J. mar. biol. Ass. India, 1994, **36** (1 & 2) : 267-275

THE REPRODUCTIVE BIOLOGY OF THE WARM WATER SQUID *LOLIGO DUVAUCELI* D' ORBIGNY : 3. FORMATION OF SPERMATOPHORE

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ABSTRACT

The anterior vasdeferens (AVD) shows histologically four different regions. The histological details of these regions along with their luminal contents reveal that atleast three events are involved during the passage of spermatids through the AVD : (a) Aggregation of spermatids into a 'coiled rope' along with the formation of a coat with the help of a granular secretion, (b) subsequent dissolution of the coat with the help of a fluid secretion and (c) Transformation of spermatids into spermatozoans at the distal end of AVD where it enters into the spermatophoric gland aided by the secretion of this gland.

The spermatophoric gland is the most prominent portion of the male reproductive tract. When the outer covering is removed this gland has three lobes, *viz.* 'A' 'B' and 'C'. The spermatophoric gland reveals the sequential manner in which the spermatophores are formed. The fully mature spermatozoans pass into the 'sperm tract' and get concentrated at the blind ends of the diverticula 'A'. The contributions of the three lobes of the spermatophoric gland for the formation of spermatophores are evident. The mid vasdeferens (MVD) plays the role in quick transfer of fully formed spermatophore for effective storage in the Needham's sac. Through the posterior vasdeferens (PVD) the spermatophores reach the terminal region of the reproductive tract.

INTRODUCTION

MANY spermatophores are multilayered structures which undergo a programmed sequence of expansions before liberation of sperm (Khalifa, 1949; Feldman-Musham *et al.*, 1973; Gadzma and Happ, 1974). The spermatophores are elaborate structures enclosed in an elastic proteinaceous coat (Mann *et al.*, 1966, 1970); in life they are stiff and turgid when discharged. The spermatophore of each species of cephalopod has a distinctive structure and since they are hard and readily preserved, they can be used as a taxonomic character (Marchand, 1913). But little is known of the formation and transfer of spermatophores in cephalopods.

MATERIAL AND METHODS

The squid *Loligo duvauceli* for the present study were collected from mechanised boats which reach Royapuram Fishing Harbour (13° 06′ N — 80° 18′ E) of North Madras. Fresh squids were dissected and the different regions of the entire reprodutive system were cut and transferred to small tubes containing Bouin's fluid. Sections were cut at 5 — 8 μ and stained with Ehrilch's haematoxylin with aquous eosin as the counter stain.

RESULTS

Anterior vasdeferens

The anterior vasdeferens (AVD) which runs from the testicular end to the tripartite spermatophoric gland is highly convoluted so as to give the appearance of epididymis. The convoluted tube is packed into a tangled mass, but not enclosed in the genital sac while bound to the gland. However, the vasdeferens is not of uniform width, but appears to widen in the middle, only to narrow down again before entering the gland and continue as a 'sperm tract'.

The AVD is divided atleast into 4 regions. Though these regions cannot easily be distinguished externally, mainly because of the impossibility of uncoiling the highly coiled narrow anterior vasdeferens, they differ widely in their contents and width.

The first region starting as a wide funnel-like opening (1200 x 400 μm) is thick walled and constituted by the usual three layers, namely outer connective tissue which ranges in thickness from 20 to 110 μm, the middle muscular tissue which varies from 10 to 20 μm and the inner epithelial lining whose thickness varies from 20 to 30 μm. The epithelial lining composed of flattened cuboidal cells is thrown out into typhlosole-like folds, some of them reach the centre of the lumen and are secretory. The outer margin of the folds contain secretory granules of diameter ranging from 8-15 μm which appear to be pinched off from the secretory epithelium and membrane bound (Pl. I B).

They are found throughout the interior of the lumen, densely packed around the cylindrical spermatid aggregation. The spermatids at this region occur in the form of a coiled rope and in cross section as discs with a diameter ranging from 150-250 μm. Each 'disc' is covered by a thin (8-10 μm) noncellular covering which may be a secretory coating, probably help to maintain the aggregation. There is a less dense central area in each 'disc' giving a false appearance of a central lumen. The spermatids of this stage are characteristically round with a diameter of 9-11 μm (Pl. I C, D).

Even in the immature males, the formation of typhlosoles (atleast five in number) from the epithelial layer is distinct. The wall, at this stage of maturity of the animal has the connective tissue of thickness varying from 20 to 30 μm and the epithelial layer has the same thickness while the muscular layer is poorly developed. Even secretory granules ranging from 8-10 μm occur. The spermatid aggregations are however absent (Pl. I A).

The second region of the AVD is of medium diameter (550 × 450 μm) and slightly elliptical in cross-section. When compared to the next region of the vasdeferens, it is relatively thick walled, consisting of connective tissue layer of thickness 20-30 μm and an inner loose layer of epithelial cells with thickness of 25-35 μm. They do not appear to be secretory in function. The typhlosole-like folds so prominent in the previous region is absent. However, some secretions in the form of a fluid is available in the lumen. Only 1 or 2 spermatid 'discs' occur in this portion of AVD which may be due to the fact that the sperm rope is less coiled here. The characteristic coating around the spermatid aggregation of the previous region is almost absent here. However, the aggregations have not lost their shape except that they are slightly flattened which gives an elliptical appearance in cross-section (Pl. I E).

The third region of the AVD is more elliptical (620 × 400 μm) and its inner layer of the wall is produced into a few small folds. While the wall is compact, the outer connective tissue has a thickness of about 10 μm which is followed by a prominent muscular layer of thickness ranging from 10-20 μm. The inner secretory lining is however not prominent, but in places where it is thrown out into folds contains secretory granules of bigger size. The thickness of these secretory granules range from

8-11 μm in diameter. It is very interesting to note that the entire lumen of the duct is filled with spermatids and the spermatid aggregation has lost its cylindrical shape due to the dissolution of the noncellular covering seen in the previous regions of the AVD, though the spermatids occur in a denser proportion at the centre. The diameter of the spermatid in this region ranges from 8-12 μm and intermingled with them occur secretory granules in larger proportion. The maximum diameter of this region is between 450 and 620 μm with the spermatids occupying the central region to an extent of 350-530 μm (Pl. I F).

In immature animal the epithelial cells have not developed in this region while a few germ cells occur in the lumen, which have a thickness of 5-8 μm. However, the connective and muscular layers are distinct.

The fourth and terminal region of the AVD which enters into the gland is the narrowest portion (500 × 400 μm) and thin walled. The usual three layers are not clearly seen especially the muscular layer. However, the entire interior of the lumen is packed with spermatozoans which have almost completely transformed from the spermatids of the previous region. While in some sections stained secretory products are found competing with the spermatozoans to occupy the lumen, in certain other sections the spermatozoans appear to have been penetrated by the secretions from the gland and divided into 3 to 4 longitudinal masses. The spermatozoans are deeply stained while the secretions are not so. These secretions are not produced by the wall of the duct since there is no evidence of secretory cells at this region, but have come from elsewhere, probably from the spermatophoric gland as this region, is lodged almost inside the above said gland. However, it is certain that the spermatids undergo metamorphosis only in this region.

Spermatophoric gland

The complex tripartite spermatophoric gland appears to be an over growth of glandular mass around a 'sperm tract' which is the continuation of the AVD. This tract which is cylindrical and thick walled is provided with villi protruding into an empty lumen during the younger stages. The duct is located on one side of the section and on the other side is situated the glandular mass which to begin with consists of only one region and is neatly laminated like that of the gill (Pl. II A). The gland at the next stage of development can be differentiated into two regions, one continues to give the laminated appearance, while the other gives the appearance of cristae (Pl. II B). The organisation and appearance of the 'sperm tract' at this stage of development is closely similar to that of the AVD. In the subsequent stage of development of the gland, the tissue around the 'sperm tract' develops and penetrates into the interior and forms a wedge between the laminated region and the cristae-like region (Pl. II C).

In the mature animal, the tripartite organisation of the gland is clear when the covering membrane is removed while obviously evident histologically (Pl. II D).

Lobe 'A' of the gland into which the AVD enters and from which the MVD emerges consists of two portions.

1. A thick walled duct which runs in various directions in the different regions of the lobe thereby revealing its coiled nature leads to a number of diverticula. Sections at the distal end show the double walled nature of this region clearly (Pl. II E, F). The outermost layer is composed of connective tissue and forms 20-120 μm in thickness. The inner layer consists of tall columnar cells. It is because of the infolding of the inner columnar layer

the duct gets the double walled nature. The size of the outer wall consisting columnar cells vary between 30-70 μm, while that of the inner wall is 30-50 μm in thickness. The secretion of this layer is fluid in nature, which appears to spread to the other parts of the gland as well as the interspace. For Mallory, the epithelium appear in orange, while the fluid 'secretion gives light blue. 2. The second portion is a mass of glandular tissue surrounding the convoluted 'sperm tract' and its diverticula. The glandular tissue are provided with cuboidal epithelial cells, with a thickness verying from 15-20 μm (Pl. II D, Pl. III A). This layer is covered peripherally by connective tissue having a thickness of 20-40 μm. The secretion of this region is granular and the granules are found in the interior region. Narrow ductular pathways lead from the glandular tissue towards the 'sperm tract'. The diverticula enclosed by the glandular mass of tissue are provided with epithelial cells, which appear to be ciliated. It is appropriate, to consider them as having 'brush border' or 'microvilli' (Pl. III B). These diverticule contain various stages of the developing spermatophores, right from accumulation of spermatozoans (Pl. III B, C, D) to almost completely formed spermatophores (Pl. III D). The developing spermatophores are present only inside these diverticula (Pl. III B). The spermatozoans in these diverticuia measure to a length of 7-15 μm and a breadth of 3-4 μm. The epithelium of glandular mass stain light blue and the glanules appear in dark red for Mallory.

Lobes 'B' and 'C' of the tripartite gland are more plain regions with irregular secretory epithelium at the periphery (Pl. III E, F). The epithelial cells are of columnar type in C, while cuboidal in 'B'. The thickness of the epithelium of 'C' region range from 15-35 μm. The mode of secretion appears to be apocrine and the central lumen is filled with secretory granules. These granules stained red in Mallory's and have a thickness of 5-10 μm. The secretory epithelial layer is covered peripherally by connective tissue whose thickness is about 10 μm. The secretion of these lobes appear to spread to the other regions through the interspace and finally reach the diverticula where the spermatophores are formed. For Mallory the epithelium of these two regions stains light blue while the granules appear in dark red.

It appears that all these three regions of the tripartite gland contribute to the formation of spermatophores.

Mid vasdeferens

The mid vasdeferens (MVD) takes its origin from lobe 'A' of the tripartite gland close to the entry of the AVD. It runs backward for a short distance and enlarges into the Needham's sac. The MVD is thin walled, though consisting of the usual three layers. The lumen does not appear to contain any secretion (Pl. IV A). It is likely that this short and wide duct is meant only to pass the fully formed spermatophores down from the tripartite gland to the Needham's sac. It is further likely that the passage of the spermatophores is a passive process indicated by the very thin muscular layer of the wall. The wide lumen of the MVD contains innumerable (about 100) fully formed spermatophores (Pl. IV A, B).

Needham's sac

The Needham's sac is a crescent shaped thick walled region with an outer connective tissue of thickness 20-40 μm, a middle faint muscular layer with 5-10 μm thickness and an inner secretory epithelial cells of cuboidal shape of thickness 20-30 μm, which on the other hand is well developed. Further, the lumen contains numerous spermatophores (Pl. IV C, D) arranged in parallel rows which in transverse section appear round, surrounded by secretory granules of thickness 8-11 μm. The presence of these secretory materials may indicate that some portion such as cap and cap thread of the spermatophores may be formed in this region. Apart from storing the spermatophores, till they are released, the sac may play a role in giving a finishing touch to them.

The thickness of the spermatophoric wall at this region is 10-12 μm and spermatozoans are present inside, arranged in a compact manner. The sperms are rod shaped and a refractile fluid bathes them, often called the spermatophoric plasma or matrix. While the diameter of the spermatophore including the wall is between 110-220 μm, the rod shaped spermatozoans have a length of 10-15 μm and a breadth of 3 to 5 μm. In larger males (dorsal mantle length of 9 cm) the length of the spermatozoa varies from 15-20 μm and a breadth of 4-6 μm.

Even in the immature males, the three layers of the Needham's sac are distinct : the outer connective tissue has a thickness of 20-30 μm the middle muscular layer 10-15 μm and the inner epithelial layer ranging from 20-35 μm. The secretory granules present in the lumen has a thickness of 5-7 μm. The spermatophores are evidently absent.

Posterior vasdeferens

The last region of the male reproductive system is the posterior vasdererens (PVD) which is a lengthy tube running towards the oral end from the Needham's sac. The diameter of the PVD increases at the terminal region and is distinctly flattened. The wall of the PVD is much prominent with a peripheral connective tissue of 40-60 μm thickness, followed by a thick muscular layer - unlike the wall of the Needham's sac having a thickness of 60-90 μm and the inner most epithelial layer of thickness ranging from 30-40 μm composed of cuboidal cells (Pl. IV E).

While the portion of the PVD closer to the Needham's sac is round in TS, the same near the terminal opening is elliptical. The musculature of the wall of the PVD at the distal region is still thicker suggesting a function, eventhough it may not be protrusible (Pl. IV F). The lumen is not so wide as in the MVD or Needham's sac, but has a diameter of 20-35 μm containing only a few spermatophores. The diameter of the spermatophore including its wall is 60-70 μm while the wall alone has a thickness of 15-20 μm. A few secretory granules of 10-15 μm diameter occur in the lumen of the PVD.

The lumen of the distal portion is so much compressed it appears that only by slight force it may be opened. In this region, the connective tissue has a thickness of 30-60 μm and the muscular layer extends to a thickness of 60-100 μm while the inner epithelium has thickness of 30-70 μm. The narrow lumen is only 5-30 μm wide.

Discussion

The results of the histological study of AVD are very interesting in that it could be divided at least into four regions. Because of the highly coiled nature of the narrow AVD one and the same section may show at least three regions. Further, the various transformations the spermatids undergo and finally metamorphose into spermatozoans are clearly revealed. The funnel shaped beginning of the AVD gives a clue to its role in the collection of spermatids released from the ventral opening of the testis. The epithelial lining consisting of flattened cuboidal cells with its typhlosole like folds along with the secretory ducts, plays the vital role of aggregating the spermatids into a 'coiled rope' which in CS appear as discs and is covered by a non-cellular

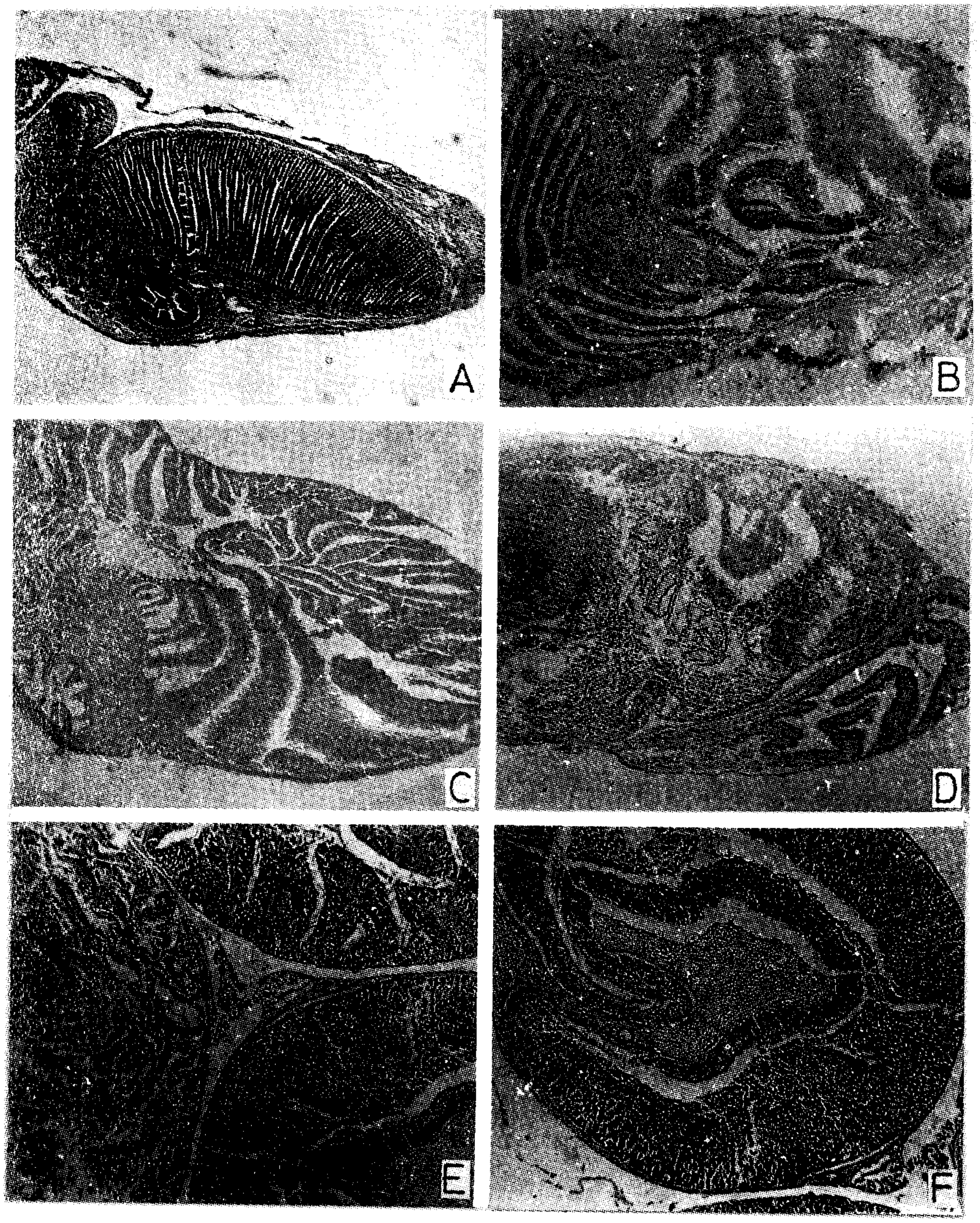

PLATE II. Cross sections of : A. Undifferentiated laminated glandular region with the distinct 'Sperm tract' (× 80), B. Second stage of the development of the immature spermatophoric gland showing the two differentiated regions of the gland (laminated and cristae-like regions), C. Third stage of development of the immautre spermatophoric gland showing the development of the tissue around the 'Sperm tract' and the formation of a wedge between the laminated and cristae region (× 80), D. Fourth stage of development of the spermatophoric gland showing the tripartite nature (× 80), E. Tripartite gland showing the lobes A and C (× 80) and F. Lobe A of the tripartite gland (× 80).

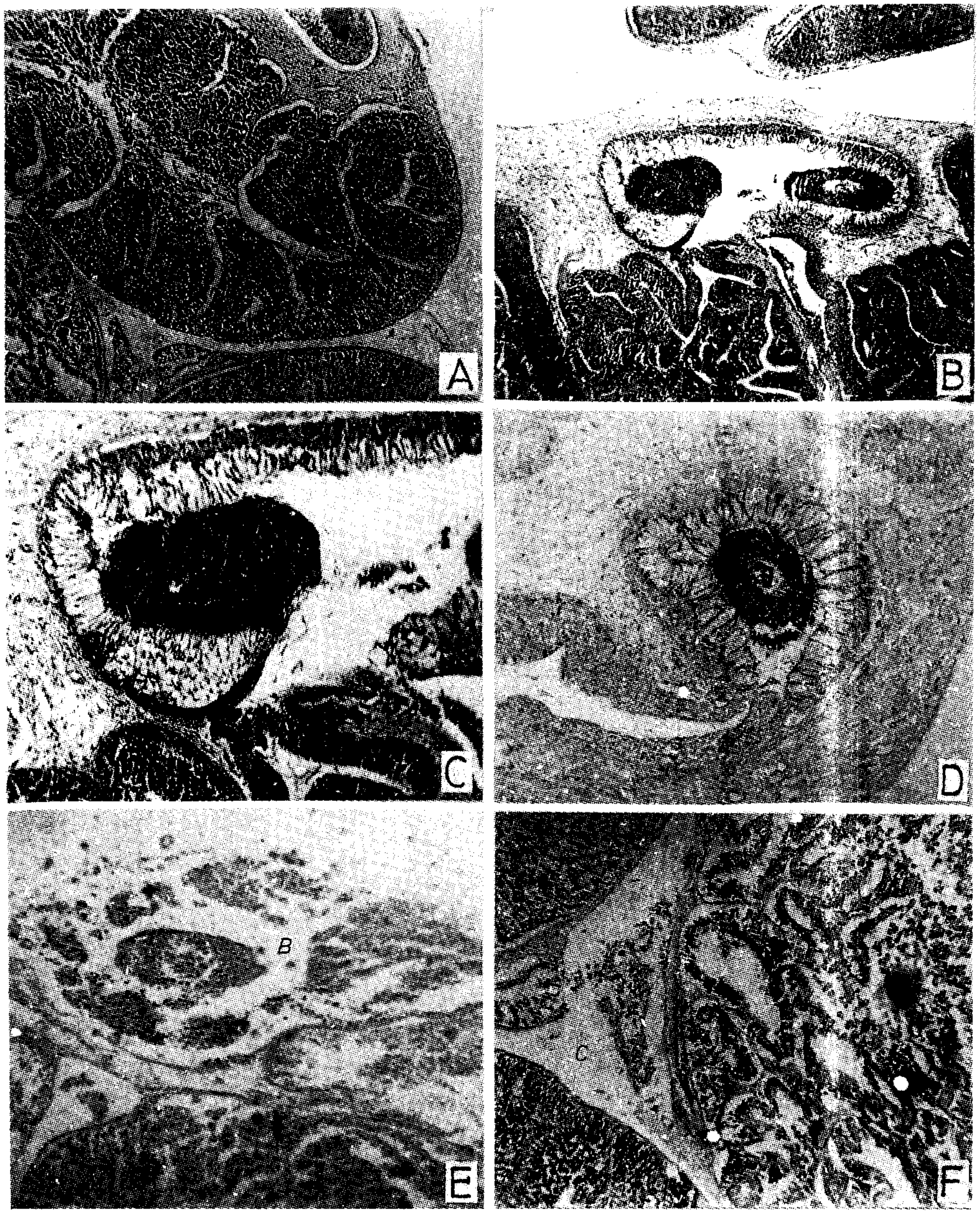

PLATE III. Cross sections of : A. Glandular portion of lobe 'A' of the tripartite gland (× 80), B. Formation of two spermatophores surrounded by 'refractive fluid' inside the diverticulum, C. Spermatophore surrounded by 'Brush border' or 'Microvilli' (× 200), D. Formation of a single spermatophore in a diverticulum (× 80), E. Tripartite gland showing lobe 'B' (× 80) and F. Tripartite gland showing lobe 'C'.

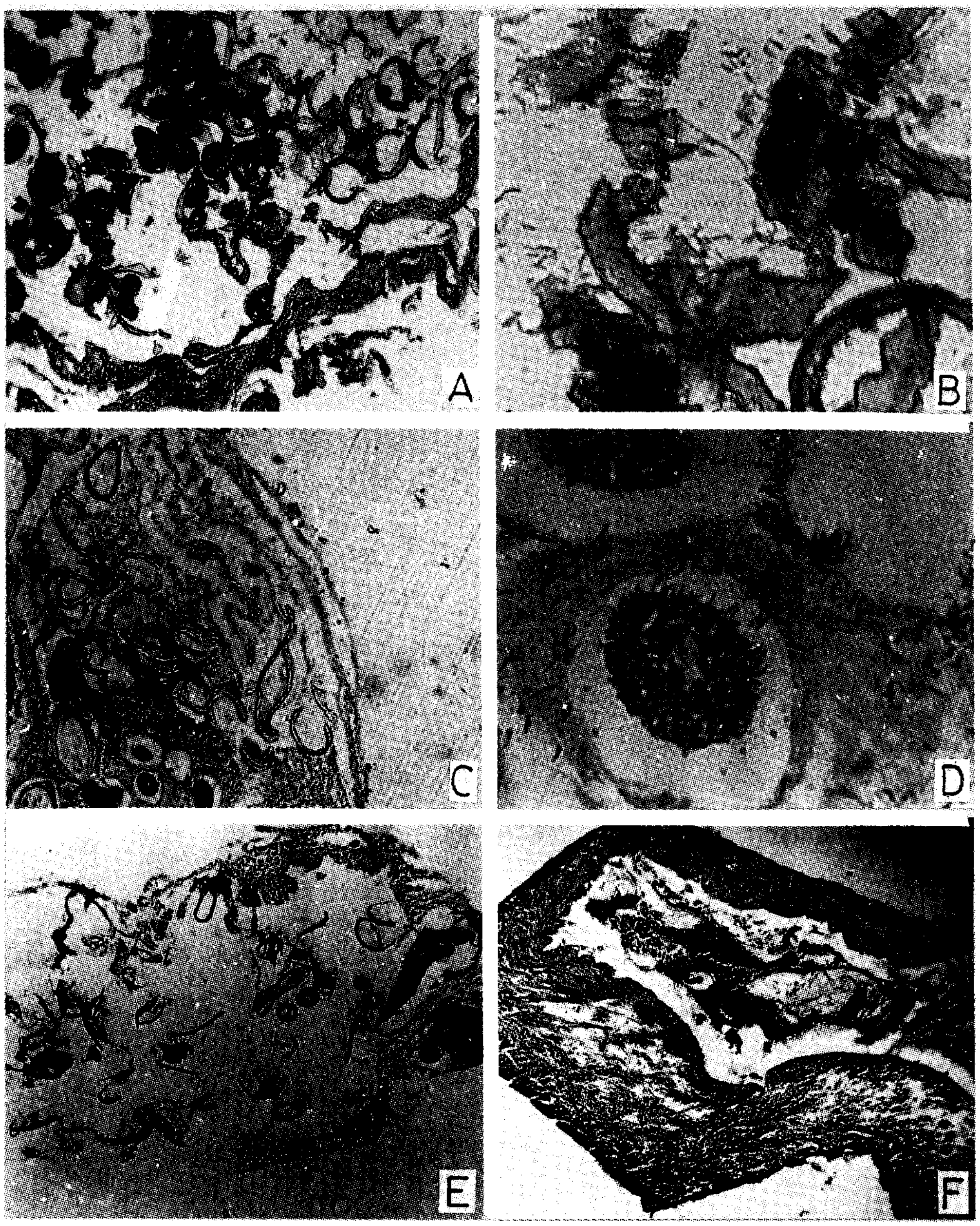

PLATE IV. Cross sections of : A. MVD showing fully formed spermatophores (× 80), B. Portion of lumen of MVD showing the rod shaped spermatozoans (× 900), C. Needham's sac showing spermatophores (× 80), D. Same in higher magnification (× 900), E. Proximal region of PVD showing spermatophores (× 80) and F. Distal region of PVD showing well developed musculature and compressed lumen (× 80).

not only of mature sperm, but also of developing spermatocytes and spermatogonia. Further, it is reported that when the male gametes leave the accessory gland they are all mature sperms suggesting that the immature male gametes might have completed their maturation inside the accessory gland. Similar cases of sperm maturation in the reproductive tract have been reported in some strains of honey bees (Adiyodi and Adiyodi, 1974). In the copepod parasite *Xernanthropus kryoyeri*, Coste *et al.* (1980) have reported that the late stages of spermiogenesis take place in the first part of the vasdeferens. On the contrary Suleiman and Brown (1978) reported that in the dog tick, *Dermacentor variabilis*, the male gametes are transported to the female in the form of spermatids and the spermatogenesis is accomplished only on exposure to secretion of the female reproductive tract. In oligochaetous annelids too the seminal vesicles form the site of sperm maturation (Stephenson 1930; Zaman, 1982). The implication is that such a necessity of a secretion in sites other than testis for sperm maturation is not uncommon among invertebrates.

The fully mature spermatozoans appear to pass into the 'sperm tract' which is nothing but the continuation of the AVD and reach the various diverticula and get concentrated at the blind ends. The diverticula are lined with columnar epithelial cells which are highly secretory and appear to be ciliated. Owing to their intense secretory activity and mode of extrusion of their products it is appropriate to describe them as having 'brush border' or 'microvilli'. The secretory products accumulate around the sperm mass in a stellate fashion. The refractive fluid secreted by the ductular portion of lobe 'A' was seen spreading to various interspaces of lobe 'A' and also around the epithelial lining of the diverticula. The secretion appears to be different from that of the other secretions of the gland in not only not being granular membrane bound or otherwise, but also in the staining properties. It is probable that this refractive fluid may percolate into the lumen of the diverticula and form the matrix of the spermatophores. This should happen before the tunics are formed. The spermatophores appear to be stationary till all the components are formed while in the diverticula. Though clear cut details about the formation of the successive tunics are not revealed by the histological sections, the fact that fully formed spermatophores leave the lobe 'A' of the gland through the MVD indicates that all the tunics are formed while in lobe 'A' to which process lobes 'B' and 'C' would have contributed by their secretions. The portion of lobe 'C' opposing the diverticula containing developing spermatophores, shows intense staining thereby, revealing its contribution to the final stages of spermatophore formation. The path of exit could only be the 'sperm tract' which continues as the MVD. The fact that sections did not show fully formed spermatophores inside the 'sperm tract' may also indicate that the spermatophores when formed are quickly evacuated to the MVD.

The details about the formation of spermatophores in *Sepia* are meagre. Blancquaert's (1925) account provides only preliminary information regarding the contribution of the vesicles of the tripartite spermatophoric organ. However precise details are conspicuously absent. Similarly, Drew's (1919) brief description of the development of spermatophore in *L. pealii* is also devoid of details.

In *Octopus* however, it is said that the 'sperm tract' passes into the intercalary piece of the first spermatophoric gland where the middle tunic is formed. Individual sperm movement ceases at this point (Belonoschkin, 1929). In the next two sections of the first spermatophoric gland there is a curled ridge, extending into the lumen and the developing spermatophore travels down the tract within the curl. It is during the passage through these sections that the further spermatophore sheaths are secreted together with a gel column and the main core of the final ejaculatory apparatus (Mann *et al.*, 1970). The function of the second spermatophoric gland is uncertain. However, the developing spermatophore passes to the blind end of the gland and then reverse down the lumen so that it leaves the ejaculatory apparatus pointing towards the penis. Arnold and Arnold (1977) opine that the ejaculatory apparatus of the spermatophore is completed within the second spermatophoric gland and during the spermatophore's subsequent passage down the distal vasdeferens. In *L. duvauceli* the possibility of the spermatophore entering into either lobe 'B' or 'C' is very remote as they do not contain any tubules and hence if they contribute anything for the spermatophore formation, it may be achieved only through secretion which in turn may reach the diverticula or 'sperm tract' of the lobe 'A'. However, the spermatophore formation of the squid *L. duvauceli* is different and unique, especially from that of the insects where it is a simple process by which a variety of accessory secretions from the male reproductive glands are mixed together in the terminal portion of the male tract just before or even during the mating process (Landa, 1960; Rudiger 1970; Pickford and Padgham, 1973; Carminda and Ferreira, 1977; Gadzma *et al.*, 1977; Dailey *et al.*, 1980). Even in the scorpion *H. fulvipes* it is not very complex (Mahalingam, 1982). Here the mixing up of the seminal products of the testis with the accessory gland secretion leads to the formation of sperm bundles. On the contrary, in the case of the squid the complex spermatophoric organ takes the entire responsibility of forming the spermatophore with all the components from out of their own secretions.

Both the anatomical as well as the histological details of MVD indicate the role of MVD as a connecting tube between the spermatophoric organ and the Needham's sac for the transfer of fully formed spermatophores for effective storage. The absence of glandular epithelium in the MVD as well as the secretory products in its lumen indicate that the spermatophore may not undergo any developmental change in this portion of the reproductive tract. Unlike the case of the colder water squids, where the fully mature spermatophores are transferred one at a time, *L. duvauceli* appears to transfer numerous spermatophores at a time which is evident from the occurrence of innumerable spermatophores in the wide lumen of MVD. The notable absence of a thick muscular layer may indicate the absence of an active role by the MVD in this transfer process.

The Needham's sac on the other hand possesses secretory epithelial lining of considerable thickness along with the darkly stained secretory granules surrounding the spermatophores in the lumen. The spermatophores of the Needham's sac appear to be different from those of MVD atleast in two ways which are revealed better in longitudinal sections. 1. The presence of cap and cap thread. 2. Slight reduction in size. The Needham's sac in *Octopus* is a blind tube, which does not appear particularly modified; however, the cap and the cap thread of the spermatophores are believed to be formed in this organ (Peterson, 1959). Further, there is general finishing and hardening or dehydration in this organ. It appears the same is true in *L. duvauceli*. However, the PVD is provided with a thick muscular wall and an inner secretory epithelium. Along with the limited number of spermatophores a few secretory granules are also seen in the lumen of the PVD. When compared to the other cephalopods *L. duvauceli* has more lengthy and elaborate PVD suggesting a role during mating. However, it may not be protrusible. Unlike the case of *Octopus*, where (Arnold and Arnold, 1977) spermatophores are passed one at a time from Needham's sac into the diverticulum of the penis, *L. duvauceli* appears to pass a few spermatophores at a time. The relative size of the lumen of the PVD and the spermatophores indicate that the spermatophores might be forced through the narrow lumen during exit which role the PVD itself might perform as indicated by its thick musculature. However, in *L. pealei* the penis lies to the left of the anus as a muscular extension of the spermatophoric sac through which small bunches of spermatophores are passed during copulation (Arnold and Arnold, 1977).

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