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# **Phlebotomine sandfly Reproduction: Fine Structure and Function of the Spermathecae**

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## Abstract

In contrast to most blood sucking flies, the structure of the spermathecae in phlebotomine sandflies exhibits extraordinary diversity. The objective of this study was to investigate the fine structure of the spermathecae in relation to mating of phlebotomine sandflies.

Light microscopy and electron (scanning and transmission) microscopy were used to examine species of four representative subgenera from the Old World *Sergentomyia* (*Parrotomyia*) *babu*, *Phlebotomus* (*Euphlebotomus*) *argentipes*, *P.* (*Phlebotomus*) *papatasi* and *P.* (*Larroussius*) *langeroni*. The spermathecae of *P. papatasi* is composed of a pair long spermathecal ducts, a cylindrical spermatheca surrounded by a visceral muscle and a spherical gland attached by an epithelial layer. The internal histology of the spermathecae in all the four species includes: a simple epithelial layer of class 1 epidermal cells, elaborate glandular cells (class 3 epidermal cells) each having receiving and conducting ductules ("end apparatus") and a cytological apodeme, which is a new finding for the insect epidermis. The glandular cells are connected to the lumen of the spermatheca by cuticular ductules passing through a cuticular block which has a high resilin content. The spermathecal complex is enveloped in a supercontracting visceral muscular system and has a myoneural junction. Based on this study a new terminology for the spermathecal morphology is proposed.

The spermathecae undergo substantial physiological changes during the female gonotrophic cycle based on studies with *Phlebotomus papatasi* and *P. argentipes*. A histochemical study revealed a mucopolysaccharide secretory-mass in the newly emerged fly. During insemination, spermatophores are deposited in the spermathecal ducts. Previous reports of mating plugs in sandflies appear to be artifacts. The histology of the spermatophore is described and the anatomical evidence for sperm competition and

displacement presented. Blood-feeding stimulates the release of sperm from spermatophores and then they migrate to the lumen of the spermatheca. The fine structure of spermatozoa in *P. papatasi* and *P. argentipes* during their morphological change, such as acrosomal membrane casting off<sup>is</sup> described. Physical evidence suggests that *P. papatasi* is inseminated more than once in each gonotrophic cycle, and that further insemination is necessary for subsequent cycles.

The lock-and-key hypothesis has been suggested as an important mechanism for species evolution. To test this, the length of the spermathecal duct and aedeagal filament were examined from 28 species of 13 subgenera of the Old World phlebotomine sandflies. In general, there was a positive correlation between these variables but in most taxa the aedeagal filaments were long for direct insemination of the spermathecal proper. These findings indicate<sup>that</sup> sperm competition takes place.

Given the structural diversity of the spermathecae and its significance in mating strategies, and the importance of spermathecae in phlebotomine systematics, a comparative morphological study of spermathecal variation with male genitalic variation was made to classify the Old World phlebotomines. Twenty nine taxa representative<sup>of</sup> all the Old World subgenera were analyzed by cladistic methods. The phylogenetic groupings based on these characters generally confirmed the presently accepted system, and that all the vectors of human leishmaniasis are in recent terminal taxa.

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# 1. General Introduction and Aims

## 1. 1. Biology of Phlebotomine sandflies and their Medical Importance

Phlebotomine sandflies (Diptera: Psychodidae) are a group of delicate hairy flies which belong to the primitive Nematocera and are distributed in warm parts of the world. To date ca. 700 species have been described comprising 8 genera, 28 subgenera, 18 species-groups and 3 ungrouped taxa from the both Old World and New World. Of these, 81 are proven or suspected vectors of *Leishmania* (Kinetoplastida: Trypanosomatidae) protozoan parasite causing diseases known as leishmaniases which affect the skin, viscera and/ or mucocutaneous region of humans (WHO, 1990).

As the vectors of the leishmaniases, phlebotomine sandflies i.e. *Phlebotomus* spp. in the Old World and *Lutzomyia* spp. in the New World, have been studied for the last seven decades with regard to disease transmission. Studies have included systematics, isoenzymes and DNA probes for incrimination of vectors, rearing and colonization, host-parasite interaction, blood meal digestion, susceptibility to infection etc. However knowledge on several aspects of fundamental biology of phlebotomine sandflies lags ca. 20 years behind other haematophagous insects such as mosquitoes, tsetse flies, blackflies etc. largely due to the minute size of sandflies. The biology of reproduction has been very little studied in the Phlebotominae. Some basic facts on the distribution, systematics, life cycle, mating behaviour and blood feeding behaviour of sandflies in relation to disease transmission (Lane, 1993) is summarised as below.

**Distribution:** Sandflies are found mainly in the tropics and subtropics. In the Old

World, man biting sandflies (and therefore leishmaniasis) are largely confined to the subtropics and tropics, there being relatively few anthropophilic species in Africa south of the Sahara and none in South East Asia (although species of *Phlebotomus* are present). In contrast, the transmission of leishmaniasis in the New World is principally in the tropics. In the Old World most foci of leishmaniasis, particularly of cutaneous leishmaniasis, are in dry, semi-arid areas- in contrast to the New World where the disease is mainly transmitted in forests.

**Systematics:** To date, there is no general agreement on the higher classification of sandflies. The principal differences in view point lie in the ranking of taxa rather than their composition or relationships. Some specialists consider sandflies a separate family (Abonnenc and Leger, 1976, Lewis, 1973 and Williams, 1993) but most retain subfamily status within the Psychodidae (Fairchild, 1955; Lewis et al., 1977; Theodor, 1958). Hennig (1972) proposed a "natural" relationship, rather than simply grouping of the taxa, of the sandflies as a subfamily based on morphological features of the adults and larvae but he left many questions unanswered because of the lack of details including the extraordinary structural diversity of the spermathecae. His view was that the phlebotomine sandflies are not monophyletic in origin but regarded the Old World and New World sandfly fauna as sister groups. The departure from the single subfamily status of sandflies was made by Abonnenc and Leger (1976), primarily based on Hennig's (1972) work, who proposed a new rational classification in which the family Phlebotomidae is divided into three subfamilies, one Old World, one Old and New World and one New World only.

**Life history:** Sandflies are terrestrial breeders about which very little is known. Available information on immature stages comes mainly from laboratory-rearing of eggs obtained from wild caught females. A female lays between 30 and 70 eggs and hatching occurs one to two weeks later. There are four larval stages; they are terrestrial, diapause and their food includes organic detritus. The pupa is inactive and usually hatches within five to ten days. The period from oviposition to adult eclosion is 20-40 days, but up to several months in diapausing species. In males, the terminalia rotate through 180° during the 24 hours immediately after emergence. Most species in temperate regions have only one generation per year but the same species can have two or three generations per year in climatically more favourable areas. In some tropical places, sandflies react more to rainfall than temperature cycles, as<sup>a</sup> result they are more abundant in either the wet or dry season depending on species. Several anthropophilic species can be present in any one area, each with its own cycle of activity and potential for parasite transmission.

**Mating:** The reproductive biology of sandflies is very little known. Mating takes place on the host during female seeking a blood-meal which occurs in a lek (eg. *Phlebotomus argentipes*, Morphospecies B, in the domestic areas) or in a swarm (eg. *P. argentipes*, Morphospecies A, in the peridomestic areas; Ilango et al., 1994). Species-specific pheromone and courtship songs are produced by males of *Lutzomyia longipalpis* to attract the females while host seeking (Ward et al., 1988). In spite of the fact that there has been a tremendous growth in understanding the morphology of animal genitalia and evolution of mating systems, there are no comprehensive comparative studies on the mating system of phlebotomine sandflies.

Blood feeding: Only females feed on blood, using the proteins for egg development. Blood taken from the host is directed into the midgut. Other liquids (e.g. sugar-meal) are directed to the crop for sterilization and then to the mid-gut. Both males and females feed on sugars. These can be obtained from aphid honeydew or from plants. The presence of sugar is essential for the full development of *Leishmania*. Most species are gonotrophically concordant, taking one blood-meal for each batch of eggs matured. However, autogeny, the ability to lay eggs without a blood-meal, is found in populations of some man-biting species (e.g. *P. papatasi*). Oviposition usually takes place 3-8 days after a blood-meal. The highest parous rates occur in populations towards the end of the 'sandfly season' (peak seasonal abundance) when sandfly infection rates are at their highest and transmission is most likely. In sandflies, little is known about the number of times a female has laid eggs and at present the method using follicular relics in the ovarioles needs to be more precise than simply distinguishing parous and nulliparous sandflies.

### **1. 2. Spermathecae in insect reproduction**

The biology of reproduction with reference to sperm storage and utilization has been of considerable interest in recent years for understanding the behaviour and evolution of animal mating systems. In higher terrestrial insects, fertilization is internal and it evolved under the pressure of intrasexual selection in species that at one time practised external placement of sperm as in the primitive terrestrial insects (Parker, 1970c). During insemination, males are prevented from depositing their sperm directly

on to the eggs and instead they are transferred to one or two separate sperm storage organs known as spermathecae. Typically, when a female insect is about to oviposit, the egg passes down the oviduct to a bursa copulatrix near the exit from the spermatheca. The female can control the position of the egg and can release exactly the number of sperm guaranteed to fertilize it. This enables her to use sperm highly efficiently so that she need not store, transport, and maintain quantities of sperm in great excess of the number of eggs she is likely to produce.

The insect spermatheca is basically a modified epidermal gland of ectodermal origin and is located in the 8th abdominal segment. Its structure has been studied since the 1970's with the aid of light and electron microscopy in various groups of insects, primarily as part of female reproductive structure or as an insect epidermal gland. Details on the ultrastructure, histology, secretion and physiological function have been gained from studies on the cockroach *Periplaneta americana* (Gupta and Smith, 1969); the yellow fever mosquito *Aedes aegypti*, (Clements and Potter, 1967, Jones and Fischman, 1970); the fruit fly *Drosophila melanogaster*, (Filosi and Perroti, 1975); the tsetse fly *Glossina morsitans* (Kokawaro et al., 1981); the liver fly *Eristalis tenax* (Sareen, et al., 1981); the honey bee *Apis mellifera*, (Poole, 1970 and Dallai, 1975); the mealworm beetle *Tenebrio molitor* (Happ and Happ, 1970); the beetle *Ips typhographus* (Hallberg, 1984), the granary weevil *Sitophilus granarius* (Tombes and Roppel, 1971) and the cotton boll weevil *Anthonomus grandis* (Grodner and Steffen, 1978). In all these cases the structure of the spermatheca consists of a duct, a capsular organ for reception and storage of spermatozoa and glandular epithelial cells. The morphology, structural organisation

and number of spermathecae differs between insect groups. However there is little difference within the insect groups, ~~more so in~~ <sup>including</sup> Diptera. However, in phlebotomine sandflies the morphology of spermathecae varies greatly between species. Because of this remarkable variation the morphology of spermathecae has been used extensively to distinguish species as in the systematics of Phlebotominae sandflies (Adler and Theodor, 1926; Sinton, 1927; Theodor, 1948; Fairchild, 1955; Leger et al., 1983; Artemiev and Neronov, 1984; Killick- Kendrick, et al 1991; Lane, 1993).

### **1. 3. Spermathecae: Structure and Function**

#### **1. 3. 1. Definition**

During insemination female terrestrial pterygote insects store sperm within spherical organs called spermathecae. Mating takes place once or several times and since the maturation of eggs extends over a prolonged period, the spermatheca allows internal fertilization of eggs from time to time (Imms, 1957). The spermathecal complex of an insect is derived from ectoderm, and forms an epidermal gland lined with cuticle continuous with the body surface. It lies in the 8th abdominal segment and usually consists of 3 parts; namely the long duct which opens into the dorsal wall of the vagina or genital cavity, the secretory glandular region, capsular region for reception and storage of spermatozoa, and the secretory glands made of several glandular cells.

#### **1. 3. 2. Embryology of the spermathecal complex in Nematoceros Diptera**

Christophers (1923) studied the development of spermathecae and spermathecal ducts in the fourth instar larva of the mosquito. In the 8th segment, three finger-like

rudimentary elements, lying dorsal to the common oviduct initially appear and become cylindrical, develop globular dilations at their hind ends and eventually form the spermathecae and their ducts. In *Phlebotomus*, Christophers and Barraud (1926) observed the development of spermathecae and spermathecal ducts during nymphal-ecdysis; on the dorsal wall of the rudimentary oviduct a small packet-like invagination between the 8th and 9th sternites becomes bilobed and ultimately forms the two spermathecae.

### 1. 3. 3. Classification of insect epidermal glands

Insect epidermal glands, which include spermathecae, are so diverse in their location, their degree of complexity and their functions, that a general classification of these glands seems very difficult and not very useful. However the terminology and classification of insect epidermal glands are currently in use from two sources. The basic structure of insect epidermal glands, including spermathecae consists, of two general types of ectodermal exocrine glands: (i) simple glands which are made up of an aggregation of uniform cells, and (ii) compound glands which contain a variety of cell types, including secretory cells and duct cells, and an associated aperture and reservoir (Richard and Davies, 1977). Although this system has been in use for a long time, it can now be replaced in line with a functional point of view as outlined below.

In their revised terminology and classification of insect epidermal glands Noirot and Quennedey (1991) considered three main classes (Fig., 5; A, B & C) on the basis of not only their morphology (relationship with the cuticular covering and mode of secretion (Noirot and Quennedey, 1974) but also the gland's morphogenesis. They proposed three types of terminology:-

(i) **Gland:** any discrete glandular structure, from an isolated gland cell to a complex assemblage of glandular units, sometimes with associated differentiation for the storage, the emission or the evaporation of the secretion;

(ii) **Gland cell:** a cell which produces a secretion or a part of it;

(iii) **Glandular unit:** the unit must at the same time be structural, functional and ontogenic. *Structural:* several cells are assembled in an *organule*, a set of cells which can be divided into two or more identical subsets.

*Functional:* a single secretion, even when more than one gland cell is present.

*Ontogenetic:* all the cells of the unit are the offspring of one epidermal cells (stem or mother cell) as a result of one or more mitotic cycles. The unit is thus an isogenic group (clone).

Furthermore they also proposed classification for epidermal glands on the basis of a comparative approach.

**Class I gland cells:** the epidermal cell is simply covered by a cuticle which is secreted by the gland cell itself. The cuticle is modified to varying degrees in relation to the egress or the storage of the secretion. Due to the differences in secretion, the apical surface of the cell resulted in the production of a pore. These cells differentiate during the pupal-adult transformation and are the descendants of one epidermal cell by a series of mitoses. Pheromone gland in Lepidoptera and "pit-glands" of adult mealworm beetles are included under <sup>this</sup> heading.

**Class II gland cells:** The epidermal gland cell is not in contact with the cuticle and its secretion can not reach the outside directly but passes either through the adjacent

cell or via the haemolymph. Examples are epidermal oenocytes and internal oenocytes involved in synthesis of sex pheromones, and other cuticular hydrocarbons.

**Class III gland cells:** In this class of epidermal cells, the secretion of glandular units is poured through a cuticular ductule or canal. It may be isolated or associated with other units forming extraordinary diversity. Three sub-classes of cells are included: terminal (III<sub>t</sub>), intercalary (III<sub>i</sub>) and canal. The terminal cell is glandular and is connected at the internal end of the cuticular ductule or a canal secreted by canal cells. The terminal cell has an invagination, the extracellular reservoir, lined with microvilli and connected with a canal from a canal cell. The canal/ductule may either abut simply at the opening of the reservoir, or extend into it with loose type of cuticle, sponge-like or perforated. This structure is known as an *end apparatus*. Very often another cell (III<sub>i</sub>) is intercalated between these two, lying around the base of the canal cell.

Morphogenesis of class III gland cells is similar to that of class I cells but mitoses occur twice giving rise to a cluster of 4 cells or tetrads. During the process of ontogenesis one or more cells of the tetrads may degenerate resulting in only 3, 2, or even 1 cell. In some cases, one or more cells produce one or several temporary *cilia* (of the 9+0 type) around which the canal wall is secreted. Examples include the "androconia" of some Scutelleridae, "milk-glands" of the tsetse fly, "socket glands" of *Dysdercus*. Basically, the spermathecal complex of insects is derived from the epidermis and accordingly the spermathecae of Phlebotominae can therefore be included under all classes of epidermal glandular cells as proposed by them except class (i) cell.

### 1. 3. 4. The spermathecae in Dipteran classification

In several insect orders the spermathecae have been used as important features for systematics. The spermathecae vary in size, shape, number, and structural organisation in different orders of insect. In the Diptera the number of spermathecae varies from one (most non-phlebotomine Psychodids, *Anopheles*, *Dixa* and *Simulium*) or two (the Phlebotominae, *Mansonia*, *Culicoides*, *Glossina* or three (Tipulids, *Culex*, *Aedes*, Syrphids, Pipunculids, Tabanids and most Calypterates).

Spermathecae in Diptera: Until the work of Sturtevant (1925-26), the details of the spermathecae of different families of Diptera had not been studied. He surveyed and used modern terminology of spermathecae in many families of Diptera for classification and phylogenetic information with special reference to Acalypterate families. The phylogenetic relationships were a major problem for a number of species groups in *Drosophila* and the study of spermathecal morphology has had an important role in interpreting the evolution of that genus (Throckmorton, 1962). Harris (1966) used spermathecae to differentiate 8 genera of British Pipunculids and one of the most complicated subfamilies of Syrphidae, the *Eristalinae* was established mainly on the basis of morphological studies of spermathecae (Borisova, 1981). Very recently in his study on the cladistics and classification of the Bombyliidae (Asiloidea) Yeates (1994) used spermathecae with other morphological features.

Spermathecae in Phlebotmine classification: The initial use of the buccal cavity, pharynx and spermathecae were used to put Phlebotomine sandfly classification on the basis of natural relationships (Adler and Theodor, 1927). Subsequently Sinton (1927, 1932) used the morphology of spermathecae in combination with the pharynx and

cibarium for distinguishing the Old World species of *Phlebotomus*. With the exclusive use of spermathecal morphology and cibarial armatures, Foch and Abonnenc (1952) identified and classified all the phlebotomine sandflies from French Guiana. The interrelationship of phlebotomine sandflies with other non-phlebotomine Psychodids was proposed for the first time by Fairchild (1955) with modern evolutionary concepts. Unfortunately he left many open questions regarding the origin and evolution of spermathecae due to the non availability of internal details. Using light microscopy, Theodor (1965) furnished more details on the morphology of spermathecae, especially the segmentation v s non-segmentation and single duct v s separate ducts. The founding father of phylogenetic analysis, Willi Hennig (1972), investigated the phylogenetic systematics of psychodids and concluded that phlebotomine sandflies are a monophyletic group. In several aspects concerning the evolution and interrelationship of morphology of spermathecae, he agreed with most of the views of Theodor (1958 & 1965) but disputed the exclusion of the genera *Heritigia* & *Warileya* from the phlebotomines. Recently, Leger et al (1983) and Killick-Kendrick et al (1991) have used the variation in the basal dilation of the distal part of the spermathecal ducts to identify Mediterranean species of the subgenus *Phlebotomus (Larroussius)*. Even now the systematic position and classification of both New World and Old World phlebotomine sandflies relies on spermathecae e.g., Lewis et al (1977) and Lewis (1977), Artemiev and Neronov, (1984) and Lane (1993).

#### **1. 4. The ultrastructure, histology and histochemistry of the spermathecal complex**

**in various insect orders**

Studies of the structure of the spermathecal complex have been undertaken since the 1970's with the aid of the electron microscope and this review will concentrate on this aspect. The ultrastructure of the spermathecae and its connection with nerves, muscles, and tracheoles have been described in detail both as an important part of the female reproductive system and as an example of insect epidermal glands in different insects: the cockroach *Periplaneta americana* (Gupta and Smith, 1969); the yellow fever mosquito *Aedes aegypti* (Clements and Potter, 1967 and Jones and Fischman, 1970); the fruit fly *Drosophila melanogaster* (Filosi and Perroti, 1975); the tsetse fly *Glossina moristans* (Kokwaro et al 1981); *Eristalis tenax* (Sareen, et al 1981); the honey bee *Apis mellifera* (Poole, 1970 and Dallai, 1975); the mealworm beetle *Tenebrio molitor* (Happ and Happ, 1970); *Ips typhographus* (Hallberg, 1984); the granary weevil *Sitophilus granarius* (Tombes and Roppel, 1971) and the cotton boll weevil *Anthonomus grandis* (Grodner and Steffen, 1978). In each case the spermathecae consists of secretory glandular epithelium, a tubular storage organ for the reception of spermatozoa and a duct connected to the bursa copulatrix.

Investigations using the Scanning Electron Microscopy have often been associated with Transmission Electron Microscopy studies to confirm the detailed outer surface of spermatheca and their attachment to other tissues. The following findings have been made with these techniques: (1). a thin irregular surface of the spermatheca and close contact between the two spermathecal chambers in *Glossina* (Kokwaro et al, 1981); (2). the tracheal network on the basement membrane and cuticular aperture penetrated by the

tracheolar process in *Eristalis tenax* (Sareen et al, 1989); (3). the tracheolar network but no aperture being penetrated by the tracheolar process in *Apis mellifera* (Poole, 1970), 4. attachment of nerve and muscle fibres, short collecting ducts between the glandular cells and spermathecal lumen and vascular sinuses and pores on the surface of the glands in *Sitophilus granarius* (Tombes et al, 1971) and *Anthonomus grandis* (Grodner, 1979).

TEM study: Based on the ultrastructure of the glandular epithelial cells in spermatheca, the nature of secretory products such as polysacchrides and mucopolysacchrides, and cytoplasmic organelles such as mitochondria, Golgi bodies, rough endoplasmic reticulum, smooth endoplasmic reticulum, and various transporting vesicles involved in the secretions it is suggested that: (i) the secretion provides a source of energy for the maintenance of the spermatozoa (Clements & Potter, 1967; Davey & Webster, 1967; Grodner, 1979; Jordan, 1972; Monga, 1972; Sareen et al, 1989; Tombes et al, 1972); (ii) the secretion might induce sperm motility and provide a chemotactic substance to direct spermatozoa towards the spermatheca after copulation (Clements & Potter, 1969; Detinova, 1962; Grodner & Steffens, 1978); (iii) the maximum production of secretion coincides with the first occurrence of successful mating and precedes by several days the onset of oviposition (Happ & Happ, 1970); (iv) the secretion is necessary for spermathecal filling and might play some role in the sperm storage prior to egg fertilization, (Clements & Potter, 1967; Filossi & Perroti, 1975; Grodner, 1979; Hallberg, 1984).

Both the secretory cells and non-secretory cells of the spermathecal gland are lined with cuticle. The ultrastructure and chemical composition of the cuticle was studied with

special emphasis on the presence of resilin in the spermatheca of *Periplaneta americana* (Gupta and Smith, 1969), and *Aedes aegypti* (Clements and Potter, 1967; Jones and Fischman, 1970). Furthermore, detailed study was made of the end-apparatus of glandular cells and cuticular ductule in the duct cell; and its role in transporting the secretion to the spermathecal lumen. The presence of a loosely-knit felt work of fibres and absence of epicuticle in the ductule wall has been revealed (Gupta and Smith, 1969).

Although the spermatheca is derived from the epidermis, like many visceral organs, it is surrounded by haemolymph. Like any other internal organ the spermatheca is supplied with nerve and visceral muscle fibres and tracheoles. The ultrastructure of muscle fibres, their innervating nerve axons and synaptic junctions, neurosecretory material and its probable role in the efficient coordination of egg expulsion and the sperm release at oviposition and tracheation have been studied in the spermatheca of *Periplaneta americana* (Gupta and Smith, 1969), *Sitophilus granarius* and *Hypera postica* (Tombes, 1976).

Other ultrastructural studies of the spermatheca include (i) the structure of spermatozoa in the spermatheca of *Aedes aegypti* (Clements and Potter, 1967) and *Bombyx mori* (Myia, 1984), (ii) the sphincter-like valve of the spermatheca regulating sperm supply at ovulation in *Glossina* (Roberts, 1973), (iii) the cytoskeletal role of microtubules (Dallai, 1975), (iv) the quantitative relationship between age and height of epithelial cells, and (v) the preservation of spermatozoa in *Apis mellifera* (Poole, 1970).

#### **1. 5. Previous studies on the histology of spermathecae in Phlebotomine sandflies**

In order to present the classification of American Phlebotomine sandflies, Theodor (1965) studied the relative importance of morphology of spermathecae in the Old World Phlebotomine sandflies. He included a precise description on the morphology and histology of the glandular epithelial cells, sclerotized segmented spermatheca, nature of cuticle, muscle fibres and the ducts. Furthermore, he compared spermathecal segmentation with that of New World Phlebotomine sandflies and proposed his new classification.

From the anatomical drawing of female *Ph. papatasi* by Jobling (1987) the following interpretation has <sup>been</sup> made based on the knowledge gained from this study. The spermathecal complex lies below the rectum, flanked by a pair of large accessory glands on either side. It consists of a round spermathecal gland, elongated spermatheca, and a long spermathecal duct. The spermathecal 'glands' are an aggregation of epidermal glandular cells with cuticular ductules and they are opened into a round cavity flanked by a thin circular cavity at the anterior region of the lumen. The lumen of the spermatheca is lined by a thin cuticular intima which folds and connects the lumen as septate chambers. Outside the lumen is a thin layer of epithelium and a thick contractile muscular layer. The posterior region of the lumen connected by a long duct which opens into the anterior dorsal side of the bursa copulatrix. The duct is composed of a thick cuticular intima and a thin layer of epithelium interconnected with contractile muscle.

Recently Maroli et al. (1991) claimed to have found a "mating plug" in the spermathecae of wild caught *Phlebotomus perniciosus*<sup>5</sup> and *P. papatasi*. Its formation was related to differences in the physiological state of mated females.

## 1. 6. The spermathecae in the reproductive physiology of the insect

Sperm activity: The nerve and muscle supply to the spermatheca are responsible for the motility and fertilizing capacity of spermatozoa in the boll weevil, *Anthonomus grandis*, (Villavaso, 1977). The most significant role of the spermatheca in *Drosophila melanogaster* is to receiving and transmission of the sperm stimulus to the brain and therefore improve the survival capacity of sperm (Merle, 1977). The spermathecal glands, their secretion and muscle and the individual movements of the spermatozoa all take part in the transfer of spermatozoa from the oviduct to the spermatheca of <sup>the</sup> honey bee queen (Ruttener and Koeniger, 1971). Sperm transfer, its mechanism and absorption of fluid in the spermatheca have been extensively studied in *Aedes aegypti* ( Jones & Wheeler, 1965a & 1965b). The transfer of spermatozoa with male accessory fluids into the spermathecae in *Culicoides melleus* is effected by two complementary processes; the influx of fluid, by osmosis, into the spermatophore and simultaneous withdrawal of fluid from the spermathecae (Lin<sup>e</sup>ly, 1981)

Activation of sperm for egg penetration: The changes in the morphology of sperm in the spermatheca which may facilitate sperm penetration of the eggs have been studied in two insect species. The plasma membrane loosens from the sperm in the spermatheca and is probably shed before sperm descends the spermathecal ducts of the house fly (Degrugiller and Leopold, 1976). Similarly it has been reported that after migration of the sperm into the spermatheca, some eupyrene (nucleate) sperm shed their cell membrane in the lower part of the large lobe and the upper part of the spermathecal ducts in *Bombyx mori* (Miya, 1984).

Yolk formation, ovulation, insemination, fertilization and oviposition: The presence of sperm in the spermatheca, in combination with other factors, has been shown to produce specific changes in the reproductive behaviour of insects. The formation of yolk following the ingestion of large quantities of blood in *Anopheles subpictus* was due to the presence of sperm in the spermatheca which presumably induces a specific hormone (Roy, 1940). In *Aedes aegypti*, the inception of ovulation was preceded by the presence of blood in the stomach and the sperms in the spermatheca (MacGregor, 1931); which triggers simultaneously neurosecretion in the brain and seminal fluid in the spermatheca (Gillet, 1955).

The relative amount of semen in the spermatheca influences female receptivity to subsequent matings in *Glossina* (Tobe & Langley, 1978). The fertilization of *Glossina* is controlled by the supply of sperm from the spermatheca. The spermathecal valve at its junction is known to regulate the entry of sperm when the oocyte micropyle is appropriately positioned between the spermathecal ducts (Roberts, 1973).

Insemination of the spermatheca has been observed to trigger changes in the oviposition behaviour of *Rhodnius prolixus*, *Pycnoscelus indicus* and *Leucophaea maderae*. However, in *R. prolixus* the reception of such a change occurs via a blood-borne factor produced by the spermatheca and acting on the neuro-secretory cells of the brain (Davey, 1965 & 1967) while in the cockroaches the information is transmitted neuronally from the spermatheca to the brain via the ventral nerve cord (Stay and Gelperin, 1966). In the field cricket, *Gryllus bimaculatus* spermathecatomy failed to increase the egg production and showed the spermatheca is the receptive site for male

stimulants (Simmons, 1987).

### **1. 7. Adaptive value of Spermathecae**

In primitive insects such as Collembola and Thysanura, males deposit a spermatophore or sperm packet on the substrate ( i.e. without copulation) and the females acquire and store it internally for fertilizing their eggs. The change from external to internal fertilization evolved under the pressure of intrasexual selection in insects that once used external placement of sperm in association with the invasion of terrestrial habitats (Engelman, 1970). In pterygotes sperm are prevented from travelling directly to the eggs, instead they are often shunted into the special sperm storage structure, the spermathecae.

In their analysis of the evolution of insect mating systems, Thornhill and Alcock, (1983) suggested several hypotheses for the role of spermathecae in the evolution of insect reproduction. With the exception of the extremely short lived mayflies, in which the spermatheca is absent, for females with a long adult life the spermatheca has two important virtues: (i) the female can use the stored sperm at the precise moment it is required and avoid mating immediately prior to each oviposition and thus save costs (time, energy and risk) associated with copulation; (ii) control over the fertilization process is heightened, which means that a female can release the exact number of sperms needed to fertilize the eggs and enable sperm to be used more efficiently so that she need not store, transport, and maintain quantities of sperm in great excess of the number of eggs she is likely to produce.

In a single copulation, a male *D. melanogaster* transfers about 2 to 4 thousand sperms and the female wastes about 75% of them in fertilising her eggs. It means females need more sperm than they can store. The storage process selects against inferior gametes. To maintain a large number of sperm in the spermatheca, entails a metabolic cost and the female must supply the nutrients to transport and maintain the sperm in a viable state. In addition, the female ensures that the supply of sperm in the spermathecae is not depleted during oviposition in order to maintain a high level of fertility. To minimise the cost as well as allow continued oviposition, sperm replenishment is required, which enables multiple mating, which therefore is a by-product of small, energy conserving storage organs. Such selective pressures could perhaps have acted on the spermathecae of phlebotominae and these might have resulted in such a bewildering diversity. In the following paragraphs I briefly outline <sup>the</sup> hypotheses which could have generated variation in the spermathecae of Phlebotominae.

### 1. 7. 1. Darwin and his explanation for sexual selection

The diversity and complexity of the male mating system is an extraordinarily widespread trend in animals with internal fertilization. In contrast, genitalia involved in external fertilization are consistently simple and uniform. The association between internal fertilization and diversity of genitalia is so strong that it suggests there must be some extremely general explanation having to do with the act of copulation. In his "On the Origin of species", Charles Darwin (1859:75) first hinted at the explanation. He wrote that this form of (sexual) selection depends, not on a struggle for existence, but on a struggle between the males for possession of females, the result is not death to the

unsuccessful competitors, but few or no offspring. He also made a distinction between selection for traits that enhance survival (natural selection) and selection for traits that increase an individual's success in acquiring mates (sexual selection). Furthermore, he proposed that sexual selection consists of two different pressures: (1) a constantly recurrent struggle between males for possession of females and (2) a process of mate choice, usually by females that select those males which are vigorous and well-armed, and in other aspects most attractive. The former pressure, i.e. male-male competition to fertilize the egg is known as sperm competition (Parker, 1970) and the latter mechanism called sexual selection by female choice as proposed by Darwin (individuals which are more successful in obtaining mates leave more offspring and are thereby "fitter"). In most insect matings, females choose among males, putting selective pressures on males to be more attractive to their prospective mates. Changes in mating behaviour, and consequent changes in shape and form, are often driven by such sexual selection. Both strategies i.e. male-male competition and sexual selection by female choice could well be applied to the genitalia of phlebotomine sandflies, when one would consider the diversity of external morphology of male genitalia and internal sperm storage organs, the spermathecae of females. Male genitalia are not considered in this thesis except in the cladistic analysis, because the main focus will be on the review of structure and function of spermathecae with reference to sexual selection by female choice.

### **1. 7. 2. Fisher's runaway and female choice hypotheses**

Competition to fertilize eggs is complicated by the ability of females to receive and store sperm within a spermatheca. In many insects the male's sperm must have the

ability to reach the spermatheca and adopt a position within it such that the female is likely to 'select' his sperm for use when fertilising her eggs. For any organism, the fundamental evolutionary interest is passing genes to future generations. Nevertheless, males and females pursue this goal through different strategies because of physical constraints. One conspicuous asymmetry between the sexes is that females invest more heavily in offspring than the males do; for example, in mammals the females gestate and lactate while males are free to inseminate other females (Small, 1992). The strategy of receptive females is therefore not only to efficiently store the sperm but also to be extremely careful in selecting between potential fathers in order to pass the best genes to their descendants. Mate selection by females, however, might be a significant evolutionary force acting on males, affecting particular genes into future generations. Fisher (1929) held that females choose males because of particular traits even though the traits probably have little effect on their offspring. The trait in the male and choice by the female pass through generation after generation, co-evolving in a runaway manner. Natural selection stops the exaggeration only when the trait becomes a hindrance. In theory, female insects should favour (1) males that are of the same species and possess sufficient quantities of sperm, (2) males of superior genetic quality, and (3). males that provide exceptional material benefits for the female or parental investment for the female progeny. Receptive females are able to identify suitable members of their own species and thereby avoid hybrid matings, with the production of sterile or low-viability offspring.

### **1. 7. 3. Genital recognition and character displacement**

Eberhard (1985) summarized the significance of genitalic recognition and species isolation in animal mating systems. According to him the female determines species identity on the basis of species-specific characters either in the form of complicated cuticular structures or neural stimuli. She avoids fertilization if the structures or stimuli are not appropriate. Several recent works such as correlation between sperm and spermathecae in featherwing beetles *Bambara*, internal genitalia of the moths *Apamea* are examples of lock-and-key mechanisms; neural behaviour also belongs to this category. Both the lock-and-key and genital recognition hypotheses support the view that genital differences result in species-isolation. According to the character displacement hypothesis (Brown and Wilson, 1956) morphology, including the genitalia of closely related species, should tend to be more different in zones of sympatry than in zones of allopatry and is therefore another species-isolating mechanism. However, it is hardly applicable to genitalia of closely related species where either their geographic ranges are uncertain or where isolation is not always complete.

#### 1. 7. 4. Pleiotropism

Some authors suggests that animal genitalia are not subject to selection and their various forms are due to pleiotropic effects (Edwards, 1993). This view otherwise known as the neutral theory which contents that 1. sperm can be transferred equally well by members of closely related species, 2. genitalic variation is determined, at least in part, by genes that also influence other body characters. There are several arguments against the way in which selective neutrality is applied to genitalic variation. One example whcih emphasises this is the baculum, a small bone in the mammalian penis; its variation

is often used in taxonomic revisions with phenetic context, regarded as neutral and uninfluenced by the selection (Edwards, 1993). But studies of copulatory behaviour involving anatomy, neural and physiological studies and genital soft-tissue features used in the Hennigian cladistics confirm that the morphology of mammalian genitalia is functional i.e. subject to selection. By this arguments, the structure of spermathecae in phlebotomine sandflies is extremely unlikely to be neutral and uninfluenced by selection.

## 1. 8. AIMS

### 1. 8. 1. Objectives and Rationale

From the above discussion, it is clear that spermathecae are important structures in the life of many insects for in addition to the short term benefits of sperm storage they also provide the evolutionary opportunity for sperm competition. In sandflies, with their exceptional variation in spermathecae, little is known about either the structure or function of these structures.

Therefore, the objectives of this study are three fold:

**Objective 1. to determine the histology and ultrastructure of the spermathecae in different groups of Old World sandflies so that homology of the parts can be established and their function deduced.**

To do this, a detailed study of *Phlebotomus papatasi* will be made at the light and electron microscope level and compared to two other species of *Phlebotomus*, *P. (Euphlebotomus) argentipes*, and *P. (Larroussius) langeroni*, and one species of *Sergentomyia*, *S. (Parrotomyia) babu*.

**Objective 2. To determine the changes which take place in the spermathecae during the gonotrophic cycle and how sperm are implanted in the spermathecal complex and subsequently utilised.**

The changes in the spermathecae of *Phlebotomus papatasi* at different times after

emergence in relation to mating and blood feeding will be studied with special attention to the histology of the tissues.

The role of spermatophores in insemination will be compared in two species: *P. papatasi* and *P. argentipes*.

To assess whether potentially different methods of sperm implantation are used in Old World sandflies, a comparative study will be made of the spermathecal ducts and the male intromittant organs in different genera and subgenera.

**Objective 3. To deduce the role of the development of the spermathecae has had on the phylogeny of the Old World phlebotomines.**

A cladistic analysis using ultrastructural and comparative morphological data from females, and morphological data from males in particular the genitalia, will be used to develop a phylogeny of the Old World sandflies.

## **2. A Comparative Study on the Ultrastructure of Spermathecae of Phlebotomine sandflies**

### **2. 1. INTRODUCTION**

#### **2. 1. 1. The spermathecae in insects and in phlebotomine sandflies**

In phlebotomine sandflies the structure of the spermathecae varies both rapidly and divergently (i.e. it acquires a new form in each new species). Hence spermathecal structure has been a consistently useful feature in the taxonomy of phlebotomine sandflies (Adler and Theodor, 1926; Fairchild, 1955; Theodor, 1965; Lewis, 1977; Killick-Kendrick, et al 1991, Lane, 1993). To date the only information on sandfly spermathecae is the great diversity revealed after treatment in corrosive media like Berlese's fluid or Potassium hydroxide (KOH) prior to the identification of sandflies. This processing leaves only the cuticle, which is chemically and physically highly resistant. Not only is the detailed structure of sandfly spermathecae unknown but also the underlying causes for the extraordinary structural variation of spermathecal cuticle is poorly explored. The terminology used to describe spermathecae is ill-defined, and takes no account of the current classification of insect epidermal histology.

Spermathecal structure plays a critical role in insect reproduction, as it is here that sperm can be maintained in a viable condition for a long time, ensuring efficient utilization of stored sperm until fertilization (Parker, 1970). The ultrastructure of spermathecae in various insect species has been studied either as important reproductive structures in their own right or as specialised epidermal glands (Clement &

Potter, 1967; Gupta & Smith, 1969; Jones & Fishman, 1970; Happ & Happ, 1970; Dallai, 1975; Filosi & Perotti, 1975; Grodner, 1978; Kokwaro, et al 1981; Sreen, et al, 1989). However, in recent years attention has shifted from structural studies alone to include functional morphology of insect spermathecae to include, for example, the relationship between morphological variation and sperm displacement (i.e. the replacement at a subsequent mating of the stored sperm of a previous mating) (Walker, 1980; Smith, 1984; Ridley, 1989).

A thorough understanding of spermathecal structure and function in phlebotomine sandflies is important for two reasons. Firstly, in contrast to most other groups of Diptera in which the spermathecal structure is highly conserved, sandflies exhibit an extraordinary diversity in the shape of spermathecae. There is great variation between the species but very little within species. The reasons for this and the selective advantages to the females are completely unknown. Secondly, the spermatheca has a central role in reproduction as sperm are held in it, hyperactivated, and perhaps modified in some way to gain entry through the micropyle and fertilize the ovum (Davey and Webster, 1967; Degrugillier & Leopold, 1976; Miya, 1982).

### 2. 1. 2. The relationship between systematics, biology and spermathecae

Theodor (1965) proposed that the primitive form of spermathecae in the Old World species seems to be that of a wide membranous tube as found in *Sergentomyia babu*; subsequent evolution could have occurred in two different ways: 1. The apical part of the tube become more heavily sclerotised and the ducts narrower, forming a capsule (eg. *S. africana*). The capsule might vary in form and even develop "spines" (eg. *Grassomyia*). 2. The tube becomes irregularly crinkled and the ducts narrow (eg.

*Phlebotomus chinensis*, *S.squamirostris*). Finally, segmentation becomes regular and the accordion form results varying in the number and relative size of the segments and in the form and size of the apical process.

### 2. 1. 3. Selection of forms for detailed study

The genus *Sergentomyia* Franca & Parrot is distributed widely in the Old World tropics and sub-tropics with 276 valid species or subspecies (Seccombe et al, 1993). Members of this genus are vectors of *Sauroleishmania*, the parasites of reptiles. Within *Sergentomyia*, the subgenus *Parrotomyia* Theodor is the largest with ca. 51 described species or subspecies distributed in the Oriental, Afrotropical and Australasian regions. Members of this taxon are characterised by the presence of elliptical capsular spermathecae, comb-like cibarial teeth and a lamp-glass-shaped pharynx. They are placed in the *africana*, *denticulata*, *babu* and *grekovi* series. Except the *grekovi*-series in which the spermatheca is spherical, the remaining taxa have oblong spermathecae. In the *babu*-series, 4 species are recognised from the Oriental region of which *Sergentomyia babu* and *Sergentomyia insularis* are parapatric species in southern India (Ilango et al., 1994) and *Sergentomyia shortii* and *Sergentomyia baghdadis* are distributed in North-Eastern India and Western and North-Western India respectively. *Sergentomyia babu* was examined in this study.

The genus *Phlebotomus* Rondani and Berte is distributed exclusively in the Old World and includes the subgenera *Idiophlebotomus*, *Sepeophlebotomus*, *Euphlebotomus*, *Anaphlebotomus*, *Phlebotomus*, *Synphlebotomus*, *Adlerius* and *Larroussius*. Females of *Phlebotomus* are characterised by segmented spermathecae, cibaria without teeth and the

abdominal tergites 2-6 with many erect hairs. The subgenus *Euphlebotomus* is a primitive subgenus (characterised by <sup>^</sup>three lobed paramere according to Hennig, 1972), with 8 species and 2 subspecies. *Phlebotomus argentipes* is distributed from Afghanistan to Borneo and is an important vector of visceral leishmaniasis in North and South India (Lewis, 1977). Four species are included in the subgenus *Phlebotomus* and all of them are vectors of either visceral and/or cutaneous leishmaniasis in the Old World (Killick-Kendrick, 1991). *P. papatasi*, chosen for this study, has a wide distribution and is <sup>^</sup>proven vector of *Leishmania major*. *Larroussius* is the largest subgenus of *Phlebotomus* with ca. 24 species and 5 subspecies. The members of this subgenus are recognised by the characteristic variation in the distal extremities of the spermathecal common duct. *P. langeroni* chosen for this study is a suspected vector of *L. infantum* in Egypt (El Sawaf *et al.* 1985).

The objectives of this chapter are to (1) resolve the ultrastructure of the spermathecae, (2) deduce the function of the various parts and (3) establish homologies of the structures (4) propose a uniform terminology for spermathecae of the subfamily phlebotomine sandflies. As indicated above, four representative species from the Old World were examined: *Phlebotomus (Phlebotomus) papatasi*, *Phlebotomus (Euphlebotomus) argentipes*, *Phlebotomus (Larroussius) langeroni* and *Sergentomyia (Parrotomyia) babu*.

## 2. 2. MATERIALS AND METHODS

### 2. 2. 1. Sandfly samples

Material used in this study came from two sources: laboratory colonies and the field. The

developmental work was carried on *P. papatasi* because this was the most abundant material available.

Laboratory colonies: *Phlebotomus papatasi* and *Phlebotomus langeroni* originally from Israel and Egypt respectively were maintained at 26° C & 75% RH at the London School of Hygiene and Tropical Medicine following the methods of Dillon and Lane (1993). For this part of the study three day old adult flies were used which were sugar-fed (30% (w/v) and had access to males and 2 days later were given a blood feed. A detailed study on the spermathecae of these species was made from freshly prepared specimens using a compound microscope and subsequently a Polyvar phase contrast microscope.

Field samples: Specimens of *Sergentomyia babu* and *P. argentipes* were collected from Poonamalle village, 25 km away from Madras, southern India. Samples of *S. babu* were collected at night by aspirator from the walls of houses and of *P. argentipes* from the cattle while swarming and blood feeding. When the flies were collected the temperature and R.H. were recorded as  $27^{\circ}_{\lambda}$ <sup>C</sup> and 80% respectively. These wild caught flies were of unknown age in contrast to the laboratory colonised samples.

Preliminary processing: All flies were immobilised at 0°C for 5 minutes to inactivate them, dipped in 0.05% Tween 80 detergent soap solution to wet them, washed 3 times in distilled water and excess water removed with tissue paper. All dissections were in normal saline (0.15 M NaCl) solution. The terminal abdominal segments were removed to expose the spermathecae with fine blades cut from razor blades or micro-entomological pins attached to wooden handles. Subsequently, material was processed for either light microscopy or electron microscopy as detailed below. Movements and other

physical properties of the spermathecae were also observed in normal saline solution.

### 2. 2. 2. Light microscopy

To resolve the 3 dimensional structure of the spermathecae within the abdomen as a prelude to electron microscopy a histological study by light microscopy was done. The terminal abdominal segments were removed, serially dehydrated in an ethanol series and embedded in wax. Transverse sections  $1\mu\text{m}$  thick were cut, dewaxed and stained with haematoxylin and eosin.

In addition, the Toluidine Blue test (Neville, 1969; Jones and Wheeler, 1972) was used to detect the presence of resilin in cuticle. The terminal abdominal segments were fixed in formal-saline solution, dehydrated in ethanol and embedded in paraffin wax. Sections ( $1\mu\text{m}$  thick) were cut and stained with 1% toluidine blue in 1% aqueous borax for one minute, dehydrated and mounted in DPX.

Photographs were taken with a Polyvar phase-contrast light microscope using Kodak colour and Black and White films.

### 2. 2. 3. Electron Microscopy

#### 2. 2. 3. 1. Scanning Electron microscopy (SEM)

Flies were dissected at room temperature in normal saline (0.15 M NaCl) solution to expose the spermatheca and furca, and overlying tissues, such as the accessory glands and ovaries were carefully removed. The specimens were fixed by gradually replacing the saline with a solution of 3% glutaraldehyde in saline at room temperature, to avoid sudden contraction of the spermatheca, before finally fixing in 3% glutaraldehyde in 0.075M sodium cacodylate buffer at  $4^{\circ}\text{C}$  for one hour. After washing in 0.075M sodium cacodylate buffer/ 0.2M sucrose (pH 7.4) at  $4^{\circ}\text{C}$  for one hour,

specimens were post-fixed in 1% osmium tetroxide (in 0.075M cacodylate buffer) followed by distilled water, each at 4°C for 30 minutes. The preparation was then dehydrated in graded acetone solutions at room temperature (70%, 80%, 90%, 100%, 100%: 10 minutes in each grade), before careful transfer to small porous pots in acetone for critical point drying (CPD750 critical point drier). The specimens were mounted on aluminium stubs with double-sided cellotape and sputter-coated on an Eduardo S150 Sputter coater with a thin layer of gold. The spermatheca were examined with a JEOL25 III Scanning Electron Microscope and photographed with Kodak Technical Pan film TP120.

### 2. 2. 3. 2. Transmission Electron Microscopy (TEM)

Colony and field-collected material were fixed differently: specimens of *Phlebotomus papatasi* and *Phlebotomus langeroni* were fixed in 3% glutaraldehyde in 0.075M sodium cacodylate buffer (pH 7.4) at 4°C overnight, whereas *Phlebotomus argentipes* and *Sergetomyia babu* were fixed in a specially prepared field fixative in order to withstand storing at high temperature (30°C) in the field prior to storing at 4°C in the laboratory. The field-collected material was fixed in 3% glutaraldehyde held at about 27°C before 0.075M sodium cacodylate was added 15 days later.

After fixation, colony and field-collected material were processed as follows: washed in washing buffer (at 4°C); post fixation in 1% osmium tetroxide in 0.075M sodium cacodylate buffer at 4°C for 1-2 hours; washed in distilled water and block stained (2% uranyl acetate in 30% methanol). Specimens were then dehydrated in graded methanol (30%, 60%, 70%, 80%, 90%, 100%, 100%; 10 minutes in each grade) and transferred to propylene oxide (=1,2-epoxy propane) for 20 minutes. Because the

spermathecal complex is lined with a cuticular intima which could cause infiltration problems, the following combinations of epoxy resin and propylene oxide were used: 25% + 75% for 30 minutes, 50% + 50% for 1 hour, 75% + 25% for 1 hour before the 100% resin infiltration. Infiltration with 100% resin was overnight (at 4°C), before embedding in fresh resin and polymerising at 60°C for 48-72 hours. Sections 35-40nm thick were cut on an LKB Ultratome III using glass knives, placed on 200 mesh copper grids (with and without formvar film) and stained with Reynold's Lead citrate for 5 minutes before examination with a JEOL 100CX microscope.

## 2. 3. RESULTS

### 2. 3. 1. The spermathecae of *Phlebotomus papatasi*

#### 2. 3. 1. 1. The light microscope study

The spermathecae of *P. papatasi* consist of a pair of golfclub-like structures situated between abdominal segments 7-9, lying below the rectum (Figs., 1 & 4). A short spermathecal duct runs anteriorly from the bursa copulatrix and is attached to the base of a small cylindrical chitinous structure - the spermatheca proper. The duct and the spermatheca are covered by a thin epithelial layer and the apical part of the spermatheca is surrounded by a gland of large secretory cells.

The spermathecal ducts have separate origins at the bursa copulatrix (Figs., 8) and are 0.13 mm long and 4  $\mu$ m wide, the narrow lumen of each duct is constant over the duct's entire length. The body of the spermatheca proper (Fig. 7) is a chitinous, capsular structure on top of which lies a spherical mass of cells, presumably a gland of secretory cells. The spermathecal duct, spermatheca proper and the presumed gland are collectively

the spermathecal complex. Their fine structure is described below.

### 2. 3. 1. 2. SEM of the spermathecae

Details on the surface of an organ can be as important as the internal histology. External features of the spermatheca can not be resolved easily with light microscopy but are well illustrated by the SEM (Figs., 9 & 10). For example, the well differentiated epithelial cells and muscle fibres of the spermatheca are contiguous with the bursa copulatrix, and therefore presumed of dermal origin, and the muscle fibres are alternately arranged with epithelial layers to form an interwoven network (Fig., 9).

The spermathecal body is a sub-cylindrical structure (18  $\mu\text{m}$  long) at an angle of ca.  $90^\circ$  to the spermathecal duct. Both the epithelial layer and muscle fibres are continuous with the spermathecal duct and thus the bursa copulatrix. The epithelial cells are round, 2.6- 3.6  $\mu\text{m}$  in diameter and arranged in rows, running parallel to the muscle fibres. The basement membrane of the epithelial layer is deeply convoluted and appears nodular. The thick muscle fibres, 2.5- 5  $\mu\text{m}$  wide, run <sup>the</sup> length of the spermathecal body and are striated with repeating units occurring at 2.5  $\mu\text{m}$  intervals.

A pair of peripheral motor-nerve fibres, originating from the seventh abdominal nerve ganglion, is connected to each spermatheca near the junction of the spermathecal gland and the spermathecal body (Fig., 10). Each nerve fibre is divided into several branches which spread over the spermatheca and spermathecal duct to connect with the muscle fibres. A network of tracheoles covers the spermathecal body and spermathecal duct. In addition, a tubular structure, 1  $\mu\text{m}$  wide, runs in a zig-zag shape from the spermatheca to the base of the gland. This tube is known as the "vascular sinus" and has been found in granary weevils (Tombs and Roppel, 1972).

The spermathecal 'gland' is a spherical structure, 30- 36  $\mu\text{m}$  in diameter. On the dorsal surface of the gland, the basement membrane is superficially convoluted and is free from any tissue attachment. The ventral surface of the gland is closely applied to the apex of the spermatheca and is penetrated by muscle and nerve fibres, tracheoles and vascular sinuses that originate from the spermatheca.

### 2. 3. 1. 3. Transmission Electron Microscopy of the spermathecae

Gross internal structure: The spermatheca, like the integument of insects, is composed of cuticle, a multilayered extracellular composite material, and an underlying epidermis, from which several layers of cuticle are secreted. The spermathecal complex (Figs. 11 & 12) revealed by electron microscopy includes (i) a spermathecal gland with a cluster of elaborate secretory cells, (ii) a sub-cylindrical hollow spermatheca, with a solid spongy cuticular mass at its apex, and (iii) a long, narrow tubular duct. Both the duct and spermatheca are lined by varying thicknesses of cuticle, with an epithelial covering, ramified by a muscular system, nerve fibres and tracheoles. The sandfly spermatheca shares many features with other insect spermathecae but retains a uniqueness and specialization. A distinctive feature is the extraordinarily variable pattern of the cuticular intima which is not only quite different from one part of the spermatheca to the other but also varies between the species studied. Hence, the following description of the histology of the spermathecal complex is organised in the following sequence.

1. Spermathecal duct: structure and physical properties of the cuticle found in the duct and spermatheca, including the structure of the epithelial layer and muscle fibres around the duct and spermatheca;

2. Spermatheca: the cuticular mass and its tubules and canals, the body of the

spermatheca with its cuticular wall and its folding, and the basal sphincter valves;

3. Spermathecal gland: secretory glandular cells, receiving and conducting ductule (= end apparatus) and cellular junctions;

4. Associated structure: the muscular system, neuromuscular junctions, tracheoles and vascular sinuses.

A detailed description is given of the ultrastructure of the *P. papatasi* spermathecal complex as a model for sandflies. Details of other species (*S. babu*, *P. argentipes* and *P. langeroni*) are presented only when they are different to *P. papatasi*. Based on these findings a summary of the spermathecal structure is given, together with a revised terminology for sandfly spermathecae. The terminology and classification of the cuticle, epidermal glands, cell junctions, muscle fibres and neuromuscular junction follow that of Filshie (1982), Noirot and Quenedey (1974, 1991), Satir and Gilula (1973), Elder (1975) and Osborne (1975).

### 2. 3. 1. 3. 1. The spermathecal duct

The spermathecal duct is circular or oval in cross-section (Fig., 12) with a diameter of 4  $\mu\text{m}$ . The lumen is lined by cuticle and around this is an epithelial layer interlaced with muscle fibres. The duct cuticle and its epithelial layer and muscular system are actually an extension of the bursa copulatrix. The thickness of the cuticle including its associated derivatives differs from one part of the spermathecal complex to another i.e. between the duct base and spermathecal base, spermathecal base and spermathecal apex, spermathecal apex and cuticular mass with the terminal cavities, the cuticular ductules of glandular units, and modification/ specialization within the spermatheca including the septal folding.

### 2. 3. 1. 3. 2. Structure of cuticle

The duct (wall) cuticle (Fig. 13) is 1  $\mu\text{m}$  thick along its entire length until it joins the base of the spermatheca. The cuticle appears similar to that described for other examples of untanned insect cuticle. There are two main horizontal layers in the duct and spermatheca cuticle: The thin and densely stained epicuticle (EPI) is a non-chitinous outer layer facing the duct lumen, and thick fibrous layer of procuticle (PRO) composed of protein and the polysacchride  $\alpha$ -chitin next to the epidermal cells. The epicuticle is 77 nm thick and one can differentiate, in order of their deposition, the cuticulin layer, the inner epicuticle, the outer epicuticle and superficial layers. The cuticulin layer is a 10 nm thick, electron-dense membrane made up of lipoprotein that is the first layer of the cuticle to be secreted. The inner epicuticle is relatively much thicker (25- 90 nm) and stained less for protein than the cuticulin layer and is situated between this and the procuticle. Furthermore, the epicuticle contains fine granular substances presumably protein, lipid, lipoprotein, and oxidised phenols and extends deeply into the procuticle, an electron-lucent layer forming an alternate dark and light radiating pattern. The outermost layer of cuticle is the outer epicuticle which is ca. 10 nm thick and lipoidal in nature.

The procuticle (Figs., 13 & 23), lying below the epicuticle, is fibrillar (385- 500 nm) and is formed of protein and polysacchride  $\alpha$ -chitin. The presumed longitudinal orientation of microfibrils along the axis of the duct revealed that the microfibrils are poorly stained (due to the negative staining uranyl acetate), with uniform diameter (approximately 3 nm) and are embedded in an electron-dense matrix (arrows). Hence it is considered that the electron-dense material was protein and electron-lucent microfibrils were chitin. Microfibrils were found only in the chitin-containing regions. The packing

arrangements of microfibrils and matrix are outlined in detail below under the structure of the spermatheca (wall) cuticle (Fig. 14) and cuticular mass at the apex (Fig. 23).

Cuticle in the spermathecal wall (Figs. 14) is less thick than in the ducts. It covers a large area because of the repeated infolding, which increases the inner surface of the spermatheca. Structurally it includes a thin, electron-dense cuticulin layer (63- 156 nm) of epicuticle and relatively thick fibrous electron-lucent layer (1 nm) of procuticle. The cuticulin layer of epicuticle alone extends outwardly to form a series of finger like projections within the lumen of the spermatheca. The heavy staining property with osmium/ uranyl acetate suggests that this layer is either lipoidal or lipoprotein.

At the apex of the spermathecal body/lumen (Fig. 23), there is a hemispherical block or cuticular mass (ca. 9  $\mu\text{m}$  deep & 18  $\mu\text{m}$  width), made of soft procuticle containing rubber-like chitin and a thin layer of epicuticle ( 0.5  $\mu\text{m}$ ). The cuticular mass appears to be composed of long microfibrils and their packing arrangement within the matrix can be deduced from the arcuate patterns which appear as alternating light and dark bands running parallel or nearly parallel to the outer surface (i.e. the lumen of primary and secondary canals of the cuticular mass). There are two contrasting models proposed for insect cuticle (Fig. 6, from Filshie, 1984) to account for the arcuate pattern:

1. The dark bands contain filaments oriented horizontally, and the light, intermediate zones contain arched filaments lying in parallel, oblique planes and meeting neighbouring dark bands ( Fig., 6, a);
2. Arched, interband fibres do not exist but the appearance of arcs in sections is produced by the projections of short lengths of fibres lying in horizontal planes. Fibres are parallel to one another in a single plane, and the fibre orientation within successive planes rotates anticlockwise by a small constant angle

downward through the cuticle (Fig., 6, b). A 180° rotation of the fibre direction produces a single lamella. This is commonly known as the helicoidal model and is applied to many other biological materials, and is universally favoured as a description of insect and other arthropod cuticle.

Yet another important finding in the cuticular mass is the presence of networks of pore canals which maintain a direct connection between the epidermis and the surface (=lumen of the spermatheca). The pore canals are connected to epicuticular channels in the epicuticle. Pore canals are initially direct cytoplasmic extensions of the epidermal cells but often these extensions are withdrawn, leaving an apparently clear channel containing one or more electron-dense pore canal filaments. In some instances, microfibrils and matrix material fill or partially fill the channel so that these microfibrils become oriented perpendicularly to the rest of those in the cuticle.

To confirm the presence of resilin in the spermathecal complex, the histochemical technique of alkaline toluidine blue staining was used (Neville, 1970). The cuticle of both the spermathecal wall and cuticular mass (Fig., 2) and the spermathecal duct (Fig., 3) were stained blue by this agent: the outer layer pale blue and the inner layer dark. The elasticity of resilin allows the spermatheca and spermathecal duct to move under pressure from the muscular system. When the spermatheca is treated with chemicals such as chloroform, or sudden large temperature changes occur, an irreversible contractile state results (often seen in taxonomic preparations). Following milder temperature changes the spermatheca readily recovers and returns to the original position. Under mechanical pressure (exerted by a needle) the spermathecal duct extends about 1.5-2 times in length confirming the elasticity of the duct cuticle.

### 2. 3. 1. 3. 3. Structure of epidermal cells in the spermathecal complex

Three types of epithelial cells have been recognised in insect epidermis including spermathecae (Noirot and Quennedey, 1974 and 1991; Fig., 5; A, B & A). Those found around the duct and spermathecal body are typical of class 1 cells while the "gland" (described below) is composed of class 3 cells. These last cells are often associated or interspersed with class 1 cells.

The epidermal cells in the spermathecal duct (Fig., 11) are simple. The apical cell membrane is tortuously folded and attached to the base of the endocuticle. Belt desmosomes are often seen and act as anchoring points for microfilaments, which are commonly encountered between the apical membrane and the cuticle. The lateral surfaces of adjacent cells are linked by several kinds of specialized junctions. The most common contact sites between cells are septate junctions but where two adjacent cell membranes are looped at regular intervals they are bound by hemidesmosomes (Fig., 12).

The basal membrane exhibits a limited degree of infolding and is ramified with tracheoles and nerve fibres. The cytoplasm contains mitochondria of various sizes and membrane-limited vacuoles with unknown contents. No traces of Golgi bodies were observed. At the basal region of the cell is a relatively large, elongated nucleus with a well defined nucleolus and nuclear membrane.

### 2. 3. 1. 3. 4. Spermathecal body

The body of the spermatheca constitutes an important structure both in taxonomy and reproductive physiology of phlebotomine sandflies and it needs to be examined and described very precisely in order to understand the structural and functional roles. Light microscopy of KOH treated specimens suggests the spermathecae are segmented and

apically there are structures resembling a "collar" and a pit with "hairs" (Fig., 7). TEM in this study has revealed that the spermatheca in fact has a hemispherical cuticular mass (Fig, 23). In longitudinal (Fig. 15, 16 & 23) and cross sections (Fig., 22), the cuticular mass is penetrated by several tubules from the glandular units of the spermathecal gland. At the bottom of the cuticular mass (Fig., 15), there is a deeply excavated circular cavity, which I shall term the primary cavity, circumscribed by a collar-shaped peripheral cavity, here termed the secondary cavity (Fig., 16). Each tubule of the cuticular mass terminates in tiny, oval-shaped pits, the terminal pit, opening into the primary cavity. Both cavities are continuous with the lumen of the spermatheca. Thus, the 'head' of the spermatheca seen in KOH treated specimens is a solid cuticular mass and the 'collar' is infact a space, not a flange as often described (see Fig., 29).

In transverse section (Fig., 17), the cylindrical spermatheca has a series of regular, finger-like foldings protruding from the wall into the lumen. There are two types of cuticular wall folding: 1. slender septal folding 1  $\mu\text{m}$  long with blunt tips, and 2. stout, conical interseptal processes 0.25  $\mu\text{m}$  long. The two types of projection are arranged alternately. Under transmission light microscopy of either KOH prepared specimens or fresh preparations (Fig., 7), the spermatheca appear segmented with transverse septa. Clearly, these transverse septa are the slender septal foldings which do not completely separate the spermatheca into segments. In three dimensions, the spermatheca would be like a stack of empty car tyres with a large central lumen.

At the junction of the spermathecal duct and the main body of the spermatheca there is folding of cuticle supported by a ring of muscle which could act as a sphincter (Fig., 17). Such a valve could control the passage of incoming sperm and their later

release.

### 2. 3. 1. 3. 5. Spermathecal gland

The spermathecal gland (Figs., 18 & 19 ) is a bulb like structure consisting of clusters of glandular units (class 3 glandular cells) 16-27  $\mu\text{m}$  long. In between the glandular cells are a few small epithelial cells (class 1 epidermal cells). Each glandular unit consists of a large primary terminal cell 22  $\mu\text{m}$  long and a small duct cell 5  $\mu\text{m}$  attached to the upper surface of the cuticular mass of the spermatheca. A small cuticular ductule, leads from the primary glandular cell through the cuticular mass to the lumen of the spermatheca. From light microscopic observation, these cuticular ductules observed here are seen as "hairs" or hair-like processes on the head of the spermatheca.

The primary gland cell (Fig., 18 & 19) has an invaginated extracellular reservoir of 9-13  $\mu\text{m}$  long lined with microvilli, into which a cuticular ductule is protruded from the duct cell. They are collectively formed an end apparatus. The cuticular ductule that abuts with the microvilli is a loose type of sponge-like or perforated cuticle. The term "end apparatus" is currently being replaced with the original names i.e. the receiving ductule, that extends into the reservoir of the gland cell and the conducting ductule surrounded by the duct cell leading up to the surface of the cuticle. Longitudinal and cross sections of the cuticular ductule (Figs., 20 & 21) revealed that it has two distinct structural components: a thin electron dense inner layer of epicuticle (3-7 nm) which limits the cavity and a thicker electron-lucent fibrillar outer layer (8-15 nm). The thin superficial layer is interrupted at the junction where it meets the receiving canal; so the outer layer is continuous with the epicuticle of the cuticular mass. In the thick fibrillar layer, the constituent filaments are tubular, exhibit a tortuous course and constitute a

loose spongy meshwork. The relative resistance of this ductule to a corrosive medium such as Berlese's or KOH digestion suggests the presence of unsaturated lipids (Filshie, 1984) and is seen to bind with osmium as an electron-dense deposit. The diameter of the ductule is 40 nm and the lumen is filled with fibrous secretory material irrespective of the physiological stage of the fly (see chapter 4).

At the apical region (Fig., 25) of the terminal cells i.e. abutting the cuticular mass, the plasma membrane that forms an interface between the cytoplasm and the extracellular reservoir is remarkably complex. There are two important cellular characteristics of this interface: i, the plasma membrane is repeatedly folded to form microvilli, extracellular channels for the transportation of secretory, water and other metabolites from the cytoplasm into the reservoir ii, the plasma membrane along with the cuticle of <sup>the</sup> receiving ductule around the reservoir acts like a cytological 'apodeme', an anchor point for the cuticular ductule, and thus a structure analogous to the muscular attachment of the cuticular apodeme (Figs, 20 & 21). Cross sections of the microvilli show they have a central electron-lucent core covered by a thin electron-opaque layer. Transverse sections and cross sections of the cytological 'apodeme' revealed that the plasma membrane is folded into several layers and firmly attached on the opening of the receiving ductule. Plasma membrane contact and modification within the interface indicate that the desmosomal elements are alternately arranged with the different cell junctions. Among the desmosomal elements, is a girdle-like belt desmosome supported by an extracellular tonofibril. A patch of microtubules is present between the intercellular membrane. Next to the belt desmosome is a continuous junction characterised by parallel membranes with the intercellular space either filled with a homogenous, finely grained

material or septate junctions. Satir & Gilula (1973) suggested that the presence of a continuous junction is an indication for the presence of continuous epithelial regeneration. This finding of a complicated structure on the interface between the plasma membrane and cuticular ductule is reported for the first time in an insect epidermal gland. Hence a new name is proposed- "cytological apodeme"

The lateral surfaces of the secretory cells are separated by cytoplasmic extensions with intercellular spaces (Figs. 24 & 25) 2-4  $\mu\text{m}$  long, often with septate junctions connected by microtubules. Hemidesmosomes are present on the apical half of the cell.

The basement membrane (Fig. 24) is folded in at regular intervals and extends only a short distance between the adjoining secretory cells. These spaces are occupied by vascular sinuses, perhaps for exchanging fluid between glandular cells and the haemolymph.

The cytoplasm of the secretory cells is rich in microtubules around the reservoir (Fig. 25); along the long axis of the cells. In addition to their role in the cytoskeletal network and membrane component translocation, they are possibly involved, in association with microfilaments, in the apical constriction and changing the volume of the cells (Fristrom & Rickoll, 1982).

At the base of each glandular cell (Fig., 24) is a large nucleus with a well defined nuclear double membrane. The cytoplasm around the nucleolus is concentrated with cisternae of rough endoplasmic reticulum, and free ribosomes. On either side of the nucleus are the Golgi bodies including stacks of the "classical apparatus" and two populations of very small multivesicular bodies (3  $\mu\text{m}$ ) and larger irregular vesicles (5  $\mu\text{m}$ ). Between the nucleus and the apical area of the glandular cells are different sized

electron-dense secretory lysosome droplets (Fig. 7) which presumably migrate towards the reservoir. The various shapes and sizes of mitochondria and vesicles, with evenly distributed electron-lucid particles, are typical of secretory cells.

Between the class 3 cells and the rim of the spermathecal gland are a few non-secretory cells (Fig. 18) with relatively smaller nuclei surrounded by thin cytoplasm.

The duct cells are 4  $\mu\text{m}$  long (Fig, 20) and are relatively much smaller than the gland cells and lie between the latter and the cuticle of the spermathecal body. The duct cells surround cuticular ductules (0.7  $\mu\text{m}$  in diameter) which extend into the end apparatus of the glandular cells at one end and penetrate down through a massive block of cuticle to the lumen of the spermatheca at the other. The most conspicuous fine structural feature of the cuticular ductule is that it is devoid of epicuticle and consists of an electron dense thin outer layer and a thicker fibrillar or filamentous inner layer (Fig. 21). The apical plasma membrane of the secretory glandular cell and the basal plasma membrane of the duct cells are tightly packed with desmosomes and well defined tonofibrils. The apical plasma membrane of the duct cells is deeply folded in the microvilli and firmly anchored to the cuticle.

### 2. 3. 1. 3. 6. Spermathecae and supercontracting visceral muscles

The muscular system (Figs., 17, 26 & 27) consists of about 5-6 fibres which run longitudinally along the spermatheca intermeshed with the epithelial layer. At the "head" they are attached to a cuticular apodeme. The fibres are small (2-3  $\mu\text{m}$  diameter) with unusually short sarcomeres, in contrast to the long fibres of other insect visceral-muscle fibres. They have irregular Z-lines and ill-defined A- and I-bands. There is a single large nucleus which is laterally distributed in the endoplasmic reticulum. Mitochondria are

few, relatively large and randomly disposed. However no ER or Golgi bodies were found.

The ultrastructure of the muscle fibres found here is of the supercontracting, visceral muscle type reported by Rice (1970) in the oesophagus, midgut and aortic muscles of *Glossina morsitans*. In transverse and slightly oblique thin section (Fig. 17) each fibre consists of short, narrow (2-4  $\mu\text{m}$ ) sarcomere, invested by a well differentiated sarcolemma, but the separation of myofibrils is incomplete, since the Z-discs are perforated with many holes, a characteristic feature of supercontracting fibres. The sarcomere of this type of muscle does not consist of isolated contractile units and, during supercontraction, the myofilaments do not shorten, but slide through the holes in the Z-discs into the adjacent sarcomere. No M-bands are present and the H-band is apparent in the middle of the A-band. The I-band is composed of thin myofilaments (6  $\mu\text{m}$ ) bound into the Z-disc material from which they radiate, interdigitating with the thick myofilaments (6-10  $\mu\text{m}$ ) of the A-band. The visceral muscles are therefore presumably composed of thin filaments of myosin and thick filaments of actin. In cross section (Fig. 25) the myofibrils are not aligned in the same direction; some run circularly, some longitudinally, and some are diagonally orientated. The filament lattice shows myosin filaments in poor hexagonal array surrounded by 8-9 actin filaments. The transverse tubule system (TB) is well developed and is frequently associated with the sarcoplasmic reticulum (SR) forming the diads (DI). Hemidesmosomes (HD) attach Z-discs to the plasma membrane and overlie the thick basal lamina. The motor nerve terminal forms a neuromuscular junction with the muscle fibres near the junction of the gland and the spermatheca (described below).

The ultrastructural features of supercontracting muscles are related to the slow and rhythmic contractions of the insect visceral muscular system. The advantage of having these muscle fibres is that they accommodate asymmetric contractions which occur around tubular structures like the spermatheca allowing them to become greatly distended (Rice, 1969). Such rhythmic and peristaltic movements were observed <sup>in</sup> spermathecal ducts in saline squashes. The muscular system of the spermathecal duct perhaps gives mechanical support during copulation.

### 2. 3. 1. 3. 7. The Spermatheca myoneural junction

Between the cuticular apodeme of the spermatheca and the spermathecal gland (Fig., 18), the muscle is covered with nerve branches supplied by a pair of motor-nerves from the 7th abdominal ganglion. A typical insect myoneural junction is present where these nerves contact muscle fibres (Tombes, 1976; Gupta & Smith, 1969). The classification and terminology of insect neuromuscular junction used here follows that of Osborne (1975).

In the myoneural junction (Fig., 27) the axon lies in a shallow groove on the muscle surface and is "naked" i.e. without a Schwann cell sheath. The most important anatomical features of the synapsing axon include the synaptic vesicles, the presynaptic membrane and electron-dense presynaptic structure (= presynaptic membrane projection of vertebrate synaptic contacts), the synaptic cleft and the postsynaptic membrane. The axon terminal has a diameter of 2.5  $\mu\text{m}$  and the axoplasm contains a large number of spherical electron-lucent synaptic vesicles with an average diameter of 40 nm. According to Osborne (1975), these vesicles could arise either from the smooth endoplasmic reticulum or from the mitochondrion and are the sources of acetylcholine or L-glutamate.

A single large mitochondrion is found by the vesicles. The presence of the presynaptic membrane and presynaptic dense projection suggests that they are the attachment points for the synaptic vesicles and release sites for a neurotransmitter, either by means of a membrane-gating mechanism or by exocytosis. A "gap", known as the synaptic cleft, 12-15 nm wide, is found between the axolemma and sarcolemma and is filled more or less uniformly by electron-opaque material. This material usually consists of acid mucopolysacchrides and functions as a variable molecular sieve. The postsynaptic membrane is relatively "straight" compared with the rest of the sarcolemma and functions as an endplate containing enzymes such as acetylcholinesterase for breaking down the transmitter substances. The axon is naked and, in effect, is directly exposed to nearby haemolymph and the basement membrane offers little or no resistance to the flow of ions.

### 2. 3. 1. 4. Summary of the *P. papatasi* spermathecae

As in other insect species, the spermathecae of *Phlebotomus papatasi* is a typical insect epidermal gland and is reconstructed as in the Figure, 28;

It consists of cuticle, epithelial cells, glandular secretory cells, supercontracting visceral muscles and a neuromuscular junction.

The cuticle of the spermathecal body is developed as septate finger-like projections and at the apex of the spermatheca it forms a cuticular mass rich in resilin.

Glandular secretory cells contain receiving and conducting canals and a apical apodeme.

Muscular system is characterised by perforated Z-disc for supercontracting activity and is associated with a myonuclear junction for neurosecretion.

### 2. 3. 2. Comparative ultrastructure of the spermathecae of *S. babu*, *P. argentipes* and *P. langeroni*

#### 2. 3. 2. 1. Light microscopy

In contrast to *P. papatasi*, *S. babu*, *P. argentipes* and *P. langeroni* have a common spermathecal duct (i.e. a short duct that connects the bursa copulatrix and the individual ducts). The common duct is a simple and long structure in *S. babu*, ca. 50  $\mu\text{m}$  long, but in the two *Phlebotomus* species it has a complex organisation and is a useful taxonomic feature.

The common duct of *P. argentipes* has a broad base (Fig., 33), narrowing further up to form a cone-like structure leading to the long narrow individual duct. In *P. langeroni*, the common duct (Figs., 43 & 44) is much wider and more cylindrical before the duct bifurcates. At the start of the individual spermathecal duct (Fig., 44). In *P. langeroni* there is an extraordinary modification of the proximal lateral wall- a solid deposition of cuticle. Perhaps this is a distinctive feature of the subgenus *Larroussius*. The proximal end of each duct is connected to a capsular spermatheca.

The spermatheca of *S. babu* (Fig., 29) is tubular, unsegmentated with a pair of indistinct cuticular foldings on either side. But in *P. argentipes* and *P. langeroni* the segmentation is well pronounced and the apical part variously modified in the different species. In *P. papatasi*, there is a hemispherical structure traversed by a "hairy process" (the cuticular ductules from the secretory cells of the spermathecal gland). Below the hemispherical structure, there is a circular cavity flanked by a peripheral collar-like space. The apex of the spermatheca is less specialised in *S. babu* and *P. argentipes* but

it is extended into a tubular structure in *P. langeroni* (Fig., 45). The duct and spermatheca are lined by a thin cuticle and externally covered by an epithelial layer together with muscles, motor nerves, and tracheoles that extend right up to the spermatheca.

### 2. 3. 2. 2. Transmission Electron Microscopy of the spermathecae

#### 2. 3. 2. 2. 1. Spermathecal duct

Unlike *P. papatasi*, in which the duct is simple and its histology remains unmodified for its entire length, the ducts of *S. babu*, *P. argentipes* and *P. langeroni* show considerable variation in the structure of the cuticle and its underlying epidermal lining. The only common feature that all three species share is that the epicuticle in the common duct opening is much thinner, presumably composed of lipoidal cuticulin. In *S. babu* (Fig., 31) it is very thin (38-76 nm) and is irregularly folded and projected into the lumen. The epithelial layer is pushed slightly towards the dorsal <sup>aspect</sup> and the muscular system lies on the opposite side i.e. ventral.

In *P. argentipes* (Fig., 35) the base of the common duct is ovoidal in cross section but with a circular lumen (4  $\mu\text{m}$  diameter) with lateral extensions (4  $\mu\text{m}$  long) forming a wing-like structure- the lateral grooves. These lateral grooves are reduced as they ascend and eventually merge with the lumen of <sup>the</sup> common duct. Furthermore, it is very difficult to apply Filshie's (1984) classification for the subdivision and fibrillar arrangement of cuticle, because its differing thickness around the common duct. The thickness of the procuticle around the lumen is 2.3  $\mu\text{m}$  but it is only 1-5  $\mu\text{m}$  thick covering the lateral grooves. The packing arrangement of the fibrils and the matrix in the procuticle are clearly differentiated and the cuticle is subdivided into lamellae by layers

of alternating electron-transparent and electron-dense bands oriented perpendicularly to the axis of the duct. Below the cuticle, the epithelial layer differs from *P. papatasi* in that it lacks muscle fibres and is arranged into two layers. The apical layer appears non-nucleated, its apical membrane is tortuously folded attached by a belt desmosome to the cuticle and contains numerous mitochondria. The basal nucleated layer is tightly packed covering the apical layer of the epithelium. At the proximal end of the common duct opening where the lateral grooves taper into the duct, a muscle layer comes and joins to the common spermathecal duct. At this point the duct's thickness is reduced to 0.5  $\mu\text{m}$ .

The structural organisation of the common duct in *Phlebotomus argentipes* (Figs., 37 & 38) presumably performs two vital functions during mating: Firstly, accommodate and hold the trilobed paramere firmly in the lateral grooves and secondly the aedeagal filament could protrude and retract in the circular lumen like a syringe. If alternately executed these two complementary process could constitute an injection mechanism.

The common duct in *P. langeroni* is short and broader; in cross section (Fig., 46) it is elliptical with the thickness of the fibrillar procuticle increased by 50% at the sides. Around the cuticle, is a thin epithelial layer, itself surrounded by thick muscle fibres. The sarcomeres of these muscle cells are longer (5  $\mu\text{m}$ ) than in *P. papatasi* but the myofibrillar arrangements are the same. The main part of the duct shares all the features of the common duct and the basal part of the individual duct, except that the muscle fibres are alternately arranged with the epithelial layers.

### 2. 3. 2. 2. 2. Spermatheca

Like the ducts, the ultrastructure of the spermatheca in all the three species differs from one another.

The spermatheca of *S. babu* (Fig., 30) is simpler in the organisation of the spermathecal gland and the spermatheca proper than in the *Phlebotomus* species examined. Unfortunately details were only available from the cross section at the apex and at the centre of the spermatheca from a single successfully fixed and cut specimen; other features are deduced from high-power light microscope observations. The apex of the spermatheca proper is less specialised with a cup shaped cuticular mass (Fig., 32). Canals that connect the cuticular ductules and the lumen of spermatheca found in *P. papatasi* are not present in *S. babu*. However, there is a ring-like cuticular apodeme for muscular attachment. A cross section of the spermathecal apex revealed that the receiving canals from the glandular cells are few and are located quite distally. The epicuticle of the cuticular ductule is thicker and more granulated and the procuticle more strongly stained than in *P. papatasi*. The fibrils of the cuticular mass are clearly oriented. A cross section through the body of the spermatheca (Fig., 30) shows that the cuticle wall that covers the body of the spermatheca is membranous and much thinner (30-50 nm) than in the three *Phlebotomus* species examined. Undulating, irregular projections into the lumen are present in contrast to the rigid septa. The epithelial covering is compact (0.2 to 1.3  $\mu\text{m}$  thick) and lies between the cuticle and the muscle fibres. The apical area of the epithelium is less specialised than in *Phlebotomus* and is superficially attached to the cuticle ie. no desmosomal components visible. The muscle fibres are relatively thicker (3-4.5  $\mu\text{m}$ ) but with shorter sarcomeres (2  $\mu\text{m}$ ) than the *Phlebotomus* species. The muscle fibres have their nucleus and mitochondria in the middle of the sarcomere.

Transverse sections of the spermathecal body of *P. argentipes* (Fig., 34) revealed the following differences to *P. papatasi*: its lumen is narrower, interseptal folding is

absent, the terminal segment is larger with thicker cuticle than other segments; the cuticular mass has a single triangular/ columnar channel and is filled with microvilli (Figs., 37 & 39), the ductules connecting the secretory cells and the spermathecal lumen are much smaller, with no apparent differentiation between the epicuticle and procuticle; the procuticle ( $0.5 \mu\text{m}$ ) on the wall is fibrillar with high resilin contents; the epithelial layer lies more outwardly from the muscle fibres.

On the spermathecal body wall, the fibrillar procuticle ( $0.5 \mu\text{m}$ ) is more chitinous (high resilinous); the epithelial layer lies below muscle fibres; the sarcomeres of visceral fibres are thinner ( $3 \mu\text{m}$ ) and shorter ( $3 \mu\text{m}$ ); the apical membrane is strongly attached to the back of the cuticle with a well developed system of apical microvilli and cellular junctions including belt desmosome, hemidesmosomes.

Apart from the cuticular architecture, the histology of the spermatheca of *P. langeroni* (Fig., 48) is similar to that of *P. papatasi*. The epicuticle around the lumen of the spermatheca is modified into uniform septal folds  $140 \text{ nm}$  long which project into the lumen. They are longer, thinner and more numerous than in *P. papatasi* but the interseptal folding found in *P. papatasi* is absent. The septal folding of the epicuticle is extended into a narrow (Fig., 50) canal ( $23 \mu\text{m}$  long and  $30 \text{ nm}$  wide) derived from the spermathecal body and retains all the histological features of the spermatheca. This canal is probably homologous with the primary canal of *P. papatasi* and from the nature of the procuticle it might have been derived from the the apex of the cuticular mass. However, the secondary canal of the *P. papatasi* is not found in *P. langeroni*. The epithelial cells around the spermatheca are cuboidal and interspersed with visceral muscle fibres. Although the cuticular mass is relatively smaller ( $1.5\text{-}2 \mu\text{m}$  in diameter) than *P. papatasi*,

the arrangement of cuticular ductules and the glandular cells is similar. A preterminal motor nerve which runs to the visceral muscle around the spermatheca has the axon terminal capped with a Schwann cell (Fig., 48). The function of the Schwann cell, according to Osborne (1975) is to maintain a low sodium ion concentration and to extract nutrients from the haemolymph (L-glutamate).

### 2. 3. 2. 2. 3. Spermathecal gland

The information obtained on the spermathecal gland of *S. babu* was fragmentary and unfortunately was only from a cross section at the apical region of the gland (Fig., 32). However, there are clearly fewer but larger glandular cells and the receiving and conducting canals are shorter than in the three *Phlebotomus* species. The attachment of the glandular cells to the cuticular mass is less developed (little plasma membrane infolding) in contrast to in the other three species.

In *P. argentipes* (Figs., 40 & 42), the glandular cells are long and narrow and lie much closer together. Around the extracellular reservoir and at the base of the microvilli, a definite row of electron-dense secretory (presumably polysaccharide droplets) were observed. In addition, a few large vesicles of unknown structure and function were also noticeable at the centre of the cells. The mitochondria are relatively small and the majority of them are found behind the reservoir. The basal membranes of the secretory cells are extremely convoluted, as in the primary cells of the Malpighian tubules. This convoluted basal plasma membrane and high concentration of mitochondria suggests this glandular unit is a component of an active transport system; for transporting either ions and, or macromolecules from the haemolymph into the spermathecal gland.

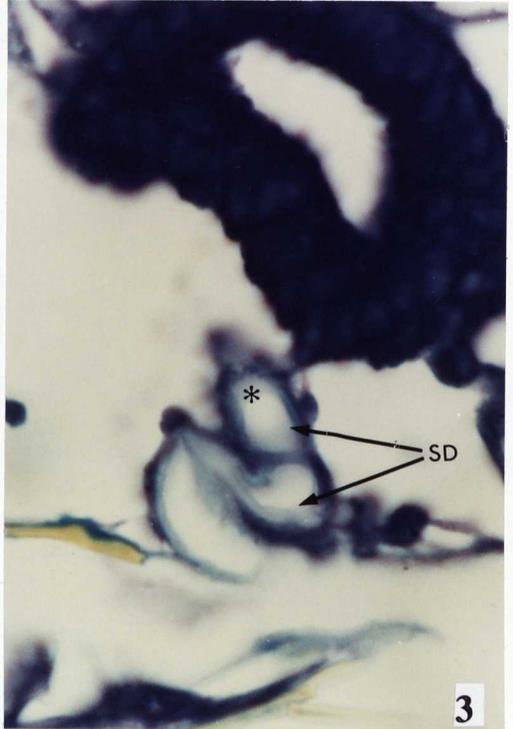
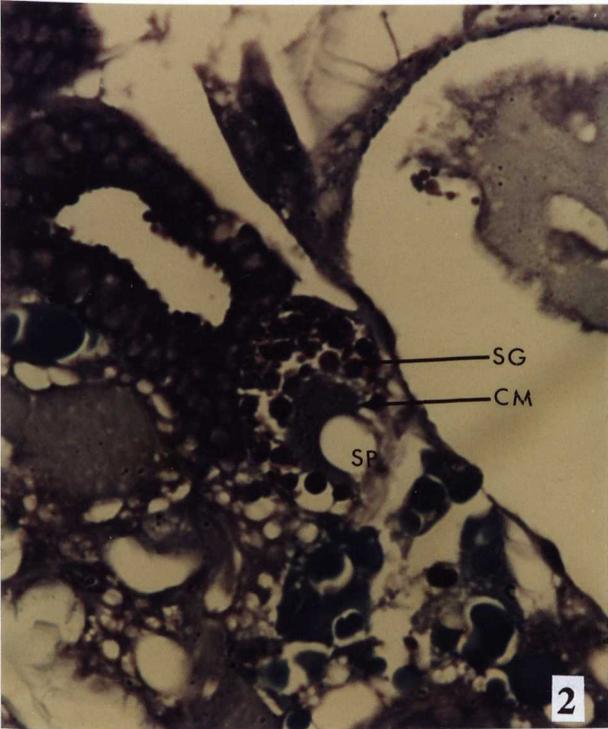
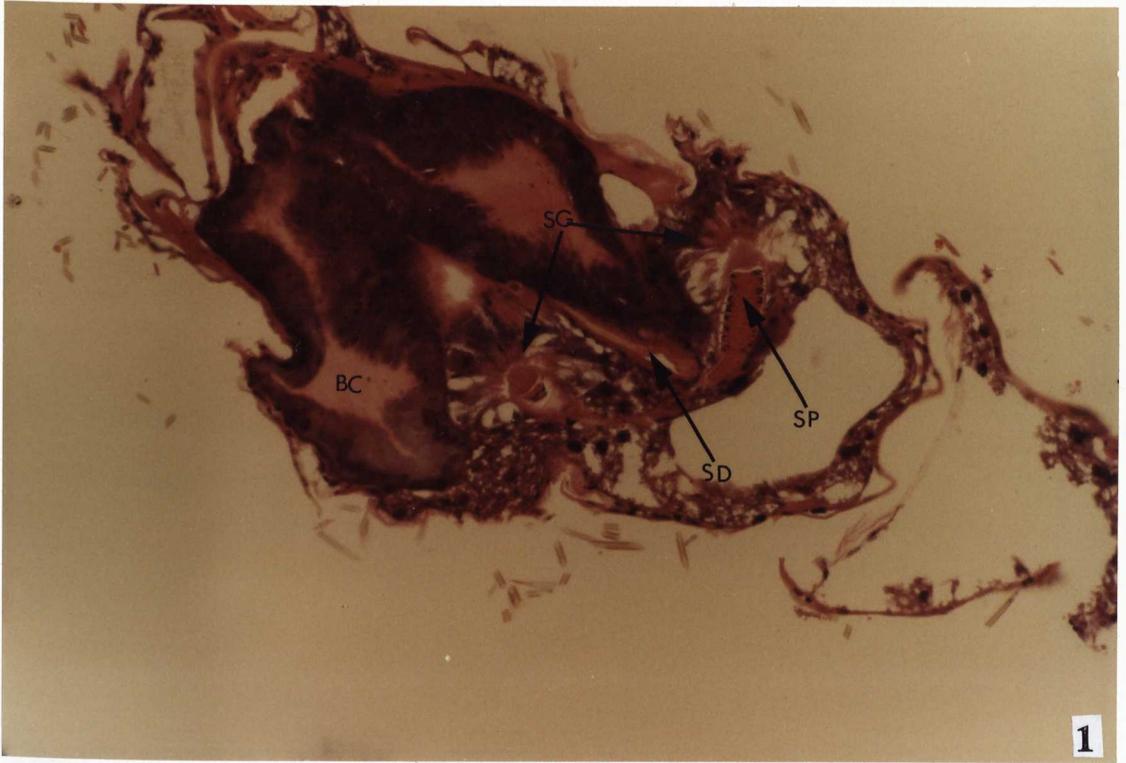
The histology of the gland cells in *P. langeroni* (Figs., 51 & 52) is similar in

many respects to that of *P. papatasi* but it differs in the following aspects: the gland as a whole is smaller and more compact, the glandular cells (15  $\mu\text{m}$ ) are regularly spaced and radially arranged, their apex narrower than the base. The receiving and conducting ductules (2.5-3  $\mu\text{m}$  long) are relatively short, have fibrillar procuticle and a specialised opening into the primary canal as found in *P. papatasi*. The basal membrane has no obvious infolding. The cuticular mass is reduced to 1  $\mu\text{m}$  in diameter. The duct cell is typical of *P.papatasi*.

Figure, 1. Longitudinal section of posterior abdominal segments, stained in haematoxylin and eosin, showing internal organs including the spermathecae and other details as in fig. 4. (X 600). BC, bursa copulatrix; SG, spermathecal gland; SP, spermatheca; SD, spermathecal duct.

Figure, 2. Longitudinal section of cuticular mass (CM), which is located between the spermathecal gland (SG) and the apex of the spermatheca (SP), stained with Toluidine blue for the presence of resilin (X 750).

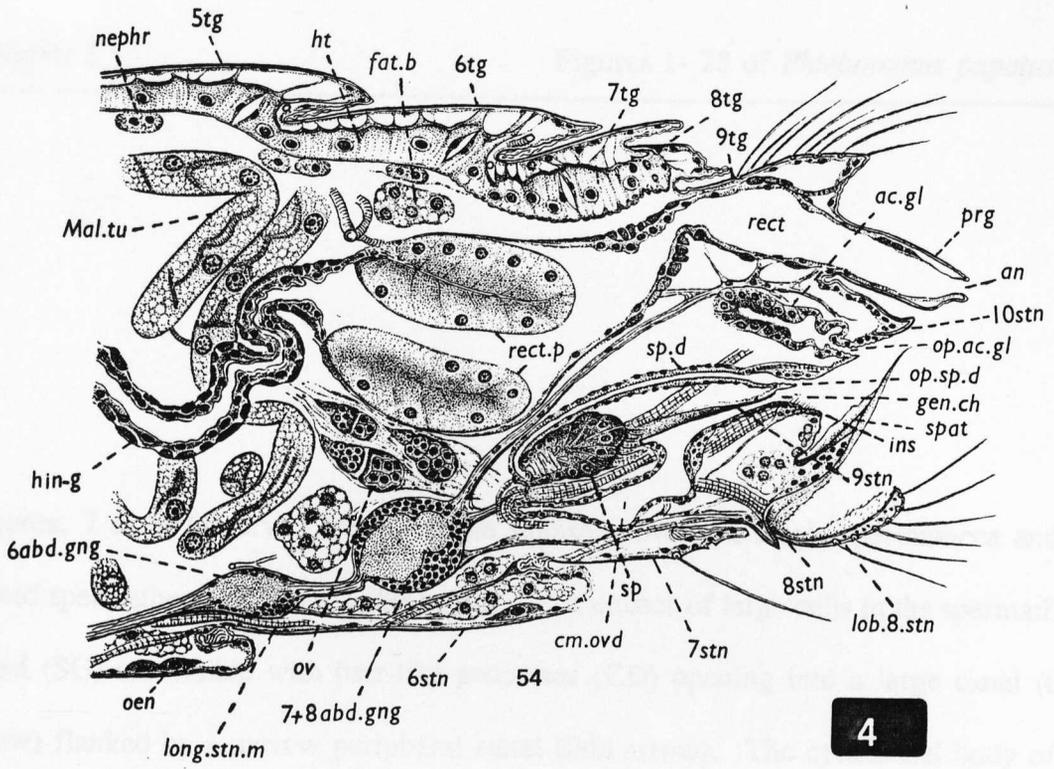
Figure, 3. Cross section of spermathecal ducts (SD) showing the presence of resilin (asterisk) stained with Toluidine blue (X 1, 800).



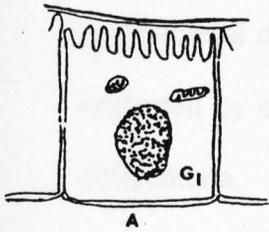
Figure, 4. Drawing of Jobling (1986) showing the longitudinal section of the posterior end of the abdomen. Op.sp.d=opening of spermathecal duct; sp=spermatheca; sp.d=spermathecal duct; for other details see Jobling (1986).

Figure, 5. Classification of epidermal cells (redrawn after Noirot & Quennedey, 1974 & 1992). (A) G1 Class 1 gland cell; (B) G2 Class 2 gland cell; (C) G3 Class 3 gland cell; ct=Perforated cuticle; D=Duct cell; E=End apparatus; R=Reservoir; Sp=Subcuticular space.

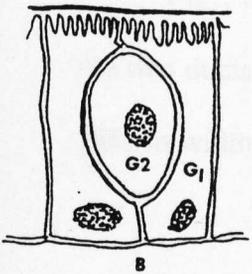
Figure, 6. Diagrams of two interpretations of the arcuate patterns in cuticular lamellae (after Filshie, 1982). (a) Continuous curved filaments pass from one lamella to the next. (b) Filaments are packed parallel to one another in sheets, the latter being stacked helicoidally. In straight sections, projections of small lengths of straight microfibrils lead to arcuate patterns.



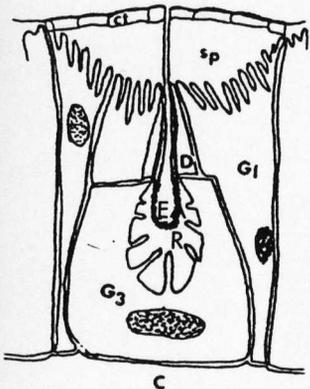
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A

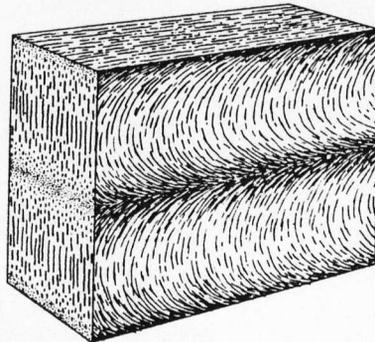


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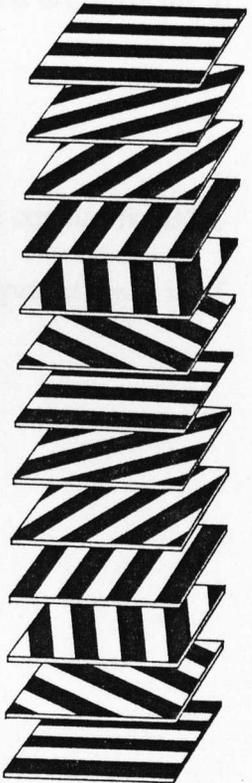


C

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a

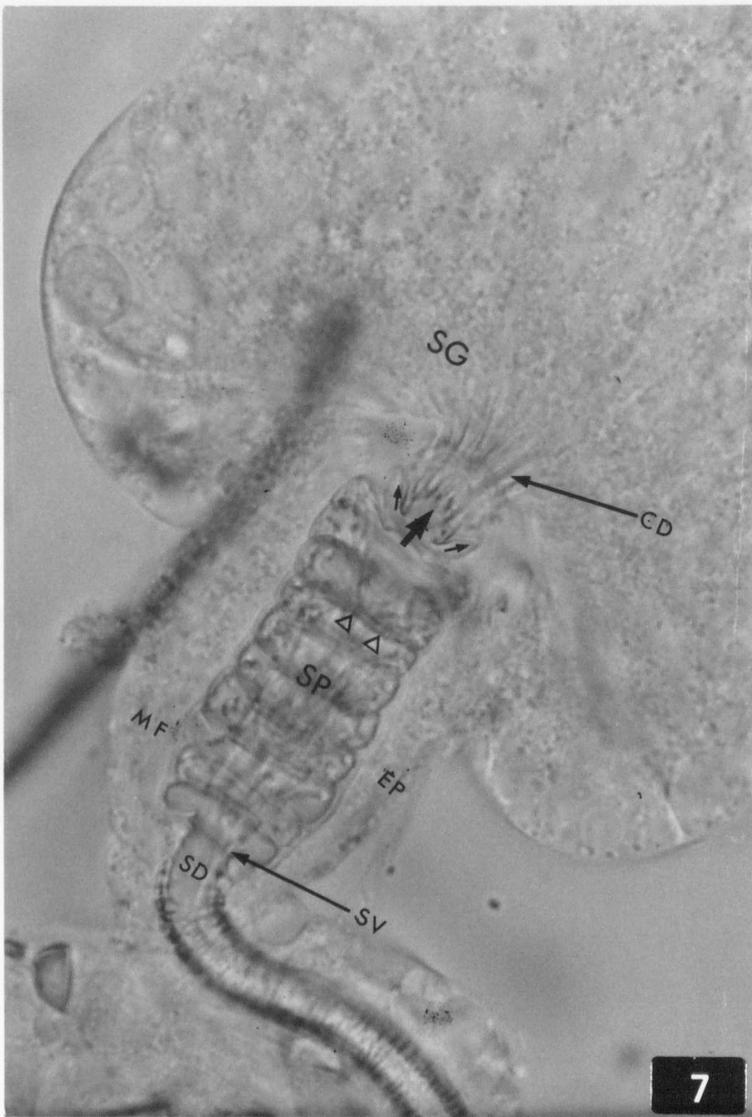


b

6

Figures, 7 & 8. Observed from a saline squash showing a single spermatheca and the paired spermathecal ducts (X 1, 800). Fig., 7: A cluster of large cells in the spermathecal gland (SG) are visible with hair-like processes (CD) opening into a large canal (thick arrow) flanked by a narrow peripheral canal (thin arrow). The cylindrical body of the spermatheca (SP) is segmented (open triangles) and its base is connected to a long narrow spermathecal duct (SD) through a guarded sphincter valve (SV). The whole structure is supported by a thin epithelial layer (EP) and muscle fibres (MF) which are difficult to differentiate.

Fig., 8: A pair of spermathecal ducts (SD) attached to the bursa copulatrix (BC). The two ducts are separated (twin headed arrow) and the duct lumen (open arrows) is of uniform width throughout.



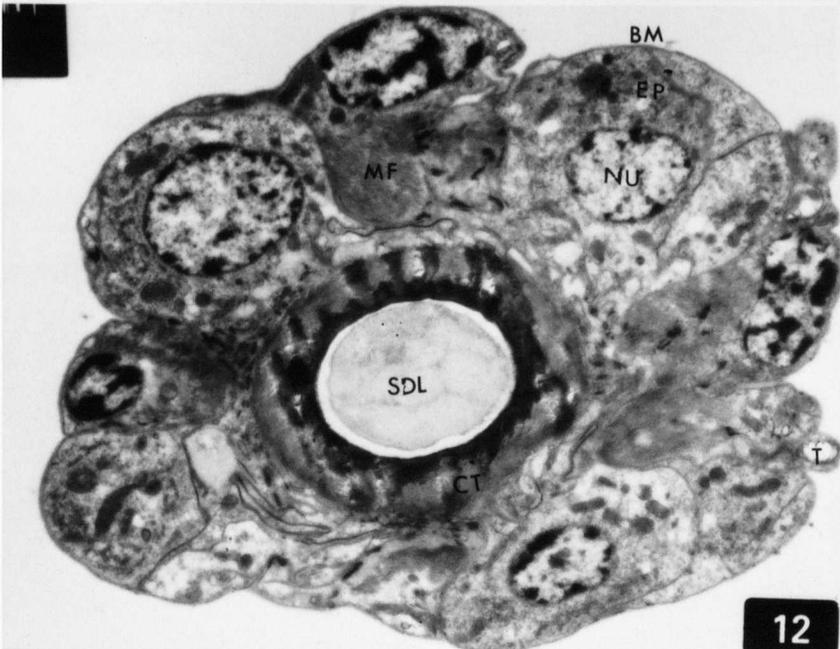
Figure, 9. SEM, of the spermathecal complex (X 1, 540). The spermathecae (SP) are capped with spherical glands (SG) and epithelial cells (EP) which also cover the spermathecal ducts (SD). Each spermatheca is supplied by corrugated tracheoles (T), and a pair of straight motor nerves (N).

Figure, 10. SEM of gland and spermatheca (X 6, 600). The outer surface, the basal membrane, of the gland is smooth. The basal region of the gland, the spermatheca and the duct are supplied by nerves (N), tracheoles (T) and a dilated thick vascular sinus (v). Note the thick visceral muscle fibres (MF) and globular epithelial cells (EP) supporting the spermatheca and duct.



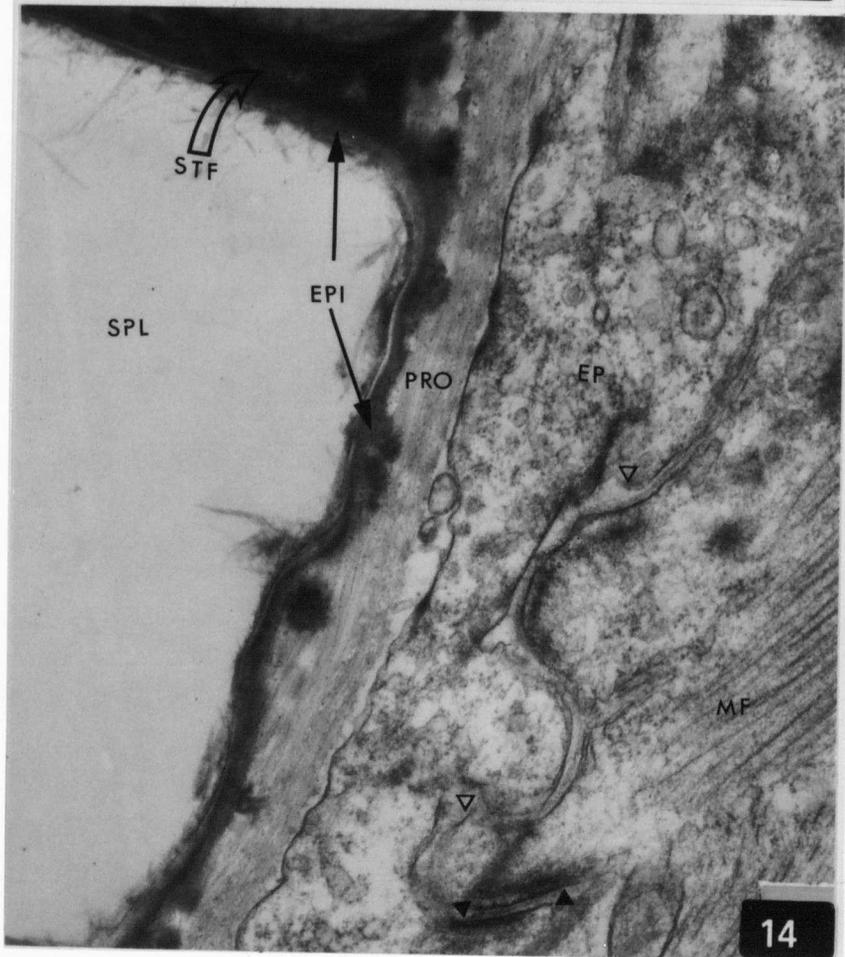
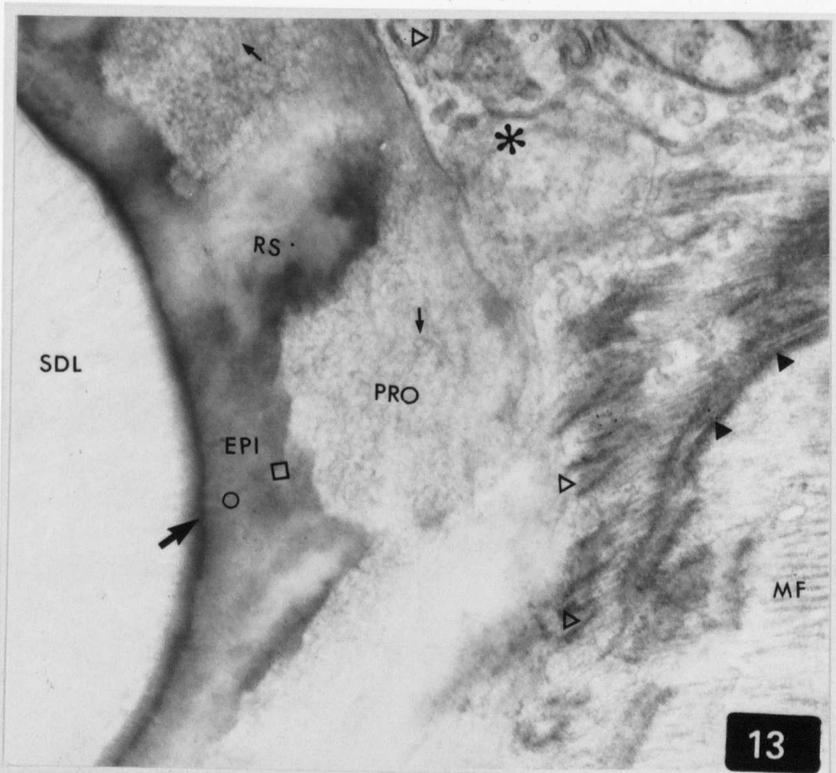
Figure, 11. TEM, Transverse but slightly oblique section showing the gross anatomy of a complete spermathecal gland and the upper part of the spermatheca (X 3, 200). Note the row of secretory glandular cells (twin head arrow mark), on a hemispherical-shaped cuticle-mass (CM) with a tiny cavity (PC, primary canal). Beneath is the lumen (SPL) of segmented spermatheca (SP) packed with spermatozoa (SPR). On either side of the cuticle-mass is a cuticular apodeme (ca) to which are attached longitudinal muscle fibres (MF).

Figure, 12. TEM, Cross section of the spermathecal duct showing the entire histology of the cuticle (CT) and epidermis (EP) (X 6, 600). Note a circular lumen (SDL) surrounded by a thin cuticle lining, and a single layer of epithelial cells interlaced with muscle fibres (MF) and connections of tracheoles (T). BM, basement membrane; N, nucleus.



Figure, 13. TEM, Cross section of cuticle of the spermathecal duct (X 40, 000). The electron-dense thin epicuticle (EPI) lying next to the lumen (SDL) is differentiated into outer epicuticle (thick arrow), a middle layer of inner epicuticle (open circle) followed by the cuticulin layer (open square) which is continuous with the resilin layer (RS). Note the thick layer of procuticle (PRO) in which the microfibrils of chitin appear as electron-opaque white patches surrounded by an electron-dense matrix of protein (small arrows). Applied to the cuticle (asterisk) is the apical membrane of the epithelial cells supported by hemidesmosomes (open triangles) and belt desmosomes (closed triangles) and below that myofibrils (MF).

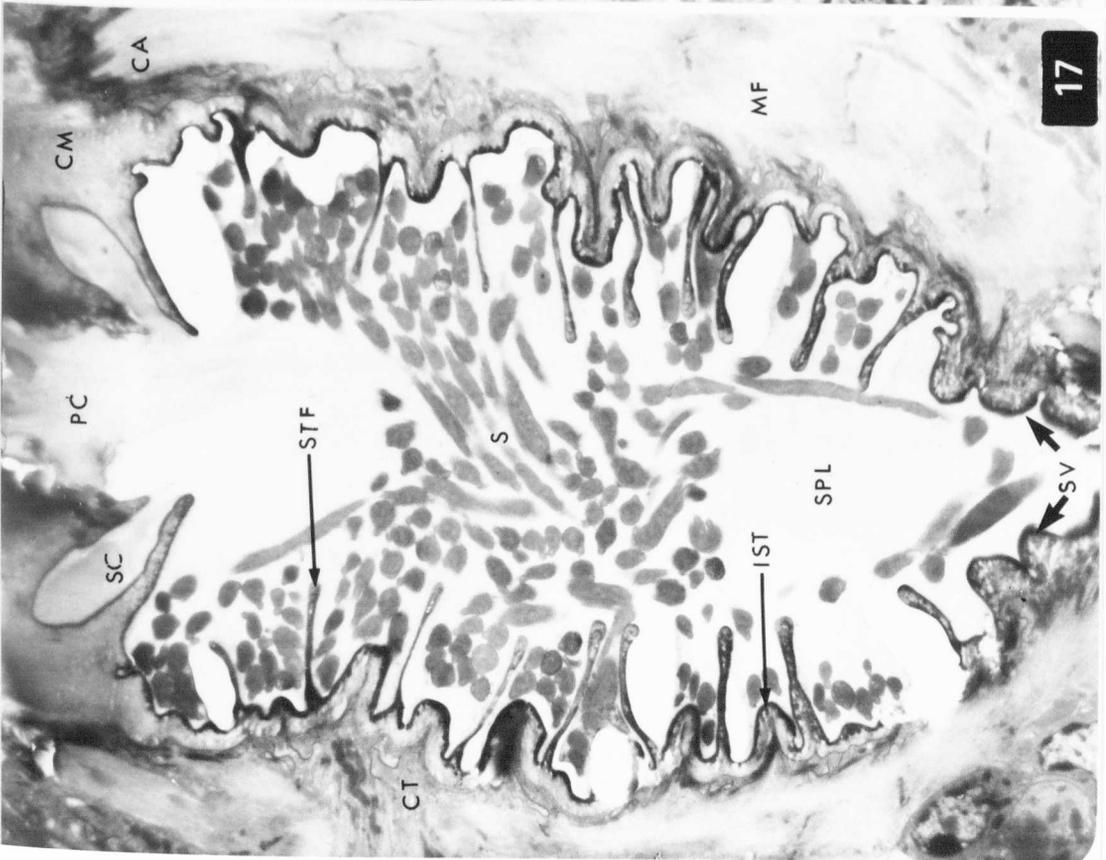
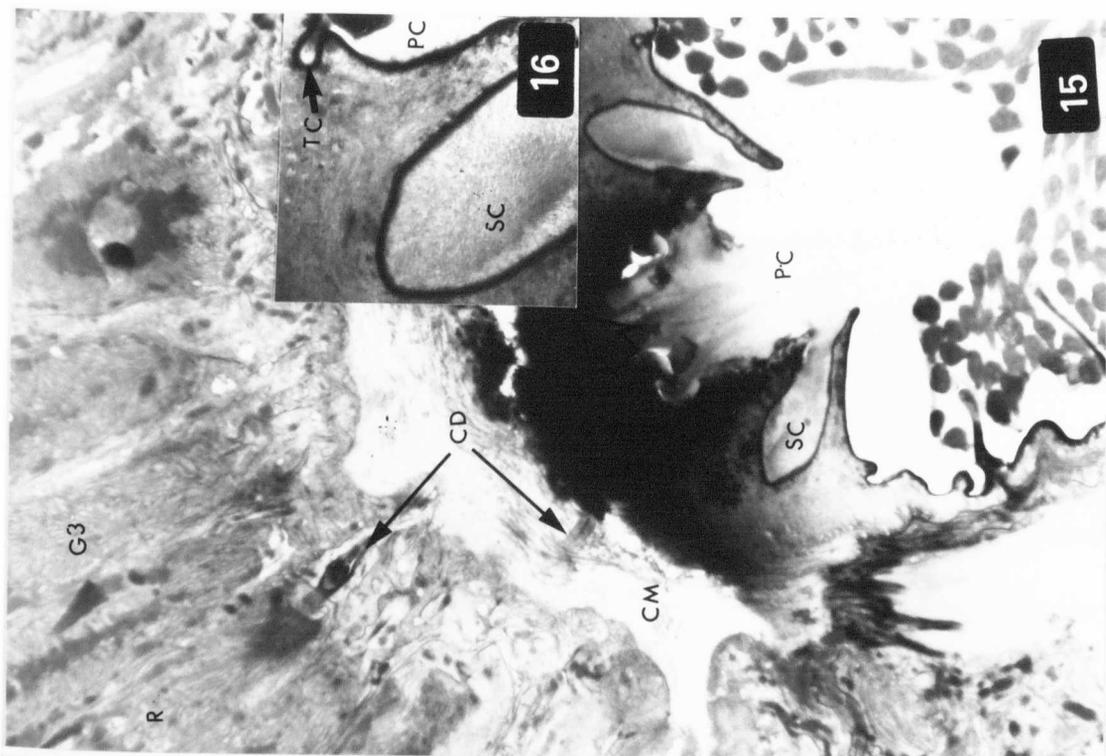
Figure, 14. TEM, Cross section of cuticle from the spermathecal body wall (X 32, 000). The epicuticle (EPI) is relatively thicker than found in the duct and is thrown up from the procuticle (PRO) to form the finger-like projections of the septa (STF) within the lumen of the spermatheca (SPL). The procuticle (PRO) is relatively thin, within it the microfibrils can be seen running perpendicular to the axis of body of the spermatheca. Note the underlying epidermal cell (EP) and muscle fibres firmly attached to the cuticle by belt desmosomes (closed triangles).



Figure, 15. TEM, Longitudinal section through the apex of the spermatheca (X 5, 200) showing a hemispherical cuticular mass, underneath which is a dome-like primary canal (pc) flanked by a secondary canal (sc).

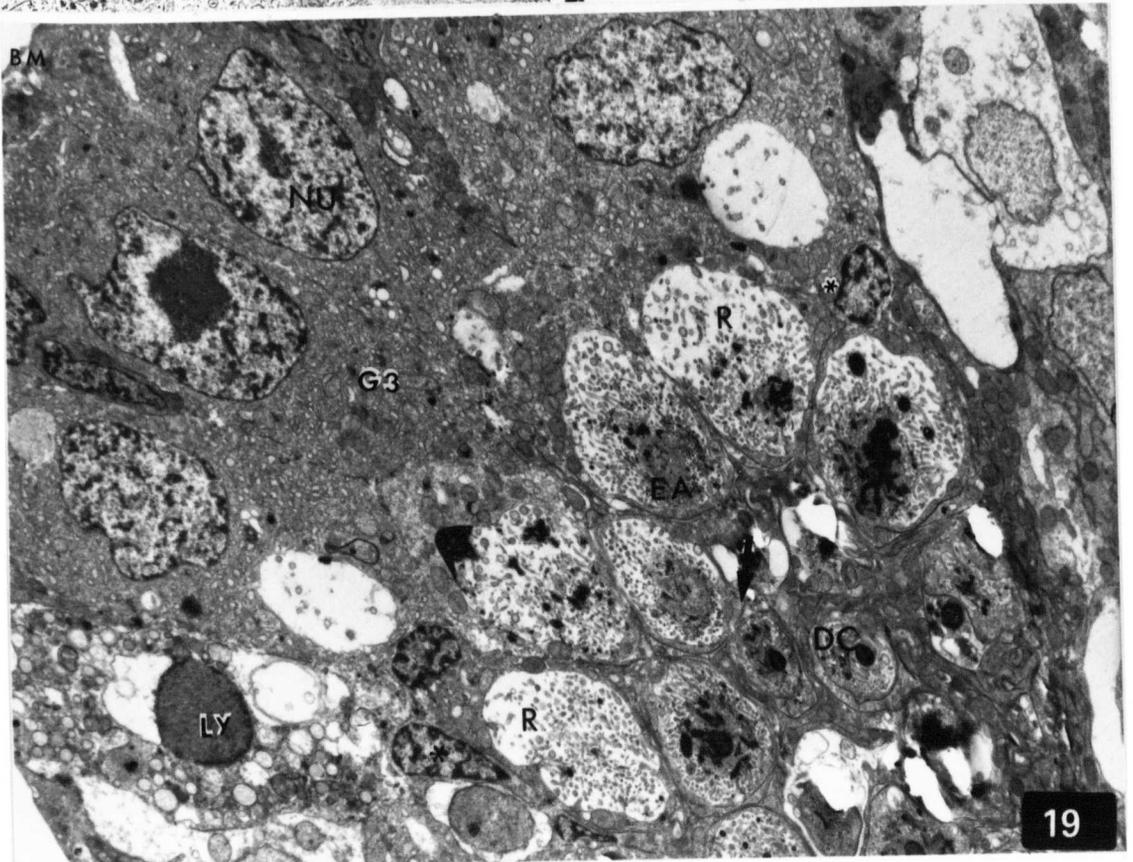
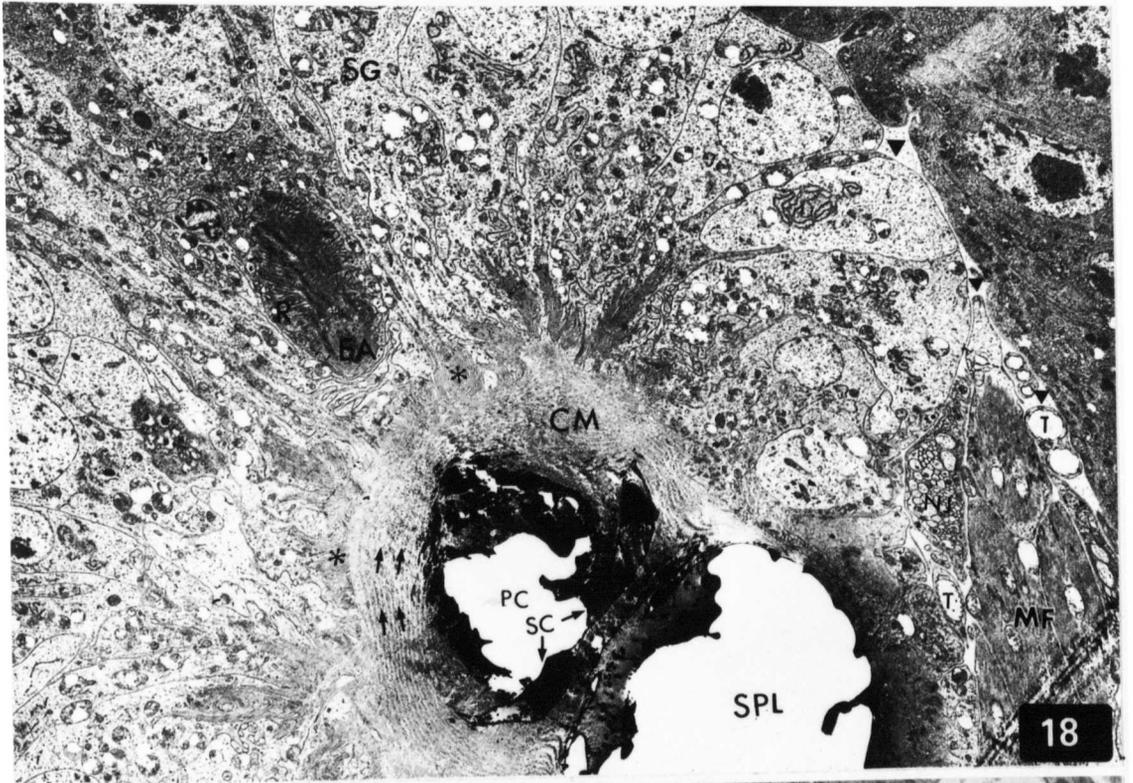
Figure, 16 (inset). Cuticular ductules (CD) from glandular cells (G3) run through the cuticular mass (CM) and enter the primary canal via terminal pits (TC) (X 1, 300).

Figure, 17. TEM, Longitudinal section of the spermathecal body (X 5, 000). The cuticle (CT) of the body wall is folded at regular intervals and projects into the lumen either as long folding (STF) or short interseptal conical processes (IST) compartmentalising the lumen. Thick, striated, visceral muscle fibres runs along side the cuticle wall terminating at an apodeme at the apex. At the base, the body wall converges with a relatively thick folding of the cuticle supported by circular muscle fibres forming a sphincter-like valve separating the spermathecal lumen from the spermathecal duct. Ca, cuticular apodeme; cm, cuticular mass; mf, muscle fibres; pc, primary canal; s, sperm; sc, secondary canal; spl, spermathecal lumen; sv, sphincter-like valve.



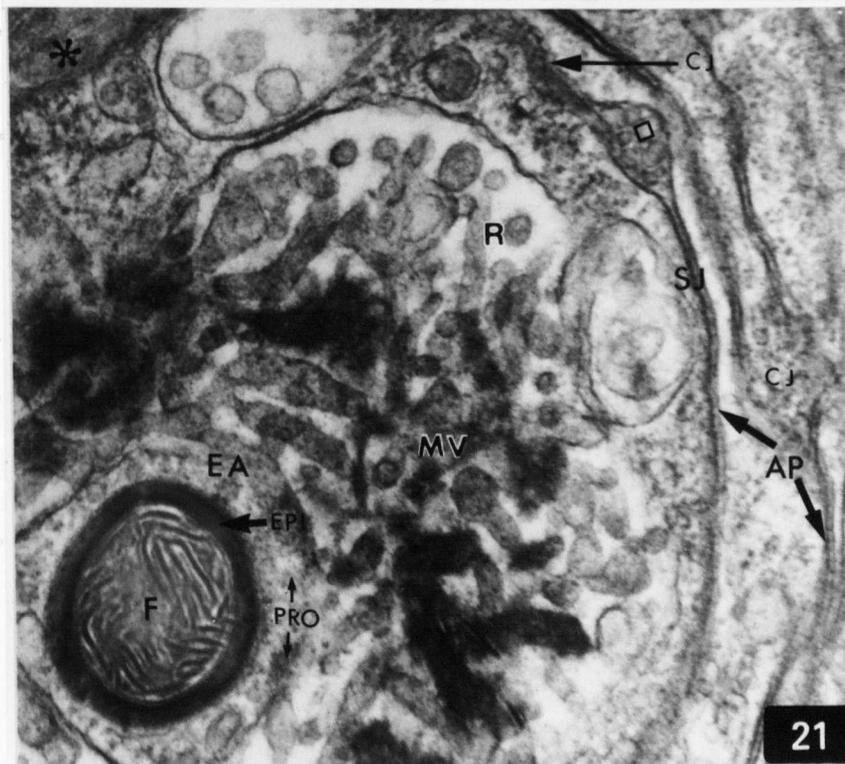
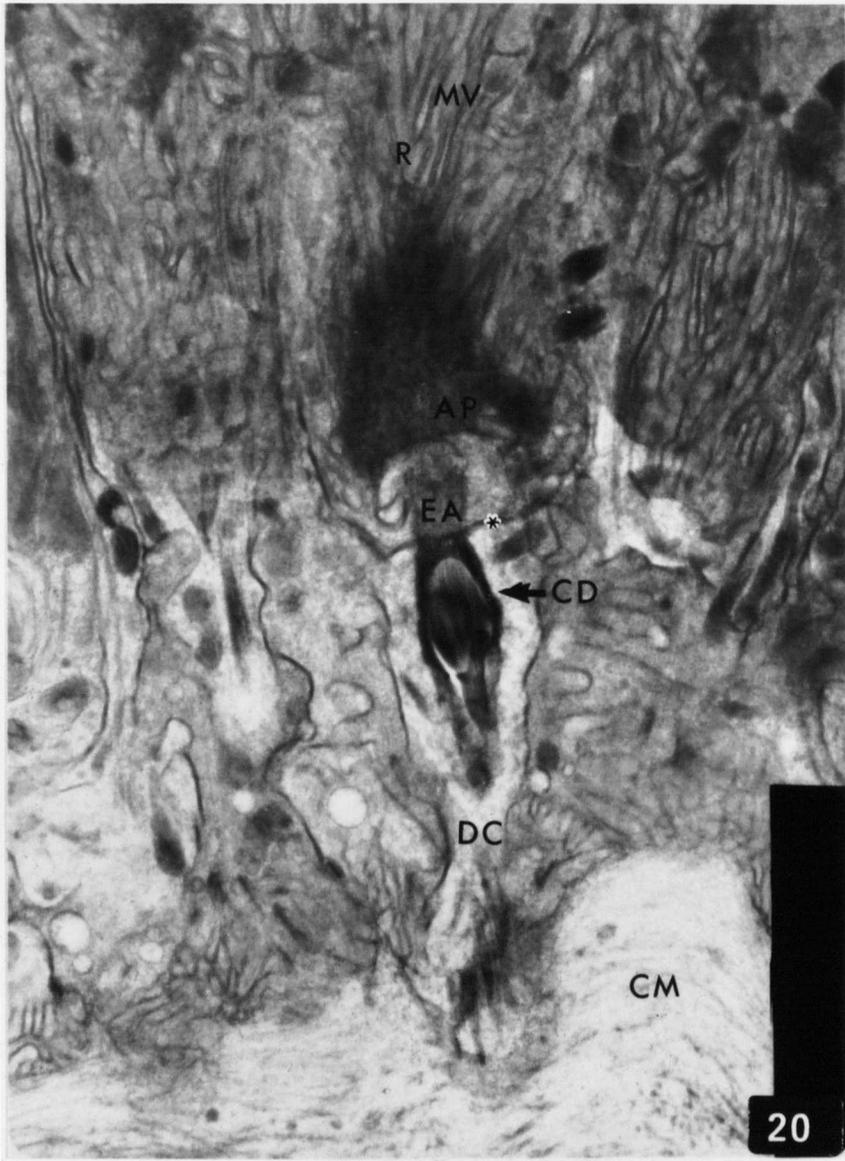
Figure, 18. TEM, Transverse section at low magnification of the spermathecal gland (X 3, 200). The spermathecal gland is composed of a cluster of type 3 glandular cells interspersed with simple epithelial cells (see Figure 5 for the classification of epidermal glands). The basal membrane of the glandular cells is in direct contact with the haemolymph (closed triangles). Below the gland is the cuticular mass in which the microfibrils are formed into arcuate patterns (small arrows) and below that the primary and secondary canals of the spermatheca. The apical membrane of the gland cells develops into microvilli (asterisk). On the basal margins of the gland, motor nerves and muscle fibres (MF) form a neuromuscular junction (NJ). Ca, cuticular mass; ea, receiving canal; fa, folded plasma membrane; lm, lamellation in cuticular mass; spl, spermathecal lumen; r, reservoir.

Figure, 19. TEM, Transverse section of the spermathecal gland at about 45° showing class 3 glandular cells (X 5, 000). Each cell has a well defined central reservoir (r) and receiving canal (ea), duct cell (dc). Between the glandular and duct cells are compact epidermal cells with a small nucleus (asterisk). Within the glandular cells is a lysosome-like structure (LY). Arrow indicates the margin between the glandular cell and the duct cell.



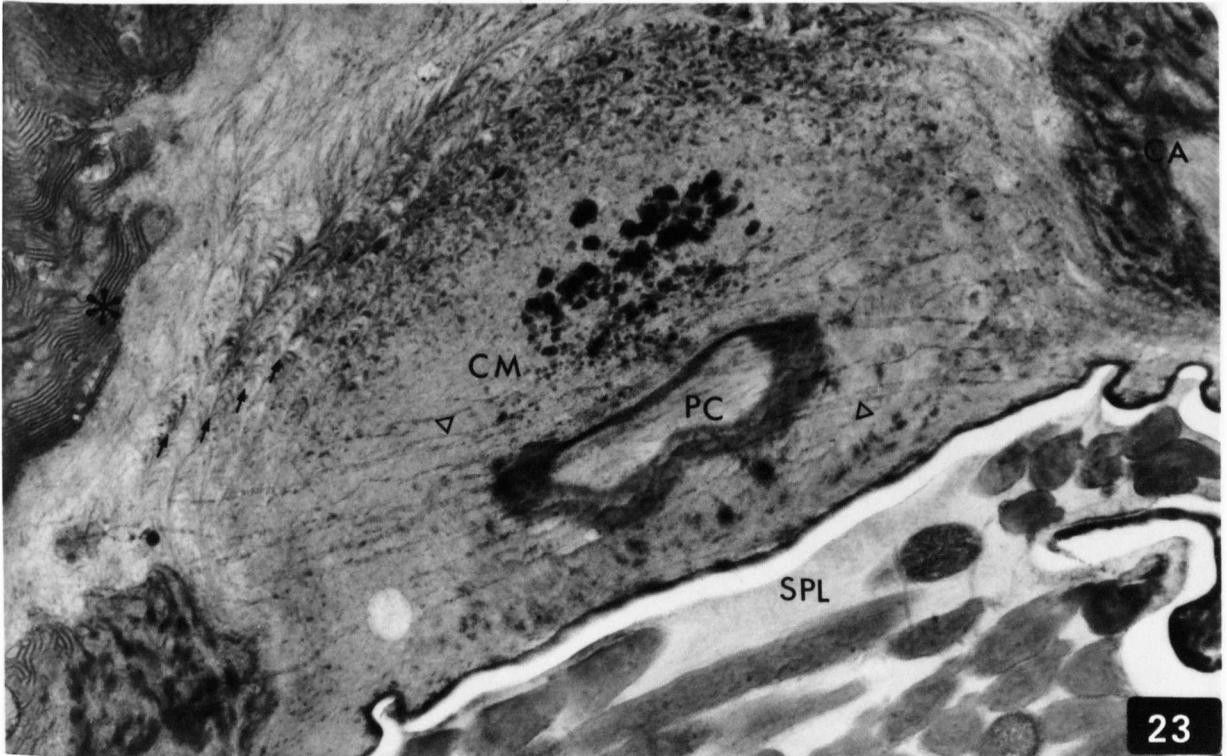
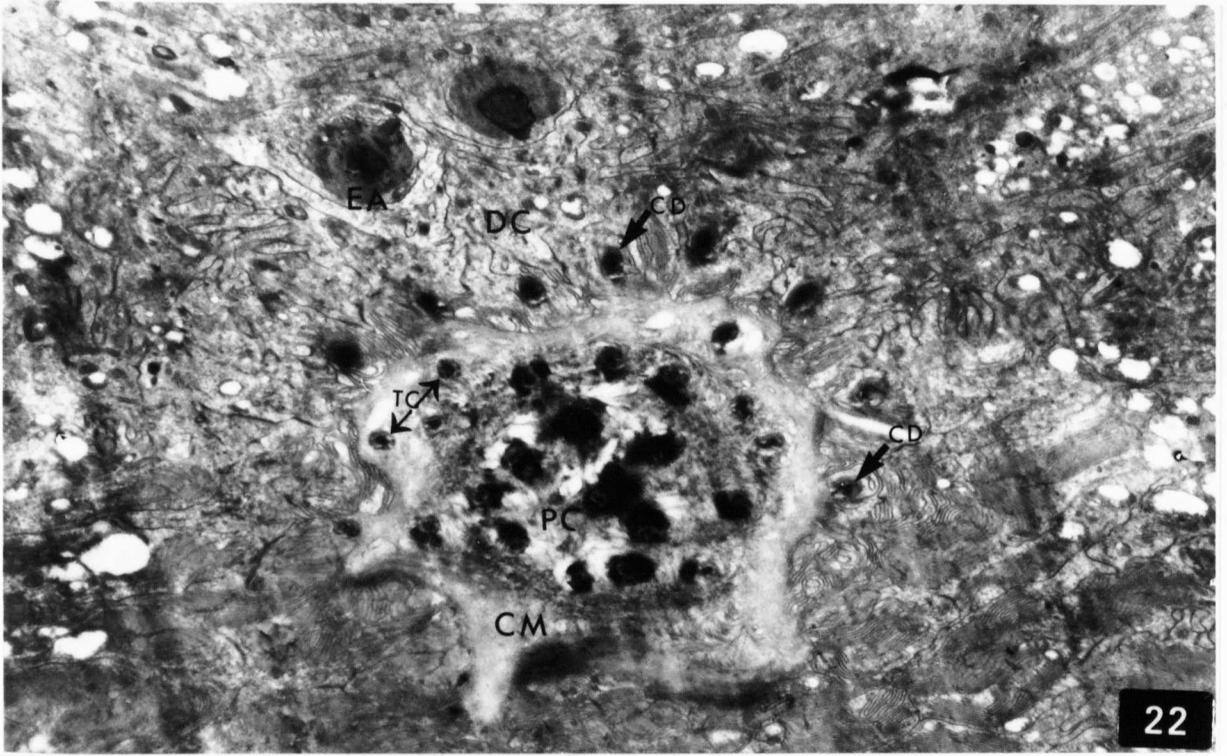
Figure, 20. TEM, Longitudinal section of the receiving canal (EA) and conducting canal (cd) (see the text for revised terminology) of the glandular cell (X 16, 600). The microvilli (MV) from the reservoir (R) and the apical membrane (\*) unite to form a cytological apodeme (AP) in which the receiving canal (EA) is connected and leads to a conducting cuticular canal (cd). Note the cuticular ductule is distinctly enveloped in the duct cell (dc) and the latter is deeply inserted into the cuticular mass (CM) of the spermatheca.

Figure, 21. TEM, Cross section of the receiving canal (X 16, 600). The microvilli (MV) of the reservoir (R) abut on the receiving canal (EA). The receiving canal is differentiated into an electron-dense thin layer of epicuticle (EPI) and a thick filamentous layer of procuticle (PRO). The lumen is filled with a fibrous secretory material (F). The cell membrane of the cytological apodeme (AP) is supported by the septate junction (SJ), continuous junction (CJ), microtubules (open square) and tonofibrils (\*).



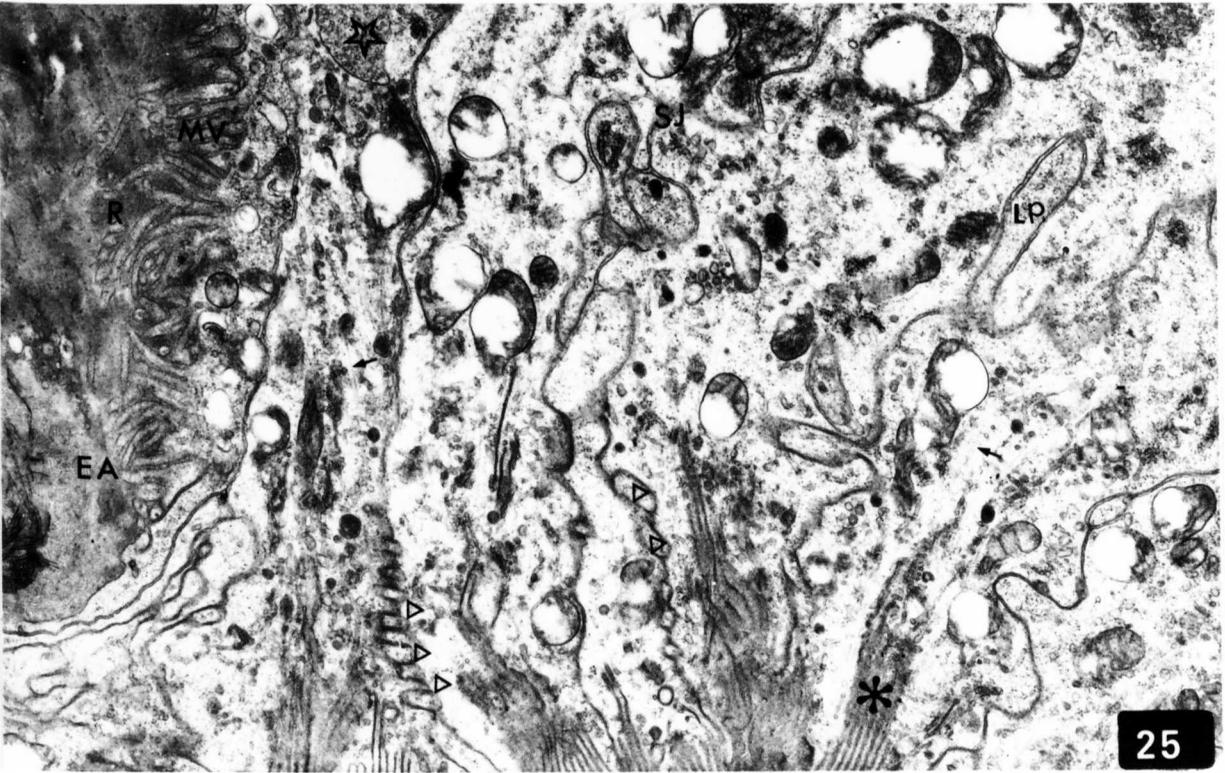
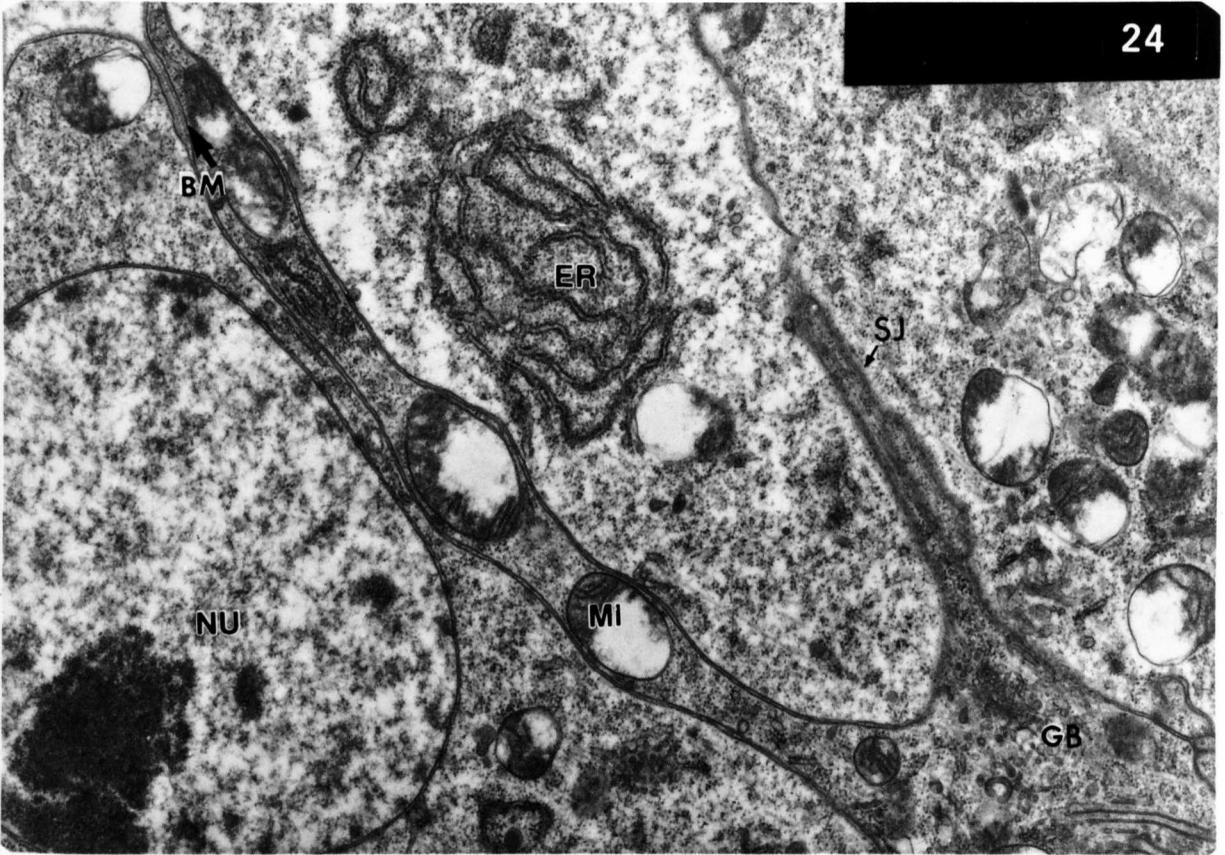
Figure, 22. TEM, Cross section of the apex of the spermatheca (X 6, 600). Details as in the Figures, 15 & 16.

Figure, 23. TEM, The apex of the spermatheca showing the arrangement of microfibrils in the cuticular mass, cut at 45° to the vertical (X 10, 000). Note the fibres are arranged in an arcuate pattern in lamellae (parallel arrows) which correspond with Figure, 6. The presence of pore canals (open triangles) in the cuticular mass (CM) which lead to the spermathecal lumen (SPL) emphasises the chitinous nature of the procuticle. The whole structure is supported by muscle fibres and the cuticular apodeme (CA).



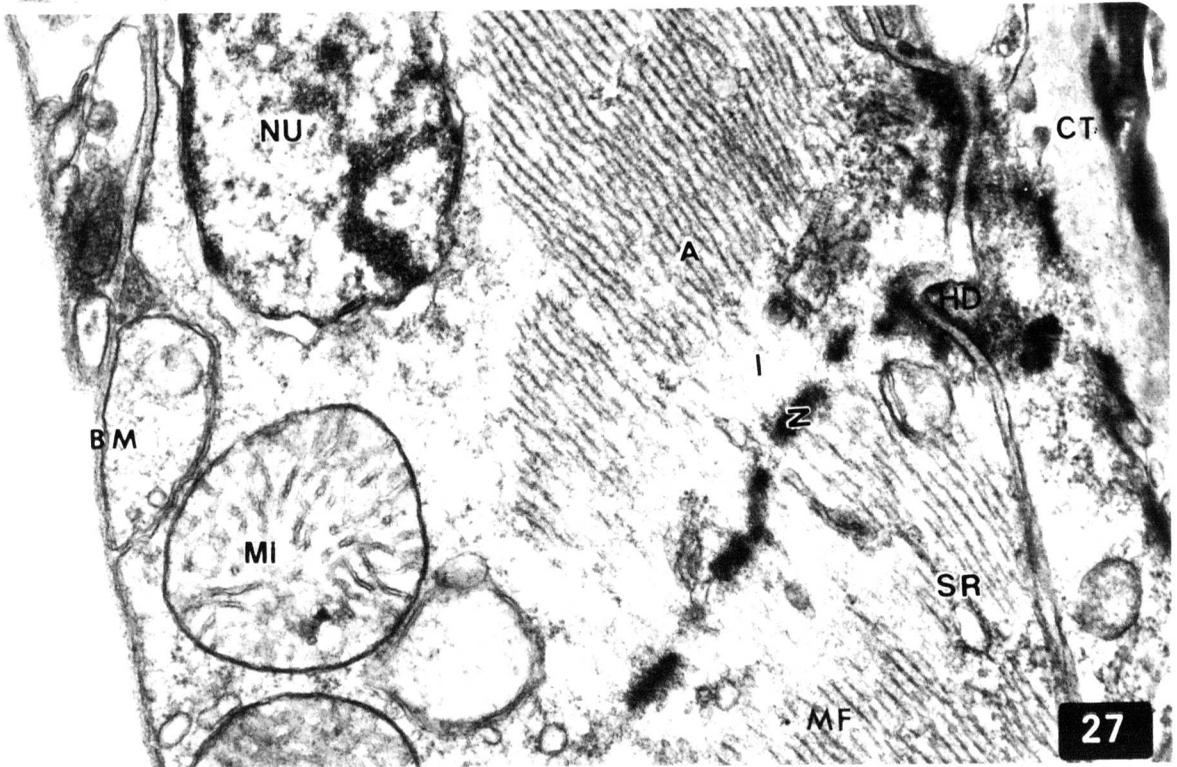
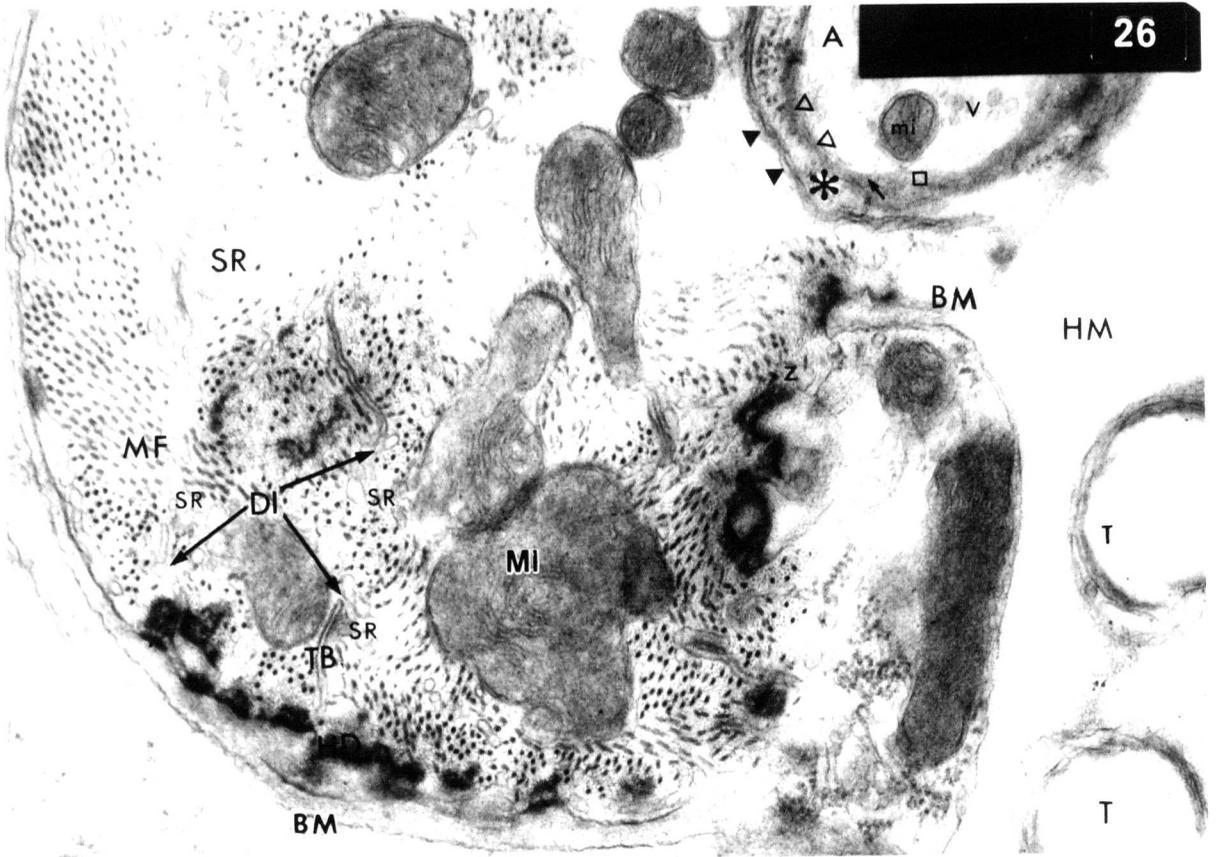
Figure, 24. TEM, Basal region of glandular cell (X 16,600). The large nucleus is placed towards the base of the gland cells. On either side and anterior to the nucleus is the mass of rough endoplasmic reticulum (ER) and Golgi bodies (GB). A large number of mitochondria are randomly distributed. The lateral wall of the plasma membrane is folded with a septate junction (SJ). The basement membrane (BM) of the glandular cells are deeply folded at the points where the vascular sinuses enter.

Figure, 25. Longitudinal section showing the apical part of the gland cell and receiving canal (EA) (X 13, 200). In addition to the microvilli (MV) found in the reservoir (R) of the gland cell, the apical membrane is repeatedly folded (asterisk) for firmly fixing the cell into the cuticular mass. Below this interdigitating region, the apical membrane is modified into a series of hemidesmosomes (open triangles). The lateral plasma membrane of the cell is folded and looped (LP) and formed with septate junctions (SJ). Another important feature of this region is the presence of long microtubules (arrows) and membrane limited tubules (open star) in patches, septate junction (SJ).



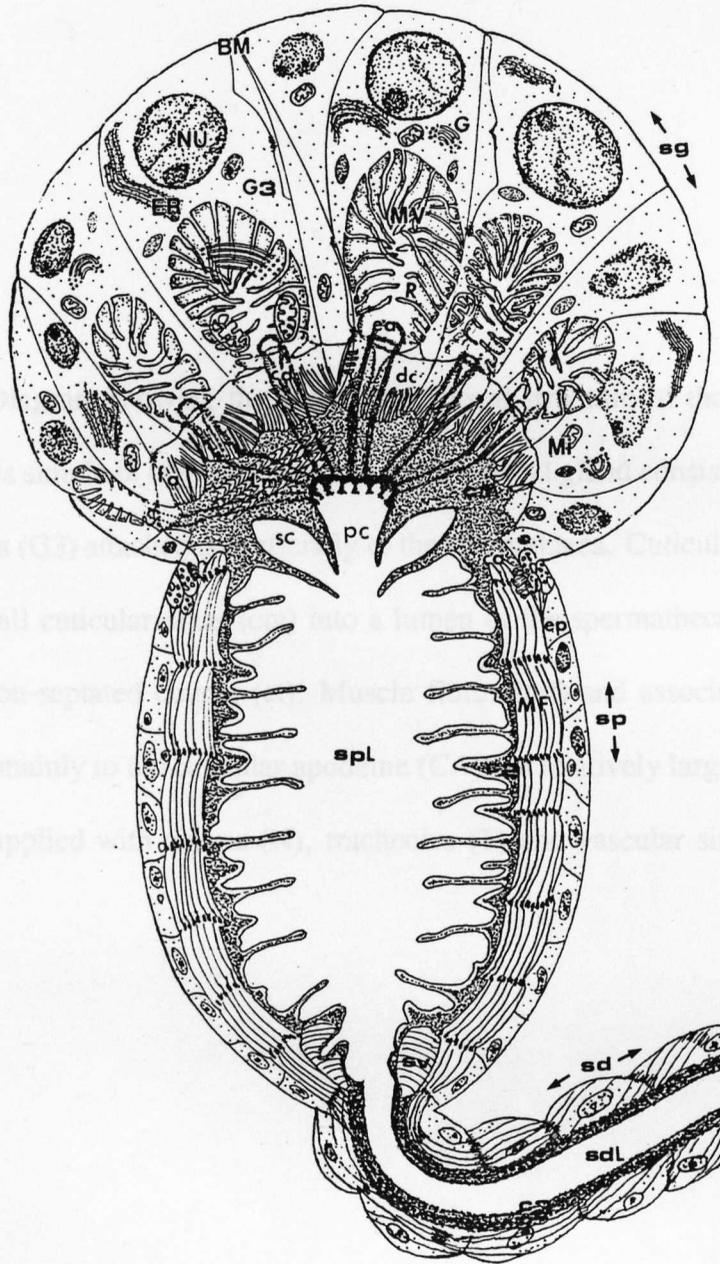
Figure, 26. TEM, Cross section of a neuromuscular junction (X 40, 000). The naked axon (A) lies in a shallow groove on the surface of the muscle that faces the haemolymph (HM) and tracheoles (T). Within the axon are electron-lucent, spherical, synaptic vesicles (v) and a large mitochondria. On the presynaptic membrane (open triangles) there is a presynaptic dense projection (small arrow/ open square) which is seen to be attached to the synaptic vesicles. In the synaptic cleft (asterisk), electron-dense particles can be seen. The postsynaptic membrane (closed triangles) is associated with sarcoplasm on one side and the basal membrane on the other. The muscle fibre shows the multidirectional arrangement of the filaments (fm), A- and i- bands, the sarcoplasmic reticulum (SR), transverse tubules (TB), dyad (DI) and hemidesmosomes (HD).

Figure, 27. TEM, Oblique section of visceral muscle on the spermathecal wall (X 32, 000). Note the perforated Z-line, a characteristic feature of supercontracting intrinsic visceral fibres, which separates the sarcomere (SM). The I-band, representing the thin filaments of myosin, is close to the Z-discs and interdigitates with the neighbouring thick actin filaments (A). The sarcoplasmic reticulum (SR) is seen along the filamentous (FM) course and the transverse tubules (TB) are continuous with actin filaments and the hemidesmosomes. The sarcoplasmic membrane is closely apposed (by hemidsmosomes, HD) to the body wall cuticle (CT).



Figure, 28. A diagrammatic reconstruction of the histological organisation of a spermatheca.

Abbreviations. BM: basement membrane; BC: bursa copulatrix; CA: cuticular apodeme; CD: cuticular ductule; CM: cuticular mass; CT: cuticle on the duct & spermathecal wall; DC: duct cell; EA: end apparatus; ER: rough endoplasmic reticulum; FA: highly folded apical membrane (= microvilli); G: Golgi bodies; G3: secretory gland cell; NJ: neuromuscular junction; LM: lamellar pattern of cuticular microfibrils; Mi: mitochondria; MF: muscle fibres; MV: microvilli; N&n: nerves; NU: nucleus; PC: primary canal; R&r: reservoirs; SC: secondary canal; SG: spermathecal gland; SP: spermatheca; SPL: spermathecal body lumen; SD: spermathecal duct; SDL: spermathecal duct lumen; SV: sphincter valve; T&t: tracheoles; V: vascular sinuses; Z: z-band.



Figure, 29. Diagram showing the three dimensional structure of the spermathecae. The spermatheca is simple in construction. The spermathecal gland consists of a relatively few secretory cells (G3) attached superficially to the spermatheca. Cuticular ductules (cd) pass through a small cuticular mass (cm) into a lumen of the spermatheca (SP) having a thin walled and non-septated cuticle (ct). Muscle fibres (mf) and associated epidermal cells (ep) attached mainly to the cuticular apodeme (CA) are relatively large. The spermathecal complex is supplied with nerves (N), tracheoles (T) and vascular sinuses (VS).

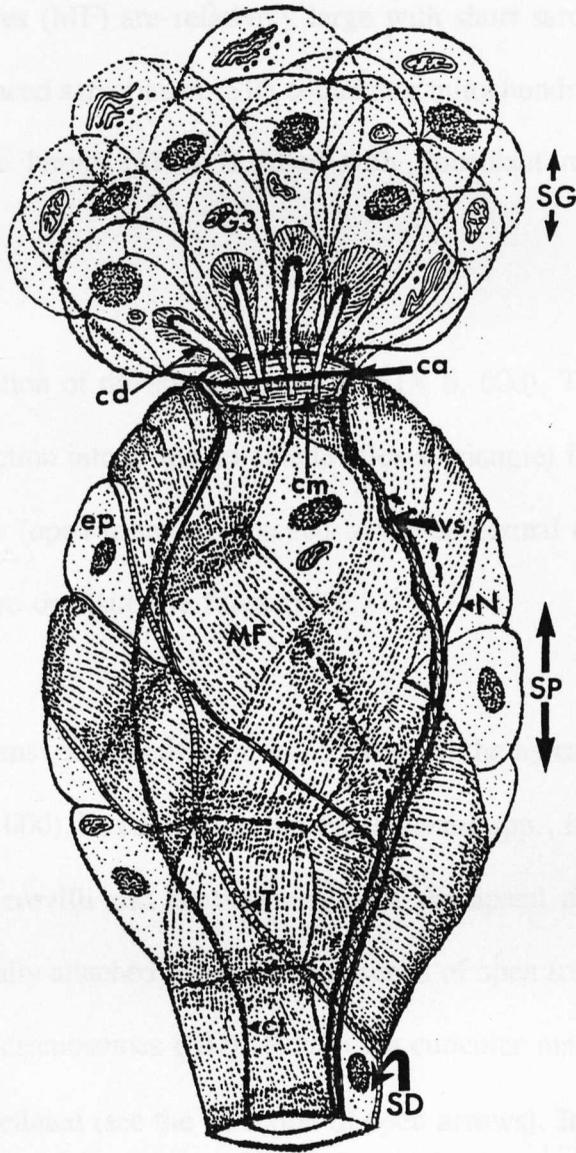
11. Cross section of the body of the organism (X 10, 1000). Like the first in  
view, the histology of the middle of the stem and the prostrate is fibrous  
tissue. The epidermal layer that is occasionally attached to the surface and the

middle part of the stem are composed of several layers of cells, perforated by  
pores. The middle part of the stem is composed of several layers of cells and  
is highly porous.

12. Cross section of the body of the organism (X 10, 1000). Like the first in  
view, the histology of the middle of the stem and the prostrate is fibrous  
tissue. The epidermal layer that is occasionally attached to the surface and the

middle part of the stem are composed of several layers of cells, perforated by  
pores. The middle part of the stem is composed of several layers of cells and  
is highly porous.

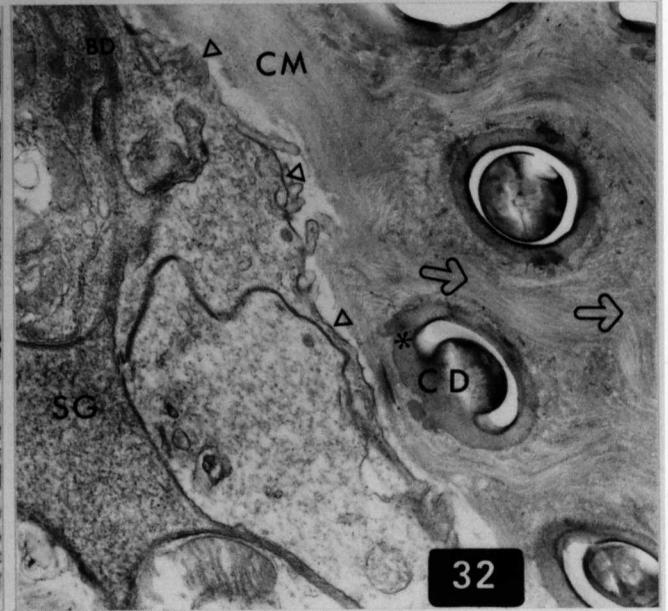
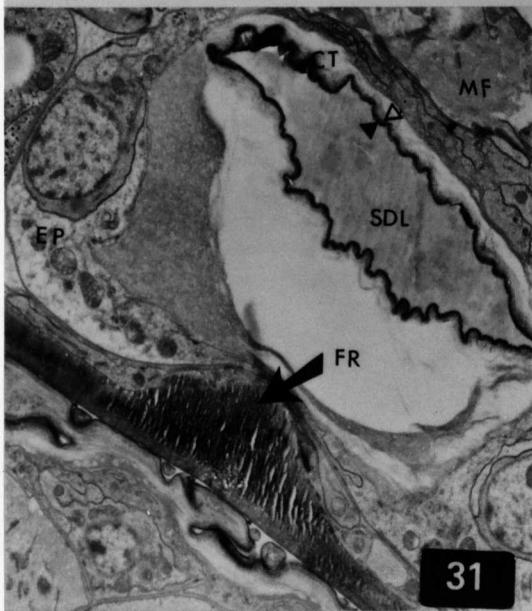
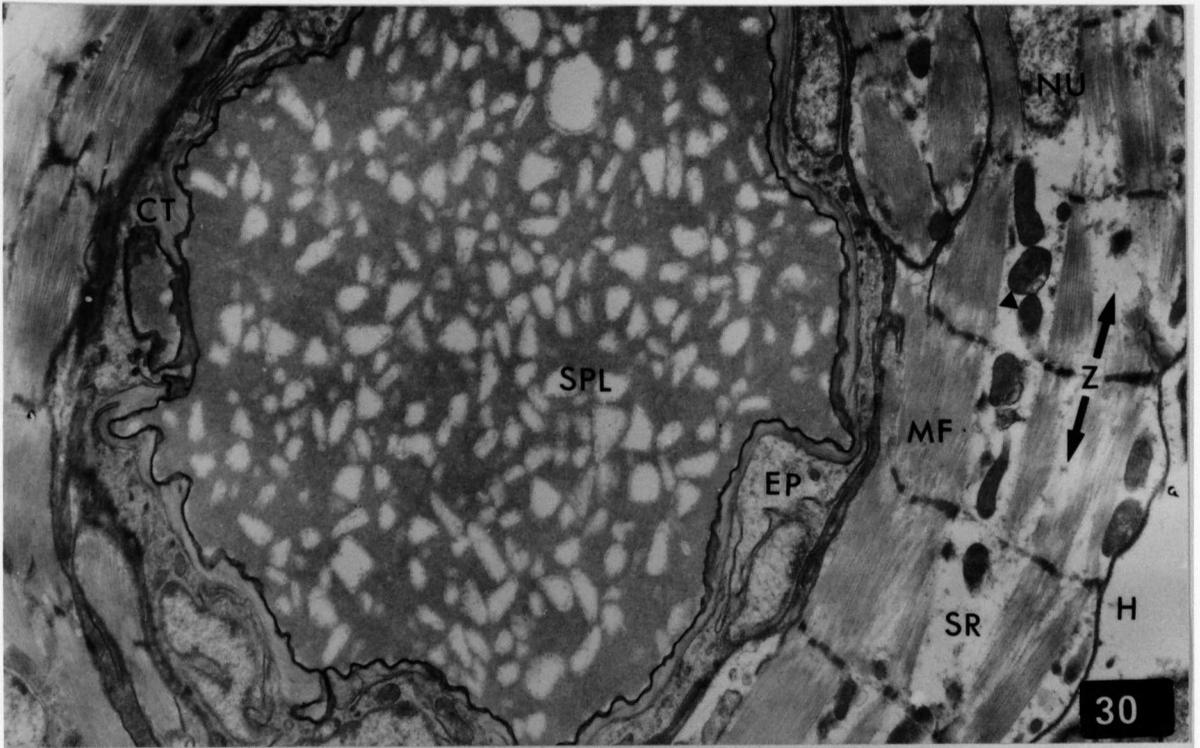
13. Cross section of the body of the organism (X 10, 1000). Like the first in  
view, the histology of the middle of the stem and the prostrate is fibrous  
tissue. The epidermal layer that is occasionally attached to the surface and the



Figure, 30. Cross section of the body of the spermatheca (X 10, 1000). Like the duct in this species, the histology of the cuticle (CT) is simple, and the procuticle is fibrillar. Septa are absent. The epithelial layer (EP) is superficially attached to the cuticle and the overlying muscle fibres (MF) are relatively large with short sarcomeres, perforated Z-discs and centrally placed sarcoplasm (SR) containing mitochondria (closed triangle) and a nucleus (NU). The lumen (SPL) is filled with flocculant material, presumably a mucopolysacchride.

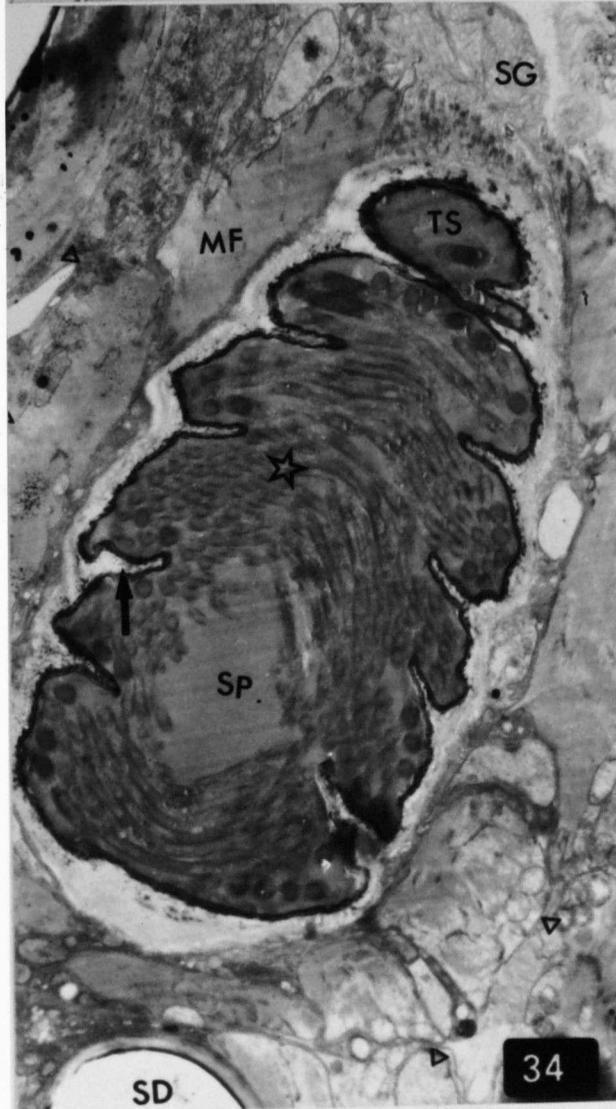
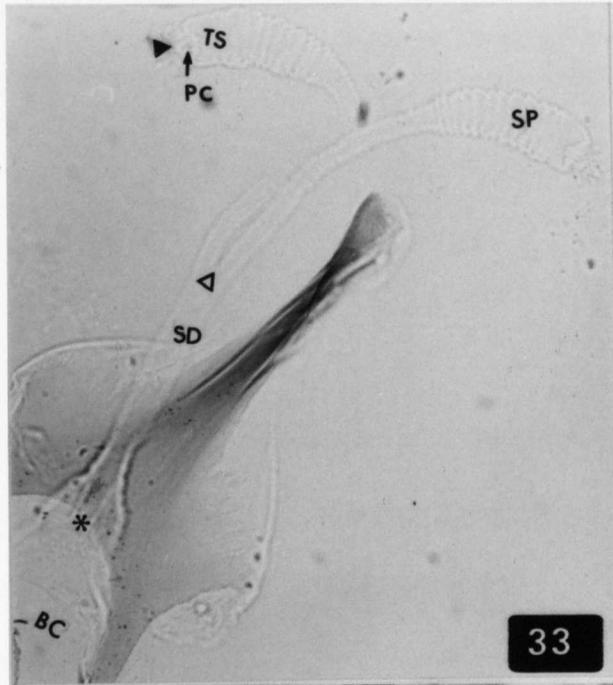
Figure, 31. Cross section of the spermathecal duct (X 6, 600). The irregular wall (CT) shows little differentiation into a thin epicuticle (closed triangle) facing the lumen (SDL) and fibrous procuticle (open triangle) attached to the epidermal cell (EP). The visceral muscle fibres (MF) are opposite the furca (FR).

Figure, 32. TEM, Transverse section cut at 45° through the apical glandular region and cuticular mass (X 20, 000). In contrast to the *Phlebotomus* spp., in which the apical area is firmly fixed by microvilli and hemidesmosomes, the apical membrane of the gland (SG) is very superficially attached (shown by the series of open triangles) to the cuticular mass (CM) with belt desmosomes (BD). Within the cuticular mass, the microfibrils are long, arcuate and lamellated (see the direction of open arrows). In the cuticular ductules (CD) passing through the cuticular mass, the layer of epicuticle is completely obliterated, being occupied by overlying procuticle (asterisk).



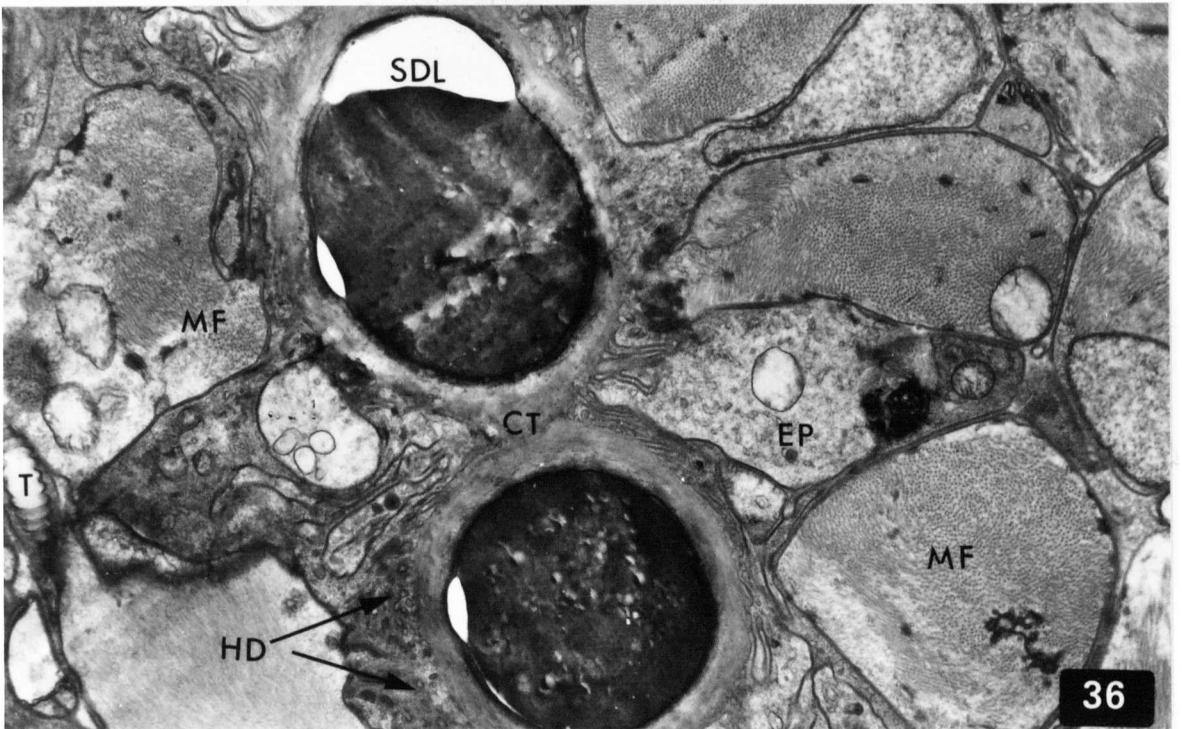
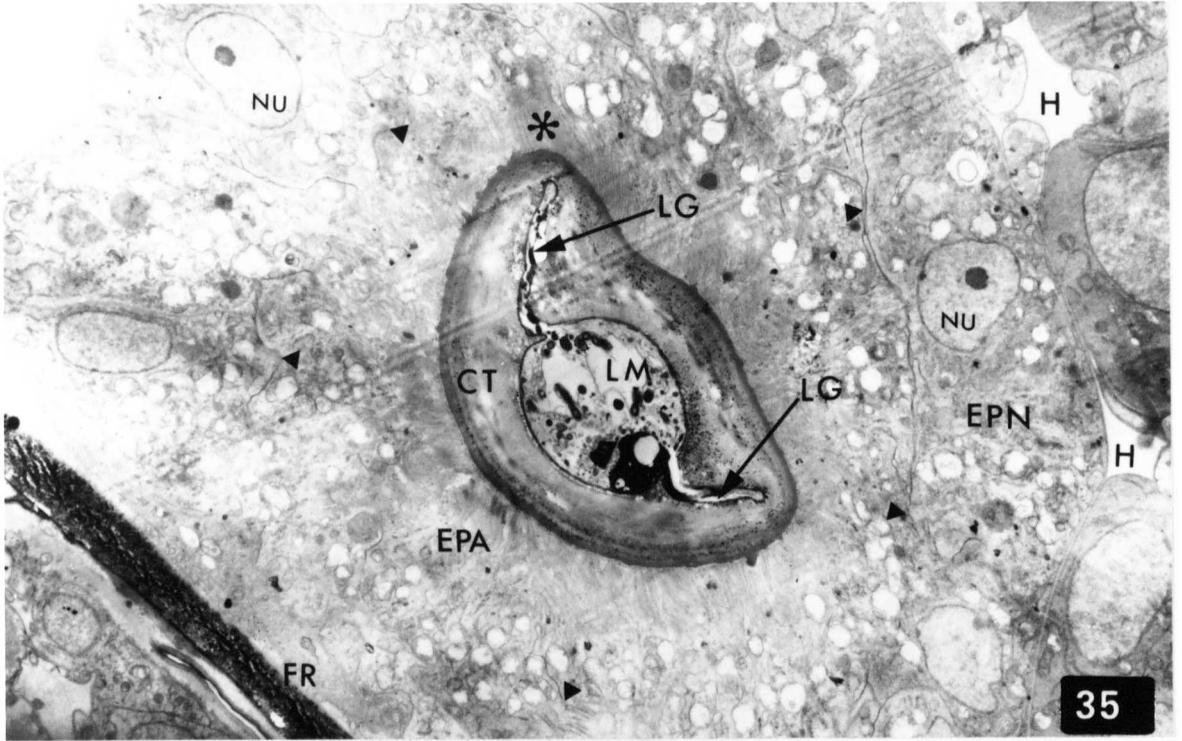
Figure, 33. The spermathecae of *Phlebotomus argentipes* mounted in the Berlese's medium (X 750). A single common duct (SD) originating from the bursa copulatrix (BC) is connected to a pair of carrot shaped spermathecae (SP) through individual ducts. The basal section of the common duct is cone shaped and its lumen is restricted in the middle (asterisk). The terminal segment (TS) of the spermatheca is larger than the other segments and is deeply inserted by a cone shaped primary canal (PC) from which the remnants of cuticular ductules (closed triangle) leading to the spermathecal gland can be seen.

Figure, 34. TEM, Longitudinal section cut at 45° through the spermatheca (X 2, 600). The spermatheca proper (SP) is situated compactly in the viscera (the series<sup>of</sup> open triangles delimit the spermatheca from the other posterior visceral organs). The septal folding (arrow) is relatively short. The terminal segment (TS), under the spermathecal gland, is partly exposed. The lumen is filled with spermatozoa (star). Below the spermatheca is the duct (SD).



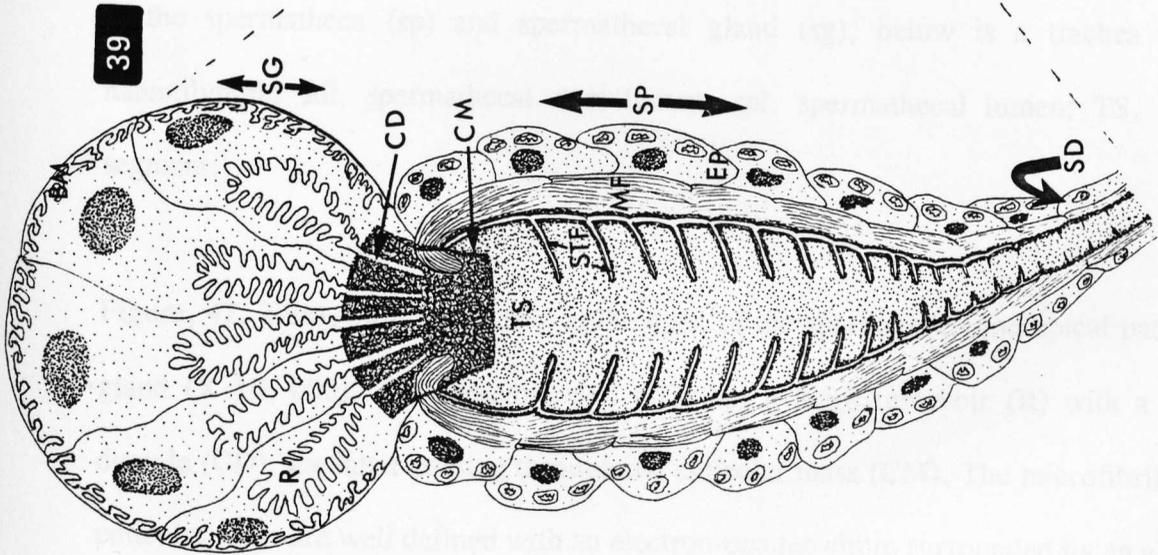
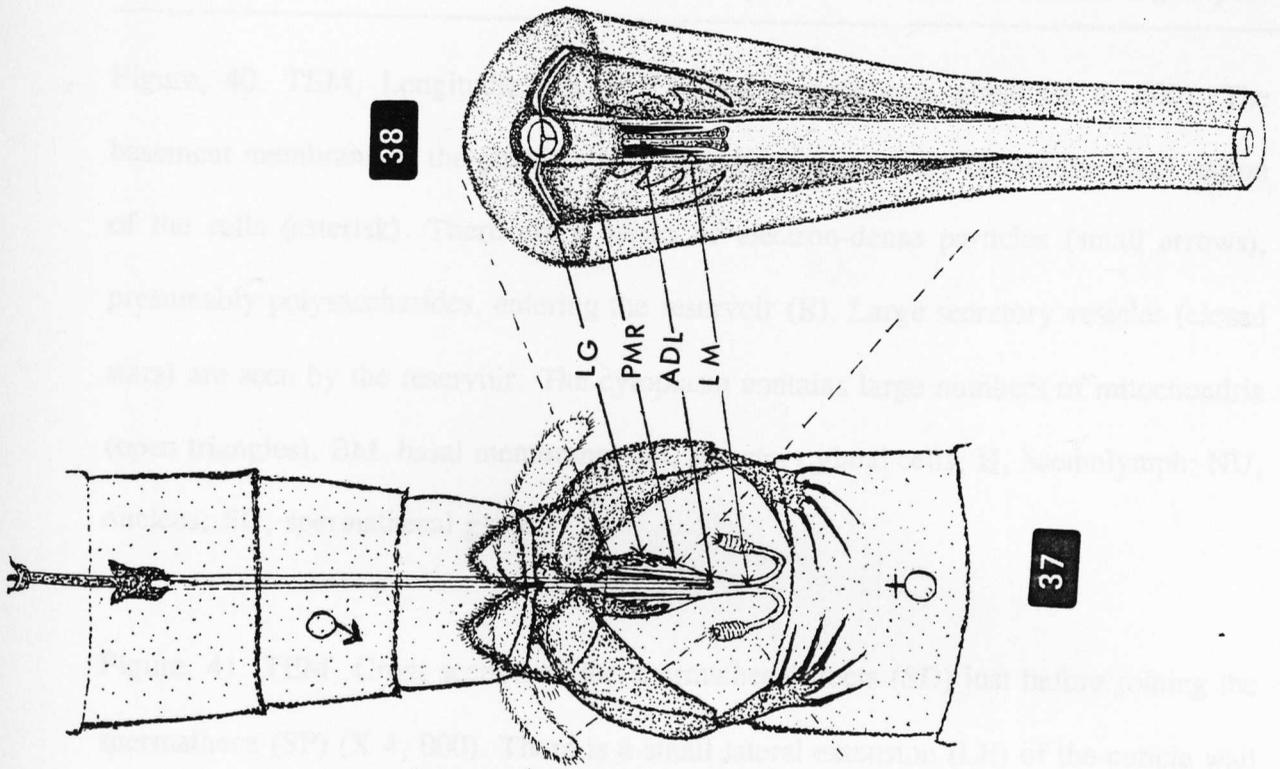
Figure, 35. TEM, Cross section of the basal section of the common duct (X 5, 200). The multilayered fibrillar procuticle (CT) is semicircular and encloses a circular lumen (LM) which gives rise to a pair of lateral grooves (LG) (see also figure, 32). Outside the cuticle is a double-layered epithelial lining (closed triangle shows demarkation) lacking muscle fibres. The inner layer (EPA), closest to the cuticle, is non-nucleated and its apical membrane (FA) is heavily folded and fixed to the cuticle for maximum mechanical support. The outer layer (EPN), in contact with the haemolymph (H), is clearly nucleated (NU) and resting on the furca (FR).

Figure, 36. TEM, Cross section of individual spermathecal duct (SDL) at the point they arise from the common duct (X 10, 000). The thin cuticle lining (CT) on both ducts is firmly supported by an elongated epithelial cellular layer (EP) and thick muscle fibres (MF) through well defined hemidesmosomal (HD) cellular junctions.



Figures, 37 & 38. (37) Diagram showing the male genitalia and spermathecae of female during mating; (38) Three dimensional reconstruction of the basal part of the common duct showing the possible functional correspondence with parameres and aedeagus of male. LM, lumen of duct; LG, lateral grooves; ADL, aedeagal filament; PMR, trilobed paramere.

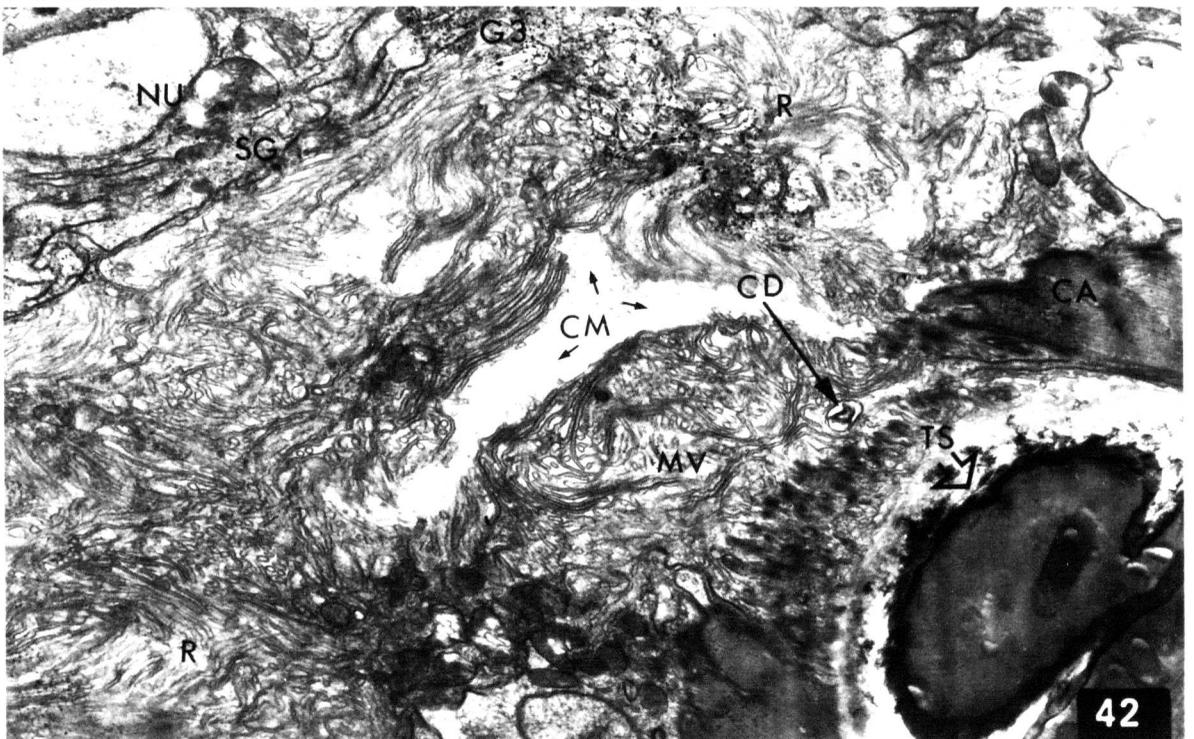
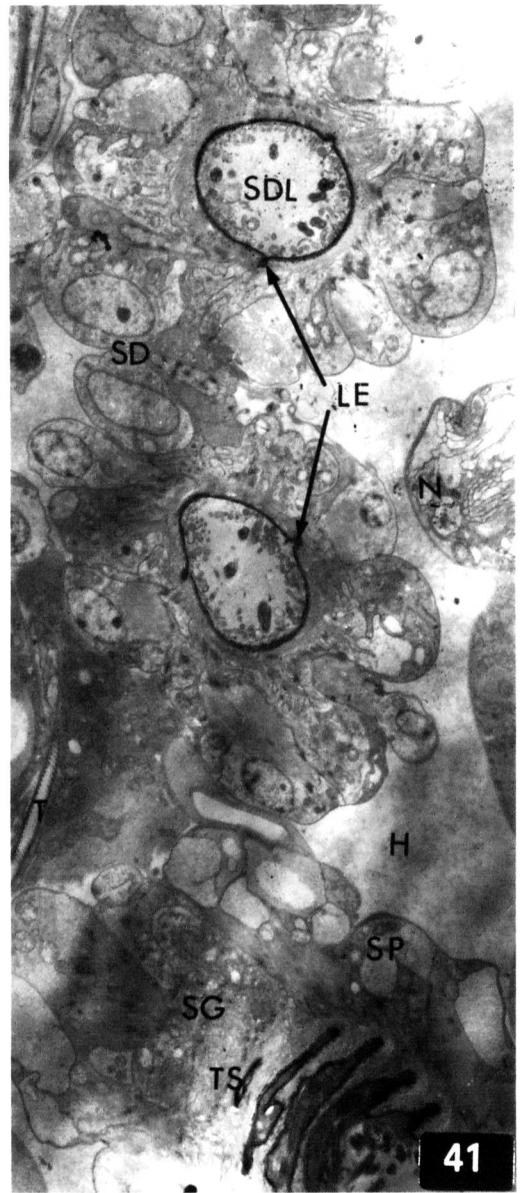
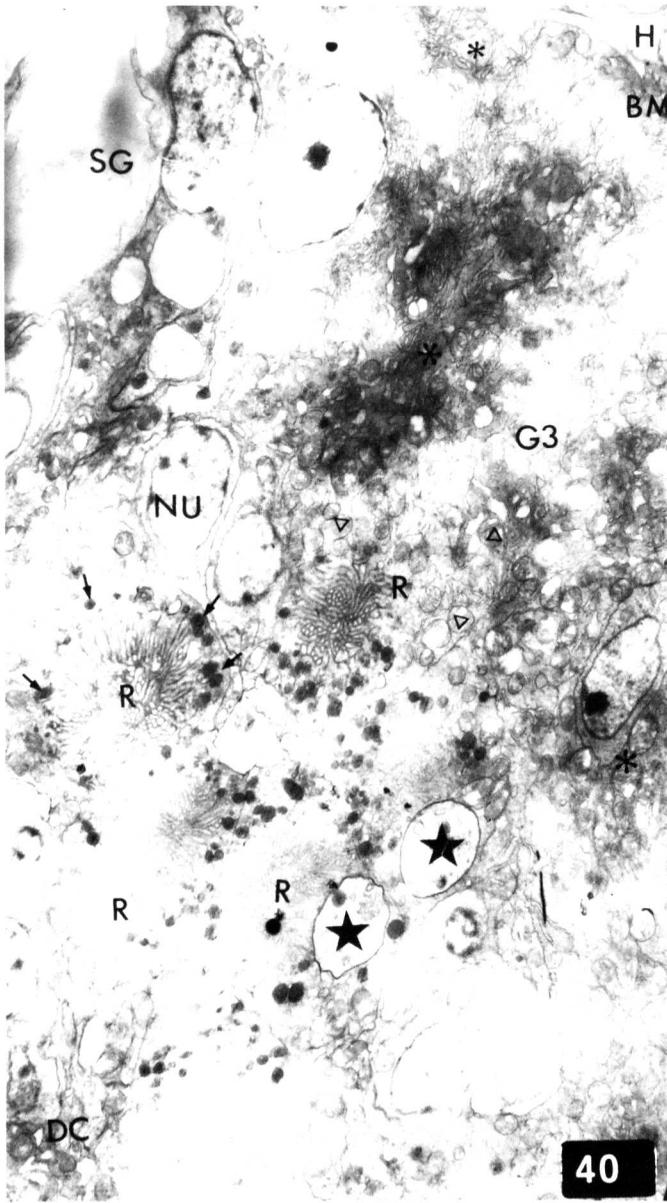
Figure, 39. Reconstruction based on electron microscopy showing the different parts of the spermatheca. The basal membrane (BM) of the secretory cells of the spermathecal gland (SG) are deeply folded. Attached to the terminal segment (TS) is a cuticular mass (CM) penetrated by cuticular ductules (CD) and microvilli of the apical region of the spermathecal gland cells (G3). There is no clear demarcation between the duct (SD) and spermathecal proper (SP) in contrast to *P. papatasi*. The lumen of the spermatheca is projected by a series of uniform septal foldings (STF) derived from the cuticle wall.



Figure, 40. TEM, Longitudinal section of the spermathecal gland (X 6, 600). The basement membrane of the gland cells is folded and extensively covers the basal region of the cells (asterisk). There are a series of electron-dense particles (small arrows), presumably polysaccharides, entering the reservoir (R). Large secretory vesicles (closed stars) are seen by the reservoir. The cytoplasm contains large numbers of mitochondria (open triangles). BM, basal membrane; G3, secretory gland cells; H, haemolymph; NU, nucleus; SG, spermathecal gland.

Figure, 41. TEM, Cross section of the spermathecal ducts (SD) just before joining the spermatheca (SP) (X 4, 000). There is a small lateral extension (LE) of the cuticle wall on either side of the duct which appears crenulated under the light microscope (see figure, 30). Above the ducts is a motor nerve (N) supplying the spermatheca, on the right is the spermatheca (sp) and spermathecal gland (sg), below is a trachea (T). H, haemolymph; sdl, spermathecal duct lumen; spl, spermathecal lumen; TS, terminal segment,

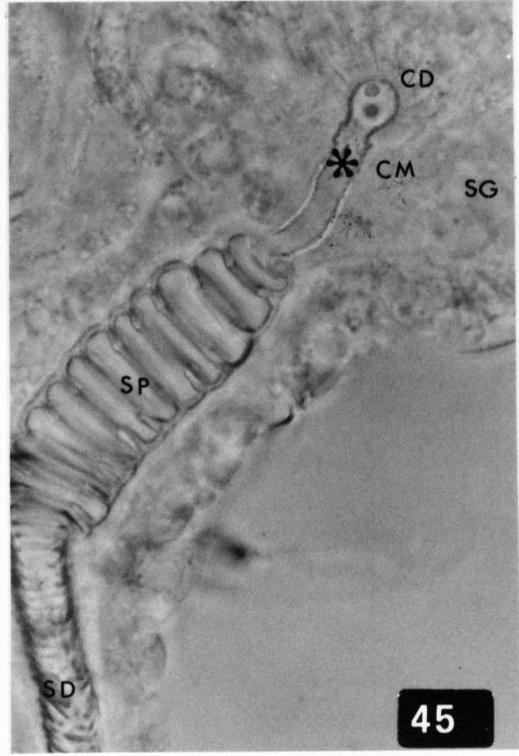
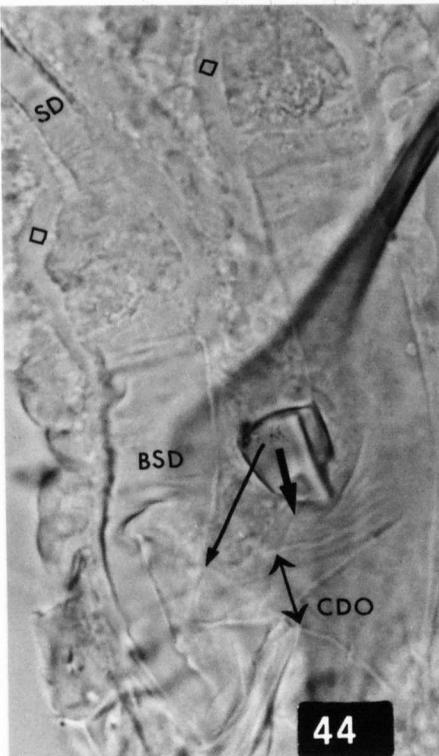
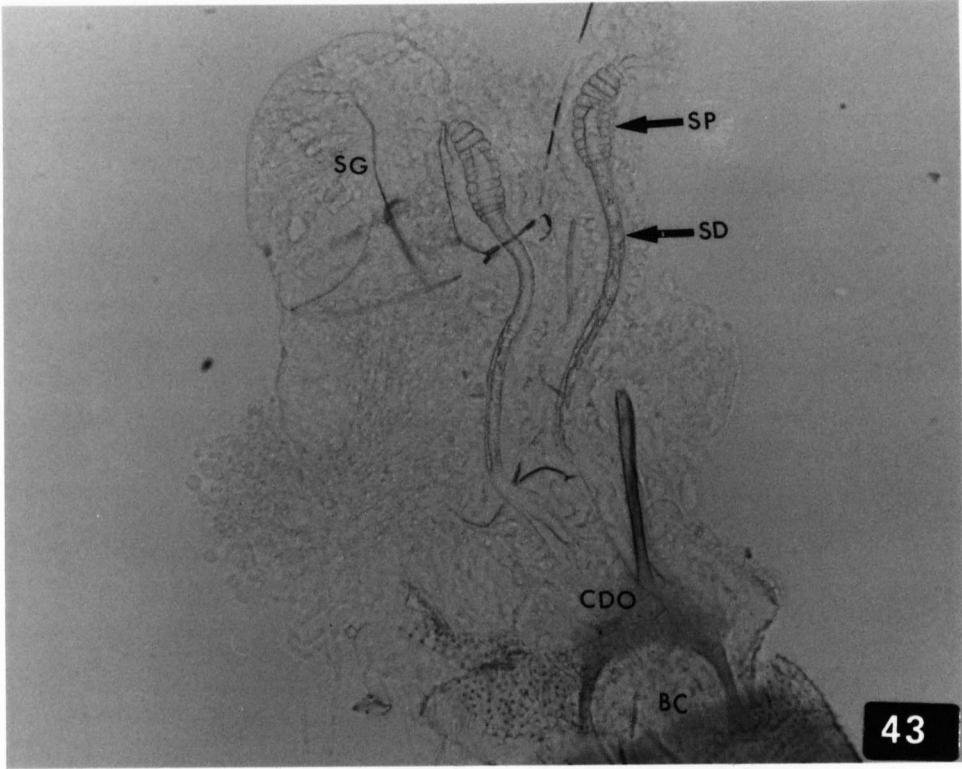
Figure, 42. TEM, Oblique section at the apex of the spermatheca and apical part of the gland (X 10, 000). The secretory cell (G3) has a large reservoir (R) with a narrow ductule (CD), the latter passing through the cuticular mass (CM). The microfibrils in the cuticular mass are well defined with an electron-opaque chitin surrounded by an electron-dense protein matrix (small arrows). The terminal segment (open arrow) is large and extends to the apical chamber. CA, cuticular apodeme; FA, plasma membrane foldings; NU, nucleus; SG, spermathecal gland; TS, terminal segment.



Figure, 43. Saline squashes showing the spermathecae (X 600). From the bursa copulatrix (BC), a short common duct (CDO) is bifurcated to form a pair of individual spermathecal ducts (SD) each leading to a spermatheca (SP) and associated spermathecal gland (SG).

Figure, 44. The common duct and basal section of individual spermathecal ducts (X 1, 800). The common duct opening (twin headed arrow) is slightly distorted but its depth and the origin of individual basal ducts (BSD) is well marked (long and short arrow). On the outer side of the distal part of the individual basal ducts from where the spermathecal duct (SD) starts, there is an unequal cuticle deposit (open square).

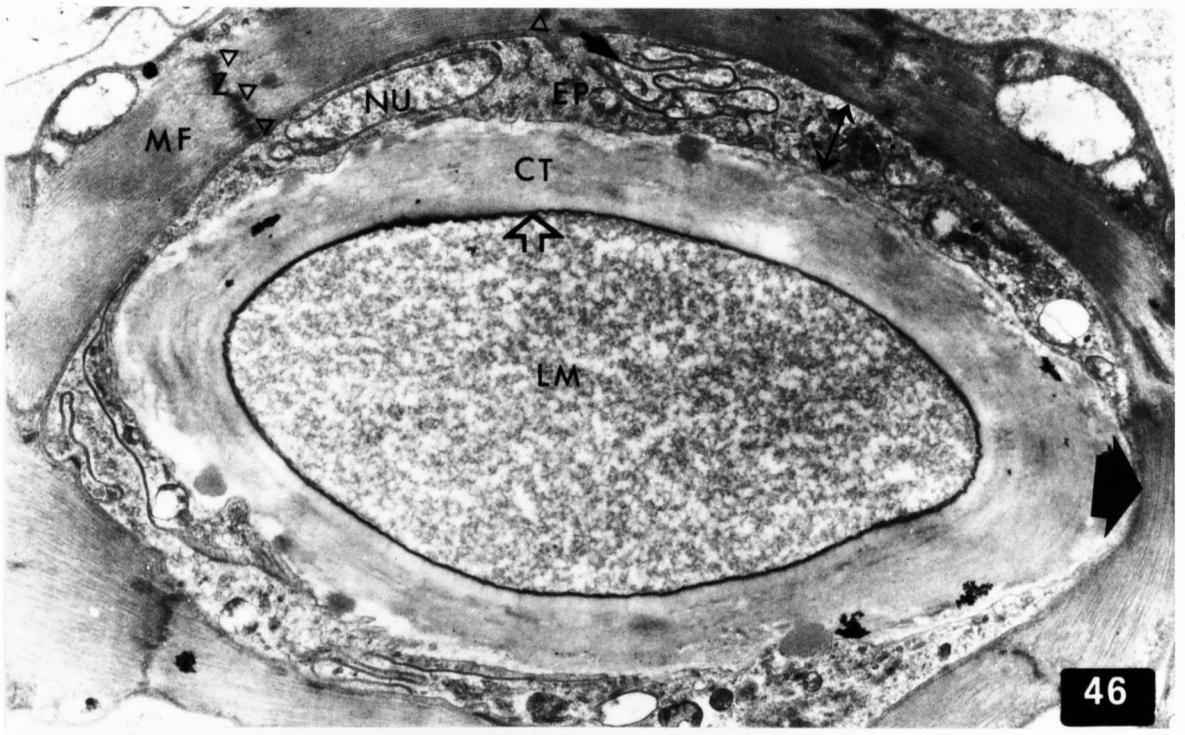
Figure, 45. The spermatheca and spermathecal gland (X 1, 800). The primary canal extends from the spermatheca to form a long tube (asterisk) into which the cuticular ductules (CD) from the spermathecal gland cells enter (SG). Surrounding the primary canal (asterisk) is the large cuticular mass (CM).



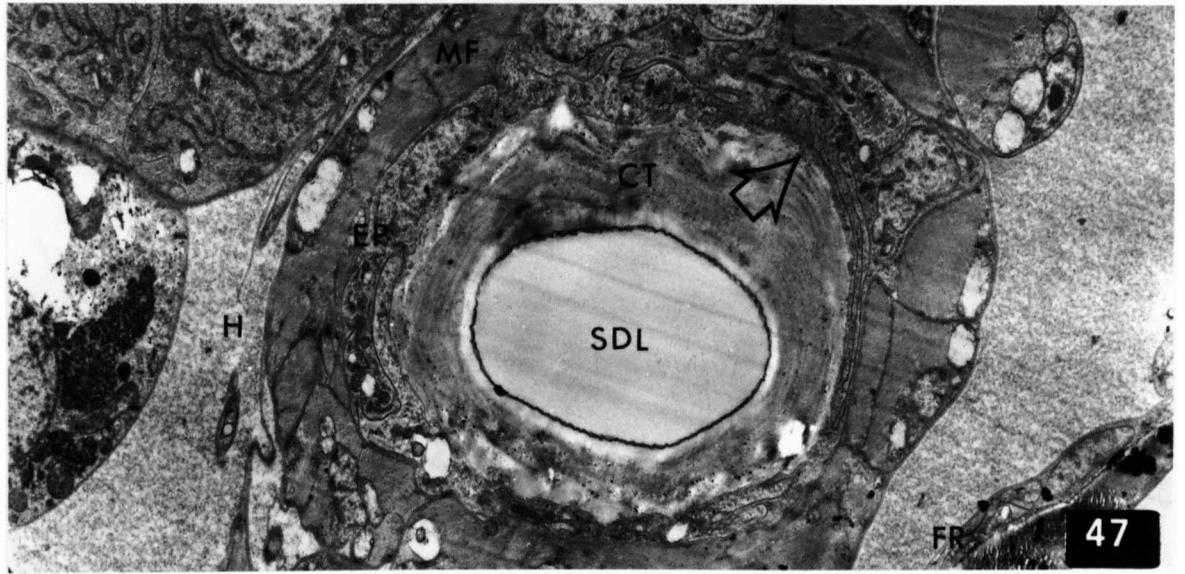
Figure, 46. TEM, Cross section of common duct (X 10, 000). The thin cuticle (CT) is mainly composed of fibrous procuticle with a thin epicuticle (open arrow) facing the lumen (LM). The epithelial layer (EP) is very narrow (twin headed arrow), with adjacent cells closely attached by desmosomal elements (arrow) leaving little point of contact between the overlying muscle fibres (MF) and the cuticle. Laterally, cell covering (thick arrow) is much reduced. The sarcomeres ( oppositely facing open triangles) of the muscle fibres are short and the myofibrils run through perforated Z-discs (Z).

Figure, 47. TEM, Cross section through an individual spermathecal duct (see also figure, 40) (X 6, 600). The procuticle (CT) is unevenly thick (open arrow). The underlying epithelial layer (EP) and muscle fibres (MF) are slightly different to that around the common duct opening. FR, furca; H, haemolymph; SDL, spermathecal duct lumen.

Figure, 48. TEM, Cross section of the spermatheca proper (X 20, 000). The thin epicuticle layer is formed into septa (STF) from the cuticle wall (CT) which appears to be scrambled. The epithelium and muscle fibres are alternate, quite different to the arrangement around the ducts and are attached by belt desmosomes (open triangles); the basal membrane (BM) of the epithelium is repeatedly infolded. A preterminal motor nerve fibre (MN) supplies the spermatheca; its axon (A) is capped by a Schwann cell layer (SN); and it has a relatively few straight mesoaxon folds (MSX); mi, mitochondria; NU, nucleus.



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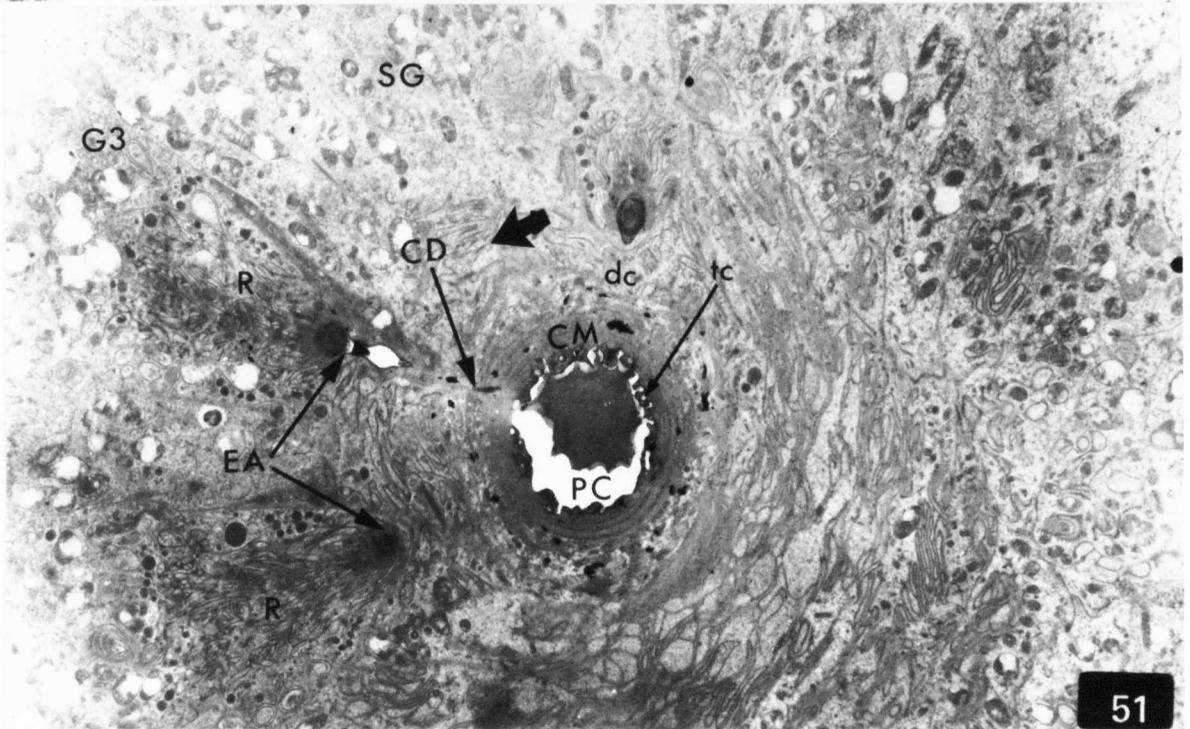
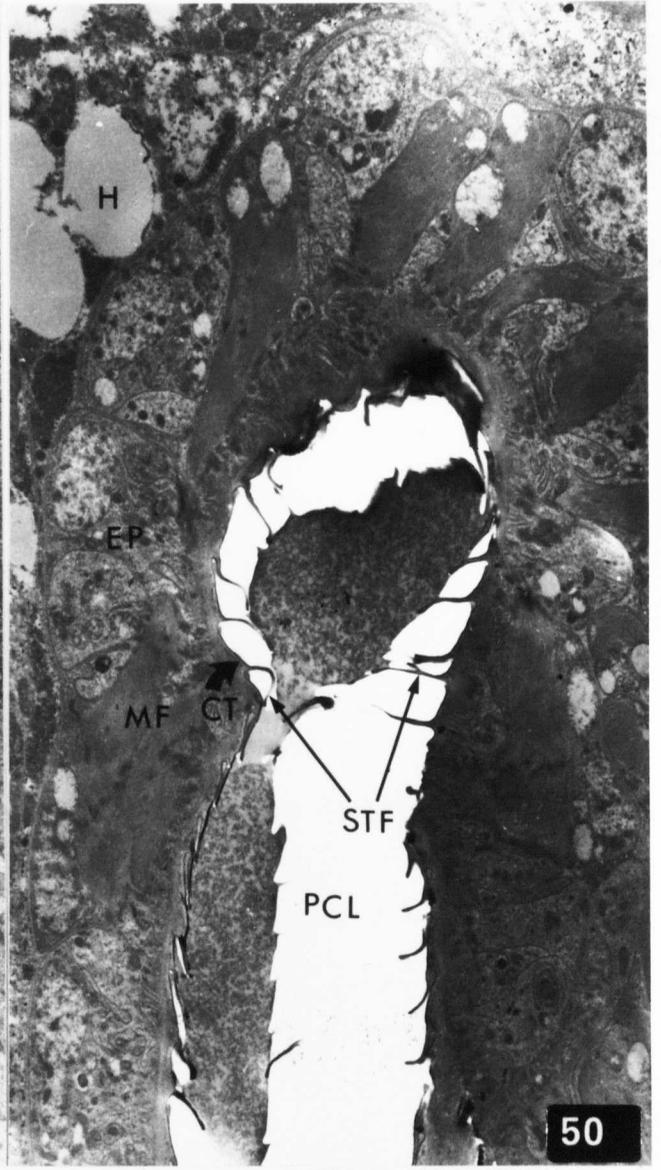
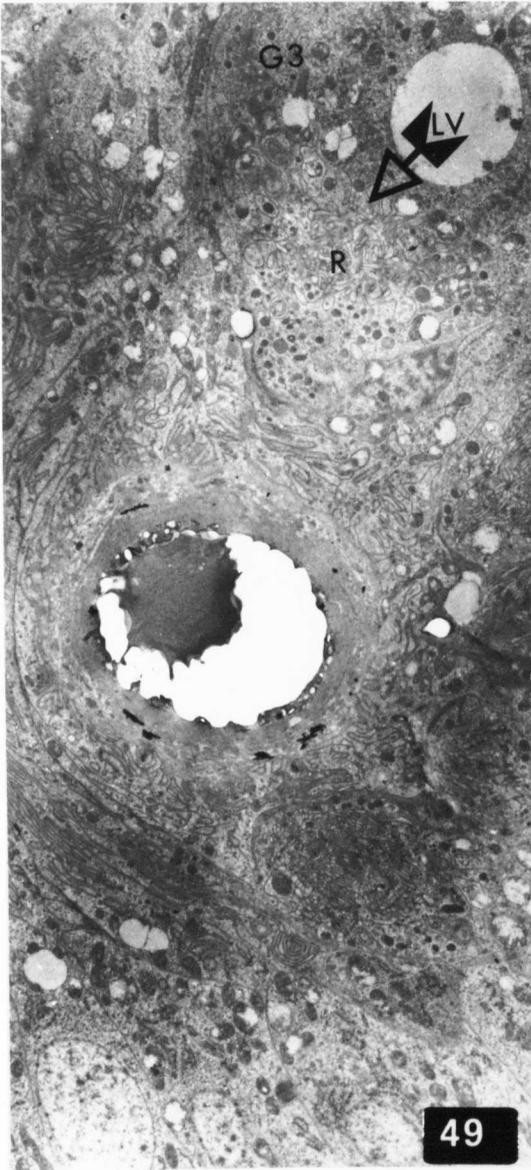


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Figure, 49. TEM, Longitudinal section of a gland cell (X 5, 200). A large electron-lucent vesicle (LV), probably derived from the endoplasmic reticulum, containing droplets of secretory material is being discharged at the base of microvilli through the process of exocytosis.

Figure, 50. TEM, Oblique section of the primary canal of the spermatheca (X 5, 200). The very thin layer of cuticle (CT), which is an extension of the spermatheca, gives rise to a series of finger-like projections or septal foldings (STF) into the lumen of the primary cavity (PCL). EP, epithelial layer; H, haemolyph; MF, muscle fibres.

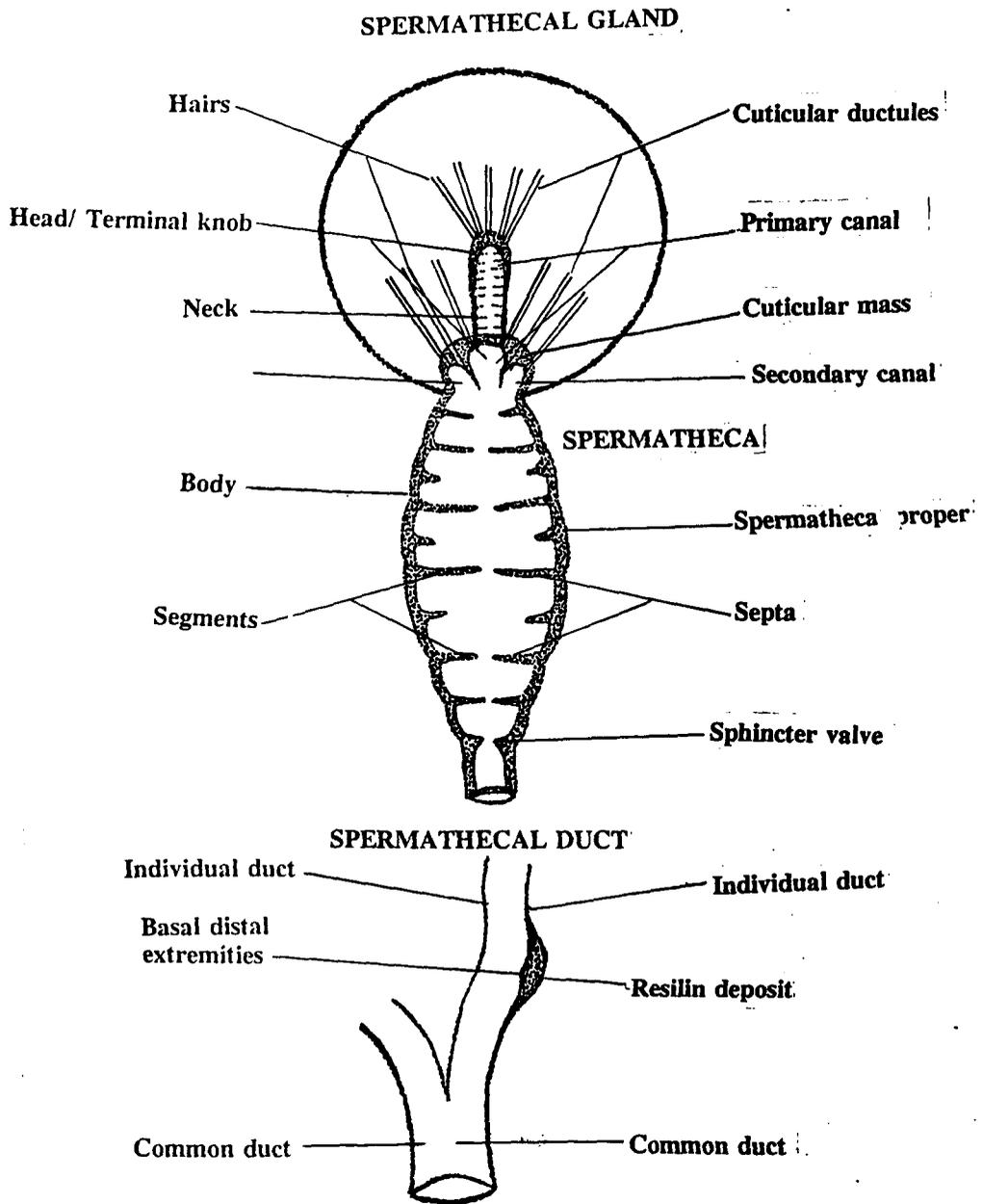
Figure, 51. TEM, Cross section of the spermathecal gland (X 5, 200). The organisation of the gland is similar to that in *P. papatasi*. The secretory glandular cells (G3) are arranged radially to the axis of the gland. The receiving canals (EA =receiving canal) from the reservoir (R) are connected by conducting canals (CD) running through duct cells (dc) and the cuticular mass (CM) into the lumen of the primary cavity (PC). Like *P. papatasi*, the apical region of cells (thick arrow) is deeply folded to give mechanical support to the gland.



Figure, 52. Diagrams showing the old and new terminologies in different parts of the spermathecal gland, spermatheca proper and spermathecal duct for phlebotomine sandflies.

**OLD TERMINOLOGY**

**NEW TERMINOLOGY**



## 2. 4. Discussion on spermathecal structure

From the ultrastructural study on the spermathecae of four Old World phlebotomine sandflies, conclusions can be drawn along three lines: 1. a comparison between species to deduce the homology of the spermathecal parts which results in the proposal of a new terminology; 2. a comparison with other insect spermathecae; 3. Physiological role and possible evolutionary function of different parts of spermathecae of the sandflies.

### 2. 4. 1. Comparison between species, homology and new terminology

In all the four species examined, the pattern of cuticular architecture varies and dominates the spermathecal duct and spermatheca proper. In the spermathecal ducts, the pattern of cuticle variation, especially the thickness of resilin deposit in the common duct, differs from one species to another. The thickness of the cuticle is relatively thinner in *Sergentomyia babu* and presumably in other species of *Sergentomyia* and closely related taxa such as *Sycorax*, some species of *Idiophlebotomus* and *Spelaeophlebotomus*. However, in *Phlebotomus argentipes* and *P. langeroni*, the cuticle is relatively thicker, and is more modified. For example, with the lateral extension in the common duct of *P. argentipes*, completely separated individual ducts of *P. papatasi* and the development of distal extremities at the base of the common spermathecal duct in *P. langeroni*.

Like the duct, the structural modification of cuticle in the spermatheca proper exhibits diversity. In *S. babu*, there is no folding of the cuticular wall but all the three species of *Phlebotomus* possess septal folding, which is usually described as "segmentation" in the taxonomic works based on light microscopy. The 'segmentation' is well defined in all the subgenera of *Phlebotomus* except *Adlerius* in which the segmentation is less obvious because of the increased number of relatively much smaller

interseptal foldings.

The apex of the spermatheca proper is developed less in *S. babu* than the three species of *Phlebotomus* studied. The cuticular mass presumably supports the cuticular ductules from the spermathecal gland and acts as a site of attachment for spermathecal gland cells. Thus in *S. babu* which has a few gland cells and it is not well developed. Even in the few species examined there is a positive relationship between the size of cuticular mass and the number of gland cells. Furthermore, the primary canal, which is derived from the cuticular ductules of gland cells, is short and compact in most of the subgenera of sandflies and is often laterally extended with secondary canals (eg., members of the subgenera *Phlebotomus* and *Paraphlebotomus*) but it is elongated into a tubular form in the members of *Adlerius* and *Larrousius*.

Such variations as basic pattern, for example: the small v s. large cuticle deposit in the spermathecal ducts, non-segmentation v s. segmentation and less developed cuticular mass with a few glandular cells v s. massively developed cuticular mass with a large glandular cells, are evident that these are homologous structures which have evolved from an early time.

Although the morphology of spermathecae has been used for nearly 70 years or so in the taxonomy of phlebotomine sandflies, the structural basis of the variation in cuticular structure was hardly known. The various parts of spermathecae described with light microscopy are defined entirely on their relative position and superficial appearance eg., head, neck, body, hairs, rather than any more solid basis. Hence a new terminology is proposed for different parts of spermathecae (Fig., 52) based on their structural relationship. Further comments on the homology of the different parts of spermathecae

and their counter parts in the male genitalia are dealt in chapter 6, "Comparative morphology and cladistic analysis of spermathecae of the Old World Phlebotomine sandflies".

### 2. 4. 2. Sandfly spermathecae as typical insect epidermal glands

Spermathecal ultrastructure has been studied in 8 insects from 4 orders: *Aedes aegypti* (Clements and Potter, 1967), *Glossina morsitans* (Odiambo, 1984), *Drosophila melanogaster* (Filosi and Perotti, 1975), *Periplaneta americana* (Gupta and Smith, 1969), *Tenebrio molitor* (Happ and Happ, 1970), *Sitophilus granularis* (Villavaso, 1975 a,b), and *Apis mellifera* (Dallai, 1975). In all cases the histology of the spermathecae is typical of insect epidermal glands, particularly in the ultrastructure of the procuticle. But the four species of sandflies studied here show a remarkable variation from the basic structure, not previously observed in this organ, especially the pattern of cuticle variation in the spermathecal duct and spermathecal body, the presence of supercontracting visceral muscle fibres and the provision of a neuromuscular junction. Variations in the spermathecae of the four species studied, and presumably in other phlebotomine sandfly species too, suggests that the structure of the spermathecal complex in each species is adapted to meet the particular needs of sperm storage and has evolved in relation to, perhaps, different mating systems.

The ultrastructural study of the muscle system supporting the duct and spermatheca revealed that it consists of supercontracting intrinsic visceral fibres. These fibres are characterised by their capacity for long, isotonic contractions around curved surfaces of visceral tubes and bags (Rice, 1969). There are two advantages in having supercontracting visceral muscles: 1, they are well adapted to accommodate the

asymmetric contractions which are bound to occur in muscles that operate around tubes; 2, they enable the visceral tubes and bags to become greatly distended. During mating, the resilin rich-cuticle of the spermathecal duct which would allow great distensibility, together with the ability to maintain adequate tension over long contractions are important factors in accommodating the non-flexible, aedeagal filaments of sandflies.

The existence of both a myoneural junction and neurosecretory endings has been reported in the spermathecae of cockroaches (Gupta and Smith, 1969) and granary weevils (Tombes, 1976). Such a neuromuscular system has also been found in the present study in *P. papatasi*, *P. argentipes* and *P. langeroni*. The presence of neuromuscular junctions is most likely to be important in promoting the secretion of material from the glandular cells immediately after bloodfeeding, which would in turn encourage the sperm to migrate into the spermathecal body.

Details of the ultrastructure of the neurosecretory endings shows differences between *P. papatasi* and *P. langeroni* in the presence or absence of Schwann cells; they are absent in *P. papatasi* and present in *P. langeroni*. According to Osborne (1975), the absence of Schwann cells (i.e. naked terminal) in the insect neuromuscular junction is an adaptation for high sodium ion content in haemolymph so that action potentials are conducted in abnormal ionic environments. Whilst this difference might be seen in insects with different diets (blood versus plant material) it is very difficult to see why two relatively similar insects with approximately the same diet should have anatomical differences.

### 2. 4. 3. Comparison of sandfly spermathecae with other insects

Sandfly spermathecae differ from other insect spermathecae in that they have

considerable modification of the spermathecal ducts and compartmentalisation of the spermathecal body and supercontracting visceral muscle systems which make phlebotomine sandfly spermathecae unique.

In non-compartmentalised spermatheca there would be ample opportunity for sperm to mix, if these sperm came from different males there could be sperm precedence. But even if they came from one male there would be competition from sperm. The compartmentalised spermathecae on the other hand would minimise the mixing of sperm and hence competition between them. If the sperm entered the 'segments' in the order they arrived in the spermatheca and remained curled up in the lumen there would be relatively little opportunity for competition between them.

From the ultrastructural evidence presented, the cuticular mass at the apex of the spermatheca appears to have a dual role:

(a) it serves as a cuticular apodeme for the visceral muscle fibres running along the axis of the spermatheca and (b) it provides support for the cuticular ductules of <sup>the</sup> glandular cells. Thus the spermathecae could change in volume depending upon the requirements of the individual species for the reception and storage <sup>of</sup> spermatozoa.

The presence of pore canals in the cuticular mass in *P. papatasi*, is an exceptional feature of the rubber-like cuticle and that could have served as direct connections between the gland and lumen of the spermatheca in an early stages of the fly but <sup>here</sup> retained as non-functional as the fly attained the pharate stage. The presence of primary and secondary canals at the base of the cuticular mass, perhaps not only increase the volume of the spermatheca but also form the collection part for the cuticular ductules and hence any secretion that comes from them. Interestingly enough, there is a relationship, perhaps

coincidence in the number of these canals and the form of the spermathecal duct in *Phlebotomus*: Primary and secondary canals are present in the spermatheca where long bifurcated spermathecal ducts occur and primary canal alone are present in those species with <sup>a</sup>common spermathecal duct.

The structure of epithelial cells in the spermathecal duct and spermatheca proper shows exceptional diversity among insect epidermal glands. Their location and morphology on the duct and spermatheca coincide with the pattern of cuticular variation which is perhaps designed to meet different needs for sperm nourishment and storage. Since the epithelial cells are embryologically of epidermal origin they are responsible for the formation and maintenance of the cuticle and its derivatives. In *S. babu*, the apical plasma membrane of epithelial cells attached to the cuticle of the spermatheca proper is simple and superficial but supported strongly by the multiple layers of the visceral muscular system. In *P. argentipes*, there is extensive apical membrane folding with a two-tier system of epithelial cells around the opening of the common duct. The epithelial cells are alternately arranged with the muscular system around the duct of *P. papatasi*. And finally, in the duct of *P. langeroni* there is a relatively thin, compact epithelial lining without any space between the cells and covered by a thick layer of visceral musculature.

The structural complexity of the spermathecal gland is much more elaborate than the simpler organisation of epithelial cells and indicates quite clearly that the cells forming this gland must be producing secretory products. The histology of the terminal cells with reservoir and duct cells resemble that in other insect spermathecae, but there is an obvious difference in the spatial distribution of terminal cells among the species studied. In *S. babu*, there <sup>are</sup> ~~a~~ relatively few cells than in the *Phlebotomus* studied. Whether

this means that fewer sperm are stored or that sperm are kept for a shorter period is unknown (assuming that all cells are equally productive in any secretion)

In the glandular cells, the differentiation of the plasma membrane includes deep folding (microvilli), apical apodemes, continuous belt desmosomes, septate junctions together with the tonofibrils and the extensive distribution of microtubules. These cellular differentiations and their inclusions are general properties of mechanical significance for insect epidermal glands but in the spermathecae of phlebotomine sandflies, besides their mechanical role, they could be contributory factors to the extraordinary structural variation of the spermathecal gland cells.

The gland cells are separated from the haemolymph by basal lamellae of variable thickness. The plasma membrane underneath <sup>the</sup> basal lamellae is not differentiated in *P. papatasi* and *P. langeroni*, but in *P. argentipes* it is invaginated to form extracellular channels, as in the vertebrate kidney, for facilitating the exchange and reabsorption of ions and other metabolites.

To summarise, the histological variation of the spermathecal complex in phlebotomine sandflies affords a mechanism where competition between sperm can take place to the advantage of the female.

### **3. Histological changes in the spermathecae of *Phlebotomus papatasi* during the gonotrophic cycle**

#### **3. 1. INTRODUCTION**

In insect reproduction, the function of the spermathecae, the sperm storage organs, is to receive the sperm from the male for later use in the fertilization of eggs. During the process of reception and storage of sperm, histological changes take place which subsequently lead to sperm utilization. Earlier works focused on these histological changes of the spermathecae, including ultrastructure and histochemical studies, in order to develop the sterile-insect technique for controlling pests of medical, veterinary and agricultural importance. However, recently the structure and shape of the spermathecae have been of special interest in comparative biology, with reference to sperm transfer and utilization in the evolution of insect mating systems.

Ultrastructural and histochemical studies of insect spermathecae provided evidence that glandular secretory cells, a constituent of the spermathecae, produce glycoproteins or mucopolysaccharides (Happ and Happ, 1970). A chemotactic substance such as the mucopolysaccharide secreted by the spermathecal gland, has been shown to be responsible for the transport of sperm from the bursa copulatrix to the spermatheca (Grodener and Steffens, 1978). The motility and fertilising capacity of spermatozoa are affected by very small amounts of the secretion from the spermathecal gland. The

importance of these secretions is shown by the fact that excision of the external musculature of the spermatheca does not reduce sperm movement to the structure. It does however prevent sperm movement from the organ and reduces sperm displacement from 66% to 22 % in the boll weevil, *Anthonomus grandis* (Villavaso, 1975). When studying sperm movement in response to the spermathecal gland secretion, it is important to understand how sperm are deposited in the female and the morphological changes that take place in the sperm before reaching the bursa copulatrix. The answer to that question differs for different insects (Davey, 1985).

Many insects deposit their sperm in a spermatophore within the bursa copulatrix. In Diptera, spermatophores are known to occur in five families- four Nematoceran viz. *Culicoides* spp, Ceratopogonidae (Linley, 1981), *Glytostendipes paripes*, Chironomidae (Nielsen, 1959), *Simulium* spp, Simuliidae (Wenk, 1965) and *Plecia neartica*, Bibionidae (Lepla, et al., 1975) and one higher Dipteran *Glossina austeni*, Glossinidae (Pollock, 1970). In anopheline mosquitoes, the "mating plug" regarded as a vestige of the dipteran spermatophore is in the common oviduct of newly-mated females (Giglioli and Mason, 1966), but Davey (1985) disputes the terminology used and considered it was actually a spermatophore as found in other nematoceros Diptera. Maroli et al. (1991) claimed to have found the formation of a mating plug in phlebotomine sandflies.

Dallai *et al* (1984) studied the sperm ultrastructure, which include the presence of <sup>1K2</sup>perforatorium, a double layered acrosome, in three species of phlebotomine sandflies, and suggested that phlebotomine sandflies are not only one of the most primitive nematoceran families but also rank it as a distinct family.

From the study of sperm transfer in *Culicoides melleus*, Linley (1980) observed

that the transfer was effected by two complementary processes; an influx of fluid by osmosis into the spermatophores and simultaneous withdrawal of fluid from the spermatheca. During the process of transformation and storage in insect spermathecae, the sperm undergo considerable changes, including the loss of the acrosomal membrane (Degrugillar and Leopold, 1976) detachment of the glycocalyx from the plasma membrane and by hyperactivation ( Selmi, 1992).

### 3. 1. 2. Spermathecae and sperm competition

In the recent past, no part of the insect genitalia has undergone such intensive investigation in comparative biology as the shape and function of the spermathecae in relation to the evolution of insect mating systems. The provision of spermatheca is a consequence of internal fertilization and it could have evolved under the different selective pressures on females. Parker (1970) coined the term "sperm competition" for the competition between the sperm from two or more males for the fertilization of the ova within a single female. It is important to note that sperm competition can only occur if the female mates again before her initial supply is exhausted (Pruzan-Hotchkiss et al., 1981). Sperm precedence (i.e. non-random sperm use) and sperm displacement (i.e. the replacement at a subsequent mating of the stored sperm of previous matings) are two important components of sperm competition (Gromko and Pyle, 1978). Dybas and Dybas (1981) studied the sperm and spermatheca in featherwing beetles (*Bambra* spp.) as a species-specific character and suggested it was a lock-and-key mechanism for species isolation and evolution, but they did not find sites in the spermathecae corresponding to the screw-thread structures of sperm. Parker and Simmons (1991) proposed a model for sperm displacement in which each unit of seminal fluid transferred to the sperm stores

displaces one unit from the stores, and that the fluid displaced is a random sample of that stored. However they concluded that the anatomical mechanism underlying sperm displacement is not yet understood. Furthermore in his analytical paper on the incidence of sperm displacement in insects, Ridley (1989) hypothesised that species with spheroid spermathecae should show low displacement and species with elongate spermathecae high displacement.

The objectives of this chapter therefore are to investigate *P. papatasi* and *P. argentipes* comparatively to determine:

1. The histological changes taking place in the spermathecae during the gonotrophic cycle of the fly;
2. How sperm are implanted, whether spermatophores are produced, and whether there is any anatomical evidence for sperm competition or displacement in sandflies;
3. How the sperm are transported into the spermatheca and subsequently utilised in oviposition;
4. If there <sup>are</sup> any relationships between the shape and volume of sperm and spermatheca.

### 3. 2. MATERIALS AND METHODS

Throughout the next series of experiments laboratory reared *P. papatasi*, from a colony originating in Israel were used. Flies were maintained at 26°C and approximately 75% RH at the London School of Hygiene and Tropical Medicine.

#### 3. 2. 1. Experimental design for histological changes

The age and condition of the flies examined at different periods are appended in Table 1. To determine if there were any changes in the spermathecal

complex throughout the gonotrophic cycle flies were kept under different conditions and examined at different times. The main factors tested were female access to males and blood feeding. Control flies were females kept apart from males and fed on 5% sugar solution only. To ensure that females and males were virgin, newly emerged adults were separated within 12 hours of emergence i.e. before the male genitalia rotate.

Unless stated otherwise, the methods for light and electron microscopy are as given in chapter 3. A minimum of 10-15 flies were sectioned and 20-30 grids usually examined from each group. In the case of studies on the secretory nature of each gland, spermatophore and sperm<sup>from</sup> more than 10 blocks were sectioned and 50 grids examined from each group.

For comparison, some samples of *P. argentipes* were used: They were caught from peridomestic areas near Madras, South India; because the flies were wild-caught the exact age is unknown. They were collected from cattle while *in copula*, and the abdomen was filled with a fresh blood meal and the age, therefore, would be at least 5-7 days old; at the temperature and RH were 27°C and 80% respectively late<sup>at</sup> night; and they were fixed them in a specially prepared field fixative to withstand the changing temperature in transportation. The results obtained from the ultrastructure include spermatophore, sperm and physiological condition of spermatheca.

### 3. 2. 2. Test for Alcian blue-PAS for Acid and Neutral Mucin

To determine whether mucopolysaccharides<sup>a</sup> were present in secretions from the spermathecal gland, the Alcian blue-PAS test for acid and neutral mucin was used. The four terminal abdominal segments were fixed in formal-saline solution, dehydrated in eth<sup>a</sup>anol and embedded in paraffin wax. Transverse sections were cut 1 μm thick, dewaxed,

and stained with Alcian blue solution (Alcian blue 1gm, 3% acetic acid 100 ml; Schiff's reagent BDH, (Bancroft and Stevens, 1982) for 5 minutes, washed in water and then in 1% aqueous Periodic acid (5 minutes), rinsed in water (5-10 minutes), stained lightly with Harris Haematoxylin solution, washed in water, dehydrated and mounted in DPX.

### 3. 2. 3. Transfer of spermatophore and sperm

To determine when sperm were transferred, in addition to the histological studies, flies were observed mating (*in copula*) at different times in the gonotrophic cycle and carefully removed from the large cages and allowed to complete mating and then dissected to see whether sperm transfer had actually taken place. The structure and movement of spermatophores and sperm were observed and photographed with a Polyvar phase-contrast microscope at X1000.

### 3. 2. 4. Oviposition, fecundity and sperm utilization

In order to see the movement and behaviour of sperm at oviposition, inseminated, bloodfed flies were kept individually in a container with no facility for egg-laying. After 24 hours, individual flies were fixed carefully by the thorax on a clean glass slide with glue. In order to persuade the flies to lay eggs, the tip of the posterior terminal segment was grasped gently with the terminalia from this group of male flies. When the fly started egg-laying, it was dissected carefully in saline solution and the movement of sperm was observed in the spermatheca and ducts.

In a separate experiment to determine if all the sperm are used at oviposition, bloodfed flies were kept individually in a small tube (ca. 1cmx4cm), covered with muslin cloth and a sugar meal on top of it and the bottom of the tube moisten<sup>ed</sup>(with tissue paper for egg- laying. The major problem with laboratory reared sandflies is that very few

(hardly<sup>any</sup> survive oviposition. Of the 46 flies kept in this way, 24 laid eggs and a lone fly survived oviposition with 27 eggs. The fly was dissected very carefully<sup>and</sup> the contents of the spermatheca and ducts examined.

The length of sperm was measured by light microscopy and <sup>the</sup> diameter by TEM. The volume of the spermatheca proper and spermathecal duct was calculated from both light and Transmission Electron Microscopy.

### 3. 3. RESULTS

#### 3. 3. 1. Immediately post-emergence (Table. I)

In newly emerged flies, the lumen of the spermatheca proper is filled with a filamentous-like mass (Figs., 53, & 56) which appears<sup>a</sup> as a pile of stalks. The reservoirs and cuticular ductules of the gland are also found with these substances (Fig., 54). The presence of such substances in the lumen of the spermatheca and reservoir and cuticular ductules of the gland coincides with the cytological condition of the glandular cells inferring<sup>r</sup> that these substances are secreted by the glands. It was confirmed from transmission electron microscopy that the presence of well developed RER and abundance of free ribosomes and Golgi bodies throughout the cytoplasm and membrane-bound vesicles presumably moving towards the reservoir strongly suggests an active synthetic phase of the gland (Fig., 55). However, the shape of <sup>the</sup> reservoir and the microvilli abutting the receiving canal are intact. Happ and Happ (1970) reported that in the spermathecae of the mealworm beetle, *Tenebrio molitor* amino acids/ protein produced from the RER in the gland cells moves to the Golgi bodies where carbohydrate is added<sup>that</sup> and then they are packed as secretory droplets for extrusion. A similar mechanism could also take place in

the spermathecae of *P. papatasi*.

The secretory substances (Fig., 54) in the spermatheca, and in the reservoir and cuticular ductules of the gland are strongly PAS positive, which indicates that this glandular product is an Acid mucopolysaccharide. Furthermore, the long fibrous nature of <sup>the</sup> secretory material <sub>λ</sub> shows it is presumably insoluble and <sup>of</sup> a fairly low molecular weight.

### 3. 3. 2. Pre-copulation

As the fly becomes older, ca. 12 hours after emergence, further changes are seen in the spermathecal gland and in the lumen of spermatheca and ducts. In the first instance, the discharge of the secretory mass from the spermatheca is essential; if not, this would cause either mechanical obstruction to the upcoming sperm or prevent the sperm from lodging within the spermatheca during the process of transfer. In the case of ca. 12 hour-old sugarfed flies, the secretory substance tends to diffuse and is often seen helically coiled, or loose in the spermathecal duct ( Fig. 60).

Females <sup>of</sup> 12 to 24 hours, fed only on sugar and with access to males were found *in copula* which lasted for about five minutes. These paired flies were removed very carefully from population cages while still *in copula* and the spermathecal ducts found to contain spermatophores (Fig. 58). The ultrastructure of spermatophores and spermatozoon are dealt with below.

### 3. 3. 3. Post-copulation changes

#### 3. 3. 3. 1. The fine structure and histological changes of spermatophore

The spermatophore of *P. papatasi*.

Immediately after copulation in one day-old sugarfed flies <sup>with</sup> access to males, a single spermatophore was found in each spermathecal duct (Fig., 58). Each

spermatophore is a cylindrical structure 44  $\mu\text{m}$  long, completely filling the diameter of the spermathecal duct and occupying about one third the length of the duct. It contains an electron-dense fibrous material loosely packed around the sperm which are intact. At this stage, the spermatozoa in the spermatophores are inactive. Furthermore the placement of the spermatophores in both ducts is not identical in any of the flies examined; and is usually basally positioned.

Up to three spermatophores were seen in a single spermathecal duct (Fig., 59), lying one above the other with a considerable gap between them. The spermatophores remained intact until the flies had a blood meal.

There were drastic changes in the behaviour of implanted spermatophores in the spermathecal ducts within 20-30 hours of the female bloodfeeding. The spermatophores were seen breaking down and the spermatozoa were moving towards the spermatheca leaving behind the remnants of materials in the spermatophore which were descending towards the bursa copulatrix.

The spermatophore of *P. argentipes*. (Figs., 63 & 64).

At the base of the common duct, there is evidence that three spermatophores containing sperms were present and <sup>that</sup> they were alongside one another. Unlike the spermatophore of *P. papatasi*, the ultrastructure of the spermatophore in *P. argentipes* is well defined. Each unit has a double membranous layer, the inner layer is an electron-dense thin layer and the outer an electron-lucent thick layer.

### 4. 3. 3. 2. The fine structure of spermatozoa

The spermatozoa of *P. papatasi*.

The spermatids in the newly implanted spermatophore are clustered together and

it<sup>is</sup> hard to see them in their entirety by light microscopy. However, the liberated spermatid from the spermatophore, as found in the spermathecal ducts of a bloodfed fly, are relatively shorter than the fully developed, mature spermatozoan. The fully matured sperm, at the time of fertilization is 17  $\mu\text{m}$  long (Figs., 71: A, B & C) and 0.7  $\mu\text{m}$  wide (Fig., 62). Dallai *et al*, (1984) described the ultrastructure of spermatozoa from both the spermatheca and vas deferens, however, mostly from longitudinal sections. Detailed examination of the spermatozoan shows it has: a head with an elongated nucleus capped by an acrosomal complex, the acrosome has a flattened, concave cisternum limited by parallel membranes and homogeneous, electron-dense contents; the concavity of the acrosome has a plug of fine, translucent material with granules and filaments, collectively known as the perforatorium. Furthermore, in their figures, Dallai *et al*, (op. cit.) overlooked an important difference between the sperm in the vas deferens and sperm in the spermathecae:- the sperm has a acrosomal outer membrane in the vas deferens but this is lost in the spermatheca. Cross sections of the post-nuclear region (Fig., 62) show the axoneme with a "9+9+0" pattern; the doublets have both dynein arms and the 9 peripheral singlets lack intertubular (coarse fibre) material; the axoneme is supported by a single conspicuous mitochondrial derivative, rich in longitudinally arranged cristae and containing, on the side close to the axoneme, a 20 nm thick longitudinal crystalline axis. The spermatozoan of *P. argentipes*.

The spermatozoan is much smaller than the spermatozoan of *P. papatasi* being ca. 15  $\mu\text{m}$  long and 0.5  $\mu\text{m}$  in wide (Figs., 66, 67 & 68). Because there are several differences between the sperm of *P. argentipes* and those of other species, the ultrastructure of the *P. argentipes* sperm is described in detail.

**Head region (Figs. 67 & 68):** The head is occupied by a solid, electron dense cylindrical nucleus 2.25  $\mu\text{m}$  long and capped by a multilayered acrosomal complex. The acrosome, 0.2  $\mu\text{m}$  wide and 0.25 in  $\mu\text{m}$  depth, has concave, multilayered cisternae containing electron-lucent substances. The concavity of the acrosome is filled by a conical structure /or projecting from the prenuclear area of the fine granular body, some 0.15  $\mu\text{m}$  long, known as the axial rod or perforatorium (Jameison, 1987). The nucleus is indented posterolaterally to house the anterior ends of the mitochondrial derivatives and posteriorly to receive the flagellum. The derivatives and axoneme are not in line with the central axis of the nucleus. The spermatozoan is limited by a well defined undulating plasma membrane from head to tail.

**Post-nuclear region (tail) (Figs. 63, 64 & 66):** Transverse and cross sections of the postnuclear region revealed that the axoneme is of the classical "9+9+1" type, in contrast to the known spermatozoan of phlebotomine sandflies in which the pattern is "9+9+0". The peripheral singlets and the nine doublets are not parallel to the longitudinal axis of the axoneme but are slightly angled. Furthermore, instead of 2 central singlets, there is a single double-layered, translucent ring-like structure forming the central elements. The inner subtubules (B) are united by rays (Afzelius rays) with the central elements. The intramitochondrial material shows incomplete crystallization. The centriole adjunct surrounds, asymmetrically, the bases of the mitochondrial derivatives and the axial filament complex where all of these structures attach to the posterior end of the nucleus. The function of the centriole adjunct, according to Jamieson (1987), is a head-tail attachment for the upper ends of the mitochondrial derivatives, needed for cushioning the robust flagellar movements. In addition to the mitochondrial derivatives,

the axoneme is flanked by a single accessory body, (*sensu* Jamesion, 1987) which is an extension of the centriole adjunct from which it may or may not become separated. This tube is primarily proteinaceous with a carbohydrate core rich in ATPase (presumably indicating a role in motility).

As in *P. papatasi*, in which the cast off acrosomes were found in the base of spermathecal duct, the horse-shoe shaped externum- the outer laminated part of acrosomes and an unidentifiable electron-dense large mass were found in the base of the spermathecal duct in *P. argentipes*.

### 3. 3. 4. Changes in post-blood feeding

In those flies which had access to males (inseminated) and a subsequent blood meal, there were significant changes in the histology of the sperm, spermathecal gland, the lumen of the spermatheca and in ducts.

#### 3. 3. 4. 1. Changes in glandular cells and lumen

To differentiate between the changes caused by blood feeding rather than simply ageing , control flies of the same age but given access only to sugar were examined. The first group were 7 days old fed only on sugar-water; the second were also 7 days old but had had a blood-meal 24 hours previously. Neither group had access to males. In the first control group (sugar water only) the cuticular ductules, lumen of the spermatheca and ducts were filled with a very fine, fibre-like secretory residue (Fig. 73) in contrast to long filamentous secretory material found in the experimental groups. In the second control group blood-fed similar residues were seen as in blood-fed flies with access to males (as in the Fig. 72).

In blood-fed flies with access to males, the reservoirs in the glandular cells

became filled and distended laterally with a very electron-dense material ( Fig., 69), different to that seen in newly emerged and 3-days old flies. The apical area around the reservoir of the gland cells is dotted with dark vesicles. The lumen of the spermatheca and ducts were found with a well defined outer light zone and dark (fluid) zone (Fig.72).

### 3. 3. 4. 2. Entry and arrangement of sperm within the spermatheca

The motility of spermatozoan of *P. papatasi*.

The spermatozoa found in the spermatophore immediately after insemination in the sugarfed fly are inert but, as mentioned earlier, in flies 5 days old and 20-30 hours post bloodfeeding (Fig., 59), the spermatozoa were seen leaving the spermatophores and moving with their characteristic serpentine lashing movements towards the spermatheca. During the process of transformation in the spermathecal ducts, the sperm shed the acrosomal membranes (Figs. 57 & 61) which are found among the remains of the spermatophore at <sup>the</sup> base of the spermathecal duct.

Having undergone considerable morphological changes in the spermathecal ducts, the spermatozoa were not allowed to freely enter into the spermatheca. Instead, they were regulated by the sphincter valve at the base of the spermatheca. After entering into the lumen of the spermatheca, the sperm were found occupying the interseptal areas and at the centre of the lumen. There are a few but sharp differences between the two species

*P. papatasi* and *P. argentipes*, such as the number, distribution and placement of spermatozoa. Both species were blood fed and the spermatheca had spermatozoa suggesting that they are perhaps the same age groups. *P. papatasi*, there are 5-9 in the interseptal 'segments', and their distribution is totally unorganised (Table, II & Fig., 70).

There are 5-7 sperm able to move in the lumen of <sup>the</sup> duct. In *P. argentipes* ( Fig., 65), in

which the spermatozoa are more organised with the head facing towards the interior of the lumen and the tail approaching the wall of the spermatheca.

### 3. 3. 5. Fertilisation and oviposition

When flies 72 hours post-blood feeding were induced to oviposit, individual sperm were seen passing down the spermathecal ducts (Figs. 71; A, B & C). Spermulation (the process of releasing mature spermatozoa from the spermatheca) was presumably regulated by the sphincter valves at the base of the spermatheca. Spermatozoa were not released simultaneously from both the spermathecae; rather, this process took place alternately. When a released spermatozoan from one spermatheca was about to reach the base of the spermathecal duct (near the bursa copulatrix), the next spermatozoan was released from the other spermatheca. The spermatozoa seen in the ducts were fully matured and much longer than the sperm previously observed ascending the spermathecal ducts. Sperm moved with characteristic lashing movements at the same speed until they reached the egg micropyle.

### 3. 3. 6. Post-ovipositional changes

Post-ovipositional change is a crucial phase, as far as sperm storage is concerned, in the reproductive life of *P. papatasi*. When the spermatheca of the lone fly surviving oviposition was examined very carefully under high power, no spermatozoa were seen in the spermatheca or spermathecal ducts. Instead, the lumen was filled with a secretory mass, although the latter was not solid and compact and very similar to that in the virgin fly. From this very slim evidence it appears that sperm storage in the spermatheca of *P. papatasi* is "gonotrophically concordant" and that sperm replenishment occurs when the next reproductive phase of the insect begins. A similar finding was

also reported by Yuval and Schlein (1986).

### 3. 3. 7. Spermatheca and sperm utilisation

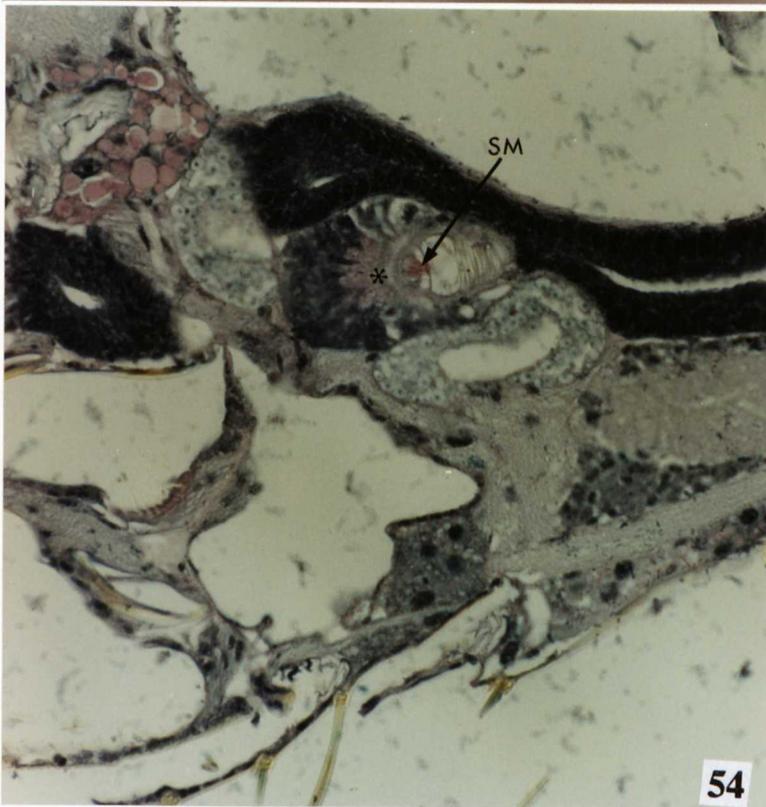
As an alternative to direct observation of the spermathecae from flies surviving oviposition in order to find out whether females need to mate more than once each gonotrophic cycle, a volumetric approach was undertaken. The volume of the spermathecal lumen was calculated and the maximum number of sperm able to fill it estimated. Table II gives data for these estimates. The estimates of spermathecal volume and the spermatozoan strongly suggests that *P. papatasi* is polyandrous.

Table, I. Histological changes in the spermathecae during the gonotrophic cycle of *Phlebotomus papatasi*

After emergence of	Condition of flies	No. of flies sectioned/ examined	No. of flies positive for * = Secretory mass, + = Spermatophores, != sperms, @ = Secretory residue		
			Spermatheca	Spermathecal duct	No. of flies not scored (= @)
< 12 Hours	Sugar fed no access to ♂♂	15	9*	--	6
24 Hours	Sugar fed access to ♂♂	14	5*	1+	9
48 Hours	Sugar fed access to ♂♂	10	5*	1+	5
72 Hours	Sugar fed access to ♂♂	10	3*	2+	7
5 Days	4 hrs post bloodfed access to ♂♂	16	4*	3+	12
5 Days	24 hrs post bloodfed access to ♂♂	18	3*, 1! (upward movement)	6*, 12+, 6! at oviposition	6
7 Days	72 hrs post bloodfed access to ♂♂	15	1!(downward movement)	As in the spermatheca	14
7 Days	sugarfed no access to ♂♂	10	In all, residues of * found	As in the spermatheca	--
7 Days	24 hrs post bloodfed no access to ♂♂	10			
30 Days	24 days postblood fed at oviposition	46	1* found, as in the newly emerged/virgin	As in the spermatheca	24/46 laid eggs & one survived oviposition

Fig., 53. *Phlebotomus papatasi*. A saline squash specimen of a newly emerged fly showing the spermatheca in which the lumen is filled with the secretory mass (SM) (X 1, 800).

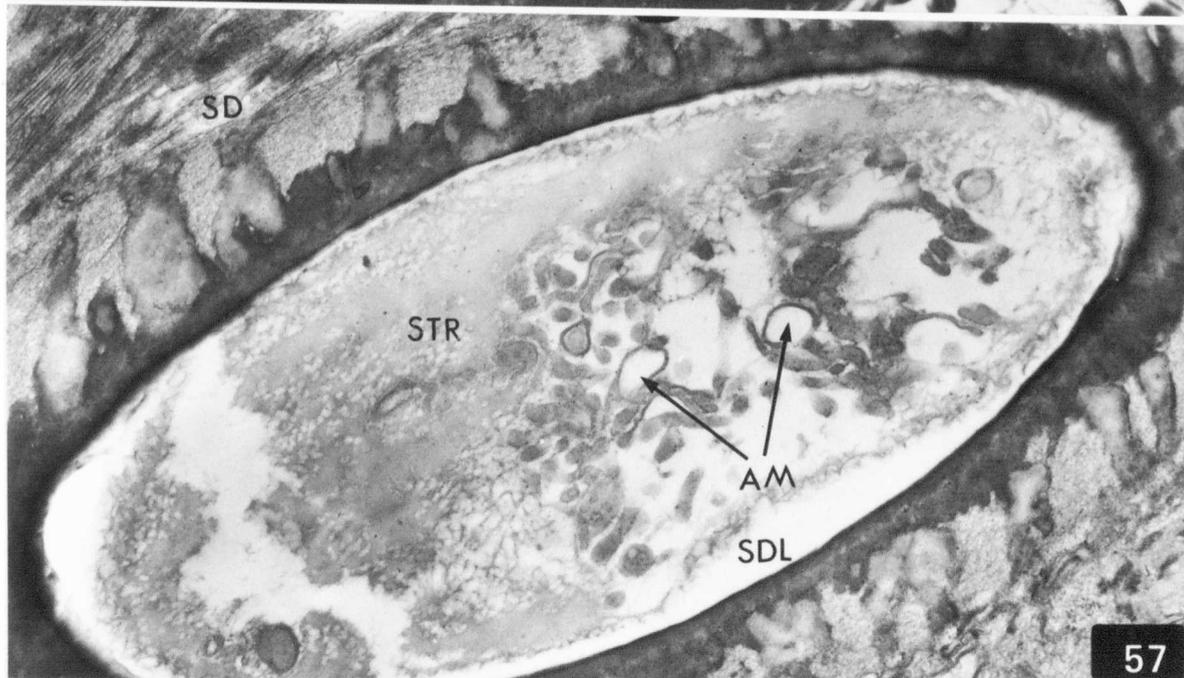
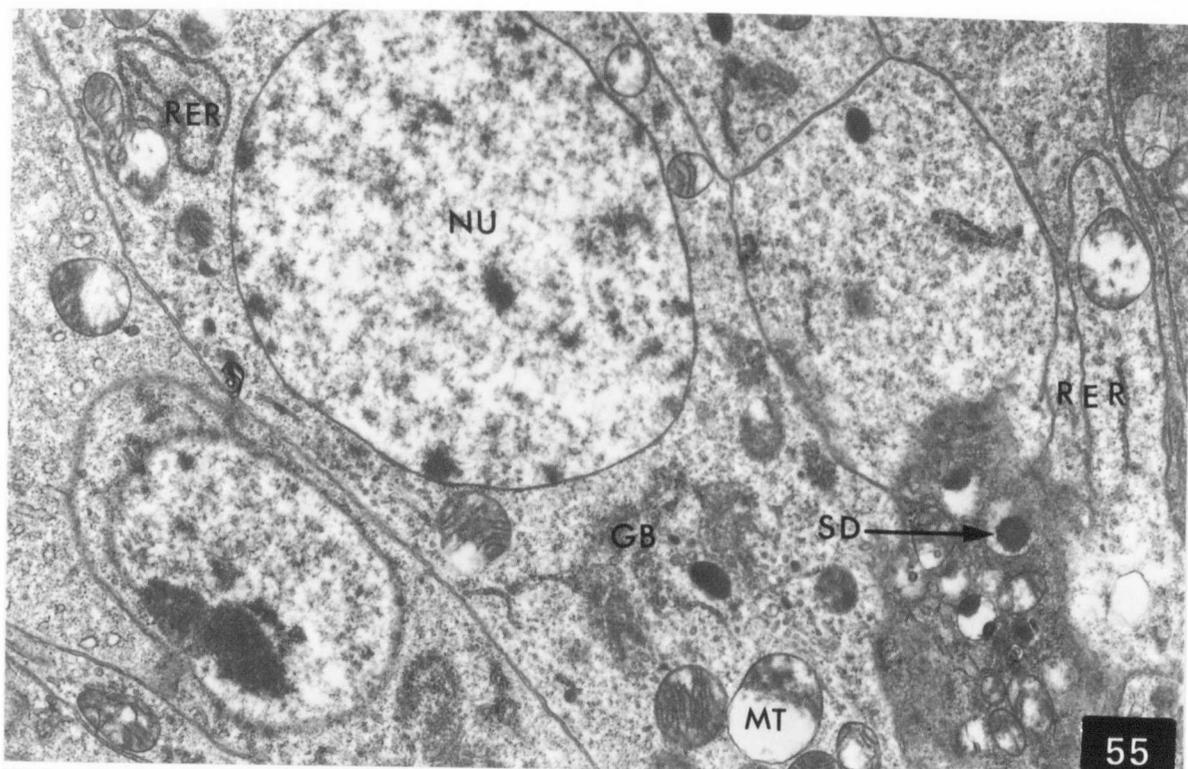
Fig., 54. *Phlebotomus papatasi*. Transverse section of the spermatheca showing positive staining of the secretory material in the reservoir and cuticular ductule of spermathecal gland cells (asterisk) and the lumen of spermatheca proper of a newly emerged fly indicating the mucopolysacchride nature of the secretion. Cytoplasm of the gland cells (open star) shows the production of amino acid/ protein exclusively with Alcian blue (X 750).



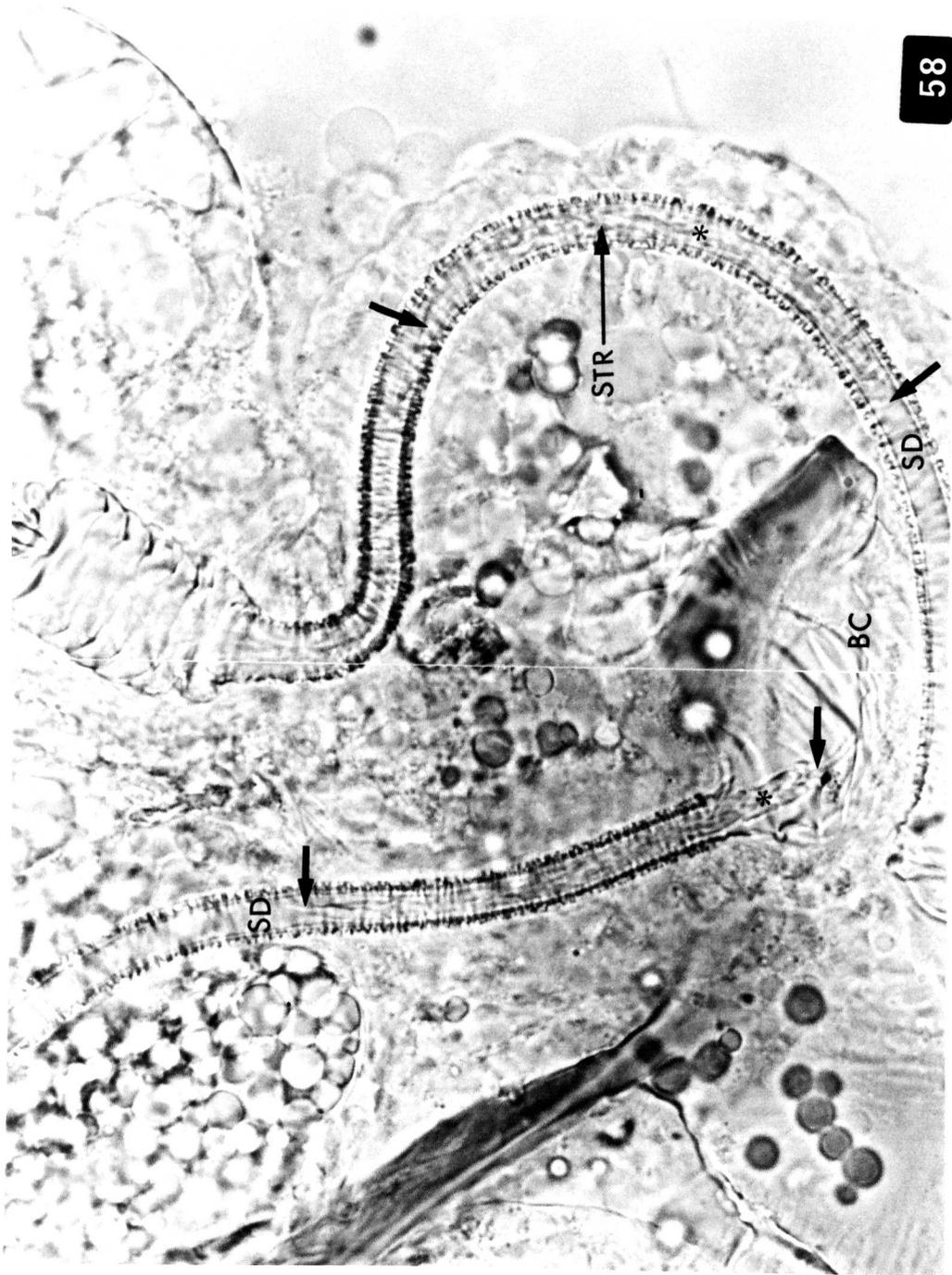
Figure, 55. *Phlebotomus papatasi*. TEM, Longitudinal section of the spermathecal gland from a newly emerged fly. Electron-dense secretory drops (SD) synthesised from by the Rough Endoplasmic Reticulum (RER) and Golgi bodies (GB) are presumably migrating towards the reservoir of the gland cells (X 13, 200). MT, Mitochondria; NU, Nucleus.

Figure, 56. *Phlebotomus papatasi*. TEM, Longitudinal section of the spermatheca showing the fibrous secretory mass (SM) between the septal foldings (STF) of the upper lumen (SPL) (X 13, 200).

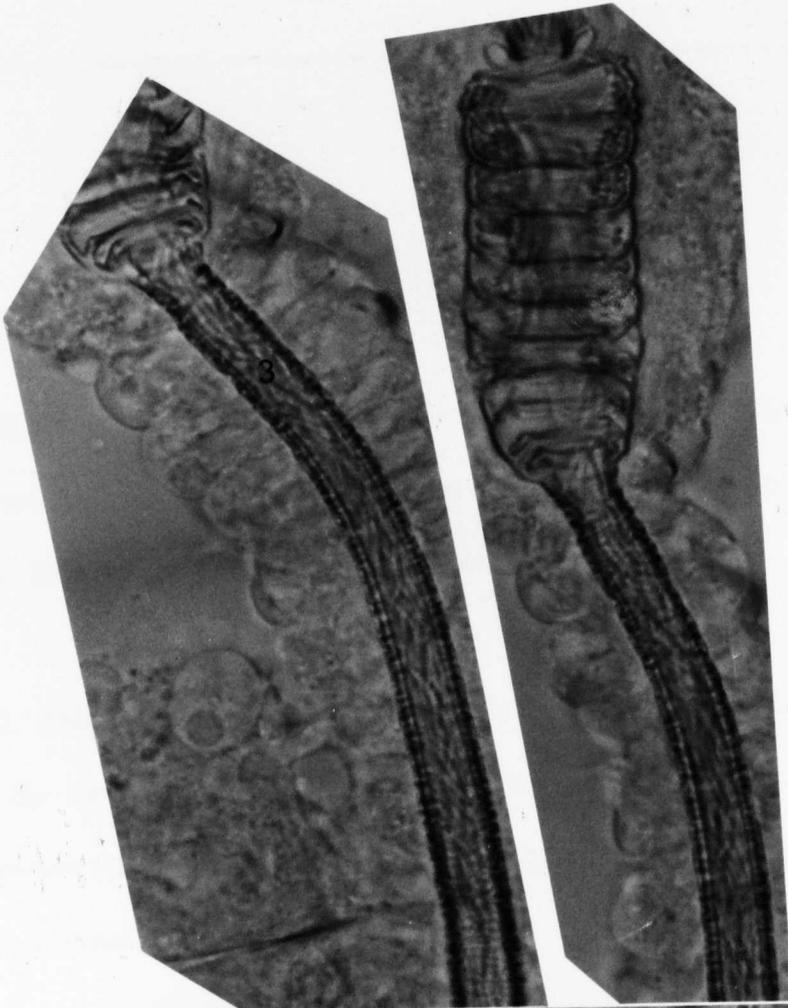
Figure, 57. Cross section of the basal spermathecal duct in a blood-fed fly with access to males showing the lumen (SDL) filled with the remnants of a spermatophore (STR) and acrosomal membranes (AM) cast from the spermatozoa after migrating to the spermatheca (X 20, 000).



Figure, 58. *Phlebotomus papatasi*. Saline squash of a 24-hour old sugar-fed fly immediately after insemination. One spermatophore (STR) is placed in each spermathecal duct (STR). Note the lower and upper limit (short arrows) of the spermatophores, showing they are not located in the same position relative to the bursa copulatrix (BC). The spermatozoa (asterisk) are immobile in the spermatophore (X 1, 800).



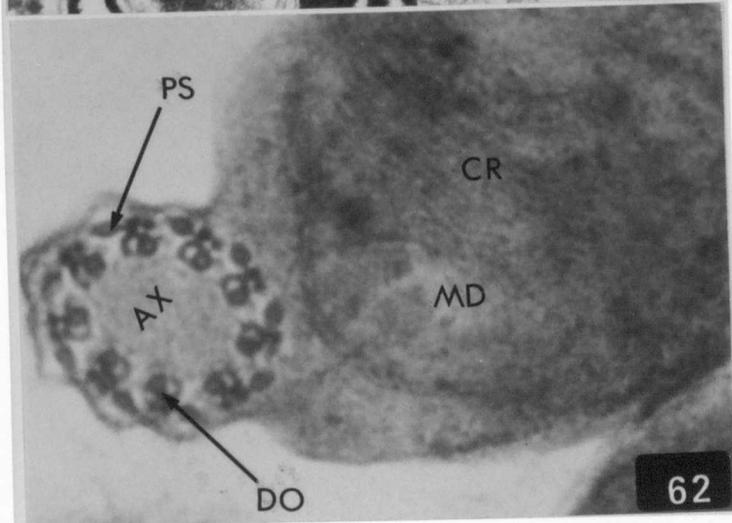
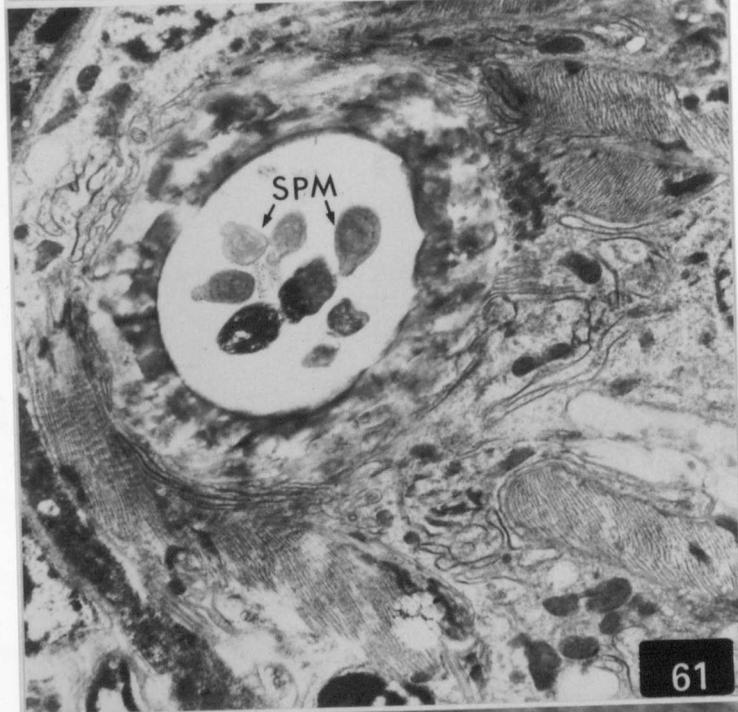
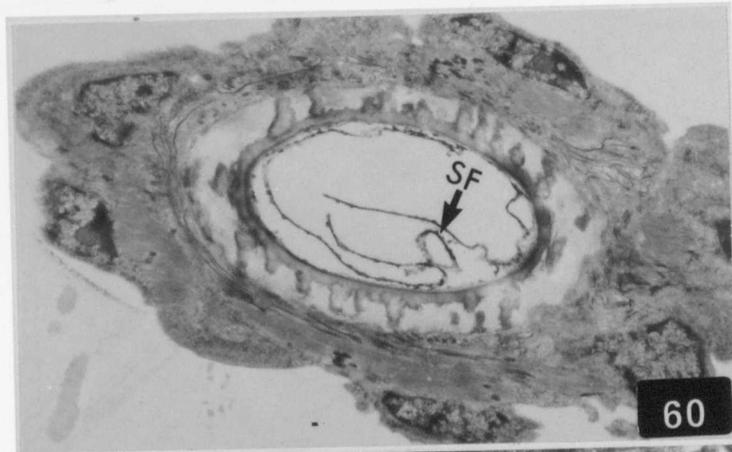
Figure, 59. *Phlebotomus papatasi*. Saline squash of a 7 day old fly 24-hours after bloodfeeding showing spermatozoa in spermatophores and the spermatheca. In one duct, there are 3 spermatophores (1, 2, 3) lying one above the other with a considerable gap (open arrow) between them suggesting a number of inseminations took place. At this stage the sperm (SPM) are active, leaving the spermatophores and moving towards the spermatheca (X 1, 800).



Figure, 60. *Phlebotomus papatasi*. TEM, Cross section of the basal section of the spermathecal duct showing the extrusion of helically coiled fibrous secretory material (SF) in ca. 24 hour old fly, but before insemination (X 6, 600).

Figure, 61. *Phlebotomus papatasi*. TEM, Cross section of the spermathecal duct of a 7 day old fly 24-hours after bloodfeeding showing the sperm released from the spermatophores ( X 10, 000) (other details as in fig. 59). SDL, spermathecal duct lumen; Open star, spermatozoan.

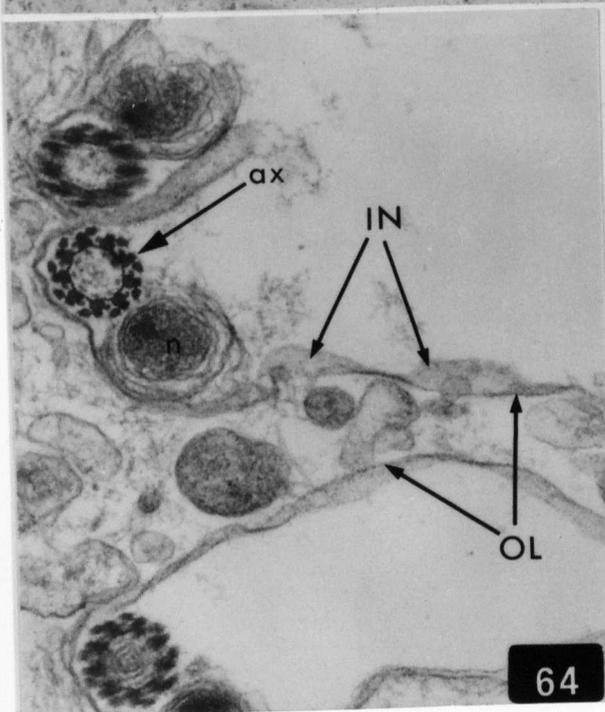
Figure, 62. *Phlebotomus papatasi*. TEM, Cross section of a spermatozoan at the same stage as Fig., 61. (X1, 50, 000). AX, axoneme; cr, cristae; DO, doublets; MD, mitochondria; PS, peripheral singlets.



Figure, 63. *Phlebotomus argentipes*. TEM, Cross section of the common spermathecal duct (CDO) showing spermatophores. Each spermatophore (1, 2, and 3) contains a few spermatozoa (short arrows). Very dark unrecognisable electron-dense masses (open arrow) are found around the spermatophores (X 10, 000).

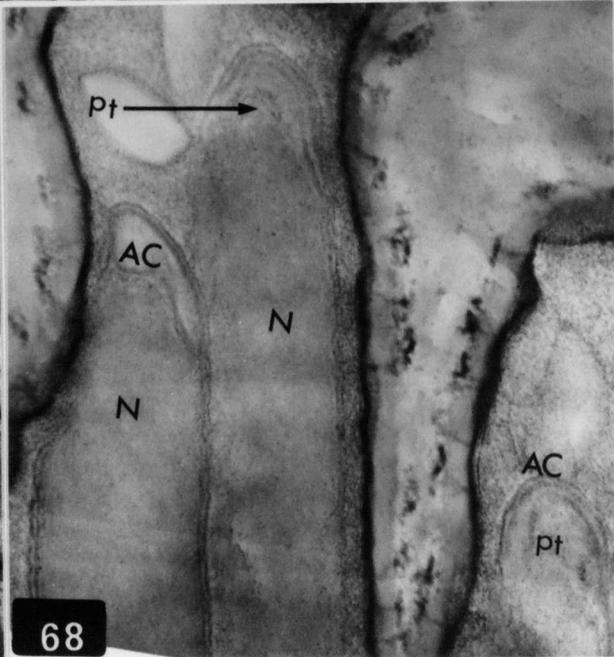
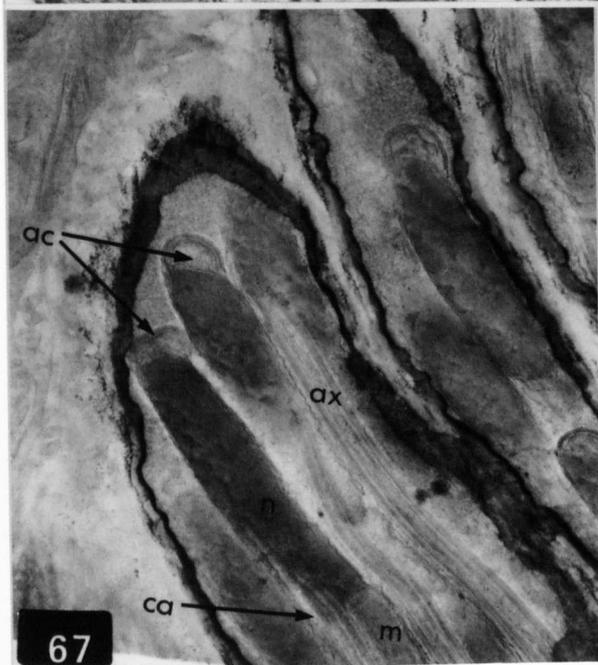
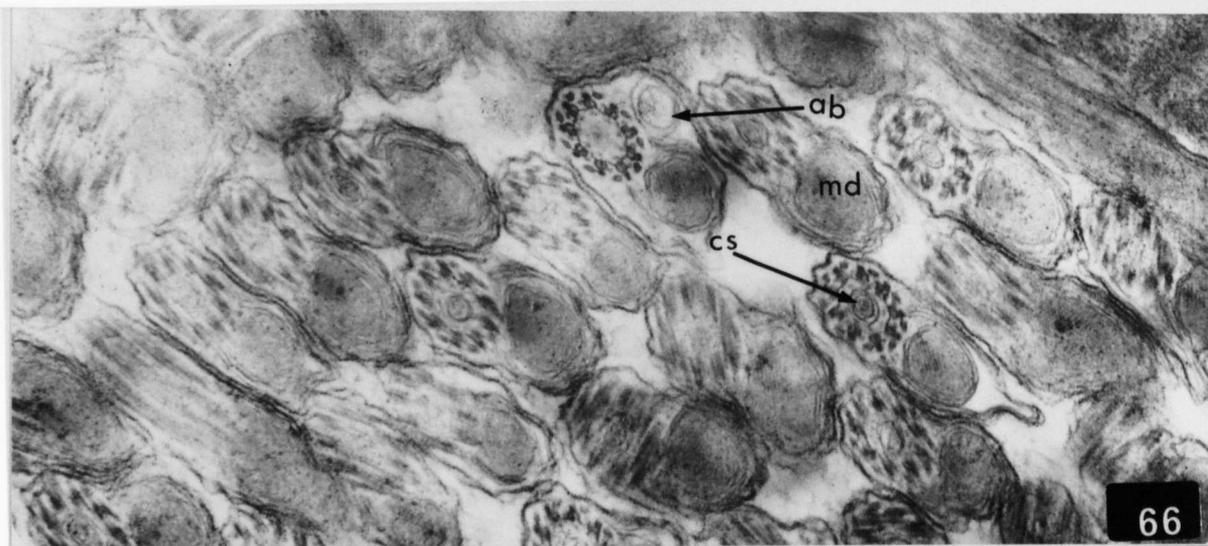
Figure, 64. *Phlebotomus argentipes*. TEM, Cross section of the spermatophore showing that it has a double layered membrane- the outer layer is thin and electron-dense (OL) and the inner layer thick and electron-lucent (IN). The axoneme (ax) and nucleus (n) of the sperm can be clearly seen (X 52, 000).

Figure, 65. *Phlebotomus argentipes*. TEM, Longitudinal section of the spermatheca of a wild caught, bloodfed fly showing the lumen (SP) is tightly packed with spermatozoa (X 6, 600).



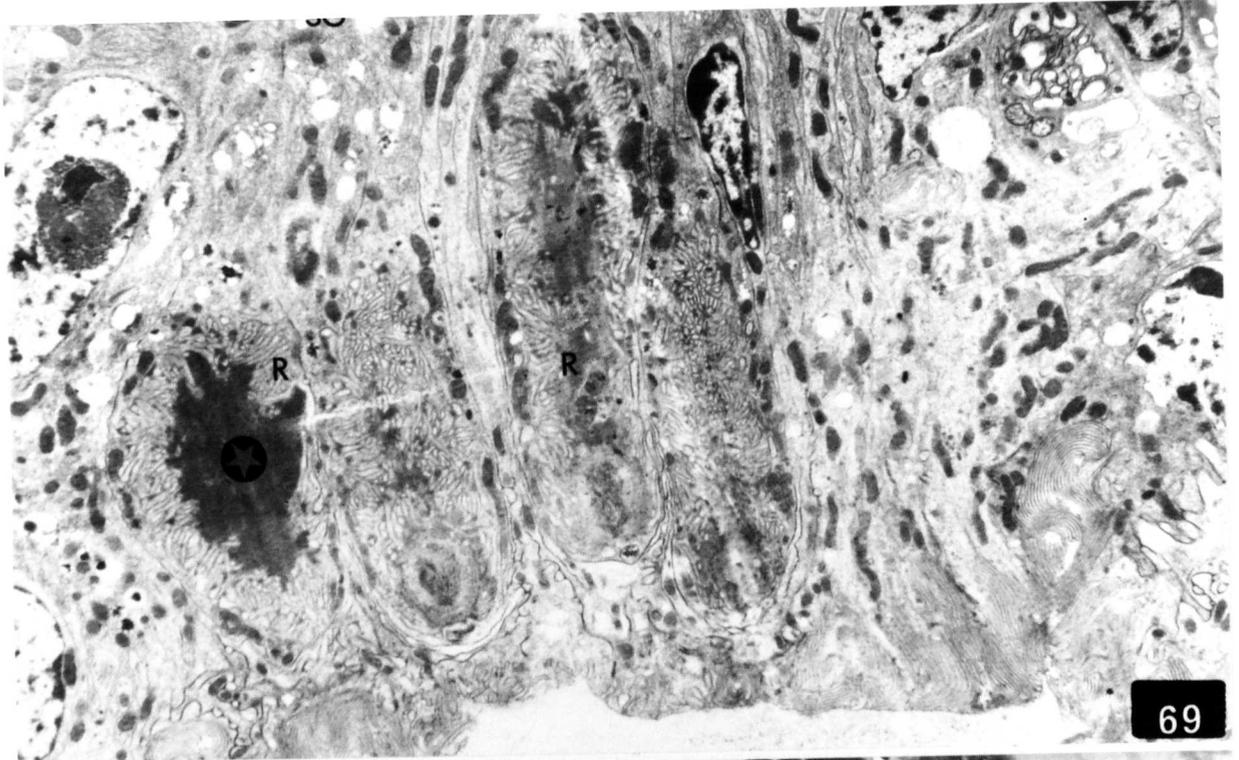
Figures, 66, 67 & 68. *Phlebotomus argentipes*. TEM, Cross section (Fig., 66; X 10, 000) and longitudinal section ( Figs., 67 & 68; X 52, 000) of spermatozoa in the spermatheca.

ab, accessory body; ac, acrosome; ax, axoneme; ca, centriole adjunct; cs, central singlet; m, mitochondria; n, nucleus; pt, perforatorium.

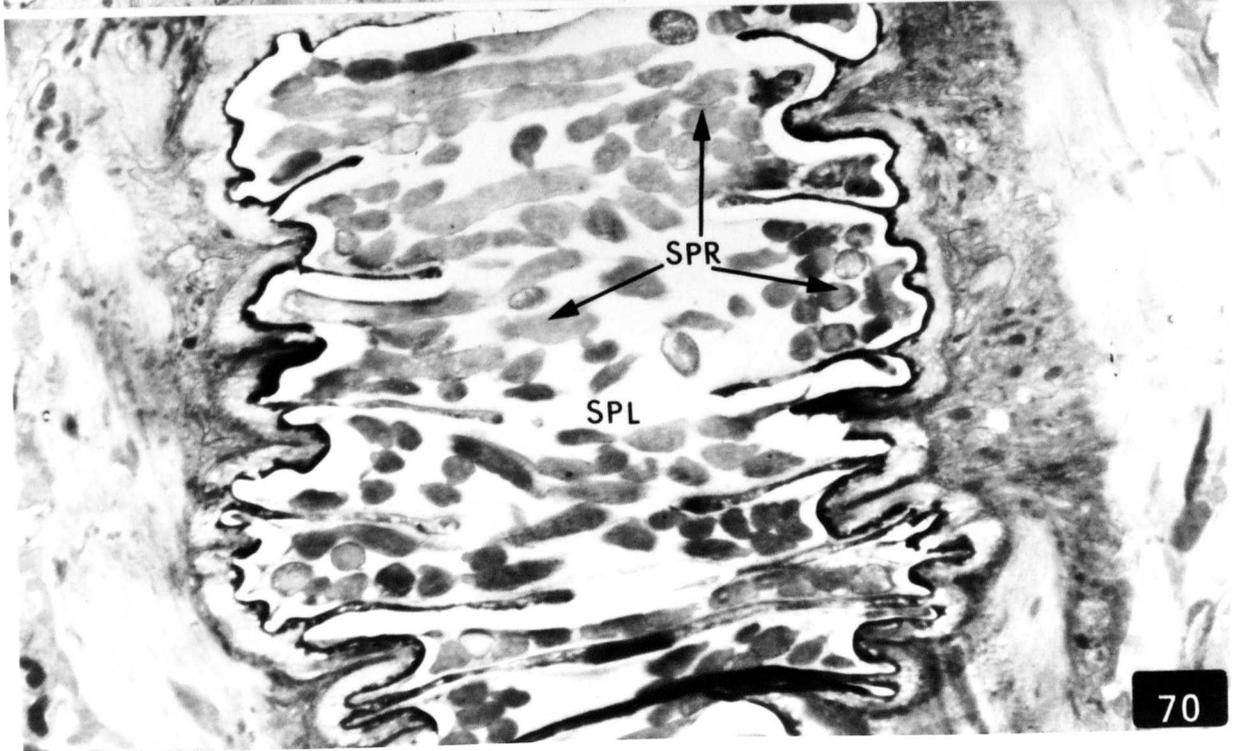


Figure, 69. *Phlebotomus papatasi*. TEM, Longitudinal section of the spermathecal gland (SG) of a bloodfed fly. The reservoir (R) of the gland cell is filled with an electron-dense material (closed star) (X 6, 600).

Figure, 70. *Phlebotomus papatasi*. TEM, Longitudinal section of the spermatheca in a bloodfed fly. The lumen (SPL) is full with spermatozoa (SPR) which are loosely packed ( X 6, 600).

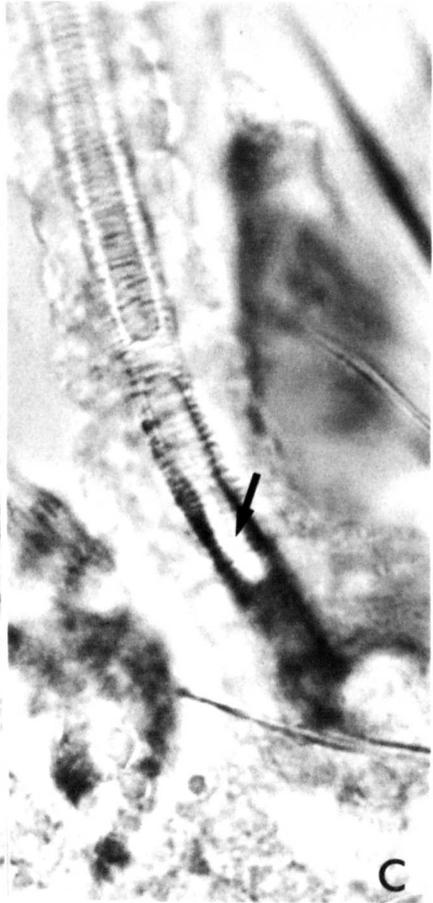
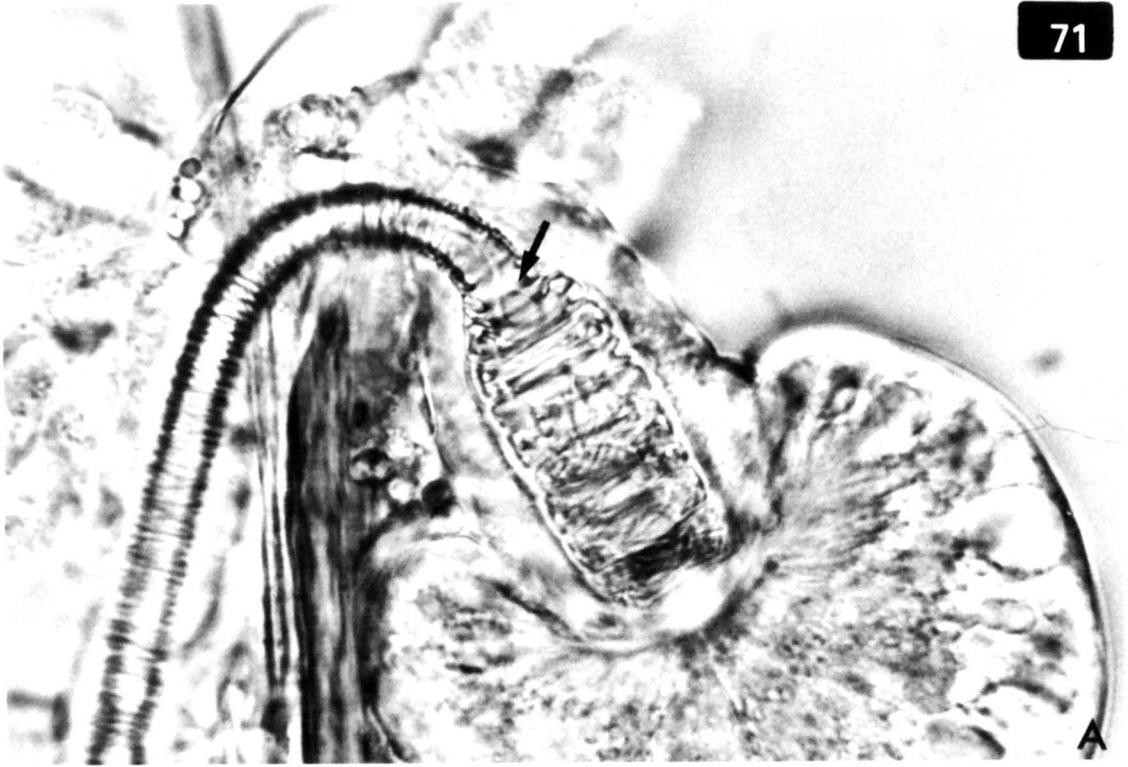


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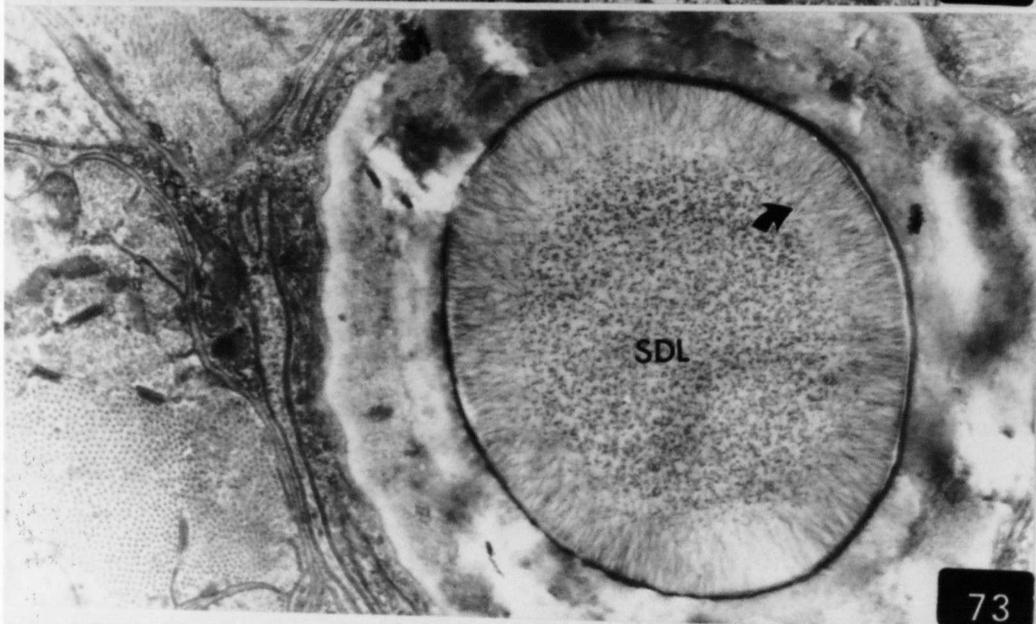
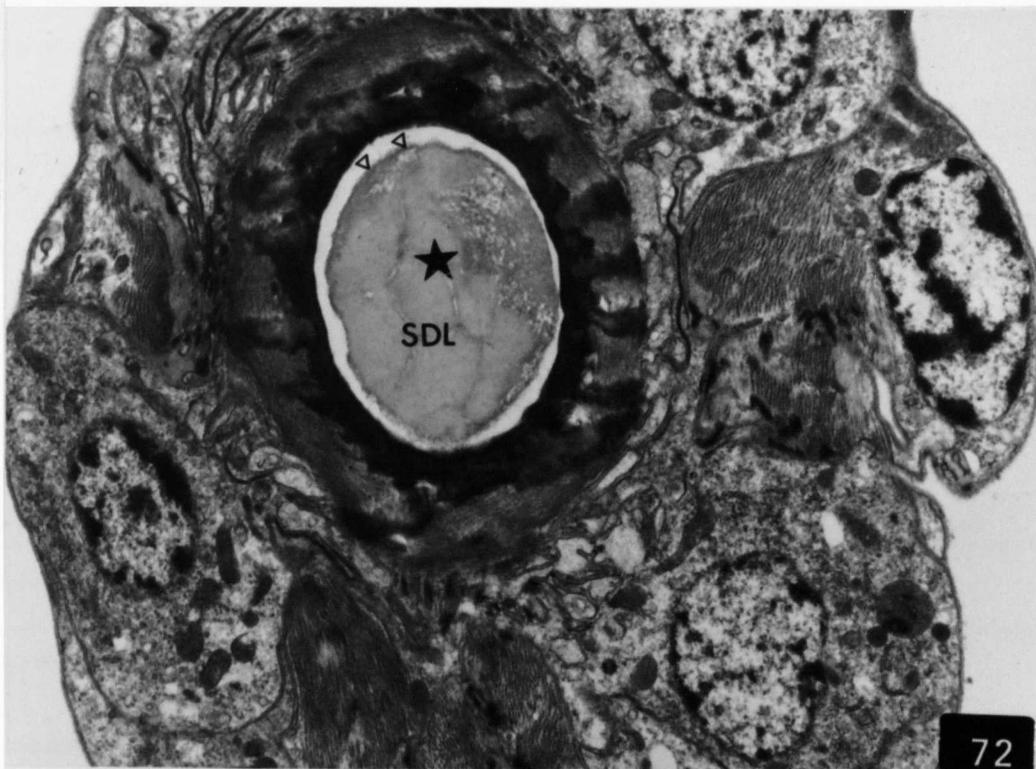
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Figure, 71. *Phlebotomus papatasi*. Saline squashes showing the spermatheca and the movement of spermatozoa (short arrows) during oviposition: (A) the sperm is released from the spermatheca by a sphincter valve at the base of spermatheca; (B) sperm moving down the middle of the duct; (C) sperm reaching the bursa copulatrix where it subsequently enters the micropyle of the egg which is not seen (X 1, 800).



Figure, 72. *Phlebotomus papatasi*. TEM, Cross section of the spermathecal duct in a bloodfed fly not given access to males. Like the reservoir of the gland cells and the lumen of the spermatheca, the lumen of the spermathecal duct (SDL) is filled with an electron-dense material (closed star) surrounded by a thin electron-opaque layer (small open triangle) (X 10, 000).

Figure, 73. *Phlebotomus papatasi*. TEM, Cross section of the spermathecal duct in one of the control group of sugar fed flies of the same age as the fly in fig. 72 (see the text for details). The lumen of the duct is occupied by a fibrous material (curved arrow) (X 20, 000).



Table, II. The relationship of spermathecal and sperm volume in *Phlebotomus papatasi*

Character	No. of samples	Mean ( $\mu\text{m}$ )	Std.D.	Range ( $\mu\text{m}$ )	Volume ( $\mu\text{m}^3$ )
Length of the spermathecal duct	10	113.75	2.072	108.5-116	
Diameter of the spermathecal duct	12	3.957	0.293	3.341-4.409	
Estimated volume of the spermathecal duct					1400
Length of the spermatheca	10	30.27	1.796	27.27-32.727	
Diameter of the spermatheca	10	17.44	1.916	14.4-20	
Estimated volume of the spermatheca					6400
Length of spermatophore	10	43.78	3.993	33-46.75	
Estimated volume of spermatophore					540
Length of sperm	12	16.841	0.78	15.815-17.996	
Diameter of sperm	12	0.714	0.118	0.573-0.918	
Estimated volume of sperm					6.75
Estimated no. of spermatophores/ duct (= access to no. of mates)		2.6 (=3)			
Estimated no. of sp'ores that can occupy a spermatheca		11.877 (=12)			
Estimated no. of sperms/ spermatophore		79.84			
Estimated no. of sperms/ duct		207.424			
Estimated no. of sperms/ spermatheca		948.345			
Observed no. of sperms in a given point of (@ c.s.) duct	10	7.8	1.317	6.10	
No. of eggs laid	24 <sup>1</sup>	37.917	25.387	2-96	
Estimated or actual no. of sperms utilised/ egg at oviposition <sup>2</sup>		50.019			

<sup>1</sup>24/46 of flies laid eggs

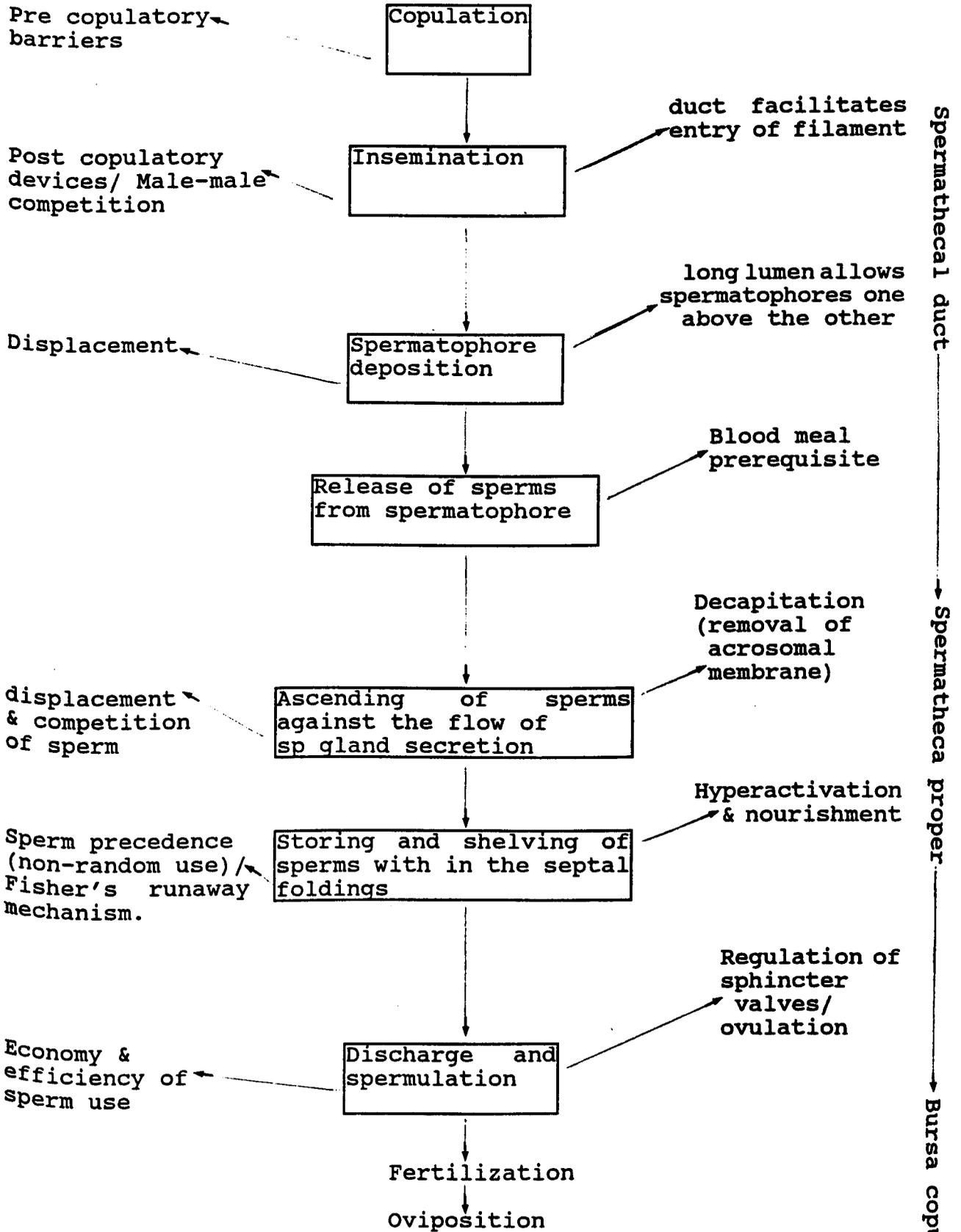
<sup>2</sup>Of 24 flies a single fly survived oviposition with 28 eggs.

Figure, 73. A

Control of spermathecae in the mating system of P. papatasi

Evolutionary function

Reproductive (physiological) function



### 3. 4. DISCUSSION

From the examination of spermathecae at emergence, the mass of loose material in the spermathecal lumen and ducts suggests that the secretory phase of the glandular cells could have started at an early stage in development, perhaps in the late pupal stage (pharate adult). As the fly ecloses and starts to develop, the already stored material (i.e. filamentous material) is evacuated from the spermathecal lumen by physiological changes in the organ.

Once the spermatophores are implanted in the spermathecal ducts, the spermatids within the spermatophore undergo real growth i.e. <sup>the final stages of</sup> spermatogenesis. During the growth phase, they have to face two major challenges before they can transfer as free spermatozoa to the spermathecae and later fertilize the eggs: 1. the spermatozoa must leave the spermatophore presumably under the control of the spermathecal gland secretion triggered by blood feeding 2. compete with the neighbouring sperm for access to the lumen of the spermatheca where they become activated resulting <sup>in</sup> the acrosomal reaction and capacitation. These two important events have not been reported before in the insect sperm transfer mechanism. There have been a number of processes, reviewed by Kaulenas (1992), responsible for sperm motility in various groups of insects: (a) spermathecal gland secretion and maintenance of correct ion balance in the spermatheca activate sperm motility (chemotaxis); (b) by propulsive mechanisms built into the spermatophore, in which case the sperm play no direct active part; (c) as a result of contractions by the common oviduct, induced by male accessory gland secretions and sperm motility is secondary to the sperm transport mechanism provided by the oviduct contractions; (d) there is a withdrawal of fluid from the spermatheca, with a resulting

aspiration of sperm in to the spermatheca.

After the initial evacuation of the loose secretory mass from the spermatheca, the spermathecal gland continues to be secretory in all the subsequent stages of the fly, irrespective of whether the female has had access to males or not, <sup>whether</sup> and <sub>↳</sub> sugar fed or bloodfed. The continuous production of secretion may or may not be alone responsible for the motility and transfer of spermatozoa. Unfortunately, in insect sperm transfer mechanisms there is no concrete evidence which could support the impact of the spermathecal gland on morphological changes of spermatozoa, as found in the mammalian female reproductive tract, before they are used for fertilization. However, Degrugillier and Leopold (1976) found changes in the morphology of spermatozoa in testes of male and spermathecae of female *Musca domestica*. In mature testicular sperm, the acrosomal cavity is surrounded by an inner acrosomal membrane and an outer sheath (plasma membrane) that encloses the sperm. Within the spermatheca, the plasma membrane loosens, separates from the sperm body and is eventually shed. This loss of the plasma membrane, they suggested, must occur while sperm are stored within the spermatheca. In mammals around the time of ovulation, spermatozoa acquire a type of motility, described as hyperactivation, which is an efficient way of forward propulsion within the viscous fluids encountered in the female reproductive tract prior to the "acrosomal reaction" and thus release or expose the hyaluronidase to facilitate sperm penetration of the egg vestments (Roldan et al., 1992). The capacitation process, i.e the acrosomal reaction, involves important membrane modifications and changes in cell metabolism particularly affecting sperm motility (Selmi, 1993). Similar situations must also operate in the species studied but only after blood feeding of phlebotomine sandflies.

Blood-meal feeding invokes a multitude of well-choreographed physiological events in blood sucking insects. Blood meal stimulates secretion of FMRF (Phe-Met-Arg-Phe-amide)-like immunoreactivity peptides from the neurosecretory cells of the midgut for blood meal digestion, secretion of enzymes, nutrient transport and diuresis in mosquitoes, cockroaches and *Rhodnius* (Elia et al, 1993). Furthermore, the blood-meal is a prerequisite for the presence of sperm in the spermathecae of *Anopheles subpictus* (1940) and *Aedes aegypti* (MacGregor, 1931). A similar system is most likely used in the regulation of sperm motility in phlebotomine sandflies. As mentioned earlier, sperm transfer i.e. the release of spermatozoa from spermatophores and movement towards the spermatheca, is found only in the bloodfed sandfly. The bloodborne factors regulated by the neurosecretory peptides (the evidence of neurosecretory substances from the myoneural junction of the spermatheca is reported in (chapter 3) <sup>in</sup> association with the spermathecal gland secretion, <sup>is</sup> perhaps involved in not only triggering the release of sperm from the spermatophores, but also providing an appropriate media for the motility, transfer and hyperactivation of spermatozoa.

The impact of blood feeding on motility, transfer and hyperactivation, including removal of acrosomal membrane, of sperm is yet another example of physiological prezygotic isolating mechanisms in bloodfeeding *Phlebotomus*. Although the ultrastructure of the spermatozoon of *P. argentipes* shares many histological features, such as the acrosomal complex and perporatorium in the head, with the spermatozoa of other phlebotomine sandflies, it differs in the axonemal pattern and the presence of accessory bodies in the postnuclear region. Such differences in sperm ultrastructure perhaps evolved as alternatives for access to the spermatheca.

The fine structure, number and placement of spermatophores in the spermathecae of *P. papatasi* and *P. argentipes* suggest that they are adapted to two different type of mating strategies, as precopulatory isolating mechanism.

Maroli et al (1991) claimed the formation of a "mating plug" (a physical block which prevents further insemination in other insect mating system) in phlebotomine sandflies but from information gathered in this present study this is considered a misinterpretation (see Figs., 58 & 59).

Dyabas and Dybas (1981) suggested that in the featherwing beetle, *Bambara*, the diameter and the length of the spermatheca and spermathecal duct correlates with the dimensions of the giant sperm cells of the males. This may or may not be correct (Eberhard, 1985), because they found no sites in the spermathecae corresponding to the screw-thread structures and they did not eliminate alternative possibilities such as stimulation from the spermathecae. The point is that forces of selection may some times be on the spermathecae rather on the male genitalia/ spermatozoa. The differences in the arrangement of sperm in the spermathecae of the two species of sandflies provide an alternative explanation, from the female selection point <sup>of</sup> view, <sup>in</sup> which the structure of spermathecae is under the influence of sexual selection by female choice.

#### 3. 4. 1. Anatomical evidence for sperm competition/ displacement

During the process of spermatophore transfer, there should be competition or displacement between the spermatozoa while trying to reach the spermatheca. Furthermore, it was that the release of the spermatozoa from spermatophores, started from spermatophores displaced or pushed down near base of the ducts, rather than the <sup>most</sup> recently placed (close to the base of the spermatheca).

Anatomical evidence for sperm displacement in the spermathecal ducts of *P. papatasi* and *P. argentipes* and the absence of sperm after oviposition in the spermatheca of *P. papatasi* suggests that the structural diversity of spermathecae could be related to the degree of polyandry (receiving sperm from more than one male during one breeding season) in *Phlebotomus* species. These species can accrue two particular kinds of advantage from being polyandrous (Thornhill and Alcock, 1983):

(1). Genetic-benefit polyandry- (a). The female replaces sperm of a genetically inferior mate with the gametes of a genetically superior individual, (b). The female adds sperm from a genetically different male to her sperm supplies to increase the genetic diversity of her offspring;

(2). Sperm replenishment polyandry- (a). The female adds to her depleted or inadequate sperm supplies, (b). the female avoids the costs of storing and maintain large quantities of sperm from a single donor (Thornhill and Alcock, 1983).

Phlebotomine sandflies exhibit great diversity in their spermathecae- from spheroid spermathecae in *Grassomyia* and *Idiophlebotomus* to elongate spermathecae in *Sergentomyia*, *Phlebotomus* and *Lutzomyia*. Ridley (1991) hypothesised that species with spheroid spermathecae should show low sperm displacement, and species with elongate spermathecae high displacement. Applying this hypothesis to the variety of sandfly spermathecae suggests a variety of mating strategies in sandflies.

A flow diagram (Fig. 73, A) summarises the events which the spermathecae could control in the mating system of *P. papatasi*, both physiologically and in evolutionary terms.

## 4. Coadaptation of male and female genitalia of the Old World Phlebotomine sandflies

### 4. 1. INTRODUCTION

The morphology of insect genitalia is used consistently as an important taxonomic feature, because of its bewildering structural variation. For example, the structural variation in the aedeagus and spermathecae was used exclusively to differentiate species of robber flies Asilidae: Diptera; Theodor, 1972). However, genital differentiation has been explored in a wide variety of animals to unravel the evolution of species. Studies on insect mating systems have concentrated on the lock-and-key mechanism. Females have evolved under selection favouring those individuals that avoided wasting eggs by having them fertilized by the sperm of other species; elaborate, species-specific female genitalia (locks) admit only the genitalia of conspecific males (keys), enabling females to avoid mistakes in fertilization (Eberhard, 1990).

To test the lock-and-key hypothesis in animal mating systems, groups of insect species have been studied. For example, in the featherwing beetle *Bambaria invisibilis* (Coleoptera: Ptiliidae), Dyabas and Dyabas (1981) discovered a strong correlation between the lengths of both the spermathecal lumen and the spermathecal duct, and sperm length. They also suggested that sperm competition might have resulted in morphological coadaptation between the sizes of male sperm and female spermathecae. In *Apamea* moths (Noctuidae: Lepidoptera), the sperm transferring organs in males and sperm storing

organs in females have been shown to form a postcopulatory, but prezygotic isolation mechanism (Mikkola, 1992). Because of their high species-specificity in clearly seen morphological details, insect genitalia are considered as reproductive isolating mechanisms.

The length of the sperm storage tubules in birds (Briskie and Montgomerie, 1992) and of the genital tracts in mammals (Harcourt and Gardiner, 1994) have been found to be correlated with the length of spermatozoa. These studies not only contribute to explanations of the pattern of reproductive strategies but also underline the contribution animal mating systems make to species isolating mechanisms.

In Old World phlebotomine sandflies, the female has two spermathecae and their associated ducts lead to either a common duct (in most subgenera) or independent ducts (in the subgenera, *Phlebotomus*, *Paraphlebotomus* and *Synphlebotomus*) opening into the bursa copulatrix. In the male, the aedeagus includes a short ejaculatory or sperm pump to which the testes are connected. This pump is a conspicuous syringe-like organ with a pair of long bifurcated aedeagal filaments. During mating, the aedeagal filaments are closely applied to the spermathecal duct for sperm transfer (Fig:74 & 75 A & B). The length of the spermathecal duct and its complementary aedeagal filament varies between members of the subfamily Phlebotominae. For example, *Idiophlebotomus* and *Spelaophlebotomus* species have spermathecal ducts which are very short and narrow with male aedeagal filaments supported by abdominal rods; whereas in most of the subgenera of both the Old World and New World the length of the spermathecal ducts is approximately equal to the length of the aedeagal filaments. In the extreme case of *Phlebotomus (Anaphlebotomus) rodhaini*, the length of spermathecal duct is exactly

twice the length of the aedeagal filaments. To date, only three observations have investigated the physical relationship of aedeagal filaments and spermathecal ducts: the published figures of *Phlebotomus major* (Sinton, 1925), *Phlebotomus perfiliewi* (Hertig, 1949), and *Lutzomyia migonei* (Ortiz and Marque, 1963). In the last case the aedeagal filaments occupy 2/3 of the duct length and in the first two species the filaments are inserted right up to the base of the spermathecae.

In the previous chapter, the long length of the spermathecal duct in *Phlebotomus papatasi* and *P. argentipes* provided physical evidence for sperm displacement. Not only can the long lumen of the duct accommodate many spermatophores but also it can allow the spermatozoa, released from the spermatophore after bloodfeeding, to compete with one another in order to monopolise the available storage space in the spermatheca. Apart from this fragmentary note, little is known about the relationship between of the spermathecal ducts and the aedeagal filaments and the underlying causes for the structural variation of the spermathecal duct in phlebotomine sandflies.

The objective of this chapter is to investigate the interrelationship between spermathecal ducts and aedeagal filaments to identify any lock-and-key mechanism which could have a bearing a sperm transfer.

### 4. 2. MATERIALS AND METHODS

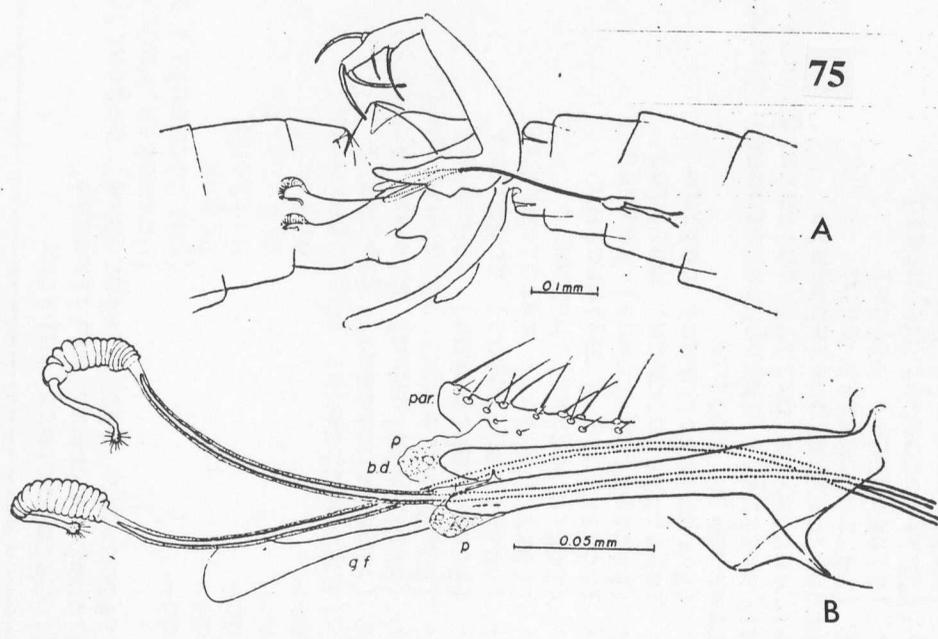
This study was mostly based upon slide-mounted specimens in the collection of the Department of Entomology, The Natural History Museum, London. Twenty-eight taxa, representing 13 subgenera of Old World phlebotomine sandflies were examined (Table, III). Measurements (in mm) were made: (i) of the spermathecal duct, from the base of

the spermatheca to the base of the spermathecal duct- either the common duct or the individual duct; (ii) of the aedeagal filaments, from the tip to the base where they join the sperm pump. The structure of the spermathecae was often very poorly visible in mounted specimens. Such specimens were either remounted or the Berlese's medium rewetted. Data on the spermathecae and aedeagal filaments of the closely related species *Phlebotomus papatasi* and *P. bergeroti*; *P. martini* and *P. celiae* and *P. argentipes* (sp. complex ?) were from Abonnenc (1959) and Parrot (1937); Gebre-Michael and Lane (1993); and Ilango et al. (1994) respectively. Measurements from the figures of Abonnenc (1967), Kirk and Lewis (1951), Lewis and Lane (1976) and Lewis (1977), Quate (1965) were also used.

Regression analysis was used for the statistical analysis using the UNISTAT statistical package.

Figure, 74. *Phlebotomus papatasi* showing the genitalia during copulation.

Figure, 75 (a & b). *Phlebotomus perfiliewi* (after, Hertig, 1949), (a). *in copula*. (b) details showing aedeagus, genital filaments filling ducts of spermathecae; b. d., base of spermathecal duct; g. f. stem of genital fork; p. pouches with granular debris; par., tip of paramere.

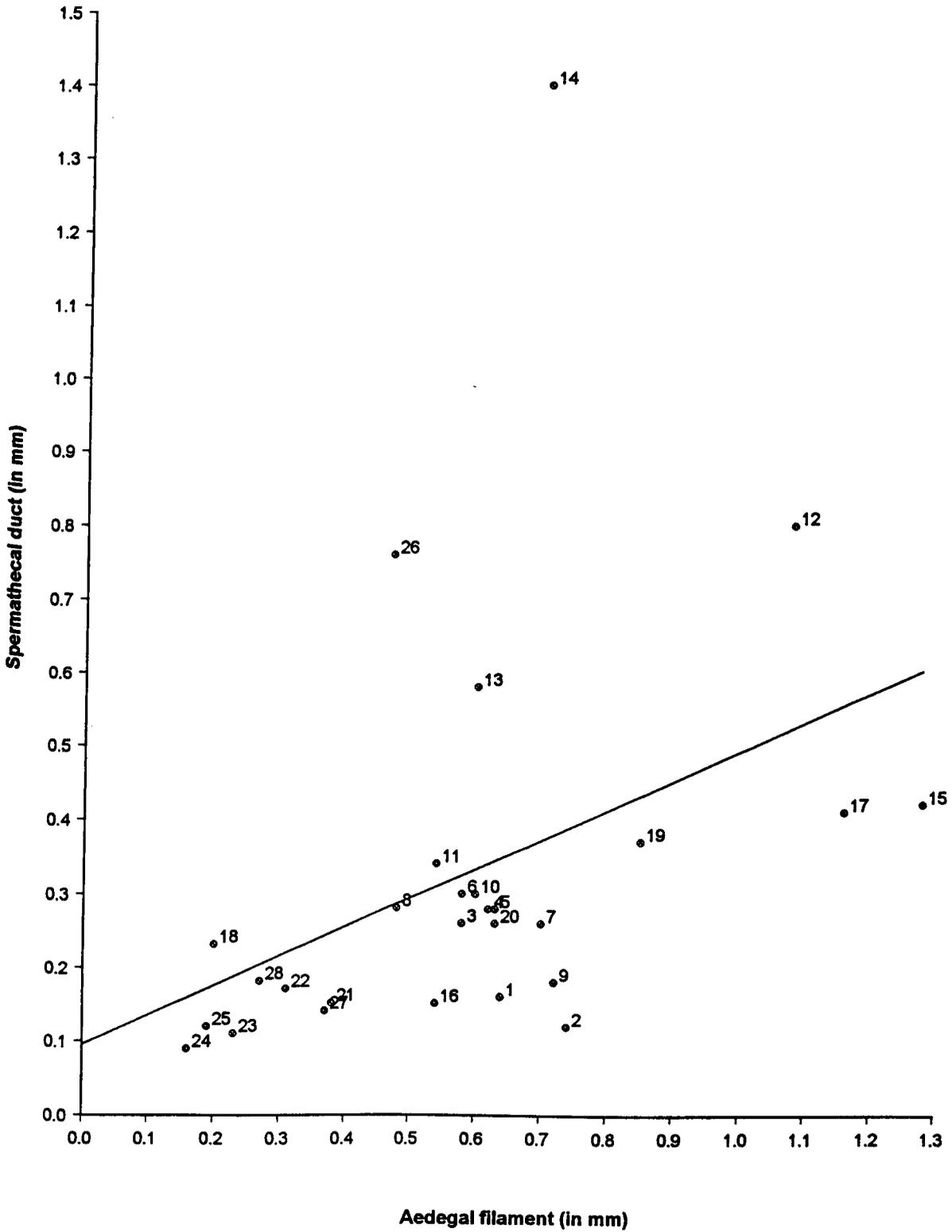


Table, III. The relative lengths of spermathecal ducts and aedeagal filaments in a range of Old World sandfly species.

Sl.no.	Species	No. of specimens*	Spermathecal duct (in mm)	Aedeagal filament (in mm)
1.	<i>Idiophlebotomus tubifer</i>	1	0.16	0.64
2.	<i>Idiophlebotomus wellingsae</i>	1	0.12	0.74
3.	<i>Phlebotomus (Euphlebotomus) argentipes</i> (domestic, Madras)	6♂, 7♀	0.26	0.58
4.	-do-	10♂, 5♀	0.28	0.62
5.	-do-	1	0.28	0.63
6.	-do-	1	0.30	0.58
7.	-do-	1	0.26	0.70
8.	-do-	1	0.28	0.48
9.	<i>P. (Euphebotomus) philippinensis</i>	1	0.18	0.72
10.	<i>P. (Euphlebotomus) mesghali</i>	1	0.30	0.60
11.	<i>P. (Euphlebotomus) kiangsuensis</i>	1	0.34	0.54
12.	<i>P. (Euphlebotomus) yunshengensis</i>	1	0.80	1.08
13.	<i>P. (Anaphlebotomus) stantoni</i>	1	0.58	0.60
14.	<i>P. (Anaphlebotomus) rodhaini</i>	1	1.40	0.70
15.	<i>P. (Larroussius) keshishiani</i>	1	0.42	1.28
16.	<i>P. (Larroussius) burneyi</i>	1	0.15	0.54
17.	<i>P. (Adlerius) longiductus</i>	1	0.41	1.16
18.	<i>P. (Paraphlebotomus) kazeruni</i>	1	0.23	0.20
19.	<i>P. (Synphlebotomus) martini</i>	10♂, 4♀	0.37	0.85
20.	<i>P. (Synphlebotomus) celiae</i>	12♂, 10♀	0.26	0.63
21.	<i>Grassomyia indica</i>	1	0.15	0.38
22.	<i>Sergentomyia (Neophlebotomus) dhandai</i>	1	0.17	0.31
23.	<i>Sergentomyia bailyi</i> (nicnic group)	1	0.11	0.23
24.	<i>Sergentomyia punjabensis</i>	1	0.09	0.16
25.	<i>S. (Parrotomyia) babu</i>	1	0.12	0.19
26.	<i>S. (Sintonius) clydei</i>	1	0.76	0.47
27.	<i>P. (Phlebotomus) papatasi</i>	9♂, 18♀	0.14	0.37
28.	<i>P. (Phlebotomus) bergeroti</i>	3♂, 1♀	0.18	0.23

(\* Unless mentioned otherwise, 1 indicates one male and one female examined)

**Fig., 76. Relation between the spermathecal duct length and aedeagal filament length**



R2 = .1606278

SE = .2577148

C0 = 9.522156E-02

C1 = .3959259

### 4. 3. RESULTS

The results (Figure,76) show that there is a strong positive correlation between the length of the spermathecal duct and the conspecific aedeagal filament across the species investigated ( $r = 0.16$ ,  $p = 0.035$ ). Furthermore the results also suggest that there is clear differences in the length of the spermathecal duct and aedeagal filament both between and within closely related taxa (i) subgenera of *Phlebotomus* v s. *Synphlebotomus* and (ii) species of *Phlebotomus papatasi* v. s. *P. bergeroti*, *P. martini* v. s. *P. celiae* and among the populations of *P. argentipes* (complex ?).

### 4. 4. DISCUSSION

The patterns of intraspecific and interspecific variation in spermathecal duct length (0.09- 0.140 mm) and aedeagal filament length (0.23- 1.28 mm) in all the 28 species studied, clearly demonstrates that spermathecal duct length did not simply evolve to accommodate the length of the aedeagal filament. The presence of a strong correlation suggests that a reproductive isolating mechanism could have occurred in the mating system of phlebotomine sandflies.

Six major hypotheses have been proposed to explain the wide variation in morphology of genitalia: 1. lock-and-key mechanism (LKM), 2. sexual selection by female choice, 3. sperm competition 4. Fisher's runaway theory, 5. character displacement and 6. pleiotropism. In his elegantly structured book on "Sexual selection and animal genitalia", Eberhard (1985) presented evidence both for and against these hypotheses for the development of reproductive isolation mechanisms and subsequent speciation. From these hypotheses, the lock-and-key hypothesis (LKM) and

sexual selection by female choice are considered further to explain the relationship between spermathecal duct length and aedeagal filament length of phlebotomine sandflies.

The LKM hypothesis as mentioned earlier that "...females avoid having their eggs fertilized by the males of other species by evolving complicated genitalia that permit insemination only by the corresponding genitalia of their own species". If the length of spermathecal duct serves mainly a lock function, they could theoretically be homogenous but it shows a great species-specific diversity. LKM has been shown as <sup>a</sup> reproductive isolation mechanism between, but not within, the moth species of *Apamea* (Mikkola, 1992) and in featherwing beetles, (Dyabas and Dyabas, 1981). In the latter example, the conclusions may not be correct because they did not find any sites in the lumen of the spermatheca where the screw-thread like sperms could fit, and they did not eliminate alternate possibilities such as stimulation from the sperm or exclusion of other sperm (Thornhill and Alcock, 1983).

According to the theory of sexual selection by female choice, females discriminate among males of their own species on the basis of the males' genitalia, and that males with favoured genitalic morphologies sire more offspring than others. A female could discriminate among males' genitalia on the basis of their ability to fit mechanically into hers, or through other sensations occurring in her genital region. Eberhard (1990) advanced convincing evidence, including the diversity of female genitalia associated with multiple insemination, on how females are very selective and pursue strategies to pass genes to future generations.

In support of both arguments (LKM, female choice), I present evidence for the pattern of variation in the lengths of the spermathecal duct that the female not only puts

selection pressure on the wide variation of the aedeagal filament length and sperm length but also, possibly, on sperm speed.

Variation in sperm length: Species of the subgenera *Idiophlebotomus* and *Spelaeophlebotomus* have very thick aedeagal filaments supported, often, with heavy abdominal rods, which can not be inserted into a short spermathecal duct during mating. Their close relatives (see the cladistic section for the character states) in non-Phlebotomine psychodids, such as *Sycorax*, also have short spermathecal ducts which are also unlikely to accommodate the aedeagus during mating. However, the spermatozoa of these taxa, such as *Telmatoscopus* and *Psychoda* (Baccetti et al., 1973) and possibly *Sycorax* and *Idiophlebotomus*, lack a flagellum. Hence, perhaps the spermatozoa of these taxa are non-motile and do not need to swim in a short and narrow spermathecal duct as found in the other members of *Phlebotomus*. The large sac-like spermathecal body of these taxa may serve only as storage of secretions from the spermathecal gland, and it is unlikely that they receive and store large quantities of sperm, which is very costly to females for maintenance, in contrast to the species of *Phlebotomus*. In sperm transfer, for example *P. papatasi*, the spermatozoan with long flagellum was found actively swim in the spermathecal duct to reach the lumen of <sup>the</sup> spermatheca proper. Hence the female sandfly could exercise its control over the spermatozoa, notably of their length and speed, in *Phlebotomus* spp. by providing an opportunity for sperm competition to take place.

Variation in the length of the aedeagal filaments. In those species in which the length of the aedeagal filaments are either longer than the spermathecal ducts or both are equal, sperm competition and lock-and-key mechanisms are likely to be in operation. As

a result, the females could force males not only to deposit their sperms as layers within the spermathecal ducts but increase the speed of sperm to travel the long route of the duct to outcompete other sperm.

In the previous chapter I reported layers of spermatophores occurring on the basal spermathecal ducts of *P. papatasi* and *P. argentipes*. Thus a female could influence which sperm occupy the spermathecal lumen and thereby control the paternity of her eggs simply. The female again exercises control in selecting the sperms not only from the last inseminated spermatophores but also faster sperm with long flagella that will be the first to occupy the spermatheca proper. Males, however, counter this tactic by evolving sperm long enough to fill and travel the duct thereby again excluding sperm from subsequent inseminations. If no agreement between the sexes over the pattern of sperm storage is reached, selection on the spermathecal duct may result into an 'arms race'. Only such processes can explain the differences in the spermathecal duct, even between closely related species.

### **4. 4. 1. Sexual selection and recognition of speciation in disease vectors of phlebotomine sandfly species**

*Phlebotomus argentipes* occurs from Iran and Afghanistan in the west, through the Indian subcontinent and Malaysia to Indonesia. It is the vector of kala-azar in three main foci: in north-east India (West Bengal and Bihar), southern India (Tamil Nadu), western India (Gujarat). The species shows geographical variation in several morphological characters such as wing and colour (Annandale, 1911), antennal ascoids (Lane, 1988) and in cuticular hydrocarbons (Kamhawi et al., 1992). Lane (1988) associated the length of

antennal ascoids (female's secondary sexual traits presumably involved in the emission of pheromones) with that of population differences i.e., as possible siblings in a complex of species or of variations in a single species. Recently, there are two morphological species described based on the differences in morphology, mating behaviour and habitat (Ilango et al., 1994).

Genitalic differences evolve as result of reproductive isolation mechanisms which have been reported in a wide variety of animal mating systems (Eberhard, 1985). Sexual selection by female choice and reproductive character displacement are considered to be the most important speciation mechanisms in closely related species. Such reproductive isolation is stronger in sympatry than in allopatry: eg ., Sonoran fruitflies *Drosophila mojavensis* and *D. arizonensis* (Wasserman and Koepfer, 1977); damselflies *Calopteryx maculata* and *C. aequabilis* (Wagge, 1979). Even the small samples of *Phlebotomus argentipes* drawn from different geographical regions in Southern Asia, suggest that they are reproductively isolated as different forms/ species i.e. <sup>as a</sup> species complex.

## **5. Cladistics of the Old World Phlebotomine sandflies with special reference to the spermathecae**

### **5. 1. INTRODUCTION**

Many proposals have been made on the systematics of phlebotomine sandflies, including their general classification and relationships with other groups (Fairchild, 1955; Lewis et al., 1977; Williams, 1993), the classification of American phlebotomine sandflies (Theodor, 1965), insect fossils with particular reference to the phylogenetics of the Psychodidae (Hennig, 1972), evolution of phlebotomine sandflies (Lewis, 1982) and finally, phenetic analysis and the phylogeny of phlebotomine sandflies (Risipal and Leger, 1991). However, there is no comparative study which has critically evaluated evolutionary relationships among the phlebotomine sandflies.

The evolutionary relationships of the psychodid subfamilies Bruchomyiinae Trichomyiinae, Sycoracinae, Psychodinae and Phlebotominae have been debated for many years. Some workers consider the phlebotomine sandflies are a subfamily of the Psychodidae (Duckhouse, 1973; Lewis, 1977), others proposed the phlebotomine sandflies to have separate family status (Abonnenc and Leger, 1976; Dallai et al., 1984, Williams, 1993), or even that it is <sup>a</sup> family consisting of three subfamilies, one Old World, one Old and one New World and one New World only (Abonnenc and Leger, 1976). Hennig (1972) considered the phlebotomine sandflies of the New World and Old World to be sister taxa with a monophyletic origin.

As discussed earlier, among the morphological features of phlebotomine sandflies the structure of the spermathecae presents particular diversity. Its ultrastructure and changes during the gonotrophic cycle have been dealt with in previous chapters.

Structural variation and morphological changes in the reproductive system of various animal taxa have been used to deduce their evolution and phylogeny, for example: the ultrastructure of insect spermatozoa in insect phylogeny (Jamieson, 1987); changes in the head morphology and overall dimension of spermatozoa in the evolution and phylogeny of eutherian mammals (Roldan et al., 1992); and female reproductive histomorphology in a cladistic analysis and phylogeny of Phyllostomatoid bats (Hood and Smith, 1982). Given the structural diversity of spermathecae in sandflies, potentially, they are a useful means of studying the phylogeny of the phlebotomines.

Hence, the objective of this chapter is to use the structural variation of the spermathecae and their counterparts in the male genitalia to investigate the interrelationships of the Old World phlebotomine sandflies and to reconstruct their phylogeny using cladistic analysis (Hennig, 1966; Forey et al., 1993).

### 5. 2. MATERIALS AND METHODS

Twenty-nine taxa representing all the Old World subgenera of phlebotomine sandflies (Seccombe et al., 1993) were selected (Table, IV). Twenty-two characters were examined, 11 derived from the morphology of spermathecae and 11 from the male genitalia (Table, V). The ultrastructure of the spermathecae obtained from 4 species representing 4 subgenera: *S. (Parrotomyia)*, *P. (Euphlebotomus)*, *P. (Phlebotomus)* and *P. (Larroussius)* was used to determine homologies. The terminologies derived from

both light and electron microscopic observations were applied to all the subgenera including the out-group. Wherever possible, specimens in the Natural History Museum, London were examined but some times details were obscured (eg., length of the spermathecal ducts) so illustrations had to be used, such as those in Fairchild (1955), Theodor (1965), Quate (1964 and 1965), Lewis (1967, 1978), Kirk and Lewis (1951), Lewis and Buttiker (1982), Lewis and Dyce (1988) and Artemiev and Neronov (1984).

### 5. 2. 1. Selection of the out group.

The membranous tubular spermatheca (eg. *Sergentomyia minuta*; Theodor, 1965) and the style with a single terminal spine in male genitalia are considered to be plesiomorphic (primitive forms). Since they also occur in other Psychodidae (eg. *Sycorax silacea*, Trichomyiinae). *S. silacea* was thus used as the outgroup.

### 5. 2. 2. Data coding

Plesiomorphic states are coded as zero while more apomorphic (derived/ recent) states were coded as 1. If there were more than two character states, these were coded as ordered multistate transformation series (0-n).

### 5. 2. 3. Data analysis

The data matrix (Table, VI) was analyzed using the computer program Hennig86, version 1.5 (Farris, 1988), applying the mhennig\* and bb\* options for calculating trees. Trees can be constructed using 4 methods in Hennig86: IE, HENNIG, MHENNIG and BB. These methods are increasingly fast methods, but the chances of obtaining the most parsimoni<sup>o</sup><sub>λ</sub> solutions for homoplasious data sets concomitantly decrease. For small data sets, option IE\* is recommended. Option BB\* is the most useful for medium to large data sets but should be based upon trees generated by MHENNIG\* to minimize run times.

The successive approximations character weighting procedure (SACW), which calculates weights from the best fits of the characters on the most parsimonious cladograms, was then applied. When the analyses yielded more than one cladogram, a strict consensus tree was calculated ('nelsen' option of Hennig86).

### 5. 3. Results and discussion

The cladistic analysis using SACW produced 7 equally parsimonious trees (trees with the shortest lengths and therefore with the minimum number of evolutionary steps). Each had a length of 153 steps, consistency indices of 0. 49, and a retention indices of 0. 86. These indices are measures of homoplasy in cladistic data, and their values reduce as the number of homoplastic, or extra steps, increases. Membranous spermatheca (character state, 10) and <sup>a</sup>single terminal spine on the style are the pleisomorphic states, <sup>which</sup> characterise the taxa at the base of all the 7 most parsimonious cladograms.

The spermathecae of the taxa under study (collected from published figures) is superimposed on a strict consensus tree calculated with the 'nelsen' option (Figs. 77 & 78 A-Zc) to show the changes in these structures during the phylogeny of the Old World sandflies.

Complete node diagnoses output and individual character fits can be found in Annex, 1.

The seven most parsimonious trees (Annex, 1) differ in their resolution of the clades. For example, in tree 4, polytomies occur in clades 34, 36, 38, 42, 43 and 48. Such polytomies also occur in the consensus tree in clades 33, 35, 36, 38 and 43.

Throckmorton (1962) proposed a phylogeny for *Drosophila* from a detailed light

microscopic study of the spermathecal structure alone. A similar proposal is made here for the phylogenetic grouping of Old World phlebotomine sandflies from the ultrastructural and light microscopic study of spermathecal variation. The term phylogenetic groupings for phlebotomines used here strictly refer to those found from a cladistic analysis of the structure of spermathecae and male terminalia only. When other morphological features such as those from the head, wing, etc., are included, the position of the taxa may well be altered but probably rather minimally. Although the general topology of the cladogram suggests that the taxa under the study are of monophyletic origin, unfortunately, polytomies occur between the clades 35 and 38 in the consensus tree.

**Table, IV. List of taxa and their acronym for Table VI & Figure 77, code letter for Figure 78 and geographic distribution used in the cladistic analysis.**

1. Out group (= *Sycorax silacea*); out group (A); Europe, Palaeartic
2. *Idiophlebotomus asperulus*; asperulus (B); Malaysia- Oriental
3. *Idiophlebotomus tubifer*; tubifer (C); Maharhastra-India, Oriental
4. *Idiophlebotomus erebicolus*; erebicolus (R); Philippines, Oriental
5. *Phlebotomus (Spelaeophlebotomus) gigas*; gigas (E); Zaire-Africa, Afrotropical
6. *Chinius junlianensis*; chinius (S); Sichuan Province-China, Palaeartic
7. *Phlebotomus (Euphlebotomus) argentipes*; argentipes (T); India- Oriental
8. *Phlebotomus (Anaphlebotomus) stantoni*; stantoni (Y); Malaysia, Oriental
9. *Phlebotomus (Anaphlebotomus) rodhaini*; rodhaini (N); Zaire, Afrotropical
10. *Phlebotomus (Australophlebotomus) brevifilis*; brevifilis (O); New South Wales, Australia
11. *Phlebotomus (Kasaulius) newsteadi*; kasaulius (U); Punjab-India, Oriental
12. *Phlebotomus (Larroussius) major*; major (Z); India, Oriental
13. *Phlebotomus (Larroussius) keshishiani*; keshishiani (Za); pakistan, U.S.S.R, Oriental & Palaeartic
14. *Phlebotomus (Larroussius) burneyi*; burneyi (Zb); Pakistan, Oriental
15. *Phlebotomus (Adlerius) longiductus*; adlerius (Zc); U.S.S.R, Palaeartic
16. *Phlebotomus (Phlebotomus) papatasi*; papatasi (V); Palaeartic, Afrotropical and Oriental
17. *Phlebotomus (Paraphlebotomus) alexandri*; kazeruni (W); U.S.S.R, Palaeartic
18. *Phlebotomus (Synphlebotomus) martini*; martini (X); Afrotropical & Palaeartic
19. *Grassomyia indica*; grassomyia (P); India, Oriental
20. *Sergentomyia (Parvidens) lesleyae*; parvidens (G); Sudan, Afrotropical
21. *Sergentomyia (Neophlebotomus) hodgsoni*; hodgsoni (H); India, Oriental
22. *Sergentomyia (Neophlebotomus) quatei*; quatei (J); India, Oriental
23. *Sergentomyia (Neophlebotomus) kirki*; kirki (F); Sudan, Afrotropical
24. *Sergentomyia (Spelaeomyia) mirabilis*; mirabilis (D); Zaire, Afrotropical
25. *Sergentomyia (Parrotomyia) babu*; babu (K); India, Oriental
26. *Sergentomyia pugifera*; pugifera (L); Queensland, Australasia
27. *Sergentomyia (Sintonius) clydei*; clydei (Q); India, Oriental
28. *Sergentomyia anodontis*; anodonti (I); Malaysia, Oriental
29. *Sergentomyia (Sergentomyia) punjabensis*; punjabensi (M); India, Oriental

**Table, V. Character states of the spermathecae and male genitalia of the Old World Phlebotominae**

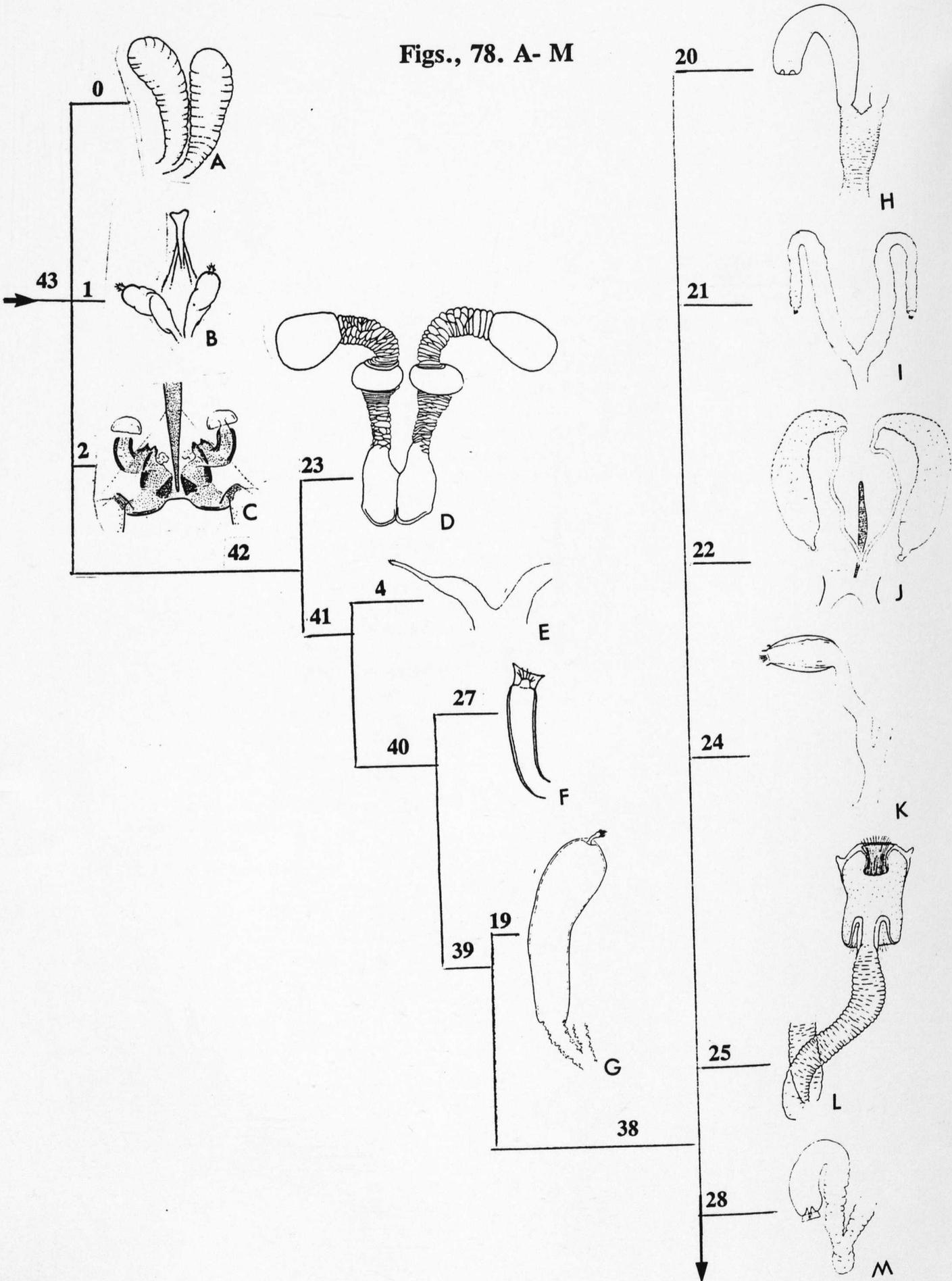
Character	Plesiomorphy (0)	Apomorphy (1,.....)
1. Spermathecal duct	common	distal extremities united 1 separated 2
2. Duct opening	broad	narrow 1
3. Thickness of cuticle as in 2	thin	thick 1 +lateral extension 2 + accessory 3
4. Length of duct	short	medium 1 long 2
5. Segmentation of spermatheca	Unsegmented	segmented 1
6. Apex of cuticle block in SP	undifferentiated	differentiated within the apex 1 elongate beyond the apex 2
7. Glandular ductules	one/few	several 1
8. Glandular cells	long/few	short/many 1
9. Muscle fibres	thick	thin 1
10. Wall cuticle	thin	thick 1 heavily sclerotised 2
11. Differentiation of SP & duct	no	differentiated 1
12. Sperm pump	supported by abdominal rod	not supported 1
13. Base of pump	broad	simple 1
14. Genital filament	short	long 1
15. Aedeagus tip	divided into 2 lobes	undivided 1
16. Aedeagus base	normal	truncated 1
17. Paramere	simple	peaked 1 bi/trifurcated 2
18. Coxite	short or = to style	long 1
19. Coxite lobes	absent	present 1
20. Style	long	short 1
21. Style spines	1 terminal with/ without 1 or 2 basal & sub terminal	2 terminal 1/2 subterminal & 1/2 basal 1 4 terminal 2
22. lateral lobes	long	short 1

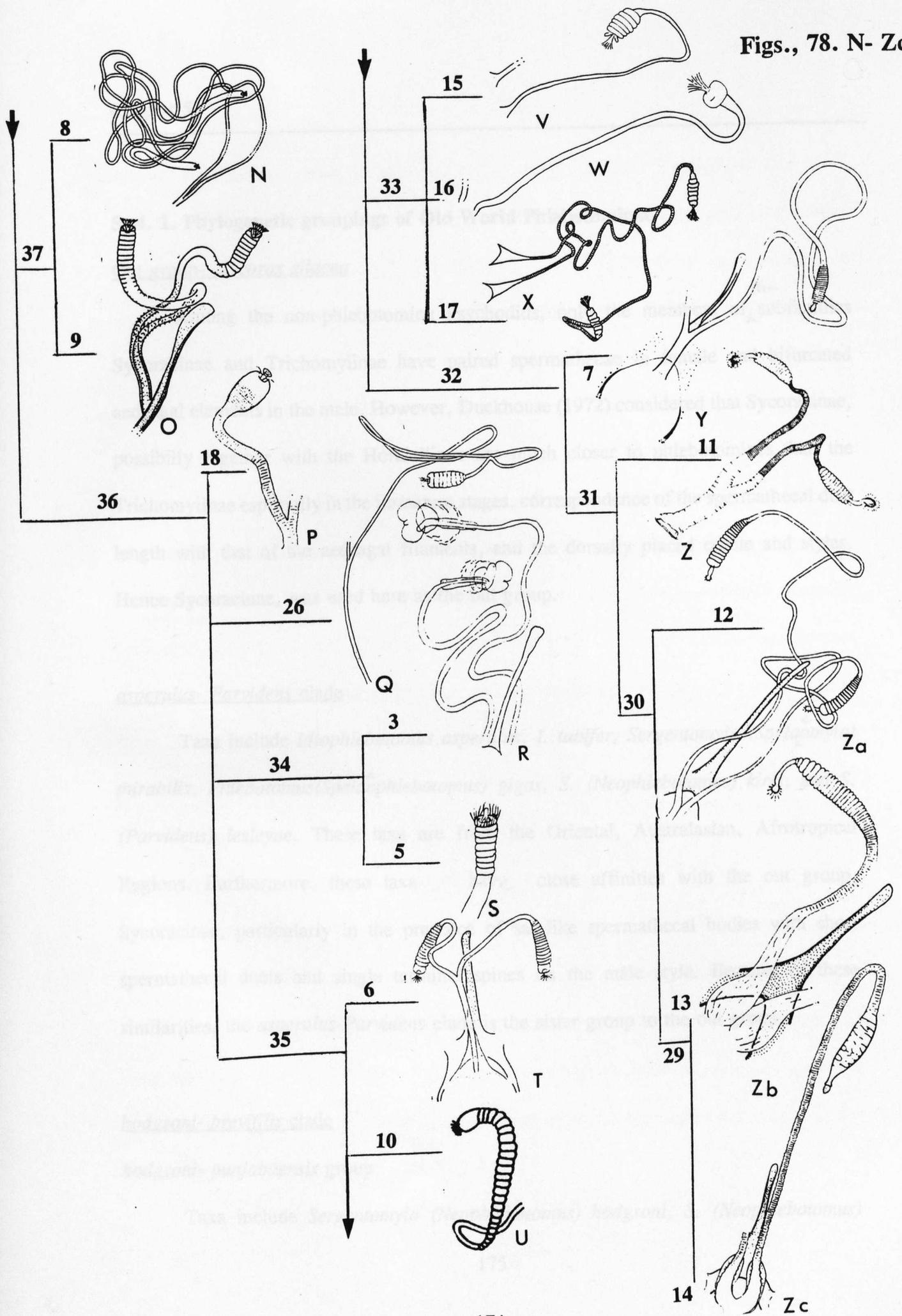
**Table, VI.** Data Matrix for 29 taxa used in the cladistic analysis  
(Characters and their states are given in Table, V).

Outgroup	00000	00000	00000	00000	00
asperulus	00000	00000	00000	00101	00
tubifer	00200	00100	00000	02000	01
erebicolus	00021	10012	10100	00100	00
gigas	00001	10000	00101	02110	00
Chinius	21001	10011	11010	00001	01
argentipes	00211	11111	11101	02101	10
stantoni	00111	21111	11001	02101	10
rodhaini	01021	11100	01011	11101	10
brevifilis	10021	11100	01001	01001	01
Kasaulis	01111	11111	1??01	02001	11
major	00111	21111	11101	10101	10
keshishiani	10121	21111	11101	10101	10
burneyi	10221	21111	11101	12101	10
Adlerius	11221	21111	11101	11101	10
papatasi	21111	11111	11101	02110	11
alexandri	20111	11111	11101	01111	00
martini	20121	11111	11101	01111	11
Grassomyia	01010	11112	?1101	00001	21
Parvidens	01000	11100	01101	02001	11
hodgsoni	00000	11100	01101	12001	21
anodonti	01010	11100	01101	01001	21
quatei	01012	21100	01101	01001	21
mirabilis	20000	00000	01100	00110	00
babu	01010	11100	01101	01111	21
pugifera	00010	11100	01100	11001	21
clydei	01021	11111	11111	10001	21
kirki	00010	11100	01101	01100	00
punjabensi	00000	11100	01101	01001	21



Figs., 78. A- M





#### 5. 4. 1. Phylogenetic groupings of Old World Phlebotominae

##### Out group- *Sycorax silacea*

Among the non-phlebotomine Psychodids, only the members of <sup>the</sup> subfamilies Sycoracinae and Trichomyiinae have paired spermathecae in female and bifurcated aedeagal elements in the male. However, Duckhouse (1972) considered that Sycoracinae, possibly together with the Horaiellinae are much closer to phlebotomines than the Trichomyiinae especially in the immature stages, correspondence of the spermathecal duct length with that of the aedeagal filaments, and the dorsally placed coxite and styles. Hence Sycoracinae, was used here as the out group.

##### *asperulus- Parvidens* clade

Taxa include *Idiophlebotomus asperulus*, *I. tubifer*, *Sergentomyia (Spelaomyia) mirabilis*, *Phlebotomus (Spelaophlebotomus) gigas*, *S. (Neophlebotomus) kirki*, and *S. (Parvidens) lesleyae*. These taxa are from the Oriental, Australasian, Afrotropical Regions. Furthermore, these taxa have close affinities with the out group, Sycoracinae, particularly in the presence of sac-like spermathecal bodies with short spermathecal ducts and single terminal spines on the male style. Because of these similarities, the *asperulus-Parvidens* clade is the sister group to the out group.

##### *hodgsoni- brevifilis* clade

##### *hodgsoni- punjabaensis* group

Taxa include *Sergentomyia (Neophlebotomus) hodgsoni*, *S. (Neophlebotomus)*

*quatei*, *S. anodontis*, and *S. (Sergentomyia) punjabensis*. Throughout this level, members of the taxa typically exhibit the plesiomorphic membranous tubular spermatheca found in the out group. Except for *pugifera*, which is the lone representative of Australasian Region, other taxa are from the Afrotropical, Palaearctic and Oriental regions.

*rodhaini-brevifilis* group

Taxa include *Phlebotomus (Anaphlebotomus) rodhaini* and *Phlebotomus (Australophlebotomus) brevifilis*. The former is from the Afrotropical Region and the latter is from the Australasian region. The spermathecae of both taxa have apomorphic features, such as a long spermathecal duct. The inclusion of *P. rodhaini* within the subgenus *Anaphlebotomus* is questionable, because, except for the long length of the spermathecal duct, other spermathecal features such as the unsegmented spermatheca and thick muscle fibres are autapomorphic.

*Grassomyia-Chinius* clade

*Grassomyia-clydei* group

Lewis (1984) considered the absence of ventral maxillary sensilla in *Grassomyia* as plesiomorphic, but the sclerotised capsular spermatheca is a unique and an autapomorphic feature. The segmentation of the spermatheca in *clydei* and other members of the subgenus *Sintonius* is due to homoplasy, an explicit apomorphic state found in the subgenera of *Phlebotomus*.

*erebicolus-Chinius* group

Although *Idiphlebotomus erebicolus* is included within the subgenus *Idiophlebotomus*, the spermatheca is segmented which is a derived feature. Hence a separate status may be warranted for this species. *Chinius* is considered to be a primitive taxon (Seccombe et al, 1993), but the spermathecal morphology and the male genitalia clearly show a trend towards derived features.

*argentipes-martini* clade

*argentipes-Kasaulius* group

Both taxa in the *argentipes-Kasaulius* group are very similar in their genitalic morphology and <sup>are</sup> distributed exclusively in <sup>the</sup> Oriental Region. Originally, Theodor (1948) and Artemiev (1979) considered *Phlebotomus newsteadi*, a member of *Euphlebotomus* because of the resemblance of the lateral spines of the aedeagus, shape of the paramere, coxite and style, long moniliform, segmented spermatheca. But Lewis (1982) removed *P. newsteadi* from the subgenus *Euphlebotomus* and erected a new subgenus based only on its narrow wing and a comparison of its leg segments with those of other species. The aedeagal filament supported by pointed rods in *P. newsteadi* closely resembles that of *Phlebotomus caudatus* in particular, a member of the subgenus *Euphlebotomus*, for which Lewis did not give any explanation. If *P. caudatus* is <sup>a</sup> close relative of *P. newsteadi*, then either both could be included in *Euphlebotomus* or separated off to form a new genus. Secondly, until the complete female morphology of *P. caudatus* is known, the status of *P. newsteadi* will remain an open question.

*papatasi-martini* group

Members of this group represent three different subgenera. In all the taxa, the genital morphology has synapomorphic features, such as the complete basal separation of the spermathecal ducts, development of a massive cuticular mass with secondary canals in the spermathecal body, a distinct coxite lobe and consistent number of 4 spines on the style. They also have an *symplesomorphic* feature like long lateral lobes relative to the coxite and style length. Hence, this clade can be considered in one new group which is above the generic level.

*stantoni-Adlerius* clade

Members of this clade could have been the most recent taxa in sandfly evolution. All the taxa within this clade except *stantoni* (which shows homoplasy in its genital morphology with that of the *argentipes-Kasulius* group) could have originated from a common stock with a spermathecal morphology in which the shared derived features (*synapomorphies*) of genital morphology include: variation in the distal extremities of the basal spermathecal ducts, spermathecal body with long primary canal (=neck), the shape of the aedeagus in *Larroussius* and the pattern of hairs distributed on the coxite lobes in *Adlerius*. Like the *papatasi-martini* group, the status of this taxa could be also considered above the generic level.

**5. 4. 2. Conclusion**

The phylogenetic study based on the spermathecal and male genitalic characters suggests that (i) the Old World phlebotomines are monophyletic in origin and (ii) the

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recent taxa (crown group) including the clades between 29 and 53, contain the vectors of leishmanises in the Old World.

**Annexure,1**

```
procedure c:\wp51\text\matrix *
xread
! Phylogeny of spermathecae and male terminalia in the Old World Phlebotominae
xread 0.0 seconds
procedure --
mhennig length 103 ci 29 ri 65 trees 4
mhennig 14.7 seconds
xsteps file 0 from mhennig 4 trees
tree/length
    0    1    2    3
  103  103  103  103
xsteps 0.1 seconds
xsteps file 0 from mhennig 4 trees
xsteps 0.1 seconds
mhennig length 206 ci 45 ri 83 trees 9
mhennig 16.9 seconds
xsteps file 0 from mhennig 9 trees
xsteps 0.3 seconds
mhennig length 153 ci 49 ri 86 trees 7
mhennig 12.1 seconds
xsteps file 0 from mhennig 7 trees
xsteps 0.2 seconds
mhennig length 153 ci 49 ri 86 trees 7
mhennig 12.0 seconds
bb file 0 from nelsen 1 tree
bb length 153 ci 49 ri 86 trees 102
bb 76.1 seconds
mhennig length 153 ci 49 ri 86 trees 7
mhennig 12.0 seconds
bb file 0 from mhennig 7 trees
bb length 153 ci 49 ri 86 trees 102
bb 75.0 seconds
xsteps file 0 from bb 102 trees
xsteps 2.3 seconds
bb file 0 from bb 102 trees
bb length 153 ci 49 ri 86 trees 102
bb 75.7 seconds
xsteps file 0 from bb 102 trees
xsteps 2.3 seconds
bb file 0 from bb 102 trees
bb length 153 ci 49 ri 86 trees 102
```

## Chapter 5

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bb 75.7 seconds

xsteps file 0 from bb 102 trees

tree/length

0	1	2	3	4	5	6	7	8	9
153	153	153	153	153	153	153	153	153	153
10	11	12	13	14	15	16	17	18	19
153	153	153	153	153	153	153	153	153	153
20	21	22	23	24	25	26	27	28	29
153	153	153	153	153	153	153	153	153	153
30	31	32	33	34	35	36	37	38	39
153	153	153	153	153	153	153	153	153	153
40	41	42	43	44	45	46	47	48	49
153	153	153	153	153	153	153	153	153	153
50	51	52	53	54	55	56	57	58	59
153	153	153	153	153	153	153	153	153	153
60	61	62	63	64	65	66	67	68	69
153	153	153	153	153	153	153	153	153	153
70	71	72	73	74	75	76	77	78	79
153	153	153	153	153	153	153	153	153	153
80	81	82	83	84	85	86	87	88	89
153	153	153	153	153	153	153	153	153	153
90	91	92	93	94	95	96	97	98	99
153	153	153	153	153	153	153	153	153	153
100	101								
153	153								

xsteps 2.7 seconds

xsteps file 0 from mhennig 7 trees

tree/length

0	1	2	3	4	5	6
153	153	153	153	153	153	153

xsteps 0.2 seconds

xsteps file 0 from mhennig 7 trees

tree 0 length 153 ci 49 ri 86

character/steps/ci/ri

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
---	---	---	---	---	---	---	---	---	---	----	----	----	----	----	----	----	----	----

## Chapter 5

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8 7 5 9 4 3 2 3 1 3 1 3 4 3 3 4 15 7 4  
25 14 40 22 50 66 50 33 100 66 100 33 25 33 33 25 13 14 25  
50 40 76 53 81 87 83 60 100 92 100 50 50 0 66 57 18 50 40

19 20 21  
4 7 6  
25 28 16  
50 68 61

tree 1 length 153 ci 49 ri 86

character/steps/ci/ri

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18  
8 9 5 8 4 3 2 3 1 3 1 3 4 3 3 5 15 8 4  
25 11 40 25 50 66 50 33 100 66 100 33 25 33 33 20 13 12 25  
50 20 76 60 81 87 83 60 100 92 100 50 50 0 66 42 18 41 40

19 20 21  
4 7 6  
25 28 16  
50 68 61

tree 2 length 153 ci 49 ri 86

character/steps/ci/ri

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18  
8 9 5 9 4 3 2 3 1 3 1 3 4 3 3 4 15 8 4  
25 11 40 22 50 66 50 33 100 66 100 33 25 33 33 25 13 12 25  
50 20 76 53 81 87 83 60 100 92 100 50 50 0 66 57 18 41 40

19 20 21  
4 7 6  
25 28 16  
50 68 61

tree 3 length 153 ci 49 ri 86

character/steps/ci/ri

0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18  
8 9 5 8 4 3 2 3 1 3 1 3 4 3 3 5 15 8 4  
25 11 40 25 50 66 50 33 100 66 100 33 25 33 33 20 13 12 25  
50 20 76 60 81 87 83 60 100 92 100 50 50 0 66 42 18 41 40

19 20 21  
4 7 6  
25 28 16  
50 68 61

## Chapter 5

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tree 4 length 153 ci 49 ri 86

character/steps/ci/ri

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
8	9	5	8	4	3	2	3	1	3	1	3	4	3	3	5	15	8	4
25	11	40	25	50	66	50	33	100	66	100	33	25	33	33	20	13	12	25
50	20	76	60	81	87	83	60	100	92	100	50	50	0	66	42	18	41	40

19 20 21

4 7 6

25 28 16

50 68 61

tree 5 length 153 ci 49 ri 86

character/steps/ci/ri

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
8	9	5	8	4	3	2	3	1	3	1	3	4	3	3	5	15	8	4
25	11	40	25	50	66	50	33	100	66	100	33	25	33	33	20	13	12	25
50	20	76	60	81	87	83	60	100	92	100	50	50	0	66	42	18	41	40

19 20 21

4 7 6

25 28 16

50 68 61

tree 6 length 153 ci 49 ri 86

character/steps/ci/ri

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
8	9	5	9	4	3	2	3	1	3	1	3	4	3	3	4	15	8	4
25	11	40	22	50	66	50	33	100	66	100	33	25	33	33	25	13	12	25
50	20	76	53	81	87	83	60	100	92	100	50	50	0	66	57	18	41	40

19 20 21

4 7 6

25 28 16

50 68 61

best fits

character/steps/ci/ri

0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
8	7	5	8	4	3	2	3	1	3	1	3	4	3	3	4	15	7	4
25	14	40	25	50	66	50	33	100	66	100	33	25	33	33	25	13	14	25
50	40	76	60	81	87	83	60	100	92	100	50	50	0	66	57	18	50	40

19 20 21

4 7 6

## Chapter 5

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25 28 16  
50 68 61

worst fits

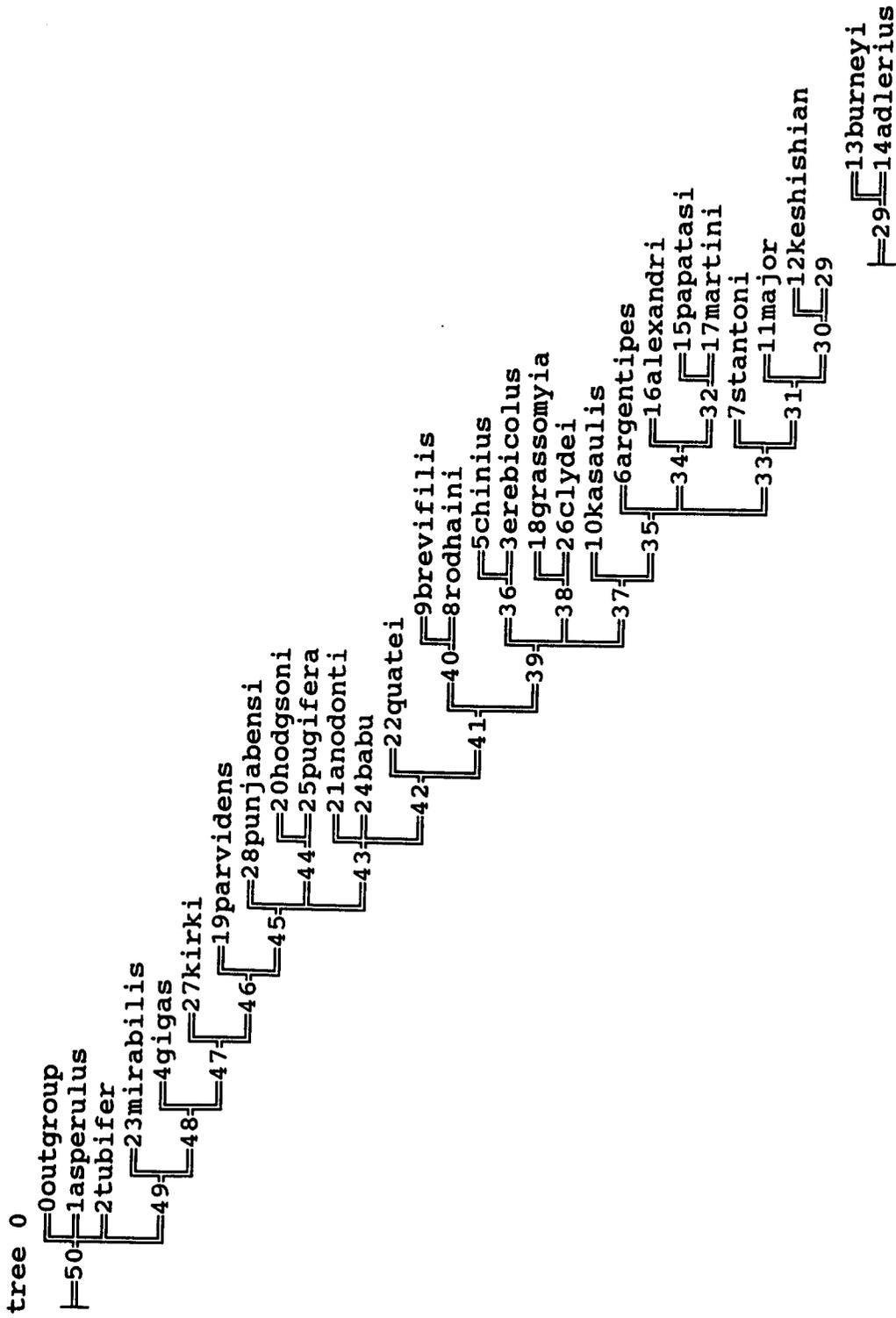
character/steps/ci/ri

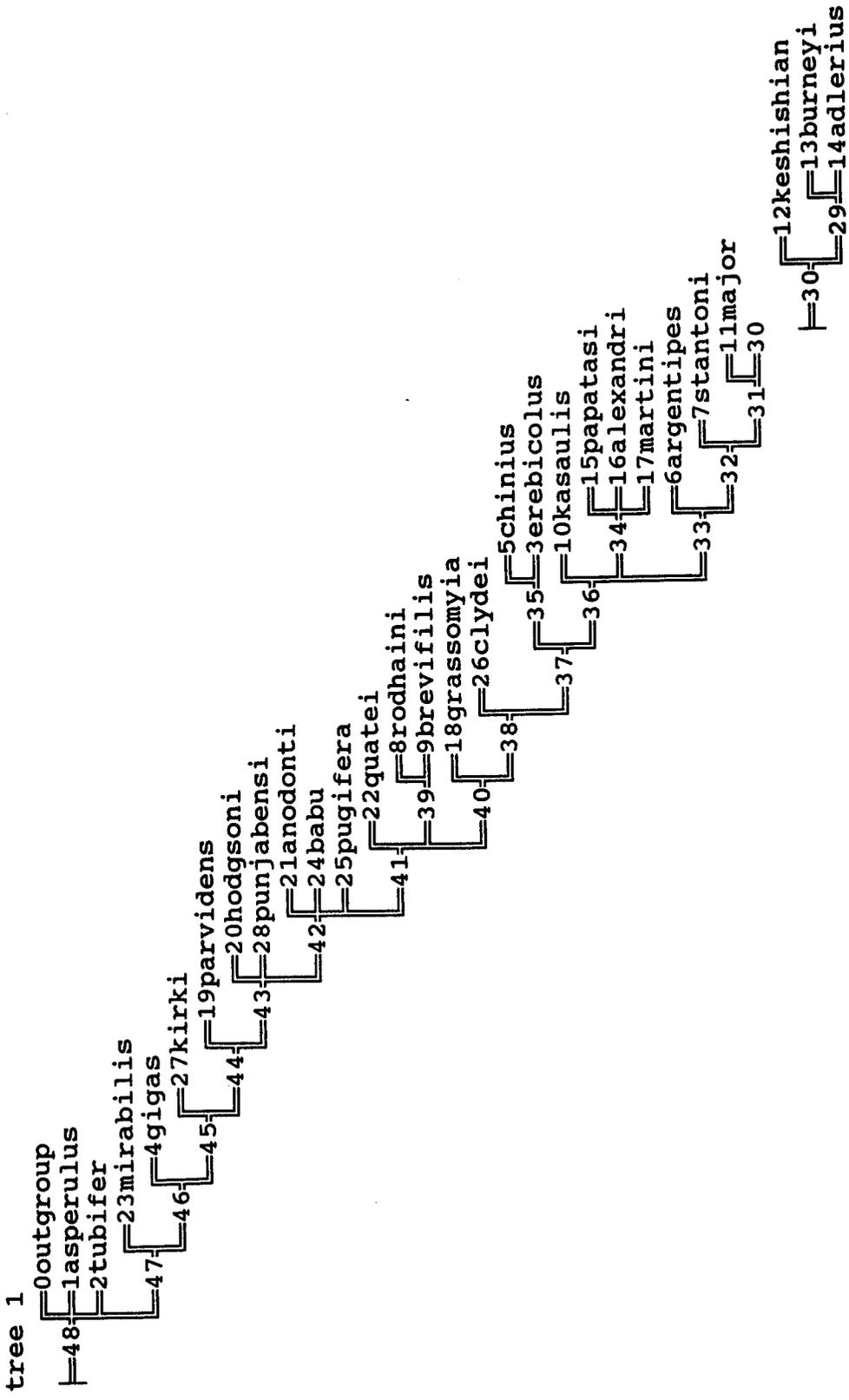
0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
8	9	5	9	4	3	2	3	1	3	1	3	4	3	3	5	15	8	4
25	11	40	22	50	66	50	33	100	66	100	33	25	33	33	20	13	12	25
50	20	76	53	81	87	83	60	100	92	100	50	50	0	66	42	18	41	40

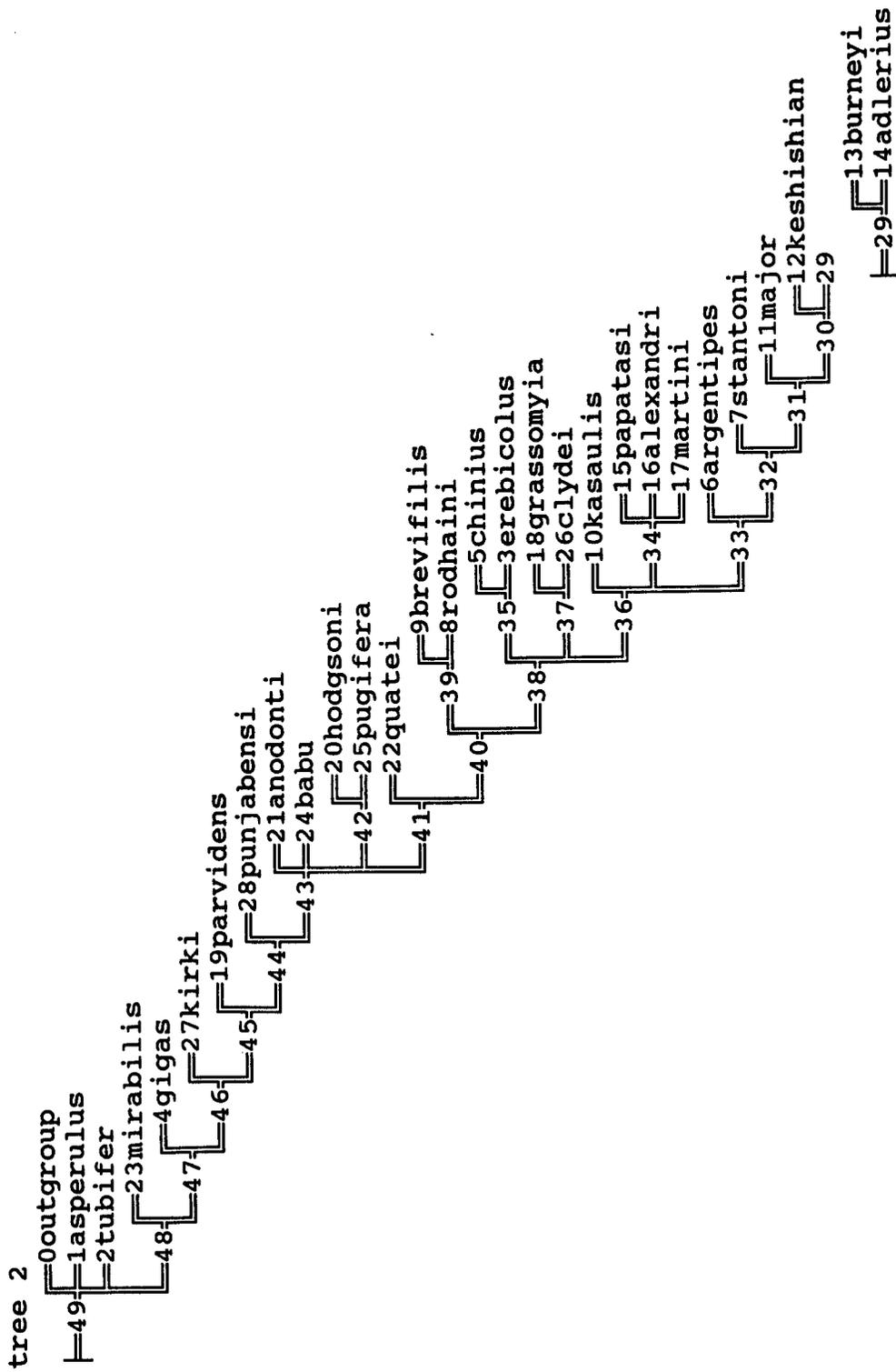
19 20 21  
4 7 6  
25 28 16  
50 68 61

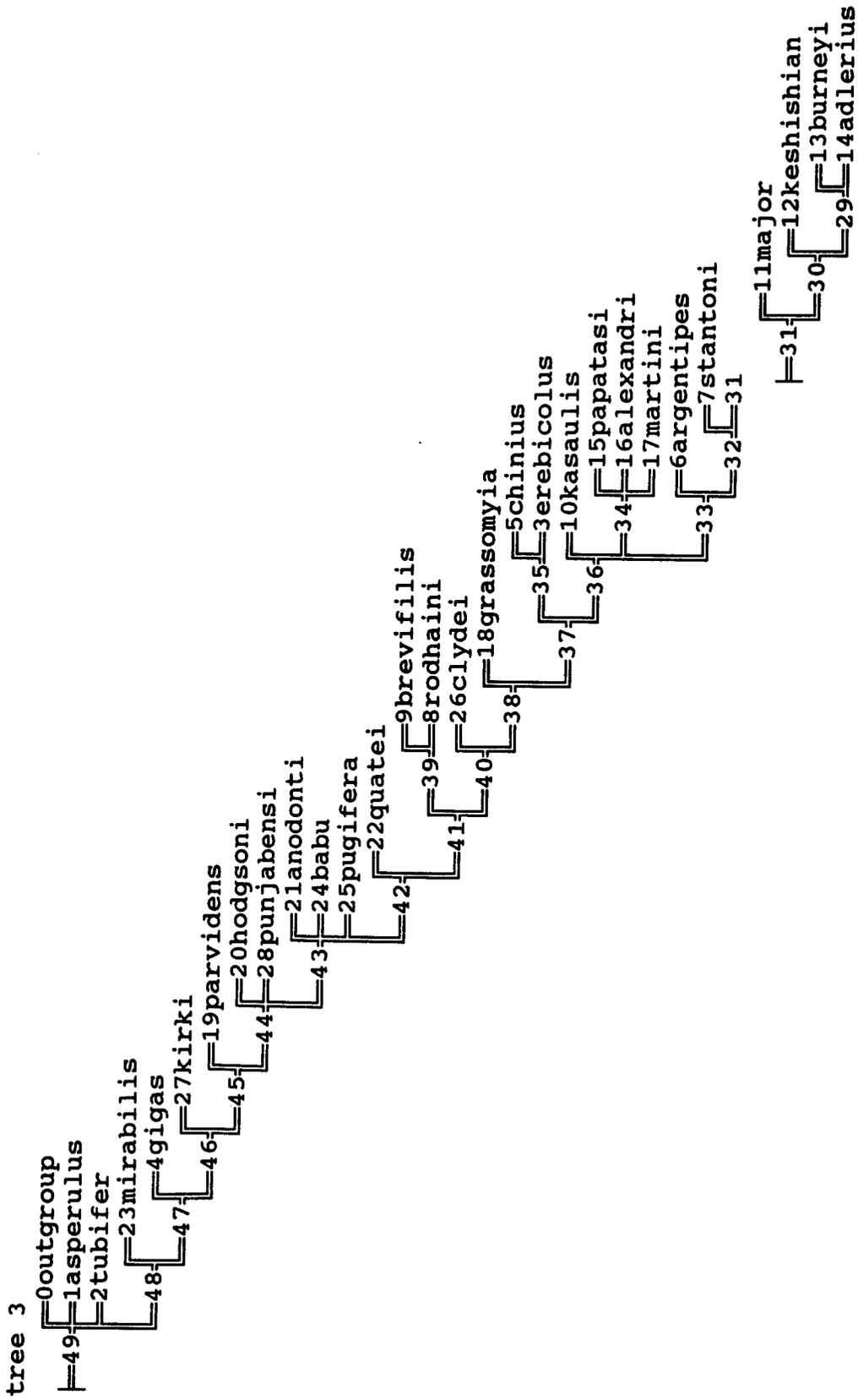
xsteps 1.7 seconds

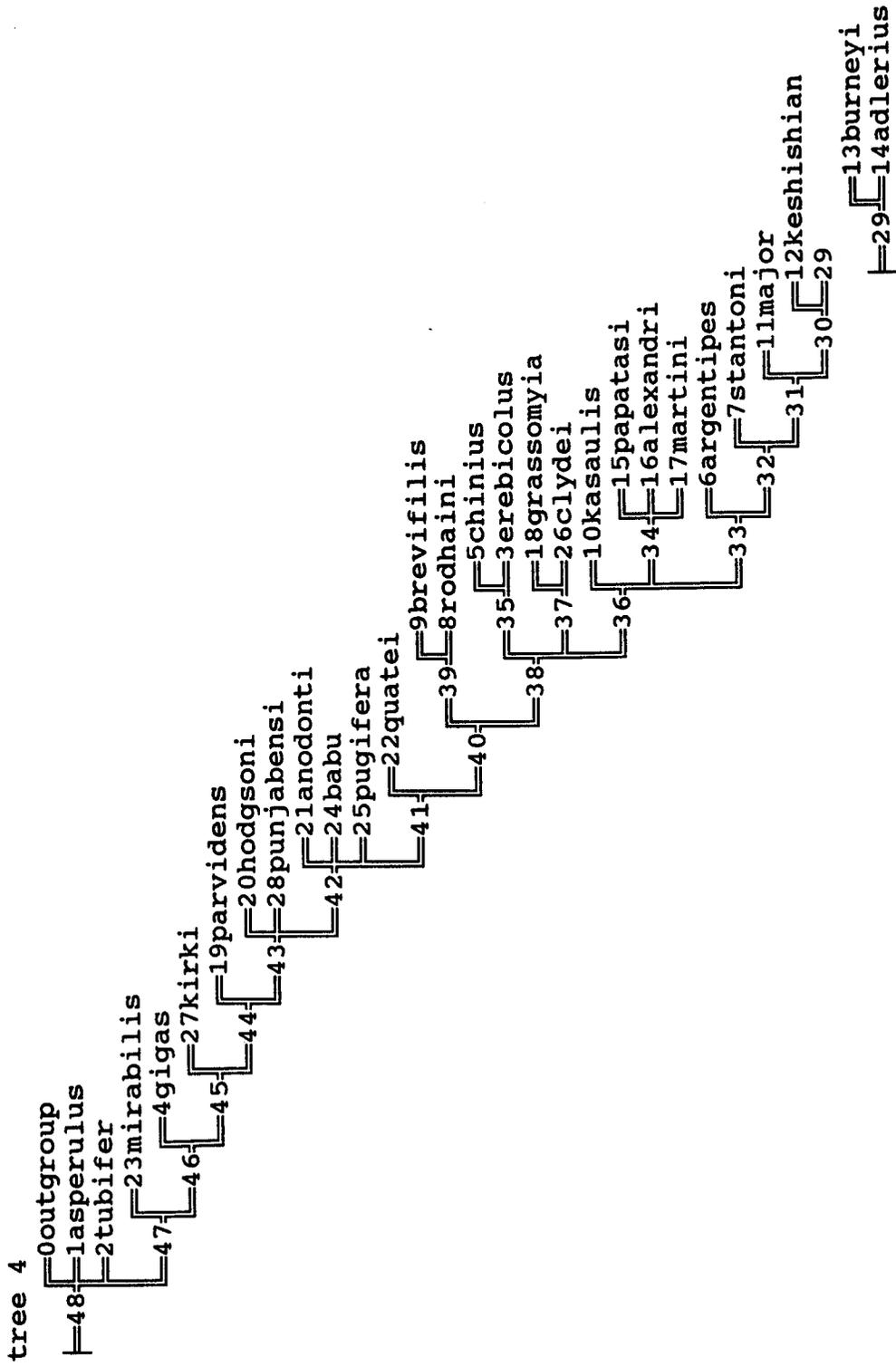
tplot file 0 from mhennig 7 trees



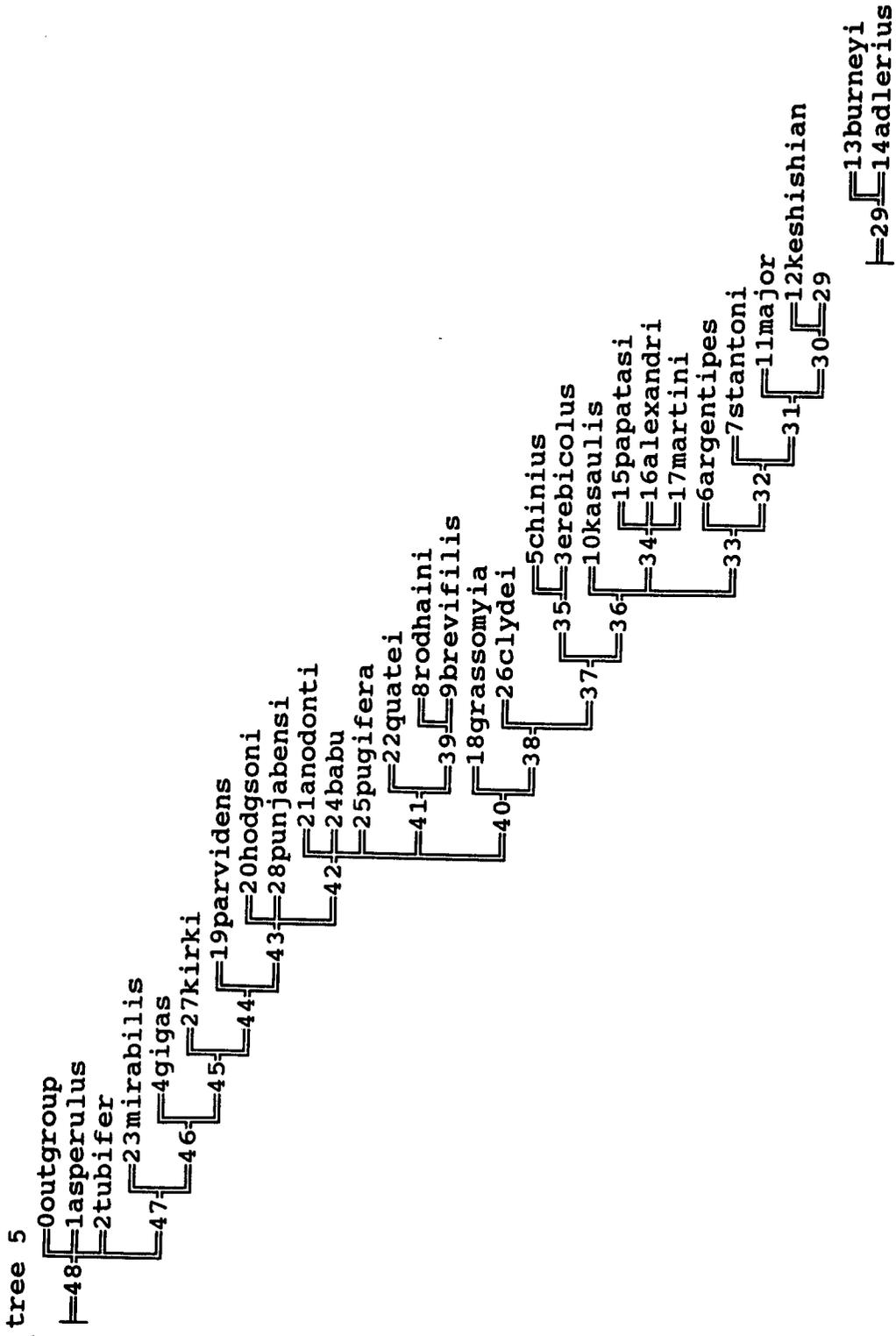


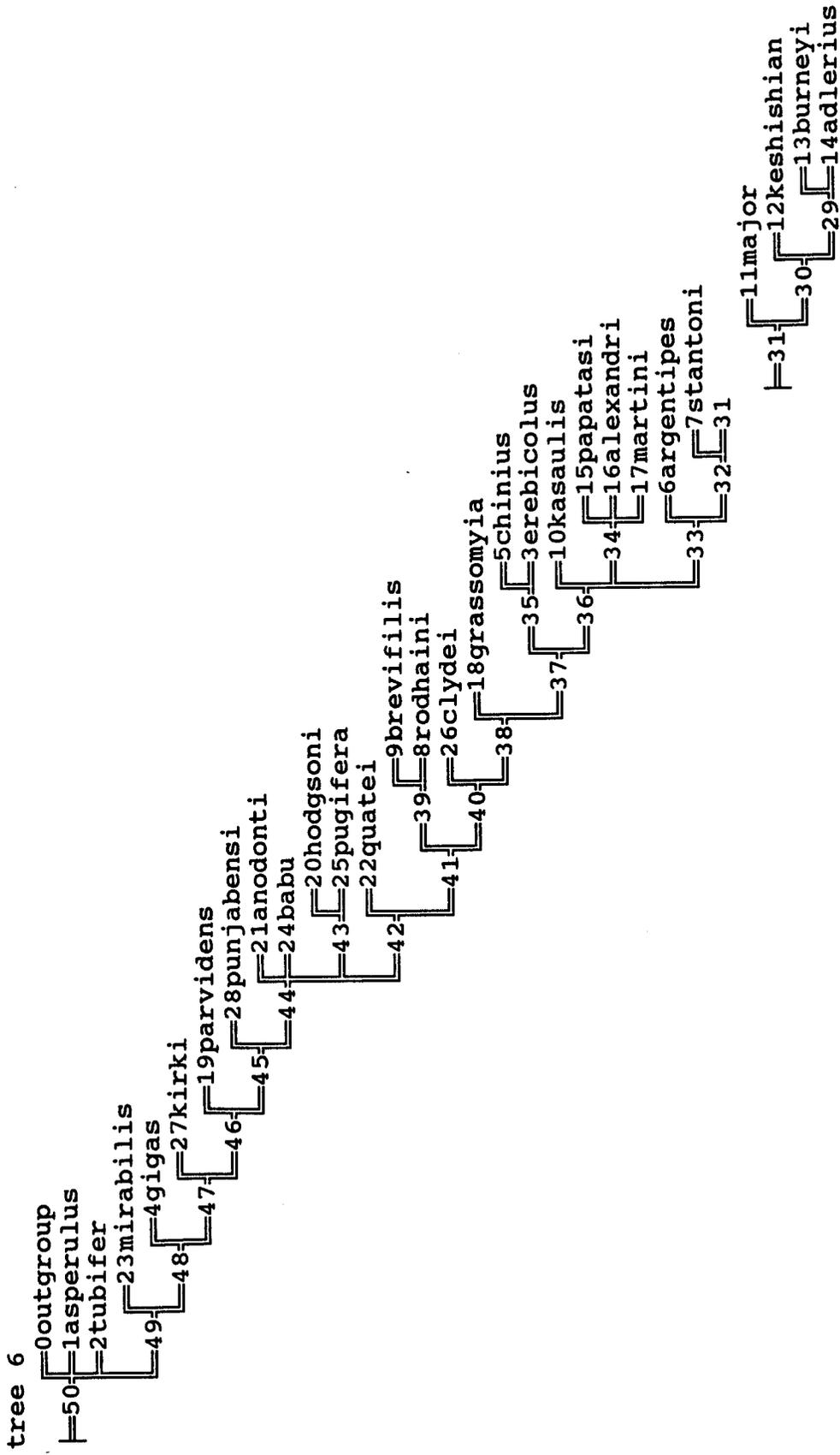




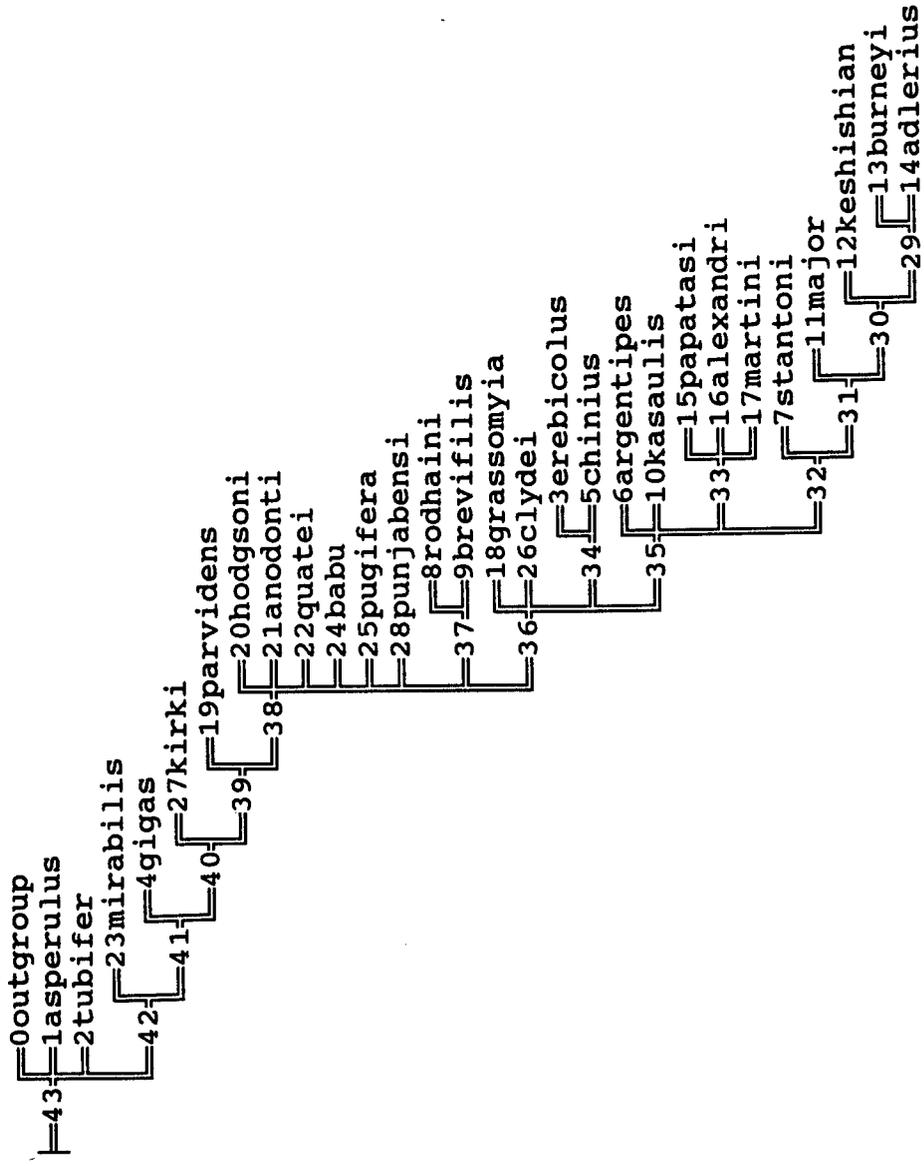


Chapter 5





```
tplot 3.8 seconds
nelsen file 0 from mhennig 7 trees
nelsen 0.0 seconds
xsteps file 0 from nelsen 1 tree
tree/length
0
165
xsteps 0.1 seconds
tplot file 0 from nelsen 1 tree
```



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