark follicle cells the matrix is less electron dense and there are fewer cristae (Fig. 6D).

With further development the thecal layers become more stretched, resulting in fewer fibroblasts and more abundant collagen fibrils. The follicle cells become flatter and more elongated. The intercellular space between the follicle cells and the oocyte increases and becomes more prominent, but is yet to become continuous since there are a few gap junctions between the follicle cells and the oocyte. All the follicle cells are of the dark type

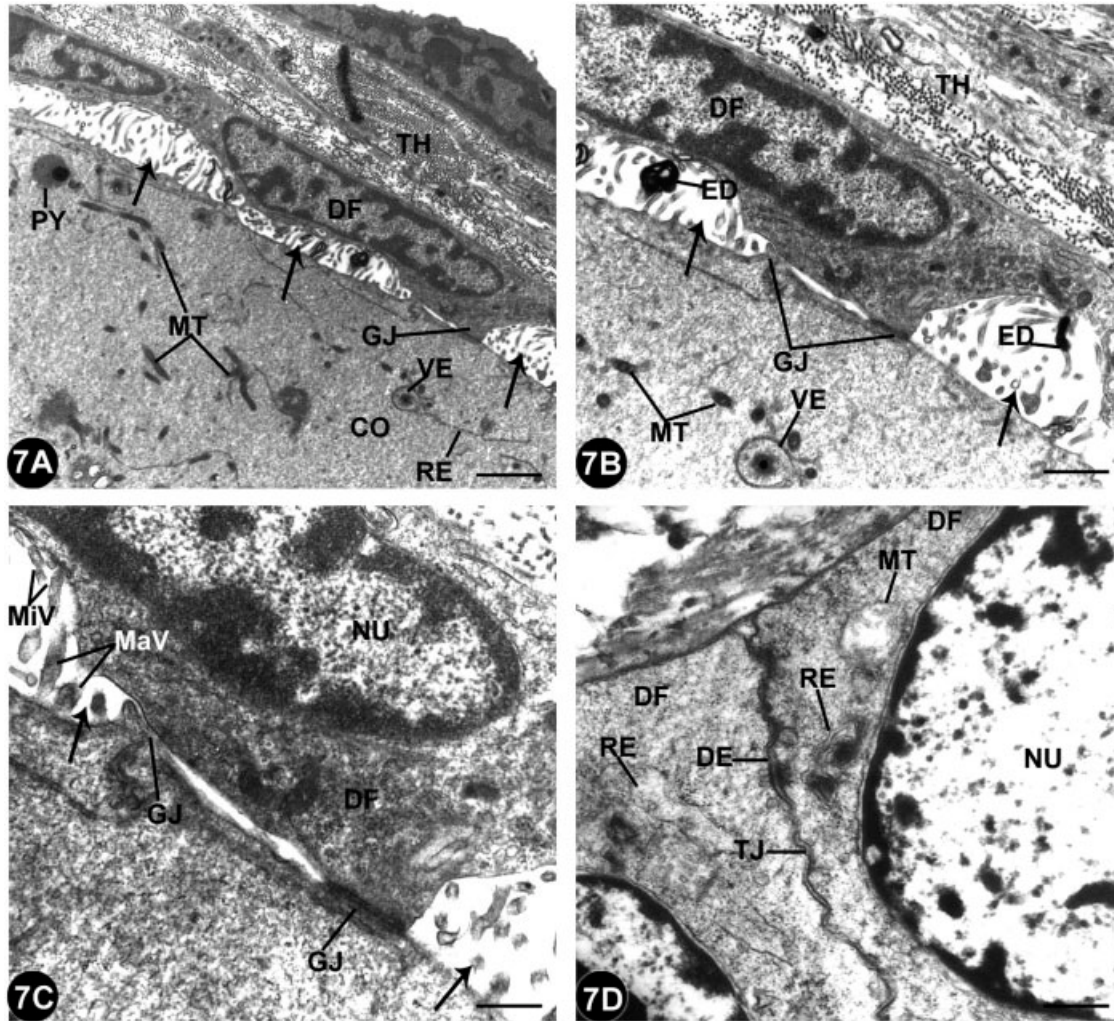


Fig. 7. A–D: The follicle cell–oocyte interface of a somewhat more advanced previtellogenic follicle (*Gegeneophis ramaswamii*).—TEM. **A:** The developing perivitelline space is almost continuous (arrows) except at the regions of gap junctions between the oolemma and the membrane of the follicle cell. All follicle cells are of the dark type. **B:** A portion of (A) magnified to show electron-dense bodies in the forming perivitelline space. **C:** A portion of (B) further magnified to show the gap junctions. **D:** Intercellular junction between follicle cells with a desmosome (DE) and a tight junction (TJ). CO, cortical ooplasm; DF, dark follicle cell; ED, electron-dense vesicle; GJ, gap junction; MaV, macrovilli; MiV, microvilli; MT, mitochondria; NU, nucleus; PY, primordial yolk platelet; RE, rough endoplasmic reticulum; TH, theca; VE, membrane-bound vesicle in cortical cytoplasm. Scale bar = 6 μm in (A); 2.8 μm in (B); 1.7 μm in (C); 0.5 μm in (D).

and contain a continuous patch of dense heterochromatin below the nuclear envelope (Fig. 7A). The macrovilli of the follicle cells and the microvilli of the oocyte are prominent in any area where a junction between the follicular cells and oocyte is not present. There are a few electron-dense granules in the intercellular space (Fig. 7A,B). The intercellular junction between follicle cells remains tight with desmosomes and tight junctions (Fig. 7D). The cytoplasm of the follicle cells contains mitochondria characteristic of the dark type, and RER with an electron-dense material in the cisternae (Fig. 7D). The ooplasm maintains the cortico-medullary differentiation. The cortical cytoplasm has abundant elongated mitochondria and RER, a few yolk precursors and also a few vesicles with

an electron-dense boundary and a less dense core (Fig. 7A).

As development proceeds the differentiation of theca into theca externa and theca interna is less obvious and it contains an almost single layer of theca cells with underlying dense collagen fibrils (Fig. 8A). The follicle cells consist only of the dark type but the nuclear heterochromatin is not as prominent as previously (Fig. 8 A–C). The intercellular space between the follicle cells and the oocyte has become a continuous perivitelline space (Fig. 8A). The macrovilli of the follicle cells and the microvilli of the oocyte dominate the space (Fig. 8A,B,D–F). The macrovilli extend up to the oocyte and associate with the oolemma (Fig. 8B,E). Also, the macro- and micro-villi contact each other. The

perivitelline space contains a few large electron-dense granules (Fig. 8A) and also a diffuse granular material, which is perhaps the precursor of the vitelline envelope (Fig. 8B,E,F). The outer surface of the oocyte occasionally has projections into the perivitelline space from which microvilli also originate (Fig. 8D). The ooplasm maintains the cortico-medullary differentiation (not shown). The cortical region has abundant mitochondria, RER, yolk precursors and dense vesicles (Fig. 8A) and Golgi complexes (Fig. 8F). The cytoplasm of the follicle cells contains abundant mitochondria and RER (Fig. 8C–E) and a prominent Golgi complex (not shown). The intercellular space between the follicle cells is prominent, continuous, and less tortuous and there is always a desmosome towards the oocyte end (Fig. 8B,C).

In the early stages of the previtellogenic follicles the mitochondria appear peripherally as clouds (Fig. 9A,B). The cloud contains a dense aggregation of membrane-bound vesicles glued in a cement-like matrix. There are yolk precursors with a heterogeneous appearance in the neighborhood of these clouds. There are a few larger vesicles containing an electron-opaque material comparable to the lipid droplets shown in the previtellogenic oocyte of *Bufo arenarum* by Vилlecco et al. (1999). In subsequent development, the mitochondria separate to become dense aggregates themselves. The matrix, free from mitochondria, breaks away into small islets when some of the large membrane-bound vesicles are almost empty (Fig. 9C). A few such large vesicles appear to associate among themselves, forming larger aggregates in the vicinity of matrix clouds. Another kind of vesicle containing an outer electron-dense boundary, with a beaded appearance, and an electron-lucent core is also present in the vicinity of these clouds (Fig. 9D,E). Later, the mitochondria spread out and are distributed randomly in the cortical cytoplasm. A few vesicles appear in the ooplasm among the mitochondria (Fig. 9F,G). They appear to be produced as blebs of the mitochondria and later pinch off (Fig. 9H).

DISCUSSION

This is the first TEM description of the previtellogenic follicle of caecilians. It is also perhaps the first report of sequential and ultrastructural changes towards transformation of previtellogenic follicles into vitellogenic follicles for any amphibian. The latest reviews of ovary and oogenesis in the Urodela (Uribe, 2003), the Anura (Sanchez and Vилlecco, 2003) and the Gymnophiona (Exbrayat, 2006) indicate the occurrence of oogonial nests, oocytes and prefollicular cells in the ovaries, irrespective of the month of the year, the season or the reproductive status. However, the recruitment of the follicles (i.e., the assembly of prefollicular

cells and oocytes to establish the primordial follicles), accumulation of yolk precursors and the subsequent changes in the follicles towards previtellogenesis, vitellogenesis and postvitellogenesis would depend upon the seasonal reproductive pattern characteristic of each species, some are a-seasonal though. An understanding of the ultrastructural features of ovarian follicles in amphibians during different periods of oogenesis is important since the structural details are likely to underlie follicular cells–oocyte intercellular signaling (Sanchez and Vилlecco, 2003).

The previtellogenic follicle consists of an oocyte arrested in diplotene and surrounded by a single layer of follicular cells as reported earlier (Sanchez and Vилlecco, 2003). We found early previtellogenic follicles, which have assembled due to the association between follicle cells and the oocytes, throughout the year in both *Ichthyophis tricolor* and *Gege-neophis ramaswamii*. The elaborate structural organization in the follicle cells as well as the oocyte begins in September and the postvitellogenic follicles are found between November and April, the period which coincides with low-ambient temperature and high-moisture content of the soil. The two species, though differing slightly in distribution in the Western Ghats (Oommen et al., 2000; Bossuyt et al., 2004), follow a similar seasonal pattern in ovarian changes. Aspects of seasonality of female reproduction in these caecilians will be reported elsewhere.

The successive layers of a fully developed anuran previtellogenic follicle consist of theca externa, theca interna, basement membrane of the follicle cells, the follicle cells themselves, the perivitelline space, which will later develop into the vitelline envelope, and the plasma membrane of the oocyte. The zona pellucida and the vitelline envelope appear later (Stifani et al., 1988, 1990; Aybar et al., 1996; Vилlecco et al., 1996, 1999, 2000). The organization of the previtellogenic follicles in the caecilians almost matches that of the frogs described. The transformation of the theca into a thin layer, losing the distinction as externa and interna and its thinning out is perhaps a consequence of the increase in the size of the oocyte. Corresponding with this transformation the collagen material of the previtellogenic follicle theca forms into dense-collagen fibrils, which would provide for the tensile strength of the outer envelop layer. The cortico-medullary differentiation of oocyte cytoplasm seen in caecilians is the same as in other amphibians (Sanchez and Vилlecco, 2003; Uribe, 2003).

As is typical of anurans and urodeles (Sanchez and Vилlecco, 2003), the oocyte has only one nucleus in the caecilians as well. Amplification of the nucleoli is another characteristic feature of amphibians in general (Sanchez and Vилlecco, 2003). This amplification occurs during the trans-

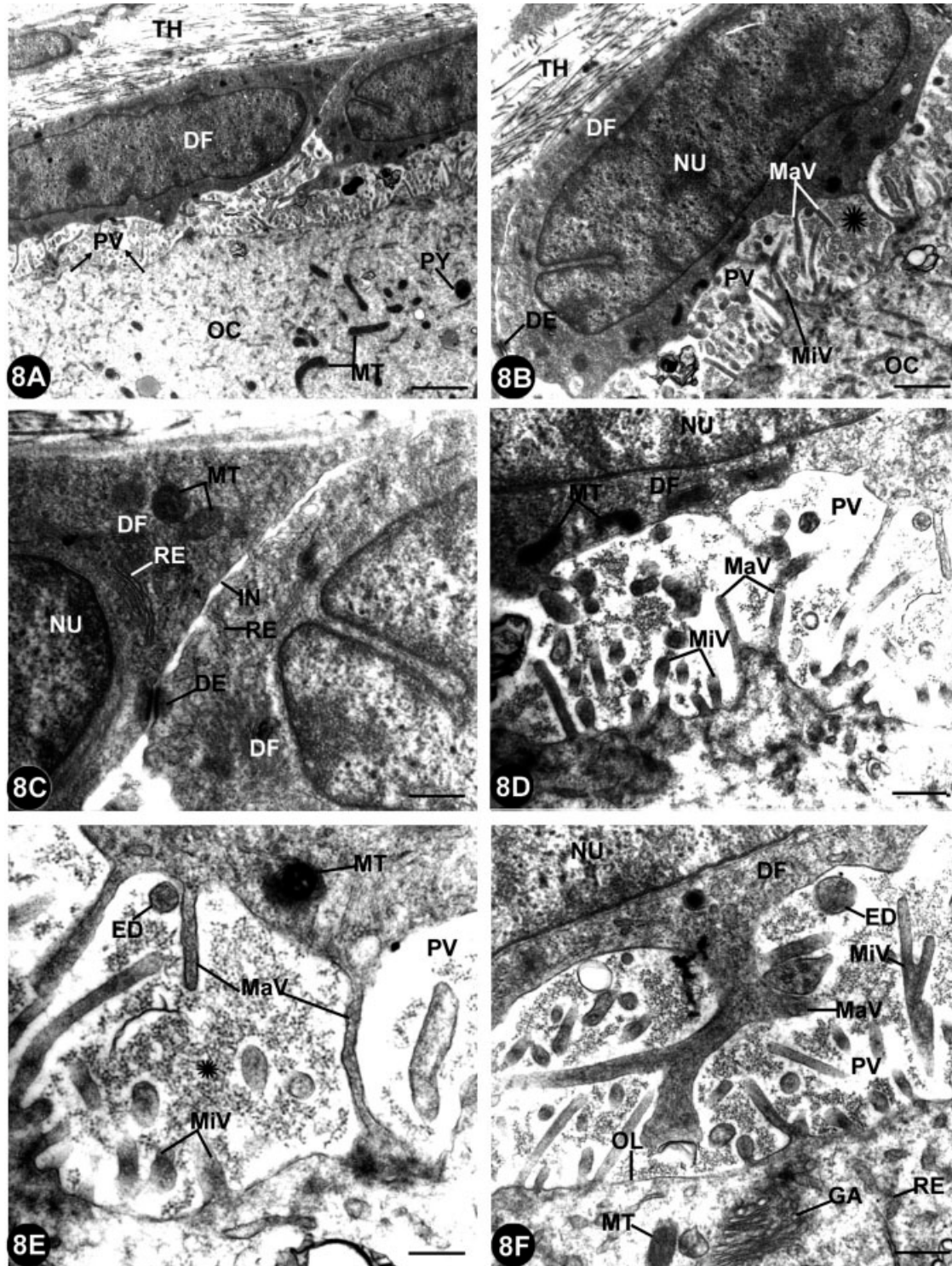


Fig. 8. A–E: A terminal phase previtellogenic follicle (*Ichthyophis tricolor*). TEM. **A:** The perivitelline space has become continuous (arrows), and exhibit numerous macro- and micro-villi. All follicle cells are of the dark type. **B:** A portion of (A) magnified to show the abundance of macro- and micro-villi and the accumulation of a dense granular material in the perivitelline space (asterisk). **C:** Intercellular junction between dark follicle cells showing an almost continuous intercellular space (IN) and a desmosome (DE). **D:** A portion of (B) magnified to show the perivitelline space. **E:** Another portion of (B) highly magnified, showing the interrelationship between macro- and micro-villi. **F:** A highly branched macrovillus. A Golgi apparatus (GA) is shown in the cortical ooplasm. DF, dark follicle cell; ED, electron-dense granule; MaV, macrovilli; MiV, microvilli; MT, mitochondria; NU, nucleus; PY, primordial yolk platelet; OC, oocyte; OL, oolemma; PV, perivitelline space; RE, rough endoplasmic reticulum; TH, theca. Asterisks indicate electron dense precursor material of vitelline envelope. Scale bar = 6 μm in (A); 3.7 μm in (B); 1.2 μm in (C); 1.4 μm in (D); 1.1 μm in (E); 1.3 μm in (F).

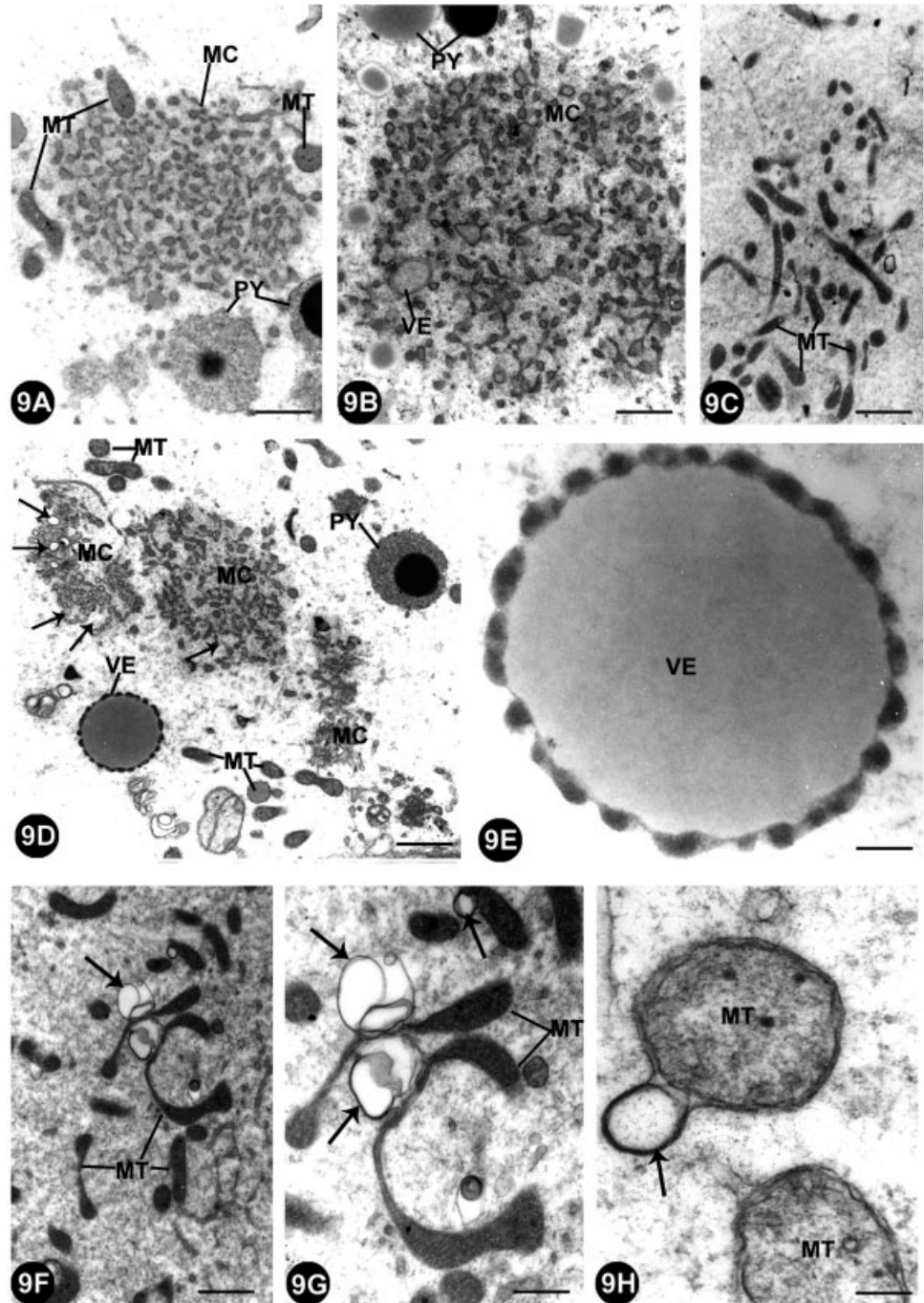


Fig. 9. A–H. The mitochondrial cloud and related development (*Ichthyophis tricolor*). TEM. **A:** Mitochondrial cloud of an early previtellogenic oocyte showing the cementing matrix and peripheral mitochondria. **B:** In subsequent development the mitochondria have moved completely away from the mitochondrial cloud. The cementing matrix contains membrane-bound vesicles, whose content is probably lipid. **C:** A cluster of mitochondria completely free from the mitochondrial cloud. **D:** The cementing matrix contains clear vesicles (arrows). There is a large vesicle (VE) having an outer electron-dense boundary, with a beaded appearance, and an electron-lucent core is also present in the vicinity of these clouds with large aggregates in the vicinity of the matrix clouds. **E:** The vesicle in (D) magnified (VE) to show its electron-dense beaded boundary and an electron-opaque content. **F:** Long filamentous mitochondria, near vacuoles (arrows). **G:** A portion of (F) magnified. **H:** The vacuoles appear as blebs in the mitochondria (arrow). MC, cementing matrix of a mitochondrial cloud; MT, mitochondria; PY, primordial yolk platelets; VE, vesicle filled with lipid. Scale bar = 1.4 μm in (A–D); 0.3 μm in (E); 1.1 μm in (G); 0.4 μm in (H).

formation of the early previtellogenic oocyte into a later phase in the caecilians. The amplification indicates transcription of ribosomal RNA. In *Xenopus* primary oocytes RNA transcription begins during diplotene in early previtellogenic stage. The main period for the selective replication of rRNA is the pachytene of meiotic prophase and is completed during early diplotene (Sanchez and Vilecco, 2003). Thus, the pattern of nucleolar amplification in the caecilian previtellogenic oocytes is comparable to that in the anurans. However, in

the growing oocytes of anurans the amplified nucleoli are distributed throughout the periphery of the nucleus and are often firmly attached to the inner nuclear membrane by a network of fibrillar strands (Frankie and Skeer, 1970; Sanchez and Vilecco, 2003). We did not observe such a translocation of the amplified nucleoli or any fibrillar connection of the nucleoli and the inner nuclear membrane in caecilian previtellogenic oocytes. The organization of the nucleolus as a fibrillo-granular structure is classical (Sanchez and Vilecco, 2003).

The nuclear pores observed in this study are again a classical feature and provide for trafficking of materials between the nucleus and the cytoplasm (Sanchez and Vилlecco, 2003).

We have limited our observation to changes in the follicles prior to accumulation of yolk in the primordial yolk platelets. The cytoplasm of the previtellogenic oocytes contains only primordial yolk platelets. It is in these platelets that the yolk precursors, consisting of vitellogenin, glycogen and lipids, will accumulate (Sanchez and Vилlecco, 2003). The lipids due for deposition in the yolk platelets appear and are stored as lipid droplets in the vicinity of the mitochondrial cloud well before vitellogenin starts arriving at the oocyte in caecilians. The lipid droplets appear to be produced within the oocyte in the residual mitochondrial cloud, which consists of the membrane-bound vesicles cemented together. Future studies applying tracers may show if this interpretation is correct.

Our study traces the development of the ultrastructural complexity at the follicle cell–oocyte interface. At the earliest phase of differentiation of the previtellogenic follicle there is no trace of the perivitelline space. There are intermittent junctional complexes between the membrane of the follicle cells and the oolemma. Vилlecco et al. (2000, 2002) observed heterologous gap junctions between oocyte and follicle cells in *Bufo arananum*. The presence of intercellular bridges during oogenesis may represent a crucial event of the active cooperation between follicle cells and the oocyte (Andreuccetti et al., 1999). The perivitelline space is initiated as discontinuous troughs at the junctional points of the follicle cells, where macro- and microvilli appear. Once initiated, the perivitelline space gradually spreads due to the disengagement of the tight junctions between the follicle cell membrane and the oolemma. The macrovilli of the follicle cells are initially simple protuberances. Later, they associate with microvilli of the oolemma and develop clatherin-coated vesicles for translocation of yolk precursors into the oocyte cytoplasm.

The material of the vitelline envelope in amphibians accumulates as granules and fibrils in the perivitelline space (Stifani et al., 1988, 1990; Aybar et al., 1996; Cabada et al., 1996; Vилlecco et al., 1999, 2000, 2002; Sanchez and Vилlecco, 2003). In caecilians examined, until the commencement of translocation of yolk materials into the oocyte, the material of the vitelline envelope remains finely granular. Perhaps the transformation of the granular material into the lattice-work of the fibrillar vitelline envelope will occur later, concomitant with or subsequent to the translocation of yolk precursors.

In the literature the terms granulosa cells and follicle cells have been used synonymously. We adopt the term follicle cells. Two kinds of follicle cells, dark and light, in the early previtellogenic

follicle are already known for anurans (Sanchez and Vилlecco, 2003). In caecilians these two variants of follicular cells differ not only in electron density but in the ultrastructural organization of the mitochondria, endoplasmic reticulum, and nucleus. However, well before the transformation of previtellogenic follicles into vitellogenic follicles all the follicle cells become the dark type, suggesting a developmental transition. The prominent and peculiar mitochondria of the follicle cells in general, and those of the darker variant in particular, would reflect the role of these cells in the transport of yolk precursors across the follicle cells into the oocyte (Sanchez and Vилlecco, 2003). However, the extensively developed RER and Golgi complex of the dark follicle cells would suggest a role more than sequestration of yolk from the blood to the oocyte.

The occurrence of enormous mitochondria as dense aggregates during the early previtellogenic phase of the caecilian oocyte, and their moving away from the clouds to become evenly distributed in the cortical ooplasm leaving behind the cementing matrix during late previtellogenesis are interesting observations. The presence of large mitochondrial clusters during the previtellogenic stage of the amphibian oocytes has been described and reviewed (Guraya, 1979; Tourte et al., 1981, 1984; Mignotte et al., 1987; Zarnescu, 2004). They are believed to originate from the Balbiani body (Belairs, 1964; Guraya, 1979). In *Xenopus laevis* the mitochondrial cloud increases during previtellogenesis (Callen et al., 1980; Mignotte et al., 1987) and the cloud contains many intermingled mitochondria and small vesicles (Billet and Adam, 1976; Tourte et al., 1981). Most of the mitochondria are gathered in the middle of the cell as single mass, held together by an intermitochondrial cement in the vicinity of the nucleus in *X. laevis* (Tourte et al., 1981, 1984; Mignotte et al., 1987). Thus, the situation prevailing in the caecilian oocytes corresponds to the latter. It was proposed that the cement contains RNA and proteins and attracts mitochondria to provide nuclear information for an intense biogenesis (Eddy and Ito, 1971; Wegnez et al., 1978; Tourte et al., 1984). According to Tourte et al. (1981), in *Xenopus*, during diplotene, the mitochondrial cloud enlarges rapidly and the association of mitochondria with the cement is rare inside the cloud.

Sanchez and Vилlecco (2003), in their review, related the mitochondrial cloud in anuran oocytes to translocation of mRNA into the vegetal cortex. These authors described a METRO (message transfer organizer) pathway, in which transcripts are localized in the mitochondrial cloud before redistribution to the germ plasm at the vegetal cortex. The transcripts in this pathway include the nanos family member Xcat2 (Mosquera et al., 1993), which may be involved in axial patterning,

the germ-cell associated protein Xpat (Hudson and Woodland, 1998), and the signaling factor Wnt11 (Ku and Melton, 1993). According to Chan et al. (2001) RNA's such as fat Vg follow a pathway, using both the mitochondrial cloud and elements of the pathway, to reach their localization in the vegetal cortex. The formation of cortical vesicles in the previtellogenic oocyte has been observed earlier in previtellogenic and early vitellogenic oocytes of the frog *Ceratophrys cranwelli* (Sanchez and Vilecco, 2003).

In conclusion this article describes the sequential changes in the transformation of early previtellogenic follicles into late previtellogenic follicles in two species of caecilians. During this transformation the perivitelline space originates and is fully established. The follicle cells develop macrovilli and the oolemma develops microvilli. The macro- and microvilli interdigitate and the mRNA is amplified in the oocyte as revealed by the numerous nucleoli. Mitochondria disperse out from the cementing matrix of the mitochondrial cloud. Several membrane-bound vesicles, probably the lipid precursors of yolk, appear in the cement of the mitochondrial cloud.

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