**รายงานโครงการวิจัยฉบับสมบูรณ์**

# ศึกษาโครงสร้างระดับจุลทรรศน์อิเลคตรอนของไข่ และตรวจสอบหาเซลล์สร้างเซอโรโทนิน ในระหว่างรอบการเจริญพันธุ์ของแม่พันธุ์กุ้งก้ามกราม

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**นักวิจัยที่ปรึกษา**


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สนับสนุนโดยทบวงมหาวิทยาลัย และสำนักงานกองทุนสนับสนุนการวิจัย

**บทคัดย่อการวิจัย**

การศึกษาวิจัยครั้งนี้เป็นการวิจัยพื้นฐาน ทำการศึกษาโครงสร้างระดับจุลทรรศน์ และจุลทรรศน์อิเลคตรอนของไข่และรังไข่ และตรวจสอบหาเซลล์สร้างเซอโรโทนินของแม่พันธุ์กุ้งก้ามกรามในระหว่างรอบการเจริญพันธุ์ จากการศึกษาลักษณะของไข่ ทั้งขนาด การกระจายของโครมาติน และปริมาณการสะสมลิปิดและยอล์ค สามารถจำแนกระยะการพัฒนาของไข่ได้เป็น 6 ระยะ คือ โอโอโกเนีย (Oog) ปรีวิทีโลยีนิกโอโอซัยท์ระยะต้น (Oc1) ปรีวิทีโลยีนิกโอโอซัยท์ระยะปลาย (Oc2) วิทีโลยีนิกโอโอซัยท์ระยะต้น (OC3) วิทีโลยีนิกโอโอซัยท์ระยะปลาย (Oc4) และโอโอซัยท์เต็มวัย (Oc5) โดยโอโอโกเนียที่เพิ่มจำนวนจากการแบ่งตัวแบบไมโตซีสในชั้นเยิร์มของรังไข่จะเดินทางไปด้วยกลไกที่ยังไม่ทราบแน่ชัด แล้วสะสมที่บริเวณกลางรังไข่ในส่วนปลายของโอโอยีนิกเพาช์ และเริ่มแบ่งตัวแบบไมโอซีสระยะที่1 ซึ่งจัดเป็นโอโอซัยท์ปฐมภูมิ จากนั้นจึงเข้าสู่การแบ่งตัวแบบไมโอซีสระยะที่2 ซึ่งจัดเป็นโอโอซัยท์ทุติยภูมิ (Oc1-Oc4) ซึ่งโอโอซัยท์ทุติยภูมิจะหยุดการแบ่งตัวที่ระยะไดโปรทีนของไมโอซีสระยะที่2 เพื่อสังเคราะห์และสะสมลิปิดและยอล์คขณะอยู่ในระหว่างรอบการเจริญเติบโตของร่างกาย การสะสมของโอโอซัยท์ระยะต่างๆในรังไข่สามารถใช้เป็นเกณฑ์ในการแบ่งพื้นที่ในรังไข่ออกเป็น 4 บริเวณ คือ โอโอยีนิก ปรีวิทีโลยีนิก วิทีโลยีนิก และเต็มวัย ตามลำดับ นอกจากนี้ การสะสมของโอโอซัยท์ระยะต่างๆในรังไข่ของแม่พันธุ์แต่ละตัวยังสามารถใช้เป็นเกณฑ์ในการจำแนกระยะของรังไข่ในรอบการเจริญพันธุ์ได้เป็น 5 ระยะ คือ 0 (ทันทีหลังวางไข่- 48 ชม.) I (หลัง 48 ชม.) II III และ IV (ก่อนและหลังลอกคราบครั้งสุดท้าย) ตามลำดับ โดยรังไข่ระยะ I II และ III จะเป็นการพัฒนาของรังไข่ในขณะที่อยู่ระหว่างรอบการเจริญเติบโตของร่างกาย เมื่อรังไข่และไข่พัฒนาถึงระยะเต็มวัย เนื้อเยื่อประสานโดยรอบไข่จะแตกออกและผลักไข่ไปสู่ท่อนำไข่ที่มีผนังเชื่อมต่อกับผนังของรังไขด้านข้างในส่วนท้ายของรังไข่แต่ละข้าง หลังจากนั้นรังไข่จะกลับสู่ระยะ 0 ซึ่งพบโอโอโกเนียรุ่นใหม่รวมตัวอยู่ในส่วนกลางรังไข่ และมีเซลล์เยิร์ม และเซลล์โฟลลิเคิลกระจายเป็นกลุ่มๆตามโอโอยีนิกเพาท์ที่ว่างลงจำนวนมาก สำหรับเซลล์โฟลลิเคิล พบว่าในระหว่างรอบการเจริญพันธุ์มี 2 ชนิด คือ เซลล์โฟลลิเคิลชนิดที่1 และเซลล์โฟลลิเคิลชนิดที่2 ซึ่งเซลล์โฟลลิเคิลชนิดที่2 จะปรากฏครั้งแรกในการพัฒนาของไข่ระยะ Oc2 การตรวจหาเซลล์สร้างเซอโรโทนินในระหว่างรอบการเจริญพันธุ์ พบว่ามีการสะสมเซอโรโทนินในไข่ตั้งแต่ระยะ Oc1และ Oc2 แล้วลดลงในระยะ Oc3 และ Oc4 และไม่พบในระยะ Oc5 แต่จะพบในเซลล์โฟลลิเคิลชนิดที่1และ2 และในหลอดเลือด ซึ่งสอดคล้องกับผลการศึกษาโครงสร้างระดับจุลทรรศน์อิเลคตรอน ที่พบถุงคัดหลั่งชนิดทึบแสงอิเลคตรอนตรงแกนกลางในซัยโตพลาสมของไข่ระยะ Oc1และ Oc2 จำนวนมาก และพบมีการของพัฒนาไมโครวิลไลที่ผิวของไข่ระยะ Oc3 และ Oc4 การศึกษานี้แสดงให้เห็นว่าไข่ของกุ้งก้ามกรามมีการถูกยับยั้งการพัฒนาชั่วคราวในระยะโปรเฟสของไมโอซีส2 และเชื่อว่าเซอโรโทนินมีบทบาทสำคัญในการสังเคราะห์ลิปิดและยอล์คในระยะต้น และส่งเสริมการขนส่งลิปิดและยอล์คจากเลือดเข้าสู่ไข่ในระยะเจริญเต็มวัย

**Abstract**

This research was performed microscopics and immunohistochemical studies the morphological changes and location of serotonin secreting cells of ovary and oocytes during ovarian cycle of giant freshwater prawn, *Macrobrachium* rosenbergii. Based on the microscopics observation of cells' sizes, chromatin pattern, amount of lipid droplets and yolk granules, the female germ cells could be classified into 6 different steps, which are oogonia (Oog), early previtellogenic oocyte (Oc1), late previtellogenic oocyte (Oc2), early vitellogenic oocyte (Oc3), late vitellogenic oocyte (Oc4) and mature oocyte (Oc5). The Oog developed from germ cells proliferation in germarium surrounding the developing oocytes and aggregating with unknown mechanism to tip of oogenic pouch on central ovarian core. The Oog entered primary oocyte stage when undergoing 1$^{st}$ meiotic division in oogenic zone. The ovarian zones are defined according to the stage of developing oocyte that are present, i.e., oogenic, previtellogenic, vitellogenic and maturation zones, respectively. The ovarian cycle was divided into 5 stages based on the number and types of oocytes present in each stage. Stage 0 and I are spawned (within 48 hr after spawn) and spent (after 48 hr from spawn) stages. Stage II and III are proliferative and prematuration stages, while stage IV (before and after premating molt) is maturation stage. The ovary during stage I, II and III are occurred in somatic growth cycle, which varies in length form prawn to prawn. During spawning, the oogenic pouch breaks with only connective sheaths and hemolymph sinus remaining. Right after spawning, the ovulating oocytes pushed into subcapsular space and leaved to the oviduct on ventral-posterolateral site of each ovarian lobe. Island of subsequence oogonia in spent ovary and germ cells in spent oogenic pouches could be observed on the central core of stage 0 ovary. 2 types of follicular cells are characterized during the ovarian cycle. The ultrastructures and immunolocalization for 5HT-producing cells of developing oocyte during ovarian cycle and in other organs are discussed. This study revealed that, the germ cells undergo mitotic proliferation in germinal epithelium and aggregate in oogenic pouch for 1$^{st}$ and 2$^{nd}$ meiotic division. The developing oocytes complete 1$^{st}$ meiotic division in oogenic zone and arrest at diplotene stage of 2$^{nd}$ meiosis was proposed. Presences of serotonin in developing oocyte and follicular cell reflex an importance roles in synthesis, transportation and accumulation of lipid and proteinatious yolk preparing for embryo.

**สรุปย่อการวิจัย (Executive summary)**

**แผนการดำเนินงานวิจัยตลอดโครงการในแต่ละช่วง**

| | ก.ค. 46 - ธ.ค. 46 | ม.ค. 47 - มิ.ย. 47 | ก.ค. 47 - ธ.ค. 47 | ม.ค. 48 - พ.ย. 48 |
|---|---|---|---|---|
| 1. Microscopic structure of developing oocyte. | ——— | | | |
| 2. Ultrastructure of developing oocyte. | | ——— | | |
| 3. Localization of 5-HT reactive cells in nervous and reproductive tissues. | | | ——— | |
| 4. Localization of 5-HT reactive cells in develioping oocyte. | | | | ——— |

**การดำเนินการวิจัยตามวัตถุประสงค์**

การดำเนินการวิจัยเป็นไปตามวัตถุประสงค์ตามแผนการวิจัยที่วางไว้ กล่าวคือ

1. ศึกษาโครงสร้างระดับจุลทรรศน์ของไข่ (oocytes) และรังไข่ (ovary) ระยะต่างๆระหว่างรอบการเจริญพันธุ์ (ovarian cycle) ของแม่พันธุ์กุ้งกามกราม

2. ศึกษาโครงสร้างระดับจุลทรรศน์อิเลคตรอน (ultrastructure) ของ oocytes และ ovary ระยะต่างๆระหว่าง ovarian cycle ของแม่พันธุ์กุ้งกามกราม
3. ศึกษาตำแหน่งของเซลล์สร้างเซอโรโทนิน ในก้านตา สมอง ประสาทส่วนอก และกล้ามเนื้อ ของแม่พันธุ์กุ้งก้ามกรามระหว่าง ovarian cycle
4. ศึกษาตำแหน่งของเซลล์สร้างเซอโรโทนิน ในรังไข่กำลังพัฒนา

**ผลงานวิจัยที่ได้รับ**

1. โครงสร้างระดับจุลทรรศน์ และจุลทรรศน์อิเลคตรอน (ultrastructure) ของ oocytes และ ovary ระยะต่างๆระหว่าง ovarian cycle ของแม่พันธุ์กุ้งกามกราม
2. ตำแหน่งของเซลล์สร้างเซอโรโทนิน ระหว่าง ovarian cycle ของแม่พันธุ์กุ้งกามกราม

**เนื้อหางานวิจัย**

**ความสำคัญและที่มาของปัญหา (Introduction)**

The giant freshwater prawn, *Macrobrachium rosenbergii* de Man belong to phylum; Arthropoda, class; Crustacea, order; Decapoda, suborder; Natantia, family; Palaemonidae, genus; *Macrobrachium*, species; *rosenbergii* and subspecies; de Man. This prawns are found in a variety of freshwater and brackishwater of the tropical and subtropical area of Southeast Asia. In the present, it is consider as one of the crustacean species with increasing potential for aquaculture, therefore it was introduced worldwide. The female prawns become reproductively mature about 6 months of age. Mating can occur between hard-shell males and the females which just complete premating molt (soft-shelled). A premating molt female is determined by the presence of large orange ovarian mass on the dorsal side of cephalothorax beneath the carapace. The male deposits a spermatophore, gelatinous mass containing spermatozoa, between the walking leg of female. Egg laying occurs within a few hours and fertilized externally. The number of eggs produced at each spawn is directly proportional to the size of the female. A fully mature female may lay 80,000-100,000 eggs per spawning, but the first broods lay less egg at only about 5,000-20,000 eggs, average 1,000 eggs/g of wet weight. The ovaries often continue to develop in the berried (egg carrying) females. Eggs remain attached to the brood chamber for about 18 to 23 days after spawning. The newly spawned egg is characterized by bright-yellow to orange color which gradually change to brown and finally gray color about 2-3 days before hatching. In *M. rosenbergii* and most other species of decapod crustacean, reproduction is thought to be under

regulation of various hormones. The complex interactions between several neuroendocrines and endocrine organs which play a key role in control of gonads development and secondary sexual characteristics have been identified in males as well as females. At least two antagonistic neurohormones regulate crustacean gonadal maturation. Firstly, gonad inhibiting hormone (GIH) is stored and released from the sinus gland in eyestalk optic lobes of both sexes. Secondly, gonad stimulating hormone (GSH), a hormone found in brain and thoracic ganglia of male and female.

Several neurotransmitters have been identified to affect the release of the reproductive hormone in crustaceans. For instance, dopamine (DA) stimulates GIH release whereas, serotonin (5-hydroxytryptamine; 5HT) stimulates GSH release. Many evidences indicate that, GSH acts directly on ovarian maturation but, indirectly via androgenic hormone (AH) on testicular maturation and male secondary sexual characteristics. Moreover, AH play an important role on differentiation to the testes of the undifferentiate primordial gonad and maintenance of maleness. Meeratana has been demonstrated that, 5HT 1μg/g BW could increased ovarian index to about 5 times of the control value, which was in agreement with the reports by Chantapreeda and other. 5HTalso stimulated ovarian maturation and shortened the period for ovarian maturation and embryonic development. These findings, which were supported by LM morphology of the ovaries and mean diameter of the oocytes, have never been reported in literature.

Serotonin has played an importance role as master hormone in central nervous system of invertebrates. It acts as a central and peripheral neurotransmitter of the nervous system of some species. On the other hand, serotonin has also been known to be involved in the release of hormones and to act as neurohormone in crustacea. Thus serotonin is one of the most important biologically active substance not only in vertebrates, but also in invertebrates.

In marine and freshwater bivalves, 5HT exists in neural ganglia, germinal epithelium and gonoduct, and has been shown to stimulate reproductive process by initiate meiotic division, germinal vesicle breakdown (GVBD) and spawning. 5HT contents in the testes and nervous ganglia were increase at the time of spawning and decrease after spawning in the bivalve *Chlamys farreri nipponensis*), suggesting that 5HT plays an important role in the spawning process of this hermaphroditic species.

In the freshwater crayfish, *Procambarus clarkii,* serotonin immunoreactive cells were found in the superior lateral part of the cerebral ganglion and subesophageal ganglion.

5HT was synthesized in fertilized eggs and in early embryonic stage and was necessary for the process of early embryogenesis in many type of invertebrates. The early appearance of

5HT makes the biologist realize the significant role on embryogenesis and named it “the embryonic hormone”. Thus 5HT may takes part in any aspect during ovarian development, gametogenesis and embryogenesis.

There were few reports concerned with research relating to the improvement of the aquaculture production via the hormonal manipulation. A possible way to the establishment of increasing the harvest and look forward for monosex breeding, which could be performed in many species of fishes. Thus, this study aims to elucidate the ultrastructure of developing oocytes and the distribution of 5HT immunoreactive cells in nervous system and reproductive system during reproductive cycle of giant freshwater prawn, *M. rosenbergii* de Man, broodstock .

## วัตถุประสงค์ (Objectives)

It is of interest to elucidate the reproductive mechanism and sex differentiation of the giant freshwater prawn, *M. rosenbergii*. The results may be useful not only in understanding and controlling their production and sex, but also provide information in a model system to investigation of reproductive mechanism in invertebrates. Therefore, this research is designed to study the ultrastructure of developing oocyte in various stages of ovarian cycle. Thus, the objectives of this research are intended to:

1. Study the microscopic and ultrastructure of oocytes in various stages of ovarian cycle in giant freshwater prawn, *M. rosenbergii* broodstock.
2. Study the location of serotonin immunoreactive cells in the central nervous system and reproductive system during various stages of reproductive cycle.
3. Localization of serotonin immunoreactive cells in developing oocytes.

## ระเบียบวิธีการวิจัย (Materials and Methods)

### 1. Experimental Animals

Adult female *M. rosenbergii* de Man were obtained from a commercial farm; they were used in the experiment as soon as they had stage 0 ovarian development. The stages of ovarian maturation were determined by visual observation through the carapace, according to the technique described by Damrongphol et al. The animals were kept in in-door concrete tanks with adequate aeration and 20% water exchange daily. The tanks were circular with 1.50 m diameter and the water depth was 0.80 m. They were acclimated under natural light-dark cycles for two

weeks before experiment. Commercial prawn feed was provided at 3% body weight daily. To allow mating, blue-claw males at a ratio of 1 to 5 females were stocked in the same tank.

**2. Morphological studies of the ovarian tissue**

Ovaries of the giant freshwater prawn broodstocks at ovarian stage 0, I, II, III and IV were collected. The tissue samples were cut into small blocks about 1 $mm^3$ size, fixed in Davidson's fixative for light microscopy (LM), in 4% glutaraldehyde, 3% paraformaldehyde in PBS for transmission electron microscopy (TEM) and in 4% paraformaldehyde, 0.5% glutaraldehyde in PBS for 5HT-immunohistochemical studies. Under light and electron microscopic studies, the cycle of developing ovary and oocytes have been classified.

Study on morphological changes of the ovarian tissue samples during ovarian cycle was performed by LM (Nikohn BLP50) and EM (Hitachi H-300 TEM) using tissue sections of standard paraffin and epon502 embedded as follows:

**2.1 Paraffin section.** The tissues were cut into about 1 $mm^3$ size, immediately washed in IPS and fixed for 72 hours in Davidson's fixative. They were then dehydrated through a series of ethanol solutions, cleared in xylene, infiltrated with soft paraffin and embedded in hard paraffin at 58 $^{o}$C. The paraffin block was sectioned at 3-5 μm thick (Tissue-Tek 200) and the sections were stained with Harris's hematoxylin and counter stain with eosin stain (H&E stain). Periodic acid Schiffs (PAS) stain was also used to determine the presence of glycogen and other periodate reactive carbohydrate.

**2.2 Semithin and ultrathin section.** The tissues immediately fixed in 5% glutaraldehyde and 3% paraformaldehyde in 0.1 M phosphate buffer saline (PBS; pH 7.4) at 4 $^{o}$C overnight. After the overnight fixation at 4$^{\circ}$ C, the tissues were washed in cold 0.1 M phosphate buffer saline pH 7.2 with three exchanges and post-fixed with 1% osmium tetroxide ($OsO_4$) in 0.1 M phosphate buffer saline pH 7.2 at 4 $^{o}$C for 5 hrs. The osmified samples were then washed in cold 0.1 M phosphate buffer saline three times and stained with 1% uranyl acetate (UA) in 0.23 M sucrose for 20 min, washed in cold 0.1 M phosphate buffer saline pH 7.2 and dehydrated 10 min each in graded series ethanol of 50%, 70%, 80% and 90% at 4 $^{o}$C. They were then passed through three changes of 95% ethanol, 15 min each and three changes of 100% ethanol, 20 min each, at room temperature (RT). The dehydrated tissues were infiltrated with propylene oxide (PO) for 15 min, RT; PO:Aradite resin 2:1 for 1 h and 1:2 overnight, RT; and embedded in flat mold with Epon/Aradite 502 resin. Semi-thin section of 0.5-1 μm and thin section of 600 - 900 Angstrom were performed on Porter Blum MT-2 ultramicrotome.

**3. Localization of serotonin reactive cells in nervous and reproductive tissues**

The following tissues were isolated from the previous staging of reproductive cycle of each *M. rosenbergii*: broodstock; ovary, eyestalk optic lobes, brains, thoracic ganglia and muscle strips. Individual tissue was washed with isotonic physiological saline (IPS). The ovary was rapidly dissected out, washed with IPS and cut more or less equally into three parts; the middle part was used for study. The sampled tissues were fixed in 4% paraformaldehyde 0.5% glutaraldehyde and in 0.1 M Phosphate buffer saline pH 7.4 at 4 $^{o}$C overnight. The tissues were dehydrated, embeded and sectioned as mention previously. The sections were used for immunoreactive and absorption tests of peroxidase-antiperoxidase (PAP) method. Sections were incubated with 3% hydrogen peroxide for 15 min and then with 0.01 M phosphate buffer saline (PBS; pH 7.4) for 15 min. Then they were incubated with normal swine serum at dilution of 1:20 form 30 min at room temperature. After that, sections were wash in PBS for 15 min and immerge overnight in serotonin antiserum in a moist chamber at room temperature. Antiserum against serotonin (Immuno Nuclear Corp) was use at a dilution of 1:1600. Sections were then wash in PBS for 15 min and immersed in anti-rabbit IgG serum (1:40) for 1 hr at room temperature. There after, they were washed in PBS for 15 min and incubate in PAP complex (1:100) for 30 min at room temperature. Finally sections were washed in PBS for 15 min and were reacted with 3, 3-diaminobentizine containing 0.005% hydrogen peroxide for 5 - 20 min at room temperature. Specificity of serotonin immunoreactive was checked by an antiserum previously inactivated with excess serotonin (10 ug/mi diluted antiserum) in the same procedure.

## ผลการวิจัย (Results)

**1. Histology of the developing ovary and oviduct.**

Ovary of the giant freshwater prawn has two lobes situated underneath the carapace dorsal to the hepatopancreas and ventral to the heart. Histologically, the developing ovary is invested by ovarian capsule made of connective and muscular tissues. The paired ovarian lobes connected by the connective and muscular tissues bridge. The fibromuscular tissue of the ovarian capsule extended interiorly as trabeculae. It divided the ovarian tissue into conical profiles of ovarian lobules called oogenic pouch, containing groups of developing oocytes. Each pouch contains various steps of oocyte depending on stage of ovarian development. The central core of each ovarian lobe contains the main convoluted hemolymph vessel, connective and muscular tissues. The hemolymph vessel branched radially as hemolymph sinuses, which pass into

trabeculae and form network around the pouches (fig. 1A-C), and finally drained into subcapsular hemolymph sinuses (fig. 1H). The conical profile of each oogenic pouch is invested by connective tissue and interposed with germarium where the germinal cells located, in which they undergo mitotic proliferation during Oc2 and Oc3 (late previtellogenic to early vitellogenic stages) and migrate to the tip of the oogenic pouch by unknown mechanism. The space between each oogenic pouch connected to the subcapsular space at the periphery, which finally open into the oviduct on the ventrolateral side of the ovarian lobe (fig. 2C).

Based on the number of differentiating oocytes, the ovarian tissue could be divided into four parts of oogenic, previtellogenic, vitellogenic and maturation zones. Oogenic zone is the inner region surrounding the central ovarian core. This zone contained 1$^{st}$ meiotic dividing oogonia known as primary oocytes, and its predominant in ovarian stage I (fig. 3A). The previtellogenic zone is located next to the oogenic zone and contains previtellogenic (secondary) oocytes (Oc1 and Oc2). This zone is predominated in the ovarian stage II (fig 1A, 1B, 3B). The vitellogenic zone is located on peripheral area of the ovaries and contained vitellogenic oocytes (Oc3 and Oc4). This zone is predominant in stage III ovary (fig. 1C, 3C). In stage IV ovarian stage, the maturation zone expands through out the ovary and contained fully mature oocytes (Oc5 distinguished by deep acidophilic stain), which deeply eosinophilic with H&E stain due to the accumulation of cytoplasmic lipid and yolk platelets (fig. 3D).

In the stage 0 (spawned) ovary, it contained diversify amount and stage of oogonia and previtellogenic oocytes (fig. 2A). The spent oogenic pouches could be seen among the subsequence developing pouches after spawned (fig. 2A, 2B). Island of follicular cells and germ cells are predominantly seen among the spent oogenic follicles, which returning from thin spindle shape to their original spherical shape. The connective tissue trabeculae and the ovarian capsule become thickened during this stage of ovarian cycle, but it gradually attenuated as the ovary progressing from stages I into stages II, III and IV. The multidirections muscular fibers on the ovarian capsule are obviously seen in ovarian stage IV and become multi-layers at oviductal connection (fig. 2C).

During the ovarian cycle, the follicular cells could be classified in to two types. Type I follicular cell (Fc1), enclosed within vitelline layer of the oocyte. It is not frequently seen during oogonial and Oc1 stages of the developing oocytes. Fc1 become prominent spheroid in shape during Oc2, Oc3 and Oc4 stages and more elongated and flattened while the oocytes developed more advance to Oc5 stage. It exhibited prominent spindle nucleus with deep stained globular

chromatin during Oc5. Type II follicular cell (Fc2) is first appeared during the Oc2 stage. They exhibited less change in shape and size during Oc2 to Oc4. The Fc2 is not different in size comparing to the Fc1, lighter stained nuclei and locates more periphery to the Fc1 are remarkable (fig 5D-F). During the Oc5 stage, the nuclei of both types of follicular cells are quite similar, however the Fc1 nuclear is more spindle in shape and the Fc2 nucleoplasm is became quite darker.

The oviduct is a dilated fibromuscular sac continues from the ovarian capsule on ventrolateral side of each ovarian lobe. Its luminal surface lined with the simple columnar ciliated epithelium resting on the basal lamina (fig. 2C, 2D). The epithelial cells characterized by indistinct boundaries with light blue stained cytoplasm and no granular structures are observed. The prominent round nuclei are centrally located with largely occupied euchromatin. The nucleoli are frequently observed among few globular chromatins. The epithelial lining of oviduct is thickening and folding at proximal part. The stratified cuboidal to squamous epithelial cells are seen instead of the simple columnar one. Numerous of fibillar network on the surface of proximal part of oviduct are seen in the broodstock sampling during premating molt (fig. 2E, 2F). Transitions of columnar epithelium to simple cuboidal and squamous types with gradually lessen of cilia and fibillar structure is observed when going distally. Connective tissue and smooth muscle sit underneath the basal domain of lining epithelium.

**2. The differentiating oocytes**

Germ cells (Gc)

This study revealed typical transformation of developing oocytes, which begins from germ cells. They are prominent proliferated during Oc3 and Oc4 stage oocytes. The germ cells commence in germinal epithelial cord accompanying trabeculae and connective tissue surrounding the oocyte among the stromal cells (fig. 4E, 4F), which characterized by an oval shape nucleus (4-6 μm in diameter) with deep stained globular heterochromatin and a thin rim of faint blue cytoplasm. The nucleolus is not seen. The cells are frequently seen hidden in stroma between outer surfaces of Oc5 of mature ovary (fig. 4F). The germ cells aggregate in oogenic pouch and rapidly increase in size on entering 1$^{st}$ meioses.

Oogonia (Oog)

An oogonia enter 1$^{st}$ mieotic division in the oogenic zone of ovary. In this zone, they are which characterized by small blocks of heterochromatin distribute in typical pattern of meiotic

prophase of leptotene, zygotene, pachytene, diplotene, diakinesis, metaphase and telophase (fig 4A-D). The follicular cells are not frequently seen. It tightly attached on oogonial surface as small cytoplasm, with large nucleocytoplasmic ratio. The developing oogonia characterized by increase in size (ranging from 8.5-10 μm in diameter) and less decreased nucleocytoplasmic ratio. The very thin rim cytoplasm is in faint blue stained with H&E. They are distributed dominantly in central area surrounding the central core of the ovary. The ultrastructure during oogenesis characterized by small blocks of heterochromatin distribute in typical pattern of meiotic prophase of leptotene, zygotene, pachytene, diplotene and diakinesis (fig 2A-E). The nucleopores are numerous along the nuclear membrane. The nucleoli are high condensed on periphery of the nucleus and mostly more than one (fig 2C). Moderate number of ribosomes are observed. The endoplasmic reticulumn are observed as dilated vesicles distribute broadspread in area of cytoplasm especially surrounding the nuclear membrane. The mitochondria are exhibited among the dilated vesicles of endoplasmic reticulum (fig. 2E), while the other organelles have not yet appeared. The follicular cells are sparsely seen and tightly attach on interoogonia space as small cytoplasm, with large nucleocytoplasmic ratio. It characterizes by ovoid in shape on longitudinal plane and triangular on cross section view with highly condense marginal heterochromatins (fig 2A and 2F).

Early previtellogenic oocyte (Oc1)

The Oc1 is characterized by increase in size and more decreased nucleocytoplasmic ratio (ranging from 10-30 μm in diameter). The round cytoplasm showed a little bit enlarged with deep blue stained with H&E. Their nuclei are characterized by the presence of the deep blue stained heterochromatin, which are congregated in different patterns of the meiotic prophase figure. The Oc1 enlarged in size and moving to the periphery of oogenic pouch. Their centrally prominent round shape nuclei almost occupied by euchromatin. The nucleolus is prominent with densely packed materials, which located on the periphery (fig. 5B). Under electronmicroscopy, the Oc1 cytoplasm is characterized by conspicuous accumulation of secretory granules. It seems various in size and homogeneities of dense core and homogeneous secretory granules (fig. 3A-3F). The mitochondria and endoplasmic reticulum are prominent among the cloudy secretory granules. The nucleopores are numerous and dilated with the figure of nucleolar materials moving to the cytoplasm. The ribosomes and other organelles are not quite prominent change as seen in from oogenic stage (fig. 1). The follicular cells are elongated spheroid in shape and prominent increase in number and size. It shows a little bit decrease in nucleocytoplasmic ratio. The prominent nucleus exhibits decrease in marginal heterochromatin (fig. 3B and 3C).

The oogonia and Oc1 are predominantly seen at ovarian stage I (fig. 3A).

Late previtellogenic oocyte stage (Oc2)

The Oc2 is characterized by increase in size and more decreased nucleocytoplasmic ratio (ranging from 30-100 μm in diameter). The cytoplasm exhibited more enlargement with deep blue stained with H&E. Their nuclei are characterized by the presence of the deep blue stained globular heterochromatin, which are distributed in the meiotic prophase figure of diplotene. The Oc2 oocytes exhibited increasing in size and moving to the periphery. Their prominent centrally round nuclei and the nucleolus is prominent with densely packed materials, which located centrally. Ultrastructure of Oc2 cytoplasm is similar to Oc1, but characterized by increase in accumulation of secretory granules and cell size. Size and homogeneities of dense core and homogeneous secretory granules are in crease (fig. 3A-3F). The mitochondria and endoplasmic reticulum are prominent among the cloudy secretory granules. The nucleopores are numerous and dilated with the figure of electron dense materials moving to the cytoplasm. The nucleolus is prominent with densely packed materials, which located centrally. The ribosomes and other organelles are not quite prominent change as seen in from oogenic stage (fig. 1). The follicular cells are more elongated and prominent increase in number and size. It shows small decrease in nucleocytoplasmic ratio (fig. 1D, 5C).

The Oc1 and Oc2 are located in previtellogenic zone next to the oogenic zone of the developing ovary and occupied predominantly in ovarian stage II (fig. 3A).

Early vitellogenic oocyte (Oc3)

The Oc3 is characterized by basophilic cytoplasm with first occurring of small lipid doplets. The lipid droplets distributed randomly on the periphery of the cytoplasm (fig. 1E, 5D). The size of this stage is about 100 to 200 μm in diameter. The nucleus is enlarged, rounded and peripheral located, which exhibits more globular heterochromatin. Ultrastructure of Oc3 exhibits accumulation of ribosomes, RER and golgi apparatus develop. Its nucleus exhibits fully euchromatin and more homogeneous (fig. 4F). Moving of nuclear material across nuclear membrane via nucleopores is also seen. The commencement of endogenous yolk accumulation is seen as small granules, which concentrated around the Golgi bodies. Secretory granules aggregation is seen throughout the oocyte cytoplasm. The oocyte membrane becomes folding and exhibit finger like cytoplasmic protrusion (fig. 4B, 4C and 4D). The endocytotic figures of extracellular proteinatious granules transport across oocyte membrane are observed (fig. 4B, 4D). Mitochondria, ribosomes and endoplasmic reticulum are also observed among secretory granules (4E). This step of oocyte differentiation found predominant proliferation of type II follicular cells with prominent oval nuclei. The type I follicular cells are tightly attached surrounding each oocyte and remain unchanged from the previous stage. Island of germinal cells proliferation between oocytes space are also observed (fig. 1E).

Late vitellogenic oocyte (Oc4)

The Oc4 displayed peripheral acidophilic cytoplasm with increasing amount of lipid droplets and proteinaceous yolk plaques. The acidophillic cytoplasm on periphery exhibited more intense and wide spread in cytoplasm except the area surrouding the nuclear membrane. It's sizing between 150-250 μm in diameter. At this stage of oocyte, the lipid droplets increased in side and arrange themselves on the periphery of the cytoplasm along the cytoplasmic rim. Under electron microscopy, figure of lipid droplets, yolk vesicles and proteinatious yolk granules incorporation are observed throughout the cytoplasm (fig. 5A-5F). Mitochondria are prominent underneath the cytoplasm (fig. 5D). The cytoplasmic protrusions are replaced by multidirectional microvilli, which distribute throughout the oocyte membrane (fig. 5A- 5C). The nucleus contains almost euchromatin and prominent nucleolus (fig. 5G, 5H). Moving of nuclear material across nuclear membrane via nucleopores is also seen. Small granules and granular mass aggregation are observed around nuclear membrane. The follicular cells type I and II are apparent and more elongated in shape (fig 1F, 5E). They are elongated and increase in nucleocytoplasmic ratio. Its nuclear still prominent with mostly euchromatin (fig. 5A-5D).

The Oc3 and Oc4 are located in vitellogenic zone next to the previtellogenic zone of the developing ovary and occupied predominantly in ovarian stage III (fig. 3C).

Mature oocyte (Oc5)

The Oc5 is characterized by tremendous increasing in size, more than ten times that of the early previtellogenic oocytes. Most of them are around 300-550 μm in diameter and having a hexagonal in shape. The cytoplasm is highly acidophilic and filled with large lipid droplets and proteinaceous yolk plaques. Accumulation of deep purple stained materials in perivitelline space are seen in the ovary of broodstock sampling during premating molt. The nuclear membrane is less prominent and some time disappeared (germinal vesicle breakdown). Mostly, the nucleus filled with euchromatin with prominent nucleolus (fig. 1G, 3D, 5F). The microvilli on cytoplasm, seen under TEM, are disappeared. Accumulation of small and medium yolk granules are also seen on the periphery of cytoplasm (fig. 6C-6E). The mitochondria found numerously among the yolk granules. They exhibited elongate and cylindrical in shape. The mature oocyte undergoes 2$^{nd}$ meiotic metaphase when maturation is progressed and ovulation will occur (fig. 6C). The follicular cells are fully elongation and seldom seen. The tubular network develops in their cytoplasm. The less electron dense secretory vesicles are increased. Type I and type II follicular cells were seen with fully elongation.

No cortical rod was observed in any step of developing oocytes.

**3. The ovarian cycle.**

Histologically, the ovarian cycle could be classified in to five stages of stage 0 (spawned), I (spent), II (proliferative), III (prematuration) and IV (maturation) respectively.

Stage 0 (spawned), ovaries on the date of spawn to 48 hr after spawn are emptied containing collapsed oogenic pouches and islands of follicular cells. Boundary of each follicle indicated by the remaining follicular cells. Almost of the follicular cells are returned to their original ovoid shape. The main hemolymph vessel is apparently seen. The hemolymph sinus boundaries are not apparently seen and dispersed throughout ovarian tissue. Groups of oogonia on central ovarian core are frequently seen. Folding and thickening of ovarian capsule is apparent overall the ovary (fig. 2A, 2B). The germinal epithelium appearing as cord connected with the germinal cells along with trabeculae.

Stage I (spent), ovary after 48 hr spawned, predominately filled with oogonia resting at 1$^{st}$ meiotic division. Previtellogenic oocytes during Oc1 to Oc2 are frequently seen. Follicular cells are seldom seen among this stage of oocytes. Spent oogenic pouches are contracted to small spaces seen on periphery. The hemolymph vessels and sinuses are recovered to distinctly observed (3A).

Stage II (proliferative), the ovarian size is small with faint orange in color and apparent dark-green band along dorsal side. The oocytes are mainly in the late previtellogenic (Oc2) and some early vitellogenesis (Oc3). Germinal cells are increased in size and proliferate during this stage. Follicular cells are increased in number and apparently seen (fig. 3B).

The stage III ovary (prematuration) is bright orange in color and more increases in size. Most of the occupying oocytes are late vitellogenesis (Oc4). The follicular cells are elongated to spindle shape and less frequently seen among the oocytes (fig. 3C).

Stage I, II and III ovaries are found during somatic growth phase of prawn, which varies in length from prawn to prawn.

Stage IV ovary (maturation) is deep orange and tremendously increase in side comparing to stage 0. The ovary fully contained mature oocytes (Oc5). Follicular cells are seen decreasing in number with thin spindle shape surrounding oocyte (fig. 3D). The ovarian capsule and trabeculae are very thin and the oogenic pouch boundaries are hardly to define. The oviducts are seen on ventrolareral side of each ovarian lobe. Their walls continued with the thin ovarian capsule (fig 2C).

On electron microscopic study, it was revealed typical transformation of developing

oocytes, which starts from oogonia. The oogonium (Og) ultrastructure during oogenesis is characterized by small blocks of heterochromatin distribute in typical pattern of meiotic prophase of leptotene, zygotene, pachytene, diplotene and diakinesis (fig 2A-E). The nucleopores are numerous along the nuclear membrane. The nucleoli are highly condensed and located on periphery of the nucleus and mostly more than one (fig 2C). Moderate numbers of ribosomes are observed. The endoplasmic reticulum is observed as dilated vesicles distribute broadly spread in area of cytoplasm especially surrounding the nuclear membrane. The mitochondria are exhibited among the dilated vesicles of endoplasmic reticulum (fig. 2E), while the other organelles have not yet appeared. The follicular cells are sparsely seen and tightly attach on interoogonial space within vitelline envelope as small cytoplasm, with large nucleocytoplasmic ratio. It characterizes by ovoid in shape on longitudinal plane and triangular on cross section view with highly condense marginal heterochromatins (fig 2A and 2F).

The early and late vitellogenic oocytes (Oc1 and Oc2) are characterized by the presence of abundant ribosomes and decreased in nucleocytoplasmic ratio. The nuclei are characterized by the chromosomes, which are distributed in the pattern of diplotene or diakinesis steps of the first meiotic prophase. The Oc1 oocytes exhibited centrally prominent ellipsoidal or ovoid shape nucleus, which almost occupied by euchromatin. The nucleopores are numerous and dilated with the figure of nucleolar materials moving to the cytoplasm. The nucleolus is prominent with densely packed materials, which located centrally. The ribosomes and other organelles are not quite prominent change as seen in from oogenic stage (fig. 1). Under electron microscopy, the Oc1 and Oc2 cytoplasm is characterized by conspicuous accumulation of dense core secretory granules. It seems vary in size and homogeneities of dense core and homogeneous secretory granules (fig. 3A-3F). The mitochondria and endoplasmic reticulum are prominent among the cloudy secretory granules. The follicular cells are elongated and prominent increase in number and size. It shows a little decrease in nucleocytoplasmic ratio. The prominent nucleus exhibits decrease in marginal heterochromatin (fig. 3B and 3C).

The early vitellogenic oocyte stage (Oc3) ribosomes and rough endoplasmic reticulum are still abundant. The lipid droplets are randomly accumulated on the periphery of the cytoplasm. Oocytes during this stage also surrounded by follicular cells with prominent round nuclei. The Oc3 exhibits accumulation of ribosomes and RER develops. Its nucleus exhibits fully euchromatin and more homogeneous (fig. 4F). Moving of nuclear materials across nuclear membrane via nucleopores is also seen. The commencement of endogenous yolk accumulation is

seen as small granules, which concentrated around the Golgi bodies. Secretory granules aggregation is seen throughout the oocyte cytoplasm. The dense core secretory granules are decreased during this stage. The oocyte membrane becomes folding and exhibit finger like cytoplasmic protrusion (fig. 4B, 4C and 4D). The endocytotic figures of extracellular proteinatious granules transport across oocyte membrane are observed (fig. 4B, 4D). Mitochondria, ribosomes and endoplasmic reticulum are also observed among secretory granules (4E). The follicular cells tightly surrounded each oocyte and remain unchanged from previous stage (fig. 4B).

The late vitellogenic oocyte (Oc4) displayed increasing in amount of lipid droplets and yolk plaques. The figure of lipid droplets, yolk vesicles and proteinatious yolk granules incorporation are observed throughout the cytoplasm (fig. 5A-5F). Mitochondria are prominent underneath the cytoplasm (fig. 5D). The dense core secretory granules are less frequently seen. The cytoplasmic protrusions are replaced by multidirectional microvilli, which distribute throughout the oocyte membrane (fig. 5A- 5C). The nucleus contains almost euchromatin and prominent nucleolus (fig. 5G, 5H). Moving of nuclear materials across nuclear membrane via nucleopores is also seen. Small granules and granular mass aggregation are observed around nuclear membrane. The follicular cells are elongated and increase in nucleocytoplasmic ratio. Its nuclear still prominent with mostly euchromatin (fig. 5A-5D).

The mature oocyte (Oc5) is characterized by disappear of cytoplasmic microvilli, while germinal vesicle breakdown is seldom seen (fig. 6A, 6B). Accumulation of small and medium yolk granules are also seen on the periphery of cytoplasm (fig. 6C-6E). The mitochondria found numerously among the yolk granules. They exhibited elongate and cylindrical in shape. The dense core secretory granule is not seen. The mature oocyte undergoes 2$^{st}$ meiotic metaphase when maturation is progressed and ovulation will occur (fig. 6C). The follicular cells are fully elongation and seldom seen. The tubular network develops in their cytoplasm. The less electron dense secretory vesicles are increased.

Among the mature oogenic follicles, the spent oogenic follicles could be seen (fig. 6F-6H). The developing oocytes are absorbed and disappeared. The follicular cells seem to return from flattened form to their original spherical shape.

**4. Ultrastructure of developing ovary and oocytes**

**5. The 5HT immunolocalization**

Ovary and Oocyte

The 5HT immunohistochemistry using AEC as chromogen revealed that, stroma of ovary at any stages of development did not give positive reaction. Except, the hemolymph in vessels and sinuses (fig ). The positive reaction of hemolymph is intensely during ovarian stage IV, while the other stages are exhibited less intensity. Clone of oogonia in oogenic pouch of stage I ovary exhibit negative stained, while a strong positive reaction is seen in hemolymph sinus. The distinction of reactive 5HT immuno-stain occurred in ooplasm at the dense core of secretory granules during Oc1 and Oc2 of ovarian stage II and III. The Oc1 and Oc2 in ovary at stage 0, I and IV are also exhibited positive 5HT immuno-stain, if they occupied. The oocytes at Oc3 and Oc4 are gradually decreased in number and intensity of positive 5HT immuno-stain, while faintly homogeneous positive stained in nuclei, nucleoli and lipid droplets are demonstrated (fig ). The positive 5HT immuno-stained is disappeared at Oc5 when lipid droplets and proteinatious yolk are accumulated in ooplasm. However, transition of reddish-brown staining of 5HT to follicular cells and hemolymph sinusduring ovarian maturation are demonstrated (fig ).

Ventral nerve cord

The positive 5HT immunohistochemical reaction of nervous tissue is distinct in hemolymph, but scantly in neuron of all segments of ventral nerve cord. The intensity of reaction in hemolymph is peaked during ovarian stage IV, while the positive reaction is frequently occurred in small size neurons (fig ). It is found that, only 1 or 2 giant neurons of each segment of ventral nerve cord exhibited positive 5HT immuno-stain. There are unchanged in number and intensity of positive 5HT immuno-stain in giant neurons throughout the ovarian cycle. The positive 5HT immuno-stain in neuroglia and neuropiles are did not found during ovarian cycle (fig ).

Eyestalk and Muscular tissue

The positive 5HT immuno-stain in eyestalk is present as granules in x-organ sinus gland complex of both eyes. The number and intensity of positive reaction are not different during ovarian cycle.

The muscular tissue exhibited positive 5HT immuno-stain as granular spots on sarcolema of muscle fibers. The number and intensity of positive reaction are not different during ovarian cycle.

## วิจารณ์ผลการวิจัย (Discussion)

### Histological appearance of developing ovary

The results from histological studied on ovarian tissue via light microscope revealed morphological changes in according with the development of oocytes as in other crustacea (Harrison, 1992). The relatively thick connective and muscular tissues framework of ovary composed of collagenous and smooth muscle fibers forming fibromuscular capsule and trabecular sheets, from which the germinal cells appear as to generate. The trabecular sheets divided the ovary into a number of conical profiles of ovarian lobules called oogenic pouch as called ovarian subunit or cyst in *Penaeus vannamei* (Krol et al., 1992) or oogenic pouch in crayfish (Ando and Makioka, 1998) and freshwater crab *Potamon dehaani* (Ando and Makioka, 1999). The present of different stages of oocytes and asynchronous in their yolk deposition and development, rendering them capable of being continued fecundity at short time. Each oogenic pouch contains various stages of differentiating oocytes surrounding with stromal cells. The germinal cells from which the oogonia are derived, lies inside this wall and more aggregated on the tip of oogenic pouch pointing to central ovarian core. In lobster, *Homarus Americanus,* the germarium aggregated between ovarian cyst which served as oogonia reserved (Talbot, 1981). By this reason, sometime the previtellogenic core of ovary is not located in the middle part of the ovary as found in some prawn of this study. The germinal cells undergo mitotic division with successive karyokinesis not followed by cytokinesis within germinal layer in the same way as reported in female crustacea, *Eoleptestherita ticinensis* Balsamo-Crivelli (Scanabissi Sabelli and Tommasini, 1990). Each germ cell nucleus consequently develops and forms its membrane and cytoplasm, then separates from oogenic cord entering oogenic zone and undergo $1^{st}$ meiotic proliferation in oogenic pouch. The central core of ovary is made of connective and muscular tissues that runs along the longitudinal axis of the ovary. The oviduct is not present in central ovarian core of this prawn as reported in other *Penaeus* species (Bell and Lightner, 1988). The main hemolymph vessel are convoluted and run within the central core. It branches into trabeculae on inter-oogenic pouch space and terminated as hemolymph capillaries plexus surrounding each oocyte. Muscular wall surrounding the ovary and oogenic pouches is, unusual

in crustacean, not striated as reported in lobster's ovary (Federic W Harrison. 1992) and *Homarus Americanus* (Talbot and Helluy, 1995). In crayfish *Procambarus paeninsulanus*, the contraction of ovarian tissue during oviposition was induced by prostaglandins (Spaziani et al., 1995). In this study, the muscle cells are smooth muscle, which their nuclei have a corkscrew appearance in longitudinal section distributed within trabeculae and thickening in capsule and central ovarian core. It appears to be contractile contributing to forcing ovulated oocytes during oviposition into oviduct and broodchamber.

Accumulation of oogonia on the tip of oogenic pouch is differed from other decapods. Factors and mechanisms contributing to migration and aggregation of germ cells on tip of oogenic pouch, which undergo mitosis in germarium have to be elucidate. As episode of oocytes developed, they moved to periphery entering previtellogenic zone, while the oogenic pouch becomes expanded. Each oogenic pouch of *M. rosenbergii* exhibited various stage of developing oocytes, which are Oog, Oc1, Oc2, Oc3, Oc4 and Oc5, respectively depending on ovarian stage. After spawning, the ovaries returned either to stage 0 or 1 depending on the period of somatic growth (Damrongphol et. al., 1991).

**Fate of differentiating oocytes**

The oocyte development of crustacean starts with the process of oogenesis, which characterized by mitosis and meiosis of germ cells have been reported (Van Herp and Soyez, 1997, Krol et al., 1992, Kleckner et al., 1994). The oogonia enter $1^{st}$ meiotic division and become oocytes (Okumura, 2004). In this study, It has not yet been confirmed and according to previous reports, the Oog is classified as primary oocytes, which undergoing $1^{st}$ meiotic division and completed within oogenic zone. This suggestion is supported by the evidences of dividing germ cells in germarium and the occurrence of meiotic figure of oogonia especially during metaphase and telophase in oogenic zone. Therefore, the previtellogenic, vitellogenic and mature oocytes (Oc1, Oc2, Oc3, Oc4 and Oc5) are secondary oocytes undergoing $2^{nd}$ meiotic prophase. The secondary oocytes are almost arrest at diplotene stage of $2^{nd}$ meiotic prophase during early vitellogenic phase, meanwhile their cycle shift to somatic growth during intermolt stage of molting cycle. When the reproductive growth is triggered by the premating molt, the diplotene stage of $2^{nd}$ meiotic prophase is recruited, which exhibited enormously prolong period of cell

growth during which the chromosomes are decondensed and very active in transcription. At this moment, the vitellogenic phase occurred in accompanied with progressing of 2nd meiotic prophase and restored again at 2nd meiotic metaphase, meanwhile the ooctyes synchronously progressed and reaching full maturation. The 2nd meiosis is progressed at the time of ovulation. During these developmental periods, the polar body could not be seen, thus the oogenesis likely to be similar to the development of the male gametes, each primary oocyte giving rise to four mature oocytes. So, the final number of mature ovulated oocytes are numerous between 80,000 – 100,000 in each brood. This suggestion is unlike other crustacea, which have a long 1st meiotic prophase for preparation of DNA replication. In most organisms prophase II is brief and the nuclear envelop breaks down as entering of metaphase II. During this long period, the oocytes accumulate ribosomes, glycogen, lipid, yolk and mRNA for early embryonic growth (Kleckner et al., 1994). At this stage, the oocytes tremendously increase in size and entering full maturation (Oc5), which synchronize to the ovarian stage IV. The deposition of yolk in the Oc3 is slowly and the oocytes undergo gradual increased in size, which is produced by endogenous source (Van Herp, 1992, Tsutsui et al., 2000). The Oc4 significantly increases its volume due to accumulation of proteinatious reserves, which might account for the acidophilia. When the Oc4 and Oc5 are entered, the rate of size increment increases by accumulation of exogenous yolk, which are imported from extraovarian sources (Chang and Shih, 1995, Sagi et al., 1995). The cortical rod could not seen in any steps of developing oocyte as reported by Chang and Shih, 1995. The hepatopancreas and adipose tissue was proposed as an exogenous sources of yolk proteins (Lee and Chang, 1999., Tsutsui et al., 2000, Yang et al., 2000, Jasmani et al., 2004). This is followed by recommendation of 2nd meiosis, disintegration of nuclear membrane and ovulation. The arrest of 1st meiosis prophase during early vitellogenesis was recruited by ecdysone synthesized from follicular cells and the released of 2nd meiosis metaphase was by fertilization (Subramoniam, 2000). The mechanism of mature oocytes released from their follicle into the oviduct is resulted from tremendous rising of pressure from increment of yolk accumulation by which breaking of thin connective tissue septa. The shutter mechanism in released of eggs from their pouches was reported in *Artremia* (Criel, 1989). Fate of ovulated oocytes until fertilized and forming of zygote need to be investigate.

**The ovarian cycle**

Most decapods crustaceans continue molting after reaching puberty. Generally they undergo reproductive maturation during the period of intermolt. Molting and reproduction are alternative events of cyclical mobilization of metabolic reserves to the epidermis and gonad respectively. The size and the color of the ovaries depend on their degree of development. In 1984, O'Donovan et. al., divided the developing ovary of giant freshwater prawn, *M. rosenbergii* during intermolt period in captivity into 6 stages, while Damrongphol et al. 1991, classified in to four stages of 0, 1, 2 and 3. Lately in 1995, Chang and Shih characterize stages of *M. rosenbergii* ovary by observing through carapace into 5 stages. The immature ovaries are predominately filled with pre-, primary and secondary vitellogenic oocytes synchronized with the period leading to regular molt to the period leading to last regular molt of somatic growth consecutively. The mature ovaries are the full ovarian maturation, which are synchronized to the time of premating molt. The ovaries are fully contained mature oocytes. After spawning, the ovaries returned either to stage 0 or 1 depending on the period of somatic growth (Damrongphol et. al., 1991). In this study, five stages of developing ovary were characterized by histological appearance according to stained structures and stage of occupying oocytes. The spawned ovary (stage 0), has more enlarged in size than the spent one (stage I). The stromal and parenchymal tissues among empty oogenic pouches are loosed and flaccid, while the spent ovary is snuggled and contained more oogonia and previtellogenic oocytes. The collapse oogenic pouches could be seen in the spent ovary. The accumulation of previtellogenic oocytes undergoing ribosomal and RER-golgi phases in preparation of protein synthesis to enrichment embryonic nutrition enabled deep blue stain with H&E of proliferative ovary (stage II). Progressive in red color of H&E staining in prematuration (stage III) and maturation (stage IV) ovaries referred to yolk (lipocarotinoglycoprotein) accumulating phase of the oocytes. The island of oogonia and previtellogenic oocytes among full maturation oocytes of stage IV ovary, suggesting the occurring of multiple spawning as reported in other decapods (Dumont and D'Incao, 2004).

**Localization of serotonin**

Serotonin mediates a wide variety of physiological and behavioral effects including peripheral and central actions of both vertebrates and invertebrates. It has been implicated in induction of oocyte maturation, facilitates the fertilizability of oocyte by sperm and spawning of various species of clam and crustacean (Alvarado-Alvarez et. al., 1996; Chen et. al., 2003;

Masseau et. al., 2002; Meeratana, 2001; Vaca and Alfaro, 2000). This work is the first to report presence of serotonin in ovary and oocytes during gonadal development cycle of *M. rosenbergii* broodstock. The positive 5HT-immunostain in dilate hemolymph sinuses, but negative in other areas of ovary during stage 0 (spawned) reflex former high hemolymph level of serotonin during ovarian maturation (stage IV) and spawning. Contributing of serotonin on induction of ovarian maturation and spawning has been reported (Martinez and Rivera, 1994; Sarojini et. al., 1995; Meratana, 2001). During the period of ovarian maturation, significant increase serotonin level in gonadal and nervous tissues has been reported and maintain at this level for a week after spawned (Martinez and Rivera, 1994). It has been demonstrated that, serotonin by the doses of $2.5x10^{-6}$ to $2.5x10^{-8}$ mol\prawn induce vitellogenin synthesis in dose dependence manner in *M. rosenbergii* (Chen et. al., 2003). During this ovarian stage, the egg yolk protein was transported into early vitellogenic oocyte via the surrounding follicular cells, while the late vitellogenic and mature oocytes were uptake the yolk by pinocytotic mechanism through the microvilli and subsequently incorporate to form large yolk platelets in ooplasm (Yano, et. al. 1996). It was suggested that, the 5HT-immunoreactivity in hemolymph during maturation and spawned stage of ovary reflex an importance role of serotonin on ovarian maturation and spawning. There has been proposed that, mechanism of serotonin was via surface receptor on oocyte resulting in increase intra-oocyte cAMP, a consequence of adenylate cyclase activation, and subsequently enhanced glycogen phosphorylase activity leading to an increase lipid and yolk uptake for gametogenesis (Martinez and Rivera, 1994). In bivalve, *Mactra chinensis,* $5HT1_A$ and 5HT2 agonist induced spawning (Fong, et. al. 1996) and germinal vesicle breakdown in *Crassostrea gigas* (Kyozuka et. al., 2005), but the 5HT1 did not found in ovary and much lower profile of gene expression in eyestalk, heart, nervous tissue, hepatopancrease and muscle of female than male shrimp, *Metapenaeus ensis* (Tiu, et. al., 2005). Recently, the 5HT2 receptor antagonist, cyproheptadine, effectively inhibited the effect of serotonin induces ovarian maturation in *M. rosenbergii* (Meeratana, 2001), It is possible that, the stimulatory action of serotonin on fecundity in *M. rosenbergii* was via 5HT2 surface receptor on oocyte and ovary.

The occurrence of 5HT-immunoreactivity on follicular cells during Oc4 and Oc5 of premature and mature ovaries supported the role of follicular cell on transportation of proteinatious yolk into the oocyte as proposed in kuruma prawn (Yano, et. al. 1996). Serotonin has been proved as a key role on release of meiotic prophase arrest and undergoing of germinal vesicle breakdown in *Tivela stultorum* (Alvarado-Alvarez et. al., 1996) and *Spisula* oocytes

(Kadam and Koide, 1989), which could inhibit by cyproheptadine. More recently, serotonin has been recognized to cause proliferation of a variety of cells. Most information proposed that, the mitogenic effect of serotonin was through 5HT2 receptor on cell surface to initiate specific cellular transduction process via protein phosphorylation and regulation of cyclic nucleotide in signaling process (Franburg and Lee, 1997; Lee et. al. 2001). In this study, the intense 5HT-immunoreactive in Oc1 and Oc2 ooplasm, predominantly in dense core granules indicate that, early and late previtellogenic oocytes could synthesize serotonin. The 5HT-immunoreactive in varicosities of nerve fibers innervate on surface of oocyte and germinal epithelium as reported in surf clam was not seen. There was no evidence of 5HT-immunoreactive in oocyte secretory granules been reported in this prawn. It is the first finding, which may put some light on studying of reproductive biology in this species. This finding proposed that, the oocytes produce serotonin to contribute in gametogenesis during synthetic phase of diplotene oocytes in preparation of trophic and metabolic reserves for embryo. There was reported that, serotonin is synthesized in fertilized egg and is necessary for the process of early embryogenesis (Choi et. al., 1997).

The 5HT level in nervous tissue during gametogenic cycle of invertebrates has been studied (Martinez and Rivera, 1994). Those results reflex importance role of endogenous serotonin regulating the reproductive process of invertebrates. The presence of serotonin in central nervous system of crustaceans has been confirmed and varieties of serotonergic function have been proposed (Chen et. al. 2003). Distribution of 5HT-immunoeactive neurons in the central nervous system of squat lobster has been reported. The positions, morphologies and projections of serotonergic neurons are conserved among crustaceans, even between distantly related species (Antonsen and Paul, 2001). In this study, the presence of 5HT-imminoreactive cells in eyestalk, brain, thoracic ganglia and muscle is similar, reflecting their roles on contribution in modulatory system of motor and sensory mechanism. The 5HT-immunoreaction surrounding the neuronal soma of brain and thoracic ganglia speculated that, clusters of serotonergic synapses on motor or neurosecretory neuros subject to neurotransmitters or neurohormonal modulation by serotonin. In crayfish, the fifth thoracic and first abdominal neural ganglia were proposed as being the origins of circulatory 5HT playing roles on postural control as well as physiological regulation (Kravitz, 1988). It has been found that, 5HT induced the changes of electrical activity of X-organ cells in crayfish and crab, which suggesting 5HT play an important role on controlling of hormonal secretion in the eyestalks (Kiehn and Warrick, 1992; Nagano and Cooke, 1981). The present of

5HT-immunoreactivity in X-organ sinus gland complex in this study, indicating site of 5HT neurosecretory neurons and serotonergic terminals in this organ.

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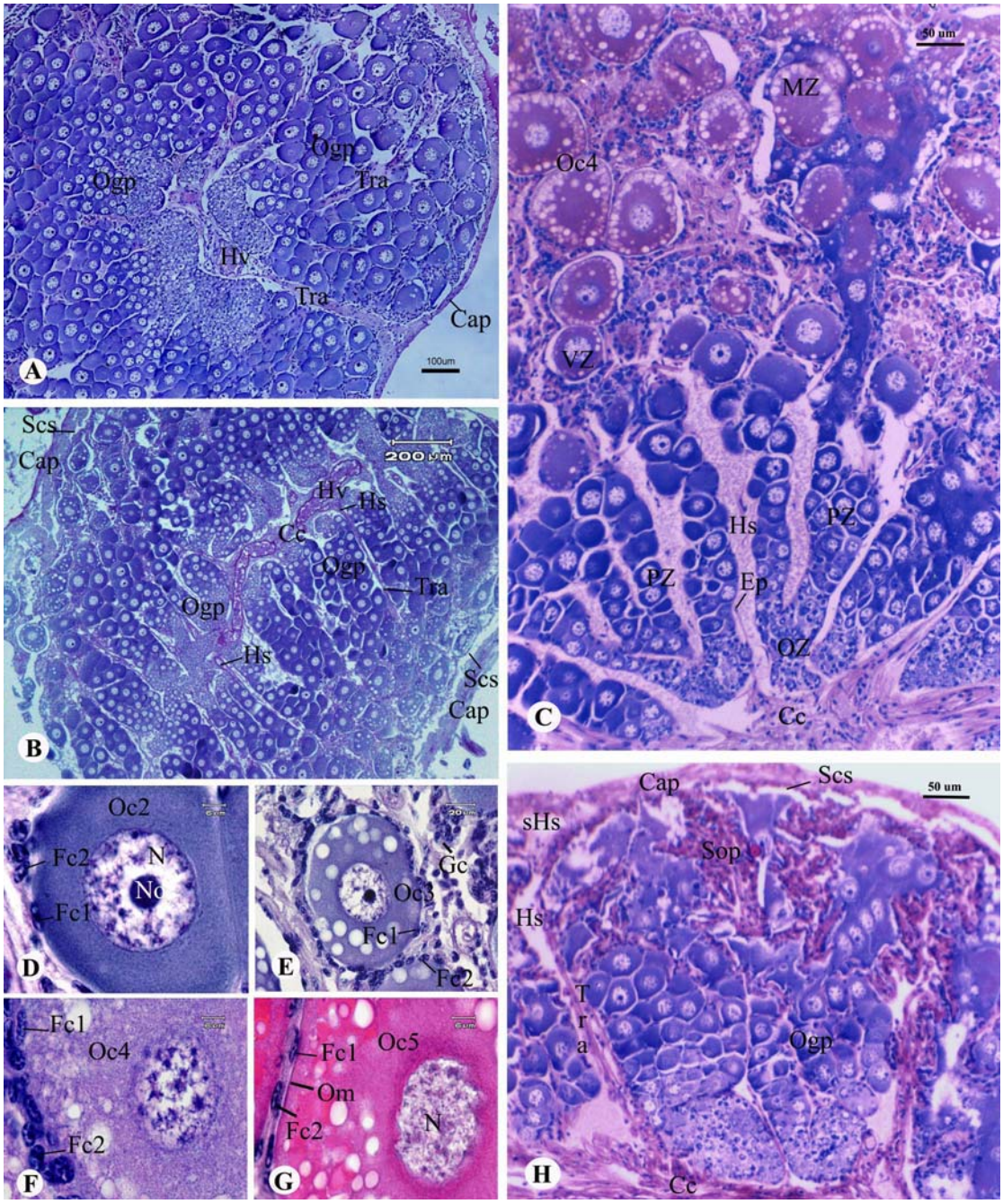
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**Figure 1** Light micrograph of paraffin section stained with PAS, showing the general histology of giant freshwater prawn ovary. It invests with fibromuscular capsule (Cap), which centrally traverses ovarian parenchyma as trabeculae (Tra) forming conical oogenic pouches (Ogp) (**A**, **B**). Combination of trabeculae occurs on central ovarian core (Cc), which lines longitudinally accompanying the main hemolymph vessel (Hv). The main hemolymph vessel is radially branches to periphery companion with fibromuscular trabeculae as hemolymph sinuses (Hs) straight to subcapsular hemolymph sinuses (sHs) and networking around the developing oocytes on its way (**C**). Clones of developing oocytes gather in Ogp assembled the ovary into oogenic zone (OZ), occupied by oogonia; previtellogenic zone (PZ), occupied by previtellogenic oocyte (Oc1 and Oc2) (**D**); vitellogenic zone (VZ), occupied by vitellogenic oocyte (Oc3 and Oc4) (**E**, **F**) and maturation zone (MZ), 0ccupied by mature oocyte (Oc5) (**G**). During early stage of ovarian development, spent oogenic pouch (Sop) could be seen on its periphery (**H**) and the subcapsular space (Scs) is also seen. The picture C and H are higher magnification of cross and longitudinal section in picture A and B respectively. Fc1- type I follicular cell, Fc2- type II follicular cell, Gc- germinal cell, N- nucleus, No- nucleolus, Om- oocyte membrane.

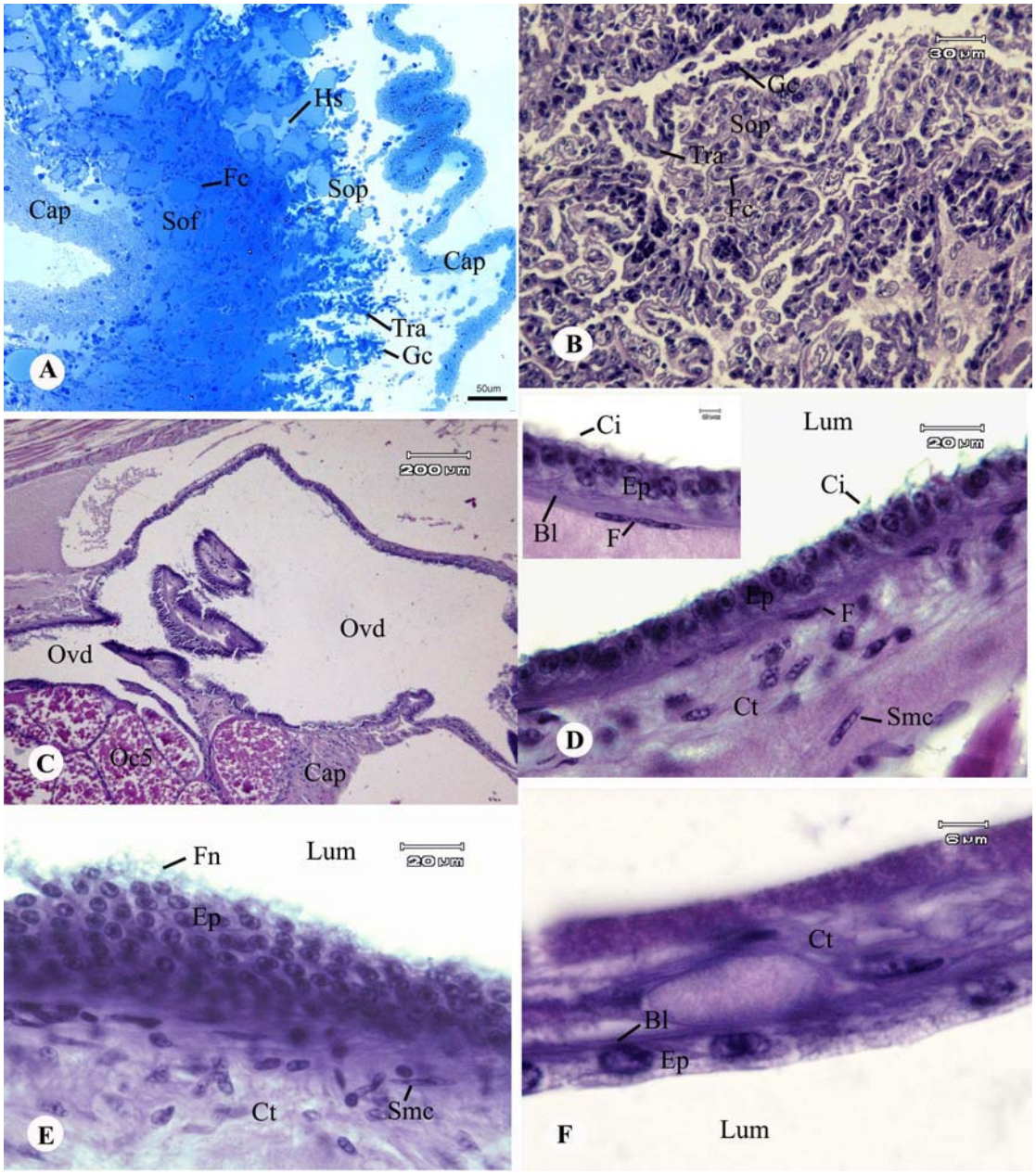
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**Figure 2** Light micrograph of semithin section stained with toludine blue (A) and paraffin sections stained with H&E (B-F), showing the spawned ovary (stage O) (A, B), mature ovary (stage IV) (C) and oviduct (D-F) during premating molt broodstock. The ovary of broodstock following spawned is empty (**A**). Spent oogenic pouches (Sop) and spent oocyte follicle (Sof) are seen throughout the ovary. Dilatation of subcapsular space and hemolymph sinus (Hs) are also seen. Breaking trabeculae (Tra) are appeared as cord companion with germarium, which connect and line with germinal cells (Gc) (**B**). Remnant of follicular cells (Fc) around Sof and grouping in Sop are frequently seen. Ovarian capsule (Cap) is thickening and highly folding. The upside down full maturation ovary (**C**) during premating molt broodstock, showing continuation of oviduct (Ovd) with thickening of fibromuscular ovarian capsule (Cap). Myriad of ovulating oocytes (Oc5) breaks their connective tissue sheaths pour into dilating oviduct on ventrolateral side of each ovarian lobe. The oviductal wall during this stage is lined by simple columnar ciliated epithelium (**D**) resting on basal lamina (Bl) and loose connective tissue (Ct). The fibroblast like cells (F) are lined immediately beneath the Bl in the thin intense blue stained layer, while the stromal cells are ovoid in shape distribute randomly in the loose thicker fibilar layer beneath the previous one. The smooth muscle fibers and cells (Smc) are located underneath the losse connective tissue, characterized by deep purple stained and cork screw – nuclear profile. Different forms of oviductal epithelium lining (Ep) along the oviduct could be classified in to proximal, middle and distal parts. In proximal part, the epithelium is stratified cuboidal layer covering with fibilar network (Fn) (**E**) along luminal surface (Lum). Transition of epithelial lining to simple ciliated columnar type is found in the middle part and to simple cuboidal or simple squamous in the distal part (**F**).

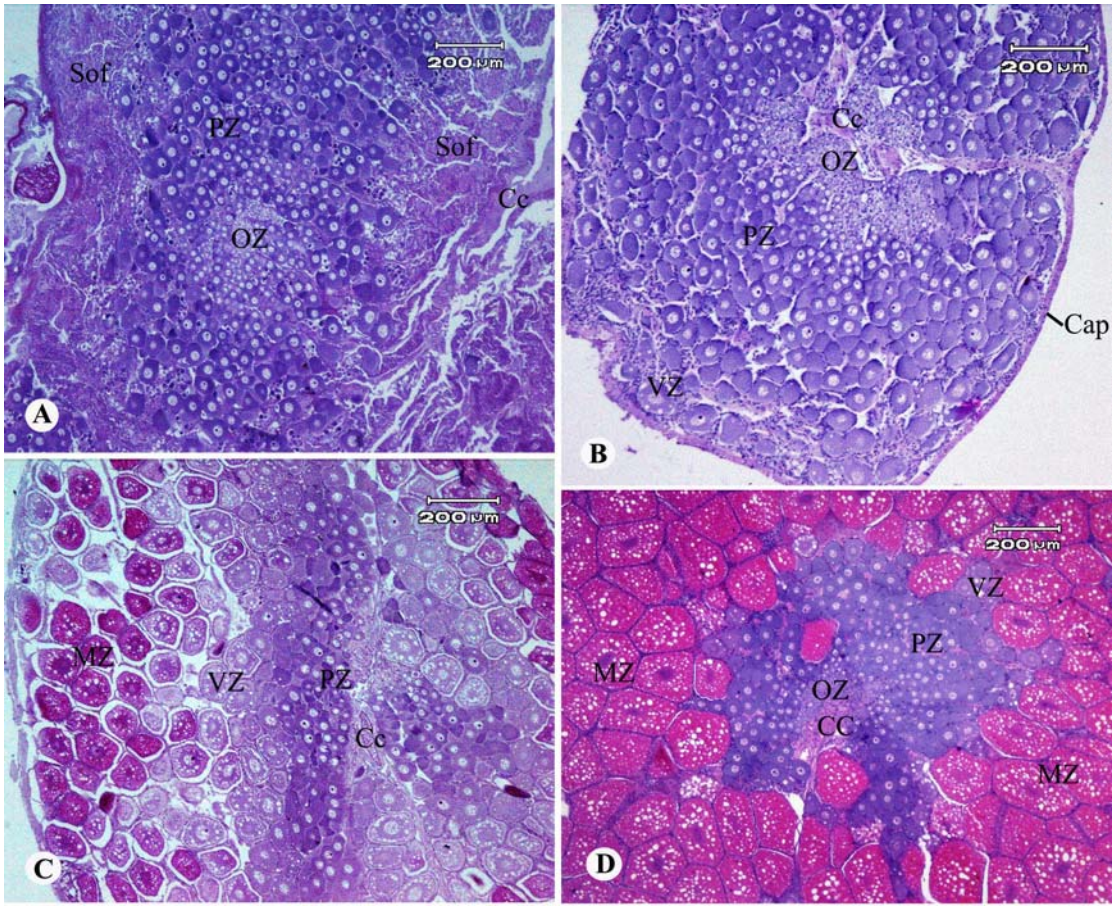
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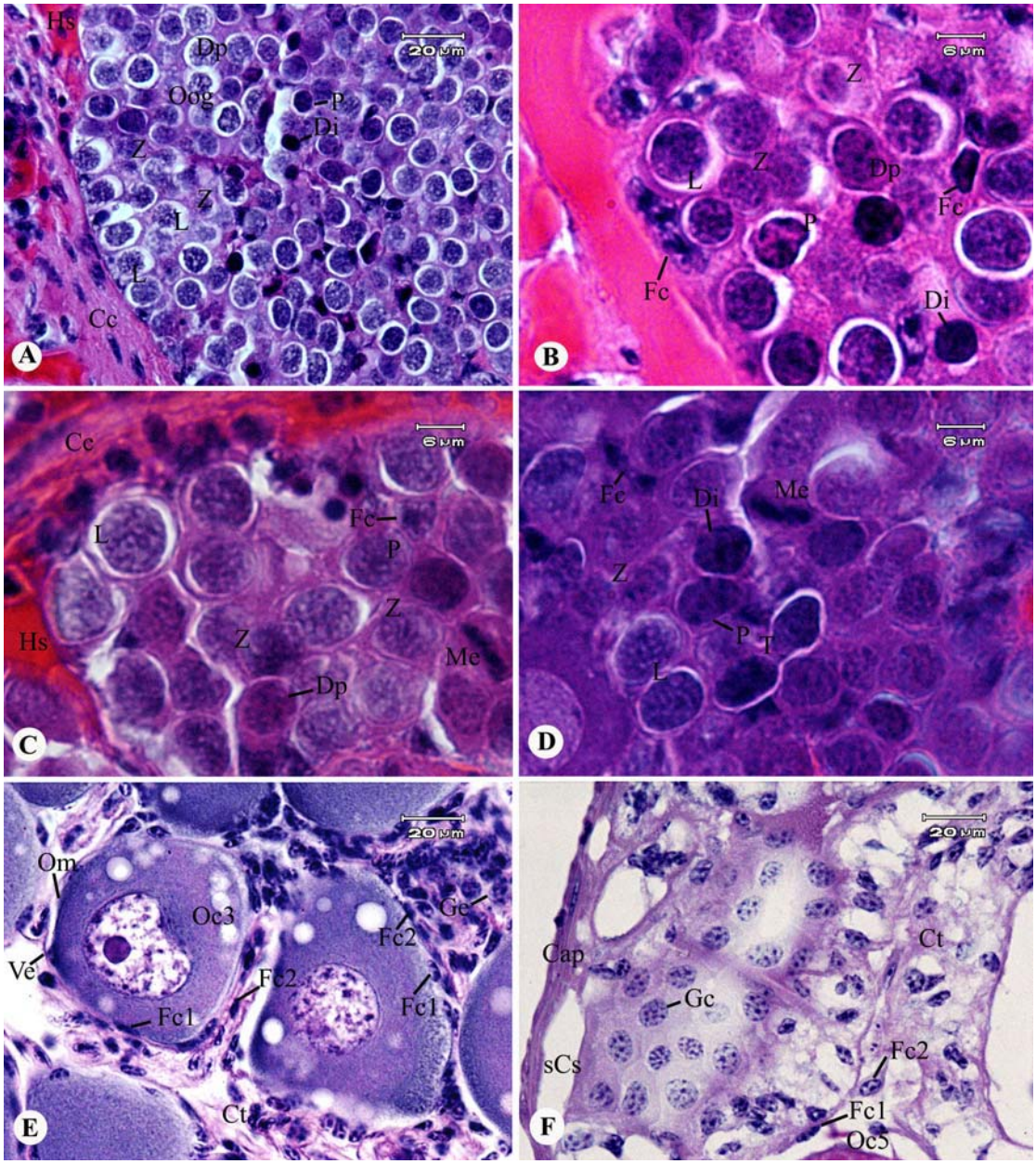
**Figure 3.** Light micrograph of paraffin sections stained with H&E, showing histological characteristic of developing ovaries. Stages of developing ovary are classified according to external morphology, histology and period of molting. By this means, 5 stages of ovarian development in giant freshwater prawn are defined. The ovarian stage 0 (spawned) is the ovary during 1 hr following spawned, which already depicted in previous figure 2. Stage I ovary (spent) (**A**) is developing ovary sampling a day to week after spawned. This recovering ovary characterized by centrally deep blue stained is consequence of collection of previtellogenic oocytes on this area including oogenic (Oz) and previtellogenic zones (Pz). The eosinophiic stained on the periphery ensuring from collapse of spent oogenic pouches and spent oocyte follicles (Sof). The ovarian stage II (proliferation) (**B**) characterized by overall deep blue stained except the ovarian capsule (Cap) and stromal tissue. The oocytes occupying this ovarian stage found advance to early vitellogenic oocyte. Ovary of this stage occurred after a week following spent. This stage of ovary is found during intermolt stage of somatic growth. The decrement of deep basophilic stained and replacing with eosinophilic stained on the periphery of ovary is occurred when the ovary progresses its stage into the III (prematuration) (**C**). At this stage, it fills with developing oocytes at late vitellogenic stage and the mature oocytes are also seen, but do not reaching their full size. The vitellogenic zone is most area of this stage and the OZ would be disappeared. The ovarian stage IV (maturation) (**D**) is mostly occupied by the full maturation oocytes seeing as deep eosinophilic stained called maturation zone (MZ). Island of previtellogenic oocytes surrounding central ovarian core (Cc) could be seen in most mature ovaries. This stage of ovary may be occur after 3 to 8 weeks after spawned.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F

**Figure 4.** Light micrograph of paraffin section stained with PAS, showing various stage of developing germ cells (Gc) and oogonia (Oog). Aggregation of various stages of Oog during 1st meiotic prophase on medial tip of the oogenic pouch pointing to central ovarian core (Cc) is depict in picture **A.** All stages of leptotene (L), zygotene (Z), pachytene (P), diplotene (Dp) and diakinesis (Di) in meiotic prophase are found. The follicular cell is not frequently seen among this developing stage (**B**). Oogonia during metaphase (Me) (**C**) and telophase (T) (**D**) of meiotic prophase are also seen. The meiotic oogonia are derived from mitotic proiferation of germ cells during Oc3 and Oc4 oocyte stages (**E**). They located on germarium accompanying trabeculae and networking around the developing oocytes on inter oocyte space. During ovarian stage IV, germinal cells hidden in the perioocyte space on interposed germarium and stromal tissue (**F)**. The germinal cells characterized by thin rim of cytoplasm with faint blue stained. Their ovoid nuclei typically light blue stained containing deep blue stained globular heterochromatin with typical pattern (**F**). Follicular cell could be classified into 2 types during Oc3 and Oc4 oocytes. Type I follicular cell (Fc1) characterized by dark blue stained spindle shape nucleus laying within vitelline envelope (Ve) close to oocyte membrane (Om). Type II follicular cell (Fc2) is not quite different in shape from the type I, but more ovoid and light blue stained nucleoplasm. It locates periphery to the typeI external to the vitelline envelope on the connective tissue (Ct) surrounding the oocyte. Type II follicular cells are larger in number, while typeI is not frequently seen (**E**, **F**).

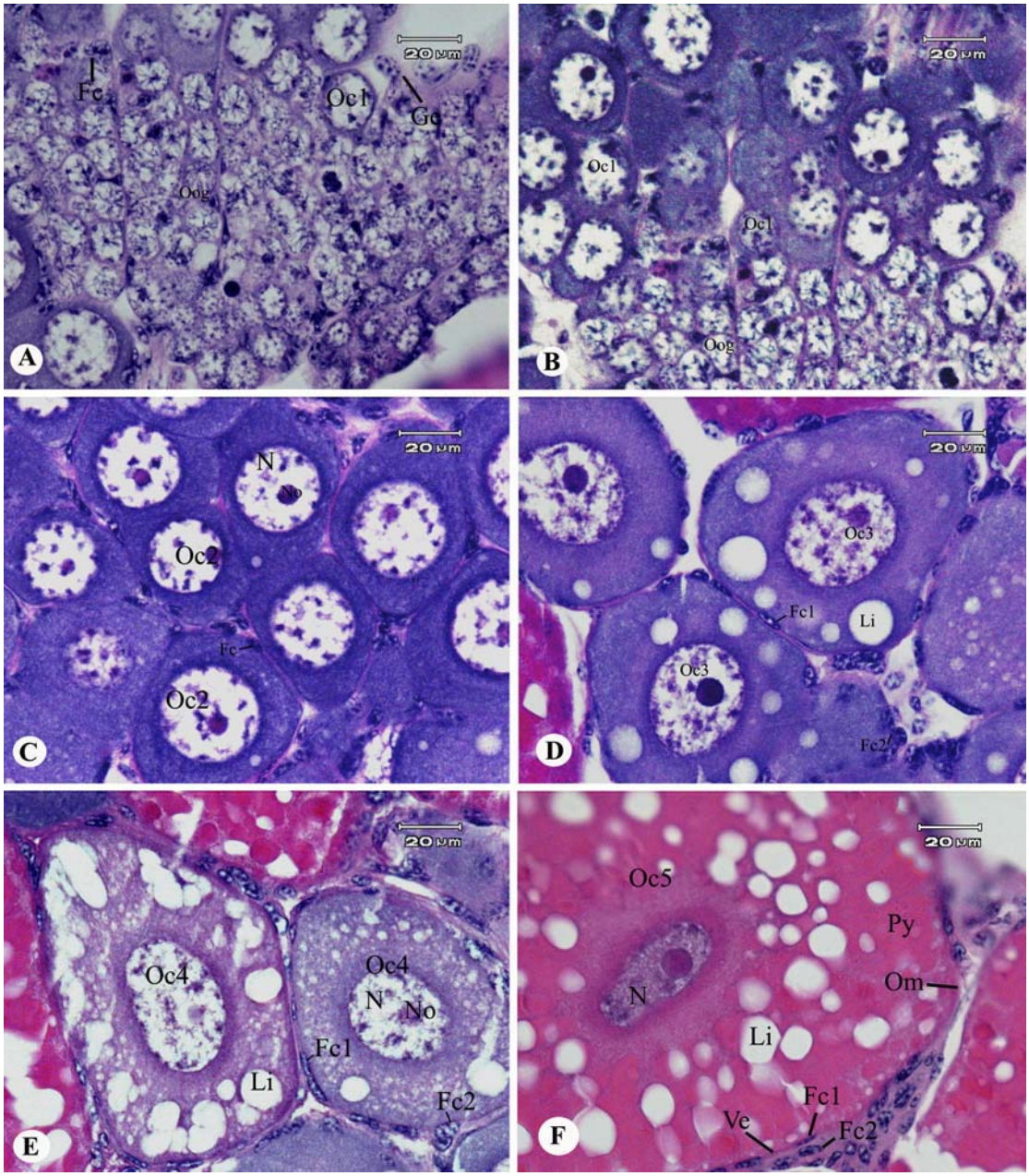

A
Fc
Oc1
Ge
Oog
20 µm
B
Oc1
Oc1
Oog
20 µm
C
N
Oc2
Fc
Oc2
20 µm
D
Oc3
Fc1
Li
Oc3
Fc2
20 µm
E
Oc4
Oc4
N
No
Fc1
Li
Fc2
20 µm
F
Oc5
Py
N
Om
Li
Fc1
Ve
Fc2
20 µm

**Figure 5.** Micrograph of developing oocytes attained from paraffin section staining with H&E, showing different steps of oocytes from oogonia (Oog) to mature oocyte (Oc5). Oogonium is characterized by small round cell with very thin cytoplasm. Large nucleocytoplasmic ratio is seen (**A**). The oogonia aggregate on tip of oogenic pouches of developing ovary. Follicular cell (Fc) is seldom seen. Oogonium entered $2^{nd}$ meiotic division during early previtellogenic oocyte (Oc1) (**B**), which characterized by deep blue stained of round thin rim cytoplasm. The nuclei (N) are round and rest on diplotene stage of $2^{nd}$ meiotic prophase containing large area of euchromatin. Globular heterochromatin is also seen. When the oocyte progressing to late previtellogenesis (Oc2) (**C**), intensely blue stained cytoplasm is seen. It increases in size and transforming to ovoid or polygonal in shape. An enlarge round concentric nucleus is prominent with nucleolus (No). More than one nucleols are frequently observed. The follicular cell is more distinct as deep blue stained nucleus with ovoid profiles. The early vitellogenic oocyte (Oc3) (**D**) is characterized by first present of random distribution of lipid droplet (Li) in deep blue stained cytoplasm. The cell size is more increase with poly gonal in shape and nucleus become ovoid with more granular heterochromatin and eccentric nucleolus. The follicular cell is increasing in number and the prominent ovoid nucleus with light blue stained is seen. Two types of type I (Fc1) and type II (Fc II)follicular cells could be defined at this stage. The late vitellogenic oocyte (Oc4) (**E**) is prominent with enlarge size and peripheral arrangement of lipid droplets. The proteinaceous yolk accumulates on cytoplasmic rim, resulting in eosinophilic stain on periphery. Deep blue stained of cytoplasm on perinuclear area is prominent.The nucleus is more ovoid in shape and nucleolus is seldom seen. Follicular cell is still prominent with elongating shape. The mature oocyte (Oc5) (**F**) is exhibited prominent eosiniphilic cytoplasm resulting from accumulation of proteinatious yolk (Py), except nuclear area. The nucleus is more flatten ovoid containing less heterochromatin. Type I follicular cell is more flatten closing to oocyte membrane (Om) and seldom seen, while type II is more prominent and more frequently seen. The vitelline envelope is seen as a very line closes to Om.

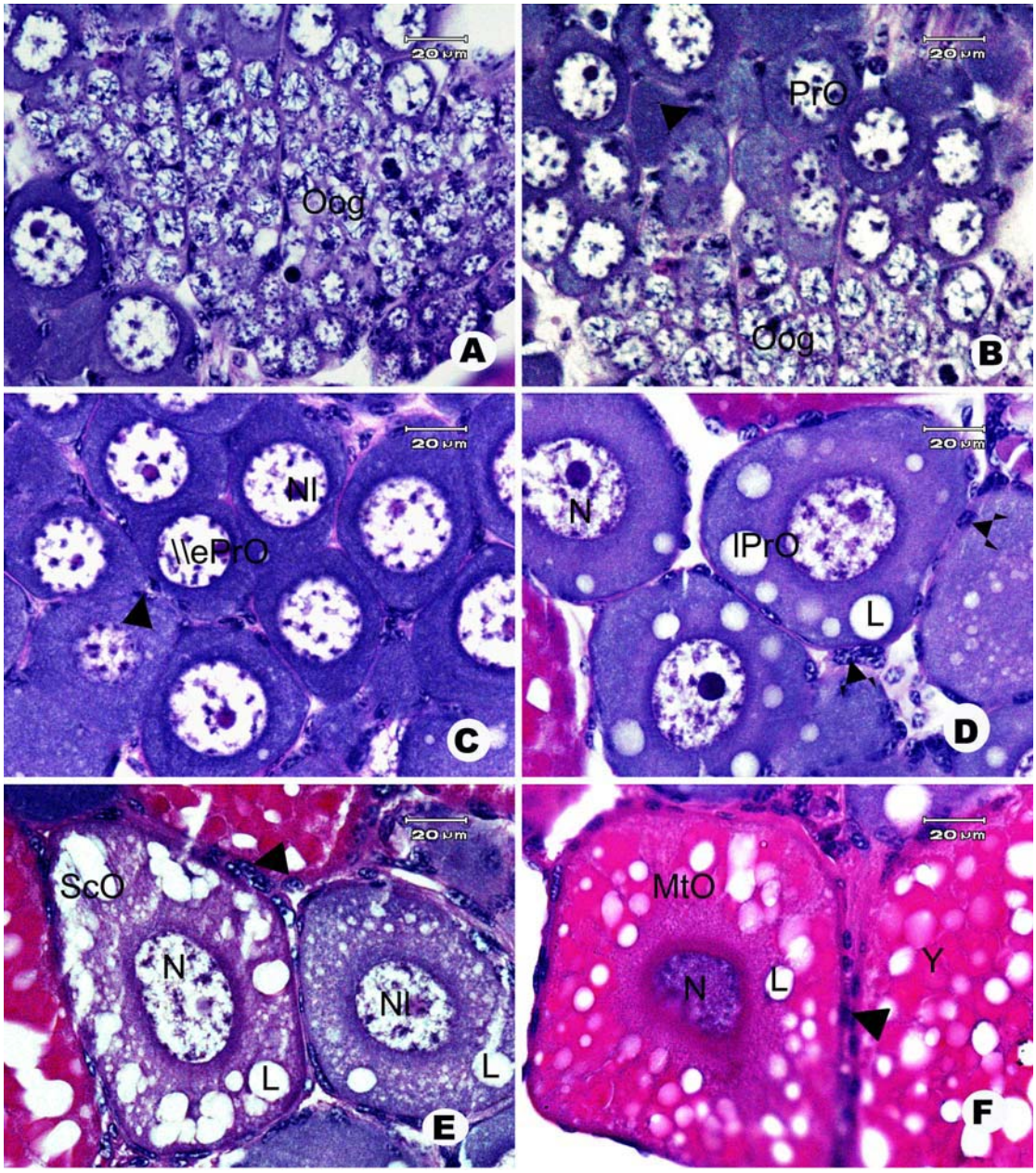

20 µm
Oog
A
20 µm
PrO
Oog
B
20 µm
Nl
llePrO
C
20 µm
N
lPrO
L
D
20 µm
ScO
N
Nl
L
L
E
20 µm
MtO
N
L
Y
F

**Figure 6.** Light micrograph of oogonia (Oog) (**A**) in oogenic zone and developing oocytes in previtellogenic zone of developing ovary of *Macrobrachium rosenbergii* broodstock. The oocytes in previtellogenic zone are early previtellogenic oocyte (Oc1) (**B**) and late previtellogenic oocyte (Oc2) (**C**), while the vitellogenic zone contained early vitellogenic oocyte (Oc3) (**D**) and late vitellogenic oocyte (Oc4) (**E**) and the maturation zone fully occupied by mature oocyte (Oc5) (**F**).

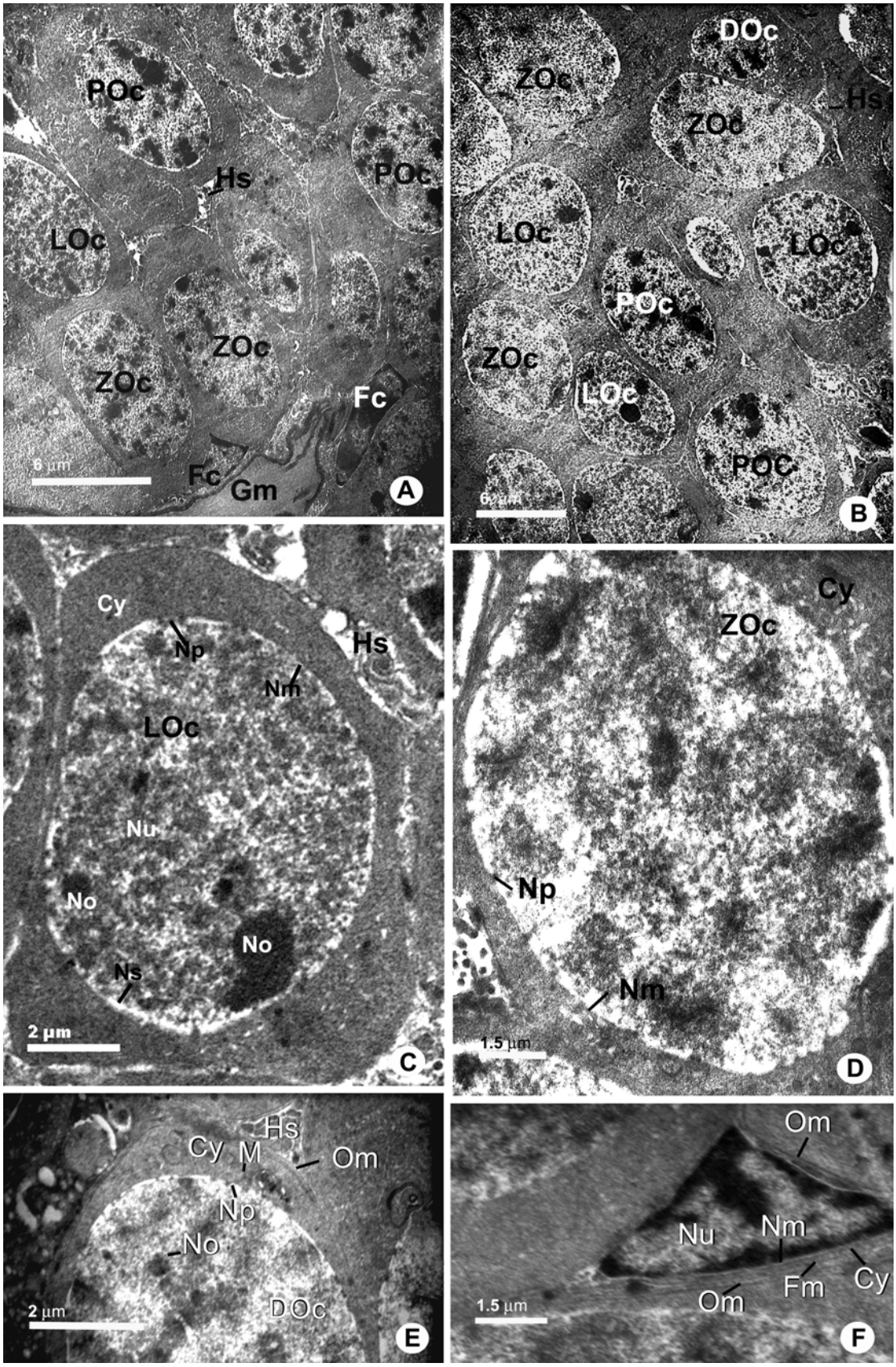

POc
Hs
POc
LOc
ZOc
ZOc
Fc
Fc
Gm
6 µm
A
DOc
ZOc
Hs
ZOc
LOc
LOc
POc
ZOc
LOc
POC
6 µm
B
Cy
Np
Hs
Nm
LOc
Nu
No
No
Ns
2 µm
C
Cy
ZOc
Np
Nm
1.5 µm
D
Hs
Cy
M
Om
Np
No
2 µm
DOc
E
Om
Nu
Nm
Fm
Cy
Om
1.5 µm
F

**Figure 7.** Electron micrograph of oogonia oocytes in oogenic zone of developing ovary. Different stages of oogonia during 1st meiotic division are seen (**A, B**). High magnification of leptotene (LOc), zygotene (ZOc) and diplotene (DOc) stage of 1st meiosis are shown in figure **C**, **D** and **E** respectively. The follicular cells (Fc) are shown in figure **A** and **F**. They are tightly intervene the oocytes and contain thin rim of cytoplasm (Fm). The nucleus is highly condensed with maginate heterochromatin. Cy, cytoplasm; Fm, follicular cell membrane; Gm, germinal membrane; Hs, hemolymph sinus; Nm, nuclear membrane; Np, nucleopore; No, nucleolus; NU, nucleus; Om, oocyte membrane; Poc, pachytene oogonia.

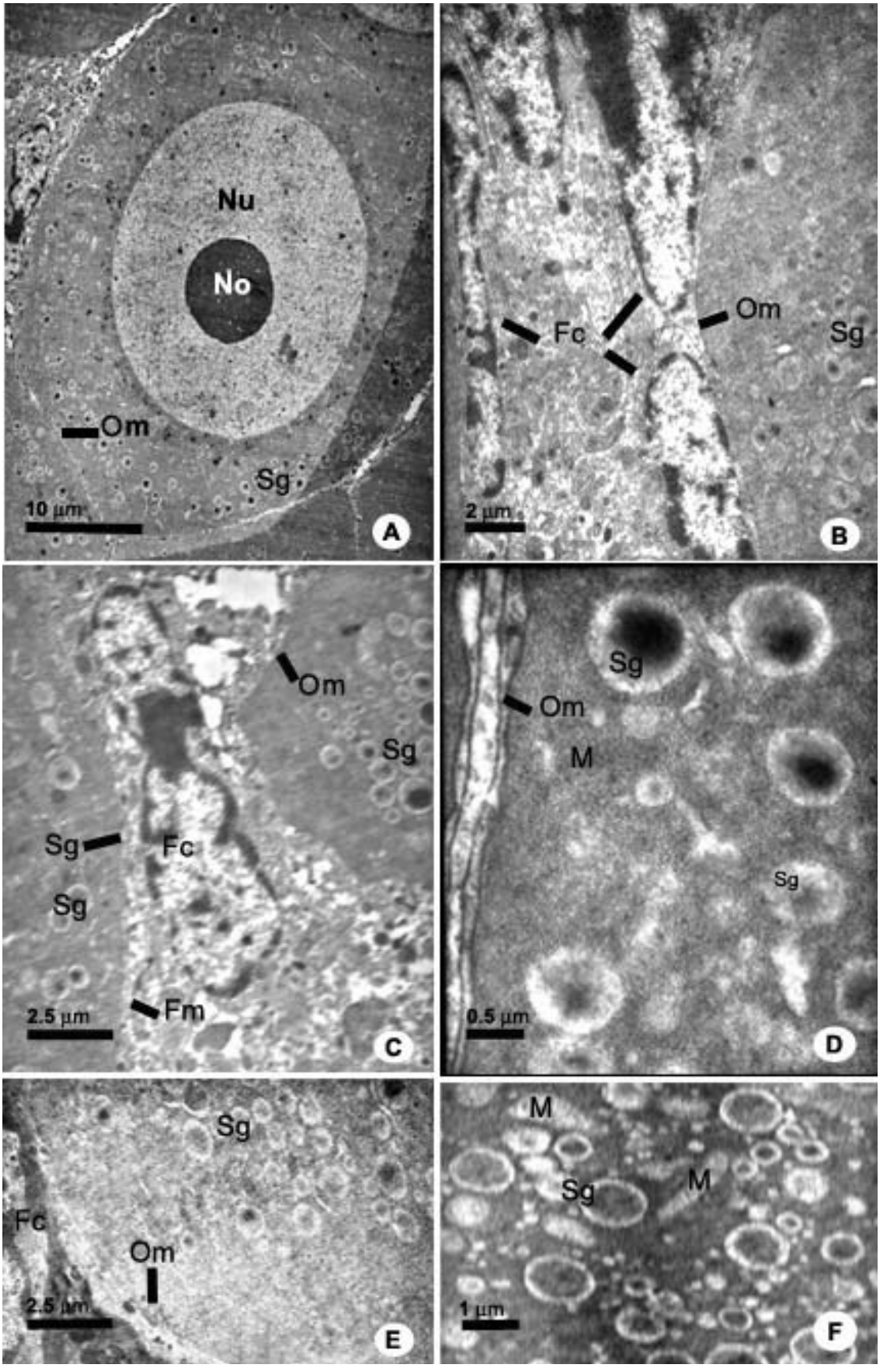
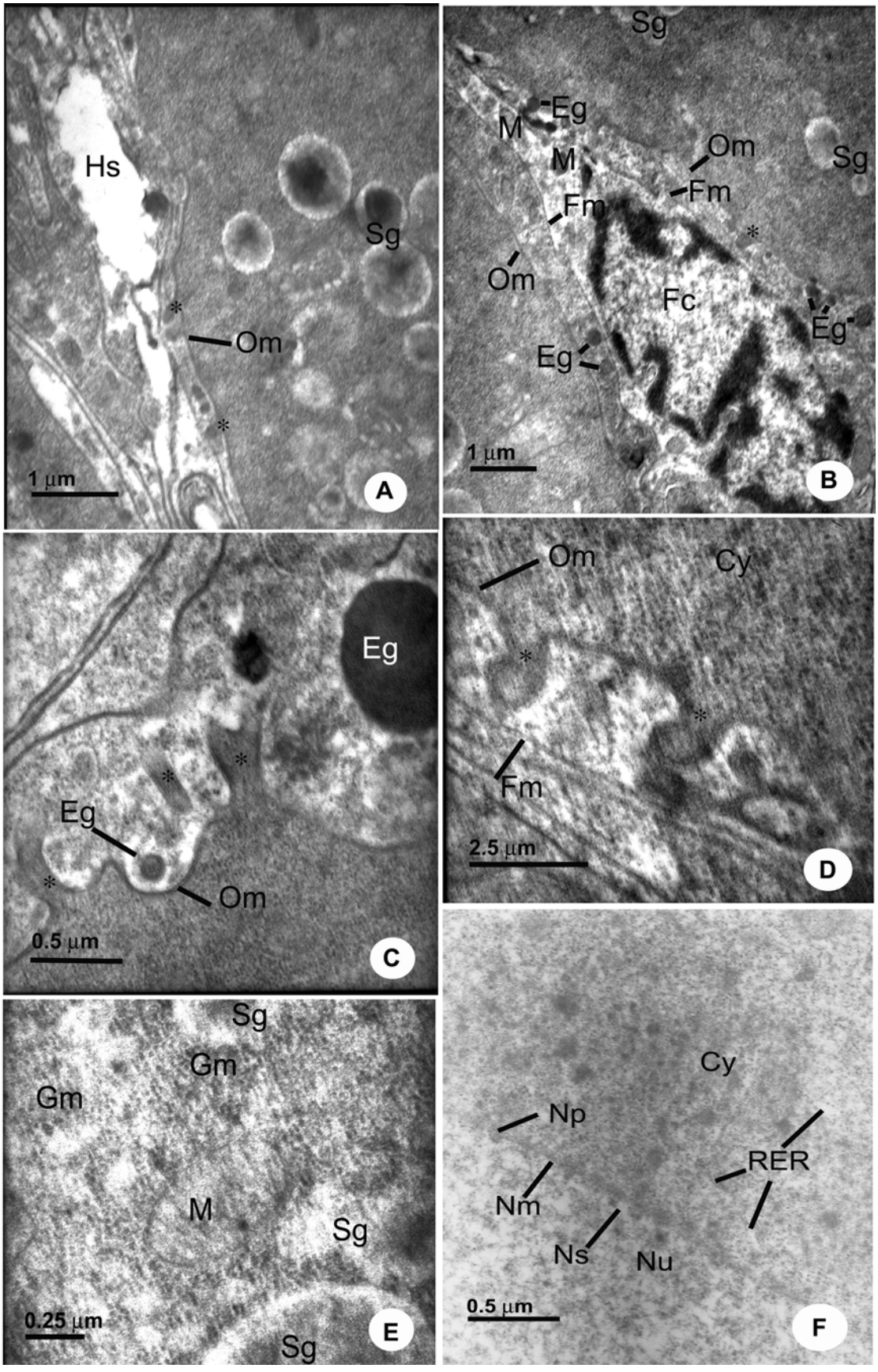

Nu
No
Om
Sg
10 µm
A
Fc
Om
Sg
2 µm
B
Om
Sg
Sg
Fc
Sg
2.5 µm
Fm
C
Sg
Om
M
Sg
0.5 µm
D
Sg
Fc
Om
2.5 µm
E
M
Sg
M
1 µm
F

**Figure 8.** Electron micrograph of late previtellogenic oocyte (Oc2) reveling different in size, but similar ultramorphology as found in early previtellogenic oocyte (Oc1). They have prominent central nucleus (Nu) with broadspread euchromatin and highly condense nucleolus (No) (**A**). Cytoplasm is fully contained various sizes of secretory granules (Sg) (**A**-**F**). Mitochondria (M) are also seen between the secretory granules (**D**, **F**). The oocyte membrane (Om) is smooth on both surface (**B**, **D**). No secretory granule situates in close contact with the oocyte membrane. The follicular cells (Fc) are proliferated and elongation, but still tightly contact to the oocytes (**B**, **C**). Their cytoplasmic volume and organelles are increased. Fm, follicular cell membrane.


Hs
Sg
Om
1 μm
A
Sg
Eg
M
M
Om
Sg
Fm
Fm
Om
Fc
Eg
Eg
1 μm
B
Eg
Eg
Om
0.5 μm
C
Om
Cy
Fm
2.5 μm
D
Sg
Gm
Gm
M
Sg
Sg
0.25 μm
E
Cy
Np
RER
Nm
Ns
Nu
0.5 μm
F