The *Drosophila* seminal Sex Peptide can associate with rival as well as own sperm and provide function for SP in polyandrous females

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Short title: Seminal sex-peptide binds to and benefits rival male's sperm

Abstract

In populations in which females tend to mate with more than one male, sperm competition and cryptic female choice can occur, triggering biases in sperm use and influencing males' paternity share outcome of the mating. This competition occurs in the context of molecules and cells of male and female working interdependently towards the common goal of optimal fertilization. For example, a male's seminal fluid molecules modify the female's physiology to increase reproductive success. However, since some of these modifications induce long-term changes in female physiology, this can indirectly benefit rival males. Indeed, rival males can tailor their ejaculates accordingly, minimizing the energy cost of mating. Here we investigate the direct benefits that seminal fluid proteins from an ejaculate of one male can confer to sperm of a rival. We report that Sex Peptide (SP) that a female receives from one male can bind to sperm from a prior mate, that were already stored in the female. Moreover, the second male's SP can restore fertility and facilitate efficient sperm release or utilization of sperm received from the first male that had been stored in the female. Thus, SP from one male can directly benefit another and as such is a key molecular component in the process of inter-ejaculate interaction.

Keywords: Sperm competition, *Drosophila*, Seminal fluid protein, Fertility, Copulation complementation

49 ***Introduction***

50 In many animal species, females mate with more than one male. This polyandry lays the
51 foundation for sperm competition, in which ejaculates from rival males compete for
52 fertilization opportunities [1,2]. These conflicts and associated cryptic female choice can
53 drive the evolution of male traits including optimal sperm numbers, morphology, and
54 seminal protein sequences [3–5].

55 Against the backdrop of these conflicts, male and female molecules and/or cells must
56 also work together to ensure reproductive success. How efficiently sperm interact with
57 the egg and instigate successful fertilization or embryo support (where relevant) is key
58 to successful fertility. Accordingly, males have evolved molecular mechanisms to trigger
59 physiological changes in females that increase the reproductive success of the mating
60 pair. Seminal fluid proteins (SFPs) are crucial regulators of these changes. SFPs are
61 produced within glandular tissues in the male reproductive tract and are transferred to
62 females along with sperm during mating [6–11]. Within a mated female, SFPs mediate
63 an array of post-mating responses such as, in insects, changes in egg production,
64 elevated feeding rates, higher activity or reduced sleep levels, long-term memory,
65 activation of the immune system and reduced sexual receptivity [12–18].

66 The ability of a male's SFPs to induce long-term changes in the mated female enhances
67 that male's reproductive success. For example, the seminal Sex Peptide (SP) of male
68 *Drosophila* binds to his sperm stored in the female, persisting there for approximately
69 ten days [19]. This binding of SP to sperm is aided by the action of a network of other
70 SFPs- "LTR-SFPs" [10,20,21]. The active region of SP is then gradually cleaved from
71 sperm in storage, dosing the females to maintain high rates of egg laying, decreased
72 receptivity to remating [19], increased food intake, and slower intestinal transit of the
73 digested food to facilitate maximum absorption and production of concentrated faeces
74 [12,22–24]. However, induction of these changes can also indirectly benefit his rival, as

75 the female's physiology will have already been primed for reproduction by her first
76 mate's SFPs. Such indirect benefits to the second male have been suggested to explain
77 the tailoring of the ejaculate by males that mate with previously mated females [11,25–
78 27]. For example, the *Drosophila* seminal protein ovulin increases the number of
79 synapses that the female's Tdc2 (octopaminergic) neurons make on the musculature of
80 the oviduct above the amount seen in unmated females [28]. This is thought to sustain
81 high octopaminergic (OA) signalling on the oviduct musculature of mated female,
82 allowing increased ovulation to persist in mated female, even after ovulin is no longer
83 detectable in the female. Therefore, males mating with previously mated females need
84 transfer less ovulin than males mated to virgin females, presumably because it may be
85 less necessary, as they benefit from the ovulation stimulating effect of ovulin from the
86 prior mating. In another example, prior receipt of Acp36DE can rescue sperm storage of
87 a male that lacks this SFP [29,30].

88 The benefits to the second male described above are indirect consequences of the first
89 male's SFPs effect on female's physiology. The second male is thus the lucky beneficiary
90 of the first male's SFPs effect on the female. However, it is unknown whether a male
91 could directly benefit from a rival's SFPs, for example, whether the latter could associate
92 with and improve the success of another male's sperm. There was some suggestion that
93 this might occur from the phenomenon of "copulation complementation"[31] in which a
94 female *Drosophila* singly-mated to a male lacking SFPs did not produce progeny unless
95 she was remated to a male who provided SFPs. This suggested that something from the
96 second mating allowed the first male's sperm to be used. However, the molecular basis
97 for this phenomenon was unknown. Also suggestive that a male could benefit from a
98 rival's SFPs, Avila et al [6] reported that *SP*-null males were better at defensive sperm
99 competition than controls: mates of SP null males sired significantly more progeny
100 (higher P1) than mates of control males when females were mated with the *SP*-null, or

control male, and then with a wild type (wt) competitor. The higher P1 of *SP*-null males in this situation occurred because their mates had retained more of their sperm than of control males, due to the requirement for SP for release of sperm from storage, and then the release and use of those sperm when the females received ejaculate from the second, wt male.

Here, we report that SP, a *Drosophila* SFP received from a second male can bind to a prior male's SP-deficient sperm and restore his fertility, including sperm release from storage and changes in the female's behavior. Our results reveal direct benefits that previously stored sperm from the first (or prior) male can receive from the second (or last) male's ejaculate during the course of successive matings. Our results also establish SP as a crucial long-term molecule that facilitates this inter-ejaculate interaction, and SP-sperm binding as the molecular mechanism that underlies copulation complementation in *Drosophila*. We discuss these findings in relation to sperm competition and the possibility of copulation complementation in nature.

Results:

1. Sex peptide from one male can associate with sperm from another

In matings with wt males, SP binds to sperm that it enters the female with. However, we wondered if sperm stored by mates of *SP*-null males, that lack bound SP, could become decorated with SP from a second male even if he did not provide sperm. If so, this would mean that SP from a second (spermless) male can bind to sperm from a prior male, already present in female tract (Fig 1. Cartoon).

To test whether SP from a second male can bind to SP-deficient sperm stored by mates of *SP*-null males, we first confirmed that no SP was detectable on sperm stored in females that had singly-mated to *SP*-null males (Fig 1A). We then examined whether SP was detected on such sperm if the female subsequently remated to a spermless male (who provided SP). We observed SP bound to the stored sperm following such

127 rematings at either 1d (Fig 1B) or 4d (Fig 1C) after the original SP-less mating. We
128 confirmed these findings with western blotting. Sperm stored in seminal receptacles of
129 females that had mated to *SP*-null males and subsequently remated to spermless males
130 were dissected and probed for the presence of SP. Consistent with our immunostaining
131 data, SP was detected in samples of *SP*-null male's sperm from females that had remated
132 to spermless males at 1d or 4d after the start of first mating (ASFM; Fig 1D, lanes 7 and
133 8). Thus, SP from a second male can bind to SP-deficient sperm stored from a prior male.
134 To see if the mating order was important, we carried out the reciprocal cross, i.e. testing
135 if SP deposited by a first male (spermless, in this scheme) could bind to sperm that were
136 subsequently introduced by a second (*SP*-null) male (Fig 2. Cartoon). Spermless males
137 transfer SP to the female tract after mating [32], but we did not detect any SP in females
138 mated to spermless males by 1d after the start of mating (ASM; Fig 1D. lane 4). We saw
139 no SP signal in sperm samples isolated from females that had mated to spermless males,
140 and then subsequently to *SP*-null males at 1d ASFM (Fig 1D. lane 5). Our
141 immunofluorescence data were consistent with our western blots: we saw no SP-sperm
142 binding in females that mated first with a spermless male and a day later with *SP*-null
143 male (Fig 2B). Therefore, if SP entered the female without sperm, it was unavailable to
144 bind to sperm from a subsequent SP-deficient male.
145 We hypothesized that we did not see SP bound to sperm in this second (reciprocal)
146 crossing scheme because by the time of the second mating SP from the spermless male
147 was no longer present in the female at 1d ASFM, since it could not be retained without
148 binding to sperm [19] and no sperm were being supplied by these first males. To
149 circumvent this, we attempted to remate females that had previously mated to
150 spermless males as soon as 3-6hrs ASFM. However, few females remated, likely due to
151 the recent experience of copulation, or to the effects of pheromones from the previous
152 mating [33,34]. In the few females that did remate, no SP-sperm binding was observed
153 (Fig S1). Since the simplest explanation for these results was that SP transferred without

154 sperm had disappeared from females by the time of the second mating, we performed
155 western blotting to determine how long SP persists in the reproductive tract of females
156 in absence of sperm. Females mated to spermless males were flash frozen at 0'(min)
157 immediately after mating, 35'(min), 1hr, and 3hr ASM and their bursa (B) and seminal
158 receptacle (SR) were dissected and probed for the presence of SP. We detected SP in the
159 bursa protein samples at 0'(min) after mating, 35'(min), and 1hr ASM. (Fig 2D. lanes 5, 7,
160 9). However, SP was undetected in bursa or seminal receptacles of females at 3hr ASM
161 (Fig 2D. lane 11, 12). Thus, we could not determine whether SP from mating with a
162 spermless male could bind a second male's sperm, because SP received from the first
163 mating was lost from the female reproductive tract before a second mating could occur.
164 Xue and Noll [31] reported that a similar cross (females mated first to spermless males
165 and then to *Prd* males) also gave no progeny (showed no copulation complementation)
166 which they proposed to be due to inactivation or early loss of SFPs in the absence of
167 sperm. Our results, showing that SP can bind to stored sperm from a prior male, provide
168 the molecular explanation for their observation.

169 ***2. SP from a second male restores fertility, inhibits receptivity and regulates***
170 ***optimal release of the first male's sperm from storage***

171 SP is needed for efficient sperm release and utilization from the female sperm storage
172 organs [6]. We tested whether SP from a second male could restore the use of a first
173 male's sperm. Females mated to spermless males have no progeny (Fig 3A). Females
174 singly-mated to *SP*-null males have significantly reduced numbers of progeny (Fig 3A. SE
175 of diff = 8.043; p***=<0.001) relative to females mated to control males (Fig 3A), likely
176 because lack of SP prevents increase in egg production [17,35,36] and release of sperm
177 from storage [6]. However, females mated to *SP*-null males and then remated to
178 spermless males at 1d (Fig 3B; p=0.2487) and 4d (Fig 3C; p=0.8618) ASFM had progeny
179 levels similar to those of females that had mated to control (SP^+) males and were
180 subsequently remated to spermless males at the same time points. Thus, SP from a

181 second (SOT) male could rescue the fertility defects that resulted from the lack of SP
182 from an *SP*-null first male.
183 Reducing the likelihood of mated females to remate is another crucial postmating
184 response regulated by SP [35,37]. Females that do not receive SP generally fail to exhibit
185 this reluctance, and remate readily. We tested whether SP from a second male could
186 delay the receptivity of females that had previously mated to *SP*-null males. Females
187 singly-mated to *SP*-null males or spermless males show a significantly higher tendency
188 to remate at 1d ASM (Fig 3D; p***=<0.001) or 4d ASM (Fig 3E; p***=<0.001) relative to
189 females mated to wt (CS) males (Fig 3D and 3E). In contrast, females mated to *SP*-null
190 males and then remated to spermless males at 1d ASFM (Fig 3D; p=0.43) showed
191 receptivity similar to mates of control males at 1d after the start of second mating
192 (ASSM). The effect, however, did not persist as long as after a mating to a wt male. At 4d
193 ASSM (Fig 3E; p***=<0.001) doubly-mated females exhibited higher receptivity relative
194 to females mated to wt males but lower than those mated to spermless males. This could
195 be either because less SP from the second (spermless) mating is able to bind to stored
196 sperm from the previous mating and thus SP levels have been more depleted by 4 days
197 ASSM than after a control mating where the sperm-SP enter the female together.
198 Alternatively, the active portion of SP received from a rival male, bound to first male's
199 sperm might be released from the sperm at a higher rate. We performed western
200 blotting to determine how long SP received from the second (spermless) male persists
201 in the reproductive tract of females previously mated to *SP*-null males. Females singly-
202 mated to CS males and those doubly-mated to *SP*-null males and spermless males at 1d
203 ASFM, were flash frozen at 2hr, 1d or 4d ASM/ASSM, respectively. SP signals were
204 detected in females mated to CS males at 2hr, 1d or 4d ASM (Fig 3F. lanes 3, 4, 5). SP was
205 detected in females mated to *SP*-null males and then remated to spermless males at 2hr
206 and 1d ASSM (Fig 3F. lanes 6, 7) but not (or very weakly) at 4d ASSM (Fig 3F. lane 8).
207 Taken together, our results show that SP from a second male can rescue the receptivity

208 defects that resulted from the first male's of lack of SP but that sufficient SP for such an
209 effect is not retained for as long as in a control situation (e.g. a mating with a wt male).
210 SP is also needed for release of sperm from storage within the mated female [6]. Thus,
211 females mated to *SP*-null males retain significantly more sperm in their seminal
212 receptacle at 4d ASM. To test whether SP acquired from a spermless male in a second
213 mating could also rescue this defect, we counted sperm in storage after a single mating
214 with *SP-null*; *ProtB-eGFP* males and after mates of *SP-null*; *ProtB-eGFP* males had
215 remated with spermless males. As expected, females mated to control (SP^+; *ProtB-eGFP*)
216 males had fewer sperm in their seminal receptacle (average of 192; Fig 3G and 3J)
217 relative to mates of *SP-null*; *ProtB-eGFP* males, which had significantly higher sperm
218 counts, indicating poor release of stored sperm (Fig 3H and 3J; p**=<0.01; average of
219 304 at 4d ASM). However, mates of *SP-null*; *ProtB-eGFP* males that had remated with
220 spermless males retained sperm in numbers similar to those observed in females mated
221 to control males (average of 212; Fig 3I and 3J; p=ns). We also counted sperm stored in
222 seminal receptacle of females mated to *SP-null*; *ProtB-eGFP* males at 5d ASM (Fig 3J) to
223 make sure that the evident decline in sperm counts or release of stored sperm in doubly
224 mated females (*SP-null; ProtB-eGFP* mates remated to spermless males that were frozen
225 at 5d ASFM or 4d ASSM) was not dependent on days after mating, rather on receipt of SP
226 from spermless males. Thus, SP from a second male can rescue the sperm release
227 defects of prior matings to males that lacked SP.

228 ***3. SP from a second male can bind to stored sperm from a previous male, while still***
229 ***binding strongly to his own sperm***

230 In the experiments described above SP was provided by a spermless second male, but in
231 nature females are much more likely to encounter a male who has his own sperm,
232 capable of binding his SP. To test whether SP from a male with sperm can still bind to
233 sperm from another male, we modified our experimental protocol such that females
234 were mated to *SP*-null males as described earlier, but rather than spermless males, we

235 now used *ProtB-dsRed* males [38] as the second male (Fig 4I. Cartoon). These second
236 males have a full suite of SFPs, sperm and their sperm-heads are labeled with ProtB-
237 dsRed. This allowed us to distinguish between sperm received from *SP*-null males (blue
238 heads) and those received from *ProtB-dsRed* males (red heads). Females were frozen at
239 2hrs ASSM and sperm dissected from their seminal receptacles were probed for SP. We
240 observed anti-SP staining along the entire sperm (head and tail) from *ProtB-dsRed* males
241 (Fig 4B). Sperm received from the *SP*-null males (blue heads) were also stained with
242 anti-SP along their length (head and tail; Fig 4B). Therefore, a control (wt) male with a
243 complete suite of SFPs and sperm of his own can also provide SP to bind to SP-deficient
244 sperm from another male.
245 The likelihood of finding an *SP*-null male in nature is very low. However, multiple-
246 mating has been shown to deplete SFP reserves [39], so it is possible that inter-ejaculate
247 interaction (or copulation complementation) could occur if the first male had depleted
248 his SFP reserves. To test whether this could occur, we performed a crossing scheme in
249 which we substituted multiply-mated control (CS) males with exhausted seminal
250 reserves [39] for the *SP*-null males used in Fig. 4A. We carried out western blotting to
251 determine the levels of SP in accessory glands (AG) of such multiply mated (CS) males
252 and the amount of SP in their mates at 2hr ASM. We observed relatively weak SP signals
253 in the AG of multiply-mated males (Fig S2. A, lane 4) and a very faint SP signal in females
254 mated to these males (Fig S2. A, lane 5) compared to relatively strong SP signal in virgin
255 (unmated) males and the females mated to these males (Fig S2. A, lane 2, 3 respectively).
256 Our immunofluorescence data showed no (or extremely weak) SP-sperm binding in
257 sperm dissected from the seminal receptacle of females mated to SFP-depleted males
258 (Fig S2. C). Females mated to SFP-depleted CS males were then subsequently remated at
259 4d ASFM (long enough to have lost any SP signal from their first multiply-mated, mates)
260 to *ProtB-dsRed* males. Sperm were dissected from the seminal receptacles of these
261 females at 2hrs ASSM, and probed for SP (Fig 4II. Cartoon). We did not observe any

262 detectable SP signal on sperm stored in females singly-mated to SFP-depleted CS males
263 at 4d ASM (Fig 4C). However, we observed anti-SP staining along the entire sperm (head
264 and tail) received by the doubly-mated female from the SFP-depleted CS male (blue
265 heads; Fig 4D) and *ProtB-dsRed* males (red+ blue heads; Fig 4D).
266 Thus, in a normal mating the amount of SP that a male transfers is sufficient to bind not
267 only his own sperm but also to remaining sperm from a rival. Moreover, SP from an
268 unmated control male can bind to previously stored sperm of a male that had his SFP
269 reserves depleted prior to mating with the female.
270
271 ***4. Sex peptide binding to sperm of a prior male does not require receipt of LTR- SFPs***
272 ***from the second male.***
273 SP binding to sperm requires the action of a network of other SFPs- "LTR-SFPs" [10,21].
274 Most of the known LTR-SFPs bind to sperm transiently (CG1656, CG1652, CG9997 and
275 antares) [20], while others do not bind to sperm (CG17575 or seminase) [40]; the latter
276 facilitate the localization of other LTR-SFPs, and SP, to the seminal receptacle. However,
277 no LTR-SFPs are detectable on sperm or in female RT at 1d ASM (Fig 5 & Fig S3). We
278 wondered whether LTR-SFPs were required from the second male in order to bind his
279 SP to the first male's sperm.
280 We carried out experiments similar to those previously described, in which females
281 were first mated to *SP*-null males and then remated to spermless males at 1d ASFM. We
282 froze females at 2hr ASSM and immunostained stored sperm dissected from their
283 seminal receptacles, for the presence of LTR-SFPs that had been received from second
284 (spermless) males.
285 Females mated to CS males and frozen at 2hr ASM served as positive controls for the
286 sperm-binding of LTR-SFPs, CG1656 (Fig 5A), CG1652 (Fig 5E) and CG9997 (Fig 5I).
287 Females singly mated to *SP*-null males, frozen at 2hr ASM, exhibited normal sperm-
288 binding of LTR-SFPs, CG1656 (Fig 5B), CG1652 (Fig 5F) and CG9997 (Fig 5J), confirming

289 that loss of SP affects neither the transfer nor the sperm-binding of other LTR-SFPs [20].
290 By 1d ASM, stored sperm from females singly-mated to *SP*-null males showed no signal
291 for the LTR-SFPs, CG1656 (Fig 5C), CG1652 (Fig 5G) and CG9997 (Fig 5K), as expected
292 given the transient sperm-binding of these proteins [20]. Thus, by the time these
293 females remated with spermless males (1d ASFM), all known LTR-SFPs received from
294 the first (*SP*-null) male were undetectable on sperm.
295 Interestingly, although females that mated to *SP*-null males and then to spermless males
296 showed SP signal on their sperm (as in Fig 1) at 2hrs ASSM, we detected no signal of
297 LTR-SFPs, CG1656 (Fig 5D), CG1652 (Fig 5H) and CG9997 (Fig 5L) on those sperm at
298 2hrs ASSM. This could be because LTR-SFPs from the second male could not enter the
299 sperm storage organs in the absence of sperm or, alternatively, that their binding sites
300 on sperm had been modified prior to the second mating (perhaps by the action of LTR-
301 SFPs received from the first mating) to make them incapable of binding.
302 We verified these observations by western blots. Consistent with immunofluorescence
303 data as in Fig 5A-L, LTR-SFP signals for CG1656, CG9997, CG1656 and Antares were
304 detected in sperm dissected from females mated to CS and *SP*-null males at 2hr ASM
305 (Fig 5M. lanes 3, 4). No LTR-SFP signals were detected in sperm dissected from females
306 mated to *SP*-null males at 1d ASM (Fig 5M. lane 5) or in sperm dissected from females
307 mated to *SP*-null males, remated to spermless males at 1d ASFM, and frozen 2hrs ASSM
308 (Fig. 5M. lane 7). However, as expected, SP signals were detected in sperm dissected
309 from females that mated to *SP*-null males, remated to spermless males at 1d ASFM and
310 frozen 2hrs ASSM (Fig 5M. blot probed for SP, lane 7).
311 Thus sperm no longer detectably bind new LTR-SFPs after they have bound LTR-SFPs
312 from their own (*SP*-null) male. That LTR-SFPs are needed for SP-sperm binding, and that
313 SP from spermless male binds the first male's sperm, further suggests that the first
314 male's sperm (or the female RT) had already been primed with its own LTR-SFPs during
315 storage in the female tract.

316 Unlike the four LTR-SFPs assessed above, the two other LTR-SFPs, CG17575 and
317 seminase, do not bind to sperm, yet are crucial for SFP-sperm binding. In the absence of
318 CG17575 or seminase, SP fails to bind to sperm [10,40]. To determine if these proteins
319 were required for a second male's SP binding to a first male's sperm, we first crossed
320 females to *SP*-null males and then to *CG17575*-null or *seminase*-null males at 1d ASFM
321 (Fig 6. Cartoon). In this situation, CG17575 and seminase had entered the female with
322 the first male's sperm, but by the time of the second mating, were undetectable in the
323 female (Fig S3). We examined whether in this situation SP transferred by *CG17575*-null
324 (or *seminase*-null) males would still bind to the *SP*-null sperm stored in the female. We
325 made use of ProtB-eGFP labelled *SP*-null males to differentiate between sperm received
326 from first (cyan (DAPI+ eGFP) sperm heads) and second (blue (DAPI) sperm heads)
327 males. Immunostaining and western blots for detection of SP on sperm dissected from
328 females mated to *SP-null; ProtB-eGFP* males and then remated to *seminase*-null (Fig 6. A
329 and C, lane 4) or *CG17575*-null (Fig 6. B and C, lane 5) males showed that SP received
330 from the second male bound to sperm (head and tail) received from *SP-null; ProtB-eGFP*
331 males. Sperm dissected from females singly-mated to *SP-null; ProtB-eGFP* males gave no
332 SP signal, as expected (Fig 6. D, lane 3 and E) and sperm dissected from females singly-
333 mated to *seminase*-null (Fig 6. D, lane 4 and F) or *CG17575*-null (Fig 6. D, lane 5 and G)
334 males also showed no SP-sperm binding, as expected, due to lack of the LTR-SFP.
335 Therefore, sperm no longer require even CG17575 or seminase from the second male's
336 ejaculate, after they have received the LTR-SFPs from their own (*SP*-null) male.

337 ***Discussion***

338 Ejaculate molecules, particularly the seminal fluid proteins (SFPs) that are received by
339 females during mating, play crucial roles in successful fertilization. In *Drosophila* they
340 induce striking changes in the physiology and behavior of females, instigating a wide
341 array of post mating responses [6,14,28,29,40–42]. Some of these responses persist

342 long-term, due to binding of a male's SP to his sperm and gradual release of the SP's
343 active C-terminal region [19]. This important process is mediated by a cascade of "LTR-
344 SFPs" that are needed to bind SP to sperm [10,20,21,40]. While all of the above can be
345 seen as facilitating reproductive success of the mating pair (particularly from the male's
346 perspective), SFPs also play roles in conflicts between males in species where females
347 are polyandrous. Den Boer et al [43] investigated sperm survival in monandrous and
348 polyandrous ants and bees. They observed that while seminal fluid enhanced the
349 survival of "self" sperm, it preferentially killed the sperm of rival males. In other words,
350 while SFPs worked in a cooperative interdependent way with "self" sperm, they harmed
351 rival sperm when in a situation of conflict (and cryptic female choice). Previous studies
352 have shown that males respond to threat of rivals by altering the allocation of both
353 sperm as well non-sperm components of their ejaculate (e.g for *Drosophila*: [11,25,26]).

354 Studies of SFP functions have tended to investigate how a male's SFPs can
355 promote the interests of his own (self) sperm. However, some data suggest that one
356 male's SFPs (ovulin, ACP36DE) can indirectly benefit a subsequent male within a
357 polyandrous female [27–30,44]. Here, we tested for direct effects of one male's SFPs on
358 another male's sperm and/or fertility. Specifically, we show that SP from a second male
359 can bind to and act with sperm received from a previous mating. Sperm stored in
360 females mated to *SP*-null males show no SP-sperm binding (as expected), but if these
361 mated females subsequently remate to a spermless male, his SP can bind to stored
362 sperm from the prior male. This binding of SP to the *SP*-null sperm restores his fertility
363 and proper sperm release dynamics. Even if a second male transfers sperm, he transfers
364 sufficient SP to bind to his own and rival sperm. Finally, our data suggest that the LTR-
365 SFPs (that usually assist in binding of SP to sperm) are not required from the second
366 male for the association of his SP with sperm received from the first male (who had

367 already provided LTR-SFPs). The first male's sperm appear to be sufficiently "primed"
368 by prior receipt of their own LTR-SFPs to be able to bind SP from a second male.

369 ***SP from a second male can associate with a prior male's sperm that were stored***
370 ***within the female***

371 Xue and Noll [31] reported that sperm transferred to females by *Prd* mutant males (that
372 lack the entire suite of SFPs) were capable of fertilizing a few eggs to yield progeny, but
373 only after the females were subsequently remated to spermless males. They coined the
374 term "copulation complementation" to describe this phenomenon, and proposed that
375 SFPs from the second male might interact with the first male's sperm to yield this result.
376 Consistent with the idea of copulation complementation, several reports suggested that
377 first-males that lacked particular SFPs [6,8,45–47] can have reproductive advantage in
378 terms of their higher paternity share in competitive situations; they were better
379 competitors, compared to controls, in defensive sperm competition assays [6,29,48]. A
380 simple explanation for these results, based on the findings that we report here, is that
381 the deficiency in the first male led to impaired release/use, and thus retention, of his
382 sperm, and this was complemented by receipt of the second male's SFPs, as we have
383 now shown here, for SP.

384 SP is the only SFP thus far known to persist within the *Drosophila* female (for 10-14 days
385 post-mating), eliciting long-term post mating responses through gradual release of its C-
386 terminal portion [19]. The long-term persistence of SP on sperm made it an excellent
387 candidate to examine for interaction with rival sperm. Here, we report that SP
388 subsequently received from a spermless male binds to a first male's sperm (*SP*-null).
389 This association is apparent even if the second mating occurs at 1d or as long as 4d
390 ASFM, indicating that binding of SP to the first male's sperm occurs irrespective of how
391 long sperm have been in the storage organs. It remains unclear how SP received from
392 spermless (second) male enters the sperm storage organs, where sperm from the first

393 mating had been stored. However, Manier et al [38] reported that 60-90 min after the
394 start of a second mating, 26% of the resident sperm (received from the previous mating)
395 are moved from storage back into the bursa where they mix with the second male's
396 ejaculate before moving back into storage. Therefore, it is possible that SP received from
397 the spermless male binds to the first male's sperm that relocated to the bursa, and the
398 newly SP-bound sperm are then transferred back into storage in the seminal receptacle.

399 ***The binding of SP received from one male to sperm of another can restore defects***
400 ***that resulted from lack of SP from the first male***

401 In the absence of sperm, or if SP is not bound to sperm, females do not maintain post-
402 mating responses and fail to efficiently release sperm from storage resulting in fewer
403 sperm available for fertilization and fewer progeny [6,10,17,35]. We observed that these
404 defects were restored when SP was received by females in a remating with spermless
405 males. Thus, the second male's SP bound to the first male's sperm is functional. The
406 rescue of the phenotype, however, was not as long lasting as in a normal single mating
407 with SP transfer, wearing off by 4d postmating rather than the normal ~10d. This could
408 be because only fewer sperm relocated from storage to the bursa [38], so they may not
409 carry sufficient SP back into storage to associate with *SP*-null sperm. Consistent with
410 this, the levels of SP that we see stained in these situations are lower than those in a wild
411 type mating.

412 ***An unmated male transfers sufficient SP to bind to his own as well rival sperm***

413 We did not know whether the amount of SP that is transferred during mating is more
414 than the available binding sites on sperm. Here, we observed that an unmated control
415 male does transfer enough SP to bind his own as well as pre-stored sperm (*SP*-null) in a
416 previously mated female. Consistent with our findings, several reports suggest that in
417 response to potential threats of sperm competition and conflicts, males allocate the
418 levels of SFPs and transfer more SP, yet less ovulin, to previously mated females [11,26].

419 Rubinstein et al [28] demonstrated that ovulin induces ovulation, acting through
420 octopamine (OA) neuronal signalling and increases the number of synapses that the
421 female's Tdc2 neurons make on the musculature of the oviduct. This latter effect
422 persisting could benefit rivals too, so males may thus be able to mitigate the levels of
423 ovulin in their ejaculate. But the question remains that if SP from one male's ejaculate
424 can bind to and assist another's sperm, why do males not lower the amount of SP
425 transferred while mating? A potential explanation is that a male would still benefit by
426 transferring enough SP to ensure that his own sperm remains saturated with SP, even at
427 a cost of part of his SP binding to another male's sperm.

428 SP binds to sperm through its N-terminal region, and this region remains bound to
429 sperm long-term [19]. The bound N-terminal region of SP on sperm stored in a mated
430 female does not allow any further binding of SP coming from rival male's ejaculate.
431 Therefore under what circumstances might SP-mediated copulation complementation
432 occur in nature? In polygynous males, SFPs are depleted faster than sperm [39]. This
433 could result in a situation in which a female who mated with a male with low levels of
434 SFPs might not receive enough SP to saturate his sperm. In these circumstances, SP
435 received from another male would help compensate for the lower amount of SP from the
436 depleted first male's ejaculate. SFP depletion would, of course, not only affect the levels
437 of SP, but also all the other crucial LTR-SFPs. However, while other LTR-SFPs enable SP
438 to bind sperm, it is the quantity of bound SP that correlates with the duration of post-
439 mating responses. In line with this hypothesis, we subjected a control male to recurrent
440 matings (providing six virgins) over the span of two days, with an intent to exhaust their
441 SFPs. We observed that sperm stored by subsequent (7th) females mated to these
442 multiply-mated males had undetectable SP signals. However, when these females were
443 remated to unmated control males, strong SP signals were detected on both the SFP-
444 depleted sperm received from the previous mating and the newly received rival sperm.

445 Therefore, our results support the idea that in nature males who have multiply-mated
446 might get some help from the SFPs of subsequent, less depleted, males. Interestingly,
447 this inter-ejaculate interaction might confer an added advantage to the second male.
448 More of the second male's SP will be retained in the female reproductive tract, for even
449 longer, if it binds to previously-stored sperm in addition to his own sperm. This could
450 allow the post-mating responses in polyandrous females to be maintained for longer
451 than in singly-mated females.

452 ***Association of a second male's SP to sperm received from a prior male does not***
453 ***require the receipt of LTR-SFPs from the second male***

454 Binding of SP to sperm is facilitated by a network of LTR-SFPs [10]. Two LTR-SFPs,
455 CG17575 and seminase, do not themselves bind to sperm, whereas other LTR-SFPs bind
456 sperm transiently (CG1652, CG1656, CG9997, antares). CG17575 and seminase localize
457 the other LTR-SFPs, and SP, to sperm storage organs [10,20,40,42,49]. We found that SP
458 from a second male (spermless or control) can associate with sperm from the first male
459 (*SP*-null) even if it enters the female in absence of its own LTR-SFPs. This suggested that
460 *SP*-null sperm (or the mated female RT) had already received modifications ("priming")
461 from its own LTR-SFPs that were required for SP binding. This further suggests that
462 once primed, a sperm can bind SP from a rival's ejaculate without the need for
463 additional LTR-SFPs, and can restore its own post-mating dynamics.

464 Thus, we find that a critical SFP from one male can associate and offer direct benefits to
465 sperm from another male, restoring the SP function to the previously stored sperm. Our
466 work shows that SP is a crucial candidate for copulation complementation in *Drosophila*,
467 and that sperm in storage (or the female RT) are primed for SP binding by the first
468 male's LTR-SFPs. Thus, despite potential competition between males, there could be
469 subtle cooperation between males as well. In addition, the allocation of resources by,

470 and effects on, rival males that mate to polyandrous females, should be viewed in light of
471 not only sexual conflicts, but also both direct and indirect effects of SFPs.

472 ***Materials and Methods***

473 ***1. Fly strains***

474 Spermless males, [*sons of tudor,* (*sot*) that lack sperm but produce and transfer a
475 complete suite of SFPs] were the progeny of *bw sp tud*^{1} females [50] mated to control,
476 *Canton S* (CS) males. *Sex peptide* null mutant males (*Δ325/Δ130*; which have sperm and
477 the entire suite of SFPs except for SP) [35] were generated by crossing the SP knockout
478 line (*Δ325/TM3, Sb ry*) to a line carrying a deficiency for the *SP* gene (*Δ130/TM3, Sb ry*).
479 Control males were the TM3 siblings of *SP*-null mutants. Matings were conducted with
480 wild type *D. melanogaster* females (CS). To determine sperm numbers, we generated a
481 line carrying the *SP*-null mutation and Protamine B-eGFP tagged sperm (*ProtB-eGFP/Y;*
482 *Δ325/Δ130*) by series of crosses between the *SP* knockout line (*Δ325/TM3, Sb ry*) and
483 *ProtB-eGFP (X); TM3/TM6* [38]. The *TM3* siblings of these males, (*SP*$^{+}$*; ProtB-eGFP)*
484 served as controls. Sperm-heads of these control males were tagged with ProtB-eGFP,
485 but the males had normal levels of SP (Fig S4). All flies were reared under a 12:12h light-
486 dark cycle at 22±1°C on standard yeast-glucose medium. Mating experiments were
487 carried out by single-pair mating 3-5 day old virgin CS females to 3-5 day old unmated
488 males of genotypes indicated in the text and remating the same female 1 day or 4 days
489 after the start of first mating (ASFM) to age matched unmated males of the genotypes
490 indicated in the text.

491 ***2. Crossing scheme to study first male's sperm and rival's SP binding***

492 Xue and Noll [31] reported copulation complementation in females mated to *Prd* males
493 (which produce sperm but lack SFPs) remated to spermless males (*sot*, which produce
494 SFPs). We followed a similar scheme but to focus on SP specifically, we used *SP*-null

495 males as the first male. As described in Results, we then remated these females to
496 spermless males, which make SFPs but not sperm. We attempted to do the reciprocal
497 experiment, where females were mated to spermless males and then remated to *SP*-null
498 males, but consistent with what was reported by Xue and Noll [31], we could not detect
499 copulation complementation in this direction for technical reasons: SP from the
500 spermless male did not persist long enough in the mated female to interact with the
501 second male's sperm (see Results). We carried out rematings at three time points, 3-6
502 hrs, 1d, and 4d AFSM. We assessed results at 2hr after the start of the second mating
503 (ASSM).

504 ***3. Fertility***

505 The reproductive performances of singly-mated or doubly-mated females were assayed
506 by analyzing fertility (numbers of progeny eclosed over ten days) [32]. Briefly, the
507 fertility assays were carried out with (A). "Single matings": Females were singly mated
508 to (i) spermless males, (ii) *SP*-null males, or their (iii) *TM3* siblings (genetically-matched
509 control males) in three individual sub-batches, and (B). "Rematings": Females were
510 mated to *SP*-null males or their *TM3* siblings (SP^+) and were then subsequently remated
511 to spermless males at 1d and 4d ASFM. Matings that lasted 15 mins or more were
512 considered successful. At the end of a mating, males were removed from the vials and
513 females were allowed to lay eggs for 10 days after the start of mating (ASM) in the first
514 batch and after the start of second mating (ASSM) in the second batch. Females were
515 transferred to fresh food vials every three days. Flies emerging from each vial were
516 counted. Fertility is represented as total progeny number produced by each female over
517 a period of 10 days. The differences in fertility were analyzed through One way Analysis
518 of Variance (ANOVA) followed by Tukey's multiple comparison tests for single-matings
519 and Mann Whitney U-tests for rematings. All assays were repeated more than two times

520 and comprised of two technical replicates, with each group consisting of a minimum
521 sample size of 15-20.

522 ***4. Receptivity***

523 To determine the propensity of females to remate, receptivity assays [17] were set for
524 females singly mated to *SP*-null, spermless or CS males and females mated to *SP*-null
525 males and then subsequently remated to spermless males at 1d ASFM. For the assay,
526 females from singly-mated and doubly-mated groups were then provided with (CS)
527 males at 1d and 4d ASM or ASSM, respectively. We determined the number of females
528 that mated within 1hr from when the CS male was introduced within the vial. Assays
529 were repeated more than two times, with each group consisting of a minimum sample
530 size of 15-20. The data were analyzed by Fisher exact tests and Chi-squared group
531 analyses.

532 ***5. Sperm utilization/ release from sperm storage organs in females***

533 To study the effect of first male's sperm and rival male's SP binding on sperm utilization
534 and release, we generated *SP*-null males whose sperm-heads are labelled with ProtB-
535 eGFP [38]. Females were mated to *SP-null*; *ProtB-eGFP* or SP^{+}*; ProtB-eGFP* (control)
536 males. Some of the mated females were frozen at 4d ASM (or 5d ASM) for sperm counts.
537 The remaining mates of *SP-null*; *ProtB-eGFP* males were remated to spermless males at
538 1d ASFM. These flies were flash-frozen at 4d ASSM. Subsequently, seminal receptacles of
539 females singly-mated to *SP-null; ProtB-eGFP* and SP^{+}*; ProtB-eGFP,* or doubly-mated to *SP-*
540 *null; ProtB-eGFP* and spermless males, were dissected and eGFP sperm were counted (at
541 a total magnification of 200X, with FITC filter on an Echo-Revolve microscope). Mature
542 sperm in the seminal receptacles of mated females were counted twice and groups were
543 blinded to ensure reproducibility and avoid bias. The percent accuracy was 90-94%.
544 Assays were repeated more than two times, with two technical replicates. Every group

545 contained a minimum sample size of 15-25. Differences in the sperm counts between
546 groups were analyzed statistically through One way ANOVA followed by Tukey's
547 multiple comparison tests.

548 ***6. Brood matings***

549 Control (CS) males were subjected to brood matings [51,52] to deplete SFPs, as their
550 levels are known to become exhausted at a higher rate than sperm numbers [39].
551 Briefly, three day old control males were mated to CS females in two broods (each
552 consisting of three virgin females) over two days. The first mating of both broods was
553 observed. On the third day, previously mated females were removed and the male was
554 provided with an additional virgin female (7th mate), matings were observed and
555 depleted CS males were removed. Half of the 7th mated females were frozen at 4d ASM,
556 while the others were subsequently remated to control (*ProtB-dsRed*) males at 4d ASFM,
557 and then frozen at 2hr ASSM. Sperm stored in the seminal receptacle of the frozen flies
558 were dissected and immunostained for SP.

559 **7. *Immunofluorescence***

560 Immunofluorescence was performed to detect SP-sperm binding [10,19,20]. Sperm
561 dissected from seminal receptacles of experimental or control females were attached to
562 poly-L-Lysine (Sigma) coated slides. Sample processing was carried out according to the
563 protocol of Ravi Ram and Wolfner [10] with minor modifications. Samples were blocked
564 with 5% bovine serum albumin, BSA in 0.1% PBX for 30min. Subsequently, samples
565 were incubated overnight in rabbit anti-SP(1:200), CG1656(1:100), CG1652(1:50),
566 CG9997(1:50) [20], in 0.1%BSA at 4°C overnight. Samples were then washed in PBS and
567 incubated at room temperature for 2h in mouse anti-rabbit IgG coupled to alexa fluor
568 488 (green) or 594 (red; Invitrogen) at a concentration of 1:300 in 1xPBS at room
569 temperature in the dark. Samples were then washed in PBS, incubated in 0.01% DAPI

570 for 3 min at room temperature in the dark, rewashed and mounted using antifade (0.2%
571 N-propyl gallate in 75% glycerol; Sigma). The fluorescence was visualized under an
572 Echo-Revolve fluorescence microscope at a magnification of 200X. A minimum of three
573 independent immunostaining batches, with a minimum sample size of 10, were
574 analyzed for each group.

575 ***8. Sample preparation and Western blotting***

576 To further examine transfer, persistence or binding of SP to sperm stored in singly-
577 mated or doubly-mated females, the lower reproductive tract (RT) or sperm stored (SS)
578 in seminal receptacles of mated female were dissected. The dissected tissues (lower RT,
579 n=5-10 or sperm, n =20-30) were suspended in 5µl of homogenization buffer (5% 1M
580 Tris; pH 6.8, 2% 0.5M EDTA) and processed further according to the protocol of Ravi
581 Ram and Wolfner [10]. Proteins from stored sperm or lower female reproductive tract
582 were then resolved on 12% polyacrylamide SDS gel and processed further for western
583 blotting. Affinity purified rabbit antibodies against SP(1:2000), CG1656(1:1000),
584 CG1652(1:500), antares(1:500), CG9997(1:1000), CG17575(1:1000), seminase(1:1000)
585 [10,20,40] and mouse antibody against actin (as a loading control; Millipore Corp., cat
586 no. #MAB1501MI at 1:3000) were used as primary antibodies. HRP conjugated
587 secondary anti-rabbit and anti-mouse antibodies (Jackson Research) were used for
588 detection of SFPs at a concentration of 1:2000.

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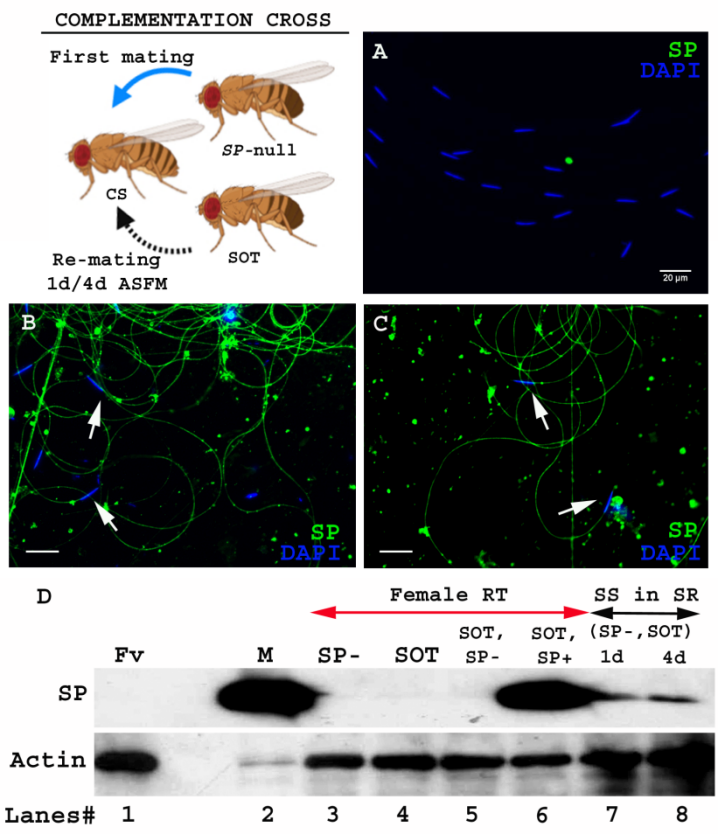
593 ***Author contributions***

S.M. and M.F.W. designed the experiments; S.M. carried out the experiments; S.M. and M.F.W analyzed the results. S.M. and M.F.W. wrote and revised the manuscript.

Conflict of interest statement

The authors declare no conflict of interests.



616

617 **Figure 1.** ***SP from a second male can bind to SP deficient sperm of previous male***
618 ***stored within a mated female.***

619 **Cartoon**: Pictorial representation of the crossing scheme (fly images from Biorender).
620 Wild type (CS) females were first mated to an *SP*-null male and then, at the indicated
621 time, to a spermless (*sot*) male. Sperm heads were stained with DAPI (blue) and SP
622 visualized with Alexa fluor 488, staining the sperm tail (green) and sperm head (cyan;
623 overlapping blue/green). **(A)** Sperm from females singly mated to *SP*-null males, 1d
624 ASM. **(B)** Sperm from females mated to *SP*-null males, remated to spermless males at 1d
625 ASFM and **(C)** at 4d ASFM, both frozen 2h ASSM. White arrows indicate sperm heads.
626 Bar=20µm **(D)** Western blot lanes# **1:** Fv, reproductive tract (RT) of virgin female
627 (negative control; n=5), **2:** M, a pair of male accessory gland (positive control; n=1), **3:**
628 SP-, reproductive tracts of females mated to *SP*-null males, 2h ASM (n=5), **4:** SOT,
629 reproductive tracts of females mated to spermless males, 1d ASM (n=5), **5:** SOT, SP-,
630 reproductive tract of females mated to spermless males and then remated to *SP*-null
631 males, 1d ASFM (n=8 RT), **6:** SOT, SP+, reproductive tract of females mated to spermless
632 males and then remated to control (SP+) males at 1d ASFM, frozen 2h ASSM (positive
633 control; n=8 RT), **7:** (SP-, SOT), 1d and **8:** (SP-, SOT), 4d sperm isolated from the seminal
634 receptacle of females mated to *SP*-null males and then remated to spermless males at 1d
635 ASFM and 4d ASFM, frozen 2h ASSM (n=15 SS). Actin served as loading control.

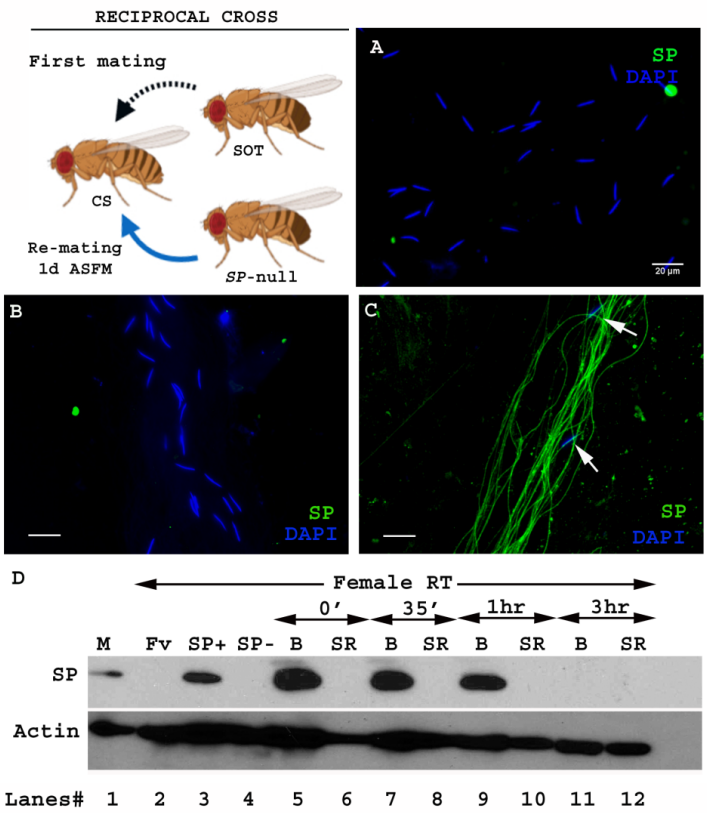


Figure 2. ***Sperm from second male are not bound to SP from a prior spermless male.*** **(Cartoon):** Pictorial representation of cross (fly images from Biorender), that is reciprocal of that in Fig 1. Females mated first with spermless (*sot*) male and then a day later with *SP*-null male that provided sperm. Sperm heads were stained with DAPI (blue) and SP visualized with Alexa fluor 488, staining the sperm tail (green) and sperm head (cyan; overlapping blue/green). **(A)** Sperm from females singly mated to *SP*-null males, 2hr ASM. **(B)** Sperm from females mated to spermless males and then remated to *SP*-null males, 1d ASFM. **(C)** Sperm from females mated to spermless males and then remated to SP+ males, 1d ASFM, serve as positive controls. Flies were frozen 2h ASSM. White arrows indicate sperm heads. Bar=20µm **(D)** Western blot lanes# **1:** M, a pair of male accessory gland (positive control; n=1), **2:** Fv, reproductive tract (RT) of virgin female (negative control; n=5), **3:** SP+, reproductive tract of females mated to control males (TM3 siblings of *SP*-null males; n=5; positive control), **4:** SP-, reproductive tract of females mated to *SP*-null males (n=5; negative control). **5-12:** Proteins from Bursa (B) or seminal receptacle (SR) from females mated to spermless males frozen at 0'(min) immediately after mating, 35'(min), 1hr, and 3hr ASM, respectively (n=15). Actin served as loading control.

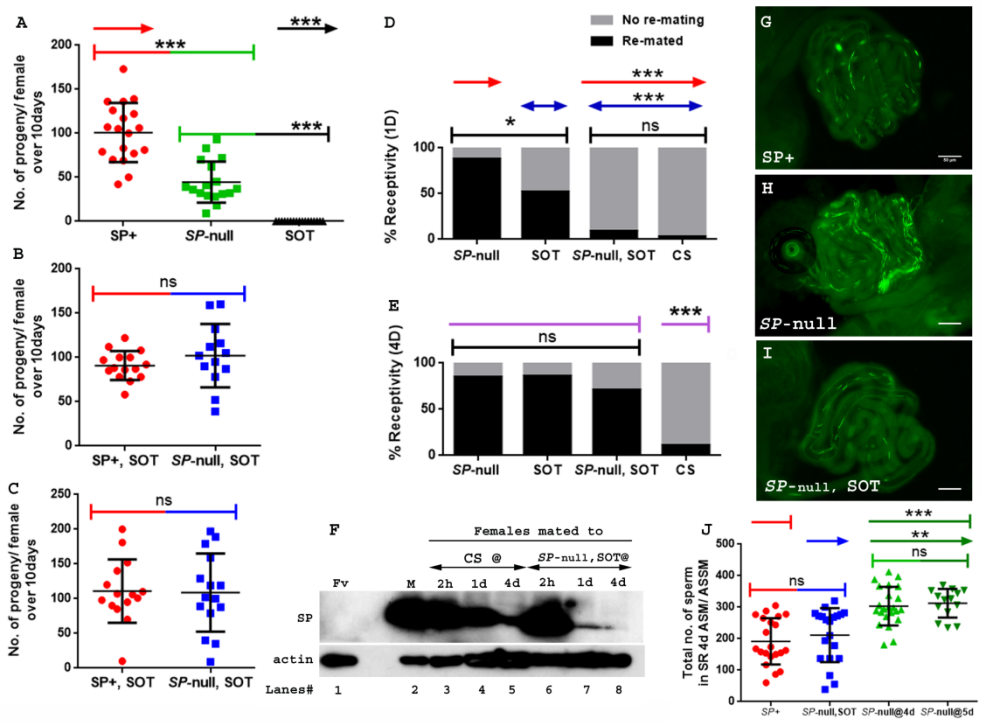


Figure 3. ***Remating with spermless males restores fertility, delays receptivity and optimizes efficient sperm release in females that previously mated to SP-null males***
(A) Graphical representation of numbers of progeny produced by each female over the span of ten days, following mating to control (TM3 siblings of *SP*-null males: SP+; red), *SP*-null males (*SP*-null; green), or spermless males (SOT), p***=<0.001; n=15-20. **(B)** Fertility of females mated to *SP*-null males and then remated to spermless males at 1d ASFM (*SP*-null, SOT; blue, n=15-20) and **(C)** Fertility of females mated to *SP*-null males and then remated to spermless males at 4d ASFM (*SP*-null, SOT; blue, n=15-20) compared to females mated to control males and then remated to spermless males (SP+, SOT, red, ns=non significant). **(D)** Percentage receptivity of females mated to *SP*-null males and then remated to spermless males (*SP*-null, SOT) at 1d ASFM, when compared to females singly mated to *SP*-null males (red arrows), spermless (SOT, blue arrows) or CS males, 1d ASM (p*=<0.05; p***=<0.001; n=15-20). **(E)** Percentage receptivity of females mated to *SP*-null males and then remated to spermless males (*SP*-null, SOT) at 4d ASFM, when compared to females singly mated to *SP*-null males, spermless (SOT) or CS males (purple arrows), 4d ASM (p***=<0.001; n=15-20). **(F)** Western blot lanes# **1:** Fv, reproductive tract (RT) of 5 virgin females (negative control) **2:**, M, a pair of male accessory gland (positive control), **3,4,5:** RT of females mated to CS males, flash frozen at 2hrs (n=5), 1d (n=15) and 4d (n=15) ASM respectively, **6,7,8**: RT of females mated to *SP*-null males and then subsequently mated to spermless males at 1d ASFM, flash frozen 2hrs (n=5), 1d (n=15) and 4d (n=15) ASSM, respectively. Actin served as loading control. **(G)** Sperm in the seminal receptacle (SR) of a typical females mated to a control male (*SP+; ProtB-eGFP*) at 4d ASM. **(H)** Sperm in the SR of a typical female mated to *SP-null ;ProtB-eGFP* male at 4d ASM. **(I)** Sperm in the SR of a typical female, mated to *SP-null; ProtB-eGFP* and subsequently remated to a spermless male at 1d ASFM, and frozen at 4d ASSM. In G-I sperm heads are green due to eGFP. Bar=50µm. **(J)** Graphical representation of sperm counts in SRs of females singly-mated to control (SP+, red, TM3 siblings of *SP-null; ProtB-eGFP*), *SP*-null (green) or doubly-mated to *SP*-null and spermless male (*SP*-null, SOT, blue) represented in G, H, I panels (p**=<0.01; p*=<0.05; ns=non significant; n=15-20).

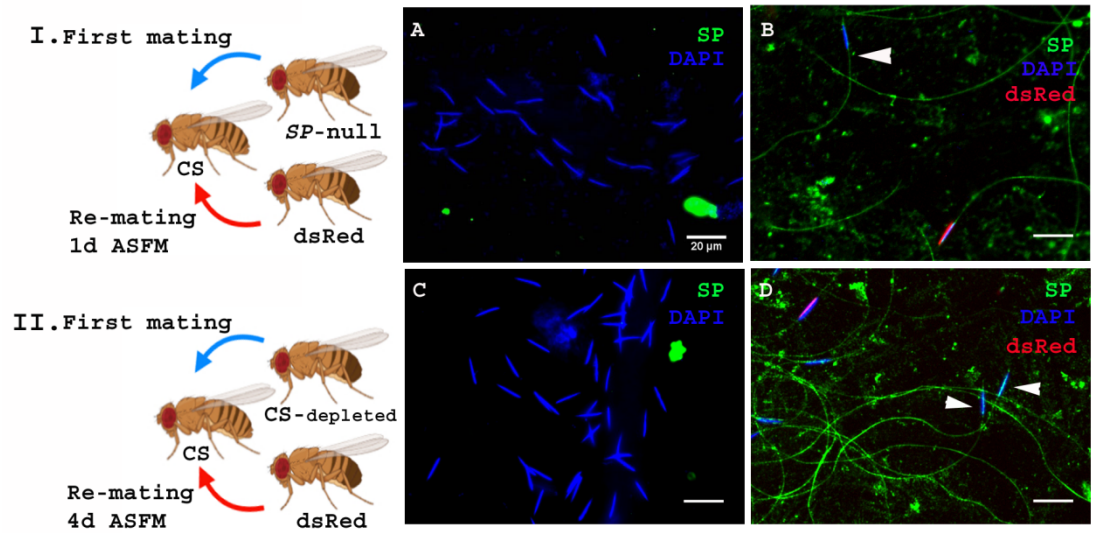


Figure 4. ***SP from a male who also provides sperm can bind to SP-deficient sperm as well as to the donor's sperm.*** **Cartoon (I):** Pictorial representation of the experimental cross (fly images from Biorender). Females mated to *SP*-null males were remated to control (*ProtB-dsRed*) males at 1d ASFM. **(A)** Sperm from females singly mated to *SP*-null males, 2hr ASM (blue sperm-head). **(B)** Sperm from females mated to *SP*-null males (blue sperm-head) remated to *ProtB-dsRed* (red+ blue sperm-head) males at 1d ASFM. SP visualized with Alexa fluor 488, staining the sperm (head+ tail; green). Flies were frozen 2h ASSM. White arrows indicate sperm heads (n=10; Bar = 20µm). **Cartoon (II):** Pictorial representation of the substitute cross (fly images from Biorender). Females mated to SFP depleted control (CS) males were remated to control (*Prot B-dsRed*) males at 4d ASFM. **(C)** Sperm from females singly-mated to SFP depleted CS males at 4d ASM (blue sperm-head). **(D)** Sperm from females mated to SFP depleted CS males (blue sperm-head), remated to *ProtB-dsRed* (red+ blue sperm-head) males at 4d ASFM. SP visualized with Alexa fluor 488, staining the sperm (head+ tail; green). Flies were frozen 2h ASSM. White arrows indicate sperm heads (n=10; Bar = 20µm).

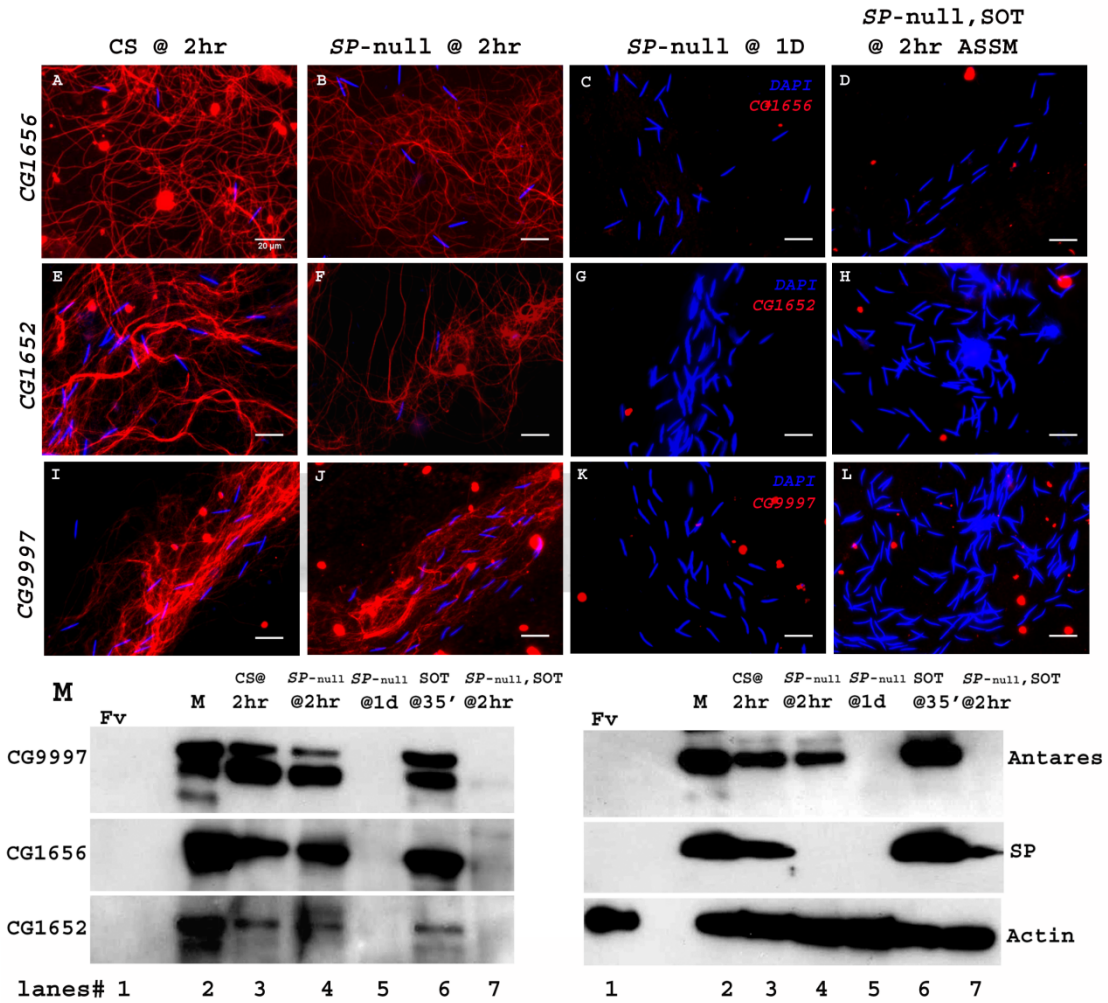


Figure 5. ***Sperm do not bind detectable LTR-SFPs from a second male.*** Females mated to wild type (CS) males at 2hr ASM show LTR-SFPs bound to sperm, CG1656 **(A)**, CG1652 **(E)**, CG9997 **(I)**. Females mated to *SP*-null males show the same **(B,F,J)** but by 1d postmating LTR-SFPs' signal were no longer detected on sperm **(C,G,K)** confirming previous reports (please see [20]). Females mated to *SP*-null males and then remated to spermless males also do not show detectable signal for sperm-LTR-SFP binding for CG1656 **(D)**, CG1652 **(H)** and CG9997 **(L)**, 2hrs ASSM, although they have SP bound (Fig 1). Sperm stained for the indicated LTR-SFP detected with Alexa fluor 594 (red) and sperm-head stained with DAPI (blue). Bar=20μm **(M)** Western blot probed for indicated LTR-SFPs. Lanes/samples are, **1:** Fv, reproductive tract (RT) of 3 virgin females (negative control), **2:** M, 1 pair of male accessory glands (positive control), **3:** CS@2hr, sperm dissected from SR of 20 females mated to wild type (CS) males at 2hr ASM, **4:** *SP*-null @ 2hr, sperm dissected from SR of 20 females mated to *SP*-null males at 2hr ASM, **5:** *SP*-null @1d, sperm dissected from SR of 20 females mated to *SP*-null males at 1d ASM, **6:** SOT@35', reproductive tract of 3 females mated to spermless males at 35'ASM (positive control), **7:** *SP*-null, SOT @ 2hr, sperm dissected from SR of 20 females mated to *SP*-null males and then remated to spermless males at 1d ASFM, and frozen at 2hr ASSM. Lanes were probed for LTR-SFPs CG9997, CG1656, antares and CG1652 and SP as described in the text. Actin served as loading control.

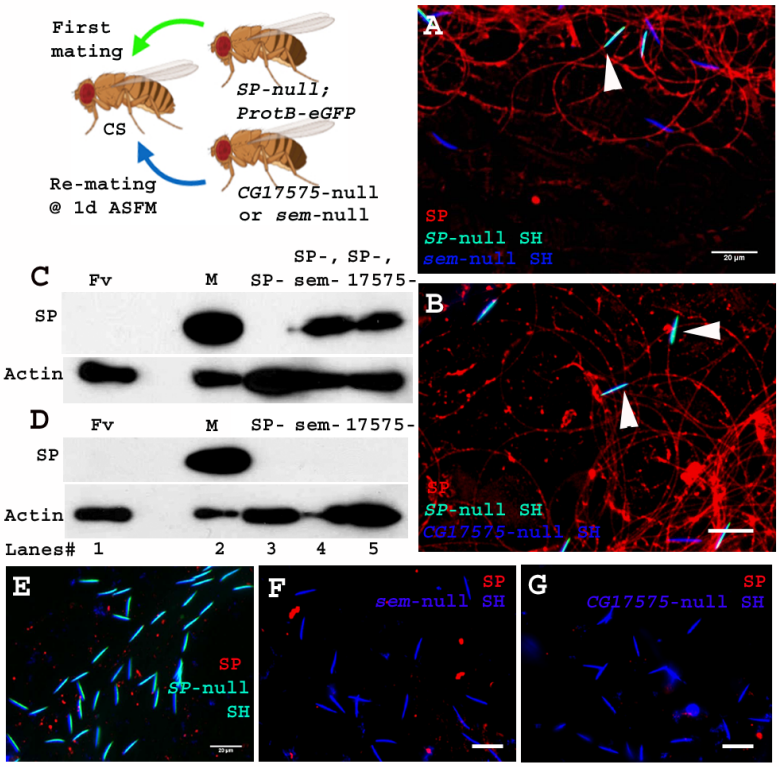
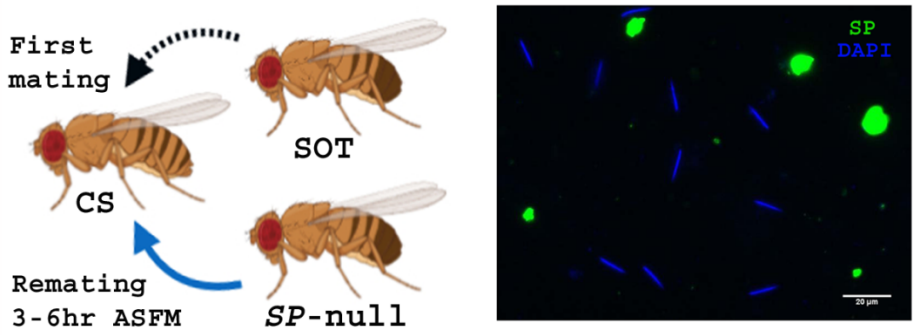


Figure 6. ***Sperm received from SP-null males do not require CG17575 or seminase from a second male to bind SP from that male.*** **Cartoon:** Pictorial representation of the experimental cross (fly images from Biorender). Females mated first with *SP-null; ProtB-eGFP* male [cyan sperm-head; DAPI(blue)+eGFP(green)] and then a day later with *CG17575*-null or *seminase*-null male (blue sperm-head; DAPI stained) and frozen, 2hrs ASSM. SP was visualized with Alexa fluor 594, staining the sperm (head+ tail) red. **(A)** Sperm from females mated to *SP-null; ProtB-eGFP* males and then remated to *seminase*-null males, 1d ASFM. **(B)** Sperm from females mated to *SP-null; ProtB-eGFP* males and then remated to *CG17575*-null males, 1d ASFM. **(C)** Western blot probed for SP. Lanes/samples are, **1:** Fv, reproductive tract (RT) of 3 virgin females (negative control), **2:** M, 1 pair of male accessory glands (positive control), **3:** SP-, sperm dissected from 20 females mated to *SP-null; ProtB-eGFP* males at 2hr ASM, **4:** SP-, sem-, sperm dissected from 20 females mated to *SP-null; ProtB-eGFP* males and subsequently to *seminase*-null males at 1d ASFM, frozen at 2hrs ASSM, **5:** SP-, 17575-, sperm dissected from 20 females mated to *SP-null; ProtB-eGFP* males and subsequently to *CG17575*-null males at 1d ASFM, frozen at 2hrs ASSM. **(D)** Western blot probed for SP. Lanes/samples are, **1:** Fv, reproductive tract (RT) of 3 virgin females (negative control), **2:** M, 1 pair of male accessory glands (positive control), **3:** SP-, sperm dissected from 20 females mated to *SP-null; ProtB-eGFP* males at 2hr ASM, **4:** sem-, sperm dissected from 20 females mated to *seminase*-null males at 2hr ASM **5:** 17575-, sperm dissected from 20 females mated to *CG17575*-null males at 2hr ASM. Actin served as loading control. **(E)** Sperm isolated from females singly mated to *SP-null; ProtB-eGFP* males, 2hr ASM. **(F)** Sperm isolated from females singly mated to *seminase*-null male, 2hr ASM. **(G)** Sperm isolated from females singly mated to *CG17575*-null male, 2hr ASM. White arrows indicate sperm heads (represented as SH, n=10; Bar=20μm).

752 ***Supplementary figures***



753

754 **Fig S1. Cartoon:** Pictorial representation of cross (fly images from Biorender). Females
755 mated first with spermless (*sot*) male and then 3-6 hr ASFM with *SP*-null male that
756 provided sperm. **Panel**: Sperm from SR of females mated to spermless males and then
757 remated to *SP*-null males, 3-6hrs ASFM, frozen at 2hrs ASSM. Sperm heads were stained
758 with DAPI (blue) and SP (green) probed with Alexa fluor 488 (n=5; Bar=20μm).

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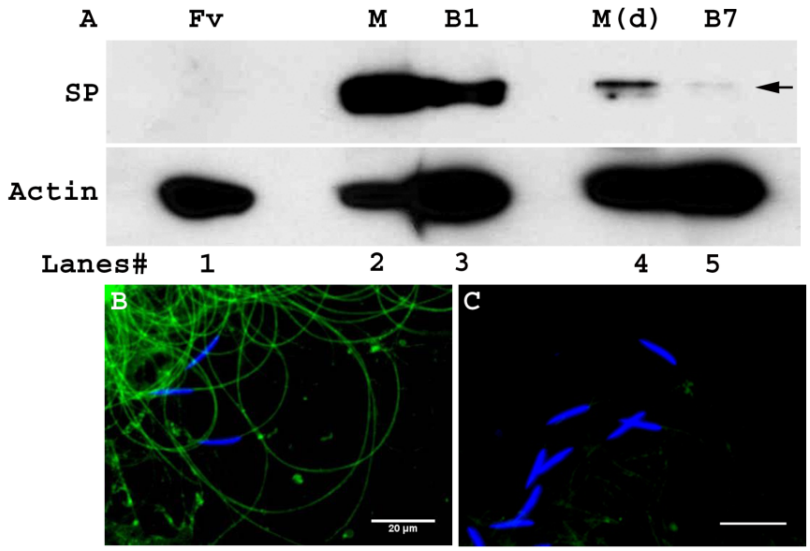
760



761

762 **Fig S2: (A)** Western blot probed for SP. Lanes/samples are, **1:** Fv, reproductive tract
763 (RT) of 2 virgin females (negative control), **2:** M, 1 pair of male accessory glands from a
764 3 day old unmated virgin male, **3:** B1, RT of 4 females mated to control unmated virgin
765 males, frozen at 2hr ASM, **4:** M(d), 1 pair of male accessory glands dissected from a
766 multiply mated male (previously mated with six virgin females), **5:** B7, RT of 4 females
767 mated to multiply-mated males, frozen at 2hr ASM. Actin served as loading control. **(B)**
768 Sperm dissected from females mated to unmated males, frozen at 2hrs ASM. **(C)** Sperm
769 dissected from females mated to multiply mated males, frozen at 2hrs ASM. Sperm
770 heads were stained with DAPI (blue) and presence of SP (green) detected with Alexa
771 fluor 488 (n=5; Bar=20μm).

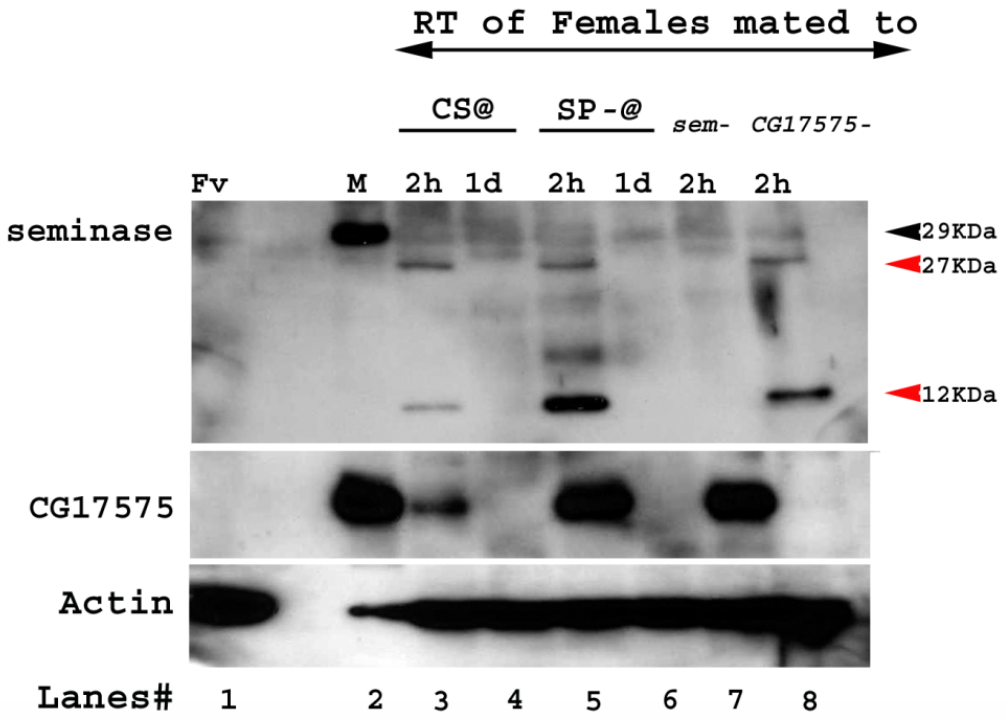


Fig S3: Western blot probed for seminase and CG17575. Lanes/samples are, **1:** Fv, reproductive tract (RT) of 3 virgin females (negative control), **2:** M, 1 pair of male accessory glands (positive control), **3-4:** RT of 5 females mated to wild type (CS) males at 2hr and 1d ASM, respectively, **5-6:** RT of 5 females mated to *SP-null; ProtB-eGFP* males at 2hr and 1d ASM, respectively **7:** RT of 5 females mated to *seminase*-null males at 2hr ASM **8:** RT of 5 females mated to *CG17575*-null males at 2hr ASM. Black arrows indicate full length seminase, red arrows indicate the cleavage products of seminase, post-mating in the female RT. Actin served as loading control.

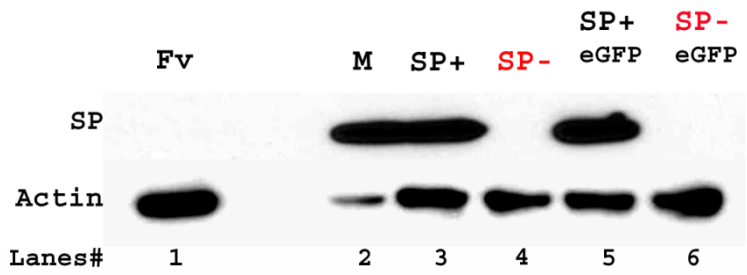


Fig S4: Western blot probed for SP. Lanes/samples are, **1:** Fv, reproductive tract (RT) of 3 virgin females (negative control), **2:** M, 1 pair of male accessory glands (positive control), **3:** SP+, RT of 3 females mated to control (TM3 siblings of *SP*-null males; positive control) males at 2hr ASM, **4:** SP-, RT of 3 females mated to *SP*-null males at 2hr ASM, **5:** SP+ eGFP, RT of 3 females mated to control (TM3 siblings of *SP-null; ProtB-eGFP* males; positive control) males at 2hr ASM **6:** SP- eGFP, RT of 3 females mated to *SP-null; ProtB-eGFP* males at 2hr ASM. Actin served as loading control.

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