Impacts of sperm competition on mating behaviour and life history traits in a simultaneous hermaphrodite

Impacts of sperm competition on mating behaviour and life history traits in a simultaneous hermaphrodite

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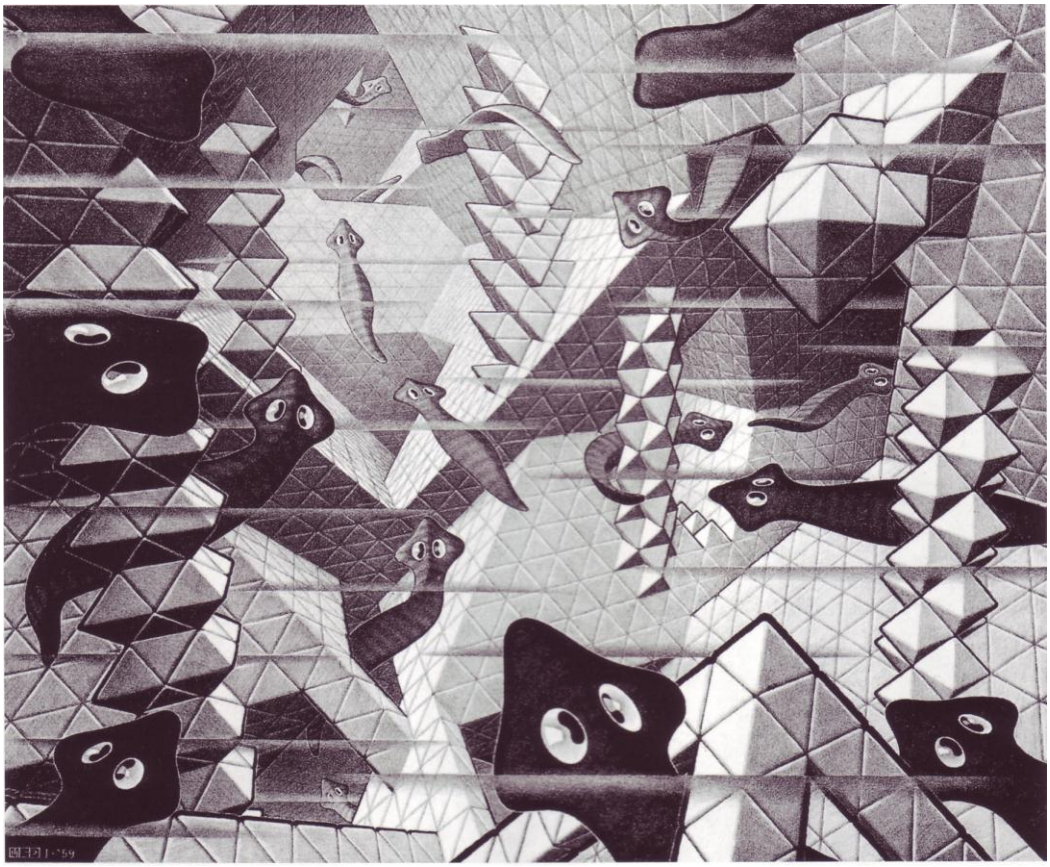

“Bricks are mostly rectangular (...). However, if one is interested in arrangements of non-cubic elements, one will find other possibilities. For instance, one can use tetrahedrons and octahedrons alternately. The building depicted above is composed of these two basic geometric shapes. For human inhabitants it is rather impractical because it contains neither vertical walls nor horizontal floors. However, if it is filled with water flatworms can live in it.”

M. C. Escher (1959)

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Abstract

Evolutionary theory suggests that post-copulatory sexual selection plays an important role in the evolution of reproductive traits of sexually reproducing animals. But despite its alleged universality empirical evidence is scarce for sexual selection operating in simultaneous hermaphrodites. I therefore investigated the potential for post-copulatory sexual selection in such an organism. Sexual selection can also act on phenotypic plasticity of traits. Flexible adjustments of an individual's own sex allocation have been proposed to be a major advantage of hermaphrodites compared to separate-sexed organisms. The simultaneous hermaphrodite *M. lignano* flexibly adjusts its sex allocation to group size. I aimed to narrow down the cues on which this flatworm relies to make this adjustment, and I measured the costs of such phenotypically plastic responses to group size. I tested for mate limitation in a natural population of this outcrossing hermaphrodite as one possibile condition where simultaneous hermaphroditism is advantageous.

In a double mating experiment I revealed genetic variation in paternity success and in five traits. One of them, mating rate, significantly predicted paternity success. This trait has recently been shown to be phenotypically plastic. I here demonstrate that it also exhibits genetic variation. Hence, it might be subjected to sexual selection. The findings of multiple paternity and genetic variation in paternity success clearly suggest that there is an opportunity for sexual selection in this simultaneous hermaphrodite. I discuss possible mechanisms of sexual selection (sperm competition, female bias in favour of one sperm donor) and random paternity skews that may underlie the paternity patterns observed in this species.

Further results suggest that the well-documented phenotypically plastic response in sex allocation was based on indirect cues for sperm competition such as tactile cues of group size rather than direct cues such as assessment of the partner's mating status. I also demonstrate that this response incurred significant production costs of phenotypic plasticity. However, since the magnitude of these costs was relatively low, I argue that flexible adjustments of sex allocation may still convey a net benefit to simultaneous hermaphrodites.

Mate availability did not appear to seriously limit female fitness in a natural habitat of *M. lignano*. This is consistent with classical sexual selection theory, originally developed for separate-sexed species. Specifically, one aspect

of Bateman's principle states that female fecundity is not limited by the availability of mating partners but by resources available for egg production, which seems to apply to this simultaneous hermaphrodite.

I conclude that sexual selection occurs in this simultaneous hermaphrodite. I rule out two presumptive cues for the phenotypically plastic response to group size and demonstrate production costs of this plasticity. Finally, I judge the significance of phenotypic plasticity and mate availability for the evolution of simultaneous hermaphroditism.

Zusammenfassung

Die Evolutionstheorie besagt, dass post-kopulatorische sexuelle Selektion eine wichtige Rolle in der Evolution von Fortpflanzungsmerkmalen sich sexuell vermehrender Tiere spielt. Trotz ihrer angenommenen Allgemeingültigkeit gibt es wenig empirische Belege für sexuelle Selektion bei Simultanzwittern. Ich untersuche deshalb hier das Potential für post-kopulatorische sexuelle Selektion in einem solchen Organismus. Sexuelle Selektion kann auch auf die phänotypische Plastizität von Merkmalen wirken. Flexible Einstellung der Investition in beide Geschlechter gilt als ein bedeutender Vorteil von Zwittern gegenüber getrenntgeschlechtlichen Organismen. Der Simultanzwitter *M. lignano* ändert die Investition in das männliche und weibliche Geschlecht je nach Gruppengröße. Ich grenze hier die möglichen Auslöser ein, nach denen sich die Plattwürmer bei diesen Reaktionen richten, und ich messe die Kosten für solch phänotypisch plastische Reaktionen auf eine veränderliche Gruppengröße. Außerdem teste ich Plattwürmer in ihrem natürlichen Lebensraum auf Partnermangel, eine Bedingung unter der das Simultanzwittertum als vorteilhaft gilt.

In einem Doppelpaarungs-Experiment fand ich genetische Variation im Vaterschaftserfolg und in fünf Merkmalen. Eines davon, die Paarungsrate, prädizierte den Vaterschaftserfolg. Vor kurzem wurde phänotypische Plastizität in diesem Merkmal gefunden. Ich zeige hier, dass es auch genetische Variation aufweist. Also kann sexuelle Selektion möglicherweise darauf wirken. Die Feststellung von multipler Vaterschaft und genetischer Variation in Vaterschaftserfolg weisen darauf hin, dass sexuelle Selektion bei diesem Simultanzwitter wirken kann. Ich diskutiere mögliche Mechanismen der sexuellen Selektion (Spermienkonkurrenz, weibliche Wahl) und zufällige Verzerrungen der Vaterschaft, die bei dieser Art vorliegen können.

Weitere Ergebnisse dieser Arbeit legten nahe, dass die gut dokumentierte phänotypisch plastische Reaktion in der Investition sexueller Ressourcen auf indirekten Indikatoren für die Gruppengröße, wie z.B. Berührungsreizen, beruht statt auf direkter Wahrnehmung etwa des Paarungsstatus' von Partnern. Ich demonstriere auch, dass diese Reaktion signifikante Kosten phänotypischer Plastizität nach sich ziehen kann. Da die gefundenen Kosten aber relativ niedrig waren, argumentiere ich, dass die

flexible Einstellungen der Investition sexueller Ressourcen immer noch einen Netto-Vorteil für Simultanzwitter darstellen könnten.

Partnermangel schien die weibliche Fortpflanzung in einem natürlichen Lebensraum nicht ernsthaft zu begrenzen. Dies entspricht der klassischen Theorie der sexuellen Selektion, die ursprünglich für getrenntgeschlechtliche Organismen entwickelt wurde. Ein Aspekt von Bateman's Prinzip besagt, dass die weibliche Fortpflanzung nicht von der Partnerverfügbarkeit entscheidend abhängt, sondern von der Ressourcenverfügbarkeit für die Eierproduktion. Das scheint auf diesen Zwitter zuzutreffen.

Ich trage hiermit zu der Ansicht bei, dass sexuelle Selektion bei Simultanzwittern vorkommt. Ich schließe zwei mögliche Auslöser für phänotypisch plastische Reaktionen auf die Gruppengröße aus und demonstriere Produktionskosten von Plastizität. Schließlich erörtere ich die Bedeutung von phänotypischer Plastizität und Partnerverfügbarkeit für die Evolution von Simultanzwittertum.

Introduction

Introduction

In this introduction I outline the general theme of my thesis, namely sexual selection in simultaneously hermaphroditic animals. After giving a definition of hermaphroditism I introduce the resource allocation model that underlies most optimality models of sex allocation. This connects to sexual selection, which is predicted to influence optimal sex allocation. Next I therefore introduce the concept of sexual selection, which includes processes that take place prior to and after copulation. Although the potential for pre-copulatory sexual selection in simultaneous hermaphrodites has been predicted to be small compared to species with separate sexes, I briefly review the evidence that is currently available for both pre- and post-copulatory sexual selection. Thereafter, I discuss some conditions that may contribute to the evolutionary stability of simultaneous hermaphroditism. Specifically, both a net benefit of phenotypically plastic or flexible responses of sex allocation and low opportunities for mating are considered to offer a benefit for simultaneous hermaphroditism compared to separate sexes. Finally, I outline the objectives of this thesis and present the model organism.

What's a hermaphrodite?

The biological term "hermaphrodite" originates from the Greek mythology. According to Ovid (Metamorphoses, Book IV), Hermaphroditos, the son of Hermes and Aphrodite, fused with a nymph called Salmacis. This resulted in Hermaphroditos having physical traits of both sexes. Modern biologists use "simultaneous hermaphrodite" to refer to an individual that possesses both male and female sex functions at the same time for at least part of its life. In contrast, "sequential hermaphrodite" means an individual that starts its reproductive life as a male (protandry) or as a female (protogyny) and changes sex later in life (Charnov 1982). Simultaneous hermaphrodites, the object of this thesis, occur in all animal phyla, except insects and vertebrates other than bony fishes (Anthes 2010; Ghiselin 1969; Jarne and Auld 2006; Michiels 1998). In this reproductive mode each individual produces male and female gametes at the same time. Broadcast-spawning simultaneous hermaphrodites release male and female gametes, spermcast organisms, e.g. marine invertebrates and land plants release only male gametes into the environment but retain female gametes, and copulating animals inseminate their partner during a copulation, followed by

internal fertilization. Copulations can be uni- or bi-directional (reciprocal). In contrast to a common preconception, many simultaneous hermaphrodites are incapable of self-fertilization (e.g., Jarne and Auld 2006). In this thesis I focus on obligately outcrossing simultaneous hermaphrodites with copulation and internal fertilization.

For a simultaneous hermaphrodite, which produces sperm and eggs at the same time, it is a pivotal decision how to divide the resources that are available for reproduction between the male and the female sex function. Sex allocation theory for simultaneous hermaphrodites predicts the optimal sex allocation, i.e. the proportion of all reproductive resources devoted to the male function that maximizes the sum of male and female fitness, depending on the shapes of so-called fitness gain curves of each sex function (Charnov 1979, 1982). It also specifies conditions influencing the shapes of these curves (e.g. local sperm competition, Schärer 2009). This body of theory is a success story because it combines several fields of evolutionary biology and it has been successfully tested in a number of species. Several empirical studies have manipulated mating group size via social group size and reported phenotypically plastic responses in male allocation that went in the predicted direction (reviewed in Schärer 2009).

A basic assumption of sex allocation theory for simultaneous hermaphrodites is the trade-off between reproductive resources allocated to the male and to the female function (Charnov 1982), if both functions draw on a finite common pool of resources: an upregulation of the investment of resources into the male function is linked to decreasing investment into the female function and *vice versa*. Empirical evidence for the trade-off in the sex allocation of a simultaneous hermaphrodite comes from a marine flatworm (Janicke and Schärer 2009b; Janicke and Schärer 2010; Schärer et al. 2005; reviewed in Schärer 2009).

Pre-copulatory sexual selection

In species with separate sexes (hereafter called gonochorists), pre-copulatory sexual selection is known to have played a central role in shaping the great variety in ornaments, courtship, and mating behaviour that is observed in species with this reproductive mode (Darwin 1871). In stark contrast to the situation in gonochorists several theoretical studies suggest that there is a relatively low potential for pre-copulatory sexual selection in copulating hermaphrodites (reviewed in Arnqvist and Rowe 2005). However, some bizarre mating behaviours have also been reported for this group of animals (Baur 1998; Charnov 1979; Koene et al. 2005; Koene and Schulenburg 2005;

Michiels 1998), which indicates that sexual selection very likely operates in simultaneous hermaphrodites. And indeed, there is some evidence for pre-copulatory mate choice in simultaneous hermaphrodites (reviewed by Anthes 2010). Several simultaneously hermaphroditic species prefer larger partners (e.g., Anthes et al. 2006; Lüscher and Wedekind 2002; Michiels et al. 2001; Vreys and Michiels 1997), presumably because insemination is costly and body size is a predictor of female fecundity in these species. A sea slug has been shown to avoid mating with a partner carrying a spermatophore, i.e. possibly because this indicates that this individual has recently mated (e.g., Haase and Karlsson 2004), and that the sperm donor would thus face sperm competition. But also internally fertilizing flatworms and sea slugs can discriminate against previously mated partners (Anthes et al. 2006; Michiels and Bakovski 2000). This behaviour may reduce sperm competition or avoid partners that are depleted in sperm they can donate. Genotypes of the unilaterally inseminating freshwater snail *Biomphalaria glabrata* that are resistant to infections with *Schistosoma mansoni* discriminate, at least when mating in the female role, against partners infected with or susceptible to this parasite. Such partners would pass the susceptibility genes to their offspring (Webster and Gower 2006). Two freshwater snail species of another genus exert mate choice based on the relatedness between the partners, thereby avoiding inbreeding (e.g., Facon et al. 2006; McCarthy and Sih 2008).

However, pre-copulatory sexual selection in simultaneous hermaphrodites does not seem to lead to exaggerated ornaments, as is seen in many separate-sexed species (Fisher 1930; Lande 1981; van Doorn et al. 2004). The opportunity for pre-copulatory sexual selection in simultaneous hermaphrodites might be reduced because both partners show a mutual willingness to mate (Anthes et al. 2010; Anthes et al. 2006; Charnov 1979). This would, e.g., be true if simultaneous hermaphrodites engaged in multiple matings mainly in order to donate rather than receive sperm, as is often assumed (e.g., Charnov 1979; but see Leonard 1990).

Post-copulatory sexual selection

Unlike pre-copulatory sexual selection, post-copulatory sexual selection has been suggested as the major evolutionary agent shaping reproductive traits in simultaneous hermaphrodites (Angeloni et al. 2002; Charnov 1979; Charnov 1996; Greeff and Michiels 1999; Greeff et al. 2001; Michiels 1998; Michiels et al. 2009; Pen and Weissing 1999; van Velzen et al. 2009; reviewed in Schärer 2009).

The presumed general willingness to mate, which I mentioned above, probably leads to the receipt of sperm from several partners, on the one hand including sperm that may be unwanted for fertilization of the own eggs. On the other hand, the simultaneous presence of sperm of different sperm donors in a female genital tract provides ample opportunities for post-copulatory sexual selection, which is still fostered by high mating rate with multiple partners, internal fertilization and sperm storage (Janicke and Schärer 2009a; Koene et al. 2009; Michiels 1998). Post-copulatory sexual selection can operate via sperm competition, defined as "the competition between the sperm of two or more males for the fertilization of a given set of ova" (Parker 1998), or via cryptic female choice (Charnov 1979; Thornhill 1983), defined as "nonrandom paternity biases resulting from female morphology, physiology, or behaviour that occur after coupling" (Pitnick and Brown 2000). Sperm competition in simultaneous hermaphrodites has not been studied extensively, but it seems common in some species (for reviews see Anthes 2010; Baur 1998; Michiels 1998). Evidence for cryptic female choice is almost absent in simultaneous hermaphrodites. In the oviduct of the spermcast mating colonial ascidian *Diplosoma listerianum* only non-self sperm of certain genotypes are accepted, while other sperm are phagocytosed (Bishop et al. 1996). A cruder mechanisms of female choice in a mobile simultaneous hermaphrodite with copulation might be the suck behaviour in the flatworm *M. lignano*, which is performed by one or both partners following a copulation (Schärer et al. 2004a), but the actual function of this behaviour remains unclear.

Overall, solid empirical evidence for post-copulatory sexual selection comes mainly from gonochorists, and is relatively scarce for other reproductive modes. Cases for sperm competition have been reported from several simultaneous hermaphrodite species, but hardly any traits have been suggested to be subject to post-copulatory sexual selection (but see Janicke and Schärer 2009). This starkly contrasts with the central role that is commonly attributed to sexual selection for the evolution of sexually reproducing organisms, including simultaneous hermaphrodites (e.g., Arnold 1994; Charnov 1979; Ghiselin 1969; Morgan 1994). Expanding the evidence of post-copulatory sexual selection to simultaneous hermaphrodites would underline the alleged universal importance of sexual selection.

For simplicity, most sex allocation models explicitly assume random mating and equal chances of all sperm to fertilize an egg. However, local sperm competition, a crucial predictor of optimal male allocation, can not only result from pre-copulatory sexual selection (e.g., Anthes 2010; Arnqvist and Rowe

2005), mating behaviour (e.g., Eberhard 1996; Petersen 1991), and post-copulatory sexual selection (Eberhard 1996; Parker 1970; Thornhill 1983), but also from random paternity skews (e.g., Greeff et al. 2001; see Schärer 2009). Random paternity skews involve stochastic effects on paternity due to mate encounter probability, imperfect sperm mixing inside the female genital tract, or sperm loss (e.g., Greeff et al. 2001; Harvey and Parker 2000).

Why to be a hermaphrodite?

Since simultaneous hermaphrodites combine two sexes in the same body, and since there are probably some fixed costs to be paid for maintaining both sexual functions at the same time (Charnov 1979, 1982), it is an important question to ask why simultaneous hermaphroditism should be advantageous compared to gonochorism, i.e. separate sexes. Michiels (1998) has argued that a major advantage of simultaneous hermaphroditism compared to gonochorism is the possibility of phenotypically plastic adjustment of sex allocation. Also gonochorists can plastically adjust the sex ratio of their offspring, but while the effect of the adjustment is always shifted by one generation in gonochorists, it concerns the own current reproduction of simultaneous hermaphrodites via the male or the female function. This presumed advantage requires that simultaneous hermaphroditites can perceive a reliable signal for the crucial parameters, e.g. the level of local sperm competition or local resource competition (Charnov 1982; Lloyd 1982; reviewed in Schärer 2009), and that the benefit of opportunistic sex allocation is large enough to outweigh any fix costs of having two sex functions (Charnov 1979, 1982). A response to sexual selection is also possible if there is variation in phenotypic plasticity of a reproductive trait. If there are sufficient fluctuations in the level of local sperm competition this can also favour simultaneous hermaphroditism because of its advantage of phenotypically plastic or flexible responses in sex allocation. However, phenotypic plasticity might come at a cost and would have to provide a net benefit for simultaneous hermaphrodites to be stable, i.e. any potential costs of phenotypic plasticity would have to be outweighed by benefits (Pigliucci 2001; St. Mary 1997; West and Sheldon 2002). As a rule, simultaneous hermaphroditism is stable if the male or the female fitness gain curve saturates, so that the fitness set of a simultaneous hermaphrodite is convex, i.e. higher than the fitness of a pure male or a pure female. A simultaneously hermaphroditic population can then not be invaded by a pure male or a pure female (the resource allocation model, Charnov 1982).

A classical argument for the stability of simultaneous hermaphroditism involves low opportunities for mating (Darwin 1876; Ghiselin 1969; Tomlinson 1966). These can result from a sedentary lifestile, low mobility, low density, or small, genetically isolated populations (the low density model, Ghiselin 1969). In this view, being a hermaphrodite could avoid the problem of encountering a conspecific that does not have the opposite sex. If an unstable population in a frequently disturbed environment is, e.g., reduced to only two individuals it will have twice the chances to survive if it is simultaneously hermaphroditic compared to a gonochoristic population. The same is true for two internal parasites that find themselves in the same host or two specimens of a reef-dwelling fish species settling on a small reef. Such conditions are likely to bring about mate limitation for the individuals living there.

Objectives

(1) In the experiment presented in CHAPTER 1 the potential for post-copulatory sexual selection was investigated in the simultaneous hermaphrodite *Macrostomum lignano*. I aimed at testing for genetic variation in body size, one behavioural and three morphological traits, and for genetic variation in paternity success in a double mating experiment. As evolutionary theory suggests that post-copulatory sexual selection plays an important role in the evolution of reproductive traits, I aimed at testing to what extent the four measured reproductive traits predict paternity success in the chosen competitive situation. In the same experiment I aimed at studying other mechanisms of paternity skew.

(2) *M. lignano* responds to changes in social group size with a phenotypically plastic change in testis size and/or sex allocation (Brauer et al. 2007; Janicke and Schärer 2009a, 2010; Schärer and Ladurner 2003; Schärer et al. 2005), testicular activity (Schärer et al. 2004b), sperm production rate (Schärer and Vizoso 2007), and mating rate (Janicke and Schärer 2009b). Responses in male allocation such as these are predicted for increasing mating group size, but the mechanisms by which these flatworms assess changes in the social group size or mating group size are unknown. It is important to know these mechanisms when one aims at manipulating mating group size, all else being equal. If other factors are manipulated at the same time (e.g., density) these will thereafter be confounded with the intended treatment. Therefore the following presumptive signals for the flatworm *M. lignano* to increase male allocation in larger groups were evaluated in CHAPTER 2: partner identity (this requires individual recognition), and mating status of the partner (monogamy vs. polygamy).

(3) Phenotypically plastic sex allocation is considered to be an advantage of simultaneous hermaphroditism compared to gonochorism because it allows an immediate adjustment of sex allocation to current conditions. However, this would have to be a net advantage after any possible costs of phenotypic plasticity have been taken into account. Environment-dependent costs such as production costs of phenotypic plasticity are commonly assumed, but have rarely been demonstrated experimentally. In CHAPTER 3 the hypothesis was tested that the response of *M. lignano* to changing group size incurs some costs in a fitness-proxy, i.e. hatchling production. To my knowledge such costs have not been reported to date in a simultaneous hermaphrodite, presumably because the expected costs are small.

(4) Simultaneous hermaphroditism is predicted to be advantageous if, e.g., population density of an outcrossing species is very low and reproduction is limited by availability of mating partners. A test for mate limitation in a natural population of *M. lignano* is presented in CHAPTER 4. The effects of supplementation of field-caught worms with an additional mating partner on hatchling production were investigated to test for mate limitation in the studied population. Body size, morphology, mating status, and mating behaviour of the field-caught worms were compared to worms grown in the laboratory.

Model organism

The free-living flatworm *Macrostomum lignano* (Fig. 1) is a member of the family Macrostomidae (Platyhelminthes, Rhabditophora, Macrostomorpha). It is a simultaneous hermaphrodite and a member of the interstitial meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). Mass cultures are kept in the laboratory at 20°C in glass Petri dishes containing f/2 medium (Andersen et al. 2005), and fed *ad libitum* with the diatom *Nitzschia curvilineata* (Rieger et al. 1988). Under these conditions worms reach about 1.5 mm in body length, lay about 1.5 eggs per day, and have a generation time of around 18 days. *M. lignano* is outcrossing with reciprocal and very frequent copulation and internal fertilization (Schärer et al. 2004a; Schärer and Ladurner 2003). The transparent body of *M. lignano* allows *in vivo* measurement of the size of the paired testes and ovaries (Schärer and Ladurner 2003), the number of received sperm (Janicke et al. 2011), and the morphology of the male copulatory organ, called stylet (Janicke and Schärer 2009a). All relevant details of its morphology, physiology, and behaviour are described in the method sections of chapters 1-4.

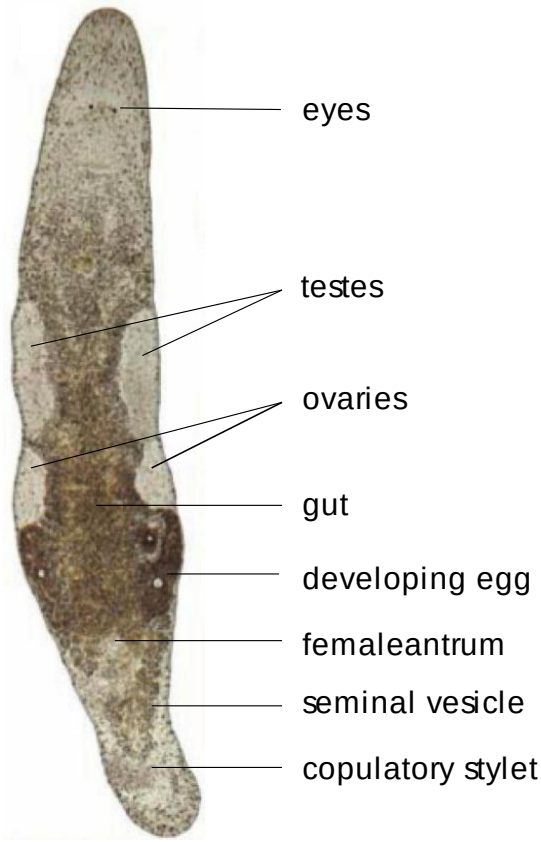

Fig. 1 *Macrostomum lignano*. The size of the whole worm is approximately 1.5mm. (Photo: L. Schärer)

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CHAPTER 1

Genetic variation in mating rate and paternity success in a flatworm

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Abstract

Evolutionary theory suggests that post-copulatory sexual selection plays an important role in the evolution of reproductive traits, which in turn requires genetic variation in such traits within a population. We tested for the presence of genetic variation in an array of morphological and behavioural traits among three standardized focal genotypes of the free-living flatworm *Macrostomum lignano*. Using molecular paternity analysis, we then investigated whether variation in these traits can predict the paternity success of these genotypes in competition against standardized competitors for the fertilization of the eggs of standardized recipients. We found genetic variation in body size, testis size, ovary size, male copulatory organ size (but not shape), mating rate, and paternity success. Our data suggest that only the behavioural trait, mating rate, but none of the measured morphological traits, significantly predicted paternity success. This result suggests that sexual selection might be responsibe for the high mating rates we observe in this simultaneous hermaphrodite. Our results further suggest that there is second male sperm precedence in *M. lignano*, with a mean P_2-value of 0.64 and a U-shaped P_2-distribution. We discuss possible mechanisms of sperm displacement, sperm aggregation, and female choice, which may underlie the observed variation in P_2.

Introduction

Post-copulatory sexual selection (e.g., Charnov 1979; Eberhard 1985, 1996) plays a central role in the evolution of reproductive traits, such as reproductive morphology and mating behaviour, and it can operate via sperm competition *sensu* Parker (1970a, 1998) and/or via cryptic female choice *sensu* Thornhill (1983) and Eberhard (1996). But despite this alleged central role, empirical evidence for post-copulatory sexual selection operating in sexually reproducing animals stems primarily from species with separate sexes, while evidence is much more restricted in species with other reproductive modes, such as sequential and simultaneous hermaphroditism. Empirical support for post-copulatory sexual selection ideally requires, first, studies that quantify intraspecific variation in reproductive traits, second, experiments that show that this variation has a heritable basis, third, investigations that determine to which extent this variation predicts paternity success (i.e., the proportion of offspring sired by a sperm donor), and fourth, experimental manipulation of the relevant traits to show that they causally determine paternity success.

For each of these points there is considerable empirical evidence from studies on separate-sexed species (e.g., Arnqvist and Danielsson 1999; House and Simmons 2003; Fedina and Lewis 2004; Schulte-Hostedde and Millar 2004; Andrade et al. 2009; Hoch 2009; Ramm et al. 2010). However, as we outline below, empirical support for post-copulatory sexual selection coming from studies on simultaneous hermaphrodites is limited with respect to the first point, and almost completely absent with respect to the other three points. We here therefore aim to provide support for the first three points in a simultaneous hermaphrodite in order to contribute to an important expansion of the evidence for sexual selection to this reproductive mode.

In order to understand the mechanisms of post-copulatory sexual selection it is crucial to identify all sources of variation in paternity success. However, such potential sources do not only include variation in reproductive traits, but also the mating order, e.g., to be the first or the second sperm donor (Birkhead and Møller 1998; Simmons 2001), a point we therefore also aim to address here.

Evidence for sexual selection in simultaneous hermaphrodites

In simultaneously hermaphroditic animals with copulation (hereafter called copulating hermaphrodites) complex reproductive morphologies and bizarre mating behaviours are widespread (Charnov 1979; Baur 1998; Michiels 1998;

Koene et al. 2005; Koene and Schulenburg 2005), which makes it very likely that sexual selection operates in these organisms. Several theoretical studies have suggested that there is a relatively low potential for pre-copulatory sexual selection in copulating hermaphrodites (reviewed in Arnqvist and Rowe 2005). Pre-copulatory mate choice in copulating hermaphrodites (reviewed by Anthes 2010) has been found to be based on the partner's body size (e.g., Vreys and Michiels 1997; Michiels et al. 2001; Lüscher and Wedekind 2002; Anthes et al. 2006), its mating history (e.g., Haase and Karlsson 2004; but see Sandner and Schärer 2010), its infection status (Webster and Gower 2006), or the degree of relatedness between the partners (e.g., Facon et al. 2006; McCarthy and Sih 2008). But pre-copulatory sexual selection does not generally appear to lead to exaggerated ornaments, as is seen in many separate-sexed species (Fisher 1930; Lande 1981; van Doorn et al. 2004).

In contrast, post-copulatory sexual selection has been suggested as the major evolutionary agent shaping reproductive traits in copulating hermaphrodites (Charnov 1979; Charnov 1996; Michiels 1998; Greeff and Michiels 1999; Pen and Weissing 1999; Greeff et al. 2001; Angeloni et al. 2002; Michiels et al. 2009; van Velzen et al. 2009; reviewed in Schärer 2009). If, as is often assumed, reproduction in copulating hermaphrodites is limited not by access to sperm to fertilize the own eggs, but by resources available for egg production (Charnov 1979), a mutual willingness to mate in the male role by both partners is expected (Charnov 1979; Anthes et al. 2006; Anthes et al. 2010). This may reduce the opportunity for pre-copulatory sexual selection and increase the mating rate, leading to intense sperm competition if sperm recipients mate multiply (Parker 1970a; Parker 1998). However, solid empirical evidence for post-copulatory sexual selection in copulating hermaphrodites is also relatively scarce (for reviews on sperm competition in molluscs and other copulating hermaphrodites see Baur 1998; Michiels 1998; Anthes 2010). In the following three paragraphs we summarize what is currently known about the influence of reproductive morphology, mating behaviour, and mating order on paternity success in copulating hermaphrodites.

Despite the fact that variation in reproductive morphology is crucial for post-copulatory sexual selection to shape this morphology, there is currently only little quantitative evidence for such intraspecific variation in copulating hermaphrodites, particularly with respect to genital morphology (e.g., Ostrowski et al. 2003; Jordaens et al. 2006; Koemtzopoulos and Staikou 2007; Janicke and Schärer 2009a; Garefalaki et al. 2010), and sperm morphology (e.g., Minoretti and Baur 2006; Janicke and Schärer 2010), while there is more

information on variation in gonad size (generally studied in the context of sex allocation, reviewed in Schärer 2009). Moreover, such variation has rarely been shown to have a heritable basis or to covary with paternity success. Penis morphology predicts paternity success in the barnacle *Semibalanus balanoides* (Hoch 2009). In the garden snail *Cornu aspersum* (formerly called *Cantareus aspersus* and *Helix aspersa*), the length of the epiphallus, the organ forming the head of the spermatophore, is positively correlated with paternity success (Garefalaki et al. 2010). In the free-living flatworm *Macrostomum lignano*, variation in testis size and in the shape of the copulatory organ significantly predicted sperm transfer success (Janicke and Schärer 2009a), and so these traits are good candidates for also predicting paternity success in this species, and may thus be subject to sexual selection.

Regarding mating behaviour, there is also only scarce evidence for intraspecific variation and for its influence on paternity success in copulating hermaphrodites. A particular behavioural trait that has been shown to vary, at least phenotypically, and to affect paternity success in the garden snail *Cornu aspersum* is the shooting of a mucus-delivering "love dart" prior to copulation. If successful, this behaviour enhances paternity success in competition with a poorer dart-shooter (Landolfa et al. 2001; Rogers and Chase 2002; Chase and Blanchard 2006).

Regarding the mating order, only few P_2-values are currently known for copulating hermaphrodites compared to the more extensive knowledge on separate-sexed species (e.g., for insects see Simmons and Siva-Jothy 1998). By convention, P_2 denotes the proportion of offspring that is sired by the second sperm donor in a double mating experiment. P_2-values that vary around a mean of 0.5 indicate that sperm of both partners are equally likely to fertilize, and sperm competition thus conforms to a "fair raffle" (Parker 1990). On the other hand, sperm precedence occurs when the first or the second donor tends to sire more offspring, which is termed first ($P_2 < 0.5$) or second ($P_2 > 0.5$) donor precedence, respectively. P_2 is thought to be somewhat species-specific and to depend on, e.g., the anatomy of the female reproductive tract, its interaction with the received sperm, and the morphology of the male copulatory organ (Birkhead and Møller 1998). The planarian *Schmidtea polychroa* appears to exhibit intermediate P_2-values (Pongratz and Michiels 2003), while last male precedence ($P_2 = 0.73$) has been found in the sea slug *Aplysia californica* (Angeloni et al. 2003). First male precedence has been shown for the land snail *Arianta arbustorum* ($P_2 = 0.34$, Baur 1994) and the garden snail ($P_2 = 0.24$, Evanno et al. 2005; Chase and Blanchard 2006). However, the pattern of sperm

precedence in the garden snail can change when a third competitor gets involved: in a triple mating experiment the first sperm donor achieved lower paternity success than the third donor, but a higher paternity success than the second donor (Garefalaki et al. 2010).

Aims of the present study

In this study we aim at (1) documenting variation in reproductive morphology, mating behaviour, and paternity success, (2) showing that this variation has a genetic basis, and (3) identifying morphological and behavioural predictors of paternity success in the free-living flatworm *Macrostomum lignano* (thereby identifying traits that could be studied experimentally in follow-up studies). To this end we quantify variation in reproductive traits (both morphological and behavioural) among different focal genotypes, and investigate the extent to which this variation predicts differences in the paternity success in a sperm competition experiment, in which two worms compete as sperm donors for the eggs of a sperm recipient. In a 3×2 factorial design the focal donors (members of one of three focal genotypes, created by pair-wise crossing of two inbred lines), are allowed to mate either in the first or second mating order with a recipient (a member of a fourth genotype) in competition with a competitor (a member of a fifth genotype). A significant effect of the genotype of the focal donor on its paternity success would indicate genetic variation in paternity success, a significant effect of the mating order on paternity success would indicate sperm precedence in *M. lignano*, and a significant interaction between genotype and mating order would indicate different sperm defence (mating first) or sperm offence (mating second) abilities among the different genotypes. Finally, we describe the distribution of the P_2-values and discuss our results in the context of possible post-copulatory mechanisms of sexual selection.

Materials and Methods

Study organism

The free-living flatworm *Macrostomum lignano* (Platyhelminthes, Macrostomorpha) is a copulating simultaneous hermaphrodite and a member of the meiofauna of the Northern Adriatic Sea. Study animals are descendants of individuals collected in the same general area near Lignano Sabbiadoro (Italy) between 1995 and 2003 (Ladurner et al. 2005). Mass cultures are kept in the laboratory at 20°C in glass Petri dishes containing f/2 medium (Andersen et al.

2005), and fed *ad libitum* with the diatom *Nitzschia curvilineata* (Rieger et al. 1088). Under these conditions worms reach about 1.5 mm in body length, lay about 1.5 eggs per day, and have a generation time of around 18 days. *M. lignano* is outcrossing with reciprocal and very frequent copulation and internal fertilization (Schärer and Ladurner 2003; Schärer et al. 2004). After about two thirds of all copulations the so-called *suck* behaviour occurs. It consists of a stereotypical posture assumed by one or both partners, which may allow the worms to suck sperm or ejaculate components out of the own female antrum (i.e., the sperm-receiving organ) (Schärer et al. 2004). Received sperm tend to anchor themselves in the antrum by means of a specialized structure, but unanchored sperm can often be seen (Vizoso et al. 2010). The transparent body of *M. lignano* allows *in vivo* measurement of the size of the paired testes and ovaries, the number of received sperm (Janicke et al. 2010), and the morphology of the male copulatory organ, called stylet (Janicke and Schärer 2009a). Individuals that are isolated after mating for 24h can store received sperm in the female antrum for more than 14 days, first laying about one egg per day and eventually running out of sperm (Janicke et al. 2010).

Generating experimental genotypes

Paternity success is a relative measure for sperm competitiveness since it can involve random effects introduced by different competitors (Garcia-Gonzalez 2008) and sperm recipients (Clark et al. 1999; Miller and Pitnick 2002). Specifically, simulations by Garcia-Gonzalez and Evans (2010) suggest that using a random competitor leads to a serious underestimation of genetic variation in sperm competitiveness, while using a standardized competitor yields unbiased estimates of this variation. In order to minimize such random effects in our experiment, we created standardized genotypes for the competitor, the recipient, and the three focal donor genotypes, by making use of highly inbred lines we have established in our laboratory. Each of these inbred lines was started by crossing two virgin worms extracted from our cultures, subsequently using maternal offspring of one of the worms, and thereafter crossing among full- or half-siblings. During the first 15 generations two offspring (full-sib inbreeding), from generation 16 to 24 three offspring (full- or half-sib inbreeding), and since generation 25 ten offspring (high level of inbreeding to maintain the lines) were used to initiate the next generation.

While the use of inbred lines allows tight control of the genotypes, there could be potential negative effects due to inbreeding depression. To avoid such effects, pairs of inbred lines (n = 10) were crossed to generate the five

standardized genotypes that were used in our experiment. Specifically, juveniles of each inbred line produced in mid-January 2009 (i.e., at generation 39) were used as parentals of our experimental animals. Pairs comprised of two juvenile worms from two different inbred lines were assembled in 24-well plates to create the three focal donor genotypes A, B, and C (by crossing lines DV47×DV22, DV28×DV75, and DV71×DV84 respectively), the competitor genotype D (lines DV61×DV69) and the recipient genotype E (lines DV3×DV49). After 17 days, these pairs were allowed to lay eggs in new wells for 12 days, and F1-hatchlings were collected when 0-11 day-old (i.e., 0-11 days after hatching, while still being immature). Two hatchlings per well were isolated as virgins and 'virtually' pooled per genotype so that maximally two worms per genotype had the same mother.

Since worms of each genotype were F1-hybrids of two different inbred lines they were at the same time outbred, statistically independent, and genetically uniform, while having a large number of different mothers, thereby minimizing potential maternal effects that could otherwise be confounded with genotype. The usage of F1-hybrids between inbred lines represents a standard procedure in quantitative genetics, and such hybrids are generally expected to exhibit hybrid vigour (heterosis) if the crossed inbred lines stem from the same general population (Lynch and Walsh 1998, pp. 205-226), as is the case for our inbred lines. We are therefore confident that using F1-hybrids between inbred lines was sufficient to avoid problems of inbreeding depression.

Colouring of recipients

The recipients were coloured before the main experiment by placing them into a solution of 10mg of a red food colourant (New Coccine, E124, Werner Schweizer AG, Wollerau, Switzerland) per ml f/2 for three days, which made them visually distinguishable from the donors after mating. Prior to the main experiment we tested the effect of the colourant on the mating behaviour of *M. lignano* by comparing pairs of two uncoloured worms with mixed pairs (one coloured and one uncoloured worm). Coloration neither significantly affected the mating rate during two hours (all means are given ± 1 s.e. throughout the manuscript unless stated otherwise: mixed pairs, 32.33 ± 2.80; uncoloured pairs, 36.89 ± 2.27; *t*-test, $t = -1.27$, $n = 37$, $P = 0.21$), nor female fecundity measured as total number of viable offspring produced during 20 days (mixed pairs, 2.50 ± 0.61; uncoloured pairs, 1.79 ± 0.47; Wilcoxon test, $\chi^2 = 0.59$, $n = 37$, $P = 0.44$). This suggests that this method of marking individuals has no strong effects on their reproductive performance.

Sperm competition experiment

30-48 days old recipients were mated successively to two sperm donors. Previous studies on *M. lignano*, which involved the microscopic observations of received sperm in the female antrum using methods described elsewhere (Janicke et al. 2010), have shown that (1) during the first copulation between two virgin worms typically only 12.5 ± 2.9 sperm are stored, (2) this first copulation results on average in only 1.7 ± 0.3 hatchlings, and (3) not all copulations lead to successful sperm transfer (n = 34; P. Sandner, unpublished data). Because an accurate estimation of P_2 requires larger numbers of offspring, we allowed all recipients to mate with the first donor for two hours and 18 ± 1 min later with the second donor for another two hours, expecting an average of about 12 copulations during each mating interval based on earlier observations (Schärer et al. 2004). This also allowed us to test for quantitative variation in mating behaviour among the focal genotypes, which, given the high mating rates we generally observe in *M. lignano*, we expected to be an important reproductive trait.

The mating behaviour was recorded as described in detail elsewhere (Schärer et al. 2004). Briefly, a pair of worms was placed in a drop of 4µl of fresh medium into an observation chamber made of two siliconized microscope slides (using Sigmacote, Sigma-Aldrich, St. Louis, USA). A total of 14 observation chambers containing twelve pairs each were observed. The treatments of the focal worms were alternated spatially in each chamber to avoid position effects. Directly after the assembly we recorded the behaviour of the worms for two hours at 1 frame $\cdot$ s^{-1} using a SONY DFW-X700 digital FireWire c-mount camera (SONY Broadcast & Professional, Köln, Germany) and BTV Pro 6.0b1 (available at http://www.bensoftware.com/). The number of copulations was scored by frame-by-frame analysis of the resulting digital movies, the observer being blind with regard to the genotype and order of each donor.

Morphometric measurements

Directly after the sperm competition experiment we measured the morphological traits of the focal donors, the competitor and the recipient (data for competitor and recipient not shown) according to a standard procedure (Schärer and Ladurner 2003). Briefly, we took digital images of the whole worm at 40x, and of both testes, both ovaries, and the stylet at 400x magnification using a digital FireWire c-mount camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) mounted on a DM 2500

compound microscope (Leica Microsystems, Wetzlar, Germany), and the software BTV Pro 6.0b1. Body and gonad size was measured using ImageJ 1.39u (available at http://rsb.info.nih.gov/ij/). Stylet size and shape was determined using a geometric morphometrics approach described in detail elsewhere (Janicke and Schärer 2009a). The first relative warp score of the copulatory stylet (a variable that explained about 77% of the variation in stylet shape) was used as a measure of the stylet curvature, and stylet size was approximated by the centroid size, which is the square root of the sum of squared distances between all landmarks of the stylet to their common centroid (Zelditch et al. 2004). During all measurements the experimenters were blind with regard to the genotype and treatment group of the worms.

Microsatellite genotyping and exclusion-based paternity assignment

To assess paternity success, the recipients were allowed to produce offspring in isolation directly after the morphometric measurements. They were transferred every four days to new enclosures until they stopped producing hatchlings. During twelve days the recipients produced a total of 678 offspring (mean: 7.79 ± 0.41, range: 2-17). All hatchlings per recipient were genotyped as follows: 6-10 day-old hatchlings were individually transferred in 0.9µl of f/2 medium to 0.2 ml tubes, to which 1.5 µl 100% Ethanol was added, and stored at -20°C for up to 10 days. For DNA extraction, the ethanol was evaporated and 19.5 µl of 1x PCR buffer containing 1.5 mM $MgCl_2$ (Qiagen, Venlo, Netherlands), plus 0.5 µl of Proteinase K (20 mg/ml; Boehringer, Mannheim, Germany) was added. Tubes were first shaken and centrifuged, then frozen at -80°C for 1h to break up the worm tissue, followed by 1h of digestion at 50°C and15 min of proteinase denaturation at 95°C (*Caenorhabditis elegans* single worm DNA isolation method by H. Schulenburg, pers. comm.). 2 µl of this isolated DNA solution were used as the template in a 10 µl polymerase chain reaction (PCR). Primers were designed in collaboration with ecogenics GmbH (Zürich-Schlieren, Switzerland) to amplify a microsatellite locus with a CAG-repeat (locus Macro21), which was identified in clone ANGU1234 of a large EST project (http://flatworm.uibk.ac.at/macest/) using the Tandem Repeats Database (http://tandem.bu.edu; Benson 1999). The primers used were Macro21F (5'-TTCATCAACATCAGCCTTATCC-3'), 5'-labelled with the fluorescent dye Yakima Yellow, and Macro21R (5'-CTGCTGCTGAGGTGTTTGG-3'). PCR reactions were carried out in 10 µl containing 0.5 U Hotstar Taq Polymerase (Qiagen, Venlo, Netherlands), 1x PCR buffer, 150 µM dNTPs (Promega, Madison WI, USA), 0.3 µM of each primer, and 2µl of the DNA solution, using a Mastercycler ep gradient S (Eppendorf, Hamburg, Germany). Cycling

conditions were as follows: denaturation and polymerase activation at 95°C for 15 min, followed by 35 cycles of 30 s at 94°C, 1 min 30 s at 53°C, 60 s at 72°C, and 30 min extension at 60°C. PCR products were size-separated on an AB3130xl genetic analyzer and genotyped using Genemapper4.0.

The recipient and the competitor genotype were monoallelic at this locus (allele size: 90bp), while the focal genotypes had alleles with different sizes (allele sizes: 87bp and/or 93bp). All offspring homozygous for the allele 90bp were thus sired by the competitor, whereas offspring carrying an allele other than 90bp had to have been sired by the focal donor. Hence, paternity could be assigned unambiguously for all hatchlings (assuming that no mutation or genotyping errors occurred).

Statistical analysis

We first compared the three focal genotypes in terms of body, testis and ovary size, stylet shape and size, and number of copulations. Testis size and ovary size were correlated positively with body size, so we controlled for this by using the residuals from the respective linear regression fits (testis size vs. body size: $R^2 = 0.25$, $F_{1, 85} = 28.10$, $P < 0.001$; ovary size vs. body size: $R^2 = 0.08$, $F_{1, 85} = 7.30$, $P = 0.01$). All data were tested for normality using Shapiro-Wilk tests. If they significantly deviated from a normal distribution and could not be transformed accordingly, nonparametric Kruskal-Wallis tests and subsequently Wilcoxon tests with strict Bonferroni correction for multiple comparisons were used, otherwise we used ANOVAs and subsequently Tukey's HSD tests (with significance reported at the 0.05 level).

To identify the determinants of paternity success we calculated a generalized linear model (GLM) using the odds ratio of paternity success (i.e. the number of offspring sired by the focal donor vs. the number of offspring sired by the competitor) as the target variables (Sokal and Rohlf 1995, pp. 760-778). We used the focal genotype, the order, and their interaction as fixed factors, and body size, the four morphological traits, and Δ copulations as covariates. Δ copulations equals the difference in the number of copulations between the focal donor and its competitor and was used to control for the mating rate of the competitor. We assumed a quasibinomial distribution and specified a logit link function. By adding the covariates to the model as first terms, we first analysed the variance explained by morphological and behavioural traits and then fitted the model with the factors genotype and order and their interaction to the residual variance. Nonsignificant terms were eliminated in a stepwise fashion from the full model (all $P > 0.29$), so that the

reduced model only contained the statistically significant terms. To test for sperm precedence, P_2 was compared to the random expectation $P_2 = 0.5$ using a Z-test.

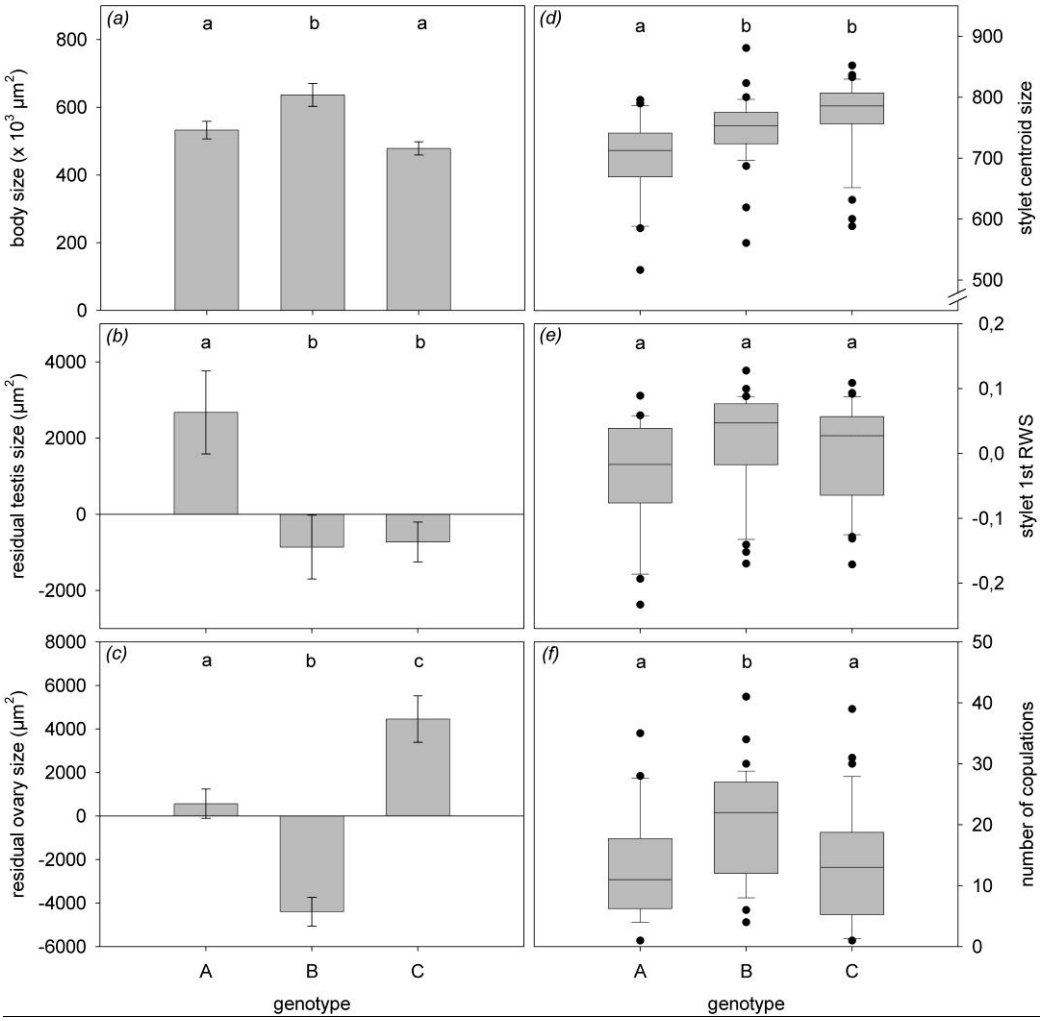

Fig. 1 Comparison among the focal genotypes A, B, and C in body size (*a*), residual gonad sizes (*b* and *c*), stylet morphology (*d* and *e*), and the number of copulations (*f*). 'Stylet centroid size' is a measure of the size of the copulatory organ and 'stylet first relative warp score (RWS)' measures its curvature. We present bar plots (means ± 1 s.e.) for parameters that fulfilled the assumptions of parametric tests, and otherwise box-and-whisker plots (median, first and third quartiles, ninth and 91st percentiles, outliers). Genotypes marked with different small letters differ significantly according to parameteric Tukey's HSD tests and non-parametric Wilcoxon tests (strictly Bonferroni corrected), respectively.

The GLM was calculated using the package 'car' in R 2.10.1 (R Development Core Team 2009). All other analyses were carried out using JMP 7.0.1 (SAS Institute 2007). Means are given ± 1 s.d. for P_2 and ± 1 s.e. otherwise. The initial sample size was $n = 168$ (3 genotypes × 2 mating orders × 28 replicates each). However, the final sample size was reduced because some recipients did not copulate with both donors ($n = 53$) or produced fewer than two offspring ($n = 23$). Moreover, two recipients and one donor died in the course of the experiment. One donor had no copulatory stylet and for one donor the information on body size was lost. This resulted in the final sample size of $n = 87$, i.e. genotype A: $n = 20$ (10 mating first /10 mating second); genotype B: $n = 35$ (16/19); genotype C: $n = 32$ (16/16).

Results

Morphological and behavioural differences between the focal genotypes

Genotype had a significant effect on all measured morphological traits except for the stylet shape (stylet first relative warp score, Kruskal-Wallis test: $\chi^2_{2, 84} = 4.7$, $P = 0.09$; Fig. 1e). Body size varied significantly (ANOVA: $F_{2, 84} = 9.0$, $P < 0.001$; Fig. 1a). Genotypes also differed significantly in residual testis size genotypes (ANOVA: $F_{2, 84} = 6.4$, $P < 0.01$; Fig. 1f). In contrast to this, the

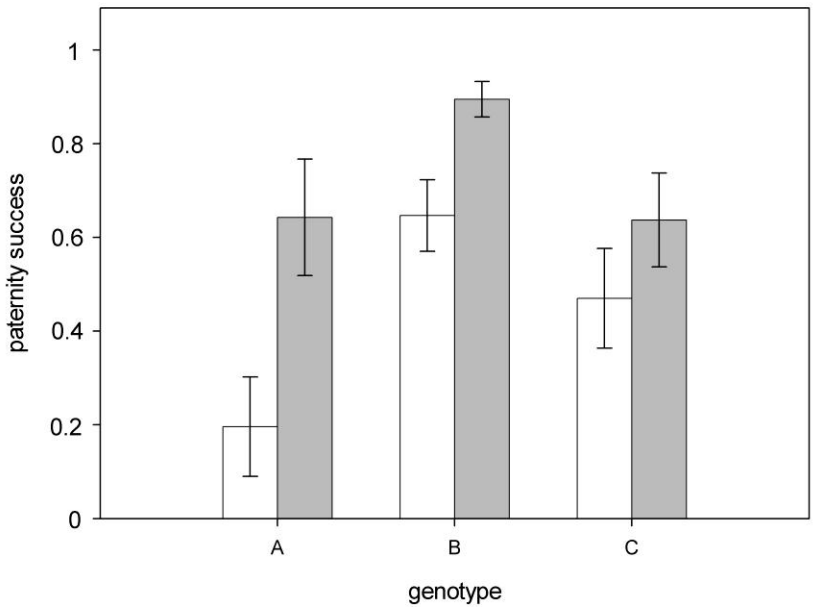

Fig. 2 Paternity success for the three focal genotypes split up by mating order (open bars, first; filled bars, second). Data are shown as means ± 1 s.e..

(ANOVA: $F_{2, 84} = 5.0$, $P < 0.01$; Fig. 1b), residual ovary size (ANOVA: $F_{2, 84} = 30.2$, $P < 0.001$; Fig. 1c), and stylet size (stylet centroid size, Kruskal-Wallis test: $\chi^2_{2, 84} = 19.6$, $P < 0.001$; Fig. 1d). The square-root-transformed number of copulations also differed significantly between the three focal number of copulations of the competitor genotype did not vary significantly when in competition with the different focal genotypes (26.1 ± 2.0, 26.2 ± 1.5, and 28.8 ± 1.6 copulations against A, B, and C respectively; ANOVA: $F_{2, 84} = 0.85$, $P = 0.43$).

Determinants of paternity success

The full model suggested that none of the measured morphological covariates had a significant effect on paternity success (Table 1). As the χ^2-values of these covariates were relatively small and *P*-values far from significant, these parameters were successively excluded from the analysis. The behavioural measure Δ copulations was the only covariate that explained a significant part of the variance in paternity success and which therefore stayed in the reduced model. The focal genotype significantly affected paternity success, even when we corrected for all the measured reproductive traits, with genotype B being the most successful sperm donor (Table 1; Fig. 2). Mating order also had a

Table 1 Effects of genotype, mating order, mating behaviour and reproductive morphology on paternity success. We report both the statistics of the full and the reduced model (GLM, see statistical analysis for details). The full model was reduced in six steps by excluding each time the least significant term. The terms are listed in reverse order of their exclusion, and the reduced model only contains the three significant terms.

Term	DF	Full model χ^2	Full model *P*	Reduced model χ^2	Reduced model *P*
Genotype	2	6.605	0.037	15.623	< 0.001
Role	1	7.371	0.007	10.175	0.001
Δ copulations	1	4.914	0.027	4.814	0.028
Stylet first RWS	1	1.023	0.312	-	-
Role × Genotype	2	1.418	0.492	-	-
Body size	1	0.294	0.588	-	-
Res. testis size	1	0.084	0.772	-	-
Stylet centroid size	1	0.003	0.954	-	-
Res. ovary size	1	< 0.001	0.983	-	-

significant effect regardless of the genotype (i.e., the genotype × order interaction term was nonsignificant and was thus excluded), with higher paternity success for focal donors when mating second (Table 1; Fig. 2), despite the fact that the number of copulations achieved by the first and second focal donor was comparable (*t*-test: $n = 87$, $t = -0.31$, $P = 0.76$).

Sperm precedence

The mean proportion of offspring sired by the second sperm donor (P_2) was 0.64 ± 0.38 s.d. and differed significantly from the 0.5 expectation under random paternity (*Z*-test: $n = 87$, $Z = 3.47$, $P < 0.001$). This suggests that *M. lignano* has second donor sperm precedence under the conditions that we tested here. Moreover, P_2-values showed a U-shaped distribution (Fig. 3) and were highly variable, with 33 focal donors that mated second achieving complete paternity success (38%), and 14 of them achieving no paternity success (16%).

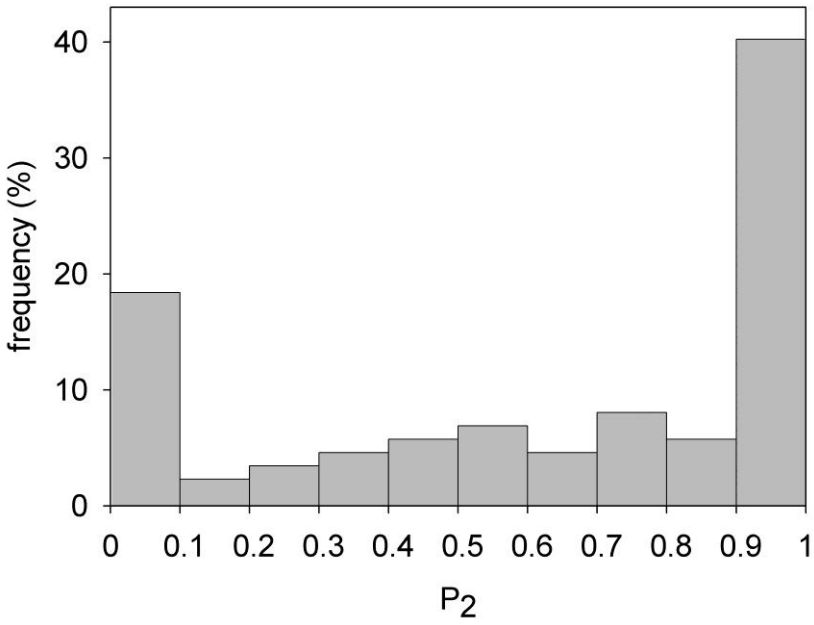

Fig. 3 Histogram showing the frequency distribution of P_2-values over all genotypes. Mean: 0.64 ± 0.38 s.d., $n = 87$.

Discussion

Reproductive morphology and paternity success

Our study is one of the first to document genetic variation in a range of reproductive morphology traits in copulating hermaphrodites (e.g., Ostrowski et al. 2003; Jordaens et al. 2006; Koemtzopoulos and Staikou 2007; Janicke and Schärer 2009a; Garefalaki et al. 2010), and it is, to our knowledge, the first study to demonstrate a genetic component underlying paternity success. However, none of the measured morphological traits significantly predicted paternity success, and we in the following discuss possible reasons for this, particularly for the traits where we had a priori expectations based on earlier results (Janicke and Schärer 2009a).

While testis size significantly predicted sperm transfer success in an earlier study (Janicke and Schärer 2009a) it did not significantly predict paternity success in the study we report here. This discrepancy can probably be explained by important differences in the way these two experiments were performed. In Janicke and Schärer (2009a) all worms had grown up in large groups and were therefore probably depleted with respect to the amount of sperm available for transfer (Schärer and Ladurner 2003; Schärer and Vizoso 2007; Janicke et al. 2010). In this case the previously shown positive relationship between testis size and sperm production rate (Schärer and Vizoso 2007) is likely to have led to a ongoing replenishment of the already depleted sperm reserves during the 24h long mating trials. In contrast, the worms we used here had grown up in isolation and therefore probably had large amounts of accumulated sperm available for transfer (Schärer and Vizoso 2007), which they probably did not deplete during the much shorter 2h mating trials. The testis size of a sperm donor was therefore less likely to affect the outcome of the experiment we report here.

Another reproductive morphology trait that significantly predicted sperm transfer success in the earlier study is the shape of the copulatory stylet (measured as the first relative warp score; Janicke and Schärer 2009a). In our dataset the three focal genotypes did not differ significantly in stylet shape (P = 0.09), and it did not significantly predict paternity success. This could be due to the fact that, although stylet shape has previously been shown to be repeatable within individuals (intraclass correlation coefficient: r_i = 0.60, $F_{49, 50}$= 3.9, P < 0.001; Janicke and Schärer 2009a), this estimate has a considerable

measurement error. Moreover, the advantage of a certain stylet shape may vary depending on the genital morphology of the recipient and the particular competitor it encounters. Effects of stylet shape on paternity success might therefore not be visible in the genetically restricted set of lines we used in the present experiment.

The fact that the focal genotype remained a significant predictor even when we corrected for all the measured reproductive morphology traits, may suggest that there were unquantified traits that affected paternity success. These may include male traits, such as ejaculate size, ejaculate composition, sperm size, and sperm morphology (e.g., Radwan 1996; LaMunyon and Ward 1998; Simmons and Kotiaho 2002; Wolfner 2007; Koene et al. 2010), or female traits, such as the morphology and physiology of the female genitalia (e.g., Pitnick et al. 1999; Miller and Pitnick 2002; Garcia-Gonzalez and Simmons 2007).

Mating behaviour and paternity success

The only covariate that significantly predicted paternity success in this study was Δ copulations: the more the focal donors copulated relative to their competitor, the higher the paternity success they achieved. Elgar et al. (2003) hypothesized that high mating rates are an option to increase paternity success in terrestrial invertebrates, and Birkhead et al. (1987) predicted high copulation frequencies in birds that are colonially breeding and where extra-pair copulations are likely. Indeed, high numbers of copulations have been shown to increase paternity success in a number of insect, spider, and bird species (e.g., Smith 1979; Müller and Eggert 1989; Birkhead and Møller 1992; Otronen 1994; Schneider et al. 2000; but see Lewis 2004).

Here we further develop a hypothesis that has also been considered by Birkhead et al. (1988) and Harvey and May (1989), namely that high copulation frequencies coevolve with second male sperm precedence. As we discuss in the next section, the results of our study suggest that there may be second donor sperm precedence in *M. lignano*, at least under the conditions tested here. Given the very high mating rates we generally observe in this species (Schärer et al. 2004; Janicke and Schärer 2009b), it is interesting to speculate that high mating rate could be an adaptation to this pattern of sperm precedence. If there is sperm precedence and the donors have incomplete information about the sperm competition risk (as seems to be the case in *M. lignano*, Sandner and Schärer 2010), lower ejaculate expenditure is predicted (Parker et al. 1997). If, furthermore, donors trade off ejaculate expenditure for expenditure on gaining matings (Parker 1998), this could lead to an increase in mating rate. High

mating rate may thus provide a donor with some degree of paternity assurance, even when ejaculate size declines with increasing mating rate. It might help win over sperm deposited by previous sperm donors via sperm displacement, and might avoid subsequent sperm competition by physically engaging with the partner and temporarily monopolising it. High mating rates can also help maximize the chances to mate close to the optimal time with respect to fertilization (recall that worms lay about one egg per day).

The hypothesized link between mating rate and sperm precedence is consistent with a number of studies. When there is second male precedence high numbers of copulations increase the paternity success (e.g., Smith 1979; Birkhead et al. 1988; López-León et al. 1993; this study). When, on the other hand, P_2 is lower we observe low mating rates (e.g., Curtis 1968; Watson 1991). It would be worthwhile to make a comparative analysis of mating rate among related species that differ in P_2, and to model the co-evolution of mating rate and sperm precedence.

However, note that our current interpretation of the measured mating rate of the three focal genotypes as traits of these genotypes could be problematic, because mating behaviour is an interacting phenotype between the two mating partners (Wolf et al. 1999). The high mating rates of the focal line B with our recipient could also be the result of a preference of the recipient for this very line.

Second donor sperm precedence

We found that the mating order in our sperm competition experiment had a significant effect on the paternity of the sperm donors, and that the average P_2-value deviated significantly from the value expected under random paternity. Together these results suggest that *M. lignano* has a second donor sperm precedence under the chosen conditions. It is important to keep in mind that each donor was placed for two hours with the recipient when we now discuss possible mechanisms that could have led to this pattern, such as adjustment of mating effort, ejaculate allocation, sperm displacement, and sperm aging.

The observed second male sperm precedence could have resulted if the sperm donors that mated in the second role would have increased their mating effort and/or sperm allocation. While our data suggest that the number of copulations of focal donors in the first and second role were similar, it is more difficult to exclude strategic sperm allocation. Models of sperm competition intensity (Parker 1998) predict that the highest ejaculate allocation per mating occurs when there is only one competitor.

Thus, the observed P_2-values might result from a strategic adjustment of ejaculate allocation as a response to sperm competition. However, a previous experiment suggested that *M. lignano* was not able to assess the mating status of single partners (Sandner and Schärer 2010). Moreover, a recent sperm-tracking experiment suggested that the sperm transfer success per donor was not increased in a competitive versus a non-competitive situation (T. Janicke, M. Eichmann and L. Schärer, unpublished data), making it unlikely that there is strategic sperm allocation.

Alternatively, second donor sperm precedence might result from sperm displacement. Indeed, the same sperm-tracking experiment mentioned above suggested that the second sperm donor reduced the number of rival sperm stored in their partner's antrum by about 50%, when two sperm donors were allowed to mate for 1h each with an initially virgin recipient (T. Janicke, M. Eichmann and L. Schärer, unpublished data), clearly suggesting sperm displacement in *M. lignano*. The effects of mating order and Δ copulations on paternity success across the genotypes studied here further support this idea. Sperm displacement can be achieved by removing sperm via morphological adaptations (Waage 1979; Gage 1992) and/or by volume displacement (Moreira et al. 2007; Takami 2007). In *M. lignano* for instance the stylet might be used to remove or damage anchored sperm from previous copulations (Vizoso et al. 2010), and unanchored sperm might be removed by volume displacement. In both cases, sperm displacement is likely to increase with mating rate. The high mating rates observed in this species (Schärer et al. 2004; Janicke and Schärer 2009b) might therefore be the result of sperm competition.

Finally, second donor sperm precedence could occur simply because the sperm of the first donor ages or gets lost earlier than the sperm of the second donor (Baur 1994; Eady 1994). This is unlikely to be the case in this study because the period between last copulation of the first and first copulation of the second donor was on average only 63 ± 4 minutes, and both donors fertilized eggs until up to twelve days after the last copulation.

To better understand the mechanisms of sperm precedence it would be interesting to compare sperm allocation, sperm transfer success, and the resulting paternity success, representing successive stages in the conversion of reproductive investment to fitness. While it is tempting to equate sperm allocation and sperm transfer success, it is clear that there are many more sperm being produced than we find in storage (Schärer and Vizoso 2007; Janicke et al. 2010), further pointing to the importance of sperm displacement. In contrast, the hypothetical S_2-value (i.e., the proportion of sperm successfully stored by the

second sperm donor) resulting from the above-mentioned sperm tracking study is 0.66, which is very close to the estimate of paternity success we report here ($P_2 = 0.64$). It therefore appears that once sperm have been successfully transferred and stored, the remaining processes do not lead to any further systematic biases.

Potential mechanisms underlying U-shaped P_2-distribitions

The P_2-distribution observed in this study was strongly U-shaped and the variance was comparatively large (e.g., literature in Simmons and Siva-Jothy 1998; but see Corley et al. 2006). Variation in P_2 likely depends on how the sperm is mixed in the female tract and how it is used for fertilization. U-shaped P_2-distributions are expected in the following scenarios: (1) one of the donors fails to inseminate the recipient, (2) the sperm of one donor, but not of the other, are lost, (3) a mating plug prevents sperm displacement, (4) the ejaculates break into a small number of packets instead of being thoroughly mixed, or (5) the female strongly biases fertilization in favour of one donor. In the following we briefly discuss these scenarios.

Failed insemination (e.g., Hockham et al. 2004) is unlikely in our experiment since we excluded recipients where one donor failed to mate, and the remaining focal donors and competitors achieved on average 15.9 ± 1.0 (Fig. 1f) and 27.1 ± 1.0 copulations respectively, which is probably enough to transfer at least some sperm.

Differential sperm loss may happen, for example, during egg laying, with large groups of sperm of one donor spilled out and groups of another donor's sperm remaining anchored. In this case we might expect more extreme P_2-values if egg laying occurred during the mating period. However, whether eggs were deposited during the mating period (which occurred in eight replicates) or not, did not significantly affect the variance in P_2 (Levene's test for unequal variances: $F_{1, 85} = 0.20$, $P = 0.65$).

Mating plugs are known from a range of organisms (e.g., Barker 1994; Simmons and Siva-Jothy 1998; Uhl and Busch 2009). They can lead to complete first male precedence or to second male precedence, when removed. However, mating plugs have never been observed in *M. lignano*.

Harvey and Parker (2000) predict bimodal P_2-distributions if the ejaculate of a donor breaks into a small number of packets and unimodal or flat P_2-distributions if it breaks into a large number of packets. The former scenario, termed 'sloppy mixing', occurs in a number of insect species (literature in Harvey and Parker 2000). In recently mated *M. lignano* one can often observe groups of sperm that are anchored in the epithelium of the female antrum and

perform a joint undulating movement (P. Sandner, pers. obs.). If we assume that sperm of the same donor form groups and thus do not mix randomly with other groups of sperm, we could expect the resulting P_2-distribution to be U-shaped (Parker 1970b; Harvey and Parker 2000). However, grouping prior to anchoring is unlikely in *M. lignano*, because sperm are probably transferred individually (Vizoso et al. 2010). Whether or not sperm usually have multiple anchoring points and whether anchored sperm groups are composed of sperm from single or multiple donors awaits further investigations.

Finally, a U-shaped P_2-distribution could also result from cryptic female choice (Charnov 1979; Thornhill 1983), defined as 'non-random paternity biases resulting from female morphology, physiology or behaviour that occur after coupling' (Pitnick and Brown 2000), if the recipients choose the sperm of certain sperm donors over others. A potential mechanisms of post-copulatory female choice in *M. lignano* is the *suck* behaviour, which is performed by one or both partners following copulation (Schärer et al. 2004). Although we would not necessarily expect strong effects of the mating order and of the number of copulations if female choice were the predominant determinant of paternity success in our study, we cannot rule out some effect of female choice on paternity, because our experiment was not specifically designed to disentangle mechanisms of sperm competition and cryptic female choice.

Conclusions

We found genetic variation in morphology and mating behaviour across three genotypes of *Macrostomum lignano* that also exhibited genetic variation in paternity success. Part of the variation in paternity success could be accounted for by the differences in mating rate, but, contrary to our expectations, we found no effect of any of the measured morphological traits. We propose that post-copulatory sexual selection may be a selective agent shaping mating rate in this species. We further show that there is second male precedence under the conditions studied. A likely mechanism for second male precedence in *M. lignano* is the displacement of previously inseminated ejaculates by subsequent sperm donors. Finally, we discuss a number of post-copulatory processes that may help to explain the U-shaped P_2-distribution found in this free-living flatworm. In order to quantify the relative importance of these post-copulatory processes, not only the genotypes of the focal donors, but also the genotypes of the recipients should be varied (Lynch and Walsh 1998, p. 598; Clark et al. 1999; Neff and Pitcher 2005). Male effects would indicate variance in sperm competitiveness, female effects would indicate female choice, and a significant

male × female interaction would indicate that male and female effects depend on the specific genotype combinations. This is clearly an interesting direction for future research with the established inbred lines.

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CHAPTER 2

No plastic responses to experimental manipulation of sperm competition *per se* in a free-living flatworm

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Abstract

In the absence of sperm competition evolutionary theory predicts low mating rates and low ejaculate expenditure per mating, and sex allocation theory for simultaneous hermaphrodites predicts a strongly female-biased sex allocation. In the presence of sperm competition a shift towards a more male-biased sex allocation and a higher ejaculate expenditure are predicted. The free-living flatworm *Macrostomum lignano* has been shown to respond plastically in mating rate, testis size, and sperm transfer to manipulation of the social group size, a proxy of the strength of sperm competition. However, manipulation of social group size may manipulate not only sperm competition, but also other factors, such as food supply and metabolite concentration. In this study we therefore manipulated sperm competition *per se* by repeatedly exposing individuals to partners that have either mated with rivals or not, while keeping the social group size constant. Our results suggest that *M. lignano* does not have the ability to detect sperm competition *per se*, as worms experimentally exposed to the presence or absence of sperm competition did not differ in sex allocation, sperm transfer or mating behaviour. A response to our manipulation would have required individual recognition, the ability to detect self-referencing tags, or tags or traces left by rivals on or in the mating partners. We first discuss the possibility that highly efficient sperm displacement may have decreased the difference between the treatment groups and then propose three alternative cues that may allow *M. lignano* to respond plastically to the social group size manipulation used in earlier studies: assessment of the mating rate, chemical cues, or tactile cues.

Introduction

Sex allocation in simultaneous hermaphrodites

Sex allocation theory for outcrossing simultaneous hermaphrodites predicts that sex allocation depends on the mating group size K+1, whereby K is the number of sperm donors individuals receive sperm from at the time the eggs are fertilized (Charnov 1982). When $K = 1$, there is no sperm competition and Charnov's model predicts marginal investment in sperm production and a strongly female biased sex allocation. This is a situation of maximal 'local sperm competition' (Schärer 2009) because here only related sperm are in competition with each other, in analogy to local mate competition in gonochorists (Hamilton 1967), where related males compete with each other. When $K > 1$, not only related but also unrelated sperm are competing for fertilizations, thus decreasing local sperm competition and increasing sperm competition. This leads to an increase in the optimal male allocation and thus a shift towards a more male-biased sex allocation. Consistent with this theory, studies on several simultaneously hermaphroditic animals have reported a phenotypically plastic increase in testis size in response to increasing social group size (e.g. Raimondi and Martin 1991; Schärer and Ladurner 2003; Tan et al. 2004; Trouvé et al. 1999, reviewed in Schärer 2009), which at least in some cases is clearly associated with higher levels of sperm competition (Janicke and Schärer 2009a).

Manipulating sex allocation in Macrostomum lignano

In *M. lignano* there is a well documented effect of the social group size on testis size (e.g., Brauer et al. 2007; Janicke and Schärer 2009b; Schärer and Ladurner 2003; Schärer et al. 2004b; Schärer et al. 2005; Schärer and Vizoso 2007; several unpublished data sets). Testis size is a meaningful measure of male allocation and sperm production (Schärer et al. 2004b; Schärer and Vizoso 2007), and bigger testes are correlated to higher sperm transfer success in *M. lignano* (Janicke and Schärer 2009a). This system thus corresponds qualitatively to the predictions of basic sex allocation theory.

Schärer and Ladurner (2003) for the first time dissected the effects of group size from density effects by simultaneously manipulating group size and enclosure size. They varied social group size by raising worms in groups of 2, 3, 4, and 8 individuals, respectively. In a fully-factorial design they kept all groups in both small and large enclosures and used testis size as a measure of male allocation. They found a positive effect of group size on male allocation, but no effect of enclosure size.

They interpreted the plastic increase in male allocation as a response to sperm competition, in agreement with Charnov's original prediction (Charnov 1982). As an explanation for the absence of enclosure size effects the authors speculated on a potential mechanism for individual recognition in *M. lignano*. Such a mechanism would enable the worms to differentiate between repeated encounters with the same individual and a real increase in group size. The ability to distinguish between familiar and unfamiliar mating partners may lead to a so-called Coolidge effect (first reviewed by Dewsbury 1981), which refers to an individual's decreasing propensity to mate with the same partner and a resuscitation of its sexual interest when presented with a new partner. Recently a Coolidge effect has been shown in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. Mated snails were significantly more likely to inseminate a novel partner than their previous one (Koene and Ter Maat 2007). This behavioural response has further been documented for many other animal taxa such as beetles (Steiger et al. 2008), fishes (Kelley et al. 1999), lizards (Tokarz 1992), birds (Pizzari et al. 2003), and mammals (references in Dewsbury 1981), and it has been suggested to allow sperm reserves to be conserved for additional reproductive opportunities (Wedell et al. 2002). We here aimed to examine whether a possible differentiation between partners is the reason for the response in male allocation to social group size in *M. lignano* (see references above).

Manipulating sperm competition per se

Schärer and Ladurner (2003) expected that a higher social group size leads to a higher mating group size and therefore to a higher level of sperm competition, and this expectation was recently confirmed (Janicke and Schärer 2009a). Their observed effect of group size on testis size is consistent with the predictions regarding the optimal investment towards the production of ejaculates, summarized by Parker (1998). When males have access to more than one female in a polygamous mating system, then they should build larger testes and produce more sperm in order to counteract sperm competition as long as the fertilization chances are fair.

In the present study we compared two different situations in which individuals either encountered sperm competition every day or in which sperm competition was completely absent. We here manipulated the degree of sperm competition *per se* by using a monogamy (M) and a polygamy (P) treatment. In the M treatment we kept each flatworm with the same partner for the duration of the experiment, and transferred both worms together to a new well every day. In the P treatment we also transferred each worm to a new well, but presented it every day with a different partner out of a set of eight worms, i.e. each P replicate consisted of four pairs, newly mixed every day (see below for details). Unlike a manipulation of social group size this manipulation of sperm competition *per se* is not confounded with density and factors associated with it (e.g. food level, metabolite accumulation, encounter probability). However, it fulfils Parker's (1998) definition of sperm competition as

"competition between the sperm of two or more males for the fertilization of a given set of ova", given the fact that ejaculates of at least two, and possibly more, partners are present in the female antrum of a worm in the P treatment when it has been presented with a different partner. We hereby test whether the response to social group size in male allocation of *M. lignano* reported by Schärer and Ladurner (2003) is based on a detection of sperm competition *per se*.

We hypothesize that (i) worms mate more often with a different partner than with a familiar partner, (ii) worms transfer more sperm to a different partner than to a familiar partner, and / or (iii) worms allocate more resources to testes when presented with a different partner than when presented with the same partner every day. Moreover, given that a trade-off plays between male and female allocation (Janicke and Schärer 2009b; Schärer et al. 2005) we expect a smaller ovary size and lower female fecundity as a correlated response to an increased allocation to testes in the P treatment.

Materials and Methods

Study organism

Macrostomum lignano (Platyhelminthes, Macrostomorpha) is a simultaneously hermaphroditic free-living flatworm and a member of the meiofauna of the Northern Adriatic Sea (Ladurner et al. 2007). Experimental animals are the descendants of individuals collected near Lignano Sabbiadoro (Italy) in 2003. Mass cultures are kept in the laboratory in glass Petri dishes containing f/2 medium (Andersen et al. 2005) and with the diatom *Nitzschia curvilineata* as an *ad libitum* food source (Rieger et al. 1988). Under these conditions and at a temperature of 20 °C worms reach 1.5 mm in body length and have a generation time of about 18 days. *M. lignano* is outcrossing (Schärer and Ladurner 2003) with frequent, reciprocal copulation and internal fertilization (Schärer et al. 2004a). Mating rates can reach 30 times per hour and microsatellite analysis has revealed multiple paternity (P. Sandner and L. Schärer, in preparation). Its transparent body wall allows to morphometrically measure the size of the paired testes and ovaries, the size of the seminal vesicle as a measure of the number of sperm ready for ejaculation and the amount of received sperm *in vivo* (Schärer and Ladurner 2003). When first mated and then isolated, individuals can store received sperm in the female antrum for up to twelve days, first laying about one egg per day and eventually running out of sperm (P. Sandner, pers. obs.). Induced variation in testis size in *M. lignano* has been shown to correlate positively with a dynamic measure of investment in sperm production (Schärer et al. 2004b), and with

the number of sperm produced by a worm (Schärer and Vizoso 2007). A phenotypically plastic increase in testis size hence leads to an increase in sperm production. Higher sperm transfer can be estimated by emptier and hence smaller seminal vesicles, i. e. the sperm source, associated with higher amounts of received sperm in the female antrum, i. e. the sperm sink (Schärer and Ladurner 2003). Note that small seminal vesicles alone do not necessarily reflect low sperm production but can also be caused by recent high sperm expenditure.

Experimental procedure

All 320 experimental animals had the same age (±1 day) because the eggs from which they hatched were laid by individuals from the stock population within 48h. Nine days after hatching, i.e. before sexual maturation, the worms were randomly distributed from a common pool to 32 24-well tissue culture plates, such that five wells of the top line of every well contained two worms (Fig. 1). All wells were filled with 1.5ml f/2 medium and supplied with diatoms *ad libitum*, the standard procedure in studies on plasticity of testis size in *M. lignano* (but see Schärer et al. 2005). Every four days new plates were prepared in the same way. For two weeks all worms were transferred daily to a well located one line further down on the plate. In the M treatment each worm was transferred together with the same partner every day (see Fig. 1), and there was therefore no possibility for sperm competition. In contrast, in the P treatment each partner was transferred so that it encountered a different member of a set of eight worms every day. The transfer was done in a way that the novel partner was different from at least the ultimate and penultimate partner (Fig. 1).

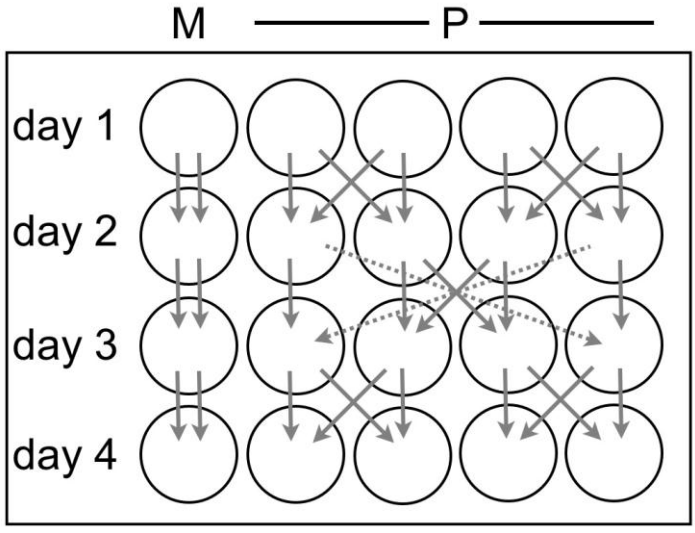

Fig. 1 Schematic representation of the experimental treatment. The fate of two worms forming one monogamy treatment replicate (M), and eight worms forming one polygamy treatment replicate (P) is depicted for four consecutive days.

A recent study showed that 90% of the pairs assembled from two mated *M. lignano* successfully mated *and* stored sperm in the antrum of the partner when they were placed in a 24-well plate for one day (Janicke and Schärer 2009a). This suggests that a period of one day usually allows for at least some matings with the partner (probably many given the high mating rates), and that our manipulation therefore produced sperm competition in the P treatment replicates. *M. lignano* is able to adjust testis size within ten days when the level of sperm competition has changed (Brauer et al. 2007). Assuming that the worms in the P treatment indeed perceived higher sperm competition, higher sperm allocation for the duration of our experimental procedure, which was 14 days, was therefore expected to be reflected in a phenotypically plastic increase in testis size.

Behavioural measurements

On day 15 of the experimental procedure, worms were not transferred to a new well but instead two worms per replicate were transferred into an observation chamber and their mating behaviour was recorded as described in detail elsewhere (Schärer et al. 2004a). Briefly, two worms were placed in a drop of 4µl of fresh medium into an observation chamber. We filmed eight observation chambers with eight pairs each. Each chamber contained as many P replicates as M replicates and the positions of both treatments were spatially balanced. Directly after the assembly we recorded the behaviour of the worms for 1h at 1 frame $\cdot$ s^{-1} using a SONY DFW-X700 digital FireWire c-mount camera and the software BTV Pro 5.4.1. (available at http://www.bensoftware.com/). Later we used BTV Pro 6.0b1 to score the mating rates by frame-by-frame analysis, with the observer being blind with regard to the treatment of the individual pairs.

Morphometric measurements

After the one hour mating trial we randomly chose one worm of each observed pair in order to measure it according to a standard procedure (Schärer and Ladurner 2003). We took digital images of the whole worm at 40x, and of both testes and both ovaries as well as of the seminal vesicle at 400x using a digital FireWire c-mount camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) mounted on a DM 2500 compound microscope (Leica Microsystems, Germany) and using the software BTV Pro 6.0b1. For image analysis we used ImageJ 1.39u (available at http://rsb.info.nih.gov/ij/). We also estimated the amount of sperm received by the partner(s) and stored in the female antrum on a scale from 0 (no sperm visible) to 3 (many sperm visible) according to Schärer and Ladurner (2003). During all measurements the experimenter was blind with regard to the treatment groups of the worms.

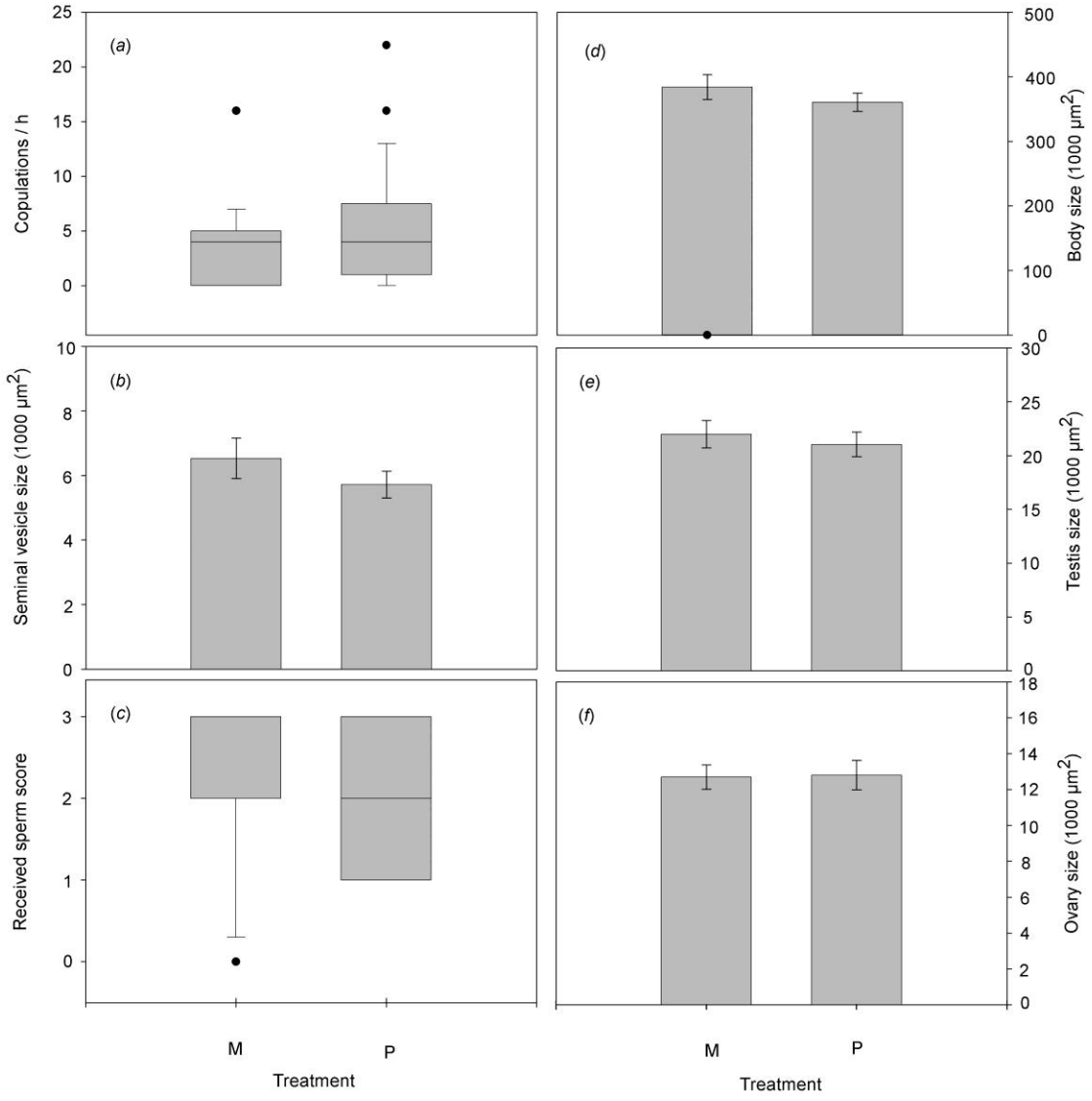

Fig. 2 Bar plots and box plots depicting the responses to the experimental treatment in mating rate (a), the size of the seminal vesicle as a measure of available own sperm (b), the received sperm score (c), the body size (d), the total size of both testes (e), and the total size of both ovaries (f). M refers to replicates presented with the same partner every day; P refers to replicates presented with changing partners.

Statistical analysis

The initial sample size was $n = 32$ P and $n = 32$ M replicates. One P replicate and four M replicates were lost because of developmental problems of one of the worms (two were immature when they were measured morphometrically, two had very few sperm in their seminal vesicle and one was lacking the whole tailplate). Further, two P replicates and four M replicates were lost during the preparation of the observation chambers. The received sperm of 12 M and 15 P replicates could not be scored because of an egg in the female antrum that was ready to be deposited, and the body size of one worm could not be determined because of a missing image. This yielded a final sample size of $N = 26$ for the received sperm score (12 M; 14 P), $n = 52$ for the body size (24 M; 28 P), and $n = 53$ (24 M; 29 P) for all other variables.

Nonparametric Wilcoxon tests were used for the mating rate and received sperm score. All other variables met the assumptions of parametric tests and therefore two-sample *t*-tests could be used. Data were analysed with JMP 7.0.1 (SAS Institute 2007).

Results

The mating rate was not significantly different between the treatment groups ($Z_{52} = 1.15$, $p = 0.25$; Fig. 2a). We did also not find a significant treatment effect on seminal vesicle size as a measure of available own sperm ($t_{52} = 1.10$, $p = 0.27$; Fig. 2b), or on the amount of received sperm as an estimate of sperm transfer ($Z_{25} = 0.33$, $p = 0.74$; Fig. 2c). Moreover, the treatment groups also did not differ significantly in body size ($t_{51} = 0.99$, $p = 0.34$; Fig. 2d), testis size ($t_{52} = 0.56$, $p = 0.58$; Fig. 2e), or ovary size ($t_{52} = 0.10$, $p = 0.92$; Fig. 2f).

Discussion

In this experiment we manipulated sperm competition *per se* in the free-living flatworm *M. lignano* and found differences in neither mating rate, received sperm score and seminal vesicles size, nor testis or ovary size. This is in contrast to the predictions of evolutionary models and to the well-documented potential of *M. lignano* to respond to different social group sizes. In the following we first discuss the possibility of relaxed sperm competition in our P treatment. We then discuss the mechanisms for an assessment of sperm competition *per se* and finally we discuss

three alternative cues for a response to sperm competition in *M. lignano*. This is done by comparisons between the experimental procedure used in this study and the manipulation of social group size used by e.g. Schärer and Ladurner (2003).

Did sperm displacement relax sperm competition?

One possibility that might explain the lack of responses in our experiment is that sperm displacement could be highly efficient (Charnov 1996). In that case there would be little rival sperm left after just a few matings when presented with a different partner, and the matings that would follow later in the daily period would therefore entail weak sperm competition. Unfortunately, we still know little about ejaculate stratification and sperm displacement in M. lignano. A first study in which worms were each mated sequentially for one hour to two partners in drops of 4µl suggests that there is a relatively weak second male precedence in sperm transfer success (T. Janicke and L. Schärer, unpublished data). Concerning the mating rates observed in this study the results are unlikely to be influenced by the proportion of sperm displaced. Since mating rate was measured during the first hour after the encounter with the same or different partner, sperm competition was almost certainly inevitable in the P treatment. Even when mating rates are comparable it is possible that worms in the P treatment would transfer more sperm in order to displace rival sperm, which would be seen in a higher sperm allocation. However, our observation of similar seminal vesicle sizes and received sperm scores in both treatment groups also does not indicate higher sperm allocation in the P than in the M treatment.

Possible mechanisms for an assessment of sperm competition per se

An adequate response to the level of sperm competition would be a Coolidge effect, i.e. an increased propensity to mate with a novel partner and decreasing propensity to mate with a familiar partner. This can, firstly, be based on individual recognition as in burying beetles (Steiger et al. 2008) or, secondly, on self-referencing tags left on the partner's body surface during mating, as has been reported for female decorated crickets (Ivy et al. 2005). A third possible way to assess sperm competition *per se* is the detection of mating traces or tags left by rivals on the partner or in its genital tract. For instance, the nudibranch *Aeolidiella glauca* discriminates against individuals as mating partners that carry an external spermatophore stemming from a recent mating (Haase and Karlsson 2004). Moreover, there is now growing empirical evidence for correct assessment of the partner's mating state in other organisms (Anthes et al. 2006; Loose and Koene 2008; Thomas and Simmons 2009; Velando et al. 2008; Wedell and Cook 1999).

The similar mating rates we observed in both P and M treatments give no indication for a response promoted by one of these three mechanisms in *M. lignano*. As stated by Dewsbury (1981, p. 473) it is also possible that individuals, when presented with novel partners, do not mate more often but transfer more sperm per copulation. Such

strategic ejaculate allocation is known from Adélie penguins that withhold ejaculates from their social partner in order to donate more sperm in extra-pair copulations (Hunter et al. 2000). However, there is also no support for either mechanism coming from the seminal vesicle size and the received sperm scores in our study. Worms did not receive more sperm in the P treatment than in the M treatment. One could argue that one would not necessarily find such a difference when most of the received sperm was displaced or lost, but the similar seminal vesicle sizes in both treatment groups give no indication to higher sperm allocation in the P than in the M treatment.

At least three other studies found no Coolidge effect or discrimination between mating states of the partner. Male decorated crickets, unlike their female conspecifics, do not identify and discriminate against previous mates (Gershman and Sakaluk 2009). The snail *Arianta arbustorum* does not adjust sperm expenditure or mating rate to the mating state of its partner (Baur et al. 1998). A recent study on the snail *Biomphalaria glabrata* shows that this snail does not discriminate former partners against novel partners in a second mating event that took place one hour after the first (Häderer et al. 2009). Beyond the lack of sensory devices and long-term memory, the authors also consider low costs of male matings as a possible reason for indiscriminate mating. Another explanation stated by the authors is that large groups and high population densities in nature make discrimination mechanisms obsolete. A similar reason might account for the absence of a Coolidge effect in male decorated crickets (Gershman and Sakaluk 2009). Here, selection for male discrimination mechanisms might be relaxed because of the strong female preference for novel males (Ivy et al. 2005).

Alternative cues for a response to sperm competition

The ability of *M. lignano* to respond to manipulations of the social group size in earlier studies can possibly hinge on differences in mating rate, chemical cues, or tactile cues, which we will discuss in turn in the following.

The first alternative trigger is the actual mating rate of an individual. When an individual gets involved in matings very frequently this might trigger a response in male allocation. It is known from *Lymnaea stagnalis*, that the fill-state of the prostate gland is detected by the brain via the penial nerve, which controls sexual activity (De Boer et al. 1997). In *M. lignano* a covariation between sex allocation and mating rate has been shown with higher mating rates in pairs formed by more male-biased individuals (Janicke and Schärer 2009b). In our study, mating rate was just like testis size not significantly different between M and P treatment groups, which is consistent with these findings. The response to sperm competition in Schärer and Ladurner's study (2003) could well be mediated by higher mating rates in larger groups.

However, if mating rate was correlated to encounter rate and encounter rate was higher in smaller enclosures, then Schärer and Ladurner (2003) should have detected this as an effect of enclosure size on male allocation. However, such an effect was not found.

Chemical cues can either be soluble signals or metabolites accumulating in the medium, as indicated by a study on the polychaete *Ophryotrocha diadema* (Schleicherova et al. 2006). Such conditioning of the medium - or the substrate - was minimized in this setup by the daily transfer of the worms to new wells. However, the lack of an enclosure size effect observed by Schärer and Ladurner (2003) also questions a role for soluble signals or metabolites.

Finally, tactile cues can be used by animals to sense a risk of sperm competition, e.g. when they mate with one individual and at the same time a third individual interferes with the copulating pair. Physical contact with other individuals within a short period of time might be a similar trigger. In earlier studies sperm competition was manipulated via the social group size: no sperm competition in pairs, intermediate levels of sperm competition in groups of three or four individuals, strong sperm competition in groups of eight individuals. In those groups the rivals were allowed to compete physically with each other and the intensity of physical contact presumably increased with social group size. In the present study only the sperm of different donors were competing and there was no possibility for the worms to sense the physical presence of rivals. Tactile cues are therefore likely involved in the documented response in male allocation. However, such cues cannot be seen as strictly opposing to sperm competition as the ultimate reason for a positive response in testis size: one can control for tactile cues under laboratory conditions (this study), but in all other cases high sperm competition will coincide with high tactile cue intensity. As a consequence, tactile cues could serve as a rule-of thumb-indicator for sperm competition in the natural habitat of *M. lignano*.

Conclusions

To conclude, we did not find any behavioural or phenotypically plastic response of *M. lignano* when we manipulated the level of sperm competition *per se*. Such a response in the predicted direction is possible in our system and has been observed repeatedly and reliably when social group size was manipulated. Thus, unless our experimental treatment was ineffective due to highly efficient sperm displacement in the P treatment, we can conclude that *M. lignano* can estimate the number of partners and competitors only in their presence, e.g. mediated by tactile cues. Less likely but still possible are the perception of chemical cues or of the mating rate for an estimation of sperm competition by *M. lignano*. There is hence a need for further experiments to define the exact underlying mechanism.

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CHAPTER 3

Costs of plastic responses to the social situation in a simultaneous hermaphrodite

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Abstract

Phenotypic plasticity is widespread but not pervasive in the kingdoms of life. One possible reason for this is that its evolution is constrained by costs. However, there currently exists little evidence for costs of phenotypic plasticity. A possible reason for the scant empirical evidence for such costs is that many experiments were conducted under benign conditions in the laboratory that allow the individuals to perform all functions unlimitedly. The simultaneously hermaphroditic flatworm *Macrostomum lignano* can plastically adjust its sex allocation to its current social group size. In this experiment we test for costs of such responses using hatchling production as a fitness proxy. We put the flatworms under nutritional stress and further increased the visibility of costs by repeatedly exposing the individuals to changes in group size (alternating environment), or keeping group size stable (stable environment). We found lower hatchling production in alternating environments compared to stable environments, suggesting the existence of costs of adjustments to the social environment. We argue that these costs are most likely due to modulation of gonad activity rather than gonad size, and that they include a time-lag of phenotype-environment mismatch when facing a new social situation. As predicted by sex allocation theory, hatchling production *per capita* was significantly lower in octets than in pairs. This group size effect was considerably larger than the stability effect, which suggests that selection on trait value may still be stronger than selection on trait plasticity, and that phenotypic plasticity may hence be adaptive, when *M. lignano* is exposed to changing social environments.

Introduction

Temporal and spatial variation in the abiotic, biotic, and social environment is frequently encountered by all kinds of organisms (Chapman et al. 2008; Merilä et al. 2004; Relyea 2002), potentially leading to a mismatch between their currently expressed phenotype and the optimal phenotype in the current environment. Phenotypic plasticity, defined as environment-dependent phenotype expression (DeWitt and Scheiner 2004), is a widespread strategy to cope with this kind of variation and is a very active field of research (for recent reviews see, e.g., Aubin-Horth and Renn 2009; Auld et al. 2010). However, organisms do not always respond in a phenotypically plastic way to such environmental changes, which can lead to maladaptation. Hence the question arises whether phenotypic plasticity may incur some costs, which could explain the absence of plasticity in some species or traits. Pigliucci (2005) stated that "research of costs of plasticity is still in its infancy, but is both theoretically important and empirically challenging, and should become a major area of future inquiry." That is why it is useful to be more specific about the nature of the above-mentioned costs. Auld et al. (2010) differentiate between maintenance costs and production costs of phenotypic plasticity. Maintenance costs (environment-independent) are costs that result from the potential to respond in a phenotypically plastic way to environmental conditions and include, e.g. costs of a regulatory mechanism, or costs of a flexible development. Production costs (environment-dependent) include energy expenses for morphological, physiological and behavioural changes. The currency in which all costs should ideally be measured is fitness. We now briefly review the evidence for both maintenance and production costs in plants and animals. From the next subsection onwards we focus on production costs only.

In plants, phenotypic plasticity is common, e.g., the defence reactions against herbivory or parasites (Heil 2010 and literature therein), and the evidence for costs is somewhat better than in animals. Van Buskirk and Steiner (2009) found that 15 out of 21 plant studies report costs of phenotypic plasticity. However, note that there probably is a general publication bias against experiments that did not find costs (reviewed in Van Buskirk and Steiner 2009). In animals, less than half of all published studies report costs of phenotypic plasticity (Van Buskirk and Steiner 2009). While there is mixed

evidence for maintenance costs of phenotypic plasticity, evidence for production costs of phenotypic plasticity is particularly wanting (see Van Buskirk and Steiner 2009). In the following we therefore focus on production costs. For instance, costs of phenotypically plastic defence against predators appear to be absent in amphibians and snails (DeWitt 1998; Relyea 2002; Scheiner and Berrigan 1998; Steiner and Van Buskirk 2008; but see Black and Dodson 1990), presumably because they have been purged by selection.

The acquisition-allocation problem

Another reason why it seems non-trivial to demonstrate production costs of phenotypic plasticity might be the acquisition-allocation problem. Large variation in resource budgets across individuals can conceal production costs of traits as well as trade-offs between two life history traits that compete for the same pool of resources. This can happen if the variation in total budget across individuals is larger than the variation in the corresponding traits within individuals (Van Noordwijk and De Jong 1986). To reveal production costs or trade-offs it might therefore be necessary to control for the total per capita supply of resources, thereby standardising the resource budget for all individuals. Feeding *ad libitum* would be one way to do so, but under nutritionally rich diets in the laboratory the individuals are likely to perform all functions maximally, and costs of phenotypic plasticity may not be visible under such benign conditions (e.g., Black and Dodson 1990; Dorn et al. 2000; Riessen and Sprules 1990; Steiner 2007; Walls et al. 1991). As phenotypic differences between individuals are supposed to be increased by stressful conditions (see references in Hoffmann and Merilä 1999), costs of phenotypic plasticity might mainly make an appearance when individuals are limited in their resources. Especially in order to reveal small costs of phenotypic plasticity it might therefore be necessary not only to standardize resource budgets but also to restrict the total amount of available resources and thereby put the organisms under nutritional stress.

Phenotypic plasticity in sex allocation of simultaneous hermaphrodites

A potential advantage of simultaneous hermaphrodites compared to separate-sexed organisms is that they can opportunistically shift reproductive resources between the male and the female function depending on the current social situation (Michiels 1998). Such an adjustment is in accordance with sex allocation theory following the concept of ‘local mate competition’ (Hamilton 1967), which has been modified for simultaneous hermaphrodites (Charnov 1980, 1982; Fischer 1981, 1984; reviewed in Schärer 2009, who proposed to

call this 'local sperm competition'). The theory predicts that male (sperm) allocation should increase with increasing mating group size, at a cost to female allocation. Classical sex allocation theory for simultaneous hermaphrodites assumes a trade-off between male and female allocation (Charnov 1979, 1982) but does not incorporate potential costs of phenotypic plasticity. Recently, Schärer (2009) mentioned that considering such costs might be important when thinking about sex allocation, and several models that included costs of phenotypic plasticity found substantial effects on the outcome of evolution (Lively 1986; Padilla and Adolph 1996; Van Tienderen 1991). The magnitude of costs of phenotypic plasticity in sex allocation may be crucial for the evolution and maintenance of simultaneous hermaphroditism (St. Mary 1997).

To our knowledge there is only one empirical study that has investigated costs of phenotypic plasticity in a simultaneous hermaphrodite. Lorenzi et al. (2008) made an experiment in the polycheate *Ophryotrocha diadema* to test for production costs of phenotypic plasticity in sex allocation in response to a change in the mating regime. They either changed the mating regime from monogamy to polygamy or *vice versa*, or kept it stable as a control. As a measure of sex allocation they quantified the focal hermaphrodite's offspring via the male and female functions, thereby assuming that investment into one function was directly proportional to the number of offspring produced through this function. They did not find lower offspring production in the changing mating regime and concluded that sex allocation adjustments are not costly in simultaneous hermaphrodites. However, the majority of the focal hermaphrodites in their experiment did not produce any cocoons, and the mean number of offspring was only about ten, which may be far from the resolution required to detect small differences. Also, the authors neither restricted nor controlled the resource budget of the polychaetes, and they changed the mating regime only once. The reasons outlined above may reduce the likelihood to discover costs of a phenotypically plastic reaction.

Phenotypic plasticity in response to changes in social group size is known to occur, e.g., in the free-living flatworm *Macrostomum lignano* (e.g., Schärer and Ladurner 2003, reviewed in Schärer 2009). In this species, the response to increased social group size encompasses an increase in testis size (Brauer et al. 2007; Janicke and Schärer 2009b; Schärer and Ladurner 2003; Schärer et al. 2005), an upregulation of testicular cell proliferation activity (Schärer et al. 2004b), an increased mating rate (Janicke and Schärer 2009b), an increased sperm production rate (Schärer and Vizoso 2007), a decreased ovary size (Janicke and Schärer 2009b; Janicke and Schärer 2010; Schärer et al.

2005), and a decreased egg production (Schärer et al. 2005). This phenotypically plastic response has been shown to be, at least to some degree, reversible and was accordingly called 'flexible' (Brauer et al. 2007, 'flexible' *sensu* Piersma and Drent 2003). However, such a response would only be adaptive if the benefits predicted by sperm competition theory (Parker 1970; Parker 1998) and sex allocation theory for simultaneous hermaphrodites (Charnov 1979, 1982) are not outweighed by costs of phenotypic plasticity. We here hypothesize that hatchling production of the stable treatment groups (both octets and pairs) is higher than in the alternating treatment groups (both starting as octets and starting as pairs). This would indicate production costs of phenotypically plastic adjustments to fluctuating social group size (maintenance costs cannot be addressed here because they are environment-independent and equally paid in both stable and alternating treatment groups). If we find such costs we aim at evaluating whether they might be outweighed by a potential benefit of the phenotypically plastic response.

Materials and Methods

Study organism

The free-living flatworm *Macrostomum lignano* (Platyhelminthes, Macrostomorpha) is a simultaneous hermaphrodite and a member of the meiofauna of the Northern Adriatic Sea (Schärer and Ladurner 2003). It is outcrossing with frequent, reciprocal copulations and internal fertilization (Schärer et al. 2004a). Experimental animals are the descendants of individuals collected near Lignano Sabbiadoro (Italy) in 2003 (Ladurner et al. 2005). Mass cultures are kept in the laboratory at 20°C in glass Petri dishes containing f/2 medium (Andersen et al. 2005). The diatom *Nitzschia curvilineata* is offered *ad libitum* as food (Rieger et al. 1988). Under these conditions worms reach about 1.5 mm in body length and have a generation time of about 18 days. Their transparent body wall allows to morphometrically measure the size of the paired testes and ovaries *in vivo* (Schärer and Ladurner 2003, for details see below).

Experimental setup

To obtain worms of similar age 600 adult *M. lignano* from a mass culture were allowed to lay eggs in six petri dishes with algae. On day 1 the worms that were used for the experiment hatched from these eggs. On day 8 the hatchlings were pooled, and 960 of them were randomly assigned to 24-well plates, so that all

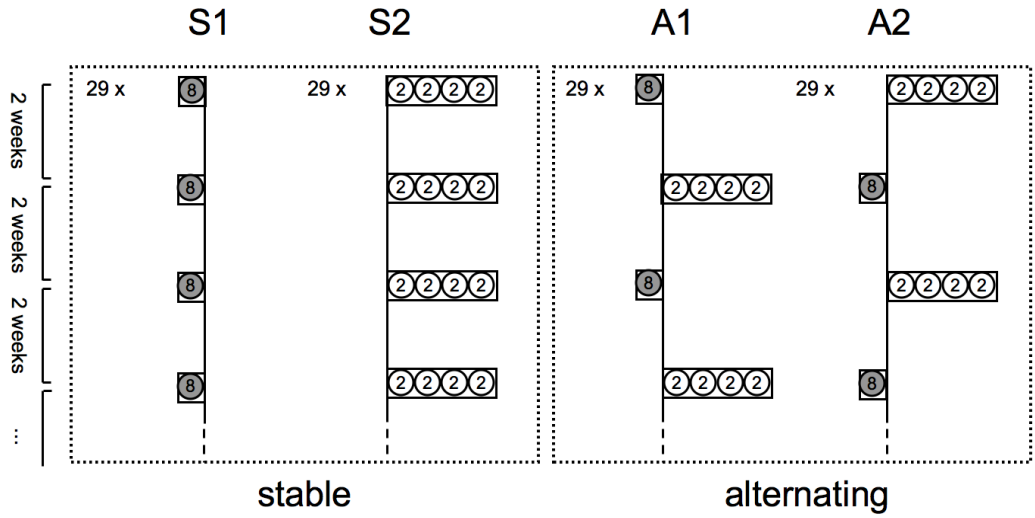

Fig. 1 The experimental design was fully factorial with group size (pair vs. octet) and stability (stable vs. alternating) as fixed factors. Group size was changed every two weeks in alternating replicates (A1, A2) and remained the same in stable replicates (S1, S2). Note that both stability levels were always balanced with respect to group size: Half of the stable treatment replicates were permanently kept in octets (S1) and half were permanently kept in pairs (S2), while half of the alternating treatment groups started in octets (A1) and half started in four pairs (A2), which were then split into four pairs or joined into one octet every two weeks.

plates contained one replicate of each treatment combination, and each replicate consisted of eight worms. To allow them to mature quickly, the worms were supplied with algae *ad libitum* in f/2 medium until the age of 3 weeks, when we alternated the group size of the alternating replicates for the first time. From the age of 3 weeks until the age of 13 weeks the worms were supplied with a restricted number of algae per week and capita in artificial seawater (hw Meersalz, Wiegandt GmbH, Krefeld, Germany). Artificial seawater does not contain any silicate, which diatoms require to build their frustule, thus preventing diatom growth. Every week worms were transferred to a new well and supplied with the same number of algae per capita. To this end, we made a homogeneous diatom suspension and determined the concentration by means of six hemocytometer counts. Using the appropriate amount of this suspension we then each time added ~28,000 diatoms per well for pairs and ~112,000 diatoms for octets. This corresponds to an average of ~14,000 diatoms per capita and week; this amount was entirely consumed in the course of six days, leading to a mild degree of food restriction.

In order to force the worms to go through several cycles of adjustments to group size we repeatedly manipulated social group size. The experimental design was fully factorial with group size (pairs vs. octets) and stability (stable vs. alternating) as fixed factors. Group size was changed five times, i.e. after week 3, 5, 7, 9, and 11 (for alternating treatment groups), or remained the same

(for stable treatment groups). The stable treatment consisted of two levels: octets that were permanently kept in groups of eight (S1, Fig. 1), and four pairs that were permanently kept in pairs (S2, Fig. 1). Within each pair replicate we pooled the four pairs every week and randomly assembled four new pairs. This guaranteed that group size and stability were manipulated but that the worms of all treatment groups encountered a range of partners throughout the experiment (as was the case in the alternating treatment groups). In order to balance group size across both stability levels, one half of the alternating treatment group consisted of groups of eight individuals that matured in an octet (A1, Fig. 1) and were randomly assigned to four pairs two weeks later, i.e., they alternated group size every other week, and the other half consisted of eight individuals that matured in four pairs (A2, Fig. 1) and were joined to form an octet two weeks later, also alternating group size every other week. Two weeks seem to be enough time for *M. lignano* to at least partially adjust sex allocation after group size has changed (Brauer et al. 2007).

Body size and sex allocation measurements

Body size and sex allocation of one worm of each replicate was measured when the worms were 3, 5, 11, and 13 weeks old. Since we did not know their identity, different worms of the same replicate might have been measured each time. Ideally, sex allocation would be calculated based on all reproductive resources that are allocated to the male and the female reproductive function, respectively (Schärer 2009). The proxy of sex allocation we use here is testis size divided by the sum of testis size and ovary size (Vizoso and Schärer 2007). All measurements were done in a standard manner described elsewhere in more detail (Schärer and Ladurner 2003). For body size we took digital images of the whole worm at 40x, and for testis and ovary size at 400x using a digital FireWire c-mount camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) mounted on a DM 2500 compound microscope (Leica Microsystems, Wetzlar, Germany) and using the software BTV Pro 6.0b1 (available at http://www.bensoftware.com/btv/dlbeta.html). For image analysis we used ImageJ 1.39u (available at http://rsb.info.nih.gov/ij/). During all measurements the experimenters were blind with regard to the treatment groups of the worms.

Fitness estimate

To estimate fitness approximately, we counted the number of hatchlings per week produced by all eight worms within the same replicate. This estimates mean fitness per replicate via the female function. Mean fitness via the male

function, not measured here, is necessarily the same within each replicate as mean female fitness (Fisher 1930), although variances likely differ (Bateman 1948; Charnov 1979). A sex allocation trade-off (e.g., demonstrated by Schärer et al. 2005) leads to decreasing female fecundity with increasing male allocation in larger groups. Hatchling production was recorded from the age of three weeks onwards. Hatchlings were counted always 12-15d after the adult worms had been removed and transferred to a new well. At that time the formation of the gonads had started but none of the hatchlings had already reproduced. Within one week the wells were checked a second time for hatchlings potentially missed in the first count. Again the observers were blind both times with respect to the treatment group of the replicates.

Statistical analysis

We calculated a generalized linear mixed model (GLMM) using stability and group size as fixed factors, replicate ID and time (in weeks) as random factors, and hatchling production per replicate and week as the response variable. We assumed a poisson distribution (appropriate for count data) and specified a log link function. To test for effects of the factors we used this model as a reference model and calculated alternative models by excluding the term that was to be tested (or adding it in case of the group size × stability interaction). We compared each alternative model to the reference model in two ways; first using Likelihood ratio tests, and second using a penalized likelihood measure of the goodness of fit, the Akaike Information Criterion (AIC). This measure favours models with a high goodness of fit and a low number of terms entered. The larger the difference in AIC between the alternative model and the reference model, the more variance is explained by the term being tested (Burnham and Anderson 2004; Sullivan and Joyce 2005). If the factor stability explains a significant part of the variance in hatchling production this would suggest production costs of phenotypic plasticity.

To see whether our manipulation of social group size indeed induced a response in terms of sex allocation we applied 2×2 ANOVAs to study the effects of group size, stability, and their interaction at four time points (weeks 3, 5, 11, and 13).

The original sample size was reduced by four replicates to a final sample size of 116 replicates. Two replicates were excluded because worms were lost during the measurement. One replicate was excluded because of a pipetting mistake, and another replicate was excluded because two worms died during the experiment. Two replicates were not excluded even though one worm was lost during the experiment. In one case the worm was replaced with a worm from a

replicate of the same treatment group that we excluded on that day. In the other case we lost one worm in a stable octet. To correct for the per capita estimates, we multiplied this replicate's food supply by 0.875 and its hatchling counts by 1.143 from that day onwards. We tested all terms for normality using Shapiro-Wilk tests. The GLMMs were calculated using R 2.5.1 (R Development Core Team 2005) and the package "lme4", all other analyses were carried out using JMP 9.0 (SAS Institute 2010). All data are presented as means ± 1 s.e..

Results

Hatchling production was significantly affected by the factor stability (Table 1, Fig. 2), with stable treatment groups producing significantly more offspring than alternating ones. Hatchling production was also significantly affected by group size (Table 1), with pairs producing significantly more offspring than octets, and this effect was considerably stronger than the effect of stability, based on the AIC differences between the 'stability model' and the reference model, and between the 'group size model' and the reference model, respectively (Table 1).

Table 1 Model comparisons to test which of the factors explained a significant part of the variance in hatchling production. The reference model is a GLMM with group size (2 or 8 worms) and stability (stable or alternating group size) as fixed factors, replicate ID and time as random factors, and the number of hatchlings produced per 8 worms and 7 days as the response variable. The goodness of fit is given by the Akaike Information Criterion (AIC), and likelihood ratio tests (LRT) are presented to test which term significantly affected the quality of the model fit.

Model parameters				Likelihood ratio test (LRT) with reference model		
Model	Term tested	AIC	Δ AIC	χ^2	d.f.	*P*
Reference model	-	1675.69				
Full model	Group size × Stability	1646.42	29.27	31.27	1	< 0.0001
Stability model	Stability	1677.74	2.05	4.06	1	0.04
Group size model	Group size	1689.71	14.02	16.02	1	< 0.0001

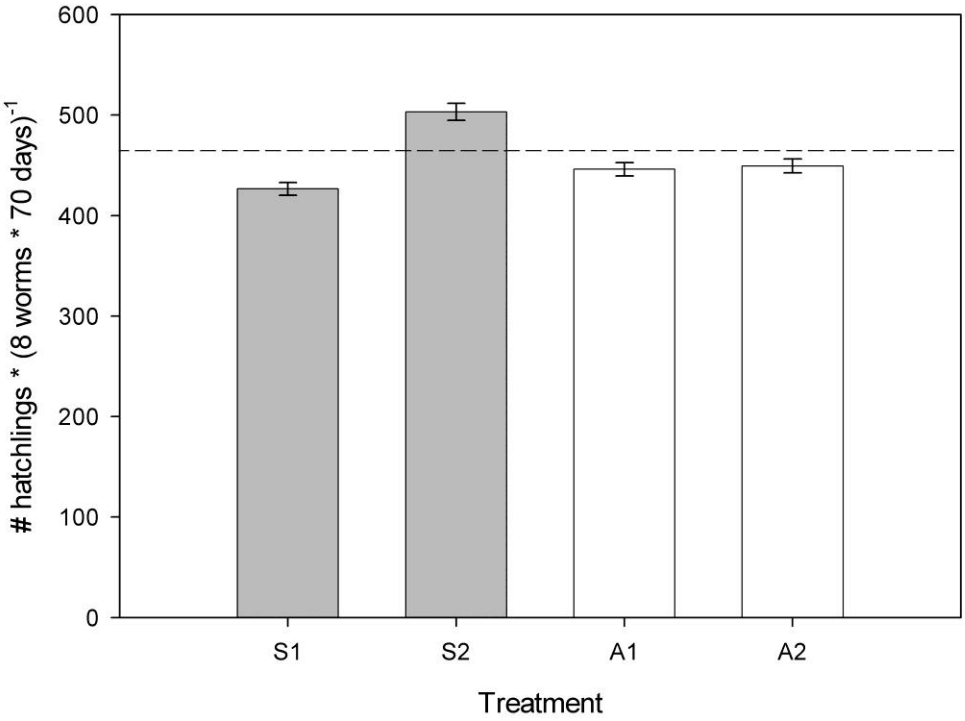

Fig. 2 Total hatchling production during 70 days (per replicate of eight worms) for each treatment group (S1, stable octets; S2, stable pairs; A1, alternating group sizes starting as octets; A2, alternating group sizes starting as pairs). Bars and whiskers represent means ± 1 s.e..

The significant effect of the group size × stability interaction on hatchling production (Table 1) suggests that the group size effect was more pronounced in stable treatment groups than in alternating treatment groups.

Body size was never affected by stability or group size (Table 2).

Our measure of sex allocation was significantly more male-biased in current octets than in current pairs in week 5, but, surprisingly, it was not affected by the factor group size in weeks 3, 11 and 13 (Table 3). Stability never affected sex allocation significantly (Table 3). Only at the first measurement there was a significant effect of the group size × stability interaction on sex allocation.

Hatchling production in all treatment groups and all replicates started before the worms were 3 weeks old. Mean hatchling production across all treatment groups was 456.2 ± 4.4 hatchlings per 8 worms and 70 days (range: 326-578), i.e. a mean of 0.8 hatchlings per worm and day. Hatchling production varied by a factor of 1.77 over the ten weeks when it was recorded (Fig. 3).

Table 2: Effects of group size, stability, and their interaction on body size at four different time points (week 3, 5, 11, and 13, respectively). A separate two-way ANOVA was calculated for each time point.

	Week 3			Week 5			Week 11			Week 13		
Factor	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Group size	0.26	1, 111	0.61	2.41	1, 111	0.12	0.04	1, 111	0.84	0.98	1, 111	0.32
Stability	1.09	1, 111	0.30	0.58	1, 111	0.45	1.67	1, 111	0.20	0.70	1, 111	0.41
Group size × Stability	0.36	1, 111	0.55	0.15	1, 111	0.70	0.06	1, 111	0.80	0.15	1, 111	0.70

Table 3: Effects of group size, stability, and their interaction on sex allocation at four different time points (week 3, 5, 11, and 13, respectively). A separate two-way ANOVA was calculated for each time point.

	Week 3			Week 5			Week 11			Week 13		
Factor	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*	*F*	d.f.	*P*
Group size	0.18	1, 111	0.68	4.10	1, 111	0.05	0.04	1, 111	0.84	0.39	1, 111	0.53
Stability	0.13	1, 111	0.72	1.75	1, 111	0.21	0.08	1, 111	0.78	2.62	1, 111	0.11
Group size × Stability	4.20	1, 111	0.04	1.20	2, 111	0.28	0.57	2, 111	0.45	0.74	2, 111	0.39

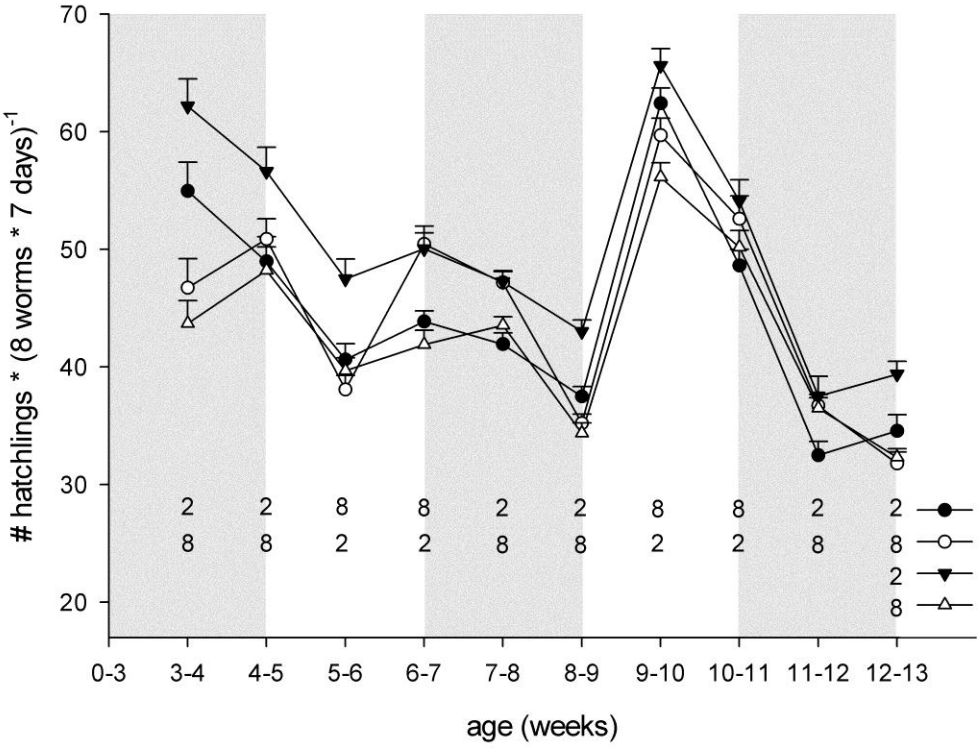

Fig. 3 Mean number of hatchlings produced per replicate and week as a function of age, for all treatment groups (triangles, stable group size; circles, alternating group size). Current group sizes for the two alternating treatment groups (open and filled circles) are given below each data point. Stable octets are indicated as filled triangles, stable pairs as open triangles. Data are presented as means ± 1 s.e..

Discussion

Stability effect

We are here first concerned with effects of stability on hatchling production. These are independent of group size effects since both stability levels were balanced for group size. Group size effects will be discussed in the next section. We found that worms produced significantly fewer offspring when they were exposed to alternating group sizes than when they experienced stable group sizes. This significant effect of environmental stability on hatchling production is consistent with the hypothesis that phenotypic plasticity in response to group size incurs some production costs. These may be due to energy expended for

repeated up- and down-regulation of reproductive tissues or gamete production in the alternating environment.

The observed costs may also be caused by behaviours performed to assess the social situation or to establish social hierarchies or territories, which might, e.g., apply for pair formation and territoriality in the simultaneously hermaphroditic reef fish *Serranus tigrinus* (Pressley 1981). Both types of behaviours could be necessary when the composition of the group changes, and may involve energy for sampling and reduce feeding or mating efficiency (DeWitt 1998). However, we did not quantify any social behaviours in this experiment and also do not know whether or how *M. lignano* populations are socially or spatially structured under natural conditions.

Group size effect

We found a significant effect of social group size on hatchling production with octets having considerably lower hatchling production than pairs but contrary to our expectation our measure of sex allocation was rarely affected by group size in this study. We therefore did not apply the classical analysis of costs of phenotypic plasticity for this trait (DeWitt 1998; DeWitt et al. 1998; Scheiner and Berrigan 1998; Van Tienderen 1991). The manipulation of social group size yielded effects on testis size or sex allocation in most but not all of the experiments conducted so far (Brauer et al. 2007; Janicke and Schärer 2009b; Janicke and Schärer 2010; Schärer and Ladurner 2003; Schärer et al. 2005, but see Schärer et al. 2005 and three unpublished datasets excluding this study). However, the sex allocation data used here were based on the measurement of a single randomly chosen member of each replicate and this very individual is only in one out of eight cases expected to be measured again in the subsequent measurement. Thus assuming there is individual variation in both sex allocation and phenotypic plasticity this might have introduced considerable noise into the sex allocation data, making it less likely to find the expected effect in all four measurements. The decreased hatchling production in larger groups has been reported previously for *M. lignano* (e.g., Schärer et al. 2005) and is likely due to a trade-off between female and male allocation in this simultaneous hermaphrodite (Schärer et al. 2005; reviewed in Schärer 2009). Concerning a trade-off with sperm production it is important to point out that testis size, the numerator in our sex allocation estimate, has been shown to be a good but incomplete predictor of sperm production rate (measured as the increase in seminal vesicle area in worms that were kept in isolation after a social group size treatment, Schärer and Vizoso 2007). Schärer and Vizoso (2007) found that sperm production rate was on the one hand predicted by testis size but at the

same time also by group size. This significant part of variance in sperm production rate not explained by testis size suggets that there is more to sperm production than testis size alone. Dynamic measures of male allocation such as sperm production rate (Schärer and Vizoso 2007) or testicular activity (Schärer et al. 2004b), might therefore offer additional information on sex allocation compared to the more static measure testis size and might be a more sensitive measure of short-term variation in male allocation. Higher cell proliferation by testicular stem cells probably requires more energy, not to mention subsequent steps of spermatogenesis (e.g., Dewsbury 1982). Hence, a trade-off between egg production and sperm production is still a possible explanation for the group size effect on hatchling production in our study. We in the following discuss possible trade-offs with other components of male allocation (seminal fluid production and expenditure on gaining matings), mating behaviour (competition for mating partners and mate choice), and social interactions (non-reproductive behaviours) that we did not quantify here.

A trade-off with seminal fluid production by accessory glands is possible but seminal fluids could not be quantified to date in *M. lignano*. Egg production could also trade off with allocation towards expenditure on gaining matings (Parker 1998). Mating rate is higher in worms originating from larger groups of *M. lignano* (Janicke and Schärer 2009b) and could potentially be costly (Daly 1978). Mate acquisition might be more costly in larger groups not due to time or energy costs of mate searching but due to scramble competition with other group members (e.g., Millesi et al. 1998; Verrell and Krenz 1998) or costly mate choice (Boorman and Parker 1976; Heisler et al. 1987; Maklakov and Arnqvist 2009). Finally, non-sexual interactions might be more frequent in larger groups and egg production could also trade off with them. Aspects of social interactions have rarely been quantified in simultaneously hermaphroditic populations. Reduced egg laying in snails at higher densities has been attributed to increased tactile interference (e.g., Dan and Bailey 1982; reviewed in Jordaens et al. 2007), or to increased food competition (Baur and Baur 1990; Mooij-Vogelaar and Van der Steen 1973). Tactile interference beyond mating interactions may be independent of density if animals tend to form clusters, while competition for evenly distributed food is likely density-dependent. In our study the food regime was standardized per capita in order to minimize group size effects due to different food availabilities. Competition for food might therefore have increased the variance in hatchling production, but probably not the means of pairs compared to octets. Another potential factor linked with density is the accumulation of harmful metabolites. In this study worms were

transferred to wells with fresh medium every week in order to minimize the accumulation of metabolites. We therefore consider the group size effect on hatchling production more likely to be caused by a trade-off with ejaculate production, mate acquisition, or social interactions than by food competition or metabolite accumulation.

Group size × stability interaction

We found a significant effect of the group size × stability interaction on hatchling production. This is expected if phenotypic plasticity is in some way limited. Limits of phenotypic plasticity can occur, e.g., due to a time-lag between experiencing the new environment and the realization of the new phenotype and due to imperfect cue reliability or inadequate responses, leading to a phenotype-environment mismatch when confronted with a new environment (Auld et al. 2010; DeWitt et al. 1998). We see some indication for a time-lag of the response to a new group size. The lines representing the offspring production of the two alternating treatment groups were expected to cross after the exposure to a new group size. They indeed crossed every other week, but they did so only during the second week of being in a new group size (Fig. 3). This means that adjustment of hatchling production to the new group size was completed only between day 8 and 14 after experiencing a new social situation. The duration of such time-lags in relation to the environmental variability presumably is important for the evolution of phenotypic plasticity (Padilla and Adolph 1996), but we currently have no information about the temporal patterns of such variation under field conditions in *M. lignano*.

The significant group size × stability interaction effect on sex allocation in the first measurement was surprising because the alternation of group size in the alternating treatment had not even begun at that time. It is therefore difficult to interpret.

Controlled feeding and hatchling production

The lack of effects of stability, group size, or their interaction on body size in all measurements suggests that the worms were comparable in body size across both group size and stability levels. The presence of testes and ovaries in all worms at the first measurement shows that all worms were sexually mature at the age of three weeks. The mean hatchling production in this study was only 58% of that in a recent study by Janicke et al. (2011), where worms were constantly fed *ad libitum* and achieved a mean of 1.4 hatchlings per capita and day during the first two days after they had been isolated. The considerably lower value we observed here is very likely due to the different food regimes in

the two experiments. For the above-mentioned reasons we think that the standardization and the restriction of the food regime lead to comparable and limited resource budgets, as intended. The overall large fluctuation in hatchling production was, however, unexpected. This might theoretically be due to fluctuations in food quantity. However, algae number was carefully controlled. More likely are therefore fluctuations in food quality. This view is supported by a temporal decline in hatchling production and its restoration coinciding with the moment when we switched to a new batch of algae (weeks 3 and 9, Fig. 3). Future experiments with food restriction should therefore aim at using algae of the same age.

Costs vs. benefits of phenotypic plasticity

A cost-benefit approach for the evolution of sex ratio adjustment (West and Sheldon 2002) suggests that "facultative sex ratio variation will only be favoured when the fitness benefits of this behaviour are greater than its costs". So it is interesting to ask whether in simultaneous hermaphrodites the costs of phenotypic plasticity of sex allocation are outweighed by a putative benefit of a phenotypically plastic response to changing social situation, which would represent a net benefit of phenotypic plasticity. Given the small magnitude of costs measured in the laboratory (this study) and the frequent observation and the high degree of phenotypic plasticity in testis size and/or sex allocation (e.g., Brauer et al. 2007; Janicke and Schärer 2009; Schärer and Ladurner 2003; Schärer et al. 2005), it is likely that there is a net benefit of phenotypically plastic responses to the social situation in *M. lignano*. Such benefits would likely depend on the relative frequencies of the habitats with their diverging selective forces, i.e. spatio-temporal changes in the environment. While the social environments were balanced in the alternating treatment of our experiment, in nature the more common environments will have the stronger selective influence on the evolution of phenotypic optima (Relyea 2002, Via and Lande 1987). Additional experiments are necessary in order to quantify the benefits of phenotypic plasticity.

Since there is evidence that the cost-benefit ratio of phenotypic plasticity varies among environments and species (Steiner 2007; Van Buskirk 2002), modelling it would be very useful in order to predict the stability of simultaneous hermaphroditism as a function of the magnitude of plasticity costs and, e.g., the fluctuations in mating group size. To model the evolution of phenotypic plasticity it would, however, be necessary to take into account both production costs and maintenance costs. Currently we have no reliable

information on the magnitude of maintenance costs of phenotypic plasticity in *M. lignano*. Overall, quantitative genetics (e.g., Dufty et al. 2002; Pletcher et al. 2002; reviewed in Piersma and Drent 2003), experimental evolution (reviewed in Kassen 2002), and, in conjunction with these, theoretical models will greatly improve our understanding of the evolution of phenotypic plasticity in the future.

Conclusions

We provide experimental support for the hypothesis that phenotypic plasticity in response to group size incurs a production cost although our proxy for sex allocation rarely corresponded to group size. We therefore expect that more dynamic measures of sex allocation are necessary to get a complete picture of the amount of energy that is invested into each sex function and that is lost through reallocations from one sex function to the other. A more complete estimate of resource allocation and behavioural observations may help to identify the traits that drive the costs paid by *M. lignano* when the social environment fluctuates.

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CHAPTER 4

Mate availability does not limit female fecundity in a natural population of free-living flatworms

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Sandner P., T. Janicke, K. Sekii, D. B. Vizoso, and L. Schärer. Mate availability does not limit female fecundity in a natural population of free-living flatworms

Abstract

One aspect of Bateman's principle states that female fecundity is more often limited by access to resources rather than mates, and, in spite of notable exceptions, there is considerable evidence in support of this notion in species with separate sexes. Here we test for the influence of mate limitation on female fecundity in a natural population of a copulating simultaneous hermaphrodite, a reproductive mode for which such evidence is very limited. Specifically, we investigated the effect of mate supplementation on the female fecundity of freshly field-caught specimens of the free-living worm, *Macrostomum lignano*, an obligate outcrosser. We found no effect of mate supplementation on female fecundity and hence no strong evidence for mate limitation in the natural population of this free-living flatworm. The results therefore suggest that this aspect of Bateman's principle applies in this simultaneous hermaphrodite. We discuss possible implications of this result for female choice and sperm digestion. Moreover, we, for the first time present data on body size, gonad size, number of received sperm stored, fertilization efficiency of these sperm, and mating rate in freshly field-caught *M. lignano*.

Introduction

In species with separate sexes (hereafter called gonochorists) female fitness is usually assumed to depend more strongly on the availability of resources rather than mates (Bateman 1948). Specifically, Bateman's experimental results with the fruit fly, *Drosophila melanogaster*, suggested that, while the fitness for males increased approximately linearly with the number of mates, the female fitness increased either little or not at all beyond one mating (Bateman 1948). While Bateman's principle was initially formulated for gonochorists, it was later extended to simultaneous hermaphrodites by Charnov (1979), who, at the time, made it clear that this extension was done in the absence of quantitative evidence. Moreover, as was recently pointed out (Janicke et al. 2011), Charnov framed this principle somewhat differently, namely by stating "that fertilized egg production by an individual is limited not by the ability to get sperm, but by resources allocated to eggs" (Charnov 1979). It is not clear whether he made this emphasis on sperm rather than mates on purpose or whether it is the unintentional result of a different phrasing. In the literal sense Charnov (1979) can be considered to be concerned with repeated matings instead of the number of mating partners.

Whether Bateman's principle actually applies to simultaneous hermaphrodites has been subject to a long-standing debate (e.g., Anthes et al. 2010; Arnold 1994; Janicke et al. 2011; Janicke and Schärer 2009; Leonard 1990; Michiels 1998), and it is an important focus of current research on sexual selection in simultaneous hermaphrodites (Anthes et al. 2010). At any rate, studies relating mate availability to female fecundity remain scarce in simultaneous hermaphrodites, especially among animals.

Simultaneous hermaphroditism is classically predicted to be favoured at low population density, with a sessile lifestyle, or with low mobility (Ghiselin 1969, Schärer 2009). This could result in female fitness being limited by the availability of mates, which would question the classical assumptions of Bateman's principle. Indeed, simultaneous hermaphroditism has been seen to offer an advantage in this context, as it reduces the problem that gonochorists face when encounter rates are low, i.e., that two partners that encounter each other can be unable to mate because they have the same sex (Ghiselin 1969; Tomlinson 1966). Similarly the possibility of self-fertilization in simultaneous hermaphrodites may be seen as a possible solution in cases of mate limitation,

an argument already put forward by Darwin (1876) and refereed to as reproductive assurance. Simultaneous hermaphroditism might therefore be selected to avoid the potentially severe risk of female infertility due to mate limitation.

Reduction in fecundity through mate or sperm limitation is probably widespread in both hermaphroditic and gonochoristic taxa (see, e.g., Levitan and Petersen 1995; Wedell et al. 2002). They are most prevalent at low population densities or low encounter rates due to low mobility. Severe sperm limitation commonly occurs in broadcast-spawning organisms, e.g. cnidarians and echinoids, that release both types of gametes into the water, where female fertilization rates (i.e., the percentage of eggs that get fertilized) can be low due to gamete dilution (see literature in Levitan 1998). It is less severe in organisms spawning synchronously or in close contact (Brawley 1992; Petersen et al. 1992; Sewell and Levitan 1992; Shapiro et al. 1994), and in 'spermcast' organisms, e.g. marine invertebrates and land plants, that release and receive sperm / pollen from the environment, but retain eggs / ovules (e.g., Bishop and Ryland 1991; Burd 1994), some of which can efficiently use even very dilute sperm. Even for animals with copulation and internal fertilization that often store sperm of different partners, mate limitation has been reported (Baur 1988; Michiels et al. 2003; Yusa 1994, see Anthes et al. 2006). The latter idea has partly been supported experimentally (e.g., Baur 1988) or by the lack of conspecifics observed in the vicinity of the individuals at the time of collection (Yusa 1994). In copulating animals sperm limitation may often be a consequence of mate limitation, both are presumably strongly interconnected and it may only be possible to fully disentangle them in controlled lab experiments that start out with virgin animals. However, as they do not necessarily coincide, we will use the term 'sperm limitation' if information on the amount of stored sperm is available, and 'mate limitation', if only this was manipulated.

Wedell et al. (2002) called for more detailed studies that establish the reproductive importance of mate limitation for natural levels of female fertility. We here conducted such a study, which was inspired by the classical and very insightful 'hand pollination' studies in plants (e.g., Burd 1994; Kolb 2005). Specifically, we manipulated mate availability in freshly field-caught specimens of the free-living flatworm *Macrostomum lignano*, an outcrossing simultaneous hermaphrodite, and tested for an effect of this treatment on female fecundity. A positive effect of mate availability on female fecundity could suggest mate limitation in the natural environment of *M. lignano*, and would argue against

the classical Bateman's principle in this simultaneous hermaphrodite. We further present data on body size, gonad morphology, number of received sperm, and mating behaviour of freshly field-caught worms, and determined whether any of these traits are correlated with female fecundity. We also calculated the fertilization efficiency of sperm received in natural copulations (disregarding embryo mortality, which is usually very low), and we compare these field data to data collected from laboratory populations of the same species. If there actually is mate limitation in the field we may also expect that worms that have fewer received sperm when collected would copulate more eagerly in order to compensate for the scarcity of received sperm. We discuss possible implications of our results for aspects of the mating system, sexual selection and sexual conflict in *M. lignano*.

Materials and Methods

Study organism

The free-living flatworm *Macrostomum lignano* (Platyhelminthes, Macrostomorpha) is a copulating simultaneous hermaphrodite and a member of the interstitial sand meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). Very little is currently known about the ecology of this species, and all published data about reproduction and behaviour has been collected in the laboratory, where the worms are kept in enriched artificial sea water at 20°C and are fed *ad libitum* with the diatom *Nitzschia curvilineata* (Rieger et al. 1988). Under these conditions, they reach about 1.5 mm in body length, lay approximately 1.4 eggs per day, and have a generation time of around 18 days (Janicke et al. 2011; Schärer and Ladurner 2003). *M. lignano* is outcrossing with reciprocal and very frequent copulation and internal fertilization (Schärer et al. 2004; Schärer and Ladurner 2003). After about two thirds of all copulations a so-called *suck* behaviour occurs. It consists of a stereotypical posture assumed by one or both partners, which may allow the worms to suck sperm or ejaculate components out of the own female antrum (i.e., the sperm-receiving organ) (Schärer et al. 2004). Sperm tend to anchor themselves in the antrum by means of a specialized structure, but unanchored sperm can often be seen (Vizoso et al. 2010). Completely developed eggs can also be observed in the female antrum before they are deposited. The transparent body of *M. lignano* allows *in vivo* measurements of the size of the paired testes and ovaries, the seminal vesicle (a measure of the number of sperm ready to be transferred;

Schärer and Vizoso 2007), and the amount of received sperm (Janicke et al. 2011). Individuals that are isolated after they were allowed to mate for 24h can store received sperm in the female antrum for at least 14 days, initially laying about one egg per day and eventually running out of sperm (Janicke et al. 2011).

Extraction of worms from field samples

Samples of sediment (about 1 cm deep) were collected in 100ml plastic cups, from a $10m^2$ area in a protected beach near Bibione, Italy (45.6338°N, 13.0754°E), between May 21th and May 27th 2009. The samples were brought to our field house, where the worms were either extracted immediately, or where samples were stored in a refrigerator at 4°C prior to extraction for up to 72 hours. To extract worms from a sample, ~50ml sand was put into a 1000ml beaker and covered with ~150ml of a 3:5 mixture of seawater (25‰ salt content) and a 7.14% $MgCl_2$ solution. After 10 min the beaker was gently shaken, and the supernatant liquid was poured through a net with a mesh width of 63µm or 100µm. The net was then placed into a plastic Petri dish containing pure seawater for observation with a dissecting microscope at 4x or 10x magnification. As soon as a worm was discovered under the dissecting microscope it was collected with an Eppendorf® pipette and individually transferred to a well filled with 1ml of seawater. We aimed at separating the worms as quickly as possible in order to avoid copulations during the extraction process. If the sample contained the target species, all extraction steps were repeated.

Morphometric measurements

Within 12 hours after extraction we checked all worms for species identity based on the morphology of the copulatory stylet and for sexual maturity based on the presence of sexual organs. We discarded specimens of other species and juveniles, and measured the morphology of adult *M. lignano* using the standard procedure described elsewhere (Schärer and Ladurner 2003). Briefly, we relaxed the worms with a solution of $MgCl_2$ in sea water (5:3), transferred them to a glass slide and covered them with a cover slip of a haemocytometer, compressing them dorsoventrally to a fixed thickness of 35µm. We then took digital images of the whole worm at 40x, and of both testes, both ovaries, and the seminal vesicle at 400x magnification using a digital FireWire c-mount camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) mounted on a Leica DME microscope (Leica Microsystems, Wetzlar, Germany), and the software BTV Pro 6.0b1 (available at

http://www.bensoftware.com/btv/dlbeta.html). Also at 400x magnification we focussed through the organ that receives the sperm of a mating partner, i.e. the female antrum, and directly counted the number of sperm that were stored therein. We later measured body size, gonad size, and seminal vesicle size using ImageJ 1.39u (available at http://rsb.info.nih.gov/ij/).

Mating experiment

After measurement the worms were randomly placed into a mating arena either as singles (0 additional mates) or in pairs (1 additional mate) and filmed for four hours using time-lapse video recording. Mating arenas consisted of drops of 4µl of seawater (25‰ salinity) placed between two microscope slides (for a detailed description and a figure of the setup, see Schärer et al. 2004). From May 22th to May 30th, 15 mating chambers were assembled containing twelve mating arenas each. The number and the spatial distribution of the arenas within each mating chamber were balanced with respect to the treatment of the worms. Filming started directly after the assembly of the chambers and was performed at 1 frame $\cdot$ s^{-1} using a SONY DFW-X700 digital FireWire c-mount camera (SONY Broadcast & Professional, Köln, Germany) and BTV Pro 6.0b1 (available at http://www.bensoftware.com/). The number and the duration of all copulations was scored by frame-by-frame analysis.

Directly after the observation period the mating chambers were disassembled and all worms were transferred individually to wells of 24-well plates that were filled with f/2 medium (Andersen et al. 2005), and they were allowed to produce offspring until they stopped laying fertilized eggs. During this period, the diatom *Nitzschia curvilineata* was supplied as an *ad libitum* food source. Temperature fluctuated between ~16°C and ~25°C during the days in the field house. On the 4th June, all individuals were brought to our laboratory and thenceforth kept at 20°C. The worms were transferred to new wells 6, 16, and 24 days after they underwent the mating experiment, and the resulting hatchlings were always supplied with *ad libitum* food. We counted the hatchlings by removing them from their wells on the 4th June (day of transport to the lab), and always 10 days after their mothers had been transferred to new wells. While counting the observer was naive with regard to the treatment group of each replicate.

Statistical analysis

To test for the effect of additional mating opportunities and other traits on female fecundity we calculated generalized linear models (GLMs) on the number of hatchlings produced. We initially calculated a GLM with a poisson

error distribution and specified a log link function, as is customary for count data. We included the fixed factors mating chamber, treatment (0 or 1 additional mating partners), body size, ovary size, testis size, seminal vesicle size, the number of received sperm (in the female antrum before the experiment), and all possible interactions. As this model showed strong overdispersion we instead used a negative binomial GLM, keeping the logarithmic link (Zeileis et al. 2008). A negative binomial GLM can capture overdispersion and has the advantage over quasi-poisson GLMs that it has a likelihood function, which makes model selection possible (Zuur et al. 2009). We initially fitted the model with all factors as above, but then removed the factor mating chamber and all possible interaction terms, because they did not explain a significant part of the variation in female fecundity and were also not 'biologically reasonable' (Grueber et al. 2011). Thus the full model contained treatment (0 or 1 additional mating partners), body size, ovary size, testis size, seminal vesicle size and number of received sperm (in the female antrum before the experiment). From there we continued the model selection procedure based on the Akaike Information Criterion (AIC) to drop terms in turn, until all remaining terms were significant. Replicates were based on a single worm (treatment 0) or on a pair (treatment 1), whereby values of pairs were averaged between both worms. If a trait was significantly correlated to body size we controlled for this in the GLMs by taking the residuals of a linear regression fit of the trait onto body size (ovary size, $R^2 = 0.52$, $n = 151$, $P < 0.0001$; testis size, $R^2 = 0.52$, $n = 151$, $P < 0.0001$; seminal vesicle size, $R^2 = 0.20$, $n = 151$, $P < 0.0001$).

To further describe these field-caught worms we calculated the egg production rate at the time of extraction based on the percentage of developed eggs present in the female antrum. We also calculated the fertilization efficiency (i.e., the number of viable eggs produced divided by the number of received sperm in storage prior to the experiment) in worms without additional partners (treatment 0 only), and compared this fertilization efficiency with that estimated from a previous laboratory study (Janicke et al. 2011). We calculated a Spearman's correlation coefficient between received sperm and copulation number in the pairs to test whether worms attempted to compensate for the lack of received sperm by mating more often (treatment 1 only).

In 12 replicates (treatment 1, nine replicates; treatment 0, three replicates) the number of received sperm could not be determined because there was an egg in the antrum, or no antrum could be found, and these replicates were therefore excluded. Four replicates were excluded because drops accidentally fused in the observation chambers. Four replicates were excluded

because the seminal vesicle of one worm was empty, and we wanted worms to be able to donate and receive sperm in this treatment. One replicate of treatment 0 was excluded because the worm was lost while handling. One worm of a treatment 1 replicate was lost after mating, so hatchling production was based on the value of the other worm. Pairs of treatment 1 that did not copulate during the mating experiment were not excluded in order to avoid a bias for mating motivation in treatment 1 versus treatment 0. After all exclusions we had 86 replicates of treatment 0 and 65 replicates of treatment 1 (i.e., total sample size was n = 151). Means are given ± 1 s.e.. Negative binomial GLMs were calculated using the glm.nb() function implemented in the package 'MASS' (Venables and Ripley 2010) in the program R 2.5.1 (R Development Core Team 2009). All other analyses were carried out using JMP 9.0 (SAS Institute 2010).

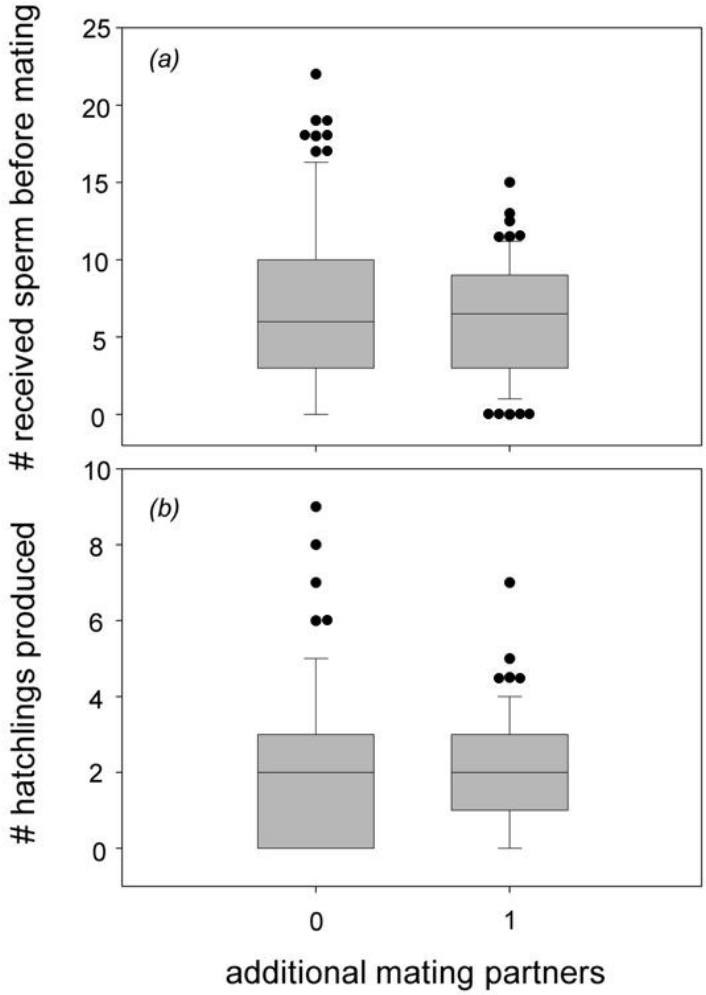

Fig. 1 Number of received sperm in storage before the experimental treatment (*a*) and number of hatchlings produced (*b*) are presented by treatment (0 or 1 additional mating partners). Box-and-whisker plots show medians, first and third quartiles, 10th and 90th percentiles and outliers.

Table 1 Summary of general linear models testing the effect of treatment (mate supplementation), the number of received sperm in storage prior to the experiment, body size, seminal vesicle size, ovary size, and testis size on female fecundity. The goodness of fit is given by the Akaike Information Criterion (AIC). Automatic backward selection based on the AIC led to the reduced model.

Model	AIC	Source	*Z*	d.f.	*P*
Full model	760.42	Treatment	0.52	1, 144	0.61
		Received sperm in storage	4.19	1, 144	< 0.001
		Body size	2.73	1, 144	0.01
		Seminal vesicle size	1.19	1, 144	0.23
		Ovary size	0.61	1, 144	0.54
		Testis size	0.15	1, 144	0.88
Reduced model	755.00	Received sperm in storage	4.24	1, 148	<0.001
		Body size	2.59	1, 148	0.01

Results

We found no significant effect of mate supplementation on female fecundity, measured as hatchling production, suggesting that worms are not mate-limited under field conditions (Fig. 1b). However, the analyses revealed that body size, as well as the number of received sperm, explained significant parts of the variation in female fecundity (Table 1). Specifically, larger animals and animals that had more received sperm in storage prior to the experiment produced more offspring, while none of the measured morphological traits explained a significant portion of variance in female fecundity (Table 1).

The field-caught worms were very small, but their morphology roughly corresponded to the proportions reported for *M. lignano* raised in the laboratory (Table 2). Only 3% had a ripe egg in the female antrum. Assuming that the time for eggs to pass the antrum is the same as in the laboratory this would result in an egg production rate of 0.1 eggs per day at the time of extraction. 82% of all worms had received sperm. The number of sperm received prior to the experiment did not differ significantly between both treatment groups (Wilcoxon-test: Z = -0.82, n = 151, P = 0.41, Fig. 1a). The fertilization

Table 2 Comparison of body size, three morphological traits, and the number of received sperm between field-caught *M. lignano* and worms reared in the laboratory (data from Janicke et al. 2011). Note that we measured more worms from this specific sample site than were used in the present experiment.

Parameter	Field-caught worms		Lab-reared worms	
	n	mean ± s. e. (% of whole body)	*n*	mean ± s. e. (% of whole body)
Body size ($\times 1000\mu m^2$)	354	103.78 ± 2.67	56	592.36 ± 16.87
Testis size ($\times 1000\mu m^2$)	354	6.66 ± 3.12 (6.42%)	56	19.63 ± 0.77 (3.31%)
Ovary size ($\times 1000\mu m^2$)	354	4.05 ± 1.85 (3.90%)	56	19.61 ± 0.72 (3.31%)
Seminal vesicle size ($\times 1000\mu m^2$)	354	1.90 ± 0.93 (1.83%)	56	6.08 ± 0.59 (1.03%)
Number of received sperm	347	6.36 ± 0.28	37	28.57 ± 2.09

efficiency of these sperm was relatively high, both in the field and laboratory (see Table 3).

Worms with fewer received sperm prior to the experiment did not copulate more often than worms with more received sperm, which we could have expected if they would attempt to make up for a limited sperm supply (Spearman's correlation between number of received sperm and number of copulations for treatment 1, $\rho = 0.03$, $n = 65$, $P = 0.72$). 63% of the assembled pairs used the opportunity to copulate, and the ones that copulated (41 replicates) exhibited on average 7.86 ± 1.10 copulations and a mean copulation duration of 14.24 ± 0.56s.

Discussion

Our results suggest that mate limitation is not a significant constraint on female fecundity in the studied natural population of *M. lignano*, as we did not find a significant increase in the number of hatchlings produced by freshly-collected worms when we supplied them with an additional mating partner. It is interesting to relate this result to that of a previous laboratory study on the same organism. Janicke et al. (2011) simultaneously manipulated the number of

Table 3 Comparison of fertilization efficiency (number of viable eggs produced divided by the number of received sperm) between a lab and a field study on *M. lignano*. Only recipients that had visible received sperm were included in this analysis. The origin of the experimental animals and the sample size are presented as well as the food regime used, a potential determinant of fertilization efficiency. Data are given as means ± s.e..

Origin	Sample size	Food supply (before / during egg laying)	Number of sperm in storage	Number of viable eggs produced	Fertilization efficiency	Source
lab	36	*ad libitum* / *ad libitum* or none	29.36 ± 1.99	5.19 ± 0.84	0.18 ± 0.02	Janicke et al. 2011
field	74	unknown / *ad libitum*	8.31 ± 0.56	2.45 ± 0.22	0.29 ± 0.04	present study

available mating partners of formerly virgin worms during 24h (1, 2, or 15 mating partners) and food availability during the subsequent isolation period (no food or *ad libitum*). They found that female fecundity during this isolation period significantly decreased with time after mating, that it was significantly predicted by food availability and the number of received sperm in storage (counted after the day in different group sizes), but not by the number of mating partners. This means that having one mating partner for 24h was sufficient to produce as many offspring as having 15 potential mating partners, hence there was no sperm limitation in smaller groups.

That female fitness in our field study as well as in the laboratory was independent on mate availability is consistent with classical sexual selection theory. Bateman (1948) predicted that the male fitness depends on mate availability, whereas the female fitness depends only on the amount of resources available for egg production. We found female fecundity to be positively predicted by body size, which is likely correlated with the resources available for egg production. Also similarly to the laboratory study (Janicke et al. 2011), we found that female fecundity could be predicted by the number of received sperm. At first sight, this positive correlation seems to indicate sperm limitation and to conflict with the lack of a significant mate supplementation effect. However, it is probably inherent to the experimental set-up we used here and does not represent the situation in the field. Recall that we probably reversed the field situation after the mating experiment, firstly by providing

food *ad libitum*, and secondly by keeping the worms isolated and preventing further mating occasions. By lifting food limitation, which, judging from both the small size of the worms (see below) and the scarce food particles in the stomach (L. Schärer, pers. obs.), is probably severe in the field, we surely created a very artificial situation that is bound to result in sperm limitation sooner or later (as it eventually did in all treatment groups of Janicke et al. 2011). However, this eventual sperm limitation may be irrelevant in the field situation, if resources for the female function would be more limiting than mate or sperm availability. In the following we compare field-caught and lab-raised worms in terms of egg production rate and number of received sperm in storage, among other traits, which further suggests that resource limitation in the field is stronger than sperm limitation.

Field-caught worms were almost six times smaller than worms previously measured in the lab, which suggests that there is probably food limitation in this population. Relative to body size, the testes, ovaries, and seminal vesicles were roughly similar in size to those previously measured in laboratory studies. Notably, the egg production rate was eleven times lower than observed in the laboratory (3% in this study vs. 34% in Janicke et al. 2011 had an egg in their antrum). We have currently no information about the food conditions in the natural habitat of *M. lignano* but another experiment performed simultaneously suggests that very few worms were able to produce even a single hatchling without food supplementation (K. Sekii, pers. obs.). This sign of very low energy reserves is consistent with the small body size of field-caught worms. Together they indicate strong resource limitation in the field.

The percentage of field-caught worms that had received sperm from previous copulations was somewhat lower than that in the laboratory (82% in this study vs. 97% in Janicke et al. 2011), and the number of received sperm in the field was only 22% of that counted in the laboratory. However, the fertilization efficiency of the sperm in field-caught worms was remarkably high. It was considerably higher than documented for *M. lignano* in the laboratory (Table 3) (and much higher than in other species, e.g., 0.03 in *Aplysia parvula*, Yusa 1994). Fertilization efficiency might depend on the number of received sperm and/or food supply: if the antrum is full and/or if the resource level is high, as it was the case in the lab-raised animals, this might reduce fertilization efficiency through sperm loss, while sperm might be more carefully stored otherwise. The high percentage of worms that had some received sperm in storage and the high fertilization efficiency indicate that sperm limitation is

probably low in the studied population. Given the very low egg production rate, which is expected to be strongly restricted by resources available in the field, this number of received sperm observed here is relatively high and probably sufficient to fertilize the eggs produced in the field up until the next mating opportunity. This reasoning would be even more meaningful if we would have included food level as an additional factor in our experiment; it is however difficult to decide which food regime corresponds to the natural food conditions.

The mating behaviour of field-caught *M. lignano* was very stereotypic. Qualitatively it corresponded to that previously observed in the laboratory (described and illustrated in Schärer et al. 2004), but there were some quantitative differences: the mating rate was only a third of the one measured in the lab, but the copulation duration was 162% of that measured in laboratory cultures (8.8s ± 0.4, Schärer et al. 2004). That *M. lignano* did not appear to compensate low numbers of received sperm with increasing numbers of copulations when given a mating opportunity (e.g., as reported for snails, McCarthy 2004) either means that these flatworms cannot sense the amount of received sperm they have, or it indicates that mate availability does not usually set a limit to female fecundity. Fertilized egg production and oviposition in *M. lignano* generally start soon after mating and cease when worms run out of received sperm. We therefore suspect that received sperm can at least qualitatively be perceived by the recipient. In the following we will briefly explore what the observed results mean for the potential of sexual selection and sexual conflict.

Mating rate of freshly field-caught worms was not as high as observed in the laboratory but probably largely exceeded what was required to assure female fecundity. This might be the case because the worms copulate more in order to donate sperm rather than to replenish sperm stores, as has been previously suggested for the situation in a planarian flatworm (Michiels and Streng 1998). This would be consistent with Charnov's hypothesis (1979) that Bateman's principle is also valid in simultaneous hermaphrodites. We do not know whether the received sperm stored by the field-caught worms came from one or several mating partners. Insemination by several partners has been found in the laboratory (Janicke and Schärer 2009a) and likely also occurs in natural populations. This would provide the opportunity for pre- and post-copulatory mate choice, which would be constrained under severe mate limitation (Charnov 1979; Thornhill 1983). If mate availability is not critical possible partners might well be rejected, e.g. because mating is costly (Daly 1978).

Considering that worms probably have sufficient amounts of received sperm to fertilize their eggs, and given the strong food restriction in the field there might even be a possibility for sperm digestion in this simultaneous hermaphrodite (reviewed in Michiels 1998; Anthes et al. 2006). This would imply a sexual conflict between sperm donor and sperm recipient about the fate of the sperm being transferred (Charnov 1979; Schärer and Janicke 2009; Schärer et al. 2011). The 'suck' behaviour (Schärer et al. 2004) that was also observed after some copulations between field-caught worms might be an adaptation to remove or even ingest sperm that have just been received in the own antrum. However, we have currently no experimental evidence for such a function of this behaviour (see Schärer et al. 2011 for comparative evidence). A common solution for simultaneous hermaphrodites in cases of mate limitation is self-fertilization (Jarne and Auld 2006). However, selfing does not usually occur in *M. lignano* (Schärer and Ladurner 2003), which may be seen as another indication that mate limitation probably does not impose a strong selection pressure on *M. lignano*.

A possible caveat with our study is that the additional mating opportunities we offered to the worms did not in fact increase the number of sperm in storage. This appears unlikely because the mate supplementation (treatment 1) resulted in an average of 7.86 ± 1.10 copulations. However, recently sperm displacement has been shown to occur in *M. lignano* (Sandner et al. in preparation, cf. CHAPTER 1 of this thesis), which might lead to a removal or replacement of sperm, potentially without a net increase in sperm number. To judge this possibility we would have had to count the received sperm before and after the experimental treatment.

Conclusions

Severe mate limitation appears unlikely in the studied natural population of *M. lignano,* as we found that most field-caught worms had received sperm and mate supplementation did not significantly increase female fecundity. On the other hand one quarter of the field-caught worms had no received sperm and worms that had more received sperm produced more offspring when put under *ad libitum* food conditions. One might conclude some role for sperm limitation from these two findings. However, this could be misleading, as low resource availability seems to strongly limit female fecundity in the field, as suggested by the small size of the worms and the low egg production rate as compared

with lab-reared worms. We therefore argue that the documented number of received sperm is generally more than adequate to fertilize the eggs being produced by *M. lignano* in the field. That female fitness in the field is more limited by resources allocated to egg production than by received sperm to fertilize the eggs would support earlier conclusions that this aspect of Bateman's principle probably operates in *M. lignano.* A final test of Bateman's principle would still require to measure both the male and the female Bateman gradients and to compare the two statistically (Anthes et al. 2010).

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General discussion

General discussion

This PhD-thesis spans a broad array of topics in evolutionary reproductive biology, including sperm competition (e.g., Parker 1998), phenotypic plasticity (e.g., Pigliucci 2005), the theory of sex allocation (e.g., Charnov 1982), and the evolution of mating systems (e.g., Ghiselin 1969). The presented experiments are original and tackle established theories or their predictions in a novel context. For instance, this work has clearly demonstrated multiple paternity in a simultaneous hermaphrodite, which extends the currently limited findings in other hermaphroditic species (e.g., Baur 1994; Pongratz and Michiels 2003; Kupfernagel et al. 2010). The project thereby helped to establish a novel model organism for research on sexual selection. Because most studies to date were focused on sexual selection in gonochorists, this work also contributes to an important expansion of the significance of sexual selection to another mode of sexual reproduction. Part of the experiments has led to straightforward conclusions. Other experiments yielded negative results that are nevertheless useful to narrow down the possible answers to my research questions. Testable hypotheses for follow-up studies are often formulated in the different Chapters, and also below in the Perspective section.

Conclusions

(1) The potential for post-copulatory sexual selection in this simultaneous hermaphrodite was confirmed by the multiple paternity found in about half of the clutches analyzed for the experiment presented in CHAPTER 1. Paternity success and five traits were found to have a genetic basis, which is a prerequisite for sexual selection to occur. The significant effect of the sperm donor's genotype on paternity success might either be caused by its superior sperm competitiveness or by a female preference in favour of this genotype. Mating rate, which also predicted paternity success significantly, is a good candidate trait to be shaped by sexual selection, because it showed genetic variation and predicted paternity success. Although I here did not determine the proportions of sperm stored in the recipient for each sperm donor separately, my results suggest that additional paternity skews occurred. They most probably included, firstly, sperm displacement leading to second donor sperm precedence, and potentially sperm grouping in the female genital tract biasing paternity according to the position of sperm inside the antrum (Greeff et al. 2001; Harvey and Parker 2000).

(2) When I manipulated sperm competition *per se*, i.e. with social group size being constant, but worms actually experiencing sperm competition, I neither found the increased mating rate previously reported for individuals originating from larger groups (Janicke and Schärer 2009b) nor the phenotypically plastic response in testis size reported for manipulations of social group size (e.g., Janicke and Schärer 2009a; Schärer and Ladurner 2003; Schärer et al. 2005). These results conflict with the hypotheses of individual recognition of mating partners, the detection of mating status of, and signs of mating on the partners. CHAPTER 2 has the merit of ruling out those hypothetical mechanisms for *M. lignano* to assess the current mating group size. This restricts the possible mechanisms for such an assessment to soluble chemical cues, physical contact, and the actual mating rate as signals affecting the sexual habits of *M. lignano*, all of which are probably correlated to social group size. This makes sense insofar as social group size is correlated with mating group size in this multiply mating organism (Janicke and Schärer 2009a).

(3) Exposure of groups of *M. lignano* to fluctuating group size for ten weeks lead to significantly lower offspring numbers than exposure to stable group size for the same time period. This significant cost of a response to changing social group size can be interpreted in the context of costs of phenotypic plasticity. Further investigations are necessary to refine the search for traits underlying these costs, as contrary to earlier findings, we did not obtain strong evidence for changes in sex allocation in this experiment. There are to date only few pieces of evidence for production costs of phenotypic plasticity, and this would be the first in a simultaneous hermaphrodite. The magnitude of this cost is small and it can probably be outweighed by the presumptive benefits of adjustments of sexual habits to changes in social group size – at least if group size is viscous enough but fluctuates eventually like in the experiment presented in CHAPTER 3. These conditions (set by West and Sheldon 2002) would select for phenotypic plasticity in this species.

(4) One scenario where simultaneous hermaphroditism is expected to be advantageous is mate limitation, e.g. due to low population density. The experiment presented in CHAPTER 4 did not yield an effect of mate supplementation on female fecundity of specimens originating from a natural population. Hence, there was no indication that the female reproductive output was limited by mate availability in the field. The question why this flatworm is a simultaneous hermaphrodite could not be answered with mate limitation. Female fecundity seemed to be rather limited by resource availability than mate availability in the natural habitat. This would be consistent with Bateman's principle (Bateman 1948), originally formulated for gonochorists and later extended to simultaneous hermaphrodites (Charnov 1979).

Synthesis

This work has proven *M. lignano* to be a well suited and highly tractable model organism for studies of sexual selection in simultaneous hermaphrodites. It thereby

contributed to expand the scope of sexual selection theory to a range of sexual systems, an important current focus in sexual selection research (e.g. Anthes et al. 2010; Jones 2009; Jones et al. 2000; Nieuwenhuis et al. 2011). Sexual selection may shape morphology, behaviour, and sex allocation in simultaneous hermaphrodites. Under the tested conditions there was a potential for mating rate to be subjected to sexual selection.

Sexual selection (e.g., sperm competition, cryptic female choice) and random paternity skews (e.g., 'joint anchoring') can skew paternity in a way that leads to considerable local sperm competition and may saturate the male fitness gain curve. Both mechanisms may be relevant to account for the stability of simultaneous hermaphroditism.

The costs associated with exposure to changing group size are a first hint on costs of phenotypic plasticity in sex allocation. The small magnitude of these costs suggests that the hypothetical advantage that flexible sex allocation conveys to simultaneous hermaphrodites (Michiels 1998) may actually be a valid point to explain the stability of simultaneous hermaphroditism in mobile animals with frequent copulations.

Perspective

This work has raised at least three testable hypotheses:

The finding of a U-shaped P_2-distribution and the observation of sperm groups anchored in the female antrum have given rise to a hypothetical mechanism that leads to such biased fertilization success, i.e. 'joint anchoring'. So far no means have been available to investigate whether anchored sperm groups are composed of sperm from single or multiple sperm donors. Thanks to upcoming technologies such as GFP-transformation (K. De Mulder and E. Berezikov, pers. comm.) it is now possible to determine S_2 *in vivo*. The 'joint anchoring' hypothesis can be tested by comparing the observed representation of both sperm donors in each anchored group of sperm to random expectations that are based on the overall S_2-value. If the observed variances were significantly larger than the expected variances this would support the 'joint anchoring' hypothesis.

It is possible to determine the relative importance of sperm competition and cryptic female choice. In order to disentangle both mechanisms a North Carolina II design should be suitable, which uses multiple donor and multiple recipient genotypes in a double mating experiment. Donor effects would indicate variation in sperm competitiveness, recipient effects would indicate female choice, and a significant donor × recipient interaction would indicate that reproductive success depends on the specific genotype combinations.

Flatworms may assess group size and therewith approximately the level of sperm competition via chemical cues, tactile cues, and/or their own mating rate. In the experiment in CHAPTER 2 these three possible cues for group size were removed, so that only characteristics of the individuals themselves, their mating status or tags left on their surfaces by mating partners were left as possible cues. It is necessary to independently manipulate chemical cues, tactile cues, and mating rate in a follow-up experiment in order to find the factor(s) that induce the phenotypically plastic responses to changes in social group size.

To identify the causes underlying the maintenance of simultaneous hermaphroditism in *M. lignano* (under the adaptivity paradigm) it might be worthwhile to investigate the shape of the male fitness gain curve. Also more ecological data on *M. lignano* would be highly desirable in this context, e.g. on population density and mating group size in natural populations.

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Appendices

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