Sexual selection and sex allocation in a simultaneous hermaphrodite

INAUGURALDISSERTATION

zur
Erlangung der Würde eines Doktors der Philosophie
vorgelegt der
Philosophisch-Naturwissenschaftlichen Fakultät
der Universität Basel

von
Tim Janicke
aus Dresden, Deutschland

Basel, 2010

Namensnennung-Keine kommerzielle Nutzung-Keine Bearbeitung 2.5 Schweiz

Sie dürfen:

das Werk vervielfältigen, verbreiten und öffentlich zugänglich machen

Zu den folgenden Bedingungen:

Namensnennung. Sie müssen den Namen des Autors/Rechteinhabers in der von ihm festgelegten Weise nennen (wodurch aber nicht der Eindruck entstehen darf, Sie oder die Nutzung des Werkes durch Sie würden entlohnt).

Keine kommerzielle Nutzung. Dieses Werk darf nicht für kommerzielle Zwecke verwendet werden.

Keine Bearbeitung. Dieses Werk darf nicht bearbeitet oder in anderer Weise verändert werden.

- Im Falle einer Verbreitung müssen Sie anderen die Lizenzbedingungen, unter welche dieses Werk fällt, mitteilen. Am Einfachsten ist es, einen Link auf diese Seite einzubinden.
- Jede der vorgenannten Bedingungen kann aufgehoben werden, sofern Sie die Einwilligung des Rechteinhabers dazu erhalten.
- Diese Lizenz lässt die Urheberpersönlichkeitsrechte unberührt.

Die gesetzlichen Schranken des Urheberrechts bleiben hiervon unberührt.

Die Commons Deed ist eine Zusammenfassung des Lizenzvertrags in allgemeinverständlicher Sprache: http://creativecommons.org/licenses/by-nc-nd/2.5/ch/legalcode.de

Haftungsausschluss:
Die Commons Deed ist kein Lizenzvertrag. Sie ist lediglich ein Referenztext, der den zugrundeliegenden Lizenzvertrag übersichtlich und in allgemeinverständlicher Sprache wiedergibt. Die Deed selbst entfaltet keine juristische Wirkung und erscheint im eigentlichen Lizenzvertrag nicht. Creative Commons ist keine Rechtsanwaltsgesellschaft und leistet keine Rechtsberatung. Die Weitergabe und Verlinkung des Commons Deeds führt zu keinem Mandatsverhältnis.

Quelle: http://creativecommons.org/licenses/by-nc-nd/2.5/ch/ Datum: 3.4.2009

Genehmigt von der Philosophisch-Naturwissenschaftlichen Fakultät auf Antrag von

Fakultätsverantwortlicher: Prof. Dr. Dieter Ebert, Basel
Dissertationsleiter: Prof. Dr. Dieter Ebert, Basel
Dissertationsbetreuer: Dr. Lukas Schärer, Basel
Korreferent: Dr. Joris M. Koene, Amsterdam

Basel, den 25. Mai 2010

Prof. Dr. Eberhard Parlow, Dekan

Sexual selection and sex allocation in a simultaneous hermaphrodite

Tim Janicke - PhD thesis

Table of contents

Abstract

Since Darwin's pioneering work, sexual selection theory has become a unifying framework in evolutionary biology successfully explaining the variation in sexual dimorphism, reproductive strategies and mating systems. Although, it has long been argued that sexual selection is an important evolutionary agent in all sexually reproducing organisms, the vast majority of empirical studies on sexual selection in animals focus on separate sexed organisms. However, given that hermaphroditism is a widespread reproductive strategy among animals, empirical work on sexual selection in this group of organisms is required to evaluate the general importance of sexual selection for animals of all types of gender expression. In my PhD project, I aimed to determine the characteristics of sexual selection that operates in the outcrossing simultaneously hermaphroditic flatworm *Macrostomum lignano*, with a particular focus on the conditions that have been proposed to affect the sex allocation (i.e., the resource allocation to the male versus the female sex function) in simultaneous hermaphrodites. Moreover, I was interested in the consequences of sex allocation for the mating behaviour in simultaneously hermaphroditic animals.

Sex allocation theory predicts that the average mating group size (i.e., the number of mating partners plus one) within a population is one of the main parameters that affect the sex allocation in simultaneous hermaphrodites. I studied the determinants of mating group size and sperm transfer success in *M. lignano* and found substantial between-individual variation in both traits. My results suggest that mating group size is primarily affected by the number of available mating partners, but I also found that worms with relatively larger testes acquired more mates. Moreover, sperm transfer success of an individual was correlated positively with testis size and was affected by the shape of the male copulatory organ. Apart from this, I also explored the role of mate choice for its potential to restrict the number of mating partners in *M. lignano*. I found that sequentially mated worms copulated more frequently with well-fed worms compared to starved worms. This suggests that worms have a preference to mate more well-fed partners, presumably because well-fed partners have a higher female fecundity and therefore represent more attractive sperm recipients. Moreover, contrary to theoretical work on strategic sperm allocation, my results indicate that worms do not adjust their sperm expenditure prudently in response to the female fecundity of the mate.

Simultaneous hermaphrodites are predicted to allocate more reproductive resources into the male sex function if the mating group size increases. Until now, empirical studies testing this prediction primarily focussed on phenotypic plasticity in testis size as an estimate of male allocation. However, sperm competition theory predicts that sperm competition does not only select for larger testes but also for the production of bigger sperm. I experimentally tested for a phenotypically plastic response in sperm length to different levels of sperm competition in *M. lignano*. Despite that fact that my experimental manipulation of the level of sperm competition induced a phenotypically plastic response in testis size and ovary size, I found no difference in the sperm length between individuals that either experienced no or intense sperm competition. Thus, there seems to be no phenotypic plasticity in sperm length in response to the level of sperm competition in *M. lignano*.

One crucial assumption of sexual selection theory for simultaneous hermaphrodites is that Bateman's principle applies to this group of organisms. Consequently, the reproductive success of the female sex function is expected to be primarily limited by the resources available for egg production rather than the number of mating partners. In support of this prediction, I could demonstrate that the food availability but not the number of mating partners has an effect on the number of offspring produced by the female sex function in *M. lignano*.

Previous research on sex allocation in simultaneous hermaphrodites mainly focused the factors that influence the resource allocation towards the male versus the female sex function, but very few attempts have been made to understand the behavioural consequences of differences in the sex allocation among individuals. I hypothesised that sex allocation has an effect on the mating behaviour in simultaneously hermaphroditic animals and predicted that individuals adopt a mating strategy that is most beneficial to the sex function that is relatively more pronounced compared to the average sex allocation in a population. An experimental test of this hypothesis revealed that more male-biased individuals mate more frequently compared to more female-biased individuals, which has been predicted if Bateman's principle applies to *M. lignano*.

In conclusion, my studies suggest a high potential for pre- and post-copulatory sexual selection in the simultaneously hermaphroditic flatworm *M. lignano*. Interestingly, my findings are in support of other studies indicating that mating group size can be high in simultaneous hermaphrodites. This body of empirical data violates a central prediction of sex allocation theory stating that simultaneous hermaphroditism is only an evolutionary stable strategy if the mating group size is small. I argue that future research should particularly focus on this disagreement between sex allocation theory and empirical data, and I propose some promising approaches to explain the evolutionary stability of simultaneous hermaphroditism in spite of large mating groups.

Zusammenfassung

Die Lehre der sexuellen Selektion hat sich seit Darwins Grundlagenwerk zu einem einheitlichen Konzept in der Evolutionsbiologie entwickelt, welches es ermöglicht die Geschlechtsunterschiede sowie die Variation in den bestehenden Fortpflanzungsstrategien und Paarungssystemen des Tier- und Pflanzenreichs erfolgreich zu erklären. Seit langem gilt es als erwiesen, dass die sexuelle Selektion eine bedeutende Selektionsart für alle sich sexuell vermehrenden Organismen darstellt. Allerdings bezieht sich der überragende Anteil der zoologischen Studien zur sexuellen Selektion auf getrennt geschlechtliche Arten und das obwohl das Zwittertum im Tierreich sehr weit verbreitet ist. Angesichts dessen sind Studien zur sexuellen Selektion bei Zwittern zwingend erforderlich, um tatsächlich beurteilen zu können, ob die sexuelle Selektion für die Evolution von Organismen verschiedenster Geschlechtsausprägungen eine ähnlich entscheidende Rolle spielt.

In meinem Doktorat untersuchte ich die Charakteristika der sexuellen Selektion bei einem sich auskreuzenden Simultanzwitter, dem Plattwurm *Macrostomum lignano*. Ein besonderes Augenmerk legte ich dabei auf jene Faktoren, die vermutlich die geschlechtsspezifische Ressourcenverteilung (d.h. die Verteilung reproduktiver Ressourcen in die männliche und die weibliche Geschlechtsfunktion) bei simultanen Zwittern festlegen. Des Weiteren interessierte ich mich für die Konsequenzen der geschlechtsspezifischen Ressourcenverteilung für das Paarungsverhalten.

Die Theorie der geschlechtsspezifischen Ressourcenverteilung für Simultanzwitter sagt voraus, dass die Anzahl der Kopulationspartner einen entscheidenden Einfluss auf die Verteilung reproduktiver Ressourcen in die männliche und die weibliche Geschlechtsfunktion hat. Eines meiner Forschungsziele war es, wichtige Einflussgrößen für die Anzahl der Kopulationspartner sowie für den Erfolg bei der Spermienübertragung zu identifizieren. Meine Ergebnisse deuten daraufhin, dass bei *M. lignano* die realisierte Anzahl der Kopulationspartner in erster Linie von der Anzahl verfügbarer Partner abhängt. Ferner fand ich heraus, dass Würmer, die größere Hoden besitzen, einen höheren Paarungserfolg haben und gleichzeitig mehr Spermien im Speicherorgan ihrer Partner hinterlassen können. Außerdem zeigte sich, dass die Morphologie des männlichen Kopulationsorgans einen Teil der Variation im Erfolg bei der Spermienübertragung erklärt. Unabhängig davon untersuchte ich auch inwiefern die Partnerwahl die Anzahl der Kopulationspartner bei *M. lignano* beeinflusst. Hierbei fand ich heraus, dass hintereinander verpaarte Würmer häufiger mit gut gefütterten als mit leicht gehungerten Partnern kopulieren. Es ist daher anzunehmen, dass die Würmer eine Präferenz für Paarungen mit gut gefütterten Partnern haben, vermutlich weil diese im Vergleich zu leicht gehungerten Partnern mehr Eier produzieren können und somit attraktivere Spermienempfänger darstellen. Ich fand jedoch keine Evidenz für eine erhöhte Spermieninvestition in gut gefütterte Partner, wie es von theoretischen Modellen zur strategischen Spermienallokation prognostiziert wird.

Theoretische Modelle sagen vorher, dass Simultanzwitter bei zunehmender durchschnittlicher Anzahl der Kopulationspartner innerhalb einer Population mehr reproduktive Ressourcen in die männliche Geschlechtsfunktion investieren. Empirische Tests dieser Modelle beschränkten sich bisher in erster Linie auf die Messung der Hodengröße als ein Maß investierter Ressourcen in die männliche Geschlechtsfunktion. Jedoch kann neben einem Effekt auf die Hodengröße auch die Produktion längerer Spermien in größeren Gruppengrößen angenommen werden, da unter diesen Bedingungen eine intensivere Spermienkonkurrenz vorherrscht und diese unter Umständen auf größere Spermien selektieren kann. Daher untersuchte ich den Einfluss der Gruppengröße auf die Spermienlänge bei *M. lignano*. Obwohl meine Manipulation der Gruppengröße einen starken Effekt auf die Hoden- und die Ovariengröße hatte, fand ich keinen Unterschied in der Spermienlänge zwischen Würmern, die keiner bzw. einer intensiven Spermienkonkurrenz ausgesetzt waren. Demzufolge wird die Spermienlänge bei *M. lignano* nicht phänotypisch plastisch an die Intensität der vorherrschenden Spermienkonkurrenz angepasst.

Für simultane Zwitter wird zudem vorhergesagt, dass Batemans Prinzip Anwendung findet, d.h. dass der Reproduktionserfolg der weiblichen Geschlechtsfunktion primär durch die Verfügbarkeit von Ressourcen für die Eierproduktion und weniger durch die Anzahl verfügbarer Kopulationspartner bestimmt wird. In Übereinstimmung mit dieser Annahme, konnte ich experimentell zeigen, dass die Ressourcenverfügbarkeit, aber nicht die Anzahl der Kopulationspartner den weiblichen Reproduktionserfolg bei *M. lignano* positiv bedingt.

Bisherige Studien zu geschlechtsspezifischen Ressourcenverteilung bei Simultanzwittern beschäftigten sich in erster Linie mit den Faktoren, die eine optimale Verteilung reproduktiver Ressourcen in die männliche bzw. die weibliche Geschlechtsfunktion bestimmen. Im Gegensatz dazu, wurde die Hinterfragung der Konsequenzen der geschlechtsspezifischen Ressourcenverteilung für das Paarungsverhalten weitestgehend vernachlässigt. Ich stellte die Hypothese auf, dass die geschlechtsspezifische Ressourcenverteilung einen Einfluss auf das Paarungsverhalten besitzt. Diese Hypothese sagt voraus, dass ein simultaner Zwitter einer Verhaltensstrategie nachgeht, die am profitabelsten für diejenige Geschlechtsfunktion ist, die relativ stärker ausgeprägt ist, bezogen auf die mittlere Ressourcenverteilung der anderen Individuen in einer Population. In einem experimentellen Test konnte ich zeigen, dass Individuen die relativ mehr Ressourcen in die männliche Geschlechtsfunktion investieren, überdurchschnittlich häufiger paaren. Unter der Annahme, dass Bateman's Prinzip für *M. lignano* zutrifft, entspricht der gefundene Effekt der geschlechtsspezifischen Ressourcenverteilung auf die Paarungsrate den Erwartungen der getesteten Hypothese.

Zusammenfassend deuten meine Studien auf ein hohes Potential für prä- und postkopulatorische sexuelle Selektion bei *M. lignano* hin. Interessanterweise bekräftigen meine Ergebnisse andere Arbeiten, die ebenfalls eine relativ hohe Paarungsgruppengröße für andere Simultanzwitter belegen. Dieser Tatbestand verletzt jedoch ein zentrales theoretisches Modell, in dem simultanes Zwittertum nur dann eine evolutionär stabile Reproduktionsstrategie darstellt, wenn die durchschnittliche Anzahl der Kopulationspartner klein ist. In meiner Arbeit weise ich abschließend auf diese Diskrepanz zwischen empirischen Daten und den theoretischen Modellen hin und stelle Ansätze vor, die helfen sollen zu klären, weswegen simultanes Zwittertum eine evolutionär stabile Reproduktionsstrategie trotz großer Paarungsgruppengrößen sein kann.

CHAPTER I

Thesis Introduction

THESIS INTRODUCTION

In this introduction, I review the theoretical framework of and the empirical support for the key aspects of the study of sexual selection in simultaneously hermaphroditic animals. Primarily, I focus on research fields that relate to the issues that I addressed in my thesis project, including (1) sexual selection and sex role preferences, (2) pre-copulatory sexual selection, (3) post-copulatory sexual selection and (4) the link between sexual selection and sex allocation in simultaneous hermaphrodites (for a review on sexual conflict in simultaneously hermaphroditic animals see e.g., Arnqvist and Rowe 2005, pp. 185). Thereafter, I specify the main objectives of my PhD project and introduce the model organism that was used to address the outlined questions.

Sexual selection in simultaneous hermaphrodites

Darwin proposed the concept of sexual selection as the „struggle between the males for the possession of the females" (Darwin 1859; specified later in Darwin 1871; for more recent definitions see e.g., Andersson 1994; Clutton-Brock 2007; Carranza 2009). Since then, sexual selection theory has become a unifying theoretical framework in evolutionary biology successfully explaining the variation in sexual dimorphism, reproductive strategies and mating systems (Pizzari et al. 2006; Clutton-Brock 2007; Jones and Ratterman 2009). Although it has long been accepted that sexual selection can be an important evolutionary agent in all sexually reproducing organisms with all types of gender expression (Ghiselin 1969; Charnov 1979; Arnold 1994b; Morgan 1994), relatively little effort has been made to test sexual selection theory in simultaneous hermaphrodites, i.e. organisms that produce male and female gametes at the same time (e.g., Andersson 1994). This is especially unfortunate, since hermaphroditism is widespread among animals comprising about 30% of all species and 24 out of 34 animal phyla, when excluding the very speciose class of insects (Jarne and Auld 2006).

Sexual selection and sex role preferences

Darwin explicitly doubted that sexual selection occurs in simultaneous hermaphrodites (Darwin 1871), presumably because he considered sexual selection primarily in terms of pre-copulatory male-male competition and female choice, which have been argued to be less intense in simultaneous hermaphrodites (e.g., Morgan 1994; Greeff and Michiels 1999a; see also next section). In contrast to Darwin, Charnov (1979) was aware that sexual selection continues after mating when he proposed that Bateman's principle also applies to simultaneously hermaphroditic animals. Bateman's principle states that the reproductive success of males is primarily limited by the number of mating partners whereas the reproductive output of females primarily depends on the resources available for egg production (Bateman 1948; for critical remarks on Bateman's original experiment and the concept itself see Sutherland 1985; Snyder and Gowaty 2007). Accordingly, Charnov (1979) argued that for simultaneous hermaphrodites "fertilized egg production by an individual is limited not by the ability to get sperm, but by the resources allocated to eggs". Therefore, sexual selection is predicted to be more intense for the male sex function compared to the female sex function, which corresponds to the classically assumed difference in the strength of sexual selection between males and females in separate sexed organisms (e.g., Andersson 1994; Cunningham and Birkhead 1998).

Over the last decades, several approaches have been proposed to quantify the strength of sexual selection (e.g., reviewed in Arnold and Wade 1984a, 1984b; Arnold 1994a; Jones 2009; Klug et al. 2010). Empirical studies on separate sexed animals primarily used the 'opportunity for sexual selection' and 'Bateman gradients' to measure sexual selection (e.g., Jones et al. 2002; Bjork and Pitnick 2006; Brown et al. 2009). The opportunity for sexual selection describes the upper limit of the strength of sexual selection (measured as the square of the coefficient of variation in mating success for a given sex; Wade 1979; Arnold and Wade 1984b), whereas Bateman gradients explore the strength of sexual selection on mating success (measured as the slope of a least-squares regression of reproductive success on mating success; Arnold and Duvall 1994). Until now, very few attempts have been made to quantify the strength of sexual selection in simultaneously hermaphroditic animals. A recent study on the polychaete *Ophryotrocha diadema* indicated that the opportunity for selection can be similar to what is known from gonochorists (Lorenzi and Sella 2008). This study also suggested that in *O. diadema* the opportunity for selection is slightly higher for the female sex function compared to the male sex function, but the data are difficult to interpret since the experiment was replicated only twice (Lorenzi and Sella 2008). Contrary to this, two current studies on the strength of sexual selection in the freshwater snails *Physa acuta* and *Biomphalaria glabrata* independently found that the Bateman gradient is steeper for the male sex function compared the female sex function, suggesting that sexual selection is more intense in the male sex function (B. Pélissié and N. Anthes, pers. comm.; Anthes et al. accepted). Nevertheless, based on these studies it is not possible to infer if there is a general pattern in the strength of sexual selection in simultaneous hermaphrodites and whether Bateman's principle actually holds for this group of organisms as originally proposed by Charnov (1979).

Studies assessing the strength of sexual selection are certainly needed to resolve a longstanding debate on the preferable mating role in simultaneous hermaphrodites (e.g., Anthes 2010). Here, the preferable mating role refers to the sex function that provides on average a higher fitness benefit from an additional mating (Anthes et al. accepted). Contrary to Charnov's hypothesis that simultaneous hermaphrodites "copulate not so much to gain sperm to fertilize their eggs as to give sperm away" (Charnov 1979), it has also been hypothesised that hermaphrodites mate preferentially in the female sex function (Leonard 2005, 2006). Until now, empirical tests of both hypotheses primarily aimed to demonstrate whether individuals trade male or female gametes in order to infer indirectly in which sex role individuals prefer to mate. There are studies indicating that simultaneous hermaphrodites trade eggs during mating, which suggests an overall preference to donate sperm to fertilize the partners eggs, i.e. to mate in the male role (e.g., Fischer 1980; Sella 1985). But contrary to Bateman's principle, there are also studies that provide evidence for sperm trading, which may suggest that individuals copulate primarily in order to get the own eggs fertilized and/or to digest sperm, i.e. to mate in the female role (e.g., Leonard and Lukowiak 1991; Vreys and Michiels 1998; Anthes et al. 2005).

Taken together, our current knowledge of the strength of sexual selection that operates on the male and the female sex function of simultaneous hermaphrodites is only anecdotal and it is far from clear in which sex role these organisms do preferentially mate.

Pre-copulatory sexual selection

Theoretical work predicts pre-copulatory sexual selection to be less intense in simultaneous hermaphrodites compared to gonochorists. On the one hand, genes that enhance the fitness of one sex function at the cost of viability (e.g., costly ornaments) will cause the same viability cost for the other sex function but without any fitness gain for the latter sex function. Quantitative genetic models suggest that this makes the expression of sex-specific traits relatively unlikely compared to gonochorists, which limits the opportunity for Fisherian runaway selection in hermaphrodites (Morgan 1994). On the other hand, given that simultaneous hermaphrodites can copulate in the male and the female sex function, both mating partners of a hermaphroditic pair are likely to invest equally in mate acquisition. This has been shown theoretically to lead to a twofold decrease in resources invested for acquiring mating partners in simultaneous hermaphrodites compared to gonochorists, because in the latter type of organisms only one half of the individuals typically invest in mate acquisition (Greeff and Michiels 1999a).

Until now, evidence for pre-copulatory mate-mate competition is scarce (but see e.g., Baeza 2007a), but there are several lines of empirical support for pre-copulatory mate choice in simultaneously hermaphroditic animals (reviewed in Michiels 1998; Leonard 2006; Anthes 2010). The best studied trait that mate choice decisions can rely on is body size. On the assumption that body size is correlated with female fecundity in simultaneous hermaphrodites, and that individuals are to some extent limited by the sperm reserves available for donation, it is predicted that large individuals are preferred mating partners, which leads to size-assortative mating if inseminations are reciprocal (Michiels 1998). Indeed, there are experimental studies demonstrating that the body size of the partner affects mate choice decisions in simultaneous hermaphrodites (e.g., Michiels et al. 2001; Lüscher and Wedekind 2002; Ohbayashi-Hodoki et al. 2004; Anthes et al. 2006a). Moreover, field studies often revealed that the body sizes of mating partners are positively correlated with each other, supporting size-assortative mating to operate (e.g., Vreys and Michiels 1997; Gianguzza et al. 2004; Monroy et al. 2005; Pal et al. 2006). However, in other studies mate choice has been found to be random with respect to body size (Peters and Michiels 1996; Koene et al. 2007; for a review on size-assortative mating see Chaine and Angeloni 2005).

Other traits that have been demonstrated to predict pre-copulatory mate choice in simultaneous hermaphrodites include the relatedness, the level of heterozygosity, the mating status and the parasite infection status of the partner (reviewed in Anthes 2010). Since matings with closely related individuals often lead to inbreeding depression (Charlesworth and Charlesworth 1987), matings with unrelated conspecifics should be favoured (Pusey and Wolf 1996). Such inbreeding avoidance has been found in the hermaphroditic freshwater snail *P. acuta* (Facon et al. 2006). In contrast, mate choice experiments in the cestode *Schistocephalus solidus* revealed an incestuous mating preference despite a strong inbreeding depression, which might be due to indirect fitness benefits that outweigh the costs of inbreeding depression in this species (Schjørring and Jäger 2007). Interestingly, in the same cestode species, it has also been shown that focal individuals preferred to mate with outcrossed rather than selfed individuals, but only if the focal individual itself was outcrossed and not if it was selfed (Schjørring 2009). Furthermore, mating preferences in simultaneous hermaphrodites can also depend on the mating status of the partner, because this is likely to predict the level of sperm competition (reviewed in Wedell et al. 2002). In order to avoid sperm competition, one would expect that sperm donors reject already mated individuals as mating partners, which is supported by a study on the marine sea slug *Aeolidiella glauca* (Haase and Karlsson 2004). Finally, mate choice in simultaneous hermaphrodites has also been found to be influenced by the parasite infection load of the mate. For instance, in the hermaphroditic freshwater snail *B. glabrata* resistant individuals do copulate less often in the female role with infected individuals compared to uninfected individuals (Webster et al. 2003; Webster and Gower 2006).

To summarize, the occurrence of pre-copulatory sexual selection was originally assumed to be less likely in simultaneous hermaphrodites compared to gonochorists, but recent work revealed that these organisms are also choosy among mating partners. However, in contrast to gonochorists, studies on mate choice in simultaneous hermaphrodites have mainly focused on traits that are supposed to be associated with mating preferences in the male sex function rather than in the female sex function.

Post-copulatory sexual selection

Inspired by the pioneering work of Parker (1970) and Thornhill (1983), great strides have been made over the last four decades in our understanding of how sexual selection acts at the post-copulatory stage. On the one hand, male-male competition can continue after copulation in terms of sperm competition, which is defined as the competition between the sperm from two or more males for the fertilization of a given set of ova (Parker 1970, 1998). On the other hand, female choice can also take place after insemination, which is often termed cryptic female choice (originally coined as the "post-mating female choice […] of sperm of preferred mates for fertilizing eggs" and as the biased provisioning "toward zygotes fertilized by superior mates", Thornhill 1983; for more recent definitions see Eberhard 1996; Birkhead 1998, 2000). Nowadays it is widely acknowledged that these two processes are important evolutionary agents that affect the behaviour, morphology and physiology of both males and females (reviewed in Eberhard and Cordero 1995; Birkhead and Pizzari 2002; Wigby and Chapman 2004; Eberhard 2009; Pizzari and Parker 2009; Birkhead 2010).

In contrast to pre-copulatory sexual selection, there is no reason to assume that post-copulatory sexual selection is less intense in simultaneous hermaphrodites compared to gonochorists. On the assumption that Bateman's principle also applies to simultaneous hermaphrodites, a conflict of interest between mating partners may arise since all individuals have a preference for adopting the male sex role during mating, which is to inseminate the partner rather than to receive sperm. This conflict over sex roles during mating is probably often resolved by reciprocal copulations (i.e., both mating partners donate and receive sperm at the same time), which is relatively common in simultaneous hermaphrodites (e.g., Charnov 1979; Michiels 1998; Anthes et al. 2006b). On the assumption that Bateman's principle applies, matings are reciprocal and all else being equal (e.g., density), it follows that the mating rate in a population of simultaneous hermaphrodites is higher than in a population of gonochorists because all hermaphroditic individuals have on average a stronger interest to mate (Michiels 1999). Hence, simultaneous hermaphrodites are expected to be more promiscuous than gonochorists (Edlund and Korn 2007), which potentially generates a relatively higher mean sperm competition intensity (defined as the average number of competing ejaculates from different sperm donors *sensu* Engqvist and Reinhold 2005). Moreover, given that all hermaphroditic individuals are inclined to accept sperm in order to give sperm away, there is only limited scope for pre-copulatory mate choice through the female function, which restricts the ability of sperm recipients to choose among sperm donors primarily to the post-copulatory level. Remarkably, Charnov (1979) already speculated that the fitness gain of the male function "is also affected by how a sperm recipient uses its partner's gametes" and he defined female choice as "the non-random use of sperm from different partners". Therefore, he stressed the importance of post-copulatory sperm choice years before Thornhill (1983) introduced the concept of cryptic female choice, which is widely neglected in the recent literature on post-copulatory sexual selection (discussed in Schärer and Janicke 2009). Taking these theoretical considerations together, it appears that post-copulatory sexual selection in simultaneous hermaphrodites by means of sperm competition and cryptic female choice might be more intense compared to gonochorists.

In accordance to this prediction, empirical studies indicate that sperm competition is rather common in simultaneously hermaphroditic animals (Baur 1998; Michiels 1998). Multiple paternity has been documented for clutches laid by field caught individuals (Baur 1994; Angeloni 2003; Kupfernagel et al. 2010) and also in lab experiments in which individuals where kept in groups of more than two individuals (e.g., Pongratz and Michiels 2003). Compared to gonochorists, relatively little is known about the sources of individual variation in paternity success in simultaneous hermaphrodites. Empirical work on the effect of the mating order on paternity share suggests no general pattern in simultaneously hermaphroditic animals. While some studies revealed first male sperm precedence to operate (e.g., Evanno et al. 2005), other studies indicate that the second sperm donor has advantage to fertilize the partner's eggs (e.g., Rogers and Chase 2002; Angeloni 2003; Pongratz and Michiels 2003) or found that sperm competition conforms to a fair raffle (*sensu* Parker 1990; e.g., Koene et al. 2009). Intraspecific variation in sperm precedence has been found to depend primarily on the time between different matings (Baur 1994). This is presumably the reason why a recent study on sperm competition in the land snail *Cornu aspersum* found last male sperm precedence in mating trials with three competitors (Garefalaki et al. 2010), although another study in the same species clearly indicated first male sperm precedence in situations where two snails competed for the fertilization (Evanno et al. 2005). Furthermore, morphological traits of the sperm donor have clearly been demonstrated to influence paternity success. For instance, testis size has been shown to have a positive effect on the paternity share in the free-spawning ascidian *Botryllus schlosseri* (Yund 1998; Johnson and Yund 2009). Moreover, the length of the male copulatory organ has been shown to correlate positively with paternity success in the land snail *C. aspersum* (Garefalaki et al. 2010). In contrast, the body size of the sperm donor seems to have no effect on paternity success (e.g., Baur 1994; Rogers and Chase 2002; Angeloni et al. 2003; Evanno et al. 2005; Garefalaki et al. 2010). Finally, a very intriguing mechanism that predicts paternity success in some simultaneous hermaphrodites is the unique dart shooting behaviour found in helicid land snails. During mating, each member of a courting pair tries to push a calcareous 'love dart' into its partner and it has been shown that successful dart shooters have a higher paternity success (Landolfa et al. 2001). This is because dart shooting increases the proportion of sperm that is stored in the female sperm storage organ (Rogers and Chase 2001), which is mediated by the injection of mucus from a gland that is associated with the dart (Koene and Chase 1998; Chase and Blanchard 2006).

Within-species variation in paternity success has most often been attributed to traits that characterise the sperm competitiveness of the sperm donor. However, the vast majority of the available datasets can not exclude cryptic female choice as the underlying mechanism for the observed biases in paternity success (for pitfalls in demonstrating sperm competition *versus* cryptic female choice see Birkhead 1998). Experimental attempts to demonstrate cryptic female choice in simultaneous hermaphrodites are very rare. Probably the best evidence for female control over paternity comes from the spermcast mating compound ascidian *Diplosoma listerianum*. Data on sperm movements in the female reproductive tract and paternity analysis from crosses between genetically different clones indicate that in addition of blocking self sperm, the oviduct can reject sperm from a particular donor while simultaneously accepting it from another (Bishop 1996; Bishop et al. 1996). Moreover, the data suggest a weak negative correlation between mating success and genetic similarity between mating partners, which has been argued to be under female control (Bishop et al. 1996). However, in these experiments the authors could not control for potential differences in sperm allocation between clones and also had to assume the sperm competition conforms to a fair raffle (Bishop et al. 1996), which makes it difficult to interpret the observed biases in paternity exclusively in the context of cryptic female choice.

Facultative selfing simultaneous hermaphrodites often exhibit a strong preference to outcross rather than to fertilize their own eggs with their own sperm (e.g., Hughes et al. 2009; Koene et al. 2009; but see Lüscher and Milinski 2003). This non-random sperm selection can also be considered as a special form of cryptic female choice. However, a preference to outcross is often inferred from paternity biases towards outcrossed offspring, which might also arise from differences in fertilisation success due to genetic incompatibilities between closely related gametes (e.g., Scofield et al. 1982; for genetic incompatibilities between unrelated gametes see e.g., Harper and Hart 2005) and differences in post-zygotic survival. Therefore, the observed paternity biases might be the result of the interaction between the gametes of both sexes and not necessarily the outcome of cryptic female choice.

To conclude, theoretical and empirical work suggests that post-copulatory sexual selection is widespread in simultaneous hermaphrodites and probably even more intense than expected for gonochorists. To date, high levels of sperm competition have been demonstrated for several species, but unequivocal evidence for cryptic female choice is still lacking. Furthermore, traits that explain variation in sperm competitiveness and cryptic female choice are barely studied in simultaneous hermaphrodites.

Sex allocation in simultaneously hermaphroditic animals

Simultaneous hermaphrodites face the unique challenge of allocating their reproductive resources to the production of sperm and eggs within the same individual. For these organisms, sex allocation theory provides evolutionary criteria to predict the relative investment into the male versus the female sex function (e.g., Charnov 1979; Fischer 1981; Charnov 1982, 1996). By this, sex allocation theory also aims to identify the conditions that need to be met so that simultaneous hermaphroditism is an evolutionary stable reproductive strategy, which is resistant against the invasion of pure males and females (Charnov et al. 1976).

Models of sex allocation for simultaneous hermaphrodites typically predict the evolutionary stable sex allocation of an organism under varying environmental and social conditions (reviewed in Schärer 2009). For this, sex allocation is defined as the resource allocation towards the male versus the female sex function and therefore does not only include resources required for gamete production but also for the development and maintenance of reproductive organs and sex-specific costs associated with the acquisition of mating partners (Charnov 1982). Most of the models assume a fixed reproductive resource budget and that there is a trade-off between the allocation of resources towards the male and the female sex function. Until now, both these assumptions are only poorly supported by empirical data. For instance, several studies indicate that the overall reproductive resource budget is not fixed, but trades-off with other life-history traits (e.g., Yund et al. 1997; Koene and Ter Maat 2004; Koene et al. 2006; Aira et al. 2007; Baeza 2007c). Moreover, empirical evidence for a trade-off in the resource allocation between the male and the female sex function is limited (but see Yund et al. 1997; Schärer et al. 2005), which is probably to some extent due to difficulties in measuring sex allocation accurately (Schärer 2009).

Another crucial assumption of sex allocation theory is that the sex functions differ in the shape of the fitness gain curve, which relates the resource allocation into a sex function to the fitness that is achieved by that allocation. Sex allocation theory predicts simultaneous hermaphroditism to be an evolutionary stable strategy only if the fitness gain curve of one of the two sex functions shows diminishing returns, i.e. decreasing marginal returns for any additional investment into a sex function (Charnov 1982). Typically, the female fitness gain curve is assumed to be linear whereas the male fitness gain curve is thought to be saturating (Figure 1a). Linear female fitness gain curves are expected if Bateman's principle applies to simultaneous hermaphrodites, because this means that the fitness of the female sex function is primarily limited by the resources available for egg production (Charnov 1979; for processes that promote the female fitness gain curve to be nonlinear see e.g., Charnov 1982; Schärer 2009). The rationale for assuming a saturating male fitness gain curve was originally put into the context of 'local mate competition', which usually describes the competition between related males (e.g., brothers) for the access to females leading to diminishing fitness return for the production of additional sons and therefore to female-biased sex ratios in gonochorists (Hamilton 1967). Similarly, 'local mate competition' can generate a diminishing male fitness gain curve in simultaneous hermaphrodites, but here it is because of competition between related sperm from the same hermaphroditic parent rather than competition between related males (Charnov 1980, 1982; Fischer 1984; Greeff et al. 2001). To clarify this, local mate competition in terms of competition between related sperm in simultaneous hermaphrodites was recently coined 'local sperm competition' (Schärer 2009), which is essentially the opposite of sperm competition *sensu* Parker (1970).

Several conditions can cause local sperm competition to be intense and thereby induce diminishing returns for any further investment towards sperm, which results in a saturating male fitness gain curve (reviewed in Schärer 2009). These include selfing or monogamy, small mating group sizes (i.e., a small average number of mating partners in a population) and paternity skews (e.g., due to sperm displacement or cryptic female choice; Charnov 1982, 1996; Pen and Weissing 1999; van Velzen et al. 2009). Here, monogamy or small mating group sizes might either results from low densities or pre-copulatory sexual selection, i.e. mate-mate competition or mate choice. Despite the large body of theoretical work, empirical evidence in support of saturating male fitness gain curves is very scarce and currently limited to spermcast mating marine simultaneous hermaphrodites (Yund and McCartney 1994; McCartney 1997; Yund 1998; Johnson and Yund 2009).

If the above mentioned assumptions are met, sex allocation theory for simultaneous hermaphrodites makes two major predictions. First, sex allocation is biased towards the sex function with the less strongly saturating fitness gain curve (typically the female function; Charnov 1982). Second, with increasing local sperm competition, simultaneous hermaphrodites should increase their resource allocation into the female sex function at the cost of the male sex function (Charnov 1980, 1982; Figure 1b). This is because in situations with high local sperm competition it does not pay to invest a lot of the limited reproductive resources into the male sex function, since this only leads to competition between related sperm of the same sperm donor (e.g., under selfing and strict monogamy all sperm have an average relatedness of $r = 0.5$). Instead, simultaneous hermaphrodites should reallocate the resources into the female sex function in order to maximise the overall number of offspring produced. Conversely, if local sperm competition decreases (e.g., with increasing mating group size) the fitness gain curve of the male sex function becomes more linear because sperm competition favours individuals that invest more resources into sperm production (e.g., Parker 1998). Finally, as soon as the fitness gain curves of both sex functions become linear, a mutant that invests all of its resources into either the male or the female sex function is expected to have the same reproductive output as an individual that invests into both sex functions and therefore males and females can invade a population of simultaneous hermaphrodites. Moreover, in contrast to gonochorists, simultaneous hermaphrodites have to build and to maintain reproductive organs of both sex functions and therefore are predicted to pay higher so-called fixed costs compared to separate sexed organisms (Charnov 1979, 1982). Therefore, it has been argued that simultaneous hermaphroditism "is favoured if and only if male reproductive success shows the law of diminishing returns with the shunting of resources from female to male function" (Charnov 1982, pp. 225).

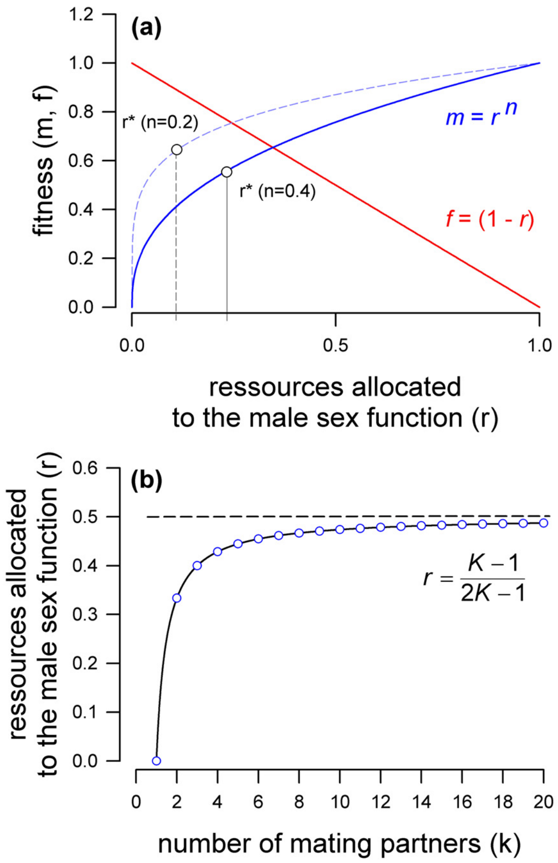

Figure 1. Theoretical foundations of sex allocation theory for simultaneous hermaphrodites. Panel (a) depicts the fitness gain curves and their mathematical expressions for the male (blue line and blue equation) and the female (red line and red equation) sex functions. Male (*m*) and female (*f*) fitness is plotted as a function of the resource allocation to the male sex function. The male fitness gain curve is assumed to saturate as described by a simple power function. Increasing the exponent n generates less saturating fitness gain curves (solid line: *n* = 0.4; dashed line: *n* = 0.2), which results in highest overall fitness returns when allocating more resources to the male sex function. Open circles show the evolutionary stable allocation to the male sex function (*r**) for a given *n* (modified from Charnov 1982). Panel (b) shows the model by Charnov (1980, 1982) on the link between male allocation and the number of mating partners (a high number of mating partners refers to a low level of local sperm competition). Note that the model predicts that the male allocation does never exceed 50%.

Empirical evidence for a female-biased sex allocation is only descriptive or correlational, and experimental evolution studies testing for shifts in sex allocation in response to varying levels of local sperm competition are currently lacking (Schärer 2009). However, many simultaneously hermaphroditic animals are capable of adjusting their sex allocation in a phenotypically plastic way, which offers another opportunity to test experimentally the evolutionary models described above at the level of phenotypic plasticity. In fact, several experimental studies in which the number of individuals within a group was manipulated confirmed the prediction of a more male-biased sex allocation in larger groups (e.g., Trouvé et al. 1999; Schärer and Ladurner 2003; Tan et al. 2004; Schärer et al. 2005; Schärer and Vizoso 2007; but see Koene et al. 2006; Baeza 2007b).

Apart from cases in which the male fitness gain curve is less saturating than the female one (e.g., Charnov 1982; Schärer 2009), more recent theoretical work identified other conditions that might also favour a more balanced or even a male-biased sex allocation. For instance, it has been shown that sperm digestion can lead to a more male-biased sex allocation, since it reduces the competitiveness of the sperm donor and therefore selects for higher investment into sperm production (Greeff and Michiels 1999b). Similarly, cryptic female choice can also result in a more male-biased sex allocation if recipients remove a fixed amount of less preferred sperm (van Velzen et al. 2009). Finally, alternative male investments other than sperm (e.g., costly copulatory organs) can also generate a male-biased sex allocation if assumptions that guarantee the stability of simultaneous hermaphroditism are relaxed (Michiels et al. 2009). To my knowledge, there are no empirical studies supporting the effect of sperm digestion, cryptic female choice or accessory male investment on sex allocation in simultaneous hermaphrodites.

In conclusion, sex allocation theory predicts that pre- and post-copulatory sexual selection should have a profound effect on sex allocation in simultaneous hermaphrodites. However, validations of crucial assumptions and empirical tests of the main predictions are still scarce.

Objectives of the thesis project

In my PhD project I studied several aspects of sexual selection in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. Until now, research on sexual selection in this species primarily explored phenotypic plasticity in testis and ovary size in order to test sex allocation theory for simultaneous hermaphrodites (e.g., Schärer and Ladurner 2003; Schärer et al. 2004b; Schärer et al. 2005; Brauer et al. 2007; Schärer and Vizoso 2007; Vizoso and Schärer 2007; Sandner and Schärer 2010). However, in order to understand the evolutionary and phenotypically plastic responses of sex allocation in simultaneous hermaphrodites, more basic insights into the processes of sexual selection in these organisms are certainly needed. Therefore, the main objective of my PhD project was to determine the presence and the characteristics of sexual selection that can operate in a simultaneous hermaphrodite, with a special focus on the conditions that are predicted to influence the resource allocation towards the male and the female sex function. Moreover, I was interested in the consequences of phenotypic plasticity in sex allocation for the mating behaviour in simultaneous hermaphrodites.

A central prediction of sex allocation theory for simultaneous hermaphrodites is that small mating group sizes lead to a more female-biased sex allocation (Charnov 1980, 1982). Empirical studies addressing this hypothesis usually manipulate social group size (i.e., the number of potential mating partners in a population) to test for a phenotypically plastic adjustment of sex allocation (e.g., Trouvé et al. 1999; Schärer and Ladurner 2003; Tan et al. 2004). However, social group size does not necessarily translate directly into mating group size (originally defined as $k + 1$, where k is the number of mating partners of one individual; Charnov 1982). For instance, if there is intense pre-copulatory mate-mate competition and/or mate choice, social group size becomes an unreliable predictor of mating groups size and consequently of the level of local sperm competition (Schärer 2009). Therefore, I explored the determinants of mating group size in *M. lignano.* In **chapter II,** I present data that demonstrate how social group size is linked to mating group size and reveal the morphological determinants of the number of mating partners an individual obtains. In the same study, I was also interested in explaining variation in sperm transfer success in order to identify morphological traits that are sexually selected in the male sex function.

In **chapter III**, I provide an experimental test of pre-copulatory mate choice in *M. lignano*. For this, I examined mate choice preferences in response to the feeding status of the mate, which is usually correlated with female fecundity and therefore presumably affects the attractiveness for being a sperm recipient in simultaneous hermaphrodites (e.g., Leonard 2006; Anthes 2010). At the same time, I also studied whether *M. lignano* is able to allocate sperm prudently as predicted by sperm competition theory (e.g., Reinhold et al. 2002). Specifically, I tested the hypothesis that focal individuals mate more often with and allocate more sperm to well-fed worms compared to starved worms.

Until now, empirical tests for the effect of mating group size on sex allocation (as predicted by Charnov 1980, 1982) primarily focused on phenotypic plasticity in testis size, which is often used as an estimate of male allocation (Schärer 2009). This is because testis size is assumed to be a strong predictor of sperm production rate, although empirical support for this is rare (but see Schärer and Vizoso 2007; Ramm and Stockley 2009). However, variation in the mating group size may also induce phenotypic plasticity in other reproductive traits, which may also induce varying costs of male reproduction. For instance, phenotypic plasticity has been reported for the size of male genitalia in the simultaneously hermaphroditic acorn barnacle *Semibalanus balanoides* (Hoch 2008, 2009). Moreover, sperm competition theory predicts that under certain conditions sperm competition favours bigger sperm (Parker 1993; Parker et al. in press). Therefore, sperm competition in larger mating groups might not only induce a phenotypic response in the number but also in the morphology of the produced sperm. A previous study in *M. lignano* studied changes in the size of the seminal vesicle and found a positive effect of group size on the size of the produced sperm mass, which was independent of testis size (Schärer and

Vizoso 2007). The authors hypothesised that a phenotypically plastic increase in sperm size in larger groups might explain the observed effect. In **chapter IV,** I present an experimental test of this hypothesis. In particular, I manipulated the level of sperm competition by raising individuals under strict monogamy or polygamy and compared the sperm morphology between these two treatments.

One crucial assumption of sexual selection theory for simultaneous hermaphrodites is that Bateman's principle applies to these organisms (Charnov 1979). This means that the reproductive output of the female sex function is primarily limited by the resources available for egg production rather than by the number of mating partners. However, for gonochorists there is accumulating evidence that females can benefit substantially from multiple mating, either by repeated matings with the same male (direct benefits) or by mating with different males (indirect benefits) (reviewed in Reynolds 1996; Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Knight 2002). In contrast to gonochorists, studies testing for costs and benefits of multiple mating in simultaneous hermaphrodites are scarce. In **chapter V,** I report a study on the effect of polyandry and food availability on the female reproductive output in *M. lignano*. Assuming that Bateman's principle predominates in simultaneous hermaphrodites, I hypothesised that food availability but not the number of mating partners has an effect on the female fecundity.

Previous research on sex allocation in simultaneous hermaphrodites primarily focused on investigating the factors that cause variation in sex allocation within species (Schärer 2009). Surprisingly, very few theoretical and empirical attempts have been made to understand the behavioural consequences of intra-specific variation sex allocation. In **chapter VI,** I propose a hypothesis, which predicts that the sex allocation has an effect on the mating behaviour in simultaneous hermaphrodites. I argue that within-species variation in sex allocation causes differences between individuals in the fitness gain that is derived from each sex function. If both sex functions differ in their optimal mating rate, I predict that individuals should adopt a mating strategy that is most beneficial to the sex function that is relatively more pronounced. For instance, if Bateman's principle applies to simultaneous hermaphrodites, individuals of a more male-biased sex allocation should mate more frequently than individuals that have a more female-biased sex allocation. To test this hypothesis, I conducted an experiment in which I manipulated the sex allocation of individuals by raising them in different group sizes and examined how this manipulation affected the mating behaviour in *M. lignano*.

Study organism

I studied sexual selection and sex allocation in the obligate outcrossing simultaneous hermaphrodite *Macrostomum lignano* (Ladurner et al. 2005). This free-living flatworm is a member of the Macrostomorpha, the most basal subtaxon of the Rhabditophora (Lophotrochozoa, Platyhelminthes; Ladurner et al. 2005; Egger et al. 2009). Until now, it has only been described to occur in the meiobenthos of the Northern Adriatic Sea, Italy (Ladurner et al. 2005). In all experiments of my PhD project, I used worms from lab cultures that were initiated with specimens collected in 2003 near Lignano Sabbiadoro, Italy (45.7°N, 13.1°E).

Recently, *M. lignano* has emerged as a model organisms for the study of developmental biology (Ladurner et al. 2008), gerontology (Mouton et al. 2009) and sex allocation in simultaneously hermaphroditic animals (Schärer 2009). The main advantage of this organism for the latter field of research is its transparency allowing non-invasive measurement of the sex allocation (in terms of testis size and ovary size), the morphology of the genitalia and the observation of sperm that is stored in the female sperm storage organ (e.g., Schärer and Ladurner 2003). Moreover, this organism can be easily cultured in the lab, where worms can be kept in glass Petri dishes filled with f/2 medium (Andersen et al. 2005) at 20°C on a 14:10 day night cycle and fed with the diatom *Nitzschia curvilineata*. Under these conditions the generation time is about 18 days (from egg laying to maturation in both sex functions). Recently, powerful tools have been developed to study several aspects of sexual selection and sex allocation in this species including a sperm tracking technique (Schärer et al. 2007), a RNAi knock-down approach to manipulate sex allocation experimentally (Sekii et al. 2009) and microsatellites for paternity analysis (Sandner et al. in prep.).

Worms that are raised under *ad libitum* food conditions reach a length of approximately 1.5 mm and lay about one egg per day (Schärer and Ladurner 2003). The paired testes are located in the central region of the animal anterior to the paired ovaries. The ovaries are posteriorly connected with a growth zone, where oocytes are provisioned with yolk. The growth zone is also connected with the sperm receiving organ (also called 'antrum'), where the received sperm can be stored for several days. The antrum also stores the egg before it is laid and is presumably the place where fertilization takes place (Vizoso et al. 2010). The tail plate of the worm contains the male copulatory organ (also called the 'stylet') and the seminal vesicle, which stores the produced sperm that are ready to be transferred to mating partners (for a more detailed description of the anatomy see Ladurner et al. 2005). Sperm morphology in *M. lignano* is relatively complex including several appendages (Willems et al. 2009), which are assumed to have coevolved with reproductive traits of the female sex function (Vizoso et al. 2010; Schärer et al. in prep.).

Worms copulate frequently under lab conditions and mating involves a sequence of different behaviours (Schärer et al. 2004a). Typically, after the so-called pre-copulatory 'circling' and 'reeling' postures, both partners form a tight disc and mutually insert their stylet into the antrum of the partner. Hence, copulations are reciprocal, which means that both partners receive and donate sperm at the same time. Within five seconds after copulation, worms often bend themselves and attach their pharynx to their own female genital opening, which is directly connected to the antrum. Afterwards sperm can sometimes be observed sticking out of the female genital opening, so that this behaviour has been termed 'suck behaviour' (Schärer et al. 2004a). However, it is far from clear whether the worms really suck sperm out of the antrum and what the adaptive significance of this behaviour actually is (but for hypotheses see Vizoso et al. 2010).

Very little is known about the ecology and the reproductive biology of *M. lignano* under natural conditions. The majority (i.e., about 75 %) of freshly field-caught worms possess sperm that is stored in the antrum (T. Janicke et al., unpublished data), suggesting that individuals regularly mate in the field, which is a prerequisite for post-copulatory sexual selection to occur. Moreover, systematic sampling revealed that density can vary spatially over a relatively small scale (K. Sekii et al., unpublished data).

References

Aira, M., J. Dominguez, F. Monroy, and A. Velando. 2007. Stress promotes changes in resource allocation to growth and reproduction in a simultaneous hermaphrodite with indeterminate growth. Biol. J. Linn. Soc. 91:593-600.

Andersen, R. A., J. A. Berges, P. J. Harrison, and M. M. Watanabe. 2005. Recipes for freshwater and seawater media. Pp. 429-538 *in* R. A. Andersen, ed. Algal Culturing Techniques. Elsevier, Amsterdam.

Andersson, M. 1994. Sexual Selection. Princeton University Press, Princeton.

Angeloni, L. 2003. Sexual selection in a simultaneous hermaphrodite with hypodermic insemination: body size, allocation to sexual roles and paternity. Anim. Behav. 66:417-426.

Angeloni, L., J. W. Bradbury, and R. S. Burton. 2003. Multiple mating, paternity, and body size in a simultaneous hermaphrodite, *Aplysia californica*. Behav. Ecol. 14:554-560.

Anthes, N. 2010. Mate choice and reproductive conflict in simultaneous hermaphrodites. Pp. 329-357 *in* P. Kappeler, ed. Animal Behaviour: Evolution and Mechanisms. Springer, Berlin.

Anthes, N., P. David, J. R. Auld, J. N. Hoffer, P. Jarne, J. M. Koene, H. Kokko, C. M. Lorenzi, B. Pélissié, D. Sprenger, A. Staikou, and L. Schärer. accepted. Bateman gradients in hermaphrodites: An extended approach to quantify sexual selection. Am. Nat.

Anthes, N., A. Putz, and N. K. Michiels. 2005. Gender trading in a hermaphrodite. Curr. Biol. 15:R792-R793.

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

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

Arnold, S. J. 1994a. Bateman principles and the measurement of sexual selection in plants and animals. Am. Nat. 144:S126-S149.

Arnold, S. J. 1994b. Is there a unifying concept of sexual selection that applies to both plants and animals. Am. Nat. 144:S1-S12.

Arnold, S. J., and D. Duvall. 1994. Animal mating systems - a synthesis based on selection theory. Am. Nat. 143:317-348.

Arnold, S. J., and M. J. Wade. 1984a. On the measurement of natural and sexual selection - applications. Evolution 38:720-734.

Arnold, S. J., and M. J. Wade. 1984b. On the measurement of natural and sexual selection - theory. Evolution 38:709-719.

Arnqvist, G., and T. Nilsson. 2000. The evolution of polyandry: multiple mating and female fitness in insects. Anim. Behav. 60:145-164.

Arnqvist, G., and L. Rowe. 2005. Sexual Conflict. Princeton University Press, Princeton, NJ, USA.

Baeza, J. A. 2007a. Male mating opportunities affect sex allocation in a protrandric-simultaneous hermaphroditic shrimp. Behav. Ecol. Sociobiol. 61:365-370.

Baeza, J. A. 2007b. No effect of group size on sex allocation in a protandric-simultaneous hermaphroditic shrimp. J. Mar. Biol. Assoc. U.K. 87:1169-1174.

Baeza, J. A. 2007c. Sex allocation in an simultaneously hermaphroditic marine shrimp. Evolution 61:2360-2373.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. Heredity 2:349-368.

Baur, B. 1994. Multiple paternity and individual variation in sperm precedence in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Behav. Ecol. Sociobiol. 35:413-421.

Baur, B. 1998. Sperm competition in molluscs. Pp. 255-305 *in* T. R. Birkhead, and A. P. Møller, eds. Sperm competition and sexual selection. Academic Press, London, England.

Birkhead, T. R. 1998. Cryptic female choice: criteria for establishing female sperm choice. Evolution 52:1212-1218.

Birkhead, T. R. 2000. Defining and demonstrating postcopulatory female choice - Again. Evolution 54:1057-1060.

Birkhead, T. R. 2010. How stupid not to have thought of that: post-copulatory sexual selection. J. Zool. (in press).

Birkhead, T. R., and T. Pizzari. 2002. Postcopulatory sexual selection. Nat. Rev. Genet. 3:262-273.

Bishop, J. D. D. 1996. Female control of paternity in the internally fertilizing compound ascidian *Diplosoma listerianum*. I. Autoradiographic investigation of sperm movements in the female reproductive tract. Proc. R. Soc. B-Biol. Sci. 263:369-376.

Bishop, J. D. D., C. S. Jones, and L. R. Noble. 1996. Female control of paternity in the internally fertilizing compound ascidian *Diplosoma listerianum*. II. Investigation of male mating success using RAPD markers. Proc. R. Soc. B-Biol. Sci. 263:401-407.

Bjork, A., and S. Pitnick. 2006. Intensity of sexual selection along the anisogamy-isogamy continuum. Nature 441:742-745.

Brauer, V. S., L. Schärer, and N. K. Michiels. 2007. Phenotypically flexible sex allocation in a simultaneous hermaphrodite. Evolution 61:216-222.

Brown, G. R., K. N. Laland, and M. Borgerhoff Mulder. 2009. Bateman's principles and human sex roles. Trends Ecol. Evol. 24:297-304.

Carranza, J. 2009. Defining sexual selection as sex-dependent selection. Anim. Behav. 77:749-751.

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

Charlesworth, D., and B. Charlesworth. 1987. Inbreeding depression and Its evolutionary consequences. Annu. Rev. Ecol. Syst. 18:237-268.

Charnov, E. L. 1979. Simultaneous hermaphroditism and sexual selection. Proc. Natl. Acad. Sci. U. S. A. 76:2480-2484.

Charnov, E. L. 1980. Sex allocation and local mate competition in barnacles. Mar. Biol. Lett. 1:269-272.

Charnov, E. L. 1982. The Theory of Sex Allocation. Princeton University Press, Princeton, NJ, USA.

Charnov, E. L. 1996. Sperm competition and sex allocation in simultaneous hermaphrodites. Evol. Ecol. 10:457-462.

Charnov, E. L., J. M. Smith, and J. J. Bull. 1976. Why be an hermaphrodite. Nature 263:125-126.

Chase, R., and K. C. Blanchard. 2006. The snail's love-dart delivers mucus to increase paternity. Proc. R. Soc. B-Biol. Sci. 273:1471-1475.

Clutton-Brock, T. 2007. Sexual selection in males and females. Science 318:1882-1885.

Cunningham, E. J. A., and T. R. Birkhead. 1998. Sex roles and sexual selection. Anim. Behav. 56:1311-1321.

Darwin, C. R. 1859. On the Origin of Species by Means of Natural Selection. John Murray, London.

Darwin, C. R. 1871. The Descent of Man, and Selection in Relation to Sex. John Murray, London.

Eberhard, W. G. 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton.

Eberhard, W. G. 2009. Postcopulatory sexual selection: Darwin's omission and its consequences. Proc. Natl. Acad. Sci. U. S. A. 106:10025-10032.

Eberhard, W. G., and C. Cordero. 1995. Sexual selection by cryptic female choice on male seminal products - a new bridge between sexual selection and reproductive physiology. Trends Ecol. Evol. 10:493-496.

Edlund, L., and E. Korn. 2007. Hermaphroditism: what's not to like? J. Theor. Biol. 245:520-527.

Egger, B., D. Steinke, H. Tarui, K. De Mulder, D. Arendt, G. Borgonie, N. Funayama, R. Gschwentner, V. Hartenstein, B. Hobmayer, M. Hooge, M. Hrouda, S. Ishida, C. Kobayashi, G. Kuales, O. Nishimura, D. Pfister, R. Rieger, W. Salvenmoser, J. Smith, U. Technau, S. Tyler, K. Agata, W. Salzburger, and P. Ladurner. 2009. To be or not to be a flatworm: the acoel controversy. Plos One 4:e5502.

Engqvist, L., and K. Reinhold. 2005. Pitfalls in experiments testing predictions from sperm competition theory. J. Evol. Biol. 18:116-123.

Evanno, G., L. Madec, and J. F. Arnaud. 2005. Multiple paternity and postcopulatory sexual selection in a hermaphrodite: what influences sperm precedence in the garden snail *Helix aspersa*? Mol. Ecol. 14:805-812.

Facon, B., V. Ravigne, and J. Goudet. 2006. Experimental evidence of inbreeding avoidance in the hermaphroditic snail *Physa acuta*. Evol. Ecol. 20:395-406.

Fischer, E. A. 1980. The relationship between mating system and simultaneous hermaphroditism in the coral-reef fish, *Hypoplectrus nigricans* (Serranidae). Anim. Behav. 28:620-633.

Fischer, E. A. 1981. Sexual allocation in a simultaneously hermaphroditic coral-reef fish. Am. Nat. 117:64-82.

Fischer, E. A. 1984. Local mate competition and sex allocation in simultaneous hermaphrodites. Am. Nat. 124:590-596.

Garefalaki, M.-E., A. Triantafyllidis, T. J. Abatzopoulos, and A. Staikou. 2010. The outcome of sperm competition is affected by behavioural and anatomical reproductive traits in a simultaneously hermaphroditic land snail. J. Evol. Biol. 23:966-976.

Ghiselin, M. T. 1969. Evolution of hermaphroditism among animals. Q. Rev. Biol. 44:189-208.

Gianguzza, P., F. Badalamenti, K. R. Jensen, R. Chemello, S. Cannicci, and S. Riggio. 2004. Body size and mating strategies in the simultaneous hermaphrodite *Oxynoe olivacea* (Mollusca, Opisthobranchia, Sacoglossa). Funct. Ecol. 18:899-906.

Greeff, J. M., and N. K. Michiels. 1999a. Low potential for sexual selection in simultaneously hermaphroditic animals. Proc. R. Soc. B-Biol. Sci. 266:1671-1676.

Greeff, J. M., and N. K. Michiels. 1999b. Sperm digestion and reciprocal sperm transfer can drive hermaphrodite sex allocation to equality. Am. Nat. 153:421-430.

Greeff, J. M., J. D. Nason, and S. G. Compton. 2001. Skewed paternity and sex allocation in hermaphroditic plants and animals. Proc. R. Soc. B-Biol. Sci. 268:2143-2147.

Haase, M., and A. Karlsson. 2004. Mate choice in a hermaphrodite: you won't score with a spermatophore. Anim. Behav. 67:287-291.

Hamilton, W. D. 1967. Extraordinary sex ratios. Science 156:477-488.

Harper, F. M., and M. W. Hart. 2005. Gamete compatibility and sperm competition affect paternity and hybridization between sympatric Asterias sea stars. Biol. Bull. 209:113-126.

Hoch, J. M. 2008. Variation in penis morphology and mating ability in the acorn barnacle, *Semibalanus balanoides*. J. Exp. Mar. Biol. Ecol. 359:126-130.

Hoch, J. M. 2009. Adaptive plasticity of the penis in a simultaneous hermaphrodite. Evolution 63:1946-1953.

Hughes, R. N., P. J. Wright, G. R. Carvalho, and W. F. Hutchinson. 2009. Patterns of self compatibility, inbreeding depression, outcrossing, and sex allocation in a marine bryozoan suggest the predominating influence of sperm competition. Biol. J. Linn. Soc. 98:519-531.

Jarne, P., and J. R. Auld. 2006. Animals mix it up too: the distribution of self-fertilization among hermaphroditic animals. Evolution 60:1816-1824.

Jennions, M. D., and M. Petrie. 2000. Why do females mate multiply? A review of the genetic benefits. Biol. Rev. 75:21-64.

Johnson, S. L., and P. O. Yund. 2009. Effects of fertilization distance on male gain curves in a free-spawning marine invertebrate: a combined empirical and theoretical approach. Evolution 63:3114-3123.

Jones, A. G. 2009. On the opportunity for sexual selection, the Bateman gradient and the maximum intensity of sexual selection. Evolution 63:1673-1684.

Jones, A. G., J. R. Arguello, and S. J. Arnold. 2002. Validation of Bateman's principles: a genetic study of sexual selection and mating patterns in the rough-skinned newt. Proc. R. Soc. B-Biol. Sci. 269:2533-2539.

Jones, A. G., and N. L. Ratterman. 2009. Mate choice and sexual selection: what have we learned since Darwin? Proc. Natl. Acad. Sci. U. S. A. 106:10001-10008.

Klug, H., J. Heuschele, M. D. Jennions, and H. Kokko. 2010. The mismeasurement of sexual selection. J. Evol. Biol. 23:447-462.

Knight, J. 2002. Sexual stereotypes. Nature 415:254-256.

Koene, J. M., and R. Chase. 1998. Changes in the reproductive system of the snail *Helix aspersa* caused by mucus from the love dart. J. Exp. Biol. 201:2313-2319.

Koene, J. M., K. Montagne-Wajer, D. Roelofs, and A. Ter Maat. 2009. The fate of received sperm in the reproductive tract of a hermaphroditic snail and its implications for fertilisation. Evol. Ecol. 23:533-543.

Koene, J. M., K. Montagne-Wajer, and A. Ter Maat. 2006. Effects of frequent mating on sex allocation in the simultaneously hermaphroditic great pond snail (*Lymnaea stagnalis*). Behav. Ecol. Sociobiol. 60:332-338.

Koene, J. M., K. Montagne-Wajer, and A. Ter Maat. 2007. Aspects of body size and mate choice in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. Anim. Biol. 57:247-259.

Koene, J. M., and A. Ter Maat. 2004. Energy budgets in the simultaneously hermaphroditic pond snail, *Lymnaea stagnalis*: a trade-off between growth and reproduction during development. Belg. J. Zool. 134:41-45.

Kupfernagel, S., H. P. Rusterholz, and B. Baur. 2010. Variation in multiple paternity and sperm utilization patterns in natural populations of a simultaneous hermaphrodite land snail. Biol. J. Linn. Soc. 99:350-361.

Ladurner, P., B. Egger, K. De Mulder, D. Pfister, G. Kuales, W. Salvenmoser, and L. Schärer. 2008. The stem cell system of the basal flatworm *Macrostomum lignano*. Pp. 75-95 *in* T. C. G. Bosch, ed. Stem Cells: From Hydra to Man. Springer Science + Business Media B.V., Berlin.

Ladurner, P., L. Schärer, W. Salvenmoser, and R. M. Rieger. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha). J. Zool. Syst. Evol. Res. 43:114-126.

Landolfa, M. A., D. M. Green, and R. Chase. 2001. Dart shooting influences paternal reproductive success in the snail *Helix aspersa* (Pulmonata, Stylommatophora). Behav. Ecol. 12:773-777.

Leonard, J. L. 2005. Bateman's principle and simultaneous hermaphrodites: a paradox. Integr. Comp. Biol. 45:856-873.

Leonard, J. L. 2006. Sexual selection: lessons from hermaphrodite mating systems. Integr. Comp. Biol. 46:349-367.

Leonard, J. L., and K. Lukowiak. 1991. Sex and the simultaneous hermaphrodite - testing models of male-female conflict in a sea slug, *Navanax inermis* (Opisthobranchia). Anim. Behav. 41:255-266.

Lorenzi, M. C., and G. Sella. 2008. A measure of sexual selection in hermaphroditic animals: parentage skew and the opportunity for selection. J. Evol. Biol. 21:827-833.

Lüscher, A., and M. Milinski. 2003. Simultaneous hermaphrodites reproducing in pairs self-fertilize some of their eggs: an experimental test of predictions of mixed-mating and Hermaphrodite's Dilemma theory. J. Evol. Biol. 16:1030-1037.

Lüscher, A., and C. Wedekind. 2002. Size-dependent discrimination of mating partners in the simultaneous hermaphroditic cestode *Schistocephalus solidus*. Behav. Ecol. 13:254-259.

McCartney, M. A. 1997. Sex allocation and male fitness gain in a colonial, hermaphroditic marine invertebrate. Evolution 51:127-140.

Michiels, N. K. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. Pp. 219-254 *in* T. Birkhead, and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, London.

Michiels, N. K. 1999. Sexual adaptations to high density in hermaphrodites. Invertebr. Reprod. Dev. 36:35-40.

Michiels, N. K., P. H. Crowley, and N. Anthes. 2009. Accessory male investment can undermine the evolutionary stability of simultaneous hermaphroditism. Biol. Lett. 5:709-712.

Michiels, N. K., A. Hohner, and I. C. Vorndran. 2001. Precopulatory mate assessment in relation to body size in the earthworm *Lumbricus terrestris*: avoidance of dangerous liaisons? Behav. Ecol. 12:612-618.

Monroy, F., M. Aira, A. Velando, and J. Dominguez. 2005. Size-assortative mating in the earthworm *Eisenia fetida* (Oligochaeta, Lumbricidae). J. Ethol. 23:69-70.

Morgan, M. T. 1994. Models of sexual selection in hermaphrodites, especially plants. Am. Nat. 144:S100-S125.

Mouton, S., M. Willems, B. P. Braeckman, B. Egger, P. Ladurner, L. Schärer, and G. Borgonie. 2009. The free-living flatworm *Macrostomum lignano*: a new model organism for ageing research. Exp. Gerontol. 44:243-249.

Ohbayashi-Hodoki, K., F. Ishihama, and M. Shimada. 2004. Body size-dependent gender role in a simultaneous hermaphrodite freshwater snail, *Physa acuta*. Behav. Ecol. 15:976-981.

Pal, P., J. Erlandsson, and M. Skold. 2006. Size-assortative mating and non-reciprocal copulation in a hermaphroditic intertidal limpet: test of the mate availability hypothesis. Mar. Biol. 148:1273-1282.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. Biol. Rev. 45:525-567.

Parker, G. A. 1990. Sperm competition games - raffles and roles. Proc. R. Soc. B-Biol. Sci. 242:120-126.

Parker, G. A. 1993. Sperm competition games - sperm size and sperm number under adult control. Proc. R. Soc. B-Biol. Sci. 253:245-254.

Parker, G. A. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Pp. 3-54 *in* T. Birkhead, and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, Cambridge.

Parker, G. A., S. Immler, S. Pitnick, and T. R. Birkhead. in press. Sperm competition games: Sperm size (mass) and number under raffle and displacement, and the evolution of P2. J. Theor. Biol. (doi:10.1016/j.jtbi.2010.03.003).

Pen, I., and F. J. Weissing. 1999. Sperm competition and sex allocation in simultaneous hermaphrodites: a new look at Charnov's invariance principle. Evol. Ecol. Res. 1:517-525.

Peters, A., and N. K. Michiels. 1996. Do simultaneous hermaphrodites choose their mates? Effects of body size in a planarian flatworm. Freshw. Biol. 36:623-630.

Pizzari, T., T. R. Birkhead, M. W. Blows, R. Brooks, K. L. Buchanan, T. H. Clutton-Brock, P. H. Harvey, D. J. Hosken, M. D. Jennions, H. Kokko, J. S. Kotiaho, C. M. Lessells, C. Macias-Garcia, A. J. Moore, G. A. Parker, L. Partridge, S. Pitnick, J. Radwan, M. Ritchie, B. C. Sheldon, L. W. Simmons, R. R. Snook, P. Stockley, and M. Zuk. 2006. Debating sexual selection and mating strategies. Science 312:690-690.

Pizzari, T., and G. A. Parker. 2009. Sperm competition and sperm phenotype. Pp. 207-245 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm Biology: An Evolutionary Perspective. Academic Press, Burlington, MA, USA.

Pongratz, N., and N. K. Michiels. 2003. High multiple paternity and low last-male sperm precedence in a hermaphroditic planarian flatworm: consequences for reciprocity patterns. Mol. Ecol. 12:1425-1433.

Pusey, A., and M. Wolf. 1996. Inbreeding avoidance in animals. Trends Ecol. Evol. 11:201-206.

Ramm, S. A., and P. Stockley. 2009. Adaptive plasticity of mammalian sperm production in response to social experience. Proc. R. Soc. B-Biol. Sci. 276:745-751.

Reinhold, K., J. Kurtz, and L. Engqvist. 2002. Cryptic male choice: sperm allocation strategies when female quality varies. J. Evol. Biol. 15:201-209.

Reynolds, J. D. 1996. Animal breeding systems. Trends Ecol. Evol. 11:A68-A72.

Rogers, D. W., and R. Chase. 2001. Dart receipt promotes sperm storage in the garden snail *Helix aspersa*. Behav. Ecol. Sociobiol. 50:122-127.

Rogers, D. W., and R. Chase. 2002. Determinants of paternity in the garden snail *Helix aspersa*. Behav. Ecol. Sociobiol. 52:289-295.

Sandner, P., and L. Schärer. 2010. No plastic responses to experimental manipulation of sperm competition per se in a free-living flatworm. Ethology 116:292-299.

Sandner, P., D. B. Vizoso, T. Janicke, K. Sekii, and L. Schärer. in prep. Genetic variation in copulation frequency predicts paternity success in a flatworm.

Schärer, L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. Evolution 63:1377-1405.

Schärer, L., and T. Janicke. 2009. Sex allocation and sexual conflict in simultaneously hermaphroditic animals. Biol. Lett. 5:705-708.

Schärer, L., G. Joss, and P. Sandner. 2004a. Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms suck. Mar. Biol. 145:373-380.

Schärer, L., and P. Ladurner. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 270:935-941.

Schärer, L., P. Ladurner, and R. M. Rieger. 2004b. Bigger testes do work more: experimental evidence that testis size reflects testicular cell proliferation activity in the marine invertebrate, the free-living flatworm *Macrostomum* sp. Behav. Ecol. Sociobiol. 56:420-425.

Schärer, L., D. T. J. Littlewood, A. Waeschenbach, Y. Wataru, and D. B. Vizoso. in prep. Mating behaviour, sexual conflict and the evolution of sperm design.

Schärer, L., P. Sandner, and N. K. Michiels. 2005. Trade-off between male and female allocation in the simultaneously hermaphroditic flatworm *Macrostomum* sp. J. Evol. Biol. 18:396-404.

Schärer, L., and D. B. Vizoso. 2007. Phenotypic plasticity in sperm production rate: there's more to it than testis size. Evol. Ecol. 21:295-306

Schärer, L., J. Zaubzer, W. Salvenmoser, C. Seifarth, and P. Ladurner. 2007. Tracking sperm of a donor in a recipient: an immunocytochemical approach. Anim. Biol. 57:121-136.

Schjørring, S. 2009. Sex allocation and mate choice of selfed and outcrossed *Schistocephalus solidus* (Cestoda). Behav. Ecol. 20:644-650.

Schjørring, S., and I. Jäger. 2007. Incestuous mate preference by a simultaneous hermaphrodite with strong inbreeding depression. Evolution 61:423-430.

Scofield, V. L., J. M. Schlumpberger, L. A. West, and I. L. Weissman. 1982. Protochordate allorecognition is controlled by a Mhc-like gene system. Nature 295:499-502.

Sekii, K., W. Salvenmoser, K. De Mulder, L. Scharer, and P. Ladurner. 2009. Melav2, an elav-like gene, is essential for spermatid differentiation in the flatworm *Macrostomum lignano*. BMC Dev. Biol. 9:62.

Sella, G. 1985. Reciprocal egg trading and brood care in a hermaphroditic polychaete worm. Anim. Behav. 33:938-944.

Snyder, B. F., and P. A. Gowaty. 2007. A reappraisal of Bateman's classic study of intrasexual selection. Evolution 61:2457-2468.

Sutherland, W. J. 1985. Chance can produce a sex difference in variance in mating success and explain Bateman's data. Anim. Behav. 33:1349-1352.

Tan, G. N., F. R. Govedich, and M. Burd. 2004. Social group size, potential sperm competition and reproductive investmnent in a hermaphroditic leech, *Helobdella papillornata* (Euhirudinea: Glossiphoniidae). J. Evol. Biol. 17:575-580.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122:765-788.

Trouvé, S., J. Jourdane, F. Renaud, P. Durand, and S. Morand. 1999. Adaptive sex allocation in a simultaneous hermaphrodite. Evolution 53:1599-1604.

van Velzen, E., L. Schärer, and I. Pen. 2009. The effect of cryptic female choice on sex allocation in simultaneous hermaphrodites. Proc. R. Soc. B-Biol. Sci. 276:3123-3131.

Vizoso, D. B., G. Rieger, and L. Schärer. 2010. Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking. Biol. J. Linn. Soc. 99:370-383.

Vizoso, D. B., and L. Schärer. 2007. Resource-dependent sex allocation in a simultaneous hermaphrodite. J. Evol. Biol. 20:1046-1055.

Vreys, C., and N. K. Michiels. 1997. Flatworms flatten to size up each other. Proc. R. Soc. B-Biol. Sci. 264:1559-1564.

Vreys, C., and N. K. Michiels. 1998. Sperm trading by volume in a hermaphroditic flatworm with mutual penis intromission. Anim. Behav. 56:777-785.

Wade, M. J. 1979. Sexual selection and variance in reproductive success. Am. Nat. 114:742-747.

Webster, J. P., and C. M. Gower. 2006. Mate choice, frequency dependence, and the maintenance of resistance to parasitism in a simultaneous hermaphrodite. Integr. Comp. Biol. 46:407-418.

Webster, J. P., J. I. Hoffman, and M. Berdoy. 2003. Parasite infection, host resistance and mate choice: battle of the genders in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 270:1481-1485.

Wedell, N., M. J. G. Gage, and G. A. Parker. 2002. Sperm competition, male prudence and sperm-limited females. Trends Ecol. Evol. 17:313-320.

Wigby, S., and T. Chapman. 2004. Sperm competition. Curr. Biol. 14:R100-R103.

Willems, M., F. Leroux, M. Claeys, M. Boone, S. Mouton, T. Artois, and G. Borgonie. 2009. Ontogeny of the complex sperm in the macrostomid flatworm *Macrostomum lignano* (Macrostomorpha, Rhabditophora). J. Morphol. 270:162-174.

Yund, P. O. 1998. The effect of sperm competition on male gain curves in a colonial marine invertebrate. Ecology 79:328-339.

Yund, P. O., Y. Marcum, and J. Stewart-Savage. 1997. Life-history variation in a colonial ascidian: broad-sense heritabilities and tradeoffs in allocation to asexual growth and male and female reproduction. Biol. Bull. 192:290-299.

Yund, P. O., and M. A. McCartney. 1994. Male reproductive success in sessile invertebrates - competition for fertilizations. Ecology 75:2151-2167.

CHAPTER II

Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite

Manuscript published as:
Janicke, T., and L. Schärer. 2009. Determinants of mating and
sperm-transfer success in a simultaneous hermaphrodite.
J. Evol. Biol. 22:405-415.

Abstract

The number of mating partners an individual has within a population is a crucial parameter in sex allocation theory for simultaneous hermaphrodites because it is predicted to be one of the main parameters to influence sex allocation. However, little is known about the factors that determine the number of mates in simultaneous hermaphrodites. Furthermore, in order to understand the benefits obtained by resource allocation into the male function it is important to identify the factors that predict sperm-transfer success, i.e. the number of sperm a donor manages to store in a mate. In this study we experimentally tested how social group size (i.e. the number of all potential mates within a population) and density affect the number of mates and sperm-transfer success in the outcrossing hermaphroditic flatworm *Macrostomum lignano*. In addition, we assessed if these parameters covary with morphological traits, such as body size, testis size and genital morphology. For this we used a method, which allows tracking sperm of a labelled donor in an unlabelled mate. We found considerable variation in the number of mates and sperm-transfer success between individuals. The number of mates increased with social group size, and was higher in worms with larger testes, but there was no effect of density. Similarly, sperm-transfer success was affected by social group size and testis size, but in addition this parameter was also influenced by genital morphology. Our study demonstrates for the first time that the social context and the morphology of sperm donors are important predictors of the number of mates and sperm-transfer success in a simultaneous hermaphrodite. Based on these findings, we hypothesise that sex allocation influences the mating behaviour and the outcome of sperm competition.

Introduction

Our understanding of how sexually reproducing organisms allocate their resources to male versus female offspring has increased remarkably over the last decades making sex allocation theory to one of the best examples for Darwinian adaptation in evolutionary biology (Klinkhamer et al. 1997; Frank 2002). However, there are still fundamental assumptions and important predictions of that theory that remain to be tested (Reece et al. 2008). Regarding the reproductive strategy an organism should possess, sex allocation theory predicts that hermaphroditism is favoured if the reproductive success of at least one sex function shows a law of diminishing returns, favouring the reallocation of resources from that function to the other (Charnov 1982). This implies that the fitness increase in one sex function must be greater for the first resources allocated into it than for any further resources, which is depicted by a saturating fitness gain curve. The classical resource allocation model for simultaneous hermaphrodites by Charnov (1979) assumes that the female gain curve is linear and that the male gain curve saturates. The rationale for this is Bateman's principle, which states that the females reproductive success is primarily limited by the energy available for producing gametes, whereas the reproductive output of males is primarily governed by the number of mates (Bateman 1948). Therefore, the female fitness is supposed to be proportional to the resource input into eggs and the male gain curve is expected to saturate because of limitations in male mating opportunities (Charnov 1982). As a consequence of a lowered average number of mating partners a form of local mate competition among sperm from the same donor might arise, which decelerates the fitness gain of any additional resource devoted to the male function (Hamilton 1967; Charnov 1979; Fischer 1981). Since the saturation of the male gain curve occurs more rapidly when the number of mating partners is low, it follows that hermaphroditism is a stable reproductive strategy only if the average number of mates is relatively small (Charnov 1982).

In simultaneous hermaphrodites the number of mates is usually expressed in terms of mating groups size, which is defined as the number of mating partners an individual has within a reproductive period plus one (Charnov 1982). As this term is specific for hermaphrodites, we will use 'the number of mates' instead of 'mating group size' throughout this paper. However, we would like to stress that the only difference between 'mating group size and 'the number of mates' is the fact that the former term includes the focal individual whose mating partners were counted whereas the latter does not.

Despite the central role of the number of mates in sex allocation theory, only very little is known about the absolute number of mating partners simultaneous hermaphrodites can acquire and on how this number varies between different individuals. Moreover, our knowledge about the factors that are responsible for the variation in reproductive success via each sex function is very scarce for hermaphrodites. However, this information is certainly needed to understand variation in sex allocation in simultaneous hermaphrodites, i.e. in the allocation of reproductive resources to the male versus the female function. As outlined above, the fitness of the male function might primarily be governed by the number of mating partners (Bateman 1948). Yet, in species where individuals are promiscuous, sperm from different individuals compete for fertilization and thus the number of offspring sired by the male function will also depend on the outcome of sperm competition (Parker 1970). Moreover, fertilization success is not only a function of male-male competition. In many species, females are able to manipulate paternity at the pre- and/or post copulatory level (Eberhard 1996). There is increasing evidence that females choose among sperm from different males post-copulatorily and that this so called cryptic-female choice (Thornhill 1983) may also affect paternity significantly (e.g. Pizzari and Birkhead 2000; Bussière et al. 2006). Therefore, a complete understanding of the pay-offs obtained by resource allocation into the male function requires not only insights into the factors that predict the number of mating partners, but also the traits that influence the number of sperm an individual is able to successfully get into storage in the partners sperm storage organ.

Here we demonstrate for a hermaphroditic flatworm how social group size (i.e. the number of all potential mates within a population) translates into the number of mates and we identify a number of key factors that determine the number of mates and sperm-transfer success - an issue that has never been addressed explicitly in simultaneous hermaphrodites. *Macrostomum lignano* is an outcrossing simultaneous hermaphrodite (Schärer and Ladurner 2003) that copulates very frequently (Schärer et al. 2004). Copulations always occur reciprocally, with partners mutually inserting their male copulatory organ into the female genital pore (Schärer et al. 2004). Furthermore, it has been shown for this species that testis size is phenotypically plastic in response to different social group sizes, which is thought to be related to the average number of mates and therefore sperm competition intensity (Schärer and Ladurner 2003). These findings indicate a high potential for sexual selection in *M. lignano* that is presumably mainly driven by post-copulatory mechanisms, i.e. sperm competition and cryptic female choice (*sensu* Thornhill 1983).

In this study we experimentally tested how social group size and density affect the number of mates and sperm-transfer success in *M. lignano*. Although, very little is known about correlates of multiple mating in hermaphrodites we hypothesized that the number of mates increases with increasing social group size but that it would be unaffected by density since worms do not change their sex allocation when exposed to different densities (Schärer and Ladurner 2003). Furthermore, we expected that worms transfer in total more sperm in larger social groups because this would explain why individuals enlarge their testes with increasing social group size, which has been shown several times for *M. lignano* (Schärer and Ladurner 2003; Brauer et al. 2007; Schärer and Vizoso 2007). Additionally, we tested whether morphological traits do explain variation in the number of mates and sperm-transfer success. Here, we focused only on traits that are potentially sexually selected. In particular, the analysis included body size, which has been shown to be involved in pre-copulatory mating decisions in other hermaphrodites by serving as a cue for fecundity of the mate (Vreys and Michiels 1997; Anthes et al. 2006; but see Peters and Michiels 1996;

Koene et al. 2007). Furthermore, we tested the effect of ovary size and testis size, which have been demonstrated to reflect gonad productivity in *M. lignano* (Schärer and Vizoso 2007; P. Sandner, unpublished data). Since worms enlarge their testes in larger social groups (e.g. Schärer and Ladurner 2003), we expect that individuals with larger testes are better competitors. In addition, we measured the size of the seminal vesicle, which has been shown to be highly correlated with the number of sperm that are available for subsequent matings (Schärer and Vizoso 2007). Finally, we also studied the role of the male genital morphology, which has been demonstrated to be important for fertilization success in gonochorists (e.g. House and Simmons 2003).

Methods

Study organism

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a free-living flatworm of the intertidal meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). In culture, it reaches 1.5 mm in body length and has a generation time of about 18 days. The worm is completely transparent allowing non-invasive measurement of internal morphology. The paired testes that occupy together about 6% of the body are located anterior to the paired ovaries (Ladurner et al. 2005). The male genitalia include a false seminal vesicle, a muscular seminal vesicle, prostate gland cells, a vesicula granulorum, and a sclerotized stylet (Fig. 1a). During copulation the stylet is inserted into the female genital pore of the mating partner, which opens into the female antrum where the sperm is stored.

In mass cultures, worms are maintained at 20 °C in glass Petri dishes containing f/2 medium and fed with the diatom *Nitzschia curvilineata* (Andersen et al. 2005). Worms used for this experiment originate from cultures of *M. lignano* that were initiated with individuals collected in 2003 near Lignano Sabbiadoro, Italy by L. Schärer and D. B. Vizoso.

Morphometry

Morphological traits of focal worms were measured *in vivo* in a standardized way as described elsewhere (Schärer and Ladurner 2003). We observed worms with a Leica DM 2500 microscope (Leica Microsystems, Germany) and took digital photos at 40x for body size and 400x for gonad size, seminal vesicle size and stylet morphometry with a digital video camera (Sony DFW-X700, Sony Broadcast & Professional, Köln, Germany). Image acquisition was done using the software BTV Pro 6.0b1 (available at http://www.bensoftware.com/beta.html) and pictures were analysed with the image analysis software ImageJ 1.38x (available at http://rsb.info.nih.gov/ij/).

Morphometry of the stylet was analysed using the geometric morphometrics approach (Zelditch et al. 2004). This landmark based method allows quantifying the variation in stylet shape and size while removing information on position and orientation of the object. In total, we defined 24 landmarks that were superimposed for each stylet image (Fig. 1b) using tpsDig 2.10 (F. James Rohlf, 2006, Department of Ecology and Evolution, SUNY at Stony Brook; available at http://life.bio.sunysb.edu/morph/). Stylets of *M. lignano* typically exhibit a curvature that can be either orientated towards the false seminal vesicle or away from it whereas all intermediate shapes exist. By convention, we always placed the first landmark at the side of the base that was orientated towards the false seminal vesicle (Fig. 1b). Landmarks with the same notation should represent homologous points on the different stylets. Therefore, we could only define six fixed landmarks, including two at the base and four at the tip of the stylet (Fig. 1b). All other points were located along the two curved sides of the stylet and were classified as so-called semi-landmarks (Zelditch et al. 2004). To make these semi-landmarks comparable between individual stylets we followed the suggestions by Zelditch *et al.* (2004) and drew two combs with equally spaced perpendicular lines between fixed landmarks on all stylet images using the public domain software MakeFan 6 (H. David Sheets, 2003, available at http://www3.canisius.edu/~sheets/morphsoft.html), prior to superimposition. Semi-landmarks were then arranged at the intersection points of these lines with the stylet (Fig. 1b). We used the minimal bending energy method to slide the semi-landmarks along the outline curves using tpsRelw 1.45 (also available at http://life.bio.sunysb.edu/morph/). We used the same program for relative warp analysis. Relative warps are principal components of a distribution of shapes. Each relative warp score describes the direction of a particular shape change from the consensus form. The relative warp analysis yielded 44 relative warp scores from which the first three explained 83.0% of all variance in the shape of the stylet. The first relative warp score, which explained 57.7% of the variance in shape, described the most drastic change in stylet shape, namely the extent of the stylet curvature (Fig. 1c) and was used as the only stylet shape variable in our final models. Based on landmark data, we also extracted the centroid size of the stylet, which is the square root of the sum of squared distances between landmarks to their common centroid (Zelditch et al. 2004) and a good estimator of overall stylet size.

A previous study has shown that measurements of body size, testis size, ovary size and seminal vesicle size are highly repeatable (Schärer and Ladurner 2003). In order to verify the repeatability of our measurements of male copulatory organ shape and size we randomly selected 50 individuals from the mass cultures and conducted two complete runs of morphological measurements as outlined above for each single worm. Between the two runs, worms were allowed to relax for 12.9 ± 4.7 min (mean ± SD) in f/2 medium. The analysis of these repeated measurements confirmed that our measurements for stylet shape and size are repeatable (intraclass correlation coefficient ri: first relative warp score, ri = 0.60, F49,50 = 3.9, $P < 0.001$; centroid size, ri = 0.97, F49,50 = 73.4, $P < 0.001$).

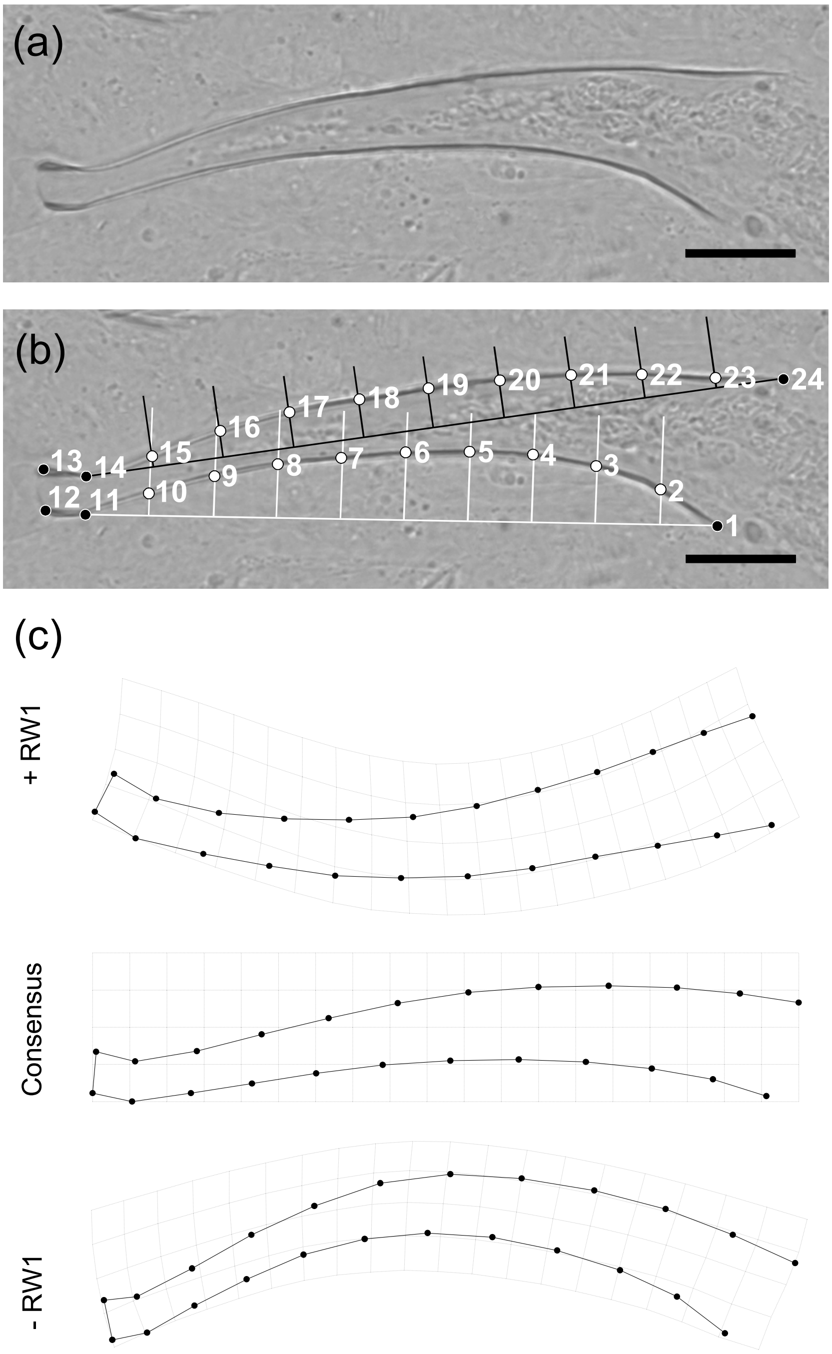

Figure 1. Morphology and geometric morphometrics of the stylet of *Macrostomum lignano*. (a) Interference contrast micrograph of a typically shaped stylet. (b) Placement of fixed landmarks (black circles) and semi-landmarks (white circles). Numbers indicate the notation and the order in which all landmarks were placed. Standardized placement of semi-landmarks was achieved by drawing combs with equally spaced perpendicular lines between fixed landmarks (from 1 to 11 for white comb; from 14 to 24 for black comb) on each stylet image. (c) Thin-plate splines of the stylet derived from the relative warp score analysis. Panels show changes in the shape along first relative warp score. The consensus shape (middle) represents the average stylet shape. Stylets with high positive values of the first relative warp score are curved away from the seminal vesicle (above the consensus) whereas negative values refer to stylets that are curved strongly towards the seminal vesicle (below the consensus). For all stylets shown the seminal vesicle would be located below the stylet. The scale bars represent 10 µm.

Sperm tracking

Sperm tracking was based on DNA-labelling of focal worms with a halogenated pyrimidine and the localization of the label using immunocytochemical staining. Worms were exposed to 5-bromo-2'-deoxyuridine (hereafter called BrdU), which is incorporated instead of thymidine into cells that undergo DNA replication (i.e. cells in s-phase). Thereby, spermatozoa of focal worms become labelled with BrdU when these cells differentiate into sperm during spermatogenesis. Using this approach allows tracking the sperm of a labelled donor (hereafter called focal worms) in an unlabelled recipient.

The protocol was adapted from the method described in detail elsewhere (Schärer et al. 2007). Focal worms were labelled by incubation in a solution of 0.5 mM BrdU (Sigma, B5002-16) in f/2 medium. A previous study showed that spermatogenesis in *M. lignano* takes about 6 days (Schärer et al. 2007). In our study focal worms were continuously exposed to BrdU for 9 days, in order to assure that all sperm in the seminal vesicle of focal worms were labelled. Afterwards, focal and unlabelled worms were washed three times in artificial sea water before they were assigned to their experimental treatments. Fixation and immunocytochemical staining was done in tissue-culture plates while worms remained in their original social groups. After the mating trials (described below), worms were relaxed in a 5:3 mixture of 7.14% $MgCl_2$ and f/2 for 25 min and then fixated for 60 min in 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS) with 10% sucrose. Fixated worms were washed three times with PBS-T (i.e. PBS plus 0.5% Triton X-100), followed by an additional 60 min wash with PBS-T and then permeated with 0.15 µg/ml Protease XIV at 37 °C for 35 min. Protease activity was stopped with cooled 0.1 N HCl. Subsequently, animals were transferred to 2 N HCl for 1 h at 37 °C, then washed three times with PBS-T and blocked with BSA-T (i.e. PBS-T plus 1% bovine serum albumin) for 60 min. BrdU labelled cells were localised using a monoclonal rat anti-BrdU antibody (ab6326, Abcam Limited, Cambridge, UK) at a 1:100 dilution in BSA-T overnight at 4 °C. After four wash steps in PBS-T, the secondary goat-anti-rat FITC-conjugated antibody (ab6115, Abcam Limited, Cambridge, UK) was applied in the dark for 1 h at room temperature at 1:200 in BSA-T. After three further wash steps in PBS-T, animals were mounted on microscope slides using Vectashield (Vector Laboratories), and stored at -20°C until observation. BrdU-labelled sperm were visualised under epifluorescence on a Leica DM 5000 B microscope (Leica Microsystems, Germany). All sperm counts were done blind with regard to the experimental treatment or the morphology of focal worms. Repeated examination of 50 randomly selected unlabelled sperm recipients confirmed a high repeatability of our sperm counts (intraclass correlation coefficient: $r_I = 0.99$, $F_{49,50} = 205.3$, $P < 0.001$).

Experimental set-up

On the first day we collected 1,200 adult worms from mass cultures, distributed them equally among 12 glass Petri dishes and allowed them to lay eggs. After 72 hours all worms were removed and the resulting hatchlings grew in f/2 medium until they had reached maturity (day 22), yielding worms of similar age. We pooled all fully grown worms and randomly distributed 100 individuals to one Petri dish that was filled with a solution of 0.5 mM BrdU in f/2 medium (focal worms) and 100 worms to each of five Petri dishes filled with pure f/2 medium. Five days later we refreshed the BrdU solution and the f/2 medium, respectively. After a total of nine days of BrdU exposure we photographed focal worms for morphological measurements and allowed them to recover overnight in isolation. Until the mating trials, all worms were kept under *ad libitum* food conditions (i.e. with a dense layer of diatoms on the bottom of the wells).

On day 32 we conducted the mating experiment. We manipulated social group size by forming social groups of 2, 3, 4, 8 and 16 individuals. Social groups were composed of one randomly selected focal worm and filled up with the respective number of unlabelled worms. Prior to the assignment of unlabelled worms into their final social group, they were pooled in order to balance any possible differences between worms kept in different Petri dishes. Density was manipulated by replicating all social group sizes in two different enclosure sizes (small or large, i.e. 24-hole and six-hole tissue culture plates containing 1.5 and 6.0 ml of f/2 medium, respectively). Focal worms were allowed to mate in their social group and density treatment for 24 hours and afterwards all worms were fixated and stained as described before. Due to time constraints, the whole experiment was split into two blocks that were separated by four days. Each block comprised six replicates of all factor combinations. As blocking the experiment had no significant effect on any of the variables measured (t-tests and Mann-Whitney U-tests: all $P > 0.05$) it was ignored in the final analysis. Several focal worms were lost due to pipetting errors during antibody staining (n = 9) or excluded because of malformation of the gonads or the copulatory organ (n = 7). The final data set included 648 individuals in 104 replicates with each factor combination being replicated on average 10.4 ± 1.6 times (mean ± SD; range: 8 - 12).

Statistical analysis

We used General Linear Models (GLM) in order to explain variation in the number of mates and sperm-transfer success. The number of mates was defined as the number of unlabelled individuals within a social group that had at least one labelled sperm in the female sperm storage organ. Therefore, the actual number of mates may be underestimated in this study since it is possible that there were some worms that mated but did not transfer any sperm (in case of non-reciprocal copulations) or did not succeed in storing any sperm in the sperm storage organ of their mate (due to sperm displacement or cryptic female choice). However, since we were more interested in explaining variation in the number of mates and less in reporting absolute values we expect that our conclusions are robust despite this underestimation. Sperm-transfer success was described by two variables, namely the mean sperm-transfer success and total sperm-transfer success. Mean sperm-transfer success refers to the average number of labelled sperm the donor managed to get into storage per mate whereas total sperm-transfer success represents the sum of such sperm in a given social group. Measurements of sperm-transfer success presumably reflect the sperm allocation of a focal worm but also the outcome of sperm competition and cryptic-female choice.

For all three response variables we calculated GLMs with density and social group size as fixed factors and included body size, testis size, ovary size, seminal vesicle size, first relative warp score of the stylet and centroid size of the stylet as covariates. Since body size was correlated with testis size, ovary size and seminal vesicle size (Pearson correlation: testis size: $r = 0.56$, *d.f.* = 102, $P < 0.001$; ovary size: $r = 0.61$, *d.f.* = 102, $P < 0.001$; seminal vesicle size: $r = 0.20$, *d.f.* = 102, $P = 0.037$) we used the residuals of these traits derived from a linear regression fit against body size.

In simultaneous hermaphrodites, some authors combine estimates of male and female reproductive investment into a single variable to assess whether an individual allocates relatively more reproductive resources into one sex function in comparison to other individuals (e.g. Lorenzi et al. 2005; Vizoso and Schärer 2007). In a separate run of all models, we included such a composite measure of sex allocation as a covariate instead of testis size and ovary size. Sex allocation was defined as testes size of an individual divided by the total size of the gonads (ovaries and testes). Therefore, high values of sex allocation refer to more male-biased individuals. In this study, sex allocation of focal worms was correlated positively with residual testis size ($r = 0.71$, *d.f.* = 102, $P < 0.001$) and negatively with residual ovary size ($r = -0.52$, *d.f.* = 102, $P < 0.001$). The models that included sex allocation did explain slightly less variation in our target variables compared to models testing gonad sizes separately. Furthermore, since the size of the male and the female gonads were highly correlated with sex allocation, we only report statistics with residual testis size and residual ovary size as covariates in the results. For models including sex allocation as a covariate see Table A1 in the online supplementary material.

In the models with the mean and the total sperm-transfer success as response variables we excluded focal worms whose number of mates was zero. This did not change any of the results reported qualitatively. Values of mean sperm-transfer success were log-transformed and total sperm-transfer success was square rooted. Residuals of all reported models did not deviate significantly from a normal distribution (Kolmogorov-Smirnov: all $P > 0.05$). Values are given as means ± SE, unless otherwise stated. All statistics were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) or JMP 7.0.1. (SAS Institute Inc., Cary, NC, USA).

Results

Morphological measurements

As intended by our random assignment of focal worms to the various treatments, there was no difference in morphological traits between social groups or densities (two-way ANOVA: all $P > 0.05$), except in centroid size of the stylet, which differed between social groups ($F_{4,94} = 2.9$, $P = 0.026$). Moreover, none of the morphological traits that entered the final models were correlated with each other (Pearson correlation: *d.f.* = 102, all $P > 0.05$), except centroid size of the stylet, which covaried negatively with residual testis size ($r = -0.22$, *d.f.* = 102, $P = 0.023$). Despite this collinearity between residual testis size and centroid size of the stylet we left both covariates together in each model because centroid size of the stylet did not explain any variation in the response variables when included instead or in combination with residual testis size. Nevertheless, because of the difference in centroid size between social groups the results regarding centroid size have to be considered with some caution.

Number of mates

The number of mates varied considerably in all social group sizes, ranging from zero to the maximum number of mates possible, except in social groups of 16 individuals where focal worms mated at most with 10 individuals. Out of 104 focal individuals 11 (10.6%) could not get any sperm into storage in any of the potential mates. The number of mates was significantly affected by social group size and residual testis size (Table 1). With increasing social group size the number of mates increased (mean number of mates, pairs: 0.9 ± 0.1; trios: 1.5 ± 0.7; quartets: 2.2 ± 0.9; octets: 2.8 ± 0.5; groups of 16 individuals: 5.4 ± 0.7; Fig. 2 a, b). Furthermore, the residual testis size had a positive effect on the number of mates (Fig. 2b). The density and all other morphological traits that we measured did not significantly explain the remaining variance in the number of mates (Table 1).

Mean sperm-transfer success

Focal worms had a mean sperm-transfer success of 6.4 ± 0.7 sperm (range: 1 - 38 sperm). Mean sperm-transfer success was affected by social group size, residual testis size, residual ovary size and the first relative warp score of the stylet (Table 1). Worms that were kept in larger social groups had a lower mean sperm-transfer success (Fig. 2c). Moreover, worms that had larger testes, smaller ovaries and stylets with high values of the first relative warp score (i.e. stylets that are curved away from the false seminal vesicle) had a higher mean sperm-transfer success (Fig. 2d). Density and all other morphological traits had no effect on mean sperm-transfer success (Table 1).

Total sperm-transfer success

Total sperm-transfer success was on average 13.3 ± 1.3 sperm (range: 1 - 61 sperm). There was no overall difference in total sperm-transfer success between social groups (one-way ANOVA: $F_{4,93} = 0.40$, $P = 0.809$). According to the full model, the total sperm-transfer success was only affected by residual testis size and the first relative warp score of the stylet (Table 1). Worms with larger testes and with stylets that were curved away from the false seminal vesicle had an increased total sperm-transfer success (Fig. 2f). Density and social group size did not predict total sperm-transfer success (Table 1; Fig. 2e).

Table 1. Results of General Linear Models performed to explain variation in the number of mates, mean sperm-transfer success (mean number of labelled sperm stored per mate) and total sperm-transfer success (total number of labelled sperm stored within a social group). Standardized beta (Std β) is the parameter estimate scaled to be dimensionless and indicates the relative importance of each covariate in explaining variation in the response variable (negative values indicate negative effects).

response	source	*d.f.*	Std β	*F*-value	*P*-value
number of mates [a]	density	1	-	2.09	0.152
	social group size	4	-	23.74	<0.001
	density x social group size	4	-	0.74	0.567
	body size	1	0.01	0.01	0.968
	residual testis size	1	0.26	10.88	0.001
	residual ovary size	1	-0.01	0.02	0.900
	residual seminal vesicle size	1	0.03	0.20	0.658
	first relative warp score	1	0.06	0.61	0.435
	centroid size of stylet	1	0.08	1.05	0.309
mean sperm-transfer success [b]	density	1	-	0.51	0.474
	social group size	4	-	10.82	<0.001
	density x social group size	4	-	0.08	0.987
	body size	1	0.12	1.89	0.174
	residual testis size	1	0.21	5.20	0.025
	residual ovary size	1	-0.18	3.97	0.049
	residual seminal vesicle size	1	0.13	2.10	0.151
	first relative warp score	1	0.27	7.54	0.008
	centroid size of stylet	1	-0.02	0.04	0.839
total sperm-transfer success [c]	density	1	-	0.03	0.870
	social group size	4	-	0.80	0.529
	density x social group size	4	-	0.41	0.800
	body size	1	0.10	1.08	0.302
	residual testis size	1	0.34	10.39	0.002
	residual ovary size	1	-0.14	1.74	0.191
	residual seminal vesicle size	1	0.13	1.65	0.203
	first relative warp score	1	0.32	8.32	0.005
	centroid size of stylet	1	-0.07	0.46	0.500

[a] Full Model: $R^2 = 0.55$; $F_{15,88} = 7.05$; $P < 0.001$

[b] Full Model: $R^2 = 0.47$; $F_{15,77} = 4.55$; $P < 0.001$

[c] Full Model: $R^2 = 0.29$; $F_{15,77} = 2.06$; $P = 0.021$

Discussion

This study focused on the degree of multiple mating and the causes of variation in the number of mates and sperm-transfer success in the simultaneously hermaphroditic flatworm *M. lignano*. We demonstrated that the number of mating partners is highly variable between individuals and showed that it is positively related to the social group size a worm is exposed to. In most of the social groups some worms managed to mate with all potential mating partners (except in groups of 16 individuals). This high degree of multiple mating violates the central prediction of sex allocation theory that simultaneous hermaphroditism should only occur when the average number of mates is small (Charnov 1982). High numbers of mating partners

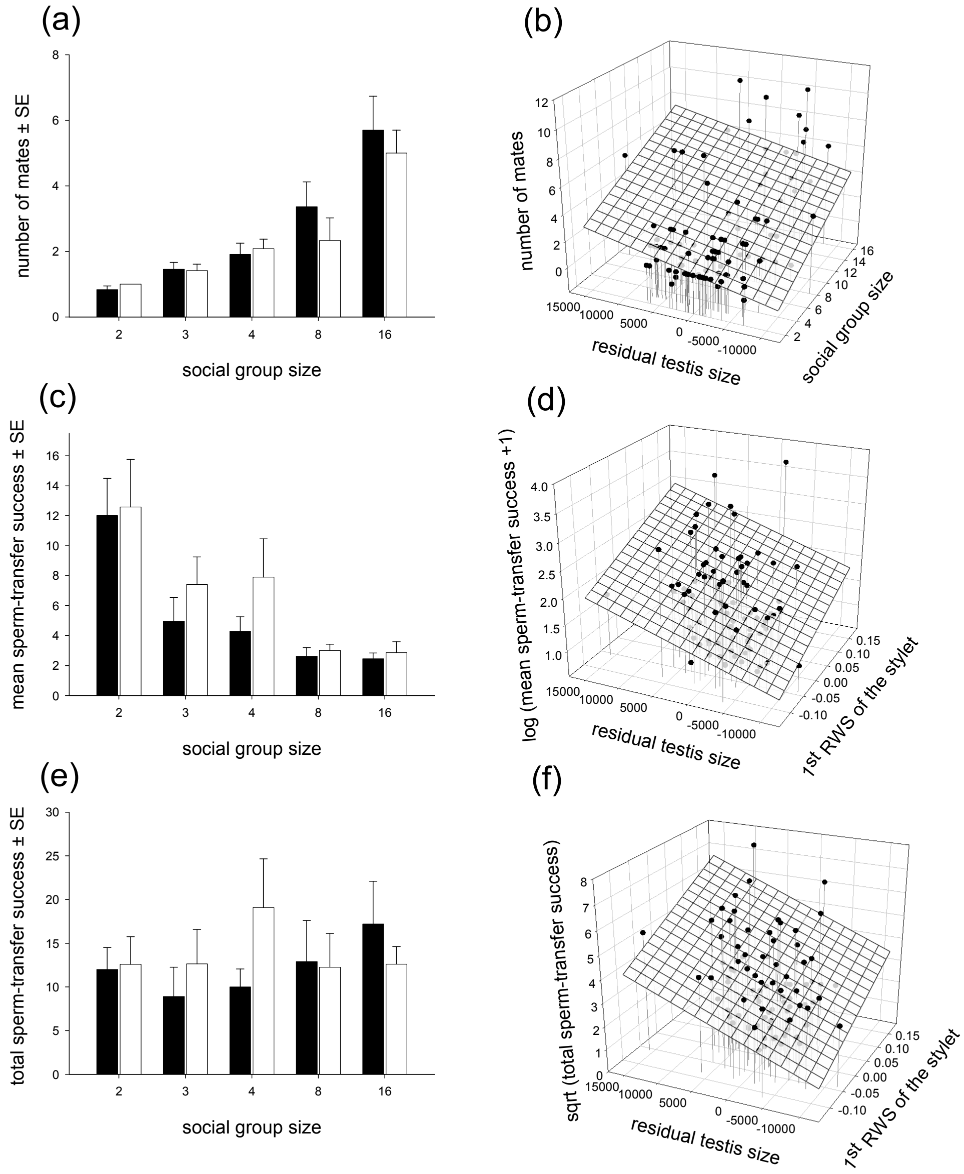

Figure 2. Effects of social group size, density and morphology of sperm donors on the number of mates (a, b), mean sperm-transfer success (c, d) and total sperm-transfer success (e, f). Social group size includes the labelled focal worm. In the bar plots filled and open bars refer to the high- and low density treatment, respectively. The plane grids in the three-dimensional scatter plots represent linear regression fits of both explanatory variables. 1st RWS refers to the first relative warp score obtained from the relative warp score analysis of the stylet. Note that response variables in bar plots are given with untransformed values. For statistics see Table 1.

represent cases of high sperm competition intensity and therefore result in a lowered local mate competition in terms of competition between related sperm. However, local mate competition is assumed to be responsible for a saturating male fitness gain curve, which is required for hermaphroditism to be an evolutionary stable strategy (Charnov 1979, 1982). Since, our measure of the number of mates is potentially an underestimation of the actual number of mating partners (see methods), this discrepancy between the number of mates in *M. lignano* and sex allocation theory for simultaneous hermaphrodites may even be more severe. The maximum number of mates in social groups of 16 individuals was ten, which suggests that there is an upper threshold of multiple mating in this species. However, this may also be caused by the relatively short period of only 24 hours in which the worms were allowed to mate in our experiment.

We are aware of only one study that also assessed the number of mates in a simultaneously hermaphroditic animal. For the planarian flatworm *Schmidtea polychroa* it has been shown that adults produce offspring from an average of four mates when kept in groups of ten individuals for four weeks (Pongratz and Michiels 2003), which also indicates a fairly high degree of multiple mating. However, in this study social group size was not manipulated and analysis of paternity and maternity always ignores matings that failed to produce offspring (e.g. due to post-copulatory sexual selection or low hatching success), which would likely underestimate multiple mating more than it is the case in our data. However, since both studies assessed multiple mating under laboratory conditions, there is a clear need for data on multiple mating in the field, in order to evaluate if it is equally high under more natural conditions. No such data seems currently available.

Studies on sex allocation in hermaphrodites often manipulate social group size to produce situations with different levels of sperm competition, simply assuming that both are closely linked without explicitly testing this assumption (e.g. Trouvé et al. 1999; Schärer and Ladurner 2003; Tan et al. 2004; Lorenzi et al. 2005). Our study provides evidence that an increase in social group size indeed results in a higher average number of mates and therefore in a higher sperm competition intensity. In contrast, despite a fourfold increase in density, this factor had no statistically significant effect on the number of mates. Assuming an increased encounter rate at higher densities, this suggests that a higher encounter rate does not automatically lead to a higher degree of multiple mating. Alternatively, worms may gather in groups and may therefore not be randomly distributed in space. Consequently, individuals would not use all the available space, which would make them unaffected by enclosure size. The lack of an effect of density agrees with earlier findings showing that *M. lignano* adjusts its sex allocation irrespective of density (Schärer and Ladurner 2003; but see Schärer et al. 2005).

In hermaphrodites, advantages in pre-copulatory mate competition and mate choice decisions have often been attributed to body size, with larger individuals being preferred mating partners because body size is often correlated with fecundity (for a review see Leonard 2006). In this study we found no relationship between body size and the number of mates. Hence, in *M. lignano* larger individuals are not capable of acquiring more mating partners. However, since we have no information on the body size of the unlabelled mates, we can not rule out that there is size-assortative mating, which has previously been documented for other simultaneous hermaphrodites (Vreys and Michiels 1997; but see Peters and Michiels 1996; Michiels et al. 2001; Koene et al. 2007).

Interestingly, we found a positive effect of residual testis size on the number of mates. There are at least two possible explanations for this finding. First, since testis size reflects sperm production rate in *M. lignano* (Schärer and Vizoso 2007), one could expect that individuals with larger testes are able to transfer sperm more often and/or in higher numbers per copulation. Since our estimate of the number of mates is based on the occurrence of labelled sperm that is stored by mating partners and may therefore be subject to post-copulatory sexual selection, it is possible that higher numbers of transferred sperm could also lead to higher numbers of mating partners. However, the size of the seminal vesicle, which is a good estimate of the sperm reserves that are available for mating in *M. lignano* (Schärer and Vizoso 2007), had no effect on the number of mates. Moreover, testis size of focal worms did not covary with the size of the seminal vesicle. Consequently, it seems unlikely that individuals with larger testes had more mating partners simply because they were able to transfer more sperm. However, this explanation can not be ruled out because morphological measurements were done one day before the mating trials started and focal worms were kept in isolation during this time. Thus, focal worms could refill their sperm reserves during this isolation period, which may have rendered our measurement of seminal vesicle size as an estimate of sperm reserves unreliable.

As a second reason for the detected effect of testis size on the number of mates, we suspect that individuals varying in testis size behave differently. Sex allocation was highly correlated with testis size implying that worms with larger testes were also more male-biased. Furthermore, an additional analysis including sex allocation as a composite measure of the relative allocation towards the male function confirmed that more male-biased individuals had more mates (see supplementary material). Following the logic of Bateman's principle (see introduction) more male-biased individuals may gain more from multiple mating than more female-biased worms. Therefore, individuals with larger testes may be more eager to copulate. Since testis size is phenotypically plastic in *M. lignano* (Schärer and Ladurner 2003) it is possible to manipulate this trait in this system, which offers the opportunity to test the positive relationship between testis size and the number of mates found in this study experimentally.

Our study also revealed large variation in sperm-transfer success, which was partly explained by social group size, residual testis size, residual ovary size and genital morphology. We found that focal worms had a lower mean sperm-transfer success in larger social groups. On the one hand, this could mean that worms reduce their ejaculate expenditure in larger groups as a response to an increased immediate sperm competition intensity as predicted by theory on optimal sperm allocation (Parker et al. 1996; Engqvist and Reinhold 2005). On the other hand, it could simply be a consequence of repeated matings with the same partner in small social groups, with a constant number of sperm transferred in each mating irrespective of social group size. Additionally, another hypothesis is that only a limited number of sperm can be stored in the female sperm storage organ and that excess sperm is actively removed or gets lost passively (e.g. during egg laying). With a greater number of competitors this would also lead to a decrease in mean sperm-transfer success. Based on our data we are unable to distinguish between these non-exclusive hypotheses.

Surprisingly, the total sperm-transfer success remained constant across social groups. This may suggest that worms spent in total about equal amounts of sperm during the mating trials regardless of the number of potential mates and the level of sperm competition they experienced. Furthermore, together with our finding on the mean sperm-transfer success this indicates that worms do not adjust their male reproductive effort in terms of sperm investment according to the Coolidge effect, which predicts that males invest less reproductive resources when re-mated with the same mating partner (Dewsbury 1981). On the assumption that our measure of total sperm-transfer success is closely related to the total sperm investment in *M. lignano*, one would expect a higher total sperm-transfer success in large groups compared to pairs because in the latter case worms can only mate with the same partner and should therefore invest less reproductive resources. However, in our study we found no difference in the total sperm-transfer success between all social group sizes. Even in groups of 16 individuals, where the average number of mates was about five individuals, worms had an equal total sperm-transfer success as they had in pairs. If sperm-transfer success is positively linked with sperm allocation these findings suggest that worms do not allocate sperm prudently with respect to the Coolidge effect.

Sperm-transfer success was also affected by residual testis size. Both, the mean and the total sperm-transfer success were positively related to testis size. This provides the first hint of ultimate benefits of having larger testes in *M. lignano*. One crucial assumption of sex allocation theory for simultaneous hermaphrodites is that the fitness of the male function is positively related to the proportion of resources allocated to the male function, depicted by a saturating fitness gain curve (Charnov 1982). However, to our knowledge this relationship has never been empirically demonstrated for internally fertilizing hermaphroditic animals yet (for sperm-casting species see McCartney 1997; Yund 1998). Assuming that the number of mates and sperm-transfer success are closely linked with the fitness of the male function, our study provides evidence that an increased male allocation leads to a higher reproductive success through the male function. In *M. lignano*, the effect of residual testis size on sperm-transfer success may be due to a higher mating frequency of more male-biased individuals, which would automatically lead to a higher number of stored sperm in the sperm storage organ of their mates (see discussion above). Another reason could be that more male-biased worms are better sperm competitors compared to more female-biased individuals because of differences in sperm displacement abilities or ejaculate quality. Interestingly, residual ovary size was negatively related with mean sperm-transfer success. This indicates that a higher reproductive success of the male function can only be achieved on the cost of the female function, which is probably a consequence of a trade-off in resource allocation to the male and the female function, which has been demonstrated for *M. lignano* (Schärer et al. 2005). So far, very little is known about the effect of sex allocation on mating frequency and male competitiveness in simultaneous hermaphrodites, which highlights the need for experimental studies testing this hypothesis.

Another morphological trait that explained variation in sperm-transfer success was the shape of the stylet. To our knowledge, this study provides the first empirical evidence that the morphology of the male copulatory organ is an important predictor of sperm-transfer success in a simultaneous hermaphrodite. However, the reason why individuals with stylets that are curved away from the seminal vesicle (indicated by positive scores of the first relative warp) have a higher sperm-transfer success remains unknown. One potential mechanism is that specifically shaped stylets prevent sperm from being subject to cryptic female choice, e.g. by modifying the shape of the sperm storage organ or by optimal positioning of sperm within the female genitalia. Alternatively, stylet shape may also be important to out-compete sperm inseminated by other worms via sperm displacement, which would be expected to generate second male sperm precedence. A detailed understanding of the processes during and after copulation with special emphasis on stylet intromission, sperm release, sperm displacement and accompanied changes in the conformation of the female sperm storage organ would help to identify the underlying mechanisms that lead to an advantage of specifically shaped stylets in sperm-transfer success.

In conclusion, this study demonstrates that individuals of *M. lignano* differ considerably in the number of mates and sperm-transfer success. We reveal that social group size and morphological traits of the sperm donor are important factors to explain variation in these parameters in a simultaneous hermaphrodite. Our findings support the idea that more male-biased individuals are able to acquire more mating partners and have a higher sperm-transfer success. Additionally, we show for the first time in hermaphrodites that genital morphology is an important predictor of sperm-transfer success. Further studies should experimentally test how sex allocation affects mating behaviour and explain how stylet shape influences sperm-transfer success in *M. lignano* mechanistically.

Acknowledgements

We thank Peter Sandner for assistance during the experiment and are grateful to Dita B. Vizoso, Peter Sandner and Ralph Dobler for fruitful discussions. Jürgen Hottinger, Viktor Mislin and Urs Stiefel kindly provided technical support. We also thank Andy Gardner and one anonymous reviewer for constructive comments on a previous draft. This project was funded by the Swiss National Fond (3100A0-113708).

References

Andersen, R. A., J. A. Berges, P. J. Harrison, and M. M. Watanabe. 2005. Recipes for freshwater and seawater media. Pp. 429-538 in R. A. Andersen, ed. Algal Culturing Techniques. Elsevier, Amsterdam.

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

Bateman, A. J. 1948. Intra-sexual selection in Drosophila. Heredity 2:349-368.

Brauer, V. S., L. Schärer, and N. K. Michiels. 2007. Phenotypically flexible sex allocation in a simultaneous hermaphrodite. Evolution 61:216-222.

Bussière, L. F., J. Hunt, M. D. Jennions, and R. Brooks. 2006. Sexual conflict and cryptic female choice in the black field cricket, Teleogryllus commodus. Evolution 60:792-800.

Charnov, E. L. 1979. Simultaneous hermaphroditism and sexual selection. Proc. Natl. Acad. Sci. U. S. A. 76:2480-2484.

Charnov, E. L. 1982. The Theory of Sex Allocation. Princeton University Press, Princeton, NJ, USA.

Dewsbury, D. A. 1981. Effects of novelty on copulatory behavior - the Coolidge effect and related phenomena. Psychol. Bull. 89:464-482.

Eberhard, W. G. 1996. Female Control: Sexual Selection by Cryptic Female Choice. Princeton University Press, Princeton.

Engqvist, L., and K. Reinhold. 2005. Pitfalls in experiments testing predictions from sperm competition theory. J. Evol. Biol. 18:116-123.

Fischer, E. A. 1981. Sexual allocation in a simultaneously hermaphroditic coral-reef fish. Am. Nat. 117:64-82.

Frank, S. A. 2002. A touchstone in the study of adaptation. Evolution 56:2561-2564.

Hamilton, W. D. 1967. Extraordinary sex ratios. Science 156:477-488.

House, C. M., and L. W. Simmons. 2003. Genital morphology and fertilization success in the dung beetle Onthophagus taurus: an example of sexually selected male genitalia. Proc. R. Soc. B-Biol. Sci. 270:447-455.

Klinkhamer, P. G. L., T. J. deJong, and H. Metz. 1997. Sex and size in cosexual plants. Trends Ecol. Evol. 12:260-265.

Koene, J. M., K. Montagne-Wajer, and A. Ter Maat. 2007. Aspects of body size and mate choice in the simultaneously hermaphroditic pond snail Lymnaea stagnalis. Anim. Biol. 57:247-259.

Ladurner, P., L. Schärer, W. Salvenmoser, and R. M. Rieger. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: Macrostomum lignano, n. sp. (Rhabditophora, Macrostomorpha). J. Zool. Syst. Evol. Res. 43:114-126.

Leonard, J. L. 2006. Sexual selection: lessons from hermaphrodite mating systems. Integr. Comp. Biol. 46:349-367.

Lorenzi, M. C., G. Sella, D. Schleicherova, and L. Ramella. 2005. Outcrossing hermaphroditic polychaete worms adjust their sex allocation to social conditions. J. Evol. Biol. 18:1341-1347.

McCartney, M. A. 1997. Sex allocation and male fitness gain in a colonial, hermaphroditic marine invertebrate. Evolution 51:127-140.

Michiels, N. K., A. Hohner, and I. C. Vorndran. 2001. Precopulatory mate assessment in relation to body size in the earthworm Lumbricus terrestris: avoidance of dangerous liaisons? Behav. Ecol. 12:612-618.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. Biol. Rev. 45:525-567.

Parker, G. A., M. A. Ball, P. Stockley, and M. J. G. Gage. 1996. Sperm competition games: individual assessment of sperm competition intensity by group spawners. Proc. R. Soc. B-Biol. Sci. 263:1291-1297.

Peters, A., and N. K. Michiels. 1996. Do simultaneous hermaphrodites choose their mates? Effects of body size in a planarian flatworm. Freshw. Biol. 36:623-630.

Pizzari, T., and T. R. Birkhead. 2000. Female feral fowl eject sperm of subdominant males. Nature 405:787-789.

Pongratz, N., and N. K. Michiels. 2003. High multiple paternity and low last-male sperm precedence in a hermaphroditic planarian flatworm: consequences for reciprocity patterns. Mol. Ecol. 12:1425-1433.

Reece, S. E., D. R. Drew, and A. Gardner. 2008. Sex ratio adjustment and kin discrimination in malaria parasites. Nature 453:609-U601.

Schärer, L., G. Joss, and P. Sandner. 2004. Mating behaviour of the marine turbellarian Macrostomum sp.: these worms suck. Mar. Biol. 145:373-380.

Schärer, L., and P. Ladurner. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 270:935-941.

Schärer, L., P. Sandner, and N. K. Michiels. 2005. Trade-off between male and female allocation in the simultaneously hermaphroditic flatworm Macrostomum sp. J. Evol. Biol. 18:396-404.

Schärer, L., and D. B. Vizoso. 2007. Phenotypic plasticity in sperm production rate: there's more to it than testis size. Evol. Ecol. 21:295-306

Schärer, L., J. Zaubzer, W. Salvenmoser, C. Seifarth, and P. Ladurner. 2007. Tracking sperm of a donor in a recipient: an immunocytochemical approach. Anim. Biol. 57:121-136.

Tan, G. N., F. R. Govedich, and M. Burd. 2004. Social group size, potential sperm competition and reproductive investmnent in a hermaphroditic leech, Helobdella papillornata (Euhirudinea: Glossiphoniidae). J. Evol. Biol. 17:575-580.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly Harpobittacus nigriceps. Am. Nat. 122:765-788.

Trouvé, S., J. Jourdane, F. Renaud, P. Durand, and S. Morand. 1999. Adaptive sex allocation in a simultaneous hermaphrodite. Evolution 53:1599-1604.

Vizoso, D. B., and L. Schärer. 2007. Resource-dependent sex allocation in a simultaneous hermaphrodite. J. Evol. Biol. 20:1046-1055.

Vreys, C., and N. K. Michiels. 1997. Flatworms flatten to size up each other. Proc. R. Soc. B-Biol. Sci. 264:1559-1564.

Yund, P. O. 1998. The effect of sperm competition on male gain curves in a colonial marine invertebrate. Ecology 79:328-339.

Zelditch, M. L., H. D. Swiderski, H. D. Sheets, and W. L. Fink. 2004. Geometric Morphometrics for Biologists: A Primer. Elsevier Academic Press, London.

Electronic Supplementary Material

Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite

Tim Janicke and Lukas Schärer

Table S1. Results of General Linear Models performed to explain variation in the number of mates, mean sperm-transfer success and total sperm-transfer success. Models include sex allocation as a composite measure of reproductive resources allocated to the male versus the female function. Standardized beta (Std β) is the parameter estimate scaled to be dimensionless and indicates the relative importance of each covariate in explaining variation in the response variable (negative values indicate negative effects).

response	source	*df*	β	*F* value	*P* value
number of mates [a]	density	1	-	1.87	0.175
	social group size	4	-	21.53	<0.001
	density x social group size	4	-	0.60	0.661
	body size	1	-0.01	0.02	0.898
	sex allocation	1	0.18	5.55	0.021
	residual seminal vesicle size	1	0.06	0.54	0.465
	first relative warp score	1	0.07	0.99	0.324
	centroid size of stylet	1	0.06	0.61	0.438
mean sperm-transfer success	density	1	-	0.34	0.561
	social group size	4	-	10.89	<0.001
	density x social group size	4	-	0.12	0.975
	body size	1	0.10	1.37	0.246
	sex allocation	1	0.25	7.95	0.006
	residual seminal vesicle size	1	0.12	1.94	0.167
	first relative warp score	1	0.24	7.06	0.010
	centroid size of stylet	1	-0.03	0.11	0.739
total sperm-transfer success	density	1	-	0.01	0.948
	social group size	4	-	0.58	0.679
	density x social group size	4	-	0.33	0.861
	body size	1	0.08	0.63	0.429
	sex allocation	1	0.31	8.91	0.004
	residual seminal vesicle size	1	0.14	1.91	0.171
	first relative warp score	1	0.33	9.50	0.003
	centroid size of stylet	1	-0.11	0.93	0.337

[a] Full Model: $R^2 = 0.52$; $F_{14,89} = 6.86$; $P < 0.001$

[b] Full Model: $R^2 = 0.47$; $F_{14,78} = 4.87$; $P < 0.001$

[c] Full Model: $R^2 = 0.26$; $F_{14,78} = 2.00$; $P = 0.028$

CHAPTER III

Strategic mating effort in a simultaneous hermaphrodite: the role of the partner's feeding status

Manuscript in preparation:
Janicke, T., H. Kesselring, and L. Schärer. in prep. Strategic mating effort in a simultaneous hermaphrodite: the role of the partner's feeding status.

Abstract

Sexual selection theory for simultaneously hermaphroditic animals predicts an overall preference for mating with larger individuals. This is because body size is often correlated with female fecundity, which may increase the fitness gain derived from inseminating larger mating partners. Empirical evidence for such size-dependent mate choice in simultaneous hermaphrodites is equivocal, possibly due to the fact that studies on mating preferences have usually made use of existing variation in body size among potential mating partners instead of attempting to manipulate this trait. In this study we experimentally manipulated the feeding status of potential mating partners and tested for effects of this manipulation on mating rate and on the number of sperm allocated per mating in an outcrossing simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*. We found that the manipulation of the feeding status had a strong effect on the body size of the potential mating partners and that focal worms copulated more frequently with well-fed partners compared to unfed partners. However, we found no evidence for a bias in the number of sperm allocated per mating towards well-fed mating partners. Our results suggest that *M. lignano* adjusts its mating effort in response to the feeding status of the mating partner, but that this is presumably mediated by pre-copulatory mate choice rather than by a strategic sperm allocation per mating.

Introduction

In the 'Descent' Darwin argued that mate choice in simultaneously hermaphroditic animals (i.e., organisms that produce sperm and eggs at the same time) should be rare or absent, because 'secondary sexual characters can not be developed' in these animals and because they 'have too imperfect senses and much too low mental powers to appreciate each others beauty' (Darwin 1871). Although it is now generally acknowledged that sexual selection also occurs in simultaneous hermaphrodites (e.g., Charnov 1979; Michiels 1998; Leonard 2006), recent theoretical work suggests that pre-copulatory sexual selection may be less intense in simultaneous hermaphrodites compared to separate-sexed organisms. For instance, quantitative genetic models have shown that the absence of sex-limited trait expression reduces but does not eliminate the opportunity for Fisherian runaway selection in simultaneous hermaphrodites (Morgan 1994). Moreover, if we assume that all individuals in a hermaphroditic population have an interest to mate, the optimal investment in mate acquisition is lower than in separate-sexed species, where usually only one sex (typically the males) invests in mate acquisition (Greeff and Michiels 1999). Based on the expected low investment for pre-copulatory processes and given that simultaneous hermaphrodites often mate multiply (e.g., Pongratz and Michiels 2003; Evanno et al. 2005; Janicke and Schärer 2009a), one might expect that post-copulatory sexual selection (in terms of sperm competition *sensu* Parker 1970 and cryptic female choice *sensu* Thornhill 1983; Eberhard 1996) is the predominant processes of sexual selection in simultaneous hermaphrodites (Charnov 1979; Schärer and Janicke 2009).

Despite the apparently low potential for pre-copulatory sexual selection in simultaneously hermaphroditic animals, there are at least four traits that have been argued to be involved in mate choice decisions in this group of organisms (reviewed in Leonard 2006; Anthes 2010). Specifically, it has been shown that the mating status (e.g., Haase and Karlsson 2004; but see Koene et al. 2008), the relatedness (e.g., Facon et al. 2006; Schjørring and Jäger 2007; but see Peters and Michiels 1996b), and the infection status of the mate (e.g., Webster et al. 2003) can be important predictors for mate choice decisions in simultaneous hermaphrodites.

However, the trait that has most often been suggested to affect the mating behaviour in simultaneous hermaphrodites is body size. Here, a preference for mating with larger partners can evolve, because larger individuals often have more resources available for gamete production, which is thought to be the main predictor of female fecundity in simultaneous hermaphrodites (Charnov 1979). Moreover, sex allocation theory for simultaneous hermaphrodites predicts that large individuals should generally allocate relatively more resources towards their female sex function compared to small individuals (Klinkhamer et al. 1997; Angeloni et al. 2002; Schärer 2009; for empirical support see e.g., Schärer et al. 2001). This so-called 'size-dependent sex allocation' might lead to an additional advantage of inseminating large individuals, because a more female-biased sex allocation is predicted to translate into a higher female fecundity (Charnov 1982). Consequently, if we assume that body size correlates positively with female fecundity and that the costs associated with sperm production are non-trivial (Dewsbury 1982), individuals are predicted to have a preference for inseminating larger partners, since this likely increases the number of sired offspring. Once a preference for mating with larger individuals has evolved, size-assortative mating is expected in species with reciprocal sperm transfer (Michiels 1998).

Empirical studies on size-dependent mate choice and size-assortative mating in simultaneously hermaphroditic animals have not revealed consistent patterns across species (Anthes 2010). While some studies provide support for an overall preference to mate with larger individuals (e.g., Michiels et al. 2001; Lüscher and Wedekind 2002; Ohbayashi-Hodoki et al. 2004; Anthes et al. 2006), other studies indicate that the mating propensity is random with respect to the partner's body size (e.g., Peters and Michiels 1996a; Koene et al. 2007; Dillen et al. 2008). Similarly, size-assortative mating in simultaneous hermaphrodites is documented for flatworms, annelids and molluscs, (e.g., Vreys and Michiels 1997; Gianguzza et al. 2004; Monroy et al. 2005; Pal et al. 2006), but can also be absent in species that belong to the same taxa (reviewed in Chaine and Angeloni 2005). These different findings across species might be due to the fact that the employed experimental approaches have usually used existing variation in body size to test for size-dependent mating strategies, instead of attempting to manipulate this trait experimentally. Using natural variation in size bears the risk that body size in these experiments is confounded by other factors, such as age, condition, or overall quality. For instance, in the freshwater snail *Lymnaea stagnalis* mating frequency and sex role preferences are age-dependent, and it has been argued that some of the evidence for size-dependent mating strategies in simultaneous hermaphrodites might partly be due to age rather than size effects, because body size is often confounded with age (Hermann et al. 2009).

The body size of a potential mating partner may not only affect pre-copulatory mate choice, but also the number of sperm that is transferred during mating. Sperm competition theory predicts that individuals should allocate relatively more sperm per mating to mating partners that are more fecund (e.g., Reinhold et al. 2002; for review see Wedell et al. 2002). This so-called 'strategic sperm allocation' in response to the body size of females has been suggested for many separate-sexed organisms, including humans (e.g., Baker and Bellis 1993; Shapiro et al. 1994; Rubolini et al. 2006; but see Sevgili and Reinhold 2007). By contrast, relatively few studies have attempted to explore strategic sperm allocation in simultaneous hermaphrodites (reviewed in Anthes 2010).

Here we tested for a strategic mating effort in response to the partner's body size in the free-living flatworm *Macrostomum lignano*. These worms are highly promiscuous (Janicke and Schärer 2009a) and copulate frequently (Schärer et al. 2004; Janicke and Schärer 2009b). Copulations are always reciprocal and the received sperm is stored in a sperm storage organ (Ladurner et al. 2005; Vizoso et al. 2010). So far, very little is known about pre-copulatory mate choice and strategic sperm allocation in *M. lignano*.

In this study we experimentally manipulated the feeding status and thereby the body size of individuals in order to test the hypothesis that worms bias their mating effort towards mating partners that are larger and therefore have more resources available for egg production. Manipulating the feeding status in *M. lignano* has previously been shown to have a strong effect on the number of offspring produced by the female sex function and therefore on female fecundity (Janicke et al. 2010). In this study, we measured mating effort in terms of the number of copulations, copulation duration, and the number of stored sperm in the mating partner's sperm storage organ. Variation in the number of copulations that a focal individual has with particular mating partners can be considered as a kind of pre-copulatory mate choice (e.g., Peters and Michiels 1996b; Haase and Karlsson 2004; Ohbayashi-Hodoki et al. 2004; Dillen et al. 2010), whereas variation in copulation duration and the number of stored sperm in the partner's sperm storage organ can indicate biases in the male mating effort per mating. In many animals copulation duration correlates positively with the number of sperm transferred during mating (e.g., Engqvist et al. 2007) and it is therefore often used as an estimate of the number of sperm that is allocated per mating (e.g., Anthes et al. 2006; Bretman et al. 2009). For *M. lignano*, such a positive relationship between copulation duration and the number of transferred sperm has not been shown yet. The number of sperm that is stored in the female sperm storage organ of the mating partner can also serve as an estimate of the number of sperm that the sperm donor actually transferred during mating (e.g., Velando et al. 2008). Although the latter measure probably represents a more accurate estimate of sperm allocation compared to the copulation duration, it relies on the assumption that sperm recipients do not have much control over the number of received sperm retained after sperm receipt.

If *M. lignano* adjusts its mating effort in response to the feeding status of the mate, we expect that focal worms copulate more frequently and/or on average longer with well-fed worms. Moreover, we expect that focal worms transfer more sperm (and thus manage to successfully store more sperm) in well-fed partners compared to unfed partners.

Methods

Study organism

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

Experimental setup

To test whether the feeding status of the mating partner has an effect on the mating effort, we consecutively mated sperm-labelled focal individuals with one well-fed individual and one unfed individual (and vice versa). Sperm of focal worms was labelled with 5-bromo-2'-deoxyuridine (hereafter called BrdU) (for details see section 'Sperm tracking' below).

Due to time constraints, we split the experiment into two blocks that were separated by two days. On day 1 of each block, we allowed 400 adult worms from the mass cultures to lay eggs in 4 Petri-dishes filled with f/2 medium and a concentrated algae solution. After 3 days all adult worms were removed yielding offspring that did not differ by more than 72 hours in age. On day 23 we pooled all fully grown worms from 2 randomly selected Petri dishes and transferred 60 randomly selected worms to one Petri dish that contained a solution of BrdU in f/2 medium and a concentrated algae solution (hereafter called sperm-labelled focal worms). The remaining worms (unlabelled mating partners) were transferred to a fresh Petri dish (i.e., f/2 medium and a dense algae layer). On day 27, we refreshed the BrdU solution of the focal worms and assigned 192 unlabelled worms into 96 pairs and kept them in 24-well tissue culture plates. Wells were filled with 1.5 ml of f/2 medium and either 0.1 ml of a concentrated algae solution or 0.1 ml of f/2 medium. This yielded 48 pairs that had *ad libitum* food conditions (hereafter called well-fed worms) and 48 pairs that had no access to food (hereafter called unfed worms), respectively. To discriminate focal worms from potential mating partners during the mating trials we colourised the mating partners using the red food dye Neococcine (E124; Werner Schweizer AG, Wollerau, Switzerland). For this we transferred all well-fed and all unfed pairs on day 29 to new wells with the appropriate food treatment but filled with 1.5 ml of colourised f/2 medium (10 mg Neococcine per ml f/2 medium). On day 32 we measured the body size of 36 sperm-labelled focal worms and of 72 colourised worms, which had been raised in pairs under *ad libitum* food conditions (n = 36) or without any access to food (n = 36). Out of each pair we measured only one randomly chosen individual. After the measurement of body size (see section 'Measurement of body size' below) all individuals were kept in isolation in their original food treatment until the mating trials (see section 'Mating trials' below), which were carried out on the subsequent day. Hence, our food level manipulation of the potential mating partners lasted 6 days. Immediately after the mating trials, the worms were fixated, and BrdU-labelled sperm were localised using an immunocytochemical staining protocol (see section 'Sperm tracking').

Measurement of body size

In order to evaluate the morphological consequences of our food level manipulation we measured the body size of all individuals one day prior to the mating trials. For this, we anesthetized worms by exposing them to a 5:3 mixture of 7.14 % $MgCl_2$ and f/2 medium for 10 min and compressed them dorsoventrally to a fixed thickness of 35 µm between a microscope slide and a cover slip of a haemacytometer (Schärer and Ladurner 2003). We observed worms with a Leica DM 2500 microscope (Leica Microsystems, Germany) and took digital photos at 40x with a digital video camera (Sony DFW-X700, Sony Broadcast & Professional, Köln, Germany). Image acquisition was done using the software BTV Pro 6.0b1 (available at http://www.bensoftware.com) and pictures were analysed with the image analysis software ImageJ 1.43h (available at http://rsb.info.nih.gov/ij/). Body area of the worms was measured with the 'wand (tracing) tool' in ImageJ and was used as an estimate of body size (Schärer and Ladurner 2003).

Mating trials

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

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

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

Sperm tracking

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

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

Effect of the feeding status on mating behaviour

The food level manipulation might have had an effect on the mating behaviour of the potential mating partners. This would have automatically affected the mating behaviour of our focal worms since matings are always reciprocal in *M. lignano*. Therefore, we tested whether well-fed worms behave similar as unfed worms. For this we used the remaining unlabelled individuals from the pairs that had either *ad libitum* food conditions or no access to food. We formed pairs of two well-fed worms and pairs of two unfed worms and assessed the mating behaviour for 60 min in observation chambers as described above. In total we assembled 5 observation chambers containing 8 pairs each (4 well-fed pairs and 4 unfed pairs) and one chamber containing only 6 pairs (3 well-fed pairs and 3 unfed pairs). One pair of well-fed worms was lost due to a pipetting error so that the final sample size was 22 pairs of well-fed worms and 23 pairs of unfed worms. These mating trials were carried out on day 32.

Statistical analyses

The first and the second block included initially 33 and 36 focal worms, respectively. Several focal worms were lost due to pipetting errors during the assembly of the observation chambers ($N = 15$) or had to be excluded from the analyses because they were injured ($N = 2$). Furthermore, 6 focal worms did not mate with any of the offered mating partners and were therefore also excluded from the analyses. Consequently, our final sample size for testing mating preferences was 46 focal worms (26 first paired with a well-fed worm; 20 first paired with an unfed worm). Additionally, 4 worms were lost during antibody staining, so that our sample size for comparing the number of stored sperm between treatment groups was 42 focal worms (23 first paired with a well-fed worm; 19 first paired with an unfed worm).

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

Finally, we examined the effect of the feeding status of the potential mating partners on the mating effort of the focal worms using Generalized Linear Mixed Models (GLMMs) with Poisson error distributions and log-link functions (Venables and Ripley 2002). We fitted GLMMs for all response variables (i.e., the number of copulations, the average copulation duration, and the number of stored sperm in the partner's sperm storage organ), and used the feeding status of the mating partner (i.e., well-fed or unfed) and the mating order (i.e., the order in which well-fed and unfed worms were offered) as fixed factors, and the focal individual as a random factor (in order to take into account the repeated measures on the same focal worm). In an additional GLMM we tested for strategic sperm allocation by adding the number of copulations as a covariate to the model with the number of sperm stored as the dependent variable. In this model we only included focal worms that copulated with both well-fed and unfed partners. Thereby we tested whether focal worms adjust their sperm allocation strategically while statistically controlling for differences in the copulation number.

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

Results

The food level manipulation had a considerable effect on the body size of the worms, as intended by our experiment. Unfed worms were on average only half as big as well-fed worms (*t*-test: $t = 14.08$, *d.f.* = 90, $P < 0.001$; Figure 1a). The body size of focal worms (mean ± SE: 499.6 ± 15.9 x $10^3 \mu m^2$) did not differ statistically from that of well-fed worms (*t*-test: $t = 0.63$, *d.f.* = 90, $P = 0.531$) but was significantly higher than that of unfed worms (*t*-test: $t = 13.96$, *d.f.* = 90, $P < 0.001$). Despite this considerable difference in body size between well-fed and unfed worms, there was no significant effect of the feeding status on the intrinsic mating behaviour of the potential mating partners. Pairs formed by two well-fed individuals and pairs formed by two unfed individuals behaved similarly with respect to the number of copulations (Wilcoxon rank sum test: $W = 284.5$, $P = 0.473$, $N = 45$; Figure 1b) and the average copulation duration (Wilcoxon rank sum test: $W = 151.0$, $P = 0.115$, $N = 30$; Figure 1c).

Out of the 46 focal worms, 12 individuals copulated only with the well-fed partner whereas 9 individuals copulated only with the unfed partner. Focal worms copulated significantly more frequently with well-fed worms than with unfed worms (Table 1; Figure 2a). The mating order also had a significant effect on the mating rate of focal worms, as indicated by more copulations with the first mating partner than with the second mating partner (Table 1; Figure 2a). In contrast, the average copulation duration was not significantly affected by the feeding status and the mating order of the mating partner (Table 1; Figure 2b).

Overall, focal worms managed to store significantly more sperm in well-fed worms compared to unfed worms (Table 1; Figure 2c). However, this effect disappeared when we corrected statistically for differences in the number of copulations between the treatments. In the GLMM including the number of copulations as a covariate, neither the feeding status nor the mating order had an effect on the number of stored sperm (Table 1). The number of copulations tended to be positively correlated with the number of sperm stored, but this relationship was not statistically significant either (Table 1).

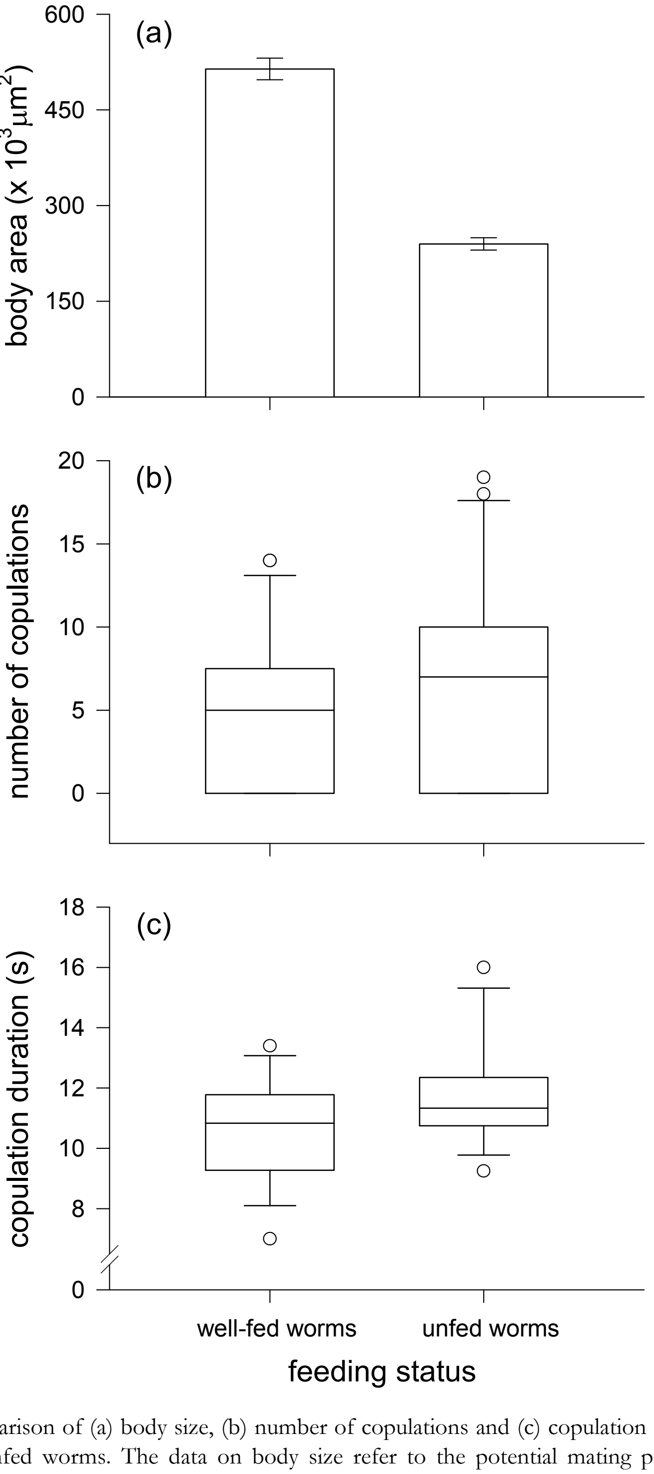

Figure 1. Comparison of (a) body size, (b) number of copulations and (c) copulation duration between well-fed and unfed worms. The data on body size refer to the potential mating partners that were offered to focal worms in the main mating trials (see paragraph "Mating trials" in the "Methods" section).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

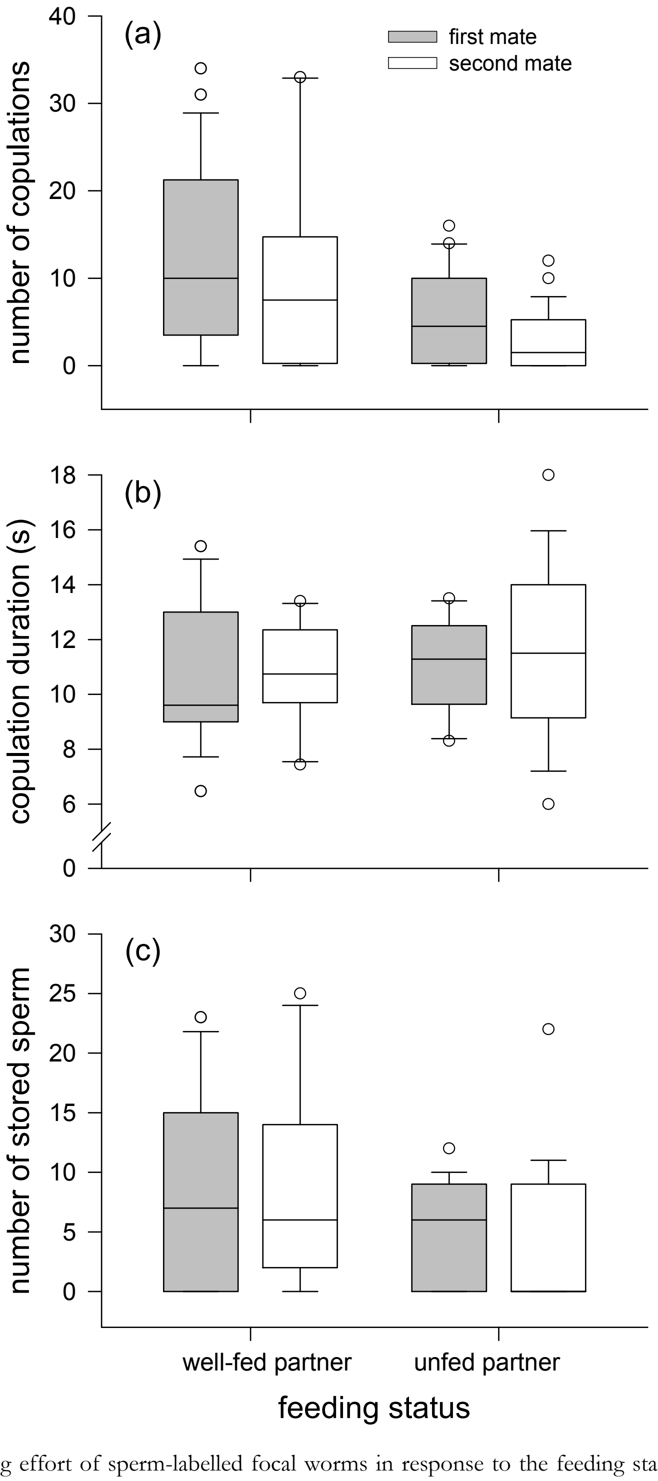

Figure 2. Mating effort of sperm-labelled focal worms in response to the feeding status of the mating partner and to the mating order in which the partner was offered. Data are shown for (a) the number of copulations, (b) the average copulation duration and (c) the number of stored sperm in the partner's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

Table 1. Summaries of Generalized Linear Mixed Models exploring the effects of the partner's feeding status and the mating order on the mating effort of focal worms. Mating effort was measured in terms of the number of copulations, the average copulation duration, and the number of stored sperm in the partner's sperm storage organ. See text for a detailed description of the statistics.

response	source	*d.f.*	*F*-value	*P*-value
copulation number[a]	feeding status	1,43	30.661	< 0.001
	mating order	1,43	6.690	0.013
	feeding status x mating order	1,43	0.305	0.584
copulation duration[b]	feeding status	1,22	2.139	0.158
	mating order	1,22	0.230	0.636
	feeding status x mating order	1,22	0.003	0.960
sperm number[c]	feeding status	1,39	7.347	0.010
	mating order	1,39	0.046	0.831
	feeding status x mating order	1,39	0.059	0.809
sperm number[d]	feeding status	1,17	1.248	0.277
	mating order	1,17	0.425	0.522
	feeding status x mating order	1,17	0.370	0.550
	copulation number	1,17	3.709	0.069

[a] Model includes all focal worms (*N* = 46).

[b] Model includes focal worms that copulated with both offered mating partners (*N* = 25).

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

[d] Model includes focal worms for which the number of stored sperm could be assessed for both offered mating partners and that copulated with both of them (*N* = 21). To test for a strategic sperm allocation the number of copulations was added as a covariate to correct statistically for differences in mating rate (estimate ± SE for copulation number: 0.02 ± 0.02).

Discussion

This study provides evidence for a strategic mating effort in response to the partner's feeding status in the simultaneously hermaphroditic flatworm *M. lignano*. We could show that focal worms copulated more frequently with well-fed partners compared to unfed partners. This difference was probably not triggered by intrinsic differences in the mating motivation of the partners, because pairs of two well-fed worms and pairs of two unfed worms did not differ in their mating behaviour. Therefore, our findings suggest a preference for mating with well-fed individuals in *M. lignano*. This preference also resulted in a higher number of sperm that focal worms managed to store in well-fed partners. However, this effect disappeared when we considered only focal worms that copulated with both mating partners and corrected statistically for differences in the number of copulations. On the assumption that the number of stored sperm correlates with the actual number of sperm transferred during mating, our data do not provide any support for a strategic sperm allocation (i.e., in terms of an adjustment of the number of sperm transferred per mating) in response to the feeding status of the mating partner. Moreover, we found that the average copulation duration did not differ between matings with well-fed and unfed worms, which would support that absence of a strategic sperm allocation if copulation duration was correlated with the number of sperm transferred during mating in *M. lignano*.

In the following, we first discuss an alternative explanation for the observed effect of the partner's feeding status on the number of copulations and we allude to the morphological and reproductive consequences of our experimental manipulation of the feeding status. After this we speculate on how mate assessment operates in *M. lignano* and whether a preference for mating with larger individuals is driven by only one sex function. Finally, we discuss two other important findings of this study, namely the absence of an effect of the partner's feeding status on our estimates of sperm allocation per mating and the effect of the mating order on the number of copulations.

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

To our knowledge, this is the first study on mating preferences in simultaneously hermaphroditic animals in which the body size was manipulated experimentally. Previous studies have usually used the naturally occurring variation in body size within field populations or lab cultures in order to produce treatment groups that differ in body size for testing size-dependent mating strategies (e.g., Lüscher and Wedekind 2002; Chaine and Angeloni 2005; Anthes et al. 2006; Dillen et al. 2008). Consequently, responses in the mating behaviour to this kind of manipulation may be confounded by traits that are correlated with body size, such as age or overall quality (Hermann et al. 2009). In our study we controlled for age effects and manipulated the feeding status of the potential mating partners by exposing them to two different food levels. This manipulation affected the body size of the mating partners and has previously been shown to influence the number of offspring produced by the female sex function in *M. lignano* (Janicke et al. 2010). Specifically, well-fed mating partners were considerably larger and thus presumably more fecund in their female sex function compared to unfed mating partners.

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

On the basis of our data, it is not possible to infer whether the preference for mating with well-fed individuals is primarily driven by only one sex function in *M. lignano.* An overall preference for mating with larger individuals has most often been attributed to a mating drive of the male sex function. Specifically, simultaneous hermaphrodites are predicted to preferentially inseminate larger partners because this will lead to an increased siring success if body size is correlated with female fecundity (Leonard 2006; Anthes 2010). However, current evidence for a male preference to mate with larger individuals is restricted to species with unilateral matings (e.g., Ohbayashi-Hodoki et al. 2004; Anthes et al. 2006), since it is very difficult to assess which sex role (if any) dominates the mate choice decision in reciprocally mating simultaneous hermaphrodites.

In theory, the female sex function might also benefit from mating with larger individuals and therefore size-dependent mate choice could also be female-driven. For instance, if body size correlates with overall ejaculate production, larger sperm donors could provide more sperm and seminal fluid to the recipient. This might not only be beneficial for the female sex function in terms of fertilization insurance, but also in terms of the receipt of additional nutrients if ejaculates can be efficiently digested. Sperm digestion has been argued to be frequent in simultaneous hermaphrodites (Charnov 1979; Sluys 1989; Baur 1998), but until now, there is no evidence that sperm digestion occurs in *M. lignano.* Another, direct benefit of mating with larger partners for the female sex function arises if body size correlates positively with the intensity of paternal care, which is likely to translate into a better offspring performance and therefore a higher fitness. However, paternal care provided by the sperm donor seems to be rather rare in simultaneously hermaphroditic animals (but see Sella 1991; Johnston and Lee 2008), and based on how *M. lignano* behaves under laboratory conditions it seems unlikely that there is any paternal care in these worms. Finally, if body size correlates with the genetic quality of the partner, both the male and the female sex function are expected to show a preference to mate with larger partners in order to obtain indirect genetic benefits (for reviews on genetic benefits see e.g., Reynolds 1996; Neff and Pitcher 2005). Consequently, there clearly exists a potential for the female sex function to have a preference for larger mating partners in *M. lignano* and other simultaneous hermaphrodites.

In this study we did not measure sperm allocation directly, which would have required to assess the number of sperm that is transferred in every single copulation (see Introduction). However, our findings that the average copulation duration and the number of stored sperm in the partner's sperm storage organ were unaffected by the feeding status of the partner, suggest that there is no strategic allocation of sperm per mating in response to this parameter in *M. lignano*. In agreement with this result, no effect of the partner's body size on sperm allocation has been found in the hermaphroditic land snail *Arianta arbustorum* (Baur et al. 1998). In contrast, a recent study on sperm allocation in the land snail *Succinea putris* suggested that large individuals donate more sperm to similar-sized mating partners than to smaller mating partners (Dillen et al. 2010; but see Jordaens et al. 2005). Furthermore, a higher sperm allocation towards larger mating partners has been demonstrated in the simultaneously hermaphroditic earthworm *Eisenia andrei* (Velando et al. 2008). Like in our study, the authors used the number of stored sperm in the partner's sperm storage organ as an estimate of sperm allocation and they could show that this number did not only depend on the body size, but also on the mating status of the mating partner (Velando et al. 2008). Moreover, longer inseminations in matings with larger individuals do also suggest a higher sperm allocation to larger mates in hermaphroditic sea slugs (e.g., Angeloni 2003; Anthes et al. 2006).

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

To summarize, to our knowledge this is the first experimental evidence that the feeding status of the mating partner has an effect on the mating behaviour in a simultaneous hermaphrodite. Our data suggest that there is a preference to mate with well-fed individuals in *M. lignano*, which ultimately also translates into an overall higher sperm allocation towards well-fed partners. However, this overall higher sperm allocation is apparently solely due to a higher mating rate with well-fed individuals and not due to a strategic sperm allocation per mating. Therefore, we presume that a strategic mating effort is primarily mediated by pre-copulatory mate choice rather than by biasing the sperm allocation per mating towards more fecund mating partners. Whether the preference for mating with more well-fed and therefore more fecund individuals is driven by the male or the female sex function remains to be tested.

Acknowledgements

We are grateful to Ralph Dobler, Matthew D. Hall and Lucas Marie-Orléach for constructive comments on an earlier version of this manuscript. Furthermore, we thank Jürgen Hottinger, Viktor Mislin and Urs Stiefel for technical support. This study was funded by grants from the Swiss National Science Foundation to L.S. (3100A0-113708 and 3100A0-127503).

References

Andersen, R. A., Berges, J. A., Harrison, P. J. & Watanabe, M. M. 2005. Recipes for freshwater and seawater media. In: *Algal Culturing Techniques* (Ed. by R. A. Andersen), pp. 429-538. Amsterdam: Elsevier.

Angeloni, L. 2003. Sexual selection in a simultaneous hermaphrodite with hypodermic insemination: body size, allocation to sexual roles and paternity. *Animal Behaviour*, 66, 417-426.

Angeloni, L., Bradbury, J. W. & Charnov, E. L. 2002. Body size and sex allocation in simultaneously hermaphroditic animals. *Behavioral Ecology*, 13, 419-426.

Anthes, N. 2010. Mate choice and reproductive conflict in simultaneous hermaphrodites. In: *Animal Behaviour: Evolution and Mechanisms* (Ed. by P. Kappeler), pp. 329-357. Berlin: Springer.

Anthes, N., Putz, A. & Michiels, N. K. 2006. Hermaphrodite sex role preferences: the role of partner body size, mating history and female fitness in the sea slug *Chelidonura sandrana. Behavioral Ecology and Sociobiology*, 60, 359-367.

Baker, R. R. & Bellis, M. A. 1993. Human sperm competition - ejaculate adjustment by males and the function of masturbation. *Animal Behaviour*, 46, 861-885.

Baur, B. 1998. Sperm competition in molluscs. In: *Sperm competition and sexual selection* (Ed. by T. R. Birkhead & A. P. Møller), pp. 255-305. London, England: Academic Press.

Baur, B., Locher, R. & Baur, A. 1998. Sperm allocation in the simultaneously hermaphroditic land snail *Arianta arbustorum. Animal Behaviour*, 56, 839-845.

Breslow, N. E. & Clayton, D. G. 1993. Approximate inference in Generalized Linear Mixed Models. *Journal of the American Statistical Association*, 88, 9-25.

Bretman, A., Fricke, C. & Chapman, T. 2009. Plastic responses of male *Drosophila melanogaster* to the level of sperm competition increase male reproductive fitness. *Proceedings of the Royal Society B-Biological Sciences*, 276, 1705-1711.

Chaine, A. & Angeloni, L. 2005. Size-dependent mating and gender choice in a simultaneous hermaphrodite, *Bulla gouldiana*. *Behavioral Ecology and Sociobiology*, 59, 58-68.

Charnov, E. L. 1979. Simultaneous hermaphroditism and sexual selection. *Proceedings of the National Academy of Sciences of the United States of America*, 76, 2480-2484.

Charnov, E. L. 1982. *The Theory of Sex Allocation*. Princeton, NJ, USA: Princeton University Press.

Darwin, C. R. 1871. *The Descent of Man, and Selection in Relation to Sex*. London: John Murray.

Dewsbury, D. A. 1982. Ejaculate cost and male choice. *American Naturalist*, 119, 601-610.

Dillen, L., Jordaens, K., Dieleman, W. & Backeljau, T. 2008. Effects of isolation and body size on the mating behaviour of the hermaphroditic land snail *Succinea putris*. *Animal Behaviour*, 75, 1401-1411.

Dillen, L., Jordaens, K., van Dongen, S. & Backeljau, T. 2010. Effects of body size on courtship role, mating frequency and sperm transfer in the land snail *Succinea putris*. *Animal Behaviour*, 79, 1125-1133.

Eberhard, W. G. 1996. *Female Control: Sexual Selection by Cryptic Female Choice*. Princeton: Princeton University Press.

Engqvist, L., Dekomien, G., Lippmann, T., Epplen, J. T. & Sauer, K. P. 2007. Sperm transfer and paternity in the scorpionfly *Panorpa cognata*: large variance in traits favoured by post-copulatory episodes of sexual selection. *Evolutionary Ecology*, 21, 801-816.

Evanno, G., Madec, L. & Arnaud, J. F. 2005. Multiple paternity and postcopulatory sexual selection in a hermaphrodite: what influences sperm precedence in the garden snail *Helix aspersa*? *Molecular Ecology*, 14, 805-812.

Facon, B., Ravigne, V. & Goudet, J. 2006. Experimental evidence of inbreeding avoidance in the hermaphroditic snail *Physa acuta*. *Evolutionary Ecology*, 20, 395-406.

Gianguzza, P., Badalamenti, F., Jensen, K. R., Chemello, R., Cannicci, S. & Riggio, S. 2004. Body size and mating strategies in the simultaneous hermaphrodite *Oxynoe olivacea* (Mollusca, Opisthobranchia, Sacoglossa). *Functional Ecology*, 18, 899-906.

Greeff, J. M. & Michiels, N. K. 1999. Low potential for sexual selection in simultaneously hermaphroditic animals. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 266, 1671-1676.

Haase, M. & Karlsson, A. 2004. Mate choice in a hermaphrodite: you won't score with a spermatophore. *Animal Behaviour*, 67, 287-291.

Hermann, P. M., Genereux, B. & Wildering, W. C. 2009. Evidence for age-dependent mating strategies in the simultaneous hermaphrodite snail, *Lymnaea stagnalis* (L.). *Journal of Experimental Biology*, 212, 3164-3173.

Janicke, T., Sandner, P. & Schärer, L. 2010. Determinants of female fecundity in a simultaneous hermaphrodite: the role of polyandry and food availability. *Evolutionary Ecology*, (doi: 10.1007/s10682-010-9402-5).

Janicke, T. & Schärer, L. 2009a. Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite. *Journal of Evolutionary Biology*, 22, 405-415.

Janicke, T. & Schärer, L. 2009b. Sex allocation predicts mating rate in a simultaneous hermaphrodite. *Proceedings of the Royal Society B-Biological Sciences*, 276, 4247-4253.

Johnston, E. L. & Lee, K. M. 2008. Brooding behaviour and reproductive success in two species of free-living simultaneous hermaphrodites. *Marine Biology*, 155, 555-561.

Jordaens, K., Pinceel, J. & Backeljau, T. 2005. Mate choice in the hermaphroditic land snail *Succinea putris* (Stylommatophora : Succineidae). *Animal Behaviour*, 70, 329-337.

Klinkhamer, P. G. L., deJong, T. J. & Metz, H. 1997. Sex and size in cosexual plants. *Trends in Ecology & Evolution*, 12, 260-265.

Koene, J. M., Loose, M. J. & Wolters, L. 2008. Mate choice is not affected by mating history in the simultaneously hermaphroditic snail *Lymnaea stagnalis*. *Journal of Molluscan Studies*, 74, 331-335.

Koene, J. M., Montagne-Wajer, K. & Ter Maat, A. 2007. Aspects of body size and mate choice in the simultaneously hermaphroditic pond snail *Lymnaea stagnalis*. *Animal Biology*, 57, 247-259.

Ladurner, P., Schärer, L., Salvenmoser, W. & Rieger, R. M. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha). *Journal of Zoological Systematics and Evolutionary Research*, 43, 114-126.

Leonard, J. L. 2006. Sexual selection: lessons from hermaphrodite mating systems. *Integrative and Comparative Biology*, 46, 349-367.

Lüscher, A. & Wedekind, C. 2002. Size-dependent discrimination of mating partners in the simultaneous hermaphroditic cestode *Schistocephalus solidus*. *Behavioral Ecology*, 13, 254-259.

Michiels, N. K. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. In: *Sperm Competition and Sexual Selection* (Ed. by T. Birkhead & A. P. Møller), pp. 219-254. London: Academic Press.

Michiels, N. K. & Bakovski, B. 2000. Sperm trading in a hermaphroditic flatworm: reluctant fathers and sexy mothers. *Animal Behaviour*, 59, 319-325.

Michiels, N. K., Hohner, A. & Vorndran, I. C. 2001. Precopulatory mate assessment in relation to body size in the earthworm *Lumbricus terrestris*: avoidance of dangerous liaisons? *Behavioral Ecology*, 12, 612-618.

Monroy, F., Aira, M., Velando, A. & Dominguez, J. 2005. Size-assortative mating in the earthworm *Eisenia fetida* (Oligochaeta, Lumbricidae). *Journal of Ethology*, 23, 69-70.

Morgan, M. T. 1994. Models of sexual selection in hermaphrodites, especially plants. *American Naturalist*, 144, S100-S125.

Neff, B. D. & Pitcher, T. E. 2005. Genetic quality and sexual selection: an integrated framework for good genes and compatible genes. *Molecular Ecology*, 14, 19-38.

Ohbayashi-Hodoki, K., Ishihama, F. & Shimada, M. 2004. Body size-dependent gender role in a simultaneous hermaphrodite freshwater snail, *Physa acuta*. *Behavioral Ecology*, 15, 976-981.

Pal, P., Erlandsson, J. & Skold, M. 2006. Size-assortative mating and non-reciprocal copulation in a hermaphroditic intertidal limpet: test of the mate availability hypothesis. *Marine Biology*, 148, 1273-1282.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. *Biological Reviews*, 45, 525-567.

Peters, A. & Michiels, N. K. 1996a. Do simultaneous hermaphrodites choose their mates? Effects of body size in a planarian flatworm. *Freshwater Biology*, 36, 623-630.

Peters, A. & Michiels, N. K. 1996b. Evidence for lack of inbreeding avoidance by selective simultaneous hermaphrodite. *Invertebrate Biology*, 115, 99-103.

Pongratz, N. & Michiels, N. K. 2003. High multiple paternity and low last-male sperm precedence in a hermaphroditic planarian flatworm: consequences for reciprocity patterns. *Molecular Ecology*, 12, 1425-1433.

R Development Core Team. 2009. R: A language and environment for statistical computing. Vienna: R Foundation for Statistical Computing.

Reinhold, K., Kurtz, J. & Engqvist, L. 2002. Cryptic male choice: sperm allocation strategies when female quality varies. *Journal of Evolutionary Biology*, 15, 201-209.

Reynolds, J. D. 1996. Animal breeding systems. *Trends in Ecology & Evolution*, 11, A68-A72.

Rubolini, D., Galeotti, P., Ferrari, G., Spairani, M., Bernini, F. & Fasola, M. 2006. Sperm allocation in relation to male traits, female size, and copulation behaviour in a freshwater crayfish species. *Behavioral Ecology and Sociobiology*, 60, 212-219.

Schärer, L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. *Evolution*, 63, 1377-1405.

Schärer, L. & Janicke, T. 2009. Sex allocation and sexual conflict in simultaneously hermaphroditic animals. *Biology Letters*, 5, 705-708.

Schärer, L., Joss, G. & Sandner, P. 2004. Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms suck. *Marine Biology*, 145, 373-380.

Schärer, L., Karlsson, L. M., Christen, M. & Wedekind, C. 2001. Size-dependent sex allocation in a simultaneous hermaphrodite parasite. *Journal of Evolutionary Biology*, 14, 55-67.

Schärer, L. & Ladurner, P. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 935-941.

Schärer, L. & Vizoso, D. B. 2007. Phenotypic plasticity in sperm production rate: there's more to it than testis size. *Evolutionary Ecology*, 21, 295-306

Schärer, L., Zaubzer, J., Salvenmoser, W., Seifarth, C. & Ladurner, P. 2007. Tracking sperm of a donor in a recipient: an immunocytochemical approach. *Animal Biology*, 57, 121-136.

Schjørring, S. & Jäger, I. 2007. Incestuous mate preference by a simultaneous hermaphrodite with strong inbreeding depression. *Evolution*, 61, 423-430.

Sella, G. 1991. Evolution of biparental care in the hermaphroditic polychaete worm *Ophryotrocha diadema*. *Evolution*, 45, 63-68.

Sevgili, H. & Reinhold, K. 2007. No evidence for strategic male mating effort in response to female weight in a bushcricket. *Behaviour*, 144, 1179-1192.

Shapiro, D. Y., Marconato, A. & Yoshikawa, T. 1994. Sperm economy in a coral-reef fish, *Thalassemia bifasciatum*. *Ecology*, 75, 1334-1344.

Sluys, R. 1989. Sperm resorption in triclads (Platyhelminthes, Tricladida). *Invertebrate Reproduction & Development*, 15, 89-95.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. *American Naturalist*, 122, 765-788.

Velando, A., Eiroa, J. & Dominguez, J. 2008. Brainless but not clueless: earthworms boost their ejaculates when they detect fecund non-virgin partners. *Proceedings of the Royal Society B-Biological Sciences*, 275, 1067-1072.

Venables, W. N. & Ripley, B. D. 2002. *Modern Applied Statistics with S*, 4th edn. New York: Springer.

Venables, W. N. & Ripley, B. D. 2010. MASS: Main Package of Venables and Ripley's MASS, R package.

Vizoso, D. B., Rieger, G. & Schärer, L. 2010. Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking. *Biological Journal of the Linnean Society*, 99, 370-383.

Vreys, C. & Michiels, N. K. 1997. Flatworms flatten to size up each other. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 264, 1559-1564.

Webster, J. P., Hoffman, J. I. & Berdoy, M. 2003. Parasite infection, host resistance and mate choice: battle of the genders in a simultaneous hermaphrodite. *Proceedings of the Royal Society of London Series B-Biological Sciences*, 270, 1481-1485.

Wedell, N., Gage, M. J. G. & Parker, G. A. 2002. Sperm competition, male prudence and sperm-limited females. *Trends in Ecology & Evolution*, 17, 313-320.

CHAPTER IV

Sperm competition affects sex allocation but not sperm morphology in a flatworm

Manuscript published as:
Janicke, T., and L. Schärer. in press. Sperm competition affects sex allocation but not sperm morphology in a flatworm. Behav. Ecol. Sociobiol.

Abstract

Sperm competition has been shown to be an important evolutionary agent affecting the behaviour, physiology and morphology of both males and females. One morphological trait that is particularly likely to be affected by sperm competition is sperm size because it is thought to influence the competitiveness of sperm by determining sperm longevity, motility and/or their ability to displace competing sperm. The majority of comparative studies across taxa have found a positive relationship between the level of sperm competition and sperm length, but very few studies have tested for a phenotypically plastic adjustment of sperm morphology in response to sperm competition. In this study we experimentally tested for an effect of sperm competition on phenotypic plasticity in sperm morphology in an obligately outcrossing simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*, by either raising worms in monogamous pairs (no sperm competition) or in promiscuous groups (intense sperm competition). Worms in groups produced larger testes and smaller ovaries as predicted by sex allocation theory, and as previously documented in this species. However, we found no evidence for an effect of group size on sperm morphology, measured as total sperm length, sperm body length, and the length of two different sperm appendages. We conclude that *M. lignano* may either be incapable of adjusting the sperm morphology in a phenotypically plastic way and/or that there might be no benefit of phenotypic plasticity in sperm traits in this species.

Introduction

Sperm competition occurs when sperm from different males compete to fertilize the same set of ova (Parker 1970, 1998). Over the last decades, this form of post-copulatory sexual selection has emerged as one of the most important processes to explain the evolution of reproductive traits in animals. It is now clear that sperm competition is a potent evolutionary agent that can affect the behaviour, morphology and physiology of both males and females (Wigby and Chapman 2004; Pizzari and Parker 2009). The most prominent trait that is affected by sperm competition is the number of produced sperm. Assuming that sperm production is costly and that sperm compete numerically, theoretical models predict that males should invest proportionally more in spermatogenesis at high levels of sperm competition, in order to gain a higher paternity share (reviewed in Parker 1998). This is supported by numerous comparative studies, which show that males of species that generally experience high levels of sperm competition have relatively larger testes (e.g. Hosken 1997; Stockley et al. 1997; Byrne et al. 2002; Pitcher et al. 2005). Similarly, intraspecific studies have demonstrated evolutionary responses in testis size to different levels of sperm competition (e.g. Hosken and Ward 2001; Pitnick et al. 2001) and there is also evidence that sperm competition induces a phenotypically plastic response in testis size (e.g. Schärer and Ladurner 2003) or sperm production rate (e.g. Schärer and Vizoso 2007; Ramm and Stockley 2009).

Beyond an increased sperm production rate, sperm competition might also select for other sperm or ejaculate traits that enhance the paternity share of a sperm donor (in this paper we preferentially use the term ‘sperm donor’ instead of ‘males’ since it also applies to hermaphrodites). By far the most frequently studied sperm trait assumed to be under selection by sperm competition is sperm size (Snook 2005). On the one hand, sperm competition may select for smaller sperm if sperm size trades-off with sperm number and if sperm of different donors compete in a fair raffle (Parker 1982). However, empirical evidence for this trade-off is equivocal (for reviews see Snook 2005; Pizzari and Parker 2009). On the other hand, sperm competition may select for bigger sperm, if sperm size is positively linked to sperm competitiveness through, for example, a higher longevity, motility and/or ability to displace smaller sperm from other males out of the females’ reproductive tract (Parker 1993; Snook 2005).

There are many comparative studies that support a positive relationship between the level of sperm competition and sperm size (e.g. Gomendio and Roldan 1991; Gage 1994; Lüpold et al. 2009; Montgomerie and Fitzpatrick 2009). However, there are also many studies that have found no effect of sperm competition on sperm size across species (e.g. Briskie and Montgomerie 1992; Gage and Freckleton 2003; Minder et al. 2005), which suggests that there is no general pattern even within taxa such as insects, birds and mammals (Pizzari and Parker 2009). Similarly, evidence for a link between sperm competition and sperm size derived from experimental evolution studies is also equivocal. Although it has been demonstrated experimentally that sperm competition can lead to the evolution of larger sperm in *Caenorhabditis elegans* (LaMunyon and Ward 2002), no such response has been found in four insect species (Hosken et al. 2001; Pitnick et al. 2001; Crudgington et al. 2009; Gay et al. 2009).

In contrast to the large body of evidence outlined above, studies focussing on a phenotypically plastic response in sperm morphology to different levels of sperm competition are very scarce (but see Awata et al. 2008; Crean and Marshall 2008; Immler et al. in press). If sperm competitiveness increases with sperm size, we may expect that under certain conditions individuals should produce bigger sperm when facing higher levels of sperm competition (Parker 1993; Snook 2005).

In this study we tested whether sperm competition affects the sperm morphology in a simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*. Individuals of this species are capable of adjusting their sex allocation (i.e. the reproductive investment into the male versus the female sex function) in response to the social group size (i.e. the number of potential mates) that they experience. Such an adjustment is in agreement with a central prediction of sex allocation theory for simultaneous hermaphrodites (for a review see Schärer 2009) and several studies have demonstrated for *M. lignano* that individuals that were raised in larger groups have bigger testes (e.g. Schärer and Ladurner 2003; Schärer et al. 2005; Schärer and Vizoso 2007; Schärer and Janicke 2009).

One of these studies hypothesised that the change in sex allocation is accompanied by a phenotypically plastic response in sperm morphology (Schärer and Vizoso 2007). Specifically, it has been shown that individuals raised in groups (i.e. intense sperm competition) not only have larger testes, but also produce a bigger total sperm mass compared to worms in pairs (i.e. no sperm competition). Sperm production rate in this study was inferred from an increase over time in the size of the sperm mass in the seminal vesicle, which is the organ containing the sperm that are ready to be transferred to mating partners (Schärer and Vizoso 2007). Interestingly, worms that had grown up under high levels of sperm competition refilled their seminal vesicle at a faster rate, even after statistically controlling for the effect of testis size. From this, the authors concluded that the phenotypically plastic adjustment of sperm production rate includes a component that is independent of testis size. Beside the possibility that sperm competition led to a faster spermatogenesis, it was hypothesised that this unknown component could be the production of bigger sperm under high levels of sperm competition (Schärer and Vizoso 2007). In the current study we aimed to test this hypothesis. Specifically, we predicted that individuals that are raised in groups produce bigger sperm compared to individuals that are raised in pairs.

Sperm cells of *M. lignano* carry several unusual appendages, including a rapidly undulating feeler anterior to the sperm body, a pair of stiff lateral bristles anchored at the junction of the sperm body and the shaft, as well as a terminal brush posterior to the shaft (Figure 1; Vizoso et al. 2010). The nucleus is located inside the shaft (Willems et al. 2009). So far, the function of the feeler, the bristles and the brush are not well understood. It has been hypothesized that the feeler allows the sperm to anchor itself in the epithelium of the female sperm receiving organ and that the bristles prevent the removal of sperm out of the sperm receiving organ during a post-copulatory behaviour (Vizoso et al. 2010). After insemination, sperm become anchored in the epithelium of the sperm storage organ close to the site where fertilization is likely to take place (Vizoso et al. 2010). Therefore, it seems possible that sperm are competing for access to the anchoring site with the highest likelihood of fertilization. In this study we focused on phenotypic plasticity in four morphological traits of the sperm, namely total sperm length, sperm body length, sperm bristle length, and sperm brush length.

Methods

Study organism

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is an obligately outcrossing simultaneous hermaphrodite, which belongs to the intertidal meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). In our laboratory mass cultures, adult worms reach approximately 1.5 mm in body length and have a generation time of about 18 days. In mass cultures worms are maintained at 20 °C in glass Petri dishes containing f/2 medium (Andersen et al. 2005) and fed with the diatom *Nitzschia curvilineata*. Under laboratory conditions worms mate frequently and are highly promiscuous when kept in groups (Schärer et al. 2004; Janicke and Schärer 2009a). The worms are transparent allowing non-invasive measurement of morphological traits, such as testis and ovary size (Schärer and Ladurner 2003). Spermatogenesis takes about six days (Schärer et al. 2007) after which the sperm is stored in the seminal vesicle, which is located in the tail plate of the worm, before it is transferred to mating partners via the copulatory stylet.

Manipulation of the sperm competition level

To manipulate the level of sperm competition we raised worms in different social group sizes, namely in groups of two individuals (hereafter called pairs) and in groups of eight individuals (hereafter called octets). Given that *M. lignano* is an obligately outcrossing simultaneous hermaphrodite (Schärer and Ladurner 2003), there is no sperm competition in pairs. In contrast, a previous study demonstrated that worms in octets experience a high level of sperm competition (Janicke and Schärer 2009a).

On day 1 of the experiment we collected 1,200 adult worms from our mass culture and distributed them equally to 12 Petri dishes, filled with f/2 medium and a dense layer of algae, where they could lay eggs. After 48 hours we removed all adult worms, which limited the range in laying date to two days. On day 11, we collected all produced hatchlings and allocated them randomly in pairs and octets into wells of 24-hole well plates. We balanced the number of treatments per plate and alternated the positions of the treatments on the plate to control for position effects. All wells contained 2 ml of f/2 medium and a dense algae layer that guaranteed *ad libitum* food conditions. We transferred all worms three times (i.e. on days 21, 28 and 35) to fresh wells to ensure that the manipulated social group size was not influenced by the produced offspring (worms usually hatch 5 days after egg laying and do not mature before 13 days after hatching; Schärer and Ladurner 2003). Each treatment was replicated 50 times.

Morphological measurement of sex allocation and sperm morphology

We had to verify if worms actually responded to the manipulation of the sperm competition level by shifting their sex allocation, as previously shown for *M. lignano* (see introduction). For this we measured body size, testis size and ovary size of worms *in vivo*, by randomly selecting one individual out of each pair and each octet. The remaining worms were used for another experiment (published elsewhere; Janicke and Schärer 2009b). Image acquisition was carried out from day 36 to 41 according to the standard protocol (Schärer and Ladurner 2003; Janicke and Schärer 2009b). Afterwards, we amputated the tail plate of the worm with a scalpel in order to make the sperm that is stored in the seminal vesicle accessible for imaging. For this we ruptured the tail plate by transferring it with only 1 µl medium on a microscope slide and covered it with a cover slip (21 x 26 mm) causing sperm to flow out of the seminal vesicle. The small amount of medium led to a very thin water film, in which the sperm cells were strongly restricted into two dimensions, greatly facilitating the measurement of the sperm morphology.

We used a Leica DM 2500 microscope (Leica Microsystems, Germany) to which we connected a digital video camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) and took digital micrographs at 40x for body size, 400x for gonad size and 1000x for sperm morphology. For image acquisition we used the software BTV Pro 6.0b1 (http://www.bensoftware.com/) and we analysed micrographs using ImageJ 1.42k (http://rsb.info.nih.gov/ij/). Morphological measurements of each sperm included total sperm length, sperm body length, the mean length of the two sperm bristles and sperm brush length using the 'Segmented Lines' tool in ImageJ. Total sperm length included the length of the sperm body and the length of the sperm shaft (for terminologies and description of the sperm measurements see also Figure 1). The length of the feeler was not included into the analysis since the rapid movement of this structure did not allow for accurate measurements. Sperm traits from 48 individual sperm (each from a different individual worm) were measured twice to assess the repeatability of our measurements. This revealed a high repeatability for total sperm length ($r_1 = 0.96$, $F_{47,48} = 46.23$, $P < 0.001$) and moderate but significant repeatabilities for the other sperm traits (sperm body length, $r_1 = 0.47$, $F_{47,48} = 2.80$, $P < 0.001$; sperm bristle length, $r_1 = 0.46$, $F_{47,48} = 2.72$, $P < 0.001$; sperm brush length, $r_1 = 0.49$, $F_{47,48} = 2.90$, $P < 0.001$).

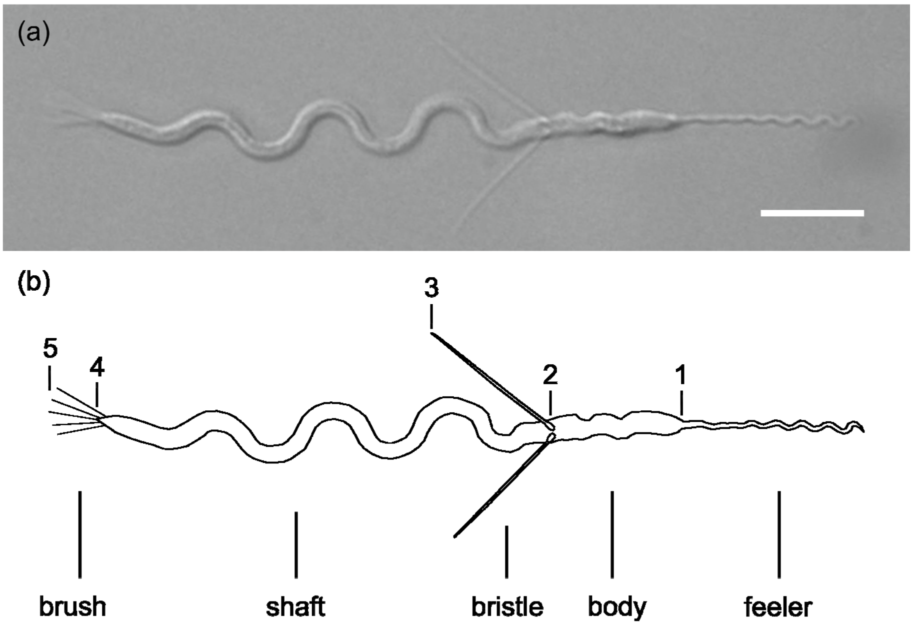

Figure 1. Micrograph (a) and schematic illustration (b) of a mature sperm of *Macrostomum lignano*. The scale bar represents 10 µm. Numbers in (b) indicate the landmarks used for the measurement of the morphological sperm traits. 'Total sperm length' was defined as the length of a segmented line drawn along the outline of the sperm between the basis of the feeler (1) and the basis of the brush (4), 'sperm body length' as the length of a segmented line drawn along the outline between the basis of the feeler (1) and the basis of the bristles (2), 'sperm bristle length' as the straight-line distance between the basis (2) and the tip of the bristle (3), and 'sperm brush length' as the straight-line distance between the basis of the brush (4) and the central tip of the brush (5).

Statistical analyses

We first assessed the number of sperm per individual that needs to be measured to obtain a reliable estimate for each individual in all morphological sperm traits. For this we used a random subset of 15 individuals from which we measured 20 sperm each. Following the method described by Pattarini *et al.* (2006), we calculated Pearson correlation coefficients of correlations between the individual means of the complete dataset (n = 20) and a randomly reduced dataset (n = 1 to n = 19) and then iterated this procedure 10 times for each individual. This analysis indicated that within-individual variation in all measured traits was low compared to the between-individual variation, a result that is commonly found in other species (e.g. Morrow and Gage 2001; Pattarini et al. 2006). Based on this assessment, we decided to include only those individuals from which we had measured at least 10 sperm in the final analysis, since this sample size is sufficient to capture more than 97% of the within-individual variation in all sperm traits inferred from measuring 20 sperm per individual (Figure 2). This reduced our final sample size to 48 individuals (24 from pairs, 24 from octets). From these individuals we randomly selected 10 sperm and used the mean values of each sperm trait in the final analysis. Including individuals for which we had measured less than 10 sperm (n = 30) did not change our results qualitatively.

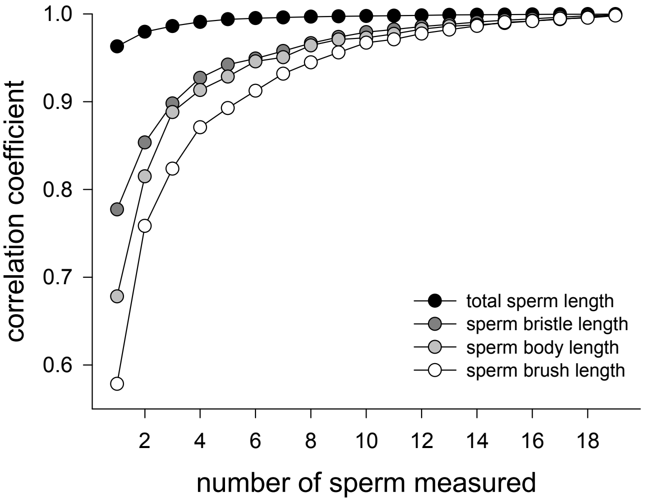

Figure 2. Relationship between the number of sperm measured and the accuracy of the estimation as described by Pearson correlation coefficients for all sperm traits measured. Note that measuring more than 10 sperm per individual does only marginally improve the accuracy of the estimates. See the method section for a detailed description of the analysis.

To test if the worms from pairs and octets were comparable in their overall resource budget (cf. Schärer et al. 2005), we tested if our treatment had an effect on body size using a Student's *t*-test. We then assessed whether our treatment induced a phenotypically plastic response in sex allocation, as already shown for *M. lignano* (e.g. Schärer and Ladurner 2003; Janicke and Schärer 2009b). For this we used ANCOVAs with testis size and ovary size as dependent variables, social group size as a fixed factor and body size as a covariate (because testis size and ovary size are usually correlated with body size). In all these analyses the interaction terms between social group size and body size were not statistically significant and were therefore excluded from the final models.

Additionally, we assessed the relationships between sperm morphology, body size, and gonad size by correlating all sperm traits with body size as well as residual testis size and residual ovary size (both calculated from a linear regression fit on body size; testis size: $R^2 = 0.15$, $F_{1,46} = 8.24$, $P = 0.006$; ovary size: $R^2 = 0.12$, $F_{1,46} = 6.16$, $P = 0.017$). To test the main hypothesis of this study, we compared all sperm traits between the treatment groups using Student's *t*-tests.

Finally, a power analysis was performed to explore the differences in each sperm trait that would have been detectable between the treatments using our experimental setup. This was done using the pwr package in R v.2.9.1 (R Development Core Team 2009). Based on the overall mean values and the standard deviations of all sperm traits (calculated from individual means) we assessed the relatively smallest significant differences between pairs and octets that we were able to detect with our sample size ($n = 48$, $\alpha = 0.05$, power = 0.8, two-tailed *t*-test).

All statistical tests were carried out in R v.2.9.1 (R Development Core Team 2009). Assumptions of normality and homogeneity of variance were met for all parametric tests presented. All statistical tests were carried out at $\alpha = 0.05$ Values are given as means ± SE unless otherwise stated.

Results

Worms that were raised in pairs and octets were comparable in body size (*t*-test: $t = -0.22$, $d.f. = 46$, $P = 0.825$; see Table S1 for descriptive statistics), suggesting that the overall resource budget was similar between the two treatments. As intended, the manipulation of the social group size had a significant effect on the sex allocation: individuals raised in octets had larger testes (ANCOVA: social group size, $F_{1,45} = 10.60$, $P = 0.002$; body size, $F_{1,45} = 9.96$, $P = 0.003$) and smaller ovaries (ANCOVA: social group size, $F_{1,45} = 24.04$, $P < 0.001$; body size: $F_{1,45} = 9.25$, $P = 0.004$) compared to worms from pairs (Table S1 for descriptive statistics). Thus, worms from octets clearly had a more male-biased sex allocation compared to worms from pairs.

We found significant between-individual variation in all sperm traits measured (Table 1; for total sperm length see also Figure 3), but none of the sperm traits were significantly correlated with body size, residual testis size or residual ovary size (Table 2). However, total sperm length, sperm body length and sperm bristle length all covaried positively with each other (Table 2), while sperm brush length was not correlated with any sperm trait.

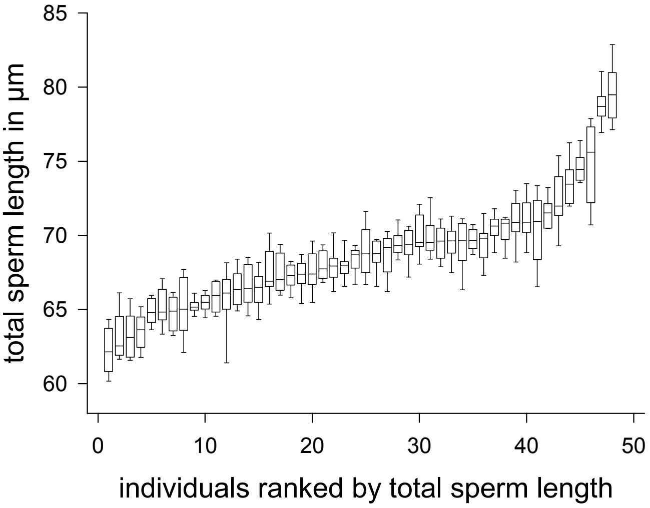

Figure 3. Box-plots showing the between-individual variation in total sperm length among 48 adult worms (10 sperm measured per individual). Individuals are ranked in order of the median in total sperm length. For statistics see Table 1.

Table 1. Descriptive statistics of sperm traits (based on all sperm cells measured, n = 480) and Kruskal-Wallis ANOVAs on ranks testing for variation in sperm morphology between individuals (n = 48).

	mean ± S.D.	minimum	maximum	*chi*-square	*d.f.*	*P*-value
total sperm length (μm)	68.6 ± 3.8	60.2	83.0	410.5	47	< 0.001
sperm body length (μm)	14.1 ± 0.9	11.8	16.9	192.1	47	< 0.001
sperm bristle length (μm)	13.2 ± 0.7	11.3	15.7	232.4	47	< 0.001
sperm brush length (μm)	4.6 ± 0.7	2.8	6.9	174.6	47	< 0.001

Table 2. Pearson correlation matrix showing correlation coefficients for body size, gonad sizes and all sperm traits based on individual mean values (n = 48).

	body size	residual testis size	residual ovary size	total sperm length	sperm body length	sperm bristle length
residual testis size	-					
residual ovary size	-	0.070				
total sperm length	-0.031	0.186	0.315†			
sperm body length	-0.156	0.041	-0.005	0.713 ***		
sperm bristle length	-0.084	0.107	0.044	0.584 ***	0.655 ***	
sperm brush length	0.195	0.034	0.097	0.272	0.180	0.102

*** $P < 0.001$, after correcting for multiple testing using the Benjamini-Hochberg method (Benjamini and Hochberg 1995).

† $P < 0.05$ before correcting for multiple testing, but > 0.05 after correction.

Finally, there was no effect of social group size on total sperm length (*t*-test: $t = -0.62$, *d.f.* = 46, $P = 0.540$; Figure 4a), sperm body length (*t*-test: $t = 0.67$, *d.f.* = 46, $P = 0.505$; Figure 4b), sperm bristle length (*t*-test: $t = 0.601$, *d.f.* = 46, $P = 0.551$; Figure 4c), or sperm brush length (*t*-test: $t = 0.11$, *d.f.* = 46, $P = 0.916$; Figure 4d).

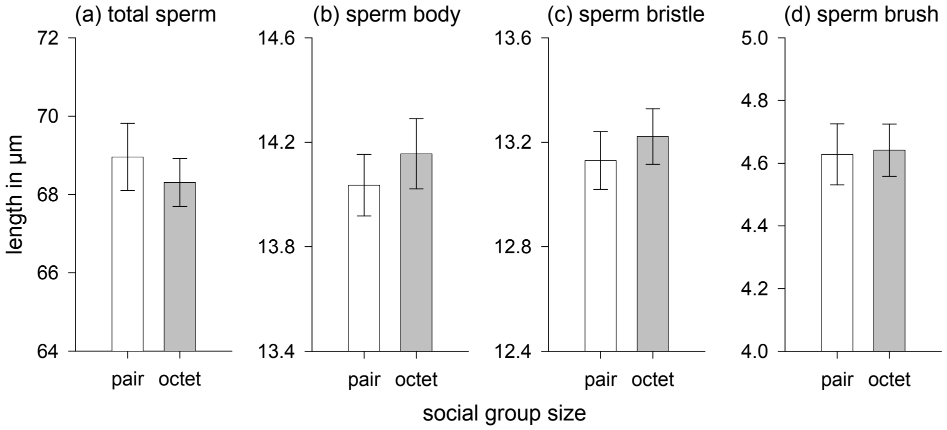

Figure 4. Comparison of the individual means of (a) total sperm length, (b) sperm body length, (c) sperm bristle length, and (d) sperm brush length between worms raised in pairs (n = 24) and octets (n = 24). See text for statistics.

The power analysis revealed that we had sufficient statistical power (0.8) to detect a difference of 5, 4, 4 and 8 % in total sperm length, sperm body length, sperm bristle length, and sperm brush length, respectively, between individuals raised in pairs and octets (n = 48, α = 0.05, two-tailed *t*-test).

Discussion

Our study suggests that a phenotypically plastic adjustment of the sex allocation in response to varying levels of sperm competition is not accompanied by phenotypically plastic changes in sperm morphology in *Macrostomum lignano*. We could show that worms in octets produced larger testes and smaller ovaries and that they were therefore more male-biased compared to individuals in pairs, as previously documented for *M. lignano* (e.g. Schärer and Ladurner 2003; Schärer et al. 2005; Schärer and Vizoso 2007; Janicke and Schärer 2009b). Despite this increase in male reproductive investment, there was no effect of the level of sperm competition on any of the sperm morphology traits we did measure. Neither total sperm length, nor sperm body, sperm bristle or sperm brush length differed between worms that were raised in pairs and octets. Based on the power analysis, the sample size was high enough to detect relatively small differences in sperm morphology between both treatment groups (i.e. 4 - 8%) compared to the large variation that we observed between individual worms (relative maximum differences: total sperm length 25%; sperm body length 19%; sperm bristle length 16%; sperm brush length 42%). However, it is unclear whether our power was sufficient to capture the smallest biologically relevant differences, since we currently lack any quantitative data on the relationship between these sperm traits and the siring success in *M. lignano*.

Schärer and Vizoso (2007) found a positive effect of group size on sperm production rate, which was independent of testis size in *M. lignano*. They hypothesised that a phenotypically plastic increase in sperm size in larger groups might explain the observed effect. However, given that the group size effect (corrected for testis size) on sperm production rate was rather strong in that study and that we should have been able to detect relatively small differences in sperm length between groups in the current study, it seems unlikely that changes in sperm length are responsible for the increased sperm production rate that the authors observed in the larger groups. Consequently, the alternative hypothesis proposed by Schärer and Vizoso (2007), i.e. that it is a faster spermatogenesis in larger groups what causes the additional effect of group size on sperm production rate, probably represents a better explanation for the observed pattern. Experiments to test this hypothesis are currently ongoing.

The absence of phenotypic plasticity in sperm size in response to sperm competition is consistent with an experimental study in the cooperatively breeding cichlid *Julidochromis transcriptus*, which also found a positive effect of sperm competition risk on testis size but not on sperm size (Awata et al. 2008). Likewise, larval density (a proxy for a higher sperm competition level in the future) had no effect on sperm length in the bruchid beetle *Callosobruchus maculatus* (Gay et al. 2009;

but note that in this study there was no effect of larval density on testis size either). Nevertheless, there are a few studies that suggest a phenotypically plastic response in sperm length to the level of sperm competition. Morrow et al. (2008) studied sperm length in *D. melanogaster* and showed that besides a large additive genetic component some variation in sperm length could be explained by the larval environment. Male flies that were exposed to a higher level of larval competition produced slightly smaller sperm (Morrow et al. 2008). Additionally, Immer et al. (in press) reported pronounced within-individual plasticity in sperm morphometry in Gouldian finches (*Erythrura gouldiae*). Among other effects, the authors could show that males increase the relative length of the sperm midpiece when placed from an intermediate into a highly competitive social environment (Immler et al. in press). The currently best evidence for a phenotypically plastic adjustment of sperm length in response to sperm competition comes from the broadcast spawning ascidian *Styela plicata* (Crean and Marshall 2008). Individuals of this simultaneous hermaphrodite produce longer sperm heads when experimentally exposed to high densities, with a relative difference in head length between individuals from high and low densities of about 7% (Crean and Marshall 2008). According to our power analysis, a difference of that magnitude would have been detectable with our experimental setup.

We found relatively low within- but high between-individual variation in all sperm traits we measured. This is consistent with other studies, indicating that sperm length is often male specific (e.g. Ward and Hauschteck-Jungen 1993) and repeatable between successive ejaculates (e.g. Morrow and Gage 2001). None of the sperm traits covaried with body size or the residual testis size. Such an absence of allometric relationships between sperm traits and body size has been found in both inter-specific (e.g. Ward and Hauschteck-Jungen 1993; for review see Pitnick et al. 2009a) and intra-specific studies (e.g. Minoretti and Baur 2006; Gay et al. 2009; but see Amitin and Pitnick 2007). If we assume that body size is a fitness-related trait in *M. lignano*, our findings suggest that sperm morphology is not strongly condition-dependent in this species, confirming findings in other organisms (reviewed in Pitnick et al. 2009a).

One potential reason for a lack of a phenotypically plastic adjustment of sperm morphology in response to a varying sperm competition level is that most of the between-individual variation is due to genetic variation rather than environmental factors (e.g. Morrow et al. 2008). In agreement with this notion, a recent meta-analysis across many animal taxa found a relatively high average heritability for sperm morphology compared to other sperm traits such as sperm motility (Simmons and Moore 2009). Detailed studies focussing on the heritability of sperm morphology traits in *M. lignano* are now needed.

Another explanation for the absence of an effect of sperm competition on sperm morphology could be that the length of the sperm, and its body, bristles and brush only play a minor role for the outcome of sperm competition in *M. lignano*. So far, we lack any data showing a direct relationship between sperm morphology and sperm competitiveness or cryptic female choice in *M. lignano*. However, a recent study demonstrated that within the genus *Macrostomum* sperm bristles only occur in species in which sperm from different donors interact in the female sperm receiving organ, but not in species with hypodermic impregnation, suggesting that the bristles and the brush are important traits in post-copulatory sexual selection (Schärer et al. unpublished data). In order to test directly how sperm morphology affects sperm competitiveness in *M. lignano*, one could use the large between-individual variation in all sperm morphology traits and assess paternity shares from mating experiments in which individuals producing consistently different sperm phenotypes compete for fertilization against each other.

Furthermore, selection on sperm morphology can also be driven by sperm-female interactions (Pitnick et al. 2009b). For instance, it has been shown that the relationship between sperm length and the rate of extra-pair paternity (a proxy for the level of sperm competition) in birds arises only indirectly through a positive correlation of extra-pair paternity with the length of sperm storage tubules in females (Briskie et al. 1997). Indeed, there are many comparative studies, which show that sperm length covaries with the morphology of the female sperm storage organ (reviewed in Pitnick et al. 2009a; Pitnick et al. 2009b). Moreover, an artificial selection experiment in *Drosophila melanogaster* revealed that the evolution of sperm length can occur as a correlated response to selection on the morphology of the female reproductive tract (Miller and Pitnick 2002).

Finally, ejaculate traits other than sperm morphology might be more important for the outcome of sperm competition. For instance, in the Atlantic Salmon (*Salmo salar*) sperm velocity, but not sperm length, is positively correlated with fertilization success (Gage et al. 2004). Therefore, *M. lignano* may not adjust the sperm morphology in response to sperm competition, but may instead adjust dynamic sperm traits, such as sperm velocity or longevity, some of which could be mediated by adjusting the composition of seminal fluids rather than the morphology of the sperm (Poiani 2006).

In conclusion, we found no phenotypically plastic effect of sperm competition on sperm morphology in *M. lignano*, despite the presence of a phenotypically plastic response in male reproductive investment. Although our data reveal considerable between-individual variation in sperm morphology, none of the sperm traits were correlated with the gross morphology of the sperm-producing individual, as measured by body size, residual testis size and residual ovary size. The functional significance of sperm length variation and of the various sperm appendages for sperm competition in *M. lignano* remains unclear and should be addressed in further studies.

Acknowledgements

We thank Peter Sandner and two anonymous referees for thoughtful comments on an earlier version of the manuscript. We are also grateful to Ralph Dobler for fruitful discussions and Jürgen Hottinger, Viktor Mislin and Urs Stiefel for technical support. This study was funded by grants from the Swiss National Science Foundation to L.S. (3100A0-113708 and 3100A0-127503).

References

Amitin, E. G., and S. Pitnick. 2007. Influence of developmental environment on male- and female-mediated sperm precedence in *Drosophila melanogaster*. J. Evol. Biol. 20:381-391.

Andersen, R. A., J. A. Berges, P. J. Harrison, and M. M. Watanabe. 2005. Recipes for freshwater and seawater media. Pp. 429-538 *in* R. A. Andersen, ed. Algal Culturing Techniques. Elsevier, Amsterdam.

Awata, S., T. Takeyama, Y. Makino, Y. Kitamura, and M. Kohda. 2008. Cooperatively breeding cichlid fish adjust their testis size but not sperm traits in relation to sperm competition risk. Behav. Ecol. Sociobiol. 62:1701-1710.

Benjamini, Y., and Y. Hochberg. 1995. Controlling the false discovery rate - a practical and powerful approach to multiple testing. J. R. Stat. Soc. Ser. B-Stat. Methodol. 57:289-300.

Briskie, J. V., and R. Montgomerie. 1992. Sperm size and sperm competition in birds. Proc. R. Soc. B-Biol. Sci. 247:89-95.

Briskie, J. V., R. Montgomerie, and T. R. Birkhead. 1997. The evolution of sperm size in birds. Evolution 51:937-945.

Byrne, P. G., J. D. Roberts, and L. W. Simmons. 2002. Sperm competition selects for increased testes mass in Australian frogs. J. Evol. Biol. 15:347-355.

Crean, A. J., and D. J. Marshall. 2008. Gamete plasticity in a broadcast spawning marine invertebrate. Proc. Natl. Acad. Sci. U. S. A. 105:13508-13513.

Crudgington, H. S., S. Fellows, N. S. Badcock, and R. R. Snook. 2009. Experimental manipulation of sexual selection promotes greater male mating capacity but does not alter sperm investment. Evolution 63:926-938.

Gage, M. J. G. 1994. Associations between body-size, mating pattern, testis size and sperm lengths across butterflies. Proc. R. Soc. B-Biol. Sci. 258:247-254.

Gage, M. J. G., and R. P. Freckleton. 2003. Relative testis size and sperm morphometry across mammals: no evidence for an association between sperm competition and sperm length. Proc. R. Soc. B-Biol. Sci. 270:625-632.

Gage, M. J. G., C. P. Macfarlane, S. Yeates, R. G. Ward, J. B. Searle, and G. A. Parker. 2004. Spermatozoal traits and sperm competition in Atlantic salmon: relative sperm velocity is the primary determinant of fertilization success. Curr. Biol. 14:44-47.

Gay, L., D. J. Hosken, R. Vasudev, T. Tregenza, and P. E. Eady. 2009. Sperm competition and maternal effects differentially influence testis and sperm size in *Callosobruchus maculatus*. J. Evol. Biol. 22:1143-1150.

Gomendio, M., and E. R. S. Roldan. 1991. Sperm competition influences sperm size in mammals. Proc. R. Soc. B-Biol. Sci. 243:181-185.

Hosken, D. J. 1997. Sperm competition in bats. Proc. R. Soc. B-Biol. Sci. 264:385-392.

Hosken, D. J., T. W. J. Garner, and P. I. Ward. 2001. Sexual conflict selects for male and female reproductive characters. Curr. Biol. 11:489-493.

Hosken, D. J., and P. I. Ward. 2001. Experimental evidence for testis size evolution via sperm competition. Ecol. Lett. 4:10-13.

Immler, S., S. R. Pryke, T. R. Birkhead, and S. C. Griffith. in press. Pronounced within-individual plasticity in sperm morphometry across social environments. Evolution.

Janicke, T., and L. Schärer. 2009a. Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite. J. Evol. Biol. 22:405-415.

Janicke, T., and L. Schärer. 2009b. Sex allocation predicts mating rate in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 276:4247-4253.

Ladurner, P., L. Schärer, W. Salvenmoser, and R. M. Rieger. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha). J. Zool. Syst. Evol. Res. 43:114-126.

LaMunyon, C. W., and S. Ward. 2002. Evolution of larger sperm in response to experimentally increased sperm competition in *Caenorhabditis elegans*. Proc. R. Soc. B-Biol. Sci. 269:1125-1128.

Lüpold, S., G. M. Linz, and T. R. Birkhead. 2009. Sperm design and variation in the New World blackbirds (Icteridae). Behav. Ecol. Sociobiol. 63:899-909.

Miller, G. T., and S. Pitnick. 2002. Sperm-female coevolution in Drosophila. Science 298:1230-1233.

Minder, A. M., D. J. Hosken, and P. I. Ward. 2005. Co-evolution of male and female reproductive characters across the Scathophagidae (Diptera). J. Evol. Biol. 18:60-69.

Minoretti, N., and B. Baur. 2006. Among- and within-population variation in sperm quality in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Behav. Ecol. Sociobiol. 60:270-280.

Montgomerie, R., and J. L. Fitzpatrick. 2009. Testes, sperm, and sperm competition. Pp. 1-53 *in* B. G. M. Jamieson, ed. Reproductive Biology and Phylogeny of Fishes (Agnathans and Bony Fishes). Science Publishers, Enfield, New Hampshire.

Morrow, E. H., and M. J. G. Gage. 2001. Consistent significant variation between individual males in spermatozoal morphometry. J. Zool. 254:147-153.

Morrow, E. H., A. Leijon, and A. Meerupati. 2008. Hemiclonal analysis reveals significant genetic, environmental and genotype x environment effects on sperm size in *Drosophila melanogaster*. J. Evol. Biol. 21:1692-1702.

Parker, G. A. 1970. Sperm competition and its evolutionary consequences in insects. Biol. Rev. 45:525-567.

Parker, G. A. 1982. Why are there so many tiny sperm - Sperm competition and the maintenance of two sexes. J. Theor. Biol. 96:281-294.

Parker, G. A. 1993. Sperm competition games - sperm size and sperm number under adult control. Proc. R. Soc. B-Biol. Sci. 253:245-254.

Parker, G. A. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Pp. 3-54 *in* T. Birkhead, and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, Cambridge.

Pattarini, J. A., W. T. Starmer, A. Bjork, and S. Pitnick. 2006. Mechanisms underlying the sperm quality advantage in *Drosophila melanogaster*. Evolution 60:2064-2080.

Pitcher, T. E., P. O. Dunn, and L. A. Whittingham. 2005. Sperm competition and the evolution of testes size in birds. J. Evol. Biol. 18:557-567.

Pitnick, S., D. J. Hosken, and T. R. Birkhead. 2009a. Sperm morphological diversity. Pp. 69-149 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm Biology: An Evolutionary Perspective. Academic Press, Burlington, MA, USA.

Pitnick, S., G. T. Miller, J. Reagan, and B. Holland. 2001. Males' evolutionary responses to experimental removal of sexual selection. Proc. R. Soc. B-Biol. Sci. 268:1071-1080.

Pitnick, S., M. F. Wolfner, and S. S. Suarez. 2009b. Ejaculate-female and sperm-female interactions. Pp. 247-304 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm Biology: An Evolutionary Perspective. Academic Press, Burlington, MA, USA.

Pizzari, T., and G. A. Parker. 2009. Sperm competition and sperm phenotype. Pp. 207-245 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm Biology: An Evolutionary Perspective. Academic Press, Burlington, MA, USA.

Poiani, A. 2006. Complexity of seminal fluid: a review. Behav. Ecol. Sociobiol. 60:289-310.

R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Ramm, S. A., and P. Stockley. 2009. Adaptive plasticity of mammalian sperm production in response to social experience. Proc. R. Soc. B-Biol. Sci. 276:745-751.

Schärer, L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. Evolution 63:1377-1405.

Schärer, L., and T. Janicke. 2009. Sex allocation and sexual conflict in simultaneously hermaphroditic animals. Biol. Lett. 5:705-708.

Schärer, L., G. Joss, and P. Sandner. 2004. Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms suck. Mar. Biol. 145:373-380.

Schärer, L., and P. Ladurner. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 270:935-941.

Schärer, L., P. Sandner, and N. K. Michiels. 2005. Trade-off between male and female allocation in the simultaneously hermaphroditic flatworm *Macrostomum* sp. J. Evol. Biol. 18:396-404.

Schärer, L., and D. B. Vizoso. 2007. Phenotypic plasticity in sperm production rate: there's more to it than testis size. Evol. Ecol. 21:295-306

Schärer, L., J. Zaubzer, W. Salvenmoser, C. Seifarth, and P. Ladurner. 2007. Tracking sperm of a donor in a recipient: an immunocytochemical approach. Anim. Biol. 57:121-136.

Simmons, L. W., and A. J. Moore. 2009. Evolutionary quantitative genetics of sperm. Pp. 405-434 *in* T. R. Birkhead, D. J. Hosken, and S. Pitnick, eds. Sperm Biology: An Evolutionary Perspective. Academic Press, Burlington, MA, USA.

Snook, R. R. 2005. Sperm in competition: not playing by the numbers. Trends Ecol. Evol. 20:46-53.

Stockley, P., M. J. G. Gage, G. A. Parker, and A. P. Møller. 1997. Sperm competition in fishes: The evolution of testis size and ejaculate characteristics. Am. Nat. 149:933-954.

Vizoso, D. B., G. Rieger, and L. Schärer. 2010. Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking. Biol. J. Linn. Soc. 99:370-383.

Ward, P. I., and E. Hauschteck-Jungen. 1993. Variation in sperm length in the yellow dung fly *Scathophaga stercoraria* (L). Journal of Insect Physiology 39:545-547.

Wigby, S., and T. Chapman. 2004. Sperm competition. Curr. Biol. 14:R100-R103.

Willems, M., F. Leroux, M. Claeys, M. Boone, S. Mouton, T. Artois, and G. Borgonie. 2009. Ontogeny of the complex sperm in the macrostomid flatworm *Macrostomum lignano* (Macrostomorpha, Rhabditophora). J. Morphol. 270:162-174.

Electronic Supplementary Material

Sperm competition affects sex allocation but not sperm morphology in a flatworm

Tim Janicke and Lukas Schärer

Table S1. Morphological comparison between worms that were raised in different social group sizes, i.e. in pairs and in octets.

morphological parameter	pair (n=24)			octet (n=24)		
	mean ± S.D.	min	max	mean ± S.D.	min	max
body size (x $10^3 \mu m^2$)	693.5 ± 153.4	475.2	995.7	703.1 ± 144.4	395.5	938.0
testis size (x $10^3 \mu m^2$)	31.3 ± 10.4	15.6	52.2	45.5±19.9	20.4	92.3
ovary size (x $10^3 \mu m^2$)	20.4 ± 5.2	13.0	35.8	14.7 ± 3.7	9.7	24.1

CHAPTER V

Determinants of female fecundity in a simultaneous hermaphrodite: the role of polyandry and food availability

Manuscript accepted for publication as:
Janicke, T., P. Sandner, and L. Schärer. Determinants of female fecundity in a simultaneous hermaphrodite: the role of polyandry and food availability. Evol. Ecol.

Abstract

Classical sexual selection theory assumes that the reproductive success of females is primarily limited by the resources available for egg production rather than by the number of mating partners. However, there is now accumulating evidence that multiple mating can entail fitness costs or benefits for females. In this study we investigated the effect of polyandry (i.e., the mating with different mating partners) and food availability on the reproductive output of the female sex function in an outcrossing simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*. We exposed virgin worms to different group sizes, a treatment that has previously been shown to affect the level of polyandry in this species. Moreover, we manipulated the food availability throughout the subsequent egg laying period, during which the worms were kept in isolation. The number of offspring produced was used as an estimate of female fecundity. We found that food availability, but not group size, had a significant effect on female fecundity. Additionally, female fecundity was positively correlated with the number of stored sperm in the female sperm-storage organ at the time of isolation, but it was not correlated with body or ovary size of the worms. Our results suggest that female fecundity in *M. lignano* is primarily determined by the resources available for egg production, and not by the level of polyandry, confirming classic sexual selection theory for simultaneous hermaphrodites.

Introduction

Classical sexual selection theory assumes that the reproductive output of females is primarily limited by the resources available for egg production rather than by the number of mating partners (Bateman 1948). Consequently, females are expected to copulate only once or a few times to obtain sufficient sperm to fertilize their eggs, especially if mating entails costs, such as time and energy expenses (Daly 1978), a higher predation risk (Rowe 1994), an increased exposure to parasites and infections (Thrall et al. 1997), a reduced immune function (Rolff and Siva-Jothy 2002), physical injuries (Crudgington and Siva-Jothy 2000) or a reduced lifespan caused by male accessory gland products (Chapman et al. 1995; Green and Tregenza 2009; but see Priest et al. 2008; Reinhardt et al. 2009). However, over the last decades empirical studies have revealed that multiple mating in females is widespread across many animal taxa. There is now accumulating evidence that multiple mating in females is not only driven by male promiscuity, but may in fact represent an adaptive behavioural strategy that increases female fitness and often outweighs the costs of mating (for reviews see Arnqvist and Nilsson 2000; Jennions and Petrie 2000; Hosken and Stockley 2003; Andersson 2005; Simmons 2005).

Hypotheses for the evolution and maintenance of multiple mating in females either rely on material (direct) or genetic (indirect) benefits (Reynolds 1996). Material benefits refer to fitness gains mediated by resources that are provided by males and can therefore be obtained both from multiple mating with different males (hereafter called polyandry) or with the same male (hereafter called repeated matings), as long as males do not become depleted in the commodity that is beneficial to females. In contrast, genetic benefits are related to genetic diversity among male gametes, which can only be augmented considerably by polyandry (but see Yasui 1997 for an argument that considers genetic diversity within ejaculates). To avoid confusion we here use ‘multiple mating’ as a general term for individuals that mate more than once, irrespective of whether this involves ‘repeated matings’ or ‘polyandry’.

Material benefits of multiple mating encompass resources that females obtain from males, such as nutrients (e.g., prey items, seminal products), parental investment and/or protection against conspecifics or predators (e.g., Gwynne 1984; Vahed 1998; Engqvist 2007; reviewed in Reynolds 1996; Arnqvist and Nilsson 2000). Furthermore, the receipt of sperm can represent a material benefit, if female reproductive success is limited by the number of sperm that is available to fertilize all the eggs (e.g., Pitnick 1993; Levitan and Petersen 1995; Fjerdingstad and Boomsma 1998; Diaz et al. 2010). Several empirical studies across a wide range of taxa have demonstrated that repeated matings are advantageous to females (e.g., Wagner et al. 2001; Fedorka and Mousseau 2002; Schwartz and Peterson 2006; Klemme et al. 2007), which suggests that direct benefits may promote the evolution of multiple mating. Likewise, a meta-analysis of 122 studies focussing on female fitness consequences of multiple mating indicated that direct benefits alone can explain the evolutionary maintenance of multiple mating in insects (Arnqvist and Nilsson 2000).

Genetic benefits derived from polyandrous mating can also be manifold (for reviews on genetic benefits see Yasui 1998; Jennions and Petrie 2000; Zeh and Zeh 2001; Simmons 2005). For instance, females might benefit from polyandry by means of a bet-hedging strategy that lowers the probability of mating only with inferior males (in case of error-prone mate choice abilities) or with males that carry genes that are maladapted to future environments (in case of unpredictable environments) (Watson 1991). Furthermore, it has been argued that females are polyandrous in order to minimize the risk and the associated fitness cost of fertilization by genetically incompatible sperm (Zeh and Zeh 1996, 1997). Contrary to the other genetic benefit models the 'genetic incompatibility hypothesis' assumes that the interaction between maternal and paternal haplotypes involves fitness consequences that are non-additive. Given that genetic compatibility is expected to be required for a normal embryogenesis, the 'genetic compatibility hypothesis' not only predicts a positive effect of polyandry on the quality of the offspring, but also on the number of viable offspring produced (Zeh and Zeh 1996).

Empirical studies indicate that there is no consistency in the effect of polyandry on female fecundity across taxa. For instance, mating with multiple males has been shown to be beneficial to females in echinoderms (Evans and Marshall 2005), insects (Tregenza and Wedell 1998; Fedorka and Mousseau 2002; Dunn et al. 2005), fishes (Evans and Magurran 2000) and reptiles (LaDage et al. 2008), but it can also be associated with a reduction of the female's reproductive output, as reported for many insect species (e.g., Orsetti and Rutowski 2003; Bybee et al. 2005; Ronkainen et al. 2010). At the same time, several studies found no effect of polyandry on female fecundity in insects (e.g., Baker et al. 2001; Schwartz and Peterson 2006; House et al. 2009).

Compared to this large body of empirical studies on separate-sexed organisms, very little attention has been placed on the fitness consequences of polyandry in simultaneous hermaphrodites, i.e. organisms that produce sperm and eggs at the same time. In his seminal paper on sexual selection in simultaneous hermaphrodites, Charnov (1979) assumed that Bateman's principle (Bateman 1948) is also valid for simultaneously hermaphroditic animals. However, his line of reasoning that "fertilized egg production by an individual is limited not by the ability to get sperm, but by resources allocated to eggs" (Charnov 1979) differs slightly from Bateman's principle, because it only refers to an effect of sperm availability on female fecundity, which is not necessarily related to the number of mating partners (unless sperm donors get sperm depleted). Therefore, Charnov (1979) primarily made a prediction for the effect of repeated matings, but not for the effect of polyandry on female fecundity. Charnov (1979) did not clarify if this difference was in any way intentional or just the result of a slightly different phrasing. Either way, empirical tests of the validity of both Bateman's principle and Charnov's hypothesis are still scarce in simultaneous hermaphrodites (but see e.g., Marshall and Evans 2007; Sprenger et al. 2008).

Studying the costs and benefits of multiple mating in simultaneous hermaphrodites reveals several interesting differences to separate-sexed organisms.

First, theoretical analyses suggest that matings in simultaneous hermaphrodites may often be more harmful to the female function than expected for separate-sexed animals (Michiels and Koene 2006). This is because in contrast to females, simultaneous hermaphrodites should remate even if mating entails severe fitness costs for the female function, as long as they can compensate these costs by a sufficiently high male fitness benefit (Michiels and Koene 2006). Second, in many simultaneously hermaphroditic species mating occurs reciprocally (i.e., each partner both donates and receives sperm during each mating), which inevitably links the mating strategies of both sex functions (Michiels 1998). Given that multiple mating might be beneficial for one sex function, but costly for the other function, a trade-off between the optimal male and female mating strategies might arise within one individual, i.e. being eager to mate in one sex function versus being reluctant in the other sex function (Bedhomme et al. 2009; Janicke and Schärer 2009b). Third, empirical data on female fitness consequences of multiple mating are crucial to resolve a longstanding debate on the preferred mating role in simultaneous hermaphrodites. Here, the preferred mating role normally refers to the sex function that on average yields a higher expected benefit from an additional mating. Contrary to Charnov's (1979) prediction that simultaneous hermaphrodites "copulate not so much to gain sperm to fertilize their eggs as to give sperm away", it has also been hypothesised that hermaphrodites mate preferentially in the female sex function (Leonard 2005, 2006). To date, empirical tests of these hypotheses have primarily aimed to demonstrate whether individuals trade male or female gametes, in order to infer indirectly in which sex role they mate preferentially. On the one hand, there are studies indicating that simultaneous hermaphrodites trade eggs during mating, which suggests an overall preference to donate sperm to fertilize the partner's eggs, i.e. to mate in the male role (e.g., Fischer 1980; Sella 1985). But on the other hand, there are also studies that provide evidence for sperm trading, which may suggest that they copulate primarily in order to receive sperm, i.e. to mate in the female role (e.g., Leonard and Lukowiak 1991; Vreys and Michiels 1998; Anthes et al. 2005).

In order to shed light on the importance of Bateman's principle for simultaneous hermaphrodites, we studied the effect of polyandry and food availability on female fecundity in the outcrossing simultaneously hermaphroditic flatworm *Macrostomum lignano*. Copulations in this species are reciprocal (Schärer et al. 2004) and it has been shown that worms are highly promiscuous when they are exposed to multiple potential mating partners (Janicke and Schärer 2009a). Furthermore, worms that allocate relatively more resources towards the male sex function mate more frequently and it has been argued that multiple mating in this species may primarily be driven by the male sex function (Janicke and Schärer 2009b). However, whether multiple mating causes either benefits or costs for the female sex function is currently unknown. We manipulated the group size and the food availability of worms to infer how both factors affect female fecundity (measured as the number of offspring produced). Furthermore, we studied how morphological traits of the worms and the amount of received sperm in storage relate to female fecundity.

Methods

Study organism

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is an outcrossing simultaneous hermaphrodite of the intertidal meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). In mass cultures worms are maintained at 20 °C in glass Petri dishes filled with f/2 medium (Andersen et al. 2005) and fed with the algae *Nitzschia curvilineata*. Under these conditions, body length of a fully grown worm reaches on average 1.5 mm and the generation time is about 18 days. The worms are transparent allowing non-invasive measurement of internal morphological traits, such as testis size, ovary size and the size of the seminal vesicle (which stores the produced sperm before it is transferred to the mating partners). The transparency also makes it possible to count the sperm that is stored in the female sperm-storage organ (hereafter called 'antrum', plural 'antra') *in vivo*. The antrum contains the fertilized egg until it is released through the ciliated vagina (Ladurner et al. 2005; Vizoso et al. 2010).

Experimental design

To test for an effect of polyandry and food availability on female fecundity we used a fully factorial design, in which we manipulated both the group size and the food level. On day 1 we pooled 1,200 adult worms from mass cultures and distributed them equally to 6 Petri-dishes filled with f/2 medium and a dense algae layer and allowed them to lay eggs. On day 3 we removed all adults from the Petri-dishes, which assured that their offspring did not differ by more than two days in age. On day 8 we pooled all hatchlings from the 6 Petri-dishes and isolated 840 individuals in wells of 24 well-plates, which were filled with 1.5 ml f/2 medium and 0.1 ml of a concentrated algae solution that guaranteed *ad libitum* food conditions. On day 29, when all individuals were at least 26 days old, we used these virgin worms to assemble different group sizes in order to manipulate the level of polyandry. Specifically, we placed virgin worms for 24 hours into groups of two individuals (hereafter called pairs), groups of three individuals (hereafter called triplets) and groups of 16 individuals in 24 well-plates under *ad libitum* food conditions. For each well-plate we balanced the number of treatments and their positions on the plate.

On day 30 (i.e., one day after group formation), we randomly selected one worm out of each group, took morphological measurements and assessed the number of stored sperm in the antrum (see next section for methods). Next, each worm was isolated in a well of a 24 well-plate. In order to manipulate the food availability, worms were randomly assigned either to wells with a dense algae layer or wells without any algae. Therefore, our manipulation of the food availability consisted of *ad libitum* food conditions and a complete lack of any food resources after mating. Again, the number of treatments and their positions on the well-plates were balanced among the different well-plates that were used. On days 32, 34, 36, 38 and 44 we transferred all worms to fresh wells, which allowed us to determine the number of the produced offspring on a temporal scale and which prevented the interaction of adult worms with their offspring (embryonic development takes 5 days). After transferring worms to fresh wells we added 0.1 ml of a concentrated algae solution to each old well to guarantee *ad libitum* food conditions for the developing offspring after hatching. The whole experiment was split into two blocks that were temporally separated by 24 hours. Each block initially comprised six replicates for all factor combinations. Blocking had no effect on any of the parameters that we measured (*t*-tests and Wilcoxon rank sum tests: all $P > 0.4$) and it was therefore ignored in all further analyses.

Female fecundity was defined as the number of offspring produced per day after the isolation of the worms from the group until day 50. Offspring counts were carried out always 10 days after removal of the parental worm from the well, which ensured that all offspring had hatched but that none had matured yet to produce their own offspring.

Rationale for group size manipulation

In an earlier study we had placed sperm-labelled focal worms together with one, two or 15 unlabelled individuals and these focal worms were allowed to mate for 24 hours under similar conditions as in the current study (e.g., *ad libitum* food conditions, same enclosure size). We could demonstrate that the number of mating partners of the focal worms was positively affected by the group size. Specifically, the average number of individuals that had received labelled sperm from the focal worm was 0.9, 1.5 and 5.4 in pairs, triplets and groups of 16 individuals, respectively (Janicke and Schärer 2009a). Given that copulations in *M. lignano* are always reciprocal, the number of individuals that had received labelled sperm should correspond exactly to the number of individuals with which focal individuals copulated in their female sex function. However, the inferred numbers of mating partners probably represent conservative estimates of the level of polyandry since the presence of labelled sperm in the antrum of a worm might underestimate the amount of actually received sperm due to cryptic female choice (Thornhill 1983), sperm displacement (e.g., Waage 1979) or passive sperm loss (e.g., Birkhead and Biggins 1998). Furthermore, one difference in the experimental setup between this study and Janicke and Schärer (2009a) is that we here used virgin worms instead of mated individuals. Given that virgins copulate more frequently than already mated individuals (T. Janicke; unpublished data), we expect that the difference in the level of polyandry between the different group sizes was, if anything, higher in the present study than that reported by Janicke and Schärer (2009a). Based on these earlier findings it is very likely that our group size treatment affected the level of polyandry in the present study. However, it is unclear to which extent our manipulation also had an effect on the number of repeated matings with the same individuals.

A number of previous studies on *M. lignano* showed that these worms are capable of adjusting their sex allocation (i.e., the resource allocation towards the male versus the female function) in response to the group size (e.g., Schärer and Ladurner 2003; Janicke and Schärer 2009b; reviewed in Schärer 2009). In larger groups worms invest more resources into the male function (in terms of larger testes) at a cost to the female function (in terms of smaller ovaries), which reduces the female reproductive output (Schärer et al. 2005; Janicke and Schärer 2009b). However, a recent study indicated that it takes several days of exposure to a specific group size to observe a sex allocation response (Brauer et al. 2007), which suggests that the group size manipulation of only 24 hours in the current study is unlikely to have an effect on the sex allocation of the worms.

Morphological measurement and counts of stored sperm

We took morphological measurements 24 hours after group formation (directly before isolating worms into single wells) to test whether worms in different treatment groups were similar with regard to their morphology and to examine whether the morphology of a worm correlates with its female fecundity. In particular, we measured body size, testis size and ovary size. We also measured the size of the seminal vesicle, which can be used as an estimate of the number of sperm allocated by worms in previous matings (Schärer and Ladurner 2003). Moreover, we assessed the number of stored sperm in the antrum to test whether this parameter was affected by our group size manipulation and if it was related to female fecundity. On day 50 (20 days after the mating trials) we again counted the number of stored sperm to confirm that all worms had run out of sperm and therefore where unable to produce any further offspring.

All morphological measurements were obtained *in vivo* in a standardized way (Schärer and Ladurner 2003; Janicke and Schärer 2009b). First, worms were anesthetized by immersing them in a 5:3 mixture of 7.14 % $MgCl_2$ and f/2 medium for 10 min. Overview pictures of the entire body, the testes, ovaries and the seminal vesicle were taken after compressing worms dorsoventrally to a fixed thickness of 35 μm between a microscope slide and a cover slip of a haemocytometer (Schärer and Ladurner 2003). We used a Leica DM 2500 microscope (Leica Microsystems, Germany) to which we connected a digital video camera (DFK 41AF02, The Imaging Source Europe GmbH, Germany) and took digital micrographs at 40x for body size and 400x for testis size, ovary size and seminal vesicle size. For image acquisition we used the software BTV Pro 6.0b1 (http://www.bensoftware.com/) and we analysed micrographs using ImageJ 1.42k (http://rsb.info.nih.gov/ij/). All these morphological measurements are repeatable within individuals (Schärer and Ladurner 2003).

Counts of the number of stored sperm in the antrum were carried out directly after morphological measurements using the same optical devices. First, we gently compressed worms between a 24 x 50 mm and a 21 x 26 mm cover slip using small plasticine feet on each corner of the smaller cover slip as a spacer. Then, we mounted the cover slips with the worm on a modified microscope slide fitted with two raised supports on which the cover slips could be placed. Thereby, the observer could easily flip the compressed worm from the dorsal to the ventral view, which is required to properly count all the sperm that are stored in the antrum. We then recorded QuickTime movies of each antrum at 1000x magnification (using immersion oil) by focussing two times slowly through the entire organ. Using these movies we later counted the number of stored sperm. All counts were done by the same observer (T.J.) who was blind with regard to the treatment group. In order to assess the repeatability of the sperm counts, the number of stored sperm was assessed twice for all antrum movies except the ones that were recorded 20 days after the mating trials. The analysis of these repeated sperm counts confirmed a high repeatability of the number of stored sperm in the antrum using the method described above (intraclass-correlation coefficient: $r_i = 0.89$, $F_{36,37} = 17.656$, $P < 0.001$).

Statistical analyses

Initially we aimed to replicate each factor combination 12 times. However, losses during measuring and pipetting errors reduced our final sample size to an average of 9.3 ± 1.0 replicates per factor combination (pairs/no food, $N = 12$; pairs/*ad libitum*, $N = 8$; triplets/no food, $N = 9$, triplets/*ad libitum*, $N = 6$; groups of 16 individuals/no food, $N = 12$; groups of 16 individuals/*ad libitum*, $N = 9$).

We tested whether worms exposed to different group sizes were morphologically similar with respect to body size, testis size and ovary size, as intended by our experimental setup. Furthermore, we assessed the effect of group size on the size of the seminal vesicle (our estimate of the amount of sperm allocated during the mating trials) and the number of received sperm. This was done using one-way ANOVAs (or Kruskal-Wallis ANOVAs in case the assumptions for parametric tests were not met). Additionally, we tested whether body size was correlated with the number of stored sperm in order to explore if this trait affected the amount of sperm that an individual is capable of storing or able to obtain from mating partners.

The determinants of female fecundity were assessed using Generalized Linear Mixed Models (GLMMs) with Poisson error distributions and log-link functions (Venables and Ripley 2002) to account for deviations from normality and unequal variances between the treatment groups. In a 'basic model', we included group size, food availability and time since mating as fixed factors, the individual as a random factor and body size and ovary size as covariates. In addition, we fitted an 'extended model', in which we added the number of stored sperm in the antrum (counted 24 hours after group formation) as an additional covariate to the 'basic model'. The reason for running two separate models was that the number of stored sperm could only be assessed from a fraction of all individuals considered in the 'basic model'. This was because many antra contained a ripe egg, which made it impossible to reliably count the number of stored sperm. Therefore, the sample size in the 'extended model' (number of observations = 222, number of individuals 37) was considerably lower compared to the 'basic model' (number of observations = 336, number of individuals = 56), which means that we presumably had more statistical power to explain variation in female fecundity using the 'basic model'. None of all possible two-way and three-way interaction terms in both the 'basic model' and the 'extended model' explained a significant amount of variation in female fecundity. Therefore, we excluded all interaction terms in the final analyses.

All statistical analyses were carried out in SPSS 17.0 (SPSS Inc. 2008) or R v. 2.10.1 (R Development Core Team 2009). We applied the penalized quasi-likelihood method (PQL) for both GLMMs (Breslow and Clayton 1993) by using the glmmPQL function implemented in the package MASS for R (Venables and Ripley 2010). Values are given as means ± 1 SE.

Results

Worms exposed to different group sizes did not differ significantly in body size, testis size and ovary size when measured directly after the mating trials (one-way ANOVAs: body size, $F_{2,53} = 0.02$, $P = 0.977$; testis size, $F_{2,53} = 0.52$, $P = 0.600$; ovary size, $F_{2,53} = 0.06$, $P = 0.943$; Figure 1). This suggests that worms in the different groups were initially similar with regard to these morphological traits as intended by our random assignment to the treatment groups. Moreover, this also confirms that the group size manipulation for 24 hours had no significant effect on the sex allocation of the worms. In contrast, seminal vesicle size was affected by group size (Kruskal-Wallis ANOVA: $\chi^2 = 10.52$, *d.f.* = 2, $P = 0.005$; Figure 2a). Post-hoc comparisons using Mann-Whitney U tests with Bonferroni correction revealed that worms from groups of 16 individuals had smaller seminal vesicles compared to worms from pairs ($z = -3.23$, $P = 0.004$, $N = 41$), but that there was no statistically significant difference between pairs and triplets ($z = -1.77$, $P = 0.232$, $N = 35$) or between triplets and groups of 16 individuals ($z = -1.14$, $P = 0.764$, $N = 36$).

Sperm counts conducted directly after the mating trials revealed a high between-individual variation in the number of stored sperm in the antrum (mean: 28.6 ± 2.1, range: 0 - 52, $N = 37$). There was only one individual that did not have any sperm in storage. The number of stored sperm did not differ between individuals that were exposed to different group sizes (Kruskal-Wallis ANOVA: $\chi^2 = 1.02$, *d.f.* = 2, $P = 0.601$; Figure 2b) and it was not correlated with the body size of the recipient (Pearson correlation coefficient: $r = 0.07$, $P = 0.688$, $N = 37$). Sperm counts that were carried out 20 days after the mating trials showed that not a single individual still had sperm in storage.

The sum of the offspring produced per worm was on average 5.2 ± 0.7 (range: 0 - 20). In total, there were 11 individuals that did not produce any offspring. Excluding these individuals from the analyses did not qualitatively change the results. We, therefore, report the statistical results from analyses that include these 11 individuals. GLMMs revealed that female fecundity was significantly affected by food availability, the time since mating and the number of stored sperm (Table 1). Specifically, female fecundity was higher in worms that were fed *ad libitum* and that had more sperm in storage after mating (Table 1; Figure 3a). Moreover, female fecundity decreased rapidly over time (Table 1; Figure 3a and b). Group size, body size and ovary size had no significant effect on female fecundity (Table 1; Figure 3b).

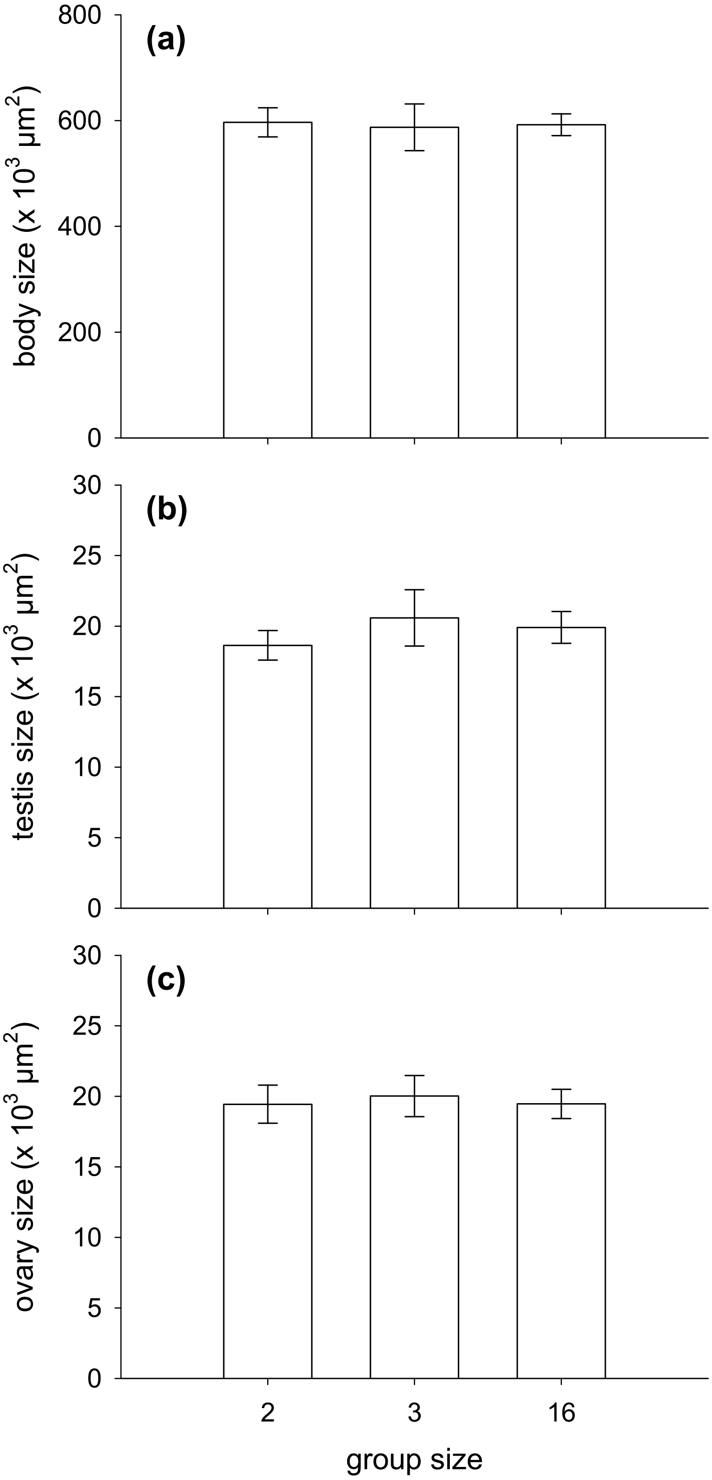

Figure 1. Comparison of (a) body size, (b) testis size and (c) ovary size between individuals exposed for 24 hours to different group sizes, i.e. pairs, triplets and groups of 16 individuals. Bars indicate means ± 1 SE. Body size, testis size and ovary size do not statistically differ between treatment groups ($P > 0.05$). See text for statistics.

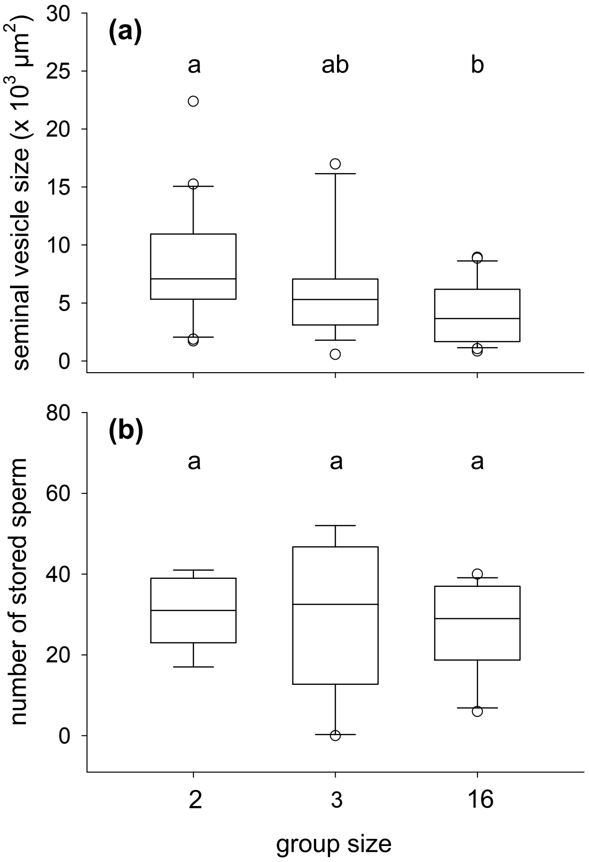

Figure 2. Comparison of (a) seminal vesicle size and (b) the number of stored sperm in the antrum (i.e., the female sperm-storage organ) between individuals exposed for 24 hours to pairs, triplets and groups of 16 individuals. Boxes show the 25th percentile, the median and the 75th percentile, whiskers denote the 10th and the 90th percentiles and open circles indicate outliers. Different letters indicate significantly different groups ($P < 0.01$). See text for statistics.

.Table 1. Summary of Generalized Linear Mixed Models testing the effect of group size, food availability, time since mating, body size, ovary size and the number of stored sperm on female fecundity. Given that the number of stored sperm could only be assessed for a fraction of all replicates, we report a 'basic model', which does not include the number of stored sperm and an 'extended model' in which the number of stored sperm was added as a covariate (see statistical analyses for details).

model	source	estimate ± SE	*d.f.*	*F*-value	*P*-value
basic model	group size	-	2,50	0.44	0.647
	food availability	-	1,50	10.14	0.003
	time since mating	-	5,275	26.79	< 0.001
	body size	0.32 ± 1.11	1,50	0.14	0.713
	ovary size	-43.29 ± 27.60	1,50	2.46	0.123
extended model	group size	-	2,30	0.13	0.880
	food availability	-	1,30	7.36	0.011
	time since mating	-	5,180	16.80	< 0.001
	body size	0.49 ± 1.30	1,30	0.24	0.627
	ovary size	-30.52 ± 31.94	1,30	0.44	0.510
	number of stored	0.06 ± 0.02	1,30	14.66	< 0.001

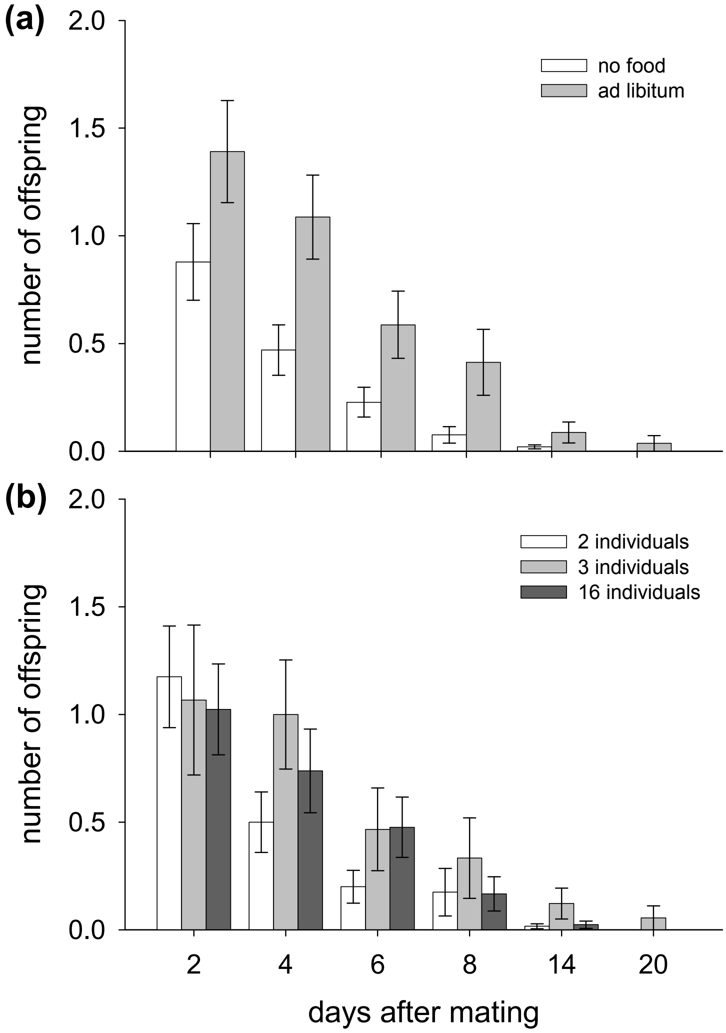

Figure 3. Daily female fecundity after mating shown for (a) the feeding levels and (b) the different group sizes. Data shown are lumped across group sizes in panel (a) and across feeding levels in panel (b). Note that x-axes are not linearly scaled. Bars indicate means ± 1 SE. See text for statistics.

Discussion

The results of our study suggest that it is not the number of mating partners but the food availability that has an effect on female fecundity in the simultaneously hermaphroditic flatworm *M. lignano*. Worms that were allowed to mate with several different sperm donors produced a similar number of offspring via their female sex function as individuals that could mate with only one sperm donor. In contrast, food resources that were available for egg production had a strong positive effect on female fecundity as predicted by sexual selection theory for simultaneous hermaphrodites (Charnov 1979).

Given that we used only the number of offspring produced under lab conditions as an estimate of female fitness, our results are limited to this measure of hatchling production. It has been argued that non-additive genetic benefits of polyandry are more likely to affect the number of offspring produced rather than offspring quality (Zeh and Zeh 1996; see introduction), and we thus should have been able to detect such genetic benefits. However, there might also have been genetic benefits of polyandry that we were not able to detect in this study. Specifically, genetic benefits derived from mating with many (including overall superior) mating partners might only be apparent when measuring offspring performance, e.g. offspring size, growth rate, or the offspring's own reproductive success (e.g., Ojanguren et al. 2005; Fisher et al. 2006).

In this study we manipulated the level of polyandry by exposing worms to different group sizes, which has been previously shown to affect the number of mating partners in *M. lignano*. Based on these earlier findings, we expect that the average number of mating partners in pairs, triplets and groups of 16 individuals was at least 0.9, 1.5 and 5.4 individuals, respectively (Janicke and Schärer 2009a; see also paragraph on the 'Rationale for group size manipulation' in the Methods section). Therefore, we are confident that we managed to manipulate the level of polyandry substantially in the current study. Nevertheless, the group size manipulation might not only have influenced the number of mating partners, but also the number of repeated matings with the same partners, for which we were unable to control with our experimental setup. Therefore, we can not exclude that our manipulation of polyandry was confounded by an additional effect of group size on the number of matings. Until now, we have no data on the effect of group size on the mating rate for the particular situation in which worms were kept in our experiment. Previous mating experiments, which were conducted in so-called observation chambers (in which worms are allowed to copulate in very small drops of culture medium; for details see Schärer et al. 2004), revealed no difference in the per capita mating rate between groups of 2, 3 and 4 individuals (T. Janicke, unpublished data). On the one hand, this previous study shows that repeated matings with the same mating partner clearly do occur in small groups. On the other hand, the results suggest that the group size has no direct effect on the mating rate in *M. lignano*. However, we have to clarify that in the current study all mating trials were carried out in much larger enclosures and the maximum number of worms was considerably higher, which limits the comparability of both studies.

In the current study we found that the size of the seminal vesicle was smaller in worms that were exposed to larger groups, which means that worms spent more sperm when they had the opportunity to mate with more mating partners. The most parsimonious explanation for this effect is a higher mating rate in larger groups. However, the observed effect could also indicate that worms allocated more sperm per mating in more competitive situations, as predicted by sperm competition theory (e.g., Parker 1998). Consequently, it is not possible to infer from our data, whether the group size manipulation had an effect on the mating rate and therefore on the number of repeated matings. Irrespective of possible effects on the frequency of repeated matings, we are certain that our experimental setup induced variation in the level of polyandry between the different group sizes. On the assumption that the mating rate was constant across all groups, our data indicate that polyandry has no effect on female fecundity in *M. lignano*. If the worms mated more frequently in larger groups, our results suggest that both polyandry and repeated mating have no positive or negative effect on our measure of female reproductive output. Only if polyandry and repeated matings have opposing fitness consequences for the female sex function (e.g., mating with different partners is beneficial but mating several times with the same partner incurs fitness costs) and if the per capita mating rate differed between groups, we might have been unable to detect an effect of polyandry on the female fecundity with our experimental setup.

Studies on the fitness consequences of multiple mating for the female sex function in simultaneously hermaphroditic animals have primarily focused on the role of repeated matings rather than the level of polyandry. For instance, in the land snail *Arianta arbustorum* repeated matings in the female role lead to an increased number of eggs laid, but not to a difference in the number of hatchlings produced (Chen and Baur 1993). In contrast, in the hermaphroditic freshwater snail *Lymnea stagnalis* it has been shown that individuals that were allowed to mate in groups lay fewer eggs than isolated individuals and an experimental manipulation of the number of copulations indicated that this difference was due to costs associated with mating (van Duivenboden et al. 1985; but see Koene et al. 2006). Recently, it has been demonstrated that at least a part of the negative effect of mating rate on female fecundity in *L. stagnalis* is due to the receipt of seminal fluids containing male accessory gland products, which presumably suppresses egg laying (Koene et al. 2009). Although these studies on *L. stagnalis* did not explicitly differentiate between effects of polyandry and repeated matings, they suggest that mating with the same partner can be costly for the female sex function in this simultaneously hermaphroditic snail.

Probably the most conclusive study on the influence of polyandry on female reproduction has been carried out in a simultaneously hermaphroditic opisthobranch *Chelidonura sandrana* (Sprenger et al. 2008). Similar to our findings, polyandry had no effect on the total number of egg masses produced and the proportion of fertile eggs. However, egg capsule volume and larval length was higher in individuals that mated once with four different sperm donors compared to individuals that mated four times with the same sperm donor, suggesting that the level of polyandry affected maternal provisioning (Sprenger et al. 2008). A positive effect of polyandry on female fecundity has also been reported for the broadcast spawning hermaphroditic ascidian *Pyura stolonifera,* in which a mixture of ejaculates from different donors increased the hatching success as a result of an elevated fertilization success compared to ejaculates from single sperm donors (Marshall and Evans 2007).

The positive effect of food availability on female fecundity found in our study is not very surprising, since resources available for egg production are expected to be crucial for the reproductive output of the female sex function (Charnov 1979). More surprising is that the initial body size and ovary size did not significantly predict the female reproductive output. This might either mean that both traits are indeed not correlated with female fecundity or that the variation in both morphological traits was too small to find an effect. The latter explanation is supported by a previous study in *M. lignano*, which suggests that if one induces variation in ovary size experimentally, a positive effect on the female reproductive output becomes apparent (Schärer et al. 2005).

The only covariate that was correlated with female fecundity was the number of stored sperm. Individuals that managed to store more sperm produced more offspring. However, whether this effect is relevant under more natural conditions is questionable, since fecundity was assessed in isolated worms, which could not replenish their sperm reserves after the mating trials. Given that the mating rate in *M. lignano* is relatively high (Schärer et al. 2004) and that worms can occur at relatively high densities in the field (K. Sekii et al., unpublished data) it remains unclear whether access to received sperm can constrain female fecundity under natural conditions, a question that should be studied in the field.

Remarkably, the number of stored sperm was unaffected by the group size. Therefore, the female sex function does not seem to gain direct benefits from multiple mating in terms of replenishing the own sperm reserves. Instead, variation in the number of stored sperm was presumably induced by factors that are not linked to multiple mating. For instance, the size of the female sperm-storage organ might constrain the amount of sperm an individual is capable of storing and may thereby affect the number of offspring produced. Moreover, quality traits of the sperm recipient might have an effect on the number of sperm that sperm donors transfer during copulations as predicted by theoretical models on strategic sperm allocation (e.g., Reinhold et al. 2002). So far, very little is known about how intrinsic traits of recipients influence the number of stored sperm in *M. lignano*. In this study, we found no correlation between the body size and the number of stored sperm, which suggests that body size itself and other traits that are size-dependent do not affect the number of sperm an individual is able to store or obtain from its mating partners.

Although the number of stored sperm was unaffected by group size, individuals in larger groups allocated more sperm during the mating trials (as inferred from the size of the seminal vesicle). This clearly suggests that not all sperm that are transferred during mating are finally stored in the partner. First of all, this could simply be due to passive sperm loss during egg laying, because fertilized eggs have to pass through the antrum before they are laid. Similarly, the capacity of the antrum is finite and therefore some of the transferred sperm may never become stored and therefore get lost passively. Another potential explanation is sperm displacement, in which individuals actively displace some of the stored sperm from previous mates. Alternatively, the recipient itself may remove the sperm out of its own antrum in order to digest them or to bias paternity towards favoured mating partners. Indeed, after copulating, worms often bend themselves in order to touch their female genital opening with their pharynx and then appear to suck sperm out of the antrum (Schärer et al. 2004). So far we know very little about sperm displacement and cryptic female choice in *M. lignano* (for a description of morphologies and behaviours that might facilitate both processes see Vizoso et al. 2010).

To summarize, in our study food availability but not group size (used as a proxy for the level of polyandry) had an effect on female fecundity in the hermaphroditic flatworm *M. lignano*. This finding is consistent with classical sexual selection theory (Bateman 1948), which predicts that the female reproductive output primarily depends on the resources that are available for egg production rather than on the number of mates. Therefore, our results support the hypothesis formulated by Charnov (1979), that Bateman's principle can also be applied to simultaneously hermaphroditic animals. However, in order to provide an ultimate test of Bateman's principle for *M. lignano*, one needs to assess the fitness benefits of multiple mating for both the female and the male sex function.

Acknowledgements

We thank Dita B. Vizoso for technical assistance. Ralph Dobler and two anonymous referees provided constructive comments on an earlier version of the manuscript. We also thank Lucas Marie-Orléach and Matthew D. Hall for statistical advice. Finally, we are grateful to Jürgen Hottinger, Viktor Mislin and Urs Stiefel for technical support. This study was funded by grants from the Swiss National Science Foundation to L.S. (3100A0-113708 and 3100A0-127503).

References

Andersen, R. A., J. A. Berges, P. J. Harrison, and M. M. Watanabe. 2005. Recipes for freshwater and seawater media. Pp. 429-538 *in* R. A. Andersen, ed. Algal Culturing Techniques. Elsevier, Amsterdam.

Andersson, M. 2005. Evolution of classical polyandry: three steps to female emancipation. Ethology 111:1-23.

Anthes, N., A. Putz, and N. K. Michiels. 2005. Gender trading in a hermaphrodite. Curr. Biol. 15:R792-R793.

Arnqvist, G., and T. Nilsson. 2000. The evolution of polyandry: multiple mating and female fitness in insects. Anim. Behav. 60:145-164.

Baker, R. H., R. I. S. Ashwell, T. A. Richards, K. Fowler, T. Chapman, and A. Pomiankowski. 2001. Effects of multiple mating and male eye span on female reproductive output in the stalk-eyed fly, *Cyrtodiopsis dalmanni*. Behav. Ecol. 12:732-739.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. Heredity 2:349-368.

Bedhomme, S., G. Bernasconi, J. M. Koene, A. Lankinen, H. S. Arathi, N. K. Michiels, and N. Anthes. 2009. How does breeding system variation modulate sexual antagonism? Biol. Lett. 5:717-720.

Birkhead, T. R., and J. D. Biggins. 1998. Sperm competition mechanisms in birds: models and data. Behav. Ecol. 9:253-260.

Brauer, V. S., L. Schärer, and N. K. Michiels. 2007. Phenotypically flexible sex allocation in a simultaneous hermaphrodite. Evolution 61:216-222.

Breslow, N. E., and D. G. Clayton. 1993. Approximate inference in Generalized Linear Mixed Models. Journal of the American Statistical Association 88:9-25.

Bybee, L. F., J. G. Millar, T. D. Paine, K. Campbell, and C. C. Hanlon. 2005. Effects of single versus multiple mates: monogamy results in increased fecundity for the beetle *Phoracantha semipunctata*. Journal of Insect Behavior 18:513-527.

Chapman, T., L. F. Liddle, J. M. Kalb, M. F. Wolfner, and L. Partridge. 1995. Cost of mating in *Drosophila melanogaster* females is mediated by male accessory-gland products. Nature 373:241-244.

Charnov, E. L. 1979. Simultaneous hermaphroditism and sexual selection. Proc. Natl. Acad. Sci. U. S. A. 76:2480-2484.

Chen, X. F., and B. Baur. 1993. The effect of multiple mating on female reproductive success in the simultaneously hermaphroditic land snail *Arianta arbustorum*. Can. J. Zool. 71:2431-2436.

Crudgington, H. S., and M. T. Siva-Jothy. 2000. Genital damage, kicking and early death - The battle of the sexes takes a sinister turn in the bean weevil. Nature 407:855-856.

Daly, M. 1978. Cost of mating. Am. Nat. 112:771-774.

Diaz, S. A., D. T. Haydon, and J. Lindstrom. 2010. Sperm-limited fecundity and polyandry-induced mortality in female nematodes *Caenorhabditis remanei*. Biol. J. Linn. Soc. 99:362-369.

Dunn, D. W., J. P. Sumner, and D. Goulson. 2005. The benefits of multiple mating to female seaweed flies, *Coelopa frigida* (Diptera : Coelpidae). Behav. Ecol. Sociobiol. 58:128-135.

Engqvist, L. 2007. Nuptial food gifts influence female egg production in the scorpionfly *Panorpa cognata*. Ecol. Entomol. 32:327-332.

Evans, J. P., and A. E. Magurran. 2000. Multiple benefits of multiple mating in guppies. Proc. Natl. Acad. Sci. U. S. A. 97:10074-10076.

Evans, J. P., and D. J. Marshall. 2005. Male-by-female interactions influence fertilization success and mediate the benefits of polyandry in the sea urchin *Heliocidaris erythrogramma*. Evolution 59:106-112.

Fedorka, K. M., and T. A. Mousseau. 2002. Material and genetic benefits of female multiple mating and polyandry. Anim. Behav. 64:361-367.

Fischer, E. A. 1980. The relationship between mating system and simultaneous hermaphroditism in the coral-reef fish, *Hypoplectrus nigricans* (Serranidae). Anim. Behav. 28:620-633.

Fisher, D. O., M. C. Double, and B. D. Moore. 2006. Number of mates and timing of mating affect offspring growth in the small marsupial *Antechinus agilis*. Anim. Behav. 71:289-297.

Fjerdingstad, E. J., and J. J. Boomsma. 1998. Multiple mating increases the sperm stores of *Atta colombica* leafcutter ant queens. Behav. Ecol. Sociobiol. 42:257-261.

Green, K., and T. Tregenza. 2009. The influence of male ejaculates on female mate search behaviour, oviposition and longevity in crickets. Anim. Behav. 77:887-892.

Gwynne, D. T. 1984. Courtship feeding Increases female reproductive success in bush-crickets. Nature 307:361-363.

Hosken, D. J., and P. Stockley. 2003. Benefits of polyandry: a life history perspective. Evol. Biol. 33:173-194.

House, C. M., C. A. Walling, C. E. Stamper, and A. J. Moore. 2009. Females benefit from multiple mating but not multiple mates in the burying beetle *Nicrophorus vespilloides*. J. Evol. Biol. 22:1961-1966.

Janicke, T., and L. Schärer. 2009a. Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite. J. Evol. Biol. 22:405-415.

Janicke, T., and L. Schärer. 2009b. Sex allocation predicts mating rate in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 276:4247-4253.

Jennions, M. D., and M. Petrie. 2000. Why do females mate multiply? A review of the genetic benefits. Biol. Rev. 75:21-64.

Klemme, I., J. A. Eccard, and H. Ylonen. 2007. Why do female bank voles, *Clethrionomys glareolus*, mate multiply? Anim. Behav. 73:623-628.

Koene, J. M., A. Brouwer, and J. N. A. Hoffer. 2009. Reduced egg laying caused by a male accessory gland product opens the possibility for sexual conflict in a simultaneous hermaphrodite. Anim. Biol. 59:435-448.

Koene, J. M., K. Montagne-Wajer, and A. Ter Maat. 2006. Effects of frequent mating on sex allocation in the simultaneously hermaphroditic great pond snail (*Lymnaea stagnalis*). Behav. Ecol. Sociobiol. 60:332-338.

LaDage, L. D., W. H. N. Gutzke, R. A. Simmons, and M. H. Ferkin. 2008. Multiple mating increases fecundity, fertility and relative clutch mass in the female leopard gecko (*Eublepharis macularius*) Ethology 114:512-520.

Ladurner, P., L. Schärer, W. Salvenmoser, and R. M. Rieger. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha). J. Zool. Syst. Evol. Res. 43:114-126.

Leonard, J. L. 2005. Bateman's principle and simultaneous hermaphrodites: a paradox. Integr. Comp. Biol. 45:856-873.

Leonard, J. L. 2006. Sexual selection: lessons from hermaphrodite mating systems. Integr. Comp. Biol. 46:349-367.

Leonard, J. L., and K. Lukowiak. 1991. Sex and the simultaneous hermaphrodite - testing models of male-female conflict in a sea slug, *Navanax inermis* (Opisthobranchia). Anim. Behav. 41:255-266.

Levitan, D. R., and C. Petersen. 1995. Sperm limitation in the sea. Trends Ecol. Evol. 10:228-231.

Marshall, D. J., and J. P. Evans. 2007. Context-dependent genetic benefits of polyandry in a marine hermaphrodite. Biol. Lett. 3:685-688.

Michiels, N. K. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. Pp. 219-254 *in* T. Birkhead, and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, London.

Michiels, N. K., and J. M. Koene. 2006. Sexual selection favors harmful mating in hermaphrodites more than in gonochorists. Integr. Comp. Biol. 46:473-480.

Ojanguren, A. F., J. P. Evans, and A. E. Magurran. 2005. Multiple mating influences offspring size in guppies. J. Fish Biol. 67:1184-1188.

Orsetti, D. M., and R. L. Rutowski. 2003. No material benefits, and a fertilization cost, for multiple mating by female leaf beetles. Anim. Behav. 66:477-484.

Parker, G. A. 1998. Sperm competition and the evolution of ejaculates: towards a theory base. Pp. 3-54 *in* T. Birkhead, and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, Cambridge.

Pitnick, S. 1993. Operational sex-ratios and sperm limitation in populations of *Drosophila pachea*. Behav. Ecol. Sociobiol. 33:383-391.

Priest, N. K., D. A. Roach, and L. F. Galloway. 2008. Cross-generational fitness benefits of mating and male seminal fluid. Biol. Lett. 4:6-8.

R Development Core Team. 2009. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna.

Reinhardt, K., R. A. Naylor, and M. T. Siva-Jothy. 2009. Ejaculate components delay reproductive senescence while elevating female reproductive rate in an insect. Proc. Natl. Acad. Sci. U. S. A. 106:21743-21747.

Reinhold, K., J. Kurtz, and L. Engqvist. 2002. Cryptic male choice: sperm allocation strategies when female quality varies. J. Evol. Biol. 15:201-209.

Reynolds, J. D. 1996. Animal breeding systems. Trends Ecol. Evol. 11:A68-A72.

Rolff, J., and M. T. Siva-Jothy. 2002. Copulation corrupts immunity: A mechanism for a cost of mating in insects. Proc. Natl. Acad. Sci. U. S. A. 99:9916-9918.

Ronkainen, K., A. Kaitala, and S. M. Kivelä. 2010. Polyandry, multiple mating, and female fitness in a water strider *Aquarius paludum*. Behav. Ecol. Sociobiol. 64:657-664.

Rowe, L. 1994. The costs of mating and mate choice in water striders. Anim. Behav. 48:1049-1056.

Schärer, L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. Evolution 63:1377-1405.

Schärer, L., G. Joss, and P. Sandner. 2004. Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms suck. Mar. Biol. 145:373-380.

Schärer, L., and P. Ladurner. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 270:935-941.

Schärer, L., P. Sandner, and N. K. Michiels. 2005. Trade-off between male and female allocation in the simultaneously hermaphroditic flatworm *Macrostomum* sp. J. Evol. Biol. 18:396-404.

Schwartz, S. K., and M. A. Peterson. 2006. Strong material benefits and no longevity costs of multiple mating in an extremely polyandrous leaf beetle, *Chrysochus cobaltinus* (Coleoptera : Chrysomelidae). Behav. Ecol. 17:1004-1010.

Sella, G. 1985. Reciprocal egg trading and brood care in a hermaphroditic polychaete worm. Anim. Behav. 33:938-944.

Simmons, L. W. 2005. The evolution of polyandry: Sperm competition, sperm selection, and offspring viability. Annu. Rev. Ecol. Evol. Syst. 36:125-146.

Sprenger, D., N. Anthes, and N. K. Michiels. 2008. Multiple mating affects offspring size in the opisthobranch *Chelidonura sandrana*. Mar. Biol. 153:891-897.

SPSS Inc. 2008. SPSS for Windows. SPSS Inc., Chicago.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122:765-788.

Thrall, P. H., J. Antonovics, and J. D. Bever. 1997. Sexual transmission of disease and host mating systems: within-season reproductive success. Am. Nat. 149:485-506.

Tregenza, T., and N. Wedell. 1998. Benefits of multiple mates in the cricket *Gryllus bimaculatus*. Evolution 52:1726-1730.

Vahed, K. 1998. The function of nuptial feeding in insects: review of empirical studies. Biol. Rev. 73:43-78.

van Duivenboden, Y. A., A. W. Pieneman, and A. Termaat. 1985. Multiple mating suppresses fecundity in the hermaphrodite freshwater snail *Lymnaea stagnalis* - a laboratory study. Anim. Behav. 33:1184-1191.

Venables, W. N., and B. D. Ripley. 2002. Modern Applied Statistics with S. Springer, New York.

Venables, W. N., and B. D. Ripley. 2010. MASS: Main Package of Venables and Ripley's MASS, R package.

Vizoso, D. B., G. Rieger, and L. Schärer. 2010. Goings-on inside a worm: functional hypotheses derived from sexual conflict thinking. Biol. J. Linn. Soc. 99:370-383.

Vreys, C., and N. K. Michiels. 1998. Sperm trading by volume in a hermaphroditic flatworm with mutual penis intromission. Anim. Behav. 56:777-785.

Waage, J. K. 1979. Dual function of the damselfly penis - sperm removal and transfer. Science 203:916-918.

Wagner, W. E., R. J. Kelley, K. R. Tucker, and C. J. Harper. 2001. Females receive a life-span benefit from male ejaculates in a field cricket. Evolution 55:994-1001.

Watson, P. J. 1991. Multiple paternity as genetic bet-hedging in female Sierra Dome Spiders, *Linyphia litigiosa* (Linyphiidae). Anim. Behav. 41:343-360.

Yasui, Y. 1997. A "good-sperm" model can explain the evolution of costly multiple mating by females. Am. Nat. 149:573-584.

Yasui, Y. 1998. The 'genetic benefits' of female multiple mating reconsidered. Trends Ecol. Evol. 13:246-250.

Zeh, J. A., and D. W. Zeh. 1996. The evolution of polyandry I: Intragenomic conflict and genetic incompatibility. Proc. R. Soc. B-Biol. Sci. 263:1711-1717.

Zeh, J. A., and D. W. Zeh. 1997. The evolution of polyandry II: Post-copulatory defences against genetic incompatibility. Proc. R. Soc. B-Biol. Sci. 264:69-75.

Zeh, J. A., and D. W. Zeh. 2001. Reproductive mode and the genetic benefits of polyandry. Anim. Behav. 61:1051-1063.

CHAPTER VI

Sex allocation predicts mating rate in a simultaneous hermaphrodite

Manuscript published as:
Janicke, T., and L. Schärer. 2009. Sex allocation predicts mating rate in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 276:4247-4253.

Abstract

Sexual selection theory for separate-sexed animals predicts that the sexes differ in the benefit they can obtain from multiple mating. Conventional sex roles assume that the relationship between the number of mates and the fitness of an individual is steeper in males compared to females. Under these conditions, males are expected to be more eager to mate, whereas females are expected to be choosier. Here we hypothesize that the sex allocation, i.e. the reproductive investment devoted to the male versus female function, can be an important predictor of the mating strategy in simultaneous hermaphrodites. We argue that within-species variation in sex allocation can cause differences in the proportional fitness gain derived through each sex function. Individuals should therefore adjust their mating strategy in a way that is more beneficial to the sex function that is relatively more pronounced. To test this we experimentally manipulated the sex allocation in a simultaneously hermaphroditic flatworm and investigated whether this affects the mating behaviour. The results demonstrate that individuals with a more male-biased sex allocation (i.e. relatively large testes and small ovaries) are more eager to mate compared to individuals with a more female-biased sex allocation (i.e. relatively small testes and large ovaries). We argue that this pattern is comparable to conventional gender roles in separate-sexed organisms.

Introduction

Darwin was puzzled by the observation that throughout the animal kingdom the female seems to be "less eager than the male" and suggested that the "exertion of some choice on the part of the female seems a law almost as general as the eagerness of the male" (Darwin 1871). The most cited explanation for this observation is Bateman's principle. Based on mating experiments with *Drosophila,* Bateman (1948) argued that the relationship between reproductive success and the number of mates is steeper in males than in females. Consequently, males gain more from mating with different partners and thus should show an "undiscriminating eagerness" whereas females are expected to display a "discriminating passivity" (Bateman 1948). Although there is an ongoing debate on the validity of Bateman's original study and its implications (e.g. Sutherland 1985; Snyder and Gowaty 2007; Jones 2009), it nevertheless holds that in many animal species (including humans) males and females differ in the benefit they derive from an elevated mating success (e.g. Jones et al. 2000; Jones et al. 2002; Brown et al. 2009).

No such general statement can be made for the male and female function of simultaneously hermaphroditic animals (i.e. organisms that produce sperm and eggs at the same time). Here copulations often occur reciprocally (i.e. both mating partners donate and receive sperm; Charnov 1979; Michiels 1998; Anthes et al. 2006) so that the mating rate of one sex function is directly linked to that of the other. On the assumption that the male and the female function have different optimal mating rates (Anthes et al. 2006), a trade-off between two mating strategies within an individual could result, e.g. being eager to mate in one function versus being choosy in the other function (at least at the pre-copulatory stage).

Recent research on mating strategies in simultaneous hermaphrodites primarily asked whether individuals of a species have an overall preference for mating in the male role (i.e. to donate sperm in order to fertilize eggs) or the female role (i.e. to receive sperm in order to get the own eggs fertilized and/or to benefit from sperm digestion) (reviewed in Michiels 1998; Anthes et al. 2006). However, theoretical studies suggest that the preferred sex role can also be flexible within a species and predict that the preference to mate in one sex function may depend on factors such as body size, the quality of the partner, the sperm precedence pattern and the mating history of available mates (Angeloni et al. 2002; Anthes et al. 2006). In particular, it has been proposed, that the preferred sex role of an individual should depend on the relation of its own body size to that of the partner (Angeloni et al. 2002) and there is empirical evidence that small individuals mate preferentially in the male reproductive role whereas large individuals prefer the female role (e.g. Ohbayashi-Hodoki et al. 2004; Norton et al. 2008; but see Gianguzza et al. 2004).

A widely unexplored parameter that might have a direct effect on the preferred mating strategy of an individual is its own sex allocation, i.e. the allocation of resources towards the male versus the female function. Schärer (2009) suggested that between-species variation in sex role preferences might be explained by differences in the average sex allocation. Based on the Fisher condition (Houston and McNamara 2002) the overall fitness return per unit investment is expected to be higher for the sex function with the lower allocation. Therefore, in species with an overall female-biased sex allocation individuals should prefer to mate in the male function (Schärer 2009). Beyond that, sex allocation might also explain variation in sex role preferences within a species. The only study providing correlational support for the link between sex allocation and mating behaviour within a species was carried out on blue-banded gobies (*Lythrypus dalli*) showing that egg laying individuals had a more female-biased sex allocation compared to those individuals that fertilized eggs (St. Mary 1994). However, theoretical and experimental work focusing on the effect of sex allocation on mating rates in simultaneous hermaphrodites is lacking.

Here we propose that within a species the sex allocation directly affects the mating strategy independently of body size and provide the first experimental evidence in support of this hypothesis. In many simultaneously hermaphroditic animals there is considerable within-species variation in sex allocation (for review see Schärer 2009). Due to this variation, individuals are expected to differ in the proportional fitness gains they derive from either sex function (Charnov 1982). When we now assume that both sex functions differ in their benefit obtained from a higher mating success, we hypothesize that the sex allocation has an effect on the mating rate an individual exhibits. Whether a more male- or female-biased sex allocation leads to a higher mating rate depends on which sex function has a higher benefit of multiple mating, which brings us back to Bateman's principle.

Charnov (1979) argued that Bateman's principle also applies to hermaphrodites and hypothesized that "individuals copulate not so much to gain sperm to fertilize eggs as to give sperm away". In contrast, it has also been claimed that it is the female role that should be preferred during mating, since the female function has a lower risk of a total reproductive failure (Leonard 2005). If the relationship between reproductive success and mating success (i.e. the Bateman gradient) is steeper for the male function, one would expect that the reproductive success of more male-biased individuals depends more on the number of mates compared to more female-biased individuals. Therefore, more male-biased individuals should be more eager to mate, so that their increased investment into their male function pays off. Conversely, individuals with a more female-biased sex allocation should adopt a mating strategy that is more discriminating and that selects for mating partners that provide the highest direct or indirect benefits. In contrast, if the Bateman gradient is steeper for the female function we expect that more female-biased individuals gain more from multiple mating and should therefore be more prone to mate.

Here we compared the mating rates of pairs that where either formed by joining two more male-biased individuals or two more female-biased individuals of the outcrossing simultaneously hermaphroditic flatworm *Macrostomum lignano*. In these worms sex allocation is phenotypically plastic and adjusted in response the number of potential mates, i.e. the social group size (e.g. Schärer and Ladurner 2003; Brauer et al. 2007; Schärer and Janicke 2009). This offers the opportunity to manipulate the sex allocation experimentally and to test how this affects the mating behaviour. If *M.*

lignano mates primarily in order to donate sperm as predicted by sexual selection theory for simultaneous hermaphrodites (Charnov 1979), we expect that more male-biased individuals show higher mating rates. In addition, we tested whether sex allocation also influences other aspects of the mating behaviour, namely the copulation duration and the frequency of a post-copulatory behaviour.

Methods

Study organism

Macrostomum lignano (Macrostomorpha, Platyhelminthes) is a simultaneously hermaphroditic free-living flatworm of the intertidal meiofauna of the Northern Adriatic Sea (Ladurner et al. 2005). In culture, it reaches 1.5 mm in body length and has a generation time of about 18 days. In mass cultures worms are maintained at 20 °C in glass Petri dishes containing f/2 medium (Andersen et al. 2005) and fed with the diatom *Nitzschia curvilineata*. The worm is fairly transparent allowing non-invasive measurement of morphological traits such as body size, testis size, ovary size and seminal vesicle size (Schärer and Ladurner 2003). The seminal vesicle represents the sperm storage organ of the male function and is located in the tail plate of the worm. A previous study showed that the size of the seminal vesicle is a good proxy for the number of sperm it contains (Schärer and Vizoso 2007).

Mating is reciprocal and often accompanied by a post-copulatory ‘suck’ in which the worm bends itself in order to touch its own female genital opening with the pharynx (Schärer et al. 2004). After this behaviour a bundle of sperm often sticks out of the own female genital opening, suggesting that the recipient may suck some of the received ejaculate out of its female sperm storage organ. Therefore this behaviour may represent a mechanism to select among sperm from different sperm donors, i.e. cryptic female choice (*sensu* Thornhill 1983). By sucking worms may remove unfavoured ejaculates out of their sperm storage organ and/or enable favoured sperm to get anchored in a part of a tissue that leads most likely to fertilisation. Moreover, sucking sperm might also prevent costs associated with polyspermy (Arnqvist and Rowe 2005). However, until now there is no clear evidence showing that this behaviour is actually linked to post-copulatory female choice.

Manipulation of sex allocation

To test whether sex allocation has an effect on the mating rate we manipulated the sex allocation of our focal worms prior to the mating trials using the approach outlined in the introduction (i.e. by raising worms in different social groups). On day 1 we collected 1,200 adult worms and distributed them equally to 12 Petri dishes filled with f/2 medium and a dense layer of algae where they could lay eggs. After 48 hours we removed all adult worms limiting the difference in laying date to two days. On day 11, we pooled all resulting hatchlings and distributed them randomly into their different social group sizes, namely pairs and octets (i.e. groups of two or eight individuals, respectively) into wells of 24-hole well plates. The treatments were arranged to balance any possible position effects (i.e. always 2 replicates of both treatments per plate, and positions on the plate alternated). Wells were filled with 2 ml of f/2 medium and a dense algae suspension that guaranteed *ad libitum* food conditions. On days 21, 28 and 35 we transferred only adult worms to fresh wells. With this setup we assured that the manipulated social group size was not influenced by the produced offspring because worms usually hatch after 5 days after egg laying and do not mature until 13 days after hatching (Schärer and Ladurner 2003). Each treatment was replicated 54 times.

Morphological measurement of sex allocation

To check whether the treatment successfully manipulated the sex allocation of the worms, we took images for morphological measurements *in vivo* prior to the mating trials. This was done by compressing anesthetized worms dorsoventrally to a fixed thickness of 35 µm between a microscope slide and a cover slip of a hemacytometer (Schärer and Ladurner 2003). Image acquisition was carried out from day 36 to 41. We used a Leica DM 2500 microscope (Leica Microsystems, Germany) to which we connected a digital video camera (DFK 41BF02, The Imaging Source Europe GmbH, Bremen, Germany) and took digital micrographs at 40x for body size and 400x for gonad size and seminal vesicle size. Recent studies have shown for *M. lignano* that testis size is a good proxy for sperm production rate (Schärer and Vizoso 2007) and that ovary size covaries positively with female fecundity (P. Sandner, unpublished data). For image acquisition we used the software BTV Pro 6.0b1 (http://www.bensoftware.com/) and we analysed micrographs using ImageJ 1.38x (http://rsb.info.nih.gov/ij/). The time period between imaging and the start of the mating trials was on average 140.3 ± 7.9 min (mean ± SE) and did not differ between the treatment groups (*t*-test: $t_{106} = 0.50, p = 0.640$).

Mating trials and quantification of mating behaviour

From day 36 to 41 we filmed the mating behaviour of pairs formed by joining individuals that originated from the social group size treatments described above. In particular, these mating pairs were either composed of two individuals selected randomly from two different pairs (hereinafter called 'Ps') or from two different octets (hereinafter called 'Os'). Consequently, we offered to all individuals one mating partner that had experienced the same social group size before, but which came from a different replicate. By using only one worm from each replicate we assured that all mating pairs were completely independent.

We conducted mating trials in observation chambers as described in detail elsewhere (Schärer et al. 2004). We placed two worms in a drop of 6 µl artificial seawater (salinity of 32 ‰) between two microscope slides. In these drops worms seem to behave similarly as they do in mass cultures but are somewhat restricted into two dimensions, which allows a better observation and quantification of the mating behaviour. Mating trials lasted 2 hours during which no food was provided.

In total we assembled 17 chambers each with 2 or 4 mating pairs. For each chamber we balanced the number of treatments (i.e. 10 chambers with 2 mating pairs of each treatment and 7 chambers with 1 mating pair of each treatment) and we alternated the positions of the drops within the chamber to avoid any position effects.

We filmed each chamber for 2 hours at 1 frame s^{-1} using a digital video camera (DFK 31BF03, The Imaging Source Europe GmbH, Bremen, Germany) and recorded movies in QuickTime format using BTV Pro 5.4.1. (http://www.bensoftware.com/). Movie capture started within 5 min after chamber assembly. We scored mating movies using BTV Pro 6.0b1. By manual frame-by-frame analysis of the QuickTime movies we assessed for the entire observation period of 2 hours the following parameters: the number of copulations, the average copulation duration and the number of sucks.

Statistical analyses

Of 54 mating pairs that we filmed 7 were excluded from the analyses. Specifically, in three cases one individual of the mating pair lacked the seminal vesicle or the male copulatory organ (a condition that can be found rarely but regularly in lab cultures but also in field caught worms), two replicates were excluded because of pipetting errors, one because one worm was injured, and one because one worm was not mature. The final data set included 47 mating pairs, i.e. 24 Ps and 23 Os. For both individuals within each mating pair we measured all morphological traits except for one individual for which it was not possible to get accurate photographs of its ovaries.

To test the effect of social group size on morphological traits we used General Linear Models with social group size as a fixed factor and body size as a covariate since gonad size is usually positively correlated with body size in *M. lignano* (e.g. Schärer and Ladurner 2003). As a combined measure of the resource allocation towards the male vs. the female function we defined sex allocation as testis size divided by the sum of testis and ovary size (Vizoso and Schärer 2007). Sex allocation thus represents a relative measure that allows comparing the resource allocation between individuals. However, it does not represent an absolute measure of the sex allocation since it does not account for potential differences in the energy demand per unit of testicular and ovarian tissue (for details see Schärer 2009). By definition, individuals with a relatively high sex allocation are considered more male-biased. Statistical comparisons of behavioural parameters were done using two-sample *t*-tests. Copulation duration refers to the mean value over all copulations recorded within a mating pair. All statistics were carried out using SPSS 13.0 (SPSS Inc., Chicago, IL, USA) or JMP 7.0.1. (SAS Institute Inc., Cary, NC, USA). Values are given as means ± SE unless otherwise stated.

Results

Exposing worms to different social group sizes had no effect on body size (Welch's *t*-test: $t_{84.6} = 0.72$, p = 0.473; Figure 1a; electronic supplementary material Table S1), which suggests that worms grew equally well in both treatments. Overall, body size was positively related to ovary size and showed a strong trend to predict variation in testis size (Table 1). However, there was no relationship between body size and measures of sex allocation and seminal vesicle size (Table 1).

Table 1. General linear models testing the effect of the social group size and body size on morphological parameters. Residuals of all models did not deviate significantly from a normal distribution (Kolmogorov-Smirnov, all p > 0.05).

morphological parameter	model fit	factor: social group size			covariate: body size		
	r^2	*F*	*d.f.*	*P*	*F*	*d.f.*	*P*
testis size	0.15	13.7	1,91	<0.001	3.8	1,91	0.053
ovary size	0.16	9.7	1,90	0.002	6.1	1,90	0.016
sex allocation	0.27	33.9	1,90	<0.001	0.7	1,90	0.407
seminal vesicle size	0.14	14.3	1,91	<0.001	0.4	1,91	0.514

Table 2. Comparison of the mating behaviour between worms that originated from pairs (more female-biased individuals) and worms that originated from octets (more male-biased individuals). Statistics refer to Student's *t*-tests. For the comparison of copulation number the Welch's *t*-test was used since variances were not equal.

behavioural parameter	pair			octet					
	mean ± SE	min	max	mean ± SE	min	max	*t*	*d.f.*	*P*
copulation number	6.7 ± 1.2	0	27	15.5± 2.2	0	44	-3.47	34.3	0.001
copulation duration (sec)	12.1± 0.5	8.8	17.1	9.8 ± 0.5	7.0	14.4	3.43	40	0.001
sucks per copulation	0.9 ± 0.1	0.2	1.7	0.5 ± 0.1	0	1.5	2.62	40	0.013

Social group size had a strong effect on sex allocation. Worms that originated from octets had larger testes, smaller ovaries and consequently a higher sex allocation (Table 1 and S1; Figure 1). Worms kept in octets had 31.7% larger testes but 19.2% smaller ovaries compared to worms that were kept in pairs. Therefore, worms from octets were clearly more male-biased compared to those worms from pairs. In addition, the size of the seminal vesicle was smaller in individuals originating from octets (Table 1 and S1).

Out of the 47 mating pairs that we observed, five did not copulate during the mating trials (four Ps and one O). The number of sucks was highly correlated with the number of copulations (Spearman, $rho = 0.83$, $p < 0.001$, $n = 47$). Therefore, we used the number of sucks divided by the number of copulations as a relative measure of the frequency of the suck behaviour. The number of copulations, the average copulation duration and the relative number of sucks were not correlated among each other (Spearman; all $p > 0.1$, $n = 42$).

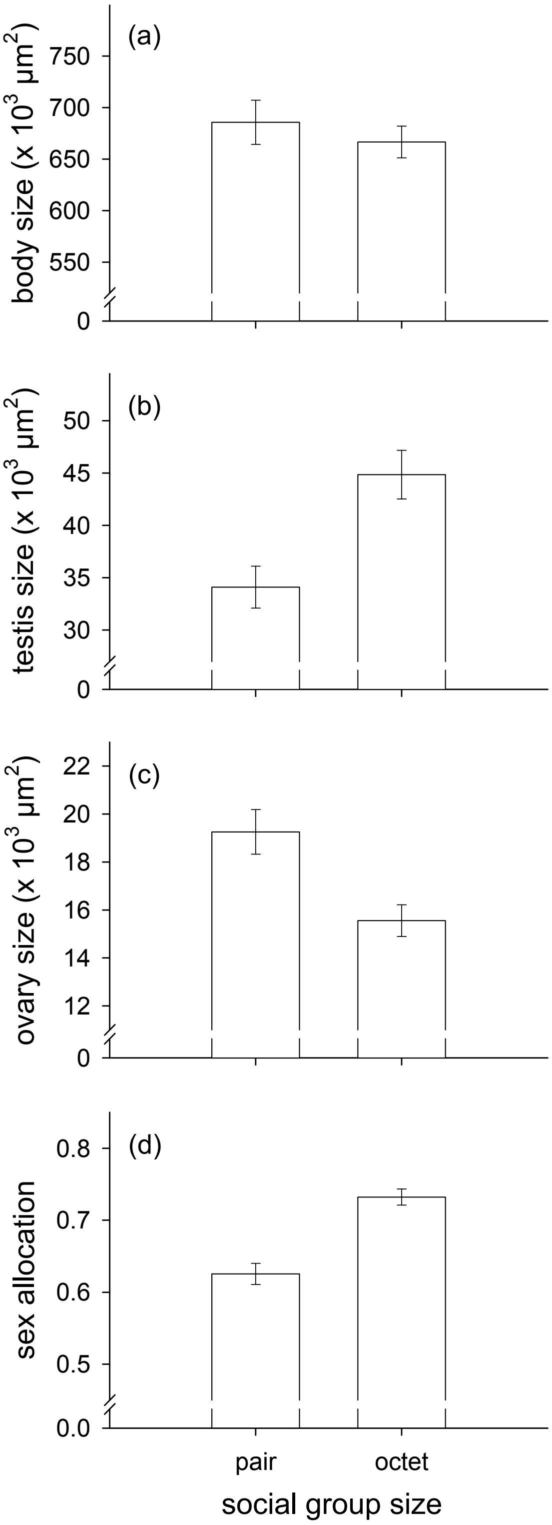

Figure 1. Effect of social group size on sex allocation in *Macrostomum lignano*. Differences in (a) body size, (b) testis size, (c) ovary size and (d) sex allocation are shown for individuals that were raised in pairs and octets. Sex allocation refers to testis size divided by the sum of testis size and ovary size. Bars indicate means ± SE.

Individuals that differed in their original social environment and consequently in their sex allocation behaved differently with respect to all behavioural parameters we tested. In particular, Os (more male-biased worms) copulated more than twice as often compared to Ps (more female-biased worms; Table 2; Figure 2a). Even when excluding mating pairs that did not copulate at all (*n* = 5) this difference remained significant (number of copulations; Ps: 8.1 ± 1.8, Os: 16.2 ± 1.8; Welch's *t*-test: $t_{33.2} = -3.21$, $p = 0.003$).

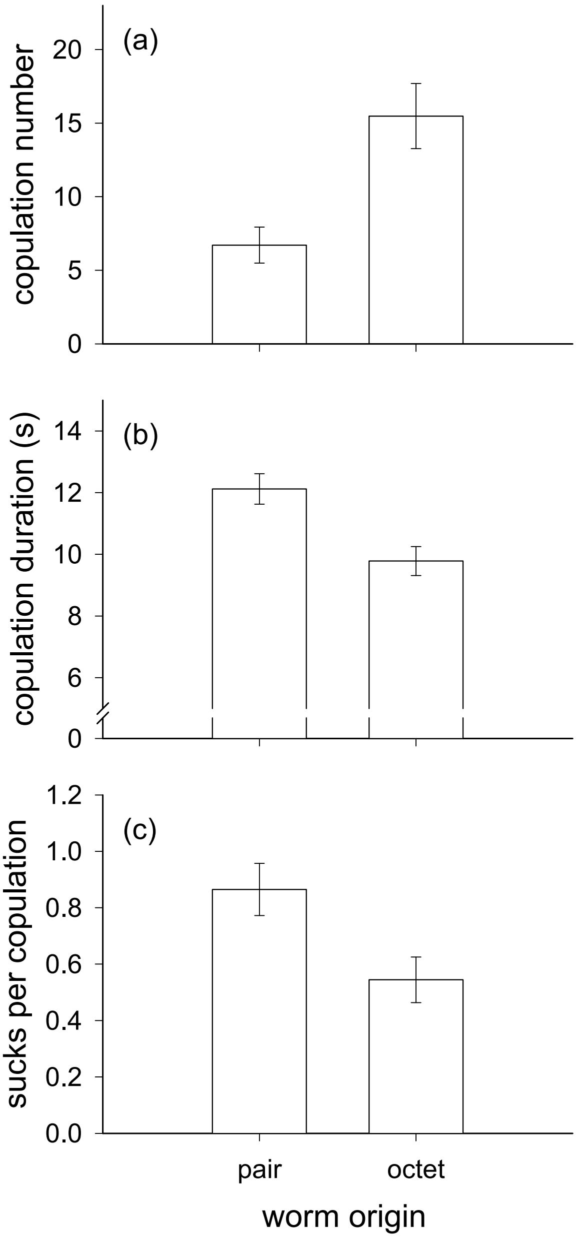

Figure 2. Comparison of mating behaviour between more female-biased pairs (both individuals originated from pairs) and more male-biased pairs (both individuals originated from octets). Means for (a) copulation number, (b) the average copulation duration and (c) the relative number of sucks (i.e. corrected for the number of copulations) are depicted from mating trials that lasted 2 hours. Bars indicate means ± SE.

Overall, there was also a positive correlation between the average sex allocation of both mating partners and their copulation number (Spearman, *rho* = 0.36, *p* = 0.014, *n* = 47), but this relationship was not significant within both treatments (within pairs: Pearson, *r* = -0.18, *p* = 0.397, *n* = 24; within octets: Spearman, *rho* = 0.28, *p* = 0.193, *n* = 23).

There was also a difference in copulation duration. Copulations in Ps lasted longer than copulations in Os (Table 2; Figure 2b). Furthermore, we found an effect of social group size on the relative number of sucks, which was higher in Ps compared to Os (Table 2; Figure 2c).

Discussion

In this study we (a) confirmed that the social group size has an effect on the sex allocation in a simultaneous hermaphrodite, as predicted by sex allocation theory and (b) demonstrate for the first time that this manipulation leads to changes in the mating behaviour. We thus provide the first experimental evidence that more male-biased simultaneous hermaphrodites show an increased mating rate, which is consistent with the view that simultaneous hermaphrodites mate primarily in order to donate sperm (Charnov 1979). We discuss these two findings in turn.

Social group size and reproductive morphology

Worms that were raised in octets had larger testes and smaller ovaries compared to individuals that were raised in pairs. This finding confirms earlier studies on *M. lignano* and other simultaneously hermaphroditic animals showing that individuals in larger social groups invest relatively more reproductive resources into the male function (reviewed in Schärer 2009) as predicted by sex allocation theory (Charnov 1982). More importantly, our data represent one of the clearest examples for a trade-off between the reproductive investment to the male versus female function, which is a fundamental (Charnov 1982), but poorly supported, assumption of sex allocation theory for simultaneous hermaphrodites (reviewed in Schärer 2009). A previous study also found a trade-off in sex allocation in *M. lignano*, but there the trade-off was only visible under specific conditions and the sample size was relatively small (Schärer et al. 2005). We are aware of only two other empirical studies that also support the existence of a trade-off between male and female allocation in simultaneous hermaphrodites (De Visser et al. 1994; Yund et al. 1997).

In our experiment social group size had no significant effect on body size so that the final test of the effect of social group size on mating behaviour is not confounded by differences in body size. However, there was a difference in seminal vesicle size between the two treatments. Worms from octets had smaller seminal vesicles compared to worms from pairs, which corresponds to previous findings (Schärer and Ladurner 2003; Brauer et al. 2007). Given that the size of the seminal vesicle correlates with the number of sperm it contains in *M. lignano* (Schärer and

Vizoso 2007), individuals in octets presumably transferred more sperm and had consequently smaller seminal vesicles than individuals that were raised in pairs.

Sex allocation and mating behaviour

The main aim of this study was to test whether sex allocation has an effect on the mating rate. Our results show that individuals that differ in their sex allocation behave differently. Pairs formed by two individuals that had a more male-biased sex allocation copulated more than twice as much compared to pairs formed by more female-biased partners. This suggests that the sex allocation of a simultaneous hermaphrodite can be a strong predictor of the mating strategy an individual adopts. Our result corresponds to a previous study in *M. lignano* that showed that testis size is positively correlated with the number of mating partners at a given group size (Janicke and Schärer 2009). Similar to the conventional gender roles in gonochorists, it appears that more male-biased individuals are relatively more eager to mate whereas more female-biased individuals are more reluctant. Therefore, we speculate that an elevated mating rate is more beneficial to the male than to the female function.

Since we did not manipulate sex allocation directly, it is possible that factors other than sex allocation could have caused the observed effects on mating rate. First, there might have been carry-over effects in our experimental setup. In particular, worms from octets may have copulated more frequently in the mating trials because they were used to mate more often due to higher encounter rates and/or higher levels of sperm competition in their originally larger social groups. This could explain why we failed to show a positive correlation between sex allocation and mating rate within both treatments. However, this lack could also be due to a relatively low variation in sex allocation within the treatments, which makes it more difficult to detect such a correlation. Furthermore, a previous study on the effect of social group size on sex allocation in *M. lignano* showed that the size of the seminal vesicle decreases within 24 hours after transferring a worm from a pair into an octet (Brauer et al. 2007). The authors interpreted this result as a direct adjustment of the mating rate to different social group sizes. However, the observed decrease in the seminal vesicle size in octets does not necessarily have to reflect a change in the mating rate but could also be caused by an increased sperm allocation per mating as predicted by sperm competition theory (for review see Wedell et al. 2002).

Second, raising worms in different social group sizes also had an effect on the size of the seminal vesicle and therefore the amount of sperm that was available during mating (Schärer and Vizoso 2007). Assuming that the number of available sperm affected the mating rate, it would be more intuitive to predict that individuals with larger sperm reserves copulate more frequently. However, our results indicate the opposite, namely that the worms with less sperm to allocate were more eager to mate. Therefore, it seems unlikely that the size of the seminal vesicle can explain the higher mating rate in worms from octets that we observed in our experiment.

Beside the effect on mating rate, we also observed a difference in copulation duration among our treatments. Pairs of more male-biased worms copulated shorter compared to more female-biased pairs. For many species it has been shown that copulation duration correlates with the number of transferred sperm (e.g. Engqvist and Sauer 2003) and this is also the case in *M. lignano* (P. Sandner, personal communication). Therefore, we suppose that the reduced copulation duration we found was an effect of the smaller seminal vesicle size of more male-biased individuals rather than a consequence of an increased sex allocation *per se*.

Additionally, individuals that originated from pairs and octets also differed in the relative number of the sucks. Interestingly, worms that were raised in pairs that copulated with a new partner sucked more often (relative to the number of copulations) compared to worms that were raised in octets that also mated with a new partner. On the assumption that the suck behaviour is a form of cryptic female choice (see introduction) and that Bateman's principle applies, one would expect worms that invest more into the female function to be choosier and therefore suck more often. This corresponds exactly to the pattern we have found. However, worms from pairs also had larger seminal vesicles and might have received more sperm from their mate during the mating trials. Therefore, individuals in pairs may have sucked more frequently only to remove surplus ejaculate without discriminating among sperm donors.

To conclude, our study demonstrates that the sex allocation might help to explain inter-individual variation in mating strategies of simultaneous hermaphrodites. We show that individuals with a more male-biased sex allocation copulate more frequently, which corresponds to conventional gender roles in separate-sexed animals. There is a clear need for more empirical and theoretical studies investigating how sex allocation affects the mating strategies and gender roles of simultaneous hermaphrodites. Particularly the causal relationship between sex allocation and mating rate requires further testing. Future experiments might investigate existing variation in sex allocation among individuals that were exposed to the same conditions to test whether sex allocation correlates with various aspects of the mating behaviour.

Acknowledgements

We thank Nils Anthes, Ralph Dobler, Peter Sandner and two anonymous referees for constructive comments on an earlier version of the manuscript. Jürgen Hottinger, Viktor Mislin and Urs Stiefel kindly provided technical support. This project was funded by the Swiss National Science Foundation (3100A0-113708).

References

Andersen, R. A., J. A. Berges, P. J. Harrison, and M. M. Watanabe. 2005. Recipes for freshwater and seawater media. Pp. 429-538 *in* R. A. Andersen, ed. Algal Culturing Techniques. Elsevier, Amsterdam.

Angeloni, L., J. W. Bradbury, and E. L. Charnov. 2002. Body size and sex allocation in simultaneously hermaphroditic animals. Behav. Ecol. 13:419-426.

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

Arnqvist, G., and L. Rowe. 2005. Sexual Conflict. Princeton University Press, Princeton, NJ, USA.

Bateman, A. J. 1948. Intra-sexual selection in *Drosophila*. Heredity 2:349-368.

Brauer, V. S., L. Schärer, and N. K. Michiels. 2007. Phenotypically flexible sex allocation in a simultaneous hermaphrodite. Evolution 61:216-222.

Brown, G. R., K. N. Laland, and M. Borgerhoff Mulder. 2009. Bateman's principles and human sex roles. Trends Ecol. Evol. 24:297-304.

Charnov, E. L. 1979. Simultaneous hermaphroditism and sexual selection. Proc. Natl. Acad. Sci. U. S. A. 76:2480-2484.

Charnov, E. L. 1982. The Theory of Sex Allocation. Princeton University Press, Princeton, NJ, USA.

Darwin, C. R. 1871. The Descent of Man, and Selection in Relation to Sex. John Murray, London.

De Visser, J. A. G. M., A. Termaat, and C. Zonneveld. 1994. Energy budgets and reproductive allocation in the simultaneous hermaphrodite pond snail, *Lymnaea stagnalis* (L) - a trade-off between male and female function. Am. Nat. 144:861-867.

Engqvist, L., and K. P. Sauer. 2003. Determinants of sperm transfer in the scorpionfly *Panorpa cognata*: male variation, female condition and copulation duration. J. Evol. Biol. 16:1196-1204.

Gianguzza, P., F. Badalamenti, K. R. Jensen, R. Chemello, S. Cannicci, and S. Riggio. 2004. Body size and mating strategies in the simultaneous hermaphrodite *Oxynoe olivacea* (Mollusca, Opisthobranchia, Sacoglossa). Funct. Ecol. 18:899-906.

Houston, A. I., and J. M. McNamara. 2002. A self-consistent approach to paternity and parental effort. Philos. Trans. R. Soc. B-Biol. Sci. 357:351-362.

Janicke, T., and L. Schärer. 2009. Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite. J. Evol. Biol. 22:405-415.

Jones, A. G. 2009. On the opportunity for sexual selection, the Bateman gradient and the maximum intensity of sexual selection. Evolution 63:1673-1684.

Jones, A. G., J. R. Arguello, and S. J. Arnold. 2002. Validation of Bateman's principles: a genetic study of sexual selection and mating patterns in the rough-skinned newt. Proc. R. Soc. B-Biol. Sci. 269:2533-2539.

Jones, A. G., G. Rosenqvist, A. Berglund, S. J. Arnold, and J. C. Avise. 2000. The Bateman gradient and the cause of sexual selection in a sex-role-reversed pipefish. Proc. R. Soc. B-Biol. Sci. 267:677-680.

Ladurner, P., L. Schärer, W. Salvenmoser, and R. M. Rieger. 2005. A new model organism among the lower Bilateria and the use of digital microscopy in taxonomy of meiobenthic Platyhelminthes: *Macrostomum lignano*, n. sp. (Rhabditophora, Macrostomorpha). J. Zool. Syst. Evol. Res. 43:114-126.

Leonard, J. L. 2005. Bateman's principle and simultaneous hermaphrodites: a paradox. Integr. Comp. Biol. 45:856-873.

Michiels, N. K. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. Pp. 219-254 *in* T. Birkhead, and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, London.

Norton, C. G., A. F. Johnson, and R. L. Mueller. 2008. Relative size influences gender role in the freshwater hermaphroditic snail, *Helisoma trivolvis*. Behav. Ecol. 19:1122-1127.

Ohbayashi-Hodoki, K., F. Ishihama, and M. Shimada. 2004. Body size-dependent gender role in a simultaneous hermaphrodite freshwater snail, *Physa acuta*. Behav. Ecol. 15:976-981.

Schärer, L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. Evolution 63:1377-1405.

Schärer, L., and T. Janicke. 2009. Sex allocation and sexual conflict in simultaneously hermaphroditic animals. Biol. Lett. 5:705-708.

Schärer, L., G. Joss, and P. Sandner. 2004. Mating behaviour of the marine turbellarian *Macrostomum* sp.: these worms suck. Mar. Biol. 145:373-380.

Schärer, L., and P. Ladurner. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 270:935-941.

Schärer, L., P. Sandner, and N. K. Michiels. 2005. Trade-off between male and female allocation in the simultaneously hermaphroditic flatworm *Macrostomum* sp. J. Evol. Biol. 18:396-404.

Schärer, L., and D. B. Vizoso. 2007. Phenotypic plasticity in sperm production rate: there's more to it than testis size. Evol. Ecol. 21:295-306

Snyder, B. F., and P. A. Gowaty. 2007. A reappraisal of Bateman's classic study of intrasexual selection. Evolution 61:2457-2468.

St. Mary, C. M. 1994. Sex allocation in a simultaneous hermaphrodite, the Blue-Banded Goby (*Lythrypnus dalli*) - the effect of body size and behavioral gender and the consequences for reproduction. Behav. Ecol. 5:304-313.

Sutherland, W. J. 1985. Chance can produce a sex difference in variance in mating success and explain Bateman's data. Anim. Behav. 33:1349-1352.

Thornhill, R. 1983. Cryptic female choice and its implications in the scorpionfly *Harpobittacus nigriceps*. Am. Nat. 122:765-788.

Vizoso, D. B., and L. Schärer. 2007. Resource-dependent sex allocation in a simultaneous hermaphrodite. J. Evol. Biol. 20:1046-1055.

Wedell, N., M. J. G. Gage, and G. A. Parker. 2002. Sperm competition, male prudence and sperm-limited females. Trends Ecol. Evol. 17:313-320.

Yund, P. O., Y. Marcum, and J. Stewart-Savage. 1997. Life-history variation in a colonial ascidian: broad-sense heritabilities and tradeoffs in allocation to asexual growth and male and female reproduction. Biol. Bull. 192:290-299.

Electronic Supplementary Material

Sex allocation predicts mating rate in a simultaneous hermaphrodite

Tim Janicke and Lukas Schärer

Table S1. Morphological comparison between worms that were raised in different social group sizes, i.e. in pairs and in octets. Sex allocation was defined as testis size divided by the sum of testis and ovary size. Note that for one individual the ovary size could not be measured.

morphological parameter	pair (n = 48)				octet (n = 46)			
	mean ±	SD	min	max	mean ±	SD	min	max
body size (x $10^3 \mu m^2$)	685.7 ±	148.6	325.1	975.0	666.7 ±	104.9	400.1	975.1
testis size (x $10^3 \mu m^2$)	34.1 ±	13.9	6.3	81.6	44.9 ±	15.8	19.7	99.1
ovary size (x $10^3 \mu m^2$)	19.3 ±	6.0	10.1	35.5	15.6 ±	4.5	8.1	23.7
sex allocation	0.63 ±	0.10	0.37	0.86	0.73 ±	0.08	0.57	0.89
seminal vesicle size (x $10^3 \mu m^2$)	8.4 ±	4.9	2.6	27.8	5.4 ±	2.1	2.0	13.0

CHAPTER VII

General Discussion

Synthesis

In my PhD project, I studied pre- and post-copulatory sexual selection in the flatworm *Macrostomum lignano* with a special focus on the conditions that are predicted to affect the sex allocation in simultaneous hermaphrodites.

In chapter II, I demonstrated how social group size translates into mating group size and identified, to my knowledge, for the first time factors that determine mating group size and sperm transfer success in a simultaneously hermaphroditic animal. The data of this study revealed substantial variation in the number of mating partners between individuals and indicated that mating group size can be relatively high in large social groups. High levels of multiple mating have also been found in several other simultaneous hermaphrodites (e.g., Angeloni et al. 2003; Pongratz and Michiels 2003; Kupfernagel et al. 2010). Consequently, there is accumulating empirical evidence, which violates the central prediction of sex allocation theory stating that simultaneous hermaphroditism is only an evolutionary stable strategy if the mating group size is small (Charnov 1982). The same study also provided novel insights into the determinants of mating group size and sperm transfer success. I found that individuals with relatively larger testes acquired more mating partners and achieved a higher sperm transfer success. This suggests that a relatively more male-biased sex allocation translates into a higher male reproductive success in *M. lignano*, and therefore represents one of the few available empirical examples for the link between the resource allocation towards the male sex function and the male fitness gain in simultaneous hermaphrodites (e.g., Yund and McCartney 1994; Yund 1998). Apart from this, I found that the morphology of the male copulatory organ was correlated with the sperm transfer success, which has been measured as the number of stored sperm per mate and in total within a social group. To my knowledge, this is the first documented evidence, that the shape of the male genitalia is a fitness related trait in a simultaneous hermaphrodite (for an effect of the size of the male copulatory organ see Garefalaki et al. 2010). The mechanism of how genital morphology affects sperm transfer success and how it translates into paternity in *M. lignano* is unclear and remains to be addressed in future studies.

Despite the overall high level of multiple mating, the mating group size in the study reported in chapter II was on average considerably smaller than the social group size, which suggests that there are processes that limit the number of mating partners of an individual. Therefore, I was interested in whether pre-copulatory mate choice has the potential to restrict the number of mating partners in this species. The results of the study presented in chapter III indicate that worms discriminate between partners based on their feeding status. Worms appear to have a preference to mate with more well-fed worms compared to starved partners, probably because well-fed individuals are more fecund in their female sex function. Since the feeding status covaried with the body size of the potential mates, mating decisions in *M. lignano* potentially rely on the assessment of body size as a cue for female fecundity, which has been shown previously for other simultaneous hermaphrodites (e.g., Vreys and Michiels 1997; Ohbayashi-Hodoki et al. 2004; Anthes et al. 2006). Owing to the fact that copulations are always reciprocal (Schärer et al. 2004), I expect that there is size-assortative mating in *M. lignano*, because large individuals are more likely to mate with similar-sized individuals leaving only smaller individuals to mate with each other (Michiels 1998). This might also explain, why I did not find a positive correlation between body size and the number of mating partners in the study presented in chapter II. In contrast to the preference to mate with well-fed worms, I found no evidence for a strategic sperm allocation in response to the feeding status of the mate. This suggests that *M. lignano* seems to be unable to adjust its sperm expenditure prudently although these worms apparently possess the sensory capabilities to assess the female fecundity of their mates.

A previous study on phenotypic plasticity in sperm production rate in *M. lignano* hypothesised that individuals might also adjust the sperm size in response to varying levels of sperm competition. In chapter IV, I present an experimental test of this hypothesis, which provided no support for phenotypic plasticity in sperm morphology in *M. lignano*. Although my manipulation of the sperm competition level induced a phenotypically plastic response in sex allocation, there was no difference in the sperm morphology between individuals that experienced either no or intense sperm competition. A power analysis confirmed that I was able to detect relatively small differences in sperm length with the experimental setup employed (compared to the substantial variation found between individuals). Hence, I am confident that there is little scope for substantial phenotypic plasticity in sperm length in response to sperm competition in *M. lignano*. Nevertheless, there might be phenotypically plastic responses to different levels of sperm competition in other traits that also incur varying costs for the male sex function, such as the speed of spermatogenesis, the production of seminal fluids and/or the size of the male copulatory organ. Studying phenotypic plasticity in these traits would not only widen our view on adaptive responses to sperm competition but would also help to get more accurate measurements of the total resource allocation to the male sex function, which is essential for testing sex allocation theory in simultaneous hermaphrodites (Schärer 2009).

In my opinion one of the most important issues that needs to be addressed in order understand sexual selection in simultaneous hermaphrodites concerns the validity of Bateman's principle, i.e. the question of which sex function is under stronger sexual selection (Charnov 1979; Leonard 2005, 2006; Anthes 2010). In my PhD project, I tested one of the predictions of Bateman's principle stating that the reproductive success of the female sex function is primarily constrained by the resources available for egg production rather than the number of mating partners. The results presented in chapter V support this prediction. I could demonstrate that the food availability has a strong effect on female fecundity whereas social group size, which I showed in chapter II is correlated positively with the number of mating partners, did not affect the number of offspring produced by the female sex function. Certainly, this study does not represent a complete test of Bateman's principle since it only comprises reproductive data of the female sex function and female fecundity was exclusively measured as the number of offspring produced (without regard of offspring quality). But still, the study suggests that polyandry is not particularly beneficial to the female sex function. Therefore, the relatively high level of multiple mating reported in chapter II and the high mating rates usually observed in *M. lignano* (Schärer et al. 2004) do not seem to be primarily driven by the female sex function but to be the outcome of an interest to mate multiply in the male sex role. Nevertheless, the absence of an effect of multiple mating on female fecundity needs to be confirmed by additional studies. Further work should, for example, aim at disentangling effects of mating with multiple partners from effects of repeated matings with the same partner. Finally, the most challenging task for future studies on this topic will be to establish more complete estimates of female fitness, such as offspring size, offspring growth and offspring's reproductive success under more natural conditions.

In the study presented in chapter II, I found a positive correlation between testis size and the number of mating partners and I speculated that more male-biased individuals copulate more frequently in *M. lignano*. In chapter VI, I formulate the hypothesis that the sex allocation has an effect on the mating behaviour in simultaneously hermaphroditic animals. This hypothesis predicts that individuals adopt a mating strategy that is most beneficial to the sex function that is relatively more pronounced compared to other individuals in a population. In particular, if Bateman's principle applies to simultaneous hermaphrodites, more male-biased individuals are expected to copulate more frequently. To provide an experimental test of my hypothesis, I compared the mating behaviour of individuals that differed in their sex allocation. In accordance to what was expected if Bateman's principle holds in *M. lignano*, I could demonstrate that more male-biased individuals copulate more frequently compared to more female-biased individuals. However, since sex allocation in this experiment was manipulated indirectly via raising the worms in different group sizes, this study does not provide an unequivocal experimental proof of the tested hypothesis. Until now, there is no method available, which allows to manipulate directly the sex allocation in *M. lignano*, but the RNAi knock-down approach of sex-specific genes provides a promising route for further research on the link between sex allocation and mating behaviour in simultaneous hermaphrodites.

To summarise, my studies revealed a high potential for pre- and post-copulatory sexual selection to operate in the simultaneously hermaphroditic flatworm *M. lignano*. Despite a preference for mating with larger individuals, I found that the mating group size can be high, which probably leads to intense sperm competition in this species. Furthermore, I could show that worms are capable of responding to high levels of sperm competition by increasing their testis size at the cost of the ovary size, but I found no evidence for a phenotypically plastic adjustment of sperm morphology. Moreover, in accordance with Bateman's principle, the level of polyandry does not seem to have an effect on the reproductive output of the female sex function. Finally, I proposed that sex allocation has an effect on the mating strategy in simultaneous hermaphrodites and provided the first experimental support for this hypothesis by showing that more male-biased individuals mate more frequently compared to more female-biased individuals.

The main scientific contributions of my thesis are the identification of the determinants of mating group size and sperm transfer success in *M. lignano*, and the proposal of a new hypothesis on the link between sex allocation and the mating behaviour in simultaneous hermaphrodites. Thereby, my studies not only provide novel insights into sexual selection in the model organism *M. lignano* and simultaneous hermaphrodites in general, but also lay the foundation for a new direction in the research on mating behaviour in simultaneously hermaphroditic animals.

Outlook

Sex allocation theory predicts simultaneous hermaphroditism to be an evolutionary stable strategy only if the fitness gain curve of at least one sex function saturates (Charnov 1982). Originally, it has been argued that high selfing rates and small mating group sizes induce a saturating male fitness gain curve and consequently stabilise simultaneous hermaphroditism as a reproductive strategy. However, my studies and other recent research suggest that mating group size can be high in outcrossing simultaneous hermaphrodites (reviewed in Michiels 1998, 1999; Leonard 2006; Anthes 2010). Even though pre-copulatory mate choice potentially limits the mating group size in *M. lignano* and other outcrossing simultaneous hermaphrodites (e.g., Haase and Karlsson 2004; Ohbayashi-Hodoki et al. 2004; Anthes et al. 2006; Schjørring and Jäger 2007), it may not be stringent enough to evoke a strongly saturating male fitness gain curves. Thus, in my opinion one of the most evident challenges of future research on sexual selection in simultaneous hermaphrodites will be to resolve this apparent discrepancy between sex allocation theory and empirical data.

First of all, there is an urgent need for more empirical data describing the shape of the male and the female fitness gain curves. To date, our knowledge of male fitness gain curves is mainly restricted to two species of sessile, spermcast mating species, i.e. the ascidian *Botryllus schlosseri* and the bryozoan *Celleporella hyalina* (Yund and McCartney 1994; McCartney 1997; Yund 1998; Johnson and Yund 2009). Hence, it would be of great interest to have also some information on the fitness gain curves of mobile, outcrossing and copulating species. Preferably, one would like to assess the fitness gain curves at first in the field (covering the natural range of variation in density), before one starts to study how certain environmental factors affect the shape of these curves under more controlled conditions.

Having demonstrated that the male fitness gain curve of a given species saturates and that the female fitness gain curve is more linear, one can go further and study the underlying mechanisms that cause diminishing fitness returns for any additional resources invested into the male sex function. Apart from selfing and small mating group sizes, other mechanisms have been proposed to generate intense local sperm competition and thereby promote a saturating male fitness gain curve (reviewed in Schärer 2009). Specifically, skews in paternity success are thought to decelerate the fitness gain of an increasing resource allocation into the male sex function (Greeff et al. 2001). Here one can distinguish between random and non-random paternity skews. Random paternity skews refer to biases in siring success due to stochastic, non-heritable effects, such as imperfect mixing of sperm from different donors, which is especially likely to occur in spermcast mating organisms. For instance, paternity analysis in a simultaneously hermaphroditic plant species revealed that such random skews can lead to an intense local sperm competition despite a high number of pollen donors (Greeff et al. 2001). To my knowledge, the prevalence of random paternity skews in simultaneously hermaphroditic animals has not been studied so far, but I expect sessile, marine spermcast mating species to be especially prone to show random paternity skews, since sperm dispersal in these organisms is likely to depend on the strength and the direction of the water current.

Non-random paternity skews can be caused by cryptic female choice and/or sperm precedence. Based on the little that is known about the occurrence of cryptic female choice in simultaneous hermaphrodites, it is not possible to evaluate its potential for inducing a saturating male fitness gain curve as predicted by theory (van Velzen et al. 2009). Therefore, experimental studies testing cryptic female choice in these organisms are clearly needed. Here, a combination of tools including paternity analysis, sperm tracking and artificial insemination will definitely help to overcome methodological problems in discerning cryptic female choice from sperm competition (Birkhead 1998).

In contrast to cryptic female choice, there is evidence for both first and second donor sperm precedence in several simultaneously hermaphroditic animals (outlined in chapter I). Although the advantage of being the first or the second sperm donor has usually been found to be moderate rather than extreme, sperm precedence is probably a potent mechanism that induces local sperm competition in some hermaphroditic species. In *M. lignano* the proportion of sperm that is stored by the second sperm donor in situations with one competitor has been found to be on average 0.68 (T. Janicke, M. Eichmann, L. Schärer; unpublished data). Similarly, a paternity analysis in *M. lignano* revealed a second mate sperm precedence with a proportion of offspring sired by the second sperm donor of 0.64 (Sandner et al. in prep.). Consequently, paternity skews due to sperm precedence may indeed account for a saturating male fitness gain curve in *M. lignano* and other simultaneously hermaphroditic animals.

Experimental evidence for a link between paternity skews due to sperm precedence and the shape of the male fitness gain curve will be very difficult to obtain, since this requires the manipulation of the sperm precedence pattern within a species experimentally (e.g., in the course of an experimental evolution experiment). Instead, I propose to test the association between paternity biases and the shape of the male fitness gain curve using a comparative approach. Ideally, one would like to correlate standardised P2-values (i.e., the paternity share of the second sperm donor measured as the deviation from a paternity share of 0.5, which corresponds to a fair raffle *sensu* Parker 1990) with the exponent of a power function, which provides the best fit for the relationship between male fitness and male allocation in a given species. Unfortunately, obtaining fitness gain curves for many species is very laborious and it will be difficult to conduct standardised experiments that allow for species comparison. Alternatively, one might explore the link between standardised P2 values and the naturally occurring sex allocation across species. Given that a more female-biased sex allocation is likely to rely on a saturating male fitness gain curve, I expect a positive correlation between female allocation and standardised P2 values.

To my knowledge, a positive correlation between the resource allocation to the female sex function and the standardised P2 values would represent the first empirical indication that paternity skews can affect sex allocation and hence the shape of the fitness gain curves in simultaneously hermaphroditic animals. The validity of such a phylogenetic correlation will mainly depend on whether the measurements of sex allocation allow for species comparison. As pointed out recently (Schärer 2009), the energetic costs needed to produce the same number of gametes or to maintain an equally sized gonadal tissue might vary substantially between species, which can make it questionable to use gamete production rate or gonad size as estimates of sex allocation. Therefore, a comparative study should focus on a taxonomic group of simultaneously hermaphroditic animals, in which many species show a high variation in the sex allocation and the sperm precedence pattern but similar modes of gamete production and a comparable complexity of the gonads. Here, the speciose group of the genus *Macrostomum* might provide a suitable taxon for testing the evolutionary link between sex allocation and paternity skews, because the measurement of sex allocation can be done in a standardized way and microsatellites required for estimating paternity success are available for several species of this genus (P. Sandner, unpublished data). However, to measure sex allocation in this genus entails difficulties that are associated with phenotypic plasticity in this trait, which has been tested and found within one single species so far (e.g., Schärer and Ladurner 2003; see also chapter IV and VI of this thesis). Potentially, this could make it challenging to obtain comparable measurements of sex allocation within this group.

In conclusion, I think that the future progress in the study of sexual selection in simultaneously hermaphroditic animals will primarily depend on two achievements. First, we need to explore if there are differences in the strength of sexual selection between the male and the female sex function and thereby test the validity of Bateman's principle for this group of organisms. Second, we need to determine the processes that generate a saturating fitness gain curve in one of the two sex functions, which is required for simultaneously hermaphroditism to be an evolutionary stable strategy. Flatworms of the genus *Macrostomum* probably represent some of the most suitable organisms that are currently available to address these two issues.

References

Angeloni, L., J. W. Bradbury, and R. S. Burton. 2003. Multiple mating, paternity, and body size in a simultaneous hermaphrodite, Aplysia californica. Behav. Ecol. 14:554-560.

Anthes, N. 2010. Mate choice and reproductive conflict in simultaneous hermaphrodites. Pp. 329-357 in P. Kappeler, ed. Animal Behaviour: Evolution and Mechanisms. Springer, Berlin.

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

Birkhead, T. R. 1998. Cryptic female choice: criteria for establishing female sperm choice. Evolution 52:1212-1218.

Charnov, E. L. 1979. Simultaneous hermaphroditism and sexual selection. Proc. Natl. Acad. Sci. U. S. A. 76:2480-2484.

Charnov, E. L. 1982. The Theory of Sex Allocation. Princeton University Press, Princeton, NJ, USA.

Garefalaki, M.-E., A. Triantafyllidis, T. J. Abatzopoulos, and A. Staikou. 2010. The outcome of sperm competition is affected by behavioural and anatomical reproductive traits in a simultaneously hermaphroditic land snail. J. Evol. Biol. 23:966-976.

Greeff, J. M., J. D. Nason, and S. G. Compton. 2001. Skewed paternity and sex allocation in hermaphroditic plants and animals. Proc. R. Soc. B-Biol. Sci. 268:2143-2147.

Haase, M., and A. Karlsson. 2004. Mate choice in a hermaphrodite: you won't score with a spermatophore. Anim. Behav. 67:287-291.

Johnson, S. L., and P. O. Yund. 2009. Effects of fertilization distance on male gain curves in a free-spawning marine invertebrate: a combined empirical and theoretical approach. Evolution 63:3114-3123.

Kupfernagel, S., H. P. Rusterholz, and B. Baur. 2010. Variation in multiple paternity and sperm utilization patterns in natural populations of a simultaneous hermaphrodite land snail. Biol. J. Linn. Soc. 99:350-361.

Leonard, J. L. 2005. Bateman's principle and simultaneous hermaphrodites: a paradox. Integr. Comp. Biol. 45:856-873.

Leonard, J. L. 2006. Sexual selection: lessons from hermaphrodite mating systems. Integr. Comp. Biol. 46:349-367.

McCartney, M. A. 1997. Sex allocation and male fitness gain in a colonial, hermaphroditic marine invertebrate. Evolution 51:127-140.

Michiels, N. K. 1998. Mating conflicts and sperm competition in simultaneous hermaphrodites. Pp. 219-254 in T. Birkhead, and A. P. Møller, eds. Sperm Competition and Sexual Selection. Academic Press, London.

Michiels, N. K. 1999. Sexual adaptations to high density in hermaphrodites. Invertebr. Reprod. Dev. 36:35-40.

Ohbayashi-Hodoki, K., F. Ishihama, and M. Shimada. 2004. Body size-dependent gender role in a simultaneous hermaphrodite freshwater snail, Physa acuta. Behav. Ecol. 15:976-981.

Parker, G. A. 1990. Sperm competition games - raffles and roles. Proc. R. Soc. B-Biol. Sci. 242:120-126.

Pongratz, N., and N. K. Michiels. 2003. High multiple paternity and low last-male sperm precedence in a hermaphroditic planarian flatworm: consequences for reciprocity patterns. Mol. Ecol. 12:1425-1433.

Sandner, P., D. B. Vizoso, T. Janicke, K. Sekii, and L. Schärer. in prep. Genetic variation in copulation frequency predicts paternity success in a flatworm.

Schärer, L. 2009. Tests of sex allocation theory in simultaneously hermaphroditic animals. Evolution 63:1377-1405.

Schärer, L., G. Joss, and P. Sandner. 2004. Mating behaviour of the marine turbellarian Macrostomum sp.: these worms suck. Mar. Biol. 145:373-380.

Schärer, L., and P. Ladurner. 2003. Phenotypically plastic adjustment of sex allocation in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 270:935-941.

Schjørring, S., and I. Jäger. 2007. Incestuous mate preference by a simultaneous hermaphrodite with strong inbreeding depression. Evolution 61:423-430.

van Velzen, E., L. Schärer, and I. Pen. 2009. The effect of cryptic female choice on sex allocation in simultaneous hermaphrodites. Proc. R. Soc. B-Biol. Sci. 276:3123-3131.

Vreys, C., and N. K. Michiels. 1997. Flatworms flatten to size up each other. Proc. R. Soc. B-Biol. Sci. 264:1559-1564.

Yund, P. O. 1998. The effect of sperm competition on male gain curves in a colonial marine invertebrate. Ecology 79:328-339.

Yund, P. O., and M. A. McCartney. 1994. Male reproductive success in sessile invertebrates - competition for fertilizations. Ecology 75:2151-2167.

Acknowledgements

The research presented in this thesis would not have been possible without the help of many people.

First and foremost, I want to express my sincere thank my supervisor Lukas Schärer for his continuous support and guidance during the project. He introduced me into the fascinating field of sexual selection in hermaphrodites and developed all the nice experimental tools for *Macrostomum lignano*, which made the work with these worms very enjoyable.

I am also indebted to Peter Sandner, Dita Vizoso, Kiyono Sekii and Lucas Marie-Orléach for the generous support and the stimulating atmosphere that I experienced within the Macrostomum Research Group.

Furthermore, I benefited a lot from discussions with and advices from Valentin Amrhein, Nils Anthes, Nayden Chakarov, Ralph Dobler, David Duneau, Dieter Ebert, Thomas Fabbro, Matthew Hall, Matthias Kölliker, Peter Ladurner, Jarkko Routtu, Willi Salvenmoser and Thomas Zumbrunn.

It has also been a real pleasure to work together with Micha Eichmann, Halil Kesselring, Delia Marrosu, Katja Stähli and Valentina Vrancini.

Nils Anthes, Ralph Dobler, Matthew Hall, Lucas Marie-Orléach, Steve Ramm, Jarkko Routtu and Peter Sandner kindly provided critical comments on parts of this thesis.

Gregor Schulte was indispensable on our joint field trip to Lignano-Sabbiodoro.

I would also like to thank Jürgen Hottinger, Viktor Mislin, Christine Parry, Yasmin Picton, Urs Stiefel and Lukas Zimmermann for their administrative and technical support throughout the years.

Moreover, I am grateful to Dieter Ebert for being the head of my thesis committee, and I wish to thank Joris Koene who kindly accepted to be the external referee of my thesis.

Last, but never least, I thank my parents Ute and Wolfgang for their support and reliance over the last years. And finally, I thank Katharina and Samuel for their love, patience and encouragement. Thank you.

The thesis project was funded by grants of the Swiss National Science Foundation to Lukas Schärer (3100A0-113708 and 3100A0-127503). During my thesis I was also financially supported by the 'Reisefonds' of the University of Basel and a 'Conference Grant' of the Association for the Study of Animal Behaviour.

Curriculum vitae

Name	Tim Janicke	
Born	25th January 1980 in Dresden, Germany	
Nationality	German	
Private Address	Im Proli 29, D-79639 Grenzach-Wyhlen	
Office Address	University of Basel, Zoological Institute, Vesalgasse 1, CH-4051 Basel	
Email	tim.janicke@unibas.ch	
Education	2007 - 2010	PhD supervised by Dr. Lukas Schärer, University of Basel
	2006	Diploma in ecology, genetics and zoology, University of Jena (Diploma thesis ‘Communication in brown skuas: Functions of multimodel signalling’ supervised by Dr. Hans-Ulrich Peter, University of Jena
	1999 - 2006	Studies in biology, University of Greifswald and University of Jena
	1997	Abitur, Fritz-Löffler Gymnasium, Dresden

Publications

Janicke, T., and L. Schärer. in press. Sperm competition affects sex allocation but not sperm morphology in a flatworm. Behav. Ecol. Sociobiol. (doi: 10.1007/s00265-010-0951-y).

Janicke, T., and L. Schärer. 2009. Sex allocation predicts mating rate in a simultaneous hermaphrodite. Proc. R. Soc. B-Biol. Sci. 276:4247-4253.

Janicke, T., and L. Schärer. 2009. Determinants of mating and sperm-transfer success in a simultaneous hermaphrodite. J. Evol. Biol. 22:405-415.

Schärer, L., and T. Janicke. 2009. Sex allocation and sexual conflict in simultaneously hermaphroditic animals. Biol. Lett. 5:705-708.

Janicke, T., and S. Hahn. 2008. On the performance of brown skua, *Catharacta antarctica lonnbergi*, vocalizations: reply. Anim. Behav. 76:E3-E5.

Janicke, T., S. Hahn, M. S. Ritz, and H.-U. Peter. 2008. Vocal performance reflects individual quality in a non-passerine. Anim. Behav. 75:91-98.

Janicke, T., M. S. Ritz, S. Hahn, and H.-U. Peter. 2007. Sex recognition in Brown Skuas: Do acoustic signals matter? Journal of Ornithology 148:565-569.

Janicke, T., and N. Chakarov. 2007. Effect of weather conditions on the communal roosting behaviour of common ravens *Corvus corax* with unlimited food resources. J. Ethol. 25:71-78.

Hahn, S., K. Reinhardt, M. S. Ritz, T. Janicke, D. Montalti, and H.-U. Peter. 2007. Oceanographic and climatic factors differentially affect reproduction performance of Antarctic skuas. Marine Ecology-Progress Series 334:287-297.

Ritz, M. S., S. Hahn, T. Janicke, and H.-U. Peter. 2006. Hybridisation between South Polar Skua (*Catharacta maccormicki*) and Brown Skua (*C. antarctica lonnbergi*) in the Antarctic Peninsula region. Polar Biology 29:153-159.

Conferences	2010	Simultaneously Hermaphroditic Animal Meeting, Amsterdam (talk)
	2009	Summer Meeting of the ASAB, Oxford, U.K. (talk)
	2009	12th Congress of the ESEB, Torino (poster)
	2008	2nd International Macrostomum Meeting, Basel (talk)
	2007	9th Biology of Spermatozoa Conference, Sheffield (poster)
	2007	1st International Macrostomum Meeting, Basel (talk)
	2005	138th Annual Meeting of the German Ornithologists' Society, Stuttgart (poster)
	2005	22nd International Polar Meeting of the DGP, Jena (poster)
	2004	137th Annual Meeting of the German Ornithologists' Society, Kiel (talk)
	2003	136th Annual Meeting of the German Ornithologists' Society, Halberstadt (poster)
	2003	4th Conference of the European Ornithologists' Union, Chemnitz (poster)
Invited talks	2010	Research Seminar, Department of Evolutionary Biology, University of Bielefeld
	2009	Research Seminar, Department of Evolutionary Ecology, University of Neuchâtel
Grants/Awards	2010	Emilia Guggenheim-Schnurr-Stiftung
	2005	Art Award of the Rector of the University of Jena
	1999	'Bundesumweltwettbewerb', Ministry of Education and Research of Germany
	1998	'Jugend forscht', Ministry of Education and Research of Germany
Memberships	since 2009	Association for the Study of Animal Behaviour (ASAB)
	since 2009	European Society for Evolutionary Biology (ESEB)
	since 2003	European Ornithologists' Union
	since 2002	German Ornithologists' Society
	since 2000	German Society for Orthopterology
	since 1995	Verein Sächsischer Ornithologen
External Reviewer		Animal Behaviour, Auk, Biological Journal of the Linnean Society, Behavioral Ecology and Sociobiology, Journal of Ethology

List of lecturers that were involved in my training and education at the University of Basel

Prof. Dr. Dieter Ebert
Dr. Thomas Fabbro
Prof. Dr. Mathias Kölliker
Prof. Dr. Walter Salzburger
Dr. Lukas Schärer