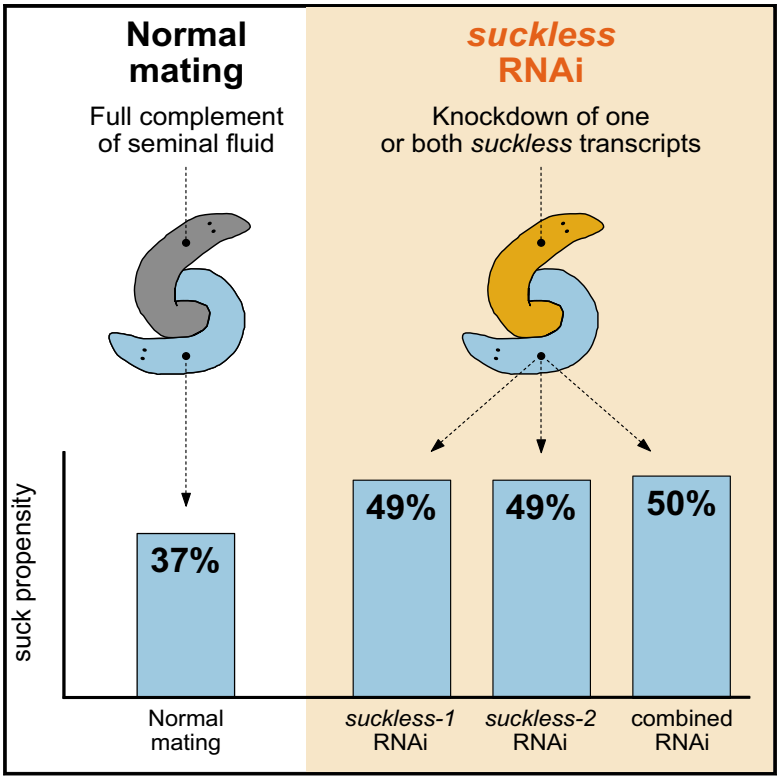
Report

Current Biology

Seminal Fluid-Mediated Manipulation of Post-mating Behavior in a Simultaneous Hermaphrodite

Graphical Abstract



Authors

Bahar Patlar, Michael Weber, Tim Temizyürek, Steven A. Ramm

Correspondence

bahar.patlar@uni-bielefeld.de (B.P.), steven.ramm@uni-bielefeld.de (S.A.R.)

In Brief

Patlar et al. identify two proteins in the seminal fluid of the flatworm *Macrostomum lignano* that manipulate the subsequent behavior of mating partners, making it less likely they perform the so-called "suck" behavior that likely influences ejaculate retention after mating. This suggests these proteins mediate sexual conflict over ejaculate fate.

Highlights

- *Suckless-1* and *suckless-2* are putative seminal fluid transcripts
- Their expression is negatively genetically correlated with partner suck propensity
- Knockdown of either transcript increases the likelihood of sucking after mating
- They may therefore mediate sexual conflict over ejaculate fate

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Seminal Fluid-Mediated Manipulation of Post-mating Behavior in a Simultaneous Hermaphrodite

Bahar Patlar,[1,2,*] Michael Weber,[1] Tim Temizyürek,[1] and Steven A. Ramm[1,3,*]
[1]Evolutionary Biology, Bielefeld University, Konsequenz 45, 33615 Bielefeld, Germany
[2]Present address: Department of Biology, University of Winnipeg, 515 Portage Avenue, Winnipeg, MB R3B 2E9, Canada
[3]Lead Contact
*Correspondence: bahar.patlar@uni-bielefeld.de (B.P.), steven.ramm@uni-bielefeld.de (S.A.R.)
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SUMMARY

Seminal fluid proteins (SFPs) are uniquely positioned to mediate post-mating sexual selection and sexual conflict [1–3]. This role may be especially important in simultaneous hermaphrodites, in which individuals will often agree to receive sperm in order to be able to donate it, shifting the arena of sexual selection to post-mating reproductive interactions [4–7]. Nevertheless, as in separate-sexed organisms, identifying individual SFPs responsible for specific post-mating effects is difficult, owing to the complexity, rapid evolution, and functional redundancy of seminal fluid [8–11]. Here, we sought to identify SFPs that influence one striking post-mating behavior of the simultaneously hermaphroditic flatworm *Macrostomum lignano*, the so-called "suck behavior," in which worms respond to ejaculate receipt by placing their pharynx over their female genital opening and seemingly attempt to remove sperm and/or other ejaculate components [12–14]. We hypothesized that sucking is counter to the sperm donor's interests, potentially selecting for SFPs that reduce the suck propensity of mating partners. We tested this using a combination of quantitative genetics and RNA interference (RNAi) knockdown. As predicted, we found negative genetic correlations between the expression levels of six (out of 58) seminal fluid transcripts and partner suck propensity. RNAi knockdown confirmed that two of these transcripts, designated *suckless-1* and *suckless-2*, indeed caused mating partners to suck less often. We suggest that these proteins are male counter-adaptations to recipient suck behavior, which itself is likely a female counter-adaptation in the ongoing evolutionary conflict to (re)gain control over ejaculate fate after mating in this hermaphroditic organism.

RESULTS AND DISCUSSION

In simultaneous hermaphrodites, any two potential mating partners could mate as either male or female, but because there may often be greater motivation to donate sperm rather than to receive it, this leads to evolution of "reciprocal mating" strategies that have been commonly observed in these organisms, in which both mating partners perform both roles either concurrently or in quick succession [4, 6, 15, 16]. Although seminal fluid-mediated effects have been primarily investigated in species with separate sexes (e.g., [17–20]), that they might also play a central role in mediating post-mating sexual selection and sexual conflict in simultaneous hermaphrodites has long been predicted [4, 6, 21, 22]. Indeed, seminal fluid may be especially important in simultaneous hermaphrodites, because of an expected shift in the arena of sexual selection to post-mating interactions in the absence of pre-mating sexual selection [4, 6]. This should create strong selection on sperm donors for high sperm competitive ability and counter-selection on sperm recipients to retain or regain control over processing received ejaculate components, either because (excess) sperm receipt itself is costly [5, 6, 23] or because the ejaculate contains manipulative seminal fluid proteins [24, 25]. This will in turn favor counter-adaptations in donors to circumvent such control. The precise adaptive significance of the suck behavior in *M. lignano* (see Video S1) has not yet been established, but it is hypothesized to have evolved as a response to sexual conflict over control of received ejaculates [12–14]. Here, we sought to test whether this has also led to the evolution of donor counter-adaptations in seminal fluid to reduce recipient suck propensity.

Consistent with the large number of seminal fluid proteins (SFPs) in other taxa (e.g., [26–30]), we already know that seminal fluid in *M. lignano* is complex, with at least 76 prostate-limited transcripts recently identified [31]. A large-scale RNAi screen provided some preliminary evidence that loss of specific SFPs can affect reproductive success [32], but importantly only for a minority of proteins investigated and without addressing their potential specific functions. To overcome the general problem of narrowing down the number of seminal fluid candidates with the goal of screening for specific functions, we therefore first designed an approach to more efficiently target a relevant subset of candidate SFPs in *M. lignano* that could manipulate the suck behavior.

In the absence of genome-wide sequence information, one promising approach based on quantitative genetics recently advocated by Ayroles et al. [33] is to use genetic variation in gene expression to annotate novel genes based on genetic correlations with genes whose functions have already been established. Here, we extended this approach for identifying candidate genes by instead directly estimating genetic correlations

between gene expression and the phenotype of interest. More specifically, to identify genes that might function as donor counter-adaptations that seek to prevent the suck response, we employed a two-step genetic correlation analysis. First, we used a seminal fluid transcript expression network [34] to initially home in on major axes of seminal fluid variation in ejaculate donors associated with their partner's suck propensity, and second, we estimated genetic correlations between transcripts loaded on these axes and suck propensity using the same donor/recipient approach in order to identify specific candidate transcripts. We then tested the functional role of these candidates using RNAi gene knockdown of donors followed by a behavioral assay of recipient suck propensity.

Quantitative Genetics Identifies Candidate Seminal Fluid Transcripts Mediating Sexual Conflict over Ejaculate Fate

We used quantitative genetics data from a fully crossed experimental design consisting of a panel of 12 inbred *M. lignano* lines, measured in two different social environments, for which we recently described sources of significant genetic and environmental variation in the expression of 58 seminal fluid transcripts (summarized by four principal components) [34]. Environmental effects were estimated as differential expression at two social group sizes (pairs and octets) differing in sperm competition intensity (e.g., [35–40]). We measured the suck propensity of the standardized virgin mating partner (i.e., an ejaculate recipient that always belonged to a single genotype and was of the same age and environmental background) as the proportion of copulations that were followed by a suck by that recipient worm in a standardized behavioral assay of mating pairs, where the donor worm came from one of these inbred line/group size combinations. To justify our approach of evaluating genetic correlations between recipient suck propensity and seminal fluid transcript expression in donors, we first tested for genetic variation in partner suck propensity using the donor's inbred line identity (i.e., genotype) as a fixed effect and, in addition, group size and genotype × group size interaction effects. Social group size had no effect on suck propensity of the recipient (two-way ANOVA; $F_{1,254} = 0.01$; $p = 0.91$) nor was there a genotype-by-group size interaction ($F_{11,254} = 1.6$; $p = 0.11$), but we found a highly significant genotype effect ($F_{11,254} = 3.2$; $p < 0.001$), indicating that recipients differed markedly in their suck propensity depending on the donor's genotype.

Having established genetic variation in both seminal fluid transcript expression [34] and partner suck propensity, we proceeded to identify candidate transcripts that affect the occurrence of suck behavior of mating partners based on testing for negative genetic correlations between seminal fluid expression and mating partner suck propensity (based on mean values of the traits per inbred line and group size level). First, we calculated the genetic correlation between partner suck propensity and four major axes of seminal fluid transcript expression variation reported in Patlar et al. [34]. We did this in each of the two social group sizes, i.e., as measured in either pairs or octets. This revealed evidence for a negative genetic correlation between the first principal component (PC1) of variation in seminal fluid transcript expression (which explained 42% of total variation) and partner suck propensity in both environments, with overlapping 95% confidence intervals (CIs) (CI(pairs): −0.72, 0.07; CI(octets): −0.81, −0.23), suggesting this may be a consistent effect, but the correlation appeared on average stronger and significant in octets (octets: $r_G = -0.55$; $p < 0.01$; Figures 1A and 1B). Given the higher intensity of sperm competition in octets [37], and thus presumably the greater premium on maintaining ejaculates in storage, this environment may be functionally more relevant to manipulating suck propensity, and we therefore prioritized this environment for subsequent analyses. We note here that one of the minor principal components, PC3 (which explained 10% of total variation in seminal fluid transcript expression), was actually positively correlated with partner suck propensity but only in pairs. This is the opposite pattern expected for the candidates we sought to identify but suggests that some other ejaculate components could even act as cues to trigger suck behavior [41]. This presumably occurs as a by-product of some other function that benefits sperm donors, selecting for their inclusion in the ejaculate, and in fact, we have now identified one protein that is likely to be responsible for this effect [41].

Focusing on PC1 as the axis of seminal fluid variation that exhibited the predicted negative genetic correlation with suck propensity, the majority of seminal fluid transcripts are significantly loaded on this principal component, which thus describes overall seminal fluid transcript abundance [34]. This indicates that partners of genotypes with higher investment in overall seminal fluid expression suck less often. We therefore next estimated the genetic correlations between suck propensity and expression level of each of 58 individual seminal fluid transcripts in octets. Genetic correlations coefficients were negative for the majority of the transcripts (ranging between −0.69 and 0.40; Table S1) but were especially strong—and significant after false discovery rate (FDR) correction—for six transcripts: *Mlig-pro5*, *Mlig-pro6*, *Mlig-pro30*, *Mlig-pro31*, *Mlig-pro32*, and *Mlig-pro33* (Figure 1C) (see Weber et al. [31] for details regarding seminal fluid transcript nomenclature in *M. lignano*). We thus prioritized these six candidates as potentially affecting partner suck behavior and next tested whether targeted knockdown of any of these six candidate transcripts, individually or in combination, indeed affected recipient suck propensity.

RNAi Confirms Two Seminal Fluid Transcripts Affect Partner Suck Propensity

To evaluate the hypothesized effect of the six candidate SFP transcripts on partner suck propensity in a manipulative experiment, we used RNAi to generate donor worms lacking these proteins in their ejaculates. To do so, we took advantage of the regenerative capacity of this flatworm [43, 44] to amputate the posterior tail region of adults, thereby "resetting" their seminal fluid production. We then allowed them to regenerate while soaking in (1) double-stranded RNA (dsRNA) probes specific to one of the six candidate SFP transcripts (Table S2), (2) a seventh "combined" treatment dsRNA from all six candidate SFP transcripts, or (3) a negative control treatment (i.e., in the absence of dsRNA). Following RNAi treatment, the knockdown and control donors were tested for their effects on partner suck propensity using a behavioral assay in which the (treated) donor worm was paired with a standardized (partner) recipient worm for 2.5 h in a 3.4-μL drop of artificial sea water squeezed between

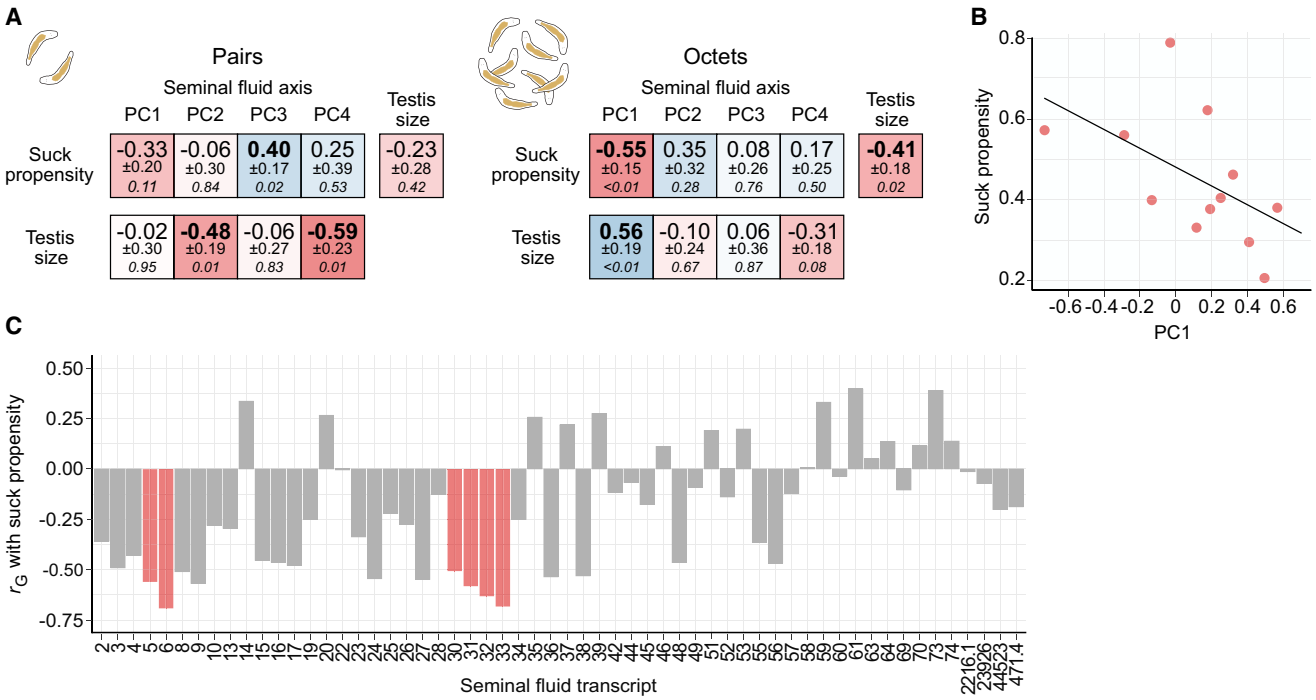

Figure 1. Genetic Correlations (r_G) between Seminal Fluid Expression of the Donor and Suck Propensity of the Recipient

(A) Genetic correlations (r_G) with (±) SEMs, and p values (italics) for main axes (principal components) of variation in seminal fluid transcript expression, testis size, and suck propensity of mating partners in pairs and octets calculated as Pearson's product-moment correlation coefficients based on average genotype values at each group size.

(B) Scatterplot of the average suck propensity and PC1 scores of the 12 genotypes in octets ($r_G = -0.55$; $p < 0.01$).

(C) Genetic correlations calculated between expression of individual transcripts and suck propensity of recipients for 58 seminal fluid transcripts (Pearson's product-moment correlation coefficients based on average genotype values in octets). Red bars show significant correlations (candidate transcripts) derived from adjusted p values after performing false discovery rate corrections. Numbers on the x axis represent the identity of the different seminal fluid transcripts (*Mlig-pro1* – *Mlig-pro76*) from [31]. In addition, four prostate-limited transcripts (2216.1, 23926, 44523, and 471.4) are depicted in the figure (with their transcript IDs from the MLRNA110815 transcriptome assembly) [42] that were not initially identified as putative seminal fluid transcripts [34].

See also Video S1 and Table S1.

two glass slides, a well-established technique in *M. lignano* that enables the continuous monitoring and recording of mating behavior [13]. We then scored numbers of copulations and the frequency with which they were followed by a partner suck during the whole 2.5-h period. We used this as the dependent variable to test whether any of the RNAi treatments differed from controls in the suck propensity of their partners.

As predicted, we found that knockdown of two of the six candidates indeed resulted in a significantly higher suck propensity of partners, based on comparing each RNAi knockdown treatment to the control (Figure 2; independent Welch's two sample t tests, followed by FDR correction, *Mlig-pro31*: $t_{54.84} = 2.60$, $P_{adjusted} = 0.03$; *Mlig-pro32*: $t_{46.55} = 2.92$, $P_{adjusted} = 0.02$; all other single knockdowns $p > 0.6$). Because worms mated to donors lacking *Mlig-pro31* and *Mlig-pro32* in their ejaculate are more likely to perform the suck behavior, these results strongly imply that these two seminal fluid transcripts normally function to reduce partner suck propensity, and so we would now designate them as *suckless-1* and *suckless-2*, respectively. Based on bioinformatic analyses of their sequence, both are likely to be secreted [31] and they are highly positively correlated in their expression in *M. lignano* [34]. However, these transcripts show no sequence similarity when blasted against the *M. lignano* genome assembly ML2 [45]. In addition, they align to two different genomic regions, which further suggests that they indeed belong to different genes. BLAST and BLASTX searches did not reveal significant matches in other organisms for either *suckless-1* or *suckless-2* [46].

The combined knockdown of all six candidates had a comparable effect on suck propensity to the single knockdown of either *suckless-1* or *suckless-2* ($t_{50.96} = 3.18$; $p = 0.02$). This could indicate that these transcripts do not impact additively on recipient suck behavior but rather may act together, although one caveat here is that we observed some residual signal of *suckless-2* expression in combined knockdown worms in the *in situ* hybridization screening we performed to verify RNAi efficiency (Figure S1). Note also that none of the RNAi treatments differed from controls for either total mating frequency or the donor's own suck propensity (Table 1), indicating that the effects we have identified act specifically on recipient suck propensity. These effects are also consistent with a previous report in *M. lignano* that showed worms suck less often after copulating with virgin partners than sexually experienced partners [47]. Because virgin worms were found to have a higher fill grade of

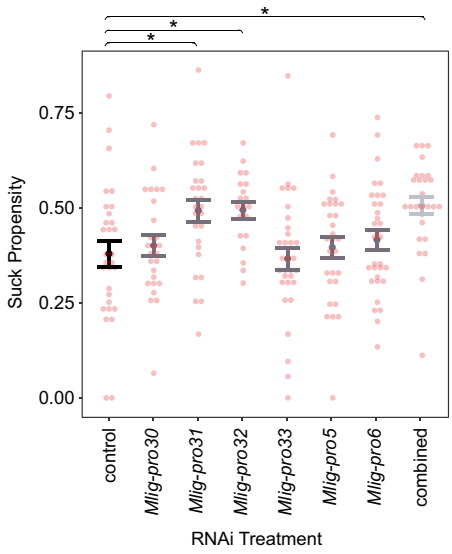

Figure 2. Identification of Two Key Seminal Fluid Transcripts Influencing Partner Suck Behavior following RNAi Knockdown of Candidates

The figure shows suck propensity (the propensity of mating partners to perform the suck behavior in 2.5 h) of each RNAi knockdown treatment and the treatment of combined knockdown of all candidate transcripts versus the negative control group. Error bars represent SEMs suck propensity for each treatment. Jittered red points are the underlying raw data, depicting the observed suck propensity of each individual sample. See also Figure S1, Tables S2 and S3, and Video S1.

their prostate gland (i.e., more seminal fluid available), they presumably transferred more seminal fluid compared to sexually experienced worms [47].

Given the likely impact of suck behavior on received ejaculates, we would expect that the ability to manipulate this behavior could have direct consequences for the donor's competitive fertilization success (paternity share) if it affects the total or relative number of donor sperm present at fertilization. As a first test of this, after the behavioral assay was completed, we paired each recipient worm with a standardized GFP-transformed competitor for 2 h, using the GFP phenotype of offspring to assign paternity and thereby estimate "defensive" sperm competitive ability of the donor (P_1) [48–50]. Somewhat surprisingly, we saw no marked effect of RNAi knockdown on paternity success (Table S3). This may have been due to our specific experimental design, and the fact that the high mating rate of *M. lignano* might have meant all donors maximized sperm in storage by the end of the extended 2.5-h mating period, irrespective of their RNAi treatment and thus partner suck propensity. According to earlier studies that used a mass laboratory culture of *M. lignano*, approximately 50%–70% of copulations observed were followed by a suck [13, 35]. In our study, we also found that the occurrence of suck behavior varies depending on the genotype of the partner (see also [48, 51]); this behavior is thus common in nature and shows substantial variation, suggesting that the effect size of knockdown we demonstrated may actually represent quite a marked change in behavior: just over one-third of control matings were followed by a suck, but this increased to 50% in the knockdown worms. Although perhaps not dramatic in the sense that sucking never approached 100%, we nevertheless should consider that this effect, coupled with the variation we have described, certainly implies that the effects could be significant in nature. Importantly, a separate study investigating natural variation in the expression of *suckless-1* and *suckless-2* generated through genotype × environment interactions has now revealed a strong positive correlation between *suckless-1* expression and defensive sperm competitive ability, P_1 [52], supporting the notion that this protein has functionally significant effects under sperm competition.

Suck Propensity Is Genetically Correlated with Ejaculate Investment

In addition to our main hypothesis, we were also interested to explore the interrelationships between seminal fluid investment, suck propensity of the recipient, and the other key aspect of male allocation, i.e., sperm production, as estimated by testis size of donors (e.g., [35–38, 53]). For testis size, we observed significant genotype ($F_{11,257} = 9.0$; $p < 0.001$) and social group size ($F_{1,257} = 11.4$; $p = 0.001$) effects but no interaction ($F_{11,257} = 1.1$; $p = 0.25$). We found average testis size increases with increasing group size (mean testis area in pairs = $14.2 \pm 0.6 \times 10^3$ μm^2; in octets = $16.8 \pm 0.7 \times 10^3$ μm^2) as expected from previous studies (e.g., [37, 38, 40, 54]). Within octets, testis size was positively genetically correlated with overall seminal fluid investment (PC1; Figure 1A; $r_G = 0.56$; $p < 0.01$), which could explain why we also observed a negative correlation between testis size and partner suck propensity (Figure 1A; $r_G = -0.41$; $p = 0.02$). These patterns mean that genotypes with larger testes (and thus probably more sperm) possibly also transfer more manipulative seminal fluid proteins in competitive environments, serving to emphasize the integrated nature of the male ejaculate (e.g., [42, 55, 56]). If sperm and seminal fluid investment are positively correlated [42], this could also help explain a previous pattern observed in *M. lignano* whereby suck propensity depended on the sex allocation (i.e., the relative investment into sperm and egg production) of mating pairs [35].

Conclusions

Although seminal fluid is a potentially crucial mediator of reproductive interactions with important implications for fertility [57–60], post-mating sexual selection [61–65], sexually antagonistic coevolution [17, 18, 20, 66], and ultimately even speciation [29, 30, 67, 68], its rapid evolution [69–71] and highly complex nature [9, 26–28, 72] mean that only a small subset of seminal fluid components have been functionally well characterized in any experimental system. To overcome this difficulty of linking seminal fluid functions to specific proteins contained within it, we here demonstrate the utility of using quantitative genetics coupled with targeted gene knockdown, an empirical approach that could potentially be applicable to a wide range of taxa, including non-model organisms for which alternative methods are not feasible. Adopting such a strategy, we were able to identify two seminal fluid transcripts in the simultaneously hermaphroditic flatworm *M. lignano*, namely *suckless-1* and *suckless-2*, that appear to suppress the suck propensity of mating partners. This is consistent with the hypothesis that these proteins mediate sexual conflict over ejaculate fate by manipulating the partner's post-mating behavior, causing them to suck less often. Donors potentially thereby regain control over the retention of their own sperm or

Table 1. Mating Frequency and the Donor's Own Suck Propensity Are Unaffected by RNAi Knockdown of Six Candidate Seminal Fluid Transcripts Hypothesized to Influence Partner Suck Propensity

Trait	Treatment	n	Mean	SD	*t*	df	p	Adj. p
Mating frequency	Control	30	22.97	9.13				
	Mlig-pro5	29	28.10	9.35	2.14	56.80	0.04[a]	0.26
	Mlig-pro6	32	22.03	7.69	−0.44	56.88	0.67	0.81
	Mlig-pro30	26	25.31	9.07	0.96	52.97	0.34	0.81
	Mlig-pro31	27	23.59	10.08	0.25	52.77	0.81	0.81
	Mlig-pro32	20	23.65	7.34	0.29	46.20	0.77	0.81
	Mlig-pro33	31	22.32	8.07	−0.29	57.61	0.77	0.81
	Combined	27	22.30	8.68	−0.28	54.82	0.78	0.81
Donor suck propensity	Control	30	0.31	0.15				
	Mlig-pro5	29	0.31	0.13	0.01	56.49	0.99	0.99
	Mlig-pro6	32	0.33	0.16	0.50	59.96	0.62	0.95
	Mlig-pro30	26	0.32	0.14	0.24	53.59	0.81	0.95
	Mlig-pro31	27	0.35	0.15	1.08	54.52	0.28	0.95
	Mlig-pro32	20	0.32	0.11	0.34	47.65	0.74	0.95
	Mlig-pro33	31	0.30	0.12	−0.30	56.18	0.77	0.95
	Combined	27	0.42	0.17	2.53	53.05	0.02[a]	0.10

Descriptive statistics of RNAi knockdown/control treatments, together with results of Welch two sample t tests comparing the control group to each knockdown treatment are shown for average mating frequency during the 2.5-h period of pairing and the suck propensity of the donor itself. Adjusted p values are derived from false discovery rate corrections.
[a]$p < 0.05$

other ejaculate components and in so doing enhance their own reproductive success. This suggests that the evolution of these proteins was a further step in the ongoing cycles of sexually antagonistic coevolution driving seminal fluid diversification.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Testis size and seminal fluid expression measurements
  - RNA interference knockdown treatment and behavioral assays
  - Defensive sperm competitive ability (P_1) assay
- QUANTIFICATION AND STATISTICAL ANALYSIS
- DATA AND CODE AVAILABILITY

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cub.2019.11.018.

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AUTHOR CONTRIBUTIONS

B.P. and S.A.R. conceived the study; B.P., M.W., and T.T. conducted the experiments; B.P. analyzed the data; and B.P. and S.A.R. drafted the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited Data		
https://doi.org/10.17632/cbdpzgsc82.1 (Mendeley Data)	This paper	
Experimental Models: Organisms/Strains		
Macrostomum lignano DV inbred lines	Zoological Institute at University of Basel, Switzerland, Lukas Schärer	
Macrostomum lignano GFP-expressing strain BAS1	Zoological Institute at University of Basel, Switzerland, Lukas Schärer	
Oligonucleotides		
Mlig-pro5 forward primer (Sp6-promotor underlined): CATTTAGGTGACACTATAGAAGTGATTATTCTCGTTGCTGCTC	This paper	
Mlig-pro5 reverse primer (T7-promotor underlined): GGATCCTAATACGACTCACTATAGGATATTGGGTCACCGCAGTTG	This paper	
Mlig-pro6 forward primer (Sp6-promotor underlined): CATTTAGGTGACACTATAGAAGAATCTGTGCTCAAGCAGACTGG	This paper	
Mlig-pro6 reverse primer (T7-promotor underlined): GGATCCTAATACGACTCACTATAGGCGGTCGCTCAAGTCGGTATC	This paper	
Mlig-pro30 forward primer (Sp6-promotor underlined): CATTTAGGTGACACTATAGAAGCTGTTTTAAGTCTGACGCATCG	This paper	
Mlig-pro30 reverse primer (T7-promotor underlined): GGATCCTAATACGACTCACTATAGGCTTGCATAGGCAATCACTGG	This paper	
Mlig-pro31 forward primer (Sp6-promotor underlined): CATTTAGGTGACACTATAGAAGAAACGAGCAATCAACAGAACTAC	This paper	
Mlig-pro31 reverse primer (T7-promotor underlined): GGATCCTAATACGACTCACTATAGGGGAGATCATATCTTTCCAGTCAGC	This paper	
Mlig-pro32 forward primer (Sp6-promotor underlined): CATTTAGGTGACACTATAGAAGAAATGCTACTGCGGATTTACG	This paper	
Mlig-pro32 reverse primer (T7-promotor underlined): GGATCCTAATACGACTCACTATAGGGCTCATGTCGTATTTGTTTTGC	This paper	
Mlig-pro33 forward primer (Sp6-promotor underlined): CATTTAGGTGACACTATAGAAGATAGAAATAAGCCACCAACTCAGC	This paper	
Mlig-pro33 reverse primer (T7-promotor underlined): GGATCCTAATACGACTCACTATAGGCATACTCTTGCACGATCACTACG	This paper	
Software and Algorithms		
R version 3.4.2	[73]	https://cran.r-project.org
ImageJ	[74]	https://imagej.nih.gov/ij/
Kinovea video player, version 0.8.15		https://www.kinovea.org
Debut Video Capture Software Professional, version 2.02		https://www.nchsoftware.com/index.html
Other		
RNALater solution	Ambion, Austin, USA	R0901-100ML
Sigmacote	Saint Louis, USA	SL2-100ML
Colorant Alimentaire Grand Blue	Les Artistes, Paris, France	

LEAD CONTACT AND MATERIALS AVAILABILITY

This study did not generate new unique reagents. Further information and requests for resources should be directed to and will be fulfilled by the Lead Contact, Steven Ramm (steven.ramm@uni-bielefeld.de).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Macrostomum lignano

Macrostomum lignano (Rhabditophora, Platyhelminthes) is a simultaneous hermaphroditic, outcrossing and reciprocally copulating, free-living marine flatworm [75]. It is cultured in the laboratory in artificial sea water (32‰ salinity) at 20°C, 14:10 hr light:dark cycle, under 60% humidity and fed with the diatom *Nitzschia curvilineata*. Worms copulate frequently under laboratory conditions (ca. ten matings per hour) and sperm is transferred together with seminal fluid via the stylet (male copulatory organ) to the partner's female antrum (sperm storage organ) [13, 14]. Seminal fluid is produced by prostate gland cells located around the stylet [14, 76], and includes a complex mixture of proteins [31]. The worms used in this experiment originated from 12 different inbred lines 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 [77]. These inbred lines are called DV lines, and we here used the lines DV1 [37, 45], DV3, DV8, DV13, DV28, DV69 and DV71 [78] and DV47, DV49, DV61, DV75 and DV84 [77].

METHOD DETAILS

Social group size manipulation

In our previous study, we described the social group size manipulation in detail [34]. Briefly, we raised 3-4 days old juveniles from 12 inbred lines for 51 days either in groups of two worms or groups of eight worms (referred to here as pairs and octets, respectively). The juvenile worms were randomly distributed into the pair or octet treatment level with a fully crossed design where all inbred line/group size combinations were represented. At age 51 days old, the morphological and behavioral measurements reported here were carried out as described below before the same worms were prepared for the seminal fluid transcript expression measurements reported in the previous study [34].

Observation of suck behavior

One individual (hereafter focal individual) was randomly chosen from 18 independent pair replicates and 18 independent octet replicates from each inbred line and paired with a standardized mating partner (recipient) to evaluate genetic variation in the suck propensity of partners. Note that we expect suck number to also reflect the total time spent sucking, since a previous report showed this behavior to be both frequent and that each individual sucking event is relatively consistent in its duration [13]. The standardized recipients originated from one inbred line (DV1). To ensure their virginity, recipients were grown under strictly isolated conditions (in individual wells of 24-well tissue culture plates [TPP AG, Trasadingen, Switzerland] filled with 1 mL of 32‰ ASW and *ad libitum* food). Before the pairing, we labeled recipients by using food color to distinguish them (and their suck behavior) from the focal donor [47]. For labeling, we transferred the virgin worms to fresh 60 well HLA Terasaki Plates (Greiner Bio-One, Frickenhausen, Germany) including 3 μL color solution (5 mg of Colorant Alimentaire Grand Blue, Les Artistes, Paris, France per one ml 32‰ ASW) and 7 μL 32‰ ASW with *ad libitum* food for 24 hr. Then worms were transferred to fresh 24-well plates without color solution and food (including only 1 mL of 32‰ ASW) for a few minutes to wash away color solution residues. The coloring has no effect on worms' survival, fecundity or mating behavior [47].

For the suck behavior observations of the recipients, each focal worm and its labeled partner was transferred into mating observation chambers described in detail elsewhere [13]. Briefly, the labeled partner was taken and transferred with a 1.7 μL drop of ASW to the center of a coated (Sigmacote®, Saint Louis, USA) microscope slide which had four HERMA photo stickers (Filderstadt, Germany) adhered to the sides (two per side). Then immediately one focal was transferred with 1.7 μL ASW onto the top of the colored partner, merging the two drops. After ca. 12 drops were placed on the same slide, we sealed around the drops with a thin line of Vaseline to protect drops from evaporation and added a second slide on top of the drops, forming each drop into a shallow three-dimensional pool that the worms could swim inside. Immediately after preparation of one observation chamber, it was placed under a camera for a video recording of 2 hr for suck behavior genetic correlation analyses or 2.5 hr for the knockdown behavior assays described below. The video recordings were done with a DFK 41AF02 Camera (Imaging Source GmbH, Bremen, Germany) connected to a computer running the Debut Video Capture Software Professional, version 2.02. The videos were captured at one frame per second, with a frame rate of ten (a video of 2 hr was zipped into 12 mins) and saved in .mov format with 1920 × 1080 HD resolution. Videos were analyzed by using Kinovea video player, version 0.8.15. Recordings were analyzed by an experimenter blind with respect to the different group size treatments and inbred lines, to count both the number of copulations and the occurrence of subsequent suck behavior of colored recipients. Any mating pairs in which no copulation occurred were excluded from the dataset (ca. 35% of the data).

Testis size and seminal fluid expression measurements

The transparent body of *M. lignano* allows non-invasive measurements of internal reproductive organs [54]. Briefly, after the video recordings, focal worms were taken out of the mating chamber and anesthetized in a 5:3 mixture of 7.14% $MgCl_2$ and 32‰ ASW for 10 minutes. Thereafter, we squeezed the focal donor dorsoventrally to a standard thickness between a microscope slide and a coverslip and took pictures acquired under 400x magnification using a Nikon Ni-U microscope (Nikon GmbH, Düsseldorf, Germany) connected to a ProgRes MFcool camera (Jenoptik GmbH, Jena, Germany). Images were processed for the testis area measurements, representing testis size, using the free-draw tools of the software ImageJ [74], blind with respect to group sizes and identity of inbred lines.

Immediately after imaging, focal worms were transferred individually to 24-well plates including 500 μL 32‰ ASW for about five minutes to wash out $MgCl_2$ and allow worms to recover from this anesthetic. Afterward, individual worms were transferred to 1 mL tubes containing 25 μL of RNALater solution (Ambion, Austin, USA), which immediately stabilizes RNA needed for SFP transcript expression measurements as described in detail in our previous study [34]. Here, we reused the expression data of single SFP transcripts, as well as data on principal component analyses derived from these that were originally reported in Patlar et al. [33], to test our hypothesis that the expression of some genes could negatively correlate with partner suck propensity.

RNA interference knockdown treatment and behavioral assays

In total, 576 juveniles from a stock culture of the DV1 inbred line were collected at 3-4 days old, and randomly distributed into three glass Petri dishes with *ad libitum* food. Taking advantage of the regenerative capacity of *M. lignano* [43], at age 50 days, we amputated half of the adult worms (equally and randomly picked from each Petri dish) to remove received and stored sperm and seminal fluid contents from their previous matings, as well as eggs produced, by cutting away their tail plate just posterior to the end of the ovaries. Afterward, we isolated these worms for 15 days to be used as standardized recipients (mating partners) of the knockdown focal donors (see next paragraph). Isolation was conducted in 10 μL 32‰ ASW with *ad libitum* food in 60 well Terasaki plates to let them regenerate and produce fresh eggs. Worms were regularly fed until the mating observations. These worms were labeled blue as described above on day 14.

We amputated the second half of the adult worms at age 55 days to prepare RNA interference knockdown treatment groups. RNAi was performed as previously described [79]. Briefly, a group of double-stranded RNA (dsRNA) probes was generated for the candidate transcripts using specific primer pairs (Table S2). The dsRNAs were diluted in 32‰ ASW including algae to a final concentration of 25 ng/μl. The second half of the amputated worms were treated with dsRNA in Terasaki plates, where we put one worm in each well and randomized RNAi treatment across wells and plates. The dsRNA treatment was done by transferring worms to fresh wells with fresh dsRNA solution every second day for ten days, sufficient to guarantee complete regeneration but knockdown of the target transcript(s) [43]. In this way, we treated 36 worms with dsRNA for each of six candidates, plus another 36 worms were treated with a combined dsRNA solution (a mixed dsRNA solution of all candidates, hereafter referred to as the combined treatment). We also included 36 worms as a negative control group treated at the same time, under the same conditions but with the solution including only 32‰ ASW with algae. Some worms (42 out of 288) died during this process, seemingly independent of RNAi treatment or control group.

We performed video recordings on surviving individuals after the regeneration period. On day 65, the video recordings were performed in exactly the same way as described above for control and knockdown worms as focals, paired randomly with one of the tail-amputated and regenerated labeled worms. Control and knockdown worms were randomly assigned into mating chambers and observations were done blind to RNAi and control treatment groups (15/246 pairs where mating did not occur were excluded). To verify the success of RNAi knockdown treatments, we performed *in situ* hybridization (ISH) screening for all RNAi knockdown treatments of candidate transcripts individually, as well as for the combined knockdown of all candidates (Figure S1). ISH screening was performed as described in a recent study of seminal fluid in *M. lignano* [31].

Defensive sperm competitive ability (P_1) assay

After 2.5 hr video recording of mating pairs of focal and labeled worms, we transferred labeled recipients into single wells of Terasaki plates and let them mate with standardized competitors for a further 2 hr. The half-an-hour difference between mating pairs of focal and competitor was preferred because there is on average high sperm displacement and thus second male precedence in *M. lignano* [80]. Therefore, we aimed to equalise sperm competitiveness of the first and second partners to some extent, since we were here only interested in differences between treatment groups as first mates. We used transgenic worms that carry a Green-Fluorescent Protein (GFP) marker as competitors. These came from the outbred transgenic BAS1 culture, obtained from a stock maintained at the Zoological Institute at University of Basel, Switzerland [7, 77], which was produced by backcrossing the GFP-transformed HUB1 line [81]. The GFP allele is a dominant marker that is expressed in all somatic and gametic cell types and therefore allows for easily genotyping offspring and assigning paternity following double mating experiments of a wild-type, GFP(-) focal worm (here the RNAi knockdown or control) and a GFP(+) competitor worm [45, 80].

GFP(+) competitors were prepared in the same way as recipient worms, i.e., first amputated and then isolated until forming the mating pairs for ten days. We scored paternity by counting GFP(-) offspring sired by focal and GFP(+) offspring sired by competitors by following the first ten days of egg laying by recipients, until all laid eggs had eventually hatched. In addition, we isolated GFP(+) competitors after pairing them with recipients to assess the offspring number produced by them, to exclude data of where a competitor worm did not lay eggs (assuming therefore that mating did not occur unless recipient also did not produce GFP(+) offspring). In total, nine out of 233 competitors did not produce any offspring, and their recipients also did not produce GFP(+) offspring; these were excluded.

QUANTIFICATION AND STATISTICAL ANALYSIS

The effect of social group size and genotype on recipient suck propensity and testis size was assessed using two-way ANOVA with interaction after testing for homogeneity of variance for social group size using Levene tests. Variances were found to be similar for both suck propensity ($p = 0.50$) and testis size ($p = 0.16$).

We estimated all the genetic correlations (r_G) by calculation of Pearson product-moment correlations of the inbred line means, which were measured from the same set of individuals for each gene and suck behavior and calculated standard errors from

10,000 replicates of bootstrapped coefficients. We tested for statistical significance by comparing the *z*-scores to two-tailed significance levels derived from a standard normal distribution.

We performed Welch two sample t tests, which is a more reliable test when the variances and sample size are unequal across groups, to compare mean partner suck propensity (and of donor as supplemental data, as well as mating frequency) of the different RNAi knockdown groups to the control group. We calculated adjusted *P*-values by performing Benjamini-Hochberg False Discovery Rate corrections to decrease the chance of Type I errors while identifying candidate transcripts and where multiple tests were applied.

For the comparison of paternity success of RNAi knockdown groups with the control group, we used a generalized linear model with a quasi-binomial error distribution and logit link function (for handling proportional data including many zeroes and ones), in which the response is a matrix where the first column is the number of the focal donor's offspring and the second column is the number of GFP(+) offspring. All statistical analyses were performed with R version 3.4.2 [73].

DATA AND CODE AVAILABILITY

The datasets generated during this study are available at Mendeley Data (https://doi.org/10.17632/cbdpzgsc82.1).