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Animal Behaviour 85 (2013) 453–461

Contents lists available at SciVerse ScienceDirect

Animal Behaviour

journal homepage: www.elsevier.com/locate/anbehav

Effects of mating status on copulatory and postcopulatory behaviour in a simultaneous hermaphrodite

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ARTICLE INFO

Article history:
Received 2 August 2012
Initial acceptance 12 September 2012
Final acceptance 26 November 2012
Available online 11 January 2013
MS. number: 12-00603R

Keywords:
accessory gland secretion
Macrostomum lignano
mating behaviour
mating history
mating motivation
partner manipulation
postcopulatory sexual selection
simultaneous hermaphrodite
sperm allocation

Mating status is one of the most important predictors of the mating propensity of an individual. This is because mating lowers the amount of sperm cells and seminal fluids available to donate for males and increases the amount of ejaculate received by females, which may both have an effect on the mating propensity. In simultaneous hermaphrodites with reciprocal copulation, the mating status is expected to affect the mating propensity in both the male and the female sex function within a single individual, but empirical evidence is scarce. We experimentally tested the effect of the mating status of an individual and its partner on copulatory and postcopulatory behaviour in the free-living flatworm *Macrostomum lignano*, an outcrossing simultaneous hermaphrodite. These worms have frequent reciprocal copulations and often display a postcopulatory suck behaviour, potentially involved in removing ejaculate components from their own sperm-receiving organ. Virgin pairs copulated more, earlier and for longer than sexually experienced pairs. Moreover, we observed fewer sucks in virgin than sexually experienced pairs, all consistent with a higher willingness both to donate and to receive sperm in virgins. We investigated whether the lower suck frequency in virgin pairs depends on the mating status of the focal individual or on that of its partner. Surprisingly, the results suggested that the suck frequency depends on the mating status of the partner. We discuss these results in the context of potential sexual conflicts over the performance of the suck behaviour.
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In copulating animals, matings are crucial events in which males and females are expected to allocate their reproductive resources strategically over multiple matings and partners to maximize their own fitness (Jennions & Petrie 2000; Wedell et al. 2002; Kokko & Mappes 2005; Parker & Pizzari 2010; Edward & Chapman 2011). The mating propensity of an individual is expected to depend on the costs and benefits of copulating, which may vary between the sexes and also across different mating opportunities, for example, because of varying amounts of available gametes and varying attractiveness of the available partners.

During copulation, males donate an ejaculate, which is usually composed of both sperm cells and seminal fluids. An important determinant of male reproductive success is the amount of transferred sperm cells, since males transferring more sperm cells have been shown to outcompete the sperm cells of competing males (e.g. Gage & Morrow 2003; but see Snook 2005). In addition, seminal fluids may interact with sperm, and thereby also influence male reproductive success (reviewed in Chapman 2001; Arnqvist & Rowe 2005), notably by manipulating female physiology and behaviour (e.g. Chen et al. 1988; Heifetz et al. 2000). Although males are expected to gain fitness benefits from inseminating numerous females with large ejaculates, the ejaculate also represents a costly investment, which requires time and energy to produce and to replenish (e.g. Nakatsuru & Kramer 1982; Royer & McNeil 1993; Schärer & Vizoso 2007). Hence, given that male reproductive success depends on the amount of ejaculate transferred (e.g. Gage & Morrow 2003; Wigby et al. 2009) and that the amount of available ejaculate is influenced by previous mating events (e.g. Brauer et al. 2007; Hettyey et al. 2009), sexually deprived males are expected to have a higher mating propensity than recently mated males.

During copulation, females receive an ejaculate, which is often stored and provides the sperm required for the fertilization of the eggs (reviewed in Orr & Zuk 2012). On the one hand, female reproductive success might be limited by the amount of sperm available to fertilize the eggs, for example because of difficulties in obtaining sufficient sperm or in finding mates (Wedell et al. 2002; Kokko & Mappes 2005), and females may benefit from multiple matings (Jennions & Petrie 2000). On the other hand, the receipt of ejaculate may also have detrimental effects on female reproductive success, which are likely to increase with repeated copulations, for

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http://dx.doi.org/10.1016/j.anbehav.2012.12.007

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example because of the risk of polyspermy (reviewed in Birkhead et al. 1993) or seminal fluid-mediated costs (reviewed in Chapman 2001; Arnqvist & Rowe 2005). Therefore, female mating propensity is expected to vary according to the amount of sperm stored to optimize the eggs' fertilization and the female's reproductive success. In addition, female mating propensity may also be manipulated by previous mating partners (Johnstone & Keller 2000), notably through the seminal fluid transferred during copulation (e.g. Chen et al. 1988). Consequently, female mating propensity can also be expected to depend on the amount and the composition of the ejaculates received from previous mating partners.

In addition to its mating status, an individual's mating propensity may also vary according to the attractiveness of the partner. When mates vary in their reproductive quality, both sexes are expected to be choosy about their mating partners, and thus display higher mating propensity with partners that are expected to provide higher fitness benefits (reviewed in Dewsbury 1982; Jennions & Petrie 2000; Edward & Chapman 2011). For instance, it has been shown that males mate preferentially with more fecund and/or virgin females (e.g. Johnson & Hubbell 1984; Schneider et al. 2011) and/or tailor the ejaculate size to the level of sperm competition (e.g. Wedell 1992; Gage & Barnard 1996; reviewed in Parker 1998). Similarly, females may preferentially mate with males providing material and/or genetic benefits (Jennions & Petrie 2000; Møller & Jennions 2001).

Consequently, since the costs and benefits of copulating can depend on the previous mating events of both mating partners, the mating status of both is expected to contribute significantly to mating propensity in both males and females. The effect of mating status on mating propensity has mainly been studied in species with separate sexes (Kokko & Mappes 2005; Edward & Chapman 2011), whereas fewer studies have focused on species with different sexual systems.

In simultaneously hermaphroditic animals (hereafter called hermaphrodites), individuals produce ejaculates and eggs at the same time and so both partners can donate and receive ejaculates. Hermaphrodites are therefore expected to allocate their ejaculate strategically over multiple matings and partners, while simultaneously aiming to ensure an optimal supply of sperm to fertilize their own eggs. Hence, mating propensity may depend on both the amount of sperm (hereafter called autosperm) and seminal fluids available to inseminate a partner and on the amount of received sperm available to fertilize the eggs (hereafter called allosperm; Anthes et al. 2006), which are both likely to vary according to the previous mating activity and social context (Schärer & Ladurner 2003).

To date, effects of mating status on mating propensity have been mainly studied in hermaphrodites with unilateral copulation, especially snails (reviewed in Anthes et al. 2006) while, to our knowledge, there are currently no experimental studies in reciprocally mating species (but see Tomiyama 1996 and Kupfernagel & Baur 2011 for correlational studies). For instance, sexual isolation has been shown to increase both female (Facon et al. 2007) and male mating propensity (Koene & Ter Maat 2005; Dillen et al. 2008). It has been argued that in some snail species male mating propensity may be regulated by the filling status of glands producing the seminal fluids, which appears to increase the fertilization success of a given amount of donated sperm (e.g. Koene & Chase 1998; Koene et al. 2005; Chase & Blanchard 2006).

In hermaphrodites with reciprocal copulation, mating events are expected simultaneously to replenish the amount of allosperm stored and to deplete the amount of autosperm and seminal fluids. Therefore, mating status is expected to have multiple effects on mating behaviour for hermaphrodites with reciprocal copulations, namely sexually isolated individuals are expected to display higher mating propensity to gain both male and female reproductive success than already mated individuals.

In this study, we tested experimentally the effect of mating status on both copulatory and postcopulatory behaviours in the free-living flatworm *Macrostomum lignano*. This species has reciprocal mating and performs a postcopulatory behaviour, the so-called suck behaviour, which is possibly involved in removing ejaculate components received during copulation (Schärer et al. 2004, 2011; Vizoso et al. 2010). In addition, it has recently been suggested that mating status affects mating propensity, since previously isolated worms that were offered two mating partners consecutively copulated more frequently with the first than with the second mate (Janicke et al. 2012).

We experimentally manipulated the mating status of worms, leading to virgin individuals and to individuals that were sexually experienced in both sex functions (i.e. in reciprocally mating species the mating status necessarily changes in both sex functions upon mating). In a first experiment, we observed pairs of virgin worms (called virgin pairs) and pairs of sexually experienced worms (called sexually experienced pairs) and compared their copulation frequency, the time to the first copulation, as well as the average copulation duration, and the suck frequency over the first five copulations. Since virgins have a lot of available ejaculate (see Appendix 1 for previously unpublished data on autosperm and seminal fluid of an experiment reported in Schärer & Janicke 2009) and lack allosperm (L. Marie-Orleach, personal observation), we expected that virgin individuals would show greater interest in both donating and receiving sperm and that they would therefore be likely to copulate more often and for longer. As we found that individuals within virgin pairs sucked less frequently than individuals within sexually experienced pairs, we performed additional experiments to test whether the suck frequency depends on the mating status of the focal worm or, alternatively, on the mating status of the partner. We expected the virgin individuals would show greater willingness to receive allosperm and so to suck less frequently than the sexually experienced individuals.

METHODS

Study Organism

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a free-living flatworm and a member of the meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005b). Individuals used here stem from a genetically outbred laboratory mass culture (called LS1) descending from worms collected in 2003 in Lignano Sabbiadoro and Bibione, Italy (Ladurner et al. 2005b). Worms in mass cultures are kept at 20 °C in petri dishes in f/2 medium (Andersen et al. 2005) and fed ad libitum with the diatom *Nitzschia curvilineata*. Under these conditions body size reaches about 1.5 mm, generation time is about 18 days and worms have a median life span of about 200 days (Mouton et al. 2009). While young worms tend to be more male biased than older worms (i.e. worms are slightly protandrous, Vizoso & Schärer 2007), the worms we used in the experiments reported below were old enough to be mature in both sex functions. *Macrostomum lignano* is an outcrossing simultaneous hermaphrodite that copulates frequently (on average about 6 copulations/h, Schärer et al. 2004) and is highly promiscuous (Schärer & Ladurner 2003; Janicke & Schärer 2009). Copulation consists of reciprocal insertion of the male copulatory stylet into the female genital organ (the antrum) of the partner (Schärer et al. 2004), generally leading to the transfer of sperm and seminal fluid from a prostate-like accessory gland (Doe 1982; Ladurner et al. 2005a, b; Vizoso et al. 2010). The sperm reserves are

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not depleted after just a few matings (see also Schärer & Ladurner 2003 and Janicke et al. 2011 for data on the size of the sperm reserves in paired worms). Recipients may store sperm from several sperm donors, leading to sperm competition (Janicke & Schärer 2009). Subsequent mates can displace previously stored sperm (L. Marie-Orleach, T. Janicke, M. Eichmann, K. De Mulder, E. Berezikov, P. Ladurner, D.B. Vizoso & L. Schärer, unpublished data), leading to second-male sperm precedence (P. Sandner, D.B. Vizoso, T. Janicke & L. Schärer, unpublished data), and stored sperm may be used to fertilize eggs for up to 20 days after mating (Janicke et al. 2011). A facultative postcopulatory behaviour often follows immediately after a copulation, in which the worm bends onto itself and places its pharynx over its own vagina (termed the suck behaviour). During this the pharynx appears to perform a sucking behaviour, after which sperm are often seen sticking out of the female antrum (Schärer et al. 2004; Vizoso et al. 2010). It has been hypothesized that the suck behaviour is involved in removing ejaculate components from the female antrum (Schärer et al. 2004, 2011; Vizoso et al. 2010).

Experiment 1

On day 1 we distributed 900 adult worms from the mass cultures over 10 petri dishes with f/2 and a dense algae layer, and allowed them to lay eggs. On day 3 we removed the adults so that the age of all the resulting offspring did not differ by more than 2 days. On day 9 we isolated the resulting juveniles by transferring each to an individual well of a 24-well tissue culture plate (TPP, Trasadingen, Switzerland). Each well was filled with 1.5 ml of f/2 and a concentrated algae solution, which guaranteed ad libitum food. In total we used 216 worms for this experiment.

To manipulate the mating status of the focal worms we transferred all worms on day 70 into fresh wells (1.5 ml of f/2 and dense algae layer) either alone (hereafter called virgin worms; $N = 72$) or paired together with a randomly chosen worm (hereafter called sexually experienced worms; $N = 72$ pairs) for either 24 or 48 h. To avoid pseudoreplication, only one worm per pair was used for the mating trials. To ensure that all sexually experienced focal worms had actually copulated within the 24 or 48 h, we assessed the offspring production of its nonfocal partner. For this we checked each well with the remaining nonfocal partner for offspring production on day 80. Given that *M. lignano* is obligatorily outcrossing and that copulations are always reciprocal, the production of offspring by nonfocal partners indicates that the corresponding focal worm must have copulated in both sex functions. If a nonfocal partner did not produce any offspring, we excluded the corresponding focal worm from the analysis (see below).

We examined the mating behaviour of virgin and sexually experienced worms in observation chambers by pairing two randomly chosen virgin worms (virgin pairs) and two randomly chosen sexually experienced worms originating from two independent pairs (sexually experienced pairs), so that both virgin and sexually experienced worms encountered an unfamiliar worm as a partner. Observation chambers were made by placing each pair into a 3 μl drop of artificial sea water between two siliconized microscope slides separated by 210 μm (as described in more detail in Schärer et al. 2004). Each observation chamber contained six pairs. Observation chambers were then filmed under transmitted light for 1 h at 1 frame/s with a digital video camera (DFK 31BF03, The Imaging Source) in QuickTime format using BTV Pro 5.4.1 (http://www.bensoftware.com/). Mating movies were then scored frame-by-frame throughout the entire hour of observation by using BTV Pro 6.0b1 (http://www.bensoftware.com/).

We assessed the number of copulations and the time to the first copulation performed over the hour of observation. Moreover, we assessed the average copulation duration and the number of postcopulatory sucks performed over the first five copulations. We decided a priori to restrict the observation window to the first five copulations for two reasons. On the one hand, we needed to focus on the first few copulations because each copulation changes the mating status of a given individual, which ultimately dilutes the differences between virgin and sexually experienced individuals induced by our experimental manipulation. On the other hand, we intended to include more than one copulation to get a more accurate estimate for each individual. This was mainly because *M. lignano* copulates very frequently and because preliminary data suggested that not all matings lead to sperm transfer, so that information obtained from only a single copulation might be misleading. Given that we could not distinguish the worms within pairs in the first experiment (but see below), the number of sucks was assessed as the total number of sucks performed by both individuals in a pair. Although the suck behaviour is primarily a postcopulatory behaviour, it can also occur outside copulation events (Schärer et al. 2004). Because we were here interested in the postcopulatory suck behaviour, we only considered sucks occurring within 5 s after the end of a copulation (Schärer et al. 2004).

Initially, we aimed at 36 replicates for each treatment group. However, eight sexually experienced pairs were excluded because the previous nonfocal partner of one of those worms did not produce any offspring. In addition, during the assembly of the observation chambers we lost two replicates owing to pipetting errors (one virgin pair and one sexually experienced pair) and one virgin pair was excluded because one individual encysted during the mating trial. Consequently, the sample size for which the behaviour could be assessed was 34 virgin pairs and 27 sexually experienced pairs. Because three virgin pairs and five sexually experienced pairs did not copulate, the final sample size was further reduced to 31 virgin pairs and 22 sexually experienced pairs for the time to first copulation. Furthermore, within the pairs that copulated, two virgin pairs and seven sexually experienced pairs failed to copulate at least five times over the mating trial; therefore tests on the average copulation duration and the number of sucks rely on a sample size of 29 virgin pairs and 15 sexually experienced pairs.

As outlined below, the results showed that individuals within virgin pairs sucked less often than individuals within sexually experienced pairs (see Results). However, experiment 1 does not allow us to disentangle the effect of the mating status of the focal individual from that of its mating partner. Therefore we performed additional experiments including mixed pairs (i.e. virgin individuals paired with sexually experienced individuals) in which we could visually distinguish the two individuals. With these experiments we could investigate whether the suck frequency depended on the mating status of the focal worm and/or on the mating status of its mating partner.

Experiment 2a

We obtained individuals as explained in experiment 1. From day 1 to day 3 we distributed 600 adult worms into six petri dishes, and on day 9 we isolated 720 of the resulting offspring into well plates filled with 1 ml of f/2 and ad libitum algae.

The mating trials lasted over 4 days starting on day 45, and 48 pairs were observed each day. To distinguish the worms within pairs visually, we dyed 48 randomly chosen worms by exposing them over 24 h to the food colour Ponceau 4R (10 mg/ml of f/2; also called E-124 or New Coccine; Werner Schweizer AG, Wollerau, Switzerland) 2 days before the mating trials. The use of the food colour Ponceau 4R does not affect the mating behaviour and the female fecundity of the worms (P. Sandner, D.B. Vizoso, T. Janicke &

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L. Schärer, unpublished data) and was not expected to influence the results because the dye was completely balanced in the experimental design. One day before the mating trials we manipulated the mating status. For this we transferred 144 worms into 96 individual wells in the following way: 24 undyed isolated worms, 24 dyed isolated worms, 24 pairs of undyed worms, and 24 pairs each of one undyed and one dyed worm. As in experiment 1, only one focal worm per pair was used for the mating trials, a randomly chosen individual for the undyed pairs and the dyed individual for the undyed/dyed pairs. This resulted, for each of the 4 days, in 48 virgin worms and 48 sexually experienced worms, of which one half was dyed and the other half was not.

We then created four treatment groups, that is, two virgin worms (V × V), one virgin focal and one sexually experienced partner (V × E), one sexually experienced focal and one virgin partner (E × V), and two sexually experienced worms (E × E); the first letter always indicates the mating status of the dyed focal worm. We did both treatments, V × E and E × V, to avoid potential effects of the dye. Pairs were placed in the observation chambers with eight drops per chamber (as described for experiment 1) and filmed with a digital video camera (Sony DFW-X700), using a fibre-optic ring light placed beside the mating chamber to provide a 'dark-field' illumination enabling the worms' dye to be seen. Mating trials were recorded for 90 min using Security Spy 2.0.5 (http://www.bensoftware.com/).

The focus of this experiment was the performance of the post-copulatory suck behaviour of the dyed focal individuals. As in experiment 1, we only considered the sucks occurring within 5 s after the end of a copulation, and we only considered the first five copulations.

The expected sample size was 48 pairs in each of the four treatments. However, we had to discard 50 pairs because the previous partner of at least one sexually experienced individual did not produce offspring (V × E: $N = 17$; E × V: $N = 11$; E × E: $N = 22$). Moreover, 44 pairs failed to copulate at least five times during mating trials (V × V: $N = 17$; V × E: $N = 11$; E × V: $N = 11$; E × E: $N = 5$), and we lost nine pairs because of pipetting errors (V × V: $N = 2$; V × E: $N = 2$; E × V: $N = 2$; E × E: $N = 3$). The final sample size was therefore V × V: $N = 29$; V × E: $N = 18$; E × V: $N = 24$; E × E: $N = 18$.

Experiment 2b

Because experiment 2a suggested a strong tendency for the mating status of the mating partner but not that of the focal worm to have an effect on the suck frequency (see Results), we repeated the entire experiment, this time using the food colour Patent blue V (also called E-131; Werner Schweizer AG, Switzerland) instead of Ponceau 4R. Patent blue V does not affect the mating rate (see Appendix 2) and allowed us to manipulate the mating status and dye the worms simultaneously (see Appendix 3).

As before, from day 1 to day 3 we distributed 1200 adult worms into 12 petri dishes. On day 10, we isolated 672 of the resulting hatchlings into individual wells (24-well tissue culture test plate) filled with 1.5 ml of f/2 and ad libitum algae. On days 19, 27 and 35, we transferred the worms to fresh wells.

The mating trials lasted for 3 days, from day 38 to day 40. We performed mating trials for 60 pairs on days 38 and 39 and for 72 pairs on day 40. One day before the mating trials, we simultaneously manipulated the mating status and dyed the appropriate number of worms. We transferred worms into fresh wells, either isolated or in pairs. Half of these wells contained the food colour Patent blue V (0.25 mg/ml of f/2). We therefore had 96 undyed isolated worms, 96 dyed isolated worms, 96 undyed paired worms and 96 dyed paired worms. As before, we used only one focal worm per pair in the mating trials. We then performed the mating trials, filmed the observation chambers, and recorded and scored the mating movies as in experiment 2a.

The expected sample size was 48 per treatment. However, we had to discard 35 pairs because the previous partner of at least one sexually experienced individual did not produce offspring (V × E: $N = 7$; E × V: $N = 10$; E × E: $N = 18$). Moreover, we lost 13 replicates because the pairs failed to copulate at least five times during the mating trials (V × V: $N = 4$; V × E: $N = 3$; E × V: $N = 2$; E × E: $N = 4$). The final sample size was V × V: $N = 44$; V × E: $N = 38$; E × V: $N = 36$; E × E: $N = 26$.

Data Analysis

In experiment 1 we compared the copulatory and post-copulatory behaviour of virgin and sexually experienced pairs. Sexually experienced pairs formed by worms previously paired for 24 h did not differ from those previously paired for 48 h in any of the measured mating behaviours (all $P > 0.4$). Therefore we ignored the pairing time in the subsequent analysis. We compared the mating behaviour between virgin and sexually experienced pairs using Wilcoxon rank-sum tests for the number of copulations, the time to first copulation and the average duration of the first five copulations. To compare the number of sucks we used a generalized linear model (GLM) with a Poisson error distribution, a log-link function and a correction for overdispersion. In experiments 2a and 2b, we used fully factorial GLMs to test the effect of the mating status of the focal individual (i.e. virgin or sexually experienced), the mating status of the mating partner (i.e. virgin or sexually experienced) and their interaction on the number of sucks. In addition, we combined the P values of the two independent data sets (i.e. experiments 2a and 2b) using Fisher's combined probability test. All statistical analyses were carried out in JMP 9.0.0 (SAS Institute Inc., Cary, NC, U.S.A.). Values are given as means ± SE.

RESULTS

Experiment 1

Over the hour of observation, mating behaviour measurements indicated mating propensity was higher in virgin pairs than in sexually experienced pairs. Virgin pairs copulated more often (Wilcoxon rank-sum test: $Z = 3.15$, $N = 61$, $P = 0.002$; Fig. 1a) and started to copulate earlier than sexually experienced pairs (Wilcoxon rank-sum test: $Z = 2.93$, $N = 53$, $P = 0.003$; Fig. 1b). Over the first five copulations, virgin pairs had a higher average copulation duration than sexually experienced pairs (Wilcoxon rank-sum test: $Z = 3.63$, $N = 44$, $P < 0.001$; Fig. 1c). Moreover, individuals within virgin pairs exhibited significantly fewer sucks than individuals within sexually experienced pairs (GLM: $\chi^2_1 = 3.96$, $P = 0.047$; Fig. 1d).

Experiment 2

The average number of sucks observed over the first five copulations was 0.81 ± 0.11 (experiment 2a) and 1.05 ± 0.09 (experiment 2b) per individual. The results of experiment 2b confirmed the unexpected results of experiment 2a (Fig. 2), in that the mating status of the focal worm had no effect in either experiment 2a (GLM: $\chi^2_1 = 0.12$, $P = 0.734$) or experiment 2b (GLM: $\chi^2_1 = 0.50$, $P = 0.478$; Fisher's combined probability test: $\chi^2_4 = 2.10$, $P = 0.718$). However, the number of sucks differed according to the mating status of the mating partner, nearly significantly in experiment 2a (GLM: $\chi^2_1 = 3.51$, $P = 0.061$; Fig. 2a) and statistically significantly in experiment 2b (GLM: $\chi^2_1 = 5.23$,

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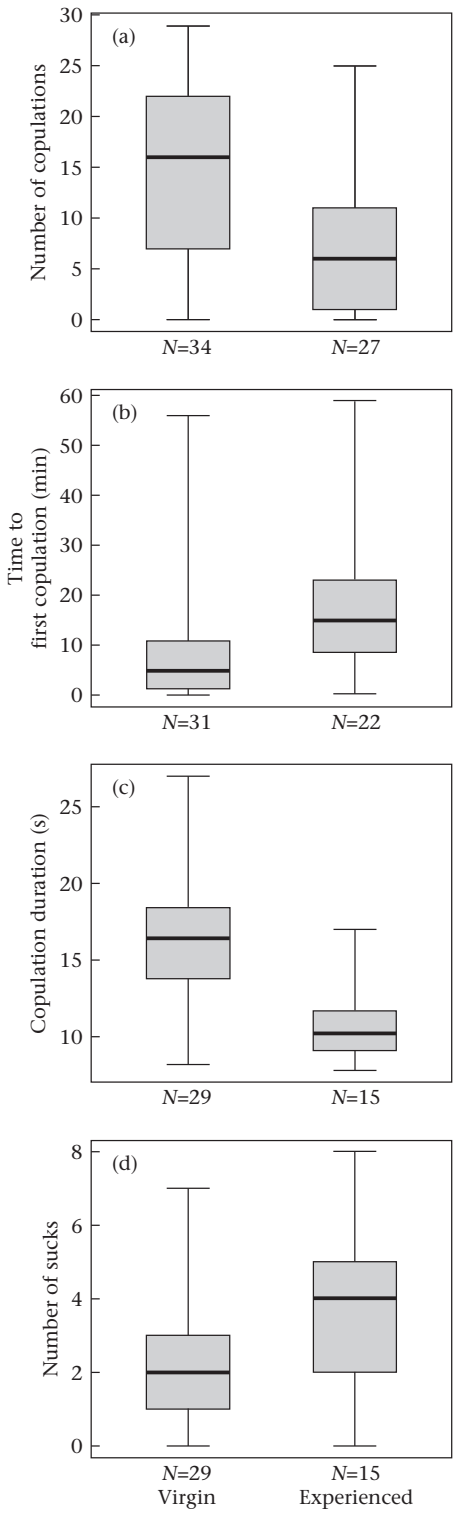

Figure 1. Effect of mating status on copulatory and postcopulatory behaviour. Comparison of (a) the number of copulations observed over the 1 h mating trial, (b) the time to the first copulation, (c) the average copulation duration of the first five copulations, and (d) the number of sucks performed over the first five copulations between virgin and sexually experienced pairs. Box plots show the median, the quartiles and the extreme values; *N* values are the sample sizes. See the Results for statistics.

$P = 0.022$; Fig. 2b), jointly leading to a significant effect of the mating status of the mating partner (Fisher's combined probability test: $\chi^2_4 = 13.23$, $P = 0.010$). Focal worms sucked less frequently after copulating with a virgin than with a sexually experienced worm, irrespective of their own mating status. The number of sucks was not affected by the interaction between the mating status of the focal worms and their partner, in either experiment 2a (GLM: $\chi^2_1 = 0.13$, $P = 0.720$) or experiment 2b (GLM: $\chi^2_1 = 1.66$, $P = 0.199$; Fisher's combined probability test: $\chi^2_4 = 3.85$, $P = 0.427$).

DISCUSSION

This study shows that mating status affects the copulatory and postcopulatory behaviour in the reciprocally copulating hermaphrodite *M. lignano*. By experimentally manipulating the mating status of individuals, we found that virgin pairs copulated more, earlier and for longer than sexually experienced pairs, consistent with higher mating propensity in virgin than in sexually experienced individuals. We further showed that the suck frequency depends on the mating status of the mating partner, but not on that of the focal individual. Worms sucked less frequently after copulating with a virgin than a sexually experienced worm, suggesting manipulation of the suck behaviour by the partner.

Mating Propensity

We found that virgin pairs had higher mating propensity than sexually experienced pairs. Similar results have been reported for hermaphroditic species with unilateral matings (e.g. Michiels & Streng 1998; Facon et al. 2007; Dillen et al. 2008), suggesting that virgin individuals have a higher willingness to donate and/or receive sperm than sexually experienced individuals. From a sperm donor's perspective, copulation probably reduces the amount of autosperm and seminal fluid available to inseminate further partners, and both of these parameters have been shown to depend strongly on the immediate social environment in *M. lignano*. Specifically, worms that have grown up in isolation have substantially larger seminal vesicles and more seminal fluid stored than worms that have grown up in pairs (see Appendix 1). Moreover, the size of the seminal vesicle approximately doubles within 2 days of isolation (Schärer & Vizoso 2007) and drops drastically within just 1 day when worms are transferred from small to large groups (Brauer et al. 2007). Thus, virgin individuals have more ejaculate available than sexually experienced individuals, so that the latter might allocate ejaculate more prudently, for example by reducing their copulation rate and the average copulation duration. The evolution of ejaculate economics can be interpreted as a trade-off between current mating and future mating opportunities (Wedell et al. 2002; Parker & Pizzari 2010). From this perspective, this could suggest that virgin individuals might allocate more sperm once given a mating opportunity and solicit more copulations with the same partner because, based on their long previous isolation period, they expect fewer future mating opportunities. Alternatively, it seems possible that aged sperm and seminal fluid may be of lower quality, thus requiring less prudent allocation.

From a sperm recipient's perspective, we expect that, in this obligatorily outcrossing species, the primary mating interest of virgin individuals is to receive sperm to fertilize their own eggs. In *M. lignano* individuals that are isolated for a long period usually have many developing eggs ready to be fertilized, but lack allosperm (L. Marie-Orleach, personal observation). Since *M. lignano* has a reciprocal copulation, the high mating propensity observed in virgin individuals may be driven by the willingness to donate and/or receive ejaculate. These concomitant effects cannot be

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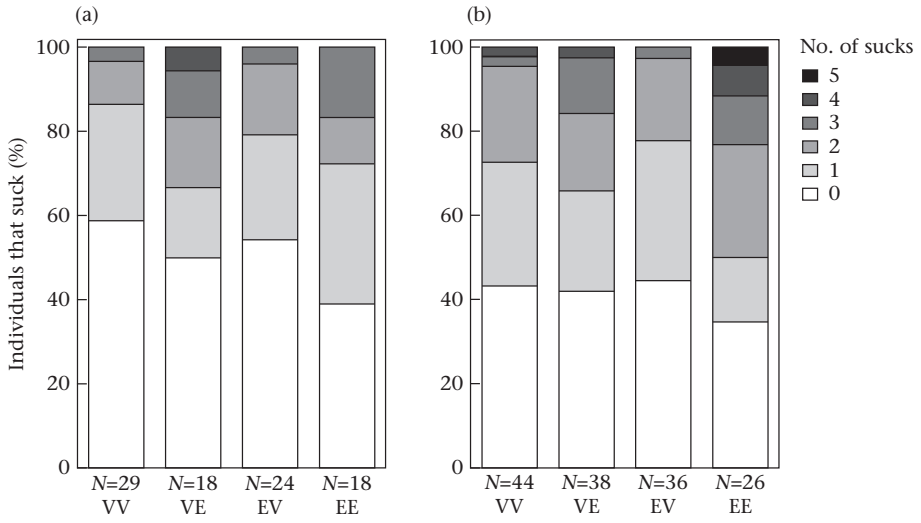

Figure 2. Effect of mating status of the focal individual and its partner on the postcopulatory behaviour of the focal individual. The percentages of worms that did not suck or sucked one to five times in the first five copulations during the mating trials are shown. Results are shown for the two independent experiments (a) 2a and (b) 2b. The letters below the X axis designate the pair type. The first-mentioned letter indicates the mating status of the focal individual and the second-mentioned letter indicates the mating status of its mating partner (V = virgin, E = sexually experienced). *N* values are the sample sizes. See Results for statistics.

disentangled in the current study. However, exposing virgin worms to male-sterile mating partners, for example by using the approach of Sekii et al. (2009), could probably yield individuals that have donated ejaculate but nevertheless lack allosperm, and may thus lead to a better understanding of the determinants of mating propensity in *M. lignano*.

In addition to its own mating status, the mating status of the mating partner is also expected to influence how much ejaculate a donor should transfer (Parker 1970; Wedell et al. 2002; Engqvist & Reinhold 2006). Theoretical models suggest contrasting predictions on whether a sperm donor should transfer bigger ejaculates to already mated recipients and overcome the sperm of competing sperm donors, or rather to conserve ejaculate to mating opportunities with low sperm competition by transferring bigger ejaculates to virgin recipients (reviewed in Parker & Pizzari 2010). This is expected to depend on various parameters, including sperm limitation faced by the sperm recipient, the sperm precedence pattern (i.e. precedence of the first or the last sperm donor) and the average level of sperm competition (see Engqvist & Reinhold 2006; Ball & Parker 2007). Empirical evidence suggests that sperm donors indeed allocate more ejaculate to already mated recipients (e.g. Gage & Barnard 1996; Velando et al. 2008) or, in contrast, allocate more ejaculate to virgin recipients (e.g. Wedell 1992; Loose & Koene 2008). At present, the lack of knowledge on the mating system in natural conditions does not allow us to determine whether the above conditions may be met in *M. lignano*. Therefore, it is possible that worms transfer bigger ejaculates to virgin individuals by copulating more often and for longer.

Displaying higher mating propensity when mating with a virgin individual would require the ability to detect cues of the mating status of the partner. A study in the pond snail, *Lymnaea stagnalis*, showed that individuals preferred to inseminate a new partner, but this effect vanished when the trails of mucus produced by the snails were removed (Koene & Ter Maat 2007). From this finding the authors concluded that the mating status might be signalled through a chemical component. There is currently no evidence for the presence of a cue that reveals the mating status in *M. lignano*, since worms do not differ behaviourally or show phenotypic plasticity when they are repeatedly exposed to either the same partner or to novel and already mated partners (Sandner & Schärer 2010). However, it has been suggested that mate assessment may be estimated through tactile cues during the circling and reeling behaviours often performed before copulations (Schärer et al. 2004). Such behaviours involve close proximity that could allow individuals to sense the presence of developing eggs carried by the partner, which are likely to be more abundant in virgin individuals, thereby enabling worms to sense the mating status of the potential partners prior to mating.

Postcopulatory Suck Behaviour

A striking outcome of our study is that the frequency of the postcopulatory suck behaviour depended primarily on the mating status of the mating partner, and not on the mating status of the individual that sucks. Namely, individuals sucked significantly less frequently after copulating with a virgin than with a sexually experienced individual. Virgin individuals differed from sexually experienced worms in the amount of autosperm and seminal fluid stored (see Appendix 1). Thus, having a virgin as a mating partner might have two consequences: receiving more sperm and more seminal fluid. Although the function of the seminal fluids is at present not known in *M. lignano*, several studies across various taxa have shown that seminal fluids can confer higher fertilization success by manipulating the physiology and/or the behaviour of the recipient (reviewed in Chapman 2001; Arnqvist & Rowe 2005; e.g. Wigby et al. 2009). For instance, in the garden snail *Helix aspersa*, individuals often shoot their mating partners with the so-called 'love dart' during copulation. Mucus, which is attached to the dart, triggers muscle contractions in the recipient (Koene & Chase 1998) and thereby favours the uptake of the spermatophore and reduces the risk of sperm digestion (Chase & Blanchard 2006). Sperm digestion seems to be widespread in hermaphrodites (see Baur 1998; Michiels 1998), and from a sperm donor perspective, sperm digestion is likely to be extremely costly. Therefore, a manipulative strategy favouring the use of sperm for fertilization rather than digestion would be advantageous (Anthes 2010). Thus, under the assumption that the postcopulatory suck behaviour of the partner decreases the fertilization success of the sperm donor, it might be beneficial for a donor to prevent it. Consequently, our results may indicate that virgin worms may be more effective at

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preventing their partner from sucking, by transferring ejaculates containing larger amounts of prostate gland secretions and/or a higher proportion of prostate gland secretions per unit sperm than sexually experienced worms.

An alternative hypothesis for the observed effect of the partner's mating status on the suck behaviour would be that individuals suck less after copulating with a virgin individual, because virgin individuals may donate larger ejaculates. Since ejaculate size might be an important determinant of siring success in *M. lignano* under sperm competition, recipients favouring donors that transfer large ejaculate may yield progeny with the selective advantage of producing large ejaculates ('sexy son hypothesis', Weatherhead & Robertson 1979). However, ejaculate size depends not only on sperm production rate but also on recent mating activity, which presumably makes ejaculate size an unreliable indicator of genetic quality. Hence, a preference for large ejaculates might not necessarily be beneficial for recipients.

The two hypotheses on the observed effect of mating status on the suck behaviour outlined above assume that the sucking decreases the fertilization success of the sperm donor (e.g. by removing ejaculate components), but at present we cannot exclude other potential functions of the suck behaviour. For instance, if the digestion of ejaculate components boosts the female fecundity of the recipient (e.g. egg production and/or egg quality), then the suck behaviour might to some degree be beneficial to the sperm donor (Yamaguchi et al. 2012), although the likelihood of such nuptial gifts in hermaphrodites has been questioned (Michiels 1998). Therefore, further experiments are clearly needed. First, we need a better understanding of the function of the suck behaviour in general (Vizoso et al. 2010). Second, an experimental set-up is required that allows the manipulation of the suck behaviour to identify its effect (e.g. remating rate, sperm use and female fecundity).

Conclusions

Our study shows that the copulatory behaviour of *M. lignano* depends on its mating status: virgin pairs mated more often, earlier and for longer than sexually experienced pairs. In contrast to our initial expectations, individuals performed fewer sucks after copulating with a virgin worm. Since virgin individuals are likely to transfer more seminal fluids to their mating partners, our finding suggests that seminal fluid could potentially inhibit the suck behaviour. Thus, sperm donors may manipulate the postcopulatory suck behaviour of their mating partner to increase the fertilizing success of the transferred sperm.

Acknowledgments

We thank Matt Hall and Joël Meunier for statistical advice; Nadja Burri for assistance during the experiment and Jürgen Hottinger, Viktor Mislin and Urs Stiefel for technical support. We are also grateful to Göran Arnqvist and Steve Ramm for fruitful discussion and to the two anonymous referees for thoughtful comments on the manuscript. This project was funded by a grant of the Swiss National Science Foundation to L.S. (SNF grants 3100A0-127503 and 31003A-143732).

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Appendix 1

Effect of Isolation on Amount of Stored Autosperm and Seminal Fluid

Methods

We here present previously unpublished data of an experiment described in more detail in Schärer & Janicke (2009), where same-age individuals were raised from juveniles either in isolation or in pairs. Seminal vesicle area, a reliable estimate of the amount of autosperm (Schärer & Vizoso 2007), was measured following the usual procedure (Schärer & Ladurner 2003). In addition, the amount of seminal fluid was assessed from pictures of the tail plate containing the prostate-like accessory glands (see e.g. Figures 2n, 4b and 4c in Ladurner et al. 2005b), using a visually estimated ordinal scale with four categories representing 0 (no gland product visible), 1 (few gland products visible), 2 (intermediate gland products visible) to 3 (many gland products visible). To avoid pseudoreplication, we used one randomly chosen individual per pair in the data analysis. The final sample size was 55 virgins and 62 pairs.

Results

The worms that grew up in isolation had larger seminal vesicles than worms that grew up in pairs (Wilcoxon rank-sum test: $Z = 6.13$, $N = 117$, $P < 0.001$; Fig. A1a). In addition, virgin individuals appeared to have more stored seminal fluid than paired individuals since the prostate-like accessory glands were significantly more prominent (Wilcoxon rank-sum test: $Z = 5.39$, $N = 117$, $P < 0.001$; Fig. A1b).

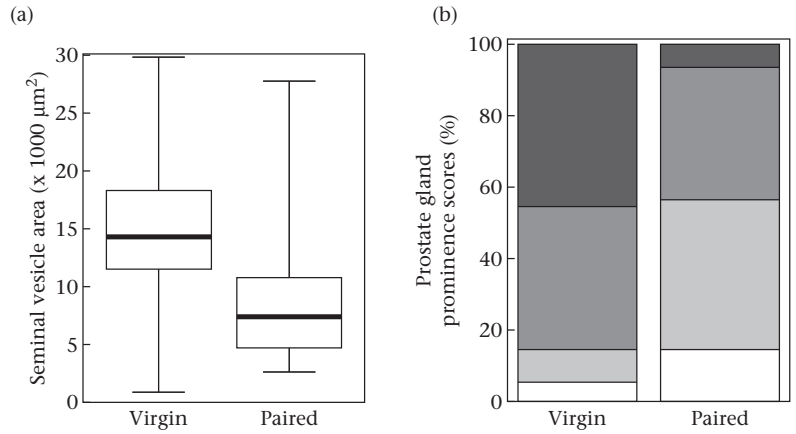

Figure A1. Effect of isolation on the amount of stored autosperm and seminal fluid. (a) Comparison of the seminal vesicle area between virgin and paired worms. Box plots show the median, the quartiles and the extreme values. (b) Percentage of virgin and paired individuals that had a prostate-like accessory gland assessed as class 0 (white), class 1 (light grey), class 2 (dark grey) and class 3 (black). See Results for statistics.

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Discussion

The results clearly suggest that worms that grow up in isolation have more autosperm and larger amounts of seminal fluids available to donate to their mating partners than worms that grow up in a pair.

Appendix 2

Effect of Patent blue V on Mating Rate

Methods

We tested the potential effects of the vital dye patent blue V (also called E-131; Werner Schweizer AG, Switzerland) on mating rate. On day 1, 300 adult worms were distributed into three petri dishes to lay eggs until day 3. On day 12, we isolated 80 of the resulting hatchlings. We performed the mating trials on days 36 and 37. One day before the mating trials, we transferred 40 individuals into fresh wells, of which 10 contained patent blue V dye (0.25 mg/ml of f/2 medium). Each day, we assembled 20 pairs (10 pairs containing two undyed individuals and 10 pairs containing one undyed individual and one dyed individual) in mating chambers following the procedure described in Schärer et al. (2004) and filmed the mating interactions for 2 h. The sample size was 20 undyed pairs and 20 undyed/dyed pairs.

Results and discussion

The numbers of copulations/h of undyed pairs (mean $\pm$ SE $= 15.5 \pm 1.2$) and undyed/dyed pairs (18.2 ± 2.0) did not differ significantly (t test: $t_{31.4} = -1.16$, $P = 0.255$). Hence, a 24 h exposure to the dye Patent blue V before a mating trial did not affect the mating rate of the worms.

Appendix 3

Effect of Patent Blue V on Allosperm Storage and Offspring Production

Methods

To test for potential effects of the vital dye patent blue V, we used a subset of the worms produced for experiment 2b. Briefly, on day 1 to day 3, 1200 adult worms were placed in petri dishes to lay eggs. On day 10, we isolated 80 of the resulting hatchlings for the purpose of this dye experiment. On day 27, we paired the worms into 40 wells, of which half contained the vital dye Patent blue V (0.25 mg/ml of f/2 medium) leading to 20 undyed pairs and 20 dyed pairs. On day 28 (i.e. 1 day after pair formation), we randomly picked one individual of each pair and assessed the number of stored allosperm following the procedure described in Janicke et al. (2011). We then isolated both individuals of each pair and assessed the number of offspring produced until day 48. We calculated the offspring production of the pair by summing the number of offspring laid by the two isolated partners. The initial sample size was 20 undyed and 20 dyed, but we lost five replicates from manipulation errors (one undyed and four dyed) and 12 worms had an egg in the sperm-receiving organ (six undyed and six dyed), preventing an accurate count of received sperm. The final sample size for sperm counts therefore was 13 undyed and 10 dyed and for pair offspring production 19 undyed and 16 dyed.

Results

Virgin worms that were paired for 24 h with or without dye did not differ in the number of allosperm received (median [25% quartile–75% quartile]; without dye: 18 [4–29.5]; with dye: 18 [13.75–29.75]; Wilcoxon rank-sum test: $Z = 0.28$, $N = 23$, $P = 0.779$) or in offspring production (median [25% quartile–75% quartile]; without dye: 5 [2–10]; with dye: 5.5 [3–10.25]; Wilcoxon rank-sum test: $Z = 0.28$, $N = 35$, $P = 0.777$).

Discussion

Pairing virgin worms with or without Patent blue V did not affect the number of allosperm stored or offspring production. This suggests that the presence of the vital dye Patent blue V does not affect mating activity and so this enabled us to manipulate the mating status and dye the worms simultaneously.