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The benefits of male ejaculate sex peptide transfer in *Drosophila melanogaster*

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Abstract

The accessory gland protein (Acp) ejaculate molecules of male *Drosophila melanogaster* mediate sexual selection and sexual conflict at the molecular level. However, to date no studies have comprehensively measured the timing and magnitude of fitness benefits to males of transferring specific Acps. This is an important omission because without this information it is not possible to fully understand the strength and form of selection acting on adaptations such as Acps. Here, we measured the fitness benefits to males of ejaculate sex peptide (SP) transfer. SP is of interest because it is a candidate for mediating sexual conflict: its frequent receipt reduces female fitness. In single matings with virgin females SP is known to increase egg laying and decrease receptivity. Hence, we predicted that SP could: (i) boost a male's absolute paternity by increasing offspring production and delaying female remating and/or (ii) boost relative paternity share. We tested these predictions using two different lines of SP-lacking males, in both two-mating and free-mating assay conditions. SP transfer conferred higher absolute, but not relative, male reproductive success. In matings with virgin females, SP transfer increased mating productivity and delayed remating and hence the onset of sperm competition. In already mated females, SP transfer did not elevate absolute progeny production, but did increase intermating intervals and hence the period over which a male could gain paternity. Consistent with this, under free-mating conditions over an extended period, we detected a 'per-mating' fitness benefit for males transferring SP. These benefits are consistent with a role for SP in mediating conflict, with SP acting to maximize short-term fitness benefits for males.

Introduction

The study of ejaculate molecules transferred along with sperm by males of *Drosophila melanogaster* provides a unique case study of evolution in action and reveals the influence of sexual selection and sexual conflict at the molecular level (Chapman, 2001; Wolfner, 2002; Chapman *et al.*, 2003; Gillott, 2003). It also exemplifies the extraordinarily rapid evolutionary change that can be driven by selection (e.g. Swanson *et al.*, 2001; Haerty *et al.*, 2007; Wong *et al.*, 2008). Accessory gland proteins (Acps) play roles likely to determine the overall reproductive success of males in competition. For example, Acps boost egg laying and decrease female willingness to remate in matings with virgin females, and appear to facilitate the displacement of stored sperm from previous males in matings with already mated females (Harshman & Prout, 1994; Herndon & Wolfner, 1995; Heifetz *et al.*, 2000; Prout & Clark, 2000; Chapman *et al.*, 2003; Liu & Kubli, 2003). In addition, specific Acps play a role in sperm storage and retention in the female sperm storage

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organs, and hence sperm competition (Neubaum & Wolfner, 1999; Chapman *et al.*, 2000; Bloch Qazi & Wolfner, 2003; Ram & Wolfner, 2007a). Sequence variation associated with Acps also correlated with a male's success in aspects of sperm competitive ability such as sperm displacement (Clark *et al.*, 1995), female refractoriness and P1 and P2 (the proportion of offspring sired by the first and second male to mate, respectively, see Boorman & Parker, 1976) (Fiumera *et al.*, 2005, 2007).

The genes encoding Acps are predicted to respond to selection arising from sexual conflict (Parker, 1979, 2006; Swanson & Vacquier, 2002; Arnqvist & Rowe, 2005) because, in addition to the functions listed above, the receipt of high levels of Acps can harm females. For example, some Acps may have toxic effects (Lung *et al.*, 2002; Mueller *et al.*, 2007) and receipt of Acps can cause a reduction in female lifespan and reproductive success (Chapman *et al.*, 1995; Wigby & Chapman, 2005). There are also associations between sequence variation in or near Acp genes and a male's ability to shorten female lifespan in single mating tests (Fiumera *et al.*, 2006). The male accessory gland sex peptide (SP) (Chen *et al.*, 1988) has been highlighted as important in determining female mating costs (Wigby & Chapman, 2005). This conclusion is also supported by the finding of a significant positive association between a male's ability to reduce female lifespan following single matings, and his ability to reduce female receptivity (i.e. one of the functions of SP) (Civetta & Clark, 2000). Thus, there is evidence that receipt of Acps, perhaps particularly SP, can decrease female fitness.

To conclude that Acps are subject to selection arising from sexual conflict, we need evidence that selection acts in opposing directions in males and females (Rowe & Day, 2006). Hence, although selection on females should act to reduce the costs of Acp receipt (e.g. Holland & Rice, 1999; Wigby & Chapman, 2004), the potential benefits to males should select for increased efficiency or magnitude of Acp effects. Consistent with these predictions, results from experimental evolution show that when males were allowed to adapt against a static female phenotype, they evolved greater fitness and exacted greater mating costs from females following single matings, consistent with the idea that male ejaculate composition had changed (Rice, 1996, 1998). Further, it is important to consider the temporal nature of any benefits accrued to males. For example, short-term benefits to males that cause longer term costs to females could be selected, because under polyandry in nonsocial insects the sexes have little shared interest in reproduction after the current mating bout is over. We have knowledge of the function of Acps from work with single gene mutants (reviewed by Chapman, 2001; Wolfner, 2002; Ram & Wolfner, 2007b) and demonstrations of the potential importance of Acps in determining male fitness from association tests and from experimental evolution (Clark *et al.*, 1995; Rice, 1996, 1998; Fiumera *et al.*, 2005, 2007). However, no studies have yet comprehensively measured the timing and magnitude of fitness benefits for males of individual Acp transfer.

In this study, we conducted fitness tests of a male ejaculate protein, SP, that is implicated in mediating sexual conflict. We tested whether SP transfer increases male reproductive success, and estimated the timing and magnitude of any benefits. To do this we employed two independently produced types of SP-lacking males (i.e. knockout; Liu & Kubli, 2003 or knockdown males; Chapman *et al.*, 2003) and we examined the effects of SP on male fitness in two experimental environments. We first measured male sperm competitive ability in a two-mating experimental design, and second, estimated male reproductive success over an extended period in a 'free-mating' competitive environment. We tested the following specific predictions:

(i) *SP transfer will increase absolute male reproductive success*. SP is predicted to increase the number of offspring produced before remating, because it reduces female receptivity in matings with virgin females (Chapman *et al.*, 2003; Liu & Kubli, 2003). Hence, males that transfer SP are predicted to produce a higher absolute number of progeny and delay the onset of sperm competition and thus the displacement of their sperm, in comparison with SP-lacking males. In combination, this could increase the 'per-mating' rate of offspring production.

(ii) *SP transfer will increase relative male reproductive success*. SP could boost a male's relative paternity share if SP increases the efficiency with which a given number of sperm are used, or if sperm competitive ability is positively associated with SP induced increases in mating productivity.

Materials and methods

Fly stocks

Sex peptide-lacking males

Two types of SP-lacking males were used: *SP* gene knockouts (Liu & Kubli, 2003) and SP knockdown males produced by RNAi (Chapman *et al.*, 2003). SP knockout males were produced by crossing SP^0*/TM3,Sb,ry* males to Δ*130/TM3,Sb,ry* females. The resulting SP^0/Δ*130* (SP^0) males produce no SP (Liu & Kubli, 2003). Control males were generated by crossing SP^0,SP^+*/TM3,Sb,ry* males to Δ*130/TM3,Sb,ry* females to generate SP-producing SP^0,SP^+/Δ*130* (SP^+) males. To control for genetic background, we backcrossed the strains: the Δ*130/TM3,Sb,ry* stock was backcrossed for three generations, and chromosomes 1, 2 and 4 of the SP^0*/TM3,Sb,ry* and SP^0,SP^+*/TM3,Sb,ry* stocks were backcrossed for four generations into the Dahomey wildtype background. SP knockdown males were produced by crossing SP-inverted repeat lines (*UAS-SP-IR1* and *UAS-SP-IR2*)

to an X-linked accessory gland-specific *Gal4* driver (*Acp26Aa-P-Gal4*) (Chapman *et al.*, 2003), i.e. SP1 knockdown (kd) males (*Acp26Aa-P-Gal4; UAS-SP-IR1*) and SP2 kd males (*Acp26Aa-P-Gal4; UAS-SP-IR2*). SP in these males is undetectable in Western blots (Chapman *et al.*, 2003). We chose one of these lines at random for most of the tests (SP2 kd). The *UAS-SP-IR* lines were in an outbred w^1 genetic background and we made a driver control (DR-C) for the X chromosome *Acp26Aa-P-Gal4* insertion by taking the male offspring of *Acp26Aa-P-Gal4* females and w^1 males (i.e. *Acp26Aa-P-Gal4/+*). To control for the inverted repeat, we crossed w^1 females with *SP-IR-2* males (IR2-C). In the extended assay period experiment where we used both *UAS-SP-IR* lines, we produced inverted repeat controls from reciprocal (r) crosses (IR1r-C; IR2r-C), using the sons of *UAS-SP-IR1* or *UAS-SP-IR2* mothers and *Acp26Aa-P-Gal4* fathers (i.e. +; *UAS-SP-IR1* and +; *UAS-SP-IR2* respectively). This allowed us to control for the inverted repeat and the autosomal background concurrently.

Females and competitor males

To assign paternity, we used females and competitor males carrying the recessive eye colour marker *sparkling poliert (spa)* in a wild-type genetic background. *spa* males were found to produce SP at levels similar to that of control males in Western blots (data not shown).

Fly culturing

Flies were cultured at 25 °C on a 12 : 12 light : dark cycle in humidified rooms. Stocks were cultured in glass bottles (189 mL each) containing 70 mL of sugar–yeast (SY) food [100 g autolysed yeast powder, 100 g dextrose, 20 g agar, 30 mL Nipagin (10% w/v solution), 3 mL propionic acid and 1 L water]. All experiments were performed in glass vials (75 mm height × 25 mm diameter) containing 7 mL of SY food with *ad libitum* live yeast granules or paste. To generate SP-lacking and control males, three each of parental males and females for each cross were housed together in vials and transferred onto fresh food every day. Ten days later, male offspring were collected and housed in groups of 10 in vials until used in the experiments. To generate experimental females and competitor males, we allowed *spa* females to oviposit on Petri dishes filled with a grape juice–agar mix [50 g agar, 600 mL red grape juice, 42.5 mL Nipagin (10% w/v solution) and 1.1 L water] smeared with live yeast paste. We grew flies at standard densities either by picking larvae and placing them 100 per vial or by pipetting eggs into bottles using a standard density method (Clancy & Kennington, 2001). Females and males eclosing from these bottles or vials were collected (females as virgins, males within 2 days of eclosion) and held in same sex vials at 10 flies per vial. Flies entered the experiments when they were 4–5 days post-eclosion. For mating experiments, males were anaesthetized and transferred into the mating vials 1 day prior to the start of the experiments. For egg counts, oviposition vials also contained 4 g L^{-1} charcoal powder, to increase the contrast and facilitate counting.

Two-mating assays of first and second mating male reproductive success

To test the reproductive success of SP-lacking or control males under two-mating assay conditions, we made SP-lacking or control males either the first or second males to mate with a female, and measured mating productivity (eggs and progeny production), female refractoriness and male sperm competitive ability, as described below.

First male reproductive success

We mated 80 replicates each of SP^0 and SP^+ males and 100 each of SP2-knockdown and IR2-C and DR-C control males to *spa* females, followed by rematings of half of each group of females either 6 or 24 h later to *spa* males. In each case, mating vials comprised a single female with two males. Females were individually aspirated into vials and the time of introduction, the start and end of copulation, were recorded over the subsequent 3 h. After mating, females were transferred to oviposition vials by aspiration. Fecundity and progeny counts between the first and second matings allowed us to assess, and control for, differences in fecundity and egg to adult survival between male genotypes. After 6 or 24 h, females were transferred into remating vials and given the opportunity to remate for 2 h. After a successful second mating, females were transferred individually to fresh vials daily. Vacated vials were incubated at 25 °C for 12 days at the end of which the offspring were counted and paternity assessed. Females that did not remate in the 24-h remating assays were given a second chance to remate 48 h after their first matings. For these females we also scored the number of emerging offspring in the 24- to 48-h period. In our first experiment using SP knockout and control males, very few females initially mated to control males remated after 24 h. We therefore repeated this treatment, starting with 50 females for each group.

In these tests, absolute reproductive success of SP-lacking or control males is given by the number of eggs and progeny produced after the first mating, the likelihood of female remating and the extra progeny gained by females not remating. Relative reproductive success is given by the extent of P1 paternity (the proportion of offspring fathered by the first male to mate, Boorman & Parker, 1976).

Second male reproductive success

Using similar procedures to those described above, we mated *spa* females first to *spa* males and then 24 h later 40 each of these females were given the opportunity to remate with SP^0 or SP^+ males and 50 each with SP2-knockdown, IR2-C or DR-C males. We recorded mating latency and duration of the matings, the fecundity of the

females for the 4 days following the second mating and the fertility and paternity of the eggs laid during that period. In this test, the absolute reproductive success of SP-lacking and control males is given by the number of progeny fathered after the second mating. Relative reproductive success is given by the P2 paternity share.

Free-mating tests of male reproductive success in 12-day or lifetime assays

We tested the reproductive success of SP-lacking and control males in a free-mating environment in which males were in competition for matings and fertilizations with *spa* marker males. We ran two sets of assays: over 12 days and an extended assay over the whole female's lifetime.

12-day assays

We used SP^0 and SP2-knockdown males and their respective controls, as above and tested male competitive reproductive success. We placed single 1- to 2-day-old SP-lacking or control males in vials containing three 1- to 2-day-old *spa* males and two 2-day-old *spa* females (n = 30 replicates set up). To score male mating success we checked vials for matings each morning after lights on, every 20 minutes for 3 h. We identified the genotype of the mating male by scoring eye colour. Flies were transferred onto fresh food with light CO_2 anaesthesia every day for 12 days, and any dead or lost *spa* males or females were replaced with *spa* virgins. We counted and scored paternity of the offspring that emerged 12 days later from each vial from each day of the 12-day period, except for the day 4 vials (where samples were frozen a day or two late, and the food had deteriorated, making reliable paternity assignment impossible).

Lifetime assays

We used both SP knockdown lines and their reciprocal cross controls (i.e. SP1-knockdown and SP2-knockdown, and IR1r-C and IR2r-C) and monitored reproductive success over the whole lifetime of the females housed with SP-lacking or control males. For this assay, we chose to use two knockdown lines (rather than one knockdown and one knockout line as above) to allow us to compare male reproductive success directly across two replicate experiments. Two SP knockdown or control males were placed in vials containing two *spa* males and two *spa* females (n = 20 replicates for each treatment). Flies were transferred to fresh vials every 1 or 2 days until day 17 and every 3 days from then on. Any dead males were replaced with spare males of the same age and all males were replaced on day 14 with fresh 3-day-old males. The first matings of 125 of 160 females were observed. Matings were recorded on a further 13 days spread throughout the experiment, as described above and the experiment was terminated when all females were dead.

In these tests, absolute reproductive success for SP-lacking and control males is given by the total number of progeny fathered and by mating frequency estimates. We estimated male 'per-mating' reproductive success as another measure of absolute reproductive success by controlling for differences in mating rate. Relative reproductive success is given by a male's paternity share.

Data analysis

We analysed data for the SP knockout and knockdown lines separately. Where appropriate we used generalized linear models (GLM) with the appropriate error distributions. To correct for overdispersion in GLM with binomial errors, we used the quasi-binomial function (Crawley, 2005). The significance of a factor in our models was tested in an analysis of deviance through subtraction of each factor from the full model, followed by a comparison of the full and reduced models. The deviance (G^2) (−2 times the difference between the log-likelihood of the reduced model and the log-likelihood of the full model) was tested for significance by comparing it with a chi-squared distribution, or an F-distribution when using quasi-binomial errors (Crawley, 2005). Where appropriate, we combined probabilities as described in Sokal & Rohlf (1995, pp. 794–797). Mean values for proportion data were calculated from total counts of the raw data, as recommended by Crawley (2005). All analyses were carried out in R v2.5.1 (Ihaka & Gentleman, 1996). Data are represented as average values ± SE throughout the text unless stated otherwise. For proportions (p), standard errors were calculated as $\mathrm{sqrt}[p(1-p)/n]$. To analyse the per-mating reproductive success of males in the free-mating assays, we calculated the mean percentage of the total offspring that were fathered by SP-lacking or control males, and the mean percentage of the total number of matings that were with SP-lacking or control males. The ratio of those two values gave an index of the 'per-mating' male reproductive success for SP-lacking and control males. The use of populations instead of vials as the unit of replication in this analysis of male post-mating success created a conservative test. Too few matings were observed in each individual vial to perform an effective analysis using 'per-vial' data.

Results

Two-mating assays of first and second mating male reproductive success

First male reproductive success

Absolute reproductive success. As expected, SP transfer significantly decreased female sexual receptivity to remating in matings with virgin females. Females previously mated to SP knockout and SP knockdown males remated more frequently and sooner than did females

mated to the respective control males (Fig. 1a,b). There were significant effects of male genotype, remating period and the number of eggs laid after the first mating on female remating receptivity (Table 1). The duration of the first copulation had a significant effect on receptivity to remating in the SP knockout experiment (Table 1) but not in the SP knockdown experiment (Table 1); however, the combined *P*-value ($P = 0.027$) indicates an overall positive relationship.

As expected, females mated to SP-lacking males laid significantly fewer eggs in the period between their first and second matings (SP knockout: $F_{1,154} = 10.66$, $P = 0.001$; SP knockdown: $F_{2,283} = 11.90$, $P < 0.001$) and there were also significant effects on egg production due to remating interval (SP knockout: $F_{1,154} = 250.79$, $P < 0.001$; SP knockdown: $F_{1,283} = 389.07$, $P < 0.001$; Fig. 1c,d). SP-transferring control males produced on average 10–19 more eggs during the 24-h intermating interval than SP-lacking males (eggs laid within 24 h SP knockout: $SP^0 = 57.51 \pm 2.76$, $SP^+ = 68.2 \pm 3.59$, difference = 10.69; SP knockdown: SP2 knockdown = 49.7 ± 4.11, IR2-C = 60.0 ± 3.67, DR-C = 69.35 ± 3.28, difference = 19.65). Only 29.3% of females mated to SP^+ control males remated after 24 h, with an additional 53% of the remaining females remating after 48 h. The induction of the longer refractory period to 48 h resulted in males gaining on average an extra 34.44 ± 3.73 offspring.

To test for differences in absolute progeny production between SP-lacking and control males, we analysed progeny fathered by SP-lacking or control males following a successful second mating with *spa* competitor males. The results for the number of offspring fathered following the 24-h remating tests (there were few or no control rematings after 6 h) gave contradictory results for SP knockout vs. SP knockdown experiments. In the SP knockout experiment, overall significantly more offspring were fathered by SP^0 than by control males ($SP^0 = 93.16 \pm 10.42$, $SP^+ = 33.23 \pm 8.44$; $G^2_1 = 879.0$, $F = 18.21$, $P < 0.001$) with a nonsignificant trend in the opposite direction, i.e. for higher progeny production by SP-transferring males, in the SP knockdown experiment (SP2 knockdown = 11.77 ± 2.92, IR2-C = 13.00 ± 9.31, DR-C = 38.13 ± 21.46; $G^2_2 = 399.1$, $F = 2.06$, $P < 0.136$).

The results of the experiments testing first male reproductive success overall show consistent benefits of SP transfer in absolute reproductive success in terms of the lowering of female receptivity, stimulation of egg production and hence the gain of extra progeny fathered before remating by females. However, these benefits were apparent in matings with virgin females, and disappeared after these females remated: there were no

Table 1 Female remating propensity after mating to SP-lacking or control males in two mating assays.

	SP knockout experiment			SP2 knockdown experiment		
Source	d.f.	Deviance	*P*	d.f.	Deviance	*P*
Male genotype	1	64.50	< 0.001	2	6.95	0.031
Remating interval	1	35.21	< 0.001	1	38.29	< 0.001
Number of eggs	1	4.96	0.026	1	12.36	< 0.001
Duration of mating 1	1	4.68	0.030	1	2.15	0.143

Results of a generalized linear model with binomial errors. The SP knockout experiment compared SP^0 and SP^+ males, and the SP2 knockdown experiment compared SP2 knockdowns with IR2-C and DR-C males. Male genotype and remating interval (6 or 24 h) were treated as fixed factors, whereas duration of the first mating and number of eggs laid between matings were included as covariates.

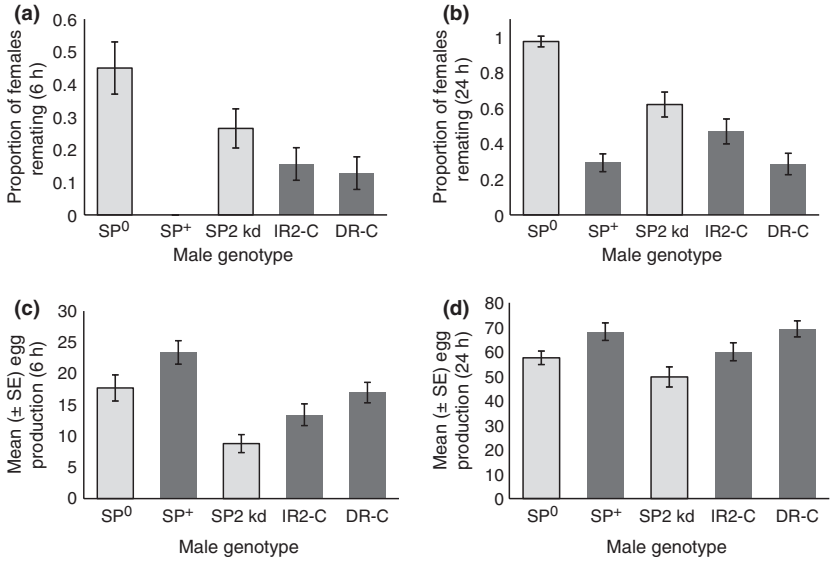

Fig. 1 Female receptivity and egg production following matings to SP-lacking or control males in the two mating assays. (a,b) Proportion (±SE) of females remating (a) 6 h and (b) 24 h after single matings to SP-lacking (SP^0, SP2 knockdown) or control (SP^+ IR2-C, DR-C) males. (c,d) Mean (±SE) female egg production over (c) 6 h and (d) 24 h following single matings to SP-lacking or control males. SP-lacking males – light grey bars; SP-producing control males – dark grey bars. SP knockout experiment: $n = 40$ and SP knockdown experiment: $n = 50$ per treatment.

Table 2 P1 first male paternity share for SP-lacking and control males in two mating assays with rematings after 24 h.

	SP knockout experiment (SP^0 vs. SP^+)				SP2 knockdown experiment (SP2 knockdown vs. IR2-C, DR-C)			
Source	d.f.	Deviance	*F*	*P*	d.f.	Deviance	*F*	*P*
Male genotype	1	576.9	10.40	0.002	2	438.7	3.45	0.039
Intermating egg production					1	9.6	0.15	0.700
Duration of mating 1	1	0.03	0.0005	0.982	1	232.0	3.65	0.061
Duration of mating 2	1	155.1	2.80	0.100	1	248.0	3.90	0.053

Results of GLM analysis. Male genotype was a fixed factor, and duration of the first and second matings (24 h later) and the number of eggs laid between the two matings were covariates. Dispersion parameters, SP knockout experiment = 55.46; SP2 knockdown experiment = 63.59.

consistent differences in the number of progeny fathered by SP-lacking or control males following a second mating by a competitor male.

Relative reproductive success. There was no evidence that SP-transferring control males achieved a higher first male P1 paternity share. In experiments with SP knockout males there was even significantly higher P1 paternity share for SP^0 males (Table 2, Fig. 2b). However, this was not apparent in the experiments with SP knockdown males where the significant difference in P1 paternity share was driven entirely by the driver control DR-C males, which gained high paternity (Table 2). Exclusion of this group showed that there were no differences between SP2-knockdown and IR2-C males ($G_1^2 = 4.67$, $F = 0.09$, $P = 0.762$). The number of eggs laid after the first mating also had no significant influence on P1 paternity share. Our results are not confounded by different egg-adult survival for the different male genotypes used here (see Supplementary Data 1). Across both experiments there were significant trends for longer second matings to lead to lower P1 paternity share (SP knockout experiment $r = -0.34$, $P = 0.009$; SP knockdown experiment $r = -0.19$, $P = 0.12$, Table 2, combined value of $P = 0.033$). There were no differences in P1 attributable to whether females remated after 6 or 24 h (Table 3, Fig. 2), but again a consistent effect was noted of the second mating duration on P1 (Table 3, combined value of $P = 0.031$). There were significant positive correlations between P1 paternity share and mating productivity (Supplementary Data 3) but no evidence for any differences in this respect between SP-transferring or lacking males. Overall the results from the P1 experiments show no evidence for effects of SP transfer on relative male reproductive success.

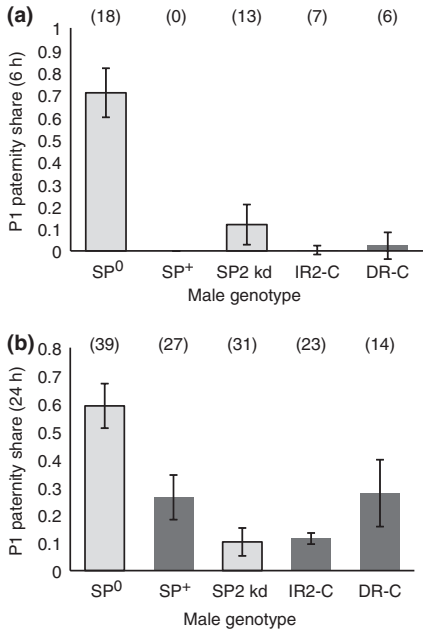

Fig. 2 Mean (±SE) male P1 paternity share following first matings to SP-lacking (SP^0, SP2 knockdown) or control (SP^+, IR2-C, DR-C) males, and second matings to *spa* males. SP-lacking males – light grey bars; SP-producing control males – dark grey bars with sample sizes in parentheses above bars. (a) Females remating after 6 h (b) after 24 h.

Table 3 P1 first male paternity share for SP-lacking and control males gained following female rematings after either 6 or 24 h.

	SP knockout experiment				SP2 knockdown experiment			
Source	d.f.	Deviance	*F*	*P*	d.f.	Deviance	*F*	*P*
Male genotype					2	296.6	2.26	0.111
Remating interval	1	10.5	0.17	0.680	1	28.0	0.43	0.515
Male genotype × remating interval					2	288.1	2.63	0.078
Intermating egg production	1	30.1	0.49	0.486	1	18.6	0.28	0.596
Duration of mating 1	1	28.2	0.46	0.500	1	200.3	3.06	0.084
Duration of mating 2	1	171.6	2.82	0.100	1	259.4	3.96	0.050

Results of GLM analysis. Male genotype and remating interval were fixed factors and copulation duration and the number of eggs laid between the two matings were covariates. No females first mated to SP^+ control males in the SP knockout experiment remated after 6 h and thus only the SP^0 treatment was tested in this analysis. Dispersion parameter, SP knockout experiment = 60.94, SP2 knockdown experiment = 65.56.

Second male reproductive success

Absolute reproductive success. We tested for differences in the absolute number of progeny fathered by SP-lacking and control males after females successfully mated twice. In the SP knockout experiment, the number of progeny fathered by SP-lacking and control males did not differ significantly (SP^0 = 127.56 ± 22.32, SP^+ = 144.67 ± 17.90; G_1^2 = 20.83, P = 0.551). In the SP knockdown experiment, there were significant differences, with control SP-transferring males having the highest and lowest progeny production (SP2 knockdown = 92.44 ± 13.47, IR2-C = 33.26 ± 9.32, DR-C = 101.68 ± 19.67; G_2^2 = 963.8, P = 0.001). In contrast to the results from the P1 experiments when SP-transferring males were the first to mate with twice mated females, there was no evidence here for increased absolute reproductive success for SP-transferring males when they mated with nonvirgin females previously mated once to *spa* competitor males. It is not possible to determine how many eggs, if any, were produced in response to SP transfer in these second matings of females, but our data suggest that any such effect was minimal. Hence, the data suggest that male fitness benefits arising from the boost to egg production caused by SP are primarily observed in matings with virgin but not with recently mated females. Note, however, that we have no data from this experiment on the magnitude of any benefits arising from delaying remating by SP transfer to already mated females. Overall, our results from the P2 experiments provide no evidence that transfer of SP resulted in increased absolute reproductive success.

Relative reproductive success. Control and SP-lacking males did not differ significantly in their P2 paternity share (Table 4 and Fig. 3). Neither copulation duration of the *spa*, SP-lacking or control males, nor the number of eggs laid after the first mating had a significant effect on P2 values (Table 4). In contrast to the P1 experiment, there were mostly no correlations between paternity share and mating productivity (Supplementary Data 3). Overall, the results from the P2 experiments show no significant benefits in relative reproductive success in terms of SP transfer.

Table 4 P2 second male paternity share for SP-lacking and control males in two mating assays.

	SP knockout experiment				SP2 knockdown experiment			
Source	d.f.	Deviance	F	P	d.f.	Deviance	F	P
Male genotype	1	57.90	0.002	0.964	2	27.91	0.06	0.569
Intermating egg production	1	184.39	0.01	0.935	1	57.65	2.35	0.130
Duration of mating 1	1	30.62	0.01	0.974	1	17.4	0.71	0.403
Duration of mating 2	1	1619.33	0.06	0.809	1	11.72	0.48	0.492

GLM analysis results. The SP knockout experiment compared SP^0 and SP^+ males, and the SP2 knockdown experiment compared SP2 knockdowns with IR2-C and DR-C males. Male genotype was a fixed factor and durations of the first and second copulations and the number of eggs laid between the two matings were covariates. Dispersion parameter, SP knockout experiment = 27 231.23, SP2 knockdown experiment = 24.55.

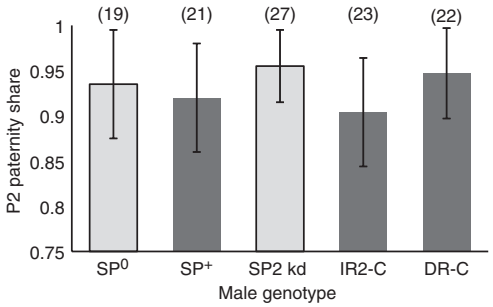

Fig. 3 Mean (±SE) male P2 paternity share following first matings to *spa* males and second matings to SP-lacking (SP^0, SP2 knockdown) or control (SP^+, IR2-C, DR-C) males. SP-lacking males – light grey bars with sample sizes in parentheses above bars; SP-producing control males – dark grey bars.

Free-mating tests of male reproductive success in 12-day or lifetime assays

We tested for differences in reproductive success for SP-lacking or control males in two experiments. In the first, we tested the reproductive success of SP^0 and SP2 knockdown males vs. their controls (SP^+, IR2-C and DR-C) when all males were in competition with *spa* males over a period of 12 days. In the second experiment, we tested the reproductive success of SP1 and SP2 knockdowns vs. IR1r-C and IR2r-C controls in competition with *spa* males over the whole lifetime of the females in the assays. In an independent test, we confirmed that the SP-lacking and control males used did not differ significantly in their courtship rate, whereas mating rates differed as expected with SP-lacking males mating more frequently, even though this last difference was not statistically significant (Supplementary Data 2).

Absolute reproductive success

Twelve-day assay. To determine absolute reproductive success, we calculated the total paternity per male for SP-lacking or control males and the total number of matings observed per vial during the entire 12-day assay period. In the SP knockout experiment, more progeny were fathered by SP-transferring males during the 12-day assay period, with SP^0 males fathering an average of 377.00 ± 33.78 and SP^+ control males 414.43 ± 45.20 offspring, but this effect was not significant ($F_{1,58}$ = 0.44, P = 0.51, see Fig. 4a). There were more matings, as expected, in the SP^0 treatment, but this difference was not significant (average total mating frequency per vial

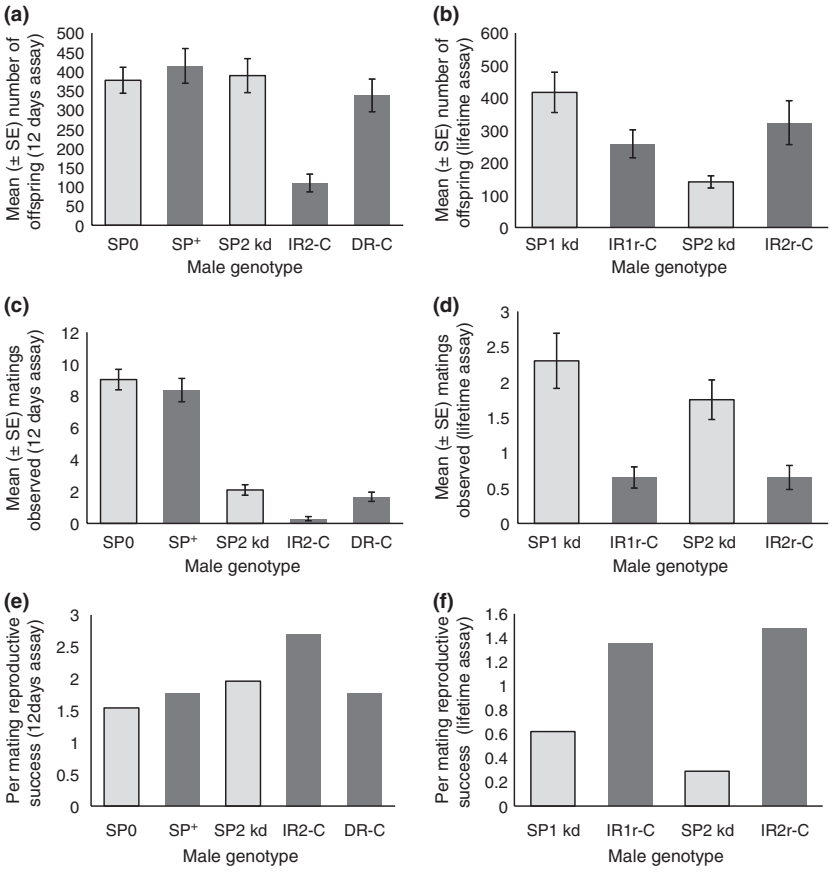

Fig. 4 Absolute reproductive success of SP-lacking or control males under free-mating conditions. SP-lacking males – light grey bars; SP-producing control males – dark grey bars. (a,b) Mean total number of progeny sired by SP-lacking or control males during the (a) 12-day (n = 30 per treatment) and (b) lifetime (n = 20 per treatment) assays. (c,d) Mean mating frequency for SP-lacking or control males during the (c) 12-day and (d) lifetime assays. (e,f) Mean per-mating reproductive success for SP-lacking or control males during the (e) 12-day and (f) lifetime assays. Male per-mating reproductive success was calculated as the ratio of the mean percentage of the total offspring fathered by SP-lacking or control males divided by the mean percentage of the total number of matings with SP-lacking or control males.

SP^0 treatment = 9.03 ± 0.64, SP^+ treatment = 8.37 ± 0.73 matings, $F_{1,58}$ = 0.47, P = 0.50, Fig. 4c). In the SP knockdown experiment, there were significant differences in absolute progeny production by SP-lacking or control males, mainly due to the low number of progeny fathered by IR2-C males (mean progeny fathered by: SP2 knockdown = 389.20 ± 44.20, IR2-C males = 109.90 ± 23.02, DR-C males = 337.67 ± 42.50; $F_{2,87}$ = 15.45, $P < 0.001$, Fig. 4a). SP-lacking replicates in the SP2 knockdown treatment showed the highest mating frequency, as expected (average total mating frequency per vial: SP2 knockdown = 2.10 ± 0.33, IR2-C = 0.30 ± 0.13, DR-C = 1.67 ± 0.29; $F_{2,87}$ = 11.52, $P < 0.001$, Fig. 4c). Combining the probabilities from these independent experiments, showed that, in general, treatments with SP-lacking males had a higher mating rate over the 12-day assay period than did their controls (combined P = 0.007).

Lifetime assay. A nested ANOVA revealed that the number of offspring produced in the lifetime assay was not different between SP-lacking males and their controls (treatment: $F_{1,76}$ = 0.06, P = 0.816; SP1 knockdown = 416.80 ± 62.14, IR1r-C = 258.20 ± 43.22, SP2 knockdown = 140.65 ± 18.79, IR2r-C = 323.40 ± 67.72; line replicate: $F_{2,76}$ = 7.55, P = 0.001, Fig. 4b). Significantly more matings were observed in treatments with SP-lacking males than with their controls (SP1 knockdown = 2.3 ± 0.39, IR1r-C = 0.65 ± 0.15, SP2 knockdown = 1.75 ± 0.28, IR2r-C = 0.65 ± 0.17; treatment: $F_{1,76}$ = 4.54, P = 0.036, Fig. 4d).

Taken together the results from the 12 day and lifetime assays show no consistent evidence that the transfer of SP led to higher absolute progeny numbers. However, there was evidence that mating frequency was lower in groups where control, SP-transferring, males were present. This suggests that intermating intervals were longer when all males present were transferring SP. SP transfer generally resulted in higher per-mating reproductive success (Fig. 4e,f), but because of the lack of directly comparable replicates we were able to analyse statistically only the data for the lifetime assay. This analysis showed that control males did indeed have significantly higher per-mating reproductive success than SP knockdown males (ANOVA, $F_{1,2}$ = 31.02, P = 0.031). SP1 and SP2 knockdown males gained an index of 0.62 and 0.29 offspring per mating per female, respectively, whereas IR1r-C and IR2r-C males gained 1.35 and 1.48 offspring per mating per female respectively. These results suggest an additional absolute 'per-mating' fitness benefit resulting from SP transfer.

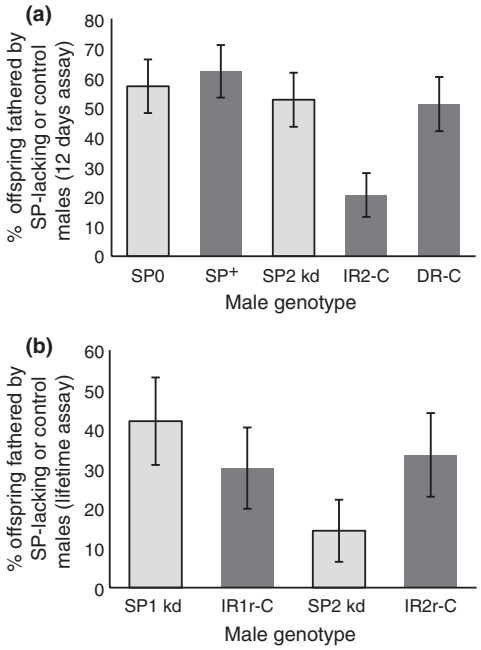

Fig. 5 Relative reproductive success of SP-lacking or control males in the free-mating 12-day and lifetime assays. Percentage of offspring produced by SP-lacking or control males during the (a) 12-day or (b) lifetime assay periods. SP-lacking males (SP^0, SP1 knockdown, SP2 knockdown) – light grey bars; controls (SP^+, IR2-C, DR-C, IR1r-C and IR2r-C) – dark grey bars.

Table 5 Relative reproductive success of SP-lacking and control males in free-mating assays.

(a) 12-day assay period

	SP knockout experiment				SP2 knockdown experiment			
Source	d.f.	Deviance	*F*	*P*	d.f.	Deviance	*F*	*P*
Male genotype	1	132.9	1.21	0.276	2	2345.7	7.53	< 0.001
Mating frequency	1	166.7	1.52	0.223	1	1395.4	8.96	0.004

(b) Lifetime assay period

	SP1/SP2 knockdown experiment			
Source	d.f.	Deviance	*F*	*P*
Male genotype	1	887.2	4.47	0.038
Replicate	3	3972.8	6.67	< 0.001
Mating frequency	1	884.8	4.46	0.038

GLM analysis results. Male genotype was a fixed factor and the total number of matings achieved by SP-lacking or control males was a covariate. (a) The 12-day assay period experiment. We compared SP^0 vs. SP^+ males and SP2 knockdowns vs. IR2-C and DR-C males. Dispersion parameter, SP knockout experiment = 109.83; SP2 knockdown experiment = 155.73. (b) The lifetime assay period experiment. We compared SP1 and SP2 knockdowns with IR1r-C and IR2r-C males. In this analysis replicate lines (i.e. SP knockdowns 1 and 2 and IR1r-C and IR2r-C) were nested within male genotype. Dispersion parameter = 198.46.

Relative reproductive success

We analysed the proportion of total offspring produced by SP-lacking or control males in both the 12-day and lifetime assays (Fig. 5).

Twelve-day assay. In the 12-day assay, there was no significant difference in relative male reproductive success for SP^0 vs. their control SP^+ males (Table 5a). SP^0 males fathered 57.2% and SP^+ males 62.3% of offspring (Fig. 5a). For the SP knockdown males there were significant differences in relative reproductive success between SP2 knockdown, IR2-C and DR-C males (Table 5a); however, this was attributable to the low paternity share attained by IR2-C males (average 20.6% offspring). DR-C males gained 51.2% paternity, which was very similar to SP2 knockdown males (52.7% offspring).

Lifetime assay. There were significant differences in the paternity share obtained by SP1, SP2 knockdown and control males (Table 5b); however, there was no consistent pattern with respect to the receipt of SP. SP2 knockdown males gained lower paternity share than their controls (SP2 knockdown = 14.4%, IR2-C_2 = 33.5%), but SP1 knockdown males gained a higher paternity skew than their controls (SP1 knockdown = 42.0%, IR1-C = 30.2%, Fig. 5b). There were again significant correlations between paternity share and mating productivity (Supplementary Data 3) but no evidence for any differences between SP-transferring or lacking males.

Overall the results show no evidence for increased relative reproductive success in the free-mating conditions for males transferring SP.

Discussion

Our results show that SP results in higher absolute, but not higher relative, reproductive success for the males that transfer it. Interestingly, however, the absolute reproductive success benefits were context dependent. Benefits were clearly apparent in matings with virgin females, where SP transfer increased the number of offspring produced before remating and delayed the onset of sperm competition. However, we saw no increases in the absolute reproductive success of SP-transferring males with once mated females in the P2 experiments. This suggests that SP does not boost egg production significantly in already mated females. Our results from the free-mating assays conducted over an extended period support the idea that SP does not elevate productivity, but that it does decrease receptivity, in already mated females: we found no evidence for increased absolute progeny numbers, but did find evidence for significantly decreased remating frequency in environments with elevated SP. Consistent with this, in the extended, free-mating assays, we found signifi-

cantly increased 'per-mating' reproductive success for males. This suggests that in mated females, the benefits of SP transfer arise primarily from decreasing receptivity, which increases the per-mating share of paternity. Taken together, the results show that SP benefits males because, in matings with virgin females, it stimulates productivity and decreases receptivity, and in already mated females it decreases receptivity and hence increases per-mating paternity. We discuss these findings in more detail below.

In the two-mating experiments, SP-transferring males benefitted by increasing their absolute reproductive success in matings with virgin females by gaining on average 10–20 more offspring than SP-lacking males during the first 24 h after mating. After this period, SP^+ males gained on average ~35 extra progeny by preventing females from second matings, thus delaying the onset of sperm competition. These benefits, however, ceased once those females mated again, with a male's absolute reproductive success after a second mating being unaffected by the presence of SP. There was no evidence that the productivity benefits from matings with virgin females persisted into overall progeny fathered, as there were no correlations between the number of offspring produced in the intermating interval and the number of progeny fathered following remating (data not shown). There was also no evidence that the transfer of SP increased the relative share of paternity: there were no differences between the P1 or P2 paternity share of SP-lacking or control males. Thus, transfer of SP did not increase the efficiency of sperm use. Although the degree of paternity share was related to the overall number of progeny fathered, with more productive males gaining a higher share of paternity, these effects were independent of receipt of SP (Supplementary Data 3). We also found that, in accordance with other studies, mating duration significantly influenced the outcome of sperm competition (Parker, 1970; Arnqvist & Danielsson, 1999; Simmons, 2001; Nilsson *et al.*, 2003; A. Bretman, C. Fricke and T. Chapman, unpublished data). Taken together, these results show that the key benefits of SP comprised absolute boosts to paternity before female remating in matings with virgin females.

In the free-mating experiments, matings occurred at a relatively high frequency, resulting in any fitness benefits arising from increased productivity following SP transfer to virgin females being quickly overridden by the effects of subsequent matings. Whether there are Acps that can increase egg production above the high level seen in already mated females is not yet known. Females in groups with SP-lacking males mated at higher frequencies, and therefore received elevated levels of other Acps, some of which may have counterbalanced lower levels of SP. This is especially likely given the functional degeneracy of Acps, with seminal fluid proteins such as Acp26Aa having effects on egg laying via a different mechanism to that exploited by SP (Herndon & Wolfner, 1995; Heifetz *et al.*, 2000).

In the two-mating assays, SP-transferring males gained ~10–20 more offspring than SP-lacking males, whereas, in the free-mating experiments, SP-transferring males in the SP knockdown experiment produced two to five times as many offspring per mating per female as did SP-lacking males (Fig. 4e,f). Estimates from our previous data (Chapman *et al.*, 1995) show that up to 200 offspring can potentially be lost to a female through direct costs of mating over her lifetime under optimal conditions (although Acps other than SP probably also contribute to this direct cost of mating in females). The benefits of SP to males hence seem rather low compared with the high fitness costs to females. It would be interesting in future work to address theoretically the magnitude of the per-mating benefits to males of SP transfer that are necessary in order for selection to favour the evolution of SP despite the lifetime mating costs to females caused by it.

The significant per-mating benefits of SP transfer that we identified suggest that such benefits of SP for any particular male are relatively short lived. This is important in the context of SP as a target of selection arising from sexual conflict because it exemplifies the lack of shared interest of mating partners in future reproductive events. A male can gain a short-term benefit from transferring SP in terms of increased absolute numbers of progeny fathered before the female remates, but he has no interest in her future reproductive prospects beyond that remating. Therefore, if there are costs to the female of SP transfer that reduce her longer term reproductive prospects, it is of little selective consequence to the current mating male. The results are therefore in general consistent with a role for SP in mediating sexual conflict. Our study, however, focuses on the effects of SP only, and the temporal benefits for other Acps, for example those which potentially increase paternity share following remating (Fiumera *et al.*, 2005, 2007) might occur over the longer term. As yet, the identity of the full set of Acps subject to selection arising from sexual conflict is not clear and it will be interesting in the future to compare the nature of the benefits for Acps subject to contrasting forms of selection.

Our study shows the importance of using different genetic reagents and different independent lines of mutant strains and control lines. Although we found consistent benefits of SP transfer across both types of SP-lacking males and all experimental designs, there were inconsistencies. For example, the results from the SP2 knockdown experiments showed variability between the inverted repeat (IR2-C) and driver controls (DR-C). This could partly be due to the low mating rate of the IR2-C males (Supplementary Data 2), possibly because of their paler eye colour. The degree of expression of the w^+ eye colour marker might affect male mating frequency directly through reduced visual perception, or could affect the willingness of females to mate with such males, by raising the courtship threshold necessary for matings

with IR2-C males to occur. Our results show that mating rate differences can therefore occur even when otherwise controlling for genetic background; hence, real caution should be applied to observations of phenotypes made with single mutant lines and that consistency across multiple lines of evidence may be required in similar studies.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Data 1 Egg–adult viability of eggs laid by females mated to SP-lacking and control males.

Data 2 Premating reproductive success of SP-lacking, control and *sparkling* competitor males.

Data 3 Correlations between paternity share and mating productivity.

Table S1 Premating reproductive success for SP-lacking, control and spa competitor males. Courtship frequency is the number of observed courtship attempts/number of observations and mating frequency is the number of matings/number of observation days. The number of all courtship attempts observed (A) and total number of matings observed (C) are given as well as the opportunities not taken. (B) courtship observations (D) observation days without matings.

Table S2 Correlations between the progeny productivity of a mating productivity and paternity share.

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