Sexual Selection and Sex Allocation in a Simultaneous Hermaphrodite: Examining Phenotypic and Genetic Influences

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Nikolas Vellnow

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Fakultätsverantwortlicher:	Prof. Dr. Dieter Ebert, Basel
Dissertationsleiter:	PD Dr. Lukas Schärer, Basel
Korreferent:	Dr. Nils Anthes, Tübingen

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Prof. Dr. Martin Spiess, Dekan

Sexual Selection and Sex Allocation in a Simultaneous Hermaphrodite: Examining Phenotypic and Genetic Influences

Nikolas Vellnow – PhD Thesis

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Abstract

The evolution of anisogamy resulted in a cascade of unique phenomena and evolutionary consequences. Among those phenomena are sexual selection, the problem of sex allocation and genomic conflict over sex allocation. During my PhD project I studied aspects of these evolutionary consequences of anisogamy using the reciprocally copulating, simultaneously hermaphroditic flatworm *Macrostomum lignano*.

Sexual reproduction, especially when it involves reciprocal copulation and internal fertilization, requires close interactions of at least two mating partners. In **Chapter 2** I report an experiment testing for effects of sperm donor genotype by sperm recipient genotype interactions on i) mating behaviors and ii) pre- as well as postcopulatory fitness components. Two mating behaviors, but not the pre- and postcopulatory fitness components were affected by such genotype-by-genotype interactions, while almost all variables were influenced by the genotype of the donor. The sperm donor by sperm recipient genotype interactions on mating behaviors reveal that there is genetic variation for both sexual selection and selection arising from sexual conflict to act on during this precopulatory stage. The lack of these interaction effects on the pre- and postcopulatory fitness components could indicate that sexual conflict and sexual selection is shifted towards later stages, namely the stage between sperm storage in the recipient, and the fertilization of eggs. This conclusion may not only hold for *M. lignano* but possibly also more generally for other reciprocally copulating hermaphrodites.

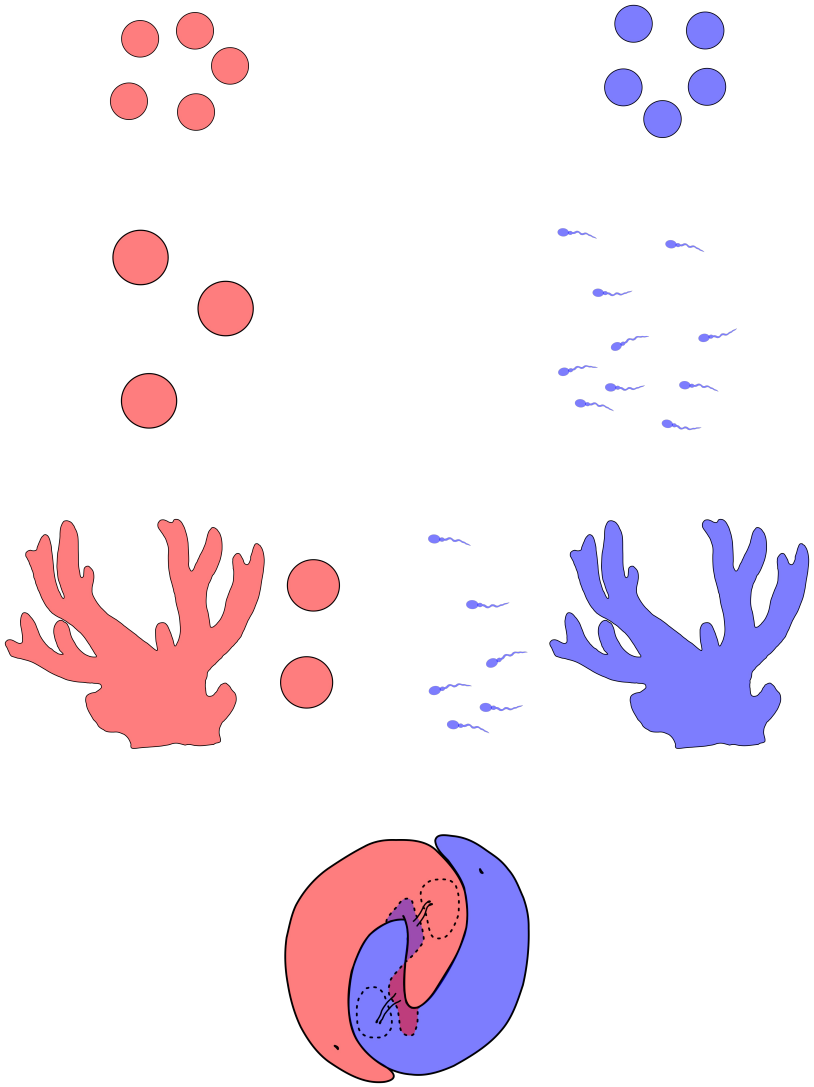
The local sperm competition model predicts not only the selection for a more female-biased sex allocation due to competition between related sperm. It also specifies the mechanism by which this change in sex allocation is selected, namely diminishing fitness returns for investment into the male function, due to competition between related sperm. I present results in **Chapter 3** that confirm a positive relationship between testis investment and paternity success. However, the predicted diminishing fitness returns for testis investment in smaller group sizes, i.e. group sizes which should have resulted in strong local sperm competition, could not be confirmed. Since there are no other, more plausible hypotheses to explain the phenotypically plastic shifts in sex allocation, I conclude that the local sperm competition model could still be valid, but that an improved experimental design, increasing the range of local sperm competition, may be used in future studies.

Nuclear genes and cytoplasmic genetic factors are not equally transmitted via eggs and sperm, potentially leading to cytonuclear conflict over the optimal sex allocation. Cytonuclear conflict involving mitochondria can therefore be expected to be widespread, but it has mainly been documented in plants rather than animals. In **Chapter 4** I report the results from a quantitative genetic breeding experiment testing for cytotype effects on sex allocation traits, as predicted under an ongoing cytonuclear conflict over sex allocation. Contrary to this prediction, we did not find evidence for strong cytonuclear conflict over sex allocation. I propose two possible explanations: namely i) that the nuclear genome in animals ‘won’ the coevolutionary arms race and ‘domesticated’ the mitochondrion during the course of coevolution or ii) that the studied population was not polymorphic for loci involved in cytonuclear conflict.

The different aspects of the male-female phenomenon, which I studied during my PhD are quite diverse but interconnected. Sexual selection, sex allocation and genomic conflict over sex allocation all influence each other, because ultimately they are all consequences of the evolution of anisogamy. I therefore suggest that it may often be necessary to study how these different aspects of the male-female phenomenon are connected, rather than focusing on them in isolation.

Chapter 1

Thesis Introduction



Thesis Introduction

The presence of the male-female phenomenon, i.e., **anisogamy** (see Glossary at the end of the Thesis introduction), and its various consequences include some of the most intriguing adaptations in the living world. This size dimorphism of fusing gametes—with males producing numerous small gametes (sperm) and females producing fewer, bigger gametes (eggs)—is thought to lead to different selection pressures on males and females in **gonochorists** and the male and the female reproductive functions in **hermaphrodites** (Bateman 1948; Parker et al. 1972; Schärer et al. 2012; Parker 2014; Lehtonen et al. 2016b; but see Ah-King and Nylin 2010 for an opposing view). In this introduction I first describe the evolution of the male-female phenomenon from first principles (i.e., starting with the evolution of anisogamy), following Parker's (2014) idea of the **sexual cascade**. In the sexual cascade the evolution of the male-female phenomenon and its consequences are thought of as a logical succession of stages leading—if certain conditions are met—ultimately to the emergence of **copulation** with internal fertilization and **'classical' sex roles**. Note that I focus on the evolution of the male-female phenomenon in metazoans, although many conclusions may also apply to plants and other anisogamous organisms. I then explain how the male-female phenomenon results in sexual selection in gonochorists as well as simultaneous hermaphrodites. Subsequently, I outline how the optimal **sex allocation** in simultaneous hermaphrodites is influenced by so-called **fitness gain curves**, with special regards to processes occurring during sexual selection. I then introduce how, as a consequence of anisogamy, differently inherited genetic factors residing within the same organism can be in conflict over an individual's optimal sex allocation. Note that I do not treat plants or sequential hermaphrodites in much detail in my thesis and use the term 'hermaphrodite' as meaning 'simultaneous hermaphrodite' throughout.

The Fisher Condition and the Evolution of Anisogamy

Fisher Condition

Isogamy is probably the ancestral state for sexual reproduction (Lessells et al. 2009; Lehtonen and Parker 2014; Parker and Pizzari 2015), in which each parent makes an equal resource contribution to the resulting zygote. During sexual reproduction each parent also usually contributes half of its nuclear genome to the zygote (but see the **Genomic conflicts over sex allocation** subsection). Because each parent contributes equally, both types of parents (be it mating types or sexes) necessarily have the same average fitness at the population level. This characteristic condition for systems with sexual reproduction is often referred to as the **Fisher condition** (Fisher 1930; Houston and McNamara 2006). As a consequence of the Fisher condition, negative-frequency dependent selection will tend to lead to an equal investment into both mating types (or both sexes), because the rare type has a fitness advantage as long as it is rare. It only loses this advantage once equal investment is attained in the population (Düsing 1884; Fisher 1930; Queller 2006). However, the Fisher condition will only lead to an equal investment into the two types as long as the, greatly simplifying, assumptions of random **mating** and large population size hold (Hamilton 1967; Charnov 1982; Queller 2006).

Evolution of Anisogamy

Although isogamy is widespread in unicellular organisms (Lehtonen et al. 2016a), it is not the only way of gamete fusion. For instance, anisogamy evolved several times independently, mostly in multicellular organisms, e.g., in the ancestor of metazoans, of land plants, in different groups of algae, and two times in the Ectocarpales within the brown algae (Bell 1978; Silberfeld et al. 2010; Parker 2014). As a consequence of the evolution of anisogamy the two sexual functions emerged: male and female (Lessells et al. 2009; Parker 2011). Although the resolution of **genomic conflict** has been proposed as a cause for the evolution of anisogamy as well (Cosmides and Tooby 1981; Hurst 1990), it is now widely accepted that 'gamete competition', possibly in combination with 'gamete limitation', is mainly responsible for its evolution (Parker et al. 1972; Lessells et al. 2009; Lehtonen and Kokko 2010). These 'gamete competition' models show that gamete competition will lead to the evolution of anisogamy, if one assumes i) a trade-off between gamete number and gamete size and ii) a positive relationship between (at least some range of) zygote size and zygote fitness (Parker et al. 1972; Parker 2011, 2014). In particular, disruptive selection will, on the one hand, select the producers of the small gamete type (the proto-males) to maximize the number of fusions with the larger gamete type, by making the sperm tinier and more numerous. On the other hand, it will select the producers of the larger gamete type (the proto-females) to make gametes even larger, to maximize the number of surviving offspring (Parker et al. 1972). During this 'primordial conflict' the proto-males are then essentially exploiting the investment into eggs provided by the proto-females (Parker et al. 1972; Parker 2014).

Sex Allocation

As a consequence of the evolution of anisogamy, organisms are selected to strategically invest their finite resources into the male versus the female reproductive function, i.e., to express the optimal sex allocation (Charnov 1982). As mentioned above, the Fisher condition will tend to lead to an equal investment into both sex functions under many conditions. Equal investment can therefore be viewed as the *a priori* prediction for sex allocation, unless the assumptions of random mating and large population size are violated (see later sections for a discussion of how these assumptions affect sex allocation).

The two sex functions can be housed in different, specialized kinds of individuals in the case of gonochorism, housed in the same individual but temporally separated in sequential hermaphrodites, or housed simultaneously in the same individual in simultaneous hermaphrodites, where both sperm and eggs are produced during at least part of the lifetime of a single individual (Hamilton 1967; Charnov 1982; Munday et al. 2006; Schärer 2009; Weeks 2012). As a consequence, every hermaphroditic individual can act in its male role (as a sperm donor) as well as in its female role (as a sperm recipient). At the moment, we actually do not know whether the first anisogamous multicellular organisms were gonochorists or hermaphrodites. But it can certainly not be excluded that they may have been hermaphroditic, since several *Volvox* species, which are often used as model organisms for understanding the evolution of multicellularity, are actually hermaphroditic (e.g., Isaka et al. 2012). Moreover, it is not known either what the ancestral mating system among the metazoans was (Ghiselin 1969; Eppley and Jesson 2008; Iyer and Roughgarden 2008; Riesgo et al. 2014; Schärer et al. 2014). Nevertheless, I first explain the further evolutionary consequences of the emergence of anisogamy focusing on gonochorists, because in the literature these consequences have been mostly worked out for that sexual system (e.g., Jennions and Kokko 2010; Parker 2014). Subsequently, I point out the characteristics for hermaphrodites.

Emergence of Pre- and Postcopulatory Sexual Selection and 'Classical' Sex Roles

Initially, anisogamous multicellular organisms may have been either sessile or fairly immobile marine organisms, which reproduced by releasing their gametes into the seawater. Under these conditions, anisogamy—in conjunction with equal sex allocation—leads to the numerous sperm competing for the few ova (Jennions and Kokko 2010 p. 350; Parker 2014). Because sessile, **broadcast-spawning** animals do not have many other means by which to increase their reproductive success, sexual selection at this stage acted mainly on gametic investment, so that males invested heavily into the production of sperm and females into the production of eggs (cf. Table 7.1 in Parker and Pizzari 2015 p. 142). At that stage, sexual selection was thus mainly 'postejaculatory' (*sensu* Parker 2014) and this remains true in extant sessile or weakly mobile species with broadcast spawning, as for example many sponges, corals or echinoderms. As animals evolved means to move around more efficiently, this made **female-targeted sperm release** by males possible. And since such targeted release may have increased the proportion of eggs that the males were able to fertilize, selection led males to seek out females and ejaculate their sperm closer to where the eggs are released.

Parker (2014) therefore called the evolution of mobility the "catalyst" that permitted sexual selection to lead to the evolution of traits other than pure ejaculate size. The reason is that—if not only the mere quantity of sperm determines male reproductive success, but also when and where the ejaculate is released—resources may also be allocated towards mate search and fending off other rival males. Therefore, resources previously mainly invested into testes and sperm production may instead have been allocated into sensory abilities and mobility (Jennions and Kokko 2010; Parker 2014). This stage can still be observed in fishes and many amphibians, where males seek out spawning opportunities with females and try to monopolize matings with them.

Males that ejaculate their sperm even closer to the eggs than under simple female-targeted sperm release can potentially increase their fertilization success even further. This may lead to the evolution of copulation with internal fertilization, if females also benefit or at least do not suffer costs from copulations (Parker 1970). An additional fitness benefit of copulation, also benefitting the females, might have been that both male and female gametes have a higher survival, because inside the female body they are better protected from pathogens, parasites and predators. Immobile species with spermcast mating, in which females retain eggs and only sperm is broadcasted (Bishop and Pemberton 2006), might be an intermediate evolutionary step during which female gametes are protected from external threats and males might then have been selected to evolve intromission and copulation to

increase their fertilization success. It has also been pointed out that the evolution of internal fertilization was associated with the colonization of the land and could protect gametes against drying out (Dawkins and Carlisle 1976), although the existence of internally fertilizing aquatic animals (e.g., many marine invertebrates, some fishes and newts) casts doubt on the generality of this explanation.

But irrespective of how copulation and internal fertilization evolves, once it does, sexual selection will now act during two distinct **selection episodes**: precopulatory and postcopulatory. Because of the Fisher condition, and the resulting equal sex allocation, there will generally be a similar number of males and females present in the population. However, because of anisogamy the males will tend to compete for access to the females and their eggs. More specifically, during the precopulatory episode, males have to compete for matings with females, because, assuming no substantial postzygotic paternal investment, the larger parental investment provided by females removes them from the mating pool for longer than the males (Queller 1997; Jennions and Kokko 2010). Consequently, at any given moment, there are likely to be more sexually active males than sexually receptive females (i.e., the **operational sex ratio** is male-biased), forcing the males to compete for matings with the few receptive females. During the postcopulatory episode, different males continue to compete for the fertilization of the eggs from polyandrous females via their numerous sperm (**sperm competition**; Parker 1970, 1982) and females may bias fertilization in favor of preferred males (**cryptic female choice**; Charnov 1979; Thornhill 1983).

Sexual Selection in Simultaneous Hermaphrodites

Bateman's Principle in Simultaneous Hermaphrodites

Although at first glance, sexual selection might not seem to apply to hermaphrodites, because there are no male and female individuals, sexual selection in gonochorists and hermaphrodites actually acts according to similar principles. This is because sexual selection arises ultimately from anisogamy and hermaphrodites are, of course, also anisogamous (Charnov 1979; Anthes et al. 2010; Jennions and Kokko 2010). Charnov (1979) was the first to point out that **Bateman's principle** may also apply to hermaphrodites and that the production of fertilized eggs via an individual's own female function is therefore not limited by the ability to get enough sperm to fertilize them, but by the resources allocated to the production of eggs. Sexual selection may thus lead to hermaphrodites preferring to mate and donate sperm in their male role and to be choosier with whom to mate in their female role (i.e., the optimal mating rate for the male function may be higher than that of the female function). And, as in gonochorists, the sperm transferred by different sperm donors will compete for the fertilization of eggs after copulation, if the sperm recipient has mated with more than one sperm donor.

Shift towards Postcopulatory Sexual Selection

Since hermaphrodites can act either in their male or female role during mating and, as just explained, they may prefer to mate in their male role, there are unique opportunities for **sexual conflict** that arise over who takes on which role (Charnov 1979; Michiels 1998; Schärer et al. 2014). This sexual conflict over mating roles can lead to different evolutionary outcomes: unilateral mating, alternating unilateral mating and reciprocal mating (Michiels 1998; Schärer et al. 2014).

In hermaphrodites with alternating unilateral mating or reciprocal mating, the resulting mating rate will likely be intermediate between the (higher) male optimum and the (lower) female optimum. The reason for this is that individuals then cannot exhibit strong mate choice in their female function without sacrificing sperm transfer opportunities for their own male function. Therefore, hermaphrodites are often thought to mate more often than is optimal for their female function and as a result sexual selection may be shifted more towards the postcopulatory stage (Schärer et al. 2014). One might wonder who is then more in control over the fate of the ejaculates in the sperm recipient's

body: The sperm donor or the sperm recipient. In **Chapter 2** I present a study testing the effects of the genotype of the sperm donor, the genotype of the sperm recipient and their interaction on pre- and postcopulatory fitness components.

Sex Allocation in Hermaphrodites and the Local Sperm Competition Perspective

Since, also in hermaphrodites, sperm and egg contribute an equal amount of nuclear genetic material to the zygote, the Fisher condition applies equally. Negative frequency-dependent selection will therefore tend to lead to equal investment into both sex functions under many conditions.

However, there are reasons to believe that the conditions that lead to the evolution and evolutionary maintenance of hermaphroditism can favor an uneven sex allocation. Charnov made extensive use of fitness gain curves (see Glossary) in his theoretical work to explain the evolution and evolutionary maintenance of hermaphroditism (1979, 1982). Charnov's insight was that, as long as either one or both sex functions show diminishing fitness returns for investment into those sex functions (and there is a trade-off between the investment into the male and the female sex function), simultaneous hermaphroditism will be favored (Charnov 1979, 1982 pp. 219–227).

There have been different reasons proposed for why fitness gains might show diminishing returns for the male or female function. For example, the female function may show diminishing fitness returns in the case of local resource competition, which was first conceptualized for gonochorists (Clark 1978), but later also applied to hermaphrodites (Charnov 1982; Lloyd 1982). Here, female-derived offspring will compete for resources more strongly if they are more clumped in space compared to the offspring derived from the male function. In that case reproductive resources may be more profitably allocated to the male function and hermaphroditism with a male-biased sex allocation will be favored. Local resource competition seems to be most likely in sessile or weakly mobile animals and plants, where the male gametes (sperm or pollen) may travel further than the female gametes (locally settling larvae or seeds). Another situation that can lead to a diminishing female fitness gain curve is brooding with limited brood space (Heath 1979; Charnov 1982). In this case, fitness returns for investment into the female function increase linearly until the limited brood space is completely filled up. But every additional egg produced will then show no more fitness returns and again hermaphroditism with a male-biased sex allocation will be favored. Finally, **local sperm competition**, the competition between related sperm for the fertilization of a given set of ova, will lead to diminishing fitness returns for investment into the *male* function as explained in the following.

Local Sperm Competition

One of the key assumptions that needs to be fulfilled, in order for the Fisher condition to lead to equal investment in the male and female function in a hermaphrodite, is that sperm of every given sperm donor in the population is equally likely to be represented in every sperm recipient's receiving organ (according to the sperm donor's ejaculate investment) (Hamilton 1967; Queller 2006; Schärer 2009). This assumption is arguably rarely fulfilled. Indeed, for many species only a limited number of possible mating partners might be available for any given focal individual, either because of small groups being spatially clustered in their environment or because of limited mobility allowing only few partners to be reached. These individuals with which a focal individual is able to mate can be considered its 'mating group' (Charnov 1980). While assuming no subdivision in the population would mean that the sperm of a focal sperm donor competes with an equal proportion of sperm from all the other sperm donors in the population, in a small mating group it only competes with the sperm from the other donors of that same mating group. As the mating group becomes smaller, the sperm of a focal donor does no longer only compete with sperm from other donors, but increasingly with the

own and therefore related sperm. As a consequence, investment into sperm becomes more and more wasteful with a decreasing mating group size and resources are more profitably invested into the own female function (or possibly into male traits other than sperm; Michiels et al. 2009; Preece et al. 2009; Schärer and Pen 2013). A decreasing mating group size will therefore result in a more and more diminishing male fitness gain curve, because of increasing local sperm competition (Fig. 1). Consequently, the most intense local sperm competition occurs under selfing and monogamy, where any sperm that is not necessary to fertilize the own or the partner's eggs is wasted, and a very female-biased sex allocation will be favored (Charnov 1980; Greeff et al. 2001; Schärer and Wedekind 2001; Schärer 2009; Schärer and Pen 2013).

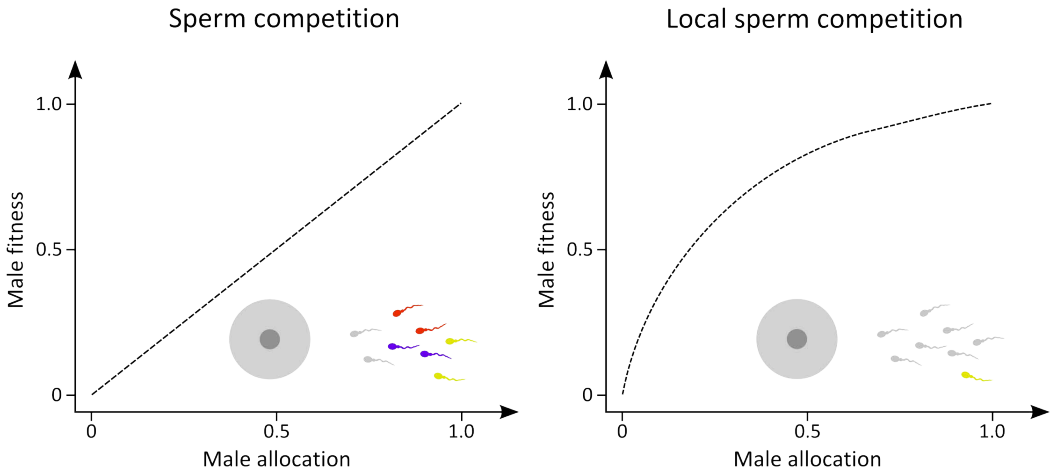


Figure 1. Effect of local sperm competition on the male fitness gain curve. When unrelated sperm (indicated by different colors) compete, investment into the male function in the form of more sperm will tend to yield linear fitness returns (left hand side). When, in contrast, mainly related compete for fertilization, the fitness gains for increased male allocation will tend to show strongly diminishing fitness returns (right hand side).

The prediction that increasing local sperm competition leads to more diminishing fitness returns for investment into the male function has, to my knowledge, only been tested once in a hermaphroditic animal (Yund 1998). In **Chapter 3** I present the results of an experiment testing this prediction in the hermaphroditic flatworm *Macrostomum lignano*.

Genomic Conflicts over Sex Allocation

Although I wrote in the previous sections that both sperm and egg contribute exactly half of the genetic information to the genome of the resulting zygote, that is actually only true for autosomal nuclear genes. There are two groups of genes that are not equally transmitted via sperm and eggs. First, in gonochoristic species genes residing on the sex chromosomes (if present) are not transmitted to the zygote at the same rate via sperm and eggs (Hamilton 1967). Second, since in anisogamous eukaryotes the egg contributes the main share to the zygote cytoplasm, the mother contributes most of the cytoplasmic genetic factors (including the genomes from some of her intracellular organelles), while this is rarely the case for males (Birky 1995, 2001; but see Breton et al. 2011). Therefore, there will be an evolutionary conflict over the optimal sex allocation between the autosomal nuclear genome on the one hand and the genes residing on sex chromosomes and cytoplasmic genetic factors on the other hand (Hamilton 1967; Cosmides and Tooby 1981; Charnov 1982 p. 121; Hurst et al. 1996). In particular, sex ratio distorters emerging on the sex chromosome of the heterogametic sex (e.g., on the Y-chromosome in mammals or the Z-chromosome in birds) are selected to bias sex allocation (e.g., towards sons in mammals and towards daughters in birds) (Hamilton 1967). In contrast, the maternally

inherited cytoplasmic sex allocation distorters will always be selected to bias the organism's sex allocation towards the female function, because the male function is usually an evolutionary dead end for them (Cosmides and Tooby 1981).

Cytonuclear conflict over sex allocation is common, since cytoplasmic genetic factors are mostly maternally inherited. Cytoplasmic genetic factors include mitochondria, cytoplasmic endosymbionts, vertically transmitted parasites and chloroplasts (Cosmides and Tooby 1981).

Any mutation arising in the cytoplasmic genetic factor that increases female allocation will spread, because such cytoplasmic sex allocation distorters will be overrepresented in the next generation (Cosmides and Tooby 1981; Charnov 1982 p. 121) (Fig. 2). The spread of such a sex allocation distorter may even lead to the extinction of the population, if the whole population ends up consisting of pure females (Hamilton 1967; Cosmides and Tooby 1981; Hurst et al. 1996). On the opposite side of this genomic conflict, any mutation in the nuclear genome that restores the sex allocation towards the optimum for the nuclear genome will spread as a consequence. The result can be a coevolutionary arms race of newly emerging cytoplasmic sex allocation distorters that are countered by nuclear sex allocation restorers (Cosmides and Tooby 1981; Hurst et al. 1996).

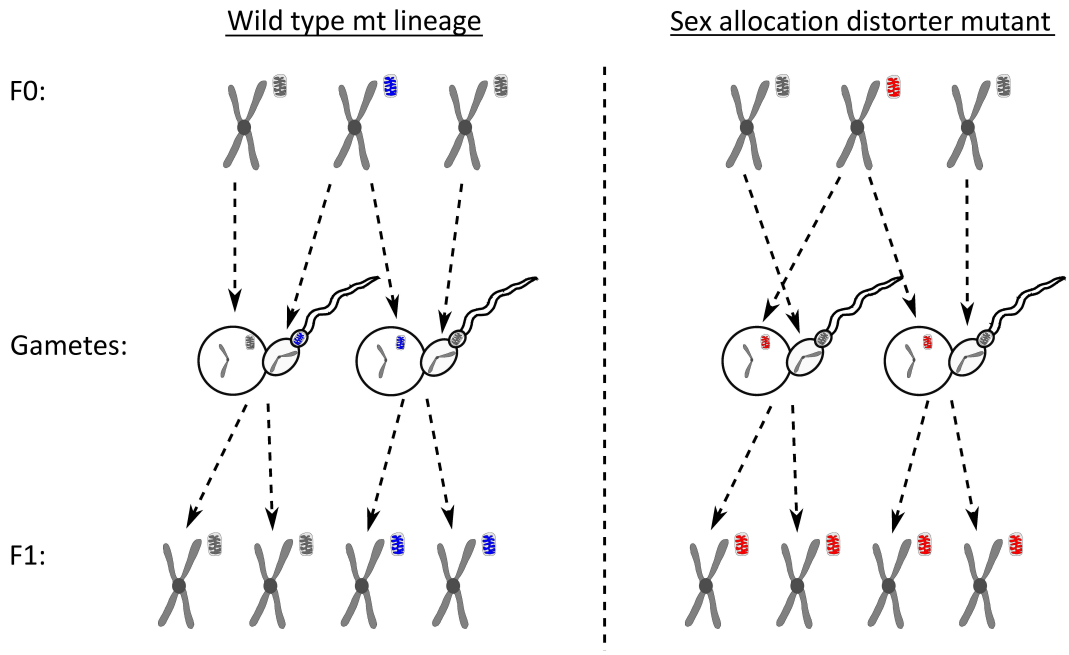


Figure 2. Increased fitness of a cytoplasmic sex allocation distorter in a simultaneous hermaphrodite (a mitochondrial distorter in this example). If the cytoplasmic sex allocation distorter mutant (red mitochondrion on the right hand side) manipulates the F0 parent into producing only female gametes it doubles its representation in the F1 generation compared to the wild type mitochondrion lineage (blue mitochondrion on the left hand side).

There is ample empirical evidence for mitochondrial sex allocation distorters leading to cytoplasmic male sterility in plants, where 10% among angiosperm species show cytoplasmic male sterility (Delannay 1978; Burt and Trivers 2008 pp. 161–181), but not in animals (Weeks 2012). There is, however, ample evidence that the vertically transmitted, intracellular symbiont *Wolbachia* manipulates the sex allocation of its arthropod hosts in many different ways (Werren et al. 2008). Why there is so much more evidence for mitochondrial sex allocation distorters in plants than in animals is puzzling and I discuss this question in some detail in **Chapter 4**.

Objectives of the Thesis

My PhD project covered quite diverse aspects of the male-female phenomenon. Specifically, I studied how phenotypic and genetic factors can influence sexual selection and sex allocation in the simultaneously hermaphroditic flatworm *Macrostomum lignano*.

In **Chapter 2** I examine sexual selection in a simultaneous hermaphrodite. The outcome of sexual selection is always influenced by the phenotypes of both mating partners. In fact, the outcomes of sexual selection episodes can be thought of as **interacting phenotypes** (Moore et al. 1997; Schneider et al. 2016 pp. 5–8). If those phenotypes have underlying genetic variation, this can have important consequences for the evolutionary response to sexual election (Moore et al. 1997). By using a full factorial 6x6 design I examined how the genotype of the sperm donor, the genotype of the sperm recipient, and their interaction influence different mating behaviors and fitness components of the sperm donor during pre- and postcopulatory sexual selection episodes.

In **Chapter 3** I test two important predictions from sperm competition and sex allocation theory, respectively. First, I test whether *M. lignano* individuals with big testes sire more offspring in their male role than individuals with small testes. Second, I test an important prediction from the local sperm competition perspective, namely, that fitness gains for testis investment diminish under local sperm competition. This prediction is crucial for two main reasons. Namely, it provides a mechanism that allows understanding i) the evolutionary maintenance of hermaphroditism (Charnov 1982 pp. 242–251; Schärer 2009; Schärer and Pen 2013) and ii) the reason why the optimal sex allocation changes according to the prevailing group size in the population (Charnov 1980; Greeff et al. 2001; Schärer and Ladurner 2003; Schärer 2009).

In **Chapter 4** I present a quantitative genetic breeding study using pair-wise crosses of 2x15 independent inbred lines to examine cytonuclear conflict over sex allocation. More specifically, we made use of the fact that in simultaneous hermaphrodites the offspring from the cross of two inbred lines will have (almost) identical nuclear genomes, but different cytotypes depending on who the maternal parent is. This permitted to partition variation in sex allocation into (among other) its nuclear and cytoplasmic components. In this study we test for manifestations of cytonuclear conflict over sex allocation in a simultaneously hermaphroditic animal, a group for which, to my knowledge, cytoplasmic sex allocation distorters have never been reported before.

Glossary

Anisogamy	Gamete dimorphism, especially with regards to size, together with binary fusion and disassortative mating between gamete size classes (Lessells et al. 2009).
Bateman's principle	The stronger correlation between number of matings and reproductive success in males than in females due to anisogamy (Bateman 1948; Arnold 1994).
Broadcast spawning	A method of reproduction in which sperm and eggs are released into the water and fertilization takes place externally (Bishop and Pemberton 2006).
'Classical' sex roles	Sex roles described by Darwin (1871) and Bateman (1948), where males are more eager to mate than females and therefore more likely to compete for matings, while females tend to be choosier with whom they mate.
Copulation	A special case of mating in which an intromittent organ is used to deliver sperm to the female sperm receiving organ of the mating partner, usually followed by internal fertilization.
Cryptic female choice	"Nonrandom paternity biases resulting from female morphology, physiology, or behavior that occur after mating" (Schärer 2009; Pitnick and Brown 2000).
Cytonuclear conflict	Conflict of evolutionary interests between cytoplasmic genetic factors and the nuclear genome. It is a special case of genomic conflict (Cosmides and Tooby 1981).
Genomic conflict	Conflict of evolutionary interests between genes within the same organism. Also often called 'intragenomic conflict', although *different* genomes residing inside the same organism can also be in conflict (Cosmides and Tooby 1981; Hurst 1992).
Gonochorist	An organism that produces either sperm or eggs, but not both, i.e., a separate-sexed organism that can be either male or female.
Hermaphrodite	An organism that produces both sperm and eggs during its life time. In this thesis the term is used to mean simultaneous hermaphrodite: an organism that produces both sperm and eggs at the same time during at least part of its life time.
Interacting phenotype	A trait that requires or is influenced "by interactions with a conspecific social partner or neighbor" (Moore et al. 1997).
Female-targeted sperm release	A process during which males move into close proximity to a target female before ejaculation (Parker 1970, 2014). Assumed to be an intermediate evolutionary step between broadcast spawning and copulation with internal fertilization.
Fitness gain curve	Relationship between the investment into a sex function and the resulting fitness through that sex function (Schärer 2009).
Fisher condition	Represents the fact that the average fitness of the male and female function must be equal in sexual organisms (Houston and McNamara 2006). This is only true regarding autosomal nuclear genes.
Isogamy	The fusing gametes have the same morphology, especially with regards to size (Lessells et al. 2009).

Local sperm competition	"Competition between related sperm for the fertilization of a given set of ova" (Schärer 2009).
Mating	The pairing of opposite-sexed or hermaphroditic animals, usually to reproduce sexually. Mating can happen in quite different manners, e.g., broadcast spawning or copulation.
Operational sex ratio	"Instantaneous ratio of sexually active males to sexually receptive females" (Jennions and Kokko 2010).
Selection episode	An arbitrarily chosen segment of an organism's life cycle resulting from the partitioning of overall selection into multiplicative parts. Permits to compare strength and direction of selection between those different selection episodes (Arnold and Wade 1984).
Sex allocation	The allocation of reproductive resources to male versus female reproductive function in sexual organisms (Charnov 1982; Schärer 2009).
Sexual cascade	The succession of transitions flowing from the early evolution of syngamy to the evolution of copulation and classical sex roles (Parker 2014).
Sexual conflict	"A conflict between the evolutionary interests of a sperm donor and a sperm recipient" (Schärer et al. 2014).
Sperm competition	"Competition between the sperm of two or more (unrelated) individuals for the fertilization of a given set of ova" (Schärer 2009).

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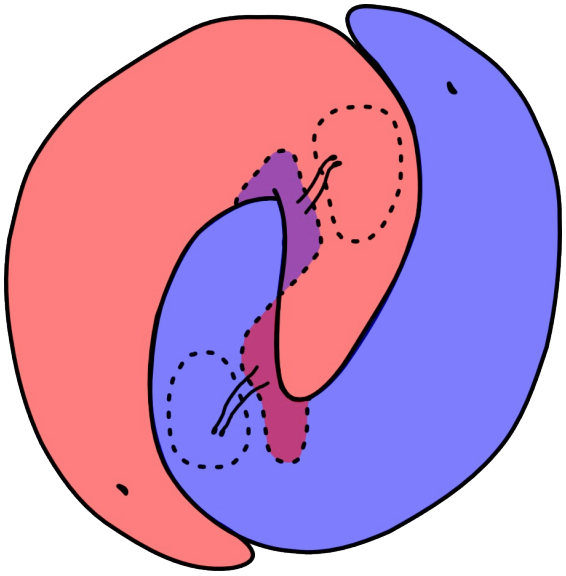
Chapter 2

Effects of Sperm Donor and Sperm Recipient Genotypes along Episodes of Pre- and Postcopulatory Sexual Selection



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Abstract

Sexual reproduction is by necessity influenced by at least two individuals (plus possible interfering competitors). Thus, an individual's reproductive success results from an interaction between its own phenotype and that of its mating partners, and ultimately from their genotypes (assuming the phenotypes have underlying genetic variation). Here we study how in a simultaneous hermaphrodite—the transparent free-living flatworm *Macrostomum lignano*—the genotypes of the sperm donor and the sperm recipient both influence the sperm donor's success during mating and sperm transfer. Specifically, we paired six transgenic GFP-expressing genotypes in the role as the sperm donor with six wild-type genotypes in the role as the sperm recipient, in a replicated full-factorial design (to permit testing for interaction effects). We further added a third worm from one additional wild-type genotype to each replicate, to determine the success of our sperm donor in the presence of competition. Sperm donor genotype significantly affected mating and sperm transfer success, while the genotype of the sperm recipient and the sperm donor x sperm recipient genotype interactions had no significant effects on these variables. With respect to mating behavior we found significant genotype main effects on mating latency and donor x recipient genotype interactions on a peculiar postcopulatory suck behavior, which may be involved in sexual conflict in this species. The sperm donor by sperm recipient genotype interactions on mating behaviors reveal that there is genetic variation for both sexual selection and selection resulting from sexual conflict to act on at this stage. The lack of these interactions for mating success and sperm transfer success could indicate that sexual conflict and sexual selection is shifted towards later stages, namely the stage between sperm storage in the recipient and the fertilization of eggs.

Introduction

Selection may act quite differently during separate stages of an organism's life cycle. In order to understand how selection affects lifetime reproductive success it is therefore often useful to divide the life cycle into consecutive segments, so-called episodes of selection (Arnold and Wade 1984). One simple but useful division of the life cycle in a sexually reproducing organism might, for example, be into juvenile survival, adult survival, and reproduction, because it can reasonably be assumed that selection acts differently during these three episodes.

An important type of selection acting during the last episode, namely sexual selection, has been intensively studied (Andersson 1994; Andersson and Iwasa 1996; Birkhead and Møller 1998). This research was initially concerned with how males compete for matings and how females choose with whom to mate (Darwin 1871; Bateman 1948), but has since been extended to include processes that occur after copulation (Parker 1970; Charnov 1979; Eberhard 2009; Birkhead 2010). More specifically, ejaculates of different sperm donors may compete for the fertilization of eggs of a sperm recipient during sperm competition (Parker 1970, 1998) and sperm recipients may bias fertilization towards sperm of specific partners during cryptic female choice (Charnov 1979; Thornhill 1983). Since females in many taxa routinely mate with more than one male, sperm competition and cryptic female choice are expected to be widespread phenomena (Birkhead and Møller 1998; Jennions and Petrie 2000; Parker and Birkhead 2013; Taylor et al. 2014).

Traits that are sexually selected during pre- and postcopulatory episodes may be quite different. For example, larger body size is selected in males of many species, because it allows to overpower other males during contest competition and therefore permits to monopolize matings with females (Andersson 1994 pp. 247–293). In contrast, a higher sperm production rate, often achieved by a bigger testis mass, is often selected during sperm competition after copulation (e.g., Parker et al. 1997; Preston et al. 2003; Awata et al. 2006; Simmons and García-González 2008). In fact, selection on the same traits might even act in different directions during pre- and postcopulatory episodes, because there are likely to be trade-offs between investment into traits important for pre- versus postcopulatory sexual selection (Parker and Pizzari 2010; Kvarnemo and Simmons 2013; Parker et al. 2013; Lüpold et al. 2014), making a conceptual separation between pre- and postcopulatory sexual selection all the more necessary. The quacking frog *Crinia georgiana* is an interesting example for the different selection pressures imposed by pre- and postcopulatory sexual selection. In this species, males with thicker arms achieved higher mating success at low densities, presumably because they were better able to monopolize matings. But at high densities, those males could not prevent other males from mating (Buzatto et al. 2015) and consequently selection on relative testis size became predominant (Buzatto et al. 2017).

An important feature of pre- and postcopulatory sexual selection is that selection is not imposed on the organism by constant environmental factors, but rather by other, evolving members of the same species. In fact, fitness components arising during sexual reproduction are, by their very nature, the result of an interaction between at least two mating partners and possibly involving competitors as well, similar to 'interacting phenotypes' (Moore et al. 1997; Schneider et al. 2016). Fitness components as mating success and sperm transfer success can analogously to, for instance aggression, be viewed as a single trait influenced by an interaction with reciprocal effects (cf. Fig 2c in Moore et al. 1997). Therefore, the evolutionary response to selection will not only depend on selection and genetic variation for traits in one sex, but also on selection and genetic variation for traits in the other sex and possible competitors (Moore et al. 1997; Moore and Pizzari 2005; Schneider et al. 2016). A complete understanding of lifetime reproductive success, and the resulting evolutionary response, may therefore necessitate the inclusion of the interacting phenotype perspective.

The importance of taking the interacting phenotype perspective into account does not depend on whether the organism is a gonochorist (separate-sexed organism) or a simultaneous hermaphrodite (Marie-Orleach et al. 2017). The success of a sperm donor in a simultaneously hermaphroditic species at achieving matings, at transferring sperm and at subsequently fertilizing eggs might, similarly, not only depend on the phenotype of the sperm donor itself, but also on that of the sperm recipient and potential sperm competitors.

The presence of interacting phenotypes can, theoretically, alter the rate of evolutionary change (Moore et al. 1997), if there is genetic variation underlying the interacting phenotypes, i.e., if there are genotype-by-genotype interactions (GxG) affecting the phenotype. Testing whether there is indeed genetic variation that has an interactive effect on fitness components therefore is an important empirical question. Considerable empirical research to find effects of interacting genotypes has been conducted in the field of maternal effects (Mousseau and Fox 1998). However, despite the fact that the importance of these GxG interactions have been pointed out several times (e.g., García-González 2008; Engqvist 2013), only a limited number of studies explicitly tested for male x female genotype interactions on fitness components relevant during different sexual selection episodes. For example, Castillo and Delph (2016) found male x female genotype interactions to affect interaction latency (i.e., the time between start of the mating trial and the first interaction between the pair) in the gonochoristic nematode *Caenorhabditis remanei*, which could have important implications for speciation. Clark et al. (1999) found male x female genotype interaction effects on P_1- and P_2-values in *Drosophila melanogaster*. And male x female genotype interaction effects on fertilization success and egg choice by sperm were also found in the broadcast spawning blue mussel *Mytilus galloprovincialis* (Evans et al. 2012). In the field cricket *Teleogryllus oceanicus* there are apparently no such interactions present, but the female genotype affects P_2-values (Simmons et al. 2014). And while not specifically aiming at male x female genotype interactions, some other studies tried to find interacting phenotypes involved in sexual selection. For example, Garía-González and Simmons (2007) found that an interaction between sperm and spermatheca length influenced P_2-values in the dung beetle *Onthophagus taurus*. In contrast, there seems to be no sperm by ovarian fluid interaction on paternity success in chinook salmon *Oncorhynchus tshawytscha* (Evans et al. 2013), although some effects in this study might have been missed, because in their experimental design the researchers did not control for variation in sperm number and egg genotype.

Simultaneous hermaphrodites often exhibit very complex mating behaviors with an arguably high degree of interaction between the mating partners, especially when mating occurs reciprocally. Examples include the 'penis fencing' behavior in polyclad flatworm *Pseudoceros bifurcus* (Michiels and Newman 1998) and 'love' dart shooting in reciprocally mating garden snail *Cornu aspersum* (=*Cantareus aspersus*=*Helix aspersa*) (Davison et al. 2005). The correlated evolution of male and female reproductive morphology in gastropods of the Aglajidae and Gastropteridae (Anthes et al. 2008) also seems to suggest that the close interaction during copulation are important for mating in these hermaphrodites. Furthermore, the reciprocally mating flatworm *Macrostomum lignano* exhibits an intricate mating behavior during which both partners reciprocally influence each other as well (Schärer et al. 2004; Vizoso et al. 2010). Because hermaphrodites are often assumed to prefer the male over the female role during mating interactions, which forces reciprocally copulation species to mate more often than is optimal for the female function, sexual selection and sexual conflict may be shifted more towards the postcopulatory episode in these species (Charnov 1979; Michiels 1998; Schärer et al. 2014).

In summary, there seems to be a lack of studies that explicitly test for male x female genotype interactions during different sexual selection episodes and we have even less information on how these interactions differ between pre- and postcopulatory episodes. Moreover, we are aware of no such studies in copulating simultaneous hermaphrodites. This is unfortunate since we expect sperm donor x

sperm recipient genotype interactions to play an important role during sexual selection, especially since highly interactive, reciprocal mating behaviors are common in simultaneous hermaphrodites (Michiels and Newman 1998; Schärer et al. 2004; Davison et al. 2005; Anthes et al. 2008; Vizoso et al. 2010).

Objective

In our study we paired different sperm donor and sperm recipient genotypes of the simultaneously hermaphroditic flatworm *Macrostomum lignano* in a replicated full-factorial 6x6 design, to test whether the genotype and GxG interactions influence the success of the sperm donor during a pre- and a postcopulatory selection episode, namely mating and sperm transfer. Additionally, we also test for genotype and GxG interaction effects on several mating behaviors.

Methods

Study Organism and Culture Lines

We performed the present experiment with the free-living meiobenthic flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes), which occurs in the intertidal zone of the Northern Adriatic Sea and the Eastern Mediterranean basin (Ladurner et al. 2005, L. Schärer pers. obs.). *M. lignano* is a small (adult length ~1.5mm), transparent, obligatorily outcrossing, simultaneous hermaphrodite with a generation time of ~18 days. The eggs hatch ~5 days after laying and worms reach maturity in both sex functions ~13 days after hatching (Schärer and Ladurner 2003). Laboratory cultures of *M. lignano* can be maintained in glass Petri dishes in artificial sea water (ASW) of 32‰ salinity or f/2 algal medium (Andersen et al. 2005) at 20 °C, 14:10h light:dark, 60% humidity and with the diatom *Nitzschia curvilineata* as the sole food source.

These flatworms copulate promiscuously and frequently (mean number of copulations in 4h period: 24; range: 5-55; in Schärer et al. 2004). During the copulation, mating partners reciprocally insert their male copulatory organ into each other's (female) antrum, the female sperm-receiving and sperm-storing organ (Schärer et al. 2004; Vizoso et al. 2010). After some copulations the worms perform a peculiar sucking behavior, during which they place their pharynx over their own female genital opening and appear to suck out substances from the antrum, although there is no clear evidence yet concerning what exactly (if anything) is removed (Schärer et al. 2004; Vizoso et al. 2010). It has been hypothesized, however, that allosperm or prostate secretions are removed during this process, possibly allowing the sperm recipient to choose sperm from preferred mates or to remove potentially harmful prostate secretions (Schärer et al. 2004; Vizoso et al. 2010).

The individuals used in this study came from either the wild-type inbred DV lines (see **Chapter 4**), or the transgenic inbred LM lines (Marie-Orleach et al. 2017). Briefly, the DV lines were initiated by sampling individuals from outbred cultures established from several natural populations and inbred with 15 generations of maximal biparental inbreeding, followed by 9 generations of still substantial biparental inbreeding, and are now kept at small effective population sizes. The LM lines were generated by backcrossing worms from the green fluorescent protein (GFP)-expressing HUB1 line (Demircan 2013; Marie-Orleach et al. 2014; Wudarski et al. 2017) onto several DV lines for eight generations (see **Chapter 4** and Marie-Orleach et al. 2017 for a more detailed account of the establishment of the DV and LM lines, respectively). Since the GFP allele is dominant, LM individuals carrying at least one GFP allele will express GFP in all cells of their body, which makes it possible to observe and count their GFP-positive sperm cells after they have been transferred to the antrum of the (transparent) mating partner.

Experimental Design

We assigned experimental worms to one of three different roles that they can adopt during mating interactions: sperm donor, competitor or sperm recipient. Even though any one worm might adopt each of these roles during its mating interactions, we only observed, measured and analyzed it from the perspective of its pre-assigned role. Each sperm donor belonged to one of six independent fixed genotypes, each sperm recipient belonged to one of six different independent fixed genotypes, and each competitor belonged to the same fixed genotype that was again different from all sperm donor and sperm recipient genotypes. More specifically, the sperm donor genotypes were offspring from crosses between two LM lines (i.e., LM10xLM68, LM12xLM18, LM20xLM33, LM35x81, LM67xLM69 and LM71xLM84), the sperm recipient genotypes were each offspring from crosses between DV lines (DV8xDV22, DV65xDV83, DV44xDV61, DV31xDV50, DV28xDV29 and DV26xDV46), and the competitor genotype was the offspring from the line cross DV1xDV13. We then grouped the mature sperm donor and sperm recipient genotypes in a full-factorial design together with the standardized competitor and measured the success of the sperm donor along two subsequent pre- and postcopulatory episodes of sexual selection, allowing us to test for effects of sperm donor genotype, sperm recipient genotype and their interaction. For the sake of brevity we will from now on use the terms donor, recipient and competitor for sperm donor, sperm recipient and competitor, respectively.

Experimental Procedures

In order to raise outbred, fixed genotypes for the experiment, we initially transferred replicated triplets of adult worms of each DV and LM line (mean number of replicates: 13.1; range:7-14) from the laboratory cultures into a single well in 24-well tissue culture plates (TPP AG, Switzerland) with fresh algae (generation F0, see Fig. 1). After 28 days, during which these F0 triplets were transferred to new wells twice and produced offspring (generation F1, see Fig. 1), we then always paired F1 hatchlings from two DV lines or two LM lines in new wells. Then we let those pairs grow up together for 27 days (again transferred twice). Subsequently, we transferred the pairs again into new wells to lay eggs (mean number of pairs per cross: 27.2; range: 14-37) and produce offspring with fixed genotypes, which we then used as our experimental animals (F2, see Fig. 1). In order to reduce confounding effects of the parental environment on F2 individuals' phenotype and to therefore permit testing for genotype effects, all animals from the point of taking them out of the laboratory cultures experienced standardized conditions and we distributed the different inbred lines and fixed genotypes over the 24 wells per plate using a spatially-balanced restricted randomization.

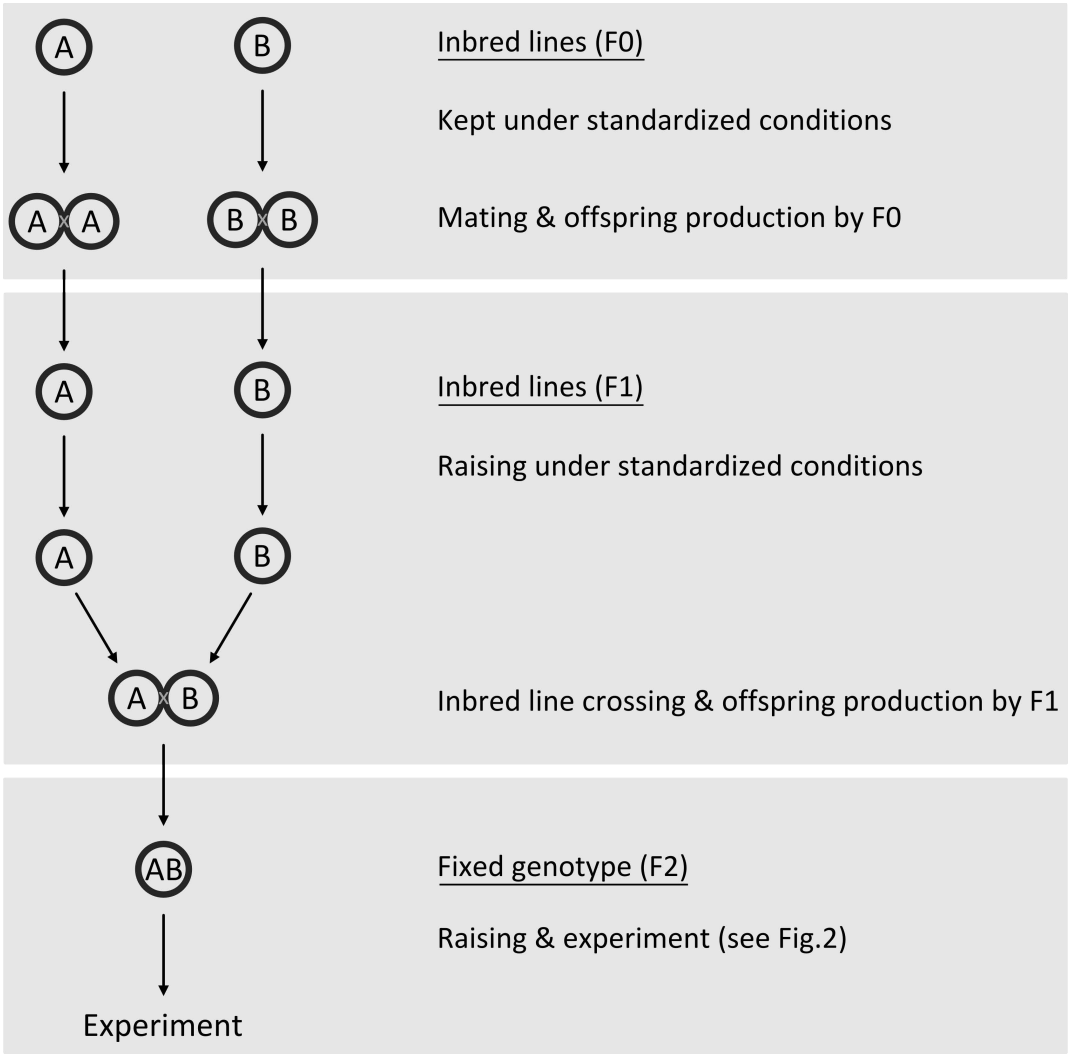

Figure 1. Schematic illustration of the crossing design to raise animals used in the experiment. Shown here is one cross between two inbred lines, A and B, resulting in the fixed genotype AB. This procedure was repeated for six LM line crosses, used as the sperm donors, and seven DV line crosses, of which one served as the competitor and the other six as the sperm recipients.

Due to time limitations, we divided the experiment into 10 blocks that were processed on separate days, each initially containing one complete set of all donor genotype x recipient genotype combinations (10 blocks x 6 donor genotypes x 6 recipient genotypes = 360 replicates in total). Within each block the time schedule was as follows. We let the adult F1 worms lay eggs from day 1 to 2, and paired donors with competitors and isolated recipients in 24-well plates on day 9. We then transferred the paired donors and competitors as well as the isolated recipients to wells with new algae on days 17 and 25. On day 32 we transferred the competitor and the recipient to new wells with red and blue food dye (7.1 mg/mL ASW New Coccine and 0.4 mg/mL Patent Blue, respectively) to make it possible to visually distinguish and separate them later. Both New Coccine and Patent Blue have been shown in previous experiments to not affect mating rate or offspring production significantly after 24h of dyeing (Sandner 2011; Marie-Orleach et al. 2013).

We then started the mating trials with the donor, the competitor and the virgin recipient on day 33. We assembled the triplets in drops of 4 µL ASW in observation chambers (Schärer et al. 2004) with 6 replicates per chamber and recorded a time lapse video (1 frame s^{-1}) of their behavior for the next 90 min with a digital video camera (DFK 41AF01, The Imaging Source, Bremen, Germany). From those mating movies we later determined (i) the number and the order of matings between the different worms (i.e. donor-recipient, donor-competitor, and competitor-recipient), (ii) the number of suck

behaviors performed by the recipient and the donor after donor-recipient matings, and (iii) the mating latency until the first donor-recipient mating in seconds.

Immediately after the mating trials we isolated the recipient, and transferred the competitor and donor together back to their original well. Subsequently, in order to estimate sperm transfer success, a postcopulatory fitness component, we recorded a movie of the antrum of the recipient to score the number of GFP-positive and GFP-negative sperm), and assessed the number of sperm in the recipient's antrum as described elsewhere (Janicke et al. 2011; Marie-Orleach et al. 2016). Briefly, we carefully squeezed worms between a 24 x 50 mm and a 21 x 26 mm cover slip separated by small plasticine feet and recorded a movie while focusing through its antrum under differential interference contrast illumination to count the total number of sperm stored. We then recorded a second movie while focusing through the antrum under epifluorescence illumination to count the number of GFP-positive sperm. For this we used a Leica DM 5000 B microscope (Leica Microsystems, Heerbrugg, Switzerland), with an epifluorescence light source and a digital microscope camera (Leica DFC360 FX, Leica Microsystems). We recorded movies with Leica Application Suite 4.1.0 (Leica Microsystems) and analyzed all data extracted from mating and antrum movies blindly with regards to treatments.

Although it is also possible to estimate paternity success by using the GFP marker (Marie-Orleach et al. 2014), long progeny arrays are needed to estimate paternity success with a reasonable precision, because of binomial sampling error (Marie-Orleach et al. 2016). However, this was not possible within the time frame of this experiment.

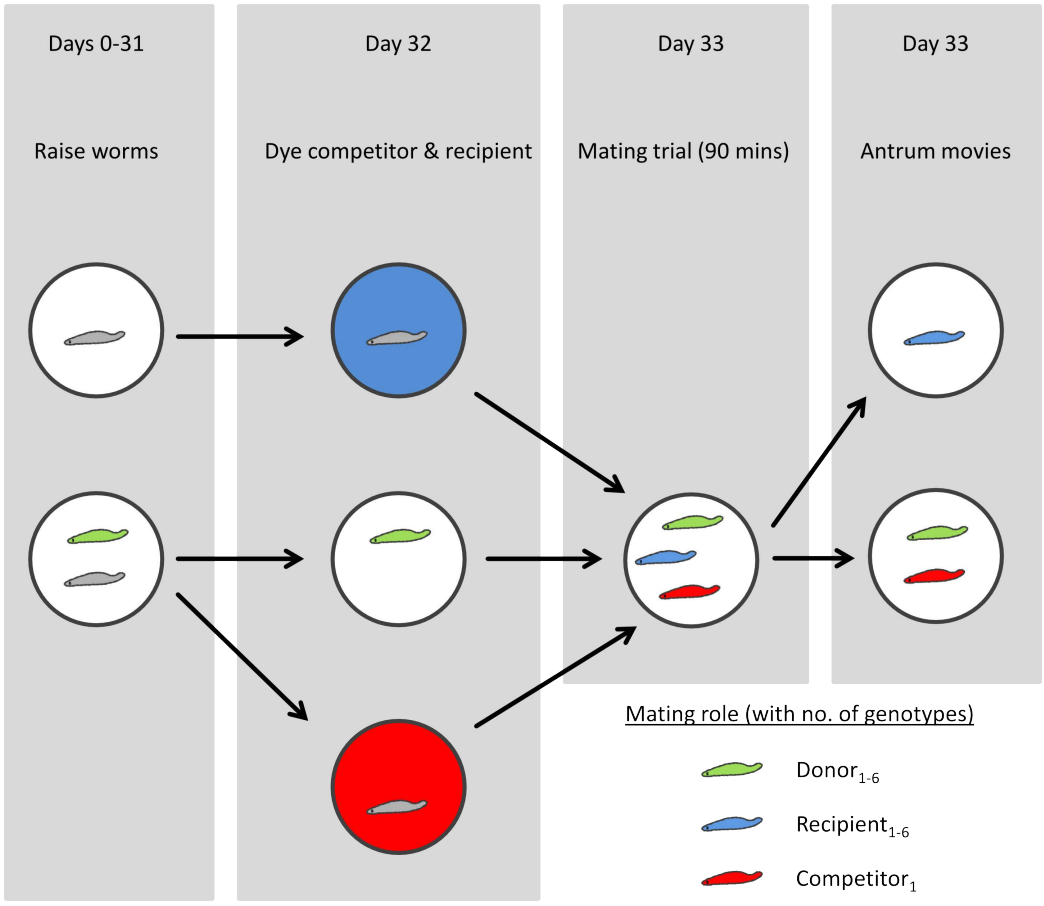

Figure 2. Schematic illustration of the experimental procedure. Note how the competitor and the virgin recipient were dyed on day 32 (with red and blue food dye, respectively) so that all worms could be identified afterwards.

Rationale for the Mating Behavior and Fitness Component Estimates

Mating Behavior Estimates

In addition to the number of matings we also quantified whether donors tended to mate with the recipients more at the beginning or the end of the mating trial, by calculating a 'relative mating rank' as follows. First, we calculated the average donor mating rank as,

$$\overline{donor\ mating\ rank} = \frac{sum\ of\ ranks\ of\ donor\ matings\ among\ all\ recipient\ matings}{Total\ number\ of\ donor-recipient\ matings}$$

and analogously for the competitor as,

$$\overline{competitor\ mating\ rank} = \frac{sum\ of\ ranks\ of\ competitor\ matings\ among\ all\ recipient\ matings}{Total\ number\ of\ competitor-recipient\ matings}$$

and then the relative mating rank of the donor as,

$$relative\ mating\ rank = \frac{\overline{donor\ mating\ rank}}{\overline{donor\ mating\ rank} + \overline{competitor\ mating\ rank}}$$

Values for the relative mating rank can vary between (but cannot adopt) zero and one and in replicates with a high relative mating rank value the donor-recipient matings occurred more towards the end of the mating trial compared to the competitor-recipient matings. We included relative mating rank because sperm displacement occurs in *M. lignano* (Marie-Orleach et al. 2014) and we therefore hypothesized that matings later in the sequence might contribute more to our measure of sperm transfer success. The mating latency can be viewed as a proxy for the preference that the mating partners have for each other and has been used in previous studies to describe mating interactions (e.g., Marie-Orleach et al. 2013; Castillo and Delph 2016). We therefore included it in the analysis. And since the postcopulatory suck behavior has been hypothesized to be involved in cryptic female choice or sexual conflict (Schärer et al. 2004; Vizoso et al. 2010)—for which donor and recipient genotype effects can be predicted—we also calculated the proportions of donor-recipient matings after which either the donor or recipient sucked.

Fitness Component Estimates

In this study one of our main interests was how donor and recipient genotypes affect both pre- and postcopulatory fitness components. Fitness components can be calculated directly from the estimated data (raw fitness components) or from other fitness components (derived fitness components) (Marie-Orleach et al. 2016). First, we calculated mating success and sperm transfer success directly from the data. For this we defined mating success, the first (precopulatory) fitness component for each sperm donor, as the proportion of donor-recipient matings of the total number of matings that the recipient had (median: 32.3, range: 1-72). We estimated sperm transfer success of the donor, the second (postcopulatory) fitness component, as the proportion of the donor's (GFP-positive) sperm of the total number of sperm in the antrum of the recipient (median: 24, range: 0-66).

Sperm transfer success can be expected to be, to some extent, due to the mating success achieved, but it is also of interest whether and how donor and recipient genotypes influence the translation of mating success into sperm transfer success. Marie-Orleach et al. (2016) proposed the term 'sperm transfer efficiency' and calculated this derived fitness component by dividing sperm transfer success by mating success (cf. Fig. 1 in Marie-Orleach et al. 2016). Although this is an informative fitness component, this variable was unfortunately distributed in such a way for our data that assumptions of statistical tests were violated. Thus, we followed another approach. Namely, we fitted a binomial GLM with sperm transfer success as response and mating success as predictor variable, extracted the residuals and used them as an estimate of sperm transfer efficiency. Sperm transfer efficiency in our study therefore shows how much more (or less) sperm transfer success a donor achieved than would have been expected based on the achieved mating success alone.

Statistics

We analyzed mating behaviors with either linear models (LMs) or generalized linear models (GLMs) using the software R (version 3.2.4). We used a LM to model the effects on the relative mating rank and the square-root transformed mating latency (i.e., time in seconds until the first donor-recipient mating). The proportions of donor-recipient matings that were followed by a donor or recipient suck behavior, respectively, were modelled with GLMs using a binomial error distribution and a logit-link function, in which the variance is given by the product of the mean and the dispersion parameter φ (i.e., with multiplicative overdispersion) (R Development Core Team 2008). For all mating behavior models the effects included were donor genotype, recipient genotype and their interaction.

To study pre- and post-copulatory success, we also fitted analogous binomial GLMs to model the two fitness components, namely mating and sperm transfer success, with donor genotype, recipient genotype and their interaction as predictor variables. For sperm transfer success as the response we also included, in separate models, mating success and mating success together with the relative mating rank of the donor as additional predictor variables (Table 2). This was done to test whether donors that mated more often and more towards the end of the mating trial had a higher sperm transfer success, as could be expected because of the previously documented sperm displacement (Marie-Orleach et al. 2014).

For all of the above LMs and GLMs we tested the significance of effects by performing F-tests using "type III" sum of squares using the 'Anova()' function of the 'car' package in R (Fox and Weisberg 2011). We chose "type III" sum of squares for the F-test because they are recommended for unbalanced data and control for the effect of the interaction while testing for the significance of main effects (Quinn and Keough 2002). Finally, we assessed whether the assumptions for all models were fulfilled by visually inspecting residuals versus predicted values plots and normal quantile-quantile plots (Faraway 2016).

We had to exclude a number of replicates because of handling and pipetting errors and furthermore, different replicates had to be excluded depending on which response variable we analyzed (e.g., sperm transfer success can only be estimated in replicates in which no egg in the antrum obscured the sperm count and in which the recipient received at least one sperm cell, etc.). Therefore we provide short information about the respective data sets used in Table 1 and 2. All confidence intervals reported are 95% confidence intervals. In order to test whether donors had a relative mating rank significantly different from 0.5, whether donors had a significantly different sperm transfer and mating success and whether donors started mating at a significantly different time from the competitor we used Wilcoxon signed rank tests in R (R Development Core Team 2008).

Results

Mating Behaviors

The matings of the donors occurred slightly but significantly more often towards the end of the mating trial compared to the competitor (mean relative mating rank: 0.549, CI: 0.539-0.560; Wilcoxon signed rank test: $V=26353$, $p<0.0001$). The donor genotypes differed in their relative mating rank, and the interaction between donor and recipient genotype had a significant effect, while the recipient genotype was not significant (Fig. 3a & Table 1). The median latency until the first donor-recipient mating was 1434s (interquartile range: 715-2316s). Mating latency was significantly affected by both donor and recipient genotype, but not by their interaction (Fig. 3b and Table 1). On average donors and recipients, respectively, sucked after a proportion of 0.290 (CI: 0.262-0.319) and 0.401 (CI: 0.380-0.423) of their matings with the donor, and the values were influenced by their own genotype (Fig. 3cd

and Table 1). However, the GxG interaction was only significant for the proportion of sucks by the recipient (Fig. 3d and Table 1).

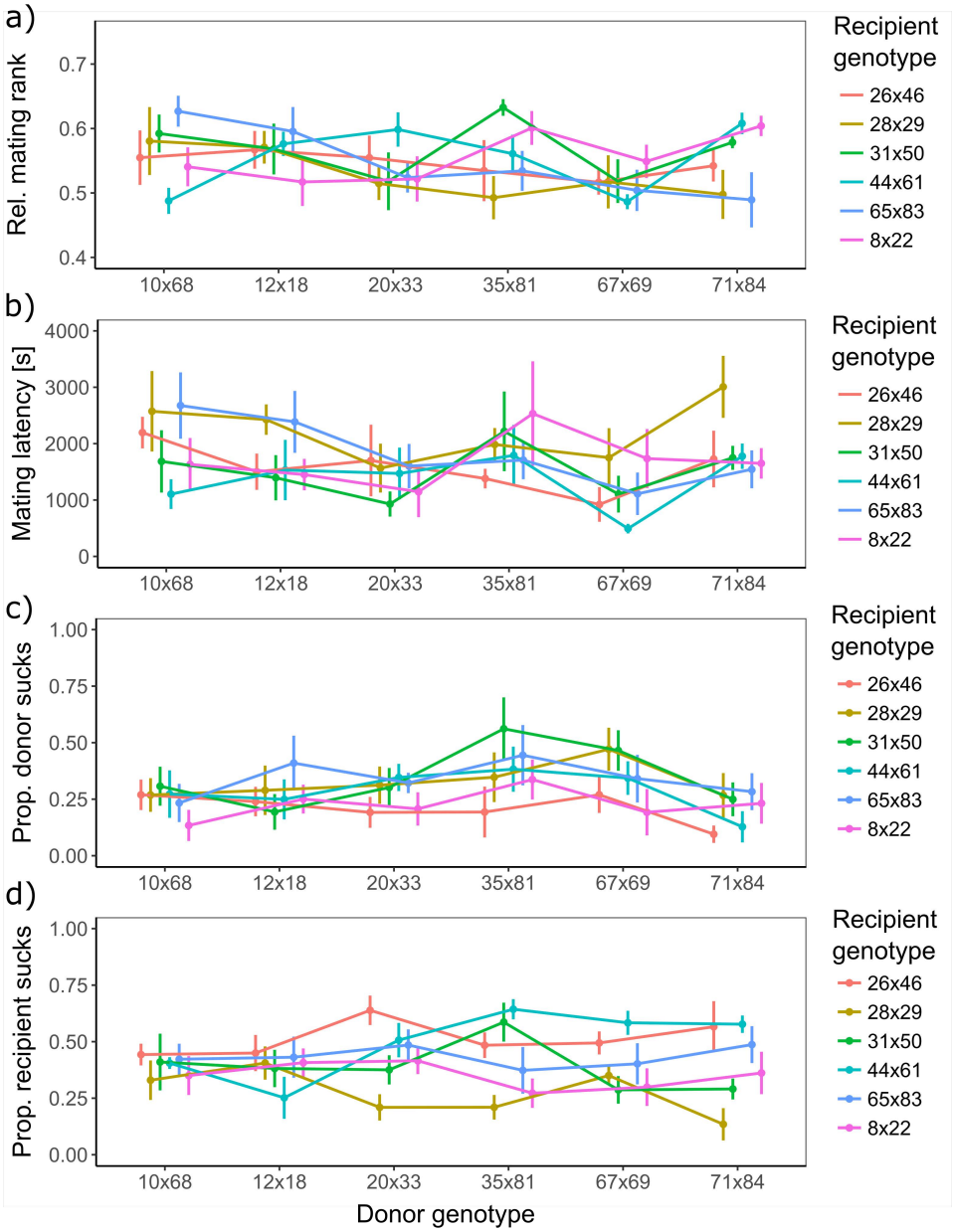

Figure 3. Effects of the sperm donor and sperm recipient genotype on the mating behaviors: a) relative mating rank (i.e., larger values mean the donor tended to mate later than the competitor); b) mating latency (i.e., time until first donor-recipient mating); c) the proportion of donor-recipient matings followed by a donor suck behavior; and d) the proportion of donor-recipient matings followed by a recipient suck behavior. Plotted are the means (±SE) for each genotype combination and the values with the same recipient genotype are connected with colored lines.

Table 1. LMs and GLMs testing for donor (D) and recipient (R) genotype effects on mating behaviors. The relative mating rank and mating latency (square-root transformed) were analyzed with LMs. The proportions of matings followed by suck behaviors were analyzed with GLMs assuming a binomial distributions correcting for overdispersion (with dispersion parameter φ). Different replicates had to be excluded for the analyses of the respective mating behaviors and exclusion criteria are explained in the "Dataset" column.

Response	Effect	SS	d.f.	F-ratio	P-value	φ	Dataset
Relative mating rank	Donor genotype	0.086	5	2.346	**0.042**	-	Replicates where the donor and competitor mated at least once with the recipient (n=263)
	Recipient genotype	0.035	5	0.972	0.435		
	DxR interaction	0.304	25	1.663	**0.029**		
	Residual	1.657	227	-	-		
Mating latency	Donor genotype	4675	5	4.372	**<0.001**	-	Replicates where the donor mated at least once with the recipient (n=265)
	Recipient genotype	3075	5	2.876	**0.015**		
	DxR interaction	3991	25	0.746	0.805		
	Residual	48975	229	-	-		
Proportion of matings followed by donor suck	Donor genotype	41.45	5	3.022	**0.012**	2.74	Replicates where the donor mated at least once with the recipient (n=265)
	Recipient genotype	21.04	5	1.534	0.180		
	DxR interaction	52.2	25	0.761	0.788		
	Residual	628.07	229	-	-		
Proportion of matings followed by recipient suck	Donor genotype	5.498	5	0.800	0.551	1.37	Replicates where the donor mated at least once with the recipient (n=265)
	Recipient genotype	66.71	5	9.708	**<0.0001**		
	DxR interaction	59.32	25	1.726	**0.020**		
	Residual	314.73	229	-	-		

Pre- and Postcopulatory Fitness Components

Donors were on average able to secure a proportion of 0.356 (CI: 0.332-0.382) of the recipient's matings, and donor genotype significantly affected mating success, while recipient genotype and their interaction did not (Fig. 4a and Table 2).

Donors were able to achieve an average sperm transfer success of 0.588 (CI: 0.528-0.588), which was significantly higher than their mating success (Wilcoxon signed rank test: $V=22079$, $p<0.0001$). Sperm transfer success was also only significantly affected by donor genotype (Fig. 4b and Table 2). Donors that mated more often relative to their competitors transferred significantly more sperm (Table 2 and Fig. 5). Interestingly, the donor genotype LM71xLM84 had a much lower sperm transfer success than the others, although worms of that genotype had a mating success that was comparable to the other genotypes (cf. Fig. 4ab). In contrast to our prediction, whether matings occurred towards the end of the mating trial did not significantly affect sperm transfer success (Table 2).

Sperm transfer efficiency was strongly and significantly affected by donor genotype, but not by recipient genotype or their interaction (Fig. 4c and Table 2).

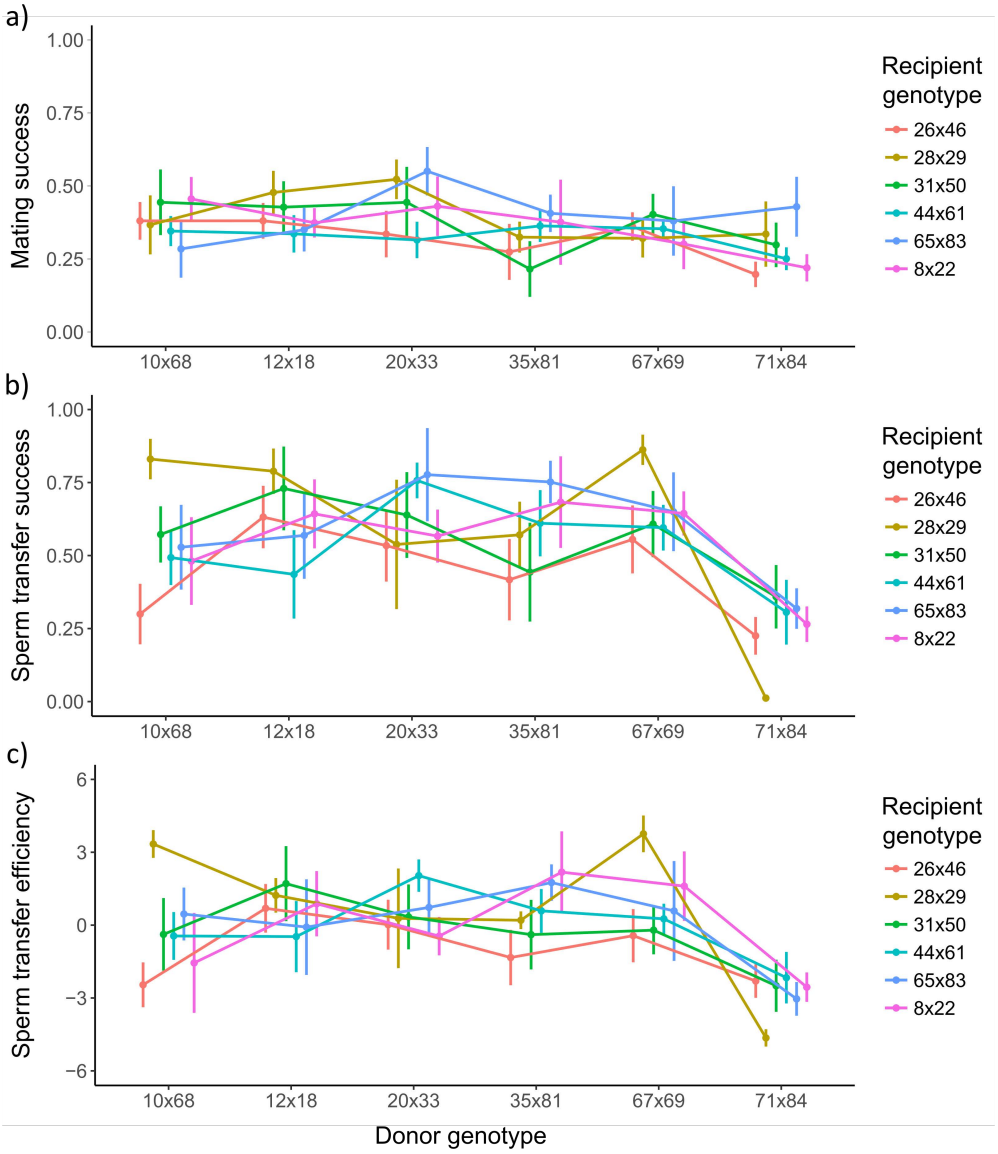

Figure 4. Effects of sperm donor and sperm recipient genotype on pre- and post-copulatory fitness components: a) mating success, b) sperm transfer success and c) sperm transfer efficiency. Plotted are the means (±SE) for each genotype combination and the values with the same recipient genotype are connected with colored lines. Note that the SE of the 71x84-28x29 combination in b) is so small that it is hidden behind the filled circle.

Table 2. GLMs (for mating success and sperm transfer success) and linear model (for sperm transfer efficiency) testing for donor (D) and recipient (R) genotype and mating behavior effects. GLMs assumed a binomial distribution and corrected for overdispersion with dispersion parameter **φ**. Different replicates had to be excluded for the analyses of the respective fitness components and exclusion criteria are explained in the "Dataset" column.

Response	Effect	SS	d.f.	F-ratio	P-value	φ	Dataset
Mating success	Donor genotype	97.16	5	3.296	**0.007**	5.90	Replicates where the recipient mated at least once (n=277)
	Recipient genotype	31.17	5	1.057	0.385		
	DxR interaction	184.0	25	1.248	0.199		
	Residual	1421.1	241	-	-		
Sperm transfer success	Donor genotype	554.9	5	11.352	<**0.0001**	9.78	Replicates where the recipient received at least one sperm cell (n=252)
	Recipient genotype	45.12	5	0.923	0.467		
	DxR interaction	253.8	25	1.038	0.419		
	Residual	2111.9	216	-	-		
Sperm transfer success	Donor genotype	396.7	5	8.745	<**0.0001**	9.07	Replicates where the recipient mated at least once with the donor and received at least one sperm cell (n=244)
	Recipient genotype	26.3	5	0.580	0.716		
	Mating success	414.8	1	45.717	<**0.0001**		
	DxR interaction	259.8	25	1.145	0.295		
	Residual	1878.3	207	-	-		
Sperm transfer success	Donor genotype	402.1	5	9.662	<**0.0001**	8.32	Replicates where the recipient mated at least once with the donor and recipient and received at least one sperm cell (n=235)
	Recipient genotype	26.8	5	0.644	0.667		
	Mating success	269.4	1	32.363	<**0.0001**		
	Rel. mating rank	17.1	1	2.056	0.153		
	DxR interaction	246.5	25	1.185	0.257		
	Residual	1631.4	196	-	-		
Sperm transfer efficiency (residual)	Donor genotype	413.9	5	8.774	<**0.0001**	-	Replicates where the recipient mated at least once with donor and received at least one sperm cell (n=244)
	Recipient genotype	59.3	5	1.257	0.284		
	DxR interaction	279.9	25	1.187	0.254		
	Residual	1962.5	208	-	-		

Discussion

While we found evidence for donor x recipient genotype interactions for two mating behaviors—relative mating rank and the proportion of recipient sucks—we, in contrast to our expectations, found no evidence for such interactions affecting either the pre- and postcopulatory fitness components (Table 1 and 2). Moreover, there were significant, and in some cases strong, effects of the genotype of the donor on almost all variables considered, including the fitness components. In the following we discuss the implications of these results for sexual selection and sexual conflict in *M. lignano* and copulating simultaneous hermaphrodites in general.

Mating Behaviors

Relative Mating Rank

Donors mated significantly more towards the end of the mating period, presumably because they started mating later than the competitor (Wilcoxon signed rank test: $V=23436$, $p<0.0001$). It is possible that the (single) competitor genotype started to mate earlier than the donor genotypes because it happened to have a very high intrinsic mating rate due to a sampling effect when choosing the inbred lines. Moreover, donor genotypes differed in their relative mating ranks, which could mean that they need more or less time to acclimatize to the new conditions in the mating chamber or that they are more or less bold in approaching novel mating partners. Interestingly, whether donors and recipients tended to mate more at the beginning or the end of the mating trial was significantly affected by the interaction of their genotypes. This could indicate that certain recipient genotypes resist mating attempts by certain genotypes for longer than others (or that they prefer some over others).

Mating Latency

This donor x recipient genotype interaction for relative mating rank does, however, not seem to affect mating latency, possibly indicating that these interactions manifest themselves only later during the mating trial, because the worms need some time to acclimatize to the condition. In contrast to our results, Marie-Orleach et al. (2017) did find significant GxG interactions for mating latency in a different experiment with *M. lignano*. However, the experimental designs differed in two aspects: Marie-Orleach et al. used a somewhat different subset of lines to breed their fixed genotypes (and in different combinations) and mating behavior was not tested in the presence of a competitor, but rather in pairs. The results may therefore not be directly comparable. Mating latency and even 'interaction latency' (the latency until the first behavioral interaction between male and female) have been proposed as informative measures having implications for assortative mating and speciation (Castillo and Delph 2016). In the same study, the authors found a significant male x female genotype interaction effect on 'interaction latency' although they did not find such an effect on mating latency.

Proportion of Matings Followed by Donor and Recipient Sucks

Both the proportion of matings followed by donor, and recipient sucks was mainly influenced by the genotype of the individual performing the suck, indicating that the worms have strong control over their own behavior. However, because the sucking behavior has been hypothesized to be involved in sexual conflict over the fate of transferred sperm or potentially harmful seminal fluids, we also expected there to be interaction effects (Schärer et al. 2004; Vizoso et al. 2010; Marie-Orleach et al. 2013). We did indeed find a significant interaction for the proportion of recipient sucks, but not for the proportion of donor sucks (Table 1). As above, donors could either be different from recipients in this respect because of sampling effects when assigning the six donor and recipient genotypes or because recipients were virgins before the mating trial. In particular, Marie-Orleach et al. (2013) found that *M. lignano* individuals sucked significantly less after mating with a virgin mating partner compared to a sexually-experienced partner, which could be linked to receiving more sperm and seminal fluids

from a virgin. Specifically, virgin worms may be more successful at preventing their partner from sucking, because, not having previously expended them, they have more densely filled prostate glands, and might therefore be able to transfer larger amounts of manipulating prostate gland secretions (Marie-Orleach et al. 2013). In our experiment, the donors also sucked less often after mating with the *virgin* recipient compared to the recipient after mating with the *experienced* donor (Wilcoxon signed rank test: V=17470, p<0.0001; Fig. 3cd). Possibly, GxG interactions manifest themselves more easily in how often the recipient sucks than in how often the donor sucks because the rather weak interaction effects are steamrolled by the possibly very strong effect of the presumably more copious prostate gland secretions transferred by virgins.

In their study, Marie-Orleach et al. (2017) did not find significant interactions for suck behavior although the results might, again, not be directly comparable. Namely, the authors used the absolute number of sucks performed after the first five matings as the trait of interest, while we used the proportion of all matings followed by a suck in this study (see methods section). The donor x recipient genotype interaction effect on the recipient's suck behavior we found is consistent with a role of this suck behavior during sexual conflict. Sexual conflict between sperm donor and sperm recipient interests is likely an important driver of mating interactions in simultaneous hermaphrodites (Michiels 1998; Schärer et al. 2014). Sexual conflict over the fate of received sperm and/or seminal gland products have been proposed as a potential explanation for the evolution of this behavior (Schärer et al. 2004; Vizoso et al. 2010). Conflicts over sperm receipt arise from the fact that hermaphrodites will likely tend to prefer the male role during a mating interaction (Charnov 1979) and conflicts over receiving seminal gland products are assumed to occur over the possibility that those substances manipulate the partner's future fecundity, e.g., by manipulating their sex allocation towards more female allocation (Charnov 1979; Michiels 1998; Schärer and Janicke 2009; Schärer et al. 2014). Follow-up experiments are now needed to determine what exactly the presumed sexual conflict involving the suck behavior is about, including a characterization of the biological activity of seminal fluid.

Fitness Components

A new contribution of this study to the field of interacting phenotypes and GxG research is the estimation of these interaction effects on fitness components in a simultaneous hermaphrodite. Testing for these interaction effects in simultaneous hermaphrodites offers a broader picture with respect to the number of phylogenetic groups and mating systems in which these interactions may occur. Furthermore, a shift of sexual conflict and sexual selection to the postcopulatory arena has been predicted in reciprocally copulating simultaneous hermaphrodites compared to gonochorists (Schärer et al. 2014), which could have been detected with our experimental design.

Mating Success

Donors secured less than half of the recipient matings, presumably because it took them longer to start mating with the recipient than it did the competitors (Wilcoxon signed rank test for difference between mating latency of competitor and donor: V=23436, p<0.0001).

Since donor genotypes differed in their mating latency and relative mating rank (Table 1), starting later and generally mating later may have caused them to achieve a lower mating success during the entire mating trial. It seems unlikely, however, that this is the only reason for the donor genotype effect, because it remains significant even when mating latency and relative mating rank are included in the model (GLM: $F_{5,225}$=3.76, p=0.003). Although mating success is an important component of a sperm donor's reproductive success, to our knowledge, we are the first to test mating partner GxG effects on this selection episode separately from the postcopulatory selection episodes. Even though Castillo and Delph (2016) found interaction effects on 'interaction latency' this measure is arguably still quite far removed from mating success, which is the relevant fitness component in this context.

Sperm Transfer Success

Despite the donor's moderate mating success of just over a third of the matings of the recipients, they managed to achieve a significantly higher sperm transfer success of close to 60% of the sperm stored by the recipients (Fig. 4ab). One possible caveat for this comparison is that we can only make this claim if we do not overestimate the count of the GFP+ sperm, which could result in higher sperm transfer success values. However, counts of GFP+ sperm seem to be underestimated rather than overestimated compared to GFP- sperm counts (cf. Experiment 6 in Marie-Orleach et al. 2014). Since sperm displacement has been described in this species, mating more towards the end of the mating trial could make it possible for the donors to displace a high proportion of the competitor's sperm, leading to a higher sperm transfer success. Interestingly, however, in this experiment variation *within* the donors in whether they mated late during the mating trial did not significantly influence sperm transfer success (Table 2). In contrast, there was a strong and highly significant effect of mating success on sperm transfer success within the donors (Table 2 and Fig. 5); an effect that has been previously shown in *M. lignano* (Marie-Orleach et al. 2016). Pélissié et al. (2014) showed that the mating rank affected the number of offspring a sperm donor sired in the snail *Physa acuta*, indicating first donor sperm precedence. We predicted a mating rank effect in *M. lignano* because of an assumed last donor sperm precedence (Marie-Orleach et al. 2014). The lack of a mating rank effect in our study could be due to the fact that the mating order of only the last few matings before the end of the mating trial actually affects sperm transfer success. A re-analysis focusing only on the last few mating interactions therefore appears worthwhile.

The sperm donor genotype LM71&LM84 seems to be an interesting outlier with regards to the sperm transfer success it achieved (Fig. 4b). Namely, it had a much lower sperm transfer success than all the other donor genotypes despite having a comparable mating success, which is also illustrated by the very low sperm transfer efficiency of this genotype (Fig. 4c). Furthermore, it did not have an particularly long mating latency or low relative mating rank, which could have explained its low sperm transfer success. Therefore, it seems that LM71&LM84 worms managed to successfully transfer substantially less sperm per copulation and/or that they displaced much less sperm in comparison to the competitor than the other donor genotypes. An analysis of the morphology of those worms with respect to testis and seminal vesicle size would be interesting to confirm this hypothesis, because worms with bigger testis size have a higher sperm transfer efficiency (Marie-Orleach et al. 2016).

In contrast to the lack of studies examining GxG interactions for mating success, there are, to our knowledge, three studies estimating such interaction effects for fertilization or paternity success in gonochorists (Clark et al. 1999; Evans et al. 2012; Simmons et al. 2014). Of these three studies only the first two found these interactions to be significant. Therefore, it seems that GxG interactions during pre- and/or postcopulatory episodes could be important in gonochorists. But are they also important in hermaphrodites? Although we strongly expect that these GxG interactions should also be present in hermaphrodites, especially reciprocally copulating ones (see Introduction), we neither are aware of any such findings in the literature nor did we find such interactions in our study. *M. lignano* copulates reciprocally, in a closely interlocking posture (think of two interlocking "G"s; cf. Fig. 5 in Schärer et al. 2004). Thus, there is arguably a lot of scope for interactions between the mating partners to influence mating success as well as sperm transfer success. It has been argued that sexual conflict and sexual selection are shifted more towards the postcopulatory episode in reciprocally copulating hermaphrodites (Schärer et al. 2014, see also **Chapter 1**). Assuming this to be true, one might expect the strongest GxG interactions to be manifested during the postcopulatory episode, where ejaculates of the sperm donor can interact with the female morphology and behavior of the sperm recipient. Our measure of sperm transfer success captures some part of this episode, but since we did not estimate paternity success in this study we have no information about the influence of GxG interactions on

what happens after sperm is stored in the antrum. Testing for GxG interaction effects on the remaining part of the postcopulatory fitness component of the donor, 'sperm fertilization efficiency' (*sensu* Marie-Orleach et al. 2016), which could be estimated with another experimental design, seems to be an interesting study to conduct next. One way to compare the importance of different selection episodes is with the measurement 'opportunity for selection', which can be considered an upper bound for the strength of selection (Crow 1958; Arnold and Wade 1984). Interestingly, Marie-Orleach et al. (2016) found the opportunity for selection for mating success to be smaller than for the remaining postcopulatory success. In particular, sperm fertilization efficiency contributed most to male reproductive success.

Sperm Transfer Efficiency

An interesting pattern that emerges when comparing mating and sperm transfer success is that few individuals achieved a mating success of 0 or 1, but that a high proportion achieved a sperm transfer success of 0 or 1 (Fig. 5). Therefore, there must be unknown factors introducing this variation. Such factors could include i) strong sperm displacement by the last sperm donor, ii) a strong manipulation of the ejaculate by recipient controlled processes (e.g., sperm digestion, cryptic female choice or egg laying).

Concerning the first point, as already mentioned, we know that *M. lignano* exhibits sperm displacement (Marie-Orleach et al. 2014). However, we do not know the exact mechanism by which this happens. Mechanical removal does not seem very likely, because in contrast to the presence of special aedaegus appendages in members of Odonata (Waage 1979; Córdoba-Aguilar et al. 2003) the intromittent stylet in *M. lignano* does not exhibit striking appendices that would facilitate removal (Ladurner et al. 2005; Vizoso et al. 2010). Instead it seems more likely that the sperm can be removed volumetrically by the competitor's ejaculate, as has been shown in *D. melanogaster* (Price et al. 1999; Manier et al. 2010). Concerning the second point, the suck behavior could potentially remove a substantial amount of the sperm in the antrum, so that the next donor after an effective suck behavior would achieve a sperm transfer success of 1. However, to date we never found significant effects of the suck behavior on sperm transfer success in this species, possibly because the behavior might be more directed at other ejaculate components, such as seminal fluid. Egg laying may also lead to sperm loss from the antrum since the eggs can push some sperm out of the antrum when it is laid (L. Schärer, pers. obs.). However, only very few recipients laid eggs during the mating trials in this experiment so that egg laying alone cannot explain such a high number of 0's and 1's in sperm transfer success as visible in Fig. 5 (NV, pers. obs.).

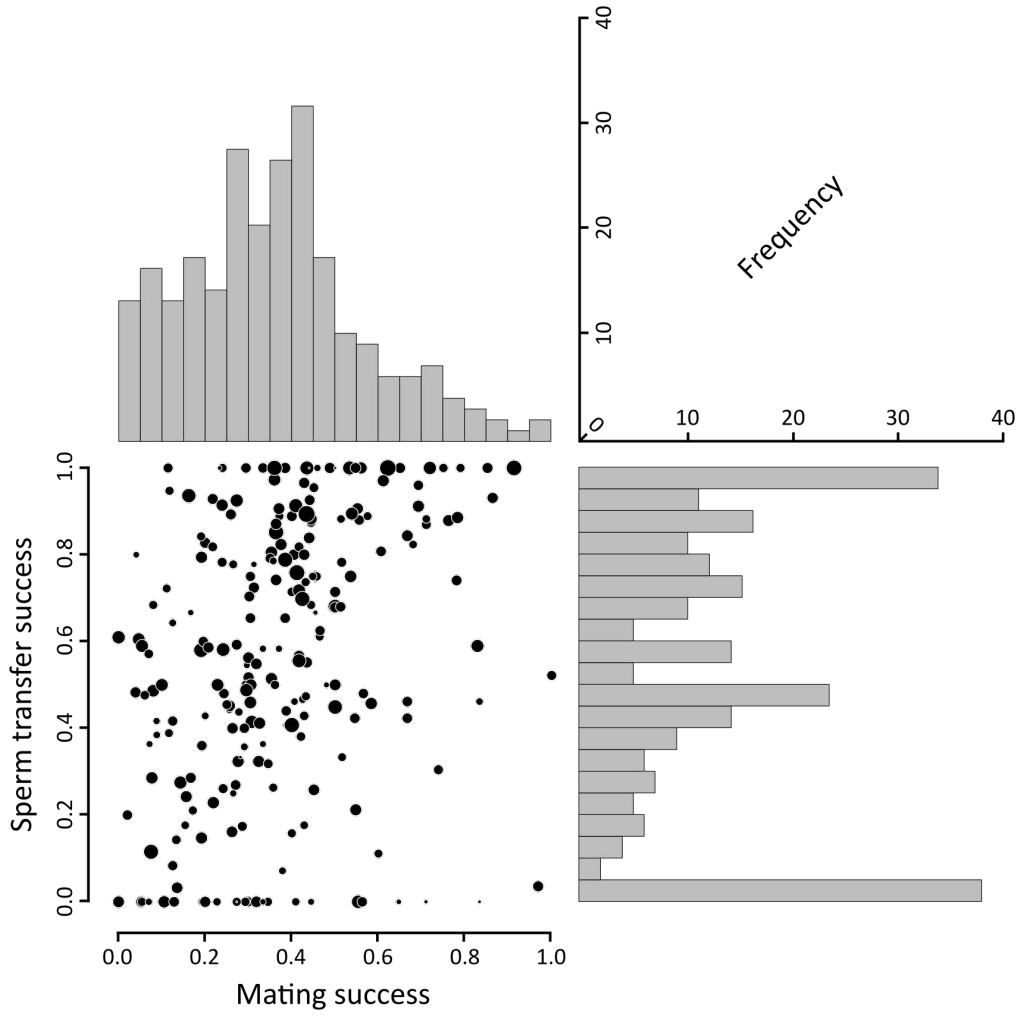

Figure 5. Sperm transfer success as a function of mating success. Areas of black circles indicate the total sperm number in the recipient's antrum (i.e., sperm transfer success of replicates with big circles are estimated with less binomial sampling error). A frequency histogram was added for mating success at the top and for sperm transfer success at the right hand side. Note the high proportion of very low (mainly 0's) and very high (mainly 1's) values for sperm transfer success.

Conclusions

We found GxG interaction effects on two mating behaviors, but not on the pre- and postcopulatory fitness components measured, although there were strong effects of the sperm donor genotype on almost all traits measured. Since strong interactions can be expected in reciprocally copulating hermaphrodites it seems worthwhile to test for them in follow-up experiments using slightly different designs and/or genotypes. For instance, since sexual selection and sexual conflict has been predicted to be shifted more towards the postcopulatory arena in reciprocally copulating hermaphrodites (Schärer et al. 2014) a more complete estimation of fitness components spanning the whole postcopulatory episode could possibly detect interaction effects. Furthermore, being able to quantify the currently unknown factors influencing mating success, sperm transfer success and the resulting paternity success will be helpful to pinpoint where during the different episodes of sexual selection GxG interactions (if present) manifest themselves.

Acknowledgements

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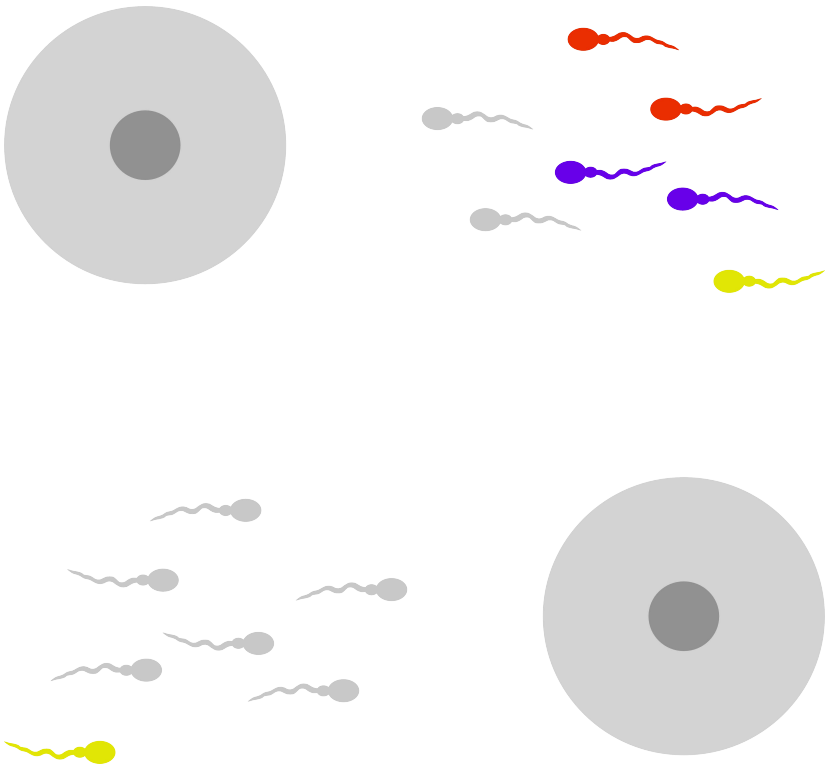
Chapter 3

Bigger Testes Increase Paternity in a Simultaneous Hermaphrodite, Independently of the Sperm Competition Level



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Bigger testes increase paternity in a simultaneous hermaphrodite, independently of the sperm competition level

N. VELLNOW* , L. MARIE-ORLEACH†, K. S. ZADESENETS‡ & L. SCHÄRER*

*Zoological Institute, Evolutionary Biology, University of Basel, Basel, Switzerland
†School of Biology, University of St Andrews, Fife, UK
‡Institute of Cytology and Genetics SB RAS, Novosibirsk, Russia

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sperm competition;
testis size.

Abstract

Hermaphroditic animals face the fundamental evolutionary optimization problem of allocating their resources to their male vs. female reproductive function (e.g. testes and sperm vs. ovaries and eggs), and this optimal sex allocation can be affected by both pre- and post-copulatory sexual selection. For example, local sperm competition (LSC) – the competition between related sperm for the fertilization of a partner's ova – occurs in small mating groups and can favour a female-biased sex allocation, because, under LSC, investment into sperm production is predicted to show diminishing fitness returns. Here, we test whether higher testis investment increases an individual's paternity success under sperm competition, and whether the strength of this effect diminishes when LSC is stronger, as predicted by sex allocation theory. We created two subsets of individuals of the simultaneously hermaphroditic flatworm *Macrostomum lignano* – by sampling worms from either the highest or lowest quartile of the testis investment distribution – and estimated their paternity success in group sizes of either three (strong LSC) or eight individuals (weak LSC). Specifically, using transgenic focal individuals expressing a dominant green-fluorescent protein marker, we showed that worms with high testis investment sired 22% more offspring relative to those with low investment, corroborating previous findings in *M. lignano* and other species. However, the strength of this effect was not significantly modulated by the experienced group size, contrasting theoretical expectations of more strongly diminishing fitness returns under strong LSC. We discuss the possible implications for the evolutionary maintenance of hermaphroditism in *M. lignano*.

Introduction

General aspects of sex allocation

All sexually reproducing organisms face the evolutionary optimization problem of how much of their limited resources they should invest into male vs. female reproduction, that is, the problem of sex allocation (Charnov, 1982). Consider, for example, producing the optimal offspring sex ratio in gonochoristic (separate-sexed) animals. As during sexual reproduction both parents contribute the same amount of nuclear genetic material to the zygote, the total fitness of males and females – on the population level – has to be equal (Düsing, 1884; Fisher, 1930; Houston & McNamara, 2006; Queller, 2006). Therefore, negative frequency-dependent selection will tend to select for the production of more daughters when females are rare in the population and of more sons when males are rare. Given certain, arguably strong, simplifying assumptions, including random mating (i.e. any male gamete in the population has the same probability of fusing with any female gamete) and large population size (i.e. mates and competitors are always unrelated) (Hamilton, 1967; Schärer & Ramm, 2016), this is expected to lead to equal investment into sons and daughters over evolutionary time (Düsing, 1884; Fisher, 1930; Charnov, 1982).

Although the principle of equal investment resulting from this so-called Fisher condition was initially

Correspondence: Nikolas Vellnow, Zoological Institute, Evolutionary Biology, University of Basel, Vesalgasse 1, CH-4051 Basel, Switzerland.
Tel.: +41 61 267 03 75; fax: +41 61 207 03 62;
e-mail: nikolas.vellnow@unibas.ch

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formulated with gonochoristic animals in mind, it actually applies more generally. Under the same simplifying assumptions, an equal investment into male and female function could also be expected in (nonselfing) simultaneous hermaphrodites, because also here every zygote gets half of its nuclear genetic material from each parent (though, as we outline below, these assumptions are likely often broken in hermaphrodites). In these organisms, it is not the amount of investment into daughters and sons that is selected to be equal. Instead, the balance between investment into the male function (e.g. sperm, male-specific tissues and male-specific behaviours) on the one hand, and investment into the female function (e.g. eggs, female-specific tissues and female-specific behaviours) on the other hand, is the target of selection (Charnov, 1982, pp. 7–9; Schärer, 2009).

In spite of this general tendency towards equal investment, there are many conditions under which equal investment into the male and female function is not the evolutionary stable strategy. For instance, Hamilton (1967) drew attention to local mate competition (i.e. competition between related individuals for access to mates) in gonochoristic animals, in which – in the extreme case – the sons of a single female compete for the fertilization of their own sisters. Such local mate competition violates the random mating and large population size assumptions and selects for increased investment into daughters, because investment into more than just a few sons will not increase the number of grandchildren the mother produces. Investment into additional sons is therefore wasteful, favouring the production of more daughters and fewer sons (Charnov, 1982; West, 2009). Analogously, nonrandom mating can also affect the optimal sex allocation in simultaneous hermaphrodites, which we explore in the following section.

Sexual selection influences sex allocation

Although in anisogamous species, both parents contribute an equal amount of nuclear genetic material to the zygote, the mother contributes much more resources via her egg than the father via his sperm. In the absence of substantial post-zygotic paternal investment, this is expected to lead to 'classical' sex roles (Darwin, 1871; Bateman, 1948; Dewsbury, 2005; Parker & Birkhead, 2013; Lehtonen *et al.*, 2016), with males competing for access to females and the fertilization of their eggs, whereas females may choose which males to give access to their unfertilized eggs. In copulating organisms, sexual selection can be subdivided into precopulatory and post-copulatory episodes. During the precopulatory episode, males will tend to compete among each other for matings and females will tend to choose with whom to mate. When a female has mated with two or more males, they will often continue to compete for the fertilization of her eggs via their ejaculates – termed sperm competition (Parker, 1970) – and the female may bias which male's sperm to fertilize her eggs with – termed cryptic female choice (Thornhill, 1983). These episodes of pre- and post-copulatory sexual selection take place both in gonochorists and in simultaneous hermaphrodites, whereas in the latter, individuals may simultaneously compete and chose during the pre- and post-copulatory episodes in their respective roles as sperm donors and sperm recipients (Charnov, 1979).

In his influential work on sex allocation, Charnov proposed that the evolution of sex allocation is influenced by the shape of so-called fitness gain curves for the investment into the respective sex functions (Charnov, 1979, 1982), which in turn can be influenced by sexual selection (Charnov, 1979, 1980). One such case is local sperm competition (LSC), that is the competition between related sperm for the fertilization of ova of the mating partner (Greeff *et al.*, 2001; Schärer & Wedekind, 2001), which was named in analogy to local mate competition that we discussed above (Schärer, 2009; Schärer & Pen, 2013). Here, investment into the male function in the form of sperm production confers diminishing fitness gains when related sperm compete for fertilizations. A hermaphrodite that experiences LSC may therefore achieve higher fitness returns when it re-allocates resources to its own female function, in which the fitness returns are often expected to be more linear (Schärer, 2009). Alternatively, it may re-allocate those resources to other forms of male investment, such as seminal fluids, love darts or male-specific behaviours like mate searching or courtship (Michiels *et al.*, 2009; Schärer, 2009; Schärer & Pen, 2013), provided that these show higher returns than investment in its own female function.

When the sperm of every given sperm donor in a population of hermaphroditic animals is not equally likely to be represented in every recipient's sperm receiving organ, then the random mating assumption is broken. This occurs, for instance, when individuals only mate and compete within a small subset of the population (i.e. if they compete in small mating groups), leading to strong LSC, because (related) sperm from the same sperm donor will then compete for fertilizations. In the extreme case of a mating group of just two individuals, all sperm in a mating partner's female reproductive tract will be from the same sperm donor. Consequently, the production of more sperm than necessary to assure the fertilization of the partner's eggs can be considered wasteful. Instead, resources could be more profitably invested into an individual's own female function. Therefore, a decrease in mating group size is predicted to lead to stronger LSC and hence to a more female-biased sex allocation, provided that other forms of male investment are not available or also show diminishing returns (Charnov, 1980; Schärer, 2009; Schärer & Pen, 2013). If, in contrast, the sperm from a

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given individual compete with unrelated sperm from other sperm competitors, producing additional sperm may indeed pay off and result in higher paternity success (Parker *et al.*, 1990). In this case, it may not be beneficial to re-allocate resources towards the female function and sex allocation is predicted to be less female-biased (Charnov, 1980; Schärer, 2009; Schärer & Pen, 2013).

Besides a small mating group size, other processes can also lead to an increase in LSC and favour a more female-biased sex allocation. In particular, processes leading to the different mating partners in a mating group having unequal chances to fertilize the eggs are likely to make the male fitness curve more saturating (Schärer, 2009; Schärer & Pen, 2013). For instance, sperm displacement can lead to higher LSC than expected by the number of sperm donors alone (Charnov, 1996). Similar effects can result from cryptic female choice, if a fixed proportion of sperm from a disfavoured sperm donor is removed and/or rejected by the sperm recipient (van Velzen *et al.*, 2009). And finally, random paternity skews resulting from stochastic effects can also lead to stronger LSC and therefore favour a more female-biased sex allocation (Greeff *et al.*, 2001; Schärer, 2009; Schärer & Pen, 2013).

Empirical evidence for LSC

Phenotypically plastic sex allocation adjustments that correspond to the predictions of the mating group size model have been observed in several hermaphroditic animals and corroborate the LSC perspective (reviewed in Schärer, 2009). For example, the trematode *Echinostoma caproni* increases its male allocation, measured as a composite of the size of the testes and the cirrus sac, when spending its metacercarial stage in larger groups compared to when it occurs in pairs or in isolation (Trouvé *et al.*, 1999). Another parasite, the intestinal trematode *Gyliauchen volubilis*, showed more female-biased sex allocation with decreasing mating group size in field-caught rabbitfish *Siganus rivulatus* (Al-Jahdali, 2012). And finally, the free-living simultaneously hermaphroditic flatworm *Macrostomum lignano* changes its phenotype according to different experimentally manipulated mating group sizes. For example, it increases testis size (Janicke *et al.*, 2013), testicular stem cell proliferation activity (Schärer *et al.*, 2004b) and sperm production rate (Schärer & Vizoso, 2007), presumably in part by increasing the speed of spermatogenesis (Giannakara *et al.*, 2016), when growing up in larger mating groups where sperm competition between unrelated sperm is stronger (and LSC thus weaker).

There is, however, only very limited empirical evidence that fitness gains for the male function indeed show more strongly diminishing returns under strong LSC. To our knowledge, the only study that has unequivocally documented a relationship between the level of LSC and the shape of the male fitness gain function was performed in the spermcast mating colonial ascidian *Botryllus schlosseri*. Here, the male fitness gain curve – measured as the relationship between the cross-sectional testis area of a focal colony and the percentage of available eggs fertilized by that colony in nearby partner colonies – did change according to the predictions of sex allocation theory. Namely, colonies placed under strong LSC had a gain curve with more strongly diminishing returns compared to those placed under weaker LSC (Yund, 1998). The scant empirical support for diminishing male fitness gains is problematic, because it is a key component of the theoretical foundation to explain the evolution of hermaphroditism and sex allocation (Charnov, 1979, 1980; Schärer, 2009; Schärer & Pen, 2013) and currently lacks empirical support in copulating hermaphrodites.

Objective

In this study, we use the copulating simultaneously hermaphroditic flatworm *M. lignano* to test (i) whether greater testis investment actually increases paternity success under sperm competition, and (ii) whether, as predicted by the LSC perspective, the gains in paternity success for greater investment into testes are more substantial in large compared to small mating groups (i.e. in a situation with presumed low or high LSC, respectively).

Materials and methods

Study organism and cultures

The experiment was conducted with *Macrostomum lignano* (Macrostomorpha, Platyhelminthes), a free-living flatworm that lives between sand grains in the intertidal zone of the Northern Adriatic sea and the Eastern Mediterranean basin (Ladurner *et al.*, 2005). It can be cultured in the laboratory (at 20 °C, 14 : 10 h light : dark and 60% humidity) in glass Petri dishes filled with artificial sea water or nutrient-enriched f/2 algal culture medium (Andersen *et al.*, 2005) and with the diatom *Nitzschia curvilineata* as the sole food source. It is small (adult length ~1.5 mm) and has a generation time of ~18 days, with eggs hatching ~5 days after laying and individuals reaching maturity in both sex functions ~13 days after hatching (Schärer & Ladurner, 2003). *Macrostomum lignano* is an obligatorily outcrossing simultaneous hermaphrodite with frequent and reciprocal copulation (Schärer *et al.*, 2004a) and, because of its highly transparent body, detailed measurements of internal reproductive structures are possible and noninvasive measures of testis and body size (among others) can be obtained (Schärer & Ladurner, 2003; Marie-Orleach *et al.*, 2016).

The worms used as mating partners in this experiment came from LS1, an outbred wild-type culture

(Marie-Orleach *et al.*, 2013). Focal worms came from the outbred transgenic BAS1 culture, which carries a green-fluorescent protein (GFP) marker and expresses GFP in all cell types (Marie-Orleach *et al.*, 2016). As some lines and cultures of *M. lignano* exhibit a karyotype polymorphism (Zadesenets *et al.*, 2016, 2017), we established a new BAS1 culture that exclusively included individuals whose karyotype was 2n=8 and that were homozygous for the GFP allele, increasing stable inheritance of this marker. More specifically, we performed metaphase chromosome preparation to count the number of chromosomes for 277 worms from the original BAS1 culture (Zadesenets *et al.*, 2016). Subsequently, we paired some of them with worms from the LS1 culture to assess the penetrance of the GFP allele (i.e. the proportion of GFP expressing offspring produced). To found the new BAS1 culture, we used only those 76 individuals (i) that showed the 'normal' 2n=8 karyotype (Zadesenets *et al.*, 2016), (ii) for which we could phenotype a progeny array of ≥ 17 offspring (mean: 48.7, range: 17–92) and (iii) for which all offspring expressed the GFP allele, thus indicating that their BAS1 parent was homozygous for the GFP allele. This culture is now being kept in a meta-population structure and at a total population size of 1200 individuals to maintain its genetic diversity.

As the GFP allele is dominant and as we expect it to be fixed within the BAS1 culture, offspring from BAS1 × LS1 crosses will always show GFP expression and can thereby be distinguished from the offspring of LS1 × LS1 crosses, which will lack GFP expression (Marie-Orleach *et al.*, 2014). Both LS1 and BAS1 are maintained at the Zoological Institute in Basel.

Experimental design

The rationale of the experimental design was to divide focal worms into two subsets with either a large or small testis size (while excluding individuals with intermediate trait values) and to then measure their resulting paternity success in two different mating group sizes, which each included a GFP-positive BAS1 individual as the focal worm and either two (hereafter called 'triplets') or seven (hereafter called 'octets') GFP-negative LS1 individuals as the partners (i.e. a full-factorial 2 × 2 design). Note that here 'mating group size' refers to the number of worms able to interact (sometimes also called the social group size); in this system, a social group size of three vs. eight is known to lead to a substantial difference in the mating group size (Janicke & Schärer, 2009a; Janicke *et al.*, 2013).

This experimental design permitted us to investigate the effect of testis size on paternity success and whether the magnitude of this effect was modulated by the mating group size in which a focal resided. Following the LSC rationale, we predicted that the same increase in testis size should confer a higher relative increase in paternity success in octets than in triplets, as we outline in more detail in the 'Statistics' section below. But before we do so, it is helpful to generate some theoretical expectations to which we could compare the observed paternity successes, which we do in the following section.

Theoretical expectations

We calculated the expected paternity successes of focal worms in the respective treatments, making the following, highly simplifying assumptions: (i) every individual in the mating group mates with every other individual, (ii) paternity success is the outcome of a fair raffle sperm competition, weighted by an individual's sperm production, (iii) sperm production is proportional to testis investment (measured as testis size), and (iv) focal worms from the BAS1 culture have, on average, a similar testis investment as the competitors from the LS1 culture.

We set the sperm production of a BAS1 focal with low testis investment (L) to, a, and with high testis investment (H) to, $2a$, because their testis size turned out to be approximately twice as high (Section 'Results' in Table 1). The sperm production of a LS1 competitor was set to, $1.5a$, because worms of this culture are expected to have, on average, an intermediate sperm production (as the BAS1 culture is derived from the LS1 culture; Marie-Orleach *et al.*, 2016). The expected paternity successes for the L and H focal worms in the triplets (3) and octets (8) are then as follows:

$$P_{L3} = \frac{a}{a+b} = \frac{a}{a+1.5a} = \frac{a}{2.5a} = 0.4$$

$$P_{H3} = \frac{2a}{2a+b} = \frac{2a}{2a+1.5a} = \frac{2a}{3.5a} \approx 0.5714$$

$$P_{L8} = \frac{a}{a+6b} = \frac{a}{10a} = 0.1$$

$$P_{H8} = \frac{2a}{2a+6b} = \frac{2a}{11a} \approx 0.1818$$

Thus, the paternity success of worms with high vs. low testis investment, respectively, is expected to be higher by 42.9% in the triplets (i.e. 0.5714 vs. 0.4) and by 81.8% in the octets (i.e. 0.1818 vs. 0.1). Note that the higher relative increase in the larger mating group size is consistent with the LSC scenario. Furthermore, it is important to note that we do not necessarily expect the outcomes to exactly match this simplified scenario, but these expectations serve as a useful comparison to our observed results and allow us to explore which of the assumptions may not have been met.

Experimental procedures

For logistic reasons, we divided the experiment into three blocks, each processed 1 day apart, but otherwise

Table 1 Shown are medians (and interquartile ranges) for measured morphological traits ($\times 10^3$ μm^2) in the different experimental groups.

	Triplets		Octets	
	Low testis investment	High testis investment	Low testis investment	High testis investment
Testis size	15.7 (12.6–22.3)	31.8 (19.6–40.2)	15.0 (10.4–18.5)	26.9 (19.4–40.2)
Ovary size	9.8 (7.1–12.7)	10.0 (8.2–12.1)	9.5 (7.6–12.9)	9.1 (6.8–11.4)
Seminal vesicle size	13.0 (9.1–20.5)	14.1 (10.7–18.9)	11.1 (8.0–14.9)	11.4 (8.2–19.3)
Body size	464.5 (344.1–496.6)	394.6 (336.4–465.9)	422.6 (323.3–491.7)	373.1 (315.6–483.7)

Note the approximately two-fold higher testis size in the subset with high testis investment compared to the one with low testis investment.

treated the worms in the same way. In the following, we explain the experimental procedures for one block only.

We kept all worms used here either in (i) glass Petri dishes in groups of one hundred during the growth phase or in (ii) triplets or octets in wells of 24-well tissue culture plates during isolation and mating trials (TPP AG, Switzerland), in 20 mL or 1.5 mL of f/2 medium (Andersen *et al.*, 2005), respectively. Except for a 24-h period of food deprivation to which the focal worms were submitted to facilitate the morphological measurements, all worms received *ad libitum* diatom algae during the whole experiment. Partner worms were raised under the same conditions as the focal individuals, except that they were not measured (and therefore also never food deprived).

On day 0, we put the parents (F0) of the focal worms into three Petri dishes (100 adult worms per dish) allowing them to lay eggs and then removed them on day 2, so that all focal worms (F1) used in this experiment were of similar age. On day 19, we transferred 100 focal worms to a new Petri dish, and on day 28, we transferred them into new Petri dishes *without* algae (in order to allow them to regurgitate the consumed algae in their gut, thus facilitating morphometry). On the following day, we isolated focal worms in 24-well plates, took their morphological measurements (block 1: $n = 81$; block 2: $n = 72$; block 3: $n = 85$) and then put them back into isolation in 24-well plates with algae until the next day.

To take morphological measurements, we performed a noninvasive squeeze preparation on the focal worms (Schärer & Ladurner, 2003; Vizoso & Schärer, 2007). Briefly, we anesthetized worms with a 2:1 mixture of 7.14% $MgCl_2$ and f/2 medium and squeezed them dorsoventrally between a glass slide and a haemocytometer cover glass separated by a 35-μm plastic spacer. Then, we captured digital micrographs at 40–400× magnification with a digital video camera (DFK 41BF02, The Imaging Source, Bremen, Germany) attached to a DM 2500 microscope (Leica Microsystems, Heerbrugg, Switzerland). We used BTV Pro 6.0b7 (http://www.bensoftware.com/) to acquire the images.

During the evening of the same day, we measured body and testis size (and later also ovary and seminal vesicle size) of all focal worms using ImageJ 1.47v (http://imagej.nih.gov/ij). For testis size and ovary size, we used the sum of the areas of both testes and ovaries, respectively (note that for 23 and 26 of the 103 focals, respectively, two testes and ovaries could be seen in the pictures, but only one could be properly imaged, in which case that gonad was measured twice to estimate total gonad size). Then, we performed a linear regression of testis on body size to determine residual testis size, which we used as a measure of relative testis investment that is uncorrelated to body size. Subsequently, we retained only worms belonging to the lowest and highest quartiles of the distribution of residual testis size for the further experiment (called the *low testis investment* and *high testis investment* subsets, respectively; see Figs 1 and 2).

The rationale for controlling for body size in this way is that bigger worms can be expected to have a higher resource budget available and could therefore have higher paternity success for that reason alone. As we were interested in the effect of testis size *per se*, we used residual testis size to assign each worm to their subset, which, as we show in the Results, nevertheless resulted in an approximately two-fold difference in absolute testis size between the subsets.

On day 30, we assigned GFP-positive focal worms of the low and high testis investment subsets to one of the two mating group size treatments (Fig. 1). For this, we grouped focal worms in wells with either two (triplets) or seven (octets) GFP-negative partner worms taken directly from a Petri dish with 100 worms, thus forming the first mating group. After 24 h, we then transferred the focal worm to a new mating group (with the same number of partner worms as in the first one) and isolated the partners from the first mating group in 24-well plates for 7 days to allow them to lay eggs and produce offspring. In total, we repeated this four times, so that every focal worm passed through four consecutive mating groups of the same size, thereby increasing the number of offspring based on which the paternity success of the focal could be estimated (Fig. 1). Note that, although *M. lignano* can phenotypically adjust its testis size and sperm production rate according to the mating group size, it was previously shown that it needs > 4 days to do so (Brauer

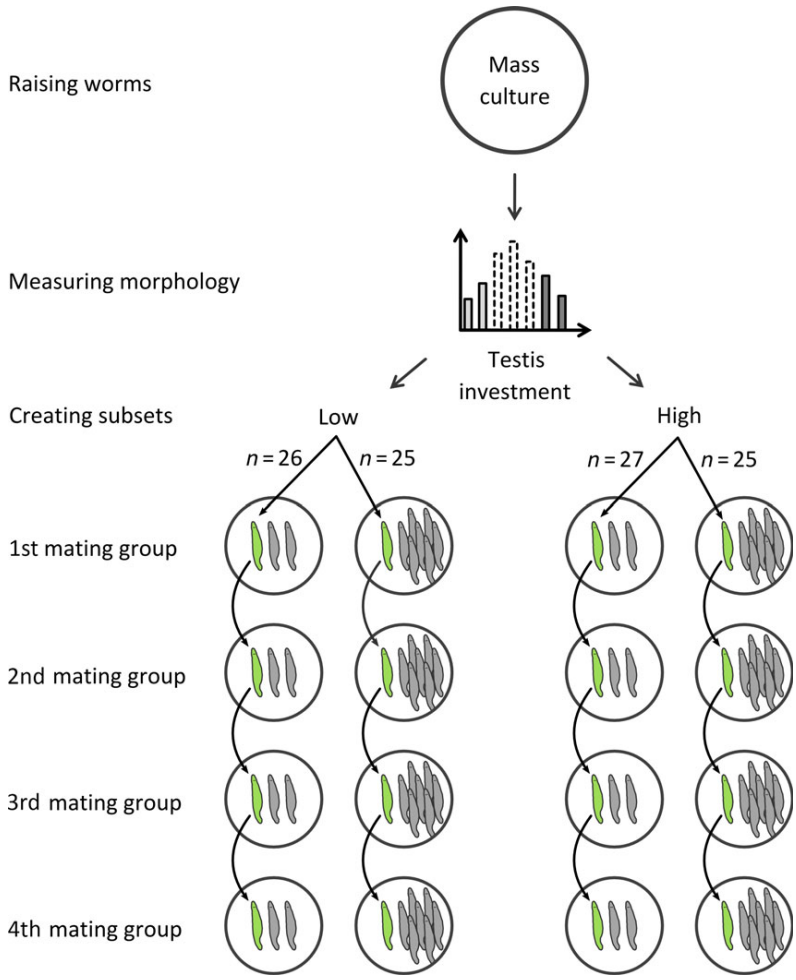

Fig. 1 Schematic illustration of the experimental design. Note how each GFP-positive focal worm (green) had to compete with wild-type worms (grey) in four consecutive mating groups. The sample sizes (n) refer to the final number of focal worms in the respective treatment combinations.

et al., 2007; Schärer & Vizoso, 2007). The isolated mating partners were removed from the wells after 7 and 10 days later the by now hatched offspring were genotyped by checking their GFP status under a MZ10 F stereo microscope with epifluorescence illumination (LEICA Microsystems).

Statistics

We first tested whether the assignment of worms to the different treatment groups resulted in the intended distributions of morphological trait values, by testing for effects of the subset, the mating group size and their interaction, as well as the block on the respective morphological traits using linear models in R (version 3.4.0; R Development Core Team, 2016; also used for all following statistical analyses). We log-transformed the values for the morphological traits to fulfil the normality assumption for residuals.

To assess the effect of the subset, the mating group size and their interaction (but see below) on paternity success, and to statistically control for block effects and account for the detected overdispersion, we fitted a quasi-binomial generalized linear model (GLM), in which the variance is given by the product of the mean and φ, the dispersion parameter. For this, we used the function 'glm' with family 'quasi-binomial' and a logit-link. Paternity was estimated as the number of offspring that expressed GFP among the total number of offspring over all partners from the four mating groups.

For all fitted models, the factors subset and mating group size were centred by encoding their levels as either −0.5 or 0.5 to facilitate hypothesis testing in the presence of interaction terms (Schielzeth, 2010). As the levels of both subset and mating group size were chosen as extreme values that presumably occur at both ends of the distribution in worm populations, it is preferable to estimate the interaction term at the intermediate levels (Schielzeth, 2010). We tested the significance of effects by removing single effects from the model and comparing the reduced to the complete model using an F-test. Model assumptions for all

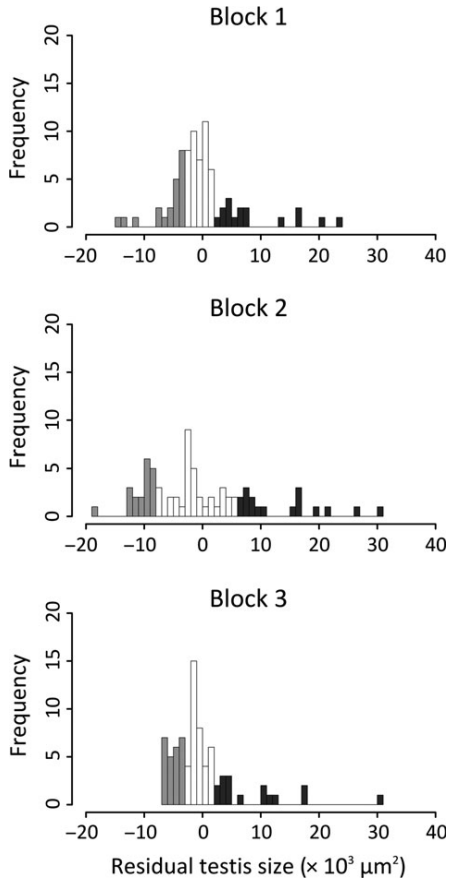

Fig. 2 Distributions of the residual testis size according to which we assigned the worms into subsets with low (light grey) or high (dark grey) testis investment for each of the three experimental blocks.

models were assessed by visually inspecting residuals vs. predicted values plots and normal quantile–quantile plots (Faraway, 2016).

Although the quasi-binomial GLM permits us to test for the effects of the subset and the mating group size, the biological interpretation of the interaction term is less clear in the light of our theoretically derived hypotheses (cf. 'Theoretical expectations' section). Specifically, we wanted to test whether the increase in paternity success in (i) the *triplets* and (ii) the *octets* was significantly different from the theoretical expectations, and whether (iii) the *relative* (rather than absolute) increase in the octets was significantly higher compared to that in the triplets, as predicted by the LSC perspective.

To test these hypotheses, we implemented permutation tests in R, by sampling n paternity success values for *each* mating group size treatment from our empirical data without replacement, and randomly assigned them to either the low or high subset (where n is the sample size for the mating group size treatments in our experiment). To test hypotheses (i) and (ii), we calculated, as our test statistic, the increase in paternity success for triplets (i.e. $\text{mean}_{H3} - \text{mean}_{L3}$) and octets (i.e. $\text{mean}_{H8} - \text{mean}_{L8}$), respectively. And to test hypothesis (iii), we first calculated the relative increase in paternity success due to larger testes in triplets $[(\text{mean}_{H3} - \text{mean}_{L3})/\text{mean}_{L3} \cdot 100]$ and in octets $[(\text{mean}_{H8} - \text{mean}_{L8})/\text{mean}_{L8} \cdot 100]$, and then the difference between them, as our test statistic. We repeated this sampling 10 000 times to generate null distributions of these test statistics. Then, we estimated the *P*-value as the proportion of those permutations where, for hypotheses (i) and (ii), the test statistic was lower (lower limit in triplets: 0.075; lower limit in octets: 0.023) or higher (upper limit in triplets: 0.268; lower limit in octets: 0.140) than expected under the theoretical calculations and where, for hypothesis (iii), the absolute value of the test statistic was greater than or equal to the absolute observed test statistic (note that using absolute values yields a two-sided test). Additionally, we used 10000 bootstrap iterations with replacement to estimate the 95% confidence intervals (as the 95 percentile confidence interval) for the relative increase in the respective mating groups.

To test whether the paternity success of a focal individual was repeatable across its four consecutive mating groups, we estimated repeatability with the R package 'rptR' (Nakagawa & Schielzeth, 2010; Schielzeth & Nakagawa, 2013). For this, we used the function 'rpt.remlLMM' to calculate repeatabilities from a linear mixed-effects model fitted with restricted maximum likelihood. Because the variance for binomial proportions is a quadratic function of the mean and this violates assumptions for linear models, the logit-transformation, which removes this mean-variance relationship, is often recommended for analysing binomial proportions (Warton & Hui, 2010; Engqvist, 2013). Therefore, we logit-transformed paternity values according to the formula $\log[(p + 0.01)/((1 - p) + 0.01)]$, where p is the paternity success value in each mating group (Warton & Hui, 2010). We added the constant of 0.01 to paternity values of 0 and −0.01 to paternity values of 1 to permit their inclusion, in the analysis, for which the logit would otherwise not be defined. Here, repeatability is the proportion of the total variance in paternity success explained by interindividual differences between paternity values in the four consecutive mating groups.

Finally, we tested whether the repeatability estimates between the two mating group size treatments differed statistically with a two-tailed permutation test. For this, we (i) randomly reassigned replicates to the two mating group sizes 10 000 times and calculated the differences between the repeatability estimates (repeatability for triplets minus repeatability for octets) for each

permutation and ii) estimated the *P*-value as the proportion of those permutations where the absolute difference was greater than or equal to the absolute observed difference.

Results

Morphology and assignment over treatments

As intended, worms from the high testis investment subset had testes that were almost twice as large in absolute terms compared to the worms from the low testis investment subset (high testis investment, median: 29 000 μm^2; low testis investment, median: 15 300 μm^2; Tables 1 and 2a), but there was no significant difference in testis size between the mating group size treatments, nor was there a significant interaction between the testis investment subset and mating group size treatments. The assignment of individuals over the treatment groups was also successful in so far as they did not differ in either body or ovary size (all $P > 0.3$; Table 2a). The only exception was seminal vesicle size, in that worms allocated to the octets had significantly smaller seminal vesicles (with the difference being approximately 0.17 ± 0.07 standard deviations), whereas worms with low and high testis investment did not differ in seminal vesicle size (cf. Tables 1 and 2a). We do not think, however, that this initial difference in seminal vesicle size should have had a strong effect on the amount of sperm transferred, because the focal worms were exposed to many mating partners over the four consecutive 24-h time periods. Therefore, not the initial amount of sperm in the seminal vesicle, but rather the sperm production rate during these periods will likely have determined the amount of sperm available for transfer (Schärer & Vizoso, 2007).

Testis investment and mating group size effects on paternity

Overall, worms with higher testis investment had a 22.3% (95% CI: −12.9% to 74.0%) higher mean paternity success relative to worms with low testis investment, but although worms in smaller groups (which of course had fewer competitors) had higher paternity success, the interaction between subset and mating

Table 2 Treatment effects of the (a) linear models for morphological traits and (b) quasi-binomial generalized linear model for paternity success.

(a) Response	Effect	$\beta \pm$ SE	d.f.	AIC	*F*-value	*P*-value
Testis size	None	–	–	−239.4	–	–
	Testis investment	0.65 ± 0.06	1	−159.0	118.88	**<0.0001**
	Mating group size	−0.05 ± 0.06	1	−240.5	0.82	0.37
	Testis investment × mating group size	0.01 ± 0.12	1	−241.4	0.01	0.94
	Block	–	2	−176.6	44.21	**<0.0001**
Ovary size	None	–	–	−195.1	–	–
	Testis investment	0.08 ± 0.07	1	−196.0	1.07	0.303
	Mating group size	−0.07 ± 0.07	1	−196.1	0.96	0.331
	Testis investment × mating group size	−0.06 ± 0.15	1	−197.0	0.14	0.706
	Block	–	2	−168.1	17.04	**<0.0001**
Sem. vesicle size	None	–	–	−194.4	–	–
	Testis investment	0.04 ± 0.07	1	−196.0	0.36	0.549
	Mating group size	−0.17 ± 0.07	1	−191.2	5.03	**0.027**
	Testis investment × mating group size	0.02 ± 0.15	1	−196.4	0.02	0.899
	Block	–	2	−150.3	28.89	**<0.0001**
Body size	None	–	–	−308.8	–	–
	Testis investment	−0.04 ± 0.04	1	−309.6	1.08	0.302
	Mating group size	−0.04 ± 0.04	1	−310.0	0.72	0.398
	Testis investment × mating group size	0.01 ± 0.09	1	−310.7	0.03	0.869
	Block	–	2	−259.3	33.01	**<0.0001**
(b) Response	Effect	$\beta \pm$ SE	d.f.	Deviance	*F*-value	*P*-value
Paternity success	None	–	–	616.1	–	–
	Testis investment	0.38 ± 0.16	1	648.2	5.06	**0.027**
	Mating group size	−2.17 ± 0.16	1	1653.4	163.30	**<0.0001**
	Testis investment × mating group size	−0.10 ± 0.32	1	616.5	0.08	0.781
	Block	–	2	659.7	3.43	**0.036**

F-tests were performed by removing single effects and comparing the full with the reduced model and significant *P*-values are written in bold face. Note that the parameter estimate β is on the log scale for the morphological traits and on the logit scale for paternity success.

group size was not significant (Fig. 3, Table 2b; but see the 'Statistics' section for caveats in interpreting the interaction term). Worms in triplets had a relative increase in paternity success of 18.7% (95% CI: −6.6% to 53.4%) when doubling their testis investment, a substantial and significant deviation from the theoretical expectation of 42.9% (two-tailed permutation test: $P = 0.013$; Fig. 3). In octets, the worms increased their paternity success by 33.1% (95% CI: −15.4% to 107.6%) with increased testis investment, which was also substantially lower than the expected 81.8%, although the difference between the observed and the expected increase was not statistically significant (two-tailed permutation test: $P = 0.132$). Although the relative increase in paternity was more pronounced in octets, as predicted by the LSC perspective, it was not significantly different between triplets and octets (two-tailed permutation test: $P = 0.453$; Fig. 3). In particular, worms with high testis investment tended to have lower paternity success than theoretically expected (Fig. 3).

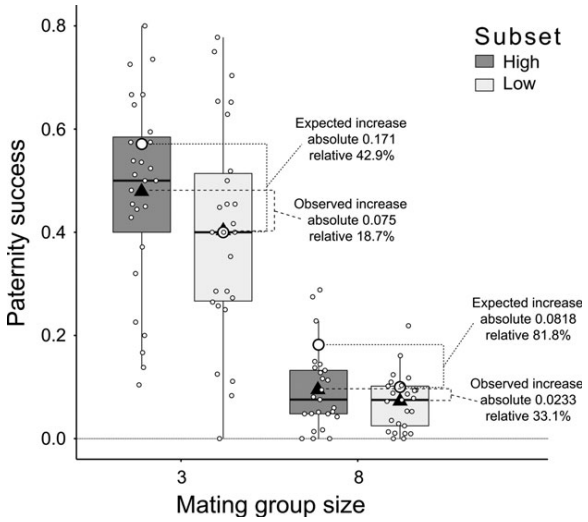

Fig. 3 Paternity success achieved across all four subsequent mating groups by focal worm of low testis investment (light grey) and high testis investment (dark grey) tested in two mating group sizes, either triplets or octets. Small circles represent the individual measurements (and are jittered along the *x*-axis for better visibility). The box plots show medians, and the 25th and 75th percentiles, respectively, and whiskers extend to 1.5 times the interquartile range. The large white circles represent theoretical expectations (see the 'Theoretical expectations' section), and the black triangles represent treatment means (see the 'Methods' section). Note that, although the focal worms with larger testes are expected to increase their paternity success more in triplets than in octets in absolute terms (0.171 vs. 0.0818), focal worms are expected to benefit more in octets in relative terms (42.9% vs. 81.8%).

Repeatability of paternity success

The repeatability of paternity success across the four mating groups was significant and moderate in octets ($R = 0.360$, CI = 0.197–0.499, $P = 0.004$) and substantially lower and not quite statistically significant in triplets ($R = 0.093$, CI = 0.000–0.227, $P = 0.061$). The difference in repeatability between the two mating group sizes was significant (two-tailed permutation test: $P = 0.040$).

Discussion

In the present experiment, we found that worms with higher testis investment sired a higher proportion of offspring in an environment where sperm competitors were present. We found the relative gain in paternity success resulting from higher testis investment to be lower than our theoretical expectations, and there was no strong evidence that the observed effect of testis investment was more pronounced under low LSC (i.e. in octets as opposed to triplets), a result that does not match what we predicted based on the LSC perspective. Moreover, the within-individual repeatability for paternity success was significantly higher in octets. In the following, we discuss these points in turn and explore implications for the evolutionary maintenance of hermaphroditism in *M. lignano*.

Testis investment effect

The positive effect of testis investment on paternity success found here confirms previous correlative findings that suggested an increased male reproductive success for individuals with larger testes in *M. lignano* (Marie-Orleach *et al.*, 2016), although worms tended not to benefit as much from higher testis investment as predicted under our simplifying theoretical assumptions. Moreover, this testis investment effect is in line with empirical results from other species and confirms predictions from sperm competition theory, as we discuss in the following.

In *M. lignano*, previous studies have shown that worms with larger testes also have more active testes, produce more sperm per unit time and also produce sperm more quickly (Schärer *et al.*, 2004b; Schärer & Vizoso, 2007; Giannakara *et al.*, 2016). This leads to an increase in sperm transfer success (Janicke & Schärer, 2009a; Marie-Orleach *et al.*, 2016), which in turn increases paternity success (Marie-Orleach *et al.*, 2016). Our results confirm this mechanism, because we can attribute the increased paternity success of the high subset to their larger testes (while they did not differ in body size). Interestingly, a recent and well-replicated experimental evolution study in *M. lignano* showed no evolutionary response for testis size in selection lines that were either kept in monogamous pairs or

polygamous octets for 20 generations (Janicke *et al.* 2016). But this lack of response to selection might potentially be linked to the pronounced phenotypic plasticity that *M. lignano* exhibits in many reproductive traits, including testis size (Schärer *et al.*, 2004b; Schärer & Vizoso, 2007; Janicke *et al.*, 2013; Giannakara *et al.*, 2016), which may have shielded the available genetic variation from selection (Price *et al.*, 2003; Kopp & Matuszewski, 2014; Ghalambor *et al.*, 2015). Overall, however, there is strong evidence that testis size influences male reproductive success and is therefore under selection in this species.

The observed effect of testis investment in our study is unlikely due to differences in the overall resource budget between the subsets, because individuals with low and high testis investment were deliberately chosen to have a similar body size. Similarly, an effect of ovary size is unlikely, as it did not differ between the testis investment subsets either. The significantly lower seminal vesicle size of worms from octets compared to triplets seems to be an unfortunate sampling effect, because this effect was present only in the first block of the experiment ($F_{1,33} = 5.017$, $P = 0.032$), but not in the other two blocks (second block: $F_{1,27} = 1.444$, $P = 0.240$; third block: $F_{1,31} = 0.100$, $P = 0.754$). Having said that, we do not think that it strongly influenced the results, because (i) as already mentioned above, we expect the initial fill grade of the seminal vesicle to be less important than the sperm production rate during the four consecutive 24 h mating trials and (ii) the worms with low and high testis investment did not differ in their seminal vesicle size, nor was there a subset by mating group size interaction effect on seminal vesicle size. This unwanted seminal vesicle size effect could, however, have somewhat reduced the difference between the octet and triplet treatment groups.

Given that our experiment did not directly manipulate testis size, any unmeasured traits that correlate with residual testis size and themselves affect paternity success could, at least in theory, have been responsible for, or contributed to, the observed relationship between residual testis size and paternity success. Sensible candidates for such traits might be seminal fluid proteins, which have been shown to interact with sperm and the female reproductive system and thereby influence male reproductive success in other species (Chapman, 2001; Arnqvist & Rowe, 2005; Wigby *et al.*, 2009). However, we currently have no evidence for seminal fluid effects in *M. lignano*, nor do we know whether there is a correlation between seminal fluid production and residual testis size. Mating rate may also correlate with testis size, as worms raised in octets with consequently larger testes mated more often and for longer than worms raised in pairs with smaller testes (Janicke & Schärer, 2009b). On the one hand, a higher mating rate can lead to increased paternity success simply because more sperm are transferred. On the other hand, if a sperm donor with a higher mating rate also displaces more sperm of his competitors, this could be an alternative mechanism for how testis investment led to increased paternity success in our study. However, we cannot distinguish between these two alternative hypotheses with the present data.

As we found lower paternity benefits due to higher testis investment than expected under our theoretical predictions, processes that deviate from a fair raffle sperm competition, such as sperm displacement (Charnov, 1996), cryptic female choice with a removal of a fixed proportion of the ejaculate (van Velzen *et al.*, 2009) or simply random paternity skews resulting from stochastic effects (Greeff *et al.*, 2001; Schärer, 2009; Schärer & Pen, 2013), may play a role in this system. However, the paternity success that our BAS1 focal did not achieve must have been achieved by its LS1 competitors. Therefore, these processes can only explain our results if they affected the focals in a different way than the competitors, for which we have no evidence (see also next section).

Looking at a broader range of taxa, three different types of studies lend support to the important role of relative testis size during sperm competition. First, there is correlational evidence for males with larger testes achieving higher paternity success in both laboratory studies and natural populations (e.g. Preston *et al.*, 2003; Schulte-Hostedde & Millar, 2003; Awata *et al.*, 2006; Holleley *et al.*, 2006). Second, there is evidence for the importance of testis size during sexual selection from experimental evolution studies under different enforced sexual selection regimes (e.g. Hosken & Ward, 2001; Pitnick *et al.*, 2001; Simmons & García-González, 2008), although some studies generated inconclusive results regarding the role of testis size (Crudgington *et al.*, 2009; Firman & Simmons, 2010). And third, many comparative studies also suggest this link between testis size and paternity success (e.g. Parker *et al.*, 1997; Parker & Pizzari, 2010; Simmons & Fitzpatrick, 2012). Altogether, these studies suggest that relative testis size may be a valid proxy for sperm production.

The testis investment effect on paternity success that we found for *M. lignano* in this study, and evidence from other taxa, confirms predictions from sperm competition theory. In particular, it is often assumed that paternity success of a sperm donor is to some degree proportional to the number of sperm it ejaculates, that is, that sperm production rate is in many cases the target of selection. This is especially true when sperm competition operates like a 'fair raffle', in which every transferred sperm cell has the same chance to fertilize an egg (Parker, 1990; Parker *et al.*, 1990). And empirical data from several taxa show that a higher number of sperm in the ejaculate have a positive effect on paternity success (e.g. Martin *et al.*, 1974; Gage & Morrow, 2003; Garcı́a-González & Simmons, 2005; Stoltz & Neff, 2006; Boschetto *et al.*, 2010). But although the

speed of spermatogenesis and the amount of spermatogenic tissue *per se* are likely the most important determinants of sperm production rate (and therefore the available sperm number in the ejaculate), the majority of studies use a measure of relative testis size as a proxy for sperm production rate (Parker *et al.*, 1997; Ramm & Schärer, 2014).

In summary, the positive effect of testis investment on paternity success found in our experiment shows, in combination with previous findings, that testis size, sperm production and increased paternity success during sperm competition are indeed linked in *M. lignano*. This confirms the theoretical prediction that increased investment into ejaculates is selected due to sperm competition, although the paternity benefits arising from increased investment were somewhat lower than expected under a model with pure fair raffle sperm competition.

Mating group size effects on relative paternity gains for testis investment

It has been suggested that LSC causes diminishing fitness gains for investment into the male sex function – and hence testis size and sperm production – which may be one reason why hermaphroditism is favoured over gonochorism in some animals (Charnov, 1980; Schärer, 2009; Schärer & Pen, 2013). Therefore, we hypothesized that the fitness gain curve for investment into the male function would show more sharply diminishing returns already for lower values of male allocation in smaller mating groups (with strong LSC) compared to larger mating groups (with weak LSC). Our results do not strongly support this prediction, because the worms in octets did not benefit significantly more from bigger testes than worms in triplets did. This was true both when we used the GLM approach, which tested for the difference between triplets and octets in the benefit for worms with high testis investment in *absolute* terms, and when we used a permutation test to detect differences between triplets and octets in the increase in high testis investment individuals *relative* to low testis investment individuals.

We see three possible explanations for the observed similarity between the paternity gains for increased testis investment in triplets vs. octets. First, the fitness gain curves do differ in shape, but our experimental design was not optimal for detecting this difference. Second, we did not detect a difference because of insufficient statistical power in our study. Third, the fitness gain curves do not differ in shape in the different group sizes, which would question the LSC perspective. We explore these explanations in turn.

Possible limitations of the experimental design

Concerning the first point, it is possible that we were not able to detect the difference between the paternity gains for increased testis investment in triplets vs. octets because our experimental design was not ideal to test for it. One possibility may be that within the range of LSC that we explored in our experiment (i.e. one and six unrelated competitors), the fitness gains resulting from higher testis investment are already quite high (but see 'Difference in strength of LSC between triplets and octets' section for a discussion of the possibility that LSC was *high* in both treatments). Then, the fitness that can be gained for the same testicular investment might not be so different in these two mating group sizes and we may therefore not have been able to detect a difference. We are aware of only one study that provides evidence for a changing male fitness gain curve in response to different levels of LSC in simultaneous hermaphrodites (Yund, 1998). In that study on the marine, spermcast mating ascidian *Botryllus schlosseri*, the author compared three treatment groups of experimentally assembled mating arrays. These involved a focal (male phase) sperm donor colony (of varying testis size) competing for fertilizations in two (female phase) sperm recipient colonies, in either a (i) 'high intensity sperm competition' group with two competing sperm donors having high testis investment, (ii) an 'intermediate intensity sperm competition' group with two competing sperm donors having low testis investment, and (iii) a 'competitor-free' group in which no competing sperm donors were placed in close proximity to the focal. However, as these mating arrays were placed in the field, there were also other nonexperimental competing sperm donors that provided low levels of 'exogenous sperm' and sometimes achieved a considerable paternity success, especially in the 'competitor-free' group. Yund (1998) found that male fitness gains diminished more strongly in the treatments with lower level of sperm competition and by far the most strongly saturating fitness gain curve was found for the 'competitor-free' treatment, whereas the other two treatments were more linear and differed much less. Therefore, one could expect the strongest effect on the shape of the male fitness gain curve when the conditions change from *very* strong LSC to intermediate levels of LSC. A situation with only one permanently present competitor, as in the triplets in our study, might already have substantially lower than maximal levels of LSC, so that the difference in the level of LSC experienced in octets might be fairly small. Instead, it might have been preferable to choose an experimental design that introduces a situation with even higher LSC than is possible with one permanently present competitor in a mating group, as that might have brought us into the range where the effects of LSC on the fitness gains become more easily detectable. One possible experimental design that could achieve this would be one with a 'part-time competitor' that can mate with the sperm recipient only a fraction of the time, whereas the focal worm is allowed to mate the rest of the time. An

experimental treatment like that might increase LSC to an amount where its effects, compared to the presence of one or several 'full-time competitors', is more easily detectable.

Moreover, the explored range of testis investment is crucial for detecting differences in fitness gain curves. In our experiment, we already used the most extreme quartiles of the phenotypic distribution of residual testis size, but maybe it would have been preferable to aim at creating an even broader range of testis investment values to estimate diminishing effects on male fitness. A possible way to generate a broader range of testis investment or rather sperm production rate might be dose-dependent RNA interference, which has been successfully used in *M. lignano* in an earlier study (Sekii *et al.*, 2013).

In addition, testis investment (as measured in our experiment) likely is an incomplete proxy for the sperm production rate. Although testis investment surely does not reflect sperm production perfectly, there is considerable empirical evidence that it does so reasonably well. Namely, the number of testicular stem cells in S-phase, a dynamic measure of testicular activity of a focal worm, is positively correlated with the mean testis investment of the worms in the mating group in which the focal worm was raised ($r^2 = 0.32$, $P < 0.001$; Schärer *et al.*, 2004b). Furthermore, as seminal vesicle area is strongly and positively correlated with number of sperm it contains ($r^2 = 0.77$, $t = 10.9$, $P < 0.001$; see Schärer & Vizoso, 2007) and as the increase in seminal vesicle size during isolation can be predicted by testis size (ANCOVA, including also the factor group size: $r^2 = 0.56$; see Schärer & Vizoso, 2007), we consider testis investment a valid proxy for sperm production (see also 'Testis investment' section). It is, however, possible that worms with high testis investment can plastically down-regulate the amount of sperm they transfer so that they do not profit as much from their bigger testes as one would expect from their testis size. This could also explain the fact that especially worms with high testis investment tended to have lower paternity success than expected (Fig. 3). Although we have no knowledge of such a phenomenon *per se*, we know that the relationship between testis size and sperm production rate can be influenced by the mating group size in which *M. lignano* is raised (Schärer & Vizoso, 2007).

Finally, if the competitors (LS1 culture) for some reason had a different testis size than the average focals (BAS1 culture), then the expected effect sizes could vary as a result. When we explored this possibility by simulating different testis sizes for the competitors (data not shown), we found the lowest deviations between our data and those expectations when the competitors had a testis size very similar to the focals with high testis investment (i.e. when competitors had a testis size of around 29×10^3 μm^2). On the one hand, we have little reason to expect them to differ in testis size because (i) the BAS1 culture was established by backcrossing the transgenic inbred HUB1 line onto the LS1 culture (Marie-Orleach *et al.*, 2016), so that they should be very similar genetically, (ii) a comparison between a transgenic and wild-type inbred line that should otherwise be genetically similar showed no differences in siring ability (Marie-Orleach *et al.*, 2014), and (iii) the focals and competitors were raised under similar conditions until the mating trials started (cf. 'Methods' section). On the other hand, as we raised competitors and focals in different petri dishes, we cannot exclude the possibility that these slightly different raising conditions led to different testis sizes in the competitors compared to the focals. This could have led to a decreased effect size and could therefore explain that we did not find the expected effect of mating group size on paternity gains. Furthermore, if the assumptions of random mating, fair raffle sperm competition and sperm production proportional to testis investment were broken in a way that was different between the focals and the competitors, this could have led to a smaller effect size.

Possible limitations of statistical power

Concerning the second point, it appears possible that there is actually a difference in the effect of testis investment on paternity between triplets and octets in *M. lignano*, but that we could not detect it because of insufficient statistical power. Although we cannot exclude this possibility, each treatment combination had at least 25 replicates, and paternity values were calculated on a fairly high number of total offspring that the partner worms produced (triplets: median = 32, range = 14–58; octets: median = 118.5, range = 78–194). However, the low and moderate within-individual repeatabilities for paternity (triplets: $R = 0.093$; octets: $R = 0.360$) suggest that, particularly in triplets, there may have been quite substantial unknown sources of paternity variation that changed from one mating group to the next. This variation could have affected the estimation of paternity success for the focal worm and therefore obscured the effects we tried to detect here. Possible examples of such unmeasured factors are the mating rate, mating order and/or effects of variation in seminal fluid composition. Also, effects of the genotype of the mating partners and/or competitors could potentially have strong effects on the paternity of the focal (Clark *et al.*, 1999; Evans *et al.*, 2013; Simmons *et al.*, 2014; Travers *et al.*, 2016), and in the triplets, such stochastic variation might have been more pronounced, while it averaged out in the larger groups.

Difference in strength of LSC between triplets and octets

Concerning the third point, it may be possible that the fitness gain curves are not different between triplets and octets. As we did not record mating behaviour directly, we do not know whether the focal worm

actually mated with all possible mating partners. We do know, however, that the realized mating group size is usually lower than the (possible) social mating group size, that is, the focal worm does not successfully store sperm in every member of its mating group (cf. Figs. 2A and 3B in Janicke & Schärer, 2009a and Janicke *et al.*, 2013; respectively). In the present study, focal worms in octets and triplets sired offspring in on average 4.9 (± 2.3 SD) and 0.8 (± 0.8 SD) partners, respectively (compared to worms that managed to store sperm in 2.8 and 1.5 partners, respectively, in a previous study that also joined focals and recipients for 24 h; Janicke & Schärer, 2009a), and the paternity representation in different partners was often highly skewed (i.e. very high in some but very low in other partners; Fig. 4). If this within-focal skew is somewhat representative of the actual among-competitor skew in paternity success, this might indicate a reduced effective number of mates and therefore a higher than otherwise expected level of LSC even in octets. Possible reasons for an elevated LSC in octets could be sperm displacement (Charnov, 1996), cryptic female choice during which a fixed proportion of the ejaculate is removed by the recipient (van Velzen *et al.*, 2009) or simply random paternity skews resulting from stochastic effects (Greeff *et al.*, 2001; Schärer, 2009; Schärer & Pen, 2013). Of these three we have only evidence for sperm displacement in *M. lignano* (Marie-Orleach *et al.*, 2014), although the relatively low repeatability for paternity success suggests substantial variation, be it due to stochasticity or cryptic female choice, as a contributing factor to the relatively high levels of LSC in octets. However, considering that focal worms in our experiments that were tested in octets had on average 4.9 (± 2.3) partners to whom they transferred sperm whereas focals in triplets transferred sperm to only 0.8 (± 0.8) partners, we think that there was still a substantial difference in the number of effective mating partners between triplets and octets and thus a difference in the level of LSC between the mating group sizes.

In summary, we think it would be premature to conclude that there is no difference in the level of LSC between triplets and octets, before we have more data about how the mating group size translates into the effective number of mates when paternities are skewed

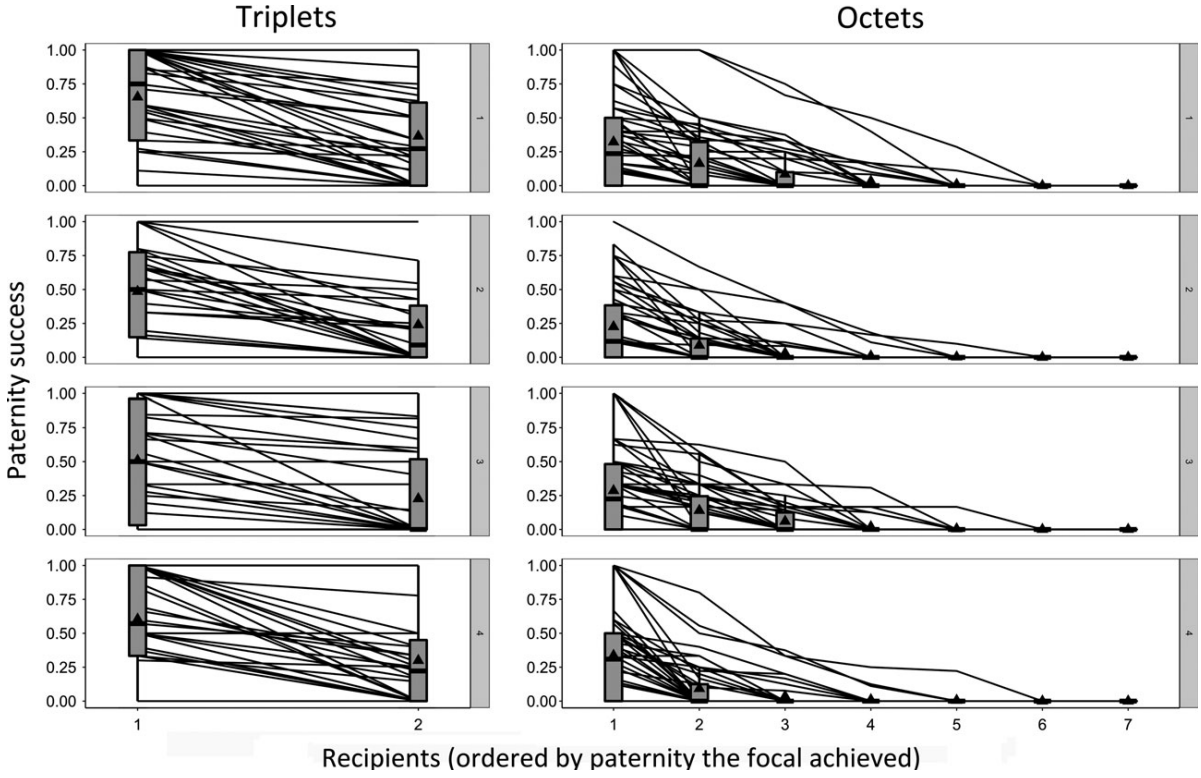

Fig. 4 Paternity success achieved by each focal worm in all of its possible mating partners, ordered by the paternity success that the focal achieved in a given partner. The black lines connect the values for individual focal worms. The box plots show medians, and the 25th and 75th percentiles, respectively, and whiskers extend to 1.5 times the interquartile range. The black triangles indicate mean paternity values, and the four different panels represent the four consecutive mating groups that the focals were tested in.

and how that in turn affects LSC. Furthermore, it seems unclear how, if not based on the LSC perspective, one would explain the well-documented phenotypic plasticity in sex allocation in response to mating group size in *M. lignano* (Schärer *et al.*, 2004b; Schärer & Vizoso, 2007; Janicke *et al.*, 2013; Giannakara *et al.*, 2016), as well as the evolutionary maintenance of hermaphroditism in this and other hermaphrodites, which we discuss next.

Local sperm competition and the evolutionary maintenance of simultaneous hermaphroditism

Although we measured surprisingly modest paternity gains in response to a two-fold difference in testis investment – itself suggesting diminishing fitness returns for testis investment – we were not able to confirm experimentally that LSC leads to diminishing fitness returns for male allocation in *M. lignano* and to show that LSC therefore can contribute to the evolutionary maintenance of simultaneous hermaphroditism in these and other copulating simultaneous hermaphrodites (Schärer & Pen, 2013). We thus briefly mention some other hypotheses besides LSC that have been considered to explain the evolution and maintenance of hermaphroditism, some of which are not mutually exclusive.

According to the fitness gain curve perspective, it is required that either the male and/or the female fitness gain curve shows diminishing returns for hermaphroditism to be the evolutionarily stable strategy. It is not necessary that it is the male fitness gain curve that shows diminishing returns, as long as the female fitness gain curve does so sufficiently (Charnov, 1979, 1982). Therefore, diminishing fitness gains for investment into the female function can also favour and maintain hermaphroditism. For example, in species that exhibit brooding and a limited brood space, the female fitness gain curve can saturate as soon as enough eggs have been produced to fill up the entire brood space (Heath, 1979; Charnov, 1982). Every additional egg produced will then result in much lower fitness returns, because there is no more room for it in the brood space. However, although there seems to be some evidence for a correlation between brooding and hermaphroditism in certain taxa (Ghiselin, 1969), evidence for brooding constraints on egg production is generally weak (Sewell, 1994) and we have no evidence for brooding behaviour, or any other forms of brood care, in *M. lignano*. Similarly, local resource competition, in which related individuals compete for resources in a local area, can also lead to diminishing female fitness gains, namely when offspring produced via the female function have a more clumped distribution in space than offspring produced via the male function, and when female function derived offspring therefore compete more strongly among each other (Charnov, 1982; Lloyd, 1982). Although this could possibly be an alternative explanation for why hermaphroditism is maintained in *M. lignano*, we lack any empirical evidence for sex-specific spatial clustering among offspring at present.

Finally, it has long been argued that hermaphroditism can be favoured over gonochorism when there is no strong trade-off between the male and the female function, for example due to a low overlap between resource requirements of the two sex functions (Charnov *et al.*, 1976), either because organs belonging to the two functions are built at different times or because they require different kinds of limiting resources and/or nutrients. But again, we have no empirical evidence that this is the case in *M. lignano*. On the contrary, we do have clear evidence for a trade-off between male and female allocation, at least under some conditions (Schärer *et al.* 2005; Janicke & Schärer 2009b). Furthermore, there seems to be no *a priori* reason to assume that testis tissue and ovary tissue should be built up by different resources. And while sex allocation is slightly more male-biased at early age in this species (Vizoso & Schärer, 2007), investment in male vs. female reproductive tissues cannot be considered separated in time either.

In summary, although there are several potentially plausible alternative hypotheses to explain the evolution and maintenance of hermaphroditism, none of them seems particularly likely in *M. lignano* given our current knowledge of the biology of this free-living flatworm. The LSC perspective therefore still seems a likely scenario and should be explored further.

Conclusions

Accumulating evidence from several studies, including the current one, suggests a clear, positive relationship between allocation into testes (be it in terms of phenotypic plasticity or standing variation), and the resulting sperm transfer and paternity success in *M. lignano*, which is in line with predictions from sperm competition and sex allocation theory. In contrast, we did not find strong evidence for more sharply saturating male fitness gains under LSC, another core prediction of sex allocation theory. Nevertheless, we think that it would be premature to reject the LSC hypothesis, because there are no plausible and empirically supported alternative hypotheses that could explain the evolutionary maintenance of hermaphroditism in this flatworm. Experiments with lower levels of sperm competition (e.g. with a competitor that is only present some of the time) and a broader range of variation in testis investment might be needed to get a more complete view of LSC and how it influences the male fitness gain curve.

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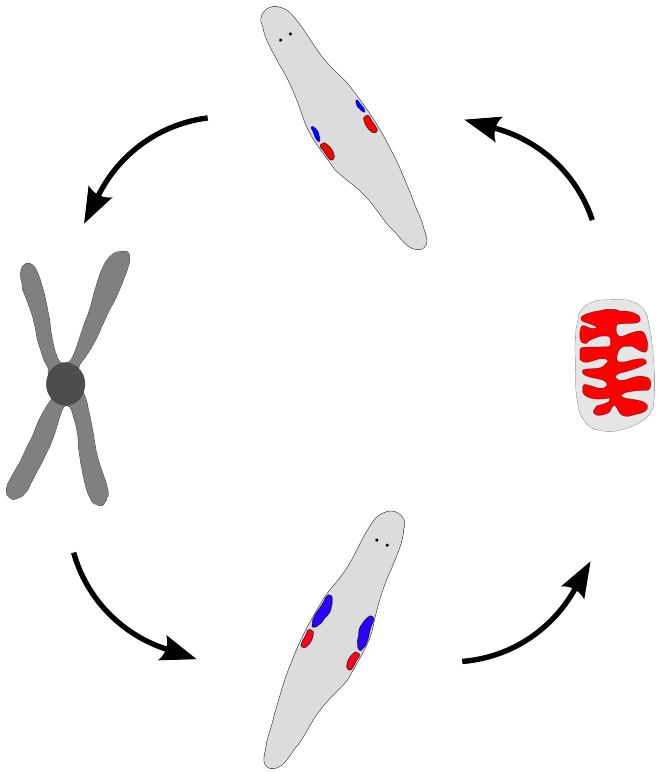
Chapter 4

No Evidence for Strong Cytonuclear Conflict over Sex Allocation in a Simultaneously Hermaphroditic Flatworm



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No evidence for strong cytonuclear conflict over sex allocation in a simultaneously hermaphroditic flatworm

Nikolas Vellnow[1*], Dita B. Vizoso[1,2], Gudrun Viktorin[1] and Lukas Schärer[1,2]

Abstract

Background: Cytoplasmic sex allocation distorters, which arise from cytonuclear conflict over the optimal investment into male versus female reproductive function, are some of the best-researched examples for genomic conflict. Among hermaphrodites, many such distorters have been found in plants, while, to our knowledge, none have been clearly documented in animals.

Methods: Here we provide a quantitative test for cytonuclear conflict over sex allocation in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. We used a quantitative genetic breeding design, employing pair-wise crosses of 2 × 15 independent inbred lines, to partition the phenotypic variance in several traits (including sex allocation) into its nuclear and cytoplasmic components.

Results: Although the nuclear genetic background had a significant effect on all traits analyzed, we found significant cytoplasmic genetic variation only for ovary size, there explaining just 4.1% of the variance. A subsequent statistical power analysis showed that the experimental design had considerable power to detect cytonuclear interactions.

Conclusion: We conclude that there were no strong effects of cytonuclear conflict in the studied populations, possibly because the usually compact mitochondrial genomes in animals have a lower evolvability than the large mitochondrial genomes in plants or because the sampled populations currently do not harbor variation at putative distorter and/or the restorer loci.

Keywords: Genomic conflict, Cytonuclear conflict, Sex allocation, Cytoplasmic male sterility, Animal, Simultaneous hermaphrodite

Background

It has been recognized since the late 1960s that many evolutionary phenomena cannot be understood except in the light of genes being selected to selfishly increase their own representation in a population, also called the gene-centered view of evolution [1–3]. In fact, over the course of the last decades it has become evident that even genes within the same individual can be in conflict with each other [4–6]. Empirical examples for such genomic conflict—sometimes called 'intragenomic conflict', although that term poorly reflects that genes within one individual may reside on different genomes—are now manifold and range from driving B-chromosomes [7], over transposable elements [8] and driving sex chromosomes [9], to cytoplasmic sex allocation distorters [4, 10, 11].

Cytoplasmic sex allocation distorters emerge from cytonuclear conflict over optimal investment into male versus female reproductive function [4, 12, 13]. They are a common phenomenon, since they result from the almost ubiquitous maternal inheritance of cytoplasmic genetic factors, including mitochondria and chloroplast genomes, cytoplasmic endosymbionts, and vertically-transmitted parasites [14]. Because of their (near) exclusive transmission through female gametes [15], any newly emerging cytoplasmic factor that increases the fitness via maternally derived offspring will increase in frequency in the population, even if it at the same time harms male reproduction [4, 12]. In fact, in cases where

* Correspondence: nikolas.vellnow@unibas.ch
[1]University of Basel, Zoological Institute, Evolutionary Biology, Basel, Switzerland
Full list of author information is available at the end of the article

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the fitness via maternally derived offspring trades off with the fitness via paternally derived offspring, any cytoplasmic mutation that will reduce investment into the male function will spread, because it simultaneously increases investment into the female function and therefore its own fitness. For nuclear genes, however, equal investment into male and female function often is the evolutionary stable strategy—at least under the commonly made assumptions of random mating and large population size [16]—resulting in cytonuclear conflict over sex allocation.

As a result of cytonuclear conflict, cytoplasmic sex allocation distorters are thought to emerge and spread, only to be counteracted by nuclear restorer alleles that restore sex allocation towards the nuclear optimum. According to Hurst et al. [13] cytonuclear conflicts (and genomic conflicts in general) may bring about different outcomes. The conflict can disappear when a suitable restorer allele goes to fixation and the distorter allele is lost as a result, either via drift or via pleiotropic costs. Subsequently, even the restorer allele can be lost again if it bears fitness costs itself, so that no signs of the conflict will remain visible in the population and it will be hard to tell that the conflict had ever manifested itself. Another possible outcome is a stalemate, in which the distorter does not manage to "win", but neither is the arisen restorer powerful enough to push the sex allocation all the way back to the nuclear optimum and hence to drive the distorter to extinction. Instead the population will be polymorphic for the distorter and restorer loci [11]. Moreover, cytonuclear conflict can also lead to the extinction of the whole population if a distorter spreads too fast for a restorer allele to emerge in time before extinction and the absence of male function comprises population fertility. This outcome seems to be most likely for those selfish elements that also have a strong negative effect on population growth rate, as is the case for y-linked meiotic drive genes [1], but it can also occur in populations of self-incompatible hermaphrodites in which a cytoplasmic sex allocation distorter rapidly spreads to fixation [17].

Empirically, it will be difficult to find evidence of conflict unless the studied population is in a transitional state or finds itself in a stalemate situation, with polymorphisms at either the distorter or the restorer loci (or possibly both). Since changes in sex allocation caused by cytoplasmic sex allocation distorters can have strong fitness effects, both distorters and restorers may sweep quickly to fixation and no traces of conflict will be found, despite the cytonuclear conflict being latently ever-present in species with uniparental inheritance of cytoplasmic genes.

Cytonuclear conflict over sex allocation manifests itself in different forms in gonochorists (separate-sexed organisms) and hermaphrodites. While in gonochorists the bone of contention is the investment into male versus female offspring (i.e. the sex ratio), it is the resource investment into an individual's male versus female function that is at stake in hermaphrodites.

In gonochoristic animals most known examples of cytonuclear conflict over sex allocation are caused by cytoplasmic genetic elements other than mitochondria [10, 13]. For instance the intracellular alphaproteobacterium *Wolbachia*, which is common in many arthropod species, is transmitted vertically through the cytoplasm of the egg and thus also has uniparental inheritance. It can cause diverse phenotypes in its hosts, such as feminization of offspring, induction of parthenogenesis or killing of sons for the benefit of the daughters, in order to improve its own transmission at the cost of host fitness [18].

To our knowledge there is only one clear example of complete male sterility induced by mitochondria in animals, namely in *Drosophila melanogaster*, in which crosses with the cytoplasm from one specific population, but nuclear genomes from different populations, are male-sterile due to an impaired sperm differentiation [19, 20]. However, it is currently unclear whether this mutation—a single amino acid change in the cytochrome b protein—is just plain deleterious, or whether it conveys a transmission advantage to the mutant mitochondria. Furthermore, Patel et al. [21] found a mutation in subunit II of the mitochondrial cytochrome c oxidase gene that reduces male fertility in *D. melanogaster*, presumably because it impairs sperm development and motility, with no detectable effect on female fecundity. This reduced male fertility could be restored by several different nuclear genetic backgrounds, consistent with a 'sex-specific selective sieve' [22]. It seems unlikely, however, that this is the result of cytonuclear conflict over sex allocation, since there was no detectable transmission advantage to the mutant mitochondria [21].

Among simultaneous hermaphrodites, most of the evidence for cytonuclear conflict has been discovered in plants. Here it is usually a mitochondrial sex allocation distorter that leads to a male sterile phenotype, which is often evidenced as a failure to develop and/or mature the male reproductive tissues. This phenomenon is also called cytoplasmic male sterility (CMS) and male sterility has been found to be associated with an increase of ovule production in male sterile plants, presumably because of trade-offs between male and female function [23–25]. CMS is a common occurrence in angiosperms with 10% of the species exhibiting high frequencies of male sterile individuals due to one or several mitochondrial male-sterility genes [26]. Furthermore, as a result of CMS the sexual system of plants can change from hermaphroditism to gynodioecy, i.e. with the simultaneous

presence of hermaphrodites and females (essentially male-sterile hermaphrodites), but no males in the population [27]. Gynodioecy has been described in 350 species from 39 plant families [10].

CMS can manifest itself in widely different morphologies, such as the complete absence of male reproductive organs, meiotic failure, or abortion of pollen at diverse developmental stages. And while many studies find very strong effects of mitochondrial sex allocation distorters, leading to complete male sterility [28, 29], these effects can also be of a more quantitative nature, in which the allocation into the male function is only partially reduced [11]. In fact, every sex allocation distorter is expected to spread, no matter how strong its effect on sex allocation, as long as it results in increased seed production, so that one can expect many small (and thus possibly difficult to observe) negative effects on male allocation in natural populations [10].

Given how many times CMS has been observed among hermaphroditic plants, it is surprising that mitochondrial CMS has never been documented in hermaphroditic animals, particularly since the same fundamental cytonuclear conflict over sex allocation should also be present in these organisms. In fact, gynodioecy, which is thought to be the result of CMS in plants (see above), is very rare in animals. In his careful review on sexual systems among animals Weeks [30] mentions only nine gynodioecious animals, despite a comprehensive literature research. Most of these species are not well studied, so even their classification as gynodioecious should be viewed with some caution, as it can be difficult to distinguish gynodioecy from sex change or from simultaneous hermaphroditism with pure-sex life-stage. One interesting and plausible example of CMS occurs in the Caribbean reef coral, *Porites astreoides*, which has been described as gynodioecious [31]. It is, however, not known whether mitochondria or other cytoplasmic endosymbionts are involved in this case, because in *P. astreoides* not only mitochondria, but also ectoderm-associated bacteria [32], endosymbiotic dinoflagellates [33] and possibly also apicomplexans [34] are transmitted vertically from mother to offspring. It could arguably be in the interest of all of these symbionts to decrease their hosts' male allocation in favor of an increased female allocation. So on balance, there is a striking paucity of mitochondrial CMS as well as gynodioecy in general in the literature about hermaphroditic animals.

Several explanations for the lack of mitochondrial CMS in hermaphroditic animals have been proposed [35], of which the two main ones are that (i) either hermaphroditic animals have not received enough scientific attention in this regard or that (ii) the mitochondrial genome is less likely to generate sex allocation distorters in animals compared to plants, possibly because of its smaller size, lack of recombination, and lack of gene rearrangements [13, 24]. With respect to the first point, sexual morphology is indeed readily observable in the flowers of angiosperms, as these organs are very extrovert and evident, while in hermaphroditic animals ovaries and testes are often hidden inside the body, and thus more difficult to observe. Therefore male-sterile phenotypes are probably easier to spot in angiosperms (by lack of stamen and pollen) than in most hermaphroditic animals, although some transparent hermaphrodites, such as ctenophores, chaetognaths and many flatworms should also allow for easy identification of male-sterile phenotypes (e.g. lack of testes and/or male copulatory organs). Also, many angiosperms are of commercial interest and plant breeders commonly cross different varieties or even species in order to produce male-sterile individuals with increased seed production, which makes the detection of sex allocation distorters more likely and lucrative [36]. However, many male-sterile phenotypes in plants have been found in natural populations as well, a phenomenon of which already Darwin was aware [23].

Concerning the second point, animal mitochondrial genomes are indeed small compared to those of plants. They commonly only contain 37 encoded genes (range: 14–53) and have a rather narrow size range between 11 and 32 kb [37]. In contrast, the size of plant mitochondrial genomes ranges from 200 to 11,000 kb and large parts of these genomes have been shown to consist of non-coding regions [38]. And although non-coding regions also exist in animals, we still have a fairly limited understanding of the function of these regions [37]. Furthermore, the mitochondrial genome in plants experiences more gene rearrangements [39] and recombines more often [40]. It therefore appears possible that a greater evolutionary potential of plant mitochondria might make the emergence of new sex allocation distorter mutations more likely in plants compared to animals.

Objective

Here we test for the presence of cytoplasmic sex allocation distorters in a simultaneously hermaphroditic animal, by performing a quantitative genetic breeding study in the free-living flatworm *Macrostomum lignano*. Specifically, we partitioned the phenotypic variance in several morphological traits (including sex allocation) into its nuclear and cytoplasmic components. The experiment was conducted under the rationale that, even if there are no drastic signs of CMS, such as complete male sterility, there might still be quantitative effects of cytoplasmic genetic elements on sex allocation, as different cytoplasmic distorters and nuclear restorers interact to determine the resulting sex allocation.

Methods

Study organism

The free-living flatworm, *Macrostomum lignano* Ladurner, Schärer, Salvenmoser, Rieger 2005 (Macrostomorpha, Rhadbitophora, Platyhelminthes) is a member of the intertidal meiofauna of the Adriatic Sea and the Eastern Mediterranean basin [41]. These small (adult length ~ 1.5 mm) worms can be cultured in the laboratory (at 20 °C, 14:10 h light:dark, and 60% humidity) in glass Petri dishes, in either artificial seawater (ASW) or the nutrient-enriched f/2 algal culture medium [42], with the diatom algae *Nitzschia curvilineata* as the sole food source [41, 43]. *M. lignano* is an obligatory outcrossing simultaneous hermaphrodite with an ~18d generation time [44] and shows frequent reciprocal mating [45]. Its highly transparent body permits detailed measurements of internal reproductive structures (see below), and thus allows us to obtain non-invasive estimates of sex allocation [44, 46].

Establishment and maintenance of the inbred DV lines

We have previously given brief accounts of the establishment of certain inbred lines used here, namely DV1 [46]—the line used for the *M. lignano* genome project [47]—and DV3, DV8, DV13, DV28, DV69, and DV71 [48]. Here we provide a fuller account of the establishment of these and many additional lines.

We initiated the inbred lines in January 2004 by assembling 240 pairs of virgin worms from a range of outcrossed laboratory cultures started from different source populations (see Table 1), each pair from the same source population. The straight-line distances between these source populations range from ~0.2 km (PS to X), over ~2.5 km (PS to P1), to ~10 km (PS to UV). Although a larger geographic sampling range might have been preferable to maximize the diversity of cytoplasmic vs. nuclear genetic backgrounds, this range covered the entire confirmed distribution of *M. lignano* at that time [41]. From each founding pair, a line was initiated from the maternal offspring of only one member, which—assuming maternal transmission and no heteroplasmy (see the "Sequencing of mitochondrial *nad2* and *cox1-cytb* fragments" section in the Results)—associates one specific cytoplasmic genotype (cytotype) with one subsequently inbred nuclear background. In order to reduce the probability of a line dying out, we maintained up to six copies of each line.

During the first 15 generations of inbreeding each copy was started with only two juvenile offspring per parental cross, so that the resulting parents of the next generation were necessarily full-sibs and the resulting offspring therefore stemmed from maximal biparental inbreeding. Moreover, in order to maximize the loss of genetic diversity, all copies of each line were made from a single parental pair whenever possible. Despite this replicated breeding scheme we lost a substantial number of lines over several generations. From generation 15 onwards we therefore started a second breeding scheme with each copy within a line being started with three, rather than just two, juvenile offspring from the same parental group. So while the resulting parents of the next generation could have either been full- or half-sibs, the resulting offspring still stemmed from substantial biparental inbreeding. The experiment reported here was initiated in generation 20 by taking worms from this second inbreeding scheme (see next section). As a result of these two breeding schemes we can expect inbreeding coefficients of $F_t > 0.97$ [49], representing a very high level of inbreeding.

For completeness, we also briefly report how these lines have since been kept, and which lines are still being maintained to date (Table 1). The second inbreeding scheme was kept until generation 24, when we switched to maintaining two copies (A and B) from then onwards, each generation being initiated with up to ten offspring from each parental group, thus maintaining a small effective population size and a high level of inbreeding. Again, whenever possible, both copies descend from the same parental group. Moreover, at irregular intervals we further pass these lines through a population bottleneck by starting the populations with only up to five individuals. Specific inbred lines are then expanded whenever they are needed for experiments, with the stock cultures always maintained at these small population sizes. The inbred lines (and also the experimental crosses) were cultured in 6- or 24-well tissue culture plates (TPP, Switzerland), with parental pairs and triplets held in the latter and larger parental groups in the former.

Crossing design

The rationale of the experimental design takes advantage of the fact that (i) in a simultaneous hermaphrodite we can obtain maternal offspring from both parents (and thus potentially different cytotypes) of a specific cross and (ii) recombinant F1 offspring resulting from a cross between parents from two highly inbred lines inherit (almost) identical nuclear genetic material (as segregation and recombination cannot introduce new combinations among the gametes of these parents). Together this means that we can obtain individuals that may differ in the maternally inherited cytoplasmic genes, but are (nearly) identical in their nuclear genes. This permits to study if the maternal background affects any traits, as expected under cytonuclear conflict over reproductive allocation. One potential caveat of this approach, however, is that there could also be maternal effects linked to a common rearing environment if the parents from one line have grown up together (i.e. maternal effects could be confounded with cytotype effects). The design

Table 1 The number of initiated inbred DV lines, those used in the experiment, and those surviving until the current moment, grouped by their source populations in the Northern Adriatic Sea [41]

Source (Site)	Initiated (n) (in 2004)	Used DV lines (in 2006)	Surviving DV lines (currently)
Lignano Sabbiadoro 1995 (P1)	30	22[f], 39[g], 57[g]	18[g], 22[f], 27[g], 39[g], 41[g], 57[g], 71[e], 83[g]
Lignano Sabbiadoro 2002 (P1)	7	na	25[d]
Isola di Martignano 2002 (X)	8	47[i]	47[i]
Isola di Martignano 2003 (PS)	90	26[c], 28[c], 33[c], 49[j], 67[a], 68[b], 72, 81[c], 84[j]	16[c], 26[c], 28[c], 33[c], 49[j], 51[c], 65[c], 67[a], 68[b], 81[c], 84[j]
Bibione 2003 (UV)	105	1[c], 3[d], 6[d], 8[d], 12[d], 13[c], 14, 29[c], 35[d], 40, 44[d], 46[d], 50[d], 61[d], 69[d], 75[h], 76	1[c], 3[d], 6[d], 8[d], 10[c], 12[d], 13[c], 20[g], 29[c], 31[c], 35[d], 37[c], 44[d], 46[d], 50[d], 61[d], 69[d], 75[h]
Total (n)	240	30	39

The letters indicate the different mitochondrial haplotypes of DV lines, with the following polymorphic bases and resulting amino acid substitutions (base positions are given with respect to the start of the sequences for each fragment):

Haplotype	350	373	408	421	587	40	127	148	386	441	528
a	A	C	C	A	G	A	A	C	T	C	T
b	A	C	C	T	G	A	A	C	C	C	T
c	A	C	C	T	G	A	A	C	T	C	T
d	A	C	T	T	G	A	A	C	T	C	T
e	A	T	C	A	A	A	A	C	T	C	T
f	A	T	C	A	G	G	A	C	T	A	C
g	A	T	C	A	G	G	A	C	T	C	C
h	A	T	C	A	G	G	A	C	T	C	T
i	A	T	C	T	G	A	A	T	T	C	T
j	G	C	C	T	G	A	G	C	T	C	T
Encoded amino acids	I/V	F	A/V	L/F	V/I	L	W	R	F/S	F/L	V
Open reading frame	*nad2*	*nad2*	*nad2*	*nad2*	*nad2*	*cox1*	*cox1*	*cox1*	*cytb*	*cytb*	*cytb*

Underlined DV lines were used in the experiment but are no longer available. Note that DV stands for Dita Vizoso, who was mainly responsible for their establishment. The table footer indicates the observed haplotypes in the sequenced genes (see also main text)

that we employed here takes all these points into consideration (Fig. 1).

In order to control for maternal effects, we used a split brood design to produce, initially, up to six independently grown pairs per DV line (up to four of which were actually used, see below). Specifically, we took, for each line, up to 12 offspring from a single parental group (containing two or three parents) and distributed them in up to six pairs in wells on 24-well plates. These pairs constitute our line replicates, having the same cytotype but potentially different maternal effects. We then collected up to six offspring per line replicate and allowed them to grow in isolation in separate plates (day 1). These worms were to become the parents in our crossing experiment. We randomly selected pairs of DV lines that had enough worms and enough line replicates to become our line crosses (note that only one line cross is depicted in Fig. 1). Within each line cross, we chose randomly two to four (of the initially up to six) line replicates to use in the further experiment, which were in turn replicated up to six times, in what we call the actual cross replicates (see Fig. 1). In order to distinguish the two parents within a pair, we dyed one parent by immersing it for 24 h in a 5.6 mg/mL solution of the food dye New Coccine in ASW (day 19), a dosage at which no adverse effects on the mating behavior are observed [50]. Which worm was dyed was decided using a restricted randomization approach. We produced and randomly distributed 277 pairs of 20 line crosses onto 24-well plates (day 20) and allowed the parents (one dyed and one undyed) to mate for three days, after which time we isolated them for egg laying (day 23) in order to be able to collect offspring separately from each maternal cytotype. These offspring were to become our focal worms (Fig. 1). We collected up to two such focal worms per laying parent (i.e. up to four per pair) and isolated them in 24-well plates (day 34).

In order to allow these focal worms to interact with a partner while keeping the resulting variation to a minimum, we used worms from what we call the tester DV line (DV20) from the same generation. We dyed said testers using the procedure described above, and added them to the focal worms (day 39). After five days (day 44), we re-dyed the testers (as the dye washes out eventually), and placed them with the focals in fresh plates (day 45). Finally, we measured the morphology of the

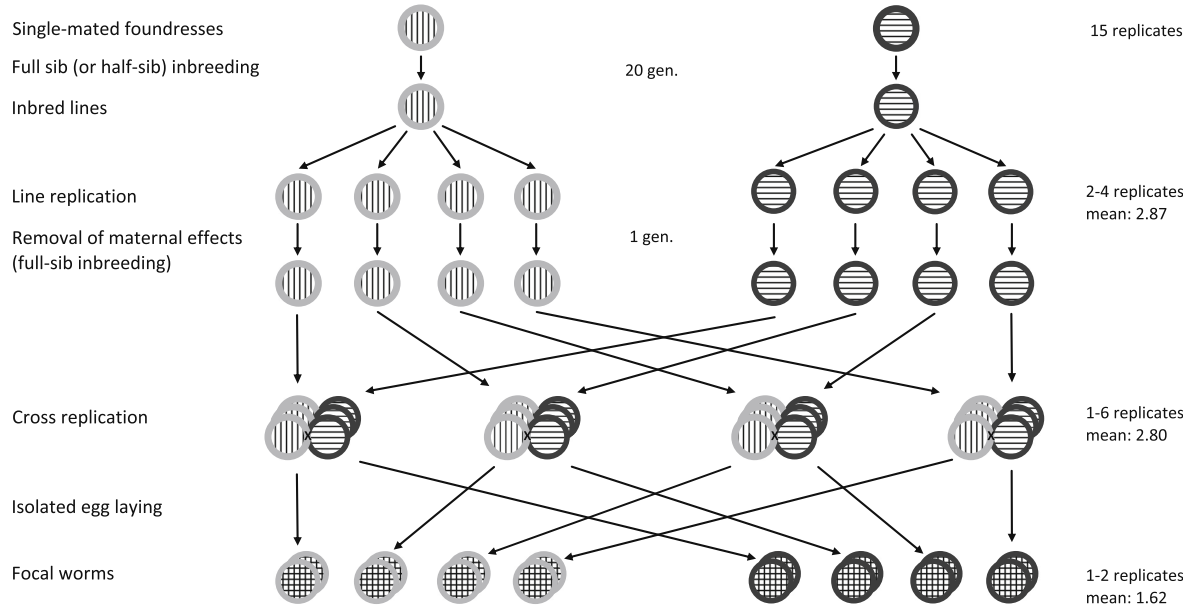

Fig. 1 Crossing scheme used in this study (shown here for one of the 15 independent pairs of inbred line crosses) in which the *stripe pattern* of the inner circular area represents the nuclear genome and the shade of *gray* of the outer rim indicates the cytotype. Note how both lines were replicated and grown independently for one generation to account for maternal effects in the analysis and how the resulting offspring of the line crosses share the (approximately) identical nuclear genome, but differ in the cytotype depending on which line is the maternal parent.

focals, as described in the following subsection (days 50 and 51). In the end, we obtained enough focal worms (n = 391) for 15 of our initial 20 line crosses stemming from 241 crossings (i.e. actual parental pairs) of which in nine cases only one parent produced offspring.

Morphological measurements

The morphological measurements were taken as previously described [44, 51]. Briefly, we anaesthetized worms with $MgCl_2$ and squeezed them dorsoventrally between a glass slide and a hemocytometer cover glass, separated by a 35 μm plastic spacer. We then took digital micrographs at 40-400× with a digital video camera (DFW-X700, Sony Broadcast & Professional, Köln, Germany) attached to a Leitz Diaplan compound microscope (Leica Microsystems, Wetzlar, Germany). We used the image capture software BTV Pro (Bensoftware) to acquire the images and the pictures were analyzed with the public-domain image-analysis software Object-Image 2.09 (developed by Norbert Vischer, University of Amsterdam). Specifically, body size (the total body area), testis size (the sum of both testis areas), ovary size (the sum of both ovary areas), seminal vesicle size (the area of the seminal vesicle) and eye size (the mean of the area of both eyes) were measured this way. Sex allocation was calculated by dividing testis size by the sum of testis size and ovary size [51].

Statistics

To test for effects of the cytotype as well as the nuclear genetic and maternal background on the different morphological measures a linear mixed model was fitted with the *lme4* package in R (version 3.2.4) using restricted maximum likelihood in order to extract unbiased variance estimates of the random effects [52, 53]. Note that because of the details of the used algorithms the estimate for a random effect variance will be zero in cases when the true random effect variance is very small or negative. In these cases the random effect can be considered as contributing no significant amount of variance [54]. Response variables were body size, testis size, ovary size, sex allocation, seminal vesicle size and eye size. All response variables were log- and z-transformed to meet the normality assumption for residuals and to facilitate model convergence, respectively. Only an intercept was used as fixed effect and random effects were fitted according to the nested structure of the experimental design, with cross replication nested within line replication, line replication nested within cytotype, and cytotype nested within line cross (Fig. 1 and Table 2). Random effects were removed in a stepwise fashion and the significance of the removed effect was tested by comparing the simpler model to the more complex model with a parametric bootstrap test with 20,000 iterations using the R package 'pbkrtest' [55].

Table 2 Shown are the percentages of explained variance by the different random effects (and their respective *p*-values) for the measured morphological traits

Factors	Body size	Testis size	Ovary size	Sex allocation	Seminal vesicle size	Eye size
Line cross	**28.0 (<0.001)**	**35.0 (<0.001)**	**24.4 (<0.001)**	**24.1 (<0.001)**	**28.8 (<0.001)**	**67.0 (<0.001)**
Cytotype	0.6 (0.17)	0.1 (0.66)	**4.1 (0.046)**	0.0 (1)	0.0 (0.68)	0.0 (0.83)
Line replication	4.4 (0.09)	0.0 (0.65)	0.0 (0.70)	0.6 (0.52)	1.4 (0.36)	**2.0 (0.019)**
Cross replication	2.3 (0.50)	0.2 (0.62)	0.0 (0.66)	0.0 (0.68)	0.0 (1)	4.9 (0.06)
Plate ID	0.8	1.6	1.1	3.0	5.2	0.0
Residual	63.9	63.2	70.4	72.3	64.6	26.1

The percentages of explained variance were calculated from the variance estimates in the full model and bold values indicate significant effects

Additionally, a plate ID random effect was included to account for variance among the well plates in which worms were held. The variance components to calculate the percentages of explained variance of the different factors were taken from the full model. The model assumptions of normally distributed and homoscedastic residuals were checked with normal q-q plots and residuals vs. fitted plots, respectively. For the eye size analysis 5 replicates had to be excluded because of missing eye size measurements. We chose not to correct for body size when analyzing testis, ovary, seminal vesicle and eye size as well as sex allocation since we assumed the absolute size of these organs to be more closely related to fitness than their body size corrected values (but see the Additional file 1 for an analysis that includes a body size correction, which is qualitatively very similar to the analysis presented in the main text). We included body and eye size as traits in the analysis to compare the results of sex allocation traits with and because mitochondria have been reported to be important components of the eyes of some flatworms including the Macrostomida [56].

In order to estimate the magnitude of the cytotype effect sizes that we could have detected with our experimental design, a post-hoc power analysis was performed. For this we i) simulated datasets for specific chosen values of the random effects (see below) using the same number of replicates for each factor level as in the original dataset, ii) tested for each of those simulated datasets the significance of the cytotype effect by comparing the model with cytotype nested within line cross with the reduced model only containing the line cross random effect by parametric bootstrapping (this time with 600 bootstrap iterations to work more time efficiently) and iii) calculated the proportion of tests that resulted in a significant *p*-value as an estimate of our statistical power. The chosen values for the variance components used for the simulations were assumed to be those from the model for ovary size (i.e. the values in the ovary column in Table 2), except that several different values for the cytotype effect size (i.e. the proportion of variance explained by the cytotype) were used (see the x-axis in Fig. 3) and consequently the residual variance changed as well. Here we chose to use the empirical values for ovary size because ovary size is a priori the most likely trait to be influenced by cytonuclear conflict, since it should translate most directly into an altered maternally derived fitness (note that when using the variance components from the model with testis size, very similar results were found). For each of the 11 different effect size values 1000 datasets were simulated, which adds up to a total of 11 × 1000 = 11,000 datasets. These simulations were also conducted in R version 3.2.4 [52].

In our discussion of effect sizes and statistical power we adhere to the conventions suggested by Cohen [57]. More specifically, we consider 0.01, 0.09 and 0.25 of the variance explained to represent small, medium and large effect sizes, respectively [57, 58] and we consider Hedge's g values of 0.2, 0.5 and 0.8 to represent small, medium and large effect sizes, respectively [59].

Mitochondrial genetic diversity of DV lines

In order to test whether worms were monomorphic within and showed mitochondrial genetic variation among the different DV lines, two mitochondrial DNA fragments, one within the *nad2* gene and one spanning the *cox1* and *cytb* genes, were amplified by PCR and sequenced. For each available DV line at least two individuals were sequenced. Moreover, in order to verify our assumption of maternal inheritance of the mitochondrial genome, two virgin individuals from inbred lines that differed in their *nad2* fragment were crossed and the parents as well as the offspring were sequenced. In some pairs one of the parents failed to produce maternal offspring, in which case we still analyzed the maternal offspring of the other parent. In total these added up to 40 instances of mitochondrial transmission from line crosses DV1xDV75 (six times two, and three times one maternal offspring per pair), DV39xDV69 (six times two, and six times one maternal offspring per pair) and DV71xDV84 (one time two, and five times one maternal offspring per pair).

Genotyping was performed as follows: For DNA isolation, individuals were immersed in 30 μl 100% Ethanol and kept at −20 °C for at least an hour. Ethanol was

evaporated at 80 °C until dry, and 20 μl 10 mM Tris-HCL pH 8.0 containing 1 mg/ml Proteinase K (Roche, Mannheim, Germany) were added. Samples were incubated at 65 °C over night, followed by 15 min at 95 °C to deactivate Proteinase K. 0.5–1 μl of this isolate was used as template for a 20 μl PCR reaction (modified from *Caenorhabditis elegans* single worm DNA isolation method by H. Schulenburg, pers. comm.). PCR was performed using Q5 polymerase (New England Biolabs, Ipswich, MA, USA) using specific primers for the *nad2* fragment (MacNad2-F: TAAGATTAGTGGGAAAGATG GGAAG; MacNad2-R: AACAAACATAGAAAATGGGG GAATACC; fragment size: 380 bp) and the *cox1-cytb* fragment (MacCox1-F: GGTTTATCTGGTATGCCTCG TCG; MacCytb-R: CGCTCCTCAAAAAGACATCTG; fragment size: 680 bp). Cycling conditions were the same for both fragments: 30 s 98 °C, 35× (7 s 98 °C, 30 s 64 °C, 30 s 72 °C), 2 min 72 °C. Fragments were sequenced using the MacNad2-F and MacCytb-R primers, respectively.

Results

Effects of line cross, cytotype and other variance components

Our analyses revealed significant line cross effects for all measured traits, as evidenced by the stepwise removal of lower level random effects and comparison of reduced to unreduced models (Fig. 2 and Table 2). This suggested that there was considerable genetic variation for all these measured traits, including sex allocation, among the nuclear genomes within/among the founding populations (Table 1), explaining between 24.1 and 67.0% of the observed phenotypic variance (Table 2). In contrast, the effect of the cytotype was not significant for most of the traits, except for ovary size (parametric bootstrap test, $p = 0.046$), where it explained only 4.1% of the variance (cf. ovary panel in Fig. 2). There was no apparent difference in the magnitude between the cytotype effects of traits measuring reproductive morphology, over which cytonuclear conflict seems more likely (i.e. testis size, ovary size, sex allocation and seminal vesicle size), and those measuring other morphological traits (i.e. body size and eye size, Fig. 2). Neither line replication, nor cross replication contributed a significant amount of variance for any of the traits, except for eye size, where line replication contributed 2.0% variance (parametric bootstrap test, $p = 0.019$), suggesting that there are no strong maternal effects for these traits.

Post hoc power analysis

Given the number of replicates we used for each factor level combination in this study and given the variance components that we have empirically found for ovary size, the cytotype effects would have needed to explain about 9% of the variance to be detectable with a power of 0.8 (Fig. 3). This is considered the desired power by convention in the statistical literature [57, 58] and we should thus have been able to reliably detect medium to large effect sizes of cytonuclear conflict with our experimental design.

Sequencing of mitochondrial *NAD2* and *COX1-CYTB* fragments

Five and six polymorphisms were detected within the sequenced *nad2* and *cox1-cytb* fragments, respectively. Taken together, these 11 polymorphisms can be used to subdivide the 39 currently maintained DV lines into at least 10 groups with regard to their mitochondrial haplotype (see footer of Table 1). Six of the polymorphisms cause amino acid substitutions, of which four lie within the predicted *nad2* coding region, and two within the predicted *cytb* coding region (Table 1). Moreover, individuals belonging to the same DV line always carried the same *nad2* and *cox1-cytb* sequence, suggesting no mitochondrial variation within lines.

Many DV lines that were paired for the main crossing experiment differed in their *nad2* and/or *cox1-cytb* sequence, so that eight (DV8xDV22, DV33xDV50, DV35xDV81, DV46xDV67, DV6xDV47, DV49xDV57, DV39xDV69 and DV75xDV84) out of the 15 crosses used in this study were done between lines that clearly differed in their mitochondrial haplotype (irrespective of whether considering base or amino acid sequence), at least with respect to these gene regions. From the seven remaining crosses, five did not differ in the considered gene regions (DV1xDV13, DV28xDV29, DV3xDV12, DV40xDV76, DV44xDV61) and in the other two (DV14xDV26 and DV68xDV72) only one line could be genotyped, because the other line had in the meantime been lost.

Finally, in all of the 40 instances in which both the mother and her maternal offspring were sequenced for the *nad2* gene, the maternal mitochondrial genotype was inherited. This suggests exclusive maternal inheritance (or at least a low frequency of paternal inheritance) of the mitochondrial genome in *M. lignano*, thus fulfilling an important assumption of the predicted cytonuclear conflict.

Discussion

Short summary of results

Our study confirmed that there was, for all traits measured, considerable and statistically significant nuclear genetic variation in the sampled populations from which the inbred lines used here were derived [60]. Conversely, no significant variation due to the cytotype could be found in either sex allocation or the other measured traits, except for a fairly small, but statistically significant effect on ovary size. This was the case in spite of the fact

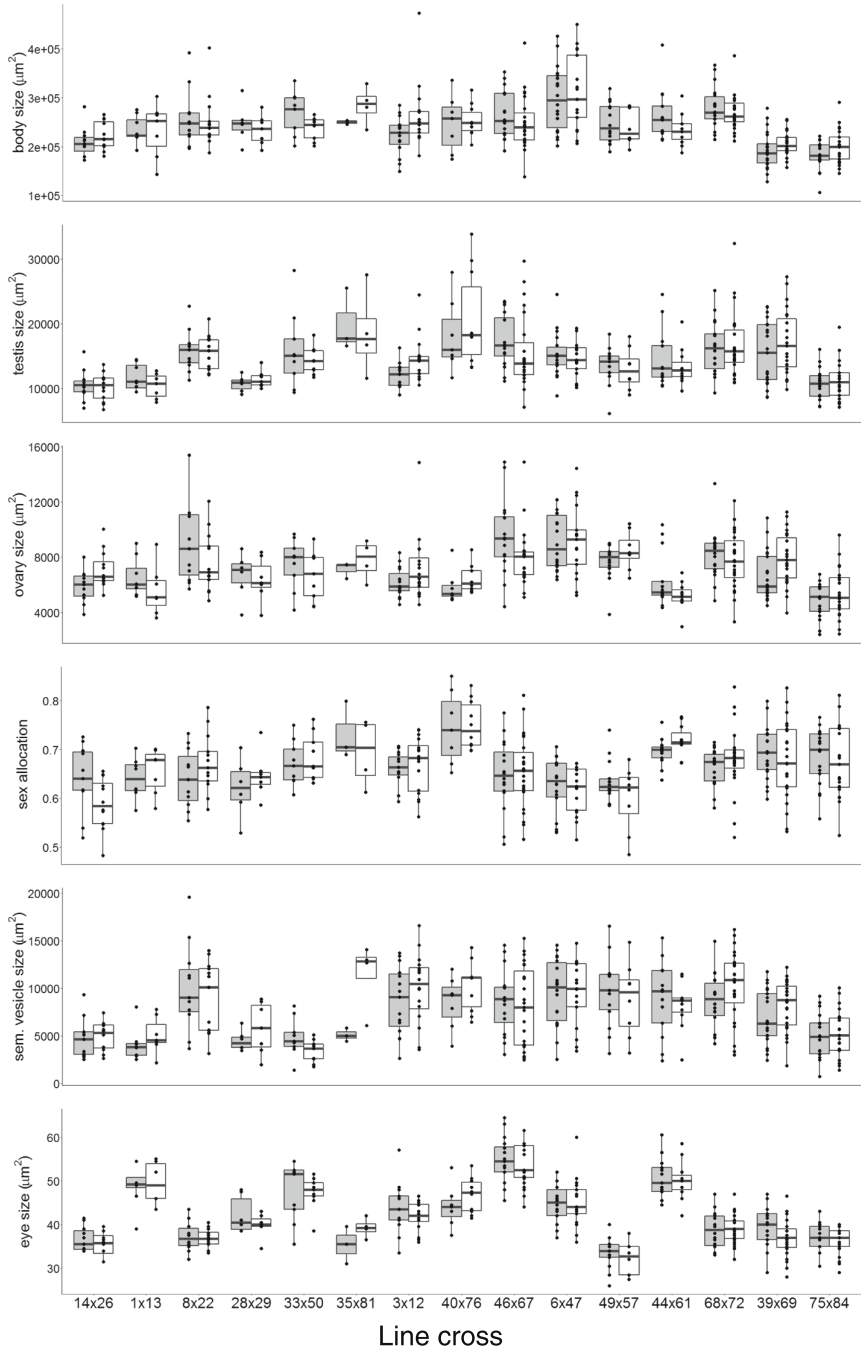

Fig. 2 Effect of the specific line cross (e.g. DV14 x DV26) and their two respective cytotypes (box plots are *gray* for the first and *white* for the second line in each cross) on all the other measured traits. *Black dots* represent the individual measurements, the bottom and the top of the box represent the 25th and 75th percentiles, respectively, and whiskers extend to 1.5 times the interquatile range. Note that all traits are plotted on their original measurement scales for visualization (see also the "Statistics" section in the Methods).

that the experiment was statistically powerful enough to detect effects of medium to large size (i.e. cytotype effects that explain 9% of the observed variance or more), while the observed ovary size effect did not reach that threshold. Therefore we did not find evidence for strong sex allocation distorters in our experiment, which were

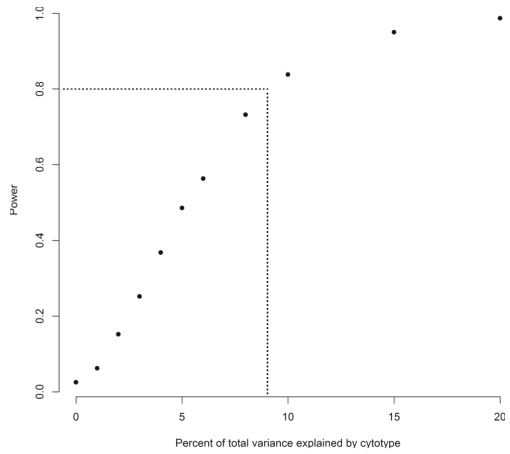

Fig. 3 Power analysis for different simulated cytotype effect sizes (x-axis) assuming the same variance components as those empirically found for ovary size (see main text for rationale). Each *black dot* represents the proportion of 1000 simulations that resulted in a significant cytotype effect (with a significance threshold of 0.05). To achieve a power of 0.8 one needs a cytotype effect size of about 9% or more (*dotted line*).

predicted under the above-mentioned scenarios of cytonuclear conflict over sex allocation. We do not interpret the detected cytotype effect on ovary size as strong evidence for cytonuclear conflict over sex allocation, because of three reasons. Firstly, the effect was fairly small, especially when compared to the line cross effects. Secondly, the relevant *p*-value was just below the significance threshold of 0.05 and since we performed many tests in this study we cannot exclude the possibility that this represents a type I error (and any approach to correct for multiple testing would surely have rendered this effect non-significant). And lastly, the cytotype effect on ovary size might in part have been due to variation in body size, because for some of the crosses where the cytotypes differed most strongly, body size actually differed in the same direction (e.g. DV14xDV26, DV39xDV69, DV44xDV61 and DV46xDV67; cf. body size and ovary size panel in Fig. 2). In fact, when controlling statistically for body size the cytotype effect on ovary size disappeared, because of the strong correlation between body and ovary size (see Additional file 1). However, we think the fact that we did not find strong evidence for cytonuclear effects on sex allocation is very interesting and important, bearing in mind that there are, to our knowledge, no published studies looking at this phenomenon in simultaneously hermaphroditic animals so far and that the publication of such "negative" results is crucial for scientific progress [61–63]. In the following we discuss different aspects of our results in some more depth.

Statistical power

Assuming that the magnitude of cytoplasmic effects on sex allocation is comparable to that of other cytoplasmic genetic effects recently summarized in a meta-analysis [59] we had good statistical power to detect cytotype effects. In their study Dobler et al. [59] found the average effect size to be medium (Hedges' g: 0.49; 95% CI: 0.30–0.79), although many studies they analyzed also reported small cytoplasmic genetic effects. It is therefore possible that some small effects went undetected in our study. However, medium to large effects, or even cases of complete male sterility, which are often reported in the CMS literature for plants [11, 28, 29], should clearly have been detectable in our study with a high power, especially since we measured sex allocation quantitatively and with a well-established method that has previously revealed theoretically predicted phenotypically plastic adjustments of sex allocation to changes in the social environment [44, 46]. Our study also had considerable statistical power compared to many other published studies, as most studies in the behavioral ecology and animal behavior literature have a lower power to detect small or medium sized effects [64], compared to the power observed here (Fig. 3).

Measured traits

Sex allocation distorters should be selected to increase the investment into the female function, for example, by reducing the investment into male function when there is a trade-off between the two sex functions. In *M. lignano* accumulating evidence suggests that there is indeed a trade-off between ovary and testis size [65, 66]. These morphological measures of sex allocation seem to reflect allocation to male and female function well, and testis size has been shown to correlate with the cell proliferation activity in that organ [67] and with the amount of sperm produced [68]. Moreover, testis size also predicts male sperm transfer success [69] and paternity success [70], presumably because worms with bigger testes manage to transfer more sperm per mating [70]. Given all these reasons one could therefore have expected a cytotype effect on testis size, on ovary size, and/or on sex allocation, if there indeed was variation at a sex allocation distorter locus in the sampled populations.

Establishment of inbred lines

Of the 240 initiated inbred lines almost 200 went extinct because the worms failed to reproduce sufficient numbers of offspring to maintain the lines (cf. Table 1). The most parsimonious explanation for this seems to be inbreeding depression, which is a common phenomenon in the establishment of inbred lines [71, 72], especially if outbreeding is the normal reproductive mode, as likely is the case in *M. lignano* [44]. However, complete or

extremely strong CMS could in theory also cause a failure to reproduce, e.g. if all individuals within a line were male sterile, so that there were no functioning sperm available to fertilize the eggs. In such cases any sex allocation distorter alleles causing a complete shut-down or extremely strong reduction of male fertility might have been lost due to the extinction of those lines, thus potentially explaining why we did not find complete male sterility in the surviving lines. Given that we almost never see completely male-sterile individuals in our outbred cultures stemming from the same source population as the DV lines, this, however, seems a rather unlikely scenario (LS and NV, pers. obs.), particularly since only a few functioning sperm cells would likely have been necessary to fertilize enough offspring to produce the next generation under the non-competitive inbreeding system we employed here.

Stage of the evolutionary arms race

The lack of evidence for cytonuclear conflict on sex allocation could, of course, have resulted from the fact that we happened to sample the population(s) at a stage of the evolutionary arms race when there was no variation at either putative cytoplasmic sex allocation distorter loci or nuclear restorer loci [13]. Or in other words, the population(s) studied could have been sampled at a time point when either a nuclear restorer or a partial cytoplasmic distorter had just swept to fixation. To test if this is true it would have been useful to cross more distant populations (or even incipient species) of *M. lignano* and to investigate whether the resulting offspring suffers from reduced male allocation caused by cytoplasmic distorters. A period of independent evolutionary history of multiple populations might have made it more likely that putative nuclear restorer alleles from one population fail to restore sex allocation back to the nuclear optimum in response to putative sex allocation distorters of another population. Generating such crosses has revealed many latent CMS genes in plants [24, 29, 36] and cytoplasmic genetic effects studied with interspecies crosses are typically larger than in intraspecies crosses [59].

The inbred lines used in our experiment were sampled from 3 sites (Table 1) at only a few kilometers distance from each other. We currently have no knowledge about whether and, if yes, how much gene flow happens between those sites and when their most recent common ancestor lived. Assuming these to be partially diverged sub-populations with somewhat independent histories of cytonuclear conflict, several of the crosses might have shown cytotype effects, since they were established between inbred lines from different sites (e.g. crosses DV14xDV26, DV8xDV22, DV28xDV29 and DV33xDV50; cf. Fig. 2). However, our results suggested this was not the case. Furthermore, eight crosses we tested differed in their mitochondrial haplotype for the *nad2* and/or *cox1-cytb* gene fragments as well as the encoded amino acid sequence, of which two were sampled from different sites (DV8xDV22 and DV33xDV50), of which DV8xDV22 might possibly have showed some degree of cytonuclear conflict (Fig. 2).

Alternatively, crossing *M. lignano* worms from these Italian populations in the Northern Adriatic Sea with *M. lignano* we have recently been able to sample from Greek populations from the Sithonia peninsula in the Northern Aegean Sea might potentially be more promising for detecting cytoplasmic sex allocation distorters, since these populations likely have had more time to diverge. This was unfortunately not possible when our experiment was conducted, because the worms from Greece were sampled for the first time in 2013 (i.e. several years after our experiment was performed), and the establishment of new inbred lines is a lengthy and highly laborious process. Any follow-up experiments should ideally be informed by a better understanding of the geographical distribution of *M. lignano* and the connectivity between different populations of this species. However, since we do not know much about the connectivity between populations and dispersal abilities of this species there was no reason a priori to expect that cytoplasmic sex allocation distorters are not detectable within the geographic scale of the Italian populations.

Low evolvability of mitochondrial genome

Another possible explanation for the lack of strong cytotype effects is that cytonuclear conflict over sex allocation may not easily manifest itself in animals in general, due to the generally rather compact mitochondrial genome of most metazoans. After the acquisition of mitochondria into the cells of eukaryotes and the subsequent coevolution between the mitochondrial and nuclear genomes, many mitochondrial genes were transferred to the nuclear genome, so that today most metazoan mitochondrial genomes are small in size (~16 kb with a range between 11 and 32 kb) and usually contain only 13 protein-coding genes, 22 tRNA genes, two ribosomal RNA genes and only very few non-coding sequences [73, 37, 74]. In such a compact, "streamlined" and non-recombining mitochondrial genome new mutations are more likely to affect essential functions, while mitochondrial genomes in plants have the "space and opportunity to form new coding sequences among which sterility-inducing genes may emerge" [24]. However, although metazoan mitochondrial genomes are often viewed as being small, having invariant gene content, and being solely responsible for ATP production, evidence is emerging for their involvement in cell signaling and differentiation, fertilization and apoptosis [75]. And

despite being more evolutionarily conserved than plant mitochondrial genomes, at least some animal mitochondrial genomes show striking deviations from typical architectures, with introns and linear or multicircle mitochondrial DNA, as well as variable gene content (mainly due to number of tRNAs). Breton et al. [75] make a good case for a stronger appreciation of the taxonomic variability in metazoan mitochondrial gene content and organization, and their evolutionary implications.

Nevertheless, except for lacking the atp8 gene and having a slightly modified genetic code [37, 75, 76], flatworm mitochondrial genomes studied to date are fairly representative of other Metazoa, and preliminary data suggest that the mitochondrial genome of *M. lignano* closely matches that of other flatworms (A. Waeschenbach and T. Littlewood, pers. comm.). But, animal mitochondrial genomes have been reported to evolve over evolutionary times as short as ten generations in a seed beetle [77] and cytoplasmic genetic effects in general do not seem to be stronger in plants than in animals [59]. Therefore it seems premature to flat-out refuse the possibility of the emergence of sex allocation distorters in animals, solely based on the argument of their compact mitochondrial genome.

Implications for evolutionary transitions between sexual systems

Explaining the apparent lack of mitochondrial CMS in animals may not only shed light on the evolution of genomic conflict, but it may also have implications for our understanding of the evolution of sexual systems. In particular, CMS has been invoked as a "kick starter" of the evolutionary transition from monoecy via gynodioecy to dioecy in plants [78–80], while the rarer evolutionary transition from hermaphroditism to separate sexes in animals seems to happen more often via androdioecy rather than gynodioecy [30]. If, as our results seem to suggest, cytonuclear conflict in animals indeed leads to CMS less often and/or less easily than in plants, because sex allocation distorters may evolve less often in the compact metazoan mitochondrial genomes, then this could help to explain the different patterns for evolutionary transitions in sexual systems between animals and plants.

Conclusions

In our study we did not find evidence for strong cytonuclear conflict over sex allocation in a simultaneously hermaphroditic animal. It would be tempting to use this evidence as confirmation for the hypothesis that the compact metazoan mitochondrial genome is less prone to evolve selfish sex allocation distorters compared to those of plants, resulting in different patterns of evolutionary transitions between sexual systems in animals and plants. This conclusion would be somewhat premature, however, given how little we currently know about the reproductive biology, sexual systems and mitochondrial genomes in many metazoan phyla. It has become apparent in recent years that mitochondrial genomes are not just "passive bystanders of adaptive evolution" [75] and the highly conserved stretches of DNA as which they were seen until a few decades ago. Thus further studies of mitochondrial genomes and their evolutionary effects seem clearly worthwhile.

For example, crosses between more diverged populations (or incipient species) of hermaphroditic animals could potentially reveal latent cytonuclear conflicts, as they have often done in plants. Furthermore, effects of mitochondrial genetic variation on reproductive phenotypes could be explored with gene silencing approaches, which are beginning to be used for editing the mitochondrial genome [81]. Moreover, elegant experimental evolution studies, similar to the approach used by Kazancıoğlu and Arnqvist [77], could be used in suitable model organisms to follow the spread of sex allocation distorters and restorers in real-time.

Additional file

Additional file 1: Statistically controlling for body size. (DOCX 21 kb)

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Availability of data and materials

The dataset generated and analyzed during the current study is available in the Dryad Digital Repository, http://dx.doi.org/10.5061/dryad.f468h

Authors' contributions

LS and DV designed and performed the experiment. GV sequenced the mitochondrial NAD2 and COX1-CYTB gene fragments. NV analyzed the data and performed the power analysis. NV wrote the main part of the manuscript with all other authors writing parts of and providing comments on the manuscript. All authors read and approved of the final manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Author details
[1]University of Basel, Zoological Institute, Evolutionary Biology, Basel, Switzerland. [2]University of Innsbruck, Institute of Zoology, Innsbruck, Austria.

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SUPPORTING INFORMATION

Statistically controlling for body size

Since ovary size and all other traits were correlated with body size we repeated the analysis using body size as a fixed effect in a linear mixed model while keeping the random effects structure as reported in the main text. Random effects were again removed in a stepwise fashion and the significance of the removed effect was tested by comparing the simpler model to the more complex model with a parametric bootstrap test with 20000 iterations using the R package 'pbkrtest' (Halekoh and Højsgaard 2014) but with body size as fixed effect remaining in the model. The results (Table S1) were very consistent with the analysis in the main text (Table 2). The only important difference was that the cytotype here explained even less of the variance in ovary size (excluding variance explained by the fixed effect of body size) and that this difference was thus no longer statistically significant.

Table S1. Shown are the percent of variance explained (and p-values in brackets) by different random effects while controlling for body size as fixed effect in the model. For body size the standardized slope β and the corresponding p-value are reported. There are no major changes compared to the original analysis (Table 2 in manuscript) except that here the cytotype explains almost no variance for ovary size either. Note that here the percent of variance explained by the random effects are calculated based on the variance that is not already explained by the fixed effect, i.e. the variance after controlling for body size is scaled to 100 percent.

Factors	Testis	Ovary	SA	Seminal vesicle	Eye size
Body size (fixed effect)	**β=0.42 (<0.001)**	**β=0.59 (<0.001)**	**β=-0.21 (0.001)**	**β=0.47 (<0.001)**	**β=0.13 (<0.001)**
Line cross	**31.8 (<0.001)**	**21.9 (<0.001)**	**26.0 (<0.001)**	**26.4 (<0.001)**	**66.6 (<0.001)**
Cytotype	0.0 (1)	0.6 (0.53)	0.0 (1)	0.0 (1)	0.0 (0.28)
Line replication	0.0 (1)	0.0 (1)	0.3 (0.59)	2.3 (0.20)	**2.2 (0.02)**
Cross replication	0.9 (0.54)	0.0 (1)	0.0 (1)	0.0 (0.68)	4.9 (0.06)
Plate	1.3	1.1	3.2	5.3	0.0
Residual	66.0	76.4	70.6	66.0	26.3

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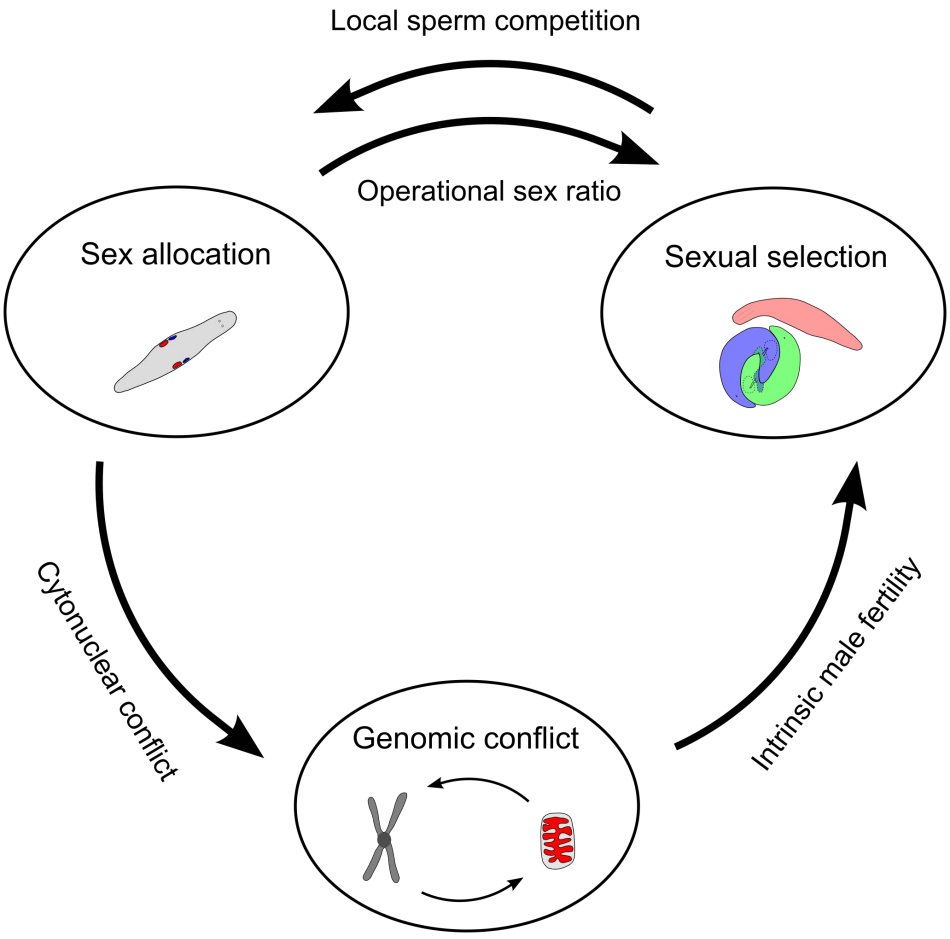
Chapter 5

General Discussion and Outlook



The evolution of anisogamy led to a cascade of new phenomena and evolutionary consequences that did not exist before (Parker 2014). Among others it led to: i) sexual selection, ii) the problem of sex allocation and iii) cytonuclear conflict over sex allocation. During my PhD project I studied aspects of all these evolutionary consequences of anisogamy using the simultaneously hermaphroditic flatworm *Macrostomum lignano*.

Chapter 2: Sexual Selection and GxG

Sexual reproduction, especially when it occurs by copulation and internal fertilization, requires interactions of at least two mating partners, i.e. it involves interacting phenotypes (Moore et al. 1997; Schneider et al. 2016). In reciprocally copulating hermaphrodites one can arguably expect matings to be strongly influenced by interactions between the mating partners, because of the often very intricate, reciprocal mating behaviors (e.g., Michiels and Newman 1998; Schärer et al. 2004; Davison et al. 2005; Anthes et al. 2008; Vizoso et al. 2010). In **Chapter 2** I described an experiment, in which we tested for sperm donor genotype x sperm recipient genotype interaction effects on both mating behaviors and pre- and postcopulatory fitness components. We found these kinds of GxG interactions to affect two mating behaviors, but not the pre- and postcopulatory fitness components, whereas almost all traits considered were influenced by the sperm donor's genotype.

It is possible that the outcomes of sexual selection during pre- and postcopulatory processes up until sperm storage in the female antrum are mainly under control of the sperm donor. The influence of the sperm recipient may only manifests itself during the remaining episode until fertilization, during which the 'sperm fertilization efficiency', a measure of how sperm transfer success translates into male reproductive success (Marie-Orleach et al. 2016), takes effect. Interestingly, the opportunity for selection, an upper bound for the strength of selection (Crow 1958; Arnold and Wade 1984), was also larger for the postcopulatory fitness components—especially sperm fertilization efficiency—than for mating success in the study of Marie-Orleach et al. (2016). However, since the recipient genotype and its interaction with the donor genotype affected some mating behaviors, it might be premature to conclude that such interaction effects do play no role during the selection episodes tested in our experiment. In our experiment we may have been able to estimate mating behaviors with less measurement error than the fitness components mating success and sperm transfer success. If that is true, it could explain why we only found GxG interactions for mating behaviors. Binomial sampling error may have obscured some patterns in the analysis of the fitness components, while the mating behavior mating latency, for which we found significant GxG interactions, is not affected by binomial sampling error. However, the proportion of matings followed by a recipient suck, another mating behavior, was significantly affected by GxG interactions, although it should also be affected by binomial sampling error. Furthermore, there are still important processes taking place during these pre- and postcopulatory selection episodes that strongly influence the resulting fitness components, but which we do not know about or cannot measure at the moment. Especially during the transition from mating success to sperm transfer success there must be processes that contribute strongly to the unexplained variation in sperm transfer success and presumably as a result also in paternity success (cf. the strongly U-shaped distribution of sperm transfer success in Fig. 5, **Chapter 2**). Explaining what process caused this finding and correcting experimentally or statistically for it in future experiments might increase the power to detect treatment effects on paternity success. For instance, the exact order in which matings take place might strongly affect postcopulatory fitness components when a species exhibits first donor sperm precedence (Pélissié et al. 2014) or last donor precedence (Marie-Orleach et al. 2014) and can be taken into account in the analysis. Another source of unexplained variation in postcopulatory fitness components might be manipulations of the ejaculate by the recipient during sperm digestion (Michiels 1998) and cryptic female choice (Charnov 1979). In general, processes that lead to large variation in the size of transferred ejaculates whether under

control of the sperm donor, the sperm recipient or influenced by their interaction may result in strongly skewed sperm precedence patterns.

Chapter 3: Sex Allocation and Local Sperm Competition

Certain aspects of sexual selection can influence the optimal sex allocation. In hermaphrodites, local sperm competition (Schärer 2009), can favor a more female-biased sex allocation. Previous studies showed that i) increased testis investment resulted in higher sperm transfer success and paternity success via the male function (Janicke and Schärer 2009; Marie-Orleach et al. 2016) and ii) that *M. lignano* changes its sex allocation according to the prediction from the local sperm competition model (e.g., Schärer and Ladurner 2003; Janicke et al. 2013). However, except for one study in a spermcast mating, colonial ascidian (Yund 1998), evidence for the mechanism by which local sperm competition leads to a change of optimal sex allocation, namely by making the male fitness gains for male allocation more diminishing, was still lacking. In accordance with predictions from sperm competition theory, I presented results in **Chapter 3** that confirm the positive relationship between testis investment and paternity success. However, in contrast to predictions from the local sperm competition hypothesis, we did not find diminishing fitness returns for testis investment in smaller group sizes, i.e. group sizes which should have resulted in strong local sperm competition.

Although several species, including *M. lignano*, change their sex allocation in accordance with the mating group size model (e.g., Trouvé et al. 1999; Al-Jahdali 2012; Janicke et al. 2013), we could not experimentally verify local sperm competition, the mechanism by which this phenotypically plastic change supposedly evolved. This is surprising given that hypotheses other than the group size model and the local sperm competition hypothesis, which could have helped to explain the evolutionary maintenance of hermaphroditism in *M. lignano*, do not seem likely either (see discussion of **Chapter 3**).

It is possible that the statistical power to detect the predicted effect on paternity was low because of *M. lignano*'s rather low fecundity and the resulting substantial binomial sampling error (Marie-Orleach et al. 2016). Furthermore, concluding from my PhD project, there seem to be many processes, about which we do not yet know much or which we cannot quantify easily, that also introduce variation in paternity success (see previous section). A more detailed understanding of the pre- and postcopulatory processes influencing fitness components for the male function would clearly benefit any future study trying to estimate treatment effects on paternity success.

Concerning future experiments testing for the effects of local sperm competition on the shape of the male fitness gain curve, I propose two ways to improve the experimental design. First, generating greater variation in testis size and/or in sperm production rate—for example by using RNAi to knock down genes involved in testis function and sperm production (Sekii et al. 2013)—may make the detection of the predicted effects easier. Second, aiming to generate differences in local sperm competition large enough to be detected may improve the experimental design. For example, a design, in which a focal worm is paired with a recipient only some of the time, while the recipient is paired with a competitor the rest of the time, could increase the range of local sperm competition compared to levels experienced in triplets vs. octets.

Chapter 4: Cytonuclear Conflict over Sex Allocation

Since cytoplasmic genetic factors and nuclear genes are not inherited equally via female- and male-derived offspring, the cytoplasm and the nuclear genome may be in conflict over the optimal sex allocation. Cytonuclear conflict over sex allocation can therefore be expected in all sexually reproducing eukaryotes that exhibit uniparental inheritance of the cytoplasmic genetic factors.

Although this conflict is present in plants as well as in animals, evidence for mitochondrial sex allocation distorters currently is restricted to the plant literature. In **Chapter 4** I reported the results from a quantitative genetic breeding experiment testing for cytotype effects on traits related to sex allocation, as predicted under an ongoing cytonuclear conflict over sex allocation. In contrast to our predictions, we did not find evidence for strong cytonuclear conflict over sex allocation in *M. lignano*.

After the incorporation of a symbiotic α-proteobacterium (the proto-mitochondrion) by an Archaea host and during the following coevolution, the proto-mitochondrion lost and transferred many genes to the nuclear genome of its host. The streamlining of the mitochondrial genome during this 'domestication' process was particularly strong in animals and fungi, but not so much in plants (Gray et al. 1999; Adams and Palmer 2003; Gray 2012). This can be viewed as a resolution of the cytonuclear conflict in animals and fungi during which the nuclear genome 'won' (Burt and Trivers 2008). It would be very interesting to study a case where a symbiont is at an earlier stage of the 'domestication' period. I predict that the symbiont at this earlier stage, while its genome is not yet strongly reduced, may still have more evolutionary 'power' in the conflict and the manifestation of the conflict might thus be more readily visible. An interesting case study could also be the gynodioecious mustard hill coral (*Porites astreoides*) (Chornesky and Peters 1987), one of the very few known animals that show this sexual system (Weeks 2012). As gynodioecy is often caused by cytoplasmic sterility in plants, the range of vertically transmitted symbionts of this coral—namely ectoderm-associated bacteria (Sharp et al. 2012), endosymbiotic dinoflagellates (Thompson et al. 2015), and possibly also apicomplexans (Kirk et al. 2013)—may prove to be responsible for sex allocation distortions.

The lack of evidence in our experiment presented in **Chapter 4** could also be due to the coevolutionary arms race being at a stage in which both the cytoplasmic sex allocation distorter and the nuclear sex allocation restorer are fixed in the population. Crosses between more distant populations could be used to reveal dormant cytonuclear conflicts in animals, as has been done successfully already in plants (Burt and Trivers 2008) and in other fields of evolutionary conflict research (e.g., Ting et al. 2014). The fact that ongoing cytonuclear conflicts, especially involving mitochondria, are so common in plants, but rare in animals, still remains puzzling and is worth further research.

Interconnection between Aspects of Male-Female Phenomenon

The different aspects of the male-female phenomenon covered in my thesis (i.e., sexual selection, sex allocation and genomic conflict) can potentially influence each other (Fig. 1). First, sexual selection affects the optimal sex allocation via the process of local sperm competition (Charnov 1980; Schärer 2009) or when females bias their offspring sex ratio towards sons after mating with an attractive male (Burley 1981). Sex allocation may also influence the strength of sexual selection, if changes in sex allocation affect the operational sex ratio (Fawcett et al. 2011). Second, genomic conflicts in the form of cytoplasmic sex allocation distorters (Cosmides and Tooby 1981) or sex allocation distorters on sex chromosomes (Hamilton 1967) influence sex allocation in an obvious way. And lastly, genomic conflicts may also influence sexual selection (Price and Wedell 2008; Engqvist 2012). One example is cytonuclear conflict, which may lead to the accumulation of deleterious mutations on cytoplasmic genetic factors that only affect male fitness (Frank 2012). These mutations may be a common source of variation in intrinsic fertility of the male function. Intrinsic differences in fertility caused by male-deleterious mutations on cytoplasmic genetic factors may affect the optimal male sperm investment. In particular, males should overall spend fewer resources on ejaculate investment when there is variation in intrinsic fertility in the population, because success during sperm competition will then be less influenced by differences in ejaculate investment, but instead by differences in intrinsic fertility

(Engqvist 2012). It therefore seems worthwhile to further study those connections between different aspects of the male-female phenomenon instead of focusing on them in isolation.

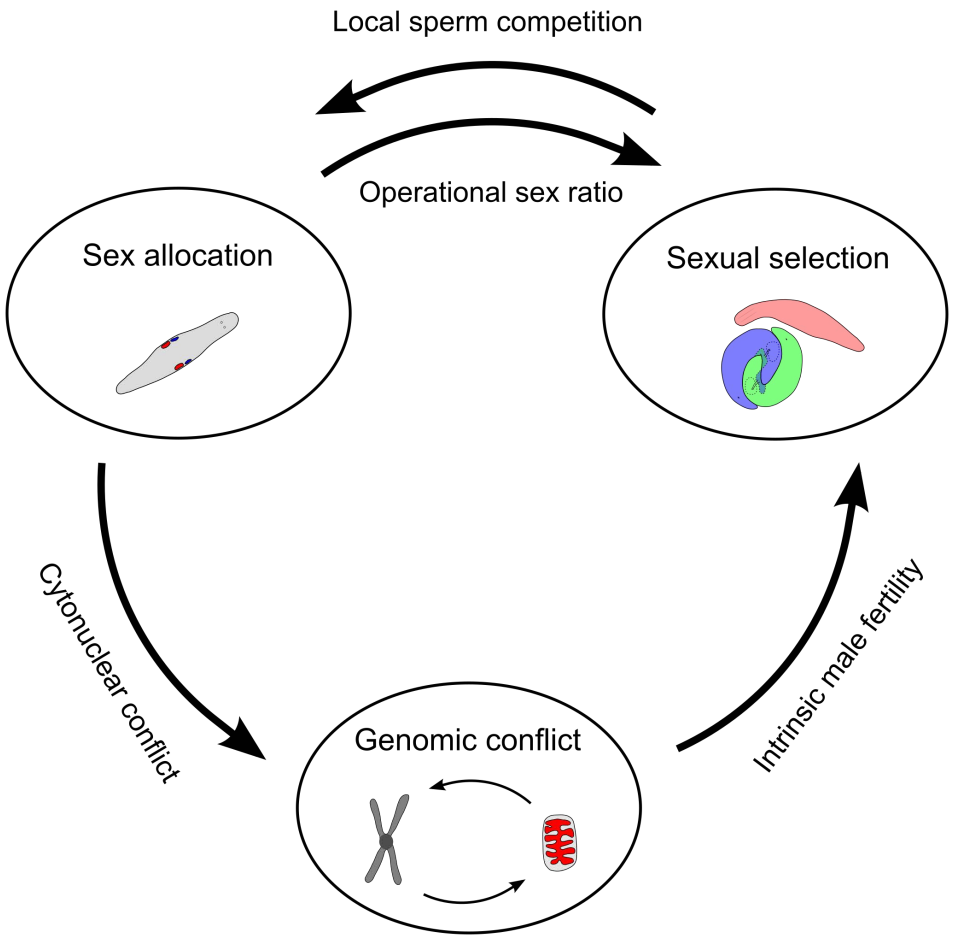


Figure 1. Schematic illustration of how the different aspects of the male-female phenomenon covered in my thesis are connected to each other.

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