Sexual Selection Mechanisms in a Simultaneously Hermaphroditic Flatworm

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Abstract

Male and female animals often display striking differences in shape, behaviour, or reproductive strategies. For the most part, this was attributed to evolution under sexual selection where males and females are thought to be under different selection regimes. Although initially described to occur mainly through contests between males for mates and mate choice by females, sexual selection is now widely accepted to occur after copulations through sperm competition and/or cryptic female choice. However, studying these post-copulatory processes proved to be challenging because they occur internally and are often difficult to observe and measure. Therefore, advancements in understanding post-copulatory sexual selection greatly depend on the development of techniques that facilitate the observation of internal processes. Moreover, measuring the reproductive success of individuals under competitive conditions hinges on techniques that allow easy identification of the parentage of offspring.

During my PhD project, I used the simultaneously hermaphroditic flatworm *Macrostomum lignano* to study aspects of pre- and postcopulatory sexual selection. The establishment of a transgenic line of worms expressing a green fluorescent protein (GFP) in all cells provides a unique opportunity to differentiate and track sperm under competitive scenarios within the female reproductive tract *in vivo*. Additionally, a suite of transgenic lines (NL lines), each expressing fluorescent proteins localized to specific tissues of the body or nuclei of cells, provide distinct inheritable markers that enable efficient identification of parentage in offspring from mating crosses. I tested and validated the reliability of the NL lines, mating crosses between lines, and other GFP techniques, which I could then effectively use to study central aspects of sexual selection in *M. lignano*.

The NL lines were instrumental in my experiment designed to measure the strength of sexual selection in the male and female sex functions in *M. lignano*. The difference in the strength of sexual selection between the sexes, i.e., stronger sexual selection in males than in females, has been attributed to the difference in costs of producing male and female gametes (i.e., anisogamy). Therefore, theoretical considerations of simultaneous hermaphrodites that also exhibit anisogamy predict stronger sexual selection in the male compared to the female sex function. *M. lignano* exhibits a reciprocal mating system (i.e., worms donate and receive sperm simultaneously), where the copulations in the male and female sex functions are strictly linked. My measurements of standardised selection metrics in *M. lignano* revealed substantial scope for sexual selection in both male and female sex functions with no significant difference between the same. My findings thus suggest that although anisogamy may lead to a difference in the reproductive interests of the male and female sex functions, the actual strength of sexual selection may be influenced by other factors, such as the mating system.

Previous studies in *M. lignano* have shown that sexual selection predominantly occurs in post-copulatory episodes. My investigation into the role of sperm size in a post-copulatory episode of selection revealed a non-linear association with a competitive advantage for intermediate sperm size. I speculate that the observed selection for intermediate size may arise due to the trade-off between sperm size and the number of sperm produced. That is, an increase in sperm size may be associated with fewer sperm produced and transferred to the partner, leading to fewer sperm represented in sperm competition. Therefore, the selective forces for more competitive larger sperm and that for numerous but smaller sperm may reach a balance at an intermediate optimum.

In conclusion, I took advantage of several features and powerful techniques established in *M. lignano* to obtain novel insights into the operation of sexual selection. My PhD works further signify the importance of sexual selection as an evolutionary agent in simultaneous hermaphrodites.

Chapter I

Thesis Introduction

Sexual selection

Sexual selection has become an unifying framework in evolutionary biology that explains the striking differences in size, colour and behaviour between males and females. Darwin formulated the theory of sexual selection which posited that the expression of elaborate traits represents their advantage in competition to acquire mates, either by outcompeting rivals of the same sex or by attracting members of the opposite sex (Darwin, 1871). Since then, research into sexual selection has been successful in explaining the wide variety of mating systems and reproductive strategies observed among animals, plants and fungi (reviewed e.g., in Andersson, 1994; Arnold, 1994; Birkhead and Møller, 1998; Skogsmyr and Lankinen, 2002; Arnqvist and Rowe, 2005a; Jennions and Kokko, 2010; Nieuwenhuis and Aanen 2012). Bateman's seminal study (1948) using *Drosophila melanogaster* was the first formal presentation of how sexual selection operates in males and females. From mating trials and assessment of parental contributions, Bateman's observations suggested that males and females may obtain different benefits from having multiple mating partners. In particular, Bateman (1948) found that the reproductive success of males was more strongly dependent on mating success than that of females. This led him to argue that in promiscuous species, female reproductive success was mainly limited by egg production and male reproductive success was mainly limited by access to females, explaining why males are typically more eager to mate than females in many species (Bateman, 1948). Later advancements in understanding phenomena such as parental investment (Trivers, 1972) and polyandry (Parker and Birkhead, 2013) have revealed the significance and evolutionary implications of Bateman's proposition.

Darwin considered sexual selection primarily in terms of pre-copulatory male-male competition and female choice. However, in promiscuous species, sexual selection was shown to continue after copulation, in terms of competition between sperm and ejaculates of different males for fertilisations (i.e., sperm competition; Parker, 1970) and female preference for sperm from some males over others (i.e., cryptic female choice, Eberhard, 1996). Recent decades have seen a shift in the perception of sexual selection from selection on traits influencing mate acquisition to that of biasing fertilization success (Jennions and Kokko, 2010), as a consequence of the prevalence of the above-mentioned post-copulatory processes. Initially, post-copulatory selection on males was found to favour the production and transfer of large ejaculates to outcompete those of the rivals in the post-copulatory stages. More detailed observations however revealed the costs involved in producing large ejaculates favouring males to allocate their ejaculates strategically among mating opportunities to maximize fitness returns (Wedell et al., 2002; Parker and Pizzari, 2010). Additionally, the outcome of post-copulatory sexual selection has been shown to be determined by ejaculate characteristics such as the morphology and behaviour of sperm (reviewed in Snook,

2005; Pizzari and Parker, 2009), the composition of seminal fluid transferred along with sperm (Chapman, 2001; Arnquist and Rowe, 2005b) and how ejaculates interact with the female reproductive tract (Pitnick et al., 2009). Thus, understanding sexual selection in a species requires investigations into all the consecutive pre- and post-copulatory episodes of sexual selection that can potentially influence reproductive success.

Sexual selection in simultaneous hermaphrodites

It is widely accepted that sexual selection is an evolutionary agent common to all sexually reproducing organisms. Despite that, the body of research on sexual selection is strongly biased towards species in which the male and female sex is expressed separately among individuals (gonochoristic species). Simultaneous hermaphroditism, where individuals possess male and female sex functions at the same time, is a widespread sexual system present in 70% of all animal phyla (Jarne and Auld, 2006). Simultaneous hermaphrodites exhibit unique sexual phenomena such as self-fertilization, conflict over mating roles, and plasticity in resource allocation to their own male and female sex functions (reviewed in Michiels, 1998; Anthes, et al., 2006a; Schärer, 2009; Anthes, 2010). Yet, research on sexual selection in simultaneous hermaphrodites is relatively limited.

Sex role preferences

Charnov (1979) recognized the potential for sexual selection in simultaneous hermaphrodites and proposed that Bateman's principle can be applied to the male and female sex functions in simultaneous hermaphrodites. He stated that in hermaphrodites, "fertilized egg production by an individual is limited not by the ability to get sperm, but by the resources allocated to eggs" (Charnov, 1979). This proposition implied that individuals mate not primarily to get sperm to fertilize their eggs but to give sperm away to mating partners, leading to all individuals in a population preferring the male role and conflict of interest between partners over the mating role. However, it has also been hypothesized that hermaphrodites may prefer mating in the female sex function under certain conditions (Leonard, 2005; 2006). Empirical studies attempting to determine the preferred sex role have found evidence for both sperm trading (e.g., Vreys and Michiels, 1998; Anthes et al., 2005) and egg trading (Fischer, 1980; Sella, 1985) among hermaphroditic species, and hence the preferred sex role is thought to differ among hermaphroditic species.

Preferred sex roles in a species could also be determined by comparing the fitness returns to each additional matings between the two sex functions (i.e., Bateman gradient), such that the sex function that offers higher returns may be preferred. The few studies that have performed this assessment in hermaphrodites have found a higher benefit of multiple mating for the male sex function that supports a possible sex role

preference for the same (Anthes et al., 2010; Pélissié et al., 2012; Hoffer et al., 2017; but also see Pongratz and Michiels, 2003 and Janicke et al., 2015), although direct empirical evidence for the same is still lacking. More recent studies suggest sex role preferences may also be influenced by life-history traits, depending for example on the age of the animals. In the hermaphroditic snail, *Lymnaea stagnalis,* younger and smaller snails were found to prefer mating in the male role, while older individuals did not exhibit a sex role preference in general (Nakadera et al., 2015). Additionally, recently inseminated snails were reluctant to accept another mating in the female role, preferring to mate in the male sex role instead (Moussaoui et al., 2018).

Pre-copulatory sexual selection

The union of the two sex-functions in the same body in simultaneous hermaphrodites has significant implications for pre-copulatory sexual selection. For example, if a trait functions to enhance mating success in one sex role at the cost of survival, the survival cost is also experienced by the other sex function however without the associated fitness benefits. Additionally, as simultaneous hermaphrodites can copulate in both male and female functions, all individuals in a population can be expected to on average invest equally into mate acquisition, unlike higher investment by the males in gonochorists. Considering these features in comparison to gonochorists, theoretical models suggest a two-fold decrease in the resources invested into acquiring mates (Greeff and Michiels, 1999) and consequently the evolution of sex-specific traits is relatively unlikely in simultaneous hermaphrodites. These findings predict pre-copulatory sexual selection in simultaneous hermaphrodites to be less intense compared to gonochorists.

However, empirical studies have demonstrated mate choice and strategic mating effort in simultaneous hermaphrodites based on the body size of the partners (e.g., Ohbayashi-Hodoki et al., 2004; Anthes et al., 2006b, Loose and Koene, 2008; Janicke et al., 2012), where body size is thought to be an indicator of female fecundity. Moreover, there is also some evidence supporting size-assortative mating in hermaphrodites (Gianguzza et al., 2004; Monroy et al., 2005; Pal et al., 2006; see Graham et al., 2015 for a meta-analysis). Mating preference has also been shown to depend on the mating status of the partner in the hermaphroditic sea slug *Aeolidiella glauca*, which was suggested to be a strategy to avoid sperm competition in already mated individuals (Haase and Karlsson, 2004). Relatedness between partners has been demonstrated to influence pre-copulatory mate choice in the hermaphroditic snail, *P. acuta*, where individuals preferred unrelated partners as an inbreeding avoidance strategy (Facon et al., 2006). These studies indicate that in hermaphrodites the eagerness to mate may vary among mating partners, providing the opportunity for the occurrence of pre-copulatory sexual selection. However, the eagerness to mate may also depend on the sex allocation of individuals, i.e., the relative allocation of resources between the two sex functions. Individuals with a more male-biased sex allocation were found to be more eager to mate compared to

individuals with a more female-biased allocation in the flatworm *Macrostomum lignano* (Janicke and Schärer 2009b), which may also influence the choosiness of the individuals.

Post-copulatory sexual selection

On the assumption that Bateman's principle holds in simultaneous hermaphrodites, all individuals can be expected to be more eager to mate in the male role to donate sperm and less so in the female role, leading to conflict between mating partners over sex roles. This conflict is often resolved by reciprocal copulation (i.e., mating partners donate and receive sperm at the same time) or reciprocal exchange of gametes (sperm or egg trading), which is commonly observed in simultaneous hermaphrodites (e.g., Charnov, 1979; Michiels, 1998; Anthes et al., 2006a, Schärer et al., 2015). Thus, all individuals in a population of simultaneous hermaphrodites have on average a stronger interest to mate in order to give sperm away and are inclined to accept sperm in female sex function in return. This leads to a relatively higher mating rate in simultaneously hermaphrodites compared to gonochorists and limits the scope for pre-copulatory mate choice through the female function. The higher mating rate could potentially generate more intense sperm competition in simultaneous hermaphrodites and the ability of the female sex function to exert choice on the sperm donors may be restricted primarily to the post-copulatory stages, as already speculated by Charnov (1979) in his seminal paper. These theoretical considerations suggest that post-copulatory sexual selection through sperm competition and cryptic female choice may play a crucial role in simultaneous hermaphrodites.

In line with the above theoretical suppositions, variance in male reproductive success has been shown to be strongly explained by post-copulatory components influencing fertilisations in the hermaphroditic snail *Physa acuta* (Pélissié et al., 2014) and the flatworm *Macrostomum lignano* (Marie-Orleach et al., 2016; 2021). Empirical assessments also indicate sperm competition and multiple paternity are common in simultaneously hermaphroditic animals (Michiels, 1998). However, studies investigating patterns of sperm precedence have found fertilization precedence for the first sperm donor to mate (e.g., Evanno et al., 2005), second sperm donor (Angeloni, 2003; Pongratz and Michiels, 2003) and also indicated fertilization success through fair raffle choice of sperm (e.g., Koene et al., 2009). The paternity success of sperm donors has also been shown to be influenced by their morphological traits. For example, paternity success was positively correlated to testis size in the free-spawning ascidian, *Botryllus schlosseri* (Yund, 1997; Johnson and Yund, 2009) and the free-living flatworm, *M. lignano* (Vellnow et al., 2018). The length of the male copulatory organ in the land snail *C. aspersum* (Garefalaki et al., 2010) and the shape of the male copulatory stylet in *M. lignano* (Janicke et al., 2009a) have been shown to positively influence success in post-copulatory sexual selection. Additionally, accessory gland

proteins have been suggested to play a crucial role in post-copulatory sexual selection in hermaphrodites (Schärer et al., 2015). Remarkably, hermaphroditic land snail species exhibit a unique “love-dart” shooting behaviour, which was found to inject accessory gland proteins into the mating partner using a calcareous appendage (Landolfa et al., 2001). This behaviour has been shown to influence the physiology, morphology and behaviour of the partner with the ultimate consequence of higher paternity success for the dart shooter (reviewed in Lodi and Koene, 2016).

To summarize, current theoretical and empirical knowledge suggests that post-copulatory sexual selection may be more intense than its pre-copulatory counterpart in simultaneous hermaphrodites. Sperm competition has been demonstrated to be intense, but evidence for the occurrence of cryptic female choice in hermaphrodites is still lacking. Furthermore, detailed investigations of the traits and mechanisms involved in explaining post-copulatory sexual selection in hermaphrodites are scarce.

Study Organism

I studied sexual selection in the obligately outcrossing simultaneous hermaphrodite *Macrostomum lignano*, a free-living flatworm that is a member of Macrostomorpha of the subphylum, Rhabditophora (Lophotrochozoa, Platyhelminthes; Ladurner et al. 2005; Egger et al. 2009). *M. lignano* has so far been described to occur among the marine meiofauna of the Northern Adriatic and the Aegean Sea, Italy (Ladurner et al. 2005, Schärer et al., 2020). In the last two decades, *M. lignano* has emerged as a suitable model organism in a variety of fields, including research into ageing (e.g., Mouton et al., 2009; 2018), regeneration (e.g., Nimeth et al., 2007), developmental biology (e.g., Ladurner et al., 2008), sex allocation (e.g., Schärer and Ladurner, 2003; Janicke et al., 2013) and sexual selection (Marie-Orleach et al., 2016; 2021). This species has been easily cultured under laboratory conditions, where worms are kept in glass Petri dishes filled with f/2 medium or artificial sea water and fed with the diatom, *Nitzschia curvilineata*. Under laboratory conditions, *M. lignano* has a short generation time of approximately 3 weeks and shows an adult body size of about 1.5 mm (Ladurner et al., 2005).

The small size and highly transparent body of the worm are highly advantageous for observing and studying internal reproductive structures and processes in detail (Figure 1.1; Visozo et al., 2010). Individuals have paired testes located in the central region of the body anterior to the pair of ovaries (Figure 1.1). The sperm cells produced in the testes are transported through the *vasa deferentia* to the seminal vesicle located in the posterior tail plate of the worms, from where they are transferred to the mating partner via the male copulatory organ, the stylet (Figure 1.1). The ovaries are posteriorly connected to the growth zone, where developing oocytes are provisioned with yolk.

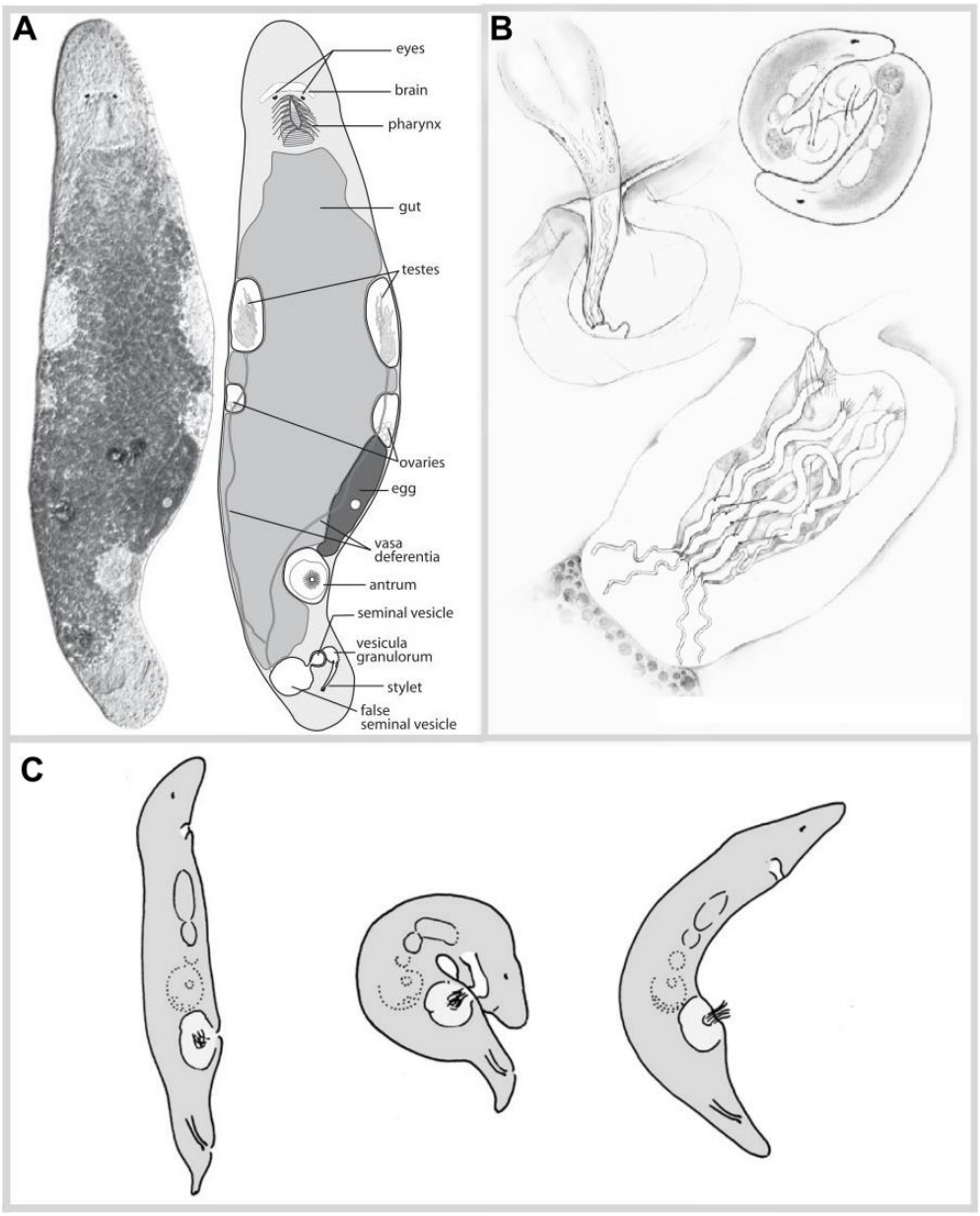


Figure 1.1. A) Micrograph and line drawing of a live adult specimen of *M. lignano* (~1.8 mm) (figure from Vizoso et al., 2010). B) An artistic rendering of the mating postures, the process of sperm transfer, and the location and morphology of the received sperm in the female antrum in *M. lignano* (artwork by Dita B. Vizoso). C) Depiction of the stages of the suck behaviour in *M. lignano* (figure from Vizoso et al., 2010).

Fertilisation is presumed to occur when the mature oocytes enter the sperm-receiving and storage organ, the female antrum (Vizoso et al., 2010). The morphology of the sperm in *M. lignano* is found to be relatively complex including several appendages (Willems et al., 2009), which are thought to have coevolved with reproductive traits associated with the female sex function (Vizoso et al., 2010; Schärer et al., 2011).

Under laboratory conditions, *M. lignano* is highly promiscuous (Janicke and Schärer, 2009) and worms exhibit a high copulation rate (~ 6 copulations per hour, Schärer et al., 2004). During copulation, both partners form a tight disc and reciprocal intromission of the male stylet into the female antrum of the mating partner occurs, which means both partners receive and donate sperm simultaneously. Immediately after copulation, worms facultatively perform the so-called "suck" behaviour, where worms bend over to place their pharynx over their own female genital opening and appear to suck out the sperm that was just received from a partner (Schärer et al., 2004). It has been posited that the stiff lateral bristles in the sperm morphology have evolved in order to prevent the sperm from being removed from the antrum by the sucking behaviour (Vizoso et al., 2010; Schärer et al., 2011).

The histological and molecular techniques, including *in situ* sperm tracking using immunocytochemical approaches (Schärer et al., 2007) and experimental manipulation of traits using phenotypic engineering (Sekii et al., 2009) have enabled studies addressing evolutionary questions using *M. lignano* as a model. More recently, a suite of transgenic lines of *Macrostomum lignano* has been established that express green or red fluorescent proteins (GFP/RFP). Transgenic worms expressing GFP ubiquitously in all cells have greatly aided studies on sex allocation and sexual selection, as GFP+ sperm cells from a transgenic sperm donor could be differentiated from GFP- sperm from a wildtype worm (e.g., Janicke et al., 2013, Marie-Orleach et al., 2014). This feature allowed more fine-scaled studies on post-copulatory aspects of sexual selection in *M. lignano*, for example, the relative importance of sperm storage efficiency and sperm fertilizing efficiency for male reproductive success (Marie-Orleach et al., 2016; 2021). Another set of transgenic inbred lines has been established (Wudarski et al., 2017) that express GFP/RFP in specific tissues that provide distinct phenotypic markers and can be used as identifiers of parentage, broadening the scope for experimental designs studying sexual selection.

Thesis outline

In my PhD thesis, I study several aspects of sexual selection in the simultaneously hermaphroditic flatworm, *Macrostomum lignano*. A major hurdle to understanding sexual selection in a species has been the challenge of observing internal processes occurring after copulation. Due to its small size and transparent body, one can perform a range of measures *in vivo*, including observation of received sperm in the sperm storage organ. Worms expressing GFP ubiquitously in all cells, including sperm cells, have provided an opportunity to observe and differentiate sperm from different donors within the female reproductive tract of sperm recipients, non-invasively (Marie-Orleach et al., 2014). This enabled several very informative investigations into the nature of pre- and post-copulatory sexual selection in *M. lignano* (e.g., Janicke et al., 2016; Marie-

Orleach et al., 2016; 2021). A new set of inbred lines (collectively called NL lines) expressing GFP/RFP markers in specific tissues or localised to the nucleus of all cells broadens the range of possible experimental techniques in *M. lignano*. In **chapter II,** I describe the observable characteristics of the phenotypic markers in the seven NL lines and validate the reliability of using them. I compare the life-history and behaviour traits with their wild-type ancestor line, to confirm that the introduction of the transgene marker had negligible negative effects on the functioning of the worms. (NL stands for 'Netherlands', the lines were established in Groningen, Netherlands).

A major portion of the quantitative study of sexual selection has focused on gonochoristic species (species with separate sexes), that most often exhibit sexual dimorphism. Despite being widespread across animals, simultaneous hermaphroditic species have received relatively little attention in terms of sexual selection. Moreover, the union of the two sex functions in the same body and the prevalent absence of sexual dimorphism among hermaphrodites offer unique sexual systems for the study of sexual selection. A new approach made possible by the NL lines described in chapter II allows us to quantify sexual selection in the simultaneous hermaphrodite, *Macrostomum lignano.* In **chapter III,** I use several NL lines in combination to conduct mating experiments aimed at measuring mating success and reproductive success of individuals in groups. The heritable transgenic markers in *M. lignano* acted as reliable identifiers of parentage. I quantified sexual selection using widely used metrics, including opportunity for selection, opportunity for sexual selection, Bateman gradient and Jones index, while also discussing the limitations associated with these metrics. Additionally, I also investigated the effect of sampling effort on these metrics of sexual selection.

Sexual selection is now widely accepted to also occur in the post-copulatory stages in competition among rival ejaculates to acquire fertilisations. In species where individuals mate with multiple partners, the efficiency to outcompete rival ejaculates can become a major predictor of male reproductive success. Therefore, understanding the phenotypic effect of ejaculate traits on the outcome of post-copulatory sexual selection became an important part of understanding sexual selection in general. One of the most important ejaculate traits is sperm size, which is found to vary tremendously not just within ejaculates but also among males and among species. Sperm size has been documented to be a crucial predictor of the competitiveness of sperm through its influence on sperm motility and longevity. Comparative studies across species have found a positive association between sperm size and strength of post-copulatory sexual selection, but the relationships between sperm size and post-mating success within species are mixed. In **chapter IV**, I investigated linear and non-linear relationships between sperm size within a species and post-mating success in *M. lignano*, measured as the proportion of sperm stored in the female antrum. By using inbred lines of *M.*

lignano expressing GFP in sperm cells, I explored how sperm competition generates selection on standing genetic variation in sperm size.

Chapter II

Description and Validation of Fluorescent Markers to Study Sexual Selection

Abstract

Although sexual selection was initially thought to occur exclusively at the pre-copulatory stages (i.e., through contests among males and female mate choice), in the last decades it has been widely observed to continue also after copulation in the form of sperm competition and/or cryptic female choice. However, studying sexual selection remains challenging, as the post-copulatory processes involved in sexual selection occur internally and are therefore often impossible to observe. Moreover, tracking the parentage of offspring in order to measure the reproductive fitness of individuals in a population can also be a logistically demanding endeavour. The techniques developed in the transparent flatworm *Macrostomum lignano* using transgenic lines expressing a green fluorescent protein (GFP) in all cells including sperm cells have been used to effectively circumvent these challenges. Here we present a set of seven transgenic lines (NL lines) expressing fluorescent proteins localized to specific tissues in the body or nucleus of all cells, that offer novel opportunities for the study of sexual selection. We describe the characteristics of the fluorescent markers in the different NL lines and present tests indicating negligible negative effects of the introduction of transgenic markers into the worms. We found that worms from the transgenic NL lines do not differ from wild-type worms in their morphological development and reproductive ability. We also validated mating crosses between NL lines that produced offspring expressing fluorescent markers of both parents, providing a useful tool that can be used to track parentage and measure reproductive fitness in competitive scenarios. Finally, we briefly discuss the utility and limitations of the features in the NL lines for the efficient study of sexual selection and other subjects in evolutionary biology.

Introduction

Darwin described sexual selection as arising from differences in reproductive success resulting from the competition between individuals of one sex to acquire mates of the other sex (Darwin, 1871). His original insights provided the first proximate explanation for the prevalence of elaborate weapons and ornaments commonly observed in males and the visible phenotypic differences between males and females. These traits appeared to function not to increase the survival of an individual, but to be involved in intrasexual competition among rivals of the same sex or to attract members of the opposite sex solely in the interest of reproduction (Darwin, 1871, Clutton-Brock, 2007). Thus, such secondary sexual characters came to be associated with contest competition or mate choice as mechanisms of sexual selection determining the relative mating success of the individuals, and ultimately their reproductive success. However, the realisation that sexual selection can also occur after copulation created new avenues in the study of processes that influence the reproductive successes of individuals. Specifically, it is widely observed that females mate with and store sperm from multiple male partners, creating an opportunity for competition between the ejaculates from different mating partners for fertilizations (sperm competition; Parker, 1970). Males may also manipulate the female partners, for example through the transfer of seminal fluids in their ejaculates, by influencing females' future behaviour and/or fecundity in an attempt to improve their own reproductive success, leading to a conflict of interests between the sexes (sexual conflict). Moreover, females may also play an active role in the post-copulatory stages by biasing sperm transfer and fertilizations success towards certain partners (cryptic female choice; Eberhard, 1996). In recent decades, post-copulatory sexual selection has been recognized as an important evolutionary force that shapes male and female reproductive traits (Birkhead and Pizzari, 2002), such as the "giant sperm" in *Drosophila hydei* (Pitnick and Markow, 1994). Thus, understanding the underlying mechanisms involved in post-copulatory sexual selection became a crucial focus in sexual selection research.

Historically, post-copulatory aspects of sexual selection have been inferred from experimental manipulation of copulations combined with the assessment of skews in paternity. For example, females were artificially double-mated and the offspring were analysed to measure the proportion of offspring sired by the second male to mate (so-called P_2 values) (e.g., Dunn, 1927; Sumption, 1961; Dziuk, 1965; Lefevre and Jonsson, 1962). Initially, such studies involved using genetic markers with simple inheritance patterns or radio-sterilization of males to infer the paternity success of the competing males. However, such tools required accounting for potential biases in paternity induced by artificial factors, for example, selective bias towards specific phenotypically visible genetic markers. More recent studies have used DNA analysis, particularly using microsatellite markers, to determine the paternity of offspring (e.g., Sheldon and

Ellegren, 1999; Radespiel et al., 2002; Kraaijeveld-Smit et al., 2003). However, the analysis of patterns in paternity only allowed inferences about the overall fitness outcome of post-copulatory sexual selection and failed to provide insights into the underlying episodes of selection driving the observed skews in paternity.

Detailed understanding of post-copulatory sexual selection requires studying how ejaculates from competing sperm donors interact with each other and how sperm stored in the female reproductive tract are differentially used for fertilizing eggs. Yet, observing and quantifying such post-copulatory processes is challenging, because 1) these processes occur inside the female reproductive tract and cannot be visualized easily and 2) it is difficult to differentiate sperm from different males inside the female reproductive tract. These challenges have been overcome to different extents using certain molecular techniques. For example, sperm have been experimentally labelled using amino acids or nucleotides carrying radioisotopes, which could then be quantified in the recipient by scintillation counting or autoradiography (e.g., Simmons et al., 1999). In another technique, DNA in sperm cells is labelled with a halogenated pyrimidine, bromodeoxyuridine (BrdU), during spermatogenesis, which can later be tracked in the recipient using immunocytochemical staining techniques (Janicke et al., 2009b). The establishment of transgenic lines of *Drosophila melanogaster* to express fluorescent proteins in the sperm enabled the real-time visualisation of competing ejaculates and quantification of sperm behaviour *in situ* for the first time (e.g., Manier et al., 2010; Lüpold et al., 2012). The use of these techniques to study sperm stored in the recipient greatly contributed to our understanding of post-copulatory sexual selection. However, the common limitation of the above techniques is the need for destructive sampling, either to fixate sperm recipients or to dissect the female reproductive tract for the required observations so that the reproductive success of sperm recipients cannot be tracked. Thus, any quantification of sperm behaviour or patterns observed in sperm storage and use cannot be directly linked to the reproductive success of the two sexes, failing to demonstrate the actual selection on the stored sperm and/or the female.

Recently, these challenges were overcome by the ability to non-invasively visualize labelled sperm from a sperm donor under sperm competitive conditions, in vivo, in the reproductive tract of the free-living hermaphroditic flatworm, *Macrostomum lignano* (Marie-Orleach et al., 2014). This breakthrough was made possible due to the establishment of a transgenic line of *M. lignano*, which expresses green fluorescent protein (GFP) in all cells, including sperm cells. The non-invasive procedures established in this system enabled quantitative assessments of different post-copulatory stages of sexual selection, greatly improving the resolution with which post-copulatory sexual selection could be studied. For example, the pool of received sperm in the reproductive tract of a single recipient could be observed repeatedly enabling the study

of the dynamics of sperm displacement over time (Marie-Orleach et al., 2014). By measuring the reproductive performance of GFP+ focal individuals in competitive scenarios, sexual selection could be partitioned into its different episodes and quantified separately in terms of selection arising from the mating success of individuals, the ability to store sperm in the recipient and the fertilizing efficiency of sperm (Marie-Orleach et al., 2016; 2021). Thus, the strength and relative importance of the different episodes in determining the reproductive success of an individual could be assessed quantitatively. Furthermore, the form of selection arising from each episode of sexual selection (linear or non-linear) on different life-history and reproductive traits could be determined, providing a detailed picture of the operation of sexual selection in *M. lignano* (Marie-Orleach et al., 2024).

Here we present a new toolbox in *M. lignano* that adds to the previously established techniques by providing opportunities to conduct more sophisticated experiments in the study of sexual selection. The NL lines are a set of 7 transgenic inbred lines of *M. lignano*, each expressing a unique and distinct fluorescent phenotypic marker. Thus, in addition to the GFP marker expressed ubiquitously in all cells, the NL lines provide 6 new inheritable fluorescent markers localized either in the nucleus of all cells or in specific tissues in the body of the worms. Given the distinct fluorescent markers, mating crosses between lines can be conducted to produce offspring carrying fluorescent markers of both parents. This allows for experimental designs that require tracking the maternity and paternity success of multiple individuals in competitive mating groups, which was not possible in previous approaches where only one GFP+ focal worm could be tracked (Marie-Orleach et al., 2016; 2021). Thus, the NL lines are expected to expand the scope for experimental designs and offer novel opportunities to study the mechanisms of post-copulatory sexual selection and to obtain new insights into sexual selection in general. The establishment of the NL lines has been discussed in detail in Wudarski et al., 2017, but the lines and their distinct transgenic phenotypes have not yet been phenomenologically described, in terms of their observable features and aspects relevant to reproduction and sexual selection. Moreover, before they can be reliably used in experiments, we need to evaluate if traits associated with reproduction differ between the NL lines and their wild-type ancestral line and to assess if the fluorescent markers are reliable identifiers of individual worms and sperm from transgenic worms. Here, we describe the phenotypic characteristics of 7 NL lines and report comparisons of certain life-history and behavioural traits between the NL lines and the ancestral DV1 line. Thereby, we validate the NL lines by testing if the introduction of the transgene has affected the NL lines negatively to evaluate the suitability of the NL lines for sexual selection research. We further discuss their potential advantages and limitations for future experiments.

Materials and methods

Model organism

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is an obligatorily outcrossing simultaneous hermaphrodite found in the intertidal zones of the Northern Adriatic sea and the Aegean sea (Ladurner et al., 2005; Schärer et al., 2020). Laboratory cultures of *M. lignano* are kept at 20°C in glass Petri dishes with artificial sea water (ASW) at a salinity of 32 ‰ and fed with the diatom *Nitzschia curvilineata*. Under laboratory conditions, *M. lignano* are found to be highly promiscuous and mate frequently when kept in groups. Copulation in this species involves reciprocal intromission of the male copulatory organ into the female sperm-receiving organ, the antrum (Schärer et al., 2004; Vizoso et al., 2010). *M. lignano* exhibits a post-copulatory suck behaviour, in which worms bend down and place their pharynx on top of their own genital opening and appear to suck, which may have the function to remove sperm out of the antrum (Vizoso et al., 2010). The transparent body of the worms allows observation and measurement of internal structures *in vivo,* such as testis and ovary size (Schärer and Ladurner 2003), stylet morphology (Janicke and Schärer, 2009a) and sperm stored inside the female antrum (Marie-Orleach et al., 2016, 2021).

The recent establishment of transgenic lines expressing GFP in *M. lignano* enables *in vivo* sperm tracking and assigning parentage to offspring from mating experiments (Janicke et al., 2013; Marie-Orleach et al., 2014; 2021; Wudarski et al., 2017). A detailed description of the methods used to generate the NL lines transgenic line has been described by Wudarski et al., 2017. Briefly, plasmid or linear DNA were constructed carrying fluorescent markers associated with promoters of genes expressed ubiquitously in all cells or specific tissues of the worm body. The DNA constructs were then microinjected into fertilized one-cell stage eggs, followed by a low dose of irradiation, which resulted in random integration of the transgenes into the genome and stable transmission through the germline (Wudarski et al., 2017).

Characterisation of the NL lines

NL1 – ubiquitous GFP expression in all cells

NL1 was generated by introducing GFP fused to the promoter region of the elongation factor 1 alpha gene (EFA::eGFP), which is expected to be translated in all cells of the body. Thus, the GFP transgene in NL1 (hereafter called *all-gfp* line) is expressed ubiquitously in all cells of the body in each worm (Figure 2.1A). Under epifluorescence illumination, most morphological features of the worms can be observed in detail. The GFP marker is most strongly illuminated in the relatively large gastrodermal cells of the gut epithelium. In the tail plate of the worms, the prostate gland cells can be

identified as exhibiting strong GFP illumination, along with the vesicula granulorum present in the proximal end of the copulatory stylet, that contain the prostate secretion granules (Figure 2.1D). The GFP signal is relatively weakly illuminated in the rostrum (i.e., region anterior to the eyes) and the gonad regions (Figure 2.1B & C). Although weakly illuminated, elongated sperm can be observed in the testes of the worms (Figure 2.1C). Sperm produced by the *all-gfp* worms express GFP throughout the sperm cells and can be distinguished from GFP- wildtype sperm in the female antrum of mating partners, as previously possible with the GFP+ HUB1 line.

NL9 – muscle tissue-specific GFP expression

NL9 was generated by introducing transgene containing GFP fused to the promoter for the muscle-specific *MYH6* gene (MYH6::oGFP), which is expected to be translated in the muscle cells in the body (Figure 2.2A, hereafter called *muscle-gfp* line). The musculature of *M. lignano* consists of circular muscles running around the body wall, inner longitudinal muscles running along the length of the body and an intermediate layer of dorso-ventral diagonal muscle cells (Ladurner et al., 2005). The GFP signal under epifluorescence is much weaker compared to the *all-gfp* line and requires more intense illuminating light or longer exposure to visualize the marker well. Given the described musculature in *M. lignano*, the observed patterns of GFP suggest that maybe not all muscle cells carry the marker and we observe some variation between *muscle-gfp* worms in the patterns of muscle cells illuminated. Broadly, however, the circular muscle cells that run around the body wall of the worms are strongly and most consistently illuminated (Figure 2.2B). The dorso-ventral diagonal muscle cells can also often be found to be illuminated (Figure 2.2B). However, cells from the inner-most longitudinal muscle layer are more rarely observed under epifluorescence illumination. Additionally, specialized muscle structures such as pharyngeal muscles (Figure 2.2C), muscles surrounding the copulatory stylet involved in the mating behaviour (Figure 2.2D) and muscle cells surrounding the female antrum can be clearly visualized and may enable more detailed observations of how these structures function (Figure 2.2E).

NL20 – GFP localised in the nucleus of all cells

NL20 was generated by introducing GFP fused to the promoter for the histone 2B gene (EFA::H2B::oGFP), which localised GFP to the nucleus of all cells in the body (hereafter called *nucleus-gfp* line). Under epifluorescence illumination, the GFP markers in the nuclei are visualized as green dots that are scattered throughout the body, representing the distribution of all cells in the body of the worm (Figure 2.3A). The density of GFP expression is highest in the rostrum and in the tail plate of the worms. Within the testes of a worm, the smaller roundish nuclei along the periphery are likely to be those of the spermatogenic cells, that differentiate into spermatids through meiotic

division (Figure 2.3B). The nuclei of elongated sperm cells are localized in their long tail shafts, condensed into interconnected units resembling beads in a string, and can be visualized in the testes of *nucleus-gfp* worms (Figure 2.3B). Moreover, the sperm with GFP localized in the nuclei provide a new sperm phenotype that can be differentiated from GFP+ sperm and GFP- wildtype sperm within the female antrum of a partner. The relatively large nuclei of undeveloped oocytes can be clearly visualized and easily counted within the ovaries of *nucleus-gfp* worms (Figure 2.3B). Additionally, the relatively larger nuclei of the prostate gland can be distinguished from nuclei of other cells in the tail plate, allowing us to count the number of prostate gland cells in the tail plate of the *nucleus-gfp* worms (Figure 2.3D).

NL21 – GFP in the testis

NL21 worms express the GFP transgene fused to the promoter of the ELAV4 gene, localized in the testicular tissue of their body (ELAV4::oGFP). Under epifluorescence illumination, the entire regions of the pair of testes are brightly illuminated by GFP (Figure 2.4A, hereafter, called *testis-gfp* line). However, given the relatively strong GFP signal, it is challenging to visualize in detail the cellular structures and developing sperm cells within the testes. Similar to *all-gfp* worms, sperm produced by *testis-gfp* worms express GFP in the cells. Differentiated GFP+ sperm can be observed being transported through the vas deferens to the seminal vesicle present in the tail plate of the worms (Figure 2.4A), where sperm can be seen stored until being transferred to the mating partner during copulation (Figure 2.4C). As the rest of the body is not illuminated under epifluorescence illumination, GFP+ sperm received from a mating partner can also be visualized in the female antrum of the *testis-gfp* worms and their behaviours can be observed *in vivo* (Figure 2.4D).

NL22 – GFP in the gut epithelium

NL22 worms express GFP fused to the promoter of the APOB gene, expected to show tissue-specific expression to the gut (APOB::oGFP; hereafter, called *gut-gfp* line). The gut lumen in *M. lignano* is surrounded by a single-layered epithelium of gastrodermal cells, mainly consisting of phagocytes and glandular cells. As observed in the *all-gfp* line, gastrodermal cells are relatively large and show strong GFP illumination in regions of the body posterior to the pharynx and anterior to the tail plate (Figure 2.5A), excluding the gonads. Posterior regions of the gut consistently showed relatively stronger GFP illumination compared to the anterior regions. The cells of diatoms ingested by the *gut-gfp* worms can be observed in dark within the gut of the worms (Figure 2.5C).

NL23 – GFP in the Ovaries

The worms from the NL23 line express GFP fused to the promoter of the CABP7 gene (CABP7::oGFP). Under epifluorescence illumination, the GFP transgene in NL23 (hereafter, called *Ovary-gfp* line) shows tissue-specific expression to the pair of ovaries (Figure 2.6A). The GFP transgene is also found illuminated in the developing oocytes and their nuclei during yolk formation in the growth zone, posterior to the ovaries (Figure 2.6B). The fertilized eggs in the antrum of *ovary-gfp* worms also express the GFP marker (Figure 2.6C). However, the transgenic GFP marker has not been observed and reported in eggs after they are laid by the *ovary-gfp* worms.

NL24 – GFP in the testes and RFP in the ovaries

NL24 line was established by introducing a double-reporter construct with tissue-specific expressions to the gonads. Thus, worms from the NL24 line (hereafter called double-GFP) express a GFP marker in the testis (ELAV4::oNeonGreen) and a red fluorescent protein (RFP) marker in the ovaries (CABP7::oScarlet-I) of the worms (Figure 2.7A). Under epifluorescence illumination, the expression patterns of the GFP marker are similar to that of the *testis-gfp* line, including GFP+ sperm (Figure 2.7B & D). The expression patterns of the RFP marker resemble that of the *Ovary-gfp* line but are illuminated in red in the *double-gfp* line (Figure 2.7C).

NL line crosses

Given the distinct transgene constructs and expression in the NL lines, we expected that crosses between NL lines may result in offspring that carry and express the transgene markers of both parent NL lines. Therefore, we formed mating pairs representing all possible crosses (21 crosses) between the 7 NL lines in wells of a 24-well plate and observed the offspring under epifluorescence to verify if transgene GFP (or RFP) markers of both parents are expressed in the offspring. In total, 14 of the 21 mating crosses resulted in offspring where transgene markers of both NL line parents could be observed reliably (Table 2.1). The other seven combinations could not be verified, mainly because the expression patterns of the two NL lines overlapped strongly in these combinations, making it difficult to differentiate the two markers (Table 2.1). This observation means that the transgenic markers of the NL lines can be used in combination as identifiers in tracking maternity and paternity in mating experiments.

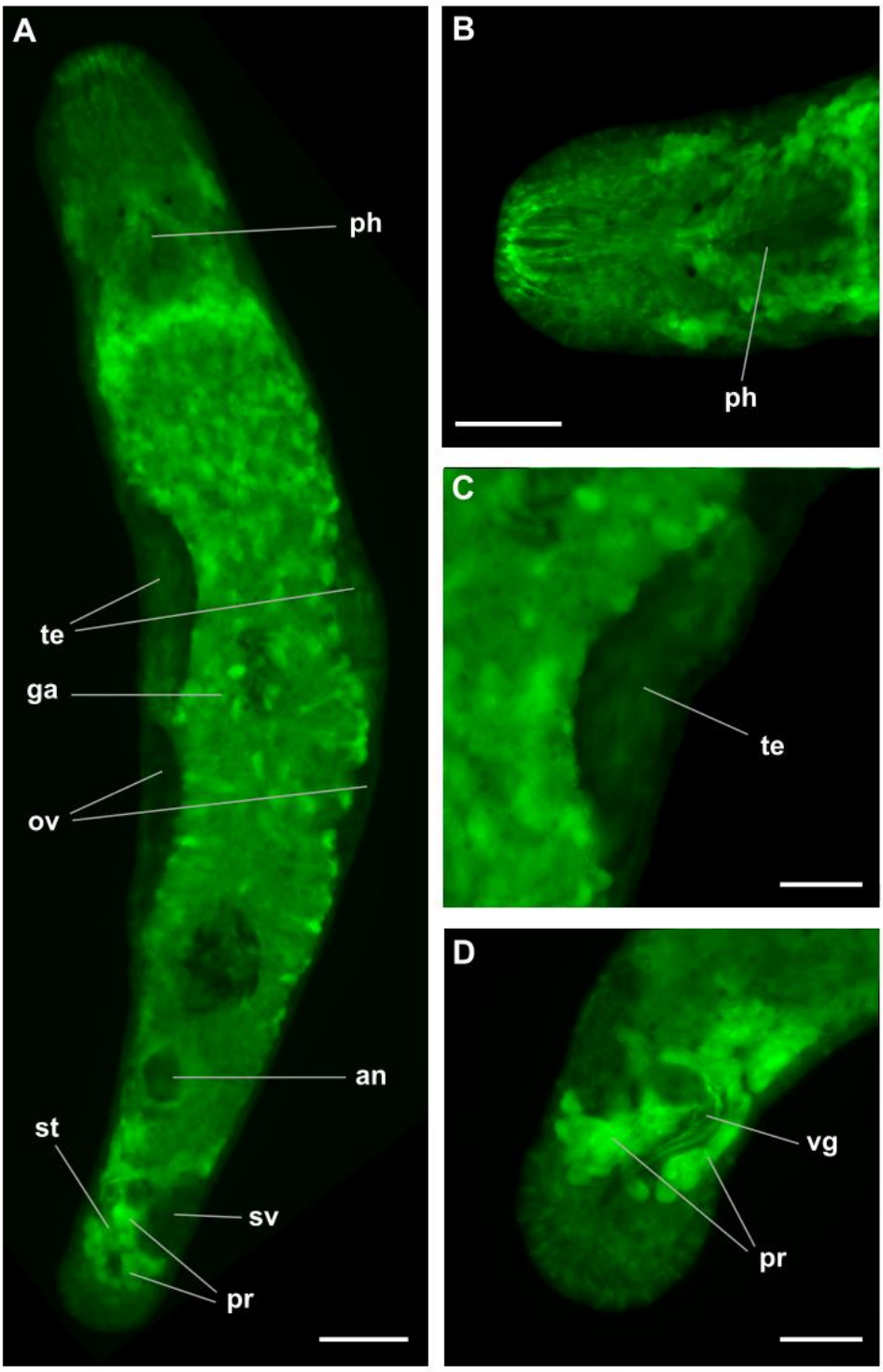


Figure 2.1 - An NL1 worm expressing GFP ubiquitously in all cells of the body under the promoter of EFA gene, visualized under epifluorescence illumination. A) the GFP expression in the whole body of the worm. B) The head end of the worm showing the rostrum. C) The weakly illuminated testis where developing sperm could be seen. D) The tail plate of the worm, showing clusters of strongly illuminated prostate gland cells and the vesicula granulorum. White bars indicate approximate scales: A & B - 0.1 mm, C & D - 0.05 mm. (an - female antrum, ga - gastrodermis, ph - pharynx, pr - prostate gland cells, ov - paired ovaries, te - paired testes, st - male copulatory stylet, sv - seminal vesicle carrying auto-sperm, vg - vesicula granulorum).

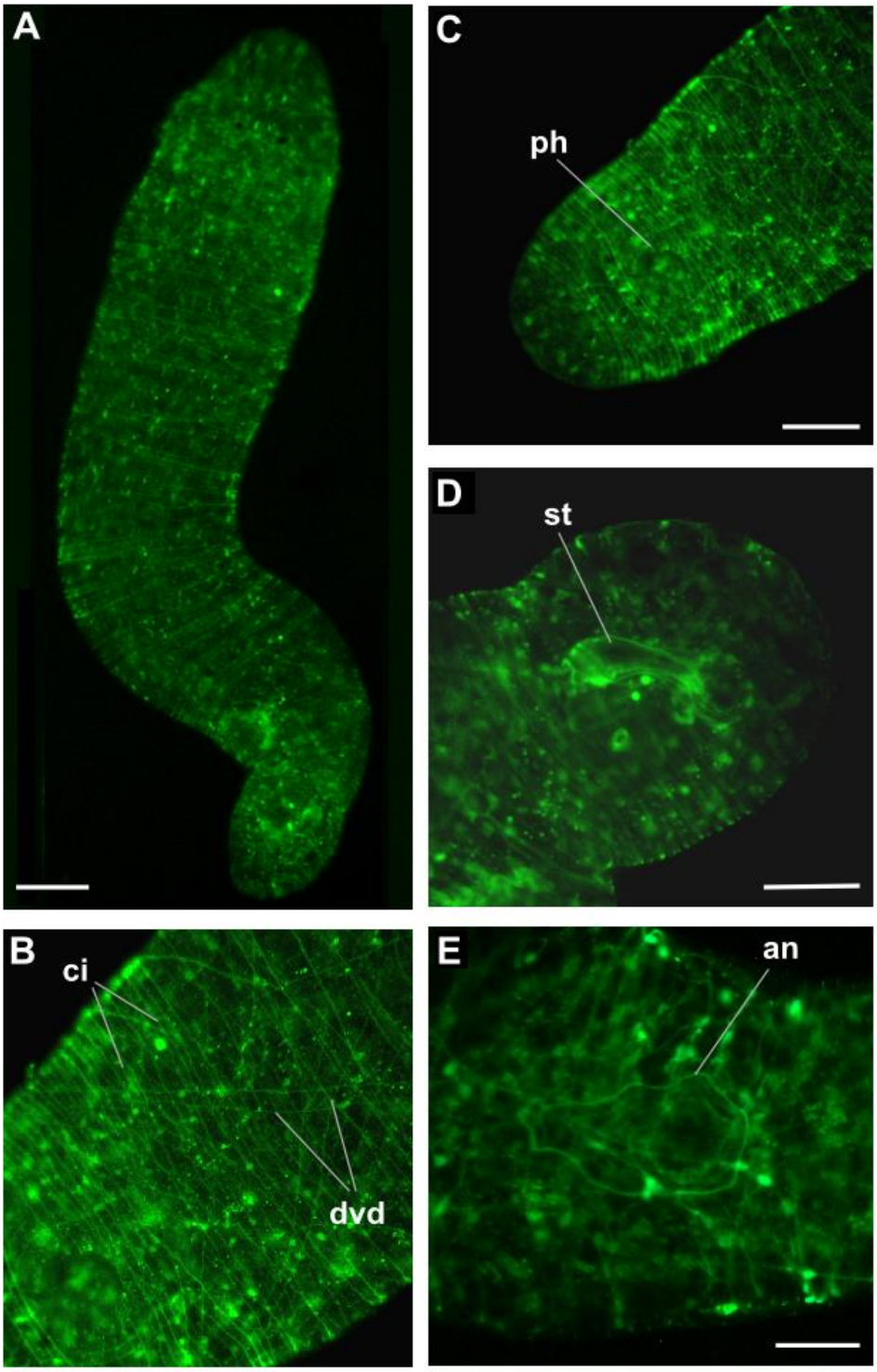


Figure 2.2 - An NL9 worm expressing GFP under a muscle-specific promoter of the MYH6 gene. A) the GFP expression in the whole body of an NL9 worm. B) a section of the body showing circular and dorso-ventral diagonal muscle cells. C) The head section with the pharynx. D) The tail plate of a worm showing muscles surrounding the male stylet brightly illuminated by GFP, likely plays a functional role in copulations. E) The concentric muscles along the wall of the female antrum White bars indicate approximate scales: A & B - 0.1 mm, C & D - 0.05 mm. (an – female antrum, ci – circular muscle cells, dvd – dorso-ventral diagonal muscle cells, ph – pharynx, st – male copulatory stylet)

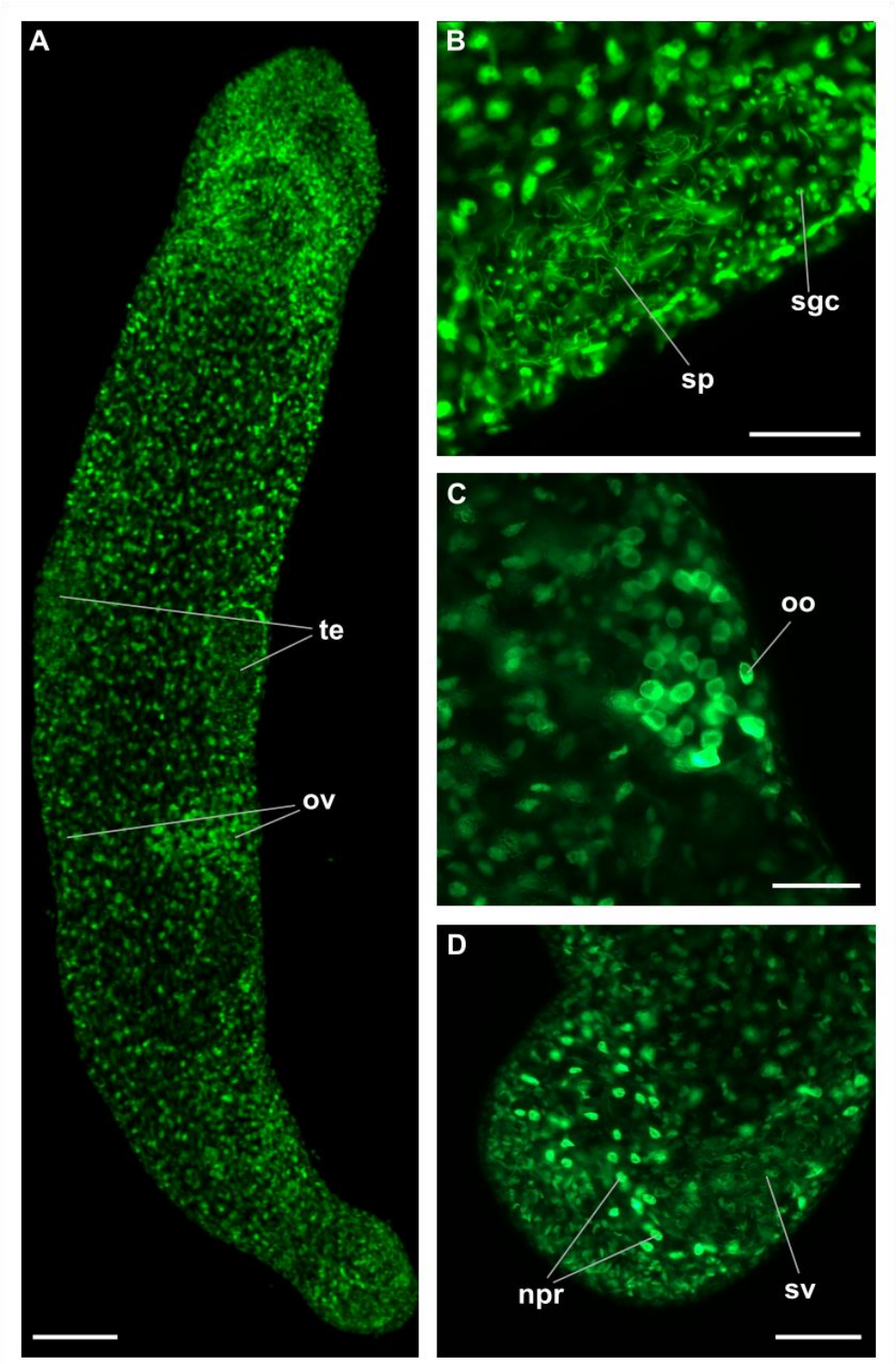


Figure 2.3 - An NL20 worm expressing GFP fused to the histone 2B gene. A) The nucleus of all the cells in the body expressing GFP. B) The testis region nucleus of spermatogenic and differentiated sperm cells. C) The ovary region showing large oocyte nuclei. D) The tail plate of a worm showing the relatively larger nuclei of prostate gland cells. White bars indicate approximate scales: A - 0.1 mm, B - 0.05 mm, C & D - 0.03 mm. (npr - nuclei of prostate gland cells, sgc - spermatogenic cells, sp - sperm cell nucleus, sv - seminal vesicle with sperm, te - testes, oo - oocyte nucleus, ov - ovaries)

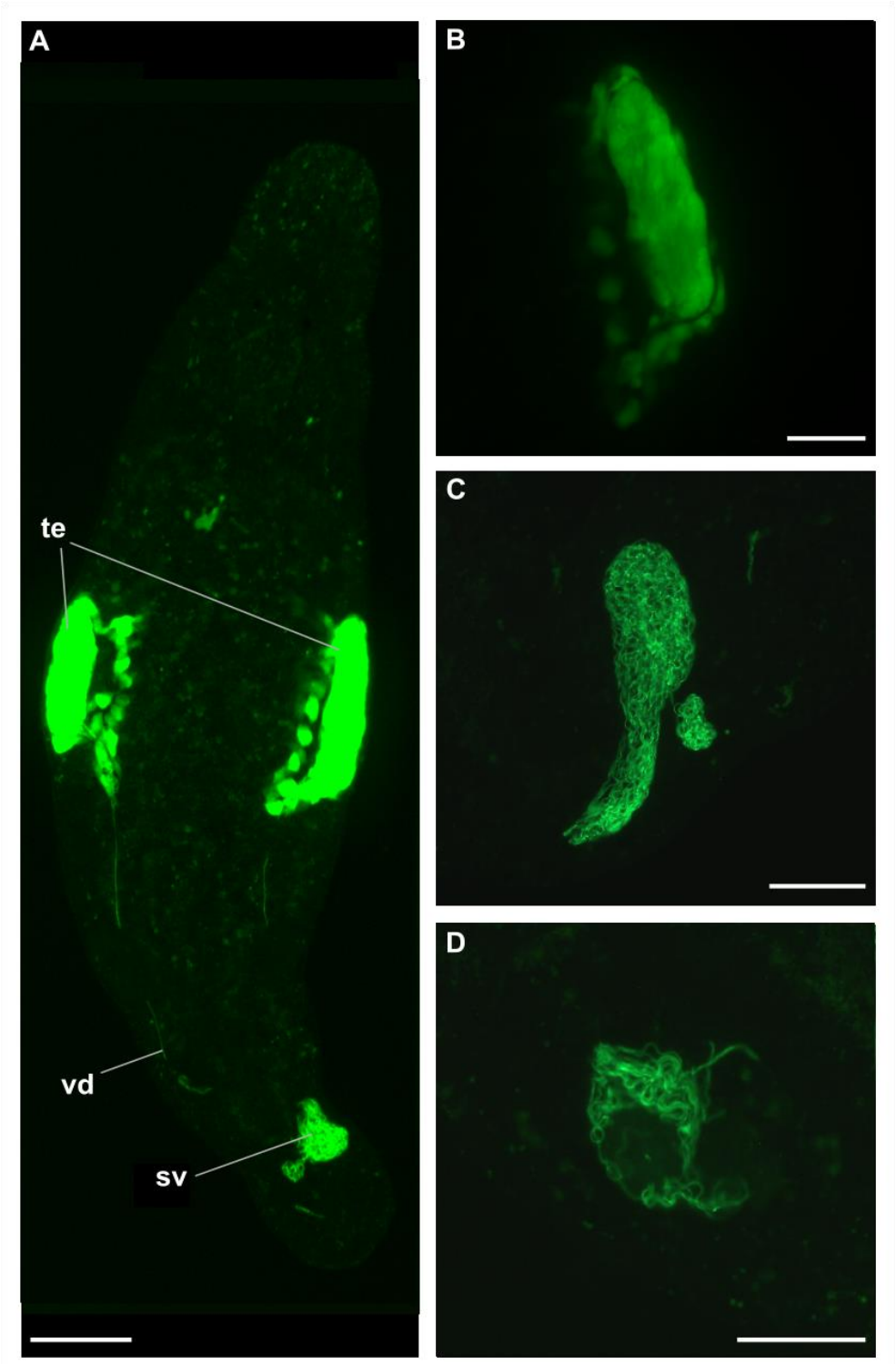


Figure 2.4 - An NL21 worm expressing GFP fused to the ELAV4 gene. A) The whole body of an NL21 worm, B) A testis from an NL21 worm expressing GFP. C) True and false seminal vesicle of NL21 where GFP+ sperm are stored until copulation D) The GFP+ sperm in the female antrum received from an NL21 mating partner. White bars indicate approximate scales: A - 0.1 mm, B - 0.05 mm, C & D - 0.03 mm. (te - testes, sv - seminal vesicle, vd - vas deferens carrying sperm from testes to the seminal vesicle)

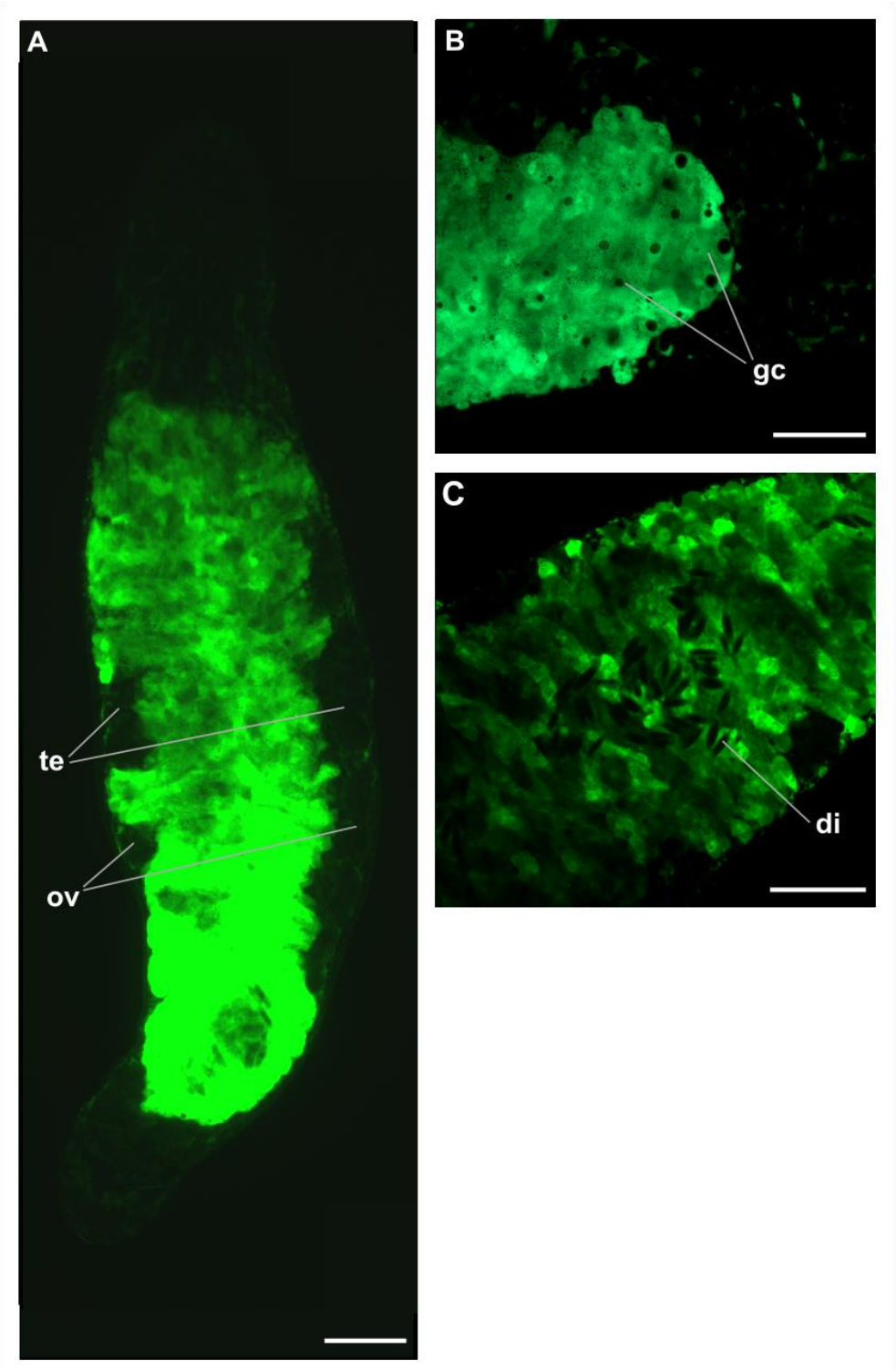


Figure 2.5 - An NL22 worm expressing GFP fused to the APOB gene in the gut epithelium. A) The whole body of an NL22 worm, B) the posterior end of the gut. C) Diatoms in the gut lumen. White bars indicate approximate scales: A & C - 0.1 mm, B - 0.05 mm. (te - testes, ov - ovaries, gc - gastrodermal cells expressing GFP, di - diatom cells)

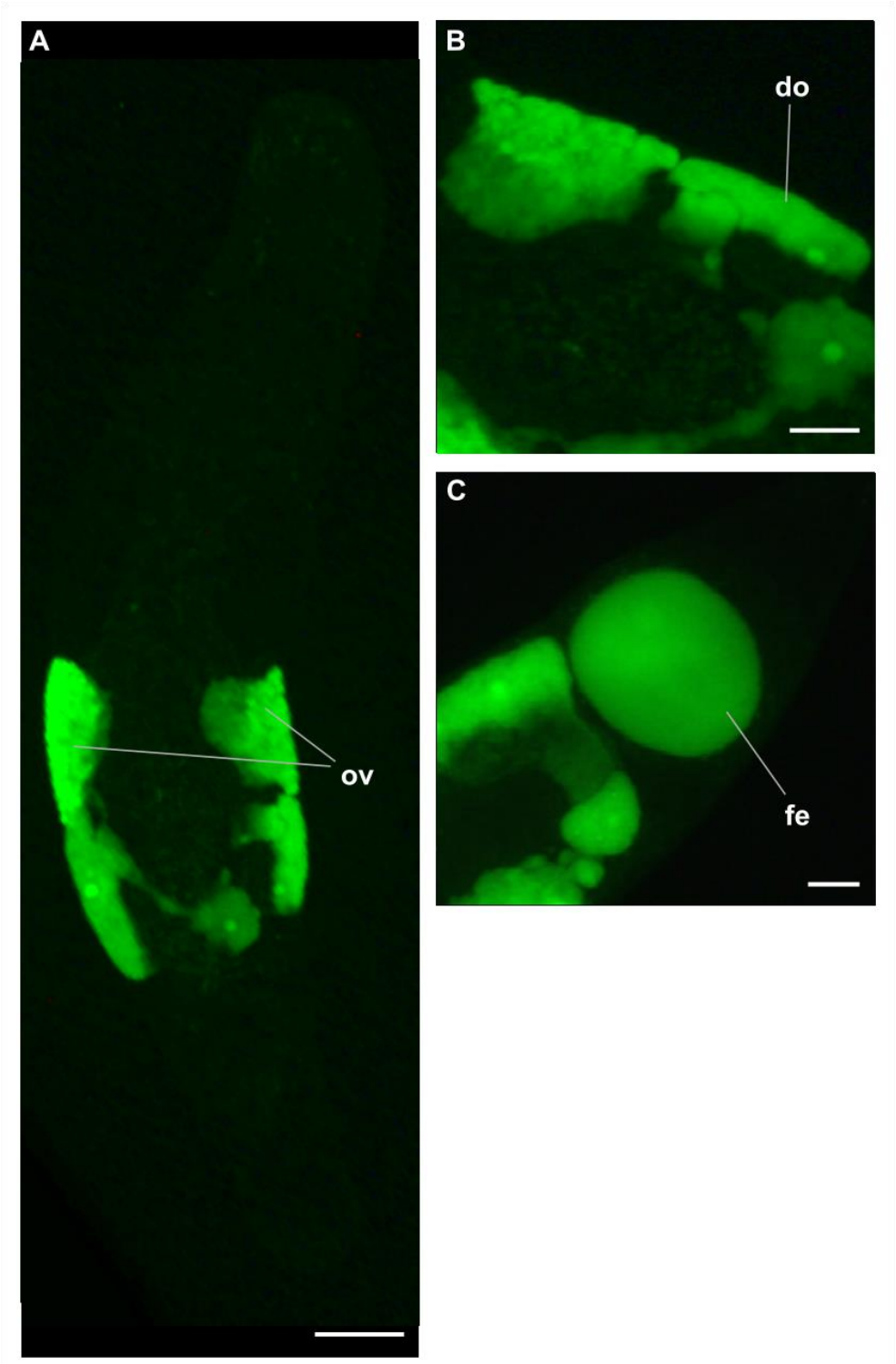


Figure 2.6 - An NL23 worm expressing GFP fused to the CABP7 gene in the ovaries. A) The whole body of an NL23 worm, B) developing oocyte in the growth zone posterior to the ovary C) a fertilized egg in the female antrum. White bars indicate approximate scales: A - 0.1 mm, B & C - 0.05 mm. (ov - ovaries, do - developing oocyte, fe - fertilized egg)

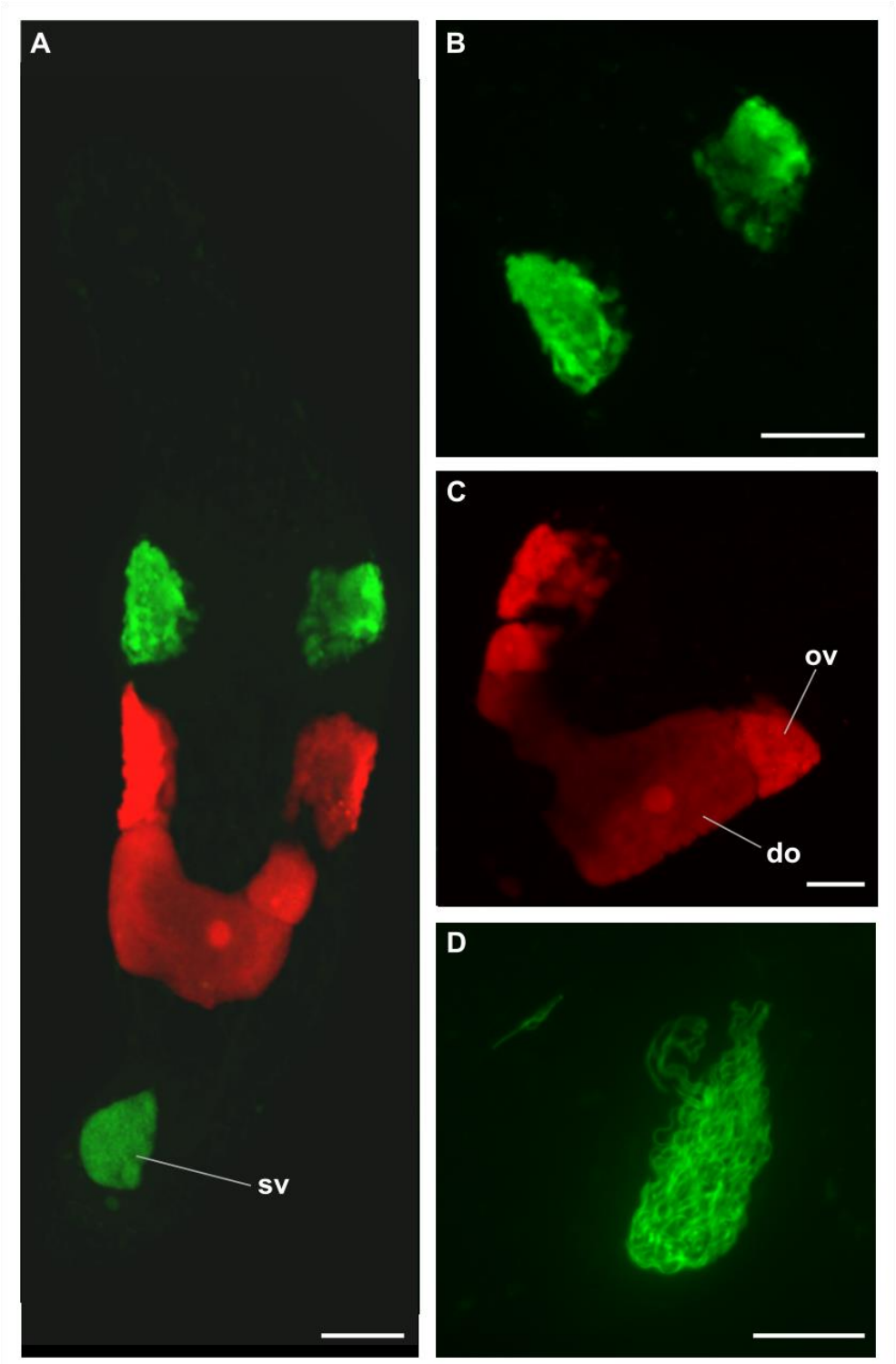


Figure 2.7 - An NL24 worm expressing GFP fused to the ELAV4 gene (ELAV4::oNeonGreen and RFP fused to the CABP7::oScarlet-I in the ovaries. A) The whole body of an NL23 worm, B) testes expressing GFP, C) Ovaries and developing oocytes expressing RFP, D) Seminal vesicle in the tail plate storing GFP+ sperm. White bars indicate approximate scales: A & B - 0.1 mm, C & D - 0.05 mm. (ov - ovaries, do - developing oocyte, sv - seminal vesicle)

Line ID	NL1	NL9	NL20	NL21	NL22	NL23
NL9	NO					
NL20	YES	NO				
NL21	NO	YES	YES			
NL22	NO	YES	YES	YES		
NL23	NO	YES	YES	YES	YES	
NL24	NO	YES	YES	NO	YES	YES

Table 2.1. Verification of offspring from NL line crosses. YES (Green): markers of both parents could be reliably observed in the offspring. NO (Red): markers of the parents could not be reliably observed/distinguished.

Experiments: NL lines vs DV1 line

To validate the NL lines, we performed experimental tests where we compared between the NL lines and the ancestral DV1 line the 1) mating behaviours, 2) morphological trait size and 3) measures of reproductive success.

Rearing conditions

On day 1, 200 adults from each of the 7 NL lines and 1200 adults of the DV1 line were distributed into Petri dishes, 100 adults in each, for egg laying with 20 ml ASW and ad libitum algae. On day 3, we removed all adults from the dishes controlling the age of the resulting juveniles in the petri dishes to less than 48 hours. On day 9, we sampled the hatchlings to form rearing pairs of 2 hatchlings with 1 worm from each NL line (focal worms) paired with 1 wild-type DV1 worm. DV1 rearing pairs were formed, as control pairs, with two worms from the DV1 inbred line. In total, 66 pairs were formed from each NL line and the DV1 line and were placed in wells of 24-well plates with 1.5 ml of 32‰ ASW and ad-libitum algae. To control for any environmental effects during development, every 24-well plate contained 3 pairs from each of the 8 inbred lines placed in randomly assigned wells. All pairs were transferred to fresh ASW and algae every 6-10 days. All the following experiments were performed using the worms from these rearing groups.

All statistical analyses for the following experiments were performed in R (Version 4.2.3).

Experiment 1. Mating behaviour of NL lines vs DV1 line individuals.

Experimental setup

On days 42-44, we tested if individuals of the GFP+ NL lines have similar mating behaviour to the ancestral GFP- DV1, by measuring the willingness of worms to initiate mating (time taken until the first mating) and their mating rates. For this, we conducted mating trials in pairs of worms. Focal NL worms or a randomly chosen DV1 worm from experimental rearing groups (focal worms) were paired with a DV1 partner worm of a similar age, and also reared in pairs independently. The mating behaviour of the pairs was recorded for a period of 2 hours using standardized mating chambers (Scharër et al., 2004) placed under a digital video camera (Imagingsource DFK31BF03). A total of 22 pairs were formed representing each line (seven NL lines and one DV1). Each mating chamber was made with 8 drops of 32% ASW each with one replicate pair from each of the 8 lines under study. The movies were then analysed in VLC media player to measure the time taken for the first copulation to occur (mating latency) and the total number of copulations performed by the pair while being blind to the line identity of the worm.

Statistics

In total, mating behaviours were measured for a total of 163 worms, across all lines. 13 pairs were lost due to handling errors during mating chamber preparation or during recording. To compare the NL lines and the ancestral DV1 line, the mating latency data was square-root transformed for analysis. We tested the effect of NL line identity on mating latency by fitting a linear model with NL line identity as the fixed factor. Pair-wise comparisons were performed using the 'DunnettTest' function from the DescTools package in R using the DV1 line as control. For the number of matings, we fitted generalized linear models (GLM) assuming a quasipoisson error distribution and log-link function using reproductive behaviour measures as the dependent variable and the NL line identity as the fixed factor. Pairwise comparisons between the NL lines and the control DV1 line were performed using Dunnett's method in the 'contrast' function of *emmeans* package using the DV1 line as control.

Results

43 worms did not mate during the period of observation. Mating latency could not be calculated for these worms and hence they were omitted from our analysis. However, individuals with no matings were included in the analysis of the number of matings. We found a significant effect of line identity in mating latency ($F = 3.012$, df = 7, $R^2 = 0.106$, $P = 0.006$) and number of matings ($\chi^2 = 33.87$, df = 7, $R^2 = 0.165$, $P = 1.8e^{-5}$). The pairwise comparisons of mating latency revealed that 3 out of 7 NL lines took significantly longer to mate for the first time compared to the DV1 line (Figure 2.8).

We observed that the same 3 NL lines also exhibited a significantly lower number of matings compared to the DV1 line.

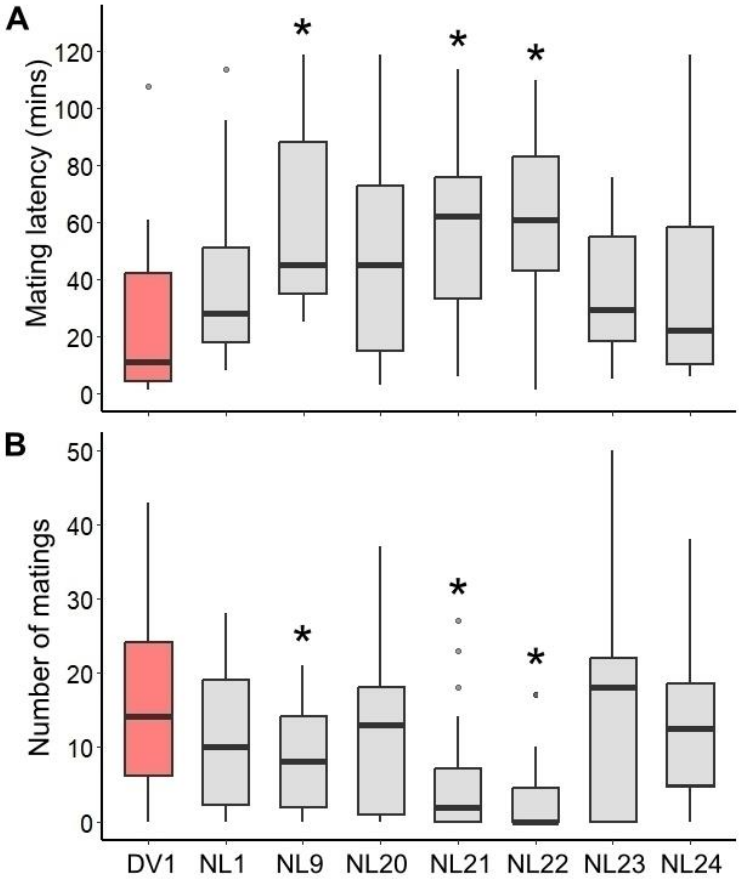


Figure 2.8 - Reproductive behaviour of worms from the NL lines and the control DV1 line. Comparisons of mating latency (A) and number of matings (B) between NL and the wildtype control DV1 line individuals raised in pairs of 2 worms. The measurements of the control DV1 inbred line are shown by the first boxplot from the left (in red). * indicates comparisons with the DV1 line that revealed significant differences ($P < 0.05$). See text for statistics.

Experiment 2. Morphological traits of NL lines vs DV1 line individuals.

Experimental setup

To test if individuals of the GFP+ NL lines have similar morphologies to the ancestral GFP- DV1, we measured a suite of morphological traits from the NL worms from the NL rearing groups and a randomly chosen worm from the DV1 rearing groups on days 69 - 71. A total of 25 worms were measured from each line. Worms were prepared using a standard protocol (described in Schärer and Ladurner 2003) and were observed under a Leica DM 2500 microscope (Leica Micro-systems, Germany). Digital images

were acquired using a video camera (The Imaging Source, DFK 41BF02) connected to the imaging software BTV Pro 6.0b7 (http://www.bensoftware.com/), at 100x for the total body size and 400x for all other morphological traits. Measurements of the morphological traits were performed in ImageJ 1.53c using the ObjectJ plugin (https://sils.fnwi.uva.nl/bcb/objectj/). From each worm, we measured the areas of total body size, pairs of testes and ovaries, and the seminal vesicle, while being blind to the line identity of the worm. Measurements were skipped if a morphological trait could not be observed reliably in the sample.

Statistics

Of the total 200 worms prepared, 3 worms were lost due to handling errors. Testes could not be measured for 3 worms, and seminal vesicles could not be photographed for 12 worms. For 8 worms each, only one of the testes and ovaries could be measured. The total area of the testes and ovaries was calculated as the sum of the areas of each pair. In worms where only one testis or ovary could be measured, the total area was taken to be twice the area of the one measured. All morphological traits were log-transformed for statistical analysis. To test for morphological differences between the lines, we fitted linear models independently for the 4 morphological traits, with NL line identity as the fixed effect. Pair-wise comparisons were performed to compare all the NL lines to the control DV1 line, using a Dunnett's test from the *DescTools* package (V0.99.49) in R.

Results

We found a significant effect of line identity in body size ($F_{7,189}$ = 11.958, R^2 = 0.281, P = 1.36e^{-12}), testes ($F_{7,189}$ = 6.8, R^2 = 0.174, P = 3.42e^{-07}), ovaries ($F_{7,189}$ = 2.89, R^2 = 0.063, P = 0.007) and seminal vesicle ($F_{7,189}$ = 2.49, R^2 = 0.054, P = 0.018). Dunnett's tests for the morphological traits revealed several significant differences in pairwise comparisons (Figure 2.9), but all significant comparisons represented a larger size of the morphological trait in the respective NL line. None of the NL lines showed a significantly smaller size in any morphological traits (Figure 2.9), suggesting the transgene introduction has not negatively affected any morphological development in the NL lines.

Experiment 3. Reproductive success

Experimental setup

To test if the reproductive capacity of NL line worms was similar to that of the ancestral DV1 line, we measured the reproductive success of the NL worms in two ways. First, we measured the reproductive success as the total number of offspring of the male and female sex function, separately, by isolating all worms from rearing pairs for 7 days (days 93 – 100). Female reproductive success or female fecundity produced by rearing pairs (i.e., NL worm paired with a DV1 partner worm), during a 2-week period

(between days 33 – 47). Second, we measured reproductive success was measured as the number of offspring produced by the focal worms through their female function (NL worms or one randomly chosen DV1 worm). Male reproductive success or male siring success was measured as the number of offspring produced by the DV1 partner worm from the rearing pairs.

Statistics

The reproductive success of pairs was measured for a total of 528 pairs, i.e., 66 pairs for each inbred line. At the time of measuring male and female reproductive separately, 51 pairs could not be measured as one or both worms of the pairs were lost during the rearing. To compare if the measures of reproductive success (reproductive success in pairs, male siring success and female fecundity) were different between the NL lines and the ancestral DV1 line, we fitted generalized linear models (GLM) assuming a quasipoisson error distribution and log-link function with the number of offspring produced as the dependent variable and the NL line identity as the fixed independent factor. Pairwise comparisons against the control DV1 line were performed using Dunnett's method in the 'contrast' function of *emmeans* package.

Results

We found a significant effect of line identity in the reproductive success of pairs ($\chi^2 = 126.12$, df = 7, $R^2 = 0.18$, $P = < 2.2e^{-16}$), male siring success ($\chi^2 = 24.67$, df = 7, $R^2 = 0.048$, $P = < 8.7^{-4}$) and female fecundity ($\chi^2 = 163.61$, df = 7, $R^2 = 0.232$, $P = < 2.2e^{-16}$). Dunnett's pairwise comparisons revealed that almost all NL lines had significantly higher measures of reproductive success in pairs and fecundity in the female function (Figure 2.10). 4 of 7 NL lines showed significantly higher male siring success. None of the pairwise comparisons showed a significantly lower reproductive success in the NL lines compared to the DV1 line (Figure 2.10).

Discussion

The results of the experiments overall suggest that the integration and expression of the different fluorescent protein constructs did not negatively affect the development or reproductive functions of the NL lines. Therefore, our findings suggest that the transgenic NL lines represent a valuable and reliable tool for the study of sexual selection. The different transgenic markers are inherited by respective offspring and can be used as identifiers to assess maternity and/or paternity of worms efficiently. Here we discuss the utility of the NL lines and biological applications of techniques that are now possible with these inbred lines. We also discuss the considerations and limitations of using NL lines.

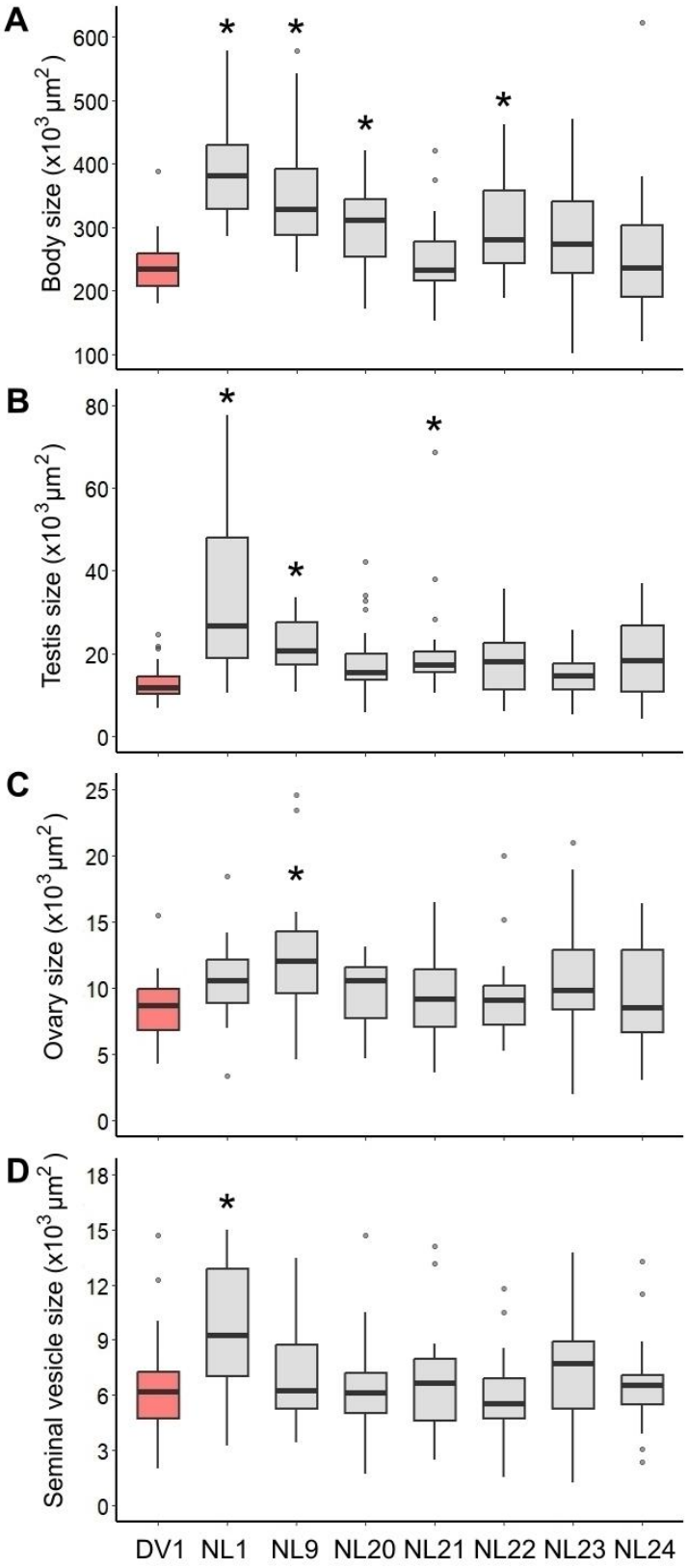


Figure 2.9 - Morphological measurements of worms from the NL lines and the control DV1 line. The Comparisons of body size (A), testis size (B), ovary size (C), and seminal vesicle (D) between NL and the DV1 line individuals raised in pairs of 2 worms. The measurements of the control DV1 inbred line are represented by the first boxplot from the left (in Red).

* indicates comparisons with DV1 line that revealed significant differences ($P < 0.05$). See text for statistics.

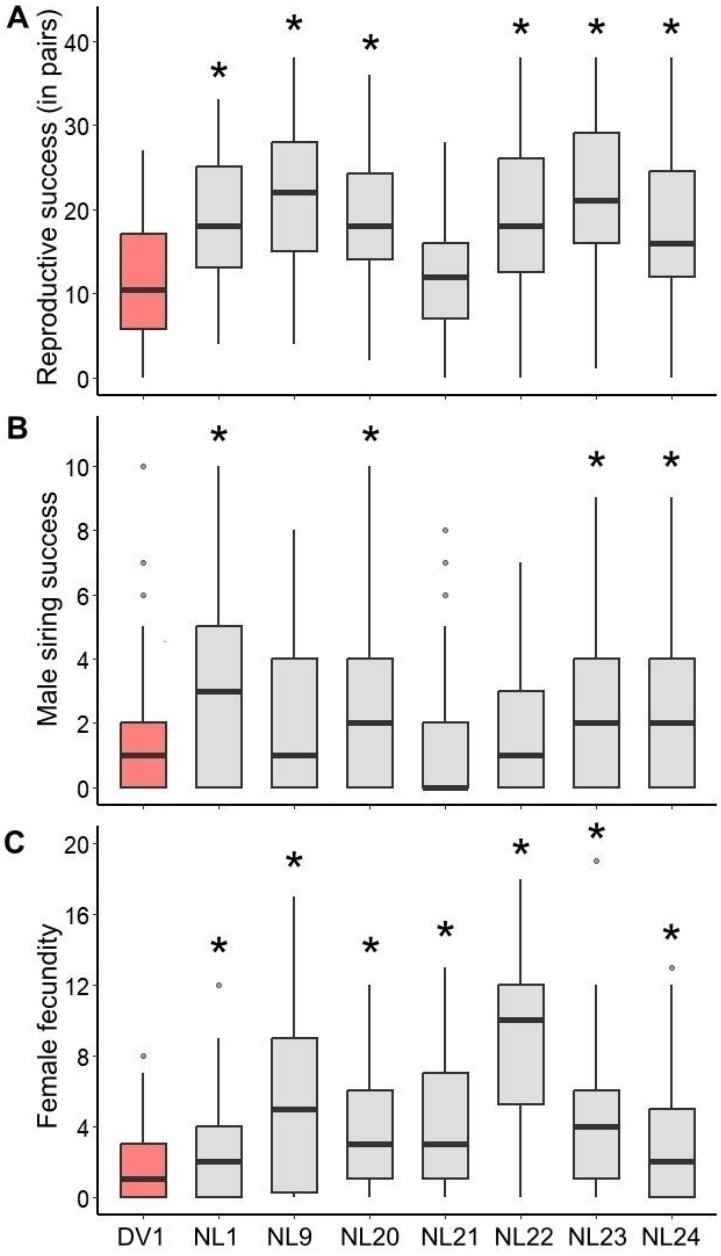


Figure 2.10 - Reproductive success of worms from the NL lines and the control DV1 line. Comparisons of reproductive success of pairs (A), male siring success through the partners (B) and female fecundity of focal worms (C) between NL and the wildtype control DV1 line individuals raised in pairs of 2 worms. The measurements of the control DV1 inbred line are represented by the first boxplot from the left (shown in Red). * indicates comparisons with the DV1 line that revealed significant differences ($P < 0.05$). See text for statistics.

Validation of the transgenic lines

The transgene DNA constructs introduced into single-cell stage eggs are presumably integrated at random sites in the genome. This may have detrimental effects on the NL lines by affecting important genes at the integration sites or due to phototoxicity

resulting from high levels of fluorescent protein expression (e.g., Dixit and Cyr, 2003; Tinevez et al., 2012). In *Drosophila melanogaster*, the transgenic lines expressing GFP or RFP were found to perform less well compared to the wildtype in some reproductive assays (Manier et al., 2010). Similarly, also in *M. lignano*, certain other transgenic lines (HUB2 and HUB4) have been found to take longer time to develop compared to the wildtype worms (Demircan, 2013. PhD Thesis, Unpublished.). In this study, pairwise comparisons between the NL lines and the ancestral DV1 line of morphological traits revealed significantly larger trait sizes in some NL lines (Figure 2.10). This does not represent a negative effect in the NL lines, however, this result is still surprising as all the NL lines, except NL24, were generated from the wildtype inbred DV1 line and are expected to be genetically very similar. Body size has been shown to not influence reproductive success in both male and female sex functions in *M. lignano* (Janicke and Scharër, 2009; Janicke et al., 2011; Marie-Orleach et al., 2016), and neither does ovary size on female fecundity (Janicke et al., 2011). However, testes size and seminal vesicle size have been found to be positively associated with sperm transfer success and male reproductive success in *M. lignano* (Marie-Orleach et al., 2016). Hence, the significantly larger testes size and seminal vesicle size in some NL lines have to be taken into consideration when using these NL lines, especially in experiments studying aspects of sexual selection.

Four NL lines took significantly longer to copulate for the first time compared to the wild-type DV1 line in our 2-hour-long mating behaviour observations. This could, at least partly, also explain the lower number of copulations in these lines (especially NL21 and NL22), as they had a shorter duration after the first copulation to accumulate additional copulations. Mating success has been found to be positively associated with fitness components such as sperm transfer success but was a poor predictor of overall male reproductive success in *M. lignano* (Marie-Orleach et al., 2016). In line with this supposition, the fewer number of copulations during our mating trials was not reflected in a lower reproductive success in these lines. In fact, a majority of pairwise comparisons of reproductive success measures showed significantly higher reproductive performance in the NL lines, with none of the comparisons showing significantly lower values compared to the DV1 line. Therefore, it is slightly unclear whether the lower mating rate observed in some lines is an artefact of our methods, in that worms from certain lines took longer to acclimate to the mating chambers and the new mating partner. Yet, we believe that this is unlikely to be the primary explanation as we minimized any environmental effects on the life-history of the NL lines and mating partners were assigned randomly to the focal NL lines in our experiment. Nevertheless, our results recommend caution in using especially *testis-gfp* and *gut-gfp* lines in certain experimental designs where their mating success may be a significant factor.

In this study, we did not assess the inheritance patterns of the transgene markers. The inheritance patterns in a different transgenic line of *M. lignano*, the GFP+ HUB1 line, were found to largely follow Mendelian segregation assuming a dominant allele on a single diploid locus (Marie-Orleach et al., 2014). However, a rare occurrence of deviations has been observed in this line (Vizoso D.B., Marie-Orleach L., and Schärer L., unpublished observations). Additionally, the integration sites of the transgene constructs in the genome have not been identified yet for the NL lines, except for the *testis-gfp* line. The DV1 wildtype has been observed to carry a chromosomal polymorphism, with two, three or four copies of the largest chromosome. The NL lines derived from the DV1 line are hence also expected to carry similar karyotype polymorphisms. If the transgene integration site in any of the lines sits on the large chromosome, this polymorphism could potentially affect inheritance patterns and dosage of the transgene DNA construct in the NL line worms. Detailed molecular analysis and studying multi-generational crosses are needed in order to better understand the expression of the transgenes and their transmission to offspring.

Outlook

The transgenic NL lines expressing distinct fluorescent markers provide a new toolkit for research in sexual selection, which will greatly improve the possibilities for experimental designs in the model organism, *M. lignano*. The GFP+ worms of *M. lignano* have already been effectively used to study various aspects of sexual selection. GFP+ sperm from a transgenic line could be differentiated from the GFP- sperm of a competitor within the female sperm receiving and storage organ, the female antrum *in vivo*. This, for example, made it possible to disentangle social group size from mating group size and to study the relationship between mating group size and sex allocation in *M. lignano* (Janicke et al., 2013). GFP techniques developed in *M. lignano* also enabled quantitative analysis investigating the relative significance of the pre- and post-copulatory episodes of sexual selection in *M. lignano* (Marie-Orleach et al, 2016, 2021) and the form of selection arising from these episodes on morphological traits (Marie-Orleach et al., 2024).

The herein-described *nucleus-gfp* (NL20) worms showing GFP localised in the nucleus of sperm cells provide a third distinguishable sperm phenotype. Specifically, three sperm phenotypes (GFP+, GFP- and GFP only in the sperm nucleus) can now be distinguished within the female antrum. This feature enables opportunities for a more fine-scaled quantitative study of post-copulatory processes such as sperm displacement in *M. lignano*. For example, sperm transfer success inferred from distinguishing sperm inside the female antrum can now be measured from 2 focal worms (i.e., if they express GFP in sperm or the sperm nucleus) in a mating group, unlike other studies that used 1 focal worm (e.g., Marie-Orleach et al., 2016; 2021). Similarly, the transgenic markers can be transmitted to offspring through either the male or the female sex function.

Mating crosses between NL lines (14-21 crosses tested) resulted in offspring where transgenic markers of both parents could be observed. This means that the transgene markers of the NL lines can be used as reliable identifiers of both parents in mating experiments that require parentage analysis. For example, if an experimental mating group of 4 worms were formed with one worm each representing the *nucleus-gfp*, *testis-gfp*, *ovary-gfp* and *gut-gfp* lines, both maternal and paternal parents of all resulting offspring can be identified with ease. The *nucleus-gfp* line provides certain other possibilities. The ability to visualize and count nuclei of spermatogenic cells in the testes and oocytes in the nucleus could, for example, be used as proxy estimates of the functioning of the respective gonads or investment into the male and female sex functions. This may also enable a more informative assessment of sex allocation plasticity, by assessing the plastic variation in the number of spermatogenic and oocyte nuclei in response to mating group size or other factors. Moreover, the relatively larger nuclei of prostate gland cells in the tail plate can also be visualized and counted in *nucleus-gfp* worms, providing an opportunity to quantitatively account for another investment into the male sex function in *M. lignano*.

However, given the little knowledge available on the inheritance patterns of the NL lines, it is possible that some offspring of the NL do not inherit and express the transgene marker. This has been observed in the past and also as part of this study, and could constrain some experimental designs. A potential solution to circumvent this issue would be to identify the subset of worms that reliably transmit the marker to offspring or are homozygous for the marker. These worms can be identified by pairing NL line worms to a wildtype DV1 partner and testing if all offspring produced by the pair express the transgene marker (with a representative minimum number of offspring tested). Experiments using NL lines can be designed to include homozygote identification and then only use those worms that have been validated to be homozygous for the marker.

Conclusion

The phenotypic descriptions and the experiments performed in this study show that the NL lines of *Macrostomum lignano* are a reliable and useful tool. Overall, we did not find any pervasive negative effects of the transgene introduction in any of the NL lines. The NL lines add possibilities to an already powerful toolkit of experimental approaches used in *M. lignano* with the help of other transgenic lines, in terms of observing post-copulatory processes and paternity analyses. We conclude that the availability of NL lines and associated transgene markers in a transparent organism is a powerful tool to acquire new insights into sexual selection as well as in other fields of biology.

Chapter III

Bateman's Principles in a Reciprocally-Mating Simultaneous Hermaphrodite

Abstract

Sexual selection theory posits that anisogamy (the sex difference in gamete size) gives rise to varying reproductive interests in the two sexes and, consequently, to sex-specific selection on morphological, behavioural, physiological and life-history traits. Thus, males producing smaller, abundant sperm have been shown to experience stronger selection on mating activity compared to females producing costly eggs. In hermaphrodites, however, the male and female sex functions are tied together in the same body with no possibility for sexual dimorphism to evolve. Additionally, reciprocal mating behaviours in some hermaphrodites strictly link the mating activity in the two sex functions, further limiting the evolution of sex-specific mating behaviour. Here we tested for sex differences in sexual selection in the reciprocally mating hermaphroditic flatworm, *Macrostomum lignano*. We used inbred lines expressing distinct GFP markers that enabled paternity assignment and conducted mating experiments using small groups of worms to measure the mating and reproductive success of the worms. Our results did not support the prediction of stronger sexual selection in the male compared to the female sex function. We conclude that sexual selection in *M. lignano* deviated from conventional expectations, suggesting anisogamy alone may not be sufficient to explain the sex-difference in the strength of sexual selection. However, a meta-analysis of recent studies suggests that the conventional expectation of stronger sexual selection on male sex function may still hold across hermaphrodites.

Introduction

Sexual selection is thought to arise from competition for access to mating partners and/or their gametes (Kokko et al. 2006; Jones and Ratterman 2009) and has been posited to play a crucial role in the evolution of reproductive traits in animals and plants (Clutton-brock, 2007). When Darwin (1871) introduced the concept, he argued that males are typically eager to copulate, whereas females are coy and choosy with respect to mating. However, it took decades before researchers began to explore the underlying causes for Darwin's postulated difference between the sexes. In his landmark paper, Bateman (1948) presented results of mating experiments with fruit flies, *Drosophila melanogaster*, showing that male but not female reproductive success is highly correlated with mating success, which he argued is the *cause* for higher intra-sexual competition for mates among males. In accordance with these findings, male fitness is often considered to be primarily limited by access to mating partners whereas female fitness depends primarily on the availability of resources that can be used for egg production – a concept that is often termed the Bateman's principle (Arnold 1994). Importantly, Bateman also speculated on the ultimate reason for the observed difference between the sexes, arguing that anisogamy (i.e., production of discrete types of gametes by the sexes) drives the difference in the relationship between reproductive success and mating success between males and females (Bateman 1948). Recent theoretical works provide strong support for Bateman's idea that anisogamy promotes sex differences in the strength of sexual selection (Lehtonen et al., 2016; Parker, 2014; Lehtonen, 2022) and the evolution of Darwinian sex roles in terms of male-male competition, female choosiness and female-biased parental care (Schärer et al., 2012). Empirical work suggests that not only males but also females of many species benefit from having multiple mating partners (e.g., Arnqvist & Nilsson, 2000; Fromonteil et al., 2012) and might therefore be also subject to pre-copulatory sexual selection (Hare & Simmons, 2019). Yet, even if sexual selection in females might be more widespread than envisioned by the pioneers of the field, evidence from meta-analysis suggests that males are typically more prone to compete for mating partners (Janicke et al., 2016), illustrating that the so-called Darwin-Bateman paradigm (Dewsbury, 2005) predominates across the animal tree of life.

The vast majority of theoretical and empirical work in the study of sexual selection has focussed on gonochoristic organisms (i.e., species in which sexes are expressed separately in individuals), attempting to explain the tremendous diversity in dimorphism between males and females. Yet, despite being widespread across animals (and plants) (Jarne & Auld, 2006), simultaneous hermaphrodites (i.e., species in which both sexes are expressed within the same individual at the same time) have received relatively little attention in the study of sexual selection. Phenomena commonly associated with sexual selection such as complex courtship and copulatory behaviour,

multiple mating, sperm competition and elaborate genitalia are common and highly developed in hermaphrodites (reviewed in Leonard, 2006; Michiels, 1998; Anthes 2010). Additionally, theoretical studies have synthesized extensions to sexual selection models developed by gonochorists and concluded that sexual selection can play a crucial role in hermaphroditic systems (e.g., Charnov, 1979; Morgan, 1994; Willson, 1994). The male and female sex functions are tied together in the same body in hermaphrodites leaving a limited opportunity for sex-specific trait expression and sexual dimorphism. Yet, experimental studies testing for sex-differences in simultaneous hermaphrodites suggest that anisogamy alone, without sexual dimorphism, may be sufficient to generate stronger sexual selection in the male compared to the female sex function (Pelisse et al., 2012). However, unilateral mating behaviour (i.e., one partner assumes the male role and donates sperm, while the other partner copulates in the female role receiving sperm) occurs in some simultaneous hermaphrodites and may allow for flexibility in sex role preferences and variation in the number of copulations in each role (e.g., Nakadera et al., 2015).

In simultaneous hermaphrodites, conflict of interest between mating partners over sex-roles have been suggested to be resolved through strategies such as reciprocal copulation (Leonard, 2005; Schärer et al., 2015), in which each individual mates in both sex roles, donating and receiving sperm, simultaneously. Thus, copulations in the male and female sex functions are strictly linked and are constrained to be equal in number and variance under reciprocal mating, which can be more relaxed in unilaterally mating simultaneous hermaphrodites and gonochorists. Therefore, sex-differences in sexual selection in reciprocally mating hermaphrodites are expected to arise more from the differences in how copulations translate into offspring production through the partners, which may foster a shift in sexual selection from the pre- towards the post-copulatory arena in terms of pronounced sperm competition and cryptic-female choice. Previous studies quantifying episodes of sexual selection in the reciprocally mating flatworm, *Macrostomum lignano*, suggest that sexual selection might indeed operate primarily at post-copulatory stages of selection in simultaneous hermaphrodites (Marie-Orleach et al., 2016, 2021). Given the absence of sexual dimorphism and variation in copulatory mating success between the sex functions, measuring sexual selection in reciprocally mating hermaphrodites may contribute to a better understanding of whether anisogamy alone is sufficient to drive stronger sexual selection in the male sex function. To our knowledge, only one study has until now investigated sex-specific sexual selection in a reciprocally mating hermaphrodite and found a positive correlation between the number of mating partners and reproductive success for both male and female sex functions, suggesting no difference in the strength of sexual selection (Pongratz & Michiels, 2003).

Quantifying sexual selection in simultaneous hermaphrodites requires accounting for potential interactions between the two sex functions within an individual. Between-individual variation in resource allocation towards the two sex functions may determine the relative reproductive effort through each sex function (including the preferred sex role during mating) (Janicke and Schärer, 2009; Schärer et al., 2015), such that reproductive success in the two sex functions are inherently non-independent. For example, under the assumption of a sex allocation trade-off, mating frequently in the male role may increase male reproductive success, but may reduce egg production through the female role. Thus, such cross-effects and the potential for sexually mutualistic or antagonistic selection between the two sex functions are important aspects of sexual selection in simultaneous hermaphrodites. Anthes et al. (2010) provided the conceptual framework for quantifying sexual selection in simultaneous hermaphrodites by assessing such cross-sex effects, which have been applied in only a few recent studies in unilaterally mating hermaphrodites (Hoffer et al., 2017; Pélissié et al., 2012).

In the present study, we aim to quantify sexual selection on the male and female sex functions of the reciprocally-mating hermaphroditic flatworm, *Macrostomum lignano*. We conducted mating experiments using transgenic GFP+ lines of *M. lignano,* which allowed us to track parentage and to measure male and female reproductive success reliably. Following the approach suggested by Anthes et al. (2010) we used standardised metrics of sexual selection to test if 1) sexual selection operates in both sex functions of *M. lignano* and 2) if the male sex function is subject to stronger sexual selection (as predicted by Bateman's principle). Finally, 3) we performed a meta-analysis of the few existing studies to place our results in a broader context and to test for general patterns of sexual selection in simultaneous hermaphrodites.

The variance-based metrics of sexual selection (described below) were recently shown to be sensitive to sampling effort and may result in an overestimation and misinterpretation of sexual selection under insufficient sampling (Carleial et al., 2023). Our experimental design involved repeated sampling of mating and reproductive success in multiple mating trials. This allowed us to, 4) test the impact of sampling duration on metrics of sexual selection by comparing the 'instantaneous' estimates measured within each mating trial to 'cumulative' patterns (derived by summing mating success and reproductive success across all preceding mating trials).

Methods

Model organism

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is an obligatorily outcrossing simultaneous hermaphrodite found in the intertidal zones of

the Northern Adriatic sea and the Aegean sea (Ladurner et al., 2005; Schärer et al., 2020). Laboratory cultures of *M. lignano* are kept at 20°C in glass Petri dishes with artificial sea water (ASW) at a salinity of 32 ‰ and fed with the diatom *Nitzschia curvilineata*. Under laboratory conditions, *M. lignano* are found to be highly promiscuous and mate frequently when kept in groups. Copulation in this species involves reciprocal intromission of the male copulatory organ into the female sperm-receiving organ, the antrum (Schärer et al., 2004; Vizoso et al., 2010). The transparent body of the worms allows us to observe and measure internal structures *in vivo*, such as testis and ovary size (Schärer and Ladurner 2003), stylet morphology (Janicke and Schärer 2009) and sperm stored inside the female antrum (Marie-Orleach et al., 2016, 2021). The recent establishment of transgenic lines expressing GFP in *M. lignano* enables *in vivo* sperm tracking and efficient assignment of parentage after mating experiments (Janicke et al. 2013; Marie-Orleach et al. 2014, 2021; Wudarski et al. 2017).

Experimental setup

In this study, we aimed to i) quantify sexual selection for both sex functions in *M. lignano* and ii) assess sex differences in the strength of sexual selection. We conducted mating experiments to measure the relative mating success (MS) and reproductive success (RS) achieved through the male and female sex functions of each worm when sexually interacting in small mating groups of 4 individuals. Following the logic of Bateman's original experiment, we assessed the gMS and RS of worms based on the identification of mothers and fathers of all offspring produced in the group. This was accomplished by using variable GFP (green fluorescent protein) markers expressed in the transgenic lines of *M. lignano*, called the NL lines (Wudarski et al. 2017). NL lines are a set of inbred lines of *M. lignano* each expressing GFP (and RFP in line 'NL24') in regions of the body that are unique to each inbred line. The GFP marker in the genetically related HUB1 transgenic line, developed using the same technique as NL lines, was found to have a genetically dominant pattern of inheritance and is stably transmitted through the germ line to offspring, and thus could be used as identifying markers to track maternity and paternity of offspring (Marie-Orleach et al., 2014). Offspring from crosses between two NL lines are expected to carry GFP markers from both parents, and offspring produced when crossed with a wild-type worm carry the GFP marker of the NL parent.

In this study, we used three NL lines each exhibiting fluorescence either ubiquitously in all cells (NL1), in the nucleus of all cells (NL20) or in the gonads (NL24, GFP in testes and RFP in the ovaries) in combination with the wildtype, DV1 inbred line. By making pairwise test crosses, we have previously confirmed that all possible crosses between these 4 inbred lines result in offspring where GFP markers of both parents (or one NL line parent in crosses with DV1 line) could be observed. However, NL line

worms were required to be homozygous for the GFP marker to be used in the mating experiment so that all offspring inherit and express the GFP marker of their NL line parent(s). Therefore, we conducted a preliminary penetrance analysis to identify NL line worms that were homozygous for the GFP marker and only those worms were used in the mating experiment.

Rearing conditions

On day 1, 200 adults from each of the 7 NL lines and 1200 adults of the DV1 line were distributed into Petri dishes, 100 adults in each, for egg laying with 20 ml ASW and ad libitum algae. On day 3, we removed all adults from the dishes controlling for age differences of the resulting juveniles to be at maximum 48 hours. On day 9, we sampled the hatchlings to form rearing pairs of 2 hatchlings with 1 worm from each NL line (focal worms) paired with 1 wild-type DV1 worm. DV1 rearing pairs were formed with two worms from the DV1 inbred line. In total, 66 pairs were formed from each NL line and the DV1 line and were placed in wells of 24-well plates with 1.5 ml of 32‰ ASW and ad-libitum algae. To control for any environmental effects during development, every 24-well plate contained 3 pairs from each of the 8 inbred lines placed in randomly assigned wells. All pairs were transferred to fresh ASW and algae every 6-10 days.

Penetrance Analysis

Eggs laid by rearing pairs in their respective wells between days 30 and 51 were collected and resulting offspring were fed fresh algae to be reared into adulthood. Approximately 3-4 weeks old offspring were observed using a Leica M205 FA microscope (Leica microsystems, Germany) at 200-400x magnification under epifluorescence illumination to screen for the presence of fluorescent markers of their respective NL parent worm. An NL line parent worm was determined to be homozygous for the GFP marker when all offspring produced with a wildtype DV1 partner expressed the marker, with a minimum of 15 offspring screened. As a negative control, we screened a subset of offspring produced by DV1 pairs and did not find GFP expression in any of the offspring screened. Only NL worms that were determined to be homozygous for their respective GFP markers were used in the mating experiment. On day 93, once homozygous worms were identified, NL worms from the rearing pairs and one randomly chosen worm from DV1 pairs were separated from their rearing partners and isolated in wells of a 24-well plate for one week. This was done in order for the worms to use up sperm that they might have received from previous matings so that they do not have any sperm from their previous mates in storage at the start of the mating experiment.

Mating experiment

On day 101, we formed mating groups of 4 worms with one randomly chosen homozygous worm from each of the three NL lines (NL1, NL20 or NL24) and one wildtype DV1 worm. Thus, a mating group consisted of 4 worms each with their own unique identifiable GFP marker of the NL worms and absence of the same in the wildtype DV1 worm. Overall, 44 replicated mating groups were formed to be studied in the experiment. The mating groups were allowed to interact for a period of 4 hours in wells of a 24-well plate with ASW and ad-libitum algae. Given the high mating rate observed in *M. lignano*, this period is expected to be adequate for each worm to mate repeatedly with their potential partners in the group. At the end of the mating trial, all worms were isolated in fresh wells with algae for egg-laying.

As *M. lignano* lack a specialized means to store sperm for long durations (Vizoso et al., 2010), isolating the worms may cause a substantial reduction in the number of stored sperm available to fertilize eggs, which may result in sperm limitation. Such sperm limitation may influence the fecundity of worms and in turn our measures of gMS and RS. To minimise the effect of sperm limitation, the mating groups with the same sets of worms were re-formed every four days for a period of 4 hours enabling worms to mate and replenish sperm available to fertilize eggs, followed by isolation in fresh wells for egg laying. In total, we performed twelve cycles of mating trials and egg laying, lasting over a period of 48 days. As worms were transferred to fresh wells for egg laying after each mating trial, male and female gMS and RS of the worms were measured in each mating cycle, separately. Thus, we could obtain measures of instantaneous sexual selection from each mating trial as well as an overall estimate of sexual selection by summing up values of mating and reproductive success over cycles. Isolating the worms for egg-laying enabled us to directly identify the respective maternal parent of all resulting offspring. 3-4 weeks old offspring were observed using a Leica M205 FA microscope at 200-400x magnification under epifluorescence illumination to identify the GFP markers expressed. The paternal parent was then indicated by the GFP marker observed in the offspring other than that of the maternal parent. If only the GFP marker of the maternal parent was observed, the offspring was determined to be sired by the wildtype DV1 worm.

Statistical Analysis

All statistical analyses for this study were performed in R (Version 4.2.3). Due to a handling error, we could not use data collected from one mating group in our analysis and thus the final analysis was performed using data collected from 43 replicates.

Measuring gMS and RS

We measured gMS and RS separately from each of the 12 mating cycles (instantaneous measures). The female reproductive success (RS_f) of each worm was measured as the total number of surviving offspring produced by each worm in isolation. Male reproductive success (RS_m) of each worm was calculated as the sum of all offspring sired by the worm with all partners in the mating group. Male and female 'genetic' mating success (gMS_m and gMS_f, respectively) of each worm were measured as the number of partners who share at least one offspring through the male and female sex function of the worm, respectively. We obtained our measures of the total male and female RS of each worm in the experiment by summing the RS measured over all 12 mating cycles. We calculated the total male and female gMS of each worm as the total number of unique mating partners that share at least one offspring with each worm over all 12 cycles. Calculations of sexual selection metrics were performed on these cumulative measures of gMS and RS.

Focal sampling

gMS and RS were measured within closed mating groups meaning that every offspring produced in the group has one maternal and one paternal parent from the same group. Thus, average gMS and RS within mating groups were constrained to be equal for both sex functions. Estimates of gMS and RS obtained from worms of the same group are not independent. To account for this source of non-independence, we generated 10000 bootstrapped datasets by randomly sampling one worm from each mating group as the focal worm (focal sampling), resulting in 43 independent measures of gMS and RS in each dataset. Computations of the sexual selection metrics described below were performed on these simulated focal datasets.

Quantifying sexual selection

We quantified the pre-copulatory sexual selection of both sex functions using standardized metrics that are based on Bateman's pioneering work. In particular, variance-based metrics of sexual selection namely opportunity for selection (I) and opportunity for sexual selection (I_s) are measured as standardized variance in reproductive success (RS) and genetic mating success (gMS), respectively (Crow, 1989; Wade & Arnold, 1980; Jones, 2009). These two metrics are related to the maximum possible intensity of selection or upper bound on the selection differential, but not the realised selection. Thus, they may over-estimate sexual selection, as some of the observed variance in gMS and RS may be due to stochastic factors or fecundity of partners, respectively, which are unlinked to sexual selection (Henshaw et al., 2016; Carleial et al., 2023). In contrast, the so-called Bateman gradient (β_{ss}) is the slope of a linear regression of relative RS on relative gMS (Bateman, 1948; Arnold & Duval, 1994) and measures the benefit of having an additional mating partner and thus the net

return of outcompeting rivals, which is the essence of Darwinian sexual selection. The Jones index (s'_{max}), also called 'the maximum standardized sexual selection differential' combines both I_s and β_{ss} in one measure (Jones, 2009) and has been found to outperform all other metrics and to be especially effective in assessing sex differences in the strength of sexual selection (Henshaw et al., 2016).

Anthes et al., (2010) proposed an extension to the Bateman gradient approach to quantify sexual selection in simultaneous hermaphrodites. In addition to the simple regression between RS and gMS in the same sex, this includes testing cross-sex effects in multiple regressions, e.g., the effect of mating success in the male function (gMS_m) on the reproductive success in the female function (RS_f) and vice versa. Additionally, the suggested decomposition of the covariance in RS_m and RS_f into its components allows us to characterize sexually mutualistic or antagonistic selection that may or may not exist between the sex functions.

Computing Opportunity for selection and sexual selection (I and I_s)

For each bootstrapped dataset, we relativized gMS and RS values of both sex functions by dividing the absolute values by their sample means. We computed male and female opportunities for overall selection (I) as the variance in relative RS. Opportunity for sexual selection (I_s) was calculated as the variance in relative gMS. 95% confidence intervals were calculated as the 2.5^{th} and 97.5^{th} percentiles from the 10000 bootstrapped estimates of I and I_s. We tested differences in I and I_s between the two sex functions were significantly different from zero using permutation tests.

We generated 10000 permutated datasets where we assigned observed gMS and RS measures from each worm randomly to one of the two sex functions. For each dataset, we relativized the gMS and RS values within each sex by their respective mean values. We then calculated the variances in relative gMS and RS within each sex function and the numeric difference in the variances between the same. *P*-values were computed as the proportion of permutated values of the sex-difference in I and I_s that was higher than the absolute value of the observed sex-difference in the actual dataset.

Computing Bateman gradients and Jones index

From each bootstrap sample, Bateman gradients (β_{ss}) were calculated in two ways. Firstly, we calculated Bateman gradient as the slopes of a linear regression of relative RS on relative gMS within each sex, e.g., the effect of male mating success (gMS_m) on male reproductive success (RS_m). Secondly, as suggested for hermaphroditic systems by Anthes et al. (2010), we performed multiple regression of RS on both gMS in the same sex and the gMS in the opposite sex included as the cross-sex effect (e.g., RS_m on gMS_m and gMS_f). In outcrossing sexually reproducing organisms, mating is required to produce offspring such that a higher reproductive success of mated individuals

compared to those that did not mate has limited relevance for quantifying sexual selection (Anthes et al. 2017). Therefore, we computed all Bateman gradients including (complete dataset) and excluding (reduced dataset) individuals with zero gMS.

gMS of worms was quantified as genetic mating success, which implies that RS values were constrained to be equal to or higher than the gMS values, which may lead to an autocorrelation between gMS and RS by design (Anthes et al. 2017). To account for this, we estimated the strength of this autocorrelation by using a permutation approach and subtracted the obtained slopes from the observed Bateman gradients. Specifically, for each bootstrap dataset, we randomized the absolute values of RS against the gMS values within each sex to generate a dataset that fits the only condition that RS values are equal to or greater than gMS values. We then calculated the slope of linear regression of relativized RS on relativized gMS in this permutated dataset (β_{rand}). This slope is expected to represent the portion of the actual Bateman gradient that is due to the above-mentioned autocorrelation. We then corrected our estimates of Bateman gradients by subtracting β_{rand} from β_{ss}. We applied the same correction procedure to the complete and reduced dataset.

As mentioned above, Jones index is a measure of pre-copulatory sexual selection that combines Bateman gradient and variance in mating success, calculated as, $s'_{max} = \beta_{ss}\sqrt{I_S}$. To calculate Jones index, we used estimates of β_{ss} obtained from the simple linear regression of RS on gMS. Moreover, we only used β_{ss} of the complete dataset as I_s was only calculated from the complete dataset. Additionally, using the I_s, within- and cross-sex β estimates, we calculated the covariance in RS_m and RS_f and its components using eq. 5 in Anthes et al., 2010, shown below.

$$Cov(RS_m, RS_f) = \beta_{mm}\beta_{fm}Var(MS_m) + \beta_{mf}\beta_{ff}Var(MS_f) + (\beta_{mm}\beta_{ff} + \beta_{mf}\beta_{fm})Cov(MS_m, MS_f) + Cov(\varepsilon_m, \varepsilon_f)$$

These terms identify the types of selection that contribute to covariation between RS_m and RS_f. The first two terms account for the combined effect of gMS in one sex function on RS via both sexes, such that a positive term represents a sexually mutualistic selection and a negative term means sexually antagonistic selection arising from gMS in that sex function. The third term represents the component of covariance in RS_m and RS_f due to the covariance in gMS_m and gMS_f. The last term describes the residual covariance that is not linked to gMS and represents other forms of non-sexual selection.

Effect of sampling duration

The data collected from the mating experiment at the resolution of each mating cycle (4 days) allowed us to study the effect of sampling duration in measuring the strength of sexual selection. First, we computed the instantaneous estimates of I, I_s, β_{ss} and s'_{max}

using gMS and RS values measured within each of the 12 mating cycles, using the same methods described in the sections above. Secondly, we generated a cumulative dataset by summing gMS and RS values measured in each cycle to that of all the preceding mating cycles. Thus, the cumulative dataset represented measures of gMS and RS obtained from varying sampling durations (i.e., 4, 8, 12 until 48 days). Computing the strength of sexual selection along cumulative datasets, allowed us to explore how metrics of sexual selection are influenced by increasing sampling duration. However, we believe that sexual selection metrics obtained from the cumulative dataset after 48 days are biologically most representative and we interpret our findings primarily in the context of this dataset.

Meta-analysis

In an attempt to put our results in a more global context, we synthesised previous studies reporting standardised metrics of sexual selection in simultaneous hermaphrodites. This was done taking advantage of a database on sexual selection metrics (Janicke et al. 2016; Fromonteil et al. 2023), which is continuously updated and includes 3 unilaterally mating freshwater snails (Anthes et al., 2010; Pélissié et al., 2012; Janicke et al., 2015; Hoffer et al., 2017) and one other reciprocally mating flatworm (Pongratz et al., 2005). A formal meta-analysis to test for an overall sex-difference in I, I_s, β_{ss} was done following an approach described in detail elsewhere (Janicke et al. 2016). In brief, we computed effect sizes for the sex difference in I and I_s using the log coefficient of variation ratio *lnCVR* (Senior et al. 2020) and the sex difference in the Bateman gradients as Hedges' g with positive values indicating a male bias in the given sexual selection metric. Global effect sizes for each metric were computed from linear mixed-effects models implemented in the *metafor* package in R (V4.4-0; Viechtbauer 2010) in which we included study identifier and species identifier as random terms to account for statistical non-independence of repeated measures taken form the same species and/or the same study.

Results

Characterization of mating system

In total, 172 individuals were studied to measure gMS and RS over a total sampling duration of 48 days (12 mating cycles). Overall, 171 of 172 worms were observed to have reproduced during the experiment, that is had at least one offspring through either sex function. 8 worms sired no offspring through the male function, 18 worms failed to produce any offspring through the female function and 1 worm failed to reproduce through both sex functions.

A total of 3089 offspring were screened for GFP markers in the experiment. Parentage could not be reliably identified for 11 offspring due to the absence of a GFP marker or developmental defects. 129 of the 172 worms produced offspring through the female function sired by at least 2 partners, translating into a rate of multiple paternity of 75%. Due to reciprocal copulation, the average gMS in the male and female sex functions were constrained to be equal within mating groups at 2.16 mating partners (SD = 0.82 and 1.03 for the male and female sex function, respectively). The average number of offspring sired through the male function was 16.98 (SD = 13.17) offspring and the offspring produced by the female function was 17.16 (SD = 14.39) offspring.

Measures of sexual selection

We found that the variances in the relative RS and gMS did not differ between the two sex functions, indicating that the opportunity for overall selection (I) and opportunity for sexual selection (I_s) were similar for the male and female sex functions (Table 3.1). After correcting for the potential autocorrelation described in the methods, Bateman gradients (β_{ss}) calculated as the slope of simple least-squares regression of relative RS on relative gMS were found to be positive, and significantly different from zero, for both sex functions in the complete dataset (Table 3.1, Figure 3.1). This indicated that individuals who had more genetic mating partners also had more offspring through either sex function. However, Bateman gradients in the two sex functions were not significantly different, as revealed by the permutation test (Table 3.1). Removing null values of individuals with zero gMS (reduced dataset) affected neither our estimates of Bateman gradients nor the difference between the male and female sex functions. Similar to the results of Bateman gradients, the Jones index was significantly positive in, but did not differ between the two sex functions (Table 3.1). Our results reveal that the strength of sexual selection acting on the male function is not different from that of the female function in *M. lignano*.

Cross-sex effects

Bateman gradients calculated from multiple regression including cross-sex terms are presented in Table 3.2. The within-sex terms in the multiple regression were similar to the results from the simple regressions above. However, the effects of mating through one sex function on the reproductive success of the opposite sex function (cross-sex effects) were substantially smaller and not significantly different from zero for 3 out of the 4 effects estimated (Table 3.2). Only the effect of the MS_f on RS_m was found to be marginally significant in the complete dataset. Additionally, the decomposition of the positive covariance in RS_m and RS_f revealed a positive mutualistic effect of gMS in the female sex function on RS of both sex functions (Table 3.2). However, both estimates were not significantly different from zero in our results from the reduced dataset, when

Table 3.1 - Measures of sexual selection based on relative mating success (MS; divided by its sample mean) and relative reproductive success (RS; divided by its sample mean) and differences between the male and female sex functions based on non-parametric permutation tests. Mean estimates and 95% confidence intervals (in brackets) were obtained from 10000 bootstrap focal samples. I = Opportunity of selection, I_s = Opportunity for sexual selection, β_{ss} = Bateman gradient (calculated from simple regressions of relative RS on relative MS), β_{ss}* = Bateman gradient calculated on the reduced dataset (gMS > 0), s'$_{max}$ = Jones index, maximum selection differential due to sexual selection. Bold values indicate significantly positive (>0) estimates of Bateman gradients.

Estimates	Sex function		Sex differences	
	Male	Female	Difference	*P* value
I	0.61 (0.43, 0.83)	0.72 (0.47, 1.03)	-0.11(-0.46, 0.22)	0.24
I_S	0.15 (0.09, 0.22)	0.23 (0.12, 0.38)	-0.08(-0.25, 0.06)	0.13
β_{ss}	**0.90** (0.24, 1.57)	**1.09** (0.63, 1.62)	-0.19(-1.01,0.59)	0.27
β_{ss}^*	**0.82** (0.15,1.69)	**1.29** (0.52, 2.09)	-0.47(-1.36, 0.37)	0.15
s'_{max}	0.69 (0.19,1.25)	0.91 (0.51,1.38)	-0.22(-0.89, 0.45)	0.11

the null values of gMS were removed.

Sexual selection across hermaphrodites – meta-analysis

In line with observation in gonochoristic species, the opportunity for selection (I) showed a strong trend, although not significantly, to be higher in the male sex-function across hermaphrodite species studied so far (ΔI: lnCVR ± SE = 0.373 ± 0.194; z = 1.92, P = 0.055, Figure 3.2). The Bateman gradient (β_{ss}) was found to be significantly higher for the male sex function compared to the female sex function ($\Delta\beta_{ss}$: Hedge's g ± SE = 0.364 ± 0.169; z = 2.15, P = 0.032, Figure 3.2). These results are in line with the prediction of stronger sexual selection in the male sex function in hermaphrodites (Charnov, 1979). By contrast, the opportunity for sexual selection (I_s) was found to be significantly higher for the female function across hermaphrodites (ΔI_s: lnCVR ± SE = -0.201 ± 0.032; z = -6.34, P = <0.001, Figure 3.2), suggesting stronger sexual selection in the female function arising from differential mating success.

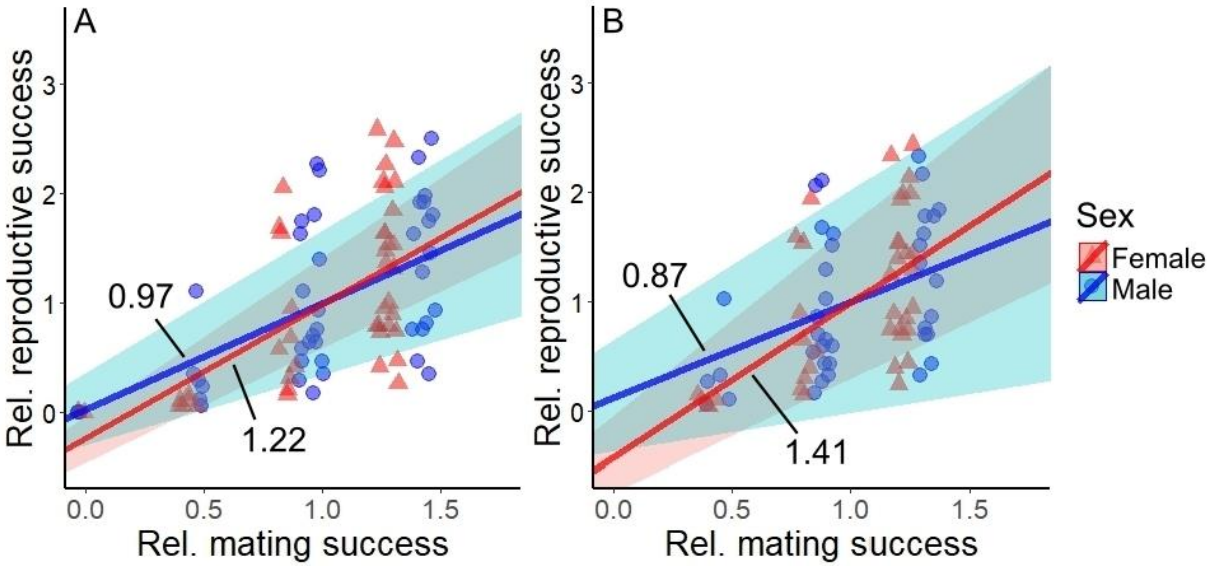


Figure 3.1 - Bateman gradients (β_{ss}) based on least-squares regressions of relative reproductive success (RS) on relative mating success calculated from the complete dataset (panel A) and the reduced dataset (panel B) with null values of gMS removed. The jittered points represent gMS and RS values from one focal sample, i.e. data from one randomly chosen individual from each mating group. The shaded regions represent 95% confidence intervals obtained from 10000 bootstrap estimates of β_{ss}. Note: the Bateman gradient values presented in the plots have not been corrected for the artificial confound of RS ≥ gMS as done for the values presented in Table 3.1.

Effect of sampling duration

Our analysis of the cumulative dataset demonstrated a reduction in the variance in relative gMS and RS with the initial increase in sampling duration in both sex functions, which stabilized over longer sampling durations (Figure 3.3). The instantaneous estimates of I and I_s within each mating cycle (short durations) were consistently higher than the cumulative estimates, particularly compared to estimates from longer sampling durations (Figure 3.3). Estimates of Jones index showed similar results with instantaneous estimates being higher compared to the cumulative estimates. The cumulative estimates of Bateman gradients exhibited larger confidence intervals but did not differ from instantaneous estimates in either sex function (Figure 3.3).

Discussion

Empirical studies of sexual selection have often been closely associated with sex-specific expression and sexual dimorphism and were therefore carried out primarily on gonochoristic species. Studying sexual selection in a hermaphrodite allowed us to address the fundamental question about the nature of sexual selection in the absence of sexual dimorphism, which we assessed for the first time comprehensively in a reciprocally copulating species. In contrast to the general pattern of stronger sexual selection found in males of separate-sexed organisms, we found no differences between the male and female sex function in the intensity of sexual selection in the hermaphroditic flatworm, *M. lignano*. However, a meta-analysis of the few existing studies measuring sexual selection in hermaphrodites reveals that some but not all predictions from the Darwin-Bateman paradigm may still hold for simultaneous hermaphrodites. We discuss the implications of our findings below.

Our results suggest that sexual selection is acting on both male and female sex functions of *M. lignano*. Yet we are reluctant to draw strong conclusions given the limitations of Bateman's metrics discussed below. We observed substantial variance in gMS and RS in both sex functions, indicating a similar potential for both sexual and non-sexual selection (I_s and I, respectively) in both sex functions. Likewise, the statistically similar Bateman gradients (and Jones indices) between the sex functions showed that the RS in both sex functions increased proportionally with the respective MS, supporting an advantage of mating with (copulatory MS) or producing offspring with (genetic MS) more mating partners in both sex functions in this species. The applied estimate of mating success has been argued to be a crucial aspect of interpreting Bateman gradients (e.g., Anthes et al., 2010, Pélissié et al., 2012, Marie-Orleach et al., 2016). We used genetic mating success (gMS) obtained from parentage analysis (e.g., Bateman 1948; Jones et al. 2000; Pischedda and Rice 2012), and not copulatory mating success (cMS), which is usually inferred from direct observation of mating behaviour and measured as the number of partners that individuals copulate with (e.g., Collet et al. 2012; Pélissié et al. 2012; Fritzsche and Arnqvist 2013. Copulatory mating success (cMS) primarily considers copulations and the number of mating partners as the central target of sexual selection. The strictly reciprocal copulation in *M. lignano* means that cMS in the two sex functions are constrained to be identical, and so is the variance in cMS. Thus, there cannot be any sex-difference in pre-copulatory components of sexual selection arising from variation in cMS in the two sex functions. However, sex-difference in post-copulatory components of sexual selection is still possible, as how cMS translates into RS can vary between the two sex functions. Moreover, *M. lignano* has been observed to copulate very frequently, potentially in attempts to donate sperm rather than to receive sperm, as suggested for simultaneous hermaphrodites (Charnov, 1979). A substantial portion of copulations, however, may not result in successful sperm

Table 3.2 - Within-sex and cross-sex Bateman gradients based on multiple regressions of relative reproductive success (RS) on relative genetic mating success (MS) (Upper part of the table) and decomposition of the covariance in reproductive success between the two sex functions into its components according to Anthes et al., 2010 (Lower part of the table). The estimates were calculated both in the complete dataset and when discarding individuals with null MS. Bold values indicate significant estimates.

		Multiple regressions	
		Complete	Reduced (MS>0)
Within-sex terms			
Effect of MS_m on RS_m	β_{mm}	**0.82** (0.14, 1.49)	**0.74** (0.05, 1.43)
Effect of MS_f on RS_f	β_{ff}	**1.09** (0.63, 1.62)	**1.30** (0.54, 2.10)
Cross-sex terms			
Effect of MS_f on RS_m	β_{mf}	**0.34** (0.001, 0.68)	0.41 (-0.18, 0.98)
Effect of MS_m on RS_f	β_{fm}	0.03 (-0.42, 0.45)	-0.05 (-0.67, 0.54)
Decomposition of Covariance in RS			
Covariance between RS_m and RS_f	$\mathrm{Cov}(RS_m, RS_f)$	**0.17** (0.03, 0.33)	0.097 (-0.03, 0.24)
Effect of MS_m on RS_m and RS_f	$\beta_{mm}\,\beta_{fm}\,\mathrm{Var}(MS_m)$	0.004 (-0.05, 0.06)	-0.003 (-0.05, 0.04)
Effect of MS_f on RS_m and RS_f	$\beta_{ff}\,\beta_{mf}\,\mathrm{Var}(MS_f)$	**0.09** (0.002, 0.19)	0.058 (-0.02, 0.15)
Effect of covariation in MS_m and MS_f	$(\beta_{mm}\,\beta_{ff} + \beta_{mf}\,\beta_{fm})$ $\mathrm{Cov}(MS_m MS_f)$	0.037 (-0.01, 0.09)	0.016 (-0.01, 0.06)
Covariance between residuals	$\mathrm{Cov}(\varepsilon_m,\varepsilon_f)$	0.031 (-0.05, 0.13)	0.026 (-0.05, 0.12)

exchange between the partners (Santhosh, unpublished data.) and do not affect reproductive success in the two sex functions. In line with these observations, the number of copulations was found to be a relatively poor predictor of reproductive success through the male function in *M. lignano* (Marie-Orleach et al., 2016).

On the other hand, genetic mating success (gMS) is more directly associated with reproductive success or fertilizations and thus captures also post-copulatory effects arising from sexually selected traits such as ejaculate size, sperm motility, or cryptic female choice (Arnold and Wade 1984; Birkhead and Pizzari, 2002). As sexual selection has been shown to predominantly occur in the post-copulatory stages of selection in *M. lignano*, gMS may therefore be a more relevant measure. Although gMS has been used frequently (e.g., Jones et al. 2004, 2005; Mills et al. 2007; Levitan 2008), recent discussions have suggested caution in interpreting the results derived from gMS. For example, gMS of male function fails to consider the portion of mating success that did not result in offspring sired, which may result in an overestimation of male Bateman gradients. As parentage is determined from reproductive success itself, gMS may partially confound the predictor (MS) to the response (RS) resulting in an autocorrelation between the variables and an over-estimation of the strength of sexual selection. Our analysis accounted for this limitation of using gMS in studies of sexual selection by estimating Bateman gradients that account for the autocorrelation between gMS and RS using permutation tests. We believe that our statistical analysis of Bateman gradients provides a favourable approach for assessing Bateman gradients in studies using gMS and can be applied in all model systems.

Another crucial implication of using genetic mating success is in the interpretation of the female Bateman gradient. We found a significantly positive Bateman gradient for the female function in *M. lignano*. This might represent an adaptive consequence where mating with additional males results in an increment in female fecundity, as observed in many polyandrous breeding systems (reviewed in Arnqvist and Nilsson, 2000; Clutton-Brock, 2009). Additionally, species exhibiting sex-role reversal with female-female competition for mates and male-biased parental care have been observed to show positive female Bateman gradients (e.g., Jones 2005; Fritzsche and Arnqvist, 2013). This may also occur from the sperm donors' manipulation of recipients' fecundity through seminal fluids, a widespread phenomenon in simultaneous hermaphrodites (e.g., Koene et al., 2010; reviewed in Schärer et al., 2015). Alternatively, more fecund individuals may inherently attract more mates, giving rise to a higher rate of multiple paternity and positive female Bateman gradient (Fromonteil et al. 2023). In *M.* lignano, unfed worms have been shown to have lower fecundity compared to well-fed worms. Janicke et al., 2012 found that worms adjusted their mating effort by copulating more frequently with and storing more sperm in well-fed partners compared to unfed partners. The precopulatory *circling* and *reeling* behaviour

observed in *M. lignano* has been suspected to be linked to mate assessment (Schärer et al., 2004), which could potentially include the fecundity of the mate. Additionally, this may also explain the cross-sex effect of MS_f on RS_m, as the more fecund worms attract more mating partners, reciprocal copulation assures mating success for the male sex function and an increase in reproductive success and vice versa. However, based on our results, we cannot identify the underlying cause for the observed positive relationship between gMS and RS in the female sex function.

Thirdly, a positive female gradient may also represent a spurious correlation arising from the use of gMS. That is, even if cMS was identical among individuals, more fecund individuals that produce more eggs inherently have a higher probability of producing offspring sired by more partners. By correcting Bateman gradients for autocorrelation, we at least partially, i) account for the copulations that did not result in fertilizations and missed out by gMS and ii) account for the spurious correlation described above arising from obtaining mating success from parentage. Finally, an increase in genetic mating success from 0 to 1 is invariably linked to an increase in RS by at least 1 offspring, creating a strongly positive Bateman gradient between 0 and 1 mating partners. The female Bateman gradient was found to be significantly influenced by this confound in the hermaphrodite, *P. acuta* and removing individuals with zero gMS from analysis altered the female Bateman gradient estimate from positive to non-significant. However, reducing the dataset in our analysis to only individuals with gMS > 0 did not affect the steepness of the female Bateman gradient. Overall, the above considerations fail to negate the possibility that the positive female Bateman gradient is indeed driven by a causal relationship between gMS and RS.

While the female Bateman gradient is often found to be positive (Fromonteil et al. 2023), the corresponding male Bateman gradients are typically steeper in many species (e.g., *Drosophila*, Bjork and Pitnick, 2006; Bank voles, Mills et al., 2007; Wild turkey, Krakauer, 2008; also see Janicke et al., 2016 for a meta-analysis). This asymmetry between the sexes was attributed to anisogamy, i.e., differences in the cost of male and female gametes, and thus posited to hold true for hermaphrodites. The male sex function was observed to experience stronger sexual selection compared to female sex function in the hermaphroditic snails, *B. glabrata* (Anthes et al., 2010), *L. stagnalis* (Hoeffer et al., 2017) and *P. acuta* (Pélissié et al., 2012), but a second study in *P. acuta* found no statistical difference between the sex functions (Janicke et al., 2015). In our study, the male Bateman gradient was not steeper than the female gradient. We suspect the reason may lie in the difference in the mating modes, i.e., unilateral copulations in snails above vs strictly reciprocal copulations in *M. lignano*. The only other study measuring Bateman gradients in the reciprocally mating hermaphroditic flatworm, *Schmidtea polychroa*, found a similar lack of sex difference in the intensity of sexual selection (Pongratz and Michiels, 2003). However, the question of why male and female function

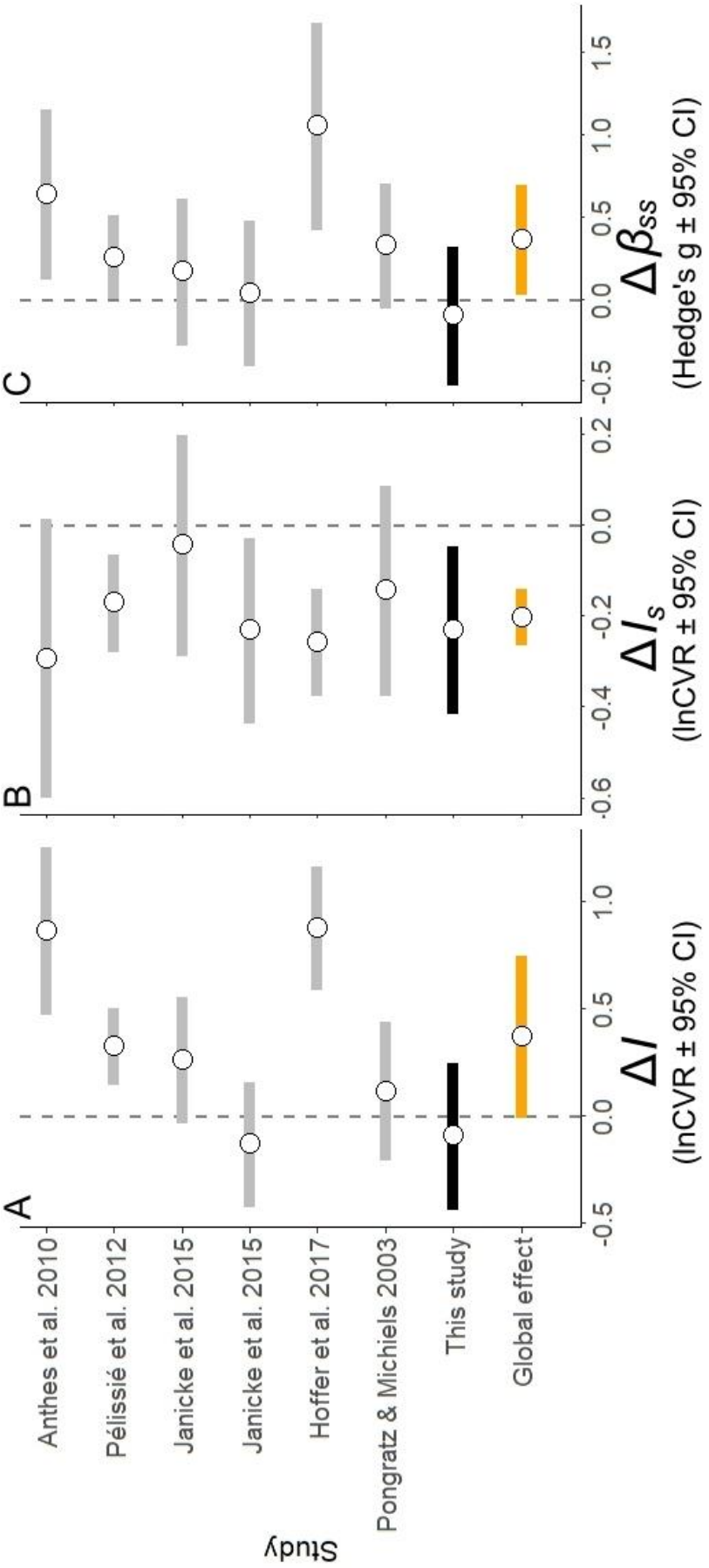


Figure 3.2 - Sex-biased sexual selection across hermaphrodites. Forest plots showing estimates of the sex bias in (A) the opportunity for selection (I), (B) the opportunity for sexual selection (I_s), and (C) the Bateman gradient (β_{ss}). Effect sizes (lnCVR and Hedges' g; see Materials and Methods) are shown as white points with their 95% confidence intervals (CIs). Positive values indicate male-biased sexual selection parameters and vice versa. Black line indicates effect sizes from this study and the yellow line indicates global effect size from the meta-analysis.

benefit equally from mating in reciprocally mating species, but not in unilaterally mating species, remains yet to be answered.

Anisogamy is thought to generate sex-specific selection and sexual dimorphism in secondary sexual traits, mating rate, re-mating latency and life-histories (Schärer et al., 2012). The union of the two sex functions in one body limits the potential for independent sex-specific selection between the sex functions, thus influencing the intensity of sexual selection in hermaphrodites. However, sex role preferences during mating in unilaterally mating hermaphrodites can vary within and between individuals when potential fitness gain per mating differs between the sex functions (Anthes et al., 2006), which is predicted to be usually higher for the male sex function (Charnov, 1979, Anthes et al., 2006). For example, the preferred sex role has been shown to be linked to body size and age in *L. stagnalis* and *P. acuta* (Wethington and Dillon 1993; Nakadera et al., 2015), with smaller snails, preferring to copulate in the male role. Thus, the flexibility in sex role preferences may contribute to sex-differences in the variance in mating success and the pre-copulatory stages of sexual selection in the two sex functions. Evidently, the precopulatory components explained a substantial portion of the variance in male reproductive success in *P. acuta* (Pélissié et al., 2014). In contrast, copulations in the two sex functions are strictly constrained to be equal in reciprocally mating hermaphrodites, with restricted scope for sex role preferences during copulations. Consequently, the variance in cMS is also constrained to be equal. Thus, reciprocal copulation can be expected to constrain sexual selection in *M. lignano* to post-copulatory stages and this was supported by post-copulatory components explaining a major portion of the variance in male reproductive success in earlier studies (Marie-Orleach et al., 2016, 2021). In this context, a potential limitation of our experimental design, which might have influenced the estimated metrics of sexual selection, is the small mating group size of only 4 worms. Specifically, gMS could only vary between 0 and 3, which may have limited the amount of possible variance in gMS and may therefore lowered our chances of observing sex-difference in I_s and the Bateman gradient.

Meta-analysis of sexual selection metrics in simultaneous hermaphrodites

Given the very small number of studies used, we acknowledge that our results are preliminary and do not allow us to draw strong conclusions about the generality of simultaneous hermaphrodites. A previous meta-analysis focussing primarily on gonochorists showed that the Darwin-Bateman paradigm of stronger sexual selection in males compared to females can be generalized across the animal kingdom (Janicke et al. 2016). Our results are, at least partially, in line with these previous findings and support the hypothesis that sexual selection is stronger in the male sex function compared to the female sex function in simultaneous hermaphrodites in terms of more male-biased estimates of I and β_{ss}. The higher opportunity for total selection (I), defined

as the sum of natural and sexual selection (Arnold, 1994; Hosken and House, 2011), in the male sex function may at least partly be a consequence of the higher sexual selection in the same, as indicated by the male-biased sex difference in Bateman gradient (Winkler et al., 2021). However, surprisingly, we observed a strongly female-biased sex difference in the opportunity for sexual selection (I_s). It might be too early to speculate on potential reasons driving this unexpected sex-difference. Yet, stronger sexual selection on the male sex function might erode genetic (and therefore also phenotypic) variance in traits affecting male mating success such that the variance in mating success varies more in the female sex function in which selection on mating success is more relaxed.

Effect of sampling duration

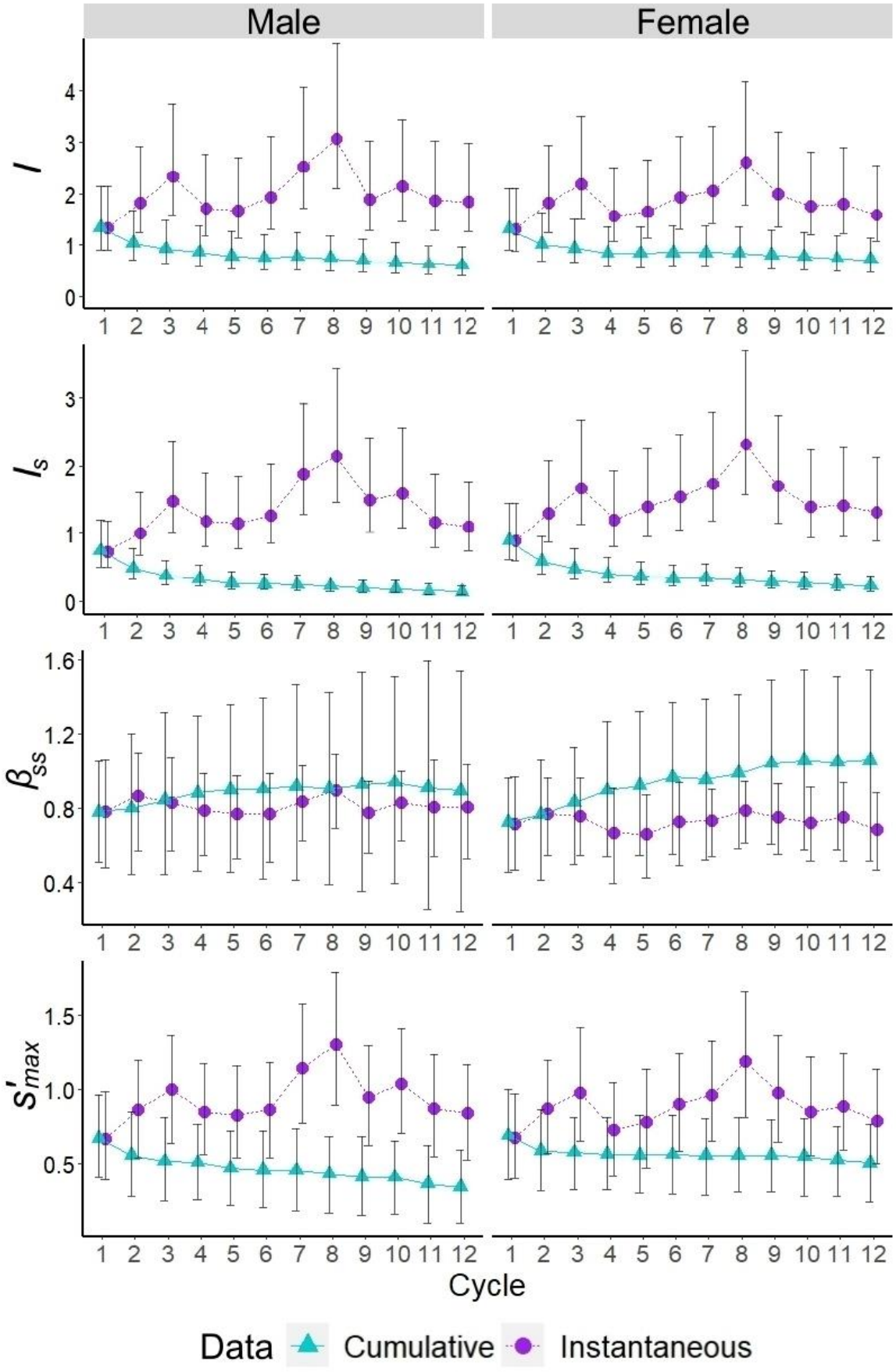
We observed a reduction in the cumulative estimates of I and I_s, showing an initial decrease with an increase in sampling duration that appeared to level-off at longer sampling durations. The instantaneous estimates of I and I_s were consistently higher than the cumulative estimates, and showed relatively wider confidence intervals. Although relatively less pronounced, a similar pattern observed in the Jones index estimates could be expected given that this metric depends on estimates of I_s by its definition. The initial reduction in cumulative estimates suggests that the variance in mating and reproductive success eroded over time as worms acquired more mating partners and produced more offspring. A similar reduction in I and I_s was observed with an increase in polyandry in the red jungle fowl, *Gallus gallus* (Collet et al., 2012). This could occur as even the less competitive individuals, that may fail to attain mating or reproductive success in short sampling durations, may be observed to do so during longer sampling durations. Alternatively, similar patterns could also result from worms preferentially mating and producing offspring with multiple partners, resulting in a reduction in any mating skew and variance over longer sampling durations. The cumulative sexual selection estimates remained more or less unchanged at longer sampling durations, suggesting that accurate estimates may require a sufficient sampling duration representing a significant fraction of the entire breeding period. Our results are in support of the findings of Carleial et al. (2023) showing that variance-based sexual selection estimates (I and I_s) measured over a short sampling duration may be affected by stochastic factors which may lead to an over-estimation of sexual selection metrics.

Conclusion

This study extends our understanding of sexual selection in animals by testing for sex differences in the standardised selection metrics in a reciprocally mating simultaneous hermaphrodite. Contrary to earlier findings reported for unilaterally mating hermaphrodites, our results do not support the Darwin-Bateman Paradigm in the

flatworm *Macrostomum lignano*. More studies on reciprocally mating simultaneous hermaphrodites are clearly needed to evaluate whether the predictions by the pioneers of the field, Darwin and Bateman, also hold for organisms in which both sexes are expressed in the same individual.

Figure 3.3 - Temporal dynamics in the measures of sexual selection in the male and female sex functions (Left and right panels, respectively). Green points (triangles) represent means of estimates measured cumulatively over all preceding mating cycles, and purple points (circles) show means of instantaneous estimates assessed independently for each mating cycle. Error bars show 95% confidence intervals calculated from 1000 bootstrap estimates. I = Opportunity of selection, I_s = Opportunity for sexual selection, β_{ss} = Bateman gradient (calculated from simple regressions of relative RS on relative MS), s'_{max} = Jones index, maximum selection differential due to sexual selection.


Male
Female
I
I_S
β_{ss}
s'_{max}
Cycle
Data
Cumulative
Instantaneous

Chapter IV

Sperm Competition Favours Intermediate Sperm Size in a Hermaphrodite

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Abstract

Sperm competition is a potent mechanism of post-copulatory sexual selection that has been found to shape reproductive morphologies and behaviours in promiscuous animals. Especially sperm size has been argued to evolve in response to sperm competition through its effect on sperm longevity, sperm motility, the ability to displace competing sperm and ultimately fertilization success. Additionally, sperm size has been observed to co-evolve with female reproductive morphology. Theoretical work predicts that sperm competition may select for longer sperm but may also favour shorter sperm if sperm size trades off with number. In this study, we studied the relationship between sperm size and post-mating success in the free-living flatworm, *Macrostomum lignano*. Specifically, we used inbred isolines of *M. lignano* that varied in sperm size to investigate how sperm size translated into the ability of worms to transfer and deposit sperm in a mating partner. Our results revealed a hump-shaped relationship with individuals producing sperm of intermediate size having the highest sperm competitiveness. This finding broadens our understanding of the evolution of sperm morphology by providing empirical support for stabilizing selection on sperm size under sperm competition.

Introduction

Ever since Parker's pioneering work on sperm competition, the study of post-copulatory sexual selection has drastically improved our understanding of male and female reproductive biology (Parker, 1970, 1982; Birkhead and Moller, 1999; Simmons, 2001). In species where individuals mate multiply with different partners, post-mating fertilisation success determined by the efficiency to outcompete rival ejaculates can become a major predictor of male reproductive success (Collet et al., 2012; Pélissié et al., 2014; Marie-Orleach et al., 2021). Consequently, sperm competition is expected to promote post-copulatory intrasexual selection on various traits that influence fertilization success. Over the last decades, great efforts have been made to gain a better understanding of such phenotypic effects of ejaculate traits on the outcome of sperm competition. Especially, sperm size has received substantial attention (Simmons and Siva-Jothy, 1998; Simmons,2001; Pizzari and Parker, 2009) and has been documented to have highly variable relationships (positive, negative and no association) with sperm motility, sperm longevity and the defensive behaviours of an ejaculate across species (Snook 2005; Fitzpatrick and Lüpold 2014). In addition, sperm size has been found to vary tremendously not only within ejaculates but also among males and among species, which is often considered to result from selection arising from sperm competition and/or cryptic female choice (Pitnick et al., 2009). Theoretical work often assumes a positive effect of sperm size on its fertilization success under sperm competition (Parker, 1993; Parker et al., 2010; Pizzari and Parker, 2009). Yet, sperm size is also considered to trade-off with sperm number, which complicates the prediction of how sperm size is selected under varying sperm competition risks and intensities. Specifically, if sperm size trades-off against the number of sperm produced or resources required for pre-copulatory mate acquisition, selection arising from sperm competition may favour ejaculates with sperm of intermediate size (Parker, 1993; Parker et al., 2010; Parker and Begon, 1993).

Although variation in ejaculate performance traits, such as sperm size, is generally intrinsic to males, it has also been shown that females play an active and crucial role in post-copulatory sexual selection and that competitive fertilization success of males is influenced by interactions between sperm and the female reproductive tract (Lüpold et al., 2013; Miller and Pitnick, 2002; Pitnick et al., 2009). Notably, sperm size has been found to covary with the length of female reproductive organs, such as a seminal receptacle or spermatheca (Minder et al., 2005; Morrow and Gage, 2000; Pitnick et al., 1999, 2003; Briskie and Montgomerie 1992, Briskie at al., 1997). Moreover, using an experimental evolution study, Miller and Pitnick (2002) showed a correlated evolution in sperm length as a result of an evolutionary increase in seminal receptacle length in *Drosophila melanogaster*. Thus, selection on sperm size may also arise from selection

imposed by properties of the female reproductive tract.

Empirical tests on the relationship between sperm size and sperm competitiveness provided mixed results within and across species (Pizzari and Parker, 2009). Comparative studies indicate that higher levels of sperm competition are associated with longer sperm in internally fertilizing species (e.g., fish: Balshine et al., 2001, birds: Briskie et al., 1997, Lepidoptera: Gage 1994, mammals: Gomendio and Roldan, 1991 & Tourmente et al., 2011; but also see, Stockley et al., 1997) suggesting that sperm size is determined, at least partly, by the intensity of sperm competition. By contrast, a negative or no relationship between sperm competition and sperm size has been reported for Sylvian warblers (Immler and Birkhead, 2007) and mammals (Gage and Freckleton, 2003). Another line of across-species comparisons suggests that higher levels of sperm competition are associated with reduced intra-specific and intra-male variation in sperm size across birds (Calhim et al., 2007, Immler et al., 2008; Kleven et al., 2008), and such results have been speculated to arise from stabilizing selection for an optimum sperm size (Calhim et al., 2007; Lüpold et al., 2009).

The most direct evidence for selection acting on sperm size comes from studies quantifying how phenotypic variation in sperm size translates into sperm competitive success within species. Empirical studies attempting to establish such associations within species have found positive (Radwan, 1996), negative (Gage and Morrow, 2003; Garcìa-González and Simmons, 2007) or no clear relationship (Cascio Sætre et al., 2018; Kahrl et al., 2021) between sperm size and male reproductive success (or paternity share). In addition, studies comparing relative fertilization success between extreme sperm sizes (e.g. large vs small) also provided mixed results (Bennison et al., 2015; Morrow and Gage, 2001). These contrasting results might, at least partly, result from substantial within-individual variation in male post-mating success and the associated uncertainty in estimating individual differences in sperm competitiveness. Marie-Orleach et al. (2021) took measures of reproductive performance of the same individual over multiple mating events and detected low repeatabilities for different proxies of post-mating success, indicating the significance of stochastic variation in measures of post-mating success. Importantly, the vast majority of empirical studies assume a linear relationship between sperm size and reproductive (or post-mating) success and do not test for non-linear associations which limits our understanding of how selection operates on sperm size. To our knowledge, only five studies have tested for such non-linear associations, but none of the studies found any significant non-linear associations between sperm length and estimates of fitness (Cramer et al., 2013; Lymbery et al., 2018; Kahrl et al., 2021; Rowe et al., 2022; Marie-Orleach et al., 2023).

Here, we report an experimental study exploring the relationship between sperm size and post-mating success within a species. Specifically, we tested the hypothesis that sperm size predicts sperm transfer success in the simultaneously hermaphroditic

flatworm, *Macrostomum lignano,* a species in which sperm competition has been shown to be intense (Janicke et al., 2013; Marie-Orleach et al., 2021). In *M. lignano*, a recent study explored how sperm morphology predicts male reproductive success (and other fitness components) (Marie-Orleach et al., 2024), focussing on purely phenotypic relationships between sperm traits and male fitness components. By using inbred lines, we aimed to extend previous findings by exploring how sperm competition generates selection on standing genetic variation in sperm length.

An outbred lab culture and a set of 12 inbred lines of *M. lignano* expressing a green fluorescent protein (GFP) in all cell types, including sperm cells, allowed us to distinguish between sperm from a focal GFP-positive (hereafter denoted as GFP+) sperm donor from those of wildtype competitors with no GFP expression (hereafter GFP-). We used these inbred lines to conduct mating trials testing how variation in sperm size among the lines predicted sperm transfer success (i.e., the number of sperm stored in the partner's sperm storage) in the presence of sperm competition. While we hypothesized an association between sperm size and sperm competitiveness, we did not have a clear prediction for the shape of this relationship as the factors determining its exact form (e.g., resource allocation among ejaculate components, sperm displacement propensity and spatial constraints for sperm storage) are largely unknown for *M. lignano*.

Materials and Methods

Model organism

The free-living flatworm *Macrostomum lignano* (Macrostomorpha, Platyhelminthes) is an obligatorily outcrossing simultaneous hermaphrodite found in the intertidal zones of the Northern Adriatic Sea and the Aegean Sea (Ladurner et al., 2005; Schärer et al., 2020). Laboratory cultures of *M. lignano* are kept at 20°C in glass Petri dishes with artificial sea water (ASW) at a salinity of 32 ‰ and fed with the diatom *Nitzschia curvilineata*. Under laboratory conditions, *M. lignano* is found to be highly promiscuous and to mate frequently when kept in groups. Copulation in this species involves reciprocal intromission of the male copulatory organ into the female sperm-receiving and -storage organ called the antrum (Schärer et al., 2004; Vizoso et al., 2010). *M. lignano* exhibits a post-copulatory suck behaviour, in which worms bend down and place their pharynx on top of their own genital opening and appear to suck, which may have the function to remove sperm out of the antrum (Vizoso et al., 2010). The transparent body of these worms enables the observation of numerous internal structures and processes *in vivo* such as testis and ovaries, stylet morphology and sperm (both GFP+ and GFP-) stored in the female antrum (Janicke et al., 2011; Marie-Orleach et al., 2016, 2021).

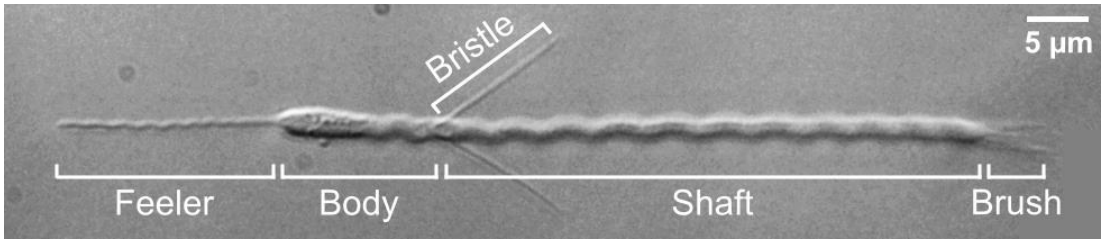

Figure 4.1 - Morphology of a mature sperm cell in *Macrostomum lignano*.

The morphology of sperm in *M. lignano* is complex with two structures hypothesized to have specialized functions in the post-copulatory stages of reproduction (Visozo et al., 2010; Ladurner et al., 2005; Willems et al., 2009). The lateral bristles have been hypothesized to reduce the likelihood of sperm being removed from the antrum during a post-copulatory suck behaviour (Vizoso et al., 2010). The feeler has been observed to anchor sperm cells to the epithelium at the anterior region of the antrum (called cellular valve), where fertilization is hypothesized to occur (Vizoso et al., 2010). Given their functions, we predict selection for longer lateral bristles and feelers. Longer bristles may enable better resistance from being sucked out of the antrum and longer feelers may allow better positioning of the sperm into the cellular valve to increase the chance of fertilizing an egg. The sperm body carries a core of mitochondria and the nucleus is present in the extended sperm shaft, which also contains mitochondria. The posterior brush is made of microtubules protruding at the end of the sperm shaft. No apparent functions have been observed for sperm body, shaft and posterior brush in the female antrum so far, thus we had no clear predictions for the form of selection of these sperm components. Therefore, in addition to total sperm length, we only test our predictions for feeler length and bristle length in this study.

Experimental setup

In this study, we aimed (i) to quantify between-individual variation in sperm size and (ii) to assess the relationship between sperm size and sperm transfer success in *M. lignano*. To this end, we measured the sperm size of adult worms (N = 39) from the outbreeding BAS1 culture to quantify the natural phenotypic variation in sperm morphology in *M. lignano*. The BAS1 culture is an outbred GFP+ culture derived from backcrossing the GFP marker from a GFP+ inbred line (HUB1) onto a GFP- outbred culture for 9 generations (Marie-Orleach et al., 2016). BAS1 worms express GFP ubiquitously in all cells, including sperm cells. For our second aim, we conducted mating experiments and measured the relative sperm competitiveness of worms known to vary in sperm size. The sperm size of worms cannot be measured before mating experiments, as it is a destructive process harming the worms (see next section). Hence, we used inbred lines of *M. lignano*, which are expected to show low within- but large

between-line variation in sperm size. Using inbred lines also allowed us to estimate the amount of variation in sperm size attributable to genetic variance across lines.

We investigated variation in sperm size among LM lines, a set of 12 GFP+ inbred lines of *M. lignano* derived from the inbred GFP- DV lines by backcrossing the GFP marker from the HUB1 inbred line onto themselves for several generations (Marie-Orleach et al., 2017). For the sperm competition experiment, we chose 8 of the 12 LM lines with relatively narrow variations in sperm size, competing against common competitors from a single GFP- inbred line called DV1. Like other DV lines, the DV1 inbred line was initiated by crossing two virgin worms taken from an outbred culture, followed by 15 generations of full- or half-sib inbreeding (For more details, refer Janicke et al., 2013 & Vellnow et al., 2017). Using worms from the GFP- DV1 line as common competitors of all LM lines allowed us to observe and distinguish the GFP+ sperm of LM sperm donors from the GFP- sperm of their DV1 competitors. Sperm competitiveness was estimated by calculating sperm transfer success as the proportion of GFP+ focal sperm relative to the total number of sperm stored in the antrum. Sperm transfer success has been shown to positively covary with mating success and paternity share in *M. lignano* (Marie-Orleach et al., 2016), thus representing an important fitness component of the male sex function. As mating rates have been shown to be high (Schärer et al., 2004), using unmated (virgin) worms would not represent a realistic scenario to study reproductive traits in this species. Therefore, worms were raised in small groups of 3 worms before the mating trials so that they were sexually experienced and had reached a biologically realistic steady state of sperm and egg reserves ready to be donated and fertilized, respectively.

Sperm size measurements

Measurements taken from each sperm cell included the total sperm length, measured from the anterior tip of feeler to the posterior tip of brush (Figure 4.1), and the length of anterior feeler and lateral bristles (mean length of the two bristles). Measurements were done using an established technique previously used in *M. lignano* (Janicke and Schärer, 2010). Adult worms were isolated for a day in wells of a 24-well plate to allow them to restore sperm reserves in their seminal vesicles, where they are stored until the sperm is transferred to a partner during copulation. The tail plates of the worms consisting of the seminal vesicle were amputated and transferred onto a microscopic slide with 1 µl of ASW medium, rupturing the seminal vesicle and causing sperm to spill out into the surrounding medium. This restricted the movement of sperm cells greatly, facilitating the imaging of sperm morphology with a digital camera (The Imaging Source, DFK 41BF02) connected to a light microscope (Leica DM2500, Leica Micro-systems, Germany) and imaging software BTV Pro 6.0b7 (http://www.bensoftware.com/).

We took digital micrographs of sperm cells at 1000x magnification and the micrographs

were analyzed in ImageJ 1.53c using the ObjectJ plugin (https://sils.fnwi.uva.nl/bcb/objectj/). About 15 sperm cells were measured from all individuals that were measured as part of this study. This number has been shown to be adequate to provide an accurate estimate of the individual mean values of sperm length (Janicke and Schärer, 2010). Additionally, we estimated the measurement error in this method by calculating the repeatability when the total sperm length, bristle length and feeler length of the same sperm cell was measured thrice for a total of 10 randomly chosen sperm cells each from different worms.

Rearing conditions

On day 1, 200 GFP+ adults from each of the 12 LM lines and 2400 GFP- adults of the DV1 line were distributed into Petri dishes, 100 adults in each, for egg laying with 20 ml ASW and ad libitum algae. On day 3, we removed all adults from the dishes controlling the age differences in the resulting juveniles to less than 48 hours. On day 9, we sampled the hatchlings to form rearing groups of three hatchlings, all from the same inbred line. In total, we formed 30 such groups for each LM line and 360 groups from the DV1 line. The groups were placed in wells of 24-well plates with 1.5 ml of 32‰ ASW and ad libitum algae. To control for any environmental effects during development, every 24-well plate contained 2 groups from each of the LM lines in randomly assigned wells. All groups were transferred to fresh medium and algae every 6-10 days until adulthood. During this period, the sperm size of the 12 LM lines was measured from a different set of adult worms (56 - 63 days old) and 8 lines with relatively narrow within-line variation in sperm morphology were chosen for the sperm competition experiment.

Sperm competition experiment

On days 35 through 55, we assessed the sperm transfer success of worms from the 8 LM lines when competing against the common competitor DV1 line. On every experiment day, we formed ‘mating groups’ by moving one randomly picked adult from a LM rearing group, verified for GFP expression, to a fresh well along with three DV1 worms. Thus, a mating group consisted of one GFP+ focal worm from one of the 8 LM lines and three GFP- worms of a DV1 rearing group, and each focal LM worm competed against two DV1 competitors for access to eggs of a third DV1 worm. On the following day, the focal LM worm was removed from the experimental group.

Immediately after the 24-hour mating trial, the number of GFP- and GFP+ sperm in the female antrum of the three DV1 partners was assessed, as previously reported (Janicke et al., 2013). We used a digital video camera (ORCA-Flash 4.0 V2: C11440-22CU, Hamamatsu corporation) connected to a Leica DM2500 microscope (Leica Micro-systems, Germany) and the software Leica Applications Suite X (LAS X, Leica Micro-

systems, Germany) to make movies of the female antrum. For each DV1 partner, we recorded a movie of the female antrum by focusing through the antrum under differential interference contrast illumination to be able to see all the sperm in the antrum (movie 1). Immediately after, we recorded a second movie of the female antrum under epifluorescence illumination to visualize the GFP+ sperm that were transferred and stored by the focal worm (movie 2). We analysed the movies using VLC media player and counted, for each focal donor, the total number of sperm (from movie 1) and the number of GFP+ sperm (from movie 2) from the focal donor in the female antrum of all its potential partners.

Similar to previous studies with *M. lignano*, we encountered worms with an egg (or eggs) in the antrum (i.e., 263 out of 720 DV1 worms used in the experiment). In the presence of eggs in the antrum, it is not possible to reliably count all sperm that remain in the antrum under interference contrast illumination, which meant we could only count the GFP+ sperm in these partners. Previous studies extrapolated the total number of sperm in such worms by using the average value of the total number of sperm in all the other worms (Marie-Orleach at al., 2016, 2021). In this study, the number of GFP+ sperm was sometimes greater than the total number of sperm resulting in proportion values higher than 1. Therefore, we performed all analyses excluding worms that had eggs in their female antrum.

Statistical analysis

All statistical analyses for this study were performed in R (Version 4.2.3).

Variation in sperm size

The total natural variation in sperm length in *M. lignano* was assessed by calculating the coefficient of variation for each sperm morphological trait in the outbred BAS1 culture. We tested for between-individual differences on all sperm traits using Kruskal Wallis rank sum tests with individual identifier as the predictor variable. Repeatabilities for the measurement method were calculated for 3 measurements of each sperm cell with the rptGaussian function in the *rptR* package (V0.9.22, Stoffel et al., 2017), using 1000 parametric bootstraps to evaluate the uncertainty in the repeatability estimate. All further analyses were done using the arithmetic mean of sperm measurements obtained from each individual. We estimated the proportion of phenotypic variation in sperm size that is explained by the LM line identity, i.e., the variance that can be attributed to the genotype of the male, using a linear mixed model with LM line identifier and individual worm identifier as random effects and trait length as dependent variable. The linear mixed models were built using the 'lmer' function in the *lme4* package in R (V1.1-32, Bates et al., 2015), assuming the individual worm identifier to be nested within the LM

line identifier.

Relationship between sperm length and sperm transfer success

Initially, we intended to run 240 sperm competition trials (30 per line), but we lost 4 replicates due to handling errors. Moreover, 263 (36.5%) of the 720 DV1 partners had one or more eggs in their antrum, resulting in the loss of 24 replicates where all partners had eggs in their antrum at the time of observation. Previous studies have reported comparable frequencies for the occurrence of eggs in the female antrum in *M. lignano* (Marie-Orleach at al., 2016, 2021). Thus, the final analysis was performed with a total of 212 replicates (24 to 29 replicates per line, mean = 26.5). For each replicate, we summed the total number of sperm and the number of focal GFP+ sperm found in the three partners (only 2 or 1 in cases of eggs or worms were lost). The sperm transfer success of a GFP+ focal worm was calculated as the proportion of GFP+ sperm among the total number of sperm stored in the antrum of all the partners.

Firstly, we assessed the differences in sperm transfer success among the LM lines. We fitted binomial Generalized linear models (GLM) using the *lme4* package (V1.1-32, Bates et al., 2015), with line identity as a categorical fixed effect and the relative number of GFP+ and GFP- sperm with the 'cbind' function as the response variable representing sperm transfer success. We observed over-dispersion in the binomial GLM (dispersion ratio = 9.344), which may arise from the variation in the total number of sperm observed in the partners' antrum (mean ± standard deviation = 44 ± 24). Therefore, we refitted the models assuming a quasibinomial data distribution. Additionally, we tested if the total number of sperm observed in the partners' antrum, sampled to estimate sperm transfer success differed between the LM lines, as this may suggest that systematic difference in sperm production between mating groups of different LM lines. For this, we built a generalized linear model with the total number of sperm sampled as the dependent variable and the LM line identifier as the categorical predictor variable, assuming a quasipoisson data distribution to account for over-dispersion (dispersion parameter = 13.42).

Secondly, we assessed the relationship between sperm transfer success and total sperm length. Given their functional significance in post-copulatory sexual selection, we performed the assessment also for lateral bristle lengths and anterior feeler lengths. As mentioned earlier, sperm length measurements are invasive requiring the amputation of the tail plate so we could not measure the sperm morphology of focal worms used in the mating experiments. Therefore, we used the mean value of each line (hereafter, called "line means") as our measure of trait length. This meant that all focal worms from the same LM line were assumed to express similar length, for all three traits. However, to account for the variation within lines such that mean values of lines with higher precision are given relatively higher weight compared to those with lower

precision, we used the inverse of the standard deviation in sperm length within lines as a weighing factor in our models.

We built two quasibinomial regression models, one first-order linear and one including, in addition, a second-order quadratic term, with individual sperm transfer success as the response variable and line means of sperm traits as a continuous predictor variable. In order to assess the potential for a hump-shaped relationship between sperm length and sperm transfer success, we used a quadratic polynomial of line means as the independent predictor variable. We performed a likelihood ratio test to infer whether adding the quadratic term provides a better model fit. In addition, we compared the goodness of fit of the two models by using the Akaike information criterion accounting for the over-dispersion in the data (quasi-AIC), as implemented in the *bbmle* package (V1.0.25, Bolker, 2022) in R.

Results

The measurement repeatabilities were high for all three sperm traits measured (Table S4.1). The total and bristle length were highly repeatable whereas repeatability was lower for the feeler length (Table S4.1). This was expected as undulations of the feelers caused difficulties in accurate measurement of the length.

Variation in sperm size among individuals and inbred lines

In the outbred BAS1 culture, we observed a large variation in sperm length ranging from 70 to 90µm, approximately. We found significant between-individual variation in all sperm traits measured (Table S4.1, also see Figure S4.1 for variation in total sperm length). The coefficients of variation (CV) of all sperm traits were comparable (4.31 to 6.10 %). The variation in sperm size within individuals (WCV) was on average lower than the overall variation observed in the culture (Table S4.1).

As expected, the combined variation in sperm size observed in the LM lines approximately covered the overall phenotypic variation measured from the outbred culture (Figure 4.2). The sperm size of the DV1 line (i.e. used as common competitors in the mating trials) corresponded to the average sperm size found in LM lines (Figure 4.2). Therefore, LM lines had larger, similar and smaller sperm size to DV1, allowing us to study the potential role of competitor's sperm size on sperm transfer success. We found a significant between-line variation in all sperm traits measured (total sperm length: Kruskal-Wallis test, $\chi^2 = 65.34$, df = 11, $P = 9.3e^{-10}$). The linear mixed models revealed that a substantial portion of phenotypic variation in all sperm traits could be explained by LM line identity (Table S4.2), explaining 45 % of the variation in total sperm length.

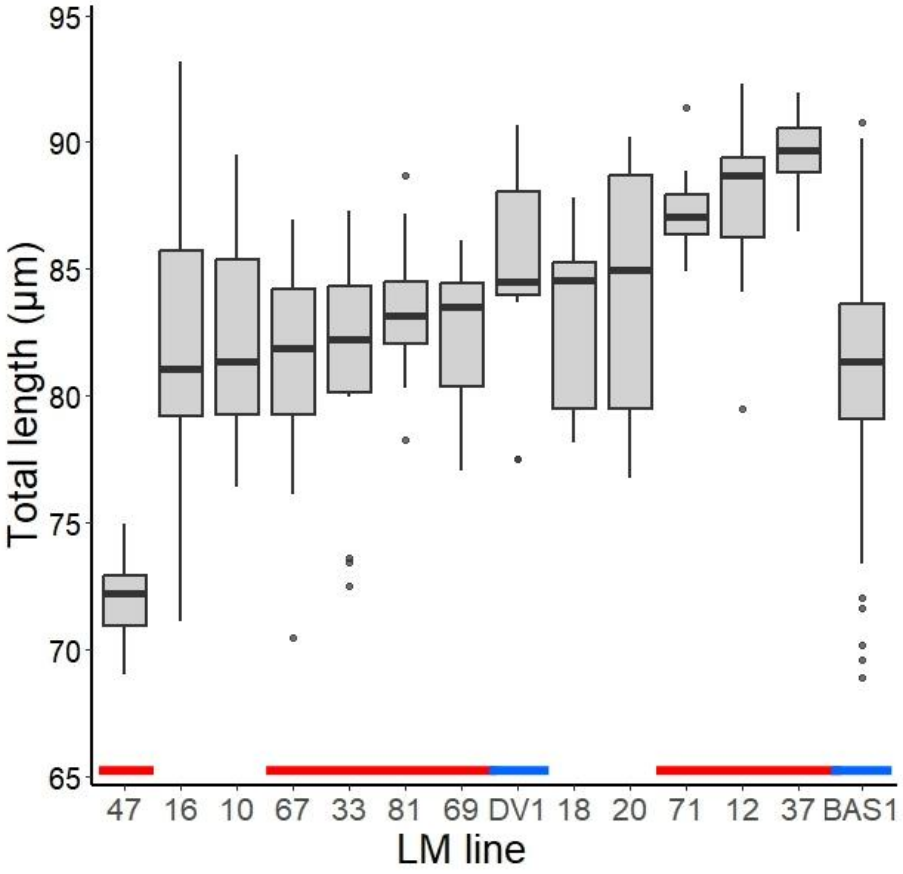

Figure 4.2 - Variation in total sperm length among the 12 LM lines, DV1 line and the outbred BAS1 culture. LM lines are arranged in increasing order of median total sperm length. Red bars indicate the 8 LM lines chosen for the sperm competition experiment and the blue bars indicate the DV1 and BAS sperm size.14-15 worms were measured for each line and 39 worms for the BAS1 culture. About 15 sperm cells were measured for each worm in both BAS1 and LM lines.

Sperm size and sperm transfer success

The generalized linear model testing differences between mating groups of LM lines revealed that the total number of sperm sampled did not differ between the mating groups of LM lines ($\chi^2 = 7.30$, df = 7, $P = 0.40$), showing that our measures of sperm transfer were not biased by the number of sperm sampled. We observed a significant between-line variation in sperm transfer success of LM focal worms (line identifier: $\chi^2 = 52.07$, df = 7, $P = 5.65e^{-9}$). We detected significant negative relationships in the first-order linear models between sperm transfer success and all three sperm traits modelled (Table 4.1). The second-order quadratic models revealed a significant non-linear relationship with intermediate lengths having higher sperm transfer success for all three sperm traits (Table 4.1, Figure 4.3 and S4.2). Additionally, likelihood-ratio tests suggest that the quadratic regression models provided significantly better fits to the data compared to the linear regression models (Table 4.1).

Discussion

Sperm size has repeatedly been found to evolve in response to sperm competition across species (Balshine et al., 2001; Godwin et al., 2017; LaMunyon and Ward, 2002; Tourmente et al., 2011). However, our understanding of how sperm size affects sperm competitiveness and hence male reproductive performance within species is limited. In this study, we observed a hump-shaped relationship between post-mating success and sperm size with sperm of intermediate size having on average the highest competitiveness in the simultaneously hermaphroditic flatworm *M. lignano*. Our results provide support for the hypothesis that sperm competition may favour the evolution of intermediate sperm size (Calhim et al., 2007; Parker, 1993). In the following, we discuss our findings and point to the strengths and limitations of our study.

Sperm transfer success and sperm size

Non-linear selection towards an optimal sperm size has been suggested mainly by comparative studies (Kleven et al., 2008; Lüpold et al., 2009), inferred from a reduction in sperm size variation in species with higher levels of sperm competition. Lymbery et al. (2018) observed non-linear selection with an intermediate peak on canonical axes of multiple sperm traits including sperm length, swimming velocity and swimming path linearity in the mussel, *Mytilus galloprovincialis*. In *M. lignano*, a recent study tested whether various sperm traits predict male reproductive performance (Marie-Orleach et al., 2023) and found no significant non-linear relationship between sperm morphology and sperm transfer efficiency - a fitness component comparable to sperm transfer success assessed in this study. This previous study did not explore the importance of total sperm length and studied purely phenotypic effects of sperm morphological traits on male fitness components, which does not allow inference of the evolutionary trajectories. In the present study, we aimed to fill this gap by exploring how genetic variance in total sperm length and other traits relates to sperm transfer success among inbred LM lines (discussed further below). Estimates of mean sperm length obtained from inbred lines correspond to breeding values, which allowed us to investigate how sperm competition selects on standing genetic variance in sperm length. Our approach demonstrates that selection arising from sperm transfer success favours the evolution towards intermediate length for all three sperm traits most relevant to post-copulatory sexual selection.

In our study, we focused on sperm transfer success to quantify potential selection on sperm size arising from competition to deposit sperm in the female reproductive tract, which is a crucial step in order to obtain reproductive success through male function. In *M. lignano*, sperm transfer efficiency (i.e., sperm transfer success per successful mating) has been estimated to explain 27% of the variation in male reproductive success

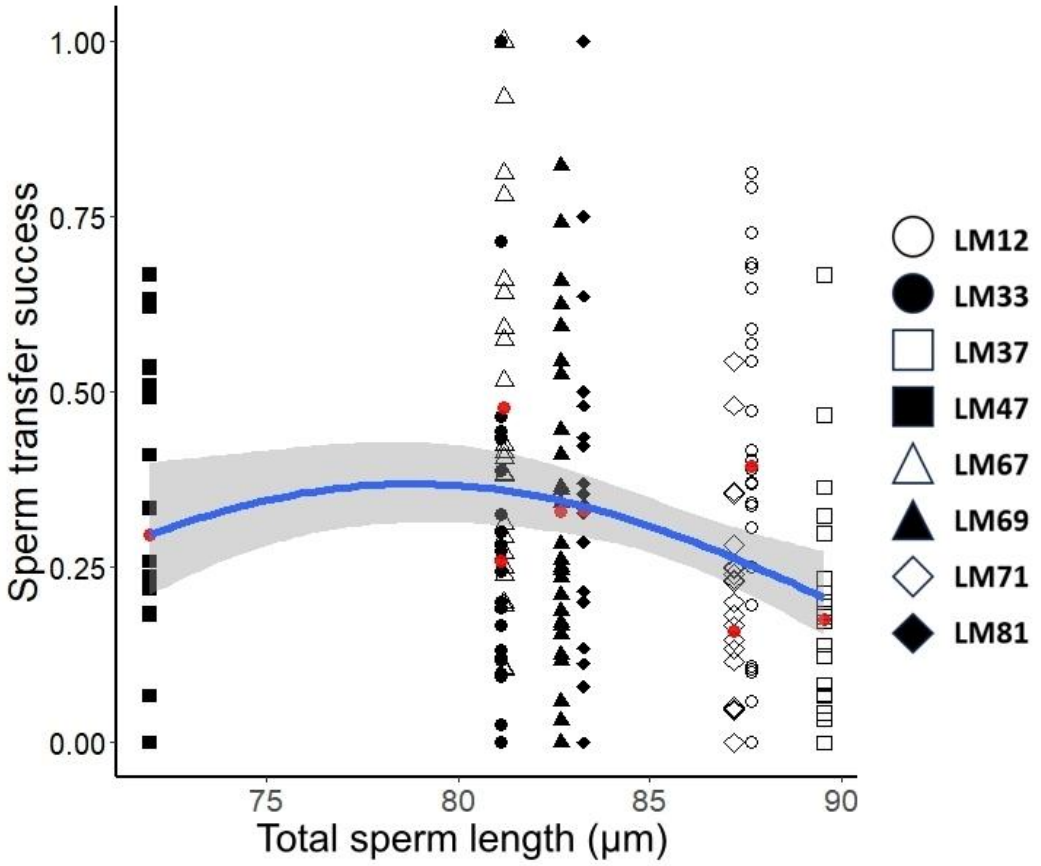

Figure 4.3 - Relationship between total sperm length and sperm transfer success. Symbols indicate different LM lines (see legend). Red dots indicate mean sperm transfer success in each line. The solid blue line shows the fit of the quadratic model using all data points, and grey regions indicate the 95% confidence intervals.

(Marie-Orleach et al., 2021). The fitness component that explained, with 37%, the most variation in male reproductive success was sperm fertilizing efficiency (i.e., the proportion of sired offspring relative to the number of sperm stored in the female sperm storage organ). However, sperm transfer success has been shown to strongly predict the proportion of sired offspring in *M. lignano* (Marie-Orleach et al., 2016) indicating that sperm transfer success is a good proxy for post-mating performance in the species. Nevertheless, we acknowledge that other aspects, such as sperm fertilizing efficiency, may also affect the selection of sperm traits and remain unexplored in our study.

All three sperm traits exhibited significant negative linear relationships with sperm transfer success. Such negative associations have been suggested to support the theory that sperm competition generates selection for increased number of sperm, which trades-off with sperm size resulting in smaller sperm (Gage and Morrow, 2003; Garcìa-González and Simmons, 2007). However, hump-shaped relationships provided the best fit for sperm transfer success for total length, bristle length and feeler length, suggesting selection for intermediate sperm size.

Table 4.1 - Statistical analysis of the relationship between sperm traits and sperm transfer success. Results of ANOVA Wald tests for linear and quadratic polynomial regressions are shown together with likelihood ratio tests comparing the two models for all sperm morphological traits. Significant *P* values are shown in bold.

Sperm trait	**Linear model**				**Quadratic model**				**LRT**	
	χ^2	*P*	R^2	qAIC	χ^2	*P*	R^2	qAIC	χ^2	*P*
Total sperm length	9.497	**0.002**	0.039	300.88	7.859	**0.005**	0.073	294.45	37.10	**0.003**
Total sperm length²					8.415	**0.004**				
Feeler length	8.903	**0.003**	0.037	291.77	3.560	0.059	0.054	289.72	55.33	**0.043**
Feeler length²					4.010	**0.045**				
Bristle length	4.866	**0.027**	0.020	300.99	6.547	**0.011**	0.049	296.01	170.00	**0.008**
Bristle length²					6.790	**0.009**				

Selection for increased sperm number alone cannot explain this result satisfactorily. Selection for intermediate sperm size, however, was predicted by theoretical models when the fertilization efficiency of sperm increased with a decreasing gradient in relation to size (Parker 1993, Parker and Begon, 1993, Parker et al., 2010). These models assumed that larger sperm have a fertilization advantage over smaller sperm but an increase in mass of each sperm traded off against other male reproductive expenditures, such as sperm number and mate acquisition. We suspect that the lower success of individuals with particularly long sperm observed in our study may be explained by this trade-off between sperm size and number and that selection for intermediate sperm size may provide a balance between the selective forces for more competitive, but fewer, large sperm and numerous, but less competitive, small sperm.

The selection for intermediate sperm size could also arise from the female environment where sperm compete, as the sperm size of DV1 fell in the intermediate range of the LM sperm size distribution. Sperm size has been shown to covary with the female reproductive tract (or its components) across diverse taxa (Minder et al., 2005; Morrow and Gage, 2000; Pitnick et al., 1999, 2003; Briskie and Montgomerie 1992) and evolve in response to selection on the female reproductive tract in *Drosophila melanogaster* suggesting a role of female sperm choice for shaping sperm morphology (Miller and Pitnick, 2002). Cryptic female choice by DV1 recipients in terms of a choice for sperm size that was similar to their own may have favoured intermediate sperm size in our experiment. However, the female antrum in *M. lignano* is a simple organ with a single chamber to receive and store sperm. The cell membrane along the wall of the antrum is convoluted and highly flexible, which is required for the passage of eggs (Vizoso et al., 2010). Given the simple structure and flexibility in size, we do not have a clear mechanistic explanation for how the antrum may exert female choice on sperm in relation to size.

Different components of sperm morphology may have very different consequences for sperm competitiveness, such that different traits show divergent associations to male reproductive success within species. For example, in the red deer (*Cervus elaphus*), sperm head and tail length were positively associated with sperm swimming velocity and male fertility, but mid-piece length showed a negative correlation with male fertility (Malo et al., 2006). In *M. lignano*, bristle and feeler length have been hypothesized to play active roles in post-copulatory stages, preventing the removal of sperm from the female antrum and enabling anchoring of sperm to the cellular valve, respectively (Vizoso et al., 2010). Selection arising from sperm competition can be expected to be stronger on these two traits compared to others and we predicted linear selection for longer bristles and feelers. Our results showed non-linear selection for an intermediate length of these traits. This finding might be the consequence of strong positive correlations between the length of both traits and total sperm length (Janicke et al., 2010), for which we also found support for stabilising selection.

Using inbred worms provided us with groups of individuals that are expected to be genetically similar and we expected them to have a relatively narrow range of sperm size within lines and significant differences among lines. While we found clear differences among lines, we also observed substantial variation in sperm length within lines. This might explain the high variation in sperm transfer success within lines, reiterating the significance of stochastic and non-genetic sources of variation in the measurements of post-mating success (Marie-Orleach et al., 2021). Moreover, *M. lignano* exhibits a high mating rate, such that the focal worms are expected to have copulated repeatedly with each mating partner in the 24-hour mating trials. Our experimental setup did not allow us to control the number of copulations and we acknowledge that variation in sperm transfer success may also represent varying levels of multiple mating.

The inbred lines used in the study were derived from four geographically distinct populations of *M. lignano* (Vellnow et al., 2017) and are expected to differ genetically, resulting in variation between the LM lines. Therefore, phenotypic differences other than sperm size arising from the focal LM genotype and/or its interaction with the partner DV1 genotype could have contributed to between-line variation in sperm transfer success in the LM lines. Moreover, partner genotypes have been shown to affect reproductive morphology and behaviour in *M. lignano* (Marie-Orleach et al., 2017) but evidence for the influence of partner genotype on fitness components, such as mating success and sperm transfer success, is still lacking. Thus, as true for most studies on selection gradients, our results do not allow us to establish a causal link between sperm size and sperm transfer success.

A shortcoming of our study was a discontinuous gradient in sperm size among LM lines with a particularly big difference between the line with the shortest (LM47) and the line with the second shortest sperm (LM67). This resulted in the LM 47 being the only representative of the lower end of the spectrum in sperm size. However, we decided not to remove LM47 from the analysis because our aim was to cover the largest possible variation in sperm size that could be observed in the outbred BAS1 culture of *M. lignano*, which itself is likely to harbour only a fraction of the variation in sperm size compared to natural populations. Another potential shortcoming of our study is that the variation in sperm length within inbred lines was unexpectedly high. We did not measure the actual sperm size of the focal LM worms used in the mating experiments. Given that focal worms were taken from inbred lines with a relatively narrow range of variation, we expect the actual sperm size of the focal worms would not differ substantially from the values used in our analysis. We also accounted for among-line differences in the variance of sperm length by adding the inverse of standard deviations in sperm size as a weighting factor in our model. Our results remained qualitatively unaffected without the weighting factor in the model.

Intra-specific variation in sperm size

Intra-specific variation in sperm size is a frequently observed phenomenon across animal taxa (reviewed in Pitnick et al., 2009). We also observed substantial variation in sperm size in both BAS1 culture and among the LM lines. The inbred line identity explained a substantial fraction of sperm trait variation (45% variation in total sperm length). This portion of variation is expected to be attributable to the genetic variance between the tested LM lines and is an estimate of the total genetic variance (equivalent to the broad-sense heritability) in *M. lignano*. This result is in line with the commonly observed high heritability in sperm size across animals (Simmons and Moore, 2009). In addition, we also observed substantial variation in sperm size within LM lines, with some lines expressing a range of sperm length equivalent to that of BAS1. The unusual genetic system of *M. lignano* might contribute to this relatively large within-line variation with karyotype polymorphisms frequently observed. Worms of this species have been reported to carry 2n = 8, 2n = 9 or 2n = 10 chromosomes with rare cases of other karyotypes (Zadesenets et al., 2016, 2020). The BAS1 culture has been generated such that all worms carry the normal karyotype of 2n=8 (Vellnow et al., 2018). However, the karyotypes of the LM lines have not yet been explored. Ancestral DV lines were derived from wild populations and karyotype polymorphisms may still persist in the LM lines, and thus contribute to the observed phenotypic variation. Karyotype polymorphism could result in increased variation in mRNA dosages of genes influencing sperm morphology and additional copies of chromosomes may account for more genetic material that has to be physically packed within the sperm shaft.

Conclusion

We used *in vivo* sperm counting to assess how genetic variance in sperm size translates into post-mating success in an outcrossing simultaneous hermaphrodite. Our finding of a hump-shaped association between sperm size and post-mating success suggests that sperm competition may favour an intermediate sperm size. We speculate that such stabilizing selection most likely results from a trade-off between sperm size and sperm number in *M. lignano*, as predicted by theory. Our study is one among the few to explore and present evidence for non-linear selection arising from post-copulatory sexual selection on sperm size and calls for future studies to consider divergent forms of selection on ejaculate traits.

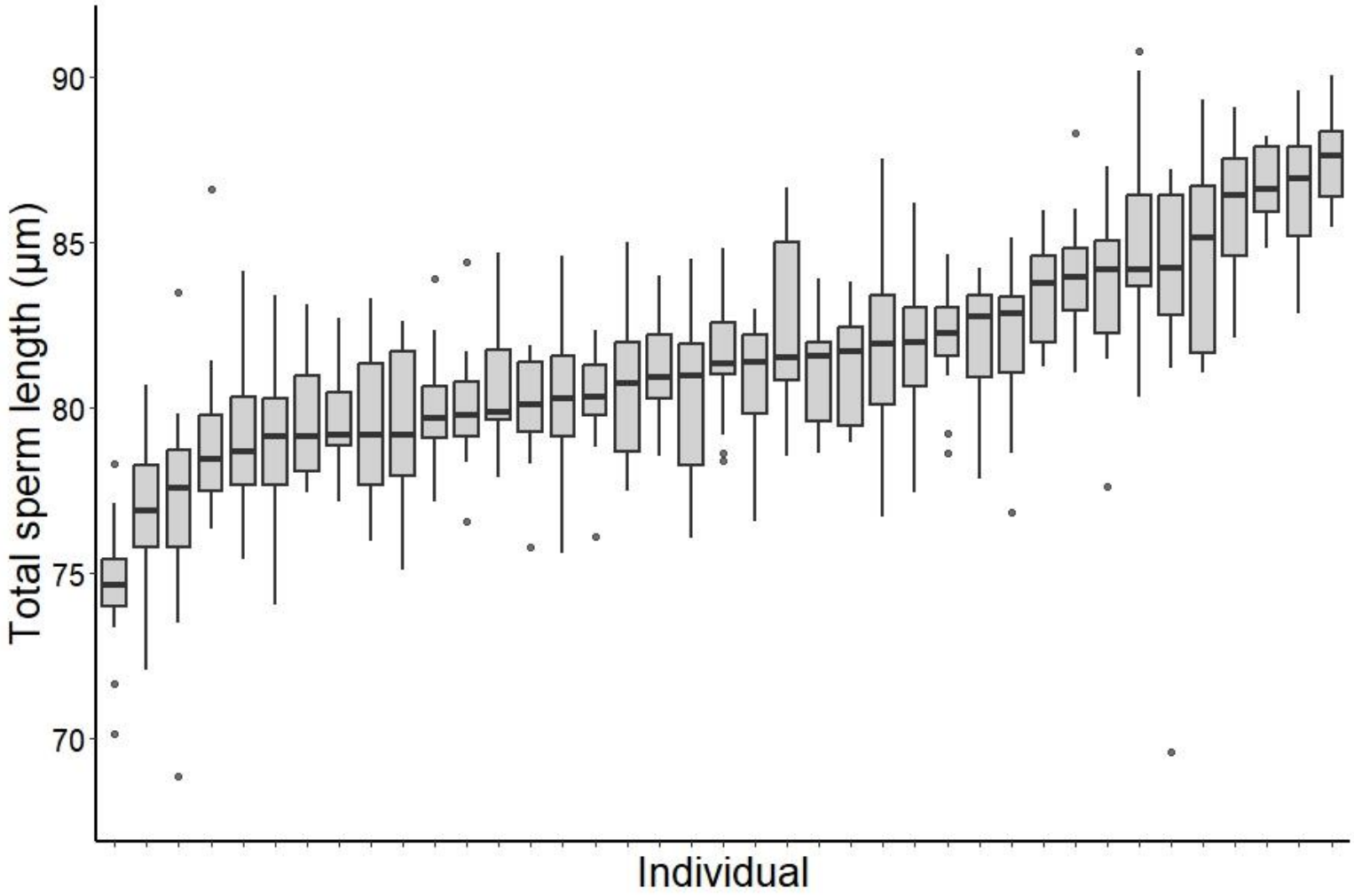

Fig S4.1 - Between-individual variation in total sperm length in the outbred BAS1 population (n = 39). Individuals are arranged in increasing order of their median total sperm length (see Table S4.1 for statistical tests).

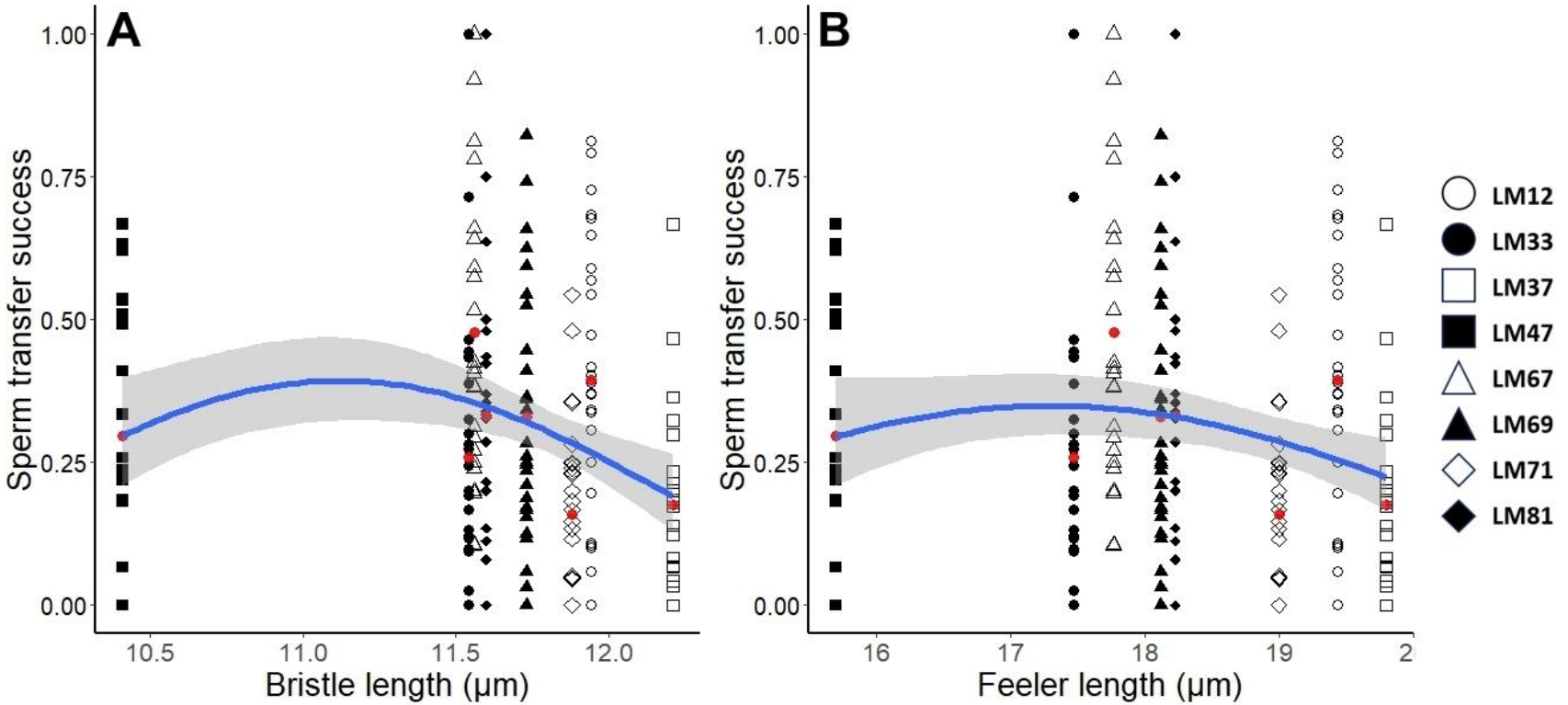

Fig S4.2 - Relationship between sperm transfer success and (A) lateral bristle length and (B) anterior feeler length. Symbols indicate different LM lines. Red dots indicate the mean sperm transfer success of each line. The solid blue line shows the fit of the quadratic model and grey regions indicate the 95% confidence intervals.

Table S4.1 - Statistical tests and variation in sperm traits in the BAS1 culture. (based on all sperm cells measured, n = 591) Results of Kruskal-Wallis analysis of variance testing for variation in sperm morphology between individuals (n = 39). (SD -Standard deviation; SE - Standard error; CV - Total coefficient of variation; WCV - Mean within-ejaculate coefficient of variation)

Sperm trait	Mean ± SD (µm)	Repeatability ± SE	CV (%)	WCV (%)	χ^2	*P*
Total sperm length	79.4 ± 3.51	0.962 ± 0.03	4.31	2.65	347.5	$<2.2e^{-16}$
Feeler length	16.92 ± 1.03	0.671 ± 0.167	6.10	4.87	202.54	$<2.2e^{-16}$
Bristle length	10.91 ± 0.6	0.911 ± 0.065	5.52	4.30	231.42	$<2.2e^{-16}$

Table S4.2 - Overall phenotypic variance in sperm traits and the variance due to LM line identity and individual worm identity, from linear mixed models (Proportions in brackets).

Sperm trait	Phenotypic Variance	Mixed model effects - variance		
		LM identity	Worm identity	Residual variance
Total sperm length	38.259	18.292 (0.45)	14.748 (0.37)	7.201 (0.18)
Feeler length	3.085	1.138 (0.36)	0.999 (0.31)	1.073 (0.33)
Bristle length	0.723	0.178 (0.24)	0.295 (0.40)	0.270 (0.36)

Table S4.3 - Information on the cultures and inbred lines of *Macrostomum lignano*.

Culture/ inbred line	Origin/ derived from	Method used	GFP+/GFP-	Use in this study	Reference
LS1 (outbred)	Field caught worms	Field sampling	GFP-	Not used	Marie-Orleach et al., 2013
DV1 (inbred)	LS1	Full- and half-sib crossing from pairs	GFP-	Common competitors in mating trials	Janicke et al., 2013; Vellnow et al., 2017
HUB1 (inbred)	DV1	Transgene (DNA) injection into single-cell embryo	GFP+	Not used	Janicke et al., 2013
BAS1 (outbred)	LS1	Backcrossing HUB1	GFP+	Measuring natural variation in sperm size	Marie-Orleach et al., 2016
LM lines (inbred)	DV lines (similar to DV1)	Backcrossing HUB1	GFP+	Focal worms in mating trials	Marie-Orleach et al., 2017

Chapter V

General Discussion

My PhD project addressed several aspects of sexual selection, with a special focus on its operation in a simultaneous hermaphroditic flatworm, *Macrostomum lignano*.

The GFP techniques established in *M. lignano*, a transparent organism, have already become a powerful tool for the study of sexual selection. A suite of transgenic lines, called 'NL lines', with distinct phenotypic markers expands the range of possible GFP techniques and enables more fine-scaled investigations into sexual selection. In **chapter II,** I provide phenotypic descriptions of the fluorescent markers in the seven NL lines and additionally, I tested and validated the reliability of the NL lines to be used as tools in experimental studies. Notably, the aim of the tests was not to determine whether all traits in NL line worms were similar to those in the wildtype DV1 worms. Instead, I aimed to establish that any potential negative effects associated with the integration of the artificial DNA constructs into the genome and expression of the fluorescent markers in the NL line worms can be considered negligible. Overall, my results showed that the NL worms did not face any costs in terms of their morphological development or reproductive ability. Certain features of the fluorescent markers, for example, the GFP+ nuclei in the gonads of the *nucleus-gfp* line, provide unique opportunities to measure internal processes in the worms and draw insights into evolutionary subjects such as sex allocation and sexual selection.

I have now described a new sperm phenotype with GFP localised in the sperm nucleus (in the *Nucleus-gfp* line) that can be differentiated from GFP- and GFP+ sperm. The GFP+ nucleus could potentially aid in karyotyping of the worms. That is, *M. lignano* has been found to exhibit polymorphism for the number of copies of its large chromosome (Zadesenets et al., 2016; 2017). More copies of the large chromosome mean more genetic material has to be packaged within the sperm shaft and may lead to longer nuclei in the sperm cells of individuals carrying more copies. I performed a preliminary study testing this possibility however using a Hoechst nuclear stain to stain and measure the length of the sperm nucleus, my results were inconclusive. The GFP marker can potentially be used to observe and measure the length of sperm nuclei accurately. Thus, the possibility of determining karyotype through the length of sperm nuclei could be tested again using the GFP+ sperm nuclei of the *nucleus-gfp* line. Additionally, I verified 14 different combination crosses between NL lines that result in offspring expressing fluorescent markers of both parents. This feature could thus be used as identifiers of parentage in mating groups representing different fluorescent markers. The study presented in Chapter III using the NL lines was possible mainly due to this feature and exemplifies the potential benefits of the advantages and limitations of the NL lines identified in the chapter.

In **Chapter III**, I used the fluorescent markers in the NL lines in combination with an experimental design to quantify sexual selection in *M. lignano.* I measured widely used metrics of sexual selection to test Charnov's (1979) prediction, that Bateman's

principles suggesting stronger sexual selection occurring in male compared to female animals also hold true for the male and female sex functions in simultaneous hermaphrodites. Measuring sexual selection in *M. lignano* importantly added to a very small body of studies testing Bateman's principles directly in simultaneous hermaphrodites. The absence of sexual dimorphism, plasticity in sex allocation within individuals and reciprocal copulation present unique conditions in which the occurrence of sexual selection has been rarely investigated. Recent studies in unilaterally mating hermaphroditic snails presented evidence supporting Charnov's prediction, revealing stronger sexual selection in the male function compared to the female function in these snail species (Anthes et al., 2010; Pélissié et al., 2012; Hoffer et al., 2017). To our knowledge, ours is the first study measuring Bateman's metrics in a reciprocally-copulating simultaneous hermaphrodite (but see Pongratz and Michiels, 2003 for a very similar test).

Contrary to the snails, my results showed no difference between the sex functions in the strength of sexual selection based on the standardized metrics of pre-copulatory sexual selection. Although all the metrics revealed a slight bias towards a stronger sexual selection in the female function in *M. lignano*, the metrics were not significantly different in our analysis. In addition to the absence of sexual dimorphism, reciprocal copulation in *M. lignano* leads to strictly equal mating success in the male and female sex functions. This is expected to restrict and shift the action of sexual selection from pre- towards post-copulatory episodes. Previous studies in *M. lignano* have indeed revealed a substantial opportunity for selection on the male sex function, which also appeared to occur predominantly during the post-copulatory episodes (Marie-Orleach et al., 2016; 2021). Thus, my findings are interesting as they potentially imply that Bateman's principle may not hold true in reciprocally-copulating hermaphrodites. However, the limitations of my experimental design and the metrics of sexual selection used prevent me from drawing strong conclusions based on my results. Clearly, more studies measuring sexual selection in reciprocally mating hermaphrodites are required in order to confirm the implications of my results.

A meta-analysis of 7 studies (including my own) presenting metrics of sexual selection in simultaneous hermaphrodites revealed an overall male bias in opportunity for selection and Bateman gradient. However, our dataset is very small and predominantly represented by hermaphroditic snail species, which may bias our results towards the unilaterally mating system and not simultaneous hermaphrodites in general. Therefore, more investigations on simultaneous hermaphrodites that also represent other mating systems, for example, reciprocal copulation and hypodermic insemination, are required to infer more general patterns for simultaneous hermaphrodites. Additionally, I also tested the effect of sampling duration on the metrics of sexual selection. My results are very similar to a previous study in multiple species showing that the commonly used

variance-based metrics of sexual selection (i.e., opportunity for selection, opportunity for sexual selection and Jones index, a derived estimate) are all sensitive to sampling duration and can be easily overestimated under short sampling duration. This is an important observation as experiments aimed at measuring sexual selection metrics often do so in short time intervals, mainly due to logistical reasons. These results recommend caution for any future studies to consider sampling duration as an important factor when measuring sexual selection metrics.

One of the most important traits involved in the post-copulatory stages of sexual selection is sperm size. Sperm size has been shown to influence the outcome of post-copulatory sexual selection through its associations with for example sperm motility and sperm longevity (Snook 2005; Fitzpatrick and Lüpold 2014). Currently, there is substantial evidence explaining the variation in sperm size among species, i.e., sperm size is positively associated with the level of sperm competition in species (e.g., Balshine et al., 2001; Briskie et al., 1997; Tourmente et al., 2011). However, sperm size is also found to vary substantially among males within species and empirical studies investigating the association between intra-specific variation in sperm size and sperm competitiveness provide mixed results. By using inbred lines of *M. lignano* expressing differing sperm size, in **Chapter IV,** I present data revealing a non-linear association between standing genetic variation in sperm size and sperm transfer success, measured as the proportion of sperm stored in the female antrum. My results suggest that sperm size may be under stabilizing selection towards an intermediate sperm size under sperm competition. This result is in line with theoretical predictions made under the assumption that sperm size trades off with other male reproductive investments, such as number of sperm produced or mate acquisition (Parker, 1993; Parker et al., 2010; Pizzari and Parker, 2009). Our study is one of the few that have tested non-linear associations between sperm size and sperm competitiveness.

The two sperm morphological traits, sperm feeler and lateral bristles, that have been posited to have active roles within the female storage organ were also found to exhibit the same non-linear association to sperm transfer success as total sperm size. This supports the proposition that all sperm traits directly involved in the outcome of sperm competition may face stabilizing selection. Alternatively, the association between the two sperm traits could just be a consequence of the strong correlation between the two sperm traits and total sperm size. Additionally, sperm transfer success represents only one potential source of selection acting on sperm size, i.e., their ability to be stored in the female sperm storage organ. Sperm size may also be selected by their relative ability to fertilize eggs, i.e., sperm fertilizing efficiency. The form of selection arising from sperm fertilizing efficiency is yet to be determined and an obvious next step in understanding sexual selection on sperm size in *M. lignano*. Acknowledging the limitations in the experimental design and that the observed association is correlational,

more studies that test for linear and non-linear associations are required in order to better understand the operation of sexual selection on sperm size.

Moreover, certain crucial features of the mating behaviour and sexual reproduction in *M. lignano* are yet to be fully understood and can provide novel insights. For example, the suck behaviour is a very interesting component of the mating behaviour in this species. It is posited to be associated with female resistance from being inseminated and may aid the female sex function to exert choice on the received ejaculates. Moreover, the lateral bristles that are part of the complex sperm design in *M. lignano* have been hypothesised to have evolved to aid the sperm to resist being sucked out of the female antrum by the suck behaviour. Although the suck behaviour has been described and addressed by a few studies, the evolutionary implication of the behaviour in terms of its influence on the overall outcome of sexual selection is yet to be understood. Additionally, I observed in a preliminary study that a substantial portion of copulations do not result in insemination, either by one partner or both partners. This could potentially indicate a high rate of mating failure in the species and can play an important role in sexual selection. Alternatively, this could also potentially represent a strategic investment of the male sex function into different mating partners, indicating a form of pre-copulatory mate choice in the species. Although reciprocal copulation is said to limit the action of pre-copulatory sexual selection, the latter scenario could be a mechanism for the action in the pre-copulatory sexual selection in *M. lignano*.

To summarize, my PhD studies are in support of other studies signifying the importance of sexual selection in simultaneous hermaphrodites. *M. lignano* as a model organism continues to prove to be a rich source of novel insights into the operation of sexual selection. I believe my contributions to the study of sexual selection through my PhD works suggest alternative ways to think about certain commonly assumed ideas in evolutionary biology. For example, by presenting an advantage for intermediate sperm size, I suggest that presuming larger sperm size always provides higher post-copulatory success may result in erroneous inferences. The absence of sex difference in the strength of sexual selection in a reciprocally-mating hermaphrodite species clearly warrants further investigations into this unique mating system, that may not fit into the classic paradigms of evolutionary biology.

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Curriculum Vitae

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Education

2020 – 2024	PhD in University of Basel, Switzerland Supervisors: Prof. Dr. Dieter Ebert, Dr. Lukas Schärer and Dr. Tim Janicke
2015 – 2020	Integrated BS-MS Dual Degree in Biological sciences, IISER Pune, India.
2015	Sainik School, Amaravathi nagar, Tamilnadu, India.

Publications

Santhosh. S., Ebert, D., and Janicke, T. (2024). Sperm competition favours intermediate sperm size in a hermaphrodite. *Journal of evolutionary biology*, accepted April 2024.

Santhosh. S., Ebert, D., and Janicke, T. (2024). Bateman gradients in a reciprocally-mating hermaphrodite. Manuscript under preparation.

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Conferences and Workshops.

2023 Film-making for scientists, University of Basel.

2023 Simultaneous Hermaphroditic Organism Workshop (SHOW), Amsterdam

2022 European Society for Evolutionary Biology (ESEB) Conference, Prague, 2022 – presented a poster.

2022 Evolutionary biology workshop, Guarda.

2022 Simultaneous Hermaphroditic Organism Workshop (SHOW), Thessaloniki.

2021 Biology22 Conference, University of Basel.

2021 Simultaneous Hermaphroditic Organism Workshop (SHOW), Basel.

2020 13th International Macrostomum meeting, online event.

2019 The First conference of the Indian Society of Evolutionary Biologists, Bangalore.

Student supervision

Jeanine Fluri and Flurina Schlöth, 2021. (3rd year Bachelor*). It's a numbers game: behavioural and morphological correlates of sperm transfer success in Macrostomum cliftonensis*. University of Basel, Switzerland.

Alina Lavater and Gian Schupbach, 2022. (3rd year Bachelor). *The link between sperm morphology and sperm transfer success in Macrostomum lignano.* University of Basel, Switzerland.