



Experimental Study

Ultrastructure of the male accessory glands of sesarmid crab, *Parasesarma plicatum* (Latreille, 1803)



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ABSTRACT

The structure of accessory glands (AGs) in the male sesarmid crab, *Parasesarma plicatum*, is described by light and transmission electron microscopy. Adult males of carapace of width 1.6–2.2 cm were collected from along the estuarine regions of Kanyakumari District, India. Posteriorly, the male reproductive system receives several sac-like structures, referred to as AGs. Histologically, the AG is internally lined by cuboidal epithelium and the lumen encloses eosinophilic vesicular secretions, apparently glycoproteinaceous in nature. Ultrastructurally, the epithelium shows the signs of typical infrastructure for synthetic activity, as demonstrated by the prolific presence of rough endoplasmic reticulum, free ribosomes, and Golgi complex. The cytoplasm is manifested with electron-dense, electron-lucent, and medium-density secretory vesicles, and the mode of release into the lumen is both merocrine and apocrine. Within the lumen, these secretory vesicles coalesce and aggregate into large heterogeneous masses of varying sizes, which may play an important role post-copulation within the female duct.

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1. Introduction

Parasesarma plicatum [1], of the family Sesarmidae inhabits the muddy substratum of estuarine and mangrove environment and has a wide range of distribution in the tropics. These crabs are economically and environmentally significant, playing a pivotal role in the food chain and maintenance of ecological balance. Sesarmid crabs are “ecosystem engineers” as they change the availability and quality of food, create new habitats, refuge, and shelter for other constituent species [2]. In spite of their ecological

importance, these crabs are unnoticed, as most of the physiological studies are attributed towards the edible crabs.

Most crustaceans, unlike insects, do not possess well-defined male accessory reproductive glands. Instead, the epithelial lining of the male reproductive tract has undergone extensive modification giving rise to glandular areas, which are highly specialized and compartmentalized for the production of spermatophores and seminal substances [3–5]. Rarely, the male reproductive tract of some decapods such as, *Ocypode platytarsis* [6], *Uca triangularis Bengali* [7], *Metopograpsus messor*, *Sesarma quadratum* [8], *Maja brachydactyla* [9], *Eriocheir sinensis* [10,11], and *Sarmatium punctatum* [12] possess glandular accessory structures referred to as accessory glands (AGs)/accessory sex glands (ASGs), which are positioned appendicular to the posterior part of the vas deferens (VD) [8–12].

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The ASG is known to have significance in mammals [13], and its secretions contain a variety of bioactive molecules. These molecules exert wide-ranging effects on female reproductive activity [14]. In insects, the male AG products have attained great importance in reproduction as they are a means of transport for sperm and can form a mating plug. Within the female, AG secretion is suggested to help sperm activation, nourishment, and the supply of materials to the female [15,16]. The peptides and proteins secreted by the ASGs, together with spermatophores, enter the spermatheca during mating and play important roles in the synthesis of membrane components, and the acrosome reaction [17,18]. In brachyurans, the ASG materials of *E. sinensis* [10,11] and *M. metopograpsus* [8] assist spermatophore breakdown, and in the former, the spermathecal and ASG, protein homogenates could increase the sperm enzyme acrosin vitality. Studies on AGs warrant more research.

Although considerable progress has been made in the glandular function of the VD, there have been few studies on AGs concerning their role in reproduction in brachyurans or indeed in decapods. So, evaluation of the occurrence or function of AGs within these taxa necessitates extensive research. The present study describes in detail the histology and ultrastructure of the AGs of *P. plicatum*, and provides basic information for further studies on the role of AGs in male and female reproduction.

2. Materials and methods

2.1. Collection and rearing of animals

Adult males of *P. plicatum* with carapace width 1.6–2.2 cm were collected from Manakudy Estuary (latitude 8°4' N; longitude 77°26' E) of Kanyakumari District, Tamil Nadu, India. Collections were handpicked and or made by bait on a weekly basis. After examining the molt stages [19], crabs were reared in a laboratory in plastic cisterns and were fed *ad libitum* on clam meat and (boiled) egg white. Intermolt crabs were used for light microscopic studies. Adequate care was taken to maintain them in near-natural condition to minimize or avoid stress to the animals.

2.2. Dissection

The male reproductive system of *P. plicatum* was dissected by cutting open the dorsal portion of the carapace. The dissection was performed under a dissection microscope in a medium of 0.9% physiological saline. A portion of the tissue to be observed was taken on a clean glass slide. A drop of the staining solution (toluidine blue) was placed on the tissue and gently pressed with the coverslip and the smear was observed under a trinocular microscope (Labomed, India) and photographed.

2.3. Histology and histochemistry

For histological examination, a minimum of seven specimens were taken and the tissues were fixed in Bouin's Fluid, dehydrated in a graded alcohol series, and cleared

in xylene for 15 minutes. The tissues were embedded in paraffin wax, sectioned at 5–7 μm thickness, and stained with hematoxylin and eosin [20]. The stained sections were viewed and photomicrographed by CosLab (India) bright field transmission microscope. For histochemical studies, the paraffin sections (5–7 μm thick) were used to test the chemical nature of the AGs. The presence of proteins was demonstrated by mercury bromophenol blue (MBB) staining [21]. Neutral polysaccharides with 1-2-glycol groups were detected by periodic acid Schiff (PAS) staining. PAS was also conjugated to Alcian Blue at pH 2.5 to stain acidic polysaccharides [22].

2.4. Ultrastructural study

AG tissue, after being fixed in 3% buffered glutaraldehyde for 24 hours, was washed thoroughly with 0.1 M phosphate buffer and post-fixed in osmium tetroxide for 1–2 hours at 4 °C. After a brief wash in 0.1 M phosphate buffer, AG tissue was dehydrated in a graded series of ethanol (70–90%). Following dehydration in 90% ethanol, the sample was incubated in (freshly prepared) 2% ethanolic uranyl acetate (*en bloc* staining) and subsequently dehydrated with 100% ethanol. Propylene oxide was used as the clearing agent. The tissue was left for infiltration for a minimum of 6 hours in a 1:1 mixture of propylene oxide and araldite, followed by pure araldite for a further 8 hours. The tissue was then embedded in araldite and kept in an oven maintained at 60 °C undisturbed for 2 days for polymerization. Semi-thin sections (1 μm), stained with 1% toluidine blue, were used to ensure the exact positioning of the tissue. Ultra-thin (600 Å) sections contrasted with uranyl acetate and lead citrate were scanned under a Jeol (USA) transmission electron microscope and photographed.

3. Results

The male reproductive system of *P. plicatum* was bilaterally symmetrical, located in the antero-lateral portion of the cephalothorax, and consisted of a pair of tubular testes, VD, AGs, and ejaculatory ducts (Figure 1). Throughout its length, the posterior VD received several sac-like structures, the AGs; the tubules of which were not uniform in length. The tubules in the proximal region of the posterior VD were smaller (0.7–1.2 mm long) than those in the middle or distal region of the posterior VD (2.2–3.2 mm long) and more voluminous. Thickness of the tubules increased at the base and each tubule opened separately into the lumen of the posterior VD. Few tubules bifurcated at their free ends.

3.1. Histology

Although the sac-like AG tubules of *P. plicatum* were extensions of the posterior VD, they exhibited histological differences from the posterior VD. The wall of the AG tubule was lined with cuboidal epithelium (7–17 μm) and each cell possessed a deeply basophilic, elongated, and/or oval-shaped nucleus (Figure 2). A thin layer of muscle tissue (1 μm) overlaid the epithelium. The lumen of the AG tubule

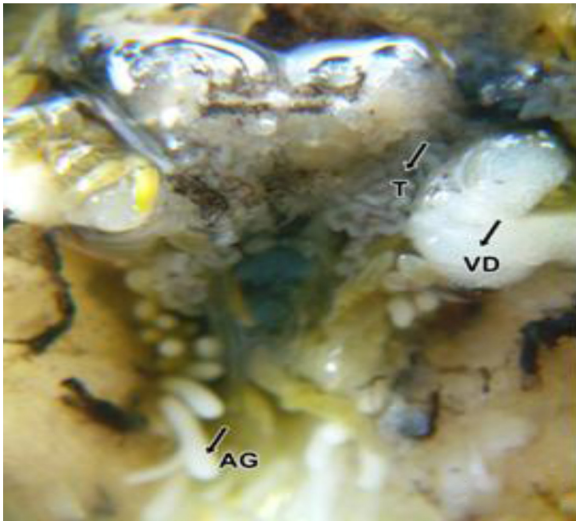


Fig. 1. Dissected display of male reproductive system of *P. plicatum*
 T - Testis
 VD- Vas deferens
 AG - Accessory gland.

was extensive, which encompassed eosinophilic homogeneous materials (5–30 μm), which were either in granular or vesicular forms. Although AG secretions appeared vesicular, they enclosed small granules (0.5–4 μm), which were found towards the periphery, and in the central portion of the lumen, these granules gradually coalesced to form larger eosinophilic vesicles (Figure 3). In spite of the lumen of the posterior VD showing continuity with the AG lumen, it appeared that the secretions of both the tubular glands intermingled only at the junction.

3.2. Histochemistry

The secretion in the lumen stained strongly for MBB, and was stronger in the peripheral region in contact

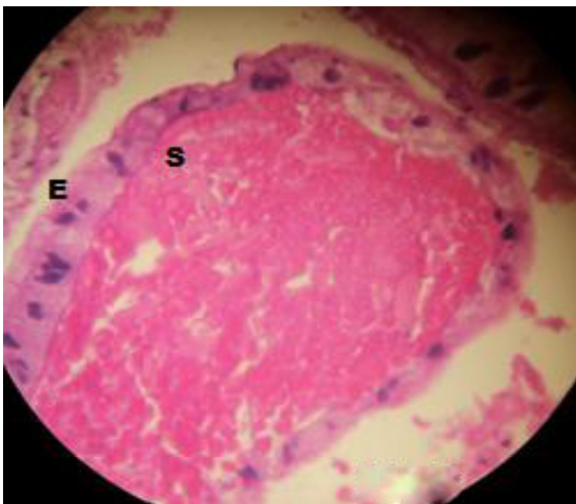


Fig. 2. C.S of single AG tubule with cuboid epithelium (E).The lumen encompasses eosinophilic secretion (S) x 200.

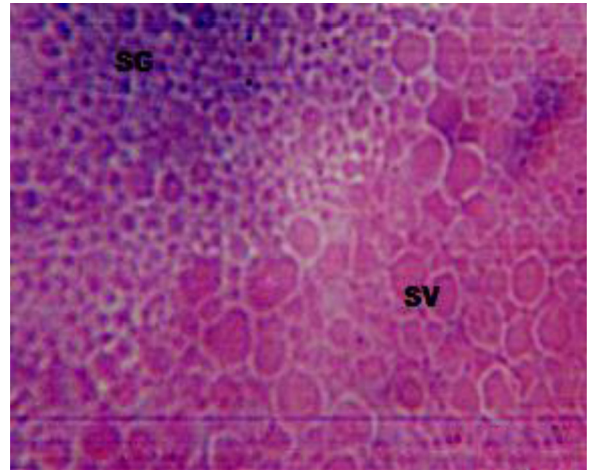


Fig. 3. The lumen of AG tubule exhibiting coalescence of secretory granules (SG) into secretory vesicles (SV) x 1000.

with the epithelium (Figure 4). Alcian Blue moderately stained the epithelial and connective tissue layers, but the inner amorphous masses were negative, indicating a lack of alcianophilia in the lumen. The luminal secretion stained strongly for MBB, revealing its proteinaceous nature, although the reaction varied along the entire length of the gland. PAS showed uniform staining throughout the glandular secretion, whereas the outer epithelial layer showed less positivity (Figure 5). The granular and vesicular entities of the glandular secretion showed deep sensitivity to MBB, and PAS indicated their glycoproteinaceous nature (Figures 4 and 5).

3.3. Ultrastructural study

Semi-thin section of AG tubules clearly exhibited the epithelial wall and lumen (Figure 6). Under transmission electron microscopy, the wall of the AG presented a connective tissue layer interdigitated with the thin muscle layer. The cubic epithelium showed signs of secretory activity, according to the presence of prolific rough

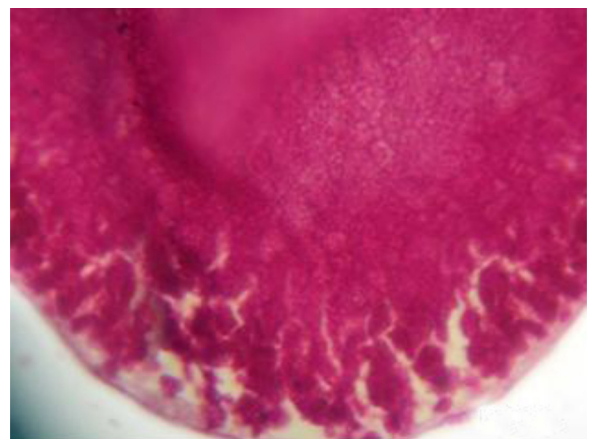


Fig. 4. Secretions of AG showing strong positivity to PAS x 500.

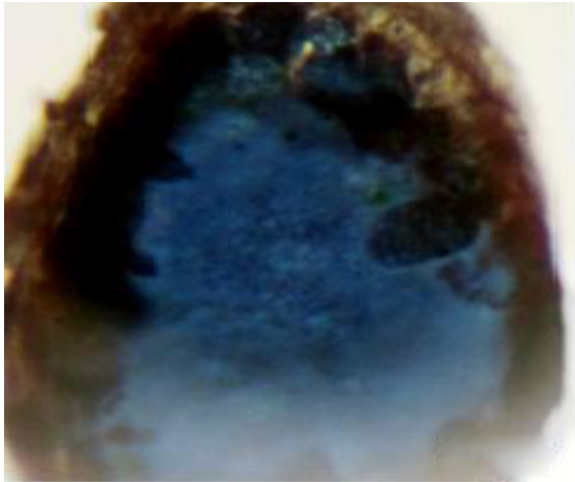


Fig. 5. Secretions of AG showing positivity to MBB. Note the MBB negative epithelium x 200.

endoplasmic reticulum, free ribosomes, and Golgi complex (Figure 7). The epithelial cytoplasm possessed irregular lobed nuclei, with electron-dense heterochromatin and dense granules distributed along the periphery. It appeared that the chromatin material diffused from the nucleus to the surrounding region (Figure 8). The cisternae of the endoplasmic reticulum were bulbous and dilated with medium electron-dense granular material. The Golgi complexes were randomly distributed and consisted of highly electron-dense vesicles. Elongated mitochondria were distributed throughout the cytoplasm. Distinct cellular junctions and membranous infoldings were seen in the epithelium. The apical border of the epithelium was brush-bordered (with irregular microvilli) and aided in the release of secretory materials.

The cytoplasm contained three types of secretory masses: highly electron dense, electron lucent, and medium electron dense (Figure 9). Scanning through

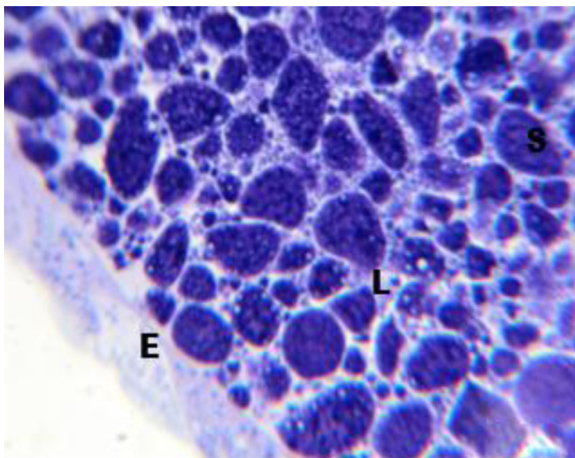


Fig. 6. Semi thin section of the accessory gland tubule showing the secretory masses x 800
E- Epithelium
L- Lumen.

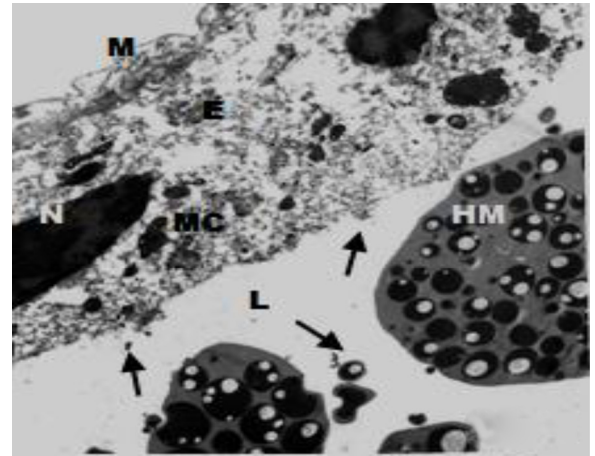


Fig. 7. Transmission electron micrograph shows a general view of the AG wall; the epithelial (E) and the muscular layer (M). Note the nuclei (N) containing heterochromatin. The epithelium displays the electron dense secretory vesicles and mitochondria (MC). Lumen exhibits the heterogeneous large mass (HM) of secretory vesicle. Arrow indicates the microvilli and the secretory globule x 10000.

various fields, it was obvious that the highly electron dense masses pinched off into small dense forms/vesicles widely seen in the cytoplasm (Figure 10). Sporadically, these electron-dense vesicles were seen towards the vicinity of the apex and exocytosed into the lumen through microvilli (merocrine). The distribution of electron lucent masses frequently surrounded the nucleus and appeared to be the precursors of secretion. This substance then fragmented into small lucent vesicles (Figure 11) in the cytoplasm. In some instances, the cytoplasm exhibited the spherical dense core surrounded by an electron-lucent halo. The medium electron-dense granular masses were sparse in the cytoplasm. Electron-lucent granular substances and medium electron-dense masses were released into the lumen through an apocrine mode, demonstrated by

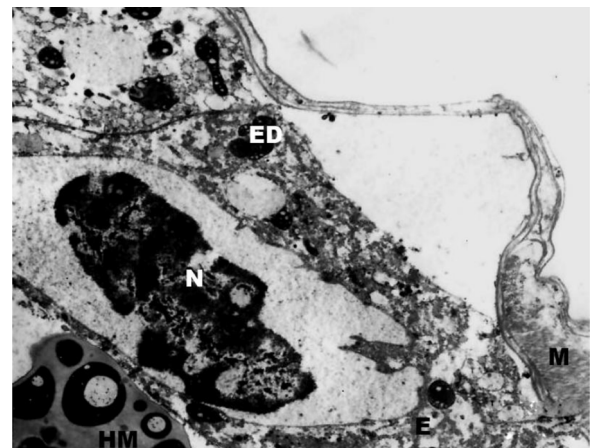


Fig. 8. The accessory glandular epithelium (E) showing nucleus (N) surrounded by electron lucent region and the lumen enclosing the secretory masses (HM) x 10000
ED- Electron dense granules
M-Muscle layer.

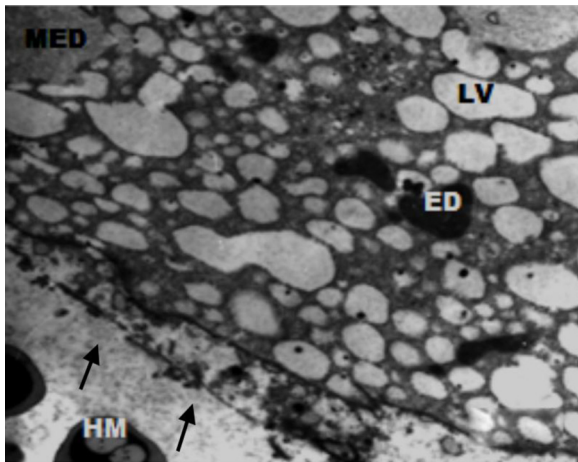


Fig. 9. The AG epithelium showing three types of secretion; Electron dense (ED), electron lucent (LV) and medium electron dense (MED). The arrow indicates the mode of secretion x 45000.

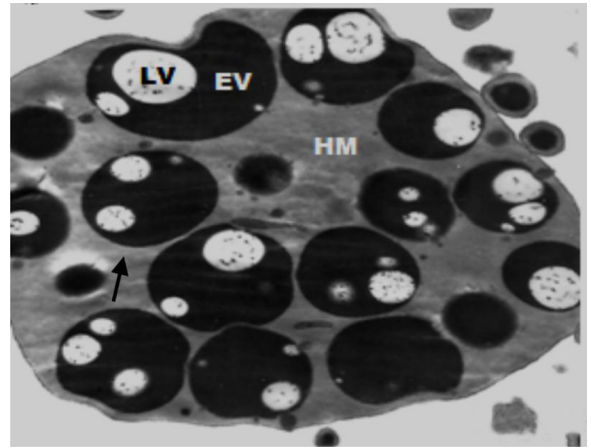


Fig. 12. A single large heterogeneous mass of secretory vesicles encompasses electron dense (EV), electron lucent (LV) secretions. Arrow indicates the medium electron dense secretion (MED) x 5000.

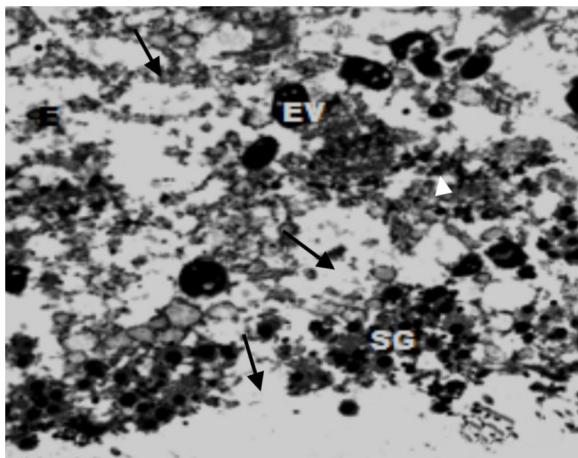


Fig. 10. The AG epithelium exhibits the degeneration of large electron dense vesicles into smaller granules (SG) and their discharge into the lumen. The arrow indicates the breakdown and apical puncture (apocrine) mode of secretion x 4500.

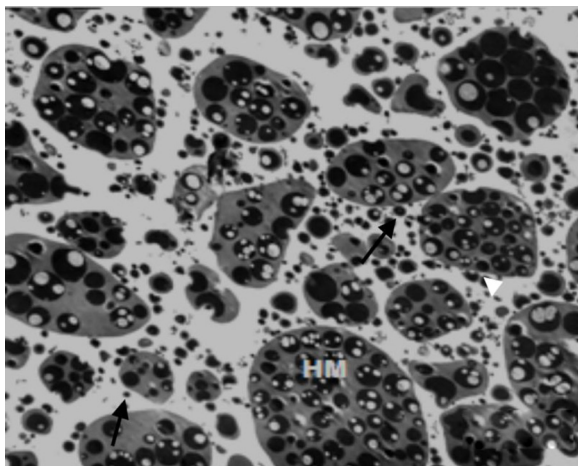


Fig. 11. The AG lumen displaying the heterogeneous mass of secretory vesicles (HM). Arrow indicates the coalescence of small vesicles x 2000.

rupture of the apical membrane and subsequent discharge of the contents into the lumen. Upon reaching the lumen, all three types of secretions coalesced to form large heterogeneous masses (Figure 12). These masses subsequently aggregated and were modified in such a way that the medium electron-dense masses encapsulated the highly electron-dense vesicles and electron-lucent masses (Figure 12).

4. Discussion

In *P. plicatum*, the AG tubules are short, sac-like structures arising through the entire distance of the posterior VD. In *M. messor*, the AG appears as fasciculated long tubules [8], and coral-shaped with bifurcating tubules in *O. platytarsis* [6] and *Ocypode ceratophthalmus* [23]. The ASGs of *M. brachydactyla* are composed of seven or eight highly ramified, enlarged diverticula connected to the dorsal region of the posterior VD [9].

The AG wall is trilayered and the epithelium is cuboidal in *P. plicatum* as previously reported in *O. platytarsis* [6], *O. ceratophthalmus* [23], *M. messor*, *S. quadratum* [8], and *M. brachydactyla* [9]. In *P. plicatum*, the basophilic granular secretions are seen amidst the eosinophilic vesicular secretions. Both types are positive for PAS and MBB staining and negative for Alcian Blue, revealing the presence of glycoproteins, akin to the pattern of *S. quadratum* [8], but contrary to the glycoproteinaceous and mucoproteinaceous nature of *M. messor* [24,25] and *O. platytarsis* [6].

The epithelial cells of the AG of *P. plicatum* show an elongated nucleus with condensed chromatin and several nucleoli, rough endoplasmic reticulum, Golgi complex, and ribosomes, demonstrating their secretory nature. The significance of membranous infoldings in the epithelium of *P. plicatum* may be involved in promoting the transport of materials. This was already reported in the AG epithelium of *M. messor* and *S. quadratum* [8] and in the VD epithelium of the cray fish, *Cherax albidus* [26]. Furthermore, the microvilli seen at the luminal surface of the epithelium in the present study suggest the transport of secretory

materials as in *O. ceratophthalmus* [23], *M. messor*, *S. quadratum* [24], and *Helothis armigera* [27].

The lumen shows several secretory vesicles of varying sizes, encompassing electron-dense mass in the electron-lucent material. The nature and mode of release of these masses of *P. plicatum* attracts considerable interest. The highly electron-dense vesicles, the electron-lucent, and the medium electron-dense vesicles that amass at various regions of the epithelial cytoplasm appear to be secretory precursors, which are partially discharged by merocrine and apocrine secretion. The microvilli seen at the luminal surface of the epithelium in the present study strongly suggests the merocrine mechanism as recorded in *O. ceratophthalmus* [23], *M. messor*, *S. quadratum* [24], and *H. armigera* [27]. The electron-dense vesicles accumulated at the apical epithelium, and were released into the lumen with mechanical damage to the apical surface. The microvilli on the luminal plasma membrane diminished, probably due to apical damage, which confirmed the apocrine mode in comparison to *O. ceratophthalmus* [23,28]. Different groups of crustaceans exhibit different modes of secretion. For example, in *Squilla holoschista*, the AG epithelial secretion is released by a macroapocrine process [29], whereas in the fiddler crab, *Uca triangularis bengali*, the secretion is meroapocrine [7]. In *M. brachydactyla*, the secretory material produced by the endoplasmic reticulum seems to accumulate in the cytoplasm, forming granules released through an apocrine mechanism [9].

After the release into the lumen, the secretory products of the glandular epithelial cells of *P. plicatum* apparently undergo structural and/or chemical changes in the lumen. The contents of the electron-dense vesicles present in the apical cytoplasm differ from those in the large secretory units found in the lumen. Such post-release changes were noted in the ASGs of *O. ceratophthalmus* [23], *M. messor*, and *S. quadratum* [24]. Therefore, we presume that the highly organized structure of secretory units is achieved largely within the lumen. The large globular and heterogeneous secretory mass seen within the AG lumen is the result of aggregation of electron-dense granules interspersed with electron-lucent ground substance. The portion of the lumen in close vicinity to the apical portion of the epithelium also shows the presence of numerous dense granules and electron-lucent materials, which are from aggregates of the granular entities. It seems likely that the lucent materials are subsequently amassed and modified into a large globular secretory mass.

The exact role of the AG gland secretion is enigmatic. However, it is obvious from the present study that the AG secretion does not directly contribute to the spermatophore matrix; nor does it play any significant role in spermatophore wall formation, inasmuch as the spermatophore formation is seen to occur before the semen arrives at the posterior part of the male duct where the AG is present. Beninger and Larocque [30] have suggested that acid mucopolysaccharides of gonopod tegumental glands protect the male genetic investment from opportunistic microbes following copulation, sperm competition, and paternity assurance, whereas the neutral mucopolysaccharides may function as a lubricant to reduce mechanical wear of the ejaculatory duct and reduce the viscosity of the

ejaculate as it enters the ejaculatory canal. In the present study, the glycoproteinaceous secretion of the AG may have alimentary/or metabolic roles, and may give mechanical support to aid in the indehiscence of the spermatophore wall, which corresponds with *M. messor* and *E. sinensis* [11,31]. In our previous study, the organic and inorganic constituents of AG secretions of *P. plicatum* may be involved in sperm viability and prolonged storage of spermatozoa within the male and female tract [32]. An antimicrobial role for the AG secretion of various microbes cannot be negated at this point inasmuch as the proteinaceous seminal secretion of *Penaeus monodon* and *Scylla serrata* has been shown to have antimicrobial activity [33,34].

It is supposed that in mammals the ASG is known to have a variety of bioactive molecules [13]. These molecules exert a wide-ranging effect on female reproductive activities. A portion of ASG proteins make a nutritional contribution in spermatozoa and they also exert an influence on spermatozoa vitality [35,36] and fertilization capacity [13,37], and can induce a cascade of membrane alterations [38]. We presume that the AG secretion of *P. plicatum* may also have the aforementioned functions, which warrants more research.

In conclusion, the AG is an important component of the male reproductive system in crabs, which functions to enhance the fertility of spermatozoa, digest the spermatophore wall, provide nutrition to the spermatozoa, and is involved in antibacterial activity and spermatozoal viability. Surveying the presence of AG among the brachyuran crabs, it becomes apparent that the AG is present in several of the mangrove and estuarine crabs: Grapsidae, Majidae, and Ocypodidae crabs (*M. messor*, *S. quadratum*, *O. ceratophthalmus*, *M. brachydactyla*, and *E. sinensis*). This suggests an additional adaptive role in their respective ecosystems and phylogenetic significance, although AG materials are an independent and important component of the male reproductive system of brachyuran crabs that warrants further research in all taxa.

Conflict of interest

The authors declare that there is no conflict of interest.

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