Structure and function of the spermathecal complex in the phlebotomine sandfly *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae): II. Post-copulatory histophysiological changes during the gonotrophic cycle

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The spermathecal complex of *Phlebotomus papatasi* Scopoli (Diptera: Psychodidae) undergoes histological and physiological changes during its gonotropic cycle. The present histochemical study revealed a mucopolysaccharide secretory mass in the spermathecae of the newly emerged sandfly. Sperm competition occurs when two or more males compete to fertilize an ovum in the female reproductive tract. In this study, spermatophores of two or more competing males were deposited at the base of the spermathecal ducts, which originate from the female bursa copulatrix. This suggests that females play a role in sperm displacement, which is defined as any situation in which the last male to mate with a female fertilizes maximum number her eggs. A blood meal ingested by the female for ovary development and egg laying stimulates the release of sperm from the spermatophore. The spermatozoa then migrate to the lumen of the spermatheca. The ultrastructure of spermatozoa comprises a head with double-layered acrosomal perforatorium, an elongate nucleus, and the axoneme with a 9 + 9 + 0 flagellar pattern. This axomene differs from the aflagellate axoneme of other Psychodinae. Morphological changes, such as the casting off of the acrosomal membrane, and histological changes in the spermatophore are also described. Mating plugs that have been described previously in sandflies appear to be artefacts. Females of *P. papatasi* may be inseminated more than once during each gonotrophic cycle, and additional inseminations may be necessary for each cycle. The relationships between the volumes of the sperm and the spermatheca were calculated to determine sperm utilization and fecundity of *P. papatasi*. As the females of *P. papatasi* mate polyandrously, the anatomical and physiological complexity of the spermathecal complex may be related to post-copulatory sexual selection.

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1. Introduction

Female insects store sperm in their spermathecae for later use in the fertilization of eggs. During the process of reception and storage of sperm, histological changes often take place in the spermathecae (Davey 1985). Earlier studies of these changes focussed on the ultrastructure and histochemistry to develop sterile-insect techniques for controlling pests of medical, veterinary and agricultural importance (Happ and Happ 1970; Davey 1985). Recently, however, the structure and shape of the spermathecae have also been the objects of special interest in comparative biology in reference to the concept of post-copulatory sexual selection in the evolution of insect mating systems (Walker 1980; Ridley 1989; Simmons and Siva-Jothy 1998; Pitnick *et al* 1999).

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Insect spermathecae produce glycoproteins or mucopolysaccharides through their glandular secretory cells (Happ and Happ 1970). The mucopolysaccharide secreted by the spermathecal gland has been shown to contain a chemotactic substance responsible for the transport and migration of spermatozoa from the female bursa copulatrix to the spermatheca in the boll weevil, *Anthnomous grandis* (Grodner and Steffens 1978). Very small amounts of the secretion from the spermathecal gland affect the motility and fertilizing capacity of spermatozoon (Grodner and Steffens 1978). The importance of these secretions for fertilization is demonstrated by the fact that the removal of spermathecal gland led to gradual loss of sperm motility and spermathecal emptying (Villavaso 1975, and also see Kaulenas 1992 and Gillott 2003). When studying sperm movement in response to the spermathecal gland secretion, it is important to understand how sperms are deposited in the female and what morphological changes take place in the sperm before reaching the bursa copulatrix. For example, studies have shown in the house fly, *Musca domestica*, that the acrosomal membrane of a spermatozoon was separated in the spermathecae (Degrugillier and Leopold 1976) and in the mosquito, *Anopheles subpictus*, that a blood meal was a prerequisite for the presence of sperm in its spermathecae (Roy 1940). Such studies, however, have not been done yet on *Phlebotomus*.

Many insects deposit their sperm within a spermatophore in the bursa copulatrix of the female. The formation of spermatophores is known to occur in seven families of Diptera, namely in four families of Nematocerans such as (i) the biting midges, the Ceratopogonidae (Linley 1981), e.g. *Culicoides* spp.; (ii) the midges, Chironomidae (Nielsen 1959), e.g. *Glytotendipes paripes*; (iii) the black flies, Simuliidae (Wenk 1965), e.g. *Simulium* spp; and (iv) the bibonid fly, Bibionidae (Leppla *et al* 1975), e.g. *Plecia neartica*; and in three major families of Dipterans such as (i) the tsetse fly Glossinidae (Pollock 1970), e.g. *Glossina austeni*; (ii) the stalk-eyed flies, Diopsidae (Kortaba 1996), e.g. *Cyrtodiopsis whitei*; and (iii) the sepsid flies, Sepsidae (Eberhard and Huber 1998), e.g. *Archsepsis* spp. In anopheline mosquitoes (Culicidae), a mating plug is placed by the male into the common oviduct of newly mated females (Giglioli and Mason 1966). This mating plug is formed through gelation, coagulation and solidification of parts of the ejaculates of the male accessory glands which is regarded as a vestige of the dipteran spermatophore. Davey (1985), howevere, disputed this terminology and argued that the mating plug was actually a spermatophore similar to that found in other Nematocerous Diptera. For vinegar fly species of *Drosophila*, the term "mating plug" (suggested by Alonso-Pimental *et al* 1994 based on ultrastructural studies) is restricted to the dense, compact, elastic structures that form within the uterus and distend it, but do not contain a sperm mass. Polak *et al* (2001) described the mating plug in *Drosophila hibisci* as serving not only as a nutritional gift and chastity device to prevent subsequent insemination, but also to hold sperm mass at the opening of the spermatheca and to facilitate its entry into it. Maroli *et al* (1991) described the formation of a mating plug in phlebotomine sandflies. The formation of a mating plug in Phlebotomine sandflies is improbable for two reasons: (i) The term "mating plug" in related families of Dipterans as cited above is now considered as spermatophore; and (ii) the spermatophore does not make any obstruction and allows subsequent inseminations to occur in the female reproductive tract but a mating plug prevents back flow of deposited spermatozoa from the female genital tract and puts up in the female an effective barrier against re-mating and re-insemination (Mann 1984). In Diptera, sperms are, therefore, transferred either to bursa copulatrix or to the base of the spermathecal openings (Linley and Simmons 1981). They are subsequently transported to one or more sclerotized spermatheca through a narrow, long spermathecal duct for storage. During the process of maturation and storage of sperm in insect spermathecae, the sperm sometimes undergo considerable structural changes, including the loss of the acrosomal membrane (Degrugillar and Leopold 1976) by detachment of the glycocalyx from the plasma membrane and hyperactivation (Selmi 1992). These studies have particular bearing on the spermatozoa and their movements in the female reproductive tract of *Phlebotomus* because its spermathecae show extreme variation and it takes blood-meal for ovary growth and egg-laying. Hence, the spermathecal variation in *Phlebotomus* is not only important to see how sperm are deposited and used in subsequent gonotrophic cycles but also a potential source of post-copulatory sexual selection.

In the recent past, the shape and function of the spermathecae in relation to the evolution of insect mating systems has been extensively studied (Walker 1980; Ridely 1989; Pitnick *et al* 1999). The diversity of sperm-storage organs is particularly prominent in taxa, such as *Drosophila* spp., in which post-copulatory sexual selection is likely to occur (Pitnick *et al* 1999). Post-copulatory sexual selection mechanisms may occur due to: (i) competition for ovum fertilization among the sperm of different males present in the genital tract of a female (Parker 1970); (ii) cryptic female choice in which non-random paternity biases result from the morphology, physiology, or behaviour of females after copulation (Pitnick and Brown 2000); and (iii) evolutionary arms races in which there is a conflict between the sexes for the control of reproduction and speciation (Lloyd 1979; Holland and Rice 1998; Arnqvist *et al* 2000). In his analysis of the incidence of sperm displacement in insects, Walker (1980; but see Ridley 1989) hypothesised that species with spheroid spermathecae should show

low sperm displacement, and species with elongate spermathecae high sperm displacement. The sperm displacement is often being referred as P^2 – the proportion of eggs fertilized by the last of two or more males. Simmons and Siva-Jothy (1998) found that the multiple spermathecae found in Diptera revealed low sperm displacement, while primitive insect taxa with a single spermatheca exhibited high displacement of sperm. Cryptic female choice was demonstrated in the yellow dung fly *Scathophaga stercoraria* in which sperm of rival males are stored in different portions of the spermathecal compartments during copulation (Hellriegel and Bernasconi 2000) and only large females may benefit from increased number of the spermathecae by being able to act against male interests as the female need not store sperm from each male separately (Hosken and Ward 2000; Ward 2000). Sexual conflict has been invoked to explain the correlation of the evolution of female reproductive morphology with either the length of the spermatozoa, as in stalk-eyed flies *Cyrtodiopsis* sp. (Diopsidae) (Presgraves *et al* 1999) and in *Drosophila* spp. (Pitnick *et al* 1999), or with the length of the male genital tract as in phlebotomine sandflies (Ilango and Lane 2000). Yet the interactions between male characteristics, such as the structure of the penis and sperm and the composition of the accessory fluid, and the female reproductive morphology and physiology remain to be demonstrated (Pitnick *et al* 1999).

Phlebotomine sandflies provide a potentially rich source of information on the interactions between male and female reproductive characters because the morphology of their sperm-storage organs is extraordinarily diverse. Females of *Phlebotomus papatasi* and almost all phlebotomine sandfly species typically possess a pair of spherical, sclerotized spermathecal bodies, each of which is connected to a bursa copulatrix through a long, narrow spermathecal duct. The spermathecal body is capped by a large glandular secretory cell. The spermathecal body and duct are lined by a non-porous and impermeable resilin-rich cuticle supported by simple epithelial cells and visceral muscle fibres (Ilango 2005).

The objectives of the present study are to provide a baseline study of the spermathecae of *P. papatasi* to determine: (i) how sperm are transferred from the male to the female, whether a spermatophore is produced by the males, and whether there is any anatomical evidence for sperm competition or sperm displacement in the female; (ii) the histophysiological changes that take place in the spermatheca during the gonotrophic cycle of the female; (iii) how the sperm are transported into the spermatheca of the female and subsequently utilized in the fertilization of the eggs; and (iv) whether there are any correlations between the volume of sperm and spermatheca.

2. Materials and methods

Phlebotomus papatasi is a vector of *Leishmania major* and is distributed widely across the Old World (Killick-Kendrick 1990). The colony of *P. papatasi* that was used for the experiments originated from Israel and was maintained at 26°C and approximately 75% relative humidity at the London School of Hygiene and Tropical Medicine. The sugar solution and blood-meals provided to the sandflies are mentioned in the following experiments.

2.1 *Experimental design for the documentation of histological changes of the female reproductive tract*

The age and condition of the sandflies used in these experiments are given in table 1. To determine whether there were any structural changes in the spermathecal complex throughout the gonotrophic cycle, the sandflies were kept under a variety of conditions and examined at different times (see table 1). The main factors determining the presence of spermatozoa in the spermathecae that were studied were female access to males and a blood meal. Control females were kept separately from males and fed only on a 5% sugar solution. To ensure that females and males were virgin for all the experiments, newly emerged adults were separated within 12 h of emergence, i.e. before the male genitalia rotate. The latter condition is found in most flies belonging to the suborders (Nematocera, Diptera), in which the male genitalia is inverted either permanently after emerging from the pupa or temporarily during mating. This inverse interlock facilitates the male to copulate the female from the bottom her genitalia (Bickel 1990).

2.2 *Preliminary processing of adult sandflies*

All dissected sandflies were immobilized prior to the dissection of their reproductive organs at 0°C for 5 min, dipped in a solution of 0·05% Tween 80 detergent solution to wet them and ensure their immersion, and washed three times in distilled water. Excess water was removed with tissue paper. All dissections of the sandflies were performed in normal saline (i.e. 0·15 M NaCl solution). To study the bursa copulatrix, spermathecal body and duct, the four terminal abdominal segments were removed to process them subsequently for either light microscopy or transmission electron microscopy as detailed below.

2.3 *Test for acid and neutral mucin*

To determine whether mucopolysaccharides were present in the 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 normal-saline solution to avoid a sudden contraction of the spermathecae and the rest of the female reproductive tract, dehydrated in a graded ethanol series, and embedded in paraffin wax. Transverse sections were cut 1 µm thick, dewaxed, and stained with an Alcian blue solution of 1 g Alcian blue in 100 ml, 3% acetic acid and Schiff's reagent (the commercial reagent provided by the British Drug House, UK) (Bancroft and Stevens, 1990) for 5 min, washed in distilled water, and then washed in 1% aqueous periodic acid for 5 min, rinsed in distilled water for 5–10 min, stained lightly with Harris haematoxylin solution, again washed in distilled water, dehydrated in alcohol series, and mounted in Canada balsam/DPX.

2.4 *Transmission electron microscopy*

The entire specimens were first fixed by gradually replacing the saline with a solution of 3% glutaraldehyde in saline at room temperature over the span of 2 h to avoid a sudden contraction of the muscle fibres that surround the spermatheca. The specimens were subsequently fixed overnight in 3% glutaraldehyde in 0·075 M sodium cacodylate buffer at 4°C. After fixation, the material was successively washed in washing buffer at 4°C, post-fixed in 1% osmium tetroxide in 0·075 M sodium cacodylate buffer at 4°C for 1–2 h, washed in distilled water, and block stained in 2% uranyl acetate in 30% methanol. Specimens were then dehydrated in graded methanol (30%, 60%, 70%, 80%, 90%, 100%, 100%; 10 min in each grade) and transferred to 1,2-epoxy propane for 20 min. Because the spermathecal complex is lined with an impermeable cuticular intima that could hinder infiltration, the following combinations of epoxy resin and propylene oxide to facilitate the infiltration were used: 25% + 75% for 30 min, 50% + 50% for 1 h, 75% + 25% for 1 h before infiltration with resin. Infiltration with 100% resin was performed overnight at 4°C, before embedding the sections in fresh resin and polymerising them at 60°C for 48–72 h. Sections 35–40 nm thick were cut using glass knives, placed

Table 1. Chain of events occurring in spermathecal body and duct during the gonotriphic cyles of *P. papatasi.*

Hours after emergence	Condition of flies	No. of flies examined	No. of flies positive for secretory mass, spermatophores and sperms					
			Spermatheca		Spermathecal duct			
			Secretory mass	Sperms	Secretory mass	Spermato phores	Sperms	No. of flies not scored
< 12	Sugar fed no access to ♂♂	15	9					6
24	Sugar fed access to ♂♂	14	5			1		9
48	Sugar fed access to ♂♂	10	5			1		5
72	Sugar fed access to ♂♂	10	3			2		7
120	4 h post-bloodfed access to ♂♂	16	4			3		12
120	24 h post-bloodfed access to ♂♂	18	3	1 (upward movement)	6	12	6	
168	72 h post-bloodfed access to ♂♂	15		1 (downward movement)			1 (downward movement)	14
168	Sugarfed no access to ♂♂	Each group with 10 as a control group	Residues found		Residues found			
168	24 h post-bloodfed no access to ♂♂							
720	600 h post-bloodfed access to ♂♂ at ovipostion	46	1 (as in virgin/newly emerged)		1 (as in virgin/newly emerged)			24/46 laid eggs and one survived oviposition.

on 200 mesh copper grids (with and without formvar film), and stained with Reynold's lead citrate for 5 min before examination. Specimens from at least 10–15 sandflies were sectioned, and usually 20–30 grids were examined from each sandfly. For the study of the secretory nature of each spermathecal gland, spermatophores and sperm from more than 10 sandflies were sectioned and 50 grids were examined from each sandfly.

2.5 *Study of the transfer of spermatophore and sperm*

To determine the timing of sperm transfer during the gonotrophic cycle, sandflies were observed mating at different times before oviposition, removed from the large cages, and then dissected in saline solution. The structure and movement of spermatophores and sperm were observed and documented with a Polyvar phase-contrast microscope at X1000.

2.6 *Study of oviposition, fecundity and sperm utilization*

To observe the movement and behaviour of sperm at oviposition, inseminated blood-fed female sandflies were kept individually in separate containers with no substrate for egg laying. After 24 h, individual sandflies were fixed carefully by the thorax on a clean glass slide with glue. In order to induce female sandflies to lay eggs, the tip of her posterior terminal segment was grasped gently with the male genitalia by holding a live male sandfly. When egg laying began, the live female was dissected carefully in saline solution, and the movements of sperm were observed in the spermatheca and its ducts.

To determine whether all the sperm are used at oviposition, blood-fed sandflies were kept individually in a small tube (ca. 1 cm in length × 4 cm in width). The tube was covered with muslin cloth and sugar meal was placed on top of it and its bottom was moistened with tissue paper for egg-laying. The major problem with laboratory-reared sandflies is that hardly any of them survived oviposition, which may be a result of experimental condition observed among phlebotomine sandflies in many experimental studies in the past. Of the 46 sandflies, 24 laid eggs, but only one sandfly survived the oviposition of 28 eggs. The lone surviving female sandfly was dissected carefully and the contents of its spermatheca and ducts examined.

The length of the spermatozoa and spermathecal ducts was measured by light microscopy and the diameter of spermatozoa by transmission electron microscopy (TEM). The volumes of the spermatheca proper, spermathecal duct, spermatophore, and spermatozoa were calculated from both light microscopy and TEM images by using the equation for a cylinder $pr^2 h$, where $r = 0{\cdot}5$ diameter and h = length.

3. Results

3.1 *Processes immediately following emergence*

In newly emerged sandflies, the lumen of the spermatheca proper is filled with a filamentous mass (figures 1A and 3A, B) that appears as a pile of stalks. The secreted substances in the spermatheca and in the reservoir and cuticular ductules of the spermathecal gland are strongly PAS positive, which indicate that this glandular product is an acid mucopolysaccharide (figure 1B, table 1). Furthermore, the long fibrous nature of the secreted substances shows that it is presumably insoluble in nature and of a fairly low molecular weight.

3.2 *Pre-copulation condition*

Within 12 h after emergence of a sandfly, changes occur in its spermathecal gland and in the lumen of the spermatheca and its ducts. Initially, the secretory mass from the spermatheca is discharged. This discharge apparently must occur first or else it would cause either mechanical obstruction to the upcoming spermatozoa from the basal spermathecal ducts or prevent the spermatozoa from lodging within the lumen of the spermathecal body in which sperms were stored. In 12 h old sugar-fed sandflies, the secreted substance tends to dissociate and is often seen helically coiled, or loose in the spermathecal duct (figure 3A).

Females of 12 to 24 h of age, which were fed only sugar, were observed when the females were introduced into a cage with males. Five to ten males congregated, chasing one another on the ceiling or sides of the cage. One of the males copulated with a female, and the copulation lasted from a few seconds to 3 min. The virgin females that copulated once moved to another male aggregate for a subsequent mating. Inseminated females were removed carefully from the population cages while still *in copula*, and the spermathecal ducts and components of the spermatophores were examined.

3.3 *Post-copulatory changes*

3.3a *The ultrastructural and histological changes of the spermatophore*: Immediately after copulation in one-day-old sugar-fed females, a single spermatophore was observed in each spermathecal duct (figure 1C). Each spermatophore is a cylindrical structure 44 µm long, completely filling the cross-sectional area of the spermathecal duct and occupying about one-third of its length. It contains an electron-dense fibrous seminal fluid, which surrounds the live spermatozoa. At this stage, the spermatozoa in the seminal fluid are inactive. Furthermore, the

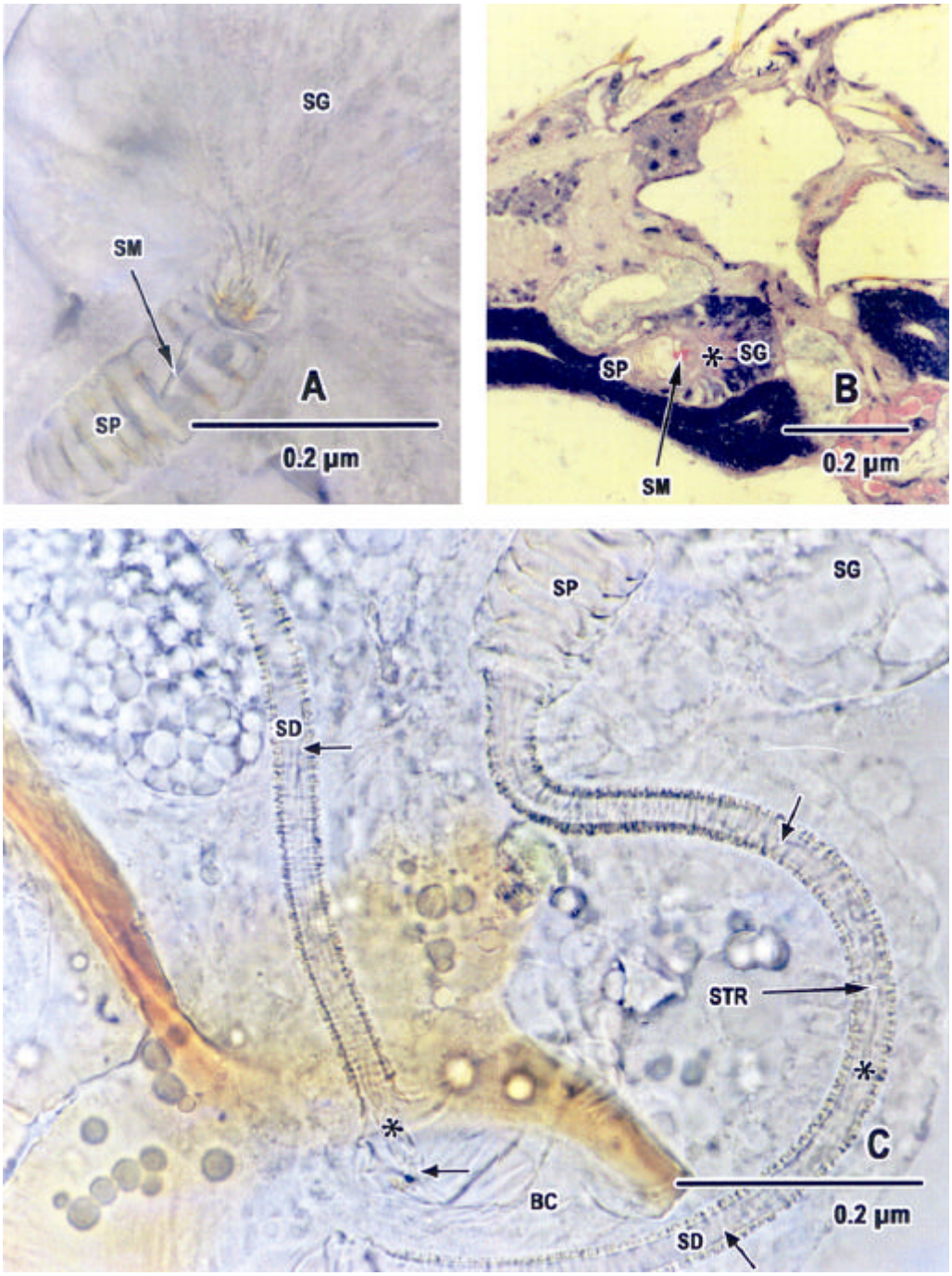

Figure 1. Light microscopic photograph of the spermatheca of *P. papatasi*. (**A**) Saline squash specimen of a newly emerged sandfly showing a spermatheca, in which the lumen of the spermathecal body (SP) is surrounded by the spermathecal gland (SG) and is filled with the secretory mass (SM). (**B**) Transverse section through the posterior abdominal region showing the reservoir and cuticular ductule of spermathecal gland cells (SG) and the lumen of spermatheca proper (SP) of a newly emerged sandfly indicating the mucopolysacchride nature of the secretion. The cytoplasm of the gland cells (*) and spermathecal body show positive staining, and the production of amino acid/protein exclusively with Alcian blue. (**C**) Saline squash of a 24 h old sugar-fed sandfly immediately after insemination. One spermatophore (STR) has been placed in each spermathecal duct (SD). Note the lower and upper end (short arrows) of the spermatophores, showing that they are not located in the same position relative to the spermathecal duct (SD). The spermatozoa (*) are immobile within the spermatophore. SP, spermathecal body; SG, spermthecal gland.

placement of the spermatophore in both spermathecal ducts was not identical in any of the sandflies examined, although the spermatophores were usually positioned at the entrance of the spermathecal ducts. In one sandfly, three spermatophores that had presumably been inserted by three different males were seen in a single spermathecal duct (figure 2A) lying one above the other with considerable gaps between them. All spermatophores remained intact until the female sandflies had been fed a blood meal.

There were drastic changes in the behaviour of spermatophores within the spermathecal ducts within 20–30 h of a blood meal by the female. The spermatophores were seen breaking down, and the spermatozoa moved towards the spermatheca, leaving behind remnants of seminal fluid. This fluid then flowed towards the bursa copulatrix. These processes were inferred from both saline squash dissections (figure 2A) and serial section of TEM (figure 3C). In untreated control experiments, female sandflies that were fed a sugar solution and a blood meal, but had no access to males, continued to produce an aqueous fluid in their spemathecae. The secreted fluid, however, was as electron-lucent in sugar-fed sandflies and electron-dense in blood-fed sandflies.

3.3b *The ultrastructure of the spermatozoon*: The spermatids in freshly transferred spermatophores were immobile, clustered together, and difficult to observe. However, once released from the spermatophore, the spermatids that could not be observed individually were shorter (figure 1C) than the fully developed mature spermatozoa (figure 2B). The mature spermatozoon was 17 µm long (figure 2B) and 0·7 µm wide (figure 4B) at the time of fertilization. Dallai *et al* (1984) described the spermatozoon of *P. papatasi* as follows: Spermatozoon contains an elongate nucleus in its head that is capped by an acrosomal membrane. The acrosome has a flattened, concave cisternum limited by parallel membranes and homogeneous, electron-dense contents. The concavity of the acrosome is filled by a plug of a translucent substance with granules and filaments, collectively known as the perforatorium. These observations were confirmed in the present study, but Dallai *et al* (1984) seem to have overlooked the differences of the spermatozoa in the vas deferens and the spermathecae. In the vas deference, a spermatozoon has an acrosomal outer membrane, which is lost when it reaches the spermatheca. Cross sections of the post-nuclear region (figure 4B) revealed the following features: The axoneme of flagellar tubes were composed of protofibrils with a "9 + 9 + 0" pattern; the doublets of protofibrils comprised inner and outer arms (i.e. dynein arms); the nine peripheral singlets that lie peripherally between the doublets lack intertubular (coarse fibre) material; and 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.

3.4 *Changes in post-blood feeding of the sandfly, entry, movement and arrangement of spermatozoa within the spermatheca*

The spermatozoa found in the spermatophore immediately after insemination in sugar-fed sandflies were immobile. But in 5 days old sandflies and 20–30 h after a blood meal (figure 2A), the spermatozoa were seen leaving the spermatophore and moving with their characteristic serpentine lashing movements towards the spermatheca. During this migration, the spermatozoa shed the acrosomal membrane (figures 3D and 4B), the remains of which were in the spermatophore at the base of the spermathecal duct.

Entry of spermatozoa into the spermatheca may be regulated by the sphincter valve at the base of the spermatheca that is surrounded by super-contracting visceral muscle fibres (Ilango 2005). After a spermatozoon enters the lumen of the spermatheca, it occupies the interseptal areas and the centre of the lumen. Five to nine spermatozoa were observed in interseptal area, and the distribution of the spermatozoa was unorganized (figure 3D); 5–7 spermatozoa were able to move into the lumen of the spermathecal duct (figure 4A).

3.5 *Fertilization and oviposition*

When sandflies were induced to oviposit 72 h after their blood meal, individual spermatozoa passed through the spermathecal ducts (figure 2B). The sphincter valves at the base of the spermatheca presumably regulated the release of mature spermatozoa from the spermatheca. Spermatozoa were not released simultaneously from both the spermathecae, but rather alternately. When a released spermatozoon from one spermatheca was about to reach the base of the spermathecal duct near the bursa copulatrix, the next spermatozoon was released from the other spermatheca. The spermatozoa seen in the spermathecal ducts were longer than the spermatozoa that were observed entering the spermathecal ducts. The spermatozoa moved with characteristic lashing movements at the same speed until they reached the egg micropyle.

3.5a *Post-oviposition changes in the spermathecae of the sandfly*: When the spermatheca of the only sandfly that had survived oviposition was examined under the microscope, no spermatozoa were seen in its spermatheca or spermathecal ducts. Instead, the lumen was filled with a secreted substance that differed from that of a virgin

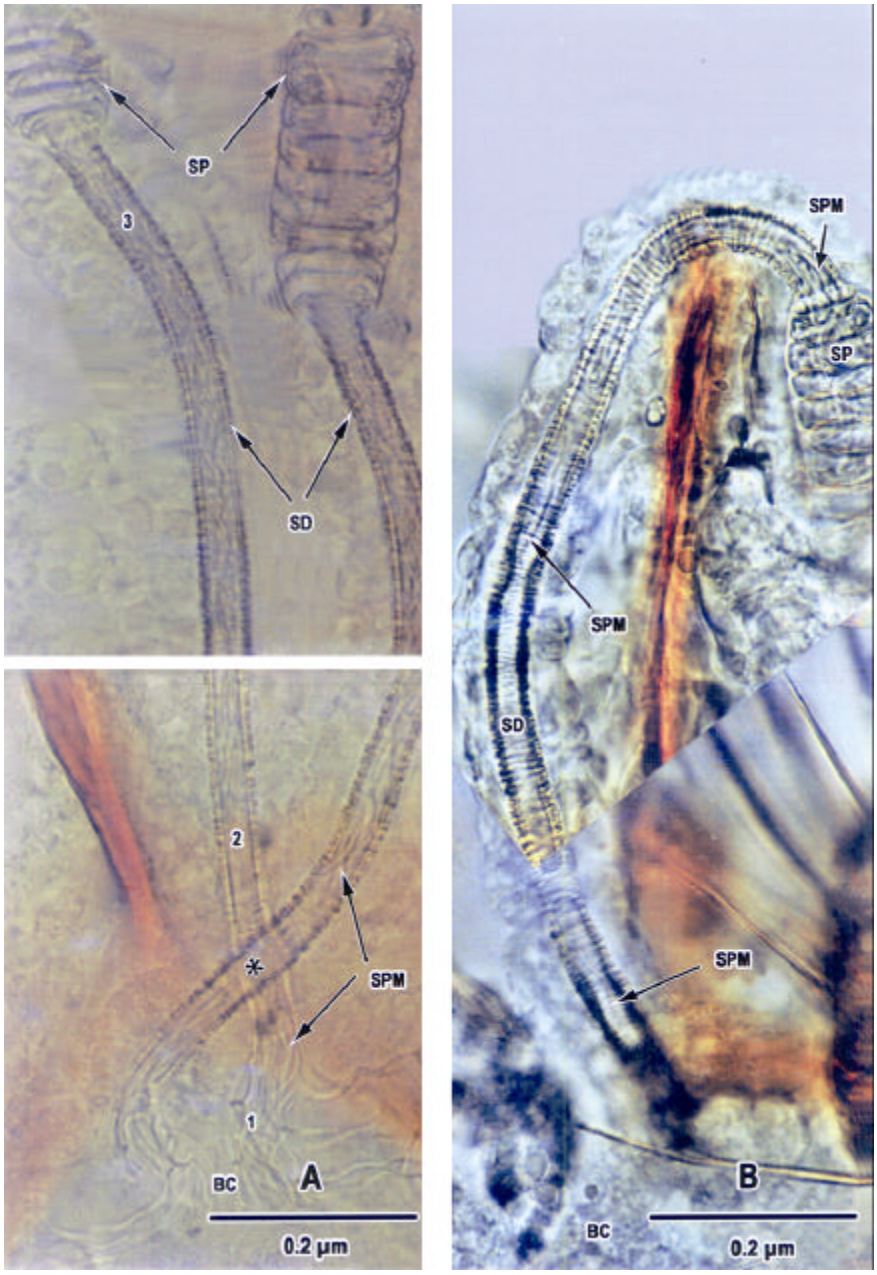

Figure 2. Light microscopic photograph of the spermatheca of *P. papatasi.* (**A**) Saline squash of 7 days old sandfly, 24 h after a blood meal, showing spermatozoa in spermatophores and the spermathecae. In one spermathecal duct, there are three spermatophores (1, 2, 3) lying one above the other with a considerable gap between them suggesting that a number of inseminations took place. At this stage, the sperm (SPM) are active, leaving the spermatophores in the spermathecal duct (SD) and moving towards the spermathecal body (SP). (*Note*: This photograph is a composite of two pictures due to the large space occupied by the spermathecae in this magnification). (**B**) Saline squashes of the spermatheca and the movement of spermatozoa (SPM) during oviposition: From the spermathecal body (SP), spermatozoa are presumably released by a sphincter valve at the base of the spermatheca; a spermatozoon moving down in the middle of the spermathecal duct (SD) and reaching the bursa copulatrix (BC) where it subsequently enters the micropyle of an egg that is not visible in this picture. (*Note*: This photograph is a composite of three pictures due to the large space that occupied by the spermathecae in this magnification).

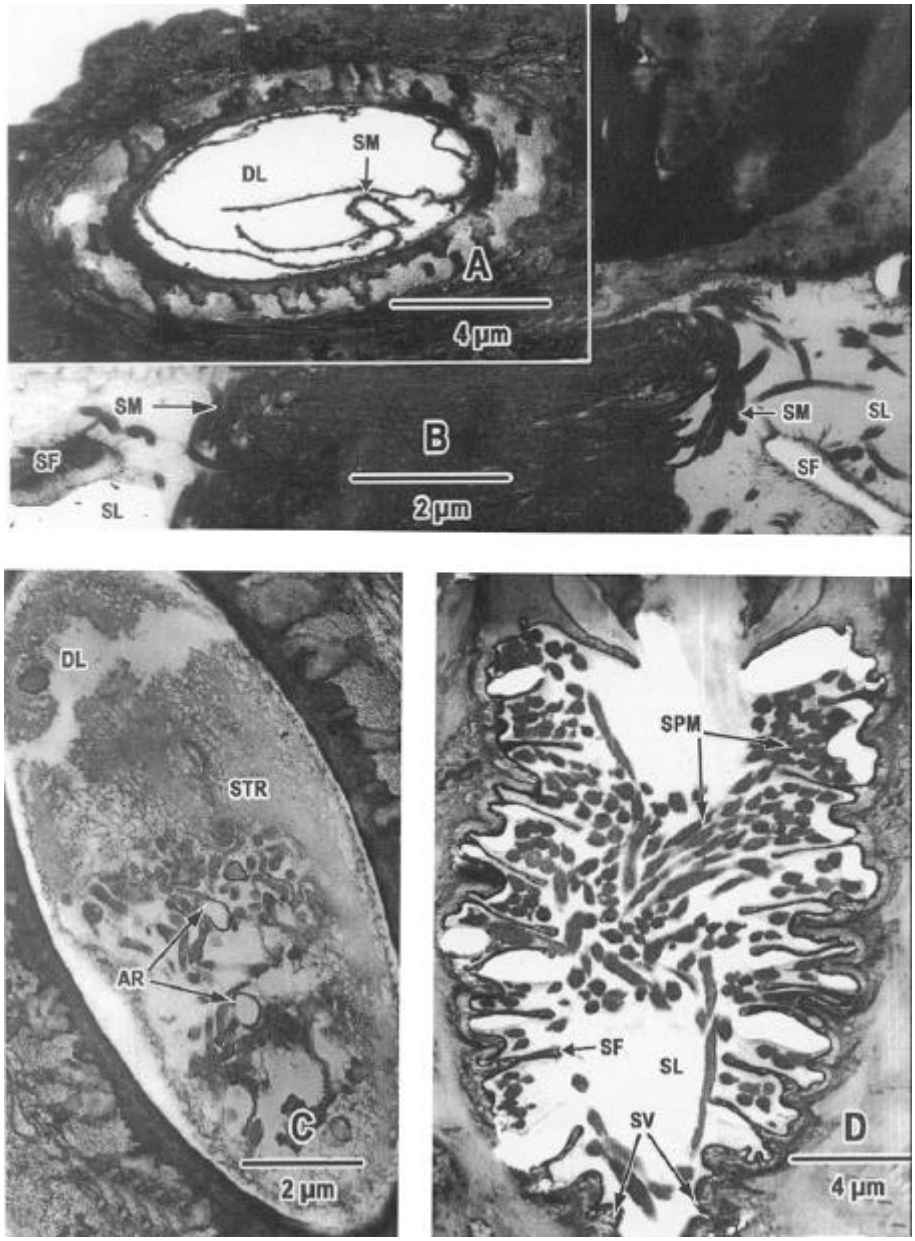

Figure 3. TEM photograph of the spermatheca of *P. papatasi*. (**A**) Cross section through the basal section of the lumen (DL) showing the extrusion of helically coiled fibrous secretory material (SM) in the spermathecal duct (SD) in a ca. 24 h old sandfly before insemination. (**B**) Longitudinal section through a spermatheca of a sandfly showing the fibrous secretory mass (SM) between the septal foldings (SF) of the upper lumen (SL) of the spermathecal body. (**C**) Cross section through the basal spermathecal duct in a bloodfed sandfly that had access to males, showing the lumen (DL) filled with seminal fluids of the spermatophore (STR) and acrosomal membranes (AR, see also figure 4B) cast from the spermatozoa after migrating to the spermatheca. (**D**) Longitudinal section through the spermatheca in a bloodfed sandfly that had access to males. The lumen (SL) of the spermathecal body is filled with spermatozoa (SPM), which are loosely packed. The cuticle of the spermathecal wall is folded at regular intervals and projects into the lumen either as long foldings (SF) or short interseptal conical processes compartmentalising the lumen. Thick striated muscle fibres (MF) cover the cuticle wall and terminate at an apodeme (CA) at the apex. At the base, the body wall converges with relatively thick foldings of the cuticle supported by circular muscle fibres forming a sphincter-like valve (SV) that separates the spermathecal lumen from the spermathecal duct.

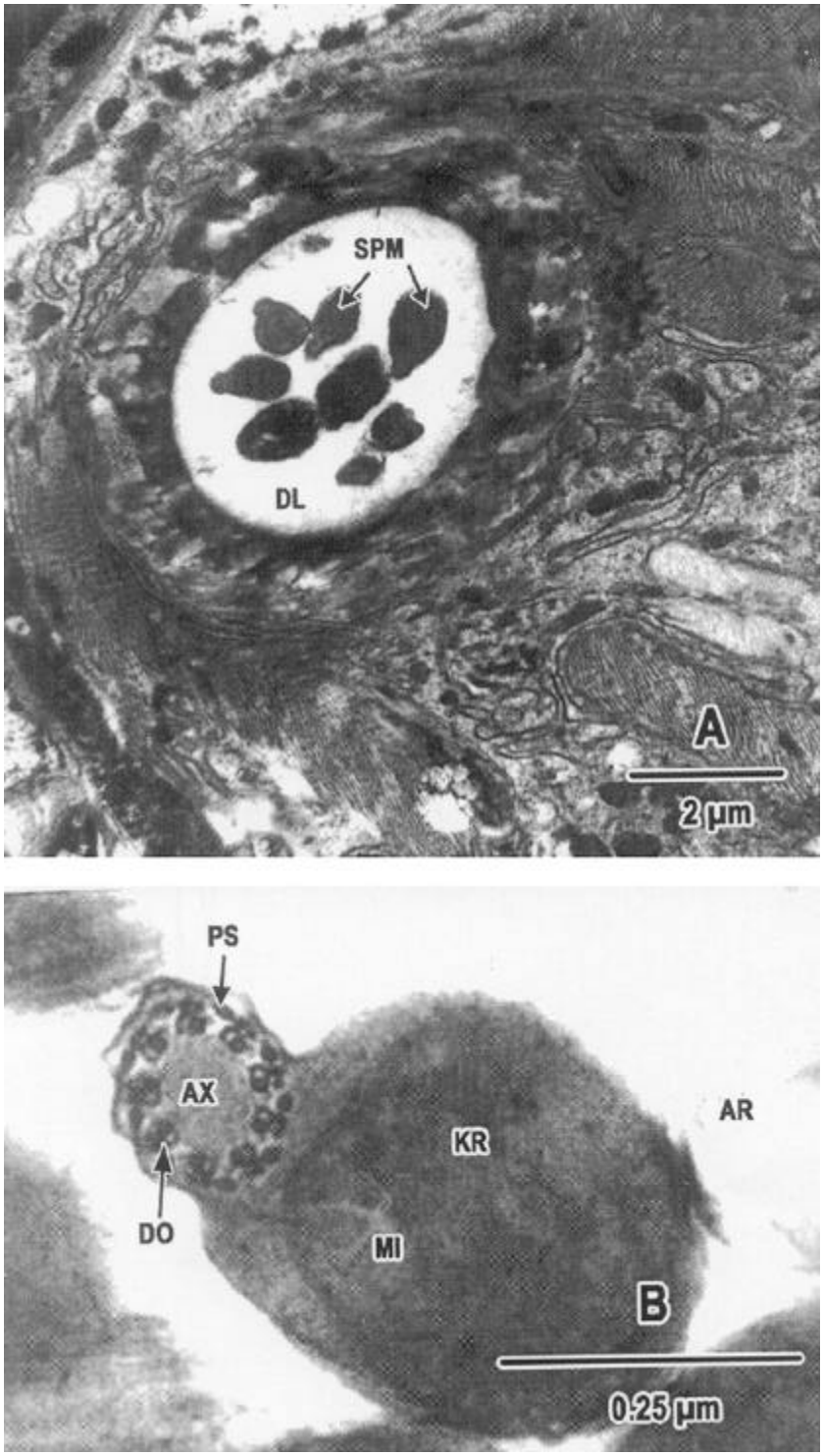

Figure 4. TEM photograph of the spermatheca of *P. papatasi*. (**A**) Cross section through the spermathecal duct (DL) of a 7-day-old sandfly 24 h after a blood meal, showing the spermatozoa (SRM) released from the spermatophores. At this stage, active spermatozoa (SPM) are moving towards the spermathecal body. (**B**) Cross section of a spermatozoan at the same stage as in figures 2A, 4A. AX, axoneme; AR, acrosome (in duct lumen as in figure 3C); KR, cristae; DO, doublets; MI, mitochondria; PS, peripheral singlets.

sandlfy by not being solid and compact. From this single evidence, it appears that sperm storage in the spermatheca of *P. papatasi* needs a blood meal immediately after insemination in each ovarian cycle, i.e. gonotrophically concordant, and that further sperm replenishment prior to the blood meal in the spermathecae occurs when the next reproductive phase of the insect begins. Unfortunately, the problem with laboratory-reared phlebotomine sandfly is that the females rarely survive oviposition, which may be either due to non-availability of a natural host for blood meal or due to environmental stress. Further studies on the post-insemination in the spermathecae of different species of phlebotomine sandflies using both colony-based and wild-caught samples should clarify this question.

3.5b *Spermatheca and sperm utilization*: As an alternative to direct observation of the spermathecae of sandflies that survived oviposition to find out whether females need to mate more than once during each gonotrophic cycle, a volumetric approach provides data in which the volume of the spermathecal lumen was calculated and the maximum number of sperm able to fill it was estimated (table 2).

The spermathecal duct can accommodate 2–3 spermatophores, and each spermatophore contains about 80 spermatozoa. Similarly, the spermathecal body, in which sperm is stored, has the capacity to hold 948 spermatozoa in each capsule. This means that the capacity of each spermathecal body can accommodate the sperm from 12 spermatophores. Another intriguing finding of this study is that a female needs a total of 50 spermatozoa (25 in each capsule) to fertilize an ovum in each gonotrophic cycle. These results indicate that the females were not only polyandrous, but that their sperm-storing organs control the fertilization processes after insemination. The narrow spermathecal duct and the segmented spermathecal body, for example, may provide an opportunity for the competing male aedeagus and spermatozoa that the "last in and first out" may be a mechanism of cryptic female choice, and the structure of the spermathecae may be related to this goal.

4. Discussion

4.1 *The physiological function of the spermathecae in* P. papatasi

From the examination of spermathecae at eclosion, i.e. at the emergence of a nymph from the pupa, the mass of a loose substance in the spermathecal lumen and ducts

Table 2. The relationship of spermathecal structure and sperm utilization/fecundity in *P. papatasi.*

Character	No. of samples	Mean (μm)	SD	Range (μm)	Volume (μm^3)
Length of the spermathecal duct	10	113·750	2·072	108·5–116	–
Diameter of the spermathecal duct	12	3·957	0·293	3·341–4·409	–
Volume of the spermathecal duct	–	–	–	–	1399·420
Length of the spermatheca	10	30·272	1·796	27·27–32·727	–
Diameter of the spermatheca	10	17·44	1·916	14·4–20	–
Volume of the spermatheca	–	–	–	–	6397·252
Length of spermatophore	10	43·78	3·993	33–46·75	–
Volume of spermatophore	–	–	–	–	538·608
Length of sperm	12	16·841	0·78	15·815–17·996	–
Diameter of sperm	12	0·714	0·118	0·573–0·918	–
Volume of sperm	–	–	–	–	6·746
Estimated No. of spermatophores/duct (= access to No. of mates)	–	2·598 (= 3)	–	–	–
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	–
Actual No. of sperms/duct	–	207·44	–	–	–
Actual No. sperms/spermatheca	–	948·283	–	–	–
No. of eggs laid	24[1]	37·917	25·387	2–96	–
Estimated or actual No. of sperms utilized/egg at oviposition[2]	–	50·019	–	–	–

[1]24/46 of flies laid eggs.
[2]Of 24 flies a single fly survived oviposition with 28 eggs.

suggests that the secretory phase of the glandular cells could have started at an early stage in development, perhaps in the late pupal stage (i.e. the pharate adult). As a sandfly develops and starts to eclose, the stored substance (i.e. filamentous material) is evacuated from the spermathecal lumen by physiological changes in the organ. These changes, especially the enhancement of secretory activity of glandular cells, may provide as source of chemotactic substances and of nutrients for the spermatozoa that are deposited at the entry of the spermathecal ducts.

Once the spermatophores are introduced into the spermathecal ducts, the spermatids within the spermatophores undergo the final stages of spermiogenesis called spermatid maturation. During the growth phase, they have to face two major challenges before they can move to the spermathecae and subsequently fertilize eggs: the spermatozoa must (i) leave the spermatophore presumably under the control of the spermathecal gland secretion, which is triggered by a blood meal of the female and results in the removal of the acrosome from the spermatozoon, i.e. capacitation, and (ii) compete with spermatozoa from any previous matings for access to the lumen of the spermatheca where they will be activated. These two crucial processes are reported in this study for the first time in the insect sperm-transfer mechanism. There have been a number of processes responsible for sperm motility in various groups of insects (Chen 1996). For example, the spermathecal gland secretion and the maintenance of the correct ion balance in the spermatheca activate sperm motility (chemotaxis); and there is a withdrawal of fluid from the spermatheca that causes an aspiration of sperm into the spermatheca. These findings are consistent with the following observations made in *P. papatasi* as well.

After the initial evacuation of the loose secreted substance from the spermatheca, the spermathecal gland continues to be secretory in all the subsequent stages that were studied, irrespective of whether the females were fed sugar or blood. The continuous production of secretion may be responsible for the motility and movement of spermatozoa. In addition to the motility and movement of spermatozoa in the female spermathecae, changes in the morphology of spermatozoon are also reported. For example, Degrugillier and Leopold (1973) found changes in spermatazoon morphology in male testes and in female spermathecae of the house fly *Musca domestica*. In mature testicular spermatozoa, the sperm head is covered by an acrosomal concavity, which is formed by an inner acrosomal membrane, and an outer plasma membrane. Within the spermatheca, the plasma membrane is separated from the acrosomal membrane of spermatozoon and is eventually shed. Degrugillier and Leopold (1976) suggested that the loss of the plasma membrane must occur while the spermatozoa are stored within the spermatheca. In mammals around the time of ovulation, sperm acquire a type of motility described as hyperactivation (Roldan *et al* 1992). It is an efficient way of forward propulsion within the viscous fluids encountered in the female reproductive tract near the egg prior to the capacitaion of the spermatozoa (i.e. acrosomal reaction) and, thus, releases or exposes the hyaluronidase to facilitate sperm penetration of the egg coat (Gomendio and Roldan 1991). The capacitation process is a phenomenon of sperm maturation prior to the fertilization of eggs in the female reproductive tracts, which involves significant membrane modifications and changes in cell metabolism that affect sperm motility (Selmi 1992). Similar situations may also operate in *P. papatasi*, but only after a blood meal.

Blood feeding invokes a multitude of well-choreographed physiological events in blood sucking insects (Rudin and Heckes 1982). A blood meal in mosquitoes and *Rhodnius* stimulates the secretion of FMRF (Phe-Met-Arg-Phe-amide)-like immunoreactivity peptide from the neurosecretory cells of the midgut for blood meal digestion, the secretion of enzymes, nutrient transport, and diuresis (Elia *et al* 1993). Furthermore, a blood meal after the insemination is a prerequisite for the presence of sperm in the spermathecae of *Anopheles subpictus* (Roy 1940) and *Aedes aegypti* (MacGregor 1931). Similarly, the ingestion of a blood-meal after matings is most likely used in sperm migration in the spermathecae of *P. papatasi*. As mentioned earlier, the release of spermatozoa from spermatophores and the movement towards the spermatheca occurs only in sandflies that have been fed blood. These findings agree with those of *P. papatasi* (Yuval and Schlein 1986) who found not only blood feeding is essential for the presence spermatozoa in the spermathecae of inseminated females but also females mate polyandrously. Of 89 female parous sandflies trapped in a fallow field, 65 had no sperm in their spermathecae while 26 of 34 sandflies found in burrows of the rodent host *Psammomys obesus* had spermatozoa in their spermathecae, indicating an association between host blood meal and the presence of spermatozoa in the spermathecae.

The spermathecal gland secretion provides an appropriate media for the motility, transfer and hyperactvation of spermatozoa. However, the blood-borne factors, such as FMRF (Phe-Met-Arg-Phe-amide)-like immunoreactivity peptide regulated by the neurosecretory substance (evidence for the neurosecretory substances of myoneural junction in the spermatheca, see Ilango 2005) in association with the spermathecal gland secretion, are perhaps involved in triggering the release of spermatozoa from the spermatophores. Maroli *et al* (1991) described the formation of a mating plug, which is a physical block that

prevents additional inseminations in some other insect mating systems, in phlebotomine sandflies. However, this may be a misinterpretation. Perhaps the spermatophores were mistaken for mating plugs. Evidence in this study revealed the presence of multiple spermatophores found in the spermathecal duct as a result of female inseminated more than once. This observation is consistent with the finding of Yuval and Schlein (1986) that the females of *P. papatasi* are polyandrous.

4.2 *Phlebotomine sandfly spermathecae and post-copulatory sexual selection*

The pattern of structural variation in the spermathecae and the mechanism of histological changes, such as the spermathecal gland secretion, insemination, sperm storage, and sperm utilization, clearly indicate that the spermathecae of *P. papatasi*, and possibly of other sandfly species, are not just passive sperm stores. Modulations of their activities are likely to provide opportunities for post-copulatory sexual selection.

The aedeagal filaments of the male are inserted into the female spermathecal ducts for spermatophore deposition, and the spermathecal ducts are less than half the length of the aedeagal filaments (Ilango and Lane 2000). This significant difference between the length of the aedeagal filaments and the length of the spermathecal ducts provides opportunities for both males and females to control reproductive processes. Males can displace rival spermatophores, and females can use more spermatophores and sperm for fertilizing their eggs from males with longer aedeagal filaments to maximize her reproductive success. This is consistent with my findings that multiple spermatophores are present lying one above the other in the spermathecal duct. There was also a positive correlation measured in millimetre between the length of the spermathecal ducts and the length of the aedeagal filaments ($r^2 = 0{\cdot}27$, $r = 0{\cdot}52$ i.e. correlation coefficient, $P = 0{\cdot}005$, significant at 0·5% level) found in 26 species of phlebotomine sandfly (Ilango and Lane 2000). There has certainly been co-evolution of male and female reproductive tracts, with the aedeagal filaments showing patterns of co-evoultion with the spermathecal ducts across species. For the coevolution of male and female reproductive tracts, such as in the Stalk-eyed flies (Presgraves *et al* 1999) and the vinegar flies (Pitnick *et al* 1999), environmental influence and pleitropy are improbable casual factors. Consistent with these findings, the female spermathecal duct of phlebotomine sandflies is an important agent that selects to enhance the competitive fertilization success of male genital filament. Because aedeagal filament evolution tracks the change in the length of the spermathecal duct, the female reproductive tract almost certainly mediate selection on male aedegal filaments by influencing opportunities for sperm competition and sexual conflict.

In a variety of insect taxa, females have complex sperm storage organs with multiple compartments (Eberhard 1996). A possible function of such multiple compartments is to enable differential sperm storage, that is, the spatial separation of competing sperm in independent compartments. This separation, in turn, may allow females to use sperm differentially at fertilization. According to Eberhard (1998), sperm competition almost never occurs without females influencing the outcome, if only through the morphology of her reproductive organs, which may determine how much sperm is stored and to what degree males have access to sperm stores. Although my study did not determine paternity, it did show some evidence that the anatomy of the sandfly spermathecae sets the rules for sperm storage. The spermathecal duct and body of *P. papatasi* have the capacity not only to store different volumes of spermatozoa in the spermathecal duct and body but also to provide multiple compartments to increase the space available for spermatozoa. For example, each spermathecal body is capable of accommodating 948 spermatozoa. This means that to fill each spermathecal body, each spermathecal duct should have been filled with 948 spermatozoa in 12 spermatophores, but my results show little over 2·5 spermatophores in each duct. This is possible because the difference of spermatozoa stored in the spermathecal duct and the body is likely to be caused by the quantity of spermatophore or spermatozoa displaced or pushed down by the competing spermatophores or spermatozoa to the bursa copulatrix.

Another intriguing finding of my study is that of the 38 eggs fertilized (see table 2), a female needs 50 sperm (25 in each capsule) to fertilize an ovum in each gonotrophic cycle. These results support the hypothesis that sperm-storing organs of females have the ability to affect the reproductive processes after insemination by providing opportunities for sperm competition, cryptic choice of sperm, and sexual conflicts. Sexual conflict (i.e. evolutionary conflict between the sexes), will occur whenever there is not strict genetic monogamy and reproduction is costly. Given the widespread occurrence of both these conditions, sexual conflicts is rife and can favour traits that increase the fitness of one sex even when it is costly to the other (Hosken and Stockley 2004). Hence, sperm competition and sexual conflict select for characters in both sexes that are beneficial in a evolutionary struggle over paternity. Future studies, using sterile-insect autoradiographic techniques or DNA fingerprinting technique, will help reveal the exact pattern of sperm use, i.e. P^2 (proportion of offspring sired by the last of two or more male spermatozoa) in the spermathecae of different phlebotomine sandfly taxa. Such studies will eventually be

helpful for the development of genetic control of the leishmaniasis vectors.

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