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Traumatic mating causes strict monandry in a wolf spider

DEAR EDITOR,

We identified strict monandry in a wandering wolf spider species (*Pardosa pseudoannulata*) and revealed the underlying mechanisms of this mating structure. Evidence indicated that males damaged the inner walls of the female genital tract with the sharp part of their intromittent organ during insemination. The traumatic mating caused hemolymph hemorrhage, which mixed with seminal fluid and gradually formed an impermeable amorphous mating plug after about 15 days, completely blocking the female copulatory opening. The wounds also significantly decreased the willingness of females to mate again. Thus, strict monandry in *P. pseudoannulata* was caused by traumatic mating, whereby genital trauma suppressed the willingness of females to mate again and the development of mating plugs prevented re-mating.

Although polygamy is widespread in animals, monandry is comparatively rare, and strict monandry is extremely uncommon (Taylor et al., 2014). As such, the mechanisms underlying strict monandry remain poorly studied. While traumatic mating is reported to delay female re-mating (Crudgington & Siva-Jothy, 2000) and reduce re-mating duration (Tong et al., 2021), its impact on strict monandry is unclear (Lange et al., 2013; Reinhardt et al., 2015).

The wandering wolf spider (*P. pseudoannulata*) is widely distributed in East Asia and is a multivoltine species, with 3–4 generations produced each year. In the current study, the *P. pseudoannulata* male-to-female sex ratio during the mating season was approximately 1.35 (*n*=6 530 surveyed in April 2020). We observed that females almost never re-mated after initial coupling (Figure 1A), and that blackened amorphous mating plugs gradually formed in the female epigyne after mating (Figure 1B, C). We therefore wondered (i) whether females were strictly monandrous; (ii) how mating plugs were produced; and (iii) what mechanisms supported strict monandry.

We randomly selected unmated males to mate with females under eight different reproductive states: i.e., 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). Results showed that none of the mated females mated again, confirming that adult *P. pseudoannulata* females are strictly monandrous.
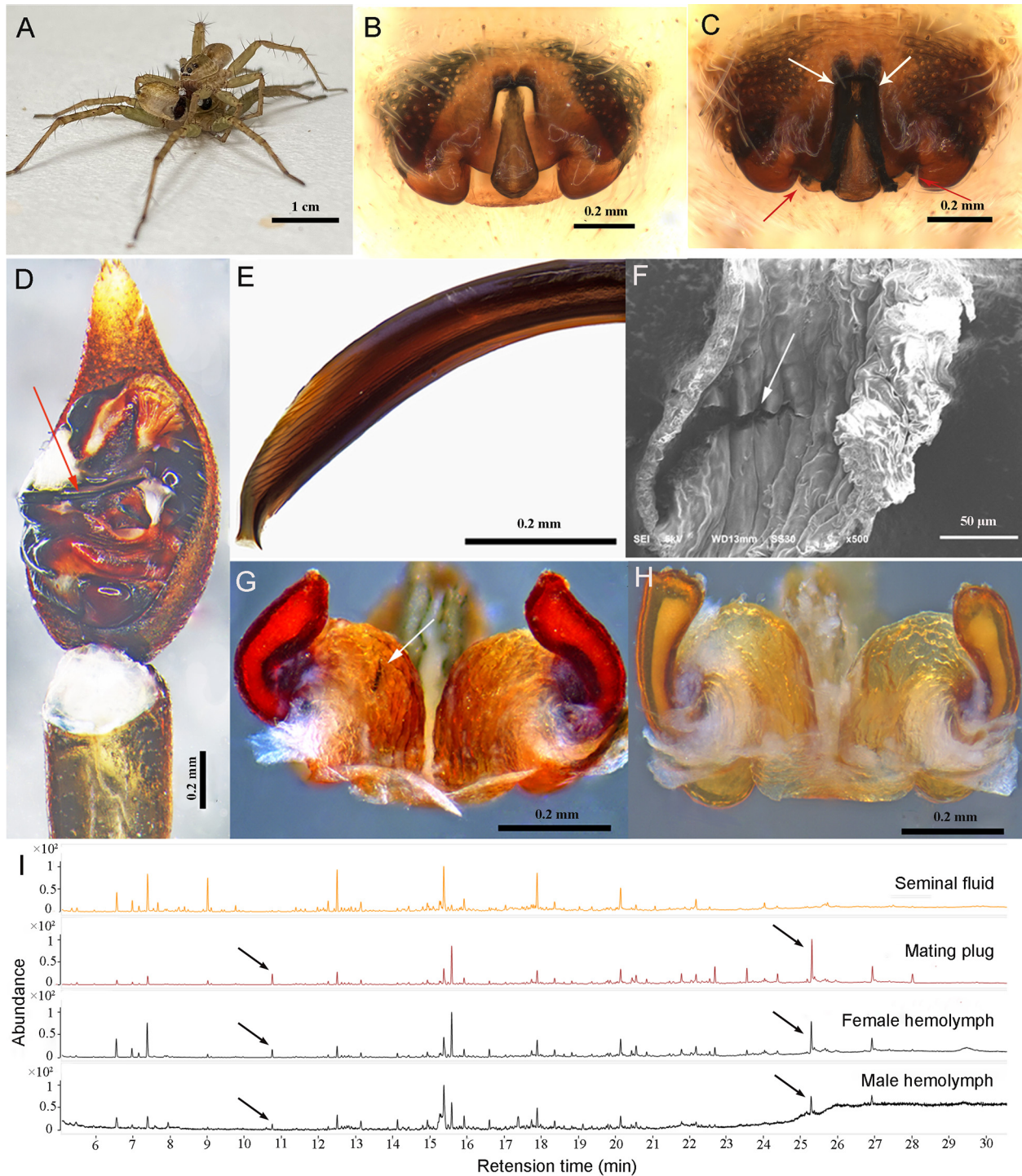
After manipulation of adult female mating, we collected and dissected their genital tracts. We also collected and dissected the copulatory organs of adult males (Supplementary Materials). As found in males of other spider species, the intromittent organ (Figure 1D) of the adult *P. pseudoannulata* male bore a strongly sclerotized and sharp embolus (Figure 1E). During copulation, the embolus (mean±standard error of the mean (*SEM*): 0.70±0.02 mm length of inner arc, *n*=20) was inserted into the female genital tract to transfer sperm, with the sharp tip of the embolus injuring the inner wall of the genital tract (Figure 1F, G; Supplementary Figure S1). The epigyne (external genital structure in female spiders) and spermatheca of the mated females were darker (Figure 1G) than those of the unmated females (Figure 1H), and scars due to injury by the male embolus were easily observed (100% occurrence in every copulation, *n*=50) (Figure 1G; Supplementary Figure S1). After embolus insertion and injury, the females secreted hemolymph, which mixed with seminal fluid and subsequently (around 15 days) solidified to form an amorphous mating plug that blocked the female genital tract (Figure 1C; Supplementary Figure S3). Consequently, the genital tract could not be re-penetrated by a male. The quantity of secreted liquid may depend on the severity of damage caused to the genital tract. Notably, we observed that even when males only inserted into one copulatory opening, excess fluid leaked into the other genital tract, filling the

Figure 1 Mating pairs, dissection of male and female copulatory organs, and GC-MS spectra of seminal fluid, mating plugs, and female hemolymph in the wolf spider *P. pseudoannulata*

A: Mating (male inserts left palp into female right copulatory opening). B: Epigyne of unmated female. C: Epigyne of mated female (red arrows indicate blackened amorphous mating plugs projected from copulatory openings, white arrows indicate substance that filled the furrows on both sides of the median septum to form mating plugs). D: Male palp (red arrow indicates embolus); E: Detached embolus (hooked conductor bears a sharp tip). F: Scanning electron microscopy (SEM) image of inner wall of female genital tract (white arrow indicates a wound by male embolus). G: Dissected epigyne of mated female (3 d after mating, white arrow indicates melanized scar). H: Dissected epigyne of an unmated female. I: GC-MS profiles of hexane extracts from seminal fluids, mating plugs, female hemolymph, and male hemolymph (arrows indicate two components shared in mating plugs, female hemolymph, and male hemolymph, but absent in seminal fluid).

furrows on both sides of the median septum (Figure 1C).

To identify the origin of the amorphous mating plugs, we used gas chromatography-mass spectrometry (GC-MS) to examine the chemical components, and high-performance chromatography-tandem mass spectrometry (HPLC-MS/MS) to investigate amino acid compositions in seminal fluid, mating plugs, and female hemolymph (Supplementary Materials). Results showed that the main organic compounds in the mating plugs were the same as those in the female and male hemolymph samples but differed slightly from those in seminal fluid (Figure 1I; Supplementary Table S1). The HPLC-MS/MS results showed that the concentration (percentage) of each free amino acid in total free amino acids between mating plugs and female hemolymph was very similar (70%, 14 of 20 amino acids), with no significant differences. However, the similarity between mating plugs and seminal fluid was relatively low (10%, 2 of 20 amino acids) (Supplementary Table S2), showing a significant difference (χ^2_1=45, P<0.001). In addition, 11 of the 20 (55%) free amino acids showed no significant difference in concentration between mating plugs and female hemolymph but showed significant differences between mating plugs and seminal fluid (Supplementary Table S2). These results suggest that mating plugs originate primarily from the female hemolymph. Previous research has suggested that mating plugs in spiders originate from the embolic conductor of the male palps (Kuntner et al., 2015). Thus, to the best of our knowledge, this study provides the first evidence of female hemolymph-produced mating plugs.

To test whether seminal fluid and traumatic insertion can suppress female mating, we simulated mating by artificial microinjection. Females were injected with seminal fluid without genital tract damage (seminal fluid group), injected with nothing with genital tract damage (genital tract penetration group), injected with seminal fluid with genital tract damage (genital tract penetration+seminal fluid group), injected with phosphate-buffered saline (PBS) without genital tract damage (PBS group), manipulated by penetration of the metatarsus of the first pair of legs (leg penetration group), or unmanipulated. Manipulated females were subjected to mating trials at different time points (Supplementary Materials). Results showed that seminal fluid did have some, but not significant, impact on the suppression of female mating (Supplementary Figure S2A). However, genital tract injury greatly suppressed female willingness to mate. In both injured groups, no females mated within 12 h. Moreover, the cumulative mating rates of females in these two groups (8% and 6%) were significantly lower after 1 d compared with those in the control groups (100%) (P<0.001) (Supplementary Figure S2A).

In addition, mating latency of females in the two penetrated groups (genital tract penetration group: 106.9±15.5 h, n=22; genital tract penetration+seminal fluid group: 120.0±17.1 h, n=20) were significantly longer than that of other groups (seminal fluid group: 3.7±0.7 h, n=50; PBS group: 2.4±0.6 h, n=20; leg penetration group: 3.8±0.8 h, n=20; unmanipulated group: 1.6±0.2 h) (P<0.001), further confirming that genital tract injury greatly suppressed female willingness to mate.

Although injury caused by microinjection also caused hemorrhage and the formation of impermeable mating plugs after two weeks, the females did not obtain sperm and remained willing to mate. This differed from natural mating, after which the females turned aggressive and refused to mate again.

Traumatic mating in spiders has been previously reported in *Harpactea sadistica* (Dysderidae), in which males use their emboli to pierce the female abdominal integument to reach the spermatheca during mating, but do not insert the embolus into the copulatory openings (Řezáč, 2009). Furthermore, in some spider species, such as the wolf spider *Schizocosa ocreata*, males perform coercive mating by biting resistant females with their fangs, resulting in hemolymph loss and scar tissue formation, with bite wounds appearing in the same place in different females (Johns et al., 2009). In contrast, we found that *P. pseudoannulata* males used their emboli to cause intragenital wounding, similar to traumatic mating found in insects, with the wounds occurring anyplace within the female genital tract (Supplementary Figure S2A). Unlike our findings, traumatic mating in *H. sadistica* does not result in strict monandry (Řezáč, 2009). Thus, we propose that traumatic mating may have evolved in two distinct ways in spiders.

During mating, the *P. pseudoannulata* males held the females tightly by their legs, such that the females could not easily detach the males (Supplementary Movie S1). Once the female was successfully mounted, the mating process was controlled by the male, allowing males to potentially mate with both palps consecutively without being attacked by the female, although they caused traumatic injury during the mating process. After mating, the males quickly dismounted from the female abdomen, and were subsequently attacked by the female in most cases (95% of n=300). Those females that struggled more during mating tended to show greater wounding of the genital tracts. Thus, females may try to avoid greater injury by struggling less.

Although traumatic mating was not fatal to females, we found that it significantly affected female lifespan (one-way analysis of variance (ANOVA), $F_{2,57}$=37.52, P<0.001). Average lifespan of females with insertion in both copulatory openings was 142.6±3.9 days (n=20), which was significantly shorter than that of unmated females (187±3.3 days; n=20) (Tukey multiple comparisons of means, P<0.001), but not significantly different from females with insertion in only one copulatory opening (152.5±4.3 days; n=20) (Tukey multiple comparisons of means, P=0.168). This result is consistent with previous research of other species that undergo traumatic insemination, such as the warehouse pirate bug *Xylocoris flavipes* (Backhouse et al., 2012). By engaging in traumatic mating, males may reduce the likelihood of females surviving subsequent mating attempts, thereby enticing females to invest more in current reproduction.

Strict monandry in *P. pseudoannulata* is likely an adaptation to intense competition among males due to the male-biased sex ratio, as physically superior males may mate multiple times. We expect this mechanism will be revealed in additional arthropod species in the future, especially those that produce mating plugs.

DATA AVAILABILITY

Raw data from this study are available at https://doi.org/10.

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SUPPLEMENTARY DATA

Supplementary data to this article can be found online.

COMPETING INTERESTS

The authors declare that they have no competing interests.

AUTHORS' CONTRIBUTIONS

S.C.Z. conceived and designed the study. N.N.M., D.Y.G., and Y.Z. collected spiders in the field. N.N.M., D.Y.G., and A.J.M. performed the research. X.G.J., J.L., and Y.P. advised on the experiment and discussed the results. S.C.Z., N.N.M., and D.Y.G. analyzed the data. S.C.Z. wrote the first draft. All authors gave final approval for publication. All authors read and approved the final version of the manuscript.

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