# Experimental tests of sex allocation theory in a simultaneous hermaphrodite using phenotypic engineering

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# Abstract

Sex allocation theory can provide successful predictions about how individuals allocate resources into male and female reproduction. However, in simultaneous hermaphrodites, experimental support for some fundamental aspects of sex allocation theory are, so far, limited, and many studies are conducted as correlational analyses without experimental trait manipulation. This limitation is especially true in animals, where gonads are usually internal, making it difficult to experimentally manipulate the traits of a specific sex function. Therefore in my PhD project I established and performed phenotypic engineering in the free-living simultaneous hermaphroditic flatworm *Macrostomum lignano*, and experimentally tested two fundamental aspects of sex allocation theory, namely a saturating male fitness gain curve and the existence of a trade-off between male and female allocation.

First, in order to identify candidate genes that could be involved in sex allocation, I searched sex-specific genes based on existing gene annotations from other organisms using available gene sequence databases in *M. lignano*. Next, I examined gene expression patterns of such candidate genes using *in situ* hybridization. After confirming sex specificity, I knocked down gene expression using RNA interference (RNAi), enabling me to obtain a number of male-sterile phenotypes. In **Chapter II**, I report one of the identified testis-specific genes, named *melav2*, as an example of how to confirm sex specificity, an important aim for specific manipulation of one sex function in a simultaneous hermaphrodite.

Having identified male-specific genes, I next investigated how the manipulation of sperm production using one of these genes impacts on male fitness. In sex allocation theory the shape of the fitness gain curve has an important role to predict optimal sex allocation. In many simultaneous hermaphrodites saturating male fitness gain curves are predicted, but studies experimentally testing this prediction are scarce. Therefore in **Chapter III**, I quantitatively manipulated sperm production using dose-dependent testis-specific RNAi knock-down of the *macbol1* gene, and the results provide clear experimental evidence of a saturating male fitness gain curve in *M. lignano*, to my knowledge the first such evidence in copulating simultaneous hermaphrodites.

Another fundamental but to date poorly supported assumption of sex allocation theory is the existence of a trade-off between male and female resource allocation. In theoretical models individuals are assumed to have a limited amount of reproductive resources, and an increase in allocation in one sex function necessarily leads to a decrease in the other sex function, imposing some constraints on the individual's sex allocation decisions. In **Chapter IV**, in support of this assumption, I show that experimentally hindering spermatogenesis via testis-specific RNAi knock-down of the *mac-C3H-zfn* gene results in an increase in ovary size, as assumed by theory.

In conclusion, my thesis provides experimental evidence for two fundamental aspects of sex allocation theory, namely a saturating male fitness gain curve and the existence of a trade-off between male and female allocation in a copulating simultaneous hermaphrodite. These two findings confirm an important theoretical prediction, namely that selection favors lower male allocation when there are diminishing male fitness returns and that freed reproductive resources can flow from the male to the female function.

# CHAPTER I

Thesis Introduction

# Thesis introduction

## Sex allocation theory

How organisms allocate their resources into male and female reproduction is an important aspect of their life history, for example, the sex ratio of offspring in gonochorists, the timing of sex change in sequential hermaphrodites, and the resource allocation to male and female function in simultaneous hermaphrodites. Sex allocation theory has provided a powerful predictive framework for this intriguing question (e.g., Charnov, 1982; Fisher, 1930; Hamilton, 1967; Schärer, 2009; Sheldon and West, 2004; Trivers and Willard, 1973; West, 2009; West et al., 2000; Leigh et al., 1976; Warner et al., 1975). Historically, after the first mathematical argument by Düsing (Düsing, 1884), Fisher was the first to provide a succinct, influential key framework for sex allocation theory (Fisher, 1930). He noted that each individual has one father and one mother and thus genetic contributions through male and female function to the next generation are necessarily equal. Therefore, if the cost of producing a son and a daughter is equal and mating occurs randomly, the optimal sex ratio produced in gonochorists will become 50:50 at equilibrium due to frequency dependent selection that provides a fitness advantage to the rare sex. However, if there is a difference in costs of producing sons and daughters, for example due to competition or cooperation among relatives of the same sex, selection will favor biased sex allocation producing the more profitable sex (e.g., Clark, 1978; Emlen et al., 1986; Hamilton, 1967; West, 2009). The basic concept of this was first described by Hamilton as "local mate competition", providing a plausible explanation for extremely female-biased sex ratio in insects and mites (Hamilton, 1967). When reproduction takes place in a small subdivided population and competition for mates arises between related males, a mother produces offspring with a female-biased sex ratio, because fitness returns for the production of additional males is diminishing (less profitable). Nowadays it is realized that local mate competition is a special case of "local resource competition", namely competition between relatives for limiting resources (Clark, 1978), and biased sex allocation attributed to local resource competition is widely observed (e.g., West, 2009). Although these concepts were first formulated to explain variation in the sex ratio in gonochorists, they are also applicable to sex allocation in hermaphrodites, namely resource allocation into male and female function within one individual (e.g., Bulmer and Taylor, 1980; Campbell, 2000; Charnov, 1982; Schärer, 2009). In the following I briefly outline sex allocation theory for simultaneous hermaphrodites.

In the models of sex allocation theory for simultaneous hermaphrodites, there are two important components, the trade-off between male and female reproductive allocation and the shape of the so-called fitness gain curves (Charnov, 1979; Charnov, 1982). In theoretical models individuals are expected to have a limited amount of reproductive resources and an increase in resource investment into one sex function is assumed to lead to a proportional decrease in the other sex function. In order to maximize their overall fitness, individuals therefore have to optimally allocate their limited reproductive resources into male and female functions within the constraint imposed by this sex allocation trade-off. Optimal sex allocation is often predicted based on the shape of the fitness gain curves, which describe how much reproductive fitness can be gained through a given level of resource allocation into the male and female functions. If the fitness gain curve in at least one of the sex functions is saturating (i.e., shows diminishing returns) it is, at a certain point, more advantageous to reallocate the investment towards the other sex function, rather than to keep investing into the saturating sex function. The rule for optimal sex allocation is that resources should always be invested into the sex function with the higher marginal returns for further investment and the optimal switch point therefore occurs when the slope of the saturating function drops below that of the other sex function. An individual with such a combination of fitness gain curves would be expected to allocate to both sex functions, and would thus be predicted to be a simultaneous hermaphrodite (Charnov, 1982; Schärer, 2009).

In many simultaneous hermaphrodites a saturating fitness gain curve is generally predicted for the male rather than the female function (Charnov, 1982; Schärer, 2009), since female reproduction is often thought to be limited by resource availability and thus results in (nearly) constant marginal fitness returns for a given further resource allocation (i.e., a linear fitness gain curve) (Bateman, 1948; Charnov, 1979). Under some particular conditions, however, a saturating female fitness gain curve can be expected, for example, if there is a limited capacity for brooding the own eggs (Charnov, 1982; Heath, 1979), if there is local resource competition among individuals due to limited dispersal of the offspring produced through the female function (Charnov, 1982; Clark, 1978; Lloyd, 1982), or if there are insufficient male gametes to fertilize all of the available eggs (Charnov, 1982) (see Schärer, 2009 for a review). On the other hand, male fitness often depends on the level of sperm competition. Sperm competition occurs whenever several sperm donors copulate with the same recipient (Parker, 1970) and is thus a widespread phenomenon in various organisms (e.g., Birkhead and Møller, 1998). Theory predicts that sperm competition favors individuals that allocate more resources into sperm production, since the paternity share obtained will depend on the relative amounts of sperm transferred by each sperm donor (Parker, 1990; Parker, 1998). Therefore the shape of the male

fitness gain curve is generally expected to vary as a function of the level of sperm competition. A saturating male fitness gain curve is predicted when fitness returns for additional male allocation become diminishing. The concept is analogous to "local mate competition" (Charnov, 1980; Charnov, 1982; Fischer, 1981; Fischer, 1984; Hamilton, 1967), but recently the term "local sperm competition" has been suggested (Schärer, 2009), thus combining the concepts of "local mate competition" and "sperm competition". Local sperm competition refers to the competition between genetically related sperm. When local sperm competition is intense, for example due to a small mating group size (Charnov, 1980; Fischer, 1981), high selfing rate (Charlesworth and Charlesworth, 1981; Charnov, 1987), efficient sperm displacement (Charnov, 1996), or cryptic female choice (Van Velzen et al., 2009), one would predict a strongly saturating male fitness gain curve and relatively few resources should be allocated to the male function (Greeff et al., 2001; Petersen, 1991; Schärer, 2009; Schärer and Wedekind, 2001). In contrast, when the mating group size is large and sperm competition approaches a fair raffle (Charnov, 1982; Charnov, 1996), local sperm competition becomes less intense and competition between unrelated sperm increases. In this case the male fitness gain curve is expected to become less strongly saturating and more linearized (Charnov, 1979; Petersen, 1991; Schärer, 2009), because the individual continues to obtain benefits from increasing its investment into the male function to win in sperm competition against other individuals. In this scenario, the predicted optimal sex allocation would shift to become more male-biased, approaching a 50:50 allocation (Charnov, 1980; Charnov, 1982; Petersen, 1991).

**Empirical studies**

The theoretical framework outlined above provides a number of plausible predictions about the dynamics of sex allocation in simultaneous hermaphrodites, but its validity of course needs to rely on empirical support. Some of the predictions are supported by considerable numbers of studies, for example, a shift towards more male-biased sex allocation in response to increasing levels of sperm competition (e.g., Schärer and Ladurner, 2003; Tan et al., 2004; Trouvé et al., 1999). However, studies supporting some of the fundamental aspects of sex allocation theory, such as the trade-off between male and female allocation and the saturating shape of the male fitness gain curve, are still scarce, especially in simultaneously hermaphroditic animals. For example, empirical studies on the male fitness gain curve have been conducted mainly in plants (e.g., Broyles and Wyatt, 1990; Campbell, 1998; Devlin et al., 1992; Rosas and Domínguez, 2009; Schoen and Stewart, 1986; Snow and Lewis, 1993), revealing a variety of shapes, including both saturating (e.g., Campbell, 1998; Rosas and Domínguez, 2009) and linear male fitness gain curves (e.g., Broyles and

Wyatt, 1990). In contrast, studies on simultaneous hermaphroditic animal are much fewer, which is unfortunate given that hermaphroditism is also very widespread in animals (Jarne and Auld, 2006; Schärer, 2009). I am aware of studies on only two species of sessile marine invertebrates where the fitness gain curves have been studied, showing both a saturating and a linear male fitness gain curve in a colonial ascidian (Yund, 1998) and linear male fitness gain curves in a colonial bryozoan (McCartney, 1997). Moreover, these studies, and also most of the plant studies, are based on correlational analyses. Although such descriptive studies are a good place to start for understanding what is happening in nature and may be useful for estimating which traits are important, they cannot prove causality. For this we need to manipulate the phenotypic traits of interest and to eliminate possible effects of unmeasured correlated traits. Such phenotypic manipulation has always been a powerful approach in evolutionary biology and has yielded profound insights into the function of the traits in question (e.g., Andersson, 1982; Maklakov and Arnqvist, 2009; Møller, 1988; Polak and Rashed, 2010).

Experimental trait manipulation may also be a powerful approach for testing the existence of a trade-off between male and female allocation. So far this assumption is poorly supported and empirical studies are often controversial in both plants (reviewed in Campbell, 2000) and animals (reviewed in Schärer, 2009). Although some studies report the expected negative correlation (e.g., Garnier et al., 1993; Yund et al., 1997), there are numerous studies showing a positive correlation between male and female allocation (e.g., Ågren and Schemske, 1995; Campbell, 1997; Locher and Baur, 2000; O'Neil and Schmitt, 1993; Schärer et al., 2005). Several models have identified potential factors responsible for such a paradox. For example, the existence of variation in resource acquisition ability among individuals may cause a positive correlation between traits (Schärer et al., 2005; van Noordwijk and De Jong, 1986). Similarly, if allocation is made in a hierarchical manner (e.g., resources are first subdivided into reproduction and other functions, and only then allocated into the male and female reproduction), variation in early level of the hierarchy may obscure the trade-off (de Laguerie et al., 1991). Moreover, the presence of phenotypic plasticity in resource allocation may also mask the trade-off (Malausa et al., 2005).

An alternative way of demonstrating the trade-off is thus required, for example, by manipulating one sex function (e.g., decrease of male allocation) and examining its effect on the other sex function (e.g., increase of female allocation) (Schärer, 2009). Studies with such approaches are expected to be relatively easy in plants because the male and the female functions (stamen and pistil in flowers) are expressed towards the outside and can thus be manipulated. In contrast, a large part of the reproductive allocation in animals occurs in the testis and the ovary (i.e., internal organs within the body), and it thus has been notoriously difficult to manipulate these tissues. A notable

exception is a study on the pond snail, which showed that the prevention of male copulation activity by removing a particular part of the vas deferens led to an increased number of eggs (De Visser et al., 1994), concluding that male resources not used in the focals were reallocated to the female function because of the trade-off. However, as pointed out by others (Koene et al., 2009; Schärer, 2009), recent studies suggest that the observed increase in egg production may be due to the absence of negative effects of received seminal fluids in the focal snails, and thus not due to a trade-off revealed by the manipulation, as initially suggested. Thus studies supporting the trade-off assumption with such sex-specific manipulations are still scarce in simultaneous hermaphroditic animals.

**Phenotypic engineering using RNA interference**

One possible approach to achieve sex-specific manipulation in simultaneous hermaphrodites is phenotypic engineering using RNA interference (RNAi). The phenomenon of RNAi was first described in the nematode *Caenorhabditis elegans* (Fire et al., 1998). Since then it has been found in a broad range of organisms (reviewed in Hannon, 2002; Ketting, 2011; Sifuentes-Romero et al., 2011), and has established itself as a widely applicable and relatively easy research tool to specifically manipulate individual gene expression. RNAi can be triggered by introducing double stranded RNA (dsRNA) molecules into cells. Briefly, the protein machineries within cells recognize the introduced dsRNA molecules, degrade them into small segments called small interfering RNA (siRNA), and then use these siRNAs as a template to search and destroy corresponding mRNA expressed within cells (reviewed in Downward, 2004). Therefore, the gene knock-down via RNAi occurs in a sequence-specific manner, which, given appropriate knowledge of gene function, enables one to phenotypically engineer specific traits. Moreover, one could also expect that the effect of RNAi gene-knock down can be varied by changing the amount of dsRNA that is introduced, which is a powerful way to manipulate the resulting phenotypes quantitatively.

**Objectives of the thesis**

In my PhD project I experimentally examined two fundamental aspects of sex allocation theory, namely the predicted saturating male fitness gain curve and the assumption of a trade-off between male and female allocation, using the simultaneously hermaphroditic free-living flatworm *Macrostomum lignano*. For the manipulation of particular sexual traits, I took advantage of available molecular techniques in *M. lignano* and established a suitable workflow to manipulate sexual traits by manipulating sex-specific genes via RNAi.

Firstly, I searched for sex-specific genes in *M. lignano* using a candidate gene approach based on existing gene annotations for other organisms. Next, I confirmed the expression patterns of these candidate genes using *in situ* hybridization. After confirming sex specificity, I knocked down these genes using RNAi and investigated the resulting phenotypes. In **Chapter II**, I report one of the candidate genes obtained using this workflow and show its essential role in spermatogenesis in *M. lignano*. Moreover, I also show that sperm that were prevented from proper spermatogenesis are not transferred into the sperm storage organ, i.e., the seminal vesicle, resulting in male sterility. This finding is a key point in the next following studies, since it means that the amount of sperm in the seminal vesicle reflects only properly differentiated sperm. In **Chapter III**, I tested one of the fundamental predictions of sex allocation theory, a saturating male fitness gain curve under low levels of sperm competition. To study the shape of the male fitness gain curve, it is important to have enough variation in the male function. For this I established dose-dependent RNAi to create large variation in sperm production rate (a proxy for male allocation), as estimated from changes in seminal vesicle size. In this study I show that the male fitness gain curve of *M. lignano* is clearly saturating under low levels of sperm competition, as predicted by sex allocation theory. In **Chapter IV**, I tested the presence of a trade-off between male and female allocation, a fundamental assumption of sex allocation theory. I show that hindered sperm production via the testis-specific RNAi gene knock-down leads to an increase in ovary size, which is consistent with the idea of a resource trade-off between male and female allocation.

**Study organism**

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is a simultaneous hermaphrodite and a member of the interstitial sand fauna of the Northern Adriatic Sea (Ladurner et al., 2005). In the laboratory, we maintain the worms in nutrient-enriched artificial seawater (Guilard's f/2 medium, Andersen et al., 2005) in glass petri dishes, feed them *ad libitum* with the diatom *Nitzschia curvilineata*, and keep them at 20°C on a 14:10 h day-night cycle (Rieger et al., 1988). Under these conditions, generation time is about 18 days: 5 days from egg laying to hatching and 13 days from hatching to adult (Schärer and Ladurner, 2003). The size of adult worms reaches approximately 1.5 mm. From anterior to posterior within the body, the paired testes, the paired ovaries, developing eggs, and the sperm receiving organ (the female antrum with its genital opening) are located. The male copulatory organ (the stylet) and the sperm storage organ (the seminal vesicle) are located in the tail plate. As the worms are transparent, non-invasive measurements of these different morphological traits are possible (Schärer and Ladurner, 2003). Reproduction is purely by outcrossing and self-fertilization has not been observed

(Schärer and Ladurner, 2003). Copulation occurs frequently and reciprocally, in which worms form a tight disc and mutually insert their stylets into the partner's female genital opening (Schärer et al., 2004).

*M. lignano* is an excellent model organism for developmental and evolutionary biology (e.g. Janicke and Schärer, 2009; Kuales et al., 2011; Ladurner et al., 2008; Schärer and Janicke, 2009; Schärer and Ladurner, 2003; Sekii et al., 2009), and allows us to employ powerful experimental approaches to study reproductive allocation. Sex allocation of *M. lignano* shows phenotypic plasticity in response to the intensity of sperm competition: the worms become more female-biased when they grow up in smaller mating group size and become more male-biased when raised in larger mating group sizes (Janicke and Schärer, 2009; Schärer and Ladurner, 2003; Schärer et al., 2005). Moreover, it has a slightly protandrous gonad development and allocates more resources into the female function under higher resource availability (Vizoso and Schärer, 2007), which is predicted if the female fitness gain curve is, at least, less saturating than the male fitness gain curve.

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# CHAPTER II

## *Melav2*, an *elav*-like gene, is essential for spermatid differentiation in the flatworm *Macrostomum lignano*

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# *Melav2*, an *elav*-like gene, is essential for spermatid differentiation in the flatworm *Macrostomum lignano*

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## Abstract

Failure of sperm differentiation is one of the major causes of male sterility. During spermiogenesis, spermatids undergo a complex metamorphosis, including chromatin condensation and cell elongation. Although the resulting sperm morphology and property can vary depending on the species, these processes are fundamental in many organisms. Studying genes involved in such processes can thus provide important information for a better understanding of spermatogenesis, which might be universally applied to many other organisms.

In a screen for genes that have gonad-specific expression we isolated an elav-like gene, melav2, from Macrostomum lignano, containing the three RNA recognition motifs characteristic of elav-like genes. We found that melav2 mRNA was expressed exclusively in the testis, as opposed to the known elav genes, which are expressed in the nervous system. The RNAi phenotype of melav2 was characterized by an aberrant spermatid morphology, where sperm elongation often failed, and an empty seminal vesicle. Melav2 RNAi treated worms were thus male-sterile. Further analysis revealed that in melav2 RNAi treated worms precocious chromatin condensation occurred during spermatid differentiation, resulting in an abnormally tightly condensed chromatin and large vacuoles in round spermatids. In addition, immunostaining using an early-spermatid specific antibody revealed that melav2 RNAi treated worms had a larger amount of signal positive cells, suggesting that many cells failed the transition from early spermatid stage.

We characterize a new function for elav-like genes, showing that melav2 plays a crucial role during spermatid differentiation, especially in the regulation of chromatin condensation and/or cell elongation.

# Background

Failure of spermatogenesis is one of the major causes of male sterility. Many cases of human infertility are associated with low sperm production (oligozoospermia), poor sperm motility (asthenozoospermia) and abnormal sperm morphology (teratozoospermia) (Hirsh, 2003). Also, it has been shown that abnormalities of sperm chromatin, which is important for properly transmitting genetic information to offspring, can often be observed in cases of infertility (Agarwal and Said, 2003). Although spermatid cells undergo a complex metamorphosis in a species-specific manner, resulting in various types of sperm morphology and other traits depending on species, fundamental processes such as the reorganization of the nucleus, cell organelles, and cell shape are found in most organisms (Pitnick et al., 2009). Thus studying genes involved in such processes can provide important information for a better understanding of spermatogenesis, which may also be applied to many other organisms.

*Macrostomum lignano* (Macrostomorpha, Rhabditophora, Platyhelminthes) is a simultaneously hermaphroditic flatworm, namely with male and female gonads within one individual, and a suitable model organism for gametogenesis research. An EST database is available as a source for gene information (http://flatworm.uibk.ac.at/macest/) (Morris et al., 2006). In addition, basic experimental techniques for gene analysis such as *in situ* hybridization and gene specific RNA interference (RNAi) are already established (Pfister et al., 2008; Pfister et al., 2007). Monoclonal antibodies against various types of cells are also available for detailed analysis of tissues (Ladurner et al., 2005b). The biggest advantage of *M. lignano* for spermatogenesis research is its transparency, which allows non-invasive observation of cells and tissues in live animals using light microscopy and thus allows very efficient screening of RNAi phenotypes. Finally, their frequent copulation and short generation time make it easy to examine mating behavior and the degree of reproductive contribution to the next generation (Schärer et al., 2004). Also a lot of research has been done in terms of evolutionary biology, such as sex allocation adjustment, sexual conflict, and sperm competition (Janicke and Schärer, 2009; Schärer and Janicke, 2009; Schärer and Ladurner, 2003; Schärer et al., 2005)). Thus studying *M. lignano* allows the comprehensive understanding of spermatogenesis, not only at the developmental level but also its significance in evolutionary aspects.

In *M. lignano*, the testes mainly consist of male germ cells such as spermatogonia, spermatocytes, spermatids, and sperm. A thin layer of tunica cells encloses the testes. After meiosis, spermatids remain in four-cell clusters with cytoplasmic connections until just before the completion of sperm maturation (Willems et al., 2009)). Mature sperm have a complex morphology, with distinct parts

easily observed in the microscope. From anterior to posterior, sperm have a feeler, a body which ends in a pair of bristles, and a shaft which ends in a brush (Ladurner et al., 2005b)). Within the shaft the nucleus is condensed into connected packages of compact chromatin. The overall appearance of the sperm nucleus can be compared to connected railway carriages (Willems et al., 2009). This morphology will be referred to as "train-shape" in this manuscript. Sperm differentiation starts with the development of the anterior part which is followed by the elongation of spermatids and then the nucleus becomes enclosed in the shaft of the mature sperm. Here we reserve the term ‘sperm’ for cells that have completed spermatogenesis and call the aberrant cells of the RNAi phenotype ‘aberrant spermatids’.

In the process of spermiogenesis, post-transcriptional control of mRNA is very important (Kleene, 2003; Steger, 2001). In an elongating spermatid, the nucleus has to be condensed into a compact shape, which causes cessation of transcription. Therefore, transcription of genes that are necessary for later stages has to be completed before chromatin condensation, but their translation needs to be controlled until they are needed. For this post-transcriptional control, such as transport, translational repression and storage of mRNAs, the involvement of various types of RNA binding proteins has been reported (Kleene, 2003; Steger, 2001; Tay and Richter, 2001; Yang et al., 2005; Zhong et al., 1999). For example, during spermiogenesis DNA-binding histones are gradually replaced by transition proteins (Tnp1 and Tnp2) and then protamines (Prm1 and Prm2), and it is suggested that protamine-1 mRNA binding protein (PRBP) has a role for proper translational activation of *prm-1* mRNA (Zhong et al., 1999).

*Elav* genes are RNA binding proteins that are characterized by three RNA recognition motifs (RRMs) and a hinge region between the second and the third RRM (Pascale et al., 2008; Soller and White, 2004). RRMs are the most common protein domains found in all kingdoms and each RRM consists of 80-90 amino acids containing two conserved sequences called RNP-1 and RNP-2 (Burd and Dreyfuss, 1994; Kenan et al., 1991; Maris et al., 2005; Samson, 2008). Structurally, a RRM has two α helices and four anti-parallel β strands, forming two β-α-β structures. It is suggested that proteins containing one or several RRMs are capable of interacting with RNA molecules (Kenan et al., 1991; Maris et al., 2005; Samson, 2008). Molecular functions of the *elav* gene family are quite diverse, including mRNA stability, splicing, translatability and transport (Good, 1997; Pascale et al., 2008).

In this paper, we study a *Macrostomum elav*-like gene, *melav2 (**M**acrostomum* **elav**-like gene **2**), using differential interference contrast microscopy, *in situ* hybridization, monoclonal antibodies, histology, and electron microscopy. We found that *melav2* is expressed exclusively in the testes. Moreover, we show that *melav2* RNA interference causes aberrant spermatid morphology, abnormal chromatin

condensation, and an empty seminal vesicle leading to male-sterility. We thus prove that *melav2* plays a crucial role for proper sperm differentiation and male fertility in *M. lignano*.

# Results

### *Melav2* is an *elav*-like gene encoding three RNA recognition motifs

*Melav2* was identified and isolated during the process of screening for gonad-specific genes in *M. lignano*. We found this gene in our EST database with the keyword '*sex-lethal* (*sxl*)'. *Sxl* is a well-known gene involved in sex determination in *Drosophila melanogaster*, although its function is not conserved among all insects (Dubendorfer et al., 2002; Harrison, 2007; Schutt and Nothiger, 2000; Serna et al., 2004; Traut et al., 2006). However, we found that, as described in detail below, *melav2* had a higher similarity with *elav* genes than with *sxl*. We therefore named it *melav2* (***M****acrostomum* ***elav***-like gene **2**) to distinguish it from *melav1*, another *elav*-like gene present in our EST database. Given the scope of our paper we focused on *melav2*. The entire open reading frame of this gene was obtained from the EST clone ANGU919. This gene was predicted to encode 422 amino acids.

Using BLASTX analysis, we found that *melav2* has a high similarity with the Elav/Hu gene family, which is characterized by three RRMs with a hinge region between the second and the third RRM. Multiple alignments with other *elav*-like genes (Figure 1A) revealed that the first and second RRM of *melav2* are relatively conserved whereas the third RRM does not show such a high homology.

The structure of the RRM is important for the interaction with RNA. We therefore examined the structural similarity of *melav2* and other *elav* genes. An RRM contains two α helices and four anti-parallel β strands, forming two β-α-β structures (Burd and Dreyfuss, 1994; Kenan et al., 1991; Maris et al., 2005; Samson, 2008). Structural analysis using the SWISS-MODEL program revealed that *melav2* has two such β-α-β structures in each RRM region and that they correspond to the β-α-β region of other *elav* genes (Figure 1A). In the third RRM of *melav2*, however, the β-α-β pattern was less distinct. We further analyzed *melav2* using the SMART program for identification and annotation of protein domains (Figure 1B). According to this program, *melav2* was considered to have three RRMs, corroborating the similar overall gene structure to other *elav* genes. The e-value of the first and second RRM was 4.89e-18 and 2.16e-10, respectively, and the e-value of the third RRM was 4.61e-05. All these e-values were for the RRM in the SMART database (SMART accession number SM00360).

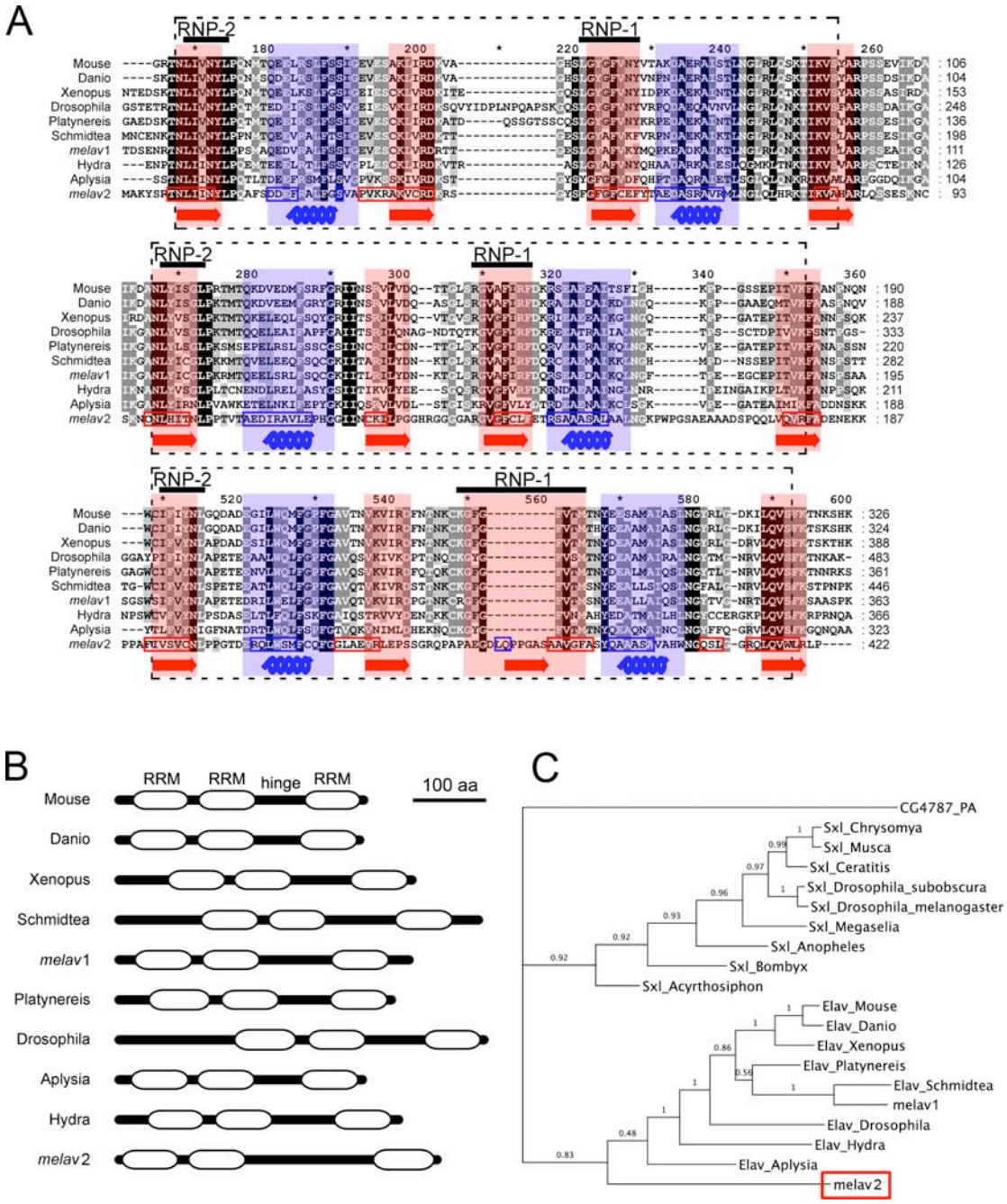


**Figure 1: Comparison of *melav2* and other *elav* family genes.** (A) Alignments of the *melav2* amino acid sequence with other *elav*-like genes from different organisms. Three RNA recognition motifs (RRM) are present in all *elav*-like genes (each indicated by a dashed line box). Each RRM has two conserved sequences RNP-1 and RNP-2. Structurally, RRM consists of two alpha helixes (highlighted in blue and blue coil) and four beta sheets (highlighted in red and red arrow). The predicted corresponding sequence of *melav2* is shown in blue and red boxes, respectively. (B) Predicted domain structure by the SMART program. Each box indicates an RRM. *Melav2* had a similar gene structure that is similar to other *elav* family genes, namely three RRMs with a hinge region between the second and the third RRM. (C) Phylogenetic tree of *melav2*, other *elav* genes, and *sxl* genes from different organisms. *Melav2* was categorized into the *elav* gene family. Numbers show the Bayesian posterior probability (with values >0.95 representing good nodal support). For accession numbers see materials and methods. Mouse: Mus musculus, Xenopus: Xenopus laevis, Danio: Danio rerio, Drosophila: Drosophila melanogaster, Aplysia: Aplysia californica, Platynereis: Platynereis dumerilii, Schmidtea: Schmidtea mediterranea, Hydra: Hydra magnipapillata, melav1: another elav-like gene from M. lignano (see the discussion), Musca: Musca domestica, Ceratitis: Ceratitis capitata, Chrysomya: Chrysomya rufifacies, Megaselia: Megaselia scalaris, Anopheles: Anopheles gambiae, Acyrthosiphon:

Acyrthosiphon pisum, Bombyx: Bombyx mori, CG4787-PA: a protein from D. melanogaster as an outgroup.

BLASTX analysis also showed a certain similarity between *melav2* and another *elav* related gene, *sxl* (data not shown). Structurally, *sxl* has two RRMs. The third RRM of *melav2* was not well conserved compared to other *elav*-like genes, but despite the similarity with *sxl*, the phylogenetic analysis revealed that *melav2* seemed to belong to the *elav*-like gene family (Figure 1C).

### *Melav2* is expressed exclusively in the testes

In order to investigate the expression pattern of the *melav2* mRNA, we performed whole mount *in situ* hybridizations (Figure 2). 1-day old hatchlings possess a gonad anlage of 4-6 primordial germ cells (Pfister et al., 2008). However, expression of *melav2* was not yet detected at this stage (Figure 2A). *Melav2* expression was also lacking in 4-days old worms (Figure 2B), which already have spermatogonia and spermatocytes but not yet spermatids. In subadult worms, which start to have maturing sperm, several cells in the testis were *melav2* positive (Figure 2C). In fully mature worms, which have an increasing number of maturating sperm and also developing eggs, *melav2* expression became strong in the testes (Figure 2D). Cells on the edge of testis had no or only weak *melav2* expression, while the signal around the center of the testis was stronger (Figure 2E), suggesting that the expression level increased as spermatogenesis progressed. Semi-thin sectioning after whole mount *in situ* hybridization revealed that spermatogonia and probably spermatocytes I did not express *melav2* (Figure 2F). Furthermore, it seems that a weak signal was present in spermatocytes II and a strong signal was observed in spermatids (Figure 2F). Mature sperm did not seem to express *melav2*, considering that no expression was detected in fully mature sperm within the seminal vesicle (Figure 2D). These results suggest that *melav2* starts to be expressed at the late stage of spermatogenesis, but not anymore after spermatogenesis was completed.

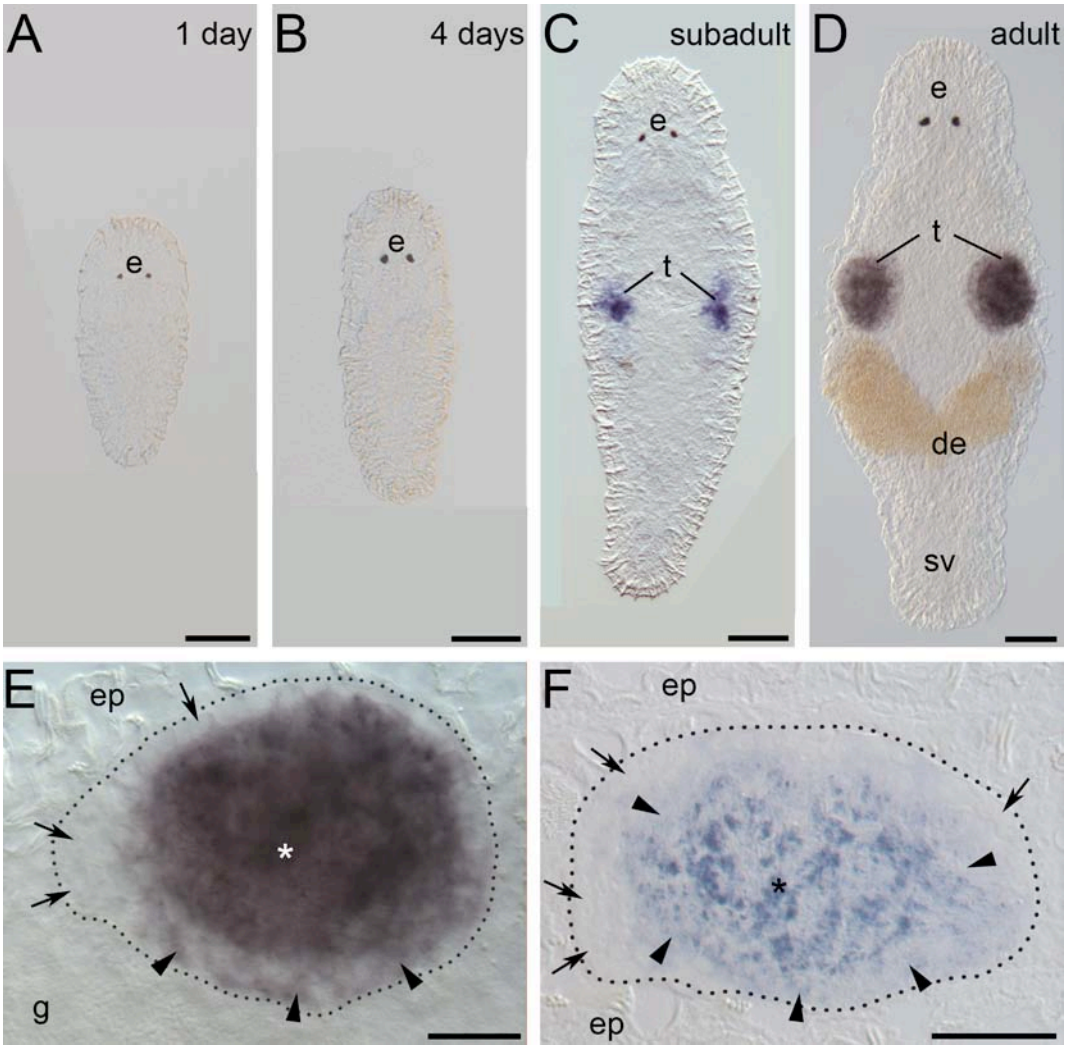


**Figure 2: Expression pattern of *melav2* mRNA.** (A, B) In 1-day old hatchlings (A) and 4-days old juveniles (B) no *melav2* signal was detected. (C) Subadult worms. The signal was present in several cells of the developing testes (t). (D) Mature adult worms. Strong expression was detected in testes (t), but not in the seminal vesicle (sv). (E) Magnified image of testis of a mature adult worm (D). Cells on the edge of testis had no or only weak *melav2* expression (arrow and arrowhead, respectively). Strong expression was detected mainly in the centre of testis (asterisk). (F) Sagittal section of the testis after whole mount *in situ* hybridization. The head is left and the dorsal side is up. Spermatogonia, and probably also spermatocyte I were *melav2* negative (arrow). Weak signal was detected in spermatocyte II (arrowhead) and strong expression was detected in spermatids (asterisk). e, eyes; de, developing eggs; ep, epidermis; g, gut. Scale bars: A-D, 50 μm; E, F, 20 μm.

### *Melav2* RNAi causes an aberrant spermatid morphology

By performing RNAi gene knock-down experiments, we found that *melav2* is essential for spermatogenesis in *M. lignano*. Apart from the differentiating spermatids, we did not find remarkable morphological defects in other tissues such as spermatogonia and spermatocytes (Additional file 1), neuropile and nerve cords (Additional file 2), epidermis, muscles and gland cells (Additional file 3), and various stages of oocyte development (Additional file 4) of *melav2* RNAi treated worms. These observations are consistent with the testis-specific *melav2* gene expression pattern (Figure 3). The animals had ovaries and developing eggs (Figure 3A, B), and the other reproductive organs such as the female genital opening, the antrum (Figure 3C, D), and the male copulatory stylet (Figure 3E, F) exhibited a normal phenotype. However, *melav2* RNAi treated worms did not have any received sperm in their female antrum (Figure 3D) and had no sperm in the seminal vesicle (Figure 3F). In controls, on the contrary, both organs were full of sperm (Figure 3C, E). In a few exceptional cases (4 out of 48), we observed one or two aberrant spermatids in the seminal vesicle in the *melav2* RNAi treated worms (Figure 3G), suggesting that the formation of the vas deferens was not affected by RNAi. We therefore conclude that the empty seminal vesicle resulted, not from a lack of a vas deferens, but from a failure of sperm maturation, as described later. *Elav* genes are known to function in the nervous system in other organisms, but the neuropile structure and the nerve cords of *melav2* RNAi treated worms appeared unaffected (Additional file 2). Furthermore, behaviors, such as swimming or feeding, also appeared to be normal in *melav2* RNAi treated worms.

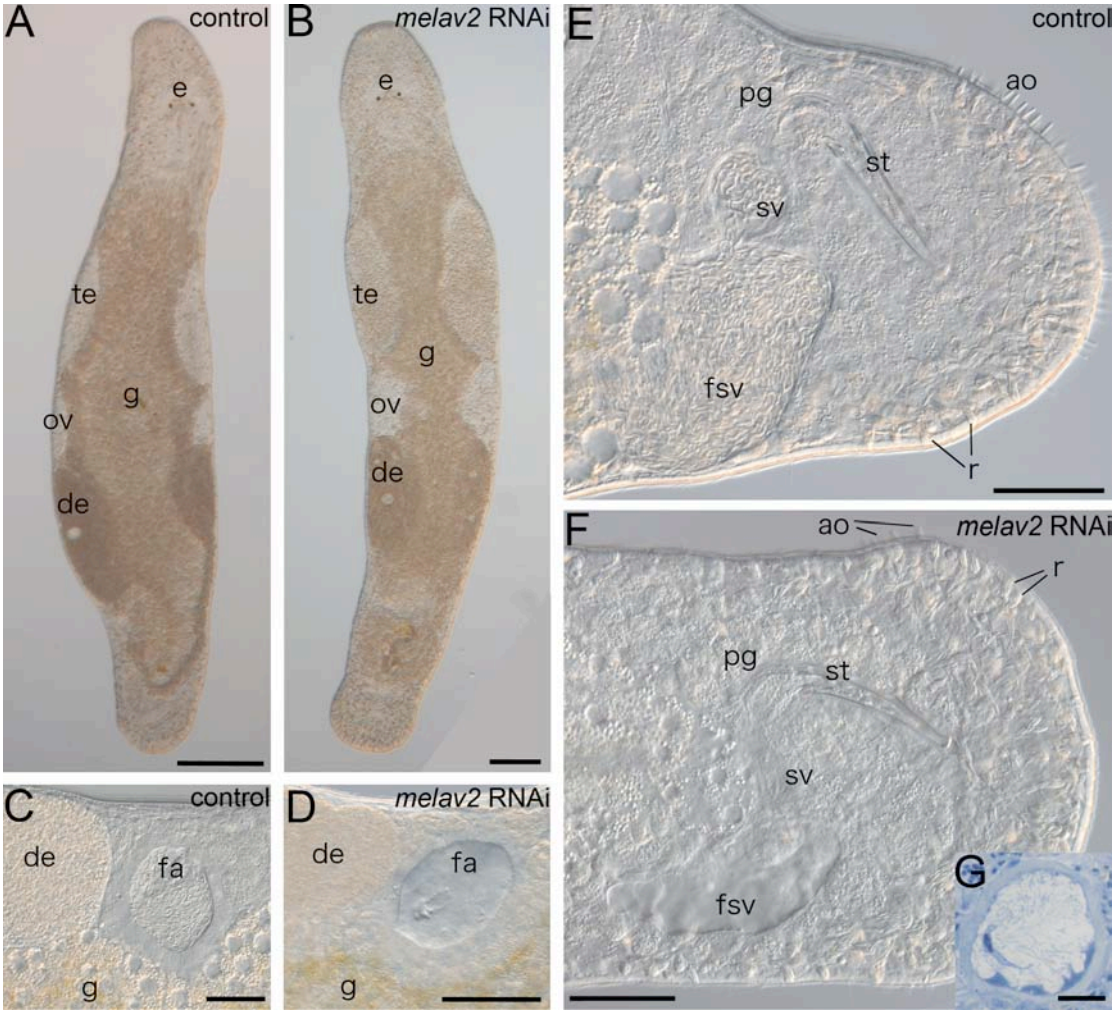


**Figure 3: Comparison of the overall morphology of control and *melav2* RNAi treated *M. lignano*.** (A, B) The overall of morphology of control animals (A) was comparable with that of *melav2* RNAi treated animals (B) except for the morphology of the testis (te). Eyes (e), gut (g), ovaries (ov), developing eggs (de), and copulatory stylet (st) were not affected by the *melav2* RNAi treatment. (C, D) The female antrum (fa) was filled with received sperm in the control animals (C), while no sperm was present in the *melav2* RNAi treated animals (D). (E, F) In the control animals (E), the false seminal vesicle (fsv) and the seminal vesicle (sv) were filled with sperm, while no sperm was usually found there in the *melav2* RNAi treated animals (F). The copulatory stylet (st), prostate glands (pg), rhabdites (r), gut (g) and adhesive organs (ao) were not affected by the *melav2* RNAi treatment. Note that only a few adhesive organs are in the focal plane in this picture. (G) In a few cases, the *melav2* RNAi treated animals had some aberrant spermatids in the seminal vesicle (G), suggesting that the vas deference was connected to the testis normally. Scale bars: A, B, 100 µm; C-F, 50 µm; G, 10 µm.

The testes of *melav2* RNAi treated worms showed severe defects (Figure 4). The testis of control worms contained many maturating sperm, which were elongated and well organized in the central region of the testis (Figure 4A). In live control animals the sperm were moving vigorously inside the testis. In the *melav2* RNAi treated worms, however, the content of the testis looked strongly disorganized (Figure 4B), and internal movements occurred rarely. Furthermore, testis components of control animals contained normal differentiating sperm (Figure 4C), while in the *melav2* RNAi treated worms an accumulation of aberrant cells was present (Figure

4D). Note that not many mature sperm can be seen in control worms because they are transferred to the seminal vesicle as they complete spermatogenesis.

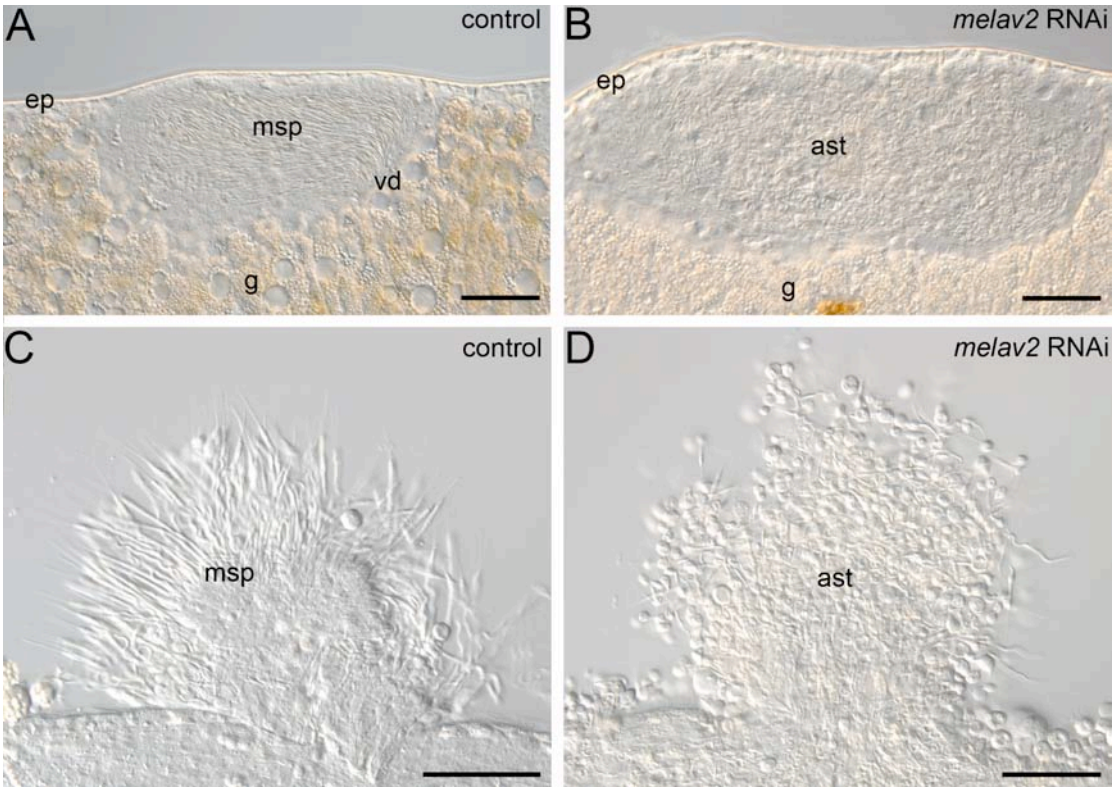


**Figure 4: Comparison of the testis of control and *melav2* RNAi treated *M. lignano*.** (A, B) The testis of control animals (A) had maturating sperm (msp) that were elongated and well organized towards the vas deferens (vd), while the inside of the testis in *melav2* RNAi treated animals (B) looked disorganized and contained aberrant spermatids (ast). (C, D) Testis components from squeezed animals. In the control animals (C), many elongated maturating sperm (msp) were observed, while only aberrant spermatids (ast) were observed in the *melav2* RNAi treated animals (D). g, gut; ep, epidermis. Scale bars: 50 µm.

We next analyzed the morphology of single cells in detail (Figure 5). In control worms, spermatid cells were often seen as clusters of four cells until they finished spermiogenesis (Figure 5A-C). Spermiogenesis begins with the development of the feeler, then the body (Figure 5A, B) and the bristles, followed by the formation of the shaft (Figure 5C). After the the split of the tetrad the brush is formed (Figure 5D). In *melav2* RNAi treated worms, however, most of cells were not in four-cell clusters, even before elongation was completed (Figure 5E). In addition, many spermatids showed an aberrant morphology (Figure 5F-I). In some cases, for example, round immature cells had feelers with the wavy morphology characteristic of developed sperm (Figure 5F), while the feeler of normal spermatids in controls remained straight at that stage of development (Figure 5A, B). In other cases, spermatids of *melav2* RNAi treated animals had a feeler and a pair of bristles but failed to elongate the shaft (Figure 5G-I). This aberrant morphology was never observed in controls. Taking all

these features together, the morphological defects induced by *melav2* RNAi occurred mainly in the posterior parts, which are formed in the later stages of spermiogenesis.

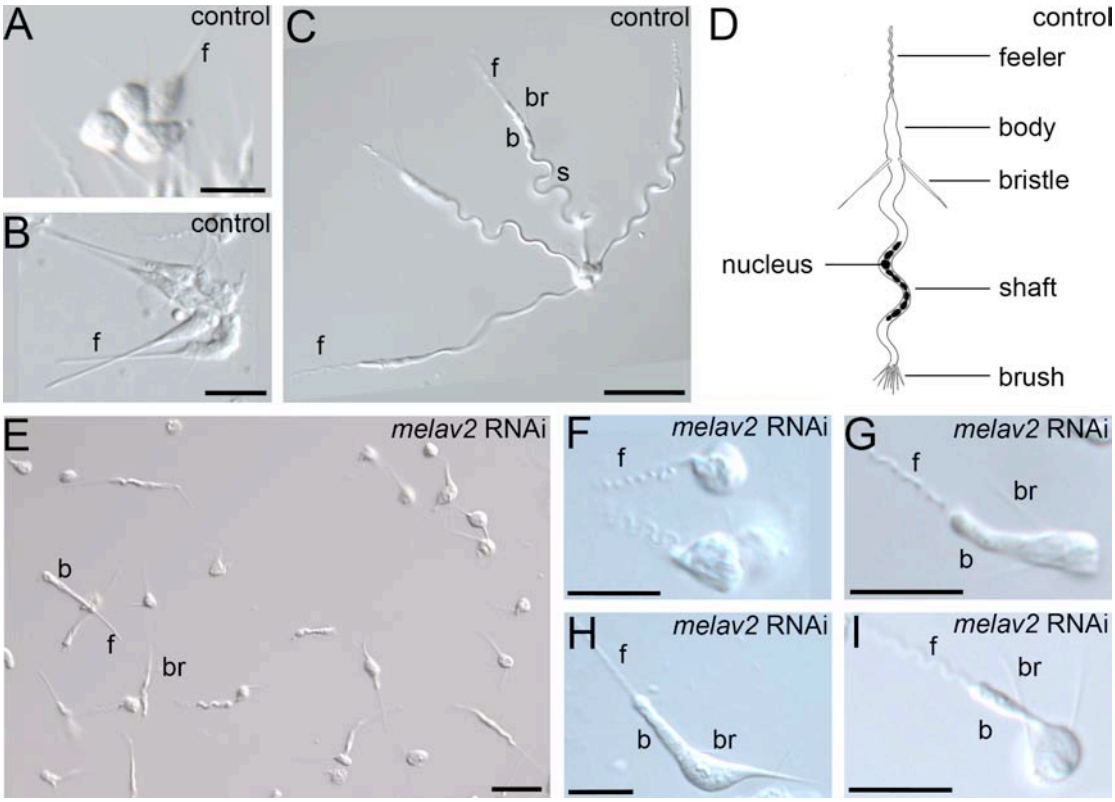


**Figure 5: Comparison of the sperm morphology of control and *melav2* RNAi treated *M. lignano*.** (A-C) Normal spermatid differentiation starts with the development of the anterior part. Note that spermatid cells are arranged in clusters of four cells until just before the completion of sperm maturation. (D) Schematic illustration of normal mature sperm. Note that the direction of bristles is toward posterior in mature sperm. (E) In the *melav2* RNAi treated animals no clusters of four cells were found. (F-I) Examples of aberrant spermatid morphology in the *melav2* RNAi treated animals. Note that the posterior part of the developing spermatids was affected by the *melav2* RNAi treatment in all cases. b, body; br, bristles; f, feeler; s, shaft. Scale bars: A, B, F-I, 10 µm; C, E, 20 µm.

### *Melav2* is necessary for spermiogenesis

In order to determine exactly which stage of spermatogenesis was disturbed by *melav2* RNAi, we compared semi-thin sections of control and *melav2* RNAi treated worms. We found that spermatogenesis started to fail at the spermatid stage in the *melav2* RNAi treated worms (Figure 6). In the testes of control worms (Figure 6A), spermatogonia and spermatocytes were observed at the peripheral regions, whereas elongating spermatids and sperm were located at the center. In the *melav2* RNAi treated worms (Figure 6B), spermatogonia and spermatocytes seemed likely to be normal (Additional file 1). However, from the spermatid stage onwards, cells started to become aberrant. The center of the testis was full of these aberrant cells with large vacuoles (Figure 6B).

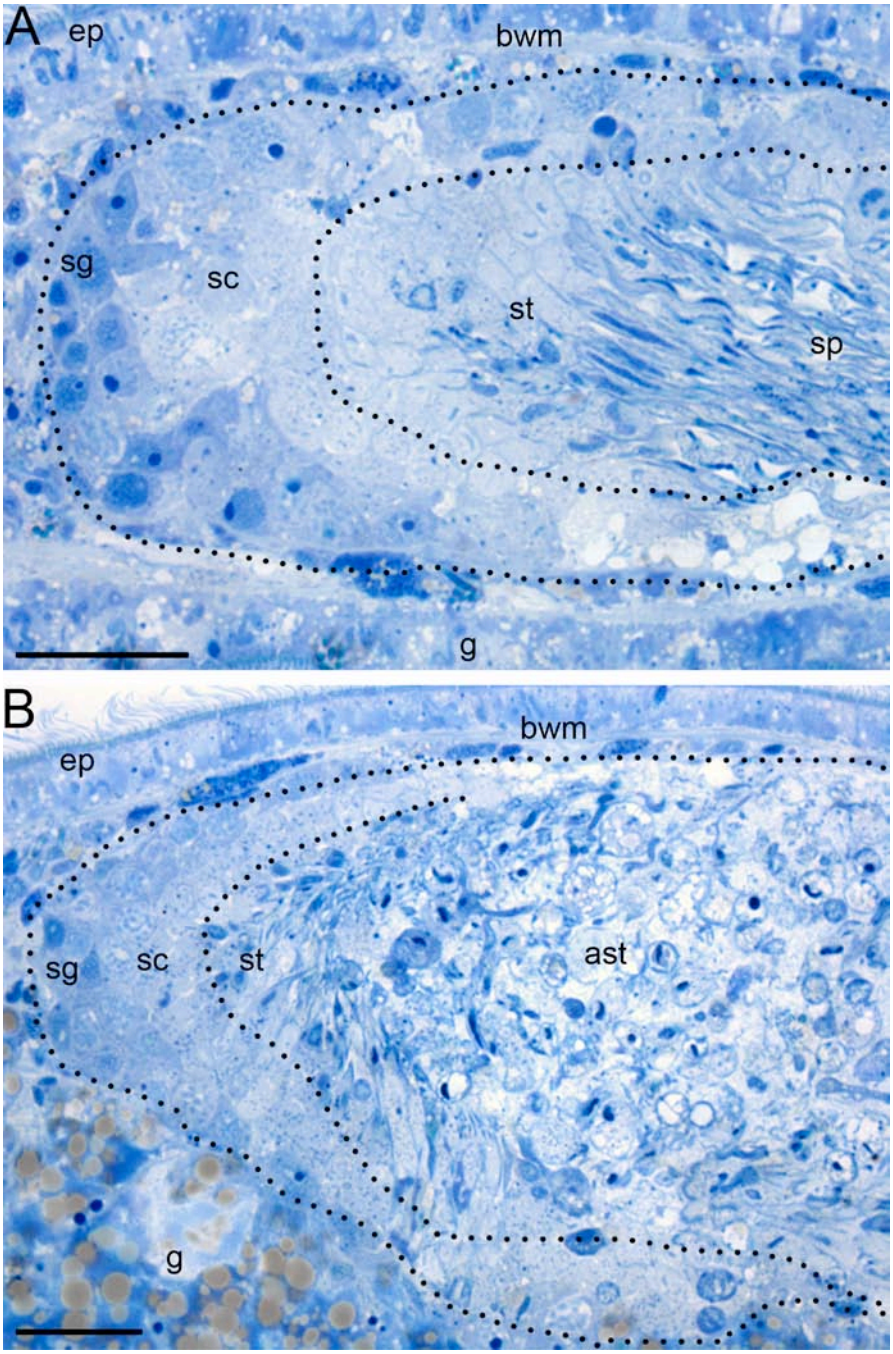


**Figure 6: Comparison of the spermatogenesis of control and *melav2* RNAi treated *M. lignano*.** In the control animals (A), spermatogonia (sg) and spermatocytes (sc) were observed in the peripheral region of the testis, and elongating spermatids (st) and sperm (sp) were present in the center. In the *melav2* RNAi treated animals (B), spermatogonia (sg) and spermatocytes (sc) were also observed to be of normal morphology, but the center region of the testis was filled with aberrant spermatids (ast) with large vacuoles. Dotted lines roughly indicate the regions that contain the respective cell types. g, gut; ep, epidermis; bwm, body wall musculature. Scale bars: 20 µm.

Transmission electron microscopy revealed a failure of nucleus reorganization between the middle and the late stages of spermatid development (Figure 7). First, spermatogonia and spermatocytes I and II looked normal in both control and *melav2* RNAi treated worms (Additional file 1). In control worms (Figure 7A), chromatin

started to condense gradually during spermatid elongation and became reorganized into the train-shaped morphology in later stages (Figure 7C). In the *melav2* RNAi treated worms, the early stage of spermatid development appeared to be normal, except for a precocious condensation of the chromatin (Figure 7B). Around the middle stages of spermatid development, we often observed already tightly condensed patches of chromatin (Figure 7D), which was never found in the control worms. In addition, these cells often had large vacuoles in the cytoplasm (Figure 7D and also Figure 6B). In some cases, cell components such as mitochondria and other organelles were observed outside of cell (Figure 7D), suggesting that cells were dying by necrosis. Cells with train-shaped nuclei were never observed in the *melav2* RNAi treated worms.

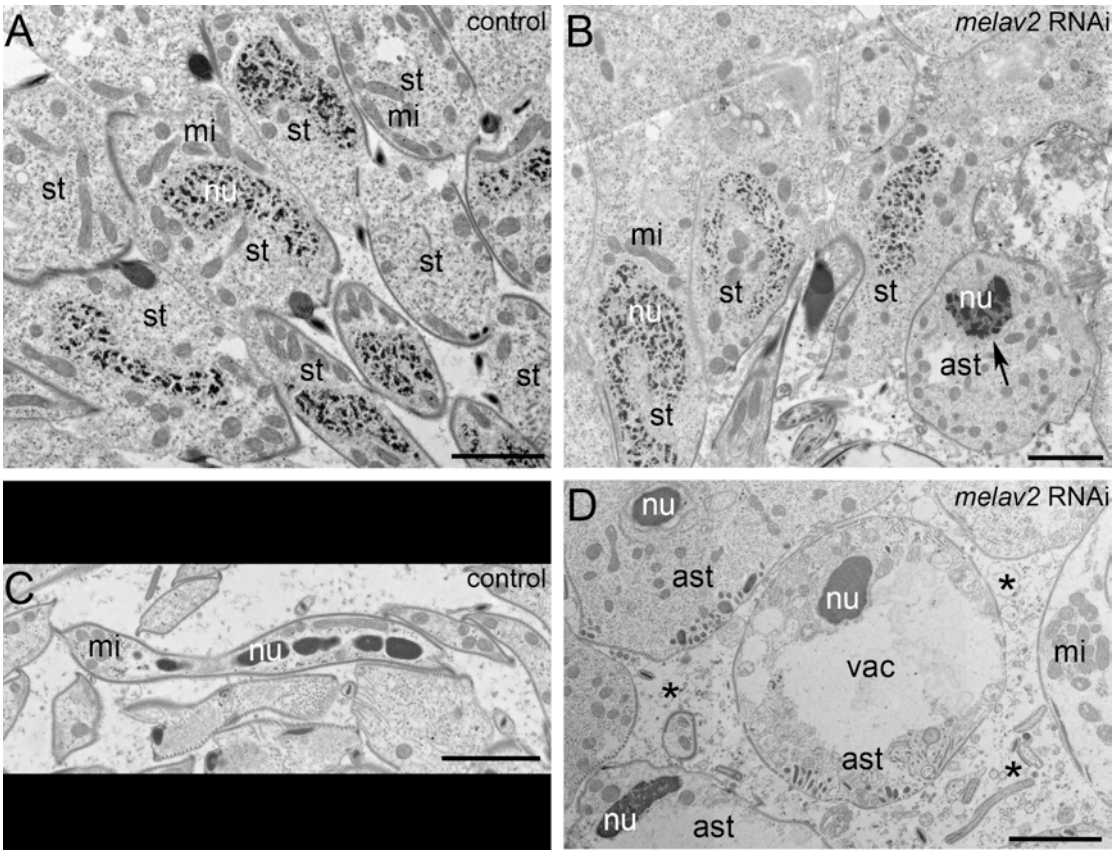


**Figure 7: Comparison of spermatid differentiation of control and *melav2* RNAi treated *M. lignano* by TEM.** (A, B) In the control animals (A), the nucleus of a spermatid was never tightly condensed until quite a late stage of spermiogenesis, while in the *melav2* RNAi treated animals (B), some spermatids started to show a condensed nucleus precociously (B, arrow). (C, D) In the control animals (C), mature sperm had an elongated morphology and the nucleus was condensed into the train-shape, while in the *melav2* RNAi treated animals (D), the train-shaped nuclei were never observed, and the cells often had large vacuoles (vac). Some cell components were also observed outside of the cells (D, asterisk), suggesting cells were dying by necrosis. st, spermatid; ast, aberrant spermatid; mi, mitochondria; nu, nuclei. Scale bars: 2µm.

### *Melav2* RNAi interrupts the transition from spermatid to sperm

Immunocytochemical staining with the monoclonal MSp-1 antibody, which recognizes only early spermatids in *M. lignano* (Ladurner et al., 2005a), revealed that many cells did not complete the transition from early spermatid stage properly in *melav2* RNAi treated worms (Figure 8). In control worms the signal was always detected in clusters of four cells and the number of MSp-1-positive cells did not exceed a few dozens (Figure 8A, B). In the *melav2* RNAi treated worms, however, a considerably larger amount of MSp-1-positive cells was observed (Figure 8C, D), suggesting an accumulation of these cells in the testis. In some cases, the signal looked normal and cells were present in a cluster of four cells, but most of the MSp-1 signal looked disorganized (Figure 8D'). These results suggest that the spermatid differentiation until early spermatid stage was normal but the transition to a later spermatid stage was not completed properly. Therefore many later spermatids remained to hold a certain level of Msp-1 protein in an aberrant appearance, probably because they were partially arrested, differentiating and /or dying.

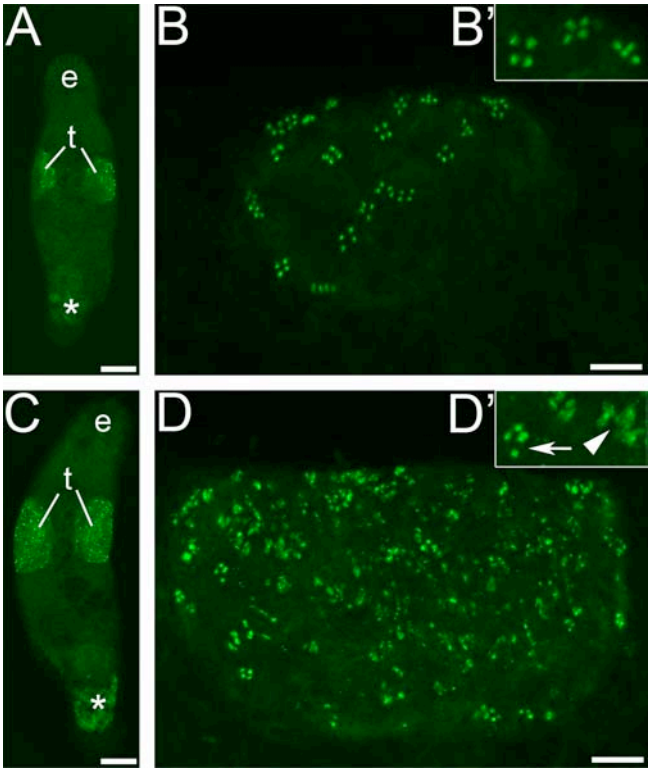


**Figure 8: MSp-1 antibody staining for early spermatid cells in control and *melav2* RNAi treated *M. lignano*.** The testis of control animals (A overview, B detail) had normal MSp-1 signal present in clusters of four cells (B, B'), while the testis of *melav2* RNAi treated animals (C overview, D detail) had a considerably larger number of MSp-1 positive cells (D, D'). Note that the *melav2* RNAi treated animals had some normal MSp-1 signal (D' arrow), but also a lot of disorganized signal (D' arrowhead). e, eyes; t, testes. Asterisk indicates non-specific signal. Scale bars: A, C, 100 µm; B, D, 20 µm.

# Discussion

### *Melav2* encodes for an *elav*-like gene

Our results clearly show a new biological function of an *elav*-like gene, namely the involvement of *melav2* in spermatid differentiation in *M. lignano*. *Elav* family genes have been mainly described to be involved in neural processes, such as the transition between proliferation and differentiation, maturation, maintenance of neurons, and learning (Good, 1997; Pascale et al., 2008; Pascale et al., 2004; Ratti et al., 2006; Robinow et al., 1988; Yao et al., 1993). Recently, however, also other biological functions have been reported. For example, RBP9, a *Drosophila* paralog of *elav*, is expressed during oogenesis and is required for female fertility (Kim-Ha et al., 1999). EXC-7, a *C. elegans* ELAV homologue, is involved in the developent of excretory canals (Fujita et al., 2003). And some *elav* family members are expressed in other tissues or ubiquitously (Good, 1995), suggesting non-neuronal functions. In our study, we did not find remarkable effects of *melav2* RNAi on the nervous system, although we cannot exclude the possibility of *melav2* functions in neural cells in pre-hatching stages. However, we found another *elav*-like gene in *M. lignano*, *melav1* (Figure 1). As previously outlined, the focus of the current study was on the testis-specific expression of *melav2*, and we therefore did not explore the function of *melav1*. We speculate that it may have a role in nervous system similar to other organism's *elav*-like genes (particularly given that it appears to more closely resemble these genes, Figure 1C).

*Elav* genes are characterized by three RRMs with a hinge region between the second and the third RRM. RRM is a quite common protein domain in eukaryotes (Burd and Dreyfuss, 1994; Kenan et al., 1991; Maris et al., 2005). For example, a recent analysis of ESTs in the planarian *Schmidtea mediterranea* revealed that RRM was the second most common domain (Zayas et al., 2005). It is considered that slight changes in RRM can lead to different interactions with other proteins, leading to various types of functions (Maris et al., 2005). In fact, recent findings revealed diverse molecular functions for members of the *elav* gene family. For example, *elav* has been shown to regulate alternative splicing of *neuroglian, erect wing* and *armadillo* transcripts in *Drosophila* (Koushika et al., 1996; Koushika et al., 2000; Lisbin et al., 2001). The human *elav* gene Hel-N1 (HuB) has been suggested to stabilize *GLUT1* mRNA and to increase its translational efficiency (Jain et al., 1997). In *melav2*, the similarity of the third RRM was not well conserved compared to the first and the second RRM. Recently, it has been indicated that the Elav protein can form multimeric complexes (Kasashima et al., 2002; Soller and White, 2005), and *in vitro* experiments suggested that the third RRM has a role for the multimerization, although this is not its only function (Toba and White, 2008). It might be possible that

the difference in the third RRM of *melav2* contributes to a new interaction with the target mRNAs involved in spermatogenesis.

### *Melav2* is necessary for spermatid differentiation

The *melav2* RNAi phenotype showed severe defects in spermatids and an abnormally condensed chromatin. We do not consider these features as signs of an apoptotic process, but instead we found several indications for necrosis. First, cell components such as mitochondria were observed outside of cells (Figure 7D), suggesting that cells were dying in a necrotic way. Second, we observed neither nuclear fragmentation nor an apoptotic body, which are typical characteristics of apoptosis also in *M. lignano* (Nimeth et al., 2002). Third, cells often showed cytoplasmic vacuolization (Figure 7D), which is unusual for apoptosis. Fourth, we observed an accumulation of cells that failed to proceed properly into the later spermatid stages (Figure 8B), suggesting that the elimination of such failed cells by programmed cell death did not occur.

We propose two possible explanations for the abnormal chromatin condensation and the failure of spermatid elongation. One possibility is that *melav2* regulates the genes that mediate chromatin condensation, and chromatin condensation occurred too early as a result of *melav2* RNAi, leading to cessation of further sperm maturation, possibly due to an early stop in the transcription of essential genes. A similar phenotype was shown in spermiogenesis of transgenic mice where pre-mature translation of *prm-1* occurred (Lee et al., 1995). *Prm-1* should be repressed until a later spermatid stage for a proper transition from nucleohistones to nucleo-protamines. Premature translation of *prm-1* caused early chromatin condensation in round spermatids, a subsequent arrest in spermiogenesis and an aberrant spermatid morphology. These features correspond well with the *melav2* RNAi phenotype shown here (Figure 7, 8 and 5, respectively) and therefore it appears possible that *melav2* has a function in regulating *prm-1* or other comparable genes.

Another possible explanation is that cells retained their round shape because of the disruption of spermatid elongation. It has been reported that a human neuronal Elav protein, Hel-N1 (HuB) gene, upregulates the translation of neurofilament M (NF-M) mRNA by using human embryonic teratocarcinoma cells (hNT2) transfected with Hel-N1 (Antic et al., 1999). Overexpressed Hel-N1 did not affect the level of NF-M mRNA expression but instead recruited it into heavy polysomes more efficiently, resulting in the formation of neurites. If a similar molecular function is performed by *melav2*, it is possible that *melav2* is involved in promoting translation of some cytoskeletal elements such as sperm-specific intermediate filaments. The shortage of these elements caused by *melav2* RNAi could result in cessation of elongation, as is often seen in the posterior part at later spermatid stage (Figure 5F-I)

and the failed chromatin reorganization, in which the chromatin was condensed but never built in a train shape (Figure 7D).

Although *melav2* has three RRMs, we have no direct evidence whether *melav2* really functions as RNA-binding protein and, if so, what kinds of mRNA are targeted. Thus, identifying the target genes of *melav2* and their interaction might be important follow-up experiments. Recently, a transcriptome and a genome sequencing project have been initiated in *M. lignano*. Therefore it will soon be possible to identify *melav2* downstream genes by microarrays or *in silico* comparison of transcriptomes of control animals with *melav2* RNAi treated worms.

### *Melav2* RNAi treated worms are male-sterile

The seminal vesicles of *melav2* RNAi treated worms were empty, and in consequence they cannot transfer sperm to a mating partner, suggesting they are male-sterile. However, *M. lignano* is hermaphroditic and the female reproductive function of *melav2* RNAi treated worms was normal (Figure 3). So a *melav2* RNAi treated animal (or a loss of function mutant) would still be able to contribute offspring to next generation as a female after copulation with normal worms. Thus, future experiments aimed at following their reproductive success of such 'females' and determining if such functional females could spread within a hermaphroditic population, would provide a better understanding of the evolution of different reproductive modes. In addition, the formation of the stylet and the male accessory glands in *melav2* RNAi treated worms was normal (Figure 3), which offers interesting possibilities for studies on sexual conflicts. Recent experiments have suggested that male accessory glands and their secretions have important roles for increasing male reproductive success by manipulating the female reproductive physiology and behavior, such as increasing egg production, decreasing female's motivation toward further copulations with other partners, and decreasing female's longevity (Chapman et al., 1993; Gillott, 2003; Price et al., 1999). Although we still need to analyze if *melav2* RNAi treated worms can in fact transfer ejaculates without sperm, such worms have a great potential to study the effects of seminal fluids in hermaphroditic organisms, by removing the effect of the transferred sperm itself.

## Conclusions

We found that *melav2* has a similarity with the *elav* gene family at the protein sequence level. *Elav* genes are mainly known to be involved in the nervous system in other organisms, but our study clearly shows that *melav2* plays a crucial role during spermatid differentiation in *M. lignano*. *Melav2* RNAi disturbed the proper regulation of chromatin condensation and/or the cell elongation, resulting in aberrant spermatid morphology and male sterility.

# Methods

### Animal culture

The free-living flatworm *M. lignano* (Ladurner et al., 2005b) was cultured in glass Petri dishes with nutrient-enriched artificial seawater (Guillard's f/2 medium (Andersen et al., 2005)) and fed with the diatom *Nitzschia curvilineata*. The worms were kept in a climate chamber with 60 % humidity at 20 °C in a 14:10 h day-night cycle (Rieger et al., 1988). Animal experimentation was carried out in accordance to Austrian legal and ethical standards.

### Gene isolation and analyses

The full length open reading frame of the clone ANGU919 (http://flatworm.uibk.ac.at/macest/) was sequenced by GATC (Konstanz, Germany) using M13 standard primers. Sequences were analyzed by BLASTX searches at the EMBL-EBI database (http://www.ebi.ac.uk/Tools/blast2/). Conserved protein domains were identified using the SMART databases (http://smart.embl-heidelberg.de/) (Letunic et al., 2006). Protein structural analysis was performed using the SWISS-MODEL program (http://swissmodel.expasy.org/) (Arnold et al., 2006; Kopp and Schwede, 2004).

### Alignment and phylogenetic analyses

Amino acid alignments were performed using the Multiple Sequence Alignment Program CLUSTALW of the EMBL-EBI database (http://www.ebi.ac.uk/Tools/clustalw2/) with default alignment parameters. The phylogenetic tree was calculated using the MrBayes 3.2.2 (Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck, 2003). Each run was performed using default parameters and comprised 5,000,000 generations.

Elav sequences were from *Mus musculus* ELAV-like protein1 [UniProtKB:P70372], *Xenopus laevis* ELAV-like2 [GenBank:NP_001081035], *Danio rerio* ELAV-like 1 [GenBank:NP_571527], *Drosophila melanogaster* Protein elav [UniProtKB:P16914], *Aplysia californica* ELAV 2-like protein [GenBank:AAY42042], *Platynereis dumerilii* Elav [GenBank:ABO93208]. Elav sequences of *Schmidtea mediterranea* and *Hydra magnipapillata* were generated by computational work using available EST database, SmedGD v1.3.14 (http://khan.neuro.utah.edu/index.html) and dbEST_HMAG070214 (www.compagen.org), respectively. The *Schmidtea* sequence was generated from contig ec1.03596.005, and the *Hydra* sequence was generated by assembling three clones, tai96e09.y2 [CX056199], tai87h08.y1 [CV182751] and tai87h08.x1

[CV182482]. *Melav1*, another *elav*-like gene from *M. lignano* was obtained from EST clone Ml_aw_006_D03 in a same way as the *melav2* gene.

Sex lethal sequences were from *Drosophila melanogaster* Protein sex-lethal [UniProtKB:P19339], *Drosophila subobscura* Protein sex-lethal [UniProtKB:Q24668], *Musca domestica* Sex-lethal homolog [UniProtKB:O17310], *Ceratitis capitata* Sex-lethal homolog [UniProtKB:O61374], *Chrysomya rufifacies* Sex-lethal homolog [UniProtKB:O97018], *Megaselia scalaris* Sex-lethal homolog [UniProtKB:O01671], *Anopheles gambiae* TPA:sex-lethal [GenBank:CAJ55784], *Acyrthosiphon pisum* sex-lethal [GenBank:NP_001119609], *Bombyx mori* sex-lethal [GenBank:NP_001036780]. As an outgroup for the calculation of phylogeny, *Drosophila melanogaster* CG4787-PA [UniProtKB:Q9VAX1] was used. Square brackets indicate accession number.

**Whole mount *in situ* hybridization**

The sequence region for the *in situ* RNA probe was amplified with the *melav2* specific primers 5'-GGC CTT CTC AGA TGA CGA GT-3' and 5'-GGA CAG ATG TTG ATG GAC CTG-3'. The PCR condition was 2 min at 94 °C, 35 cycles (30 sec at 94 °C, 30 sec 55 °C, 90 sec at 72°C), 7 min at 72 °C. Obtained PCR products were sub-cloned into pGEM®-T (Promega). Then PCR with M13 standard primers was performed to generate the template for RNA probe synthesis, including SP6 and T7 RNA polymerase promoter sequences. Digoxygenin-labeled RNA probe was generated using DIG RNA Labeling KIT SP6/T7 (Roche), following the manufactor's protocol. Whole mount *in situ* hybridization for *M. lignano* was performed as previously described (Pfister et al., 2008). The signal was developed at 37 °C using the NBT/BCIP system (Roche). Specimens were examined with a Leica DM5000 microscope. Image acquisition and analysis were performed using a Leica DFC490 digital camera, the Leica Application Suite 2.8.1 software, and the Adobe® Photoshop® 7.0 software.

**RNA interference**

By adding T7 RNA polymerase promoter sequence to the primers that were used to generate the templates for *in situ* RNA probe, two different templates for dsRNA probe were generated: one had a T7 promoter at the 5' end for producing sense RNA, and the other had it at the 3' end for producing anti-sense RNA, respectively. The PCR condition was 2 min at 94 °C, 35 cycles (30 sec at 94 °C, 30 sec 55 °C, 90 sec at 72 °C), 7 min at 72 °C. The *in vitro* synthesis of dsRNA was performed using T7 RiboMax TM Express RNAi System (Promega). As a negative control, firefly *luciferase* dsRNA was produced from the pGEM®-luc Vector (Promega). RNAi treatment for *M. lignano* was performed by soaking as previously

described (Pfister et al., 2008). We used 31 animals for control and 55 animals for *melav2* RNAi treatment. One-day old hatchlings were maintained in 24-well plates (20 worms per well) and incubated with 250 µl dsRNA solution (3.0 ng/µl) in f/2 medium containing the antibiotics Kanamycin and Ampicillin (50 µg/ml, respectively) and diatoms. dsRNA solution was changed twice a day. After 3-4 weeks of RNAi treatment, the specimens were examined with a Leica DM5000 microscope using DIC optics. Image acquisition and analysis were performed using the same imaging set-up as described in the "Whole mount *in situ* hybridization" section. For the observation of testis components and sperm morphology, live worms were squeezed under a cover slip until the testis ruptured and the contents of the testis were directly observed.

**Histology**

Control and *melav2* RNAi treated animals were relaxed in 7.14% $MgCl_2$ and fixed for 1 hour at 4°C in 2.5% glutardaldehyde in 0.1 M cacodylate buffer (pH 7.4) containing 9% sucrose. After several washes with buffer, specimens were postfixed in 1% osmium tetroxide in 0.05 M cacodylate puffer (pH 7.4) for 1 hour. After washing in buffer and subsequent dehydration in an ethanol series, animals were embedded in SPURR´s low viscosity resin (Spurr, 1969). Complete series of 1.5 µm thick semi-thin sections were cut with a prototype of a Butler diamond knife (Diatome) and mounted on glass slides. After drying the sections were stained for 2 minutes in a methylen blue Azur II mixture after Richardson (Richardson et al., 1960) and mounted in cedar wood oil. One control animal and one *melav2* RNAi treated animal were examined with a Leica 5000B microscope. Image acquisition and analysis were performed using the same imaging set-up as described in the "Whole mount *in situ* hybridization" section.

**Histology after whole mount *in situ* hybridization**

After performing *melav2* whole mount *in situ* hybridizations as described above, specimens were fixed with BOUIN´s fluid overnight. After subsequent dehydration in an ethanol series, animals were embedded in SPURR´s low viscosity resin. Complete series of 2 µm thick semi-thin sections were cut with the same sectioning set-up as described in the "Histology" section. Two animals were examined with a Leica 5000B microscope. Image acquisition and analysis were performed using the same imaging set-up as described in the "Whole mount *in situ* hybridization" section.

**Transmission electron microscopy**

Control and *melav2* RNAi treated animals were fixed and embedded as described above. Semi-thin and ultra-thin sections were cut with a diamond knife on

an Ultracut S (Leica) ultramicrotome, double stained with uranyl acetate and lead citrate. One control animal and one *melav2* RNAi treated animal were examined with a ZEISS Libra 120 energy filter electron microscope. Image acquisition and analysis were performed using a 2k Vario Speed SSCCD camera (Droendle) and the iTEM software (TEM imaging platform, Olympus).

**Immunocytochemistry**

Immunocytochemistry for *M. lignano* was performed as previously described (Ladurner et al., 2005a; Pfister et al., 2008). As the primary antibody, the spermatid-specific mouse monoclonal MSp-1 antibody (Ladurner et al., 2005a) was used (1:200). As the secondary antibody, a FITC-conjugated goat-anti-mouse antibody (1:250, DAKO) was used. Two control animals and three *melav2* RNAi treated animals were examined with a Zeiss LSM 510 confocal laser scanning microscope system. Image acquisition and analysis were performed using the LSM510 ver.3.2 software, the LSM image browser ver.4.2.0.121 software and the Adobe® Photoshop® 7.0 software.

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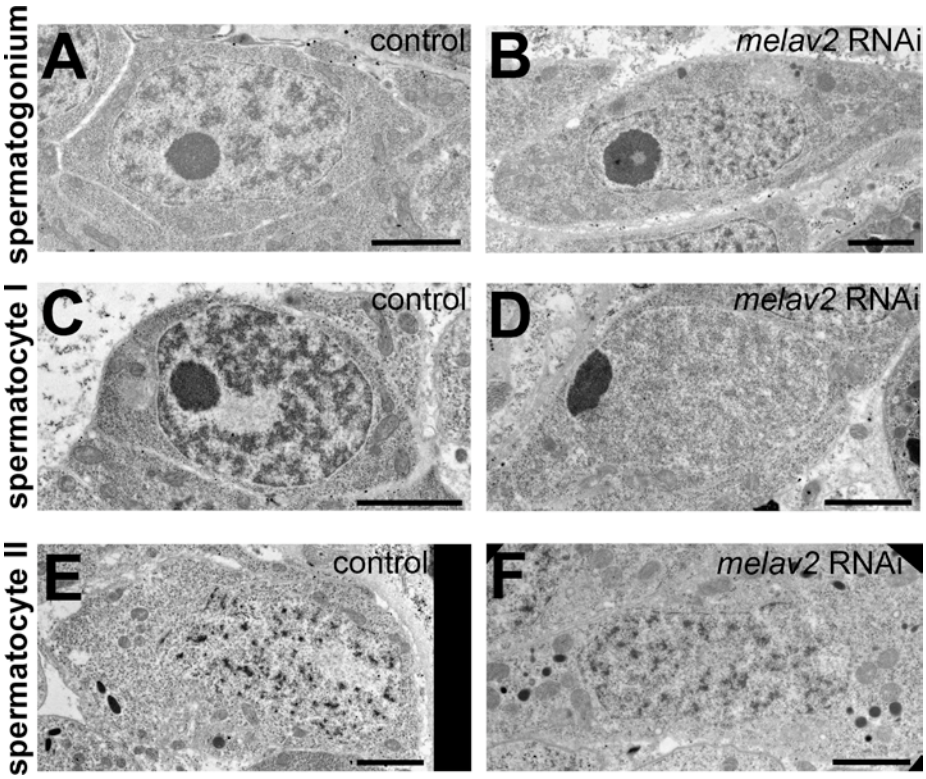
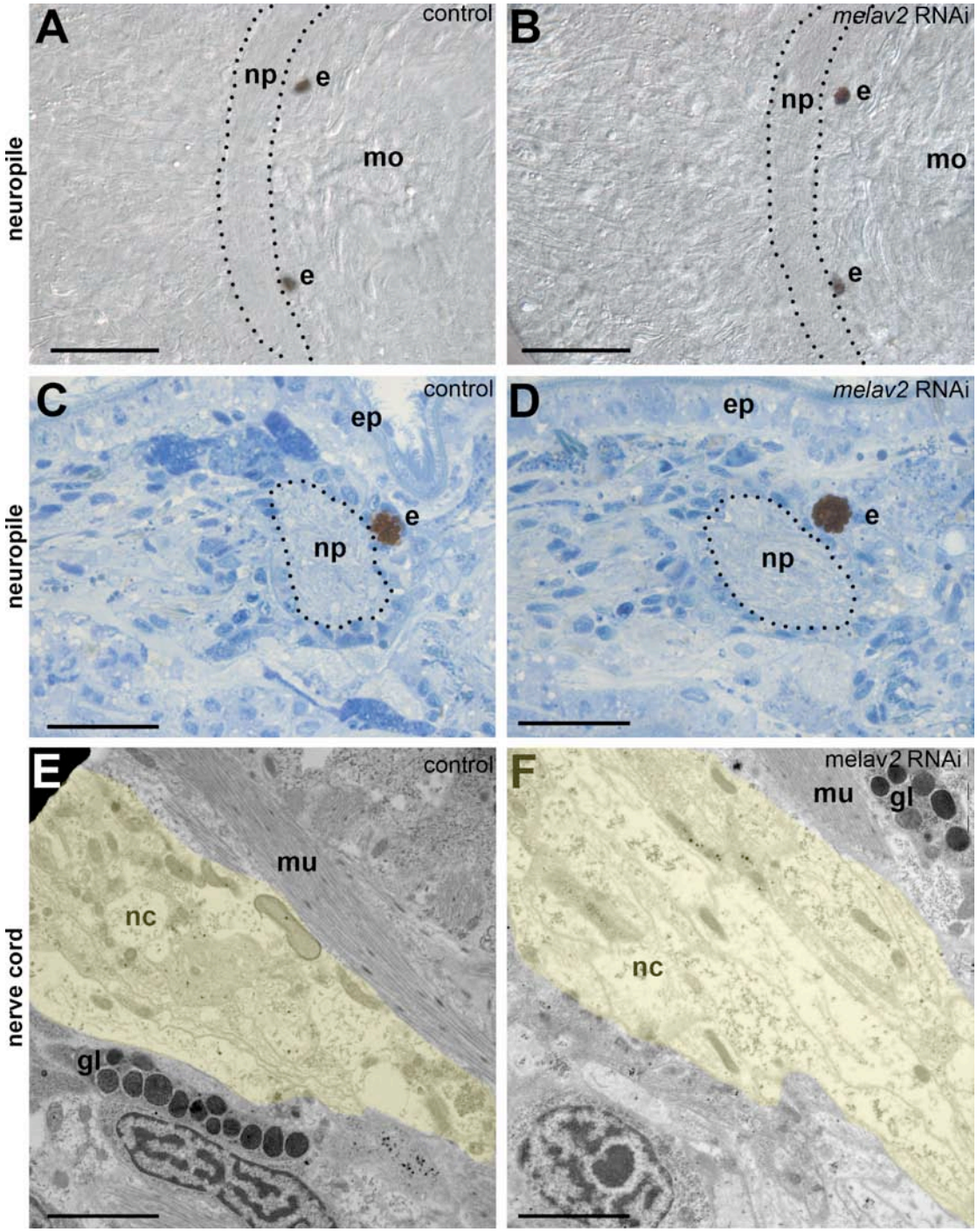
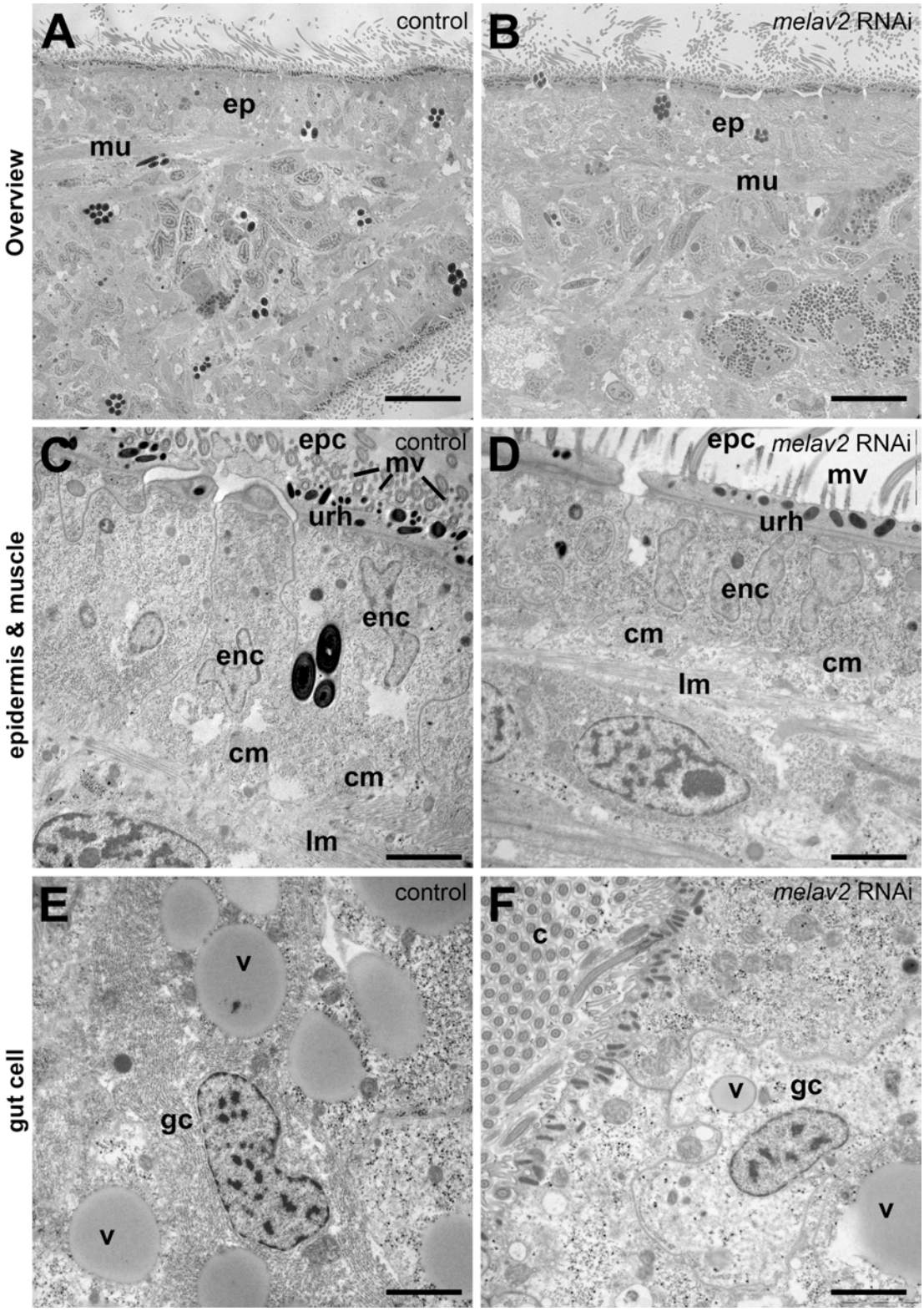
# Additional files



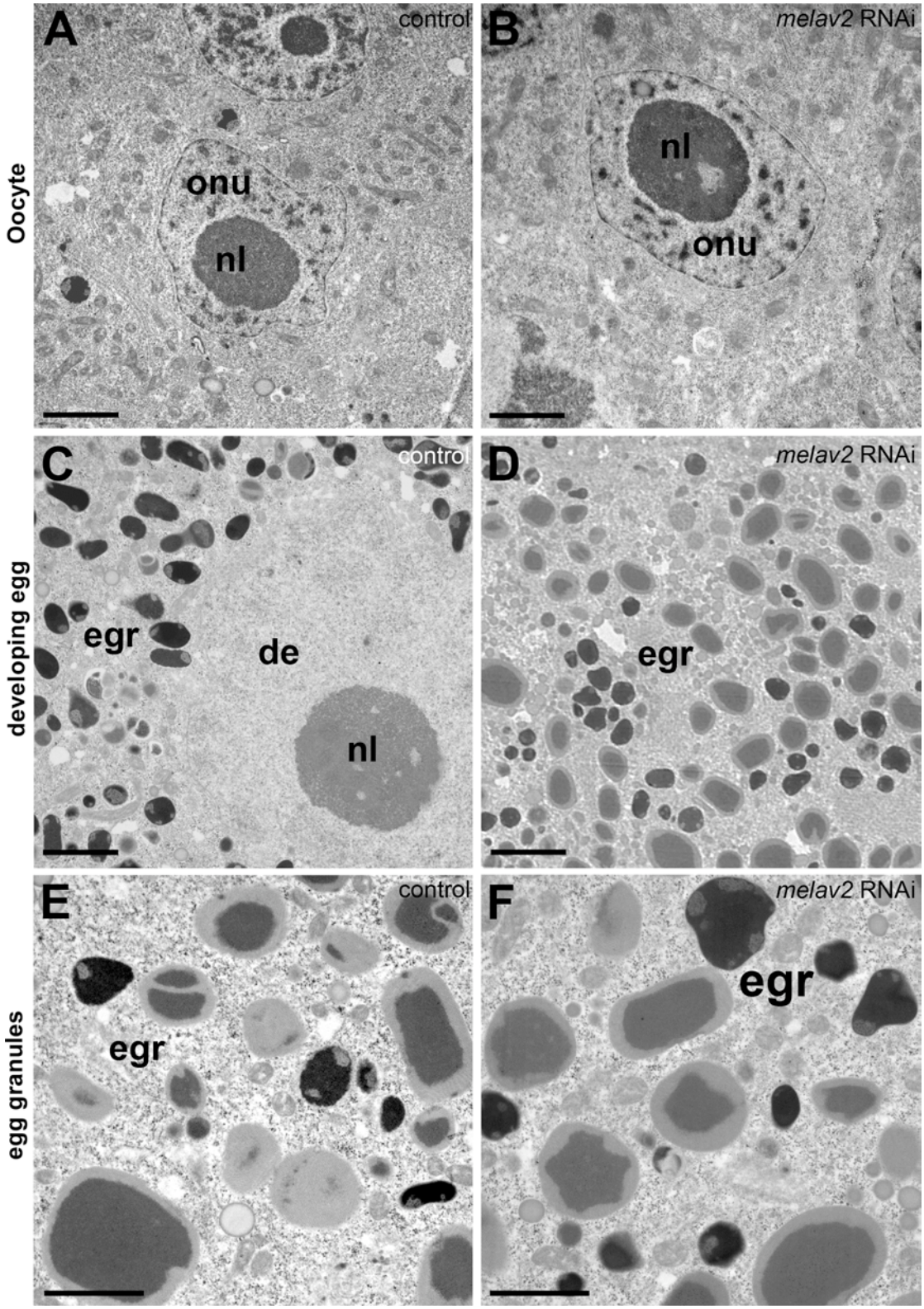

**Additional file 1: Comparison of early spermatogenesis of control and *melav2* RNAi treated *M. lignano* by TEM.** (A-F) The appearance of the spermatogonia and spermatocytes I and II of the control animals (A, C, D, respectively) was comparable to that of the *melav2* RNAi treated animals (B, D, F, respectively). Scale bars: 2 μm.




**Additional file 2: Comparison of the neuropile and nerve cord morphology of control and *melav2* RNAi treated *M. lignano*.** (A, B) The appearance of the neuropile of control animals (A) was comparable to that of the *melav2* RNAi treated animals (B) in interference contrast microscopy. (C, D) The tissue structure of the neuropile of control animals (C) was also comparable to that of the *melav2* RNAi treated animals (D) in semi-thin sections. Dotted lines roughly indicate the regions of the neuropile. (E, F) Morphology of nerve cord of control (E) was compatible to that of the *melav2* RNAi treated animals (F). e, eye; ep, epidermis; gl, gland; mo, mouth opening; mu, muscle; nc, nerve cord; np, neuropile. Scale bars: A, B, 50 μm; C, D, 25 μm; E, F 2μm.




Additional file 3 :Comparison of tissue organization, epidermal-, muscle, and gut cell morphology of control and *melav2* RNAi treated *M. lignano*. Overview demonstrates that tissue integrity is comparable in control (A) and *melav2* RNAi treated (B) *M. lignano*. Likewise, the ultrastructure of epidermal cells (C, D) and gut cells (E, F) was not affected by *melav2* RNAi treatment. c, cilia of gut cell; cm, circular mucscle; enc, epidermal cell nucleus; ep, epidermal cell; epc, epidermal cell cilia; gc, gland cell; mu, muscle cell; mv, microvilli; lm, longitudinal muscle; uhr, ultrarhabdites; v, storage vesicle. Scale bars: A, B, 10 μm; C-F, 2 μm.




**Additional file 4: Comparison of oogenesis of control and *melav2* RNAi treated *M. lignano*.** The oocyte of control (A) and *melav2* RNAi treated (B) *M. lignano* exhibited comparable morphology. The ultrastructure of developing eggs (C, D) egg granules (E, F) was not affected by *melav2* RNAi treatment. de, developing egg; egr, egg granules; nl, nucleolus; onu, oocyte nucleus. Scale bars: A-D 2 μm; E, F, 1 μm.

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# CHAPTER III

## Phenotypic engineering of sperm-production rate confirms evolutionary predictions for fitness returns of male allocation

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# Phenotypic engineering of sperm-production rate confirms evolutionary predictions for fitness returns of male allocation

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## Abstract

Sperm production is a key male reproductive trait and an important parameter in sperm competition and sex allocation theory. For example, under sperm competition paternity success is predicted to directly depend on the male allocation towards sperm production. Moreover, a saturating relationship between male allocation and paternity success (male fitness gain curve) is a key prediction of sex allocation theory for simultaneous hermaphrodites. However, male allocation towards sperm production has been difficult to manipulate in animals and thus experimental studies have been scarce. Here we phenotypically engineered sperm-production rate using dose-dependent RNA interference (RNAi) of a spermatogenesis-specific gene, *macbol1*, and examined the resulting paternity success in a simultaneous hermaphrodite, the free-living flatworm *Macrostomum lignano*. We demonstrate (i) that our novel dose-dependent RNAi approach leads to a broad range of sperm-production rates, (ii) that higher sperm-production rate increases paternity success, as predicted by sperm competiton theory, and (iii) that the male fitness gain curve is saturating, as predicted by sex allocation theory. Our study clearly documents the potential of phenotypic engineering via dose-dependent RNAi to test quantitative predictions of evolutionary theory.

# Introduction

Male allocation towards sperm production is a key parameter in sperm competition and sex allocation theory. When several sperm donors copulate with the same recipient this can lead to sperm competition between their ejaculates (Parker, 1970), an important aspect of post-copulatory sexual selection (Birkhead and Pizzari, 2002; Parker, 1998). Under these circumstances sperm competition theory predicts allocation towards sperm production to be an important male trait, since the paternity share obtained will depend on the relative amounts of sperm transferred by each sperm donor (Parker, 1990; Parker, 1998). In support of this prediction, many comparative studies have shown a positive correlation between relative testis size (a frequently used morphological proxy for male allocation towards sperm production) and the mating system (a proxy for the level of sperm competition) in a large range of animal taxa (e.g., Ginsberg and Rubenstein, 1990; Harcourt et al., 1981; Møller, 1991; Møller and Briskie, 1995; Pitcher et al., 2005; Stockley et al., 1997; reviewed in Parker and Pizzari, 2010). In contrast to the considerable comparative evidence, more direct evidence for the selective pressure on sperm production under sperm competition is less abundant. While there are a fair amount of studies that have found a positive correlation between intraspecific variation in relative testis size and sperm transfer and/or paternity success (e.g., Awata et al., 2006; Bercovitch and Nurnberg, 1996; Janicke and Schärer, 2009a; Preston et al., 2003; Schulte-Hostedde and Millar, 2004), few studies have involved experimental manipulation of sperm production. These include experimental evolution studies where individuals were kept under conditions with more or less sperm competition over many generations, leading to the expected changes in relative testis size and paternity success (e.g., Hosken et al., 2001; Hosken and Ward, 2001; Pitnick et al., 2001; Simmons and Garcia-Gonzalez, 2008). But as we outline below, we are aware of no studies that have directly manipulated sperm production.

How fitness returns are linked to male allocation towards sperm production is also an important aspect of sex allocation theory (Charnov, 1982; Schärer, 2009; West, 2009). In simultaneous hermaphrodites the shape of the fitness gain curves is important to predict optimal resource allocation into each sex function. If the male fitness gain curve is saturating (i.e., shows diminishing returns) it may, at a certain point, be more profitable to reallocate remaining resources towards the female sex function, rather than to keep investing into the saturating sex function. In many simultaneous hermaphrodites the shape of the male fitness gain curve is predicted to be saturating (Charnov, 1982; Schärer, 2009), but this has rarely been tested

empirically in animals. Male fitness gain curves were examined only in colonial marine invertebrates (e.g., an ascidian, Johnson and Yund, 2009; Yund, 1998; and a bryozoan, McCartney, 1997), in which the authors used standing variation in male allocation (i.e., total cross-sectional area of the testes and the number of male zooids, respectively), as estimates of male allocation towards sperm production. We are aware of no studies on simultaneously hermaphroditic animals that have examined the male fitness gain curve by experimentally manipulating male allocation.

Thus a common problem in both sperm competition and sex allocation research has been that it is difficult to experimentally manipulate male allocation and sperm-production rate in animals, because the testis is internal and often difficult to assess, let alone manipulate *in vivo*. However, manipulating phenotypic traits and examining the link between trait values and their fitness effects is a powerful approach in evolutionary biology, and has yielded capital insight into the function of a range of different traits (e.g., Andersson, 1982; Maklakov and Arnqvist, 2009; Møller, 1988; Polak and Rashed, 2010). It would therefore be highly desirable to obtain control over testicular function, thus allowing to phenotypically engineer different levels of sperm production in an otherwise similar background of sperm donors. Here we have established a method to experimentally and quantitatively manipulate sperm-production rate in the free-living flatworm *Macrostomum lignano*, and have used it to test two key predictions of evolutionary theory: 1) that paternity success scales positively with sperm production, a central prediction from sperm competition theory, and 2) that the shape of this relationship (the male fitness gain curve) shows diminishing returns, a prediction from sex allocation theory for simultaneous hermaphrodites.

*M. lignano* is an excellent model for both developmental (e.g., Kuales et al., 2011; Ladurner et al., 2008) and evolutionary biology (e.g., Schärer and Janicke, 2009; Schärer and Ladurner, 2003), as it permits powerful experimental approaches to study reproduction. For example, its transparent nature allows us to measure sperm-production rate *in vivo* (Schärer and Vizoso, 2007) and molecular genetic tools allow us to manipulate organ specific gene expression (Pfister et al., 2008; Sekii et al., 2009). We experimentally manipulated sperm-production rate over a broad range of values using a novel dose-dependent RNA interference (RNAi) gene knock-down approach. Specifically, we modulated the expression of *macbol1*, an essential gene for spermatogenesis in *M. lignano* (Kuales et al., 2011). We show that different doses of double-stranded RNA (dsRNA) led to different sperm-production rates and thus different amounts of sperm in the seminal vesicle (i.e., the organ where sperm ready to be donated are stored prior to copulation). Moreover, by using microsatellite paternity analysis, we then show that sperm-production rate had a positive effect on paternity success, as predicted by sperm competition theory, and that the resulting

male fitness gain curve was indeed saturating, as predicted by sex allocation theory for simultaneous hermaphrodites. This, to our knowledge, is the first study to use phenotypic engineering of sperm-production rate to test evolutionary predictions from sperm competition and sex allocation theory in animals.

# Materials & Methods

### (a) Study organism

*Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is a purely outcrossing simultaneous hermaphrodite (Schärer and Ladurner, 2003) and a member of the interstitial sand fauna of the Northern Adriatic Sea (Ladurner et al., 2005). Worms are transparent allowing non-invasive measurements of body, testis, ovary, and seminal vesicle size (Schärer and Ladurner, 2003). Copulation is reciprocal and worms mutually insert their copulatory stylets into the partner's female genital opening (Schärer et al., 2004). In the laboratory, we maintain these worms in Guillard's f/2 medium (Andersen et al., 2005) in glass petri dishes, feed them *ad libitum* with the diatom *Nitzschia curvilineata*, and keep them at 20°C on a 14:10 h day-night cycle (Rieger et al., 1988).

### (b) Experimental design

To phenotypically engineer the sperm-production rate, we modulated the expression of the testis-specific *macbol1* gene by using a novel dose-dependent RNAi approach. *Macbol1*, a *boule* gene belonging to the *DAZ* gene family (Shah et al., 2010; Yen, 2004), is expressed specifically in the testis, and the complete RNAi knock-down phenotype of this gene results in the arrest of spermatogenesis and male sterility due to an empty seminal vesicle (Kuales et al., 2011). By using different dsRNA doses, we aimed at obtaining intermediate *macbol1* RNAi phenotypes, allowing us to engineer a broad range of sperm-production rates (see section "RNAi treatment"). To quantify different sperm-production rates we examined changes in seminal vesicle size as a morphological measure of sperm-production rate. Seminal vesicle size reflects the amount of sperm contained in it and has been shown to change in response to different sperm-production rates (Schärer and Vizoso, 2007). As sperm that are experimentally prevented from completing spermatogenesis do not reach the seminal vesicle (Kuales et al., 2011; Sekii et al., 2009), changes in seminal vesicle size caused by the *macbol1* RNAi treatment can be attributed to differences in the amount of fully differentiated sperm produced, thus providing a proxy for sperm-production rate (i.e., a larger seminal vesicle size corresponds to a worm with a higher sperm-production rate and thus a higher male allocation towards sperm production)

(see section “Morphological measurements”). Finally, to test the consequences of this experimentally induced variation in sperm-production rate, we performed sperm competition experiments using focals, competitors, and recipients (see section “Experimental animals”), while using competitors as first sperm donors and focals as second sperm donors in sperm competition to father the eggs of recipients (see section “Sperm competition experiment”). The paternity of the resulting offspring was assessed using a microsatellite marker (see section “Paternity analysis”). The timeline of the experiment is shown in electronic supplemental material, figure S1.

**(c) Experimental animals**

To reduce undesired genetic variation, we used fixed genotypes for the focals, recipients and competitors, making use of our established inbred lines. Inbred lines were started by crossing two virgins and thereafter crossing full- or half-siblings, and have been maintained for over 40 generations (D. B. Vizoso, unpublished data). We obtained recipients by crossing DV13 line worms as fathers and DV8 line worms as mothers. On day 1 (electronic supplemental material, figure S1) we paired virgin worms and let them copulate for 24 hours. Then we separated them and kept the mothers individually. To visually distinguish the parents, the fathers were colorized beforehand in f/2 medium with a red food colorant (6.25 mg/ml, E124, Werner Schweizer AG) for 12 hours. On day 8, we collected the resulting 1-day old offspring (age is in days posthatching) into a Petri dish, and individually distributed them on day 15 into 24-well plates (Techno Plastic Products). We obtained focals as above but using DV71 and DV28 line worms as fathers and mothers, respectively. On day 12, we distributed the resulting 1-day old offspring individually into 60-well microtest plates (Greiner Bio-One) and immediately initiated the RNAi treatment (see section “RNAi treatment”). As the competitor we used pure DV69 line worms because many eggs of an intended cross between DV69 and DV3 line worms failed to hatch. While potential inbreeding effects in the DV69 line may have made this a particularly weak competitor, these worms were clearly able to sire viable offspring in the recipients (see section “Results”). On day 24, adult worms were collected from a DV69 line mass culture and kept individually until they were paired to copulate with the recipients.

**(d) RNAi treatment**

We used 7 doses for the RNAi treatment. The control treatment (T1) did not contain any dsRNA probe, and was expected to produce a normal phenotype, namely a large seminal vesicle filled with sperm. In the highest dose (T7), the dsRNA probe concentration was 4.85 ng/µl, and was expected to produce a phenotype with an empty seminal vesicle. The intermediate 5 doses were made in a 2.3× dilution series

(i.e., T6, 2.11 ng/µl; T5, 0.917 ng/µl; T4, 0.399 ng/µl; T3, 0.173 ng/µl; T2, 0.0754 ng/µl), and these were expected to produce intermediate seminal vesicle sizes.

The dsRNA probe was synthesized *in vitro* as previously described (Kuales et al., 2011), using the T7 RiboMax™ Express RNAi System and T7 RiboMax™ Express Large Scale RNA Production System (both from Promega). Briefly, we obtained PCR products of the probe region of *macbol1* (accession number HM222645) containing T7 promoters at the both ends, and used them as a template for dsRNA synthesis, producing sense and anti-sense RNA. We estimated the concentration of dsRNA probe both using a NanoDrop-1000 v.3.7.1 (Thermo Scientific) and gel electrophoresis in comparison with a DNA standard (Quick-Load 100 bp DNA Ladder, BioLabs), in which the amount of DNA in each band is known. The difference between the two estimates was within a two-fold margin. Probe concentrations given here use the latter approach, as recommended by the protocol of the kit used.

During RNAi treatment, focals were kept individually on 60-well microtest plates,and their positions were spatially balanced for all treatment groups. Each worm was kept in 10 µl of dsRNA solution (in f/2 with 50 µg/ml of Kanamycin and Ampicillin, Pfister et al., 2008) with an *ad libitum* amount of diatoms and transferred daily into a new well with fresh dsRNA solution and diatoms. The initial sample size was n = 168 (i.e., 24 replicates for each of the 7 dsRNA doses).

**(e) Morphological measurements**

On days 22, 25, and 28 (i.e., first, second, and third measurement) we measured the seminal vesicle size of the focals (i.e., when 11, 14, and 17 days old, respectively) to confirm successful manipulation of the sperm-production rate (electronic supplemental material, figure S1). Having grown up in isolation, the size of the seminal vesicle should reflect the complete sperm production of these worms up to that point. We also measured body, testis, and ovary size, which might be important factors for paternity success. The measurements were performed as previously described (Schärer and Ladurner, 2003). Briefly, the worms were relaxed with a solution of $MgCl_2$ and squeezed dorsoventrally between a microscope slide and a hemacytometer coverslip. We took images of the worms and organs using a Leica DM 2500 microscope (Leica Microsystems), a digital video camera (DFK 41BF02, The Imaging Source Europe), and the software BTV Pro 6.0b1 (http://www.bensoftware.com/). Images for body size were taken at 40× magnification, and the images for testis, ovary, and seminal vesicle size were taken at 400× magnification. We then measured the area of the traits using ImageJ 1.37v (http://rsb.info.nih.gov/ij/) and also visually scored the fill grade of the seminal vesicle (see section “Statistical analyses”).

**(f) Sperm competition experiment**

On days 29 and 30 (electronic supplemental material, figure S1) (i.e., when focals were 18 or 19 days old, recipients were 22 or 23 days old, and competitors were approx. 2 months old), we performed the sperm competition experiment. All copulations were set up in "mating chambers" and filmed as previously described (Schärer et al., 2004). Briefly, we placed a pairs of worms in drops of 4 µl of f/2 medium into a mating chamber built from two siliconized microscope slides with spacers. Each observation chamber contained 14 drops and positions were spatially balanced for all treatment groups. Recipients were first allowed to copulate with a competitor for 2 hours, after which they were recovered and immediately (within 25 minutes) allowed to copulate with a focal for an additional 2 hours. To visually distinguish the recipients, we colorized them as described above. We filmed the mating behavior using digital video cameras (DFK 31BF03, The Imaging Source or DFW-X700, SONY) and the SecuritySpy 2.0.3 (http://www.securityspy.com) as a time-lapse with 1 frame per second in QuickTime format. We scored the copulation frequency by visual frame-by-frame analysis. Here we defined a copulation as worms forming a tight disk for 5 seconds or more (Schärer et al., 2004).

**(g) Paternity analysis**

After the sperm competition experiment we transferred recipients individually into 24-well plates. When the recipients' offspring were approximately 7 days old (day 40 onwards), we started to collect them individually in 75% ethanol in f/2 medium and stored them at -20°C until genotyping using one microsatellite marker. After removing ethanol by evaporation, we extracted DNA by adding 20 µl of $MgCl_2$-free PCR buffer containing 0.5 µg/µl of Proteinase K (Sigma Aldrich), breaking up tissues by freezing at -80°C for 1 hour, digesting at 50°C for 1 hour, and inactivating the Proteinase K at 95°C for 15 minutes. We used 2 µl of this extraction as a template for the following 10 µl PCR reaction. We amplified the microsatellite locus Macro21 by PCR with a fluorescent conjugated forward primer 5'-TTC ATC AAC ATC AGC CTT ATC C-3' and a reverse primer 5'-CTG CTG CTG AGG TGT TTG G-3'. The PCR conditions were 15 min at 95°C, 35 cycles of 30 sec at 94°C, 90 sec 53°C and 60 sec at 72°C, and 30 min at 60°C. We used 1.5 µl of the PCR product for genotyping, which was performed using an AB3130xl Genetic Analyzer (Applied Biosystems) and the software Genemapper 4.0 (Applied Biosystems). Competitors and recipients were monoallelic at the Macro21 locus (allele size 90 bp), while focals never carried that allele (they had alleles of 87 bp and/or 97 bp). Therefore, paternity of offspring carrying alleles other than the 90bp allele could be unequivocally assigned to the focal (assuming no genotyping errors or mutations occurred).

**(h) Statistical analyses**

We performed statistical analyses using JMP 8.0.2 (SAS Institute) or R 2.10.1 (R Development Core Team). Analyses aimed at testing three main questions, namely (i) is the RNAi treatment successful at manipulating sperm-production rate (as measured by an increase in seminal vesicle size over time), (ii) has the RNAi treatment affected the final allocation into sperm and any other morphological or behavioural traits in the focals (as measured at the third measurement and during the sperm competition assays, respectively), and (iii) does our manipulation lead to the predicted effect on paternity success (i.e., a positive, but nonlinear, relationship between sperm production and paternity success)?

Firstly, we tested if the different dsRNA doses actually led to differences in sperm-production rates, as indicated by changes in seminal vesicle size from the second to the third measurement (we excluded the first measurement, because about 20% of the worms were still immature). We performed a repeated measures ANOVA using seminal vesicle size as the response variable and dsRNA dose as the fixed factor. When individuals did not yet have a seminal vesicle its size was considered zero (only 2 of 147 individuals at the second measurement). Note that the size of an empty seminal vesicle is not zero, although they are generally very small. To account for this we also assessed the fill grade of the seminal vesicles at the third measurement. The seminal vesicle consists of a true and a false seminal vesicle (Ladurner et al., 2005), and we judged it as "full" if both were completely filled with densely packed sperm, "fairly full" if individually distinguishable sperm were seen in both, "half full" if only the true seminal vesicle contained ample sperm, "fairly empty" if only a few sperm were seen, and "empty" if both were empty. The fill grade was estimated blind with respect to the treatment group. We tested whether the fill grade differed between different dsRNA doses using a Pearson's chi-square test.

Secondly, we examined whether the dsRNA dose had an effect on the final allocation into sperm, as indicated by seminal vesicle size at the time of the third measurement (i.e., immediately before the worms entered the sperm competition experiment). Moreover, we also examined its effect on other morphological traits such as body, testis and ovary size, and on copulation frequency of focals during the sperm competition experiment. For this we handled the dsRNA dose as a continuous variable by using the $\log_e$-transformed dsRNA probe concentration and performed a linear regression analysis. As the probe concentration of the control (T1) was 0 ng/µl, we added 0.1 to all the concentrations before $\log_e$-transformation. In this analysis seminal vesicle size was also $\log_e$-transformed to improve the distribution of the data. Note that the previous analysis confirms that the RNAi treatment worked as intended, by testing whether the increase of seminal vesicle size (our estimate of sperm-production rate) is different between dsRNA doses, while the analysis conducted here

describes how the final phenotype of the focals in the sperm competition experiment was affected as a function of dsRNA doses, to then include those phenotypes as predictors in the following analysis to examine the fitness gain curve.

Thirdly, we tested the shape of the fitness gain curve. Most models of sex allocation theory use power functions to describe the fitness gain curves (e.g., Charnov, 1982), because power functions allow one to describe a great range of shapes (e.g., saturating, linear, and accelerating) by changing a single parameter and they are thus convenient to handle mathematically; but there is no specific biological reason to use power functions (Schärer, 2009). Therefore, fitting power functions is not the only way to examine fitness gain curves, and nonlinearity has often been examined by fitting linear regressions with a quadratic term (e.g., Broyles and Wyatt, 1990; Devlin et al., 1992; Schoen and Stewart, 1986) instead of a power function (e.g., Campbell, 1998; McCartney, 1997; Rosas and Domínguez, 2009; Yund, 1998). Here we fitted generalized linear models (GLMs) with a quadratic term, using a logit link function, where a significant negative quadratic term would indicate saturation in fitness returns. Logistic regression-based GLMs have become the standard statistical tool for handling proportional data such as brood sex ratios, hatching success, and paternity success (Krackow and Tkadlec, 2001; Wilson and Hardy, 2002), especially if the data include many zeroes and ones, as is the case in our data set. Because our data were overdispersed we used quasi-GLMs, in which the variances are corrected by the degree of overdispersion (Wilson and Hardy, 2002; Zuur et al., 2009). For model selection, we first fitted a full model including all morphological or behavioral traits that were affected by our RNAi treatment (see Results), in addition to the copulation frequency of the competitors, which could potentially also have influenced the paternity success. Thus the full model contained the seminal vesicle size (at the third measurement), the quadratic term of seminal vesicle size (to test for saturation), the copulation frequency of the focals, and the copulation frequency of the competitors. Then, by removing non-significant parameters one by one from the full model, we selected the reduced model containing only the parameters with significant effects on the paternity success. To statistically test the effects of these terms in the reduced model, we then performed an analysis of deviance with F-statistics, as recommended when using a quasi-GLM (Crawley, 2005; Zuur et al., 2009).

Initial sample size was n = 168 (i.e., 24 replicates for each of the 7 dsRNA doses), but 21 worms had to be excluded because they did not mature or died during the experiment (losses were balanced over the treatment groups). Thus, to confirm that different dsRNA doses led to different sperm-production rates and fill grades of the seminal vesicle, 147 individuals were used. To describe the phenotype of the focals at the time of the sperm competition experiment (i.e., the examination of the effect of dsRNA doses on the morphological traits at the third measurement and the

copulation frequency of the focals) and to describe the resulting fitness gain curve, a further 81 individuals had to be excluded and the final sample size was n = 66 (i.e., T1: n = 8, T2: n = 11, T3: n = 10, T4: n = 12, T5: n = 8, T6: n = 10, T7: n = 7). Specifically, 4 individuals were lost during the sperm competition experiment, 60 were excluded because either the focal or its competitor did not copulate in the competition experiment, and 17 were excluded because the recipients produced none or only one offspring.

# Results

At the highest dose, *macbol1* RNAi generally caused an empty seminal vesicle (electronic supplemental material, figure S2) as previously described (Kuales et al., 2011). Based on the size of the seminal vesicle, our data show that the sperm-production rate was strongly affected by the dose-dependent RNAi treatment, as indicated by the significant treatment and time effects on seminal vesicle size of the focals between the second and third measurement (figure 1a, repeated-measures ANOVA: treatment, $F_{6, 140} = 31.41$, $P < 0.0001$; time, $F_{1, 140} = 24.05$, $P < 0.0001$; time*treatment, $F_{6, 140} = 11.41$, $P < 0.0001$). Specifically, seminal vesicle size was significantly lower at higher dsRNA doses and generally tended to increase over time due to the accumulation of produced sperm, but this increase was seen only at the lower RNAi doses. The frequencies of the different fill grades of the seminal vesicle also differed significantly between the treatment groups (figure 1b), in that the worms at the higher doses had less filled seminal vesicles much more often (Pearson's chi-square test: chi-square = 99.47, d.f. = 24, $P < 0.0001$).

The dsRNA dose significantly affected the final seminal vesicle size at the third measurement (figure 2a), presumably as a result of different sperm-production rates (figure 1a). We did not find any significant effects of the dsRNA dose on either body, testis, or ovary size (linear regressions: body size, slope = 185.3, t = 0.025, P = 0.98, $R^2 < 0.01$; testis size, slope = 676.2, t = 1.72, P = 0.090, $R^2 = 0.04$; ovary size, slope = -173.1, t = -0.59, P = 0.56, $R^2 < 0.01$). Unexpectedly, the dsRNA dose also affected copulation frequency of the focals (figure 2b) and this leads to a significant correlation between the seminal vesicle size and the copulation frequency of the focals (figure 2c). However, as this correlation only explained 18% of the variation, we decided to include both parameters in the subsequent analyses.

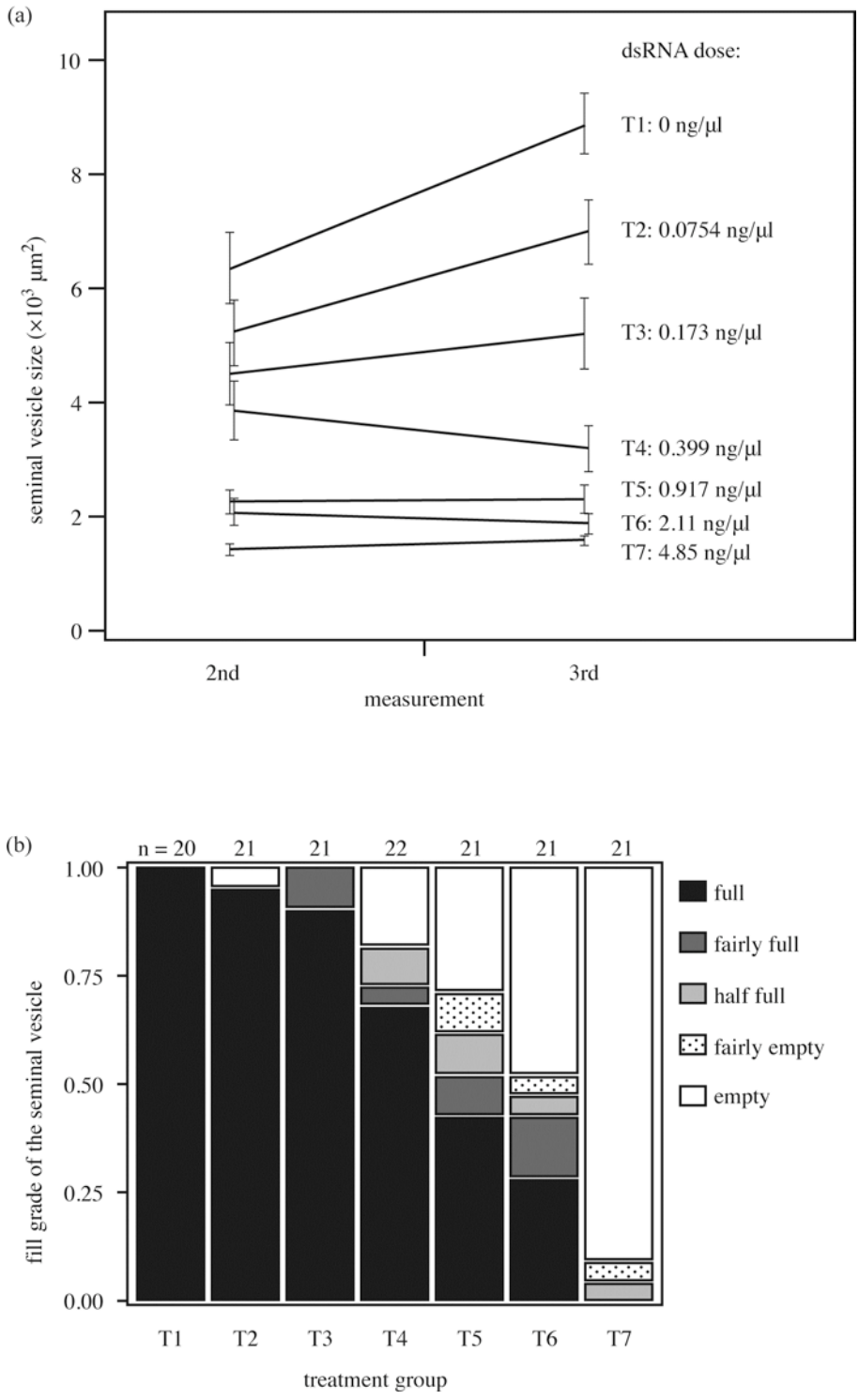


**Figure 1: Effect of dsRNA dose on the sperm production.** (a) The change in seminal vesicle size from the second to the third measurement. Seminal vesicle size at lower dsRNA doses increased more quickly than at higher doses. Error bars indicate standard errors. (b) The fill grade of the seminal vesicle at the third measurement. At lower dsRNA doses the seminal vesicle was completely filled with sperm, while at higher doses the seminal vesicle contained fewer sperm significantly more often. n = 147. (See section "Results" for statistical analyses.)

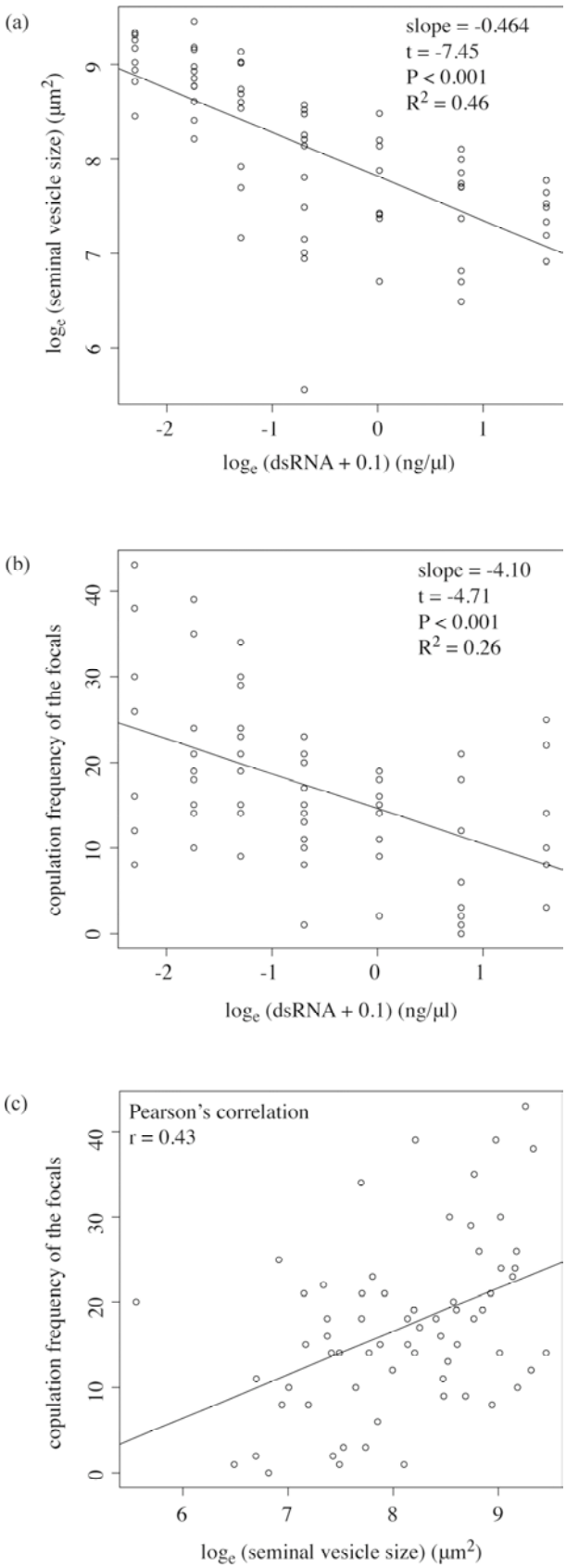


**Figure 2: Effects of dsRNA dose on final seminal vesicle size and copulation frequency.** (a) The seminal vesicle size of the focals at the third measurement. (b) The copulation frequency of the focals during the sperm competition experiment. (c) The correlation between the seminal vesicle size and the copulation frequency of the focals. n = 66. (See section "Results" for statistical analyses.)

To investigate the fitness gain curve resulting from the phenotypically engineered sperm-production rates, we first fitted the full model (see full model in table 1). By then sequentially dropping non-significant terms we obtained a reduced model with only the linear and the quadratic terms of seminal vesicle size (see reduced model in table 1, and figure 3), suggesting that seminal vesicle size is the most important predictor for gaining paternity success. The model containing the quadratic term explained the data significantly better (analysis of deviance: linear term, difference in deviance = 131.7, $F_{1,64} = 52.7$, $P < 0.001$; quadratic term, difference in deviance = 10.8, $F_{1, 63} = 4.33$, $P = 0.042$). Given that the estimated coefficient of the quadratic term in the reduced model was negative (see table 1), this suggests that the odds of paternity success increased significantly with seminal vesicle size, but that this increase gradually decelerated. In other words, the seminal vesicle size had a positive relationship with paternity success, as predicted by sperm competition theory, but this effect was saturating due to a non-linear effect of sperm-production rate, as predicted by sex allocation theory.

**Table 1.** Model selection of the effects of the linear and quadratic terms of the seminal vesicle size, and the copulation frequency of the focals and the competitors on paternity success.

| source | estimate | s.e. | t-value | residual deviance | dispersion parameter |
|---|---|---|---|---|---|
| **full model** | | | | | |
| intercept | -2.76 | 0.836 | -3.30*** | 151.42 | 2.31 |
| seminal vesicle size | 9.82E-04 | 2.80E-04 | 3.50*** | | |
| (seminal vesicle size)$^2$ | -4.85E-08 | 2.28E-08 | -2.13* | | |
| copulation frequency focals | 0.0366 | 0.0276 | 1.33 | | |
| copulation frequency competitors | -0.0340 | 0.0248 | -1.37 | | |
| **reduced model** | | | | | |
| intercept | -2.99 | 0.712 | -4.20*** | 161.80 | 2.50 |
| seminal vesicle size | 0.00110 | 2.94E-04 | 3.76*** | | |
| (seminal vesicle size)$^2$ | -5.47E-08 | 2.43E-08 | -2.25* | | |

$P < 0.05$ (*) and $P < 0.001$ (***).

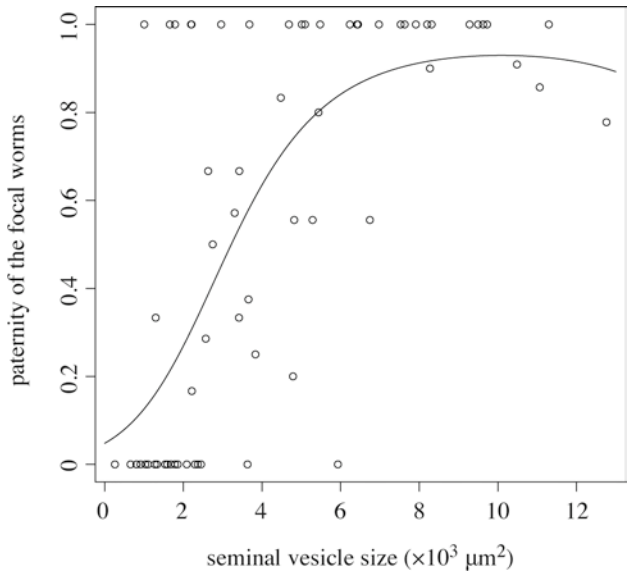


**Figure 3: Fitness gain curve describing the relationship between seminal vesicle size and paternity success.** The reduced model with both the linear and quadratic terms of seminal vesicle size was used. n = 66. (See table 1 for statistical analyses.)

# Discussion

In this study, we (i) successfully manipulated the sperm-production rate in the free-living flatworm *M. lignano* using a dose-dependent RNAi gene knock-down approach, (ii) found experimental support for a positive effect of sperm-production rate on paternity success, as predicted by sperm competition theory, and (iii) provide experimental evidence for a saturating male fitness gain curve in a copulating simultaneously hermaphroditic animal, as predicted by sex allocation theory. In the following we discuss these three findings in more detail.

### (a) Phenotypic engineering of sperm-production rate

Our novel experimental approach succeeded in producing the expected outcome of a change in male allocation towards sperm production, namely different sperm-production rates, as reflected by different rates of increase in the seminal vesicle size over time at different dsRNA doses. In addition, our approach allowed us to obtain a very broad range of variation in the number of sperm available to sperm donors (estimated as variation in seminal vesicle size). Such broad variation may be more difficult to achieve with other approaches, such as artificial selection (e.g., Johnson et al., 1994; Rathje et al., 1995), experimental evolution (Hosken et al., 2001; Hosken and Ward, 2001; Pitnick et al., 2001), or phenotypic plasticity (Schärer and Ladurner, 2003; Schärer and Vizoso, 2007; Tan et al., 2004; reviewed in Schärer, 2009).

The RNAi treatment could potentially also have affected traits other than the target trait of sperm production that we focused on in this study. While our data suggest that none of the other morphological traits was significantly affected by the RNAi treatment, testis size in fact tended to be larger at higher doses. As the *macbol1* gene functions during meiosis, it seems that the premeiotic proliferation of the male germ line was not halted by the RNAi treatment, leading to some accumulation of malformed sperm in the testis, presumably because immature or malformed sperm are not transferred to the seminal vesicle (Kuales et al., 2011). One might have expected that targeting a testicular gene would lead to a smaller testis size, which we clearly did not observe here. To achieve this one may have to target earlier-acting testicular genes. Unexpectedly, the dsRNA dose affected the copulation frequency, with worms at lower doses (and thus with larger seminal vesicles) copulating more frequently. However, considering that the *macbol1* gene shows testis-specific expression and acts during male meiosis, it seems unlikely that its decreased expression can directly affect mating behavior. The observed changes in copulation frequency are therefore more likely an indirect effect associated with sperm production, possibly linked to the fill grade of the seminal vesicle, as has, for example, been suggested for the fill grade of the prostate gland in a snail (Koene, 2006; Koene and Ter Maat, 2005). Moreover, our analyses suggest that copulation frequency here did not significantly contribute to paternity success (see table 1). Finally, we cannot exclude the possibility that *macbol1* RNAi affected other unmeasured traits of sperm competitiveness such as sperm morphology, mobility or viability. It appears, however, that fertilization ability was not strongly affected, because in each treatment group, including the highest dsRNA dose, there were always some individuals that successfully sired offspring (including one individual in the highest dose and more than 3 individuals in every other dose).

In summary, phenotypic engineering via dose-dependent RNAi allows us to manipulate a specific trait, to create novel variation quantitatively, and to disentangle the fitness effect of the trait in question from other traits. Dose-dependent RNAi may therefore be a powerful approach to study, not only the consequences of variation in sperm production, but also other traits that may otherwise be difficult to manipulate quantitatively Tatar, 2000, such as physiological traits.

**(b) Higher sperm-production rate increases paternity success**

We experimentally demonstrated that sperm-production rate had a positive effect on paternity success. Sperm competition is a common phenomenon in many species and an important aspect of post-copulatory sexual selection (Birkhead and Pizzari, 2002; Parker, 1998). Although many comparative studies have suggested that an increased level of sperm competition favors an increased expenditure on sperm production, as assessed by testis size (e.g., Ginsberg and Rubenstein, 1990; Harcourt

et al., 1981; Møller, 1991; Møller and Briskie, 1995; Pitcher et al., 2005; Stockley et al., 1997), only few studies have shown directly that a higher allocation into sperm production is actually beneficial for paternity success within a single species. For example, it has been shown that the testicular circumference of Soay sheep (Preston et al., 2003) and external testis length of yellow-pine chipmunk (Schulte-Hostedde and Millar, 2004), both proxies for testis size, are positively correlated with paternity success. However, these results may need to be interpreted with some caution, given the fact that the testes of mammals also produce male hormones that can affect other male traits, such as behavior or ornaments (e.g., Moreira et al., 1997). Moreover, very few studies have manipulated sperm production experimentally to look at its effect on the sperm competitiveness. In some studies testis size was manipulated indirectly, namely as a result of an experimental evolution regime (i.e., monogamous and polygamous), and it was shown that males from polygamous lines have larger testes and sire more offspring than monogamous lines in the yellow dung fly (Hosken et al., 2001), while in a fruit fly the smaller testis of monogamously selected males did not in fact lead to a consistent reduction in competitiveness (Pitnick et al., 2001). However, experimental evolution could potentially lead to changes in other traits influencing paternity success, which could confound the actual fitness effects of the trait in question. In our study we directly manipulated one particular gene involved in spermatogenesis in individuals that otherwise had an identical genetic background (see section "Materials & Methods"), which have been suggested as a powerful way of studying traits (e.g., Tatar, 2000). Our results represent some of the most direct evidence that higher allocation to sperm production is indeed beneficial to increase sperm competitiveness and to achieve higher paternity success in copulating animals. Our technique offers considerable promise to test more quantitative aspects of sperm competition theory.

**(c) Evidence for a saturating male fitness gain curve**

So far empirical studies on the shapes of the male fitness gain curve have mainly focused on plants (e.g., Rosas and Domínguez, 2009; reviewed in Campbell, 2000), in which hermaphroditism is the most common reproductive system (Jarne and Auld, 2006; Renner and Ricklefs, 1995). In contrast, simultaneously hermaphroditic animals have rarely been studied, which is unfortunate given that hermaphroditism is also very widespread among animals (Jarne and Auld, 2006; Schärer, 2009). We are aware of studies on only two species of sperm-casting sessile marine invertebrates, in which male gametes are passively dispersed to sessile mates in a plant-like mating system (Bishop and Pemberton, 2006; Pemberton et al., 2004), and there are so far no studies in any of the many copulating simultaneous hermaphrodites (reviewed in Schärer, 2009). In a colonial ascidian a saturating male fitness gain curve has been

shown (Yund, 1998), at least under certain conditions, while a linear male fitness gain curve has been found in a colonial bryozoan (McCartney, 1997; Yund and McCartney, 1994). In both studies the authors used standing variation in testis size or male zooid number (as proxies for male allocation towards sperm production), respectively (McCartney, 1997; Yund, 1998), and male allocation was thus not experimentally manipulated. Thus our study is the first to experimentally manipulate sperm-production rate (as a proxy for male allocation towards sperm production) to show a saturating male fitness gain curve in simultaneous hermaphroditic animals.

The shape of the male fitness gain curve is predicted to vary as a function of the level of sperm competition (Charnov, 1982; Schärer, 2009). When sperm competition is weak, for example due to a small mating group size (Charnov, 1980; Fischer, 1981), high selfing rate (Charlesworth and Charlesworth, 1981; Charnov, 1987), efficient sperm displacement (Charnov, 1996), or cryptic female choice (Van Velzen et al., 2009), sex allocation theory predicts a saturating male fitness gain curve (Charnov, 1979; Petersen, 1991; Schärer, 2009). In contrast, when sperm competition is strong and approaches a fair raffle, an individual continues to obtain benefits from increasing male allocation to overcome sperm competition, resulting in a less saturating and more linearized male fitness gain curve (Charnov, 1979; Petersen, 1991; Schärer, 2009). In our experiment the focals always competed with sperm from one competitor only and they were thus exposed to relatively weak sperm competition (see section "Materials & Methods"). In this situation the observed male fitness gain curve was clearly saturating (i.e., showed diminishing returns for increasing investment), as predicted by sex allocation theory. This finding suggests that selection should favor a low male allocation when sperm competition is weak, since higher allocation would not provide additional fitness returns after a certain amount of investment due to increasing fruitless competition among related sperm (termed local sperm competition by Schärer, 2009). This is supported by the fact that *M. lignano* has a small testes and low sperm-production rate when grown up in a small mating group, while growing up in a large mating group leads to a phenotypically plastic increase in these traits (Janicke and Schärer, 2009b; Schärer and Ladurner, 2003; Schärer et al., 2005; Schärer and Vizoso, 2007). The presence of such phenotypic plasticity in *M. lignano* suggests that the level of sperm competition that a worm experiences might indeed be variable under field conditions and selection has thus favored plasticity over a fixed evolutionarily stable sex allocation. In future studies it would be important to examine whether the male fitness gain curve in *M. lignano* becomes linearized with increasing sperm competition, as has been shown for the colonial ascidian (Johnson and Yund, 2009; Yund, 1998), or whether it retains a saturating shape also in large mating groups. The latter might occur because in the case of copulating animals, different processes of post-copulatory sexual selection,

such as efficient sperm displacement or cryptic female choice, have been predicted to weaken the effect of sperm competition (Charnov, 1996; Pen and Weissing, 1999; Schärer, 2009; Van Velzen et al., 2009). This might suggest that a saturating gain curve is retained even in situations in which sperm competition would intuitively appear to be strong (e.g., at high population density).

**(d) Conclusions**

We successfully manipulated sperm-production rate using dose-dependent RNAi, and showed that higher sperm-production rate is beneficial to achieve a higher paternity success, as predicted by sperm competition theory. Moreover, we showed that the male fitness gain curve is indeed saturating in a situation of relatively weak sperm competition, as predicted by sex allocation theory. Our study provides much needed evidence on fitness gain curves in copulating simultaneous hermaphrodites and shows that phenotypic engineering of sperm-production rate allows us to perform detailed quantitative experimental studies on predictions of evolutionary theory for fitness returns of variation in male allocation.

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# Electronic Supplementary Materials

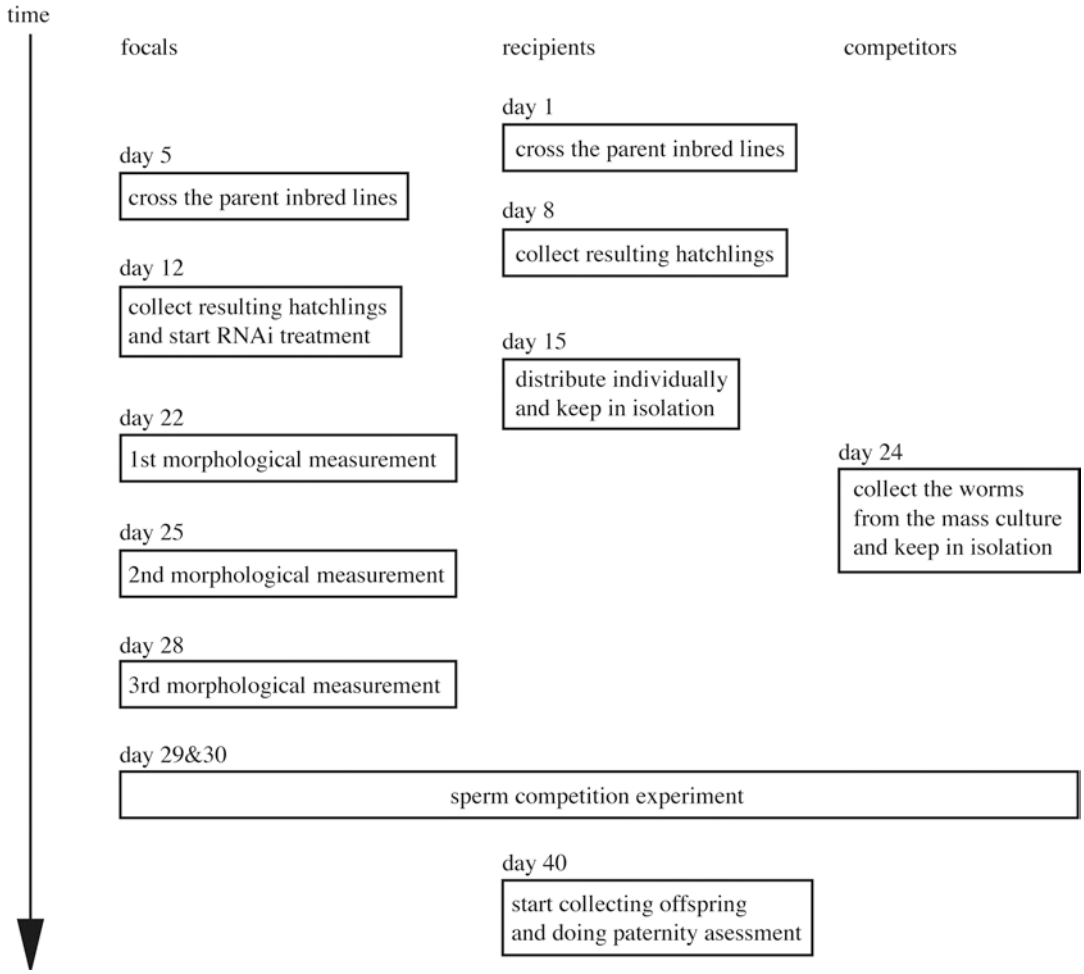


**Figure S1: Timeline of the entire experiment.**

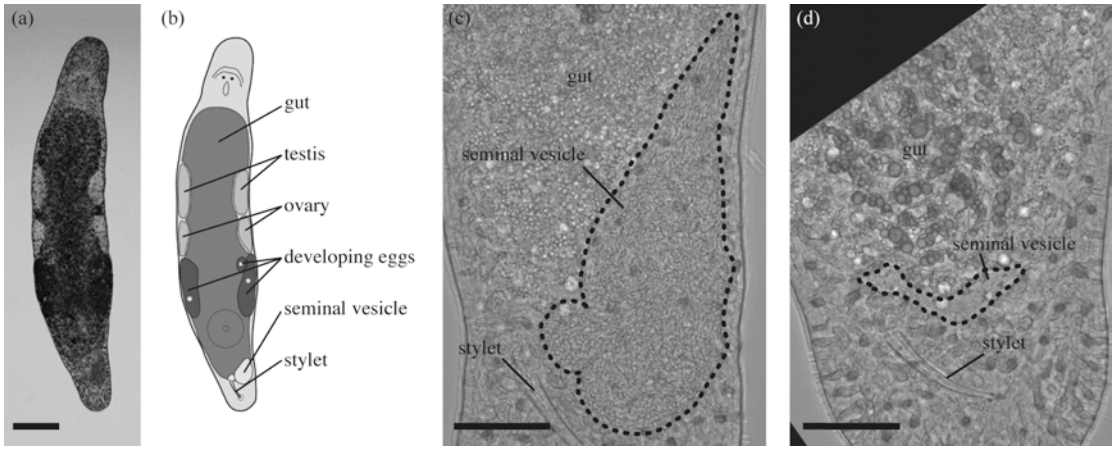


**Figure S2: The seminal vesicle of *M. lignano* in *macbol1* RNAi.** (a) Image of *M. lignano* and (b) corresponding schematic drawing. (c) Image of the seminal vesicle of a control animal filled with sperm. (d) Representative image of an empty seminal vesicle of a focal at the highest dsRNA dose. The images were taken during the third measurement. Scale bars: (a) 200 µm; (c, d) 50 µm.

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# CHAPTER IV

## Experimental suppression of male function leads to increased female allocation in a simultaneous hermaphrodite

**Manuscript in preparation**

# Experimental suppression of male function leads to increased female allocation in a simultaneous hermaphrodite

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## Abstract

Trade-offs are an important concept in evolutionary biology, since they impose constraints on phenotypic evolution. In sex allocation theory for simultaneous hermaphrodites, a trade-off between male and female reproductive allocation is a central and almost universal assumption, stating that an increase in resource investment into one sex function necessarily leads to a decrease in the investment into the other sex function. To maximize overall fitness, individuals therefore need to optimally partition their reproductive resources into each sex function within this constraint. Despite the central importance of this assumption, empirical evidence for the existence of a sex allocation trade-off is surprisingly scarce and often controversial, both among animals and plants. In this study we verified the trade-off assumption by experimentally manipulating the male function and by examining the consequences for the female function in the free-living flatworm *Macrostomum lignano*. To manipulate the male function we disrupted the expression of a testis-specific candidate gene, recently identified in a planarian flatworm, using RNA interference (RNAi). We show (i) that the *Macrostomum* homolog of this gene, *mac-C3H-zfn* is also specifically expressed in the testis, (ii) that RNAi of *mac-C3H-zfn* successfully hinders sperm production, and (iii) that this manipulation leads to an increased female allocation, as assessed by ovary size. Our results therefore provide direct experimental evidence for the presence of a trade-off between male and female reproductive allocation.

# Introduction

The concept of trade-offs has been a central focus in evolutionary biology because trade-offs can constrain the evolution of phenotypic traits and therefore affect evolutionary trajectories. In order to predict optimal sex allocation in simultaneous hermaphrodites, sex allocation theory generally assumes a trade-off between male and female allocation (e.g., Charnov, 1979; Charnov, 1982; Charnov, 1996; Pen and Weissing, 1999; reviewed in Schärer, 2009). Specifically, an increase in resource investment into one sex function is assumed to lead to a proportional decrease in the other sex function. In order to maximize their overall fitness individuals therefore have to optimally allocate their limited reproductive resources into the male and female functions within the constraint imposed by this sex allocation trade-off.

However, despite the central role of this assumption in most sex allocation models, empirical evidence for the sex allocation trade-off is surprisingly scarce and controversial in both animals (reviewed in Schärer, 2009) and plants (reviewed in Campbell, 2000). For example, a positive correlation between male and female allocation is often reported (e.g., Ågren and Schemske, 1995; Campbell, 1997; Locher and Baur, 2000; O'Neil and Schmitt, 1993; Schärer et al., 2005), instead of the expected negative correlation (e.g., Garnier et al., 1993; Yund et al., 1997). On the one hand one might consider the lack of negative correlation as evidence against the trade-off assumption, for example, because there may be no strong resource competition if male and female reproductions rely on different resource pools. This may, for example, occur due to temporal separation of investment (e.g., substantial female investment into maturation of seeds and fruits after male allocation to fertilization has effectively ceased in plants) or due to different limiting nutrients for producing male and female gametes (Charnov et al., 1976; Geber and Charnov, 1986; Schärer, 2009). But on the other hand, the generally acknowledged difficulties in detecting phenotypic trade-offs may be a more likely explanation. For example, trade-offs can be obscured if there is variation in resource acquisition ability among individuals (Schärer et al., 2005; van Noordwijk and De Jong, 1986), variation at different levels in the resource allocation hierarchy (de Laguerie et al., 1991), and phenotypic plasticity in resource allocation (Malausa et al., 2005). Therefore, the observation of a positive or non-significant correlation needs to be interpreted with some caution, and does not necessarily refute the existence of the trade-off.

An alternative way of examining the trade-offs is the experimental manipulation of the traits of interest (Schärer, 2009). In a number of studies the presence of a trade-off could be inferred from a change in the resource allocation to the female function

as a response to changes in the male function resulting from different levels of sperm competition (e.g., Janicke and Schärer, 2009b; Schärer et al., 2005; Trouvé et al., 1999, but see Schärer and Ladurner, 2003; Tan et al., 2004). In this scenario, individuals are expected to allocate more resources into the male function in larger mating groups in response to the increased sperm competition that growing in larger groups entails (Janicke and Schärer, 2009a; Schärer and Wedekind, 2001), and, given the trade-off assumption, the female allocation is expected to decrease. The disadvantage of this approach, however, is that such mating group size manipulation could potentially affect many other traits, and the observed results might be due to the independent response of male and female function to different environmental conditions, not necessarily revealing life-history trade-offs. For example, the change in mating group size may also affect mating frequency and the amount of seminal fluids received in individuals. If received seminal fluids have a suppressing effect on the female function (e.g., Koene et al., 2009), the decrease in female allocation in larger mating group sizes may not be due to the allocation trade-off, but due to the negative effects of the received seminal fluids. Therefore a more powerful approach would be to directly manipulate specific traits without changing the environment, for example, by manipulating resource allocation via prevention of the investment towards one sex function and examining resulting changes in the other sex function (Schärer, 2009).

In this study we experimentally manipulated sperm production, a key trait of the male reproductive function, and examined whether this manipulation leads to an increased female allocation. We used the simultaneously hermaphroditic free-living flatworm *Macrostomum lignano*, an excellent model organism for developmental and evolutionary biology that allows us to employ powerful experimental approaches to study reproductive allocation (e.g., De Mulder et al., 2009; Janicke and Schärer, 2009b; Kuales et al., 2011; Schärer et al., 2005; Sekii et al., 2009; Vizoso and Schärer, 2007). We applied RNA interference (RNAi) to a testis-specific gene that was identified as a homologous gene from a recent functional genomics screen of another free-living flatworm species, a freshwater planarian (Wang et al., 2010). Here we show (i) that the *M. lignano* homologue of this gene is also specifically expressed in the testis, (ii) that RNAi knock-down of this gene hinders sperm production, and (iii) that this manipulation results in an increased female allocation, as assessed by ovary size, thus providing experimental support for the sex allocation trade-off.

# Materials & Methods

### Study organism

The free-living flatworm *Macrostomum lignano* (Macrostomida, Macrostomorpha, Platyhelminthes) is a simultaneous hermaphrodite and a member of the interstitial sand fauna of the Northern Adriatic Sea (Ladurner et al., 2005). The worms are transparent and thus non-invasive measurements of different morphological traits, such as the size of the body, testis, ovary, and seminal vesicle are possible (Schärer and Ladurner, 2003). Reproduction is purely by outcrossing (Schärer and Ladurner, 2003). In the laboratory, we maintain these worms in a nutrient-enriched artificial seawater (f/2 medium, Andersen et al., 2005) in glass petri dishes, feed them *ad libitum* with the diatom *Nitzschia curvilineata*, and keep them at 20°C on a 14:10 h day-night cycle (Rieger et al., 1988). Under these conditions, generation time is about 18 days: 5 days from egg laying to hatching and 13 days from hatching to adult (Schärer and Ladurner, 2003). For the experiment we used worms from a genetically diverse mass culture called LS1.

### Gene isolation and protein domain search

For our experimental approach we used the *mac-C3H-zfn* gene, which was identified by a candidate gene approach using information from a recent functional genomic screen of germ cell development in the freshwater planarian *Schmidtea mediterranea* (Tricladida, Platyhelminthes) (Wang et al., 2010), from a free-living flatworm order distantly related to the Macrostomida. Wang et al. (2010) identified the gene *Smed-C3H-zfn-1* (GenBank accession number BK007101) as being involved in spermatogenesis (and specifically spermatid elongation). We performed a BLAST search (tblastn) of this gene against the available transcriptome data in *M. lignano* (http://www.macgenome.org/), and obtained a single highly conserved homologue (RNA918_7605 in assembly version MLRNA100918) as the first hit with the e-value 3e-70 (while the second hit was only 5e-05 and not investigated).

Based on the obtained sequence information we designed *mac-C3H-zfn* specific primers, 5'-GAC CGT CAA TCC AAG AGA GC-3' and 5'-TTG TGG CCG TTT CAA TCT C-3', and isolated the DNA fragment of *mac-C3H-zfn* from cDNA. cDNA was synthesized from total RNA using the High Capacity RNA-to-cDNA kit (Applied Biosystems). The PCR condition was 2 min at 94°C, 10 cycles (30 sec at 94°C, 30 sec 60°C, 90 sec at 72°C), 20 cycles (30 sec at 94°C, 30 sec 55°C, 90 sec at 72°C), and 7 min at 72°C.

We performed a protein sequence alignment between *mac-C3H-zfn* and *Smed-C3H-zfn-1* using the ClustalW2 (http://www.ebi.ac.uk/Tools/msa/clustalw2/). To identify conserved protein domains, we performed computational analysis of the *mac-*

*C3H-zfn* protein sequence using the SMART databases (http://smart.embl-heidelberg.de/) (Letunic et al., 2006).

**Whole mount *in situ* hybridization**

In order to study tissue specificity using *in situ* hybridization we amplified the probe region of the *mac-C3H-zfn* by PCR using primers 5'-GAC CGT CAA TCC AAG AGA GC-3' and 5'-TAA TAC GAC TCA CTA TAG GTT GTG GCC GTT TCA ATC TC-3'. The PCR condition was 2 min at 94°C, 22 cycles (30 sec at 94°C, 30 sec 65°C, 90 sec at 72°C), and 7 min at 72°C. The resulting PCR products had the *mac-C3H-zfn* specific sequence with T7 promoters at the 3' end, and were used as a template for *in situ* probe synthesis. Digoxygenin (DIG)-labeled anti-sense *in situ* probe was synthesized *in vitro*, using DIG RNA Labeling Mix (Roche) and T7 RNA polymerase (Roche). We estimated the concentration of *in situ* probe using the Quant-iT RNA Assay kit (Invitrogen) and a fluorometer (Qubit® 2.0, Invitrogen).

We performed whole mount *in situ* hybridization for adult worms as previously described (Pfister et al., 2008). The final concentration of *in situ* probe used was 0.1 ng/µl. The signal was developed at room temperature using the NBT/BCIP system (Roche). We examined specimens and took the images using a Leica DM 2500 microscope (Leica Microsystems), a digital video camera (DFK 41BF02, The Imaging Source Europe), and the software BTV Pro 6.0b1 (http://www.bensoftware.com/).

**RNAi treatment**

The dsRNA probe for the RNAi treatment was synthesized *in vitro*, using the T7 RiboMax™ Express RNAi System and the T7 RiboMax™ Express Large Scale RNA production System (both kits from Promega). We amplified the probe region of the *mac-C3H-zfn* by PCR using primers 5'-TAA TAC GAC TCA CTA TAG GGA CCG TCA ATC CAA GAG AGC-3' and 5'-TAA TAC GAC TCA CTA TAG GTT GTG GCC GTT TCA ATC TC-3'. The PCR condition was 2 min at 94°C, 22 cycles (30 sec at 94°C, 30 sec 65°C, 90 sec at 72°C), and 7 min at 72°C. The resulting PCR products had the *mac-C3H-zfn* specific sequence with T7 promoters at both ends, and were used as a template for dsRNA synthesis, producing sense and anti-sense RNA. As a negative control treatment we used dsRNA of a gene that does not occur in *M. lignano*, namely firefly *luciferase*, to control for possible unspecific effects of exposing worms to dsRNA, a standard procedure in RNAi experiments (De Mulder et al., 2009; Pfister et al., 2008; Sekii et al., 2009). *Luciferase* dsRNA was produced from the pGEM®-luc Vector (Promega). We estimated the concentration of the dsRNA probes both using a spectrophotometer (NanoDrop-1000 v.3.7.1, Thermo Scientific) and from the image of a gel electrophoresis in comparison with a DNA

standard (Quick-Load 100 bp DNA Ladder, BioLabs), in which the amount of DNA in each band is known. As the difference between the two estimates was within a two-fold margin, we took the estimation from the gel electrophoresis image, which is a recommended protocol in the kit used.

Hereafter we call the worms exposed to the *mac-C3H-zfn* dsRNA the focals, and the worms exposed to the *luciferase* dsRNA the controls. We started RNAi treatment within 1 day posthatching. During the RNAi treatment, the focals and the controls were kept individually in 60-well microtest plates (Greiner Bio One). The positions on the plate were spatially balanced for the treatments. We kept the worms in 10 µl of dsRNA solution, which is f/2 medium containing 4.1 ng/µl of dsRNA probe, 50 µg/ml of Kanamycin and Ampicillin, and an *ad libitum* amount of diatoms (Pfister et al., 2008; Sekii et al., 2009). We transferred the worms into new wells with fresh dsRNA solution every 24 hours.

**Morphological measurements**

When the worms were 14 or 15 days posthatching, we measured the size of body, testis, ovary, and seminal vesicle. Having grown up in isolation, the size of the seminal vesicle is expected to reflect the complete sperm production of these worms up to that point. The measurements were performed as previously described (Schärer and Ladurner, 2003). Briefly, the worms were relaxed with a solution of $MgCl_2$ (a mixture of 7.14% $MgCl_2$ and f/2 medium in a ratio of 5:3) and squeezed dorsoventrally to a thickness of 35 µm between a microscope slide and a hemacytometer coverslip. We took the images of the worms and organs as described above (see section "Whole mount *in situ* hybridization"). The images for body size were taken at 40× magnification and the images for testis, ovary, and seminal vesicle size were taken at 400× magnification. We then measured the area of these traits using the software ImageJ 1.37v (http://rsb.info.nih.gov/ij/). In a few cases worms were laying such that images of the testes or ovaries could only be obtained for one side (but we could visually confirm that these organs were present). In this case we estimated total testis or ovary size by doubling the value of the available side (in one focal and one control for testis size, and in two focals for ovary size).

**Statistical analyses**

We performed all statistical analyses using JMP 8.0.2 (SAS Institute). To compare the size of traits between the focals and the controls we either performed ANOVA (i.e., for body size) or ANCOVA for the traits that were positively related to body size (i.e., for testis, ovary, and seminal vesicle size) using body size as a covariate. To investigate the trade-off between male and female function, we addressed two main questions, namely (i) whether the RNAi treatment was successful

at reducing the investment into male function, and (ii) whether it led to increased investment into the female function. We initially started with 30 replicates for each RNAi treatment. After excluding worms that were either lost or treated wrongly during the experiment (3 focals and 2 controls), were immature at the time of measurement (12 focals and 15 controls), or had an incomplete measurement data set (2 focals and 1 control), the final sample size was n = 13 for the focals and n = 12 for the controls (the losses were balanced over the treatment groups).

# Results

The SMART program (Letunic et al., 2006) for identification and annotation of protein domains suggested that *mac-C3H-zfn* has two C3H-type zinc finger domains (i.e., matching ZnF_C3H1, SMART accession number SM00356), as also observed in *Smed-C3H-zfn-1* (supplementary figure 1). The e-values of the first and second domain in *mac-C3H-zfn* were relatively low, 2.43e-04 and 5.99e-04, respectively. In both domains, however, a unique conserved structure is found (see supplementary figure 1). To study *mac-C3H-zfn* gene expression, we performed whole-mount *in situ* hybridization in adult animals. We found strong expression of this gene exclusively in the testis (figure 1a), especially in the center of the testis (figure 1b), but no expression in other organs, including the ovary and developing eggs (figure 1a).

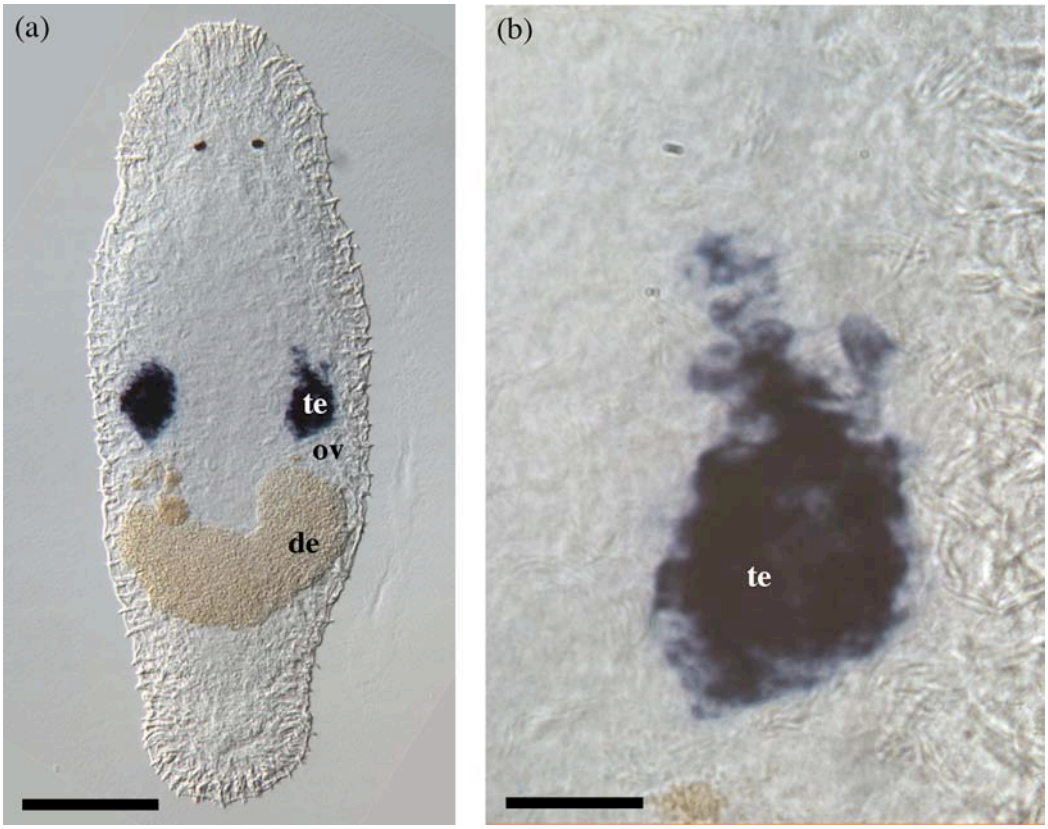


**Figure 1: Testis-specific gene expression pattern of the *mac-C3H-zfn* gene.** (a) Whole-mount *in situ* hybridization of the *mac-C3H-zfn* gene on the entire animal. Strong expression (dark color) is observed only in the testis. (b) Detail of the testis. te, testis; ov, ovary; de, developing egg. Scale bars: (a) 100 µm; (b) 20 µm.

The results of the RNAi knock-down suggested that *mac-C3H-zfn* is essential for spermatogenesis in *M. lignano* (figure 2). We did not find any severe overall effects in either the focals (figure 2a) or the controls (figure 2b). However, the testes of the focals were often brownish and contained no elongated sperm (figure 2c), while we observed normally elongated sperm in the testes of the controls (figure 2d). Moreover, the focals generally had an empty seminal vesicle (figure 2e), while that of the controls was always full of sperm (figure 2f). In contrast, the general appearance of the ovaries of both the focals (figure 2g) and the controls (figure 2h) appeared to be completely normal and unaffected by the RNAi treatment. These observations suggest that *mac-C3H-zfn* is essential for the male function, but not for female function, which is consistent with the testis-specific expression pattern of the *mac-C3H-zfn* gene (figure 1). The effect of RNAi knock-down was also examined by doing *in situ* hybridization and we visually confirmed that the testis-specific signal of the focals disappeared after the RNAi treatment (data not shown).

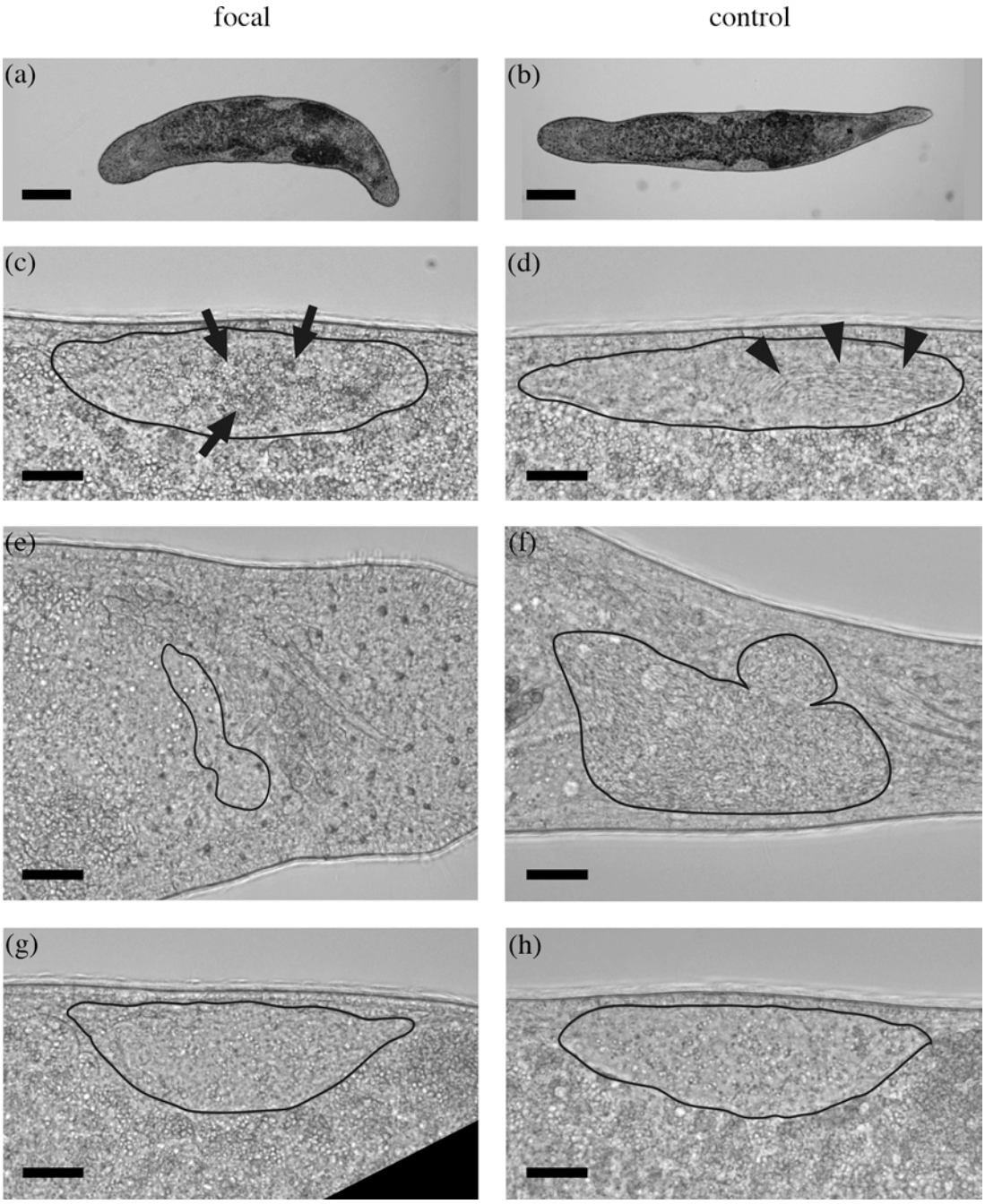


**Figure 2: Phenotypes of the *mac-C3H-zfn* RNAi knock-down.** Overall, the focal (a) and the control (b) showed a very similar morphology. However, the testis of focals (c) were often brownish and did not contain elongated sperm (shown by the arrows), while the testis of the control (d) contained normally elongated sperm (shown by the arrowheads). Moreover, the seminal vesicles of the focals (e) were generally empty, while the seminal vesicles of the controls were always filled with sperm. The general appearance of the ovaries of the focals (g) and the controls was normal, although they differed in mean size (see main text). Scale bars: (a, b) 200 µm; (c-j) 25 µm.

The RNAi treatment had no significant effect on body size (figure 3a; ANOVA, treatment, $F_{1,23} = 0.880$, $P = 0.358$), suggesting that the focals and the controls grew equally well. However, as body size was positively related to testis, seminal vesicle, and ovary size (table 1), we included body size as a covariate in the following analyses. With respect to the male function, we found that the RNAi treatment had a significant effect on sperm production. Specifically, the size of the seminal vesicle in this species can change greatly depending on the amount of sperm contained in it (Schärer and Vizoso, 2007), and the focals had a drastically smaller seminal vesicle size than the controls (table 1; figure 3b). We, however, did not find a significant difference in testis size (table 1; figure 3c), as might have been expected if male allocation was completely prevented by the RNAi knockdown. With respect to the female function, we found that the focals had a significantly larger ovary size than the controls (table 1; figure 3d), suggesting that the resource allocation into female function increased as expected from the trade-off assumption.

Table 1. ANCOVA testing the effect of the RNAi treatment and body size on different morphological traits. Bold indicates statistically significant values.

| | | factor: RNAi treatment | | | covariate: body size | | |
|---|---|---|---|---|---|---|---|
| morphological parameter | $R^2$ | F | d.f. | p-value | F | d.f. | p-value |
| testis size | 0.41 | 0.0103 | 1, 22 | 0.920 | 15.2 | 1, 22 | **<0.001** |
| seminal vesicle size | 0.67 | 42.5 | 1, 22 | **<0.001** | 7.4 | 1, 22 | **0.012** |
| ovary size | 0.66 | 11.2 | 1, 22 | **0.003** | 23.7 | 1, 22 | **<0.001** |

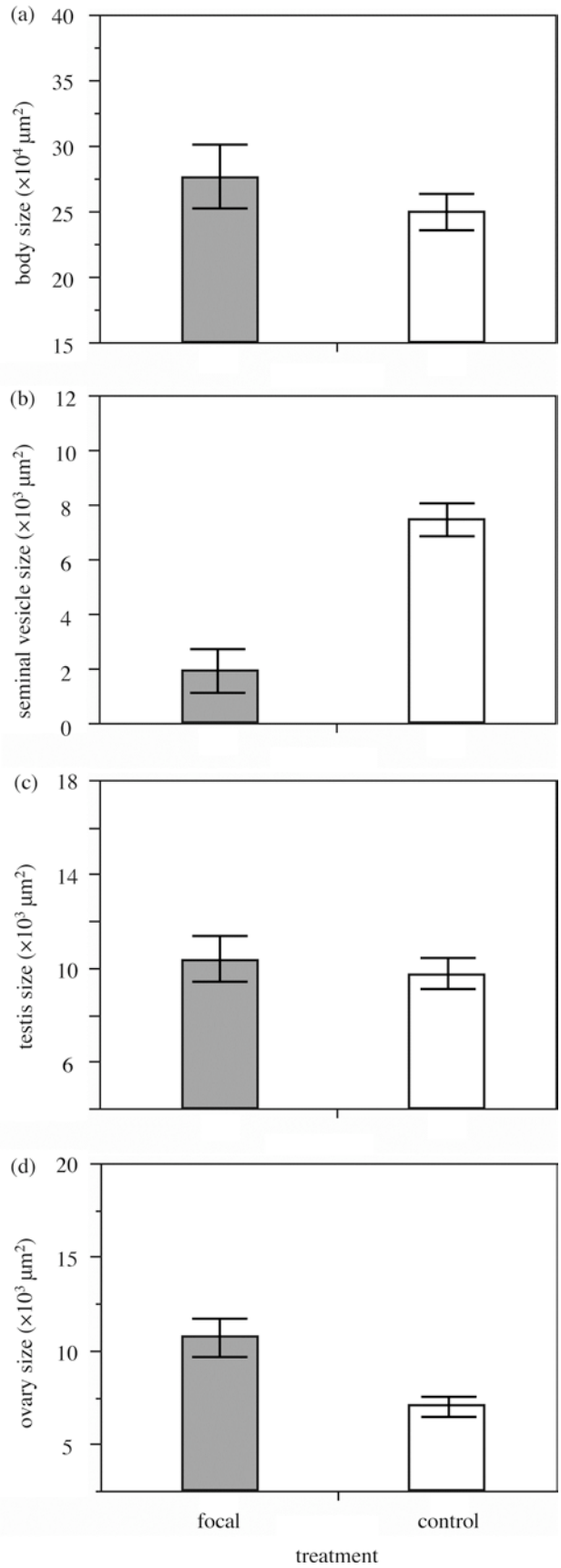


**Figure 3: Effect of *mac-C3H-zfn* RNAi on different morphological traits.** Differences in (a) body size, (b) seminal vesicle size, (c) testis size, and (d) ovary size are shown for the focals and the controls. Error bars indicate standard errors.

# Discussion

In this study we phenotypically engineered sperm production by using RNAi of a testis-specific gene, *mac-C3H-zfn,* and showed that the suppression of sperm production leads to an increased female allocation, as measured by ovary size.

**Phenotypic engineering via RNAi knock-down approach**

In previous experimental studies the trade-off between male and female function has been often examined indirectly, through the manipulation of the mating group size (e.g., Janicke and Schärer, 2009b; Schärer et al., 2005; Trouvé et al., 1999), where we expect an increase in male allocation in larger mating groups due to sperm competition and a decrease in female allocation due to the trade-off. However, we have to interpret these results with some caution, given the fact that such environmental manipulation might also affect other traits (e.g., amount of received seminal fluids), which in turn may affect each sex function independently. The observed changes in the female function may thus be a response to other traits being affected by the different mating group sizes, and not necessarily due to the trade-off. Compared to such environmental manipulations, the specific manipulation of traits performed in this study has the advantage that it allows keeping individuals in the same environmental conditions, with only the targeted traits (e.g., male function) being manipulated. A few experimental studies showing the trade-off in simultaneous hermaphrodites with such approaches have been done, for example, by removing the stamens or the style in plants (e.g., Andersson, 2003), or by removing a particular part of the vas deferens and thus by preventing male copulation activity (De Visser et al., 1994, but see Koene et al., 2009; Schärer, 2009). In addition to these approaches, here we showed that RNAi gene knock-down is also a powerful method to specifically manipulate one sex function in a simultaneous hermaphrodite. This is useful especially in animals for which it has been difficult to manipulate male allocation (sperm production), which in animals, unlike in plants, occurs inside of the body. Another powerful advantage of RNAi for future experiments is that it may allow us to quantitatively manipulate a range of traits by using dose-dependent RNAi treatment (Sekii et al., in prep.). Such a quantitative manipulation may allow to further examine the shape of the trade-off relationship between male and female allocation, which can also be an important factor influencing sex allocation strategy (Burd and Head, 1992).

When using such approaches we of course have to be careful that our manipulation occur within a meaningful physiological range and do not cause side effects unrelated to the trade-offs, thus ensuring that the observed trade-offs are not due to deleterious effects of the manipulation (Sinervo and Svensson, 1998). For example, it is important to consider the possibility that the RNAi treatment may cause

some harmful overall effects that disturb sex allocation, may directly cause some damage to the female function, or may indirectly influence the female function if a functional testis is also required for the normal maintenance of the ovary, for example, via a signal interaction between the testis and the ovary. We think that we likely avoided these problems for the following reasons. Firstly, such deleterious side effects are more likely when one experimentally enhances the performance of a target trait and expects a negative effect on the other trait. Here we did the opposite manipulation, namely we reduced sperm production and expected an increase in the female function, which is unlikely to result from a deleterious side effect. Next, we show that the gene we manipulated via RNAi treatment has testis-specific expression and no expression was found in other tissues, including ovary and developing eggs. Moreover, we did not detect any severe RNAi phenotype except in the testis, and also the RNAi treatment did not have any significant effects on overall body size, suggesting that our manipulation was very specific and that both the focal and control worms were in a good condition. Finally, the change in ovary size was in the expected direction. So we think that it is reasonable to assume that our treatment freed resources from the male function due to hindered spermatogenesis and that these resources therefore became available to allocate into female function, as expected under the trade-off assumption.

One can consider phenotypic engineering via RNAi as a way of mimicking a mutation resulting in male sterility, and such mutations could potentially occur in a range of genes involved in spermatogenesis (although not all will necessarily lead to a trade-off, cf. Sekii et al. 2009). Since every experimental approach has its strengths and weaknesses, different approaches can contribute evidence in different ways, and thus lead to a more convincing and robust empirical evaluation of theoretical assumptions or predictions. We therefore think that our study, which directly shows that a resource trade-off is possible between male and female function (the change in one sex function leads to the change in the other sex function), strengthens the existing evidence from the previous studies done in this system, which used manipulation of the mating group size (Janicke and Schärer, 2009b; Schärer et al., 2005).

**Manipulation of the male function**

The *C3H-zfn* RNAi treatment successfully affected spermatogenesis and caused an empty seminal vesicle in the treated focal worms. Assuming a complete disruption of the male function, we could have expected that the RNAi treatment might lead to a greatly reduced testis size. However, we did not find a significant difference in this trait between the focals and the controls. Conversely, an accumulation of disrupted spermatogenic cells, which appears to have led to bigger testes in a previous

experiment under RNAi of another testis-specific gene (Sekii et al. in prep), also did not seem to occur with the gene we used here, at least at the age when the current experiment was conducted. The male allocation of the focals up to the point of measurement can be thus considered to be reduced compared to that of the controls, by an amount roughly proportional to the difference in seminal vesicle size (i.e., the difference in the amount of sperm produced).

Regarding the mechanism for the observed trade-off between male and female resource allocation, several possibilities can be considered. One possibility is that *mac-C3H-zfn* is a relatively early-acting gene during spermatogenesis and that the *mac-C3H-zfn* RNAi therefore caused the cessation of the proliferation of early stage spermatogenesis stages, such as the spermatogonia (male germ-line stem cells) or early spermatocytes. However, we consider this unlikely, because *mac-C3H-zfn* gene expression was not observed in the anterior tip of the testis, where most of the spermatogonia and many spermatocytes are located (Kuales et al., 2011). Moreover, given that we started the RNAi treatment within 1 day posthatching (i.e, when the worms did not possess distinct gonads yet) and given that the testis was still successfully established at the time of measurement, we think that *mac-C3H-zfn* acts after the mitotic and/or meiotic cell proliferation in spermatogenesis. Indeed, *mac-C3H-zfn* showed expression mainly in the center of the testis, in which later stages of spermatogenesis take place (i.e., differentiation of spermatids into mature sperm). This also matches the function of the homologous gene in the planarian, where it was found to be involved in spermatid elongation (Wang et al., 2010). An alternative possibility is that the *mac-C3H-zfn* RNAi influenced other gene functions to hinder spermatogenesis. According to the computational analysis of the *mac-C3H-zfn* sequence fragment, this gene seems to have two C3H-type zinc finger domains with a conserved structure. C3H-type zinc finger proteins are known to be involved in gene regulation, in many cases with a role in RNA metabolism, such as processing, stability, translational control or export (e.g., Li et al., 2001; Tenenhaus et al., 2001; Wang et al., 2008). Thus it seems possible that *mac-C3H-zfn* also plays such an role during spermatogenesis and that the disruption of the gene function caused severe effects to retard spermatogenesis and the corresponding resource consumption in sperm production, leading to preferential allocation into female function instead.

**Consequences of the trade-off for reproductive success via female function**

While our results show an increase in ovary size as a result of the disruption of spermatogenesis, we did not measure the resulting egg production, since the animals were always kept in isolation and thus never had the opportunity to copulate, receive sperm, and lay eggs. One of important aspect of sex allocation theory is to which extent a mutant phenotype that suppresses the male function can compensate for the

loss in male fitness by an increase in female fitness, thus affecting the stability of a hermaphroditic population (Charnov, 1982). Thus in future experiments it would be important to examine how the increase in ovary size translates into egg production and the total female reproductive output. Given the fact that simultaneous hermaphroditism is extremely stable and widespread in flatworms (Schärer, 2009), we may expect that the increase in ovary size may not efficiently translated into female fitness enough to compensate the loss in male fitness, even given the existence of the trade-off between male and female function.

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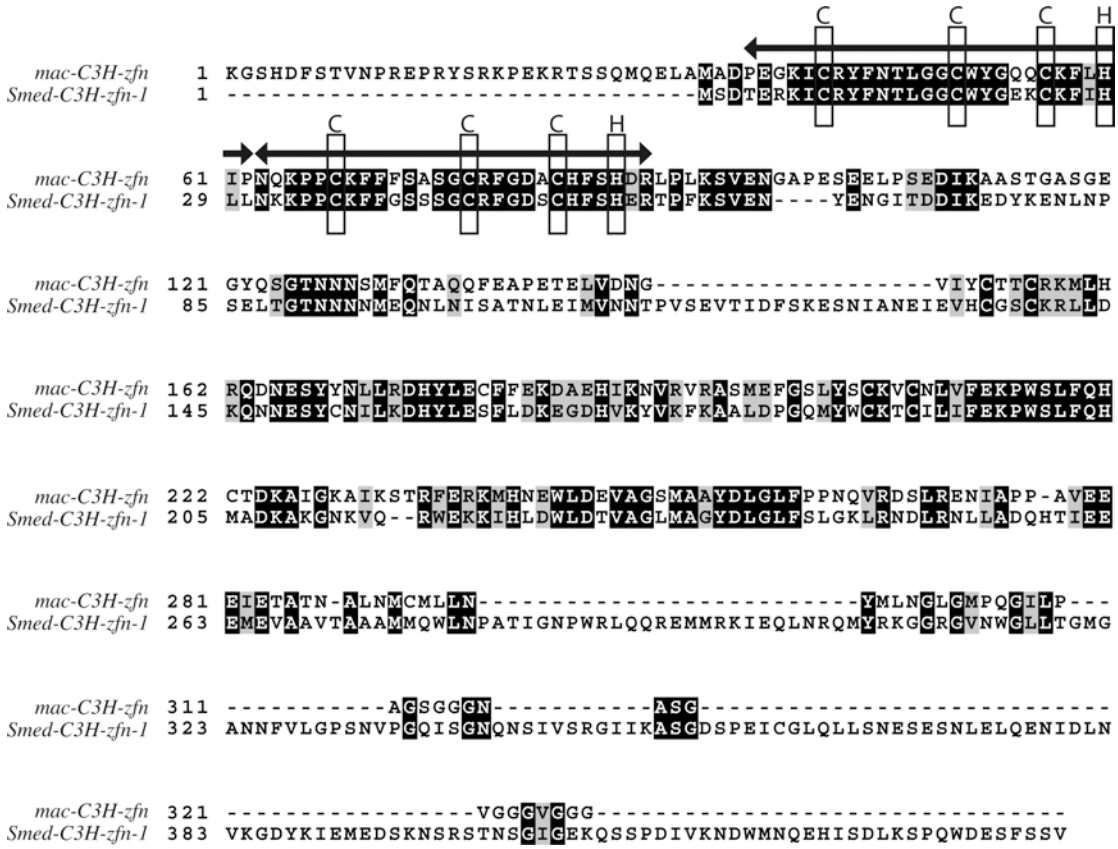
# Electronic Supplementary Materials




**Supplementary figure 1: Comparison between *mac-C3H-zfn* and *Smed-C3H-zfn-1*.** Both genes contain two C3H-type zinc finger domains (shown by the arrows). In each domain a conserved unique structure C-$X_8$-C-$X_5$-C-$X_3$-H (X represents any kind of amino acid) is found (the three Cs and one H are shown in a box, respectively). Black color highlights when same amino acids are shared between the two sequences, and grey color highlights when the amino acids in two sequences are not same but still have similar chemical properties.

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# CHAPTER V

General Discussion and Conclusions

# General Discussion and Conclusions

## Summary of studies

**Chapter II** shows that one of the genes I screened, *melav2*, has a crucial role in spermatogenesis, which is different from the conventional role of *elav* gene-family members that are usually involved in neurogenesis. Although different genes were used in further tests of sex allocation theory, a basic workflow to obtain male-specific genes is shown in this chapter. The study also provided an important insight into the biology of *M. lignano*. The *melav2* RNAi treatment disrupted spermatid differentiation, resulting in aberrant sperm morphology and an empty seminal vesicle. This finding suggests that aberrant sperm remain in the testis and are not transferred into the seminal vesicle, which contrasts to the case of teratozoospermia in some mammals (Poongothai et al., 2009; Pukazhenthi et al., 2001), i.e., sperm with aberrant morphology in male ejaculate. Therefore, when we create RNAi phenotypes intermediate between an empty and a full seminal vesicle, it is likely that the seminal vesicle still contains only normally differentiated sperm. As seminal vesicle size greatly changes depending on the amount of sperm contained in it (Schärer and Vizoso, 2007), we can expect that different sperm production rates can be measured and demonstrated based on seminal vesicle size. Accordingly the study in **Chapter III** shows that, as expected, the manipulation using dose-dependent RNAi of the testis-specific *macbol1* gene successfully created quantitative variation in sperm production. The resulting fitness gain curve was clearly saturating under weak sperm competition, as predicted by sex allocation theory. The same study also provided evidence for an important prediction of sperm competition theory, namely a positive relationship between sperm production and male sperm competition ability. The evidence for a saturating male fitness gain curve suggests that selection should favor a low allocation into male function when sperm competition is weak, corresponding to the previous studies showing that worms develop a more female-biased sex allocation under such conditions (e.g., Janicke and Schärer, 2009; Schärer and Ladurner, 2003; Schärer et al., 2005). Connecting these two findings, in **Chapter IV** I demonstrate that, in the case of the testis-specific *mac-C3H-zfn* RNAi, resources not used for the male function can be reallocated to the female function, providing evidence for a trade-off between male and female allocation. In the following I discuss some interesting aspects of my research that could not be covered in **Chapters II** to **IV**.

**Failure of testis size manipulation**

Although sperm production was successfully affected by the RNAi treatments, the size of the testis was not reduced in any of the studies performed during my PhD project. This seems to be due to the accumulation of immature sperm in the testis, for example, as clearly shown in the study of *melav2* gene. The testis contained many spermatogenesis-arrested cells, as indicated by the accumulation of spermatid-specific antibody staining signal. Thus in a strict sense our manipulation is not a manipulation of male allocation *per se*, but just a mimic of the expected outcome of a reduced male allocation, which is a suitable manipulation for examining a short period of reproductive performance, or just a delay of spermatogenesis which probably resulted in slower resource consumption. To more directly manipulate male allocation, we would need to target earlier-acting genes in spermatogenesis to prevent the initial establishment and the subsequent maintenance of the testis.

However, such genes were difficult to obtain via the candidate gene approach I used here. A general expectation in a candidate gene approach is that tissue or organ development is controlled by gene regulatory cascades, in which master regulatory genes at the top of the cascade are more highly conserved than the genes further downstream, for example, as seen in the case of eye development controlled by the highly conserved gene *pax6* (e.g., reviewed in Kozmik, 2005). However, recent studies have revealed that earlier-acting genes in different sex determining cascades are highly divergent among organisms, with different genes having been recruited upstream of the cascade, and thus some conservation is only found relatively at the bottom of the cascade (Marín and Baker, 1998; Schütt and Nöthiger, 2000; Zarkower, 2001), as hypothesized by bottom-up theory (Wilkins, 1995). Moreover, sex-related genes tend to show rapid evolution (e.g., Haerty et al., 2007; de Bono and Hodgkin, 1996; Tucker and Lundrigan, 1993; Whitfield et al., 1993). Thus a candidate gene approach has some difficulties when searching sex-specific reproductive genes including early-acting genes, since sex-related genes in one species may not have a same function in closely related species (e.g., Schütt and Nöthiger, 2000), let alone between flatworms (lophotrochozoans) on the one hand and other organisms such as mammals (deuterostomes), and insects and nematodes (ecdysozoans) on the other hand, in which sex determination has mainly been studied.

Such difficulties due to divergence in reproductive genes may also be supported by the three genes used in my PhD project. For example, *melav2* was shown to function during spermatogenesis in *M. lignano*, while the conventional role of many genes in the *elav* family is in neurogenesis (e.g., Pascale et al., 2008; Pascale et al., 2004; Ratti et al., 2006; Yao et al., 1993) and in a few cases in other functions such as oogenesis (Kim-Ha et al., 1999) and excretory canal development (Fujita et al., 2003). *Macbol1* is involved in spermatogenesis in *M. lignano* and its function in

spermatogenesis is relatively conserved among other organisms, but other paralogs in *M. lignano*, *macbol2* and *macbol3*, seem to have other functions (*macbol2* has testis-specific expression but no conspicuous RNAi phenotype, and *macbol3* is expressed in the ovary and developing eggs and its RNAi phenotype shows aberrant egg maturation), implying more diverse functions of this gene family (Kuales et al., 2011). *Mac-C3H-zfn* was obtained based on a screen of germ cell development in the freshwater planarian flatworm *S. mediterrannea* (Wang et al., 2010). In a BLAST search other genes showing high similarity with *mac-C3H-zfn* are only found within Platyhelminthes including *S. mediterrannea* (data not shown), suggesting that it has uniquely evolved in the flatworms.

To overcome these weaknesses of the candidate gene approach when looking for sex-specific genes, a recently established transcriptome data set in *M. lignano* (Arbore et al., in prep.), to which I also contributed substantially, might prove to be a powerful tool. The fact that the testes and ovaries of *M. lignano* are situated in a distinct region of the body and easily distinguishable under a microscope due to the transparency of the body, enabled us to physically cut the worms based on the anatomical positions and to obtain the fragments containing different combinations of tissues (i.e., the first fragment containing the head; the second containing the head and the testis; the third containing the head, the testis and the ovary; the fourth containing the head, the testis, the ovary, and the tail plate). RNA-Seq transcriptome analysis with this well designed fragment comparison now allows an efficient estimation of tissue specificity of the transcripts, namely testis- or ovary-specific gene expression. This allows a more efficient approach in which we can directly start from the genes already showing sex specificity and potential importance in spermatogenesis or oogenesis, without relying on *a priori* knowledge based on gene functions in other organisms, which may often be unreliable in the case of sex-related genes.

**Confirmation of two fundamental aspects in the sex allocation theory and potential future studies**

In my PhD study I confirmed two fundamental aspects of sex allocation theory: the saturating male fitness gain curve and the existence of the trade-off in *M. lignano*. Previous studies show that sex allocation in *M. lignano* is highly phenotypically plastic, becoming more female-biased in smaller mating group sizes (weaker sperm competition) than in larger mating group sizes (stronger sperm competition) (e.g., Janicke and Schärer, 2009; Schärer and Ladurner, 2003; Schärer et al., 2005). The more female-biased sex allocation under weaker sperm competition seems to be explained by the saturating male fitness gain curve and the existence of the trade-off: any further allocation into the male function would not provide additional fitness returns after a certain amount of investment due to the saturation, and thus resources

are allocated into the female function due to the trade-off. Although the plasticity in sex allocation suggests more linearized male fitness gain curves in stronger sperm competition, further studies are required to confirm this prediction. In the case of copulating animals, different processes of post-copulatory sexual selection, such as efficient sperm displacement or cryptic female choice, have been predicted to weaken the effect of sperm competition (Charnov, 1996; Pen and Weissing, 1999; Schärer, 2009; Van Velzen et al., 2009). Therefore it would be important to examine to which extent the saturation is retained under stronger sperm competition, together with the studies revealing the involvement of sperm displacement or cryptic female choice. Considering that in many simultaneously hermaphroditic taxa population density can be high and multiple mating is common (Michiels, 1998; Michiels, 1999), which is in contrast to the more conventional perspective that simultaneous hermaphrodites live in low density, such studies would shed light on an important aspect of hermaphroditic mating systems.

In all three of my main studies, the RNAi knock-down led to an empty seminal vesicle because of the failure of sperm transfer. These results suggest that the loss of function in one single gene resulting in malformed sperm may easily lead to complete loss of male reproductive success. The RNAi treatments in all studies were started from the hatchling stage in which the worms do not yet possess any gonads, but the testis could still be established when they grew up. This suggests that, even if some deleterious mutations occur during spermatogenesis, the worms do not seem to have a mechanism to control aberrant spermatogenesis, for example, via feedback signaling from the seminal vesicle to the testis, which otherwise would be beneficial to efficiently prevent resources from being allocated into non-profitable sperm production and to maximize reallocation into the female function. Only if mutations occur in fairly early-acting genes involved in spermatogenesis and thus lead to the complete loss of the testis, we might expect that all resources used for sperm production will be liberated for the female function. In sex allocation theory, whether the mutants with suppressed male function can compensate the loss of male fitness by the female function and thus can invade a hermaphroditic population is one of key arguments for hermaphroditism being evolutionary stable strategy (Charnov, 1982). In future studies it should be examined how the observed increases in ovary size are translated into the total female reproductive success. Given that simultaneous hermaphroditism is extremely stable and widespread in flatworms (Schärer, 2009), we may expect that the loss of the male reproductive success might be too big to be compensated by the female function, unless the testis is eliminated and reallocation into the female function is maximized, or such compensation might be impossible even with maximized reallocation.

**The female function's side**

In my PhD project I primarily focused on the male side of this model organism, but it would also be important to investigate the female aspects in further studies. For example, the shape of the female fitness gain curve in *M. lignano* has not yet been examined, although it is likely linear, or at least less saturating than the male one. This is indicated in a previous study showing that female allocation increases with resource availability, which is expected if the female reproductive success is limited by the resource availability, which tends to linearize the female fitness gain curve (Vizoso and Schärer, 2007). Unfortunately, since little is known about the ecology and reproduction under natural conditions in *M. lignano*, I can not provide conclusive arguments about the presence or absence of factors that likely make the female fitness gain curves more saturating, such as a limited capacity for brooding (Charnov, 1982; Heath, 1979), local resource competition among offspring (Charnov, 1982; Clark, 1978; Lloyd, 1982), and male gamete limitation (Charnov, 1982). So far, we do not have evidence for brooding behavior in *M. lignano*. Moreover, recent studies on freshly-caught worms from the field provided some suggestive data that male gamete limitation does not seem to be very important under natural condition (Sandner et al., unpublished data). However, studies on the shape of the female fitness gain curve are still needed.

A study on the trade-off resulting from direct manipulation of the female function is also required. Intuitively, we expect that suppressing the female function will lead to an increased investment into the male function. However, it is also possible that the manipulation might reveal an intriguing possibility, namely that the trade-off between male and female allocation may be asymmetric. For example, if the female fitness is limited by resource availability, it may suggest that the selection acting on the mechanism to reallocate extra resources from male function to female function might be stronger than in the opposite direction of the resource reallocation (i.e., from female to male function) under the saturating male fitness gain curve. Thus it might be possible that experimental suppression of the female function might not lead to an increase in the male function, but might result in an increase in other traits that contribute to other fitness components. Moreover, we might expect that the reallocation of resources into the male function might start to appear only under strong sperm competition, if the male fitness gain curve gets more linearlized. To test these potential asymmetric resource flows between male and female function, one possible experiment would be to examine the effect of suppressing the female function on the male function under different levels of sperm competition.

Obtaining female-specific genes has proved to be more challenging in the candidate approach I used here. In addition to the problems of the sex related genes as discussed above, the transcriptome data in *M. lignano* suggests that the female-

specific genes are much fewer than the male-specific genes. Such a bias appears to be a general phenomenon, e.g., as reported for *D. melanogaster* (Parisi et al., 2004). Therefore, obtaining female-specific candidate genes seems more difficult than obtaining male-specific genes. The further screening of the transcriptome data may be a more promising approach to efficiently search the female-specific genes. Recently a large-scale RNAi screening with such data has already revealed one promising candidate gene, RNAi knock-down of which suppresses egg production and causes female-sterile worms (Arbore et al., in prep.).

**Conclusions**

In my PhD project I established and performed phenotypic engineering of sperm production and confirmed two fundamental aspects of sex allocation theory in a simultaneous hermaphrodite, namely the presence of a saturating male fitness gain curve, to my knowledge for the first time in a copulating simultaneous hermaphrodite, and the presence of a trade-off between male and female allocation. These two findings confirm important predictions of sex allocation theory, that selection favors lower male allocation when having saturating male fitness returns and that freed resources can flow from the male to the female function, making hermaphroditic sex allocation an efficient strategy and causing more female-biased sex allocation under weak sperm competition. My studies also contributed to provide some powerful research tools for future studies in *M. lignano*. I established a workflow for identifying sex-specific genes and succeeded in the production of male-sterile worms using the testis-specific RNAi gene knock-down approach. I also demonstrated the quantitative manipulation of a sperm production trait using dose-dependent RNAi. Such sex-specific and quantitative manipulations in a simultaneous hermaphrodite will be very useful for approaching important questions in evolutionary biology such as sexual selection and sex allocation theory.

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