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Mating experience affects male mating success, but not female fecundity in the wolf spider *Pardosa pseudoannulata* (Araneae: Lycosidae)

Deyong Gong[a,1], Shichang Zhang[a,1], Xiaoguo Jiao[a], Zhiqing Hu[a], Xichen Sha[a], Shu Zhang[b,*], Yu Peng[a,*]

[a] State Key Laboratory of Biocatalysis and Enzyme Engineering, School of Life Sciences, Hubei University, Wuhan, 430062, China
[b] Institute of Plant Protection and Soil Science, Hubei Academy of Agricultural Sciences, Hubei University, Wuhan, 430064, China

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ABSTRACT

In a mating system in which females are monandrous and males are polygynous, females may incur a risk by mating with males with possible sperm depletion following consecutive matings. Here, we examined the effects of male mating history on male mating success and female reproductive fitness in the wolf spider *Pardosa pseudoannulata* by performing mating trials and sperm counting experiments. Results showed that male mating history had a significant negative impact on subsequent copulation success but had little effect on courtship duration and courtship intensity. In addition, neither male courtship intensity nor morphological measurements of males and females had significant effects on male mating success. Furthermore, male mating history had no obvious impact on the fecundity of inseminated females, with no significant differences observed in the oviposition rate of females, the numbers and the carapace width of the second-instar spiderlings between treatments. Results showed that the number of sperm decreased significantly after mating but could be replenished, with no significant differences observed between groups in which males had rested for 7 d. These findings suggest that polygynous male spiders may recharge their sperm during the mating season, but how females differentiate the mating status of males remains unknown.

1. Introduction

Polyandry is widespread in the animal kingdom (Andersson, 1994). Females may benefit from polyandry in many aspects, such as fertilization assurance, provision of resources, parental care for their offspring, and increased offspring viability (Arnqvist and Nilsson, 2000; Simmons, 2005). To obtain mating opportunity, males in polyandrous species have evolved a variety of exaggerated sexual characters, and/or arm to win advantage in male-male competition (Andersson, 1994), and have evolved some strategies to protect their paternity, such as mate guarding (Parker and Pizzari, 2010; Schneider and Andrade, 2011; Simmons, 2005). However, it has been reported that females have several mechanisms that can affect the results of mating and thus affect male reproductive success, including cryptic female choice (i.e. discrimination among males during or after copulation) (Eberhard, 1996).

For species without mate guarding, a male's reproductive success is largely determined by the number of females he can fertilize (Schneider and Andrade, 2011; Simmons, 2005). However, the quantity of sperm produced/used may restrict male's reproductive success as fewer sperm may be a disadvantage in male-male competition and could be overwhelmed by rival male sperm (Birkhead and Moller, 1998). Therefore, sperm replenishment is crucial for these species. To date, however, replenishment has been primarily described in mammals (Birkhead and Moller, 1998), with few reports on arthropods, especially arthropod annual species (Lemaître et al., 2009). Mating history may have a considerable impact on future reproductive success of annual arthropods if sperm replenishment does not occur. In the seed beetle *Callosobruchus maculatus*, for example, the quantity of ejaculate passed from a male to a female declines dramatically with successive matings (Savalli and Fox, 1999). Therefore, mating history can greatly influence later mating and reproductive success on males.

Male mating history can greatly affect female fecundity. Previous studies in insects (e.g. moth *Cnephasia jactatana*) have indicated that females who mated with sexually experienced males had lower lifetime fecundity than those that mate with virgin males due to declines in sperm quantity and quality (Jiménez-Pérez and Wang, 2004), which are crucial to female fitness (Wigby et al., 2009). In many species, females

* Correspondence authors.
E-mail addresses: Ricezs6410@163.com (S. Zhang), pengyu@hubu.edu.cn (Y. Peng).
[1] These authors contributed equally to this work.

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are able to differentiate a male's mating status, showing a preference for males with less mating experience (Ruther et al., 2009; Sato and Goshima, 2007). However, in other species, male mating history has little impact on female reproductive success due to new sperm replenishment (e.g., beetles and moths) (Jones and Elgar, 2004). For instance, *Drosophila melanogaster* males become depleted in seminal fluid proteins with repeated matings but can transfer "virgin" levels of seminal fluid proteins after 3 d of rest (Sirot et al., 2009). It has also been reported that male mating history in Lepidoptera species can strongly affect female reproductive performance, fecundity, and longevity (reviewed in Torres-Vila and Jennions, 2005). To enhance their fertility, females in some species, such as the New Zealand seed bug (*Nysius huttoni*), can mate as many as 68 times to replenish sperm (Wang and Davis, 2006). However, the impact of male mating history on female fecundity in spiders has received little attention, especially in wandering spiders (Schneider and Andrade, 2011). Costa (1998) reported in wolf spider *Lycosa malitiosa* that the stored sperm from a single sperm induction was enough to inseminate two consecutive females, which has little impact to female fecundity. Furthermore, studies on web-building spiders have primarily focused on mating history and its impact on sexual behavior in both males and females, but not on the reproductive potential of females (Michalik and Rittschof, 2011; Molina and Christenson, 2008).

Spiders are good models to study sexual behaviors (Foelix, 2011; Schneider and Andrade, 2011). Adult spiders possess two reproductive organs, with males having two palps (highly modified appendages that are used to insert into female reproductive tracts for sperm transfer) and females having two copulation openings (Foelix, 2011). After maturity, male spiders eject semen from the genital pores, and then collect it with the paired palps (i.e., sperm induction) (Robinson, 1982). Thus, in theory, spiders can mate with one or both palps multiple times. To maximize reproductive benefit, a male may recharge palps with newly produced sperm after mating (Huber, 1998). To date, however, empirical evidence to support this assertion is rare, especially in wandering spiders (Costa, 1998; Schneider and Andrade, 2011). Michalik and Rittschof (2011) reported in the golden orb-web spider *Nephila clavipes* that sperm depletion is permanent and it is common in web-building spiders. However, it is largely unknown whether males would recharge their palps with new sperms after mating and how often they recharge their palps (Schneider and Andrade, 2011).

The wolf spider *Pardosa pseudoannulata* (Araneae: Lycosidae) is widely distributed in China, Japan, and India (Platnick, 2017) (Fig. 1A). It mainly lives in rice fields and can move fast on the surface of water (Zhang's observation). In our preliminary experiments, we observed that males can mate with multiple females with no physical damage to their palps, whereas females are essentially monandrous, as reported in other wolf spiders such as *P. astrigera* (Jiao et al., 2011) and *Schizocosa ocreata* (Norton and Uetz, 2005).Therefore, males have to mate with as many females as possible to achieve maximal reproductive benefit. Conversely, females need to discriminate male mating status as previously mated males may suffer from sperm depletion, which may, in turn, result in a reduction of female reproductive fitness.

We performed mating experiments in the laboratory using wolf spider *P. pseudoannulata*. We aimed to test the impact of male mating history to their subsequent mating success and to the fecundity of the females. We predicted that male mating history has negative impact to both of them.

2. Materials and methods

2.1. Collection and maintenance of spiders

We collected sub-adult (i.e., one molt before adulthood) spider *P. pseudoannulata* from rice fields at Huazhong Agricultural University in Wuhan, Hubei Province, China (30°52′N, 114°31′E) from April to June 2017 and May 2019. The spiders were maintained individually and

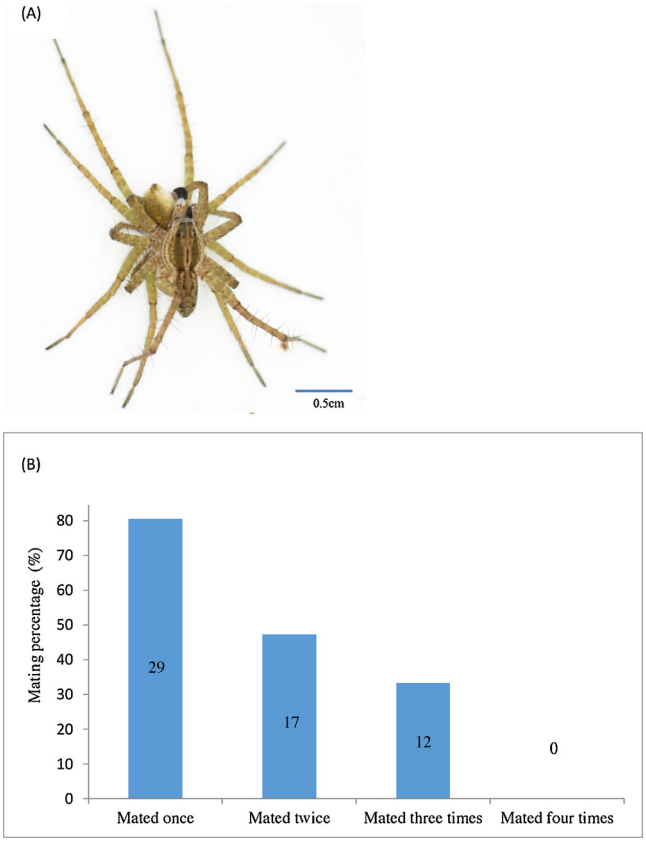

Fig. 1. Wolf spider *Pardosa pseudoannulata* mating (male inserts right palp into female copulation opening) (A) and impact of male mating history on subsequent mating success (B). Numbers on bars indicate number of spiders. Picture is credited to S. Zhang.

were visually isolated from each other in glass tubes (10 cm in length and 2 cm in diameter) under 14 h:10 h L:D photoperiod, 25 °C, and 50%–70% relative humidity in the laboratory. A piece of sponge with absorbed water was placed at the bottom of the tube to provide water for the spider, and the tube was covered by cotton wool. We fed the spiders with 20˜30 adult *Drosophila melanogaster* and one *Tendipes* sp. every 3 d. The developmental status (molting) of the spiders was checked twice daily (09:00 and 21:00).

2.2. First experiment: consecutive male mating

The effects of *P. pseudoannulata* male mating history on male remating behavior and female fitness were investigated by sequentially presenting four virgin females to each male at an interval of 7 d (i.e. a male can mate at most 4 times with 4 different females). The 7 d interval was selected as it is unknown whether or how often polygynous male wandering spiders recharge their palps, and 7 d may be sufficient to refresh and produce sperm. However, Costa (1998) found that male wolf spider *Lycosa malitiosa* did not recharge their palps within 10 d interval of mating, suggesting it may take more time before the male spider needs a new sperm replenishment, but it is unknown whether this species would recharge their palps in later life.

We used a petri dish (diameter 12 cm, height 1.8 cm) as the mating arena. The bottom of the petri dish was covered by a piece of qualitative filter paper, and a glass cylinder (diameter 10.5 cm, height 12 cm, with both ends open) was placed on the paper so one end was covered. Spiders were introduced into the glass cylinder from the other end. According to our preliminary observation, we found that females were much more aggressive than males, and it seems that female has territorial consciousness in the petri dish, they attack whatever was

introduced into the mating arena as long as they have seen it. Therefore, in each mating trial of our experiment, we introduced males into the arena first and allowed 20 min for adaptation, and then we introduced a randomly chosen female into it. The spiders were given 1 h to mate; if no mating occurred, the female was swapped for another until the male mated or was cannibalized.

All mating trials were performed between 08:00 and 12:00. After mating, we observed the pair for an additional 20 min to determine the occurrence of sexual cannibalism, after which the spiders were returned to their tubes. The mating trials were replicated another three times using the same male and different females, with an interval of 7 d given to the male between mating trial and sufficient prey provided. The filter paper was changed after each mating trial and the glass was cleaned using 100% ethanol. Body condition of the spiders, including body length, carapace width, and body weight, were measured before the mating trials. The carapace width is an often-used tool for evaluating the developmental rate of spiders (Hagstrum, 1971).

All courtship and mating behaviors were recorded using a Sony video camera (DSCRX1, Sony, Japan) as Jiang et al. (2018). If a female attacked or grabbed a courting male before copulation, they were separated immediately with a fine brush and returned to their tubes for other mating experiments (if the male was intact and had survived). In each mating trial, if the male and female spider failed to mate within an hour, it was regarded as copulation failure, and we provided another female to the male until the male had mated or had been killed by the female. Male courtship repertoire in this species includes approaching female carefully, raising a pair of forelegs, performing pushup and percussing substrate by forelegs (Zhang's observation). In our experiment, three behavioral variables were recorded: courtship duration (i.e., time interval between onset of male raising foreleg and mounting female), copulation duration (i.e., interval between onset of male mounting female and male dismounting female), and number of times male raised palps during courtship. Courtship intensity was calculated as the ratio of the number of times a male raised palps to courtship duration.

After successful copulation, spiders were returned to their original tubes, and each male was fed 10 adult *D. melanogaster* and one *Tendipes* sp. daily before the next trial. The mated females were maintained as described above and monitored daily until their death. The number of offspring and carapace width of the second-instar spiderlings were used to estimate female fecundity (Zuo et al., 2015). Oviposition and egg hatching were recorded every 12 h (09:00 and 21:00), with females that escaped or died naturally before oviposition not included in data analysis. After the second-instar spiderlings climbed onto their mother's back, we counted the number from each clutch. In addition, we randomly selected 10 second-instar spiderlings and fixed them in 75% alcohol to measure carapace width (from left to right) using an ocular micrometer under a microscope (DFC495; Leica, Solms, Germany).

2.3. Second experiment: sperm quantification

We performed another set of mating trials, in which virgin males were randomly assigned into four groups. The mating procedure was the same as in the mating trials above. Group A consisted of male virgins (control) ($N = 12$) who had their palps removed after 7d; Group B consisted of male who mated once ($N = 15$), after which their palps were immediately removed. Group C consisted of males who mated once ($N = 11$), 7 d after which their palps were removed. Group D consisted of males who mated twice ($N = 12$) with two different virgin females, with an interval of 7 d between matings, and their palps were removed 7 d after the second mating. We anaesthetized the males with CO_2 before surgically removing both palps, which were immediately placed in individual Eppendorf tubes (300 µl) and stored at −20 °C for later sperm counts.

The sperm counting methods followed Schneider et al. (2006) and Gabel and Uhl (2013). During sperm counts, we first separated the genital bulb from the rest of the palp to avoid contamination of the sample with non-sperm storage material. We transferred the genital bulb into an Eppendorf tube with 50 µl of buffer solution (0.9% saline + 10% triton-X detergent), ruptured it with clean, fine forceps, and then ground it with a pair of forceps to release the capsulated sperm. The sample was then vortexed for 20 s and ultrasonicated for 20 s to ensure highly homogeneous distribution of sperm. Two samples (4 µl) were extracted and placed on a 0.1-mm Neubauer improved double-chamber hemocytometer (Watson Biolab, Japan). The sperm cells were observed under a compound microscope at 400× magnification, which showed that they were rarely clumped. Three randomly chosen quadrates were counted per sample. The average of the three quadrates was calculated for each of the two sampling fields. We calculated the mean of the two sampling chambers, and then estimated the total number of sperm present in each palp using dilution factors. We averaged the sperm number in the two palps as the sperm number of a male. The mated females were maintained and monitored daily until their death.

2.4. Data analysis

Data were first checked for normality using the Kolmogorov-Smirnov test. The effects of male mating history on male courtship duration, copulation duration, and number of second-instar spiderlings were tested by one-way analysis of variance (ANOVA), and heteroscedasticity was checked by the Breusch-Pagan test with R package "lmtest". The carapace width of second-instar spiderlings in the different groups and sperm number among males under different treatments were compared by non-parametric Kruskal-Wallis tests. We used a binary logistic regression to examine the determinants of successful mating. We used mating success (fail = 0, success = 1) as the binomial response variable, and included body condition of both males and females (including age, carapace width/body weight, and body length) and male courtship intensity as predictors. In the second experiment, we used a general linear regression to test the correlation between copulation duration and number of sperm remaining in the palps (i.e., palps in Groups C and D, 46 palps in total). Sperm number between each group was compared by the non-parametric Mann-Whitney U test. Statistical tests were carried out in the R programming environment v3.6.0 (R Core Team, 2019) and SPSS (v13.0; SPSS Inc., Chicago, IL, USA).

3. Results

Being attacked did not deter a male's further mating attempts with other females, with courting behavior observed with new females after attacks by a previous female. Eventually, all males were cannibalized during courtship after their consecutive expositions to females.Male mating experience had a significant impact on subsequent copulation success (Pearson chi-square, $\chi^2 = 29.86$, $df = 3$, $P < 0.001$) (Fig. 1B). Male mating history had no significant effect on male courtship duration ($df = 2$, $F = 1.081$, $P = 0.306$), courtship intensity ($df = 2$, $F = 1.155$, $P = 0.322$), or copulation duration ($df = 2$, $F = 3.162$, $P = 0.056$) (Fig. 2).

Logistic regression showed that male courtship intensity and morphological measurements of both males and females (i.e., age, carapace width/ body weight, and body length) had no significant effects on male mating success in the three matings ($P = 0.346$, $P = 0.561$, and $P = 0.378$ for the first, the second and the third mating, respectively).

There were no significant differences in the female oviposition rate between the different male mating experience groups (Pearson chi-square, $\chi^2 = 0.059$, $df = 2$, $P = 0.971$). In addition, there were no differences in the number ($df = 2$, $F = 0.718$, $P = 0.495$) or carapace width ($\chi^2 = 0.710$, $df = 2$, $P = 0.871$) of second-instar spiderlings between treatments (Table 1), suggesting that male mating experience had almost no detectable effect on female fecundity.

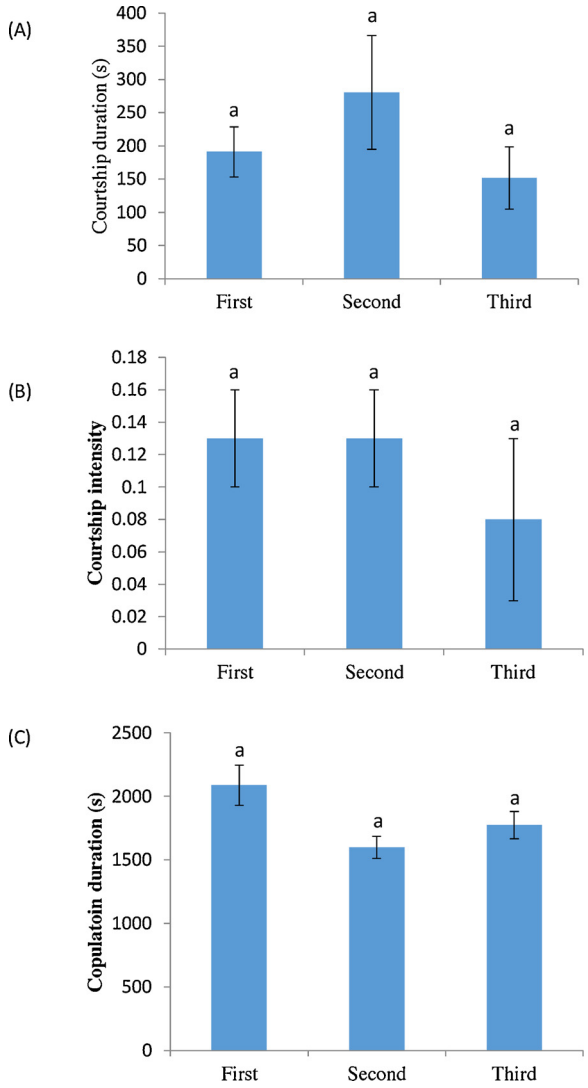

Fig. 2. Effects of mating experience (first, second, and third mating) on mean (± SE) (A) courtship duration, (B) courtship intensity, and (C) copulation duration of male *Pardosa pseudoannulata* (one-way ANOVA). Same lowercase letters on error bar indicate no significant differences between groups.

Sperm number varied between males in different groups. The sperm number in Group B was significantly lower than that in all other groups (Mann-Whitney U test, Group A vs Group B: $W = 178$, $P < 0.001$; Group C vs Group B: $W = 42$, $P = 0.036$; Group D vs Group B: $W = 44$, $P = 0.025$). However, there were no significant differences between Group A, C, or D (Kruskal-Wallis $\chi^2 = 0.095$, $df = 2$, $P = 0.954$) (Fig. 3), implying that sperm number in male palps had significantly decreased after mating, and that males can recharge their palps within the 7-d interval after mating. In addition, a simple linear regression showed a significant negative correlation between copulation duration and number of sperm left in the palps ($R^2 = 0.08$, $df = 44$, $P = 0.046$) (Supplementary Fig. 1).

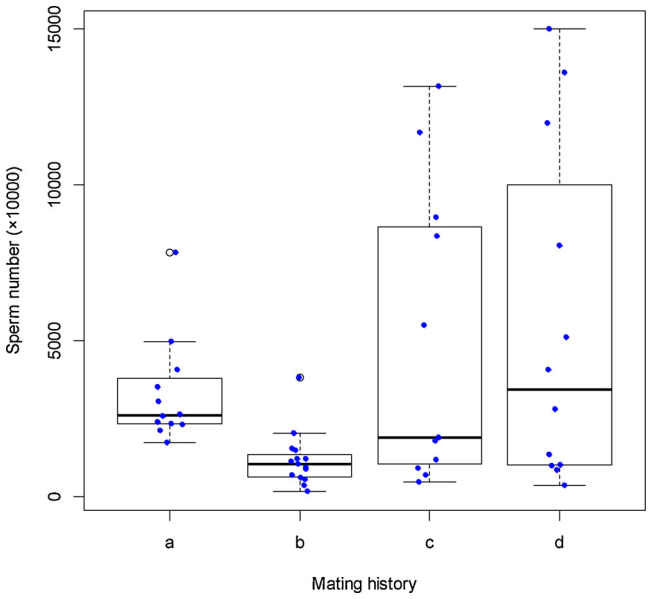

Fig. 3. Impact of mating history on sperm number in palps of *Pardosa pseudoannulata* males (a. 7 d after maturity; b. just mated; c. 7 d after first mating; d. 7 d after second mating). Each blue dot represents an average number of sperm contained in two palps of a male spider.

4. Discussion

In this study, we tested the impact of male mating history on their subsequent mating success and the fecundity of females in the wolf spider *P. pseudoannulata*. Results showed that male mating history significantly affected future mating success negatively, but not the fecundity of females that were inseminated. Molina and Christenson (2008) reported that mating history has little impact on the sexual behavior or future performance of the cobweb spider (*Nesticodes rufipes*), including sperm release. This is consistent with our study. We did not find a significant decline in sperm number in males with different mating histories (Fig. 3); on the contrary, the sperm number of some twice-mated males was even greater than that of virgin males, which may be due to the variance in body condition of different males (Fig. 3), and males in good body condition may produce more sperms during sperm replenishment. For wandering spiders, direct evidence showing palp recharge remains limited (Costa, 1998; Huber, 1998; Schneider and Andrade, 2011).

Different from web-building spiders, male wandering spiders usually do not exhibit mate guarding behavior. To maximize their reproductive interests, they instead continue to seek out and mate with as many new females as possible. In *P. pseudoannulata*, males would continue to court all females (including the female they have just mated with), regardless of mating history, even after only a short period of rest (~10 min) between matings (Zhang's observation). However, the females were monandrous and refused further copulation after initial mating (Zhang's observation); therefore, the reproductive success of males is largely dependent on the obtained number of matings. Contrary to our results, Jiao et al. (2011) reported that mating experience in male wolf spiders (*P. astrigera*) can significantly affect the

Table 1
Effects of male mating history on oviposition rate and number and carapace width of second-instar spiderlings of *Pardosa pseudoannulata*.

Mating order	% Oviposition rate (n)	Number of spiderlings	Carapace width of spiderlings (mm)
First	75 (18/24)	66.6 ± 6.5 (n = 18)[a]	0.46 ± 0.08 (n = 10)[a]
Second	68.75 (11/16)	71.2 ± 8.4 (n = 11)[a]	0.44 ± 0.01 (n = 10)[a]
Third	70 (7/11)	56.1 ± 7.9 (n = 7)[a]	0.44 ± 0.01 (n = 10)[a]

Data are means ± standard error. Differences were compared using one-way ANOVA. Values ending with same letter denote no significant difference ($P > 0.05$).

reproductive output of females. This result may be because the 24-h interval between male matings was insufficient to produce new sperm and recharge palps, leading to sperm depletion and the inability to fertilize all eggs of a female. In invertebrates, the reproductive output of females mated to virgin males is usually significantly higher than that of females mated to sexually experienced males (Torres-Vila and Jennions, 2005) because sperm production is costly to males, especially as males age (Ceballos et al., 2015; Dean et al., 2010; Sasson et al., 2012). As *P. pseudoannulata* males can fertilize females even after living in the laboratory for three months (Zhang's observation), we believe that males possess adequate energy to refresh sperm storage and mate multiple times without substantial declines in sperm quantity or quality in the first few weeks after maturity.

It has been reported in various animals that mating with a multiply-mated male can decrease female longevity (Perez-Staples et al., 2008; Edward et al., 2011). It may be that the components of ejaculates (e.g., seminal proteins, macromolecules) are beneficial to the female (Perry et al., 2013), and the depletion of these components in multiple-mated males can result in negative effects on female longevity. This may also be the reason why females were not willing to mate with mated males in our study. We did not record the longevity of females in our study, but this should be investigated in the future. However, it has been reported in wolf spider *Schizocosa malitiosa* that male may transfer receptivity-inhibiting substances to females during mating, which may inhibit females to accept further mating in a certain period of time (Aisenberg and Costa, 2005; Michalik et al., 2013). We may investigate in future whether male *P. pseudoannulata* can also transfer such kind of receptivity-inhibiting substances to females during mating, which caused female monandry. In addition, it is not known whether spiders refresh their seminal fluid proteins during sperm recharge. Thus, the dynamics and functions of seminal fluid proteins in polygynous spiders during mating require further research.

In our study, females were more likely to mate with males with less mating experience, indicating that females can differentiate the mating status of the courting males. However, why would females discriminate against males that mated before, since they had already replenished their sperm, and mating with them had no negative effects? It may be because females can discriminate between males with different previous mating histories, but cannot assess whether the males had enough time to replenish their sperm reserves. In addition, we did not find any significant differences in male courtship duration or intensity or in any morphological measurements between the three groups, indicating that the energy spent by a male during courtship did not significantly vary, and previous mating had little impact on the physical condition of males after 7 d of rest. Nevertheless, no males were able to mate four times or more in our experiment (Fig. 1B), probably because of age, and future research should investigate how many times and how often males can recharge their palps by dissecting male palps at different intervals (e.g., daily rather than after 7 d). In addition, no morphological measurements in either males or females (e.g., age, body length, body weight, carapace width) had significant effects on male mating success. These results indicate that females may assess male mating status by other means, e.g., chemical cues. It has been widely reported that females can assess male functional fertility by selecting secondary sexual traits, such as male ornamentation, coloration, chemical cues, and body size (Andersson, 1994). Furthermore, in fish, the male courtship rate can co-vary with sperm quantity, quality, and fertilization efficiency (Weir and Grant, 2010). This is consistent with our results, which demonstrated that sperm quantity, courtship duration, and courtship intensity did not change substantially between the three matings in males. Female animals are known to use chemical cues to adjudge the mating status or quality of males, and this is commonly observed in spiders (Rypstra et al., 2003; Schneider and Andrade, 2011). Jiao et al. (2011) found that mating history in male wolf spiders *P. astrigera* has little impact on subsequent copulation success, with half the tested males mating successively with five virgin females at an interval of 24 h. In our study, females apparently discriminated against mated males, which may be via chemical cues released from male bodies. This has also been reported in some insects, such as the parasitic wasp *Nasonia vitripennis* (Ruther et al., 2009), and in the stone crab *Hapalogaster dentata* (Sato and Goshima, 2007). In the future, we will investigate whether *P. pseudoannulata* females respond differently to the body extracts of males with different mating statuses and what factors play the most important roles during male courtship.

5. Conclusions

In the wolf spider *P. pseudoannulata*, mating history of the polygynous male had a significant effect on subsequent mating success, but little impact on female fecundity, with no differences observed in the number and carapace width of second-instar spiderlings between treatments. Sperm counting results showed there were no significant differences in sperm number between males with different mating histories, indicating that males may recharge their palps with newly produced sperm after mating. However, how virgin females differentiate the mating status of males remains unknown, and whether male mating history significantly affects female longevity needs further investigation. Our study may provide a new perspective to the study of sexual selection and sexual conflict of monandrous animals, especially in taxon of spiders.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.beproc.2019.103921.

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