The Natural History Journal of Chulalongkorn University 5(1): 21-30, May 2005

Ultrastructural Changes in the Ovarian Follicular Wall During Oocyte Growth in the Nile Tilapia, *Oreochromis niloticus* Linn.

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ABSTRACT.–Ultrastructural study of cytodifferentiation in the ovarian follicle of the Nile tilapia, *Oreochromis niloticus*, was carried out during the period of oocyte growth with special reference to the vitelline envelope, granulosa and thecal cell layers. In the perinucleolar stage, a simple layer of flattened granulosa cells was observed. The cells possess multivesicular bodies, RER and free ribosomes. In the cortical alveolar stage, granulosa cells also show a well developed dilated tubular RER and electron-dense materials. At this stage the vitelline envelope is observed as a single electron-dense mesh pattern layer, becoming thicker during the vitellogenic stage. From the early vitellogenic stage, the amount of electron-dense materials in granulosa cells increased. The granulosa cells proliferate and form a multicellular layer. The cells are organelle-rich, with elongate mitochondria, free ribosomes, dilated tubular RER and a Golgi system. The theca of the perinucleolar oocyte is a stratified squamous layer. At this stage, the thecal cells show ultrastructural steroidogenic features including mitochondria with tubular cristae, abundant globular SER and transport vesicles with an electron-dense content. In the vitellogenic stage, the thecal cells still show steroidogenic characteristics, but with more abundant mitochondria with tubular cristae as well as some pleomorphic mitochondria. Overall, these results describing the ultrastructure of the developing *O. niloticus* oocyte focusing on the morphology of the follicular cells suggest a primary role for thecal cells in production and secretion of steroid hormones at this stage of ovarian development in this species.

KEY WORDS: Ovarian follicle, Vitelline envelope, Granulosa cell, Thecal cell, *Oreochromis niloticus* Linn.

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INTRODUCTION

The study of the normal development, differentiation, structure, and function of various components of developing follicles in the ovaries of numerous fish species have been

a consistent focus of comparative reproduction (Guraya, 1986). Recently, the fish screening assay has been selected as a component of the Tier 1 screening by the Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) to evaluate the potential toxicity of chemicals and mixtures on the endocrine system (US EPA, 2002). A comparative study of cellular, subcellular and endocrine biology of ovarian follicle growth is thus essential for a better understanding of biology of fish eggs, and of the effects of xenobiotic factors on egg production.

The ovary is a primary target organ in any assessment of reproductive toxicity in female and in other studies on female reproductive biology. In teleosts, the anatomy of the ovary and the pattern of follicular development are diverse, but all share the ultimate goal of producing and supporting germ cells for successful reproduction. Folliculogenesis in teleosts begins after the transformation of oogonia to primary oocytes and continues throughout the growth and maturation of the oocyte (Khan and Thomas, 1999). The ovarian follicle has vital functions in the support of oogenesis, steroidogenesis and vitellogenesis. During oocyte growth and maturation, follicular somatic cells undergo cytodifferentiation and cooperate in the production of steroidal mediators (Nagahama, 1988). Although the basic structure and developmental processes share major similarities in teleosts, there is considerable variation at the cytological levels.

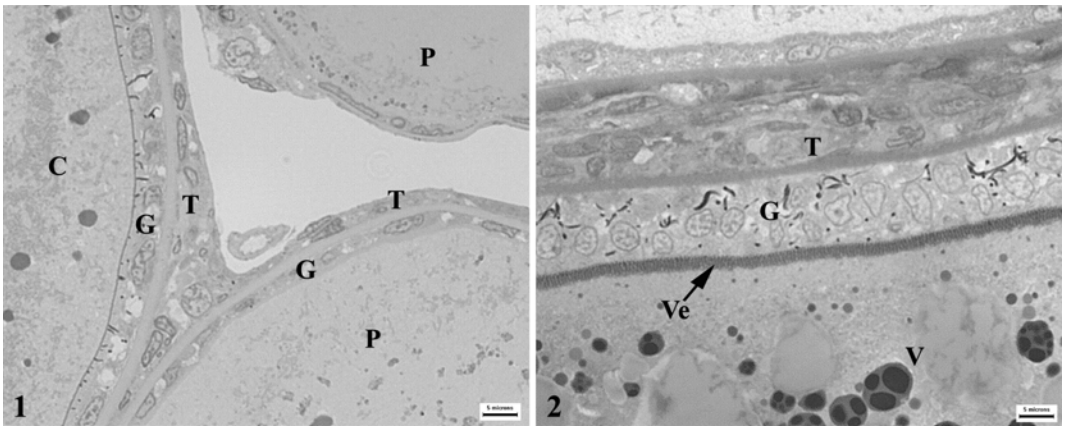
The Nile tilapia, *Oreochromis niloticus* Linn. (Perciformes: Cichlidae) is an economically important teleost adapted to freshwater, and numerous studies using this model are documented in aquaculture, reproductive biology, endocrinology and aquatic toxicology. *Oreochromis* spp. possess a cystic type ovary with asynchronous follicular development. Nakamura et al. (1993) has reported the ultrastructure of the early (40-100 d post hatch) ovary, and reported evidence for steroidogenesis. In a previous study, we reported the histological characteristics of oocytes at different developmental stages in *O. niloticus* (Srijunngam and Wattanasirmkit, 2001). In the present study, we further elucidate ultrastructural changes in the ovarian follicular components, including vitelline envelope, granulosa cell layer and thecal cell layer in *O. niloticus* during the period of oocyte growth.

MATERIALS AND METHODS

Oreochromis niloticus brood stock was obtained from the Aquatic Animal Breeding Research Station at Pathumtani, Department of Fisheries, the Ministry of Agriculture and Cooperatives of Thailand. The brood stock was acclimated and raised in 325-L glass aquaria (300 fish/aquarium) with an aerated water supply and fed with commercial fish food pellets (15.5% protein; CP Company, Thailand) twice daily. The aquarium was filled with 200 liters carbon-resin filtered water. A static renewal system was used and the holding water of every aquarium was renewed every 4 days. Water temperature ranged between 27 and 29 ^{o}C and pH ranged between 6.6 and 7.5. The fish were maintained on a 14 h light: 10 h dark photoperiod. The fish were sampled (n=20) every month at the age of sexual maturation begin from 6 to 9 months.

Histology and ultrastructure

Eight ovaries (2 ovaries from each month) were sampled and fixed in 4% glutaraldehyde at 4 ^{o}C and post-fixed in 2% osmium tetroxide, and then processed through steps in the rapid protocol for TEM processing (Rowden and Lewis, 1974). The fixed tissues were stained with 2% aqueous saturated uranyl acetate, dehydrated in graded ethanol solutions and propylene oxide, and embedded in epoxy resin (Epon 812). Semi-thin sections (150-200 nm) were cut with an ultramicrotome (RMC MT-XL), stained with 0.5 % toluidine blue and observed by light microscopy (Zeiss Axioskop 40). From eight semi-thin samples, four samples were selected as a representative of every month to process for thin sections. Thin sections (90-100 nm) were stained with lead



FIGURES 1-2. **(1)** Light micrograph of ovarian follicles of *O. niloticus* shows flattened granulosa and thecal cells of perinucleolar oocytes and low cuboidal granulosa and flattened thecal cells of cortical alveolar oocyte. **(2)** Light micrograph of ovarian follicle of *O. niloticus* shows thick vitelline envelope and proliferation of granulosa cell of vitellogenic oocyte. **C**: cortical alveolar oocyte, **G:** granulosa cell, **P**: perinucleolar oocyte, **T:** thecal cell, **V:** vitellogenic oocyte, **Ve:** vitelline envelope. Bars: 5 μm.

citrate and uranyl acetate and were examined with transmission electron microscope (Jeol JEM-2010) in the Department of Biology, Boston University.

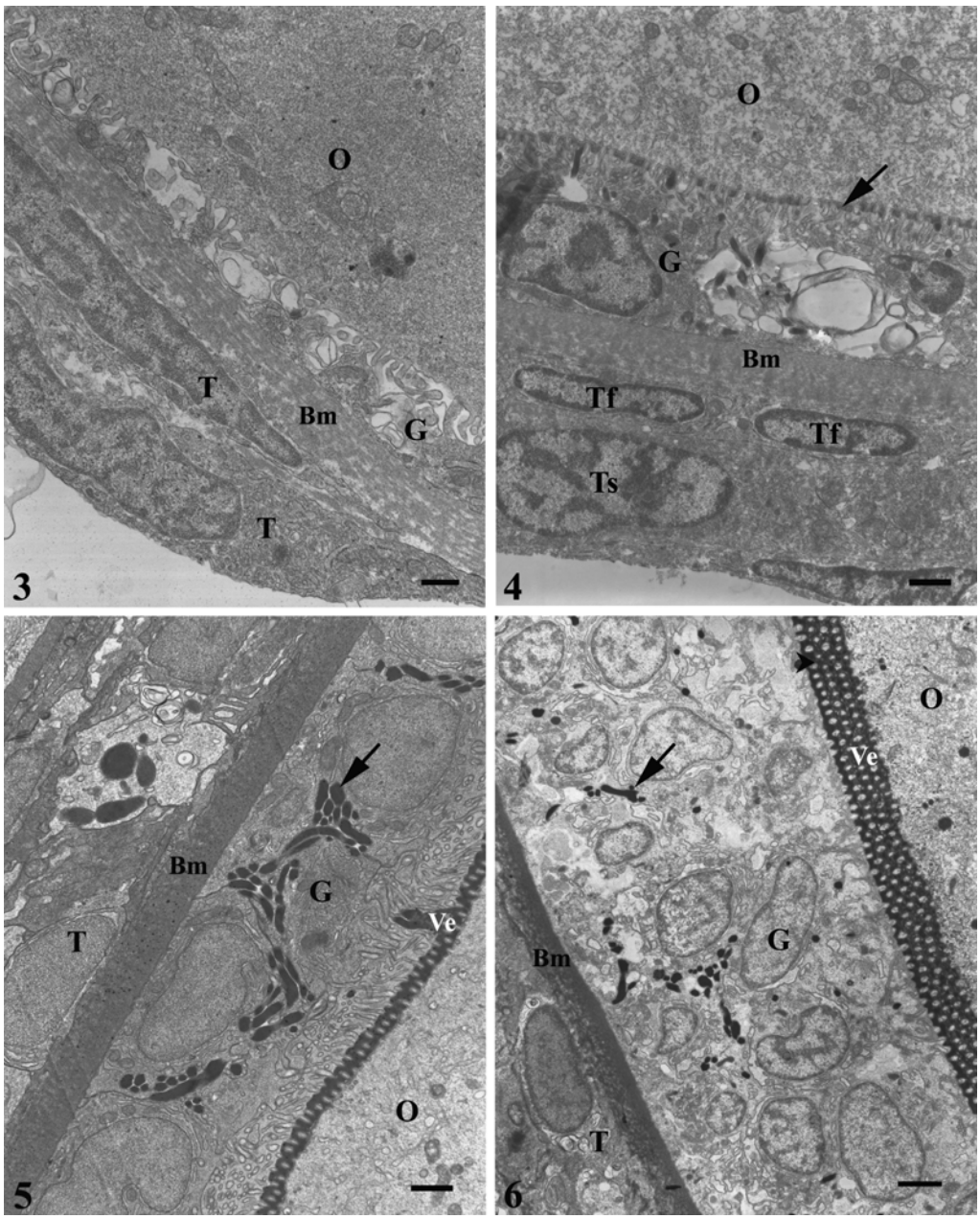
RESULTS

Ovarian follicular organization

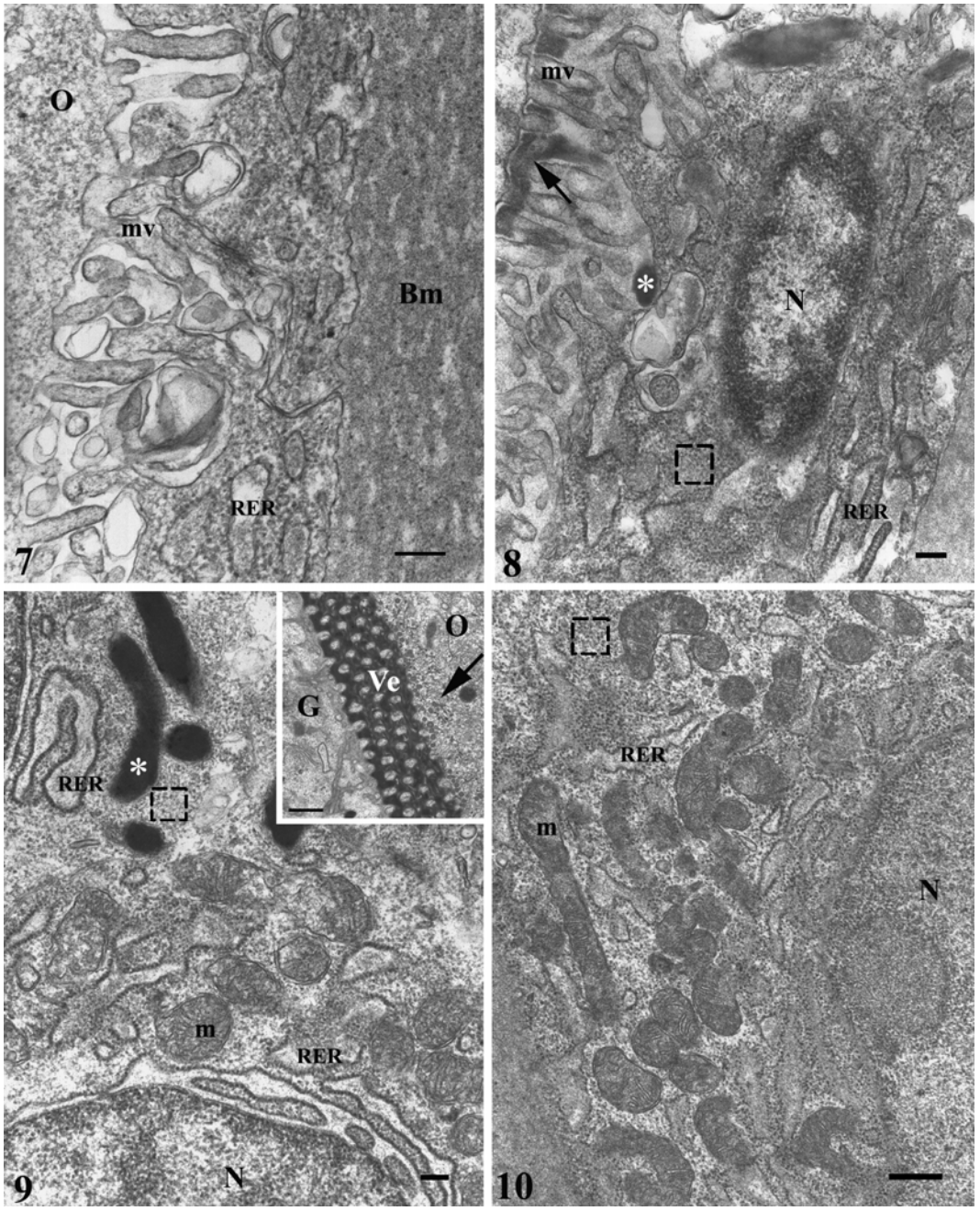
The ovary of *O. niloticus* consists of oocytes at different stages of development including 1) the chromatin nucleolar stage, 2) perinucleolar stage, 3) cortical alveolar stage, 4) vitellogenic stage and 5) ripe (preovulatory) stage (Alves et al., 1983; West, 1990). Each ovarian follicle consists of the developing oocyte surrounded by two layers of somatic cells, the inner granulosa cells and the outer thecal cells. During early oocyte growth (perinucleolar stage), the oocyte was surrounded by a simple layer of flattened (squamous) granulosa cells. An outer layer of simple or stratified thecal cells was also present (Fig. 1). The two cell types were separated by noncellular basement membrane (Fig. 3). A vitelline envelope was first observed at the cortical alveolar stage as a layer of electron-dense amorphous material in the area of interdigitation of cytoplasmic processes (microvilli) of the oocyte and the granulosa cells. At this stage, the granulosa cells differentiated from squamous to low-cuboidal shape (Figs 1 and 4). At the early vitellogenic and vitellogenic stages, the somatic cell layers of the follicle became thicker with evidence of granulosa cell proliferation (Fig. 2). The vitelline envelope also became thicker after entering the vitellogenic stage, forming a mesh pattern of electron-dense, homogeneous material (Figs 5 and 6). Higher magnification electron micrographs showed that this pattern may be a result of collective deposition of electron-dense material in the perivitelline space where the cytoplasmic processes of the oocyte and granulosa cells were interdigitated (Figs 5 and 8). There were a number of diffuse electron-dense granules in the ooplasm near the vitelline envelope (Fig. 9).

The granulosa cells

The granulosa cells of perinucleolar oocytes possessed a number of multivesicular bodies, rough endoplasmic reticulum and free ribosomes. Mitochondria were rarely seen at this stage. Interdigitation of cytoplasmic processes between the oocyte and granulosa cells was evident. Multivesicular bodies were found near the interdigitation area (Fig. 7). At the cortical alveolar stage, the RER was dilated and well developed. Electron-dense materials



FIGURES 3-6. Electron micrograph of ovarian follicles of *O. niloticus*. **(3)** Follicle of perinucleolar oocyte consists of squamous granulosa cells with microvilli and squamous thecal cells. ×10,000. Bar: 500 nm. **(4)** Follicle of cortical alveolar oocyte consists of low cuboidal granulosa cells, fibroblast-like thecal cells and special thecal cells. Note the thin vitelline envelope (arrow). ×5,000. *Bar*: 1 μm. **(5)** Follicle of early vitellogenic oocyte consists of cuboidal granulosa cells with electron-dense materials (arrow) and squamous thecal cells. ×5,000. Bar: 1 μm. **(6)** Follicle of vitellogenic oocyte consists of thick proliferate granulosa cell layer with electron-dense materials (arrow). Note the thick vitelline envelope. ×5,000. Bar: 1 μm. **Bm**: basement membrane, **G**: granulosa cell, **O**: oocyte, **T**: thecal cell, **Tf**: fibroblast-like thecal cell, **Ts**: special thecal cell, **Ve**: vitelline envelope.



FIGURES 7-10. Electron micrograph of granulosa cells of *O. niloticus*. **(7)** Granulosa cell of perinucleolar oocyte shows extensive interdigitation of microvilli and tubular RER. ×20,000. Bar: 200 nm. **(8)** Granulosa cell of cortical alveolar oocyte contains abundant free ribosomes (square) and tubular RER. Note the deposition of electron-dense material in the area of microvillus interdigitation (arrow). ×20,000. Bar: 200 nm. **(9)** Granulosa cell of vitellogenic oocyte contains abundant free ribosomes (square), tubular RER and electron-dense materials (*). ×20,000. *Bar*: 200 nm. Inset: area of vitelline envelope, showing diffuse electron-dense granules (arrow). ×8,000. Bar: 1 μm. **(10)** Granulosa cell of late vitellogenic oocyte contains abundant free ribosomes (square), tubular RER and elongate mitochondria. ×15,000. Bar: 500 nm. **Bm**: basement membrane, **G**: granulosa cell, **m**: mitochondria, **mvb**: multivesicular bodies, **mv**: microvilli, **N**:

were found throughout the cytoplasm and in the area of vitelline envelope (Fig. 8). A number of multivesicular bodies and free ribosomes were also evident. The amount of electron-dense materials in the cytoplasm increases from the early vitellogenic stage (Fig. 5). The cells begin to show abundant mitochondria with electron-dense matrices, free ribosomes, well developed tubular RER and a Golgi system. At the vitellogenic stage, the granulosa cells proliferated resulting in thickening of this layer (Fig. 6). Cells continued to be organelle-rich with mitochondria, free ribosomes, dilated tubular RER and Golgi system. The RER cisternae were filled with an amorphous electron-lucent material (Fig. 9). Mitochondria were elongate with electron-dense matrix at the late vitellogenic stage (Fig. 10).

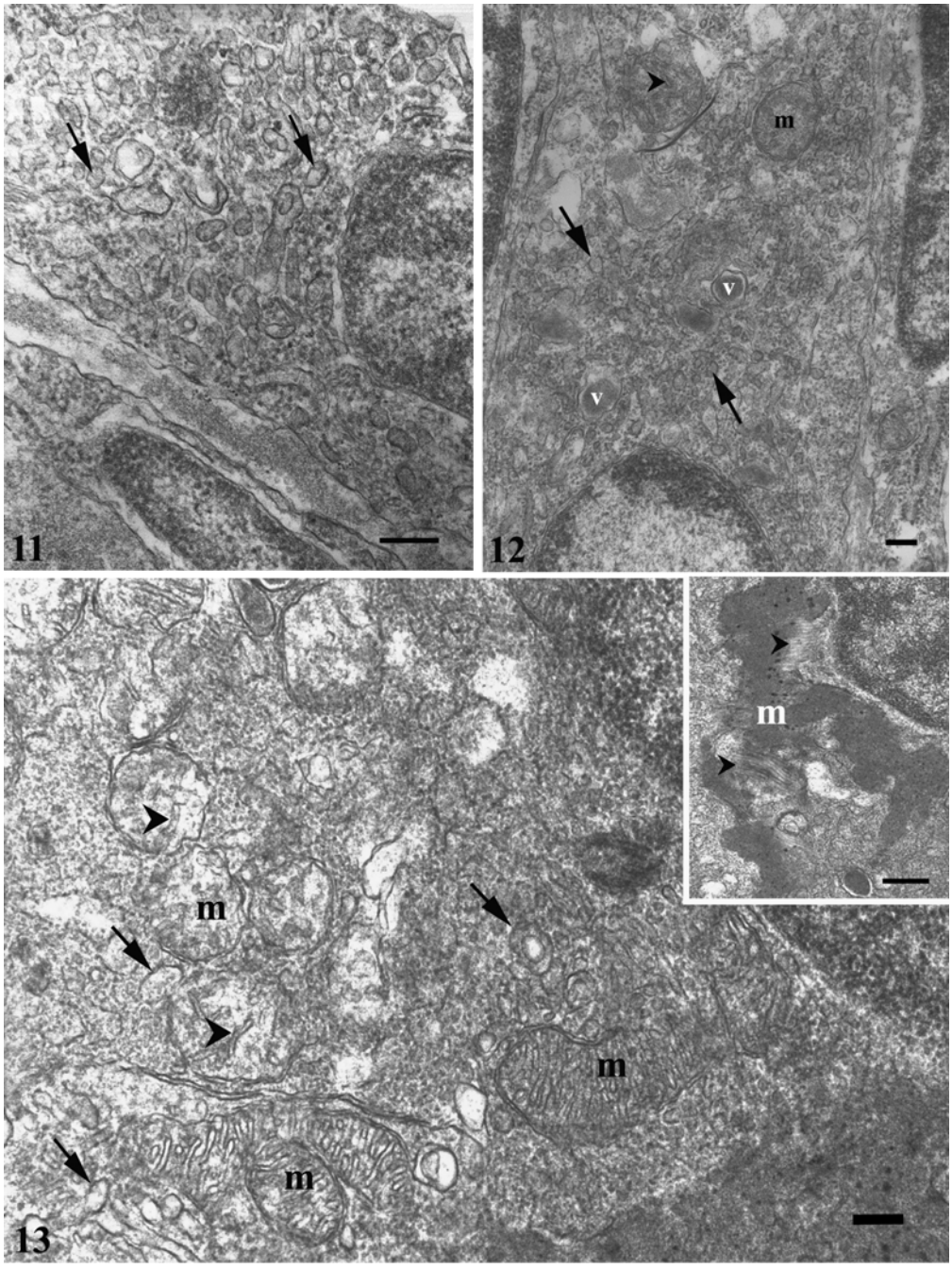
The thecal cells

The thecal layer consisted of two cell types as shown in Figure 4, (a) squamous fibroblast-like thecal cells with poorly developed cytoplasm and (b) a larger thecal cell with organelle-rich cytoplasm. We focused on the special thecal cells, which had the characteristics of active steroid producing cells. The cells had abundant round to oval mitochondria with tubular cristae, a globular smooth endoplasmic reticulum and transport vesicles with an electron-dense content (Figs 11-13). In the vitellogenic oocytes, both round-oval and elongated mitochondria were more abundant. At this stage thecal cells had pleomorphic mitochondria (Nicholls and Maple, 1972; Gresik et al., 1973) (Fig. 13).

DISCUSSION

Ovarian follicular growth is the result of complex processes of hormonal controlled development and differentiation which involve cellular and molecular changes of the oocyte, ooplasmic components, egg envelopes and somatic cells (Guraya, 1986). In this study, we report the morphological differentiation of the vitelline envelope and the follicular walls of *O. niloticus* ovary during primary (perinucleolar stage) and secondary (cortical alveolar–vitellogenic stages) oocyte growth.

The vitelline envelope (VE) is evident once the oocyte enters the cortical alveolar stage, becoming thicker as the oocyte grows and deposits yolk. Its structure is a single electron-dense homogeneous mesh pattern layer. Although the origin of the electron-dense components of the VE is unclear, a number of reports, including this one, indicate that it originates from the collective deposition of electron-dense amorphous material in the perivitelline space between cytoplasmic processes of the oocyte and granulosa cells. The VE of *O. niloticus* is different from that of other teleosts. For example the common carp VE is thick and has a complex structure composed of 4 layers (Linhart et al. 1995). Ravaglia and Maggese (2003) studied the ultrastructure of VE in *Synbranchus marmoratus* and reported that in the late perinucleolar stage it consists of a zona externa and zona interna, becoming thicker and multilaminar as the oocyte reaches the vitellogenic stage. In a marine teleost *Pagrus major*, the VE has a similar structure composed of 2 major layers (ZRE and ZRI) and it becomes more complex with seven membranous reticular lamellae for ZRI when the oocyte enters vitellogenic phase (Matsuyama et al., 1991). In icefish, *Chionodraco hamatus*, an antarctic species, the VE is composed of several concentric layers (Baldacci et al., 2001). The ultrastructural studies of a lentic piscivorous teleost *Serrasalmus spilopleura* show that VE in the late previtellogenic stage is similar to that of *O. niloticus* (Guimarães and Quagio-Grassiotto, 2001). These differences in VE structure in various species of teleost fish may be the result of differences in habitat and/or diet. Thus fish living in harsh environmental conditions (marine or Antarctic environments) appear to possess thicker and more complex VE than freshwater fish.



FIGURES 11-13. Electron micrograph of thecal cells of *O. niloticus* shows steroidogenic features. **(11)** Thecal cell of perinucleolar oocyte contains abundant globular SER (arrows). ×40,000. Bar: 200 nm. **(12)** Thecal cell of cortical alveolar oocyte contains abundant globular SER (arrows), mitochondria with tubular cristae (arrowhead) and transport vesicles. ×20,000. Bar: 200 nm. **(13)** Thecal cell of vitellogenic oocyte contains abundant globular SER (arrows), mitochondria with tubular cristae (arrowheads) and elongate mitochondria with lamellar cristae. ×20,000. Bar: 200 nm. Inset: pleomorphic mitochondria with electron-dense and filamentous inclusions (arrowheads). ×15,000. Bar: 500 nm. **m**: mitochondria, **v**: transport vesicle.

Morphological and functional differentiation of somatic cells in the fish ovarian follicle during oocyte growth and maturation has previously been described. The ovarian follicular somatic cells of *O. niloticus* undergo morphological changes during this period. The granulosa cell layer differentiates from a simple flattened cell layer in the perinucleolar stage to a stratified cuboidal cell layer in the vitellogenic stage. As in the present study, Nakamura et al. (1993) reported that the granulosa cells of perinucleolar oocytes in *O. niloticus* have a poorly developed cytoplasm with a few organelles. From the early vitellogenic stage, the granulosa cells possess organelles typical of active protein-secreting cells including electron-dense materials, well developed tubular RER, free ribosomes, and a Golgi system. Reports of *O. niloticus* fry indicate that the granulosa cells of vitellogenic oocytes proliferate and are hypertrophic with well developed organelles (Nakamura et al., 1993). In a marine teleost, *Pagrus major*, the granulosa cells also have similar organelles in vitellogenic phase, but dilation of the RER is evident during the maturational phase when nuclear migration has occurred (Matsuyama et al., 1991).

In tilapia *O. niloticus*, the studies focusing on steroid-producing cells (SPCs) suggest that steroid synthetic capacity occurs close to the onset of gonadal sex differentiation (Nakamura and Nagahama, 1985). In early life stage (40-80 d after hatching), the SPCs are present outside the follicle in the ovarian cavity and in the interstitial region near blood vessels. They begin to infiltrate the thecal layer of early vitellogenic oocytes when the fish reaches 100 d post hatch) (Nakamura et al., 1993). In the present study, the special thecal cells are evident in the thecal layer from the perinucleolar stage (Fig. 11). The cells have abundant SER and mitochondria with tubular cristae, characteristics of steroid-producing cells. This difference in our observation is probably dues to the difference in the age of the fish used. In young amago salmon *Oncorhynchus rhodurus*, both thecal cells and granulosa cells possess similar organelles including agranular and granular ER, free ribosomes and some mitochondria with tubular cristae, but they are poorly developed possibly due to low steroid production in early ovarian developmental stages (Nakamura and Nagahama, 1993).

The granulosa and thecal cells play different roles in the two-cell hypothesis of steroidogenesis (Young et al., 1986; Nagahama, 1997). The thecal cell is behaved to produce steroid precursors (androgens) and the granulosa cell converts androgens to estradiol-17β or other steroid mediators (Nagahama, 1988; Khan and Thomas, 1999; Devlin and Nagahama, 2002). The results from this study suggest that thecal cell of this species has steroidogenic capacity. The granulosa cells possess organelles typical of protein-secreting cells which suggests additional functions in synthesis of the envelope. Similar results have also been reported in marine teleosts such as the mackerel *Scomber scomber*, zebra fish *Brachydanio rerio*, red sea bream *Pagrus major* (Bara, 1965, Yamamoto and Onozato 1968 cited in Guraya, 1976; Matsuyama et al., 1991). The protein-secreting character of the granulosa cell correlates with the fact that there is high production of enzymes involved in the conversion of steroid precursors (Guraya, 1986).

Overall, these results provide further insight into the cellular and subcellular organization of the developing *O. niloticus* oocyte and follicle with emphasis on the morphological background of ovarian somatic cells responsible for the synthesis and secretion of steroid hormones.

ACKNOWLEDGEMENTS

The authors are very grateful for technical support from staff at the EM Lab, Department of Pathology, Faculty of Medicine at Siriraj Hospital, Mahidol University and Professor William Eldred and Dr. Todd Blute at the Department of Biology, Boston University. Valuable suggestions and comments from anonymous reviewers are fully acknowledged.

Financial support for this work was provided by a Fulbright Junior Research Scholarship Program, TUSEF and MUA-CU thesis grant, Ministry of University Affairs, Thailand.

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Received: 11 January 2005
Accepted: 24 April 2005