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## Costs and benefits of multiple mating in a species with first male sperm precedence

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## Summary

1. Different patterns of sperm precedence are expected to entail different costs and benefits of mating for each sex that translate into distinct predictions regarding mating system evolution. Still, most studies addressing these costs and benefits have focused on species with mixed paternity or last male precedence, neglecting first male sperm precedence. We attempted to understand whether this latter pattern of sperm precedence translates into different costs and benefits for each sex in the haplodiploid spider mite *Tetranychus urticae*, a species in which female multiple mating is prevalent but most offspring is sired by first males.
2. First, we assessed the stability of the sperm precedence pattern. To do so, we measured offspring paternity after exposing females to a different number of matings and mating intervals. Next, to determine the potential costs or benefits of multiple mating for females under different contexts, we measured the fecundity and survival of females that re-mated at different time points. To measure the potential costs of multiple mating for males, we analysed male survival in the presence of different numbers of virgin or mated females. We also tested whether males can reduce offspring production of their competitors, by reducing the production of fertilized offspring of mated females.
3. We found no change in the pattern of sperm precedence, independently of the mating interval between matings and the number of matings. Females paid a cost of mating, as multiply mated females laid fewer eggs than once-mated females. However, while males had reduced survival when exposed to an intermediate number of virgin females, they paid no additional costs of mating with mated females. Moreover, females that mated multiple times produced fewer fertilized offspring than females that mated once. Thus, males that copulated with mated females reduced the fitness of other males, potentially leading to a relative fitness benefit for themselves.
4. Our results show that complex costs and benefits may arise in males in species with first male sperm precedence. How these costs and benefits affect the maintenance of selection for polyandry remains an open question.

## Keywords

Multiple mating; sperm competition; mating costs; polyandry, arthropods; spider mites

## Introduction

Multiple matings are prevalent in species with internal fertilization (Simmons, 2001). However, this behaviour is expected to entail negative consequences for both sexes, such as increased risk of predation and energy expenditure (Arnqvist & Nilsson, 2000; Arnqvist & Rowe, 2005). Consequently, for multiple mating to be selected, the reproductive advantage gained by this behaviour should be higher to the costs incurred and the costs and benefits obtained by each sex with multiple mating should depend on the pattern of sperm precedence of the species.

In species with mixed paternity, the number of offspring sired by males is expected to increase with each additional mating (Bateman, 1948), hence selection in this sex should favour increased mating frequency, as a result of offensive and defensive adaptations to sperm competition (Ridley, 1989b). Furthermore, multiple mating can be advantageous for females whenever they obtain direct and/or indirect benefits from mating. Direct benefits, such as increased fecundity and survival, can be obtained via nuptial gifts or nutritious ejaculates (Snook, 2014). Indirect (or genetic) benefits, can occur via sperm choice or by using sperm from more than one male, and they translate into increased offspring quality and diversity, respectively (Kvarnemo & Simmons, 2013; Snook, 2014). Therefore, in these species, the balance between costs and benefits obtained with each mating is generally not the same for both sexes (Bateman, 1948), giving rise to sexual conflict. In these cases, one sex employs reproductive tactics to enforce matings while the other resists them, depending on which sex will benefit the most from each additional mating (Arnqvist & Rowe, 2005; Parker, 2006).

Alternatively, under complete first male sperm precedence, only the first mating of a female generates fertilized offspring. In such case, one can expect males to only mate with virgin females. Furthermore, females cannot obtain genetic benefits, since they cannot choose which sperm to use or decide to use sperm from more than one male. Thus, in the absence of direct benefits for females, selection in these species should favour monandry, as both sexes are expected to invest all resources in copulations involving virgin females only (Thomas, 2011), thereby limiting the scope for sexual conflict (Hosken, Stockley, Tregenza, & Wedell, 2009). Consequently, one would expect first male sperm precedence to be uncommon, as monandry seems to be beneficial for both sexes. Yet, at least 10% of insect species have been classified as having this pattern of sperm precedence (Simmons, 2001) and it seems to be even more frequent in arachnids, namely in spiders (Austad, 1984; Wedell, Gage, & Parker, 2002). One possible explanation for its occurrence would be that this pattern is evolutionarily transient (Dougherty, Simmons, & Shuker, 2016), and species will rapidly evolve to monandry or to a system in which multiple males sire the offspring of one female. Alternatively, the pattern of sperm precedence may be context dependent. Indeed, in several species, it has been shown to vary according to multiple factors, such as the number of matings (Zeh &

Zeh, 1994), the interval between mating events (Bullini, Coluzzi, & Bianchi Bullini, 1976) or the effectiveness of the first mating (Weldingh, Toft, & Larsen, 2011). Finally, multiple mating, even without offspring fertilization, might be beneficial for one of the sexes. On the one hand, females might gain direct benefits with multiple mating, despite using the sperm of the first male only. On the other hand, males may be able to increase their relative reproductive success without obtaining any paternity share. Indeed, it has been suggested that males may displace or kill the sperm of the male that would inseminate the female, this way increasing their own relative fitness (Harshman and Prout 1994, Macke et al. 2012).

Because of the potential lability of sperm precedence patterns and their ecological and evolutionary implications, it is important to know (a) if such patterns are maintained under different contexts and (b) how they translate into cost and benefits for both sexes. As studies concerning species with first male sperm precedence are remarkably scarce (but see Fisher et al. 2013; Boulton and Shuker 2015, 2016), our knowledge on these issues remains incomplete.

We study the consequences of multiple mating for both sexes in the two-spotted spider mite, *Tetranychus urticae*. Spider mites are haplodiploid, with females stemming from diploid, fertilized eggs and males emerging from haploid, unfertilized eggs. Typically, the adult sex ratio in this species is female biased (Mitchell, 1972), but it can change with several factors such as temperature and different scales of mate competition (Macke, Magalhães, Bach, & Olivieri, 2011; Roy, Brodeur, & Cloutier, 2003). An earlier study suggest that only the first copulation of a female results in fertilized offspring (Helle, 1967). This leads to the expectation that males should only mate with virgin females to avoid unnecessary costs. Accordingly, males actively guard juvenile quiescent females and mating occurs as soon as females moult into virgin adults (Potter, Wrensch, & Johnston, 1976), a behaviour that is consistent across species with first male sperm precedence (Ridley, 1989a). In addition, when given the choice between mated and virgin females, males prefer to mate with virgins, basing their decision upon volatiles and chemical trails (Oku, 2010; Rodrigues, Figueiredo, Varela, Olivieri, & Magalhães, 2017). Nevertheless, matings involving mated females are frequently observed (Clemente, Rodrigues, Ponce, Varela, & Magalhães, 2016; Oku, 2010). Here, we provide a comprehensive account of potential costs and benefits of multiple mating for both sexes in spider mites. First, we performed paternity tests, using a recessive mutation that codes for resistance to a pesticide as a genetic marker, in order to describe the pattern of sperm precedence. To test whether this pattern varies with the female mating history, we varied the number of matings and the interval between mating events. We then measured the fecundity and survival of females that re-mated at different time points, to assess potential costs or benefits of multiple mating for females. In addition, we assessed potential costs and benefits of multiple mating for males, taking into account that these should vary depending on the mating status of the females involved.

## Materials and Methods

### Spider mite populations and rearing conditions

To study the costs of multiple mating in males, we used a population collected in Carregado, Portugal, established at the University of Lisbon in 2010 from approximately 300 individuals (TuTOM; Clemente et al., 2016). To study the other traits, we used the EtoxR strain, resistant to etoxazole (Uesugi, Goka, & Osakabe, 2002), and the LondonS strain (Grbić et al., 2011), susceptible to the same pesticide, both established at the University of Lisbon in 2013 from approximately 2000 individuals. Etoxazole is a pesticide that interferes with chitin synthesis, affecting spider mite embryos and juvenile stages at the time of hatching or ecdysis (i.e., at the quiescent stage; Van Leeuwen et al. 2012). In the EtoxR strain, resistance to Etoxazole is recessive and conferred by a single chitin synthase 1 (CHS1) amino acid change (Van Leeuwen et al., 2012). We used this trait as a marker for paternity by applying pesticide to the offspring of resistant females mated to resistant and susceptible males. Because in haplodiploids males only inherit the genetic material of their mothers, only daughters are affected by pesticide application. Thus, after pesticide application we could identify three categories of offspring: alive sons, alive daughters and dead juveniles; alive daughters were sired by resistant males, while dead juveniles were sired by susceptible males (see details in “Data Collection”).

All populations were exposed to an antibiotic treatment to eliminate symbionts, using a protocol adapted from Breeuwer (1997). Prior to the experiment, we confirmed that resistance was fixed in the EtoxR strain and absent in the LondonS strain, following a protocol adapted from Van Leeuwen et al. (2012). We compared the fitness of resistant females mated with resistant or susceptible males, to account for potential male genotype effects, and no significant differences were found (Fig. S1).

All spider-mite populations were reared in large numbers (>2000) on bean plants (*Phaseolus vulgaris*, Fabaceae, var. *Enana*; Germisem Sementes Lda, Oliveira do Hospital, Portugal), under controlled conditions (25°C, photoperiod of 16L: 8D).

### Experimental Setup

#### Mating protocol

Randomly selected virgin females from the EtoxR strain were allowed to mate once, twice or multiple times (O, T and M, respectively) and the mating interval between the first and subsequent matings was either 0 or 24 hours (re-mated immediately, I or re-mated later, L, respectively; Fig. 1a). Females that mated only once mated either with a resistant or with a susceptible male. In treatments with two or more

matings, females mated either first with a resistant and then with a susceptible male, or the opposite. Thus, these females were allocated to 5 different treatments: O, TI, MI, TL and ML (Fig. 1a).

A description of the methods is provided in Figure 1. Briefly, EtoxR quiescent females were isolated for 24 hours on leaf discs on water-saturated cotton without males. Once they became adult (one day later), groups of 5 females were placed with 5/6 susceptible - or resistant - males on 0.8 cm<sup>2</sup> leaf discs (to ensure food *ad libitum*). The patches were observed for 2 hours and once a female had successfully mated, she was transferred to a new patch, either empty or with males from the other strain, in the same proportion (5 females: 6 males). Half of the females placed with males on the second patches were observed for two more hours and isolated when mated (TI). The other half was left unobserved on the patch with males for 24 hours (MI), which, in this species, is a sufficiently large time interval to ensure the occurrence of multiple matings (authors Pers. Obs., Krainacker and Carey 1989; Magalhães et al. 2007). The females left without males after the first mating on the first day were either left in the same patches for one more day (O) or transferred to patches with males of the alternative strain. Again, half the females were observed for 2 more hours and isolated if mated (TL) and the other half was left unobserved on the patch with males for 24 hours, thus allowing for multiple matings (ML). After mating, two- to three-days old females were isolated on a 2.55 cm<sup>2</sup> leaf disc placed on water-soaked cotton, in order to measure the life-history traits mentioned below. To maximize the success of the first mating, we (a) isolated males prior to testing them, (b) limited their copulations to 5 females, a value below their daily reproductive limit (Krainacker & Carey, 1989) and (c) discarded first matings interrupted by other individuals on the patch. Pre-copulatory choice cannot be excluded from this setup but should be reduced owing to (b) and (c). Due to considerable experimental effort, this experiment was done across 22 separate days, all treatments being represented at each day.

#### Data Collection

##### *The effect of mating history on the pattern of sperm precedence*

To measure the lability of the pattern of sperm precedence, we performed a paternity test on the offspring of females that mated with different numbers of males at different time intervals. For this purpose, females isolated on leaf discs were transferred every three days to a new disc where they would lay eggs. To assess paternity, the eggs laid by the isolated females were allowed to develop until they reached the first juvenile stage (three days after female transfer to a new disc), then leaf discs were transferred to water-soaked cotton with diluted etoxazole (500 ppm; Fig. 1b). Six days later, the number of adult daughters, adult sons and dead juveniles on each leaf disc was recorded. Spider mites are haplodiploid, producing haploid sons, which result from unfertilized eggs, and diploid daughters, stemming from fertilized eggs (Helle & Sabelis, 1985). Because males only inherit the genetic material of their mothers, the survival of sons should not be

affected by pesticide application, since all females were resistant to etoxazole (two alleles, RR), being their sons resistant themselves (one allele R). Daughters, on the other hand, could be homozygous for resistance (two alleles, RR), if their father was resistant (one allele, R), or heterozygous (two alleles, RS), if their father was susceptible (one allele, S). Because resistance is recessive, the number of alive daughters and dead juveniles after pesticide application indicates the number of offspring sired by resistant and by susceptible males, respectively. Note that natural juvenile mortality is not totally absent in treatments involving single matings with resistant males, and the penetrance of the pesticide is not 100% hence some mortality is still present. Therefore, our treatments involving double matings are compared to results stemming from these controls, and not to a theoretical expectation of 100% or 0% mortality, respectively. In total, we assessed the paternity of offspring from 377 females.

#### *Does multiple mating entail costs or benefits in females?*

We tested potential costs or direct benefits of mating multiple times for females, by measuring the effect of the number of matings and the interval between matings on offspring number and female survival. To this aim, the same females used to assess paternity were used to measure daily fecundity and female survival (Fig. 1b). Females were transferred every three days to a new disc and the eggs laid on the old leaves were counted. The survival of mated females was followed daily. Daily fecundity was measured as the total number of eggs laid per female divided by the number of days the female was alive. In total, we analysed the daily fecundity and the survival of 485 females.

#### *Does mating with mated females entail costs or benefits in males?*

Because matings with mated females are not expected to generate fertilized offspring in species with first male sperm precedence, male benefits are dependent on the mating status of the females they mate with. The same might be expected in term of costs, assuming mating investment varies with female mating status as well (Rodrigues et al., 2017). To account for this possibility, male survival was measured in the presence of virgin or in the presence of mated females.

To this aim, males and females were isolated separately at the quiescent stage, to control their age and ensure virginity prior to the experiment. When these individuals became adults (*circa* 24 hours later), groups of ten females were either left isolated (virgin – V) or placed with 15 males (mated – M). The latter were left with males for 24 hours to ensure the occurrence of multiple matings (authors Pers. Obs., Krainacker & Carey 1989; Magalhães et al. 2007). The next day, focal virgin one-day old males were placed on a new leaf disc with either 1, 5 or 20 virgin (V1, V5, V20) or mated females (M1, M5, M20) from the previous day. To normalize densities across treatments, patch size varied according to the number of individuals (0.38 cm<sup>2</sup>, 2.55 cm<sup>2</sup> or 9.1 cm<sup>2</sup> for patches receiving 1, 5 or 20 females, respectively). The

focal male was then transferred daily to a new patch with the same number of (mated or virgin) females in every treatment except for the ones with 20 females. In this last treatment, as male mating capacity decreases with age (Krainacker & Carey, 1989), from the third day onwards, the focal male was placed with 12, instead of 20 females (size of the patch: 6.25cm<sup>2</sup>). Male survival was recorded every day until death. In total, the survival of 180 males was analysed. Due to considerable experimental effort, and the very high number of females required for each replicate, this experiment was carried out across 66 separate days.

Mating with mated females may provide a relative increase in the fitness of males, despite first male sperm precedence. For example, by mating with mated females, males may displace or kill the sperm of another male inside the female and thereby increase their relative reproductive success (Harshman & Prout, 1994; Macke et al., 2012). To test this hypothesis, we compared the total number of daughters (i.e. male genetic contribution to the next generation) produced by females with different number of matings and mating intervals. The same females used to assess paternity, daily fecundity and survival were used to measure this trait (Fig. 1b). Because the aim was to study lifetime fecundity, females who died due to artificial causes (drowning in water-soaked cotton) were excluded from the analysis. In total, we analysed the total number of daughters produced by 427 females.

### Statistical analyses

All analyses were carried out using the R statistical package (v. 3.0.3). Maximal models were simplified by sequentially eliminating non-significant terms from the highest- to the simplest-order interaction, with the highest p-value to establish a minimal model (Crawley 2007; see Table S1), and the significance of the explanatory variables was established using chi-squared tests, in the case of discrete distributions or Wald F tests, in the case of continuous distributions (Bolker et al. 2008; see Table S2). *A posteriori* contrasts with Bonferroni corrections were done to interpret the significant effect of factors with more than two levels (glht, multcomp package): comparisons were done between treatments with single matings, or single females in the case of male survival, and all other treatments (Table S3).

To analyse the effects of mating on female survival, daily fecundity, total number of daughters and offspring paternity, the same model structure was followed: the mating treatment (i.e. 0: once-mated, TI: twice-mated immediately, MI: multiply-mated immediately, TL: twice-mated later, ML: multiply-mated later) was fitted as fixed explanatory variable, whereas day and male type order (female mated first with a resistant and then with susceptible males, or the opposite) were fitted as random explanatory variables.

To analyse the proportion of offspring sired by the first male, we redistributed the data of offspring survival into two variables called contribution of the 1<sup>st</sup> male (1P) and contribution of the second male (2P) to

offspring. 1P corresponds to the number of dead juveniles or the number of alive daughters, depending on whether the first male was susceptible or resistant, 2P corresponds to the number of alive daughters or the number of dead juveniles, depending on whether the first male was susceptible or resistant. These parameters were computed using the function `cbind`, with 1P, 2P and the number of sons as arguments. Since the model was greatly overdispersed, we used a generalized linear mixed model with a beta-binomial error distribution and added the term `ziformula=~1` to the model (`glmmTMB`, `glmmTMB` package) (Brooks et al., 2017). Offspring sex-ratio, i.e. the proportion of sons, was also computed using the function `cbind` with the same parameters but reorganized (see Table S1). A generalized linear mixed model with a beta-binomial error distribution was used to account for overdispersion (`glmmTMB`, `glmmTMB` package) (Brooks et al., 2017).

Daily fecundity per female was transformed to improve normality (Box-Cox transformation; Crawley 2007) and subsequently analysed using linear mixed-effect models (`lmer`, `lme4` package).

Female survival was analysed using a Cox proportional hazards mixed-effect models (`coxme`, `coxme` package).

To analyse the effects of mating on male survival, the female status (i.e., M: mated; V: virgin) and the number of females on each patch (1, 5, 20) were fitted as fixed explanatory variables, and day was fitted as a random explanatory variable. Male survival was analysed using a Cox proportional hazards mixed-effect models (`coxme`, `coxme` package). Because the interaction between the fixed factors was significant, we analysed separately each level of female status for the effect of female number.

The total number of daughters sired by the first male was analysed using the variable “contribution of the 1<sup>st</sup> male” (1P). This parameter was analysed using a model with negative binomial distribution (`glmer.nb`, `lme4` package) to account for data overdispersion.

## Results

The effect of mating history on the pattern of sperm precedence

First male sperm precedence is virtually complete and no differences in sex-ratio were found across treatments. Overall, there was no significant effect of the mating treatment on the proportion of offspring sired by the first males ( $X^2_4=1.411$ ,  $P=0.842$ ) and the proportion of sons remained the same across treatments ( $X^2_4=3.78$ ,  $P=0.437$ ; Fig. 2, Table S2).

Does multiple mating entail costs or benefits in females?

The mating treatment affected daily fecundity significantly ( $F_{4,389,95}=8.633$ ,  $P<0.001$ ). Contrast analyses revealed that females that mated multiple times 24h after their first mating had significantly lower fecundity compared to once-mated females, while females from all other treatments laid the same number of eggs (O vs TI:  $Z=-0.025$ ,  $P=1.00$ , O vs TL:  $Z=-0.725$ ,  $P=1.00$ , O vs MI:  $Z=-1.976$ ,  $P=0.193$  and O vs ML:  $Z=-4.151$ ,  $P<0.001$ ; Fig. 3a, Table S3).

Multiple mating did not affect significantly the survival of females. Indeed, while the survival of females with different mating histories varied significantly ( $X^2_4=10.899$ ,  $P=0.0277$ ) it was never significantly different from that of the once-mated control (O vs TI:  $Z=-0.203$ ,  $P=1.00$ , O vs TL:  $Z=1.235$ ,  $P=0.867$ , O vs MI:  $Z=1.379$ ,  $P=0.671$  and O vs ML:  $Z=-1.719$ ,  $P=0.343$ ; Fig. 3b).

Does mating with mated females entail costs or benefits in males?

Male survival varied with both the mating status and the number of females he was placed with on each patch ( $X^2_2=7.198$ ,  $P=0.027$ ). Indeed, males placed with virgin females survived less in the presence of 5 females than in presence of 1 female per day (V1 vs V5:  $Z=2.349$ ,  $P=0.038$ ; V1 vs V20:  $Z=0.353$ ,  $P=1.00$ ; Fig. 4a, Table S3). However, males placed with mated females exhibited the same survival rate, independently of their number ( $X^2_2=0.497$ ,  $P=0.78$ ; Fig. 4b).

The total number of offspring sired by the first male depended of the female's mating treatment ( $X^2_4=15.956$ ,  $P=0.003$ ). Indeed, multiply mated females with an interval of 24 hours between first and subsequent matings produced fewer fertilized offspring, compared to once mated females (O vs ML:  $Z=3.174$ ,  $P=0.006$ ; Fig. 4c, Table S3). However, females belonging to all other treatments produced the same number of fertilized offspring as once-mated females (O vs TI:  $Z=-0.024$ ,  $P=1.00$ , O vs TL:  $Z=-0.315$ ,  $P=1.00$ ; O vs MI:  $Z=-0.367$ ,  $P=1.00$ ; Fig. 4c, Table S3).

## Discussion

In this study, we found that, in *T. urticae*, nearly all fertilized offspring were sired by the first male, independently of the mating interval and the number of matings. In addition, a decrease in fecundity, but not in survival, was found in females with multiple mating opportunities. Males, however, did not suffer increased costs when placed with mated females, even though mating with 5 virgin females was costly. In addition, females mated multiple times produced fewer eggs fertilized by first males, which suggests that males may increase their relative fitness when mating with mated females.

We found a consistent pattern of first-male precedence in our system. In our set-up, we cannot disentangle complete from nearly complete first male sperm precedence, because natural death in the quiescent stage may be confounded with death by pesticide exposure. Thus, there is a non-null threshold of detection for fertilization by second males and we cannot exclude the possibility that second males contribute to siring less than 5% of the offspring. Whether this small percentage is an artefact of our design and may help explain the existence of polyandry in this species remains unclear.

Other factors besides the number of matings and the mating interval could influence the pattern of sperm precedence. For instance, in *Drosophila pseudoobscura*, a mostly monandrous fly, females use the sperm from the second mating whenever the first mating opportunity fails (Fisher et al., 2013). The authors make a distinction between true polyandry and pseudopolyandry, which occurs when females remate but no sperm competition takes place, owing for instance to lack of sperm transfer. This could be the case in spider mites as well. Indeed, sperm depletion, or incomplete sperm transfer in the first mating may result in some paternity share (Krainacker & Carey, 1989). Unfortunately, the frequency of sperm-depleted matings in natural populations of spider mites is unknown.

The fact that we could not find unequivocal evidence for mixed paternity points to the absence of indirect benefits of multiple mating for females. Indeed, these type of benefits are only possible when females can use the sperm from more than one male or chose which sperm to use (Snook, 2014). Additionally, we show that females that mated multiply paid a cost in terms of fecundity. This is surprising since, of the few studies that explored the costs and benefits of polyandry in species with first male sperm precedence, most show that male ejaculates provide benefits to the females, thereby indicating the existence of direct benefits of multiple mating for females (e.g. Thailayil et al. 2011; Helinski & Harrington 2012; but see Rebecca A. Boulton & Shuker, 2015). The mating costs observed here are, most likely, a reflection of the negative effects of multiple mating coupled with increased number of mating attempts. The fact that we only found a cost when the interval between the first and subsequent matings was of 24 hours may be explained by differences in female receptivity across different mating intervals. Indeed, females that mate 24 hours after the first mating, independently of the number of matings, generally take longer to mate than females that re-mate immediately after the first mating (authors personal observations, Clemente et al. 2016). This suggests that females become more resistant to mating sometime after the first copulation. As a consequence, males may intensify their mating attempts with those females, possibly leading to increased costs for them.

The fact that we did not detect clear benefits of multiple mating in females suggests that polyandry is maintained in this species because it is beneficial for males. However, the pattern of sperm precedence observed indicates that males virtually do not sire offspring when mating with mated females. In several

species, the cost of mating for males varies with the mating status of females. Matings with mated females may entail fewer costs, if males allocate sperm differently according to the reproductive value of females (“strategic ejaculates”; Simmons 2001; Kelly & Jennions 2011). Accordingly, in species with first male sperm precedence, we expect males to invest more in matings with virgin females, as these have the highest reproductive value. Our results are in line with this prediction, as they show that only males mating with virgins pay a cost of mating. This suggests that males invest more in reproduction when placed with these females, either increasing their mating rate or transferring more sperm at each copulation, although it is intriguing that fewer costs were detected at the highest female density. One such investment may be the energy spent in approaching females. Indeed, video recordings suggest that males approach females, and not the opposite, and that females reject males’ mating attempts by moving away from them (Fig. S2). In addition, previous results show that copulations with virgin females occur at a faster rate and last longer than copulations with mated females (Rodrigues et al., 2017). Nevertheless, and regardless of the mechanism, it seems that males may engage into matings with virgins, which result in high offspring yield but also a survival cost, or into matings with mated females, yielding no offspring but also fewer costs. Owing to its low costs, matings with mated female may take place either because there is no selection pressure to eliminate such behaviour or to guarantee that they do not miss opportunities of mating with virgins owing to a mistake in mating status discrimination (Rodrigues et al., 2017).

Despite being mostly ineffective, matings with mated females may still yield some benefits to males. A decrease in the total fecundity of multiply-mated females has been observed in spider mites (Macke et al. 2012), a result that we replicate here. This may translate into fewer offspring being sired by first males (the Relative Fitness hypothesis, Macke et al. 2012). Here, we validated this hypothesis by showing that males produced fewer offspring (i.e., daughters) when they were the first mating with females that mated multiply 24 hours later. Because the proportion of daughters remained unchanged, this decrease in the number of daughters is probably due to a decrease in fecundity of females owing to costs of mating with several males. Therefore, mating with mated females could increase the relative reproductive success of subsequent males, by reducing the genetic contribution of the first males to the following generations.

The benefits of reducing the fitness of other males are most likely context dependent. Indeed, this strategy requires that the males mating with mated females (or their brothers) produce some descendants and pay a low penalty with this behaviour. Apart from the life-history costs of the behaviour, which we showed here to be low, they could lose mating opportunities with virgin females. Moreover, the uncovered benefits should be dependent on population structure. Indeed, in large populations, benefits should be mitigated as they are shared by all other males of the population, while in small populations, relatedness can be high, in which case, reducing the fitness of other related males in the population may not be advantageous. Indeed, theory predicts that relatedness among males should select for males inducing less

harm to the females they mate with, as this would undermine the reproductive success of related individuals (Pizzari, Biernaskie, & Carazo, 2015; Rankin, 2011). Accordingly, it has been empirically shown that related males inflict less harm to females than unrelated males (e.g., Carazo et al. 2014). Yet, if competition occurs locally, males are not expected to behave differentially towards related males, as these are their only competitors (Pizzari et al. 2015). Finally, the effectiveness of this behaviour relies on a collective action, as one mating is not enough to reduce the fitness of the first. Therefore, the population structure in which this behaviour will be beneficial should roughly correspond to a situation of budding dispersal (Gardner, Arce, & Alpedrinha, 2009), in which related males arrive together in a patch occupied by unrelated individuals, and collectively reduce the fitness of unrelated males for the next generation. Spider mite populations do go through a dispersal-colonization phase during which their population structure should be very similar to the one described. This phase is followed by an expansion phase, during which waves of virgin females should be followed by waves of mated females. Males may thus display both strategies, first mating with virgin females, then later, when these become scarce, copulating with mated females. Thus, spider mites are expected to face conditions for this behaviour to be selected. Still, it is not clear whether such conditions occur often enough for selection to occur. It is thus important to design experiments varying population structure to test these ideas.

Altogether, our results show that multiple mating is costly for females but that matings with mated females are potentially beneficial for males. The consequences of polyandry in species with first male sperm precedence have seldom been explored, leaving a gap in our knowledge (but see Dougherty et al. 2016). Indeed, if we had found no benefits of polyandry in both sexes, we could speculate that selection would be favouring monandry. Conversely, if males and females benefited from polyandry, we could expect that selection would maintain polyandry, which in turn, would open the door for an evolution of the sperm precedence pattern itself. Because we found that some males may benefit from mating with mated females, but that females suffer costs with polyandry, conflicts between sexes should be present and the direction of selection on polyandry will depend on sexually antagonistic coevolutionary responses, e.g. male ability to overcome female resistance.

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#### **Authors Contributions**

Resources provisioning: SM, TVL; Experimental conception and design: LRR, IO, SM; acquisition of data: LRR, ARTF; statistical analyses: LRR; paper writing: LRR, SM, with input from all authors. All authors have read and approved the final version of the manuscript.

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**Figure 1. Protocol followed to assess sperm precedence and female fecundity and survival. a) Mating Protocol.** Groups of 5 females and 5/6 males were placed together on patches until they mated. Females mated once (O), twice (T) or multiply (M), with a mating interval between the first and subsequent matings of either 0 hours (I, Immediately) or 24 hours (L, later). Females are bigger than males and are always resistant to the pesticide (white). Black males are susceptible and white males are resistant to the pesticide. In this scheme only one male type order is represented (resistant males first). However, both orders were performed. Dotted arrows: females were transferred immediately from one patch to the next; full arrows: females were maintained on a patch for 24 hours with males; dashed arrows: females were maintained on a patch for 24 hours without males. **b) Data Collection.** Each female was isolated on a leaf disc placed in water-soaked cotton and her survival was checked daily. Every 3 days, the female was transferred to a new leaf disc where she could continue to lay eggs. The number of eggs laid by the female on each leaf disc was measured after each transfer. On the 6<sup>th</sup> day, the leaf disc was moved into a container with cotton soaked in diluted pesticide. On the 12<sup>th</sup> day offspring sex-ratio and survival were measured, to extract offspring paternity and the total number of daughters sired by the first male. X represents death owing to pesticide.

**Figure 2. Effect of multiple mating on male paternity.** Females mated once, twice, or multiply. Re-mating was set immediately (0h) or 24 hours after the first mating. Fertilized offspring (i.e. daughters) is divided into the proportion of daughters sired by the first (light grey) and by the second male (dark grey). Note that natural death in the quiescent stage may be confounded with death by pesticide exposure, in both bars representing fertilized offspring. Unfertilized offspring (i.e. sons) is represented in white. Vertical bars correspond to standard errors of the mean.

**Figure 3. Effect of multiple mating on female fecundity and survival.** Females mated once, twice, or multiply. Re-mating was set immediately (0h) or 24 hours after the first mating. **a) Mean number of eggs laid by females per day.** Vertical bars correspond to standard errors of the mean. The asterisk (\*) represents significant differences between treatments ( $P < 0.05$ ). **b) Female survival curve.** Dashed line, immediate re-matings (0 hours interval); Dotted line, later re-matings (24 hours interval). Grey circle, two matings; White circle, multiple matings; Continuous line and black circle, one mating.

**Figure 4. Effect of multiple mating on male survival and benefits of mating with mated females. a) and b) Male survival curves.** Males were placed in patches with 1, 5 or 20 virgin (a) or mated (b) females every day and their survival was followed. Distinct colours represent different number of females per patch:

black, one female; grey, 5 females; white, 20 females. **c) Total mean number of fertilized offspring sired by the first male.** Females mated once, twice, or multiply. Re-mating was set immediately (0h) or 24 hours after the first mating. Vertical bars correspond to standard errors of the mean. The asterisk (\*) represents significant differences between treatments ( $P < 0.05$ ).















