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Ovarian development of freshwater crab *Paratelphusa lamellifrons* (Alcock) (Potamidae: Decapoda)

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Abstract: The gonad of freshwater crabs was located in the cephalothorax above the hepatopancreas. The female reproductive system consists of a pair of ovaries, oviducts and spermathecae. The study of the ovarian development was to describe the structure of the ovary and oogenesis in the *Paratelphusa lamellifrons* and the morphological changes in the female reproductive developmental stages overtime. Qualitative analyses of female gonads were made by describing the structure of the oocytes and determining the developmental stages of the oocytes from oogonia to full grown oocytes. The gonadal stages were observed macroscopically by volume and colour were validated through histological analysis and proved to be useful method for the rapid identification of sexual maturity in the species. Four gonadal developmental stages were found for females such as oogonia, previtellogenic oocytes, vitellogenic oocytes and full grown oocytes. Stages of maturity were described as immature, early mature, late mature and ripe.

Key words: *Paratelphusa,* Ovary, Oogonia, Spermathecae*,* Oocytes

Introduction

Decapod crustacean gonads are partly or completely connected paired and typically elongated organs located in the dorsal part of the body (Brusca & Brusca, 1990). The reproductive system of the female consists of a pair of ovaries, a pair of oviducts and, in some groups, a pair of spermathecae (Krol *et al*. 1992). The entire ovary is bounded by fibrous connective tissue which is bounded organ from the surrounding hemocoel. The ovary is 'H' shaped and located just beneath the carapace.

The horns of the ovary extend antero-laterally from either side of the gastric mill and lie dorsally to the hepatopancreas. Two posterior horns, which lie ventral to the heart, extend posteriorly on either side of the intestine. The male reproductive system consists of testis, Vas deferences, Spermatophore and male gonopods (Sarker *et al*., 2011).

Following the pubertal-molt mating, the female enters a prolonged period without ecdysis known as anecdysis and normally will not molt or mate again. Sperm are stored in the seminal receptacles and used during both ovulations that normally occur in this species (Steele & Bert, 1994). Eggs are laid and held together by adhesion to the setae of the endopodites of the abdominal segments and the maturing egg mass (Johnson, 1980).

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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 zone 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 (Brown, 2009).

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 (Brown, 2009). 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. The colour of

ovaries changes transparent to yellow to deep orange with the progression of maturation of ovary for the *P. lamellifrons.* However, it could be considered that the ovarian colour was not strictly to body size. Therefore, colour changes with ovaries maturation and ovarian development stages for the specific-species is necessary. The present study examined ovarian development and describes the stages based on the external and histological characteristics of ovary of *P. lamellifrons.*

Materials and Methods

Collection of specimen

A total of 25 crabs were collected from the Ponds of the adjacent area of Rajshahi city in Rajshahi district from April 2012 to December 2012 for laboratory rearing. Crabs were collected by the help of cast net (Khepla jal), Khulson and by using hand. After collection the crabs were kept in banana bark to transport them from pond to the laboratory.

Crab identification and histology of the ovary

In the laboratory, the specimens were placed on tray and were washed with clean water. Common experience were used in identifying males and females. In case of male crab, it has a long, narrow and inverted 'T' shaped abdomen whether females has an inverted 'U' shaped abdomen. Live crabs were transported to the Ecology research laboratory, where they were placed on deep freeze for 20 minutes for anesthetizing prior to dissection. All crabs were weighed on an electric balance. Carapace width (CW) was defined as the distance between the two anterior lateral spines. Carapace length (CL) was defined as the distance between the centers of the frontal interorbital carapace margin and the posterior margin. Carapace width and length were measured to the nearest millimeter (mm) digital caliper. Crabs with missing limbs, broken carapaces, or any signs of disease were not used. The first carapace cut was made from the dorsal articulation above the right posterior leg, lateral to the frontal margin at the head. A second cut was made following to the first cut, on the left side. The lateral cuts were then joined by a transverse cut at the posterior margin of the carapace. The freshwater crabs *P. lamellifrons* ovaries are located covering the cardiac stomach and under the spines, intermingled with the hepatopancreas. Gills are located to the side of the ovaries. Ovaries were removed from the abdominal cavity and sectioned for histology. Each section was processed in order to identify the reproductive stage of the ovary. According to Silva *et al.,* (2012), on his macroscopic and microscopic on the gonadal development in the freshwater crab the gonadal stages were classified as immature or previtellogenic, ripening or vitellogenesis, mature or complete vitellogenesis and spawned.

Results and Observations

1. Anatomy of the female reproductive system

The size of the dissected female ranged from 33.75 mm to 49.16 mm. The female reproductive system of *P. lamellifrons* consists of a pair of ovaries, oviducts and spermathecae or seminal receptacle. The entire ovary was bounded by fibrous connective tissues which separate the organ from the surrounding Haemocoel. The ovaries were elongated organs located dorsally in the cephalothorax and interconnected only by a short transversal expansion posteriorly to the stomach and ventrally to the heart (Fig. 1). The ovaries extend posteriorly from the transversal expansion in the form of two parallel lobes positioned laterally to the mid-digestive tract. Depending on the stage of maturation, the posterior lobes may extend as far as the third abdominal segment (Fig. 1).

Around the middle of each posterior lobe an extension was seen projecting ventrally and connecting to the underside of the spermathecae. The spermathecae were spherical or ovoid sacs below the middle intestine, filled with a thick, milky liquid. The walls of the organ were thin, whitish and easily disrupted with the tweezers. Ventrally the spermathecae were coupled to a pair of short, thin and translucent oviducts which open onto the sternite of the sixth thoracic segment through an operculated pore called the gonopore. The ovary was almost 'H'- shaped and located dorsally just beneath the carapace (Fig. 1).

The gonads vary in colour, shape, consistency and volume as the female matures. The colouring changes from white through orange to dark orange. White-coloured ovaries were cylindrical, slender and flaccid and extend to the first abdominal segment. Orange-coloured gonads were likewise cylindrical and slightly compressed dorsoventrally, but were firmer to the touch and reach the second or third abdominal segment. Dark brown ovaries were considerably larger and compressed dorsoventrally; the anterior lobes cover the entire hepatopancreas, while the

posterior lobes may extend as far as the extremity of the third abdominal segment.

2. Stages of Maturity

Based on colour change, external morphology and histology, the ovary was divisible into five maturity stages, namely, immature, early maturing, late maturing, ripe and spent. Present study of the freshwater crabs *P. lamellifrons* those stages have the following characteristics.

2.1 Immature: The ovary is thin, tubular, filiform shape and translucent appearance and has a small anterior terminal bulging. The ovary started to form primary oocytes including the presence of vacuolated globules in the cytoplasm and follicle cells on the periphery of the cytoplasm (Fig. 1A&B)

2.2 Early Mature: The ovary was easily visible macroscopically, ivory or light yellow in colour and occupies about one half of the volume of hepatopancreas dorsally (Fig. 1C). At this stage, the ovary had a lumen and well developed germinal strand with oogonial cells. These oogonial cells were characterized by large nucli and small amounts of ooplasm. The early developing or primary vitellogenesis stage is the initiation of vitellogenesis. The ovary changes to yellow colouration (Fig. 1C). The vacuolated globules disappeared from the cytoplasm.

2.3 Late Mature: Ova were conspicuous when the ovary was viewed macroscopically. The colour of the ovary varies from yellow to yellowish orange (Fig. 1D&E). Oogonial cells develop into primary oocytes. During the late-maturing or secondary vitellogenic stage, the lobules were developed prominently in sterno carapace and upper digestive gland.

2.4 Ripe: The ovary was the dominant visible organ obscuring the hepatopancreas dorsally with dark orange colouration (Fig. 1F). The enclosing fibrous connective tissue was highly stretched, often to the point of bursting during dissection and occupying one quarter (1/4) of the thoracic cavity. The nucleus was solid and centrally placed, with small yolk droplets appearing in the peripheral region of the ooplasm (Fig. 4). On the other hand, the spent stage was not studied in this experiment.

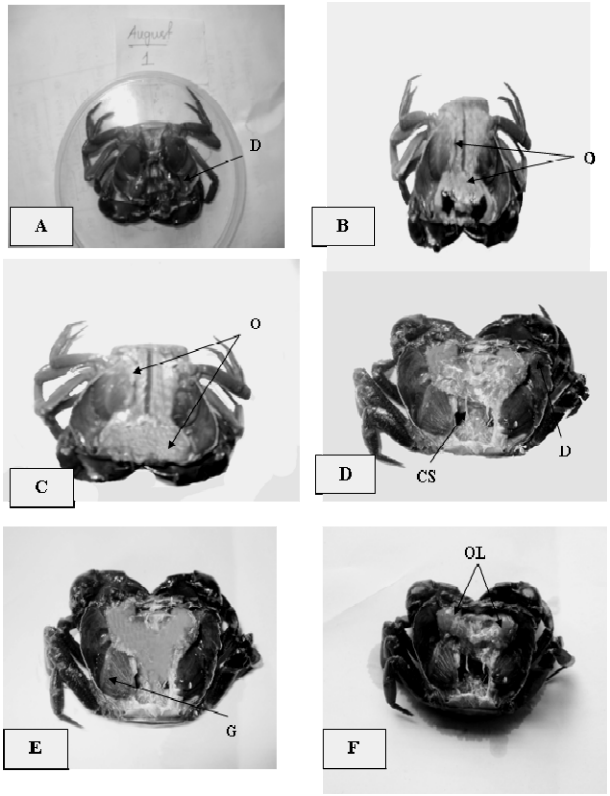

Fig. 1. External observation of ovary of *Paratelphusa sp.* The ovarian developmental stages were determined. A. Immature; B. Developing; C. Early maturing; D. Late maturing; E. Maturation and F. Ripe ovary. Digestive gland (D); Ovary (O); Cardiac stomach (CS); Gill (G) & ovarian lobe (OL).

3 Histological description of the ovary of immature crabs

The immature or proliferating ovary (Fig. 2) showed transparent to translucent colour that was sometimes difficult to recognize. The ovary was made up of oogonia that concentrated mostly at the periphery of the ovary. Spherical oogonium was observed and cytoplasm was rare in the oogonium (Fig. 2). In this stage, ovary started to form primary oocytes including the presence of vacuolated globules in the cytoplasm and follicle cells on the periphery of the cytoplasm. Immature ovary contains somatic cells, well developed germinal strand. Oogonial cells having large nulci and small amount of ooplasm. The proliferating oogonia trend to go rapidly to primary oocytes by mitosis division of the germ cells. There was presence of vacuolated globules in the cytoplasm and follicle cells on the periphery of the cytoplasm (Fig. 2).

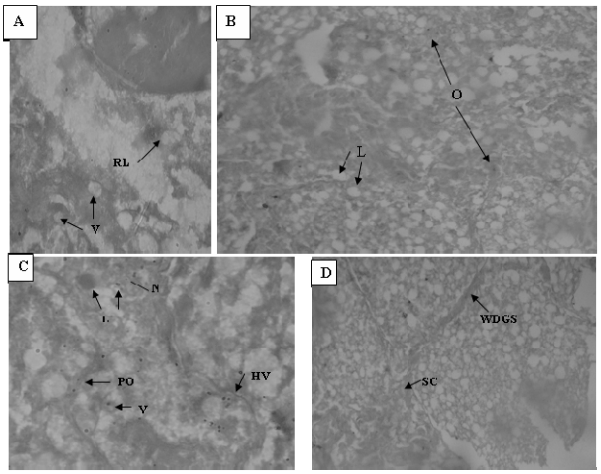

Fig. 2. Histological slide of immature ovary; A. vacuolated globule (V), reducing lumen (RL); B. oogonium (O), conspicuous lumen (L); C. nucleus (N), late primary oocyte (LPO), primary oocyte (PO), vacuolated globule initial form(V), hemal vessel(H); D. well developed germinal strand(WDGS), somatic cell(SC).

4 Histological description of the ovary of mature crab

The mature ovary contains the somatic components include the lining of the ovary, hemal vessels, sinuses, hemocytes, oocytes, follicular cells and fibrous materials (Fig. 3) later constituting the ovarian stoma. The periodical invaginations in the lining gave rise to cysts in which the germ cells develop (Fig. 3). The follicular cells were remaining irregularly around the germcells. The hemal vessels were lined by a thin and weavy membrane and contain a granular substance, the haemolymph (Fig. 3). The hemal sinuses appear as spaces filled with hemolymph between the ovary fibres. The hemolymph contained spherical or ovoid cells, the hemocytes, which were also found in the ovarian stoma (Fig. 3). Cortical alveoli (CA) were found in the periphery of the oocytes (Fig. 3).

5 Histological description of the ovary of ripe crab

Hemal vessels were moving weaving along the basal lamina. Basal lamina was the structure located at the base of all epithelium and acts as a barrier between connective tissue and epithelium. Cortical alveoli were present (Fig. 4). Inclusion of yolk globules in the ooplasm was occurred. Somatic cells were found outside the basal lamina (Fig. 4). Mature ovum was found in the ripe stage (Fig. 4). There was lack of somatic cells and no lumen was found in the ripe stage ovary.

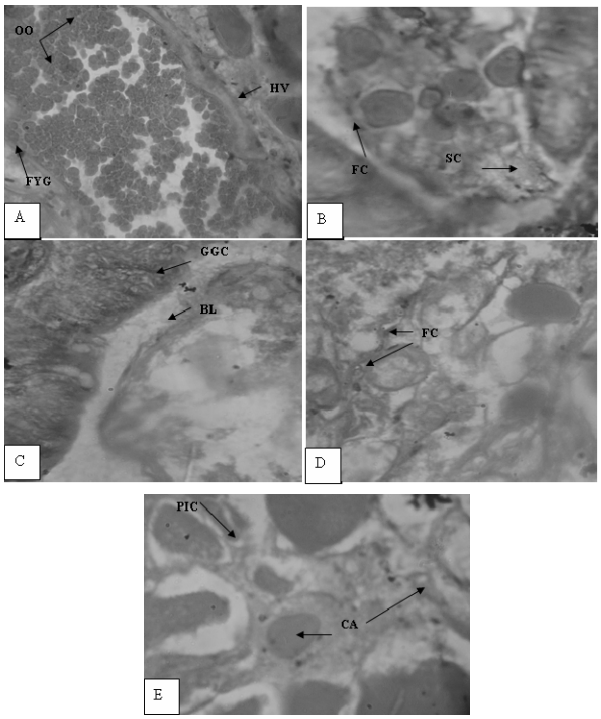

Fig. 3. Histological feature of mature ovary. A. oocytes (OO), hemal vessel (HV), fused yolk globules (YG). B. follicle cell (FC), somatic cell (SC). C. growing germ cell (GGC), basal lamina (BL). D. follicle cell (FC). E. periodical invaginations cyst (PIC), cortical alveoli (CA).

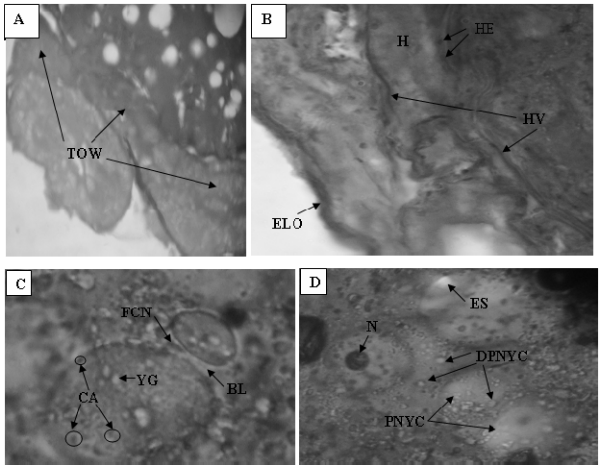

Fig. 4. Histological slide of ripe ovary. A. Thick ovarian wall (TOW); B. External lining of ovary (ELO), Hemolymph (H), Hemal vessel (HV), Hemocyte (HE); C. Cortical alveoli (CA), Yolk globule (YG), Basal lamina (BL), Follicle cell nucleus (FCN); D. Nucleus (N), Perinuclear yolk complex (PNYC), Dispersing Perinuclear yolk complex (DPNYC), Empty space (ES).

6 Stages of ovary development

The ovary was contained inside units called ovarian lobes or ovarian pouches (Fig. 2).The ovarian lobes encompassed the oocytes. Most oocytes inside the ovarian lobes were at about the same developmental stage. Oogenesis is a continuous process in which oocytes undergo development from primary growth to secondary growth in a somewhat rapid fashion. Some lobes were configured with two main stages, but they may also have a small percentage of a third stage in them. Development was a fluid process and oogonia were always present, but they may not always be evident.

Early primary growth oocytes were also always present because along with oogonia, they compose the germinal zone, and all ovarian lobes have a germinal zone. Therefore, there can be more than one developmental stage inside one ovarian lobe, but mostly the ovarian lobes were in the same developmental stage per crab. The structure of the freshwater crab's ovary consists of oocytes that develop from the center of the ovarian lobe to the periphery as oogenesis advances. This distribution of germinal cells in the ovarian lobes was characterized by the developmental progress of cells from oogonia, which were found in a central germinal zone also known as a germaria to late secondary growth stage, which were found in the periphery of the ovarian lobes. The germinal zone was a row of germinal cells at the center of the ovary. During primary growth, there were no well-defined germinal zones and oogonia were difficult to recognize. In secondary growth, the germinal zones were well-defined and consist mostly of primary growth oocytes. Germinal zones provide new cells for continual development of oocytes as cells mature when they reached the periphery.

6.1 Oogonia

Small oval cells were found in the germinal zone. These cells could not be classified as oocytes because oogonia were capable of dividing via mitosis to form other oogonia or via meiosis to form an oocyte. They were diploid cells in which meiosis has not started. Oogonia have a characteristic scant, very clear ooplasm. The nucleus was pale, and a single nucleolus was prominent.

6.2 Early Primary Growth or Previtellogenic Oocytes

Early primary growth oocytes have begun meiosis, and therefore the cell has become a primary oocyte. The appearance of the early primary growth oocytes was similar to oogonia. When the ooplasm changes from the clear, scant appearance that it had during oogonia to a blue hue that was the indication that the oogonia was now in early primary growth. These cells were larger than oogonia and can no longer divide via mitosis again. Therefore, the early primary growth oocyte was an oocyte and remains as such throughout the rest of its development, until full-grown.

6.3 Late Primary Growth or Vitellogenic Oocyte

The beginning of late primary growth was characterized by gradual basophilia of the ooplasm and by the absence of yolk. The basophilic ooplasm indicates that the cell was active with production of organelles, which increase the volume of the ooplasm, another marker for this stage. 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. During this stage, the germinal zone was not as evident as it will be in more developed oocytes, yet the germinal zone with the oogonia and the early primary growth oocytes was always present. Hemolymph was evident, and follicular cells and somatic cells were also present but not as obvious.

The perinuclear yolk complex was found (Fig. 3.). The function of the perinuclear yolk complex may be to assist in assembling the proteins that later form the yolk globules. The perinuclear yolk complex disappears as the oocyte develops and growth continues into the next stage. In late primary growth, somatic cells and follicular cells were evident. These cells didn't disappear during secondary growth and had always been present, but it was 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 were distinguishable because of their elongated nucleus, and they could be found surrounding the oocytes. The elongated nucleus of the follicle cells was more apparent in the later stages of development. Somatic cells were found outside

the basal lamina and within the germinal compartment. Somatic cells can become follicle cells, but until they were not found in the germinal compartment and surround the follicle, they were not described as follicle cells but only as somatic cells.

Basal lamina was the structure located at the base of all epithelium and acts as a barrier between connective tissue and epithelium. Cortical alveoli were another morphological characteristic that emerge during late primary growth, and they occur immediately prior to the onset of secondary growth. 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 became visible in the perimeter of the ooplasm. Secondary Growth or vitellogenesis, began with the inclusion of yolk globules in the ooplasm.

However, the follicle cells still surround the oocyte. During mid-secondary growth, the yolk globules were more evident in the ooplasm as they grow larger. There was also a well-differentiated germinal zone that contains only primary growth oocytes. Somatic cells were found outside the basal lamina and the germinal compartment, and follicle cells were found within the germinal compartment. Follicle cells surround the oocytes, and their nuclei were clearly seen around oocytes. Basal lamina was located at the base of the epithelium, separating the connective tissue from the epithelium and acting as a barrier. Full-grown oocytes have reached their maximum size.

These oocytes were found mostly on the perimeter of the ovarian lobe at first; then when development continues, the whole ovarian lobe will appear with fused yolk oocytes.

6.4 Full-Grown Oocytes

Full-grown oocytes have reached their maximum size. Most of the ooplasm retains a bright-pink stain. With the absence of large accumulation of cell organelles, yolk becomes fused in full-grown oocytes (Fig. 4). These oocytes were found mostly on the perimeter of the ovarian lobe at first; then when development continues, the whole ovarian lobe will appear with fused yolk oocytes.

Discussion

The reproductive system and the ovary of different maturing stages were studied. A pair of ovary, a pair of oviducts and a pair of spermathecae were found. The anterior part of the ovary was swollen and extended towards the width of the carapace. The gonad varied in colour, shape, consistency and volume as the female matures.

During the maturation process, there are histological and morphological alterations. The gonads change in volume and are easily seen by macroscopic examination. The colouration of the ovaries in *Sylviocarcinus pictus* ranged from transparent when immature to white and yellow tones in stages II (in vitellogenesis) and IV (spawned). The sequence in the change of colour was similar to that described for the freshwater crabs *Eudaniela garmani* and *Sinapotamon yangtsekiense*, in which transparent ovaries change to a white colour until acquiring a cream colour, followed by yellow and ending up bright orange, evidenced by the accumulation of yolk (Rostant *et al*., 2008 and Chen *et al*., 1994).

According to Silva (1999), the presence of highly developed nuclei, as observed in *G. cruentata*, indicates that the nuclear components are ready for vitellogenesis. In crustaceans, this type of cell is only found in the germinative zone, where it is produced by mitosis throughout the egg-bearing life of the female (Adiyodi & Subramoniam, 1983; Krol *et al.,* 1992). Groups of oogonia with compacted chromatin, possibly undergoing mitosis, were observed in the specimens of *G. cruentata* collected for this study. According to Adiyodi & Subramoniam (1983), the rapid succession of mitotic stages in oogonia makes them difficult to observe. In fact, although mitotic oogonia were identified in our specimens, the specific stages could not be determined.

The histological examination of the ovaries of *G. cruentata* revealed four types of germ cells: oogonia, previtellogenic oocytes, vitellogenic oocytes and mature oocytes. The description of cellular stages agrees with the characteristics described by Adiyodi & Subramonian (1983). A similar characterization was adopted for the brachyurans *Uca rapax* (Castiglioni *et al.,* 2007), *Ucides cordatus* (Santana, 2002), and *Chasmagnathus granulate* (López *et al.,* 1997) and for *Panulirus* spiny lobsters (Silva, 1999). Germ cells are classified according to a range of criteria, such as cell diameter and nucleus appearance (Mota & Tome, 1965) and degree of vitellogenesis (Kulkarni *et al.,*1991). In the present study the classification was based on degree of vitellogenesis.

One of the most significant processes in the reproductive biology of any animal species is the development of oogonia into mature oocytes. Present investigations into oocyte maturation have classified the process into discrete stages based upon microscopic characteristics, from oogonia to mature oocytes (Brown, 2009). This embedding technique allows the tissue to be sectioned at 5 µm. For this research, the process of oocyte maturation in freshwater crabs was described and a classification system for stages was developed to best categorize the morphological changes occurring throughout the maturation process of the oocytes.

In freshwater crabs, oocytes are contained within an ovarian lobe. The connective tissue that surrounds the ovarian lobe and retains all the oocytes inside is visible. As oocytes mature, they move to the periphery of the ovarian lobe, and only oogonia and early primary growth oocytes are found closer to the center of the ovarian lobe. This area, where the younger stages originate, is known as the germinal zone or germaria, which is represented as specialized germ cell areas. In a study by Hinsch (1972), it was observed that in the spider crab, *Libinia emarginata,* the oogonia and primary growth oocytes were found in central regions of the ovary.

In the freshwater crab, *Potamon dehaani,* when the oogonia reach about 20 µm in diameter, they move out of the germinal zone. Ando & Makioka (1999) noted that in *P. dehaani,* the oogonia are not enclosed in the same regions where larger oocytes are found. Ando & Makioka (1999) observed that oocytes larger than 100 µm in diameter are enclosed within their own regions. The authors call these regions the oogenetic lobes. Hence, in *P. dehaani,* there are large oogenetic lobes that contain only mature eggs (Ando & Makioka, 1999).

With the more mature stage germinal cells encompassing the majority of the oocytes present, the younger developmental stage oocytes are represented in smaller percentages of the total oocyte count (Brown, 2009). Oogenesis is a fluid process, and the different developmental stages of oocytes inside the ovarian lobes correspond to the different times in the annual reproductive cycle in which the crab will spawn (Brown, 2009).The younger oocytes, occupying less of the total percentage of the ovarian lobe, will eventually develop to occupy most of the ovarian lobe as they become mature (Brown, 2009).

In the freshwater crab, *P. dehaani,* Ando & Makioka (1999) recognized a similar germinal zone, which they called “germaria.” In *P. dehaani,* Ando & Makioka (1999) noticed that germaria are located in an ovarian epithelium.

The germinal zone was found in the center of the ovarian lobe, and the germinal zone contained the oogonia and early preprimary growth. In the freshwater crab, *P. dehaani,* oogonia were defined as being basophilic and smaller than 20 µm in diameter. Basal lamina can also be observed near the oogonia (Brown, 2009). Ando & Makioka (1999) observed that in *P. dehaani,* the oogonia are found only in the germaria, which in 34 turn is found inside an ovarian epithelium. This similarity is shared by the freshwater crab, *P. dehaani,* and blue crabs (Brown, 2009). In both species, the oogonia are noted to be found only inside the germinal zone.

Throughout development, one nucleus and one nucleolus are present (Brown, 2009).These were particularly evident during primary growth. A perinuclear yolk complex, also known as the PAS positive body, appears during primary growth. This structure is observed as one of the early stages of late primary growth. This perinuclear yolk complex, or PAS positive body, aids in the future production of yolk. When it is present in the primary growth oocyte, it is assembled of organelles, not vitelline. The PAS positive bodies are noted first near the nucleus and later disappear by dispersing in the ooplasm as the oocyte develops further. Follicle cells and somatic cells were always present in the oocyte; however, they are more apparent during the later stages of development and Nuclei of follicle cells can be observed surrounding the oocytes and are found inside the germinal compartment (Brown, 2009). The follicle cells remain in the ovarian lobe, and the oocyte comes out of the ovarian lobe, where it is fertilized and embryonic development begins.

The appearance of a full-growth oocyte is then similar to the appearance of the ovulated eggs (Plate- 3.5). Histology provides the only means to determine oocyte developmental stages. Some females with larger carapace widths were found to have ovarian lobes in earlier developmental stages. Ovigerous females with oocytes in primary growth were found not only in this study but also in studies by Lee *et al.,* (1996) and Hinsch (1972).

The beginning of late primary growth was characterized by gradual basophilia of the ooplasm and by the absence of yolk. 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 (Souza & Silva, 2009).

In the observation, oogonium, vaculated globule and lumen were conspicuous in the immature stages. Hemal vessels, follicle cells, basal lamina and periodical invagination cyst were observed in mature stages. The ripe ovary was characterized with the full grown oocytes, yolk globules, cortical alveoli and perinuclear yolk complex.

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