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Effects of two seminal fluid transcripts on post-mating behaviour in the simultaneously hermaphroditic flatworm *Macrostomum lignano*

Michael Weber | Bahar Patlar | Steven A. Ramm

Department of Evolutionary Biology, Bielefeld University, Bielefeld, Germany

Correspondence
Michael Weber, Department of Evolutionary Biology, Bielefeld University, Konsequenz 45, 33615 Bielefeld, Germany.
Email: michael.weber1@uni-bielefeld.de

Present address
Bahar Patlar, Department of Biology, University of Winnipeg, Winnipeg, MB, Canada

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Abstract

The seminal fluid proteins (SFPs) transferred to mating partners along with sperm often play crucial roles in mediating post-mating sexual selection. One way in which sperm donors can maximize their own reproductive success is by modifying the partner's (sperm recipient's) post-copulatory behaviour to prevent or delay re-mating, thereby decreasing the likelihood or intensity of sperm competition. Here, we adopted a quantitative genetic approach combining gene expression and behavioural data to identify candidates that could mediate such a response in the simultaneously hermaphroditic flatworm *Macrostomum lignano*. We identified two putative SFPs—*Mlig-pro46* and *Mlig-pro63*—linked to both mating frequency and 'suck' frequency, a distinctive behaviour, in which, upon ejaculate receipt, the worm places its pharynx over its female genital opening and apparently attempts to remove the received ejaculate. We, therefore, performed a manipulative experiment using RNA interference-induced knockdown to ask how the loss of *Mlig-pro46* and *Mlig-pro63* expression, singly and in combination, affects mating frequency, partner suck propensity and sperm competitive ability. None of the knockdown treatments impacted strongly on the mating frequency or sperm competitive ability, but knockdown of *Mlig-pro63* resulted in a significantly decreased suck propensity of mating partners. This suggests that *Mlig-pro63* may normally act as a cue in the ejaculate to trigger recipient suck behaviour and—given that other proteins in the ejaculate have the opposite effect—could be one component of an ongoing arms race between donors and recipients over the control of ejaculate fate. However, the adaptive significance of *Mlig-pro46* and *Mlig-pro63* from a donor perspective remains enigmatic.

KEYWORDS
mating rate, multiple mating, seminal fluid, sexual conflict, sexual selection, sperm competition

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1 | INTRODUCTION

In polyandrous species, if females store sperm from multiple males, post-mating sexual selection can occur when sperm from different males compete with each other for fertilization (i.e. sperm competition; Parker, 1970) and/or when females choose specific sperm to fertilize eggs (i.e. cryptic female choice; Eberhard,1996; Thornhill, 1983). As a consequence, several male adaptations have evolved to increase relative fertilization success, including displacing sperm of previous males (Harshman & Prout, 1994; Marie-Orleach, Janicke, Vizoso, Eichmann, & Schärer, 2014; Waage, 1986), preventing females from re-mating (Abraham et al., 2016; Radhakrishnan & Taylor, 2007, 2008; Uhl, Nessler, & Schneider, 2009) or possessing morphologically more competitive sperm (Birkhead, 1995; Birkhead & Pizzari, 2002). This can lead to sexual conflict over the optimal fitness strategies concerning reproduction, often resulting in cycles of sexually antagonistic coevolution (Chapman, 2018; Chapman et al., 2003; Parker, 1979; Pischedda & Stewart,2016).

The seminal fluid proteins (SFPs) found in the ejaculate play crucial roles in reproduction and can modulate the mating partner's behaviour and physiology such that they affect the reproductive success of both partners (reviewed in Avila, Sirot, Laflamme, Rubinstein, & Wolfner, 2011; Chapman, 2001; Hopkins, Sepil, & Wigby, 2017; Sirot, Wong, Chapman, & Wolfner, 2015), making these proteins key mediators of post-mating sexual selection (Cameron, Day, & Rowe, 2007; Chapman, 2001; Hodgson & Hosken, 2006; Poiani, 2006; Ram & Wolfner, 2007a). Additionally—because they can modulate reproductive behaviour and physiology in many ways that are favourable to the seminal fluid-donating individual (in gonochores, the male), but not necessarily to the seminal fluid-receiving individual (the female)—they can cause sexual conflict (Chapman, Liddle, Kalb, Wolfner, & Partridge,1995; Pischedda & Stewart,2016; Sirot et al., 2015). SFPs influence subsequent female physiology and behaviour in various ways (reviewed in Avila et al., 2011; Hopkins et al., 2017; Poiani, 2006; Sirot et al., 2015). For example, specific SFPs are known to modulate egg production, ovulation and/or egg-laying rates (Avila et al., 2011; Gillott, 2003; Poiani, 2006; Ram & Wolfner, 2007a) while other SFPs can affect sperm storage (Chapman, Neubaum, Wolfner, & Partridge, 2000; Neubaum & Wolfner, 1999; Qazi, 2003). Such functions do not inevitably lead to sexual conflict, but a good example of an SFP that acts in a sexually antagonistic manner is sex peptide in *Drosophila melanogaster*, which decreases female receptivity and stimulates egg production but lowers females' fitness and lifetime reproductive success (Wigby & Chapman, 2005).

Another example of a potential sexual conflict mediated through the seminal fluid is a mating-induced change of the partners' sexual receptivity. Decreased sexual receptivity of mated females occurs in a wide range of insects, and it is suggested that males benefit from this change because it decreases the likelihood or intensity of sperm competition. In *D. melanogaster*, mated females actively reject courting males and the SFP 'sex peptide' received with the ejaculate plays a central role in inducing this change (Chapman et al., 2003; Häsemeyer, Yapici, Heberlein, & Dickson, 2009; Liu & Kubli, 2003; Ram & Wolfner, 2009; Yang et al., 2009; Yapici, Kim, Ribeiro, & Dickson, 2008): females mated to sex peptide null males remain highly receptive to re-mating (Chapman et al., 2003; Liu & Kubli, 2003).

Although *Drosophila* has been the main model species to date for studying such seminal fluid-mediated effects, there are several other reported cases in a broad range of insects where females show a similarly reduced sexual receptivity after receiving full ejaculates or specific SFPs (reviewed in Avila et al., 2011; Simmons, 2001). For example, females of the Queensland fruit fly *Bactrocera tryoni* show no difference in sexual receptivity when mated to irradiated males (deficient in sperm) compared with mates of nonirradiated males, suggesting that components of the seminal fluid, not sperm, are responsible for the decreased post-mating receptivity observed (Harmer, Radhakrishnan, & Taylor, 2006). Moreover, the injection of extracts obtained from male reproductive tracts to *B. tryoni* females leads to a decrease in sexual receptivity and shorter copulation times when subsequently mated, similar to behaviours seen in previously mated females (Radhakrishnan & Taylor, 2007). In *Anopheles gambiae*, injection of male accessory gland homogenates into virgin females results in a decreased likelihood of re-mating (Shutt, Stables, Aboagye-Antwi, Moran, & Tripet, 2010). Other examples of SFPs affecting post-mating behaviour include lady beetles (Perry & Rowe, 2008a, 2008b), seed beetles (Moya-Laraño & Fox, 2006; Rönn, Katvala, & Arnqvist, 2006; Yamane, Miyatake, & Kimura, 2008) and ground beetles (Takami, Sasabe, Nagata, & Sota, 2008). Furthermore, there is some evidence for similar seminal fluid-mediated effect in vertebrates; for example, the beta-endorphin found in rat seminal fluid has the capacity to suppress female receptivity (Forsberg, Bednar, Eneroth, & Södersten, 1990). Depending on any direct or indirect benefits females might accrue from additional matings, such effects may often be counter to female interests (Chapman et al.,1995; Hollis et al., 2019; Wigby & Chapman, 2005).

Charnov (1979) first recognized that sexual conflict also occurs in hermaphrodites, since sperm donors can also potentially benefit from transferring ejaculate components manipulating sperm recipients and affecting the outcome of sperm competition (Charnov, 1979; Koene, 2006; Michiels, 1998; Schärer, Janicke, & Ramm, 2015). Moreover, as we explain in more detail below, simultaneous hermaphroditism might also create unique targets for seminal fluid action (Charnov, 1979; Schärer et al., 2015; Schärer & Ramm, 2016). Such seminal fluid-mediated effects, with a potential beneficial effect for one but a potential harmful effect for the other partner, have already been detected in the pond snail *Lymnaea stagnalis*. The intravaginal injection of one *L. stagnalis* SFP (LyAcp10) led to a decrease in egg laying (Koene et al., 2010), and the injection of two other SFPs (LyAcp8b and LyAcp5) reduced the number of sperm transferred by the recipient in a subsequent mating as a donor and, as a result, decreased their paternity success (Nakadera et al., 2014; see also Schärer, 2014). This latter effect emphasizes that it is a potentially adaptive strategy in simultaneous hermaphrodites to steer your partner away from its male function and that the action of seminal fluid may be one means of doing so (Schärer & Ramm, 2016). There

is also evidence for the manipulation of the re-mating frequency in a simultaneously hermaphroditic species, namely the love-dart shooting snail *Euhadra quaesita*. Before exchanging sperm, both mating partners attempt to drive their mucous-coated love-dart into their respective partner. In stabbed snails, the intermating interval is longer than in not-stabbed individuals, and so snail pairs injected with mucous subsequently mate less often than control pairs (Kimura, Shibuya, & Chiba, 2013).

In our model species *Macrostomum lignano*, the complement of SFPs has only just been characterized (Weber et al., 2018). Nevertheless, there are already some indications for potential effects of SFPs. An initial screen of 18 putative SFPs selected without any prior functional information revealed that the RNAi-induced knockdown of at least some SFPs may modulate aspects of fertility and sperm competitiveness (Weber, Giannakara, & Ramm, 2019). However, given that this screen identified only a minority of candidate SFPs modulating fertility or sperm competitiveness, and that all of these effects have not yet been verified after controlling for multiple testing, this suggests that more targeted methods may be beneficial to first prioritize candidates mediating specific effects. Patlar, Weber, Temizyürek, and Ramm (2020) adopted precisely such an approach, using quantitative genetics to identify six candidate SFPs likely to affect the post-mating 'suck' behaviour of sperm recipients, based on a negative genetic correlation between SFP transcript expression (Patlar, Weber, & Ramm, 2019) and suck propensity. The suck behaviour in *M. lignano* is a striking and frequent response to ejaculate receipt (occurring after *c*. 50% of copulations), in which the worm places its pharynx over its female genital opening and appears to attempt to suck out its contents, suggesting this trait has evolved in the context of sexual conflict over control of the received ejaculate (Marie-Orleach, Janicke, & Schärer, 2013; Schärer, Joss, & Sandner, 2004; Schärer, Littlewood, Waeschenbach, Yoshida, & Vizoso, 2011; Vizoso, Rieger, & Schärer, 2010). Whether worms are attempting to remove sperm or seminal fluid, or both, by this sucking behaviour, as well as the resulting fitness consequences, is so far unclear. RNAi-induced knockdown of two of the six SFP candidate genes, *Mlig-pro31* and *Mlig-pro32* (designated as *'suckless'* genes), caused a substantially increased suck propensity of mating partners, suggesting that these transcripts indeed manipulate mating partners and may thereby mediate sexual conflict over ejaculate fate, but no clear effect on paternity outcomes in a standardized defensive sperm competition assay (Patlar et al., 2020, but see Patlar & Ramm, 2020).

The quantitative genetics approach adopted by Patlar et al. (2020), linking transcript expression to downstream phenotypes, provides a powerful framework to identify candidates for subsequent screening of SFP-mediated effects in a targeted manner. We therefore adopted this same method, estimating genetic correlations between seminal fluid expression and partner behaviour to identify two candidate SFPs, *Mlig-pro46* and *Mlig-pro63*, potentially affecting two behavioural phenotypes of interest: partner re-mating behaviour and the propensity for partners to exhibit the suck behaviour (see Materials and Methods). In order to test whether these transcripts indeed influence these behaviours and have downstream impacts on fitness, we then used RNAi to test these two putative SFPs for their effects on mating frequency in pairings with untreated partners, on the suck propensity of mating partners, as well as sperm competitive ability. Both putative SFPs were already included in the above-mentioned naïve RNAi screen (Weber et al., 2019), in which *Mlig-pro46* showed some evidence for a reduced offensive sperm competitive ability (P_2, see below), making it an even more promising candidate for more detailed characterization.

2 | MATERIALS AND METHODS

2.1 | Study organism and experimental subjects

Macrostomum lignano is a free-living, outcrossing simultaneous hermaphrodite found in the Northern Adriatic Sea and Eastern Mediterranean (Ladurner, Schärer, Salvenmoser, & Rieger, 2005; Zadesenets et al., 2016). The worms reach *c*. 1.5 mm in body length, and the testes and ovaries are located in the central part of the body on either side of a medial gut. The male copulatory organ, the female reproductive organ and the prostate gland cells (where seminal fluid is produced) are all located in the posterior part of the worms (Hyman,1951; Ladurner, Pfister, et al., 2005; Ladurner, Schärer, et al., 2005; Weber et al., 2018). *M. lignano* is obligately outcrossing; that is, self-fertilization does not occur, and copulations are reciprocal: both partners receive and donate sperm at the same time (Schärer & Ladurner, 2003). *M. lignano* shows an average mating rate of about 6–15 copulations per hour (Janicke & Schärer, 2009b; Marie-Orleach et al., 2013; Schärer, Joss, et al., 2004), and the high mating rate may lead to suck behaviour because individuals likely prefer donating sperm more than receiving (Schärer, Joss, et al., 2004; Vizoso et al., 2010). The worms are kept in cultures in glass petri dishes filled with artificial sea water (ASW, 32‰) or nutrient-enriched artificial sea water (Guillard's f/2 medium) (Guillard & Ryther, 1962) and fed with diatoms (*Nitzschia curvilineata*). They are kept under standard conditions on a 14:10 light:dark cycle at 60% relative humidity and a constant temperature of 20°C. All the animals used in this experiment as knockdown/control donors and as recipients (see below) belonged to the highly inbred DV1 line (Janicke et al., 2013) that was previously used to identify and functionally characterize putative seminal fluid candidates (Patlar et al., 2020; Weber et al., 2018, 2019).

For the identification of transcripts that are genetically correlated with mating frequency, we analysed mating behaviour recorded in an earlier experiment by Patlar et al. (2019). Briefly, the worms used in this experiment originated from 12 highly inbred lines (hereafter genotypes) that belong to a larger set of inbred lines which was originally generated at the University of Innsbruck and is now maintained at the University of Basel (for details, see Vellnow, Vizoso, Viktorin, & Schärer, 2017).

To assign paternity to offspring of competing ejaculate donors (i.e. what would be competing males in separate-sexed animals), we used an outbred transgenic BAS1 line of *M. lignano* that expresses GFP ubiquitously (Marie-Orleach, Janicke, Vizoso, David, & Schärer,

2016; Vellnow, Marie-Orleach, Zadesenets, & Schärer, 2018) as sperm competitors. Thereby, the resulting offspring could be unambiguously assigned as being sired by either the DV1 (GFP^-) or BAS1 (GFP^+) worm (see also Janicke et al., 2013; Marie-Orleach et al., 2014). BAS1 was produced by backcrossing the GFP-transformed HUB1 line (Wudarski et al., 2017) and offspring production, mating frequency and morphology were previously found not to differ between individuals from HUB1 (GFP^+) and DV1 (GFP^-) lines (Marie-Orleach et al., 2014).

2.2 | Putative seminal fluid transcripts in *M. lignano*

Transcripts encoding putative SFPs in *M. lignano* were previously identified in two stages. First, Ramm et al. (2019) used an RNA-Seq approach in combination with data on tail-limited expression from an earlier study (Arbore et al., 2015) to define a set of putatively tail-specific transcripts that are differentially expressed between an environment with a high sperm competition level (group of eight worms) compared to a no sperm competition level (isolated worms) (Social group size is a good predictor of mating group size and thus sperm competition intensity in *M. lignano* [Janicke & Schärer, 2009a; Schärer & Ladurner, 2003; Schärer, Ladurner, & Rieger, 2004]). The vast majority of these transcripts (140/150) exhibited higher expression in octets, meaning they likely represent an aspect of male allocation in this species. Based on *M. lignano's* anatomy, their most likely site of expression would be the prostate gland cells located around the male genital opening (stylet) that are responsible for seminal fluid production.

In a second step, Weber et al. (2018) then performed an in situ hybridization (ISH) screen to define which of these plastic tail-specific transcripts really are prostate-limited in their expression, identifying a subset of 76 transcripts expressed exclusively in the prostate gland cells making these putative SFP transcripts. Fifty-eight of these transcripts were included in a subsequent gene expression study characterizing genotypic, environmental and GEI sources of variation in transcriptional expression of seminal fluid (Patlar et al., 2019), and we selected candidates for this study from among these 58 transcripts.

2.3 | Selection of candidate transcripts

Based on this information, we could then identify candidate transcripts that affect mating frequency for subsequent RNAi. To do so, we re-used data on SFP transcript expression reported in Patlar et al. (2019) together with novel data on mating frequency collected but not reported as part of that earlier experiment (Patlar et al., 2019). Briefly, 12 inbred lines of *M. lignano* were raised in two different social group sizes, namely groups of two (no sperm competition) or eight (high sperm competition) individuals. All genotype/group size combinations were raised under controlled conditions for *c*. 7 weeks until behavioural and SFP expression measurements were carried out (see details Patlar et al., 2019).

To examine the mating frequency, mating pairs were formed as one partner originated from a single inbred line (DV1) to be used as the virgin, standardized recipient grown under strict isolated conditions, and the other partner, the donor, originated from one of the different genotype/group size combinations. In total, 18 independent pair replicates and 18 independent octet replicates of each genotype were coupled with standardized recipients to observe mating behaviours. To be able to distinguish partners in mating pairs under normal light during observations, the recipient worms were exposed to the food colouring dye Grand Bleu [E131, E151] (Les Artistes—Paris), diluted to a concentration of 0.25 mg/ml in ASW, for 24 hr beforehand. Such a 24-hr exposure enables us to easily distinguish coloured from noncoloured worms and has previously been shown not to affect the mating frequency (Marie-Orleach et al., 2013). Observations were done by video recording, and each donor worm and its coloured recipient partner was transferred into two-dimensional, mating observation chambers described in detail elsewhere (Schärer, Joss, et al., 2004). Briefly, the recipient worm was transferred with a 1.7 μl drop of ASW to the centre of a coated (Sigmacote®) microscope slide which has four HERMA photo stickers as spacers adhered to the sides (two per side). Then, the donor worm was transferred with 1.7 μl ASW to the recipient, merging the two drops. Each observation chamber consisted of 16 such pairs, each made by 3.4 μl drops of ASW, on a single glass slide. Once all mating pairs had been placed on the slide, another coated microscope slide was placed upon the first, to form each drop into a shallow three-dimensional pool that the worms can swim inside (Figure S1). To protect the drops from evaporation, the area was sealed with a thin line of Vaseline around the perimeter prior to adding the second slide. Immediately after the observation chamber was ready, it was placed under a camera for a video recording of 2.5 hr. Video recordings were obtained with a DFK 41AF02 camera (Imaging source GmbH) connected to a computer running the software Debut Video Capture Professional, version 2.02. The videos were captured at one frame per second, with a frame rate of 10 (a video of 2 hr was zipped into 12 min), in .mov format with 1920 × 1080 HD resolution. Videos were analysed using the Kinovea video player, version 0.8.15. For analysis of mating frequency and suck propensity, the number of copulations for each mating pair and the number of suck events of the recipient worm were counted throughout the full 2.5-hr period based on the videos recorded.

We then identified candidates in two steps. First, we used the first four principal components (PCs) describing variation in seminal fluid transcript expression from Patlar et al. (2019) to estimate which (if any) of these were genetically correlated with mating frequency, in both pairs and octets. Together, these four PCs explained *c*. 76% of the total variation in seminal fluid transcript expression. Because PC1 accounted for *c*. 42% of the total variance, including positive loadings for the majority of transcripts, with no corresponding negative loadings, it was interpreted as capturing overall investment in seminal fluid transcript expression. By contrast, PC2–PC4 each exhibited a mixture of positive and negative loadings for a smaller subset of SFP transcripts and therefore

describe instead variation in seminal fluid composition (Patlar et al., 2019). Based on identifying one such PC, namely PC4, which was consistently negatively correlated with mating frequency at both social group sizes, we then investigated further those transcripts that were significantly loaded on PC4. We directly assessed how SFP expression level correlated with mating frequency, predicting that candidate mediators of a reduced mating frequency in partners should exhibit a negative genetic correlation between expression level and mating frequency. Two such candidates (*Mlig-pro46* and *Mlig-pro63*, see Results) were then investigated further in an RNAi knockdown experiment.

Because the same two candidate transcripts, *Mlig-pro46* and *Mlig-pro63*, were also positively loaded on an additional axis of seminal fluid variation, PC3, and this was positively correlated with suck propensity in the pairs environment (Patlar et al., 2020), we further investigated whether RNAi knockdown also impacted on partner suck behaviour.

2.4 | Raising conditions for the RNAi knockdown experiment

Six to eight days post-hatching, a batch of same-age hatchlings (to be used as donors and recipients) was collected and distributed in glass petri dishes filled with ASW and fed ad libitum with algae at a density of *c*. 150 individuals per dish. Individuals were transferred once per week to new glass petri dishes filled with ASW and ad libitum algae until they underwent tail amputation (days 40 and 36 for the doors and recipients, respectively—see below).

2.5 | RNA interference

RNAi was performed as previously described (Kuales et al., 2011), largely following the same procedures as in Weber et al. (2019) to ensure comparability of results. Briefly, for both seminal fluid candidates, a double-stranded RNA (dsRNA) probe was generated by an in vitro transcription system using primer pairs with T7 and SP6 promoter regions (T7 and SP6 Ribomax™ large scale RNA kit; Promega) (*Mlig-pro46*: forward primer: CTGCACGGTTGTTACCTTCG, reverse primer: TCATCTTCATAATTGCGGTGAAAG; *Mlig-pro63*: forward primer: ACAACTGACAATGCGATTAGC, reverse primer: CTGCTCGTACACAACCATCG). In the control treatment, we added just water instead of dsRNA; control individuals were otherwise treated identically to the knockdown individuals. Previous studies with dsRNA for firefly luciferase indicate receipt of dsRNA per se does not affect worms (Arbore et al., 2015; Lengerer et al., 2018; Pfister et al., 2008; Sekii, Salvenmoser, De Mulder, Schärer, & Ladurner, 2009; Weber et al., 2019), so here we followed several other *M. lignano* studies in using a no dsRNA probe as the negative control (Kuales et al., 2011; Lengerer et al., 2014; Ramm et al., 2019). The efficacy of RNAi knockdown was verified by performing whole-mount ISH in a preliminary test (Figure S1).

Before beginning the RNAi (or control) treatment, donor animals were tail-amputated between the antrum and ovaries. This takes advantage of the regenerative capacity of *M. lignano* (Egger, Ladurner, Nimeth, Gschwentner, & Rieger, 2006), allowing us to remove the antrum, with all potential previously received ejaculate in it, the seminal vesicle, with potential (own) stored sperm as well as the SFP-producing prostate gland cells. Through amputation and subsequent regeneration, we ensured that seminal fluid production was 'reset' prior to the RNAi/control treatment and the individuals have an equal amount and age of stored sperm and seminal fluid reserves and further that individuals also contained no received sperm or seminal fluid at the beginning of the mating trials (see below). After amputation, individuals were randomly allocated to one of four treatment groups: either the negative (no dsRNA) control or one of three RNAi treatments containing the relevant dsRNA solution (*Mlig-pro46* only; *Mlig-pro63* only; or *Mlig-pro46* and *Mlig-pro63* combined). They were maintained in these treatment groups throughout the regeneration process, kept individually in a well of a 60-well microtest plate (Greiner Bio-One™ 60-well HLA Terasaki Plates). Each worm was placed in 10 µl dsRNA solution (*c*. 25 ng/µl dsRNA for the specific transcript (in combined treatment in total 50 ng/µl dsRNA) in ASW-algae mix). Throughout the whole experiment, animals were fed ad libitum with algae and were maintained under standard culture conditions. On days 41, 43, 45, 47 and 49, 2 µl of the relevant dsRNA solution was added to each RNAi well. On days 42, 44, 46 and 48, the worms were transferred to a new well containing 10 µl of new dsRNA solution to ensure a constant exposure to dsRNA. The first mating trial was conducted on day 10 post-amputation, which is sufficient time to allow complete regeneration (Egger et al., 2006; Lengerer et al., 2018).

The GFP^+ donor worms used in the experiment as sperm competitors to the experimental subjects were also tail-amputated on the same day as the knockdown/control worms at the age of 40 (±1) days. Thereafter, they were also each kept individually in one well of a 60-well microtest plate in 10 µl ASW with ad libitum algae. GFP^+ worms were transferred to a new well containing 10 µl ASW with ad libitum algae once on day 45.

The recipient worms used in the experiment were 50 (±1)-day-old adult worms and were also tail-amputated, 14 days prior the mating assay at age 36 (±1) days. After amputation, they were also each kept individually in one well of a 60-well microtest plate in 10 µl ASW with ad libitum algae. Recipient worms were transferred to a new well containing 10 µl ASW with ad libitum algae once on day 43.

2.6 | Observation of mating and suck frequency of knockdown

For each RNAi/control treatment, we then conducted two separate assays with separate batches of donor, competitor and recipient worms, to measure (a) mating frequency (in both batches), (b) suck propensity (in both batches) and (c) either defensive (P_1) or offensive (P_2) sperm competitive ability (depending on the batch: in batch 1 we

measured only P_1 and in batch 2 only P_2). Initially, worms in both assays (P_1 and P_2) were treated identically. The mating frequency and suck propensity were calculated by counting the number of copulations and the number of suck events per mating by the recipient during the 2.5-hr mating period which was filmed during the sperm competitive ability assays described in the next subsection. Video recording was performed using mating chambers using the same procedure as described above, with the only exception being that the mating period lasted for 2.5 hr here instead of the 2 hr used for the earlier trials identifying candidates.

2.7 | Sperm competitive ability assays (P_1 and P_2)

All mating trials were conducted on day 11 post-amputation. Recipients were kept after their mating trial in 60-well plates in ASW with ad libitum algae and were transferred to a new well every second day until day 11 (6 wells in total), where they remained until day 21 (after which no further offspring were detected). The resulting offspring were counted and categorized as either GFP^- (sired by first knockdown or control donor) or GFP^+ (sired by the competitor donor) until day 21, based on expression of GFP assessed at age 7–10 days using a Nikon SMZ-18 stereomicroscope with a C-HGFI Intensilight fluorescence light source and GFP filter cube (Nikon GmbH).

To estimate defensive sperm competitive ability (P_1), either knockdown or control worms were mated and filmed for 2.5 hr with a randomly selected recipient worm in the above-described mating observation chamber. After 2.5 hr the recipient worms were each put individually in a well of a 60-well microtest plate. 30 min after separating the mating partners, a GFP^+ sperm competitor worm was added to the well containing the already-mated recipient worm, and the pair was allowed to mate for a further 2.5 hr. After the 2.5-hr mating period, the recipient and the GFP^+ sperm competitor were separated into an individual well as described above.

To estimate offensive sperm competitive ability (P_2), the sperm competition assay was carried out exactly like the P_1 assay, except that the GFP^+ worm was paired with the recipient first and the knockdown/control worm second. Again, the mating period with the knockdown/control individual was conducted in the mating observation chamber and filmed.

Each treatment group started with 36 donor worms at the beginning of the RNAi treatment. With some replicates excluded due to death of the donor before the mating trial, the absence of resulting offspring or the observation of five or fewer successful matings, the final realized sample sizes for each treatment group ranged from 22–34 (see Table 1).

2.8 | Statistical analysis

The effect of genotype on mating frequency was assessed by performing two-way ANOVA using genotype as a fixed effect and, in addition, group size and genotype × group size interaction effects. Genetic correlation estimates were based on the Pearson correlation coefficients calculated between mean trait values of genotypes. We calculated the corresponding genetic correlations as r_G = Cov(x1, x2)/[Var(x1) × Var(x2)] and tested for statistical significance by comparing the *z*-scores to two-tailed significance levels derived from a standard normal distribution.

For analysis of the P_1 and P_2 assays, the paternity share of knockdown and control individuals (GFP^-) were compared against the GFP^+ competitor using a generalized linear model with a quasibinomial distribution and a logit link function (Engqvist, 2013). For analysis of the mating frequency, we compared the number of copulations of knockdown and control mating pairs, using a separate linear model for each of the three treatment-control comparisons. For analysis of suck propensity, the frequency of sucking events (i.e. the proportion of matings followed by a suck) of the mating partners of knockdown and control individuals were compared using a generalized linear model with a binomial distribution and a logit link function. Analyses were conducted using the lme4 package (Bates, Maechler, Bolker, & Walker, 2015) in R (R version 3.1.3,2015).

3 | RESULTS

3.1 | Genetic correlations between SFP expression, mating frequency and suck propensity

We estimated genetic correlations between mating frequency and the overall seminal fluid investment (PC1) and relative composition (PC2–4) axes reported in Patlar et al. (2019) at two different group sizes (pairs and octets). We found that mating frequency was not genetically correlated with overall seminal fluid investment, but there was a highly significant negative genetic correlation between mating frequency and PC4 in both pairs and octets (Figure 1a).

Having established that mating frequency is highly negatively correlated with PC4, we therefore next estimated the genetic correlations between mating frequency and the five seminal fluid transcripts which were significantly loaded on PC4 (Patlar et al., 2019). Among these, we found three transcripts which exhibit a significant negative correlation between mating frequency and SFP transcript expression: *Mlig-pro63*, *Mlig-pro46* and *Mlig-pro37* (Figure 1b; here we illustrated these correlations only for octets, which is anyway the more relevant environment for sperm competition, because both group sizes show a very similar pattern). In fact, according to their highly similar sequence with overlapping regions, and the fact that when blasted against the *M. lignano* genome assembly ML2 (Wasik et al., 2015) they align to the same regions within the same protein-coding gene in the genome, *Mlig-pro37* and *Mlig-pro46* appear to belong to the same gene. For that reason, for our RNAi screen we selected just *Mlig-pro46*, which was already investigated in the previous screen (Weber et al., 2019), plus the independent candidate *Mlig-pro63*.

Notably, these same candidates were also significantly loaded on PC3, as reported previously in Patlar et al. (2019),

TABLE 1 Descriptive statistics and tests for treatment effects on mating rate, suck propensity and sperm competitive ability following RNAi knockdown of two seminal fluid transcripts (*Mlig-pro46*; *Mlig-pro63*; *Mlig-pro46* & *Mlig-pro63* combined)

	P_1						P_2					
Treatment	***n***	**Mean paternity share (P_1)**	***SE***	***df***	**t-value**	***p*-value**	***n***	**Mean paternity share (P_2)**	***SE***	***df***	**t-value**	***p*-value**
Control	32	0.18	0.05				27	0.88	0.05			
Mlig-pro46	28	0.20	0.05	58	1.01	.32	22	0.92	0.03	47	0.77	.44
Mlig-pro63	30	0.21	0.07	60	0.37	.71	25	0.90	0.04	50	0.44	.66
Combined	30	0.23	0.05	60	0.62	.54	26	0.86	0.05	51	0.37	.71
	n	**Mean number of matings**	***SE***	***df***	**t-value**	***p*-value**	***n***	**Mean number of matings**	***SE***	***df***	**t-value**	***p*-value**
Control	33	20.06	1.74				30	15.27	1.13			
Mlig-pro46	31	21.13	1.43	62	0.48	.63	23	16.74	1.35	51	0.85	.40
Mlig-pro63	34	22.03	1.44	65	0.87	.39	28	17.11	1.34	56	1.02	.31
Combined	32	23.06	1.41	63	1.37	.18	28	16.29	1.40	56	0.57	.57
	n	**Mean suck propensity**	***SE***	***df***	**t-value**	***p*-value**	***n***	**Mean suck propensity**	***SE***	***df***	**t-value**	***p*-value**
Control	33	0.54	0.02				29	0.47	0.03			
Mlig-pro46	31	0.51	0.02	62	−1.135	.26	24	0.44	0.04	51	−0.768	.45
Mlig-pro63	34	0.44	0.03	65	−3.032	.0035	26	0.41	0.03	53	−1.543	.1290
Combined	32	0.47	0.03	63	−1.821	.07	27	0.41	0.03	54	−1.758	.08

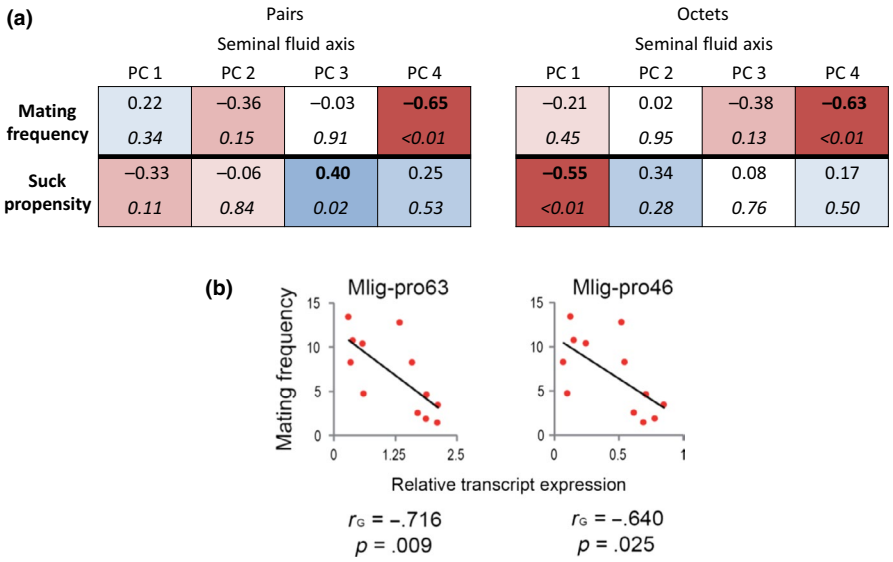

FIGURE 1 Genetic correlations (r_G) between seminal fluid of the donor and mating rate. (a) Genetic correlation coefficients and *p*-values (italic) for seminal fluid axes (principal components) and mating rate/suck propensity in pairs and octets (data from Patlar et al., 2019). (b) The relationship between the average mating rate and relative transcript expression with genetic correlation coefficients and respective *p*-values in octets (the transcripts are labelled according to their Mlig-pro [number] identifier assigned in Weber et al., 2018)

which was found to be positively correlated with suck behaviour (Figure 1a), at least in the pair group size without rivals (Patlar et al., 2020).

3.2 | RNAi knockdown effects on mating frequency

When we compared the mating frequency of mating pairs including an SFP knockdown donor to those including a control donor, none of the knockdowns impacted strongly on mating frequency, as measured by the total number of copulation events in the 2.5-hr mating period (all $p \geq .1$, Figure 2; for full statistical details for each knockdown, see Table 1).

3.3 | RNAi knockdown effects on suck propensity

When we compared the mean suck propensity of mating pairs including an SFP knockdown donor to those including a control donor, one of the individual knockdowns exhibited a strongly reduced suck propensity by the mating partner (*Mlig-pro63*, $t = -3.032$, $p = .0035$; Figure 2; Table 1), as measured by the

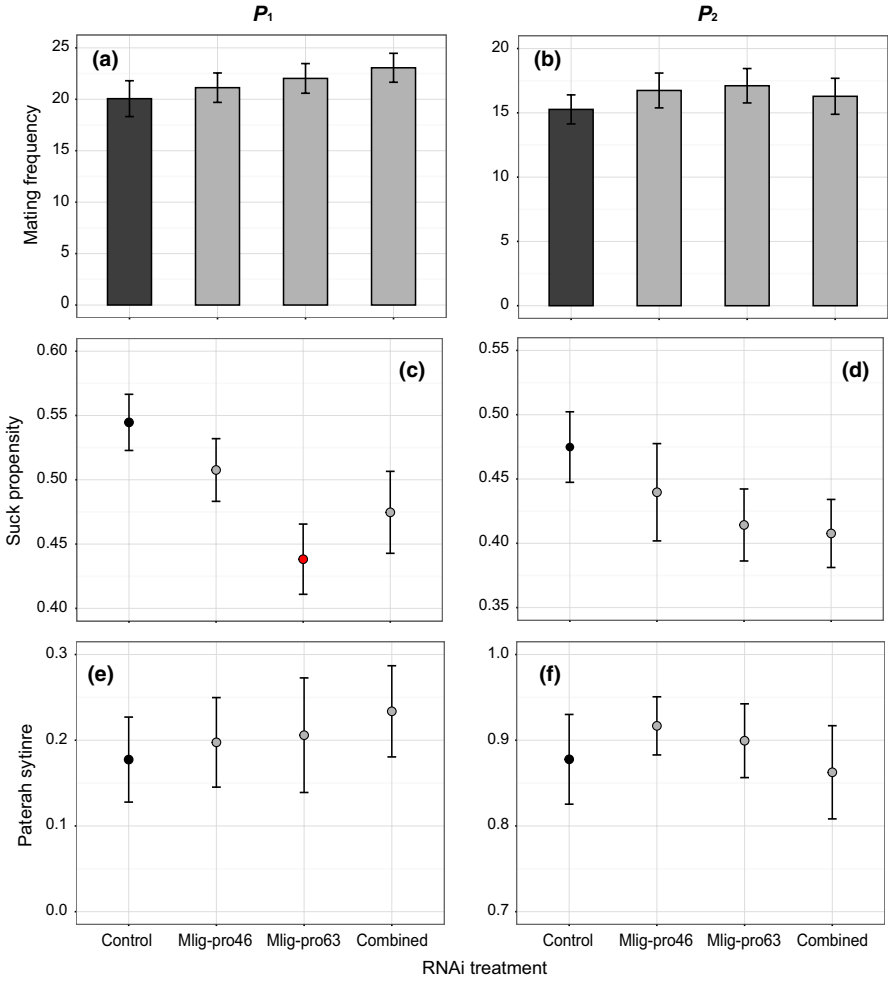

FIGURE 2 The effect of RNAi knockdown of two different seminal fluid transcripts (*Mlig-pro46*; *Mlig-pro63*; *Mlig-pro46* & *Mlig-pro63* combined). The transcripts are labelled according to their Mlig-pro [number] identifier assigned in Weber et al. (2018). (a) Mean mating rate ± *SE* of knockdown versus control mating pairs when the RNAi worm mated first. (b) Mean mating rate ± *SE* of knockdown versus control mating pairs when the RNAi worm mated second. (c) Mean suck propensity ± *SE* of the ejaculate receiving individual of knockdown versus control mating pairs when the RNAi worm mated first. (d) Mean suck propensity ± *SE* of the ejaculate receiving individual of knockdown versus control mating pairs when the RNAi worm mated second. (e) Mean paternity share (P_1) ±*SE* of knockdown versus control individuals mated with a partners when the RNAi worm mated first. (f) Mean paternity share (P_2) ±*SE* of knockdown versus control individuals mated with a partners when the RNAi worm mated second

sucking events per mating in the 2.5-hr mating period, although only in the P_1 assay (i.e. when they mated as the first partner with a recipient worm who subsequently mated with a rival sperm donor). None of the other knockdowns had a significant effect on suck propensity, although in both the P_1 and P_2 assays the combined RNAi showed a nonsignificant trend towards reduced suck propensity.

3.4 | RNAi knockdown effects on P_1 and P_2

When we compared recipient worms mated to SFP knockdown donors to those mated to the control donors, none of the knockdowns impacted strongly on defensive sperm competitive ability (P_1) or offensive sperm competitive ability (P_2), as measured by the paternity share between knockdown/control individuals and the competitor (all $p \geq .3$, Figure 2; Table 1).

4 | DISCUSSION

By selecting two putative seminal fluid transcripts with prostate-limited expression (Weber et al., 2018) that exhibit genetic correlations with both mating frequency and partner suck propensity (Patlar et al., 2019, 2020), and subjecting these to RNAi knockdown (individually and combined) followed by behavioural and competitive paternity assays, we aimed to test whether these transcripts directly influence partner behaviour. In fact, we did not detect any significant seminal fluid-mediated effect on mating frequency. However, we did find that one of the seminal fluid transcripts, *Mlig-pro63*, appears to impact positively on the frequency of the post-copulatory suck behaviour often exhibited by ejaculate recipients in *M. lignano*. Finally, we also did not detect any difference in paternity share between knockdown and control individuals, neither when the knockdown individuals were the first mating partners (P_1) nor when the knockdown individuals were the second mating partners (P_2) in controlled sperm competition assays. In the following, we discuss each of these three main results in turn.

Firstly, one reason why RNAi knockdown of *Mlig-pro46* and/or *Mlig-pro63* had no detectable impact on mating frequency could be that the SFPs tested in our assay have a long-term effect on subsequent behaviour, and not a more or less immediate one as we tested for here. In our experimental design, we could only detect possible effects between the first copulation and the end of the 2.5 hr recording time. While we thus checked for immediate effects on re-mating, there are for example SFPs in *Drosophila* known to act only over

the longer term. In a screen of 25 *D. melanogaster* SFP knockdowns, none appeared to modulate the receptivity of the mated female at 24 hr post-mating (Ram & Wolfner, 2007b), with an equally low receptivity to re-mating regardless of whether females mated to control or knockdown males. But three of these SFP knockdowns showed a significant long-term effect on female receptivity, with females mated to males from these three knockdown treatments being significantly more receptive to re-mating at 4 days post-mating than were mates of control males. Nevertheless, the way that we identified candidates in *M. lignano* involved testing for correlations between transcript expression and mating frequency over a similarly short timescale, so the lack of effect in our assay is still surprising. Perhaps another explanation could be that while Patlar et al. (2019) used 12 different inbred lines, differing in their genotypes, we used in our experiment only one genotype. The 12 inbred lines could differ in other, correlated ways, which in combination with *Mlig-pro46* and/or *Mlig-pro63* affect mating frequency but are not incorporated in our RNAi experimental design.

Our results indicate that *Mlig-pro63* appears to play a role in promoting the suck behaviour of the mating partner. Although it can occur by various taxon-specific means, ejection of previously received ejaculates by recipients (females) could be quite a common phenomenon (see, e.g., Pizzari & Birkhead, 2000; Rodriguez, 1995; Snook & Hosken, 2004) and is usually interpreted in the context of cryptic female choice and/or sexual conflict over ejaculate fate (Arnqvist & Rowe, 2005; Simmons, 2001). Our results suggest that seminal fluid plays an important role in mediating this response, at least in *M. lignano* (cf. Snook & Hosken, 2004). Nevertheless, that there is a decrease of the suck propensity with the loss of this SFP from the ejaculate is still at first glance also a surprising result, at least from a donor perspective. Indeed, Patlar et al. (2020) have recently identified two SFPs in *M. lignano* that have the opposite effect, apparently manipulating partner behaviour to reduce the frequency of sucking and therefore presumably gaining greater control over the fate of the transferred ejaculate. Consistent with this, previous work had indicated that individuals mated to virgin partners (which presumably transfer bigger ejaculates) exhibit a lower frequency of the suck behaviour (Marie-Orleach et al., 2013). By contrast, the effect we detected—of a decrease in suck propensity upon the RNAi-induced loss of one ejaculate component—suggests that other SFPs might act as cues used by the ejaculate recipient to trigger suck behaviour. This would seem to imply that they have evolved and are still included in the ejaculate for some other, as yet unknown, reason that still provides a net benefit to the ejaculate donor, since—assuming the suck behaviour benefits recipients at the expense of donors—their inclusion would otherwise seem to be a maladaptive strategy. The inclusion of *Mlig-pro63* in the ejaculate could be explained by such pleiotropic effects. While we could rule out a short-term impact on mating frequency, it is unclear whether *Mlig-pro63* knockdown instead affects longer term (re)mating behaviour and/or egg production parameters (ovary size, egg-laying rate). Hopkins et al. (2019) recently showed such an evolutionary prioritization of some functions, at the expenses of other, for *Drosophila* secondary-cell secretions. While a normal secondary-cell activity reduces male defensive sperm competition performance, it is required to reduce female receptivity to re-mating. Most likely the loss due to the reduced defensive sperm competition performance is outweighed by the benefits of a suppressed female receptivity to re-mating. The presence of SFPs with apparently opposing effects is itself interesting and may be evidence of ongoing arms races between donors (males) and recipients (females) over the control of ejaculate fate, potentially contributing to seminal fluid's complexity.

Also with respect to suck propensity, we note that the fact we observed an effect of *Mlig-pro63* knockdown on suck propensity in the P_1 but not the P_2 assay suggests that *Mlig-pro63* presumably transferred first by the sperm competitor individual in the P_2 assay was still affecting partner behaviour at the time of pairing with the *Mlig-pro63* knockdown worm. The SFP Acp36DE in *D. melanogaster* shows such an effect. If a male fails to transfer Acp36DE, both his own sperm and those transferred by the next male show decreased storage, despite the second male presumably transferring Acp36DE himself (Chapman et al., 2000). This highlights one methodological difficulty with performing such double mating assays, in that SFP-mediated effects presumably intended to influence the utilization of own sperm can, under certain study designs that may not well reflect the situation in nature, actually influence that of rivals, and vice versa. In *Drosophila*, the ejaculates of the first and the second male to mate can both contribute to the second male advantage. Second male SFPs contribute to the ejection or incapacitation of residing first male's sperm, whereas SFPs from the first male still increase the longevity of also the second male's sperm (Nguyen & Moehring, 2018; see also Hodgson & Hosken, 2006).

The absence of detectable impacts of SFP knockdown on sperm competitiveness could be a direct result of the fact the time window we investigated does not well reflect the action of these transcripts. Ram and Wolfner (2007b) tested 25 SFPs knockdowns in *Drosophila* for their impact on re-mating, none of them appeared to modulate the receptivity of the mated female at 24 hr post-mating. Females showed equally low receptivity to re-mating independently of being mated to either control or knockdown males. But they could identify three SFPs which showed an effect on long-term receptivity of females: at 4 days post-mating, mates of these three knockdown males were significantly more receptive to re-mating than mates of control males. Also, because in our experiment the competitor was introduced either very soon following the knockdown/control individuals (P_1 assay), or else there was no competitor following the knockdown/control individuals (P_2 assay), there was perhaps no opportunity to influence sperm competition outcomes via (eventually) reduced receptivity. The surprising result that we did not, contrary to the previous RNAi screen (Weber et al., 2019), find an effect of the *Mlig-pro46* knockdown on paternity share, certainly suggests we should remain cautious about interpreting the role of *Mlig-pro46* in sperm competition until we have gained a greater understanding of its mechanism of action. However, the different outcomes of the two studies could perhaps be due to the slightly different

experimental designs regarding mating duration and length of the break between the two competitors. While in the previous experiment there was 1 hr between removing the first donor and adding the second, as well as a longer mating period of 3 instead of 2.5 hr, in this experiment this gap between pairings was just half an hour. The shorter mating period presumably reduced the opportunity for cumulative effects on sperm competitiveness, and the shorter gap is likely to have exacerbated any carry-over effects alluded to above, meaning effects of SFPs presumably intended for own ejaculates actually also impact on rival ejaculates, or vice versa, tending to equalize paternity success between competitors.

In conclusion, we found evidence for seminal fluid-mediated effects on suck propensity in the simultaneously hermaphrodite *M. lignano*, but no indication that the two candidate transcripts *Mlig-pro46* and *Mlig-pro63* affect mating frequency. Further research will be needed to investigate the potential impact of seminal fluid on the long-term receptivity of mating partners in *M. lignano*. Overall, by using a combination of quantitative genetics and behavioural data to first identify seminal fluid components with potential effects on post-mating behaviour and subsequent RNAi knockdown assays, we have gained some novel insights about seminal fluid action in the flatworm *M. lignano*. The combination of quantitative genetics and behavioural data together with subsequent RNAi is a promising approach for future investigation of SFP function in this and other nonmodel organisms.

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CONFLICT OF INTEREST

SAR is a member of the editorial board of Journal of Evolutionary Biology.

AUTHOR CONTRIBUTIONS

MW and SAR conceived the study. MW performed the RNAi knockdown and MW and BP the mating assays.MW analysed results and drafted the manuscript together with SAR. All authors contributed to manuscript revisions and approved the final manuscript.

ORCID

Michael Weber https://orcid.org/0000-0002-2545-4370
Bahar Patlar https://orcid.org/0000-0002-0442-9061
Steven A. Ramm https://orcid.org/0000-0001-7786-7364

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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