RESEARCH ARTICLE

Figures and figure supplements

Drosophila seminal sex peptide associates with rival as well as own sperm, providing SP function in polyandrous females

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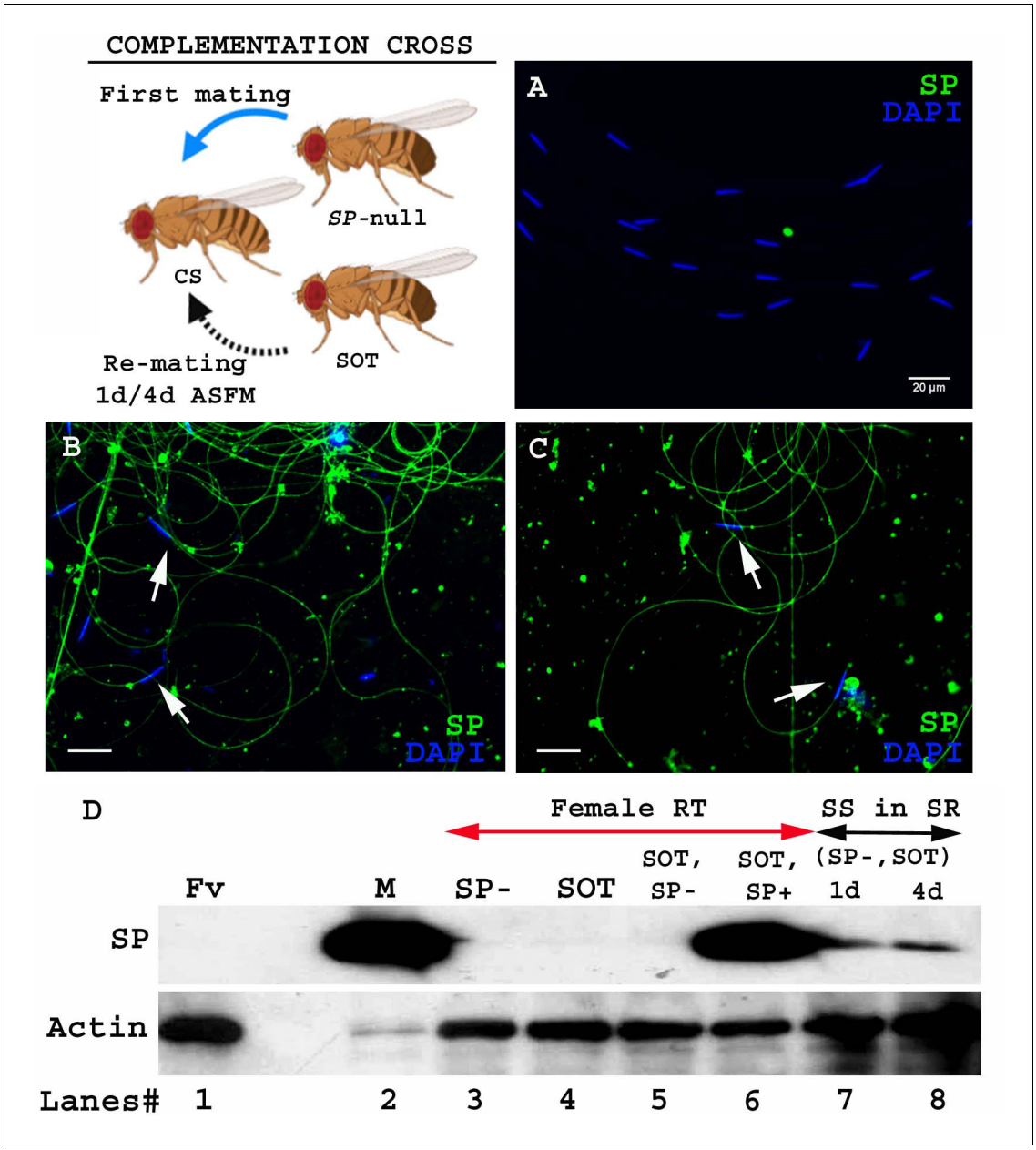

Figure 1. SP from a second male can bind to SP-deficient sperm of previous male stored within a mated female. Cartoon: Pictorial representation of the crossing scheme (fly images from Biorender). Wild type (CS) females were first mated to an *SP*-null male and then, at the indicated time, to a spermless (SOT) male. Sperm heads were stained with DAPI (blue) and SP was 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, 1d ASM. (**B**) Sperm from females mated to *SP*-null males, remated to spermless males at 1d ASFM and (**C**) at 4d ASFM, both frozen 2 hr ASSM. White arrows indicate sperm heads. Bar = 20 µm (**D**) Western blot lane numbers 1: Fv, reproductive tract (RT) of virgin female (negative control; n = 5), 2: M, a pair of male accessory gland (positive control; n = 1), 3: SP-, reproductive tracts of females mated to *SP*-null males, 2 hr ASM (n = 5), 4: SOT, reproductive tracts of females mated to spermless males, 1d ASM (n = 5), 5: SOT, SP-, reproductive tract of females mated to spermless males and then remated to *SP*-null males, 1d ASFM (n = 8 RT), 6: SOT, SP+, reproductive tract of females mated to spermless males and then remated to control (SP+) males at 1d ASFM, frozen 2 hr ASSM (positive control; n = 8 RT), 7: (SP-, SOT), 1d and 8: (SP-, SOT), 4d sperm isolated from the seminal receptacle of females mated to *SP*-null males and then remated to spermless males at 1d ASFM and 4d ASFM, frozen 2 hr ASSM (n = 15 SS). Actin served as loading control.

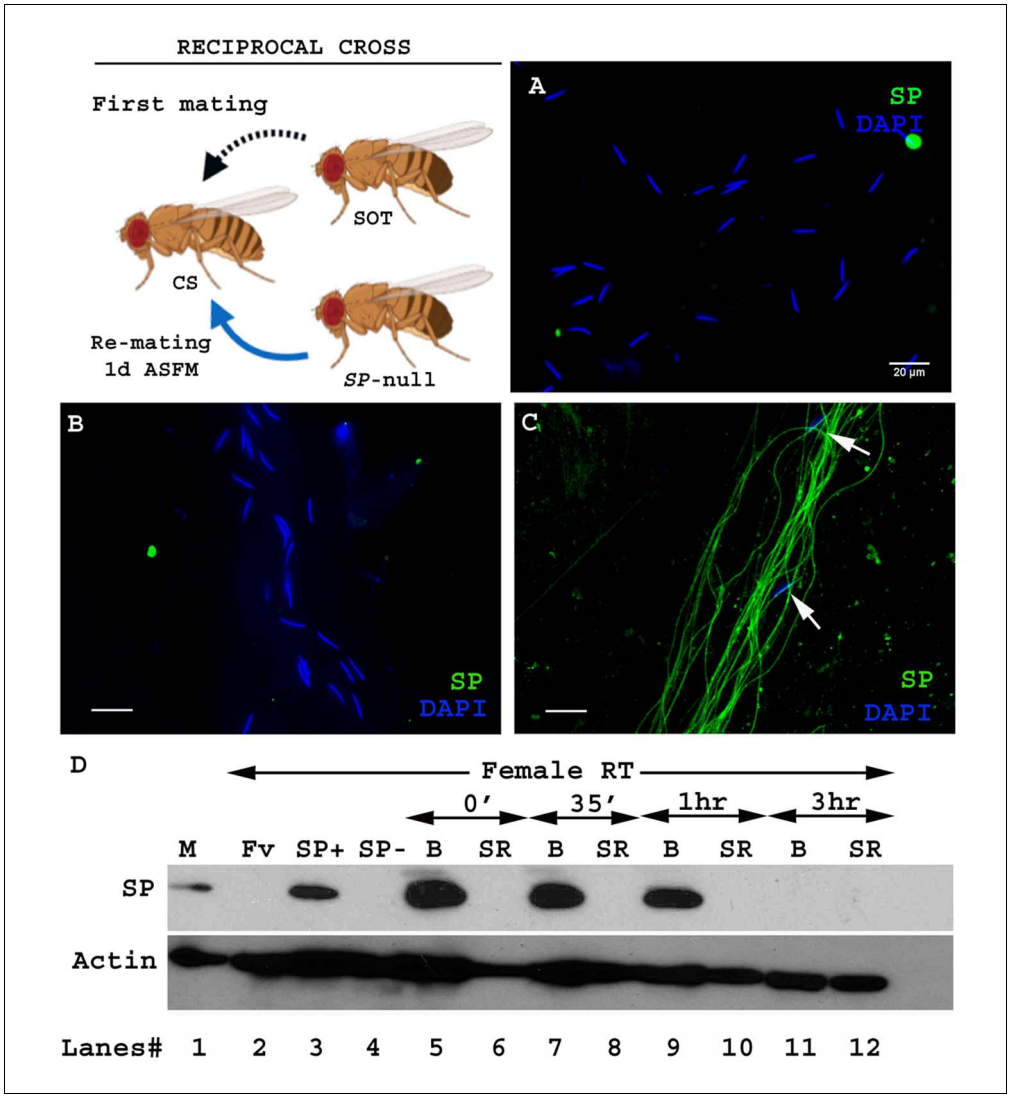

Figure 2. Sperm from a second male are not bound to SP from a prior spermless male. (Cartoon): Pictorial representation of the cross (fly images from Biorender); it is reciprocal of that in ***Figure 1***. Females mated first with spermless (SOT) males and then a day later with *SP*-null males that provided 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, 2 hr 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 2 hr ASSM. White arrows indicate sperm heads. Bar = 20 µm (**D**) Western blot lane numbers 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, 1 hr, and 3 hr ASM, respectively (n = 15). Actin served as loading control.

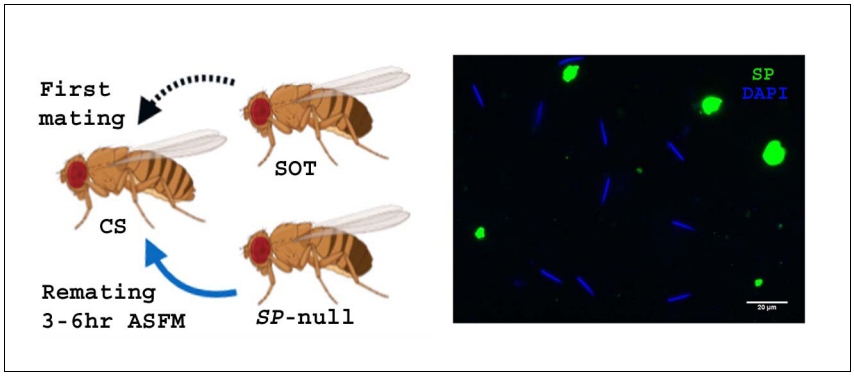

Figure 2—figure supplement 1. Cartoon: Pictorial representation of cross (fly images from Biorender). Females mated first with spermless (*sot*) male and then 3–6 hr ASFM with *SP*-null male that provided sperm. Panel: Sperm from SR of females mated to spermless males and then remated to *SP*-null males, 3–6 hr ASFM, frozen at 2 hr ASSM. Sperm heads were stained with DAPI (blue) and SP (green) probed with Alexa fluor 488 (n = 5; Bar = 20 μm).

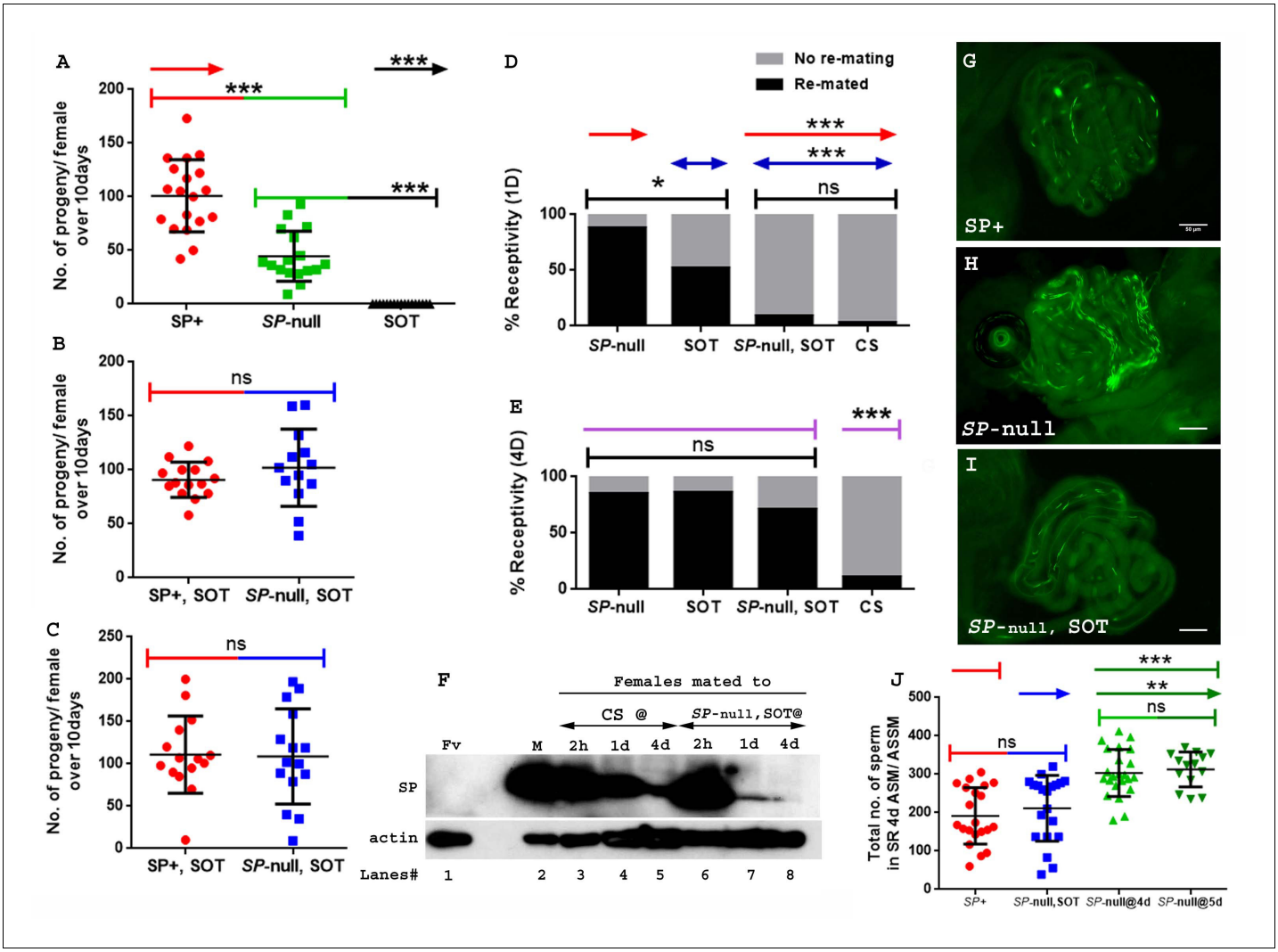

Figure 3. Remating with spermless males restores fertility, delays receptivity and optimizes efficient sperm release in females that previously mated to SP-null males. (**A**) Graphical representation of numbers of progeny produced by each female over the span of 10 days, following mating to control (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 for each technical replicate). (**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 for each technical replicate). (**F**) Western blot lane numbers 1: Fv, reproductive tract (RT) of five 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 2 hr (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 2 hr (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 female 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 = 50 µm. (**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).

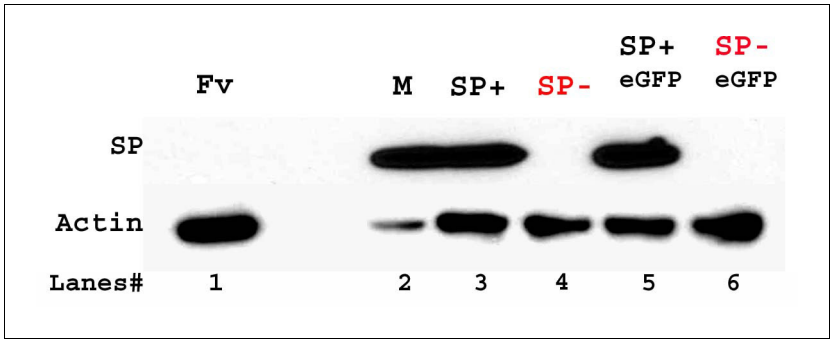

Figure 3—figure supplement 1. Western blot probed for SP. Lanes/samples are 1: Fv, reproductive tract (RT) of three virgin females (negative control); 2: M, one pair of male accessory glands (positive control); 3: SP+, RT of three females mated to control (TM3 siblings of *SP*-null males; positive control)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

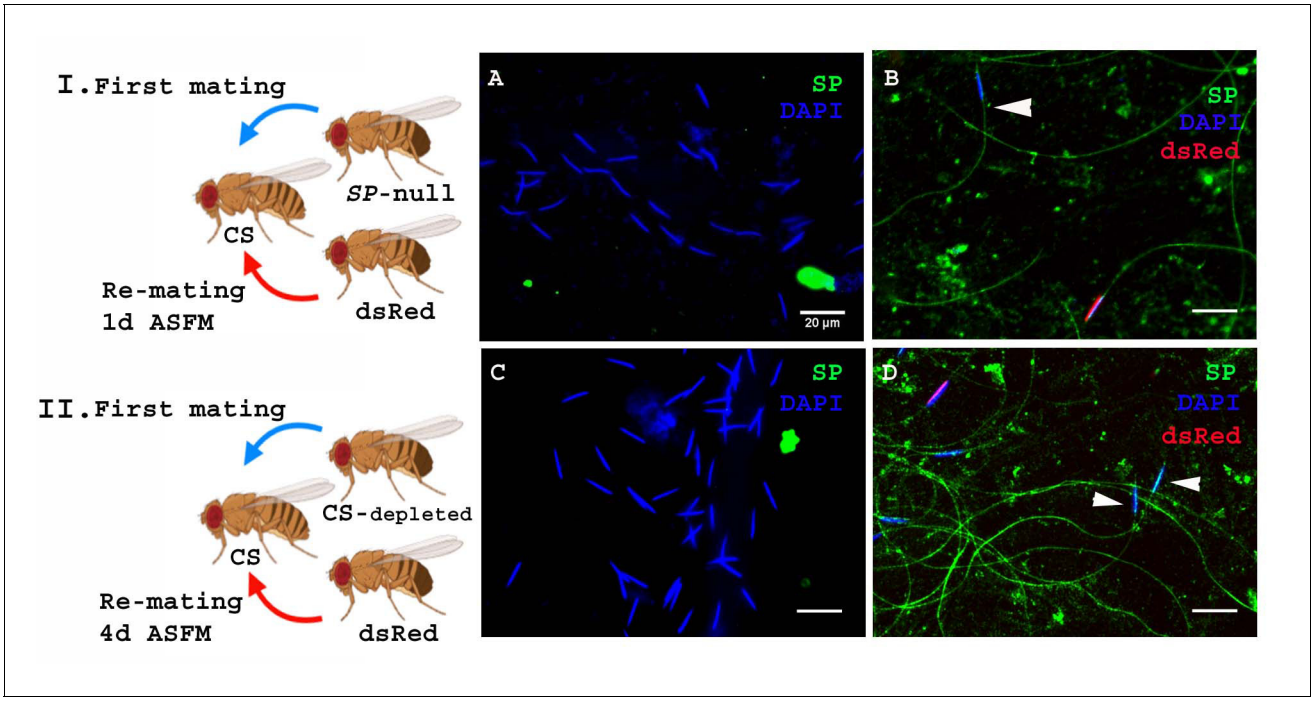

Figure 4. SP from a male who also provides sperm can bind to SP-deficient sperm as well as to the donor's sperm. Cartoon (I): Pictorial representation of the experimental cross (fly images from Biorender). Females mated to *SP*-null males were remated to control (*ProtB-dsRed*) males at 1d ASFM. (**A**)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

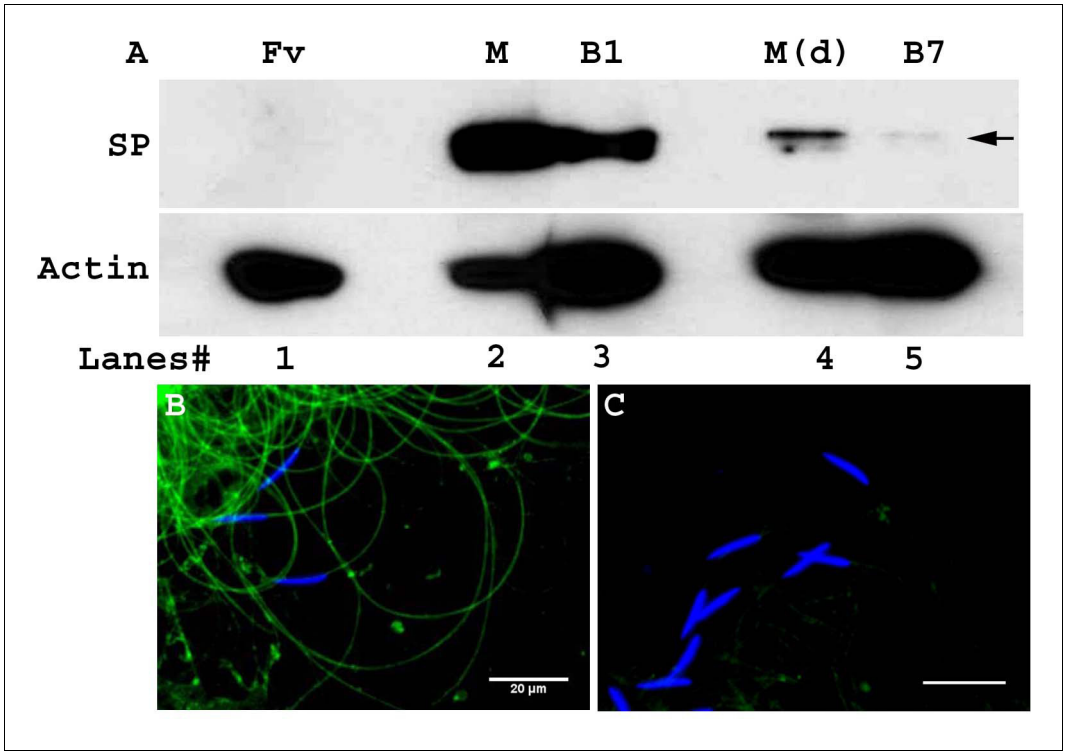

Figure 4—figure supplement 1. Little to no SP is transferred by multiply-mated males. (**A**) Western blot probed for SP. Lanes/samples are 1: Fv, reproductive tract (RT) of two virgin females (negative control); 2: M, one pair of male accessory glands from a 3-day-old unmated virgin male; 3: B1, RT of four females mated to control unmated virgin males, frozen at 2 hr ASM; 4: M(d), one pair of male accessory glands dissected from a multiply mated male (previously mated with six virgin females); 5: B7, RT of four females mated to multiply-mated males, frozen at 2 hr ASM. Actin served as loading control. (**B**) Sperm dissected from females mated to unmated males, frozen at 2 hr ASM. (**C**) Sperm dissected from females mated to multiply mated males, frozen at 2 hr ASM. Sperm heads were stained with DAPI (blue) and presence of SP (green) was detected with Alexa fluor 488 (n = 5; Bar = 20 µm).

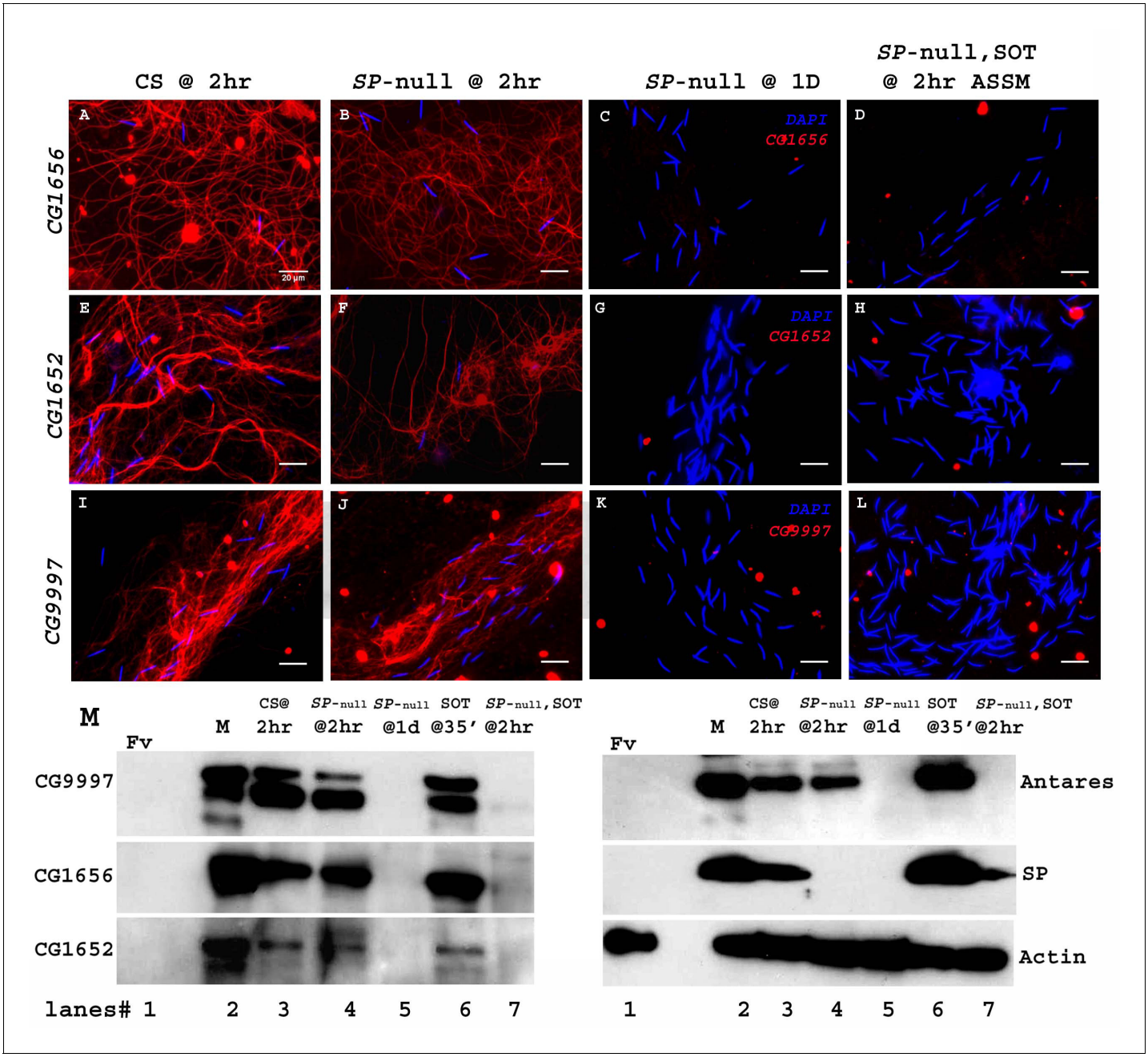

Figure 5. Sperm do not bind detectable LTR-SFPs from a second male. Females mated to wild type (CS) males at 2 hr ASM show LTR-SFPs bound to sperm, CG1656 (**A**), CG1652 (**E**), CG9997 (**I**). Females mated to *SP*-null males show the same (**B,F,J**) but by 1d postmating LTR-SFPs' signal were no longer detected on sperm (**C,G,K**) confirming previous reports (***Singh et al., 2018***). 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**), 2 hr ASSM, although they have SP bound (***Figure 1***). Sperm stained for the indicated LTR-SFP detected with Alexa fluor 594 (red) and sperm-head stained with DAPI (blue). Bar = 20 µm (**M**) Western blot probed for indicated LTR-SFPs. Lanes/samples are 1: Fv, reproductive tract (RT) of three virgin females (negative control); 2: M, one pair of male accessory glands (positive control); 3: CS @ 2 hr, sperm dissected from SR of 20 females mated to wild type (CS) males at 2 hr ASM; 4: *SP*-null @ 2 hr, sperm dissected from SR of 20 females mated to *SP*-null males at 2 hr 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 three females mated to spermless males at 35'ASM (positive control); 7: *SP*-null, SOT @ 2 hr, sperm dissected from SR of 20 females mated to *SP*-null males and then remated to spermless males at 1d ASFM, and frozen at 2 hr ASSM. Lanes were probed for LTR-SFPs CG9997, CG1656, antares and CG1652 and SP as described in the text. Actin served as loading control.

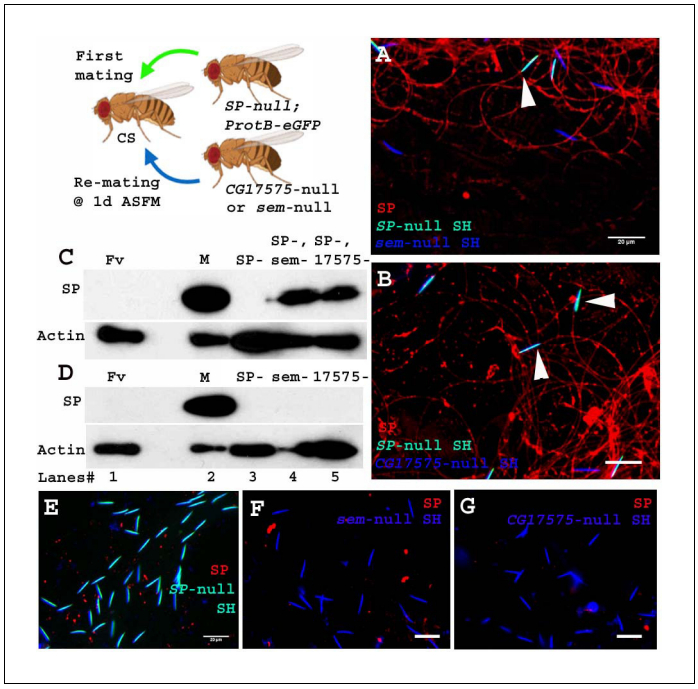

Figure 6. Sperm received from SP-null males do not require CG17575 or seminase from a second male to bind SP from that male. Cartoon: Pictorial representation of the experimental cross (fly images from Biorender). Females mated first with *SP-null; ProtB-eGFP* males [cyan sperm-head; DAPI(blue)+eGFP(green)]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

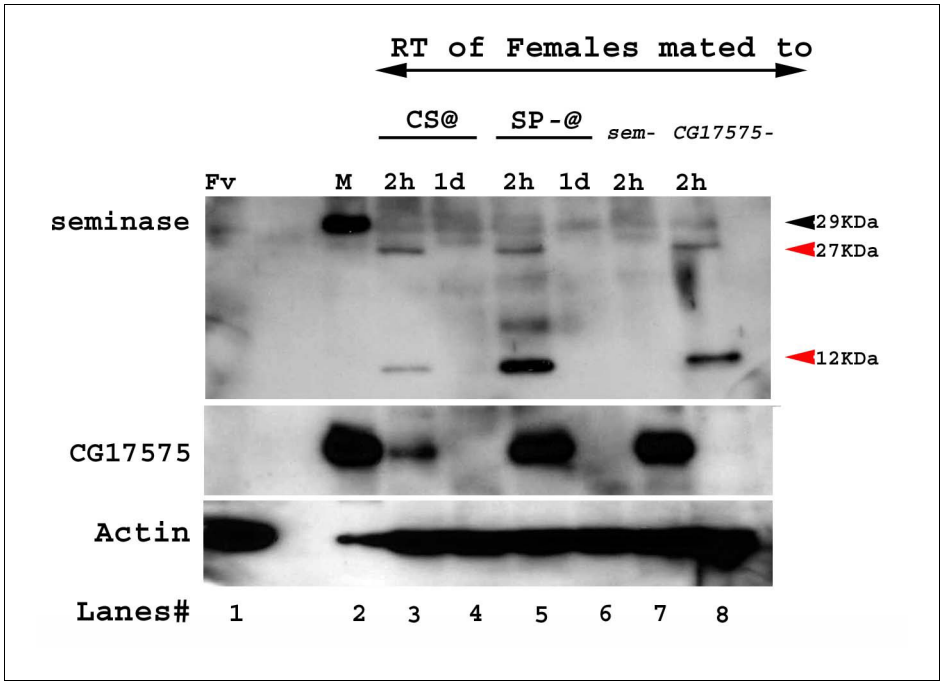

Figure 6—figure supplement 1. Western blot probed for seminase and CG17575. Lanes/samples are 1: Fv, reproductive tract (RT) of three virgin females (negative control); 2: M, one pair of male accessory glands (positive control); 3–4: RT of five females mated to wild type (CS) males at 2 hr and 1d ASM, respectively; 5–6: RT of five females mated to *SP-null; ProtB-eGFP* males at 2 hr and 1d ASM, respectively; 7: RT of five females mated to *seminase*-null males at 2 hr ASM; 8: RT of five females mated to *CG17575*-null males at 2 hr 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.