CHAPTER – 3

OOGENESIS IN *SESARMA QUADRATUM*,

A CONTINUOUS BREEDER
3.1. INTRODUCTION

Decapod crustaceans in general, exhibit varying patterns of breeding, which apparently are correlated with the efforts of the species in question to cope with the respective ecosystem it inhabits. Significantly, while a number of the decapod crustaceans are known to be annual breeders (releasing a single brood per year) such as *Barytelphusa cuncularis* (Diwan and Nagabhushanam, 1974), *Paratelphusa hydrodromous* (Anilkumar, 1980), *Ocypoda platytarsis* (Pillai, 1982), *Gecarcinus steniops* (Santhamma, 1985), there are several others which are consecutive or continuous breeders (releasing several broods a year) like *Metopograpsus messor* (Sudha and Anilkumar, 1996), *Panopeus africanus* and *Uca tangeri* (Rodrigues et al., 1997), *Piluminus vespertilio* (Litulo, 2005), and *U. triangularis* (Syama et al., 2009). And populations of a single species, but geographically separated, could show differing patterns of reproduction. The Calicut population of the field crab *Paratelphusa hydrodromous*, for example, is an annual breeder releasing the brood in April or May, while the Trivandrum population of the same species, is a consecutive breeder, releasing as many as 3-4 broods a year (Anilkumar and Adiyodi, 1983; Adiyodi, 1989). Being a pivotal component of breeding and fecundity, gametogenesis has been inviting the attention of the investigators for the past several years. There have been attempts to describe precisely the process of vitellogenesis at light and electron microscopic levels (Sudha and Anilkumar, 1996; Kao et al., 1999; Castiglioni et al., 2007) in decapod crustaceans. These investigations, however, were essentially depicting the process of yolk deposition and the biochemistry of vitelline components (Hinsch and Cone 1969; Castiglioni et al., 2007; Dutra et al., 2008). In any of these studies, however, the
programming of gemetogenesis in tune with the specific pattern of breeding in the candidate species is not given sufficient attention. The existence of this lacuna has encouraged us to undertake the present study at light and electron microscopic levels. Through the present study, we examine the cytological events occurring in the ovary in relation to consecutive breeding in the mangrove crab, *Sesarma quadratum*, a prolific breeder that releases as many as 14-16 broods a year (Chapter 2, for details). The study on the one hand, would allow us to ascertain the cytological processes involved in yolk deposition, while on the other, it explains the genesis of a successive set of oocytes that would in turn enable the crab to accomplish consecutive breeding in immediate succession.

Decapod crustaceans exhibit sexual dimorphism (Chapter 1 for details). Accordingly, the female brachyuran crab possesses a broadened abdomen suitable for holding (spawned) eggs. For those brachyurans wherein direct development (without the involvement of a larval stage) is the rule, the broadened abdomen would be used for holding the juveniles as well. The preparatory stages for establishment of the sexual dimorphism in brachyurans are initiated much before the pubertal moult. The signs of broadening the abdomen in females and enlargement of the claspers (as seen in the fiddler crabs) in males begin to appear about 3-4 months prior to the pubertal moult. Presumably, these signs of establishment of sexual dimorphism should be effected in the development of the gonads as well. Eventhough logically, the gonads get differentiated from a pair of mesomeres (originated from mesoderm cells) arranged laterally. These mesomers, as development proceeds would give rise to the primary gonial cells which in turn would be transformed into functional sex cells. The exact timing of metamorphosis
of the mesomeres (from its post-embryonic stage) to the functional gonads as seen in adults is only meagrely attempted in brachyuran crabs, although some reports are available from other groups. This lacuna has prompted us to undertake the present study as depicted in this chapter (at light microscopic levels) with an objective of defining the cytological events taking place in the gonads during the pre-pubertal moults. Although this study is related to evolution of gametes, this part of the study (gonadal development in prepubertal females) is being represented as a separate section (section-A) under this chapter (Chapter 3).

3. 1. SECTION A. GONADAL DIFFERENTIATION IN PRE-PUBERTAL CRABS

3. 2. A. MATERIALS AND METHODS

Juveniles of *S. quadratum* (CW ranging between 4-7 mm) were collected from the mangrove swamps of the intertidal regions of Nileshwar, Kasaragod district, Kerala, India, with geographical coordinates 12° 15’ North and 75° 16’ East. During the months of January to March juveniles appear in large numbers (several hundreds) at the banks of the main river where water is available at all tides. The external morphology of a juvenile crab differs from that of adult *S. quadratum*, in that the former lacks external pigmentation and possesses a thin translucent carapace. Each crab collected afresh from the field was examined thoroughly. They were categorized into three groups depending upon (1) the CW; (2) the nature of the abdomen/development of first pleopod: juveniles which do not exhibit sexual dimorphism, juvenile males and the juvenile females.
A. Juveniles that do not exhibit sexual dimorphism - CW is 6mm or less. The secondary sex characters are not visible in this group of juveniles (Fig.3.1).

B. Juvenile males - they possess 6 - 7 mm in CW. The abdomen is narrow. First pleopods are plain, slender and elongated. They are not sexually mature and have not yet undergone pubertal moult.

C. Juvenile females - the CW is 7 - 8 mm. Abdomen appears flat. Pleopods are not fully developed and are very small in size.

As the gonads of juveniles (undifferentiated) were too small to be identified and dissected out separately, the whole animals were fixed in 5% neutral formaldehyde. The gonads of prepubertal males and females were also dissected out and fixed immediately in 5% neutral formaldehyde for 24 hours, then washed thoroughly with water, subsequently dehydrated through alcohol series and embedded in paraffin wax. Microtome (paraffin) sections of 4 –7 µm thickness were stained in Heidenhans Hematoxylin- Eosin and/or Toluidine blue. Leica Research Microscope (Germany) was used for histological observations, its imaging and for photomicrography.

3. 3. A. RESULTS

3. 3. A. I. Undifferentiated gonad of juvenile crabs

In its undifferentiated state, the gonad of a juvenile crab appears as two laterally located strands of tissue extending the entire length of the cephalothorax. The gonad at this stage is characterized by the presence of a number of germinal cells (~13.87 ± 3.609µm diameter) and nucleus (9.596 ± 1.184 µm diameter). Light microscopic observations of semi-thin sections (made for electron microscopy) stained in Toluidine
blue revealed the basophilic nature of the nuclei of the germinal cells, with condensed chromatin distributed in the centre and the periphery (Fig.3.2).

3.3 A. 2. Juvenile male

Histology of a juvenile male (<7mm CW) gonad suggests the appearance of large number of germinal cells. All the cells were almost uniform in appearance. The number of gonial cells exceeds that of the female gonad (Fig.3.3.1).

3.3 A. 3. Juvenile female

The gonad (ovary) of the prepubertal females (<8mm CW) showed the presence of undifferentiated gonial cells which have already started divisional stages as evidenced by the presence of enlarged cells in the sections (Fig.3.3.2).

3.4 A. DISCUSSION

In decapods, different criteria have been used to identify sexual maturity. The juvenile phase or growth phase, and the adult or reproductive phase are separated by a prepubertal moult, after which the animal attains sexual maturity. Carapace width (size) at maturity was estimated to be 9 mm for females (50% reproductive), and 8mm for males (50% of adult). The changes in external morphology have been used by many authors to estimate sexual maturity as in the spider crab, *Maja squinado* (Sampedro et al., 1999). The morphological change that occurs in the female abdomen during the terminal moult is a good indicator of sexual maturity. Growth of chelipeds in juvenile females showed no changes with allometric significance and was comparable with that of juvenile males. Unlike juvenile females with a flat abdomen, adult females had a domed abdomen.
with well-developed pleopods. Histological analysis of the juveniles showed that the gonad contained several undifferentiated primordial germ cells (Fig. 3.2). Light microscopy reveals the existence of undividing cells in the gonad of juvenile with CW <6 mm. As the animal grows in size (CW >6 mm), the differentiation of the gonad also proceeds, indicated by the existence of the size difference in the resultant cells. It seems that the differentiation of gonads occurs at an early stage, much earlier than the external morphometric changes appear.

The morphometrically immature crabs (6-7 mm CW) showed the presence of spermatocytes. The immature females, on the other hand, had in their ovaries differentiating oocytes. Maleness is attained at a faster rate than the femaleness (8 mm CW). Juveniles having a CW of 7 mm exhibited the presence of enlarged first pleopods while femaleness (presence of flat abdomen) was seen in the juveniles with a CW of 8 mm. Juvenile females possess a gonad with comparatively less number of gonia while that of the males acquire plentiful gonia.

The objective of this study was to describe the nature of the undifferentiated and differentiating gonad inasmuch as this aspect has not been attempted in brachyuran crabs. Although many studies have been carried out on ‘juvenile allocation’ so far not much is known on its reproductive development. Juvenile survival in the intertidal environment may be regulated by changes in adult density. Adults functioned as cannibals and intraguild predators by consuming both conspecific and heterospecific juveniles (Kneib et al., 1999). It is considered that a minimum of two and a maximum of
three years are required to reach sexual maturity from the larval stages in grapsid crabs (Diesel and Horst, 1995; Fukui, 1988; Tsuchida and Watanabe, 1997; López et al., 1998).

3. 1. SECTION B. OOGENESIS IN *SESARMA QUADRATUM*, A CONTINUOUS BREEDER.

3. 2. B. MATERIALS AND METHODS

Adult females (CW ranging between 8 - 22 mm) collected from the intertidal regions were brought to the laboratory and maintained in plastic cisterns giving near-natural conditions. They were fed ad libitum on boiled mussel meat, or egg yolk. The crabs were sacrificed for histological, histochemical, ultrastructural and biochemical studies.

3. 2. B. 1. Histology

The ovaries were dissected out in saline with a pair of fine scissors and fixed immediately in 5% neutral formaldehyde for 24 hours, then washed thoroughly with water, subsequently dehydrated through alcohol series and embedded in paraffin wax. Microtome (paraffin) sections of 4 – 7 µm thickness were stained in Heidenhans Hematoxylin- Eosin. Leica Research Microscope (Germany) was used for histological and histochemical observations, its imaging and for photomicrography.

3. 2. B. 2. Electron microscopy

Ultrastructural studies were performed in a Transmission electron microscope at the National Institute of Mental Health and Neurosciences (NIMHANS), Bangalore. For electron microscopy, the tissue (ovary) was dissected out and fixed in Karnovsky’s fluid...
(4% paraformaldehyde and 3% glutaraldehyde in phosphate buffer, pH 7.2) for 24 hours. The tissue fixed in Karnovsky’s fluid was washed twice in phosphate buffer (pH 7.2-7.4) for approximately 15 minutes till the smell of the fixative disappeared. Then 1% OsO₄ was added and the tissue was kept in refrigerator (4° C) for one and a half hours. The tissue turned black in colour and hence, could be easily identified. The same was washed with phosphate buffer (pH 7.2-7.4) for half an hour. The tissue was then passed through a series of 70 %, 80% and 90% ethanol for half an hour each, and transferred to enblock stain (2% Uranyl acetate in 95% ethyl alcohol) for one hour, followed by dehydrating in absolute ethanol. Clearing of the tissue was done in propylene oxide. The tissue was then embedded in araldite and propylene oxide in the ratio 1:1 by keeping overnight in the rotator; excess osmium tetroxide got removed. The tissue was then placed in fresh pure araldite and kept for 4 hours in the rotator. Three changes were made with araldite, with a gap of 4 hours, and then embedding was done at 60 °C for 48 hours.

**Trimming**

With the help of a razor blade, the blocks were trimmed, so as to have a cutting face free from extra resin. The trimmed block was fixed on a specimen holder of the ultramicrotome (LEICA EM UC6). 6 mm thick glass pieces were cut with a glass cutting machine, then a plastic boat was fixed on to the edge of the glass piece by means of molten wax for collecting the sections. The boat was filled with water. The sections were allowed to be floated on the pool of water on the boat.
Semi-thin sections

Semi-thin sections of 1µm thickness were used for scanning the tissue under light microscope, with a view to locate the exact portion of the block to be sectioned. The floating sections were lifted with a thin glass rod on to a clean glass slide, placed on a hot plate at about 80°C and dried; sections stained with Toluidine blue for one minute, were washed in running water, dried and mounted in DPX. The slides were scanned under a light microscope to ensure the quality of preservation and localize the area of interest for the subsequent ultra-thin sectioning.

The blocks were further trimmed (if necessary) and ultra-thin sections of 500Å thickness were cut in a LEICA EM UC6 ultramicrotome. The ultrathin sections were collected on copper grids which were either meshed or slotted. Grids were stored in grid boxes. Double staining with uranyl acetate and lead citrate was done to obtain contrast during observation. The grids were observed under a Transmission electron microscope (TEM).

The juvenile crabs (<6mm CW) were also fixed in Karnovsky’s fluid and processed for electron microscopy as mentioned above. Semithin sections were cut and observed for the purpose of proper orientation of the material before preparing the ultrathin sections.

3. 2. B. 3. Biochemical analysis

3. 2. B. 3. 1. Proteins

Protein content of the ovary of *S. quadratum* was estimated by Lowry et al., (1951). The ovary was dissected out, weighed and homogenized with diethyl ether to
remove the excessive lipid fraction. The protein was precipitated in 10% cold TCA and stored in a refrigerator (at 4°C), if required. For analysis, the precipitate was collected by centrifugation (at 5000 rpm for 10-15 minutes), and dissolved in 0.1 N NaOH solution. This was used as the protein sample for quantitative analysis. To 1 ml of the sample, 5.5 ml of the Reagent C [a mixture of Reagent A (2% Na₂CO₃ in 0.1N NaOH) and the Reagent B (0.5% copper sulphate in 1% sodium potassium tartarate) in the ratio 50:1] was pipetted out. The mixture was shaken well. 0.5 ml of Folin Ciocalteau’s reagent (1N) was added to each tube and was incubated at room temperature for about 20 minutes for optional colour development. The (purple blue) colour developed was read at 620 nm using a CNL digital analyzer. Standard was prepared using Bovine serum albumin concentrations of 30, 60, 90, 120, 150 and 180 µg.

3. 2. B. 3. 2. Lipids

The ovary was dissected out, weighed and was kept in an oven to dehydrate the tissue to estimate the amount of total lipid. To estimate the total lipid of the ovary, the dried tissue (dried to constant weight) was subjected to Sohxlet extraction using chloroform as the solvent (Vogel, 1959). From the weight of the lipid fraction thus obtained, the total lipid content was estimated.

3. 2. B. 3. 3. Carbohydrates

The ovary was dissected out, weighed and homogenized in 80% ethanol and centrifuged at 3000 rpm for 15 minutes. The ethanol-soluble supernatant is referred to as the oligosaccharide fraction and the ethanol-insoluble part is referred to as the polysaccharide fraction (after Johnston and Davies, 1972). The supernatant, after
centrifugation, was collected in a small beaker and was incubated at 60°C for evaporation. When the ethanol is evaporated completely, the ethanol soluble oligosaccharide fraction may be constituted with required quantity of distilled water. The ethanol insoluble fraction was constituted by dissolving the tissue (obtained after centrifugation) in distilled water. The total carbohydrate present in the two fractions was estimated by phenol sulphuric acid method (Dubois et al., 1956). The colour developed was read at 540 nm using the CNL digital analyzer. Different aliquots of glucose 30, 60, 90, 120, 150 and 180 µg were used as the standard.

3. 2. B. 3. 4. Total free amino acids (FAA)

Total FAA were determined colorimetrically by ninhydrin method (Lee and Takahashi, 1966). The tissue (ovary) was weighed, homogenized in 80% ethanol, and centrifuged at 3000 rpm for 10 minutes. The supernatant was allowed to evaporate at 60°C in an oven and the FAA extract was constituted by adding adequate amount of distilled water. Different aliquots of the samples were taken in test tubes and made up to 1ml with distilled water. To each test tube 5.7ml of Ninhydrin Reagent (prepared by adding 1.5 ml 1% ninhydrin in 0.5 M citrate buffer + 3.6 ml glycerol (50%) + 0.6 ml 0.5 M citrate buffer). The mixture was heated in a boiling water bath exactly for 12 minutes and was cooled in tap water bath to room temperature. The resulting solution was shaken well and the OD was measured using CNL digital analyzer at 570 nm. Standard glutamic acid was used for calibrating the readings.
**3. 2. B. 3. 5. Individual FAAs**

Individual FAAs were estimated using paper and thin layer chromatography. The cold extract ovary was prepared in the same way as for total FAAs. The cold extract was run in a column of Dowex 50. The amino acids were eluted using 2N ammonium hydroxide. The extract was evaporated at 60°C in an oven and subsequently constituted with appropriate quantity of distilled water. For paper chromatography, the sample was spotted on a Whatman (No.1) chromatographic paper and was run in a mixture of n-butanol: acetic acid: water (4:2:2). After the run, the paper was sprayed with 0.1% ninhydrin in 95% acetone, and was dried at 100°C in an oven. The spots were identified by calculating the Rf values. Standard glutamic acid was also run along with the sample for the purpose of reference.

For identification of spots, authentic individual amino acids were run concurrently. Since the Rf values of authentic amino acids varied when spotted separately and in a mixture, one amino acid (authentic) was mixed at a time with the unknown sample and allowed to run side by side with the unknown sample spotted alone. With this procedure, we got fairly good comparisons for a more accurate identification of the individual FAA.

**3. 2. B. 3. 6. Quantification of amino acids from chromatogram**

The spots on the chromatogram were cut and placed into test tubes. The amino acids were eluted using 5ml 0.1 % copper sulfate in 80% ethanol as the solvent. The eluant was then read at 570 nm using a colorimeter. 40µg glutamic acid, spotted separately, was used as the standard.
3. 2. B. 3. 7. Thin layer chromatography

For thin layer chromatography, silica coated Aluminum sheets of 20 X 20 cm (Merck) were used. The sample was spotted on the pre-coated sheet using micropipette and was run in a mixture of n - butanol: acetic acid: water (4:2:2), taken in a chromatography chamber. After running for about 4 hours, the sheet was dried and sprayed with 0.3% ninhydrin in 95% acetone, and was dried at 100º C in an oven. The spots were marked as and when dried.

3. 2. B. 3. 8. Ascorbic acid

Ascorbic acid present in the ovary was determined by the method of Lowry et al., (1945). The ovary was dissected out, washed in 0.9% saline and homogenized in 0.5-1.0 ml distilled water. 0.5 ml of the sample was taken in a test tube, to which 2ml of 5% TCA, and 0.5 ml di-nitro phenyl hydrazine thiourea copper sulphate reagent was added. It was mixed well and the test tubes were capped with parafilm and placed in a water bath at 37 º C for 4 hours. The tubes were removed and allowed to chill in ice water. Subsequently 2.5 ml ice cold 65% H₂SO₄ was added and mixed thoroughly and kept at RT for 30 minutes. The reading was measured at 520 nm in a CNL digital analyzer. 1mg % of standard ascorbic acid was also prepared and used as the Reference Standard.

3. 2. B. 4. Histochemistry

Histochemical tests were employed to demonstrate the chemical constituents of inclusion bodies found in the ovary. The tissues were analyzed for the proteins, carbohydrates, lipids and nucleic acids.
3. 2. B. 4. 1. Proteins (Mercuric Bromophenol Blue method)

The paraffin sections (5-7µm) of the tissues were deparaffinized in xylene and then passed through ethanol series to water and stained in mercury bromophenol blue (1%) acetic acid solution, rinsed in 0.5% acetic acid for 5 minutes, transferred to tertiary butyl alcohol, cleared in xylene and mounted in DPX. Proteins were stained a deep clear blue colour (Pearse, 1968).

3. 2. B. 4. 2. Lipids (Sudan Black B)

Sudan black B method was used to stain the total lipids. The deparaffinised sections were brought to 70% ethanol, stained with Sudan black B saturated in 70% ethanol, washed in running water and mounted in glycerine gelly. Lipids were stained black.

3. 2. B. 4. 3. Carbohydrates (Periodic Acid Schiff’s (PAS) reagent)

The paraffin sections (5-7µm) were deparaffinized in xylene, then passed through ethanol series and were brought to water, oxidized by 1% aqueous periodic acid followed by treatment with Schiff’s reagent (Pearse, 1968). The slides were then quickly washed in water and dehydrated through ethanol series and mounted in DPX. Carbohydrates (1:2 glycol groups) were stained deep purple red.

3. 2. B. 4. 4. Nucleic acids (Feulgen Reaction)

The deparaffinised sections, brought to water, were hydrolyzed by 1N HCl exactly for 8 minutes, at 60°C rinsed in distilled water and stained with Schiff’s reagent for 2 hours. The sections were then transferred to bleaching solution (10% potassium metabisulphite solution), washed in running water, dehydrated, cleared in xylene and mounted in DPX. The nucleic acids appeared in reddish purple shade (Pearse, 1968).
3. 2. B. 5. Statistical analysis

Sampling data were primarily analyzed using standard statistical software, InStat (GraphPad InStat, Version 2.00, 1993). Mean, Standard Deviation, Standard Error and Level of Significance (testing hypothesis) were analyzed using this software.

3. 3. B. RESULTS

3. 3. B. 1. Morphology of the reproductive system in female Sesarma quadratum

The reproductive system in females of S. quadratum is comprised of a pair of ovaries located dorsolaterally underneath the carapace. Anteriorly, the two limbs were seen bent backwards. At about the middle of the body, the two limbs of the ovary are connected by a bridge, giving the ovary an ‘H’ shaped appearance. Just posterior to the connecting bridge, the ovary bears a spermatheca on each side (Fig 3.4.A). The oviduct arises from the junction of the ovarian lobe and the spermatheca. Oviduct is a thin tubular structure opening out on the ventral side at the base of the 4th thoracic segment. Spermatheca of S. quadratum is somewhat oval shaped with a stalk of ~330 µm long and ~625 µm wide (Fig 3.4.B).

3. 3. B. 2. Vitellogenesis

The process of ovarian growth in S. quadratum has been arbitrarily classified into six stages viz., I, II, III, IV, V and VI (Table 3.1). Stage I - Ovary appears colourless or white, characterized by transparency of tissues and lack of yolky oocytes. Oocytes present were small in size, with a diameter of less than 50 µm.
Stage II - Ovary appears as creamy or yellow strands. Oocytes at this stage are 50 -100 μm diameter.

Stage III - Ovaries are yellowish brown in colour, with the oocyte diameter ranging between 101 and 150 μm.

Stage IV - Ovaries are vitellogenically active, brown in colour, non-compact with oocyte diameter ranging from 150 to 200 μm.

Stage V - Ovaries are dark brown having large yolky oocytes ready for spawning (200-250 μm).

Stage VI - Ovaries (spent) are inactive, translucent, yellowish flaccid bands with residual eggs. Mean diameter of the ovary and the oocytes are 2-3 mm and 25 μm respectively. Resorbed oocytes of 25-50 μm diameters occur. The left out (residual) eggs are also seen.

3. 3. B. 3. Ovarian factor

   The ovarian factor at the different stages of maturation (Table 3.2) was calculated from the ovary weight and the cube of body weight (after Bomirsky and Klek, 1974). Ovarian factor of *S. quadratum* showed progressive increase from 0.85 to 19.13 during maturation. Spent ovary possesses an ovarian factor of 1.7. The P value of increase in ovarian factor related to various stages of maturation seems highly significant (P<0.0037).

3. 3. B. 4. Gonadosomatic index

   Gonadosomatic index (GSI) (as percentage calculated from the weight of the ovary and the body weight) of the animal varies in different stages (Table 3.1). Pearson’s correlation analysis revealed a statistically significant positive correlation (r^2 = 0.9435;
P< 0.0058) existing between GSI and ovarian maturation. GSI showed a progressive increase from 0.28 ± 0.13 (Stage I) to 7.01 ± 0.55 (Stage V). Stage VI (spent ovary) had a GSI of 0.23 ± 0.31 which closely resembled that of Stage I ovary.

3. 3.B. 5. Nucleocytoplasmic index

Based on the diameter of the oocyte nucleus and the cell as a whole, the nucleocytoplasmic index (NCI) of the oocytes at different stages of maturation was determined using the formula

\[
\text{NCI} = \frac{\text{Volume of nucleus (mm}^3\text{)}}{\text{(Volume of cell — volume of nucleus)}}
\]

As the ovary matures, the NCI gets considerably lowered. The diameter of the nucleus shows an increment from 12µm at Stage I to 25µm at Stage V. The oocyte diameter at Stage I is 25-50 µm while at Stage V, it reaches a size of 225 µm. Considerable enlargement of the oocyte results in the change in its shape from spherical to ellipsoidal or ovoid. The NCIs of maturing ovary show a descending pattern (Table 3.1). NCI at different stages are found to be considerably significant \(r^2 = 0.8316; \ P< 0.0310\).
3.3. B. 6. Histology of the ovary

Histological features of all stages of maturation of ovary of *S. quadratum* were examined. Based on the changes in the colour of the ovary, the oocyte diameter and cytological characteristics, five stages viz., Stage I, Stage II, Stage III, Stage IV and Stage V and one oosorptive or spent stage (Stage VI) have been distinguished (Table 3.1). Histologically, the ovary of *S. quadratum* consists of two functional regions (a) germinal zone and (b) maturation zone.

*Stage I ovary*

The ovary at Stage I appears as white bands and is characterized by the presence of avitellogenic oocytes, with a relatively high nucleo-cytoplasmic index (0.067). Most oocytes do not show any signs of (yolk) synthesis. The germinal zone (GZ) (Fig.3.5) appears very active and shows the presence of significant number of basophilic oogonia. Each oogonium has a large round nucleus (OD – 4.07-7.51 µm) and a thin layer of cytoplasm. The peripheral zone of the ovary is comprised of previtellogenic oocytes (PVO) surrounded by follicle cells (FC) (Fig 3.6). There is a gradual increase in size of the oocytes distributed from the centre of the ovary to its periphery (Fig.3.6). The oocytes at Stage I are generally spherical, each measuring 25-50 µm in diameter. The nucleus lies at the centre of the oocyte and the ooplasm appears finely granular (Fig.3.6). The basophilic follicle cells surrounding the oocytes are arranged as a cord and appear ovoid (Fig. 3.6).

*Stage II ovary*

Ovary at Stage II has yellowish hue and is slightly thicker (~0.64cm) than that of Stage I. GZ containing oogonia in meiotic stages occur at the centre of the ovary. Meiotic
oocytes having a diameter of 4-5 µm and belonging to different divisional stages like leptotene, zygotene and pachytene are also seen at this stage (Fig. 3.7). Previtellogenic oocytes of 50 - 100 µm, are distributed throughout the peripheral (maturation) zone of the ovary. In this stage of vitellogenesis, a layer of follicle cells becomes distinct. Oocytes with distinct basophilic nuclei (13.38-16.58 µm in diameter) are seen located almost in the centre of the cell; the nucleocytoplasmic ratio at this stage is seen to be 0.042 (Table 3.1). There is no apparent synchrony in growth of the oocytes at this stage, evidenced by the occurrence of larger ones (90-100µm in diameter) in the periphery and the smaller (52-75µm in diameter) towards the centre. Follicular cells having ovoid nuclei (2.19 – 5.36 um size) surrounding the oocytes were prominent. The follicle cells at this stage become more conspicuous and form a continuous layer encircling each oocyte. The ooplasm, in general, shows the presence of a great number of basophilic granules (Fig. 3.7). The peripheral ooplasm contains few droplets like structures (Fig 3.7) apparently precursors of yolk. The GZ bears oogonia and premeiotic oocytes (Fig. 3.8). These pre-vitellogenic oocytes, as they grow in size, move from the GZ to the peripheral maturation zone.

**Stage III ovary**

The ovary at this stage is yellowish brown in colour, the oocyte diameter being 101-150 µm and nucleocytoplasmic index being 0.039. The ovary of *S. quadratum* is comprised of compactly arranged vitellogenic oocytes of comparable sizes. Germarium is restricted to the central position. No considerable proliferative activity appeared in the GZ. The nuclei of the oocytes, in general, were centrally placed, and showed the presence of nucleoli. Considerable amounts of yolk start accumulating in the ooplasm (Fig.3.9).
Yolk globules, of 25 - 38 µm in diameter, appear in the ooplasm. Basophilic granules in the ooplasm as seen in Stage II appear concentrated at the perinuclear region. Prominent, synthetically active follicle cells have ovoid nuclei measuring 2.72-3.95µm in diameter (Fig. 3.9).

Stage IV ovary

At this stage, the ovary is brown-coloured. Oocyte diameter ranges between 150 and 200 µm and NCI is only 0.029. Germarium gets restricted and appears strand-like. In a few instances, the germarium shows signs of oogonial proliferation judged by the presence of dividing cells (Fig 3.10.A). Ovary at this stage shows the presence of two populations (peripheral (A) and central (B)) of oocytes in the maturation zone (Fig 3.10.B). Oocytes of population A are characterised by the presence of large and small yolk globules. The nucleus appears ellipsoidal. There also occur several basophilic granules in between the yolk spheres. The vitellogenic oocytes acquire different shapes apparently due to the pressure exerted by the neighboring oocytes. The population B, on the other hand are formed of differentiating oocytes that are poor in their yolk content. Population (B) oocytes resemble the oocytes at stage II. Follicular cells are still active and appear prominent. The cytoplasm of the follicle cells gets stretched out due to the enlargement of the oocytes (10.43 µm long and 4-5 µm wide).

Stage V – (Pre-spawn) ovary

Ovary at Stage V appears dark brown in colour; large vitellogenic oocytes are ready for spawning. NCI is only 0.017. Germinal zone appears as ribbon like patches along the ovary. It exhibits differentiating oocytes and premeiotic oogonial cells at different stages of division (Fig 3.11.B). Oocytes measure 200 – 255 µm in diameter.
Fully vitellogenic oocytes have closely packed yolk globules and cortical granules in the ooplasm (Fig 3.11.A). Cortical granules may be the precursor yolk granules. The nucleus is visible only in very few instances and appears dense due to the accumulated yolk (Fig 3.11.A).

Stage VI – Post-spay Ovary

The ovary at this stage appears as translucent yellowish flaccid band. Residual eggs are often seen in such ovaries. Mean thickness of the ovary is 2-3 mm. Resorbed oocytes of 25-50 \( \mu \)m diameter occur. Germinal zone is inactive, evidenced by the occurrence of non proliferating oogonia represented as a compact structure in the middle of the ovary (Fig 3.12). Some oogonial undifferentiated cells are seen scattered in the peripheral zone. The peripheral zone also displays the presence of the residual follicle cells of the preceding clutch pending lysis.

### 3.3. B. 7. Histochemistry

#### 3.3. B. 7. 1. Proteins

The yolk materials of the ovarian sections imparted a deep metallic blue colour with Mercuric Bromophenol Blue indicating strong positive signal for the presence of proteins (Fig.3.13.A).

#### 3.3. B. 7. 2. Lipids

Lipids are sudanophilic substances (SBB positive) and so are stained positively with Sudan black B. The sections of ovary also showed positive colouration with Sudan black B confirming the lipoproteinous nature of the yolk substance (Fig.3.13.B)
3. 3. B. 7. 3. Carbohydrates

It was found that the sections of the ovary gave a uniform pattern of staining (positive) with PAS indicating the presence of carbohydrate molecules in the yolk globules of the oocytes (Fig 3.13.C).

3. 3. B. 7. 4. Nucleic acids

Feulgen staining was used to identify chromosomal material (nucleic acids) in the oocyte. The nucleus of the oocyte as well as that of the follicle cells showed the presence of Feulgen positive materials (Fig. 3.13.D). The follicle cell nuclei gave a strong positive result. The ooplasm also showed a poor staining suggesting the occurrence of some nucleic acid (RNA) material.

3. 3. B. 8. ELECTRON MICROSCOPY

Stage I ovary

Electron micrograph of ovary in Stage I shows mostly undifferentiated oocytes, before the accumulation of yolk (Fig 3.14.A). The nucleus has a coarse pattern of heterochromatin concentrated in the centre and the periphery. Electron-dense granules and a few mitochondria were seen (Fig 3.14.C). Some developing oocytes that show the presence of small yolk materials were seen in the ooplasm. Golgi apparatus was seen in the juxtranuclear position of the oocyte (Fig 3.14.B).

Stage II ovary

Electron microscopy of the ovary in Stage II reveals the presence of oogonial cells in the germinal zone (Fig.3.15.A). These oogonial cells are in various stages of divisions. Oocytes undergoing meiotic stages were also seen. Some oocytes have condensed chromatin while some others have chromatin in the pachytene stage with
chiasmata. Some oogonia were in anaphase stage (Fig. 3.15.A). Oocytes have large nuclei, but lack nucleolus. The cytoplasm of oocytes in Stage II displayed the presence of a highly developed rough endoplasmic reticulum (RER) in a unique pile - like arrangement (Fig 3.15) evidencing synthetic activity of the oocytes. Ooplasm is also rich in mitochondria. A few small-sized yolk spheres appeared in the ooplasm at Stage II.

Follicle cells showed the presence of large oval nuclei. The peri-nuclear region (of FC) was seen surrounded by several mitochondria, few ribosomes and some RER (Fig 3.16). The close vicinity of plasma membrane showed the presence of many dense pinocytotic vesicles. Adjacent to the oocyte plasma membrane, these vesicles appear to be pinocytotic in nature. Pinocytotic vesicles show signs of fusion (coalescence) with the oolemma to release the contents into the ooplasm, with clear signs of exocytosis (Fig 3.16.B). Some follicle cells show the presence of smooth endoplasmic reticulum (SER), in the same cell where exocytosis was observed. The presence of SER may be an indication of the synthesis and/or further processing of the yolk materials through conjugation with lipid. RER is present in the follicle cells, but not in large numbers. Adjacent follicle cells also show interdigitations with the oocytes.

Stage III ovary

Electron microscopic observations of Stage III ovary showed clearly that the major part of the ooplasm was occupied by the yolk. The nucleus of the oocyte appeared prominent (12 µm in diameter) and spherical. Follicle cells were with oval shaped nuclei (about 5µm long). The germinal zone was also comprised of several gonial cells that were not in divisional phases. The nuclei of the oogonia in the GZ had condensed chromatin.
Stage IV ovary

Transmission electron microscopic observations revealed the occurrence of a well differentiated peripheral zone and a central germinal zone in the ovary. The peripheral zone is shown to have two populations of oocytes: Population-A, comprising of already differentiated oocytes with profuse presence of yolk globules, and Population-B, comprising of already differentiated oocytes, but having little amount of yolk materials within. The germinal zone, on the other hand, showed the presence of a number of dividing (zygotene, diplotene and diakinesis) and differentiating germ cells (Fig 3.17.A). The oocytes present at the peripheral region (Population-A), were rich in yolk globules of varying sizes (3-70 µm in diameter) and possessed well defined nucleus and nucleolus (Fig.3.18.A), indicating synthetic activity. The oolemma shows many inpushings suggesting intake of vesicular materials from the follicle cells (Fig 3.18.B).

In Population-B, on the other hand, the ooplasm is rich in mitochondria of variable sizes and shapes, present in clusters, while golgi bodies and RER were present in small numbers (Fig 3.19.A). In several occasions, RER were closely associated with the golgi (Fig.3.19.A). Some of the mitochondria were much elongated reaching about 40 µm long. The follicle cell cytoplasm of the Population-B oocytes was found to contain large number of mitochondria and some RER (Fig 3.19.B).
Stage V ovary

The oocyte is completely packed with homogenous yolk granules of various sizes. Mature oocytes have cytoplasm with spherical vacuoles, apparently due to the leaching, thus reflecting the presence of lipid droplets. Lipid droplets in the electronmicrograph are less dense and appear pale grey. Some granular materials, apparently yolk precursors, were also seen in between the yolk. Numerous pinocytotic vesicles appear to get accumulated on either sides of the oocyte membrane; some of these are found to release the contents into the cytoplasm suggesting incorporation of extra-ovarian substances. The small vesicles that were in the cortical ooplasm fuse to form larger yolk bodies. Some mitochondriae and dilated RER appeared in the follicle cell which could be considered as signs of synthetic activity (Fig 3.20).

3. 3. B. 9. BIOCHEMISTRY

3. 3. B. 9. 1. Water

The water content of the ovary throughout the vitellogenic cycle in S. quadratum was assessed through comparison of the dry weight with the wet tissue weight. The amount of water up to the mid stage ovary appeared to be about 82%. As maturation progressed, the level fell to about 45-50% (Table 3.3).

3. 3. B. 9. 2. Total Protein

A high significant fluctuation in the ovarian protein profiles of S. quadratum was noticed in relation to various stages of maturation (P<0.0001) (Table 3.4). The ovary in Stage I contained only 9.47 ± 3.07 mg /100 g body weight of total protein (TCA extractable). This gradually increased to 33.34 ± 6.31 mg /100 g body weight (3.6 fold)
(previtellogenesis period) as the ovaries attained Stage II. This was followed by rapid increase in the total protein levels to $215.32 \pm 25.58 \text{ mg /100 g body weight (6.5 fold)}$ during the following early vitellogenic Stage III. Then onwards a doubling of the protein profile was noticed in the succeeding Stages (IV and V). Stage IV was characterized by $483.01 \pm 146.55 \text{ mg /100 g body weight}$ of total protein, while a maximum of $810.04 \pm 52.32 \text{ mg /100 g body weight}$ total protein was found to occur in Stage V, as the animal was ready for oviposition. The spent ovary showed a relatively small protein profile of $15.42 \pm 2.62 \text{ mg /100 gm body weight}$.

3. 3. B. 9. 3. Lipids

During the early stages of oogenesis, the ovary of *S. quadratum* was very poor in its total lipid components (chloroform extractable). Only very small amounts of lipid appeared in the ovary at this stage. The Stage II showed a slight increase in the lipid profile ($40.55 \text{ mg /100 g body weight}$). Then onwards, the lipid fraction showed a dramatic increase to $74.26 \pm 26.31 \text{ mg /100 g body weight}$ at Stage III and $156.80 \pm 13.16 \text{ mg /100 g body weight}$ at Stage IV and $766.25 \pm 176.61 \text{ mg /100 g body weight}$ at Stage V (Table 3.4). Lipid levels appeared at its peak during the transformation of Stage IV to Stage V. Stage VI showed a deep fall in lipid levels.

3. 3. B. 9. 4. Polysaccharide fraction

Variation in the profiles of the polysaccharide fraction of the ovary of *S. quadratum* is evident from Table 3.5. It revealed a gradual increase in the profile as the maturation proceeds. The polysaccharide fraction of the ovary in the Stage I of maturation was only $0.27 \pm 0.10 \text{ mg /100 g body weight}$. This rapidly increased to $27.03$
± 10.97 mg /100 g body weight during Stage II, followed by a gradual increase to 35.64 ± 6.82 mg /100 g body weight during Stage III. Stage IV was characterized by 39.63 ± 14.10 mg /100 g body weight of polysaccharide fraction. The maximum profile was exhibited at the pre-oviposition period (Stage V) i.e., 89.25 ± 16.55 mg /100 g body weight. The spent ovary showed relatively small levels (26.87 ± 4.88 mg /100 g body weight) of polysaccharide fractions, compared to Stage V.

3. 3. B. 9. 5. Oligosaccharide fraction

Oligosaccharide fractions in the ovarian tissue of *S. quadratum* are shown in the Table 3.5. The Table revealed a gradual increase in the profile during the maturation process. The ovary in Stage I showed an oligosaccharide content of 0.20± 0.15 mg /100 g body weight. This reached to 22.24 ± 10.85 mg /100 g body weight, during Stage II. Subsequently, the level reached upto 24.80 ± 17.80 mg /100 g body weight during Stage III and 25.91 ± 3.70 mg /100 g body weight at Stage IV. A peak oligosaccharide profile of 89.25 ± 16.55 mg /100 g body weight was shown by the ovary in late Stage V. Soon after oviposition, the ovary showed a comparatively low oligosaccharide profile (10.64 ± 1.69 mg /100 g body weight).

3. 3. B. 9. 6. Total FAA

Total FAA constituted about 1-2% of the ovarian wet weight in *S. quadratum*. The Stage I ovary has FAA only in traces. In Stage II, the concentration reached 4.00 ± 1.23 mg /100 g body weight. As vitellogenesis progressed, the level showed a slight increase (4.87 ± 0.90 mg/100 g body weight) at Stage III and subsequently, at Stage IV, it rose to 8.72 ± 1.85 mg /100 g body weight. Finally, in Stage V ovarian total FAA was at
its peak i.e., 21.99 ± 6.32 mg /100 g body weight (Table 3.6). The levels of total FAA show a significant correlation (P < 0.001) with ovarian maturation.

3. 3. B. 9.7. Individual Amino acids

The amino acids of the ovary at various stages were ethanol extracted and subsequently concentrated by ion exchange chromatography (Dowex-50) and the eluted samples were run in paper as well as thin layer chromatographic plates. Chromatographic separation by both paper and thin layer chromatography yielded similar results; a number of amino acids in its free form were found to be present. The amino acids were identified based on the Rf values of Reference Standards (after Giri et al., 1956), eluted and then quantified colorimetrically using glutamic acid as the standard. Most prominent FAAs were leucine, isoleucine, tryptophan, methionine, proline, threonine, glutamic acid, lysine, histidine and serine.

3. 3. B. 9.8. Ascorbic acid

Traces of ascorbic acid were found to occur in the ovary of *S. quadratum*. In Stage I ovary, ascorbic acid appeared in traces only, and hence could not be estimated. The Stage II ovary exhibited a quantity as low as 0.78 ± 1.23 mg/100 g body weight. The ascorbic acid profile reached 2.58 ± 1.31 mg/100 g body weight at Stage III and 2.63 ± 1.49 mg/100 g body weight at Stage IV. Peak occurrence of ascorbic acid (7.47 ± 3.50 mg/100 gm body weight) was recorded in the late Stage V ovary (Table 3.6).
3. 4. B. DISCUSSION

The present examination reveals that the ovary of *S. quadratum*, in its structure and physiology, resembles very much to those of other brachyurans described in the past (Anilkumar, 1980; Adiyodi & Subramonian, 1983; Sudha and Anilkumar, 1996; López et al., 1997; Rodrigues et al., 1997; Litulo, 2005). Significant colour changes in the ovary during the process of maturation in relation to degree of yolk deposition, is well known for a number of decapod crustaceans like penaeid shrimps (Dall et al., 1990), and in brachyuran crabs (Anilkumar, 1980; Adiyodi & Subramonian, 1983; Meusy & Charniaux-Cotton, 1984; Arculeo et al., 1995; Sudha and Anilkumar, 1996). In *S. quadratum*, ovarian maturation depicts the transformation of white ovary through yellow, yellowish brown, brown to dark brown colour. The change in colour of the ovary may be attributed to modifications of carotenoid content, occurring during the oogenesis, that play an important role during embryogenesis (Goodwin, 1951). The average GSI in females progressively increased from 0.28 ± 0.13 in Stage I ovary to 7.01 ± 0.55 in Stage V (Table 3.1). The decrease in the nucleocytoplasmic ratio (Table 3.1) of the oocytes is consequent on the expansion of the cytoplasm with its synthetic machinery and the incorporation of yolk. This is in correlation with the increase in the oocyte size from Stage I to Stage V as a result of the accumulating yolk in the cytoplasm. Present histological and histochemical studies reveal that *S. quadratum* possesses two functional zones; the germinal zone or germarium and the maturation zone. The position of the germarium in *S. quadratum* is comparable to other decapod crustaceans such as *L. emarginata* (Hinsch, 1970), *Cancer pagurus* (Eurenius, 1973), *Ocypoda ceratophthalma*
Krishnakumar et al., 1979, *P. hydrodromous* (Anilkumar, 1980), *Ocypoda platytarsis* (Pillai, 1982) and *M. messor* (Sudha and Anilkumar, 1996). The germinal zone is located at the centre of the ovary while the differentiating and maturing oocytes are seen towards the peripheral region. Previous workers have shown that the proliferative activity of the germinal zone is greater in the post-ovipository period. A cyclicity in the proliferation of the germarium has been reported in *G. lateralis* (Weitzman, 1966), *P. hydrodromous* (Anilkumar, 1980), *G. steniops* (Santhamma, 1985), and *M. messor* (Sudha and Anilkumar, 1996). Cyclical changes in the germarium have been noticed with progressing ovarian maturation. Light and electron microscopic studies on oogenesis in *S. quadratum* (present study) reveal that proliferation in the germinal zone starts at a very early stage i.e., even before the extrusion of the first clutch of eggs during the active breeding season.

These observations encourage us to address the question of germinal zone activity in tune with the reproductive performance of this grapsid crab (*S. quadratum*). Our continuous observations on breeding (and moulting) physiology of the species, coupled with the present histological and ultrastructural observations prompt us to draw some interesting contentions. It may be pertinent to recall at this juncture that *S. quadratum* (the present study, as described in Chapter 2) releases as many as 12-16 broods a year. During the breeding season (August to succeeding January) the population is highly prolific i.e., during a span of six months, releasing about 12 broods. This would mean that the population releases a clutch in almost every fortnight (a correlation with lunar periodicity as discussed in the previous chapter), without any interruption. The present study reveals that the oocytes for each clutch of eggs are being proliferated, differentiated
and matured from the germinal zone (germarium) situated in the central portion of the ovary. These observations on the continuously spawning crabs reveal that as the oocytes attain Stage IV, another layer (line) of oocytes in Stage I (pre-vitellogenic) are being primed to undergo vitellogenesis for the succeeding clutch of eggs. Such a demonstration of the cyclic changes of germarium, in tune with the vitellogenic cycle, aiming at uninterrupted (continuous) breeding is hardly reported in a decapod crustacean. It is our hope that such an assessment of the cytological events taking place in a growing ovary, transcending succeeding clutches of brood (as reported in the present study), would hopefully help us answer many of the basic questions in continuous breeding biology of a decapod. Another aspect that draws our attention is the pattern of germarium activity during non-breeding season. In February – March season, for example, approximately 44% of the females engage in breeding, wherein the germarium is differentiated and primed within the succeeding 15 days, so as to release another clutch in another fortnight. However, about 35% of the females abstained from breeding activity in February. It seems that out of the 56% of the non-breeding crabs, 15.7 % preferred to enter premoult stage, and therein breeding activity was seen to be arrested. This would mean that from February- March onwards, germarium in a sizable proportion of the population showed a restraint in its differentiation and maturation. It remains to be seen what are the exact mechanisms involved in shifting the germarium activity in those females who abstained from breeding activity.

The germinal zone of the Stage I ovary shows proliferative activity as evidenced by the dividing oocytes (Fig.3.5). Moving cords of follicle cells could be observed in early Stage I ovary. These FCs later encircle the differentiated oocytes. The electron
micrograph of the oocyte at this stage exhibits presence of Golgi bodies (Fig 3.16) indicating its synthetic machinery in action. The Golgi may be involved in the synthesis of yolk, a preparative process of vitellogenesis. In *Penaeus kerathurus*, early yolk formation is endogenous and derives from the activity of the nuclear envelope, ER and Golgi complexes, and that lipid droplets are products of intracellular membrane recycling activity occurring within large multivesicular bodies (Carvalho et al., 1998).

In the ovary of the pubertal females, several differentiated follicle cells were seen concentrated in the germinal zone and moving towards the peripheral zone to encircle the differentiated oocytes (Fig.3.6.). These follicle cells appear to move as cords and several follicle cells were seen around the oocyte. Streaming movement of follicle cells have been reported in *C. maenas* (Laulier and Demeusy, 1974) and *M. messor* (Sudha and Anilkumar, 1996). The enveloping of the oocyte by the follicle cell has been described in the ovaries of *C. maenas* (Laulier and Demeusy, 1974), *M. idella* (Vijaya, 1989), *P. hydrodromous* (Anilkumar, 1980), *M. messor* (Sudha and Anilkumar, 1996). The number of follicle cells remains almost constant throughout the vitellogenic cycle. At the beginning, the follicle cells appear spherical or oval but once they envelop the oocyte, it acquires an oval shape. The changing size and shape of follicle cells were formerly reported by a number of workers in insects (Bast and Tefler, 1976), and in the brachyuran crabs *P. hydrodromous* (Anilkumar, 1980), *M. messor* (Sudha, 1993). The round FCs in the initial stages of vitellogenesis are believed to have intense biosynthetic activity (Chinzei et al., 1987; Van Herp and Payen, 1991; Tsutsi et al., 2000).

The appearance of lipid droplets and abundance of RER and mitochondria in the ooplasm indicate high autosynthetic activity at this stage. It could also be worth
mentioning in this context that our electronmicrograph showed exocytosis of vesicular materials from follicle cells to the adjacent oocytes (Fig. 3.16). Additionally, we could observe the occurrence of several pinocytotic vesicles (Fig. 3.16.B) at the membrane border of follicle cells. The pinocytotic vesicles release their contents into the oocyte (Plate 3.16.B). Further, the exocytotic vesicles emanating from the follicle cells surrounding the Stage II oocytes of *S. quadratum* signify the autosynthetic yolk formation during the early stages of oogenesis. Autosynthesis of yolk substances at early stages of vitellogenesis have been reported earlier (Desantis et al., 2001). This observation supports the follicular synthesis of ovarian yolk substances and their subsequent release into the oocytes. Extra-oocytic yolk precursors appear to pass from the hemolymph into the follicle cells and subsequently into the oocytes via micropinocytosis. Oocytes of second stage ovary indicated the presence of numerous RER, mitochondria and ribosomes giving ample proof for the ensuing yolk synthesis. Follicle cells also bear RER providing evidence for their role in the vitellogenesis. The number of RER becomes more as the vitellogenesis proceeds. RER being the machinery for the synthesis of protein yolk in the mid and late vitellogenic stages appear more dilated. Contribution of the follicle cells to the growing ovaries at the initial stages of vitellogenesis were reported earlier (Ching and Shih, 1995; Tsutsi et al., 2000). Similarly, sequestration of yolk materials from extra-ovarian sites are also reported previously (O’Donovan et al., 1984; Chinzei, 1987; Desantis et al., 2001). Thus, after having an overview of the entire mechanism of vitellogenesis, it appears quite plausible that during the early stages, the ovary of *S. quadratum* relies on the yolk materials synthesized within the ovary, while towards the later stage (Stage V), it relies on the materials incorporated
from without, a pattern akin to what has been described in some of the brachyuran crabs previously (Meusy, 1980; Adiyodi and Subramoniam, 1983; Meusy and Payen, 1988; Sudha and Anilkumar, 1996). However, it is still to be explored whether there exists distinct differences in chemistry of the yolk synthesized intraoocytically and that incorporated from extraoocytic sources.

Yolk uptakes by growing oocytes through pinocytosis were observed in *Oniscus ascellus* (Beam and Kessel, 1980) the amphipod, *Orchestia gammarella* (Zerbib, 1976, 1977) and decapods (Krishnakumar et al., 1979, Schade and Richard, 1980). Munuswamy and Subramoniam (1985) have reported the incorporation of heterosynthetic yolk into the oocyte in *Streptocephalus dichotomous*. Van Herp and Payen (1991) and Tsutsi et al. (2000) have suggested the role of follicle cells in vitellogenin production.

Extra-oocytic yolk precursors appear to pass from the hemolymph into the follicle cells and subsequently into the oocytes via micropinocytosis. Pinocytotic vesicles fuse in the cortical ooplasm to form large yolk bodies. Several papers on biochemical variations during reproduction have demonstrated that other tissues and organs, besides the hepatopancreas and ovary, can accumulate organic reserves (Palacios et al., 2000; Cavalli et al., 2001; Rosa and Nunes, 2002). In females, during secondary vitellogenesis, synchronous growth of the oocytes occurs through endocytotic uptake of vitellogenin (Charniaux-Cotton, 1985). This accumulation of yolk in the ovary during preparation for reproduction can be observed in the increase of the GSI values in females as the ovary develops. Similar results were found in other crustaceans: *Macrobrachium rosenbergii* (Cavalli et al., 2001), *Aristeus antennatus*, *Parapenaeus longirostris* and *Nephrops norvegicus* (Rosa and Nunes, 2003).
The present histological and ultrastructure studies also reveal that the germinal zone which remains inactive in the Stage III shows signs of proliferative activity, with divisional stages appearing from Stage IV onwards (Fig 3.1A). The maturation zone at this stage shows the presence of two populations of oocytes: Population-A, consisting of mature and yolk-filled oocytes and Population-B, comprising newly differentiated oocytes placed towards the centre. Oocytes of Population -A showing the vesicular intake at its oolemma suggest incorporation of yolk materials from extraovarian sources. The active follicle cells surrounding the oocytes with rich RER and mitochondria strengthen the heteronthetic yolk formation.

The oocytes of ovaries at Stage V are filled with yolk, while its cytoplasmic organelles are inconspicuous (Fig. 3.20). The oocytes at this stage also exhibit the presence of numerous pinocytotic vesicles proximal to the oolemma, strengthening the incorporation of yolk materials from extraovarian sources (heterosynthesis) towards the late stage of vitellogenesis. Pinocytotic vesicles incorporated into the oocytes fuse to form larger yolk bodies. Pinocytotic vesicles have been noticed in the cortical ooplasm to form heterosynthetically derived type 3 yolk bodies in the copepod, *Labidocera aestiva* (Palmela et al., 2005). The oocytes of Stages III to V do not show the presence of Golgi bodies. The non-occurrence of Golgi may be due to the obliteration of these structures as a result of the accumulating yolk and may impose inability for the synthetic processes.

Ovarian development in crustaceans during the annual reproductive cycle is not only marked by an increase in mass, but also changes in biochemical composition (Tesima et al., 1988; Harrison, 1990; Smith, 2004; Reppond et al., 2009). Biochemical analysis of the ovary of *S. quadratum* has revealed that the yolk is formed of proteins,
lipids, carbohydrates and water. Protein and lipid form the major constituents of yolk in *S. quadratum*. In the early stages of maturation, major part (82-88%) of the ovary is constituted by water. Water content of the ovary comes to about 55-60% in the late stage ovary. About 19% of the ovarian wet weight is constituted by protein, while 17% is composed of lipid. The presence of significant quantities of both proteins and lipids in the yolk of *S. quadratum* appears to be keeping with the need for a long period of growth and maintenance of the embryo. The lipoproteinous nature of the yolk in decapod crustaceans has been reported by earlier workers also. Protein to lipid ratio in the ovary shows wide variation in the different species. The percentage of protein and lipid comes to about 24 and 23% respectively in *P. hydrodromous* (Anilkumar, 1980) while it approximates 20 and 18% respectively in *M. messor* (Sudha, 1996). In *Ocypoda platytarsis* (Pillai, 1982) the percentage of protein is estimated to be 22.5%, whereas the lipid shows a low profile of 10.02% only. The histochemical analysis by MBB, SBB and PAS reveals that the yolk material is composed of protein, lipid and carbohydrates. Results of quantitative analysis indicate that the profiles of protein show progressive increment during the different stages as vitellogenesis proceeds (Table 3.4). During the early stages of oogenesis the ovary of *S. quadratum* is very poor in its lipid components. This is in conformity with the observations in *P. hydrodromous* (Anilkumar, 1980) and *M. messor* (Sudha, 1993). An increase in the concentration of the lipid appears during Stage II of ovarian maturation, when the synthesis of lipid yolk commences. Ovarian lipid levels during the early stages of vitellogenesis show ~ 46% increase from Stage II to III of vitellogenesis which spans about 5 days, while a 53% increment was shown in three days when it attained stage IV. The lipid profile showed a steep elevation of about 79% in 2-3 days to reach the pre-
spawn stage (Stage V). A comparative correlation between the low water content and high lipid reserve to meet high energy demand during embryogenesis has been suggested by Romanoff and Romanoff (1967). Quantitative assays (Dubois et al., 1956) showed a marked difference in the distribution of poly- and oligo-saccharide fractions in the ovarian tissue at the developing stages. Polysaccharide fractions however, predominate in the ovary. Crustacean blood is as important as the hepatopancreas for the storage and metabolism of polysaccharide (Johnston, 1971). The free amino acid content of the ovary seems to be 1-2% only. A significant increase in the free amino acid was found in the ovary during maturation. Paper and thin layer chromatography reveals that the major amino acid fractions of the ovary are leucine, isoleucine, tryptophan, methionine, proline, threonine, glutamic acid, lysine, histidine and serine. Glutamic acid being a metabolite linked to Kreb’s cycle could be suggested to play an important role in the energy production as well (Schoffeniels and Gilles, 1970). The total FAA of *S. quadratum* shows similarity to the FAA found in *M. messor* (Sudha, 1993). Alanine, Arginine and Glutamic acid appeared to be major FAA in the ovary of *M. messor* (Sudha, 1993). Utilization of FAA as a source of energy has been described in crustaceans (Huggins and Munday, 1968) and in insects by Gilmour (1965). During the last embryonic stage, decreasing concentrations of amino acids such as lysine and arginine, were glutamic acid and valine was recorded shrimp *Lysmata seticaudata* (Calado et al., 2005). Minute quantities of ascorbic acid have been detected in the ovary of *S. quadratum*. The reducing property of ascorbic acid is said to give a protective action. Ascorbic acid has been found to enhance fertility in bulls (Barnes, 1962). Protein droplets appear in the developing oocytes during
the early phase of vitellogenesis. The yellowish brown hue of the ovary suggests the presence of carotenoids.

Genesis of follicle cells in *S. quadratum*, as observed under light and electron microscope is worth discussing. It may be recalled that the differentiated oocytes are not enveloped completely by the follicle cells in Stage I. Subsequently when the oocytes get differentiated, the follicle cells invade the inter-oocytic spaces as cords (Fig. 3.6) and envelope the oocyte. Streaming movement of follicle cells prior to its encasing the oocytes have been reported in *C. maenas* (Laulier and Demeusy, 1974) and *M. messor* (Sudha and Anilkumar, 1996). The enveloping of the oocyte by the follicle cells has been described in the ovaries of *P. hydrodromous* (Anilkumar, 1980), *M. messor* (Sudha, and Anilkumar 1996). At the beginning the follicle cells appear spherical or oval but once they envelop the oocyte it acquires an oval shape. The changing size and shape of follicle cells were formerly reported by a number of workers in insects (Bast and Tefler, 1976) and brachyuran crabs like *P. hydrodromous* (Anilkumar, 1980) and *M. messor* (Sudha, 1996). The biochemical composition of the yolk determines the pattern of embryonic development and the stage at which the young ones are released. Protein lipid ratio in different groups of invertebrates as well as vertebrates differs considerably.

The structural and phylogenetic data suggests a relationship between the major egg yolk precursor protein of decapod crustaceans with insect apolipoprotein (apoLp-II/I) and vertebrate apoB and thereby recommended the name apolipocrustacein (apoCr) rather than Vitellogenin (Vtg) (Avarre et al., 2007). In the blue crab, *Callinectes sapidus*, 27.5% of the total lipids were found in the lipid droplet fraction of mature oocytes (Lee and Walker, 1995). Lipids and proteins were identified as major components (56.6 and 37.6%
respectively) and carbohydrate minor component (5.8%) of the calculated energy content in the bivalve *Nucula turgida* (Davis and Wilson, 1983). A similar biochemical composition in the spawned eggs of the mussel, *Mytilus galloprovincialis* (proteins 45%, lipids 22% and carbohydrates 3%) have been reported by Sedano (1995). Within mammals, there is a 95% identity between the corresponding proteins. Even the proteins of more distant species such as the chicken and *Xenopus laevis* share 84% and 73% identity respectively with the human VLDLR (Schneider et al., 1999). Avian egg yolk contains approximately 50% by weight of water and 35% of lipid, the rest being mostly protein. It has been reported that a balance of amino acid and fatty acid composition present in diets of *M. rosenbergii* affect the dynamics of growth and reproduction (Wilder, et al., 1994).

### 3.5. SUMMARY

1. *S. quadratum* attains sexual maturity at a CW of 9 mm. Females having a CW less than 6 mm are juveniles and showed no external secondary sexual characters. The undifferentiated gonad of the juveniles contained several germinal cells with condensed chromatin. As growth proceeds, the gonads get differentiated into testis in juvenile males and ovary in juvenile females.

2. The ovary in *S. quadratum* is ‘H’ shaped. Significant colour changes occur in the ovary during the process of maturation in relation to degree of yolk deposition.

3. The ovary at various stages of maturation shows a progressive increase in the gonadosomatic indices. Accumulation of yolk in the cytoplasm results in increase in size of the oocytes and a resultant decrease in the nucleocytoplasmic index.
4. Histological and ultrastructural observations revealed that the ovary in *S. quadratum* has two functional zones: a central germinal zone comprising oogonia and a peripheral maturation zone with oocytes. The germinal zone shows proliferative activity during vitellogenesis culminating into oocytes. Oocytes are surrounded by follicle cells. Oocytes grow in size by accumulation of yolk.

5. Yolk formation occurs in two ways A) Autosynthesis- during which the synthetic machinery of the oocytes become active. Follicle cells are also involved in the synthesis of yolk. B) Heterosynthesis- during which yolk synthesized elsewhere are sequestered to the oocytes.

6. Ultrastructure of the ovary revealed the occurrence of exocytosis from the follicle cells into the oocytes.

7. The active germinal zone with two populations of oocytes in Stage IV ovary reveals the development of a second clutch of oocytes in the ovary, evidencing continuous breeding in the species.

8. Biochemical analysis of the ovary of *S. quadratum* has revealed that significant fluctuations occur in the ovarian profiles at various stages of maturation. The major organic reserves of ovary such as lipids, proteins, FAAs, polysaccharides, oligosaccharides and ascorbic acids were found to show significant increase in relation to stages of ovarian maturation.