Sexual Selection in a Transparent Worm: Insights from Fluorescent Sperm

PhD Thesis

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Abstract

Sexual selection is a potent source of selection underlying the evolution of sexual dimorphism, reproductive strategies and mating systems. Although sexual selection was initially thought to occur exclusively at the pre-copulatory stage (e.g., contests among males, and female mate choice), it can also continue after copulation through sperm competition and/or cryptic female choice. However, the study of these post-copulatory processes remains challenging because they occur internally and so are often difficult to observe. During my PhD project, I used the simultaneously hermaphroditic flatworm *Macrostomum lignano* to study sexual selection, with a special focus on distinguishing the processes occurring at the pre- and post-copulatory stage of sexual selection.

Mating is not necessarily a harmonious union, instead partners are expected to allocate their mating resources strategically over different mating opportunities to maximise their own fitness. My results suggests that virgin pairs have a greater mating propensity, presumably because they want to obtain sperm to fertilise their own eggs and have more sperm ready to give away. Moreover, in several species sperm donors have been shown to transfer accessory gland secretions along with sperm, which can manipulate the partners to increase donor's fitness. I speculate that this may also be the case in *M. lignano* as sperm recipients behave differently after mating with virgins, which likely transfer more accessory gland secretions.

Progress in understanding post-copulatory processes of sexual selection greatly depends upon the development of techniques that facilitate the observation of internal processes. The unique opportunity to track sperm under competitive conditions inside the female reproductive tract *in vivo* has become possible thanks to a transgenic line ubiquitously expressing green fluorescent protein (GFP): fluorescent sperm in a transparent worm. I tested and validated the reliability of the GFP-techniques in *M. lignano*, from which I could take advantage to reach novel findings.

Although sexual selection is recognised be composed of pre- and post-copulatory episodes of selection, few studies provide a quantitative understanding of the relative importance of the different episodes of selection. My results suggest that in *M. lignano* a large part of the variance observed in male reproductive success arises from two post-copulatory episodes of selection, sperm-transfer efficiency and sperm-fertilising efficiency. Moreover, individuals with bigger testis gain higher paternity share, presumably because they transfer more sperm per copulation. These two findings disagree with the view that sexual selection mainly arises from differences in mating success. Instead, the results suggest that in this study system the post-copulatory episodes of selection are very important, and that the success of these episodes likely depends upon the sperm production rate.

Simultaneous hermaphrodites need to decide how they allocate their resources towards their own male and female sex functions. A fundamental theory is that this trade-off depends on the number of mating partners (i.e., mating group size). In particular, sex allocation theory predicts that individuals should allocate more energy towards their female function in small mating groups. When the mating group size increases, the intensity of sperm competition increases and so individuals are expected to increase their investment towards their male function. This is fully supported by my results, which represents the most direct test of this basic theory to date.

In conclusion, the simultaneous hermaphrodite *Macrostomum lignano* is a powerful model organism to study sexual selection and sex allocation. My studies took advantage of several of its features to provide novel insights in fundamental topics such as the operation of sexual selection along episodes of selection and sex allocation in simultaneous hermaphrodites. Overall, my PhD works suggest that the post-copulatory episodes of sexual selection may be important agents of selection.

Chapter I

Thesis Introduction

Sexual Selection

From Pre- to Post-Copulatory Sexual Selection

Males and females can display striking differences in size, shape, colour or behaviour. In particular, males often express conspicuous ornaments and behaviours that could not be explained by natural selection alone. Therefore Charles Darwin formulated the theory of sexual selection whereby the expression of such conspicuous traits would be favoured through competition for mate acquisition, either by outcompeting rivals of the same sex or by attracting members of the opposite sex (Darwin 1859, 1871). Since Darwin's seminal theory, sexual selection has become a flourishing body of research which is not restricted to the competition for mate acquisition, and spans most, if not all, living organisms, including animals, plants and fungi (reviewed e.g., in Andersson 1994; Arnold 1994b; Birkhead and Møller 1998; Skogsmyr and Lankinen 2002; Arnqvist and Rowe 2005a; Jennions and Kokko 2010; Nieuwenhuis and Aanen 2012).

A first step towards a more qualitative understanding about how males and females may be under different selection regimes was made by Bateman (1948). Bateman's study consisted in mating trials involving equal numbers of male and female *Drosophila melanogaster*, where the parental contribution to the resulting offspring was subsequently assessed. Bateman's main observation was that male reproductive success is more strongly dependent on mating success than female reproductive success (called the "Bateman's third principle"; *sensu* Arnold 1994a). Bateman then argued that in promiscuous species female fitness is primarily limited by egg production, while male fitness is primarily limited by the access to females, providing a first explanation on why in many species males are usually more eager to mate than females (Bateman 1948). However the importance and the evolutionary implications of Bateman's work have been acknowledged some decades later, when the consequences of promiscuity on sexual selection were better understood (Parker 1970; Trivers 1972; Charnov 1979; Parker and Birkhead 2013).

In promiscuous species, sexual selection can continue after copulation, in that sperm of different males can compete for fertilisation (i.e., sperm competition) and in that females may influence the fertilisation success of some males by preferentially using their sperm (i.e., cryptic female choice) (Parker 1970; Charnov 1979; Thornhill 1983; Eberhard 1996; Parker 1998; Birkhead and Pizzari 2002; Parker and Pizzari 2010). Importantly, the prevalence of these post-copulatory processes clearly emphasises that mating does not guarantee parentage, and has shifted our perception of sexual selection towards selection on traits biasing the fertilising process (Jennions and Kokko 2010). On one hand, selection on males has been shown to favour the production and transfer of large ejaculates to outcompete those of competing males. On the other hand, due to the costs involved in producing large ejaculates, males have also

been shown to strategically allocate their ejaculate over the different mates and mating opportunities (Wedell et al. 2002; Parker and Pizzari 2010). Moreover, sperm competition is not only played by numbers. The morphology and the behaviour of the sperm (reviewed in Snook 2005; Pizzari and Parker 2009), as well as the composition of the seminal fluid transferred along with sperm (reviewed in Chapman 2001; Arnqvist and Rowe 2005a) can be important determinants of sperm competition, acting notably through interactions with the female reproductive tract. In sum, sexual selection is nowadays often viewed as a complex process that encompasses consecutive pre- and post-copulatory episodes of selection that may all influence reproductive success.

Quantification of Sexual Selection

Once sexual selection had been recognised as an important agent of selection, there was a need for a theoretical framework enabling its quantification. In essence, sexual selection favours individuals that bear certain traits and thus generates non-random variance in reproductive success among individuals. Therefore, the challenges of quantifying sexual selection are to measure the variance due to sexual selection, and to quantify whether specific traits are under sexual selection. For this, several measures have been established (reviewed in Arnold and Wade 1984; Arnold and Duvall 1994; Shuster and Wade 2003; Klug et al. 2010). Some measures focus on phenotypic traits and their correlations with reproductive success (e.g., selection differential; Lande 1979). Whereas other measures are based on the variances observed either in mating success or reproductive success (i.e., opportunity for sexual selection or opportunity for selection; Wade 1979), or on the slope of linear regression of reproductive success on mating success (Bateman gradient; Arnold and Duvall 1994; *sensu* Andersson and Iwasa 1996). As stressed in a recent debate (Klug et al. 2010; Krakauer et al. 2011; Jennions et al. 2012), the above mentioned measures quantify, at best, different facets of sexual selection in a given system, and should thus be interpreted with caution.

Moreover, it is unfortunate that behavioural assays are considered in only few studies (Fritzsche and Booksmythe 2013). Instead, mating success is often inferred from parentage assignment, as the number of genetic mates (e.g., Bateman 1948; Jones et al. 2000; Gopurenko et al. 2007; Byers and Dunn 2012; Pischedda and Rice 2012), which inevitably omits the unsuccessful matings, and cannot account for repeated matings from a same pair (Wade and Shuster 2005). Therefore, a promising approach is to combine behavioural observations with parentage assignments, which elegantly permits to determine whether a sexually selected trait is mediated by pre-copulatory or post-copulatory selection (Jones 2009; see e.g., Pélissié et al. 2012; Fritzsche and Arnqvist 2013), and also to partition the variance observed in reproductive success along different pre- and post-copulatory episodes of selection to determine which episode of selection has the highest opportunity for selection (Anthes et al. 2010; Collet et al. 2012).

Sexual Selection in Simultaneous Hermaphrodites

Although research in sexual selection has long focused on species where individuals are either male or female throughout their lives (hereafter called gonochorists), sexual selection is also expected to operate in species where individuals possess both sex functions at the same time (hereafter called simultaneous hermaphrodites) (Charnov 1979; Morgan 1994; Michiels 1998; Avise 2011; Schärer and Pen 2013), which is a widespread sexual system in animals, occurring in 24 of the 34 animal phyla, and representing 5-6% of the animal species (Jarne and Auld 2006). Owing to the simultaneous expression of the two sex functions, simultaneous hermaphrodites exhibit specific sexual phenomena such as e.g., self-fertilisation, conflict over mating roles, and resource allocation to their own male or the female function (reviewed in Michiels 1998; Anthes et al. 2006; Schärer 2009; Anthes 2010).

A crucial step in the recognition that sexual selection can operate in simultaneous hermaphrodites was Charnov's (1979) proposal that the Bateman's principles can also be applied to male and female sex functions of simultaneous hermaphrodites. Charnov (1979) explicitly stated that "fertilized egg production by an individual is limited not by the ability to get sperm, but by the resources allocated to eggs". This implies that the strength of sexual selection is expected to be stronger in the male than in the female sex function, and individuals may mate preferentially to donate rather than to receive sperm. The latter prediction leads to an incompatible situation where all individuals in a population tend to prefer the male mating role (i.e., donate sperm), creating a conflict of interest between partners over the mating role (reviewed in Michiels, 1998; Anthes, 2006; see e.g., Michiels and Newman 1998). An evolutionary solution that many species seem to have taken to solve this conflict is to reciprocate matings in a way that both partners accept receiving sperm from each other in order to have the opportunity to donate their sperm (Michiels 1998; Schärer and Pen 2013). This scenario implies that all individuals are eager to engage in mating, which has several implications on the operation of sexual selection in both the male and female sex functions. From the male function perspective, individuals might not primarily be selected to acquire new matings, but rather to successfully transfer sperm to their mating partners, to make efficient sperm to fertilise eggs, and to influence sperm usage of their mating partners. From the female function perspective, individuals may be less choosy about their mating partners, which leads to a surplus of potentially unwanted sperm. It is then expected that the partner may exert choice after mating, by preferentially using sperm of certain donors for fertilisation (i.e., cryptic female choice). Therefore, sexual selection in simultaneous hermaphrodites is often argued to mainly operate at the post-copulatory episodes of selection (Michiels 1998; Schärer and Pen 2013). In accordance with this hypothesis, compelling evidence shows that post-

copulatory sexual selection may be an important agent of selection in simultaneous hermaphrodites (e.g., Koene and Schulenburg 2005; Chase and Blanchard 2006; Anthes et al. 2008; Garefalaki et al. 2010; Schärer et al. 2011). A classic example is terrestrial gastropods that shoot the so-called love dart into their mating partner before copulation. This striking behaviour has been shown to increase the siring success of the shooter (Landolfa et al. 2001), which is achieved by manipulating the sperm storage and usage of the sperm recipient (Rogers and Chase 2001; Chase and Blanchard 2006).

Some authors have questioned whether Bateman's principles also applies to simultaneous hermaphrodites arguing that it is not necessarily the male role which is the preferred mating role (debated in Leonard and Lukowiak 1984; Michiels 1998; Leonard 2005; Anthes et al. 2006; Anthes 2010). In particular, Leonard (2005) argued that the preferred mating role should be the role that confers the control over fertilisation. Thus in internally fertilizing species where the female function may potentially control the fate of the partner's sperm, the male role should be disfavoured because it does not guarantee parentage (Leonard 2005; e.g., Leonard and Lukowiak 1984). This argument is however questionable because it mainly relies on a descriptive studies rather than on manipulative studies (Anthes 2010; but see Anthes and Michiels 2005; Anthes et al. 2005 for manipulative studies). An alternative way to identify the preferred mating role in simultaneous hermaphrodites is to compare the fitness returns of each additional mating between both sex function. For this, Anthes et al. (2010) have recently developed a framework that relies on the comparison of the Bateman gradients of the male and female sex functions (but see Kokko et al. 2012). At present, few attempts have investigated Bateman gradients in simultaneous hermaphrodites (but see Pongratz and Michiels 2003 for similar test), and the results indicate that the Bateman gradients are steeper in the male than in the female sex function in the two species tested, the freshwater snails *Biomphalaria glabrata* (Anthes et al. 2010) and *Physa acuta* (Pélissié et al. 2012). Therefore, it seems too early to draw a potential general conclusion, as well as to determine the main factors influencing the male and female Bateman gradients in simultaneous hermaphrodites.

Sex Allocation in Simultaneous Hermaphrodites

Simultaneous hermaphrodites must develop and maintain both the male and the female reproductive organs, and so probably pay greater costs than pure males or pure females would (Charnov 1979). The *sine qua non* condition to compensate these additional costs is that the fitness gain curve of at least one sex function is saturating (i.e., diminishing fitness returns per unit of investment) (Charnov 1979, 1982). It is usually the male fitness gain curve which is thought to be saturating, while the female fitness is expected to be linearly proportional to the investment in egg production (following Bateman's principles; Charnov 1979, 1982; Schärer 2009; see Yund 1998; and Johnson and Yund 2009 for empirical evidence). The reason why the male fitness gain curve is expected to be saturating is that the competition between sperm donated by the same sperm donor (called local sperm competition, LSC, Schärer 2009, in analogy to the local mate competition, Hamilton 1967) leads to diminishing fitness returns for any additional investment into sperm production. This is because in situations where sperm compete mainly between related sperm for the fertilization of a given set of ova (i.e., high LSC), the production of more sperm increases this competition and consequently decreases the fitness returns per unit of investment. Thus individuals should allocate more resources towards their female function to increase their total fitness if LSC is high. Therefore, LSC is expected to be a crucial factor that shapes the male fitness gain curve and thereby affects the optimal sex allocation that individuals should adopt (Schärer 2009; Schärer and Pen 2013).

Consequently, any factor that affects the intensity of LSC is expected to influence the optimal sex allocation. Such factors include selfing rate, mating group size, sperm displacement, sperm digestion, cryptic female choice and random paternity skews (reviewed in Schärer 2009; Schärer and Pen 2013). For instance, when mating group size is small, individuals are expected to invest more resources in their female than their male sex function. In contrast, when the mating group size increases, the sperm compete more and more against unrelated sperm (i.e., LSC decrease), and so individuals are expected to produce more sperm to be competitive in sperm competition, and thus to re-allocate resources towards the male function (Charnov 1980). In accordance with these predictions, empirical studies suggest that some simultaneous hermaphrodites show a phenotypically plastic adjustment of their sex allocation in response to mating group size (reviewed in Schärer 2009). In particular, field studies show that individuals in high densities are more male-biased (e.g., Raimondi and Martin 1991) and experimental work also demonstrated that individuals in larger social groups have a more male-biased sex allocation (Trouvé et al. 1999; Schärer and Ladurner 2003; Janicke and Schärer 2009a but see Koene et al. 2006).

Thesis Outline

Throughout my PhD project, I studied sexual selection in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. I was interested in sexual selection acting on phenotypic traits and behaviours, with a special focus on distinguishing between processes involved in pre- and post-copulatory sexual selection.

In many species, mating is a prerequisite to gain fitness, but it may also incur substantial costs. Therefore, individuals are expected to strategically allocate their reproductive resources over different mating opportunities. Notably, in simultaneous hermaphrodites, the mating status is expected to be a crucial factor for the decision whether to engage in a mating. This is because mating status determines both the need for sperm receipt to fertilise the own eggs and the availability of sperm to be donated to mating partners. In **chapter II**, I investigated whether mating status affects mating propensity in *M. lignano*. Moreover I investigated whether the frequency of the post-copulatory suck behaviour, a facultative behaviour potentially involved in removing ejaculate components from their own sperm-storage organ, was affected by the mating status of the sperm recipient and/or the sperm donor. This chapter documents that complex interactions may arise between mating partners, potentially due to a conflict over sperm usage.

Internal processes occurring after copulation are often challenging to observe. Owing to its transparency, *M. lignano* enables to perform a range of measures *in vivo*, including observation of received sperm. A breakthrough has recently been realised with the generation of a line ubiquitously expressing green fluorescent protein (GFP) in all cell types, including in the sperm cells, which enables the unique opportunity to assess the proportion of sperm from a GFP-expressing donor inside the reproductive tract of recipients *in vivo* and non-invasively. In **chapter III**, I tested and validated the reliability of this promising technique. In brief, while other techniques allow peering into the post-copulatory black box only with destructive-sampling techniques, the GFP-technique in a transparent organism allows an unobstructed view into the post-copulatory black box with a non-invasive technique.

The tools I established in chapter III offer novel opportunities to quantify sexual selection. While the quantification of sexual selection often focuses on mating success (e.g., opportunity for sexual selection, Bateman gradients), **chapter IV** integrates the quantification of post-copulatory components. Specifically I investigated how mating success (i.e., copulations achieved), sperm-transfer efficiency (i.e., sperm stored per copulation), and sperm-fertilising efficiency (i.e., paternity per stored sperm) contribute to the observed variance in the resulting male reproductive success. In addition, the decomposition of sexual selection along fitness components allows to pinpoint at which episode of selection specific sexually selected traits operate. The resulting data inform us about the opportunity for sexual selection of mating

success, sperm-transfer efficiency and sperm-fertilising efficiency in this study system.

A fundamental prediction in simultaneous hermaphrodites is that individuals adjust their sex allocation according to their mating group size. This is because when the mating group size increases, the intensity of sperm competition increases and so individuals are expected to increase their investment towards their male function to be competitive in sperm competition. Therefore the theory predicts that allocation towards the male function first increases and then eventually saturates with increasing mating group size. I present, in **chapter V**, the first study that explicitly investigates the relationship between mating group size and sex allocation.

Study Organism

I studied sexual selection and sex allocation in *Macrostomum lignano,* a free-living flatworm that belongs to the Rhabditophora, the largest taxon of the phylum Platyhelminthes. *M. lignano* is a species from the marine meiofauna and has so far been described to occur in the Adriatic and Aegean Sea (Ladurner et al. 2005b). While relatively little is known about its ecology, *M. lignano* has emerged in the last decade as a highly suitable laboratory model organism to address a broad range of questions, including for example ageing (e.g., Mouton et al. 2009), developmental biology (e.g., Ladurner et al. 2008), sex allocation (e.g., Schärer and Ladurner 2003), and sexual selection (e.g., Janicke and Schärer 2009b).

The main advantages of *M. lignano* for the study of sex allocation and sexual selection are its small size and transparency, which enables to observe several internal structures *in vivo*. Moreover, this species can be easily cultured under laboratory conditions, where worms are kept in glass Petri dishes filled with f/2 medium (modified after Andersen et al. 2005) and fed with the algae *Nitzschia curvilineata*. Under these conditions, *M. lignano* has a short generation time (i.e., about 18 days) and its body size reaches about 1.5 mm (Ladurner et al. 2005b).

M. lignano is an obligately outcrossing simultaneous hermaphrodite. The paired testes are located in the central area of the body (*cf.* Figure 1). The produced sperm cells are transported through the *vasa deferentia* to the intermediate storage organ, the seminal vesicle, from where they can be transferred to the mating partner via the male copulatory organ, the stylet (*cf.* Figure 1). The paired ovaries produce the eggs, which develop in the growth zone. Posterior to the growth zone, the received sperm can be stored for several days in a female reproductive organ called the antrum (*cf.* Figure 1). Fertilisation occurs presumably when the egg enters the antrum, before egg laying (Ladurner et al. 2005b; Vizoso et al. 2010).

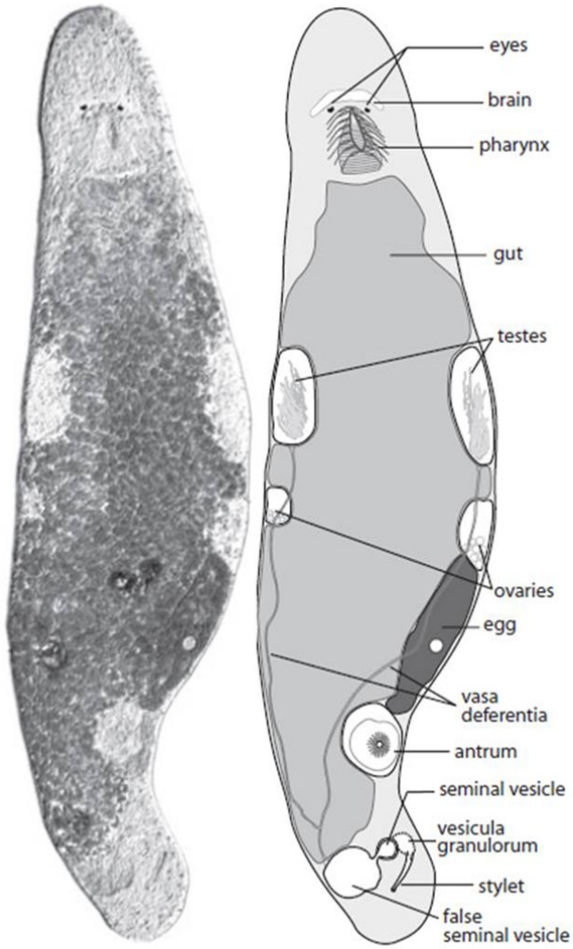

Figure 1: Micrograph and line drawing of a live *M. lignano* squeezed between two glass slides.
The length of this worm is approximately 1.8 mm.
(Vizoso et al., 2010).

M. lignano has a very high copulation rate (about 6 copulations per hour) (Schärer et al. 2004a), and is highly promiscuous (Janicke and Schärer 2009a). The copulation consists of a reciprocal insertion of the stylet into the antrum of the partner, and the transfer of sperm and prostate gland secretions (cf. Figure 2b, c; Doe 1982; Schärer et al. 2004a; Ladurner et al. 2005a; Vizoso et al. 2010). Immediately after the copulation, worms may display a facultative behaviour, the so-called suck behaviour, in which worms bend onto themselves, place their pharynx over their own female genital opening and appear to suck for about 5 s (cf. Figure 2e; Schärer et al. 2004a). It is not clear whether the suck behaviour really removes ejaculate components out of the antrum. But it has recently been argued that the complex morphology of the sperm, including stiff lateral bristles, has evolved to prevent the sperm to be removed from the antrum (Vizoso et al. 2010; and see Schärer et al. 2011 for a comparative study supporting this scenario).

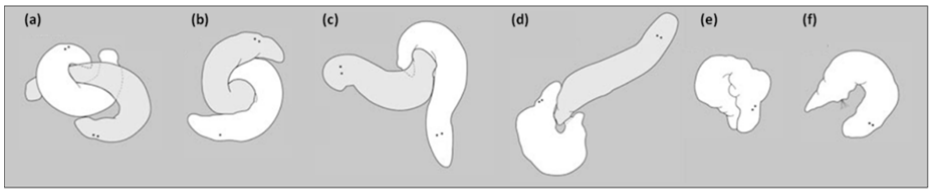

Figure 2: line drawings of key mating elements of *Macrostomum lignano*, including pre-copulatory behaviour (a), the copulation itself (b and c), and the facultative suck behaviour (e). (modified from Schärer et al. 2004)

Testis and ovary sizes show phenotypic plasticity in response to social group size. As predicted by sex allocation theory (Charnov 1980, 1982), worms are more male-biased in larger social groups (Schärer and Ladurner 2003; Schärer et al. 2005; Brauer et al. 2007). Importantly, more male biased worms have been shown to have higher sperm production rate (Schärer and Vizoso 2007), and to display a higher mating rate (Janicke and Schärer 2009b). Similarly, more male biased worms likely produce fewer eggs, as suggested by the lower number of produced offspring per capita in large social groups (Schärer et al. 2005).

Moreover, the study of sexual selection and sex allocation in *M. lignano* greatly benefits from the development of histological and molecular techniques in this species. Notably, *in situ* sperm tracking (Schärer et al. 2007), and phenotyping engineering enabling the experimental manipulation of specific traits (Sekii et al. 2009) have both provided powerful tools to address evolutionary questions (Janicke and Schärer 2009a; Sekii et al. 2013). More recently, a transgenic line of *Macrostomum lignano* that expresses green fluorescent protein (GFP) has been established by injecting a DNA construct into the single-cells egg stage. The GFP expression is driven by a housekeeping gene promoter and therefore is ubiquitous, including in the sperm cells (see Demircan et al. in prep for details on the establishment of the GFP line).

Chapter II

Effects of Mating Status on Copulatory and Post-Copulatory Behaviour in a Simultaneous Hermaphrodite

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Abstract

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 post-copulatory behaviour in the free-living flatworm *Macrostomum lignano*, an outcrossing simultaneous hermaphrodite. These worms have frequent reciprocal copulations and often display a post-copulatory 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.

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 and Petrie 2000; Wedell et al. 2002; Kokko and Mappes 2005; Parker and Pizzari 2010; Edward and 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 and 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 and Rowe 2005b), 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 and Kramer 1982; Royer and McNeil 1993; Schärer and Vizoso 2007). Hence, given that male reproductive success depends on the amount of ejaculate transferred (e.g., Gage and 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 fertilisation of the eggs (reviewed in Orr and Zuk 2012). On the one hand, female reproductive success might be limited by the amount of sperm available to fertilise the eggs, for example because of difficulties in obtaining sufficient sperm or in finding mates (Wedell et al. 2002; Kokko and Mappes 2005), and females may benefit from multiple matings (Jennions and 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 example because of the risk of polyspermy (reviewed in Birkhead et al. 1993) or seminal fluid-mediated costs (reviewed in Chapman 2001; Arnqvist and Rowe 2005b). Therefore, female mating propensity is expected to vary according to the amount of sperm stored to optimize the eggs' fertilisation and the female's reproductive success. In addition, female mating propensity may also be manipulated by previous mating partners (Johnstone and 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 and Petrie 2000; Edward and Chapman 2011). For instance, it has been shown that males mate preferentially with more fecund and/or virgin females (e.g., Johnson and Hubbell 1984; Schneider et al. 2011) and/or tailor the ejaculate size to the level of sperm competition (e.g., Wedell 1992; Gage and Barnard 1996; reviewed in Parker 1998). Similarly, females may preferentially mate with males providing material and/or genetic benefits (Jennions and Petrie 2000; Møller and 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 and Mappes 2005; Edward and 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 fertilise 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 fertilise 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 and 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 and 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 and 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 fertilisation success of a given amount of donated sperm (e.g., Koene and Chase 1998; Koene et al. 2005; Chase and 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 post-copulatory behaviours in the free-living flatworm *Macrostomum lignano*. This species has reciprocal mating and performs a post-copulatory behaviour, the so-called suck behaviour, which is possibly involved in removing ejaculate components received during copulation (Schärer et al. 2004a; Vizoso et al. 2010; Schärer et al. 2011). 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 and 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 and 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. 2004a) and is highly promiscuous (Schärer and Ladurner 2003; Janicke and Schärer 2009a). Copulation consists of reciprocal insertion of the male copulatory stylet into the female genital organ (the antrum) of the partner (Schärer et al. 2004a), generally leading to the transfer of sperm and seminal fluid from a prostate-like accessory gland (Doe 1982; Ladurner et al. 2005a; Ladurner et al. 2005b; Vizoso et al. 2010). The sperm reserves are not depleted after just a few matings (see also Schärer and 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 and Schärer 2009a). Subsequent mates can displace previously stored sperm (Marie-Orleach et al. in prep.), leading to second-male sperm precedence (Sandner et al. in prep), and stored sperm may be used to fertilise eggs for up to 20 days after mating (Janicke et al. 2011). A facultative post-copulatory 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. 2004a; 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. 2004a; Vizoso et al. 2010; Schärer et al. 2011).

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 ml drop of artificial sea water between two siliconized microscope slides separated by 210 mm (as described in more detail in Schärer et al. 2004a). 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 post-copulatory 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 post-copulatory behaviour, it can also occur outside copulation events (Schärer et al. 2004a). Because we were here interested in the post-copulatory suck behaviour, we only considered sucks occurring within 5 s after the end of a copulation (Schärer et al. 2004a).

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 & 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 fibreoptic 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: $Z = 3.15$, $N = 61$, $P = 0.002$; Figure 1a) and started to copulate earlier than sexually experienced pairs (Wilcoxon rank-sum test: $Z = 2.93$, $N = 53$, $P = 0.003$; Figure 1b). Over the first five copulations, virgin pairs had a higher average copulation duration than sexually experienced pairs (Wilcoxon rank-sum test: $Z = 3.63$, $N = 44$, $P < 0.001$; Figure 1c). Moreover, individuals within virgin pairs exhibited significantly fewer sucks than individuals within sexually experienced pairs (GLM: $\chi_1^2 = 3.96$, $P = 0.047$; Figure 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 (Figure 2), in that the mating status of the focal worm had no effect in either experiment 2a (GLM: $\chi_1^2 = 0.12$, $P = 0.734$) or experiment 2b (GLM: $\chi_1^2 = 0.50$, $P = 0.478$; Fisher's combined probability test: $\chi_4^2 = 2.10$, $P = 0.718$). However, the number of sucks differed according to the mating status of the mating partner, nearly significantly in experiment 2a (GLM: $\chi_1^2 = 3.51$, $P = 0.061$; Figure 2a) and statistically significantly in experiment 2b (GLM: $\chi_1^2 = 5.23$, $P = 0.022$; Figure 2b), jointly leading to a significant effect of the mating status of the mating partner (Fisher's combined probability test: $\chi_4^2 = 13.23$, $P = 0.010$). 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: $\chi_1^2 = 0.13$, $P = 0.720$) or experiment 2b (GLM: $\chi_1^2 = 1.66$, $P = 0.199$; Fisher's combined probability test: $\chi_4^2 = 3.85$, $P = 0.427$).

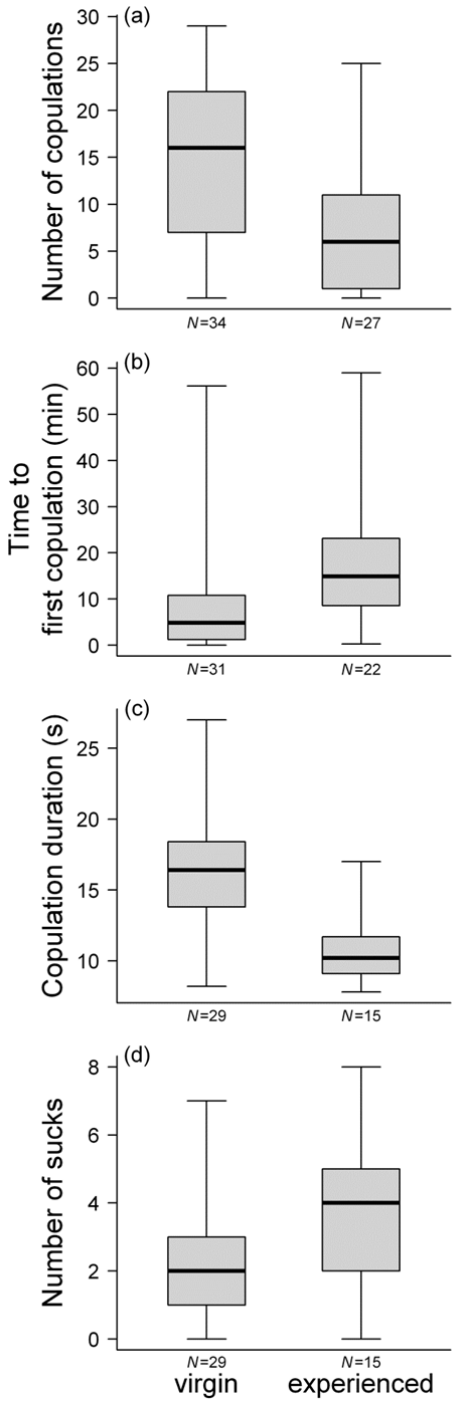

Figure 1. Effect of mating status on copulatory and post-copulatory behaviour. Comparison of (a) the number of copulations observed over the 1 h mating trial, (b) the time to the first copulation, (c) the average copulation duration of the first five copulations, and (d) the number of sucks performed 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.

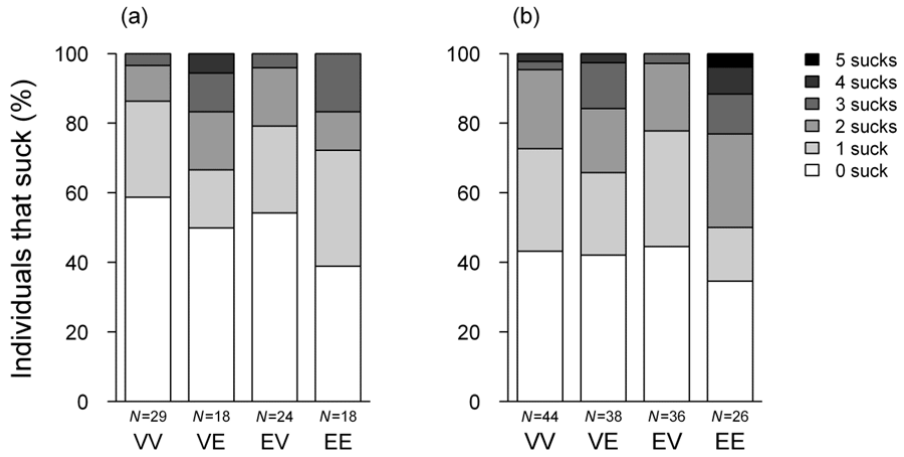

Figure 2. Effect of mating status of the focal individual and its partner on the post-copulatory behaviour of the focal individual. The percentages of worms that did not suck or sucked one to five times in the first five copulations during the mating trials are shown. Results are shown for the two independent 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.

Discussion

This study shows that mating status affects the copulatory and post-copulatory 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 and 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 and 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 and 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 fertilise their own eggs. In *M. lignano* individuals that are isolated for a long period usually have many developing eggs ready to be fertilised, 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 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 and 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 and 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 and Reinhold 2006; Ball and Parker 2007). Empirical evidence suggests that sperm donors indeed allocate more ejaculate to already mated recipients (e.g., Gage and Barnard 1996; Velando et al. 2008) or, in contrast, allocate more ejaculate to virgin recipients (e.g., Wedell 1992; Loose and 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 and 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 and 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. 2004a). 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.

Post-Copulatory Suck Behaviour

A striking outcome of our study is that the frequency of the post-copulatory 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 fertilisation success by manipulating the physiology and/or the behaviour of the recipient (reviewed in Chapman 2001; Arnqvist and Rowe 2005b; 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 and Chase 1998) and thereby favours the uptake of the spermatophore and reduces the risk of sperm digestion (Chase and 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 fertilisation rather than digestion would be advantageous (Anthes 2010). Thus, under the assumption that the post-copulatory suck behaviour of the partner decreases the fertilisation 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 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 and 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 fertilisation 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 post-copulatory suck behaviour of their mating partner to increase the fertilising success of the transferred sperm.

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Appendices

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 and Vizoso 2007), was measured following the usual procedure (Schärer and 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: $Z = 6.13$, $N = 117$, $P < 0.001$; Figure 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: $Z = 5.39$, $N = 117$, $P < 0.001$; Figure A1b).

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.

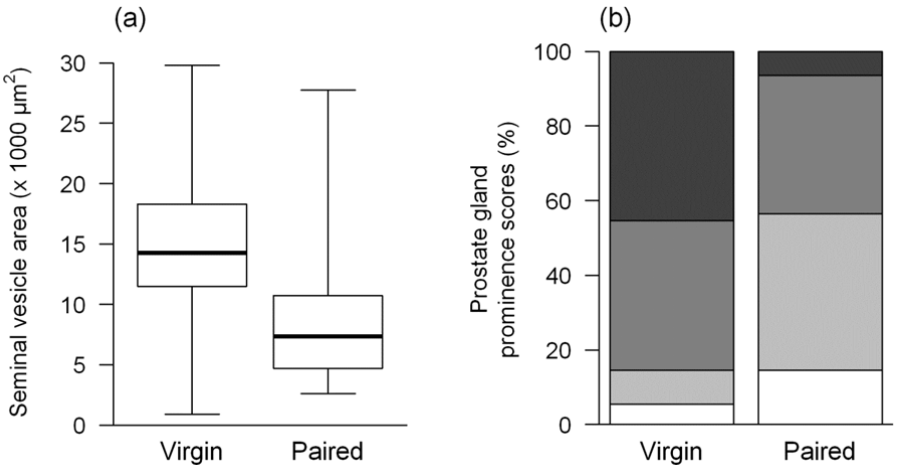

Figure A1. Effect of isolation on the amount of stored autosperm and seminal fluid. (a) Comparison of the seminal vesicle area between virgin and paired worms. Box plots show the median, the quartiles and the extreme values. (b) Percentage of virgin and paired individuals that had a prostate-like accessory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

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 36and37. 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/2medium). 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. (2004a) 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 ± SE = 15.5 ± 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.

Chapter III

Fluorescent Sperm in a Transparent Worm: Validation of a GFP Marker to Study Sexual Selection

Manuscript in preparation:
Marie-Orleach, L., T. Janicke, D.B. Vizoso, M. Eichmann, K. De Mulder, E. Berezikov, P. Ladurner, and L. Schärer. in prep. Fluorescent sperm in a transparent worm: validation of a GFP marker to study sexual selection.

Abstract

Although sexual selection was initially thought to occur exclusively at the pre-copulatory stage (e.g., contests among males, and female mate choice), in the last 40 years it has been shown that it can continue beyond copulation through sperm competition and/or cryptic female choice. However, the study of these post-copulatory processes remains challenging because they occur internally and therefore are often inaccessible. In the transparent flatworm *Macrostomum lignano* a recently established transgenic line that expresses green fluorescent protein (GFP) in all cell types including sperm offers a unique opportunity to visualise, and quantify, the contribution of GFP-expressing donor sperm inside the reproductive tract of wild-type recipients *in vivo* and non-invasively. We here present a series of tests indicating that GFP-expressing worms do not differ from wild-type worms in terms of morphology, mating rate and reproductive success. In addition, we validated that all GFP-expressing individuals reliably display a GFP signal when observed under epifluorescence illumination. However, GFP-expressing individuals produce sperm with varying intensity of GFP signal, which is presumably due to sperm ageing. The GFP marker is, with some few exceptions, inherited according to Mendel's laws. Finally, we illustrated the usefulness of the GFP-techniques by studying sperm displacement. For this, we assessed twice the number of sperm stored by a GFP-expressing donor in a recipient, before and after a wild-type second donor. The results reveal that donors can displace previously stored sperm and replace it with their own. In conclusion, the present study documents that the availability of the GFP-techniques in a transparent organism represents a novel and highly powerful tool to study sexual selection.

Sexual selection has first been defined by Darwin as the selection that "depends on the advantage which certain individuals have over others of the same sex and species solely in respect of reproduction" (Darwin 1871). Sexual selection theory intends to explain, for instance, why red deer males engage in impressive battles or why peacock males display colourful features. This likely happens because individuals that outcompete rivals (e.g., via male-male competition) and/or attract mating partners (e.g., via female mate choice) gain mating opportunities that consequently lead to a higher reproductive success (Andersson 1994). In addition to the competition for mating opportunities, it has been realised that sexual selection can continue beyond copulation. When females mate multiply, sperm of different males may compete for fertilisation (sperm competition) and females may also influence the fertilisation success of some males by preferentially using their sperm (cryptic female choice) (Parker 1970; Charnov 1979; Thornhill 1983; Eberhard 1996; Parker 1998; Birkhead and Pizzari 2002; Parker and Pizzari 2010). Therefore, sexual selection is nowadays often considered to act through several consecutive episodes of selection, occurring before, during and after the copulation event, all of which can potentially affect reproductive success (Pizzari et al. 2002; Anthes et al. 2010; see e.g., Collet et al. 2012). However, because post-copulatory processes often occur inside the female reproductive tract, they are challenging to observe. Therefore, progress in our current understanding of post-copulatory sexual selection will depend on the development of techniques that should ideally allow the observation of internal processes in the female reproductive tract *in vivo*.

Post-copulatory sexual selection is expected to ultimately affect the paternity share, and a large number of studies have inferred the mechanisms of post-copulatory sexual selection by studying the patterns of paternity skews. This has been achieved by combining behavioural manipulations and paternity analyses, based on e.g., phenotypic markers (e.g., Lefevre and Jonsson 1962; Nilsson et al. 2003), the sterile-male technique (e.g., Harano et al. 2008) or microsatellite markers (e.g., Birkhead et al. 1999). A similar approach is to use artificial insemination instead of behavioural manipulations to control for potential differences in the number of sperm inseminated (e.g., Martin et al. 1974; Evans et al. 2003; Birkhead et al. 2004; Denk et al. 2005). However, both of these approaches focus on the ultimate outcome of post-copulatory sexual selection and so, yield limited insights about the underlying mechanisms from which the skews in paternity share result.

Some established methods already allow shedding light on the cryptic nature of these internal processes and permit to assign the relative contributions of donors to a pool of sperm *in situ*, inside the reproductive tract of a recipient. To our knowledge, this can currently be achieved through the following five methods. First, when donors have a nonoverlapping range for a morphological sperm cell trait, then this trait may be used as a marker to distinguish sperm from different sperm donors (e.g., sperm length, Hellriegel and Bernasconi

2000). Second, sperm can be experimentally radiolabelled, using either amino-acids or nucleotides containing specific radioisotopes, which can later be quantified in the recipient by scintillation counting or autoradiography (e.g., Bishop 1996; Simmons et al. 1999). Third, sperm cell DNA can be labelled with a halogenated pyrimidine (such as bromodeoxyuridine, BrdU) integrated during spermatogenesis, which can later be tracked in the recipient by using immunocytochemical staining techniques (e.g., Schärer et al. 2007; Janicke and Schärer 2009a). Fourth, in *Drosophila melanogaster* transgenic lines were established that express fluorescent markers in sperm, e.g., green or red fluorescent proteins, which enables visualization and counts of sperm *in situ* (e.g., Civetta 1999; Manier et al. 2010; Lüpold et al. 2011). And fifth, via a competitive PCR approach it is possible to quantify donor-specific microsatellites in the sperm stored in the reproductive tract of a recipient (e.g., Bretman et al. 2009; Bussière et al. 2010; Hall et al. 2010; Tuni et al. 2013). These opportunities to quantify the contributions of specific donors to a pool of sperm stored within a recipient have greatly improved our understanding of the mechanisms of post-copulatory sexual selection, including sperm transfer, sperm storage, sperm displacement, sperm dynamics and cryptic female choice (Bishop 1996; Bretman et al. 2009; Janicke and Schärer 2009a; Manier et al. 2010). However, all of these methods have a common limitation because they involve destructive sampling, requiring either to dissect out the female reproductive tract or to fixate the entire sperm recipient and so, the sperm recipient cannot be used for paternity analysis or further experimental manipulations.

Here we present a study system in which we can track the sperm of a specific sperm donor *in vivo* under competitive conditions, using the non-invasive visualisation of labelled sperm inside the female reproductive tract of a transparent sperm recipient. This breakthrough has become possible due to a recently established transgenic line of the free-living flatworm *Macrostomum lignano*, which expresses green fluorescent protein (GFP) in all cell types, including the sperm cells (Figures 1, 2) (Demircan et al. in prep). This technique adds two unique opportunities to the previously established methods, both of which are linked to the non-invasive nature of the approach. First, it allows to repeatedly assess the contribution of a sperm donor within a pool of received sperm and therefore to study temporal patterns of sperm storage within recipients. Second, it allows studying how sperm transfer success translates into paternity success.

Hence, we argue that this technique offers novel opportunities to study mechanisms of post-copulatory sexual selection and thus to obtain new insights on sexual selection in general. However, to fully evaluate the usefulness of this method the reproductive performance of the transgenic individuals as well as the techniques involved in assessing the relevant phenotypes need to be studied and validated. Here, we report on a series of tests that on the one hand clearly establish the reliability of the GFP

techniques, while at the same time revealing some limitations that need to be taken into account in future experiments. Furthermore, as a proof of principle, we have applied the GFP techniques to study sperm displacement in *M. lignano*. For this, we assessed twice the number of sperm received from a GFP donor in a recipient, before and after a second sperm donor. The results unambiguously demonstrate the presence of sperm displacement in *M. lignano*.

Materials

Macrostomum lignano (Macrostomorpha, Plathylminthes) is a free-living flatworm from the intertidal zone in the Northern Adriatic Sea that is easily cultured in laboratory conditions where it reaches about 1.5 mm and has a generation time of about 18 days (Ladurner et al. 2005b). It is a simultaneous hermaphrodite that copulates reciprocally and frequently, and possesses distinct pre- and post-copulatory behaviours that can be easily observed and quantified (Schärer et al. 2004a; Marie-Orleach et al. 2013). Worms are transparent, allowing non-invasive observation and reliable measurements of the size of internal structures such as testis, ovary and seminal vesicle, as well as of the sperm cell and its appendages (Schärer and Ladurner 2003; Schärer and Vizoso 2007). The received sperm can be counted inside the storage organ (hereafter antrum) (Vizoso et al. 2010; Janicke et al. 2011). In addition, the size and the morphology of the male copulatory organ may also be measured using geometric morphometric methods (Janicke and Schärer 2009a, 2010). Finally, specific traits can be experimentally manipulated via RNA interference approaches (i.e., phenotyping engineering) to provide powerful quantitative tests for sexual selection predictions (e.g., Sekii et al. 2013). Thanks to these traits and well established methods, *M. lignano* has emerged as a suitable model organism to study sexual selection.

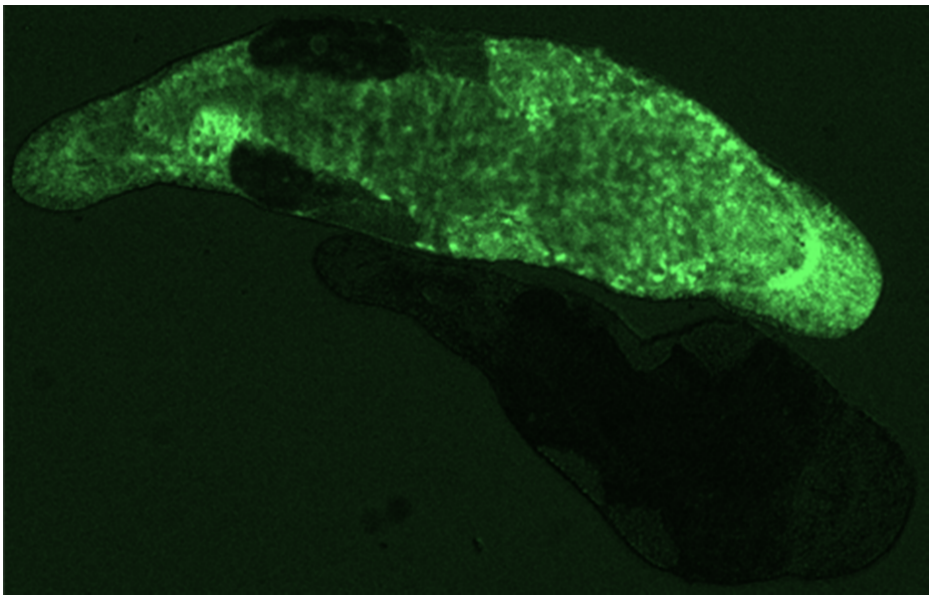

Figure 1. Picture of a GFP(+) and a GFP(-) individuals (photo © Micha Eichmann).

In this study, we investigate whether transgenic GFP-expressing individuals [hereafter GFP(+)] differ from wild-type individuals [hereafter GFP(-)] in traits other than the GFP expression, and thus whether the GFP-techniques can reliably be used to study sexual selection. For all the tests, we used two lines, a GFP line (called HUB1; Demircan et al. in prep) and a GFP(-) line (called DV1; Janicke et al. 2013). As explained in Janicke et al. (2013), the DV1 line was created via full-sib and half-sib inbreeding for 24 generations, and has been maintained in small population size to maintain inbreeding. More recently, the DV1 line was used to create a stable transgenic line expressing GFP, the HUB1 line (Demircan et al. in prep) and so, the HUB1 and DV1 lines are expected to be genetically almost identical. Briefly, transgenesis was achieved by micro-injecting a DNA construct into a single cell stage egg. The DNA construct was vehicled by a transposable element, and was composed of a housekeeping gene promoter and the coding sequence of the enhanced GFP. Thus the GFP is ubiquitously expressed, including in sperm cells. Details on the establishment of the HUB1 line are described in Demircan et al. (in prep).

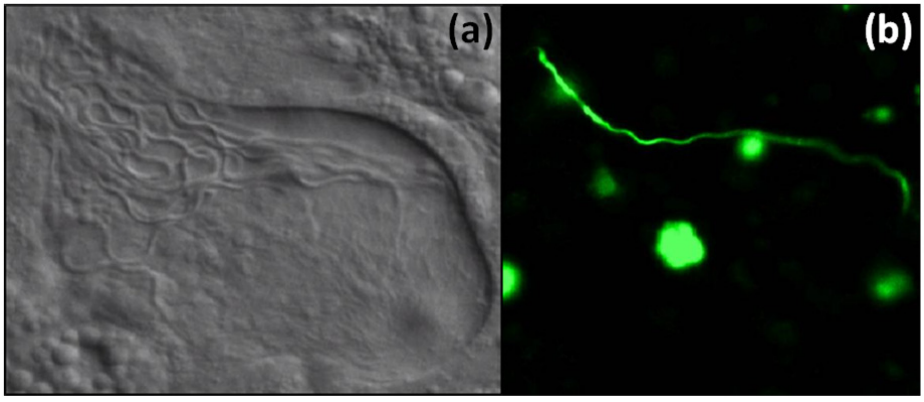

Figure 2. Pictures of the antrum of a worm mated with a GFP(+) and a GFP(-) partner. (a) under bright field illumination, we see the total number of received sperm (~14 sperm cells). (b) under epifluorescence illumination, we see the single sperm coming from the GFP(+) sperm donor.

Methods and Results

General Methods

The present study reports several independent experiments that have certain protocols in common. These protocols are reported in this section for sake of clarity.

Generating same-age individuals. To reduce experimental noise due to age differences between individuals, we use same-age individuals. For this, we transfer well fed adult individuals in glass Petri dishes filled with f/2 medium (modified after Andersen et al. 2005) and fed with the algae *Nitzschia curvilineata*, enabling individuals to lay eggs. Soon afterwards (i.e., usually 1 or 2 days) we remove the adult individuals so that the resulting hatchlings do not differ by more than 1 or 2 days.

Raising conditions. Soon after hatching, the resulting same-age hatchlings are collected and distributed in wells of 24-well tissue culture plates (TPP, Switzerland) filled with 1.5 mL of f/2 medium and fed *ad libitum*. Then, individuals are regularly transferred to new wells with fresh algae, until they reach sexual maturity.

Colouring individuals. To enable distinguishing the worms from each other, we colour the worms by using the vital dye patent blue V (also called E-131, Werner Schweizer AG, Switzerland), diluted at a concentration of 0.25 mg/mL of f/2 medium. A 24 h exposure allows colouring individuals, and has been shown to not affect the mating rate (see Marie-Orleach et al. 2013 for detailed tests).

Assessing received sperm in the female antrum. To observe the sperm received in the antrum, we follow a standard protocol (see Janicke et al. 2011; and Janicke et al. 2013 for detailed protocols). Briefly, this requires the preparation of an observation chamber where worms are squeezed in between two glass slides. Then, by using a microscope connected to a camera, we record movies of the entire antrum in which the sperm can be visualised *in vivo*. Bright field illumination allows the visualisation of the total sperm, while epifluorescence illumination restricts the visualisation to GFP(+) sperm. Thus, we count the total sperm and GFP(+) sperm in storage based on movies, blind with respect to the different treatments.

Statistics. All statistics analyses were carried in JMP 10.0.1 (SAS Institute Inc., Cary, NC, U.S.A.).

GFP(+) and GFP(-) Lines Comparisons

1. Morphology

Experimental setup. To test if individuals of the GFP(+) and GFP(-) lines have similar morphologies, we raised individuals in groups of either 2 or 8 individuals, and measured a suite of morphological traits. In details, we raised same-age individuals in pairs (i.e., 1 GFP(+) and 1 GFP(-) individual), or in octets (i.e., 4 GFP(+) and 4 GFP(-) individuals). We then took morphological measurements following the usual protocol (Schärer and Ladurner 2003), including body size, testis size, ovary size and seminal vesicle size. We measured both individuals in the pairs, and one randomly sampled individual of each line in the octets.

Statistics. The sample size was 19 pairs and 25 octets for all traits, except for seminal vesicle size for which the sample size was 18 pairs and 24 octets. To test for morphological differences between the lines, we fitted linear mixed models independently for the 4 response variables (i.e., body size, testis size, ovary size and seminal vesicle size), and used the line [i.e., GFP(+) or GFP(-)], the social group size (i.e., pair or octet) and the interaction line × social group size as fixed effects, and the group as a random effect.

Results. Individuals from the GFP(+) and GFP(-) lines did not differ in body size (LMM, $F = 0.04$, $df = 1$, $P = 0.84$), testis size (LMM, $F = 0.06$, $df = 1$, $P = 0.81$), ovary size (LMM, $F = 0.10$, $df = 1$, $P = 0.75$) and seminal vesicle size (LMM, $F = 0.24$, $df = 1$, $P = 0.63$) (Figure 3). Moreover, we found phenotypic plasticity in response to the different social group sizes for some morphological traits (LMMs, body size, $F = 16.67$, $df = 1$, $P < 0.001$; testis size, $F = 25.88$, $df = 1$, $P < 0.001$; ovary size, $F = 3.29$, $df = 1$, $P = 0.08$; seminal vesicle size, $F = 0.74$, $df = 1$, $P = 0.39$). This has previously been observed in outbred populations (e.g., Schärer and Ladurner 2003; Janicke and Schärer 2009b), and recently also in the HUB1 line (Janicke *et al.* 2013). All the interactions line × group size were not significant (LMMs, all P>0.3).

2. Mating Behaviour

Experimental setup. To test if the individuals of the GFP(+) and GFP(-) lines have similar mating rates, we performed mating trials on paired individuals. In details, we raised GFP(+) and GFP(-) individuals of two age cohorts in isolation. Then, we performed mating trials, following the usual protocol (Schärer et al. 2004a), in different crosses, GFP(+)×GFP(+), GFP(+)×GFP(-), and GFP(-)×GFP(-) in which the age cohorts were equally distributed over treatments and the partners belonged to the same age cohort. The mating behaviour was recorded for two hours during which we counted the exhaustive number of matings performed blind with respect to the treatments.

Statistics. The sample size was 14 GFP(+)×GFP(+), 11 GFP(+)×GFP(-), and 14 GFP(-)×GFP(-). To examine if the number of copulations differed between these 3 crosses, we fitted a generalized linear model (GLM), with a Poisson error distribution, a log-link function and a correction for overdispersion, and we used the cross, the age cohort and the interaction cross × age cohort as factors.

Results. We found that the 3 crosses had a similar copulation rate (GLM, $\chi^2 = 2.36$, $df = 2$, $P = 0.31$; Figure 4). Moreover, the younger cohort (1.1 ± 0.5, mean ± SE) copulated significantly less than the older one (10.8 ± 1.6, mean ± SE; GLM, $\chi^2 = 35.34$, $df = 1$, $P < 0.001$), and the interaction cross × age cohort was not significant ($\chi^2 = 2.12$, $df = 2$, $P = 0.35$) (Figure 4).

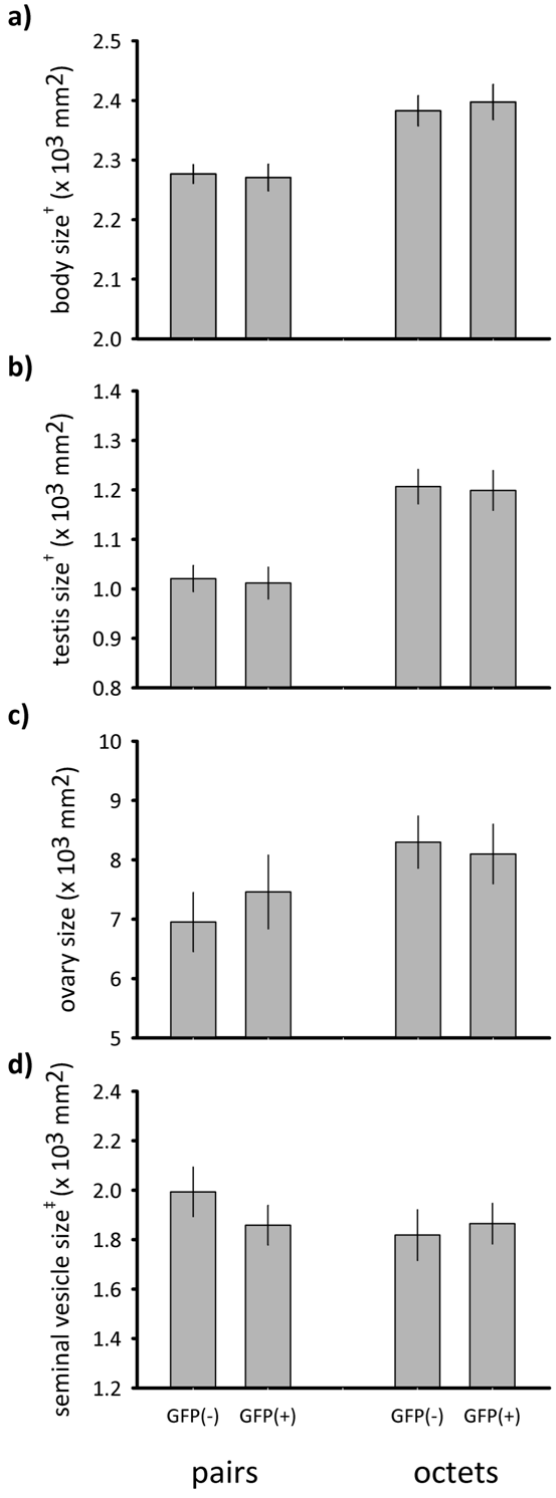

Figure 3. Morphology of the GFP(-) and GFP(+) lines. Comparisons of (a) body size, (b) testis size, (c) ovary size, and (d) seminal vesicle between GFP(-) and GFP(+) individuals raised in groups of 2 (i.e., pairs), or 8 individuals (i.e., octets). We show means (± SE). † stands for log transformation. ‡ stands for squared root transformation. See text for statistics.

3. Male and Female Reproductive Success

Exerimental setup. To test if the individuals from the GFP(+) and GFP(-) lines have similar male siring abilities and female reproductive success, we offered a partner to GFP(+) and GFP(-) individuals, and compared the number of offspring they produced through their male and female sex functions. In details, we raised same-age GFP(+) and GFP(-) individuals in isolation. We then formed pairs of GFP(+)×GFP(-) and GFP(-)×GFP(-) for 3 days, which we further separated to count the number of offspring each individual produced in isolation. We used the GFP(+)×GFP(-) pairs to estimate both the male siring ability of the GFP(+) worms (i.e., the number of offspring laid by its GFP(-) partner), and its female offspring production (i.e., the number of offspring it laid itself). In the GFP(-)×GFP(-) pairs, we randomly selected a priori one individual as a focal worm, for which we assessed the male siring ability and female offspring production as for the GFP(+)×GFP(-) pairs.

Statistics. The final sample size was 28 GFP(+)×GFP(-) and 29 GFP(-)×GFP(-). We compared the male siring ability and the female offspring production of the GFP(+) and GFP(-) individuals by using Wilcoxon signed-rank tests.

Results. The GFP(-) and GFP(+) lines did not differ in their male siring ability (Wilcoxon signed-rank test, $S = 895$, $P = 0.11$), or in their female offspring production (Wilcoxon signed-rank test, $S = 883$, $P = 0.21$) (Figure 5).

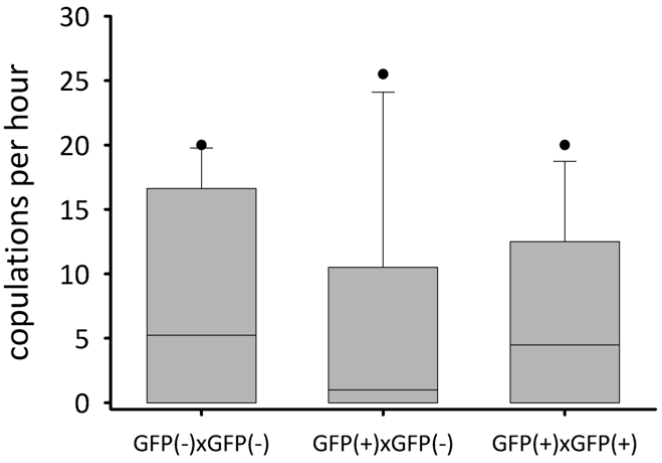

Figure 4. Mating behaviour of the GFP(-) and GFP(+) lines. Comparisons of the mating rate of the crosses GFP(-)×GFP(-), GFP(+)×GFP(-), and GFP(+)×GFP(+). The boxes show the 25th percentile, the median and the 75th percentile. The whiskers show the 10th and the 90th percentile, and the dots show the outliers. See text for statistics.

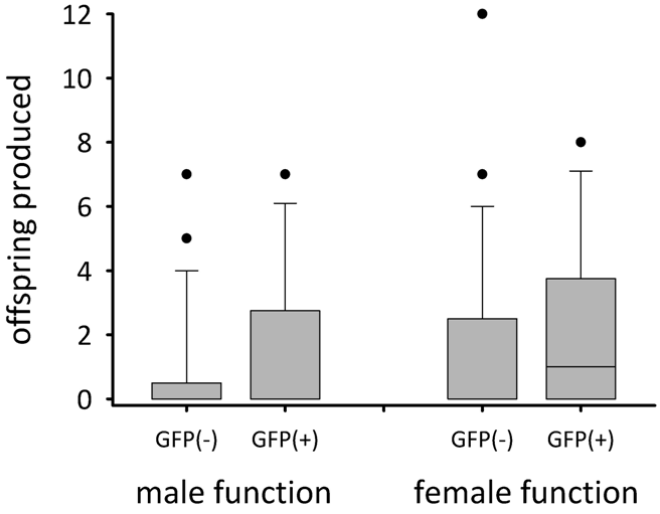

Figure 5. Reproductive success of the GFP(+) and GFP(-) lines. Comparisons of the male siring ability and the female offspring production of the GFP(-) and the GFP(+) individuals. The boxes show the 25th percentile, the median and the 75th percentile. The whiskers show the 10th and the 90th percentile, and the dots show the outliers. See text for statistics.

GFP Phenotyping Techniques

1- Worm Phenotyping

Experimental setup. To determine if the GFP expression is a reliable phenotypic marker, we raised HUB1 and DV1 individuals and assessed the GFP signal. For this, we raised 20 GFP(+) and 20 GFP(-) individuals and distributed them individually in wells of 60-well microtest plates (Greiner Bio-One, Germany). The GFP signal of the 40 worms was visually estimated by five different observers, blind with respect to the treatment, using a binary scale as 0 (no GFP signal) and 1 (GFP signal).

Results. As expected, GFP(+) and GFP(-) worms were all correctly scored as GFP(+) and GFP() individuals by the 5 observers.

2.1. Sperm Phenotyping – Repeatability

Experimental setup. To test if we can reliably count the number of GFP(+) sperm in the antrum of a GFP(-) recipient, we assessed twice the number of GFP(+) sperm in a same recipient, and computed the repeatability of this measure. For this, we raised same-age individuals as follow: GFP(+) pairs, GFP(-) pairs and GFP(-) in isolation (notice the GFP(-) individuals used here are from an outbred line). Then, we coloured the isolated GFP(-) individuals, and we formed groups of 3 individuals, including a coloured virgin GFP(-) individual used as a focal sperm recipient, 1 GFP(+) individual and 1 GFP(-) individual, and let them copulate for 100 min. Thus the virgin had two potential mating partners, a GFP(+) and a GFP(-) individual. Then, we isolated the focal sperm recipient of each group to estimate the number of total and

GFP(+) sperm received. Each sperm recipient was observed twice, in independent mating chambers, with a 30 min interval.

Statistics. The final sample size was 44 replicates. We assessed the repeatability of the first and the second counts of both the total and the GFP(+) sperm by computing the intraclass-correlation coefficients (Lessells and Boag 1987). Notice that because the recipients that did not store sperm may overestimate the repeatability, we assessed the repeatability twice, including and excluding the recipient without sperm in storage.

Results. We found that counts of both the total number of sperm ($r_i = 0.91$, $F_{43,44} = 22.0$, $P < 0.001$) and the GFP(+) sperm ($r_i = 0.91$, $F_{43,44} = 31.1$, $P < 0.001$) are highly repeatable. This persists also after excluding recipients that did not have sperm in storage (total sperm, $r_i = 0.82$, $F_{37,38} = 10.5$, $P < 0.001$; GFP(+) sperm, $r_i = 0.79$, $F_{28,29} = 8.4$, $P < 0.001$).

2.2. Sperm Phenotyping - Bright Field vs. Epifluorescence Counts

Experimental setup. To test if we assess the same number of GFP(+) sperm under bright field and epifluorescence illumination, we counted sperm under both illumination techniques in recipients that only received GFP(+) sperm. In details, we raised same-age individuals in GFP(+)×GFP(-) pairs. We then sampled the GFP(-) individual to realise movies of the antrum to assess the number of sperm under bright field and epifluorescence illumination. Hence, for each GFP(-) recipient, we obtained two sperm counts, which are expected to match because the received sperm comes exclusively from a GFP(+) donor.

Statistics. The final sample size was 57 replicates. We first tested if the numbers of sperm assessed under both illumination techniques correlate with each other, by using a Spearman's correlation. Moreover, we tested whether we counted the same values in both illumination techniques by using the Wilcoxon signed-rank test. Notice that individuals that did not receive sperm (i.e., $N = 12$) may bias these two tests, so we performed them twice, by including and excluding the worms without received sperm.

Results. Even though the number of sperm assessed under bright field illumination was highly correlated with the corresponding number assessed under epifluorescence illumination (Spearman's correlation, $r_S = 0.81$, $P < 0.001$), we counted more sperm under bright field illumination than under epifluorescence illumination (Wilcoxon signed-rank test, $S = -310.5$, $P < 0.001$) (Figure 6). These patterns remain when recipients that did not store sperm were excluded, (Spearman's correlation, $r_S = 0.67$, $P < 0.001$; Wilcoxon signed-rank test, $S = -310.5$, $P < 0.001$). This discrepancy may be due to an overestimation of the number of sperm under bright field illumination and/or an underestimation of the number of sperm under epifluorescence illumination.

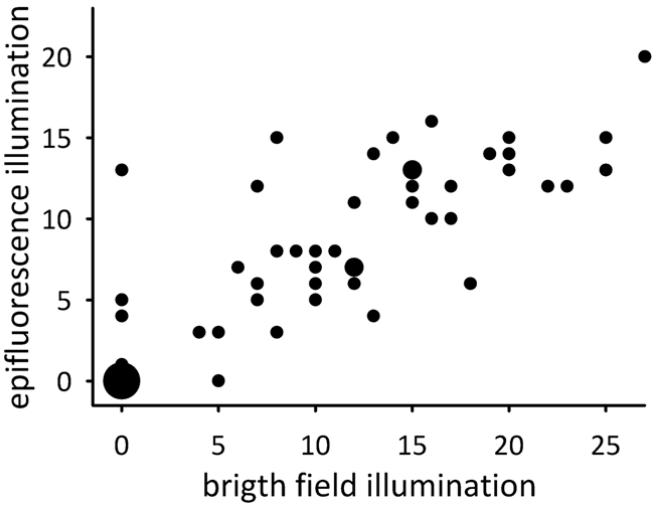

Figure 6. Scatter plot of the number of GFP(+) sperm assessed in a live GFP(-) recipient, under bright field and epifluorescence illumination. Small size dots represent individual replicates, intermediate size dots (n=2) represents two overlapping replicates, and the big size dot represent 12 overlapping replicates. See text for statistics.

2.3. Sperm Phenotyping - Sperm Ageing

Experimental setup. To investigate whether the GFP signal in the sperm is stable over time, we compared the strength of the GFP signal of young and old sperm. For sperm age manipulation, we raised individuals either in groups or in isolation, thus controlling whether the produced sperm is spent for copulation or accumulated in the seminal vesicle for several days. In details, we raised same-age individuals in 3 treatments, GFP(-) individual in isolation (hereafter called control), GFP(+) individual in isolation (hereafter called old sperm), and GFP(+) individual in octets (hereafter called young sperm). Then, we prepared sperm cells for observation, following a standard protocol (see Janicke et al. 2011 for detailed protocol), which allows assessing the GFP signal of a single sperm cell. For each individual, we realised movies of single sperm cell under epifluorescence illumination, and repeated this for 14.3 ± 0.2 sperm (mean $\pm$ SE). Then, based on these movies, we scored the strength of the GFP signal of each sperm using an ordinal scale with four categories: 0 (no GFP signal), 1 (weak GFP signal), 2 (intermediate GFP signal) and 3 (strong GFP signal).

Statistics. The sample size was 20 individuals per treatment. For each individual, we averaged the scores obtained from the different sperm. As all controls were successfully scored as 0, we compared only the GFP signal from the old GFP(+) sperm and the young GFP(+) sperm by using a *t*-test.

Results. As expected, all GFP(-) control individuals produced sperm that were all scored as no GFP signal. However, we found an age effect on the strength of the GFP sperm signal, the old sperm had a lower GFP signal than the young sperm ($t_{33} = -3.78$, $P < 0.001$). Some, but few, old GFP(+) sperm were

scored as no GFP signal (3 ± 1%, mean ± SE). Hence sperm ageing might affect the strength of the GFP signal in sperm.

Mendelian Segregation

Demircan et al (in prep) suggests that the GFP expression in *M. lignano* is dominant and that the GFP transgene was integrated at a single locus. This is indicated by the inheritance pattern observed during the first two generations following the transgene integration, as well as by the inverse PCR. Therefore, given this information, we expected the inheritance of the GFP marker to follow a Mendelian segregation assuming a single dominant locus.

Experimental setup. To test the inheritance of the GFP marker, we assessed its segregation in offspring of crosses composed of known genotypes. First, we used pairs of GFP(+)×GFP(+), GFP(+)×GFP(-), and GFP(-)×GFP(-), to create offspring that were respectively GFP(+) individuals, individuals presumably heterozygous at the GFP locus (hereafter called *het*), and GFP(-) individuals. Second, we created pairs in five treatment groups: GFP(+)×GFP(+), GFP(+)×GFP(-) *het*×*het*, *het*×GFP(-), and GFP(-)×GFP(-) in which we assessed the GFP status [i.e., GFP(+) or GFP(-)] of their offspring blind with respect to the treatment.

Statistics. The sample size was 8 GFP(+)×GFP(+), 9 GFP(+)×GFP(-), 9 *het*×*het*, 12 *het*×GFP(-), and 13 GFP(-)×GFP(-). The offspring production was on average 35.9 offspring ± 1.7 (± SE) per pair. To test if the GFP marker shows a Mendelian segregation assuming a dominant marker encoded by a single locus, we compared the proportion of GFP(+) offspring produced against the expected value within each treatment, by using Wilcoxon signed-rank tests.

Results. The GFP status of the offspring produced in the different crosses did not significantly differ from the expected values assuming a dominant GFP marker encoded by a single locus (Wilcoxon signed-rank test, all $P > 0.1$). However, although the proportion of GFP(+) individuals did not significantly deviates from 1 in the GFP(+)×GFP(-) treatment (Wilcoxon signed-rank test, $S = -3.0$, $N = 9$, $P = 0.25$), we found 2 pairs that produced 1 GFP(-) offspring out of 32 and 39 in total, and 1 pair produced 7 GFP(-) offspring out of 13 in total.

Sperm Displacement

Experimental setup. We took advantage of the GFP techniques to test whether the sperm stored in the antrum can be displaced by subsequent mating partners. For this we assessed twice the number of GFP(+) sperm in a recipient, before and after a second GFP(-) donor, which we then compared to a control treatment without second sperm donor. In details, we raised same-age individuals in pairs of GFP(+)×GFP(-) and GFP(-)×GFP(-). First, we sampled the focal sperm recipient [i.e., the GFP(-) individual from the

GFP(+)×GFP(-)], and assessed the number of total and GFP(+) sperm received in its antrum. Second, we released the focal sperm recipient, either in isolation (i.e., called control treatment), or with a coloured GFP(-) individual sampled from the GFP(-)×GFP(-) pairs (i.e., called competition treatment). Third, one day afterwards, we sampled the focal recipient and assessed a second time the number of total and GFP(+) sperm received in its antrum, with GFP(+) sperm corresponding to the first donor sperm. Hence, we assessed the number of sperm received from a first donor before and after the presence of a second sperm donor (i.e., competition treatment), that we can compare to a control treatment without second sperm donor.

Statistics. The final sample size was 13 replicates in the competition treatment, and 18 replicates in the control treatment. We statistically tested for sperm displacement by examining if the number of first donor sperm decreased more rapidly in the competition treatment than in the control treatment. For this, we used repeated-measures ANOVAs with the number of sperm (i.e., either total sperm or first donor sperm) as a response variable, and used the time (i.e., first or second observation time-point), the treatment (i.e., competition or control), and the interaction time × treatment as factors.

Results. We found unambiguous evidence for sperm displacement in *Macrostomum lignano* (Figure 7). First, the total number of sperm was stable between the two observation time-points (repeated-measures ANOVA, $F_{1,29} = 0.05$, $N = 31$, $P = 0.83$), and did not differ between the treatments (repeated-measures ANOVA, $F_{1,29} = 1.22$, $N = 31$, $P = 0.28$), but we found a significant effect of the interaction time × treatment (repeated-measures ANOVA, $F_{1,29} = 6.36$, $N = 31$, $P = 0.018$). This interaction seemed driven by the decrease of the total sperm in the control treatment ($t_{17} = -2.79$, $N = 18$, $P = 0.013$), which is likely due to sperm usage and/or sperm loss, while the total sperm was stable in the competition treatment ($t_{12} = 1.33$, $N = 13$, $P = 0.21$).

Second, in the number of first donor sperm, we found a significant time effect (repeated-measures ANOVA, $F_{1,29} = 16.32$, $N = 31$, $P < 0.001$), and no treatment effect (repeated-measures ANOVA, $F_{1,29} = 1.15$, $N = 31$, $P = 0.29$). Interestingly, we found that the number of first donor sperm was significantly influenced by a time × treatment interaction (repeated-measures ANOVA, $F_{1,29} = 9.12$, $N = 31$, $P = 0.005$). This interaction was driven by a steeper decrease of the number of sperm in the competition treatment (Wilcoxon signed-rank test, $S = -40.5$, $N = 13$, $P = 0.002$), which was not significant in the control treatment (Wilcoxon signed-rank test, $S = -15.5$, $N = 18$, $P = 0.44$).

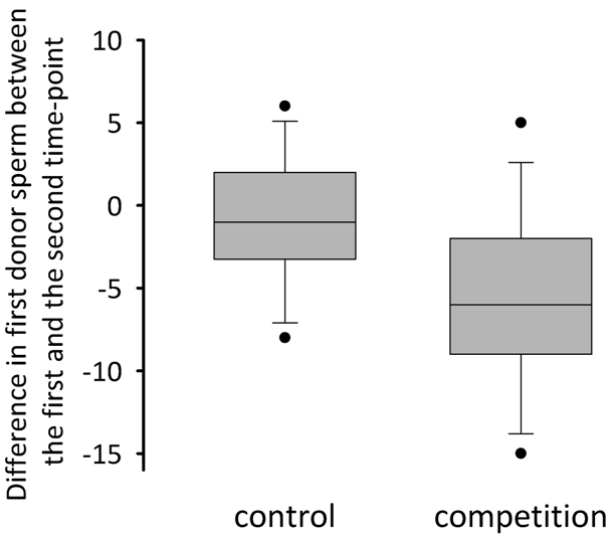

Figure 7. Sperm displacement in *M. lignano*. Comparisons of the loss of first donor sperm in a competition treatment (i.e., recipient copulated with a second donor) and in a control treatment (i.e., no second donor). The loss of first donor sperm is calculated as the difference between the sperm counted at the second observation time-point and those counted at the first observation time-point. Hence negative values indicate sperm loss. The boxes show the 25th percentile, the median and the 75th percentile. The whiskers show the 10th and the 90th percentile, and the dots show the outliers. See text for statistics.

Discussion

The results suggest that expressing GFP does not affect any of the measured traits, as shown by the comparisons between GFP(+) and GFP(-) lines. Moreover, the GFP signal is a powerful and reliable tool for the study of sexual selection, as sperm donors, their sperm and their sired offspring can easily be identified *in vivo*. The GFP marker seem to have Mendelian inheritance with a single and dominant locus, but further studies should determine if the few deviations found in our results are due to a different genetic model. Finally, we show a biological application of the GFP techniques, which unambiguously demonstrates the presence of sperm displacement in *M. lignano*.

GFP techniques: Reliability, Limitations & Using Recommendations

Overall, the tests performed show that the GFP(+) lines can be reliably used. First, the GFP(+) line had similar morphological traits, mating rate and reproductive success than the DV1 line. Thus, the integration of the transgene as well as its expression does not seem to reduce the performance of the GFP(+) worms. Second, GFP expression provides a reliable tool for experimental reproductive biology: GFP(+) individuals are visually distinguishable from the GFP(-) individuals. Moreover, the high repeatability of GFP(+) sperm counts in live recipients provides a powerful tool for studying sperm competition. Although, we found a discrepancy in the number of received sperm under bright field and epifluorescence illumination, we think

this may be due to sperm ageing, as suggested by the small percentage of sperm without GFP signal in virgin GFP(+) worms (which had accumulated sperm over several days), and it can thus be easily circumvented with an appropriate experimental design. Therefore we recommend to carefully use the GFP(+) line when used for tracking GFP(+) sperm in recipients, and to avoid the use of virgin or sexually isolated GFP(+) sperm donors.

The inheritance pattern of the GFP marker seems to follow the Mendelian segregation assuming a single dominant locus, as shown in the proportion of GFP(+) offspring from different line crosses. In a few cases we observed proportions that did not fit the Mendel's laws. Particularly, 3 out of 9 GFP(+)×GFP(-) pairs produced at least 1 GFP(-) offspring, where we would have expected none. Such rare events could be due to a phenotypic loss of expression (for instance due to silencing of the GFP marker, or to developmental problems), the HUB1 parents not being homozygous as expected, or to a different genetic model (for instance having more than one GFP locus, or a biased segregation). These possible scenarios can be disentangled with additional molecular analyses and multi-generational large scale crosses. But since the Mendelian segregation of single GFP(+) individuals can be tested by pairing it with a virgin wild type individual and assessing the GFP status of the offspring in a progeny array, we suggest following this procedure in experiments that need exact paternity share measures.

Sperm Displacement

The unique opportunity to repeatedly quantify the contribution of a sperm donor to a pool of received sperm yielded new insights on the reproductive biology of *Macrostomum lignano*: individuals displace sperm from previous donors and replace it with their own. The mechanism by which sperm is displaced in *M. lignano* is, at present, not known. Given that the antrum seems to be a limited-volume organ, we hypothesize that copulation may create a pressure in the antrum (e.g., by the copulatory organ and/or by the transferred sperm), displacing some stored sperm. If this is the case, morphological traits of the copulatory organ and the sperm may confer selective advantages in sperm offense and/or sperm defence. Interestingly, the shape of the male copulatory organ has been shown to be correlated with sperm-transfer success (Janicke and Schärer 2009a). Moreover, the complex shape of the sperm cells in *M. lignano*, including stiff lateral bristles, has been hypothesized to be involved in preventing the sperm to be displaced (Vizoso et al. 2010; Schärer et al. 2011).

Sperm displacement may be an underlying mechanism for last male sperm precedence, i.e., the last male to copulate with a sperm recipient has larger paternity share than expected under a fair raffle scenario (Parker 1970, 1998; Parker and Pizzari 2010). In *M. lignano*, recent evidence shows that, indeed, a second mating partner sires more offspring than a first mating partner (Sandner et al. in prep). When sperm displacement and last male sperm

precedence occur, increasing the likelihood to be last sperm donor of a mating partner can be achieved by increasing mating rate. Therefore we expect that, in general, sperm displacement may fuel the evolution of high mating rate. Interestingly, *M. lignano* has a very high mating rate, which has been hypothesised to be an evolutionary response to sperm displacement (Sandner et al. in prep).

Usually, sperm displacement is measured in double mating experiment in which a sperm recipient copulate once with a first sperm a donor and then, once with a second sperm donor (e.g., Simmons et al. 1999; Hall et al. 2010; Manier et al. 2010). Our experimental design differs because the focal sperm recipients most likely copulated several times with both the first and the second sperm donor and, as a consequence, it does not allow to quantify the number of sperm displaced per copulation.

Conclusion

The tests performed indicate that the GFP(+) lines in *Macrostomum lignano* may is a reliable tool when used under certain conditions. This technique permits non-invasive quantification of the contribution of a sperm donor within a pool of sperm in a recipient *in vivo*. We argue that the availability of reliable GFP techniques in a transparent organism is a powerful tool, and represents an opportunity to reveal new insights in sexual selection.

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Chapter IV

Quantifying Episodes of Sexual Selection: Insights from a Transparent Worm with Fluorescent Sperm

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Marie-Orleach, L., T. Janicke, D.B. Vizoso, P. David, and L. Schärer. in prep. Quantifying episodes of sexual selection: insights from a transparent worm with fluorescent sperm

Abstract

Sexual selection operates through consecutive episodes of selection that may ultimately contribute to the observed variation in reproductive success between individuals. Understanding the relative importance of these episodes is challenging, especially because the relevant post-copulatory processes occur internally and are therefore difficult to observe. Here we investigate consecutive pre- and post-copulatory episodes of sexual selection of the male sex function by assessing how mating success (i.e., copulations achieved), sperm-transfer efficiency (i.e., sperm stored per copulation), and sperm-fertilising efficiency (i.e., paternity per stored sperm) contribute to the resulting reproductive success of focal individuals kept in groups. Specifically, we used a transgenic line of the transparent hermaphroditic flatworm, *Macrostomum lignano*, which expresses green fluorescent protein (GFP) in all cell types, including sperm cells, thus enabling *in vivo* sperm tracking and paternity analysis. We found that most of the variance observed in male reproductive success arose from the two fitness components, namely sperm-transfer efficiency (24%) and sperm-fertilising efficiency (53%), while mating success accounted for only 6% of the variance. Next we investigated selection differentials for a whole suite of morphological traits (including gonad size, shape of the male copulatory organ and sperm morphology) to identify the episodes during which sexual selection on these traits operates. Testis size showed a positive selection differential, which was mainly due to a higher sperm-transfer efficiency of individuals with bigger testes. These results demonstrate that male reproductive success was not primarily limited by the number of matings but, instead, by the ability to successfully transfer sperm and to fertilise the partner's ova, which highlights the importance of post-copulatory episodes of sexual selection in this study system. To our knowledge, this is the first study that simultaneously quantifies the contribution of mating success, sperm-transfer efficiency and sperm-fertilising efficiency to the variance observed in male reproductive success.

Bateman (1948) famously introduced a framework for quantifying the strength of sexual selection, which is based on the linear relationship between mating success and reproductive success (called the "Bateman gradient"; Lande and Arnold 1983; Arnold and Wade 1984; Arnold and Duvall 1994; Jones 2009; Anthes et al. 2010; Kokko et al. 2012). While this view has long dominated our perception of sexual selection as a process arising only from differences in mating success (see e.g., Arnold 1994b), it is now acknowledged that in promiscuous species sexual selection often continues after mating, via sperm competition (Parker 1970, 1998) and/or cryptic female choice (Charnov 1979; Thornhill 1983; Eberhard 1996). Thus post-copulatory sexual selection needs to be integrated when quantifying sexual selection (Eberhard 2009; Birkhead 2010; Jennions and Kokko 2010).

Sexual selection is nowadays often viewed as encompassing consecutive pre- and post-copulatory episodes of selection, all of which can ultimately contribute to the observed variance in reproductive success between individuals (Pizzari et al. 2002; Jones 2009; Anthes et al. 2010). Thus, for a complete understanding of sexual selection, one ideally needs to investigate selection during all of its episodes separately, which then also allows to identify the processes during which sexual selection operates on specific phenotypic traits. The relative importance of the different pre- and post-copulatory episodes of sexual selection is rather poorly understood, in part because it remains challenging to observe and quantify all relevant pre- and post-copulatory processes in the same study system.

A few recent studies have decomposed the variance in male reproductive success along episodes of sexual selection (Collet et al. 2012; Pischedda and Rice 2012; Pélissié et al. under review). For this, the studies assessed the mating success, inferred either from behavioural observations (copulatory mating success, Collet et al. 2012; Pélissié et al. under review) or from paternity analyses (genetic mating success, Pischedda and Rice 2012), and the resulting male reproductive success. Like this, variance in male reproductive success could be partitioned into mating success and paternity share, corresponding to a pre- and a post-copulatory episode of selection, respectively.

Moreover, male success during the post-copulatory episode of selection is itself often considered to be composed of two aspects: (1) the number of sperm successfully transferred and stored per copulation (hereafter called sperm-transfer efficiency; reviewed in Parker 1998), and (2) the efficiency by which each stored sperm cell then leads to a successful fertilisation (hereafter sperm-fertilising efficiency; reviewed in Snook 2005; Pizzari and Parker 2009). These two aspects occur subsequently and presumably rely on different mechanisms so that sperm-transfer efficiency and sperm-fertilising efficiency should ideally be considered as resulting from two distinct episodes of selection. However, it is often challenging to discern between them, because it requires assessing the sperm cells successfully stored in the female reproductive tract *in vivo*. Owing to this difficulty we, to our knowledge, currently lack quantitative studies that

simultaneously consider how mating success, sperm-transfer efficiency, and sperm-fertilising efficiency affect male reproductive success.

Here, we report a study that decomposes sexual selection into these different episodes of selection in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. Individuals of this promiscuous species trade off their resource allocation between the male and the female sex functions (Schärer and Ladurner 2003; Schärer et al. 2005; Janicke and Schärer 2009b), and more male-biased individuals have an increased sperm production rate, which is well reflected by their larger testes (Schärer and Vizoso 2007). The received sperm can be stored for several days in the female sperm storage organ and can further be displaced by subsequent mating partners (Marie-Orleach et al. in prep.). Sperm cells have a relatively complex morphology, which has been argued to be an evolutionary response to sexual conflict over the fate of received sperm and/or sperm competition. In particular, sperm cells harbour appendages expected to help in preventing the sperm to be removed from the female sperm storage organ (Vizoso et al. 2010; Schärer et al. 2011). A recently established transgenic line of this species, which ubiquitously expresses green fluorescent protein (hereafter called GFP) in all cell types, including sperm cells (Demircan et al. in prep.), offers the opportunity to visualise sperm received from a GFP-expressing individual [hereafter GFP(+)] inside the female reproductive tract of a sperm recipient (Janicke et al. 2013).

In the present study we tracked the male reproductive performance of a focal GFP(+) worm in a competitive context (i.e., in a group of 5 individuals) by measuring copulatory activity, the resulting number of sperm cells successfully stored in the partners, and the resulting number of offspring sired. Thus, we could decompose male reproductive success along different episodes of selection based on mating success, sperm-transfer efficiency, and sperm-fertilising efficiency, and compare their relative importance. Moreover, we measured a suite of morphological traits in the focals (including gonad size, male copulatory stylet morphology and sperm morphology) to test their male selection differentials, which we further decomposed in selection differentials on each fitness components. To our knowledge this is the first quantitative study to assess the relative contribution of mating success, sperm-transfer efficiency and sperm-fertilising efficiency to male reproductive success.

Materials and Methods

Model Organism

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) inhabits the intertidal zone of the Northern Adriatic Sea (Ladurner et al. 2005b). Laboratory cultures are maintained at 20°C in glass Petri dishes with *f*/2 medium (Andersen et al. 2005) and are fed with the diatom *Nitzschia curvilineata*. *M. lignano* is an outcrossing and promiscuous simultaneous hermaphrodite (Schärer and Ladurner 2003; Janicke and Schärer

2009a). Copulations are frequent (about 6 copulations per hour) and consist of the reciprocal insertion of the male copulatory organ (hereafter called stylet) into the female copulatory organ (hereafter called antrum) of the partner (Schärer et al. 2004a). Received sperm is stored in the antrum and can be displaced by subsequent mating partners (Marie-Orleach et al. in prep.). Worms are transparent, which allows us to perform a range of non-lethal measurements *in vivo*, such as gonad size (Schärer and Ladurner 2003), stylet morphology (Janicke and Schärer 2009a) and sperm morphology (Janicke and Schärer 2010). Worms have been shown to adjust their sex allocation in response to the social conditions, being more male-biased (i.e., having larger testis and smaller ovaries) in larger social groups (Schärer and Ladurner 2003; Schärer et al. 2005; Janicke and Schärer 2009b). In addition, the transparency of the worm allows *in vivo* visualisation and reliable counting of the number of received sperm in storage, inside the antrum (Janicke et al. 2011).

Green Fluorescent Protein Techniques

This study requires to discriminate competing sperm of different donors inside recipients *in vivo*, and to assess the resulting paternity. This can be achieved in *M. lignano* using recently established transgenic lines that express a green fluorescent protein (GFP) (Janicke et al. 2013; Demircan et al. in prep). The GFP is ubiquitously expressed in all cell types, including the sperm cells, meaning that the sperm of a GFP(+) individual show a GFP(+) signal when observed under epifluorescence illumination. This allows us to quantify the proportion of GFP(+) sperm in a pool of received sperm cells in a multiply mated recipient *in vivo* (Janicke et al. 2013; Marie-Orleach et al. in prep.). Moreover, the GFP marker is transmitted to the offspring, so that the paternity success of the GFP(+) individual can be measured efficiently. However, preliminary studies indicate that the inheritance pattern of the GFP marker deviated somewhat from Mendel's law assuming a single dominant and homozygous locus (see Marie-Orleach et al. in prep. for more details). Therefore, we needed to account for the proportion at which each GFP(+) individual transmitted the GFP marker to its offspring (hereafter called penetrance), in order to obtain accurate estimates of the paternity success of a given individual (see below for details). Offspring production, mating rate and morphology have been shown to not differ between GFP(+) and GFP(-) individuals (Marie-Orleach et al. in prep.).

Culture Lines

Individuals used in this study are from two outbred lines, a GFP(+) and a GFP(-) line. The GFP(-) line (called LS1) is a culture that was established in 2003, based on individuals sampled in Northern Adriatic Sea, and since then maintained in the laboratory in a meta-population structure to maintain genetic diversity (Marie-Orleach et al. 2013).

The GFP(+) outcrossed culture, hereafter called BAS1, was established by backcrossing HUB1, a GFP(+) transgenic line (Janicke et al. 2013), to a genetically diverse laboratory culture kept in 12 sub-populations (LS1, Marie-Orleach et al. 2013). In order to preserve genetic diversity, 20 pairs were generated for each sub-population by crossing one randomly chosen LS1 worm with a HUB1 worm. During 9 generations, one GFP(+) offspring per family was paired with a randomly chosen worm from the same sub-population. If a pair produced no GFP(+) offspring (about 7.4% throughout), an extra offspring from another pair (of the same sub-population) was used, to maintain the number of pairs.

To produce GFP-homozygotes, GFP(+) offspring of the 9th generation (hereafter F0) were crossed to produce 20 pairs per population (240 in total), avoiding the pairing of known relatives to reduce loss of diversity. Six GFP(+) offspring (hereafter F1) from each F0 pair were then mated to a wild type worm and their offspring scored for GFP expression in order to assess their genetic status. F1 worms that produced purely green offspring (from a minimum of 16) were defined as homozygous and used to start the BAS1 sub-populations after a three-week purging of received sperm.

Experimental Set-Up

In this study, we aimed (1) to quantify the relative contributions of several episodes of sexual selection to the variance of male reproductive success, and (2) to identify the episodes during which selection on traits operates. For this, we used individuals that had reached a steady state and we tested them in a competitive context to assess selection operating in conditions assumed to be biologically relevant (e.g., a balance between sperm and egg production). Therefore each focal individual was assigned to two groups during the entire experiment, a group used to raise and maintain the focal in a steady state (hereafter called A groups), and a group to test the reproductive performances of the focal (hereafter called B groups).

The experiment consisted of three phases, the first two of which we only report for completeness, as they did not allow us to collect the expected data. Namely, we aimed to discriminate male and female reproductive success while being grouped, based on an expected delay in GFP expression of the paternally inherited GFP allele during egg development compared to the maternally inherited GFP allele. In contrast, in phase 3, we could successfully discriminate male and female reproductive success by isolating all group members.

Preparation and Phases 1 and 2

On day 1, we sampled adult GFP(+) and GFP(-) individuals from the mass cultures and distributed them for egg laying onto glass Petri dishes filled with f/2 medium and *ad libitum* algae (500 GFP(+) and 1 000 GFP(-) individuals to 5 and 10 Petri dishes, respectively). On day 3, we removed all adults, thus limiting the age differences of the resulting juveniles to 48 h. On day 9, we

sampled the juveniles to create 72 A groups comprising 1 GFP(+) and 4 GFP(-) individuals, and 72 B groups comprising just 4 GFP(-) individuals. Groups were placed in wells of 24-well tissue culture plates (TPP AG, Switzerland) filled with 1.5 mL of 32‰ artificial sea water (ASW), and maintained under specific food conditions (adjusted per capita and day by counting diatoms using a Neubauer-improved counting chamber, Marienfeld GmbH, Germany). To distribute the workload, the experiment was split in four batches, each including one quarter of the replicates. For sake of clarity, we only report the days on which the first batch was processed (the three other batches were always processed on the three subsequent days).

Phase 1. We initially provided 2 000 diatoms per capita and day, aimed at facilitating the emergence of trade-offs (cf. Schärer et al. 2005). On days 15, 22, and 29, we transferred the worms in fresh wells. On day 34, we transferred all GFP(+) individuals from their A group to their B group. The B groups were placed in wells of 60-well micro-test plates (Greiner Bio-One, Germany) and moved every day to new wells, while the remaining A groups were maintained in wells of 24-well plates as previously. On day 41, we transferred the GFP(+) individuals back into their A group, and all groups were again held in wells of 24-well plates. We observed a low offspring production (1.37 ± 0.06 offspring per group per day, average ± SE) in the 60-well plates, presumably due to food restriction.

Phase 2. We increased the algae quantity to 6 000 diatoms per capita per day, which we maintained until the end of the experiment. From day 49 to 59, i.e., after we expected the worms to have recovered from the restricted food treatment, we repeated the treatment as in *Phase 1*. For this, on day 49 we transferred the GFP(+) individuals to their B groups. On day 59, we removed all individuals and transferred the GFP(+) individuals to their A groups. We then transferred all groups to fresh wells on day 64.

Phase 3.1: Estimation of the Mating Success

We examined the mating success of each GFP(+) individual with its partners in its respective B group. For this we transferred on day 69 all A groups into fresh wells containing the food colour Patent blue V (also called E-131; Werner Schweizer AG, Switzerland; 0.25 mg/mL of ASW). A 24 h exposure allows to visually distinguish coloured from non-coloured worms, and does not affect the mating rate or the offspring production (Marie-Orleach et al. 2013). The following day we placed the now blue GFP(+) worm into its B group in observation chambers. These were built by placing groups, five per chamber, into 8 µL drops of ASW in between two siliconized microscope slides separated by 210 µm (Schärer et al. 2004a). We then filmed the mating interactions under transmitted light for 3 h, at 1 frame/s, using digital cameras (DFK 41AF02, The Imaging Source Europe GmbH) and the software BTV Pro 6.0b7 (http://www.bensoftware.com/). We used KMPlayer version 1.5.1 (http://kmplayer.com) to analyse each movie frame by frame, permitting us to

estimate the total number of copulations and the proportion of copulations performed by the GFP(+) focal.

Phase 3.2: Estimation of the Sperm-Transfer Success

Immediately after the mating trial, we assessed the number of sperm in the antrum of all individuals (Janicke et al. 2011). For this we relaxed each individual by a 10 min exposure to a 5:3 solution of 7.14% $MgCl_2$ and ASW. Then, we gently squeezed the relaxed worm between two cover slips separated with plasticine, and placed the preparation onto a microscope slide (Janicke et al. 2011). We then recorded a first movie of the antrum by slowly focusing through it at a magnification of 630x under bright field illumination, visualising the total number of sperm in storage. For the GFP(-) individuals, we recorded a second antrum movie, this time under epifluorescence illumination, to visualise only the number of GFP(+) sperm, i.e., the number of sperm that were successfully transferred by the focal GFP(+) individual. We used a Leica DM2500 microscope (Leica Microsystems) equipped with an epifluorescence light source and a digital camera (Leica DFC360 FX, Leica Microsystems). Movies were recorded using the Leica Application Suite 4.1.0 (Leica Microsystems). We analysed antrum movies using KMPlayer and counted for each antrum the total number of sperm (inferred from movies under bright field illumination) and GFP(+) sperm (inferred from movies under epifluorescence illumination). Thereby, we could assess the number and proportion of sperm that the focal GFP(+) individual successfully transferred to its potential partners (hereafter called sperm-transfer success). Counts of both total and GFP(+) sperm cells show high repeatability (Janicke et al. 2011; Marie-Orleach et al. in prep.). However, we encountered some worms in which we could only assess the number of GFP(+) sperm cells due to the presence of a ripe egg in the antrum (N = 57 out of 208 in total), which prevents reliable counts of the total number of sperm cells. For these individuals, we used the average number of total sperm cells computed from the counts of the other GFP(-) individuals.

Phase 3.3: Estimation of the Morphological Traits

Just after the acquisition of the antrum movies, we took digital micrographs of each focal GFP(+) individual to characterize a range of morphological traits (Schärer and Ladurner 2003; Janicke and Schärer 2009a). Briefly, we took digital micrographs of the body at 40x and of the testis, ovary, seminal vesicle and stylet at a 400x magnification using a microscope Leica DM2500 (Leica Microsystems) connected to a digital camera (DFK 41AF02, The Imaging Source Europe GmbH). The digital micrographs were taken using BTV Pro 6.0b7 and analysed with ImageJ 1.45s (http://rsb.info.nih.gov/ij/), yielding estimates of body size, testis size, ovary size and seminal vesicle size. In addition, we used geometric morphometrics (Zelditch et al. 2004) to assess the stylet's centroid size, and stylet shape based on the first three relative warp scores RWS (see Janicke and Schärer 2009a for the detailed protocol). RWS1,

RWS2 and RWS3 capture the general stylet curvature, the width of the stylet and the orientation of the stylet tip, respectively (see Appendix 1 for visualisations). All these measurements show good repeatabilities (Schärer and Ladurner 2003; Janicke and Schärer 2009a).

Phase 3.4: Estimation of the Reproductive Success

Afterwards, we isolated all individuals to lay eggs in wells of 24-well plates filled with 1.5 mL of ASW and algae. We transferred the individuals every day to fresh wells until day 82 (i.e., for a total egg-laying duration of 12 days), and we counted the offspring produced and assessed their GFP status. Thereby, we could assess how many offspring each focal GFP(+) individual produced through its female sex function, and through its male sex function, estimated as the proportion of GFP(+) offspring laid by GFP(-) partners (see below for data on the penetrance of the marker).

Estimation of the Sperm Morphology

On day 82, we characterized the morphology of the sperm of the GFP(+) focals, following Janicke and Schärer (2010). Briefly, we amputated the tail of the worms, which contains the seminal vesicle filled with the own sperm. We squeezed the tail between a microscope slide and a cover slip so that the sperm spread over the slide, and took pictures of about 10 sperm per individual (using the standard imaging setup). We determined 3 sperm traits, namely the total sperm length, sperm body length, and bristles length, all of which are repeatable (Janicke and Schärer 2010).

Estimation of the Penetrance of the GFP marker

Next, we let the amputated GFP(+) individuals regenerate their tails in isolation, which usually takes less than one week (Egger et al. 2006) and then estimated the penetrance of the GFP marker. For this, we paired the GFP(+) individuals with virgin GFP(-) individuals (LS1 line, Marie-Orleach et al. 2013) in wells of 24-well plates. We regularly transferred them to fresh wells and counted the GFP(+) and GFP(-) offspring produced by each pair for up to 50 offspring per pair. Thus we could correct the number of GFP(+) offspring for each focal GFP(+) individual, by dividing the number of GFP(+) offspring by the penetrance.

Data Analysis

We started with a sample size of 72 independent replicates, but lost 7 due to handling errors, 6 due to developmental problems, and 7 because they did not produce enough offspring (i.e., < 10) to reliably assess the penetrance of the GFP marker. Therefore, the final sample size was reduced to 52 replicates.

Male Gradients

In an initial analysis we investigated the linear relationship between mating success and paternity share, which we further decomposed as the linear

relationships between mating success and sperm-transfer success, and sperm-transfer success and paternity share. For this we first divided the estimates obtained for the focal by the sums of the respective group, yielding measures of mating success (MS, i.e., the focal's copulations divided by the total copulations in the group), sperm-transfer success (STS, i.e., the focal's sperm divided by the total sperm in the four partners) and paternity share (PS, i.e., the focal's offspring divided by the total offspring of the four partners). Next, we divided these proportions by the overall means, to obtain relative measures denoted as MS*, STS* and PS* (Jones 2009), in which asterisks stand for relative data (cf. Pélissié et al. under review). Finally, we performed ordinary least squares linear regressions of PS* on MS*, STS* on MS*, and PS* on STS*.

Decomposition of the Variance in Male Reproductive Success

We decomposed the variance observed in male reproductive success (hereafter called mRS) along subsequent fitness components (Arnold and Wade 1984; Collet et al. 2012; Pélissié et al. under review), by using two different deterministic models. Model 1 includes three fitness components, while model 2 includes four fitness components.

Model 1 is a decomposition of the variance in mRS along three fitness components: partner fecundity (F), mating success (MS), and post-mating success (PMS, defined as PS/MS).

$$V(mRS^*) \approx V(F^*) + V(MS^*) + V(PMS^*) + covariances$$

in which (model 1)

$$covariances = COV(F^*, MS^*) + 2COV(F^*, PMS^*) + 2COV(MS^*, PMS^*)$$

Model 2 integrates the number of sperm cells successfully stored in the partners (i.e., sperm-transfer success, STS), which allows decomposing the post-mating success into the sperm-transfer efficiency (STE, defined as STS/MS) and sperm-fertilising efficiency (SFE, defined as PS/STS). Thus:

$$V(mRS^*) \approx V(F^*) + V(MS^*) + V(STE^*) + V(SFE^*) + covariances$$

in which (model 2)

$$covariances = 2COV(F^*, MS^*) + 2COV(F^*, STE^*) + 2COV(F^*, SFE^*) + 2COV(MS^*, STE^*) + 2COV(MS^*, SFE^*) + 2COV(STE^*, SFE^*)$$

For each variance and covariance, we computed the 95% confidence interval by bootstrapping (10 000 iterations). Importantly, a part of the variances observed in PMS*, SFE* and SFE* was due to Binomial sampling errors. To account for this, we computed the variance due to Binomial sampling error

(see Appendix 2), which we further subtracted from the observed variance of their respective fitness components.

We tested for significant differences between variances by using pairwise signed difference test. Specifically, we bootstrapped the variances observed in each fitness component (10 000 iterations), and calculated the differences between the bootstrapped variances of 2 fitness components for each iteration. We then counted the occurrences of positive and negative differences, and used the less frequent occurrence to derive the P value of the pairwise comparison as: $P = (2 \times occurrence) / 10\,000$.

Finally, to test whether the covariances between two fitness components differed from zero, we performed Spearman's correlation tests.

Selection on Traits

For each measured trait, we computed the total male selection differential (i.e., effect on mRS), as well as the selection differential on each fitness component. For this, we first statistically standardized the individual values for all morphological traits so that their means equal 0 and their standard deviations equal 1 (Jones 2009). Then we performed generalized linear models to test the effects of a trait on mRS, F, MS, PMS, STE and SFE. See Appendix 3 for details.

The linear regressions and the GLMs were carried out in JMP 10.0.1 (SAS Institute Inc., Cary, NC, U.S.A.), and the statistical analyses involved in the variance decomposition were carried out in Mathematica 5.1 (Wolfram Research, Inc.).

Results

Male Gradients

We found a positive relationship between mating success and paternity share ($\beta = 1.06 \pm 0.40$, $R^2 = 0.12$, $F_{1,49} = 6.96$, $P = 0.011$; Figure 1a), suggesting that individuals that copulated relatively more sired relatively more offspring. The decomposition of this relationship showed that individuals that copulated relatively more stored relatively more sperm in their partners ($\beta = 0.68 \pm 0.26$, $R^2 = 0.12$, $F_{1,50} = 6.68$, $P = 0.013$; Figure 1b), and that individuals that stored relatively more sperm in their partners sired relatively more offspring ($\beta = 0.90 \pm 0.17$, $R^2 = 0.35$, $F_{1,49} = 26.50$, $P < 0.001$; Figure 1c). In contrast, mating success was not related to the proportion of offspring produced through the female function in the group ($\beta = 0.33 \pm 0.42$, $R^2 = 0.01$, $F_{1,50} = 0.62$, $P = 0.436$. However, we do not discuss this result because, unlike for the male function, female reproductive success was the result of the copulations performed during the mating trial but also of the previous copulations performed before the mating trial with other partners.

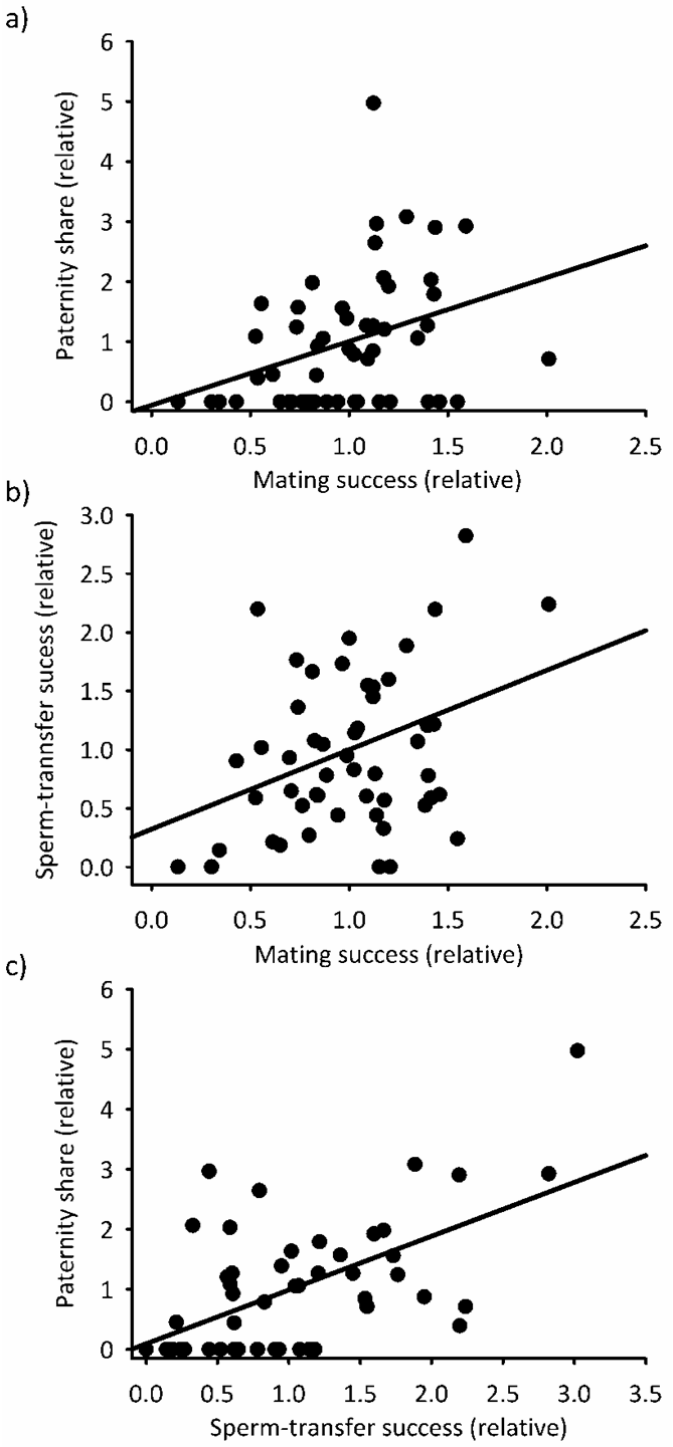

Figure 1. Linear regressions of (a) paternity share on mating success, (b) sperm-transfer success on mating success, and (c) paternity share on sperm-transfer success. All the data shown are relative (Jones 2009). See the results for statistics.

Decomposition of the Variance in Male Reproductive Success

The decomposition according to model 1 indicated that partner fecundity, mating success and post-mating success accounted for 16%, 9%, and 46% of the variance observed in male reproductive success, respectively (Figure 2a). Importantly, the variance arising from post-mating success was significantly larger than the variance arising from mating success ($P = 0.011$). Moreover, the variance arising from partner fecundity was larger than the variance arising from the variance arising from mating success ($P = 0.028$), and tended be smaller than the variance arising from post-mating success ($P = 0.076$). The remaining 29% of variance was due to the binomial error variance and the covariances between fitness components. Covariances were all not significantly different from zero.

The decomposition according to the model 2 indicated that partner fecundity, mating success, sperm-transfer efficiency and sperm-fertilisation efficiency accounted for 11%, 6%, 24% and 53% of the variance observed in male reproductive success, respectively (Figure 2b). The variance arising from sperm-transfer efficiency tended to be larger than the variance arising from partner fecundity ($P = 0.063$), and was significantly larger than the variance arising from mating success ($P = 0.002$). Moreover, although sperm-fertilising efficiency accounted for the largest portion of variance, it was not significantly different from those arising from the other components. This is probably due to the generally small numbers of stored sperm and offspring produced, which make our estimates of sperm-fertilising efficiency error-prone. The covariances were all not significantly different from zero.

The variance observed in relative male reproductive success was 1.15, while the variances predicted by models 1 and 2 were 1.48 and 2.24, respectively. The discrepancy between V(mRS*) and the variance predicted by the models arises from the skewed distributions of our data, especially in SFE, which would indicate that the model 2 should be interpreted with caution. But, because model 2 showed a similar pattern than model 1 (i.e., large variance arising from the post-copulatory fitness components), we think that model 2 also provides meaningful data.

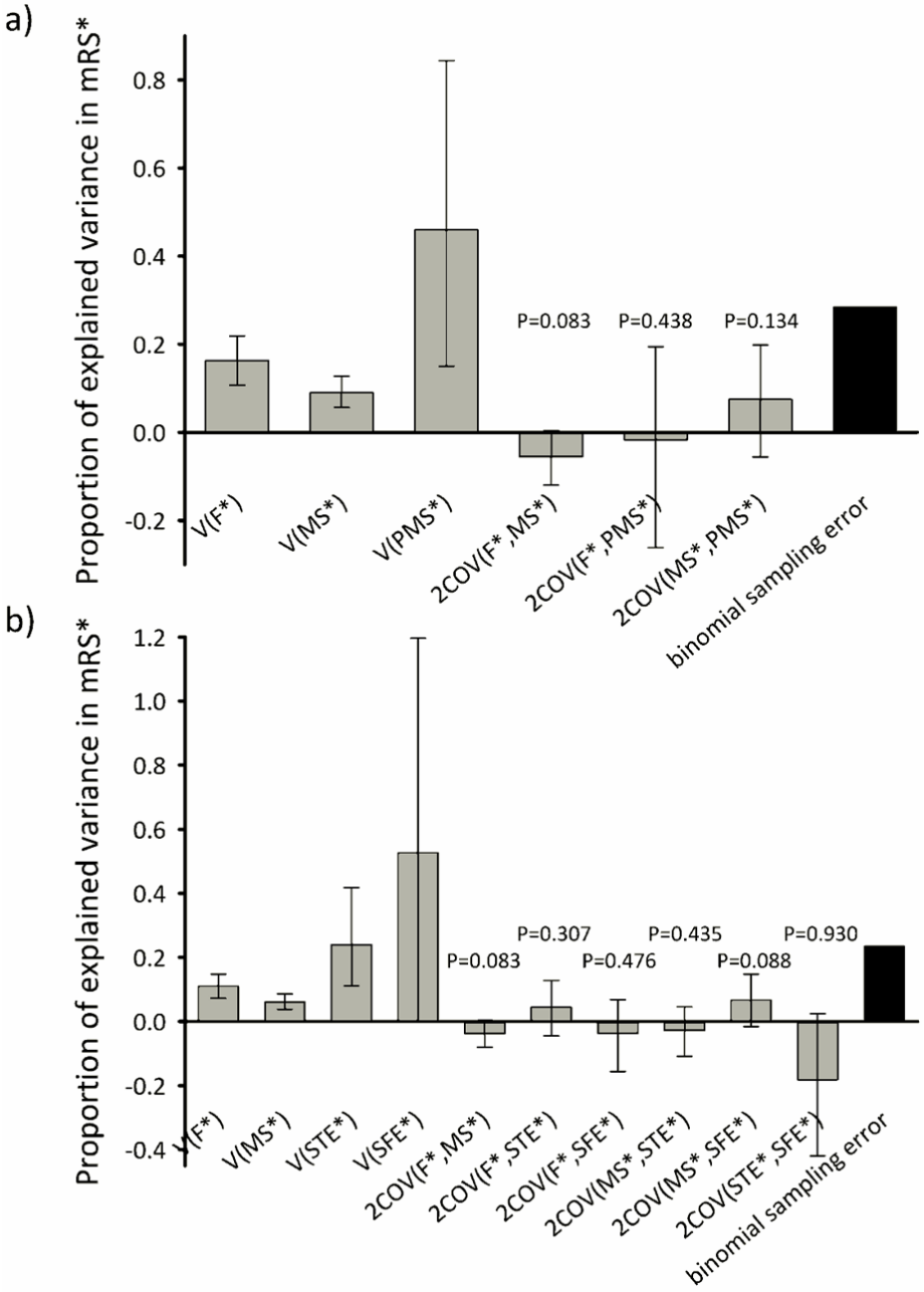

Figure 2. Decomposition of the variance in male reproductive success along different fitness components and their covariances. (a) shows the decomposition following model 1 along three multiplicative fitness components, partner fecundity (F), mating success (MS) and post-mating success (PMS). (b) shows the decomposition following model 2 along four multiplicative fitness components, partner fecundity (F), mating success (MS), sperm-transfer efficiency (STE), and sperm fertilisation efficiency (SFE).
Error bars represent the boostrapped 95% confidence intervals. *P* values test if covariances are different from zero. Asterisks stand for relative data. See the results for statistics.

Selection on Morphological Traits

Testis size showed a positive male selection differential (GLM, estimate ± SE: 0.356 ± 0.142, $\chi^2_1 = 6.09$, $P = 0.014$), which arose from the selection on partner fecundity (GLM, estimate ± SE: 0.132 ± 0.066, $\chi^2_1 = 3.90$, $P = 0.048$) and sperm-transfer efficiency (GLM, estimate ± SE: 0.235 ± 0.084, $\chi^2_1 = 7.60$, $P = 0.006$) (Figure 3). In other words, individuals with bigger testis sired more offspring, presumably because they successfully transferred relatively more sperm cells per copulation and because their partners were more fecund. Moreover, seminal vesicle size showed a nearly significant male selection differential (GLM, estimate ± SE: 0.291 ± 0.148, $\chi^2_1 = 3.76$, $P = 0.053$), which arose from the significant selection on sperm-transfer efficiency (GLM, estimate ± SE: 0.220 ± 0.089, $\chi^2_1 = 6.06$, $P = 0.014$). Finally, stylet centroid size also showed large, but not significant, male selection differentials. Selection on all the measured traits is summarised in Table 1.

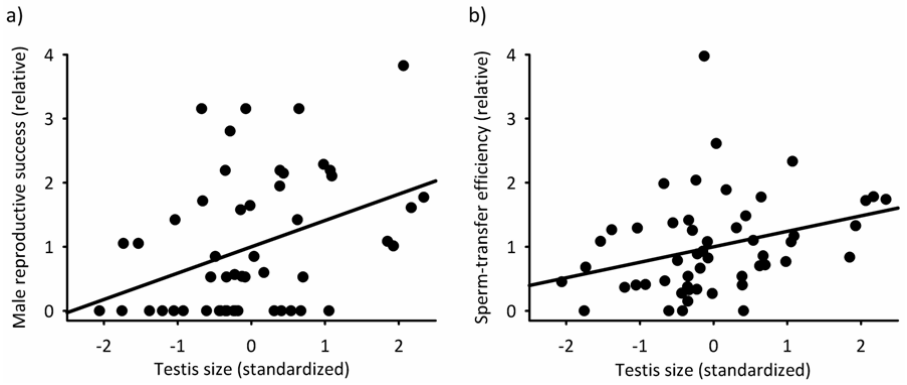

Figure 3. Effects of testis size on (a) male reproductive success, and (b) sperm-transfer efficiency. Testis size is standardized, and male reproductive success and sperm-transfer success are relative (Jones 2009). See the results and Table 1 for statistics.

Discussion

This study provides novel insights on the operation of sexual selection. First, we found that mating success had a positive effect on paternity share and that this relationship was mediated by the number of sperm successfully transferred, as mating success increases sperm-transfer success, which in turn increases paternity share. Second we found, in model 1, that most of the variance observed in male reproductive success arose from post-mating success. Model 2 suggested that the two fitness components sperm-transfer efficiency and sperm-fertilising efficiency accounted for 24% and 53% respectively, while mating success accounted for only 6%. Third, we found that individuals with bigger testis sired more offspring, mainly because they managed to successfully transfer relatively more sperm per copulation. In the following we discuss these three findings in turn.

Table 1. Selection differentials of the 11 measured morphological traits on male reproductive success (mRS), and on the fitness components, partner fecundity (F), mating success (MS), post-mating success (PMS), and its components, sperm-transfer efficiency (STE), and sperm-fertilising efficiency (SFE). Significant *P* values are indicated in bold. See the methods and Appendix C for details.

Traits	mRS	F	MS	PMS	STE	SFE
Body size	0.064 ± 0.151	0.117 ± 0.066	-0.016 ± 0.047	-0.009 ± 0.122	0.083 ± 0.090	-0.030 ± 0.146
Testis size	**0.356 ± 0.142**	**0.132 ± 0.066**	0.023 ± 0.047	0.209 ± 0.118	**0.235 ± 0.084**	0.009 ± 0.136
Ovary size	-0.055 ± 0.157	0.031 ± 0.068	0.013 ± 0.047	-0.090 ± 0.115	-0.068 ± 0.094	-0.004 ± 0.141
Seminal vesicle size	0.291 ± 0.148	0.064 ± 0.068	0.065 ± 0.046	0.115 ± 0.118	**0.220 ± 0.089**	-0.026 ± 0.146
Stylet centroid size	0.264 ± 0.162	0.081 ± 0.067	0.079 ± 0.046	0.058 ± 0.135	0.059 ± 0.091	0.022 ± 0.163
Stylet RWS 1	-0.012 ± 0.153	0.039 ± 0.068	-0.006 ± 0.047	-0.006 ± 0.116	0.100 ± 0.093	-0.034 ± 0.140
Stylet RWS 2	-0.069 ± 0.153	0.012 ± 0.068	0.043 ± 0.047	-0.141 ± 0.105	-0.059 ± 0.093	0.048 ± 0.126
Stylet RWS 3	0.173 ± 0.165	-0.013 ± 0.068	0.003 ± 0.047	0.174 ± 0.124	0.038 ± 0.095	-0.018 ± 0.150
Total sperm length	0.116 ± 0.156	0.000 ± 0.068	0.054 ± 0.048	0.057 ± 0.117	0.087 ± 0.101	-0.079 ± 0.121
Sperm body length	0.071 ± 0.161	0.031 ± 0.068	0.024 ± 0.048	0.017 ± 0.135	-0.012 ± 0.106	-0.020 ± 0.147
Sperm bristle length	0.157 ± 0.166	0.015 ± 0.068	0.081 ± 0.047	0.051 ± 0.131	0.128 ± 0.105	-0.052 ± 0.147

Male Gradients

Our results indicate that individuals that had a higher mating success obtained a higher paternity share. Moreover, we could dissect this relationship by assessing an intermediate step, the sperm-transfer success, which shows that it is what mediates the correlation between mating success and paternity share in *M. lignano*. The reproductive success of a male may be determined by many potential factors. In particular, in internally fertilising animal, the sperm recipient may potentially have control over the fate of the partner's sperm and thus may influence the fertilisation success of some males by preferentially using their sperm. This would make the number of stored sperm an unreliable predictor of paternity share. Our results do not negate the presence of cryptic female choice in *M. lignano*, but it highlights that the number of sperm stored in partners is an important determinant of male reproductive success. Thus, any traits that influence sperm-transfer success are expected to confer a selective advantage. The positive relationship we found between mating success and sperm-transfer success is of particular interest in *M. lignano* as individuals mate more frequently in response to an increased level of sperm competition (Janicke and Schärer 2009b). Hence, together with our findings, this shows that individuals may increase their mating rates to be more competitive in sperm competition, as predicted by theory (Parker 1998).

The applicability of classical Bateman gradients to simultaneous hermaphrodites was first proposed by Charnov (1979), and recently two studies assessing the copulatory mating success (Anthes et al. 2010; Pélissié et al. 2012) indeed showed steeper Bateman gradients in the male than the female sex function, which would suggest that pre-copulatory sexual selection is stronger in the male sex function. These type of data can inform the longstanding debate about the preference for mating in the male or female role (see Charnov 1979; Pongratz and Michiels 2003; Leonard 2005; Anthes et al. 2006; Lorenzi and Sella 2008; Janicke and Schärer 2009a), but at present the empirical data are too scarce to draw a general conclusion. Our study reveals a positive relationship between mating success on paternity share, but it does not allow a proper comparison with the female sex function (see Results). Therefore additional experiments allowing to compare the fitness benefits for the male and the female sex function are needed to determine which function undergoes stronger pre-copulatory sexual selection.

Importantly, the spirit behind the Bateman gradients is to measure how mating success translates into fitness, and so the estimation of mating success is of crucial importance. Measures of mating success is usually inferred either from observations of mating interactions (called copulatory mating success; e.g., Collet et al. 2012; Pélissié et al. 2012; Fritzsche and Arnqvist 2013), or from parentage analysis (called genetic mating success; e.g., Bateman 1948; Jones et al. 2000; Gopurenko et al. 2007; Byers and Dunn 2012; Pischedda and Rice 2012). In the present study, the opportunity to assess the sperm-transfer success allowed us to also infer mating success from the sperm stored in

partners (hereafter called sperm mating success). Noteworthy these three types of measures of mating success have different significations as they are inferred from different selection episodes, and thus they capture the result of sexual selection up to this particular episode (Anthes et al. 2010). Namely, copulatory mating success encompasses exclusively the selection on gaining copulation. Sperm mating success encompasses the selection on gaining copulation and also on storing sperm into partners. Genetic mating success encompasses the selection on gaining copulation, storing sperm to partners, and also on fertilising partners' ova (Anthes et al. 2010). Thus, the measure of mating success inferred from selection episode closer to reproductive success merge more components of sexual selection, and thus gradually leads to autocorrelations with reproductive success (Anthes et al. 2010; Arnqvist 2013). We illustrate this critical point in Appendix 4.

Decomposition of the Variance in Male Reproductive Success

Our results show that most of the variance observed in male reproductive success arose from post-mating success. Previous studies that decompose the variance in male reproductive success find contrasting results about the relative importance of pre- and post-copulatory episodes of selection (Collet et al. 2012; Pischedda and Rice 2012; Pélissié et al. under review). In simultaneous hermaphrodites, sexual selection has been hypothesised to mainly occur at the post-copulatory rather than the pre-copulatory level (Charnov 1979; Michiels 1998; Schärer and Pen 2013). This is because if male fitness depends more on mating success than female fitness, then individuals are expected to prefer the male role when encountering a mating opportunity (but see Kokko et al. 2012), leading to a conflict over the preferred mating role between mating partners. This specific conflict can be resolved by adopting reciprocal copulation, but this has two consequences. First, individuals have to deal with a surplus of received sperm, which may potentially be undesirable for the sperm recipient. Second, male fitness may not be limited by the number of matings achieved but rather by the ability to successfully transfer sperm to their partners and have them used for fertilisation (Charnov 1979; Michiels 1998; Schärer and Pen 2013). Therefore post-copulatory sexual selection is expected to be prevalent in simultaneous hermaphrodites (see e.g., Koene and Schulenburg 2005; Chase and Blanchard 2006; Schärer et al. 2011), which is fully supported by our data.

The major finding of our study is the decomposition of the post-mating success into sperm-transfer efficiency and sperm-fertilising efficiency. The results show that 53% of the variance observed in male reproductive success arose from sperm-fertilising efficiency and 24% from sperm-transfer efficiency. This finding shows that these two episodes of selection have a large opportunity for selection in *M. lignano*. Both of these episodes are expected to depend on interactions between ejaculates of different donors and on interactions between ejaculates and the reproductive tract of the sperm

recipients. In addition, sperm-transfer efficiency is expected to depend on the ability with which individuals transfer sperm to partners (see selection on morphological trait section), and on the success by which sperm cells resist displacement by consecutive partners. Sperm-fertilising efficiency is expected to mainly depend on interactions of the ejaculate components with the ejaculate of competing sperm donors, and possibly on potential developmental failure of the eggs fertilised by sperm from certain donors. Hence, our data suggest that these mentioned evolutionary mechanisms may be strong agents of selection in *M. lignano*.

Importantly, one may expect mutualistic or antagonistic selection on different episodes of selection, which would characterize an overall individual quality or trade-offs, respectively. In both models, we did not find positive or negative significant covariances between fitness components, which therefore suggest that selection operates independently on the different fitness components measured.

Moreover, Pischedda and Rice (2012) and Pélissié et al. (under review) emphasise that it is important to account for the mating order (i.e., being the first or the last sperm donor), which they have found to reduce the portion of variance explained by the remaining post-copulatory fitness component. These two studies were performed in the fruit fly *Drosophila melanogaster* (Pischedda and Rice 2012) and in the freshwater snail *Physa* acuta (Pélissié et al. under review), two species with a strong sperm precedence (i.e., precedence of the last or the first sperm donor, respectively). We agree that the variance in post-mating success may be inflated if strong male sperm precedence occurs. In *M. lignano*, a study in which the first and the second sperm donors were allowed to mate for a specific amount of time with a sperm recipient indicates a moderate level of last male sperm precedence (P_2=0.64; Sandner et al. in prep.). However, given that both of the sperm donors mated several times in their allotted time, we think the sperm precedence of the last copulation is rather low in *M. lignano* and so, mating order is most likely not a large source of the variance observed in post-mating success.

Importantly, the variance decomposition of reproductive success along fitness components should be interpreted with caution. This is because, first, variance arising from each fitness component depends on the measures used to characterise the episodes of selection. For example, the number of mates and the number of matings may both be used to characterise the success at the pre-copulatory episode of selection. But because they measure different facets of the operation pre-copulatory sexual selection, the variance arising from these two measures of the pre-copulatory episode of selection may substantially differ. Second, a low portion of variance arising from a fitness component does not preclude that selection operates on the episode of selection measured. For example, as mention above, Pischedda and Rice (2012) and Pélissié et al. (under review) found that a portion of the variance in the post-copulatory fitness component may be explained by mating order, which may be large

when there is a strong sperm precedence. However we think that, even if one can statistically account for sperm precedence based on the mating sequences, the processes underlying sperm precedence may encompass post-copulatory components such as sperm phenotypic and behavioural traits, seminal fluids (e.g., Price et al. 1999; Miller and Pitnick 2002; Manier et al. 2010). Thus, we think that it is misleading to consider the whole variance due to mating order as a pre-copulatory trait. In sum, to reach firm conclusions about the operation of sexual selection in a study system, in addition to the decomposition of the variance in reproductive success along fitness components, one needs to also understand the processes determining the outcome in the different episodes of selection.

Selection on Morphological Traits

Our study reveals a strong influence of testis size on male reproductive success, and shows that testis size mainly affects sperm-transfer efficiency. This is in accordance with previous studies, where testis size was found to positively correlate with sperm-transfer success (Janicke and Schärer 2009a) and offspring production (Sekii et al. 2013). Since testis size is a reliable indicator of sperm production rate in *M. lignano* (Schärer and Vizoso 2007), our data suggest that individuals with bigger testis, and hence a higher sperm production rate, transfer more sperm per copulation and also reach a higher paternity success. Surprisingly, in our study testis size did not affect mating success. This is in contrast with previous studies where testis size was manipulated by raising worms in different group sizes (Janicke and Schärer 2009b) or via phenotyping engineering (Sekii et al. 2013). In the present study we used individuals raised in a stable group size throughout the experiment. Thus we explored a narrower phenotypic range of testis sizes than in Janicke and Schärer (2009b) and Sekii et al. (2013), and this is probably why our results do not concord. We however think that it is more appropriate to measure selection on traits in a steady state, which presumably corresponds better to the selection occurring in natural conditions.

Contrary to our initial expectations (Janicke and Schärer 2009a; Vizoso et al. 2010; Schärer et al. 2011), we did not find any significant fitness effect of the stylet morphology, or sperm morphology. These negative results should however be considered with caution, as selection differentials should ideally rely on larger sample sizes than we could achieve here (Hersch and Phillips 2004). Overall, our data indicate that directional selection (if any) on the stylet and sperm morphology are weaker than on testis size, but we cannot exclude that these traits are not sexually selected. For this we need further experiments relying on a larger sample size.

Conclusions

In his seminal contribution, Bateman (1948) concludes that "In the male [...] fertility is seldom likely to be limited by sperm production but rather by the number of inseminations or the number of females available to him". Our data contradicts this statement. Instead, we found that although male fitness depends to some extent on copulation activity, selection seems stronger on the post-copulatory episodes of selection, in which sperm production is presumably a crucial determinant. Therefore, our findings supports the hypothesis that post-copulatory selection is a potent evolutionary force (Eberhard 2009; Birkhead 2010) selecting on traits that affect sperm transfer success and fertilization success.

Acknowledgments

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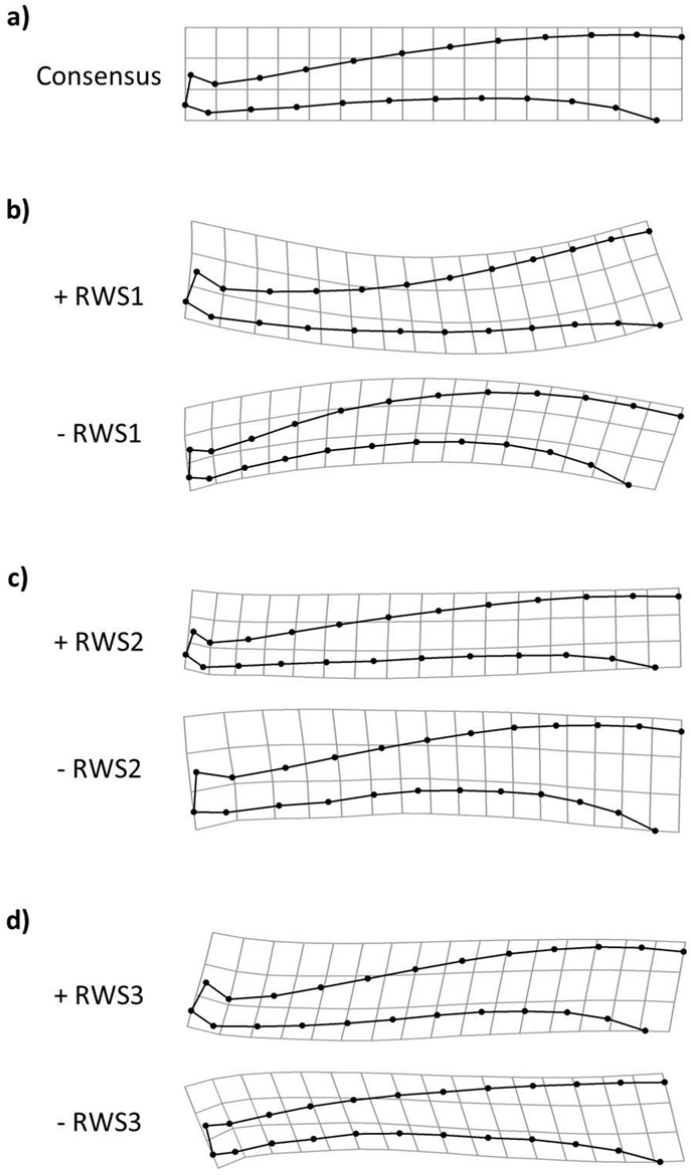
Appendices

Appendix 1: Geometric Morphometric Analysis of the Copulatory Stylet



Legend. Relative warp analysis of the landmark-based thin plate splines of the stylet. Diagrams show the configurations of a) the consensus stylet shape, b) the maximum and minimum values of the first relative warp score (RWS1) that describes the overall stylet curvature, c) the maximum and minimum values of the second relative warp score (RWS2) that describes the width of the stylet, and d) the maximum and minimum values of the third relative warp score (RWS3) that describes the orientation of the tip of the stylet. RWS1, RWS2 and RWS3 explain 51%, 14% and 13% of the variance in stylet shape, respectively.

Appendix 2: Estimations of the Variance due to Binomial Sampling Error

The variances observed in PMS*, STE* and SFE* included a part due to binomial sampling error that we denoted as $\widehat{V}(PMS^*)$, $\widehat{V}(STE^*)$ and $\widehat{V}(SFE^*)$, respectively. To account for this, we estimated them, by using the formula of variance of a Binomial distribution. In the following, we explain the procedure used to calculate $\widehat{V}(PMS^*)$.

First, we computed the variance due to Binomial sampling error in PS for each focal individual, $\hat{V}(PS_i)$, as follow: $\hat{V}(PS_i) = (PS_i) \times (1 - PS_i)$

Second, we computed the variance due to Binomial sampling error in PMS for each focal individual, $\hat{V}(PMS_i)$, as follow: $\hat{V}(PMS_i) = \frac{\hat{V}(PS_i)}{(MS_i)^2}$

Third, we computed the variance due to Binomial sampling error in PMS, $\hat{V}(PMS)$, as follow: $\hat{V}(PMS) = \frac{\sum \hat{V}(PMS_i)}{\sum F_i}$

Finally, we computed the variance due to Binomial sampling error in PMS*, $\hat{V}(PMS^*)$, as follow: $\widehat{V}(PMS^*) = \frac{\hat{V}(PMS)}{(\overline{PMS})^2}$

We followed similar procedures to compute $\widehat{V}(STE^*)$ and $\widehat{V}(SFE^*)$.

We accounted for $\widehat{V}(PMS^*)$, $\widehat{V}(STE^*)$ and $\widehat{V}(SFE^*)$ by subtracting them to the observed variance of their respective fitness components.

Appendix 3: Selection Differentials of the Morphological Traits on Male Reproductive Success and the fitness Components

For each of the measured morphological traits we calculated the selection differentials on mRS, and on each fitness component independently. Selection differentials are usually computed as coefficients of ordinary linear regressions (Arnold and Wade 1984). However, we used generalized linear models (GLMs) for three reasons: first most of the response variables rely on small numbers, second they substantially deviate from a normal distribution, and third most of the fitness components are defined as proportions. Thus, we offset the denominators of the proportions so that the response variable corresponds to the raw data of interest. For instance for sperm-transfer efficiency, STE, defined as STS/MS, which corresponds to $((focal\ sperm)/(total\ sperm))/MS$, we used $focal\ sperm$ as the response variable, and we set the offset term to be equal to the log of $total\ sperm \times MS$. Noteworthy, like the coefficients of ordinary linear regressions, the estimates of the GLMs performed here indicate the shift of the mean of the predictor variable (i.e., trait).

Hence, we used the following GLMs:

$$mRS = a \times trait \ + b \ + \text{offset: log } (penetrance) \quad (3)$$

$$F^* \ = a_1 \times trait + b \quad (4)$$

$$focal\ copulations^* \ = a_2 \times trait + b + \text{offset: } total\ copulations^* \quad (5)$$

$$mRS \ = a_3 \times trait + b + \text{offset: log } (MS \times penetrance \times F) \quad (6)$$

$$focal\ sperm = a_4 \times trait + b + \text{offset: log}(MS \times total\ sperm) \quad (7)$$

$$mRS \ = a_5 \times trait + b + \text{offset: log}(STS \times penetrance \times F) \quad (8)$$

Because F and the number of focal copulations approach normality, the models (4) and (5) assume a normal error distribution and a identity link function. Whereas because mRS and the number of focal sperm are small numbers and count data, the models (3), (6), (7) and (8) assume a Poisson error distribution and a log link function. Note that only F and the number of focal copulation require to be relativized, because by definition the log transformation in Poisson GLMs converts absolute difference into relative difference.

These models allow to assess the total male selection differential on any phenotypic trait (a), which can further be approximately decomposed in the selection due to partner fecundity (a_1), mating success (a_2), post-mating success (a_3), sperm-transfer efficiency (a_4), and sperm fertilisation efficiency (a_5). Consequently,

$$a \approx a_1 + a_2 + a_3 \quad \text{and} \quad a \approx a_1 + a_2 + a_4 + a_5$$

Appendix 4: Comparing Different Estimators of Male Mating Success

Legends. Linear relationships between male reproductive success and different estimators of male mating success. Male mating success of a focal could in theory be inferred from the observation of copulations (a, d), successfully transferred sperm (b, e), and offspring sired (c, f). The upper row (a, b, c) is based on counts of copulations, sperm cells or offspring. The lower row (d, e, f) is based on the numbers of mates with whom the focal mated (copulatory mating success, cMS), to whom it transferred sperm (sperm mating success, sMS)), and with whom it sired offspring (genetic mating success, gMS). The data are not relativized for illustrative purpose. NA stands for not available in this study.

Chapter V

Sex Allocation Adjustment to Mating Group Size in a Simultaneous Hermaphrodite

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Abstract

Sex allocation theory is considered as a touchstone of evolutionary biology, providing some of the best supported examples for Darwinian adaptation. In particular, Hamilton's local mate competition theory has been shown to generate precise predictions for extraordinary sex ratios observed in many separate-sexed organisms. In analogy to local mate competition, Charnov's mating group size model predicts how sex allocation in simultaneous hermaphrodites is affected by the mating group size (i.e., the number of mating partners plus one). Until now, studies have not directly explored the relationship between mating group size and sex allocation, which we here achieve in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. Using transgenic focal worms with ubiquitous expression of green-fluorescent protein (GFP), we assessed the number of wild-type mating partners carrying GFP+ sperm from these focal worms when raised in different social group sizes. This allowed us to test directly how mating group size was related to the sex allocation of focal worms. We find that the proportion of male investment initially increases with increasing mating group size, but then saturates as predicted by theory. To our knowledge, this is the first direct test of the mating group size model in a simultaneously hermaphroditic animal.

Sex allocation theory provides the theoretical framework to predict resource allocation to male and female reproduction in sexually reproducing organisms and is considered as a touchstone in evolutionary biology (Frank 2002; reviewed in Charnov 1982; Hardy 2002; West 2009). In particular, Hamilton's theory of local mate competition (LMC; Hamilton 1967) has become one of the best supported examples for Darwinian adaptation, by accurately predicting female-biased sex ratios in many separate-sexed organisms (West et al. 2000; Frank 2002).

The classic model of LMC, which is generally considered as competition between related individuals for the access to mating partners, predicts a female-biased sex ratio in spatially structured populations, where matings occur before the dispersal of females (Hamilton 1967). Specifically, there are two forces that contribute to biased sex ratios (Taylor 1981). First, the production of many sons in the same patch leads to competition among brothers for mating partners, which is not expected to be beneficial from the mother's perspective. Second, a female-biased sex ratio results in more mating opportunities for sons and therefore translates into a higher expected reproductive success of each produced son and an overall higher reproductive success for the mother. Only under the assumption of a large population size and random mating does LMC become negligible and only then are females expected to invest equally into sons and daughters (Hamilton 1967). The most conclusive empirical evidence for an effect of LMC on sex allocation comes from studies on parasitoid wasps, pollinating fig wasps and spider mites, which provide both qualitative and quantitative support for sex allocation theory in separate-sexed organisms (e.g., Werren 1980; Herre 1985; Hardy 2002; Macke et al. 2011; reviewed in West 2009).

The concept of LMC is also fundamental for the study of sex allocation in simultaneous hermaphrodites, that is organisms in which individuals produce male and female gametes at the same time (Charnov 1982). In contrast to separate-sexed organisms, sex allocation theory for simultaneous hermaphrodites provides the theoretical framework to predict the optimal relative investment into the male versus the female sex function within the same individual (reviewed in Schärer 2009). One central prediction of sex allocation theory for simultaneous hermaphrodites is that individuals are expected to reallocate their resources toward the female sex function if the mating group size (defined as the average number of mating partners plus one) is small (Charnov 1980, 1982). In analogy to the phenomenon of LMC in structured populations of separate-sexed organisms, a small mating group leads to competition between related sperm from a donor for the fertilisation of a given set of ova (recently termed "local sperm competition"; Schärer 2009), which leads to a decelerating fitness gain for additional investment into sperm production. Therefore, simultaneous hermaphrodites are expected to have a female-biased sex allocation if the mating group size is small (Charnov 1980; Fischer 1981; Charnov 1982), as this re-allocation reduces local sperm

competition and allows an overall higher reproductive success for a female-biased individual.

Charnov (1980) presented a resource allocation model, which explores explicitly the relationship between mating group size and the resulting optimal sex allocation in outcrossing simultaneous hermaphrodites (herein called the "mating group size model"). This model predicts that the proportion of reproductive resources r^* devoted to the production of sperm increases with mating group size according to the equation $r^* = (K - 1)/(2K - 1)$, where K is the number of (sperm) donors that a (sperm) recipient receives sperm from (Charnov 1980; Fischer 1981; Charnov 1982). Consequently, the resource allocation to the male sex function is predicted to increase with an increasing mating group size, reaching an asymptote at $r^* = 0.5$ as mating group size $(K + 1)$ becomes very large and more and more donors compete for a recipient's eggs.

Previous empirical work on the effect of mating group size on sex allocation in simultaneously hermaphroditic animals has mainly focused on phenotypically plastic responses in sex allocation to varying group sizes (reviewed in Schärer 2009). For instance, field studies have shown that male allocation is positively related to population density, which suggests that individuals invest more resources into the male sex function if competition for mating partners is high under natural conditions (e.g., Raimondi and Martin 1991; Hart et al. 2010). Similarly, experimental studies on a broad range of simultaneously hermaphroditic animal species provide evidence that individuals invest relatively more resources into the male sex function when kept in larger groups under laboratory conditions (e.g., Trouvé et al. 1999; Schärer and Ladurner 2003; Koene et al. 2006; Baeza 2007). However, in all of these studies it was unknown how density and/or social group size (i.e., the number of potential mating partners within a group) actually translated into the corresponding mating group size. In the very few cases were this relationship has been evaluated, it was shown that the mating group size can be considerably smaller than the social group size, potentially rendering social group size an unreliable estimate of mating group size (e.g., Pongratz and Michiels 2003; Janicke and Schärer 2009a). This highlights the necessity of measuring the trait that is predicted to affect the sex allocation (i.e., mating group size) rather than a proxy of it (i.e., social group size) when testing Charnov's mating group size model (see also Schärer 2009). To conclude from this, our current empirical support for the effect of mating group size on sex allocation needs to be considered as only indirect, as previous studies have not provided a direct experimental test of the relationship between mating group size and sex allocation in simultaneous hermaphrodites.

Here we report a study on the relationship between mating group size and sex allocation in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. Over the last decade, *M. lignano* has emerged as a highly suitable model organism for the study of sex allocation in simultaneously hermaphroditic

animals (Schärer 2009; Anthes 2010). Previous studies have showed that *M. lignano* adjusts its sex allocation in response to the social group size in a phenotypically plastic way, with individuals kept in larger groups having a more male-biased sex allocation (e.g., Schärer and Ladurner 2003; Schärer et al. 2005; Brauer et al. 2007; Janicke and Schärer 2009b). Furthermore, it has been documented that worms in larger social groups have on average more mating partners (Janicke and Schärer 2009a). In this study, we raised focal worms in a range of different social group sizes and estimated the actual mating group size and the sex allocation within the same experimental setup. Using individuals from a recently established transgenic line with ubiquitous expression of green-fluorescent protein (hereafter GFP) as focal worms, we could estimate the resulting mating group size in a biologically meaningful way. To our knowledge, this is the first direct test of Charnov's mating group size model in a simultaneously hermaphroditic animal.

Methods

Study Organism

The free-living flatworm *M. lignano* (Macrostomorpha, Platyhelminthes) is an obligatorily outcrossing simultaneous hermaphrodite of the intertidal meiofauna of the Northern Adriatic Sea (Schärer and Ladurner 2003; Ladurner et al. 2005b). Stock cultures in the laboratory are maintained at 20°C in glass Petri dishes filled with *f*/2 medium (Andersen et al. 2005) and fed with the algae *Nitzschia curvilineata*. The worms are transparent allowing noninvasive measurement of various morphological traits, such as testis size and ovary size (Schärer and Ladurner 2003). The transparency of the worms also enables the visualisation and assessment of the number of received sperm that are stored in the female sperm-storage organ (hereafter called "antrum") *in vivo* (Janicke et al. 2011). The antrum usually contains ≤40 sperm and the estimates of the number of stored sperm have been shown to be highly repeatable in this species (Vizoso et al. 2010; Janicke et al. 2011). Matings are always reciprocal (Schärer et al. 2004a) suggesting that an individual receives and donates sperm while copulating. As a consequence, the number of mating partners is inherently the same for both sex functions.

Culture Lines

This study focuses on a phenotypically plastic adjustment of sex allocation in response to mating group size. For the experiment, we used individuals obtained from two culture lines, which are both descendants of the same inbred line. This inbred line, hereafter called DV1, was initiated by crossing two virgin worms from our genetically diverse laboratory mass cultures. In the subsequent generations, the maternal offspring of only one worm of the pair was collected and later crossed with their full- or halfsiblings. In particular, only two offspring were crossed during the first 15 generations (full-sib

inbreeding) and three offspring from generation 16 to 24 (full- or half-sib inbreeding). Since then, always 10 offspring (moderate level of inbreeding to maintain the lines) were used to initiate the next generations. Recently, the DV1 line was used to generate a stable germ-line transmitting transgenic line expressing enhanced GFP driven by an elongation factor 1 a promotor. This was achieved by injecting a corresponding DNA construct into a single cell stage embryo (details on the construct used, its integration, and the subsequent generation of stable homozygotes will be published elsewhere; Demircan et al. in prep). The transgenic HUB1 line shows ubiquitous expression of GFP, so that this protein can also be found and visualised in the sperm cells. The transparent nature of the worms therefore allows tracking the sperm of a transgenic GFP(+) worm in a non-transgenic GFP(−) recipient *in vivo* (Figure 1; Movie S1). In this experiment we used GFP(+) worms of the HUB1 line as focals and GFP(−) worms of the DV1 line as potential mating partners. Given that both lines originate from the same line, which was inbred for many generations, we expect GFP(+) and GFP(−) worms to be genetically almost identical, except for the fact that the GFP(+) worms carry the transgenic construct. Experiments performed in our laboratory indicate that these two lines do not differ in reproductive performance (Marie-Orleach et al. in prep.). Moreover, a preliminary study showed that both lines are capable of adjusting their sex allocation in a phenotypically plastic way in response to social group size, as has previously been shown for our genetically diverse mass cultures (e.g., Schärer and Ladurner 2003; Schärer et al. 2005; Janicke and Schärer 2009b).

Experimental Design

On the first day, we collected adult worms from mass cultures of GFP(−) and GFP(+), and transferred them to glass Petri dishes filled with *f*/2 medium and a dense algae layer, to allow worms to lay eggs. In detail, we distributed 250 adult GFP(+) worms equally among two Petri dishes and 1800 adult GFP(−) worms equally among 20 Petri dishes. On day 4, all adult worms were removed from the Petri dishes so that all eggs were laid within 72 h, which guaranteed that all offspring produced were of similar age and stemmed from parents held in very similar backgrounds. On day 15, we pooled all offspring produced by GFP(+) and GFP(−) worms respectively, and distributed them randomly among the treatment groups. Specifically, we transferred one GFP(+) focal worm to wells of 24-well tissue well-plates (TPP AG, Switzerland) and added to each focal a specific number of GFP(−) worms so that the final social group size was 2, 3, 4, 5, 8, 12, or 16 worms (e.g., groups of eight individuals consisted of one GFP(+) worm and seven GFP(−) worms). All wells contained 1.5 mL of *f*/2 medium and a standard amount of an algae solution that guaranteed *ad libitum* food conditions (i.e., a dense layer of algae on the bottom of the wells). We arranged the treatments on the well-plates in a way that balanced any potential position effects. Initially, we replicated all social group sizes 20 times so that the experiment comprised overall 140 GFP(+)

worms and 860 GFP(−) worms. On days 22, 35, 47, and 55, all worms were transferred to fresh wells (i.e., 1.5 mL *f*/2 medium and a dense algae layer) to guarantee continued *ad libitum* food conditions and to reduce possible interactions of adult worms with their offspring.

Estimation of Sex Allocation and Mating Group Size

From day 62 to day 69, we took morphological measurements of the GFP(+) focals and assessed the presence of GFP(+) sperm in the antrum of each of the GFP(−) worms within each social group (Figure 1). At the same time, we also checked whether all the worms within a social group were mature, as inferred from a full development of the gonads and the male copulatory organ. To avoid time effects, we balanced the treatment groups sampled among days. Specifically, we first isolated all individuals of a given social group in wells of 60-well microtest plates (Greiner Bio-One, Germany) filled with 10 μL of *f*/2 medium. We did this to prevent gradual changes in the composition in a social group as such changes could potentially affect the sperm representation of the focal worms. Next, we identified the GFP(+) focal of each social group using a MZ12.5 stereo-microscope equipped with a epifluorescence light source (Leica Microsystems, Germany) and then took pictures for morphometry following a standard protocol with a compound microscope (Schärer and Ladurner 2003). In brief, focals were anesthetized in a 5:3 mixture of 7.14% MgCl2 and *f*/2 medium for 10 min. Thereafter, we squeezed focals dorsoventrally to a fixed thickness of 35 μm between a microscope slide and a cover slip of a hemocytometer, and took digital micrographs of the entire body, the testes, and the ovaries with a Leica DM 2500 microscope (Leica Microsystems) and a digital video camera (DFK 41AF02, The Imaging Source Europe GmbH, Germany; 40× magnification for body size and 400× magnification for testis size and ovary size). For image acquisition, we used BTV Pro 6.0b1 (http://www.bensoftware.com/) and we analyzed micrographs using ImageJ 1.42k (http://rsb.info.nih.gov/ij/). All these morphological measurements have been shown to have a high repeatability (Schärer and Ladurner 2003).

We further assessed the presence of stored GFP(+) sperm in each of the GFP(−) worms based on movies of the antrum, which were recorded as described previously (Janicke et al. 2011). Briefly, we compressed anesthetized worms between a 24 mm × 50 mm and a 21 mm × 26 mm cover slip using small plasticine feet as spacers. Afterward, we mounted this cover slip chamber on a microscope slide, so that the observer could easily flip the worm from the dorsal to the ventral view, allowing accurate assessment of the presence of sperm stored in the antrum (Janicke et al. 2011). We recorded movies of each antrum by focusing slowly through the entire organ at a 630× magnification under epifluorescence illumination to visualise the GFP(+) sperm transferred by the GFP(+) focal (Movie S1). For this we used a Leica DM 2500 microscope (Leica Microsystems) equipped with an epifluorescence light source and connected with a highly sensitive digital video camera, a Leica DFC

360 FX (Leica Microsystems). Movies were recorded using the screen-capture software CamStudio version 2.0 (http://camstudio.org) and analyzed using KMPlayer version 3.0 (http://kmplayer.com/forums).

Based on these movies we assessed the presence of stored GFP(+) sperm in the antrum of GFP(−) worms. Mating group size was assessed as the number of GFP(−) individuals in the social group that had at least one GFP(+) sperm in storage plus one so that the mating group includes the number of mates of a given focal individual and the focal individual itself (cf., Charnov 1982).

We need to clarify here that our estimate of mating group size does not necessarily reflect the actual number of mating partners that a focal individual has had over a certain time span, because it relies exclusively on the current presence of successfully stored sperm in its partners. Processes associated with the removal of transferred sperm (e.g., sperm displacement, passive sperm loss, and/or cryptic-female choice) or the usage of sperm for fertilising the eggs will lead to an underestimation of the number of mating partners (see also Janicke and Schärer 2009a), so that the total number of mating partners of the focal worms over the period of the experiment was presumably higher than our results suggest. However, the crucial trait predicted to affect the sex allocation in simultaneous hermaphrodites is not the total number of mates, but the average number of mating partners that are in competition for a given set of ova (Charnov 1982; Schärer 2009; cf., Parker 1998). Our measurement of the number of mating partners, which is based on the presence of stored sperm, corresponds to the number of mating partners in Charnov's mating group size model (termed "*K*" in the original equation; see Introduction) and is therefore an appropriate estimate of the mating group size in the context of sex allocation theory.

Statistical Analysis

From the intended total sample size of 140 replicates we lost 56 replicates, mainly because some worms did not develop properly or grew slowly. Given that the worms used for this experiment originated from an inbred line this is not surprising and matches with our experience with this and other inbred lines we are maintaining. Specifically, we lost 40 replicates due to incomplete development of either the focal or one or more of its partners (e.g., lack of the testes, ovaries, and/or male copulatory organ), 11 replicates due to pipetting errors during transfers, and five replicates due to handling errors during morphological measurements. Consequently, our final sample size was reduced to 84 replicates (pairs: $N = 16$, triplets: $N = 13$, quartets: $N = 14$, quintets: $N = 15$, octets: $N = 10$, groups of 12 worms: $N = 10$, groups of 16 worms: $N = 6$; incomplete development of individual worms is of course more likely to affect the larger social groups).

In this study, we used the proportion of testis size to overall gonad size (i.e., testis size/[testis size + ovary size]) as an estimate of sex allocation (cf., Vizoso

and Schärer 2007; Janicke and Schärer 2009b). We note that this estimate represents a relative measure of the sex allocation, which allows comparing the resource allocation toward the male and female sex function between individuals, with higher values indicating a more male-biased sex allocation. However, it does not provide an absolute measure of sex allocation, because it is exclusively based on measures of the size of male and female gonadal tissues, which, although both involved in gamete production do not necessarily equal in terms of energetic demands per unit size. Furthermore, additional traits that may also impose costs to male and female reproduction (e.g., copulatory organs, seminal fluids, yolk, egg-shell glands, sex-specific behaviours) are not considered here (cf., Schärer and Pen 2013). Consequently, our estimate of sex allocation relies on the assumption that testis and ovary size are good proxies for the reproductive investment into the male and female sex function, respectively (reviewed in Schärer 2009). For *M. lignano* this assumption has been verified directly for testis size (e.g., Schärer et al. 2004b; Schärer and Vizoso 2007), whereas evidence that ovary size reflects the resource allocation into the female sex function is less direct (e.g., Schärer et al. 2005).

The statistical test of Charnov's mating group size model was done in the following two steps. First, we tested the effect of our experimental manipulation of the social group size on sex allocation and on mating group size of the GFP(+) focals. Second, we explored the relationship between our estimates of mating group size and sex allocation among social group sizes to test Charnov's mating group size model.

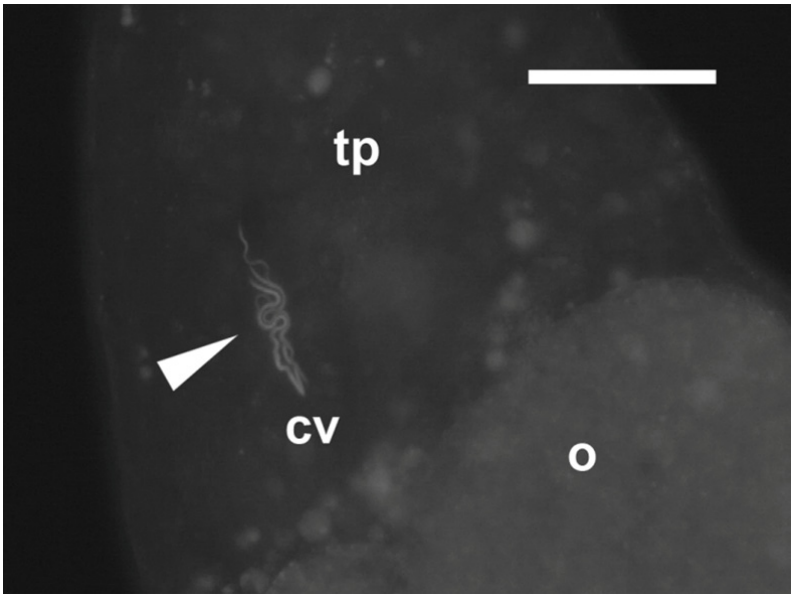

Figure 1. Micrograph of the tail region of a green-fluorescent protein (GFP)(−) worm storing four spermatozoa received from a GFP(+) worm. Image shows the GFP(+) sperm (arrowhead), the tail plate (tp), and a developed oocyte (o). Sperm are anchored with their feeler in the cellular valve (cv), which is a specialized epithelium of the sperm storage organ where the oocyte passes through before it is laid. This image is a snapshot of a monochrome movie taken under epifluorescence illumination to visualise the GFP(+) sperm (see Supporting Information). Anterior of the worm is to the bottom. Scale bar represents 50 μm.

Effect of Social Group Size on Sex Allocation and Mating Group Size

First, we tested whether the social group size affected the body size of focal worms using a Kruskal-Wallis rank sum test. This was done to infer whether the overall resource budgets differed between the social groups. In this study, we were primarily interested in effects on sex allocation, but for a more complete data representation we also tested whether social group size affected testis size and ovary size independently (as suggested by Schärer 2009) using Kruskal-Wallis rank sum tests. Finally, we tested whether social group size had an effect on the sex allocation and on the mating group size using Kruskal-Wallis rank sum tests. Post hoc tests were conducted using Wilcoxon rank sum tests with Benjamini-Hochberg adjustment of *P* values to correct for false discovery rates (Benjamini and Hochberg 1995). We used nonparametric tests to account for unequal variances across treatment groups and/or deviations from normality.

Test of Charnov's Mating Group Size Model

We fitted linear and quadratic regressions to explore how sex allocation relates to the mating group size. Quadratic regressions were applied to account for the fact that Charnov's mating group size model predicts that the relationship between sex allocation and mating group size is nonlinear. We ran log-likelihood ratio tests and obtained the Akaike information criterion (AIC) to evaluate whether the nonlinear model provides a better fit than the linear model. First, we fitted a linear and a quadratic regression on the arithmetic means of sex allocation and mating group size computed separately for each social group size. This was done to relate the experimentally induced variation in mating group size to the experimentally induced variation in sex allocation. We weighted these mean values in both models according to the number of replicates obtained for each social group size, to account for differences in the accuracy of our estimates. Second, we similarly fitted and compared linear and quadratic regressions on the individual data to provide a largely descriptive test of how individual variation in mating group size translates into sex allocation (i.e., only part of this variation is due to our experimental manipulation).

All statistical analyses were carried out in R version 2.15.2 (R Development Core Team 2012). Values are given as mean ± 1 SE.

Results

Social group size had an effect on the body size of focal worms (Kruskal-Wallis rank sum test: $\chi^2 = 33.38$, degrees of freedom [df] = 6, $P < 0.001$). Focal worms kept in larger social groups grew bigger (Figure 2A) suggesting that individuals kept in larger social groups had an overall higher resource budget compared to individuals kept in smaller social groups. Individuals of different social groups varied significantly in testis size (Kruskal-Wallis rank sum test: $\chi^2 = 36.96$, df = 6, $P < 0.001$; Figure 2B) but not in ovary size (Kruskal-Wallis rank sum test: $\chi^2 = 10.68$, df = 6, $P = 0.099$; Figure 2C). As a consequence,

social group size had a strong effect on the sex allocation of focal worms (Kruskal-Wallis rank sum test: $\chi^2 = 38.36$, df = 6, $P < 0.001$). Specifically, individuals raised in larger groups had a more male-biased sex allocation compared to individuals in smaller groups (Figure 3A).

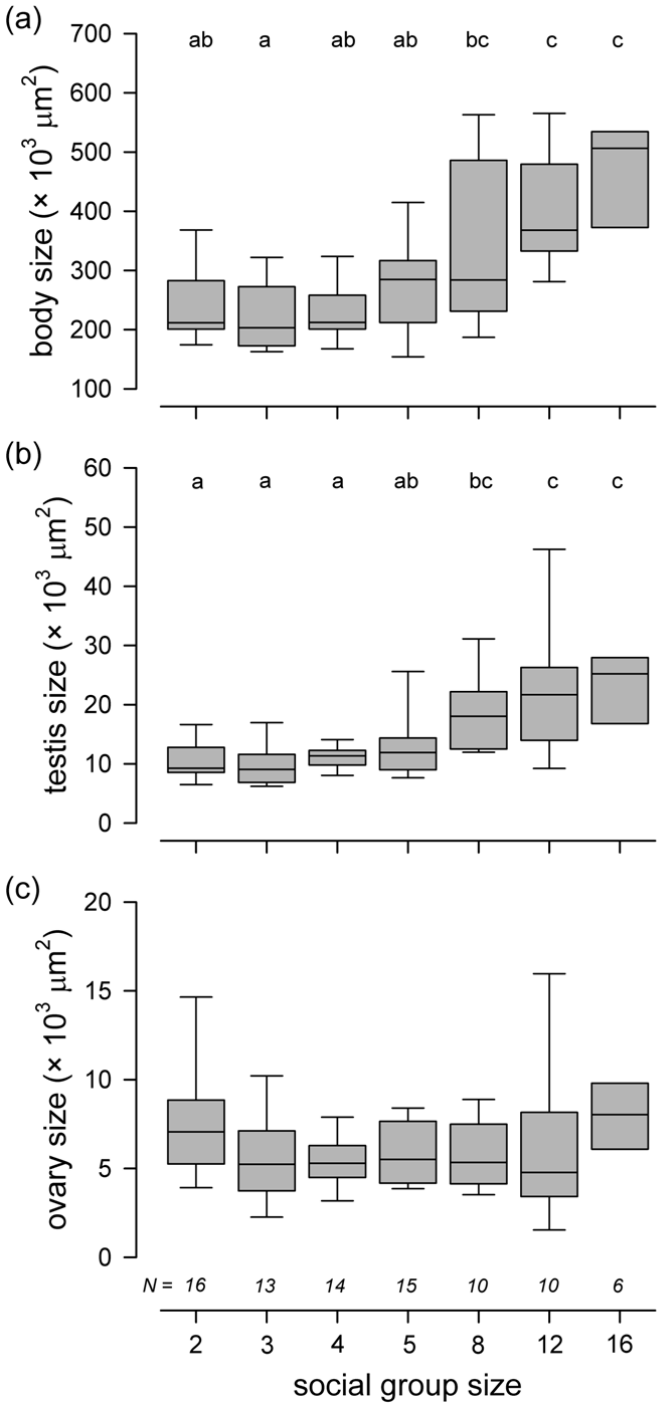

Figure 2. Effects of social group size on (A) body size, (B) testis size, and (C) ovary size. Different letters indicate significantly different treatment groups inferred from Wilcoxon rank sum post hoc tests (corrected for multiple testing, see main text). Boxplots show the 25th percentile, the median, and the 75th percentile and whiskers denote the 5th and the 95th percentiles.

Our manipulation of the social group size also induced considerable variation in the mating group size (Kruskal-Wallis rank sum test: $\chi^2 = 48.88$, df = 6, $P < 0.001$), in that focal worms in larger groups managed to store sperm in more partners than individuals in smaller groups (Figure 3B). Interestingly, the number of mating partners leveled off with increasing social group size (i.e., individuals in social groups of 8, 12, and 16 did not differ in mating group size; Figure 3B).

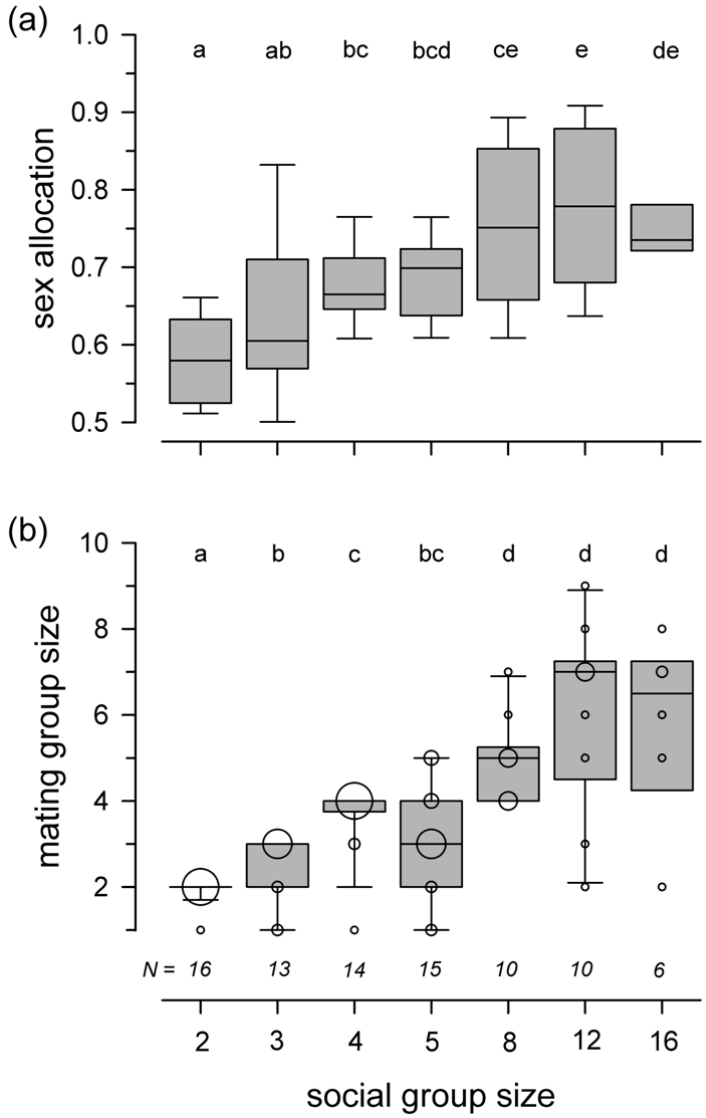

Figure 3. Effects of social group size on (A) sex allocation (i.e., testis size divided by overall gonad size) and (B) mating group size (i.e., the number of partners carrying green-fluorescent protein [GFP](+) sperm plus one). Different letters indicate significantly different groups inferred from Wilcoxon rank sum post hoc tests (corrected for multiple testing, see main text). Boxplots show the 25th percentile, the median, and the 75th percentile and whiskers denote the 5th and the 95th percentiles. Open circles in (B) are individual data points and circle size reflects the number of cases for which we observed a given mating group size.

Mean estimates of sex allocation were clearly positively related to mean estimates of mating group size obtained from each social group size (linear regression: $R^2 = 0.92$, $F_{1,5} = 56.25$, $P < 0.001$; quadratic regression: $R^2 = 0.96$, $F_{2,4} = 51.05$, $P = 0.001$) with the quadratic regression providing a significantly better fit than the linear regression (likelihood ratio test: $\chi^2 = 5.41$, df = 1, $P = 0.020$; $AIC_{\text{linear regression}} = -29.53$; $AIC_{\text{quadratic regression}} = -32.94$). Sex allocation increased with increasing mating group size in a saturating manner (Figure 4A). Very similar patterns were found in the descriptive analysis, in which we tested how individual variation in mating group size translated into sex allocation using individual data points (linear regression: $R^2 = 0.31$, $F_{1,82} = 37.49$, $P < 0.001$; quadratic regression: $R^2 = 0.37$, $F_{2,81} = 23.41$, $P < 0.001$; likelihood ratio test: $\chi^2 = 6.70$, df = 1, $P = 0.010$; $AIC_{\text{linear regression}} = -176.91$; $AIC_{\text{quadratic regression}} = -181.61$; Figure 4B).

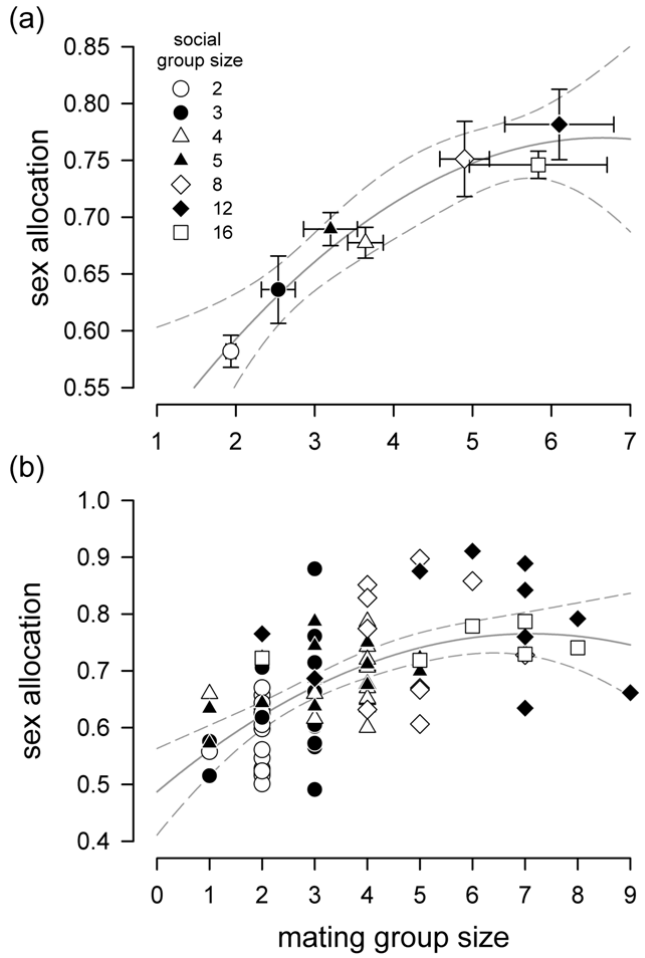

Figure 4. Relationship between sex allocation and mating group size shown for (A) group means ± 1 SE of sex allocation and mating group size obtained from each social group size and (B) individual data points. Symbols indicate the corresponding social group size (see legend). Solid line shows the fit of a quadratic regression (model fit on weighted means: $y = 0.70 + 0.17x - 0.04x^2$; model fit on individual data points: $y = 0.68 + 0.51x - 0.21x^2$), dashed lines indicate the 95% confidence intervals. Note that statistical tests on the group means were done on weighted means and that the SEs are only shown as a visual aid (see Methods section).

Discussion

This study provides the first direct test of Charnov's mating group size model for a simultaneously hermaphroditic animal. First, we show that experimental manipulation of the social group size induces variation in both sex allocation and mating group size, which confirms earlier results obtained in separate studies. Second, we demonstrate, to our knowledge for the first time, that sex allocation and mating group size are positively related in a saturating manner, as predicted by sex allocation theory. In the following we discuss these two major outcomes in more detail.

Effects of Social Group Size on Sex Allocation and Mating Group Size

Our results confirm earlier findings on the effect of social group size on sex allocation in *M. lignano*, which have also shown that worms in larger groups have a more male-biased sex allocation (e.g., Schärer and Ladurner 2003; Schärer et al. 2005; Brauer et al. 2007; Janicke and Schärer 2009b). In contrast to these previous studies, in which the social group size ranged only from 2 to 10 individuals, we here also tested social groups of 12 and 16 individuals, with the intention to explore whether the sex allocation adjustment continues or whether it reaches an asymptote. Interestingly, we found that the sex allocation of individuals kept in groups of 12 and 16 individuals did not differ from that of individuals kept in octets. This suggests that the previous studies had probably already covered the maximum variation in sex allocation that can be observed in *M. lignano* as the result of a phenotypically plastic response to differences in social group size, at least under laboratory conditions. Future studies should clearly try to assess the mating group size in the field to get an idea about the natural variation in mating group size and how it translates to estimates obtained under laboratory conditions.

We also found a strong effect of social group size on the body size of the worms, which has been previously found in some, but not all studies on plasticity of sex allocation in *M. lignano* (e.g., Schärer and Janicke 2009; but see Janicke and Schärer 2009b). In theory, this finding could have complicated our conclusions about the effect of social group size on sex allocation, because body size itself has been argued to affect the sex allocation in simultaneous hermaphrodites (reviewed in Schärer 2009). In accordance with that prediction, there is evidence for such a size-dependent sex allocation in *M. lignano*, with smaller individuals having a more male-biased sex allocation when kept in the same group size (Vizoso and Schärer 2007). However, in our study, individuals in larger groups grew bigger and had a more male-biased sex allocation, which is exactly the opposite of what is predicted by theory on size-dependent sex allocation. Therefore, we believe that size-dependent sex allocation is unlikely to explain the observed effect of social group size on the resource allocation into the male and the female sex function. Nevertheless, given the observed

positive effect of social group size on body size and the presence of size-dependent sex allocation in *M. lignano*, it remains possible that we might have underestimated the variation in sex allocation in this study.

We need to clarify here that our measure of sex allocation does not represent an absolute but only a relative estimate of the resource allocation devoted to the male versus the female sex function. This is because sex allocation was measured in terms of the size of the gonadal tissue rather than in terms of the energy invested into both sex functions. As a consequence, estimates greater than 0.5 (cf., Figure 3A) are not necessarily indicative of a male-biased sex allocation (see also Methods section). Instead, our estimate of sex allocation only provides a relative measure, which still allows us to compare changes in resource allocation toward the male and the female sex function between individuals (reviewed in Schärer 2009).

Social group size was also found to affect our estimate of mating group size. As expected, focal worms that were kept in larger social groups managed to store sperm in more mating partners. Interestingly, we found no difference in the number of mating partners between social groups of 8, 12, and 16 individuals suggesting that there is an upper threshold in the number of individuals that can be successfully inseminated by a focal worm. The average numbers of mating partners found in this study correspond largely to the results of an earlier study in which sperm-labeled focal worms were kept in social groups of 2, 3, 4, 8, and 16 individuals (using an older sperm-labeling technique; Janicke and Schärer 2009a). This is somewhat surprising, because in the earlier study focal worms were allowed to mate within their social group for only 24 h and not for several weeks as in this study. We suspect that the reason why the much longer group exposure of focal worms did not lead to a higher number of successfully inseminated partners compared to the previous study, is a high turnover rate of the sperm stored in the female sperm storage organ. Especially sperm displacement and/or passive sperm loss during egg laying are likely to reduce the time that received sperm remains stored in the female sperm storage organ in *M. lignano*. Recent studies on *M. lignano* indicate that there is second male sperm precedence caused by sperm displacement (Sandner et al. in prep; Marie-Orleach et al. in prep.). Furthermore, given that fertilised eggs have to pass through the antrum (i.e., the sperm storage organ), before being laid (Vizoso et al. 2010), it is likely that some of the stored sperm are passively lost during egg laying. In addition, active sperm removal by the recipient (e.g., cryptic-female choice) might be an additional mechanism, which limits the time that sperm remains stored (for possible mechanisms, see Vizoso et al. 2010). Finally, the usage of sperm to fertilise the eggs will also deplete the number of sperm that is stored in the sperm storage organ, which eventually also constrains the time that the sperm of a given donor remains stored in the recipient. Here it is worthwhile to note that sperm depletion due to passive sperm loss and/or sperm usage for fertilisation might be particularly important

in *M. lignano* as worms usually store relatively few sperm in their sperm storage organ (e.g., on average 29 sperm; Janicke et al. 2011).

Our finding that the mating group size does not exceed a certain threshold in *M. lignano* may have important implications for the evolutionary stability of simultaneous hermaphroditism. Sex allocation theory suggests that simultaneous hermaphroditism is an evolutionary stable strategy if the mating group size remains relatively small (Charnov 1982). This is because small mating group sizes and the associated high potential for local sperm competition lead to a saturating fitness curve for the male sex function, and such a saturating fitness gain curve in one sex function is a prerequisite for simultaneous hermaphroditism to be resistant against the invasion of pure males and females (reviewed in Charnov 1982; Schärer 2009). Our results suggest that the maximum average mating group size in *M. lignano* is approximately six, a range where the theoretically predicted sex allocation is 0.44 and thus well below 0.5. Further work is clearly needed to identify the mechanisms, which are causing the observed upper threshold of mating group size in *M. lignano*.

Test of Charnov's Mating Group Size Model

The major novel insight of this study is the documentation of a positive and nonlinear relationship between mating group size and sex allocation, as predicted by the mating group size model (Charnov 1980, 1982). Together with empirical studies on separate-sexed organisms (e.g., Werren 1980; Werren 1983; Herre 1985; reviewed in West 2009), our work suggests that Hamilton's LMC theory, which has later been extended to simultaneous hermaphrodites (Charnov 1980; Fischer 1981), provides valid predictions that are universal for animals of various gender expressions.

Previous empirical tests of the Charnov's mating group size model for simultaneous hermaphrodites have used social group size or density as proxies for mating group size and therefore provided only indirect support for the theory (e.g., Raimondi and Martin 1991; Schärer and Ladurner 2003; Tan et al. 2004; Janicke and Schärer 2009b; for an experimental evolution study on plants, see Dorken and Pannell 2009; Schärer 2009). Although social group size is presumably often positively related to mating group size, data of this and a previous study (Janicke and Schärer 2009a) suggest that this relationship can be non-linear, so that social group size becomes an inaccurate estimate of mating group size. Therefore, we argue that measuring mating group size is a crucial prerequisite to provide a more direct experimental test of the mating group size model.

Perspectives

Our study is the first to directly quantify the relationship between mating group size and sex allocation in a simultaneously hermaphroditic animal. However, we have to clarify that our experimental design might still not provide the ultimate test of Charnov's mating group size model for at least two reasons.

First, the mating group sizes model makes a number of assumptions that might not accurately match the biology of our model organism. Specifically, one important assumption of Charnov's mating group size model is that the proportion of eggs that are fertilised by a donor depends only upon the number of sperm donated by that donor in relation to the number of sperm donated by other individuals (i.e., the model assumes a fair-raffle sperm competition; Charnov 1980, 1982). However, in many organisms this assumption probably does not apply, due to both random and nonrandom processes, which have been argued to bias the fraction of sperm stored from particular donors, so that also the mating group size can become an imprecise estimate for the intensity of local sperm competition (Charnov 1996; Greeff et al. 2001; Schärer and Pen 2013). Indeed, for many simultaneously hermaphroditic animals, including *M. lignano*, there is evidence for biased sperm precedence (e.g., Angeloni et al. 2003; Pongratz and Michiels 2003; Garefalaki et al. 2010; Sandner et al. in prep), which ultimately leads to a skewed representation of a donor's sperm in the partners sperm storage organ. Therefore, mating group size, as generally considered and as measured here, might still underestimate the intensity of local sperm competition (and thus overestimate the effective mating group size) in our and other model organisms. Future studies on the link between local sperm competition and sex allocation should attempt to explicitly quantify the skewed representation of sperm stored by different donors in a recipient and test how such skews can affect the sex allocation in simultaneous hermaphrodites (Greeff et al. 2001; Schärer and Pen 2013).

Second, our data do not provide any information about causality as this would have required to manipulate the number of successful mating partners experimentally, which will be very difficult if not impossible to achieve in our and other model systems. As a consequence we cannot exclude alternative hypotheses that are also predicting a positive effect of group size on the sex allocation. For instance, an increased male allocation in larger groups might have been an adaptation to an increased mating rate rather than more sperm competition, as suggested by the "male mating rate hypothesis" (reviewed in Vahed and Parker 2012).

Having these two caveats in mind, we suggest that further work should focus on (1) quantifying skews in sperm transfer success and (2) on using alternative approaches to quantify the relationship between local sperm competition and sex allocation. In particular, one very promising direction would be to test sex

allocation theory at a microevolutionary level. To our knowledge, there is only one experimental evolution study on separate-sexed spider mites, which demonstrates strikingly how sex ratios evolve in response to LMC (Macke et al. 2011). For simultaneously hermaphroditic animals we still lack an analogous experimental proof for the evolution of sex allocation in response to local sperm competition (but see Dorken and Pannell 2009 for plants). In addition to approaches using comparisons across species (e.g., Petersen 1991), such experimental evolution studies are clearly needed to complement the currently available empirical support for sex allocation theory in simultaneous hermaphrodites.

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Supporting Information

Movie S1. Movie showing the tail region of an individual that stores four sperm received from a GFP(+) worm in its sperm storage organ. (http://onlinelibrary.wiley.com/doi/10.1111/evo.12189/suppinfo).

Chapter VI

Concluding Remarks and Perspectives

My PhD project tackled several aspects of sexual selection in simultaneous hermaphrodites. The studies I performed clearly emphasised that post-copulatory sexual selection is an important agent of selection that may contribute to shape morphology, behaviours and sex allocation. Moreover, my PhD project included the validation of a powerful technique to study sexual selection, namely the application of GFP-labelling in a transparent organism, from which I could take advantage to reach novel findings.

Chapter II showed that mating status influences mating propensity. I presume that this is likely driven by the availability of the reproductive resources (i.e., own unfertilised eggs, own sperm, partner sperm and/or prostate gland secretions). But disentangling these potential factors is difficult in *M. lignano* because it has a reciprocal copulation, and so the filling status of the respective storage organs will often change simultaneously. Interestingly, in simultaneous hermaphrodites with unilateral copulation, it has been shown that individuals can sense the receipt of an ejaculate (Anthes et al. 2005) as well as the filling status of their prostate gland (De Boer et al. 1997) and accordingly adjust their mating propensity. In *M. lignano*, the development of the RNA intereference techniques may permit producing individuals that are virgin in only one sex function. Specifically, one would ideally pair a phenotypically engineered individuals that do not produce ejaculate or that do not have a stylet with another unmanipulated individual to test how unilateral sperm donation affect a mating interaction. Such manipulative study could help to better understand whether the decision to engage in mating depends upon the male and/or the female sex function in *M. lignano*.

The most striking finding of chapter II concerns the performance of the suck behaviour, with individuals sucking less after mating with a virgin. A clear problem in interpreting the significance of this finding is that there is at present no conclusive evidence that the suck behaviour removes ejaculate components out of the antrum, or if it is detrimental for the sperm donor. Assuming the latter is true, the most plausible scenario to me is that the suck behaviour may be manipulated through the prostate gland secretions transferred along with the sperm. The transfer of substances manipulating the behaviour or the physiology of sperm recipients has been shown in gonochoristes (e.g., Chen et al. 1988; Heifetz et al. 2000), as well as in simultaneous hermaphrodites (e.g., Koene et al. 2005; Chase and Blanchard 2006). To unambiguously demonstrate such effects, one needs to experimentally inject seminal fluid in an individual (e.g., Chen et al. 1988; Koene et al. 2005; Chase and Blanchard 2006), or to generate individuals that differ in the composition of their seminal fluids (e.g., Heifetz et al. 2000). The first option seems difficult to realise in *M. lignano*, but the second option seems feasible in the near future. Indeed RNA-Seq experiments followed by *in situ* hybridisation have already permitted to identify several candidate genes expressed specifically in prostate glands. The knock-down of their expression will presumably clarify whether prostate gland

secretions can indeed prevent the sperm recipients from sucking and, more broadly, will offer exciting opportunities to investigate the functions of prostate gland secretions in *M. lignano*.

Apart from the proximal process mentioned above, chapter II also provides insights into potential sexual conflicts in *M. lignano*. A conflict is expected to arise between mating partners over the control of the sperm usage. Namely sperm donors may develop persistence traits to bias the usage of their sperm for fertilisation, while sperm recipients are expected to develop resistance trait to counter such manipulation attempts. Ongoing innovations of persistence traits and resistance traits can lead to sexually antagonistic coevolution. In populations where such sexually antagonistic coevolution is operating, genetic polymorphisms in persistence and resistance traits are expected to occur, with combinations of certain genotypes being resistant against some, but not all persistence genotypes (e.g., Nilsson et al. 2002; reviewed in Arnqvist and Rowe 2005a). Thus, one may expect the suck behaviour and the potential manipulation of the suck behaviour via prostate gland secretions to be involved in such sexually antagonistic coevolution. This hypothesis has recently been tested in a separated experiment (Marie-Orleach L., P. Mouginot, and L. Schärer, in prep). The preliminary results suggest that the genotype of the sperm donor influences the suck frequency of the sperm recipient. This interesting information is in support of my initial speculation, and further suggests that individuals may differ in the amount and/or composition of the prostate gland secretion transferred.

The availability of the GFP-techniques in a transparent organism represents a breakthrough in the study of sexual selection in *M. lignano*. In **chapter III**, I tested and validated the reliability of this tool. Importantly, the aim of this chapter was not to determine whether GFP(+) and GFP(-) worms were similar in all traits. Instead, the aim of the series of tests I performed was to establish that the costs (if any) of the integration of the DNA construct, and the expression of the GFP molecule can be considered negligible. In brief, my results satisfactorily showed that GFP(+) worms do not differ from GFP(-) worms in terms of morphology, mating rate and reproductive success Moreover, I found that GFP(+) individuals, their sperm and their offspring can easily be identified and can reliably be used under certain conditions. Thus, all studies using the GFP-techniques in *M. lignano*, including chapters IV and V, directly benefited from the advantages and limitations identified in this chapter.

Notably in **chapter IV**, I used the GFP-technique to quantify sexual selection, which represents, to my knowledge, the first study that simultaneously assesses the relative contributions of mating success, sperm-transfer efficiency and sperm-fertilising efficiency to the variance observed in male reproductive success. The major finding is that a large part of the variance observed in male reproductive success arose from the post-copulatory fitness components. This is an important finding because sexual selection has traditionally been considered as a process that arises from differences in mating success (see e.g.,

Arnold 1994b). The next logical step will be to explain the large variance arising from the post-copulatory fitness components. I found that testis size correlated with sperm-transfer efficiency. However, none of the measured morphological traits could explain the large variance arising from sperm-fertilising efficiency. This fitness component might be affected by an interaction with the sperm recipient, i.e., certain sperm may perform better in certain, but not all, sperm recipients (i.e., male × female interaction; e.g., Clark et al. 1999). Moreover, sperm-fertilising efficiency is also expected to depend on the interactions between competing sperm from different donors (i.e., male × male interaction; e.g., Clark et al. 2000). Such complex interplay between the sperm donors and the sperm recipients might be involved in explaining part of the variance observed in sperm-fertilising efficiency. These interactions may be studied by realising a three-way fully factorial experimental design in which fixed genetic lines are used as focal donors, competitors and recipients. Thus, by assessing the mating success, the sperm-transfer efficiency, the sperm-fertilising efficiency and the resulting male reproductive success of the focal individuals within the different crosses, one should be able to assess the relative contributions of the recipient's line and the competitor's line to the success of the focal's line at each of the mentioned episodes of selection. Although several GFP(+) and GFP(-) inbred lines are already available in the lab, such an experiment requires a large sample size so that it will unfortunately be difficult to overcome the technical limitations.

In addition, chapter IV indicates that testis size seems to be under sexual selection, presumably because individuals with bigger testis manage to successfully transfer more sperm per copulation. However, care should be taken before one interprets this correlation as a causal relationship. My study investigated how phenotypic traits correlate with different fitness components. Thus, the reported selection differentials represent the direct operation of selection on the trait (i.e., direct selection), and/or the consequences of selection operating on correlated traits (i.e., indirect selection). For instance, testis size may be correlated with other traits (e.g., production of prostate gland secretions) that may contribute to the positive effect of testis size on sperm-transfer efficiency. The interdependence between measured traits may be investigated by using multivariate analyses such as the principal components analysis (PCA). PCA can be extremely useful (e.g., Anthes et al. 2010; Firman and Simmons 2010), but it has the drawback to decouple the characterisation of the phenotypic traits from the fitness estimates. An alternative approach, the canonical analysis, allows determining major axes based on the fitness estimates (Blows and Brooks 2003; Blows 2007; Hall et al. 2008), and therefore might represent a promising technique to use. Moreover, the use of multivariate analyses would facilitate to test for stabilizing (or disruptive) selection, which I would expect to occur on certain phenotypic traits in *M. lignano*, such as stylet shape and sperm morphology. To summarise, the directional selection I found on testis size should be considered as a first step

of identifying traits that are sexually selected in *M. lignano*. There is a clear need for a more complete understanding of the operation of sexual selection in *M. lignano* that requires first to investigate both linear and non-linear forms of selection on combinations of traits, and second to experimentally manipulate a specific trait and test the fitness consequences to demonstrate a causal relationship (e.g., Khila et al. 2012; Sekii et al. 2013). I am convinced that all the tools available in *M. lignano* will help to reveal fascinating process in the operation of sexual selection on phenotypic traits.

Sex allocation is a fundamental concept in the evolution and maintenance of simultaneous hermaphroditism. Simultaneous hermaphrodites are expected to be more female biased in small mating groups and increase their allocation towards their male function when the mating group size increases. **Chapter V** provides the first empirical evidence explicitly testing a fundamental theory that was previously supported only by more indirect evidence (i.e., using density or social group size as proxy for mating group size). Results of this study suggest a nonlinear relationship between sex allocation and mating group size as predicted by sex allocation theory. The underlying reason for the observed phenotypically plastic response is supposed to be that (1) the male fitness gain curve shows diminishing fitness returns, while the female gain curve is thought to be linear, and (2) that the male fitness gain curve becomes more linear with increasing mating group size (Charnov 1979, 1980, 1982). It is therefore very unfortunate that, at present, there is little empirical evidence showing a saturating fitness gain curve (Yund 1998; Johnson and Yund 2009). Moreover, these two studies were performed in a single spermcast mating species. Therefore, additional empirical tests are clearly needed to understand the shape of fitness gain curves in internally fertilising species, as well as the different factors that can influence it.

Moreover, in its current form the mating group size model assumes a fair-raffle sperm competition scenario. The results of chapters III and IV show that this is not true in *M. lignano*, and this is probably the case in most sexual organisms. This has substantial implications because a complex interplay is expected to occur between sexual selection and sex allocation. Indeed, sex allocation is expected to be affected by any mechanisms of sexual selection causing paternity skews (Schärer and Pen 2013), such as sperm displacement (Charnov 1996), sperm digestion (Greeff and Michiels 1999), cryptic female choice (van Velzen et al. 2009) or random paternity skews (Greeff et al. 2001; Schärer and Pen 2013). Thus more detailed investigations on the role of these processes in driving sex allocation in *M. lignano* and, more broadly, in simultaneous hermaphrodites are clearly needed. Such experiments, however, will be challenging to realise because the mechanisms underlying paternity skews are difficult to manipulate experimentally.

The measures used to characterise the resources allocated towards the male and the female functions relies exclusively on gonad size (and thus presumably on gamete production rate; Schärer et al. 2005; Schärer and Vizoso 2007), there

are probably other traits to which individuals can allocate their resources in order to gain fitness, including pre- and post-copulatory traits (Schärer and Pen 2013). For instance, in the male function, one can expect individuals to trade-off resources towards sperm production, seminal fluid production and mating acquisition (i.e., mate searching ability), and the optimal allocation towards one or another of these traits should depend on the fitness returns. Hence it would be of great interest to empirically investigate several male and female fitness components to determine what conditions influence, for instance, the allocation towards the pre- versus post-copulatory traits (Parker et al. 2013; Schärer and Pen 2013).

In conclusion, my PhD studies are in support of other studies indicating that sexual selection is an important agent of selection in the simultaneous hermaphrodites. My studies took advantage of several features of *M. lignano* to reach novel insights on the operation of sexual selection. I presume the main contribution of my PhD works is the quantification of sexual selection along episodes of selection. This study nicely integrated post-copulatory episodes of selection into the quantification of sexual selection, and it revealed that they have a larger opportunity for selection than mating success. In accordance, my PhD studies also stressed that copulations are complex interactions in which partners develop traits to optimize their own fitness, notably I suggested that individuals can displace the sperm of previous donors, and manipulate the behaviour of the recipient to increase the fertilisation success of the transferred sperm. Finally my studies highlighted the potent role of testis size in *M. lignano* where individuals that have larger testis seem to transfer more sperm per copulation, and thereby to sire more offspring.

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Curriculum Vitae

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Education

2010-present Ph.D. in Evolutionary Biology, supervised by L. Schärer, Zoological Institute, University of Basel, Switzerland.

2007-2009 M.Sc. in Ecology and Evolutionary Biology, University of Rennes 1, France.

2004-2007 B.Sc. Degree in Biology of organisms, University of Caen, France.

Publications

Marie-Orleach L., Janicke T., Eichmann M., De Mulder K., Berezikov E., Ladurner P., Vizoso D.B. and Schärer L. (in prep) Fluorescent sperm in a transparent worm – Validation of the GFP techniques to study sexual selection.

Marie-Orleach L., Roussel J.-M., Bugeon J., Tremblay J., Ombredane D. and Evanno G. (in press) Melanin-based coloration of sneaker male Atlantic salmon is linked to the viability and emergence timing of their offspring.. *Biological Journal of the Linnean Society.*

Janicke T., Marie-Orleach L., De Mulder K., Berezikov E., Ladurner P., Vizoso D.B. and Schärer L. 2013. Sex allocation adjustment to local sperm competition in a simultaneous hermaphrodite. *Evolution.* 67: 3233-3242.

Marie-Orleach L., Janicke T. and Schärer L., 2013. Effects of mating history on copulatory and post-copulatory behaviour in a simultaneous hermaphrodite. *Animal Behaviour* 85: 453-461.

Mander L., Marie-Orleach L. and Elliott M., 2013. The value of wader foraging behaviour study to assess the success of restored intertidal areas *Estuarine, Coastal and Shelf Science* 131: 1-5.

Conferences

Marie-Orleach L., Janicke T., Vizoso D.B. and Schärer L. September 2013. Poster. Quantifying Pre- and Post-Copulatory Episodes of Sexual Selection in a Simultaneous Hermaphrodite. Biology of Spermatozoa 2011, Sheffield, UK.

Marie-Orleach L., Janicke T., Vizoso D.B. and Schärer L. September 2013. Oral Communication. Quantifying Pre- and Post-Copulatory Episodes of Sexual Selection in a Simultaneous Hermaphrodite. European Meeting of PhD Students in Evolutionary Biology, Cornwall, UK.

Marie-Orleach L., Janicke T., Vizoso D.B. and Schärer L. August 2013. Oral Communication. Quantifying Pre- and Post-Copulatory Episodes of Sexual Selection in a Simultaneous Hermaphrodite. Congress of the European Society for Evolutionary Biology, Lisbon, Portugal.

Marie-Orleach L., Janicke T., Schlatter A., Sekii K., Vizoso D.B. and Schärer L. June 2013. Oral Communication. Traits Involved along the Pre- to Post-Copulatory Axis of Selection in *Macrostomum lignano*. Simultaneous Hermaphroditic Organism Meeting, Thessaloniki, Greece.

Marie-Orleach L., Janicke T. and Schärer L. May 2013. Poster. To Suck or not to Suck? Effects of Mating Status on a Postcopulatory Behaviour in a Simultaneous Hermaphrodite. PhD Students Meeting, Neuchâtel, Switzerland.

Marie-Orleach L., Janicke T., Eichmann M., De Mulder K., Berezikov E., Ladurner P., Vizoso D.B. and Schärer L. November 2012. Oral communication. Fluorescent Sperm in a Transparent Worm – Validation of GFP Techniques to Study Post-copulatory Sexual Selection. 6th International Macrostomum Meeting, Basel, Switzerland.

Marie-Orleach L., Janicke T., Eichmann M., Vizoso D.B., De Mulder K., Berezikov E., Ladurner P. and Schärer L. February 2012. Oral communication. Fluorescent Sperm in a Transparent Worm – Post-Copulatory Sexual Selection in a Simultaneous Hermaphrodite. Simultaneous Hermaphroditic Organism Meeting, Turin, Italy.

Marie-Orleach L., Janicke T., Eichmann M., De Mulder K., Berezikov E., Ladurner P., Vizoso D.B. and Schärer L. November 2011. Oral communication. Mating Success and Reproductive Success in a Simultaneous Hermaphrodite. 5th International Macrostomum Meeting, Basel, Switzerland.

Marie-Orleach L., Janicke T., Eichmann M., De Mulder K., Berezikov E., Ladurner P., Vizoso D.B. and Schärer L. September 2011. Oral communication. Mating Success and Reproductive Success in a Simultaneous Hermaphrodite. Biology of Spermatozoa 2011, Sheffield, UK.

Marie-Orleach L., Janicke T., De Mulder K., Berezikov E., Ladurner P., Vizoso D.B. and Schärer L. August 2011. Oral communication. Mating Success and Reproductive Success in a Simultaneous Hermaphrodite. Petit Pois Déridé, Toulouse, France.

Marie-Orleach L., Janicke T. and Schärer L. February 2011. Poster. Copulatory and Post-Copulatory Mating Behaviours Depend on the Mating Status of Both Partners in a Simultaneous Hermaphrodite. Biology 2011, Zürich, Switzerland.

Marie-Orleach L., Janicke T. and Schärer L. November 2010. Oral communication. Copulation Behaviour and Mating History in *Macrostomum lignano*. 4th International Macrostomum Meeting, Basel, Switzerland.

Marie-Orleach L., Roussel J.M., and Evanno G. September 2010. Oral communication. Colouration of Atlantic salmon (*Salmo salar*) Sneaker Males Linked to the Viability and Emergence Timing of their Offspring. New Directions in Sexual Selection Research: Unifying Behavioural & Genomic Approaches, Bath, UK.

Students Supervision

Mouginot P. (2nd year Master) 2012, "*Postcopulatory Behaviour as an Interacting Phenotype*", University of Basel, Switzerland.

Baumgarten F. and Leu C. (3rd year Bachelor) 2012, "*Effect of Genetic Compatibilities on Hatching Success in Macrostomum lignano*", University of Basel, Switzerland.

Leu C. (2nd year Bachelor) 2011, "*Effect of the Green Fluorescent Protein Expression on the Male and Female Reproductive Abilities of Macrostomum lignano*", University of Basel, Switzerland.

Kälin M. and Meyer A. (3rd year Bachelor) 2011, "*Effect of Mating History on Sperm Competition and Siring Success*", University of Basel, Switzerland.

Eichmann M. and Moser D. (3rd year Bachelor) 2010, "*Function of the Post-Copulatory Suck Behaviour of Macrostomum lignano*", University of Basel, Switzerland.

Workshops and Courses

2013 Social Genetic Effects: Theory and Genetic Analysis, Wageningen, The Netherlands.

2012 Scientific Writing Clinic, Neuchâtel, Switzerland.

2012 Big Questions in Behavioural Biology, Neuchâtel, Switzerland.

2012 Writing Grant Proposals, Neuchâtel, Switzerland.

2011 Applied Statistical Regression Modelling for Biologists using R, Neuchâtel, Switzerland.

2011 Statistical Analysis of Siring Success and Relatedness, Neuchâtel, Switzerland.

2010 Introduction to Evolutionary Genetics, IST Austria, Austria.

2010 Workshop for Ph.D. students in Evolutionary Biology, Guarda, Switzerland.

Additional Information

Reviewer for: Animal Behaviour
Biological Journal of the Linnean Society
BMC Evolutionary Biology
Evolution
Journal of Ethology

Co-organizer of: The Simultaneous Hermaphroditic Organism Meeting, Basel, February 2011.
The weekly "Journal Club", Basel, spring semester 2013
The bi-weekly "Interaction Seminar", Basel (September 2010 to December 2011).

Grants: 2013 Travel grant, Interuniversity Doctoral Program in Organismal Biology (CHF 700).
2013 Travel grant, University of Basel (CHF 700).
2013 Doctoral studies completion, University of Basel (CHF 9700).
2013 Doctoral studies completion, FAG Basel (CHF 14000).
2011 Travel grant, Interuniversity Doctoral Program in Organismal Biology (CHF 800).
2011 Travel grant, University of Basel (CHF 300).
2010 Travel grant, University of Basel (CHF 650).

Membership: European Society for Evolutionary Biology.
Swiss Zoological Society.
Société Française d'Écologie.