Supplementary Materials for

Traumatic mating causes strict monandry in a wolf spider

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SUPPLEMENTARY MATERIALS AND METHODS

Ethics statement

All experiments performed in this study were carried out under the same conditions as the rearing conditions. All spiders were treated in accordance with the legal requirements of Hubei University (the site of the experiment) with regards to research ethics and animal treatment.

Study species and maintenance

We collected sub-adult (i.e., one molt before adulthood) male (*n*=1 500) and female (*n*=800) *Pardosa pseudoannulata* spiders from paddy fields in Wuhan, Hubei Province, China (30°52'N, 114°31'E) from June to October in 2020 and 2021. The spiders were kept individually and were visually isolated from each other in cylindrical glass tubes (10 cm in length, 2 cm in diameter) in the laboratory under a 14:10 h light:dark cycle at 26±0.5 °C and 50%–70% relative humidity. The spiders were fed 30 adult fruit flies (*Drosophila melanogaster*) every two days and had continuous access to water from a soaked sponge at the bottom of the tube. The tube was covered with cotton wool (Gong et al., 2019). Developmental status (molting) was recorded daily (at 09:00). Before the experiment, body size (carapace width and body length), body weight, and age at mating (days after final molting) were recorded.

Mating procedures

Mating tests were conducted in a mating arena (9 cm diameter glass Petri dish covered by a filter paper). In the mating tests, a female was placed in the mating arena first to allow her to adapt to the environment. A male was introduced 20 min later, and their interactions were observed for 20 min. Both females and males were selected randomly, and all males used for the mating tests were unmated. To obtain mated females, unmated females were allowed to mate under the same procedures. The filter paper was replaced after each pairing. Spiders are characterized by a pair of copulatory organs, whereby females have two copulatory openings and males have two palps for insertion (Foelix, 2011). Here, the *P. pseudoannulata* male first inserted one palp into one copulatory opening, then the other palp into the other copulatory opening of a female, though never at the same time. During copulation, males exhibited two types of palp insertion, i.e., single- and multiple-bout modes. In the single-bout mode (observed in approximately 96% of cases (n=300)), the two palps were inserted alternately, i.e., first one and then the other. In the multiple-bout mode (observed in approximately 4% of cases (n=300)), the two palps of the male were inserted alternately for around 10 bouts, initially lasting ~3 min, then lasting ~1 min until penetration became impossible due to the formation of mating plugs (Supplementary Movie S2).

(1) Confirmation of strict female monandry

To confirm strict female monandry in *P. pseudoannulata*, randomly chosen unmated males were mated with females under eight different reproductive states: 1 h, 3 h, 5 h, 1 d, 3 d, 7 d, ~30 d (after dispersal of the first batch of spiderlings), and 60 d after mating (n=20 females in each trial). Females carry egg sacs in their spinnerets after deposition and spiderlings stay on the dorsal part of the female abdomen after hatching. Thus, females reject any courtship and mating during the parental care stage. We considered mating to have succeeded if a male mounted the dorsal abdomen of a female and inserted his palps into the copulatory openings, with an inflated hematodocha during palp insertion. Each female was tested using three different males (at most) consecutively, with a 1 h interval between tests. If a female did not mate within three pairings, we assumed that no re-mating would occur.

(2) Dissection of copulatory organs

To investigate the mechanism underlying monandry, we dissected spiders with different mating statuses using an Olympus SZX16 stereomicroscope (Olympus, Tokyo, Japan). Unmated females (n=150) were randomly assigned to three groups: (a) insertion in both copulatory openings; (b) insertion in only one copulatory opening; and (c) no insertion in any copulatory opening. For females in group (a), males were allowed to complete both palp insertions. For females in group (b), copulation was interrupted after the male completed one palp insertion by poking the male with a fine brush. For females in group (c), no males were provided.

Males were dissected immediately after mating to examine their emboli. Females were dissected at 10 different time points: i.e., immediately after mating, 3 h, 12 h, 24 h, 3 d, 5 d, 7 d, 9 d, and 15 d after mating, and after egg deposition (n=5 at each time

point). Before dissection, the spiders were anaesthetized using CO_2. Females were first examined for the existence of mating plugs in the epigyne. Subsequently, we dissected the epigyne to remove the mating plugs, and examined the genital tracts under scanning electron microscopy (SEM) (JEM-1011, JEOL, Tokyo, Japan) to investigate wounds on the inner wall.

(3) Analyses of main components of mating plugs

To determine the origin of mating plugs, we used gas chromatography-mass spectrometry (GC-MS) to examine the chemical components of seminal fluid, mating plugs, female hemolymph, and male hemolymph. (1) To collect mating plugs, we dissected the genital tracts of females after they mated for 7 days, and carefully removed the amorphous mating plugs. Ten mating plugs were pooled as one sample in a 300 μL Eppendorf tube. (2) To collect female and male hemolymph, we used a fine blade to cut the forelegs (femur part) of the spiders and used a thin-walled capillary glass tube (0.3×100 mm, Instrument Factory, West China Medical University, Chengdu, China) to draw 5 μL of hemolymph, which was then transferred to a 300 μL Eppendorf tube. (3) To collect seminal fluid, we cut the palps of unmated males and placed them in a 300 μL Eppendorf tube, where they were crushed using a micropipette tip. For each Eppendorf tube, we added 50 μL of hexane to extract chemical compounds. The mixtures were homogenized using a vortex mixer (SCILOGEX MX-S, Haitian Youcheng Technology Co., Ltd., Beijing, China) for 3 min, then centrifuged at 10 000 rpm for 5 min at 4 ℃. The supernatant was transferred to a smaller Eppendorf tube (500 μL), then concentrated by surface exposure to nitrogen until the total volume of the extract was 5 μL.

Each extract (1 μL) was injected into the GC-MS system using a 5R-GP syringe (SGE, Ringwood, Victoria, Australia). Mass spectra were produced using an HPGC 8860 coupled with an MS 5977B detector. An HP-5 column of 30 m×250 μm×0.25 μm capillaries (Agilent, Wilmington, USA) and helium as the carrier gas (flow rate of 1.0 mL/min) were used. The injector port and detector temperatures were maintained at 250 ℃. The separation protocol, developed to ensure sufficient separation of the compounds for analysis, started at an initial temperature of 50 ℃ for 3 min, then increased to 280 ℃ at a rate of 10 ℃/min and maintained for 5 min. Subsequent comparative analyses were conducted using the NIST17 Library to identify chemical compounds. Nitrogen gas-concentrated methanol was used as the negative control to confirm that no peaks or ions similar to those of the analytes were present in the spectra.

We also investigated amino acid compositions and respective concentrations in seminal fluid, mating plugs, and female hemolymph using high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). An amino acid test kit (45+AA; MSLab, Beijing, China) was used to test free amino acids. Before analysis, the three types of samples and mixed amino acid standard solutions in a concentration gradient were processed using a derivatization procedure. Samples were prepared using the abovementioned methods for GC-MS analysis. Approximately 2 mg of each sample was first dissolved in 50 μL of ethanol (100%), then dried under nitrogen gas and eluted

in 100 μL of deionized water, followed by homogenization using a vortex mixer for 10 min. Subsequently, the mixtures were centrifuged at 12 000 rpm and 4 °C for 5 min. The supernatant was transferred to an Eppendorf tube (500 μL), and 50 μL of each sample was placed in an ampoule and hydrolyzed using 4 mL of 6 M HCl at 110 °C for 21 h, with the ampoule filled with nitrogen gas. The samples were cooled, and 5 mL of 4 M NaOH was added to each sample. After this, 50 μL of protein precipitating agent was added to 50 μL of the sample and to 50 μL of an amino acid standard solution. The mixtures were then centrifuged at 13 200 rpm and 4 °C for 4 min. The supernatant (8μL) was removed, and 42 μL of binding buffer and 20 μL of derivative reagent was added. After mixing, the reactions were incubated for derivatization at 55 °C for 15 min. After cooling, 50 μL of the mixture was used for analysis.

Separation and quantification were performed using LC-MS (LC20AD-API 3200MD TRAP; MSLab 45+AA-C18 column, 150×2.6 mm, 5 μm; Shimadzu, Kyoto, Japan). The essential amino acid (EAA) score was evaluated using reference amino acids (Food and Agriculture Organization/World Health Organization) and defined as the ratio of the content of the first limiting EAA in the sample to the content of the same amino acid in the reference standard.

The HPLC system consisted of an SRD-3600 Solvent Rack with an analytical six-channel vacuum degasser, a DGP-3600A pump, a WPS-3000TSL analytical autosampler, and a tcc-3200 column compartment. Chromatographic separations were performed using an MSLab 45+AA-C18 column (150×2.6 mm, 5 μm) with helium as the carrier gas at a flow rate of 1.0 mL/min. The mobile phase consisted of water (A) and acetonitrile (B). The solvent was delivered to the column at a flow rate of 1.0 mL/min as follows: 0–6.0 min from A–B (83:17) to A–B (83:17); 6.0–6.1 min from A–B (83:17) to A–B (76:24); 6.1–10.0 min from A–B (76:24) to A–B (73:27); 10.0–10.1 min from A–B (73:27) to A–B (3:97); 10.1–13.6 min from A–B (3:97) to A–B (88:12); and 13.6–15 min from A–B (88:12) to A–B (88:12).

The conditions for MS-MS detection were as follows: mode, positive-ion mode; ion spray voltage, +5 500 V; nebulizer gas pressure, 55 psi; curtain gas pressure, 60 psi; collision gas pressure, medium; entrance potential, 10 V; nitrogen gas as collision gas in multiple reaction monitoring mode. Data were recorded using Analyst v1.5.1 (Applied Biosystems, Foster City, CA, USA). Amino acid standard solution (Shanghai Aladdin Bio-Chem Technology, Shanghai, China) was injected for calibration of the system and amino acid quantification. Amino acids were identified by their retention time and mass spectral analyses (MS and MS/MS spectra), and their concentrations were assessed based on accurate mass, retention time, and MS/MS information in accordance with published guidelines for metabolomics studies (Sumner et al., 2007). The experiment was repeated using three replicates.

After obtaining the components and their respective concentrations in each sample, we calculated the concentration percentage of each free amino acid to total free amino acids in each sample. We then averaged the three replicates as the final percentage, which was then compared among the three sample types (i.e., seminal fluid, mating plugs, and female hemolymph).

(4) Effects of seminal fluid and wounds on female mating suppression

To test whether seminal fluid and traumatic insertions can suppress female mating, we manipulated females and simulated mating by artificial microinjection. We performed six treatments on females: (1) genital tracts were injected with seminal fluid using a micromanipulator without damaging the genital tracts (0.2 mm in depth) (seminal fluid group) (n=50); (2) genital tracts were penetrated with a micromanipulator until hemolymph was observed oozing from the copulatory openings (0.5 mm in depth) (genital tract penetration group) (n=50); (3) genital tracts were penetrated with a micromanipulator (same as that in group 2) and injected with seminal fluid (genital tract penetration+seminal fluid group) (n=50); (4) metatarsus of the first pair of female legs was pierced by a micromanipulator (leg penetration group) (n=20); (5) genital tracts were injected with phosphate-buffered saline (PBS) without damaging the genital tracts (PBS group) (0.2 mm in depth, n=20); (6) females were unmanipulated (n=20). The artificial injection procedures followed those of Minekawa et al. (2020). During manipulation, females were first anaesthetized using CO_2, then placed on a foam platform. During genital tract penetration using the micromanipulator, we found that hemolymph was secreted when the needle was inserted into the female copulatory opening at the depth of 0.5 mm, but not at the depth of 0.2 mm. Thus, we assumed that penetration at the depth of 0.5 mm would cause damage to the genital tracts.

To collect seminal fluid, we placed the palps of five unmated males (i.e., 10 palps in total) in a 1.5 mL Eppendorf tube and crushed them using a micropipette tip. Seminal fluid was then recovered using a capillary glass tube and diluted with PBS at a concentration of 5 pmol/50 nl. During artificial injection, 200 nl of seminal fluid solution or PBS was individually microinjected into both genital tracts using a micromanipulator (M3301-R, World Precision Instruments, Inc., Sarasota, FL, USA) and a Picospritzer® III (Parker Hannfin Corporation, Cleveland, OH, USA). A dose of 20 pmol/copulatory opening was considered sufficient as superfluous liquid was observed to leak from the copulatory openings.

Manipulated females were placed back in their individual vials and were subjected to mating trials at 1 h, 3 h, 12 h, 1 d, 3 d, 5 d, 7 d, 9 d, 13 d, and 15 d, respectively, after manipulation. Each female was exposed to three males (at most) at each time point, and whether females mated and mating duration (i.e., total palp insertion durations) were recorded, with the cumulative mating rate of females then calculated. Spider body size (male body length, male body weight, male carapace width, female body length, female body weight, and female carapace width) and age were recorded before the experiments.

Statistical analyses

We first tested the data for normality of residuals and homogeneity of variance using the Shapiro-Wilk and Levene tests, respectively. Normally distributed data among different groups were compared using one-way analysis of variance (ANOVA), and non-normally distributed data among different groups were compared using the

Kruskal-Wallis test. To examine the impact of seminal fluid and wounds on female mating suppression, we compared mating latency (duration from manipulation to mating) of the females and mating duration of the males among the different treatment groups using analysis of covariance (ANCOVA), with spider body size (body length, body weight, and carapace width) and age (days after maturity) as covariates. We used the chi-square test to compare the cumulative mating rates of females in different groups. All data analyses were performed using R v4.2.1 (R Core Team, 2022). All tests were two-tailed, with $P<0.05$ set for significance.

SUPPLEMENTARY RESULTS

The results of experiments (1) and (2) are reported in the main text, while some results of experiments (3) and (4) are reported here.

Experiment (4) showed that the mating rate of females in the seminal fluid group was not significantly different from that of females in the PBS and unmanipulated groups at the 1 h ($\chi^2_2=2.68$, $P=0.262$), 3h ($\chi^2_2=2.36$, $P=0.307$) and 12 h time points ($\chi^2_2=0.11$, $P=0.947$) (Supplementary Figure S2A). However, at each time point, the mating rate of females in the seminal fluid group was slightly lower than that in the PBS and unmanipulated groups. These results suggest that seminal fluid had some, but not significant impact on female mating suppression.

At the 1 h time point, the mating rate of females in the leg penetration group was significantly lower than that in the other three groups ($P<0.001$), suggesting that leg penetration suppressed female willingness to mating within 1 h of manipulation. However, they recovered and mated quickly: 85% within 3 h and 100% within 12 h (Supplementary Figure S2A), suggesting that trauma from leg penetration had an instant but not long-term impact on female mating success.

In the two female groups with injured genital tracts, a large proportion (40% and 46%) remained unmated (Supplementary Figure S2A, B). Among the unmated females, some rejected male courtship throughout the entire study (38% and 36%), while some accepted male courtship, but males were unable to successfully insert their palps (18% and 24%) into the copulatory openings, probably due to the presence of mating plugs (Supplementary Figure S3).

Female mating latency was significantly affected by female treatment (ANCOVA, $F_{5,138}=44.10$; $P<0.001$) and male body length (ANCOVA, $F_{1,138}=4.09$; $P=0.045$) (Supplementary Table S3). Although average male mating duration did not vary significantly among the different treatment groups ($\chi^2_5=5.95$, $P=0.311$) (Supplementary Table S4), it did vary significantly ($P<0.001$) among the different mating time points within the two groups with injured genital tracts (Supplementary Figure S2C). For example, there was a significant increase from 3 d to 5 d ($P<0.001$) and from 5 d to 7 d ($P<0.001$) (Supplementary Figure S2C), possibly due to the gradual formation of amorphous mating plugs (Supplementary Figure S3), which increased palp insertion difficulty and mating duration to complete sperm transfer. However, after

7 d, male mating duration in these two groups decreased significantly (P<0.001), probably due to the increased difficulty to insert palps, which became impossible at 13 d (Supplementary Figure S2C).

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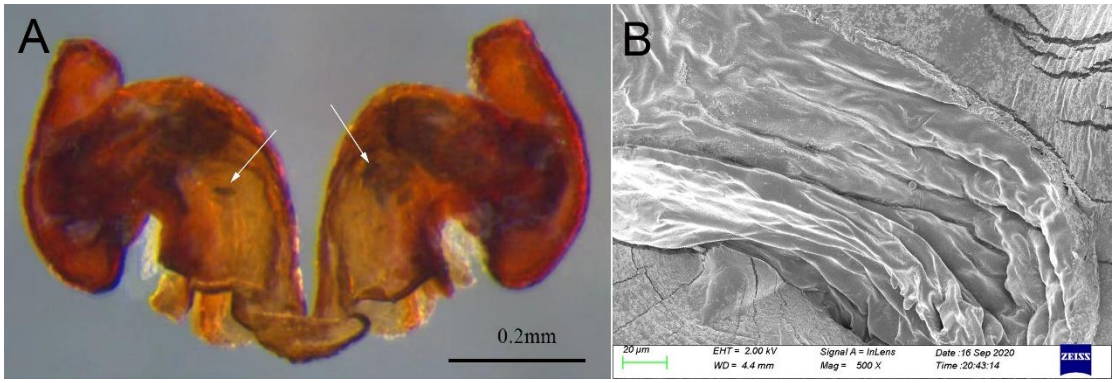
Captions for Movies

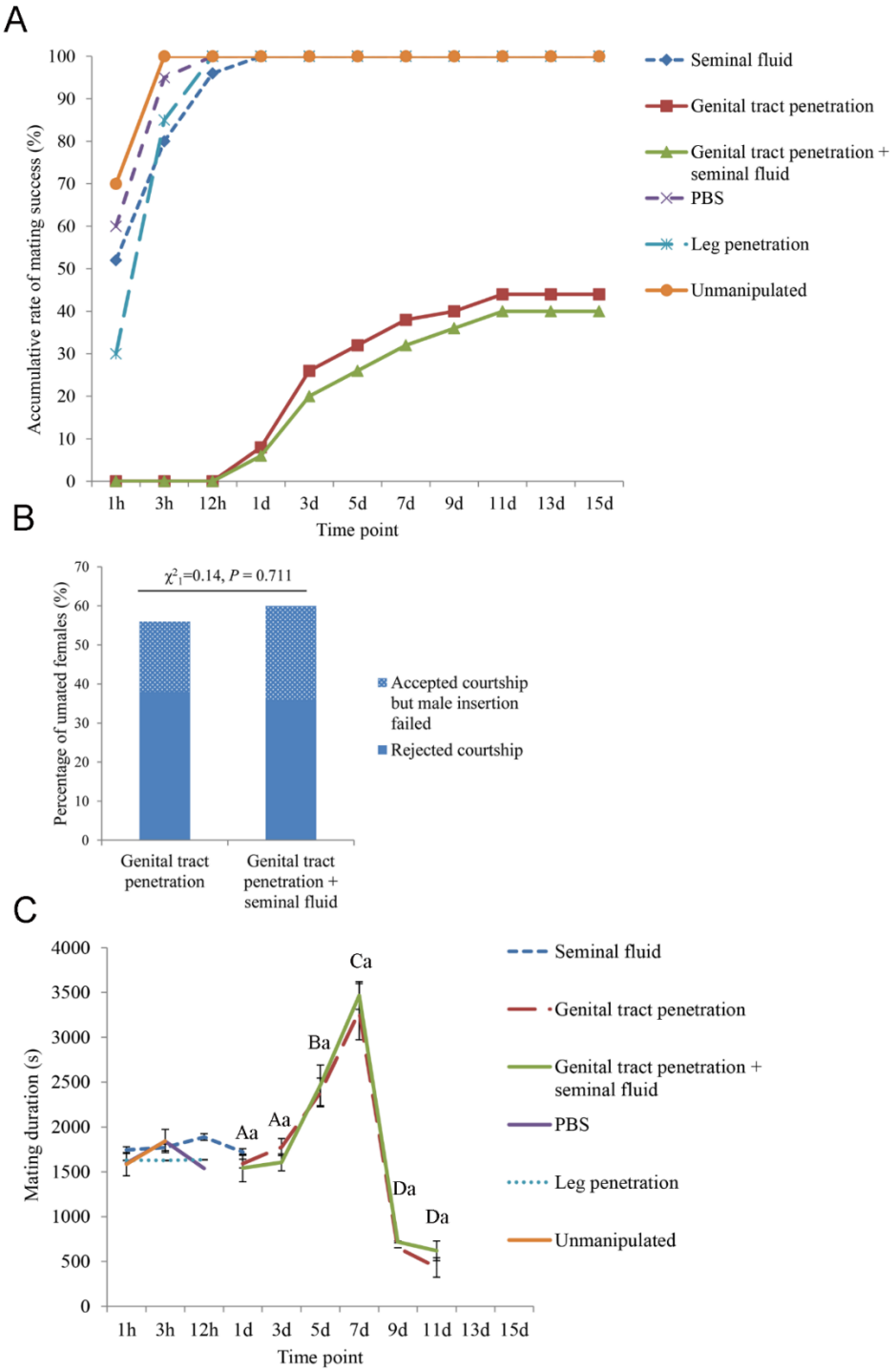
Caption for Movie S1. Female cannot detach male partner during mating.

Caption for Movie S2. Male displaying problems with palp insertions.



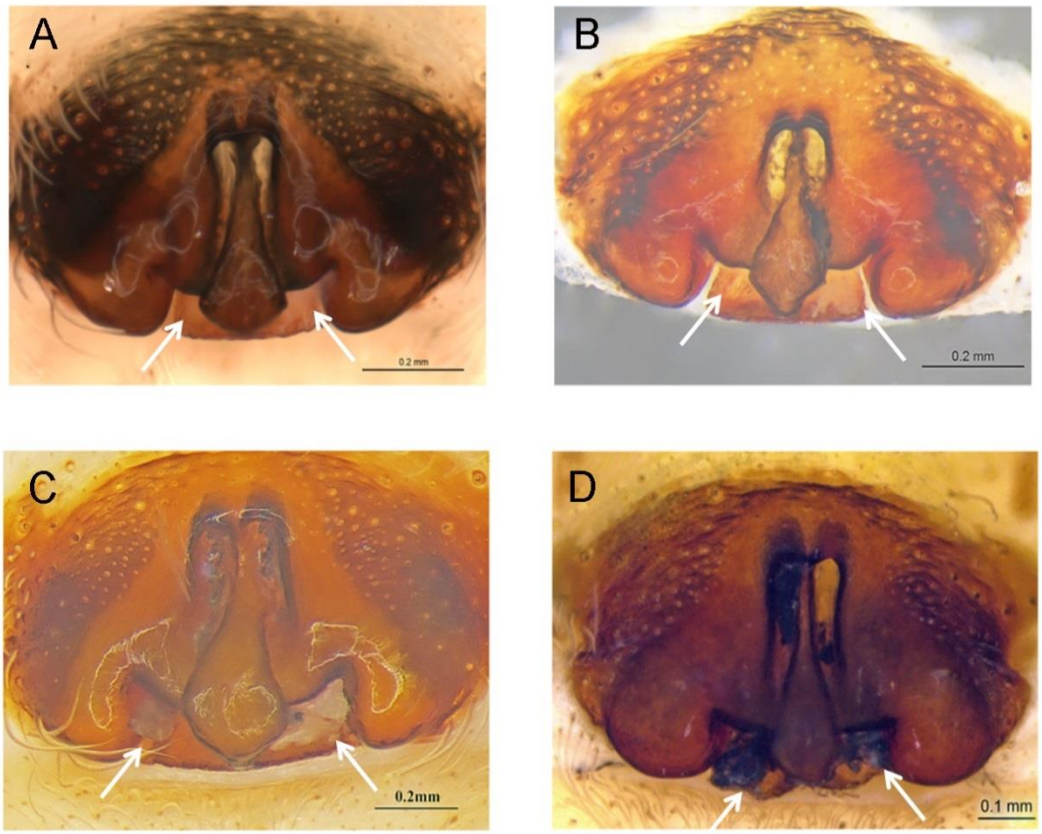
Supplementary Figure S1 Damaged and undamaged genital tracts in female spiders

A: Male embolus was inserted into both genital tracts of females (white arrows indicate scars). B: Inner wall of genital tract of an unmated female under SEM.



Supplementary Figure S2 Effect of seminal fluids and artificial wounds on suppression of female mating

A: Accumulative rate of female mating in different groups. B: Percentage of unmated females in two groups in which female genital tracts were wounded. No significant difference was observed between the groups. C: Female mating duration in different groups. Different capital letters indicate significant differences between different time points within an experimental group, and different lowercase letters indicate significant differences between different experimental groups at same time point.



Supplementary Figure S3 Formation process of amorphous mating plugs in female epigyne after mating

A: 5 h after mating. No mating plugs were observed (arrows indicate copulatory openings). B: 3 d after mating. Whitish substance (arrows) expelled from copulatory openings. C: 7 d after mating. Whitish substance (arrows) gradually turned brown and hardened. D: 15 d after mating. Whitish substance turned black (arrows) and female copulatory openings sealed and became impermeable.

Supplementary Table S1 Two main chemical compounds present in all mating plugs and female and male hemolymphs but absent in seminal fluids.

Retention time (min)		Chemical compound	Molecular formula	Molecular weight
Mating plug	11.390	Decane, 2,4,6-trimethyl-	$C_{13}H_{28}$	184.36
	25.295	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.50
Female hemolymph	11.387	Decane, 2,4,6-trimethyl-	$C_{13}H_{28}$	184.36
	25.283	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.50
Male hemolymph	11.394	Decane, 2,4,6-trimethyl-	$C_{13}H_{28}$	184.36
	25.268	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester	$C_{19}H_{38}O_4$	330.50

Supplementary Table S2 Percentage of individual free amino acid content in total free amino acids contained in seminal fluid, mating plugs, and hemolymph in female *P. pseudoannulata* spiders (means±SEM) (%) (*n*=3). Different lowercase letters indicate significant differences at *P*<0.05 between three groups based on Kruskal-Wallis test.

Free amino acid	Seminal fluid	Mating plug	Female hemolymph
Gly	14.65 ± 0.31a	7.66 ± 0.16b	7.11± 0.32b
Ala	1.75 ± 0.06a	2.54 ± 0.05b	2.23 ± 0.01c
Ser	2.25 ± 0.09a	1.82 ± 0.08b	1.82 ± 0.06b
Pro	1.26 ± 0.03a	1.12 ± 0.07a	1.25 ± 0.01a
Val	1.12 ± 0.02a	1.77 ± 0.03b	1.93 ± 0.02c
Thr	0.95 ± 0.04a	2.13 ± 0.06b	2.83 ± 0.03c
Cys	0.26 ± 0.03a	0.95 ± 0.05b	1.16 ± 0.05c
Ile	0.30 ± 0.03a	1.21 ± 0.03b	1.11 ± 0.04b
Asn	0.88 ± 0.05a	2.59 ± 0.06b	3.17 ± 0.36b
Asp	2.34 ± 0.15a	1.88 ± 0.05b	2.17 ± 0.07ab
Gln	8.24 ± 0.19a	12.12 ± 0.71b	12.21 ± 0.24b
Glu	3.33 ± 0.03a	2.82 ± 0.11b	3.10 ± 0.11ab
Met	1.14 ± 0.06a	3.54 ± 0.17b	3.81 ± 0.05b
His	6.51 ± 0.06a	5.50 ± 0.07b	6.51 ± 0.13b
Phe	0.48 ± 0.07a	1.10 ± 0.11b	1.53 ± 0.10c
Arg	14.09 ± 0.29a	8.49 ± 0.44b	9.06 ± 0.37b
Trp	0.52 ± 0.09a	1.61 ± 0.25b	1.98 ± 0.05b
Lys	0.92 ± 0.16a	4.54 ± 0.50b	5.18 ± 0.20b
Tyr	0.65 ± 0.09a	0.16 ± 0.03b	0.12 ± 0.01b
Leu	1.39 ± 0.09a	1.70 ± 0.23a	2.52 ± 0.1b

Note: Different lowercase letters indicate significant differences at $p < 0.05$ between the three groups based on Kruskal-Wallis test.

Supplementary Table S3 ANCOVA results comparing female mating latency in different treatment groups, with spider body size (body length, carapace width, body weight) and spider age (days after maturity) as covariates.

Source	*df*	*F*	*P*
Intercept	1	0.018	0.893
Treatment	5	44.099	< **0.001**
Male body length	1	0.633	**0.041**
Male body weight	1	0.057	0.812
Male carapace width	1	2.869	0.093
Male age	1	0.670	0.414
Female body length	1	0.543	0.462
Female body weight	1	0.068	0.795
Female carapace width	1	0.808	0.370
Female age	1	0.600	0.440

Supplementary Table S4 ANCOVA results comparing male mating duration in different treatment groups, with spider body size (body length, carapace width, body weight) and spider age (days after maturity) as covariates.

Source	*df*	*F*	*P*
Corrected model	13	0.813	0.646
Intercept	1	3.620	0.059
Treatment	5	1.002	0.419
Male body length	1	2.468	0.118
Male body weight	1	0.015	0.902
Male carapace width	1	2.567	0.111
Male age	1	0.483	0.488
Female body length	1	0.779	0.379
Female body weight	1	0.877	0.351
Female carapace width	1	1.120	0.292
Female age	1	1.057	0.306