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Histology and Histochemistry of the Male and Female Reproductive System of the Sesarmid Crab *Muradium Tetragonum*

Pothiappan Kumarasamy[1], Viswambaram Ganapiriya[1], Kannayiram Muthukumaravel[1], Manickam Sasipriya[1], Bharathi Santhanabharathi[2] Mohamed Saiyad Musthafa[2] and Marckasagayam Priyadharshini[2†]

[1]*P.G. and Research Department of Zoology, Khadir Mohideen College, Affiliated to Bharathidasan University, Adirampattinam, Tamil Nadu 614 701, India,* [2]*Unit of Research in Radiation Biology & Environmental Radioactivity (URRBER), P.G & Research Department of Zoology, The New College (Autonomous), Affiliated to University of Madras, Chennai, Tamil Nadu 600 014, India.*

Abstract

The sesarmid crab *Muradium tetragonum*, inhabiting the mangrove, are considered as a key consumer of litter and thereby play an important role in the detritus food chain and energy flow in the mangrove ecosystem. The present investigation was carried out with objectives to enlighten the reproductive system of *Muradium tetragonum* through histological and histochemical studies. Histological organization of the testis of *M. tetragonum* revealed that each testis has a lobular structure consisting of several testicular lobules arranged around the collecting duct. Histology of the deferens of *M. tetragonum* revealed it to be composed of three-layer of tissues along the entire length:the outer connective tissue, the middle muscular and the inner epithelial layer. Based on the histological architecture these three regions are recognized as proximal vas deferens (PVD), middle vas deferens (MVD) and distal vas deferens (DVD). Histological characteristics of the ovary of *M. tetragonum* during different phases of ovarian development were studied. Based on the colour changes of the ovary and diameter of the oocytes five stages of ovarian development can be pronounced. Histochemical analysis of the male reproductive tissues of *M. tetragonum* signifies the secretion of a different biomolecule by specifying their origin in the reproductive tissue and their possible transformation into spermatophores. In the female reproductive tissues, histochemical evaluation envisaged the secretory products during different stages of ovarian development The secretory substances of the spermatheca expound on the significance of its secretion in dehiscing the spermatophore wall and in nourishing as well as protecting the spermatozoa.

Keywords: *M.Tetragonum*, testis, vas deferens, Accessory sex gland, ovary

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† Corresponding author: mpriyadharshini001@gmail.com

1. Introduction

Decapod crustaceans exhibit different patterns of breeding, which are related with the efforts of the species to cope with the respective ecosystem it inhabits. Brachyuran crabs, considered as one of the broadest crustacean groups, occupy a variety of marine environments including coral reefs, sandy beaches, rocky beaches, mangroves, and seagrass meadows[1-2]. Ecofriendly and environmentally significant crabs are abundantly found in the mangrove forests and are highly important in assessing the mangrove ecosystem[8]. The sesarmid crabs are considered "ecological engineers" since they play a noteworthy role in the detritus food chain and energy flow[5-7]. The male reproductive organ of decapod crustaceans is composed of testis and vas deferens (VD) leading to the external gonopore. Rarely in some species, at the junction between the VD and ejaculatory duct (ED) receive tubular, saccular, rosette, coral-shaped glandular structures which are described as accessory glands (AG). Some glandular structures arising in an adventitious manner are also involved in the production and/or protection of semen. Oogenesis is an energetically expensive reproductive process that is generally divided into primary and secondary phases, the former is characterized by primary oocyte recruitment from oogonia[8-9] and the latter featuring the growth of the oocytes[10]. The histochemical organization of the ovary of the test crab *M. Tetragonum* revealed that its histological architecture has a close resemblance to those of other brachyuran crabs[11-13]. The reproductive cycle of crustaceans has been studied in detail in commercially important species such as *Macrobrachium rosenbergii*[14], *Callinectes sapidus*[15], *Scylla paramamosain*[16], *S. olivacea*[17] and in some ecologically important species such as *U. rapax*[18], *Armases rubripes*[19], *Goniopsis cruentata*[20] *Ucides cordatus*[21]. The literature describes male and female reproductive systems of crustaceans, but studies on the physiology of reproduction are far from satisfactory. The present study was aimed to elucidate the male and female reproductive system by detailed analysis of the sperm formation, the seminal secretion in the VD and with emphasis to spermatheca of the sesarmid crab *M. tetragonum* through histological and histochemical studies.

2. Materials and methods

2.1 Collection and maintenance of crabs

The experimental crab of the present study *M. tetragonum* was collected from Agniyar estuary, Tanjavur district, Tamil Nadu. Upon transport to the laboratory, Crabs were divided into male and female by observing their abdomen and chelipeds. The following day, the moulting stages of the crab were identified by observing the setagenic events of the epipodite of the maxillipedes after[22]. Crabs were fed with puffed rice and rice flakes. Water was changed on alternate days. Crabs (3.3±0.5 cm) alone were taken for study. The hard carapace was dissected, and the morphological structure of both male and female reproductive systems was observed.

2.2 Dissection

The intermolt male and female crabs with a carapace width of 2.2-3.2 cm were considered for the present study. The reproductive system

of the crab was dissected by cutting off the dorsal portion of the hard carapace. Dissection was done under a dissection microscope using 0.9% saline as the medium. The testes, the different regions of the vas deferens i.e. PVD, MVD, DVD, accessory sex gland (ASG) and the ejaculatory duct (ED) were removed from the male crab and the ovary, oviduct and spermatheca removed from the female crab were used for the experimental analyses. A portion of tissue for observation was made into a smear on a clean slide stained with methylene blue and observed under a microscope.

2.3 Histological Study

The testes of the experimental crabs were fixed in Carnoy's fixative, while the different regions of the VD, the accessory sex gland (ASG) and the ejaculatory duct (ED) and the female reproductive tissues, i.e. ovaries and spermatheca were fixed in alcohol. After 24 hours of fixation, the tissues were passed through an ascending series of alcohol for dehydration and cleared in xylene or methyl benzoate. The paraffin-block was sectioned to a thickness of 7 μm using a rotary microtome. The sections were stained with Harris' hematoxylin and then counterstained with 1% alcohol eosin[23].

2.4 Histochemical Study

For the detection of proteins, carbohydrates, and lipids by histochemistry, testes, three different regions of the VD namely PVD, MVD, DVD, the ejaculatory duct and female reproductive tissues of *M. teragonum* were fixed in 5% cold neutral buffered formalin. After fixation, they were washed in water, dehydrated in an ascending series of alcohol, and cleared in methyl benzoate, the tissues were embedded in paraffin wax (melting point 58-60℃) and 7μm thick tissue sections were taken. The tissue sections on the slide after deparaffinization and hydration were used for histochemical analysis.

To detect the basic protein, the acidic protein, free amino group, the tyrosyl group, the tryptophanyl group, the methods Aqueoeus bromophenol blue test[24], Mercuric bromophenol test[25], Ninhydrin - Schiffs test, Millona test and DMAB - nitrite[26] were adopted respectively. Periodic acid Schiffs test, Schiff's technique[26], Best's Carmine Test, Toluidine blue test[24], Alcian blue (pH - 2.5) test were adopted to detect the glycogen and Acid Mucopolysaccharides, acidic sulphated mucopolysaccharides, hyaluronic acid and sialomucins respectively. Sudan Black B Test and Oil Red O Test were adopted for the detection of lipids following the protocol of[27] and[24] respectively.

3. Results

3.1 Histological analysis of male reproductive system

3.1.1. Testis

The testes of *Muradium tetragonum* appear to be lobular and composedof several testicular lobules arranged around the centrally located collecting duct (seminiferous tubules) (Fig.1). The germ cells in different divisional stages appear to be synchronous. Each lobule of the testis, lined by a simple squamous germinal epithelium, produces a cyclic generation of spermatocytes. The Interior of the lobules is classified into two distinct zones: multiplication or germinal zone (GZ) and transformation zone (TZ). The GZ is located at the periphery of the lobules or near the seminiferous tubules with spermatogonial cells and few accessory cells (Fig.1). The spermatocytes and spermatids

in the advanced stages of their development are in the TZ.

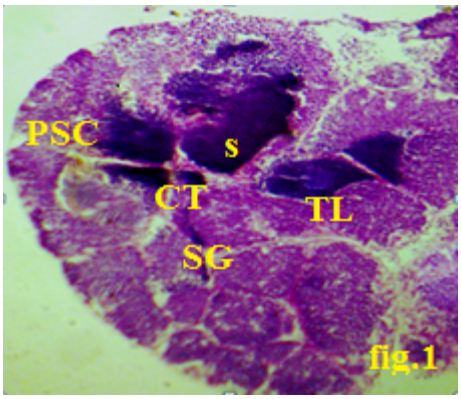


Fig. 1. T.S. of testis of the male sesarmid crab *M. tetragonum*. CT-Collecting tubule, PSC-Primary spermatocyte, S-spermatozoa, SG-Spermatogonia, TL-Testicular lobule, Scale Bar 100 μm

The germ cells of each lobule mature synchronously. The resulting sperms, after proliferation of a new generation of spermatocytes from the peripheral layer of spermatogonia proceed to the collecting tubule. Cross-sections of these tubules have revealed that the primary spermatogonia are large and spherical with an indistinct cytoplasm and a centrally positioned nucleus with one or two nucleoli. Secondary spermatogonia were smaller than primary spermatogonia with a clump of chromatin and a much-reduced cytoplasm and nucleus-plasma. The spermatocytes have a relatively small indistinct cytoplasm, with a voluminous spherical nucleus showing several stages of division. The chromatin of primary spermatocytes appears as basophilic clumps, which become more pronounced and deeply basophilic as they develop into secondary spermatocytes. The nucleus of the spermatid is initially spherical and then becomes elliptical in the advanced phase of spermiogenesis. The chromatin of each spermatid appears as a ring immediately below the nuclear membrane. Sperms are formed due to cell differentiation, resulting in the appearance of an acrosomal vesicle near the nucleus. The nucleus gradually extends over the acrosomal vesicle and surrounds almost the entire area when spermatogenesis is complete. At the end of this process, the spermatozoa (size - 4.11 ± 0.31 μm) looks like a crescent, with a cup-shaped nucleus partially containing the vesicular acrosome (Fig.1). The sertoli cells remain interspersed between the germinal cells which are irregularly shaped, variable in length, with the definite plasma membrane, nucleus and nucleolus and they undergo division.

3.1.2. Vas deferens

The vas deferens of the sesarmid crab *M. tetragonum*, begin in the collecting duct of the testis and end in the papillae of the penis. The vas deferens are classified into proximal vas deferens (PVD), middle vas deferens (MVD) and distal vas deferens (DVD). PVD, measuring 200-350μm in diameter is further differentiated into two distinct regions. Light microscopic studies of the anterior part of the PVD reveal large nuclei and columnar cells of varying heights located in the lower half of the cells. The lumen of the anterior end of the vas deferens shows a uniform distribution of sperms. The lumen contains a homogenous basophilic secretion, which surrounds the mature spermatozoids without the spermatophore. The sperms are grouped and distributed in the basophilic secretion.

3.1.3. *Proximal vas deferens (PVD)* The proximal region of the PVD is characterized by an increase in the diameter of the lumen and an associated

thinning of the walls. The posterior part of the PVD contains cuboidal columnar cells resting on a layer of muscle and connective tissue. The nuclei in the lower half of the cells are more spherical. Segmentation of the continuous sperm sheath into individual capsules occurs in the posterior part of the PVD region. The lumen comprises fully formed spermatophores of various sizes and is distributed within the eosinophilic vesicular secretion (Fig. 2, 3).

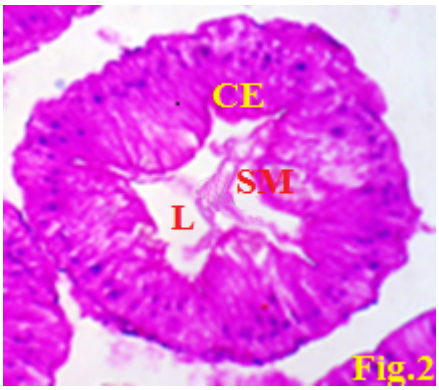


Fig. 2. T.S. of the Proximal Vas deferens (PVD) of the male sesarmid crab *M. tetragonum*. ECE-Elongated columnar epithelium, L-Lumen, SM-sperm mass. **Scale Bar 120 μm**

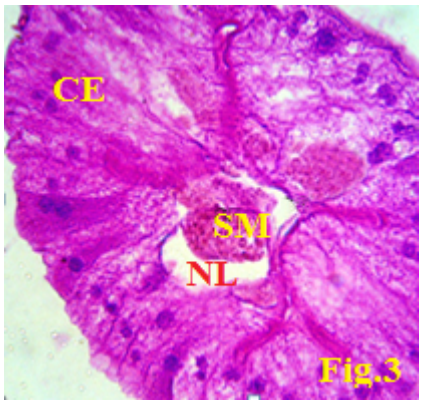


Fig. 3. T.S. of the Proximal Vas deferens (PVD) of the male sesarmid crab *M. tetragonum*. ECE-Elongated columnar epithelium, L-lumen, SM-sperm mass, NL-Narrow lumen. **Scale Bar 100 μm**

3.1.4. Middle vas deferens (MVD)

The histological organization of the proximal part of the middle vas deferens is almost similar to the distal part of PVD. The MVD is surrounded by an outer connective tissue, a middle muscle layer and an inner squamous or cuboidal epithelium. MVD secretes profuse abundant amount of vesicular eosinophilic secretion, which enrich the spermatophore's wall. The spermatozoa are organized in the form of a thick agglutinated mass inside the spermatophore. The lumen of MVD serves as a storage area for spermatophores and seminal plasma (Fig.4).

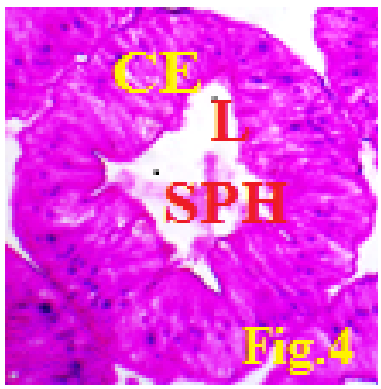


Fig. 4. T.S. of the Middle Vas deferens (MVD) of the male sesarmid crab *M. tetragonum*. CE-Columnar epithelium, L-Lumen, SPH-spermatophore, Scale Bar 80 μm

3.1.5. Distal vas deferens (DVD)

A cross-section of the DVD showed a thin cuboidal epithelium with a thick musculature, measuring approximately 4-6 μm. The epithelial layer comprises cuboidal cells with numerous folds in the lumen. The lumen contains basophilic and eosinophilic secretions intertwined with spermatophores. Spermatophores in the DVD lumen are infrequent on both PVD and MVD as presented in (Fig. 5).

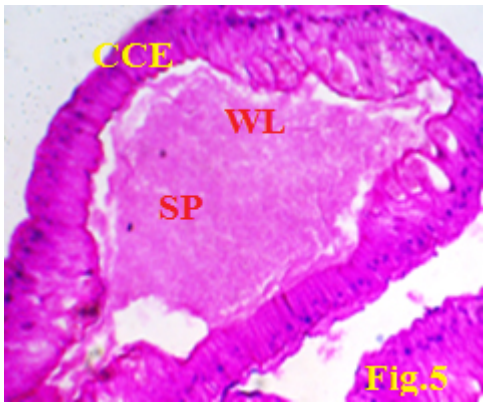


Fig. 5. T.S.of the Distal Vas deferens (DVD) of the male sesarmid crab *M. tetragonum* CCE-Cuboidal columnar epithelium, SP-Seminar plasma, WL-Wide lumen. **Scale Bar 60 μm**

3.1.6. Accessory sex gland (ASG)

The ASG tubules of *M. tetragonum* are the protrusion of the DVD as small sacs or buds. The epithelium is squamous or cuboidal with variable heights (3.5 ± 0.7 μm). The nuclei of the epithelial cells are large, spherical, or oval and sometimes lobulated. The secretory product of the ASG is highly specific in nature and consistent with that of VD secretions. The lumen of ASG is filled with eosinophilic polygonal masses bordered by eosinophilic granules. Some ASG sacs show the presence of few spermatophores (Fig.6).

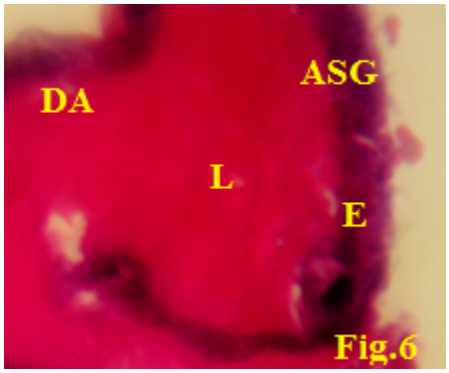


Fig. 6. T.S. of the Accessory sex gland of the male sesarmid crab *M. tetragonum* ASG-Accessory sex gland, DA-Duct of Accessory sex gland with the distal vas deferens, E.Epithelium, LS-Luminal secretion. Scale Bar 200 μm

3.2 Histological analysis of female reproductive system

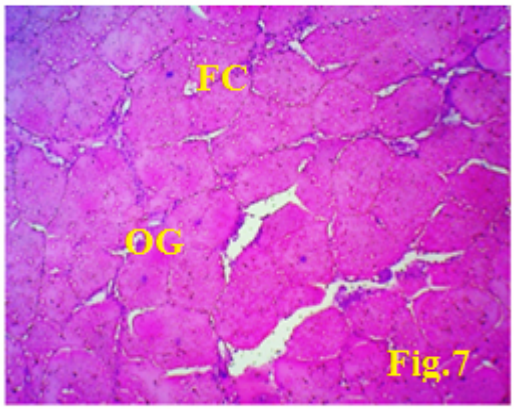
The histological characterization of the *M. tetragonum* ovary during the different stages of maturation was examined. They were recognized based on colour changes in the ovary, the diameter, and cytological characteristics of the oocyte, especially the chromatin pattern and amount of lipid vesicle in five stages viz., Stage I, Stage II, Stage III, Stage IV and Stage V and one sorptive or elapsed stage (Stage VI). Histologically, the ovary of *M. tetragonum* consists of two functional regions:

(a) germinal zone or proliferative zone;

(b) maturation zone as presented in (Fig. 7-12).

3.2.1.Stage I ovary

The ovary at Stage I appears as a white band, is characterized by the presence of vitellogenic oocytes with a relatively high nucleo-cytoplasmic index. Most of the oocytes have undergone yolk synthesis. The germinal zone (GZ) appeared very active and showed a significant number of basophilic oogonia. Each oogonium was found to possess a large round nucleus with a thin layer of cytoplasm. The ovary comprises previtellogenic oocytes (PVO) in its peripheral zone, surrounded by follicle cells (FC) (Fig. 8). The size of the oocytes gradually increased with mature ones occupying the center and smaller ones towards the periphery as shown in (fig 7-12). Stage I oocytes characterized by finely granular cytoplasm were spherical and measured 26- 52 μm in diameter.



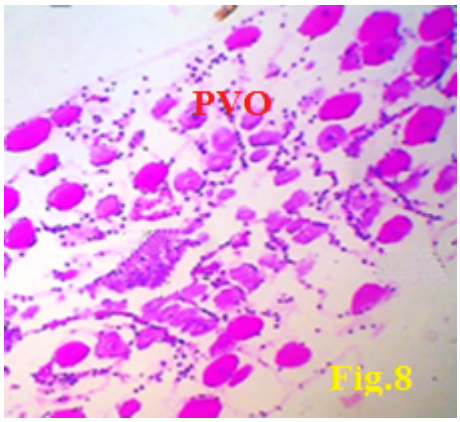
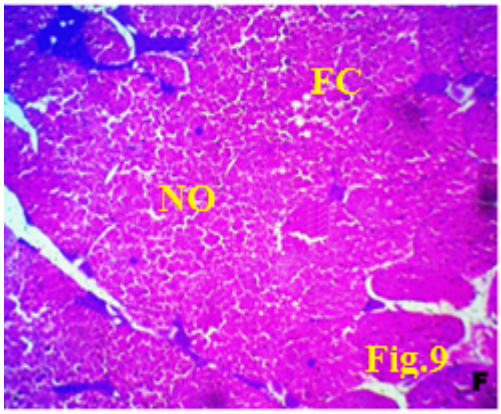


Fig. 7 and 8. T.S. of the Immature ovary of the female sesarmid crab *M. tetragonum* FC- Follicle cells, OG- Oogonia, PVO- Previtellogenic oocytes. Scale Bar 1500 μm and 150 μm

3.2.2. Stage II ovary

Stage II The ovary appears slightly thicker than Stage I with a yellowish hue. The germinal zone contains oogonia in the meiotic stages. Previtellogenic oocytes with a diameter of 46-92 µm are dispersed in the peripheral (maturation) zone of the ovary. Follicle cells, which enclose each oocyte as a continuous layer, are prominent in this stage. Many basophilic granules are witnessed in the ooplasm. Similarly a few droplet-like structures appeared on the peripheral ooplasm as shown in (Fig. 10), could be the precursors of yolk. Oogonia and premeiotic oocytes are found in GZ. After migration, previtellogenic oocytes appeared large in the peripheral maturation zone, where they grow.



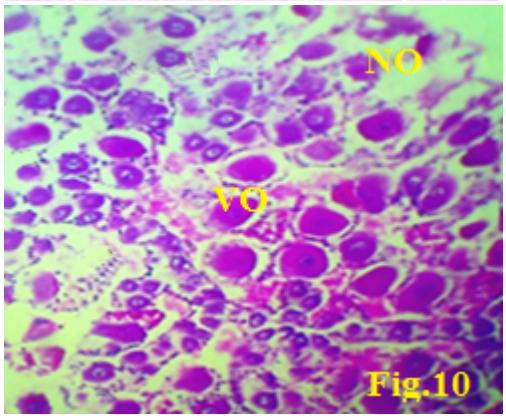


Fig. 9 and 10. T.S. of the nearly mature ovary of the female sesarmid crab *M. tetragonum* FC- Follicle cells, VO- Vitellogenic oocyte Oogonia, NO- Nearly mature oocytes. Scale Bar 1500 μm and 150 μm

3.2.3. Stage III ovary

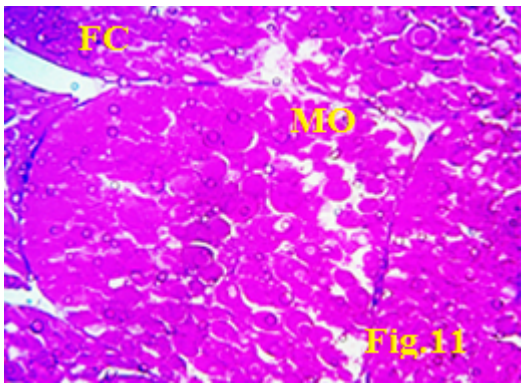
The ovary at stage III was yellowish-brown, with a diameter of 102-145 µm. At this stage, the ovary was composed of densely packed vitellogenic oocytes with a-pronounced size. No proliferative activity was observed in the germinal zone. The ooplasm had a considerable amount of yolk with centrally positioned nuclei and distinct nucleoli.

3.2.4. Stage IV ovary

The ovary at this stage exhibits brown colouration. The Oocytes, characterized by large and small yolk globules, tend to be bulky with a diameter ranging between 150 and 198 µm. The Germarium appears as a stripe like structure. The nucleus was ellipsoidal. The cytoplasm of the follicle cells is elongated due to the enlargement of oocytes.

3.2.5. Stage V – (Pre-spawn) ovary

Ovary at Stage V appeared dark brown. Large vitellogenic oocytes ready for egg-laying were displayed. The germinal zone, comprising differentiating oocytes and premeiotic oogonial cells, appeared as ribbon-like spots together with the ovary. The vitellogenic oocytes had tightly packed yolk globules and cortical granules in their ooplasm. Cortical granules may be the precursor of yolk granules. The nucleus could only be seen in very few oocytes (Fig. 12).



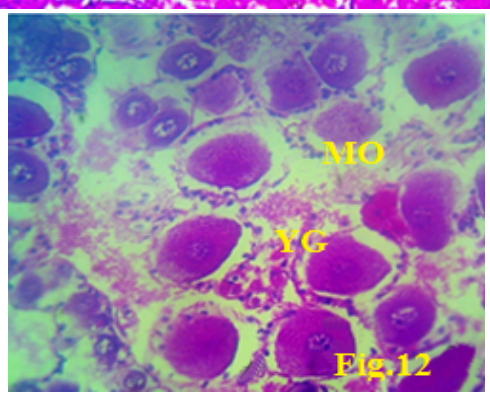


Fig. 11 and 12. T.S. of the mature ovary of the female sesarmid crab *M. tetragonum* FC- Follicle cells, YG-Yolk granules, MO- mature oocytes. **Scale Bar 1500 µm and 150 µm**

3.2.6. Stage VI – Post-spawn Ovary

The post-spawn ovary appeared as a translucent yellowish flaccid band. Small, resorbed oocytes were seen. The germinal zone was inactive, which was evidenced by the presence of non-proliferating oogonia represented as a compact structure in the center of the ovary. Some undifferentiated oogonic cells scattered in the peripheral zone had been observed. The peripheral zone also divulged the existence of the residual follicle cells of the previous clutch pending lysis.

3.2.7. Spermatheca

Compound microscopic observation of the spermatheca of *M. tetragonum* indicates that it is bounded by the muscle layer of (almost 2 µm), multilayered connective tissue (10-11 µm) and an epithelium were observed. The spermatheca can be spatially differentiated into dorsal and ventral chambers based on the morphology, position, and function of the oviduct. The dorsal chamber of the spermatheca, lined with a layer of columnar epithelium ranging from 15 to 24 µm has a basal nucleus of 2.5 ± 0.91 µm in diameter. The wall of the oviduct is composed of thin connective tissue and a cuboidal epithelium, which connects the spermatheca through the ventral chamber. The junction of these two forms a special kind of tissue consisting of densely packed cells with oval-shaped nuclei. The ventral chamber of the spermatheca is

lined by columnar epithelium, the height of the epithelium ranging between 32 and 43 μm. The luminal content that appears as homogeneous, eosinophilic, and basophilic represent the cumulative products of VD, ASG and spermatheca as well as the sperms interspersed in the luminal contents.

3.3 Histochemical analysis of male and female reproductive system

3.3.1. Testis

Histochemical studies revealed that the connective tissue layer reacts positively to carbohydrate (PAS) and lipids (SBB) but moderately to mucopolysaccharides (AB). The cytoplasm of spermatogonial cells showed negativity to PAS, but the nucleus shows moderate positivity to PAS. Moderate positivity to Best's carmine in the nucleus suggests the presence of glycogen. The germinal center shows positivity to SBB and AB. In the present investigation, the sperm mass in the testis collectior tubule was agglutinated by testis secretion. Positive reactions with the Mercuric bromophenol blue test, Ninhydrin test, Schiff's test and Millon's tests elucidate the presence of basic, acidic, amino and hydroxyl groups of proteins (Table-1). The results of carbohydrate histochemistry viz. PAS, Best carmine and Toluidine blue demonstrated positive reactions that explain the presence of Carbohydrates (Table-2). In addition to proteins and carbohydrates, lipids were also deduced by the Sudan Black B, Oil Red O and Nile blue tests (Table-2)

3.3.2. Vas deferens

Histochemical analysis of the vas deferens of *M. tetragonum* showed that the VD epithelium reacted positively for lipids and moderately to PAS and AB. The homogeneous secretion of the anterior PVD reacted intensively to PAS, MBB, and AB and moderately to glycogen (Best's carmine). The eosinophilic vesicular secretion of the distal PVD and MVD was positive to PAS, MBB and AB. The spermatophore wall exhibited intense positivity for mucopolysaccharides (AB), neutral mucopolysaccharides (Aldehyde fuchsin) and protein (MBB) that showed the presence of sulfated and carboxylated glycosaminoglycans. The PVD epithelial cells and secretory products indicated the presence of basic, acidic, amino, tyrosyl and tryphtophenyl groups of proteins. In the present study, the histochemical characteristics of the DVD epithelium and its secretions are rich in basic, acidic and tyrosyl groups of proteins. Positive reaction with toluidine blue at higher pH inferred the presence of carboxylated and sulphated mucopolysaccharides (Table-2).

3.3.3. Accessory sex glands

Histochemical evaluation of the granular secretion of ASG divulged high positivity to carbohydrate (PAS) mucopolysaccharides (AB) and protein (MBB) as presented in (Table -2).

3.4. The ovary of *M. tetragonum*

3.4.1. Mercury Bromophenol Blue test (MBB)

Oogonial cells and previtellogenic oocytes showing an intense positive reaction to MBB, have general and acidic proteins. The nucleus of the previtellogenic oocyte revealed a weaker reaction to MBB. On the other hand, the cytoplasm of early primary vitellogenic oocytes showed a positive reaction to MBB with the nucleus being highly reactive. The secondary vitellogenic oocytes also showed a very high reactivity to MBB. Follicle cells

were also seen to be intensely stained with MBB as presented in (Table- 3).

3.4.2. Periodic acid Schiff's test

Evaluation of Schiff's periodic acid on the ovary of *M. tetragonum* revealed a positive PAS reaction and clarified the presence of glycogen, while the nucleus of previtellogenic and primary vitellogenic oocytes showed a slight positivity. But their perinuclear space was intensely stained with PAS and indicated the high amount of carbohydrates. The secondary vitellogenic oocytes were more deeply stained with PAS that primary vitellogenic oocytes. Granules seen at the periphery of previtellogenic oocytes as well as primary and secondary vitellogenic oocytes showed intense positivity to PAS. The ovarian stroma of the spent ovary specified moderate positivity to PAS but the follicle cells were strongly stained with periodic acid and Schiff's reagent (Table-3).

3.4.3. Best's Carmine

The histochemical evaluation of the *M. tetragonum* ovary in the Best Carmine test showed a slight positive reaction in the Previtellogenic oocytes. The ooplasm of primary and secondary vitellogenic oocytes indicated moderate positivity showing a rational amount of glycogen.

3.4.4. Sudan black

Histochemical analysis of lipids in the *M. tetragonum* ovary by the Sudan Black B test demonstrated that oogonia and previtellogenic oocytes exhibited moderate positive reaction to Sudan black B. Follicle cells, the nucleus of the pre and primary vitellogenic oocytes displayed positivity to lipids. Yolk globules showed an intense positive reaction to Sudan Black B. Secondary vitellogenic oocytes showed greater positivity to Sudan black B stain than primary vitellogenic oocytes. In contrast, a negative reaction to Sudan Black B was observed in the ovarian stroma.

3.4.5. Alcian blue

The evaluation of sulfated mucopolysaccharides in the *M. tetragonum* ovary by Alcian blue test had the following results: Oogonial cells showed a negative reaction to Alcian blue while previtellogenic oocytes and follicle cells showed positivity to Alcian blue. The nucleus of previtellogenic, primary and secondary vitellogenic oocytes, as well as the ooplasm of primary and secondary vitellogenic oocytes and the ovarian stroma showed a moderate reaction to Alcian blue.

3.4.6. Spermatheca

Histochemical analysis of the epithelium of the spermatheca showed a positive reaction to Mercuric bromophenol blue stains, while the basal region of the epithelium had moderately positive reaction to Periodic acid Schiff's reagent and indicates the presence of general and acidic proteins as well as glycogen. The presence of sulphated mucosubstances in the epithelium is evidenced by their intense blue colour to Alcian blue staining. The connective tissue and muscle layer are rich in glycogen and can be evidenced by their positive reaction to PAS and Best Carmine. The luminal content of the spermatheca exhibited high positivity to MBB, PAS and Alcian blue which predicts the presence of protein, glycogen and sulfated mucosubstances respectively. Free spermatozoa as well as those found inside the spermatophores are strongly positive to MBB and PAS which showed the

presence of general and acidic proteins as well as glycogen. The histochemical evaluation of the spermathecal layers, luminal contents and spermatozoa are represented in the table.

4. Discussion

4.1. Histological and histochemical analysis of male reproductive system.

The cytological architecture of the testis of *M. tetragonum* shows several testicular lobules, with many sperm cells in different developmental stages. In decapods, the stages of spermatogenesis can be synchronous or asynchronous in the acini of different regions of the testis depending on the species[28]. The current study observed two zones namely germinal (GZ) and transformation (TZ) zones in the testis through light microscopic observations. Each zone has a different content and plays a specific role during spermatogenesis [29]. The germinal zone in the testis of *M. tetragonum* is thinner and is located at the periphery or close to the seminiferous tubule (collecting duct). It contains accessory cells and spermatogonia, which consequently transforms into spermatocytes, spermatids, and spermatozoa in the transformation zone. In addition, the dividing Germ cells are interspersed with non-germinal cells or accessory cells also called sustentacular cells, interstitial cells, nurse cells or nutritive cells, and Sertoli cells. The existence of such non-germinal cells has been reported by several authors[29-30]. According to[32], Sertoli cells strongly enhances the process of spermatogenesis and spermiogenesis in Decapoda. Accessory cells in *M. brachydactyla*[29] and *P. plicatum*[31] become prominent and elevate during the final stage of spermatogenesis. Thus, the observations of the current study, being in congruence with the findings of the studies, predict a similar functional role of the accessory cells in enhancing and assisting the process of spermatogenesis.

In the present investigation, the testis of *M. tetragonum* produces a cyclic generation of spermatocytes. The contents of each follicle mature synchronously, the resulting sperm showing an eccentric position (Fig. 2). The collector tubule of *M. tetragonum* encompasses only mature spermatozoa which have migrated to the VD, being differentiated into three zones according to their size and synthetic activity. Similar observations have been made in other brachyuran crabs[33-37]. The lumen of PVD in *M. tetragonum* exhibited a variation in its size and shape, being oblique and narrow. The columnar epithelium of the PVD, which shows variation in thickness on the dorsal, ventral, and lateral sides, causes fragmentation of the sperm to sheathe and form the mould for the formation of the spermatophore capsule.

Histochemical assessment of the reproductive tissues reveals their biochemical nature and that of secretory products. Positivity to Mercuric Bromophenol test, Ninhydrin test, Schiff's test and Millons test, indicates the presence of basic and acidic groups, amine groups and hydroxyl groups of proteins as presented in (Table-1). The positive results obtained in Bestcarmine, PAS and Toluidine blue test elucidate the presence of carbohydrates. In addition to proteins and carbohydrates, lipid substances were also ascertained through Sudan black B, oil Red O and Nile blue tests (Table-2). The sperm mass in the collecting tubule of the testis was found to be agglutinated

with the secretions of the testis. It is inferred that the proteins and mucopolysaccharides secreted from the posterior part of the testis might have been involved in the agglutination of sperm mass into discrete units. The above findings are in accordance with the report of[38] in S.*serrata.*

In brachyuran decapods, the epithelium of PVD performs a secretoryfunction[39]. The secretion of the PVD serves as the basic substance for the spermatophore. It helps to agglutinate sperm and serves as a nutritional source for the sperm stored in the spermatophore. However, as evidenced by the current investigation, the lumen of the PVD contains not only the substances of its own epithelial cells but also the secretory products of the testes. Histochemical analysis of epithelial cells proteins and substance "A" secreted in the PVD revealed the presence of basic, acidic, amine, tyrosyl and tryptophenyl groups (Table-1). The best carmine, PAS and toluidine blue tests specified the presence of glycogen and acid mucopolysaccharides in the substance A and epithelium (Table-2). Lipid histochemical tests indicated negative results with substance "A" and positive results with the epithelium of PVD (Table-2).

MVD of *M. tetragonum* apparently serves as a long-term storage site. The MVD of Brachyuran crabs such as *S. quadratum* [40] and *P. plicatum*[31] shows a pouch-like appearance which could allow storage of more sperms. Studies have been carried out on the MVD of brachyuran crabs to evaluate their secretory activity and storage[37, 40-41]. The secretion of MVD not only offers mechanical support but also incorporates some substances to harden the spermatophore wall. In few brachyuran decapods, the MVD epithelium has been suited for the absorption of the PVD secretory materials[31, 39-40]. The structure of the DVD in *M. tetragonum* does not vary considerably with that of MVD of the brachyuran crabs except for the thick muscular layer, which probably facilitates the combination of seminal plasma, the spermatophores and seminal ejaculation during coitus.

The male reproductive tract of some decapods consists of glandular structures, located in the posterior part of VD called ASG. Cuboidal epithelium of ASG has been previously reported by a number of authors[42], in *O. platytarsis*[43], in *O. ceratopthalmus*, and[44] in *S. quadratum*. The ASG secretion of crustaceans is presumed to be either neutral mucopolysaccharide or mucoprotein. In this work, the Lumen of the accessory sex gland tubule of *M. tetragonum* encloses eosinophilic secretion with different textures: homogenous polygonal masses and granular secretions, which showed positivity to MBB, PAS and AB test suggesting the presence of mucoprotein and glycoprotein (Table-2). Similar histochemical nature of ASG secretory products have been reported by[44] in *S. quadratum*.

4.2 Histological and histochemical analysis of female reproductive system

Histologically, the ovary of *M. tetragonum* is composed of an outer connective tissue and a very thin inner layer of epithelium. Most of the ovarian histological studies emphasize the germinative components but give little or no attention to the lining of the ovary. However, in *C. sapidus*[45], Panulirus spiny lobsters[46] and *G. cruentata*[20], the ovary was lined by a thin layer of fibrous connective tissue. Significant colour changes in the ovary of *M. Tetragonum* during the different

developmental stages can be attributed to the degree of yolk deposition.

Some crab species lack muscles in their ovary. Hence, spawning occurs with the contraction of the abdominal and cephalothoracic muscle adjacent to the ovaries[47]. Consequently, the ovary of *M. tetragonum* was observed to be without muscles. It is compatible with the results of other brachyurans such as *Ranina ranina*[48], *Ucides cordatus*[49], *M. brachydactyla*[50], *G. cruentata*[20] and *C. sapidus*[15].

In this investigation, the size of the oogonium of *M. tetragonum* was smaller than the oogonia of other brachyuran crabs *Sesarma quadratum* (4 to 8 μm)[51], *S. serrata* (5-10 μm)[16] and *C. sapidus* (9-12 μm)[15]. The variation in oogonia size between different brachyuran decapods may be related to the size of the animals, an increase in diameter of the oocytes could be attributed towards the transformation of previtellogenic into primary vitellogenic oocytes, because of vacuolization. Studies by Grapsidae[20], Majidae[50], Portunidae[16], Hymenosomatidae[52], Gecarcinidae[53] and Penaeidae[54] have suggested that such variation in diameter could be related to the accumulation of yolk, and the process of accumulation of yolk is species-specific.

In the experimental crab *M. tetragonum*, the cytoplasm of primary vitellogenic oocytes was less basophilic. They later became acidophilic in nature, due to the accumulation of vitellus in the oocyte[55]. The secondary vitellogenic oocyte of *M. tetragonum* was found to be significantly larger than that of other crabs reported so far.

Based on histological architecture of the ovary of *M. tetragonum* five developmental stages can be classified as reported by[56-57]. While a greater number of young germinative cells is observed in the Stage - 1 and Stage - 2 of the ovary, highlighting the beginning of ovarian development[55], a significant increase in the number of mature oocytes can be observed during the last stages of vitellogenesis and clarified the maturity of the ovary, being ready to spawn favouring the observation of[17].

From the carbohydrate histochemical analysis of the ovary, it is clear that the oocyte of *M. tetragonum* was highly positive to PAS. Magenta colour stained granules were observed in the periphery of the early-stage oocytes. The presence of these granules was also reported in the blue crab, *C. sapidus*[15] where they were known as cortical alveoli, whereas in *S. quadratum*[51] they are named as cortical granules. In *M. tetragonum* the oocytes of latter stages exhibited mild reaction with Alcian blue, but the early-stage oocyte and follicle cells were found to be highly alkinophilic and mucopolysaccharides were expected. These observations corresponded to the findings of[53] in brachyurans.

The spermathecae of *M.tetragonum* were histologically composed of a connective tissue capsule which encompassed the glandular epithelium. The lumen comprised sections of the spermatheca, seminal fluids and spermatophores[58-59]. Histochemical analysis of the spermatheca of *M.tetragonum* revealed that the epithelium of spermatheca exhibited high positivity to Mercuric bromophenol and moderate positivity of Periodic acid Shiff's reagent. Similar observations had been made by[45] in *Callinectes sapidus*. The connective tissue layer of the spermatheca of the present study was rich in glycogen[60]. showed a moderate positive reaction of connective tissue layer to PAS in the spermatheca of

Chionectes opilio. The luminal content of the spermatheca of the experimental crab displayed high positivity to MBB, PAS and AB which specified the high amount of protein, glycogen and sulfated mucosubstances. The above observations are in line with that of[60] in *C.opilio*. and attributed that luminal substances are high energy content and may be significant in the storage and opening (dehiscing) of the spermatophores.

5.Conclusion

The results concerning the histological and histochemical characteristics of the reproductive gonads of sesarmid crab *Muradium tetragonum* were discussed/compared with the findings of studies conducted in other brachyuran crabs. The biological structures displayed elucidate the reproductive organization of the study organism and confirm its phylogenetic origin, documenting the characteristics of the thoracotrema-heterotrema clade. The evaluation of spermatozoa formation and seminal secretion in vas deferens together with the role of spermatheca in females through histochemical studies provides a deep insight into the existing chemical constituents, the nature of secretory products and their function in the physiology of reproduction.

Table. 1. Histochemical results of the ovary and spermatheca of the female sesarmid crab *M. tetragonium*

Tests	Oocyte of Privitellogenic Ovary		Oocyte of vitellogenic Ovary		Oocyte of Secondary vitellogenic Ovary		Oocyte of Spent Ovary		Spermatheca			To Indicate
	C	N	C	N	C	N	C	N	E	LC	S	
Mercuric Bromophenol blue	+++	++	+++	+++	+++	+++	+++	++	++	+++	+++	General and Acidic Proteins
Methylation+MBB	-	-	-	-	-	-	-	-	-	-	-	
Periodic acid and Schiff	++	+	+++	++	+++	++	++	++	++	+++	+++	Free Aldehydes Glycogen, Viccny Glycol
Diastase+PAS	-	-	-	-	-	-	-	-	-	-	-	
Best Carmine	++	+	++	++	++	++	++	++	++	++	++	Glycogen and Muccopolysaccarides
Diastase + Best Carmine	-	-	-		-	-	-	-	-	-	-	
Sudan Black B	+	++	++	++	+++	++	+	+	+	+++	+	General Lipids
Acetone + SBB	-	-	-	-	-	-	-	-	-	-	-	
Alcian Blue	+	+	+	++	+	++	+	++	++	+++	++	Hyaluronic Sialomucin

\+ Moderate positive reaction
++ Greater positive reaction
+++ Intense positive reaction
\- Negative reaction

Table. 2. Histochemical results of the testis and Vas Deferens of the male sesarmid crab *M. tetragonium*

Tests	Testis		PVD		MVD		DVD		ASG	ED		To Indicate
	GE	SM	EL	SA	EL	SB	EL	SP	SC	EL	SP	
Periodic acid and Schiff	+	++	+	+++	+	++	+	++	++	+	++	Glycogen
Best' s Carmine	++	+	++	++	++	++	++	++	++	+	++	Glycogen
Toluidine Blue at Different pH	++	+	+	++	+	++	+	++	++	+	+++	Acid Mucopolysaccharides
Sudan Black B	+	+	+		+	+	+	+	+	+	++	General Lipids
Oil Red O Test	+	+	+	-	+	++	+	++	+	+	++	Neutral Lipids

\+ Moderate positive reaction
++ Greater positive reaction
+++ Intense positive reaction
\- Negative reaction

Table. 3. Histochemical results of the Ovary and Spermatheca of the female sesarmid crab

Tests	Testis		PVD		MVD		DVD		ASG	ED		To Indicate
	GE	SM	EL	SA	EL	SB	EL	SP	SC	EL	SP	
Aqueou Bromophenol blue	+	++	+	+++	+	++	+	++	++	+	++	Basic Group of Proteins
Mercuric Bromophenol blue	++	+	++	++	++	++	++	++	++	+	++	General and Acidic Proteins
Ninhydrin - Schiff' s	++	+	+	++	+	++	+	++	++	+	++	Presence of free amino group
Millon' s	+	+	+	+	+	++	+	++	+++	+	++	Hydroxyl group tyrosine
DMAB	+	+	+	++	+	++	+	++	++	+	+++	Tryptophanyl Group

\+ Moderate positive reaction
++ Greater positive reaction
+++ Intense positive reaction
\- Negative reaction

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