Receipt of Seminal Fluid Proteins Causes Reduction of Male Investment in a Simultaneous Hermaphrodite

Yumi Nakadera, Elferra M. Swart, Jeroen N.A. Hoffer, Onno den Boon, Jacintha Ellers, and Joris M. Koene
**Supplemental Table S1.** Summary of “Bioassay of SFPs on Sperm Transfer” test.

<table>
<thead>
<tr>
<th>series</th>
<th>total N</th>
<th>HFBA ± SE</th>
<th>sperm ± SE</th>
<th>LyAcp3</th>
<th>LyAcp4</th>
<th>LyAcp5</th>
<th>LyAcp7a</th>
<th>LyAcp7b</th>
<th>LyAcp8a</th>
<th>LyAcp8b</th>
<th>LyAcp10</th>
<th>Saline ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>27</td>
<td>33.33±4.44 (9)</td>
<td>34.50±3.27 (7)</td>
<td>20.06±5.24 (4)</td>
<td>32.28±5.94 (7)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>21</td>
<td>36.75±1.81 (6)</td>
<td>12.43±2.50 (7)</td>
<td>29.44±12.29 (4)</td>
<td>25.50±0.31 (4)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>52</td>
<td>22.42±4.03 (9)</td>
<td>10.98±2.26 (11)</td>
<td>9.25±1.61 (9)</td>
<td>17.02±3.33 (13)</td>
<td>17.55±2.90 (10)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>38</td>
<td>14.16±3.15 (8)</td>
<td>40.78±6.95 (8)</td>
<td>36.86±8.84 (7)</td>
<td>31.03±3.21 (9)</td>
<td>40.13±8.93 (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>34</td>
<td>31.39±5.66 (7)</td>
<td>23.88±6.11 (6)</td>
<td>24.09±5.22 (8)</td>
<td>15.21±4.69 (7)</td>
<td>37.50±7.95 (6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The upper half shows the mean ± SE and sample sizes of the SFP treatments and saline controls in each experimental series (A-E). The sample sizes are shown in parentheses. All the series contain saline treated snails (controls). The last two rows indicate the p-values of one-sample t tests, after calculating the relative number of sperm transferred (see Supplemental Experimental Procedures). For adjusting the p-values, we used the Bonferroni correction. For the significant proteins, which are indicated in bold in the last row, LyAcp5 reduced sperm transfer by 56% on average (Series C: 47.3%; Series D: 64.7%) and LyAcp8b by 29.2% (Series D: 22.7%; Series E: 35.8%).
Supplemental Experimental Procedures

Animal Collection and Maintenance

We used the laboratory strain of *L. stagnalis* that has been bred at VU University Amsterdam (The Netherlands) for about 50 years. This cultured strain originates from a wild population in a nature reserve and agricultural area near Eemnes, the Netherlands. Snails were kept in low-copper fresh water in large laminar flow tanks. The water was aerated and kept at 20 ± 1°C with a 12h:12h light:dark cycle. The animals were fed lettuce and, when juvenile, also fish flakes (Tetraphyll, Tetra GmbH).

The simultaneous hermaphrodite *L. stagnalis* copulates unilaterally, meaning that one snail performs the male role (donor) by inseminating its mate (recipient/female role) [S1]. Their unilateral mating and stereotypical mating behaviour allow us to readily discriminate, by observation, which individual performs the male or female role. Insemination, i.e., the transfer of semen (composed of sperm and seminal fluid) usually takes 20-60 min., with the partners sometimes reciprocating by swapping sex roles immediately afterwards [S2]. After insemination, recipients preferentially use and store received sperm [S3-5], even though they can self-fertilize without obvious inbreeding depression [S6-8]. On average, they produce one to three egg masses per week, each containing 50-150 eggs. Similar to many invertebrates, they do not show parental care, territoriality nor a hierarchical social structure.
Effect of SFPs on Sperm Transfer

We examined whether the receipt of an ejaculate alters the recipient’s sperm transfer in its subsequent copulation as a male. We kept donor snails in isolation for eight days, in perforated plastic containers (460 ml). This isolation period is long enough for physiological effects of previous receipts to disappear and to replenish the prostate gland [S9, 10], thereby restoring male mating motivation [S11]. After this isolation period, we intravaginally injected either an artificial ejaculate or a control solution into each snail (treatments: sperm, SFPs, sperm + SFPs; control: saline).

First, we prepared artificial ejaculates from prostate glands (seminal fluid producing organ) and seminal vesicles (autosperm storage organ). We anaesthetized 22 snails by injecting about 2 ml of 50 mM MgCl₂ through the foot and dissected out their prostate glands and seminal vesicles using microsurgical scissors and forceps (World Precision Instruments, Inc.). We collected the 22 seminal vesicles in a 1.5 ml vial with 1 ml saline solution (5.83 mM CaCl₂.2H₂O, 3.76 mM MgCl₂, 42.69 mM NaCl, 37.53 mM KCl), which was vigorously vortexed to liberate sperm from the organ’s tissue (see also [S12, 13]). After removal of the tissue, sperm numbers were counted in four subsamples with a Neubauer counting chamber (depth, 0.1 mm; area, 0.0025 mm²) to obtain an estimate of the total number of sperm present. We prepared two 1.5 ml vials each containing approximately 12.5 x 10⁶ sperm. The 22 prostate glands were collected in a vial with 0.5 ml saline solution, crushed and then vortexed to obtain seminal fluid. After tissue removal, half the seminal fluid solution was added to one of the vials with sperm, while the other half was transferred to an empty vial. Saline solution was added to the three vials (sperm, SFPs, sperm + SFPs) plus the additional clean vial (control) to a total volume of 1 ml. All samples were continuously kept on ice.

Next, we intravaginally injected 0.03 ml of the freshly-made artificial ejaculates into each donor snail by using syringes equipped with soft silicon tubing (see details in [S14, 15]). Each injected volume had approximately 37.5 x 10⁴ sperm and/or the equivalent to one third of a prostate gland content, representative of quantities present in a natural ejaculate [S14]. A numbered bee tag (Het
Bijenhuis, The Netherlands) was glued onto the shell of each treated snail for identification, before which they were returned to their individual containers to recover.

The following day, each treated donor was paired with a recipient snail of the same age; these had been isolated from the mass culture four days earlier. We recorded whether the donors inseminated their recipients, and whether they acted in the male or female role first (respectively called primary and secondary donors). A large portion of our donors (115 out of 187) engaged as primary donors in both experimental runs. The duration until intromission, insemination duration and shell length (a reliable measure of body size [S16, 17]) were also recorded. Immediately after the donors finished insemination, we dissected the recipients to count the number of sperm they received [S12]. First, we anesthetised mated recipient snails by injecting about 2 ml of 50 mM MgCl₂ through their foot by using a syringe. We removed the shell and made the first incision with fine forceps and scissors, just posterior of the female gonopore and then cutting the skin along the midline towards the head to reach the reproductive organs. Immediately after insemination, one can clearly see the expanded vaginal duct, containing just‐received ejaculate. We carefully dissected out the duct (female gonopore and vaginal duct) containing the ejaculate, and collected it into an Eppendorf tube with 200 µl of saline solution. We suspended the ejaculate in the tube by crushing and vortexing for 30s. Then, we placed the female tissue in a new tube with 200 µl of saline and vortexed for another 30s. After we repeated this procedure once more, we pooled the solution in these three tubes into the first one, without the female tissue. Then, after a final 30s vortexing, we obtained 600 µl of ejaculate suspended in saline solution. We took 30 µl of the sample to count number of sperm heads on a Neubauer counting chamber (depth 0.1 mm, area 0.0025 mm²; four subsamples counted). From this measurement, we estimated the number of sperm transferred using the formula in [S12; it is important to realise that the actual counting area for this method is 0.04 mm², 0.0025 mm² specifies the smallest squares on the counting chamber, which does not become entirely evident from the initial description of the method].
Donors that did not inseminate or engage in copulation within the eight hours of behavioural observation were excluded (24 out of 187 snails were excluded, so the overall mating rate was 88.6%).

We conducted these tests in two runs. For the first run, 92 donors (shell length 2.93 ± 0.02 cm; mean ± SEM) were inseminated, of which 24 saline, 19 sperm, 23 SFPs, and 26 sperm + SFPs. For the second run, 95 donors (shell length 2.91 ± 0.02 cm) were inseminated, of which 24 saline, 28 sperm, 23 SFPs, and 20 sperm + SFPs. The shell lengths of the recipients in the two runs were 2.86 ± 0.02 cm and 2.88 ± 0.01 cm, respectively. In addition, a few ejaculate samples did not contain any sperm (18 out of 138), though this frequency did not differ between runs ($\chi^2_1 = 0.049, p = 0.824$), nor between primary and secondary donors ($\chi^2_1 = 0.006, p = 0.937$). Copulations without sperm transfer, which can be due to an error in scoring insemination behaviour or to too few sperm transferred to detect, were excluded from the statistical analyses (16 out of 115 donors).

Because our study species sometimes swaps sexual roles immediately after the first mating is finished [S1, 2], this allows us to examine if their natural insemination immediately affects sperm transfer. Therefore, in the second run of “effect of SFPs on sperm transfer” experiment, we also counted sperm transfer of those donors that first mated as females (secondary donors) in addition to the primary donors (which were the only ones counted in the first run); we refer to this factor as Mating order.
Effect of SFPs on Paternity Success

Next, we examined whether the receipt of SFPs reduces paternity success of the recipient in a subsequent mating as a male. To test this, we raised virgin recipient snails by isolating juveniles (shell length about 1.5 cm) in perforated containers. Each snail was provided daily with a lettuce disc (19.6 cm²), which is slightly below their maximum daily food intake [S17]. After these recipients matured (judged by egg mass production via self-fertilization), additional adult snails were isolated from our mass culture and were randomly assigned to becoming donors or inseminators. After eight days of isolation, 12 inseminators were allowed to copulate with a future donor, giving us recently-inseminated donors, and 14 individuals were assigned as control donors. The following day, we paired each virgin recipient with one type of donor, control or recently-inseminated. We paired the animals randomly, but did ensure that we made combinations which could unequivocally discriminate paternity of the offspring (i.e., microsatellite genotypes of donors and recipients were different, see below). We observed each mating pair (13 out of 26 pairs mated), recording insemination duration every 15 min. and measuring shell length as proxy for body size. Note that, although we gave donors nine days of sexual isolation, which fully motivates them to assume the male role, recipient snails were virgins (i.e., they never mated before). Due to the even higher male mating motivation of virgins it is difficult to fully control mating outcomes in the experimental set-up, thus explaining why only 50% of the pairs mated in the right order. In addition, according to our follow-up experiment, being recently inseminated does not reduce their male mating motivation (Nakadera et al. in prep).

After mating, we isolated recipients again and collected their egg masses to measure paternity success of the donors, versus self-fertilized offspring of recipients [S3-S5]. Given their lack of inbreeding depression [S6-S8], this experimental setup (competition against autosperm of recipients) allows us to evaluate paternity success after being inseminated without controlling for various other factors, e.g., mating order in double mating regime.
Prior to copulation, both donors and recipients were genotyped using microsatellite markers. For whole DNA extraction, we collected their hemolymph in 1.5 ml Eppendorf tubes by gently poking their foot with a blunt plastic stick. We centrifuged the solution at max. speed for 15 min. obtaining the visible pellet at the bottom for use of DNA extraction, following [S4]. We amplified three microsatellite loci (2k11 [Accession no. EF208748], 2k68 [Accession no. EF208750], [S18], Lsc01014 [See main text]) by PCR. The reaction mixture was 17.25 µl of H2O, 2.5 µl 10×PCR buffer (MRC Holland), 2 µl of 2.5 mM dNTP, 1 µl of 5 µM labelled forward and reverse primers, 0.25 µl of taq DNA polymerase (MRC Holland), 0.025 µl of pfu (Promega) and 1 µl of DNA extract. The PCR program was initiated with a hot-start at 80°C, then an initial denaturation at 95°C for 5 min., and 30 cycles of [95°C for 5s, (58°C for 2k11, 52°C for 2k68, 56°C for Lsc01014) for 45s, 72°C for 60s] and a final extension at 72°C for 10 min. in a thermal cycler (MJ Research). After amplification the presence of the product was confirmed on agarose gel electrophoresis. For DNA fragment analysis, we made loading samples by mixing 8.1 µl of HiDi formamide (Applied Biosystems), 0.9 µl of SizeStandard™ (500LIZ™, Applied Biosystems) and 1 µl of PCR product, and the mixed samples were denatured at 95°C for 5 min. When we had too much PCR products, we diluted with H2O. We ran the DNA fragment analysis on the DNA sequencer (ABI31000, Azco Biotech, Inc.). We measured microsatellite-containing DNA fragment lengths using GeneMapper® (Applied Biosystems), allowing us to distinguish each genotype of each locus based on both length and colour signal from labelled primers. With the available genotypes of donors and recipients, we made combinations which could unequivocally discriminate paternity of the offspring.

The recipient’s eggs were genotyped to estimate donor paternity success, and to determine if offspring were outcrossed or selfed. The first egg masses were collected in Eppendorf tubes (1.5 ml) and freeze-dried. After measuring egg mass dry weight, we selected eight random embryos per egg mass using fine forceps (World Precision Instruments, Inc.) on an anti-static mat (ROMEX). Each embryo was crushed in 50 µl of 0.5 M NaOH and incubated at room temperature for 10 min. For neutralization, 5 µl of 1 M Tris buffer was added and PCR and DNA fragment analysis was carried out
by following the same procedure as described above. We obtained embryos from 9 control and 4 inseminated replicates, and succeeded to genotype 7.38 ± 0.47 (control, 7.11 ± 0.68; inseminated, 8.00 ± 0.00) embryos per egg mass (i.e., 96 offspring in total).

Bioassay of SFPs on Sperm Transfer

To identify the responsible protein, we tested the effect of single SFPs on the number of sperm transferred. We intravaginally injected a single SFP, as described above, and measured how much sperm the recipients transferred afterwards. We used eight SFPs previously purified and sequenced using a reversed-phase HPLC approach [S15]. To facilitate reference to these substances in future we coded the SFPs according to their respective peak number [S15] using the prefix LyAcp (Lymnaea Accessory gland protein). We used the same protocol as for the first experiment, with the adaptation that lyophilized and frozen (-80°C) samples of the eight proteins were dissolved separately in a saline solution in ejaculate-equivalent concentrations. Three control treatments were included: sperm and saline, saline only, and HPLC-buffer HFBA in saline in the same trace amounts as in the purified protein samples. These experiments were performed in five series. In each test series, we tested the effect of 1 - 4 types of SFPs and at least one type of controls (saline in all series, HFBA and sperm in two series; Table S1) on sperm transfer. In total, we tested 172 snails for their sperm transfer. Shell length of snails was the same for all series (recipients: 2.90 ± 0.15 cm, donors: 2.85 ± 0.19 cm).
Statistics

We tested the effect of SFPs on sperm transfer by using ANOVA with Treatment (type of test solution) as a fixed factor. For the first run, we analyzed the number of sperm transferred by treated donors, only if they inseminated first (primary donors). For the second run, we used type III ANOVA to add Mating order as a factor, thus including both primary and secondary donors (the latter acted as female first). The number of sperm transferred was square root transformed for normal distribution (Note, that all the reported number of sperm transferred in text or figures are raw data). Then, we used the Tukey’s honest significant difference method for comparing between treatments in the first run, and in the second run, samples within treatments and mating orders (i.e., eight groups).

We tested whether paternity success differed between control and inseminated donors by using a generalized linear model (glm function in R) with binomial distribution (outcrossed or selfed). As dependent variable, we entered number of outcrossed offspring and selfed offspring. The fixed factor was Donor type (control or inseminated). To examine whether overdispersion influenced the outcome of this model, we divided the residual deviance by the degrees of freedom [S19], which yielded a ratio of 0.895. Since this is close to one, we did not assume overdispersion in the main text, although the output was practically the same in the model with a quasi-binomial distribution ($\chi^2_{1} = 8.67$, $p = 0.003$). Control donors sired $6.56 \pm 0.63$ out of $7.11 \pm 0.68$ genotyped offspring, while inseminated donors sired $5.75 \pm 0.48$ out of $8.00 \pm 0.00$ genotyped offspring. In addition, we tested whether the control and inseminated donors differed in their insemination duration, dry weight of egg mass and shell length by using a t test (and Kruskal-Wallis test for insemination duration).

For the bioassay of SFPs, we tested whether each type of SFP mediated reduced sperm transfer in a subsequent mating. Because saline controls of the five experimental series showed a significant difference (Series, $F_{4, 28} = 2.92$, $p = 0.039$: Table S1), in each series, we subtracted the mean number of sperm transferred in the saline injected snails from the number in the treatments
within that series. Then, we pooled these data between series to compare if each type of SFPs and other control treatments (Sperm and HFBA) significantly deviated in relative sperm transfer from 0 by using a one sample t test. After testing all these treatments, we adjusted p-values using the Bonferroni correction (Table S1; Note that, other methods for multiple comparison did not change the major result). All statistical analyses were done in R.

**Supplemental References**


