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Sexual Reproduction in the Monogenean *Diclidophora merlangi*: Tissue Penetration by Sperms

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Summary. Copulation between pairs of adult *Diclidophora merlangi* detached from the host was frequently observed. The spined penis of one animal always attaches to a second worm at a latero-ventral position posterior to the genital openings. There is no vagina. The sperms travel between the cells of the recipient to reach the seminal receptacle. The ultrastructure of the sperm is described. Positions of adult *D. merlangi* on the gills of the host would facilitate pairing for sperm transfer.

Introduction

Cerfontaine (1895) described a vagina in several species of *Diclidophora*, but subsequently this observation was refuted and a vagina is now generally considered to be absent among diclidophorinean parasites (Sproston, 1946; Llewellyn and Tully, 1969; McCauley and Smoker, 1969). Despite intensive study, Frankland (1955) never observed copulation in *D. denticulata*. In the present study large numbers of *Diclidophora merlangi* were collected for the purpose of obtaining eggs. It was noticed that when the parasites were kept in dishes of sea water, one frequently attached to another by means of the spined penis. In view of the reported lack of a vagina and because copulation had not been previously recorded, further study of pairing parasites was made.

Materials and Methods

Specimens of adult *D. merlangi* were collected from whiting (*Merlangius merlangus*) 25–28 cm in length caught in inshore waters off the North-East coast of Scotland. The gill arch to which each parasite was attached was recorded (arches numbered I to IV from the operculum inwards) as were the positions of the parasites on the arches and the number of primary lamellae spanned by each parasite.

The worms were removed from the fish, washed clear of host mucus by rinsing in sea water and placed in crystallizing dishes (5 cm in diameter) containing filtered sea water. Four to six parasites were placed in each dish and these were maintained at 6° C, 9° C or 13° C. The water in the dishes was changed every 24 h.

Pairs of copulating parasites and individual worms which had been copulating and had subsequently separated were observed alive and were also fixed for later

study. Some material was fixed in Bouin's fluid made up in sea water, or in Carnoy's fixative and embedded in paraffin wax. Sections through the region of penis attachment were cut at 6–8 μm and stained with Haematoxylin and Eosin or with Cason's stain. Other material, consisting of worms which had received sperms, was fixed in gluteraldehyde at 4° C and post-fixed in osmic acid for examination with the electron microscope. This material was embedded in TAAB resin and sectioned transversely on a Cambridge Huxley ultramicrotome. Initially, sections 1 μm in thickness were cut and stained with 1% toluidine blue. These were used to locate the region of penis attachment by conventional light microscopy. Subsequently, sections were cut at 50 nm, collected on formvar coated copper grids and stained with uranyl acetate and Reynold's lead citrate for electron microscopy. A Philips 200 Electron microscope was used, at an accelerating voltage of 60 kv.

Results

Sperm Transfer

When adult *D. merlangi* are kept in dishes of sea water, one is frequently seen (Fig. 1) to attach to another by means of the penis. Llewellyn and Tully (1969) described the penis as a "muscular sphere armed with a ring of recurved hooks". In the present study, sections of the penis show it to be comprised of a large muscular sucker-like structure (Fig. 2) and that when it is applied to the body wall of the recipient, a plug of recipient tissue is sucked into its cavity (Figs. 2, 3). It appears that only the penis hooks penetrate the tissues of the recipient. If two attached parasites are gently separated, a stream of living sperm is released from the penis. The exact point of attachment may vary, but it always occurs at a position on the ventro-lateral margin of the body of the recipient posterior to the male and female genital openings. Attachment can occur on the left or right side of the animal. In living worms it can be seen that attachment is brought about by one animal moving the anterior portion of its body half way across the dorsal anterior portion of a second animal (the recipient). This second animal slightly raises the margin of its body and the first animal attaches in a ventro-lateral position.

When pairs of attached parasites were fixed and sectioned in paraffin wax, sperms could be seen in the penis of one animal and rod-like bodies which stained darkly with Ehrlich's Haematoxylin were seen in the tissue of the second animal near the point of attachment of the penis (Figs. 2, 3). These rod-like bodies closely resembled sperm nuclei as seen in the penis or testis. Facial sections of the recipient showed similar bodies clustered around the point of penis attachment and between this point and the seminal receptacle, a distance of between 1 and 2 mm. These rod-like bodies were found only in the tissues on the side of the body to which the penis had been attached and also around the seminal receptacle. Electron microscopy revealed that these bodies were sperma-

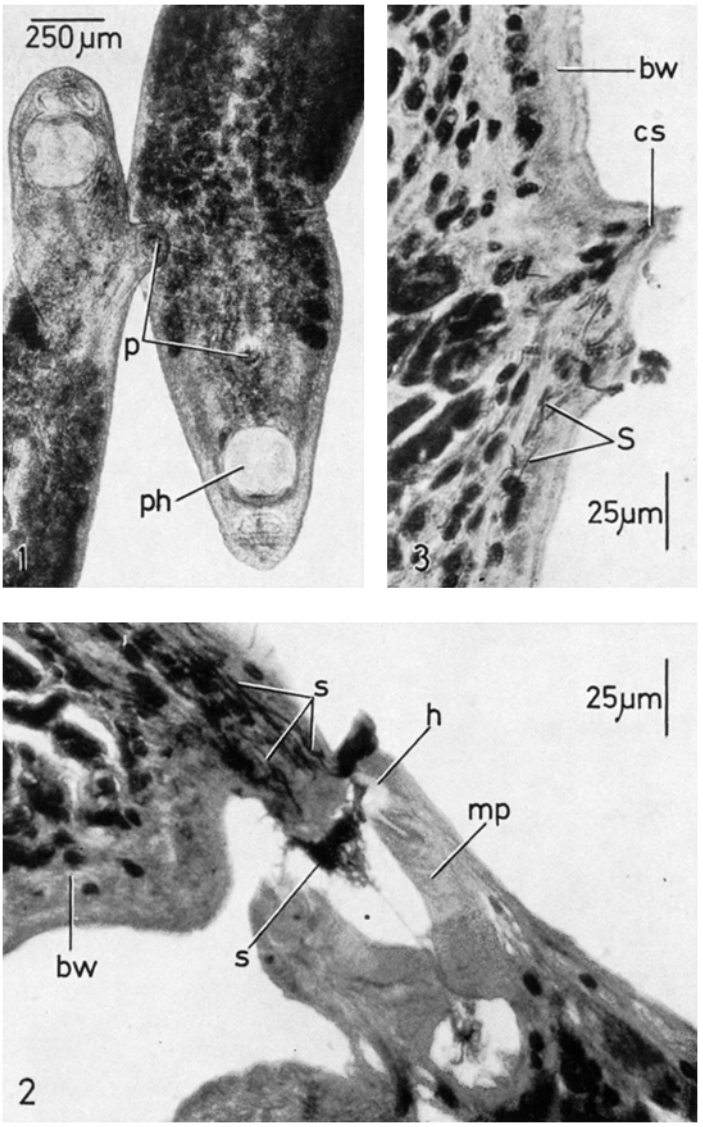


Fig. 1. Copulation between two individuals of *Diclidophora merlangi*

Fig. 2. Section through two copulating individuals, showing the sucker-like penis of one worm and the adjacent body wall of the recipient worm. Sperms are present in the lumen of the penis and within the tissues of the recipient

Fig. 3. Facial section through the body wall of a recently mated recipient worm, showing the raised scar where the penis of the other worm was attached and sperms migrating from this region through the tissues

tozoa since they can be readily recognized by their double flagellar structure (see later). If was, therfore, confirmed that, during pairing observed in *D. merlangi*, sperms had passed from one individual to the other.

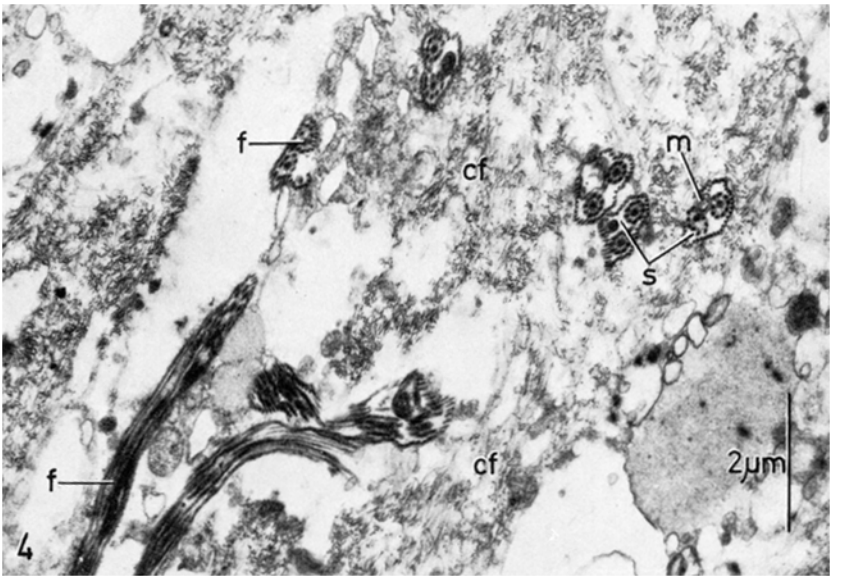
Despite intensive search in both living and sectioned animals, no vagina was found. No tube could be found at the point where the sperms entered the body and no tube was found leading from the seminal receptacle to the ventral body surface as described by Cerfontaine (1895). EM sections showed that the sperms moved through the body between the cells and in amongst collagen fibres (Figs. 4, 5). Occasionally a few sperms were found to have moved in an anterior direction after entering the body but the ultimate fate of these is not known.

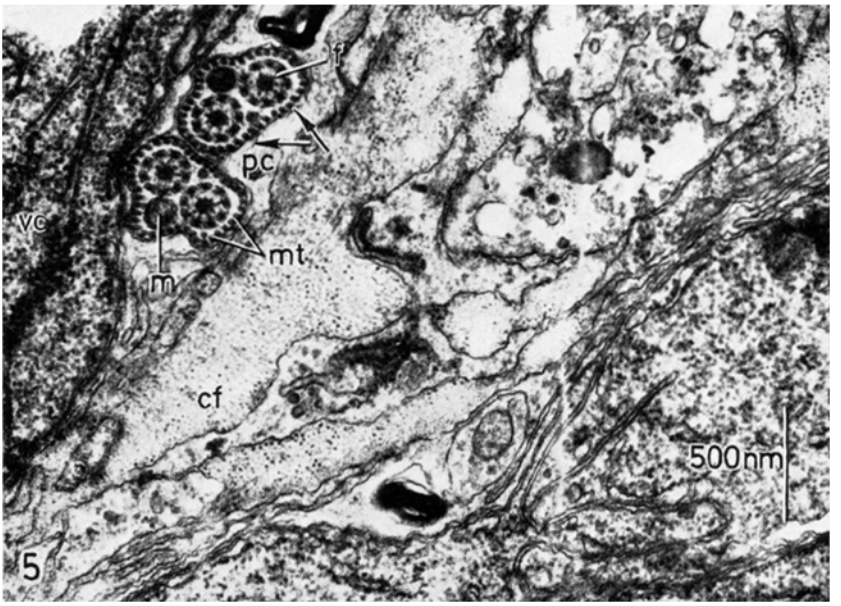
Copulation was repeatedly observed between pairs of adult *D. merlangi* irrespective of temperature (within a 6–13° C range) both in the light and in the dark. On several occasions worms were found to be copulating within 30 mins of removal from the fish and in many cases it occured even 3 or 4 days after removal from the host. The process of sperm transfer may last for at least one hour. One pair of worms was separated during copulation and had returned to their original position within 30 mins.

Reciprocal fertilization was never observed but the penis of the recipient frequently released sperm during copulation. It may be that reciprocal fertilization does occur *in vivo*. On one occasion, a parasite was seen to fertilize another while itself being fertilized by a third parasite. Copulation was never observed between adult and juvenile *D. merlangi* nor between juvenile worms, although they were frequently placed together in the same dish. Self-fertilization was not seen. Egg laying and the formation of eggs in the ootype were both observed during copulation.

The Spermatozoon of *D. merlangi*

As stated previously, when copulating parasites are gently separated a stream of living sperm is released. These fully-formed sperms were examined under oil immersion with the light microscope using phase contrast. In each sperm there are two flagella one of which appears to follow a relatively straight course along the lenth of the sperm "body" while the other coils three times around the body. A dark "acrosomal" cap is seen at the anterior end of the body. The greater part of the flagella lie posteriorly to the sperm body where they appear to be bound together for most of their length, separating and becoming free from each other only at their most posterior tips. The total length of the sperm is between 170 and 200 μm with the posterior portions of the flagella comprising about 70% of this total length.





Figs. 4 and 5. Electron micrographs of sections through the anterior body of a recently mated *Diclidophora merlangi*

Fig. 4. Shows sperms amongst collagen fibres ×12200

Fig. 5. Shows sperms between cells; arrows mark plasmalemma of adjacent parenchymal cell ×41600

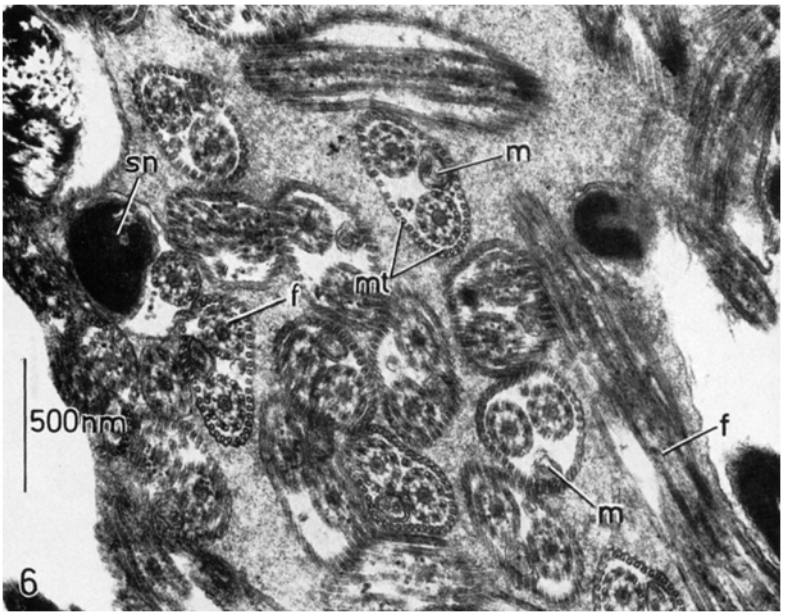


Fig. 6. Electron micrograph of sperm in the seminal receptacle. The double flagellar structure and enclosing ring of microtubules of the sperm can be seen ×47000

EM sections of sperms from the seminal receptacle (Fig. 6) showed that they closely resemble those described by Ktari (1971) from a species of *Diclidophora* from *Gadus capelanus*. The body of the sperm consists of an elongated nucleus and mitochondrion. In some sections the nuclear diameter is very large confirming that most of the thick body of the sperm seen with light microscopy is occupied by the nucleus. Sections show that the mitochondrion extends beyond the nucleus. The size of the mitochondrion also varies suggesting that it too may taper at one or both ends. An acrosome was not found in the EM sections.

Transverse sections of the sperm body showed that the distance between the two flagella varied and that in cases where one flagellum was cut transversely the other often appeared at an oblique angle. This supported the light microscope observations of one flagellum running along the sperm body while the other coils around it.

The sperm body is limited by microtubules orientated parallel to the long axis and these are contained within an outer membrane which surrounds the sperm body. The sections suggest that the outer membrane binds the two flagella for most of their length posteriorly and that they only become free and separate at their most posterior tips.

Position of Adult *D. merlangi* on the Gills of its Host

Copulation *in vivo*, would, of course, necessitate the close proximity of the parasites to each other and so the exact positions of the worms on the gills of whiting were examined. A total of 310 whiting infected with adult *D. merlangi* were examined and 417 attached adult parasites collected. Of these parasites 75.6% were found on gill arch I, 17.4% on gill arch II, 4.4% on arch III and 2.6% on arch IV. The number of parasites on the left and right side of the fish was almost exactly the same, with 50.7% being found on the left side. Detailed examination showed that 393 worms (94.3%) were found on the outer hemibranchs and 24 (5.7%) on the inner hemibranchs (all gill arches included). Of the 310 whiting, 165 (53.2%) carried single adult worm infections, 60 fish (19.3%) carried two adult parasites, 36 fish (11.6%) had three adult worms and the rest (15.9%) bore between four and twelve adult parasites. Although 60 fish carried 2 adult parasites, in 22 of these fish the worms were found on opposite sides of the host. On 118 individual gill arches two or more adult parasites were found and on 87 (73.7%) of these arches the worms were found in close proximity to each other. These worms either occupied adjacent lamellae or else were separated by no more than two primary lamellae. On four other occasions pairs of worms were found lying directly opposite to each other, one on the outer and one on the inner hemibranch of the same gill arch. Adult parasites were found along the straight anterior sector of the gill and on its posterior curved region and no particular region of the gill arch appeared to be preferred by attached parasites. When adult and juvenile worms were found on the same gill arch, their relative positions were noted and it was found that only 31% of the juvenile worms were located on lamellae adjacent to those occupied by adult worms.

In order to calculate the chances of two parasites attaching at random on adjacent segments of the gill, counts were made of the number of primary lamellae on the outer hemibranch of each gill arch in 10 whiting, 25–28 cm in length. The hemibranch of the first gill arch (I) bore an average of 72 (range 71–75) lamellae. The hemibranch of gill arch II bore 73 (71–75) arch III bore 66 (61–71) and IV bore 56 (50–63) individual primary lamellae. The number of primary lamellae grasped by individual adult worms was also noted for 30 parasites. The majority of worms spanned three lamellae but three of the thirty examined grasped five lamellae. *D. merlangi* is found most commonly on the first and second gill arches of its host and shows no apparent preference for any particular region of the gill arch. If it is assumed that at least 70 of the lamellae of these two arches are available for attachment and that a parasite will seldom occupy more than five lamellae, it appears that there are 66 potential sites for attachment on the average first or second gill arch

of a whiting 25–28 cm in length. In view of the apparently large number of available sites it is significant that in 73.7% of all gill arches bearing two or more adult worms the parasites were found on adjacent lamellae or separated by one or two primary filaments. In the latter case the lateral edges of the parasites were frequently in direct contact.

Parasites from both single and multiple infections will lay eggs after removal from the hosts and parasites from both types of infection contain sperm in their seminal receptacles.

Discussion

Copulation between pairs of adult *D. merlangi* after removal from the host was frequently observed. The spined penis of one animal attaches to a second parasite at a latero-ventral position posterior to the genital openings. It has been shown that the sperms travel through the tissues (between the cells) of the recipient to the seminal receptacle. The individual sperm consists of an elongated nucleus/mitochondrion complex with two flagella and possibly an acrosomal cap. Beneath the outer membrane the sperm body is bounded by microtubules. The position of adult *D. merlangi* on the gills of the host would facilitate pairing for sperm exchange.

A vagina is generally supposed to be absent among the Diclidophorinae according to Sproston (1946) and Llewellyn and Tully (1969). Cerfontaine (1895) reported the presence of a short vagina leading from the seminal receptacle directly to the ventral surface in four species of *Diclidophora* including *D. merlangi*. Frankland (1955) could find no vagina in *D. denticulata* but described a scar on the ventral body wall of the animal and she suggested that the 'cirrus' penetrated the body wall to make contact with the seminal receptacle. Llewellyn and Tully (1969) suggested that copulation could take place among young parasites and that the vagina might then close. In the present study sperm exchange was never observed between juvenile parasites nor between adults and juveniles.

It has been suggested that in the absence of a vagina sperm entry may take place via the uterus (Bychowsky, 1957). The present work shows that in future studies consideration should be given to another potential means of sperm entry, namely the entry of sperms through the body surface and their migration through the tissues. Morris and Halton (1971) showed that there are probably no cell junctions in the adult epidermis of *D. merlangi*. If the syncytium is continuous in the region of sperm entry then it would be necessary to breach the epidermis to permit access to the body tissues. It seems likely that the penis hooks perforate the tegument and such a breach may serve for sperm entry.

Alternatively, suction pressure generated by the penis sucker or histolytic secretion of the penis or of the sperms themselves may be responsible for breaching the epidermis. Provided that the seminal receptacle is not bounded by a syncytium it is probable that the epidermis is the only membranous barrier since the EM sections showed the sperm travelling between the cells on their migration to the receptacle. The sperms must travel a distance of 1 to 2 mm from the point of insertion to the seminal receptacle. Nothing is known of the way in which the sperm orientates in order to reach the receptacle but it may be that chemo-attraction is involved. Very few sperm appeared to move anteriorly after entering the tissues.

Copulation involving sperms penetrating the body wall has been previously reported among several invertebrate groups but never before among the Monogenea. Manton (1938) in her study of the Onychophora found that spermatophores were placed anywhere on the body surface of *Peripatopsis*, and the sperms passed through the haemocoel by their own activity to reach the ovary where they penetrated the ovarian wall. Penetration of the body wall by sperms has also been reported in *Cimex* by Abraham (1934), among leeches by Meyers (1935) and in archiannelids by Ax (1969). Westheide (1967) studying polychaetes, suggested that the sperms of *Microphthalmus aberrans* bored through the body wall with the aid of a histolytic secretion.

The basic structure of the sperm of *D. merlangi* resembles that described by Ktari (1971) and Tuzet and Ktari (1971) for a species of *Diclidophora* from *Gadus capelanus*. The body of the sperm is bounded by microtubules which may serve to strengthen it while travelling through the body tissues. Ktari (1971) showed that microtubules were present in the sperm of several polyopisthocotylineans but absent in the monopisthocotylinean *Trochopus pini*.

In many EM sections of the sperm of *D. merlangi* the diameter of the nucleus was very large, confirming that the prominent body of the sperm seen with light microscopy was occupied largely by the nucleus. Ktari (1971) claimed that such nuclei were hypertrophic and abnormal but there was no reason to suppose that this was so in *D. merlangi*. The acrosomal cap seen with the light microscope was not found in the EM sections. It is generally known that acrosomal secretions have a lytic function and it may be that acrosomal secretions in the sperm of *D. merlangi* aid penetration either through the epidermis or between cell boundaries.

Cerfontaine (1898) noted that *D. merlangi* occured most frequently on the first gill arch of its host and this observation was subsequently confirmed by Llewellyn (1956), Smith (1969) and Arme and Halton (1972). It is confirmed again in the present study. There was no difference

in infection between the right and left sides of the fish and this again confirms the findings of Arme and Halton (1972). The present study established that where more than one adult parasite occupied an individual gill arch the majority of worms (on 73.7% of these arches) were attached to adjacent filaments or were separated by no more than two primary lamellae. It was also shown that the outer hemibranch is more frequently parasitized (94.3%) than the inner hemibranch (5.7%). Thus, in cases where two or more adult parasites occured on the same gill arch they most frequently occupied neighbouring sites of the same hemibranch. However, when a juvenile worm was located on the same gill arch as an adult, the parasites occupied adjacent sites in only 31% of the cases. Thus there appears to be a movement along the gill arch at the late juvenile or early adult stage.

Llewellyn (1956) suggested that the apparent gill preference by species of *Diclidophora* might be due to gill ventilating mechanisms. This may be so but in view of the apparently large number of available sites for attachment on the preferred gills, the recorded positions of adult *D. merlangi* suggest that worms are attracted to each other. It seems likely that the frequent occurrence of parasites on neighbouring lamellae is linked to their reproductive habits. Of the 310 whiting infected with *D. merlangi* 165 carried single worm infections. Sixty fish bore two adult parasites but on 22 of these fish the worms were found on opposite sides. It appears, therefore, that for maximum chance of sperm exchange to take place within the population, some mechanism may exist to attract worms to each other and thus increase their chances of making direct contact. This requirement may be reflected in the frequency of parasites on neighbouring lamellae and in the preference for the outer hemibranch. In actively swimming fish the tips of the hemibranchs are held apart (Hughes, 1963) and since the parasites attach with their anterior ends near the distal ends of the lamellae, copulation between a parasite on the inner and one on the outer hemibranch may prove difficult. In the present study self-fertilization was never observed but since about 60% of the fish carried single parasites or only one parasite on each side, the possibility of self-fertilization cannot be ruled out. Parasites from single infections do lay eggs and sperms are present in their seminal receptacles but it is not possible to know whether self-fertilization has occured or whether a second parasite was at one time present on the gill.

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Key to lettering of Figs. 1–6

b.w.	body wall of recipient worm	mt.	microtubules
c.f.	collagen fibres	p.	penis with ring of hooks
c.s.	copulatory scar	p.c.	parenchymal cell
f.	flagellum of sperm	ph.	pharynx
h.	penis hook	s.	sperm
m.	mitochondrion	s.n.	sperm nucleus
m.p.	muscular sucker-like region of penis	v.c.	vitelline cell

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