



Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking

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Different interests between mating partners regarding the fate of their gametes can lead to sexual conflicts in many species. Although these conflicts can sometimes be dealt with pre-copulatorily (e.g. by choosing with which partners to mate), they often extend beyond copulation. Post-copulatory sexual conflicts are expected to be particularly strong in simultaneous hermaphrodites because an individual may have to accept sperm in order to obtain an opportunity to donate sperm, reducing the effectiveness of pre-copulatory conflict resolution. The present study investigates the post-copulatory interactions between male and female sexual traits of a highly promiscuous simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*. Using light and electron microscopy, we show the different levels of complexity of the sperm and the genitalia, and derive hypotheses about how the different traits may represent evolutionary responses to such sexual conflicts. © 2010 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2010, 99, 370–383.

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INTRODUCTION

Despite the fact that mating partners may appear to cooperate when producing common offspring, most reproductive interactions are considered to involve sexual conflicts (Charnov, 1979; Parker, 1979; Arnqvist & Rowe, 2005; Parker, 2006). For example, a sperm donor (hereafter donor) will profit most if multiple sperm recipients (hereafter recipients) use only its sperm to fertilize all the available eggs (note that, in species with separate sexes, the terms donor and recipient are equivalent to male and female). A recipient, on the other hand, may benefit from mating with multiple donors and choosing post-copulatorily which sperm to use to fertilize its eggs, aiming to maximize direct or indirect benefits (a sperm donor's choosiness is of course also possible; Kokko, Jennions & Brooks, 2006).

Choosiness, however, need not arise from a female strategy to secure direct or indirect benefits. In their urge to secure paternity, donors often transfer more sperm than is required to ensure fertility (often as a consequence of sperm competition; Birkhead & Møller, 1998). This may lead to costs for the recipient because it increases the risk of polyspermy (Frank, 2000). Recipients may thus be selected to evolve costly adaptations to restrict the access to their eggs to fewer sperm, leading to the rejection (pre- or post-copulatorily) of many potential donors. Donors are then likely to evolve counter-adaptations that increase their chances of fertilization, again leading to choosiness and sexual conflict. Sexual conflicts will occur whenever there is any form of rejection of some donors (Parker, 2006) and can result in the evolution of complex morphological traits (Birkhead & Pizzari, 2002; Arnqvist & Rowe, 2005, Parker, 2006), which are often but not always restricted to reproductive traits (Parker, 1979; Parker, 2006). In the present study, we focus on sexual conflicts arising from

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disagreements over the reproductive outcome of mating encounters (i.e. how the available gametes will be used).

When the transfer of sperm occurs unilaterally, as is the case not only in gonochorists, but also in many hermaphrodites, sexual conflict may be dealt with pre-copulatorily, as recipients may choose with which donors to mate (e.g. based on conspicuous display traits) and avoid the other donors. Examples abound, however, where conflicts over reproduction extend beyond the initiation of copulation (Arnqvist & Rowe, 2005), encompassing traits that are behavioural (e.g. mate guarding: Alcock, 1994; Dillen, Jordaens & Backeljau, 2009; nuptial gifts: Sauer *et al.*, 1998), morphological (e.g. male–female genital interactions; Rönn, Katvala & Arnqvist, 2007), and chemical (e.g. ejaculate–female interactions: Pitnick, Wolfner & Suarez, 2009; substances transferred by love darts: Koene & Schulenburg, 2005).

In simultaneous hermaphrodites, each recipient can also be a donor. This poses the problem of potentially having to receive unwanted ejaculates to gain opportunities to donate sperm, and can thereby shift the weight of sexual conflict towards the post-copulatory arena. On the other hand, individuals may compensate possible costs of mating in the female function by increasing their paternity, potentially allowing for higher mating costs and larger potential for sexual conflicts compared to gonochorists (Michiels & Koene, 2006). We expect that when reciprocal mating is obligatory (i.e. both partners act as donors and recipients simultaneously), this shift to post-copulatory conflicts will be most pronounced. Simultaneous hermaphrodites without obligate reciprocal copulation also have a risk of getting unrequited inseminations, if simply as a result of the physical proximity to the mate during sperm donation. However, there is still scope for pre-copulatory conflicts on which role each partner will play in a particular mating encounter (Anthes, Putz & Michiels, 2006; Anthes & Michiels, 2007).

A donor may further increase its male-derived fitness by inducing the recipient to down-regulate its allocation towards male function, and/or to up-regulate the the female function. This may increase the amount of resources invested into the offspring resulting from this particular mating, and additionally decrease the intensity of sperm competition. Thus, ejaculate components that manipulate the partner's resource allocation may be beneficial for the donor but detrimental for the recipient (Charnov, 1979; Michiels, 1998; Michiels & Streng, 1998; Schärer, 2009; Schärer & Janicke, 2009).

Although they probably have stronger sexual conflicts than gonochorists, simultaneous hermaphrodites were regarded as unable to develop secondary

sexual traits by Darwin (1882), perhaps because he concentrated on the more conspicuous pre-copulatory displays of gonochorists.

The free-living flatworm *Macrostomum lignano* (Ladurner *et al.*, 2005) is able to adjust its sex allocation according to the number of competitors (Schärer & Ladurner, 2003), which suggests that sperm competition is common. It has internal and reciprocal copulation, with both partners acting as donors and recipients at the same time, and exhibits high mating rates (Schärer, Joss & Sandner, 2004). It is quite promiscuous, mating with up to ten partners within 24 h when kept in groups of 16 worms (Janicke & Schärer, 2009). It is thus a good model organism to look for potential post-copulatory conflicts and to study morphological and behavioural adaptations linked to these conflicts. Indeed, *M. lignano* exhibits an intriguing post-copulatory suck behaviour, during which the worms bend down and place their pharynx over their own female genital opening, and then appear to suck. Although it was proposed that this behaviour may be an adaptation to manipulate sperm or secretions received during copulation (Schärer *et al.*, 2004), its actual function still remains unclear.

In the present study, we provide a detailed account of the morphology and behaviour of *M. lignano* sperm and its intimate interaction with the sperm-receiving organ. From these observations, we derive hypotheses for the possible functions of different sexual traits in the light of the post-copulatory sexual conflicts suggested by the high levels of sperm competition and the suck behaviour.

MATERIAL AND METHODS

ORGANISM

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a free-living flatworm of the intertidal sand meiofauna of the Northern Adriatic Sea (Ladurner *et al.*, 2005). In the laboratory, mass cultures of *M. lignano* are kept in f/2 (an artificial seawater medium, Andersen *et al.*, 2005) and fed with the diatom *Nitzschia curvilineata* (Rieger *et al.*, 1988). It is an outcrossing, simultaneous hermaphrodite (Schärer & Ladurner, 2003), conveniently small (adults are approximately 1.7 mm in length) and transparent, allowing non-invasive observation and measurement of the internal morphology (Fig. 1). The paired ovaries are located on the sides of the gut in the central region of the body, together occupying approximately 3.5% of the body (Ladurner *et al.*, 2005). There is no clearly defined oviduct, with the ovaries gradually changing into a growth zone where developing oocytes are provisioned with yolk (eggs are entolecithal; Morris *et al.*, 2004). Posterior to the growth zones is a

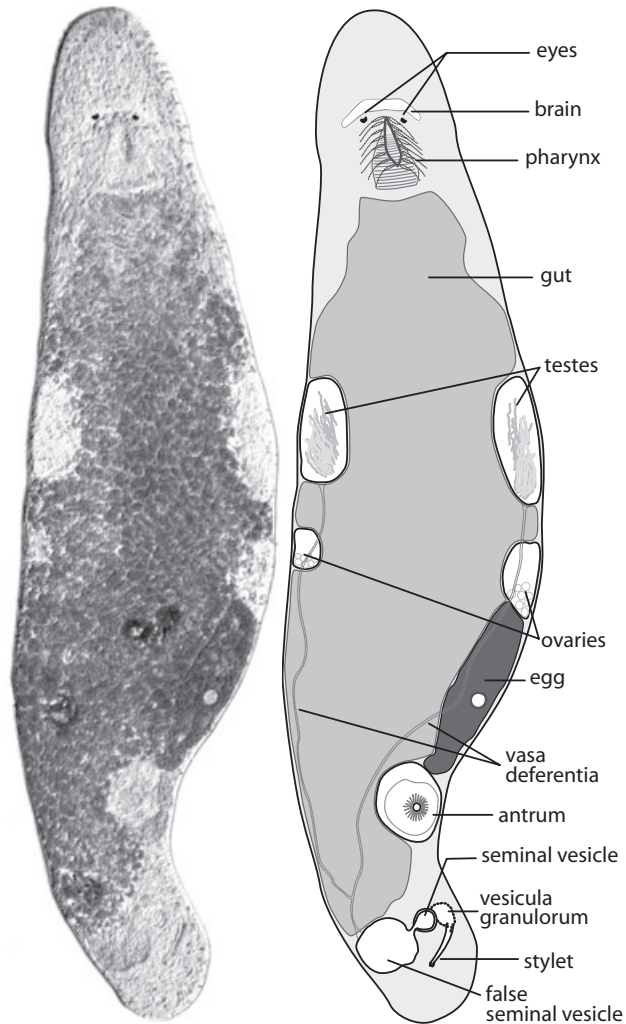


Figure 1. Micrograph and line drawing of a live adult specimen of *Macrostomum lignano* squeezed between two glass slides. The posterior part of the body is slightly twisted to better show the female antrum. The total length of this worm is approximately 1.8 mm.

single sperm-receiving and egg-laying structure, the female antrum (Fig. 2B), which communicates with the outside via a short, ciliated vagina. The paired testes, which together occupy approximately 5.5% of the body (Ladurner *et al.*, 2005), are also located on the sides of the gut, anterior to the ovaries. Single vasa deferentia leave each testis, merging shortly before joining the false seminal vesicle in which sperm ready to be transferred accumulate (Fig. 1). Next comes the smaller, muscular, seminal vesicle, followed by the vesicula granulorum, where the necks of the prostate gland cells are collected (Fig. 2C). The stylet or male copulatory organ (Fig. 2C), through which sperm (Fig. 2D) and prostate secretions are transferred during copulation, is located towards the

end of the tail plate, with the base usually on the opposite side of the false seminal vesicle and the tip central.

LIGHT MICROSCOPY

Prior to observation, worms were relaxed using diluted magnesium chloride, which selectively inactivates muscles without affecting cilia or sperm, and placed between a glass slide and either a haemocytometer glass cover slip or a normal cover slip, kept apart by plastic or plasticine spacers. These different methods were applied when different degrees of squeezing or manipulation were required. The worms were first documented without being fully squeezed aiming to observe the natural shape of the internal organs, and were rotated and further squeezed when necessary to observe details at higher magnification. Observations were carried out with a compound microscope, and digital micrographs and movies were taken with a digital camera connected to a computer, as in Ladurner *et al.* (2005). Notes and drawings were made to clarify features of the structures, or whenever the direct observation was difficult to capture in the micrographs and movies. Dimensions of the drawings were adjusted based on the digital documentation. Over 50 worms were used for these detailed observations. They were taken from our standard cultures, which had been kept for up to 20 generations in the laboratory and originated from different localities in the Northern Adriatic (Ladurner *et al.*, 2005). We have confirmed our findings over several years in hundreds of worms used in experiments, freshly collected from the field, and serially sectioned for other studies (e.g. Ladurner *et al.*, 2005). Observations were also made on numerous specimens without anaesthetic, to verify that the magnesium chloride does not affect the morphologies under study.

ELECTRON MICROSCOPY

Juvenile worms were removed from mass cultures and kept isolated until they reached maturity. Eight pairs were assembled and placed in observation chambers for 1 h so that they could copulate (Schärer *et al.*, 2004), and then immediately relaxed using magnesium chloride. Worms were then fixed individually with a glutaraldehyde-osmium tetroxide double fixation (2.5% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.2 containing 10% sucrose, post-fixed with 1% osmium tetroxide in 0.1 M cacodylate buffer), dehydrated via an alcohol series, and embedded in Spurr's low viscosity resin (Spurr, 1969). Only worms that actually copulated were used for sectioning (a total of five pairs, one worm randomly chosen from

each pair). The same procedure was carried out with eight virgin worms, to compare the state of the antrum before and after copulation. Ultra-thin sections were cut using a Reichert Ultracut UCT. Sections were double-stained with uranyl acetate and lead citrate and examined with a Zeiss LIBRA 120 transmission electron microscope. Detailed ultra-structure was carried out for three freshly mated (two longitudinally and one transversally sectioned), and two virgin individuals in which the ultra-thin sections provided sufficient coverage and quality for the pictures.

MORPHOLOGY AND BEHAVIOUR OF THE GENITALIA AND SPERM

FEMALE ANTRUM

The female antrum of *M. lignano* is composed of a single chamber, spherical and antero-posteriorly elongated (Figs 2B, 3A). The entrance to the antrum is marked by the ciliary tuft, a small region containing cilia distinctly longer than those of the vagina (Figs 2B, 3E). The rest of the antrum lacks cilia but is covered with microvilli protruding directly from the antral epithelium (Fig. 3F), reminiscent of absorptive tissues (such as in the gut; Alberts *et al.*, 1983). The cells are rich in vesicles and microtubules,

and contain several conspicuous Golgi apparatuses, suggesting a high metabolic activity (Fig. 3B, C). The cell membranes are convoluted and irregular in shape, reflecting the extreme flexibility required for the passage of the egg. The antrum is well innervated and surrounded by a net of muscles (Figs 2B, 3A), which define the antrum boundary. At the anterior end of the antrum, the epithelium gives way to the cellular valve ('Durchgangsapparat' or 'Verschlussapparat'; Ladurner *et al.*, 2005), a specialized epithelium connecting with the growth zone characterized by cells arranged in a fan-like shape (Figs 2B, 3A). The developed oocyte or egg passes through the cellular valve into the antrum, although the exact mechanism is still unknown. The presence of numerous gaps between the valve cells (Fig. 3D) and their large and convoluted membranes suggest that a duct can be formed by a temporary separation of the cells, which would re-adhere after the passage of the egg. A typical antrum without an egg measures approximately 70 μm in length (measured at the muscle boundary), but it is very flexible and can contract and elongate, as well as stretch to accommodate the egg, which typically measures approximately 120 μm in diameter (Ladurner *et al.*, 2005). The antral lumen contains electron-dense substances mainly in the form of fibres (Fig. 3F) that are already present before copulation (Fig. 4A). After copulation, small globules

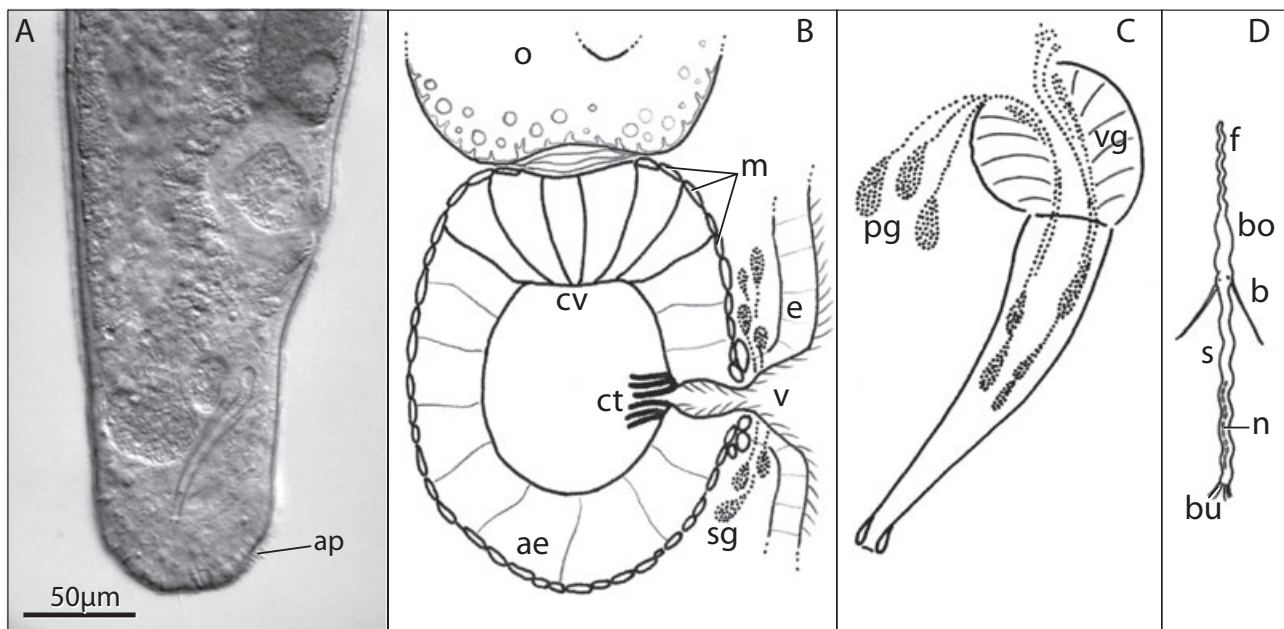


Figure 2. Main reproductive characters of *Macrostomum lignano*. A, micrograph of the tail region. B, schematic representation of the female genitalia. C, schematic representation of the male genitalia. D, schematic representation of the sperm. The adhesive papillae (ap), antral epithelium (ae), ciliary tuft (ct), epidermis (e), cellular valve (cv), muscles (m), oocyte (o), prostate gland cells (pg), shell glands (sg), sperm body (bo), sperm bristles (b), sperm brush (bu), sperm feeler (f), sperm nucleus (n), sperm shaft (s), vagina (v), and vesicula granulorum (vg) are highlighted.

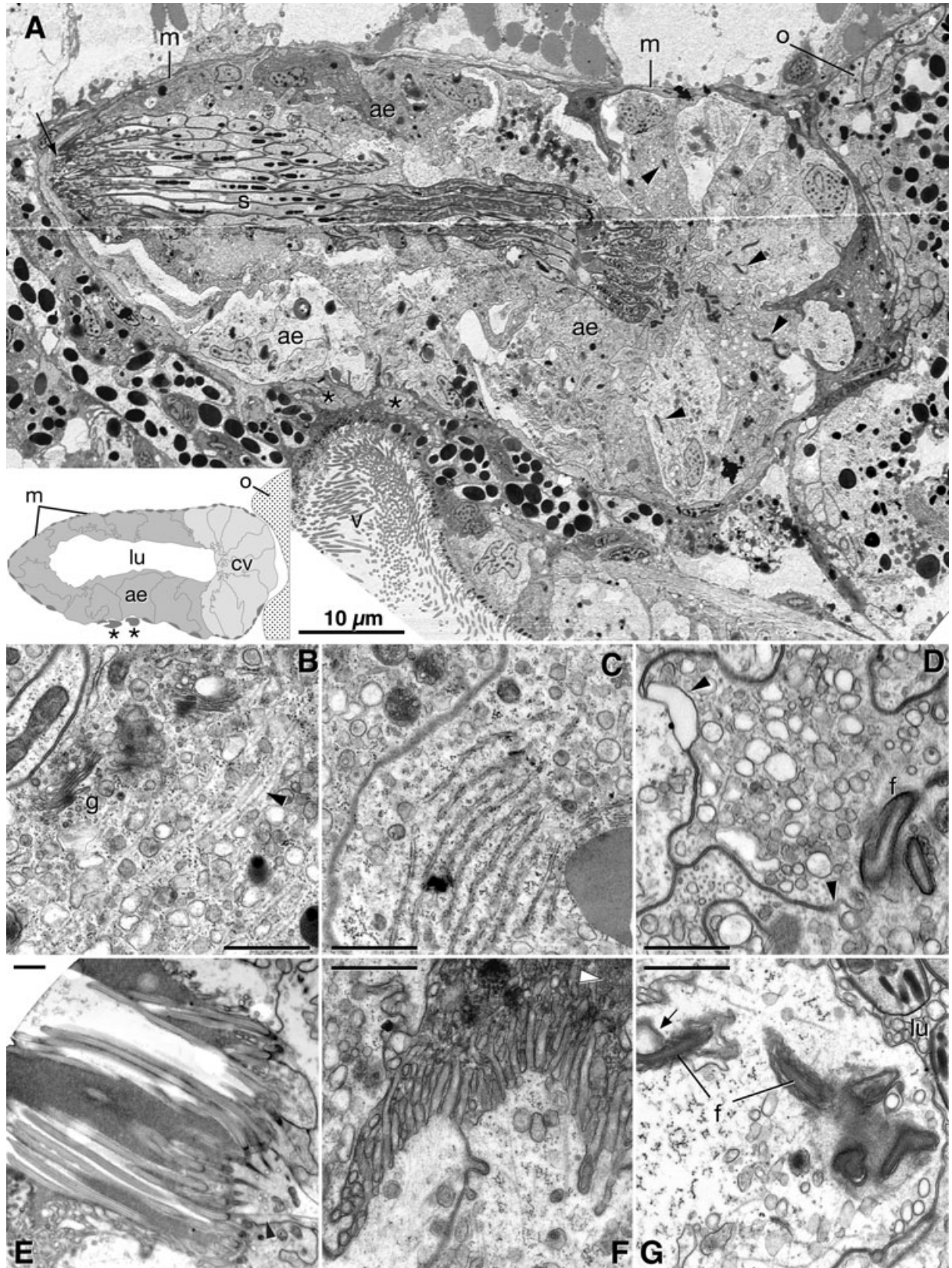


Figure 3. Ultrastructure of the female antrum of *Macrostomum lignano* (transmission electron micrographs). A, longitudinal section and inset with a simplified diagram of the antrum (anterior is on the right side). Note the fan shape of the cellular valve (cv, inset), located on the right side, after the oocyte (o). The antrum lumen (lu) is filled with sperm (s), which are anchored with their feelers in the cellular valve (arrowheads). The sperm are clearly polarized, and strongly compress the antral epithelium on the posterior side (arrow). Note the muscular layer forming the antrum boundary (m), which forms a strong ring (asterisk) above the vagina. B, Golgi apparatuses (g) in an antrum epithelium cell. C, endoplasmic reticulum in an antrum epithelium cell. B, C, note the abundance of vesicles, microtubules (arrowhead), and ribosomes (arrow). D, intercellular gaps in the cellular valve. The membranes of three adjoining cells separate locally (arrowheads). Note the anchored feeler (f). E, ciliary tuft at the entrance of the antrum. Note the long ciliary roots (arrowhead). F, surface of the antral epithelium showing the dense microvilli. Note the electron-dense fibres in the lumen (arrowhead). G, protein-like fibres surrounding the feelers anchored in the cellular valve. The feelers (f) are extracellular anchored, producing folds in the membrane of the antral cell (arrow). The antral epithelium (ae), feeler (f), Golgi apparatus (g), antral lumen (lu), muscular layer (m), oocyte (o), sperm (s), and vagina (v) are highlighted. Scale bar 1 μm in (B, C, D, E, F, G).

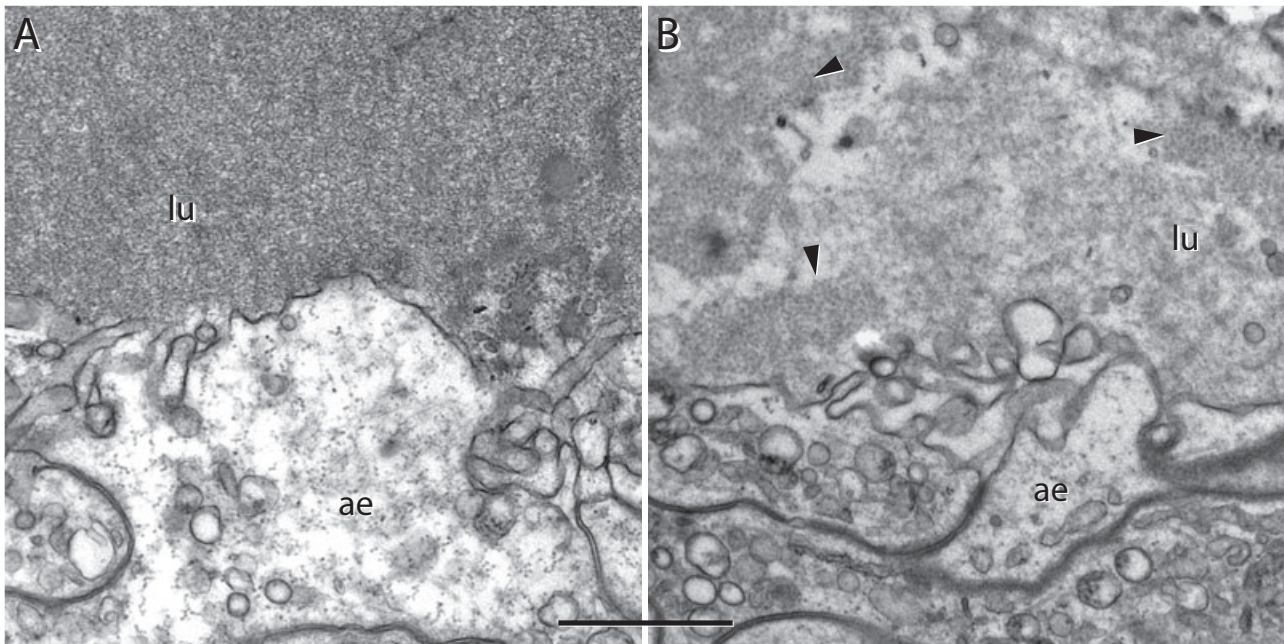


Figure 4. Detail of the antrum lumen of virgin and mated *Macrostomum lignano* (transmission electron micrographs). A, fibres fill the lumen (lu) of a virgin individual. B, besides the fibres, large clusters of granules (arrowheads) are present in the lumen of a mated individual. antral epithelium (ae), lumen (lu). Scale bar = 1 μm .

can be seen in addition to the fibres (Fig. 4B), plus large conglomerates visible with light microscopy.

STYLET

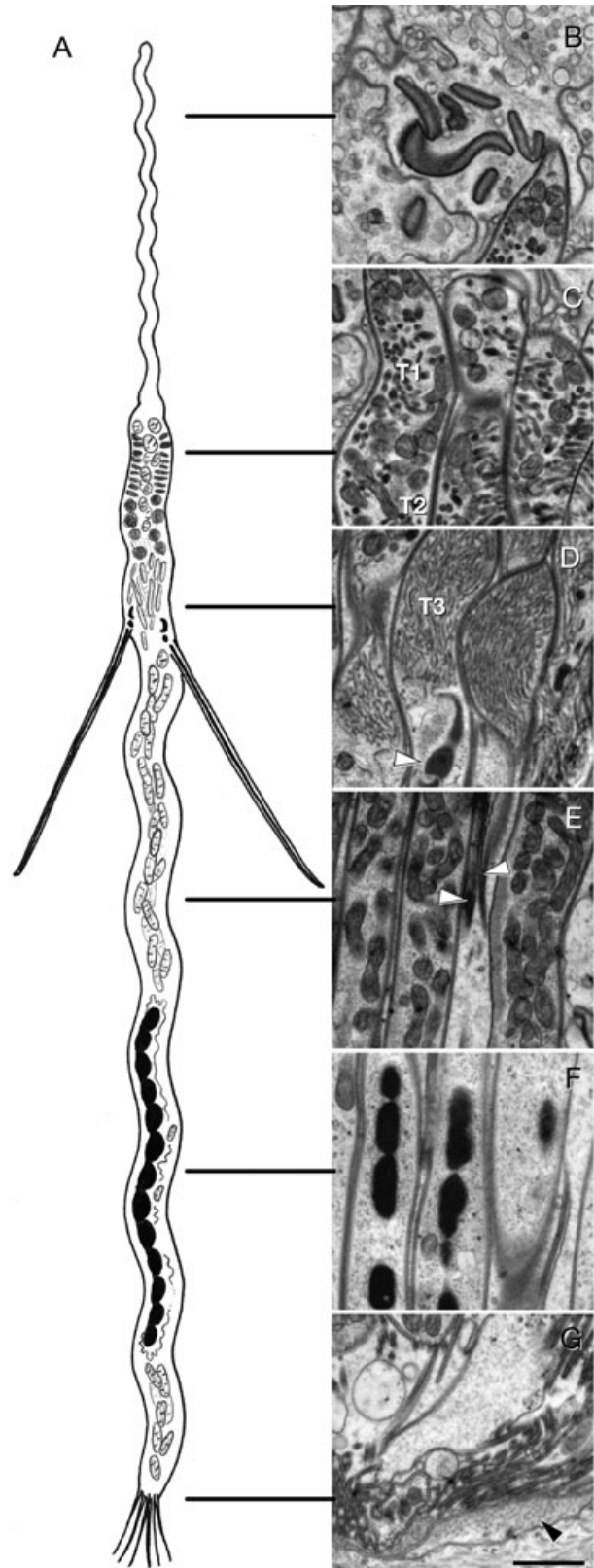
The male copulatory organ is a sclerotized tubular structure, which is intracellularly secreted by a syncytium (Doe, 1982). The stylet can be roughly divided into base, shaft, and tip (Fig. 2C). The base is slightly spherical, sometimes partly occupied by the vesicula granulorum. It is at the base where the stylet is anchored to the muscles responsible for its movements. The shaft decreases gently in diameter and follows a mild, broad spiral until it reaches the tip.

Part of its curvature is along the longitudinal axis and can be easily measured (Fig. 2C). At the end of the shaft, a more or less pronounced curvature marks the start of the tip, where the stylet wall thickens to form a blunt rim (often called distal thickening). The stylet shape varies among individuals, and it has been shown to affect sperm transfer success (Janicke & Schärer, 2009).

SPERM

The sperm of *M. lignano* is highly complex (Fig. 2D) and capable of astonishing behaviour (Movies S1–3). Unlike ‘typical’ sperm (*sensu* Morrow, 2004), it com-

Figure 5. Morphology of the mature sperm of *Macrostomum lignano*. A, line drawing of a mature sperm. Note the backwards pointing position of the bristles, which point forwards in developing sperm. B, C, D, E, F, G, transmission electron micrographs. B, profiles of the sperm feelers embedded in the cells of the cellular valve. C, mitochondria, T1 vesicles, and T2 vesicles in the anterior part of the sperm body. D, T3 vesicles and anchoring point of the bristles (i.e. the bristle complex) in the posterior part of the sperm body (arrowhead). E, densely packed mitochondria in the anterior part of the sperm shaft. Note the sections through two bristles between the sperm (arrowheads). F, interconnected units of the sperm nucleus in the posterior part of the sperm shaft. G, sperm brushes pressing against the posterior antral epithelium. Note the cross-section of a muscle cell, which delimits the antrum (arrowhead). Scale bar 1 μm in (B, C, D, E, F, G).



pletely lacks an axoneme and flagella, but it is by no means less motile (Movie S1), with movement being generated by arrays of cortical microtubules that span the full length of the cell (Newton, 1980). Distinct parts are easily recognized with light microscopy (Fig. 2D), and ultrastructure reveals a corresponding differentiation of the intracellular structure (Fig. 5), which changes during spermiogenesis (Willems *et al.*, 2009). The anterior end (i.e. the one which is forward regarding movement and function) is called feeler (Ferguson, 1940; also 'distal process': Newton, 1980; Willems *et al.*, 2009), probably as a result of a characteristic 'probing' behaviour (Movie S2). The feeler lacks organelles and vesicles (Fig. 5A, B), and is characterized by a swift, short-wave undulating movement (Movie S1). The start of the body (also referred to as the head; Willems *et al.*, 2009), is marked by an increase in width and a marked reduction in the undulation's wavelength. It contains a core of mitochondria and three types of vesicles (also called 'dense bodies' T1–T3; Willems *et al.*, 2009), arranged in three anteroposterior layers (Figs 5, 6). T1 vesicles are found at the start of the body. They are elongated and lay in contact with the sperm's membrane, on two sides of a core of mitochondria (Figs 5A, C, 6). Further down the body, T2 vesicles start to appear. They are round and denser than T1 vesicles. Many lay in contact with the sperm's membrane, but they also spread into the centre of the sperm, displacing the mitochondria (Figs 5A, 6B, C). The last third of the body is densely packed with the lighter T3 vesicles, which are very thin and tubular in shape (Figs 5A, D, 6B). They lay approximately aligned with the body's axis (Fig. 5A, D).

The shaft (also referred to as 'tail'; Ferguson, 1940) is very active and undulates at a lower but variable wavelength (Supplementary Movie S1). It also contains mitochondria (Fig. 5A, E) and, in the posterior

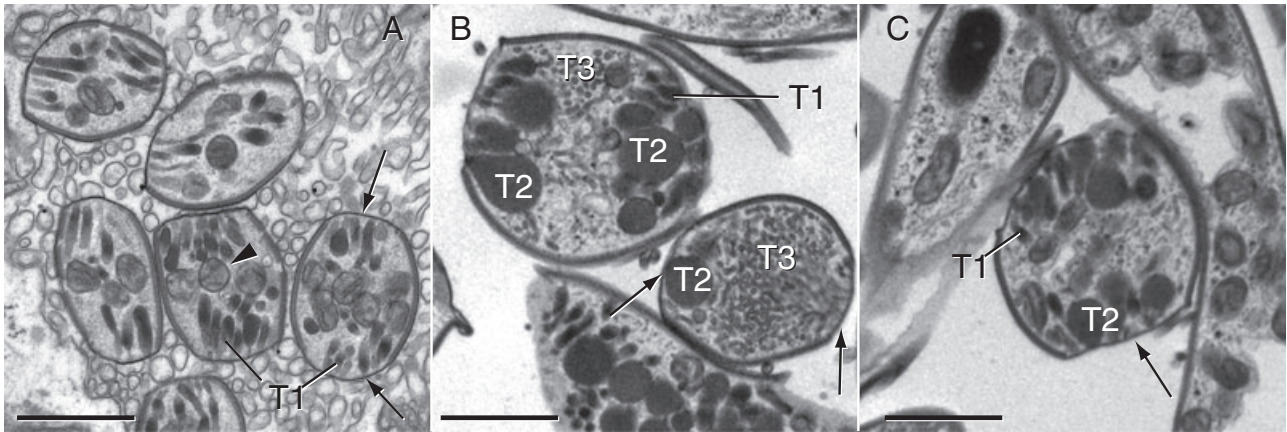


Figure 6. Transmission electron micrographs of transversal sections in the region of the sperm body, showing the gaps between the cortical microtubules' sets (arrows), and the different vesicles in close proximity with the sperm membrane. A, sperm in the lumen of the female antrum. Section across the frontal part of the sperm body, showing T1 vesicles and mitochondria (arrowhead). B, C, sperm in the seminal vesicle. Sections across the middle and posterior part of the sperm body, showing mainly T2 and T3 vesicles. Sections in (B) and (C) are slightly oblique. Scale bars = 1 μ m.

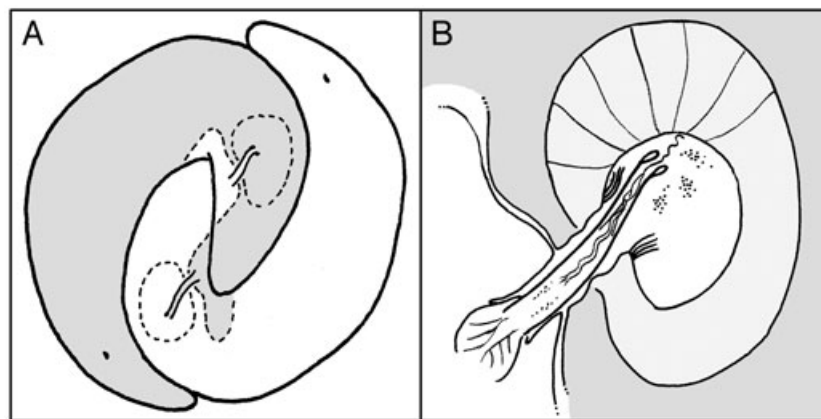


Figure 7. Copulation in *Macrostomum lignano*. A, line drawing of two copulating worms. Note the reciprocal insertion of the stylet into the partner's antrum. B, detail of the position of the stylet in the antrum of the partner during copulation. Note that the stylet might be able to reach the sperm anchoring point in the cellular valve.

half, the nucleus, which is condensed into interconnected units (Fig. 5A, F) and can be seen with light microscopy (Fig. 2D, Movie S1). We consider that a rod-like nucleus would probably be stiffer, and therefore this nuclear arrangement may allow the great flexibility of the aflagellate sperm typical of these flatworms. Between the body and the shaft, two straight bristles extend backwards (for a detailed study on their structure and development during spermiogenesis, see Willems *et al.* 2009). The cortical microtubules are arranged in two sets on opposite sides of the sperm (Fig. 6) (Newton, 1980). The two sets are separated by gaps, where the vesicles lay in direct contact with the sperm membrane (Figs 5C, 6). The microtubules protrude, enveloped in cell mem-

brane, at the end of the shaft leading to the appearance of the brush (Fig. 5G).

MALE-FEMALE INTERACTIONS

MATING

Copulation in *M. lignano* is reciprocal, with both worms engaging in precopulatory whirl-like behaviours ('circling' and 'reeling'; Schärer *et al.*, 2004) until they tightly wrap around each other when simultaneously inserting the stylet into the other worm's antrum (Fig. 7A). The stylet, which is as long as the antrum (Fig. 2), is inserted so that the blunt tip is close to the cellular valve, where it deposits sperm and prostate secretions (Fig. 7B). Although the

copulation time is short (on average 8.8 s; Schärer *et al.*, 2004), it is possible that the stylet removes or manipulates the position of previously received sperm. However, the muscular and flexible nature of the antrum could potentially prevent the stylet from reaching the cellular valve. Intriguingly, during copulation, the two tail plates are kept tightly pressed together by the frontal part of the worms (Fig. 7A), which may in turn constrain free movements of the antra. This tight 'embrace' becomes evident if one of the partners loses interest in the ongoing copulation and pulls away with the frontal part of its body, while the other remains firmly attached. Forced copulations, however, appear unlikely, because, as long as one worm remains attached to the substrate with its adhesive papillae (Fig. 2A), the other can not assume the appropriate copulatory posture. Prostate secretions are transferred together with sperm, and we suspect that they produce the conglomerates frequently observed in the antral lumen, perhaps by interacting with some of the female-derived substances (e.g. those shown in Fig. 4).

Approximately two-thirds of the copulations are directly followed by a behaviour where either one or both worms place their pharynx onto their own female opening and appear to suck, after which sperm shafts can be seen sticking out of the vagina (Schärer *et al.*, 2004). This suck behaviour may be a trait involved in manipulating the received ejaculate components; for example, by removing prostate secretions or sperm, or changing the position of sperm (either those freshly received or those previously stored) within the antrum.

SPERM-ANTRUM INTERACTIONS

Sperm in the antrum can be seen 'probing' the antral epithelium, i.e. tapping with the point of the feeler on the surface of cells (Movie S2). The feelers can also penetrate the antral epithelium, and they preferentially do so in the cellular valve, where they radiate outwards from a central area (Fig. 3A, Movie S3). Because sperm are often seen attached to cellular valve cells, it was originally postulated that these cells have a nourishing function (Luther, 1905). However, as early as 1947, Luther (1947) suggested that the cellular valve may regulate sperm in their access to oocytes. On the basis of the lack of vesicles and other cytoplasmic elements in the feelers (Fig. 5B), we consider that transfer of nutrients directly from the cellular valve to the sperm is unlikely.

No cellular valve has been observed in species with hypodermic insemination, which inject sperm directly in the body instead of transferring it into the antrum (e.g. *M. hystrix*, *M. viride* Luther, 1905; *M. appen-*

diculatum, Luther, 1947; *M. rubrocinctum* Ax, 1951). This underlines that the cellular valve's function is related to the presence of sperm in the antrum. Cellular valves of other *Macrostomum* species shown by Luther (1947) can have a higher tissue differentiation, for instance protruding from the antral epithelium, or separated from the antral lumen by a muscular diaphragm. This suggests that the interaction between sperm and recipient can be more complex than in *M. lignano*.

The penetration of the feeler into the cellular valve is extracellular, forming depressions on the cell membranes as the sperm anchors itself into the valve (Fig. 3G). The antrum reacts by producing protein-like fibres that accumulate in the cytoplasm around the anchored feelers (Fig. 3G). The function of these fibres is unknown, but, based on their proximity to the feelers, we hypothesize that they may modulate the interaction between the membranes of the two cells, potentially influencing the anchoring strength. The amount of fibres varies widely among feelers, even within the same antral cell. This may simply be a result of differences in the time of anchoring (i.e. time since sperm-epithelium contact). More interestingly, it may be a reaction to the identity of the sperm (i.e. a response to sperm surface molecules) or of the donor (e.g. via ejaculate substances).

Nourishment by the recipient may still happen in the antral lumen, perhaps via some of the aforementioned electron-dense substances (Fig. 4). The high levels of metabolic activity in the antral epithelium, as suggested by the abundance of vesicles, Golgi apparatuses, and ribosomes (Fig. 3B, C), could indeed indicate nourishment of stored sperm. Alternatively, this cellular activity can be an indication of sperm (or ejaculate) digestion, a phenomenon that has been suggested for many flatworm groups (An-der-Lan, 1939; Sluys, 1989) and other hermaphrodites (Michiels, 1998; Westheide, 1999), or the production of substances as a response to the received prostate secretions.

SPERM BRISTLES AND SUCK BEHAVIOUR

Despite the suck behaviour, at least some sperm manage to remain in the antrum. Once sperm feelers are deep in the epithelium, this may be explained by the anchoring. The suck behaviour, however, usually occurs immediately after sperm transfer, and thus presumably before sperm have had the time to fully anchor themselves. Moreover, at least some unanchored sperm can usually be found in the antrum (Movie S2), even days after copulation. We hypothesize that the bristles assist in preventing the sperm from being pulled out by sticking to the antral epithelium (Fig. 8). An alternative but not incompatible

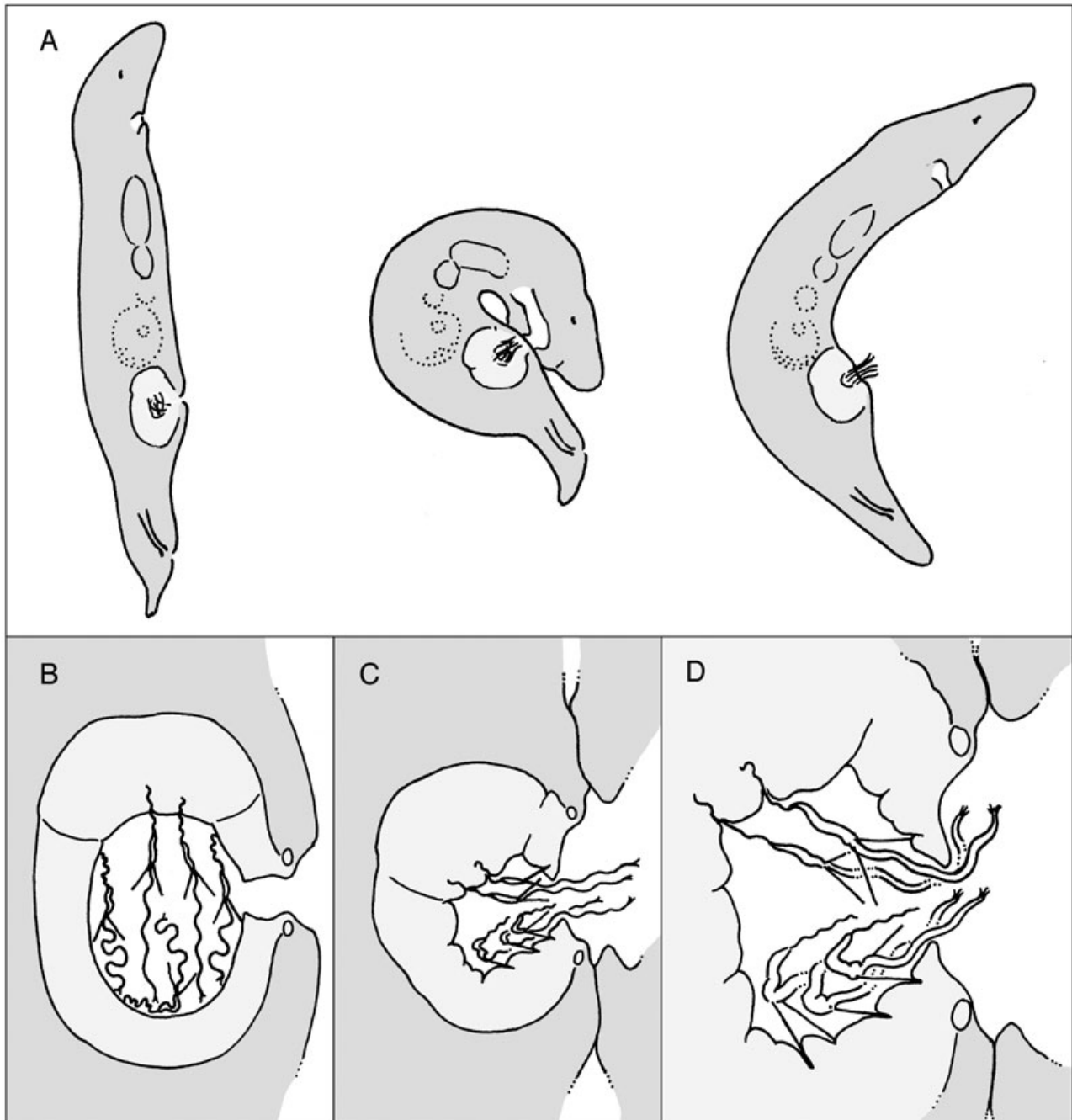


Figure 8. Hypothesis for the function of the complex sperm morphology during the suck behaviour in *Macrostomum lignano*. A, overview of behavioural stages. Immediately after copulation generally no sperm can be seen sticking out of the vagina. Then the worm places its pharynx over the vagina and appears to suck. Afterwards sperm are often seen sticking out of the vagina. B, immediately after copulation sperm feelers may start the anchoring process into the cellular valve. C, during the suck behaviour the female antrum is strongly compressed, and thus the cellular valve is expected to be in close proximity to the vagina. Sperm bristles may prevent the sperm from being sucked out. D, detail of (C), showing how the bristles may interact with the antral epithelium. Once the antrum relaxes, the sperm probably glide back into the antrum again (not shown).

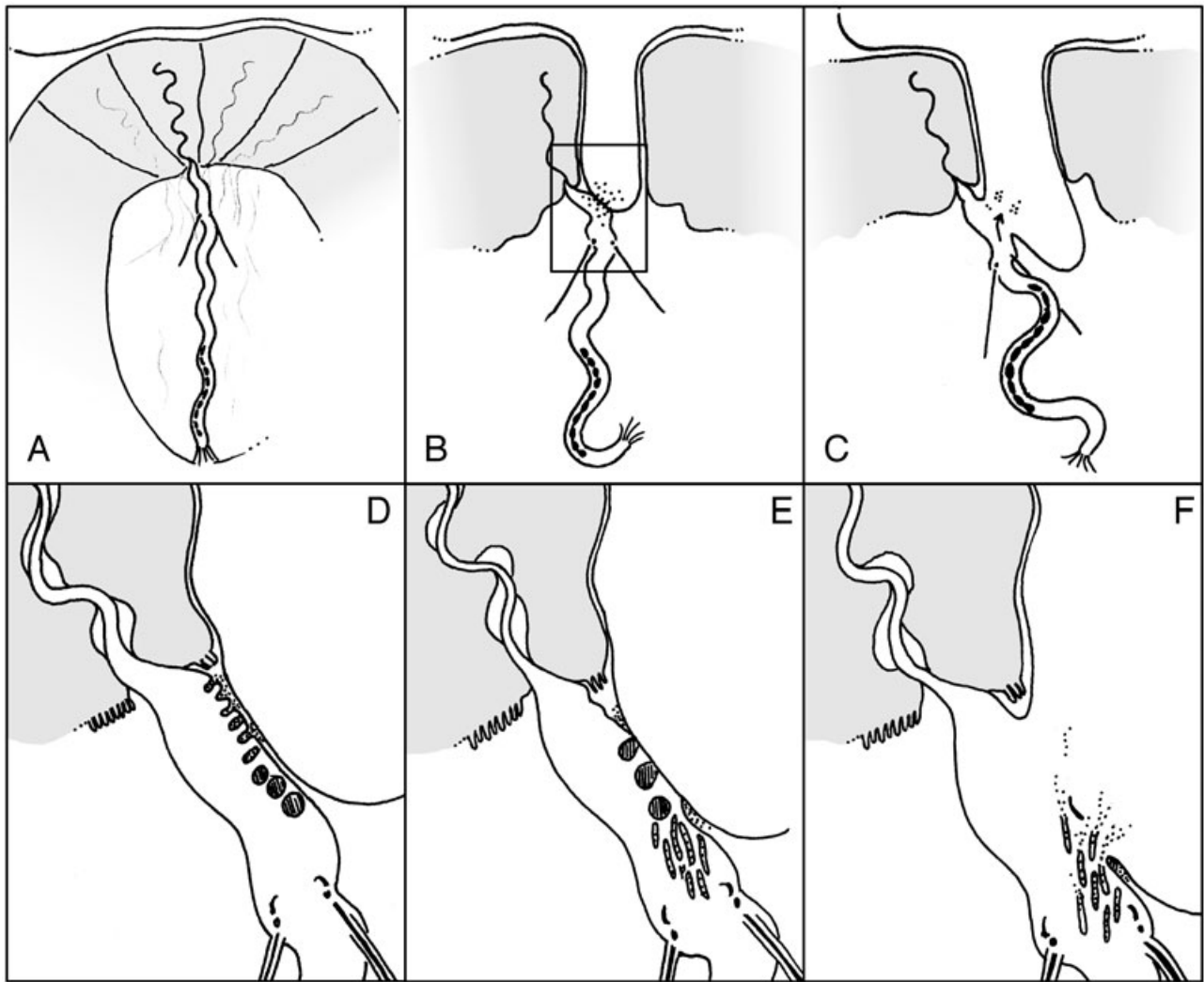


Figure 9. Hypotheses for sperm-egg contact and fertilization in *Macrostomum lignano*. A, overview of the closed cellular valve before the egg enters the antrum. Note how the sperm feelers radiate out into the different cells of the cellular valve from a central attachment point. Only one sperm is drawn completely for clarity. B, the egg enters the antrum after the cells of the cellular valve have temporarily separated, and first contacts the sperm at the anterior part of the sperm body; for detail, see (D). C, syngamy between the sperm and the egg occurs at this point and the contents of the sperm (including the nucleus) can enter the egg; for details, see (E) and (F). D, detail of the first contact point between egg and sperm in the region of the T1 vesicles, triggering syngamy. E, T2 vesicles function as acrosomal bodies and induce some type of acrosomal reaction. F, T3 vesicles are involved in activating the egg.

hypothesis for the function of the bristles is that they are an adaptation for sperm-sperm conflicts, by hindering the anchoring of freshly-arrived sperm.

FERTILIZATION

The mechanisms of fertilization are still unknown for all members of the genus *Macrostomum*. Based on different sperm and antrum features, we here propose a scenario for fertilization in *M. lignano* (Fig. 9), and presumably other species with reciprocal mating. Specifically, we hypothesize that the oocyte meets the

anchored sperm during its passage through the cellular valve. Because the feeler is deeply anchored in the cellular valve (Fig. 9A), the anterior part of the body would be the first part of the sperm to come into contact with the oocyte membrane (Fig. 9B). Precisely in this region, the gap between the cortical microtubules sets in the sperm is wide, and many vesicles are in close contact with the sperm membrane (Figs 5, 6). We postulate that the sperm fuses with the oocyte at this point, in a lateral fashion, as previously shown for other flatworms (trematodes: Burton, 1967;

Table 1. Explicit hypotheses proposed for the function of the studied traits in the context of sperm behaviour, post-copulatory conflicts, and fertilization

Hypotheses

Post-copulatory conflicts

- Stylet can interfere with previously received sperm during copulation (see section: Mating) (Fig. 3B)
- Antrum epithelium is involved in degradation of ejaculate components (see section: Female antrum)
- Cellular valve regulates the access of sperm to oocytes* (see section: Sperm–antrum interactions)
- Protein-like fibres in cellular valve cells modulate anchoring of feelers (see section: Sperm–antrum interactions)
- Bristles prevent sperm from being pulled out during suck behaviour (see section: Sperm bristles and suck behaviour) (Fig. 8)
- Bristles hinder the anchoring of later-arriving sperm (see section: Sperm bristles and suck behaviour) (Fig. 8)

Fertilization

- Intercellular gaps in the cellular valve open to allow the passage of the oocyte (see section: Female antrum)
- Sperm anchor themselves in the cellular valve in a way that facilitates the contact with the incoming oocyte (see section: Fertilization) (Fig. 9A)
- Sperm–oocyte fusion occurs laterally, via the sperm body contacting the oocyte (see section: Fertilization) (Fig. 9B)
- Sperm vesicles T1, T2, and T3 act sequentially to induce syngamy, acrosomal-like reaction, and oocyte activation (see section: Fertilization) (Fig 9E, F)

*Also suggested by Luther (1947).

Justine & Mattei, 1984; Orido, 1988; monogeneans: Justine & Mattei, 1986). The most anterior vesicles, T1, may be responsible for the syngamy: the binding of sperm and egg membranes (Fig. 9D). The subsequent vesicles, T2, resemble acrosomal bodies (Willems *et al.*, 2009) and may thus elicit some type of acrosomal reaction (Fig. 9E). Once the fusion is established, the cytoplasm and nucleus of the sperm can enter the oocyte (Fig. 9F). The third vesicle type, T3, may potentially be involved in activating the oocyte and preventing the entry of other sperm.

Although feelers are able to penetrate different types of epithelia (D. B. Vizoso, unpubl. data), we do not think that they go beyond the cellular valve and penetrate the egg. First, the way that they radiate out from the centre of the cellular valve leads them away from the place where the oocyte passes, so that they actually do not contact the egg. Second, we have followed the sperm transferred by sperm-labelled individuals using a sperm tracking technique (Schärer *et al.*, 2007), and have never observed labelled sperm nuclei beyond the antrum, suggesting that sperm do not normally traverse the cellular valve in *M. lignano*.

Anchoring in the cellular valve may not only allow sperm to be in the right position to fuse with the oocyte, but also it may also prevent sperm that failed to fertilize the oocyte from being flushed out by the passage of the egg.

CONCLUSIONS

The detailed observation of the morphological and behavioural traits investigated in the present study

allows us to formulate explicit hypotheses about their role in post-copulatory processes (Table 1). The present study shows that, in *M. lignano*, opportunities for female control and sperm competition exist at subsequent stages on the way to fertilization, acting like a series of hurdles a successful sperm has to overcome. We propose that this series of selection events are the likely cause of the complex structure of genitals and sperm. The ultrastructure of both sperm and antrum also reveals a subtle chemical interplay, which may further lead to antagonistic interactions between ejaculate and recipient. An enthralling variation of these traits exists across the different *Macrostomum* species, and ongoing comparative studies may shed some light on the evolution of post-copulatory sexual conflicts in simultaneous hermaphrodites and its relationship to mating strategies. A short generation time and its outcrossing nature make *M. lignano* amenable to long-term breeding experiments (e.g. artificial selection and experimental evolution). Its gene expression can be easily manipulated (e.g. RNA interference by soaking; Pfister *et al.*, 2008) and its size and transparency allow straightforward observation of internal traits. Expanding our knowledge on the genetics of *M. lignano* (e.g. via genomic approaches, mutation screens and controlled breeding experiments) will allow us to pinpoint genes involved in these and other traits involved in post-copulatory interactions. We therefore consider that *M. lignano* is an excellent system where experimental approaches to trait manipulation, such as selection experiments (e.g. on longer versus shorter sperm bristles), gene knockdowns (e.g. by RNA interference), and direct manipulation (e.g. prevention of the suck behaviour)

will allow testing the actual functions and adaptiveness of the discussed traits.

The evolution of reciprocal mating, so common in hermaphrodites, may have caused a lack of external ornaments and behaviours involved in pre-copulatory struggles and displays. At the same time, however, it appears to have resulted in a fascinating assortment of mostly internal, sexually selected traits. With the appropriate equipment, Darwin could have seen peacock's tails, deer antlers, clasps, nuptial gifts, and all manners of courtship songs squeezed into the deceptive simplicity of simultaneous hermaphrodites.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

- Movie S1.** A free-swimming sperm of *Macrostomum lignano*. Note the undulating movements of the feeler and the shaft. The nucleus can be seen in the posterior half of the shaft, forming a chain-like structure.
- Movie S2.** ‘Probing’ behaviour of the feeler of *Macrostomum lignano* sperm in the female antrum. Most sperm are polarized and only their brushes can be seen (right-bottom quadrant). Some sperm are unanchored and move in the antral lumen. Note the sperm in the middle, which actively touches the antral epithelium with its feeler, the so-called ‘probing’ behaviour.
- Movie S3.** Sperm of *Macrostomum lignano* anchored in the cellular valve of the female antrum. Note the feelers radiating towards the sides of the cellular valve.

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