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Strategic mating effort in a simultaneous hermaphrodite

The role of the partner's feeding status

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Abstract Sexual selection theory for simultaneously hermaphroditic animals predicts an overall preference for inseminating partners that have a relatively higher female fecundity. Previous work on the link between male mating decisions and female fecundity has primarily focused on the effect of the partners' body size using existing variation in this trait within a study population. On the assumption that the body size is positively correlated with female fecundity, sperm donors should preferentially inseminate relatively larger individuals to obtain a higher fitness gain through their male sex function. However, empirical evidence for such size-dependent mate choice in simultaneous hermaphrodites is equivocal, possibly because of confounding variables. We studied the mating behavior of the simultaneously hermaphroditic flatworm *Macrostomum lignano* and tested for a strategic mating effort in response to the feeding status of the partner. We experimentally manipulated the feeding status of potential mating partners in order to generate variation in female fecundity among them and tested whether this affected the copulation number and the number of sperm that the focal worm managed to store in the partner's sperm storage organ. We found that the manipulation of the feeding status had a strong effect on the body size of the potential mating partners and that focal worms copulated more frequently with, and stored more sperm in well-fed partners compared to unfed partners. Our results suggest that *M. lignano* adjusts its mating effort in response to the feeding status of the mating partner.

Keywords Body size · Female fecundity · Food availability · Mate choice · Mating rate · Size-assortative mating · Sperm allocation

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Introduction

Darwin argued that mate choice in simultaneously hermaphroditic animals (i.e., animals that produce sperm and eggs at the same time) should be rare or absent, because 'secondary sexual characters cannot be developed' in these animals and because they 'have too imperfect senses and much too low mental powers to appreciate each other's beauty' (Darwin 1871). And although it is now generally acknowledged that sexual selection also occurs in simultaneous hermaphrodites (e.g., Charnov 1979; Michiels 1998; Leonard 2006), recent theoretical work indeed suggests that pre-copulatory sexual selection may be less intense in simultaneous hermaphrodites compared to separate-sexed organisms (reviewed by Arnqvist and Rowe 2005). For instance, quantitative genetic models have shown that the lack of sex-limited trait expression in simultaneous hermaphrodites reduces the opportunity for Fisherian runaway selection in these organisms (Morgan 1994). Moreover, if we assume that all individuals in a hermaphroditic population generally have an interest to mate (Charnov 1979), the optimal investment in mate acquisition is expected to be lower than in separate-sexed species, where usually only half of the individuals in a population (typically the males) invest in mate acquisition (Greeff and Michiels 1999; Puurtinen and Kaitala 2002). Although this does not necessarily imply an overall reduced investment into traits

associated directly with precopulatory mate choice, it has been argued that post-copulatory sexual selection (in terms of sperm competition *sensu* Parker 1970, and cryptic female choice *sensu* Thornhill 1983; Eberhard 1996) may be the predominant process of sexual selection in internally fertilizing simultaneous hermaphrodites (Charnov 1979; Schärer and Janicke 2009).

However, despite the apparently low potential for pre-copulatory sexual selection in simultaneously hermaphroditic animals, several traits have been identified to be involved in mate choice decisions in this group of organisms (reviewed by Leonard 2006; Anthes 2010). Among other traits, it has been shown that the mating status (e.g., Haase and Karlsson 2004; but see Koene et al. 2008), the relatedness (e.g., Facon et al. 2006; Schjørring and Jäger 2007; but see Peters and Michiels 1996b), and the infection status of the mate (e.g., Webster et al. 2003) can affect mate choice in simultaneous hermaphrodites.

Another trait that is likely to affect pre-copulatory mating preferences in simultaneous hermaphrodites is the fecundity of the partner (reviewed by Anthes 2010). Especially from the perspective of the sperm donor, it might be preferable to inseminate individuals that have a relatively high female reproductive output. In analogy to males in separate sexed organisms, which often bias their mating effort towards more fecund females (e.g., Wedell et al. 2002), a hermaphroditic sperm donor may sire more offspring by preferentially inseminating partners that are relatively more fecund in their female sex function. Such a biased mating effort is particularly expected if the costs associated with insemination are nontrivial so that sperm donors have to adjust their ejaculate allocation strategically (Dewsbury 1982). Previous work on the link between mate choice and female fecundity in simultaneous hermaphrodites primarily focused on studying the role of the partner's body size for mating decisions (DeWitt 1996; Leonard 2006; Anthes 2010). The rationale behind this is the fact that body size is often positively correlated with the resources available for egg production, which is assumed to translate proportionally into female fecundity in simultaneously hermaphroditic animals (Charnov 1979, 1982). Moreover, sex allocation theory for simultaneous hermaphrodites predicts that large individuals should generally allocate relatively more resources towards their female sex function compared to small individuals (Klinkhamer et al. 1997; Angeloni et al. 2002; Schärer 2009; for empirical support see, e.g., Schärer et al. 2001). This so-called 'size-dependent sex allocation' might lead to an additional advantage for inseminating large individuals due to a relatively higher female reproductive output of larger individuals.

Empirical studies on size-dependent mate choice in simultaneously hermaphroditic animals have, however, not revealed consistent patterns across species (Anthes 2010). While some studies provide support for an overall preference to mate with, or to increase the mating effort towards larger individuals (e.g., Michiels et al. 2001; Lüscher and Wedekind 2002; Ohbayashi-Hodoki et al. 2004; Anthes et al. 2006a), other studies indicate that the mating propensity is random with respect to the partner's body size (e.g., Peters and Michiels 1996a; Jordaens et al. 2005; Koene et al. 2007). Unfortunately, it is unclear whether these inconsistent findings reflect interspecific variation or whether they are due to the fact that the employed experimental approaches have used existing variation in body size, instead of attempting to manipulate this trait experimentally. As was recently pointed out, using natural variation in size can be problematic if body size is confounded by other potentially important factors, such as age and/or parasite load (Hermann et al. 2009). Essentially, body size may serve as a proximate cue on which mating decisions rely on. However, the underlying ultimate trait, which is expected to affect mate choice decisions in simultaneous hermaphrodites, is the female fecundity of the partner.

In this study, we used a novel approach to test for a strategic adjustment of the mating effort in response to the female fecundity of the mating partner in a simultaneously hermaphroditic animal. Specifically, we manipulated experimentally the feeding status of potential mating partners and tested whether focal individuals bias their mating effort towards well-fed or unfed mating partners. Manipulating the feeding status in our study species, the hermaphroditic flatworm *Macrostomum lignano*, has been previously shown to have a strong effect on both female fecundity (Janicke et al. 2011) and body size (Vizoso and Schärer 2007). Specifically, worms that have relatively more access to food are significantly more fecund in their female sex function and grow to a larger size. Moreover, a higher food availability has been demonstrated to generate a more female-biased sex allocation relative to body size in these worms (Vizoso and Schärer 2007). Finally, the feeding status could affect the overall body condition of the worms, which could have an additional impact on the reproductive output and therefore on the attractiveness of the mating partners. Consequently, manipulating the feeding status of potential mating partners in *M. lignano* induces variation in body size, female fecundity and possibly body condition, which is expected to translate into varying fitness returns that can be obtained by a sperm donor from mating with differentially fed partners.

In particular, we tested the hypothesis that sperm donors bias their mating effort strategically towards well-fed partners compared to unfed partners in *M. lignano*. We measured mating effort in terms of the number of copulations and the number of stored sperm in the mating partner's sperm storage organ. Variation in the number of copulations that a focal individual has with particular mating partners can be considered as a kind of pre-copulatory mate choice

(e.g., Peters and Michiels 1996b; Haase and Karlsson 2004; Ohbayashi-Hodoki et al. 2004; Dillen et al. 2010). However, in reciprocally mating simultaneous hermaphrodites, such a variation in the mating rate does not necessarily indicate a mating preference in the male sex function since it could also be driven by the female sex function or by both functions. For this reason, we also assessed the number of stored sperm in the partner's sperm storage organ, which likely indicates biases in the mating effort of only the male sex function. However, this relies on the currently untested assumption that the number of sperm that is stored in the female sperm storage organ of the mating partner is correlated with the number of sperm that the sperm donor actually transferred to its partner during mating. We predicted focal worms to copulate more frequently with well-fed worms and to transfer more sperm, and thus manage to successfully store more sperm in well-fed partners compared to unfed partners.

Methods

Study organism

The free-living flatworm *M. lignano* (Macrostomorpha, Platyhelminthes) is an obligately outcrossing simultaneous hermaphrodite of the intertidal meiofauna of the Northern Adriatic Sea (Schärer and Ladurner 2003). In mass cultures, worms are maintained in glass Petri dishes filled with f/2 medium (Andersen et al. 2005) at 20°C on a 14:10 h day–night cycle and fed with the diatom *Nitzschia curvilineata*. Under these conditions, body length of a fully grown worm reaches on average 1.5 mm, and the generation time is about 18 days. Worms are highly promiscuous (Janicke and Schärer 2009a) and copulate frequently with an average 6 matings/h (Schärer et al. 2004; Janicke and Schärer 2009b). For this experiment, we used worms from cultures that were initiated with individuals collected near Lignano Sabbiadoro (Italy) in 2003.

Experimental setup

To test whether the feeding status of the mating partner has an effect on the mating effort, we consecutively mated focal worms first with a well-fed individual and then with an unfed individual (or vice versa). Sperm of focal worms were labeled with 5-bromo-2′-deoxyuridine (hereafter called BrdU) (for details, see section 'Sperm tracking').

Due to time constraints, we split the experiment into two blocks that were separated by 2 days. On day 1 of each block, we allowed 400 adult worms from the mass cultures to lay eggs in four Petri dishes filled with f/2 medium and a concentrated algae solution. After 3 days, all adult worms were removed, yielding offspring that did not differ by more than 72 h in age. On day 23, we pooled all fully grown worms from two randomly selected Petri dishes and transferred 60 randomly selected worms to one Petri dish that contained a solution of BrdU in f/2 medium and a concentrated algae solution (hereafter called sperm-labeled focal worms). The remaining worms (unlabelled mating partners) were transferred to a fresh Petri dish (i.e., f/2 medium and a dense algae layer). On day 27, we refreshed the BrdU solution of the focal worms and assigned 192 unlabelled worms into 96 pairs and kept them in 24-well tissue culture plates. Wells were filled with 1.5 ml of f/2 medium and either 0.1 ml of a concentrated algae solution or 0.1 ml of f/2 medium. This yielded 48 pairs that had *ad libitum* food conditions (hereafter called well-fed worms) and 48 pairs that had no access to food (hereafter called unfed worms), respectively. To discriminate focal worms from potential mating partners during the mating trials, we colorized the mating partners using the red food dye Neococcine (E124; Werner Schweizer AG, Wollerau, Switzerland). For this, we transferred on day 29 all well-fed and all unfed pairs to new wells with the appropriate food treatment but filled with 1.5 ml of colorized f/2 medium (10 mg Neococcine per ml f/2 medium). On day 32, we measured the body size of 36 sperm-labeled focal worms and of 72 colorized worms, which had been raised in pairs under *ad libitum* food conditions (n=36) or without any access to food (n=36). Out of each pair we measured only one randomly chosen individual. After the measurement of body size (see 'Measurement of body size' section), all individuals were kept in isolation in their original food treatment until the mating trials (see section 'Mating trials'), which were carried out on the subsequent day (day 33). Hence, our food level manipulation of the potential mating partners lasted 6 days. Immediately after the mating trials, the worms were fixed, and BrdU-labeled sperm were localized using an immunocytochemical staining protocol (see section 'Sperm tracking').

Measurement of body size

To evaluate the morphological consequences of our food level manipulation, we measured the body size of all individuals 1 day prior to the mating trials (see section 'Mating trials'). For this, we anesthetized the worms by exposing them to a 5:3 mixture of 7.14% $MgCl_2$ and f/2 medium for 10 min and compressed them dorsoventrally to a fixed thickness of 35 μm between a microscope slide and a cover slip of a haemacytometer (Schärer and Ladurner 2003). We observed worms with a Leica DM 2500 microscope (Leica Microsystems, Germany) and took digital photos at 40× with a digital video camera (Sony DFW-X700; Sony Broadcast & Professional, Köln, Germany). Image acquisition was done using the software BTV Pro 6.0b1 (available at http://

www.bensoftware.com) and pictures were analyzed with the image analysis software ImageJ 1.43 h (available at http://rsb.info.nih.gov/ij/). Body area of the worms was measured blind with regard to the treatment groups using the 'wand (tracing) tool' in ImageJ and served as an estimate of body size (Schärer and Ladurner 2003).

Mating trials

Mating trials were conducted in observation chambers in which worms were placed in drops of 3 μl artificial seawater (32‰ salinity) between two microscope slides (for a detailed description, see Schärer et al. 2004). In these chambers, worms are to some extent restricted to move into two dimensions, which allows a better observation and quantification of the mating behavior.

Focal worms were allowed to mate consecutively with one well-fed and one unfed mating partner for 60 min each. After focal worms were exposed to a first potential mating partner, we opened the observation chamber and assembled a second chamber in which each focal worm was offered a second mating partner of the other food level treatment as the previous one. The time between the two mating trials that was needed for the assembly of the second observation chamber was on average 18.3±1.7 min (mean±SE). All observation chambers comprised 12 drops, each containing one focal worm and either one well-fed or one unfed worm. We balanced the number of treatments in each observation chamber and also the mating order in which the two differently treated worms were offered to the focal worms. Furthermore, we also balanced the drop position of the treatments in the chambers between all observations chambers. During the mating trials, no food was provided.

We filmed each chamber at 1 frame s^{-1} using a digital video camera (DFK 31BF03; The Imaging Source Europe GmbH) and recorded movies in QuickTime format using BTV PRO 5.4.1 (http://www.bensoftware.com). Movie capture started within 5 min after chamber assembly. Mating behavior was scored by manual frame-by-frame analysis of the QuickTime movies using BTV PRO 6.0b1. For each focal worm, we assessed the total number of copulations within both of the 60-min mating trials.

Sperm tracking

Sperm tracking was done by labeling the DNA of the sperm of focal worms with BrdU and by localizing the label using an immunocytochemical staining protocol (Schärer et al. 2007). BrdU is incorporated in the DNA instead of thymidine while stem cells undergo DNA replication. Thereby, sperm of focal worms become labeled with BrdU once stem cells have differentiated into sperm during spermatogenesis. This method allows tracking the sperm of a labeled donor in an unlabelled recipient.

Focal worms were labeled by continuous incubation in a solution of 0.5 mM BrdU (Sigma, B5002-16) in f/2 medium for 9 days. Fixation and immunocytochemical staining was done in tissue-culture plates. After the mating trials (described above), worms were relaxed in a 5:3 mixture of 7.14% $MgCl_2$ and f/2 for 25 min and then fixed for 60 min in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) with 10% sucrose. Fixed worms were washed three times with PBS-T (i.e., PBS plus 0.5% Triton X-100), followed by an additional 60 min wash with PBS-T and then stored in PBS overnight. On the next day, worms were permeated with 0.15 mg/ml Protease XIV at 37°C for 35 min (note that the originally published staining protocol erroneously gave the protease concentration as 0.15 μg/ml; Schärer et al. 2007). Protease activity was stopped with cooled 0.1 N HCl. Subsequently, animals were transferred to 2 N HCl for 1 h at 37°C for DNA denaturation, then washed three times with PBS-T and blocked with BSA-T (i.e., PBS-T plus 1% bovine serum albumin) for 60 min. BrdU-labeled cells were localized using a monoclonal rat anti-BrdU antibody (ab6326; Abcam Limited, Cambridge, UK) at a 1:100 dilution in BSA-T overnight at 4°C. After four wash steps in PBS-T, the secondary goat–anti-rat FITC-conjugated antibody (ab6115; Abcam Limited) was applied in the dark for 1 h at room temperature at 1:200 in BSA-T. After three further wash steps in PBS-T and one wash step in PBS, animals were mounted on microscope slides using Vectashield (Vector Laboratories, Burlingame, CA, USA), and stored at −20°C until observation. BrdU-labeled sperm were visualized under epifluorescence on a Leica DM 5000 B microscope (Leica Microsystems, Germany). All sperm counts were done blind with regard to the experimental treatment. A previous study showed that sperm counts are highly repeatable (Janicke and Schärer 2009a).

Effect of the feeding status on mating behavior

The food level manipulation might have had an effect on the mating behavior of the potential mating partners. This would have automatically affected the mating behavior of our focal worms since copulations are always reciprocal in *M. lignano*. Therefore, we tested whether well-fed worms behave similar as unfed worms. For this we used the remaining unlabelled individuals from the pairs that had either *ad libitum* food conditions or no access to food for 6 days. We formed pairs of two well-fed worms and pairs of two unfed worms and assessed the number of copulations for 60 min in observation chambers as described above. In total we assembled five observation chambers containing eight pairs each (four well-fed pairs and four unfed pairs) and one chamber

containing only six pairs (three well-fed pairs and three unfed pairs). One pair of well-fed worms was lost due to a pipetting error so that the final sample size was 22 pairs of well-fed worms and 23 pairs of unfed worms. These mating trials were carried out on day 32.

Statistical analyses

The first and the second blocks included initially 33 and 36 focal worms, respectively. Several focal worms were lost due to pipetting errors during the assembly of the observation chambers (n=15) or had to be excluded from the analyses because they were injured (n=2). Furthermore, six focal worms did not mate with any of the offered mating partners and were therefore also excluded from the analyses. Consequently, our final sample size for testing mating preferences was 46 focal worms (26 first paired with a well-fed worm; 20 first paired with an unfed worm). Additionally, four worms were lost during antibody staining, so that our sample size for comparing the number of stored sperm between treatment groups was 42 focal worms (23 first paired with a well-fed worm; 19 first paired with an unfed worm).

Blocking had no effect on any of the parameters measured and was therefore ignored in the final analyses (t-tests and Wilcoxon rank sum tests: all P>0.05). First, we tested whether our food level manipulation had an effect on the body size of the potential mating partners and whether the focal worms differed in body size from the potential mating partners using Student's t-tests. Second, we tested whether the number of copulations differed between pairs formed by two well-fed worms and pairs formed by two unfed worms using Wilcoxon rank sum tests.

Finally, we examined the effect of the feeding status of the potential mating partners on the mating effort of the focal worms using Generalized Linear Mixed Models (GLMMs) with a Poisson error distribution and a log-link function (Venables and Ripley 2002). We fitted GLMMs for both response variables (i.e., the number of copulations and the number of stored sperm in the partner's sperm storage organ), and used the feeding status of the mating partner (i.e., well-fed or unfed) and the mating order (i.e., the order in which well-fed and unfed worms were offered) as fixed factors, and the identity of the focal worm as a random factor (in order to take into account the repeated measures on the same focal worm).

All statistics were carried out in R v. 2.10.1 (R Development Core Team 2009). We applied the penalized quasi-likelihood method (PQL) for all GLMMs (Breslow and Clayton 1993) by using the glmmPQL function implemented in the package MASS v. 7.3-5 for R (Venables and Ripley 2010).

Results

The food level manipulation had a considerable effect on the body size of the worms, as intended by our experiment. Unfed worms were on average only half as big as well-fed worms (t-test: t=14.08, df=90, P<0.001; Fig. 1a). Moreover, the body size of focal worms (mean±SE: 499.6±15.9×10^3 μm^2) did not differ statistically from that of well-fed worms (t-test: t=0.63, df=90, P=0.531) but was significantly higher than that of unfed worms (t-test: t=13.96, df=90, P<0.001). Despite this considerable difference in body size between well-fed and unfed worms, there was no significant effect of the feeding status on the intrinsic number of copulations of the potential mating partners. Pairs formed by two well-fed individuals and pairs formed by two

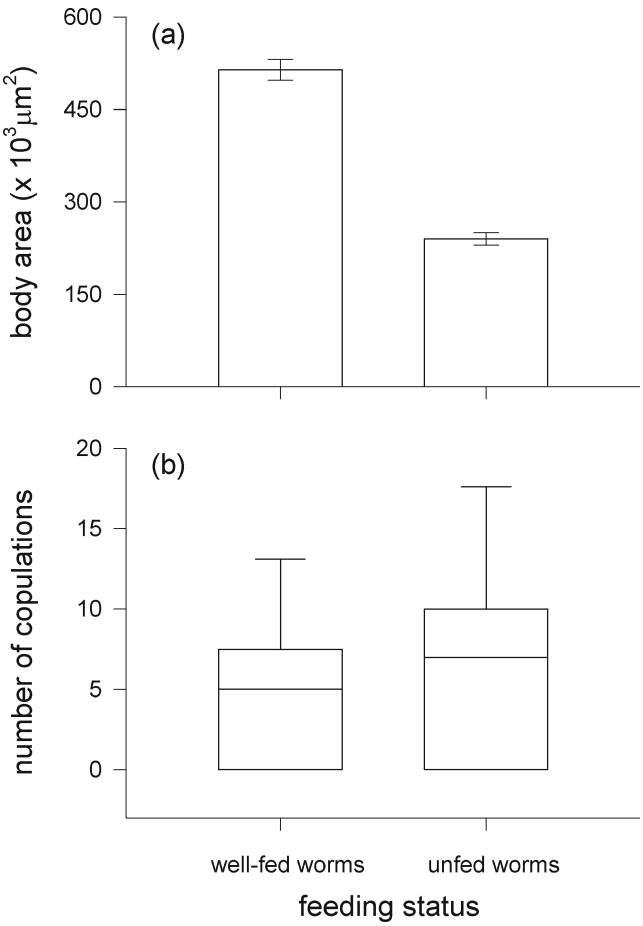

Fig. 1 Comparison of (**a**) body size and (**b**) number of copulations between well-fed and unfed worms. The data on body size refer to the potential mating partners that were offered to focal worms in the main mating trials (see 'Mating trials' section). The data on the number of copulations were obtained from additional mating trials in which the mating behavior was compared between pairs of two well-fed and pairs of two unfed individuals (see 'Effect of the feeding status on mating behavior' section). *Bars* in (**a**) show means±SE. *Boxplots* in (**b**) show the 25th percentile, the median and the 75th percentile and whiskers denote the 10th and the 90th percentiles. See text for statistics

unfed individuals behaved similarly with respect to the number of copulations (Wilcoxon rank sum test: W=284.5, P=0.473, n=45; Fig. 1b).

Out of the 46 focal worms, 12 individuals copulated only with the well-fed partner, whereas nine individuals copulated only with the unfed partner. Overall, focal worms copulated significantly more frequently with well-fed worms than with unfed worms (Table 1; Fig. 2a). The mating order also had a significant effect on the mating rate of focal worms, as indicated by more copulations with the first mating partner than with the second mating partner (Table 1; Fig. 2a). Moreover, the number of sperm that focal worms managed to store in their partners was affected by the feeding status of the partner. Specifically, focal worms stored significantly more sperm in well-fed compared to unfed partners (Table 1; Fig. 2b).

Discussion

In this study, we provide evidence for a strategic adjustment of the mating effort in response to the partner's feeding status in the simultaneously hermaphroditic flatworm *M. lignano*. We could show that focal worms copulated more frequently with well-fed partners compared to unfed partners. This difference was probably not triggered by intrinsic differences in the mating motivation of the partners, because pairs of two well-fed worms and pairs of two unfed worms did not differ in their mating rate. Furthermore, given that focal worms also stored more sperm in well-fed compared to unfed partners, our findings suggest an enhanced male mating effort towards well-fed individuals in *M. lignano*.

In the following, we first discuss the morphological and reproductive consequences of manipulating the feeding status and the observed effect on the number of copulations. Next, we consider how mate assessment may operate in *M.*

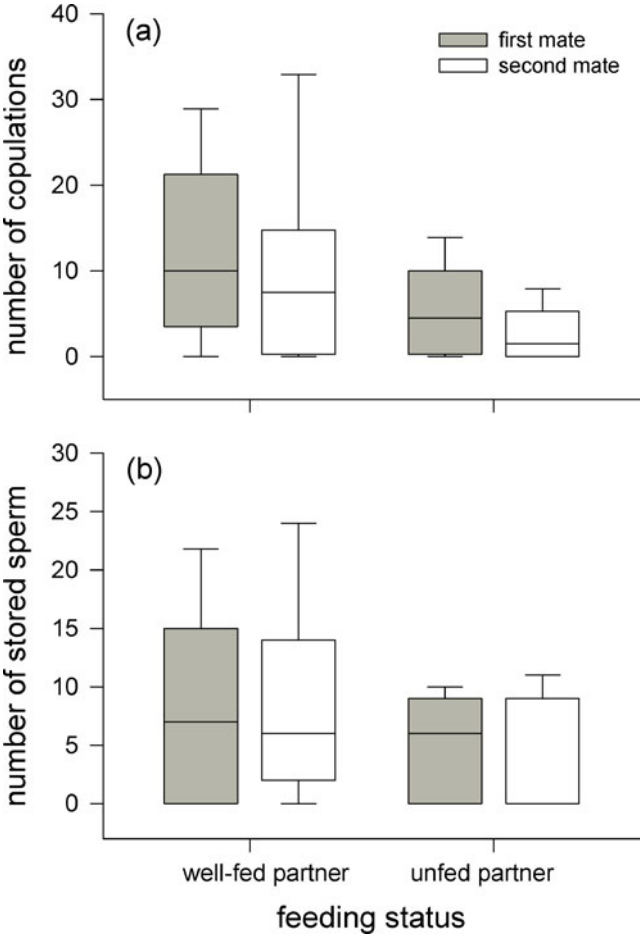

Fig. 2 Mating effort of sperm-labeled focal worms in response to the feeding status of the mating partner and to the mating order in which the partner was offered. Data are shown for (**a**) the number of copulations and (**b**) the number of stored sperm in the partner's sperm storage organ. The number of stored sperm is shown for all focal worms, irrespective of whether they mated with both partners or only with one of them. *Grey* and *white bars* refer to data of the first and second mating partner, respectively. *Boxplots* show the 25th percentile, the median and the 75th percentile and whiskers denote the 10th and the 90th percentiles. See Table 1 for statistics

Table 1 Summaries of Generalized Linear Mixed Models exploring the effects of the partner's feeding status and the mating order on the mating effort of focal worms

Response	Source	*df*	*F* value	*P* value
Copulation number[a]	Feeding status	1,43	30.661	<0.001
	Mating order	1,43	6.690	0.013
	Feeding status×mating order	1,43	0.305	0.584
Sperm number[b]	Feeding status	1,39	7.347	0.010
	Mating order	1,39	0.046	0.831
	Feeding status×mating order	1,39	0.059	0.809

Mating effort was measured in terms of the number of copulations and the number of stored sperm in the partner's sperm storage organ

[a] Model includes all focal worms (n=46)

[b] Model includes focal worms for which the number of stored sperm could be assessed for both offered mating partners (n=42)

lignano and if the preference for mating with well-fed individuals is driven by only one sex function. Finally, we discuss our finding on the number of stored sperm in the partner's sperm storage organ and the effect of the mating order on the number of copulations.

To our knowledge, this is the first study on mating preferences in simultaneously hermaphroditic animals in which variation in body size of the partners was induced experimentally. Previous studies have usually used the naturally occurring variation in body size within field populations or lab cultures in order to produce treatment groups that differ in body size for testing size-dependent mating strategies (e.g., Lüscher and Wedekind 2002; Chaine and Angeloni 2005; Anthes et al. 2006b; Dillen et al. 2010). Consequently, responses in the mating behavior to this kind of manipulation may be confounded by traits that are correlated with body size, such as age (Hermann et al. 2009). In our study, we controlled for age effects and manipulated the feeding status of the potential mating partners by exposing them to two different food levels. This manipulation affected the body size of the mating partners and has previously been shown to influence the number of offspring produced by the female sex function in *M. lignano* (Janicke et al. 2011). Specifically, well-fed mating partners were considerably larger and presumably more fecund in their female sex function compared to unfed mating partners.

Nevertheless, our manipulation of the feeding status might also have affected other traits than body size and female fecundity for which we could not control with our experimental setup. For instance, worms that had no access to food for 6 days might have represented less attractive mating partners to focal worms due to an overall lowered body condition. Although our food level limitation potentially induced stress to the unfed worms, we did not find any intrinsic differences in the number of copulations between pairs of unfed and pairs of well-fed individuals suggesting that unfed worms behaved normally. Moreover, for all unfed worms we did not observe any morphological malformations with regard to the testes, the ovaries, the seminal vesicle, the male copulatory organ and body shape (data not shown). Consequently, we think that our food level manipulation did not induce significant variation in traits other than body size and female fecundity, which could also have explained the suggested preference for well-fed partners by the focal worms.

One possible alternative explanation for the observed effect of the partner's feeding status on the mating rate of the focal worms is that unfed worms have, for some unknown reason, an aversion to mate with well-fed individuals. For instance, matings with a well-fed and therefore large individual could potentially be harmful to a small individual. In particular, the male copulatory organ of the larger individual might not fit in the female sperm storage organ of the smaller individual. However, in *M. lignano*, the size of the male copulatory organ does not correlate with body size (Janicke and Schärer 2009a), which suggests that the male copulatory organ of a relatively larger individual does not necessarily induce harm to a small recipient. Consequently, it seems unlikely that unfed worms rejected matings with well-fed worms in order to avoid harm induced by the male copulatory organ of the larger focal worms. Nevertheless, based on our data, we cannot definitively exclude the alternative hypothesis that unfed partners had an overall lower interest to mate with focal worms.

Mate choice for more fecund partners in *M. lignano* is potentially mediated by a mate assessment based on body size, as has been suggested for several other simultaneously hermaphroditic animals (e.g., reviewed by Leonard 2006; Anthes 2010). For instance, mating pairs of the flatworm *Dugesia gonocephala* show a unique pre-copulatory 'flattening' behavior, during which both mating partners seem to simultaneously signal and assess their relative size in order to decide on whether to mate or not (Vreys and Michiels 1997). Similarly, copulations in *M. lignano* are usually initiated by pre-copulatory 'reeling' and 'circling' behaviors characterized by a continuous physical contact, which might also allow the worms to assess each other's body size (Schärer et al. 2004). If mate choice really relies on body size as a proximate cue for the partner's female fecundity, we would expect that there is size-assortative mating in *M. lignano*, because matings in this species are always reciprocal. Size-assortative mating is likely to occur since large individuals should preferentially mate with other large individuals, leaving small individuals to mate only with similar sized individuals (Michiels 1998).

Based on our data, it is not possible to infer conclusively whether the preference for mating with well-fed individuals is primarily driven by only one sex function in *M. lignano*. An overall preference for mating with larger individuals has most often been attributed to a mating drive of the male sex function (Anthes 2010). Specifically, simultaneous hermaphrodites are predicted to preferentially inseminate larger partners because this will lead to an increased siring success if body size is correlated with female fecundity (Leonard 2006; Anthes 2010). However, current evidence for a male preference to mate with larger individuals is restricted to species with unilateral matings (e.g., Ohbayashi-Hodoki et al. 2004; Anthes et al. 2006a), since it is difficult to assess which sex role (if any) dominates the mate choice decision in reciprocally mating simultaneous hermaphrodites. Our finding that focal worms managed to store more sperm in well-fed compared to unfed mates may suggest that the male sex function has a preference to inseminate larger partners in *M. lignano*. Whether this observed bias in the number of stored sperm results only from a higher mating rate with well-fed partners or whether focal worms also adjust

strategically their sperm allocation per mating in response to the feeding status of the partner (as predicted by sperm competition theory; reviewed by Wedell et al. 2002) remains unknown, since we could not quantify the number of sperm transferred per mating in this study. Moreover, it has to be clarified that number of labeled stored sperm in the partner's sperm storage organ does not necessarily reflect the number of sperm that focal worms actually transferred to their mates. First, sperm recipients might have some control over the number of sperm that is stored in their own sperm storage organ allowing them to choose among different sperm donors in terms of cryptic female choice (*sensu* Thornhill 1983; Eberhard 1996). Indeed, *M. lignano* often performs a so-called 'suck behavior' after a copulation (Schärer et al. 2004), which has been speculated to allow recipients to remove sperm from a previous mating partner out of their own sperm storage organ (Vizoso et al. 2010; Schärer et al. 2011). Until now, we lack any data showing that worms really remove sperm by the suck behavior. Second, given that our food level manipulation induced variation in the body size among mating partners it might also have affected the size of the sperm storage organ, which may have caused a lower sperm storage capacity in unfed compared to well-fed mating partners. However, the sperm storage organ of *M. lignano* is relatively flexible in size (Vizoso et al. 2010), and the body size of a recipient is usually not correlated with the number of sperm stored (Janicke et al. 2011). This makes it unlikely that unfed worms were more constrained in their sperm-storage capacity compared to well-fed worms. And third, sperm digestion has been argued to be frequent in simultaneous hermaphrodites (Charnov 1979; Sluys 1989; Baur 1998), and we cannot rule out that the lower number of sperm stored by focal worms in unfed partners compared to well-fed partners is simply the result of an increased sperm digestion by unfed worms. However, there is currently no evidence that sperm digestion occurs in *M. lignano*.

In addition to the effect of the feeding status of the mating partner, we also found that the mating order affected the number of copulations. Focal worms copulated more frequently with the first compared to the second mating partner. This is probably due to the fact that focal worms were kept in isolation for 24 h prior to the first mating trial but for less than 20 min prior to the second mating trial. Therefore, focal worms had probably more sperm available to donate and less received sperm in storage in the first mating trial compared to the second mating trail (cf. Schärer and Vizoso 2007), which might have caused a higher motivation to mate with the first partner. This coincides with the observation that pairs of virgin worms copulate more frequently compared to pairs of already mated individuals in *M. lignano* (T. Janicke; unpublished data) and also with other studies, which have demonstrated that isolated individuals are relatively more eager to mate in other simultaneous hermaphrodites (e.g., Michiels and Bakovski 2000; Dillen et al. 2008). Alternatively, higher mating rates in the first mating trials might also result from a preference of non-focal worms to inseminate more isolated partners (focal worms). Such a preference is expected to be beneficial to the male function since it reduces the risk of facing sperm competition (e.g., Haase and Karlsson 2004).

In conclusion, this is, to our knowledge, the first experimental evidence that the feeding status of the mating partner has an effect on the copulation number in a simultaneous hermaphrodite. Our data suggest that there is a preference to mate with well-fed individuals in *M. lignano*, which ultimately also translates into a higher number of sperm stored in well-fed partners. Whether the observed preference for mating with more well-fed, and therefore more fecund, individuals is driven primarily by the male or the female sex function remains to be tested.

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References

Andersen RA, Berges JA, Harrison PJ, Watanabe MM (2005) Recipes for freshwater and seawater media. In: Andersen RA (ed) Algal culturing techniques. Elsevier, Amsterdam, pp 429–538

Angeloni L, Bradbury JW, Charnov EL (2002) Body size and sex allocation in simultaneously hermaphroditic animals. Behav Ecol 13:419–426

Anthes N (2010) Mate choice and reproductive conflict in simultaneous hermaphrodites. In: Kappeler P (ed) Animal behaviour: evolution and mechanisms, 1st edn. Springer, Berlin, pp 329–357

Anthes N, Putz A, Michiels NK (2006a) Hermaphrodite sex role preferences: the role of partner body size, mating history and female fitness in the sea slug *Chelidonura sandrana*. Behav Ecol Sociobiol 60:359–367

Anthes N, Putz A, Michiels NK (2006b) Sex role preferences, gender conflict and sperm trading in simultaneous hermaphrodites: a new framework. Anim Behav 72:1–12

Arnqvist G, Rowe L (2005) Sexual conflict. Princeton University Press, Princeton

Baur B (1998) Sperm competition in molluscs. In: Birkhead TR, Møller AP (eds) Sperm competition and sexual selection. Academic, London, pp 255–305

Breslow NE, Clayton DG (1993) Approximate inference in generalized linear mixed models. J Am Stat Assoc 88:9–25

Chaine A, Angeloni L (2005) Size-dependent mating and gender choice in a simultaneous hermaphrodite, *Bulla gouldiana*. Behav Ecol Sociobiol 59:58–68

Charnov EL (1979) Simultaneous hermaphroditism and sexual selection. Proc Natl Acad Sci USA 76:2480–2484

Charnov EL (1982) The theory of sex allocation. Princeton University Press, Princeton
Darwin CR (1871) The descent of man, and selection in relation to sex. John Murray, London
DeWitt TJ (1996) Gender contests in a simultaneous hermaphrodite snail: a size-advantage model for behaviour. Anim Behav 51:345–351
Dewsbury DA (1982) Ejaculate cost and male choice. Am Nat 119:601–610
Dillen L, Jordaens K, Dieleman W, Backeljau T (2008) Effects of isolation and body size on the mating behaviour of the hermaphroditic land snail *Succinea putris*. Anim Behav 75:1401–1411
Dillen L, Jordaens K, van Dongen S, Backeljau T (2010) Effects of body size on courtship role, mating frequency and sperm transfer in the land snail *Succinea putris*. Anim Behav 79:1125–1133
Eberhard WG (1996) Female control: sexual selection by cryptic female choice. Princeton University Press, Princeton
Facon B, Ravigne V, Goudet J (2006) Experimental evidence of inbreeding avoidance in the hermaphroditic snail *Physa acuta*. Evol Ecol 20:395–406
Greeff JM, Michiels NK (1999) Low potential for sexual selection in simultaneously hermaphroditic animals. Proc R Soc B-Biol Sci 266:1671–1676
Haase M, Karlsson A (2004) Mate choice in a hermaphrodite: you won't score with a spermatophore. Anim Behav 67:287–291
Hermann PM, Genereux B, Wildering WC (2009) Evidence for age-dependent mating strategies in the simultaneous hermaphrodite snail, *Lymnaea stagnalis* (L.). J Exp Biol 212:3164–3173
Janicke T, Schärer L (2009a) Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite. J Evol Biol 22:405–415
Janicke T, Schärer L (2009b) Sex allocation predicts mating rate in a simultaneous hermaphrodite. Proc R Soc B-Biol Sci 276:4247–4253
Janicke T, Sandner P, Schärer L (2011) Determinants of female fecundity in a simultaneous hermaphrodite: the role of polyandry and food availability. Evol Ecol 25:203–218
Jordaens K, Pinceel J, Backeljau T (2005) Mate choice in the hermaphroditic land snail *Succinea putris* (Stylommatophora: Succineidae). Anim Behav 70:329–337
Klinkhamer PGL, deJong TJ, Metz H (1997) Sex and size in cosexual plants. Trends Ecol Evol 12:260–265
Koene JM, Montagne-Wajer K, Ter Maat A (2007) Aspects of body size and mate choice in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. Anim Biol 57:247–259
Koene JM, Loose MJ, Wolters L (2008) Mate choice is not affected by mating history in the simultaneously hermaphroditic snail *Lymnaea stagnalis*. J Mollus Stud 74:331–335
Leonard JL (2006) Sexual selection: lessons from hermaphrodite mating systems. Integr Comp Biol 46:349–367
Lüscher A, Wedekind C (2002) Size-dependent discrimination of mating partners in the simultaneous hermaphroditic cestode *Schistocephalus solidus*. Behav Ecol 13:254–259
Michiels NK (1998) Mating conflicts and sperm competition in simultaneous hermaphrodites. In: Birkhead T, Møller AP (eds) Sperm competition and sexual selection. Academic, London, pp 219–254
Michiels NK, Bakovski B (2000) Sperm trading in a hermaphroditic flatworm: reluctant fathers and sexy mothers. Anim Behav 59:319–325
Michiels NK, Hohner A, Vorndran IC (2001) Precopulatory mate assessment in relation to body size in the earthworm *Lumbricus terrestris*: avoidance of dangerous liaisons? Behav Ecol 12:612–618
Morgan MT (1994) Models of sexual selection in hermaphrodites, especially plants. Am Nat 144:S100–S125
Ohbayashi-Hodoki K, Ishihama F, Shimada M (2004) Body size-dependent gender role in a simultaneous hermaphrodite freshwater snail, *Physa acuta*. Behav Ecol 15:976–981
Parker GA (1970) Sperm competition and its evolutionary consequences in insects. Biol Rev 45:525–567
Peters A, Michiels NK (1996a) Do simultaneous hermaphrodites choose their mates? Effects of body size in a planarian flatworm. Freshwat Biol 36:623–630
Peters A, Michiels NK (1996b) Evidence for lack of inbreeding avoidance by selective simultaneous hermaphrodite. Invertebr Biol 115:99–103
Puurtinen M, Kaitala V (2002) Mate-search efficiency can determine the evolution of separate sexes and the stability of hermaphroditism in animals. Am Nat 160:645–660
R Development Core Team (2009) R: a language and environment for statistical computing, 2.10.1. R Foundation for Statistical Computing, Vienna
Schärer L (2009) Tests of sex allocation theory in simultaneously hermaphroditic animals. Evolution 63:1377–1405
Schärer L, Ladurner P (2003) Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. Proc R Soc B-Biol Sci 270:935–941
Schärer L, Vizoso DB (2007) Phenotypic plasticity in sperm production rate: there's more to it than testis size. Evol Ecol 21:295–306
Schärer L, Janicke T (2009) Sex allocation and sexual conflict in simultaneously hermaphroditic animals. Biol Lett 5:705–708
Schärer L, Karlsson LM, Christen M, Wedekind C (2001) Size-dependent sex allocation in a simultaneous hermaphrodite parasite. J Evol Biol 14:55–67
Schärer L, Joss G, Sandner P (2004) Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms suck. Mar Biol 145:373–380
Schärer L, Zaubzer J, Salvenmoser W, Seifarth C, Ladurner P (2007) Tracking sperm of a donor in a recipient: an immunocytochemical approach. Anim Biol 57:121–136
Schärer L, Littlewood DTJ, Waeschenbach A, Wataru Y, Vizoso DB (2011) Mating behavior and the evolution of sperm design. Proc Natl Acad Sci USA 108:1490–1495
Schjørring S, Jäger I (2007) Incestuous mate preference by a simultaneous hermaphrodite with strong inbreeding depression. Evolution 61:423–430
Sluys R (1989) Sperm resorption in triclads (Platyhelminthes, Tricladida). Invertebr Reprod Dev 15:89–95
Thornhill R (1983) Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am Nat 122:765–788
Venables WN, Ripley BD (2002) Modern applied statistics with S, 4th edn. Springer, New York
Venables WN, Ripley BD (2010) MASS: main package of Venables and Ripley's MASS, R package, version 7.3-5
Vizoso DB, Schärer L (2007) Resource-dependent sex allocation in a simultaneous hermaphrodite. J Evol Biol 20:1046–1055
Vizoso DB, Rieger G, Schärer L (2010) Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking. Biol J Linn Soc 99:370–383
Vreys C, Michiels NK (1997) Flatworms flatten to size up each other. Proc R Soc B-Biol Sci 264:1559–1564
Webster JP, Hoffman JI, Berdoy M (2003) Parasite infection, host resistance and mate choice: battle of the genders in a simultaneous hermaphrodite. Proc R Soc B-Biol Sci 270:1481–1485
Wedell N, Gage MJG, Parker GA (2002) Sperm competition, male prudence and sperm-limited females. Trends Ecol Evol 17:313–320