Marine Biology (2004) 145: 373–380
DOI 10.1007/s00227-004-1314-x

RESEARCH ARTICLE

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Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms *suck*

Received: 6 November 2003 / Accepted: 16 January 2004 / Published online: 4 March 2004

Abstract Simultaneous hermaphrodites experience unique conflicts of interest during reproduction, some of which are reflected in their complex mating behaviours. We here provide the first detailed description of the mating behaviour of a marine flatworm of the genus *Macrostomum*, a cosmopolitan group of microturbellaria. Mating in this species is usually initiated by the precopulatory behaviours *circling* and *reeling*, then leads to reciprocal copulation where worms mutually insert their copulatory stylet, and often ends in an intriguing postcopulatory *sucking* behaviour. We provide detailed data on the frequencies and durations of the different behaviours, and examine some biotic and abiotic factors that could influence the mating rate. We further speculate on the function of *sucking* and suggest that it could be an adaptation for the digestion of sperm and/or the removal of seminal components, which may function as allohormones.
Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s00227-004-1314-x.

Communicated by O. Kinne, Oldendorf/Luhe

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Introduction

Turbellaria are a highly diverse group of flatworms. They represent a taxonomically ill-defined group among the Platyhelminthes, which are currently undergoing major revision (Jondelius 1998; Littlewood et al. 1998; Jondelius et al. 2002; Telford et al. 2003). Most turbellaria are simultaneous hermaphrodites (Ghiselin 1969), and they are therefore interesting study organisms to investigate questions of hermaphroditic reproduction, such as sex allocation, sperm competition, mating conflicts and mate manipulation (e.g. Charnov 1979; Michiels 1998; Schärer and Ladurner 2003). Moreover, some intriguing reproductive patterns, such as conditional gamete trading (Fischer 1980, 1987; Leonard and Lukowiak 1984; Sella 1985), hypodermic impregnation (Apelt 1969; Michiels 1998), and sperm digestion (Sluys 1989; Greeff and Michiels 1999; Westheide 1999; Bojat et al. 2001) occur in hermaphrodites. However, with a few exceptions, very little is known about their mating behaviour.

Most of the available data covers the larger turbellaria, such as triclads, and among those especially planarians (e.g. Peters et al. 1996; Vreys and Michiels 1997, 1998; Michiels and Bakovski 2000) and polyclads (Michiels and Newman 1998). For microturbellaria there is some information on Acoela (Hyman 1937; Costello and Costello 1938; Apelt 1969) and Proseriata (Giesa 1966), and a few older reports that provide some, mostly anecdotal, observations for a variety of species (e.g. Hallez 1879; Bresslau 1928; Meixner 1938). We are aware of only one report that gives a description of the mating behaviour of a species of *Macrostomum* (Ax and Borkott 1968). It consists of a short movie that shows, among other things, a copulation and sperm transfer in *M. romanicum* (called *M. salinum* in the publication).

We have recently established a member of the genus *Macrostomum* (Rhabditophora: Macrostomida) as a model organism, to address the above-mentioned questions. During this work we observed elements in the

mating behaviour that may be of interest for future studies on mating conflict and sperm digestion. These, however, require a detailed description of and clear terminology for the different behaviours, which we provide in the present study. Our description of the copulation behaviour of *Macrostomum* sp. is based on detailed microscopic observations, and provides quantitative data on the occurrence and duration of different behavioural components. We further suggest a hypothesis for the function of the observed postcopulatory behaviour.

Materials and methods

Study animal

Macrostomum sp. (Rhabditophora: Macrostomida) is a member of the interstitial sand fauna of the northern Adriatic Sea (Ladurner et al. 2000). It is an outcrossing simultaneous hermaphrodite (Schärer and Ladurner 2003), and reaches 1.5 mm in length when fully grown. It is transparent, allowing non-invasive observation of internal structures (Fig. 1). The paired testes are located anterior to the paired ovaries, and the female genital pore is anterior to the male genital pore. The female genital pore opens into the female atrium, into which sperm are transferred during copulation. The male genital pore is associated with a sclerotic stylet that serves as a copulatory organ, with a false seminal vesicle (i.e. the enlarged end of the vas deferens) that contains the sperm to be used in future copulations and with a muscular seminal vesicle that pumps the sperm. Sperm are complex (Fig. 2A), and copulations are frequent and reciprocal. Received sperm can often be observed in the female atrium, where sperm heads stick in a specialised tissue that connects to the oviduct (Fig. 2B), and sperm often move vigorously. Eggs start to form posterior to the ovary, gradually increase in size during vitellogenesis and enter the female atrium, generally one at a time, where they remain for some time before being laid. Mass cultures of *Macrostomum* sp. have been initiated at the University of Innsbruck in 1995 according to culture conditions described elsewhere (Tyler 1981; Rieger et al. 1988), and were recently established at the University of Münster. Briefly, worms are maintained at 20°C in glass petri dishes containing *f*/2 medium (a nutrient-enriched artificial seawater, Guillard and Ryther 1962), and are fed with the diatom *Nitzschia curvilineata*. Generation time under these conditions is 18 days, 5 days from egg laying to hatching and 13 days from hatching to adult.

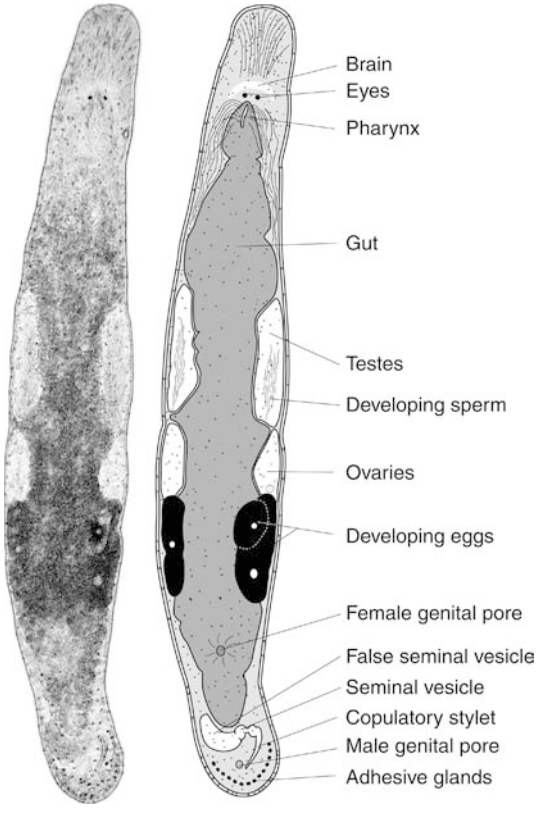

Fig. 1 *Macrostomum* sp. Photograph and line drawing of an adult, showing the main components of the reproductive system. Note the relative positions of the male and female genital openings, which is important to interpret the copulatory postures. Total length of worm is about 1.5 mm

Methods of observation

Worms usually move in three dimensions, and frequently move along the walls of culture dishes, making direct observation difficult under these conditions. We therefore devised an observation chamber that allows us to observe worms in a two-dimensional plane, by placing them in a drop of *f*/2 medium between two microscope slides (Fig. 3). Depending on the purpose of the observation we varied the distance between the slides. Thin observation chambers used a 105 μm spacer (i.e. one HERMA photo sticker), in which worms touch both slides and thereby are slightly compressed, facilitating observation of internal structures. Thick observation chambers used a 210 μm spacer (i.e. two HERMA photo stickers), in which worms would generally move on either the upper or lower slide. In order to avoid evaporation six additional drops of *f*/2 were added next to the drops containing the worms and the whole observation chamber was sealed with pure white Vaseline. We have successfully kept worms in such observation chambers for at least 10 days. Worms appear to show normal behaviour, including egg laying, normal embryonic development and successful hatching of offspring. No food was provided during the observation period, as the excreted algae can interfere with the image analysis algorithm.

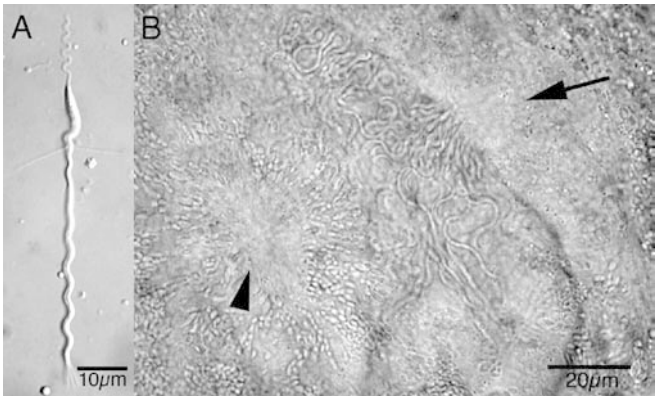

Fig. 2A, B Sperm of *Macrostomum* sp. **A** Sperm under interference contrast illumination. Note tapering end (which produces rapid undulations), two lateral bristles and blunt end (which looks like small brush). **B** Received sperm in the female atrium. Note that sperm stick in a specialised tissue with the tapering end and possibly the bristles (*arrow*). Also note the position of the female genital opening (*arrowhead*), which is surrounded by granular gland secretions

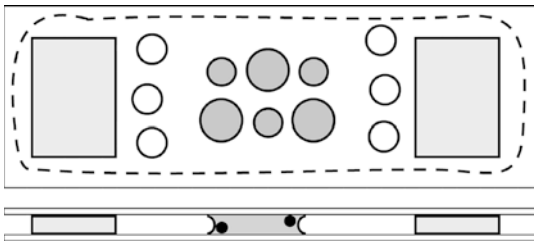

Fig. 3 Top view and longitudinal section through an observation chamber. The observation chamber consists of two microscope slides that are glued together with photo stickers (*light grey*). By varying the number of photo stickers one can vary the distance between the slides. Before joining the two slides, drops containing the worms to be observed (*six central grey circles*) and drops to reduce evaporation (*peripheral white circles*) are placed on one of the slides, and are encircled with pure white Vaseline (*dashed black line*). In the longitudinal section the space between the slides is exaggerated, and only one drop is depicted (*small black circles* indicate cross sections through the worms). Under a microscope the rim of the drop appears as a dark circle with the meniscus bowed inwards, keeping worms in the observable area

Description of the mating behaviour

In order to provide an initial description of the different elements of the mating behaviour we prepared many observation chambers that usually contained only one drop with one pair, and which were subsequently observed under an Olympus BH-2 compound microscope at various magnifications. Adult worms used for these observations usually stemmed from the mass cultures, and were chosen arbitrarily. Periods of these observations were recorded as digital QuickTime movies, using a Sony DFW-X700 digital FireWire c-mount camera connected to a PowerMac G4/450 running the shareware BTV Pro (http://www.bensoftware.com/btv-pro.html). This set-up allows digital movie capture at a maximum of about 8 frames s^{-1} at the native resolution of the camera (1,024×768). In order to achieve higher frame rates for more detailed observations some movies were made with a Sony CCD Iris video camera attached to a Sony DV Walkman, and subsequently transferred to the computer using BTV Pro.

Quantitative description of the mating behaviour

Mating could be a simple function of the frequency with which worms encounter each other, which could in turn be a function of the size of the enclosure in which the worms find themselves. In order to evaluate this possibility, the effect of enclosure size on the copulation rate was investigated. Enclosure size was varied by varying drop size in a range from 0.6 to 4.7 µl, which corresponded to drop areas of 2.6–22.0 mm^2 (median: 8.9 mm^2). Eight observation chambers with six drops, each containing one pair of worms, were made over a period of 5 days ($n = 48$). Each observation chamber was filmed for exactly 4 h at 1 frame s^{-1}, yielding 14,400 frames per QuickTime movie. Movie capture was initiated within 5 min after the observation chamber was assembled. The size of each worm was estimated as the mean of three area measurements taken from the movies.

The movies were analysed in three ways. (1) We determined the number of times worms encountered each other with a custom-made image analysis algorithm (available from the authors) that was programmed in the public domain image analysis software ImageJ (available at http://rsb.info.nih.gov/ij/). The program progressively buffers a user-specified set of frames from the movie and automatically segments the worm outlines from the enclosure and debris (e.g. eggs or excreted algae). All the outlines are recorded in a dataset and can be interactively verified against the original movie images. Encounters were scored as the times at which two separate outlines merged to one (i.e. whenever the worms came in contact). (2) We determined the number of times the different reproductive behaviours occurred by manual frame-by-frame analysis of the QuickTime movies. Behavioural elements were recorded in time slices of ten frames (i.e. 1,440 time slices), which provided a sufficiently high temporal resolution. We determined the time of copulation and the occurrence of postcopulatory behaviour. To estimate the temporal distribution of the copulatory behaviour, we calculated the median copulation time for each pair. If any eggs were laid, we further noted the time of egg deposition and also calculated the median egg-laying time. (3) We determined the durations of the different reproductive behaviours. We did this by picking one copulation for each pair at random (excluding the first and last copulations). By manual frame-by-frame analysis of the QuickTime movies we determined the duration of the precopulatory behaviour, the duration of the copulation, the occurrence and duration of the postcopulatory behaviour, and the time until the next copulation.

Statistical analysis

Of the 48 drops, 11 had to be excluded form the analysis (five because one or both worms appeared to have been injured during pipeting, three because one worm appeared to be immature, and three because the drop did not touch the upper slide). The remaining sample size was thus $n = 37$. We graphically checked if data fulfilled the assumptions of parametric test statistics, and transformed the data if necessary. If no suitable transformation could be found, we used nonparametric statistics. For all statistical tests we give two-tailed error probabilities. Averages are always given as means (± 1 SE) unless otherwise stated. Data were analysed with JMP 3.2.2. (SAS Institute 1994).

Results

Description of the mating behaviour

Worms copulated readily in the observation chambers, and all elements of the behaviour could easily be observed. The precopulatory behaviour consists of two elements, *circling* and *reeling*, that can alternate (Fig. 4). *Circling* consists of the worms mutually crawling on each other, often forming a tight ball. Proper *circling* can only occur if both partners detach from the substrate by releasing the adhesive glands on their tail plates. If one remains attached, the other may circle around the attached individual, but copulation cannot occur. During *reeling* worms take on a head to tail orientation, with the snout of one individual touching the other individual dorsally anywhere between the tip of the tail to the location opposite the female opening. In this posture the worms can often be seen "reeling" around their centre of mass in the direction of the heads. Frequently the worms will show an "erection", a raising of the tissue surrounding the copulatory stylet, which appears as a translucent cone-like shape in the tail plate (see arrowheads in Fig. 4) and which suggests that *reeling* is sexually motivated. Another characteristic of *reeling* is that there is a gap in the centre of the reel. *Reeling* is less frequent than *circling* and is not a prerequisite for copulation. Both behaviours can occur without leading to a copulation.

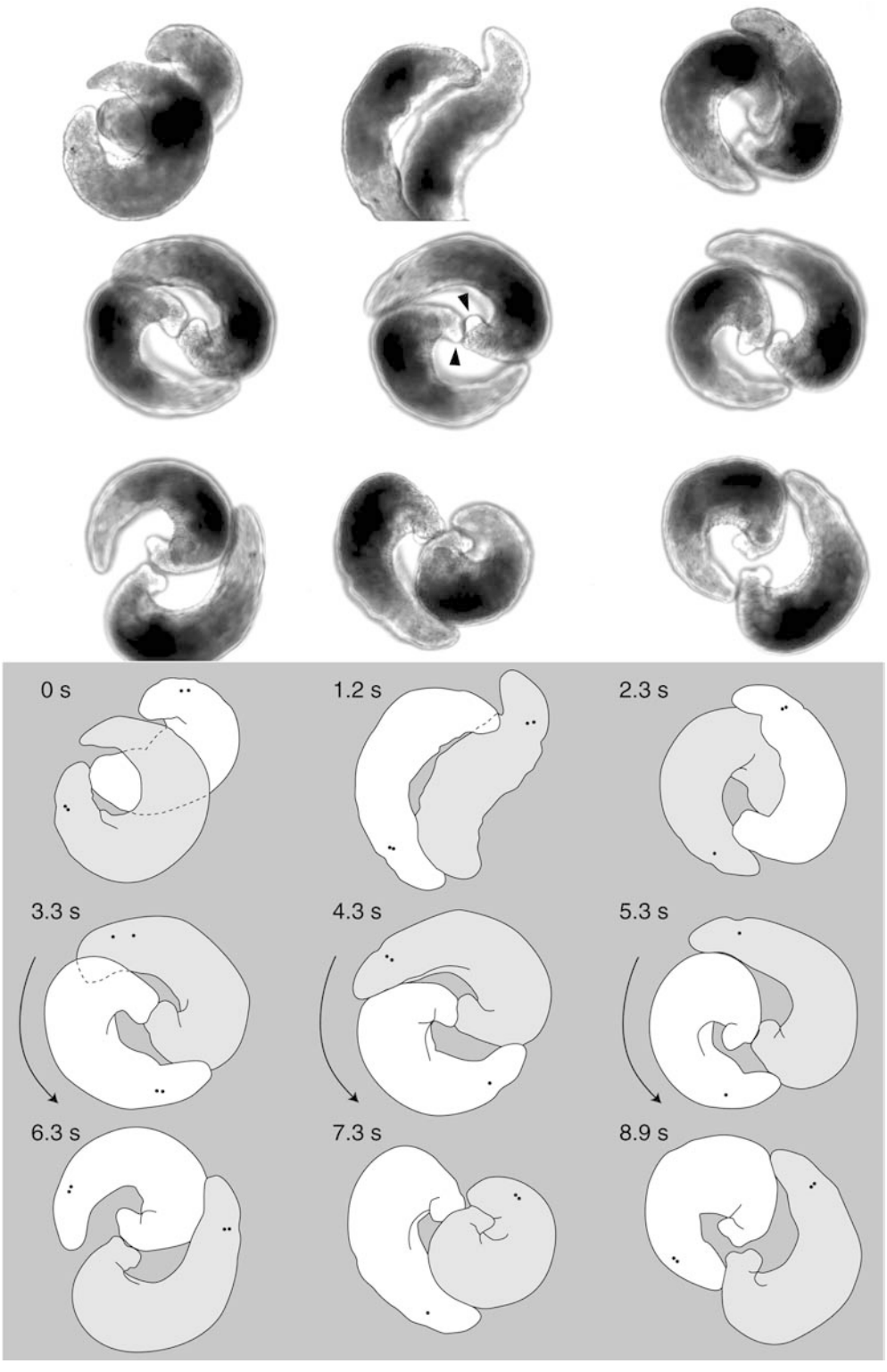

Fig. 4 *Macrostomum* sp. Key frames from a video sequence (with line drawings) of the precopulatory *circling* and *reeling* behaviours of a pair of adults. In this sequence *circling* occurs from the start to the frame at 2.3 s and *reeling* in frames from 3.3 to 8.9 s. The depicted sequence did not lead to a copulation. The *bowed arrows* in the line drawings indicate that the pair turned 360° from one frame to the next. *Arrowheads* indicate "erections", a cone-like swelling around the copulatory stylet. The full video sequence is available as a QuickTime movie (see supplementary materials S1 and S2 for a small and large version respectively)

The *copulation* consists of a posture where the tail plates touch each other ventrally in opposing directions, while the anterior ventral surface of each worm touches the posterior dorsal surface of the partner (Fig. 5). The shape of the individual worm in this posture resembles a G (Fig. 5, for a graphical representation see the two small interlocking Gs next to the worms at 9.5 s). Together the worms form a tight disc that lies on the substrate with no gap between them. In this posture worms often rotate around their centre of mass, as described for *reeling*. In thin observation chambers worms could be observed to reciprocally insert their stylet in the female opening of their partners, but sperm transfer was never clearly observed.

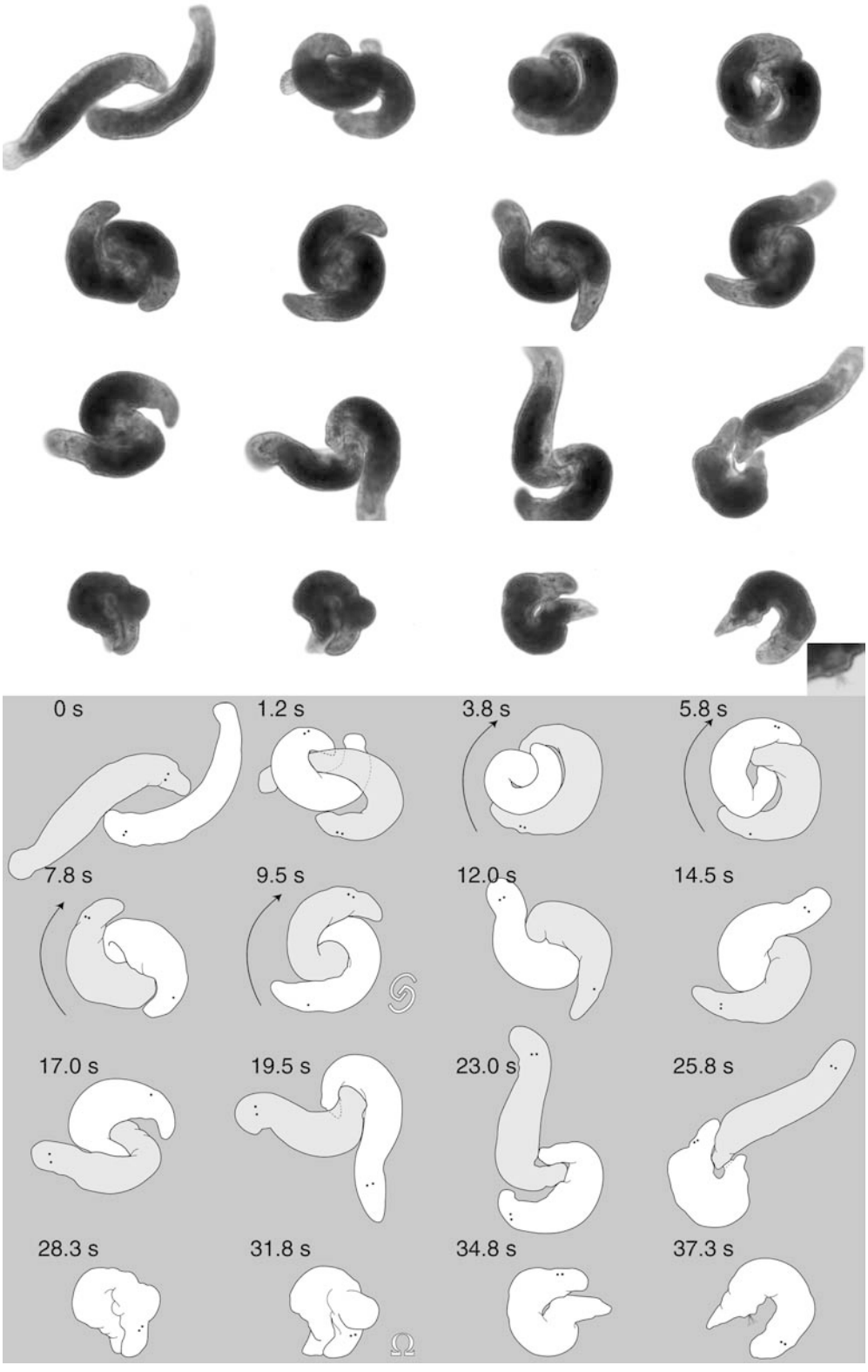

Fig. 5 *Macrostomum* sp. Key frames from a video sequence (with line drawings) of the copulatory and postcopulatory behaviours of a pair of adults. Copulation (frames at 7.8–19.5 s) is preceded by a short phase of *circling* (from the start to the frame at 5.8 s), and ends in a *suck* (frames at 28.3–34.8 s). Note: (1) the bundle of sperm sticking out of the female opening on the last picture (37.3 s, the *small inset* shows this in more detail); (2) the small graphical representation of the G-position during mating next to the worms at 9.5 s; and (3) the small graphical representation of the Ω-position during *sucking* next to the worm at 31.8 s. The full video sequence is available as a QuickTime movie (see supplementary materials S3 and S4 for a small and large version respectively)

The postcopulatory behaviour is facultative, and can be exhibited by none, one, or both worms. It consists of a stereotypical *sucking* posture (Fig. 5), which resembles an Ω-shape, and in which the worm folds back onto itself while positioning its pharynx over its female opening (Fig. 5, for a graphical representation see the small Ω next to the worms at 31.8 s). In thin observation chambers and at high resolution, one can observe that the pharynx is performing a *sucking* behaviour. After the pharynx disengages one can often

observe a bundle of sperm sticking out of the female opening (Fig. 5).

Quantitative description of the mating behaviour

Worms on average encountered each other 418 times over the 4-h period (range: 136–970, every 15–106 s), and there was a clear relationship between the size of the drop in which worms were held and the number of encounters (Spearman rank correlation, $r_s = -0.55$, $P < 0.001$).

We observed a total of 885 copulations. Worms copulated on average 24 times over the 4-h period (range: 5–55). Copulations lasted 8.8 ± 0.4 s (range: 5–16 s, coefficient of variation = 30.6, $n = 37$), and were preceded by a precopulatory phase of 15.9 ± 1.7 s (range: 5–49 s, CV = 66.5). The mean duration until the next copulation was 613 ± 158 s (range: 50–5,140 s). Despite the strong relationship between drop size and the number of encounters, we found no significant relationship between drop size and the number of copulations ($r_s = -0.17$, $P = 0.31$), nor between the number of encounters and the number of copulations ($r_s = 0.23$, $P = 0.16$), suggesting that the number of copulations is not a simple function of either of these parameters. There also appeared to be no effects of the average size of the pair ($r_s = 0.09$, $P = 0.62$) or the relative size difference between the worms in the pair ($r_s = 0.22$, $P = 0.18$) on the number of copulations.

However, we observed a clear trend for more copulations to occur later in the observation period, as measured by the total numbers of copulations observed in all pairs in the first, second, third and fourth hour (123, 223, 242, and 298 copulations, respectively). As a result, the mean of the median copulation time of each pair was 142.5 min after the start of the observation, which is significantly later than expected if copulations were spread equally over the 240-min observation period (one-sample *t*-test against the expected median copulation time of 120 min, $t = 3.7$, $P < 0.001$). Some pairs alternated active copulatory periods with periods of resting or fast swimming, whereas other pairs copulated at a relatively constant rate.

We observed a total of 1,090 *sucks*; 76% of these occurred within 5 s after the end of a copulation, and there was a strong correlation between the number of copulations and the number of *sucks* within a pair ($r_s = 0.79$, $P < 0.001$). Nevertheless, 258 *sucks* occurred independent of a copulatory event, suggesting that *sucking* is not only a postcopulatory behaviour. The average *suck* lasted for 4.9 ± 0.2 s (range: 4–7 s, CV = 18.7). The low CV suggests that *sucking* is highly stereotypic. Of the 885 copulations we observed, 33% were not followed by a *suck*, 40% were followed by one individual performing *sucking* behaviour, and in the remaining 27% cases both partners performed a *suck*.

Of the 37 pairs, 28 laid a total of 44 eggs during the 4-h observation period (i.e. 1, 2, 3, and 5 eggs were laid by 17, 8, 2 and 1 pairs, respectively). This suggests a per capita egg-laying rate of 0.6 eggs in 4 h or 3.6 eggs day^{-1}. This is substantially higher than the normally observed rate of about 1–2 eggs day^{-1} (L. Schärer, unpublished data), and suggests that the transfer to these small observation chambers stimulated egg laying. Moreover, the mean of the median egg-laying time was 50.4 min, which is significantly earlier than expected if laying were spread equally over the 240-min observation period (one-sample *t*-test against the expected egg laying time of 120 min, $t = 4.8$, $P < 0.001$, $n = 28$).

Discussion

A striking aspect of the mating behaviour of *Macrostomum* sp. is the high mating rate. Given that these worms usually lay only 1 or 2 eggs day^{-1}, and that they therefore need only a few sperm to fertilise them, the high mating rate is unlikely to be explained by assurance of fertility. We also do not think that the high mating rates are an artefact of the spatially constrained holding conditions in the observation chambers. Copulations are readily observed under the normal culture conditions, in which hundreds of worms are kept in glass petri dishes at densities that are about two orders of magnitude lower. Moreover, we found no significant effect of drop size on copulation rate, suggesting that this factor is not very important. Further, we have observed comparable copulation rates in worms that had recently been caught in the field, thus excluding a possible artefact of long-term laboratory maintenance.

Although there can be no doubt that sperm are transferred between worms (Schärer and Ladurner 2003), we have never observed sperm transfer in direct observations. One possible reason could be that many copulations do not actually lead to sperm transfer, which could partly explain the high mating rate. Alternatively, if only few sperm were transferred in each copulation, it may be difficult to see the transfer. In the video documentation of the mating behaviour of *M. romanicum* presented by Ax and Borkott (1968), hundreds, if not thousands of sperm are transferred in one copulation. This is clearly not the case in our species. A well-filled seminal vesicle probably contains only between 100 and 200 sperm (L. Schärer, unpublished data), and sperm transfer at the rate observed in *M. romanicum* would clearly be unsustainable at the observed mating rates. Moreover, we have repeatedly observed that small numbers of sperm can pass through an everted stylet after a failed copulation attempt. We thus consider it likely that sperm are transferred frequently, but in relatively small numbers.

The functions of *circling* and *reeling* could be linked to courtship, mate assessment, or may just be the outcome of an attempt to assume the copulatory posture. The need to get in close contact for copulation may require the mutual crawling on each other seen in *circling*. However, *circling* sometimes appeared to be performed

primarily by one worm on the other, as may be expected for courtship behaviour. But, as we could not clearly distinguish the individual worms in a pair, we did not attempt to analyse this. Marking the worms with vital dyes would be required to study this possibility in more detail. Regarding mate assessment, we presume that the close proximity during *circling* could allow the worms to sense the presence of developed eggs in the body of their partners, and that this could influence their attractiveness. *Reeling*, in contrast, may simply represent a failed attempt to assume the copulatory posture.

However, the most interesting finding is the peculiar postcopulatory *sucking* behaviour. Although its function remains unknown, we would like to propose a few possibilities. Since 76% of all *sucks* occurred directly after a copulation event, they must be a consequence of something that happens during copulation. When the pharynx disengages after *sucking* we have often observed a bundle of sperm that was sticking out of the female genital opening; this was never observed in any other context. We initially considered the possibility that the observed structures are the cilia of the vagina, but these are much shorter, and are thinner than sperm. One possible explanation for *sucking* could be that the worms eat the sperm they just received. Sperm digestion is a well-known phenomenon in hermaphroditic animals, and it occurs in a wide range of species (e.g. Sluys 1989; Baur 1998; Michiels 1998; Westheide 1999). However, it usually occurs via resorptive tissues in the sperm-receiving organs or via ducts that connect these organs with the gut. We are aware of only two other species where a recipient was observed to directly eat the received sperm. One is in the arrowworm *Spadella cephaloptera* (John 1933), an organism exhibiting pseudocopulation, whereby the sperm are deposited on the outside of the body and then migrate into the female opening. The other case is in the leech *Placobdella parasitica*; in this case, the spermatophores are deposited externally, then dissolve the body wall, and the sperm enter the animal hypodermically (Myers 1935).

An interesting observation about *sucking* is that the sperm in the bundle all appear to have the same length when sticking out of the female opening. One possible explanation could be that this occurs because they remain attached internally (as shown in Fig. 2B). The female atrium can contract very strongly (such as during egg laying), and it is thus conceivable that the lose ends of the attached sperm and the free sperm could be pressed out through the female opening, and any free sperm eaten. To show this convincingly will require following the fate of labelled sperm in the recipient, and determining if sperm are actually digested when taken up orally.

During copulation *Macrostomum* sp. does not only transfer sperm, but also prostate secretions. Recently mated worms sometimes have translucent granules in their female atrium, which could represent coagulated prostate secretions. The structure of the prostate glands has not been studied in detail in our species, but prostate glands are a prominent feature of the tail plate in many Macrostomidae (Doe 1982), and they sometimes reach dramatic sizes (e.g. *M. miraculicis* in Schmidt and Sopott-Ehlers 1976, called *Bradburia miraculicis* in Faubel et al. 1994). The function of the prostate secretion remains unknown. Older literature often states that it may be involved in nourishing sperm, but we are aware of no convincing evidence for this idea. However, recent ideas about mate manipulation via allohormones could apply (e.g. Michiels 1998; Koene and ter Maat 2001). Experimental studies in *Drosophila* have convincingly shown that male accessory gland proteins influence a number of female reproductive traits to the advantage of the male (Holland and Rice 1999; Pitnick et al. 2001), but that they can be harmful for the receiving female (Chapman et al. 1995). It therefore seems plausible that recipients of such manipulating allohormones attempt to remove them, and *sucking* may be involved in this.

Finally, one could imagine that the high mating frequency is linked to *sucking*. To transfer few sperm per mating may make *sucking* less rewarding and may hence be a strategy to increase the chances of depositing sperm that have a chance to fertilize the next egg.

Acknowledgements We would like to thank M. Fasel for artwork and P. Ladurner for the sperm picture. P. Ladurner, N. Michiels, R. Rieger and D. Vizoso provided helpful discussion and comments on the manuscript. L.S. would further like to thank N. Michiels for his hospitality. During this study L.S. was supported by an IHP-fellowship (SNF, Switzerland) and a L.-Meitner-fellowship (FWF, Austria). Animal experimentation was carried out in accordance to German legal and ethical standards.

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