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OOGENESIS IN THE CANCER CRAB *PARTHENOPE LONGIMANUS* (LINNAEUS, 1764): HISTOLOGICAL AND HISTOCHEMICAL PROFILE WITH STATISTICAL ALALYSIS ON THE DEVELOPMENTAL STAGES OF OOCYTE ALONG IMMATURE AND MATURE PHASES

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ABSTRACT

Five maturity stages were defined in the ovary of the cancer crab *Parthenope longimanus* namely immature, early maturing, late maturing, ripe and spent. In immature phase during winter season the ovarian lobules had a great number of oogonia (50 to 60µm in∅) and few number of previtellogenic oocyte (60 to150µm in∅). These previtellogenic oocytes had a homogeneous ooplasm. So the winter season can be interpreted as a period of multiplication phase of oogenesis and the primary growth phase began at its end. In spring season the ooplasm of the previtellogenic oocyte (60 to150µm in∅) became intensely basophilic. These oocytes have undergone two synchronously vital activities. They became follicular and grew gradually in diameter. Opaque granules are deposited in the form of a ring around the nucleus referred to as yolk nucleus. In secondary growth of the oocyte, 150 to 210µm in ∅ (vitellogenesis), maturing phase, there were increasing quantities of lipid droplets, peptide linkage, basic proteins, acid MPS, glycogen … etc. (see table 2a,b). Yolk bodies (glycoproteins) increased rapidly and replaced the vacuoles located around the periphery of oocyte. The vitelline envelope becomes conspicuous under the follicle cells. Gradually other lipid deposition appeared in the early secondary growth phase and these lipid droplets migrated to the periphery of ooplasm. In ripe phase, during summer season the full-grown oocytes have reached their maximum diameter, 180 to 220µm. The thickness of the vitelline envelope gradually increased. This indicated that the chorion is formed around the oocyte during the process of vacuolization of the follicle epithelium. In spent phase, during autumn the ovaries appeared flaccid, translucent and greatly reduced. The Post-vitellogenic oocyte is characterized by a homogenous ooplasm and the yolk material accepted a clear blue hue with the different triple stains applied. The ooplasm of the previtellogenic oocyte contained ribosomes, mitochondria, Golgi complex, and cisternae of the granular endoplasmic reticulum. In vitellogenic oocytes, the abundance of the endoplasmic reticulum is a particularly striking feature. The Golgi complex consisted of a variable number of stacked cisternae. As vitellogenesis proceeded, the oocyte surface became irregular with the formation of microvilli and micropinocytotic vesicles. A dense granular material present between the follicular cells and the oocyte appeared as detached vesicles in the cortical ooplasm. The number of the different stages of oocyte development has been counted per ovary in the four seasons of the year. This counting has been made in three crabs` ovaries per season. All data were tabulated and have been compared using a 1-way ANOVA ($p < 0.05$), with the oocyte stages of maturity as a fixed factor. Turkey`s Multiple Comparison test has been applied to compare the ratio of oocyte developmental stages along the year.

KEYWORDS:- ovarian lobule; oogonia; previtellogenic oocyte; vitellogenic oocyte; postvitellogenic oocyte; ribosomes; mitochondria; Golgi complex; granular endoplasmic reticulum.

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INTRODUCTION

Reproduction of marine organisms is adapted to environmental conditions, in particular, temperature, photoperiod, food supply, and predators. Consequently, reproduction is cyclical and based on the season of the year in which conditions are adequate for the survival of the offspring. Reproductive biology of decapod crustaceans is well documented, particularly in brachyurans. Several methods have been used to define the reproductive cycle in crabs including gonad indices (Boolootian, et al.1959 and Du Preez & MacLachlan,1984 c), incidence of ovigerous females (Fielding & Haley, 1976 and Pillay & Ono, 1978) and histological examination of the gonads (Haefner, 1977, Hartnoll,1978, Gemmell, 1979 and Jones and Simons, 1983). The general applications of these methods on invertebrate reproduction have been reviewed by Giese & Pearse (1974) and Grant & Tyler (1983a, b), whilst the ecological aspects of crustacean reproduction were reviewed by Sastry (1983). The reproductive characteristics of crab have been recently studied (Stella et al. 1996); as well as ecological aspects of its reproduction (Ruffino et al. 1994). Also , the effects of pollutants on reproductive events of have been assayed (Rodriguez & Medesani , 1994; Rodriguez, et al. 1994 & 1998). Sexual maturity in crabs has been studies by various authors as a function of size (Hartnoll, 1982; Grasse , 1994). The foundation of reproduction for any species originates from the development of germinal cells during the process of gametogenesis, Therefore, the development of oocytes in the females and of spermatozoa in the males is a fundamental function of reproduction. Oogenesis is a complex process of cellular and molecular changes that occur during the formation, growth, and maturation of the female germinal cells. The development of the oocyte is remarkable, and the study of this process is essential to the understanding of reproduction. The available literature is insufficient to describe the process of gametogenesis on histochemical and ultrastructural point of view, moreover, many genera of brachyurans are not covered yet. Previous studies have been limited to observations on the incidence of vigerous females (Pillay & Nair , 1976) and notes on larval development (Rajabai, 1959).

In this study , crabs were found capable of initiating reproductive activity following the so-called puberty molt. Several authors (Hartnoll 1974, 1982; Legrand & Juchault , 1994) associate this molt with a change in the relative growth of certain appendages, or tagmata of reproductive value (i. e., chelae, abdomen) with respect to a reference body size, such as the length or width of the carapace. Few attempts have been made, however, to associate changes in relative growth with the histological condition of the gonads. The aim of the present study is to determine the annual activity of the ovaries of the cancer crab *Parthenope longimanus* (Linnaeus, 1764) and the characterization of the developmental stages of the oocyte from both the relative growth and the histological points of view. Brachyurans produce great number of larvae that grow rapidly and attains early sexual maturity. Offsetting their fecundity are high mortality rates and a short life span (Steele, 1982). The distribution of the species varies depending mostly on season, sex, and age. Adult crabs inhabit a range of bottom types including fresh, estuarine, and shallow marine & oceanic waters. Large sized crabs are prevalent in larger bays and bayous (Oesterling,1976). Differences in salinity, temperature, and habitat are among the factors that affect brachyurans distribution and abundance (Steele and Bert, 1994). Although adults are ubiquitous throughout an estuarine system, they are distributed seasonally with respect to salinity and sex. In general, males are more prevalent in low-salinity areas, whereas females predominate in high-salinity areas (Oesterling, 1976). Some studies have shown that brachyurans populations are cyclic, and their abundance can fluctuate dramatically from year to year. These inter-annual fluctuations in abundance are thought to be due to physical, chemical, and biological factors that strongly influence the characteristics of the populations (Steele and Bert, 1994). Brachyurans are heterosexual and exhibit distinct sexual dimorphism. The female's abdomen changes from a narrow shape to a broader, wider shape as she become sexually mature. Females attain sexual maturity in 12 to 14 months, reaching adult size at app. 130-139 mm or after 18–20 molts. When they molt at maturity, growth ends; consequently, it is known as the "terminal molt" (FWC–FWRI, 2011a,b,c). Females mate only once, but males may mate numerous times. Mating takes place in brackish water after the female's terminal molt. Unlike most marine organisms, crabs mating and spawning (shedding of eggs) occur at different times (Steele, 1982). A precise analysis of the microscopic structure of the ovaries provides a better comprehension of the development of the female germ cell, the oocyte during the process of oogenesis, and its cyclical changes that define the reproduction of this species.

Oogenesis is the sequence of stages that oocytes undergo, from the oogonium to oocyte maturation. To properly describe the process of oogenesis, several essential aspects need to be studied: the examination of the germinal epithelium of the ovary, in order to distinguish where the oogonia are located; and the identification of the process through which oogonia give rise to the oocytes, including the cellular morphological characteristics during the different stages of development, from early primary growth to late secondary growth. The development of the oocyte involves active and complex increases of ooplasm, and the deposit of abundant nutrients enclosed in structures such as yolk platelets and lipid droplets in the ooplasm during a precise sequence of changes. Morphological changes in the ooplasm that occur during this process aid in identifying the stages of the oocytes throughout oogenesis and thus the phase of the reproductive cycle of the specimen. This maturation sequence is the basis of the analyses of reproduction in the species and can be identified and quantified through histological examination.

MATERIALS AND METHODS

Animals

Specimens of the cancer crab *Parthenope longimanus* (Linnaeus, 1764) were sampled regularly twice every month at depth 1- 2 meters during the four seasons in the year (2013) along the northern estruarine harbour of the Arabian Gulf - Saudi Arabia. Identification of this species under investigation was carried out according to Michael & Vassily (1998) and Reza & Alireza (2014). Each collection was transported in well-aerated sea water to the laboratory. Few amount of

magnesium sulphate was added in the aquaria as a relaxing agent. Individuals of each collection were used to prepare sections of ovaries for histological and ultrastructural studies.

Microscopic observation

Isolated ovaries from each collection were fixed in neutral 10 % formalin or Bouin. These isolated ovaries have been washed in distilled water for 24 hours, dehydration through ascending series of ethyl alcohol, alternated by another dehydration series of tertiary butyl alcohol (used as a softening agent), then tertiary butyl alcohol and paraffin oil (1:1), absolute paraffin oil. All preparations then washed carefully in tissue mate (melting paraplast) with melting point 54-58 °C and blocked in fresh paraplast. Sections for histology with thickness of 5-6µ and sections for histochemistry with thickness of 10-12 µ were somewhat successfully carried out. For the routine histological study, the sections were stained with Ehrlich haematoxylin and eosin stain (Pearse, 1968, 1980 and Pantin, 1948). A number of triple stains were tried to enable the differentiation of the tissues. A number of histochemical reactions were tried to enable the identification of the different components of yolk before and during vitellogenesis. For the demonstration of lipids, the ovaries were either fixed in Baker's calcium fixative and embedded in gelatine (Baker, 1944) or the fresh material freezed. Both the gelatine blocks and frozen material were sectioned at 12-15 µ on the cryostat (Ao cryo-cut II, 851C). Glycerol gelatine is melted at 60°C on a water bath. The mounting process of gelatine sections is carried out by dropping approx. 0.5 ml of mounting agent glycerol gelatine (2:1) onto the horizontal slide using a glass rod. By this way the space between slide and cover glassis filled. As soon as the specimen has been covered with a homogeneous solution, cover with a clean cover glass, taking care of avoiding air bubbles. Allow to dry and harden in a refrigerator over a period of 20-30 min in a horizontal position. For microscopic examination, Ortholux Leintz Wetzler Stereoscope microscope with 100, 250 and 400 magnification capacities with Lighthouse 250 with external light source of Schott KL 1500 was used. The Camera used was a full automatic microscope Camera for research and laboratory purposes. The details of the histochemical tests and other techniques employed in this work will be appropriately given in the course of the following accounts or at least the references will be mentioned. The selected sections of the ovary were then photographed to give a somewhat complete overview for the process of deutoplasmogenesis in the crab investigated.

Transmission electron microscopy (TEM)

Parts of ovaries were dissected alive in sea water and the nervous systems with a part of the mantle underneath were isolated. The nervous system was fixed in 2.5% Glutaraldehyde in 0.05 M PBS containing 0.33 M NaCl (1h, 4°C). The fixative was removed by washing specimens several times with PBS. Post-fixation was carried out using 2% Osmium tetroxide in PBS for 30-60 min at 4°C. Specimens were subsequently washed with PBS, dehydrated in a graded ethanol series, and propylene oxide and embedded in araldite resin. Semithin sections and ultrathin (60-70 nm) sections were obtained using Leica UC6 microtome equipped with diamond knives. Ultrathin sections were picked with formvar-coated singleslot copper grids, stained automatically with uranyl acetate and lead citrate in a Nanofilm TEM STAINER, and examined on a Phillips CM 120 transmission electron microscope at 60 kV. Semithin sections were placed on glass slides and stained with toluidine blue (1% toluidine, 1% Na2B4O7, 20% sucrose) for 1 min at 60°C.

RESULTS

Morphology of the ovary

The female reproductive system of the cancer crab *Parthenope longimanus* (Linnaeus, 1764) comprised a pair of ovaries, a pair of seminal receptacle, and a pair of gonopores. The ovary is H- shaped and located dorsally just beside of the gastric stomach and lied dorsally to the hepatopancreas. At the posterio-lateral border of the gastric stomach, near the origin of the posterior mandibular muscle bundles the anterior horns were joined by the commissure. Two posterior horns, which lied ventral to the heart, extend posteriorly on either side of the intestine. The posterior prolongation of the ovary was subequal, one of the horns being larger and extending further 5-6 mm beyond the other. The seminal receptacles arose from the mid-lateral border of the posterior horns. Each seminal receptacle led into a narrow genital tube which further opened outside through a small circular gonopore situated on the sixth thoracic sternite. The entire ovary is bounded by fibrous connective tissues which separate the organ from the surrounding hemocoel. It is divided into internal units called ovarian lobules which encompass the oocytes in its different developmental stages (Fig. 1). The ovaries showed several gross stages as they went from immature to mature stages (Fig. 2). The immature ovary resembled a small pink filament. The ovaries changed from the light, soft pink of the immature stage to the bright orange of the mature ovary. Mature condition of the ovaries was determined by gross analysis of the organ and by establishing a hue-coded staging system based upon the hue of the ovaries. This hue-coded system included physiologically mature females as having orange gonads, which determined that the female is gravid. It has been observed that the size at maturity is inconsistent. It was noticed that overlap in size ranges of immature and mature female cancer crabs was considerable.

Oocyte development

Primary growth of the oocyte

Most oocytes inside the ovarian lobules are at about the same developmental stage (Fig. 3). Oogenesis is a continuous process in which oocytes undergo development from primary growth to secondary growth in a somewhat rapid pattern. Therefore, the percentage of oocytes inside an ovarian lobule at a specific developmental stage can correspond with the occurrence of another developmental stage (Fig. 4). Some lobules are configured with two main stages, but they may also have a small percentage of a third stage in them. Therefore, an ovarian lobule that is mainly in mid-secondary

growth can have some oocytes in primary growth and a few oocytes representing the early primary growth stage. Early primary growth oocytes are always present because along with oogonia, they compose the germinal epithelium (Fig. 5). The position of the germinal epithelium varied widely. It may be peripheral, peripheral but confined to a particular region or in the form of germ nests distributed throughout the ovary. The oocytes develop from the center of the ovarian lobule to the periphery as oogenesis advances. This distribution of germinal cells in the ovarian lobules is characterized by the developmental progress of cells from oogonia, which are found in a central germinal epithelium (Fig. 5), to late secondary growth stage, which are found in the periphery of the ovarian lobes (Fig. 4). The germinal epithelium is a row of germinal cells at the centre of the ovary. Based on hue change, external morphology and histolology, the ovary was divisible into five maturity stages, namely immature, early maturing, late maturing , ripe and spent. In immature phase, during winter season the ovary is thin, tubular, translucent and extremely difficult to locate macroscopically. It is usually less than 2mm thick and without pronounced lobulation. The ovarian lobules had oogonia which appeared as if they are budded from the germinal epithelium. Oogonia seemed oval in shape, 50 to 60µm. The ratio of the nucleus to the ooplasm is app. 2.5:1. Affinity to stain: with Ehrlich haematoxylin & eosin and the different triple stains applied as Heidenhain's iron haematoxylin, the ooplasm accepted a red hue while the nucleus accepted blue hue (see table 1). Few number of previtellogenic oocyte are also present. The previtellogenic oocyte has an oval shape, 60 to150µm. The ratio of the nucleus to the ooplasm 1:2. The ooplasm increaseed in size gradually, therefore it is possible to differentiate the oogonia from the previtellogenic oocyte through the ratio of the nucleus to the ooplasm. Affinity to stain: with Ehrlich haematoxylin & eosin and the different triple stains applied as Heidenhain's iron haematoxylin, the ooplasm accepted a dark red hue while the nucleus accepted a deep blue hue (see table 1& Figs. 2-5). A layer of prefollicle cells enclosed the previtellogenic oocytes. The ooplasm of these stages, oogonia and previtellogenic oocytes is homogeneous and with comparatively large nucleus provided with a prominent nucleolus. The number of previtellogenic oocytes increases gradually at the expense of oogonia. Both stages have the same histological appearance and the same affinity to the different histological stains applied. Autolyzed vitellogenic oocyte appeared in the preparations of ovaries of January-February (Figs. 6-7). This indicates that this winter season can be interpreted as the interbreeding phase. However the number of oogonia increased gradually during this period. Hence these months can be interpreted as the period of multiplication phase of oogenesis and the growth phase begins at its end. The previtellogenic oocyte ooplasm is eosinoplelic and appeared homogeneous in nature. The nucleus is large and centrally located. It is acidophilic and the nucleoli are of various sizes. The nucleolus in the previtellogenic oocyte is a solid rounded body. It is highly basophilic. With the growth of the oocyte, this nucleolus increases in size, develops vacuoles inside it, and becomes acidophilic. It may bud off another nucleolus from it. Later on during this period, these previtellogenic oocyte become no longer lineary arranged along the germinal epithelium. They displaced aside while increasing in diameter. During this primary growth the germinal epithelia provided new cells for continual development of oocytes as cells mature when they reach the periphery (Fig. 8). Oogonia cannot be classified as oocytes because oogonia are capable of dividing via mitosis to form other oogonia or via meiosis to form an oocyte. They are diploid cells in which meiosis has not started. Early primary growth oocytes have begun meiosis, and therefore the cell became a primary oocyte. The early primary growth oocyte has an ooplasm characterized by ooplasmic basophilia. When the ooplasm changed from the clear, scant appearance that it had during oogonia to a blue hue, that is the indication that the oogonia is now in early primary growth (Fig. 9). Therefore, the early primary growth oocyte is an oocyte and remains as such throughout the rest of its development, until full-grown. Oocytes, unlike oogonia, are haploid cells in meiosis that have duplicated chromosomes. The beginning of late primary growth is characterized by gradual basophilia of the ooplasm and by the absence of yolk. The basophilic ooplasm indicated that the cell is active with production of organelles, which increased the volume of the ooplasm, another marker for this stage. During this stage, the germinal epithelium is not as evident as it will be in more developed oocytes, yet the germinal epithelium with the oogonia and the early primary growth oocytes is always present. Hemolymph is evident, and follicular cells and somatic cells are also present but not as obvious (Fig. 10). In the course of the beginning stages of late primary growth stage, a distinct PAS positive body was present in the ooplasm, usually near the nucleus (Fig. 11). The PAS positive body, also known as the perinuclear yolk complex, is only evident during the early stages of late primary growth, and it dispersed before the cortical alveoli can be observed. This perinuclear yolk complex, referred to preferably as yolk nucleus, became apparent proximal to the nucleus. This inclusion is further portrayed as an area with the appearance of loosely aggregated particles that begins to emerge around the nucleus during primary growth. This dense material consists mainly of fine granulo-fibrillar components and seems to be a common structure in germinal cells. In his study, it was found that the yolk nucleus appeared as a mass of lipid granules located at first beside the nucleus. As the perinuclear yolk complex becomes spherical, it migrates to the peripheral region of the ooplasm to lied just below the cell membrane. The function of the perinuclear yolk complex may be to assist in assembling the proteins that later form the yolk globules. The PAS positive body is a cellular structure that will organize the yolk. The perinuclear yolk complex disappeared as the oocyte developed and growth continued into the next stage. The PAS positive body dispersed its contents into the ooplasm as late primary growth continues. Once the perinuclear yolk complex has dispersed, filaments similar in appearance to lampbrush chromosomes are observed in the nucleus of oocytes. These structures are an indication that the oocyte is in arrested meiosis (Fig. 12).

In late primary growth, somatic cells and follicular cells are evident. These cells do not disappear during secondary growth and have always been present, but it is at the end of late primary growth and at the beginning of early secondary growth that follicular cells, basal lamina, and somatic cells become more evident. Follicle cells are distinguishable because of their elongated nucleus, and they can be found surrounding the oocytes (Fig. 12). The elongated nucleus of the follicle cells is more apparent in the later stages of development. Somatic cells are found outside the basal lamina and within the germinal compartment (Fig. 13). Somatic cells can become follicle cells, but until they are not found in the germinal compartment and surround the follicle, they are not described as follicle cells but only as somatic cells. Basal lamina is the structure located at the base of all epithelium and acts as a barrier between connective tissue and epithelium. Basal lamina had not been described in past studies of the investigated species of crab. Cortical alveoli are another

morphological characteristic that emerge during late primary growth, and they occur immediately prior to the onset of secondary growth (Fig. 14). At times, formation of cortical alveoli and the formation of yolk may proceed simultaneously in the latest stages of late primary growth. The cortical alveoli have never been described in the investigated species of crab. In this study the staining procedures indicate that these may be cortical alveoli because they are PAS positive, and cortical alveoli contain polysaccharide components and protein components that would give them a strong purple hue. The cortical alveoli are synthesized by the oocyte and become visible in the perimeter of the ooplasm. When cortical alveoli appear, they remain for the duration of development. This is the only morphological feature that was found in late primary growth and throughout secondary growth.

In early maturing phase, during spring season the ovary is easily visible macroscopically, ivory or light yellow in colour, and occupies about one half of the volume of hepatopancreas dorsally. At this stage, the ovary has a lumen and well developed germinal strand with oogonial cells. These oogianal cells are characterized by large nuclei and small amounts of ooplasm. The previtellogenic oocyte diameter ranged 30–60 µm. The ooplasm was intensely basophilic and contained a distinctive perinuclear yolk complex. Prefollicle cells migrating to the lobule periphery, appeared to be in the process of surrounding the oocytes. This stage is characterized by the presence of swollen seminal receptacles in the oviduct of the females. Oocytes with moderate diameter are present in the ovary. Autolyzed oocytes and their debris disappear. Very few number of oogonia can be seen or may be absent at all. The majority of the oocytes differ from the previtellogenic oocyte described in winter season. They gradually undergo two synchronously vital activities. They become follicular and grow gradually in diameter (Fig. 15). Opaque granules are deposited in the form of a ring around its concentric nucleus. The ooplasm is almost completely opaque so that the nucleus becomes inconspicuous under a stereomicroscope. Hence, the translucent stage oocyte can be easily differentiated from the dark opaque stage oocyte (Fig. 16). Histologically, the presence of a few rows of peripheral vacuoles seems to be the most predominant characteristics of vitellogenic oocyte. In addition, more than one nucleolus, which varyin diameter, can be observed in each cell.

Secondary growth of the oocyte (vitellogenesis, deutoplasmogenesis)

During the final stages of late primary growth, there are increasing numbers of lipid droplets in the ooplasm. The oil droplets are identified in tissue that has been infiltrated as vacuoles in the tissue. The lipids that compose these oil droplets dissipate during the infiltration process with ethanol. Oil droplets are also evident during early secondary growth but not mid- or late secondary growth (Fig. 17). Secondary growth, or vitellogenesis, begins with the inclusion of yolk globules in the ooplasm. This initialization of yolk proteins in the ooplasm indicates that the oocyte has undergone late secondary growth. Each stage was based upon the size of oocyte diameter, yolk globules, and variableness of the ooplasm, such as the polymorphism of yolk globules. When secondary growth begins, the ooplasm loses the basophilic characteristics, becoming clearer in appearance and staining less blue/purple and more a lighter pink (Fig. 18). During early secondary growth, the PAS positive body or perinuclear yolk complex that was apparent in primary growth disappears; however, the follicle cells still surround the oocyte. During mid-secondary growth, the yolk globules are more evident in the ooplasm as they grow larger. During late secondary growth, the ooplasm stains a bright pink, and the germinal epithelium is evident. When the yolk globules reach a maximum diameter, the oocyte is in late secondary growth. Vitelline becomes more fluid and lighter in hue. Large yolk globules coalesce, and the ooplasm has a more homogenous consistency (Fig. 19).

In late maturing phase, during spring season the volume of the ovary is subequal to the hepatopancreas in size. Ova are conspicuous when the ovary is viewed macroscopically. The colour of the ovary varies from yellow to yellowish orange. Oogonial cells develop into primary oocytes. The nuclei of these oocytes continue to be with uniformly distributed chromatin. The ovary is the dominant visible organ obscuring the hepatopancreas dorsally with dark orange coloration. The enclosing fibrous connective tissue is highly stretched , often to the point of bursting during dissection. The opacity of ooplasm increases. Histologically, the number of vacuoles gradually increases, and they become dispersed towards the central area. Small granules of the same nature of the ring are scattered randomly in the ooplasm. These granules consist of proteins (peptide linkage) (Fig. 20). Carbohydrates and proteins are absent in this stage of oocyte development. Large granules appear in the ooplasm and increase in number. These granules gain dark colour with the different stains. With Best's Carmine reaction they gain a red hue due to the presence of glycogen (Fig. 16). Other large granules gain a dark blue hue when Aqueuos bromophenol blue is applied (Fig. 15). These granules are basic proteins. Other large-sized granules begin to appear at the periphery of the ooplasm. These granules gain positive reaction with Alcian blue, PAS and Alcian blue- PAS reactions. This means that the components of these granules are acid MPS (see Table 2a,b). From these results it can be concluded that the large-sized granules consist of glycoproteins. The individuality of granules are visible. Yolk bodies (glycoproteins) increase rapidly and replace the central vacuoles and the remaining vacuoles are located around the periphery of oocyte. The vitelline envelope also becomes conspicuous under the follicle cells. Pigmented granules first appear in this stage and are located at the periphery of the oocyte. Mucosubstances are positively intense at the periphery of the oocyte while proteins surround the nucleus. Gradually other fat deposition appeared in the early secondary growth phase and these fat droplets migrated to the periphery of ooplasm. The vitellogenic oocyte has an oval shape, 150 to 210µm. The ratio of the nucleus to the ooplasm 1:4.5 . The ooplasm is intensely granulated . Affinity to stain: with Ehrlich haematoxylin & eosin and the different triple stains available as Heidenhain's iron haematoxylin, the granules in the ooplasm accepted a deep dark blue hue while the nucleus accepted a deep blue hue. Affinity of the ooplasmic components to Sudan black B, Nile blue sulphate and Liebermann Bürchardt stains is little and increased in September-October intensely (Fig. 17). The diameter range of these oocytes is from 130 to 180µm. The ratio of the nucleus to the ooplasm 0.5:2 (see table 2a,b).

Ripe phase, during summer season full-grown oocytes have reached their maximal diameter. Most of the ooplasm retains a bright-pink stain. With the absence of large accumulation of cell organelles, the tissue now has a greater affinity for acid stains, and as a result, eosin stains them pink. Yolk becomes fused and homogenous in full-grown oocytes (Fig. 20). These oocytes are found mostly on the perimeter of the ovarian lobule at first; then when development continues, the whole ovarian lobe will appear with fused yolk oocytes. When full-grown, oocytes retain follicle cells, and yolk platelets are at their maximum diameter of 95 µm. The histochemical study of the oocytes revealed that there are no lipid components present in the vacuoles. Most of the brown and black lipid patches are intermingled among yolk bodies and vacuoles. The distribution of lipid patches at the periphery is more concentrated than that in the inner region of the late stage oocytes. Vacuoles, positively stained with 0.1% alcian blue, are developed in the step late secondary growth oocyte, and they become scattered throughout the ooplasm in the ripe stage oocyte, and then accumulate at the periphery of the oocytes. Mallory trichrome dye stained the vitelline envelope in the ripe stage oocyte and revealed it as a thin layer. The thickness of the vitelline envelope gradually increases in ripe stage oocyte and then reaches its maximal thickness. This indicates that the chorion is formed around the oocyte during the process of vacuolization of the follicle epithelium. In addition, yolk bodies also stained positive with this dye and appeared first in the ripe stage oocyte as bright brown clusters on the cell periphery. The ripe oocyte has an oval shape, 180 to 220µm. The ratio of the nucleus to the ooplasm 1:4.5 . The ooplasm is homogenous without granulation . Affinity to stain: with Ehrlich haematoxylin & eosin and the different triple stains available as Heidenhain's iron haematoxylin, the granules in the ooplasm accepted opaque blue hue while the nucleus accepts deep blue hue. Affinity to stains: with Ehrlich haematoxylin & eosin and the different triple stains available as Heidenhain's iron haematoxylin, the patches in the ooplasm accept light blue hue while the nucleus accepts deep blue hue (see table 2).Therefore three histological overviews can be identified: (1) Individuality of ooplasmic granules is well-differentiated. The follicle cells are well-developed and applied greatly to the outer surface of the oocyte. (2) Individuality of granules disappear. The follicle cells loosen from the outer surface of the oocyte and this surface begins to accept definite colour with different stains. With Mallory triple stain, it gains a dark blue colour, i.e., deposition of chorion surrounding the oocyte. (3) Individuality of granules disappear completely and the components of the ooplasm become homogenous in appearance (Figs. 18 & 19). The follicle cells degenerate gradually and debris of these follicle cells appear around the oocyte i.e., the fully formed oocyte in the ovary is free from follicle cells. Throughout late primary growth, the formation of organelles such as mitochondria, Golgi complexes, and abundant quantities of endoplasmic reticulum, ribosome, and fragmented glycogen may contribute to the blue staining of the ooplasm (Fig. 17). For example, ribosomes are basophilic organelles that have a chemical attraction for basic stains, like hematoxylin stains, which give the cell its unique blue hue, characteristic of late primary growth (Figs. 14 & 17) (see tables 1 & 2a,b). In spent phase, during autumn the ovaries appear flaccid, translucent and greatly reduced. Unspawned ova are visible through outer fibrous connective tissue. The germ strand is well defined, with oogonial and developing oocytes radiating out from this region. The greatest part of the ovary consists of fibrous connective tissue and hemocoel space containing blood cells and phagocytes. Mature non-spawned ova undergoing resorption are often present and surrounded by phagocytes. The ovaries of egg-bearing females showed various phases of maturation and their development was almost completed when the berry was in the late secondary growth and ripe stage. This would suggest that ovarian maturation is a continuous process after attainment of maturity. The Post-vitellogenic oocyte is characterized by a homogenous ooplasm and the yolk material accepts a clear blue colour with the different triple stains applied.

Electron Microscope Observations

The previtellogenic oocyte has a large germinal vesicle and ooplasm which contained ribosomes, mitochondria, Golgi complex, and cisternae of the granular endoplasmic reticulum. The oolemma is smooth and at this stage exhibits no particular morphological specialization (Figs. 21 & 22). The nuclear envelope is interrupted by numerous pores through which nuclear material presumably passes into the perinuclear ooplasm (Fig. 22). In vitellogenic oocytes, the abundance of the endoplasmic reticulum is a particularly striking feature. Profiles of the granular form differ in their shapes and contents (Figs. 23 & 24). All these profiles contain flocculent material. In addition, most of them contain electron-opaque bodies app. 25 µm in diameter, which are similar to the intracisternal granules found in the crayfish oocyte. The reticulum undergoes progressive differentiation during which the flocculent material and intracisternal granules aggregated to form large yolk bodies. The limiting membranes of the reticulum retain some of their attached ribosomes, at least temporarily (Figs. 23-27). Certain elements of the reticulum are devoid of ribosomes and probably represent portions of the agranular reticulum. Continuity of the agranular reticulum with the granular reticulum and Golgi complex is frequently suggested (Figs. 23-27). The Golgi complex consists of a variable number of stacked cisternae. Vesicles containing a finely granular content are found at the "forming face" of the Golgi complex, and possible continuities of such vesicles with the endoplasmic reticulum are often seen (Figs. 23 - 25). Small, coated vesicles, often similar in size and density to the intracisternal granules, appear to be derived from cisternae of the "active face" of the Golgi complex (Figs. 23 and 25). As vitellogenesis proceeds, the oocyte surface becomes irregular with the formation of microvilli and micropinocytotic vesicles. A dense granular material present between the follicular cells and the oocyte collects in the forming micropinocytotic vesicles and can be seen in what appear to be detached vesicles in the cortical ooplasm. The forming and detached vesicles have on their ooplasmic surfaces "fuzzy coats" which disappear shortly after detachment and internalization. In tangential sections, the membranes of these vesicles exhibit a regular polygonal structure (Figs. 28 & 29). Once in the cortical ooplasm, some of the vesicles appear to fuse to produce smooth membranebounded yolk spheres which, at this stage, appear distinct from yolk formed within the endoplasmic reticulum (Fig. 27). The Golgi complex may also contribute substances to this micropinocytotically formed yolk material (Figs. 27-30). The number of the different stages of oocyte development were counted per ovary in the four seasons of the year. This counting has been made in three crabs` ovaries per season. All data were tabulated and have been compared using a 1-way ANOVA ($p < 0.05$), with the oocyte stages of maturity as a fixed factor. Turkey`s Multiple Comparison test has been applied to compare

the ratio of oocyte stages along the year. It is sufficient to take a quick look on the tables (3-7) and the histograms (1-8) to find out the differences and to predict the maturity peak of the ovary.

DISCUSSION

Much of the classical work on oocyte development in crustaceans was summarized by Welson (1934). However, information concerning oogenesis in the mature gonad is scanty. Histological observations of the ovary of the crayfish, Cambarus, did not mention germinal cells (Stephens,1952). Linder (1959) described the derivation of oocytes from the division of oogonia in the Anastraca but did not state whether the process is a continuing one in the adult. Aoto (1952) did describe dividing oogonia in the gonad of the adult hermaphroditic prawn, *Pandalus kessleri*, following the final discharge of spermatozoa. The results reported in the present study throw new light on the activity of germinal epithelium as related to the ovarian development in the cancer crab, *Parthenope longimanus*. The position of the germinal epithelium in the crab ovary varied widely. It may be peripheral as in amphipods and in the isopod Lysmata seticudata, peripheral but confined to a particular region as in *Eupagurus* (*Pagurus*) *bernhardus* and *Clibanatius olivaceous* or in the form of germ nests distributed throughout the ovary as in the land crab *Gecarcinus lateralis* or central as in *Carcinus means*. In Callinectes sapidus it is in the form of a central shaft of germinal tissue . In the early stage of the ovary development , this zone is full of basophilic granular masses possibly representing the dividing oogonial cells. In the periphery of this germinal epithelium, small oocytes with a prominent nucleus are seen budding of into the lumen surrounding the entire length of this germinal cord. It is of interest to note here the observations of (Ryan, 1967) on Portunus sanguinolentis that the ovarian lobule lumen is in the centre and that the germinal epithelium is placed on the border of the lumen . In Portunus pelagicus the primary oocytes proliferated from the central germinal epithelium move across the surrounding lumen into the ovarian lobules where they undergo further growth and maturation. Once the oocytes start growing in the lobules of the ovary, the release of new oocytes from the germinal epithelium ceases suggesting that such a release is cyclical. However, the germinal epithelium never shrink nor become basophilic. This study concluded that the blue crab, *Parthenope longimanus* , inhabiting the Arabian Gulf, spawned minimally during spring and has a spawning peak during summer season, the spawning success decreases dramatically during autumn season and winter season could be considered as interbreeding phase and is a multiplication phase of oogenesis. The cancer crab, *Callinectes sapidus*, spawned at least twice as an adult (Hard, 1942 ; Pyle ;Cronin, 1952 and Barreto, et al. 2006), depositing almost 2 million eggs at each ovulation (Pyle and Croin, 1950). Whether at the 2nd ovulation such a large number of eggs are derived solely from preexisting primary oocytes. Silva, et al. (2012) investigated oogenesis in freshwater crab *Sylviocarcinus pictus* and defined different developmental stages of oocyte,. Gecarcinus reproduces cyclically throughout most of its adult life, depositing thousands of eggs at each ovulation. In these animals, the continued presence of dividing oogouia indicates that, in this species at least, the enormous number of eggs spawned are accounted for in part by the continuation of oogenesis in the mature ovary. The observations that the Feulgen-nueleal reaction is limited to the germinal cells and that glycogen and lipids are not demonstrable in these primordial cells are in complete agreement with those of Linder (1959) for the fairy shrimp, Chirocephalopsis. However, Burgos (1955) was able to demonstrate a positive Feulgen reaction in the nuclei of mature, unfertilized sea urchin eggs, following removal of pigment and lipids by treatment with acetone-HC1 at 600 C. His method has not been used on crustacean oocytes. Although the origin of the germ cells is not established, the ovarian capsule may constitute the germinal epithelium, as described for the crab *Callinectes* (Cronin,1952 and Barreto, et al. 2006). Pillai (1960) reported that in the shrimp, *Cardina laevis*, the germarium first appears as a thickened region of the inner epithelium surrounding the ovary. Some cells of this epithelial layer also appear to undergo nuclear division without any corresponding ooplasmic division and invest the oocytes with a follicular layer. In the ovaries of adult *Gecarcinus* described in this paper, germ nests were distributed throughout the organ and oocytes were present in various stages of development. Ghekiere,, et al. (2004), stated that "the only acceptable histological evidence of neoformation of germ cells in the adult mammalian ovary would be the observation of mitotic division in oogonia..." Such an observation has been made in the invertebrate, Gecarcinus, and provides evidence that oogenesis continues in the ovaries of the adult of this crustacean. In the ovarian lobes of Portunus pelagicus along with the maturing oocytes , a group of young oocytes also present in the centre showing no sign of development. These young oocytes may be late arrivals and it is possible that they have not received the stimulus for further differentiation and maturation. In the present study these cells were defined as somatic cells. Hard (1942) also noticed such immature oocytes among the maturing oocytes of Callinectes sapidus but believed that their presence was due to the new egg formation from the germinal epithelium. That these young oocytes are not utilized in subsequent gametogenesis is shown by the observation that they are resorbed along with the other un- spawned oocytes after ovulation. The implication is that a fresh crop of oocytes formed from the germinal epitheium for the next gametogenesis

Maturation of the ovary and spawning process differ in different species of crustaceans and even differ in the same species according to habitat and environmental conditions. The present study showed that the ovaries of the blue crab *Parthenope longimanus* (Linnaeus, 1764) are occupied with a great number of oogonia and few number of previtellogenic oocytes, postvitellogenic oocytes during winter season , great number previtellogenic oocytes and few number of oogonia during spring season, great number of vitellogenic and ripe oocytes during summer season and great number of postvitellogenic oocytes during autumn season. Among different workers who have studied the maturation of ovaries in branchyuran crabs , there is little consistency as to the number of maturity stages recognized. Haefner (1977) recorded six stages in rock crab *Cancer irroratus* and five stages in *Chaceon quinquedens*. Dhas et al (1980) came across five maturity stages in Portunus pelagicuc ,whereas Sukumaran et al (1986) recognized four maturity stages, excluding the spent stages , in *P. sanguinolentus*. Erdman and Blake (1988) divided the developmental stages in the deep sea golden crab *Chaceon fenneri* into six. The process of deutoplasmogenesis in the crab *Portunus pelagicus* has been studied on histochemical criteria and the ovarian follicles have been classified into two stages namely

previtellogenic and vitellogenic. The vitellogenic oocyte is classified again into three steps with regard to the advancement of deutoplasmogenesis (El-Bawab and El-shereif, 1987). Instances of decapods spawning more than once in a season have been observed in Penaeus japonicus (Nakamura, 1980), P. setiferus (Lindner and Anderson, 1956) and P. duorarum (Caillouet, 1972). The seasonal distribution of M. affinis in different stages of maturity shows that non-spawning prawns dominate the catches from April to September, and that the species breeds from October to March with a peak in December. George (1958) reported the breeding time of this species as October to December in the inshore fishery and as November to February in the offshore fishery of the coast. The species of P. indicus has a prolonged breeding period in Cochin waters, with the greatest breeding activity between October and April. Panikkar and Menon (1956) recorded two breeding periods, in October to November and in May to June. Subrahmanyam (1963) reports that in Madras waters, the highest breeding activity of the species is seen during March and May to September. The seasonal distribution of mature Penaeus stylifera showed that this species breeds throughout the year in the Cochin area, with peaks during December and June to August. Menon (1953) has observed the breeding of Marsopenaeus affinis on the Malabar coast during October to December, while Shaikmahmud and Tembe (1960) recorded the species breeding throughout the year in Bombay waters, with an intensive period in March to May. George (1958) however, observed maximum numbers of mature females of Penaeus. indicus in December. Aktas and Kumlu (1999) concluded that the spatial and temporal distribution of a species is highly variable by month and year. Higher decapod densities are often found in early summer (June to July) between the latitudes of 28° and 29° degrees and extend north and south in August. In later months (September to December) one can find a relative high-density zone in the northern most part of the distribution area. In January and February smaller aggregations are found at lower latitudes of around 28° (Appendices 1 to 4). Menon (1952, 1955) recorded this species as breeding for at least 6 months of the year, both on the Malabar coast and in the Cochin area, while George and Morgan (1979) found it to breed throughout the year. It appears to be usual for the species to show two main peaks of breeding activity in the year, but the timing of these peaks may vary from place to place and from year to year. The spawning pattern of Penaeus semisulcatus in the Persian Gulf, as measured by the variation in percentage of mature females and by post larval occurrence, shows two peaks per year, one in spring and the other in autumn. These peaks vary in relative importance from year to year, although the spring peak is the more important one. Among researchers concerned with marine invertebrate species, a controversy exists on the site of vitellogenesis. Vitellogenesis in the acidians *Styela plicata* (Lesuaer,1823), *Styela partita* (Stimpson, 1852) and *Ciona intestinalis* (Linnaeus, 1767) is homosynthetic in nature (Saad, 2008). Moreover, different species of ascidians inhabit the Mediterranean Sea have different reproductive cycles. *Styela plicata* and *Styela partita* have two reproductive peaks throughout the year. One peak is shown during September to October and the other peak was observed during January till February while *Ciona intestinalis* is mature all the year round (Saad, 2002 ; Saad and Hamed, 2009). The present study concluded that vitellogenesis in the cancer crab *Parthenope longimanus* (Linnaeus, 1764) is homo- and heterosynthetic in nature. Mitochondria, Golgi complex, RER, the nucleus and the follicle cells of the vitellogenic oocyte are responsible for synthesis of proteins, carbohydrates and lipid of yolk. The main protein moiety of the yolk or vitellin is synthesized in hepatopancreas and transported to the oocyte through haemolymph and the process of active endocytosis of the vitellogenic oocyte. Advance in biomolecular analysis has led to a determination of Vitellogenin (Vg) mRNA expression in crustaceans. In marine species, exogenous Vg synthesis in the hepatopancreas of *Penaeus monodon* has been proposed (Tseng et al. 2001), of *Metapenaeus ensis* (Kung et al. 2004), of *Pandalus hypsinotus* (Tsutsui et al. 2004), while endogenous ovarian Vg synthesis has been found in the fiddler crabs *Uca pugilator* (Eastman-Reks & Fingerman, 1985); *Uca rapax* (Silveira & Silveira, 2007); in penaeid shrimps (Yano and Chinzei, 1987; Rankin et al. 1989 ; Rankin and Davis,1990), and in *Callinectes sapidus* (Lee and Watson, 1994). In other penaeid shrimps species the mRNA encoding Vg has been found in both the ovary and the hepatopancreas tissues (Averre et al. 2001 ; Tsang et al. 2003 ; Tsutsui et al. 2004 ; Kung, et al. 2004). In freshwater species the hepatopancreas seems to be the site of synthesis of Vg in Macrobrachium resenbergii (Chen and Chin, 1994; Lee and Watson, 1994; Soroka et al. 2000 ; Jasmani et al. 2000) and in freshwater crayfish Cherax quadricarinatus (Abdu et al. 2002). In this way, the objective of the present study was to determine the site(s) of mRNA expression of Vg in the freshwater crayfish at different stages of female maturation based on RT-PCR analyses. The crustacean hepatopancreas is the primary site for lipid digestion and metabolism (Chang and O'Connor, 1983). Since the lipid is carried in the hemolymph on carrier proteins, the role of yolk proteins as a lipid shuttle to the developing eggs should be further characterized (Quackenbush, 1989a,b; Sobha, et al. 2007). In addition the large number of species studies may have contributed to the variable results reported as some aspects of ovary development appear to be specific (Chen and Chen 1993, Quinitio et al .,1990, Rankin et al. 1989). For P. monodon, vitellin has been isolated in the ovaries (Quinitio et al. 1990, Tom, et al. 1987a, b) and eggs (Chen and Chen 1993) vitellogenin was identified in the haemolymph (Jasmani, et al. 2000) and in the hepatopancreas (Quinitio et al. 1990) and more recently, it has been quantified in the haemolymph (Jasmani, et al. 2000). It is often assumed, vitellogenin in the haemolymph is being transported to the ovary from exogenous sources such as the hepatopancreas (Charniaux-Cotton, 1985). Vitellogenin levels in the haemolymph have therefore been used as an indicator of when this exogenous yolk precursor is being synthesised. Recent evidence from examining the expression pattern of the vitellogenin genes during the reproduction cycle, confirmed that both the ovary and hepatopancreas play an important role in the synthesis of the yolk precursors for P. mondon (Fainzilber, et al. 1992 ; Lubzens, et al. 1997 ; Tseng et al. 2001). However, the total and relative contribution from each tissue has proven difficult to determine. Result from two studies carried out on P. monodon failed to agree on the pattern of changes in vitellogenin concentration with ovary development study by longyant et al. (2003) showed a drop in haemolymph levels when the ovary reached maturity . this drop was not shown in the study of Vincent et al. (2001). In addition, these two studies showed significant differences in the quantities detected at the various ovary development stages for P. monodon despite both studies using the same techniques (ELISA) to quantify the vitellogenin. Vincent et al. (2001) found vitellogenin in the haemolymph of what they termed '0' stage of ovary development, while Longyant et al. (2003) showed it be thus despites SOP transcripts being found at all ovary stages final synthesis was limited to the later stage. Avarre et al. (2001) found that Sinus Gland Extracts (SGE) and CHH family

peptides inhibited this final synthesis of the SOP. Interestingly, vitellin production by the ovary in P. semisulcatus , decreased significantly when cortical rods appeared (Jasmine, et al. 2000) suggesting their oocytes changes from production vitellin proteins to CR protein. Thus CHH peptides affect the production of both vitellin and CR proteins however the process is not fully understood. The appear to regulate through the inhibition of vitellogenin synthesis in the hepatopancreas (and possibly other sites) and vitellin and CR protein synthesis in the ovary. It has been proposed that GIH prevents the uptake of exogenous vitellogenin precursors. However Avarre et al. (2001) proposes that GIH or eyestalk extract have the potential to affect all stages of ovary development in penaeid prawns.

The ultrastructural studies have shown that some degree of oocytic activity is necessary in the assembly and molding of these yolk materials derived from extraoocytic sites. Although the hepatopancreas has been implicated as the source of extraoocytic yolk materials in the blue crab, *Parthenope longimanus* (Linnaeus, 1764), vitellogenesis in the crustaceans appears to involve a greater degree of intraoocytic synthesis. In the crayfish (Beams and Kessel, 1963), yolk is produced within the highly differentiated system of the endoplasmic reticulum with its complex of interconnected cisternal and tubular elements. The intracisternal granules formed within the stacks of the cisternae of the rough endoplasmic reticulum are transported through the agranular reticulum to different regions of the oocyte where they aggregate to form the definitive yolk. Those authors feel that neither the Golgi complex nor micropinocytosis assumes a significant role in vitellogenesis in the crayfish oocyte. By contrast, vitellogenesis in Libinia appears to involve both intra- and extraoocytic sources of yolk materials. The endoplasmic reticulum is not as highly oriented in its spatial arrangement as is that of the crayfish oocyte. However, the granular reticulum appears active in the synthesis of intracisternal granules which aggregate to produce yolk bodies. The Golgi complex and agranular reticulum appear also to be involved in vitellogenesis, although their precise roles have not been determined with certainty from these ultrastructural observations. They may contribute substances which complex with the materials formed in the granular reticulum. In Libinia, unlike the crayfish, micropinocytosis appears to play an important role in the incorporation of yolk materials. The origin of these yolk materials is not known. Yolk formation in Libinia resembles that in the horseshoe crab, *Limulus polyphemus*, in which yolk is initially produced by the endoplasmic reticulum and Golgi complex and later is produced by extraoocytic sources and is incorporated into the oocyte by micropinocytosis (Dumont and Anderson, 1967).

(Table 1): Morphological nature of the ooplasm and the nucleus of the oocyte of the cancer crab *Parthenope longimanus* at its different stages of development.

Stages of oocyte development / Stain / Referenes	Oogonia		Previtellogenic oocyte		Vitellogenic oocyte				Post-vitellogenic oocyte	
	Cyto-plasm	Nucleus	Cyto-plasm	Nucleus	Step 1 Small granules	Step 2 Large granules	Step 3 patches	Nucleus	Yolk material (homog-enous)	Nucleus
Ehrlich haematoxylin & eosin Pearse 1968	red	blue	blue	deep blue	Deep dark blue	opaque blue	light blue	deep blue	blue	deep blue
Heidenhain's iron haematoxylin Pearse 1968	red	blue	red	deep blue	deep dark blue	opaque blue	light blue	deep blue	blue	deep blue
Haemalum & eosin Pearse 1968	red	deep blue	dark red	deep blue	deep dark blue	opaque blue	light blue	deep blue	red	deep blue
Mallory triple stain Pearse 1968	red	deep blue	dark red	deep blue	deep dark blue	opaque blue	light blue	deep blue	red	deep blue
Masson trichrome stain Pearse 1968	red	deep blue	dark red	deep blue	deep dark blue	opaque blue	light blue	deep blue	red	deep blue

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Weigert's haematoxylin & Van Gieson stain Pearse 1968	red	deep blue	dark red	deep blue	deep dark blue	opaque blue	light blue	deep blue	red	deep blue
Alcian blue & eosin Pearse 1968	red	blue	red	dark red	deep dark blue	opaque blue	light blue	deep red	red	deep blue

(Table:2a): Showing the different histochemical stains tried and their indications.

Histochemical stain	Its indication
Mercury bromphenol blue. Mazia, *et al.* 1963	Protein
Biuret reaction. Gabe, 1976	Peptide linkage
Aqueos bromphenol blue. Mazia, *et al.* 1963	Acidic Protein
Toluidine Blue. Pearse, 1968	Basic protein
Xanthoproteic reaction. Pearse, 1968	Phenyl group
Millon's reaction (Baker modification). Pearse, 1968	Tyrosine
Ninhydrin Schiff Reaction (Yasuma & Itchikawa). Gabe, 1976	NH2 group
Oxidised Tannin Azo method (Dixon). Pearse, 1968	Amino group
Sakaguchi reaction. Pearse, 1968	Arginine
PAS. Pearse, 1968	Muco- substance
Alcian blue. Pearse, 1968	Acid mucopolys acch arides
Alcian blue and PAS. Pearse, 1968	Acidic & Neutral MPS
Best's Carmine. Pearse, 1968	Glycogen
Sudan black B. Pearse, 1968	Lipids
Nile blue sulphate. Cain,1947	Neutral & acidic lipid
Liebermann Bürchardt. Pearse, 1968	Cholesterols and its esters

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(Table 2b): The chemical nature of the different components forming the yolk in the oocyte of the cancer crab *Parthenope longimanus* during deutoplasmogenesis.

Stages of oocyte development Histoch-emical reaction & References	Indication	Previtellogenic (young) oocyte Cell size 100 µm Nucleus size35 µm	Vitellogenic oocyte Cell size 120 -180 µm Nucleus size75 µm			Post-vitellogenic oocyte Cell size 200 µm Nucleus size80 µm
		Cytoplasm	Cytoplasm			Yolk material (homogenous)
			Step 1 Small granules	Step 2 Large granules	Step 3 patches	
Mercury bromph - enol blue Mazia *et al.* 1963	Protein	bb	bbbb	bbbbb	bbbbb	bbbbbbb
Biuret reaction Gabe 1976	Peptide linkage	VV	VVVV	VVVVV	VVVVVV	VVVVVVV
Aqueos bromph-enol blue Mazia *et al.* 1963	Acidic Protein	bb	bbb	bbbbb	bbbbbb	bbbbbbbb
Toluidine Blue Pearse 1968	Basic protein	bb	bbbb	bbbbb	bbbbbbb	bbbbbbbbbb
Xanthoproteic reaction Pearse 1968	Phenyl group	OO	OO	OO	OOOOO	OOOOOOO
Millon's reaction (Baker modification) Pearse 1968	Tyrosine	PP	PPP	PPPP	PPPPP	PPPPPPP
Ninhydrin Schiff Reaction (Yasuma & Itchikawa) Gabe 1976	NH2 group	PP	PP	PPPP	PPPPPP	PPPPPPPP
Oxidised Tannin Azo method (Dixon) Pearse 1968	Amino group	PP	PP	PPPP	PPPPPP	PPPPPPPP

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Sakaguchi reaction Pearse 1968	Arginine	OO	OO	OOO	OOOOO	OOOOOOO
PAS Pearse 1968	Muco-substance	RR	RRR	RRRR	RRRRRR	RRRRRRRRR
Alcian blue Pearse 1968	Acid mucopolys acch arides	RR	RRR	RRR	RRRRRR	RRRRRRRRR
Alcian blue & PAS Pearse 1968	Acidic & Neutral MPS	B RR	BB RR	RRRR	RRRRRR	RRRRRRRRR
Best's Carmine Pearse 1968	Glycogen	PP	PPP	PPPPP	PPPPPPPP	PPPPPPPPPP
Sudan black B Pearse 1968	Lipids	-	BbBbBb	BbBbBb	BbBbBbBb	BbBbBbBbBbBbBb
Nile blue sulphate Cain,1947	Neutral & acidic lipid	-	bb	bbbb	bbbbbbb	bbbbbbbb
Liebermann Bürchardt Pearse 1968	Cholestero-ls & its esters	-	-	-	-	-

Bb- brownish black O- orange V- violet

b- blue P- pink R- red

The repetition of each symbol indicates the intensity of this colouration

(Table 3): Total sum of oocytes in its different developmental stages during the four seasons of the year. Note, 3 ovaries have been counted per each season.

Oocyte stage	During autumn				During winter				During spring				During summer			
	Ovary 1	Ovary 2	Ovary3	Mean	Ovary 1	Ovary 2	Ovary3	Mean	Ovary 1	Ovary 2	Ovary3	Mean	Ovary1	Ovary2	Ovary3	Mean
Oogonia	200	170	145	515	80	76	58	214	40	49	38	127	24	16	28	68
Previtellogenic oocytes	115	140	123	378	70	64	58	192	22	34	29	85	12	8	16	36
vitellogenic oocytes	1	1	2	4	7	10	3	20	65	50	74	189	184	140	170	494
posvitellogenic oocytes	55	48	60	163	22	15	34	71	1	2	1	4	1	1	1	3

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Turkey`s comparison tests of oocyte developmental stages along the year.

(Table 4) One-way analysis of variance and Tukey's Multiple Comparison Test for the number of oogonia present in the ovary in the four seasons of the year.

Table Analyzed					
Data Table-1					
One-way analysis of variance (ANOVA)					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	55,39				
R squared	0,9541				
ANOVA Table	SS	df	MS		
Treatment (between columns)	39570	3	13190		
Residual (within columns)	1905	8	238,1		
Total	41470	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff	
during autumn vs during winter	103,3	11,6	P < 0.001	62.99 to 143.7	
during autumn vs during spring	129,3	14,52	P < 0.001	88.99 to 169.7	
during autumn vs during summer	149	16,73	P < 0.001	108.7 to 189.3	
during winter vs during spring	26	2,919	P > 0.05	-14.35 to 66.35	
during winter vs during summer	45,67	5,126	P < 0.05	5.320 to 86.01	
during spring vs during summer	19,67	2,208	P > 0.05	-20.68 to 60.01	

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(Table 5) One-way analysis of variance and Tukey's Multiple Comparison Test for the number of previtellogenic oocytes present in the ovary in the four seasons of the year.

Table Analyzed					
Data Table-2					
One-way analysis of variance (ANOVA)					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	121,8				
R squared	0,9786				
ANOVA Table	SS	df	MS		
Treatment (between columns)	22970	3	7655		
Residual (within columns)	502,7	8	62,83		
Total	23470	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff	
during autumn vs during winter	62	13,55	P < 0.001	41.27 to 82.73	
during autumn vs during spring	97,67	21,34	P < 0.001	76.94 to 118.4	
during autumn vs during summer	114	24,91	P < 0.001	93.27 to 134.7	
during winter vs during spring	35,67	7,793	P < 0.01	14.94 to 56.39	
during winter vs during summer	52	11,36	P < 0.001	31.27 to 72.73	
during spring vs during summer	16,33	3,569	P > 0.05	-4.394 to 37.06	

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(Table 6) One-way analysis of variance and Tukey's Multiple Comparison Test for the number of vitellogenic oocytes present in the ovary in the four seasons of the year.

Table Analyzed					
Data Table-3					
One-way analysis of variance (ANOVA)					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	103,7				
R squared	0,9749				
ANOVA Table	SS	df	MS		
Treatment (between columns)	51740	3	17250		
Residual (within columns)	1330	8	166,3		
Total	53070	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff	
during autumn vs during winter	-5,333	0,7164	P > 0.05	-39.05 to 28.38	
during autumn vs during spring	-61,67	8,284	P < 0.01	-95.38 to -27.95	
during autumn vs during summer	-163,3	21,94	P < 0.001	-197.0 to -129.6	
during winter vs during spring	-56,33	7,567	P < 0.01	-90.05 to -22.62	
during winter vs during summer	-158	21,22	P < 0.001	-191.7 to -124.3	
during spring vs during summer	-101,7	13,66	P < 0.001	-135.4 to -67.95	

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(Table 7) One-way analysis of variance and Tukey's Multiple Comparison Test for the number of post-vitellogenic oocytes present in the ovary in the four seasons of the year.

Table Analyzed					
Data Table-4					
One-way analysis of variance (ANOVA)					
P value	P<0.0001				
P value summary	***				
Are means signif. different? (P < 0.05)	Yes				
Number of groups	4				
F	58,97				
R squared	0,9567				
ANOVA Table	SS	df	MS		
Treatment (between columns)	5705	3	1902		
Residual (within columns)	258	8	32,25		
Total	5963	11			
Tukey's Multiple Comparison Test	Mean Diff.	q	P value	95% CI of diff	
during autumn vs during winter	30,67	9,353	P < 0.001	15.82 to 45.52	
during autumn vs during spring	53	16,16	P < 0.001	38.15 to 67.85	
during autumn vs during summer	53,33	16,27	P < 0.001	38.48 to 68.18	
during winter vs during spring	22,33	6,812	P < 0.01	7.484 to 37.18	
during winter vs during summer	22,67	6,913	P < 0.01	7.817 to 37.52	
during spring vs during summer	0,3333	0,1017	P > 0.05	-14.52 to 15.18	

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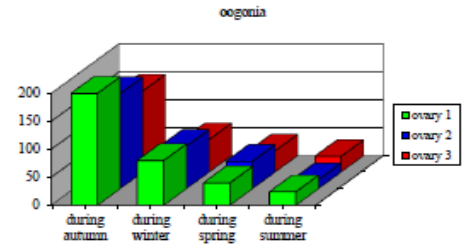
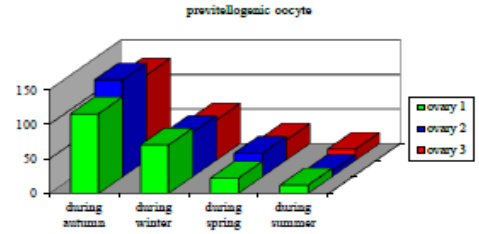
Histograms (1- 4) showing the fluctuations in the number of oocyte developmental stages during the four seasons of the year.

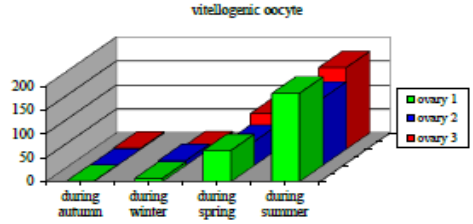
Histogram 1



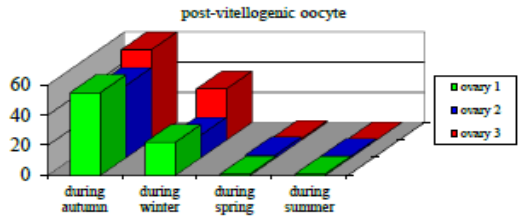
Histogram 2



Histogram 3

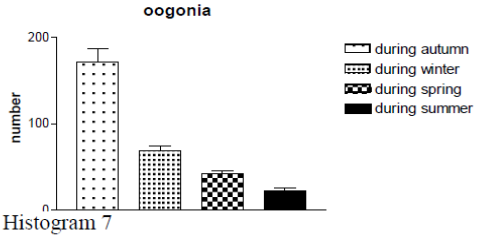


Histogram 4

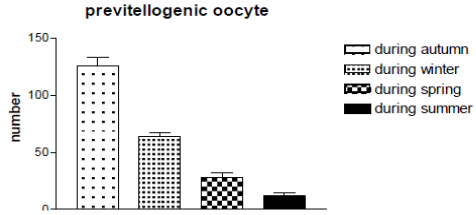


Histograms (5- 8) showing the mean of fluctuations of the number of oocyte developmental stages in the four seasons of the year.

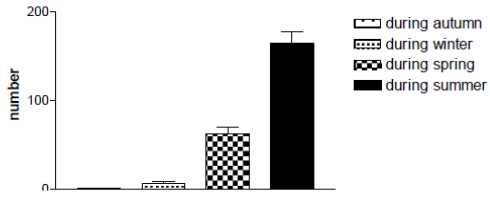
Histogram 5



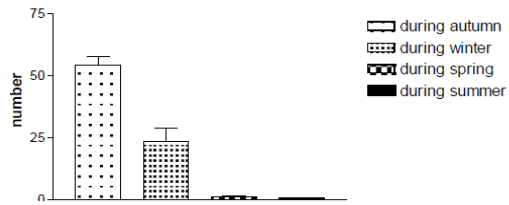
Histogram 6



Histogram 7



Histogram 8



LEGENDS

Fig. 1

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing ovarian lobules, germinal epithelium and primary growth oocytes. The specimen was collected in September. Periodic Acid Schiff.

Fig. 2

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing an enlarged ovarian lobule with secondary growth oocyte. The specimen was taken during October. Periodic Acid Schiff.

Fig. 3

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing early primary growth oocytes in the centre. Collection: any time during June to September. Aqueous Bromphenol blue.

Fig. 4

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing follicle cells nuclei surrounding the follicles. Collection: any time during June to September. Masson trichrome stain.

Fig. 5

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing late secondary growth oocytes on periphery of ovarian lobule.Strong positive reactions to tests for glycogen. Collection: any time during March-June. Weigert's haematoxylin & Van Gieson stain.

Fig. 6

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing fusion and collapsing of ooplasm among oocytes. Note: the ovary is yellow and indistinct, gut and muscles are visible. The specimen was obtained during October to December. Heidenhain's iron haematoxylin.

Fig. 7

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing autolyzed oocytes. Weigert's haematoxylin & Van Gieson stain.

Fig. 8

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing primary growth oocyte surrounded. Note, the germinal epithelia provided new cells for continual development of oocytes as cells mature when they reach the periphery. Collection: January. Alcian blue.

Fig. 9

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing vitellogenic oocytes. Note, the ooplasm changed from the clear, scant appearance that it had during oogonia to a blue hue and oil droplets appeared in the ooplasm. Collection: June. Alcian blue.

Fig. 10

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing defined germinal epitheium in late secondary growth ovarian lobule. Note, the germinal epithelium with the oogonia and the early primary growth oocytes are obvious. Collection: July -August. Masson trichrome stain.

Fig. 11

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing Perinuclear yolk complex (yolk nucleus) as seen in primary growth. Collection: July -August. Toluidine blue.

Fig. 12

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing Lampbrush chromosomes. Collection: August. Liebermann Bürchardt stain.

Fig. 13

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing basal lamina, somatic cells and follicle cells. The specimen was obtained during September-October. Toluidine blue.

Fig. 14

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing Mature egg, yolk globules are similar to those in full-grown oocytes. The specimen was obtained during October. Alcoholic Aqueuos bromophenol blue.

Fig. 15

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing late secondary growth oocyte with complete follicle. large yolk globules, germinal epitheium and somatic cells. The specimen was obtained during September-October. Weigert's haematoxylin & Van Gieson stain.

Fig. 16

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing vitellogenic oocyte. The specimen was obtained during September-October. Alcian blue-PAS.

Fig. 17

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing vitellogenic oocyte, lipids and oil droplets are distributed in the ooplasm. Sudan black B or Nile blue sulphate. The specimen was obtained during September-October.

Fig. 18

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing full grown oocytes with homogenous ooplasm. Note: the ovary yellowish white hue and translucent. The specimen was obtained during January till April.

Weigert's haematoxylin & Van Gieson stain.

Fig. 19

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing late secondary growth. Vitelline becomes more fluid and lighter in hue. Large yolk globules coalesce, and the ooplasm has a more homogenous consistency. The specimen was obtained during September-October. Periodic Acid Schiff.

Fig. 20

Photomicrograph of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing full-grown oocytes. The specimen was obtained during September-October. Toluidine blue.

Fig. 21

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing previtellogenic oocyte are present in the centre, larger previtcllogenic oocytes are located distal to the centre. The vitellogenic oocytes are peripherally. Nuclei of follicular cells may be seen in spaces between oocytes. The specimen was obtained during July.

Fig. 22

TEM of a transverse section of an ovary of the cancer crab *Parthenope longiman*us showing a portion of a previtellogenic oocyte and its encompassing follicular cells from the ovary of an immature female. The ooplasm contains ribosomles, mitochondria , Golgi complex, and cisternae of agranular endoplasmic reticulum. The specimen was obtained during August till September.

Fig. 23

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing a portion of vitellogenic oocyte with a cisterna of the granular reticulum containing, flocculent material, intracisternal granules and a nearly mature yolk sphere. Ribosomes are present on the membrane bounding the yolk sphere. The forming face of the Golgi complex is closely associated with other elements of the reticulum. The specimen was obtained during August till September.

Fig. 24

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing a portion of vitellogenic oocyte with cisternae of the reticulunm containing intracisternal granules and aggregating yolk material appear to be continuous, via vesiculation, with the Golgi complex. Microtubules are found in both oocyte and follicular cells. Micropinocytotic vesicles are forming at egg surface. The specimen was obtained during September.

Fig. 25

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing a portion of vitellogenic oocyte with stages in the aggregation of intracisternal granules and homogeneous material within the endoplasmic reticulum of the subcortical region of vitellogenic ooplasm. Vesicles containing dense mlaterial may be derived from the active faces of Golgi complex. The specimen was obtained during October.

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Fig. 26

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing a portion of vitellogenic oocyte with two attached micropinocytotic vesicles which are coated on their ooplasmic surfaces. Two internalized vesicles are devoid of such coats. The specimen was obtained during August till September.

Fig. 27

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing a portion of vitellogenic oocyte with follicular epithelium separated by dense material which is apparently incorporated into micropinocytotic vesicles. The vesicles may be derived from either micropinocytosis or from the Golgi complex. Agranular reticulum granular reticulum containing dense yolk material. The specimen was obtained during August till September.

Fig. 28

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing a portion of vitellogenic oocyte with vacuolated ooplasm. The specimen was obtained during October.

Fig. 29

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing advanced stage of oocyte development. The specimen was obtained during September.

Fig. 30

TEM of a transverse section of an ovary of the cancer crab *Parthenope longimanus* showing homogenecity of the ooplasm in the ripe phase. The specimen was obtained during September.

ABBREVIATIONS

AO	Autolyzed Oocyte
AR	Agranular Reticulum
BL	Basal Lamina
CA	Cortical Alveoli
CT	Fibrous Connective Tissue
EY	Electron-Dense Yolk Globules
FM	Flocculent Material
FN	Follicle Cell Nucleus
FE	Follicular Epithelium
GE	Germinal Epithelium
GC	Golgi Complex
GR	Granular Reticulum
H	Hemolymph
IG	Intracisternal Granule
LC	Lampbrush Chromosome
LD	Lipid Droplets
MO	Mature Ovum
MV	Micropinocytotic Vesicle
M	Microtubules
MT	Mitochondria
NE	Nucleolus
N	Nucleus
OD	Oil Droplet
O	Oogonia
OP	Ooplasm

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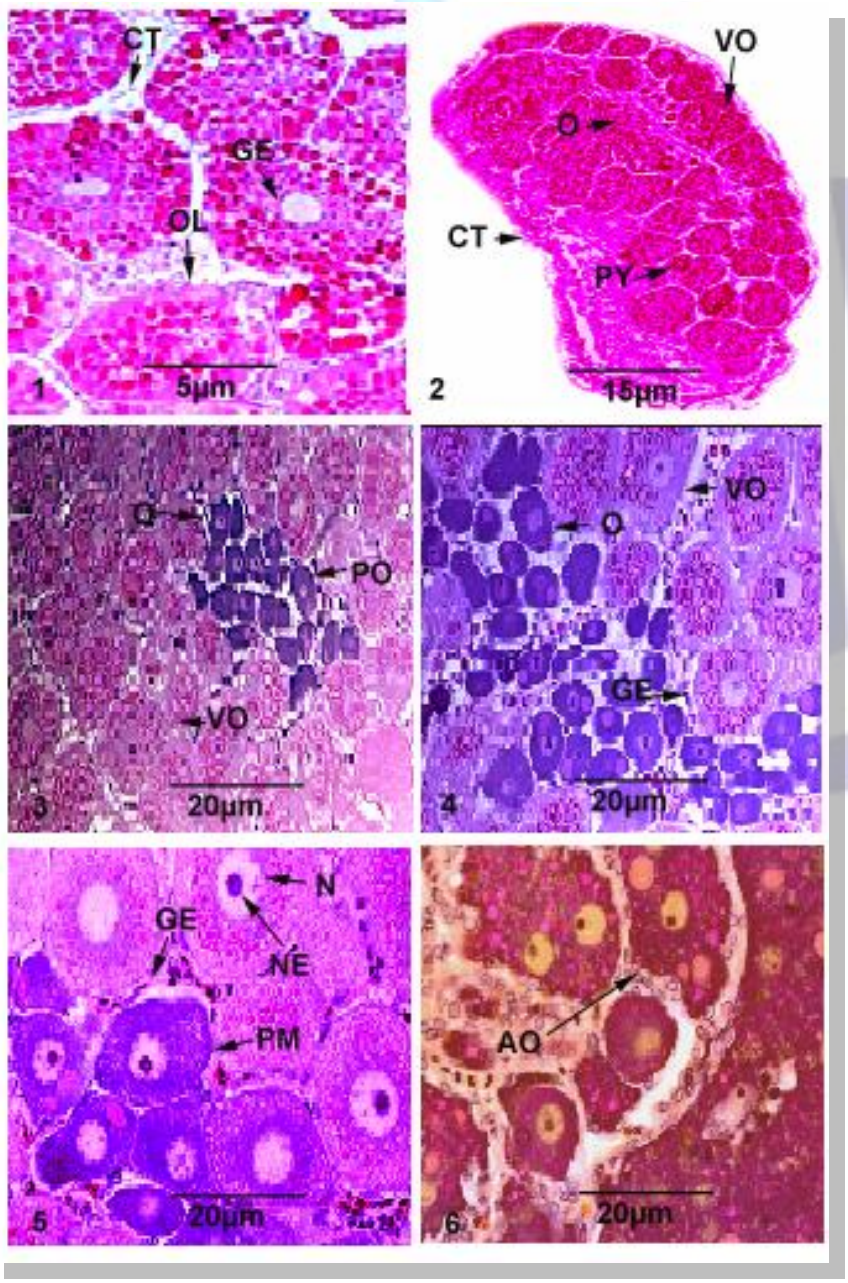
OL	Ovarian Lobule
PC	Perinuclear Yolk Complex
PM	Plasma Membrane
PY	Previtellogenic Oocyte
PG	Primary Growth Oocyte
R	Ribosomles
SO	Secondary Growth Oocyte
SC	Somatic Cell
V	Vacuole
VO	Vitellogenic Oocyte
YG	Yolk Globules



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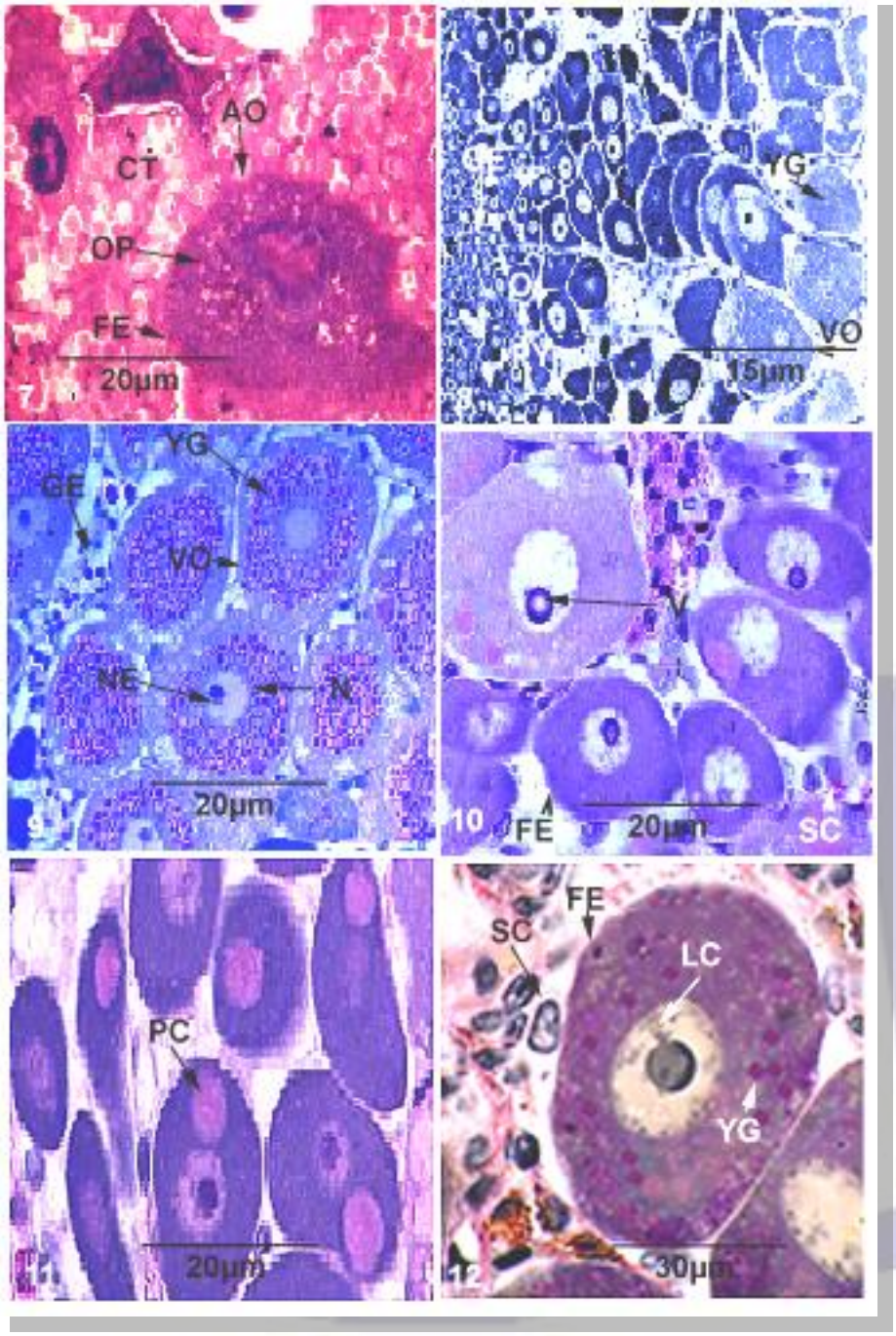

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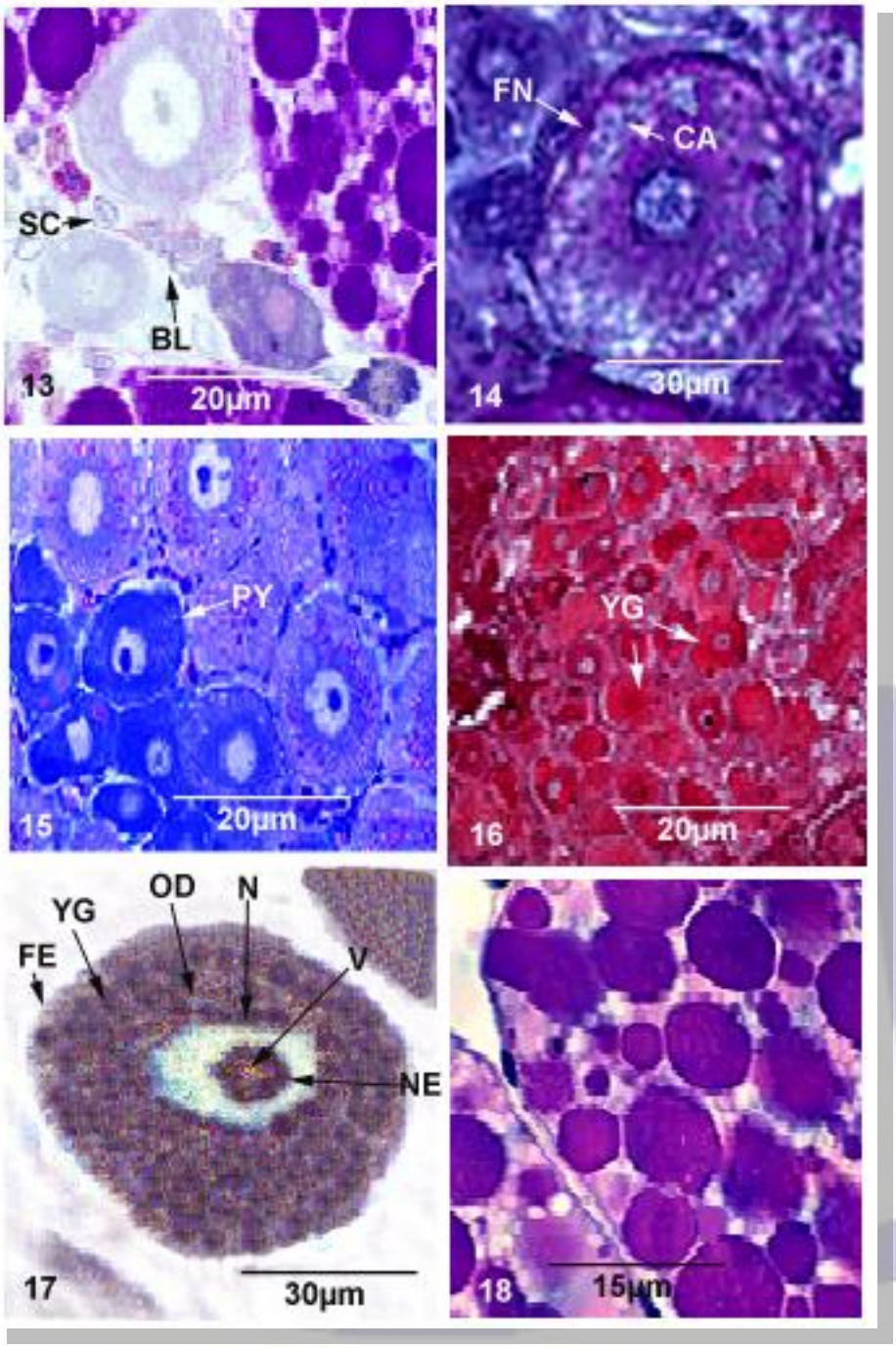

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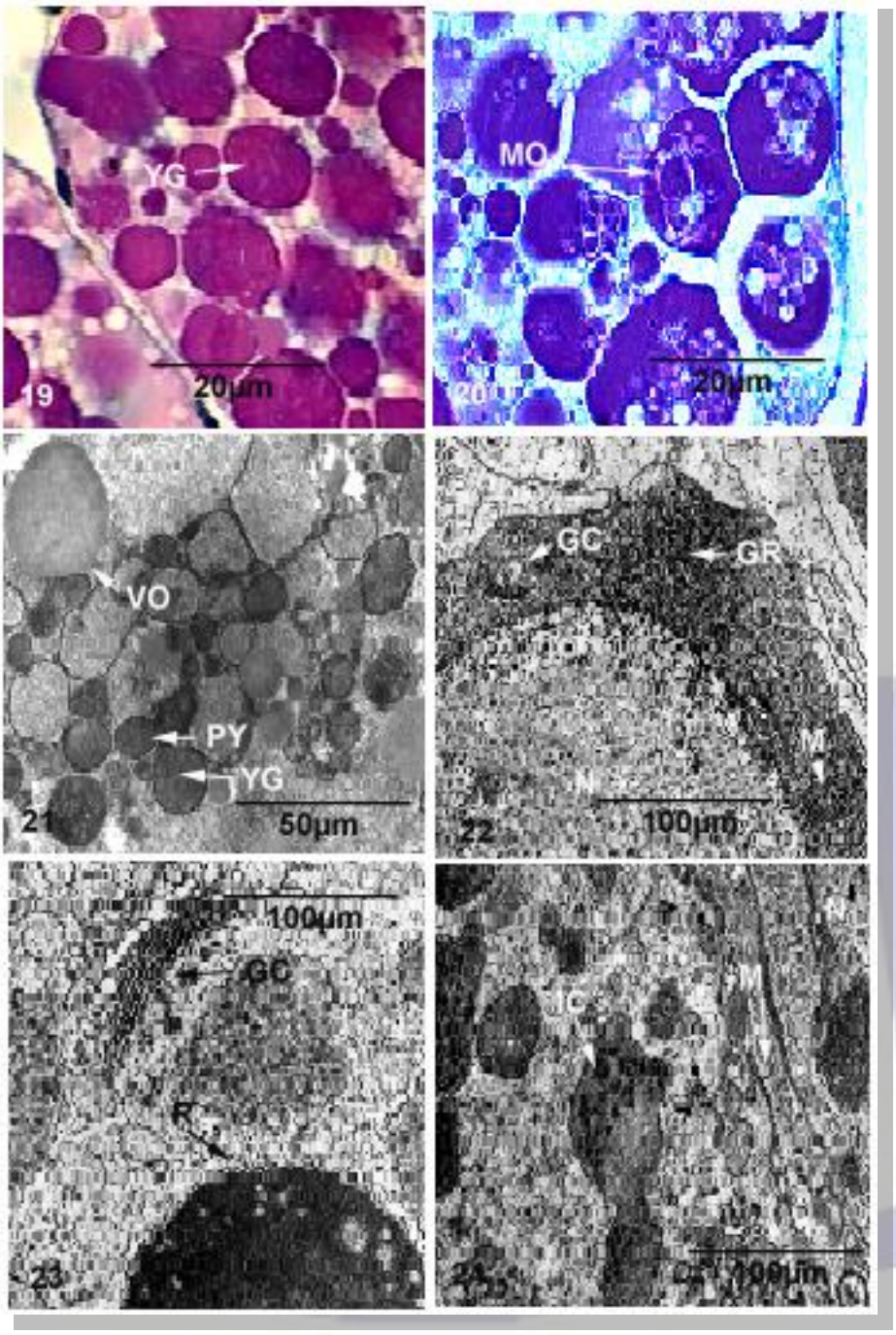

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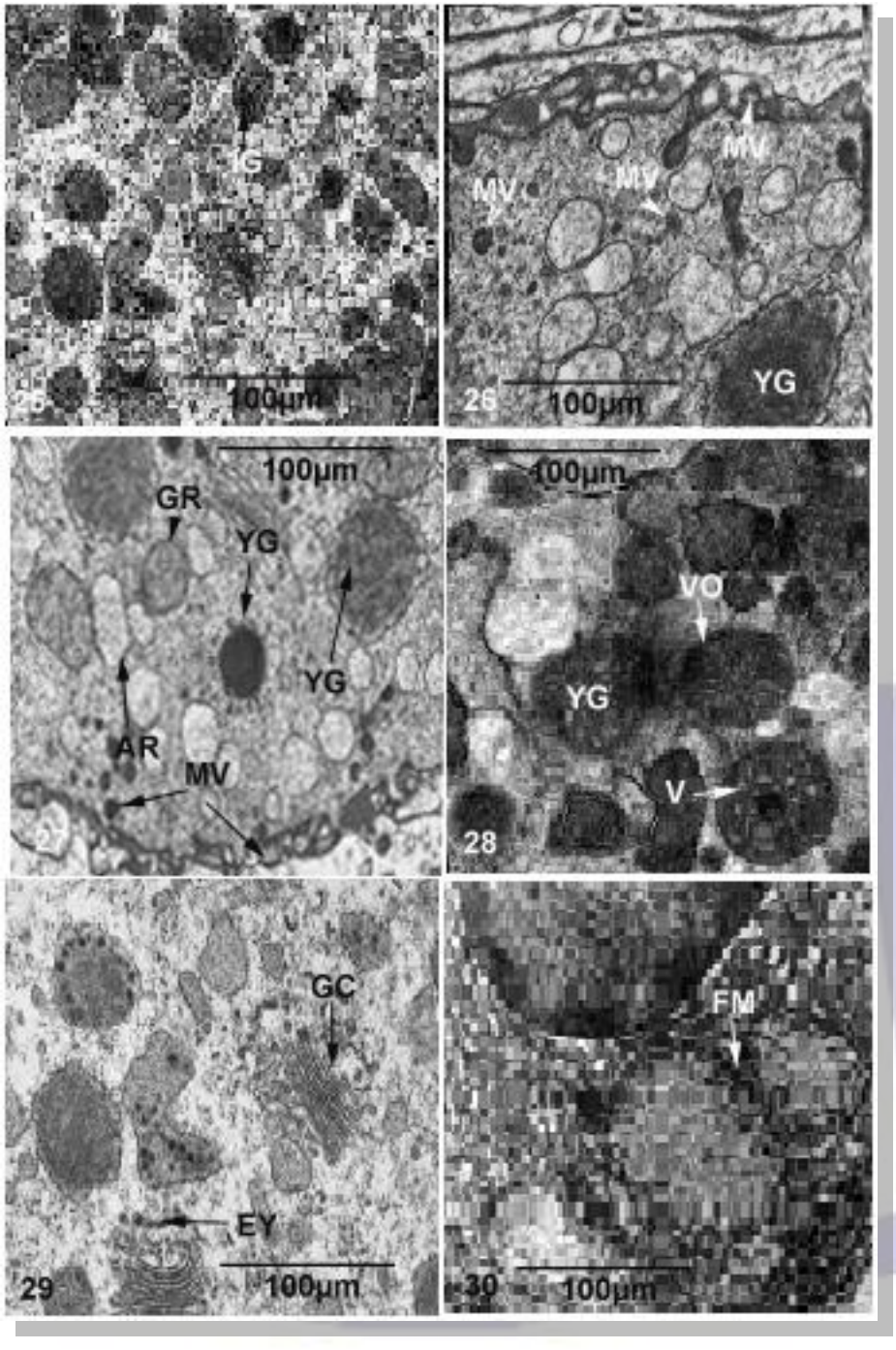

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